Therapeutic protein aggregates: biophysical characterization and in vitro modelisation of subcutaneous tissue

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Abstract

The aim of this thesis was to explore the link between stability and immunogenicity of therapeutic proteins. Using various environmental stress conditions, protein aggregates were formed, biophysically characterized, and the relationship between the degree of aggregation and the impact on the protein bioactivity highlighted. Then, in view of simulating in vitro the phenomena of in situ protein aggregation once injected in the subcutaneous tissue, we reviewed the literature on 2D and 3D assays predicting the potential immunogenicity of (new candidate) therapeutic proteins. Based on that, an immunocompetent model of the human subcutaneous tissue was developed, combining hydrogels scaffolds into which antigen-presenting cells (MUTZ-3 dendritic cells) were embedded.

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Therapeutic Protein Aggregates: Biophysical Characterization and \textit{In Vitro} Modelisation of Subcutaneous Tissue

THÈSE

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To all the inspiring people I’ve met
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FIRST use of a protein as a therapeutic dates back to the 1920’s. In those years, insulin was extracted from bovine or porcine pancreases and injected in humans to treat diabetes mellitus. However, usage of proteins of animal origin presents some disadvantages such as the limited amount of organs available, cost of the purification process, and immunogenicity risk of a non-human variant of the protein.¹ Later in the 1960’s, with growth hormone another protein is introduced as replacement therapy for short stature conditions. Its extraction from bovine or human pituitary glands however carries -as shown some decades later- the risk of disease transmission, in this case of prions causing Creutzfeldt–Jakob disease.² Fortunately, the development of recombinant DNA technology allows in the early 1980’s a safe and sustained production of human proteins from Escherichia coli. Bacteria are not the only cell type used for recombinant protein expression. Yeast and mammalian cells (e.g., CHO cells), as well as transgenic animals and plants are employed as protein expression systems that offer additional advantages such as post-translational modifications and high productivity.³ ⁴

The need of physiologically active post-translational modifications for certain proteins and cost effective production are not the only challenges encountered in protein manufacturing. Protein solubility and stability are major concerns of upstream and downstream processing in biotechnology.⁵ Indeed, accumulation of the recombinant protein in the expression system may lead to the formation of aggregates and insoluble forms of the protein (i.e., inclusions bodies).⁶ ⁷ Moreover, purification steps, formulation processes, transport and storage conditions may also cause aggregation through different mechanisms or pathways.⁸ Therefore, the development of a unique and optimal formulation is crucial in order to stabilize the therapeutic protein in its active form until injection.⁹ Appearance of aggregates, potentially leading to limited stability and bioactivity, requires performing a complete biophysical characterization of the protein structure, size and conformation using orthogonal techniques during the development process.
Due to the broad range of sizes and different types of aggregates that can be formed, a whole set of analytical methods may be employed:\textsuperscript{10, 11}

- Separation techniques like native and SDS-PAGE, size exclusion chromatography, analytical ultracentrifugation and field flow fractionation;
- Detection and size measurements using visual inspection, optical and electron microscopy, atomic force microscopy, light obscuration, multiangle and dynamic light scattering, flow imaging and nanoparticles tracking analysis;
- Spectroscopic methods for structural analysis like circular dichroism, UV-visible, fluorescence, infrared and Raman spectroscopy.

Finally, sensitivity of proteins to enzymatic degradation (e.g., gastric fluid, nasal mucosal cavity) and poor permeation through cellular membranes limits the choice of the delivery route. Thus, nowadays most of the marketed biopharmaceuticals are administered via the parenteral route.\textsuperscript{12} Conversely to intravenous or intramuscular injections, subcutaneous (SC) injection offers significant advantages such as better patient compliance for repeated dose regimens (less painful) with the possibility of self-administration at home, reducing at the same time health costs.\textsuperscript{13} Additionally, slow absorption of the therapeutic protein from the SC tissue allows for an extended residence time,\textsuperscript{14} which can also be deleterious in terms of immunogenicity. Prolonged interaction of therapeutic proteins with sentinel immune cells present in the SC tissue may increase their potential recognition as foreign molecules. This slow absorption may also enhance a depot effect leading to local adverse reactions in case of irritating compounds, and potentiate protein degradation and aggregation tendency in situ.\textsuperscript{15} Indeed, interactions with extracellular matrix components, shift of pH and temperature (storage at 4°C to body temperature), and interstitial pressure variation are factors affecting the stability of therapeutic proteins upon SC injection.\textsuperscript{15}

The aim of this thesis was to study the link between stability and immunogenicity of therapeutic proteins. More specifically, we intended to study the impact of protein aggregation on its structure and bioactivity. In a second phase, we sought to characterize mechanical properties of the human subcutaneous tissue into which therapeutic proteins are often injected. This, in order to develop an \textit{in vitro} immunocompetent model of this tissue predicting the immunogenicity of therapeutic proteins.
As shown in Fig. 1, the first step of the project was to produce and purify a soluble form of the protein of interest. Despite the existence of a commercial version of the recombinant human interferon alpha2b (rhIFNα2b), commercialized under the trade name of Intron®A (Merck MSD), the need to have large amounts of protein from the same batch and at a higher concentration justified an in-house production. A tri-dimensional representation of the secondary structure of the protein is shown in Fig. 2. After plasmid design (see vector cards in Annex 1) and small-scale preliminary assays, the protein was recombinantly expressed in E. coli using a 20 L fed-batch bioreactor. Second steps consisted in reproducing in vitro some environmental factors of stress causing protein aggregation, such as thermal variations, oxidation enhancers and agitation. Following this forced aggregation, a complete characterization of the protein’s antiviral bioactivity and biophysical properties using orthogonal methods was performed. All of these three steps and results obtained are detailed in the Chapter 1. Previous work done on stability enhancement and characterization of two other model proteins (BSA and HEWL) is included in Annex 2.

Figure 1. Objectives and plan of the thesis
Immunogenicity assessment of a protein requires usage of various methods during the development process of a new drug candidate. A comprehensive review is presented in Chapter 2, focusing more specifically on in vitro techniques available and 3D organotypic models under development.

A simplified 3D model of the human subcutaneous tissue mimicking its visco-elastic properties (in comparison to measurements performed on human ex vivo skin samples) and embedding dendritic cells is developed in Chapter 3.

Finally, some preliminary results obtained on impact of protein aggregates on the stimulation of dendritic cells are described in Chapter 4, and some perspectives are given for further studies on this project.

Figure 2. Tri-dimensional representation of the secondary structure of human interferon alpha 2b. Image from the RCSB Protein DataBase (www.rcsb.org) of PDB ID 1RH2 from reference 16.
REFERENCES


CHAPTER 1
Multi-parametric Evaluation of Therapeutic Protein Aggregation

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ABSTRACT

Stability studies are necessary for each new therapeutic protein in order to define the optimal formulation as the aggregation tendency is a protein-dependent phenomenon. In order to characterize aggregates of interferon alpha 2b (rhIFNa2b) induced by various environmental stress conditions, we used benchmarked orthogonal methods including circular dichroism (CD), fluorescence spectroscopy and anisotropy, dynamic light scattering (DLS), turbidity and asymmetric-flow field flow fractionation (AF4). The final aims of our work were (1) to characterize different aggregate species obtained and gain insight into the relationships between stress nature, aggregates characteristics and their impact on protein bioactivity; (2) to comprehensively present results defining aggregation patterns and offering a comparison between stresses at one glance. Our findings show that mechanical stress and metal-catalyzed oxidation engender the formation of small to moderate sized aggregates, which were reversible upon dilution in terms as measured by bioactivity, whereas thermal stress gave irreversible aggregates and denaturation of the protein leading to a loss of bioactivity.

KEYWORDS

protein aggregation; stability; protein structure; circular dichroism; fluorescence spectroscopy; UV/Vis spectroscopy; dynamic light scattering; asymmetric field-flow fractionation, biopharmaceutical characterization.
1. INTRODUCTION

Since the commercialization of Humulin® insulin in 1982, the pharmaceutical industry is facing a major challenge with regard to the stabilization and avoidance of immunogenicity of therapeutic proteins. Being highly complex molecules, they require a specific conformation ("native state") in order to be active. When proteins encounter environmental stress they have a tendency to unfold and refold differently, resulting in the formation of aggregates. These aggregates may appear during the production process, storage or transport, but also upon injection. As described by Kinnunen and Mrsny the microenvironment at the site of injection may not be optimal to maintain native therapeutic protein folding. Finally, aggregates formed in vitro during storage or in situ upon administration are recognized as foreign by the immune system and may trigger an immune response.

Subcutaneous (SC) injection remains the preferred route of administration for therapeutic proteins, as it allows self-administration by the patients, increasing compliance. Owing to the low vascularization of the SC tissue, a prolonged retention time of injected protein therapeutics is observed. However, SC injections have the disadvantage to be limited in volume, which requires the formulation of highly concentrated products of 1.5 to 2 mL volume. Recently, though, increase in injection volume up to 3.5 mL is possible by co-formulation with a permeation enhancer (human hyaluronidase). In addition, SC injections are generally considered to be more immunogenic than those given intramuscularly or intravenously, thus potentially leading to an increase in injection site reactions (ISRs). This is due to the prolonged exposure to the product and the high prevalence of antigen-presenting cells (APCs) that will interact with protein monomers and aggregates.

One method used to prevent protein aggregation and to reduce immunogenicity is the covalent attachment of poly(ethylene glycol) (PEG), called PEGylation. The addition of PEG creates a steric hindrance around the molecule preventing protein self-association and recognition by the immune system. However, protein bioactivity may be reduced by PEGylation, which is compensated by the positive effect of increased half-life (t1/2) and area under curve (AUC) typically achieved by PEGylated proteins. After long-term use of PEGylated therapeutic proteins, clinical studies revealed the formation of anti-PEG antibodies in some patients, correlated with a reduced efficacy and a rise in adverse events.

Proteins are influenced by various environmental parameters triggering the formation of different types of aggregates. To assess conformational stability of candidate therapeutic
proteins, stability testing and forced aggregation studies are performed. In 2011, a classification of protein aggregates was suggested by Joubert et al. who studied aggregate formation of a monoclonal antibody (mAb) by biophysical characterization. Seven classes of aggregates were defined, ranging from “not aggregated” to “large, unfolded and irreversible”, taking into account the nature of these aggregates. A broad spectrum of stress conditions was tested and the applicability of this classification was evaluated with other mAbs. As in many similar studies, the large amount of data generated is difficult to display to draw clear conclusions and to classify different types of aggregates.

Recent studies describing aggregation pathways, classification of aggregates and defining a common nomenclature for protein characterization are found in literature. However, these classifications remain either too general (nomenclature on types of aggregates without concrete application), too specific for one type of protein (using generally mAbs but not small proteins, which are more difficult to characterize), or too complex and time-consuming due to the multitude of analytical methods used. We propose here a radar chart model to present all results obtained from orthogonal characterization methods compiled in a clear, comprehensive and adaptable way, offering the possibility to extract the degree of protein aggregation from the chart surface area and patterns obtained.

Recombinant human interferon alpha 2b (rhIFN$_{\alpha}2b$) was chosen as a model protein for our multi-parametric evaluation due to its small molecular weight of 19.3 kDa. The non-PEGylated version is approved by FDA since 1986, and its SC injection three times per week may lead to milder immune reactions than other biotherapeutics (up to 40% of patients develop anti-IFN$_{\alpha}$ antibodies). ISRs appear in less than 5% of patients according to the product information for Intron® A, but various cutaneous side effects were described in the literature.

2. MATERIALS AND METHODS

2.1. Recombinant production of rhIFN$_{\alpha}2b$

The codon optimized gene sequence, rhIFN-2ab, encoding for amino acids 24–188 of the human leukocyte interferon alpha 2b precursor [IFNA2, GeneID: 3440, UniProtID: P01563] and translationally fused to a N′-terminal hexahistidine-tag was subcloned into pET23d(+) resulting in expression plasmid pET23d(+)::H6rhIFN$_{\alpha}2b$. pMJS9, encoding for sulphydryl
oxidase ErvP1 (yeast) and for human protein disulfide isomerase PDI under control of an arabinose-promoter was kindly provided by Prof. Dr. L. W. Ruddock (Department of Biochemistry, University of Oulu, Finland).

rhIFNα2b was produced in a 20 L stirred tank Biostat®C Bioreactor (Sartorius AG, Germany) in fed batch mode. To this end, Escherichia coli SHuffleT7 Express (C3029H, New England Biolabs, Germany) was co-transformed with both pET23d(+)::H6rhIFNα2b and pMJS9 (1:1, w/w ratio). 2 x 1 L LB medium containing 0.5 % (w/v) glucose, 120 µg/mL ampicillin, 34 µg/mL chloramphenicol were inoculated at OD₆₀₀nm = 0.02, 30°C (final OD₆₀₀nm = 3.46, 20 h). The bioprocess was started with 10 L LB (0.5 % w/v glucose, 75 µg/mL ampicillin, 17 µg/mL chloramphenicol, pH 7.0, 30°C) at OD₆₀₀nm = 0.5. Dissolved oxygen (DO) was limited to 60 % at 6 slm (standard liter per minute). At OD₆₀₀nm = 1.96 (t= 165 min) a bolus of 10 L process medium was added and pre-expression of co-chaperones was induced with 0.25 % (w/v) L-arabinose, followed by 1 mM IPTG after 30 min. Biomass was harvested after 7.25 h at 5,000 x g and stored at -20°C.

2.2. Purification of soluble rhIFNα2b

Cells were freeze-thawed three times and suspended in 200 mL lysis buffer (100 mM Tris, 200 mM NaCl, 20 mg lysozyme, protease inhibitor tablet, Hoffmann La-Roche AG, Basel, Switzerland; 1600 U benzonase activated in 10 mM Tris, 2.5 mM MgCl₂, pH 8.0) for 1 h at 21°C. Cell lysis was performed by ultrasonication. Subsequently, soluble and insoluble fractions were separated at 24,445 x g, 4°C. The soluble fraction was adjusted to pH 8.15 (1 M Tris base) and centrifuged to remove debris. Finally, 100 mM imidazole was added and sample was clarified using 0.45 µm polyvinylidene difluoride (PVDF) syringe filters. His₆-tagged rhIFNα2b was captured on a 5 x 5 mL stacked HisTrap™ FF Crude column (GE Healthcare Life Sciences, Germany). Equilibration occurred with 12 column volumes (CV) 20 mM Tris, 200 mM NaCl, pH 8.15) at 238.7 cm/min. 50 mL sample were followed by 28 CV of equilibration buffer to wash out unbound material. rhIFNα2b was eluted using a linear gradient from 100 mM to 500 mM imidazole (2 CV) and 2 CV 500 mM imidazole at 89.5 cm/h. 45 mL eluate per run was collected in 80 mL 20 mM Tris, 100 mM NaCl, pH 8.15. The purified protein was filtrated with 0.2 µm Sartobran® filtration unit (Sartorius).
Buffer exchange was performed by dialysis of 100 mL protein, using a 6-8’000 kDa MWCO Spectra/Por® membrane tubing, against 5 L formulation buffer (130 mM NaCl, 10 mM sodium phosphate buffer pH 6.75, 0.342 mM EDTA disodium salt, 0.1 mg/mL polysorbate 80) for 24 h at 4°C under agitation at 300 rpm followed by a second dialysis against 2 L of fresh formulation buffer for further 24 h. Protein solution was aliquotated in 15 mL Falcon tubes, which were frozen in isopropanol at -80°C to be freshly thawed once needed.

2.3. Protein analysis

SDS-PAGE, Coomassie staining and immunoblot analyses were carried out using standard protocols with 100 mM dithiothreitol (DTT) as indicated. Proteins were transferred to polyvinylidene difluoride membranes (PVDF, Carl Roth GmbH, Germany) and detected with mouse anti penta-His antibodies (34660, Qiagen, Germany, 1:1,000) in 5 % (w/v) bovine serum albumin (BSA)/Tris buffered saline with 0.1 % Tween® 20 (TBST) and peroxidase (POD)-labeled secondary goat anti mouse antibodies (A4416, Sigma-Aldrich, Germany, 5 % w/v skim milk/TBST). Purified recombinant His6-tagged EmGFP (0.5 µg/lane) served as control.

2.4. Aggregate preparation

Solutions containing rhIFNα2b were prepared at two concentrations (0.1, 0.5 mg/mL) in formulation buffer, mimicking the buffer of commercial Intron®A, m-cresol omitted to allow fluorescence characterization, and stored at 4°C. Samples of 0.5 mL were conditioned in 3 mL glass vials with rubber cap and then subjected to the following stress conditions: (i) mechanical stress by stirring at 4°C, 700 rpm, magnetic stirrer 6x6 mm, either overnight (14 h) or for three days (72 h); (ii) metal-catalyzed oxidation (MCO) by addition of 5 mM CuSO4 and 4 mM ascorbic acid, overnight at 4°C, and quenched with 5 mM EDTA (fixed protein:CuSO4 ratio of 1:100); (iii) thermal stress by incubation at 64°C (defined by CD spectra as melting temperature, Tm), and 90°C during 1 hour.
2.5. Circular dichroism

The secondary structure of the protein was determined using a Jasco J-815 CD spectropolarimeter (Jasco Inc., Easton, Maryland). Far-UV CD spectra were recorded from 260 to 190 nm at a scan speed of 100 nm/min. The response time was set to 2 sec, data pitch to 1 nm and the bandwidth to 2 nm. Samples at 0.5 mg/mL were diluted in Milli-Q® water to a final concentration of 8 µM. No dilution was applied for samples at 0.1 mg/mL (corresponding to 5.18 µM). Each result represents the average of three accumulations with blank subtraction. Data were collected at 20°C using a quartz cuvette with 1 mm path length.

2.6. Intrinsic fluorescence spectroscopy

Intrinsic tryptophan fluorescence, expressed in counts per second (CPS), was measured using FluoroMax-4 spectrofluorometer (Horiba Scientific, Jobin Yvon GmbH, Bensheim, Germany) at an excitation wavelength of 295 nm. Emission spectra were recorded at emission wavelengths from 300 to 380 nm with an integration time of 1 sec per 1 nm increment. The excitation and emission slit widths were set to 4 nm. All samples were diluted in Milli-Q® water to a final concentration of 2.85 µM and loaded in a quartz cuvette of 10 mm path length. Data were collected at 20°C. Each result represents the average of three accumulations with blank subtraction.

2.7. Fluorescence anisotropy

Fluorescence anisotropy of rhIFNα2b samples were measured by using a FluoroMax-4 spectrofluorometer (Horiba Scientific) at an excitation wavelength of 295 nm. Emission spectra were recorded from 330 to 370 nm with an integration time of 10 sec per 1 nm increment. The excitation and emission slit widths were set to 5 nm. All samples were diluted in Milli-Q® water to a final concentration of 2.85 µM and loaded in a quartz cuvette of 10 mm path length. Data were collected at 20°C. From anisotropy measurements, we calculated the mean of the whole spectra from 330 to 370 nm.
2.8. Dynamic light scattering

The hydrodynamic diameter of native and aggregated protein was determined using a Zetasizer Nano ZS (Malvern Instruments Ltd, Worcester, UK). 110 µL of undiluted sample was placed in a disposable low-volume cuvette (ZEN0040). Measurement was done at 173° backscatter angle after 60 sec of equilibration time and at a temperature of 25°C. Each result represents the average of three measurements.

2.9. UV-visible spectrometry

Absorbance measurements were recorded using a Synergy Mx microplate reader (BioTek Instruments, Winooski, Vermont) over a wavelength range of 230-500 nm at 1 nm intervals. 80 µL/well of each sample were placed undiluted in Corning UV transparent 96-well plate (ref. 3635), after shaking for 10 sec the measurement was done at 25°C. Each result represents the average of at least three wells with corresponding blank subtraction.

2.10. Asymmetric-flow field flow fractionation

Fractograms of native and aggregated rhIFNα2b were obtained using an AF2000 system (Postnova Analytics GmbH, Landsberg, Germany) coupled with a Waters 2487 UV detector (Milford, MA) set at \( \lambda = 280 \) nm, a Postnova 3150 refractive index detector, and a Postnova 3609 multi-angle light-scattering (MALS) detector. Samples were analyzed through an AF4 channel of 275 mm length and covered with a regenerated cellulose membrane with a molecular weight cut-off (MWCO) of 5 kDa and a 350 µm wide spacer. AF4 measurements were performed after equilibration with 0.9 % NaCl solution and 50 µg of 0.5 mg/mL protein samples were injected into the system (0.1 mg/mL samples were below the detection limit). The separation method used was defined as follows: for the focus step, injection flow was set at 0.2 mL/min during 4 min using a cross-flow of 4 mL/min and a focus pump flow of 4.30 mL/min; after a transition time of 1 min the elution step started with 15 min of constant 4 mL/min cross-flow, followed by a 30 min power gradient with an exponent of 0.2 and finally 20 min without cross-flow. Analyses were performed with the channel adjusted at 25°C. The detector flow-rate and the slot-flow rate were both set at 0.25 mL/min. Data analyses were
performed using the NovaFFF AF2000 software (Postnova Analytics GmbH). For each sample, the corresponding blank signal was subtracted. Molecular weight (Mw) estimation of rhIFNα2b was done using a dn/dc value of 0.185 mL/g and a theoretical extinction coefficient of 0.946 mL/(mg*cm).

2.11. Transmission Electron Microscopy (TEM)

TEM imaging was done on a Tecnai™ G2 Sphera microscope (Fei, Hillsboro, Oregon) equipped with a 2000 by 2000 pixels high-resolution digital camera. Carbon 200 mesh ultrathin grids were glow-discharged with a 350 V plasma under 0.3 Torr vacuum for 20 sec. Then 5 µL protein samples were deposited and allowed to spread on the grid for 30 sec, blotted on a Whatman® drying pad (grade 556), washed quickly on a 100 µL drop of a 2 % (w/v) freshly prepared uranyl acetate solution and stained by deposition onto a second uranyl acetate drop for 30 sec.

2.12. Bioactivity assay

To compare bioactivity of stressed vs. native proteins and to show antiviral activity of the therapeutic protein expressed and purified in-house (native protein) in comparison to the commercial product (Intron®A), we conducted a bioactivity assay using A-549 cells (human lung carcinoma cell line, ATCC ref. CCL185) infected with a vesicular stomatitis virus (VSV) after 10 h incubation with protein samples. Recombinant VSV expressing green fluorescent protein (VSV-GFP) was a kind gift from Dr. D. Garin.

The assay was performed in 96-well microtiter assay plates with a clear bottom and black opaque walls (Corning ref. 3904) seeded with $2 \times 10^5$ cells/mL (100 µL/well) in Dulbecco’s Modified Eagle Medium (DMEM, no phenol red) supplemented with 4 mM L-glutamine, 10 % (v/v) fetal bovine serum (FBS) and penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively). All products were obtained from Gibco® (Life Technologies, Grand Island, New York). After 24 hours incubation at 37°C in an atmosphere of 95 % humidity containing 5 % CO₂, serial dilutions of rhIFNα2b or Intron®A (multidose pen for injection 18 MIU, Merck MSD) were added to the cell culture medium (final vol. 200 µL/well). Ten hours later, medium containing rhIFNα2b or Intron®A was removed and cells challenged with $10^7$ VSV-GFP
particles/mL (100 µL/well - multiplicity of infection of 50) diluted in DMEM complemented with 2 % FBS and transferred into an incubator set at 33°C (95 % humidity, 5 % CO₂). Viral proliferation was measured after 12 hours by fluorescence measurements using a Synergy Mx microplate reader at an excitation wavelength of 475 nm and an emission wavelength of 509 nm (9 nm bandpass filter for both). Fluorescence area scans were performed with 10 measurements per well and at least sextuplicates of each sample were measured. 100 % infectivity, corresponding to 0 % bioactivity, was determined for each plate using a negative control containing only cells challenged by VSV-GFP in the absence of native protein or Intron®A.

The raw value obtained for each well was normalized to the corresponding negative control and expressed in percentage of infectivity depending on the protein concentration expressed in international units (IU) per mL of the commercial standard. After removing outliers using Grubbs’ method (α = 0.05), a two-way ANOVA using Dunnett’s multiple comparisons tests was performed with GraphPad Prism® software (version 7.03, GraphPad Software Inc., La Jolla, California). Results obtained for the native protein were taken as a control group to define statistical significance.

2.13. Fluorescence microscopy

In addition to fluorescence measurement of each well, cells were observed and pictures were taken using an Axiovert 200 microscope (Carl Zeiss, Feldbach, Switzerland) equipped with QIClick CCD camera (QIImaging, Surrey, Canada) and a blue excitation fluorescence filter set 09 (Ex. BP450-490; Em. LP515).

2.14. Radar charts data analysis

Results obtained from the orthogonal methods were summarized in seven axes radar charts using OriginPro® 8.6 software (OriginLab Corporation, Northampton, MD). Outliers were removed using Grubbs’ method (α = 0.05) on GraphPad Prism® software. Each axis corresponds to one parameter defined as representative of the degree of conformational change for each method used. For each concentration, statistical differences between native (control) and aggregated samples were calculated using a parametric one-way ANOVA with
Dunnett’s multiple comparisons tests. Statistics between the two concentrations for each sample were performed using an unpaired parametric t-test.

