Design, Synthesis and Evaluation of Fluorescent Probes for Biological Applications

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Abstract

We have introduced a conceptually new family of fluorophores for application in chemical biology. The concepts of planarization and polarization as it is found in natural systems like the chemistry of vision were merged. A flexible aromatic scaffold based on substituted oligothiophenes was chosen to modulate the conjugation of the probe and the insertion of an electron-donating moiety and different acceptor groups at the terminal positions introduced a permanent dipole moment in the fluorophores. The optoelectronic properties were determined and the subsequent investigations in large unilamellar vesicles (LUVs) evidenced the simultaneous ability of these fluorophores to respond to membrane fluidity and membrane potential. In parallel aromatic substrates naphthalenediimides (NDI) and perylendiimides (PDI) were functionalized with hydroxyl moieties to obtain a series of compact panchromatic probes able to cover all the visible range. These results evidenced the possibility to modulate the optoelectronic properties, to potentially employ these molecules as biological pH-probes.

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Design, Synthesis and Evaluation of Fluorescent Probes for Biological Applications

THÈSE

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La Faculté des sciences, sur le préavis de Messieurs S. MATILE, professeur ordinaire et directeur de thèse [Département de chimie organique, J. LACOUR, professeur ordinaire (Département de chimie organique) et H. ANDERSON, professeur (University of Oxford – Department of Chemistry – Oxford, United Kingdom)], autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

Genève, le 3 décembre 2012

Thèse - 4492 -

Le Doyen, Jean-Marc TRISCONÉ

N.B. - La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives aux thèses de doctorat à l'Université de Genève".
To my parents
And my brother

“Everything must be made as simple as possible. But not simpler.”

Albert Einstein
This thesis has been conducted in the Department of Organic Chemistry at the University of Geneva under the direction of Prof. Stefan Matile. I wish to express my sincere gratitude for giving me the opportunity to join his research group. During these four years I enjoyed the intriguing scientific guidance and discussion with my supervisor. My gratitude also goes to Prof. Harry Anderson, University of Oxford, and Prof. Jérôme Lacour University of Geneva who agreed to read my thesis and participate in its defense.

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Last but not least I want to warmly thank my parents and my brother for the continuous support during this experience away from home. I have never forgotten all the efforts and sacrifices you have done during years allowing me to perform my studies. My endless gratitude is for you all.
List of Publications


Summary

In the first part of the thesis, the design and the investigation of a conceptually new class of fluorescent probes for biological investigation was reported. These fluorophores merged the well-known concepts of planarization and polarization as it is found in natural systems like the chemistry of vision. The final purpose of this project was the development of a series of sensitive probes able to discriminate the bilayer membrane fluidity and the transmembrane potentials, important phenomena which are involved in many cellular activities.

![Figure S1. Structure of push-pull oligothiophene amphiphilic probes.](image)

To achieve this goal a flexible aromatic structure of an oligothiophene backbone was modified by modulating the (de)planarization properties of the
fluorescent probes in order to affect the overall \( \pi \)-conjugation. In the same time the insertion of a methoxy donor group and different acceptor moieties at the terminal positions of the principal molecular axis introduced a permanent dipole moment in the fluorophores (Figure S1).

A subsequent solvatochromic investigation (Figure S2) of these molecules was performed to quantify with Lippert, Bakishiev, Kawski and Reichardt solvent polarizabilities parameters the efficiency of the push-pull systems. The compounds \( S_1 \) and \( S_2 \) showed the best push-pull system, 25% more efficient than the trimer analogues \( S_5 \) and \( S_6 \) and almost double the dipole strengths of the hydrazone compounds \( S_3 \) and \( S_4 \).

![Figure S2. Lippert plot for quaterthiophene based fluorophores S1-S6.](image)

The simulation of the response in cellular membranes was obtained with the large unilamellar vesicles (LUVs). Two different experiments were designed to evaluate the sensitivity of the fluorophores to the membrane fluidity and to the transmembrane potential.

The probes were subjected in DPPC LUVs in which a transition from gel to liquid phase occurs at 41 °C. The temperature cycles evidenced red-shifted excitation spectra for all the amphiphiles moving from the disordered liquid to the organized gel phase suggesting that the lateral membrane constriction forced the oligothiophenes to a more planar conformation. Interestingly the
shifts (Figure S3A) were comparable for all the amphiphiles proving that the dipole moments were not involved in the planarization process.

The membrane polarization was installed in EYPC vesicles to investigate the fluorophores response to the transmembrane potentials. Differently from the membrane fluidity, only the compounds with the strongest dipole moments S1 and S2 have shown a linear response of the excitation spectra with the increased potentials. Weaker push-pull systems such S3, S4 and shorter analogues S5 and S6 were totally unaffected by the different conditions evidencing a major role of the dipole moments in the sensitivity to the membrane potentials (Figure S3B).

![Figure S3. (A) Excitation maxima in DPPC vesicles of S1 (blue), S2 (light blue), S3 (red) S4 (magenta), S5 (green) and S6 (dark green) during the temperature cycles. (B) Red shift in the $I_{ex(50)}$ of S1 (blue) and S2 (cyan) as a function of the applied transmembrane potential.](image-url)

These results have evidenced the simultaneous ability of these fluorophores to respond by different and complementary interactions to distinct environmental changes. Moreover they support further investigations with similar compounds to confirm and maximize the sensitivity achieved with this first generation of polarizable and planarizable probes.
The second part of this work was focused on the development of a series of panchromatic molecules with possible application as fluorescent probes. Well-known aromatic substrates naphthalenediimides (NDI) and perylenediimides (PDI) were functionalized, in a straightforward manner, with hydroxyl moieties to obtain a series of compact panchromatic probes (Figure S4).

**Figure S4.** Molecular structures of panchromatic probes with color variations due to the deprotonation or the *in-situ* coupling.

The deprotonation of the alcohol moieties provided access to an impressive absorbance shift able to cover all the visible range approaching 800 nm with the dianion of S9. On the other hand, the dynamic covalent chemistry was employed for the *in-situ* oxidative imination of S7 and for the formation of a fluorescent boronic ester by coupling of the catechol S8 with the arylboronic acid. These results evidenced the possibility to modulate the optoelectronic properties, to quickly build multicomponent architectures and to create devices for differential chemosensing based on these colorful probes.
Résumé

Dans la première partie de la thèse, on décrit la préparation et l’étude d’une nouvelle classe de sondes fluorescentes destinées à la recherche biologique. Ces molécules fluorescentes intègrent les mécanismes de polarisation et planarisation qui existent dans des systèmes naturels comme ceux associés à la chimie de la vision. Ces sondes doivent être capables de quantifier la fluidité de la membrane et le potentiel transmembranaire qui sont des paramètres importants dans de nombreux processus cellulaires.

Figure S1. Structures des sondes amphiphiles de type push-pull contenant le motif oligothiophène.

Pour réaliser cet objectif, on a modifié une structure flexible d’oligothiophènes de façon à moduler ses capacités de (dé)planarisation et à
perturber sa conjugaison \( \pi \). Dans le même temps, on a introduit un moment de dipôle permanent le long du fluorophore grâce l’insertion d’un groupement donneur (méthoxy) à une extrémité et de différents groupes accepteurs à l’autre extrémité de l’axe moléculaire principal (Figure S1).

Ensuite, on a étudié le solvatochromisme de ces molécules (Figure S2) afin de quantifier l’efficacité du système push-pull grâce aux paramètres Lippert, Bakishiev, Kawski et Reichardt décrivant la polarisabilité des solvants. Les meilleurs systèmes push-pull ont été obtenus avec les composés \( S1 \) et \( S2 \) qui ont une efficacité 25% plus importante que les trimères \( S5 \) et \( S6 \) et une force de dipôle quasi-doublée par rapport aux hydrazones \( S3 \) et \( S4 \).

![Graphique de Lippert obtenu avec les quaterthiophènes S1-S6.](image)

Une simulation de la réponse des sondes dans la membrane cellulaire a été obtenue grâce à une expérience réalisée sur de larges vésicules unilamellaires (LVU). On a ainsi pu évaluer la sensibilité des sondes à la viscosité de la membrane et au potentiel transmembranaire. Les fluorophores ont été mélangés avec DPPC pour créer des LVU dont la transition de phase gel-liquide a lieu à une température de 41 °C. Des cycles de chauffage et de refroidissement ont montré que les maxima d’excitation de tous les amphiphiles passant de la phase liquide (désordonnée) à celle de phase gel (ordonnée) se décalent dans les grandes longueur d’ondes, ce qui suggère qu’une contrainte latérale appliquée...
dans la membrane force le thiophène à être plus planaire. Il est intéressant de constater que le décalage observé (Figure S3A) est comparable pour tous les amphiphiles; ceci prouve que le moment de dipôle n’intervient pas dans le processus de planarisation. On a ensuite injecté ces fluorophores dans la membrane de vésicules EYPC polarisées afin d’étudier leur comportement en présence de potentiel transmembranaire. Contrairement aux résultats obtenus pour la fluidité membranaire, seuls les composés possédant un fort moment de dipôle (S1 et S2) ont montré une relation linéaire entre le spectre d’excitation et le potentiel. Les systèmes push-pull plus faibles (S3 et S4) et leurs analogues plus courts (S5 et S6) n’ont pas été affectés par les différentes conditions expérimentales ce qui suggère que la sensibilité de ces molécules face au potentiel transmembranaire est fortement affectée par leur moment de dipôle (Figure S3B).

Figure S3. Maxima d’excitation dans les vésicules DPPC (A) de S1 (bleu), S2 (bleu clair), S3 (rouge) S4 (rouge), S5 (vert) and S6 (vert foncé) pendant les cycles de température. (B) : Décalage dans le rouge pour $I_{ex(50)}$ obtenu pour S1 (bleu) et S2 (bleu clair) en fonction du potentiel transmembranaire appliqué.

Ces résultats ont mis en évidence la capacité des fluorophores étudiés à répondre aux changements de leur environnement, et ceci grâce à différentes interactions. Ces mêmes résultats encouragent des recherches supplémentaires sur des composés analogues dans le but d’augmenter la sensibilité obtenue avec cette première génération de sondes polarisables et planarisables.
La deuxième partie de ce travail s’est centrée sur le développement d’une série de molécules panchromiques, potentiellement utilisables comme sondes fluorescentes. Les naphthalènediimides (NDI) et perylènediimides (PDI), molécules aromatiques bien connues, ont été fonctionnalisées avec des groupes hydroxyles, en utilisant des synthèses directes et rapides, afin d’obtenir des sondes panchromiques compactes (Figure S4).

Figure S4. Structures moléculaires des sondes panchromatiques et représentation des produits obtenus par déprotonation et couplage *in-situ*.

La déprotonation des fonctions hydroxyles fournit une large gamme spectrale qui couvre tout le spectre visible, jusqu’au proche infrarouge avec le dianion PDI S9 dont l’absorbance dépasse les 800 nm. D’autre part, l’utilisation de liaisons covalentes dynamiques comme la formation *in-situ* d’imine après oxydation de l’hydroquinone S7 ou la préparation d’un ester boronique par condensation du catéchol S8 avec un acide boronique sont des outils attractifs pour moduler ces mêmes propriétés optoélectroniques, construire rapidement des macromolécules ou créer des senseurs chimiques.
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INTRODUCTION

1.1 General

Our ability to understand the structures and the dynamics involved in biological systems is strongly related to an accurate visualization and definition in time and space of signals coming from cells. Fluorescent spectroscopy plays a predominant role among the non-invasive techniques in the visualization of in-vivo systems allowing the collection of large and well defined series of data on structures and processes. Fluorescence-based tool with remarkable photostability and properties were developed, during years, specifically to investigate biological role of carbohydrates, cellular bilayer membrane structures, proteins activities and nucleic acids.

The design of new fluorophores for biological applications often suffers from intrinsic dichotomy by which keeping high structural homology with natural analogue molecules prevent the achievement of remarkable results in term of fluorescence properties (e.g. fluorescence quantum yield, bathochromic emission) and, conversely, more elaborated and artificial modifications of the molecular skeleton could induce perturbation of normal cellular activity or in the worst case be lethal for living systems.

In the first part of this chapter some examples from nature showing how to modulate the photophysical properties of chromophores and pigments, are briefly illustrated. In the second part an overview of various synthetic chromophores which mimic the same natural concepts are reported to illustrate the wide range of applications in which responsive dyes could be employed.
1.2. Lessons from Nature

1.2.1. Lobster Mimesis

The blue/purple ($\lambda_{\text{max}} = 632$ nm) chromatic camouflage carried out by lobster employing the red/orange pigment astaxanthin 1 (AXT, $\lambda_{\text{max}} = 472$ nm) have intrigued scientist for the last 60 years\textsuperscript{5,6}. The recovered orange color of the animal during cooking increased the oddness towards the origin of this particular phenomenon.

![Chemical structure of astaxanthin (AXT).](image)

**Figure 1.** Chemical structure of astaxanthin (AXT).

In lobsters, AXT is present as a guest into a more complex supramolecular cavity arranged by the macromolecular protein crustamycin (CR). Quite recently it was revealed that the origin of such remarkable bathochromic shift in color must be related to several interactions which take place due to the particular assembly between AXT and CR moieties\textsuperscript{7}.

Softer X-ray techniques have depicted the three dimensional structure of the AXT-CR complex (Figure 2) showing that the first contribution to the red-shifted absorption came from the extended conjugation of the molecule including the coplanar heterocyclic rings. Secondly, the carbonyl oxygen of the rings were shown to be involved in hydrogen bond patterns with various hydrophilic residues from proximal amino acids and water molecules in the first solvation shield. As a consequence, AXT acquired a permanent dipole moment because of this polarization induced by the host protein. Further
electronic delocalization was showed to be promoted by the interactions between the polylene chain and other histidine and tryptophan residues. These three factors together were considered responsible of the red-shifted absorption to 580 nm.

The final shift to 630 nm was associated to the further supramolecular assembly of protein into dimer or tetramer forms. In these structures, the AXT molecules are located in a parallel conformation in a sort of pocket surrounded by a particular protein environment.

![Figure 2](image.png)

**Figure 2.** Structure of the supramolecular complex (A) between astaxanthin molecules and $\beta$-crustacyanin residues. Detailed view (B) of the residues interactions and distances. Images from reference$^7$.

A couple of years later, Buda and co-workers$^8$ have confirmed by resonance Raman spectroscopy and solid state NMR investigation that the interaction between the AXT and the protein modified the electronical properties of the carotenoid. Nevertheless the conformational change and the induced
polarization were calculated to contribute to 30% of the overall bathochromic shift of AXT. The major contribution to the blue color come from the exciton coupling due to the proximity (Figure 3) of the two astaxanthin molecules.

**Figure 3.** Top and side views of two astaxanthin molecules in β-crustacyanin. $\alpha$ is the angle between the molecular long axis, $R$ is the unit vector connecting the center of mass and $\mu$ is the transition dipole moment. Image from reference$^9$.

The overall color change of the AXT is a paramount example of a natural supramolecular assembly. Different factors such defined spatial localization, environmental induced polarization and forced planarization play together affording unique properties to a simple carotenoid molecule.

**1.2.2. Chemistry of Vision**

Similar phenomena were found in the chemistry of vision$^9$ where a small carotenoid molecule, 11-cis-retinal (2, Figure 4) is covalently linked through a Schiff base with a lysine (Lys$^{296}$) residue of opsin, a transmembrane protein present in the retina. Different interactions between 2 with residues of opsin are
responsible for the light sensitivity of the long (L), medium (M) and short (S) wavelength visual cones which allowed the discrimination of colors\textsuperscript{10}. The photo-isomerization of 2 in all-trans-retinal by the absorption of a photon leads to a conformational change in the entire protein activating the visual process. Subsequently the trans-retinal is hydrolyzed and dissociated from opsin which regenerates the original complex with another molecule of 11-cis-retinal provided by adjacent retinal epithelial cells.

![Figure 4](image)

**Figure 4.** Chemical structure (A) 2. Spectral sensitivities (B) of human cones derived from the color matching function. Image from reference\textsuperscript{10}.

The retinylidene group located in the proximity of the extracellular site\textsuperscript{11} (Figure 5) has shown a constrict conformation of the \(\beta\)-ionone ring in a medium polarity environment due to the presence of aromatic and hydrophilic amino acid residues from the helices III and IV.

![Figure 5](image)

**Figure 5.** Schematic representations (A) of the chains surrounding 2 viewed from cytoplasmic side and (B) of the different residues interacting with the retinal Schiff base. Images from reference\textsuperscript{11}.  

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A variety of different interactions on the protonated Schiff base (PSB) side were found to take place in the different mutants of opsin inducing polarization on the carotenoid scaffold. The relative positions of Phe$^{261}$ and Ala$^{269}$ respect to PCB were identified as the main responsible for the absorption shift from red to green in the cones.

Molecular dynamic simulation together with quantum mechanical calculation$^{12}$ confirmed that the electrostatic interactions, hydrogen bonds and the restrict orientation of the $\beta$-ionone ring were responsible of the spectral tuning in the PSB-opsin complex (Figure 6). The blue-shifted absorption of the complex was mainly related to the stabilization of the ground or the destabilization of the excited state. The first one originated from the hydrogen bond with Ala$^{269}$Ser residue the latter from the lowered electrostatic interaction between the $\beta$-ionone and the hydroxyl moiety in the Thr$^{118}$Ala mutant opsin. On the other hand, a reduced energy gap was observed as result of polar interaction with Glu$^{113}$Asp which remarkably destabilized the ground state or by complementary stabilization of the excited state by stronger electrostatic interaction with the Ala$^{269}$Thr mutant.

![Figure 6](image.png)

**Figure 6.** Schematic and mechanistic representation of four selected residue interactions on the binding site of retinal Schiff base. Image from reference$^{12}$. 
Analogously to the lobster pigmentation, nature applied the same concepts in the construction of very sensitive process such vision. Simple modifications of the amino acid sequences in a protein are able to tune remarkably the optical properties of the supramolecular complex allowing a precise discrimination among colors.

1.2.3. GFP and Other Fluorescent Proteins

Completely different strategy was adopted by nature to provide a brilliant fluorescence in a simple organism such jellyfish. The bright green emission is generated by the \( \beta \)-barrel protein (GFP) in which eleven \( \beta \)-strand form almost a perfect cylinder capped at each end by a short section of \( \alpha \)-helix. The fluorophore, \textit{para}-hydroxybenzylidene-imidazoline 3, is encapsulated in the middle of this elegant structure (Figure 7) and is surrounded by a hydrogen bond network which works as shield from the bulk solvent.

\[ \text{Figure 7. Protonation equilibrium (A) between 3 and 4 and tertiary structure (B) of GFP. Image from reference}^{13}. \]
The hydrogen bonding pattern surrounding the chromophore is responsible of the double absorbance maxima of the GFP (Figure 8). The higher energy absorption ($\lambda_{\text{max}} = 395$ nm) is due to the neutral form of the phenol residue 3 of the chromophore while the less intense absorption ($\lambda_{\text{max}} = 475$ nm) is related to the anionic form 4. The excitation at both the wavelength results in a green emission ($\lambda_{\text{em}} = 503\text{-}508$ nm). A continuous irradiation generates a slowly decrease of the higher energy band in favor of the red-shifted one$^{14}$. This equilibrium is regulated by the hydrogen bond system that allowed the excited state intramolecular proton transfer (ESIPT) responsible of the green emission.

![Figure 8](image.png)

**Figure 8.** Schematic representation of the green emission and ESIPT process. Image from reference$^{13}$.

The phenolate as donor group, the supramolecular electrostatic interaction network and the hydrogen bond pattern are found in analogue fluorescent protein (e.g. RFP) of corals$^{15}$. The ability of nature to cover the whole visible spectrum (Figure 9) by merging these factors is clearly a source of inspiration for the development of synthetic fluorophores.
Figure 9. Collection of the chromophores present in the red fluorescent proteins (RFP) with the corresponding absorption and emission spectra maxima. Image from reference\textsuperscript{15}. 

\textsuperscript{15} Reference number.
1.3. Planarizable Chromophores

1.3.1. Molecular Rotors

The most diffused class of molecules which present phenomena related with the gain or the loss of planarity upon external perturbation is the molecular rotor family. These fluorophores consist of a donor and an acceptor group conjugated by flexible short π-system and are often known as twisted intramolecular charge transfer (TICT) molecules. As in a common fluorescent probe (Figure 10) the absorption of a photon promotes the molecule from the flat ground state (S₀) to the first excited state (S₁). From here the rotor can return to S₀ by fluorescent pathway from the local excited state (LE). Nevertheless for these molecules the intramolecular twist occurring upon excitation generates a second more stable excited state and also a higher energetic analogue of S₀. As a consequence the TICT energy gap is smaller compared to the one based on the original energetic levels.

**Figure 10.** Jablonski diagram describing the emission pathways for two different molecular rotors 5 and 6. Image from reference.
The smaller energy gap related to the TICT process explains the presence of a red-shifted second emission for compound like 5 and also the quenched emission in molecules as 6. For the latter, the difference in energy between the twisted excited and ground state is too small and favors the non-radiative decay processes.

As far as the overall structures consist of a donor and an acceptor moieties separated by small aromatic systems, a variety of molecular rotors were designed and investigated\textsuperscript{18,19}. The most common 7-16 (Figure 11) have an analogue backbone as the dimethylamino benzonitrile 5 or are based on the julolidine or stilbene moieties like 6 and 17. More articulated structures such 18 or ionic as 19 have shown the same particular properties.

\begin{figure}[h]
  \centering
  \includegraphics[width=\textwidth]{rotor_structures.png}
  \caption{Chemical structures of the most common molecular rotors.}
\end{figure}

It was mentioned that the energy of the TICT excited state is lower compared to the LE but the TICT formation rate is strongly dependent on solvent. Polar solvent are known to increase the TICT stability and solvent viscosity is the main variable that affect the formation of the twisted excited
state. As a consequence the most common application of the molecular rotor is related to the investigation of the viscosity in fluids.

Figure 12. Chemical structures of the molecular rotors 20-25 and fluorescence quantum yield as a function of viscosity. Image from reference 20.

Molecular rotors 20-25 are analogue of 6 and show a linear increase of fluorescence quantum yield 20 as a function of viscosity of the media (Figure 12). Mixed solvent systems like ethanol/glycerol or glycerol/water are commonly used for the evaluation of the viscosity on the emission intensity. The viscosity of such mixtures is related to the ratio of glycerol in the system while the overall polarity of the solution remains constant excluding the effect of polarity on the quantum yield and on the fluorescence shift.

The formation of aggregates and/or the partial precipitation of the probes are the main problems in the viscosity investigations. These phenomena affect the correct interpretation of the photophysical responses coming from the fluorophores. To overcome this limit, recently Theodorakis and co-workers have modified the basic scaffold of the molecular rotor into a ratiometric fluorescent probes 21,22 26 and 27. In these molecules a primary fluorophore insensitive to viscosity is linked through and hydrocarbon chain to a molecular rotor moiety. The intramolecular distance between the fluorophores is fixed to allow Förster energy transfer process (FRET). The primary fluorophore acts as a donor for the rotor and in the same time as an internal standard for the emission.
**Figure 13.** Chemical structures of the molecular rotors 26 and 27 and fluorescence spectra as a function of viscosity. Images from reference\(^\text{23}\).

Molecular rotors have been also tested to monitor and investigate the properties of polymers\(^\text{23}\). The polymerization reactions could be followed by the increase of the fluorescence intensity due to the changes in the viscosity of the bulk media (Figure 14). Moreover molecular rotors were sensitive enough to be employed also in the estimation of the polymers molecular weight. Different polymer molecular weights were identified by changes in the fluorescence quantum yields of the probes.

**Figure 14.** Chemical structures of the molecular rotors. Polymerization (A) and (B) polymer molecular weight analyzed by changes in fluorescence intensity. Images from reference\(^\text{23}\).
More recent and interesting is the use of photon-induced conformational change in biological system. Intracellular viscosity plays and key role in the diffusion of signals and metabolites. Nevertheless living cell are complex matrices and their visualization and the investigation are extremely difficult. Recently the first dual mode fluorescence molecular rotor 30 capable to visualize the intracellular viscosity was developed\textsuperscript{24} (Figure 15).

![Figure 15](image.png)

**Figure 15.** Chemical structures of the cyanine based molecular rotor 30 and confocal fluorescence ratiometric image in living cell. Image from reference\textsuperscript{24}.

