Design and synthesis of novel, functional polymer-protein nanoarchitectures

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Abstract
The work undertaken during this Ph.D. consisted in the development of the synthesis but also in the characterization of a recently discovered subclass of amphiphilic biohybrid macromolecules called Giant Amphiphiles. In this manuscript, three new synthetic routes allowed for the first time the efficient, high yielding, and easy synthesis of these Giant Amphiphiles biomacromolecules without tedious purification procedures. This breakthrough in the field allowed the further study of the full potential application of these amphiphilic biohybrid macromolecules in order to create meaningful structures. In this purpose, some of these Giant Amphiphiles have been used to create nanoreactors but also nanocarriers, demonstrating that these new subclass of amphiphilic molecules have a promising future in nanobiotechnology and nanomedicine scientific area.

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Design and Synthesis of Novel, Functional Polymer-Protein Nanoarchitectures

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Ce travail de thèse a donné lieu à la publication de quatre articles dans des revues scientifiques internationales:


Les molécules amphiphiles *i.e.* molécules constituées d’une tête polaire et d’une queue apolaire s’auto-assemblent dans la nature de manière hiérarchique et ordonnée grâce à des interactions non-covalentes de manière à créer des superstructures telle la membrane cellulaire (double couche lipidique dans laquelle sont insérées et organisées d’autres biomolécules comme les protéines, les glycoprotéines, etc.) qui possèdent des fonctions très importantes comme la protection contre l’environnement extérieur, la compartimentalisation, la mobilité, etc.

A ce jour, deux autres classes de molécules amphiphiles autres que les amphiphiles moléculaires ont été reportées. Ainsi, les super amphiphiles et les *Amphiphiles Géants* sont deux classes de macromolécules qui diffèrent de leurs analogues moléculaires par leur taille et leur constitution. En effet, les *Amphiphiles Géants* consistent en une classe de biomacromolécules constituées d’une protéine hydrophile spécifiquement attachée à un polymère hydrophobe. Ces dernières années ont connu de récentes avancées dans la préparation des ces composés hybrides. 1,2,3 Malheureusement, la synthèse de ces *Amphiphiles Géants* n’offre en général pas de bons rendements et est très limitée par des facteurs tel que l’incompatibilité de solubilité entre la protéine hydrophile et le polymère hydrophobe.

Ce travail de thèse a, entre autres buts, été de développer de nouvelles générations d’*Amphiphiles Géants* avec le seul et unique but de programmer leur auto-assemblage pour obtenir des nanostructures multifonctionnelles qui profitent à la fois des propriétés intrinsèques des parties synthétique et naturelle. En d’autres termes, cette thèse présente de nouvelles approches synthétiques mises en place au laboratoire pour surmonter les limitations inhérentes à la préparation de ces biomacromolécules amphiphiles, mais fait également l’objet

d’études sur l’auto-assemblage de ces dernières. L’attention a été surtout focalisée sur le développement de ces nouvelles approches mais aussi sur leur exploitation dans le but d’obtenir des nanoarchitectures pleinement fonctionnelles.

Dans un premier temps, nos efforts ont été consacrés à la préparation d’une petite bibliothèque d’Amphiphiles Géants en utilisant la réaction bien connue de cycloaddition dipolaire [3+2] de Huisgen (aussi couramment appelée « click » réaction). Il fut alors observé que ces Amphiphiles Géants dérivés de l’Albumine de Sérum Bovin (BSA) s’organisent de manière à créer des structures sphériques bien définies dont les dimensions (de 20 à 500 nm de diamètre) laissent à penser qu’elles sont probablement micellaires ou vésiculaires. Il fut également observé que la variation de la chaîne polymérique (2 kDa à 8 kDa) n’avait pas d’influence sur la morphologie ou la taille des agrégats obtenus. Nous avons aussi établi que cette méthode, basée sur l’utilisation d’une molécule hydrophile hétéro-bifonctionnelle comportant les fonctions maléimide et alcyne est générique pour les protéines contenant un résidu cystéine libre et accessible à la surface de la protéine. Par ailleurs, il a été démontré que le type de superstructure adopté par les Amphiphiles Géants est fonction de la nature de la protéine. Ainsi, nous avons observé que les amphiphiles issus de l’attachement d’un polystyrène sur l’hémoglobine s’auto-assemblent de manière à former des structures cylindriques avec une longueur de 1000 nm et un diamètre de 300 à 400 nm.

Dans un second temps, le travail a été consacré à la mise en place d’une nouvelle approche appelée méthode de post-fonctionnalisation. Cette nouvelle voie synthétique a été conçue pour procéder en deux étapes. La première implique le couplage sélectif d’une protéine à un polymère hydrophile fonctionnalisé de manière appropriée via une réaction d’addition 1,4 de type Michael. Aussi, ce polymère fut conçu de telle manière qu’il possède au sein de son squelette de nombreuses fonctions alcynes terminaux. Dans une seconde étape, la « click » réaction (orthogonale, en conditions douces) entre les fonctions alcynes de ce polymère et de petits azides hydrophiles organiques a permis l’introduction de l’hydrophobicité et par conséquent l’auto-assemblage des biomacromolécules amphiphiles ainsi créées en superstructures bien définies.

Enfin, la polymérisation *in situ* de monomères hydrophobes sur une protéine correctement fonctionnalisée avec un initiateur radicalaire a été développée avec succès. Grâce à cette méthode, le styrène mais également d’autres monomères plus intéressants du point de vue fonctionnel ont été polymérisés en conditions aqueuses sur la BSA. Cette dernière technique a prouvé être très intéressante dans le but de former des nano-assemblées fonctionnelles. Ainsi le développement de cette puissante méthode permit d’auto-organiser de manière hiérarchique des nano-réacteurs et des nano-containers. De façon très surprenante, l’observation de la catalyse enzymatique a permis de démontrer que ces superstructures sont perméables aux petites molécules.\(^5\) Finalement, des travaux initiaux permettent de penser que ces nano-containers ont une certaine affinité biologique pour des cellules bactériennes vivantes et plus particulièrement pour une souche de la bactérie *E. coli*.

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Abbreviations

PEO  poly(ethylene oxide)
PEE  poly(ethylethylene)
HHH  hollow hoop structure
PS   poly(styrene)
PB   poly(butadiene)
PAA  poly(acrylic acid)
PVP(ou PV2P) poly(2-vinylpyridine)
T_g  Glass transition temperature
PIC  polyion complex
PIAA poly(isocyano-L-ala-L-ala)
PPQ  poly(phenylquinoline)
PI   poly(isoprene)
PCEMA poly(cynnamoylethylethacrylate)
PMOXA poly(2-methylloxazoline)
PDMS poly(dimethylsiloxane)
LCST Low Critical Solution Temperature
UCST Upper Critical Solution Temperature
CSC  core-shell-corona
DNA  Deoxyribonucleic acid
pIPPAm poly(N-isopropylacrylamide)
BCAm  benzo-18-crown[6]-acrylamide
DTT  dithiothreitol
MRI  Magnetic Resonance Imaging
pDEAEMA poly(2-(diethylamino)ethyl methacrylate)
pVBA poly(4-vinyl benzoic acid)
TPPTS triphenylphosphine tris-(sulfonate)
ATRP Atom Transfer Radical Polymerization
THF  Tetrahydrofuran
PB (ou PBS) phosphate buffer (saline)
DMSO dimethylsulfoxide
DCM dichloromethane
r.t. room temperature
CuAAC copper-catalyzed azide alkyne cycloaddition
DEAD diethylazodicarboxylate
MWCO Molecular Weight Cut Off
PDI Polydispersity Index
NHS N-hydroxysuccinimidyld
RAFT radical Addition-Fragmentation Chain Transfer
DIFO difluorinated cyclooctyne
CMC Critical Micellar Concentration
DMF Dimethylformamide
PMMA polymethyl methacrylate
NIPPAm N-isopropylacrylamide
CF  carboxyfluorescein
TEA triethylamine
EDTA Ethylene diamine tetra-acetate sodium salt
Abbreviations

Hb  Hemoglobin
BSA  Bovine Serum Albumin
HRP  Horseradish peroxidise
BSA  Bovine Serum Albumin
EGFP  Enhanced Green Fluorescent Protein
HRP  Horseradish peroxidise
HEWL  Hen Egg White Lysozyme
HSA  Human Serum Albumin
CPMV  Cow Pea Mosaïc Virus
TGase  transglutaminase
TMV  Tobacco Mosaïc Virus
CAL B  *Candida Antartica* Lipase B
UV  Ultraviolet
FT-IR  Infrared
ESI  electrospray ionisation
MS  mass spectroscopy
MALDI-TOF  Matrix Assisted Laser Desorption Ionization – Time Of Flight
SDS-PAGE  Sodium Dodecyl sulfate – Polyacrylamide Gel Electrophoresis
TEM  Transmission Electron Microscopy
CFM  Confocal Fluorescent Microscopy
GPC  Gel Permeation Chromatography
HPLC  High Performance Liquid Chromatography
SEC  Size Exclusion Chromatography
NMR  Nuclear Magnetic Resonance
*J*  coupling constant
*δ*  chemical shift
equiv.  equivalent
ppm  part per million
mV  millivolt
Hz  hertz
°C  degree Celsius
K  degree Kelvin
M  molar
kDa  kilodaltons
amu  atomic mass unit
rpm  round per minute
nm  nanometer
µm  micrometer
AU  arbitrary units
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Chapter I
General Introduction


The structuring of matter in Nature, often proceeds through the self-assembly of amphiphilic building blocks. Fascinating examples of this process are ubiquitous in biological systems, where soluble superstructures such as biomembranes, viruses, multimeric proteins and nucleic acid multiplexes are formed at the mesoscopic level (10-1000 nm). Probably the most representative example of this self-assembling process comes from the cell membrane\(^1\) (Figure I-1) where functionality is achieved through the interplay between different, often structurally simple, monomeric units. In this natural supramolecular structure, the organization is accomplished by the hierarchical self-assembly\(^2,3\) of different types of individual functional molecules (phospholipids, glycolipids, glycoproteins, membrane spanning peptide helices, the cytoskeleton, etc). These molecules spontaneously self-assemble in aqueous media, by ingeniously employing multiple non-covalent interactions such as hydrogen bonding, \(\pi-\pi\) stacking, and Van der Waals interactions. The resulting superstructure is functional and further combines compartmentalization, order and mobility, characteristics all essential for life.
The importance of self-assembly in Nature led scientists to extensively explore its basic principles. Already more than 100 years back, the study of self-assembly processes of amphiphilic molecules had started. Initially, it primarily focused on soaps, fats and oil, a field which was the subject of colloid chemistry and has grown immensely since the early works on bilayer membranes.\textsuperscript{4,5,6} Nowadays, in the rapidly evolving fields of nanosciences (soft matter chemistry, surface chemistry and supramolecular chemistry), one of the main goals is to be able to create supramolecular systems possessing architectures with a well-defined programmed morphology and one of the main tools toward this goal is self-assembly.

Within the last decades, it has been possible to design supramolecular assemblies with various morphologies such as micelles, vesicles, tubes, disks, lamellas and even more exotic structures such as helices or inversed structures, from synthetic molecular amphiphiles, super-\textsuperscript{,} and more recently Giant- amphiphiles as classified in terms of their molecular weights (Figure I-2). The superstructures resulting from the self-assembly of these individual molecules can be of one, two or three dimensional nature\textsuperscript{6,7,8,9} and contain between $10^1$ and $10^6$ molecules (approaching therefore synthetic and biological polymers in molecular mass).
Although significant progress has been achieved, it still remains a challenge to understand precisely the principles that govern the self-organization of individual molecules leading to the formation of nanoassemblies and furthermore to be able to manipulate these nanometer superstructures and enhance their properties to create functional nanodevices.\textsuperscript{12,13,14,15,16,17,18,19,20}


Apart from the use of surfactants as soaps which is known for centuries now, these amphiphilic molecules have known an important diversification of applications during the last decades especially in the areas of food industry, cosmetics, targeted drug systems and, more recently, in nanotechnologies. Surfactants are generally described as molecules containing a hydrophobic head and one or more hydrophobic tails.
As mentioned before, amphiphilic molecules can be classified in 3 different subclasses depending on their size and relative volumes as shown in Figure I-2: molecular amphiphiles also referred to as low molecular weight amphiphiles, super amphiphiles constituted by amphiphilic block copolymers, and Giant Amphiphiles consisting of a protein specifically connected to a hydrophobic polymeric chain.

I.2.1. Low molecular weight amphiphiles.

Low molecular weight amphiphiles (also named molecular amphiphiles) are constituted by two distinct parts: a polar (“head”) and one or more apolar (“tail”) moieties. The so-called “head” is constituted by ionic, non-ionic or zwitterionic groups whereas the so-called “tail” generally consists of one or two alkyl chains. Extensive studies have focused on the parameters that govern the self-assembling properties of a wide variety of natural and synthetic molecular amphiphiles and their use in the creation of novel functional nanostructures.\(^5,6,8,21\) These low molecular weight amphiphiles are generally classified in 3 major subclasses: lipids (natural molecular amphiphiles), synthetic surfactants and bolaamphiphiles.

It has been reported that different properties of the basic building block can be expressed at the supramolecular level depending on the conditions employed but also on the individual structure of the monomer. In water, above a certain concentration which is called critical micellar concentration (CMC), the self-assembly of amphiphilic molecules is entropically favoured to that of the presence of individual molecules in solution. The major driving force for the aggregation of these amphiphiles in water is considered to be the hydrophobic effect. Upon self assembly, these molecular amphiphiles arrange and organize in such a way that the heads are orientated towards the water and the tails buried together (to avoid contact with water) leading to the formation of different superstructures like micelles, rods, planar bilayer, inverted micelles, bicontinuous structures and vesicles, multilayers and lyotropic liquid crystalline phases (at high concentrations) (Scheme I-1).\(^6\) Even more exotic morphologies were observed in the literature and involved the formation of chiral superstructures such as “cigars”,\(^22,23\) twisted ribbons, helices,\(^24\) tubes,\(^25\) braids,\(^26,27\) boomerangs,\(^28\) or superhelices.\(^29,30\)
Phospholipids are, without any doubt, the most studied subclass of molecular amphiphiles. In nature, they hierarchically self-organize in the presence of numerous other components (i.e. proteins, glycoproteins) to create the walls of living cells. These molecules have been used as model for numerous studies aimed to unknot the relationship between the supramolecular architectures and their molecular structure.

![Scheme I-1. Common aggregation morphologies observed with molecular amphiphiles upon self-assembly: (a) micelles, (b) micellar rods, (c) planar bilayers, (d) vesicles, (e) inverted micelles.](image)

The superstructures formed by amphiphiles in dilute solutions is determined by the three terms of the free energy of surfactant self-assembly:

- A favourable hydrophobic contribution, due to an entropy effect involving the solvent water.
- A surface term that deals with the tendency of the molecules to aggregate in order to shield the apolar tails from water and the tendency to spread out as a result of electrostatic repulsion, hydration and steric hindrance.
- A packing contribution, which implies that the hydrophobic segments exclude water and the polar head groups limiting therefore the type of aggregates that are geometrically possible.

In 1980, Israelachvili et al. developed a theoretical model, based on statistical mechanics of phospholipids, which predicts the structure of the aggregates obtained after self-assembly of these molecular amphiphiles. This theory is mainly based on the geometric relation between the hydrophilic and hydrophobic domains of the molecule. It predicts the type of the aggregate on the basis of the packing parameter ($P$) of the molecule, which is a
function of the volume of the molecule \((v)\), its length \((l)\) and the mean-cross sectional (effective) head group area \((a)\) as shown in Equation I-1.

\[
P = \frac{V}{l \times a}
\]

Equation I-1.

From this formula, it can be predicted that cone-shaped lipids \((P<1/2)\) aggregate in micellar structures, cylindrical lipids \((1/2<P<1)\) such as natural phospholipids form bilayered structures such as vesicles or planar bilayers upon self-assembly and finally that reversed micelles are generated when the volume of the hydrophobic tail is more important than the volume occupied by the hydrophilic head \((P>1)\) (Figure I-3). The predictions of this model are in agreement with most of the experimental results for phospholipids, but also small amphiphiles with conventional aliphatic chains,\(^3^3\) and for several more complicated molecules such as diblock copolymers (e.g. amphiphiles consisting of a dendrimer acting as polar head group connected to a polystyrene tail).\(^3^4\)

![Figure I-3](image)

**Figure I-3.** Examples of aggregation patterns depending on the head-to-tail ratio \((P)\) above the CMC: \((a)\) micelle for \(P<1/2\), \((b)\) micellar rod for \(1/3<P<1/2\), \((c)\) planar bilayer or vesicle for \(1/2<P<1\) and \((d)\) inverted micelle for \(P>1\).

Several deviations from the model have been reported for surfactants incorporating rigid segments\(^3^5\) or multiple hydrogen bonding units,\(^3^6\) while some authors have criticized this packing parameter approach for predicting the aggregate morphology.\(^3^7\)
I.2.2. Amphiphilic block copolymers.

Amphiphilic diblock copolymers *i.e.* polymers constituted from a hydrophilic (*e.g.* polyethylene oxide PEO, polyacrylic acid, *etc.*) and a hydrophobic block (*e.g.* polystyrene, polybutadiene, *etc.*), are interesting materials for nanotechnology, pharmacy and medicine and have already been utilized as drug delivery systems. They have the ability to self-assemble on the mesoscopic length scale into highly regular superstructures in manners similar to those of their low molecular weight counterparts. Interestingly, triblock and multiblock copolymers can self-assemble in water and organic solvents in more diverse superstructure morphologies than the ones observed for molecular amphiphiles. For the purposes of this thesis, this overview will predominantly focus on amphiphilic diblock copolymers as their overall structure and properties are more similar to the ones of the protein-polymer *Giant Amphiphiles* that are the focus of this thesis.

I.2.2.1. Amphiphilic linear diblock copolymers.

Whereas the design rules for the synthesis of low molecular weight amphiphiles in relation to the aggregate morphologies are well established nowadays, in the case of amphiphilic diblock copolymers, these are still in the process of being formulated.

It is important to note that the CMC of these amphiphilic block copolymers is usually much lower\textsuperscript{38} and the dynamics of the chain exchange are much slower, due to numerous factors including the high molecular weight, the possible entanglement and the low mobility of the chains in the core.\textsuperscript{39,40} Due to these mobility restrictions, the superstructures obtained by the self-assembly of amphiphilic diblock copolymers are much more robust and stable in aqueous solutions than those of their low molecular weight counterparts. This was demonstrated by Discher and coworkers who studied the aggregation behaviour of poly(ethylene oxide)-*b*-poly(ethylethylene) polymers (PEO-*b*-PE).\textsuperscript{40} It was observed that small vesicles (≤200 nm) were formed upon hydration and vitrification of this diblock copolymers whereas, giant vesicles (20-50 µm) were formed through electroformation.\textsuperscript{41,42,43,44} These vesicles appeared to be highly deformable and, in contrast to conventional lipids, have an enhanced toughness and limited permeability.
Due to these mobility restrictions, the superstructures obtained by the self-assembly of amphiphilic diblock copolymers are much more robust and stable in aqueous solutions than those of their low molecular weight counterparts (Figure I-4).45

Figure I-4. Schematic plot of typical physical properties with the molecular weight of a vesicle’s amphiphile (reprinted from reference 45).

Copolymers constituted by one polar and an apolar block can form either star or crew-cut micelles,46 depending on the block lengths. Star micelles are formed when the corona-forming blocks are much longer than the core-forming block whereas crew cut micelles consist of a large core-forming block and a small corona-forming block.

Crew-cut aggregates have been extensively studied by Eisenberg and coworkers using asymmetric amphiphilic diblock copolymers of polystyrene-b-poly(acrylic acid), polystyrene-b-poly(ethylene oxide) and polystyrene-b-poly(4-vinylpyridine).47,48 Apart from the simple micellar structure, a wide variety of other morphologies were observed including rods, lamellae, vesicles, hexagonally packed hollow hoop structures (HHH) and large compound micelles as shown in Figure I-5.
Figure I-5. Different morphologies of crew-cut aggregates obtained by self-organization of PAA-\(b\)-PS diblock copolymers: (A) small spheres from PS\(_{500-58}\)-PAA; (B) rod-like micelles from PS\(_{190-20}\)-PAA; (C) vesicles from PS\(_{410-20}\) and (D) large compound micelles from PS\(_{200-4}\) (reprinted from references 47 and 48).

It was found that the type of morphology observed is governed by three major components of the free energy of aggregation: (i) core chain stretching, (ii) surface tension between the micellar core and the solvent outside the core, and (iii) intercoronal chain interactions. \(^{49,50,51,52,53,54}\) These factors, and thereby the morphology of the aggregates, are further affected by external parameters including the solvent composition, \(^{55}\) the relative block length, \(^{50,51,56}\) the temperature, \(^{57}\) and the presence of additional species such as salts (change in the ionic strength), acids, bases, homopolymers and low molecular weight surfactants. \(^{58,59,60,61,62}\)

The present consensus on how the structure of the individual amphiphilic copolymers affects the morphology of the macromolecular architecture, takes into account the contribution of the molecular weight, relative block length, structure (e.g. branched vs. linear), conformational aspects, the presence of functional groups and the possibility of specific interactions between polymer blocks. These, however, are not the only parameters determining the morphology of block copolymer aggregates. As mentioned earlier, significant is also the role of external factors e.g. various solution conditions such as solvent nature and composition, polymer concentration and the presence of additives (e.g. ions, surfactants and homopolymers). Eisenberg and coworkers published recently an overview of the contribution of all the above mentioned parameters to the self-assembling behaviour and aggregate morphology of block copolymers. \(^{63}\)
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As mentioned in Section I.2.1, the type of aggregate morphology formed by traditional amphiphiles can be predicted using the theory developed by Israelachvili.\textsuperscript{31,32} Unfortunately, in the case of block copolymer amphiphiles, the situation is much more complex. Nevertheless, based on the same geometrical considerations, one can predict the morphology of aggregates depending on an analogous packing parameter \((P)\). Taking into account the bigger dimensions of the molecules and the curvature of the hydrophilic-hydrophobic interface,\textsuperscript{64} this packing parameter is defined as:\textsuperscript{65}

\[
P = \frac{V}{l \cdot a} = 1 - H \cdot l + \frac{K \cdot l^2}{3}
\]

\textbf{Equation I-2}

Where \((V)\) is the hydrophobic volume occupied by the amphiphile, \((a)\) the interfacial energy and \((l)\) is the chain length of the block copolymer amphiphile and where the parameters describing the hydrophobic-hydrophilic interface are the mean curvature \((H)\) and the Gaussian curvature \((K)\) (Figure I-6).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{amphiphile_shape}
\caption{Description of the amphiphile shape in terms of the surfactant parameter \((P)\) and its relation to the interfacial mean curvature \((H)\) and Gaussian curvature \((K)\) (reproduced from reference 64).}
\end{figure}

The simplest morphologies (\textit{i.e.} spheres, cylinders and bilayers) are obtained by the combination of parameters given in Table I-1. It is evident that an increase for example in the hydrophobic/hydrophilic ratio results in a change in aggregates morphology from spherical to rod-like micelles to vesicles.
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<table>
<thead>
<tr>
<th>Shape</th>
<th>P</th>
<th>H</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphere</td>
<td>1/3</td>
<td>1/R</td>
<td>1/R²</td>
</tr>
<tr>
<td>Cylinder</td>
<td>½</td>
<td>1/(2R)</td>
<td>0</td>
</tr>
<tr>
<td>Bilayer</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table I-1. Packing parameter ($P$), mean curvature ($H$) and Gaussian curvature ($K$) for different aggregation morphologies.

Another significant difference observed on the self-assembling behaviour of block copolymer amphiphiles as compared to that of traditional amphiphiles arises if not only geometrical, but also thermodynamic parameters are taken into consideration. There are two major contributions to the free energy of the system:

- The loss of entropy when flexible parts of the polymeric amphiphile are enforced in the restricted environment of the aggregates.
- The interfacial energy of the hydrophobic-hydrophilic interface.

Due to the significantly larger size of the amphiphilic block copolymers, both these contributions are considerably more important in the self-assembling process of these macromolecules. The *Glass Transition Temperature* ($T_g$)\(^{66}\) of primarily the core forming block has a particularly important role in this context. Because of the amphiphilic nature of the macromolecules, the interfacial energy is typically large, leading to the separation of these segments in the self-organized assemblies. When the interfacial energy is the predominant contribution to the free energy of the system, *i.e.* when polymers having low conformational entropy are used, the amphiphiles organize into morphologies with the lowest interfacial area per volume unit, giving therefore a preference to form planar (bilayer type) interfaces over cylindrical or spherical domains. Experimentally, a number of amphiphilic macromolecules that contain a rigid or conformationally restricted segment have been observed to form bilayer type architectures, which is in line with this prediction. Thereby, rod-coil type block copolymers have been investigated.\(^{67,68,69}\) Copolymers in which one of the constituent blocks has a specific interaction (*e.g.* hydrogen bonding, ionic interactions, etc)\(^{70,71}\) are conformationally restricted. When specific interactions are present between the solvophobic segments of the amphiphilic macromolecules, highly ordered structures have often been observed. This is the case of block copolymers where mesogenic groups are introduced in the macromolecules or where secondary interactions between these non-soluble parts are present on the block copolymer backbone. These principles also explain the change in aggregate
morphology when the ion type or concentration is changed in the case of an amphiphilic block copolymer containing a polyelectrolyte segment.\textsuperscript{70,71} For example, Kataoka and coworkers used poly(ethylene glycol)-b-poly(α,β-aspartic acid) and poly(ethylene glycol)-b-poly(L-lysine) as a pair of polymers which are oppositely charged. When pairs with a different length of the charged block were mixed, neutral polyion complexes (PIC) were formed. These complexes consisted of the minimal required number of polymers to neutralize the total charge. Pairs with charged blocks of similar length formed bimolecular complexes that grew into larger PIC micelles. Under conditions in which matched and unmatched pairs coexist, only the matched pairs formed PIC micelles whereas the remaining polymers of unmatched length remained as individual entities. It was assumed that this is a result of the increased stability of the micelles over the individual PICs.

In order to obtain structures with a high level of organization, monomers that contain the necessary information in the form of chirality, hydrogen-bonding capacity, steric demands, electrostatic properties, hydrophilic or hydrophobic character, or metal ion binding capability, are used. This principle, in Nature, is demonstrated in the case of proteins where the amino acid sequence determines the secondary structure (\textit{i.e.} α-helices and β-sheets), which in turn controls the tertiary (protein subunit) and quaternary (overall) structure of the biomacromolecule and its possible further functional aggregation/assembly. This influence of the monomer unit on the final aggregated structure has also been observed for diblock copolymers. For example, Nolte and coworkers have investigated amphiphilic rod-coil block copolymers composed from polyisocyanide and polystyrene.\textsuperscript{10,72,73} The isocyanides used in this study were derived from dipeptides and upon polymerization formed, due to hydrogen bonding between amide bonds, helical structures whose handedness was controlled by the type (D or L) of the peptide used.
Figure I-7. (A) Structure of the polystyrene-block-polyisocyanodipeptide used in Nolte group, (B) Schematic representation of the polyisocyanodipeptide block with the peptidic arms represented as blue arrows and the polyisocyanide backbone as a red helix, (C) TEM micrographs of a left-handed superhelix formed by self assembling of PS$_{40}$-b-PIAA$_{10}$ (L-ala-L-ala) amphiphiles in sodium acetate buffer at pH 5.6 (reprinted from references 10 and 72).

These polystyrene-b-polyisocyanates were found to form micellar aggregates, vesicles, bilayered structures and super helices when dispersed in aqueous solutions. The latter superhelices were observed to have handedness opposite to the handedness of the helices from which they originated. The aggregates morphologies were also found to depend on many parameters such as the relative block lengths, the pH and the isocyanide monomers used. Additionally, it was demonstrated that the flexibility of the rod-like structures could be adjusted simply by changing the counterion in the aqueous buffer.

Long range interactions between individual aggregates have also been reported by Jenekhe and collaborators who studied the self assembly of rod-coil diblock copolymers consisting of poly(phenylquinoline) (PPQ) and polystyrene (PS) (Figure I-8). The use of selective solvents for the rigid PPQ rod led to the formation of large micrometer sized aggregates with various morphologies (spheres, vesicles, cylinders and lamellae). The cylindrical and spherical aggregates contained a large hollow cavity as a result of the close packing of the rigid rod blocks. The micelle-like aggregates were observed to be able to encapsulate large amounts of fullerenes in the inner cavity and in the PS core.
On the contrary, the use of a selective solvent for the flexible PS block yielded exclusively hollow spherical micelles of several micrometers in size (Figure I-8). Further, long-range, close-packed self-ordering of the micelles produced periodic microporous materials of which the microstructures and optical properties could be tuned by the addition of small amounts of fullerenes, which were incorporated in the PS corona. The preparation of films of vesicles is not only limited to the PS-PPQ system. Ding et al. demonstrated the same principle through the evaporation of organic solutions of polyisoprene-\textit{b}-poly(2-cinnamoylethylethacrylate) (PI-\textit{b}-PCEMA) vesicles on top of a water surface. The shape of the vesicles changed from spherical to hexagonal, forming a close-packed film with a hexagonal morphology.

Recently, Van Hest and coworkers developed an amphiphilic diblock copolymer based on polystyrene-block-poly(acrylic acid) (PS-\textit{b}-PAA) suitable for bioconjugation with proteins. After polymerization, $\omega$-terminus (bromide end group) was substituted with an azide moiety. This functional diblock copolymer was allowed to aggregate into vesicular structures where the bilayer shell was covered with azide groups. Further “clicking” of the 1-alkyne modified protein EGFP (Enhance Green Fluorescent Protein) onto the polymersomes was achieved in aqueous conditions and proved to afford amphiphilic triblock protein-polymer bioconjugates through confocal laser-scanning microscopy.
I.2.2.2. Amphiphilic linear triblock copolymers.

ABC triblock copolymers have shown to give a greater even variety of structures resulting from the phase separation of the three different blocks. In this case, experiments demonstrated that the formation of the microseparated assemblies was influenced by two independent composition parameters, namely the volume fraction and the relative immiscibility between directly connected and between non-linked blocks. Triblock copolymers have proven to be extremely useful precursors for very complex self-assembled structures such as “three layer” micelles, “crew-cut” micelles and the so called “Janus” micelles. The latter for example, consist of a cross linked poly(butadiene) core and a corona constituted by poly(styrene) and poly(methylmethacrylate). The stabilization of these interesting supramolecular structures is thought to be the result of direct solubilisation of the ABC triblock copolymer in a solvent selective for one of the blocks, or, as in the case of the Janus micelles, the result of a supramolecular organization in the bulk state which is then transferred in solution. In the recent literature concerning these ABC triblock copolymers, important theoretical and experimental studies have also been carried out on poly(styrene)-b-poly(butadiene)-b-poly(methyl methacrylate). It was observed that the morphology of the aggregates is dependent on the repulsive forces between the poly(butadiene) middle block and both poly(styrene) and poly(methyl methacrylate) end blocks. More recently, an ABC triblock consisting in a poly(styrene)-b-poly(butadiene)-b-poly(ethylene oxide) has been extensively studied for its morphological behaviour.

In 2002, Meier and coworkers reported on the potential interest of these polymersomes as bionanocontainers. In this work, they reported on the synthesis of ABA triblock copolymers PMOXA-PDMS-PMOXA (poly(2-methyloxazoline)-b-poly(dimethylsiloxane)-b-poly(2-methyloxazoline)) that self assemble in water to form vesicular structures. It was demonstrated that these polymersomes are able to accomodate the LamB channel protein within the block copolymer membrane. The resulting vesicles were proven to be functional as they were shown to interact with living systems. On this direction, Meier and coworkers exposed the vesicles to the bacteriophage λ, which binds to the LamB protein, and monitored the viral DNA being loaded into the polymersomes, giving the pioneering demonstration of the possible applications of these polymersomes in living systems.
Other works from the Meier group studied also the insertion of different channel proteins like FhuA or Ompf proteins within the copolymer membrane. Upon encapsulation of catalytically active proteins within the inner cavity of these proteopolymersomes, it was demonstrated that the presence of the pore protein is necessary for the substrate to cross the membrane and be metabolized by the enzyme. The product of the reaction was subsequently by the same transport process released in the aqueous medium.

Finally, Schubert and collaborators reported on a new class of triblock copolymers that combine metallosupramolecular and polymer chemistry. These PS\textsubscript{32}-b-P2VP\textsubscript{13}-b-[Ru]-PEO\textsubscript{70} triblock copolymers possess interesting features such as reversibility of the metal-ligand complex under precise conditions, combined with the well known interesting photophysical and electrochemical properties of the ruthenium-terpyridine complex and the possibility to easily construct a library of triblock copolymers very easily.

### I.2.2.3. Stimulus responsive copolymers

Block copolymers in which the amphiphilicity can be switched “on” and “off” have also been extensively studied during the last years. These so-called stimuli-responsive or “smart” block copolymers respond with large property changes to small chemical or physical stimuli such as pH, specific ions, ionic strength, electric fields or temperature. In this category block copolymers that are constituted by a block whose monomer unit posses a lower or an upper critical solution temperature (LCST or UCST) are listed. As a result, above or below
respectively a specific temperature, the polymer spontaneously transforms from molecularly dissolved to phase separated in water. Block copolymers possessing such a temperature dependent polymer coupled with a hydrophilic polymer can thus reversibly switch from hydrophilic to amphiphilic upon temperature changes.88

Diblock copolymers consisting of two different responsive blocks can exist in three states in aqueous solutions, namely, as conventional micelles, reversed micelles, and molecularly dissolved polymeric chains.89,90,91,92,93 The composition of the micellar core of the aggregates formed by these so called schizophrenic diblock copolymers, is dictated by the conditions, allowing the inside and the outside block to change by a combined stimulus of pH and ions,89,92 pH and temperature,93 pH only,91 or temperature only.90

As an example, in the case of diblock copolymers constituted by poly(4-vinyl benzoic acid) (VBA) and poly(2-(diethylamino)ethyl methacrylate) (DEAEMA), aggregation studies showed that the micellar core is formed by VBA at low pH, DEA at high pH whereas, non-micellar aggregates that precipitate were formed at intermediate pH.91

Core-shell-corona (CSC) pH sensitive micelles have also been constructed from poly(styrene)-b-poly(2-vinylpyridine)-b-poly(ethylene oxide) (PS-b-P2VP-b-PEO) ABC triblock copolymers.94 Interestingly, these ABC triblock copolymers were found to respond to external stimuli (e.g. pH) allowing tuning of the size of the aggregated system. Temperature and pH-sensitive CSC micelles in which the shell can be selectively cross linked have also been reported by Armes and collaborators.95

The stimuli-responsive polymers are nowadays extensively studied as they might find a variety of applications in medicine as controlled-release systems. Soluble drugs, proteins, DNA multiplex can be easily encapsulated within these responsive copolymers aggregates and can be released at a desired rate at a specific time and place depending on the conditions. For example, such a system has been developed for the controlled release of the solute vitamine B12.96
Figure I-10. Schematic representation of core-shell porous membrane microcapsules formed by aggregation of the stimulus responsive copolymer \( p(\text{NIPAAm})-co-p(\text{BCAm}) \). Upon addition of \( \text{Ba}^{2+} \) ions in the medium, specific recognition of \( \text{Ba}^{2+} \) by the crown ether occurs and the grafted polymer swells and closes the pores. After dialysis against water, \( \text{Ba}^{2+} \) is removed from the medium, the grafted polymer shrinks and then the pores open.\(^9\)

On this direction, Yamaguchi and collaborators developed microcapsules consisting in of a core-shell porous membrane and linear grafted poly(\( \text{NIPPAm-co-BCAm} \)) (\( i.e. \) poly(\( \text{N-isopropylacrylamide}\))-\( b\)-poly(\( \text{benzo-18-crown[6]-acrylamide}\))) chains in the pores, which acted as molecular recognition gates. The gates could be closed by the addition of barium cations \( \text{Ba}^{2+} \) which are captured by the BCAm receptors and thereby decrease the lower critical solution temperature (LCST) of the NIPPAm. The system proved to be fully reversible after dialysis against deionized water.

I.2.3. Protein-Polymer Giant Amphiphiles.

I.2.3.1. Definition.

In the above described examples of self-assembly of macromolecules into well defined superstructures, only synthetic components were considered. Assemblies incorporating biological components are particularly attractive as supramolecular biomaterials as they
would combine the structural properties of the synthetic polymer with those of the biological moiety combined with an increased biocompatibility and possibly biological function. A new, innovative class of such biosurfactants which was recently introduced by the Nolte group, are the *Giant Amphiphiles*, consisting of a protein or an enzyme linked selectively and specifically to a hydrophobic polymer.\textsuperscript{11,97} These biohybrid polymers differ from other protein-polymer conjugates in the sense that the protein to polymer ratio is predefined and the position of the bioconjugation site is precisely known. *Giant Amphiphiles* are in fact diblock copolymers which have by design significantly higher molecular weights and volumes than their synthetic counterparts. Furthermore, taking into account that Nature synthesizes its biopolymers with high efficiency, *Giant Amphiphiles* have the intrinsic structural advantage over the synthetic block copolymers of possessing a monodisperse block (the protein).

**I.2.3.2. Methods for specific protein functionalization.**

In the particular case of protein-polymer *Giant Amphiphiles*, three different methods to functionalize a protein with a hydrophobic polymer have already been reported (Figure I-11):

A. The first one involves the direct attachment of a polymer terminated with an appropriate chemically reactive group directly on a selected functional group of the protein. This method is called direct specific coupling.

B. Secondly, the specific functionalization via cofactor reconstitution can be only used in the case of enzymes containing a prosthetic group. It involves an initial coupling of the prosthetic group onto the polymer, followed by a reconstitution between the cofactor functionalized polymer and the corresponding apoenzyme.

C. The last method involves an indirect specific functionalization and consists of two steps. In the first one, a heterobifunctional linker is attached specifically onto a specific position of the protein to create a reactive biohybrid, which is on the second step functionalized using an appropriately appended polymer.
Figure I-11. Schematic representation of the synthetic methodologies used previously to synthesize Giant Amphiphiles: (A) direct coupling of an appropriately functionalized hydrophobic polymer, (B) Specific coupling by cofactor reconstitution of the apoenzyme with the appropriate cofactor appended hydrophobic polymer, (C) Indirect specific functionalization via coupling with a heterobifunctional linker followed by the attachment of the hydrophobic polymer.

I.2.3.3. About the crucial choice of the amino-acid to target.

Choosing the appropriate way to specifically modify a protein with a polymer is not an easy task. The major difficulty in synthesizing Giant Amphiphiles (except the solubility incompatibility problem found for the hydrophilic protein and the hydrophobic polymer which will be addressed later on) comes from the choice of the targeted amino-acid. Proteins are natural polymers constituted from a sequence of a combination of 21 natural amino-acids.

Specific modification of proteins/enzymes is one of the most challenging problems in protein chemistry. Many research groups have during the last 50 years developed some new methodologies in order to avoid multiconjugation reactions nevertheless, some amino-acids cannot be targeted because of their too high proportion in the protein sequence. On the other hand, some amino-acids are present in a relative small percentage in the protein sequence and as a consequence are the ideal candidates for specific conjugation e.g. Cysteine, Tyrosine,
Lysine and Glutamine together with the α-N-terminus. Another important consideration that should be mentioned is the availability of the target functional group. This later has to be sufficiently exposed on the exterior of the protein to react with the suitable terminal reactive function of the polymer.

In the following paragraphs, the advances on the specific modifications of selected, low abundance, amino-acids will be presented.

### I.2.3.3.a. Cysteine modification.

As mentioned above, the cysteine residue (Cys) is only present in small percentage in proteins, thus it is a good candidate to link a protein with a synthetic polymer in a selective, quantitative and rapid fashion. Bioconjugate chemistry literature abounds with methods to selectively functionalize free thiols of proteins.

For example, cysteine reacts easily with α-halo carbonyl compounds or with maleimido-appended molecules and macromolecules to give respectively the corresponding thioether or 1,4-Michael thioether adduct as shown on Scheme I-2. Bioconjugation of proteins with maleimide are generally undertaken in aqueous buffer solution at neutral pHs (6.5<pH<7.5) to avoid side reactions that can occur especially with lysine residue at higher pHs. Both α-halo carbonyl and maleimide derivatives can be easily synthesized in good yields by different methods.

![Scheme I-2. Selective reaction of cysteine residue with α-halo compounds (top), and with maleimide derivatives (bottom).](image-url)

Cysteine residues can also be easily modified by reaction with pyridyl disulfide derivatives (Scheme I-3) to give the corresponding disulfide compounds and concurrently generate a pyridyl thione. This latter has unique spectral properties that allow the quantification of sulfhydryl coupling by monitoring the increase in absorbance at 343 nm.
Scheme I-3. Reaction between cysteine and pyridyl disulfide compounds.\textsuperscript{104,105,106}

In this particular case, the resulting product (a disulfide) can be easily cleaved using standard disulfide reducing agents e.g. DTT (dithiothreitol) to give back the starting material. This is a method that has been used also in the case of the synthesis of well-defined protein-polymer bioconjugates where, especially in the case of \textit{in situ} polymerization on proteins, it offered the possibility to cleave the polymer\textsuperscript{107} from the protein in order to analyze the former one.

I.2.3.3.b. Lysine and/or N-terminus modification.

The amino group of Lysine (Lys) and/or N-terminus of proteins can be easily and selectively modified. Unfortunately, modification of this amino-acid generally affords a multiplicity of different conjugates due to the proportion of lysine residues in protein structures.

The first method consists of a condensation with an aldehyde\textsuperscript{108} to give the corresponding imine (Schiff base) that can be reduced in mild conditions using sodium cyanoborohydride (NaCNBH\textsubscript{3}) to obtain the secondary amine as described on Scheme I-4.

\begin{center}
\includegraphics[width=\textwidth]{SchemeI4.png}
\end{center}

\textbf{Scheme I-4.} Modification of Lysine through reductive amination.\textsuperscript{108}

Another method which is more widely used in protein chemistry, and especially for labelling of enzymes, is based on amide bond formation using NHS-activated carboxylic acids.\textsuperscript{109,110}
1.2.3.3.c. Modification of Tyrosine residue.

Tyrosine is a mild nucleophile but its nucleophilicity can be enhanced under mildly basic conditions (pH>8.5) by deprotonation of the phenol side chain. Two different reagents were mostly used for this modification. First of all, the use of diazonium salts is frequently reported however often cross reactivity occurs with histidine and/or lysine. They can also be modified by iodination. 

Tyrosine modification has been investigated by Hermanson et al. nevertheless under quite harsh reaction conditions. Moreover, a cross-reactivity towards Cystein and/or Lysine residues was also observed. These studies have evolved significantly by the new ingenious techniques that were extensively studied for the last five years in Francis group which developed some novel methods to selectively target Tyrosine under mild conditions.

Francis and collaborators managed to selectively modify tyrosine residues by the use of *in situ* generated π-allylpalladium species. As described on Scheme I-6, upon exposure of the protein to an allylic acetate derivative, a catalytic amount of Pd(OAc)$_2$ and triphenylphosphine tris-(sulfonate) (TPPTS, a water-soluble phosphine ligand), efficient protein labeling was observed within 45 min at room temperature. Analysis of the reaction mixture consistently indicated 50-65% conversion of the starting protein to a singly alkylated product, corresponding to the addition of the π-allyl species to tyrosine after loss of the acetate group. A small amount of doubly alkylated product was also observed. Consistent with a mechanism involving the phenolate anion, the reaction was found to proceed best at pH 8.5-9.0.
In another work, Francis reported on the dual surface modification of Tobacco Mosaic Virus (TMV) coating protein. To achieve this orthogonal functionalization, they specifically modified the exposed to the solvent tyrosine residue at position 139 by electrophilic aromatic substitution in ortho position of the hydroxyl group using diverse para substituted diazo reagents at pH 9 for 2 hours at 4°C as shown on Scheme I-7. The modification was successful in all studied cases, except in the case where an amide function was employed in para of the diazo group. This specific modification of tyrosine, together with the functionalization of glutamine (using secondary amines and EDC/HOBt coupling agent), led to the formation of TMV nanoscale materials that could have potential applications as multivalent scaffolds for the display of biological ligands and for the preparation of linear arrays of chromophores and inorganic nanoparticles as well as drug and gene delivery carriers. This reaction has been recently successfully used to prepare nanosized Magnetic Resonance Imaging (MRI) Contrast Agents by selective modification of tyrosine residue at position 85 of the interior surface of bacteriophage MS2 capsids devoid of nucleic acids.

Francis and collaborators also reported on a highly selective Mannich-type reaction for tyrosine side chain specific modification that involves aliphatic aldehydes and electron-rich para-aniline derivatives. The reaction proceeds smoothly at pH~6.5 by the electrophilic aromatic substitution of \textit{in situ} generated imines onto tyrosine residues as shown on Scheme I-8.

\begin{equation}
\text{Scheme I-6. Modification of tyrosine using } \pi \text{-allyl palladium complexes.}^{112}
\end{equation}

\begin{equation}
\text{Scheme I-7. Modification of Tyrosine using para-diazocompounds.}^{113}
\end{equation}
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I.2.3.3.d. Glutamine modification.

Glutamine (Gln) is also a unique target for the selective modification of proteins. As reported by Sato, such a modification can proceed through an enzymatic reaction using the transglutaminase (TGase). This enzyme catalyzes the coupling reaction between Gln and various primary amines, including the exposed Lys side chain as shown on Scheme I-9. This enzyme is highly selective towards the Lys target and will only catalyze the coupling reaction if the primary amine is highly exposed. Practically, this approach has been used to introduce PEG-polymers and other synthetic targets onto proteins, provided they possess long enough primary amine bearing aliphatic chain.

I.2.3.4. History of Giant Amphiphiles.

The first attempt of introducing hydrophobicity on proteins through the coupling of relatively small hydrophobic chains was reported by Ringsdorf. During these studies streptavidin, (a 60 kDa protein containing 4 identical subunits that can each be bound to a biotin molecule) was attached to a modified biotin bearing apolar chain leading to an increased amphiphilic character in the protein.
During the last decade, *Giant Amphiphiles* were introduced by the Nolte group and since have flourished in the literature. The first reported example involved the specific coupling of the lipase B from *Candida Antartica* (CAL B) with a hydrophobic polymer.\textsuperscript{11} During these studies, the exposed disulfide bridge of the enzyme was reduced with dithiothreitol (DTT) and then, the modified enzyme was coupled to a maleimide appended hydrophobic 4.3 kDa polystyrene chain through a 1,4-Michael-type addition using a mixture of THF/water (9/1) as solvent.

![Scheme I-10](image)

**Scheme I-10.** Synthetic approach used for the synthesis of CAL B-polystyrene Giant Amphiphiles; (a) reduction of the disulfide bridge with DTT and (b) coupling of reduced CAL B with a maleimide appended polystyrene (n=40, PDI=1.04) (reprinted from reference 11).

This coupling led to the formation of biohybrid macromolecules that self-assemble in water to form micrometer long fibers constituted of bundles of micellar rods (Figure I-12). The conditions used in this report, took advantage of the known stability of CALB in organic solvents to achieve solubilisation of both components during the bioconjugation step. These conditions are not judged to be generic as they are quite harsh for the generality of proteins and could easily lead to denaturation. Nevertheless, CAL B in its aggregated *Giant Amphiphile* form was shown to retain part (6-7%) of its original catalytic activity. The authors explained the loss of activity by the bundling of individual rods that causes inaccessibility of the substrate to the protein head group.
Another approach, using a biotin-streptavidin system similar to what was described by Ringsdorf, was also investigated by the Nolte group. In this base, biotin was initially modified with a 9.2 kDa polystyrene chain and spread at the air/water interface in a Langmuir trough. Subsequently, streptavidin was added in the aqueous phase as shown in Scheme I-11. Using this approach, only the two streptavidin subunits located on the same side of the protein can bind the biotin appended polystyrene chains. Investigation of these systems proved that monolayers of polystyrene-Streptavidin bioconjugates were obtained upon addition of streptavidin in the sub-phase system. In a second step, it was demonstrated that the remaining two free binding sites on Streptavidin were still available to complexate other biotinylated molecules. In this direction, the authors reported on the binding to the monolayer of a biotinylated form of the iron storage protein ferritin as well as that of a modified horseradish peroxidase resulting in a catalytically active surface. Interestingly, the resulting catalytic monolayers showed an activity not dependent from the lateral pressure. In this particular case, polymeric tails are attached to the protein in a non covalent fashion, but due to the high affinity constant between streptavidin and biotin ($K_a = 10^{15}$ M$^{-1}$) the formation of the protein-polymer complex can be considered as non-reversible.
Scheme I-11. Schematic pathway developed by Nolte and coworkers to prepare Streptavidin-polystyrene Giant Amphiphiles monolayers (reprinted from reference 117).

In another approach, horseradish peroxidase-polystyrene Giant Amphiphiles were formed following the cofactor reconstitution pathway. Horseradish peroxidase (HRP) is an enzyme which contains a heme cofactor buried within its core. In this work, the heme cofactor was removed from the protein according to existent experimental procedure and the synthesis of the HRP-polystyrene Giant Amphiphiles was achieved by cofactor reconstitution between the apoenzyme and an heme appended polystyrene as can be shown on Figure I-13.

Figure I-13. (A) Structure of the heme appended polystyrene used for the cofactor reconstitution, (B) computer-generated model of the reconstituted HRP-polystyrene Giant Amphiphile, (C) SEM (top) and TEM (bottom) pictures of polystyrene-HRP Giant Amphiphiles self-assembly (scale bar = 200 nm) in aqueous solution (reprinted from reference 118).
I.3. Scope of work.

In the short history of Giant Amphiphiles, the focus has been put on their successful synthesis and the potential of creating bioconjugate systems with applicability in different areas such as materials chemistry, biomedicine, nanotechnologies. As mentioned above, these protein-polymer amphiphiles have been prepared either by direct conjugation of appropriately functionalized hydrophobic macromolecules to specific aminoacids or cofactors or through bioaffinity couplings. Nevertheless, although these compounds have attracted increasing interest during the last few years, the synthesis of Giant Amphiphiles and, as a consequence, their further study for potential applications were hampered by experimental limitations. These limitations are mainly due to the solubility incompatibility between the protein hydrophilic head and the hydrophobic polymeric tail and by constraints posed to guarantee the stability of the protein itself. These problems together with the tedious purification procedures required by the intrinsic properties of such systems, significantly limited any further studies for exploring Giant Amphiphiles’ full application potential into the creation of pre-programmed hierarchical multifunctional systems.

Participating in a concerted effort of the laboratory to design and create, novel generations of Giant Amphiphiles with the scope to program them in later steps into well-defined multifunctional nanoassemblies that make use of the intrinsic properties of both the protein and the polymer moieties, this Thesis is concerned with novel approaches designed to surpass the synthetic limitations, create multifunctional systems and study their assembly. For this reason, it was decided to focus mainly on developing novel synthetic approaches and exploiting their potential.

More specifically, following this short introduction, the Second Chapter will be dedicated to the creation of a small library of Giant Amphiphiles using the well-known [2,3]-dipolar Huisgen cycloaddition (also referred to as “click chemistry” reaction). Giant Amphiphiles have already shown to self-assemble in a manner similar to that of their molecular and super counterparts. It was thus envisioned that altering the length of the polymer would allow a controlled variation of the overall shape of the biomolecules and thus lead to extracting important fundamental information concerning the molecular
shape/aggregation relationship. Finally, tuning the protein head group was also attempted as it appeared to be a challenging task.

In the Third Chapter, the design and successful application of a novel approach, the post-functionalization approach, will be described. This new synthetic pathway was designed to proceed through two discrete steps. The first step involved bioconjugation of an appropriately functionalized hydrophilic polymer, which was designed to contain multiple 1-alkyne groups and was synthesized using ATRP polymerization in collaboration with Prof. Dave Haddleton and Dr. G. Mantovani. The orthogonal multi-clicking of various azidated compounds allowed on a second step, the introduction of hydrophobicity and therefore the synthesis of well-defined amphiphilic triblock Giant Amphiphiles.

In the Forth Chapter, the ATRP mediated, in situ polymerization of hydrophobic monomers initiated from a protein biomacroinitiator was developed and proved to be a powerful tool for the creation of meaningful, functional nanoassemblies. The hierarchical self organization of nanoreactors and nanoassemblies will be discussed as it successfully led to the formation of the first Giant Amphiphile nanoreactors. Interestingly, these nanoreactors proved to be catalytically active while, surprisingly, in initial experiments they showed affinity with living bacterial cells.

Finally, the outcome of this research and the perspectives for future studies will be briefly discussed in Chapter V and a detailed experimental section will be provided in Chapter VI.
References


[2] Generally, hierarchical self-assembly is defined as “the formation of an organized structure through different and distinct levels of self-assembly processes that decrease in strength”.


Chapter I – General Introduction

[66] The IUPAC Compendium of Chemical Terminology, 66, 583 (1997). The glass transition temperature, Tg, is the temperature at which an amorphous solid, such as glass or a polymer, becomes brittle on cooling, or soft on heating.
Chapter I – General Introduction

Chapter I – General Introduction


II.1. Background / Concept.

To proceed with selective chemical reactions on biomolecules, several are the requirements that need to be compulsorily respected to guarantee their stability and integrity. Furthermore, taking into account the multifunctional and, most of the times, chemically fragile nature of biological entities, the major challenge into this direction is a chemical one: to achieve bioorthogonal reactions under the benign reaction conditions that would preserve and respect the multifunctionality and structure of biologically derived components. The ideal coupling functional groups therefore should be highly stable in aqueous conditions and the reactions extremely chemoselective and with high yields.

Pursuing this, numerous groups developed new methodologies that are often stored under the generic term of “click” chemistry reactions. This catchy term “click”, refers to energetically favoured, specific, and versatile chemical transformations, that lead to a single reaction product. In other words, the fundamental nature of the “click” is simplicity and efficiency. This concept seems to answer perfectly the needs of scientists in areas of research as diverse as molecular biology, drug-design, biotechnologies, material science or macromolecular chemistry. Reactions of the “click” type are rather rare. Yet, the last few years saw the emergence of a rudimentary “click” toolbox.

The introduction of the “click” concept in 2001 by Sharpless, Kolb and Finn\(^1\) has defined the stringent set of criteria that a process must meet to be useful in the “click”-context as they stated that “click” reactions should be “modular, wide in scope, high yielding, stereospecific, simple to perform creating only inoffensive by-products (that can be removed without chromatography) and requiring benign or easily removed solvents, preferably
Although meeting the “click” requirements is quite difficult, several processes, such as the nucleophilic ring opening reactions, the non-aldol carbonyl chemistry, the additions to carbon–carbon multiple bonds and the cycloaddition reactions, were identified to step up to the mark. For example, among the proposed reaction pool stands the well-known Staudinger ligation. This reaction involves the chemoselective ligation between azide and phosphine, (both functional groups being quite stable in water) and has been largely used in the recent years by the Bertozzi group to selectively functionalize biomolecules or even living cells. Unfortunately, phosphines are susceptible to air oxidation and derivatives optimization aiming at increased reaction yields and improved water solubility still remains a challenging mission. This methodology has been also reported for the site-selective immobilization of proteins onto gold surfaces. Amongst the “click” reactions on biomolecules other methods like the Diels-Alder cycloaddition reaction, that has been used for example in the selective immobilization of proteins, the thiol-ene additions and oxime formation have also flourished in the literature during the last few years. Among these, cycloadditions and more specifically the copper (I) catalyzed variant of the Huisgen 1,3-dipolar cycloaddition (CuAAC, Scheme II-1) has certainly attracted the most of attention, so much that it is now often referred to as the “click chemistry reaction”. Several reports have confirmed the wealth of applications of this practical and sensible chemical approach in the areas of bioconjugation, polymer and materials sciences and drug discovery.

Scheme II-1. General scheme of “click” chemistry cycloaddition reactions.
In fact, it was Sharpless and coworkers which rediscovered and reintroduced as “click”, a reaction that was pioneered in the 60s by Rolf Huisgen. The reaction itself is, a [3+2] dipolar cycloaddition between an azide and an acetylene (Scheme II-2) and is nowadays mostly used in its copper-catalyzed version (also generally referred to as “click chemistry reaction”), as a reliable and selective method for the synthesis of useful new compounds. It was in fact during a pioneering study by Meldal and co-workers demonstrating the applicability of click chemistry in peptide synthesis, that the regiospecific copper(I)-catalyzed variant of the 1,3-dipolar Huisgen cycloaddition was introduced for peptidic terminal alkynes and azides using solid-phase synthesis. The copper catalyzed version has the advantage to afford one single regioisomer whereas the basic thermal version gave an equimolar mixture of 2 regioisomers (Scheme II-2). The alkynyl and azido functional groups involved in this reaction are highly stable and react only through this chemoselective dipolar cyloaddition reaction in water (no side product observed) to produce the corresponding 1,2,3-triazolyl compound which is itself, very stable in aqueous solution and high temperatures. This reaction, which has been initially widely utilized in pure organic synthesis, has found widespread application in the area of bioconjugate chemistry and for these reasons has also been utilized for the purposes of the study presented here.

Scheme II-2. Thermic and copper-catalyzed version of the [3+2] Huisgen dipolar azide acetylene cycloaddition.

The inspiration came from the early works in the bioconjugation field. For instance, Finn et al. reported on the decoration of virus capsids with dyes. The cowpea mosaic virus (CPMV) coating protein, which was utilized as biomolecular scaffold in this study, was decorated with azide or alkyne motifs at either of the exposed reactive lysine or cystein residues. The corresponding products were then engaged in click chemistry reactions with the corresponding complementary fluorescein derivatives. In this study, the viral capsid appeared to be very sensitive to Cu (II) which was used as Cu(I) source and even disassembled. For this
reason, tris(benzyltriazolylmethyl)amine (also referred as to TBTA) was used as a ligand in conjunction with copper, and proved to be successful in inhibiting the disassembling process.

Nevertheless, due to cytotoxicity of the mandatory copper catalyst needed for the catalytic click chemistry version of the 1,3-dipolar Huisgen cycloaddition towards both bacterial and mammalian cells but also towards the catalytic activity of numerous enzymes, catalyst-free variants of the Huisgen cycloaddition have also been developed during the last years. On this direction Bertozzi and coworkers developed a method that relies on the strain promoted [3+2] cycloaddition between a strained cycloalkyne and an azido-derivatized biomolecule. Following this method they managed to selectively modify biomolecules in living systems through a series of cyclooctyne derivatives bearing a biotin motif. This series of linkers was shown to be stable in mild acidic and basic conditions but also towards biological nucleophiles such as thiols for prolonged times. They successfully linked in a selective fashion the biotinylated cyclooctynyl derivatives to a modified glycoprotein GlyCAM-Ig at physiological conditions without any apparent toxicity. Western Blots confirmed the achievement of the reaction and confirmed the orthogonality of the reaction.

In a more recent work, they successfully applied this newly copper-free click chemistry reaction to the imaging of dynamic processes in living cells. As Staudinger ligation reaction is too slow to follow rapid biological processes and as click chemistry reaction is toxic towards living systems, they decided to apply the copper-free version of click chemistry to follow labelling of proteoglycans of cells within a short time scale. A cyclooctyne derivative capped with an Alexa Fluor 488 dye was designed and synthesized. They were able to selectively label glycans at the surface of CHO cells by using Alexa 488 DIFO (difluorinated Cyclooctyne) but more importantly, they were able to follow in vivo trafficking of labelled glycans within the cells into cell compartments (e.g. Golgi, endosome, lysosome) within the one minute time scale.

A novel, copper free tandem [3+2] cycloaddition- retro-Diels-Alder reaction involving oxanorbornadienes which affords stable 1,2,3-triazole-linked compounds was also recently reported by the Nolte group. The reaction was successfully applied to the decoration of proteins or small peptides with fluorescent tags. For example, hen egg white lysozyme (HEWL) was functionalized with a heterobifunctional linker bearing at one extremity an oxanorbornadiene moiety and a carboxylic acid in the other. Following the coupling of the carboxylic moiety to the exposed lysine residues of HEWL, the derivatized
protein was incubated with 7-azido-hydroxycoumarin and gently shaken for 36 hours at 25°C. Nevertheless, since this novel reaction releases the toxic product furan, the scope of this reaction might be limited for bioconjugation and in vivo applications.

As described above, this copper-catalyzed [3+2] Huisgen dipolar cycloaddition is a powerful reaction that can be selectively performed in mild conditions, aqueous solutions, and does not require energy (no need to heat). Moreover, it is tolerant toward a wide range of functional groups, is highly selective and gives generally excellent yields.

For all these reasons, the synthesis of Giant Amphiphiles by copper-catalyzed [3+2] Huisgen dipolar cycloaddition click chemistry reaction was envisioned. As shown on Scheme II-3, this first alternative synthetic method that was developed in the laboratory relies on a straightforward two-step synthesis. In the first step, the specific maleimide-thiol coupling is utilized to selectively functionalize a thiol containing protein by a heterobifunctional linker bearing a terminal alkyne/azide. In the second step, click chemistry mediates the coupling of a hydrophobic polymeric chain appended by the complementary corresponding functionality to the protein-alkyne/azide bioconjugate. Though this reaction lies under the category of the two step indirect specific functionalisation, we reasoned that the orthogonality of the click chemistry coupling would allow for significant improvement of the conjugation reaction yields.

![Scheme II-3. General scheme of the synthetic pathway used to create Giant Amphiphiles from “click chemistry”.](image)

II.2. Results and discussion.

In this study, the 66 kDa carrier protein Bovine Serum Albumin (BSA) which is widely used as a model protein in numerous scientific bioconjugation works was initially
utilized.\textsuperscript{27,28,29,30} Cheaper than most commercial proteins, quite stable and relatively easy to purify, BSA can be used in relatively large quantities for scientific research.

Serum Albumin is the most abundant protein in blood plasma (typical concentration of 50 g/L) and functions as a transport protein for numerous endogenous and exogenous substances. It plays an important role in the regulation of the colloid osmotic pressure of blood by providing about 80\% of the osmotic pressure as is considered to be chiefly responsible for the pH maintenance in blood.\textsuperscript{31} The primary sequence of BSA was presented in the same year as the structure of the Human Serum Albumin (HSA).\textsuperscript{32} It was proposed that BSA is composed of 582 amino acid residues with a sequence that has 17 disulfide bonds resulting in nine loops formed by the bridges. More importantly, BSA is considered to be an ideal model for specific bioconjugation reactions since this globular protein contains only one free cysteine residue, located at position 34 as shown on the Figure II-1.\textsuperscript{33} Surprisingly, in spite of the substantial information concerning BSA, its crystal structure has not yet been resolved and has led to some contradictory results and discussions. Nevertheless, its dimensions have been estimated to be 30 x 80 x 80 Å\textsuperscript{31} and its structure is considered to be homologous with that of Human Serum Albumin (HSA) with which it shares about 80\% primary sequence identity\textsuperscript{34} and very similar biological functions (Figure II-1).

![Figure II-1. A. Structure organization of BSA, location of disulphide bonds. B. Space filling model of serum albumin molecule with basic residues coloured in blue, acidic residues in red, and neutral ones in yellow. C. Three dimensional representation of HSA with the $\alpha$-helices depicted in purple, the $\beta$-turns as pale blue segments and the disulfide bridges and free cysteine (at position 34) depicted as yellow sticks.](image)

Having selected BSA as the model protein to proceed with our studies, as described in the Scheme II-3, an appropriate hydrophilic heterobifunctional linker had to be designed for the efficient and selective coupling of the protein with a hydrophobic polymer via the copper-catalyzed [3+2] Huisgen dipolar cycloaddition.
II.2.1. Synthesis of heterobifunctional linkers.

As already mentioned in the general introduction, the specific conjugation of proteins is often achieved by targeting a cysteine residue. Cysteines that are not involved in disulfide bridges are generally few or even unique in the protein sequences. This allows for their specific targeting in bioconjugation reactions by using the correct, specific chemical reagent and appropriate conjugation conditions.

Scheme II-4. Schematic representation of the design of the heterobifunctional linker aimed to be cystein biospecific, and perform Huisgen [3+2] click chemistry cycloaddition reactions.

For the purposes of this research, we focused on the synthesis of heterobifunctional hydrophilic linkers containing a maleimide moiety on one side and either an azide or an alkyne on the other terminus (Scheme II-4). We decided to utilize the hydrophilic triethylene glycol as the starting backbone material for the synthesis of this linker, as its hydrophilicity was expected to increase solubility of the final heterobifunctional linker and therefore, the specific conjugation reaction yields.

Maleimides, as mentioned in Chapter I, react as Michael acceptors specifically with Cysteine residues at mild conditions and neutral pHs (6.8–7.5) with great specificity (maleimides will only react with amines at pH >8), and more importantly with high reactivity.\textsuperscript{35,36} For these reasons, we selected to utilize maleimide for the bioconjugation of the heterobifunctional linker with thiol containing proteins. Nevertheless it can be envisioned that this moiety will be replaced in the future with other, protein specific coupling groups. Since the maleimide moiety is not stable under several reaction conditions, we designed our synthesis to proceed through an initial mono-functionalization of the triethylene glycol.
backbone with either an azide or an alkyne moiety and introduced the maleimide functionality on a later step.

Using this approach, the preliminary experiments on the preparation of maleimido-heterobifunctional linkers bearing a terminal azide were unsuccessful as the azide and maleimide moieties could easily undergo inter- and/or intra- [3+2] dipolar cycloadditions under the reaction conditions required for the synthesis of this hydrophilic heterobifunctional linker.\textsuperscript{37,38,39} Several attempts to optimize the reaction conditions and avoid cycloaddition failed and an insoluble gummy polymer was recovered in all cases while \textsuperscript{1}H NMR revealed the disappearance of the maleimide signal (6.9 ppm) together with the broadening of the peaks, thus confirming this cycloaddition reaction. It was therefore decided to focus on the synthesis of alkyne appended heterobifunctional maleimide linkers.

The introduction of a terminal alkyne onto triethylene glycol was achieved by a simple nucleophilic substitution with propargyl bromide as previously reported in the literature.\textsuperscript{40} Triethylene glycol was initially mono-deprotonated with sodium hydride in THF in dry conditions. The addition of propargyl bromide gave the resulting product in quantitative yields (Scheme II-5).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{SchemeII-5.png}
\caption{Scheme II-5. Reagents and conditions used for the synthesis of the monoalkyne triethylene glycol derivative 1.}
\end{figure}

The PEG monoalkyne compound 1 was then reacted with maleimide via a modified Mitsunobu reaction in order to synthesize the corresponding maleimido heterobifunctional linker 2.\textsuperscript{41} Briefly, the coupling proceeded through an initial reaction of triphenylphosphine with DEAD at -78°C under nitrogen atmosphere which was followed by the addition of initially compound 1 and then successively neopentyl alcohol and maleimide as mentioned in Scheme II-6.
Chapter II – Formation of Giant Amphiphiles by Click Chemistry Reaction

Scheme II-6. Reagents and conditions used for the synthesis of the heterobifunctional maleimido-alkyne linker 2.

The resulting solution was slowly let to warm up to ambient temperature (Scheme II-6) and was stirred overnight. After chromatographic purification, the hydrophilic heterobifunctional linker 2 was recovered in 70% yield.

It should be mentioned that analogous compounds were successfully generated utilizing a tetaethylene glycol and a pentaethylene glycol as hydrophilic backbones, but since similar results were obtained in all cases when tested in bioconjugation, they are not individually mentioned in this thesis.

II.2.2. Coupling of the heterobifunctional linker 2 to the native BSA.

The coupling between native BSA and the heterobifunctional maleimido-alkyne linker 2 was performed in aqueous conditions using 20 mM phosphate buffer pH 7.4 (PB). A 100 molar excess of the heterobifunctional linker 2 in DMSO was slowly added to the BSA solution and the reaction mixture was gently shaking for 24 hours at 7°C (Scheme II-7). The reaction mixture was then purified by extensive dialysis against 20 mM PB pH 7.4 using 10 kDa MWCO regenerated cellulose dialysis bags.

Scheme II-7. General reaction scheme and conditions used for the coupling of the heterobifunctional linker 2 to BSA to afford the alkyne-1-appended bioconjugate I.
Figure II-2. Characterization of the alkyne-1-appended BSA bioconjugate I. Left: Electrophoresis under denaturating conditions. Lane 1: native BSA, lane 2: purified bioconjugation reaction mixture. Right: SEC chromatographic traces at 254 nm of the native BSA (dashed grey trace) and BSA linker I (solid black trace).

SEC chromatographic analysis showed no significant difference in the retention times of native BSA and the BSA-1-alkyne I conjugate (Figure II-2). Electrophoresis under denaturating conditions on the other hand revealed a new band after Coomassie Brilliant Blue staining. Surprisingly, this new band exhibited a faster migration behaviour (i.e. was located in the lower apparent molecular weight region) when compared to native BSA. We attribute this behaviour to either the difference in the total net charge of BSA, or to a conformational change induced by the bioconjugation of the linker to the protein.

The selective conjugation of the hetero bifunctional linker 2 onto the protein to afford the mono-adduct I was finally confirmed by MALDI-TOF. A mass difference of ~ 300 uma in the molecular peaks corresponding to the native protein and I was observed, and judged to be consistent with the specific coupling of one linker molecule per protein (Figure II-3).
II.2.3. Preparation of polystyrene azides.

Three different, commercially available bromide-capped polystyrenes with molecular weights varying from 2 to 8 kDa were easily transformed to the corresponding azides by nucleophilic substitution of the bromide group with sodium azide. Briefly the reaction proceeded by stirring overnight the corresponding polystyrene (2, 4 and 8 kDa) with a 4 molar excess of sodium azide solution in DMSO at 60°C (Scheme II-8).\textsuperscript{42} After removing the solvent under reduced pressure, the azido-polystyrenes 3a, 3b, 3c which were obtained in excellent yields were characterized by GPC, MALDI, FT-IR and \textsuperscript{1}H NMR.

\begin{center}
\begin{tikzpicture}
\node at (-2,0) {\textbf{Scheme II-8.} Reagents and conditions used for the synthesis of azido-polystyrenes 3a, 3b, 3c.}
\draw[<->, thick] (-1,0.5) -- (0.5,0.5) node[midway, above] {quantitative};
\draw[<->, thick] (-1,-0.5) -- (0.5,-0.5) node[midway, above] {Na\textsubscript{3}N, DMSO, 60°C, overnight};
\draw[<->, thick] (-1,-1.5) -- (0.5,-1.5) node[midway, above] {Br};
\draw[<->, thick] (-1,-2.5) -- (0.5,-2.5) node[midway, above] {R\_n};
\draw[<->, thick] (-1,-3.5) -- (0.5,-3.5) node[midway, above] {N\textsubscript{3}\_n};
\end{tikzpicture}
\end{center}

The GPC chromatographic behaviour of the reacting and produced polystyrenes was investigated before and after the azidation reaction and is reported in Figure II-4. Unfortunately, the mass difference between azides and bromides was too small to introduce any characteristic difference in the retention times under the chromatographic conditions utilized (solvent: toluene, room temperature, 2 PL-Gel mixed columns (ThermoLabs) in
series). Nevertheless, the GPC analysis confirmed the retention of the polydispersity after the reaction as observed by the broadness of the peaks.