3. RESULTS

3.1. Recombinant production of soluble IFNα2b

The 20 L fed batch process for the production of soluble rhIFNα2b based on SHuffle® T7 Express production clone harboring pET23d(+)::H6rhIFNα2b and pMJS9 grown in the presence of low concentrations of ampicillin and chloramphenicol promoting more stable growth and high productivity was successfully implemented (Fig. S1). Volumetric amount of rhIFNα2b increased over time as determined by increasing strength of the corresponding protein band detected by the anti penta-His antibody (Fig. S1). However, no signs of massive formation of potential inclusion bodies were visible. A final wet weight biomass of 106.4 g (5.3 g/L) was obtained after 7.25 h. After initial capturing by Ni²⁺-based affinity chromatography, a significant amount of 1.11 g of soluble protein was obtained at a yield of 90.2 +/- 4.7 % (based on the soluble protein fraction purified in 3 individual column runs) at > 98 % purity. The resulting chromatogram of one representative purification run is shown in Fig. S1c. The purified rhIFNα2b tended to aggregate under elution conditions at concentrations exceeding 5 mg/mL. Hence, the elution step was performed at instant dilution of the eluted protein in 20 mM Tris, 100 mM NaCl, pH 8.15 with increasing mixing speed and immediate removal of traces of aggregated material by sterile filtration with a 0.2 µm filter to prevent further aggregation. Coomassie stained SDS-PAGE of the purified sample of rhIFN revealed an apparent size of around 20 kDa (Fig. S1e).

3.2. Aggregation analysis by radar chart presentation

The use of orthogonal methods has been for years the state-of-the-art way to fully characterize proteins, aggregates and aggregation phenomena. In order to establish a more concise method to analyze all results obtained from the extensive physical characterization encompassing CD, fluorescence and anisotropy spectroscopy, DLS and UV-Vis spectrometry, a new way of presenting this information is proposed.
For CD, the mean residue ellipticity (MRE, $[\theta]_{MR} = \text{deg.cm}^2\text{.dmole}^{-1}$) at 220 nm was selected, which is a characteristic wavelength for helical secondary structure. IFNα2b is composed of a cluster of five alpha-helices, plus a $3_{10}$ helix in the AB loop, which is stabilized in the core of the molecule by a disulfide bond.\textsuperscript{24}

Intrinsic fluorescence emission intensity, using an excitation wavelength of 295 nm, was experimentally defined as maximum at a wavelength of 338 nm for the native rhIFNα2b protein. For all samples, variations in fluorescence intensity were monitored at this wavelength. At 295 nm, W76 and W140 were primarily excited. In its native conformation, W76 is located in a coil region (between two $\alpha$-helices) rising to the surface and being easily accessible. By contrast, W140 is less exposed because it is located in an $\alpha$-helix structure starting with P137 (PDB ID: 1RH2).\textsuperscript{24} More generally, changes in the emission spectra of tryptophan often occur in response to conformational transitions, subunit association or denaturation.\textsuperscript{25}

The fluorescence emission maximum ($\lambda_{\text{max}}$ in nm) is dependent on the tryptophan residues present in the protein structure and on the polarity of their surrounding environment. During the conformational changes of the protein, if the Trp residues become more exposed to water-buffer, $\lambda_{\text{max}}$ will shift to longer wavelengths. Red shift and increase or decrease of fluorescence intensity are indirectly related to the degree of exposure of Trp and unfolding of a protein.\textsuperscript{26-28}

Monitoring polarization anisotropy provides information on the size and shape of the protein via its dependence on rotational diffusion coefficient. Anisotropy is also influenced by the solvent viscosity and the flexibility of the molecule. Increase in anisotropy is indicative of a reduced mobility of the tryptophan residues, which could be related to aggregation.\textsuperscript{29}

DLS results, i.e. hydrodynamic diameters in nm are expressed here in Number distribution, which enabled a more accurate discrimination between the different kinds of stresses applied. Z-average diameter was not used because it tends to weight larger particles more than smaller species.

From UV-Vis spectra, we defined the turbidity value as the absorbance at 450 nm since the increase in turbidity above 320 nm is typically taken as indicative of aggregation.\textsuperscript{30-32}
Aggregation index (AI) was used to quantify the degree of aggregation for each stress condition based on specific turbidity obtained at a wavelength of 350 nm and corrected by the optical density at 280 nm. It is defined according to the following equation:\[^{30}\]

\[
AI = \frac{A_{350}}{(A_{280} - A_{350})} \times 100
\]

where \(A_{280}\) and \(A_{350}\) are the measured absorbance at 280 nm and 350 nm, respectively.

The native state and the five stress conditions chosen to be applied to determine aggregation tendency of the model protein rhIFNα2b are summarized in form of radar charts (Fig. 1 and 2). Protein samples were stirred over the course of three days to simulate extreme mechanical stress occurring during shipping, manipulation of the syringe and passage through the needle during injection. MCO was performed to create aggregates formed during manufacturing when the therapeutic protein encounters various chemical treatments.\[^{9},^{32},^{33}\] Furthermore, thermal stresses were used to mimic moderate (Tm) and extreme (90°C) stress conditions leading to potential aggregation after partial or complete protein unfolding.

At initial analysis of low and high protein concentrations (Fig. 1 and 2), apparent conformational changes resulting in similarly spread patterns for both thermal stresses were observed. The aggregation obtained after MCO and stirring during 3 days was more moderate. For both durations of stirring, we noticed a similar condensed pattern. However, stirring overnight stress was the one creating the smallest effects.

At a protein concentration of 0.1 mg/mL (Fig. 1), only two axes showed significant changes for MCO and mechanically stressed overnight. A large decrease in intrinsic fluorescence revealing a refolding of the protein where Trp residues were less exposed (or more buried) or degraded after MCO, and a small increase in aggregation index indicating an aggregation prone tendency after mechanical stress were observed. Interestingly, DLS always indicated a significant size change in Number weighted means.
Figure 1. Radar chart analysis of rhIFNα2b at native state and after five different stress conditions at a concentration of 0.1 mg/mL. Each axis has an independent scale. Scale and axis legend are the same for the 6 radar charts (represented only for native state). Each point is the average of at least three independent experiments (n = 3-6, opaque area) + standard deviation (dotted line). Statistical differences are given in comparison to the native protein (control) with the corresponding p-value ** < 0.01, *** < 0.001 and **** < 0.0001.
Figure 2. Radar chart analysis of rhIFNα2b at native state and after five different stress conditions at a concentration of 0.5 mg/mL. Each axis has an independent scale. Scale and axis legend are the same for the 6 radar charts (represented only for native state). Each point is the average of at least three independent experiments (n = 3-6, opaque area) + standard deviation (dotted line). Statistical differences are given in comparison to the native protein (control) with the corresponding p-value *< 0.05, **< 0.01, ***< 0.001 and ****< 0.0001.
Figure 3. Radar chart analysis of rhIFNα2b at native state and after five different stress conditions at two concentrations of 0.1 and 0.5 mg/mL. Each axis has an independent scale. Scale and axis legend are the same for the 6 radar charts (represented only for native state). Each point is the average of at least three independent experiments (n = 3-6). Filled colored area are for 0.5 mg/mL samples and hatched for 0.1 mg/mL. Statistics are done for each stress between both concentrations, with the corresponding p-value *< 0.05, **< 0.01, ***< 0.001 and ****< 0.0001.
The patterns obtained on the radar charts were highly dependent on the type of stress applied. For example, the helical content (220 nm MRE) of the MCO treated protein sample was only reduced by half at 0.5 mg/mL concentration (Fig. 2), even though the majority of the protein was completely unfolded with almost no intrinsic fluorescence intensity. No red shift of the fluorescence was observed, but DLS results indicated the presence of aggregates with a hydrodynamic diameter up to 2 µm, corroborating the significant increase in anisotropy. Turbidity and aggregation index were only slightly increased. Thermally stressed samples displayed totally different patterns. Intrinsic fluorescence intensities were unchanged after stress but all other parameters showed significant aggregation with a loss of secondary structure, a significant λ<sub>max</sub> red shift, an increase in anisotropy, 3 µm average sized-aggregates, and higher turbidity.

Aggregation effects observed were concentration-dependent (Fig. 3). In fact, more concentrated samples showed larger aggregates and different degrees of unfolding. The whole aspect of protein folding is easily visible from the radar charts by the colored-area occupied by each stress condition at each concentration.

Aiming to classify all types of aggregates generated in different categories, total chart areas for each condition were calculated and the ratio to the reference value of the native protein, called stressed-to-native ratio (Table 1), was determined. As seen previously, the increase in the area occupied on the radar chart was smaller at 0.1 mg/mL than at the higher concentration (0.5 mg/mL). Three kinds of aggregation behaviors were observed: limited aggregation using stirring during one night, moderate aggregation obtained after stirring during 3 days and MCO, and finally strong aggregation using thermal stresses superior or equal to Tm, frequently associated with protein degradation.
Table 1. Percentage of area covered on the radar chart, corresponding stressed-to-native ratio and their statistical significance in one-way ANOVA for all native and stress conditions at both concentrations tested (0.1 and 0.5 mg/mL)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Chart area (%)</th>
<th>Stressed-to-native ratio</th>
<th>One-way ANOVA</th>
<th>Chart area (%)</th>
<th>Stressed-to-native ratio</th>
<th>One-way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>0.9</td>
<td>1.0</td>
<td>-</td>
<td>1.0</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>Stirring 14 h</td>
<td>6.7</td>
<td>7.8</td>
<td>ns</td>
<td>4.7</td>
<td>4.6</td>
<td>ns</td>
</tr>
<tr>
<td>Stirring 72 h</td>
<td>15.6</td>
<td>18.1</td>
<td>ns</td>
<td>12.4</td>
<td>12.2</td>
<td>ns</td>
</tr>
<tr>
<td>MCO</td>
<td>21.7</td>
<td>25.3</td>
<td>ns</td>
<td>7.1</td>
<td>7.0</td>
<td>ns</td>
</tr>
<tr>
<td>64°C (Tm)</td>
<td>38.4</td>
<td>44.8</td>
<td>**</td>
<td>21.0</td>
<td>20.6</td>
<td>**</td>
</tr>
<tr>
<td>90°C</td>
<td>38.8</td>
<td>45.2</td>
<td>**</td>
<td>13.6</td>
<td>13.3</td>
<td>ns</td>
</tr>
</tbody>
</table>

3.3. Separation and Mw estimation of aggregates using AF4

Fractograms recorded for native and aggregated protein samples are shown in Fig. S2. High background and low signal were obtained due to the limitation of concentration applied (0.5 mg/mL). Moreover, the formulation buffer contains polysorbate 80 above the critical micelle concentration allowing for the formation of micelles. This led to a high MALS detector signal at the end of the elution gradient, also observed for the blank measurement that was subtracted. Due to these limitations, the information given here should be considered as qualitative only. The AF4 measurement of the native protein (Fig. S2a) exhibited two peaks: peak 1 gave a high Mw of 1250 kDa but corresponds only to 0.6 µg of protein; peak 2 showed the typical Mw for the monomeric protein of 19.6 kDa and a significant amount of 8.2 µg. After stirring stress overnight (Fig. S2b), a main peak 2 was still observed, integrating for a molar mass of 19.3 kDa but corresponding to a lower mass of 4.7 µg. This might suggest that about half of the monomeric form of the protein was lost during mechanical stress. When this stirring stress was extended to 3 days (Fig. S2c), the main peak 2 was recorded at a longer elution time, giving a Mw of 55 kDa that may correspond to the protein’s trimeric form. After thermal stresses at Tm and 90°C (Fig. S2d-e), no defined peak was observed, meaning that the protein completely lost its folded structure and was
denatured during the process. Finally, after MCO stress treatment (Fig. S2f), the main peak 1 was recorded at a longer elution time with a highly dispersed molar mass between 5.6 and 44 kDa and a significant amount of 7.5 µg detected. The corresponding sample appeared to be composed of many different sub-structures that were not resolved further with this method.

3.4. Visualization of aggregates using transmission electron microscopy

To obtain information on different sizes and morphologies of rhIFNα2b aggregates, we prepared protein samples on a carbon grid using negative staining for visualization by TEM. Size was determined using ImageJ software. TEM suggested a strong concentration-dependent tendency for aggregation (Fig. S3). The formulation buffer alone produced a granular background (Fig. S3a). With native protein solutions at high concentration, large aggregates of around 250 nm were identified (Fig. S3b). Even larger aggregates (≈ 340 nm) were found enabling us to discern their subunits composition (Fig. S3c). These subunits had a mean size of around 50 nm and consisted of a combination of multimers. When we reduced the protein concentration to 0.5 mg/mL (Fig. S3d), smaller aggregates ranging from 100 nm (white arrow) to 240 nm (black arrow) in size were obtained. Finally, at a concentration of 0.1 mg/mL (Fig. S3e), which is close to the intermediate concentration for the commercialized product in Europe, only some small white dots were observed as an irregular background, having a mean size of around 8 nm, almost comparable to the DLS results for native protein (6.58 nm ± 0.76). However, this technique did not allow us to identify any specific particle morphologies generated by each stress condition tested in this aggregation study. This was probably due to the sample preparation (interaction with uranyl acetate and drying steps) inducing formation of large aggregates even with “native” protein deposition.

3.5. Bioactivity Assay

Antiviral proliferation assay was used to compare the bioactivity of stressed vs. native proteins and to validate antiviral activity (“bioactivity”) of rhIFNα2b expressed by the methodology described here against the commercial product.
The antiviral activity is displayed in Fig. 4. A comparable bioactivity of our native in-house protein and the commercial Intron®A was observed at both concentrations of 0.5 (Fig. 4a) and 0.1 mg/mL (Fig. 4d), confirming the functionality of the native protein.

The effect of aggregate formation on bioactivity was further investigated. Surprisingly, we observed that after MCO (Fig. 4a, d), stirring overnight and stirring for 3 days (Fig. 4b, e) - regardless of the concentration - the protein bioactivity was still preserved. There appeared to be a sufficient number of correctly folded and active monomers and dimers remaining in the aggregated protein solution to confer viral protection to A-549 cells. In contrast, mixtures of monomers, dimers and aggregates created by thermal stress exposure were only partially active (Tm) or totally inactive (90°C) in terms of viral protection, at high statistical significance (p ≤ 0.0001) in comparison to native protein (Fig. 4c, f).

**Figure 4.** Bioactivity of IFNα2b at native state and after different stress conditions expressed in terms of VSV-GFP infectivity (%) as a function of protein concentration (IU/mL) added to the cell culture medium. Protein in its native state (black histogram) was taken as reference on each graph. Remaining protective effect of aggregates obtained from protein at 0.5 mg/mL (a, b, c) and 0.1 mg/mL (d, e, f) are represented. Each bar is the average of at least four independent experiments (mean ± SD, n = 4-6).
These results were correlated to observations by fluorescence imaging microscopy. VSV was visualized by detecting green fluorescent protein (GFP) expressed by the virus, hence green spots on the images corresponded to a cell infected with VSV-GFP (Fig. 5). At low protein concentration (Fig. 5b), the fluorescence was only slightly diminished compared to the untreated control (Fig. 5a). Conversely, almost no fluorescence was visible while increasing the protein concentration to 1E+3 IU/mL indicating a strong antiviral protective effect of rhIFNα2b (Fig. 5c).

**Figure 5.** Fluorescence microscopy images of A-549 cells infected with VSV-GFP after no treatment (a), after incubation with a low concentration of native rhIFNα2b (2.56E-3 IU/mL, b) or a high concentration of native rhIFNα2b (1E+3 IU/mL, c). Scale bars = 50 µm.

### 4. DISCUSSION

#### 4.1. Classification of aggregates according to their degree of aggregation revealed by orthogonal techniques

Radar chart representation was used to summarize all orthogonal techniques employed in this study. These methods are well-known and benchmarked technologies in the field of biophysical characterization of proteins. However, usually the results are directly presented as individual spectra and conclusions are drawn for each technique separately. In this study, we propose to combine all these techniques and define a classification of aggregates based on combined objective data, allowing the reader to obtain an overview of the type of aggregates present. The idea was to link the axes of this radar chart to representative main parameters associated with structural and conformational changes related to aggregation phenomena. Axes and their scales were determined for rhIFNα2b, but can easily be adapted depending on the type of protein studied (small proteins, fragments of mAbs or full antibody). For example, CD values may be monitored at another wavelength for proteins primarily composed of β-
sheets, and similarly $\lambda_{\text{max}}$ can be redefined experimentally for any other protein. Moreover, we have chosen a whole set of orthogonal methods, but other techniques may be used to characterize those aggregates further. The concept might be generalized selecting any set of axes that are representative of conformation changes linked to aggregation.

We noticed the effect of concentration, in accordance with the conclusions of Wang, et al. describing three possible consequences of an increase of protein concentration: either a reduction of aggregation owing to crowding effect, or an extended aggregation due to extended chance of association, or a precipitation due to solubility limit. The present study reflects clearly the second situation obtaining a maximum stressed-to-native ratio almost twice higher for the 0.5 mg/mL concentration than for the lowest concentration tested.

From our model we were able to define three classes of aggregates at 0.5 mg/mL: limited aggregation rate with a stressed-to-native ratio below 10; moderate aggregation rate with a ratio between 10 and 30; and strong aggregation accompanied with full denaturation at ratios above 30.

As aggregation is a concentration-dependent phenomenon and taking into account the standard deviation of measurements that can be important with laser- and optical-based techniques, we were not able to establish significant classes of aggregates at a concentration of 0.1 mg/mL. The same concentration limitation was encountered for AF4 analyses of native and aggregated rhIFNα2b. However, AF4 results obtained at the concentration of 0.5 mg/mL led to similar conclusions in terms of classification of aggregates. Modifications in the separation protocol and usage of a lower MWCO membrane might further improve detection and analysis of rhIFNα2b with this technique.

### 4.2. Impact of aggregation on protein bioactivity

Antiviral bioactivity assay showed that native rhIFNα2b was as effective in an in vitro infectivity assay as the commercially available product. Evaluating aggregates created under a variety of stress conditions, it might be concluded that stresses leading to limited and moderate aggregation (stirring and MCO) conserved a good bioactivity in vitro. This might be explained by the formation of reversible aggregates that can recover their native conformation upon serial dilution, such as applied during the bioactivity assay. In fact, modification of the protein concentration, pH or salt concentration can induce important
changes in the reversible self-association equilibrium of rhIFNα2b as shown by Li, et al. More specifically for MCO stressed samples of rhIFNα2b we observed that the slight decrease in helical content did not lead to a loss of bioactivity, as described previously by Torosantucci et al. Conversely, the strong aggregation obtained upon thermal stresses showed significant loss of bioactivity even after dilution. More than aggregation, this shows evidence that the denaturation of the protein structure under these stress conditions is not reversible.

5. CONCLUSIONS

A multi-parametric characterization of a therapeutic protein and its aggregates formed upon application of various stresses is presented. From all orthogonal methods used we chose representative parameters and displayed them on radar charts. This representation may be applicable to other proteins and not exclusively to our specific model protein IFNα2b. The results allowed us to define a new simplified and easy-to-read representation of aggregation patterns. Radar charts ratio of the occupied area were determined in order to delimit a classification composed of three classes (or degree of aggregation).

Aggregation obtained from mechanically stressed samples was, depending on stress duration, limited to moderate. Oxidation by chemical treatment gave mild aggregates and thermal-stressed samples were partially or totally denatured. Limited and moderately aggregated proteins preserved their bioactivity, suggesting the reversibility of the aggregation process for these specific stress conditions. In contrast, thermal stress led to irreversible aggregation and denaturation of the protein, and therefore to a loss of antiviral activity.

Application of various stress conditions leads to completely different kinds of aggregation, evidenced by the statistically significant changes of patterns obtained on radar charts that are highly dependent on the concentration of the protein solution.

In further studies, one can imagine going beyond and decipher the link between these different classes of aggregates and their immunogenic potential in vitro using APCs, such as dendritic cells.
ACKNOWLEDGMENTS

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REFERENCES


SUPPLEMENTARY FIGURES

Figure S1. Production of recombinant human IFNα2b. (a) Process histogram plot of a 20 L fed batch process for the production of soluble rhIFNα2b in E. coli SHuffle T7 Express (pET23d(+):H6rhIFNα2b, pMJS9) in LB with 0.5 % (w/v) glucose, 75 µM ampicillin, 17 µg/mL chloramphenicol at 30°C. 10 L medium were inoculated at OD$_{600}$nm of 0.5. Employing a constant air flow of 6 slm dissolved oxygen (DO, pO$_2$) was limited to 60 % by variable stirrer speed. pH was maintained at pH 7.0 by addition of 1 M H$_2$SO$_4$ or 1 M NaOH, respectively. At OD$_{600}$nm = 1.96 (165 min) a bolus feed with 10 L process medium including glucose and antibiotics was added. The pre-expression of ErvP1 and PDI was induced by 0.25 % (w/v) L-arabinose for 30 min starting at 195 min. 1 mM IPTG was added 30 min after addition of L-arabinose (220 min). Bacteria were harvested after 7.25 h at 5,000 x g. (b) Coomassie stained SDS-gels of whole cell extract (upper panel), soluble and particulate fraction (lower panel) generated from samples taken at given time points (see OD$_{600}$nm in a) during fermentation. 2 mL samples were lysed by freeze/thawing at -80°C and subsequent incubation with 250 U activated benzonase and 0.1 µg/mL lysozyme in 500 µL lysis buffer followed by ultrasonic sound treatment and separation of soluble and particular fraction at 16,000 x g. After denaturation in reducing SDS loading buffer 20 µL were loaded per lane. (b, lower panel) Western blot with anti penta His antibody was performed to verify the identity of the recombinant molecule. (c) Exemplary FPLC chromatogram of affinity purification of 50 mL soluble protein fraction on a 5 x 5 mL stacked HisTrap™ FF Crude column. The column was equilibrated with 20 mM Tris, 200 mM NaCl, pH 8.15 at 238.7 cm/min. 50 mL soluble protein fraction was loaded. The protein was eluted using a linear gradient from 100 mM to 500 mM imidazole in 20 mM Tris, 200 mM NaCl; pH 8.15 over 2 CV at 89.5 cm/h. 45 mL eluate per run was collected in 80 mL 20 mM Tris, 100 mM NaCl, pH 8.15 at increasing stirrer speed from 100 to 125 rpm. (d) Coomassie stained SDS-gel of 4 µL flow through (FT) and 4 µL of the main eluate peak fractions (Elu1 = increasing peak, Elu2 = peak maximum including right shoulder; M, prestained protein ladder 10-180 kDa). (e) Coomassie stained SDS-gel of 4 µL 1:5 diluted purified rhIFN under denaturing, reducing conditions (Elu, elution fraction; M, prestained protein ladder).
CHAPTER 1 - Multi-parametric Evaluation of Therapeutic Protein Aggregation
Figure S2. Fractograms obtained after AF₄ analysis of rhIFNα₂b at native state (a) and after the five stress conditions (b-f). For each sample the corresponding blank signal was previously subtracted. Red line shows signal from MALS detector and blue line displays signal from UV detector.
Figure S3. Transmission electron microscopy images obtained after negative staining of formulation buffer (a) and native rhIFNα2b stored at 1 mg/mL (b) showing more condensed background and a 250 nm aggregate (white arrow). At the same concentration (1 mg/mL), another large aggregate of around 340 nm is observed (c) composed of small subunits with a mean size of 50 nm. Native rhIFNα2b at 0.5 mg/mL (d) gave a background of 20 nm size with larger structures measuring from 100 nm (white arrow) to 240 nm (black arrow), at a lower concentration of 0.1 mg/mL (e) it presented only small white dots in the background of around 8 nm. Scale bars = 200 nm.
In the previous chapter, we have described the various environmental stress conditions that can be used to create protein aggregates, and the relationship between the degree of aggregation and the impact on the protein bioactivity. This last chapter achieved the first part of our aim - to study the link between stability/aggregation and immunogenicity of therapeutic proteins. In the following chapter, we will focus on the second part of this objective looking for assays that allow to predict the potential immunogenicity of new candidate therapeutic proteins. This literature review will compile regulatory authorities’ recommendations, two-dimensional and three-dimensional in vitro assays designed for this purpose. We will examine here potential key elements, which should be taken into account in order to develop (chapter 3) our own in vitro model.
ABSTRACT

Immunogenicity assessment of therapeutic proteins is routinely performed through various techniques during the drug development process: (i) in silico to design the least immunogenic protein possible; (ii) in vitro using mainly classic 2D assays with PBMC-derived cells or immune cell lines to follow the protein uptake, immune cell maturation induced and pro-inflammatory cytokines released; (iii) in vitro increasingly using 3D models of the human immune lymphatic system or full-thickness skin; (iv) and finally in vivo with preclinical and clinical studies. This review focuses primarily on the immunogenicity assessment of therapeutic protein injected subcutaneously and new in vitro models that may be used to mimic specifically this tissue.