This molecule presented double absorption ($\lambda_{\text{max}} = 400$ and 613 nm) and double emission ($\lambda_{\text{em}} = 450$ and 650 nm) but only the red-shifted emission was sensitive to the temperature and the viscosity allowing a ratiometric investigation. Living cells treated with 30 were visualized with high resolution time and space, proving that this family of molecules is really promising for the investigation of complex environments.

Similar to 30, the bisporphyrin rotor 31 was able to determine and quantify the cytoplasmic viscosity. The relative planar or twisted conformations between the porphyrin units\textsuperscript{25} allowed a ratiometric measurements based on the relative two peaks in the emission spectra. Moreover this bulky probe afforded a real-time monitoring of dynamic process such the viscosity increase during the photoinduced cell death. Fluorophore photosensitized production of toxic singlet oxygen $O_2 (\alpha' \Delta g)$ and its subsequent decay was monitored to prove how the viscosity affected the diffusion processes in cells. These results provided the basis for a potential application of complex molecular rotors in the photodynamic therapy of cancer cells.
Figure 16. Chemical structure of the 31 and the ratiometric fluorescence images of 31 in cells as function of time during the photoinduced cell death. Images from reference 25.

1.3.2. Planarization Induced by Temperature

The temperature effect on the planarity of chemical structures was investigated in depth to understand the molecular recognition processes and for the development of sensors. Variation of temperature may cause an increase or a loss of planarity. This effect is evidenced when it appears with molecules able to absorb visible light (Figure 17).

Figure 17. Chemical structures of trans-stilbene 32, PDI 33, and spyropyran 34.
The trans-stilbene molecular structure 32 had challenged for years scientists in a dispute between a flat or a twisted conformation\textsuperscript{26}. Temperature augmentation seemed to destroy the planarity of this molecule in gas phase while it did not have any effect in solid state where the two phenyls were slightly shifted\textsuperscript{27}. In solution the loss of planarity with increasing temperature was finally proved by Raman spectroscopy\textsuperscript{28} and further confirmed by calculation\textsuperscript{29}.

An analogue effect of temperature appeared in the perylenediimides (PDI) aromatic system 33 in solution (Figure 18)\textsuperscript{30}. The absorption spectra investigation at different temperatures of diluted solutions of 33 evidenced the presence of multiple isosbestic points which were interpreted as clear signal of an interconversion between two species. Based on the experimental data and on calculation, the formation of a slightly twisted core form was proposed at higher temperatures.

![Figure 18](image)

**Figure 18.** UV-Vis spectra of 33 at different temperature and calculated equilibrium forms of PDI. Images from reference\textsuperscript{30}.

The same analysis was the base of the investigation of temperature effect on the planarity of spyrpyran 34 and some methylated derivatives\textsuperscript{31} (Figure 19). Different form the PDI, 34 had a twisted conformation which planarized upon the increase of the temperature. This clear enhancement in conjugation was observed by an impressive change of the absorption of 34 in xylene form a colorless to a purple solution. Analogues of 34 bearing methyl groups in 3, 3’
positions were unable to provide any thermochromic effect because of the steric hindrance introduced by the substituents.

![Figure 19](image1.png)

**Figure 19.** Schematic representations of the induced planarization of 34 increasing temperature. Images from reference\textsuperscript{31}.

Conformational changes due to variations of temperature are not prerogative of small molecules but are present also in macromolecules such conjugated polymers. These macromolecules have recently attracted the attention because of their unique properties as electrical conductive, easy processable and low cost materials.

![Figure 20](image2.png)

**Figure 20.** Chemical structures of the polythiophenes 35-40 and typical temperature-dependent absorption spectra. Images from reference\textsuperscript{32}.

Polythiophenes are probably one the most diffused family among the functional polymeric materials and their properties were investigated in depth.
Thermochromic investigations (Figure 20) of such molecules\textsuperscript{32} were extensively employed to evaluate the effect of the temperature on the conjugation in solution and in solid state. Different thermochromic behaviors were found to be related with the nature, the amount and the relative positions of the substituents along the polymer backbones \textbf{35-40}.

Almost in the same time Holdercroft reported a systematic investigation\textsuperscript{33} of the effects of the regioregularity and the length of the alkyl substituents on the thermochromism of the polythiophenes \textbf{41-44}. The results confirmed that the presence of defects (lack of regioregularity) along the backbone strongly affected the thermochromic behavior (Figure 21). Polythiophenes with high content of defects showed a clear isosbestic point unrelated with the length of the \(\beta\)-substituents. Regioregular polymers with short alkyl substituents exhibited a continuous blue shift of the absorption spectra with increased temperature. The same macromolecules with long \(\beta\)-alkyl moieties were depicted as the non-regioregular compounds by an isosbestic point. These results evidenced that the planarization of such molecules was simultaneously related to the nature and positions of the substituents along the backbone.

\begin{figure}[h]
\centering
\includegraphics[width=0.4\textwidth]{structures.png}
\caption{Chemical structures of the polythiophenes \textbf{41-44} and absorption maxima as function of temperature for 20\% defective polymers (A) and high regioregular polythiophene (B). Images from reference\textsuperscript{33}.}
\end{figure}

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1.3.3. Planarization Induced by Pressure

The first pure organic34 mechanofluorochromic dye35 45 was reported by Araki in 2011. Upon precipitation, this pyrene based fluorophore formed a white powder (B-form) which became yellow (G-form) once pressed (Figure 22). Contrary to the white aggregate the yellow powder was soluble in organic solvents. Crystallographic analysis evidenced that the hydrogen bonds on the imide sites conferred stability and an ordered columnar structure to the B-form. Upon application of a weak pressure, the conjugation between the pyrene core and the phenyls groups was maximized but the hydrogen bond pattern was lost giving a mismatched architecture.

![Figure 22](image)

**Figure 22.** Chemical structure of 45 and representations of columnar (blue) and distorted (green) aggregations. Images from reference35.

Similar molecules belong to the category of piezofluorochromic aggregation-induced emission (PAIE) probes36. Oligoaromatic molecules such 46 adopted a twisted conformation in solution and also in solid state. Upon application of a pressure, the twist conformer became flat allowing intramolecular interactions such π-π stacking. The aggregates have shown optical properties remarkably different from the single molecule (Figure 23). The deplanarized single molecule state was then fully recovered after annealing the sample film.
Figure 23. Chemical structure of 46 and images taken of annealed (green) and pressed (yellow) film. Images from reference36.

The same concept of an enhanced conjugation was employed in the design of smaller probes (47-50, Figure 24) based on the anthraquinone. As the molecular rotors37, these molecules were employed to follow the polymerization reactions. A systematic variation of aromatic pending groups was performed to screen the effective conjugation upon the pressure induced planarization showing considerable results in terms of sensitivity.

Figure 24. Chemical structures of anthraquinone planarizable probes 47-50 with different aromatic substituents. Image from reference37.

The investigation of mechanofluorochromic dyes and PAIE is nowadays one of the most promising research field in the development of the optical data storage systems, chemosensors and light emitting devices.
1.3.4. Planarization Induced by Self-Assembly

Another way to induce the planarization comes from the self-assembly of twisted donor-acceptor fluorophores as 51 in solution. In the single molecules state 51 was shown to adopt a twisted conformation because of the steric hindrance between the pyrene and the rhodamine acetic acid moieties. This twisted conformation rearranged to a planar structure upon addition of a non-solvent of the probe. The aggregation of 51 into stable nanoparticle occurred due to a concurrent planarization of the scaffold. The process was coupled with a bathochromic shift of the emission spectra with a simultaneous 40 times increase of the quantum yield which suggested a J-type aggregation among the molecules (Figure 25).

![Image](image_url)

**Figure 25.** Chemical structure of 51 and fluorescence spectra shift upon the formation of nanoparticles. Image from reference 38.

The same concept was applied to a longer oligoaromatic molecule 52 for the creation of fluorescent switches (Figure 26). The full planar molecular structure in nanoparticle switched on the fluorescence which was completely quenched in solution because of the non-radiative processes due to the twisted conformation. The aggregate could then be destroyed recovering the original non-fluorescent twisted state exposing the supramolecular aggregated to solvents vapors. Other than application as visible detector this kind of
aggregates were suggested to be interesting substrates for the development of nanosized optoelectronic devices.

**Figure 26.** Chemical structure of 52 and schematic representation of enhanced conjugation in nanoparticles. Images from reference 70.

### 1.3.5. Planarization Induced by Protons, Cations and Anions

Physical parameters like temperature and pressure have been shown to be very efficient in the modulation of the planarity in many substrates. Appropriate functional groups together with the environmental chemical modifications have been employed successfully to modulate the planarization of some molecules.

Fluorescent oxadiazole based polymers 53 have been reported to modulate their photoluminescence properties as a function of acidity of the media (Figure 27). The emission maxima and the quantum yield were found to change remarkably because of the protonation of the oxadiazole unit 54. A simultaneous hydrogen bond formation with the proximal oxygen atoms was assumed as a possible cause of the induced planarization of the scaffold. The initial properties were fully recovered upon evaporation of the acid from the
solution. Remarkably this molecule has been shown to be stable in limited conditions such strong acidic media together with extended photoirradiation time. Improvement of the stability in harsh conditions for polymeric material is an important feature to enlarge and optimize the applications of these materials in solar cell or biological substrates.

Figure 27. Acid mediated planarization of 53 to the protonated form 54. Emission spectra (A) and emission intensity variation (B) during acid addition-removal cycles. Images from reference 30.

A reversible modulation of the conductive property of polymers is crucial in the devices that work as molecular switches and it could be achieved by inducing changes in the effective conjugation of the molecules 41.

Swager pioneered this approach in 1993 showing how the introduction of crown ether moieties on polythiophene backbone 42 can strongly affect the conjugation of the polymer. Substituted polythiophene 55 was able to adopt spontaneously the extended conjugation structure detected by the absorption maxima around 500 nm. The addition of metal ions resulted in a less flat conformation 56 due to the metal complexation by crown ether moieties which induced a torsion of the polymeric scaffold (Figure 28). A subsequent chelation

23
of the perturbing agent reinstalled the original planarity and conductivity of the polymer.

Figure 28. Schematic representation of the deplanarization of 55 to 56 induced by metal complexation.

Similar regioregular polythiophenes 57 and 58 sensitive to thermochromism and ionochromism were developed a couple of years later by Leclerc. These compounds showed the same planarity response in solution and in solid state to the temperature effect ($\lambda_{\text{max}} = 434 - 544$ nm) as the more diffused alkyl substituted polythiophenes (Figure 29).

Figure 29. Molecular structures of the polythiophenes 57 and 58 and variation of the absorption at 544 nm as a concentration of different cations for 57 (A) and 58 (B). Images from reference 43.
The introduction of different crown ether moieties in 57 and 58 allowed the formation of the intramolecular metal complexes in which two adjacent crown ethers bound metal cations forcing the backbone to planarize ($\lambda_{\text{max}} = 544$ nm). Moreover the size of the crown ether moieties was shown to be determinant in the formation of the 2:1 ratio complexes with different cations. Compound 57 strongly planarized upon the additions of sodium cations while the larger crown ether in 58 have shown the same effect with potassium ions.

More recently Roncali has reported the complexation of lead cations by thiophene tetramers 59. A constant ipsochromic shift was observed in solution upon titration of 59 with lead perchlorate due to the formation of the 1:1 complex with inter-ring crown ether groups.

The interaction of the oligomers with the cations was also observed in solid state (Figure 30) by an increase of the electronic transport properties of gold electrodes coated with a monolayer of 59. The complexation of Pb$^{2+}$ cations by the oligothiophene units caused the shift of one of the molecular orbital close to the Fermi energy of the electrode evidencing clearly how the conductive property of the material could be reversible modulated.

![Figure 30](image-url)
Similar planarization properties were found in biological investigations involving ribonucleic acids as substrates. DNA chemistry and his detection have attracted the investigation based on a variety of different chromophores.

Zhou has introduced a naked-eye visualization of the single-stand (ss) DNA enzymatic cleavage by S1 nuclease\textsuperscript{45} which make this method fast and convenient compared to instrumental analysis. A polythiophene charged probe \textbf{60} adopted a non-conjugate conformation in aqueous media to shield the hydrophobic backbone form water and this resulted clearly in a yellow color of the solution ($\lambda_{\text{max}} = 400$ nm). In presence of ss-DNA, due to electrostatic interactions, the polymer was stretched to form a duplex which remarkably red-shifted the absorption to 530 nm. Such results were reasonably justified by the enhanced conjugation of the polymer backbone. The action of nuclease enzyme was followed by naked-eye and absorbance spectra showing a slow recovering of the original yellow color of the solution due to the destruction of the ss-DNA and the disassembly of the supramolecular complex (Figure 31).

![Figure 31](image-url)

\textbf{Figure 31}. Schematic representations on nuclease assay and chemical structure of polythiophene \textbf{60}. Images from reference\textsuperscript{45}.

A comparable visible change was observed by Leclerc employing the analogue polythiophenes\textsuperscript{46} \textbf{60} and \textbf{61} which displayed a shift from 400 to 527 nm upon the duplex formation with ss-DNA (Figure 32). The shift back of the
absorption to 420 nm was observed once the complementary DNA strand was added. The final complex was identified as a triplex in which surprisingly the electrostatic interactions between the polythiophene probe and ss-DNA were not interfering with the hydrogen bond pattern of the DNA duplex.

**Figure 32.** Chemical structures of polythiophenes 60 and 61, Schematic representation of triplex formation (A) and solutions (B) of polythiophene, duplex and triplex in water. Images from reference 46.

The sensitivity of polythiophene based molecules based on planar-twisted equilibrium forms were not limited to large molecules. Shinkai developed a colorimetric assay in which a cationic oligothiophene 62 was treated with some biological relevant anion 47 such ATP, phosphate and carbonate (Figure 33).

**Figure 33.** Molecular structure of the oligothiophene 62 and color changes in water solution of equimolar (μM) concentrations of thiophene and anions detected by absorption spectra (A) and naked eye visualization (B). Images from reference 47.
Increased concentrations of negative charged analytes were detected by a remarkable color shift from yellow to pink in solution due to the electrostatic induced planarization of the thiophene scaffold as described before for DNA analogue probes. Moreover organic anion provided more remarkable shift compared to the inorganic ions suggesting that also the hydrophobic interaction played a role in the stretching of the oligothiophene.

Recently the same polythiophene 62 was employed in the construction of a "molecular thermometer"48. Polysaccharide 63 with the cationic 62 formed a helical complex that showed structural changes in response to an external stimulus. The effective conjugation length of the oligothiophene was affected by the temperature change shifting the absorbance from 482 nm at 5 °C to 403 nm at 85 °C. The flexibility of the macromolecular complex allowed the creation of a visual thermometer in which the color shifted gradually from orange to green (Figure 34). The flexibility of this new kind of colorful complex was pointed out to be a remarkable starting point for the development of a new series of sensors, polarized materials and switching devices.

![Figure 34](image)

**Figure 34.** Molecular structure of the polysaccharide 63 and temperature dependency of UV/Vis absorption, CD and fluorescence spectra. Color changes of the molecular thermometer with temperature. Images from reference48.
1.3.6. Planarizable Oligothiophenes

The remarkable colorful properties of thiophene based materials were not limited to the long functionalized molecules. Similar effects were found and investigated in detail also in shorter analogues with smaller substituents. These results proved the extraordinary flexibility of the thiophene backbones.

Many works in the last 20 years have investigated the oligothiophenes inter-ring twists as a function of the substituents along the backbone mainly by calculation\(^{49-54}\).

In 1995 Hadziioannou reported a study\(^{55}\) in which the simulated deplanarization of bisthiophenes 64-68 was correlated with the shifts of the optical properties in solution (Figure 35).

![Figure 35. Molecular structures of the investigated bisthiophenes 64-68 with different substituents.](image)

The insertion of one alkyl substituent in 65 produced an 8 nm ipsochromic shift of the absorption compared to 64 because of the induced twist of the ground state. In the same time the emission resulted red-shifted of 6 nm due to the inductive effect and the planarity of the excited state. The substitution in 66 did not produce any steric interactions and both the absorption and the emission peaks were bathochromically-shifted of 10 nm. Finally the substituents in 3,3’ in the bisthiophenes 67 and 68 gave a 20 nm blue-shifted absorption and a comparable fluorescence patterns with two emission maxima related with the \textit{syn} and \textit{anti} conformations of the planar excited state.
A couple of years later, Leclerc and co-workers have extended the investigations\textsuperscript{56,57} based on the \textit{ab-initio} calculation and on photophysical measurements of the effects of different substituents on a terthiophene scaffolds \textbf{69-72} (Figure 36).

![Molecular structures of the investigated terthiophenes 69-72 with different substituents.]

\textbf{Figure 36.} Molecular structures of the investigated terthiophenes \textbf{69-72} with different substituents.

Structural analysis has evidenced a non-planar conformation in the gas-phase already for the unsubstituted terthiophene \textbf{69} showing an inter-ring dihedral angle of 147 ° for the more stable \textit{anti} conformer. This result differed remarkably with the almost flat conformation (170-175 °) of this oligomer in the solid state. The planarity was almost recovered by the insertion of the methoxy groups in 3, 3'' in \textbf{70}. Despite the induced steric effect of the substituents, the electron-donating character of these groups increased the electronic conjugation favoring a more planar conformation. The replacement of the methoxy with methyl groups in \textbf{71} weakened the donor effect keeping the steric hindrance. The consequence was depicted by an increase of the inter-ring twist (147 °) favoring a more deplanarized conformation. Final elongation of the substituents alkyl chain in \textbf{72} resulted in an even more pronounce twisted conformation due to the high steric hindrance introduced by the two ethyl group in 3’ and 4’ positions. These calculated results were confirmed by more pronounced blue shifts of the absorption spectra in the solution of \textbf{69}, \textbf{71} and \textbf{72} compared to the more flat \textbf{70}. Contrary the emission spectra were closer to each other suggesting that independently on the substituents all the molecules adopted the same flat conformation in the excited state.
In the same time they have reported the investigation\textsuperscript{58,59} of a series of oligothiophenes 73-80 in which the substituents were present both along the molecular scaffold and at the end of the backbone (Figure 37).

![Figure 37](image)

**Figure 37.** Molecular structures of the investigated oligothiophenes with different substituents along the major molecular axis 73-76 and also on the terminal positions 77-80.

The insertion of the alkyl substituents at the $\beta$ positions in the same ring or in two adjacent thiophenes was shown by calculation to induce an important twist of the ground state. In the same time, the spectroscopic investigation pointed out remarkable blue-shifted absorption peaks, lower extinction coefficients and larger bandwidth compared to the unsubstituted analogues. Complementary the substitution in the free $\alpha$ positions resulted in an increased molecular rigidity, bathochromic shift of absorption and emission maxima and in a decrease of the non-radiative processes. This result was interpreted as an increase of the overall conjugation length without any remarkable conformational change. Moreover the same trends were found for all the
oligothiophenes 73-80 confirming that the presence of substituents affected in
the same way all the oligothiophenes independently on the lengths.

More recently Barbarella have performed the same investigation on
tetrahiophene molecules60 81-83 varying the position along the backbone but
not the nature of the substituents.

The unsubstituted tetrahiophene showed not planar anti conformation (φ₁ =
161 °, φ₂ = 163 °) in gas phase and presented a broad absorption band in
solution at room temperature. The situation changed remarkably at low
temperature (4 K) where the absorption spectra recovered the vibronic pattern
and the mirrors symmetry with the flat emission spectra (Figure 38).

Figure 38. Molecular structures of tetrahiophenes 81-83 and normalized
photoluminescence/absorption spectra at 4 and 300 K. Images from reference60.

The insertion of methyl substituents in the terminal thiophene rings 82
increased the twist between adjacent units but did not have a remarkable long
range effect (φ₁ = 141 °, φ₂ = 159 °). A complementary effect was found for the
compound 83 in which two methyl substituents were present on adjacent
thiophenes providing a huge inter-ring torsion (φ₁ = 160 °, φ₂ = 109 °). The
absorption spectra in solution supported the theoretical results showing a blue-
shifted and broad absorption spectra for 82 and 83 at room temperature.
Analogously to 81, the backbone planarity was recovered in solution at low temperature and was detected by the presence of similar vibronic pattern and sharp peaks also for the substituted tetrathiophenes.

The conformation of oligothiophenes was also proved to be affected by the presence of donor-acceptor moieties at the edges of the backbone (Figure 39). The insertion of a dipole moment in such molecules is reflected by a geometrical equilibrium between a non-polar twisted heteroaromatic and a flat zwitterionic heteroquinonoid forms in which the electron-transfer from the donor to the acceptor group can occur. Navarrete has performed a systematic analysis61 of some modified oligothiophenes 84-88 by IR and Raman spectroscopies to evaluate the effects on the probe conformations of different groups and length of the π-bridge.

![Figure 39. Molecular structures oligothiophenes 64 and 84-88 with different terminal substituents.](image)

Upon the insertions of different acceptors in 64 a partial quinonoid character for all the thiophene backbones 84-88 was detected by the shift of the Raman signals patterns. These results could be translated in a partial planarization of the oligothiophenes due to the presence of the electron-withdrawing groups. Moreover the analysis of the extended bridges 87 and 88 suggested that the partial quinonoidization of the molecules could extent toward the middle of the molecules. On contrary the side of the molecules bearing the electron-donating groups generated the same patterns as the unsubstituted 64 retaining the typical heteroaromatic form.
Recently these results found a practical application in the fabrication of organic solar cell (OSC). Small oligothiophene molecules were found to be ideal in these devices because the remarkable electronic properties can be tuned by the straightforward insertion of different substituents. Recently Bäuerle have reported one of the highest efficiency (5.2%) for organic vacuum-deposited single junction solar cells based on oligothiophene 89 and fullerene \(^{62}\).

**Figure 40.** Molecular structures oligothiophenes common skeleton 89 and calculated morphology. Images from reference\(^{62}\).

The concomitant absence of substituent in \(\beta\)-positions and the presence of terminal dicyanovinyl moieties gave rise to almost flat oligomers with a dihedral angle smaller than 4 ° (Figure 40). Different from the herringbone crystal structures of alkyl substituted oligothiophenes, 89 showed a parallel oriented columnar stack. Moreover the presence of the cyano groups leaded to a small intermolecular distance due to relevant hydrogen bond interaction with the nitrogen atoms. The resultant well-organized packing in solid state and the strong coplanarity among the oligothiophene backbones were identified as responsible of the remarkable achievement in term of efficiency.

Different approach was reported by Roncali in the construction of organic solar cells based on 3D conjugated oligothiophenes\(^{63}\). In these devices the goal was not focused on a supramolecular planar assembly among the molecules but on the control of a well-defined 3D architecture based on the deplanarization around a bisthiophene node (Figure 41). The torsion along the main axis was produced by the presence of thiophenes as \(\beta\)-substituents. This approach
implied the advantages of the extension of the conjugation also along the branches and the modulation of the twist along the main axis as function of the substituents.

Figure 41. Molecular structures of the 3D twisted oligothiophenes 90-95.

The insertion of thiophene moieties as lateral chains caused a huge deplanarization of the oligomer scaffolds. In solution, 90 showed already a 40 nm hypochromic shifted absorption compared to the unsubstituted tetrathiophene. The extension of the oligothiophene groups as β-substituent in 91 was represented by the split of the absorption maxima in two distinct peaks
(λ_{abs} = 346, 400 nm) proving the coexistence of different conjugated systems. The removal of one substituent in 92 and 93 gave rise to a better conjugation of the system. Finally the symmetric star-shape oligothiophene 94 and 95 were affected by an impressive deplanarization of the overall molecules which was confirmed by calculation showing a dihedral angle of 84 °. Although the preliminary results as donor molecules in organic solar cells were not remarkable, this new 3D approach showed good potentiality for the development of organic field-effect transistor and solar cells. The possible straightforward modifications of the optoelectronic and conformational properties of oligothiophenes is a suitable starting point in the design of well-defined 3D architectures.

The most recent investigation of the relationship between the performances of OSC and oligothiophenes was reported by Bao. Bulk heterojunction solar cells based on polythiophenes were related to the backbone conformations of analogue dodecathiophenes 96-98.

![Figure 42. Molecular structures of dodecathiophenes 96-98 and current-voltage plot of the OSC. Images from reference\textsuperscript{64}.](image)

Quite surprisingly the most flat oligothiophene derivative 96 was not providing the best results in terms of device efficiency (Figure 42). The planarity of the backbone was found to be extremely important to increase the delocalization along the scaffold and the polymer holes mobility to achieve larger photocurrent. On the other end, a deplanarized backbone was needed to
maximize the molecular ionization potential and the device open circuit voltage, one of the key parameters in the energy conversion. The final results showed that the weak twisted 98 was providing slightly better efficiency than 96 and the too deplanarized and inefficient 97. The overall conclusion pointed out that the simple modifications of the scaffold in these conductive polymers could be the key parameter to achieve impressive results and to optimize the properties of these devices.