![Graphs showing GPC chromatograms](image)

**Figure II-4.** GPC chromatographic analysis of the different commercially available polystyrene bromides before (dashed grey traces) and after the azidation reaction (solid black traces).

IR analysis of the azidated polymers showed the characteristic stretching band of the C-N$_3$ bond around 2100 cm$^{-1}$. The $\omega$-terminus substitution was confirmed by $^1$H NMR spectroscopy through the characteristic downfield shifting of the $\alpha$ to the azide group protons as compared to those of the $\alpha$ to the bromide group of the starting materials. Finally, the MALDI-TOF spectra showed, as expected, a shift of the polymer distribution by 38 uma (corresponding to the abstraction of bromide (−80) and substitution with an azide (+42) inducing a difference of mass of 38 uma), further confirming the azidation of the polystyrene bromides.
II.2.4. BSA-PS Giant Amphiphiles through the direct, click chemistry mediated, coupling of polystyrene azides.

As already mentioned earlier, the main difficulty of the efficient synthesis of Giant Amphiphiles resides in the solubility incompatibility between the two components, i.e. the water soluble protein and the hydrophobic polymer. In the past, this problem was addressed by the incorporation in the reaction scheme of a miscible with water, selective for the hydrophobic polymer, organic solvent (such as THF). Though this solution has been successful in specific cases of proteins (such as CAL B which has a known increased tolerance for organic solvents), the percentage of this organic solvent can in the general case be destructive or harmful for the protein conformation/activity. Taking this into account, we utilized THF to solubilise the polymers during the course of this study and kept the THF / water content minimal (less than 10%). Furthermore, in order to achieve the [3+2] Huisgen “click” cycloaddition, a Cu mediated variant of click chemistry was employed. We selected to utilise as catalyst the Cu (I) generating Cu(II)SO₄ and sodium ascorbate system, as it had already been proven that it is efficient and compatible with bioconjugation reactions.

More specifically, to achieve the polystyrene azide clicking on to the BSA-1-alkyne I, the polystyrenes were initially dissolved in THF and slowly added in a 10 molar excess to a 20 mM phosphate buffer solution of BSA-alkyne I containing 2 molar excess of CuSO₄ and 4 of Na ascorbate. The final total volumes of the reactions were adjusted with 20 mM phosphate buffer, pH 7.4 in order to avoid having over 10 % organic solvent content in the aqueous solution reaction mixtures.
Scheme II-9. General synthetic scheme for the click chemistry reaction between BSA-alkyne I and the series of polystyrene azides 3a, 3b, 3c leading to the formation of the BSA-PS clicked products IIa, IIb, IIc respectively.

Unfortunately, problems of solubility drastically hampered the reaction in our initial efforts since already, the 4 kDa polystyrene azides were found to be insoluble in the reaction conditions.

The reaction was finally achieved by modifying the procedure in such a way that the protein was added last to the reaction mixture (in a concentrated solution) allowing an initial extensive sonication of the polystyrene azides while added into the buffer to achieve their optimal dispersion. As it will be shown in the next paragraphs, bioconjugation of polymers with a molecular weight upto 8 kDa was achieved with this modified protocol, nevertheless bioconjugation of higher molecular weight hydrophobic polymers than the ones utilized to couple BSA-alkyne conjugate I still remains a challenging task.

II.2.5. Characterization of the BSA-polystyrene Giant Amphiphiles.

Purification of the biohybrid amphiphiles IIa, IIb, IIc was undertaken by simple dialysis of the reaction mixture using 10 kDa molecular weight cut-off (MWCO) dialysis membranes against 10 % DMSO, 20 mM phosphate buffer pH 7.4 initially and then against 20 mM phosphate buffer pH 7.4. The resulting turbid solutions containing IIa, IIb, IIc were analyzed by SEC chromatography (solvent: 30% acetonitrile 20 mM phosphate buffer, flow
rate: 0.5 mL.min⁻¹, room temperature, column: BioBasic SEC 300 (Thermo)). The formation
of new peaks corresponding to the compounds IIa, IIb, IIc, possessing a larger hydrodynamic
volume than that of native BSA (shorter retention times, higher molecular weights), was
immediately observed (Figure II-5). No presence of the BSA-alkyne linker I peak was
detected in any of the measurements, demonstrating its efficient removal by the dialysis step.
As pointed by the SEC measurements, the reaction yields were rather high verifying thus the
efficiency of the “click” chemistry reaction for the synthesis of Giant Amphiphiles. As
expected, the BSA dimer remained in the reaction mixture even after dialysis due to its high
molecular. The difference observed in the retention times of the different adducts IIa, IIb and
IIc is not significant, probably due to the lack of sensitivity of the column under the specific
conditions utilised to analyze the samples. Further attempts to chromatographically
discriminate between these products in terms of molecular weight by changing the solvent
system (composition and pH), the flow rate and/or the column were unsuccessful.

A blank experiment was performed by incubating the BSA-1-alkyne linker I and a
mixture of the azide appended polystyrenes 3a, 3b and 3c in 20 mM phosphate buffer without
CuSO₄ and Na ascorbate. Upon gentle stirring for ~ 48 hours, the mixture was subjected to
the same dialysis protocol that was used in the case of the click chemistry reactions. The SEC
measurements upon dialysis revealed the presence of the unreacted BSA-1-alkyne I and a
successful removal of the non interacting polymers. This verified the click coupling and
excluded the possibility of non-specific interactions between the polymer and the protein.

**Figure II-5.** SEC chromatographic characterization of the blank experiment (I + 3a, 3b and
3c without CuSO₄/sodium ascorbate, black dotted trace) and BSA-polystyrene amphiphiles
IIa, IIb and IIc (respectively solid black, solid grey and dashed grey traces) after dialysis.
Electrophoresis under denaturing conditions (SDS-PAGE) confirmed the chromatographic results. As expected in the case of amphiphilic block copolymers, assembly hampers the migration in the gel and thus the BSA-polystyrene Giant Amphiphiles $\text{IIa, Iib, IIc}$ obtained did not migrate on the gels due to their amphiphilic character (Figure II-6). The same results were observed when electrophoresis was performed under native conditions. The presence of small quantities of unreacted BSA is also apparent in the gel. Unfortunately the absolute quantification of the reaction yields was not possible as SEC chromatography did not separate the unreacted protein peak from that attributed to the BSA dimer.

![Figure II-6. SDS-PolyAcrylamide Gel Electrophoresis (PAGE) under denaturating conditions stained with Coomassie Brilliant Blue; lane 1: native BSA, lane 2: BSA-PS 2 kDa $\text{Ia}$, lane 3: BSA-PS 4 kDa $\text{Ib}$, lane 4: BSA-PS 8 kDa $\text{Ic}$.](image)

The *click* chemistry derived BSA-polystyrene polymer-protein conjugates were also analyzed by MALDI-TOF (Matrix Assisted Laser Desorption Ionisation Time Of Flight mass spectrometry). Unfortunately, the results obtained were rather poor and our efforts to adapt the conditions using different mixtures of matrices were unfruitful. This is a common problem in the area of amphiphilic block copolymers. Yet, measurement of the smaller BSA-PS amphiphilic bioconjugate *i.e.* BSA-PS 2 kDa $\text{Ia}$ confirmed the formation of the biohybrid, through a new peak with a molecular weight at 68672 amu (Figure II-7).
II.2.6. Aggregation studies.

The self-assembly of the BSA-polystyrene amphiphilic biomacromolecules derived via the direct click chemistry coupling of various polystyrene-azides onto the alkyne-1-appended BSA I, was studied using Transmission Electron Microscopy (TEM). The samples were dialyzed against nanopure water prior to the measurement and were diluted ~100 times with nanopure water. A minute quantity (~10 µl) of each sample was placed on a formvar coated copper grid and after a waiting period of ~15 minutes, the solvent was drained using a filter paper. No staining or shadowing was necessary to visualize the samples as the increased electronic density of polystyrene provided enough contrast. No architectures were found in samples prepared using native BSA. TEM revealed the formation of well-defined spherical superstructures with diameters varying from 30 to 500 nm size as can be shown on Figure II-8.

Interestingly, the size of the hydrophobic polymer tail (2 kDa to 8 kDa) did not seem to have any effect on the aggregation behaviour of the Giant Amphiphiles. This observation is attributed to the fact that the difference in the hydrophobic chain lengths used in this study was not big enough, as compared to the protein size, to have any dramatic impact on the self assembly of the resulting BSA-polystyrene superstructures. Unfortunately, due to solubility problems, higher molecular weight polystyrene azides could not be utilized. It is therefore...
believed (as different architectures have already been shown for Giant Amphiphiles)\textsuperscript{43} that the appropriate approach to be followed if one wants to study the effect of the shape on the resulting aggregated architectures, is to utilize a smaller, in terms of dimensions and molecular weight, protein component.

![Figure II-8. TEM micrographs of superstructures obtained by aggregation of A. BSA-PS Ia, B. BSA-PS Ib and C. BSA-PS Ic.](image)

Finally, it should be noted that toward the end of our studies, a publication from the Nolte group\textsuperscript{27,44} also verified the efficiency of the copper catalyzed, Huisgen cycloaddition, click chemistry approach through the direct coupling of hydrophobic polymers to peptides and proteins using slightly different conditions, or the cofactor reconstitution approach.

### II.2.7. Further studies of the direct click chemistry approach utilizing other proteins.

The applicability of this, direct, click biospecific conjugation of polymers to proteins other than BSA was tested in a series of enzymes and proteins displaying an accessible
cysteine residue. We were interested to prove the generic nature of this bioconjugation method as well as to further study the role of proteins in the aggregation behaviour of the bioconjugates. For this reason the free thiol containing Cytochrome C from equine heart, (Cyt C) and Haemoglobin from bovine blood, (Hb) were utilized. 

Haemoglobin is a large (64 Kda), multiple, subunit globular protein necessary for gas exchange in living organisms. It has long been the focus of studies in molecular biology and was one of the first proteins for which structure and sequence were determined.\(^{45}\) Hemoglobin comprises two pairs of dissimilar subunits, \(\alpha\) and \(\beta\). Each \(\alpha\) chain contains one cysteine residue (\(\alpha\) 104) and each \(\beta\) chain two cysteine residues (\(\beta\) 93 and \(\beta\) 112). However, in the native state of hemoglobin, of these six sulphhydryl groups, only two (\(\beta\) 93) react with reagents specific for sulphhydryl groups.\(^{46,47,48,49}\) 

Cytochrome C, or Cyt c is a small (11 kDa) heme protein found loosely associated with the inner membrane of the mitochondrion. It is an essential component of the electron transfer chain, where it carries one electron. Cyt C is also a heme bearing protein that contains 2 free cysteine residues (one of them is coordinated with the heme cofactor and therefore not accessible) and is also implicated in the cell apoptosis.\(^{50,51}\) 

The proteins were incubated on a first step with a 100 molar excess of the maleimido heterobifunctional linker 2. After gentle shaking for 2 days at 7°C, the reaction mixtures were extensively dialyzed (using regenerated cellulose membranes with MWCO values of 10 and 50 kDa for Cyt C and Hb respectively) and the biohybrids analyzed by SEC and electrophoresis. Both SEC and electrophoresis under native conditions did not reveal any dramatic changes that would indicate the formation of the alkyne functionalized proteins. Nevertheless, since the same observation was also true in the successful case of BSA-1-alkyne I, we proceeded with the second step during which the alkyne bearing proteins III (1-alkyne functionalized Cytochrome C) and IV (1-alkyne functionalized Haemoglobin) were coupled to polystyrene azides 3a, 3b, 3c through a copper catalyzed [3+2] Huisgen cycloaddition using the CuSO\(_4\)/Na ascorbate Copper (I) generating system and the optimized for BSA reaction conditions. Following an incubation time of \(\sim\) 2 days, the reaction mixtures were dialyzed and the resulting amphiphilic macromolecules Va, Vb, Vc produced from the clicking of polystyrene azides with molecular weight 2kDa, 4 kDa and 8 kDa respectively with Cytochrome C, as well as the analogous haemoglobin bioconjugates VIa, VIb, VIc were
characterized by SEC chromatography. These measurements (solvent: 30% acetonitrile 20 mM phosphate buffer, flow rate: 0.5 mL.min\(^{-1}\), room temperature, column: BioBasic SEC 300 (Thermo)) revealed the formation of a series of new products that are shown on Figure II-9, exhibiting a behaviour similar to that of the BSA-polystyrene Giant Amphiphiles. It should be noted that in the case of Hemoglobin, the traces monitored at 405 nm (heme) were in agreement with the ones monitored at 254 nm, proving the presence of heme in the product solution.

![Figure II-9. SEC chromatographic analysis of native proteins and amphiphilic biomacromolecules (left). Left: native Cytochrome C and amphiphilic CytoC-polystyrene bioconjugates Va, Vb, Vc at 254 nm. Right: native Hb and amphiphilic Hb-polystyrene bioconjugates VIa, VIb, VIc at 406 nm.](image-url)

As mentioned earlier, obtaining MALDI-TOF spectra of such compounds is not an easy task. Though using several combinations of matrices, we were only able to obtain MALDI spectra for the cases shown in Figure II-10. Nevertheless, in the case of the conjugates Va and VIa, MALDI verified the SEC and electrophoresis results (showing no migration of the products under both native and denaturating conditions). It is good also to note that, as haemoglobin was very sensitive to MALDI conditions and a variety of (polymeric) peaks was obtained for the native enzyme itself, we utilized an alternative pathway to stabilize it which involved mixing with native BSA. Upon this, better results were obtained both in the case of the native Hb and in the case of the bioconjugate VIa as presented in Figure II-10.
The study of aggregation study was performed using TEM microscopy. Again, 10 µl of ~ 100 times diluted samples of the native enzymes and the purified reaction mixtures, were placed on a formvar coated copper grid and after a waiting period of ~15 minutes, the solvent was drained using a filter paper. Both native Cyt C and Hb did not reveal any aggregation behaviour when examined with TEM. Unfortunately the same was observed for the case of the Cytochrome C bioconjugates where a non defined, gel like pattern was only observed on the grids. Surprisingly TEM revealed that in the case of the amphiphilic Hb-polystyrene conjugates a different morphology than the one obserbed for BSA was adopted. It was observed that all Hb-PS Giant Amphiphiles formed well defined cylindrical (“worm-like”) superstructures with a quite monodisperse length of roughly 1000 nm and diameter between 300 and 400 nm (Figure II-11). Nevertheless, the images also contained unstructured material as well as different smaller (presumably micellar) architectures, and therefore further SEM experiments are in progress with the aim to clarify the aggregation patterns.
In conclusion, both Haemoglobin and Cytocrome C were successfully functionalized following the direct click chemistry coupling approach. In experiments performed in the laboratory that are not included in this thesis, the formation of Giant Amphiphiles through this approach was also studied and proved to be successful for two more proteins, the Tobacco Mosaic Virus Coating Protein (TMV CP) and papain. It is therefore considered to be a method generic for thiol containing proteins. It should also be finally noted that though the bioconjugation experiments proved to be successful, in both heme proteins a change of colour during the course of the reactions suggested the destruction of active form (probably due to the selected copper (I) generating system) and therefore a change of catalyst is judged to be necessary to ensure the stability of the proteins in further studies.

II.2.8. Study of Giant Amphiphiles in organic solvents.

The essence of our studies lies in the understanding of the factors that dictate the formation of biohybrid superstructures and in the subsequent use of this knowledge to program functional two- and three-dimensional nanometer-sized superstructures. As part of this study, we reasoned it should be possible to “reverse” the aggregation morphologies with the use of organic solvents. In a medium which would be selective for the polymeric tail, we expected that if the giant surfactants would assemble at all, this should lead to the formation of inverted structures (Figure II-12), meaning that the polymer tails would be by nature exposed and oriented to the solvent and that the hydrophilic protein heads would be clustered in the core of the superstructures.
We investigated this assumption using the BSA-polystyrene Giant Amphiphiles which were synthesized through the direct click-chemistry approach. All samples were extensively dialyzed against nanopure water to remove the excess of unreacted reagents and phosphate buffer. After ~48 hours, we freeze-dried the samples to remove water and dissolved in dry dichloromethane which was judged to be the most appropriate selective for polystyrene-solvent (blank experiments revealed no solubility of BSA). We immediately noted the formation of opaque solutions while the aggregation was verified using TEM as shown in Figure II-13. In this case, we utilized carbon coated formvar copper grids, applied a small quantity (10 µl) of the dichloromethane solution and allowed the solvent to evaporate.

From the TEM micrographs, we observed the formation of well defined self-assembled superstructures (Figure II-13) with diameters varying from 30 to 500 nm. From their appearance and dimensions, we reasoned that they are most probably micellar or vesicular in nature with the BSA being at the core of the membrane and the polymer exposed.

**Figure II-12.** Schematic representation of micellar and inverted micellar structures.

**Figure II-13.** TEM micrographs of BSA-PS 4 kDa IIb in CH$_2$Cl$_2$. The two pictures on the bottom are negative stained (Uranyl Acetate).
It should also be noted, that when experiments were performed without dialysis (i.e. without the removal of the excess unreacted polystyrene), the structures showed a very interesting dynamic behaviour with time probably due to the dynamics of the excess of polystyrene in dichloromethane (Figure II-14).

![Figure II-14. A. TEM micrographs of BSA-PS 4kDa Ib in CH$_2$Cl$_2$. Micrograph on the left immediately after addition of the CH$_2$Cl$_2$, on the right 48h later. B. TEM micrographs of BSA-PEG$_{5000}$ in dichloromethane.](image)

Blank experiments with BSA did not reveal the formation of any aggregates, while when we used as a blank BSA-polyethylene glycol 5 kDa (PEG$_{5000}$), we were able to observe only the formation of structures that we attribute to material that collapses on the grid after evaporation of dichloromethane ("coffee stain artefacts") (Figure II-14). This is the first time that aggregation of protein-polymer amphiphiles is ever observed in organic solvents.

II.2.9. Using Giant Amphiphiles to bring enzymes into organic solvents.

In a further attempt to generate meaningful superstructures, we though it would be of interest to exploit the aggregation of Giant Amphiphiles in organic solvent by using the resulting aggregates as nanoreactors through the encapsulation of an enzyme within the cavity of these inverted spherical structures. We reasoned that within such inversed superstructures
(Figure II-15), the BSA head group of the Giant Amphiphiles would most probably be in the core of the superstructures, creating a protein-friendly environment able to host other proteins and at the same time protect them from exposure to the organic solvent.

**Figure II-15.** Formation of nanoreactors by inversion of the superstructures in organic solvent.

Toward this direction, we utilized as guest protein $\beta$-galactosidase, a well known enzyme that hydrolyzes ester derivatives of $\beta$-galactose. The selection of the enzyme was only based in the presence of a commercially available fluorogenic activity assay that we planed to utilize for the monitoring of the activity.

The protocol developed to encapsulate the $\beta$-galactosidase within the Giant Amphiphile superstructures, involved the freeze drying of a sample of BSA-PS 4 kDa IIb, the addition of a minimal quantity of a concentrated aqueous solution of $\beta$-galactosidase and the repeating of a series of freeze-drying cycles followed by the intermediate addition of small quantities of dichloromethane. Upon repeating these cycles several times, the solid residue was dispersed in the initial quantity of dichloromethane.

The activity of the enzyme was verified using CFM microscopy. In order to be able to fluorescently visualize the superstructures themselves, the amphiphilic polymer-protein bioconjugates were labelled using an NHS-activated Alexa-488 dye through specific coupling with the Lysine residues of BSA, prior to being inversed into the organic solvent.

As mentioned above, for the fluorogenic assay the appropriate pro-fluorescent substrate was commercially available. After a $\beta$-galactosidase catalyzed hydrolysis two products are released in solution: $\beta$-galactose and the fluorescent product, 7-hydroxy-4-methylcoumarin, known to display a strong fluorescence at 405 nm (Figure II-16).
In order to measure the activity of the $\beta$-galactosidase encapsulating nanoreactors, a dichloromethane solution of the nanoreactors was placed between two hydrophobic glass slides and was sealed prior to the measurement. Upon visualizing the fluorescent superstructures (Figure II-17, left), the substrate was injected and the slide was independently scanned at 488 and 405 nm. It should be noted that the Giant Amphiphile superstructures were not resolved with CFM, as their dimensions were rather small for the resolution of the instrument.

**Figure II-16.** Protocol used to visualize catalysis of $\beta$-galactosidase by Confocal Fluorescent Microscopy.
Chapter II – Formation of Giant Amphiphiles by Click Chemistry Reaction

Figure II-17. Confocal Fluorescent Microscope pictures of polystyrene-BSA superstructures after entrapment of β-galactosidase before (A. 488 nm, B. optical image, C. 405 nm) and after addition of fluorogenic substrate (D. 488 nm, E. optical image, F. 405 nm).

Following a lag period, the intense blue fluorescence at 405 nm was detected (Figure II-17) and attributed to the formation of the fluorescent product. No fluorescence was detected before the addition of the fluorogenic substrate to the aggregates solution or when a blank experiment was performed in the absence of β-galactosidase within the vesicles.

II.3. Summary and Outlook.

In conclusion, in the studies presented here, we were able to successfully synthesize small libraries of *Giant Amphiphiles* that self-assemble adopting different morphologies depending on the protein used.

More specifically, the copper catalyzed, Huisgen [3+2] cycloaddition was utilized and a protocol was developed to allow its use for such bioconjugations. This polymer bioconjugation approach was comparatively utilized in different proteins, showing therefore its generic nature for free cysteine containing proteins. Furthermore, the characterization of the resulting *Giant Amphiphiles* was performed in unprecedented details.

During the characterization studies, the *Giant Amphiphiles* were shown to form well-defined spherical superstructures in aqueous medium in the case of Bovine Serum Albumin
whereas the Haemoglobin *Giant Amphiphiles* formed cylindrical types of like structures. Tuning of the length of the hydrophobic polymeric tail was shown not to change the aggregation behaviour in the case where the same protein was utilized. We attribute this to the relatively small molecular weight variation that can be achieved using polystyrene, as compared to the molecular weight of the proteins.

It should be stressed that during this study the first example of the self-assembling properties of *Giant Amphiphiles* in organic solvents is presented. This is a stimulating breakthrough in the polymer-protein biohybrid research area, one which we intend to further pursue and understand since it provides us with enormous potential for the creation of innovative nanoassemblies. To demonstrate this, a guest enzyme was successfully introduced in the superstructures and their permeability and capacity to act as nanoreactors was demonstrated through a CFM observation of its catalytic action.
References

Chapter III
Formation of Giant Amphiphiles by Post-Functionalization of Hydrophilic Triblock Protein-Polymer Conjugates

III.1. Concept / Background.

Although several methods have already been developed for the synthesis of Giant Amphiphiles, practical limitations can hamper their efficient synthesis in high yields. The main limitation may be attributed to the incompatibility (mainly in terms of solubility) of the two major components (i.e. the hydrophobic polymer and the hydrophilic protein) while several constraints are also being posed to ensure that the protein structures will remain intact. The conditions that were used in the past, took advantage of the known tolerance of specific proteins in organic solvents and the small window of solvent compatibility between the hydrophobic polymer and the protein to achieve solubilization of both components during the bioconjugation step.\textsuperscript{1,2,3} These conditions are not judged to be generic as they are quite harsh for the generality of proteins and can easily lead to denaturation. Furthermore, the concomitant to the formation of Giant Amphiphiles aggregation expressing their strong amphiphilic character, possess also an additional limitation, that of the purification as any attempt to isolate the Giant Amphiphiles from unreacted polymers trapped within the superstructures would certainly involve dissolution steps that could interfere with protein integrity.

Consequently, due to the purification and solubility problems inherent to the synthesis of Giant Amphiphiles that were reported in the precedent literature, it was decided to design a new strategy aimed to circumvent these problems in order to access quantitative amounts of products enabling us to further study possible applications of these biomacromolecules. In this work, we therefore report on a novel, generic approach for the synthesis of triblock Giant Amphiphiles in which hydrophobicity is introduced by
Chapter III – Formation of Giant Amphiphiles by Post-Functionalization of Hydrophilic Protein-Polymer Conjugates

post-functionalization of an appropriately designed, pre-functionalized protein-polymer multifunctional biohybrid (Scheme III-1).

Scheme III-1. General concept of the post-functionalization approach.

In the design of this approach, since the coupling between the hydrophilic protein and the hydrophobic polymer is the major drawback in the synthesis of Giant Amphiphiles, we envisioned a strategy involving the initial coupling of a multifunctional hydrophilic polymer to a protein followed by the transformation of the hydrophilicity of the polymer. This technique should, in principle, give better reaction yields due to the hydrophilic nature of both the synthetic and natural polymeric blocks involved in the bioconjugation. The polymer had to be designed in such a way that in a second step, its hydrophilicity could be turned to the resulting hydrophobic block needed for the introduction of amphiphilicity in the bioconjugate. To synthesize such a multifunctional polymer several requirements needed to be taken into consideration. The final polymer should combine a bioconjugation site, an overall hydrophilic character and multiple grafted, tunable functional units.

The latter, should be able to introduce, by a simple reaction, hydrophobicity in a specific, efficient and reproducible manner and should be therefore carefully chosen. Indeed, to avoid the destabilization of the protein attached to the polymer, the reaction leading to the formation of amphiphilic structures had to be a high yielding, orthogonal reaction that proceeds under mild conditions in aqueous solutions. To this end, we decided to introduce within the hydrophilic polymer backbone a gradient of pending alkyne-1 functionalities that could be easily transformed and therefore introduce hydrophobicity, in a later step under the mild conditions needed for the well-known copper catalyzed Huisgen azide-alkyne dipolar cycloaddition.
To synthesize the multifunctional hydrophilic polymer with a high level of control over molecular weight and polydispersity (PDI), it was decided to follow Atom Transfer Radical Polymerization.

### III.1.1. ATRP polymerization.

Atom Transfer Radical Polymerization (ATRP), also called transition-metal-mediated living radical polymerization (TMM-LRP), has its basis in the atom transfer chemistry used in organic synthesis, e.g. intramolecular cyclization,\(^4\) and was discovered independently by the groups of Sawamoto\(^5\) and Matyjaszewski\(^6\) in the 90s. ATRP is now well established and universally used as an efficient method for the polymerization of vinyl monomers\(^7,8,9,10,11,12,13,14\) since it has proven to be versatile and exhibit good tolerance toward a wide range of functional groups.\(^7,8,9,10,11,12,13,14\) Moreover, it allows excellent control over the polymer architecture and it is quite simple to set up in an organic chemistry laboratory. The only restrictive requirement for ATRP is the absence of oxygen atmosphere, a problem that can be easily solved by repeating freeze-pump-thaw cycles prior to the polymerization.

The ATRP polymerization process of any vinyl monomer involves the combination of an alkyl halide acting as initiator and a catalytic low valent metal complex, e.g. Cu\(^{1}\)Br–bipyridine,\(^7,8,9\) Ru\(^{II}\)Cl\(_2\) (PPh\(_3\)),\(^10\) Ni\(^{II}\)[C\(_6\)H\(_3\)(CH\(_2\)NMe\(_2\))\(_2\)-2,6]Br,\(^13\) which is capable of being oxidized to the \(n + 1\) state by addition of a halogen atom. Copper has by far proven to be the transition metal of choice, as determined by the successful investigation of a spectrum of copper and other metal complexes as catalysts for the ATRP of a broad range of monomers in diverse media by many research groups.\(^6\) However, iron\(^15,16,17,18,19\) may eventually prove to be the transition metal of choice for environmental reasons unless industrially viable procedures for internal reuse of the copper complexes are adopted. It should also be noted that Ruthenium\(^10,20,21,22\) and Osmium\(^23\) have certain advantages as a consequence of their high halidophilicity that may eventually also make them a good choice for use in protic media.

The postulated mechanism proposed by both Matyjaszewski\(^2,7\) and Haddleton\(^24\) is composed, as usual in polymerization processes, from 3 different steps (Scheme III-2):
✓ An initiation step where the abstraction of the halogen occurs on the initiator. During this step, the halide-carbon bond (generally a bromide but can also be a chloride) of the initiator is cleaved in a homolytic fashion, generating a carbon-based radical species and a new metal halide with the metal in the $n+1$ oxidation state, e.g. Cu (II).

✓ A second step, called propagation step, where the polymer grows by the sequential addition of monomeric units to the carbon-based radical species formed during initiation. This step involves a free radical attack of the polymer radical on a monomer, a step commonly observed in all free radical polymerization mechanisms. During the propagation, termination is prevented by the reverse abstraction of a halogen atom from the metal halide to give a new polymeric alkyl halide, also referred to as “dormant” species. The equilibrium between the “dormant” and “active” species (free radicals in the polymerization feed) is therefore shifted towards the former as shown in Scheme III-2, reducing thus the concentration of radical species growing chains in the polymerization feed and avoiding as a consequence the early termination process.

✓ A termination step where the final polymer is terminated by radical hetero coupling with bromine radical.

\[
\begin{align*}
\text{Cu(I)X} / L_n & \quad \text{dormant species} \\
\text{P-X} & \quad \text{active species} \\
\text{Cu(II)X} \quad L_{n-1} & \quad \text{P-P}
\end{align*}
\]

\[k_{\text{act}} \gg k_{\text{deact}}\]

\[X = \text{halide (generally a bromide)}\]

\[k_{\text{act}} \gg k_{\text{deact}}\]

\[k_{\text{deact}} \gg k_{\text{act}}\]

\[\text{Scheme III-2. General Scheme describing the Atom Transfer Radical Polymerization proposed mechanism.}\]

ATRP polymerization has been extensively studied using numerous pyridine, aliphatic tertiary polyamine, polyimine containing compounds, terpyridines and phenantrolines as ligands depending on the nature of the monomers intending to polymerize and the conditions used for the polymerization (e.g. polymerization solvent). Some of the above mentioned ligands have been extensively studied especially in Matyjaszewski\textsuperscript{25,26,27} group. As a result of these studies, bipyridines\textsuperscript{8,28} and pentamethyl diethylenetriamines (PMDETA\textsuperscript{29,30}) in
conjunction with low-valent metal ions are usually used nowadays in numerous research
groups as ligands for ATRP polymerization.

![Figure III-1](image)

**Figure III-1.** Most commonly used ligands in the ATRP polymerization process.

Furthermore, a series of $N$-$n$-alkyl-2-pyridylmethanimine derivatives that were
developed by Haddleton and collaborators, are also nowadays used with a wide range of
monomers going from hydrophilic to hydrophobic methacrylates, styrene, etc.\textsuperscript{31,32,33,34} In
recent literature, it has for example been demonstrated that the pyridine-2-carbaldehyde
imines used as ligand in association with copper(I) bromide are very effective for ATRP of
methyl methacrylate (MMA) with a variety of different initiators.\textsuperscript{14} These imine-based
ligands, whose general structure is displayed on Figure III-2, offer the advantage over other
ligands that they can be synthesized through a simple one-step procedure (condensation
between amine and pyridyl carbaldehyde) which can utilize virtually any primary amine for
the tuning of the ligand solubility while, bipyridine derivatives require rather tedious synthetic
procedures to modify the lateral alkyl chains.\textsuperscript{7}

![Figure III-2](image)

**Figure III-2.** Structures of the $N$-alkyl-pyridyl imine based ligands developed by Haddleton
and coworkers.

Taking therefore into account the above mentioned advantages and versatility of
ATRP polymerization, this method was selected for the synthesis of the well-defined,
multifunctional polymer required during this bioconjugation approach.
In conclusion, within this chapter our studies on the novel, post-functionalization approach, aiming at a high yielding and efficient synthesis of Giant Amphiphiles will be presented. We envisioned a sequence in which a functionalized hydrophilic polymer was initially specifically coupled to a protein to form a triblock hydrophilic polymer-protein bioconjugate and on a second step, post-functionalized through a copper catalyzed multi-click chemistry reaction with hydrophobic azides to convey an overall amphiphilic character to the bioconjugate. The design and ATRP mediated synthesis of the hydrophilic multifunctional polymer will be described along with the bioconjugation reaction and the final introduction of hydrophobicity yielding triblock Giant Amphiphiles.

III.2. Results and discussion.

III.2.1. Design and synthesis of the polymer.

For the purposes of our approach, we designed as mentioned above the target polymer to combine a bioconjugation site, an overall hydrophilic character and multiple grafted alkyne units aimed to act as hydrophobicity and/or multifunctionality entry points as described on Figure III-3.

Figure III-3. Schematic representation of the polymer 4 designed for cysteine containing proteins bioconjugation, displaying the different important features of the macromolecule.
The design and synthesis of the hydrophilic statistical copolymer 4 starting from the appropriate initiator and monomers is described in Scheme III-3 and Figure III-3. The maleimido-protected compound 6 was considered to be an appropriate initiator as it would lead, after deprotection, to the formation of a maleimide function known to react selectively and fast with free accessible thiols of proteins. The hydrophilicity of the final polymer 4 emerged from the glycerol units which were introduced via the ketal protected diol monomer 7 utilized during the polymerization. The choice of the pendant alkyne units as hydrophobicity precursors was based on the versatility and orthogonality of the click, copper (I) catalyzed [3+2] Huisgen cycloaddition in bioconjugation reactions (see Chapter II). For this purpose a trimethylsilyl protected propargyl monomer 8 was utilized as a gradient in the polymerization, after ca. 50% conversion of the the ketal protected diol monomer 7. The statistical nature of the polymer was pursued to ensure the overall hydrophilicity. Finally, the Hostasol yellow fluorescent marker containing monomer 9 was also added in low quantities during the early steps of the polymerization, to introduce a fluorescent identity to the final polymer, aimed at easier characterization of the bioconjugates.

Scheme III-3. Retrosynthesis of the hydrophilic multifunctional polymer 4 designed and synthesized in this study starting from the initiator 6, monomers 7 and 8 and fluorescent comonomer 9.
III.2.1.1. Synthesis of the initiator, monomers and ligand for ATRP polymerization.

The synthesis of the polymer was performed in collaboration with Prof. D. M. Haddleton and under the guidance of Dr. G. Mantovani.


α-Functional polymers containing unprotected functional groups (such as hydroxyl, amido and tertiary amine), can be easily obtained by ATRP starting from appropriate initiators.\textsuperscript{14,35} In this specific case however, this simple strategy would be problematic as the maleimido initiator is itself a polymerizable monomer, and therefore, copolymerization into the growing chain would occur.\textsuperscript{36,37} To circumvent this problem, we prepared the maleimido-protected ATRP initiator 6 following the synthetic protocol shown in Scheme III-4.\textsuperscript{38,39,40}

\begin{center}
\textbf{Scheme III-4.} Reagents and conditions for the synthesis of the maleimido-protected ATRP initiator 6.
\end{center}

As shown in Scheme III-4, the double bond of maleic anhydride was first protected with an oxanorbornene motif by reflux in toluene in the presence of furan for six hours to give 10 in 87% yield. The resulting intermediate 10 was then reacted with ethanolamine in methanol for 4 hours at reflux to give the alcohol 11 in 42% yield. The initiator 6 was subsequently obtained by esterification of 11 using bromoisobutyryl bromide in the presence
of triethylamine in methanol (room temperature, overnight) to give the resulting initiator 6. The final product was purified by chromatography column and obtained in 93% yield.

III.2.1.1.b. Synthesis of the monomers 7, 8, 9.

The monomers (protected hydrophilic monomer and protected alkyne monomer) as well as the hostasol fluorescent comonomer were synthesized according to existing literature procedures. An esterification of the respective commercially available alcohols using methacryloyl chloride was necessary to obtain the monomers 7, 8, 9 in good yields.

More specifically, the solketal monomer 7 (Scheme III-5) was obtained by esterification of (2,2-dimethyl-1,3-dioxolan-4-yl)methanol by methacryloyl chloride in the presence of triethylamine in THF in 64% yield whereas the formation of trimethylsilyl protected monomer was achieved under the same conditions (utilizing diethylether as solvent), to afford the desirable product 8 in 81% yield (Scheme III-6). The protection was judged to be necessary for solubility reasons in the case of monomer 7, and to avoid the terminal alkyne polymerization in the case of the monomer 8.

Hostasol yellow, a fluorescent tag widely used in industrial chemical companies for microscopy experiments employing an Ar ion laser as the excitation source has also recently found applicability in polymer synthesis. For the purposes of this research the precursor hostasol tag (Figure III-4, left) was functionalized with the appropriate reagents in 2 steps to
furnish the corresponding methacrylate derivative 9 shown on Figure III-4 (this fluorescent monomer was prepared by Dr. Giuseppe Mantovani in the Haddleton group). Used in a minute quantity, this marker aimed at facilitating the visualization of bioconjugation products. It should be noted that it possesses a strong absorption around 466 nm in the UV and is even distinguishable to the naked eye, allowing therefore easy optimization of the conditions used for separation of bioconjugates on resin columns and characterization of the products.

![Figure III-4. Structure of hostasol yellow precursor (left) and hostasol yellow monomer 9 (right) fluorescent tags.](image)

III.2.1.1.c. Synthesis of the ligand 12.

The ligand used during the polymerization was the pyridyl-N-propylimine 12 and was synthesized by condensation of n-propylamine and pyridylcarboxaldehyde as previously described. The resulting product 12 (Scheme III-7), obtained in 45% yield was stored at 4°C and under nitrogen atmosphere before use.

![Scheme III-7. Reagents and conditions used for the synthesis of the ligand 12.](image)

The maleimido-protected initiator 6, the solketal methacrylate monomer 7, the hostasol fluorescent comonomer 9 (depicted on the Scheme III-2 as grey spheres) in 1% molar ratio as compared to the initiator 6, the ligand 12 and the solvent anisole were placed in a Schlenk tube and subjected to 5 freeze-pump-thaw cycles to remove O\(_2\). The beginning of the polymerization was subsequently triggered by the canulation of this solution into a second Schlenk tube containing Cu(I)Br under O\(_2\) free conditions. Aliquots of the reaction feed were removed with a syringe during the course of the reaction to calculate PDI (polydispersity indice) and conversion. The conversion of the solketal monomer was followed by \(^1\text{H}\) NMR by comparing the integrals of the proton signals of the double bond to those of the dimethyl ketal functionality. In order to avoid the formation of block copolymers which would be difficult to manipulate in terms of solubility we aimed for the statistical copolymer, by adding after approximately 54% conversion, the second monomer, i.e. the trimethylsilyl protected alkyne methacrylate 8, to the polymerization feed to induce gradient copolymerization.

\[
\begin{align*}
\text{N} & \quad \text{O} & \quad \text{O} & \quad \text{Br} \\
\text{O} & \quad \text{O} & \quad \text{O} & \quad \text{O} & \quad \text{O} & \quad \text{O} & \quad \text{Si} \\
+ & \quad \text{O} & \quad \text{O} & \quad \text{Si} & \quad \text{N} & \quad \text{N} & \quad \text{H} \\
\end{align*}
\]

\text{Cu}^{0}\text{Br, anisole, r.t.}

\text{Scheme III-8.} Reagents and conditions for the ATRP mediated synthesis of 5.

The polymerization was stopped after around 80% total conversion of the solketal monomer. The molecular weight (M\(_n\)) of the polymer was calculated by \(^1\text{H}\) NMR by comparison of the integrals of solketal side chain protons with those of the oxanorbornene protected terminus. It was found that the polymer has a MW of 11.5 kDa whereas GPC
measurements indicated a PDI of 1.15. Using $^1$H NMR, by comparison of the integrals corresponding to the protons of vinyl motif of the oxanorbornene terminus, of the protons of dimethyl ketal function of solketal and those of the trimethylsilyl function protecting the alkyne side chains, it was found that the polymer 5 contains an average of 8 trimethylsilyl-protected alkyne side chains and 50 solketal monomer units per polymeric chain.

**III.2.1.1.e. Deprotection of the resulting polymer.**

For the final synthesis of the multifunctional polymer 4, the fully protected polymer 5 was engaged in post-polymerization deprotection of the different units. This was achieved in 3 straightforward steps:

*i*) Removal of oxanorbornene moiety to liberate the maleimide functionality.

![Scheme III-9](image1.png)

Scheme III-9. Reagents and conditions used for the preparation of 13.

This deprotection was achieved by refluxing the polymer 11 in toluene overnight to remove the furan protecting group by a retro Diels-Alder reaction, leading to the maleimide-terminated polymer 13.\textsuperscript{40,46} This reaction has been previously shown to also proceed even in the solid state (neat) by leaving the maleimido-protected polymer powder in a vacuum oven at
80°C overnight\textsuperscript{46} to yield the pure maleimido-polymer and therefore avoid the use of organic solvent as well as the final precipitation step of the resulting polymer. In this case nevertheless, toluene was utilized as a solvent.

The polymer 13 was characterized by \textsuperscript{1}H NMR, and, as expected, showed the disappearance of the signals of the protons of the oxanorbornene moiety along with the appearance of a singlet at 6.8 ppm corresponding to the protons of the vinyl moiety of the maleimide functionality as shown on the \textsuperscript{1}H NMR spectra of Figure III-5.

**Figure III-5.** \textsuperscript{1}H-NMR (in CDCl\textsubscript{3}) spectra of maleimido-protected polymer 5 (top) and maleimido-deprotected polymer 13 (bottom).
ii) Deprotection of the trimethylsilyl protected alkyne functions.

![Scheme III-10](image)

**Scheme III-10.** Reagents and conditions for the synthesis of polymer 14.

As previously described in the literature, this reaction proceeds with virtually 100% yield when the deprotection is undertaken in the presence of TBAF as reagent using acetic acid as buffering agent to maintain an acidic pH in the reaction mixture (Scheme III-10). The use of acetic acid in combination with TBAF is a well-established procedure in organic chemistry that is normally used when the substrate to deprotect contains a sensitive functional group (e.g., esters, thioesters) that can be cleaved when TBAF (basic species) alone is employed. Indeed, it was already shown in a previous report that the use of TBAF alone can lead to the cleavage of ester bonds onto the same type of alkyne-containing polymers. For this reason, acetic acid was also employed as a buffering agent during the TBAF mediated deprotection of polymer 14. The polymer 13 was dissolved in a mixture THF / acetic acid at -20°C and reacted with TBAF overnight under nitrogen atmosphere. Removal of the ammonium salts on a silica pad afforded the pure polymer 14 in quantitative yield.

$^1$H NMR of the resulting alkyne deprotected polymer 14 (Figure III-6) verified its structure by revealing the disappearance of the characteristic peak corresponding to the trimethylsilyl protecting group at 0.2 ppm together with the appearance of the terminal alkyne protons at 2.5 ppm. The ratio of the functional groups within the polymer remained unchanged. These results were confirmed by FT-IR spectroscopy. As expected, a
characteristic stretching band of the terminal –C≡C–H bond indicating the deprotection of trimethylsilyl functions on the resulting polymer was observed at 3308 cm\(^{-1}\) and is shown in Figure III-7.

**Figure III-6.** \(^1\)H-NMR (in CDCl\(_3\)) of the trimethylsilyl alkyne protected polymer 13 (top) and the alkyne deprotected polymer 14 (bottom).

**Figure III-7.** FT-IR analysis of polymer 13 (solid line) and polymer 14 (dashed line).
iii) Deprotection of the ketal functionalities.

\[
\begin{align*}
\text{NO}_3\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{Br} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{H} & \quad \text{O} \\
\end{align*}
\]

Scheme III-11. Reagents and conditions used for the synthesis of polymer 4.

This reaction was performed in dioxane by catalytic acid hydrolysis of the ketal function using 1 M HCl aqueous solution as shown on Scheme III-11. The deprotection of the vicinal diols on the polymer backbone altered the solubility of the produced polymer 5 as expected and therefore the polymer was recovered in methanol and immediately freeze-dried to avoid degradation of maleimide moiety which is well known to be unstable in water under non neutral pH conditions.

Final full characterization of the polymer 4, after the three deprotection steps was performed by GPC, $^1$H NMR and MALDI-TOF analysis. First, by GPC the retention of polydispersity of the polymer 4 after the three deprotection steps was confirmed (PDI~1.20) and indicated a $M_n$~8 kDa. $^1$H NMR spectrum of polymer 4 confirmed the deprotection of the ketal functions along with the preservation of alkyne functions (Figure III-8). It is also important to notice that the maleimide functionality and its ratio within the polymer, as confirmed by the presence of the peak at 6.9 ppm, was not altered by the successive reactions.
III.2.1.2. Coupling of the hydrophilic polyalkyne (4) to Bovine Serum Albumin.

In contrast to all methods previously utilized for the synthesis of Giant Amphiphiles, which comprised the direct use of the hydrophobic polystyrene for bioconjugation and therefore were hampered by solubility incompatibility problems, the bioconjugation of polymer 4 was designed to be facilitated by its hydrophilic character. This bioconjugation reaction was realized in a 20 mM PB pH 7.4 aqueous solution containing native BSA at a concentration of ca. 0.35 mM and a 100 molar excess of the polymer 4 (Scheme III-12) without the need to implement the medium with organic solvents (in additional experiments that are not mentioned within the thesis, the addition of minute quantities of DMSO as a cosolvent was also studied and found to be successful).
Scheme III-12. Reagents and conditions used for the coupling of polymer 4 to native BSA.

After gentle shaking for 24 hours at 7°C, the reaction mixture was analyzed by SEC. Similar retention times were observed for BSA (18.31 min.) and BSA-polyalkyne VII conjugation reaction (18.39 min.) at 254 nm under the conditions utilized for the analysis (solvent: 70 % phosphate buffer 5 mM pH 7.4, 30 % acetonitrile, room temperature, column: SEC-300 BioBasic, flow rate: 0.5 mL.min⁻¹, Figure III-9). Nevertheless, at 466 nm (maximum absorbance of the fluorescent hostasol yellow monomer at 466 nm),⁴¹-⁴⁴ no absorption was observed for native BSA, whereas two peaks were observed for the bioconjugation reaction mixture attributed to the bioconjugate and the unreacted polymer (Figure III-10). A blank sample containing only the hydrophilic polymer 14 was analyzed also by SEC and showed only one broad peak with a higher retention time (i.e. smaller molecular weight). This analysis was the first indication of the successful formation of the BSA-polyalkyne bioconjugate VII.
Chapter III – Formation of Giant Amphiphiles by Post-Functionalization of Hydrophilic Protein-Polymer Conjugates

Figure III-9. SEC traces at 254 nm of native BSA (black trace) and BSA-polyalkyne conjugate VII (red trace).

Figure III-10. SEC traces at 466 nm of native BSA (black trace), the BSA-polyalkyne VII conjugation reaction mixture (red trace) and polyalkyne 4 (green trace).

The success of the coupling of the polyalkyne 4 to native BSA was confirmed by electrophoresis under denaturating conditions (Figure III-11). Both visualization under the UV lamp at 366 nm and through Coomassie Blue staining showed a new (fluorescent) band possessing a higher molecular weight than that of native BSA, thus confirming our previous results observed by the chromatographic analysis of reagents and the reaction mixture. The presence of unreacted native BSA was demonstrated by its characteristic migration band in the electrophoresis.
Figure III-11. SDS-PAGE analysis of native BSA (lane 1) and the bioconjugation reaction mixture of BSA and polymer 4 (lane 2) after Coomassie Blue staining (left) and visualization under the UV lamp at 366 nm (right).

The clear single peak at ~74850 uma together with the peak corresponding to native BSA (~66590 uma) that were observed from the MALDI-TOF measurements, finally confirmed the attachment of a single hydrophilic multifunctional polymeric chain on the native BSA, demonstrating thus the selective coupling of the polymer onto the protein. As expected the peak obtained for the bioconjugate exhibited a broader distribution in mass than the native protein, due to the polydispersity of the hydrophilic polyalkyne 4.

Figure III-12. MALDI-TOF analysis of the bioconjugation reaction mixture between native BSA and polyalkyne 4.
Several different methods were investigated to purify the BSA-polyalkyne bioconjugate VII from unreacted BSA and excess of polyalkyne 4. Dialysis using 25 kDa MWCO regenerated cellulose membranes achieved the removal of the majority of the unreacted polymer. For the removal of native BSA, MPLC using a wide range of self-packed columns (going from Sephadex to Superose resins) and also preparative electrophoresis followed by electroeluting of the appropriate selected band were tested. We succeeded in removing excess polyalkyne 4 from the reaction mixture; unfortunately, after a numerous efforts in this direction, minute quantities of unreacted native BSA remained with the BSA-polyalkyne VII biohybrid product. Nevertheless the product was significantly enriched as showed by electrophoresis (Figure III-13) and we were allowed to proceed to the next steps aiming at the formation of Giant Amphiphiles.

**Figure III-13.** Electrophoresis after purification by Superdex 75 resin column of the reaction mixture VII. Lane 1: crude reaction mixture, lane 2 to lane 6: different fractions obtained after the Superdex 75 column of BSA-polyalkyne VII with the dashed box indicating the position of hydrophilic multifunctional BSA-polyalkyne VII on the gel.

**Figure III-14.** UV analysis of BSA-polyalkyne VII (solid black trace) and native BSA (grey dotted trace).
UV study of the conjugate VII (after purification on Superdex 75 resin) showed the characteristic peak at 466 nm of the fluorescent tag present on the polymer backbone (Figure III-14).

III.2.1.3. Post-functionalisation of the hydrophilic polymer with hydrophobic azides groups.

III.2.1.3.a. Synthesis of hydrophobic alkyl azides.

Several hydrophobic azides were prepared from the commercially available corresponding bromides by nucleophilic substitution of the bromide using a 0.5 M solution of sodium azide in DMSO.\textsuperscript{50}

\[
\text{R-Br} \xrightarrow{\text{NaN}_3, \ 0.5 \text{ M in DMSO}, \ 60^\circ \text{C overnight}} \text{R-N}_3
\]

with \( R = \text{n-C}_{10}H_{21} \) (15), Bz (16), F\textsubscript{5}Bz (17).

Scheme III-13. Reagents and conditions for the preparation of hydrophobic alkyl azides 15, 16 and 17.

As also observed in the literature, the yields of these reactions were found to be quantitative.\textsuperscript{50} The products of the reactions were isolated, characterized by FT-IR, NMR and mass spectroscopy, and stored at -20°C in dry ether for safety reasons (small alkyl azides are often hazardous, explosive) before use.

III.2.1.3.b. Synthesis of porphyrin hydrophobic azides.

The synthesis of structurally and functionally more complex azidated compounds was also investigated. In this direction the synthesis of azide functionalized porphyrins or an azidated thymine analogue was pursued.
The tetraphenylporphyrin 18 was prepared in collaboration with Dr. Sylvain Koeller by condensation between pyrrole and benzaldehyde using acetic anhydride/acetic acid as solvent mixture. After purification, aromatic electrophilic parasubstitution of a phenyl ring of 18 was achieved by reaction of sodium nitrite in trifluoroacetic acid, affording the resulting para-nitro tetraphenylporphyrine 19. Finally, the para-nitro tetraphenylporphyrine 19 was reduced to the amine 20 by reaction with tin chloride in hydrochloric acid. Conditions and characterization are fully presented in the experimental section.

![Scheme III-14. Reagents and conditions for the synthesis of amino-tetraphenylporphyrin 20.](image_url)

For the purposes of our synthesis, an azido-functionalized heterobifunctional triethylene glycol derivative was synthesized in two steps. The introduction of the azide group was achieved in a one-pot, 2 steps synthesis comprising mono-functionalization with mesyl chloride in THF using triethylamine to afford a triethylene glycol mesylate intermediate which was directly engaged in the second step without further purification. After evaporation of the volatiles, nucleophillic substitution of the mesylate was undertaken using sodium azide in ethanol to give the monofunctionalized azido triethylene glycol 22 in 44% yield as shown.
in Scheme III-15 below. Subsequently, the intermediate 21 was reacted with succinic anhydride in dry toluene overnight to afford the azide bearing acid 22 in 93% yield.

![Scheme III-15. Reagents and conditions utilized for the synthesis of the azido heterobifunctional triethylene glycol linker 22.](image)

In a final step, the aminoporphyrin 20 and the azido triethylene glycol acid 22, previously activated with thionyl chloride, were coupled in dichloromethane overnight to afford the azido-porphyrin 23 in 94% yield (Scheme III-16).

![Scheme III-16. Reagents and conditions for the coupling of the aminophorphyrin 20 to the azido heterobifunctional triethylene glycol acid 22.](image)

Finally, starting from azidated triethylene glycol 21, an azido thymine derivative 24 was synthesized. It was obtained by esterification of 2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)acetic acid (Scheme III-17) by compound 21 in the presence of HBTU and DIPEA at r.t. in quantitative yield.

![Scheme III-17. Reagents and conditions for the synthesis of azidated thymine 24.](image)
III.2.1.3.c. Click chemistry assays on the polymer.

After the synthesis of a small library of azido derivatives, the efficiency of the multi clicking step was tested primarily to the polymer, to optimize the reaction conditions. To avoid problems inherent to the instability of the maleimido moiety under click chemistry conditions,\textsuperscript{57,58} it was decided to selectively deprotect the polymer in such a way that only terminal alkyne side chains would be free and accessible for the multilicking reaction. For solubility reasons, the glycerol repeating units were kept in their protected ketal form. Using the conditions described previously for the full deprotection of the polymer, the polymer 4 was partially deprotected by TBAF in THF using acetic acid as buffering agent as shown in Scheme III-18.

\begin{center}
\includegraphics[width=\textwidth]{SchemeIII-18.png}
\end{center}

\textbf{Scheme III-18.} Reagents and conditions for the preparation of polymer 25 and the multiclicked polymers 26\textit{a}, 26\textit{b}, 26\textit{c}, 25\textit{d}.

Clicking of hydrophobic azides was then investigated using a 5 molar excess of the azide per polymer terminal alkyne unit, 0.6 equiv. CuBr and 1 equiv. sodium ascorbate in
10% DMSO, 20 mM PB pH 7.4. During the isolation of the pure multi-clicked products, Cu (II) was removed from the reaction mixtures (embarrassing paramagnetic species for $^1$H NMR) on neutral alumina column and the products were analyzed by GPC and NMR spectroscopy.

The compounds $26a$, $26b$, $26c$, $26d$ were discriminated from the starting material $25$ by GPC using toluene as the mobile phase and 2 PL-Gel mixed columns (in series) through a reproducible difference in the retention times thus demonstrating the formation of the multi-triazolyl polymer compounds as shown on Figure III-15. Furthermore, the PDI of the “clicked” polymers $26a$, $26b$, $26c$, $26d$ were found to be around 1.2 as proven by the GPC measurements. It should be mentioned that in all cases, the UV traces were in good agreements with the corresponding RID traces.

![Figure III-15](image)

**Figure III-15.** GPC traces of protected polyalkyne $25$ (black curve) and purified clicked products $26a$, $26b$, $26c$, $26d$.

The formation of the products was confirmed by $^1$H NMR. As shown on Figure III-16, the disappearance of the terminal alkyne protons on the polymer backbone was observed together with the downfield shifting of the $–$CH$_2$– in $\alpha$ of the triple bond position and the appearance of new broad signals corresponding to the protons of the lateral alkyl chains grafted by the copper-catalyzed Huisgen dipolar azide alkyne cycloaddition on the polymer backbone.
Figure III-16. $^1$H-NMR of polyalkyne 25 and “clicked polymers” 26a (R=C$_{10}$H$_{21}$), 26b (R=Bz), 26c (R=F$_5$Bz).

In the case of the pentafluorobenzyl azide 17, $^{19}$F NMR was also used to follow the formation of the product (Figure III-17). When the starting pentafluorobenzyl azide 17 and the clicked corresponding polymer 26c were compared, a shifting of the peaks together with a broadening of the $^{19}$F signals (due to longer relaxation periods) were observed, indicating the accomplishment of the reaction.

Figure III-17. $^{19}$F-NMR spectra (in CDCl$_3$) of compound 17 (black trace) and 26c (grey trace).
Unfortunately, in the case of the porphyrine derivative 26d, the complexity of the acquired spectra presumably due to a copper (II) complexation, did not allow the full spectroscopic characterization of the product.

III.2.1.3.d. Post functionalization of BSA-polyalkyne VII using different hydrophobic azides.

As previously shown, clicking of hydrophobic azides onto the polymer backbone was successfully achieved in aqueous solution. It was thus decided to proceed with the “clicking” of hydrophobic azides 15, 16, 17, 23 onto the BSA-polyalkyne multifunctional biohybrid VII.

Scheme III-19. Reagents and conditions for the synthesis of clicked Giant Amphiphiles VIIIa, VIIIb, VIIIc and VIIIId.

Same conditions as mentioned above were used. The reaction mixtures containing 0.12 mM of the hydrophilic BSA-PA VII in 20 mM phosphate buffer, 40 molar excess of each of the azido derivatives 15, 16, 17 or 23, and 2 mM CuSO$_4$ / 4 mM sodium ascorbate as the catalytic system were gently shaken for 48 hours at 7°C. The reaction mixtures became opaque soon after the initiation of the click reaction through the addition of CuSO$_4$/Na ascorbate, indicating the formation of amphiphilic products. After the 48h allowing for the completion of the multi-“click chemistry” reaction, all reagents were removed by a simple excessive dialysis step against 20 mM phosphate buffer pH 7.4 (using 25 kDa MWCO
regenerated cellulose membranes). The characteristic aggregation caused by the amphiphilicity of the products was observed by TEM microscopy and verified by the hampering of the electrophoretic mobility of the products as a result of their amphiphilic character in the case of reaction with azides 15, 16, 17. In the case of azido porphyrin 23, no reaction was observed, a fact attributed to the insolubility of the porphyrin into the aqueous medium.

Figure III-18. SDS-PAGE analysis of native BSA, BSA-polyalkyne VII and clicked amphiphilic bioconjugates:
- lane 1, native BSA;
- lane 2, BSA-polyalkyne VII;
- lane 3, BSA-PA@C_{10}H_{21} VIIIa;
- lane 4, BSA-PA@Bz VIIIb;
- lane 5, BSA-PA@F_{5}Bz VIIIc.

No band corresponding to the reacting hydrophilic polymer-protein conjugate VII was observed, indicating the efficiency of the copper-catalyzed dipolar cycloaddition of hydrophobic azido compounds on the hydrophilic biomacromolecule VII.

SEC measurements did not give any satisfactory results under the conditions used, a fact attributed to the aggregation of the amphiphilic polymer-protein bioconjugates. Furthermore, MALDI-TOF measurements of the hydrophilic BSA-polyalkyne conjugates VIIIa, VIIIb, VIIIc were not successful even when mixtures of matrices were used.
III.2.1.3.e. Aggregation Studies.

Figure III-19. TEM micrographs of BSA-PA@C\textsubscript{19}H\textsubscript{21}N\textsubscript{3} \textbf{VIIIa} (line A), TEM micrographs of BSA-PA@Bz \textbf{VIIIb} (line B), TEM micrographs of BSA-PA@F\textsubscript{5}Bz \textbf{VIIIc} (line C).

After dialysis of the reaction mixtures, the aggregation profiles of the Giant Amphiphiles \textbf{VIIIa}, \textbf{VIIIb} and \textbf{VIIIc} were investigated with Transmission Electron Microscopy (TEM). For this reason, the mixtures were further dialyzed against nanopure water and a 100 times diluted solution was placed onto a formvar coated copper grid. As in previous cases, no staining was necessary presumably due to the increased electronic density of the polymer moiety. The formation of well-defined spherical aggregates was observed in all cases (Figure III-19, A-D) whereas blank experiments performed under the same conditions using BSA in the presence of the polymer 4 and mixtures of the BSA-PA bioconjugate VII in the presence of the azides or the azides themselves, did not reveal any aggregation pattern.
The formation of such spherical superstructures is well in agreement with previous reports on BSA-Polystyrene Giant Amphiphiles. In the recent work reported by Nolte and coworkers on the synthesis of BSA-polystyrene Giant Amphiphiles, self-assembly of the amphiphilic biomacromolecules gave well-defined spherical aggregates consisting probably of micellar superstructures as suggested by the size of the aggregates (between 30 and 70 nm). It should be noted that spherical aggregates observed for the BSA-PA@C\textsubscript{10}H\textsubscript{21}N\textsubscript{3} Giant Amphiphiles VIII\textsubscript{a} were rather uniform in diameter (mean diameter ca. 150 nm, Figure III-19, lane A), while the diameters of the BSA-PA@Bz and BSA-PA@F\textsubscript{5}Bz Giant Amphiphiles VIII\textsubscript{b} and VIII\textsubscript{c} varied between 20 and 200 nm (Figure III-19, C-D). We attribute this difference of size dispersity of the resulting superstructures in water to the more dynamic behaviour of the decanyl and fluorobenzyl alkyl chain as compared to the benzyl residue. This is the first report of a difference in aggregation behaviour in Giant Amphiphiles arising from the different hydrophobic tail attached.

![Figure III-20](image1.png)

**Figure III-20.** CFM images of (A), the BSA-PA@C\textsubscript{10}H\textsubscript{21}N\textsubscript{3} VIII\textsubscript{a} (excitation with 514 Argon-Krypton laser line), (B), a BSA-PA@C\textsubscript{10}H\textsubscript{21}N\textsubscript{3} VIII\textsubscript{a} emulsion in a water / ethanol / decane mixture and (C), the BSA-PA VII.

Laser confocal microscopy additionally allowed the observation of the superstructures through the fluorescence arising from the Hostasol tag which is incorporated on the polymer backbone. When the conjugate BSA-PA@C\textsubscript{10}H\textsubscript{21}N\textsubscript{3} VIII\textsubscript{a} was suspended in a 1/1 (v/v) mixture of water and ethanol, upon addition of a small quantity of decane and vigorous shaking, a characteristic for amphiphilic molecules, milky emulsion was formed. The analysis of this emulsion by confocal microscopy clearly shows the biohybrid VIII\textsubscript{a} orientating itself at the decane-water interface, a fact that is attributed to its amphiphilic nature.
Chapter III – Formation of Giant Amphiphiles by Post-Functionalization of Hydrophilic Protein-Polymer Conjugates


In this chapter, the successful design and development of a novel strategy for the preparation of Giant Amphiphiles has been discussed.

The ATRP mediated synthesis proved to be efficient both in the implementation of the posed functional properties and in the creation of a well-defined relevant polymer.

The bioconjugation of BSA to this multifunctional hydrophilic polyalkyne was achieved using a chemoselective Michael addition reaction, under mild conditions that do not interfere with the integrity of the protein quaternary structure. The subsequent post-functionalization of the resulting hydrophilic polymer-protein bioconjugate was also carried out in mild conditions using a copper-catalyzed click chemistry reaction.

Following this new approach, the multiclicking of several hydrophobic azides onto the multifunctional hydrophilic polymer protein bioconjugates led to a, previously unattainable, variety of Giant Amphiphiles without any need of condition optimization or polymer synthesis.

Aggregation studies with TEM and CFM microscopy revealed the formation of well defined superstructures. For the first time, difference in aggregation was observed when changing the monomer nature (e.g. difference observed in aggregates polydispersity depending on the monomer, C$_{10}$ vs. Bz, F$_{5}$Bz).

Future studies involving the introduction of an increased number of alkyne functionalities and the multiclicking of azides expressing a catalytic functionality, certainly provide exciting opportunities in the area and should be pursued.
Chapter III – Formation of Giant Amphiphiles by Post-Functionalization of Hydrophilic Protein-Polymer Conjugates

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Chapter IV

In Situ, ATRP Mediated Hierarchical Formation of Bio Nanoreactors

IV.1. Concept / Background.

As already mentioned in previous chapters, during the synthesis of Giant Amphiphiles, the hydrophobicity of the polymer is generally the limiting factor, while purification has also proved to be difficult. In an effort to circumvent such problems, high content in organic solvents aqueous solutions have been previously applied,\textsuperscript{1,2,3} making the bioconjugation conditions quite harsh and problematic to apply in the majority of proteins. Taking into account recent developments in the field of bioconjugation and ATRP polymerization that will be discussed later on in this chapter,\textsuperscript{4,5,6,7,8,9} we envisioned that if successful, the initiated onto a protein macroinitiator (\textit{i.e.} a protein that has been functionalized with a polymerization initiator at a precise position), direct, ATRP polymerization of a hydrophobic monomer would provide a facile and efficient alternative approach leading to the quantitative \textit{in situ} formation of defined amphiphilic macromolecular structures.

For this reason, a novel, simple synthetic pathway was developed and will be discussed in this chapter aiming at the development of protein initiated ATRP polymerization of hydrophobic monomers (Scheme IV-1). Further studies on the application of this method for the creation of meaningful structures will then be discussed.

\textbf{Scheme IV-1.} General scheme of the synthetic pathway used to create Giant Amphiphiles by ATRP polymerization.
Recently, several groups have developed a new technique to synthesize protein-polymer bioconjugates which lies on the in situ polymerization of hydrophilic monomers onto protein macroinitiator through either Atom Transfer Radical Polymerization (ATRP)\textsuperscript{4,5,6,7,8} or Reversible Addition-Fragmentation Chain Transfer (RAFT)\textsuperscript{9} mediated polymerization. It was these pioneering studies and their results, that intrigued us to approach the synthesis of Giant Amphiphiles through living polymerization techniques.

**IV.1.1. Protein-polymer bioconjugates through protein initiated living polymerization.**

The pioneering work on the in situ polymerization of biomacronitiators was recently presented by Maynard and coworkers for the creation of smart polymer-protein conjugates in good yields (greater than 65\%).\textsuperscript{6} In this work, the polymerization of the pH and temperature dependant poly(N-isopropylacrylamide) (polyNIPAAm) starting directly from protein macroinitiators (e.g. BSA and lysozyme macroinitiators) was achieved. These biomacronitiators were prepared by the selective coupling of pyridyl disulfide- or maleimido-ATRP initiators onto the corresponding proteins. In the case of lysozyme, the polymer-protein bioconjugates retained the catalytic activity for the enzyme moiety.

In a more recent work, the Maynard group reported on the in situ growing of polyNIPAAm on a biotin-streptavidin macroinitiator (Scheme IV-2),\textsuperscript{5} with the retention of the bioaffinity of streptavidin for biotin. They demonstrated that the grafting of polymers onto the enzyme complex did not interfere with the strong binding interaction of biotin to streptavidin.

\begin{center}
\textbf{Scheme IV-2.} Strategy proposed by Maynard and collaborators to prepare streptavidin-poly(NIPPAm) bioconjugates.\textsuperscript{5}
\end{center}
Haddleton and coworkers reported on the *in situ* polymerization of hydrophilic monomers such as poly(ethylene glycol) methyl ether methacrylate (PEGMA) or dimethylaminoethyl methacrylate (DMAEMA) onto protein derived macroinitiators such as BSA and lysozyme.\(^7\) In this study, BSA and lysozyme were initially functionalized with respectively a maleimido- and a NHS-activated acid ATRP initiator, subsequently purified before the polymerization step (Scheme IV-3). A fluorescent comonomer derived from hostasol or rhodamine B tags was added in minute quantities together with the monomers and the obtained protein-polymer bioconjugates were successfully observed by fluorescence detection SEC-HPLC. They observed that lysozyme conjugates had a broader mass distribution than the BSA conjugates (probably due to the multi-site attachment of the initiator to the native lysozyme).

**Scheme IV-3.** Procedure introduced by Haddleton and coworkers to prepare fluorescent hydrophilic polymer-protein biohybrid macromolecules.\(^7\)
The Matyjaszewski group also took advantage of the *in situ* polymerization methodology to prepare hydrophilic chymotrypsin-polymer bioconjugates with near uniform distributions that were also found to retain part of their enzymatic activity (50-86% of the initial activity of the native enzyme). In the course of this study, 2-bromoisobutyryl bromide was coupled to chymotrypsin through a two-phase reaction mixture (phosphate buffer pH 8 / dichloromethane) that was stirred at 800 rpm and at 25 °C. After purification of the chymotrypsin macroinitiator, monomethoxy poly(ethylene glycol) (MPEG-methacrylate) was polymerized using bipyridine / CuBr as catalytic system in phosphate buffer pH 6 to afford the corresponding hydrophilic polymer-protein bioconjugates with near uniform polydispersities.

In all cases, the living polymerization mediated formation of bioconjugates allowed to bypass the multiple synthetic steps used in conventional bioconjugation techniques. Furthermore, the use of a low molecular weight reagent in the place of a polymer, significantly facilitated the purification of the resulting bioconjugates as only a simple dialysis step was necessary to remove the unreacted monomer.

Taking into account the above mentioned advances, we envisioned that if such a method would also be efficient when replacing the hydrophilic with a hydrophobic monomer, it would certainly provide an efficient alternative also for the synthesis of *Giant Amphiphiles*. Furthermore, we reasoned that since the hydrophobic monomer would be gradually grafted on the bioconjugate, ATRP could prove to be an ideal tool to generate amphiphilic structures containing a more defined structure (lower PDI). We expected that as the amphiphilic character of the overall structure gradually increased during the polymerization, aggregation into superstructures would occur in a rather precise point, thus leading to the polymerization termination and, by this process, providing more defined polymer-protein conjugates at both the molecular and supramolecular level.

Aiming at the creation of functional nanoassemblies, we thought that this method would also allow the one-pot encapsulation of small molecules or catalytically active enzymes during the *in situ* formation of the superstructures. Taking into account the nature of *Giant Amphiphiles* and the concomitant to their synthesis formation of non-dynamic aggregates, any attempt to achieve such an encapsulation post their synthesis would certainly interfere with either the integrity of the proteins and/or the overall architectures. We reasoned that following
the ATRP mediated approach the statistical encapsulation of guest proteins without compromise to their catalytic function.

In this chapter, our successful studies toward these goals will be discussed, while initial intriguing results on the interaction of such systems with living cells will also be presented.

**IV.2. Results and discussion.**

**IV.2.1. Synthesis of the protein macroinitiator.**

**IV.2.1.1. Synthesis of the initiator.**

The hetero bifunctional ATRP initiator 27 was synthesized as previously described by Haddleton\textsuperscript{10} and Velonia\textsuperscript{11} (Scheme IV-4), using a synthetic pathway similar to that utilised in the case of compound 6 (Chapter III). Upon synthesizing the precursor 6, the final deprotection step was achieved by refluxing the compound 6 overnight in toluene and led to the formation of the maleimido ATRP initiator 27 in virtually quantitative yield by a retro Diels-Alder reaction as shown on the Scheme IV-4.

![Scheme IV-4. Reagents and conditions utilized for the synthesis of the maleimido ATRP initiator 27.](image-url)
IV.2.1.2. Selective coupling to BSA.

The efficiency of the in situ ATRP protein-polymerization toward the synthesis of Giant Amphiphiles was explored using the 66 kDa globular bovine serum albumin (BSA) that contains only one free cysteine residue at position 34. Adapting the approach that was used by Maynard and coworkers for the in situ creation of smart polymer-protein conjugates, the bioconjugation reaction to BSA was performed under mild conditions and was initiated by the slow addition of a 40 molar excess solution of the initiator $27$ in DMSO, to a solution of BSA in aqueous phosphate buffer (PB), pH 7.4 as shown in Scheme IV-5. The reaction mixture was gently shaken for 2 days at 7°C to allow maximum yields.