KEYWORDS therapeutic protein; immunogenicity assessment; in vitro model; subcutaneous injection; injection site reactions

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1. INTRODUCTION

1.1. Immunogenicity definition - Human immune system

Biopharmaceuticals such as therapeutic proteins represent an increasing percentage of new drug approvals. A total of 239 therapeutic peptides and proteins were approved by the US Food and Drug Administration (FDA), formulated in 340 commercialized drug products, among which 116 (35%) are subcutaneously administered.¹ The European Medicines Agency (EMA) for her part approved around 100 biotech-derived medicines over just the last 5 years.² These therapeutic proteins are targeted treatments for chronic and/or life-threatening diseases such as diabetes, cancer, autoimmune diseases (e.g., multiple sclerosis) and enzyme deficiency replacement therapies. As all powerful tools, the use of biologics can lead to - although rare nowadays³ – serious adverse events. One of those is the stimulation of an immune response that can appear after a single or repeated injections of numerous therapeutic proteins.⁴ Conversely to vaccines, immunogenicity of therapeutic proteins is an unwanted effect as it may result in the formation of antidrug antibodies (ADAs), which could negatively impact the efficacy and safety of the treatment. Indeed, ADAs may have a neutralizing activity by binding to the active site of the protein and may induce various degrees of hypersensitivity reactions ranging from injection site reactions (ISRs) to anaphylaxis. This latter type of severe reactions, type I hypersensitivity or allergic reactions, are linked to the production of specific immunoglobulin E (IgE) and the rapid release of histamine, whereas mild type III reactions involve the formation of immune complexes of protein surrounded by ADAs.⁵ A classification of all the adverse drug reactions encountered following recombinant protein injection was already described by Murcada, et al.⁶ and Corominas, et al.⁷ As thoroughly described in the recently revised EMA guideline on the immunogenicity assessment of therapeutic proteins,⁸ the immunogenic potential of a protein is directly linked to product factors such as the protein structure and its post-translational modifications, the presence of aggregates and impurities, dose, route and frequency of administration. There are also patient-related factors like disease state, presence of pre-existing antibodies and genetic background that contribute to the immunogenicity of therapeutic proteins.⁹⁻¹¹ The presence of aggregates is one major source of immunogenicity resulting from the creation of neoepitopes in comparison to the protein in its native state. These aggregates can
be formed during the manufacturing process, transport, storage and (mis)handling. In addition, destabilization due to important changes in its microenvironment during injection can further create aggregates. While the factors related to manufacturing and handling can be easily avoided by optimization of the formulation composition, changes in the manufacturing process, detection using orthogonal characterization methods, and patient instructions. Formation of aggregates after subcutaneous injection on the other hand is much more difficult to predict and identify. The occurrence of immunogenicity of therapeutic proteins in relation to their propensity to form aggregates was detailed in a review by Moussa, et al.

The innate and the adaptive immune system are both implied in the immune response against therapeutic proteins via their recognition and uptake by professional antigen presenting cells (APCs). APCs are macrophages or dendritic cells (DCs) that will phagocytose (or endocytose) and process the protein, to finally present the antigen by virtue of MHC class II proteins at their surface, leading to the activation of CD4+ T cells. Dendritic cells are present as sentinels in all types of tissues, and once matured are able to migrate through the lymphatic system to the lymph nodes and lymphoid organs. Immunogenicity can also be raised by a T cell-independent pathway when B cells encounter directly the antigen, such as aggregate neoepitopes or post-translational modifications. B cells maturation in plasma cells is induced and will lead to the release of ADAs. In both responses, the presence of repetitive neoepitopes induces the breaking of immune tolerance. It can be either the central tolerance linked to B cells, or the peripheral one maintained by specific T lymphocytes, called regulatory T cells or Tregs.

Therapeutic protein immunogenicity prediction is currently a major issue investigated by academic and industrial research groups, and regulatory organizations. Multiple approaches have been considered to be applied during drug development, preclinical and clinical phases, and post-marketing surveillance. Among them are in silico prediction methods, in vitro cell-based assays and in vivo assessment of ADAs and NAbs from blood samples of animal and human clinical trials. In silico and in vivo methods will be briefly described in the introduction, before focusing more specifically on in vitro approaches and notably the design of 3D cell cultures to investigate subcutaneous immune reactions.
1.2. *In silico* prediction

*In silico* immunogenicity prediction tools are based on amino acid sequence analysis of new protein drug candidates. Algorithms developed are in perpetual improvement supported by their concomitant use for vaccine design. Many models now offer good estimations of the immunogenic potential of a protein, mainly identifying HLA-II peptide epitopes and evaluating their binding affinities. B cell epitope binding remains much more complex to predict because it implies the recognition of nonlinear parts exposed by the three dimensional refolding of the protein. This can be related to the neoepitopes formed by aggregates, which involve different conformational structures adopted by the aggregation of protein monomers between them or by involving excipients and/or impurities such as leachables.

Bryson, et al. comprehensively reviewed the available immune epitope database and software. To cite a few more, Epivax Inc. developed an algorithm called EpiMatrix to predict MHC class I and II epitopes, Lonza’s Epibase® *In Silico* platform allows for high throughput screening of peptides binding to the HLA receptor, and ProSentium™ database was established by ProImmune Ltd.

The (bio-)informatics field is in constant evolution and the most efficient computational tools are now also taking into account the refolding and potential conformational changes of the protein, enabling a better prediction. These software and databases are useful during the early discovery phase to select lead candidates, but also to design less immunogenic biotherapeutics. However, these computational tools still have limitations. As they tend to be overpredictive, *in vitro* confirmation of epitopes identified are needed and conversely the limited number of HLA class II alleles tested do not completely reflect the important polymorphism found in the human population.

1.3. *In vivo* preclinical and clinical assessment (ADAs and Nabs assays)

Later in the development process, immunogenicity assessment is done by detection of ADAs in blood samples collected during preclinical and clinical phases. A number of *in vivo* animal models were developed in rodents with HLA-transgenic, humanized, and human severe combined immunodeficiency (SCID) mouse models, using minipigs or non-human...
primates. Despite tremendous efforts to create better predictive animal models, a poor correlation of immunogenicity prediction was noticed between these animal models and the results of clinical trials on humans for FDA approved therapeutic proteins. Besides the fact that none of them fully reflects the complex functioning of the human immune system, the enforced application of 3R’s principles pushed more and more industries to implement new strategies (see paragraphs on in vitro assays and 3D models below).

A current approach in immunogenicity assessment during clinical investigations consists of a first row of “ADA screening assays” to determine the presence or absence of circulating ADAs after treatment with the biopharmaceutical, followed by a “confirmatory assay”, which if revealed to be positive will be later accompanied by a “characterization assay” defining the neutralizing ability of these ADAs. Various techniques are used for the readout of ADAs assays, the most common being enzyme-linked immunosorbent assays (ELISA). Evolution of immunogenicity assays using different detection methods like “direct, indirect or capture assays, electrochemiluminescence (ECL) assays and antigen-binding tests, such as radioimmunoassays” were recently summarized by Pineda, et al. The authors report on the difficulty in obtaining harmonization between clinical trials and their immunogenicity assessment results due to the inter- and intra-variability of existing assays. Moreover, regulatory authorities and pharmacopeias are now considering a risk-based approach concerning the testing for the creation of ADAs. Kinetics of appearance of ADAs should be considered, as well as whether the immune response is transient or persistent and related or not with clinical sequelae. In this way, number of patients, ADA sample collection time and duration should be carefully planned just like the design and validation of the ADA assay (format, cut-point, sensitivity, reproducibility, etc.).

In terms of clinical ADA monitoring, the present common sample collection strategy consists of a systematic collection and ADA testing of all patients in each clinical trial. But a recent paper by Amgen and Merck researchers suggests a new “event-driven” strategy for therapeutic proteins at low immunogenicity risk. In this approach, collected samples would only be analyzed by ADA assay in case of safety issues. Alternative strategies, such as a “fit-for-purpose” approach, for immunogenicity testing of biotherapeutics have also been proposed by other contributors.

Over the last couple of years, in addition to regulatory guidelines, many white papers by industry consortia and research articles had convergent interests in the elaboration of ADAs assays and development of novel techniques. Refinement of these protocols could have a
significant influence on the immunogenicity results obtained.\textsuperscript{39, 40} The necessity of having comparable approaches for immunogenicity assessment of biosimilars is frequently mentioned due to the rising number of biosimilar approvals, first in Europe and now followed by the United States.\textsuperscript{41} Those papers address problems such as the evaluation and confirmation of cut-points establishing the titer threshold between positive and negative samples in ADAs assays,\textsuperscript{38} drug-target interferences and their impact on results,\textsuperscript{34} and impact of the choice of storage buffers used for conjugated reagents on long-term performance of ADA methods.\textsuperscript{35} Advantages of emerging new technologies compared to current ADA assays,\textsuperscript{37} and comparison of injection site reactions incidence after biosimilar or reference drug administration are also discussed herein.\textsuperscript{42}

The ABIRISK (Anti-Biopharmaceutical Immunization: prediction and analysis of clinical relevance to minimize the RISK) consortium gathers universities, institutes, industrial researchers and clinicians from Europe to focus in particular on the correlation between patient factors, clinical factors and the incidence of immunogenicity.\textsuperscript{43} Recent papers from this group show clinical examples of immunogenicity assessment of biopharmaceuticals (infliximab, interferon-beta and natalizumab), attempting to establish a link between ADA responses, hypersensitivity reactions and the presence of detectable circulating drug-specific T cells.\textsuperscript{39-40, 44} This results in a new database platform (tranSMART) compiling data of multiple sclerosis cohorts and ADA test results to be compared on a European level.\textsuperscript{39}

Evaluation of immunogenicity through ADA detection and (semi-) quantification is only one part of the elucidation of the clinical manifestations, but the characterization of their neutralizing ability is also of importance regarding the safety and efficacy of therapeutic proteins. Actually, the subgroup of neutralizing antibodies (NAb's) has a direct impact on the “loss of drug efficacy by blocking the biological activity of a therapeutic product” as stated by Wu, et al.\textsuperscript{45} Two different formats exist for these assays (cell-based or non-cell-based assays) and as for the ADA assays there is currently a lack of harmonization despite the diverse national and international guidelines edited.\textsuperscript{8, 30, 31, 46-48} Although the incidence of immunogenicity is well disclosed in the prescribing information, few report on the impact of immunogenicity on the protein’s pharmacokinetic profile.\textsuperscript{49} Similarly, Shankar, et al.\textsuperscript{5} highlighted the fact that current drug package inserts do not clearly inform physicians on how to manage immunogenicity and related adverse events in the clinic.

Finally, among all clinical studies using ADA and NAb assays to assess immunogenicity, one aspect rarely taken into consideration within the same clinical trial and therefore using
exactly the same assay design is the influence of the route of administration. A well-known dogma classified subcutaneous (SC) and intramuscular (IM) injections as being more immunogenic routes than intravenous (IV) infusion. However, there is a common interest of patients, physicians and industry to develop novel subcutaneous formulations to improve compliance via self-administration, ease and rapidity of the medical intervention. Moreover, some IV administered proteins such as trastuzumab and rituximab are now approved for SC administration in Europe. Hamuro, et al. recently published perspectives on the SC route of administration as an immunogenicity risk factor for therapeutic proteins, assembling clinical immunogenicity data of six commercialized products for which a comparison of ADAs and NAb levels obtained after administration by the SC or IV route was performed. Factors affecting the immunogenicity of both routes were listed, such as formulation composition, therapeutic indication and disease state of the patient, mechanism of drug action (immunosuppressive or not), dose and frequency of dosing, concomitant medication, and blood sample timing. In their conclusion, authors highlighted the knowledge gap concerning possible differences between the two parenteral routes of injection in the pathways followed by the immune reaction. ADA incidence was observed to be similar or up to two-fold increased in SC injection compared to IV, however, formulated protein concentrations tested were not the same.

1.4. Regulatory requirements (FDA/USP – EMA/Ph.Eur.)

As already mentioned above, the regulatory authorities and organizations (EMA, FDA, European and US pharmacopoeias, ICH) publish regularly new or revised guidelines on this immunogenicity issue to help sponsors to fulfill efficacy and safety requirements to be granted marketing authorization and later to perform post-marketing surveillance.

Concerning the FDA, we have seen in the previous section that their recommendations are related to patient and product-specific factors to be considered for a reliable immunogenicity assessment during the clinical phase, and to the critical points for ADAs and NAb assay development and validation to investigate immune response during phase I clinical trials. With respect to preclinical studies, guidance mentioned that in vivo animal models are not necessarily representative of future immunogenicity in human due to species differences, although useful information such as possible consequences of inhibition of an endogenous protein or identification of aggregate species that have the potential to be immunogenic can
be obtained. Unfortunately, there are no recommendations regarding the early prediction of immunogenicity during the research and development phase, as well as no (short term) economic incentive, which could encourage the development of new reliable *in vitro* immunogenicity assays, other than assays involving peripheral blood mononuclear cells (PBMCs).

As mentioned previously, the EMA guidelines on immunogenicity assessment focus on the clinical assessment and factors that may influence the development of an immune response against a therapeutic protein (patient- and disease-related factors, product-related factors). Potential clinical consequences of immunogenicity on efficacy (ADA and NAb assays) and safety (acute, delayed, and/or autoimmune reactions) are comprehensively described. There is also an observation that non-clinical assessment (i.e. preclinical studies) of immunogenicity has limitations and their predictivity in humans is considered to be low. Moreover, it is stated that “non-clinical *in vitro* or *in vivo* studies aiming at predicting immunogenicity in humans are normally not required”, however, “ongoing consideration should be given to the use of emerging technologies […] *in vitro* assays based on innate and adaptive immune cells could be helpful in revealing cell-mediated responses”.

While the European Pharmacopeia (Ph. Eur.) considers only immunogenicity testing in the context of vaccine products, and the only defined assay for recombinant therapeutic proteins concerns the detection of host-cell proteins contaminants, the USP dedicates two complete sections (<1106> and <1106.1>) to this issue. A consensus appears between regulatory agencies that *in vitro* early assessment of immune response to therapeutic proteins using cell-based predictive assays is not yet sufficiently reliable to be incorporated in their guidelines. This motivates the search for more predictive immunoassays.

### 1.5. Need for early prediction in the drug development process

In the previous section, the *in silico* tools available to predict immunogenic epitopes and overall immunogenicity potential of a protein from its amino acid sequence were described. However, all the results obtained through these methods must still be confirmed by *in vitro* assays before engaging in preclinical studies.

Subsequently in the development process, preclinical studies are classically conducted on animal models of different species (wild type or transgenic rodents, minipigs and non-human
primates). Although useful to anticipate strong toxicity problems, regarding prediction of potential immunogenicity issues in humans they either tend to be over predictive (for most human recombinant proteins) or under predictive.\textsuperscript{57} Suitability of various animal models to correctly predict immune response in humans was discussed in detail by Brinks, et al.\textsuperscript{58} Without mentioning the important differences that were found between immune systems in human and animal models, these studies evaluated protein immunogenicity using the same ADA/NAbs assays performed during human clinical trials that were shown previously still to be in need of improvement, notably regarding their reproducibility.

In order to complement \textit{in silico} prediction and avoid over- or underestimation of safety risks in preclinical studies such as seen in the example of TGN1412,\textsuperscript{57} there is a clear need of an early and better immunogenicity prediction using reliable \textit{in vitro} models. Such models, in addition to allowing for high-throughput screening, are in line with the 3R’s principle and may also enable a better understanding of the human immune system in the long term. We will describe in this review the different standard 2D \textit{in vitro} assays currently used for immunogenicity assessment of biopharmaceuticals, their strengths and limitations, and the appearance of new 3D \textit{in vitro} models trying to better mimic the human immune system. All this keeping in mind the particular interest in the prediction of immunogenicity and clinical sequelae after injection into the subcutaneous tissue.

\textbf{2. 2D IN VITRO ASSAYS}

\textbf{2.1. Primary cell-based assays}

Most of the \textit{in vitro} biological assays performed to predict and assess immunogenicity of therapeutic proteins use human primary immune cells. Isolated from whole blood donations, PBMCs comprise lymphocytes (T cells, B cells, and NK cells), monocytes, and few dendritic cells. They are generally obtained from naïve healthy donors, however, samples from specific subpopulation of patients (antigen-exposed or not) can be of interest to study disease-related immunogenic reactions.

These primary cells can be used as whole PBMCs or be further purified to isolate specific subsets such as CD\textsubscript{4+} or CD8+ T cells. Monocytes can be isolated by positive selection using the expression of their specific marker, CD\textsubscript{14} in humans. Subsequent differentiation can be induced by addition of targeted cytokines like GM-CSF and IL-4 to obtain monocyte-derived
dendritic cells (MDDCs or MoDCs), or M-CSF to get monocyte-derived macrophages (MDM). Finally, different subpopulations derived from PBMCs can be mixed together at defined ratios to mimic the whole human immune system in one cell culture plate.

As each human donor has their own genetic background, PBMCs will express different major histocompatibility complex (MHC or HLA) proteins on their surface. Therefore, HLA typing is necessary. To reflect immune reactions from the whole population a large number of donors will be required. Usually, PBMCs from 30 to 50 donors are pooled together to cover around 80-95% of the most frequent human HLA class II haplotypes (HLA-DR, HLA-DQ, HLA-DP).

The use of whole PBMCs incubated with the therapeutic protein allows for a complete immune response simulation but is limited to drugs that will not negatively impact cell proliferation (inadequate for immunomodulatory drugs). In this specific situation, a 2-step assay format is preferred to enable first the APCs loading with the antigen, followed by an interaction between APCs and T cells.

Concerning immunogenicity assessment of biologics, we can distinguish four types of assays based on the (subsets of-) cells used:

- Whole PBMC
- CD4+ or CD8+ T cells
- Monocyte-derived dendritic cell (MoDCs)
- Mixtures of MoDCs and T cells, using different ratios

These primary cells allow to perform different types of assays reflecting various steps of the whole pathway of T cell mediated immune response from the antigen uptake by professional APCs to T cell proliferation.

### 2.1.1. HLA binding assays (class I and II)

In complement to in silico identification and evaluation of the binding affinity of HLA-II peptide epitopes, in vitro assays can be performed using purified HLA-II peptides (usually 15-mers with an overlapping region) of the protein of interest. Direct quantification of binding affinity and kinetics is realized employing surface plasmon resonance (SPR) or biochemical assays like ELISA. This latter technique used in 384-well plate format and with the assistance
of a liquid handling robot allows for the high throughput screening of peptide-MHC II binding assays.\textsuperscript{65}

Despite being more rarely set-up due to inherent difficulties linked to the high polymorphism of MHC class I molecules and structure/conformation influence on binding affinity, MHC-I peptides binding assay was designed using the same high-throughput approach combined with a technology called luminescent oxygen channeling immunoassay (LOCI or AlphaScreen\textsuperscript{66}) or FACS-based MHC stabilization assay.\textsuperscript{67}

Several other assays and techniques may be used, like competition assays allowing kinetic measurements and identification of CD4+ T cell epitopes\textsuperscript{68}, or even cell-based assays where MHC I-peptides bind directly HLA-typed human B-cell lines.\textsuperscript{59}

However, those previous approaches are biased by the presentation of all potential MHC II-peptides extracted from the protein sequence, without considering the natural enzymatic processing of the protein inside APCs. Thus, another approach consists in extracting and differentiating immature MoDCs from PBMCs and incubating them with the whole antigen. After this interaction with the protein of interest, matured MoDCs are harvested and HLA-peptides are purified and analyzed by mass spectroscopy sequencing.\textsuperscript{70}

### 2.1.2. T cell activation and proliferation

Following the T cell dependent immune response, after therapeutic protein uptake and process by APCs, the specific MHC-II peptides and co-stimulatory molecules expressed at the APC surface are available for recognition by T cells receptors (TCR) inducing the proliferation of T cells. In \textit{in vitro} PBMC assays, the antigen priming of CD4+ T cells by mature MoDCs leads to the proliferation of CD4+ T cells, which can be followed by radioactive labeling with tritiated thymidine pulsation and scintillation counter,\textsuperscript{61, 63, 71} or by using fluorochromes like carboxyfluorescein succinimidyl ester (CFSE).

MoDC and T cell activation are also commonly recorded via flow cytometry analysis of surface markers whose expression is down- or up-regulated. MoDCs exposition to antigens, such as aggregates of therapeutic proteins, may induce an up-regulation of the activation and maturation markers CD40, CD80, CD83, CD86, CD209, HLA-DR and a change in morphology.\textsuperscript{72, 73} Phenotypic changes of T cells are registered through modification of expression of co-stimulatory CD40-ligand, CD25, CD46 and CD69.\textsuperscript{74}
A recent publication from Schultz, et al.\textsuperscript{71} describes a novel \textit{in vitro} T cell assay combining CD\textsubscript{4}+ T cells purified from PBMCs and the remaining PBMCs which were irradiated. Irradiation inhibits cell division and guarantees that the proliferation and cytokines released were exclusively linked to CD\textsubscript{4}+ T cell. Four commercialized therapeutic monoclonal antibodies showing CD\textsubscript{4}+ T cell-dependent immunogenicity in the clinic were used to successfully validate this optimized PBMC:T cell assay.

\textbf{2.1.3. Cytokine release}

Consecutive to the co-activation of DCs and T cells, pro-inflammatory cytokines such as TNF\textalpha, IFN\gamma, IL-2, IL-4, IL-6, IL-8 and IL-10 are released. The detection and quantification of these cytokines can be carried out by testing cell culture supernatants with ELISA\textsuperscript{75} or cytometric bead array (Luminex\textsuperscript{R} Multiplex Assay),\textsuperscript{64,73} or by incubation of antigen-stimulated cells on enzyme-linked immunospot (ELISPOT) plates.\textsuperscript{62,64,71} The secretion of pro-inflammatory cytokines is involved in the induction of (allergic) immune reactions to foreign proteins at the injection site.\textsuperscript{75}

To conclude, \textit{in vitro} immunogenicity prediction assays like PBMC assays described above offer advantages to design high-throughput assays testing a part or the whole process of T cell dependent immune response, with more than one assay condition, and to confirm peptide-HLA complexes identified \textit{in silico}. However, to develop reliable assays it remains challenging to optimize the protein concentration and number of challenges necessary to induce T cell proliferation, as well as the number of cells and ratio between T cells and DCs.\textsuperscript{62} Moreover, it requires pooling PBMCs from a large number of donors, which is expensive and requests time-consuming standardized procedures for sampling, extraction, cell counting, freezing and quality controls. This may explain why some specialized companies offer now to supply PBMC primary cells differentiated in DCs (Poietics\textsuperscript{TM}, Lonza), or to provide services of immunogenicity prediction with their own \textit{in vitro} assay like ImmunXperts SA, Antitope Ltd (EpiScreen\textsuperscript{TM}), Lonza (EpiBase\textsuperscript{TM}), and ProImmune Ltd (REVEAL\textsuperscript{R}).\textsuperscript{71}
2.2. Immune cell lines

Challenges associated with the procurement and handling of primary cells encouraged some research groups to find a more reliable and readily available source to perform immune prediction experiments. Moreover, since the new European Union regulation (1223/2009) is abrogating animal testing for safety assessment of cosmetic products and due to the rising interest for nanomaterials, the industry has now turned to a variety of immune cell lines to identify skin sensitizers, to develop DC vaccines for cancer immunotherapy, or to detect immunogenicity of impurities in therapeutic proteins.

Most of these immune cell lines are (myelo-)monocytic cell lines obtained from patients suffering from acute or chronic leukemia. Following exposure to cytokines or other signals (PMA, DMSO, 1,25-dihydroxy-vitamin D3), they are able to differentiate into monocytes, DCs, macrophages, and granulocytes. Properties and phenotypes of THP-1, KG-1, HL-60, Mono Mac 6 (MM6), K562 and MUTZ-3 cell lines were described, and studied by Santegoets, et al. seeking for human DC cell line differentiation models to study DC vaccination. The majority of those DC models display phagocytosis and other phenotypic and functional DC characteristics. Among them MUTZ-3, which stands out by its cytokine-dependence, can be differentiated either into epidermal DCs also called Langerhans cells (LC), or into interstitial DCs (IDC) found in the dermis as well as throughout the body. MUTZ-3 cells exhibit a specific DC phenotype and are able to mature, while upregulating the expression of costimulatory molecules and maturation markers mentioned earlier (CD40, CD80, CD83, CD86 and HLA-DR).