1.4. Push-Pull Chromophores

1.4.1. General Applications

Push-pull aromatic systems as small molecules or more extended polymeric matrices has attracted the interest because of their unique electronical properties due to the simultaneous presence of an electron rich group conjugated with and an electron withdrawing moiety through variable distance related to the nature of the π-bridge (Figure 43).

Figure 43. General resonance structures of donor-acceptor conjugated molecule.

Push-pull aromatic systems present large values of the multiple order polarizabilities due to the anisotropy of the polarizability of the π electrons. Thanks to this intrinsic property these molecules, in which remarkable single or multiple charge transfer (CT) transition can occur through an extended π-system, were investigated in depth as nonlinear optics materials (NLO), two-
dimensional NLO substrates and for the application in laser and optical fibers. The remarkable charge transfer processes which occur between the donor and acceptor moieties are suitable for the production of low band gap polymers which are ideal for the construction of memory devices (Figure 44). Moreover the considerable dipole moments of these molecules favor the stability of CT state affording a non-volatile character to the stored information. The creation of donor-acceptor interdigitated surfaces is also a key requirement in the bulk polymeric solar cells to favor the exciton dissociation. A huge variety of polymeric materials bearing electron-donating and withdrawing groups were investigated in order to maximize this process and to create organic solar cells able to compete with the more diffused silicon devices.

![Figure 44](image)

Figure 44. Contribution of the donor-acceptor moieties (A) to the memory switching behavior. Band gap modulation strategy in donor-acceptor polymeric solar cells. Images from reference.

Finally the ability of the push and the pull groups to tune the electronic properties of the substrates, stabilizing or destabilizing the energy levels of the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO), were fundamental in the applications as organic light emitting diodes (OLED). The variation of the amount and the nature of the donor-acceptor systems were carried out together with many others backbone modifications to improve the lifetime of these very intriguing
devices. Nevertheless these interesting and fascinating systems are not strictly related with the main topic of this work which is focused on the application of push-pull systems in chemical biology. For this reason the main attention is focused on more recent results obtained by application of such molecules as biological probes.

1.4.2. Push-Pull Probes for Biological Topics

The push-pull fluorophore 99 and 100 were introduced by Weber as hypersensitive probes for the membranes viscosity visualization (Figure 45)\textsuperscript{70,71}. These fluorescent naphthalene based dyes have shown an impressive increase of the dipole moment upon excitation and they may induce the reorientation of the surrounding dipole moment of the solvent molecules. The interactions of the fluorophore with solvents decreased the energy of the excited state and provided a red-shifted emission.

![Figure 45](image)

\textbf{Figure 45.} One photon confocal images of 100 in giant unilamellar vesicles. Different colors correspond to different membrane bilayer phases. Images from reference\textsuperscript{72}.

When these probes are located in the lipid bilayer membranes the strong dipole moment caused the reorientation of water molecule in the first solvation shield which resulted in an environmental polarity change\textsuperscript{72,73}. The different
amount of water molecules present and the flexibility of membrane phases were discriminated by different shifts of the emission maxima which allowed the recognition of lipid bilayer phases. Additionally due to the electron transfer character of these molecules the fluorescence in water was almost completely quenched and was switched on upon the partitioning inside the lipid bilayer.

Since these pioneering results a variety of different probes were designed and applied for the same investigation but the minimal structure consisting in a push-pull system was always kept as constant. The versatility and sensitivity of such molecules 99-109 for the investigation of the membrane bilayers have provided exceptional results which contributed to a large investigation and commercialization of them with well-known names (Figure 46).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure46.png}
\caption{Chemical structures of the most investigated membrane probes based on donor-acceptor moieties.}
\end{figure}
Recent improvements in the investigation of the biological lipid bilayers were directed toward a better discrimination in time and space of membrane dynamics. In parallel, ongoing modifications of the probes skeletons were performed to maximize the fluorescence response.

On the first topic is related the work presented recently by Mély and co-workers on the visualization of liquid ordered domains\textsuperscript{74} (RAFT) and on the cellular apoptosis process. The RAFT existence in cellular membrane was evidenced by the investigation via indirect techniques\textsuperscript{75,76} but never visualized directly.

![Chemical structure 110 and emission spectra](image)

**Figure 47.** Chemical structure 110 and the emission spectra in different vesicles. The ratiometric fluorescence detection (A, B and C) in cell during cholesterol depletion. Images from reference\textsuperscript{74}.

The cholesterol enriched liquid ordered (L\textsubscript{0}) domains clearly segregate from liquid disordered phase (L\textsubscript{a}) formed by unsaturated lipid and cholesterol. L\textsubscript{0} was showed to be detectable by the Nile Red\textsuperscript{77} based probe 110 able to partition mainly in the outer leaflet of membrane bilayer (Figure 47). The investigation
in vesicles as biological membranes model has shown an emission spectra of typical for RAFT domains and easy to discriminate from all the other possible membrane phases. A subsequent ratiometric fluorescence investigation in living cells, together with a cholesterol enrichment/depletion method, has proved the ability of this probe to clearly visualize also ex-vivo the liquid order phase and discriminate it from other aggregation states of the complex cellular membrane. Comparable changes in fluorescence emission were observed with the analogue compound 111, sensitive to membranes surface charges and membrane phases. The results obtained with this probe suggested that the outer leaflet of cell membranes is enriched in L0 phase because of the high amount of sphingomyelin (Figure 48). More interesting was the observation of the same response of the probe to the induced cholesterol depletion or to the cellular apoptosis.

**Figure 48.** Chemical structure 111 and the ratiometric emission spectra of an intact and an apoptotic cell. Images from reference^{78}.

These results, based on a well-known push-pull fluorophore, suggested that during the apoptosis, the loss of the transmembrane asymmetry is followed by a decrease of L0 phase due to the transfer of the lipids form inner leaflet to the outer.

A different investigation based on the efficiency of strong push-pull systems inserted on a molecular rotors scaffold 112-114 was used by Theodorakis in the
discrimination of the amyloid-β and the prion plaques in brain cells\textsuperscript{30}. As mentioned in the previous paragraphs molecular rotors show an enhancement of the fluorescence intensity once the backbone free rotation is hindered. The combination of this property together with a strong molecular dipole afforded remarkable results in the fluorescence discrimination in brain cells. The amyloid-β (Aβ) peptides deposits associated with the Alzheimer disease were clearly distinguished from the plaques derived by the prion proteins (PrP\textsuperscript{Sc}) dysfunctions (Figure 49) related to the Creutzfeldt-Jakob diseases\textsuperscript{81,82}. Quite surprisingly, this discrimination between the different substrates was not related to different fluorescence intensities as it should be expected from molecular rotor based compound. The difference in the fluorescence spectra was proved to be associated with different environmental polarity. The compounds 112-114 displayed a planar configuration once they were bounded with different type of amyloid deposits but the dipole moment responded differently within the binding pocket of amyloids plaques. These results pointed out the ability of these small molecules, properly functionalized, to provide very detailed fluorescent visualization of brain diseases. Moreover the results were comparable with the one obtained with the more diffused investigation techniques like the positron emission tomography (PET)\textsuperscript{83}.

\textbf{Figure 49.} Chemical structures of 112-114. Real colors (A, B) of amyloid-β (A) and prion protein (B) labeled with 112. Emission spectra (C) of 112 of ex-vivo deposit of amyloid-β and PrP\textsuperscript{Sc}. Images from reference\textsuperscript{80}. 
The interest in the visualization of the brain cells diseases and processes by employing small fluorescent molecules is becoming an intriguing topic as far as this new investigation pathway have shown comparable, if not better, results in respect of the more invasive and expensive analysis. Moreover the relatively simple nature of these probes allowed ongoing modifications of the scaffold to improve the quality of the results in terms of reduced toxicity, photostability of probes and good discrimination in space and in time of the fluorescence images (Figure 50).

On this topic, recently Tsien has introduced a conceptually new fluorophore voltage sensitive dye (VSD)\textsuperscript{84} for the investigation of neuronal cell membranes combining in a single molecule the effect of electrochromic\textsuperscript{85,87} dyes and FRET probes\textsuperscript{88,89}.

![Figure 50. Mechanisms of fluorescent voltage sensing. (A) Electrochromic VSDs sense voltage through the Stark effect. (B) FRET-pair voltage sensors use lipophilic anions. (C) Molecular probe PET VSDs based of donor-acceptor probe. Images from reference\textsuperscript{84}.](image)

Electrochromic dyes sense difference in voltage by a direct interaction of the donor-acceptor system and the electric field. FRET probes are composed by a fluorophore and an apolar ion which partitions differently between the outer and the inner leaflets of membrane bilayers according to the potential. The energy transfer process occurs only when the ions are not too far away from the fluorophore, meaning that they may be located preferentially on the same side of the probe to quench the emission.
The innovative probe 115 and 116 based on the fluorescein skeleton allowed a photoinduced electron transfer process (PET) from the donor group to an acceptor fluorophore only when the electric field is aligned antiparallel to the probe orientation. As a consequence the fluorescence of the acceptor was modulated by the efficiency of the PET process which was related to the potential (Figure 51). This new concept based on the donor-acceptor probes was applied in the investigation of the voltage in neuron affording impressive results in terms of fluorescence response, kinetic of processes and sensitivy. These preliminary results appeared to be very promising for the implementation of a real-time detection of neuronal activity employing fluorophores based on commercial, easily functionalizable molecules.

![Figure 51](image)

**Figure 51.** Chemical structures of the fluorescein based fluorophores 115 and 116. Rat hippocampal neurons (A) and leech Retzius cells (B) marked with VSD probes Images from reference 84.

It has been showed that lipid bilayer membranes or proteins can be fluorescently labeled and investigated with, respectively, push-pull probes or functionalized derivatives of GFP and RFP. Nothing was yet mentioned about the “core” of the cells, the nucleic acids. Differently from the aromatic amino acids (e.g. tryptophan) or proteins, the nucleic acids are practically non-emissive as the native nucleobases 90-93. The main challenge in the design and implementation of fluorescence detectable synthetic nucleic acid is to minimize
the structural and functional perturbation when replacing a native nucleoside with a synthetic analog, while at the same time endowing the synthetic surrogates with useful photophysical features. To overcome the inherent photophysical limitation of the native heterocycles, diverse fluorescent nucleobases analogs have been explored over the years.3

Probably the most relevant recent achievement in the design of nucleobases fluorescent analogues was reported by Tor who developed a unique and complete fluorescent ribonucleoside alphabet, comprised of highly emissive purine 117,118 and pyrimidine 119,120 analogs, all derived from the thieno[3,4-d]pyrimidine as the heterocyclic nucleus94 121 (Figure 52).

The structural, biophysical and spectroscopic characteristics of these emissive RNA nucleosides illustrated highly desirable traits, including native Watson-Crick faces, unparalleled structural isomorphicity with respect to native nucleosides, minimal perturbation upon incorporation into duplexes, as well as intense visible emission. Moreover this approach gave access to synthesis of highly emissive and responsive isomorphic purines, a rare class of molecules. This new set of nucleobases opened the access to a more in depth, accurate and detailed investigation of the cellular dynamic or RNA and DNA.

Figure 52. Schematic representation of RNA fluorescent alphabet.
It has been briefly shown how suitable is the concept of push-pull molecules for the investigation of biological substrates. The possibility to install many different functional groups on a variety of substrates allowed the design and modulation of an infinite variety of probes for the detailed analysis of all the processes that take place in complex systems like living cells.

1.4.3. Push-Pull Oligothiophenes

The possibility to introduce and vary the substituents along and at the end of the backbone together with the molecular length modulation of the length has prompted the interest on oligothiophenes as high technological materials. Short push-pull oligothiophenes have been investigated in depth for applications in various devices like NLO material, OLED and solar cells because of their tunable electronic properties\cite{95,97}.

A representative collection of oligothiophenes as NLO probes was recently reported and investigated by Navarrete\cite{98}. In these molecules \textbf{122-130}, the donor and the acceptor groups or the length of the $\pi$-bridge were changed systematically to investigate theoretically and experimentally the electronic properties (Figure 53). All the molecules showed a remarkable polarization due to the presence of strong electron-donating and withdrawing groups. DFT calculation have shown the ability of these scaffold to stabilize the charge transfer state in which the net negative charge was almost totally located on the cyanovinyl moieties while the positive one was spread from the donor group along the oligothiophene bridge. In the same time the IR, Raman analysis evidenced a strong vibrational mode which confirmed the high polarization along the principal molecular axis. Moreover weak changes were observed in the Raman spectra between the solid and in solution states suggesting that the molecules adopted an almost planar conformation already in solution. The overall electronical properties of these compounds together with the
straightforward synthesis and the photostability encouraged their application as NLO material.

Figure 53. Molecular structures of NLO probes 122-130 based on push-pull oligothiophene scaffolds.

Similar probes 131-134 were recently investigated and compared with analogues in which the thiophene bridges were replaced by phenyl rings. The optoelectronical properties, the second (β) and third (γ) order polarizabilities were evaluated and related to the different molecular structures (Figure 54).

Figure 54. Molecular structures of NLO probes 131-134 based on push-pull imidazole-oligothiophene scaffolds.
The electrochemical and optical investigations pointed out the importance of the length, the planarity and the effective conjugation of the \( \pi \)-spacer for the optimization of the charge transfer state stabilization. The compounds 131 and 132 have showed values for \( \beta \) and \( \gamma \) eight and thirty times higher than the analogue phenyl derivatives. These results confirmed once more the unique properties and potentiality of donor-acceptor oligothiophenes as suitable probes for the fabrication of NLO devices.

Small soluble push-pull molecules have attracted the attention of supramolecular chemists as intriguing substrates for the fabrication of organic solar cells. According to the Bässler model\textsuperscript{100,101}, only molecules that lack of a dipole moment can be considered appropriate as charge-carrier transport. The presence of a molecular dipole moment would increase the energetic disorder preventing the charge hopping. Based on this assumption Roncali have introduced probably the smallest push-pull probes\textsuperscript{102} 135-137 in which the electron-donating and withdrawing groups were directly connected to the \( \beta,\beta' \)-positions on the same thiophene ring (Figure 55).

**Figure 55.** Molecular structures of push-pull oligothiophene probes 135-137 for solution-processed solar cells.
The nature of the spacer in the investigated molecules was shown to affect the energy of the LUMO level together with the strength of the acceptor moieties. On the other hand the HOMO level was almost comparable among all probes as expected due to the presence of the same electron-donor moiety. Moreover the high solubility of these triads suggested the future application of these semiconductors probes in the fabrication of solution-processed solar cells.

In the same time Würther disowned the Bässler model, constructing a bulk heterojunction solar cells (BHJ) based on highly dipolar donor-acceptor small molecules 138-143 as hole conducting components\textsuperscript{103}. In these devices these probes spontaneously rearranged in an antiparallel π stack neutralizing the molecular dipole moment and allowing the flux of the holes (Figure 56).

Figure 56. Molecular structures of push-pull thiophene probes 138-143 in the crystal (A), space-filling of the dimer (B), π-stack motif (C) and schematic representation of the dipole moments orientations. Images from reference\textsuperscript{103}. 

50
Solution-processed BHJ solar cells built with the short donor-acceptor thiophenes and the fullerene as electron-conductor exhibited a conversion efficiencies up to 5.1%. In particular, the compound 140 has showed better efficiency than the device made with Nguyen’s diketopyrrolopyrrole, the leading dye in the solution-processed BHJ solar cell based on small molecules. These remarkable results clearly invited to reconsider the design concepts of organic semiconductors materials particularly in the organic photovoltaics field.

Finally, extending the concept of donor-acceptor from molecular to supramolecular systems, oligothiophenes were widely employed in the design of organic solar cells104. Thanks to the tunable electronic properties and the straightforward synthesis, various oligothiophene derivatives were employed as electron donor and hole channels in combination with many different chromophores such naphthalenediimides (NDI)105,106, perylenediimides (PDI)107,108 and fullerene109,110 providing remarkable results and very elegant complex structures (Figure 57).

**Figure 57.** Schematic representation of multiple channels self-organizing surface initiated polymerization solar cells based on oligothiophene and NDI (A), ordered stacking formed by oligothiophene-PDI amphiphilic dyads (B) and spherical and fibrillar supramolecular aggregation of oligothiophene-fullerene dyads (C). Images from reference105,107,109.
The possibility to modulate the electronical properties of the oligothiophenes by insertion of donor and acceptor groups stimulated also the interest on these probes as biological markers.

Barbarella has recently extended the impressive sensitivity obtained with polythiophenes to the analogue oligomers\textsuperscript{111}. A minimal controlled elongation of the aromatic system up to four thiophene units 144-149 together with donor and acceptor terminal moieties was shown to be sufficient to tune the photophysical properties of these molecules along a wide range of the visible spectra (Figure 58A).

![Chemical structures of fluorescent oligothiophenes 144-149.](image)

**Figure 58.** Chemical structures of fluorescent oligothiophenes 144-149. Emission shifts (A) in solution extending the thiophene bridge. Antibody labeled (B) with the final oligothiophene and (C) fluorescent antigen detection in cell. Images from reference\textsuperscript{111}.

Moreover it has been showed the ability of such molecules to keep their photophysical properties once linked to biological substrates like antibodies. Moreover these fluorophores allowed a clear fluorescent visualization not only
of the bound molecules (Figure 58B) but also provided informations about the location of the corresponding antigen on the cellular cytoplasmic membranes (Figure 58C).

The same push-pull oligothiophene probes 144-146 plus some analogues 150 and 151 were employed for the detection of the molecular beacons (MB)\textsuperscript{112}. This family of molecules refers to oligonucleotides sequences that spontaneously rearrange into hairpin structures putting in close proximity the 5’ and 3’ ends. Short donor-acceptor oligothiophenes together with dabcyl were linked at the ends of the oligonucleotides sequences. In the hairpin conformation dabcyl was shown to quench almost completely the fluorescence (Figure 59) of the oligothiophenes through Förster resonance energy transfer (FRET) pathway.

![Figure 59. Chemical structures of oligothiophenes used as molecular beacon probes. Schematic representation of the enhanced oligothiophene fluorescence by hairpin opening or duplex formation (A). Fluorescence intensity (B) for probes in hairpin (yellow), duplex (green), hairpin together with dabcyl (orange), duplex together with dabcyl (red). Images from reference\textsuperscript{112}.

Upon opening of the hairpin structure by temperature increase or formation of a duplex with complementary nucleobases sequence the fluorescence of the
thiophene probes was recovered. These results were comparable for all the bisthiophene derivatives in nanomolar concentrations showing high environmental sensitivity of these biomarkers.

The same group reported a couple a years later a new series of rigid thiophene oligomers\textsuperscript{113} 152-158 able to spontaneously cross the cellular bilayer membrane of living cells and stain uniformly in the cytoplasm affording fluorescence up to 168 hours. Interestingly some of the investigated oligomers were able to locate close to the nucleus and to induce the secretion on nanostructured fluorescent fibrils (Figure 60).

![Chemical structures and images](image)

**Figure 60.** Chemical structures of fluorescent rigid oligothiophenes 152-158 and confocal fluorescence images of NIH 3T3 cells after 48 and 168 h labeled with oligothiophene fluorescent probes able to visualized cytoplasm (red) and induce fibrils secretion (green). Images from reference\textsuperscript{113}.

Comparable colorful results were obtained by Loew with a complete different class of fluorophores\textsuperscript{114}. The hemicyanine based probes 159-162 with
strong oligothiophene based donors provided an impressive red-shifted emission together with strong sensitivity to the membranes fluidity (Figure 61).

![Chemical structures of hemicyanine probes 159-162 and emission spectra in different membrane phases. Images from reference 114.](image)

**Figure 61.** Chemical structures of hemicyanine probes 159-162 and emission spectra in different membrane phases. Images from reference 114.

The thiophene moieties imparted a strong affinity to the biological environments and flexibility to the whole molecules. As a consequence, different membrane environments were clearly discriminated by concomitant shifts of the emission maxima and changes in the fluorescence quantum yields. These compounds have shown remarkable properties also as second harmonic generation 115,116 fluorophores. The compact structures and the easy synthetic pathways of these probes were identified as a promising factor for the modulation of the optoelectronic properties of these probes.

A similar bisthiophene unit 117 was employed by Swanger in the design of a small twisted fluorophore 163 with high blood brain barrier permeability in vivo. This simple push-pull probe was shown to stain selectively and quickly the senile plaques and the cerebrovascular amyloid angiopathy in living brains. The strong and easy detectable red fluorescence was related to the strong push-
pull system and also to the enriched fluorescence quantum yield due to the almost flat conformation of the probe once bound to the substrate (Figure 62).

Nevertheless, further bathochromic shift should be desirable for practical application in diagnosis of diseases (e.g. Alzheimer). Related to this point, the minimal structure of this probe appeared promising for further modulations of the optical properties. Moreover this fluorophore can be considered as a clear example pointing out how simple well-designed structures can provide remarkable results in chemical biology.

![Figure 62](image)

**Figure 62.** Molecular structure of push-pull oligothiophene 163, optimized twisted ground state geometry (A), fluorescent image of coronal section of brain (B) and multiphoton microscopy image of in vivo detection of senile plaques and amyloid angiopathy (C). Images from reference117.

Recently a push-pull bisthiophene 164 were reported as a mimic of the pyridoxal 5'-phosphate (PLP), the biological active form of the vitamin B6118. PLP is constituted by an aldehyde group linked to a pyridinium group. The activity of such molecule covers a variety of enzymatic processes such decarboxylation, racemization and transamination in living systems119. The formation of the aldimine by coupling of the PLP aldehyde with an amino acid is the key step in many reactions involving these substrates. As a consequence, the α-proton of the substrate became more acid due to the stabilization of the negative charge by the pyridinium moiety. In the same way, the irradiation with UV light was able to install a communication between the donor and acceptor
moieties of 164 generating the active PLP mimic 165. This compound was shown to catalyze the hydrogen-deuterium exchange in the amino acids. The irradiation with visible light regenerated the original inactive form 164 freezing the exchange in the substrates (Figure 63). Such method appeared as very simple way to follow the catalytic racemization of amino acids and this simple probe was the first example of photoswitchable PLP mimic.

Figure 63. Schematic representation of the photoinduced activation of the PLP mimic bisthiophene. Percent of hydrogen-deuterium exchange of L-alanine (A) in presence of 164 (black dots) and 165 (empty dots). Variation of hydrogen-deuterium percent exchange (B) during cyclic irradiation with UV and visible light. Images from reference118.

1.5. pH-Sensitive Probes for Biological Applications

1.5.1. Introduction

Precise determination and control of pH is an accurate operation in many field of chemistry and related industrial applications. More complicated is the investigation of acid-base equilibria that take place constantly in living cells.
Cytoplasmic pH is the core variable which regulates all the cellular functions and life. pH was found to regulate cellular apoptosis and proliferation\textsuperscript{120-122}, cellular processes such as endocytosis\textsuperscript{123} and phagocytosis\textsuperscript{124}, switching on of muscles contractions\textsuperscript{125,126} or synaptic transmissions\textsuperscript{127-129}. Moreover, abnormal intracellular pH was found to be related with cellular dysfunctions responsible of cancer\textsuperscript{130} and other disease like Alzheimer\textsuperscript{131}.

\textbf{Figure 64.} Most widely used intracellular pH sensitive fluorophores. Revised image from reference\textsuperscript{132}.

Observation of cellular pH modifications with good time and spatial resolution is highly desirable to provide critical informations of \textit{in-vivo} processes. Fluorescence spectroscopy together with the development of
fluorescent sensitive probes (Figure 64) is probably the most employed and powerful technique for this investigation. Such molecules, generally quite polar, must fulfill a series of requirements to be employed in living system. First of all they should have clear detectable and stable photophysical properties which are not altered by the complex cellular matrix. Secondly they must be totally biocompatible and inert to cellular substrates. Probes with remarkable emission but high cytotoxicity will induce fast cell death and, by the same point of view, fluorophore that strongly or specifically interact with cell substrates could alter or in the worst case inhibit the natural processes affording false results. The last but not least characteristic is the ability to cross the hydrophobic bilayer membrane in order to reach the aqueous cytoplasm.