![Scheme IV-5. Reagents and conditions used for the selective conjugation of the ATRP initiator 26 to native BSA.](image)

The resulting bioconjugate protein macroinitiator IX (BSA-ATRP macroinitiator IX), was easily isolated from the starting material $27$ and DMSO by a simple, extensive dialysis step (using 25kDa MWCO regenerated cellulose membranes) against 20 mM phosphate buffer pH 7.4. After purification, the reaction yield was initially quantified using the colorimetric Ellman’s assay. The absorption at 412 nm arising from the thionitrobenzoate product of Ellman’s test revealed (in agreement with what was reported in Chapter II), that the native BSA contained about 47% free cystein residues available for bioconjugation. After the coupling reaction and the purification, virtually no free cysteins were detected in the mixture, indicating that the coupling between the maleimido-ATRP-initiator $27$ and the free cystein of native BSA was practically quantitative. This observation was confirmed by both native gel electrophoresis and SEC measurements.
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Figure IV-1. Left: Native gel electrophoretic profile of native BSA (lane 1), and the purified reaction mixture after conjugation of BSA with the ATRP initiator 27 (lane 2). Right: SEC-HPLC chromatographic traces of native BSA (solid black trace) and BSA-macroinitiator IX (dashed grey trace).

On the electrophoresis gels that were obtained under native conditions, a new band was observed for the bioconjugate IX (lane 2, Figure IV-1), exhibiting different migration profile than that of the native BSA (lane 1, Figure IV-1). No band corresponding to the native BSA was observed in the enriched reaction mixture even after employing silver staining, confirming thus the results obtained by Ellman’s test and pointing to a quantitative reaction. Surprisingly, this new band migrated faster than BSA (i.e. was placed in the lower apparent molecular weight region) behaving in a manner similar to that observed also in the case of the BSA-1-alkyne bioconjugate I (Chapter II). We attribute this behaviour to a possible change in the total net charge of the protein adduct and/or conformation changes during the conjugation.

SEC chromatographic analysis also indicated the formation of the bioconjugate. The retention time of the BSA macroinitiator IX was found, as expected, based on the measurements previously performed on the biohybrid BSA-1-alkyne I (Chapter II), to be only slightly different to that of native BSA (Figure IV-1, right). No peak corresponding to the unreacted excess of the free initiator 27 was observed, indicating its total removal by the extensive dialysis purification step.

Finally, MALDI-TOF analysis (Figure IV-2) revealed masses at 66313 and 66737 uma respectively for native BSA and BSA-macroinitiator IX, hence exhibiting a difference of mass (+ 424 uma) consistent with the selective conjugation of one ATRP initiator molecule.
27 per protein. After its full characterization, the BSA macroinitiator IX was freeze dried and found to be stable for prolonged periods of time (months) when stored at -20°C.

![MALDI-TOF analysis of native BSA (grey) and BSA macroinitiator IX (black).](image)

**Figure IV-2.** MALDI-TOF analysis of native BSA (grey) and BSA macroinitiator IX (black).

### IV.2.2. *In situ* formation of Giant Amphiphiles: ATRP polymerization on BSA macroinitiator IX.

Styrene was judged to be the appropriate monomer to proceed with the experiments on the *in situ* polymerization of hydrophobic monomers initiated on the bioconjugate IX as it would lead to the formation of polystyrene Giant Amphiphiles that are known to exhibit a strong amphiphilic character. Furthermore, the use of styrene as hydrophobic monomer would allow the comparison with previous results obtained with other techniques used in the lab (such as the preparation of BSA-polystyrene Giant Amphiphiles obtained by direct click chemistry coupling in Chapter II or the BzN₃ clicked bioconjugates obtained through the post functionalisation approach in Chapter III).
The polymerization reactions were carried out using a modified version of the standard general procedures that were introduced by the Haddleton group for the synthesis of hydrophilic protein-polymer conjugates\(^7\) and by the Maynard group for the preparation of smart polymer-protein conjugates.\(^6\) Briefly, the in situ styrene polymerization onto BSA-macronitiator IX was performed in aqueous phosphate buffer solution, oxygen free conditions, ambient temperature, using the copper bromide/N-(n-propyl)-2-pyridylmethanimine 12 catalyst system and without the presence of any “sacrificial” initiator.\(^4,5,6\) Several sets of experiments were performed utilizing different monomer to BSA-macronitiator IX ratios while a series of control experiments (in the absence of the BSA-macronitiator IX, monomer, Cu(I) and in the presence of O\(_2\)) were conducted to ensure that the in situ polymerization proceeds on the predesigned BSA initiating position.

As we were aiming for the polymerization of hydrophobic monomers in aqueous solution, the monomer was initially emulsified in 10% DMSO, 20 mM phosphate buffer solution containing the ligand, and then subjected to repeating freeze-pump-thaw cycles before initiating the polymerization by canulation into the second flask containing the protein macronitiator IX and copper (I) bromide under oxygen free atmosphere. Whereas the styrene to BSA macronitiator IX ratio was varied, the CuBr / ligand 12 / BSA macronitiator IX ratio was kept constant at 41 / 70 / 1 in all experiments.\(^7\)
As mentioned above, several sets of experiments were performed utilizing different monomer to BSA-macroinitiator IX ratios (varying from 50 to 3000 times excess of the monomer over IX). These experiments aimed to study the applicability of ATRP in the synthesis of Giant Amphiphiles together with the influence of the monomer to BSA macroinitiator IX ratio on the polymerization outcome. If our initial assumptions were correct, the polymerization should control the polydispersity of the formed polymer and therefore the aggregation of the resulting superstructures. The conditions utilized in these experiments are summarized in Table IV-1. It should be noted that during the polymerization period, the dark brown copper (I) complex colour formed upon the canulation of the reagents to the BSA- macroinitiator IX /Cu (I) oxygen free flask remained stable while a gradual increase of turbidity was observed in the reaction mixtures. The polymerizations were terminated by stirring the reaction mixtures under oxygen atmosphere. Several control reactions were also performed in the absence of the biomacroinitiator IX, the monomer or Cu(I)Br and in the presence of oxygen to ensure that polymerization proceeds in the predesigned position and are also listed in Table IV-1. In this case, the dark brown copper (I) complex colour was again retained throughout the reaction time, but no visual change in the turbidity of the reaction mixtures was observed. In all cases, the resulting bioconjugate solutions were subjected to extensive dialysis to ensure that all traces of reagents (styrene, copper, N-(n-propyl)-2-pyridylmethanimine) were removed. The dialysis was initially performed against 2% EDTA, 10% DMSO, 20 mM phosphate buffer and finally against either 20 mM phosphate buffer or nanopure water depending on the analysis. All reactions were analyzed using SEC, MALDI-TOF and electrophoresis and imaged using Transmission Electron Microscopy (TEM).
### Table IV-1

<table>
<thead>
<tr>
<th>#</th>
<th>Equivalents</th>
<th>Styrene</th>
<th>BSA-macroinitiator IX</th>
<th>Cu(I)Br</th>
<th>O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td></td>
<td>50</td>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>XI</td>
<td></td>
<td>500</td>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>XII</td>
<td></td>
<td>1500</td>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>XIII</td>
<td></td>
<td>2000</td>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>XIV</td>
<td></td>
<td>3000</td>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>2000</td>
<td>0 (native BSA)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0</td>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>2000</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>2000</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table IV-1. List of the polymerization experiments performed using varying ratios between the monomer (styrene) and the BSA macroinitiator IX, and control experiments.

**Figure IV-3.** SEC-HPLC traces of different blank experiments performed under standard ATRP *in situ* polymerization conditions. Traces obtained when the reaction was performed in the absence of the biomacronitiator IX (blue), absence of Cu (green) or in the presence of oxygen (dark red).

The blank experiments using native BSA instead of the biomacronitiator IX ensured that the polymerization occurs only on the initiation predesigned position of IX as shown by the absence of reaction in the SEC chromatograph presented in Figure IV-3. Further blank
experiments performed under ATRP polymerization conditions in the absence of Cu(I)Br or in the presence of oxygen demonstrated that the reaction proceeds through ATRP.

![SEC chromatographic traces of BSA macroinitiator IX (blue trace) and samples of the different in situ styrene polymerization experiments after purification using “low” monomer to BSA macroinitiator IX ratio (ratio 50:1, black trace; ratio 500:1, red trace).](image)

Figure IV-4. SEC chromatographic traces of BSA macroinitiator IX (blue trace) and samples of the different in situ styrene polymerization experiments after purification using “low” monomer to BSA macroinitiator IX ratio (ratio 50:1, black trace; ratio 500:1, red trace).

When polymerization was conducted in the presence of “low” monomer to protein macroinitiator IX ratios (50 and 500 times excess), SEC chromatographic analysis revealed mixtures of unreacted BSA macroinitiator IX and various, rather disperse bioconjugates X or XI with hydrodynamic volumes in general slightly higher than that of native BSA (Figure IV-4) accompanied by low yields. On the contrary, when the polymerization was performed utilizing “high” monomer to biomacroinitiator IX ratios, SEC analyses demonstrated the quantitative formation of BSA-polystyrene Giant Amphiphiles possessing a hydrodynamic volume larger than that of BSA as indicated by the single peak observed with a shorter retention time than the BSA macroinitiator IX (Figure IV-5). It should be noted that in all cases, RID and UV traces were in good agreement. Interestingly, the broadness of the peaks observed for the native BSA, the BSA-macroinitiator IX and the Giant Amphiphiles XII, XIII, XIV was similar, indicating both the efficiency of the polymerization reaction and the retention of polydispersity.
Figure IV-5. SEC chromatographic traces of BSA macroinitiator IX and the different samples from in situ styrene polymerization experiments after purification using “high” monomer to BSA macroinitiator IX ratio (XII ratio 1500:1, red trace; XIII ratio 2000:1, blue trace; and XIV ratio 3000:1, orange trace).

These results were also supported by the electrophoretic analysis of the samples (Figure IV-6). In the case of the “low” monomer to biomacroinitiator IX ratio reactions X and XI, native gel electrophoresis revealed bands with slower electrophoretic mobilities than that of native BSA (higher molecular weight) along with bands corresponding to unreacted BSA macroinitiator IX, thus confirming the low yields of these reactions. In contrast, native gel electrophoresis revealed classical Giant Amphiphile behaviour for the “high” ratio reactions XII, XIII, XIV, i.e. migration hampered by the amphiphilic character of the bioconjugates. In the later cases, no trace of the starting macroinitiator was observed even when the gels were subjected to silver staining. It is worth mentioning that in all blank experiments the expected corresponding starting material was observed.
Figure IV-6. Native gel electrophoretic profile after Coomassie Brilliant Blue staining (A) and Silver staining (B) of native BSA, BSA macroinitiator IX, and different samples obtained by the in situ ATRP mediated polymerization of styrene on the BSA macroinitiator IX. The content of each lane is summarized on the right.

MALDI-TOF analysis of the polystyrene-BSA amphiphiles conclusively verified the above mentioned results (Figure IV-7). It should be noted that, as previously reported for Giant Amphiphiles prepared through conventional conjugation, MALDI-TOF spectra were difficult to obtain for all polymerization samples. Not all spectra obtained were of the clarity of those of native proteins due to the amphiphilic nature of the biohybrids. Several combinations of matrices were utilized to circumvent this problem. The mean molecular weight of the biohybrids was found to increase in general when the monomer to BSA-
macroinitiator IX ratio was varied from “low” (50 to 500) to “high” (1500 to 3000) as shown on Figure IV-7. The MALDI-TOF spectra of BSA-polystyrene conjugates X and XI showed the m/z signals corresponding to the free BSA macroinitiator IX, along with m/z signals varying from 67 to 71 kDa. In contrast, MALDI-TOF analyses showed the m/z signals varying from 71 to almost 80 kDa depending on the monomer to BSA-macroinitiator IX ratio utilized in XII, XIII, XIV. No signal corresponding to the free BSA macroinitiator IX was observed in the latter case.

![Figure IV-7](image)

**Figure IV-7.** MALDI-TOF analysis of BSA-macroinitiator IX (red trace) and the products of selected ATRP mediated in situ polymerization reactions of styrene over IX. The blue trace corresponds to styrene / IX ratio 500:1 and the green trace to 2000:1 ratio.

Though the formation of the amphiphilic biomacromolecules was demonstrated by SEC chromatography, MALDI-TOF and electrophoresis, it was further decided to also elucidate the structure of the formed polystyrene chain itself. This was achieved by digesting the proteins from the biohybrids, isolation of the polymers and subsequent spectroscopic study. To this direction, a solution of BSA-polystyrene synthesized by in situ ATRP polymerization was subjected to HCl mediated protein degradation and the resulting mixture was extracted in CH$_2$Cl$_2$ to isolate the polymer (Scheme IV-7).$^{16}$ The latter was subsequently analyzed by NMR spectroscopy and MALDI-TOF.
Scheme IV-7. Protein digestion in the BSA-polystyrene Giant Amphiphiles.

$^1$H NMR analysis (Figure IV-8), revealed peaks possessing the typical chemical shifts of polystyrene (i.e. two broad peaks in the aromatic region corresponding to the benzene ring and two broad peaks between 1 and 2 ppm corresponding to the CH and CH$_2$ in position $\alpha$- and $\beta$- of the benzene ring respectively). Furthermore, MALDI-TOF analysis revealed only two major distributions of the isolated polystyrene (4.7 kDa and 7.1 kDa). It is worth noting that the distribution of the predominant peak was found to possess a rather low polydispersity when compared to standard commercial polystyrene of known polydispersity indice (PDI ~ 1.13, Encapson, Figure IV-9). We attribute this low polydispersity to the selected synthetic approach.

Figure IV-8. $^1$H-NMR analysis of the polystyrene isolated from hydrolysis of BSA-polystyrene XIII.

Figure IV-9. Comparison of MALDI-TOF spectra of polystyrene isolated from the BSA-polystyrene conjugate XIII (black) and a standard polystyrene (grey, PDI 1.13).
IV.2.3. Aggregation studies.

The aggregation patterns of the BSA-polystyrene X-XIV superstructures obtained via the ATRP in situ polymerization of the protein macroinitiator IX were investigated by Transmission Electron Microscopy (TEM, Figure IV-10). This study revealed the in situ formation of well-defined spherical superstructures exhibiting aggregation behaviour similar to that of the conventionally synthesized BSA-polystyrene Giant Amphiphiles.\textsuperscript{15} The diameter of these aggregates was measured and found to be very regular with sizes varying from 30 to 100 nanometers in diameter as shown on Figure IV-10.

![TEM micrographs of BSA polystyrene Giant Amphiphiles X (A, B), XII (C, D), XIII (E, F) and XIV (G, H) superstructures in water.](image)

This observation is in good agreement with results obtained both by our group (Chapter II) and by the Nolte group.\textsuperscript{15} In our studies, the conventionally synthesized BSA-PS were found to form spherical aggregates with diameters varying between 30 and 100 nm, depending on the polystyrene employed. Similarly, in the Nolte studies it was found that the average diameter of BSA-polystyrene Giant Amphiphile spherical superstructures (MW\textsubscript{polystyrene} 4150, n~38) was about 30 to 70 nm. Interestingly, the diameter of the spherical superstructures did not change with the variation of the monomer to BSA-macroinitiator IX ratio, further confirming thus the results observed in the first chapter according to which no difference in aggregation was induced by variation of the conjugating polymer.\textsuperscript{17}
IV.2.4. Extension of the methodology to other hydrophobic monomers.

In order to investigate whether the in situ ATRP formation of Giant Amphiphiles is a method generic and applicable to a variety of hydrophobic vinyl monomers, the BSA-macroinitiator IX initiated ATRP polymerization was also investigated with two other hydrophobic monomers.

On this end, we thought that it would be of interest to use the trimethylsilyl propargyl methacrylate monomer 8 (synthesized and utilized for the multifunctional polyalkyne 5, Chapter III) for the in situ ATRP polymerization of proteins as, if successful, it would lead to Giant Amphiphiles possessing the protected, functional 1-alkyne moiety that could be further functionalized in later steps to afford a variety of bioconjugates. Furthermore, we also decided to utilize a perfluorinated, pentafluorobenzene derivative for the ATRP based on the unique combination of high thermal stability, chemical inertness (to acids, bases and solvents) and very interesting surface properties\textsuperscript{18,19,20} that the produced polymer moiety would possess.

The trimethylsilyl propargyl methacrylate monomer 8 was synthesized as previously mentioned (Chapter III, Chapter VI). For reasons of synthetic ease for the fluorinated monomer, on the first step, the monomer 8 was deprotected as previously described by a reaction with TBAF in THF using acetic acid to keep an acidic pH to the solution (Scheme IV-8). The pentafluorobenzyl derived monomer 29 for the ATRP was obtained in a second step by a copper catalyzed [3+2] Huisgen dipolar cycloaddition between the alkyne 28 and the pentafluorobenzyl azide 17 (synthesized in Chapter III) in excellent yields using copper sulphate and sodium ascorbate as source of Cu(I).
Scheme IV-8. Reagents and conditions used for the synthesis of the hydrophobic monomer 29.

The trimethylsilyl propargyl methacrylate monomer 8 and monomer 29 were subsequently polymerized in the presence of the BSA ATRP macroinitiator IX using the optimized conditions found for the polymerization of styrene i.e., the ratio 8 or 29 as compared to the BSA-macroinitiator IX was kept constant at 2000 / 1 (Table IV-2), while the reaction was performed in aqueous phosphate buffer solution, oxygen free conditions, ambient temperature and using the copper bromide / N-(n-propyl)-2-pyridylmethanimine 12 catalyst system and without the presence of any “sacrificial” initiator (Scheme IV-9).

Scheme IV-9. General synthetic scheme of the in situ ATRP mediated polymerization of monomers 8 and 28 on BSA macroinitiator IX.
It should be noted that during the polymerization of the fluorinated vinyl monomer 29, our attempts to optimize the reaction scheme led to addition of a larger DMSO quantity (20%) to assist with monomer dispersion in the reaction mixture.

Furthermore, in the case of monomer 8, an experiment in the presence of the non polymerizable, fluorescent dye carboxyfluorescein (CF), was also performed to facilitate the observation of the superstructures by fluorescence confocal microscopy (vide infra for further inclusion experiments). By the hierarchical self-assembly of the BSA-polyalkyne (BSA-PA) Giant Amphiphiles XVI in the presence of CF, the superstructures obtained should be statistically loaded with CF and thus could be easily observed by CFM microscopy. For this reason CF was introduced in the polymerization feed at a concentration of 5 mM.

Upon polymerization, the reactions were as previously stirred for several hours under oxygen atmosphere and the unreacted monomers and reagents were removed by extensive dialysis against initially 10% DMSO, 20 mM phosphate buffer, pH 7.4 and then against 20 mM phosphate buffer, pH 7.4 using 25 kDa regenerated cellulose dialysis membranes. All samples were analyzed as previously.

<table>
<thead>
<tr>
<th>#</th>
<th>Monomer (equiv.)</th>
<th>equivalents BSA macroinitiator IX</th>
<th>CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>XV</td>
<td>8 (2000)</td>
<td>1</td>
<td>--</td>
</tr>
<tr>
<td>XVI</td>
<td>8 (2000)</td>
<td>1</td>
<td>5 mM</td>
</tr>
<tr>
<td>XVII</td>
<td>29 (2000)</td>
<td>1</td>
<td>--</td>
</tr>
</tbody>
</table>

Table IV-2. Table summarizing the conditions utilized for the ATRP in situ polymerization realized by using the hydrophobic monomers 8 and 29 and BSA-macroinitiator IX.

In the case of the ATRP mediated polymerization of monomer 29, both GPC and electrophoresis revealed for the biomacromolecular products XVII (Figure IV-11) a behaviour typical of that previously observed for Giant Amphiphiles. Electrophoresis under denaturating conditions showed that, as already observed in the case of in situ styrene polymerization, the electrophoretic mobility was hampered by the amphiphilicity of the biomacromolecule. Furthermore, as judged from the SEC chromatographic behaviour of the dialyzed reaction mixture, the ATRP polymerization led in this case to a mixture of products with apparent higher hydrodynamic volume (shorter retention times) and a broader molecular weight distribution than that that was observed with styrene, as judged by the broadness of the
peak. Aggregation studies using TEM microscopy revealed the formation of well-defined spherical aggregates with diameters varying from ca. 50 up to 100 nm (Figure IV-12).

![SEC Chromatographic Traces](image1)

**Figure IV-11.** SEC chromatographic traces at 254 nm of native BSA (dashed grey trace) and amphiphilic macromolecule XVII (solid black trace).

![TEM Micrographs](image2)

**Figure IV-12.** TEM micrographs of the aggregates obtained by the self-assembly of the amphiphilic bioconjugates XVII.

We were unfortunately unable to characterize the products with MALDI even when using various matrices combinations. Taking into account the data presented above, it is judged that the synthesis of Giant Amphiphiles with fluorinated polymeric tails was successful, nevertheless further optimization of the reaction conditions (presumably by changing the organic cosolvent utilized to facilitate the dispersion of monomers during ATRP) will be necessary to achieve bioconjugates with the low polydispersity indices that characterized the ATRP derived polystyrene Giant Amphiphiles.
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Figure IV-13. SEC chromatographic traces of native BSA (solid black line), purified bioconjugates XV (solid grey line), and XVIII after reaction with TBAF (grey doted line), and reaction with KF (black dashed line).

In the case of the \textit{in situ} polymerization of monomer 8, SEC chromatography indicated the formation of a new peak attributed to the biomacromolecules XV (Figure IV-13). TEM micrographs of the aggregates XV revealed the formation of well-defined spherical aggregates with diameters varying from 50 to 150 nm (Figure IV-14) while CFM measurements of the reaction performed having CF incorporated in the polymerization feed (reaction XVI), demonstrated the statistical encapsulation of CF (Figure IV-14).\textsuperscript{22}

Figure IV-14. \textit{Top:} A. and B. CFM images of BSAPA structures XVI polymerized in the presence of CF (left, fluorescent image at 488 nm and \textit{right}, optical image). \textit{Bottom:} TEM micrographs of the aggregates observed after \textit{in situ} polymerization of the trimethylsilyl protected monomer 8 on BSA macroinitiator IX in the presence of carboxyfluorescein.

Since the synthesis of such multifunctional bioconjugates was attempted with the aim to further exploit the pending 1-alkyne chains, the deprotection of the trimethylsilyl groups
was pursued. A successful deprotection would unmask the protected 1-alkynes allowing the *clicking* of a variety of hydrophobic/hydrophilic azides. The deprotection step was investigated by the use of either TBAF or KF. The selection of the above mentioned reagents was done on the basis of the reported in literature reaction conditions since, in the case of biomacromolecules, a significant factor directing the choice of reagents and conditions is protein stability.

After gentle shaking a ~ 0.1 mM solution of the amphiphiles XVI with 2000 equiv. of TBAF or KF for 24 hours (Scheme IV-10), the resulting solutions were dialyzed against 20 mM phosphate buffer, pH 7.4 to remove the excess of the reagents. In this case, the SEC chromatographic traces of the deprotection reaction samples XVIII and XIX, (Figure IV-13) were similar to that obtained for the bioconjugate XVI. Both electrophoresis, MALDI and IR were also unsuccessful in clarifying the result of the deprotection. CFM microscopy revealed that the superstructures were not affected by the deprotection step, in the case of the CF containing samples.

![Scheme IV-10. Reagents and conditions used for the deprotection of the pending alkyne side chains of the bioconjugate XVI.](image)

Though the above mentioned observations were not conclusive for the deprotection step, we reasoned that an indirect proof might be given upon performing a clicking reaction onto these superstructures. We decided to include in our experiments the azides 15, 16 and 21 which were synthesized as previously mentioned. It should be noted that the triethylene glycol azide 21, was used during this investigation because of its hydrophilic nature. We envisioned that by clicking a hydrophilic azide onto the polymer moiety chains of the bioconjugates, it would induce an overall change of hydrophilicity which would be expressed in its aggregation behaviour. The multi-*clicking* of different azide residues onto the polymer backbone was investigated using both XVIII and XIX. Since similar results were obtained in both cases, we
will only report on the results obtained by derivatization of compound XIX by the copper catalyzed Huisgen [3+2] click chemistry cycloaddition reaction.

Scheme IV-11. Reagents and conditions used to click the azido-derivatives 15, 16, 21 onto the deprotected BSA-polyalkyne XIX.

<table>
<thead>
<tr>
<th>R-N₃</th>
<th>BSA-PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>XXa 15</td>
<td>XIX</td>
</tr>
<tr>
<td>XXb 16</td>
<td>XIX</td>
</tr>
<tr>
<td>XXc 21</td>
<td>XIX</td>
</tr>
</tbody>
</table>

Table IV-3. Table summarizing the different multi-clicking experiments realized on XIX.

When the clicking of hydrophobic azides 15 or 16 was investigated using SEC chromatography (Scheme IV-11), an indication that the reaction had occurred was provided through the formation of a new peak with small intensity (probably due to aggregation of the corresponding Giant Amphiphiles on the column). The clicking of hydrophilic azide 21 on the other hand, led as expected to the observation of a new peak located in the higher molecular weight than that of the BSA-PA XIX before clicking.

An indirect proof of the formation of the clicked products came as planned through CFM microscopy. Interestingly, CFM revealed the formation of disordered structures collapsed on the glass slide for the clicked product XXc, whereas only discrete fluorescent aggregates were observed after the clicking of hydrophobic azides 15 or 16 possessing structures that were in good agreement to the spherical structures previously seen for Giant amphiphiles.
More specifically, as seen with CFM microscopy (Figure IV-15), both optical and fluorescence imaging (at 488 nm) revealed the presence of fluorescent superstructures, due to the encapsulation of fluorescein in the case of clicked hydrophobic residues 15 and 16. For the reaction product XXc, it was immediately noticed that the fluorescence background of the compound solution was intense and disorganized (gel like) fluorescent material was observed on the glass slide. These results provide the first indirect proof of the formation of the clicked products and of the applicability and efficiency of our approach toward the ATRP mediated formation of a plethora of Giant Amphiphiles. The superstructures were also studied by TEM and were observed to conserve the aggregation patterns shown by CFM.
Figure IV-15. CFM images of fluorescent aggregates observed for compounds A. XVI, B. compound XIX, C. compound XXa, D. compound XXb and E. compound XXc.

Figure IV-16. TEM pictures of structures observed with compounds XXa (left) and XXb (right).
IV.2.5. Encapsulation of material within the superstructures.

After the demonstration that the ATRP mediated in situ preparation of protein-polymer Giant Amphiphiles was feasible, extremely efficient and rather simple to perform under mild conditions, the possibility to use this method for the hierarchical formation of nanocontainers and/or nanoreactors was investigated. The utilization of the protein polymer Giant Amphiphiles for the construction of nanocontainers (able to carry different guest (bio)organic molecules) and nanoreactors (performing catalysis by hosting other proteins or even organic catalysts) has long been the Holy Grail of research in the field (Figure IV-17).

![Figure IV-17. Schematic representation of a nanoreactor with catalytically active enzymes encapsulated proceeding enzymatic reactions.](image)

Several problems, inherent to both the stability of the protein structure and the amphiphilic character of the biohybrids, made this a very difficult task. Once synthesized using the conventional methods, Giant Amphiphiles concurrently aggregate into non-dynamic superstructures. Any attempt to incorporate therefore other molecules within the superstructures would most possibly need harsh conditions, endangering both the protein stability and conformation and the aggregated architectures themselves. We envisioned that the gradual formation of the biohybrid polymeric chain by using protein initiated ATRP would, on the other hand, allow the hierarchical formation of such nanostructures. We therefore initiated a study aiming to prove whether it was possible to perform the ATRP mediated polymerization in the presence of a second, non polymerizable, protein and if so, to determine whether this second protein would be trapped within the superstructures during aggregation process of the BSA-polystyrene Giant Amphiphiles. The capacity of amphiphilic bioconjugates to concurrently form hierarchically assembled nanocontainers as hypothesized was investigated by performing the polymerization reaction in the presence of various non-polymerizable species such as a fluorescent dye (carboxyfluorescein, CF) or guest proteins (papain or Horseradish Peroxidase, HRP) that are shown on Table IV-4.
IV.2.5.1. Encapsulation of dyes or fluorescently labelled proteins.

Investigations on the possibility to form hierarchically assembled nanocontainers as hypothesized was confirmed by performing the polymerization reaction in the presence of a second non-polymerizable dye or labelled protein. To this end papain, a 23 kDa enzyme extracted from *Carica Papaya*, was labelled using either the NHS-activated ester of fluorescein (Figure IV-19, *left*) or the NHS-activated ester of Atto 610 (Figure IV-19, *right*), and once the fluorescent protein was purified by extensive dialysis against the buffer used in the optimized ATRP conditions (20 mM phosphate, pH 7.4), it was incorporated in the polymerization feed.

More specifically, a 150 fold excess of carboxyfluorescein or a 10 fold excess of the fluorescently labelled papain over the macroinitiator IX was integrated into the optimized reaction scheme (reaction XIII conditions, *i.e.* 2000 times excess of the monomer styrene over the biomacroinitiator IX) and the reaction was performed under the conditions described in section IV.2.2 (Scheme IV-13). A supplementary dialysis step was added after the
polymerization to remove the non encapsulated dyes / proteins. During this step, the products were dialyzed against 2% EDTA, 20 mM phosphate buffer pH 7.4 using 250 kDa dialysis bags, to afford yellowish aggregates that sediment with time.

**Figure IV-19.** Structure of the two commercially available dyes used to label proteins in this study: NHS-fluorescein (left, 488 nm) and NHS-Atto (right, 610 nm).

**Scheme IV-13.** General procedure used to statistically encapsulate labelled enzymes within superstructures obtained from the hierarchical self-assembly of the BSA-polystyrene Giant Amphiphiles. Inclusion followed by a post-polymerization labelling of the nanoassemblies.

SEC chromatography of the dialyzed samples revealed, as shown in **Figure IV-20**, the expected formation of Giant Amphiphiles in the papain inclusion experiment XXI, whereas
practically no free papain was detected. It should be mentioned that in a blank experiment that was performed under the same conditions in the absence of BSA-macroinitiator IX, the native papain was recovered suggesting that papain itself is not affected under ATRP conditions.

Figure IV-20. SEC chromatographic measurements at 254 nm of the ATRP polymerization reaction XXI in the presence of Carica papaya papain after dialysis (solid black trace) and of native Carica papaya papain (dashed grey trace).

TEM analysis of the superstructures revealed spherical architectures similar to those previously observed (i.e. reaction XIII, without the presence of fluorescein labelled papain, Figure IV-21), demonstrating that the presence of a non polymerizable protein in the reaction feed does not disturb the overall hierarchical aggregation process.

Figure IV-21. (Left) TEM micrograph of the aggregates observed after the in situ polymerization in the presence of fluorescein labelled Carica papaya papain XXI. (Right) CFM images of the fluorescently papain loaded aggregates demonstrating the statistical encapsulation of papain within the superstructures.
To determine whether the labelled *Carica papaya* papain had been encapsulated within the superstructures, the dialyzed samples were also analyzed with Confocal Fluorescence Microscopy (CFM, Figure IV-21). The comparison of the structures observed through the optical microscope and through fluorescence revealed that the labelled, non-polymerizable, enzyme was as expected statistically trapped within the superstructures without disturbing the self-assembly process. The possibility of non specific interactions between the superstructures and *Carica papaya* papain was further excluded by adding fluorescein labelled papain to preformed, non protein containing vesicles and following the dialysis purification steps. No fluorescence was observed both in this case as well as when a labelled *Carica papaya* papain sample was dialyzed in the absence of Giant Amphiphiles superstructures. When samples of the nanocontainers of encapsulated fluorescein-papain were further externally labelled with Atto (*i.e.* reaction XXV, Scheme IV-13), CFM revealed the statistical presence of both fluorescent species in the superstructures (Figure IV-22).

**Figure IV-22.** CFM images of BSA-PS polymerized in the presence of fluorescein labelled *Carica papaya* papain after external labelling of the superstructures with Atto-NHS dye (*reaction XXV*). Lines A, B, C represent 3 different areas of the sample. The scanning was independently performed at 610 nm (Atto, *left*) and 488 nm (Fluorescein, *right*).
IV.2.5.2. Hierarchical construction of BSA-PS nanoreactors.

Since the incorporation of a non-polymerizable dye or enzyme/protein in the polymerization feed allowed statistical encapsulation of material within the spherical superstructures without disturbing the hierarchical aggregation process, we decided to explore the formation of bioconjugate nanosized reactors. For this reason we focused our research on the encapsulation of horseradish peroxidase (HRP). This heme bearing, extracellular plant peroxidase (Figure IV-23), is probably one the most studied members of the plant peroxidase superfamily that catalyzes the oxidative coupling of phenolic compounds using hydrogen peroxide as the oxidizing agent.

![Figure IV-23. 3D structure of HRP (left) with the heme cofactor depicted molecule as red stick and protohemin IX structure (right). The enzyme is 5.7 x 3.5 x 3.3 mm in size.](image)

The HRP catalytic sequence consists from a three-step cyclic reaction (Scheme IV-14) in which the enzyme is first oxidized by hydrogen peroxide and then reduced in a two electron transfer step by reducing substrates, typically a small phenol derivative but also conjugated aromatic diamine compounds such as the 3,5,3′,5′-tetramethyl-biphenyl-4,4′-diamine generally referred to as tetramethyl benzydine (TMB).
HRP[Fe(III)Porph\(_2^-\)]\(^+\) + \(\text{H}_2\text{O}_2\)  

native state  

HRP[(Fe(IV)=O)Porph\(^*\)]\(^+\) + \(\text{H}_2\text{O}\)  

compound I

HRP[(Fe(IV)=O)Porph\(^*\)]\(^+\) + \(\text{AH}\)  

compound II

HRP[(Fe(IV)=O)Porph\(^*\)]\(^+\) + \(\text{AH}\)  

HRP[Fe(III)Porph\(_2^-\)]\(^+\) + \(\text{H}_2\text{O} + \text{AH}\)

\(\text{AH} = \text{substrate molecule, Fe (III)Porph = heme}\)

Scheme IV-14. Catalytic cycle of HRP in the presence of an oxidizing agent (hydrogen peroxide \(\text{H}_2\text{O}_2\)) and a reducing substrate (AH).

For the purposes of our studies, the ATRP polymerization of styrene was initiated on the BSA macroinitiator \(\text{IX}\) in the presence of a 15 time excess of HRP over \(\text{IX}\) and the optimized ATRP polymerization reaction conditions were utilized (\textit{i.e.} 2000 times excess of styrene over \(\text{IX}\), Table IV-4). The mixture was subsequently dialyzed first against 2\% EDTA 10\% DMSO 20 mM phosphate buffer, pH 7.4 using 25 kDa MWCO regenerated cellulose membranes and then against 20 mM phosphate buffer, pH 7.4 using 250 kDa MWCO polypropylene dialysis bags. Upon dialysis, a slightly different coloration (brown red) of the resulting solution was observed as compared to previous polymerizations performed in the absence of encapsulated, non polymerizing material.

![SEC chromatographic traces of polymerization](image)

**Figure IV-24.** SEC chromatographic traces of polymerization in the presence of HRP (reaction \textit{XXIV}) after dialysis (solid black trace), and native HRP (dashed grey trace).
SEC chromatographic analysis revealed the absence of free HRP in the dialyzed reaction mixture as shown in Figure IV-24. Finally, TEM demonstrated the formation of rather monodisperse, spherical aggregates with dimensions well in agreement with the previous experiments as shown in Figure IV-25.

Figure IV-25. TEM micrographs of the aggregates observed after in situ polymerization of styrene on BSA macroinitiator IX in the presence of HRP (reaction XXIV).

The efficiency and permeability of these hierarchically formed Giant Amphiphile nanocontainers was tested using a purified HRP containing nanoreactors solution and the standard TMB/H$_2$O$_2$ chromogenic assay. TMB, as shown in Scheme IV-15, produces by oxidation in the presence of HRP a conjugated aromatic diimine whose formation can be followed by following UV absorbance at either 670 nm or at 450 nm (after stopping the reaction with hydrochloric acid).

\[
\text{H}_2\text{N} \quad \begin{array}{c} \downarrow \text{NH} \end{array} \quad \text{H}_2\text{N} + \text{H}_2\text{O} \quad \text{HRP} \quad \text{H}^+ \quad \text{HN} \quad \begin{array}{c} \downarrow \text{NH} \end{array} + 2 \text{H}_2\text{O}
\]


More specifically, increasing quantities of a ready to use 3,3',5,5'-tetramethylbenzidine (TMB)/H$_2$O$_2$ solution (Sigma Cat nr: T0440) were added to a dispersion consisting of the purified BSA-PS HRP containing nanoreactors and, following a small lag time, the intense blue colour of the soluble reaction product of the one-electron oxidation of TMB was recorded at 650 nm. The deep yellow colour read at 450 nm after stopping catalysis with an acid solution provided a final, direct proof of the capacity of the nanoreactors (Figure
The reaction was also repeated utilizing increasing nanoreactors quantities with the same outcome. Blank experiments were conducted in the presence of BSA-polystyrene amphiphiles without any encapsulated protein. In this case, as expected, no catalytic reaction was observed as indicated the absence of peak corresponding to the product of the reaction at 450 nm.

![UV study](image.png)

**Figure IV-26.** UV study of the catalytic activity of HRP loaded in BSA-polystyrene Giant Amphiphiles superstructures. (*left:* curves obtained when increasing the volume of TMB added to the HRP loaded aggregates; *right:* absorbance at 450 nm as a function of TMB volume added).

### IV.2.6. Interaction of fluorescently labelled papain loaded BSA-polystyrene Giant Amphiphiles with living systems.

Though liposomes loaded with guest molecules, such as anticancer agents, have demonstrated clinical effectiveness, amphiphilic block copolymer vesicles are also now regarded as the encapsulators of the Future. Since most of the biomimetic systems are based on bilayer-forming low molar mass lipids which are generally not stable enough, polymeric systems are proving to be extremely useful in the understanding of natural processes (such as the clearance mechanism from the blood circulation for instance) or for applications like vectorisation. In fact, compared to lipids, most polymer membranes are hyperthick and can thereby achieve greater stability than any natural lipid membrane.

Consequently, synthetic diblock/triblock copolymers -polymersomes- have been extensively studied during the last years for their encapsulation properties and are indeed reported to be much more robust and suitable for this kind of applications. Furthermore, it is already clear that several biological membrane processes can be actually reproduced by polymer-based vesicles, as for instance biocompatibility, encapsulation or protein integration.
Up to date, polymersomes have already shown to be efficacious drug delivery systems.\textsuperscript{35,36,37} For example, diblock/triblock copolymers have been used to carry different bioactive compounds such as porphyrins for photoactive therapy,\textsuperscript{38} bioactive drugs such as anticancer agents (\textit{e.g.} paclitaxel\textsuperscript{39,40} and taxol\textsuperscript{41}) and even proteins used as therapeutics for different pathologies. Numerous of \textit{in vivo} and \textit{in vitro} studies have shown that these diblock/triblock copolymers are extremely promising compounds in the drug delivery systems development field, as they avoid for example fast clearance from blood or increase the biodistribution because of the higher stability of drugs in such nanocapsules. The fact that the block copolymers can be stimuli responsive (\textit{e.g.} pH responsive)\textsuperscript{42,43} increases the interest in their efficient utilization as they could in principle be programmed to release \textit{in vivo} bioactive compounds as a function of specific physiological conditions such as the pH,\textsuperscript{42} the presence of enzymes,\textsuperscript{43} etc. To this end, many research groups are focusing their efforts in developing “intelligent”, “smart”, biodegradable block copolymers with the potential to be utilized for a programmed interaction with living systems. Another interesting area of research aiming at the same direction focuses on the creation of glycopolymers with the aim to make use of their intrinsic affinity to biological systems to achieve such interactions.

Since \textit{Giant Amphiphiles} are in fact polymersomes, with biologically relevant amphiphilic structures, we envisioned the possibility that they could also exhibit an increased -compared to synthetic polymersomes- affinity with living systems which could potentially bring them into the active arena of drug delivery or controlled release systems.

We therefore decided to investigate whether \textit{Giant Amphiphile} hierarchically assembled nanostructures could interfere with biologically active systems aiming at their further development as nanocontainers, nanocarriers, or even better as drug delivery systems. A series of experiments was performed in collaboration with the group of Prof. U. Schwanenberg, aiming at the initial investigation of our assumption. In these experiments, the fact that the hydrophobic driving force for the aggregation of such systems arises from the presence of the non-biologically acceptable polystyrene was left to be addressed at a later stage as the polystyrene is not exposed while the superstructures are in aqueous solutions. Furthermore, based on the efficiency of the \textit{in situ} ATRP formation of \textit{Giant Amphiphiles}, we were confident that polystyrene could be easily replaced using ATRP and a biocompatible vinyl derivative if the initial results were promising.

Our initial goal involved studying the interaction of \textit{Giant Amphiphile} polymersomes with mammalian and bacterial cells. Unfortunately, the study with mammalian cells could not
be realized due to practical reasons. We therefore focused on three different types of bacterial cells and more specifically cells from *Escherichia coli*, *Bacillus* sp. and Yeast. Since Confocal Fluorescent Microscopy has proven to be a unique tool for the observation of interactions dealing with biologically relevant systems such as living cells, we tested this interaction using

- fluorescein-NHS externally labelled BSA-polystyrene *Giant Amphiphiles* (XXI),

and

- doubly labelled BSA-polystyrene *Giant Amphiphiles* obtained after polymerization of IX with styrene in the presence of Atto-labelled papain and externally labelled with the fluorescein-NHS activated ester (Figure IV-19).

Initially, minute volumes of Atto-labelled *Carica papaya* papain loaded vesicles XXVI (*i.e.* reaction XXII after an external labelling with NHS-activated fluorescein) were incubated with these 3 bacterial strains, *i.e.* *Escherichia coli*, *Bacillus* sp. and Yeast at different times of their cellular growth (0, 2 hours and 8 hours). Experimentally, 50 µL of the aggregates solution (~ 100 µM) was added to 1 ml of cell culture and 3 mL of nutritive Luria–Bertani (LB) growth medium. The cell suspensions were gently shaken for 12 hours either at ambient temperature or at optimum growth temperature (~37°C), were then centrifuged and the cell pellets collected and resuspended in growth medium to be examined by CFM microscopy. A 10 µL quantity of this sample was utilized on the glass slide, while no fixative proved to be nessecary.

No interaction was noted in the case of *Bacillus* sp. Cells even upon longer incubation periods. In the case of Yeast bacteria, we observed an interesting, reproducible adhesion of the superstructures on the cell walls (Figure IV-27).
Surprisingly, *E. coli* cells showed the most promising results. The samples after this 12 hours incubation time (and after washing), showed an efficient and reproducible cell uptake of the amphiphilic biomacromolecule nanoreactors. As shown in Figure IV-28, the fluorescence coincides with the presence of the cells (viewed through the optical measurement), while “empty” cells and free supstructures were also observed. In all cases, both dyes were detected by independent scanning, while it should be mentioned that the intensities of the internal dyes were lower. No difference was observed in the experiments varying on the growth temperature, demonstrating that cell cycle has no influence on the cell uptake of the aggregates. It should be noted that extensive washing with 0.1% NaCl solution were realized (and was followed by centrifugation and resuspension of the cells before measurements) in order to exclude any possibility of non specific interactions between the bacterial cell membranes and the aggregates.
In a first effort toward elucidating these interesting observations, the vesicle uptake was monitored as a function of incubation time. More specifically, 50 µL of the aggregates solution was added to 4 mL of the cell suspension as mentioned earlier and each batch was incubated for a different period of time (1, 2, 3, 4, 8, 12 hours) in order to follow cell uptake. It was observed that the cells are fully loaded with aggregates after only two hours of incubation whereas no fluorescence (i.e. no cell uptake) was observed after 1 hour of incubation as shown in Figure IV-29. Two interesting observations were also made during these experiments. Firstly, the uninhibited growing of the cells suggested that the presence of the superstructures does not result in destroying the cells. Furthermore, no loss of fluorescence in the cells was noticed when the incubation time was prolonged, this suggesting that there is probably no diffusion of the spherical superstructures out of the cells with time.
Figure IV-29. CFM pictures of *E. coli* cells incubated with Atto-labelled papain loaded BSA polystyrene XXVI *Giant Amphiphiles* aggregates for different periods of time (left, fluorescent image at 488 nm; right, optical image).

To further elucidate the process, FACS experiments were also conducted, the collection of fluorescent cells was nevertheless unsuccessful and in progress of being repeated.

Though it is premature to extract any conclusions on the exact nature of these interactions, our experiments proved without a doubt that *Giant Amphiphile* superstructures can penetrate *E. coli* bacterial membranes without causing the death of the cells and are therefore extremely promising for possible further applications and should be further pursued.

In this chapter, we developed the first, facile, efficient, ATRP mediated in situ preparation of BSA-polystyrene Giant Amphiphiles. After synthesis of a BSA-ATRP macroinitiator, we successfully achieved the polymerization of styrene onto this macroinitiator bioconjugate in a straightforward and efficient manner. We thus prepared a family of Giant Amphiphiles with a degree of polymerization controlled by the monomer to BSA macroinitiator ratio and narrow polydispersities. The yields of the reactions were found to be quantitative when the monomer to BSA macroinitiator ratio was above 1000:1. Furthermore, our studies proved a much narrower polydispersity of the produced biohybrids that the one previously observed for hydrophilic systems, which is attributed to the nature of the synthetic approach and the intrinsic amphiphilic character of the products. Within this chapter, results on the polymerization of two more vinyl monomers are also presented proving the efficiency and generic nature of the approach.

By a simple incorporation of a second non polymerizable species (dye or enzyme) in the polymerization feed, we demonstrated that a straightforward creation of nanocontainers was possible without steps that would interfere with the integrity of the protein or of the overall hierarchical aggregated nanoarchitecture. Furthermore, we demonstrated for the first time that BSA-polystyrene Giant Amphiphile nanocontainers are permeable to small molecules and can be used as nanoreactors.

Finally, we successfully observed in this study the interactions of these fluorescently labelled papain loaded BSA-PS Giant Amphiphiles superstructures with E. coli bacterial cells that took place within a couple of hours. Due to their encapsulation and aggregation properties, Giant Amphiphiles might be regarded as very promising and interesting compounds for biomedical applications. Following this bacterial cell uptake experiment success, we can envision that in the near future, just by changing the nature of the hydrophobic polymer attached to the protein, biocompatible polymer-proteins Giant Amphiphiles could be successfully adapted to the preparation of drug delivery systems or protein nanocarriers.
Current efforts are directed toward the extension of this method to a broad range of monomers and toward the in situ preparation of nanofunctional assemblies e.g. multienzymatic nanoreactors.
References

[17] We assume this statement to be true only for relatively narrow variation in terms of molecular weights of the hydrophobic polymers utilized in these studies.
[20] Taking into account the different nature of fluorinated polymers as compared to the polymers previously studied, this assumption is only based on the observation of the broadness of the peaks. Further studies involving digestion of the protein, isolation of the polymer and further characterization, are judged to be necessary to fully support this assumption.
[21] Fluorescein was excited with the 488 line of the Argon-Krypton laser during the CFM measurements.
[44] LB medium was prepared according to the recipe of Miller: 5 g yeast extract, 10 g peptone tryptone, 10 g NaCl) Miller J.H. (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor.
Chapter V
General Conclusion & Perspectives

The work undertaken during this PhD thesis allowed the development of new synthetic pathways towards the preparation of Giant Amphiphiles under milder conditions that those previously employed and with better yields.

In a first approach, by the use of an heterobifunctional linker containing a terminal maleimide and an alkyne-1 functionality, protein bioconjugates containing a terminal alkyne function were synthesized. The coupling of a series of hydrophobic azido polymers with the alkyne-1-functionalized proteins using the copper catalyzed variant of the [3+2] Huisgen dipolar cycloaddition (“click” chemistry), led to the formation of a small library of Giant Amphiphiles. When varying the length of the polymer moiety, no change in aggregation behaviour of the resulting superstructures was observed. The click mediated method was also successfully utilized in other free cysteine containing proteins, proving its generic nature. Through this study, it was demonstrated that the aggregation pattern of such biomacromolecular systems is highly dependent on the protein nature as the BSA-polystyrene Giant Amphiphiles formed well defined spherical aggregates in water whereas cylindrical aggregates were observed with Hemoglobin-polystyrene Giant Amphiphiles. Interestingly, it was for the first time demonstrated that the Giant Amphiphiles aggregate also in organic solvents, leading to reverse superstructures in which we expect the protein to be in the core and the polymer exposed to the solvent. This exciting outcome was further utilized in order to bring an enzyme hosted by these superstructure in organic solvents and observe its catalytic action.

In the second part described within the thesis, a novel methodology was designed with the aim to bypass the incompatibility between the hydrophilic protein and the hydrophobic polymer. This new approach was based on two discrete steps where initially a hydrophilic multifunctional polymer was selectively attached to a protein through a 1,4-Michael thiol
addition and in a second step hydrophobicity was introduced by a multicking step in which small hydrophobic azides were grafted onto the polymer backbone efficiently and under mild conditions. It was demonstrated that the size distribution of the aggregates obtained after self-assembly depends on the hydrophobic azide used. The prospect of the further development of new Giant Amphiphile derivatives where the clicked moiety would express its intrinsic characteristics (i.e. catalysis, electron transfer properties, etc) and introduce multifunctionality to the amphiphilic biomacromolecules is exciting. It would for instance be interesting to multick terpyridine azides onto the polymer backbone as it would allow some further metal to complex interactions and thus create a tunable cross linking of the superstructures.

The last method that was developed relied on the in situ ATRP mediated polymerization of hydrophobic monomers onto a protein macroinitiator and afforded very interesting results. First of all, it allowed for the synthesis of Giant Amphiphiles to proceed in quantitative yields but more importantly in quantitative amounts while their final isolation was achieved by a simple dialysis step. Furthermore, it was demonstrated that an enzyme could be hosted within these hierarchical self-assembled superstructures in a one-pot procedure and without disrupting their aggregation behaviour. More importantly, using classical enzymatic tests, it was demonstrated that, when HRP was encapsulated within the superstructures, it retained its catalytic activity. This method allowed not only the inclusion of active biomolecules but also the observation of their activity as the structures surprisingly proved to be permeable. The formation of multienzymatic nanoreactors is foreseen to be one of the most exciting prospects for future studies.

Compared to the previously reported procedures for the synthesis of Giant Amphiphiles in which high organic solvent contents were used or tedious purification procedures were required, these newly synthetic methodologies were found to proceed under much milder conditions and, especially in the in situ polymerization approach, required simpler purification steps to afford products in relatively large quantities. It can be envisioned therefore that in the near Future, a collection of different active enzymes could be used to form such superstructures tuned for example in a way that they could undergo enzymatic cascade reactions.

In this thesis work, some of the properties of Giant Amphiphiles were highlighted and found to be promising to find utility in areas of bio and nanotechnologies in the near Future. The results on the interaction of such superstructures with bacterial cells are for example
intriguing and promising. Their interaction with mammalian cells should be certainly investigated. The big challenge would be to construct also systems with biocompatible polymer moieties as they should in principle allow better interactions with cell membrane and could lead to important outcomes in the area of drug delivery systems for example.
Chapter VI
Experimental Part

General Remarks

Starting Materials. All chemicals were purchased from Fluka Chemica or Sigma-Aldrich (unless otherwise specified) and used without further purification. Cu(I)Br was purified as reported by Keller and Wycoff.\(^1\) \(N\)-(\(n\)-propyl)-2-pyridylmethanimine and 2-Methyl-acrylic acid 2,2-dimethyl-[1,3]dioxolan-4-ylmethyl ester were prepared as described earlier and stored at 0 °C. Protected maleimido initiator, protected alkyne monomer, fluorescent hostasol comonomer and 1-azido-decane and benzyl-azide were synthesized according to the literature. Hostasol tag methacrylate was obtained from Dave Haddleton’s research group. NEt\(_3\) was dried over KOH pellets. Deuterated solvents were obtained from Cambridge Isotope Laboratories. Fluorescein-NHS activated ester (6-[Fluorescein-5(6)-carboxamido]hexanoic acid N-hydroxysuccinimide ester) and Atto 610 N-succinimidyl ester were purchased from Fluka Biochemica, dissolved in dry DMSO to afford a 5 mM solution and stored at -20 °C. Bovine Serum Albumin (BSA), Cytochrome C (from equine heart), Hemoglobin (Hb) were purchased from Sigma Aldrich. Papain and Horseradish Peroxidase (HRP) were purchased from Calbiochem. Polymerizations were carried out using standard Schlenk techniques under an inert atmosphere of oxygen-free nitrogen, unless otherwise stated. Yields of the reactions were not optimized.

Analytical techniques.
Aqueous size exclusion chromatography (SEC) was conducted using a Shimadzu modular system comprising a DGU-14A solvent degasser, a LC-10AD pump, a CTO-10A column oven, an SIL-10AD auto-injector, a RID-10A refractive index detector and a SPD-10A Shimadzu UV Vis. Spectrometer. The system was equipped with a Polymer Laboratories
30x7.8mm 5µm BioBasic SEC 60 guard column followed by a 300x7.8mm 5µm BioBasic SEC 300 Polymer Laboratories column, using a mixture of 70% phosphate buffer 20 mM pH 7.4, 30% acetonitrile or a mixture of 0.1% TFA, 30% MeCN in MilliQ water as the eluent at room temperature and flow rate: 0.5 mL/min (unless otherwise noted). Chromatograms were acquired at 254 nm and 280 nm wavelength and were processed with the EZStart 7.3 chromatography software.

Gel Permeation Chromatography (GPC) was conducted on the Shimadzu VP HPLC system equipped with a PL Gel Thermo column eluting with THF as an eluent (unless otherwise noted).

NMR spectra were recorded on a Bruker 300 MHz and a Bruker 400 MHz spectrometer system. All chemical shifts are reported in ppm (δ) relative to tetramethylsilane, referenced to the chemical shifts of residual solvent resonances (1H and 13C). The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, m = multiplet. The molecular weights of the polymers \( M_n \) are calculated by comparing the integrals of the chain-end signals and appropriate peaks related to the polymer backbone.

Infrared absorption spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer (using a Golden Gate diamond or a NaCl cuvette).

UV-vis spectra were recorded on a CARY 1 BIO UV-visible spectrophotometer.

Solutions were sonicated in a Bandelin Sonorex RK 100 apparatus.

Confocal microscopy experiments were performed with a Leica TCS SP2 AOBS confocal microscope using a 100x oil immersion objective. The Hostasol tag was excited with the 514 line of the Argon-Krypton laser. Fluorescein was excited with the 488 line of the Argon-Krypton laser. Atto was excited with the 543 nm Helium-Neon laser. Dual dye imaging was performed in sequential mode. Unidirectional scanning was done at 400 Hz with an image format of 512 by 512 pixels.

Transmission Electron microscopy experiments were performed using a FEI Tecnai G2 Electron Microscope. Micrographs were taken using a Tietz CCD camera at a 2048 by 2048 pixel resolution. All samples for TEM measurements were prepared on Formvar (15/95E)-Cu grids by depositing a 1000 times diluted solution of dialyzed (against nanopure water) bioconjugate samples. The solvent was drained with filter paper after a deposition period of approximately 10 minutes. No platinum shadowing was necessary for the visualization of the samples.

MALDI-TOF MS measurements were performed in the SVS-MS Mass Spectrometry Core Facility using an Axima CFR+ MALDI-TOF (Shimadzu Biotech, Manchester, UK) in
positive ionization mode and sinapinic acid as the matrix. Protein samples were mixed with matrix (1:1 volume ratio, sinapinic acid, 10 mg/ml) and air dried before analysis. Dithranol was used as matrix for the polymer samples.

Discontinuous Native PAGE\(^2\) (Ornstein-Davis) electrophoresis was run using a 4% stacking gel and a 10% resolving under standard nondenaturing conditions. Samples were dissolved in TRIS buffer containing bromophenol blue and were visualized using Coomasie Brilliant Blue or Silver Staining.
Chapter II. Formation of Giant Amphiphiles by Click Chemistry

2-[2-(2-Prop-2-ynyloxy-ethoxy)-ethoxy]-ethanol (1): In a two necked round bottom flask equipped with a cooler, to a solution of anhydrous triethylene glycol (3 g, 19.97 mmol) in dry THF (15 mL) was added under nitrogen atmosphere at 0°C sodium hydride (60% in oil) (0.160 g, 3.99 mmol). The resulting mixture was stirred 30 min. at 0°C and refluxed 2 hours at 80°C. Then, to this solution was dropwise added a solution of propargyl bromide 80% in toluene (404 µL, 3.63 mmol). The reaction mixture was refluxed at 80°C overnight under nitrogen atmosphere. Removal of the solvent under reduced pressure gave the crude product as yellow slurry. Final chromatography column with a mixture AcOEt/cyclohexane: 3/2 ($R_f = 0.23$ in AcOEt) gave the pure product as yellow oil in quantitative yield. $^1$H NMR (CDCl$_3$, 400 MHz, 298 K): $\delta$ 4.09 (d, 2H, $J = 2.1$ Hz, $\text{CH}_2\text{C}≡\text{CH}$), 3.65-3.40 (m, 12H, O$\text{C}$H$_2$C$\text{H}_2$O), 3.10 (s, 1H, $-\text{OH}$), 2.39 (t, 1H, $J = 2.2$ Hz, $-\text{C}≡\text{C}$H). $^{13}$C{1H} NMR (CDCl$_3$, 100 MHz, 298 K): $\delta$ 79.2 (1C, C$≡$CH), 74.4 (1C, C$≡$CH), 72.2 (1C, $-\text{CH}_2\text{O}$–), 70.2 (1C, $-\text{CH}_2\text{O}$–), 69.9 (1C, $-\text{CH}_2\text{O}$–), 69.9 (1C, $-\text{CH}_2\text{O}$–), 68.6 (1C, $-\text{CH}_2\text{O}$–), 61.2 (1C, $\text{CH}_3\text{OH}$), 57.9 (1C, $\text{CH}_2\text{C}≡\text{CH}$). ESI-MS (MeOH, +EI) m/z (relative intensity): 189 ([MH$^+$], <1), 162 (2), 89 (39), 45 (100).

1-[2-[2-(2-Prop-2-ynyloxy-ethoxy)-ethoxy]-ethyl]-pyrrole-2,5-dione (2): In a two necked round bottom flask, a solution of triphenylphosphine (386 mg, 1.47 mmol) in dry THF (10 mL) was cooled down to 78°C in a dry ice/acetone bath. To this mixture was dropwise added under nitrogen atmosphere at -78°C a solution of DEAD 40% in toluene (670 µL, 1.47 mmol). After 5 minutes of stirring was added the alcohol 1 (304 mg, 1.62 mmol) in solution in dry THF (10 mL). The resulting mixture was stirred 10 min. before the sequential addition of neopentyl alcohol (65 mg, 0.74 mmol) and maleimide (142 mg, 1.46 mmol). The
reaction mixture was stirred 5 additional minutes at -78°C and was then allowed to warm to ambient temperature overnight. Volatiles were removed under reduced pressure. The crude product (pink slurry) was purified by chromatography column eluting with a mixture AcOEt/cyclohexane: 2/3 \((Rf = 0.45 \text{ in AcOEt/cyclohexane: 3/1})\) to afford the compound 2 as a yellow oil (277 mg, 1.03 mmol, 70% yield). IR (neat): \(\tilde{\nu} = 3303, 3063, 2873, 1713, 1436, 1407, 1270, 1101, 828 \text{ cm}^{-1}\). \(^1\)H NMR (CDCl\(_3\), 300 MHz, 298 K): \(\delta 6.69 (s, 2H, H_C=C), 4.18 (d, 2H, J = 2.3 \text{ Hz}, CH_2−C≡CH), 3.75-3.56 (m, 12H, −CH_2O−), 2.42 (t, 1H, J = 2.4 \text{ Hz}, −C≡CH)\). \(^{13}\)C\{\(^1\)H\} NMR (CDCl\(_3\), 100 MHz, 298 K): \(\delta 170.6 (2C, CO_{imide}), 134.1 (2C, C_{vinyl}), 79.7 (1C, C==CH), 74.5 (1C, C==CH), 70.5 (1C, CH_2), 70.4 (1C, CH_2), 70.1 (1C, CH_2), 69.1 (1C, CH_2), 67.9 (1C, CH_2).\) ESI-MS (MeOH, +EI) m/z (relative intensity): 268 ([MH\(^+\)], 2), 176 (7), 149 (8), 124 (100), 104 (22), 45 (43).

General procedure for azidation of polystyrene azides

3a, 3b, 3c: To freshly prepared 0.5 M solution of sodium azide in DMSO (5 mL) were added 0.625 mmol of the bromide end-capped polystyrene (Mw 2015, 4360, 8100). After overnight stirring at 60°C, the solutions were quenched with 1 M NaOH solution. The resulting azidopolystyrenes 3a, 3b or 3c were recovered by DCM extraction and purified by filtration after precipitation in a large volume of MeOH. The pure products were analyzed by MALDI-TOF \((M_w=1978, 4322 \text{ and } 8063)\) respectively found for 3a, 3b, 3c; see Figure VI-1 for 3a), FT-IR (characteristic C−N\(_3\) stretching band observed for each polystyrene azide 3a, 3b, 3c) and \(^1\)H NMR (characteristic triplet observed at 2.71 ppm corresponding to the CH in \(\alpha\) of the azide group) and GPC (THF/triethylamine: 95/5, 2 PL Gel mixed column (Thermo), 0.5 mL.min\(^{-1}\), retention time = 16.01, 15.09 and 14.67 min. observed respectively for 3a, 3b, 3c).

Figure VI-1. MALDI-TOF spectrum of PS-N\(_3\) 2 kDa (expected \(M_w=1977\), obtained \(M_w=1978\)) obtained after azidation of PS-Br 2 kDa (2015).
Preparation of alkyne functionalized BSA (I): Native BSA (66.5 kDa) was dissolved in 20 mM PB pH 7.4 to obtain a concentration of ca. 0.3 mM. To a solution of this native protein (850 µL) in phosphate buffer pH 7.4 was added a 0.2 M solution (127 µL) of the heterobifunctional linker 2 dissolved in PBS 20 mM pH 7.4. The mixture was completed with 20 mM PBS solution until reaching a total volume of 1 mL. After gentle shaking for 24 hours at 7°C, the mixture was dialyzed against 20 mM PB pH 7.4. Finally, the biohybrids solutions were analyzed by SDS-PAGE (Figure VI-2, lane 2), SEC-HPLC (30% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min\(^{-1}\), retention time : 9.52 min., Figure VI-4, blue trace), and MALDI-TOF analysis (MW~66.9 kDa) and their aggregation behaviour was studied by TEM microscopy (no aggregation observed).

![Figure VI-2. SDS-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) under denaturating conditions stained with Coomassie Brilliant Blue: lane 1, native BSA; lane 2, purified bioconjugation reaction mixture I; lane 3, native BSA; lane 4, BSA-PS 2 kDa IIa; lane 5, BSA-PS 4 kDa IIb; lane 6, BSA-PS 8 kDa IIc.](image)

![Figure VI-3. SEC-HPLC of native BSA (dashed grey trace) and alkyne functionalized BSA I (solid black trace).](image)
Preparation of alkyne functionalized Cytochrome C (III) and alkyne functionalized hemoglobin (IV): same procedure than for the preparation of alkyne functionalized BSA (I). Here Hemoglobin Hb (64 kDa) and Cytochrom C CytoC (11 kDa) were used.

General procedure for the preparation of compounds (IIa), (IIb), (IIc): In an eppendorf, containing 276 μL of PBS 20 mM pH 7.4, 100 μL of a 15 mM solution of the polystyrene azide 3a, 3b or 3c in THF were injected while sonicating. The solution was sonicated for another 10 min. before the addition of 16 μL of a sodium ascorbate solution (40 mM), 8 μL of a copper sulfate solution (40 mM) and 600 μL of alkyne functionalized BSA solution (I) (0.25 mM). The resulting turbid solutions were gently shaken for 2 days at 7°C in dark conditions and were then extensively dialyzed using 10 kDa dialysis bags against either 20mM phosphate buffer pH 7.4 or nanopure water depending on the analysis. All reactions were analyzed by SDS-PAGE (Figure VI-2), SEC-HPLC (30% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min⁻¹, Figure VI-4) and MALDI-TOF-MS and aggregation of the biohybrids studied by TEM microscopy.

Characterization of bioconjugate IIa by SDS-PAGE (Figure VI-2, lane 4), SEC-HPLC (30% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min⁻¹, Figure VI-4, black trace, retention time : 7.33 min) and MALDI-TOF-MS (Figure VI-5, MW~68.7 kDa) analysis and their aggregation behaviour was studied by TEM microscopy (Figure VI-6, line A).

Characterization of bioconjugate IIb by SDS-PAGE (Figure VI-2, lane 5), SEC-HPLC (30% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min⁻¹, Figure VI-4, red trace, retention time : 7.66 min) and their aggregation behaviour was studied by TEM microscopy (Figure VI-6, line B).

Characterization of bioconjugate IIc by SDS-PAGE (Figure VI-2, lane 6), SEC-HPLC (30% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min⁻¹, Figure VI-4, green trace, retention time : 7.66 min) and their aggregation behaviour was studied by TEM microscopy (Figure VI-6, line C).
Figure VI-4. SEC chromatographic characterization of the blank experiment (I + 3a, 3b and 3c without CuSO$_4$/sodium ascorbate, black dotted trace) and BSA-polystyrene amphiphiles IIa, IIb and IIc (respectively solid black, solid grey and dashed grey traces) after dialysis.

Figure VI-5. MALDI-TOF analysis of BSA-PS 2 kDa IIa.
Figure VI-6. TEM micrographs of superstructures obtained by aggregation of A. BSA-PS IIa, B. BSA-PS IIb and C. BSA-PS IIc.

**General procedure for the preparation of compounds** (Va), (Vb), (Vc): Same procedure as for the preparation of compounds IIa, IIb and IIc. All reactions were analyzed by SDS-PAGE (no significative results found), SEC-HPLC (30% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min\(^{-1}\), Figure VI-7 left, red, blue and green traces), and MALDI-TOF (Figure VI-8 left, only for compound Va) analysis. No aggregation was observed by TEM microscopy.

Characterization of bioconjugate Va by SEC-HPLC (30% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min\(^{-1}\), Figure VI-7 left, red trace, retention time : 11.38 min) and MALDI-TOF-MS analysis (Figure VI-8 left, MW~13 kDa) and no aggregation behaviour was observed by TEM microscopy.

Characterization of bioconjugate Vb by SEC-HPLC (30% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min\(^{-1}\), Figure VI-7 left, green trace, retention time : 10.92 min) and no aggregation behaviour was observed by TEM microscopy.
Characterization of bioconjugate \textbf{Vc} by SEC-HPLC (30\% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min\(^{-1}\), Figure VI-7 left, blue trace, retention time : 10.82 min) and no aggregation behaviour was observed by TEM microscopy.

**General procedure for the preparation of compounds (VIa), (VIb), (VIc):** Same procedure as for the preparation of compounds \textbf{IIa}, \textbf{IIb} and \textbf{IIc}. All reactions were analyzed by SDS-PAGE (no significative results found), SEC-HPLC (30\% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min\(^{-1}\), Figure VI-7 right; red, blue and green traces), and MALDI-TOF (Figure VI-8 right, only for compound \textbf{VIb}, peak found at 68.7 kDa) analysis and their aggregation behaviour was studied by TEM microscopy (Figure VI-9).

Characterization of bioconjugate \textbf{VIa} by SEC-HPLC (30\% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min\(^{-1}\), Figure VI-7 right, red trace, retention time : 9.17 min) and their aggregation behaviour was observed by TEM microscopy (Figure VI-9).

Characterization of bioconjugate \textbf{VIb} by SEC-HPLC (30\% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min\(^{-1}\), Figure VI-7 right, green trace, retention time : 9.21 min) and MALDI-TOF-MS analysis (Figure VI-8 right, MW~68.2 kDa) and same aggregation behaviour as \textbf{VIa} was observed by TEM microscopy.

Characterization of bioconjugate \textbf{VIc} by SEC-HPLC (30\% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min\(^{-1}\), Figure VI-7 right, blue trace, retention time : 9.15 min) and same aggregation behaviour as \textbf{VIa} was observed by TEM microscopy.
Figure VI-7. SEC chromatographic analysis of native proteins and amphiphilic biomacromolecules (left). Left: native Cytochrome C and amphiphilic CytoC-polystyrene bioconjugates Va, Vb, Vc at 254 nm. Right: native Hb and amphiphilic Hb-polystyrene bioconjugates VIa, VIb, VIc at 406 nm.

Figure VI-8. MALDI-TOF spectra obtained for Left: native Cytochrome C and its 2 kDa polystyrene conjugate Va and Right: for native Hemoglobin and 2kDa polystyrene conjugate VIb when mixed with a sample of native BSA for stabilization.

Figure VI-9. TEM micrographs of aggregates observed with polystyrene-Hb Giant Amphiphiles VIa.
Observation of compound IIb in dichloromethane: 1mL of the suspension of IIb initially in 20 mM PB was freeze-dried and immediately redissolved with the same volume of pure dichloromethane. The sample was immediately either observed by TEM microscopy or negatively stained with uranyl acetate (for further study). TEM micrographs observed are shown below on Figure VI-10 and Figure VI-11.

**Figure VI-10.** TEM micrographs of BSA-PS 4 kDa IIb in CH$_2$Cl$_2$. The two pictures on the bottom are negative stained (Uranyl Acetate).

**Figure VI-11.** A. TEM micrographs of BSA-PS 4kDa IIb in CH$_2$Cl$_2$. Micrograph on the left immediately after addition of the CH$_2$Cl$_2$, on the right 48h later. B. TEM micrographs of BSA-PEG$_{5000}$ in dichloromethane.
Catalytic activity of Glucose Oxidase in BSA-PS superstructures: To 990 µL of BSA-PS conjugate were added 10 µL of a Glucose Oxidase solution. The resulting solution was immediately freeze-dried and redissolved in 990 µL of dichloromethane (with a minute quantity of milliQ water). This cycle was repeated for 3 more times and finally the solution dialyzed against dichloromethane to get ride of untrapped enzyme. Finally, the resulting dialyzed solution was observed by CFM microscopy (before and after addition of selective for GO substrate, see Figure VI-12).

**Figure VI-12.** Confocal Fluorescent Microscope pictures of polystyrene-BSA superstructures after entrapment of β-galactosidase before (A. 488 nm, B. optical image, C. 405 nm) and after addition of fluorogenic substrate (D. 488 nm, E. optical image, F. 405 nm).
Chapter III. Formation of Giant Amphiphiles by Post-Functionalization of Hydrophilic Triblock Protein-Polymer Conjugates.