One DC cell line model, U-937, can be distinguished by its histiocytic lymphoma origin. U-937 cells display monoblastic morphology and are not capable of phagocytosis. However, these cells can be differentiated into DCs or macrophages, and were successfully used to study skin sensitizers. In the field of chemical sensitizers, THP-1 and MUTZ-3 cell lines were compared to primary monocyte-derived DCs (MoDCs) in terms of biomarkers expression and cytokine release. The authors showed that CD86 DC maturation marker was expressed by all cells after stimulation with contact allergens, and concluded that dendritic cell line models “mimic primary DCs in many aspects”. Nevertheless, their use should remain a “case-by-case decision” depending on the selected biomarker measured. Another study comparing THP-1, HL-60 and MUTZ-3 human DC cell line models revealed that, with its ability to take up and present antigens through expression of MHC class I and II molecules, and to mature and
adopt a migratory phenotype, MUTZ-3 derived DCs were the ones which “most closely resemble primary DCs”.\textsuperscript{80}

One main argument against the use of cell lines instead of primary cells would be their inability to bind diverse HLA I and II peptides. However, MUTZ-3 cell line was proven to be positive for antigens HLA-A2, HLA-A3, HLA-B44, HLA-DR\textsubscript{10}, HLA-DR\textsubscript{11}, HLA-DR\textsubscript{52}, HLADQ\textsubscript{5}, and HLA-DQ\textsubscript{7}.\textsuperscript{81} More information on studies using the MUTZ-3 cell line model for detection of skin sensitizers,\textsuperscript{82-87} vaccine development,\textsuperscript{88} or to induce antitumor T cell immunity can be found in literature.\textsuperscript{89}

Other macrophages or DC cell line models exist as shown in a recent paper by Haile, et al.\textsuperscript{90} Some of them were used to detect product and process impurities present in therapeutic protein formulations that could induce innate immune response. The authors, employees of FDA, used murine macrophages (RAW 264.7), human embryonic kidney cells (HEK293) transfected with toll-like receptors (TLRs), and MM6 and THP-1 models instead of highly variable PBMCs, to detect host-cell impurities activating innate immune response present into biotherapeutics (even with immunomodulatory effect). HEK-BLUE expressing human TLRs 2, 4, 5, 7 and 9 were used to compare level of sensitivity to PBMCs and identify receptor-specific impurities. Then, monocyte or macrophage cell lines with different readouts were used to screen impurities without knowledge of their nature and TLR activation. Later their sensitivities to known impurities were compared to PBMCs, and similar sensitivity was observed when the three human cell lines were combined, except for two ligands (Poly I:C (TLR3) and MDP (MDP-NOD2)).

The outcomes obtained so far should encourage other research groups to investigate the use of these readily available and constant cell line models during drug development. They might also serve in later stages for quality assurance purposes.

2.3. Triple co-culture

As seen in the two previous sections, primary cells and immune cell line models are used in 2D \textit{in vitro} assays to predict immunogenicity of biotherapeutics and other chemical products. Another type of assay, which has a supplementary degree of complexity, is the triple co-culture of cells of different origins to recreate immune function of specific human parts, organs or epithelium. For instance, a 3D model of the human epithelial airway was designed
as a triple cell co-culture system combining lung epithelial cell lines (A549 and 16HBE14o-) and primary cells from PBMCs (MDDC and MDM). This model allows the investigation of the interaction between the different cells, as well as the cellular immune response upon xenobiotic stimulation.

Another triple co-culture model was used by Saalbach, et al. to study T cell-mediated immune response in the dermis, where primary fibroblasts obtained from skin biopsies were cultured with PBMC-derived DCs and T cells. In order to model another inflamed epithelium to study safety of nanomaterials, Susewind, et al. combined intestinal colon-colorectal carcinoma (Caco-2) cells with THP-1 and MUTZ-3 cells, which were embedded into type I collagen on an insert well.

Those co-cultures are often grown on microporous membrane, using insert wells to define a two-chamber system, and follow the migration of the immune cells after exposure to “foreign” particles by fluorescence labelling. These 2D in vitro systems are more suitable to mimic the physiological reality thanks to interactions between the different co-cultured cell types.

Major 2D and 3D in vitro models described in the previous and following sections, and their common read-outs are illustrated and summarized in Figure 1.
Figure 1. Processes of therapeutic proteins immunogenicity assessment classified in increasing complexity. Schematic representation of in vitro standard 2D assays with PBMC-derived DC or immune cell line-derived DC suspensions, major in vitro 3D models, and their common read-out technologies described in this review. Artificial lymph node (ALN) bioreactor drawing is adapted from Giese, et al.\textsuperscript{94} PBMC = peripheral blood mononuclear cells; WT = wild type.
3. 3D IN VITRO MODELS

At the interface between 2D in vitro assays described previously and animal models, 3D models are being developed that try to mimic either the lymphatic system, which allows migration of immune cells like DCs and T cells, or to mimic skin and subcutaneous models more specifically of interest for therapeutic protein immunogenicity prediction.

3.1. Artificial lymph nodes

The need for relevant human 3D models allowing to reduce animal studies and better mimicking physiology than 2D cell culture, notably in the immunotoxicology field, has brought the development of organotypic tissues such as artificial lymph node (ALN) to the fore. The main difference between these ALN and the previously described co-culture resides in the addition of a microfluidic system in the (mini-) bioreactors to control nutrient and oxygen supply, temperature and pH. This simulates more closely the physiological environment and gradients of stimuli. The fluidic circulation may also induce important variations in the cell phenotype, due to mechanical forces applied.

A research group of ProBioGen AG (Berlin, Germany) developed an in vitro human lymphatic micro-organoid model in order to perform immunological substance testing, including vaccines. Their model is a combination of a co-culture model and PBMCs based cell material with a microfluidic system. In practice, PBMC derived DCs and T cells are embedded in an agarose matrix while B cells are maintained in suspension in a continuous cycling allowing their interaction with mature DCs and primed T cells. They developed two different kinds of bioreactors allowing micro-organoid formation after 7 days, and cell maintenance over 14 to 30 days, as well as sampling for cytokine analysis and in situ imaging via two-photon microscopy. The authors suggested that their model could be improved by the addition of human cells (from human lymph nodes or bone marrow biopsy), fibroblasts or animal stromal cells. And this has been realized and published recently in a paper by Sardi, et al. where a network of mesenchymal stem cells (MSC)-derived stromal cells was added and appeared to attract PBMCs and enhance the secretion of pro-inflammatory cytokines.

Another in vitro model designed to test immunogenicity of vaccines is the MIMIC® (Modular IMMune In vitro Construct) system well-based format assay. The system is composed of four successive different steps, where the first one is to collect and conserve PBMCs from
blood samples of healthy donors, and the second step is to mimic innate immunity through the preparation of a peripheral tissue equivalent (PTE). The PTE is composed of human umbilical vein endothelial cells (HUVEC) seeded on top of a collagen matrix and upon which PBMC derived DCs are added. The media sampling and measurement of pro-inflammatory cytokine release (IL-1α, IL-1β, IL-6, IL-8, IL-10, TNFα) allow the quantification of the innate immune response. This PTE module was compared to classical PBMC assays and was shown to produce two-fold to 100-fold higher levels of cytokine secretion, thus increasing the assay sensitivity. The third step consists of the simulation of the adaptive immune response via the elaboration of a lymphoid tissue equivalent (LTE). This latter is similar to an ALN using DCs, follicular DCs, T and B cells but applied in a sequential order to mimic the in vivo series of events taking place in the lymphoid tissues. Finally, functional assays are performed to assess whether the in vitro immune response corresponds to the one observed in vivo.

These 3D tissue equivalents may be automated allowing for high-throughput testing of biopharmaceuticals. However, intrinsic inflammation or background noise could be a problem for immunogenicity testing, and ALN essentially mimic the human immune system without taking into account the specificity of the route of administration and the injection-recipient tissue characteristics (e.g., tissue composition after IM or SC injection).

3.2. (Full-thickness) skin models

Increase in the number of subcutaneously injected therapeutic proteins requires the closer examination of the composition, organization and functioning of the subcutaneous tissue. Human skin is composed of three layers: epidermis, dermis, and hypodermis. While the first two have a barrier function against the environment, the role of the subcutaneous tissue is to insulate, to provide energy and absorb physical shock. More than just composed of adipocytes, the subcutis contains also nerves, a network of lymphatic and blood capillaries, few fibroblasts secreting extracellular matrix (ECM) components, and sentinel immune cells (macrophages and DC cells). Moreover, proximity of the dermis may allow migration of dermal dendritic cells when pro-inflammatory cytokines (IL-8 and IL-6) are secreted by the subcutaneous adipose tissue after minimal trauma, such as SC injection. Indeed, defense mechanisms of the skin against pathogen infections include an array of immune cells: LC and epidermal T cells in the epidermis, and different populations of myeloid and lymphoid
immune cells, including three DC subsets, which either reside in or traffic through the dermis. These cells are in constant interaction with the commensal flora, establishing a balance between pro-inflammatory and anti-inflammatory mechanisms resulting in a protective skin immune signature unique to each human.

Human SC tissue is organized in lobules containing adipocytes, which are separated by septa of loose connective tissue in which fibroblasts reside. Its structure is slightly different across species, notably among rodents, which possess a loose connective tissue organized in numerous layers, and a specific striated muscle, called panniculus carnosus, located close to the dermis. These structural differences, which impact the spreading behavior of therapeutic protein solution after SC injection, could also influence their bioavailability, limiting the predictability of animal models. Conversely, porcine skin seems much closer to human skin in its constitution and, more importantly, with regard to immunological responses.

This last decade, the need for economically viable and standardized full-thickness skin models for cosmetic testing also led to a better understanding of skin biology and skin cancer pathology. It also supported the development of treatment strategies for chronic wounds or large burns. These three-dimensional skin equivalent models could be of interest for immunogenicity prediction of therapeutic proteins. Comprehensive reviews on 3D models of epidermis, full-thickness models consisting of combinations of epidermal and dermal equivalents, and more complex models with appendages were published these last years. A non-exhaustive list of these models currently available for clinical or research use and their corresponding references is presented in Table 1. Strictly speaking, some of the models cited here belong to the 2D models as defined in this review. Such models include reconstituted human epidermis composed of keratinocytes seeded on a scaffold, like EpiSkin®, SkinEthic®, and EpiDerm™ as predictive models; or autologous keratinocytes in the form of cell sheets or in suspension as Epicel® and Myskin™ for clinical use. For the treatment of burns, various dermal substitutes are commercially available. Some of them consist of allogenic human (AlloDerm®) or xenogenic porcine or bovine acellular dermis (MatriDerm®); other xenogenic and synthetic substitutes use mainly silicone and atelocollagen (Pelnac™). Full thickness skin models, which combine epidermal and dermal equivalents, most generally use a collagen scaffold seeded with fibroblasts, supplemented after a week by seeding of keratinocytes on top. For instance, Apligraf® is commercialized for ulcer wound healing and Phenion® is an in vitro full thickness model for safety and efficacy assessment.
However, few studies have suggested a three-layer model, i.e. adding subcutaneous fat tissue to existing “full-thickness” skin models. Hypodermis was reconstructed with adipose-derived stem/stromal cells (ASCs) using an adapted self-assembly tissue engineering approach designed by the laboratory of tissue engineering and regenerative medicine (LOEX) at Laval University (Canada), or by embedding them into a collagen scaffold. Another technique was used by Bellas, et al. to create a three-layered engineered skin. A silk scaffold was seeded with ASCs from abdominoplasty and grown for 14 days before combination with dermal and epidermal construct. Since then, many studies for three-layered skin model construction were performed using mature adipocytes, ASCs from rats to decipher their impact on full-thickness skin grafts survival, or bone-marrow derived MSCs and ASCs on a human plasma-based hydrogel.

Immunocompetent skin equivalent models were also constructed by integration of primary cells from PBMCs or immune cell lines described previously (MUTZ-3) in full-thickness dermo-epidermal models. These models allow to screen potential skin sensitizers and to better understand cell signaling and migration of epidermal DC (Langerhans cells) through the dermis.

As described previously, skin tissue engineering, often called “skingineering”, employs scaffolds of different origins (derived from animal tissues, algae, synthetic polymers) and harboring various structural and mechanical properties (pore size, viscoelasticity, biodegradability, biocompatibility). These scaffolds are used as mechanical support for cell growth and sometimes alone for the immediate protection of wounds. They may have an impact on immune cells, naturally present or added into the skin model. It was shown by Park, et al. that biomaterials can induce maturation of PBMC-derived DC, as well as pro-inflammatory cytokine secretion, which affect their endocytic ability. Once co-cultured with autologous T cells, these PBMC-derived DCs in contact with biomaterial films and antigen (ovalbumin) could lead to different immune responses polarized by the release of specific cytokines and the corresponding stimulated T helper type. Thus, careful selection of hydrogel scaffold for the creation of skin model is of importance in order to provide only a mechanical support without influencing the immune response.
### Table 1. Non-exhaustive summary of the tissue engineered skin equivalents commercially available or described in the literature. Cells are considered of human origin, unless otherwise mentioned. Pathological models and complex models containing appendages (hair follicles) are not described. 

**ASCs** = adipose-derived stem/stromal cells. **BM-MSCs** = bone marrow-derived mesenchymal stem cells.

<table>
<thead>
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<th>Layer(s) reconstructed</th>
<th>Scaffold component(s) or presentation</th>
<th>Usage</th>
<th>Cell types</th>
<th>Name/Supplier or Lab</th>
<th>Reference</th>
</tr>
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<td>Research use</td>
<td>Keratinocytes</td>
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<td>Autologous keratinocytes</td>
<td>Myskin®/Regenerlys Ltd, Sheffield, UK</td>
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<td>Spray</td>
<td>Clinical use</td>
<td>Autologous cells suspension, wound healing factors</td>
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<td>Clinical use</td>
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<td>AlloDerm®/BioHorizons, Birmingham, USA</td>
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<td>Decellularized</td>
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<td>SureDerm®/HansBiomed Co., Seoul, KR</td>
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**Epidermal and dermal construct**

| Transport-agar | Research use | Not mentioned | Phenion®/Henkel AG & Co., Düsseldorf, DE | 114, 145 |
| Inert polycarbonate filter | Research use | Keratinocytes, fibroblasts | T-Skin™/Episkin, Lyon, FR | 128 |
| Type I collagen matrix (bovine) | Clinical use | Allogeneic neonatal keratinocytes and fibroblasts | Apligraf®/Organogenesis, Inc., Canton, MA, USA | 112, 146 |
| Type I collagen matrix | Clinical use | Autologous fibroblasts and keratinocytes | denovoSkin™/Cutiss AG, Zurich, CH | 113, 147 |
## CHAPTER 2 - *In Vitro* Models for Immunogenicity Prediction of Therapeutic Proteins

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<th>Autologous fibroblasts and keratinocytes</th>
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<td>Laboratoire d’organogénèse expérimentale (LOEX), Hôpital du St Sacrement, Québec, CA</td>
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<td>IGB and University of Stuttgart, Stuttgart, DE; and School of applied chemistry, Reutlingen, DE</td>
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3.3. Subcutaneous tissue models

As mentioned earlier, human SC tissue is mainly composed of adipocytes separated by a connective tissue and few fibroblasts secreting ECM proteins. A review by Kinnunen and Mrsny recently described extensively the composition of the ECM, as well as its potential interactions with biopharmaceuticals and/or their excipients at the SC injection site. Specific conditions (temperature, pH, interstitial pressure) of this tissue induce important changes in the microenvironment of the injected therapeutic proteins, which are usually formulated in non-physiological buffers for optimal storage stability. The same research group later designed a set-up allowing to mimic SC tissue conditions, using hyaluronic acid and physiological buffer, while following the impact on the injected biopharmaceutical through changes in turbidity, pH and pressure. As claimed in their recently accepted US and European patents, the developed method and apparatus for in vitro modeling of the SC tissue could be modified to offer sterile conditions and completed by the addition of one or several cell lines.

For the immunogenicity assessment of biotherapeutics it could be of interest (i) to model precisely the SC tissue environment, in particular its mechanical properties leading to distention and mechanical constrains during the injection, (ii) to include the immune cell component reacting to the accumulation of high concentration of therapeutic proteins. In addition, tissue-remodeling signals induced by this depot effect and variations in interstitial pressure will be recognized by resident immune cells, which can then mature and activate a local immune reaction. This can be related to what happens when SC fat tissue is extensively remodeled in obese persons. Increase of fat mass alters metabolic and endocrine functions of adipocytes, inducing secretion of adipokines. Release of these cytokines leads to endothelial cell activation, enhancing diapedesis of blood monocytes. SC tissue infiltration by immune cells (mainly macrophages but also CD8+ T cells) correlated with a chronic low-grade systemic inflammation may also stimulate resident macrophages and DCs. Therefore, immunogenicity of therapeutic proteins may be increased in the specific subpopulation of obese patients. Pathologies related to obesity, such as type 2 diabetes, may require daily SC injections of GLP-1 hormone (e.g., Exenatide and Liraglutide) or of recombinant leptin to promote weight loss. This latter was shown to induce an unacceptable incidence of injection site reactions (ISRs), which was more pronounced in the obese population. Knowing that obesity is nowadays an increasing major public health
concern, it is of interest to investigate more specifically the potential immunogenicity of therapeutic proteins used in this subset of patients.

As illustrated before with full thickness skin models, attempts to reconstruct SC tissue using various approaches and techniques were realized these last years and were reviewed elsewhere. All of these studies were aiming to regenerate soft tissue in order to provide a cushioning layer for wound healing or to restore volume after resection of tumors, like mastectomy. Common basis is the use of synthetic or natural biomaterial(s) as scaffolds, on top of, or into which are seeded human ASC isolated from (autologous) liposuction. Some of them use decellularized adipose tissue from human or animal sources, to better mimic the composition of the SC fat tissue, transform them (lyophilization, foaming, micronization) and seed with human ASC.

Recent advances in bioprinting enable the creation of more complex structures adapted to cell culture conditions (oxygen and nutrients supply) and supporting cell infiltration after in vivo implantation. Various types of 3D bioprinting methods and hydrogel-based bioinks (i.e., alginate, hyaluronic acid, collagen, gelatin, fibrinogen, and tissue-derived extracellular matrix) have been developed in the last decade to print a broad range of (vascularized-) soft tissues, as reviewed in detail by Kim, et al., and Zhang, et al. These models constitute strong bases and transferable knowledge for the creation of an immunocompetent SC tissue model.

### 3.4. Full skin biopsies

Another model offering some possibilities to study immunogenicity of therapeutic proteins is full animal or human skin biopsy. Limitations of these explants are mainly (i) maintenance in culture conditions for a sufficient amount of time to enable drug testing and potentially repetitive administration, and (ii) a sufficient supply from different donors to mimic polymorphism of the human population, as for PBMC-derived cells.

Skin explants like Skimune®Pharm/Mab (Alcyomics Ltd), have been used to predict the allergenic potential and immunogenicity of new protein therapeutics or in therapeutic vaccine development (i.e. addition of adjuvants) by performing intradermal injections of drug candidates.
Another *ex vivo* human skin model, Hyposkin® is currently under development. The three-layer skin biopsy, which contains fat SC tissue in contrast to the models described previously, is maintained in culture and placed on a specific matrix insert. It enables to simulate SC injections of new pharmaceutical formulations to study compound absorption, catabolism and toxicity in the SC tissue.

**4. CONCLUSIONS & PERSPECTIVES**

In the field of immunogenicity prediction of candidate therapeutic proteins, we can distinguish three kinds of approaches: *in silico, in vitro* and *in vivo*. This review focused on the *in vitro* prediction tools currently available in standard 2D cell culture conditions or using novel 3D models. Limitations in terms of primary cell supply and representative polymorphism while using immune cell lines were discussed. In the same way, various scaffolds available commercially for clinical use or to develop research models of subcutaneous compartment or full-thickness skin were presented. Matrices are of interest in order to mimic the structure and mechanical properties of the SC tissue keeping a simple, inert and well-defined environment to seed immune cells. On the other hand, self-assembly systems composed of multiple cell types or biopsies allow reproduction of the physiological complexity and variety of cellular interactions. Three-dimensional *in vitro* models are more complex than 2D assays, aiming to improve predictability. The final objective is to develop a model sufficiently reliable to be considered by regulatory authorities as an indispensable and valuable step in the immunogenicity assessment of therapeutic proteins.

Finally, lab scale *in vitro* 3D models of various organs are now established and the complexity of the immune system could be even better mimicked when being integrated in a systemic model, as the “human-on-a-chip” concept elaborated this last decade.
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CHAPTER 3
Hydrogels in Three-Dimensional Dendritic Cell Culture as a Scaffold to Mimic Human Subcutaneous Tissue

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In the previous chapter, we established the rational to study protein immunogenicity and to develop a new in vitro assay or model for early prediction of immunogenicity. We presented existing assays mimicking physiological phenomena with various degrees of complexity. In this chapter, we will present the way we chose to model the subcutaneous route of administration. We will begin by examining potential hydrogel scaffolds to mimic mechanical properties of this tissue, and then will assess the cytocompatibility of those hydrogels with a human immune cell line called MUTZ-3.
ABSTRACT

The objective of this study was to develop a 3D cell culture model of the human subcutaneous tissue, allowing the prediction of the immunogenicity of subcutaneously injected therapeutic proteins. Several hydrogels were evaluated as scaffolds to mimic the human subcutaneous tissue in vitro. Cytocompatibility of the hydrogels with the human myelomonocytic cell line (MUTZ-3) was investigated, as well as their influence on cellular phenotype changes. Elastic Young’s moduli in compression of the hydrogels were measured by a texture analyser and compared to ex vivo human samples. MUTZ-3 cells were differentiated into dendritic cells before embedding in hydrogels. Agarose at various concentrations (0.5 %, 0.35 % and 0.25 % w/v), Geltrex® matrix and HyStem™ scaffold (1 % w/v) displayed a wide range of elastic Young’s moduli from 560 kPa to 49 kPa, compared to the reference value of 23 kPa obtained for human tissue. With the exception of HyStem™, good cytocompatibility of hydrogels was shown at the concentrations tested. An optimal combination of MUTZ-3 cells with 0.25 % agarose or Geltrex® is suggested.

KEYWORDS
3D model; MUTZ-3 cell culture; dendritic cells; subcutaneous tissue; hydrogels; immunogenicity testing.

GRAPHICAL ABSTRACT

Representation of a cell culture well containing immune cells embedded in an hydrogel scaffold. (Adapted from Medical Art Servier)
1. INTRODUCTION

3D cell culture is of increasing interest in attempting to predict the pharmacokinetics, pharmacodynamics and toxicity of drug candidates. The reason for this being that such complex physiological phenomena may better be mimicked in a 3D cell culture than under standard 2D cell culture conditions. Several such models exist, comprising co-culture of different cell types in a well insert and cell spheroids using diverse techniques. The latter is often carried out using a hydrogel matrix (such as Matrigel) to provide the mechanical support given in vivo by the extracellular matrix.

In this relatively new field, human skin models replicating both epidermis and dermis are in use to test potential skin sensitizers or to find new treatments for burns or wound healing in general. Most of the skin models include both epidermis and dermis, while full-thickness models including subcutaneous tissue are less due to its low significance during the wound healing process. Nevertheless, the increasing number of subcutaneously injected therapeutic proteins, often accompanied by adverse events such as immunogenic reaction and injection site reactions, has triggered a raised interest in better understanding this peculiar tissue. Recently, an excellent review by Kinnunen and Mrsky described the composition and physical and physiological properties of the SC tissue in detail.

Prediction of therapeutic protein immunogenicity is routinely done in vitro employing human peripheral blood mononuclear cells (PBMC) stimulation and CD4+ T cells proliferation assays. However, use of primary cells necessitates an expensive and time-consuming process to isolate, maintain and differentiate subsets of interest, as well as, to optimize PBMC storage in order to guarantee reproducibility of further analyses. In vivo, animals such as rodents, dogs or pigs are being used, although these models have limitations in their predictive power due to interspecies differences in immune system function. They are now being challenged by the need to replace, reduce and refine the recourse to animal models (3R’s principle).

In this regard, we aim to develop a 3D cell culture model encapsulating the major immune cells found in the SC tissue, namely sentinel dermal dendritic cells (DCs), in a simple but well-defined hydrogel scaffold mimicking the SC tissue mechanical properties. Our objective was to reproduce the (immune) microenvironment into which therapeutic proteins are usually injected in order to better understand their potential in situ aggregation, uptake by immune cells and pro-inflammatory cytokines release. We investigated and evaluated the
combination of several hydrogels and a human acute myelomonocytic leukemia cell line (MUTZ-3), in terms of cytocompatibility (cell viability) and potential cellular phenotype changes induced by the hydrogels used. Furthermore, elastic Young’s moduli of these hydrogels were measured and compared to those of human SC tissue samples with the intention to achieve a good representation of the mechanical properties of the SC tissue.

2. MATERIALS AND METHODS

2.1. MUTZ-3 cell line differentiation in dendritic cells

The human acute myelomonocytic leukemia cell line MUTZ-3 (ACC 295) and the human urinary bladder carcinoma cell line 5637 (ACC 35) were purchased from DSMZ (Braunschweig, Germany).

MUTZ-3 cells were maintained in a “routine” cell culture medium without phenol red composed of 60 % minimum essential medium α (MEMα) containing ribonucleosides and deoxyribonucleosides, supplemented with 20 % heat inactivated fetal bovine serum (FBS), of 5637 cell line-conditioned medium and penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively). All products were obtained from Gibco® (Life Technologies, Grand Island, NY, USA). The 5637 conditioned medium (RPMI 1640 supplemented with 10 % FBS and penicillin-streptomycin) was collected after at least 48h conditioning, centrifuged to remove cell debris and pooled. Aliquots of adequate volume were frozen and stored at -20°C after sterile filtration through 0.22 µm cellulose acetate low protein binding filters (Corning Inc., Corning, NY, USA). Cell incubation was performed at 37°C, in an atmosphere of 95 % humidity and containing 5 % CO₂.

Differentiation of MUTZ-3 into dendritic cells (MUTZ3-DCs) was induced by adding recombinant human granulocyte macrophage–colony stimulating factor (GM-CSF, 100 ng/mL), interleukin-4 (IL-4, 10 ng/mL) and tumor necrosis factor alpha (TNFα, 2.5 ng/mL, all from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) to the 80 % MEMα supplemented with 20 % heat inactivated FBS and penicillin-streptomycin, for 7 days. Cells were seeded at an initial concentration of 2 x 10⁵ cells/mL in a 12-well plate (1 mL/well). The same volume of fresh differentiation medium was added on day 3 (cell dilution 1:2). Finally, in order to check the maturation capacity of the MUTZ3-DCs, high concentration of TNFα
(75 ng/mL) was added to the medium and the cell incubated for 48 hours. This protocol was adapted from Masterson, et al. 