Many methods\textsuperscript{133-135} are adopted to facilitate the transmembrane crossing of these high polar probes but more or less all of them perturb the cell physiology. The most diffuse technique involves the chemical modification of the fluorophore hiding the charge groups of the dye by modification in neutral ester moieties\textsuperscript{120} which allowed the molecules to pass through the membrane and then get subjected to the action of cytoplasmic esterases recovering the original photophysical properties. This technique is less traumatic than others but still suffers from some disadvantages like the release of methanol and acetic acid upon the ester cleavage or drives the accumulation of the fluorophore in particular hydrophobic organelles. Nevertheless it could be applied to a large number of fluorophores allowing the cell visualization with many different probes according to specific phenomena in study.

A brief overview of the most diffused pH fluorescent probes is presented pointing out the advantages and the limitations that distinguish each class of molecules in term of sensitivity and stability.
1.5.2. Fluoresceins

Fluorescein 166 and its derivatives\textsuperscript{136,137} are probably the most used intracellular pH probes because they are quite easy to prepare and cheap. The esterification of 166 facilitates the diffusion in the cytoplasm but, upon the ester hydrolysis, fluorescein has shown a remarkable leakage out of the cells due to the not so strong polarity of the probe. The introduction of acid moieties like carboxylic 167 or sulfonic 168 in the fluorophore partially improves the properties of the fluorescein (Figure 65). The two compounds show a clear fluorescence shift upon pH changes (pK\textsubscript{a} = 6.5) but still 167 presents not negligible leakage form the cytoplasmic media while the ester derivative of 168 cannot easily diffuse across the membrane and reach the cytoplasm.

![Chemical structures of fluorescein 166 and two acid derivatives 167 and 168.](image)

Figure 65. Chemical structures of fluorescein 166 and two acid derivatives 167 and 168.

The carboxy fluorescein (CF) 166 and its derivatives are useful for an accurate pH detection thanks to the possibility to apply a ratiometric absorption or fluorescence measurement\textsuperscript{138,139}. In the first method the pH is correlated to the ratio of different fluorescence intensities generated by two distinct excitation wavelengths. Complementary, in the fluorescence method, the ratio of different emission peaks induced by same excitation is measured. Despite the increased sensitivity obtained by the ratiometric analysis 167 and 168 shows some disadvantages related to the leakage, the concentration dependent emission intensity and the photobleaching.
Further modifications (Figure 66) were introduced on the CF backbone to enhance better properties. The extension of the aromatic core in 169 gave rise to a more pronounced and red-shifted dual emission pattern which was reflected by an improved sensitivity. The presence of halides in 170 decreases the $pK_a$ values ($pK_a = 4.8$) and allows the investigations of cellular processes in a wider range of pH keeping the same advantages of CF probes. The combination of the CF and rhodamine skeleton produces 171, a useful probe for the dual excitation detection due to the increase of the extinction coefficient and the decrease of the probe photobleaching. Finally, the insertion of aromatic moieties through a flexible bond in 172 allowed the simultaneous investigation of the pH and the viscosity changes using the same probe.

1.5.3. Benzoaxanthenes

This family of probes is constituted by fluorescent derivatives based on three possible annulation isomers 173-175 (Figure 67). They are normally
classified according to the similarity with the simplest fluorophores. Compounds based on 176 bearing a carboxylic function are named SNAFLs (semi-naphtho fluorescein derivatives), while if the acid group is lost are indicated as SNAFRs (semi-naphtho fluorones) 177. Finally the third class SNARF (semi-naphtho rhodafluors) 178 and 179 is identified by affinity with the rhodamine dye.

![Diagram](image)

**Figure 67.** Basic skeleton of benzoxanthene fluorescent probes 173-179 for the intracellular pH investigation.

All these compounds and their derivatives present a double emission and excitation patterns suitable for the ratiometric investigation and a remarkable fluorescence quantum yield in basic condition. The main advantage of such molecules is referred to the unique dependence on pH of the emission ratio which is not affected by the probe concentration or by the presence of ions or different environments\(^\text{138}\). Moreover compared to fluorescein derivatives they are less subjected to photobleaching which allowed the use of such molecules
in extended-time experiments and kinetics investigations. Nevertheless they present some disadvantages which affect their overall use as biological application. The fluorescence quantum yield of the neutral form is quite low and affects the sensitivity to pH changes. The weak acidity ($pK_a = 7.5$) of these probes does not allow investigations of accurate pH values below 7. Finally the quantum yields are strongly affected by the temperature\textsuperscript{146}. In general, variation of 10 °C induces a loss in intensity of 25 % which prevents the investigation of temperature mediated processes.

1.5.4. Cyanines

Cyanine base probes other than good solubility have the advantage to shift the absorption and the emission maxima to the near infrared region (NIR). This factor increases remarkably the sensitivity and the penetration of the light in the tissues\textsuperscript{147,148}. The more pronounced disadvantage compared to the previous probes is the increased photobleaching of these molecules which limit their applications. Generally the pH sensitive cyanine could be classified into two main groups. The first class refers to mono $N$-alkylated cyanines based on 180 (Figure 68) which is almost not fluorescent in neutral form while a strong blue-shifted emission appears upon the protonation of the indolinium nitrogen. In the second group 181 an amine works as a modulator and is responsible of photoinduced electron transfer process upon protonation. The fluorescence of these molecules is then turned on or switched off according to the pH and compared to 180 the 181 derivatives do not show a blue shift upon protonation.

![Figure 68. General chemical structures of the cyanine probe 180 and 181.](image-url)
A simultaneous functionalization of the cyanine with carboxylic and sulfonic acid moieties in 182 and 183 allows a clear visualization of small pH changes in living cell (Figure 69). Further activation of the carboxylic acid was performed to link these probes to biomolecules like proteins to study pH induced activities of these substrates. The extension of the core in 184 gave remarkable results in terms of emission intensity, decreased cytotoxicity and good photostability in HepG2 cells.

![Chemical structures of polyacidic cyanines](image)

**Figure 69.** Chemical structures of polyacidic cyanines 182 and 183 and extended core probe 184.

### 1.5.5. Naphthalenediimides

Core substituted naphthalenediimides (NDI) have recently attracted the interested for the design of new fluorescent bio-probes because their unique properties. These small molecules are easy to prepare and have shown a broad solubility in many solvent systems. Moreover their optoelectronic properties have been tuned in a straightforward manner. The most diffused procedure to shift the absorption and the emission spectra over the whole visible range focused on the insertion of various electron-donor groups as in 185-188 in the electron-deficient NDI core149 (Figure 70). Similarly, the extension of the π-system150-153 together with the presence of donor and acceptor moieties like in
189-192 was largely investigated in the design of $n$ and $p$ conductive materials. An alternative way\textsuperscript{154} to shift the visible properties of the NDI molecule 193 was related to the formation of stable radical cation 194 and anion species 195 in mild conditions.

![Chemical structures of some reported NDI molecules 185-195.](image)

**Figure 70.** Chemical structures of some reported NDI molecules 185-195.

Taking advantage from these strategies, Zhu has designed a NDI based chemosensor to monitor the pH into microbioreactors\textsuperscript{155}. Piperidine functionalized NDI 196 was covalently linked to hydrophilic polymers
allowing the formation of a water soluble film avoiding the fluorescence self-quenching by aggregation.

**Figure 71.** Schematic representation of the fluorescence response of the probe 196 to pH. UV-Vis and fluorescence spectra at different pH in water. Images from reference\textsuperscript{155}.

The probe 196 has displayed a slight blue shift (\(\lambda_{\text{abs}} = 569-561 \text{ nm}\)) in the absorption spectra with a clear isosbestic point at 572 nm due to the weaker donor effect of the substituent upon protonation, moving the pH from 8 to 4.6. More interesting was the 3 times increase of emission intensity in acidic pH. Upon the protonation of the aliphatic nitrogen 197 (pK\(_a\) = 6.0) the photoinduced electron transfer process from the piperidine to the NDI core was blocked and the fluorescence was switched on. An overall linear response of the fluorescence intensity as a pH function was found in the range of pH from 4.6 to 8.0. Moreover the sample was tested during a fermentation reaction providing the pH profile of the bioreactor batch as a function of time.

Similar strategy was used by Li to develop a core extended NDI probe by fusion with a DBU unit\textsuperscript{156}. The extended core and the strong donor group provided a brilliant green color to the NDI 198 in solution. The addition of TFA
caused the protonation of the DBU 199 with a consequently impressive blue-shifted absorption providing a pale yellow solution. The original green absorbance due to the PET process was recovered by addition of TEA and this acid-base cycle was shown to be reproducible without remarkable loss of the intensity. As for the Zhu probe, also this molecule showed clear enhanced fluorescence intensity in acid conditions (Figure 72).

![Figure 72. Schematic representation of the protonation of NDI-DBU 198, UV-Vis and fluorescence spectra at different pH. Images from reference156.](image)

A comparable sensitivity but with also a remarkable shift of the absorption and emission spectra in different acidic conditions were reported by Langford157. The NDI core was functionalized with two piperidine groups 200. The simultaneous protonation of both the piperyl nitrogens 201 was observed by steady-state UV-Vis and fluorescence spectra (Figure 73). A clear isosbestic shift of 20 nm was observed in the absorption and in the emission peaks. Moreover the fluorescence quantum yield and lifetime were increased of
15% in acidic environment. A final investigation with acid-base additions showed a complete recovery of the emission intensity up to 20 cycles.

**Figure 73.** Schematic representation of the protonation of NDI 200, UV-Vis and fluorescence spectra at different pH. Images from reference\(^\text{157}\).

Remarkable results for the pH sensitivity of NDI derivatives were achieved recently by Freccero\(^\text{158}\). Based on an analogue hydrophobic NDI\(^\text{159}\) which have shown an extremely high acidity (pKa ~ 1.0 in DMSO), the functionalization with cationic moieties at the imides positions provided the water soluble probe 202. The spectroscopic, potentiometric and fluorescence titrations have shown that the NDI preserved its strong acidity in water. The first deprotonation occurred with a pK\(_\text{a} = 2.8\) providing the fluorescent monocation 203 while the second deprotonation (pK\(_\text{a} = 6.9\)) gave the zwitterionic 204 in which the fluorescence was totally quenched. Further treatment with base move to the deprotonation (pK\(_\text{a} = 11.1\)) of one ammonium group in 205 (Figure 74). Remarkably the probe behaved as an on/off fluorescence switch in the 5.0-8.5
pH range which was appropriate for the investigation in living cells. Moreover the NDIs in the monocationic 202 or zwitterionic 203 forms were able to bind and stabilize the G-quadruplex DNA due to the favorable electrostatic interactions. The cell entry and the accumulation of DNA in the nucleus of the mammalian cells were detectable due to the strong fluorescence of the probes which was completely quenched upon binding to the DNA.

![Image of chemical structures and pH profiles](image)

**Figure 74.** Schematic representation of the protonation of 202, absorption maxima profile (A), fluorescence spectra (B) and emission maxima profile as function of pH. Images from reference 128.

Compatibly of NDI with biological substrates was triggering the curiosity of chemists because of its remarkable optical properties. Due to its flat conformation and easily functionalization on the imide sides, NDIs were also employed as a labeling fluorophores for DNA investigations (Figure 75). The visualization of DNA double stand with small molecules was achieved by Iverson and co-workers employing an NDI cyclic bis-intercalator probes able to interact selectively with specific DNA sequences166-162. The experimental investigation, mainly based on 2D-NMR analysis, together with modeling and
calculations were performed to prove the formation of a stable pseudocatenane structures between the DNA backbone and the NDI probes. The connecting linkers between the NDI 206-208 was shown to play a determinant rule for the preferential binding to the minor or the major groove and to interact with specific sequences of the DNA base pairs.

![Chemical structures of the bis-NDI intercalators 206-208 and models of the pseudocatenane complex with DNA. Images from reference162.](image)

**Figure 75.** Chemical structures of the bis-NDI intercalators 206-208 and models of the pseudocatenane complex with DNA. Images from reference162.

Similar NDI with mono or double substitutions in the core together with functional groups attached at the imide sides 209 and 210 have shown an exceptional affinity to telomeric-G-quadruplex DNA and have evidenced a
potent anti-proliferase effect on cancer cell lines \textit{ex-vivo}^{161}. Different terminal moieties such as dimethylamine, morpholine, piperidine and hydroxy groups were systematically changed to investigate the bind affinity and the stability of the supramolecular adducts. The same investigation was performed elongating the spacer between the NDI core and the terminal groups. Moreover the remarkable fluorescence properties of these probes were confirmed by brilliant images in fluorescence confocal microscopy (Figure 76) during the cellular uptake processes.

![Chemical structures of substituted NDIs and 210. Molecular model of NDI binding G-quadruplex (A) and confocal fluorescence images (B) of NDIs uptake in cancer cells at different incubation time. Images from reference\textsuperscript{161}.](image)

Figure 76. Chemical structures of substituted NDIs 209 and 210. Molecular model of NDI binding G-quadruplex (A) and confocal fluorescence images (B) of NDIs uptake in cancer cells at different incubation time. Images from reference\textsuperscript{161}.

These above reports have pointed out the potentialities of the NDI probes in biological terms. The straightforward functionalization of the core or of the imide sides have been used to tune the photophysical properties and to confer a remarkable solubility in aqueous media to the hydrophobic aromatic core. NDI fluorophores should be considered suitable molecules for the development of sensitive and colorful probes to apply in chemical biology.
CHAPTER 2

OBJECTIVES

The general objective of this work is to develop innovative fluorescent probes that could be applied in biological research. The work follows two main goals related to different potential applications.

The first project focuses on the design, synthesis and evaluation of a new class of fluorescent probes in which the concepts of planarization and polarization are merged to provide sensitivity to membrane bilayer fluidity and transmembrane potential.

Among the variety of possible molecular backbones for fluorophores, we focus our attention on the oligothiophene scaffolds. The flexible aromatic nature of these molecules is ideal for the insertion of multiple modifications able to affect the planarity of the conjugated \( \pi \)-system. Moreover the optoelectronic properties can be tuned by implementation of donor-acceptor moieties on the extreme of the scaffolds.

The expected solvatochromism of the molecules will allow the visualization of different push-pull and \( \pi \)-length effects on the optical properties of the probes. Further elaboration of the same data will provide a quantification of the molecular dipole moments.

The responsiveness to membrane fluidity and transmembrane potential will be investigated in large unilamellar vesicles (LUVs) as a biological membrane model. In the first case membrane phase transitions will be induced by temperature cycles while the latter investigation will be performed in polarized vesicles.
The second project involves a completely different family of molecules and will focus on the development of panchromatic chromophores. Such molecules can have potentials applications as biological probes and building blocks for multicomponent systems.

Based on well-known naphthalenediimides (NDI) and perylenediimides (PDI), the aromatic core will be functionalized in a straightforward manner with the insertion of two or four hydroxy moieties. These groups will work as electron-donors providing remarkable panchromism to these compact molecules.

pH dependence in aqueous media is expected to generate an impressive bathochromic shift over all the visible range due to the deprotonation of the hydroxy groups. Analogously the basicity of the common organic solvents and the presence of impurities derived from the degradation will be investigated with the same probes.

On the other hand, the ability of these molecules to react with different substrates will be investigated. Potential applications as biological markers or building blocks for complex structures are directly related to the capability of these probes to react with different substrates.

Dynamic covalent chemistry will be employed to investigate the formation of a fluorescent boronic ester by coupling of the NDI catechol with the aryl boronic acid. In parallel, the oxidative imination with aryl amines will provide access to extended aromatic systems.
CHAPTER 3

RESULTS AND DISCUSSION

3.1. Synthesis of Twisted Push-Pull Oligothiophenes

Planarization and polarization of chromophores are well-known concepts that are widely employed in many different fields. Polar probes, because of their remarkable electronic conductivity and environmental sensitivity, were investigated and developed for photovoltaic applications\textsuperscript{103}, non-linear optic materials\textsuperscript{164} and as biological detectors\textsuperscript{3}. Chromophores able to modify the own degree of planarization tuning the optical properties are employed as viscosity sensor\textsuperscript{22} or to visualize polymerization dynamics\textsuperscript{23}. Individually, these two topics were investigated in depth and large libraries of probes with various structures are reported while compounds able to respond to both of these phenomena are not so developed. Nevertheless nature was able to combine these two concepts and create by supramolecular interactions systems in which induced polarization and planarization play together to gain impressive results like in the chemistry of vision\textsuperscript{165} or in the change in color of a lobster after cooking\textsuperscript{7}. Inspired by these examples we wanted to merge these two topics and create multivalent fluorophores which could be employed in the detection of bilayer membrane fluidity and potentials.

Oligothiophenes were chosen as scaffold for our probes because of their ability to cover a large range of the visible spectrum upon small modification of the backbone\textsuperscript{111} and modulate their conformational and photophysical properties according to environmental changes\textsuperscript{114}. More in detail, the choice of the oligothiophene as scaffold was justified by the examination of the aromatic
system properties compared to similar conjugated molecules, effect of the substituents on the planarization of the \( \pi \)-bridge and simple modulation of the photophysical properties by straightforward insertions of different donor-acceptor groups at the extreme of the molecules (Figure 77).

Figure 77. Representative collection of the variables involved in the design of fluorescent planarizable and polarizable probes.

A comparison of \( \pi \)-spacer among polyenes, oligophenylenes and oligothiophenes with the same length and bearing the same donor-acceptor groups have shown a strong bathochromic shift for polyenes and a weaker one for phenylenes analogues\(^{166} \). Such different shifts under the same experimental conditions must be referred to a more efficient conjugation in polyenes that improves the communication between the push and the pull groups. The weaker
red-shift in oligophenylene \( \pi \)-systems was commonly attributed to steric interaction between the \textit{ortho}-hydrogen of two consecutive aromatic rings\textsuperscript{167}. In oligothiophenes no steric hindrance is present between hydrogen atoms in the planar \textit{trans}-conformation and the lower resonance energy of thiophene (29 kcal/mol) compared to benzene (37 kcal/mol) improves the electron conductivity.

Properties such as thermocromism\textsuperscript{33} or viscochromism\textsuperscript{19} are typical for oligothiophene molecules. The ability of the flexible thiophene backbone to readapt the own conformation upon environmental changes is due to the small energetic cost (\( \sim 2 \) kcal/mol) of the rotation around the \( \alpha-\alpha' \) bond. As a model to study the more diffuse analogue macromolecules in polymeric solar cells or organic field effect transistors (OFET), the oligomers were in depth investigated both from a theoretical and experimental point of view.

Modification of the thiophene \( \beta \)-position along the all \( \pi \)-system has shown a remarkable effect on the conjugation of the molecule. This class of \( \pi \)-conjugated systems normally presents two minima of energy related to the dihedral angle between the thiophene rings. The combination of experimental measurements and calculation has shown that the most stable conformation \textit{trans} (\( \sim 150^\circ \)) is slightly lower in energy (\( \sim 1 \) Kcal/mol) than the \textit{cis} (\( \sim 42^\circ \)) one\textsuperscript{168}. The regioregular insertion of \( \beta \)-methyl groups increases the inter-ring dihedral angle in the \textit{trans} conformation to 118° and to 58° in the \textit{cis}\textsuperscript{50}. Moving to full substituted rings in \( \beta-\beta' \) positions or increasing the hindrance of the \( \beta \)-groups (e.g. isopropyl, tert-butyl) has a dramatic effect on the degree of planarization of the \( \pi \)-system.

Not only the de(planarization) of the aromatic backbone but also the possibility to develop high performance electronic and optical devices has attracted the interest during years. The modification of the electronic properties of oligothiophenes by insertion of electron-donating (EDG) and electron-withdrawing groups (EWG) along the major molecular axis was investigated in
the field of conductive polymers and second harmonic generation materials. The straightforward chemistry needed for the functionalization of thiophene backbones allowed the insertion of many different functional groups like alkoxy, EDOT, aromatic and aliphatic amines as EDG and aldehydes, ester, cyano moieties as EWG. Many of these combinations have shown remarkable results for the applications mentioned above but at the same time also physical and chemical limitations were discovered (e.g. low stability of amino-thiophene to oxidation in air, total quenching of emission in tricyanovinyl-thiophene derivatives).

In the design of probes able to combine planarization and polarization we considered all the parameters just described. As a consequence we decided to fix some of the variables and investigate a series of oligothiophenes in which:

- The methoxy moiety was fixed as EDG because the donor character of this group is remarkable but not too strong to increase the oxidative degradation of the electron-enriched oligothiophene under normal conditions. Moreover the monomer bearing such donor group was found to be commercial available.
- The length of the \( \pi \)-bridge was fixed in a first time to four thiophene units. This length is a compromise between the synthetic effort needed to get the probes and the photophysical properties in terms of absorption and emission shift reported for similar compounds. Shorter analogue molecules were synthetized in a second time.
- The regioregular position of \( \beta \)-methyl groups along the oligothiophene backbone was selected as “average deplanarized” scaffold compared to the planar unsubstituted one and the fully twisted permethylated one. The choice of the methyl group as a substituent was supported by literature reports in which this small group was already able to induce torsion between thiophene rings.
Once these points were fixed, we synthesized a series of oligothiophenes bearing different EWG to modulate the dipole moment. In the final step we introduced the amphiphilic character to achieve partitioning and orientation in lipid bilayers. Incubation of our fluorophores with hydrophilic charged amino-acid derivatives reported by our group\textsuperscript{171} allowed us to obtain a series of amphiphiles with different properties. Positively \textsuperscript{211} and negatively \textsuperscript{212} charged amphiphiles bearing cyanovinyl groups were obtained from the corresponding hydrophobic push-pull oligothiophene \textsuperscript{213} (Scheme 3). Analogously, cationic \textsuperscript{214} and anionic \textsuperscript{215} amphiphiles with hydrazone acceptor were synthesized from an aldehyde end-capped quaterthiophene \textsuperscript{216}. The series of investigated EWG was completed by the ester-capped quaterthiophene \textsuperscript{217} one of the intermediate molecules in the synthetic pathway.

The same investigation was performed on shorter analogues of \textsuperscript{211} and \textsuperscript{212} the terthiophenes \textsuperscript{218} and \textsuperscript{219} to understand whether the conjugated bridge length affects the properties of our probes or not. Finally the amphiphiles were tested in large unilamellar vesicles (LUVs) as bilayer membrane model to prove if our idea to combine the planarization and the polarization concepts could be useful for the investigation of membrane viscosity and polarizability.

\textbf{3.1.1. Synthesis of Push-Pull Quaterthiophenes}

The push-pull $\beta$-substituted quaterthiophenes \textsuperscript{213}, \textsuperscript{216} and \textsuperscript{217}, were synthesized growing the $\pi$-backbone from the EDG side (Scheme 1). Addition of thiophene units one by one allows us to control the regiochemistry of the backbone and, potentially, vary it employing isomers of the reactive monomer.

Stoichiometric iodination with NIS on the commercial monomer \textsuperscript{220} gave the selective mono-insertion of the halide in $\alpha$ position providing the aryliodide \textsuperscript{221}. Subsequent palladium catalyzed Suzuki$^{172}$ coupling with the boronic ester \textsuperscript{222} extended the aromatic bridge affording the dimer \textsuperscript{223} in good yield.
Among different possible reactions for the formation of the C-C bond, Suzuki coupling was chosen because of the mild conditions employed and the stability of the thiophene boronic ester 222. Such building block, commercially available, could also be easily synthetized and purified on a gram scale by treatment of the 2-iodo/bromo thiophene with butyl lithium and subsequent quenching with pinacolborane ester.

Implementation of the above described functionalization in $\alpha$ position and subsequent palladium catalyzed coupling allowed the synthesis of the target fluorophores passing through the iodinated dimer 224, trimer 225 and finally iodinated trimer 226.

In parallel, the ester functionalized thiophene monomer 227 was prepared by esterification\textsuperscript{173} of 228 followed by introduction of a boronate moiety by iridium catalyzed borilation in $\alpha$ position\textsuperscript{174}.