4,10-Dioxatricyclo[5.2.1.0\(^2,6\)]dec-8-ene-3,5-dione (10): Maleic anhydride (30.0 g, 306 mmol) was suspended in 150 mL of toluene and the mixture warmed to 80°C. Furan (33.4 mL, 459 mmol) was added via syringe and the turbid solution stirred for 6 h. The mixture was then cooled to ambient temperature and the stirring stopped. After 1 h, the resulting white crystals were collected by filtration and washed with 2 x 30 mL of petroleum ether to obtain 44.4 g (267 mmol, 87% yield) of the product 10 as small white needles. \(^1\)H NMR (CDCl\(_3\), 400 MHz, 298 K): \(\delta\) 6.57 (t, 2H, \(J = 1.0\) Hz, CH\(_{\text{vinyl}}\)), 5.45 (t, 2H, \(J = 1.0\) Hz, CHO), 3.17 (s, 2H, CH). \(^{13}\)C\{1H\} NMR (CDCl\(_3\), 100 MHz, 298 K): \(\delta\) 170.0 (2C, CO), 137.1 (2C, CH\(_{\text{vinyl}}\)), 82.3 (2H, CHO), 48.8 (2C, CH). ESI-MS (MeOH, +EI) m/z (relative intensity): 167 ([MH\(^+\)], <1), 121 (7), 98 (22), 94 (13), 68 (100).

4-(2-Hydroxyethyl)-10-oxa-4-azatricyclo[5.2.1.0\(^2,6\)]dec-8-ene-3,5-dione (11): The anhydride 10 (2.00 g, 12.0 mmol) was suspended in MeOH (50 mL) and the mixture cooled to 0°C. A solution of ethanolamine (0.72 mL, 12.0 mmol) in 20 mL of MeOH was added dropwise (over ~10 min) and the resulting solution was stirred for 5 min at 0°C, then 30 min at ambient temperature, and finally refluxed for 4 h. After cooling the mixture to ambient temperature, the solvent was removed under reduced pressure, and the white residue was dissolved in 150 mL of CH\(_2\)Cl\(_2\) and washed with 3 x 100 mL of water. The organic layer was dried over MgSO\(_4\) and filtered. Removal of the solvent under reduced pressure furnished an off-white residue that was purified by flash chromatography to give the product 11 (1.04 g, 5.00 mmol, 42% yield) as a white solid. \(^1\)H NMR (CDCl\(_3\), 400 MHz, 298 K): \(\delta\) 6.52 (t, 2H, \(J = 0.9\) Hz, CH\(_{\text{vinyl}}\)), 5.28 (t, 2H, \(J = 0.9\) Hz, CHO), 3.78-3.76 (m, 2H, O\(_\text{CH}_2\)), 3.72-3.69 (m, 2H, NCH\(_2\)), 2.90 (s, 2H, CH), 1.90 (bs, 1H, −OH). \(^{13}\)C\{1H\} NMR (CDCl\(_3\), 100 MHz, 298 K): \(\delta\) 177.0 (2C, CO), 136.6 (2C, CH\(_{\text{vinyl}}\)), 81.0 (2C, CHO), 60.2 (1C,
OCH₂), 47.5 (2C, CH), 41.8 (1C, NCH₂), ESI-MS (MeOH, +EI) m/z (relative intensity): 210 ([MH⁺], 16), 142 (38), 111 (43), 110 (41), 98 (29), 82 (42), 68 (100).

**2-Bromo-2-methyl Propionic Acid 2-(3,5-Dioxo-10-oxa-4-azatricyclo[5.2.1.0₂,6]dec-8-en-4-yl) Ethyl Ester (6):** A solution of the alcohol 5 (2.22 g, 10.6 mmol) and Et₃N (1.60 mL, 11.7 mmol) in 120 mL of THF (the solution remained slightly turbid) was cooled to 0°C, and a solution of 2-bromo isobutyryl bromide (1.40 mL, 11.1 mmol) in 40 mL of THF was added dropwise (30 min). The white suspension was stirred for 3 h at 0°C and subsequently at ambient temperature overnight. TLC revealed the complete disappearance of the starting material. The ammonium salt was filtered off and the solvent removed under reduced pressure to give a pale-yellow residue that was purified by flash chromatography (CC, SiO₂, petroleum ether/ethyl acetate 1:1). We obtained 3.54 g (9.88 mmol, 93% yield) of 6 as a white solid. ¹H NMR (CDCl₃, 400 MHz, 298 K): δ 6.49 (t, 2H, J = 1.0 Hz, CH vinyl), 5.23 (t, 2H, J = 1.0 Hz, CHO), 4.30 (t, 2H, J = 5.3 Hz, OCH₂), 3.78 (t, 2H, J = 5.3 Hz, NCH₂), 2.84 (s, 2H, CH), 1.86 (s, 6H, CH₃). ¹³C{¹H} NMR (CDCl₃, 100 MHz, 298 K): δ 176.0 (2C, CO imide), 171.5 (1C, CO ester), 137.0 (2C, CH vinyl), 81.0 (2C, CHO), 62.3 (1C, OCH₂), 55.8 (1C, C(CH₃)₂Br), 47.6 (2C, CH), 37.6 (1C, NCH₂), 30.6 (2C, CH₃). ESI-MS (MeOH, +EI) m/z (relative intensity): 360 ([MH⁺], 5), 358 [MH⁺], 5), 292 (13), 290 (13), 151 (6), 149 (6), 210 (13), 191 (28), 124 (67), 123 (57), 110 (41), 69 (65), 68 (100).

**2-Methyl-acrylic acid 2,2-dimethyl-[1,3]dioxolan-4-ylmethyl ester (7):** In a three necked round-bottom flask, a mixture of (2,2-Dimethyl-[1,3]dioxolan-4-yl)-methanol (10.58 g, 53 mmol) and Et₃N (22. mL, 85 mmol) in 100 mL of anhydrous THF was cooled down to 0 °C under nitrogen atmosphere, and methacryloyl chloride (9.2 g, 88 mmol) was added dropwise. The mixture was allowed to warm to ambient temperature overnight and then stirred for 2 days at ambient temperature. Triethylamine hydrochloride salt was filtered off and the solvent removed under reduced pressure. The resulting viscous oil was dissolved in dichloromethane (100 mL), washed with a saturated NaHCO₃ solution (2 × 50 mL) and water (2 × 50 mL), and finally
dried over MgSO\textsubscript{4}. After filtration, removal of the solvent under reduced pressure gave a yellow viscous oil which was distilled under vacuum in presence of Galvinoxyl\textsuperscript{®} (radical inhibitor) to give the pure solketal methacrylate (10.25 g, 34 mmol, 64\% yield) as a colourless oil. IR (neat): $\tilde{\nu}$ = 2987, 2888, 1718, 1638, 1454, 1371, 1320, 1296, 1214, 1157, 1083, 1054, 941, 845, 814, 733, 649 cm\textsuperscript{-1}.\textsuperscript{1}H NMR (CDCl\textsubscript{3}, 400 MHz, 298 K): $\delta$ 6.16-6.09 (m, 1H, C=CH\textsubscript{H}), 5.61-5.53 (m, 1H, C=C\textsubscript{H}H), 4.37-4.24 (m, 1H, (CH\textsubscript{2})C\textsubscript{H}O), 4.22 (d, 2H, $J$ = 5.3 Hz, O=CO−C\textsubscript{H}\textsubscript{2}), 4.11 (dd, 1H, $J$ = 8.3, 6.1 Hz, O−C\textsubscript{H}a\textsubscript{H}−CH), 3.81 (dd, 1H, $J$ = 8.3, 6.1 Hz, O−CH\textsubscript{a}H\textsubscript{b}−CH), 1.97 (s, 3H, C\textsubscript{H}3−C=CH\textsubscript{2}), 1.45 (s, 3H, O−C(C\textsubscript{H}3)−O), 1.38 (s, 3H, O−C(C\textsubscript{H}3)−O).\textsuperscript{13}C{1H} NMR (CDCl\textsubscript{3}, 100 MHz, 298 K): $\delta$ 167.1 (1C, CO\textsubscript{ester}), 135.9 (1C, CH\textsubscript{3}−C=CH\textsubscript{2}), 126.1 (1C, CH\textsubscript{2}=C), 126.5 (1C, CH\textsubscript{3}=C=CH\textsubscript{2}), 99.2 (1C, C≡CSi(CH\textsubscript{3})\textsubscript{3}), 92.0 (1C, C≡CSi(CH\textsubscript{3})\textsubscript{3}), 53.0 (1C, OCH\textsubscript{2}), 18.4 (1C, CH\textsubscript{3}C=CH\textsubscript{2}), 0.16 (s, 9H, Si(CH\textsubscript{3})\textsubscript{3}).

2-Methyl-acrylic acid 3-trimethylsilanyl-prop-2-ynyl ester (8): A solution of trimethylsilyl propyn-1-ol (10.0 g, 78.0 mmol) and Et\textsubscript{3}N (14.2 mL, 101.3 mmol) in Et\textsubscript{2}O (100 mL) was cooled to -20°C and a solution of methacryloyl chloride (8.8 mL, 93 mmol) in Et\textsubscript{2}O (50 mL) was added dropwise over ca. 1 h. The mixture was stirred at this temperature for 30 min, then at ambient temperature overnight; the ammonium salts were removed by filtration and the volatiles removed under reduced pressure. \textsuperscript{1}H NMR analysis of the yellow oily residue did not reveal the presence of substantial amount of any impurity, but two additional faint spots were observed by TLC (petroleum ether/Et\textsubscript{2}O 20:1) analysis, the crude product was therefore purified by flash chromatography (CC, SiO\textsubscript{2}, petroleum ether/Et\textsubscript{2}O 50:1; $R_f$ = 0.67 in petroleum ether/Et\textsubscript{2}O 20:1). 12.4 g (63.2 mmol, 81\% yield) of 8 were obtained as colourless liquid. IR (neat): $\tilde{\nu}$ = 2960, 1723, 1638, 1452, 1366, 1314, 1292, 1251, 1147, 1035, 971, 942, 842, 813, 761 cm\textsuperscript{-1}.\textsuperscript{1}H NMR (CDCl\textsubscript{3}, 400 MHz, 298 K) $\delta$ 6.14-6.12 (m, 1H, C=CH\textsubscript{H}), 5.59-5.58 (m, 1H, C=C\textsubscript{H}H), 4.73 (s, 2H, OCH\textsubscript{2}), 1.94-1.93 (m, 3H, CH\textsubscript{3}C=CH\textsubscript{2}), 0.16 (s, 9H, Si(CH\textsubscript{3})\textsubscript{3}).\textsuperscript{13}C{1H} NMR (CDCl\textsubscript{3}, 100 MHz, 298 K) $\delta$ 166.6 (1C, CO\textsubscript{ester}), 135.8 (1C, CH\textsubscript{3}C=CH\textsubscript{2}), 126.5 (1C, CH\textsubscript{3}C=CH\textsubscript{2}), 99.2 (1C, C≡CSi(CH\textsubscript{3})\textsubscript{3}), 92.0 (1C, C≡CSi(CH\textsubscript{3})\textsubscript{3}), 53.0 (1C, OCH\textsubscript{2}), 18.4 (1C, CH\textsubscript{3}C=CH\textsubscript{2}), -0.2 (3C, Si(CH\textsubscript{3})\textsubscript{3}). ESI-MS (MeOH, +EI) m/z (relative intensity): 223 ([M+Na]\textsuperscript{+}, 4), 200 ([MH\textsuperscript{+}], 7), 160 (100), 133 (47), 86 (33).
Propyl-pyridin-2-ylmethylene-amine (10): A solution of 2-acetylpyridine (10 g, 82.5 mmol) in diethyl ether (50 mL) was placed in a Schlenk tube containing activated 3 Å molecular sieves under an atmosphere of dinitrogen. n-propylamine (8 mL, 97.3 mmol) was added and the reaction mixture stirred at room temperature for 18 h. The reaction was filtered, and then ether and excess n-propylamine were removed in vacuo. N-(n-Propyl)-2-pyridylethanimine was the second fraction recovered from the reaction mixture by vacuum distillation (5.5 g, 37 mmol, 45% yield). ¹H NMR (CDCl₃, 400 MHz, 298 K): δ 8.40-8.37 (m, 1H, CH$_{para}$), 7.94-7.92 (m, 1H, CH$_{meta}$), 7.48-7.46 (m, 2H, CH$_{ortho}$), 7.05-7.02 (m, 1H, CH$_{imine}$), 3.42 (t, 2H, $J = 5.1$ Hz, NCH$_2$), 2.28 (s, 3H, CH$_3$), 1.72-1.66 (m, 2H, CH$_2$CH$_3$). ESI-MS (MeOH, +EI) m/z (relative intensity): 171 ([M+Na]$^+$, 2), 148 ([MH]$^+$, 3) 107 (100), 78 (11), 57 (47).

General ATRP mediated polymerization procedure
- Synthesis of polymer (5):
In a Schlenk tube, the initiator 6 (609 mg, 1.70 mmol), the solketal monomer 7 (9.99 g, 49.9 mmol), the fluorescent hostasol co-monomer 9 (236 mg, 0.499 mmol) and the N-(n-propyl)-2-pyridylmethanimine ligand 12 (488 mg, 3.29 mmol) were dissolved in anisole (20 mL). The mixture was subjected to 5 freeze-pump-thaw cycles and then cannulated into a second Schlenk tube containing a magnetic stirrer and Cu(I)Br (236 mg, 1.65 mmol), previously evacuated and filled with nitrogen (t = 0). The resulting mixture was stirred at ambient temperature (~20°C) and aliquots were removed at regular intervals of time in order to monitor the evolution of both the conversion (¹H NMR) and the molecular weight (SEC). At 54% conversion, the protected trimethylsilillyl alkyne 8 was added as second monomer (3.0 g, 15 mmol). The reaction was stopped at 80% overall conversion. The reaction mixture was bubbled with air for 24 hours, passed through a neutral alumina pad, and precipitated by dropwise addition to a large amount of petroleum ether (~20:1 v/v ratio with respect to anisole). The pale orange powder was dissolved in the minimum amount of dichloromethane, passed through a short neutral alumina
pad (which was subsequently washed with additional dichloromethane) and precipitated again in petroleum ether to give, after filtration, the pure polymer 5 as a pale orange powder. GPC (THF/triethylamine: 95/5, 2 PL Gel mixed columns, 0.5 mL.min⁻¹): retention time 15.32 min, PDI 1.15. MALDI-TOF analysis (sinapinic acid): 11.5 kDa.

N.B.: Conversion of the solketal monomer was followed by ¹H NMR by comparison of the signals of the protons of the double bond with those of the methyl groups of the solketal.

Figure VI-13. ¹H NMR of polymer 5 in CDCl₃.

Synthesis of polymer (13) by deprotection of polymer 5: The polymer 5 (1.06 g, 0.0922 mmol) was dissolved in toluene (10 ml) and the solution refluxed overnight. The resulting polymer was precipitated in a large amount of petroleum ether (20:1 v/v with respect to the toluene) to afford a thin pale yellow powder. Volatiles were removed under reduced pressure to give the resulting maleimido-terminated polymer 13 in
close to 100% yield (1.05 g). GPC (THF/triethylamine: 95/5, 2 PL Gel mixed columns, 0.5 mL.min⁻¹): retention time 15.20 min, PDI 1.15. MALDI-TOF analysis (sinapinic acid): 11.5 kDa. \(^1\)H NMR (\(M_n\sim11.5\) kDa).

![Figure VI-14. \(^1\)H NMR of polymer 13 in CDCl₃.](image)

**Synthesis of polymer (14) by deprotection of polymer 13:** An aqueous 1.0 M acetic acid solution (1.5 equiv. mol/mol with respect to the alkyne-trimethylsilyl groups, 1.11 mL, 1.11 mmol) was added to a solution of the maleimido deprotected polymer 13 (1.05 g, 0.0922 mmol) in THF (25 mL) in a round bottom flask. Nitrogen was bubbled through the solution (ca. 10 min) and the yellowish solution was cooled to -20°C. A 1.0 M solution of TBAF·3H₂O in THF (1.5 equiv. mol/mol with respect to the alkyne-trimethylsilyl groups, 1.11 mL, 1.11 mmol) was added dropwise over a period of ca 2-3 min. The mixture was stirred at this temperature for 30 min. and then at ambient temperature overnight. The resulting solution was passed through a silica pad (to remove the
excess of TBAF) and the pad washed with THF. Removal of the volatiles gave the product as brown oil. Finally, the polymer was precipitated in petroleum ether to give the pure polymer 14 as a fine yellow powder close to 100% yield (1.0 g). It was further characterized by GPC (PDI~1.15) and $^1$H NMR ($M_n$~11 kDa). GPC (THF/triethylamine: 95/5, 2 PL Gel mixed columns, 0.5 mL.min$^{-1}$): retention time 15.08 min, PDI 1.15. MALDI-TOF analysis (sinapinic acid): 11.5 kDa. $^1$H NMR ($M_n$~11.5 kDa).

Figure VI-15. $^1$H NMR of polymer 14 in CDCl$_3$.

N.B.: Using the same experimental procedure as for the deprotection of 13, polymer 25 was obtained directly from the trimethylsilyl deprotection of polymer 5 (see $^1$H NMR on Figure VI-16).
Synthesis of polymer (4) by deprotection of polymer 14:
Polymer 14 (1.00 g, 0.0922 mmol) was dissolved in 1,4-dioxane (80 mL) in a round bottom flask and the resulting clear solution cooled to 0°C. The mixture became turbid upon addition of 33 mL of a 1 M HCl aqueous solution, and was subsequently allowed to slowly warm up to ambient temperature overnight and stirred for a further 24 hours. The reaction medium was neutralised by using a 1 M phosphate buffer pH 7.4 solution, at 0°C. Freeze-drying of the resulting mixture gave an orange powder which was dissolved in the minimum amount of dry methanol. Final filtration through a pad of neutral alumina, washing with dry methanol and removal of the solvent under vacuum afforded the final, totally deprotected polymer 4 as an orange powder (800 mg, 0.0842 mmol, yield = 91%).

GPC (THF/triethylamine: 95/5, 2 PL Gel mixed, 0.5 mL.min⁻¹): PDI~1.2, ¹H NMR

Figure VI-16. ¹H NMR of polymer 25 in CDCl₃.
(Figure VI-17, \(M_n\approx 8\) kDa) and MALDI-TOF analysis (solvent THF/water: 1/1, \(M_W\approx 8\) kDa).

**Figure VI-17.** \(^1\)H NMR of polymer 4 in CDCl\(_3\).

1-azidodecane (15): To a 0.5 M solution of NaN\(_3\) (0.325 g, 5.00 mmol) in DMSO (10 mL) was added 1-bromo-decane (0.44 g, 2.0 mmol) at ambient temperature. The solution was stirred for 24 hours at room temperature, then 10 mL of an aqueous 1M NaOH solution were added (the mixing was slightly exothermic). After cooling down to ambient temperature, the mixture was extracted with Et\(_2\)O. The organic layers were washed with brine, dried over MgSO\(_4\), filtered, and the solvent removed under reduced pressure to afford 0.33 g of the pure 1-azidodecane 15 (4.5 mmol, 90% yield) as a colorless oil. IR (neat): \(\tilde{\nu} = 2924, 2854, 2091\) (C-N\(_3\) absorption band), 1466, 1348, 1259, 893, 721 cm\(^{-1}\). \(^1\)H NMR (CDCl\(_3\), 400 MHz, 298 K) \(\delta\) 3.26 (t, \(J = 7.0\) Hz, 2H, CH\(_2\)N\(_3\)), 1.65-1.56 (m, 2H, N\(_3\)CH\(_2\)C\(_6\)H\(_2\)), 1.20-1.40 (m, 14H, CH\(_2\)), 0.89 (t, \(J = 6.8\) Hz, 3H, CH\(_3\)). \(^{13}\)C{\(^1\)H} NMR (CDCl\(_3\), 100 MHz, 298 K) \(\delta\) 51.5 (1C, CH\(_2\)-N\(_3\)), 31.9 (1C, CH\(_2\)), 29.5 (1C, CH\(_2\)), 29.3 (1C, CH\(_2\)), 29.2 (1C, CH\(_2\)), 28.9 (1C, CH\(_2\)), 26.9 (1C, CH\(_2\)), 26.7 (1C, CH\(_2\)), 26.7 (1C, CH\(_2\)), 26.7 (1C, CH\(_2\)), 26.7 (1C, CH\(_2\)), 26.7 (1C, CH\(_2\)), 26.7 (1C, CH\(_2\)), 26.7 (1C, CH\(_2\)), 26.7 (1C, CH\(_2\)).
22.7 (1C, CH₂), 14.1 (1C, CH₃). ESI-MS (MeOH, +EI) m/z (relative intensity): 206 [M+Na]⁺, <1), 184 ([MH⁺], <1), 155 (56), 140 (100).

**1-(azidomethyl)benzene (16):** In a round bottom flask, to a 0.5 M solution of NaN₃ (0.25 g, 3.8 mmol) in DMSO (8 mL) was added benzyl bromide (0.51 g, 3.0 mmol) at ambient temperature. The solution was stirred for 24 hours at ambient temperature and then quenched with 10 mL H₂O (reaction slightly exothermic). After cooling down to ambient temperature, the mixture was extracted with Et₂O. The organic layers were washed with brine, dried over MgSO₄, filtered off and the solvent removed under reduced pressure to afford the benzyl azide 16 pure product as pale yellow oil that was used for the click step without further purification. IR (neat): \( \tilde{\nu} = 3032, 2929, 2089 \) (C-N₃ absorption band), 1738, 1496, 1455, 1349, 1252, 1202, 1078, 1029, 875, 735, 695 cm⁻¹. \(^1\)H NMR (CDCl₃, 400 MHz, 298 K) \( \delta 7.40-7.34 \) (m, 5H, CH aromatic), 4.36 (s, 2H, CH₂-N₃). \(^{13}\)C{¹H} NMR (CDCl₃, 100 MHz, 298 K) \( \delta 138.9 \) (CIV), 128.9 (2C, CHortho), 128.4 (2C, CHmeta), 128.3 (1C, CHpara), 54.9 (2C, CH₂). ESI-MS (Ether/MeOH : 1/1, +EI) m/z (relative intensity): 156 ([M+Na]⁺, <1), 134 ([MH⁺], 4), 105 (100).

**1-(azidomethyl)-2,3,4,5,6-pentafluorobenzene (17):** To a 0.5 M solution of NaN₃ (0.25 g, 3.8 mmol) in DMSO (8 mL) was added pentafluorobenzyl bromide (0.51 g, 3.0 mmol) at ambient temperature. The solution was stirred for 24 hours at ambient temperature and then quenched with 10 mL H₂O (reaction slightly exothermic). After cooling down to ambient temperature, the mixture was extracted with Et₂O. The organic layers were washed with brine, dried over MgSO₄, filtered off and the solvent removed under reduced pressure to afford the benzyl azide 17 pure product as pale yellow oil. IR (neat): \( \tilde{\nu} = 3031, 2927, 2090 \) (C-N₃ absorption band), 1735, 1494, 1455, 1347, 1251, 1202, 1078, 1029, 873, 736, 696 cm⁻¹. \(^1\)H NMR (CDCl₃, 400 MHz, 298 K) \( \delta 2.6 \) (s, 2H, CH₂-N₃). \(^{13}\)C{¹H} NMR (CDCl₃, 100 MHz, 298 K) \( \delta 140.4 \) (1C, Cortho), 138.3 (2C, Cmeta), 137.1 (2C, Cpara), 128.3 (CIV), 54.8 (1C, CH₂). ESI-MS (MeOH, +EI) m/z (relative intensity): 246 ([M+Na]⁺, 2), 224 ([MH⁺], 3), 195 (28), 181 (100).
5,10,15,20-tetraphenylporphyrin (18): Benzaldehyde (10.6 g, 0.1 mmol), acetic acid (180 mL) and acetic anhydride (20 mL) were refluxed. Pyrrole (6.7 g, 0.1 mmol), freshly distilled, was added as quickly as possible, to avoid creating an irreversible exothermic reaction. The resulting mixture was refluxing for 30 minutes. After cooling down to r.t., the resulting solution was filtered off. The solid was washed with MeOH and dried under vacuo to afford 0.75 g (5.10 \(^{-3}\) mmol, 5% yield) of tetraphenylporphyrin 18 as a purple powder. \(^1\)H NMR (CDCl\(_3\), 400 MHz, 298 K) \(\delta\) 8.85 (s, 8H, H\(_{\beta}\)-pyrrole), 8.22 (dd, \(J = 1.5\) Hz and 7.6 Hz, 8H, CH\(_{\text{meta}}\)), 7.77 (m, 12H, CH\(_{\text{ortho/para}}\)), -2.78 (s, 2H, NH\(_{\text{pyrrole}}\)). ESI-MS (CH\(_2\)Cl\(_2\)/MeOH : 95/5, +EI) m/z (relative intensity): 615 ([MH\(^+\)], 100).

5-(4-Nitrophenyl)-10,15,20-triphenylporphyrin (19): Sodium nitrite (25 mg, 0.36 mmol) was added to a solution of 5,10,15,20-tetraphenylporphyrin (18) (123 mg, 0.20 mmol) in 12 mL of TFA. The resulting solution was stirred for 3 minutes and then poured into water (120 mL). The aqueous layer was extracted with DCM and joined organic fractions were washed with 30 mL of a NaHCO\(_3\) saturated solution, 30 mL of water, dried over Na\(_2\)SO\(_4\) and evaporated. Final chromatography column on silica gel eluting with DCM afforded the pure product as a purple powder (60 mg, 0.09 mmol, 45% yield). IR (CHCl\(_3\)): \(\tilde{\nu}\) = 1597, 1521, 1474, 1348, 966 cm\(^{-1}\). \(^1\)H NMR (CDCl\(_3\), 400 MHz, 298 K) \(\delta\) 8.86 (d, 2H, J = 5.0 Hz, H\(_{\beta}\)-pyrrole), 8.85 (s, 4H, H\(_{\beta}\)-pyrrole), 8.69 (d, 2H, J = 5.0 Hz, H\(_{\beta}\)-pyrrole), 8.54 (d, 2H, J = 8.9 Hz, H\(_{\text{nitrophenyl}}\)), 8.31 (d, 2H, J = 8.9 Hz, H\(_{\text{nitrophenyl}}\)), 8.19-8.05 (m, 6H, H\(_{\text{ortho triphenyl}}\)), 7.71-7.53 (m, 9H, H\(_{\text{meta/para triphenyl}}\)), -2.74 (s, 2H, NH\(_{\text{pyrrole}}\)). ESI-MS (CH\(_2\)Cl\(_2\)/MeOH : 95/5, +EI) m/z (relative intensity): 660 ([MH\(^+\)], 100), 613 (14), 535 (2.5), 330 (26), 306 (21).
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5-(4-Aminophenyl)-10,15,20-triphenylporphyrin (20): The para-nitrophenyltriphenylporphyrin (19) (330 mg, 0.5 mmol) was dissolved in 50 mL concentrated HCl solution (37%). To this solution was added SnCl$_2$·2H$_2$O (650 mg, 0.94 mmol) and the resulting mixture was stirred at 70°C for 1h30. The solution was poured into ice-cooled water, neutralized with KOH until pH~8 and extracted with DCM until the aqueous layer became colourless. Joined organic fractions were washed with water until observation of neutral pH, dried over MgSO$_4$ and evaporated. Final chromatography column on silica gel eluting with DCM afforded the pure product as a purple powder (144 mg, 0.22 mmol, 44% yield). $R_f$ = 0.46 in DCM. $^1$H NMR (CDCl$_3$, 400 MHz, 298 K) $\delta$ 8.95 (d, 2H, $J = 5.0$ Hz, $H_{\beta}$-pyrrole), 8.84 (d, 2H, $J = 5.0$ Hz, $H_{\beta}$-pyrrole), 8.83 (s, 2H, $H_{\beta}$-pyrrole), 8.22-8.08 (m, 6H, $H_{ortho}$ triphenyl), 8.00 (d, 2H, $J = 8.2$ Hz, $H_{aminophenyl}$), 7.76-7.57 (m, 9H, $H_{meta/para}$ triphenyl), 7.07 (d, 2H, $J = 8.3$ Hz, $H_{aminophenyl}$), 4.03 (s, 2H, NH$_2$), -2.77 (s, 2H, NH$_{pyrrole}$). ESI-MS (CH$_2$Cl$_2$/MeOH : 95/5, +EI) m/z (relative intensity): 630.5 ([MH$^+$], 5), 212 (0.5), 207 (45), 149 (100).

2-(2-(2-azidoethoxy)ethoxy)ethanol (21): A solution of 1.1 g (7.33 mmol) of dry triethylene glycol, 1 mL of dry TEA, and 10 mL of dry ether was cooled down to 0°C under a nitrogen atmosphere. Methanesulfonyl chloride (0.42 g, 3.665 mmol) was added over a 1-hour period, after which the solution was allowed to warm slowly to room temperature overnight. The reaction contents were concentrated in vacuo, and 15 mL of 95% ethanol and 0.524 g (8.06 mmol) of sodium azide were added. The mixture was heated at reflux for 24 h, cooled down to ambient temperature, and concentrated in vacuo. The remaining mixture was diluted with 10 mL of ether, washed with 5 mL brine, and dried over MgSO$_4$. Concentration in vacuo afforded the crude product, which was purified by silica gel chromatography eluting with a gradient of 1:1 to 3:1 ethyl acetate/cyclohexane to afford approximately 565 mg (3.22 mmol, 44% yield) of pure compound 21 as a slight yellow oil. IR (neat): $\nu$ = 3597, 3062, 2873, 2111 (C–N$_3$ absorption band), 1737, 1455, 1346, 1267, 1121, 1062, 930, 888, 715 cm$^{-1}$. $^1$H NMR (CDCl$_3$, 400 MHz, 298 K): $\delta$ 3.80-3.60 (m, 8H, $-CH_2O-$), 3.59 (t, 2H, $J = 5.1$ Hz, $CH_2OH$), 3.37 (t, 2H, $J = 5.3$ Hz, $CH_2-N_3$). $^{13}$C($^1$H) NMR (CDCl$_3$, 100 MHz, 298 K) $\delta$ 72.7
4-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)-4-oxobutanoic acid (22): To a solution of 0.2 g (1.14 mmol) of 2-(2-(2-azidoethoxy)ethoxy)ethanol (21) in dry toluene (2 mL) was added 0.447 g (4.57 mmol) of succinic anhydride. The resulting mixture was refluxing for 24 hours. The reaction contents were concentrated under vacuo and the crude was purified by silica gel chromatography eluting with a mixture 1:2 ethyl acetate/cyclohexane to afford the pure product 22 as a white viscous solid (292 mg, 1.06 mmol, 93% yield). IR (neat): $\tilde{\nu} = 2929, 2108$ (C−N$_3$ absorption band), 1736, 1270, 1265, 1262, 1134, 716 cm$^{-1}$. $^1$H NMR (CDCl$_3$, 400 MHz, 298 K) $\delta$ 4.27 (t, 2H, $J = 4.2$ Hz, CH$_2$−OCO−), 3.60-3.80 (m, 8H, −CH$_2$O−), 3.41 (t, 2H, $J = 5.1$ Hz, CH$_2$−N$_3$), 2.67 (s, 4H, CO−C$_2$H$_5$COOH). $^{13}$C{1H} NMR (CDCl$_3$, 100 MHz, 298 K): $\delta$ 176.6 (1C, CO$_{acid}$), 172.1 (1C, CO$_{ester}$), 70.8 (1C, CH$_3$), 70.5 (1C, CH$_3$), 70.1 (1C, CH$_3$), 69.1 (1C, CH$_3$), 63.9 (1C, CH$_2$−OCO), 50.7 (1C, CH$_2$−N$_3$), 29.1 (1C, CH$_2$COOH), 28.9 (1C, CH$_3$COO−). ESI-MS (MeOH, +EI) m/z (relative intensity): 298 ([M+Na]$^+$, 93), 276.1 ([MH]$^+$), 175 (20), 117 (31).

$N$-[4-(10,15,20-Triphenylporphyrin-5-yl)-phenyl]-succinamic acid 2-[2-(2-azidoethoxy)-ethoxy]-ethyl ester (23): To a solution of the acid 22 (37.7 mg, 0.137 mmol) in 5 mL of dry DCM at 0°C was added under a nitrogen atmosphere thionyl chloride (1 mL, 13.7 mmol) and DIPEA. The reaction mixture was let to warm slowly to ambient temperature under N$_2$ atm.
Then, the volatiles were evaporated to dryness and the resulting solid subsequentially redissolved in 5 mL dry DCM. Finally was added the porphyrin \textit{20} (26 mg, 0.033 mmol) and the reaction mixture was stirred overnight. The reaction was poured in a mixture 1 / 1 (v / v): half saturated NH$_4$Cl aqueous solution / DCM. The organic layer was washed with half saturated NH$_4$Cl and water, dried over Na$_2$SO$_4$, filtered and concentrated under vacuum. TLC showed the formation of the product but also non-reacted starting tetr phenyl porphyrin amine (ESI-MS of the reaction mixture confirms this result). The crude was purified by column chromatography using a gradient CH$_2$Cl$_2$ to CH$_2$Cl$_2$/MeOH (95/5). Finally, crystallization in a mixture DCM/pentane gave the pure product as a purple powder (26 mg, 0.031 mmol, 94% yield). IR (CH$_2$Cl$_2$): $\tilde{\nu}$ = 3032, 2929, 2089 (C-N$_3$ absorption band), 1738, 1496, 1455, 1349, 1252, 1078, 1029, 875, 735, 695 cm$^{-1}$. $^1$H NMR (CDCl$_3$, 400 MHz, 298 K): $\delta$ 8.95 (d, 8H, J = 2.3 Hz, H$_{\beta$-pyrrole}), 8.84 (d, 6H, J = 6.3 Hz, H$_{ortho$ triphenyl}), 8.13 (d, 2H, J = 7.6 Hz, =CH$_{amidophenyl}$), 7.95 (bs, 1H, NH), 7.85-7.65 (m, 9H, H$_{meta$/para triphenyl}), 7.69 (d, 2H, 6.8 Hz, =CH$_{amidophenyl}$), 4.28 (t, 2H, J = 4.3 Hz, CH$_2$OCO), 3.80-3.50 (m, 8H, O$^-$C$_2$H$_5$C$_6$H$_4$O), 3.27 (t, 2H, J = 4.1 Hz, CH$_2$N$_3$), 2.70 (s, 2H, CH$_2$COO), 2.54 (s, 2H, CH$_2$CO-N), -2.74 (s, 2H, NH$_{pyrrole}$). ESI-MS (CH$_2$Cl$_2$/MeOH : 95/5, +EI) m/z (relative intensity): 887 ([MH$^+$], 100), 861 (7), 859 (10), 744 (70).

2-(2-(2-azidoethoxy)ethoxy)ethyl 2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl) acetate (24): To a solution of thymine acid (150 mg, 0.814 mmol) in 5 mL of dry DMF was added under a nitrogen atmosphere HBTU (370 mg, 0.977 mmol) and DIPEA (1 mL, 5.74 mmol). The reaction mixture was stirred under N$_2$ atm. at r.t. for 1 hour. Then were added 285 mg (1.628 mmol) of compound \textit{21} and the reaction mixture was stirred for an additional hour. The reaction contents were concentrated to dryness, and the crude (yellowish oil) was purified by chromatography column eluting with a mixture DCM/MeOH: 9/1 to afford \textit{24} in quantitative yield. $^1$H NMR (CDCl$_3$, 400 MHz, 298 K) $\delta$ 9.83 (bs, 1H, NH), 7.02 (d, 1H, J = 1.2 Hz), 4.47 (s, 2H), 4.31 (t, 2H, J = 4.62 Hz), 3.76-3.56 (m, 8H), 3.38 (t, 2H, J = 4.99 Hz), 1.88 (s, 3H). $^{13}$C{1H} NMR (CDCl$_3$, 100 MHz, 298 K) $\delta$ 167.8 (1C, CO$_{ester}$), 164.7 (1C, CO$_{amide}$), 151.2 (1C, CO$_{amide}$), 140.8 (1C, CH=), 110.1 (C$^{IV}$), 70.5 (1C, CH$_2$), 70.0 (1C, CH$_2$), 68.8 (1C, CH$_2$), 65.0 (1C, CH$_2$), 55.4 (1C, CH$_2$), 50.7 (1C, CH$_2$-N$_3$), 48.6 (1C, N-CH$_2$), 12.2
(1C, CH$_3$-C=). ESI-MS (MeOH, +EI) m/z (relative intensity): 364 ([M+Na]$^+$, 23), 341 [MH$^+$], 54), 313 (45), 183 (100), 158 (11).

![Chemical structure diagram]

26a : R = C$_{10}$H$_{21}$
26b : R = Bz
26c : R = F$_5$Bz
26d : R = O

[3+2] Huisgen cycloaddition (“MultiClick” chemistry reaction) of azide 15, 16, 17 or 23 on polymer 25 to prepare polymer 26a, 26b, 26c, 26d:

General procedure with 1-azido-decane 15: A solution of polymer 25 (22 mg, 1.3.10$^{-3}$ mmol) in 100 µL of dimethylsulfoxide was added to 900 µL of a 20 mM phosphate buffer (pH 7.4). The yellowish suspension was sonicated before the addition of 1-azido-decane 15 (9.7 mg, 5.3.10$^{-2}$ mmol). Sodium ascorbate (2.5 mg, 1.3.10$^{-2}$ mmol) and CuSO$_4$ (1 mg, 6.10$^{-3}$ mmol) were sequentially added. The mixture was stirred at ambient temperature for 24 hours in the dark and the resulting greenish slurry was extracted with dichloromethane (3 × 3 mL). The organic fractions, collected, were passed through a pad of neutral alumina that was subsequently eluted with dichloromethane and then with THF. The final product was isolated by removal of the solvents under reduced pressure. The final pure product was subsequently analyzed by GPC, $^1$H NMR, and MALDI-TOF analysis.
N.B.: Using the same experimental, Benzyl azide 16 (9.7 mg, 5.3.10^{-2} mmol), pentafluorobenzyl azide 17 (9.7 mg, 5.3.10^{-2} mmol) and the thymine derivative 24 were clicked onto the polyalkyne 25 to afford the corresponding polymers 26b, 26c, 26d.

Characterization of 26a: GPC (toluene, 2 PL Gel mixed, 0.5 mL.min^{-1}): retention time: 14.88 min., PDI~1.2 (Figure VI-18). $^1$H NMR (Figure VI-19). MALDI-TOF-MS (THF/water: 1/1), $M_w = 11$ kDa.

Characterization of 26b: GPC (toluene, 2 PL Gel mixed, 0.5 mL.min^{-1}): retention time: 14.98 min., PDI~1.2 (Figure VI-18). $^1$H NMR (Figure VI-20). MALDI-TOF-MS (THF/water: 1/1), $M_w = 10.6$ kDa.

Characterization of 26c: GPC (toluene, 2 PL Gel mixed, 0.5 mL.min^{-1}): retention time: 15.00 min., PDI~1.2 (see Figure VI-18). $^1$H NMR (Figure VI-21). MALDI-TOF-MS (THF/water: 1/1), $M_w = 11.4$ kDa.

Characterization of 26d: GPC (toluene, 2 PL Gel mixed, 0.5 mL.min^{-1}): retention time: 14.80 min., PDI~1.2 (see Figure VI-18). $^1$H NMR (not possible because of copper (II) salts complexation on porphyrinic sites. MALDI-TOF-MS (THF/water: 1/1), $M_w$ not obtained.

![Figure VI-18. GPC traces of protected polyalkyne 25 (black curve) and purified clicked products 26a, 26b, 26c, 26d.](image)
Figure VI-19. $^1$H NMR of polymer $26a$ in CDCl$_3$.

Figure VI-20. $^1$H NMR of polymer $26b$ in CDCl$_3$. 
Synthesis of the BSA-PA bioconjugate (VII): A total of 1.9 mL of a 0.3 mM solution of Bovine Serum Albumin (BSA) in 20 mM phosphate buffer (PB) pH 7.4 was added to a solution of 360 µL of 20 mM PB pH 7.4 and 112 µL of a 50 mM solution of the polymer 14 in the same buffer. The reaction mixture was stirred gently at 7°C for 1 day. Removal of the unreacted polymer was achieved by filtration of the reaction mixture using Microcon® Centrifugal Filter Units (Millipore MWCO 30 kDa). Isolation of the biohybrid VII from the unreacted BSA was carried out on a Superdex 150 column eluting with 20 mM phosphate buffer pH 7.4. The enriched fractions were freeze-dried and analyzed by SEC (mobile phase: 70% 10 mM phosphate buffer pH 7.4, 30% CH₃CN, retention time: 18.24 min., Figure VI-23, red trace), electrophoresis under native and denaturing conditions (both after visualization under the UV lamp at 366 nm or after Coomassie Blue staining, Figure VI-22) and MALDI-TOF analysis (Figure VI-24, MW~66.6 kDa (unreacted BSA) and 74.9 kDa (BSA-polyalkyne VII conjugate). Samples of the pure biohybrid were used for the coupling experiments and imaging with TEM (no aggregation observed) and confocal microscopy (Figure VI-27, C).
Figure VI-22. SDS-PAGE analysis of native BSA (lane 1) and the bioconjugation reaction mixture of BSA and polymer 4 (lane 2) after Coomassie Blue staining (left) and visualization under the UV lamp at 366 nm (right).

Figure VI-23. SEC traces at 466 nm of native BSA (black trace), the BSA-polyalkyne VII conjugation reaction mixture (red trace) and polyalkyne 4 (green trace).

Figure VI-24. MALDI-TOF analysis of the bioconjugation reaction mixture between native BSA and polyalkyne 4.
General procedure for the preparation of compounds VIIIa, VIIIb, VIIIc:

**Synthesis of VIIIa:** 48 µL of 1-azido-decane were added to 166 µL of 20 mM phosphate buffer pH 7.4 and the resulting biphasic mixture was sonicated for ca. 10 minutes. Subsequently 250 µL of a 0.24 mM solution of BSA-polyalkyne VII in 20 mM phosphate buffer pH 7.4 were added. Finally 12 µL of a 100 mM sodium ascorbate solution in 20 mM PB pH 7.4 and 24 µL of a 100 mM solution of CuSO₄ in PB 20 mM pH 7.4 were added. The reaction mixture was stirred gently for 2 days at 7°C, in the dark. The final Giant Amphiphiles VIIIa were characterized by electrophoresis (Figure VI-25, lane 3) and their aggregation patterns imaged with TEM (Figure VI-26, A.) and confocal microscopy (Figure VI-27, A.).

**Synthesis of VIIIb:** 35 µL of azidomethyl-benzene were added to 179 µL of 20 mM phosphate buffer pH 7.4, the resulting biphasic mixture was sonicated for ca. 10 min. and was “clicked” to BSA-polyalkyne VII using the same conditions as for compound VIIIa. The final Giant Amphiphiles VIIIb were characterized by electrophoresis (Figure VI-25, lane 4) and their aggregation patterns imaged with TEM microscopy (Figure VI-26, B.).

**Synthesis of VIIIc:** 58 µL of azidomethyl-pentafluorobenzene were added to 156 µL of 20 mM phosphate buffer pH 7.4, the resulting biphasic mixture was sonicated for ca. 10 min. and was “clicked” to BSA-polyalkyne VII using the same conditions as for compound VIIIa. The final Giant Amphiphiles VIIIc were characterized by electrophoresis (Figure VI-25, lane 5) and their aggregation patterns imaged with TEM microscopy (Figure VI-26, C.).

![Figure VI-25. SDS-PAGE analysis of native BSA, BSA-polyalkyne VII and clicked amphiphilic bioconjugates:](image)

- lane 1, native BSA;
- lane 2, BSA-polyalkyne VII;
- lane 3, BSA-PA@C₁₀H₂₁ VIIIa;
- lane 4, BSA-PA@Bz VIIIb;
- lane 5, BSA-PA@F₃Bz VIIIc.
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Figure VI-26. TEM micrographs of BSA-PA@C_{10}H_{21}N_{3} VIIIa (line A), TEM micrographs of BSA-PA@Bz VIIIb (line B), TEM micrographs of BSA-PA@F_{3}Bz VIIIc (line C).

Figure VI-27. CFM images of (A). BSA-PA@C_{10}H_{21}N_{3} VIIIa aggregates (excitation with 514 Argon-Krypton laser line), (B). a BSA-PA@C_{10}H_{21}N_{3} VIIIa emulsion in a water / ethanol / decane mixture and (C). a BSA-PA VII solution.
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Chapter IV. In Situ, ATRP Mediated Hierarchical Formation of Bio Nanoreactors

2-Bromo-2-methyl-propionic acid 2-(2,5-dioxo-2,5-dihydropyrrol-1-yl)-ethyl ester (27): A solution of the maleimido-protected initiator 6 (0.15 g, 0.419 mmol) was suspended in dry toluene (5 mL) and heated to reflux under nitrogen atmosphere for 8 hours. The solvent was removed under reduced pressure to give a pale-yellow residue which was subsequently purified by flash chromatography (SiO\textsubscript{2}, petroleum ether/ethyl acetate 4:1) to furnish the compound 27 as a white solid (0.109 g, 0.38 mmol, 90% yield). \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}, 298 K): \( \delta \) 6.72 (d, \( J = 1.0 \) Hz, 2H, CH\textsubscript{vinyl}), 4.32 (t, \( J = 5.3 \) Hz, 2H, OCH\textsubscript{2}), 3.84 (t, \( J = 5.3 \) Hz, 2H, NCH\textsubscript{2}), 1.88 (s, 6H, CH\textsubscript{3}). ESI-MS (MeOH, +EI) m/z (relative intensity): 292 ([MH\textsuperscript{+}], 25), 290 ([MH\textsuperscript{+}], 29), 211 (100).

Prop-2-ynyl methacrylate (28): 3-(trimethylsilyl)prop-2-ynyl methacrylate (8) (500 mg, 2.55 mmol) was dissolved in THF (25 mL) and a 1 M acetic acid solution (3.825 mL, 3.825 mmol) was added. The resulting solution was cooled down to -20°C with an ice-acetone bath and nitrogen was bubbled in for \( ca. \) 10 min. before the addition of TBAF, 3H\textsubscript{2}O (1.207 g, 3.825 mmol). The resulting solution was let to warm to r.t. overnight. The solution was passed through a pad of silica and the pad was washed with another 25 mL of THF. Removal of the volatiles under reduced pressure afforded the pure product 28 as colourless oil (307 mg, 2.47 mmol, 97% yield). \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 400 MHz, 298 K) \( \delta \) 6.17-6.15 (m, 1H, C=CH\textsubscript{H}), 5.59-5.58 (m, 1H, C=CH\textsubscript{H}), 4.63 (d, 2H, \( J = 2.5 \) Hz, OCH\textsubscript{2}), 2.52 (t, 1H, \( J = 2.3 \) Hz, C=CH\textsubscript{H}), 1.94-1.93 (m, 3H, CH\textsubscript{3}C=CH\textsubscript{2}), \textsuperscript{13}C{\textsuperscript{1H}} NMR (CDCl\textsubscript{3}, 100 MHz, 298 K) \( \delta \) 18.4 (1C, CH\textsubscript{3}C=CH\textsubscript{2}), 53.0 (1C, OCH\textsubscript{2}), 92.0 (1C, C=CH), 99.2 (1C, C=CH), 126.5 (1C, CH\textsubscript{3}C=CH\textsubscript{2}), 135.8 (1C, CH\textsubscript{3}C=CH\textsubscript{2}), 166.6 (1C, CO\textsubscript{ester}). ESI-MS (MeOH, +EI) m/z (relative intensity): 147 ([M+Na\textsuperscript{+}], 100), 125 ([MH\textsuperscript{+}], 41), 55 (31).
(1-(perfluorobenzyl)-1H-1,2,3-triazol-4-yl)methyl methacrylate (29): 300 mg (2.42 mmol) of compound 28 and 540 mg (2.42 mmol) of compound 17 were dissolved in a 1:1 mixture tBuOH/water (10 mL). Upon addition of CuSO$_4$ (19 mg, 0.121 mmol) and L-(-) ascorbic acid sodium salt (48 mg, 0.242 mmol), the resulting mixture turned orange-brown. After stirring for 24 hours, the resulting solution was extracted with ether. Joined organic fractions were washed over MgSO$_4$ and the solvent removed under vacuo. Final chromatography column on silica gel eluting with DCM afforded 706 mg (2.03 mmol, 84% yield) of the resulting clicked product 29. $^1$H NMR (CDCl$_3$, 400 MHz, 298 K) δ 7.28 (s, CH$_2$triazole), 6.16-6.14 (m, 1H, C=CHH), 5.59-5.58 (m, 1H, C=CHH), 5.71 (s, 2H, NCH$_2$), 5.12 (s, 2H, OCH$_2$), 1.94-1.93 (m, 3H, CH$_3$C=CH$_2$). $^{13}$C{1H} NMR (CDCl$_3$, 100 MHz, 298 K) δ 166.3 (1C, CO$_{ester}$), 144.8 (1C, C$_{IV}$), 141.2 (1C, C$_{para}$), 138.7 (2C, C$_{ortho}$), 137.4 (2C, C$_{meta}$), 135.8 (1C, CH$_3$C=CH$_2$), 126.5 (1C, CH$_3$C=CH$_2$), 122.3 (1C, NCH=C), 111.4 (1C, C$_{IV}$), 64.8 (1C, OCH$_2$), 35.4 (1C, NCH$_2$), 18.4 (1C, CH$_3$C=CH$_2$). ESI-MS (Ether/MeOH : 1/1, +EI) m/z (relative intensity): 370 ([M+Na]$^+$, 3), 348 ([MH$^+$], 41), 280 (100).

Preparation of Bovine Serum Albumin Macroinitiator IX: A solution of the maleimide functionalized ATRP initiator 27 (126 mM, 0.8 mL) in DMSO was slowly added to 9.0 mL of a 0.35 mM solution of native BSA in 20 mM phosphate buffer (pH 7.4). The reaction mixture was gently shaken for 48 hours at 7°C. To eliminate the excess of the ATRP initiator 27, the mixture was then extensively dialyzed initially against 2% EDTA, 10% DMSO in 20 mM phosphate buffer pH 7.4 and then against 20 mM phosphate buffer pH 7.4 using 10 kDa regenerated cellulose dialysis membranes. The resulting solution of BSA-macroinitiator IX was subsequently analyzed by aqueous SEC to confirm that the excess of the initiator 27 was removed. Samples were prepared by dissolving 50 µL of the BSA-macroinitiator IX solution in 1 mL of 0.1% TFA, 5% DMSO, 30% MeCN in nanopure water. The BSA-macroinitiator IX was freeze-dried prior to its ATRP mediated in situ polymerization. Samples of freeze-dried bioconjugate IX could be stored at -20°C and successfully utilized after prolonged periods of time (up to 3 months). ATRP-macroinitiator was characterized by native gel electrophoresis (see Figure VI-28, left), SEC-HPLC (solvent: 70 % phosphate buffer 5 mM pH 7.4, 30 % acetonitrile, room temperature, column: SEC-300 BioBasic, flow rate: 0.5
mL min\(^{-1}\), see Figure VI-28 right, retention time: 13.77 min.) and MALDI-TOF-MS (solvent: water, see Figure VI-29, MW~66.7 kDa)

**Figure VI-28.** Left: Native gel electrophoretic profile of native BSA (lane 1), and the purified reaction mixture after conjugation of BSA with the ATRP initiator 27 (lane 2). Right: SEC-HPLC chromatographic traces of native BSA (solid black trace) and BSA-macroinitiator IX (dashed grey trace).

**Figure VI-29.** MALDI-TOF analysis of native BSA (grey) and BSA macroinitiator IX (black).
General procedure for the determination of free thiols by Ellman’s assay: 3,4 4 mg of 5,5’-dithio-bis-(2-nitrobenzoic acid) (Ellman’s reagent) was dissolved in 1 mL of buffer solution (0.1 M sodium phosphate buffer pH 8.0, containing 1 mM EDTA) to prepare Ellman’s reagent solution (10.09 mM). 0.25 µL of a BSA or BSA-macroinitiator IX conjugate solution (0.3 mM), 50 µL of Ellman’s reagent and 2.5 mL of buffer solution were mixed for 15 min. at room temperature. The absorbance at 412 nm was measured by a UV-vis spectrophotometer. The thiol concentration was calculated using the Beer-Lambert’s law (molar extinction coefficient of 2-nitro-5-thiobenzoic acid = 14,150 M⁻¹.cm⁻¹ at 412 nm). The initiator itself does not absorb in UV at 412 nm and does not affect the Ellman’s assay. Native BSA, before the conjugation with the initiator, was found to contain 47% free thiols, whereas after conjugation, no free thiols were detected.

General polymerization procedure with styrene (X-XIV): Several sets of polymerization experiments were performed varying the ratio between the monomer (styrene) and the BSA macroinitiator IX (Table 1). The ratio BSA-macroinitiator IX : CuBr : ligand 12 was kept constant at 1 : 41 : 70 in all experiments. Styrene (5 µL, 44 µmol, 50 equiv. up to 500 µL, 4.4 mmol, 5000 equiv., see Table I) and N-(Propyl)-2-pyridylmethanimine 12 (~9.1 mg, 0.036 mmol, 70 equiv.) were placed in a Schlenk tube and dissolved in solvent (10% DMSO in 20 mM phosphate buffer pH 7.4, 10 mL). The mixture was deoxygenated by 5 freeze-pump-thaw cycles and sonicated for 5 minutes to emulsify the monomer. The beginning of the polymerization was triggered by the canulation of the monomer solution under nitrogen atmosphere in a second deoxygenated Schlenk tube containing the crystalline BSA-macroinitiator IX (~59 mg, 0.88 µmol, 1 equiv.) and CuBr (5.2 mg, 36 µmol, 41 equiv.) under N₂ atmosphere. A dark brown colour was immediately observed. The reaction mixture was stirred under inert atmosphere during 2 up to 24 h after which they were exposed to oxygen (Cu(I) to Cu(II)) and stirred for another 2 to 12 hours.

Several control experiments in the absence of the biomacroinitiator IX, or the monomer or Cu(I) and in the presence of oxygen were performed and are also listed in Table 1.
Table 1. Polymerization experiments with varying ratios between the monomer (styrene) and the BSA macroinitiator IX, and control experiments.

<table>
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<td>1</td>
<td>50</td>
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<td>5</td>
<td>3000</td>
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<td>+</td>
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<tr>
<td>Control</td>
<td>2000[a]</td>
<td>0 (native BSA)</td>
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<td>0</td>
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<td>Control</td>
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The crude polymerization mixtures were purified by dialysis initially against 2% EDTA 10% DMSO in 20 mM phosphate buffer pH 7.4 and then against 20 mM phosphate buffer pH 7.4 or milliQ (depending on the analyses to perform) using 10 or 25 kDa MWCO regenerated cellulose membranes to eliminate the excess of the monomer and polymerization reagents.

Figure VI-30. SEC-HPLC traces of different blank experiments performed under standard ATRP in situ polymerization conditions. Traces obtained when the reaction was performed in the absence of the biomacroinitiator IX (blue), absence of Cu (green) or in the presence of oxygen (dark red).

Characterization of samples X to XIV: All samples were further analyzed by MALDI-TOF (solvent: water, see Figure VI-34, MW from 68 up 80 kDa), native gel electrophoresis (Figure
Chapter VI – Experimental Part

VI-33) and aqueous SEC HPLC (see Figure VI-31 and Figure VI-32) and their aggregation studied by TEM microscopy (Figure VI-35) while, upon protein hydrolysis, the resulting polymers were analyzed by $^1$H NMR (Figure VI-36) and MALDI-TOF (Figure VI-37).

![Figure VI-31. SEC chromatographic traces of BSA macroinitiator IX (blue trace) and samples of the different *in situ* styrene polymerization experiments after purification using “low” monomer to BSA macroinitiator IX ratio (ratio 50:1, black trace; ratio 500:1, red trace).](image)

![Figure VI-32. SEC chromatographic traces of BSA macroinitiator IX and the different samples from *in situ* styrene polymerization experiments after purification using “high” monomer to BSA macroinitiator IX ratio (XII ratio 1500:1, red trace; XIII ratio 2000:1, blue trace; and XIV ratio 3000:1, orange trace).](image)
Figure VI-33. Native gel electrophoretic profile after Coomassie Brilliant Blue staining (A) and Silver staining (B) of native BSA, BSA macroinitiator IX, and different samples obtained by the in situ ATRP mediated polymerization of styrene on the BSA macroinitiator IX. The content of each lane is summarized on the right.
Figure VI-34. MALDI-TOF analysis of BSA-macroinitiator IX (red trace) and the products of selected ATRP mediated in situ polymerization reactions of styrene over IX. The blue trace corresponds to styrene / IX ratio 500:1 and the green trace to 2000:1 ratio.

Figure VI-35. TEM micrographs of BSA polystyrene Giant Amphiphiles X (A, B), XII (C, D), XIII (E, F) and XIV (G, H) superstructures in water.

Isolation of Polystyrene (BSA digestion): Polymerization samples (400 µL from both non-purified crude polymerization mixtures and purified bioconjugate solutions obtained after extensive dialysis) were incubated at 80°C with 6 N HCl for ~8 hours. The resulting mixtures were neutralized and polymers were then extracted in CH₂Cl₂ and analyzed by MALDI-TOF (Figure VI-37, black trace) and ¹H-NMR spectroscopy (Figure VI-36). No polystyrene was detected in the digested control experiment samples. The results of the digestion of crude and dialyzed bioconjugate solutions were in full agreement.
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Figure VI-36. $^1$H-NMR analysis of the polystyrene isolated from hydrolysis of BSA-polystyrene XIII.

Figure VI-37. Comparison of MALDI-TOF spectra of polystyrene isolated from the BSA-polystyrene conjugate XIII (black trace) and a standard polystyrene (PDI 1.13, grey trace).

General polymerization procedure with monomer 8 (XV): Trimethyl silyl protected alkyne monomer 8 (238 mg, 1.215 mmol) and $N$-(Propyl)-2-pyridylmethanimine (~8 mg, 0.057 mmol) were placed in a Schlenk tube and dissolved in solvent (10% DMSO in 20 mM phosphate buffer pH 7.4, 10 mL). The mixture was deoxygenated by 5 freeze-pump-thaw cycles. Then, the mixture was canulated in another Schlenk tube containing BSA macroinitiator IX (~62 mg, 0.93 µmol, 1 equiv.) and CuBr (4.7 mg, 33 µmol) (previously degassed) under $N_2$ atmosphere, which triggered the beginning of the polymerization (a dark brown colour of the reaction medium was observed). The mixture was stirred under inert atmosphere during 72 h. The reaction mixture was then dialyzed using a 25 kDa MWCO membrane initially against 2% EDTA 10% DMSO 20 mM PB and then against 20 mM PB pH 7.4. Resulting solutions were analyzed by means of SEC-HPLC (Figure VI-38), and MALDI-TOF analysis.
General polymerization procedure with monomer 8 in the presence of carboxyfluorescein (XVI): Trimethyl silyl protected alkyne monomer 8 (238 mg, 1.215 mmol), carboxy fluorescein (3.2 mg, 0.01 mmol) and N-(Propyl)-2-pyridylmethanimine (~8 mg, 0.057 mmol) were placed in a Schlenk tube and dissolved in solvent (10% DMSO in 20 mM phosphate buffer pH 7.4, 10 mL). The mixture was deoxygenated by 5 freeze-pump-thaw cycles. Then, the mixture was canulated in another Schlenk tube containing BSA macroinitiator (~62 mg, 0.93 µmol, 1 equiv.) and CuBr (4.7 mg, 33 µmol) (previously degassed) under N₂ atmosphere, which triggered the beginning of the polymerization (a dark brown colour of the reaction medium was observed). The mixture was stirred under inert atmosphere during 72 h. The reaction mixture was then dialyzed using a 25 kDa MWCO membrane initially against 2% EDTA 10% DMSO 20 mM PB pH 7.4 and then against 20 mM PB pH 7.4. Resulting solutions were analyzed by means of SEC-HPLC (Figure VI-38), and MALDI-TOF analysis and their aggregation and encapsulation properties imaged by TEM and CFM microscopy (Figure VI-39).

Figure VI-38. SEC chromatographic traces of native BSA (solid black line), purified bioconjugates XV (solid grey line), and XVIII after reaction with TBAF (grey doted line), and reaction with KF (dashed black line).
Figure VI-39. Top: A. and B. CFM images of BSAPA structures XVI polymerized in the presence of CF (left, fluorescent image at 488 nm and right, optical image). Bottom: TEM micrographs of the aggregates observed after in situ polymerization of the trimethylsilyl protected monomer 8 on BSA macroinitiator IX in the presence of carboxyfluorescein.

General polymerization procedure with monomer (29) to give (XVII): Fluorinated monomer (258 mg, 0.742 mmol) and N-(Propyl)-2-pyridylmethanimine (~5.1 mg, 0.659 mmol) were placed in a Schlenk tube and dissolved in 20% DMSO 20 mM PB pH 7.4 (12 mL). The mixture was deoxygenated by 5 freeze-pump-thaw cycles. Then, the mixture was sonicated for 10 min. and immediately canulated in another Schlenk tube containing BSA macroinitiator IX (~33 mg, 0.495 µmol) and CuBr (2.9 mg, 20 µmol) (previously degassed) under N2 atmosphere, which triggered the beginning of the polymerization (a dark brown colour of the reaction medium was observed). The mixture was stirred under inert atmosphere during 72 h, dialyzed using a 25 kDa MWCO membrane against 2% EDTA 10% DMSO 20 mM PB pH 7.4. Samples were analyzed by SEC-HPLC (Figure VI-40) and their aggregation studied by TEM microscopy (Figure VI-41).
Figure VI-40. SEC chromatographic traces at 254 nm of native BSA (dashed grey trace) and amphiphilic macromolecule XVII (solid black trace).

Figure VI-41. TEM micrographs of the aggregates obtained by the self-assembly of the amphiphilic bioconjugates XVII.

**BSA-PA deprotection with KF (XVIII):** To a solution of BSA-PA (2.5 mL, C = 9.3.10\(^{-5}\) M in 20 mM PB pH 7.4), 465 µL of a 1 M solution of KF in 20 mM PB pH 7.4 were added. The reaction mixture was gently stirred for 2 days at 7°C and then purified by dialysis (MWCO 10 kDa) against 20 mM PB pH 7.4 before analysis by SEC-HPLC (Figure VI-42), MALDI-TOF analysis (no significative results observed) and their aggregation studied by TEM (spherical structures observed with diameter varying from 50 to 100 nm) and CFM microscopy (Figure VI-43).

**BSA-PA deprotection with TBAF (XIX):** To a solution of BSA-PA (3 mL, C = 9.3.10\(^{-5}\) M in 20 mM PB pH 7.4), 121.6 mg of TBAF were added. 20 mM PB was added until the
volume reached 3 mL. The reaction mixture was gently stirred for 2 days at 7°C and then dialyzed against 20 mM PB pH 7.4 before analysis by SEC HPLC (Figure VI-42), MALDI-TOF analysis (no significative results observed) and their aggregation studied by TEM (spherical structures observed with diameter varying from 50 to 100 nm) and CFM microscopy (Figure VI-43).

**General procedure for the clicking of azide 15 onto compound XVIII to afford XXa:** To a solution of XVIII (0.8 mL, C~9.3.10^{-5} M in 20 mM PB pH 7.4) was added 1-azidodecane 15 (27 mg, 0.149 mmol, 2000 equiv.). Then were successively added 80 µL DMSO, CuSO₄ (56 µL of a 0.1 M solution in 20 mM PB) and sodium ascorbate (56 µL of a 0.2 M solution in 20 mM PB). The reaction mixture was gently stirred for 2 days at 7°C and then extensively dialyzed with Microcon 10 kDa dialysis cups against 20 mM PB pH 7.4 and analyzed by SEC-HPLC (Figure VI-42, left), MALDI-TOF-MS (no results obtained due to amphiphilic character of bioconjugates) and their aggregation studied by TEM (Figure VI-44, left) and CFM microscopy (Figure VI-43).

N.B. Same procedure used to prepare compound XXb (20 mg of benzyl azide 16 were added) and for compound XXc (26 mg of triethylene glycol mono-azide 21 were added).

Characterization of XXb by SEC-HPLC (Figure VI-42, left) and aggregation behaviour observed by TEM (Figure VI-44, right) and CFM (Figure VI-43) microscopy.

Characterization of XXc by SEC-HPLC (Figure VI-42, right) and aggregation behaviour observed by CFM (Figure VI-43) microscopy.

**Figure VI-42.** SEC chromatographic traces obtained after clicking with the hydrophobic azides 15 and 16 (left) and with the hydrophilic azide 21 (right).
Figure VI-43. CFM images of fluorescent aggregates observed for compounds A. XVI, B. compound XIX, C. compound XXa, D. compound XXb and E. compound XXc.

Figure VI-44. TEM pictures of structures observed with compounds XXa (left) and XXb (right).
General polymerization procedure with styrene in the presence of fluorescein labeled papain (XXI): Styrene (135 µL, 1.177 mmol, 2000 equiv.) and N-(Propyl)-2-pyridylmethanimine 12 (~6 mg, 0.041 mmol, 70 equiv.) were placed in a Schlenk tube and dissolved in solvent (10% DMSO in 20 mM phosphate buffer pH 7.4, 10 mL). The mixture was deoxygenated by 5 freeze-pump-thaw cycles. Then, the mixture was canulated in another Schlenk tube containing BSA macroinitiator IX (~39 mg, 0.59 µmol, 1 equiv.), fluorescein labeled papain (~5.6 mg, 0.24 µmol, 0.4 equiv.), and Cu(I)Br (3.4 mg, 24 µmol, 41 equiv.) previously degassed under N₂ atmosphere, which triggered the beginning of the polymerization. A dark brown colour was immediately observed for the reaction medium. The reaction mixture was stirred under inert atmosphere during 8 h after which nitrogen was stopped and the mixture was stirred for additional 8 hours (oxidation of Cu(I) to Cu (II)). Purification was performed as previously, adding an extra dialysis step with a 300 kDa MWCO membrane to remove the excess of non-encapsulated papain. A control experiment in the absence of the macroinitiator IX was performed, SEC revealed only one peak corresponding to native papain after purification. SEC-HPLC revealed that the polymerization happened and that no free papain was present after dialysis (Figure VI-45). Aggregation was observed by TEM microscopy (Figure VI-46, left) and CFM microscopy (Figure VI-46, right) revealed the statistical incorporation of papain within the superstructures.

**Figure VI-45.** SEC chromatographic measurements at 254 nm of the ATRP polymerization reaction XXI in the presence of Carica papaya papain after dialysis (solid black trace) and of native Carica papaya papain (dashed grey trace).
Figure VI-46. (Left) TEM micrograph of the aggregates observed after the in situ polymerization in the presence of fluorescein labelled Carica papaya papain XXI, (Right) CFM images of the fluorescently papain loaded aggregates demonstrating the statistical encapsulation of papain within the superstructures.

General procedure for the labeling of above superstructures (XXI) with Atto-NHS to afford compound (XXV): For the Atto labelling experiments, 50 µL of a NHS-Atto ester solution (5 mM in DMSO) was added to 200 µL of the solution of the dialyzed BSA-PS superstructures containing the fluorescein labelled papain (XXI). The reaction mixture was incubated for 2-4 hours at 7°C and was then extensively dialyzed against 20 mM phosphate buffer pH 7.4 to remove any non-reacted NHS-Atto ester prior to any measurement. Double labelling after dialysis was observed by CFM microscopy (Figure VI-47).

Figure VI-47. CFM images of BSA-PS polymerized in the presence of fluorescein labelled Carica papaya papain after external labelling of the superstructures with Atto-NHS dye (reaction XXV). Lines A, B, C represent 3 different areas of the sample. The scanning was independently performed at 610 nm (Atto, left) and 488 nm (Fluorescein, right).
General polymerization procedure with styrene in the presence of HRP (XXIV): Styrene (1.86 mmole, 194 µL, 176 mg) and \textit{N}-(Propyl)-2-pyridylmethanimine 12 (~9.6 mg, 65 µmol) were placed in a Schlenk tube and dissolved in solvent (10% DMSO 20 mM phosphate buffer pH 7.4, 10 mL). The mixture was deoxygenated by 5 freeze-pump-thaw cycles. The beginning of the polymerization was triggered by the canulation of the monomer solution under nitrogen atmosphere in a second deoxygenated Schlenk tube containing the crystalline BSA-macroinitiator IX (~62 mg, 0.93 µmol), HRP (200 UNITS/mg) (0.8 mg, 160 UNITS) and CuBr (5.5 mg, 38 µmol) under N\textsubscript{2} atmosphere. A dark brown colour was immediately observed. The reaction mixture was stirred under inert atmosphere during 48 hours. The reaction mixture was dialyzed against 20 mM PB pH 7.4 using regenerated cellulose MWCO 25 kDa dialysis bags (Spectrum laboratories). 2 mL were dialyzed using 300 kDa dialysis bags against 20 mM PB pH 7.4 (2.1 mL recovered). After SEC-HPLC analysis of the reaction mixture after dialysis (Figure VI-48) and aggregation studies of the resulting solution by TEM microscopy (Figure VI-49), the catalytic activity was investigated by UV study using TMB / H\textsubscript{2}O\textsubscript{2} as substrate.

![Figure VI-48. SEC chromatographic traces of polymerization in the presence of HRP (reaction XXIV) after dialysis (solid black trace), and native HRP (dashed grey trace).](image-url)
Figure VI-49. TEM micrographs of the aggregates observed after *in situ* polymerization of styrene on BSA macroinitiator IX in the presence of HRP (reaction XXIV).

**General polymerization procedure with styrene in the presence of carboxyfluorescein (CF) (XXIII):** Carboxyfluorescein (7.5 mg, 20 µmol), Styrene (1.86 mmole, 194 µL, 176 mg) and N-(Propyl)-2-pyridylmethanimine (~9.6 mg, 65 µmol) were placed in a Schlenk tube and dissolved in solvent (10% DMSO in 20 mM phosphate buffer pH 7.4, 10 mL). The mixture was deoxygenated by 5 freeze-pump-thaw cycles. The beginning of the polymerization was triggered by the canulation of the monomer solution under nitrogen atmosphere in a second deoxygenated Schlenk tube containing the crystalline BSA-macroinitiator IX (~62 mg, 0.93 µmol) and CuBr (5.5 mg, 38 µmol) under N₂ atmosphere. A dark brown colour was immediately observed. The reaction mixture was stirred under inert atmosphere during 48 hours. The reaction mixture was dialyzed against 2% EDTA, 10% DMSO 20 mM PB pH 7.4 using regenerated cellulose MWCO 25 kDa dialysis bags (Spectrum laboratories) until no fluorescence was detected (to the naked eye) in the dialysis tank.