2.2. Flow cytometry analyses

The immunophenotype of MUTZ-3 cells before and after differentiation was analyzed using an Accuri™ C6 cytometer and CFlow® Plus software (Becton-Dickinson Biosciences, San Jose, CA, USA). The cytometer was used at the following settings: one blue laser with an excitation wavelength (λ_ex) of 488 nm using three different filters at 533/30, 575/40 and 670LP (λ_em/BP); and one red laser with a λ_ex of 635 nm using one filter at 675/25. Cells were stained with the following mouse monoclonal antibodies (mAbs): FITC-labeled antihuman-CD14; APC-labeled antihuman-CD83; PE-labeled antihuman-langerin (CD207); and the recombinant PE-Vio770-labeled antihuman-CD209 (DC-SIGN). These antibodies and their corresponding isotype controls were used according to supplier’s instructions (Miltenyi Biotec GmbH).

Quickly, 10^5 cells were centrifuged in Eppendorf tubes at 300 x g for 10 min. The supernatant was aspirated and cells were washed with rinsing buffer (DPBS pH 7.2, 0.5 % BSA, 2 mM EDTA, degassed and filtered through 0.22 µm cellulose acetate filter) and centrifuged again. Supernatant was discard and cells were resuspended and incubated 30 min at 4°C with 1 mL per tube of rinsing buffer supplemented with 10 % human plasma to avoid non-specific Fc receptor binding of the mAbs. Cells were then centrifuged, resuspended with 100 µL rinsing buffer, and incubated with 10 µL of the appropriate surface antibody or the corresponding isotype control for 10 min in the dark at 4°C. After a last washing step, cells were centrifuged and resuspended in 1 mL rinsing buffer. As CD207 (Langerin) is expressed intracellularly, the staining was done after cell fixation with 4.2 % formaldehyde (w/w) and permeabilization with saponin using Cytofix/Cytoperm™ Kit (Becton-Dickinson Biosciences). Dead cells were excluded by forward and side scatter characteristics, and doublets (cell clumps) were removed using area forward scatter vs. height forward scatter. At least 10^4 cells were counted in the “live” gate for each sample.
2.3. MUTZ3-DCs encapsulation in hydrogels

Once differentiated, MUTZ3-DCs were embedded in three different hydrogels: a basement membrane extract Geltrex® Matrix (Gibco®), cross-linked hyaluronic acid HyStem™, and low-gelling temperature agarose (A9045 - Sigma-Aldrich, St. Louis, MO, USA). All the hydrogels were first mixed with $4 \times 10^5$ cells/mL as detailed below, poured in flat bottom 96-well plates and once set the same volume of full differentiation medium was added on top. The same procedures were followed for the preparation of the gel blank, and the same proportions of medium or DPBS were used for the standard 2D cell culture conditions used as reference.

The Geltrex® Matrix was slowly thawed overnight in the fridge (5 – 8°C) and then kept on ice before mixing at a 1:3 ratio with cells diluted in differentiation medium pre-warmed at 37°C. The final protein concentration was ≤ 9 mg/mL allowing the gelation within 30 minutes at 37°C.

The HyStem™ scaffold kit was prepared according to the manufacturer’s instructions. Briefly, hyaluronic acid and crosslinking agent stored at -20°C were allowed to warm to room temperature for 1 hour before being reconstituted and fully dissolved. Cells washed and resuspended in DPBS were then mixed with 1% (w/v) HyStem™ (thiol-modified hyaluronan) by pipetting up and down several time. Finally, thiol reactive crosslinker (PEGDA) was added and the mixture was directly poured into 96 well-plates. Gelation was observed after one hour.

For the preparation of low gelling temperature agarose, a 1% (w/v) stock solution was prepared by dissolving powdered agarose in sterile DPBS pH 7.2 in a boiling water bath, allowed to cool to 37°C before dilution and addition of the cells. Agarose was used at three different final concentrations of 0.5, 0.35 and 0.25 % (w/v). Plates were placed in a 25°C incubator for 1h for gelation before being transferred to a 37°C incubator, in a humidified (95%) atmosphere containing 5% CO₂.

2.4. Optical microscopy

In order to visualize eventual changes in the phenotype and morphology of MUTZ3-DCs once embedded in hydrogels, pictures were taken daily over 3 days with an Axiovert 200 inverted microscope (Carl Zeiss, Feldbach, Switzerland) in bright-field, equipped with a QIClick CCD.
camera (QImaging, Surrey, BC, Canada) using phase contrast objectives to better discriminate differences between cells and hydrogels.

2.5. WST-1 colorimetric viability assay

Viability of cells embedded in hydrogels was assessed by a colorimetric assay using the enzymatic conversion of tetrazolium salts (WST-1) to formazan. Thirty microliter of WST-1 (Roche, Mannheim, Germany) was added to each well of the corresponding 96-well plate for day 0, 1, 2 and 3. Absorbance at two wavelengths (450 and 690 nm, respectively) was measured after 3 hours of incubation using a Synergy Mx microplate reader (BioTek Instruments GmbH, Luzern, Switzerland). All experiments were performed in triplicate and for each well the background absorbance at 690 nm was subtracted, before the correction with the corresponding blank condition (same hydrogel without cells). On the same plate, MUTZ3-DCs were cultured under standard 2D conditions for which viability was also assessed as a reference point. Results are normalized and presented in percentage of viability compared to this reference.

2.6. Elastic Young’s modulus in compression (E)

Visco-elastic properties of the selected hydrogels were determined using a TA-XTplus Texture Analyser (Stables Micro Systems Ltd, Surrey, United Kingdom). Cell culture medium covering the hydrogel was first removed. Then, by compression using a 6 mm diameter stainless steel cylinder probe connected to a 500 g load cell, compression force (N) as a function of depth (mm) was measured. Measurements were performed at a probe velocity of 1 mm/s during and after the test. The measurement started once the trigger force of 1 mN was reached and then over 4 mm depth. Measurements done after each day were tested for outliers using Grubbs’ method (\( \alpha = 0.05 \)) on GraphPad Prism® software, and as no outlier was identified, results were pooled together to define the mean Young’s modulus for each hydrogel. The same protocol was used for ex vivo human subcutaneous samples in order to obtain a relevant reference point. The compression measurements were registered and analyzed using the best linear fit function of the Exponent software (Stables Micro Systems Ltd, Surrey, United Kingdom).
Elastic Young’s moduli were calculated based on the slope (N/mm) obtained after 3 to 6 % of distortion of the hydrogel.

The following equation was used for the calculation of elastic Young’s modulus (E):

\[
E = \frac{\text{tensile stress}}{\text{strain}} = \frac{\sigma(\varepsilon)}{\varepsilon} = \frac{F/A}{\Delta L/L_0}
\]

Where \( F \) = slope x distance of measurement, \( A \) = cross-sectional area of the probe, and \( \varepsilon = \Delta L / L_0 \) is the sample strain.

2.7. Human subcutaneous tissue sample preparation

Permission for the study on human tissues was given by the Central Committee for Ethics in Research of Geneva University Hospital (CER: 08-150 (NAC08-051)). Human skin samples were retrieved from abdominal aesthetic surgeries performed at the Geneva University Hospital. The samples were obtained from five patients (four females and one male) aged between 37 and 56 years (average age of 45 ± 4.123 years). Underlying SC fat tissue was carefully detached from dermis using a scalpel. Small pieces were cut to fit into the wells of a 24-well plate. The height and the weight were measured for each sample, and their elastic Young’s moduli were measured the same day following the protocol described above. The elastic Young’s moduli were calculated based on the slope of the full compression (4 mm, corresponding of an average 40 % of distortion) of the human SC tissue samples.

2.8. Statistical analyses

Data were compiled and analysed using GraphPad Prism software (version 7.03, GraphPad Software Inc., La Jolla, CA, USA). Outliers were removed using Grubbs’ method (\( \alpha = 0.05 \)). For elastic Young’s modulus comparison between hydrogels and human donors and analysis of SC tissue density, an ordinary non-parametric one-way ANOVA was performed using the mean value of each tested component and Tukey’s correction for multiple comparisons. For the comparison of elastic Young’s modulus intra- and inter-human donors, an unpaired two-tailed parametric t-test was used. Differences were considered statistically significant at \( p < 0.05 \).
3. RESULTS AND DISCUSSION

3.1. MUTZ-3 differentiation in dendritic cells

The immunophenotyping of MUTZ-3 cells before and after 7 days of differentiation in dendritic-like cells was confirmed by flow cytometry analysis (Fig. 1). MUTZ-3 cell line expressed the CD14 monocyte surface marker, as previously reported. Differentiation induced by addition of GM-CSF and IL-4 to the culture medium led to the downregulation of this monocytic marker. For our subcutaneous tissue model, it is important to note that the expression of CD14 is also found in human skin dermal dendritic cells.

Fig. 1. Cell surface marker expression of MUTZ-3 progenitor cells, MUTZ3-derived dendritic cells (MUTZ3-DC) after 6 days of differentiation induced by GM-CSF and IL-4, and mature MUTZ3-DC (mMUTZ3-DC) after 48 h of maturation by addition of high concentration of TNFα. Results are presented in overlaid histograms, with above-mentioned marker in red and corresponding isotype in black.

Furthermore, the dendritic cell signature CD209 was upregulated in around 35 % of the cells, confirming their differentiation into dendritic-like cells and indirectly their ability for particle uptake. In order to avoid MUTZ-3 differentiation into the Langerhans cell subtype, the CD207 antigen was used as a negative control. Only about 7 % of the cells were found to express this antigen after the 7-day differentiation protocol. Finally, the differentiated MUTZ-3 (MUTZ3-
DCs) cells were able to maturate in the presence of a high concentration of TNFα displaying an upregulation of the CD83 maturation marker in 18% of the cells.

### 3.2. Selection criteria and nature of hydrogels

Hydrogels used in this study were selected based on their ability to form a viscoelastic gel under optimal cell culture conditions (37°C) and their availability as a sterile and endotoxin-free product. Indeed, with the aim to embed dendritic cells, the key component and potential contaminants of the hydrogels should not induce any pathogen-recognition leading to maturation and activation of these immune cells. Another significant point was that these hydrogels could be diluted to the optimal concentration for cells without changing their gelling properties.

Agarose is a linear polysaccharide consisting of D-galactose and 3,6-anhydro-L-galactose blocks extracted from algae. Low gelling temperature agarose has the ability to gel below 30°C but needs temperatures higher than 60°C to be fully dissolved. This product is claimed to be suitable for cell culture. Agarose offers a panel from stiff to deliquescent gel. However, at all concentrations the separation between medium added after gelling and the gel was still clearly defined, allowing the removal of excess feeding differentiation medium.

Hystem™ gel is formed by the polymerization of a thiol-modified hyaluronan (carboxymethyl hyaluronic acid-thiopropanoyl hydrazide – CMHA-DTPH) induced by a thiol-reactive crosslinking agent (polyethylene glycol diacrylate – PEGDA). Hyaluronic acid is one the major constituents of the extracellular matrix. This cell culture scaffold is non-immunogenic, animal protein-free and fully chemically defined. Following the supplier’s protocol, a stiff gel was obtained.

Geltrex® Matrix is a basement membrane extract from murine Engelbreth-Holm-Swarm (EHS) tumor containing laminin, collagen IV, entactin, and heparin sulfate proteoglycan. This product is available in a formulation without phenol red to avoid estrogen-like effects and with a reduced content of growth factor to avoid external/exogenous stimulation of dendritic cells. Moreover, it is guaranteed to be free of any viruses (such as lactose dehydrogenase elevating virus), which is of importance when working with dendritic cells. Once thawed, the matrix gels at the eukaryotic cell culture temperature of 37°C.
3.3. Elastic Young’s modulus in compression

The mechanical properties of the hydrogels were determined with respect to their elastic Young’s modulus (E) in compression. Due to the different natures and concentrations of these hydrogels, significant differences in the elastic Young’s moduli were observed, as shown in Fig. 2. Since the aim was to develop a hydrogel scaffold mimicking the mechanical properties of the human SC tissue, we were in need of a reference value for the latter. Compression measurements done on ex vivo samples obtained from five human donors gave a mean value of $22.61 \pm 2.172$ kPa, and this was used as the reference for the development of the three-dimensional cell culture model.

HyStem™ scaffold was found to be the stiffest hydrogel tested with a Young’s modulus of 567 kPa. Agarose elasticity ranged from 49 to 210 kPa depending on the concentration used. Finally, Geltrex® matrix ($E = 66$ kPa) showed elastic properties comparable to agarose at 0.25% (w/v) concentration and the mean of the five human donors samples measured, displaying no statistical difference. Based on these results, we decided to exclude hyaluronan based Hystem™ scaffold from our hydrogel set for the cytocompatibility study with MUTZ-3 cells.

Fig. 2. Elastic Young’s moduli of the five hydrogels and human SC tissue were determined by texture analyser measurements in compression. Significant differences were observed between the hydrogels and mean of the five human tissue samples except for the least concentrated agarose 0.25% (w/v) and Geltrex®. All results are presented as means ± standard error of the mean (SEM) ($n = 16-102$, p-value ** $< 0.01$ and **** $< 0.0001$, n.s. = not significant).
3.4. Human *ex vivo* samples variability

Measurements done on the human SC tissue samples showed a large intra-individual and inter-individual variability between the five donors (Fig. 3A). For example, the samples from donors 1 and 4 showed a strong disparity in elastic Young’s moduli values compared to the samples obtained from the three other donors. In order to confirm any variation linked to the gender of the donors, we compared the data obtained for the tissue samples of the four women donors with the one obtained from the only male donor, as shown in Fig. 3B. The median points of the two subsets were distinctly diverse, with a value of 11.77 kPa for women and 35.51 kPa for man, giving a highly statistical difference (*p*-value < 0.0001). However, we would like to highlight that these conclusions were drawn on a relatively small set of samples.

![Fig. 3. Elastic Young’s modulus measured on *ex vivo* subcutaneous samples obtained from five human donors (four women and one man). (A) Intra- and inter-individual variation between donors due to the heterogeneity of the biological tissue. Each point represents one sample measurement and the bars correspond to the means ± SEM (n = 13-23/donor); (B) Influence of gender on elastic moduli. The end of the whiskers indicate the minimum and maximum of all of the data (n = 23-80, *p*-value ****< 0.0001).](image)

Moreover, all donors were overweight except the male donor that was in morbid obesity, which might be an important parameter considering the fatty composition of human SC tissue. Indeed, human SC tissue is non-homogeneous and composed of two compartments, the adipocytes and the connective tissue enveloping them (also called fibrous septa). In a recent study, it was shown in humans that overfeeding leads to a remodeling of the extracellular matrix in adipose tissue, with an increased microvascular density and connective tissue deposition. These changes in the structure of the fatty SC tissue could explain the
difference observed between women and man, which is actually not gender specific but weight-related.

Few studies have been performed on the mechanical properties of human subcutaneous tissue and none of them in the exact same experimental configuration. For example, Pailler-Mattei, et al.\textsuperscript{25} performed \textit{in vivo} elasticity measurements on human forearm skin and obtained a comparable value of 12.5 kPa. This study was realized using a conical indenter connected to a displacement sensor. Another technique was used by Nightingale, et al.\textsuperscript{26} to evaluate the Young’s modulus of fat: shear-wave images of breast tissue samples containing fibroadenoma were obtained and direct inversion methods applied to estimate the Young’s modulus. The values for fat located close to a fibroadenoma were in the range between 3.8 and 5.6 kPa.

Depending on the technique used (texture analyzer, indentation, shear wave images), the body part examined (full-thickness skin, epidermal and dermal layers only, close to a fibroadenoma) and whether the test was done \textit{in vivo} or \textit{ex vivo}, Young’s modulus values for SC tissue found in the literature are quite diverse. In our study, the choice of samples from the abdominal region was relevant because SC injections of therapeutic proteins are often made in this part of the body.

\textbf{3.5. Human subcutaneous tissue density}

From the height and weight measurements of each piece of human SC fat tissue analyzed on the texture analyser, the actual density of this tissue was calculated (\textbf{Fig. 4}).

The final mean value obtained on this panel of five donors was $0.8998 \pm 0.0465 \text{ g/cm}^3$ ($n = 108$ and coefficient of variation = 5.2 \%). This is in accordance with the various values found in literature of 0.850 to 0.9196 \text{ g/cm}^3\textsuperscript{27-29} A one-way ANOVA gave only one statistical difference (** p-value = 0.005) between the samples from donor 3 and 4, which were in the extreme with a mean value of 0.9258 and 0.8802 \text{ g/cm}^3, respectively. As mentioned earlier, donor 4 was the only male in this study, and suffered from morbid obesity.
3.6. MUTZ3-DCs embedding and hydrogel cytocompatibility

After differentiation and immunophenotyping control by flow cytometry, MUTZ3-DCs were embedded in agarose at three concentrations (0.5, 0.35, 0.25 % w/v) and in Geltrex® matrix. An indication of hydrogel cytocompatibility was obtained by optical microscopy. The phenotypic changes in the dendritic cells, from round shape to the appearance of dendrites, were used as visible proof of cell viability. MUTZ3-DCs were imaged after embedding in the various hydrogels (Fig. 5A-E) or kept under standard 2D conditions (Fig. 5F). We noticed that at all agarose concentrations (Fig. 5A-C) and Geltrex® matrix (Fig. 5D), cells exhibited dendrites, displaying their ability to spread and grow in these soft-enough hydrogels. However, the cells embedded in HyStem™ scaffold only exposed a round-like phenotype (Fig. 5E). This is in accordance with our elastic Young’s modulus results obtained for this hydrogel (Fig. 2) and show that the stiffness of this hydrogel did not allow the dendritic extension and may have led to cell death by lack of nutrients and oxygen diffusion. HyStem™ was therefore excluded from our selection. In standard 2D cell culture conditions (Fig. 5F), MUTZ3-DCs exhibited dendrites over the tissue culture treated surface and tend to gather together in clusters or cell clumps in suspension (as described by the supplier DSMZ).
Fig. 5. MUTZ-3 dendritic cells after embedding in agarose 0.5 % (A), 0.35 % (B) and 0.25 % (C), as well as in Geltrex® matrix (D) and in Hystem™ scaffold (E). Cells kept in 2D standard cell culture conditions were used as a reference (F). Arrows indicate dendritic extension. Pictures were taken with inverted optical phase contrast microscope AxioVert 200 (Zeiss) with magnification lens x20. Scale bar = 50 µm.
To further quantify their cytocompatibility with MUTZ-3, cell viability was measured every day over three days using the WST-1 assay. The results were normalized to the cell viability in 2D standard cell culture conditions over the same period. A good cytocompatibility of all hydrogels was observed over the three days (Fig. 6). Regarding the agarose results we noticed that the polymer concentration played an important role in the maintenance and/or proliferation of cells. This could be explained by the difference in access to the nutrients. A lower gel concentration would allow for a better diffusion of the medium components and oxygen vital to the cellular metabolism.

**Fig. 6.** Relative cell viability expressed in percentage of the 2D standard cell culture control. Cell viability was measured indirectly by measurement of the enzymatic mitochondrial activity using the conversion reaction of tetrazolium salts (WST-1) in formazan. All the data are expressed as means + SEM. Statistics were done using two-way ANOVA multiple comparison with day 0 as a control (n = 3, p-value * < 0.05 ** < 0.01; *** < 0.001).
4. CONCLUSIONS

The differentiation of MUTZ-3 cells into immature dendritic-like cells (MUTZ3-DCs) was achieved to provide an immune component for our tissue model. The mechanical properties of human subcutaneous tissue were mimicked using various hydrogel scaffolds. Agarose and Geltrex® exhibited a broad spectrum of elastic Young’s moduli, but revealed good cytocompatibility properties after MUTZ3-DCs embedding. Elastic Young’s modulus of human skin was measured to serve as reference and to select the optimal hydrogel for our model.

Looking at all these parameters, it appeared that Geltrex® matrix, as well as agarose at a concentration of 0.25 % (w/v) were the best candidates for 3D cell culture model to mimic human subcutaneous tissue, allowing DC extension and keeping elasticity in the range of human hypodermis. Further studies are now needed particularly regarding the cell migration capabilities given by these two hydrogels and, at a later stage, simulation of therapeutic protein injection in the model.

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CHAPTER 4
Preliminary Results on the Interaction between Protein Aggregates and Primary- or Cell Line-Derived Dendritic Cells

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1. INTRODUCTION

This chapter relates to the combination of the protein aggregates set elaborated in the first chapter and the immune cell line MUTZ-3 studied in chapter three, as dendritic cell model. Preliminary results from exposure of MUTZ-3-derived dendritic cells (DCs) to native and aggregated rhIFNα2b are presented, as well as a follow-up experiment using peripheral blood mononuclear cell (PBMC)-derived dendritic cells. Throughout those results we will try to establish a correlation between aggregate types and dendritic cell uptake, activation, and related cytokine secretion.

2. MATERIALS AND METHODS

2.1. Endotoxin removal

To avoid unspecific DC activation induced by the presence of endotoxins, they were removed from native protein using ToxinEraser™ Endotoxin Removal kit (Genscript, Piscataway, NJ, USA) according to the manufacturer’s recommendation. Modified polymyxin B (PMB) affinity matrix was regenerated before equilibration with a phosphate buffer pH 8.0. Three milliliter of freshly thawed rhIFNα2b solution at 1 mg/mL were slowly passed through the column, and fully recovered by addition of 1.5 mL equilibration buffer (corresponding to the void volume). Protein concentration was determined by absorbance at 280 nm using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and sample was diluted using endotoxin-free formulation buffer until 0.5 mg/mL concentration was reached.

2.2. Aggregates preparation in endotoxin-free materials

Materials and solutions used for the preparation of aggregates were treated to inactivate endotoxins and prepared under sterile conditions. Glass vials and spatula were extensively washed with Milli-Q® water (tested as endotoxin-free) and 70 % ethanol solution, before depyrogenation by incubation at 220°C for 1 hour.1 Rubber caps and magnetic stirrers were similarly extensively washed with Milli-Q® water and 70 % ethanol solution, followed by a “depyrogenation by dilution” consisting of five washing steps in 50 mL of water for injection
(endotoxin content ≤ 0.025 EU/mL, Corning Inc., Corning, NY, USA) each. Components were air-dried in a sterile biosafety cabinet before use. Formulation buffers and other solutions used were prepared with water for injection in sterile apyrogen disposable vials and filtered through 0.22 µm cellulose acetate filter. Stress conditions identical to those described in the first chapter were applied to endotoxin-free rhIFNα2b in order to create aggregates, applying (i) mechanical stress by stirring at 700 rpm during 14 h or 72 h; (ii) thermal stress by incubation for 1 h at 64°C (Tm) or 90°C; and (iii) metal-catalyzed oxidation by addition of CuSO₄, ascorbic acid and EDTA.

2.3. Limulus Amebocyte Lysate (LAL) test

Protein samples were tested before and after endotoxin removal, as well as after preparation of aggregates, for endotoxin content using ToxinSensor™ Endotoxin Detection System (Genscript). This assay has a limit of detection of 0.25 EU/mL and the calculated maximum valid dilution (MVD) for human injection of rhIFNα2b was determined as 1500, following the equation mentioned in the European Pharmacopeia:

\[
MVD = \frac{CLC \times C}{LOD}
\]

Where CLC = contaminant limit concentration = \( \frac{K}{M} \)

\( C \) = concentration of test solution = 1 mg/mL

LOD = limit of detection

\( K \) = threshold pyrogenic dose of endotoxin per kilogram of body mass

(for intravenous injection \( K = 5.0 \) EU/kg, and mean human body mass = 75 kg)

\( M \) = maximum recommended bolus dose of product per kilogram of body mass

(for subcutaneous injection of Intron-A®, maximum volume = 1 mL)

However, for our assays, rhIFNα2b samples were incubated with dendritic cells derived from MUTZ-3 cell line or PBMCs. Due to the high sensitivity of this cell type to endotoxins, maximum valid dilution was reduced to 4, which corresponds to an endotoxin concentration of 1 EU/mL. Actually, dendritic cells were previously shown to be sensitive to endotoxin concentrations as low as 1 ng/mL (≈ 10 EU/mL) of lipopolysaccharide from E. coli.
Calculations were based on the volume used and the final protein concentration applied onto DCs.

2.4. MUTZ-3 derived dendritic cells

As described in chapter 3, the human acute myelomonocytic leukemia cell line MUTZ-3 (ACC 295) and the human urinary bladder carcinoma cell line 5637 (ACC 35) were purchased from DSMZ (Braunschweig, Germany).