The last Suzuki coupling was performed between 226 and replacing the pinacolborane 222 with 227 to afford the ester-capped oligothiophene 217. Subsequent low temperature DIBAL reduction afforded the alcohol 229 which was oxidized under mild conditions to the key aldehyde intermediate 216 by MnO$_2$. Final replacement of the electron withdrawing group was obtained by Knoevenagel condensation\textsuperscript{175} with 230 allowing the formation of a cyanovinyl end-capped thiophene 231. Routine HBTU activated peptide coupling with 232 afforded 213 and final acetal deprotection under mild conditions with TsOH provided the other key oligothiophene 233 bearing a terminal aldehyde.
Scheme 1. a) 1. H₂SO₄, MeOH, 16 h, 70 °C, 94%; 2. [IrCODMeO]₂, DTBPY, Bis(pinacolato)diboron, octane, 2 h, 70 °C, 90%. b) NIS, DCM, AcOH, 4.5 h, 0 °C to rt, 89%. c) 222, Pd(PPh₃)₄, CsF, DMF, 16 h, 80 °C, 72%. d) NIS, DCM, AcOH, 3.5 h, 0 °C to rt, 88%. e) 222, Pd(PPh₃)₄, CsF, DMF, 16 h, 80 °C, 71%. f) NIS, DCM, AcOH, 2.5 h, 0 °C to rt, 73%. g) 227, Pd(PPh₃)₄, CsF, DMF, 16 h, 80 °C, 67%. h) DIBAL, DCM, MeOH, 2 h, -78 °C, 70%. i) MnO₂, DCM, 15 min, rt, 71%. j) 230, piperidine, ACN, 4 h, reflux, 63%. k) 232, HBTU, TEA, DMF, 1.5 h, rt, 85%. l) TsOH·H₂O, DCM, 15 h, rt, 54%.
3.1.2. Synthesis of Push-Pull Terthiophenes

These compounds were synthesized following the procedure described for the quaterthiophene. The iodo-dimer 224 was subjected to Suzuki coupling with 227 providing the ester 234. Subsequent reduction with Dibal to 235 and mild oxidation with MnO2 afforded the thioephene aldehyde end-capped 236. Knovganel condensation gave 237 and further peptide coupling yielded 238 which was deprotected to get the final push-pull terthiophene 239 (Scheme 2).

Scheme 2. g) 227, Pd(PPh3)4, CsF, DMF, 16 h, 80 °C, 65%. h) Dibal, DCM, MeOH, 2 h, -78 °C, 94%. i) MnO2, DCM, 15 min, rt, 73%. j) 230, piperidine, ACN, 4 h, reflux, 81%. k) 232, HBTU, TEA, DMF, 1.5 h, rt, 63%. l) TsOH•H2O, DCM, 15 h, rt, 84%.

3.1.3. Synthesis of Push-Pull Amphiphiles

The synthetic strategy of amphiphilic molecules was developed in our group to create in a straightforward manner a library of amphiphiles in which the
properties of the molecules can be modulated and changed in a single step. The synthesis of the hydrophobic fluorophores and the hydrophilic heads proceed on two independent and parallel pathways and met only at the end (Figure 78). In this way it becomes possible to build a huge library of similar molecules changing only the substrates to combine in the last step and not all the amphiphiles synthetic strategy. As a general result, different fluorophores can be coupled with the same head or vice-versa the same fluorophore can react with different heads to investigate the effect of the charged moieties. Moreover it is possible to modulate the hydrophobic/hydrophilic ratio of the final compounds by incubation of charged heads bearing multiple reactive sites with different fluorophores (or mixture of fluorophores) and linear hydrocarbon molecules that help the partitioning in the bilayer membrane\textsuperscript{176}.

The simple requirement needed for this strategy is the synthesis of heads and probes bearing a reactive site that works as a connector between the hydrophilic and hydrophobic moieties. The connecting site has at the same time the stability of a covalent bond but can also be cleaved under mild conditions (e.g. pH change, oxidation).

We decided to start with linear amphiphiles in which a single polar head reacts with one fluorophore. Based on previous investigations and results for
similar compounds we focus on hydrazone and oxime connections. Both of these functional groups can be slowly hydrolyzed under acidic conditions but they present a good stability at physiological pH in which the membrane bilayers investigation is performed.

Scheme 3  a) 121, DMSO, AcOH, 60 ºC, 3 h, 80%. b) 122, DMSO, AcOH, 60 ºC, 14 h, quant. c) 123, DMSO, AcOH, 60 ºC, 14 h, quant. d) 124, DMSO, AcOH, 60 ºC, 14 h, quant. e) As in c. f) As in d.

The amphiphilicity of the final compounds was obtained by insertion of hydrophilic positively and negatively charged small peptide derivatives to the hydrophobic fluorophores 216, 233 and 239 (Scheme 3). Incubation of oligothiophene 216 with hydrazides 240 and 241 gave the amphiphiles 214 and
In which the EWD aldehyde was replaced by hydrazone. In the other amphiphiles, the linkage between the fluorophores and the hydrophilic moieties was obtained by coupling of the oligothiophenes 233 and 239 with alkoxyamines 242 and 243 giving, respectively, the tetramers 211 and 212 and the trimers 218 and 219 in which the EWG cyanovinyl was kept.

3.2. Optoelectronic Properties of Quaterthiophenes

After the synthesis of all the amphiphiles, we started the investigation of their photophysical properties mainly focusing on solvatochromic properties. As mentioned in the first chapter, push-pull chromophores are sensitive to the environmental polarity and the solute-solvent interactions affect the Stokes shift. In the following paragraphs, based on the experimental absorption and emission spectra recorded in different solvents, the evaluation of the dipole moments of the above-described molecules will be discussed.

3.2.1. Screening of Electron-Withdrawing Groups

The concurrent presence in the investigated probes of an EDG and EWG conjugated by an aromatic bridge introduces a permanent polarization along the long molecular axis. The strength of the permanent dipole moment is directly related to the nature of the donor and acceptor moieties and the geometry of the aromatic bridge. By instance, the presence of stronger donor-acceptor groups in the $\alpha-\alpha'$ position is reflected in a bathochromic shift of the absorption and emission spectra due to the charge-transfer (CT) character of the transition. At the same time, the planarization degree of the aromatic bridge is affecting the conjugation of the push-pull system. Flatter backbones allow a better overlap of
the $\pi$-orbitals increasing the “communication” between the end-capped donor and acceptor groups.

As described in the beginning of this chapter, we decided to keep constant both the EDG and the length of the $\pi$-bridge and investigate how different EWG influence the properties of our probes and at the same time quantify these properties in terms of dipole moment strength.

The comparison of the absorption and emission spectra of the different molecules in the same environment can demonstrate how different EWG affect the photophysical properties. On the other hand, solvatochromic investigation is known as one of the common ways to quantify the strength of the molecules polarity, compare similar probes and investigate structure-properties relationships.

![Figure 79](image)

**Figure 79.** Normalized absorption (solid) and emission (dotted) spectra in DMF, with increasingly red-shifted emission, of 217 (ester, orange), 214 (hydrazone, red), 215 (hydrazone, magenta), 216 (aldehyde, purple), 212 (cyanovinyl, sky-blue), 211 (cyanovinyl, blue) and 213 (cyanovinyl, cyan).

For all the investigated molecules, the absorption spectra consisted of a main absorption band in the visible range (Figure 79) which is related to the promotion of a single electron from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO). In the HOMO, the electron density is mainly located on the EDG, while in the LUMO the
highest electron density is found on the EWG. This difference in electronic densities between the molecular orbitals gives to this transition a CT character along the whole molecules from the donor to the acceptor unit. Finally, according to literature\textsuperscript{166} the weak shoulder at higher energy has to be correlated with the transition to the second excited state (HOMO $\rightarrow$ LUMO+1) transition.

The shape of the fluorescence spectrum was more affected by the nature of the solvents and the shapes and shifts of the peaks will be discussed in detail in the solvatochromic experiment.

The absorption spectra in DMF of the push-pull probes showed an overall red-shift of 60 nm moving from the weaker ester 217 ($\lambda_{\text{max}}$ = 403 nm) to the stronger cyanovinyl acceptors in 211-213 ($\lambda_{\text{max}}$ $\sim$ 460 nm, Table 1). Aldehyde end-capped 216 and the hydrazone amphiphiles 214 and 215 showed an intermediate shift ($\lambda_{\text{max}}$ $\sim$ 420 nm). At the same time, no significant differences were observed for the molar extinction coefficient of the molecules.

<table>
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<th>Cpd$^a$</th>
<th>$\lambda_{\text{abs}}$ (nm)</th>
<th>$\Delta\lambda_{\text{abs}}$ (nm)</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\lambda_{\text{em}}$ (nm)</th>
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</table>

$^a$ compounds, see Scheme 1 and Scheme 3. $^b$ Absorption maxima in DMF. $^c$ Shift of absorption maxima from 217 in DMF. $^d$ Extinction coefficient. $^e$ Emission maxima in DMF. $^f$ Shift of emission maxima from 217 in DMF. $^g$ Fluorescence quantum yields in DMF.

A similar trend with much more remarkable effects was observed in the emission spectra. In DMF the ester 217 together with the hydrazone derivatives
214 and 215 gave the weaker shift ($\lambda_{em} \sim 580$ nm) whereas the cyanovinyl probes 211-213 shifted the emission to lower energies ($\lambda_{em} \sim 640$-740 nm). The overall difference of the emission in DMF was 155 nm, almost three times stronger compared to the one recorded for the absorption spectra.

The electronic structure of these push-pull oligothiophenes may be described as a linear combination of a neutral nonpolar heteroaromatic structure and a zwitterionic planar heteroquinonid one where a CT process occurs between the EDG and the EWG moieties (Scheme 4).

![Scheme 4. Representation of the resonance structures in donor-acceptor oligothiophenes.](image)

Despite the different strengths of the EWG, the weak bathochromic shift among the absorption spectra of the molecules suggests that the ground state dipole moment ($\mu_e$), or in other words, the overall conjugation between the $\alpha,\alpha'$ substituents and the $\pi$-orbital of the aromatic skeleton is strongly weakened by backbone deplanarization. As reported in literature the presence of alkyl $\beta$-substituents increases the intra-chain twists between the thiophene units without any direct electronic effect on the molecules.

As a consequence the ground state ($S_0$) of these molecules could be depicted as a twisted neutral heteroaromatic backbone. Nevertheless it has been reported that the insertion in similar scaffolds of medium-strong EWG can perturb the electronic structure already in the $S_0$ giving to the molecule a partial quinonoid character extended towards the middle of the thiophene backbone while the other moiety is still described as exhibiting pure aromatic character. This partial induced modification of the backbone could explain the weak but
consistent bathochromic shift observed when the acceptor strengths were increased.

Upon excitation to the first excited state ($S_1$), the neutral heteroaromatic character of the π-bridge is reduced in favor of the zwitterionic heteroquinonoid structure with better overlap among the π orbitals of the thiophene units. The overall consequence is the formation of a planar charge-transfer emitting state with more efficient interaction along the whole aromatic bridge between the α,α’ substituent$^{182}$. The better conjugation between the EDG and the EWG gained by planarization of the π-bridge is expressed by a more remarkable shift among the fluorescence spectra of the probes. As for the absorption spectra, stronger acceptors increase the CT efficiency and stabilize further the LUMO level, shifting the emission spectra to the lower energy region.

### 3.2.2. Solvatochromism

The CT nature of the reported probes includes them in the well-known group of molecules able to give different photophysical responses in different environments. This sensitivity to small changes is governed by the directionality and the strength of the interactions that occurs between the probes and the environment molecules. Examples of such intermolecular phenomena are hydrogen bond formation, dipole-dipole interaction, viscosity induced planarization. An estimation of the nature and the strength of the interactions that take place between a fluorophore and the surrounding media is obtained by the analysis of the shift and shape of the absorption and emission peaks in different solvents.

In general, all the investigated molecules 211-217 have shown the same solvatochromic pattern for absorption and emission spectra according to the increased polarity of the solvents (Figure 80).
Due to the torsional inter-ring mobility the absorption bands in different solvents were structurally and presented a shoulder at higher energies that seemed to be independent on solvent polarity\textsuperscript{183} while the main absorption peak showed a very feeble red-shift with increased polarity.

![Normalized absorption (solid) and emission (dotted) spectra of 213 in, with increasingly red-shifted emission, hexane (yellow), TFE (orange), dioxane (red), diethyl ether (magenta), ethyl acetate (purple), THF (blue), chloroform (cyan), acetone (dark green) and DCM (light green).](image)

**Figure 80.** Normalized absorption (solid) and emission (dotted) spectra of 213 in, with increasingly red-shifted emission, hexane (yellow), TFE (orange), dioxane (red), diethyl ether (magenta), ethyl acetate (purple), THF (blue), chloroform (cyan), acetone (dark green) and DCM (light green).

This weak sensitivity of the absorption spectra to different media is related to feeble interactions between the chromophore and the solvent molecules. Because of the major neutral aromatic form of the ground state, the weak chromophore dipole moment does not affect the orientation of the solvent molecules and the overall solute-solvent polar interactions are negligible. Moreover, by time-scale consideration, the light absorption (10\textsuperscript{-15} s) is much faster than the solvent molecules reorganization and motion (10\textsuperscript{-12} s) which means that during the excitation from the ground to the excited state the molecule is surrounded by the same local environment\textsuperscript{184}.

As discussed above for the bathochromic shift in DMF, it was possible to notice how different EWG slightly affected the absorption spectra in different solvents (Table 2). The strongest electron-withdrawing cyanovinyl group in 211-213 induced the largest shift (Δλ\textsubscript{abs} ~ 30 nm) among the different media,
followed by the aldehyde 216 ($\Delta \lambda_{\text{abs}} \sim 20$ nm) and finally the hydrazone amphiphiles 214 and 215 and the ester capped oligothiophene 217 ($\Delta \lambda_{\text{abs}} \sim 15$ nm). These weak absorption shifts confirmed that the ground state dipole moment ($\mu_g$) should be comparable among all the fluorophores despite the presence of different EWG.

Contrarily, the emission showed a remarkable batochromic shift related to the polarity of different solvents. Such a huge dependence is explained by a modulation of the strength of the solute-solvent interaction. Following excitation the donor-acceptor oligothiophenes push the own conformation to a planar zwitterionic form. As a combination of gained planarity and charge separation along the principal molecular axis, the dipole moment of the excited state in much stronger than in the ground state. Moreover, fluorescence lifetime ($10^{-9}$ s) is longer than the solvent relaxation time ($10^{-12}$ s) so the solvent dipole moment can rearrange or relax around $\mu_e$ lowering the energy of the $S_1$. As a trend, the stronger the solvent polarization, the more efficient the interaction with the dipole moment of the fluorophores.

In hexane and, more general in apolar solvents, the dipole-dipole interactions were too weak to stabilize the excited state and at the same time this feeble interaction did not broaden the vibronic transition of the emission bands. Increasing the solvent polarity, the stabilization of the fluorophores excited state became more efficient and the emission spectra shifted to lower energies while also the vibrational structures were lost. The trend of the emission spectra in function of the EWG was similar to the one described for the absorption bands but with more pronounced effects. As before, the cyanovinyl probes 211-213 showed most impressive overall shift ($\Delta \lambda_{\text{em}} \sim 170$ nm), followed by the aldehyde 216 ($\Delta \lambda_{\text{em}} \sim 120$ nm) and finally the by the weaker EWG hydrazones 214, 215 and ester 217 ($\Delta \lambda_{\text{em}} \sim 80$ nm).
## Table 2: Absorption and Emission Maxima in Different Solvents.

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<td>680</td>
<td>717</td>
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<td>588</td>
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<tr>
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<td>455</td>
<td>460</td>
<td>-</td>
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<tr>
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<td>421</td>
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<tr>
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<td>453</td>
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<td>538</td>
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<td>424</td>
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<td>552</td>
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<td>421</td>
<td>421</td>
<td>420</td>
<td>403</td>
<td>628</td>
<td>623</td>
<td>616</td>
<td>541</td>
<td>610</td>
</tr>
</tbody>
</table>

\(^a\) compounds, see Scheme 1 and Scheme 3.
By comparison with the absorption spectra the different nature of the ground and excited state becomes evident. In $S_0$ the neutral character of the molecules and the twisted geometry is reflected by a negligible interaction with the media while in the planar charged separated $S_1$ the interaction between the probes and the different environments became remarkable. Moreover, upon excitation stronger EWG stabilize better the charge separated state, increasing the $\mu_e$ and, by extension, improving dipole-dipole interaction with the solvent. The final result, as mentioned, was a double overall emission shift for the cyanovinyl molecules compared to the weaker ester and hydrazone groups.

3.2.3. Fluorescence Quantum Yields

The fluorescence quantum yields ($\Phi_f$) for the investigated compounds were calculated based on external standards Rhodamine 6G$^{185}$ and IR-140$^{186}$, by using the Equation 1$^{184}$, where $\Phi_{STD}$ is the quantum yield of the standard, $I_{STD}$, $OD_{STD}$ and $n_{STD}$ are respectively the integrated intensity, the optical density and the solvent refractive index of the standard solution and $I$, $OD$, and $n$ are the respective data measured for each probe in different solvents.

$$\Phi_f = \Phi_{STD} \frac{I}{I_{STD}} \frac{OD_{STD}}{OD} \frac{n^2}{n_{STD}^2}$$

(1)

The obtained values were comparable with similar reported probes$^{111,187}$ and as a general trend the amphiphiles showed lower values ($\Phi_f < 10$) compared to hydrophobic fluorophores (Table 3). This could be justified by the presence of a more heterogeneous skeleton in the amphiphilic molecules which gave rise to aggregation in apolar solvent and solute-solvent hydrogen bond formation in polar media.
### Table 3 Fluorescence Quantum Yields in Different Solvents.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>211</th>
<th>212</th>
<th>213</th>
<th>214</th>
<th>215</th>
<th>216</th>
<th>217</th>
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<td>9</td>
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<tr>
<td>ACN</td>
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<td>2</td>
<td>2</td>
<td>4</td>
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<td>8</td>
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<td>Chloroform</td>
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<td>23</td>
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</tr>
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<td>46</td>
<td>1</td>
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</tr>
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<td>17</td>
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<td>-</td>
<td>9</td>
</tr>
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<td>10</td>
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<td>9</td>
</tr>
<tr>
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<td>2</td>
<td>3</td>
<td>9</td>
<td>8</td>
<td>40</td>
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</tr>
<tr>
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<td>-</td>
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<td>7</td>
<td>4</td>
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<td>8</td>
</tr>
<tr>
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<td>-</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
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<td>32</td>
<td>10</td>
<td>7</td>
<td>25</td>
<td>7</td>
</tr>
<tr>
<td>Toluene</td>
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<td>5</td>
<td>16</td>
<td>5</td>
<td>2</td>
<td>11</td>
<td>13</td>
</tr>
</tbody>
</table>

<sup>a</sup> compounds, see Scheme 1 and Scheme 3.

The relationship between the $\Phi_i$ and the solvent polarity<sup>188</sup> $E_N^j$ for the three hydrophobic fluorophores showed a completely different trend for each molecule (Figure 81).

The weaker electron withdrawing ester 217 had quantum yields almost constant over the whole range of polarity. A possible explanation could imply a charge separation character upon excitation not so pronounced because of the weakness of the EWG. Polar solvents stabilized better than apolar media the charge separated $S_1$ but still this stabilization was not efficient enough to reduce the effects of the non-radiative pathways. The aldehyde 216 showed a linear increase with solvent polarity which could be interpreted as better stabilization of the zwitterion $S_1$ by dipole-dipole interactions which were lowering the effects of non-radiative processes compared with the emission from $S_1$. 

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Figure 81. Fluorescence quantum yields as a function of the solvent polarity parameter $E_{NT}$ of 211 (blue), 212 (sky blue), 213 (cyan), 214 (red), 215 (magenta), 216 (purple) and 217 (orange).

Finally the cyanovinyl derivative 213 showed an initial increase followed by a huge drop of the quantum yields with increased solvent polarity. The stronger dipole in the excited state interacted more efficiently with the more pronounced dipole of polar solvent and lowered considerably the energy gap between the excited and the ground state. The resultant smaller energy gaps increased the speed of the internal conversion from the excited state to the ground state according to the energy gap law and were reflected in a less efficient emission from the charge transfer state. The same trend was already reported in literature for other similar strong dipolar fluorophores as fluorene and dapoxyl.

3.2.4. Dipole Moments

Understand and estimate the ground-state and excited state dipole moment of push-pull oligothiophenes was one of the key points of our preliminary investigation for this new class of fluorophores. As introduced at the beginning of this chapter the (de)planarization of the $\pi$-system should play the main rule in the investigation of membrane fluidity while for the membrane polarization
experiment we needed polarizable fluorophores in order to get positive interaction. Many techniques are reported for the measurement of $\mu_g$ like Stark effect measurements$^{192}$, two-photons absorption spectroscopy$^{193}$ or high resolution electronic spectroscopy in the gas phase$^{194}$ but all of them are limited by the elevate cost of the equipment and experimental complexity. A similar situation appears for the evaluation of the $\mu_e$ by microwave conductivity$^{195}$, electric dichroism$^{196}$ or fluorescence electric polarization$^{197}$.

The solvatochromic experiment is a simple and suitable experiment for an empirical estimation of the change in dipole moment ($\Delta \mu$) in the excited state by absorption and emission measurements.

Considering the fluorophore as point dipole ($\mu$) in continuous dielectric media (solvent), the energy of the dipole ($E_D$) in an electric field ($R$) is defined as

$$E_D = -\mu \cdot R$$

where $R$ is the relative reactive field of the solvent induced by the dipole. $R$ is antiparallel to the dipole and is proportional to the magnitude of the dipole according to Equation 3:

$$R = \frac{2\mu}{a^2} \cdot f$$

where $a$ is defined as cavity radius and is generally approximated considering the probe as a sphere in which the diameter is the molecular distance between the donor and the acceptor moieties. This approximation is not valid for rod-shaped fluorophores in which the donor-acceptor charge transfer could occur not only along the $\sigma$ scaffolds but also via through-space overlap. For those molecules, like oligothiophene, the cavity radius is approximated at 40% of the linear donor-acceptor molecular distance$^{198}$. 

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Figure 82. A) Dipole of rod-shaped push-pull molecules in dielectric media. B) Stabilization effect of the solvent refractive index (n) and dielectric constant ($\varepsilon_r$) on the absorption and emission energies. Revised graphical representation from reference 184.

$f$ is the solvent polarizability and it results from the combination of the electron mobility in the solvent and the dipole moment of the solvent molecules. The fast reorientation of the electron in solvent is named high frequency polarizability $f(n)$ and is a function of the refractive index (n) while the slowest dipole reorientation of the solvent molecules around the probes is defined as low frequency polarizability $f(\varepsilon_r)$ and is related to the solvent dielectric constant ($\varepsilon_r$). Assuming no polarizability of the solute molecules, the orientation polarizability ($\Delta f$) for each solvent derives by a linear combination of the contribution of the high and low polarizability functions to the electric field surrounding the fluorophores in the ground and excited state.

\[
 f(n) = \frac{n^2 - 1}{2n^2 + 1} \quad \text{(4)}
\]
\[
 f(\varepsilon_r) = \frac{\varepsilon_r - 1}{2\varepsilon_r + 1} \quad \text{(5)}
\]
\[
 \Delta f = \frac{\varepsilon_r - 1}{2\varepsilon_r + 1} - \frac{n^2 - 1}{2n^2 + 1} \quad \text{(6)}
\]

The electric field ($R$) interacting with $\mu_e$ and $\mu_c$ can be considered as a combination of one field related with the electronic motion and another due to
the solvent reorganization in the ground and the excited state. As a consequence Equation 3 can be divided in four terms

\[ R_n^g = \frac{2\mu_g}{a^3} \cdot f(n) \]  
\[ R_\Delta^g = \frac{2\mu_g}{a^3} \cdot \Delta f \]  
\[ R_n^e = \frac{2\mu_e}{a^3} \cdot f(n) \]  
\[ R_\Delta^e = \frac{2\mu_e}{a^3} \cdot \Delta f \]

During the absorption of a photon, the solvent electronic reorientation has a comparable timescale as the change in the electron distribution in the fluorophore. On the other hand, according to the Franck-Condon principle, the solvent reorientation is too slow. The absorption process energy can be then expressed, from Equation 2 as

\[ E_{abs} = (E_{abs})_V - R_\Delta^g \cdot (\mu_e - \mu_g) - \mu_e R_n^e + \mu_g R_\Delta^e \]  

where \((E_{abs})_V\) represents the absorption energy in the gas phase.