**General procedure for the external labeling of the superstructures to afford (XXVI):** For the Fluorescein labelling experiments, 50 µL of a NHS-Fluorescein ester solution (5 mM in DMSO) were added to 200 µL of the solution of the dialyzed BSA-PS superstructures containing the Atto labelled papain. The reaction mixture was incubated for 2-4 hours at 7°C and was then extensively dialyzed against 20 mM phosphate pH 7.4 to remove any non-reacted NHS-Fluorescein ester prior to any measurement.
HRP Activity test: Ready to use 3,3’,5,5’-Tetramethylbenzidine (TMB) / H\textsubscript{2}O\textsubscript{2} solution (Sigma Cat nr: T0440) was used for kinetic measurement of TMB oxidation by the encapsulated within the BSA-PS superstructures HRP.\textsuperscript{5} Increasing quantities (from 10 to 100 \(\mu\)L) of TMB/H\textsubscript{2}O\textsubscript{2} solution were added to a 900 \(\mu\)L dispersion consisting of 10 \(\mu\)L purified BSA-PS HRP containing nanoreactors in 20 mM sodium phosphate buffer pH 7.4. The soluble blue reaction product of the one-electron oxidation of TMB was recorded at 370 nm. Further oxidation of TMB in acid solution (addition of HCl) yields a yellow diimine reaction product with an absorbance maximum at 450 nm. This end-point assay was used to determine the activity of the HRP loaded nanoreactors by terminating the HRP-catalyzed conversion of TMB after 5 min with the addition of 0.25 mM of HCl (final concentration). The resulting yellow reaction product was recorded at 450 nm after a 3 minute incubation (Figure VI-50).

\begin{figure}[h]
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\includegraphics[width=\textwidth]{Figure_VI-50.png}
\caption{UV study of the catalysis of HRP loaded aggregates as a function of the volume of aggregates added to the TMB solution, the peak increasing at 280 nm corresponding to the polystyrene chain.}
\end{figure}

Cell uptake experiments with \textit{E. coli} strain: 4 mL overnight \textit{E. coli} (DH5\(\alpha\)) culture in LB medium was prepared. 3 mL of fresh LB medium were inoculated with 1 mL of cell culture. 50 \(\mu\)L (~100 \(\mu\)M) of aggregates solution were added into the latter cell suspension. \textit{E. coli} cells and aggregates were incubated for 1 hr to 12 hrs. After incubation of \textit{E. coli} cells and Atto-labelled papain BSA-polystyrene aggregates externally labelled with fluorescein (XXVI), cells were harvested from 1 mL cell suspension. Harvested cells were washed with 1 mL of a 0.9% NaCl aqueous solution, cells solutions were vortexed, centrifuged (10000 rpm, 10 min, 4°C), the supernatant discarded. Cells pellet was resuspended in 0.5 mL of a 0.9%
NaCl aqueous solution. 10 µL of each sample were used for confocal imaging. (For some samples 5 µL 50% glycerol was used to fix the cells on the glass support). CFM microscopy clearly points out the cell uptake of Giant Amphiphiles superstructures by *E. coli* cells (Figure VI-51). Experiments on incubation time (Figure VI-52) proved that *E. coli* cells are fully loaded with Giant Amphiphiles superstructures after only 2 hours.

**Figure VI-51.** CFM images of *E. coli* cells after uptake of doubly fluorescent labelled aggregates. First column, fluorescence at 488 nm; Second column, fluorescence at 610 nm; third column, optical image; fourth column, overlaid images.
Cell uptake experiments with *Baccilus sp.* and *Yeast*: same procedure as previously followed with *E. coli* cells. Adhesion of the vesicles XXVI on the cell walls was observed in the case of Yeast (Figure VI-53, B.) whereas no interaction was observed during incubation of vesicles XXVI with *Baccilus sp.* Cells (Figure VI-53, A.).
References

Design and Synthesis of Novel, Functional Polymer-Protein Nanoarchitectures

THÈSE

présentée à la Faculté des sciences de l'Université de Genève
pour obtenir le grade de Docteur ès sciences, mention chimie

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"Design and Synthesis of Novel, Functional Polymer-Protein Nanoarchitectures"

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Genève, le 19 décembre 2008

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Ce travail de thèse a donné lieu à la publication de quatre articles dans des revues scientifiques internationales:


Résumé

Les molécules amphiphiles *i.e.* molécules constituées d’une tête polaire et d’une queue apolaire s’auto-assemblent dans la nature de manière hiérarchique et ordonnée grâce à des interactions non-covalentes de manière à créer des superstructures telle la membrane cellulaire (double couche lipidique dans laquelle sont insérées et organisées d’autres biomoléculles comme les protéines, les glycoprotéines, etc.) qui possèdent des fonctions très importantes comme la protection contre l’environnement extérieur, la compartimentalisation, la mobilité, etc.

A ce jour, deux autres classes de molécules amphiphiles autres que les amphiphiles moléculaires ont été reportées. Ainsi, les super amphiphiles et les *Amphiphiles Géants* sont deux classes de macromolécules qui diffèrent de leurs analogues moléculaires par leur taille et leur constitution. En effet, les *Amphiphiles Géants* consistent en une classe de biomacromolécules constituées d’une protéine hydrophile spécifiquement attachée à un polymère hydrophobe. Ces dernières années ont connu de récentes avancées dans la préparation des ces composes hybrides.\(^1,2,3\) Malheureusement, la synthèse de ces *Amphiphiles Géants* n’offre en général pas de bons rendements et est très limitée par des facteurs tel que l’incompatibilité de solubilité entre la protéine hydrophile et le polymère hydrophobe.

Ce travail de thèse a, entre autres buts, été de développer de nouvelles générations d’*Amphiphiles Géants* avec le seul et unique but de programmer leur auto-assemblage pour obtenir des nanostructures multifonctionnelles qui profitent à la fois des propriétés intrinsèques des parties synthétique et naturelle. En d’autres termes, cette thèse présente de nouvelles approches synthétiques mises en place au laboratoire pour surmonter les limitations inhérentes à la préparation de ces biomacromolécules amphiphiles, mais fait également l’objet

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d’études sur l’auto-assemblage de ces dernières. L’attention a été surtout focalisée sur le développement de ces nouvelles approches mais aussi sur leur exploitation dans le but d’obtenir des nanoarchitectures pleinement fonctionnelles.

Dans un premier temps, nos efforts ont été consacrés à la préparation d’une petite bibliothèque d’Amphiphiles Géants en utilisant la réaction bien connue de cycloaddition dipolaire [3+2] de Huisgen (aussi couramment appelée « click » réaction). Il fut alors observé que ces Amphiphiles Géants dérivés de l’Albumine de Sérum Bovin (BSA) s’organisent de manière à créer des structures sphériques bien définies dont les dimensions (de 20 à 500 nm de diamètre) laissent à penser qu’elles sont probablement micellaires ou vésiculaires. Il fut également observé que la variation de la chaine polymérique (2 kDa à 8 kDa) n’avait pas d’influence sur la morphologie ou la taille des agrégats obtenus. Nous avons aussi établi que cette méthode, basée sur l’utilisation d’une molécule hydrophile hétéro-bifonctionnelle comportant les fonctions maléimide et alcyne est générique pour les protéines contenant un résidu cystéine libre et accessible à la surface de la protéine. Par ailleurs, il a été démontré que le type de superstructure adopté par les Amphiphiles Géants est fonction de la nature de la protéine. Ainsi, nous avons observé que les amphiphiles issus de l’attachement d’un polystyrène sur l’hémoglobine s’auto-assemblent de manière à former des structures cylindriques avec une longueur de 1000 nm et un diamètre de 300 à 400 nm.

Dans un second temps, le travail a été consacré à la mise en place d’une nouvelle approche appelée méthode de post-fonctionnalisation. Cette nouvelle voie synthétique a été conçue pour procéder en deux étapes. La première implique le couplage sélectif d’une protéine à un polymère hydrophile fonctionnalisé de manière appropriée via une réaction d’addition 1,4 de type Michael. Aussi, ce polymère fut conçu de telle manière qu’il possède au sein de son squelette de nombreuses fonctions alcynes terminaux. Dans une seconde étape, la « click » réaction (orthogonale, en conditions douces) entre les fonctions alcynes de ce polymère et de petits azides hydrophiles organiques a permis l’introduction de l’hydrophobicité et par conséquent l’auto-assembly des biomacromolécules amphiphiles ainsi créées en superstructures bien définies.

Enfin, la polymérisation *in situ* de monomères hydrophobes sur une protéine correctement fonctionnalisée avec un initiateur radicalaire a été développée avec succès. Grâce à cette méthode, le styrène mais également d’autres monomères plus intéressants du point de vue fonctionnel ont été polymérisés en conditions aqueuses sur la BSA. Cette dernière technique a prouvé être très intéressante dans le but de former des nano-assemblées fonctionnelles. Ainsi le développement de cette puissante méthode permet d’auto-organiser de manière hiérarchique des nano-réacteurs et des nano-containers. De façon très surprenante, l’observation de la catalyse enzymatique a permis de démontrer que ces superstructures sont perméables aux petites molécules.\(^5\) Finalement, des travaux initiaux permettent de penser que ces nano-containers ont une certaine affinité biologique pour des cellules bactériennes vivantes et plus particulièrement pour une souche de la bactérie *E. coli*.

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Abbreviations

PEO    poly(ethylene oxide)
PEE    poly(ethylethylene)
HHH    hollow hoop structure
PS     poly(styrene)
PB     poly(butadiene)
PAA    poly(acrylic acid)
PVP (ou PV2P) poly(2-vinylpyridine)
$T_g$  Glass transition temperature
PIC    polyion complex
PIAA   poly(isocyano-L-alal-L-alal)
PPQ    poly(phenylquinoline)
PI     poly(isoprene)
PCEMA  poly(cyannamoylethylethacrylate)
PMOXA  poly(2-methyloxazoline)
PDMS   poly(dimethylsiloxane)
LCST   Low Critical Solution Temperature
UCST   Upper Critical Solution Temperature
CSC    core-shell-corona
DNA    Deoxyribonucleic acid
pIPPAm poly(N-isopropylacrylamide)
BCAm   benzo-18-crown[6]-acrylamide
DTT    dithiothreitol
MRI    Magnetic Resonance Imaging
pDEAEMA poly(2-(diethylamino)ethyl methacrylate)
pVBA   poly(4-vinyl benzoic acid)
TPPTS  triphenylphosphine tris-(sulfonate)
ATRP   Atom Transfer Radical Polymerization
THF    Tetrahydrofuran
PB (ou PBS) phosphate buffer (saline)
DMSO   dimethylsulfoxide
DCM    dichloromethane
r.t.   room temperature
CuAAC  copper-catalyzed azide alkyne cycloaddition
DEAD   diethylazodicarboxylate
MWCO   Molecular Weight Cut Off
PDI    Polydispersity Index
NHS    N-hydroxysuccinimidyld
RAFT   radical Addition-Fragmentation Chain Transfer
DIFO   difluorinated cyclooctyne
CMC    Critical Micellar Concentration
DMF    Dimethylformamide
PMMA   polymethyl methacrylate
NIPPAm N-isopropylacrylamide
CF     carboxyfluorescein
TEA    triethylamine
EDTA   Ethylene diamine tetra-acetate sodium salt
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>Hb</td>
<td>Hemoglobin</td>
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<td>BSA</td>
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<td>Horseradish peroxidise</td>
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<td>TGase</td>
<td>transglutaminase</td>
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<td><em>Candida Antartica</em> Lipase B</td>
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<td>MALDI-TOF</td>
<td>Matrix Assisted Laser Desorption Ionization – Time Of Flight</td>
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<td>SDS-PAGE</td>
<td>Sodium Dodecyl sulfate – Polyacrylamide Gel Electrophoresis</td>
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<td>TEM</td>
<td>Transmission Electron Microscopy</td>
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<td>CFM</td>
<td>Confocal Fluorescent Microscopy</td>
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<td>GPC</td>
<td>Gel Permeation Chromatography</td>
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<td>High Performance Liquid Chromatography</td>
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<td>SEC</td>
<td>Size Exclusion Chromatography</td>
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The structuring of matter in Nature, often proceeds through the self-assembly of amphiphilic building blocks. Fascinating examples of this process are ubiquitous in biological systems, where soluble superstructures such as biomembranes, viruses, multimeric proteins and nucleic acid multiplexes are formed at the mesoscopic level (10-1000 nm). Probably the most representative example of this self-assembling process comes from the cell membrane\(^1\) (Figure I-1) where functionality is achieved through the interplay between different, often structurally simple, monomeric units. In this natural supramolecular structure, the organization is accomplished by the hierarchical self-assembly\(^2,3\) of different types of individual functional molecules (phospholipids, glycolipids, glycoproteins, membrane spanning peptide helices, the cytoskeleton, etc). These molecules spontaneously self-assemble in aqueous media, by ingeniously employing multiple non-covalent interactions such as hydrogen bonding, $\pi$-$\pi$ stacking, and Van der Waals interactions. The resulting superstructure is functional and further combines compartmentalization, order and mobility, characteristics all essential for life.
The importance of self-assembly in Nature led scientists to extensively explore its basic principles. Already more than 100 years back, the study of self-assembly processes of amphiphilic molecules had started. Initially, it primarily focused on soaps, fats and oil, a field which was the subject of colloid chemistry and has grown immensely since the early works on bilayer membranes.\textsuperscript{4,5,6} Nowadays, in the rapidly evolving fields of nanosciences (soft matter chemistry, surface chemistry and supramolecular chemistry), one of the main goals is to be able to create supramolecular systems possessing architectures with a well-defined programmed morphology and one of the main tools toward this goal is self-assembly.

Within the last decades, it has been possible to design supramolecular assemblies with various morphologies such as micelles, vesicles, tubes, disks, lamellas and even more exotic structures such as helices or inversed structures, from synthetic molecular amphiphiles, super-, and more recently Giant- amphiphiles as classified in terms of their molecular weights (Figure I-2). The superstructures resulting from the self-assembly of these individual molecules can be of one, two or three dimensional nature\textsuperscript{6,7,8,9} and contain between $10^1$ and $10^6$ molecules (approaching therefore synthetic and biological polymers in molecular mass).
Figure I-2. Three different subclasses of amphiphiles: (a) phosphatidyl choline representing the class of low molecular weight amphiphiles (molecular volume ~0.5 nm$^3$, molecular weight ~1 kDa), (b) PS-b-PIAA diblock copolymer representing the class of amphiphilic block copolymer also called super amphiphiles (molecular volume ~6.5 nm$^3$, molecular weight ~6 kDa), (c) lipase-polystyrene (n=40) representing the class of protein-polymer Giant Amphiphiles (molecular volume ~25 nm$^3$, molecular weight > 40 kDa).

Although significant progress has been achieved, it still remains a challenge to understand precisely the principles that govern the self-organization of individual molecules leading to the formation of nanoassemblies and furthermore to be able to manipulate these nanometer superstructures and enhance their properties to create functional nanodevices.


Apart from the use of surfactants as soaps which is known for centuries now, these amphiphilic molecules have known an important diversification of applications during the last decades especially in the areas of food industry, cosmetics, targeted drug systems and, more recently, in nanotechnologies. Surfactants are generally described as molecules containing a hydrophobic head and one or more hydrophobic tails.
As mentioned before, amphiphilic molecules can be classified in 3 different subclasses depending on their size and relative volumes as shown in Figure I-2: molecular amphiphiles also referred to as low molecular weight amphiphiles, super amphiphiles constituted by amphiphilic block copolymers, and Giant Amphiphiles consisting of a protein specifically connected to a hydrophobic polymeric chain.

I.2.1. Low molecular weight amphiphiles.

Low molecular weight amphiphiles (also named molecular amphiphiles) are constituted by two distinct parts: a polar (“head”) and one or more apolar (“tail”) moieties. The so-called “head” is constituted by ionic, non-ionic or zwitterionic groups whereas the so-called “tail” generally consists of one or two alkyl chains. Extensive studies have focused on the parameters that govern the self-assembling properties of a wide variety of natural and synthetic molecular amphiphiles and their use in the creation of novel functional nanostructures. These low molecular weight amphiphiles are generally classified in 3 major subclasses: lipids (natural molecular amphiphiles), synthetic surfactants and bolaamphiphiles.

It has been reported that different properties of the basic building block can be expressed at the supramolecular level depending on the conditions employed but also on the individual structure of the monomer. In water, above a certain concentration which is called critical micellar concentration (CMC), the self-assembly of amphiphilic molecules is entropically favoured to that of the presence of individual molecules in solution. The major driving force for the aggregation of these amphiphiles in water is considered to be the hydrophobic effect. Upon self assembly, these molecular amphiphiles arrange and organize in such a way that the heads are orientated towards the water and the tails buried together (to avoid contact with water) leading to the formation of different superstructures like micelles, rods, planar bilayer, inverted micelles, bicontinuous structures and vesicles, multilayers and lyotrophic liquid crystalline phases (at high concentrations) (Scheme I-1). Even more exotic morphologies were observed in the literature and involved the formation of chiral superstructures such as “cigars”, twisted ribbons, helices, tubes, braids, boomerangs, or superhelices.
Phospholipids are, without any doubt, the most studied subclass of molecular amphiphiles. In nature, they hierarchically self-organize in the presence of numerous other components (i.e. proteins, glycoproteins) to create the walls of living cells. These molecules have been used as model for numerous studies aimed to unknott the relationship between the supramolecular architectures and their molecular structure.

**Scheme I-1.** Common aggregation morphologies observed with molecular amphiphiles upon self-assembly: (a) micelles, (b) micellar rods, (c) planar bilayers, (d) vesicles, (e) inverted micelles.

The superstructures formed by amphiphiles in dilute solutions is determined by the three terms of the free energy of surfactant self-assembly:

- A favourable hydrophobic contribution, due to an entropy effect involving the solvent water.
- A surface term that deals with the tendency of the molecules to aggregate in order to shield the apolar tails from water and the tendency to spread out as a result of electrostatic repulsion, hydration and steric hindrance.
- A packing contribution, which implies that the hydrophobic segments exclude water and the polar head groups limiting therefore the type of aggregates that are geometrically possible.

In 1980, Israelachvili et al. developed a theoretical model, based on statistical mechanics of phospholipids, which predicts the structure of the aggregates obtained after self-assembly of these molecular amphiphiles. This theory is mainly based on the geometric relation between the hydrophilic and hydrophobic domains of the molecule. It predicts the type of the aggregate on the basis of the packing parameter ($P$) of the molecule, which is a
function of the volume of the molecule \((v)\), its length \((l)\) and the mean-cross sectional (effective) head group area \((a)\) as shown in Equation I-1.

\[
P = \frac{V}{l \times a}
\]

**Equation I-1.**

From this formula, it can be predicted that cone-shaped lipids \((P<1/2)\) aggregate in micellar structures, cylindrical lipids \((1/2<P<1)\) such as natural phospholipids form bilayered structures such as vesicles or planar bilayers upon self-assembly and finally that reversed micelles are generated when the volume of the hydrophobic tail is more important than the volume occupied by the hydrophilic head \((P>1)\) (Figure I-3). The predictions of this model are in agreement with most of the experimental results for phospholipids, but also small amphiphiles with conventional aliphatic chains,\(^{33}\) and for several more complicated molecules such as diblock copolymers (e.g. amphiphiles consisting of a dendrimer acting as polar head group connected to a polystyrene tail).\(^{34}\)

**Figure I-3.** Examples of aggregation patterns depending on the head-to-tail ratio \((P)\) above the CMC: \((a)\) micelle for \(P<1/2\), \((b)\) micellar rod for \(1/3<P<1/2\), \((c)\) planar bilayer or vesicle for \(½<P<1\) and \((d)\) inverted micelle for \(P>1\).

Several deviations from the model have been reported for surfactants incorporating rigid segments\(^{35}\) or multiple hydrogen bonding units,\(^{36}\) while some authors have criticized this packing parameter approach for predicting the aggregate morphology.\(^{37}\)
I.2.2. Amphiphilic block copolymers.

Amphiphilic diblock copolymers *i.e.* polymers constituted from a hydrophilic (*e.g.* polyethylene oxide PEO, polyacrylic acid, *etc.*) and a hydrophobic block (*e.g.* polystyrene, polybutadiene, *etc.*), are interesting materials for nanotechnology, pharmacy and medicine and have already been utilized as drug delivery systems. They have the ability to self-assemble on the mesoscopic length scale into highly regular superstructures in manners similar to those of their low molecular weight counterparts. Interestingly, triblock and multiblock copolymers can self-assemble in water and organic solvents in more diverse superstructure morphologies than the ones observed for molecular amphiphiles. For the purposes of this thesis, this overview will predominantly focus on amphiphilic diblock copolymers as their overall structure and properties are more similar to the ones of the protein-polymer *Giant Amphiphiles* that are the focus of this thesis.

I.2.2.1. Amphiphilic linear diblock copolymers.

Whereas the design rules for the synthesis of low molecular weight amphiphiles in relation to the aggregate morphologies are well established nowadays, in the case of amphiphilic diblock copolymers, these are still in the process of being formulated.

It is important to note that the CMC of these amphiphilic block copolymers is usually much lower\(^38\) and the dynamics of the chain exchange are much slower, due to numerous factors including the high molecular weight, the possible entanglement and the low mobility of the chains in the core.\(^39,40\) Due to these mobility restrictions, the superstructures obtained by the self-assembly of amphiphilic diblock copolymers are much more robust and stable in aqueous solutions than those of their low molecular weight counterparts. This was demonstrated by Discher and coworkers who studied the aggregation behaviour of poly(ethylene oxide)-*b*-poly(ethylethylene) polymers (PEO-*b*-PE).\(^40\) It was observed that small vesicles (≤200 nm) were formed upon hydration and vitrification of this diblock copolymers whereas, giant vesicles (20-50 µm) were formed through electroformation.\(^41,42,43,44\) These vesicles appeared to be highly deformable and, in contrast to conventional lipids, have an enhanced toughness and limited permeability.
Due to these mobility restrictions, the superstructures obtained by the self-assembly of amphiphilic diblock copolymers are much more robust and stable in aqueous solutions than those of their low molecular weight counterparts (Figure I-4).\(^{45}\)

**Figure I-4.** Schematic plot of typical physical properties with the molecular weight of a vesicle’s amphiphile (reprinted from reference 45).

Copolymers constituted by one polar and an apolar block can form either star or crew-cut micelles,\(^ {46}\) depending on the block lengths. Star micelles are formed when the corona-forming blocks are much longer than the core-forming block whereas crew cut micelles consist of a large core-forming block and a small corona-forming block.

Crew-cut aggregates have been extensively studied by Eisenberg and coworkers using asymmetric amphiphilic diblock copolymers of polystyrene-\(b\)-poly(acrylic acid), polystyrene-\(b\)-poly(ethylene oxide) and polystyrene-\(b\)-poly(4-vinylpyridine).\(^ {47,48}\) Apart from the simple micellar structure, a wide variety of other morphologies were observed including rods, lamellae, vesicles, hexagonally packed hollow hoop structures (HHH) and large compound micelles as shown in Figure I-5.
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Figure I-5. Different morphologies of crew-cut aggregates obtained by self-organization of PAA-b-PS diblock copolymers: (A) small spheres from PS$_{500}$-b-PAA$_{58}$; (B) rod-like micelles from PS$_{190}$-b-PAA$_{20}$; (C) vesicles from PS$_{410}$-b-PAA$_{20}$ and (D) large compound micelles from PS$_{200}$-b-PAA$_{4}$ (reprinted from references 47 and 48).

It was found that the type of morphology observed is governed by three major components of the free energy of aggregation: (i) core chain stretching, (ii) surface tension between the micellar core and the solvent outside the core, and (iii) intercoronal chain interactions.49,50,51,52,53,54 These factors, and thereby the morphology of the aggregates, are further affected by external parameters including the solvent composition,55 the relative block length,50,51,56 the temperature,57 and the presence of additional species such as salts (change in the ionic strength), acids, bases, homopolymers and low molecular weight surfactants.58,59,60,61,62

The present consensus on how the structure of the individual amphiphilic copolymers affects the morphology of the macromolecular architecture, takes into account the contribution of the molecular weight, relative block length, structure (e.g. branched vs. linear), conformational aspects, the presence of functional groups and the possibility of specific interactions between polymer blocks. These, however, are not the only parameters determining the morphology of block copolymer aggregates. As mentioned earlier, significant is also the role of external factors e.g. various solution conditions such as solvent nature and composition, polymer concentration and the presence of additives (e.g. ions, surfactants and homopolymers). Eisenberg and coworkers published recently an overview of the contribution of all the above mentioned parameters to the self-assembling behaviour and aggregate morphology of block copolymers.63
As mentioned in Section I.2.1, the type of aggregate morphology formed by traditional amphiphiles can be predicted using the theory developed by Israelachvili.\textsuperscript{31,32} Unfortunately, in the case of block copolymer amphiphiles, the situation is much more complex. Nevertheless, based on the same geometrical considerations, one can predict the morphology of aggregates depending on an analogous packing parameter \((P)\). Taking into account the bigger dimensions of the molecules and the curvature of the hydrophilic-hydrophobic interface,\textsuperscript{64} this packing parameter is defined as:\textsuperscript{65}

\[
P = \frac{V}{l \cdot a} = 1 - H \cdot l + \frac{K \cdot l^2}{3}
\]

\textbf{Equation I-2}

Where \((V)\) is the hydrophobic volume occupied by the amphiphile, \((a)\) the interfacial energy and \((l)\) is the chain length of the block copolymer amphiphile and where the parameters describing the hydrophobic-hydrophilic interface are the mean curvature \((H)\) and the Gaussian curvature \((K)\) (Figure I-6).

The simplest morphologies \textit{i.e.} spheres, cylinders and bilayers) are obtained by the combination of parameters given in Table I-1. It is evident that an increase for example in the hydrophobic/hydrophilic ratio results in a change in aggregates morphology from spherical to rod-like micelles to vesicles.
Table I-1. Packing parameter ($P$), mean curvature ($H$) and Gaussian curvature ($K$) for different aggregation morphologies.

<table>
<thead>
<tr>
<th>Shape</th>
<th>$P$</th>
<th>$H$</th>
<th>$K$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphere</td>
<td>$1/3$</td>
<td>$1/R$</td>
<td>$1/R^2$</td>
</tr>
<tr>
<td>Cylinder</td>
<td>$1/2$</td>
<td>$1/(2R)$</td>
<td>$0$</td>
</tr>
<tr>
<td>Bilayer</td>
<td>$1$</td>
<td>$0$</td>
<td>$0$</td>
</tr>
</tbody>
</table>

Another significant difference observed on the self-assembling behaviour of block copolymer amphiphiles as compared to that of traditional amphiphiles arises if not only geometrical, but also thermodynamic parameters are taken into consideration. There are two major contributions to the free energy of the system:

- The loss of entropy when flexible parts of the polymeric amphiphile are enforced in the restricted environment of the aggregates.
- The interfacial energy of the hydrophobic-hydrophilic interface.

Due to the significantly larger size of the amphiphilic block copolymers, both these contributions are considerably more important in the self-assembling process of these macromolecules. The Glass Transition Temperature ($T_G$) of primarily the core forming block has a particularly important role in this context. Because of the amphiphilic nature of the macromolecules, the interfacial energy is typically large, leading to the separation of these segments in the self-organized assemblies. When the interfacial energy is the predominant contribution to the free energy of the system, i.e. when polymers having low conformational entropy are used, the amphiphiles organize into morphologies with the lowest interfacial area per volume unit, giving therefore a preference to form planar (bilayer type) interfaces over cylindrical or spherical domains. Experimentally, a number of amphiphilic macromolecules that contain a rigid or conformationally restricted segment have been observed to form bilayer type architectures, which is in line with this prediction. Thereby, rod-coil type block copolymers have been investigated.\textsuperscript{67,68,69} Copolymers in which one of the constituent blocks has a specific interaction (e.g. hydrogen bonding, ionic interactions, etc)\textsuperscript{70,71} are conformational restricted. When specific interactions are present between the solvophobic segments of the amphiphilic macromolecules, highly ordered structures have often been observed. This is the case of block copolymers where mesogenic groups are introduced in the macromolecules or where secondary interactions between these non-soluble parts are present on the block copolymer backbone. These principles also explain the change in aggregate
morphology when the ion type or concentration is changed in the case of an amphiphilic block copolymer containing a polyelectrolyte segment.\textsuperscript{70,71} For example, Kataoka and coworkers used poly(ethylene glycol)-\textit{b}-poly(\(\alpha,\beta\text{-aspartic acid}\)) and poly(ethylene glycol)-\textit{b}-poly(L-lysine) as a pair of polymers which are oppositely charged. When pairs with a different length of the charged block were mixed, neutral polyion complexes (PIC) were formed. These complexes consisted of the minimal required number of polymers to neutralize the total charge. Pairs with charged blocks of similar length formed bimolecular complexes that grew into larger PIC micelles. Under conditions in which matched and unmatched pairs coexist, only the matched pairs formed PIC micelles whereas the remaining polymers of unmatched length remained as individual entities. It was assumed that this is a result of the increased stability of the micelles over the individual PICs.

In order to obtain structures with a high level of organization, monomers that contain the necessary information in the form of chirality, hydrogen-bonding capacity, steric demands, electrostatic properties, hydrophilic or hydrophobic character, or metal ion binding capability, are used. This principle, in Nature, is demonstrated in the case of proteins where the amino acid sequence determines the secondary structure (\textit{i.e.} \(\alpha\)-helices and \(\beta\)-sheets), which in turn controls the tertiary (protein subunit) and quaternary (overall) structure of the biomacromolecule and its possible further functional aggregation/assembly. This influence of the monomer unit on the final aggregated structure has also been observed for diblock copolymers. For example, Nolte and coworkers have investigated amphiphilic rod-coil block copolymers composed from polyisocyanide and polystyrene.\textsuperscript{10,72,73} The isocyanides used in this study were derived from dipeptides and upon polymerization formed, due to hydrogen bonding between amide bonds, helical structures whose handedness was controlled by the type (D or L) of the peptide used.
These polystyrene-\textit{b}-polyisocyanates were found to form micellar aggregates, vesicles, bilayered structures and super helices when dispersed in aqueous solutions. The latter super-helices were observed to have handedness opposite to the handedness of the helices from which they originated. The aggregates morphologies were also found to depend on many parameters such as the relative block lengths, the pH and the isocyanide monomers used. Additionally, it was demonstrated that the flexibility of the rod-like structures could be adjusted simply by changing the counterion in the aqueous buffer.

Long range interactions between individual aggregates have also been reported by Jenekhe and collaborators who studied the self assembly of rod-coil diblock copolymers consisting of poly(phenylquinoline) (PPQ) and polystyrene (PS) (Figure I-8). The use of selective solvents for the rigid PPQ rod led to the formation of large micrometer sized aggregates with various morphologies (spheres, vesicles, cylinders and lamellae). The cylindrical and spherical aggregates contained a large hollow cavity as a result of the close packing of the rigid rod blocks. The micelle-like aggregates were observed to be able to encapsulate large amounts of fullerenes in the inner cavity and in the PS core.
Figure I-8. Molecular structure of the rod-coil diblock copolymer PPQ<sub>n</sub>-b-PS<sub>m</sub> and schematic illustration of its hierarchical self-assembly into hollow spherical micelles forming highly ordered microporous materials (reprinted from reference 68).

On the contrary, the use of a selective solvent for the flexible PS block yielded exclusively hollow spherical micelles of several micrometers in size (Figure I-8).<sup>68</sup> Further, long-range, close-packed self-ordering of the micelles produced periodic microporous materials of which the microstructures and optical properties could be tuned by the addition of small amounts of fullerenes, which were incorporated in the PS corona. The preparation of films of vesicles is not only limited to the PS-PPQ system. Ding et al. demonstrated the same principle through the evaporation of organic solutions of polyisoprene-<i>b</i>-poly(2-cinnamylethylethacrylate) (PI-<i>b</i>-PCEMA) vesicles on top of a water surface.<sup>74,75</sup> The shape of the vesicles changed from spherical to hexagonal, forming a close-packed film with a hexagonal morphology.

Recently, Van Hest and coworkers developed an amphiphilic diblock copolymer based on polystyrene-block-poly(acrylic acid) (PS-<i>b</i>-PAA) suitable for bioconjugation with proteins.<sup>76</sup> After polymerization, ω-terminus (bromide end group) was substituted with an azide moiety. This functional diblock copolymer was allowed to aggregate into vesicular structures where the bilayer shell was covered with azide groups. Further “clicking” of the 1-alkyne modified protein EGFP (Enhance Green Fluorescent Protein) onto the polymersomes was achieved in aqueous conditions and proved to afford amphiphilic triblock protein-polymer bioconjugates through confocal laser-scanning microscopy.
I.2.2.2. Amphiphilic linear triblock copolymers.

ABC triblock copolymers have shown to give a greater even variety of structures resulting from the phase separation of the three different blocks. In this case, experiments demonstrated that the formation of the microseparated assemblies was influenced by two independent composition parameters, namely the volume fraction and the relative immiscibility between directly connected and between non-linked blocks. Triblock copolymers have proven to be extremely useful precursors for very complex self-assembled structures such as “three layer” micelles, “crew-cut” micelles and the so called “Janus” micelles. The latter for example, consist of a cross linked poly(butadiene) core and a corona constituted by poly(styrene) and poly(methylmethacrylate). The stabilization of these interesting supramolecular structures is thought to be the result of direct solubilisation of the ABC triblock copolymer in a solvent selective for one of the blocks, or, as in the case of the Janus micelles, the result of a supramolecular organization in the bulk state which is then transferred in solution. In the recent literature concerning these ABC triblock copolymers, important theoretical and experimental studies have also been carried out on poly(styrene)-b-poly(butadiene)-b-poly(methyl methacrylate). It was observed that the morphology of the aggregates is dependent on the repulsive forces between the poly(butadiene) middle block and both poly(styrene) and poly(methyl methacrylate) end blocks. More recently, an ABC triblock consisting in a poly(styrene)-b-poly(butadiene)-b-poly(ethylene oxide) has been extensively studied for its morphological behaviour.

In 2002, Meier and coworkers reported on the potential interest of these polymersomes as bionanocontainers. In this work, they reported on the synthesis of ABA triblock copolymers PMOXA-PDMS-PMOXA (poly(2-methyloxazoline)-b-poly(dimethylsiloxane)-b-poly(2-methyloxazoline)) that self assemble in water to form vesicular structures. It was demonstrated that these polymersomes are able to accomodate the LamB channel protein within the block copolymer membrane. The resulting vesicles were proven to be functional as they were shown to interact with living systems. On this direction, Meier and coworkers exposed the vesicles to the bacteriophage λ, which binds to the LamB protein, and monitored the viral DNA being loaded into the polymersomes, giving the pioneering demonstration of the possible applications of these polymersomes in living systems.
Figure I-9. Schematic representation of bacteriophage Lambda loading DNA into PMOXA-PDMS-PMOXA triblock polymersomes in the presence of LamB proteins (left) and TEM micrographs of negatively stained complexes formed between bacteriophage Lambda and vesicles bearing LamB protein at 37°C (right) (reprinted from reference 85).

Other works from the Meier group studied also the insertion of different channel proteins like FhuA or Ompf proteins within the copolymer membrane. Upon encapsulation of catalytically active proteins within the inner cavity of these proteopolymersomes, it was demonstrated that the presence of the pore protein is necessary for the substrate to cross the membrane and be metabolized by the enzyme. The product of the reaction was subsequently by the same transport process released in the aqueous medium.

Finally, Schubert and collaborators reported on a new class of triblock copolymers that combine metallosupramolecular and polymer chemistry. These PS_{32-b-P2VP_{13-b-[Ru]}-PEO_{70}} triblock copolymers possess interesting features such as reversibility of the metal-ligand complex under precise conditions, combined with the well known interesting photophysical and electrochemical properties of the ruthenium-terpyridine complex and the possibility to easily construct a library of triblock copolymers very easily.

I.2.2.3. Stimulus responsive copolymers.

Block copolymers in which the amphiphilicity can be switched “on” and “off” have also been extensively studied during the last years. These so-called stimuli-responsive or “smart” block copolymers respond with large property changes to small chemical or physical stimuli such as pH, specific ions, ionic strength, electric fields or temperature. In this category block copolymers that are constituted by a block whose monomer unit posses a lower or an upper critical solution temperature (LCST or UCST) are listed. As a result, above or below
respectively a specific temperature, the polymer spontaneously transforms from molecularly dissolved to phase separated in water. Block copolymers possessing such a temperature dependent polymer coupled with a hydrophilic polymer can thus reversibly switch from hydrophilic to amphiphilic upon temperature changes.\textsuperscript{88}

Diblock copolymers consisting of two different responsive blocks can exist in three states in aqueous solutions, namely, as conventional micelles, reversed micelles, and molecularly dissolved polymeric chains.\textsuperscript{89,90,91,92,93} The composition of the micellar core of the aggregates formed by these so-called schizophrenic diblock copolymers, is dictated by the conditions, allowing the inside and the outside block to change by a combined stimulus of pH and ions,\textsuperscript{89,92} pH and temperature,\textsuperscript{93} pH only,\textsuperscript{91} or temperature only.\textsuperscript{90}

As an example, in the case of diblock copolymers constituted by poly(4-vinyl benzoic acid) (VBA) and poly(2-(diethylamino)ethyl methacrylate) (DEAEMA), aggregation studies showed that the micellar core is formed by VBA at low pH, DEA at high pH whereas, non-micellar aggregates that precipitate were formed at intermediate pH.\textsuperscript{91}

Core-shell-corona (CSC) pH sensitive micelles have also been constructed from poly(styrene)-b-poly(2-vinylpyridine)-b-poly(ethylene oxide) (PS-b-P2VP-b-PEO) ABC triblock copolymers.\textsuperscript{94} Interestingly, these ABC triblock copolymers were found to respond to external stimuli (e.g. pH) allowing tuning of the size of the aggregated system. Temperature and pH-sensitive CSC micelles in which the shell can be selectively cross linked have also been reported by Armes and collaborators.\textsuperscript{95}

The stimuli-responsive polymers are nowadays extensively studied as they might find a variety of applications in medicine as controlled-release systems. Soluble drugs, proteins, DNA multiplex can be easily encapsulated within these responsive copolymers aggregates and can be released at a desired rate at a specific time and place depending on the conditions. For example, such a system has been developed for the controlled release of the solute vitamin B\textsubscript{12}.\textsuperscript{96}
Figure I-10. Schematic representation of core-shell porous membrane microcapsules formed by aggregation of the stimulus responsive copolymer p(NIPAAm)-co-p(BCAm). Upon addition of Ba$^{2+}$ ions in the medium, specific recognition of Ba$^{2+}$ by the crown ether occurs and the grafted polymer swells and closes the pores. After dialysis against water, Ba$^{2+}$ is removed from the medium, the grafted polymer shrinks and then the pores open.$^{96}$

On this direction, Yamaguchi and collaborators developed microcapsules consisting in of a core-shell porous membrane and linear grafted poly(NIPPAm-co-BCAm) (*i.e.* poly(N-isopropylacrylamide)-*b*-poly(benzo-18-crown[6]-acrylamide)) chains in the pores, which acted as molecular recognition gates. The gates could be closed by the addition of barium cations Ba$^{2+}$ which are captured by the BCAm receptors and thereby decrease the lower critical solution temperature (LCST) of the NIPPAm. The system proved to be fully reversible after dialysis against deionized water.

### I.2.3. Protein-Polymer Giant Amphiphiles.

#### I.2.3.1. Definition.

In the above described examples of self-assembly of macromolecules into well defined superstructures, only synthetic components were considered. Assemblies incorporating biological components are particularly attractive as supramolecular biomaterials as they
would combine the structural properties of the synthetic polymer with those of the biological moiety combined with an increased biocompatibility and possibly biological function. A new, innovative class of such biosurfactants which was recently introduced by the Nolte group, are the Giant Amphiphiles, consisting of a protein or an enzyme linked selectively and specifically to a hydrophobic polymer. These biohybrid polymers differ from other protein-polymer conjugates in the sense that the protein to polymer ratio is predefined and the position of the bioconjugation site is precisely known. Giant Amphiphiles are in fact diblock copolymers which have by design significantly higher molecular weights and volumes than their synthetic counterparts. Furthermore, taking into account that Nature synthesizes its biopolymers with high efficiency, Giant Amphiphiles have the intrinsic structural advantage over the synthetic block copolymers of possessing a monodisperse block (the protein).

I.2.3.2. Methods for specific protein functionalization.

In the particular case of protein-polymer Giant Amphiphiles, three different methods to functionalize a protein with a hydrophobic polymer have already been reported (Figure I-11):

A. The first one involves the direct attachment of a polymer terminated with an appropriate chemically reactive group directly on a selected functional group of the protein. This method is called direct specific coupling.

B. Secondly, the specific functionalization via cofactor reconstitution can be only used in the case of enzymes containing a prosthetic group. It involves an initial coupling of the prosthetic group onto the polymer, followed by a reconstitution between the cofactor functionalized polymer and the corresponding apoenzyme.

C. The last method involves an indirect specific functionalization and consists of two steps. In the first one, a heterobifunctional linker is attached specifically onto a specific position of the protein to create a reactive biohybrid, which is on the second step functionalized using an appropriately appended polymer.
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Figure I-11. Schematic representation of the synthetic methodologies used previously to synthesize Giant Amphiphiles: (A) direct coupling of an appropriately functionalized hydrophobic polymer, (B) Specific coupling by cofactor reconstitution of the apoenzyme with the appropriate cofactor appended hydrophobic polymer, (C) Indirect specific functionalization via coupling with a heterobifunctional linker followed by the attachment of the hydrophobic polymer.

I.2.3.3. About the crucial choice of the amino-acid to target.

Choosing the appropriate way to specifically modify a protein with a polymer is not an easy task. The major difficulty in synthesizing Giant Amphiphiles (except the solubility incompatibility problem found for the hydrophilic protein and the hydrophobic polymer which will be addressed later on) comes from the choice of the targeted amino-acid. Proteins are natural polymers constituted from a sequence of a combination of 21 natural amino-acids.

Specific modification of proteins/enzymes is one of the most challenging problems in protein chemistry. Many research groups have during the last 50 years developed some new methodologies in order to avoid multiconjugation reactions nevertheless, some amino-acids cannot be targeted because of their too high proportion in the protein sequence. On the other hand, some amino-acids are present in a relative small percentage in the protein sequence and as a consequence are the ideal candidates for specific conjugation e.g. Cysteine, Tyrosine,
Lysine and Glutamine together with the $\alpha$-N-terminus. Another important consideration that should be mentioned is the availability of the target functional group. This later has to be sufficiently exposed on the exterior of the protein to react with the suitable terminal reactive function of the polymer.

In the following paragraphs, the advances on the specific modifications of selected, low abundance, amino-acids will be presented.

I.2.3.3.a. Cysteine modification.

As mentioned above, the cysteine residue (Cys) is only present in small percentage in proteins, thus it is a good candidate to link a protein with a synthetic polymer in a selective, quantitative and rapid fashion. Bioconjugate chemistry literature abounds with methods to selectively functionalize free thiols of proteins.

For example, cysteine reacts easily with $\alpha$-halo carbonyl compounds\textsuperscript{98} or with maleimido-appended molecules and macromolecules\textsuperscript{99,100,101,102,103} to give respectively the corresponding thioether or 1,4-Michael thioether adduct as shown on Scheme I-2. Bioconjugation of proteins with maleimide are generally undertaken in aqueous buffer solution at neutral pHs (6.5<$\text{pH}$<7.5) to avoid side reactions that can occur especially with lysine residue at higher pHs. Both $\alpha$-halo carbonyl and maleimide derivatives can be easily synthesized in good yields by different methods.

![Scheme I-2. Selective reaction of cysteine residue with $\alpha$-halocompounds (top),\textsuperscript{98} and with maleimide derivatives (bottom).\textsuperscript{99-103}](image)

Cysteine residues can also be easily modified by reaction with pyridyl disulfide derivatives\textsuperscript{104,105,106} (Scheme I-3) to give the corresponding disulfide compounds and concurrently generate a pyridyl thione. This latter has unique spectral properties that allow the quantification of sulfhydryl coupling by monitoring the increase in absorbance at 343 nm.
Scheme I-3. Reaction between cysteine and pyridyl disulfide compounds.\textsuperscript{104,105,106}

In this particular case, the resulting product (a disulfide) can be easily cleaved using standard disulfide reducing agents e.g. DTT (dithiothreitol) to give back the starting material. This is a method that has been used also in the case of the synthesis of well-defined protein-polymer bioconjugates where, especially in the case of \textit{in situ} polymerization on proteins, it offered the possibility to cleave the polymer\textsuperscript{107} from the protein in order to analyze the former one.

\textbf{I.2.3.3.b. Lysine and/or N-terminus modification.}

The amino group of Lysine (Lys) and/or N-terminus of proteins can be easily and selectively modified. Unfortunately, modification of this amino-acid generally affords a multiplicity of different conjugates due to the proportion of lysine residues in protein structures.

The first method consists of a condensation with an aldehyde\textsuperscript{108} to give the corresponding imine (Schiff base) that can be reduced in mild conditions using sodium cyanoborohydride (NaCNBH\textsubscript{3}) to obtain the secondary amine as described on Scheme I-4.

\textbf{Scheme I-4. Modification of Lysine through reductive amination.}\textsuperscript{108}

Another method which is more widely used in protein chemistry, and especially for labelling of enzymes, is based on amide bond formation using NHS-activated carboxylic acids.\textsuperscript{109,110}
I.2.3.3.c. Modification of Tyrosine residue.

Tyrosine is a mild nucleophile but its nucleophilicity can be enhanced under mildly basic conditions (pH>8.5) by deprotonation of the phenol side chain. Two different reagents were mostly used for this modification. First of all, the use of diazonium salts is frequently reported however often cross reactivity occurs with histidine and/or lysine. They can also be modified by iodination.\textsuperscript{111}

Tyrosine modification has been investigated by Hermanson et al.\textsuperscript{111} nevertheless under quite harsh reaction conditions. Moreover, a cross-reactivity towards Cystein and/or Lysine residues was also observed. These studies have evolved significantly by the new ingenious techniques that were extensively studied for the last five years in Francis group which developed some novel methods to selectively target Tyrosine under mild conditions.

Francis and collaborators managed to selectively modify tyrosine residues by the use of \textit{in situ} generated π-allylpalladium species.\textsuperscript{112} As described on Scheme I-6, upon exposure of the protein to an allylic acetate derivative, a catalytic amount of Pd(OAc)$_2$ and triphenylphosphine tris-(sulfonate) (TPPTS, a water-soluble phosphine ligand), efficient protein labeling was observed within 45 min at room temperature. Analysis of the reaction mixture consistently indicated 50-65% conversion of the starting protein to a singly alkylated product, corresponding to the addition of the π-allyl species to tyrosine after loss of the acetate group. A small amount of doubly alkylated product was also observed. Consistent with a mechanism involving the phenolate anion, the reaction was found to proceed best at pH 8.5-9.0.
In another work, Francis reported on the dual surface modification of Tobacco Mosaic Virus (TMV) coating protein.\textsuperscript{113} To achieve this orthogonal functionalization, they specifically modified the exposed to the solvent tyrosine residue at position 139 by electrophilic aromatic substitution in ortho position of the hydroxyl group using diverse para substituted diazo reagents at pH 9 for 2 hours at 4°C as shown on Scheme I-7. The modification was successful in all studied cases, except in the case where an amide function was employed in para of the diazo group. This specific modification of tyrosine, together with the functionalization of glutamine (using secondary amines and EDC/HOBt coupling agent), led to the formation of TMV nanoscale materials that could have potential applications as multivalent scaffolds for the display of biological ligands and for the preparation of linear arrays of chromophores and inorganic nanoparticles as well as drug and gene delivery carriers. This reaction has been recently successfully used to prepare nanosized Magnetic Resonance Imaging (MRI) Contrast Agents by selective modification of tyrosine residue at position 85 of the interior surface of bacteriophage MS2 capsids devoid of nucleic acids.\textsuperscript{114}

Francis and collaborators also reported on a highly selective Mannich-type reaction\textsuperscript{115} for tyrosine side chain specific modification that involves aliphatic aldehydes and electron-rich para-aniline derivatives. The reaction proceeds smoothly at pH~6.5 by the electrophilic aromatic substitution of \textit{in situ} generated imines onto tyrosine residues as shown on Scheme I-8.
I.2.3.3.d. Glutamine modification.

Glutamine (Gln) is also a unique target for the selective modification of proteins. As reported by Sato, such a modification can proceed through an enzymatic reaction using the transglutaminase (TGase). This enzyme catalyzes the coupling reaction between Gln and various primary amines, including the exposed Lys side chain as shown on Scheme I-9. This enzyme is highly selective towards the Lys target and will only catalyze the coupling reaction if the primary amine is highly exposed. Practically, this approach has been used to introduce PEG-polymers and other synthetic targets onto proteins, provided they possess long enough primary amine bearing aliphatic chain.

I.2.3.4. History of Giant Amphiphiles.

The first attempt of introducing hydrophobicity on proteins through the coupling of relatively small hydrophobic chains was reported by Ringsdorf. During these studies streptavidin, (a 60 kDa protein containing 4 identical subunits that can each be bound to a biotin molecule) was attached to a modified biotin bearing apolar chain leading to an increased amphiphilic character in the protein.
During the last decade, *Giant Amphiphiles* were introduced by the Nolte group and since have flourished in the literature. The first reported example involved the specific coupling of the lipase B from *Candida Antartica* (CAL B) with a hydrophobic polymer. During these studies, the exposed disulfide bridge of the enzyme was reduced with dithiothreitol (DTT) and then, the modified enzyme was coupled to a maleimide appended hydrophobic 4.3 kDa polystyrene chain through a 1,4-Michael-type addition using a mixture of THF/water (9/1) as solvent.

![Scheme I-10](image)

**Scheme I-10.** Synthetic approach used for the synthesis of CAL B-polystyrene Giant Amphiphiles; (a) reduction of the disulfide bridge with DTT and (b) coupling of reduced CAL B with a maleimide appended polystyrene (n=40, PDI=1.04) (reprinted from reference 11).

This coupling led to the formation of biohybrid macromolecules that self-assemble in water to form micrometer long fibers constituted of bundles of micellar rods (Figure I-12). The conditions used in this report, took advantage of the known stability of CALB in organic solvents to achieve solubilisation of both components during the bioconjugation step. These conditions are not judged to be generic as they are quite harsh for the generality of proteins and could easily lead to denaturation. Nevertheless, CAL B in its aggregated Giant Amphiphile form was shown to retain part (6-7%) of its original catalytic activity. The authors explained the loss of activity by the bundling of individual rods that causes inaccessibility of the substrate to the protein head group.
Figure I-12. TEM micrographs of CAL B-polystyrene aggregates (Pt shadowing) after mixing the reduced CAL B and the maleimido-appended polystyrene (A, B), smallest micellar fiber observed having a diameter of 25-30 nm (reprinted from reference 11).

Another approach, using a biotin-streptavidin system similar to what was described by Ringsdorf, was also investigated by the Nolte group. In this base, biotin was initially modified with a 9.2 kDa polystyrene chain and spread at the air/water interface in a Langmuir trough. Subsequently, streptavidin was added in the aqueous phase as shown in Scheme I-11. Using this approach, only the two streptavidin subunits located on the same side of the protein can bind the biotin appended polystyrene chains. Investigation of these systems proved that monolayers of polystyrene-Streptavidin bioconjugates were obtained upon addition of streptavidin in the sub-phase system. In a second step, it was demonstrated that the remaining two free binding sites on Streptavidin were still available to complexate other biotinylated molecules. In this direction, the authors reported on the binding to the monolayer of a biotinylated form of the iron storage protein ferritin as well as that of a modified horseradish peroxidase resulting in a catalytically active surface. Interestingly, the resulting catalytic monolayers showed an activity not dependent from the lateral pressure. In this particular case, polymeric tails are attached to the protein in a non covalent fashion, but due to the high affinity constant between streptavidin and biotin ($K_a = 10^{15} \text{ M}^{-1}$) the formation of the protein-polymer complex can be considered as non-reversible.
Scheme I-11. Schematic pathway developed by Nolte and coworkers to prepare Streptavidin-polystyrene Giant Amphiphiles monolayers (reprinted from reference 117).

In another approach, horseradish peroxidase-polystyrene Giant Amphiphiles were formed following the cofactor reconstitution pathway.\textsuperscript{118} Horseradish peroxidase (HRP) is an enzyme which contains a heme cofactor buried within its core. In this work, the heme cofactor was removed from the protein according to existent experimental procedure and the synthesis of the HRP-polystyrene Giant Amphiphiles was achieved by cofactor reconstitution between the apoenzyme and an heme appended polystyrene as can be shown on Figure I-13.

Figure I-13. (A) Structure of the heme appended polystyrene used for the cofactor reconstitution, (B) computer-generated model of the reconstituted HRP-polystyrene Giant Amphiphile, (C) SEM (top) and TEM (bottom) pictures of polystyrene-HRP Giant Amphiphiles self-assembly (scale bar = 200 nm) in aqueous solution (reprinted from reference 118).
I.3. Scope of work.

In the short history of Giant Amphiphiles, the focus has been put on their successful synthesis and the potential of creating bioconjugate systems with applicability in different areas such as materials chemistry, biomedicine, nanotechnologies. As mentioned above, these protein-polymer amphiphiles have been prepared either by direct conjugation of appropriately functionalized hydrophobic macromolecules to specific aminoacids or cofactors or through bioaffinity couplings. Nevertheless, although these compounds have attracted increasing interest during the last few years, the synthesis of Giant Amphiphiles and, as a consequence, their further study for potential applications were hampered by experimental limitations. These limitations are mainly due to the solubility incompatibility between the protein hydrophilic head and the hydrophobic polymeric tail and by constraints posed to guarantee the stability of the protein itself. These problems together with the tedious purification procedures required by the intrinsic properties of such systems, significantly limited any further studies for exploring Giant Amphiphiles’ full application potential into the creation of pre-programmed hierarchical multifunctional systems.

Participating in a concerted effort of the laboratory to design and create, novel generations of Giant Amphiphiles with the scope to program them in later steps into well-defined multifunctional nanoassemblies that make use of the intrinsic properties of both the protein and the polymer moieties, this Thesis is concerned with novel approaches designed to surpass the synthetic limitations, create multifunctional systems and study their assembly. For this reason, it was decided to focus mainly on developing novel synthetic approaches and exploiting their potential.

More specifically, following this short introduction, the Second Chapter will be dedicated to the creation of a small library of Giant Amphiphiles using the well-known [2,3]-dipolar Huisgen cycloaddition (also referred to as “click chemistry” reaction). Giant Amphiphiles have already shown to self-assemble in a manner similar to that of their molecular and super counterparts. It was thus envisioned that altering the length of the polymer would allow a controlled variation of the overall shape of the biomolecules and thus lead to extracting important fundamental information concerning the molecular
shape/aggregation relationship. Finally, tuning the protein head group was also attempted as it appeared to be a challenging task.

In the Third Chapter, the design and successful application of a novel approach, the post-functionalization approach, will be described. This new synthetic pathway was designed to proceed through two discrete steps. The first step involved bioconjugation of an appropriately functionalized hydrophilic polymer, which was designed to contain multiple 1-alkyne groups and was synthesized using ATRP polymerization in collaboration with Prof. Dave Haddleton and Dr. G. Mantovani. The orthogonal multi-clicking of various azidated compounds allowed on a second step, the introduction of hydrophobicity and therefore the synthesis of well-defined amphiphilic triblock Giant Amphiphiles.

In the Forth Chapter, the ATRP mediated, in situ polymerization of hydrophobic monomers initiated from a protein biomacroinitiator was developed and proved to be a powerful tool for the creation of meaningful, functional nanoassemblies. The hierarchical self organization of nanoreactors and nanoassemblies will be discussed as it successfully led to the formation of the first Giant Amphiphile nanoreactors. Interestingly, these nanoreactors proved to be catalytically active while, surprisingly, in initial experiments they showed affinity with living bacterial cells.

Finally, the outcome of this research and the perspectives for future studies will be briefly discussed in Chapter V and a detailed experimental section will be provided in Chapter VI.
References


[2] Generally, hierarchical self-assembly is defined as “the formation of an organized structure through different and distinct levels of self-assembly processes that decrease in strength”.


Chapter I – General Introduction

[66] The IUPAC Compendium of Chemical Terminology, 66, 583 (1997). The glass transition temperature, T_g, is the temperature at which an amorphous solid, such as glass or a polymer, becomes brittle on cooling, or soft on heating.
Chapter I – General Introduction


Chapter II
Formation of Giant Amphiphiles by Click Chemistry Reaction

II.1. Background / Concept.

To proceed with selective chemical reactions on biomolecules, several are the requirements that need to be compulsorily respected to guarantee their stability and integrity. Furthermore, taking into account the multifunctional and, most of the times, chemically fragile nature of biological entities, the major challenge into this direction is a chemical one: to achieve bioorthogonal reactions under the benign reaction conditions that would preserve and respect the multifunctionality and structure of biologically derived components. The ideal coupling functional groups therefore should be highly stable in aqueous conditions and the reactions extremely chemo-selective and with high yields.

Pursuing this, numerous groups developed new methodologies that are often stored under the generic term of “click” chemistry reactions. This catchy term “click”, refers to energetically favoured, specific, and versatile chemical transformations, that lead to a single reaction product. In other words, the fundamental nature of the “click” is simplicity and efficiency. This concept seems to answer perfectly the needs of scientists in areas of research as diverse as molecular biology, drug-design, biotechnologies, material science or macromolecular chemistry. Reactions of the “click” type are rather rare. Yet, the last few years saw the emergence of a rudimentary “click” toolbox.

The introduction of the “click” concept in 2001 by Sharpless, Kolb and Finn\(^1\) has defined the stringent set of criteria that a process must meet to be useful in the “click” context as they stated that “click” reactions should be “modular, wide in scope, high yielding, stereospecific, simple to perform creating only inoffensive by-products (that can be removed without chromatography) and requiring benign or easily removed solvents, preferably
Although meeting the “click” requirements is quite difficult, several processes, such as the nucleophilic ring opening reactions, the non-aldol carbonyl chemistry, the additions to carbon–carbon multiple bonds and the cycloaddition reactions, were identified to step up to the mark. For example, among the proposed reaction pool stands the well-known Staudinger ligation. This reaction involves the chemoselective ligation between azide and phosphine, (both functional groups being quite stable in water) and has been largely used in the recent years by the Bertozzi group to selectively functionalize biomolecules or even living cells.\textsuperscript{2,3,4} Unfortunately, phosphines are susceptible to air oxidation and derivatives optimization aiming at increased reaction yields and improved water solubility still remains a challenging mission. This methodology has been also reported for the site-selective immobilization of proteins onto gold surfaces.\textsuperscript{5} Amongst the “click” reactions on biomolecules other methods like the Diels-Alder cycloaddition reaction, that has been used for example in the selective immobilization of proteins,\textsuperscript{6} the thiol-ene additions\textsuperscript{7,8,9} and oxime formation have also flourished in the literature during the last few years. Among these, cycloadditions and more specifically the copper (I) catalyzed variant\textsuperscript{10,11} of the Huisgen 1,3-dipolar cycloaddition\textsuperscript{12,13,14} (CuAAC, Scheme II-1) has certainly attracted the most of attention, so much that it is now often referred to as the “click chemistry reaction”. Several reports have confirmed the wealth of applications of this practical and sensible chemical approach in the areas of bioconjugation,\textsuperscript{15,16,17} polymer and materials sciences\textsuperscript{18,19,20,21} and drug discovery.\textsuperscript{22}

\begin{center}
\includegraphics[width=\textwidth]{schemeII-1.png}
\end{center}

\textbf{Scheme II-1.} General scheme of “click” chemistry cycloaddition reactions.
In fact, it was Sharpless and coworkers which rediscovered and reintroduced as “click”, a reaction that was pioneered in the 60s by Rolf Huisgen. The reaction itself is, a [3+2] dipolar cycloaddition between an azide and an acetylene (Scheme II-2) and is nowadays mostly used in its copper-catalyzed version (also generally referred to as “click chemistry reaction”), as a reliable and selective method for the synthesis of useful new compounds. It was in fact during a pioneering study by Meldal and co-workers demonstrating the applicability of click chemistry in peptide synthesis, that the regiospecific copper(I)-catalyzed variant of the 1,3-dipolar Huisgen cycloaddition was introduced for peptidic terminal alkynes and azides using solid-phase synthesis. The copper catalyzed version has the advantage to afford one single regioisomer whereas the basic thermal version gave an equimolar mixture of 2 regioisomers (Scheme II-2). The alkynyl and azido functional groups involved in this reaction are highly stable and react only through this chemoselective dipolar cyloaddition reaction in water (no side product observed) to produce the corresponding 1,2,3-triazolyl compound which is itself, very stable in aqueous solution and high temperatures. This reaction, which has been initially widely utilized in pure organic synthesis, has found widespread application in the area of bioconjugate chemistry and for these reasons has also been utilized for the purposes of the study presented here.

The inspiration came from the early works in the bioconjugation field. For instance, Finn et al. reported on the decoration of virus capsides with dyes. The cowpea mosaic virus (CPMV) coating protein, which was utilized as biomolecular scaffold in this study, was decorated with azide or alkyne motifs at either of the exposed reactive lysine or cystein residues. The corresponding products were then engaged in click chemistry reactions with the corresponding complementary fluorescein derivatives. In this study, the viral capsid appeared to be very sensitive to Cu (II) which was used as Cu(I) source and even disassembled. For this
reason, tris(benzyltriazolylmethyl)amine (also referred as to TBTA) was used as a ligand in conjunction with copper, and proved to be successful in inhibiting the disassembling process.

Nevertheless, due to cytotoxicity of the mandatory copper catalyst needed for the catalytic click chemistry version of the 1,3-dipolar Huisgen cycloaddition towards both bacterial and mammalian cells but also towards the catalytic activity of numerous enzymes, catalyst-free variants of the Huisgen cycloaddition have also been developed during the last years. On this direction Bertozzi and coworkers developed a method that relies on the strain promoted [3+2] cycloaddition between a strained cycloalkyne and an azido-derivatized biomolecule. Following this method they managed to selectively modify biomolecules in living systems through a series of cyclooctyne derivatives bearing a biotin motif. This series of linkers was shown to be stable in mild acidic and basic conditions but also towards biological nucleophiles such as thiols for prolonged times. They successfully linked in a selective fashion the biotinylated cyclooctynyl derivatives to a modified glycoprotein GlyCAM-Ig at physiological conditions without any apparent toxicity. Western Blots confirmed the achievement of the reaction and confirmed the orthogonality of the reaction.

In a more recent work, they successfully applied this newly copper-free click chemistry reaction to the imaging of dynamic processes in living cells. As Staudinger ligation reaction is too slow to follow rapid biological processes and as click chemistry reaction is toxic towards living systems, they decided to apply the copper-free version of click chemistry to follow labelling of proteoglycans of cells within a short time scale. A cyclooctyne derivative capped with an Alexa Fluor 488 dye was designed and synthesized. They were able to selectively label glycans at the surface of CHO cells by using Alexa 488 DIFO (difluorinated Cyclooctyne) but more importantly, they were able to follow in vivo trafficking of labelled glycans within the cells into cell compartments (e.g. Golgi, endosome, lysosome) within the one minute time scale.

A novel, copper free tandem [3+2] cycloaddition- retro-Diels-Alder reaction involving oxanorbornadienes which affords stable 1,2,3-triazole-linked compounds was also recently reported by the Nolte group. The reaction was successfully applied to the decoration of proteins or small peptides with fluorescent tags. For example, hen egg white lysozyme (HEWL) was functionalized with a heterobifunctional linker bearing at one extremity an oxanorbornadiene moiety and a carboxylic acid in the other. Following the coupling of the carboxylic moiety to the exposed lysine residues of HEWL, the derivatized
protein was incubated with 7-azido-hydroxycoumarin and gently shaken for 36 hours at 25°C. Nevertheless, since this novel reaction releases the toxic product furan, the scope of this reaction might be limited for bioconjugation and in vivo applications.

As described above, this copper-catalyzed [3+2] Huisgen dipolar cycloaddition is a powerful reaction that can be selectively performed in mild conditions, aqueous solutions, and does not require energy (no need to heat). Moreover, it is tolerant toward a wide range of functional groups, is highly selective and gives generally excellent yields.

For all these reasons, the synthesis of Giant Amphiphiles by copper-catalyzed [3+2] Huisgen dipolar cycloaddition click chemistry reaction was envisioned. As shown on Scheme II-3, this first alternative synthetic method that was developed in the laboratory relies on a straightforward two-step synthesis. In the first step, the specific maleimide-thiol coupling is utilized to selectively functionalize a thiol containing protein by a heterobifunctional linker bearing a terminal alkyne/azide. In the second step, click chemistry mediates the coupling of a hydrophobic polymeric chain appended by the complementary corresponding functionality to the protein-alkyne/azide bioconjugate. Though this reaction lies under the category of the two step indirect specific functionalisation, we reasoned that the orthogonality of the click chemistry coupling would allow for significant improvement of the conjugation reaction yields.

Scheme II-3. General scheme of the synthetic pathway used to create Giant Amphiphiles from “click chemistry”.

II.2. Results and discussion.

In this study, the 66 kDa carrier protein Bovine Serum Albumin (BSA) which is widely used as a model protein in numerous scientific bioconjugation works was initially
Cheaper than most commercial proteins, quite stable and relatively easy to purify, BSA can be used in relatively large quantities for scientific research.

Serum Albumin is the most abundant protein in blood plasma (typical concentration of 50 g/L) and functions as a transport protein for numerous endogenous and exogenous substances. It plays an important role in the regulation of the colloid osmotic pressure of blood by providing about 80% of the osmotic pressure as is considered to be chiefly responsible for the pH maintenance in blood. The primary sequence of BSA was presented in the same year as the structure of the Human Serum Albumin (HSA). It was proposed that BSA is composed of 582 amino acid residues with a sequence that has 17 disulfide bonds resulting in nine loops formed by the bridges. More importantly, BSA is considered to be an ideal model for specific bioconjugation reactions since this globular protein contains only one free cysteine residue, located at position 34 as shown on the Figure II-1. Surprisingly, in spite of the substantial information concerning BSA, its crystal structure has not yet been resolved and has led to some contradictory results and discussions. Nevertheless, its dimensions have been estimated to be 30 x 80 x 80 Å and its structure is considered to be homologous with that of Human Serum Albumin (HSA) with which it shares about 80% primary sequence identity and very similar biological functions (Figure II-1).

![Figure II-1](image)

**Figure II-1.** A. Structure organization of BSA, location of disulphide bonds. B. Space filling model of serum albumin molecule with basic residues coloured in blue, acidic residues in red, and neutral ones in yellow. C. Three dimensional representation of HSA with the α-helices depicted in purple, the β-turns as pale blue segments and the disulfide bridges and free cysteine (at position 34) depicted as yellow sticks.

Having selected BSA as the model protein to proceed with our studies, as described in the Scheme II-3, an appropriate hydrophilic heterobifunctional linker had to be designed for the efficient and selective coupling of the protein with a hydrophobic polymer via the copper-catalyzed [3+2] Huisgen dipolar cycloaddition.
II.2.1. Synthesis of heterobifunctional linkers.

As already mentioned in the general introduction, the specific conjugation of proteins is often achieved by targeting a cysteine residue. Cysteines that are not involved in disulfide bridges are generally few or even unique in the protein sequences. This allows for their specific targeting in bioconjugation reactions by using the correct, specific chemical reagent and appropriate conjugation conditions.

For the purposes of this research, we focused on the synthesis of heterobifunctional hydrophilic linkers containing a maleimide moiety on one side and either an azide or an alkyne on the other terminus (Scheme II-4). We decided to utilize the hydrophilic triethylene glycol as the starting backbone material for the synthesis of this linker, as its hydrophilicity was expected to increase solubility of the final heterobifunctional linker and therefore, the specific conjugation reaction yields.

Maleimides, as mentioned in Chapter I, react as Michael acceptors specifically with Cysteine residues at mild conditions and neutral pHs (6.8–7.5) with great specificity (maleimides will only react with amines at pH >8), and more importantly with high reactivity. For these reasons, we selected to utilize maleimide for the bioconjugation of the heterobifunctional linker with thiol containing proteins. Nevertheless it can be envisioned that this moiety will be replaced in the future with other, protein specific coupling groups. Since the maleimide moiety is not stable under several reaction conditions, we designed our synthesis to proceed through an initial mono-functionalization of the triethylene glycol.
backbone with either an azide or an alkyne moiety and introduced the maleimide functionality on a later step.

Using this approach, the preliminary experiments on the preparation of maleimido-heterobifunctional linkers bearing a terminal azide were unsuccessful as the azide and maleimide moieties could easily undergo inter- and/or intra- [3+2] dipolar cycloadditions under the reaction conditions required for the synthesis of this hydrophilic heterobifunctional linker.\textsuperscript{37,38,39} Several attempts to optimize the reaction conditions and avoid cycloaddition failed and an insoluble gummy polymer was recovered in all cases while $^1$H NMR revealed the disappearance of the maleimide signal (6.9 ppm) together with the broadening of the peaks, thus confirming this cycloaddition reaction. It was therefore decided to focus on the synthesis of alkyne appended heterobifunctional maleimide linkers.

The introduction of a terminal alkyne onto triethylene glycol was achieved by a simple nucleophilic substitution with propargyl bromide as previously reported in the literature.\textsuperscript{40} Triethylene glycol was initially mono-deprotonated with sodium hydride in THF in dry conditions. The addition of propargyl bromide gave the resulting product in quantitative yields (Scheme II-5).

![Scheme II-5](image)

**Scheme II-5.** Reagents and conditions used for the synthesis of the monoalkyne triethylene glycol derivative 1.

The PEG monoalkyne compound 1 was then reacted with maleimide via a modified Mitsunobu reaction in order to synthesize the corresponding maleimido heterobifunctional linker 2.\textsuperscript{41} Briefly, the coupling proceeded through an initial reaction of triphenylphosphine with DEAD at -78°C under nitrogen atmosphere which was followed by the addition of initially compound 1 and then successively neopentyl alcohol and maleimide as mentioned in Scheme II-6.
Chapter II – Formation of Giant Amphiphiles by Click Chemistry Reaction

Scheme II-6. Reagents and conditions used for the synthesis of the heterobifunctional maleimido-alkyne linker 2.

The resulting solution was slowly let to warm up to ambient temperature (Scheme II-6) and was stirred overnight. After chromatographic purification, the hydrophilic heterobifunctional linker 2 was recovered in 70% yield.

It should be mentioned that analogous compounds were successfully generated utilizing a tetraethylene glycol and a pentaethylene glycol as hydrophilic backbones, but since similar results were obtained in all cases when tested in bioconjugation, they are not individually mentioned in this thesis.

II.2.2. Coupling of the heterobifunctional linker 2 to the native BSA.

The coupling between native BSA and the heterobifunctional maleimido-alkyne linker 2 was performed in aqueous conditions using 20 mM phosphate buffer pH 7.4 (PB). A 100 molar excess of the heterobifunctional linker 2 in DMSO was slowly added to the BSA solution and the reaction mixture was gently shaking for 24 hours at 7°C (Scheme II-7). The reaction mixture was then purified by extensive dialysis against 20 mM PB pH 7.4 using 10 kDa MWCO regenerated cellulose dialysis bags.

Scheme II-7. General reaction scheme and conditions used for the coupling of the heterobifunctional linker 2 to BSA to afford the alkyne-1-appended bioconjugate I.
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Figure II-2. Characterization of the alkyne-1-appended BSA bioconjugate I. Left: Electrophoresis under denaturating conditions. Lane 1: native BSA, lane 2: purified bioconjugation reaction mixture. Right: SEC chromatographic traces at 254 nm of the native BSA (dashed grey trace) and BSA linker I (solid black trace).