MUTZ-3 cells were maintained in a “routine” cell culture medium without phenol red composed of 60 % minimum essential medium α (MEMα) containing ribonucleosides and desoxyribonucleosides, supplemented with 20 % heat inactivated fetal bovine serum (FBS), of 5637 cell line-conditioned medium and penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively). All products were obtained from Gibco® (Life Technologies, Grand Island, NY, USA). The 5637 conditioned medium (RPMI 1640 supplemented with 10 % FBS and penicillin-streptomycin) was collected after at least 48 h conditioning, centrifuged to remove cell debris and pooled. Aliquots of adequate volume were frozen and stored at -20°C after sterile filtration through 0.22 µm cellulose acetate low protein binding filters (Corning Inc., Corning, NY, USA). Cell incubation was performed at 37°C, in an atmosphere of 95 % humidity and containing 5 % CO₂.

Differentiation of MUTZ-3 into dendritic cells (MUTZ3-DCs) was induced by adding recombinant human granulocyte macrophage–colony stimulating factor (GM-CSF, 100 ng/mL), interleukin-4 (IL-4, 10 ng/mL) and tumor necrosis factor alpha (TNFα, 2.5 ng/mL, all from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) to the 80 % MEMα supplemented with 20 % heat-inactivated FBS and penicillin-streptomycin, for 7 days. Cells were seeded at an initial concentration of 2 x 10⁵ cells/mL in a 12-well plate (1 mL/well). The same volume of fresh medium was added on day 3 (cell dilution 1:2). Finally, in order to check the maturation capacity of the MUTZ3-DCs, high concentration of TNFα (75 ng/mL) was added to the medium and cells were incubated for 48 h. This protocol was adapted from Masterson, et al.5 After seven days of differentiation, 5 µg/mL of Intron®A (Merck MSD) and 50 µg/mL of native or aggregated rhIFNα2b endotoxin-free samples were added to MUTZ3-DCs for 24 h. Cell culture medium was removed by centrifugation and frozen at -20°C for further ELISA analysis. Cells were stained and analyzed by flow cytometry.
2.5. PBMCs isolation, purification and differentiation to dendritic cells

Buffy coat isolated from whole blood donation of a healthy donor was obtained from the Geneva University Hospital (Centre Transfusion Sanguine - Hôpitaux Universitaires Genève, Geneva, Switzerland). The same day, buffy coat was diluted at a ratio of 1:1 in sterile Dulbecco’s modified phosphate buffer saline (DPBS), and PBMCs were isolated by separation over a Ficoll-Paque™ PLUS gradient (GE Healthcare Life Sciences, Uppsala, Sweden) of 1.078 g/ml density. After centrifugation for 20 min at 1000 x g (slow acceleration, no brake), PBMC fraction was carefully removed, washed with sterile DPBS and centrifuged for 8 min at 500 x g. Cell pellet was resuspended in sterile DPBS and cells were counted using trypan blue (dilution 1:10) using a Countess™ II FL Automated Cell Counter (ThermoFisher Scientific, Life Technologies Corporation, Bothell, WA, USA). After centrifugation for 8 min at 500 x g, cells were resuspended in cold FACS rinsing buffer (DPBS pH 7.2, 0.5 % BSA, 2 mM EDTA, degassed and filtered through 0.22 µm cellulose acetate filter). Monocytes were purified by magnetic antibody cells sorting (MACS®, Miltenyi Biotec GmbH) using human CD14+ microbeads and magnetic cell separation LS columns. In detail, 10 µL of microbeads per 10^7 total cells were mixed and incubated for 15 min at 4-8°C. The mixture was then washed with rinsing buffer and centrifuged for 8 min at 500 x g. supernatant was discarded and cell pellet was resuspended in rinsing buffer (500 µl per 10^8 cells). The LS column, placed in a magnetic field, was rinsed by 3 mL rinsing buffer and the cell suspension was introduced into the column. Unlabeled cells were washed out by 3 times addition of 3 mL of rinsing buffer, and CD14 positive cells were flushed out by firmly pushing 5 mL of rinsing buffer through the column which had been removed from the magnetic field. The purity of the fraction obtained was assessed by flow cytometry analysis. CD14 positive cells were seeded at a density of 10^6 cells/mL onto a 6-well plate, and cultured in RPMI 1640 media supplemented by 10 % heat inactivated FBS, 1 % penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively), and 1 % L-glutamine. Monocyte differentiation into dendritic cells (MoDCs) was induced by addition of 10 ng/mL of GM-CSF and IL-4 to the culture medium and incubation for 6 days. Cells were placed at 37°C, in an atmosphere of 95 % humidity and containing 5 % CO₂.

After six days of differentiation, MoDCs were carefully detached using a cell scraper and seeded in a flat bottom 96-well plate at a density of 10^6 cells/mL (100 µL/well). Twenty-four hours later, 0.5 or 50 µg/mL of native or aggregated rhIFNα2b endotoxin-free samples were
added to MoDCs medium and cells were incubated for 24 h. Cells were then transferred to a round bottom 96-well plate, centrifuged and supernatant was frozen at −80°C for further ELISA analysis. Cell pellets were resuspended in FACS rinsing buffer, stained and analyzed by flow cytometry.

2.6. Flow cytometry analyses

Cells immunophenotype was analyzed using an Accuri™ C6 cytometer and CFlow® Plus software (Becton-Dickinson Biosciences, San Jose, CA, USA), or NovoCyte™ flow cytometer (ACEA Biosciences, San Diego, CA, USA) and FlowJo® software (version 10, FlowJo LLC, Ashland, OR, USA). Cells were stained using the following mouse monoclonal antibodies (mAbs): Brilliant Violet 510 anti-human CD80, Brilliant Violet 785 anti-human CD86 from BioLegend Inc. (San Diego, CA, USA), FITC-labeled anti-human CD14, APC-labeled anti-human CD83, PE-labeled anti-human CD207 (Langerin), and the recombinant PE-Vio770-labeled antihuman-CD209 (DC-SIGN) from Miltenyi Biotec GmbH. These antibodies and their corresponding isotype controls were used according to suppliers’ instructions.

For MUTZ-3 cells experiment, $10^5$ cells were centrifuged in Eppendorf tubes at 300 x g for 10 min, the supernatant was aspirated and cells were washed with FACS rinsing buffer and centrifuged again. Supernatant was discarded and cells were resuspended and incubated for 30 min at 4°C with 1 mL per tube of FACS rinsing buffer supplemented with 10 % human plasma to avoid non-specific Fc receptor binding of the mAbs. Cells were then centrifuged and resuspended with 100 µL FACS rinsing buffer, and incubated with 10 µL of the appropriate surface antibody or the corresponding isotype control for 10 min in the dark at 4°C. After a last washing step, cells were centrifuged and resuspended in 1 mL rinsing buffer. As CD207 (Langerin) is expressed intracellularly, the staining was done after cell fixation with 4.2 % formaldehyde (w/w) and permeabilization with saponin using Cytofix/Cytoperm™ Kit (Becton-Dickinson Biosciences). Dead cells were excluded by forward and side scatter characteristics and doublets (cell clumps) were removed using area forward scatter vs. height forward scatter. At least $10^4$ cells were counted in the “live” gate for each sample. Cell viability was expressed in relative viability calculated as defined by Johannsson, et al.:

$$\text{Relative viability} = \frac{\text{fraction of viable stimulated cells}}{\text{fraction of viable unstimulated cells}} \times 100$$
For MoDCs cells experiment, $10^6$ cells/mL were centrifuged in round bottom 96-well plates at 400 x g for 5 min, the supernatant was aspirated and cells were washed twice with 200 µL FACS rinsing buffer per well. Dead cells were excluded by incubation with Zombie Violet™ viability dye (dilution 1:1000 - BioLegend, San Diego, CA, USA) for 30 min at room temperature in the dark. Cells were then washed with 150 µL FACS rinsing buffer per well and centrifuged at 400 x g for 5 min. After removal of supernatant, cells were resuspended in 50 µL per well of FACS rinsing buffer containing appropriate surface antibodies or their corresponding isotype controls (dilution 1:200) and incubated for 30 min in the dark at 4°C. Finally, cells were washed by addition of 150 µL per well of FACS rinsing buffer, centrifuged and resuspended in the same volume. Dead cells were excluded based on Zombie dye staining and doublets (cell clumps) were removed using area forward scatter vs. height forward scatter.

2.7. Cytokine production

Cytokines secreted by MUTZ3-DCs and MoDCs into the cell culture supernatants were measured using interleukin-6 (IL-6, eBioscience Inc., San Diego, CA, USA), interleukin-12p70 (IL-12p70) and interferon-gamma inducible protein-10 (IP-10, BioLegend) enzyme-linked immunosorbent assay (ELISA) following supplier’s instructions. Read-out of the assays were done by measurement of absorbance at 450 nm and 570 nm using a Synergy Mx microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

2.8. Statistical analyses

Data were compiled and analyzed using GraphPad Prism® software (version 7.03, GraphPad Software Inc., La Jolla, CA, USA).

For flow cytometry analyses, an ordinary one-way ANOVA was realized using the mean value of triplicates of each sample compared to the mean value of control formulation buffer (Control FB). Dunnett’s correction for multiple comparisons was applied. MCO protein sample having its own blank, an unpaired t-test was performed between “Blank MCO” and “MCO” samples. Differences were considered statistically significant at $p < 0.0001$. 
For ELISA results, absorbance values at 450 nm were corrected with the absorbance at 570 nm. The mean of three standard curves was used to interpolate sample concentration following a second order polynomial (quadratic). Finally, the corresponding blank value (formulation buffer or blank of metal-catalyzed oxidation) was subtracted to native and aggregated protein samples. An ordinary one-way ANOVA was realized using the mean value of triplicates and Dunnett’s correction for multiple comparisons. Differences compared to native protein were considered statistically significant at $p < 0.05$.

### 3. RESULTS AND DISCUSSION

#### 3.1. LAL test

Samples of formulation buffer, protein before and after endotoxin removal, as well as after aggregation were tested for endotoxin content at a maximum valid dilution of 4, corresponding to an endotoxin concentration of 1 EU/mL. However, samples were also tested undiluted and at dilutions of 1:2’000, 1:20’000, 1:200’000, corresponding to 0.25, 500, 5’000, and 50’000 EU/mL, respectively.

Before endotoxin removal using polymyxin B resin, rhIFNα2b had a concentration comprised between 500 and 5’000 EU/mL. This concentration dropped to less than 1 EU/mL after one run through the resin. Formulation buffer was tested with a concentration inferior to 0.25 EU/mL. After preparation of aggregates, protein samples were tested again and all complied with the limit of 1 EU/mL, except the “90°C thermal stressed sample” added onto MUTZ3-DCs. This may be due to contamination of caps via condensation or due to temperature-induced bacterial death, which led to release of LPS. To avoid this latter possibility, for the second assay, freshly thawed protein solution was filtered through 0.22 µm sterile low protein binding cellulose acetate filters before being applied onto endotoxin removal resin. Aggregate samples applied onto MoDCs had all endotoxin concentration under 1 EU/mL, except for the metal-catalyzed oxidized samples for which an intermediate results was obtained. In the test, a gel clot was not completely formed, suggesting an endotoxin concentration close to 1 EU/mL.
3.2. Differentiation of MUTZ3-DCs

As described in chapter 3, the immunophenotyping of MUTZ-3 cells before and after 7 days of differentiation in dendritic-like cells was confirmed by flow cytometry analysis (Fig. 1). MUTZ-3 cell line expressed the CD14 monocyte surface marker, as previously reported. Differentiation induced by addition of GM-CSF and IL-4 to the culture medium led to the downregulation of this monocytic marker.

![Cell surface marker expression of MUTZ-3 progenitor cells, MUTZ3-derived dendritic cells (MUTZ3-DC) after 6 days of differentiation induced by GM-CSF and IL-4, and mature MUTZ3-DC (mMUTZ3-DC) after 48 h of maturation by addition of high concentration of TNFα. Results are presented in overlaid histograms, with above-mentioned marker in red and corresponding isotype in black.](image)

Furthermore, the dendritic cell signature CD209 was upregulated in around 35% of the cells, confirming their differentiation into dendritic-like cells and indirectly their ability for particle uptake. In order to avoid MUTZ-3 differentiation into the Langerhans cell subtype, the CD207 antigen was used as a negative control. Only about 7% of the cells were found to express this antigen after the 7-day differentiation protocol. Finally, the differentiated MUTZ-3 (MUTZ3-DCs) cells were able to mature in the presence of a high concentration of TNFα displaying an upregulation of the CD83 maturation marker in 18% of the cells.
3.3. Protein aggregate effects on MUTZ3-DCs

Incubation for 24 h of native and aggregated protein samples with differentiated MUTZ-3 dendritic cells did not induce a significant change in phenotypic expression of CD83 maturation marker (Table 1). MUTZ3-DCs were shown to be able to upregulate expression of this marker upon stimulation with a high concentration (75 ng/mL) of TNFα. However, contrary to what was expected, aggregates of rhIFNα2b did not induce effects that were significantly different neither between various stress conditions applied, nor compared to the native protein.

Nevertheless, their impact on relative cell viability was noticeable with a decrease in the live cell fraction from 10 to 50 % compared to native protein (Table 1). This cytotoxic effect was also observed following the addition of the native protein (57.2 %), as well as with the commercial product Intron®A even at the lowest concentration (10 times more diluted – 53.5 %). Moreover, we noticed a more pronounced reduction of cell viability for protein samples subjected to thermal stresses. However, due to the presence of endotoxins in the 90°C stressed sample, differences between the two thermal stresses samples cannot be merely considered as being linked to the degree of unfolding or aggregation.

Table 1. Flow cytometry analyses of relative cell viability expressed in percentage of the fraction of viable unstimulated cells, and phenotypic expression of CD83 marker expressed in percentage of "singlets in live cells".

<table>
<thead>
<tr>
<th>Sample</th>
<th>Relative viability (%)</th>
<th>CD83 + (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUTZ-3</td>
<td>95.7</td>
<td>&lt;1</td>
</tr>
<tr>
<td>MUTZ3-DC</td>
<td>100</td>
<td>2.4</td>
</tr>
<tr>
<td>mMUTZ3-DC (TNFα)</td>
<td>63</td>
<td>14.0</td>
</tr>
<tr>
<td>MUTZ3-DC + Formulation b.</td>
<td>108</td>
<td>&lt;1</td>
</tr>
<tr>
<td>MUTZ3-DC + Intron®a (5 µg/mL)</td>
<td>53.5</td>
<td>1.1</td>
</tr>
<tr>
<td>MUTZ3-DC + Native rhIFNα2b</td>
<td>57.2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>MUTZ3-DC + Stirring 14 h</td>
<td>60.5</td>
<td>2.3</td>
</tr>
<tr>
<td>MUTZ3-DC + Stirring 72 h</td>
<td>42.8</td>
<td>2.9</td>
</tr>
<tr>
<td>MUTZ3-DC + Blank MCO</td>
<td>52.3</td>
<td>2.9</td>
</tr>
<tr>
<td>MUTZ3-DC + MCO</td>
<td>42.0</td>
<td>2.9</td>
</tr>
<tr>
<td>MUTZ3-DC + 64°C</td>
<td>22.2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>MUTZ3-DC + 90°C</td>
<td>7.4</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Finally, culture supernatants were tested by ELISA for IL-6 secretion from MUTZ3-DCs. No significant concentration change was observed between the samples (data not shown), and
results obtained were all in the low IL-6 concentration range comprised between 2 and 6 pg/mL with a test sensitivity of 2 pg/mL.

From this experiment, we draw two main conclusions: (i) rhIFNα2b concentration applied on cells was possibly too high and induced cell death instead of activation; (ii) phenotypic expression of CD83 maturation marker - despite being used for example in skin sensitizer assays6, 11, 12 - may be not sensitive enough to discriminate between different types of aggregates.

3.4. Preparation of human PBMCs-derived DCs

Before and after CD14 positive selection using the MACS® method, PBMC purity was checked by flow cytometry (Fig. 2). In comparison to the buffy coat sample containing only 14 % of CD14 positive cells, after purification more than 90 % of cells were CD14 positive. We observed on the FSC-A vs. SSC-A density plot that a large part of the cells was lymphocytes and few of them monocytes, but this trend is well reversed after CD14 positive selection leading to an important enrichment in monocytes.

Figure 2. Flow cytometry analyses of cells present in the buffy coat from whole blood donation before (left panel) and after CD14 positive selection using MACS® microbeads (right panel). Buffy coat is composed of a mixture of lymphocytes and monocytes containing 14 % of CD14 positive cells. After enrichment through positive selection large majority of cells are monocytes and around 90 % are CD14 positive.
3.5. Protein aggregate effects on MoDCs

After six days of incubation of monocytes with IL-4 and GM-CSF, and 24 h incubation with protein samples PBMC derived DCs were analyzed through flow cytometry. Viability of Mo-DCs was determined using Zombie violet dye, staining cells with compromised membranes (Fig. 3a). Control cells treated with the corresponding volume of formulation buffer (FB) displayed about 50 % viability. In comparison, incubation with protein samples at two concentrations of 0.5 and 50 µg/mL exhibited a reduced viability depending on the type of aggregates applied and their final concentration. More concentrated protein samples (i.e., 50 µg/mL) seemed to induce more cell death. Especially aggregates formed by 90°C thermal stress and metal-catalyzed oxidation (MCO) were shown to decrease viability below 20 %, as observed for 90°C sample on MUTZ3-DC. This value was taken as a threshold, below which results obtained were considered not representative. Therefore, results of 90°C, blank MCO and MCO samples at 50 µg/mL were removed from further analyses.

Surprisingly, CD14 expression seemed conserved after 6 days of induction of the differentiation (data not shown). However, due to missing control after induction of differentiation but before incubation with native or aggregated rhIFNα2b, we cannot conclude on a down- or up-regulation of the LPS coreceptor, CD14. Nevertheless, CD209 was expressed by almost all cells after incubation with protein samples, indicating...
differentiation of monocytes to DCs (Fig. 3b). Indeed, CD209 is a C-type lectin receptor, also called dendritic cell-specific ICAM-grabbing non-integrin. It acts as a pattern recognition receptor (PRR) of pathogens in innate immunity, and plays a role as cell-adhesion receptor during DC migration and interaction with naïve T cells. Finally, we observed a down-regulation of CD209 expression or a loss of labeled-antibody accessibility to the receptor, which followed cell viability. These results confirmed the cytotoxicity of the highest protein concentration samples (50 µg/mL) and the need to remove them from further analyses.

**Figure 4.** Flow cytometry analyses of monocyte-derived DCs: CD80, CD83 and CD86 expression represented in median fluorescence intensity (MFI) of triplicates (+ SD) after incubation with native and aggregated proteins for 24 h at (a) 0.5 µg/mL, and (b) at 50 µg/mL. Statistics were done using one-way ANOVA multiple comparisons with “Control FB” sample as control or unpaired t-test between Blank MCO and MCO samples (p-value ****< 0.0001).

The expression of costimulatory molecules CD80/CD86 and the maturation marker CD83 in a subset of CD209 positive cells is represented in Fig. 4. MoDC incubation for 24 h with rhIFNα2b samples did not induce a strong upregulation of CD80 and CD83 expression, except for aggregates formed by stirring for 14 h, which slightly but significantly upregulated CD80 when applied at a concentration of 0.5 µg/mL (Fig. 4a). Protein thermally stressed at 64°C also displayed statistical difference in expression of CD80 and CD83 when applied at a concentration of 50 µg/mL onto MoDCs (Fig. 4b). However, caution should be taken in the
CHAPTER 4 - Preliminary Results

interpretation of these latter result, as cell viability for this sample was only in the area of 22.4% (± 0.5).

Conversely, a high expression increase of CD86 was shown after incubation with all protein samples compared to the control, with the exception of the 90°C stress sample. For the latter, cell viability was low, and the gated subpopulation, based on Zombie dye staining, might be composed by cells in an intermediate apoptotic state. More generally, the absence of CD80/CD83 expression with a high expression of CD86 may be related to an immature or semi-mature state of DCs, as already described in previous studies.17-19

Culture supernatants were tested by ELISA for IL-12p70 and IP-10 (CXCL10) secretion from MoDCs. These two cytokines were chosen for their involvement in the immune response led by dendritic cells via two different pathways. By nature, type I interferons (e.g., IFNα2b) stimulate immune cells, and notably dendritic cells, through their specific receptors IFNAR1 and IFNAR2 (Fig. 5). As previously described by Gautier et al.,20 the recognition of “foreign” particles via two principal toll-like receptor (TLR) pathways, namely the nuclear factor-κB (NF-κB)–dependent and the interferon (IFN)-dependent pathways, induces IL-12 secretion and type I IFN production, respectively. They further mentioned that endogenous type I IFN secretion by plasmacytoid DCs has a synergistic effect on the NF-κB pathway and therefore on the secretion of IL-12p70 by myeloid DCs. Moreover, activation of IFN-regulated genes leads to the induction of the CXC–chemokine IP-10/CXCL10 through the JAK–STAT signaling pathway.21
Concerning the IL-12p70 ELISA results, all samples had a concentration below the detection limit of 4 pg/mL (data not shown). These results revealed that the exogenous supply of rhIFNα2b did not activate the NF-κB–dependent pathway and successive secretion of IL-12. Furthermore, incubation with rhIFNα2b could even have blocked IL-12 production due to a supraphysiological concentration as shown in other studies and summarized in Fig. 5.\textsuperscript{22-24} The fact that no IL-12 was secreted but cells upregulated CD86 may be ascribed to the semi-mature ‘limbo’ state reported by Dudek, et al.\textsuperscript{19}

However, IP-10 ELISA results displayed significant variations between samples in comparison with native protein on statistical analysis using one-way ANOVA (Fig. 6). IP-10 secretion is an evidence that exogenous addition of rhIFNα2b was taken up by MoDCs. Actually, as explained by Lande, et al.,\textsuperscript{25} CXCL10 secretion is closely linked to the presence of IFNα or β and is a chemoattractant for activated T cells.\textsuperscript{26} IP-10 concentrations obtained after 24 h incubation were highly dependent on the final concentration of rhIFNα2b added. As for IL-12 secretion, results indicated a negative impact of a high concentration of rhIFNα2b, as less IP-10 was secreted at 50 µg/mL concentration than at 0.5 µg/mL. For instance, a two-fold increase was observed for the native protein following its 100 times dilution. These results
can also be explained by the cytotoxicity at the highest concentration, as shown previously with MUTZ3-DCs and MoDCs in the flow cytometry results.

Incubation with native rhIFNα2b already induced a high secretion of IP-10 by MoDCs. Therefore, mainly negative variations were observed, with the exception of samples (50 µg/mL) stirred over 3 days. This negative impact of aggregates on their uptake by MoDCs may be explained by a loss of structure or conformational changes affecting their specific binding site on receptors IFNAR1 and IFNAR2. Finally, attention is drawn to the 90°C protein sample at 0.5 µg/mL which was shown to have a low expression of CD86 costimulatory factor (Fig. 4a). ELISA revealed no IP-10 secretion for this sample, indicating an absence of recognition and uptake by MoDCs and explaining at the same time the absence of effect on CD86 expression.

**Figure 6.** IP-10 (CXCL 10) concentration determined by ELISA in MoDCs culture supernatants after 24 h incubation with native or aggregated rhIFNα2b at two concentrations. Histograms represent means of triplicates + SD. Statistics were done using one-way ANOVA multiple comparisons with “Native” sample as control (p-value *< 0.05 **< 0.01; ***< 0.001; ****< 0.0001).

4. CONCLUSIONS AND PERSPECTIVES

Endotoxin-free samples of rhIFNα2b model protein were prepared in order to be incubated with dendritic cells. DCs were derived from two type of cells: (i) first from the MUTZ-3 cell line, already studied for its ability to differentiate into DCs and to be maintained under 3D hydrogel culture conditions (see Chapter 3); (ii) secondly, from monocytes isolated by positive selection from PBMCs contained in whole blood buffy coat. Cell line and primary cells were differentiated into DCs by incubation with GM-CSF and IL-4 cytokines for 6-7 days,
before addition of native or aggregated rhIFNα2b. Results obtained with MUTZ3-DCs revealed cytotoxicity of this protein at the concentration of 50 µg/mL, an absence of upregulation of CD83 maturation marker, and no secretion of pro-inflammatory cytokine IL-6. Therefore, the experiment was repeated with monocyte-derived DCs at two protein concentrations of 0.5 and 50 µg/mL, and in addition expression of CD80/CD86 costimulatory molecules was followed. A strong cytotoxicity was also confirmed for the most concentrated protein samples (50 µg/mL) and CD86 expression was upregulated by almost all the samples. We confirmed the uptake of rhIFNα2b samples by MoDCs through the release of IP-10 at significant concentrations. However, after 24 h incubation of protein samples no IL-12p70 secretion from MoDCs was detectable. This could be explained by an inhibition of the NF-κB-dependent pathway due to the supraphysiological protein concentration applied.

These preliminary results must be confirmed by further experiments including characterization of the CD80/CD86 expression of MUTZ3-DCs, addition of a strong positive control of maturation such as LPS for MoDCs experiment, as well as baseline measurements of all markers used (CD14, CD209, CD80, CD83, CD86) before and after differentiation. Moreover, protein concentration should be decreased in order to avoid cytotoxic effect and better determined the effects related to the formation of aggregates of the protein.