The energy of the emission process can be written by similar considerations and assuming a faster relaxation of solvent compared to the excited state lifetime. Analogously to the absorption process \((E_{em})_V\) is the emission energy in the gas phase.

\[ E_{em} = (E_{em})_V - R_\Delta^e \cdot (\mu_e - \mu_g) - \mu_e R_n^e + \mu_g R_\Delta^e \]

In the gas phase, without environmental perturbation, the energy gap between the ground and the excited state can be considered as constant \(q\). Subtracting 12 from 11 and reminding that energy is related to wavenumbers gives Equation 13
\[ \nu_{\text{abs}} - \nu_{\text{em}} = \frac{(\mu_e - \mu_g)}{hc} \cdot (R_g^0 - R_e^g) + q \quad (13) \]

where \( \nu_a \) is the maximum absorption wavenumber (cm\(^{-1}\)), \( \nu_i \) is the maximum emission wavenumber (cm\(^{-1}\)), \( h \) (erg) is the Planck constant and \( c \) (cm·s\(^{-1}\)) is the light speed. Finally, substitution from Equations 8, 10 and 6 yields the Lippert\(^{199}\) equation (Equation 14):

\[ \nu_{\text{abs}} - \nu_{\text{em}} = \frac{2(\mu_e - \mu_g)^2}{hc \alpha^3} \left( \frac{\varepsilon_r - 1}{2\varepsilon_r + 1} - \frac{n^2 - 1}{2n^2 + 1} \right) + q \quad (14) \]

Push-pull fluorophores show a linear correlation between the experimental Stokes shift in different solvents and the calculated Lippert \( \Delta f \) that allows the quantification dipole moment change in the excited state. Nevertheless, it must be mentioned that the polarizability function is an approximation and can be described by different equations. In the Lippert approximation \( \Delta f \) is expressed assuming a complete absence of fluorophore polarization, same directionality of \( \mu_g \) and \( \mu_e \) and no contribution of specific solvent effects.

Considering an isotropic polarizability of the solute Equation 14 becomes the Bakhshiev\(^{200}\) equation (Equation 15).

\[ \nu_{\text{abs}} - \nu_{\text{em}} = \frac{2(\mu_e - \mu_g)^2}{hc \alpha^3} \left[ \frac{2n^2 + 1}{n^2 + 2} \left( \frac{\varepsilon_r - 1}{\varepsilon_r + 2} - \frac{n^2 - 1}{n^2 + 2} \right) \right] + q \quad (15) \]

Moreover, if also solute parameters calculated from gas phase are considered Equation 15 becomes the Kawski-Chamma-Viallet\(^{201}\) equation (Equation 16).

\[ \frac{\nu_a + \nu_i}{2} = \frac{2(\mu_e^2 - \mu_g^2)}{hc \alpha^3} \left[ \frac{2n^2 + 1}{2(n^2 + 2)} \left( \frac{\varepsilon_r - 1}{\varepsilon_r + 2} - \frac{n^2 - 1}{n^2 + 2} \right) + \frac{3(n^4 - 1)}{2(n^2 + 2)^2} \right] + q \quad (16) \]
An alternative way to correlate the Stokes shift in different solvents reported in Equation 17 is obtained using an empirical solvent parameter scale \((E^N_T)\) developed by Reichardt\(^{188}\) in which \(\Delta \mu_b\) and \(a_b\) are change in the excited state dipole moment and the Onsager radius of the pyridinium-N-phenolate betaine dye.

\[
\nu_{\text{abs}} - \nu_{\text{em}} = 11307.6 \cdot \left[ \left( \frac{\Delta \mu}{\Delta \mu_b} \right) \left( \frac{\langle a \rangle}{a_b} \right) \right] + q
\]  

Despite the complexity of the functions involved and the approximations that could affect the results, it is appropriate to clinch that the main advantage of such an evaluation is related to a simple but accurate measurement of absorption and emission spectra in different solvents.

We decide to investigate our fluorophores with all the different approximations mentioned above. Although it could appear to be a redundant analysis, Lippert values are the most reported in literature and are needed to compare our probes with similar molecules. Bakhshiev and Kawski-Chamma-Viallet parameters together afford the estimation not only of \(\Delta \mu\) but also of the ground and excited state dipoles values. Finally Reichardt parameters give an alternative empirical way for the evaluation of \(\Delta \mu\) not related with the other three approximations.

The orientation polarizabilities were calculated according to the Lippert (\(\Delta f_L\)), Bakhshiev (\(\Delta f_B\)), Kawski-Chamma-Viallet (\(\Delta f_K\)) approximations from the physical parameters mentioned and the \(E^N_T\) were employed instead of the not-normalized \(E_T(30)\) to avoid dimensionality problems (Table 4).
Table 4. Solvents Physical Parameters, Calculated Orientation Polarizability and $E_N^T$ values

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$n^a$</th>
<th>$\varepsilon_r^d$</th>
<th>$\Delta f_L^e$</th>
<th>$\Delta f_B^f$</th>
<th>$\Delta f_K^g$</th>
<th>$E_N^T^h$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>1.357</td>
<td>20.490</td>
<td>0.285</td>
<td>0.789</td>
<td>0.638</td>
<td>0.355</td>
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<tr>
<td>ACN</td>
<td>1.342</td>
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<td>0.305</td>
<td>0.859</td>
<td>0.663</td>
<td>0.460</td>
</tr>
<tr>
<td>Chloroform</td>
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<td>4.711</td>
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<td>0.364</td>
<td>0.483</td>
<td>0.259</td>
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<tr>
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<td>-0.010</td>
<td>0.331</td>
<td>0.015</td>
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<td>0.580</td>
<td>0.309</td>
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<tr>
<td>1,4-Dioxane</td>
<td>1.420</td>
<td>2.210</td>
<td>0.021</td>
<td>0.043</td>
<td>0.307</td>
<td>0.164</td>
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<td>0.014</td>
<td>0.030</td>
<td>0.348</td>
<td>0.099</td>
</tr>
</tbody>
</table>

*a* Refractive index at 25 °C. *b* Refractive index at 25 °C. *c* Refractive index at 25 °C. *d* Relative permittivity (dielectric constant) calculated at 25 °C. *e* Lippert orientation polarizability according to the Equation 14. *f* Bakhshiev orientation polarizability according to the Equation 15. *g* Kawski orientation polarizability according to the Equation 16. *h* Reichardt normalized molar electronic transition energies values.

It must be pointed out that for some solvents remarkable deviations from the ideal behavior were observed and they were thus not considered for the final polarity evaluation. These anomalies are related to specific solute-solvent interactions which can cause misleading evaluation of the probe polarity. Briefly, a list of “anomalous solvents” with the relative specific interaction origin is reported:

- Protic solvents (e.g MeOH, TFE) can be involved in hydrogen bond formation with the fluorophore increasing the bathochromic shift. Presence of low concentration of alcohols (< 5%) in apolar environment
has been shown to shift remarkably the emission spectra\textsuperscript{184}. This concentration of protic solvent is too small to alter the bulk properties, as a consequence the observed shift must come from specific solute-solvent interactions such as hydrogen bond.

- 1,4-dioxane solvates probes as high polar solvents despite a low dielectric constant ($\varepsilon_r = 2.21$). Several explanations about this “anomalous behavior” were proposed like conformational polarity, hydrogen bond formation. The most probable reason of this strong polarity is related to the presence of a large non-ideal quadrupolar charge distribution on the entire molecule\textsuperscript{206}.

- Aromatic solvents (e.g. toluene, benzene) present similar phenomena as in dioxane with an anomalous enhancement in microscopic polarity due to the presence of quadrupole and higher multipole interactions not accounted for in the dielectric constant values\textsuperscript{207}.

- Chlorinated solvents (e.g DCM, chloroform) can show a stronger stabilization of the excited state compared to more polar solvents. A possible explanation to this specific solute-solvent interaction is related to the electron-acceptor character of these solvents\textsuperscript{208}. The solvent acceptor numbers\textsuperscript{209} are larger for chlorinated solvents compared to solvents with higher polarity (e.g. ACN).

The experimentally measured Stokes shift and the semi-sum of absorption and emission spectra were plotted as a function of the four polarity parameters (Figure 83). A linear correlation for the investigated molecules 211-217 was found for all the four data sets. Moreover, despite different polarizabilities a common trend was evident in all the graphs. The compounds 211-213 bearing cyanovinyl as EWG showed a steeper linear correlation with the polarity function and in all the three cases the trend lines were parallel. This suggests that the insertion of the polar heads in 211 and 212 was slightly affecting the solubility of the molecules in apolar media but the molecular polarity was
comparable with 213. The aldehyde end-capped thiophene 216 was represented by a trend line similar to those for the compounds just described while the ester thiophene 217 had a smaller slope. Interestingly the slope of the two hydrazone bearing molecules 214 and 215 was remarkably similar to the one of the ester on the contrary to the aldehyde. This means that the functionalization with the hydrophilic heads might also affect the polarity properties of the fluorophores in those cases.

**Figure 83.** Lippert (A), Bakhshiev (B), Kawski-Chamma-Viallet (C) and Reichardt (D) plots of 211 (blue), 212 (sky blue), 213 (cyan), 214 (red), 215 (magenta), 216 (purple) and 217 (orange).

The graphical plots provided already an intuitive trend of the dipole moment strengths for the different compounds. Nevertheless a numerical estimation of dipole values was performed reminding that the slope of the trend lines (Table 5) in Lippert ($m_L$), Bakhshiev ($m_B$), Kawski-Chamma-Viallet ($m_K$)
and Reichardt ($m_R$) are related to the $\mu_e$ and $\mu_c$ values of each molecule (Equations 18-21).

\begin{align*}
    m_L &= \frac{2(\mu_e - \mu_g)^2}{\hbar c a^3} \\
    m_B &= \frac{2(\mu_e - \mu_g)^2}{\hbar c a^3} \\
    m_K &= \frac{2(\mu_e^2 - \mu_g^2)}{\hbar c a^3} \\
    m_R &= 11307.6 \cdot \left(\frac{\mu_e - \mu_g}{81}\right)^2 \cdot \left(\frac{a}{6.2}\right)^3
\end{align*}

The cavity radius that appears in the equations was calculated by semi-empirical AM1 and taken to be equal to the push-pull group distance taking into account the rod-shaped structure of the molecules.

**Table 5.** Linear Fit Parameters for Dipole Moment Calculations.

<table>
<thead>
<tr>
<th>Cpd</th>
<th>$a$ (Å)</th>
<th>$m_L$</th>
<th>$m_B$</th>
<th>$m_K$</th>
<th>$m_R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>211</td>
<td>8.79</td>
<td>11180 ± 1710</td>
<td>3460 ± 797</td>
<td>4232 ± 713</td>
<td>7820 ± 1080</td>
</tr>
<tr>
<td>212</td>
<td>8.79</td>
<td>12485 ± 2837</td>
<td>2683 ± 409</td>
<td>3342 ± 847</td>
<td>8114 ± 1516</td>
</tr>
<tr>
<td>213</td>
<td>8.79</td>
<td>13315 ± 794</td>
<td>4249 ± 385</td>
<td>5176 ± 804</td>
<td>9494 ± 1323</td>
</tr>
<tr>
<td>214</td>
<td>7.01</td>
<td>5259 ± 270</td>
<td>1817 ± 394</td>
<td>2311 ± 296</td>
<td>3871 ± 633</td>
</tr>
<tr>
<td>215</td>
<td>7.01</td>
<td>6338 ± 489</td>
<td>1391 ± 138</td>
<td>1656 ± 139</td>
<td>3339 ± 410</td>
</tr>
<tr>
<td>216</td>
<td>7.01</td>
<td>13431 ± 1525</td>
<td>4376 ± 463</td>
<td>5537 ± 494</td>
<td>10191 ± 1071</td>
</tr>
<tr>
<td>217</td>
<td>6.92</td>
<td>7934 ± 873</td>
<td>3045 ± 238</td>
<td>3850 ± 295</td>
<td>4859 ± 475</td>
</tr>
</tbody>
</table>

*Compounds, see Scheme 1 and 3. *Distance between push and pull groups (40% of the diameter), simulated with Spartan model, calculated with semi-empirical AM1. *Slope of the linear fit of the Lippert plot. *Slope of the linear fit of the Bakhshiev plot. *Slope of the linear fit of the Kawski-Chamma-Viallet plot. *Slope of the linear fit of the Reichardt plot.
The linear combination (Equations 22-24) of the results from the Bakhshiev and Kawski-Chamma-Viallet plots allowed the estimation of ground, excited and by, difference, transition ($\Delta \mu_{\text{BK}}$) dipole moments of the fluorophores.

$$\mu_g = \frac{m_K - m_B}{2} \left(\frac{\hbar a^3}{2m_B}\right)^{1/2}$$  \hspace{1cm} (22)

$$\mu_e = \frac{m_K + m_B}{2} \left(\frac{\hbar a^3}{2m_B}\right)^{1/2}$$  \hspace{1cm} (23)

$$\Delta \mu_{\text{BK}} = \mu_e - \mu_g$$  \hspace{1cm} (24)

Analogously, change in dipole moments (Table 6) were obtained for Lippert ($\Delta \mu_L$) and Reichardt ($\Delta \mu_R$) re-adapting the Equations 18 and 21.

### Table 6. Ground-State, Excited-State and Change in Dipole Moments.

<table>
<thead>
<tr>
<th>Cpd</th>
<th>$\mu^b_g$ (D)</th>
<th>$\mu^b_e$ (D)</th>
<th>$\Delta \mu_{\text{BK}}^c$ (D)</th>
<th>$\Delta \mu_L^d$ (D)</th>
<th>$\Delta \mu_R^e$ (D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>211</td>
<td>1.7</td>
<td>17.0</td>
<td>15.3</td>
<td>27.5</td>
<td>12.7</td>
</tr>
<tr>
<td>212</td>
<td>1.7</td>
<td>15.1</td>
<td>13.5</td>
<td>29.0</td>
<td>12.9</td>
</tr>
<tr>
<td>213</td>
<td>1.8</td>
<td>18.8</td>
<td>16.9</td>
<td>30.0</td>
<td>14.0</td>
</tr>
<tr>
<td>214</td>
<td>1.1</td>
<td>9.0</td>
<td>7.9</td>
<td>13.4</td>
<td>6.3</td>
</tr>
<tr>
<td>215</td>
<td>0.7</td>
<td>7.6</td>
<td>6.9</td>
<td>14.7</td>
<td>5.9</td>
</tr>
<tr>
<td>216</td>
<td>1.6</td>
<td>13.9</td>
<td>12.2</td>
<td>21.4</td>
<td>10.3</td>
</tr>
<tr>
<td>217</td>
<td>1.3</td>
<td>11.3</td>
<td>10.0</td>
<td>16.2</td>
<td>7.0</td>
</tr>
</tbody>
</table>

- $a$ Compounds, see Scheme 1 and 3.
- $b$ Calculated from Equation 22.
- $c$ Calculated from Equation 23.
- $d$ Calculated from Equation 24.
- $e$ Calculated from Equation 18.
- $f$ Calculated from Equation 21.

The numerical estimation of the dipole moments reflected the considerations based on the experimental plots. Although the calculated values are approximations, some information could be extracted from the above table:
The different electronic distribution between the excited state and the ground state was reflected by the remarkable values of the transition dipole moments. It must be noticed that the values for the ground state dipole moment calculated by linear combination of Bakhshiev and Kawski-Chamma-Viallet equations were probably underestimated. As a consequence the $\Delta \mu$ evaluated from these data could be affected by these results. Nevertheless the $\Delta \mu_{BK}$ was in agreement with the $\Delta \mu$ obtained directly by the Lippert and Reichardt equations. Moreover the $\Delta \mu$ calculated with Lippert were in agreement with the values reported for similar compounds$^{207,210}$ and the low values of $\mu_g$ was justified assuming a remarkable effect of the deplanarized $\pi$-system on the overall donor-acceptor conjugation along the molecules.

A similar trend in the transition dipole moment was observed for all the calculated values based on different orientation polarizability parameters. Considering the hydrophobic molecules 213, 216 and 217 the strongest dipole transitions occurred for the molecule bearing the cyanovinyl group while the analogues aldehyde and ester bearing thiophenes had respectively 25 and 40% weaker strengths.

The cyanovinyl amphiphiles 211 and 212 showed comparable values with 213 which means that the functionalization with hydrophilic groups affected slightly the solubility properties in apolar media but did not affect the electronic properties of the push-pull system (Figure 84). A completely different situation was observed upon the modification of the hydrophobic aldehyde 216 into the hydrazone amphiphiles 214 and 215. An impressive loss of dipole strength ($\sim$ 45%) was observed which could be explained considering the poor $\pi$-acceptor character of the hydrazone group because of the back-donation effect of the azaenamine motif$^{211}$. As a consequence the electronic properties of these two amphiphiles were more similar to the one of the ester 217 than the starting aldehyde 216.
As a result from this investigation we have quantified the effect of different EWG on the electronic properties of a series of molecules bearing the same EDG and the same \( \pi \)-system as spacer. This quantification was a key point for our following studies in bilayer membranes to relate whether the molecular dipole moments play a determinant role in the discrimination of membrane fluidity and potential or not.

![Figure 84. Quaterthiophenes probes structures and Lippert transition dipole moments based on solvatochromic evaluation.](image)

### 3.2.5. HOMO-LUMO Levels

Further confirmation of the effect of the donor and acceptor substituents on the electronic properties of the fluorophores came from the evaluation by cyclic voltammetry (CV) of HOMO and LUMO energy values \( E_{\text{HOMO}} \) and \( E_{\text{LUMO}} \). The hydrophobic probes 213, 216 and 217 showed (Figure 85) a clear reversible oxidation peak \( E_{\text{OX}} \) while in the reduction region only the cyanovinyl derivate and the aldehyde showed a less defined signal \( E_{\text{RED}} \). CV was measured also for the amphiphilic molecules but probably because of the
low solubility in apolar solvent and aggregation it was not possible to observe a clear voltammogram.

Figure 85. Cyclic voltammograms of 213 (~1.3 mM in DCM, cyan) and 216 (~1.3 mM in DCM, magenta) and 217 (~1.5 mM in DCM, orange).

The HOMO-LUMO energy levels and the energy gap (ΔE) were evaluated from Equations 25-27 and in order to confirm the obtained data the energy gaps (ΔE_{Onset}) were also calculated based on the recorded absorption spectra following Equation 28 taking into account the values of the Planck constant \( h \) (mV·s) the speed of light \( c \) (nm·s\(^{-1}\)).

\[
E_{\text{HOMO}} = -5.1 - E_{\text{OX}} \tag{25}
\]

\[
E_{\text{HOMO}} = -5.1 - E_{\text{RED}} \tag{26}
\]

\[
\Delta E = E_{\text{LUMO}} - E_{\text{HOMO}} \tag{27}
\]

\[
\Delta E_{\text{Onset}} = \frac{hc}{\lambda_{\text{Onset}}} \tag{28}
\]
According to calculation the electron density in the HOMO is mainly located on the EDG while in the LUMO it is centered on the EWG and can spread partially along the conjugated \( \pi \)-bridge according to the strength of the acceptor group\(^9\). It means that in our molecules, where the methoxy group was kept as donor, the HOMO energies (Table 7) should be the same for all the probes while the LUMO level should be more stabilized by the cyanovinyl \( 213 \) than by aldehyde \( 216 \) and finally ester \( 217 \).

**Table 7.** Electronic Data for Quaterthiophene probes.

<table>
<thead>
<tr>
<th>Cpd (^a)</th>
<th>( E_{HOMO} )(eV)</th>
<th>( E_{LUMO} )(eV)</th>
<th>( \Delta E )(eV)</th>
<th>( \Delta E_{\text{Onset}} )(eV)</th>
<th>( E_{OX} )(V)</th>
<th>( E_{RED} )(V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 213 )</td>
<td>-5.42</td>
<td>-3.56</td>
<td>1.86</td>
<td>1.97</td>
<td>0.32</td>
<td>-1.54</td>
</tr>
<tr>
<td>( 216 )</td>
<td>-5.46</td>
<td>-3.38</td>
<td>2.08</td>
<td>2.27</td>
<td>0.36</td>
<td>-1.72</td>
</tr>
<tr>
<td>( 217 )</td>
<td>-5.43</td>
<td>-3.03</td>
<td>-</td>
<td>2.40</td>
<td>0.33</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) Compounds, see Scheme 3.1. \(^b\) Calculated from Equation 3.25. \(^c\) Calculated from Equation 3.26. \(^d\) Calculated from Equation 3.27. \(^e\) Calculated from Equation 3.28. \(^f\) First oxidation and reduction potential in V against Fe/Fe\(^{\text{+}}\).

The calculated values for the electronic levels were in agreement with data reported for similar compounds\(^18\). The HOMO level was constant for the three compounds while the LUMO energy shifted by 0.5 eV moving from the ester to the cyano acceptor with the aldehyde values sitting in the middle. Finally the \( \Delta E \) evaluated by CV was found in good agreement with the energy gap obtained from the absorption spectra of the molecules.

### 3.3. Optical Properties of Terthiophenes

The terthiophene push-pull probes were investigated based on the results described for the quaterthiophenes amphiphiles in order to compare the effect of the \( \pi \)-bridge length on photophysical properties and environmental
sensitivity of the probes. As for the tetramers we kept the same methoxy as EDG and the regioregularity of the backbone but we decided to avoid the synthesis and the investigation of the hydrazone end-capped amphiphiles because of the weak dipole moment compared to the cyanovinyl molecules.

3.3.1. Solvatochromism

The ester 234, aldehyde 236 and cyanovinyl 238 end-capped trimer probes were intermediates in the synthesis of the amphiphiles 218 and 219 analogues of 211 and 213. A solvatochromic experiment was performed on the hydrophobic probes assuming the values of 238 representatives for the two amphiphiles 218 and 219 as shown by the results of quaterthiophene analogues.

![Spectra](image)

**Figure 86.** Normalized absorption (solid) and emission (dotted) spectra in ethyl acetate, with increasingly red-shifted emission, of 234 (ester, yellow), 217 (ester, orange), 236 (aldehyde, magenta), 216 (aldehyde, purple), 238 (cyanovinyl, sky-blue) and 213 (cyanovinyl, cyan).

Similar trends as for quaterthiophenes in absorption and emission batochromic shifts (Figure 86) and comparable extinction coefficients (Table 8) were found for the trimer probes. Remarkably, the lack of one thiophene unit reduced the length of the π-system and is reflected in a general ipsochromic shift of the trimer molecules compares to the tetramer analogues.
Table 8. Spectroscopic Data of Planarizable Push-Pull Terthiophenes.

<table>
<thead>
<tr>
<th>Cpd</th>
<th>( \lambda_{\text{abs}} ) ( b ) (nm)</th>
<th>( \Delta \lambda_{\text{abs}} ) ( c ) (nm)</th>
<th>( \Delta \lambda_{\text{abs}} ) ( d ) (nm)</th>
<th>( \varepsilon ) ( e ) (M(^{-1})cm(^{-1}))</th>
<th>( \lambda_{\text{em}} ) ( f ) (nm)</th>
<th>( \Delta \lambda_{\text{em}} ) ( g ) (nm)</th>
<th>( \Delta \lambda_{\text{em}} ) ( h ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>234</td>
<td>378</td>
<td>-20</td>
<td>-20</td>
<td>16100</td>
<td>488</td>
<td>-</td>
<td>-61</td>
</tr>
<tr>
<td>236</td>
<td>399</td>
<td>21</td>
<td>-16</td>
<td>15200</td>
<td>537</td>
<td>49</td>
<td>-15</td>
</tr>
<tr>
<td>238</td>
<td>448</td>
<td>70</td>
<td>-9</td>
<td>16300</td>
<td>626</td>
<td>138</td>
<td>-74</td>
</tr>
</tbody>
</table>

\( a \) compounds, see Scheme 2. \( b \) Absorption maxima in ethyl acetate. \( c \) Shift of absorption maxima from \( 234 \) in ethyl acetate. \( d \) Shift of absorption maxima from analogues \( 213, 216 \) and \( 217 \) in ethyl acetate. \( e \) Extinction coefficient. \( f \) Emission maxima in ethyl acetate. \( g \) Shift of emission maxima from \( 234 \) in ethyl acetate. \( h \) Shift of emission maxima from analogues \( 213, 216 \) and \( 217 \) in ethyl acetate.

The results of the solvatochromic experiment (Figure 87) followed exactly the same trend as described for the longer push-pull probes. Also for the trimers the absorption spectra are not affected by different solvent polarity and the same shifts (\( \Delta \lambda_{\text{abs}} \approx 15 \) nm) among the solvents were observed for the three compounds.

Figure 87. Normalized absorption (solid) and emission (dotted) spectra of \( 238 \) in, with increasingly red-shifted emission, hexane (yellow), diethyl ether (orange), dioxane (red), ethyl acetate (magenta), DCM (purple), acetone (blue), DMSO (dark green).