SEC chromatographic analysis showed no significant difference in the retention times of native BSA and the BSA-1-alkyne I conjugate (Figure II-2). Electrophoresis under denaturating conditions on the other hand revealed a new band after Coomassie Brilliant Blue staining. Surprisingly, this new band exhibited a faster migration behaviour (i.e. was located in the lower apparent molecular weight region) when compared to native BSA. We attribute this behaviour to either the difference in the total net charge of BSA, or to a conformational change induced by the bioconjugation of the linker to the protein.

The selective conjugation of the hetero bifunctional linker 2 onto the protein to afford the mono-adduct I was finally confirmed by MALDI-TOF. A mass difference of ~ 300 uma in the molecular peaks corresponding to the native protein and I was observed, and judged to be consistent with the specific coupling of one linker molecule per protein (Figure II-3).
II.2.3. Preparation of polystyrene azides.

Three different, commercially available bromide-capped polystyrenes with molecular weights varying from 2 to 8 kDa were easily transformed to the corresponding azides by nucleophilic substitution of the bromide group with sodium azide. Briefly the reaction proceeded by stirring overnight the corresponding polystyrene (2, 4 and 8 kDa) with a 4 molar excess of sodium azide solution in DMSO at 60°C (Scheme II-8). After removing the solvent under reduced pressure, the azido-polystyrenes 3a, 3b, 3c which were obtained in excellent yields were characterized by GPC, MALDI, FT-IR and $^1$H NMR.

```
\begin{align*}
\text{Br} & \quad \text{NaN}_3, \text{DMSO, 60°C} \\
& \quad \text{overnight} \\
\rightarrow & \quad \text{N}_3 \\
\end{align*}
```

Scheme II-8. Reagents and conditions used for the synthesis of azido-polystyrenes 3a, 3b, 3c.

The GPC chromatographic behaviour of the reacting and produced polystyrenes was investigated before and after the azidation reaction and is reported in Figure II-4. Unfortunately, the mass difference between azides and bromides was too small to introduce any characteristic difference in the retention times under the chromatographic conditions utilized (solvent: toluene, room temperature, 2 PL-Gel mixed columns (ThermoLabs) in
Chapter II – Formation of Giant Amphiphiles by Click Chemistry Reaction

Nevertheless, the GPC analysis confirmed the retention of the polydispersity after the reaction as observed by the broadness of the peaks.

Figure II-4. GPC chromatographic analysis of the different commercially available polystyrene bromides before (dashed grey traces) and after the azidation reaction (solid black traces).

IR analysis of the azidated polymers showed the characteristic stretching band of the C-N$_3$ bond around 2100 cm$^{-1}$. The ω-terminus substitution was confirmed by $^1$H NMR spectroscopy through the characteristic downshield shifting of the α to the azide group protons as compared to those of the α to the bromide group of the starting materials. Finally the MALDI-TOF spectra showed, as expected, a shift of the polymer distribution by 38 uma (corresponding to the abstraction of bromide (−80) and substitution with an azide (+42) inducing a difference of mass of 38 uma), further confirming the azidation of the polystyrene bromides.
II.2.4. BSA-PS *Giant Amphiphiles* through the direct, click chemistry mediated, coupling of polystyrene azides.

As already mentioned earlier, the main difficulty of the efficient synthesis of *Giant Amphiphiles* resides in the solubility incompatibility between the two components, *i.e.* the water soluble protein and the hydrophobic polymer. In the past,\(^4^3\) this problem was addressed by the incorporation in the reaction scheme of a miscible with water, selective for the hydrophobic polymer, organic solvent (such as THF). Though this solution has been successful in specific cases of proteins (such as CAL B which has a known increased tolerance for organic solvents),\(^4^3\) the percentage of this organic solvent can in the general case be destructive or harmful for the protein conformation/activity. Taking this into account, we utilized THF to solubilise the polymers during the course of this study and kept the THF / water content minimal (less than 10\%). Furthermore, in order to achieve the [3+2] Huisgen “click” cycloaddition, a Cu mediated variant of click chemistry was employed.\(^1^0\) We selected to utilise as catalyst the Cu (I) generating Cu(II)SO\(_4\) and sodium ascorbate system, as it had already been proven that it is efficient and compatible with bioconjugation reactions.\(^2^3\)

More specifically, to achieve the polystyrene azide *clicking* on to the BSA-1-alkyne I, the polystyrenes were initially dissolved in THF and slowly added in a 10 molar excess to a 20 mM phosphate buffer solution of BSA-alkyne I containing 2 molar excess of CuSO\(_4\) and 4 of Na ascorbate. The final total volumes of the reactions were adjusted with 20 mM phosphate buffer, pH 7.4 in order to avoid having over 10 % organic solvent content in the aqueous solution reaction mixtures.
Scheme II-9. General synthetic scheme for the click chemistry reaction between BSA-alkyne I and the series of polystyrene azides 3a, 3b, 3c leading to the formation of the BSA-PS clicked products IIa, IIb, IIc respectively.

Unfortunately, problems of solubility drastically hampered the reaction in our initial efforts since already, the 4 kDa polystyrene azides were found to be insoluble in the reaction conditions.

The reaction was finally achieved by modifying the procedure in such a way that the protein was added last to the reaction mixture (in a concentrated solution) allowing an initial extensive sonication of the polystyrene azides while added into the buffer to achieve their optimal dispersion. As it will be shown in the next paragraphs, bioconjugation of polymers with a molecular weight upto 8 kDa was achieved with this modified protocol, nevertheless bioconjugation of higher molecular weight hydrophobic polymers than the ones utilized to couple BSA-alkyne conjugate I still remains a challenging task.

II.2.5. Characterization of the BSA-polystyrene Giant Amphiphiles.

Purification of the biohybrid amphiphiles IIa, IIb, IIc was undertaken by simple dialysis of the reaction mixture using 10 kDa molecular weight cut-off (MWCO) dialysis membranes against 10 % DMSO, 20 mM phosphate buffer pH 7.4 initially and then against 20 mM phosphate buffer pH 7.4. The resulting turbid solutions containing IIa, IIb, IIc were analyzed by SEC chromatography (solvent: 30% acetonitrile 20 mM phosphate buffer, flow
rate: 0.5 mL.min⁻¹, room temperature, column: BioBasic SEC 300 (Thermo)). The formation of new peaks corresponding to the compounds IIa, IIb, IIc, possessing a larger hydrodynamic volume than that of native BSA (shorter retention times, higher molecular weights), was immediately observed (Figure II-5). No presence of the BSA-alkyne linker I peak was detected in any of the measurements, demonstrating its efficient removal by the dialysis step. As pointed by the SEC measurements, the reaction yields were rather high verifying thus the efficiency of the “click” chemistry reaction for the synthesis of Giant Amphiphiles. As expected, the BSA dimer remained in the reaction mixture even after dialysis due to its high molecular. The difference observed in the retention times of the different adducts IIa, IIb and IIc is not significant, probably due to the lack of sensitivity of the column under the specific conditions utilised to analyze the samples. Further attempts to chromatographically discriminate between these products in terms of molecular weight by changing the solvent system (composition and pH), the flow rate and/or the column were unsuccessful.

A blank experiment was performed by incubating the BSA-1-alkyne linker I and a mixture of the azide appended polystyrenes 3a, 3b and 3c in 20 mM phosphate buffer without CuSO₄ and Na ascorbate. Upon gentle stirring for ~ 48 hours, the mixture was subjected to the same dialysis protocol that was used in the case of the click chemistry reactions. The SEC measurements upon dialysis revealed the presence of the unreacted BSA-1-alkyne I and a successful removal of the non interacting polymers. This verified the click coupling and excluded the possibility of non-specific interactions between the polymer and the protein.

![Figure II-5. SEC chromatographic characterization of the blank experiment (I + 3a, 3b and 3c without CuSO₄/sodium ascorbate, black dotted trace) and BSA-polystyrene amphiphiles IIa, IIb and IIc (respectively solid black, solid grey and dashed grey traces) after dialysis.](image-url)
Electrophoresis under denaturating conditions (SDS-PAGE) confirmed the chromatographic results. As expected in the case of amphiphilic block copolymers, assembly hampers the migration in the gel and thus the BSA-polystyrene Giant Amphiphiles IIa, IIb, IIc obtained did not migrate on the gels due to their amphiphilic character (Figure II-6). The same results were observed when electrophoresis was performed under native conditions. The presence of small quantities of unreacted BSA is also apparent in the gel. Unfortunately the absolute quantification of the reaction yields was not possible as SEC chromatography did not separate the unreacted protein peak from that attributed to the BSA dimer.

![Figure II-6. SDS-PolyAcrylamide Gel Electrophoresis (PAGE) under denaturing conditions stained with Coomassie Brilliant Blue; lane 1: native BSA, lane 2: BSA-PS 2 kDa Ia, lane 3: BSA-PS 4 kDa Ib, lane 4: BSA-PS 8 kDa Ic.](image)

The click chemistry derived BSA-polystyrene polymer-protein conjugates were also analyzed by MALDI-TOF (Matrix Assisted Laser Desorption Ionisation Time Of Flight mass spectrometry). Unfortunately, the results obtained were rather poor and our efforts to adapt the conditions using different mixtures of matrices were unfruitful. This is a common problem in the area of amphiphilic block copolymers. Yet, measurement of the smaller BSA-PS amphiphilic bioconjugate i.e. BSA-PS 2 kDa Ia confirmed the formation of the biohybrid, through a new peak with a molecular weight at 68672 amu (Figure II-7).
II.2.6. Aggregation studies.

The self-assembly of the BSA-polystyrene amphiphilic biomacromolecules derived via the direct click chemistry coupling of various polystyrene-azides onto the alkyne-1-appended BSA I, was studied using Transmission Electron Microscopy (TEM). The samples were dialyzed against nanopure water prior to the measurement and were diluted ~100 times with nanopure water. A minute quantity (~10 µl) of each sample was placed on a formvar coated copper grid and after a waiting period of ~15 minutes, the solvent was drained using a filter paper. No staining or shadowing was necessary to visualize the samples as the increased electronic density of polystyrene provided enough contrast. No architectures were found in samples prepared using native BSA. TEM revealed the formation of well-defined spherical superstructures with diameters varying from 30 to 500 nm size as can be shown on Figure II-8.

Interestingly, the size of the hydrophobic polymer tail (2 kDa to 8 kDa) did not seem to have any effect on the aggregation behaviour of the Giant Amphiphiles. This observation is attributed to the fact that the difference in the hydrophobic chain lengths used in this study was not big enough, as compared to the protein size, to have any dramatic impact on the self assembly of the resulting BSA-polystyrene superstructures. Unfortunately, due to solubility problems, higher molecular weight polystyrene azides could not be utilized. It is therefore...
believed (as different architectures have already been shown for Giant Amphiphiles)\textsuperscript{43} that the appropriate approach to be followed if one wants to study the effect of the shape on the resulting aggregated architectures, is to utilize a smaller, in terms of dimensions and molecular weight, protein component.

\begin{figure}
\centering
\includegraphics[width=0.7\textwidth]{figure.png}
\caption{TEM micrographs of superstructures obtained by aggregation of A. BSA-PS Ia, B. BSA-PS Ib and C. BSA-PS Ic.}
\end{figure}

Finally, it should be noted that toward the end of our studies, a publication from the Nolte group\textsuperscript{27,44} also verified the efficiency of the copper catalyzed, Huisgen cycloaddition, click chemistry approach through the direct coupling of hydrophobic polymers to peptides and proteins using slightly different conditions, or the cofactor reconstitution approach.

II.2.7. Further studies of the direct click chemistry approach utilizing other proteins.

The applicability of this, direct, click biospecific conjugation of polymers to proteins other than BSA was tested in a series of enzymes and proteins displaying an accessible
cysteine residue. We were interested to prove the generic nature of this bioconjugation method as well as to further study the role of proteins in the aggregation behaviour of the bioconjugates. For this reason the free thiol containing Cytochrome C from equine heart, (Cyt C) and Haemoglobin from bovine blood, (Hb) were utilized.

Haemoglobin is a large (64 Kda), multiple, subunit globular protein necessary for gas exchange in living organisms. It has long been the focus of studies in molecular biology and was one of the first proteins for which structure and sequence were determined. Hemoglobin comprises two pairs of dissimilar subunits, α and β. Each α chain contains one cysteine residue (α 104) and each β chain two cysteine residues (β 93 and β 112). However, in the native state of hemoglobin, of these six sulfhydryl groups, only two (β 93) react with reagents specific for sulfhydryl groups.

Cytochrome C, or Cyt c is a small (11 kDa) heme protein found loosely associated with the inner membrane of the mitochondrion. It is an essential component of the electron transfer chain, where it carries one electron. Cyt C is also a heme bearing protein that contains 2 free cystein residues (one of them is coordinated with the heme cofactor and therefore not accessible) and is also implicated in the cell apoptosis.

The proteins were incubated on a first step with a 100 molar excess of the maleimido heterobifunctional linker 2. After gentle shaking for 2 days at 7°C, the reaction mixtures were extensively dialyzed (using regenerated cellulose membranes with MWCO values of 10 and 50 kDa for Cyt C and Hb respectively) and the biohybrids analyzed by SEC and electrophoresis. Both SEC and electrophoresis under native conditions did not reveal any dramatic changes that would indicate the formation of the alkyne functionalized proteins. Nevertheless, since the same observation was also true in the successful case of BSA-1-alkyne I, we proceeded with the second step during which the alkyne bearing proteins III (1-alkyne functionalized Cytochrome C) and IV (1-alkyne functionalized Haemoglobin) were coupled to polystyrene azides 3a, 3b, 3c through a copper catalyzed [3+2] Huisgen cycloaddition using the CuSO₄/Na ascorbate Copper (I) generating system and the optimized for BSA reaction conditions. Following an incubation time of ~ 2 days, the reaction mixtures were dialyzed and the resulting amphiphilic macromolecules Va, Vb, Vc produced from the clicking of polystyrene azides with molecular weight 2kDa, 4 kDa and 8 kDa respectively with Cytochrome C, as well as the analogous haemoglobin bioconjugates VIa, VIb, VIc were
characterized by SEC chromatography. These measurements (solvent: 30% acetonitrile 20 mM phosphate buffer, flow rate: 0.5 mL.min\(^{-1}\), room temperature, column: BioBasic SEC 300 (Thermo)) revealed the formation of a series of new products that are shown on Figure II-9, exhibiting a behaviour similar to that of the BSA-polystyrene Giant Amphiphiles. It should be noted that in the case of Hemoglobin, the traces monitored at 405 nm (heme) were in agreement with the ones monitored at 254 nm, proving the presence of heme in the product solution.

![Figure II-9](image.png)

**Figure II-9.** SEC chromatographic analysis of native proteins and amphiphilic biomacromolecules (left). *Left:* native Cytochrome C and amphiphilic CytoC-polystyrene bioconjugates Va, Vb, Vc at 254 nm. *Right:* native Hb and amphiphilic Hb-polystyrene bioconjugates VIa, VIb, VIC at 406 nm.

As mentioned earlier, obtaining MALDI-TOF spectra of such compounds is not an easy task. Though using several combinations of matrices, we were only able to obtain MALDI spectra for the cases shown in Figure II-10. Nevertheless, in the case of the conjugates Va and VIa, MALDI verified the SEC and electrophoresis results (showing no migration of the products under both native and denaturating conditions). It is good also to note that, as haemoglobin was very sensitive to MALDI conditions and a variety of (polymeric) peaks was obtained for the native enzyme itself, we utilized an alternative pathway to stabilize it which involved mixing with native BSA. Upon this, better results were obtained both in the case of the native Hb and in the case of the bioconjugate VIa as presented in Figure II-10.
The study of aggregation study was performed using TEM microscopy. Again, 10 µl of ~ 100 times diluted samples of the native enzymes and the purified reaction mixtures, were placed on a formvar coated copper grid and after a waiting period of ~15 minutes, the solvent was drained using a filter paper. Both native Cyt C and Hb did not reveal any aggregation behaviour when examined with TEM. Unfortunately the same was observed for the case of the Cytochrome C bioconjugates where a non defined, gel like pattern was only observed on the grids. Surprisingly TEM revealed that in the case of the amphiphilic Hb-polystyrene conjugates a different morphology than the one observed for BSA was adopted. It was observed that all Hb-PS *Giant Amphiphiles* formed well defined cylindrical (“worm-like”) superstructures with a quite monodisperse length of roughly 1000 nm and diameter between 300 and 400 nm (Figure II-11). Nevertheless, the images also contained unstructured material as well as different smaller (presumably micellar) architectures, and therefore further SEM experiments are in progress with the aim to clarify the aggregation patterns.
In conclusion, both Haemoglobin and Cytochrome C were successfully functionalized following the direct click chemistry coupling approach. In experiments performed in the laboratory that are not included in this thesis, the formation of Giant Amphiphiles through this approach was also studied and proved to be successful for two more proteins, the Tobacco Mosaic Virus Coating Protein (TMV CP) and papain. It is therefore considered to be a method generic for thiol containing proteins. It should also be finally noted that though the bioconjugation experiments proved to be successful, in both heme proteins a change of colour during the course of the reactions suggested the destruction of active form (probably due to the selected copper (I) generating system) and therefore a change of catalyst is judged to be necessary to ensure the stability of the proteins in further studies.

II.2.8. Study of Giant Amphiphiles in organic solvents.

The essence of our studies lies in the understanding of the factors that dictate the formation of biohybrid superstructures and in the subsequent use of this knowledge to program functional two- and three-dimensional nanometer-sized superstructures. As part of this study, we reasoned it should be possible to “reverse” the aggregation morphologies with the use of organic solvents. In a medium which would be selective for the polymeric tail, we expected that if the giant surfactants would assemble at all, this should lead to the formation of inverted structures (Figure II-12), meaning that the polymer tails would be by nature exposed and oriented to the solvent and that the hydrophilic protein heads would be clustered in the core of the superstructures.
We investigated this assumption using the BSA-polystyrene Giant Amphiphiles which were synthesized through the direct click-chemistry approach. All samples were extensively dialyzed against nanopure water to remove the excess of unreacted reagents and phosphate buffer. After ~48 hours, we freeze-dried the samples to remove water and dissolved in dry dichloromethane which was judged to be the most appropriate selective for polystyrene-solvent (blank experiments revealed no solubility of BSA). We immediately noted the formation of opaque solutions while the aggregation was verified using TEM as shown in Figure II-13. In this case, we utilized carbon coated formvar copper grids, applied a small quantity (10 µl) of the dichloromethane solution and allowed the solvent to evaporate.

From the TEM micrographs, we observed the formation of well defined self-assembled superstructures (Figure II-13) with diameters varying from 30 to 500 nm. From their appearance and dimensions, we reasoned that they are most probably micellar or vesicular in nature with the BSA being at the core of the membrane and the polymer exposed.
It should also be noted, that when experiments were performed without dialysis (i.e. without the removal of the excess unreacted polystyrene), the structures showed a very interesting dynamic behaviour with time probably due to the dynamics of the excess of polystyrene in dichloromethane (Figure II-14).

**Figure II-14.** A. TEM micrographs of BSA-PS 4kDa Ib in CH$_2$Cl$_2$. Micrograph on the left immediately after addition of the CH$_2$Cl$_2$, on the right 48h later. B. TEM micrographs of BSA-PEG$_{5000}$ in dichloromethane.

Blank experiments with BSA did not reveal the formation of any aggregates, while when we used as a blank BSA-polyethylene glycol 5 kDa (PEG$_{5000}$), we were able to observe only the formation of structures that we attribute to material that collapses on the grid after evaporation of dichloromethane ("coffee stain artefacts") (Figure II-14). This is the first time that aggregation of protein-polymer amphiphiles is ever observed in organic solvents.

**II.2.9. Using Giant Amphiphiles to bring enzymes into organic solvents.**

In a further attempt to generate meaningful superstructures, we though it would be of interest to exploit the aggregation of Giant Amphiphiles in organic solvent by using the resulting aggregates as nanoreactors through the encapsulation of an enzyme within the cavity of these inverted spherical structures. We reasoned that within such inversed superstructures
(Figure II-15), the BSA head group of the *Giant Amphiphiles* would most probably be in the core of the superstructures, creating a protein-friendly environment able to host other proteins and at the same time protect them from exposure to the organic solvent.

**Figure II-15.** Formation of nanoreactors by inversion of the superstructures in organic solvent.

Toward this direction, we utilized as guest protein $\beta$-galactosidase, a well known enzyme that hydrolyzes ester derivatives of $\beta$-galactose. The selection of the enzyme was only based in the presence of a commercially available fluorogenic activity assay that we planned to utilize for the monitoring of the activity.

The protocol developed to encapsulate the $\beta$-galactosidase within the *Giant Amphiphile* superstructures, involved the freeze drying of a sample of BSA-PS 4 kDa IIb, the addition of a minimal quantity of a concentrated aqueous solution of $\beta$-galactosidase and the repeating of a series of freeze-drying cycles followed by the intermediate addition of small quantities of dichloromethane. Upon repeating these cycles several times, the solid residue was dispersed in the initial quantity of dichloromethane.

The activity of the enzyme was verified using CFM microscopy. In order to be able to fluorescently visualize the superstructures themselves, the amphiphilic polymer-protein bioconjugates were labelled using an NHS-activated Alexa-488 dye through specific coupling with the Lysine residues of BSA, prior to being inversed into the organic solvent.

As mentioned above, for the fluorogenic assay the appropriate pro-fluorescent substrate was commercially available. After a $\beta$-galactosidase catalyzed hydrolysis two products are released in solution: $\beta$-galactose and the fluorescent product, 7-hydroxy-4-methylcoumarin, known to display a strong fluorescence at 405 nm (Figure II-16).
Figure II-16. Protocol used to visualize catalysis of $\beta$-galactosidase by Confocal Fluorescent Microscopy.

In order to measure the activity of the $\beta$-galactosidase encapsulating nanoreactors, a dichloromethane solution of the nanoreactors was placed between two hydrophobic glass slides and was sealed prior to the measurement. Upon visualizing the fluorescent superstructures (Figure II-17, left), the substrate was injected and the slide was independently scanned at 488 and 405 nm. It should be noted that the Giant Amphiphile superstructures were not resolved with CFM, as their dimensions were rather small for the resolution of the instrument.
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Following a lag period, the intense blue fluorescence at 405 nm was detected (Figure II-17) and attributed to the formation of the fluorescent product. No fluorescence was detected before the addition of the fluorogenic substrate to the aggregates solution or when a blank experiment was performed in the absence of β-galactosidase within the vesicles.

II.3. Summary and Outlook.

In conclusion, in the studies presented here, we were able to successfully synthesize small libraries of Giant Amphiphiles that self-assemble adopting different morphologies depending on the protein used.

More specifically, the copper catalyzed, Huisgen [3+2] cycloaddition was utilized and a protocol was developed to allow its use for such bioconjugations. This polymer bioconjugation approach was comparatively utilized in different proteins, showing therefore its generic nature for free cysteine containing proteins. Furthermore, the characterization of the resulting Giant Amphiphiles was performed in unprecedented details.

During the characterization studies, the Giant Amphiphiles were shown to form well-defined spherical superstructures in aqueous medium in the case of Bovine Serum Albumin
whereas the Haemoglobin *Giant Amphiphiles* formed cylindrical types of like structures. Tuning of the length of the hydrophobic polymeric tail was shown not to change the aggregation behaviour in the case where the same protein was utilized. We attribute this to the relatively small molecular weight variation that can be achieved using polystyrene, as compared to the molecular weight of the proteins.

It should be stressed that during this study the first example of the self-assembling properties of *Giant Amphiphiles* in organic solvents is presented. This is a stimulating breakthrough in the polymer-protein biohybrid research area, one which we intend to further pursue and understand since it provides us with enormous potential for the creation of innovative nanoassemblies. To demonstrate this, a guest enzyme was successfully introduced in the superstructures and their permeability and capacity to act as nanoreactors was demonstrated through a CFM observation of its catalytic action.
References

Chapter II – Formation of Giant Amphiphiles by Click Chemistry Reaction

Chapter III
Formation of Giant Amphiphiles by Post-Functionalization of Hydrophilic Triblock Protein-Polymer Conjugates

III.1. Concept / Background.

Although several methods have already been developed for the synthesis of Giant Amphiphiles, practical limitations can hamper their efficient synthesis in high yields. The main limitation may be attributed to the incompatibility (mainly in terms of solubility) of the two major components (i.e. the hydrophobic polymer and the hydrophilic protein) while several constraints are also being posed to ensure that the protein structures will remain intact. The conditions that were used in the past, took advantage of the known tolerance of specific proteins in organic solvents and the small window of solvent compatibility between the hydrophobic polymer and the protein to achieve solubilization of both components during the bioconjugation step.\textsuperscript{1,2,3} These conditions are not judged to be generic as they are quite harsh for the generality of proteins and can easily lead to denaturation. Furthermore, the concomitant to the formation of Giant Amphiphiles aggregation expressing their strong amphiphilic character, possess also an additional limitation, that of the purification as any attempt to isolate the Giant Amphiphiles from unreacted polymers trapped within the superstructures would certainly involve dissolution steps that could interfere with protein integrity.

Consequently, due to the purification and solubility problems inherent to the synthesis of Giant Amphiphiles that were reported in the precedent literature, it was decided to design a new strategy aimed to circumvent these problems in order to access quantitative amounts of products enabling us to further study possible applications of these biomacromolecules. In this work, we therefore report on a novel, generic approach for the synthesis of triblock Giant Amphiphiles in which hydrophobicity is introduced by
post-functionalization of an appropriately designed, pre-functionalized protein-polymer multifunctional biohybrid (Scheme III-1).

**Scheme III-1.** General concept of the post-functionalization approach.

In the design of this approach, since the coupling between the hydrophilic protein and the hydrophobic polymer is the major drawback in the synthesis of Giant Amphiphiles, we envisioned a strategy involving the initial coupling of a multifunctional hydrophilic polymer to a protein followed by the transformation of the hydrophilicity of the polymer. This technique should, in principle, give better reaction yields due to the hydrophilic nature of both the synthetic and natural polymeric blocks involved in the bioconjugation. The polymer had to be designed in such a way that in a second step, its hydrophilicity could be turned to the resulting hydrophobic block needed for the introduction of amphiphilicity in the bioconjugate. To synthesize such a multifunctional polymer several requirements needed to be taken into consideration. The final polymer should combine a bioconjugation site, an overall hydrophilic character and multiple grafted, tunable functional units.

The latter, should be able to introduce, by a simple reaction, hydrophobicity in a specific, efficient and reproducible manner and should be therefore carefully chosen. Indeed, to avoid the destabilization of the protein attached to the polymer, the reaction leading to the formation of amphiphilic structures had to be a high yielding, orthogonal reaction that proceeds under mild conditions in aqueous solutions. To this end, we decided to introduce within the hydrophilic polymer backbone a gradient of pending alkyne-1 functionalities that could be easily transformed and therefore introduce hydrophobicity, in a later step under the mild conditions needed for the well-known copper catalyzed Huisgen azide-alkyne dipolar cycloaddition.
To synthesize the multifunctional hydrophilic polymer with a high level of control over molecular weight and polydispersity (PDI), it was decided to follow Atom Transfer Radical Polymerization.

### III.1.1. ATRP polymerization.

Atom Transfer Radical Polymerization (ATRP), also called transition-metal-mediated living radical polymerization (TMM-LRP), has its basis in the atom transfer chemistry used in organic synthesis, e.g. intramolecular cyclization, and was discovered independently by the groups of Sawamoto and Matyjaszewski in the 90s. ATRP is now well established and universally used as an efficient method for the polymerization of vinyl monomers since it has proven to be versatile and exhibit good tolerance toward a wide range of functional groups. Moreover, it allows excellent control over the polymer architecture and it is quite simple to set up in a organic chemistry laboratory. The only restrictive requirement for ATRP is the absence of oxygen atmosphere, a problem that can be easily solved by repeating freeze-pump-thaw cycles prior to the polymerization.

The ATRP polymerization process of any vinyl monomer involves the combination of an alkyl halide acting as initiator and a catalytic low valent metal complex, e.g. CuBr–bipyridine, RuCl2(PPh3), Ni[C6H5(CH2NMe2)2-2,6]Br, which is capable of being oxidized to the n + 1 state by addition of a halogen atom. Copper has by far proven to be the transition metal of choice, as determined by the successful investigation of a spectrum of copper and other metal complexes as catalysts for the ATRP of a broad range of monomers in diverse media by many research groups. However, iron may eventually prove to be the transition metal of choice for environmental reasons unless industrially viable procedures for internal reuse of the copper complexes are adopted. It should also be noted that Ruthenium and Osmium have certain advantages as a consequence of their high halidophilicity that may eventually also make them a good choice for use in protic media.

The postulated mechanism proposed by both Matyjaszewski and Haddleton is composed, as usual in polymerization processes, from 3 different steps (Scheme III-2):
An initiation step where the abstraction of the halogen occurs on the initiator. During this step, the halide-carbon bond (generally a bromide but can also be a chloride) of the initiator is cleaved in a homolytic fashion, generating a carbon-based radical species and a new metal halide with the metal in the \( n + 1 \) oxidation state, e.g. Cu (II).

A second step, called propagation step, where the polymer grows by the sequential addition of monomeric units to the carbon-based radical species formed during initiation. This step involves a free radical attack of the polymer radical on a monomer, a step commonly observed in all free radical polymerization mechanisms. During the propagation, termination is prevented by the reverse abstraction of a halogen atom from the metal halide to give a new polymeric alkyl halide, also referred to as “dormant” species. The equilibrium between the “dormant” and “active” species (free radicals in the polymerization feed) is therefore shifted towards the former as shown in Scheme III-2, reducing thus the concentration of radical species growing chains in the polymerization feed and avoiding as a consequence the early termination process.

A termination step where the final polymer is terminated by radical hetero coupling with bromine radical.

\[
\begin{align*}
\text{Cu(I)X} / L_n & \quad + \quad \text{P-X} \quad \xrightarrow{k_{\text{act}}} \quad \text{Cu(II)X}_2 / L_{n-1} + \quad \text{P} \quad \xrightarrow{k_{\text{deact}}} \quad \text{P-P} + \quad \text{M} \\
\text{Cu(I)X} / L_n & \quad + \quad \text{P-X} \quad \xrightarrow{k_{\text{act}}} \quad \text{Cu(II)X}_2 / L_{n-1} + \quad \text{P} \quad \xrightarrow{k_{\text{deact}}} \quad \text{P-P} + \quad \text{M} \\
\end{align*}
\]

**Scheme III-2.** General Scheme describing the Atom Transfer Radical Polymerization proposed mechanism.

ATRP polymerization has been extensively studied using numerous pyridine, aliphatic tertiary polyamine, polyimine containing compounds, terpyridines and phenantrolines as ligands depending on the nature of the monomers intending to polymerize and the conditions used for the polymerization (e.g. polymerization solvent). Some of the above mentioned ligands have been extensively studied especially in Matyjaszewski\(^{25,26,27}\) group. As a result of these studies, bipyridines\(^{8,28}\) and pentamethyl diethylenetriamines (PMDETA\(^{29,30}\)) in
conjunction with low-valent metal ions are usually used nowadays in numerous research
groups as ligands for ATRP polymerization.

![Figure III-1](image1)

**Figure III-1.** Most commonly used ligands in the ATRP polymerization process.

Furthermore, a series of \( N\)-n-alkyl-2-pyridylmethanimine derivatives that were
developed by Haddleton and collaborators, are also nowadays used with a wide range of
monomers going from hydrophilic to hydrophobic methacrylates, styrene, etc.\(^{31,32,33,34}\) In
recent literature, it has for example been demonstrated that the pyridine-2-carbaldehyde
imines used as ligand in association with copper(I) bromide are very effective for ATRP of
methyl methacrylate (MMA) with a variety of different initiators.\(^{14}\) These imine-based
ligands, whose general structure is displayed on Figure III-2, offer the advantage over other
ligands that they can be synthesized through a simple one-step procedure (condensation
between amine and pyridyl carbaldehyde) which can utilize virtually any primary amine for
the tuning of the ligand solubility while, bipyridine derivatives require rather tedious synthetic
procedures to modify the lateral alkyl chains.\(^{7}\)

![Figure III-2](image2)

**Figure III-2.** Structures of the \( N\)-alkyl-pyridyl imine based ligands developed by Haddleton
and coworkers.

Taking therefore into account the above mentioned advantages and versatility of
ATRP polymerization, this method was selected for the synthesis of the well-defined,
multifunctional polymer required during this bioconjugation approach.
In conclusion, within this chapter our studies on the novel, post-functionalization approach, aiming at a high yielding and efficient synthesis of Giant Amphiphiles will be presented. We envisioned a sequence in which a functionalized hydrophilic polymer was initially specifically coupled to a protein to form a triblock hydrophilic polymer-protein bioconjugate and on a second step, post-functionalized through a copper catalyzed multi-click chemistry reaction with hydrophobic azides to convey an overall amphiphilic character to the bioconjugate. The design and ATRP mediated synthesis of the hydrophilic multifunctional polymer will be described along with the bioconjugation reaction and the final introduction of hydrophobicity yielding triblock Giant Amphiphiles.

III.2. Results and discussion.

III.2.1. Design and synthesis of the polymer.

For the purposes of our approach, we designed as mentioned above the target polymer to combine a bioconjugation site, an overall hydrophilic character and multiple grafted alkyne units aimed to act as hydrophobicity and/or multifunctionality entry points as described on Figure III-3.

Figure III-3. Schematic representation of the polymer 4 designed for cysteine containing proteins bioconjugation, displaying the different important features of the macromolecule.
The design and synthesis of the hydrophilic statistical copolymer 4 starting from the appropriate initiator and monomers is described in Scheme III-3 and Figure III-3. The maleimido-protected compound 6 was considered to be an appropriate initiator as it would lead, after deprotection, to the formation of a maleimide function known to react selectively and fast with free accessible thiols of proteins. The hydrophilicity of the final polymer 4 emerged from the glycerol units which were introduced via the ketal protected diol monomer 7 utilized during the polymerization. The choice of the pendant alkyne units as hydrophobicity precursors was based on the versatility and orthogonality of the click, copper (I) catalyzed [3+2] Huisgen cycloaddition in bioconjugation reactions (see Chapter II). For this purpose a trimethylsilyl protected propargyl monomer 8 was utilized as a gradient in the polymerization, after ca. 50% conversion of the the ketal protected diol monomer 7. The statistical nature of the polymer was pursued to ensure the overall hydrophilicity. Finally, the Hostasol yellow fluorescent marker containing monomer 9 was also added in low quantities during the early steps of the polymerization, to introduce a fluorescent identity to the final polymer, aimed at easier characterization of the bioconjugates.

Scheme III-3. Retrosynthesis of the hydrophilic multifunctional polymer 4 designed and synthesized in this study starting from the initiator 6, monomers 7 and 8 and fluorescent comonomer 9.
III.2.1.1. Synthesis of the initiator, monomers and ligand for ATRP polymerization.

The synthesis of the polymer was performed in collaboration with Prof. D. M. Haddleton and under the guidance of Dr. G. Mantovani.


α-Functional polymers containing unprotected functional groups (such as hydroxyl, amido and tertiary amine), can be easily obtained by ATRP starting from appropriate initiators. In this specific case however, this simple strategy would be problematic as the maleimido initiator is itself a polymerizable monomer, and therefore, copolymerization into the growing chain would occur. To circumvent this problem, we prepared the maleimido-protected ATRP initiator 6 following the synthetic protocol shown in Scheme III-4.

![Scheme III-4. Reagents and conditions for the synthesis of the maleimido-protected ATRP initiator 6.](image)

As shown in Scheme III-4, the double bond of maleic anhydride was first protected with an oxanorbornene motif by reflux in toluene in the presence of furan for six hours to give 10 in 87% yield. The resulting intermediate 10 was then reacted with ethanolamine in methanol for 4 hours at reflux to give the alcohol 11 in 42% yield. The initiator 6 was subsequently obtained by esterification of 11 using bromoisobutyryl bromide in the presence
of triethylamine in methanol (room temperature, overnight) to give the resulting initiator 6. The final product was purified by chromatography column and obtained in 93% yield.

III.2.1.1.b. Synthesis of the monomers 7, 8, 9.

The monomers (protected hydrophilic monomer and protected alkyne monomer) as well as the hostasol fluorescent comonomer were, synthesized according to existing literature procedures. An esterification of the respective commercially available alcohols using methacryloyl chloride was necessary to obtain the monomers 7, 8, 9 in good yields.

\[\text{HO-} \xrightarrow{\text{methacryloyl chloride, TEA,} \ \text{THF, 0°C to RT, overnight}} \xrightarrow{64\%} \text{O-O} \]

Scheme III-5: Reagents and conditions for the synthesis of solketal monomer 7.

More specifically, the solketal monomer 7 (Scheme III-5) was obtained by esterification of (2,2-dimethyl-1,3-dioxolan-4-yl)methanol by methacryloyl chloride in the presence of triethylamine in THF in 64% yield whereas the formation of trimethylsilyl protected monomer was achieved under the same conditions (utilizing diethylether as solvent), to afford the desirable product 8 in 81% yield (Scheme III-6). The protection was judged to be necessary for solubility reasons in the case of monomer 7, and to avoid the terminal alkyne polymerization in the case of the monomer 8.

\[\text{HO-} \xrightarrow{\text{TEA, THF, methacryloyl chloride,}} \xrightarrow{0°C \text{ to RT, 24 hours, 81\%}} \text{O-O} \]

Scheme III-6: Reagents and conditions for the synthesis of the protected alkyne monomer 8.

Hostasol yellow, a fluorescent tag widely used in industrial chemical companies for microscopy experiments employing an Ar ion laser as the excitation source\textsuperscript{41} has also recently found applicability in polymer synthesis.\textsuperscript{42,43,44} For the purposes of this research the precursor hostasol tag (Figure III-4, left) was functionalized with the appropriate reagents in 2 steps to
furnish the corresponding methacrylate derivative 9 shown on Figure III-4 (this fluorescent monomer was prepared by Dr. Giuseppe Mantovani in the Haddleton group). Used in a minute quantity, this marker aimed at facilitating the visualization of bioconjugation products. It should be noted that it possesses a strong absorption around 466 nm in the UV and is even distinguishable to the naked eye, allowing therefore easy optimization of the conditions used for separation of bioconjugates on resin columns and characterization of the products.

![Figure III-4. Structure of hostasol yellow precursor (left) and hostasol yellow monomer 9 (right) fluorescent tags.](image)

III.2.1.1.c. Synthesis of the ligand 12.

The ligand used during the polymerization was the pyridyl-N-propylimine 12 and was synthesized by condensation of n-propylamine and pyridylcarboxaldehyde as previously described. The resulting product 12 (Scheme III-7), obtained in 45% yield was stored at 4°C and under nitrogen atmosphere before use.

![Scheme III-7. Reagents and conditions used for the synthesis of the ligand 12.](image)

Chapter III – Formation of Giant Amphiphiles by Post-Functionalization of Hydrophilic Protein-Polymer Conjugates

The maleimido-protected initiator 6, the solketal methacrylate monomer 7, the hostasol fluorescent comonomer 9 (depicted on the Scheme III-2 as grey spheres) in 1 % molar ratio as compared to the initiator 6, the ligand 12 and the solvent anisole were placed in a Schlenk tube and subjected to 5 freeze-pump-thaw cycles to remove O$_2$. The beginning of the polymerization was subsequently triggered by the canulation of this solution into a second Schlenk tube containing Cu(I)Br under O$_2$ free conditions. Aliquots of the reaction feed were removed with a syringe during the course of the reaction to calculate PDI (polydispersity indice) and conversion. The conversion of the solketal monomer was followed by $^1$H NMR by comparing the integrals of the proton signals of the double bond to those of the dimethyl ketal functionality. In order to avoid the formation of block copolymers which would be difficult to manipulate in terms of solubility we aimed for the statistical copolymer, by adding after approximately 54% conversion, the second monomer, i.e. the trimethylsilyl protected alkyne methacrylate 8, to the polymerization feed to induce gradient copolymerization.

![Scheme III-8. Reagents and conditions for the ATRP mediated synthesis of 5.](attachment:image.png)

The polymerization was stopped after around 80% total conversion of the solketal monomer. The molecular weight ($M_n$) of the polymer was calculated by $^1$H NMR by comparison of the integrals of solketal side chain protons with those of the oxanorbornene protected terminus. It was found that the polymer has a MW of 11.5 kDa whereas GPC
measurements indicated a PDI of 1.15. Using $^1$H NMR, by comparison of the integrals corresponding to the protons of vinyl motif of the oxanorbornene terminus, of the protons of dimethyl ketal function of solketal and those of the trimethylsilyl function protecting the alkyne side chains, it was found that the polymer 5 contains an average of 8 trimethylsilyl-protected alkyne side chains and 50 solketal monomer units per polymeric chain.

### III.2.1.1.e. Deprotection of the resulting polymer.

For the final synthesis of the multifunctional polymer 4, the fully protected polymer 5 was engaged in post-polymerization deprotection of the different units. This was achieved in 3 straightforward steps:

i) Removal of oxanorbornene moiety to liberate the maleimide functionality.

$$\text{Scheme III-9. Reagents and conditions used for the preparation of 13.}$$

This deprotection was achieved by refluxing the polymer 11 in toluene overnight to remove the furan protecting group by a retro Diels-Alder reaction, leading to the maleimide-terminated polymer 13.\textsuperscript{40,46} This reaction has been previously shown to also proceed even in the solid state (neat) by leaving the maleimido-protected polymer powder in a vacuum oven at
80°C overnight\textsuperscript{46} to yield the pure maleimido-polymer and therefore avoid the use of organic solvent as well as the final precipitation step of the resulting polymer. In this case nevertheless, toluene was utilized as a solvent.

\textbf{Figure III-5.} $^1$H-NMR (in CDCl$_3$) spectra of maleimido-protected polymer 5 (top) and maleimido-deprotected polymer 13 (bottom).

The polymer 13 was characterized by $^1$H NMR, and, as expected, showed the disappearance of the signals of the protons of the oxanorbornene moiety along with the appearance of a singlet at 6.8 ppm corresponding to the protons of the vinyl moiety of the maleimide functionality as shown on the $^1$H NMR spectra of Figure III-5.
ii) Deprotection of the trimethylsilyl protected alkyne functions.

\[
\text{Scheme III-10. Reagents and conditions for the synthesis of polymer 14.}
\]

As previously described in the literature, this reaction proceeds with virtually 100% yield when the deprotection is undertaken in the presence of TBAF as reagent using acetic acid as buffering agent to maintain an acidic pH in the reaction mixture (Scheme III-10). The use of acetic acid in combination with TBAF is a well-established procedure in organic chemistry that is normally used when the substrate to deprotect contains a sensitive functional group (e.g. esters, thioesters) that can be cleaved when TBAF (basic species) alone is employed. Indeed, it was already shown in a previous report that the use of TBAF alone can lead to the cleavage of ester bonds onto the same type of alkyne-containing polymers. For this reason, acetic acid was also employed as a buffering agent during the TBAF mediated deprotection of polymer 14. The polymer 13 was dissolved in a mixture THF / acetic acid at -20°C and reacted with TBAF overnight under nitrogen atmosphere. Removal of the ammonium salts on a silica pad afforded the pure polymer 14 in quantitative yield.

\[ ^1H \text{ NMR of the resulting alkyne deprotected polymer 14 (Figure III-6) verified its structure by revealing the disappearance of the characteristic peak corresponding to the trimethylsilyl protecting group at 0.2 ppm together with the appearance of the terminal alkyne protons at 2.5 ppm. The ratio of the functional groups within the polymer remained unchanged. These results were confirmed by FT-IR spectroscopy. As expected, a} \]
characteristic stretching band of the terminal $\text{C}≡\text{C}−\text{H}$ bond indicating the deprotection of trimethylsilyl functions on the resulting polymer was observed at 3308 cm$^{-1}$ and is shown in Figure III-7.

![Figure III-6. $^1$H-NMR (in CDCl$_3$) of the trimethylsilyl alkyne protected polymer 13 (top) and the alkyne deprotected polymer 14 (bottom).](image)

![Figure III-7. FT-IR analysis of polymer 13 (solid line) and polymer 14 (dashed line).](image)
iii) Deprotection of the ketal functionalities.

Scheme III-11. Reagents and conditions used for the synthesis of polymer 4.

This reaction was performed in dioxane by catalytic acid hydrolysis of the ketal function using 1 M HCl aqueous solution as shown on Scheme III-11. The deprotection of the vicinal diols on the polymer backbone altered the solubility of the produced polymer 5 as expected and therefore the polymer was recovered in methanol and immediately freeze-dried to avoid degradation of maleimide moiety which is well known to be unstable in water under non neutral pH conditions.

Final full characterization of the polymer 4, after the three deprotection steps was performed by GPC, $^1$H NMR and MALDI-TOF analysis. First, by GPC the retention of polydispersity of the polymer 4 after the three deprotection steps was confirmed (PDI~1.20) and indicated a $M_n$~8 kDa. $^1$H NMR spectrum of polymer 4 confirmed the deprotection of the ketal functions along with the preservation of alkyne functions (Figure III-8). It is also important to notice that the maleimide functionality and its ratio within the polymer, as confirmed by the presence of the peak at 6.9 ppm, was not altered by the successive reactions.
III.2.1.2. Coupling of the hydrophilic polyalkyne (4) to Bovine Serum Albumin.

In contrast to all methods previously utilized for the synthesis of Giant Amphiphiles, which comprised the direct use of the hydrophobic polystyrene for bioconjugation and therefore were hampered by solubility incompatibility problems, the bioconjugation of polymer 4 was designed to be facilitated by its hydrophilic character. This bioconjugation reaction was realized in a 20 mM PB pH 7.4 aqueous solution containing native BSA at a concentration of ca. 0.35 mM and a 100 molar excess of the polymer 4 (Scheme III-12) without the need to implement the medium with organic solvents (in additional experiments that are not mentioned within the thesis, the addition of minute quantities of DMSO as a cosolvent was also studied and found to be successful).
Scheme III-12. Reagents and conditions used for the coupling of polymer 4 to native BSA.

After gentle shaking for 24 hours at 7°C, the reaction mixture was analyzed by SEC. Similar retention times were observed for BSA (18.31 min.) and BSA-polyalkyne VII conjugation reaction (18.39 min.) at 254 nm under the conditions utilized for the analysis (solvent: 70 % phosphate buffer 5 mM pH 7.4, 30 % acetonitrile, room temperature, column: SEC-300 BioBasic, flow rate: 0.5 mL.min⁻¹, Figure III-9). Nevertheless, at 466 nm (maximum absorbance of the fluorescent hostasol yellow monomer at 466 nm), no absorption was observed for native BSA, whereas two peaks were observed for the bioconjugation reaction mixture attributed to the bioconjugate and the unreacted polymer (Figure III-10). A blank sample containing only the hydrophilic polymer 14 was analyzed also by SEC and showed only one broad peak with a higher retention time (i.e. smaller molecular weight). This analysis was the first indication of the successful formation of the BSA-polyalkyne bioconjugate VII.
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Figure III-9. SEC traces at 254 nm of native BSA (black trace) and BSA-polyalkyne conjugate VII (red trace).

Figure III-10. SEC traces at 466 nm of native BSA (black trace), the BSA-polyalkyne VII conjugation reaction mixture (red trace) and polyalkyne 4 (green trace).

The success of the coupling of the polyalkyne 4 to native BSA was confirmed by electrophoresis under denaturating conditions (Figure III-11). Both visualization under the UV lamp at 366 nm and through Coomassie Blue staining showed a new (fluorescent) band possessing a higher molecular weight than that of native BSA, thus confirming our previous results observed by the chromatographic analysis of reagents and the reaction mixture. The presence of unreacted native BSA was demonstrated by its characteristic migration band in the electrophoresis.
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Figure III-11. SDS-PAGE analysis of native BSA (lane 1) and the bioconjugation reaction mixture of BSA and polymer 4 (lane 2) after Coomassie Blue staining (left) and visualization under the UV lamp at 366 nm (right).

The clear single peak at ~74850 uma together with the peak corresponding to native BSA (~66590 uma) that were observed from the MALDI-TOF measurements, finally confirmed the attachment of a single hydrophilic multifunctional polymeric chain on the native BSA, demonstrating thus the selective coupling of the polymer onto the protein. As expected the peak obtained for the bioconjugate exhibited a broader distribution in mass than the native protein, due to the polydispersity of the hydrophilic polyalkyne 4.

Figure III-12. MALDI-TOF analysis of the bioconjugation reaction mixture between native BSA and polyalkyne 4.
Several different methods were investigated to purify the BSA-polyalkyne bioconjugate VII from unreacted BSA and excess of polyalkyne 4. Dialysis using 25 kDa MWCO regenerated cellulose membranes achieved the removal of the majority of the unreacted polymer. For the removal of native BSA, MPLC using a wide range of self-packed columns (going from Sephadex to Superose resins) and also preparative electrophoresis followed by electroeluting of the appropriate selected band were tested. We succeeded in removing excess polyalkyne 4 from the reaction mixture; unfortunately, after a numerous efforts in this direction, minute quantities of unreacted native BSA remained with the BSA-polyalkyne VII biohybrid product. Nevertheless the product was significantly enriched as showed by electrophoresis (Figure III-13) and we were allowed to proceed to the next steps aiming at the formation of Giant Amphiphiles.

Figure III-13. Electrophoresis after purification by Superdex 75 resin column of the reaction mixture VII. Lane 1: crude reaction mixture, lane 2 to lane 6: different fractions obtained after the Superdex 75 column of BSA-polyalkyne VII with the dashed box indicating the position of hydrophilic multifunctional BSA-polyalkyne VII on the gel.

Figure III-14. UV analysis of BSA-polyalkyne VII (solid black trace) and native BSA (grey doted trace).
UV study of the conjugate VII (after purification on Superdex 75 resin) showed the characteristic peak at 466 nm of the fluorescent tag present on the polymer backbone (Figure III-14).

### III.2.1.3. Post-functionalisation of the hydrophilic polymer with hydrophobic azides groups.

#### III.2.1.3.a. Synthesis of hydrophobic alkyl azides.

Several hydrophobic azides were prepared from the commercially available corresponding bromides by nucleophilic substitution of the bromide using a 0.5 M solution of sodium azide in DMSO.\(^{50}\)

\[
\begin{align*}
R-\text{Br} & \quad \text{NaN}_3, \ 0.5 \text{ M in DMSO}, \quad 60^\circ C \text{ overnight} \\
& \rightarrow R-\text{N}_3
\end{align*}
\]

with \( R = \text{n-C}_{10}H_{21} \) (15), Bz (16), F\(_5\)Bz (17).

**Scheme III-13.** Reagents and conditions for the preparation of hydrophobic alkyl azides 15, 16 and 17.

As also observed in the literature, the yields of these reactions were found to be quantitative.\(^{50}\) The products of the reactions were isolated, characterized by FT-IR, NMR and mass spectroscopy, and stored at -20°C in dry ether for safety reasons (small alkyl azides are often hazardous, explosive) before use.

#### III.2.1.3.b. Synthesis of porphyrin hydrophobic azides.

The synthesis of structurally and functionally more complex azidated compounds was also investigated. In this direction the synthesis of azide functionalized porphyrins or an azidated thymine analogue was pursued.
The tetraphenylporphyrin 18 was prepared in collaboration with Dr. Sylvain Koeller by condensation between pyrrole and benzaldehyde using acetic anhydride/acetic acid as solvent mixture.\textsuperscript{51} After purification, aromatic electrophilic parasubstitution of a phenyl ring of 18 was achieved by reaction of sodium nitrite in trifluoroacetic acid, affording the resulting para-nitro tetraphenylporphyrine 19.\textsuperscript{52,53} Finally, the para-nitro tetraphenylporphyrine 19 was reduced to the amine 20 by reaction with tin chloride in hydrochloric acid. Conditions and characterization are fully presented in the experimental section.

\[ \text{CHO} + \text{H}_2\text{N} + \text{AcOH, Ac}_2\text{O} \rightarrow \text{reflux, 30 min., 5\%} \]

\[ \text{NaNO}_2, \text{TFA, r.t., 3min., 45\%} \]

\[ \text{NH}_2 \]

\[ \text{NO}_2 \]

\[ \text{SnCl}_2, \text{HCl} \rightarrow \text{70\% , 1h30, 44\%} \]

Scheme III-14. Reagents and conditions for the synthesis of amino-tetraphenylporphyrin 20.

For the purposes of our synthesis, an azido-functionnalized heterobifunctionnal triethylene glycol derivative was synthesized in two steps. The introduction of the azide group was achieved in a one-pot, 2 steps synthesis\textsuperscript{54,55,56} comprising mono-functionalization with mesyl chloride in THF using triethylamine to afford a triethylene glycol mesylate intermediate which was directly engaged in the second step without further purification. After evaporation of the volatiles, nucleophilic substitution of the mesylate was undertaken using sodium azide in ethanol to give the monofunctionalized azido triethylene glycol 22 in 44\% yield as shown
in Scheme III-15 below. Subsequently, the intermediate 21 was reacted with succinic anhydride in dry toluene overnight to afford the azide bearing acid 22 in 93% yield.

![Scheme III-15. Reagents and conditions utilized for the synthesis of the azido heterobifunctional triethylene glycol linker 22.](image)

In a final step, the aminophorpyrin 20 and the azido triethylene glycol acid 22, previously activated with thionyl chloride, were coupled in dichloromethane overnight to afford the azido-porphyrin 23 in 94 % yield (Scheme III-16).

![Scheme III-16. Reagents and conditions for the coupling of the aminophorpyrin 20 to the azido heterobifunctional triethylene glycol acid 22.](image)

Finally, starting from azidated triethylene glycol 21, an azido thymine derivative 24 was synthesized. It was obtained by esterification of 2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)acetic acid (Scheme III-17) by compound 21 in the presence of HBTU and DIPEA at r.t. in quantitative yield.

![Scheme III-17. Reagents and conditions for the synthesis of azidated thymine 24.](image)
III.2.1.3.c. Click chemistry assays on the polymer.

After the synthesis of a small library of azido derivatives, the efficiency of the multi clicking step was tested primarily to the polymer, to optimize the reaction conditions. To avoid problems inherent to the instability of the maleimido moiety under click chemistry conditions,\textsuperscript{57,58} it was decided to selectively deprotect the polymer in such a way that only terminal alkyne side chains would be free and accessible for the multiclicking reaction. For solubility reasons, the glycerol repeating units were kept in their protected ketal form. Using the conditions described previously for the full deprotection of the polymer, the polymer 4 was partially deprotected by TBAF in THF using acetic acid as buffering agent as shown in Scheme III-18.

Scheme III-18. Reagents and conditions for the preparation of polymer 25 and the multi-clicked polymers 26a, 26b, 26c, 25d.

Clicking of hydrophobic azides was then investigated using a 5 molar excess of the azide per polymer terminal alkyne unit, 0.6 equiv. CuBr and 1 equiv. sodium ascorbate in
10% DMSO, 20 mM PB pH 7.4. During the isolation of the pure multi-clicked products, Cu (II) was removed from the reaction mixtures (embarrassing paramagnetic species for $^1$H NMR) on neutral alumina column and the products were analyzed by GPC and NMR spectroscopy.

The compounds 26a, 26b, 26c, 26d were discriminated from the starting material 25 by GPC using toluene as the mobile phase and 2 PL-Gel mixed columns (in series) through a reproducible difference in the retention times thus demonstrating the formation of the multi-triazolyl polymer compounds as shown on Figure III-15. Furthermore, the PDI of the “clicked” polymers 26a, 26b, 26c, 26d were found to be around 1.2 as proven by the GPC measurements. It should be mentioned that in all cases, the UV traces were in good agreement with the corresponding RID traces.

Figure III-15. GPC traces of protected polyalkyne 25 (black curve) and purified clicked products 26a, 26b, 26c, 26d.

The formation of the products was confirmed by $^1$H NMR. As shown on Figure III-16, the disappearance of the terminal alkyne protons on the polymer backbone was observed together with the downfield shifting of the $-\text{CH}_2-$ in $\alpha$ of the triple bond position and the appearance of new broad signals corresponding to the protons of the lateral alkyl chains grafted by the copper-catalyzed Huisgen dipolar azide alkyne cycloaddition on the polymer backbone.
Figure III-16. $^1$H-NMR of polyalkyne 25 and “clicked polymers” 26a (R=C$_{10}$H$_{21}$), 26b (R= Bz), 26c (R=F$_5$Bz).

In the case of the pentafluorobenzyl azide 17, $^{19}$F NMR was also used to follow the formation of the product (Figure III-17). When the starting pentafluorobenzyl azide 17 and the clicked corresponding polymer 26c were compared, a shifting of the peaks together with a broadening of the $^{19}$F signals (due to longer relaxation periods) were observed, indicating the accomplishment of the reaction.

Figure III-17. $^{19}$F-NMR spectra (in CDCl$_3$) of compound 17 (black trace) and 26c (grey trace).
Unfortunately, in the case of the porphyrine derivative 26d, the complexity of the acquired spectra presumably due to a copper (II) complexation, did not allow the full spectroscopic characterization of the product.

III.2.1.3.d. Post functionalization of BSA-polyalkyne VII using different hydrophobic azides.

As previously shown, clicking of hydrophobic azides onto the polymer backbone was successfully achieved in aqueous solution. It was thus decided to proceed with the “clicking” of hydrophobic azides 15, 16, 17, 23 onto the BSA-polyalkyne multifunctional biohybrid VII.

**Scheme III-19.** Reagents and conditions for the synthesis of clicked Giant Amphiphiles VIIIa, VIIIb, VIIIc and VIIIId.

Same conditions as mentioned above were used. The reaction mixtures containing 0.12 mM of the hydrophilic BSA-PA VII in 20 mM phosphate buffer, 40 molar excess of each of the azido derivatives 15, 16, 17 or 23, and 2 mM CuSO\(_4\) / 4 mM sodium ascorbate as the catalytic system were gently shaken for 48 hours at 7°C. The reaction mixtures became opaque soon after the initiation of the click reaction through the addition of CuSO\(_4\)/Na ascorbate, indicating the formation of amphiphilic products. After the 48h allowing for the completion of the multi-“click chemistry” reaction, all reagents were removed by a simple excessive dialysis step against 20 mM phosphate buffer pH 7.4 (using 25 kDa MWCO
regenerated cellulose membranes). The characteristic aggregation caused by the amphiphilicity of the products was observed by TEM microscopy and verified by the hampering of the electrophoretic mobility of the products as a result of their amphiphilic character in the case of reaction with azides 15, 16, 17. In the case of azido porphyrin 23, no reaction was observed, a fact attributed to the insolubility of the porphyrin into the aqueous medium.

Figure III-18. SDS-PAGE analysis of native BSA, BSA-polyalkyne VII and clicked amphiphilic bioconjugates: lane 1, native BSA; lane 2, BSA-polyalkyne VII; lane 3, BSA-PA@C_{10}H_{21} VIIIa; lane 4, BSA-PA@Bz VIIIb; lane 5, BSA-PA@F_{3}Bz VIIIc.

No band corresponding to the reacting hydrophilic polymer-protein conjugate VII was observed, indicating the efficiency of the copper-catalyzed dipolar cycloaddition of hydrophobic azido compounds on the hydrophilic biomacromolecule VII.

SEC measurements did not give any satisfactory results under the conditions used, a fact attributed to the aggregation of the amphiphilic polymer-protein bioconjugates. Furthermore, MALDI-TOF measurements of the hydrophilic BSA-polyalkyne conjugates VIIIa, VIIIb, VIIIc were not successful even when mixtures of matrices were used.
III.2.1.3.e. Aggregation Studies.

Figure III-19. TEM micrographs of BSA-PA@C_{18}H_{21}N_{3} VIIIa (line A), TEM micrographs of BSA-PA@Bz VIIIb (line B), TEM micrographs of BSA-PA@F_{3}Bz VIIIc (line C).

After dialysis of the reaction mixtures, the aggregation profiles of the Giant Amphiphiles VIIIa, VIIIb and VIIIc were investigated with Transmission Electron Microscopy (TEM). For this reason, the mixtures were further dialyzed against nanopure water and a 100 times diluted solution was placed onto a formvar coated copper grid. As in previous cases, no staining was necessary presumably due to the increased electronic density of the polymer moiety. The formation of well-defined spherical aggregates was observed in all cases (Figure III-19, A-D) whereas blank experiments performed under the same conditions using BSA in the presence of the polymer 4 and mixtures of the BSA-PA bioconjugate VII in the presence of the azides or the azides themselves, did not reveal any aggregation pattern.
The formation of such spherical superstructures is well in agreement with previous reports on BSA-Polystyrene Giant Amphiphiles. In the recent work reported by Nolte and coworkers on the synthesis of BSA-polystyrene Giant Amphiphiles, self-assembly of the amphiphilic biomacromolecules gave well-defined spherical aggregates consisting probably of micellar superstructures as suggested by the size of the aggregates (between 30 and 70 nm). It should be noted that spherical aggregates observed for the BSA-PA@C\textsubscripts{10}H\textsubscript{21}N\textsubscript{3} Giant Amphiphiles \textbf{VIII}a were rather uniform in diameter (mean diameter ca. 150 nm, Figure III-19, lane A), while the diameters of the BSA-PA@Bz and BSA-PA@F\textsubscript{5}Bz Giant Amphiphiles \textbf{VIII}b and \textbf{VIII}c varied between 20 and 200 nm (Figure III-19, C-D). We attribute this difference of size dispersity of the resulting superstructures in water to the more dynamic behaviour of the decanyl and fluorobenzyl alkyl chain as compared to the benzyl residue. This is the first report of a difference in aggregation behaviour in Giant Amphiphiles arising from the different hydrophobic tail attached.

Laser confocal microscopy additionally allowed the observation of the superstructures through the fluorescence arising from the Hostasol tag which is incorporated on the polymer backbone. When the conjugate BSA-PA@C\textsubscript{10}H\textsubscript{21}N\textsubscript{3} \textbf{VIII}a was suspended in a 1/1 (v/v) mixture of water and ethanol, upon addition of a small quantity of decane and vigorous shaking, a characteristic for amphiphilic molecules, milky emulsion was formed. The analysis of this emulsion by confocal microscopy clearly shows the biohybrid \textbf{VIII}a orientating itself at the decane-water interface, a fact that is attributed to its amphiphilic nature.

In this chapter, the successful design and development of a novel strategy for the preparation of Giant Amphiphiles has been discussed.

The ATRP mediated synthesis proved to be efficient both in the implementation of the posed functional properties and in the creation of a well-defined relevant polymer.

The bioconjugation of BSA to this multifunctional hydrophilic polyalkyne was achieved using a chemoselective Michael addition reaction, under mild conditions that do not interfere with the integrity of the protein quaternary structure. The subsequent post-functionalization of the resulting hydrophilic polymer-protein bioconjugate was also carried out in mild conditions using a copper-catalyzed click chemistry reaction.

Following this new approach, the multiclicking of several hydrophobic azides onto the multifunctional hydrophilic polymer protein bioconjugates led to a, previously unattainable, variety of Giant Amphiphiles without any need of condition optimization or polymer synthesis.

Aggregation studies with TEM and CFM microscopy revealed the formation of well defined superstructures. For the first time, difference in aggregation was observed when changing the monomer nature (e.g. difference observed in aggregates polydispersity depending on the monomer, C_{10} vs. Bz, F_{5}Bz).

Future studies involving the introduction of an increased number of alkyne functionalities and the multiclicking of azides expressing a catalytic functionality, certainly provide exciting opportunities in the area and should be pursued.
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Chapter IV

In Situ, ATRP Mediated Hierarchical Formation of Bio Nanoreactors

IV.1. Concept / Background.

As already mentioned in previous chapters, during the synthesis of Giant Amphiphiles, the hydrophobicity of the polymer is generally the limiting factor, while purification has also proved to be difficult. In an effort to circumvent such problems, high content in organic solvents aqueous solutions have been previously applied,1,2,3 making the bioconjugation conditions quite harsh and problematic to apply in the majority of proteins. Taking into account recent developments in the field of bioconjugation and ATRP polymerization that will be discussed later on in this chapter,4,5,6,7,8,9 we envisioned that if successful, the initiated onto a protein macroinitiator (i.e. a protein that has been functionalized with a polymerization initiator at a precise position), direct, ATRP polymerization of a hydrophobic monomer would provide a facile and efficient alternative approach leading to the quantitative in situ formation of defined amphiphilic macromolecular structures.

For this reason, a novel, simple synthetic pathway was developed and will be discussed in this chapter aiming at the development of protein initiated ATRP polymerization of hydrophobic monomers (Scheme IV-1). Further studies on the application of this method for the creation of meaningful structures will then be discussed.

![Scheme IV-1](image)

Scheme IV-1. General scheme of the synthetic pathway used to create Giant Amphiphiles by ATRP polymerization.
Recently, several groups have developed a new technique to synthesize protein-polymer bioconjugates which lies on the *in situ* polymerization of hydrophilic monomers onto protein macroinitiator through either Atom Transfer Radical Polymerization (ATRP)\(^4,5,6,7,8\) or Reversible Addition-Fragmentation Chain Transfer (RAFT)\(^9\) mediated polymerization. It was these pioneering studies and their results, that intrigued us to approach the synthesis of *Giant Amphiphiles* through living polymerization techniques.

### IV.1.1. Protein-polymer bioconjugates through protein initiated living polymerization.

The pioneering work on the *in situ* polymerization of biomacroinitiators was recently presented by Maynard and coworkers for the creation of smart polymer-protein conjugates in good yields (greater than 65%).\(^6\) In this work, the polymerization of the pH and temperature dependant poly(N-isopropylacrylamide) (polyNIPAAm) starting directly from protein macroinitiators (*e.g.* BSA and lysozyme macroinitiators) was achieved. These biomacroinitiators were prepared by the selective coupling of pyridyl disulfide- or maleimido-ATRP initiators onto the corresponding proteins. In the case of lysozyme, the polymer-protein bioconjugates retained the catalytic activity for the enzyme moiety.

In a more recent work, the Maynard group reported on the *in situ* growing of polyNIPAAm on a biotin-streptavidin macroinitiator (Scheme IV-2),\(^5\) with the retention of the bioaffinity of streptavidin for biotin. They demonstrated that the grafting of polymers onto the enzyme complex did not interfere with the strong binding interaction of biotin to streptavidin.

**Scheme IV-2.** Strategy proposed by Maynard and collaborators to prepare streptavidin-poly(NIPPAm) bioconjugates.\(^5\)**
Haddleton and coworkers reported on the \textit{in situ} polymerization of hydrophilic monomers such as poly(ethylene glycol) methyl ether methacrylate (PEGMA) or dimethylaminoethyl methacrylate (DMAEMA) onto protein derived macronitiators such as BSA and lysozyme.\textsuperscript{7} In this study, BSA and lysozyme were initially functionalized with respectively a maleimido- and a NHS-activated acid ATRP initiator, subsequently purified before the polymerization step (Scheme IV-3). A fluorescent comonomer derived from hostasol or rhodamine B tags was added in minute quantities together with the monomers and the obtained protein-polymer bioconjugates were successfully observed by fluorescence detection SEC-HPLC. They observed that lysozyme conjugates had a broader mass distribution than the BSA conjugates (probably due to the multi-site attachment of the initiator to the native lysozyme).

\begin{center}
\textbf{Scheme IV-3.} Procedure introduced by Haddleton and coworkers to prepare fluorescent hydrophilic polymer-protein biohybrid macromolecules.\textsuperscript{7}
\end{center}
The Matyjaszewski group also took advantage of the *in situ* polymerization methodology to prepare hydrophilic chymotrypsin-polymer bioconjugates with near uniform distributions that were also found to retain part of their enzymatic activity (50-86% of the initial activity of the native enzyme).\(^8\) In the course of this study, 2-bromoisobutyryl bromide was coupled to chymotrypsin through a two-phase reaction mixture (phosphate buffer pH 8 / dichloromethane) that was stirred at 800 rpm and at 25 °C. After purification of the chymotrypsin macroinitiator, monomethoxy poly(ethylene glycol) (MPEG-methacrylate) was polymerized using bipyridine / CuBr as catalytic system in phosphate buffer pH 6 to afford the corresponding hydrophilic polymer-protein bioconjugates with near uniform polydispersities.

In all cases, the living polymerization mediated formation of bioconjugates allowed to bypass the multiple synthetic steps used in conventional bioconjugation techniques. Furthermore, the use of a low molecular weight reagent in the place of a polymer, significantly facilitated the purification of the resulting bioconjugates as only a simple dialysis step was necessary to remove the unreacted monomer.

Taking into account the above mentioned advances, we envisioned that if such a method would also be efficient when replacing the hydrophilic with a hydrophobic monomer, it would certainly provide an efficient alternative also for the synthesis of Giant Amphiphiles. Furthermore, we reasoned that since the hydrophobic monomer would be gradually grafted on the bioconjugate, ATRP could prove to be an ideal tool to generate amphiphilic structures containing a more defined structure (lower PDI). We expected that as the amphiphilic character of the overall structure gradually increased during the polymerization, aggregation into superstructures would occur in a rather precise point, thus leading to the polymerization termination and, by this process, providing more defined polymer-protein conjugates at both the molecular and supramolecular level.

Aiming at the creation of functional nanoassemblies, we thought that this method would also allow the one-pot encapsulation of small molecules or catalytically active enzymes during the *in situ* formation of the superstructures. Taking into account the nature of Giant Amphiphiles and the concomitant to their synthesis formation of non-dynamic aggregates, any attempt to achieve such an encapsulation post their synthesis would certainly interfere with either the integrity of the proteins and/or the overall architectures. We reasoned that following
the ATRP mediated approach the statistical encapsulation of guest proteins without compromise to their catalytic function.

In this chapter, our successful studies toward these goals will be discussed, while initial intriguing results on the interaction of such systems with living cells will also be presented.

IV.2. Results and discussion.


IV.2.1.1. Synthesis of the initiator.

The hetero bifunctional ATRP initiator 27 was synthesized as previously described by Haddleton\textsuperscript{10} and Velonia\textsuperscript{11} (Scheme IV-4), using a synthetic pathway similar to that utilised in the case of compound 6 (Chapter III). Upon synthesizing the precursor 6, the final deprotection step was achieved by refluxing the compound 6 overnight in toluene and led to the formation of the maleimido ATRP initiator 27 in virtually quantitative yield by a retro Diels-Alder reaction as shown on the Scheme IV-4.

![Scheme IV-4](image)

**Scheme IV-4.** Reagents and conditions utilized for the synthesis of the maleimido ATRP initiator 27.
IV.2.1.2. Selective coupling to BSA.