The capacity of MUTZ3-DC and MoDC to migrate in the previously tested hydrogels (Chapter 3) should be further evaluated to validate the complete functionality of our 3D in vitro model of human SC tissue. Migration assays could be developed using a chemoattractant, such as chemokines CCL 19 (MIP-3 beta), or CCL 21 (6Ckine), or CCL 22 (MDC) binding respectively CCR 7 and CCR 4 receptors expressed by mature DC. Assays could be designed as a transmembrane Boyden chamber assay, or as a cell migration assay using cell seeding stoppers (Enzo Life Sciences, Lausen, Switzerland), both coated with the different hydrogels. Finally, therapeutic protein injections could be performed in the established 3D model to corroborate the degree of protein aggregation with pro-inflammatory signals released by DCs.
ACKNOWLEDGEMENTS

We would like to thank Prof. Carole Bourquin, Sandra Hocevar and Julia Wagner for their insightful discussions on experiment conception and technical help for isolation of monocytes. Special thanks to Emmanuelle Sublet for her assistance with cell culture and ELISA. Thanks also to Jean-Pierre Aubry and Cécile Gameiro from the flow cytometry core facility for their experts’ advices on data analysis.
REFERENCES


Summary and Conclusions

Since the 1980’s, the development of recombinant therapeutic proteins allows the treatment of chronic or acute diseases, leading sometimes to serious complications, such as diabetes or hepatitis B and C. These biopharmaceuticals are preferentially administered by subcutaneous (SC) injections, enabling (sometimes daily) self-administration by patients at home. One of the major issues of therapeutic proteins concerns their stability when exposed to environmental changes (temperature variations, agitation, light) encountered during packaging, transport and storage until their administration. Despite significant efforts to develop optimal formulations that stabilize proteins in their native and bioactive form, the transition between the formulation buffer and the physiological medium during injection can cause \textit{in situ} formation of aggregates. Besides the reduction of therapeutic efficacy induced, aggregation is one of the main causes for therapeutic proteins immunogenicity. Indeed, these aggregates, once in contact with immune cells, will be recognized due to their size and three-dimensional conformation, as foreign particles ("not self") and thus trigger an immune reaction at the injection site. These injection site reactions, although considered a minor adverse event, lead to poor patient compliance that may lead to treatment discontinuation.

The objective of this thesis was to study the correlation between the formation of aggregates of therapeutic proteins after injection into the subcutaneous tissue and the appearance of injection site reactions. More specifically, the aim was to study and characterize the formation of aggregates of therapeutic proteins and to evaluate their interactions with a model of the subcutaneous tissue.

To do this, the first step consisted in the expression of a model recombinant therapeutic protein in the bacterial expression system \textit{Escherichia coli}. The stability of the protein of interest, interferon alpha 2b (rhIFNα2b), was tested by applying the following stress conditions: stirring during 14 or 72 h, incubation at the melting temperature (64°C) and an extreme point of 90°C, and metal-catalyzed oxidation (MCO) by addition of copper sulfate and ascorbic acid. Biophysical characterization of the aggregates generated was performed using orthogonal techniques, such as UV-visible and fluorescence spectroscopy, circular dichroism, and dynamic light scattering. Moreover, the degree of antiviral bioactivity of the protein, before and after application of these various stresses, was determined in a cell-based
SUMMARY AND CONCLUSIONS

assay using the A-549 (human lung carcinoma) cell line. Cells were incubated with serial dilutions of the native or aggregated protein, and challenged after 10 h by addition of vesicular stomatitis virus (VSV). This assay allowed us to demonstrate the variability of the impact of the different types of aggregates on the bioactivity of the protein. Finally, a phenomenon of reversibility after dilution of certain types of aggregates, probably linked by weak bound interactions, was demonstrated. In order to present in a concise way the results obtained by means of these orthogonal techniques, a visualization tool based on a radar chart format was created. Implemented for this study, the proposed tool is primarily a general methodology that can be adapted to study other proteins. This tool allows having a complete overview of the biophysical characterization results, thus facilitating the comparison of aggregates and the overall understanding of their composition (size, secondary structure change, refolding).

A literature review of the in vitro cell assays available for prediction of the immunogenicity of therapeutic proteins is presented in Chapter 2. We highlighted the lack of specific recommendations from the US and European regulatory authorities for this type of assays, which are preceding the preclinical and clinical phases. Established cell-based assays, consisting of the protein incubation with dendritic cells derived from primary peripheral blood mononuclear cells (PBMCs), are described and are compared to the use of cancer cell lines (from chronic or acute leukemia). These latter offer a rapid, constant and reliable source of immune cells (dendritic cells and macrophages), without the need to perform expensive and time-consuming isolation of primary cells from whole blood. Subsequently, novel 3D cell culture models (using hydrogel scaffolds and/or combining different cell types for tissue reconstitution), currently under development are presented. Our interest was mainly focused on models that may be used to predict the immunogenicity of subcutaneously injected proteins. Specifically, the full-thickness skin models (epidermis, dermis, hypodermis) and artificial lymph nodes are detailed to imagine an ideal model serving the purpose of studying protein drug aggregation after subcutaneous injection.

In Chapter 3, a simplified in vitro model of the human subcutaneous tissue was developed. Several types of hydrogel scaffolds (agarose, Geltrex® and Hystem ™) were selected for their mechanical properties and cytocompatibility. In order to define which hydrogel best mimics the natural biomechanical properties of the SC tissue, we showed that the Young's modulus of human SC tissue has a value of about 23 kPa, which is close to the value obtained for the agarose hydrogel at a concentration of 0.25 % (w/v) and the Geltrex® extracellular matrix. In order to mimic the immune component of the SC tissue, dendritic cells derived from the
MUTZ-3 cell line were embedded in these hydrogels and maintained in culture for three days. The daily cell viability determined by a mitochondrial activity assay, and phenotypic changes observed by optical microscopy, revealed a good cytocompatibility of the agarose (at all concentrations tested), and Geltrex® hydrogels.

Finally, the results of preliminary studies of protein aggregates, formed according to the methods developed in Chapter 1, incubated with PBMCs-derived dendritic cells or dendritic cells derived from the MUTZ-3 cell line are presented. The assays carried out, although still largely to be optimized, have shown an increasing cytotoxicity of the protein at both concentrations used (0.5 and 50 μg/mL). The optimal concentration of protein, allowing activation of dendritic cells without toxicity and reversibility of the state of aggregation, still need to be defined in further studies. Nevertheless, the secretion of the IP-10 cytokine showed evidence of the uptake of the different protein samples (except for 90°C thermally stressed aggregates) by the cells, and a significant upregulation of the expression of the CD86 maturation marker of dendritic cells was observed. The significant denaturation caused by heat stress at 90°C might have prevented their recognition by dendritic cells.

However, further analyses are needed to gain a deeper understanding of the impact of the degree of aggregation of proteins on their immunogenic potential, for example by exploring the potential secretion of other pro-inflammatory cytokines. The model of human SC tissue developed in this study could be refined by a dendritic cells migration assay in the different hydrogels scaffold. Simulation of injection of therapeutic proteins into our SC tissue model and monitoring of its impact on triggering an immune system reaction would be the ultimate goal.
Résumé et Conclusions

Depuis les années 1980, l’avènement des protéines thérapeutiques recombinantes a permis le traitement de pathologies chroniques ou aiguës, aux complications parfois létales, tel que le diabète ou les hépatites B et C. L’administration de ces biomédicaments sous forme d’injection(s) sous-cutanées octroie aux patients la possibilité de s’auto-injecter à domicile leur traitement parfois quotidien. L’une des problématiques majeures des protéines thérapeutiques concerne leur stabilité face aux changements environnementaux (variations de température, agitation, lumière) rencontrés lors du conditionnement, du transport, du stockage et ce jusqu’à leur administration. Malgré des efforts importants pour développer des formulations optimales stabilisant les protéines sous leur forme native et bioactive, la transition entre le milieu de formulation et le milieu physiologique lors de l’injection peut entraîner in situ la formation d’agrégats. Outre la réduction de l’efficacité thérapeutique qu’elle entraîne, l’agrégation est l’une des causes principales de l’immunogénicité des protéines à visée thérapeutique. En effet, ces agrégats au contact des cellules du système immunitaire vont être reconnus, de par leur taille et leur conformation tridimensionnelle, comme des particules étrangères (« non soi ») et déclencher ainsi une réaction immunitaire au site d’injection. Ces réactions, bien que constituant un effet secondaire mineur, entraînent une mauvaise observance du patient vis-à-vis du traitement pouvant conduire à son arrêt définitif.

L’objectif général de cette thèse était d’étudier la corrélation entre la formation d’agrégats de protéines thérapeutiques après injection dans le tissu sous-cutané et l’apparition de réactions immunitaires au site d’injection. Plus spécifiquement, il s’agissait d’une part d’étudier et de caractériser la formation d’agrégats de protéines thérapeutiques et, d’autre part, d’évaluer leurs interactions avec un modèle du tissu sous-cutané.

Pour ce faire, la première étape a consisté en l’expression d’une protéine thérapeutique recombinante modèle, dans le système d’expression bactérien Escherichia coli. La stabilité de la protéine d’intérêt choisie, l’interféron alpha 2b (rhIFNα2b), a ensuite été testée au moyen de l’application des facteurs de stress suivants : agitation pendant 14 et 72 heures, incubation à la température de fusion de la protéine (64°C) et à une température extrême de 90°C, et
oxydation catalysée par l’ajout de sulfate de cuivre et d’acide ascorbique (MCO). Les agrégats engendrés par ces stress ont ensuite été caractérisés au moyen de différentes techniques d’analyses biophysiques, telles que la spectroscopie UV-visible et de fluorescence, le dichroïsme circulaire, et la diffusion dynamique de la lumière (DLS). De plus, le degré de bioactivité antivirale de la protéine avant et après application de ces différents stress a été déterminé via la réalisation d’un essai cellulaire sur la lignée A-549 (adénocarcinome pulmonaire humain). L’incubation des cellules avec une série de dilution de la protéine native ou agrégée, suivie à 10 h du challenge par le virus de la stomatite vésiculaire (VSV), nous a permis de démontrer la variabilité de l’impact des différents types d’agrégats sur la bioactivité de la protéine. Enfin, un phénomène de réversibilité après dilution de certains types d’agrégats, vraisemblablement liés par des interactions de forces faibles, a été mis en évidence. Afin de présenter de manière synthétique les résultats obtenus par le biais de ces techniques orthogonales, un outil de visualisation basé sur un diagramme en radar (ou de Kiviat) a été conçu. Mis en application dans notre étude sur l’interféron, l’outil proposé est avant tout une méthodologie générale pouvant être utilisée et adaptée à d’autres protéines. Il permet d’avoir un aperçu complet des résultats de la caractérisation, facilitant ainsi la comparaison des agrégats et la compréhension globale de leur composition (taille, changement de structure secondaire, modification du repliement).

Un état de l’art des essais cellulaires in vitro permettant la prédiction de l’immunogénicité des protéines thérapeutiques est dressé dans le chapitre 2. Nous avons ainsi mis en évidence l’absence de recommandations spécifiques de la part des autorités réglementaires américaines et européennes pour ce type d’essais qui précèdent les phases préclinique et clinique. Les méthodes d’essais cellulaires éprouvées, consistant en l’incubation des protéines dans une suspension de cellules primaires dendritiques (PBMCs), sont décrites et mises en perspective avec l’utilisation de lignées cellulaires cancéreuses (leucémies chroniques ou aigües). Ces dernières offrent la possibilité de disposer rapidement et de manière constante de cellules immunitaires (cellules dendritiques et macrophages), sans avoir recours au processus complexe, coûteux et chronophage d’extraction de cellules à partir de sang total. Par la suite, les nouveaux modèles de cultures cellulaires en 3D (reconstitution de tissus via l’utilisation d’hydrogels servant de structure et/ou combinant différents types de cellules), actuellement en cours de développement sont présentés. Notre intérêt s’est concentré principalement sur les modèles pouvant servir à la prédiction de l’immunogénicité des protéines injectées par voie sous-cutanée. Plus précisément, les diverses reconstitutions de
l’épithélium de la peau (épiderme, derme, hypoderme) et des ganglions lymphatiques sont détaillées afin d’imaginer l’élaboration d’un modèle idéal servant à l’étude de l’agrégation des protéines thérapeutiques après injection sous-cutanée.

Dans une troisième partie, une modélisation simplifiée du tissu sous-cutané humain a été établie in vitro. Plusieurs types d’hydrogels (agarose, Geltrex® et Hystem™) pouvant servir de matrices ont été sélectionnés pour leurs propriétés mécaniques et leur cytocompatibilité. Afin de définir l’hydrogel mimant au mieux les propriétés biomécaniques naturelles de ce tissu, nous avons montré que le module de Young de l’hypoderme humain a une valeur d’environ 23 kPa, proche des valeurs obtenues pour l’hydrogel d’agarose à une concentration de 0,25 % (m/v) et la matrice extracellulaire Geltrex®. Afin de mimer la composante immunitaire du tissu sous-cutané, des cellules dendritiques dérivées de la lignée cellulaire MUTZ-3 ont ainsi été encapsulées dans ces hydrogels et maintenues en culture pendant trois jours. La viabilité cellulaire déterminée chaque jour par le biais d’un test d’activité mitochondriale, et le suivi des changements phénotypiques observés au microscope, ont révélé une bonne cytocompatibilité des gels d’agarose (à toutes les concentrations testées) et de la matrice extracellulaire, Geltrex®.

Pour finir, les résultats d’essais préliminaires d’incubation des agrégats de protéines, formés selon les méthodes développées dans la première partie de cette thèse, sur des cellules dendritiques primaires ou issues de la lignée cellulaire MUTZ-3 sont présentés. Les essais réalisés, bien qu’encore largement perfectibles, ont permis de montrer une toxicité croissante de la protéine aux deux doses testées (0,5 et 50 µg/mL). La concentration optimale de protéine permettant une activation des cellules dendritiques sans toxicité et réversibilité de l’état d’agrégation restant encore à définir par des essais futurs. Néanmoins, la prise en charge des différents échantillons protéiques (à l’exception des agrégats produits par incubation à 90°C) par les cellules a été mise en évidence par la sécrétion de la cytokine IP-10, et une surexpression significative du marqueur de maturation des cellules dendritiques CD86 a été observée. L’importante dénaturation engendrée par le stress thermique à 90°C pourrait avoir empêché leur reconnaissance par les cellules dendritiques.

De plus amples analyses restent cependant nécessaires afin d’avoir une compréhension plus approfondie de l’impact du degré d’agrégation des protéines sur leur potentiel immunogène, en explorant par exemple la sécrétion potentielle d’autres cytokines pro-inflammatoires. Le modèle de tissu sous-cutané développé dans cette étude pourra être éprouvé via des essais de migration des cellules dendritiques dans les différents hydrogels sélectionnés. La simulation
d'injection de protéines thérapeutiques dans notre modèle de tissu sous-cutané et le suivi de son incidence sur le déclenchement d'une réaction du système immunitaire demeure à terme le but de ce projet.
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>AF₄</td>
<td>asymmetric-flow field flow fractionation</td>
</tr>
<tr>
<td>AI</td>
<td>aggregation index</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>APC</td>
<td>allophycocyanin (flow cytometry)</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>circular dichroism</td>
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<td>cluster of differentiation</td>
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<td>chinese hamster ovary</td>
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<tr>
<td>CMHA-DTPH</td>
<td>carboxymethyl hyaluronic acid-thiopropanoyl hydrazide</td>
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<td>DC</td>
<td>dendritic cells</td>
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<td>DLS</td>
<td>dynamic light scattering</td>
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<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
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<tr>
<td>DPBS</td>
<td>Dulbecco’s modified phosphate buffer saline</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>GM-CSF</td>
<td>granulocyte macrophage–colony stimulating factor</td>
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<td>ICH</td>
<td>international conference on harmonisation</td>
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<td>IFN</td>
<td>interferon</td>
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ACRONYMS

IL     interleukin
IP-10  interferon gamma-induced protein 10 (or CXCL 10)
ISRs   injection site reactions
JAK    janus activated kinase
$\lambda_{\text{max}}$ fluorescence emission maximum
LPS    lipopolysaccharide
mAb    monoclonal antibody
MCO    metal-catalyzed oxidation
MEM$\alpha$ minimum essential medium $\alpha$
MoDC   monocyte-derived dendritic cells (or MDDC)
MRE    mean residue ellipticity
Mw     molecular weight
MWCO   molecular weight cut-off
PBMC   peripheral blood mononuclear cells
PBS    phosphate buffer saline
PE     phycoerythrin
PE-Vio770™ tandem conjugate of phycoerythrin (donor) and Vio®770 dye (acceptor)
PEG    poly(ethylene glycol)
PEGDA  polyethylene glycol diacrylate
Ph. Eur. European Pharmacopoeia
PMB    polymyxin B
rhIFN$\alpha$2b recombinant human interferon alpha 2b
SC     subcutaneous
SD     standard deviation
<table>
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<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
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<td>TEM</td>
<td>transmission electron microscopy</td>
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<tr>
<td>Tm</td>
<td>melting temperature</td>
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<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
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<td>USP</td>
<td>United-States Pharmacopoeia</td>
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<td>VSV-GFP</td>
<td>vesicular stomatitis virus expressing green fluorescent protein</td>
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<td>4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate</td>
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Vector Maps

Plasmid card of pET-23d(+) rhIFNa2b
Plasmid card of pMJ9

ANNEX 1 – Vector Maps
ANNEX 2
Stabilization of Bovine Serum Albumin and Hen Egg White Lysozyme by Non-Covalent PEGylation with Tryptophan-mPEG

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ABSTRACT

Tryptophan-mPEG (Trp-mPEG) is a poly(ethylene glycol) based excipient, which has been reported previously to stabilize salmon calcitonin (sCT) by non-covalent PEGylation. In this work, we tested whether Trp-mPEG has a similar stabilizing effect on other proteins under the stress of stirring. Two model proteins, bovine serum albumin (BSA) and hen egg white lysozyme (HEWL) were formulated with a 3-fold molar excess of Trp-mPEGs and assessed with respect to an aggregate-reducing behavior. Circular dichroism (CD), turbidity measurements, dynamic light scattering (DLS), ANS (6-(anilino)naphthalene-2-sulfonic acid) fluorescence, fluorescence anisotropy and size exclusion chromatography (SEC-MALS) were applied as analytical tools.

Our results demonstrated a stabilizing effect of Trp-mPEG upon BSA and HEWL; however, this effect was not superior to unmodified PEG alone, which is also a known excipient for protein drug formulations. Moreover, Trp-mPEG had a stabilizing effect on BSA against thermal stress, but not on HEWL.

KEYWORDS

Protein aggregation, Protein formulation, Biopharmaceuticals, Non-covalent PEGylation
1. INTRODUCTION

Protein drugs represent a highly specific and active class of molecules in the pharmaceutical market. These drugs can have a big beneficial impact on health and may even be lifesaving.\textsuperscript{1} Due to the fact that proteins are highly dynamic molecules with a specific tertiary folding needed for therapeutic activity, the process of creating a stable protein drug formulation is very challenging. Proteins have a tendency to aggregate, causing a loss of pharmaceutical activity and may often result in immunogenicity and toxicity issues.\textsuperscript{2-4} Although different types and pathways for aggregation have been identified,\textsuperscript{5, 6} the underlying mechanism for protein aggregation generally includes a partial or complete unfolding, which exposes hydrophobic amino acids that are normally buried in the protein's core. These amino acids can then interact with each other in various ways resulting in protein agglomeration and aggregation.

Known factors that potentially disrupt the native folding of a protein include variation of storage temperature and pH, stirring, agitation, light exposure, variation of the ionic strength of the solvent, solvent polarity and freeze-thawing cycles.\textsuperscript{6, 7} Depending on the protein and on the stress it is exposed to, an aggregation of correctly folded protein monomers by electrostatic, hydrophobic and van der Waals forces is also possible.\textsuperscript{5}

In order to create stable protein drug formulations, several strategies are being employed, including an optimal pH, formulation with various stabilizing excipients like arginine or trehalose,\textsuperscript{8, 9} and the covalent attachment of poly(ethylene glycol) (PEG), a process widely known as PEGylation. PEGylation was first shown by Abuchowski et al. in an attempt to reduce immunogenicity of PEGylated BSA and to prolong circulating life for bovine liver catalase.\textsuperscript{10, 11}

The covalent attachment of a chain of poly(ethylene glycol) results in a hydrophilic shield, which sterically hinders protein molecules from associating, thereby reducing protein aggregation in vitro. Moreover, PEGylation leads to desirable in vivo effects by providing a steric shield against protease attack, antibody binding and macrophage uptake. This results in an increased blood circulation half-life (t\textsubscript{1/2}) while decreasing drug immunogenicity.\textsuperscript{12-13} If the protein drug surpasses a certain molecular weight after PEGylation, the t\textsubscript{1/2} is further increased as the modified protein drug is no longer excreted by glomerular filtration,\textsuperscript{14-15} as shown, e.g., in the case of PEGylated G-CSF (Neulasta\textsuperscript{®}).\textsuperscript{16, 17} However, the above-mentioned steric shielding effect can also affect the drug-receptor interaction, thereby lowering the efficacy of the drug, which represents a major disadvantage of PEGylation.\textsuperscript{18, 19}
To address the latter problem, different strategies have been followed to link PEG to a protein through a releasable linker, which dissociates in vivo, typically by hydrolysis. By this, one would keep the increased storage stability provided by PEGylation but also conserve the native protein-target affinity for the actual treatment. Furthermore, there is a small number of investigations on linking PEG to proteins by non-covalent PEGylation, which stabilizes proteins in vitro by the same mechanisms as covalent PEGylation and would in theory slowly release the unmodified protein upon administration. Although several studies on non-covalent interactions have been performed to PEGylate proteins, to our knowledge no such product has been subjected to clinical trials or introduced into the market yet.

One of these non-covalent PEGylation approaches is based on the use of the amino acid tryptophan, which is in turn covalently bound as an end group to a PEG polymer chain. The resulting Trp-mPEG non-covalently interacts with proteins through hydrophobic interactions of the tryptophan head group and hydrophobic protein groups. Unfolded proteins often display hydrophobic patches on their surface, which can present an interaction site for the hydrophobic head group of Trp-mPEG. Trp-mPEG of 2 and 5 kDa molecular weight has already been shown to stabilize one protein drug, salmon calcitonin. In order to assess if the stabilizing effect of Trp-mPEG is specific to salmon calcitonin or generally applicable to different therapeutic proteins, we examined the effect of the presence of Trp-mPEG on the aggregation tendency of two different model proteins, HEWL and BSA.

2. MATERIALS AND METHODS

2.1. Materials

HEWL and BSA (> 96 %) were purchased from Sigma (Switzerland). Methoxy-PEG (mPEGOH) 5 kDa was purchased from Iris (Germany) and methoxy-PEG 2 kDa was purchased from Fluka (Switzerland). TFA (trifluor acetic acid) and DMSO (dimethyl sulfoxide) were purchased from Acros (Switzerland).
2.2. Methods

2.2.1. Synthesis of Trp-mPEG 2- and 5kDa

Tryptophan-mPEGs of 2- and 5kDa chain lengths were synthesized as previously described. In short, mPEG-OH of 2 or 5 kDa were dissolved in anhydrous dichloromethane (DCM). Afterwards a 3-fold molar excess of p-nitrophenyl chloroformate (pnp-CF) and a 2-fold molar excess of anhydrous triethylamine (TEA) were added to the reaction mixture and dissolved. The pH was adjusted to a value between 7.5 and 8 and the mixture was allowed to react at room temperature under gentle stirring. After 24 h the reaction was stopped by drop wise addition of TFA until the solution became colorless. DCM was partially evaporated and the intermediate product (mPEG-p-nitrophenyl carbonate) was collected by precipitation from cold diethyl ether, followed by filtration. The resulting white powder was dried under vacuum.

In a second step L-tryptophan was dissolved in anhydrous DMSO, the pH adjusted to 8.3, and mPEG-p-nitrophenyl carbonate was added (at a 100:1 ratio). The mixture was left to react for 4 h under constant adjustment to keep the pH at 8.3. The reaction was stopped by cooling to 0°C and adjustment of the pH to 3 with hydrochloric acid. The product was extracted from the aqueous phase with chloroform and the organic phase was dried with anhydrous sodium sulfate and partially evaporated. The product was then purified by repeated precipitation and filtration with cold diethyl ether and cold iso-propanol. The collected colorless powder was dried under vacuum. The product’s structure was confirmed by MALDI-TOF and H-NMR analysis.

\(^1\)H NMR (300 MHz, DMSO-d-6): 3.18 ppm Trp-indole-CH\(_2\)-CH\(_2\)- (d); 3.25 ppm PEG-CH\(_3\)-O- (s); 3.5 ppm PEG-O-CH\(_2\) (m); 4.18 ppm Trp-indole-CH\(_2\)-CH\(_2\)- (q); 6.97 ppm Trp-indole (t); 7.06 ppm Trp-indole (t); 7.15 ppm Trp-indole (s); 7.32 ppm Trp-indole (d); 7.53 Trp-indole (d); 10.82 ppm Trp-COOH (s).

Maldi-TOF: 1684 Da (MeOPEG 2kDa); 1937 Da (Trp-mPEG 2kDa); 4901 Da (MeOPEG 5kDa); 5076 Da (Trp-mPEG 5kDa).