Fluorescence spectra were more affected by the nature of the environment and showed a remarkable shift that mimics perfectly the one described for the
tetramer with the more sensitive cyanovinyl probe 238 ($\Delta \lambda_{\text{em}} \sim 170 \text{ nm}$) followed by the aldehyde 236 ($\Delta \lambda_{\text{em}} \sim 130 \text{ nm}$) and finally by the ester 234 ($\Delta \lambda_{\text{em}} \sim 90 \text{ nm}$). This similarity in Stokes shift range already suggested the comparable values for the dipole moments with respect to the quaterthiophene analogues.

Table 9. Absorption, Emission Maxima and Fluorescence Quantum Yields in Different Solvents.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Cpd$^a$</th>
<th>234</th>
<th>236</th>
<th>238</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda_{\text{abs}}$ (nm)</td>
<td>$\lambda_{\text{em}}$ (nm)</td>
<td>$\Phi$ (%)</td>
<td>$\lambda_{\text{abs}}$ (nm)</td>
</tr>
<tr>
<td>Acetone</td>
<td>378</td>
<td>520</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>DCM</td>
<td>379</td>
<td>517</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>1,4-Dioxane</td>
<td>378</td>
<td>480</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>385</td>
<td>542</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>370</td>
<td>473</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>378</td>
<td>488</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Hexane</td>
<td>372</td>
<td>449</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ compounds, see Scheme 2.

3.3.2. Fluorescence Quantum Yields

The fluorescence quantum yields in different solvents followed the same trend described for quaterthiophene compounds and were again related to the strength of the push-pull system. Consistent with the data of longer analogues (Figure 88), the quantum yield of 234 was insensitive to solvent polarity, while linear and more complex behaviors were observed for 236 and 238. These results confirmed the relationship between the emission quantum yields in different solvents and the strength of donor-acceptor systems to be independent on the length of the probes.
Figure 88. Fluorescence quantum yields as a function of the solvent polarity parameter $E_N^T$ for 213 (cyan), 216 (purple), 217 (orange), 234 (yellow), 236 (magenta) and 238 (sky-blue).

3.3.3. Dipole Moments

The solvatochromic data of the trimer compounds plotted versus the calculated solvent orientation polarizabilities (Figure 89) reflected almost perfectly the linearity found for the longer analogues 213, 216 and 217. Compounds bearing the same EWG showed almost parallel interpolation lines. Despite a general decrease of the Stokes shift when moving to a shorter $\pi$-bridge, the effect of solvent polarity on the probes was comparable between quater and terthiophenes.

<table>
<thead>
<tr>
<th>Cpd</th>
<th>$a$ (Å)</th>
<th>$m_L$</th>
<th>$m_B$</th>
<th>$m_K$</th>
<th>$m_R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>234</td>
<td>5.31</td>
<td>9880 ± 1785</td>
<td>3395 ± 244</td>
<td>5018 ± 446</td>
<td>6491 ± 889</td>
</tr>
<tr>
<td>236</td>
<td>5.30</td>
<td>12297 ± 1025</td>
<td>4071 ± 281</td>
<td>5711 ± 534</td>
<td>7690 ± 1287</td>
</tr>
<tr>
<td>238</td>
<td>7.16</td>
<td>14519 ± 1088</td>
<td>4795 ± 370</td>
<td>5529 ± 691</td>
<td>9030 ± 1570</td>
</tr>
</tbody>
</table>

* Compounds, see Scheme 2. * Distance between push and pull groups (40% of the diameter). * Slope of the linear fit of the Lippert plot. * Slope of the linear fit of the Bakhshiev plot. * Slope of the linear fit of the Kawski-Chamma-Viallet plot. * Slope of the linear fit of the Reichardt plot.
The increased slope parameters (Table 10) moving from the ester 234 to the strong acceptor cyanovinyl thiophene 238 had the same magnitude as the one observed for the longer probes.

**Figure 89.** Lippert (A), Bakhshiev (B), Kawski-Chamma-Viallet (C) and Reichardt (D) plots of 213 (cyan), 216 (purple), 217 (orange), 234 (yellow), 236 (magenta) and 238 (sky-blue).

The explanations found for the donor-acceptor tetramers were still valid for the shorter oligothiophenes and again the cyanovinyl 238 showed a stronger and remarkable $\Delta \mu$ compared to 234 and 236. More important was the considerable loss in dipole moment strength comparing the tetramer and the trimer molecules. The lack of one thiophene ring seemed to affect strongly the efficiency of the charge separation that occurs upon excitation. As a consequence the difference in electronic distribution between the ground and the excited state was less pronounced and this could explain the lower $\Delta \mu$ values. Cyanovinyl trimer 238 lost 25 % of the dipole strength compared to 213 while for the aldehyde 236 and the ester 234 the loss was around 40%. These
evaluations confirmed that also the length of the \( \pi \)-system plays a decisive role for the electronic properties in push-pull fluorophores and should be considered together with the nature of the donor and acceptor moieties in the design of such probes.

Table 11. Ground-State, Excited-State and Transition Dipole Moments.

<table>
<thead>
<tr>
<th>Cpd(^{a})</th>
<th>( \mu_{g}^{b} ) (D)</th>
<th>( \mu_{e}^{c} ) (D)</th>
<th>( \Delta\mu_{g}^{d} ) (D)</th>
<th>( \Delta\mu_{e}^{e} ) (D)</th>
<th>( \Delta\mu_{L}^{f} ) (D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>234</td>
<td>1.7</td>
<td>8.8</td>
<td>7.1</td>
<td>13.8</td>
<td>7.1</td>
</tr>
<tr>
<td>236</td>
<td>1.5</td>
<td>9.3</td>
<td>7.7</td>
<td>15.0</td>
<td>7.4</td>
</tr>
<tr>
<td>238</td>
<td>1.0</td>
<td>14.2</td>
<td>13.2</td>
<td>24.0</td>
<td>11.0</td>
</tr>
</tbody>
</table>

\(^{a}\) Compounds, see Scheme 2. \(^{b}\) Calculated from equation 22. \(^{c}\) Calculated from Equation 23. \(^{d}\) Calculated from Equation 24. \(^{e}\) Calculated from Equation 18. \(^{f}\) Calculated from Equation 21.

The dipole moment values of 238, and by extension of the two amphiphiles 218 and 219, located the electronic properties of these probes in between the strong push-pull amphiphiles 211 and 212 and the weaker hydrazone functionalized 214 and 215. Moreover these dipole values came from a modification of the conjugated backbone and not of the donor-acceptor groups. This consideration suggested an investigation in bilayer membranes also with the terthiophene amphiphiles to compare the results with longer analogues in terms of planarization and polarization effects driven by the interactions with different membrane phases and polarizations.

3.4. Planarizable Push-Pull Oligothiophenes

The term “vesicles” indicates the result of a spontaneous supramolecular organizing process among amphiphilic molecules in a water media. Amphipathic molecules assemble together in a well-defined way in which the
hydrophilic moieties keep the contact with the aqueous environment and in the same time shield the hydrophobic moieties from any contact with water forming a supramolecular double layer structure with a thickness around 4-5 nm\textsuperscript{212}. The final assembly results in a single or a multiple bilayer(s) close shell(s) in which the water is present both in the inner and the outer part of the supramolecular assembly. The size and the lamellarity (number of concentric bilayers) of the vesicles could be optimized following different and various preparation pathways reported in literature\textsuperscript{213}. Generally these supramolecular aggregates can be classified based on their size and on the number of the present bilayers as:

- Small unilamellar vesicle (SUVs) in which one bilayer is present and the diameter is less than 50 nm.
- Large unilamellar vesicles (LUVs) with a diameter between about 50 and 500 nm.
- Giant unilamellar vesicles (GUVs) in which the diameter of the sphere can reach 1 \( \mu \)m.
- More complex structures like oligo (OLVs) or multilamellar vesicles (MLVs) where many concentric bilayers are arranged together.

Another common classification is based on the nature of the molecules involved in the vesicles formation. The most investigated classes of vesicles are based on amphiphilic lipid (liposomes), polymeric amphiphiles (polymersomes), sphingolipids (sphingosomes), synthetic surfactants (synthetic vesicles) and many others\textsuperscript{213}. The variety of the amphipathic molecules able to form vesicles is one of the reasons of the large use of vesicles in many different fields such cosmetics\textsuperscript{214,215}, agrochemistry\textsuperscript{216}, catalysis\textsuperscript{217-219}, polymer chemistry\textsuperscript{220,221}.

More remarkable is the possibility to form vesicles using biological lipid and phospholipid. The vesicle size and the curvature together with the
supramolecular arrangement of such molecules mimic very well the structure and the properties of the biological cell membrane. These lipid vesicles are employed in pharmacology and medicine as a drug delivery agents, in anticancer therapy and in diagnostic.

Because of the simple preparation and modulation of the properties according to the nature of the lipids, LUVs are the ideal model to test the properties of our fluorophores. Although their simpler structure compare to cells membrane, it is reasonable to think that the response in LUVs can predict well the behavior of our molecules living cells.

3.4.1. Membrane Fluidity

The assembly of phospholipids into vesicles occurs at specific thermodynamic equilibrium states which correspond to the relevant liquid crystalline phases derived from the dispersion of amphiphilic molecules in aqueous media. The nature of the phases is related to the structure of the amphiphiles and the experimental conditions. Many different liquid crystalline states exist and each one has a specific degree of order and of molecular mobility.

The liquid-disordered phase (Lα) is characterized by a rather disorganized interaction among the hydrophobic chains. A predominant gauche conformation in the saturated hydrocarbons moieties makes the bilayer fluid and similar to a liquid in which the amphiphile molecules have a rapid rotational and lateral diffusion.

In the solid-ordered state (Lβ or L′β) the individual amphiphiles are mostly with the all-trans conformation. The large stabilization effect coming from inter-chain Van Der Waals interactions increases the viscosity of the system. The motion of the molecules is remarkably restricted giving rise to a crystal-like arrangement. If the lipid molecules arrange with a tilted alkyl
chains with respect to the normal of lipid bilayer the gel-phase is abbreviated as $P'_{\beta}$ while if the chains are not tilted is indicated as $L_{\beta}$ or $L'_{\beta}$.

In general, glycerophospholipids in aqueous media self-assemble in the $P'_{\beta}$-phase at the thermodynamic equilibrium which is normally 5-10 °C below the specific lamellar chain melting temperature ($T_m$).

$T_m$ is often named also as gel-to-liquid crystalline phase transition temperature and is related with the chemical nature of hydrophobic chains. The thermodynamic stability of the gel phase is mainly related to the length of the hydrocarbon chains and the presence of the unsaturation(s) along the hydrophobic moieties228 (Table 12). Longer hydrocarbon moieties increase the inter-chains interactions stabilizing the gel phase while the presence of the unsaturation(s) along the hydrophobic chains disturbs the tails “packaging” and decrease $T_m$ values.

Very often an intermediate gel phase between the $P'_{\beta}$ and the $L_{\alpha}$-phase is observed for phospholipids. This particular phase, named ripple ($P_{\beta}'$) is present at high water content and is defined as a pretransition because of the gel to gel nature of the transition229,230. All these phase transitions are clearly indicated by the appearance of an endothermic peaks in the differential scanning calorimetry (DSC) measurement231,232. Normally the transition into ripple phase is less pronounced and defined232 compared to the proper gel to liquid transition at $T_m$.

The transition temperature values spread over a large range which simplified the experimental design for the detection of the phase transition with our probes.
<table>
<thead>
<tr>
<th>Phosphatidylcholine (PC)</th>
<th>$T_m$</th>
</tr>
</thead>
</table>
| DMPC (1,2-dimyristoyl-
  sn-glycero-3-PC) 14:0/14:0 | 23.6 ± 1.5 |
| DPPC (1,2-dipalmitoyl-
  sn-glycero-3-PC) 16:0/16:0 | 41.3 ± 1.8 |
| DSPC (1,2-distearoyl-
  sn-glycero-3-PC) 18:0/18:0 | 54.5 ± 1.5 |
| POPC (1-palmitoyl-2-oleoyl-
  sn-glycero-3-PC) 16:0/18:1 $\Delta^0$ | -2.5 ± 2.4 |
| SOPC (1-stearoyl-2-oleoyl-
  sn-glycero-3-PC) 18:0/18:1 $\Delta^0$ | 6.9 ± 2.9 |
| DOPC (1,2-dioleoyl-
  sn-glycero-3-PC) 18:1 $\Delta^0$/18:1 $\Delta^0$ | -18.3 ± 3.6 |
The nature of the phospholipids together with the experimental temperature is the key parameters that determine the fluidity of the vesicle lipid bilayer in aqueous media. Based on these parameters we designed an experiment in homogenous LUVs (0.1 µm diameter) to check whether our fluorophore can discriminate between different phases or not. We investigated both the temperature effect and the phospholipid nature as environmental discriminant parameters.

DPPC vesicles have a $T_{m}$ around 41 °C which means that above and over that temperature a viscosity sensitive probes should interact differently with the two distinct phases and as a consequence should provide two different responses.

We designed an intuitive temperature cycle starting from 25 °C and slowly rising up the temperature to 55°C before going back the starting conditions. Overcoming the $T_{m}$ the DPPC vesicles were subjected to the Pβ–Lα-phase transition and our probes should feel and visually discriminate the different environments.

In the same time the experiment was performed in DOPC vesicles. DOPC LUVs have a $T_{m}$ much lower than the experimental range of temperature which means that vesicles are stable in the Lα phase during the heating-cooling cycles. The results comparison between DPPC and DOPC LUVs should provide the informations about the behavior of our probes in different lipid vesicles and phases avoiding mistaken interpretations of the data that could come from the direct effect of temperature on the probes.

All the amphiphiles, quater and terthiophene molecules were able to partition in the membrane bilayers both in DPPC and DOPC vesicles. This proved that the molecular design of the 1:1 hydrophilic/hydrophobic moieties ratio allowed the solubilization in water (buffer) of the fluorophores and, in the same time, the partition of the amphiphilic molecules in the hydrophobic bilayer membranes.
Figure 90. Normalized emission spectra of 211 (7.6 μM, λ<sub>ex</sub> = 430 nm) in DOPC (A) and DPPC vesicles (B) at 25 °C (dashed, grey), heated at 55 °C (solid, gold), cooled down to 25 °C (solid, blue), heated to 55 °C (dotted, gold) and cooled to 25 °C (dotted, cyan).

The fluorescence spectra of 211 and 212 in DPPC (Figure 90) showed a bathochromic shift of the emission at 25°C after the first heating-cooling cycle while the red-shifted emission was kept after the second temperature cycle. This suggested that the partition into the gel phase is initially hindered, but once inside of the membrane, the probes were compatible and stable both in liquid and gel phases. In DOPC vesicles this initial blue-shifted emission was not observed at 25°C meaning that the compounds partitioned more easily in liquid phase.

For the other amphiphiles the same shift in DPPC LUVs was not present probably because of the overall smaller probes dimensions which allowed them to enter faster in the bilayer.

Nevertheless no significant shifts were observed in the emission neither at same temperature comparing liquid phase DOPC and DPPC vesicles nor upon
P$_\beta$-L$_\alpha$ phase transition during the temperature cycles in DPPC. The change in the viscosity of the system by the nature of the amphiphiles or by the temperature effect was not reflected in the emission shifts. This showed that the environmental polarity of the media surrounding the probes does not change remarkably moving from gel to liquid phases. A comparison with the solvatochromic experiment data located the emission spectra of 211 and 212 ($\lambda_{em} \sim 650$ nm), 214 and 215 ($\lambda_{em} \sim 550$ nm) and 218 and 219 ($\lambda_{em} \sim 620$ nm) in the region of the medium polarity solvents. This observation was coherent considering the probes located at the water/membrane interphase with the hydrophobic moieties inserted in the lipid bilayer and the polar groups together with the hydrophilic heads of the phospholipids.

In the same time the lack of shift of the emitting excited state could be justified reminding the planarization that occurs in such molecules upon excitation. The higher viscosity of the gel phase compared to the liquid one had no remarkable effect on the already flat excited state.

In other word, the quinonoidization of the oligothiophenes backbone upon the photon absorption occurred without any difference both in P$_\beta$ and L$_\alpha$ phases despite the different hindrance to the planarization related to different viscosity.

Finally the fluorescence quantum yields was comparable between the DPPC and the DOPC vesicles and showed in both cases a decrease (~ 25 %) at 55 °C which can be related with a temperature induced increase of the non-radiative processes compared to the emissive one$^{233}$. It should be pointed out that the quantum yield was fully recovered upon cooling down the system to 25 °C meaning that the probes were not ejected from the membrane during the transition from liquid to gel phase.
Figure 91. Normalized excitation spectra of 211 (7.6 μM, λ_{em} = 600 nm) in DOPC (A) and DPPC vesicles (B) at 25 °C (dashed, grey), heated at 55 °C (solid, gold), cooled down to 25 °C (solid, blue), heated to 55 °C (dotted, gold) and cooled to 25 °C (dotted, cyan).

The excitation spectra at 25 °C in DPPC LUVs for all the amphiphiles appear red-shifted (~ 25 nm) as compared to the DOPC (Table 13) and to the solvatochromic data. Upon increasing of the temperature over the T_m the spectra shifted back in the higher energy region, in a comparable position with the data recorded in DOPC liquid phase. Cooling down the system again to 25 °C reproduced the initial spectra at longer wavelength and this back-forward shift was observed during the heating-cooling cycles (Figure 91).

The transition from the liquid to the gel phase induced a reorganization of the lipids to a more ordered structures with stronger inter-chain interactions which caused an increase of the lateral pressure of membrane molecules on the probes scaffolds. In the same time the experiment performed in DOPC confirmed that the temperature did not induce any remarkable shift in the excitation spectra. As a consequence, the shift observed in DPPC was not related with a direct effect of the temperature on the probes but could be
induced by a gained planarization or by polarization due to the push-pull nature of probes or, finally, by the synergetic effect of the two phenomena.

Table 13. Excitation Spectra Maxima in DPPC and DOPC Vesicles During a Double Temperature Cycle.

<table>
<thead>
<tr>
<th>Cpd&lt;sup&gt;a&lt;/sup&gt;</th>
<th>DPPC LUVs</th>
<th>DOPC LUVs</th>
<th>Solv.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>λ&lt;sub&gt;ex&lt;/sub&gt; 25 °C</td>
<td>λ&lt;sub&gt;ex&lt;/sub&gt; 55 °C</td>
<td>λ&lt;sub&gt;ex&lt;/sub&gt; 25 °C</td>
</tr>
<tr>
<td>211</td>
<td>491 (nm)</td>
<td>468 (nm)</td>
<td>468 (nm)</td>
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<tr>
<td></td>
<td>491 (nm)</td>
<td>468 (nm)</td>
<td>468 (nm)</td>
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<tr>
<td></td>
<td>480 (nm)</td>
<td>462 (nm)</td>
<td>457 (nm)</td>
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<tr>
<td>212</td>
<td>480 (nm)</td>
<td>462 (nm)</td>
<td>458 (nm)</td>
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<tr>
<td></td>
<td>480 (nm)</td>
<td>- (nm)</td>
<td>460 (nm)</td>
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<tr>
<td></td>
<td>449 (nm)</td>
<td>425 (nm)</td>
<td>428 (nm)</td>
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<tr>
<td>214</td>
<td>449 (nm)</td>
<td>429 (nm)</td>
<td>428 (nm)</td>
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<tr>
<td></td>
<td>449 (nm)</td>
<td>- (nm)</td>
<td>431 (nm)</td>
</tr>
<tr>
<td></td>
<td>449 (nm)</td>
<td>428 (nm)</td>
<td>441 (nm)</td>
</tr>
<tr>
<td>215</td>
<td>449 (nm)</td>
<td>430 (nm)</td>
<td>441 (nm)</td>
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<tr>
<td></td>
<td>449 (nm)</td>
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<td>440 (nm)</td>
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<tr>
<td></td>
<td>475 (nm)</td>
<td>453 (nm)</td>
<td>454 (nm)</td>
</tr>
<tr>
<td>218</td>
<td>473 (nm)</td>
<td>454 (nm)</td>
<td>455 (nm)</td>
</tr>
<tr>
<td></td>
<td>471 (nm)</td>
<td>- (nm)</td>
<td>455 (nm)</td>
</tr>
<tr>
<td></td>
<td>468 (nm)</td>
<td>453 (nm)</td>
<td>453 (nm)</td>
</tr>
<tr>
<td>219</td>
<td>466 (nm)</td>
<td>453 (nm)</td>
<td>453 (nm)</td>
</tr>
<tr>
<td></td>
<td>467 (nm)</td>
<td>- (nm)</td>
<td>453 (nm)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Compounds, see Scheme 3. <sup>b</sup> From normalized and corrected excited spectra. <sup>c</sup> Average absorption spectra maxima from solvatochromic experiment.

As deduced from the emission spectra, no changes in polarity were detected during the Pβ-Lα transitions by the fluorescence spectra. Moreover, as showed in the solvatochromism experiment, the absorption spectra of the probes were not so affected by differences in solvents polarity. Comparable shifts upon the temperature cycles (Figure 92) were observed in DPPC for all the series of the
amphiphiles independently on the push-pull system and on the length of the $\pi$-bridge.

Figure 92. Excitation maxima in DOPC (A) and DPPC vesicles (B) of 211 (dashed, blue), 212 (solid, light blue), 214 (dashed, red) and 215 (dotted, magenta), 218 (dashed, green) and 219 (dotted, dark green).

These observations suggested that the shift in the excitation spectra in DPPC was not related with the polarization or with the different strengths of the dipole moments but should be related only with the induced backbone planarization caused by the change in the lateral constraints. The comparison of the results obtained in DOPC and DPPC was a crucial point to relate the shift to the direct effect of the membrane viscosity.

Furthermore to avoid any doubt on the origin of the excitation spectra shift we performed the temperature cycle in DPPC with different concentrations of the probes and also with a simple solution of the fluorophores in buffer (Figure 93). Lower concentrations (0.25 and 0.1% mol) of 211 in vesicles were depicted by weaker intensities in the excitation spectra but the overall shifts and positions of the peaks at 25 and 55 °C were the same as described. In the same time, the experiment performed without vesicles showed a total quench of the emission of the compound in water media. These results further confirmed the origin of the shift excluding the aggregation phenomena from the possible causes.
Figure 93. Excitation spectra of 211 (λ<sub>em</sub> = 600 nm) in DPPC at 7.6 µM (solid, blue), 3.8 µM (dotted, cyan), 1.5 µM (solid, gold) and 7.6 µM without vesicles (dashed, grey).

The results of the membrane fluidity investigation showed a good sensitivity of this new class of probes towards small environmental changes. In the same time the probes displayed good and stable photophysical properties. Furthermore these positive results seem to be related only with the planarization of the probes and not with the polarization. Simple modulations of the molecular structures appear promising for the optimization of the sensitivity towards the membrane fluidity investigation. Moreover a comparison with other reported probes (e.g. 99-109) will provide a more précised estimation of the sensitivity of these probes towards the discrimination of the bilayer membrane phases.

In biological terms the ability of our probes to visualize the differences in membrane fluidity could be employed in investigation of relevant research areas like the lipid microdomains (RAFT) stability and dynamics<sup>234</sup>, the
phagocytosis and cell signaling\textsuperscript{235}, the diseases such depression and osteoporosis generations\textsuperscript{236}.

Figure 94. Schematic representation of the membrane fluidity effect on the polarizable and planarizable probes.

3.4.3. Membrane Potential

Vesicles like cells are delimited by phospholipid bilayer membrane which serves as boundary between the internal aqueous media or cytoplasm and the external environment. The inner and outer aqueous media are not in direct communication which means nothing of what is present on one side can reach the other without passing through the lipid bilayer.

The permeability of the membrane bilayer to ions (e.g. H\textsuperscript{+}, K\textsuperscript{+}, Na\textsuperscript{+}, Cl\textsuperscript{-}, OH\textsuperscript{-}) is really low\textsuperscript{237}. Assuming a different ionic concentration on both sides of the membrane, the osmotic process is not able to equilibrate the concentrations between the inner and outer media. If a non-specific “pathway” is open through the membrane then the concentrations can be equilibrated by diffusion. For the case in which the “pathway” is highly selective for one of the ions (e.g. K\textsuperscript{+}) then it will cross the membrane till the concentrations are equal on both sides. As far as only one of charged species is involved in the membrane crossing, the
charge neutrality of the two aqueous media on the different sides of the membrane will be lost. One side will be enriched positively and the other one will compensate the overall system neutrality with an excess of negative charges. This difference of ions concentrations on opposite sides of the membrane induces a potential across the bilayer called transmembrane potential ($\Delta \psi$).