The efficiency of the *in situ* ATRP protein-polymerization toward the synthesis of *Giant Amphiphiles* was explored using the 66 kDa globular bovine serum albumin (BSA) that contains only one free cysteine residue at position 34. Adapting the approach that was used by Maynard and coworkers for the *in situ* creation of smart polymer-protein conjugates, the bioconjugation reaction to BSA was performed under mild conditions and was initiated by the slow addition of a 40 molar excess solution of the initiator 27 in DMSO, to a solution of BSA in aqueous phosphate buffer (PB), pH 7.4 as shown in Scheme IV-5. The reaction mixture was gently shaken for 2 days at 7°C to allow maximum yields.

**Scheme IV-5.** Reagents and conditions used for the selective conjugation of the ATRP initiator 26 to native BSA.

The resulting bioconjugate protein macroinitiator IX (BSA-ATRP macroinitiator IX), was easily isolated from the starting material 27 and DMSO by a simple, extensive dialysis step (using 25kDa MWCO regenerated cellulose membranes) against 20 mM phosphate buffer pH 7.4. After purification, the reaction yield was initially quantified using the colorimetric Ellman’s assay. The absorption at 412 nm arising from the thionitrobenzoate product of Ellman’s test revealed (in agreement with what was reported in Chapter II), that the native BSA contained about 47% free cystein residues available for bioconjugation. After the coupling reaction and the purification, virtually no free cysteins were detected in the mixture, indicating that the coupling between the maleimido-ATRP-initiator 27 and the free cystein of native BSA was practically quantitative. This observation was confirmed by both native gel electrophoresis and SEC measurements.
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Figure IV-1. Left: Native gel electrophoretic profile of native BSA (lane 1), and the purified reaction mixture after conjugation of BSA with the ATRP initiator 27 (lane 2). Right: SEC-HPLC chromatographic traces of native BSA (solid black trace) and BSA-macroinitiator IX (dashed grey trace).

On the electrophoresis gels that were obtained under native conditions, a new band was observed for the bioconjugate IX (lane 2, Figure IV-1), exhibiting different migration profile than that of the native BSA (lane 1, Figure IV-1). No band corresponding to the native BSA was observed in the enriched reaction mixture even after employing silver staining, confirming thus the results obtained by Ellman’s test and pointing to a quantitative reaction. Surprisingly, this new band migrated faster than BSA (i.e. was placed in the lower apparent molecular weight region) behaving in a manner similar to that observed also in the case of the BSA-1-alkyne bioconjugate I (Chapter II). We attribute this behaviour to a possible change in the total net charge of the protein adduct and/or conformation changes during the conjugation.

SEC chromatographic analysis also indicated the formation of the bioconjugate. The retention time of the BSA macroinitiator IX was found, as expected, based on the measurements previously performed on the biohybrid BSA-1-alkyne I (Chapter II), to be only slightly different to that of native BSA (Figure IV-1, right). No peak corresponding to the unreacted excess of the free initiator 27 was observed, indicating its total removal by the extensive dialysis purification step.

Finally, MALDI-TOF analysis (Figure IV-2) revealed masses at 66313 and 66737 uma respectively for native BSA and BSA-macroinitiator IX, hence exhibiting a difference of mass (+ 424 uma) consistent with the selective conjugation of one ATRP initiator molecule
27 per protein. After its full characterization, the BSA macroinitiator IX was freeze dried and found to be stable for prolonged periods of time (months) when stored at -20°C.

![Figure IV-2. MALDI-TOF analysis of native BSA (grey) and BSA macroinitiator IX (black).](image)

**IV.2.2. In situ formation of Giant Amphiphiles: ATRP polymerization on BSA macroinitiator IX.**

Styrene was judged to be the appropriate monomer to proceed with the experiments on the *in situ* polymerization of hydrophobic monomers initiated on the bioconjugate IX as it would lead to the formation of polystyrene *Giant Amphiphiles* that are known to exhibit a strong amphiphilic character. Furthermore, the use of styrene as hydrophobic monomer would allow the comparison with previous results obtained with other techniques used in the lab (such as the preparation of BSA-polystyrene *Giant Amphiphiles* obtained by direct click chemistry coupling in Chapter II or the BzN₃ clicked bioconjugates obtained through the post functionalisation approach in Chapter III).
Scheme IV-6. General synthetic scheme of the in situ ATRP mediated polymerization of styrene on BSA macroinitiator IX.

The polymerization reactions were carried out using a modified version of the standard general procedures that were introduced by the Haddleton group for the synthesis of hydrophilic protein-polymer conjugates and by the Maynard group for the preparation of smart polymer-protein conjugates. Briefly, the in situ styrene polymerization onto BSA-macroinitiator IX was performed in aqueous phosphate buffer solution, oxygen free conditions, ambient temperature, using the copper bromide/N-(n-propyl)-2-pyridyImethanimine 12 catalyst system and without the presence of any “sacrificial” initiator. Several sets of experiments were performed utilizing different monomer to BSA-macroinitiator IX ratios while a series of control experiments (in the absence of the BSA-macroinitiator IX, monomer, Cu(I) and in the presence of O2) were conducted to ensure that the in situ polymerization proceeds on the predesigned BSA initiating position.

As we were aiming for the polymerization of hydrophobic monomers in aqueous solution, the monomer was initially emulsified in 10% DMSO, 20 mM phosphate buffer solution containing the ligand, and then subjected to repeating freeze-pump-thaw cycles before initiating the polymerization by canulation into the second flask containing the protein macroinitiator IX and copper (I) bromide under oxygen free atmosphere. Whereas the styrene to BSA macroinitiator IX ratio was varied, the CuBr / ligand 12 / BSA macroinitiator IX ratio was kept constant at 41 / 70 / 1 in all experiments.
As mentioned above, several sets of experiments were performed utilizing different monomer to BSA-macroinitiator \textbf{IX} ratios (varying from 50 to 3000 times excess of the monomer over \textbf{IX}). These experiments aimed to study the applicability of ATRP in the synthesis of \textit{Giant Amphiphiles} together with the influence of the monomer to BSA macroinitiator \textbf{IX} ratio on the polymerization outcome. If our initial assumptions were correct, the polymerization should control the polydispersity of the formed polymer and therefore the aggregation of the resulting superstructures. The conditions utilized in these experiments are summarized in Table IV-1. It should be noted that during the polymerization period, the dark brown copper (I) complex colour formed upon the canulation of the reagents to the BSA- macroinitiator \textbf{IX} /Cu (I) oxygen free flask remained stable while a gradual increase of turbidity was observed in the reaction mixtures. The polymerizations were terminated by stirring the reaction mixtures under oxygen atmosphere. Several control reactions were also performed in the absence of the biomacroinitiator \textbf{IX}, the monomer or Cu(I)Br and in the presence of oxygen to ensure that polymerization proceeds in the predesigned position and are also listed in Table IV-1. In this case, the dark brown copper (I) complex colour was again retained throughout the reaction time, but no visual change in the turbidity of the reaction mixtures was observed. In all cases, the resulting bioconjugate solutions were subjected to extensive dialysis to ensure that all traces of reagents (styrene, copper, N-(n-propyl)-2-pyridylmethanimine) were removed. The dialysis was initially performed against 2\% EDTA, 10\% DMSO, 20 \text{mM} phosphate buffer and finally against either 20 \text{mM} phosphate buffer or nanopure water depending on the analysis. All reactions were analyzed using SEC, MALDI-TOF and electrophoresis and imaged using Transmission Electron Microscopy (TEM).
Table IV-1. List of the polymerization experiments performed using varying ratios between the monomer (styrene) and the BSA macroinitiator IX, and control experiments.

<table>
<thead>
<tr>
<th>#</th>
<th>Equivalents</th>
<th>Styrene</th>
<th>BSA-macroinitiator IX</th>
<th>Cu(I)Br</th>
<th>O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td></td>
<td>50</td>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>XI</td>
<td></td>
<td>500</td>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>XII</td>
<td></td>
<td>1500</td>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>XIII</td>
<td></td>
<td>2000</td>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>XIV</td>
<td></td>
<td>3000</td>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>2000</td>
<td>0 (native BSA)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0</td>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>2000</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>2000</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure IV-3. SEC-HPLC traces of different blank experiments performed under standard ATRP in situ polymerization conditions. Traces obtained when the reaction was performed in the absence of the biomacronitiator IX (blue), absence of Cu (green) or in the presence of oxygen (dark red).

The blank experiments using native BSA instead of the biomacronitiator IX ensured that the polymerization occurs only on the initiation predesigned position of IX as shown by the absence of reaction in the SEC chromatograph presented in Figure IV-3. Further blank
experiments performed under ATRP polymerization conditions in the absence of Cu(I)Br or in the presence of oxygen demonstrated that the reaction proceeds through ATRP.

**Figure IV-4.** SEC chromatographic traces of BSA macroinitiator IX (blue trace) and samples of the different *in situ* styrene polymerization experiments after purification using “low” monomer to BSA macroinitiator IX ratio (ratio 50:1, black trace; ratio 500:1, red trace).

When polymerization was conducted in the presence of “low” monomer to protein macroinitiator IX ratios (50 and 500 times excess), SEC chromatographic analysis revealed mixtures of unreacted BSA macroinitiator IX and various, rather disperse bioconjugates X or XI with hydrodynamic volumes in general slightly higher than that of native BSA (Figure IV-4) accompanied by low yields. On the contrary, when the polymerization was performed utilizing “high” monomer to biomacroinitiator IX ratios, SEC analyses demonstrated the quantitative formation of BSA-polystyrene Giant Amphiphiles possessing a hydrodynamic volume larger than that of BSA as indicated by the single peak observed with a shorter retention time than the BSA macroinitiator IX (Figure IV-5). It should be noted that in all cases, RID and UV traces were in good agreement. Interestingly, the broadness of the peaks observed for the native BSA, the BSA-macroinitiator IX and the Giant Amphiphiles XII, XIII, XIV was similar, indicating both the efficiency of the polymerization reaction and the retention of polydispersity.
Figure IV-5. SEC chromatographic traces of BSA macroinitiator IX and the different samples from in situ styrene polymerization experiments after purification using “high” monomer to BSA macroinitiator IX ratio (XII ratio 1500:1, red trace; XIII ratio 2000:1, blue trace; and XIV ratio 3000:1, orange trace).

These results were also supported by the electrophoretic analysis of the samples (Figure IV-6). In the case of the “low” monomer to biomacroinitiator IX ratio reactions X and XI, native gel electrophoresis revealed bands with slower electrophoretic mobilities than that of native BSA (higher molecular weight) along with bands corresponding to unreacted BSA macroinitiator IX, thus confirming the low yields of these reactions. In contrast, native gel electrophoresis revealed classical Giant Amphiphile behaviour for the “high” ratio reactions XII, XIII, XIV, i.e. migration hampered by the amphiphilic character of the bioconjugates. In the later cases, no trace of the starting macroinitiator was observed even when the gels were subjected to silver staining. It is worth mentioning that in all blank experiments the expected corresponding starting material was observed.
Figure IV-6. Native gel electrophoretic profile after Coomassie Brilliant Blue staining (A) and Silver staining (B) of native BSA, BSA macroinitiator IX, and different samples obtained by the in situ ATRP mediated polymerization of styrene on the BSA macroinitiator IX. The content of each lane is summarized on the right.

MALDI-TOF analysis of the polystyrene-BSA amphiphiles conclusively verified the above mentioned results (Figure IV-7). It should be noted that, as previously reported for Giant Amphiphiles prepared through conventional conjugation, MALDI-TOF spectra were difficult to obtain for all polymerization samples. Not all spectra obtained were of the clarity of those of native proteins due to the amphiphilic nature of the biohybrids. Several combinations of matrices were utilized to circumvent this problem. The mean molecular weight of the biohybrids was found to increase in general when the monomer to BSA-
macroinitiator IX ratio was varied from “low” (50 to 500) to “high” (1500 to 3000) as shown on Figure IV-7. The MALDI-TOF spectra of BSA-polystyrene conjugates X and XI showed the m/z signals corresponding to the free BSA macroinitiator IX, along with m/z signals varying from 67 to 71 kDa. In contrast, MALDI-TOF analyses showed the m/z signals varying from 71 to almost 80 kDa depending on the monomer to BSA-macroinitiator IX ratio utilized in XII, XIII, XIV. No signal corresponding to the free BSA macroinitiator IX was observed in the latter case.

![Figure IV-7](image)

**Figure IV-7.** MALDI-TOF analysis of BSA-macroinitiator IX (red trace) and the products of selected ATRP mediated in situ polymerization reactions of styrene over IX. The blue trace corresponds to styrene / IX ratio 500:1 and the green trace to 2000:1 ratio.

Though the formation of the amphiphilic biomacromolecules was demonstrated by SEC chromatography, MALDI-TOF and electrophoresis, it was further decided to also elucidate the structure of the formed polystyrene chain itself. This was achieved by digesting the proteins from the biohybrids, isolation of the polymers and subsequent spectroscopic study. To this direction, a solution of BSA-polystyrene synthesized by in situ ATRP polymerization was subjected to HCl mediated protein degradation and the resulting mixture was extracted in CH₂Cl₂ to isolate the polymer (Scheme IV-7). The latter was subsequently analyzed by NMR spectroscopy and MALDI-TOF.
Scheme IV-7. Protein digestion in the BSA-polystyrene Giant Amphiphiles.

$^1$H NMR analysis (Figure IV-8), revealed peaks possessing the typical chemical shifts of polystyrene (i.e. two broad peaks in the aromatic region corresponding to the benzene ring and two broad peaks between 1 and 2 ppm corresponding to the $CH$ and $CH_2$ in position $\alpha$- and $\beta$- of the benzene ring respectively). Furthermore, MALDI-TOF analysis revealed only two major distributions of the isolated polystyrene (4.7 kDa and 7.1 kDa). It is worth noting that the distribution of the predominant peak was found to possess a rather low polydispersity when compared to standard commercial polystyrene of known polydispersity indice (PDI ~ 1.13, Encapson, Figure IV-9). We attribute this low polydispersity to the selected synthetic approach.

Figure IV-8. $^1$H-NMR analysis of the polystyrene isolated from hydrolysis of BSA-polystyrene XIII.

Figure IV-9. Comparison of MALDI-TOF spectra of polystyrene isolated from the BSA-polystyrene conjugate XIII (black) and a standard polystyrene (grey, PDI 1.13).
IV.2.3. Aggregation studies.

The aggregation patterns of the BSA-polystyrene X-XIV superstructures obtained via the ATRP in situ polymerization of the protein macroinitiator IX were investigated by Transmission Electron Microscopy (TEM, Figure IV-10). This study revealed the in situ formation of well-defined spherical superstructures exhibiting aggregation behaviour similar to that of the conventionally synthesized BSA-polystyrene Giant Amphiphiles.\textsuperscript{15} The diameter of these aggregates was measured and found to be very regular with sizes varying from 30 to 100 nanometers in diameter as shown on Figure IV-10.

![TEM micrographs of BSA polystyrene Giant Amphiphiles X (A, B), XII (C, D), XIII (E, F) and XIV (G, H) superstructures in water.](image)

This observation is in good agreement with results obtained both by our group (Chapter II) and by the Nolte group.\textsuperscript{15} In our studies, the conventionally synthesized BSA-PS were found to form spherical aggregates with diameters varying between 30 and 100 nm, depending on the polystyrene employed. Similarly, in the Nolte studies it was found that the average diameter of BSA-polystyrene Giant Amphiphile spherical superstructures (MW\textsubscript{polystyrene} 4150, n~38) was about 30 to 70 nm. Interestingly, the diameter of the spherical superstructures did not change with the variation of the monomer to BSA-macroinitiator IX ratio, further confirming thus the results observed in the first chapter according to which no difference in aggregation was induced by variation of the conjugating polymer.\textsuperscript{17}
IV.2.4. Extension of the methodology to other hydrophobic monomers.

In order to investigate whether the *in situ* ATRP formation of Giant Amphiphiles is a method generic and applicable to a variety of hydrophobic vinyl monomers, the BSA-macronitiator IX initiated ATRP polymerization was also investigated with two other hydrophobic monomers.

On this end, we thought that it would be of interest to use the trimethylsilyl propargyl methacrylate monomer 8 (synthesized and utilized for the multifunctional polyalkyne 5, Chapter III) for the *in situ* ATRP polymerization of proteins as, if successful, it would lead to Giant Amphiphiles possessing the protected, functional 1-alkyne moiety that could be further functionalized in later steps to afford a variety of bioconjugates. Furthermore, we also decided to utilize a perfluorinated, pentafluorobenzene derivative for the ATRP based on the unique combination of high thermal stability, chemical inertness (to acids, bases and solvents) and very interesting surface properties\(^1,19,20\) that the produced polymer moiety would possess.

The trimethylsilyl propargyl methacrylate monomer 8 was synthesized as previously mentioned (Chapter III, Chapter VI). For reasons of synthetic ease for the fluorinated monomer, on the first step, the monomer 8 was deprotected as previously described by a reaction with TBAF in THF using acetic acid to keep an acidic pH to the solution (Scheme IV-8). The pentafluorobenzyl derived monomer 29 for the ATRP was obtained in a second step by a copper catalyzed [3+2] Huisgen dipolar cycloaddition between the alkyne 28 and the pentafluorobenzyl azide 17 (synthesized in Chapter III) in excellent yields using copper sulphate and sodium ascorbate as source of Cu(I).
Scheme IV-8. Reagents and conditions used for the synthesis of the hydrophobic monomer 29.

The trimethylsilyl propargyl methacrylate monomer 8 and monomer 29 were subsequently polymerized in the presence of the BSA ATRP macroinitiator IX using the optimized conditions found for the polymerization of styrene i.e., the ratio 8 or 29 as compared to the BSA-macroinitiator IX was kept constant at 2000 / 1 (Table IV-2), while the reaction was performed in aqueous phosphate buffer solution, oxygen free conditions, ambient temperature and using the copper bromide / N-(n-propyl)-2-pyridylmethanimine 12 catalyst system and without the presence of any “sacrificial” initiator (Scheme IV-9).

Scheme IV-9. General synthetic scheme of the in situ ATRP mediated polymerization of monomers 8 and 28 on BSA macroinitiator IX.
It should be noted that during the polymerization of the fluorinated vinyl monomer 29, our attempts to optimize the reaction scheme led to addition of a larger DMSO quantity (20%) to assist with monomer dispersion in the reaction mixture.

Furthermore, in the case of monomer 8, an experiment in the presence of the non polymerizable, fluorescent dye carboxyfluorescein (CF), was also performed to facilitate the observation of the superstructures by fluorescence confocal microscopy (vide infra for further inclusion experiments). By the hierarchical self-assembly of the BSA-polyalkyne (BSA-PA) Giant Amphiphiles XVI in the presence of CF, the superstructures obtained should be statistically loaded with CF and thus could be easily observed by CFM microscopy. For this reason CF was introduced in the polymerization feed at a concentration of 5 mM.

Upon polymerization, the reactions were as previously stirred for several hours under oxygen atmosphere and the unreacted monomers and reagents were removed by extensive dialysis against initially 10% DMSO, 20 mM phosphate buffer, pH 7.4 and then against 20 mM phosphate buffer, pH 7.4 using 25 kDa regenerated cellulose dialysis membranes. All samples were analyzed as previously.

<table>
<thead>
<tr>
<th></th>
<th>Monomer (equiv.)</th>
<th>BSA macroinitiator IX</th>
<th>CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>XV</td>
<td>8 (2000)</td>
<td>1</td>
<td>--</td>
</tr>
<tr>
<td>XVI</td>
<td>8 (2000)</td>
<td>1</td>
<td>5 mM</td>
</tr>
<tr>
<td>XVII</td>
<td>29 (2000)</td>
<td>1</td>
<td>--</td>
</tr>
</tbody>
</table>

Table IV-2. Table summarizing the conditions utilized for the ATRP in situ polymerization realized by using the hydrophobic monomers 8 and 29 and BSA-macroinitiator IX.

In the case of the ATRP mediated polymerization of monomer 29, both GPC and electrophoresis revealed for the biomacromolecular products XVII (Figure IV-11) a behaviour typical of that previously observed for Giant Amphiphiles. Electrophoresis under denaturating conditions showed that, as already observed in the case of in situ styrene polymerization, the electrophoretic mobility was hampered by the amphiphilicity of the biomacromolecule. Furthermore, as judged from the SEC chromatographic behaviour of the dialyzed reaction mixture, the ATRP polymerization led in this case to a mixture of products with apparent higher hydrodynamic volume (shorter retention times) and a broader molecular weight distribution than that that was observed with styrene, as judged by the broadness of the
Aggregation studies using TEM microscopy revealed the formation of well-defined spherical aggregates with diameters varying from ca. 50 up to 100 nm (Figure IV-12).

![Figure IV-11](image1.png)

**Figure IV-11.** SEC chromatographic traces at 254 nm of native BSA (dashed grey trace) and amphiphilic macromolecule XVII (solid black trace).

![Figure IV-12](image2.png)

**Figure IV-12.** TEM micrographs of the aggregates obtained by the self-assembly of the amphiphilic bioconjugates XVII.

We were unfortunately unable to characterize the products with MALDI even when using various matrices combinations. Taking into account the data presented above, it is judged that the synthesis of *Giant Amphiphiles* with fluorinated polymeric tails was successful, nevertheless further optimization of the reaction conditions (presumably by changing the organic cosolvent utilized to facilitate the dispersion of monomers during ATRP) will be necessary to achieve bioconjugates with the low polydispersity indices that characterized the ATRP derived polystyrene *Giant Amphiphiles*.
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Figure IV-13. SEC chromatographic traces of native BSA (solid black line), purified bioconjugates XV (solid grey line), and XVIII after reaction with TBAF (grey dotted line), and reaction with KF (black dashed line).

In the case of the in situ polymerization of monomer 8, SEC chromatography indicated the formation of a new peak attributed to the biomacromolecules XV (Figure IV-13). TEM micrographs of the aggregates XV revealed the formation of well-defined spherical aggregates with diameters varying from 50 to 150 nm (Figure IV-14) while CFM measurements of the reaction performed having CF incorporated in the polymerization feed (reaction XVI), demonstrated the statistical encapsulation of CF (Figure IV-14).22

Figure IV-14. Top: A. and B. CFM images of BSAPA structures XVI polymerized in the presence of CF (left, fluorescent image at 488 nm and right, optical image). Bottom: TEM micrographs of the aggregates observed after in situ polymerization of the trimethylsilyl protected monomer 8 on BSA macroinitiator IX in the presence of carboxyfluorescein.

Since the synthesis of such multifunctional bioconjugates was attempted with the aim to further exploit the pending 1-alkyne chains, the deprotection of the trimethylsilyl groups
was pursued. A successful deprotection would unmask the protected 1-alkynes allowing the *clicking* of a variety of hydrophobic/hydrophilic azides. The deprotection step was investigated by the use of either TBAF or KF. The selection of the above mentioned reagents was done on the basis of the reported in literature reaction conditions since, in the case of biomacromolecules, a significant factor directing the choice of reagents and conditions is protein stability.

After gentle shaking a ~ 0.1 mM solution of the amphiphiles XVI with 2000 equiv. of TBAF or KF for 24 hours (Scheme IV-10), the resulting solutions were dialyzed against 20 mM phosphate buffer, pH 7.4 to remove the excess of the reagents. In this case, the SEC chromatographic traces of the deprotection reaction samples XVIII and XIX, (Figure IV-13) were similar to that obtained for the bioconjugate XVI. Both electrophoresis, MALDI and IR were also unsuccessful in clarifying the result of the deprotection. CFM microscopy revealed that the superstructures were not affected by the deprotection step, in the case of the CF containing samples.

![Scheme IV-10. Reagents and conditions used for the deprotection of the pending alkyne side chains of the bioconjugate XVI.](image)

Though the above mentioned observations were not conclusive for the deprotection step, we reasoned that an indirect proof might be given upon performing a clicking reaction onto these superstructures. We decided to include in our experiments the azides 15, 16 and 21 which were synthesized as previously mentioned. It should be noted that the triethylene glycol azide 21, was used during this investigation because of its hydrophilic nature. We envisioned that by clicking a hydrophilic azide onto the polymer moiety chains of the bioconjugates, it would induce an overall change of hydrophilicity which would be expressed in its aggregation behaviour. The multi-*clicking* of different azide residues onto the polymer backbone was investigated using both XVIII and XIX. Since similar results were obtained in both cases, we
will only report on the results obtained by derivatization of compound \( \text{XIX} \) by the copper catalyzed Huisgen [3+2] \textit{click} chemistry cycloaddition reaction.

**Scheme IV-11.** Reagents and conditions used to click the azido-derivatives \( 15, 16, 21 \) onto the deprotected BSA-polyalkyne \( \text{XIX} \).

<table>
<thead>
<tr>
<th>( \text{R-N}_3 )</th>
<th>BSA-PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{XXa} )</td>
<td>15 ( \text{XIX} )</td>
</tr>
<tr>
<td>( \text{XXb} )</td>
<td>16 ( \text{XIX} )</td>
</tr>
<tr>
<td>( \text{XXc} )</td>
<td>21 ( \text{XIX} )</td>
</tr>
</tbody>
</table>

**Table IV-3.** Table summarizing the different multi-clicking experiments realized on \( \text{XIX} \).

When the clicking of hydrophobic azides \( 15 \) or \( 16 \) was investigated using SEC chromatography (Scheme IV-11), an indication that the reaction had occurred was provided through the formation of a new peak with small intensity (probably due to aggregation of the corresponding \textit{Giant Amphiphiles} on the column). The clicking of hydrophilic azide \( 21 \) on the other hand, led as expected to the observation of a new peak located in the higher molecular weight than that of the BSA-PA \( \text{XIX} \) before clicking.

An indirect proof of the formation of the clicked products came as planned through CFM microscopy. Interestingly, CFM revealed the formation of disordered structures collapsed on the glass slide for the clicked product \( \text{XXc} \), whereas only discrete fluorescent aggregates were observed after the clicking of hydrophobic azides \( 15 \) or \( 16 \) possessing structures that were in good agreement to the spherical structures previously seen for \textit{Giant amphiphiles}.
Scheme IV-12. SEC chromatographic traces obtained after clicking with the hydrophobic azides 15 and 16 (left) and with the hydrophilic azide 21 (right).

More specifically, as seen with CFM microscopy (Figure IV-15), both optical and fluorescence imaging (at 488 nm) revealed the presence of fluorescent superstructures, due to the encapsulation of fluorescein in the case of clicked hydrophobic residues 15 and 16. For the reaction product XXc, it was immediately noticed that the fluorescence background of the compound solution was intense and disorganized (gel like) fluorescent material was observed on the glass slide. These results provide the first indirect proof of the formation of the clicked products and of the applicability and efficiency of our approach toward the ATRP mediated formation of a plethora of Giant Amphiphiles. The superstructures were also studied by TEM and were observed to conserve the aggregation patterns shown by CFM.
Figure IV-15. CFM images of fluorescent aggregates observed for compounds A. XVI, B. compound XIX, C. compound XXa, D. compound XXb and E. compound XXc.

Figure IV-16. TEM pictures of structures observed with compounds XXa (left) and XXb (right).
IV.2.5. Encapsulation of material within the superstructures.

After the demonstration that the ATRP mediated \textit{in situ} preparation of protein-polymer \textit{Giant Amphiphiles} was feasible, extremely efficient and rather simple to perform under mild conditions, the possibility to use this method for the hierarchical formation of nanocontainers and/or nanoreactors was investigated. The utilization of the protein polymer \textit{Giant Amphiphiles} for the construction of nanocontainers (able to carry different guest (bio)organic molecules) and nanoreactors (performing catalysis by hosting other proteins or even organic catalysts) has long been the Holy Grail of research in the field (Figure IV-17).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figureIV17}
\caption{Schematic representation of a nanoreactor with catalytically active enzymes encapsulated proceeding enzymatic reactions.}
\end{figure}

Several problems, inherent to both the stability of the protein structure and the amphiphilic character of the biohybrids, made this a very difficult task. Once synthesized using the conventional methods, \textit{Giant Amphiphiles} concurrently aggregate into non-dynamic superstructures. Any attempt to incorporate therefore other molecules within the superstructures would most possibly need harsh conditions, endangering both the protein stability and conformation and the aggregated architectures themselves. We envisioned that the gradual formation of the biohybrid polymeric chain by using protein initiated ATRP would, on the other hand, allow the hierarchical formation of such nanostructures. We therefore initiated a study aiming to prove whether it was possible to perform the ATRP mediated polymerization in the presence of a second, non polymerizable, protein and if so, to determine whether this second protein would be trapped within the superstructures during aggregation process of the BSA-polystyrene \textit{Giant Amphiphiles}. The capacity of amphiphilic bioconjugates to concurrently form hierarchically assembled nanocontainers as hypothesized was investigated by performing the polymerization reaction in the presence of various non-polymerizable species such as a fluorescent dye (carboxyfluorescein, CF) or guest proteins (papain or Horseradish Peroxidase, HRP) that are shown on Table IV-4.
IV.2.5.1. Encapsulation of dyes or fluorescently labelled proteins.

Investigations on the possibility to form hierarchically assembled nanocontainers as hypothesized was confirmed by performing the polymerization reaction in the presence of a second non-polymerizable dye or labelled protein. To this end papain, a 23 kDa enzyme extracted from *Carica Papaya*, was labelled using either the NHS-activated ester of fluorescein (Figure IV-19, *left*) or the NHS-activated ester of Atto 610 (Figure IV-19, *right*), and once the fluorescent protein was purified by extensive dialysis against the buffer used in the optimized ATRP conditions (20 mM phosphate, pH 7.4), it was incorporated in the polymerization feed.

![Figure IV-18. 3D structure of the papain (isolated from *Carica Papaya*) used in this study with its 10 exposed Lysine residues highlighted as red sticks on the protein backbone.](image)

More specifically, a 150 fold excess of carboxyfluorescein or a 10 fold excess of the fluorescently labelled papain over the macroinitiator **IX** was integrated into the optimized reaction scheme (reaction **XIII** conditions, *i.e.* 2000 times excess of the monomer styrene over the biomacroinitiator **IX**) and the reaction was performed under the conditions described in section IV.2.2 (Scheme IV-13). A supplementary dialysis step was added after the
polymerization to remove the non encapsulated dyes / proteins. During this step, the products were dialyzed against 2% EDTA, 20 mM phosphate buffer pH 7.4 using 250 kDa dialysis bags, to afford yellowish aggregates that sediment with time.

Figure IV-19. Structure of the two commercially available dyes used to label proteins in this study: NHS-fluorescein (left, 488 nm) and NHS-Atto (right, 610 nm).

Scheme IV-13. General procedure used to statistically encapsulate labelled enzymes within superstructures obtained from the hierarchical self-assembly of the BSA-polystyrene Giant Amphiphiles. Inclusion followed by a post-polymerization labelling of the nanoassemblies.

SEC chromatography of the dialyzed samples revealed, as shown in Figure IV-20, the expected formation of Giant Amphiphiles in the papain inclusion experiment XXI, whereas
practically no free papain was detected. It should be mentioned that in a blank experiment that
was performed under the same conditions in the absence of BSA-macroinitiator IX, the native
papain was recovered suggesting that papain itself is not affected under ATRP conditions.

![SEC chromatogram](image)

**Figure IV-20.** SEC chromatographic measurements at 254 nm of the ATRP polymerization
reaction XXI in the presence of *Carica papaya* papain after dialysis (solid black trace) and of
native *Carica papaya* papain (dashed grey trace).

TEM analysis of the superstructures revealed spherical architectures similar to those
previously observed (*i.e.* reaction XIII, without the presence of fluorescein labelled papain,
Figure IV-21), demonstrating that the presence of a non polymerizable protein in the reaction
feed does not disturb the overall hierarchical aggregation process.

![TEM micrographs](image)

**Figure IV-21.** *(Left)* TEM micrograph of the aggregates observed after the *in situ*
polymerization in the presence of fluorescein labelled *Carica papaya* papain XXI. *(Right)*
CFM images of the fluorescently papain loaded aggregates demonstrating the statistical
encapsulation of papain within the superstructures.
To determine whether the labelled *Carica papaya* papain had been encapsulated within the superstructures, the dialyzed samples were also analyzed with Confocal Fluorescence Microscopy (CFM, Figure IV-21). The comparison of the structures observed through the optical microscope and through fluorescence revealed that the labelled, non-polymerizable, enzyme was as expected statistically trapped within the superstructures without disturbing the self-assembly process. The possibility of non specific interactions between the superstructures and *Carica papaya* papain was further excluded by adding fluorescein labelled papain to preformed, non protein containing vesicles and following the dialysis purification steps. No fluorescence was observed both in this case as well as when a labelled *Carica papaya* papain sample was dialyzed in the absence of *Giant Amphiphiles* superstructures. When samples of the nanocontainers of encapsulated fluorescein-papain were further externally labelled with Atto (*i.e.* reaction XXV, Scheme IV-13), CFM revealed the statistical presence of both fluorescent species in the superstructures (Figure IV-22).

![Figure IV-22](image_url)

**Figure IV-22.** CFM images of BSA-PS polymerized in the presence of fluorescein labelled *Carica papaya* papain after external labelling of the superstructures with Atto-NHS dye (reaction XXV). Lines A, B, C represent 3 different areas of the sample. The scanning was independently performed at 610 nm (Atto, *left*) and 488 nm (Fluorescein, *right*).
IV.2.5.2. Hierarchical construction of BSA-PS nanoreactors.

Since the incorporation of a non-polymerizable dye or enzyme/protein in the polymerization feed allowed statistical encapsulation of material within the spherical superstructures without disturbing the hierarchical aggregation process, we decided to explore the formation of bioconjugate nanosized reactors. For this reason we focused our research on the encapsulation of horseradish peroxidase (HRP). This heme bearing, extracellular plant peroxidase (Figure IV-23), is probably one the most studied members of the plant peroxidase superfamily that catalyzes the oxidative coupling of phenolic compounds using hydrogen peroxide as the oxidizing agent.

![Figure IV-23. 3D structure of HRP (left) with the heme cofactor depicted molecule as red stick and protohemin IX structure (right). The enzyme is 5.7 x 3.5 x 3.3 mm in size.]

The HRP catalytic sequence consists from a three-step cyclic reaction (Scheme IV-14) in which the enzyme is first oxidized by hydrogen peroxide and then reduced in a two electron transfer step by reducing substrates, typically a small phenol derivative but also conjugated aromatic diamine compounds such as the 3,5,3',5'-tetramethyl-biphenyl-4,4'-diamine generally referred to as tetramethyl benzydine (TMB).
For the purposes of our studies, the ATRP polymerization of styrene was initiated on the BSA macroinitiator IX in the presence of a 15 time excess of HRP over IX and the optimized ATRP polymerization reaction conditions were utilized \( (i.e. \ 2000 \ \text{times excess of styrene over IX, Table IV-4}) \). The mixture was subsequently dialyzed first against 2% EDTA 10% DMSO 20 mM phosphate buffer, pH 7.4 using 25 kDa MWCO regenerated cellulose membranes and then against 20 mM phosphate buffer, pH 7.4 using 250 kDa MWCO polypropylene dialysis bags. Upon dialysis, a slightly different coloration (brown red) of the resulting solution was observed as compared to previous polymerizations performed in the absence of encapsulated, non polymerizing material.

**Figure IV-24.** SEC chromatographic traces of polymerization in the presence of HRP (reaction XXIV) after dialysis (solid black trace), and native HRP (dashed grey trace).
SEC chromatographic analysis revealed the absence of free HRP in the dialyzed reaction mixture as shown in Figure IV-24. Finally, TEM demonstrated the formation of rather monodisperse, spherical aggregates with dimensions well in agreement with the previous experiments as shown in Figure IV-25.

![TEM micrographs of aggregates](image)

**Figure IV-25.** TEM micrographs of the aggregates observed after *in situ* polymerization of styrene on BSA macroinitiator IX in the presence of HRP (reaction XXIV).

The efficiency and permeability of these hierarchically formed *Giant Amphiphile* nanocontainers was tested using a purified HRP containing nanoreactors solution and the standard TMB/H$_2$O$_2$ chromogenic assay. TMB, as shown in Scheme IV-15, produces by oxidation in the presence of HRP a conjugated aromatic diimine whose formation can be followed by following UV absorbance at either 670 nm or at 450 nm (after stopping the reaction with hydrochloric acid).

![Reaction scheme](image)

**Scheme IV-15.** General reaction scheme for the HRP catalyzed oxidation of TMB followed by acidic treatment.

More specifically, increasing quantities of a *ready to use* 3,3',5,5'-tetramethylbenzidine (TMB)/H$_2$O$_2$ solution (Sigma Cat nr: T0440) were added to a dispersion consisting of the purified BSA-PS HRP containing nanoreactors and, following a small lag time, the intense blue colour of the soluble reaction product of the one-electron oxidation of TMB was recorded at 650 nm. The deep yellow colour read at 450 nm after stopping catalysis with an acid solution provided a final, direct proof of the capacity of the nanoreactors (Figure...
IV-26). The reaction was also repeated utilizing increasing nanoreactors quantities with the same outcome. Blank experiments were conducted in the presence of BSA-polystyrene amphiphiles without any encapsulated protein. In this case, as expected, no catalytic reaction was observed as indicated the absence of peak corresponding to the product of the reaction at 450 nm.

![Figure IV-26. UV study of the catalytic activity of HRP loaded in BSA-polystyrene Giant Amphiphiles superstructures. (left: curves obtained when increasing the volume of TMB added to the HRP loaded aggregates; right: absorbance at 450 nm as a function of TMB volume added).](image)

**IV.2.6. Interaction of fluorescently labelled papain loaded BSA-polystyrene Giant Amphiphiles with living systems.**

Though liposomes loaded with guest molecules, such as anticancer agents, have demonstrated clinical effectiveness, amphiphilic block copolymer vesicles are also now regarded as the encapsulators of the Future. Since most of the biomimetic systems are based on bilayer-forming low molar mass lipids which are generally not stable enough, polymeric systems are proving to be extremely useful in the understanding of natural processes (such as the clearance mechanism from the blood circulation for instance) or for applications like vectorisation. In fact, compared to lipids, most polymer membranes are hyperthick and can thereby achieve greater stability than any natural lipid membrane.

Consequently, synthetic diblock/triblock copolymers -polymersomes- have been extensively studied during the last years for their encapsulation properties and are indeed reported to be much more robust and suitable for this kind of applications. Furthermore, it is already clear that several biological membrane processes can be actually reproduced by polymer-based vesicles, as for instance biocompatibility, encapsulation or protein integration.
Up to date, polymersomes have already shown to be efficacious drug delivery systems.\textsuperscript{35,36,37} For example, diblock/triblock copolymers have been used to carry different bioactive compounds such as porphyrins for photoactive therapy,\textsuperscript{38} bioactive drugs such as anticancer agents (\textit{e.g.} paclitaxel\textsuperscript{39,40} and taxol\textsuperscript{41}) and even proteins used as therapeutics for different pathologies. Numerous of \textit{in vivo} and \textit{in vitro} studies have shown that these diblock/triblock copolymers are extremely promising compounds in the drug delivery systems development field, as they avoid for example fast clearance from blood or increase the biodistribution because of the higher stability of drugs in such nanocapsules. The fact that the block copolymers can be stimuli responsive (\textit{e.g.} pH responsive)\textsuperscript{42,43} increases the interest in their efficient utilization as they could in principle be programmed to release \textit{in vivo} bioactive compounds as a function of specific physiological conditions such as the pH,\textsuperscript{42} the presence of enzymes,\textsuperscript{43} etc. To this end, many research groups are focusing their efforts in developing “intelligent”, “smart”, biodegradable block copolymers with the potential to be utilized for a programmed interaction with living systems. Another interesting area of research aiming at the same direction focuses on the creation of glycopolymers with the aim to make use of their intrinsic affinity to biological systems to achieve such interactions.

Since \textit{Giant Amphiphiles} are in fact polymersomes, with biologically relevant amphiphilic structures, we envisioned the possibility that they could also exhibit an increased -compared to synthetic polymersomes- affinity with living systems which could potentially bring them into the active arena of drug delivery or controlled release systems.

We therefore decided to investigate whether \textit{Giant Amphiphile} hierarchically assembled nanostructures could interfere with biologically active systems aiming at their further development as nanocontainers, nanocarriers, or even better as drug delivery systems. A series of experiments was performed in collaboration with the group of Prof. U. Schwanenberg, aiming at the initial investigation of our assumption. In these experiments, the fact that the hydrophobic driving force for the aggregation of such systems arises from the presence of the non-biologically acceptable polystyrene was left to be addressed at a later stage as the polystyrene is not exposed while the superstructures are in aqueous solutions. Furthermore, based on the efficiency of the \textit{in situ} ATRP formation of \textit{Giant Amphiphiles}, we were confident that polystyrene could be easily replaced using ATRP and a biocompatible vinyl derivative if the initial results were promising.

Our initial goal involved studying the interaction of \textit{Giant Amphiphile} polymersomes with mammalian and bacterial cells. Unfortunately, the study with mammalian cells could not
be realized due to practical reasons. We therefore focused on three different types of bacterial
cells and more specifically cells from *Escherichia coli*, *Bacillus* sp. and Yeast. Since
Confocal Fluorescent Microscopy has proven to be a unique tool for the observation of
interactions dealing with biologically relevant systems such as living cells, we tested this
interaction using

* a. fluorescein-NHS externally labelled BSA-polystyrene Giant Amphiphiles (XXI), and

* b. doubly labelled BSA-polystyrene Giant Amphiphiles obtained after polymerization of IX with styrene in the presence of Atto-labelled papain and externally labelled with the fluorescein-NHS activated ester (Figure IV-19).

Initially, minute volumes of Atto-labelled *Carica papaya* papain loaded vesicles
XXVI (i.e. reaction XXII after an external labelling with NHS-activated fluorescein) were
incubated with these 3 bacterial strains, i.e. *Escherichia coli*, *Bacillus* sp. and Yeast at
different times of their cellular growth (0, 2 hours and 8 hours). Experimentally, 50 µL of the
aggregates solution (~ 100 µM) was added to 1 ml of cell culture and 3 mL of nutritive Luria–
Bertani (LB) growth medium. The cell suspensions were gently shaken for 12 hours either
at ambient temperature or at optimum growth temperature (~37°C), were then centrifuged and
the cell pellets collected and resuspended in growth medium to be examined by CFM
microscopy. A 10 µL quantity of this sample was utilized on the glass slide, while no fixative
proved to be nessecary.

No interaction was noted in the case of *Bacillus* sp. Cells even upon longer incubation
periods. In the case of Yeast bacteria, we observed an interesting, reproducible adhesion of
the superstructures on the cell walls (Figure IV-27).
Surprisingly, *E. coli* cells showed the most promising results. The samples after this 12 hours incubation time (and after washing), showed an efficient and reproducible cell uptake of the amphiphilic biomacromolecule nanoreactors. As shown in Figure IV-28, the fluorescence coincides with the presence of the cells (viewed through the optical measurement), while “empty” cells and free supstructures were also observed. In all cases, both dyes were detected by independent scanning, while it should be mentioned that the intensities of the internal dyes were lower. No difference was observed in the experiments varying on the growth temperature, demonstrating that cell cycle has no influence on the cell uptake of the aggregates. It should be noted that extensive washing with 0.1% NaCl solution were realized (and was followed by centrifugation and resuspension of the cells before measurements) in order to exclude any possibility of non specific interactions between the bacterial cell membranes and the aggregates.
In a first effort toward elucidating these interesting observations, the vesicle uptake was monitored as a function of incubation time. More specifically, 50 µL of the aggregates solution was added to 4 mL of the cell suspension as mentioned earlier and each batch was incubated for a different period of time (1, 2, 3, 4, 8, 12 hours) in order to follow cell uptake. It was observed that the cells are fully loaded with aggregates after only two hours of incubation whereas no fluorescence (i.e. no cell uptake) was observed after 1 hour of incubation as shown in Figure IV-29. Two interesting observations were also made during these experiments. Firstly, the uninhibited growing of the cells suggested that the presence of the superstructures does not result in destroying the cells. Furthermore, no loss of fluorescence in the cells was noticed when the incubation time was prolonged, this suggesting that there is probably no diffusion of the spherical superstructures out of the cells with time.
To further elucidate the process, FACS experiments were also conducted, the collection of fluorescent cells was nevertheless unsuccessful and in progress of being repeated.

Though it is premature to extract any conclusions on the exact nature of these interactions, our experiments proved without a doubt that Giant Amphiphile superstructures can penetrate E. coli bacterial membranes without causing the death of the cells and are therefore extremely promising for possible further applications and should be further pursued.

In this chapter, we developed the first, facile, efficient, ATRP mediated in situ preparation of BSA-polystyrene Giant Amphiphiles. After synthesis of a BSA-ATRP macroinitiator, we successfully achieved the polymerization of styrene onto this macroinitiator bioconjugate in a straightforward and efficient manner. We thus prepared a family of Giant Amphiphiles with a degree of polymerization controlled by the monomer to BSA macroinitiator ratio and narrow polydispersities. The yields of the reactions were found to be quantitative when the monomer to BSA macroinitiator ratio was above 1000:1. Furthermore, our studies proved a much narrower polydispersity of the produced biohybrids that the one previously observed for hydrophilic systems, which is attributed to the nature of the synthetic approach and the intrinsic amphiphilic character of the products. Within this chapter, results on the polymerization of two more vinyl monomers are also presented proving the efficiency and generic nature of the approach.

By a simple incorporation of a second non polymerizable species (dye or enzyme) in the polymerization feed, we demonstrated that a straightforward creation of nanocontainers was possible without steps that would interfere with the integrity of the protein or of the overall hierarchical aggregated nanoarchitecture. Furthermore, we demonstrated for the first time that BSA-polystyrene Giant Amphiphile nanocontainers are permeable to small molecules and can be used as nanoreactors.

Finally, we successfully observed in this study the interactions of these fluorescently labelled papain loaded BSA-PS Giant Amphiphiles superstructures with E. coli bacterial cells that took place within a couple of hours. Due to their encapsulation and aggregation properties, Giant Amphiphiles might be regarded as very promising and interesting compounds for biomedical applications. Following this bacterial cell uptake experiment success, we can envision that in the near future, just by changing the nature of the hydrophobic polymer attached to the protein, biocompatible polymer-proteins Giant Amphiphiles could be successfully adapted to the preparation of drug delivery systems or protein nanocarriers.
Current efforts are directed toward the extension of this method to a broad range of monomers and toward the \textit{in situ} preparation of nanofunctional assemblies \textit{e.g.} multienzymatic nanoreactors.
References

[17] We assume this statement to be true only for relatively narrow variation in terms of molecular weights of the hydrophobic polymers utilized in these studies.
[21] Taking into account the different nature of fluorinated polymers as compared to the polymers previously studied, this assumption is only based in the observation of the broadness of the peaks. Further studies involving digestion of the protein, isolation of the polymer and further characterization, are judged to be necessary to fully support this assumption.
[22] Fluorescein was excited with the 488 line of the Argon-Krypton laser during the CFM measurements.


[34] Yang, L.; Alexandridis, P. *Int. J. Pharm.* 2007, 342, 6-17.


[38] LB medium was prepared according to the recipe of Miller: 5 g yeast extract, 10 g peptone tryptone, 10 g NaCl) Miller J.H. (1972) *Experiments in molecular genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor.
The work undertaken during this PhD thesis allowed the development of new synthetic pathways towards the preparation of Giant Amphiphiles under milder conditions that those previously employed and with better yields.

In a first approach, by the use of an heterobifunctional linker containing a terminal maleimide and an alkyne-1 functionality, protein bioconjugates containing a terminal alkyne function were synthesized. The coupling of a series of hydrophobic azido polymers with the alkyne-1-functionalized proteins using the copper catalyzed variant of the [3+2] Huisgen dipolar cycloaddition (“click” chemistry), led to the formation of a small library of Giant Amphiphiles. When varying the length of the polymer moiety, no change in aggregation behaviour of the resulting superstructures was observed. The click mediated method was also successfully utilized in other free cysteine containing proteins, proving its generic nature. Through this study, it was demonstrated that the aggregation pattern of such biomacromolecular systems is highly dependent on the protein nature as the BSA-polystyrene Giant Amphiphiles formed well defined spherical aggregates in water whereas cylindrical aggregates were observed with Hemoglobin-polystyrene Giant Amphiphiles. Interestingly, it was for the first time demonstrated that the Giant Amphiphiles aggregate also in organic solvents, leading to reverse superstructures in which we expect the protein to be in the core and the polymer exposed to the solvent. This exciting outcome was further utilized in order to bring an enzyme hosted by these superstructure in organic solvents and observe its catalytic action.

In the second part described within the thesis, a novel methodology was designed with the aim to bypass the incompatibility between the hydrophilic protein and the hydrophobic polymer. This new approach was based on two discrete steps where initially a hydrophilic multifunctional polymer was selectively attached to a protein through a 1,4-Michael thiol
addition and in a second step hydrophobicity was introduced by a multicking step in which small hydrophobic azides were grafted onto the polymer backbone efficiently and under mild conditions. It was demonstrated that the size distribution of the aggregates obtained after self-assembly depends on the hydrophobic azide used. The prospect of the further development of new Giant Amphiphile derivatives where the clicked moiety would express its intrinsic characteristics (i.e. catalysis, electron transfer properties, etc) and introduce multifunctionality to the amphiphilic biomacromolecules is exciting. It would for instance be interesting to multick terpyridine azides onto the polymer backbone as it would allow some further metal to complex interactions and thus create a tunable cross linking of the superstructures.

The last method that was developed relied on the in situ ATRP mediated polymerization of hydrophobic monomers onto a protein macroinitiator and afforded very interesting results. First of all, it allowed for the synthesis of Giant Amphiphiles to proceed in quantitative yields but more importantly in quantitative amounts while their final isolation was achieved by a simple dialysis step. Furthermore, it was demonstrated that an enzyme could be hosted within these hierarchical self-assembled superstructures in a one-pot procedure and without disrupting their aggregation behaviour. More importantly, using classical enzymatic tests, it was demonstrated that, when HRP was encapsulated within the superstructures, it retained its catalytic activity. This method allowed not only the inclusion of active biomolecules but also the observation of their activity as the structures surprisingly proved to be permeable. The formation of multienzymatic nanoreactors is foreseen to be one of the most exciting prospects for future studies.

Compared to the previously reported procedures for the synthesis of Giant Amphiphiles in which high organic solvent contents were used or tedious purification procedures were required, these newly synthetic methodologies were found to proceed under much milder conditions and, especially in the in situ polymerization approach, required simpler purification steps to afford products in relatively large quantities. It can be envisioned therefore that in the near Future, a collection of different active enzymes could be used to form such superstructures tuned for example in a way that they could undergo enzymatic cascade reactions.

In this thesis work, some of the properties of Giant Amphiphiles were highlighted and found to be promising to find utility in areas of bio and nanotechnologies in the near Future. The results on the interaction of such superstructures with bacterial cells are for example
intriguing and promising. Their interaction with mammalian cells should be certainly investigated. The big challenge would be to construct also systems with biocompatible polymer moieties as they should in principle allow better interactions with cell membrane and could lead to important outcomes in the area of drug delivery systems for example.
General Remarks

Starting Materials. All chemicals were purchased from Fluka Chemica or Sigma-Aldrich (unless otherwise specified) and used without further purification. Cu(I)Br was purified as reported by Keller and Wycoff.\(^1\) \(N-(n\text{-propyl})\)-2-pyridylmethanimine and 2-Methyl-acrylic acid 2,2-dimethyl-[1,3]dioxolan-4-ylmethyl ester were prepared as described earlier and stored at 0 °C. Protected maleimido initiator, protected alkyne monomer, fluorescent hostasol comonomer and 1-azido-decane and benzyl-azide were synthesized according to the literature. Hostasol tag methacrylate was obtained from Dave Haddleton’s research group. NEt\(_3\) was dried over KOH pellets. Deuterated solvents were obtained from Cambridge Isotope Laboratories. Fluorescein-NHS activated ester (6-[Fluorescein-5(carboxamide)]hexanoic acid N-hydroxysuccinimide ester) and Atto 610 N-succinimidyl ester were purchased from Fluka Biochemica, dissolved in dry DMSO to afford a 5 mM solution and stored at -20 °C. Bovine Serum Albumin (BSA), Cytochrome C (from equine heart), Hemoglobin (Hb) were purchased from Sigma Aldrich. Papain and Horseradish Peroxidase (HRP) were purchased from Calbiochem. Polymerizations were carried out using standard Schlenk techniques under an inert atmosphere of oxygen-free nitrogen, unless otherwise stated. Yields of the reactions were not optimized.

Analytical techniques.
Aqueous size exclusion chromatography (SEC) was conducted using a Shimadzu modular system comprising a DGU-14A solvent degasser, a LC-10AD pump, a CTO-10A column oven, an SIL-10AD auto-injector, a RID-10A refractive index detector and a SPD-10A Shimadzu UV Vis. Spectrometer. The system was equipped with a Polymer Laboratories
30x7.8mm 5μm BioBasic SEC 60 guard column followed by a 300x7.8mm 5μm BioBasic SEC 300 Polymer Laboratories column, using a mixture of 70% phosphate buffer 20 mM pH 7.4, 30% acetonitrile or a mixture of 0.1% TFA, 30% MeCN in MilliQ water as the eluent at room temperature and flow rate: 0.5 mL/min (unless otherwise noted). Chromatograms were acquired at 254 nm and 280 nm wavelength and were processed with the EZStart 7.3 chromatography software.

Gel Permeation Chromatography (GPC) was conducted on the Shimadzu VP HPLC system equipped with a PL Gel Thermo column eluting with THF as an eluent (unless otherwise noticed).

NMR spectra were recorded on a Bruker 300 MHz and a Bruker 400 MHz spectrometer system. All chemical shifts are reported in ppm (δ) relative to tetramethylsilane, referenced to the chemical shifts of residual solvent resonances (1H and 13C). The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, m = multiplet. The molecular weights of the polymers $M_n$ are calculated by comparing the integrals of the chain-end signals and appropriate peaks related to the polymer backbone.

Infrared absorption spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer (using a Golden Gate diamond or a NaCl cuvette).

UV-vis spectra were recorded on a CARY 1 BIO UV-visible spectrophotometer.

Solutions were sonicated in a Bandelin Sonorex RK 100 apparatus.

Confocal microscopy experiments were performed with a Leica TCS SP2 AOBS confocal microscope using a 100x oil immersion objective. The Hostasol tag was excited with the 514 line of the Argon-Krypton laser. Fluorescein was excited with the 488 line of the Argon-Krypton laser. Atto was excited with the 543 nm Helium-Neon laser. Dual dye imaging was performed in sequential mode. Unidirectional scanning was done at 400 Hz with an image format of 512 by 512 pixels.

Transmission Electron microscopy experiments were performed using a FEI Tecnai G2 Electron Microscope. Micrographs were taken using a Tietz CCD camera at a 2048 by 2048 pixel resolution. All samples for TEM measurements were prepared on Formvar (15/95E)-Cu grids by depositing a 1000 times diluted solution of dialyzed (against nanopure water) bioconjugate samples. The solvent was drained with filter paper after a deposition period of approximately 10 minutes. No platinum shadowing was necessary for the visualization of the samples.

MALDI-TOF MS measurements were performed in the SVS-MS Mass Spectrometry Core Facility using an Axima CFR+ MALDI-TOF (Shimadzu Biotech, Manchester, UK) in
positive ionization mode and sinapinic acid as the matrix. Protein samples were mixed with matrix (1:1 volume ratio, sinapinic acid, 10 mg/ml) and air dried before analysis. Dithranol was used as matrix for the polymer samples.

Discontinuous Native PAGE\(^2\) (Ornstein-Davis) electrophoresis was run using a 4% stacking gel and a 10% resolving under standard nondenaturing conditions. Samples were dissolved in TRIS buffer containing bromophenol blue and were visualized using Coomasie Brilliant Blue or Silver Staining.
Chapter II. Formation of Giant Amphiphiles by Click Chemistry

2-[2-(2-Prop-2-ynyloxy-ethoxy)-ethoxy]-ethanol (1): In a two necked round bottom flask equipped with a cooler, to a solution of anhydrous triethylene glycol (3 g, 19.97 mmol) in dry THF (15 mL) was added under nitrogen atmosphere at 0°C sodium hydride (60% in oil) (0.160 g, 3.99 mmol). The resulting mixture was stirred 30 min. at 0°C and refluxed 2 hours at 80°C. Then, to this solution was dropwise added a solution of propargyl bromide 80% in toluene (404 µL, 3.63 mmol). The reaction mixture was refluxed at 80°C overnight under nitrogen atmosphere. Removal of the solvent under reduced pressure gave the crude product as yellow slurry. Final chromatography column with a mixture AcOEt/cyclohexane: 3/2 ($R_f = 0.23$ in AcOEt) gave the pure product as yellow oil in quantitative yield. $^1$H NMR (CDCl$_3$, 400 MHz, 298 K): δ 4.09 (d, 2H, $J = 2.1$ Hz, $CH_2$−C≡CH), 3.65-3.40 (m, 12H, O−C$H_2$C$H_2$−O), 3.10 (s, 1H, −OH), 2.39 (t, 1H, $J = 2.2$ Hz, −C≡CH). $^{13}$C{1H} NMR (CDCl$_3$, 100 MHz, 298 K): δ 79.2 (1C, C≡CH), 74.4 (1C, C≡CH), 72.2 (1C, −CH$_2$O−), 70.2 (1C, −CH$_3$O−), 69.9 (1C, −CH$_2$O−), 69.9 (1C, −CH$_2$O−), 68.6 (1C, −CH$_2$O−), 61.2 (1C, CH$_3$OH), 57.9 (1C, CH$_2$−C≡CH). ESI-MS (MeOH, +EI) m/z (relative intensity): 189 ([MH$^+$], <1), 162 (2), 89 (39), 45 (100).

1-[2-(2-Prop-2-ynyloxy-ethoxy)-ethoxy]-ethyl]-pyrrole-2,5-dione (2): In a two necked round bottom flask, a solution of triphenylphosphine (386 mg, 1.47 mmol) in dry THF (10 mL) was cooled down to -78°C in a dry ice/acetone bath. To this mixture was dropwise added under nitrogen atmosphere at -78°C a solution of DEAD 40% in toluene (670 µL, 1.47 mmol). After 5 minutes of stirring was added the alcohol 1 (304 mg, 1.62 mmol) in solution in dry THF (10 mL). The resulting mixture was stirred 10 min. before the sequential addition of neopentyl alcohol (65 mg, 0.74 mmol) and maleimide (142 mg, 1.46 mmol).
reaction mixture was stirred 5 additional minutes at -78°C and was then allowed to warm to ambient temperature overnight. Volatiles were removed under reduced pressure. The crude product (pink slurry) was purified by chromatography column eluting with a mixture AcOEt/cyclohexane: 2/3 ($R_f = 0.45$ in AcOEt/cyclohexane: 3/1) to afford the compound 2 as a yellow oil (277 mg, 1.03 mmol, 70% yield). IR (neat): $\tilde{\nu} = 3303, 3063, 2873, 1713, 1436, 1407, 1270, 1101, 828 \text{ cm}^{-1}$. $^1$H NMR (CDCl$_3$, 300 MHz, 298 K): $\delta$ 6.69 (s, 2H, $\text{HC}=C$), 4.18 (d, 2H, $J = 2.3 \text{ Hz}, \text{CH}_2$–$\text{C}=\text{CH}$), 3.75-3.56 (m, 12H, −$\text{CH}_2$O−), 2.42 (t, 1H, $J = 2.4 \text{ Hz}, \text{−C}=\text{CH}$). $^{13}$C{1H} NMR (CDCl$_3$, 100 MHz, 298 K): $\delta$ 170.6 (2C, CO$_\text{imide}$), 134.1 (2C, C$_\text{vinyl}$), 79.7 (1C, C=C), 74.5 (1C, C=C), 70.5 (1C, CH$_2$), 70.4 (1C, CH$_2$), 70.1 (1C, CH$_2$), 69.1 (1C, CH$_2$), 67.9 (1C, CH$_2$). ESI-MS (MeOH, +EI) m/z (relative intensity): 268 ([MH$^+$], 2), 176 (7), 149 (8), 124 (100), 104 (22), 45 (43).

General procedure for azidation of polystyrene azides

3a, 3b, 3c: To freshly prepared 0.5 M solution of sodium azide in DMSO (5 mL) were added 0.625 mmol of the bromide end-capped polystyrene (Mw 2015, 4360, 8100). After overnight stirring at 60°C, the solutions were quenched with 1 M NaOH solution. The resulting azidopolystyrenes 3a, 3b or 3c were recovered by DCM extraction and purified by filtration after precipitation in a large volume of MeOH. The pure products were analyzed by MALDI-TOF ($M_w$=1978, 4322 and 8063) respectively found for 3a, 3b, 3c; see Figure VI-1 for 3a), FT-IR (characteristic C–N$_3$ stretching band observed for each polystyrene azide 3a, 3b, 3c) and $^1$H NMR (characteristic triplet observed at 2.71 ppm corresponding to the CH in α of the azide group) and GPC (THF/triethylamine: 95/5, 2 PL Gel mixed column (Thermo), 0.5 mL.min$^{-1}$, retention time = 16.01, 15.09 and 14.67 min. observed respectively for 3a, 3b, 3c).

Figure VI-1. MALDI-TOF spectrum of PS-N$_3$ 2 kDa (expected $M_w$=1977, obtained $M_w$=1978) obtained after azidation of PS-Br 2 kDa (2015).
Preparation of alkyne functionalized BSA (I): Native BSA (66.5 kDa) was dissolved in 20 mM PB pH 7.4 to obtain a concentration of ca. 0.3 mM. To a solution of this native protein (850 µL) in phosphate buffer pH 7.4 was added a 0.2 M solution (127 µL) of the heterobifunctional linker 2 dissolved in PBS 20 mM pH 7.4. The mixture was completed with 20 mM PBS solution until reaching a total volume of 1 mL. After gentle shaking for 24 hours at 7°C, the mixture was dialyzed against 20 mM PB pH 7.4. Finally, the biohybrids solutions were analyzed by SDS-PAGE (Figure VI-2, lane 2), SEC-HPLC (30% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min\(^{-1}\), retention time : 9.52 min., Figure VI-4, blue trace), and MALDI-TOF analysis (MW~66.9 kDa) and their aggregation behaviour was studied by TEM microscopy (no aggregation observed).

**Figure VI-2.** SDS-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) under denaturing conditions stained with Coomassie Brilliant Blue: lane 1, native BSA; lane 2, purified bioconjugation reaction mixture I; lane 3, native BSA; lane 4, BSA-PS 2 kDa IIa; lane 5, BSA-PS 4 kDa IIb; lane 6, BSA-PS 8 kDa IIc.

**Figure VI-3.** SEC-HPLC of native BSA (dashed grey trace) and alkyne functionalized BSA I (solid black trace).
Preparation of alkyne functionalized Cytochrome C (III) and alkyne functionalized hemoglobin (IV): same procedure than for the preparation of alkyne functionalized BSA (I). Here Hemoglobin Hb (64 kDa) and Cytochrom C CytoC (11 kDa) were used.

**General procedure for the preparation of compounds (IIa), (IIb), (IIc):** In an eppendorf, containing 276 µL of PBS 20 mM pH 7.4, 100 µL of a 15 mM solution of the polystyrene azide 3a, 3b or 3c in THF were injected while sonicating. The solution was sonicated for another 10 min. before the addition of 16 µL of a sodium ascorbate solution (40 mM), 8 µL of a copper sulfate solution (40 mM) and 600 µL of alkyne functionalized BSA solution (I) (0.25 mM). The resulting turbid solutions were gently shaken for 2 days at 7°C in dark conditions and were then extensively dialyzed using10 kDa dialysis bags against either 20mM phosphate buffer pH 7.4 or nanopure water depending on the analysis. All reactions were analyzed by SDS-PAGE (Figure VI-2), SEC-HPLC (30% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min⁻¹, Figure VI-4) and MALDI-TOF-MS and aggregation of the biohybrids studied by TEM microscopy.

Characterization of bioconjugate IIa by SDS-PAGE (Figure VI-2, lane 4), SEC-HPLC (30% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min⁻¹, Figure VI-4, black trace, retention time : 7.33 min) and MALDI-TOF-MS (Figure VI-5, MW~68.7 kDa) analysis and their aggregation behaviour was studied by TEM microscopy (Figure VI-6, line A).

Characterization of bioconjugate IIb by SDS-PAGE (Figure VI-2, lane 5), SEC-HPLC (30% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min⁻¹, Figure VI-4, red trace, retention time : 7.66 min) and their aggregation behaviour was studied by TEM microscopy (Figure VI-6, line B).

Characterization of bioconjugate IIc by SDS-PAGE (Figure VI-2, lane 6), SEC-HPLC (30% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min⁻¹, Figure VI-4, green trace, retention time : 7.66 min) and their aggregation behaviour was studied by TEM microscopy (Figure VI-6, line C).
**Figure VI-4.** SEC chromatographic characterization of the blank experiment (I + 3a, 3b and 3c without CuSO₄/sodium ascorbate, black dotted trace) and BSA-polystyrene amphiphiles IIa, IIb and IIc (respectively solid black, solid grey and dashed grey traces) after dialysis.

**Figure VI-5.** MALDI-TOF analysis of BSA-PS 2 kDa IIa.
Figure VI-6. TEM micrographs of superstructures obtained by aggregation of A. BSA-PS IIa, B. BSA-PS IIb and C. BSA-PS IIc.

**General procedure for the preparation of compounds** (Va), (Vb), (Vc): Same procedure as for the preparation of compounds IIa, IIb and IIc. All reactions were analyzed by SDS-PAGE (no significative results found), SEC-HPLC (30% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min⁻¹, Figure VI-7 left, red, blue and green traces), and MALDI-TOF (Figure VI-8 left, only for compound Va) analysis. No aggregation was observed by TEM microscopy.

Characterization of bioconjugate Va by SEC-HPLC (30% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min⁻¹, Figure VI-7 left, red trace, retention time : 11.38 min) and MALDI-TOF-MS analysis (Figure VI-8 left, MW~13 kDa) and no aggregation behaviour was observed by TEM microscopy.

Characterization of bioconjugate Vb by SEC-HPLC (30% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min⁻¹, Figure VI-7 left, green trace, retention time : 10.92 min) and no aggregation behaviour was observed by TEM microscopy.
Characterization of bioconjugate Vc by SEC-HPLC (30% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min⁻¹, Figure VI-7 left, blue trace, retention time : 10.82 min) and no aggregation behaviour was observed by TEM microscopy.

**General procedure for the preparation of compounds (VIa), (VIb), (VIc):** Same procedure as for the preparation of compounds IIa, IIb and IIc. All reactions were analyzed by SDS-PAGE (no significative results found), SEC-HPLC (30% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min⁻¹, Figure VI-7 right; red, blue and green traces), and MALDI-TOF (Figure VI-8 right, only for compound VIb, peak found at 68.7 kDa) analysis and their aggregation behaviour was studied by TEM microscopy (Figure VI-9).

Characterization of bioconjugate VIa by SEC-HPLC (30% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min⁻¹, Figure VI-7 right, red trace, retention time : 9.17 min) and their aggregation behaviour was observed by TEM microscopy (Figure VI-9).

Characterization of bioconjugate VIb by SEC-HPLC (30% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min⁻¹, Figure VI-7 right, green trace, retention time : 9.21 min) and MALDI-TOF-MS analysis (Figure VI-8 right, MW~68.2 kDa) and same aggregation behaviour as VIa was observed by TEM microscopy.

Characterization of bioconjugate VIc by SEC-HPLC (30% acetonitrile 20 mM phosphate buffer pH 7.4, BioBasic SEC 300, 0.5 mL.min⁻¹, Figure VI-7 right, blue trace, retention time : 9.15 min) and same aggregation behaviour as VIa was observed by TEM microscopy.
Figure VI-7. SEC chromatographic analysis of native proteins and amphiphilic biomacromolecules (left). Left: native Cytochrome C and amphiphilic CytoC-polystyrene bioconjugates Va, Vb, Vc at 254 nm. Right: native Hb and amphiphilic Hb-polystyrene bioconjugates VIa, VIb, VIc at 406 nm.

Figure VI-8. MALDI-TOF spectra obtained for Left: native Cytochrome C and its 2 kDa polystyrene conjugate Va and Right: for native Hemoglobin and 2kDa polystyrene conjugate VIb when mixed with a sample of native BSA for stabilization.

Figure VI-9. TEM micrographs of aggregates observed with polystyrene-Hb Giant Amphiphiles VIa.
Observation of compound IIb in dichloromethane: 1mL of the suspension of IIb initially in 20 mM PB was freeze-dried and immediately redissolved with the same volume of pure dichloromethane. The sample was immediately either observed by TEM microscopy or negatively stained with uranyl acetate (for further study). TEM micrographs observed are shown below on Figure VI-10 and Figure VI-11.

Figure VI-10. TEM micrographs of BSA-PS 4 kDa IIb in CH₂Cl₂. The two pictures on the bottom are negative stained (Uranyl Acetate).

Figure VI-11. A. TEM micrographs of BSA-PS 4kDa IIb in CH₂Cl₂. Micrograph on the left immediately after addition of the CH₂Cl₂, on the right 48h later. B. TEM micrographs of BSA-PEG₅₀₀₀ in dichloromethane.
Catalytic activity of Glucose Oxidase in BSA-PS superstructures: To 990 µL of BSA-PS conjugate were added 10 µL of a Glucose Oxidase solution. The resulting solution was immediately freeze-dried and redissolved in 990 µL of dichloromethane (with a minute quantity of milliQ water). This cycle was repeated for 3 more times and finally the solution dialyzed against dichloromethane to get ride of untrapped enzyme. Finally, the resulting dialyzed solution was observed by CFM microscopy (before and after addition of selective for GO substrate, see Figure VI-12).

![Figure VI-12](image)

**Figure VI-12.** Confocal Fluorescent Microscope pictures of polystyrene-BSA superstructures after entrapment of β-galactosidase before (A. 488 nm, B. optical image, C. 405 nm) and after addition of fluorogenic substrate (D. 488 nm, E. optical image, F. 405 nm).
Chapter III. Formation of Giant Amphiphiles by Post-Functionalization of Hydrophilic Triblock Protein-Polymer Conjugates.

4,10-Dioxatricyclo[5.2.1.02,6]dec-8-ene-3,5-dione (10): Maleic anhydride (30.0 g, 306 mmol) was suspended in 150 mL of toluene and the mixture warmed to 80°C. Furan (33.4 mL, 459 mmol) was added via syringe and the turbid solution stirred for 6 h. The mixture was then cooled to ambient temperature and the stirring stopped. After 1 h, the resulting white crystals were collected by filtration and washed with 2 x 30 mL of petroleum ether to obtain 44.4 g (267 mmol, 87% yield) of the product 10 as small white needles. $^1\text{H}$ NMR (CDCl$_3$, 400 MHz, 298 K): $\delta$ 6.57 (t, 2H, $J = 1.0$ Hz, CH$_{\text{vinyl}}$), 5.45 (t, 2H, $J = 1.0$ Hz, CHO), 3.17 (s, 2H, CH). $^{13}\text{C}\{^1\text{H}\}$ NMR (CDCl$_3$, 100 MHz, 298 K): $\delta$ 170.0 (2C, CO), 137.1 (2C, CH$_{\text{vinyl}}$), 82.3 (2H, CHO), 48.8 (2C, CH). ESI-MS (MeOH, +EI) m/z (relative intensity): 167 ([MH$^+$], <1), 121 (7), 98 (22), 94 (13), 68 (100).