2.2.2. Experimental setup

Solutions of 2.5 mg/mL protein in Milli-Q® water were prepared with a 3-fold molar excess of either 2 or 5 kDa Trp-mPEG. Samples with mPEGOH of the same molecular weight and L-tryptophan were used as controls. 900 µl of the sample mixtures were transferred to a 4 ml glass vial containing a magnetic stir bar (10x5mm) and stirred for 24 h at 350 rpm in a
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thermostated room at 4-8°C. Afterwards the samples were transferred to 2 mL Eppendorf tubes and kept on ice while the protein aggregation was assessed with a set of different techniques within the same day. All experiments were done in triplicate in three independent experiments on different days.

2.2.3. ANS fluorescence measurements
ANS fluorescence was measured on a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon GmbH, Germany) with an excitation wavelength at the experimentally determined maxima of 380 nm. Slit widths for excitation and emission were both set to 1.5 and 3 nm for BSA and HEWL, respectively. Increment was set to 1. Spectra were recorded from 420 to 520 nm with an integration time of 1s. Samples for ANS fluorescence were prepared with 18 µM ANS. Samples were diluted to 0.25 mg/mL protein in Milli-Q® water for measurements. No backgrounds were subtracted as their signal was negligible.

2.2.4. Fluorescence anisotropy measurements
The fluorescence anisotropy was measured on a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon GmbH, Germany) with an excitation wavelength at the experimentally determined maxima of 288 nm for BSA and 295 nm for HEWL. Slit widths for excitation and emission were both set to 3 nm and increment was set to 1. Spectra were recorded from 340 to 360 nm with an integration time of 20 s. Samples were diluted to 0.25 mg/mL protein in Milli-Q® Water for measurements. No backgrounds were subtracted.

2.2.5. Dynamic light scattering (DLS) measurements
Dynamic light scattering was measured on a Zetasizer Nanoseries from Malvern (Renens, Switzerland) with a 173° backscatter angle at 25°C and an average spectrum of five repetitions was created. Samples were not diluted prior to DLS measurements.

2.2.6. Turbidity measurements
The absorbance at 400 nm was measured on a Cintra40 spectrophotometer from GBC Scientific Equipment Pty Ltd. (Hampshire IL, USA) in order to assess the turbidity. The slit widths were set to 2 nm while the intensity factor was set to 1. Measurements were recorded
as an average of three repetitions. Samples were not diluted prior to turbidity measurements. The respective backgrounds were measured as well and subtracted.

### 2.2.7. Circular dichroism (CD) measurements

A J-815 CD spectrometer from Jasco (Easton MD, USA) was used for all circular dichroism measurements. Spectra were recorded between 260 and 190 nm at scanning speed of 200 nm/min with data pitch set to 2 nm, sensitivity set to standard (100 mdeg), response set to 2 sec, band width set to 2 nm and the scanning mode set to continuous, in three accumulations. All spectra were taken at 20°C in a quartz cuvette with 1 mm path length from Starna GmbH (Pfungstadt, Germany). Samples were diluted to 0.3125 mg/mL protein in Milli-Q® water for measurements. The respective backgrounds were measured as well and subtracted.

Moreover, the melting temperatures of BSA and HEWL were determined by CD. The secondary structure content was monitored at 222 and 208 nm, respectively, with a temperature increase of 35°C/hour (from 20 to 95°C). Data pitch was set to 1°C, response was set to 2 sec, band with was set to 2 nm and sensitivity to standard. Samples were diluted to 0.3125 mg/mL protein in Milli-Q® water for measurements. No backgrounds were recorded.

### 2.2.8. SEC-MALS measurements

Size exclusion chromatography was performed with a TSKGel G200 SWXL column from Tosoh Bioscience (Lyon, France) on an alliance HT pump from Waters (Milford MA, USA) with a RI 2000 Refractometer from Schambeck SFD GmbH (Bad Honnef, Germany) and a Tristar minidawn from Wyatt (Dernbach, Germany). 50mM phosphate buffer pH 7 supplemented with 0.2 M NaCl was used as a running buffer. All running buffers were filtrated with a 0.1 µm membrane and degassed before use. Injections of 50 µl of 0.25 mg/mL protein diluted in Milli-Q® water were made with a flow rate of 0.3 mL/min. A refractive index and 2nd virial coefficient of 0.185 and 0.8x10⁻⁴ were used for BSA.²⁷ These conditions were adapted from reference ²⁸. HEWL was not analyzed by SEC-MALS as its size turned out to be too small for a good resolution on the column used here.
3. RESULTS

Figure 1. ANS Fluorescence of BSA (A,B) and HEWL (C,D) after 24 h of stirring in the absence and presence of Trp-mPEG 2 and 5 kDa, and mPEGOH.

ANS is a fluorophore sensitive to changes in polarity such as hydrophobic pockets of protein that get exposed during unfolding. ANS fluorescence will increase when faced with an apolar environment.

Figure 2. ANS Fluorescence of BSA (A,B) and HEWL (C,D) after 24 h of stirring in the absence and presence of Trp-mPEG 2 and 5 kDa, and mPEGOH.
Addition of Trp-mPEG 2kDa to HEWL resulted in a stabilized ANS fluorescence intensity (Fig. 2C). While ANS fluorescence for HEWL alone increased by a factor of 3 compared to the measurement before stirring (Fig. 1), the fluorescence intensity of ANS did not increase for HEWL with Trp-mPEG 2 kDa. Addition of Trp-mPEG 5 kDa to HEWL resulted in a stabilized ANS fluorescence intensity as well (Fig. 2D). Addition of mPEGOH 2- and 5 kDa and Trp also stabilized the HEWL ANS fluorescence (Fig. 2C-D). The addition of any of the excipients used here to BSA did not lead to a significant reduction of ANS fluorescence intensity (Fig. 2A-B). Moreover, intensity of fluorescence signals was not influenced by stirring (Fig. 1).

![Figure 3](image.png)

**Figure 3.** Anisotropy measurements of BSA (A,B) and HEWL (C,D) after 24 h of stirring.

Fluorescence anisotropy is a technique sensitive to the mobility of fluorescent particles. If a fluorophore is excited by polarized light and is not rotating during the brief period in the excited state it will emit light of the same polarization that it has been excited with.\(^{30}\) As protein aggregates are bulkier and more immobile than the monomeric form, this can be used to detect aggregates.
Fluorescence anisotropy showed a clear decrease in signal for HEWL in combination with Trp-mPEG 2 kDa (Fig. 4C). However, the signal levels before (Fig. 3) and after stirring were the same. It is possible that aggregated HEWL is reduced to monomers by the dilution prior to the anisotropy measurements. HEWL with Trp-mPEG 5 kDa exhibited a reduction of anisotropy signal after stirring (Fig. 4D), however, there were no differences in the spectra acquired before stirring (Fig. 3). MPEGOH did not influence HEWL anisotropy before or after stirring, whereas addition of Trp led to an even bigger reduction of HEWL fluorescence anisotropy (Fig. 4C-D).

Similar to HEWL, addition of Trp and Trp-mPEG 2 and 5 kDa lead to a reduction of BSA fluorescence anisotropy whereas addition of MPEGOH 2 and 5 kDa did not influence BSA fluorescence anisotropy (Fig. 4A-B). These values remained constant before and after stirring (Fig. 3).
Figure 5. Circular dichroism of BSA (A,B) and HEWL (C,D) after 24 h of stirring. Respective backgrounds were subtracted.

Protein and peptide secondary structures absorb right (R) and left (L) handed polarized light differently, resulting in an elliptical polarization of light transmitted through a sample. Circular dichroism is used to detect these differences and enable us to assess the nature and level of content of the secondary structure in a protein sample.\(^\text{31}\)

Circular dichroism showed a small increase of signal of HEWL with Trp-mPEG 2- and 5 kDa compared to HEWL alone after stirring (Fig. 6C-D). However, the level of secondary structure content remained the same as measured before stirring (Fig. 5). Addition of mPEGOH and Trp did not influence the HEWL CD signal before and after stirring. Addition of Trp-mPEG 2- and 5 kDa to BSA, as well as 2- and 5 kDa mPEGOH and Trp resulted in a slightly increased α-helical content after stirring compared to BSA alone (Fig. 6A-B). This elevated content was not observed before stirring (Fig. 5).
Figure 6. Circular dichroism of BSA (A,B) and HEWL (C,D) after 24 h of stirring. Respective backgrounds were subtracted.

Figure 7. Melting temperatures of BSA (left) and HEWL (right) in the absence and presence of excipients.

A melting temperature of 63.3°C and 76.7°C was determined for BSA and HEWL, respectively (Fig. 7). Addition of a 3-fold molar excess of Trp-mPEG 2 and 5 kDa, and 2 and 5 kDa mPEGOH resulted in an elevation of BSA melting temperature of 4, 3.8, 3.7 and 5.5°C,
respectively. Addition of a 3-fold molar excess of Trp lowered the melting temperature by 1.7°C.

Addition of a 3-fold molar excess of Trp-mPEG and mPEGOH 2 kDa resulted in an elevation of 1.6°C and 1.7°C, whereas the addition of Trp-mPEG 5 kDa and Trp resulted only in an elevated melting temperature of 0.1°C and 0.8°C. The addition of mPEGOH 5 kDa did not affect the melting temperature of HEWL.

Protein aggregates, as any kind of particles, are known to scatter light, a fact which can be used to determine their hydrodynamic radius by DLS. While there was an increase in hydrodynamic radius for HEWL after stirring, HEWL samples with Trp-mPEG 2- and 5 kDa exhibited no increase in hydrodynamic radius by stirring (Fig. 8). Stirring caused an increase in hydrodynamic radius of HEWL with mPEGOH 2 kDa and Trp. With tryptophan and mPEGOH 2 kDa this increase was comparable to HEWL alone whereas the sample with mPEGOH 5 kDa showed a much lower increase in hydrodynamic radius than HEWL alone (Fig. 8).

Figure 8. Dynamic light scattering measurements of HEWL after 24h of stirring. A one-way ANOVA analysis was performed to assess if HEWL groups with excipients are significantly lower than HEWL control group (after stress treatment only) P<0.05 is indicated with *. Turbidity has been long accepted as a hallmark of aggregation. It is related to the formation of higher order insoluble aggregate species in the size range of > 10 μm. By assessing the absorption with a spectrophotometer at 400 nm, just within the visible spectrum of light, we were able to quantify the turbidity as shown previously. The increase in turbidity is based mainly on the fact that bigger particles scatter more light than small ones and not on an
increase of absorption. There was no significant difference in turbidity after stirring for HEWL and HEWL with any of the tested excipients used (Trp-mPEG 2- and 5 kDa, mPEGOH 2- and 5 kDa and Trp, respectively) (Fig. 9).

![Figure 9](image)

**Figure 9.** Turbidity measurements of HEWL after 24 h of stirring. Respective backgrounds were subtracted. A one-way ANOVA revealed no significant differences between BSA control group and BSA groups with excipients.

Stirring lead to a huge increase of the hydrodynamic radius in the BSA samples. The addition of Trp-mPEG and mPEGOH 2 and 5 kDa largely attenuated this increase. The addition of Trp also had an attenuating effect on the increase in hydrodynamic radius of BSA, however much less pronounced and only in one experiment statistically significant (Fig. 10).
Figure 10. Dynamic light scattering measurements of BSA after 24h of stirring. A one-way ANOVA analysis was performed to assess if BSA groups with excipients are significantly lower than BSA control group (after stress treatment only) P < 0.05 is indicated with *.

There was a large increase in BSA turbidity upon stirring, which was largely suppressed by the addition of Trp-mPEG and mPEGOH of 2 and 5 kDa. The suppression of the increase in turbidity upon the addition of Trp was less pronounced and only significant in one experiment (Fig. 11).

Figure 11. Turbidity measurements of BSA after 24 h of stirring. Respective backgrounds were subtracted. A one-way ANOVA analysis was performed to assess if BSA groups with excipients are significantly lower than BSA control group (after stress treatment only) P < 0.05 is indicated with *.
SEC-MALS is suitable for the distinction between protein monomers, dimers and multimers. Dimer and multimer content of samples containing BSA, as measured by SEC-MALS, showed no significant differences between samples of any group (Fig. 12 and 13).

**Figure 12.** Ratio of dimer divided by monomer content of BSA after 24h stirring at 4°C as measured by SEC-MALS. A one-way ANOVA revealed no significant differences between BSA control group and BSA groups with excipients.

**Figure 13.** Ratio of multimer divided by monomer content of BSA after 24h stirring at 4°C as measured by SEC-MALS. A one-way ANOVA revealed no significant differences between BSA control group and BSA groups with excipients.
4. DISCUSSION

Protein aggregates come in many shapes and sizes with different characteristics.\textsuperscript{38, 39} As there is no single analytical method to cover the detection of all different types of aggregates it is recommended to use several complementary methods, based on different techniques in order to examine aggregates from orthogonal angles.\textsuperscript{33–40} The methods we employed were quite diverse and allow therefore to make a general assessment of protein aggregation with a high level of confidence. In this study we chose two model proteins, BSA and HEWL, because they were readily available and as we were investigating the broad applicability of Trp-PEG excipients for the stabilization of protein formulations we were not interested in any specific protein structures.

All results taken together (Table 1) show that stirring for 24 h at 350 rpm leads to aggregates of HEWL and BSA by increasing and renewing the number of air-water interfaces.\textsuperscript{41} These aggregates appear to be rather large as they can be detected by turbidity and DLS measurements. Moreover, they consist of protein monomers, which are slightly unfolded as detected by CD. In the case of HEWL these structural changes are also characterized by an elevated surface hydrophobicity as they are accompanied by elevated ANS fluorescence, which might be a result of a partial unfolding. An elevated surface hydrophobicity in HEWL aggregates has been reported for other stressors as well and might well be characteristic for a variety of HEWL aggregates.\textsuperscript{42} For BSA no elevated surface hydrophobicity was detected, which might mean that the aggregates consist of mostly native monomers. Finally, the aggregates are most likely of a non-covalent, reversible nature as we have been unable to detect them by SEC-MALS. Here we did not register a shift in the ratio between monomers and either dimers or multimers in any sample group before and after stirring, which leads to the conclusion that either the sample groups were all equally stable or, more likely, that aggregation was reversible and aggregates are dissolved by sample preparation and/or the experimental conditions, which represents known reasons for inaccuracy of SEC-MALS.\textsuperscript{33–37}

\textsuperscript{43} Only BSA samples were analyzed by SEC as HEWL eluted too fast for a separation due to its low molecular weight.

Trp-mPEG 2 kDa and 5 kDa successfully attenuated the aggregation of HEWL and BSA induced by 350 rpm stirring for 24 h. DLS experiments showed that the hydrodynamic radius of stress-induced proteinaceous particles is clearly reduced by Trp-mPEG for both BSA and HEWL, which relates to the presence of smaller and/or fewer aggregates.
Table 1. Summary of effects of Trp-mPEGs after 24 h of stirring at 350 rpm as compared to protein control samples without any excipients. n.d.: not determined.

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<th>ANS fluorescence</th>
<th>Anisotropy</th>
<th>CD</th>
<th>DLS</th>
<th>Turbidity</th>
<th>SEC-MALS</th>
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<tbody>
<tr>
<td><strong>BSA Trp-mPEG</strong>&lt;br&gt;2kDa</td>
<td>No reduction in surface hydrophobicity</td>
<td>Reduction in protein anisotropy</td>
<td>Slight increase in protein secondary structure</td>
<td>Reduction in protein hydrodynamic radius</td>
<td>Reduction in protein turbidity</td>
<td>No reduction in protein dimers or multimers</td>
</tr>
<tr>
<td><strong>BSA Trp-mPEG</strong>&lt;br&gt;5kDa</td>
<td>No reduction in surface hydrophobicity</td>
<td>Reduction in protein anisotropy</td>
<td>Slight increase in protein secondary structure</td>
<td>Reduction in protein hydrodynamic radius</td>
<td>Reduction in protein turbidity</td>
<td>No reduction in protein dimers or multimers</td>
</tr>
<tr>
<td><strong>HEWL Trp-mPEG</strong>&lt;br&gt;2kDa</td>
<td>Reduction in surface hydrophobicity</td>
<td>Reduction in protein anisotropy</td>
<td>Slight increase in protein secondary structure</td>
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<td>No reduction in protein turbidity</td>
<td>n.d.</td>
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For BSA, Trp-mPEGs of 2 and 5 kDa molecular weight largely suppressed the increase in turbidity seen for BSA samples without excipients. In the case of HEWL no significant increase in turbidity was detected for any sample group (protein alone and with excipients), which may indicate that these aggregates are by their nature not detectable by turbidity measurements or their concentration was too low.

Stirring induced a large increase in surface hydrophobicity as measured by ANS fluorescence in HEWL, whereas no increase was registered for BSA. This leads again to the conclusion that the aggregates created from the two model proteins show different properties. BSA, being a much larger molecule, is apparently more effective in burying its hydrophobic core than HEWL, even in the aggregated state. The increase in surface hydrophobicity of HEWL seems to be effectively suppressed by both 2 and 5 kDa Trp-mPEGs, which may be caused by an increased stability.

The content of secondary structure as measured by CD seemed largely unaffected by stirring. It is however noteworthy that the addition of Trp-mPEGs showed a slightly higher content of
secondary structure for HEWL samples, before and after stirring. The alpha-helical content of BSA was unaffected by stirring as well and the same observation of slightly increased secondary structure content with the addition of Trp-mPEGs was made, before and after stirring. This leads to the conclusion that the process of aggregation of the two model proteins does not comprise major unfolding and the resulting aggregates consist of natively folded protein monomers. Moreover, it suggests that the presence of Trp-mPEGs represents a more favorable environment than Milli-Q® water alone, even without stirring. When monitoring the melting temperature of BSA by CD an elevation by 4 and 3.8°C was observed in the presence of a 3-fold molar excess of 2 and 5 kDa Trp-mPEG, which suggests stabilization against thermal denaturation. The addition of 2 kDa Trp-mPEG to HEWL lead to an elevation of the protein melting point of 1.6°C, while 5 kDa Trp-mPEG elevated the Tm only by 0.1°C.

The level of protein fluorescence anisotropy was unchanged after the 24h period of stirring, however addition of Trp-mPEGs (2-and 5 kDa) and even more so Trp alone, lead to a significant reduction of protein anisotropy, whereas the addition of mPEGOH (2-and 5 kDa) did not have any effect. One interpretation would be that there is a significant reduction of protein particle size upon the addition of Trp and Trp-mPEGs, however, it has to be considered that these excipients are highly fluorescent particles of small molecular weight and therefore potentially influence the result obtained.

The addition of mPEGOH to our two model proteins had a similar effect as Trp-mPEG, apparently suppressing the stress-induced rise in hydrodynamic radius and turbidity to the same extent as Trp-mPEG of the same molecular weight. While their presence did not have any impact on particle size as measured by fluorescence anisotropy on both model proteins, their addition showed a similar increase in alpha helical content as Trp-mPEG. For HEWL no elevated secondary structure was recorded in the presence of MeOPEG. Similar to Trp-mPEG, a 3-fold molar excess of mPEGOH 2 and 5 kDa lead also to an elevation by 3.7 and 5.5°C of the melting temperature of BSA. The addition of mPEGOH 2 kDa to HEWL raised the melting point by 1.7°C. When adding 5 kDa MeOPEG, however, the melting point remained unaffected.

While the addition of MeOPEG did not lead to any changes in ANS fluorescence for BSA, it lead to a similar reduction in ANS fluorescence in HEWL as Trp-mPEG. This reduction was slightly more pronounced than Trp-mPEG of 2 kDa and less pronounced than 5 kDa.
Addition of Trp did not lead to a less significant attenuation of the stirring-induced increase in hydrodynamic radius and turbidity of HEWL; neither did it lead to any changes in the secondary structure as measured by CD, while it increased the melting temperature of HEWL by 0.8°C.

When added to BSA there was a certain attenuation of the stress-induced aggregation visible, however not as strong as shown with the Trp-mPEG and MeOPEG groups. This has been observed with DLS and Turbidity measurements. In one experiment the addition of Trp did not affect the secondary structure of BSA, whereas in another experiment it lead to a similar increase as Trp-mPEG 2kDa. It did, however, lower the melting temperature of BSA by 1.7°C, suggesting a slightly decreased thermal stability.

We conclude that the addition of Trp-mPEG 2 and 5 kDa at a 3-fold molar ratio does indeed protect the two model proteins BSA and HEWL against an induced stress of stirring of 24 h at 350 rpm. Moreover, a similar protective effect appears to be exerted by mPEGOH of the same chain length as Trp-mPEGs. This might be due to the fact that the methyl-end group of mPEGOH may be apolar enough to interact with hydrophobic patches in a similar fashion as the Trp-head group of Trp-mPEG, or maybe the crucial reason of stabilization is not realized by hydrophobic interactions. It is possible that both PEGylated excipients (mPEGOH and Trp-mPEG) stabilize the two model proteins non-specifically by randomly preventing protein molecules from attaching to each other by steric hindrance. Another possibility is that dissolution in plain Milli-Q® water presents an unfavorable environment for these proteins and just simply the presence of dissolved molecules leads to a better hydration pattern and more stability. The fact that a 3-fold molar excess of Trp appears to present a more stable formulation than water alone, but less stable than with PEGylated excipients, might be explained by the fact that as Trp is much smaller than PEG and would affect the hydration layer of a protein to a lesser extent. With the set of analytical techniques we employed we can assess that the stabilizing effect of Trp is inferior to the one of Trp-mPEG and mPEGOH. However, we are unable to judge whether the effect of Trp-mPEG is superior to mPEGOH. Neither can we judge if there was an effect of the PEG chain length for one of those excipients. This might be due to the fact that the formulations are equally stable or that the resolution of our analytical procedures is too low to effectively quantify the amounts of aggregates in this study. Further studies would be necessary to answer these questions.
Additionally to a stabilizing effect against stirring we observed an elevated thermal stability of BSA in the presence of a 3-molar excess of Trp-mPEG and mPEGOH. The presence of Trp, however, lead to a slightly decreased thermal stability of BSA.

Interestingly, we were able to see a small increase in thermal stability when adding 2 kDa Trp-mPEG and mPEGOH. However, when adding 5 kDa Trp-mPEG mPEGOH there was no relevant increase in stability observed. Not only was the increased thermal stability due to the PEGylated excipients smaller than for BSA, in case of HEWL there appeared to be a direct relation with the chain length of the polymers. We did neither observed any dependence on the size of the excipients on protein stabilization against stirring, nor did we see any trends when comparing the ratios between protein and excipient molecular weight in this study.

5. CONCLUSIONS

In this work we have successfully shown that the previously reported stabilizing effect of Trp-mPEG on salmon calcitonin is not limited to that specific protein. We have shown successfully that it has the potential to stabilize two other model proteins against the stress factor of stirring, results which are encouraging that these excipients may be used to protect various proteins against several stress conditions.

Given their large share of the pharmaceutical market and their potential benefits, research on novel ways of protein drug stabilization remains a topic of great interest. There is a big number of potential therapeutic proteins, each of them potentially taking different pathways of aggregation under different stress conditions. Trp-mPEG may be a “simple” new excipient able to suppress protein aggregation and improve their pharmaceutical formulation by a non-covalent-PEGylation strategy.
ACKNOWLEDGMENTS

Our special thanks go to Claudia Mueller for her input and advice and to Fabien Neuenschwander for his excellent assistance with the synthesis of the PEGylated excipients.

TABLE OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANS</td>
<td>6-(anilino)naphthalene-2-sulfonic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
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<tr>
<td>DCM</td>
<td>Dichloromethane</td>
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<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
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<tr>
<td>HEWL</td>
<td>Hen egg white lysozyme</td>
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<tr>
<td>HNMR</td>
<td>Proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix assisted laser distortion ionization – time of flight</td>
</tr>
<tr>
<td>MeOPEG</td>
<td>MethoxyPEG</td>
</tr>
<tr>
<td>Pnp-CF</td>
<td>p-nitrophenyl chloroformate</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethylamine</td>
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<tr>
<td>Trp-mPEG</td>
<td>Tryptophan-PEG</td>
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<tr>
<td>Trp</td>
<td>Tryptophan</td>
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<tr>
<td>SEC-MALS</td>
<td>Size exclusion chromatography-multi angle light scattering</td>
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REFERENCES


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Publications

- Floriane Groell, René Handrick, Mikhail Sholukh, Jürgen Hannemann, Olivier Jordan, Gerrit Borchard. *Multi-Parametric Evaluation of Therapeutic Protein Aggregation*. To be submitted.


- Christian Reichert, Floriane Groell, Michael Möller, Gerrit Borchard. *Stabilization of Bovine Serum Albumin and Hen Egg White Lysozyme by Non-Covalent PEGylation with Trp-mPEG*. To be submitted.

Oral presentations

- “The Birth, Life and Death of a Therapeutic Protein” Swiss Galenic Meeting, ETH Zurich, Switzerland - September 2016.

- “Aggregate Formation of Therapeutic Proteins: How to Characterize and Classify Them?” Pharma 2030: current and future challenges, joint meeting of the Austria Pharmaceutical Society (ÖPhG) and the Swiss Academy of Pharmaceutical Sciences (SAPhS), University of Innsbruck, Austria - April 2017.

- “Therapeutic Protein Aggregation: Causes and Consequences” 11th PhD day, Fondation Louis Jeantet, Geneva, Switzerland - June 2017.
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