4-(2-Hydroxyethyl)-10-oxa-4-azatricyclo[5.2.1.02,6]dec-8-ene-3,5-dione (11): The anhydride 10 (2.00 g, 12.0 mmol) was suspended in MeOH (50 mL) and the mixture cooled to 0°C. A solution of ethanolamine (0.72 mL, 12.0 mmol) in 20 mL of MeOH was added dropwise (over ~10 min) and the resulting solution was stirred for 5 min at 0°C, then 30 min at ambient temperature, and finally refluxed for 4 h. After cooling the mixture to ambient temperature, the solvent was removed under reduced pressure, and the white residue was dissolved in 150 mL of CH$_2$Cl$_2$ and washed with 3 x 100 mL of water. The organic layer was dried over MgSO$_4$ and filtered. Removal of the solvent under reduced pressure furnished an off-white residue that was purified by flash chromatography to give the product 11 (1.04 g, 5.00 mmol, 42% yield) as a white solid. $^1\text{H}$ NMR (CDCl$_3$, 400 MHz, 298 K): $\delta$ 6.52 (t, 2H, $J = 0.9$ Hz, CH$_{\text{vinyl}}$), 5.28 (t, 2H, $J = 0.9$ Hz, CHO), 3.78-3.76 (m, 2H, OCH$_2$), 3.90-3.87 (m, 2H, NCH$_2$), 2.90 (s, 2H, CH), 1.90 (bs, 1H, −OH). $^{13}\text{C}\{^1\text{H}\}$ NMR (CDCl$_3$, 100 MHz, 298 K): $\delta$ 177.0 (2C, CO), 136.6 (2C, CH$_{\text{vinyl}}$), 81.0 (2C, CHO), 60.2 (1C, CH$_2$O).
OCH$_2$), 47.5 (2C, CH), 41.8 (1C, NCH$_2$), ESI-MS (MeOH, +EI) m/z (relative intensity): 210 ([MH$^+$], 16), 142 (38), 111 (43), 110 (41), 98 (29), 82 (42), 68 (100).

2-Bromo-2-methyl Propionic Acid 2-(3,5-Dioxo-10-oxa-4-azatricyclo[5.2.1.0$^{2,6}$]dec-8-en-4-yl) Ethyl Ester (6): A solution of the alcohol 5 (2.22 g, 10.6 mmol) and Et$_3$N (1.60 mL, 11.7 mmol) in 120 mL of THF (the solution remained slightly turbid) was cooled to 0°C, and a solution of 2-bromo isobutyryl bromide (1.40 mL, 11.1 mmol) in 40 mL of THF was added dropwise (30 min). The white suspension was stirred for 3 h at 0°C and subsequently at ambient temperature overnight. TLC revealed the complete disappearance of the starting material. The ammonium salt was filtered off and the solvent removed under reduced pressure to give a pale-yellow residue that was purified by flash chromatography (CC, SiO$_2$, petroleum ether/ethyl acetate 1:1). We obtained 3.54 g (9.88 mmol, 93% yield) of 6 as a white solid. $^1$H NMR (CDCl$_3$, 400 MHz, 298 K): δ 6.49 (t, 2H, $J = 1.0$ Hz, CH$_2$vinyl), 5.23 (t, 2H, $J = 1.0$ Hz, CHO), 4.30 (t, 2H, $J = 5.3$ Hz, OCH$_2$), 3.78 (t, 2H, $J = 5.3$ Hz, NCH$_2$), 2.84 (s, 2H, CH), 1.86 (s, 6H, CH$_3$). $^{13}$C{1H} NMR (CDCl$_3$, 100 MHz, 298 K): δ 176.0 (2C, CO$_{imide}$), 171.5 (1C, CO$_{ester}$), 137.0 (2C, CH$_2$vinyl), 81.0 (2C, CHO), 62.3 (1C, OCH$_2$), 55.8 (1C, C(CH$_3$)$_2$Br), 47.6 (2C, CH), 37.6 (1C, NCH$_2$), 30.6 (2C, CH$_3$). ESI-MS (MeOH, +EI) m/z (relative intensity): 360 ([MH$^+$], 5), 358 [MH$^+$], 5), 292 (13), 290 (13), 151 (6), 149 (6), 210 (13), 191 (28), 124 (67), 123 (57), 110 (41), 69 (65), 68 (100).

2-Methyl-acrylic acid 2,2-dimethyl-[1,3]dioxolan-4-ylmethyl ester (7): In a three necked round-bottom flask, a mixture of (2,2-Dimethyl-[1,3]dioxolan-4-yl)-methanol (10.58 g, 53 mmol) and Et$_3$N (22. mL, 85 mmol) in 100 mL of anhydrous THF was cooled down to 0 °C under nitrogen atmosphere, and methacryloyl chloride (9.2 g, 88 mmol) was added dropwise. The mixture was allowed to warm to ambient temperature overnight and then stirred for 2 days at ambient temperature. Triethylamine hydrochloride salt was filtered off and the solvent removed under reduced pressure. The resulting viscous oil was dissolved in dichloromethane (100 mL), washed with a saturated NaHCO$_3$ solution (2 × 50 mL) and water (2 × 50 mL), and finally
dried over MgSO$_4$. After filtration, removal of the solvent under reduced pressure gave a yellow viscous oil which was distilled under vacuum in presence of Galvinoxyl$^\text{R}$ (radical inhibitor) to give the pure solketal methacrylate (10.25 g, 34 mmol, 64% yield) as a colourless oil. IR (neat): $\tilde{\nu} = 2987, 2888, 1718, 1638, 1454, 1371, 1320, 1296, 1157, 1083, 1054, 941, 845, 814, 733, 649$ cm$^{-1}$. $^1$H NMR (CDCl$_3$, 400 MHz, 298 K): $\delta$ 6.16-6.09 (m, 1H, C=CH$_2$H), 5.61-5.53 (m, 1H, C=CCHH), 4.37-4.24 (m, 1H, (CH$_2$)$_2$CHO), 4.22 (d, 2H, $J = 5.3$ Hz, O=CO−C$_2$H$_5$), 3.81 (dd, 1H, O−CH$_a$Hb−CH), 1.97 (s, 3H, CCH$_3$−C=CH$_2$), 1.45 (s, 3H, O−C(CCH$_3$)−O), 1.38 (s, 3H, O−C(CCH$_3$)−O).

$^{13}$C{1H} NMR (CDCl$_3$, 100 MHz, 298 K): $\delta$ 167.1 (1C, CO$_{\text{ester}}$), 135.9 (1C, CH$_3$−C=CH$_2$), 126.5 (1C, CH$_3$−C=CH$_2$), 109.8 (1C, C$^{\text{IV}}$), 74.0 (1C, CHO), 66.4 (1C, CHO−CH$_2$O), 64.7 (1C, CH$_2$−OCO), 26.7 (1C, CH$_3$_{ketal}), 25.4 (1C, CH$_3$_{ketal}), 18.3 (1C, CH$_3$=). ESI-MS (MeOH, +EI) m/z (relative intensity): 223 ([M+Na]$^+$, 4), 200 ([MH$^+$], 7), 160 (100), 133 (47), 86 (33).

2-Methyl-acrylic acid 3-trimethylsilanyl-prop-2-ynyl ester (8): A solution of trimethylsilyl propyn-1-ol (10.0 g, 78.0 mmol) and Et$_3$N (14.2 mL, 101.3 mmol) in Et$_2$O (100 mL) was cooled to -20°C and a solution of methacryloyl chloride (8.8 mL, 93 mmol) in Et$_2$O (50 mL) was added dropwise over ca. 1 h. The mixture was stirred at this temperature for 30 min, then at ambient temperature overnight; the ammonium salts were removed by filtration and the volatiles removed under reduced pressure. $^1$H NMR analysis of the yellow oily residue did not reveal the presence of substantial amount of any impurity, but two additional faint spots were observed by TLC (petroleum ether/Et$_2$O 20:1) analysis, the crude product was therefore purified by flash chromatography (CC, SiO$_2$, petroleum ether/Et$_2$O 50:1; $R_f = 0.67$ in petroleum ether/Et$_2$O 20:1). 12.4 g (63.2 mmol, 81% yield) of 8 were obtained as colourless liquid. IR (neat): $\tilde{\nu} = 2960, 1723, 1638, 1452, 1366, 1314, 1292, 1251, 1147, 1035, 971, 942, 842, 813, 761$ cm$^{-1}$. $^1$H NMR (CDCl$_3$, 400 MHz, 298 K) $\delta$ 6.14-6.12 (m, 1H, C=CH$_2$H), 5.59-5.58 (m, 1H, C=CCHH), 4.73 (s, 2H, OCH$_2$), 1.94-1.93 (m, 3H, CH$_3$C=CH$_2$), 0.16 (s, 9H, Si(CH$_3$)$_3$). $^{13}$C{1H} NMR (CDCl$_3$, 100 MHz, 298 K) $\delta$ 166.6 (1C, CO$_{\text{ester}}$), 135.8 (1C, CH$_3$C=CH$_2$), 126.5 (1C, CH$_3$C=CH$_2$), 99.2 (1C, C=CSi(CH$_3$)$_3$), 92.0 (1C, C=CSi(CH$_3$)$_3$), 53.0 (1C, OCH$_2$), 18.4 (1C, CH$_3$C=CH$_2$), -0.2 (3C, Si(CH$_3$)$_3$). ESI-MS (MeOH, +EI) m/z (relative intensity): 219 ([M+Na]$^+$, 100), 197 ([MH$^+$], 40).
Propyl-pyridin-2-ylmethylene-amine (10): A solution of 2-acetylpyridine (10 g, 82.5 mmol) in diethyl ether (50 mL) was placed in a Schlenk tube containing activated 3 Å molecular sieves under an atmosphere of dinitrogen. n-propylamine (8 mL, 97.3 mmol) was added and the reaction mixture stirred at room temperature for 18 h. The reaction was filtered, and then ether and excess n-propylamine were removed in vacuo. N-(n-Propyl)-2-pyridylethanimine was the second fraction recovered from the reaction mixture by vacuum distillation (5.5 g, 37 mmol, 45% yield). $^1$H NMR (CDCl$_3$, 400 MHz, 298 K): δ 8.40-8.37 (m, 1H, CH$_{\text{para}}$), 7.94-7.92 (m, 1H, CH$_{\text{meta}}$), 7.48-7.46 (m, 2H, CH$_{\text{ortho}}$), 7.05-7.02 (m, 1H, CH$_{\text{imine}}$), 3.42 (t, 2H, $J = 5.1$ Hz, NCH$_2$), 2.28 (s, 3H, CH$_3$), 1.72-1.66 (m, 2H, CH$_2$CH$_3$). ESI-MS (MeOH, +EI) m/z (relative intensity): 171 ([M+Na]$^+$, 2), 148 ([MH]$^+$, 3) 107 (100), 78 (11), 57 (47).

General ATRP mediated polymerization procedure

- Synthesis of polymer (5):
In a Schlenk tube, the initiator 6 (609 mg, 1.70 mmol), the solketal monomer 7 (9.99 g, 49.9 mmol), the fluorescent hostasol co-monomer 9 (236 mg, 0.499 mmol) and the N-(n-propyl)-2-pyridylmethanimine ligand 12 (488 mg, 3.29 mmol) were dissolved in anisole (20 mL). The mixture was subjected to 5 freeze-pump-thaw cycles and then cannulated into a second Schlenk tube containing a magnetic stirrer and Cu(I)Br (236 mg, 1.65 mmol), previously evacuated and filled with nitrogen (t = 0). The resulting mixture was stirred at ambient temperature (~20°C) and aliquots were removed at regular intervals of time in order to monitor the evolution of both the conversion ($^1$H NMR) and the molecular weight (SEC). At 54% conversion, the protected trimethylsilyl alkyne 8 was added as second monomer (3.0 g, 15 mmol). The reaction was stopped at 80% overall conversion. The reaction mixture was bubbled with air for 24 hours, passed through a neutral alumina pad, and precipitated by dropwise addition to a large amount of petroleum ether (~20:1 v/v ratio with respect to anisole). The pale orange powder was dissolved in the minimum amount of dichloromethane, passed through a short neutral alumina
pad (which was subsequently washed with additional dichloromethane) and precipitated again in petroleum ether to give, after filtration, the pure polymer 5 as a pale orange powder. GPC (THF/triethylamine: 95/5, 2 PL Gel mixed columns, 0.5 mL.min⁻¹): retention time 15.32 min, PDI 1.15. MALDI-TOF analysis (sinapinic acid): 11.5 kDa.

N.B.: Conversion of the solketal monomer was followed by ¹H NMR by comparison of the signals of the protons of the double bond with those of the methyl groups of the solketal.

Figure VI-13. ¹H NMR of polymer 5 in CDCl₃.

Synthesis of polymer (13) by deprotection of polymer 5: The polymer 5 (1.06 g, 0.0922 mmol) was dissolved in toluene (10 ml) and the solution refluxed overnight. The resulting polymer was precipitated in a large amount of petroleum ether (20:1 v/v with respect to the toluene) to afford a thin pale yellow powder. Volatiles were removed under reduced pressure to give the resulting maleimido-terminated polymer 13 in
close to 100% yield (1.05 g). GPC (THF/triethylamine: 95/5, 2 PL Gel mixed columns, 0.5 mL.min⁻¹): retention time 15.20 min, PDI 1.15. MALDI-TOF analysis (sinapinic acid): 11.5 kDa. ¹H NMR (Mₙ~11.5 kDa).

**Figure VI-14.** ¹H NMR of polymer 13 in CDCl₃.

**Synthesis of polymer (14) by deprotection of polymer 13:** An aqueous 1.0 M acetic acid solution (1.5 equiv. mol/mol with respect to the alkyne-trimethylsilyl groups, 1.11 mL, 1.11 mmol) was added to a solution of the maleimido deprotected polymer 13 (1.05 g, 0.0922 mmol) in THF (25 mL) in a round bottom flask. Nitrogen was bubbled through the solution (ca. 10 min) and the yellowish solution was cooled to -20°C. A 1.0 M solution of TBAF·3H₂O in THF (1.5 equiv. mol/mol with respect to the alkyne-trimethylsilyl groups, 1.11 mL, 1.11 mmol) was added dropwise over a period of ca 2-3 min. The mixture was stirred at this temperature for 30 min. and then at ambient temperature overnight. The resulting solution was passed through a silica pad (to remove the
excess of TBAF) and the pad washed with THF. Removal of the volatiles gave the product as brown oil. Finally, the polymer was precipitated in petroleum ether to give the pure polymer 14 as a fine yellow powder close to 100% yield (1.0 g). It was further characterized by GPC (PDI~1.15) and $^1$H NMR ($M_n$~11 kDa). GPC (THF/triethylamine: 95/5, 2 PL Gel mixed columns, 0.5 mL.min$^{-1}$): retention time 15.08 min, PDI 1.15. MALDI-TOF analysis (sinapinic acid): 11.5 kDa. $^1$H NMR ($M_n$~11.5 kDa).

![Image](image.png)

**Figure VI-15.** $^1$H NMR of polymer 14 in CDCl$_3$.

N.B.: Using the same experimental procedure as for the deprotection of 13, polymer 25 was obtained directly from the trimethylsilyl deprotection of polymer 5 (see $^1$H NMR on Figure VI-16).
Figure VI-16. $^1$H NMR of polymer 25 in CDCl$_3$.

Synthesis of polymer (4) by deprotection of polymer 14:
Polymer 14 (1.00 g, 0.0922 mmol) was dissolved in 1,4-dioxane (80 mL) in a round bottom flask and the resulting clear solution cooled to 0°C. The mixture became turbid upon addition of 33 mL of a 1 M HCl aqueous solution, and was subsequently allowed to slowly warm up to ambient temperature overnight and stirred for a further 24 hours. The reaction medium was neutralised by using a 1 M phosphate buffer pH 7.4 solution, at 0°C. Freeze-drying of the resulting mixture gave an orange powder which was dissolved in the minimum amount of dry methanol. Final filtration through a pad of neutral alumina, washing with dry methanol and removal of the solvent under vacuum afforded the final, totally deprotected polymer 4 as an orange powder (800 mg, 0.0842 mmol, yield = 91%).

GPC (THF/triethylamine: 95/5, 2 PL Gel mixed, 0.5 mL.min$^{-1}$): PDI~1.2. $^1$H NMR
Figure VI-17. $^1$H NMR of polymer 4 in CDCl$_3$.

1-azidodecane (15): To a 0.5 M solution of NaN$_3$ (0.325 g, 5.00 mmol) in DMSO (10 mL) was added 1-bromo-decane (0.44 g, 2.0 mmol) at ambient temperature. The solution was stirred for 24 hours at room temperature, then 10 mL of an aqueous 1M NaOH solution were added (the mixing was slightly exothermic). After cooling down to ambient temperature, the mixture was extracted with Et$_2$O. The organic layers were washed with brine, dried over MgSO$_4$, filtered, and the solvent removed under reduced pressure to afford 0.33 g of the pure 1-azidodecane 15 (4.5 mmol, 90% yield) as a colorless oil. IR (neat): $\tilde{\nu} = 2924, 2854, 2091$ (C-N$_3$ absorption band), 1466, 1348, 1259, 893, 721 cm$^{-1}$. $^1$H NMR (CDCl$_3$, 400 MHz, 298 K) $\delta$ 3.26 (t, $J = 7.0$ Hz, 2H, CH$_2$N$_3$), 1.65-1.56 (m, 2H, N$_3$CH$_2$CH$_2$), 1.20-1.40 (m, 14H, CH$_2$), 0.89 (t, $J = 6.8$ Hz, 3H, CH$_3$). $^{13}$C{[H]} NMR (CDCl$_3$, 100 MHz, 298 K) $\delta$ 51.5 (1C, CH$_2$-N$_3$), 31.9 (1C, CH$_2$), 29.5 (1C, CH$_2$), 29.3 (1C, CH$_2$), 29.2 (1C, CH$_2$), 28.9 (1C, CH$_2$), 26.9 (1C, CH$_2$), 26.7 (1C, CH$_2$),
22.7 (1C, CH₂), 14.1 (1C, CH₃). ESI-MS (MeOH, +EI) m/z (relative intensity): 206 [M+Na]⁺, <1), 184 ([MH⁺], <1), 155 (56), 140 (100).

1-(azidomethyl)benzene (16): In a round bottom flask, to a 0.5 M solution of NaN₃ (0.25 g, 3.8 mmol) in DMSO (8 mL) was added benzyl bromide (0.51 g, 3.0 mmol) at ambient temperature. The solution was stirred for 24 hours at ambient temperature and then quenched with 10 mL H₂O (reaction slightly exothermic). After cooling down to ambient temperature, the mixture was extracted with Et₂O. The organic layers were washed with brine, dried over MgSO₄, filtered off and the solvent removed under reduced pressure to afford the benzyl azide 16 pure product as pale yellow oil that was used for the click step without further purification. IR (neat): ν = 3032, 2929, 2089 (C-N₃ absorption band), 1738, 1496, 1455, 1349, 1252, 1202, 1078, 1029, 875, 735, 695 cm⁻¹. ¹H NMR (CDCl₃, 400 MHz, 298 K) δ 7.40-7.34 (m, 5H, CH aromatic), 4.36 (s, 2H, CH₂-N₃).

¹³C{¹H} NMR (CDCl₃, 100 MHz, 298 K) δ 138.9 (C IV), 128.9 (2C, CH ortho), 128.4 (2C, CH meta), 128.3 (1C, CH para), 54.9 (2C, CH₂). ESI-MS (Ether/MeOH : 1/1, +EI) m/z (relative intensity): 156 ([M+Na]⁺, <1), 134 ([MH⁺], 4), 105 (100).

1-(azidomethyl)-2,3,4,5,6-pentafluorobenzene (17): To a 0.5 M solution of NaN₃ (0.25 g, 3.8 mmol) in DMSO (8 mL) was added pentafluorobenzyl bromide (0.51 g, 3.0 mmol) at ambient temperature. The solution was stirred for 24 hours at ambient temperature and then quenched with 10 mL H₂O (reaction slightly exothermic). After cooling down to ambient temperature, the mixture was extracted with Et₂O. The organic layers were washed with brine, dried over MgSO₄, filtered off and the solvent removed under reduced pressure to afford the benzyl azide 17 pure product as pale yellow oil. IR (neat): ν = 3031, 2927, 2090 (C-N₃ absorption band), 1735, 1494, 1455, 1347, 1251, 1202, 1078, 1029, 873, 736, 696 cm⁻¹. ¹H NMR (CDCl₃, 400 MHz, 298 K) δ 2.6 (s, 2H, CH₂-N₃).

¹³C{¹H} NMR (CDCl₃, 100 MHz, 298 K) δ 140.4 (1C, C ortho), 138.3 (2C, C meta), 137.1 (2C, C para), 128.3 (C IV), 54.8 (1C, CH₂). ESI-MS (MeOH, +EI) m/z (relative intensity): 246 ([M+Na]⁺, 2), 224 ([MH⁺], 3), 195 (28), 181 (100).
5,10,15,20-tetraphenylporphyrin (18): Benzaldehyde (10.6 g, 0.1 mmol), acetic acid (180 mL) and acetic anhydride (20 mL) were refluxed. Pyrrole (6.7 g, 0.1 mmol), freshly distilled, was added as quickly as possible, to avoid creating an irreversible exothermic reaction. The resulting mixture was refluxing for 30 minutes. After cooling down to r.t., the resulting solution was filtered off. The solid was washed with MeOH and dried under vacuo to afford 0.75 g (5.10^{-3} mmol, 5% yield) of tetraphenylporphyrin 18 as a purple powder. $^1$H NMR (CDCl$_3$, 400 MHz, 298 K) $\delta$ 8.85 (s, 8H, H$_{\beta}$-pyrrole), 8.22 (dd, $J$ = 1.5 Hz and 7.6 Hz, 8H, CH$_{meta}$), 7.77 (m, 12H, CH$_{ortho/para}$), -2.78 (s, 2H, NH$_{pyrrole}$). ESI-MS (CH$_2$Cl$_2$/MeOH : 95/5, +EI) m/z (relative intensity): 615 ([MH$^+$], 100).

5-(4-Nitrophenyl)-10,15,20-triphenylporphyrin (19): Sodium nitrite (25 mg, 0.36 mmol) was added to a solution of 5,10,15,20-tetraphenylporphyrin (18) (123 mg, 0.20 mmol) in 12 mL of TFA. The resulting solution was stirred for 3 minutes and then poured into water (120 mL). The aqueous layer was extracted with DCM and joined organic fractions were washed with 30 mL of a NaHCO$_3$ saturated solution, 30 mL of water, dried over Na$_2$SO$_4$ and evaporated. Final chromatography column on silica gel eluting with DCM afforded the pure product as a purple powder (60 mg, 0.09 mmol, 45% yield). IR (CHCl$_3$): $\tilde{\nu}$ = 1597, 1521, 1474, 1348, 966 cm$^{-1}$. $^1$H NMR (CDCl$_3$, 400 MHz, 298 K) $\delta$ 8.86 (d, 2H, $J$ = 5.0 Hz, H$_{\beta}$-pyrrole), 8.85 (s, 4H, H$_{\beta}$-pyrrole), 8.69 (d, 2H, $J$ = 5.0 Hz, H$_{\beta}$-pyrrole), 8.54 (d, 2H, $J$ = 8.9 Hz, H$_{nitrophenyl}$), 8.31 (d, 2H, $J$ = 8.9 Hz, H$_{nitrophenyl}$), 8.19-8.05 (m, 6H, H$_{orthotriphenyl}$), 7.71-7.53 (m, 9H, H$_{meta/para triphenyl}$), -2.74 (s, 2H, NH$_{pyrrole}$). ESI-MS (CH$_2$Cl$_2$/MeOH : 95/5, +EI) m/z (relative intensity): 660 ([MH$^+$], 100), 613 (14), 535 (2.5), 330 (26), 306 (21).
5-(4-Aminophenyl)-10,15,20-triphenylporphyrin (20): The para-nitrophenyltriphenylporphyrin (19) (330 mg, 0.5 mmol) was dissolved in 50 mL concentrated HCl solution (37%). To this solution was added SnCl₂, 2H₂O (650 mg, 0.94 mmol) and the resulting mixture was stirred at 70°C for 1h30. The solution was poured into ice-cooled water, neutralized with KOH until pH~8 and extracted with DCM until the aqueous layer became colourless. Joined organic fractions were washed with water until observation of neutral pH, dried over MgSO₄ and evaporated. Final chromatography column on silica gel eluting with DCM afforded the pure product as a purple powder (144 mg, 0.22 mmol, 44% yield). $R_f = 0.46$ in DCM. $^1$H NMR (CDCl₃, 400 MHz, 298 K) $\delta$ 8.95 (d, 2H, $J = 5.0$ Hz, H β-pyrrole), 8.84 (d, 2H, $J = 5.0$ Hz, H β-pyrrole), 8.32-8.08 (m, 6H, H ortho triphenyl), 8.00 (d, 2H, $J = 8.2$ Hz, H aminophenyl), 7.76-7.57 (m, 9H, H meta/para triphenyl), 7.07 (d, 2H, $J = 8.3$ Hz, H aminophenyl), 4.03 (s, 2H, NH₂), -2.77 (s, 2H, NH pyrrole). ESI-MS (CH₂Cl₂/MeOH : 95/5, +EI) m/z (relative intensity): 630.5 ([MH⁺], 5), 212 (0.5), 207 (45), 149 (100).

2-(2-(2-azidoethoxy)ethoxy)ethanol (21): A solution of 1.1 g (7.33 mmol) of dry triethylene glycol, 1 mL of dry TEA, and 10 mL of dry ether was cooled down to 0°C under a nitrogen atmosphere. Methanesulfonyl chloride (0.42 g, 3.665 mmol) was added over a 1-hour period, after which the solution was allowed to warm slowly to room temperature overnight. The reaction contents were concentrated in vacuo, and 15 mL of 95% ethanol and 0.524 g (8.06 mmol) of sodium azide were added. The mixture was heated at reflux for 24 h, cooled down to ambient temperature, and concentrated in vacuo. The remaining mixture was diluted with 10 mL of ether, washed with 5 mL brine, and dried over MgSO₄. Concentration in vacuo afforded the crude product, which was purified by silica gel chromatography eluting with a gradient of 1:1 to 3:1 ethyl acetate/cyclohexane to afford approximately 565 mg (3.22 mmol, 44% yield) of pure compound 21 as a slight yellow oil. IR (neat): $\tilde{\nu} = 3597, 3062, 2873, 2111$ (C–N₃ absorption band), 1737, 1455, 1346, 1267, 1121, 1062, 930, 888, 715 cm⁻¹. $^1$H NMR (CDCl₃, 400 MHz, 298 K): $\delta$ 3.80-3.60 (m, 8H, –CH₂O–), 3.59 (t, 2H, $J = 5.1$ Hz, CH₂OH), 3.37 (t, 2H, $J = 5.3$ Hz, CH₂-N₃). $^{13}$C(1H) NMR (CDCl₃, 100 MHz, 298 K) $\delta$ 72.7
4-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)-4-oxobutanoic acid (22): To a solution of 0.2 g (1.14 mmol) of 2-(2-(2-azidoethoxy)ethoxy)ethanol (21) in dry toluene (2 mL) was added 0.447 g (4.57 mmol) of succinic anhydride. The resulting mixture was refluxing for 24 hours. The reaction contents were concentrated under vacuo and the crude was purified by silica gel chromatography eluting with a mixture 1:2 ethyl acetate/cyclohexane to afford the pure product 22 as a white viscous solid (292 mg, 1.06 mmol, 93% yield). IR (neat): $\tilde{\nu}$ = 2929, 2108 (C–N$_3$ absorption band), 1736, 1270, 1265, 1262, 1134, 716 cm$^{-1}$. $^1$H NMR (CDCl$_3$, 400 MHz, 298 K) $\delta$ 4.27 (t, 2H, J = 4.2 Hz, CH$_2$−OCO−), 3.60-3.80 (m, 8H, −CH$_2$O−), 3.41 (t, 2H, J = 5.1 Hz, CH$_2$−N$_3$), 2.67 (s, 4H, CO−CH$_2$CH$_2$−COOH). $^{13}$C{$^1$H} NMR (CDCl$_3$, 100 MHz, 298 K): $\delta$ 176.6 (1C, CO$_{acid}$), 172.1 (1C, CO$_{ester}$), 70.8 (1C, CH$_2$), 70.5 (1C, CH$_2$), 70.1 (1C, CH$_2$), 69.1 (1C, CH$_2$), 63.9 (1C, CH$_2$−OCO), 50.7 (1C, CH$_2$−N$_3$), 29.1 (1C, CH$_2$COOH), 28.9 (1C, CH$_2$COO−). ESI-MS (MeOH, +EI) m/z (relative intensity): 298 ([M+Na]$^+$, 93), 276.1 ([MH]$^+$, 100), 175 (20), 117 (31).

$N$-[4-(10,15,20-Triphenylporphyrin-5-yl)-phenyl]-succinamic acid 2-[2-(2-azidoethoxy)-ethoxy]-ethyl ester (23): To a solution of the acid 22 (37.7 mg, 0.137 mmol) in 5 mL of dry DCM at 0°C was added under a nitrogen atmosphere thionyl chloride (1 mL, 13.7 mmol) and DIPEA. The reaction mixture was let to warm slowly to ambient temperature under N$_2$ atm.
Then, the volatiles were evaporated to dryness and the resulting solid subsequentially redissolved in 5 mL dry DCM. Finally was added the porphyrin 20 (26 mg, 0.033 mmol) and the reaction mixture was stirred overnight. The reaction was poured in a mixture 1 / 1 (v / v): half saturated NH$_4$Cl aqueous solution / DCM. The organic layer was washed with half saturated NH$_4$Cl and water, dried over Na$_2$SO$_4$, filtered and concentrated under vacuum. TLC showed the formation of the product but also non-reacted starting tetraphenyl porphyrin amine (ESI-MS of the reaction mixture confirms this result). The crude was purified by column chromatography using a gradient CH$_2$Cl$_2$ to CH$_2$Cl$_2$/MeOH (95/5). Finally, crystallization in a mixture DCM/pentane gave the pure product as a purple powder (26 mg, 0.031 mmol, 94% yield). IR (CH$_2$Cl$_2$): $\tilde{\nu}$ = 3032, 2929, 2089 (C-N$_3$ absorption band), 1738, 1496, 1455, 1349, 1252, 1202, 1078, 1029, 875, 735, 695 cm$^{-1}$. $^1$H NMR (CDCl$_3$, 400 MHz, 298 K): $\delta$ 8.95 (d, 8H, $J$ = 2.3 Hz, H$_\beta$-pyrrole), 8.84 (d, 6H, $J$ = 6.3 Hz, H$_{ortho}$ triphenyl), 8.13 (d, 2H, $J$ = 7.6 Hz, =CH$_{amidophenyl}$), 7.95 (bs, 1H, NH), 7.85-7.65 (m, 9H, H$_{meta/para}$ triphenyl), 7.69 (d, 2H, 6.8 Hz, =CH$_{amidophenyl}$), 4.28 (t, 2H, $J$ = 4.3 Hz, CH$_2$OCO), 3.80-3.50 (m, 8H, O−C$_2$H$_4$C$_2$−O), 3.27 (t, 2H, $J$ = 5.1 Hz, CH$_2$N$_3$), 2.70 (s, 2H, CH$_2$COO), 2.54 (s, 2H, CH$_2$CO−N), -2.74 (s, 2H, NH$_{pyrrole}$). ESI-MS (CH$_2$Cl$_2$/MeOH : 95/5, +EI) m/z (relative intensity): 887 ([MH$^+$], 100), 861 (7), 859 (10), 744 (70).

2-(2-(2-azidoethoxy)ethoxy)ethyl 2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)acetate (24): To a solution of thymine acid (150 mg, 0.814 mmol) in 5 mL of dry DMF was added under a nitrogen atmosphere HBTU (370 mg, 0.977 mmol) and DIPEA (1 mL, 5.74 mmol). The reaction mixture was stirred under N$_2$ atm. at r.t. for 1 hour. Then were added 285 mg (1.628 mmol) of compound 21 and the reaction mixture was stirred for an additional hour. The reaction contents were concentrated to dryness, and the crude (yellowish oil) was purified by chromatography column eluting with a mixture DCM/MeOH: 9/1 to afford 24 in quantitative yield. $^1$H NMR (CDCl$_3$, 400 MHz, 298 K) $\delta$ 9.83 (bs, 1H, NH), 7.02 (d, 1H, $J$ = 1.2 Hz), 4.47 (s, 2H), 4.31 (t, 2H, $J$ = 4.62 Hz), 3.76-3.56 (m, 8H), 3.38 (t, 2H, $J$ = 4.99 Hz), 1.88 (s, 3H). $^{13}$C{1H} NMR (CDCl$_3$, 100 MHz, 298 K) $\delta$ 167.8 (1C, CO$_{ester}$), 164.7 (1C, CO$_{amide}$), 151.2 (1C, CO$_{amide}$), 140.8 (1C, CH=), 110.1 (C$^{IV}$), 70.5 (1C, CH$_2$), 70.0 (1C, CH$_2$), 68.8 (1C, CH$_2$), 65.0 (1C, CH$_2$), 55.4 (1C, CH$_2$), 50.7 (1C, CH$_2$-N$_3$), 48.6 (1C, N-CH$_2$), 12.2
(1C, CH$_2$-C=). ESI-MS (MeOH, +EI) m/z (relative intensity): 364 ([M+Na]$^+$, 23), 341 [MH$^+$], 54), 313 (45), 183 (100), 158 (11).

[3+2] Huisgen cycloaddition (“MultiClick” chemistry reaction) of azide 15, 16, 17 or 23 on polymer 25 to prepare polymer 26a, 26b, 26c, 26d:

General procedure with 1-azido-decane 15: A solution of polymer 25 (22 mg, 1.3.10$^{-3}$ mmol) in 100 µL of dimethylsulfoxide was added to 900 µL of a 20 mM phosphate buffer (pH 7.4). The yellowish suspension was sonicated before the addition of 1-azido-decane 15 (9.7 mg, 5.3.10$^{-2}$ mmol). Sodium ascorbate (2.5 mg, 1.3.10$^{-2}$ mmol) and CuSO$_4$ (1 mg, 6.10$^{-3}$ mmol) were sequentially added. The mixture was stirred at ambient temperature for 24 hours in the dark and the resulting greenish slurry was extracted with dichloromethane (3 × 3 mL). The organic fractions, collected, were passed through a pad of neutral alumina that was subsequently eluted with dichloromethane and then with THF. The final product was isolated by removal of the solvents under reduced pressure. The final pure product was subsequentially analyzed by GPC, $^1$H NMR, and MALDI-TOF analysis.
N.B.: Using the same experimental, Benzyl azide 16 (9.7 mg, 5.3.10^{-2} mmol), pentafluorobenzyl azide 17 (9.7 mg, 5.3.10^{-2} mmol) and the thymine derivative 24 were clicked onto the polyalkyne 25 to afford the corresponding polymers 26b, 26c, 26d.

Characterization of 26a: GPC (toluene, 2 PL Gel mixed, 0.5 mL.min^{-1}): retention time: 14.88 min., PDI~1.2 (Figure VI-18). ^1H NMR (Figure VI-19). MALDI-TOF-MS (THF/water: 1/1), \(M_w = 11\) kDa.

Characterization of 26b: GPC (toluene, 2 PL Gel mixed, 0.5 mL.min^{-1}): retention time: 14.98 min., PDI~1.2 (Figure VI-18). ^1H NMR (Figure VI-20). MALDI-TOF-MS (THF/water: 1/1), \(M_w = 10.6\) kDa.

Characterization of 26c: GPC (toluene, 2 PL Gel mixed, 0.5 mL.min^{-1}): retention time: 15.00 min., PDI~1.2 (see Figure VI-18). ^1H NMR (Figure VI-21). MALDI-TOF-MS (THF/water: 1/1), \(M_w = 11.4\) kDa.

Characterization of 26d: GPC (toluene, 2 PL Gel mixed, 0.5 mL.min^{-1}): retention time: 14.80 min., PDI~1.2 (see Figure VI-18). ^1H NMR (not possible because of copper (II) salts complexation on porphyrinic sites). MALDI-TOF-MS (THF/water: 1/1), \(M_w\) not obtained.

Figure VI-18. GPC traces of protected polyalkyne 25 (black curve) and purified clicked products 26a, 26b, 26c, 26d.
Figure VI-19. $^1$H NMR of polymer 26a in CDCl$_3$.

Figure VI-20. $^1$H NMR of polymer 26b in CDCl$_3$. 
Chapter VI – Experimental Part

Figure VI-21. $^1$H NMR of polymer 26c in CDCl$_3$.

Synthesis of the BSA-PA bioconjugate (VII): A total of 1.9 mL of a 0.3 mM solution of Bovine Serum Albumin (BSA) in 20 mM phosphate buffer (PB) pH 7.4 was added to a solution of 360 µL of 20 mM PB pH 7.4 and 112 µL of a 50 mM solution of the polymer 14 in the same buffer. The reaction mixture was stirred gently at 7°C for 1 day. Removal of the unreacted polymer was achieved by filtration of the reaction mixture using Microcon® Centrifugal Filter Units (Millipore MWCO 30 kDa). Isolation of the biohybrid VII from the unreacted BSA was carried out on a Superdex 150 column eluting with 20 mM phosphate buffer pH 7.4. The enriched fractions were freeze-dried and analyzed by SEC (mobile phase: 70% 10 mM phosphate buffer pH 7.4, 30% CH$_3$CN, retention time: 18.24 min., Figure VI-23, red trace), electrophoresis under native and denaturing conditions (both after visualization under the UV lamp at 366 nm or after Coomassie Blue staining, Figure VI-22) and MALDI-TOF analysis (Figure VI-24, MW~66.6 kDa (unreacted BSA) and 74.9 kDa (BSA-polyalkyne VII conjugate). Samples of the pure biohybrid were used for the coupling experiments and imaging with TEM (no aggregation observed) and confocal microscopy (Figure VI-27, C).
Figure VI-22. SDS-PAGE analysis of native BSA (lane 1) and the bioconjugation reaction mixture of BSA and polymer 4 (lane 2) after Coomassie Blue staining (left) and visualization under the UV lamp at 366 nm (right).

Figure VI-23. SEC traces at 466 nm of native BSA (black trace), the BSA-polyalkyne VII conjugation reaction mixture (red trace) and polyalkyne 4 (green trace).

Figure VI-24. MALDI-TOF analysis of the bioconjugation reaction mixture between native BSA and polyalkyne 4.
General procedure for the preparation of compounds VIIIa, VIIIb, VIIIc:

Synthesis of VIIIa: 48 µL of 1-azido-decane were added to 166 µL of 20 mM phosphate buffer pH 7.4 and the resulting biphasic mixture was sonicated for ca. 10 minutes. Subsequently 250 µL of a 0.24 mM solution of BSA-polyalkyne VII in 20 mM phosphate buffer pH 7.4 were added. Finally 12 µL of a 100 mM sodium ascorbate solution in 20 mM PB pH 7.4 and 24 µL of a 100 mM solution of CuSO₄ in PB 20 mM pH 7.4 were added. The reaction mixture was stirred gently for 2 days at 7°C, in the dark. The final Giant Amphiphiles VIIIa were characterized by electrophoresis (Figure VI-25, lane 3) and their aggregation patterns imaged with TEM (Figure VI-26, A.) and confocal microscopy (Figure VI-27, A.).

Synthesis of VIIIb: 35 µL of azidomethyl-benzene were added to 179 µL of 20 mM phosphate buffer pH 7.4, the resulting biphasic mixture was sonicated for ca. 10 min. and was “clicked” to BSA-polyalkyne VII using the same conditions as for compound VIIIa. The final Giant Amphiphiles VIIIb were characterized by electrophoresis (Figure VI-25, lane 4) and their aggregation patterns imaged with TEM microscopy (Figure VI-26, B.).

Synthesis of VIIIc: 58 µL of azidomethyl-pentafluorobenzene were added to 156 µL of 20 mM phosphate buffer pH 7.4, the resulting biphasic mixture was sonicated for ca. 10 min. and was “clicked” to BSA-polyalkyne VII using the same conditions as for compound VIIIa. The final Giant Amphiphiles VIIIc were characterized by electrophoresis (Figure VI-25, lane 5) and their aggregation patterns imaged with TEM microscopy (Figure VI-26, C.).

Figure VI-25. SDS-PAGE analysis of native BSA, BSA-polyalkyne VII and clicked amphiphilic bioconjugates: lane 1, native BSA; lane 2, BSA-polyalkyne VII; lane 3, BSA-PA@C₁₀H₂₁ VIIIa; lane 4, BSA-PA@Bz VIIIb; lane 5, BSA-PA@F₃Bz VIIIc.
Figure VI-26. TEM micrographs of BSA-PA@C₁₀H₂₁N₃ VIIIa (line A), TEM micrographs of BSA-PA@Bz VIIIb (line B), TEM micrographs of BSA-PA@F₃Bz VIIIc (line C).

Figure VI-27. CFM images of (A). BSA-PA@C₁₀H₂₁N₃ VIIIa aggregates (excitation with 514 Argon-Krypton laser line), (B). a BSA-PA@C₁₀H₂₁N₃ VIIIa emulsion in a water / ethanol / decane mixture and (C). a BSA-PA VII solution.
Chapter IV. *In Situ*, ATRP Mediated Hierarchical Formation of Bio Nanoreactors

2-Bromo-2-methyl-propionic acid 2-(2,5-dioxo-2,5-dihydropyrrol-1-yl)-ethyl ester (27): A solution of the maleimido-protected initiator 6 (0.15 g, 0.419 mmol) was suspended in dry toluene (5 mL) and heated to reflux under nitrogen atmosphere for 8 hours. The solvent was removed under reduced pressure to give a pale-yellow residue which was subsequently purified by flash chromatography (SiO$_2$, petroleum ether/ethyl acetate 4:1) to furnish the compound 27 as a white solid (0.109 g, 0.38 mmol, 90% yield). $^1$H NMR (400 MHz, CDCl$_3$, 298 K): $\delta$ 6.72 (d, $J = 1.0$ Hz, 2H, CH$_{\text{vinyl}}$), 4.32 (t, $J = 5.3$ Hz, 2H, OCH$_2$), 3.84 (t, $J = 5.3$ Hz, 2H, NCH$_2$), 1.88 (s, 6H, CH$_3$). ESI-MS (MeOH, +EI) m/z (relative intensity): 292 ([MH$^+$], 25), 290 ([MH$^+$], 29), 211 (100).

Prop-2-ynyl methacrylate (28): 3-(trimethylsilyl)prop-2-ynyl methacrylate (8) (500 mg, 2.55 mmol) was dissolved in THF (25 mL) and a 1 M acetic acid solution (3.825 mL, 3.825 mmol) was added. The resulting solution was cooled down to -20°C with an ice-acetone bath and nitrogen was bubbled in for ca. 10 min. before the addition of TBAF, 3H$_2$O (1.207 g, 3.825 mmol). The resulting solution was let to warm to r.t. overnight. The solution was passed through a pad of silica and the pad was washed with another 25 mL of THF. Removal of the volatiles under reduced pressure afforded the pure product 28 as colourless oil (307 mg, 2.47 mmol, 97% yield). $^1$H NMR (CDCl$_3$, 400 MHz, 298 K) $\delta$ 6.17-6.15 (m, 1H, C=CH$_2$), 5.59-5.58 (m, 1H, C=CH$_2$), 4.63 (d, 2H, $J = 2.5$ Hz, OCH$_2$), 2.52 (t, 1H, $J = 2.3$ Hz, C=CH$_2$), 1.94-1.93 (m, 3H, CH$_3$C=CH$_2$), $^{13}$C$[1H]$ NMR (CDCl$_3$, 100 MHz, 298 K) $\delta$ 18.4 (1C, CH$_3$C=CH$_2$), 53.0 (1C, OCH$_2$), 92.0 (1C, C=CH), 99.2 (1C, C=CH), 126.5 (1C, CH$_3$C=CH$_2$), 135.8 (1C, CH$_3$C=CH$_2$), 166.6 (1C, CO$_{\text{ester}}$). ESI-MS (MeOH, +EI) m/z (relative intensity): 147 ([M+Na]$^+$, 100), 125 ([MH$^+$], 41), 55 (31).
(1-(perfluorobenzyl)-1H-1,2,3-triazol-4-yl)methyl methacrylate (29): 300 mg (2.42 mmol) of compound 28 and 540 mg (2.42 mmol) of compound 17 were dissolved in a 1:1 mixture t-BuOH/water (10 mL). Upon addition of CuSO$_4$ (19 mg, 0.121 mmol) and L-(-) ascorbic acid sodium salt (48 mg, 0.242 mmol), the resulting mixture turned orange-brown. After stirring for 24 hours, the resulting solution was extracted with ether. Joined organic fractions were washed over MgSO$_4$ and the solvent removed under vacuo. Final chromatography column on silica gel eluting with DCM afforded 706 mg (2.03 mmol, 84% yield) of the resulting clicked product 29. $^1$H NMR (CDCl$_3$, 400 MHz, 298 K) $\delta$ 7.28 (s, CH$_{\text{triazole}}$), 6.16-6.14 (m, 1H, C=CHH), 5.59-5.58 (m, 1H, C=CHH), 5.71 (s, 2H, NCH$_2$), 5.12 (s, 2H, OCH$_2$), 1.94-1.93 (m, 3H, CH$_3$CH$_2$). $^{13}$C{1H} NMR (CDCl$_3$, 100 MHz, 298 K) $\delta$ 166.3 (1C, CO$_{\text{ester}}$), 144.8 (1C, C$_{\text{IV}}$), 141.2 (1C, C$_{\text{para}}$), 138.7 (2C, C$_{\text{ortho}}$), 137.4 (2C, C$_{\text{meta}}$), 135.8 (1C, CH$_3$(C=CH)$_2$), 126.5 (1C, CH$_3$(CH$_2$)$_2$), 122.3 (1C, NCH=C), 111.4 (1C, C$_{\text{IV}}$), 64.8 (1C, OCH$_2$), 35.4 (1C, NCH$_2$), 18.4 (1C, CH$_3$(CH$_2$)$_2$). ESI-MS (Ether/MeOH : 1/1, +EI) m/z (relative intensity): 370 ([M+Na]$^+$, 3), 348 ([MH]$^+$, 41), 280 (100).

**Preparation of Bovine Serum Albumin Macorinitiator IX:** A solution of the maleimide functionalized ATRP initiator 27 (126 mM, 0.8 mL) in DMSO was slowly added to 9.0 mL of a 0.35 mM solution of native BSA in 20 mM phosphate buffer (pH 7.4). The reaction mixture was gently shaken for 48 hours at 7°C. To eliminate the excess of the ATRP initiator 27, the mixture was then extensively dialyzed initially against 2% EDTA, 10% DMSO in 20 mM phosphate buffer pH 7.4 and then against 20 mM phosphate buffer pH 7.4 using 10 kDa regenerated cellulose dialysis membranes. The resulting solution of BSA-macroinitiator IX was subsequently analyzed by aqueous SEC to confirm that the excess of the initiator 27 was removed. Samples were prepared by dissolving 50 µL of the BSA-macroinitiator IX solution in 1 mL of 0.1% TFA, 5% DMSO, 30% MeCN in nanopure water. The BSA-macroinitiator IX was freeze-dried prior to its ATRP mediated in situ polymerization. Samples of freeze-dried bioconjugate IX could be stored at -20°C and successfully utilized after prolonged periods of time (up to 3 months). ATRP-macroinitiator was characterized by native gel electrophoresis (see Figure VI-28, left), SEC-HPLC (solvent: 70 % phosphate buffer 5 mM pH 7.4, 30 % acetonitrile, room temperature, column: SEC-300 BioBasic, flow rate: 0.5
mL.min\(^{-1}\), see Figure VI-28 right, retention time: 13.77 min.) and MALDI-TOF-MS (solvent: water, see Figure VI-29, MW~66.7 kDa)

![Native gel electrophoretic profile and SEC-HPLC chromatographic traces](image1)

**Figure VI-28.*** Left: Native gel electrophoretic profile of native BSA (lane 1), and the purified reaction mixture after conjugation of BSA with the ATRP initiator 27 (lane 2). Right: SEC-HPLC chromatographic traces of native BSA (solid black trace) and BSA-macroinitiator IX (dashed grey trace).

![MALDI-TOF analysis](image2)

**Figure VI-29.*** MALDI-TOF analysis of native BSA (grey) and BSA macroinitiator IX (black).
General procedure for the determination of free thiols by Ellman’s assay: 4 mg of 5,5’-dithio-bis-(2-nitrobenzoic acid) (Ellman’s reagent) was dissolved in 1 mL of buffer solution (0.1 M sodium phosphate buffer pH 8.0, containing 1 mM EDTA) to prepare Ellman’s reagent solution (10.09 mM). 0.25 µL of a BSA or BSA-macroinitiator IX conjugate solution (0.3 mM), 50 µL of Ellman’s reagent and 2.5 mL of buffer solution were mixed for 15 min. at room temperature. The absorbance at 412 nm was measured by a UV-vis spectrophotometer. The thiol concentration was calculated using the Beer-Lambert’s law (molar extinction coefficient of 2-nitro-5-thiobenzoic acid = 14,150 M⁻¹.cm⁻¹ at 412 nm). The initiator itself does not absorb in UV at 412 nm and does not affect the Ellman’s assay. Native BSA, before the conjugation with the initiator, was found to contain 47% free thiols, whereas after conjugation, no free thiols were detected.

General polymerization procedure with styrene (X-XIV): Several sets of polymerization experiments were performed varying the ratio between the monomer (styrene) and the BSA macroinitiator IX (Table 1). The ratio BSA-macroinitiator IX : CuBr : ligand was kept constant at 1 : 41 : 70 in all experiments. Styrene (5 µL, 44 µmol, 50 equiv. up to 500 µL, 4.4 mmol, 5000 equiv., see Table I) and N-(Propyl)-2-pyridylmethanimine (9.1 mg, 0.036 mmol, 70 equiv.) were placed in a Schlenk tube and dissolved in solvent (10% DMSO in 20 mM phosphate buffer pH 7.4, 10 mL). The mixture was deoxygenated by 5 freeze-pump-thaw cycles and sonicated for 5 minutes to emulsify the monomer. The beginning of the polymerization was triggered by the canulation of the monomer solution under nitrogen atmosphere in a second deoxygenated Schlenk tube containing the crystalline BSA-macroinitiator IX (~59 mg, 0.88 µmol, 1 equiv.) and CuBr (5.2 mg, 36 µmol, 41 equiv.) under N₂ atmosphere. A dark brown colour was immediately observed. The reaction mixture was stirred under inert atmosphere during 2 up to 24 h after which they were exposed to oxygen (Cu(I) to Cu(II)) and stirred for another 2 to 12 hours.

Several control experiments in the absence of the biomacroinitiator IX, or the monomer or Cu(I) and in the presence of oxygen were performed and are also listed in Table 1.
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Table 1. Polymerization experiments with varying ratios between the monomer (styrene) and the BSA macroinitiator IX, and control experiments.

<table>
<thead>
<tr>
<th>#</th>
<th>Styrene</th>
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<th>BSA macroinitiator IX</th>
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<th>O2</th>
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<tr>
<td>1</td>
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<td>1</td>
<td></td>
<td></td>
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<tr>
<td>5</td>
<td>3000</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2000[\text{a}]</td>
<td>0 (native BSA)</td>
<td></td>
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</tbody>
</table>

The crude polymerization mixtures were purified by dialysis initially against 2% EDTA 10% DMSO in 20 mM phosphate buffer pH 7.4 and then against 20 mM phosphate buffer pH 7.4 or milliQ (depending on the analyses to perform) using 10 or 25 kDa MWCO regenerated cellulose membranes to eliminate the excess of the monomer and polymerization reagents.

Figure VI-30. SEC-HPLC traces of different blank experiments performed under standard ATRP \textit{in situ} polymerization conditions. Traces obtained when the reaction was performed in the absence of the biomacroinitiator IX (blue), absence of Cu (green) or in the presence of oxygen (dark red).

Characterization of samples X to XIV: All samples were further analyzed by MALDI-TOF (solvent: water, see Figure VI-34, MW from 68 up 80 kDa), native gel electrophoresis (Figure
VI-3) and aqueous SEC HPLC (see Figure VI-31 and Figure VI-32) and their aggregation studied by TEM microscopy (Figure VI-35) while, upon protein hydrolysis, the resulting polymers were analyzed by $^1$H NMR (Figure VI-36) and MALDI-TOF (Figure VI-37).

**Figure VI-31.** SEC chromatographic traces of BSA macroinitiator IX (blue trace) and samples of the different *in situ* styrene polymerization experiments after purification using “low” monomer to BSA macroinitiator IX ratio (ratio 50:1, black trace; ratio 500:1, red trace).

**Figure VI-32.** SEC chromatographic traces of BSA macroinitiator IX and the different samples from *in situ* styrene polymerization experiments after purification using “high” monomer to BSA macroinitiator IX ratio (XII ratio 1500:1, red trace; XIII ratio 2000:1, blue trace; and XIV ratio 3000:1, orange trace).
Figure VI-33. Native gel electrophoretic profile after Coomassie Brilliant Blue staining (A) and Silver staining (B) of native BSA, BSA macroinitiator IX, and different samples obtained by the in situ ATRP mediated polymerization of styrene on the BSA macroinitiator IX. The content of each lane is summarized on the right.
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Figure VI-34. MALDI-TOF analysis of BSA-macroinitiator IX (red trace) and the products of selected ATRP mediated in situ polymerization reactions of styrene over IX. The blue trace corresponds to styrene / IX ratio 500:1 and the green trace to 2000:1 ratio.

Isolation of Polystyrene (BSA digestion): Polymerization samples (400 µL from both non-purified crude polymerization mixtures and purified bioconjugate solutions obtained after extensive dialysis) were incubated at 80°C with 6 N HCl for ~8 hours. The resulting mixtures were neutralized and polymers were then extracted in CH₂Cl₂ and analyzed by MALDI-TOF (Figure VI-37, black trace) and ¹H-NMR spectroscopy (Figure VI-36). No polystyrene was detected in the digested control experiment samples. The results of the digestion of crude and dialyzed bioconjugate solutions were in full agreement.
Figure VI-36. $^1$H-NMR analysis of the polystyrene isolated from hydrolysis of BSA-polystyrene XIII.

Figure VI-37. Comparison of MALDI-TOF spectra of polystyrene isolated from the BSA-polystyrene conjugate XIII (black trace) and a standard polystyrene (PDI 1.13, grey trace).

**General polymerization procedure with monomer 8 (XV):** Trimethyl silyl protected alkyne monomer 8 (238 mg, 1.215 mmol) and N-(Propyl)-2-pyridylmethanimine (~8 mg, 0.057 mmol) were placed in a Schlenk tube and dissolved in solvent (10% DMSO in 20 mM phosphate buffer pH 7.4, 10 mL). The mixture was deoxygenated by 5 freeze-pump-thaw cycles. Then, the mixture was canulated in another Schlenk tube containing BSA macrorinitiator IX (~62 mg, 0.93 µmol, 1 equiv.) and CuBr (4.7 mg, 33 µmol) (previously degassed) under N$_2$ atmosphere, which triggered the beginning of the polymerization (a dark brown colour of the reaction medium was observed). The mixture was stirred under inert atmosphere during 72 h. The reaction mixture was then dialyzed using a 25 kDa MWCO membrane initially against 2% EDTA 10% DMSO 20 mM PB and then against 20 mM PB pH 7.4. Resulting solutions were analyzed by means of SEC-HPLC (Figure VI-38), and MALDI-TOF analysis.
General polymerization procedure with monomer 8 in the presence of carboxyfluorescein (XVI): Trimethyl silyl protected alkyne monomer 8 (238 mg, 1.215 mmol), carboxy fluorescein (3.2 mg, 0.01 mmol) and N-(Propyl)-2-pyridylmethanimine (~8 mg, 0.057 mmol) were placed in a Schlenk tube and dissolved in solvent (10% DMSO in 20 mM phosphate buffer pH 7.4, 10 mL). The mixture was deoxygenated by 5 freeze-pump-thaw cycles. Then, the mixture was canulated in another Schlenk tube containing BSA macroinitiator (~62 mg, 0.93 µmol, 1 equiv.) and CuBr (4.7 mg, 33 µmol) (previously degassed) under N₂ atmosphere, which triggered the beginning of the polymerization (a dark brown colour of the reaction medium was observed). The mixture was stirred under inert atmosphere during 72 h. The reaction mixture was then dialyzed using a 25 kDa MWCO membrane initially against 2% EDTA 10% DMSO 20 mM PB pH 7.4 and then against 20 mM PB pH 7.4. Resulting solutions were analyzed by means of SEC-HPLC (Figure VI-38), and MALDI-TOF analysis and their aggregation and encapsulation properties imaged by TEM and CFM microscopy (Figure VI-39).

![Figure VI-38](image-url)

**Figure VI-38.** SEC chromatographic traces of native BSA (solid black line), purified bioconjugates XV (solid grey line), and XVIII after reaction with TBAF (grey doted line), and reaction with KF (dashed black line).
General polymerization procedure with monomer (29) to give (XVII): Fluorinated monomer (258 mg, 0.742 mmol) and N-(Propyl)-2-pyridylmethanimine (~5.1 mg, 0.659 mmol) were placed in a Schlenk tube and dissolved in 20% DMSO 20 mM PB pH 7.4 (12 mL). The mixture was deoxygenated by 5 freeze-pump-thaw cycles. Then, the mixture was sonicated for 10 min. and immediately canulated in another Schlenk tube containing BSA macroinitiator IX (~33 mg, 0.495 µmol) and CuBr (2.9 mg, 20 µmol) (previously degassed) under N$_2$ atmosphere, which triggered the beginning of the polymerization (a dark brown colour of the reaction medium was observed). The mixture was stirred under inert atmosphere during 72 h, dialyzed using a 25 kDa MWCO membrane against 2% EDTA 10% DMSO 20 mM PB pH 7.4. Samples were analyzed by SEC-HPLC (Figure VI-40) and their aggregation studied by TEM microscopy (Figure VI-41).
Figure VI-40. SEC chromatographic traces at 254 nm of native BSA (dashed grey trace) and amphiphilic macromolecule XVII (solid black trace).

Figure VI-41. TEM micrographs of the aggregates obtained by the self-assembly of the amphiphilic bioconjugates XVII.

BSA-PA deprotection with KF (XVIII): To a solution of BSA-PA (2.5 mL, C~9.3.10^{-5} M in 20 mM PB pH 7.4), 465 µL of a 1 M solution of KF in 20 mM PB pH 7.4 were added. The reaction mixture was gently stirred for 2 days at 7°C and then purified by dialysis (MWCO 10 kDa) against 20 mM PB pH 7.4 before analysis by SEC-HPLC (Figure VI-42), MALDI-TOF analysis (no significative results observed) and their aggregation studied by TEM (spherical structures observed with diameter varying from 50 to 100 nm) and CFM microscopy (Figure VI-43).

BSA-PA deprotection with TBAF (XIX): To a solution of BSA-PA (3 mL, C = 9.3.10^{-5} M in 20 mM PB pH 7.4), 121.6 mg of TBAF were added. 20 mM PB was added until the
volume reached 3 mL. The reaction mixture was gently stirred for 2 days at 7°C and then
dialyzed against 20 mM PB pH 7.4 before analysis by SEC HPLC (Figure VI-42), MALDI-
TOF analysis (no significative results observed) and their aggregation studied by TEM
(spherical structures observed with diameter varying from 50 to 100 nm) and CFM
microscopy (Figure VI-43).

General procedure for the clicking of azide 15 onto compound XVIII to afford XXa: To
a solution of XVIII (0.8 mL, C~9.3.10^{-5} M in 20 mM PB pH 7.4) was added 1-azidodecane
15 (27 mg, 0.149 mmol, 2000 equiv.). Then were successively added 80 µL DMSO, CuSO_4
(56 µL of a 0.1 M solution in 20 mM PB) and sodium ascorbate (56 µL of a 0.2 M solution in
20 mM PB). The reaction mixture was gently stirred for 2 days at 7°C and then extensively
dialyzed with Microcon 10 kDa dialysis cups against 20 mM PB pH 7.4 and analyzed by
SEC-HPLC (Figure VI-42, left), MALDI-TOF-MS (no results obtained due to amphiphilic
character of bioconjugates) and their aggregation studied by TEM (Figure VI-44, left) and
CFM microscopy (Figure VI-43).

N.B. Same procedure used to prepare compound XXb (20 mg of benzyl azide 16 were added)
and for compound XXc (26 mg of triethylene glycol mono-azide 21 were added).
Characterization of XXb by SEC-HPLC (Figure VI-42, left) and aggregation behaviour
observed by TEM (Figure VI-44, right) and CFM (Figure VI-43) microscopy.
Characterization of XXc by SEC-HPLC (Figure VI-42, right) and aggregation behaviour
observed by CFM (Figure VI-43) microscopy.

![Figure VI-42. SEC chromatographic traces obtained after clicking with the hydrophobic azides 15 and 16 (left) and with the hydrophilic azide 21 (right).](image)
Figure VI-43. CFM images of fluorescent aggregates observed for compounds A. XVI, B. compound XIX, C. compound XXa, D. compound XXb and E. compound XXc.

Figure VI-44. TEM pictures of structures observed with compounds XXa (left) and XXb (right).
General polymerization procedure with styrene in the presence of fluorescein labeled papain (XXI): Styrene (135 µL, 1.177 mmol, 2000 equiv.) and N-(Propyl)-2-pyridylmethanimine 12 (~6 mg, 0.041 mmol, 70 equiv.) were placed in a Schlenk tube and dissolved in solvent (10% DMSO in 20 mM phosphate buffer pH 7.4, 10 mL). The mixture was deoxygenated by 5 freeze-pump-thaw cycles. Then, the mixture was canulated in another Schlenk tube containing BSA macroinitiator IX (~39 mg, 0.59 µmol, 1 equiv.), fluorescein labeled papain (~5.6 mg, 0.24 µmol, 0.4 equiv.), and Cu(I)Br (3.4 mg, 24 µmol, 41 equiv.) previously degassed under N\textsubscript{2} atmosphere, which triggered the beginning of the polymerization. A dark brown colour was immediately observed for the reaction medium. The reaction mixture was stirred under inert atmosphere during 8 h after which nitrogen was stopped and the mixture was stirred for additional 8 hours (oxidation of Cu(I) to Cu (II)). Purification was performed as previously, adding an extra dialysis step with a 300 kDa MWCO membrane to remove the excess of non-encapsulated papain. A control experiment in the absence of the macroinitiator IX was performed, SEC revealed only one peak corresponding to native papain after purification. SEC-HPLC revealed that the polymerization happened and that no free papain was present after dialysis (Figure VI-45). Aggregation was observed by TEM microscopy (Figure VI-46, left) and CFM microscopy (Figure VI-46, right) revealed the statistical incorporation of papain within the superstructures.

**Figure VI-45.** SEC chromatographic measurements at 254 nm of the ATRP polymerization reaction XXI in the presence of Carica papaya papain after dialysis (solid black trace) and of native Carica papaya papain (dashed grey trace).
Figure VI-46. (Left) TEM micrograph of the aggregates observed after the *in situ* polymerization in the presence of fluorescein labelled *Carica papaya* papain XXI, (Right) CFM images of the fluorescently papain loaded aggregates demonstrating the statistical encapsulation of papain within the superstructures.

General procedure for the labeling of above superstructures (XXI) with Atto-NHS to afford compound (XXV): For the Atto labelling experiments, 50 µL of a NHS-Atto ester solution (5 mM in DMSO) was added to 200 µL of the solution of the dialyzed BSA-PS superstructures containing the fluorescein labelled papain (XXI). The reaction mixture was incubated for 2-4 hours at 7°C and was then extensively dialyzed against 20 mM phosphate buffer pH 7.4 to remove any non-reacted NHS-Atto ester prior to any measurement. Double labelling after dialysis was observed by CFM microscopy (Figure VI-47).

Figure VI-47. CFM images of BSA-PS polymerized in the presence of fluorescein labelled *Carica papaya* papain after external labelling of the superstructures with Atto-NHS dye (reaction XXV). Lines A, B, C represent 3 different areas of the sample. The scanning was independently performed at 610 nm (Atto, *left*) and 488 nm (Fluorescein, *right*).
General polymerization procedure with styrene in the presence of HRP (XXIV): Styrene (1.86 mmole, 194 µL, 176 mg) and N-(Propyl)-2-pyridylmethanimine 12 (~9.6 mg, 65 µmol) were placed in a Schlenk tube and dissolved in solvent (10% DMSO 20 mM phosphate buffer pH 7.4, 10 mL). The mixture was deoxygenated by 5 freeze-pump-thaw cycles. The beginning of the polymerization was triggered by the canulation of the monomer solution under nitrogen atmosphere in a second deoxygenated Schlenk tube containing the crystalline BSA-macronitiator IX (~62 mg, 0.93 µmol), HRP (200 UNITS/mg) (0.8 mg, 160 UNITS) and CuBr (5.5 mg, 38 µmol) under N\textsubscript{2} atmosphere. A dark brown colour was immediately observed. The reaction mixture was stirred under inert atmosphere during 48 hours. The reaction mixture was dialyzed against 20 mM PB pH 7.4 using regenerated cellulose MWCO 25 kDa dialysis bags (Spectrum laboratories). 2 mL were dialyzed using 300 kDa dialysis bags against 20 mM PB pH 7.4 (2.1 mL recovered). After SEC-HPLC analysis of the reaction mixture after dialysis (Figure VI-48) and aggregation studies of the resulting solution by TEM microscopy (Figure VI-49), the catalytic activity was investigated by UV study using TMB / H\textsubscript{2}O\textsubscript{2} as substrate.

![Figure VI-48. SEC chromatographic traces of polymerization in the presence of HRP (reaction XXIV) after dialysis (solid black trace), and native HRP (dashed grey trace).](image)
General polymerization procedure with styrene in the presence of carboxyfluorescein (CF) (XXIII): Carboxyfluorescein (7.5 mg, 20 µmol), Styrene (1.86 mmole, 194 µL, 176 mg) and N-(Propyl)-2-pyridylmethanimine (~9.6 mg, 65 µmol) were placed in a Schlenk tube and dissolved in solvent (10% DMSO in 20 mM phosphate buffer pH 7.4, 10 mL). The mixture was deoxygenated by 5 freeze-pump-thaw cycles. The beginning of the polymerization was triggered by the canulation of the monomer solution under nitrogen atmosphere in a second deoxygenated Schlenk tube containing the crystalline BSA-macroinitiator IX (~62 mg, 0.93 µmol) and CuBr (5.5 mg, 38 µmol) under N₂ atmosphere. A dark brown colour was immediately observed. The reaction mixture was stirred under inert atmosphere during 48 hours. The reaction mixture was dialyzed against 2% EDTA, 10% DMSO 20 mM PB pH 7.4 using regenerated cellulose MWCO 25 kDa dialysis bags (Spectrum laboratories) until no fluorescence was detected (to the naked eye) in the dialysis tank.

General procedure for the external labeling of the superstructures to afford (XXVI): For the fluorescein labelling experiments, 50 µL of a NHS-Fluorescein ester solution (5 mM in DMSO) were added to 200 µL of the solution of the dialyzed BSA-PS superstructures containing the Atto labelled papain. The reaction mixture was incubated for 2-4 hours at 7°C and was then extensively dialyzed against 20 mM phosphate pH 7.4 to remove any non-reacted NHS-Fluorescein ester prior to any measurement.
HRP Activity test: Ready to use 3,3',5,5'-Tetramethylbenzidine (TMB) / H$_2$O$_2$ solution (Sigma Cat nr: T0440) was used for kinetic measurement of TMB oxidation by the encapsulated within the BSA-PS superstructures HRP. Increasing quantities (from 10 to 100 µL) of TMB/H$_2$O$_2$ solution were added to a 900 µL dispersion consisting of 10 µL purified BSA-PS HRP containing nanoreactors in 20 mM sodium phosphate buffer pH 7.4. The soluble blue reaction product of the one-electron oxidation of TMB was recorded at 370 nm. Further oxidation of TMB in acid solution (addition of HCl) yields a yellow diimine reaction product with an absorbance maximum at 450 nm. This end-point assay was used to determine the activity of the HRP loaded nanoreactors by terminating the HRP-catalyzed conversion of TMB after 5 min with the addition of 0.25 mM of HCl (final concentration). The resulting yellow reaction product was recorded at 450 nm after a 3 minute incubation (Figure VI-50).

Figure VI-50. UV study of the catalysis of HRP loaded aggregates as a function of the volume of aggregates added to the TMB solution, the peak increasing at 280 nm corresponding to the polystyrene chain.

Cell uptake experiments with E. coli strain: 4 mL overnight E. coli (DH5α) culture in LB medium was prepared. 3 mL of fresh LB medium were inoculated with 1 mL of cell culture. 50 µL (~100 µM) of aggregates solution were added into the latter cell suspension. E. coli cells and aggregates were incubated for 1 hr to 12 hrs. After incubation of E. coli cells and Atto-labelled papain BSA-polystyrene aggregates externally labelled with fluorescein (XXVI), cells were harvested from 1 mL cell suspension. Harvested cells were washed with 1 mL of a 0.9% NaCl aqueous solution, cells solutions were vortexed, centrifuged (10000 rpm, 10 min, 4°C), the supernatant discarded. Cells pellet was resuspended in 0.5 mL of a 0.9%
NaCl aqueous solution. 10 µL of each sample were used for confocal imaging. (For some samples 5 µL 50% glycerol was used to fix the cells on the glass support). CFM microscopy clearly points out the cell uptake of Giant Amphiphiles superstructures by E. coli cells (Figure VI-51). Experiments on incubation time (Figure VI-52) proved that E. coli cells are fully loaded with Giant Amphiphiles superstructures after only 2 hours.

**Figure VI-51.** CFM images of E. coli cells after uptake of doubly fluorescent labelled aggregates. First column, fluorescence at 488 nm; Second column, fluorescence at 610 nm; third column, optical image; fourth column, overlaid images.
Figure VI-52. CFM pictures of *E. coli* cells incubated with Atto-labelled papain loaded BSA polystyrene XXVI Giant Amphiphiles aggregates for different periods of time (*left*, fluorescent image at 488 nm; *right*, optical image).

Cell uptake experiments with *Baccilus sp.* and *Yeast*: same procedure as previously followed with *E. coli* cells. Adhesion of the vesicles XXVI on the cell walls was observed in the case of Yeast (Figure VI-53, B.) whereas no interaction was observed during incubation of vesicles XXVI with *Baccilus sp.* Cells (Figure VI-53, A.).

Figure VI-53. CFM images of *A.* Bacillus sp. and *B.* Yeast cells after incubation with doubly fluorescent labelled aggregates XXVI. First column, fluorescence at 488 nm; Second column, fluorescence at 610 nm; third column, optical image; fourth column, overlaid images.
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References