In biological systems the transmembrane potential spreads around 10-100 mV$^{238}$ and is involved in the regulation of membrane protein functions and ion channels$^{239}$. In a lipid bilayer systems $\Delta \psi$ is always present together with other weaker electrical potential (Figure 95), the surface potential ($\psi_s$) and the membrane dipole potential ($\psi_d$).

![Figure 95](image)

Figure 95. Schematic representation of the electrical potential present in a typical bilayer membrane system.

$\psi_s$ is generated by the charges of the hydrophilic moieties of the lipids and the ions absorbed on them at the interface and it spans between the bilayer and the bulk media. Normally it is much weaker than $\Delta \psi$ and in biological systems reaches values of 10-30 mV$^{240}$. Moreover, $\psi_s$ is responsible for the distribution of ions very close to the membrane surface. Because of the overall negative
charge of cell membrane has the function to concentrate cations close to the bilayer and to drive away the anions.

The last electrical potential is originated from the alignment of water molecules and the dipolar residues of the lipids. The magnitude of the membrane dipole potential is related to the nature of the lipid in terms of number of unsaturation and linkage between hydrophobic and the polar moieties. As the surface potential, it is also weaker than and despite inconsistency in the reported values it can reach 300 mV. The dipole membrane potential seems to be involved in the transport of hydrophobic ions across the bilayer membrane.

Among the three different contributions to the overall membrane potential, is the one with stronger magnitude and, so far, interest from a biological point of view. Moreover, it could be quite easily installed and measured in lipid vesicles.

Differently from the membrane fluidity investigation, we needed to construct a system in which the membrane should play the role of an insulator between two different ionic solutions. For this experiment the nature of the lipid and the relative phase is much less important that the overall ability of the bilayer to avoid free diffusion on ions.

This concept together with the need of different ions concentrations between the inner and the outer aqueous media moved us to prepare egg yolk phosphatidylcholine (EYPC) vesicles. EYPC form liquid phase vesicles in standard conditions and are well-known to be a good model of the cell membrane due to the presence of different saturated and unsaturated lipids.

Moreover these vesicles were filled with potassium chloride solution and dissolved in an equimolar solution in which sodium replaced the potassium as cation. The polarization of the vesicles could be obtained employing valinomycin as selective potassium carrier in different outer Na+/K+ Cl- buffers (Table 14). The selectivity of valinomycin to transport potassium ions is
10^3 larger compared to the sodium\textsuperscript{246} which means that only K\textsuperscript+ ions will be involved in the membrane crossing under these conditions.

<table>
<thead>
<tr>
<th>( \Delta \psi ) (mV)</th>
<th>MC\textsubscript{1} (100 mM)</th>
<th>NaCl (100 mM)</th>
<th>KCl (100 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-200</td>
<td>2454:1</td>
<td>1684</td>
<td>0.7</td>
</tr>
<tr>
<td>-180</td>
<td>1124:1</td>
<td>1683</td>
<td>1.7</td>
</tr>
<tr>
<td>-160</td>
<td>515:1</td>
<td>1681</td>
<td>3.7</td>
</tr>
<tr>
<td>-140</td>
<td>236:1</td>
<td>1676</td>
<td>8.0</td>
</tr>
<tr>
<td>-120</td>
<td>108:1</td>
<td>1667</td>
<td>17.3</td>
</tr>
<tr>
<td>-100</td>
<td>49:1</td>
<td>1647</td>
<td>37.3</td>
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<tr>
<td>-80</td>
<td>23:1</td>
<td>1605</td>
<td>79.5</td>
</tr>
<tr>
<td>-60</td>
<td>10:1</td>
<td>1519</td>
<td>165.3</td>
</tr>
</tbody>
</table>

Knowing the initial concentrations of potassium on both sides of the bilayer membrane, the installed transmembrane potential was calculated by the Nerst equation (Equation 29)\textsuperscript{239}.

\[
\Delta \psi = 59 \cdot \log \left( \frac{K^+_{\text{out}}}{K^+_{\text{in}}} \right) \tag{29}
\]

The last preliminary step before testing our compounds was to prove the presence and stability of the valinomycin induced transmembrane potential in EYPC vesicles. Spectroscopically the membrane polarization can be followed with many potential sensitive dyes like safranin O\textsuperscript{247} (Figure 96).

This water soluble probe shows an increase in fluorescence intensity once the potential is installed by valinomycin and a decrease of the emission signal when the transmembrane potential is destroyed by the addition of mellitin, a small amphipathic \( \alpha \)-helix, which increases the permeability of membrane bilayer to ions (e.g. Na\textsuperscript{+}, Cl\textsuperscript{-})\textsuperscript{248}.  

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Figure 96. (A). Original kinetic data for the membrane potential calibration with safranin O Fluorescence intensity ($\lambda_{ex} = 522$ nm, $\lambda_{em} = 581$ nm) during the addition of valinomycin (1 $\mu$M at 50 s) and melittin (2 $\mu$M at 300 s) to EYPC LUVs at -200 (red), -180 (gold), -160 (yellow), -140 (green), -120 (sky-blue), -100 (cyan), -80 (blue) and -60 mV (violet). (B) Maximal fluorescence intensity as a function of the applied membrane potential between -60 and -180 mV.

The fluorescence intensities where normalized (Equation 30) assuming $I_0 = I_{em}$ right before the valinomycin addition and $I_{max} = I_{em}$ just before the melittin addition.

$$I_{em}(rel) = \frac{I_{em} - I_0}{I_{max} - I_0}$$

(30)

The safranin O test confirmed the stability of the induced potential in our vesicles and the linear dependence of the fluorescence increase according to the strength of the theoretical transmembrane electrical potentials allowing us to measure our probes in well-determined conditions. The linearity confirmed that the potential was the one expected since the Nerst equation was respected.

Similar to the investigation of the membrane viscosity, the emission spectra of our amphiphiles did not show a remarkable shift at different potentials. As before this result can be explained considering the flat character of the $S_1$ and also that the induced membrane potentials do not change the polarity of the region in which the fluorophores are located. Overall, the emission spectra
resulted insensitive in shift terms to membrane potentials for all the amphiphiles independently on the push-pull strength and the length of the π-bridge (Figure 97).

Figure 97. Normalized emission spectra of 211 (A, 7.6 μM, λex = 430 nm) and 212 (B, 8.1 μM, λex = 430 nm) in EYPC vesicles at 0 (solid, gold), -90 (dashed, yellow), -120 (dotted, sky blue), -150 (dashed, cyan) and -180 mV (solid, blue).

Nevertheless time-dependent fluorescence experiment showed clearly a detectable increase of the emission intensity (Δ$I/I$) for 211 and 212 (Figure 98) upon creation of the potential (-180 mV). This result was observed only for the two amphiphiles with stronger dipole moment while the hydrazones 214, 215 and the trimer analogues 218 and 219 did not show any change in fluorescence quantum yields. Many factors are known to affect the emission efficiency (e.g. solvent polarity, aggregation) but according to the experimental data none of them can clearly justify the observed phenomena.

As far as no emission shifts were observed we assumed that the polarity of the environment did not change remarkably upon the creation of the transmembrane potential. This implies that the increase in quantum yield is not
related with interactions of the probes with different polar media as in the solvatochromic experiment.

At the same time the emission time course were corrected for the weak increase of fluorescence coming from the slow partition of the probes in membrane which takes place over the experimental time. In this way we removed the possibility that the increase and loss of quantum yield could be related with fast partition/expulsion of the probe from the bilayer.

Another possible explanation is related with the increased lipid microviscosity due to the induced polarization as observed and reported in vesicles\cite{249} and \textit{in-vivo}\cite{250}. An increase of the microviscosity would make the environment surrounding the probes more “rigid” lowering the not radiative energy dissipation that occurs due to the interaction between the excited fluorophore and the media. Related to our results this explanation does not apply, since we observed the increase in fluorescence intensity only with two amphiphiles and not for all of them.

![Figure 98](image)

\textbf{Figure 98.} Emission time course of 211 (A, 7.6 µM) and 212 (B, 8.1 µM) at $\lambda_{em} = 630$ nm ($\lambda_{ex} = 545$ nm) during the addition of valinomycin (1 µM, at 50 s) and melittin (2 µM, at 125 s) to EYPC LUVs with inside 100 mM KCl and outside 100 mM MCl (M = Na/K 1124:1), 10 mM Tris, pH 7.

Evaluated and discarded the possible causes related with the environmental polarity, the partition process and the microviscosity changes it becomes hard
to figure out a possible cause of these results assuming an already flat excited state. Probably the interaction between the induced membrane polarization and the strong dipole moment of the amphiphiles 211 and 212 could affect the planarization of the excited state and slightly increase the weight of the fluorescence process in the way back to the ground state. More similar probes, differently twisted, should be tested in order to evaluate this hypothesis and to find the cause of such results.

**Figure 99.** Normalized excitation spectra of 211 (A, 7.6 µM, λ_{em} = 600 nm) and 212 (B, 8.1 µM, λ_{em} = 600 nm) in EYPC vesicles at 0 (solid, gold), -90 (dashed, gold), -120 (dotted, sky blue), -150 (dashed, cyan) and -180 mV (solid, blue).

In agreement with the increase in fluorescence quantum yield the excitation spectra (Figure 99) of 212 and 212 showed a red-shifted shoulder. This shifted (17 nm) bump \( I_{ex}(50) \) widened with increased potential and was not present neither for the amphiphiles with much weaker dipole moment 214 and 215 nor for the trimers 128 and 219.
The possible justification of this change could be related with an increased planarization of the probes induced by the interaction of the molecular dipole and the electric potential. The linear relation (Figure 100) that occurs between the values of $\Delta \psi$ and the relative intensity of the shoulder maximum ($\nu_{\text{ex}}$) compared to the maximum of absorption could support this explanation.

![Graph](image)

**Figure 100.** Red shift in the $I_{455nm}$ of 211 (7.6 $\mu$M, dotted, blue) and 212 (8.1 $\mu$M, solid, cyan) as a function of the applied transmembrane potential.

Complementary to the fluidity results, the membrane polarizability results suggested that the push-pull system was the key parameter to take in account for this analysis. A strong molecular dipole moment seemed to interact with the applied $\Delta \psi$ while weak donor-acceptor systems such 214 and 215 could not discriminate different electric gradient (Figure 101). Quite surprising was the lack of sensitivity of the trimer analogues in which the loss of dipole strength is only 25 % compared to the longer probes. Further investigations with terthiophene molecules with stronger dipole moment could clarify if the present result is related to the weaker dipole moment or with the shorter length of the molecules which locate the probes not so deep in the membrane bilayer.

More in general, the results of the membrane polarization have shown the importance of the dipole strength in this class of probes. A clear relation between the $\Delta \psi$ discrimination by strong push-pull systems and the insensitivity of the weaker donor-acceptor probes supports the idea of the
induced backbone planarization by the transmembrane potential. Moreover these results justify further modification and investigation with analogue fluorophores to confirm the origins of this sensitivity or suggest other possible causes like re-orientation or redistribution in the bilayer membrane.

As for the sensitivity towards the membrane fluidity, a comparison with reported voltage sensitive dyes will be useful to evaluate the sensitivity of these probes in terms of transmembrane potential.

![Figure 101. Schematic representation of the transmembrane potential effect on the polarizable and planarizable probes.](image)

In biology these sensitive probes could be employed in the visualization and quantification of changes in transmembrane potential which play a central role among the more important cell functions.

The ATP synthesis in mitochondria is mediated by the protons flow against their concentration through the mitochondrial membrane. The activity and the proper functions of various membrane proteins like ion channels, enzymes, transporters and pumps are directly and strongly dependent on the ionic gradient present across the membrane\textsuperscript{252} and many other processes and diseases are related with ions and their ability to cross membrane bilayer.
3.4.3. Final Considerations

The sensitivity of this class of probes to membrane viscosity and membrane polarization has suggested that the concept of polarization and planarization can coexist in the same molecule and operate to detect changes in membrane bilayers. The effect of different lateral constrains on the thiophene backbone seems to originate the sensitivity to membrane phases independently on the strength of the molecular dipole moment. On the other hand, the push-pull groups play the main role in the interaction with the generated electric potentials in polarized membranes.

Figure 102. Schematic representation of the membrane fluidity and potential sensitivity of oligothiophene probes generated by planarization and/or polarization.

The simultaneous ability of these fluorophores to respond by different, complementary interactions to distinct environmental changes supports further investigations with similar compounds to confirm and maximize the sensitivity achieved with this first generation of polarizable and planarizable probes.
3.5. Naphthalenediimides and Perylenediimides with Hydroquinones and Catechols in the Core

Polycyclic aromatic molecules are hydrocarbon systems which derive from the fusion of aromatic rings. The smallest member of this family is naphthalene which can generate wider aromatic system, perylene, by fusion with another naphthalene at the peri-position. Further combinations of naphthalene units with perylene enlarge the \( \pi \)-system to poly(pery-naphthalenes). Tetra-carboxylic diimides derivatives naphthalenediimide (NDI) and perylenediimide (PDI) have been widely investigated (Figure 103) by substitution on the aromatic core (bay position) which affect the electronical properties of the molecules and by modification on the imide moieties to modulate the solubility and the processability properties\textsuperscript{253,254}. Such a large set of possible modifications supported the use of these molecules as a molecular building blocks in organic light emitting diodes (OLEDs)\textsuperscript{255}, optical switches\textsuperscript{256}, photodetector\textsuperscript{257} and artificial photosystems\textsuperscript{258,259}.

![Figure 103. Schematic representation of most common rylene.]()

NDIs are flat systems in which the color and the redox properties can be easily modulated by the insertion of different EDG or EWG\textsuperscript{149}. The whole visible spectra can be covered by straightforward modifications (Figure 104). Replacing of the oxygen in the yellow NDI with sulfur shifts the absorption to the red and further change from sulfur to nitrogen gives a blue compound. On the other side the insertion of EWG increases the \( \pi \)-acidity of the NDI creating suitable molecules for the investigation of interactions such anion-\( \pi \).
PDI could be considered as big brother of NDI and because of the more extended aromaticity, the unsubstituted derivative absorbs already at 530 nm. Different from NDI, the modifications (Figure 105) at the bay positions cause a twist of the aromatic core from the planarity. The general interest on PDIs is related to the remarkable properties in terms of fluorescence quantum yield, photo and chemostability of such molecules.

**Figure 104.** Rainbow collection of NDIs with relative HOMO (bold) and LUMO (dashed) energies in eV and maximal absorption in nm. Revised graphical representation from reference149.

**Figure 105.** Bay substituents effect on the PDI emission maxima, quantum yield and twisting angle. Revised graphical representation from reference260.
Our interest in this family of fluorophores was directed mainly in the development of a series of panchromatic probes able to cover wide part of the visible spectra and in the same time be easily functionalized as potential biological probes. In parallel we wanted to test whether the amphiphile strategy described for oligothiophene probes could be applied also with more rigid molecules like PDI or not.

3.5.1. Synthesis

Inspired by the phenolate anion as a π-donor in many biological systems like the green fluorescent protein (GFP) we wondered if a similar approach could be applied to tune the photophysical properties of PDI and NDI without many synthetic efforts. We inserted the hydroxy groups which could work as electron-donor moieties and also as reactive site for further modifications in the aromatic core. The NDI substrate was modified with two OH groups to the hydroquinone analogue and to the catechol by fully substitution of the aromatic positions with hydroxyl moieties. The hydroxy PDI was prepared analogously (Scheme 5). In the same time PDI bearing a reactive aldehyde on the imide site was synthesized to test if it could be employed as a membrane bilayer probe like the oligothiophenes.

1,7-Dibromo PDI was prepared by iodine catalyzed bromination at the bay position followed by imide formation. Subsequent nucleophilic substitution with sodium hydride (NaH) activated aliphatic alcohols such allylalcohol and ethanol, provided respectively the dark red compounds and . The panchromatic target compound bearing two hydroxyl groups in the bay positions was obtained under mild condition by palladium catalyzed deallylation from .
Scheme 5. a) Allyl alcohol, NaH, 1 h, 100 °C, 55%. b) Pd(PPh₃)₄, phenylsilane, DCM, 1 h, rt, 65%. c) Ethanol, NaH, 18 h, 80 °C, 45%. d) 1. iPrOH, KOH, 4 h, 85 °C; 2. DMAc, cyclohexylamine, 3 h, 120 °C, 35%. e) DMF, Urea, 14 h, 130 °C, 72%. f) Cu(OAc)₂, TEA, 253, CHCl₃, O₂, 16 h, 40 °C, 65%. g) 240, DMSO, AcOH, 60 °C, 14 h, quant.
In parallel the hydrolysis of 250 in isopropanol with an excess of KOH, followed by condensation with stoichiometric amount of cyclohexylamine\textsuperscript{264} gave the diethoxy-monoiode derivative 251. The hydrolysis of one or both the imide(s) group(s) of 250 decreased almost completely the solubility of the compound preventing any manipulations of the dark solid before the re-insertion of the cyclohexylamine. A second condensation of 251 with urea afforded the asymettric PDI 252 in good yield. Finally, the copper-promoted N-arylation of the cyclic imide\textsuperscript{265} with the phenylboronic derivative 253 yielded the PDI 247 probe bearing the reactive aldehyde site. As reported for oligothiophenes, \textit{in-situ} capture\textsuperscript{171} of the aldehyde by the hydrazide derivative 240 afforded the PDI amphiphile 254 for the membrane investigation.

A red-shifted absorption and emission spectra are desirable because the fluorescence imaging and tracking of single molecules and processes in living cells are often complicated by intense fluorescent background signals. Such “noise” is related to the presence of endogenous molecules such flavins, collagen and porphyrins which normally absorb light in the 300-500 nm region and emit at 400-550 nm\textsuperscript{266}. To avoid this interference, a biological fluorescent probes should absorb around 500 nm and emit in the region of 600 nm.

The PDI analogue of the push-pull oligothiophene amphiphiles was designed just to test a probe which absorbs light around 550 nm due to the insertion of electron rich groups in the core and had remarkable fluorescence properties. Nevertheless the substitutions at bay positions cause torsion\textsuperscript{260} of PDI core due to steric hindrance and this could affect the interactions with the rigid gel phase in membrane bilayer.

To minimize the \(\pi\)-system torsion and in the same time to shift the absorption and emission spectra to lower energies we introduced short ethoxy groups in the core. As reported for similar compounds\textsuperscript{263}, the presence of linear substituents has a minimal effect on the planarity of the PDI when compared to more bulky substituents, inducing a small twist (9 °). Moreover the presence of the electron rich substituents was able to shift significantly the absorption and
the emission spectra (Figure 106) to lower energies ($\lambda_{\text{abs}} = 563$, $\lambda_{\text{em}} = 583$). Unfortunately the fluorescence quantum yield dropped to 12 % in chloroform, much lower compared to the almost quantitative one for similar compounds. This loss in $\Phi$ could be attributed to a photoinduced electron transfer process from the donor moieties to the electron-poor PDI unit.$^{267}$

![Normalized absorption (solid) and emission (dashed) spectra of 247 in chloroform.](image)

**Figure 106.** Normalized absorption (solid) and emission (dashed) spectra of 247 in chloroform.

Despite the drop in fluorescence quantum yield we covalently linked the fluorophore with the hydrophilic head to generate the analogue of 214 with a PDI probe 254. The combination of such rigid extended backbone and the charged head was reflected in a poorly soluble compound both in apolar and polar media. As a consequence, the investigation of 254 in LUVs did not provide any results. The most probable explanation is related with the precipitation in aqueous media of the amphiphiles because of $\pi$-$\pi$ stacking among PDI cores and consequent lack of partition in the membrane bilayers neither in gel nor in liquid phase. The PDI probe has shown much higher solubility problems in aqueous media compared to the oligothiophene analogues. Moreover the results obtained with oligothiophenes in terms of sensitivity strongly suggested to focus further efforts on this direction rather than on rigid PDIs.
3.5.2. pH Dependence

The interest in the development of such compact panchromatic probes was directed to explore whether lessons from nature could be transferred to well-known aromatic scaffolds. Moreover, the insertion of acidity sensitive moieties could trigger potential applications of these molecules as biological probes.

Intracellular pH is related to many critical processes in cells and, despite common sense, can reach very acid values (4.5-5.5) in particular organelles like endosomes and plant vacuoles. Such low values are fundamental for processes like protein denaturation or activation of particular enzymes or proteins functions which would be too slow at physiological pH. Clear and reproducible modulation of the photophysical properties of our aromatic diimides 244-246 (Figure 107) as a function of pH could be useful for fluorescent visualization of such cellular processes.

Figure 107. Molecular structures of panchromatic probes.

The absorption spectra of 244 in DMSO/water in acid pH showed a maximum at 470 nm comparable with the reported ethoxy NDI analogue. A spectacular biphasic bathochromic shift (Figure 108), able to cover all the primary colors, was observed increasing the pH. At neutral pH the compound turned red and finally in basic condition a bright blue color appeared from.
More complicated was the visualization of the slight shifts occurred from the yellow 245, to the pale orange mono deprotonated 257 further to the pink di anion 258 to the final violet 259 proper of the putative triple anionic NDI.

Similar shift to 244 but in the lower energies region was observed for the PDI 246 from an initial absorption at 570 nm passing through a greenish 260 to the final colorless solution at 760 nm of 261. Clearly these pronounced and colorful shifts were related to the deprotonation of the hydroxy groups and the consequent increased of the electron-donor efficiency of the substituents.
To quantify the acidity ($pK_a$) of our compounds the absorption maxima ($\lambda_{\text{abs}}$) data as a function of measured pH (Figure 109) were fitted with the Hill equation\(^ {271}\) (Equation 3.31)

$$
\lambda_{\text{abs}} = \lambda_{\text{AH}_m} + \frac{\left(\lambda_{\text{AH}_m} - \lambda_{\text{AH}_{m-1}}\right)}{1 + \left(\frac{pK_a}{p\text{H}}\right)^n}
$$

(31)

where $\lambda_{\text{AH}_m}$ and $\lambda_{\text{AH}_{m-1}}$ are the absorbance wavelength of the species involved in the deprotonation equilibrium and $n$ is the Hill coefficient.
As a control the pKₐ values were also determined by the deconvolution of the spectra of these chromophores by HypSpec analysis obtaining comparable results (Table 15). Nevertheless the deconvolution method did not provide good results for the data of 245 probably because of the wide overlap among the traces.

Table 15. Absorbance properties of cNDIs and cPDI

<table>
<thead>
<tr>
<th>Cpd</th>
<th>⬠abs (nm)</th>
<th>(\varepsilon) (M⁻¹ cm⁻¹)</th>
<th>pKₐ (HypSpec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>244</td>
<td>470</td>
<td>12000</td>
<td>6.3 (7.2)</td>
</tr>
<tr>
<td>255</td>
<td>565⁵⁺</td>
<td>10200⁵⁺</td>
<td>11.1 (11.5)</td>
</tr>
<tr>
<td>256</td>
<td>615</td>
<td>15200</td>
<td>-</td>
</tr>
<tr>
<td>245</td>
<td>490</td>
<td>4200</td>
<td>3.7</td>
</tr>
<tr>
<td>257</td>
<td>513⁶⁺</td>
<td>4000⁶⁺</td>
<td>9.3</td>
</tr>
<tr>
<td>258</td>
<td>570⁶⁺</td>
<td>3900⁶⁺</td>
<td>12.8</td>
</tr>
<tr>
<td>259</td>
<td>640⁶⁺</td>
<td>6900⁶⁺</td>
<td>-</td>
</tr>
<tr>
<td>246</td>
<td>570</td>
<td>6600</td>
<td>7.3 (8.0)</td>
</tr>
<tr>
<td>260</td>
<td>708⁵⁺</td>
<td>11500⁵⁺</td>
<td>10.4 (10.6)</td>
</tr>
<tr>
<td>261</td>
<td>760</td>
<td>20400</td>
<td>-</td>
</tr>
</tbody>
</table>

* Absorption maxima measured in DMSO/water 4:1 at appropriate pH.  
* Extinction coefficient.  
* pKa values in DMSO/water 4:1.  
* computed approximation from HypSpec Analysis.  
* putative value without further analysis.

The strong acidity (pKₐ 6.3) of the hydroxy group of 244 originated probably by the resonance stabilization of the conjugate base (CB) due to the electron-withdrawing imides while the weak acidity of the second hydroxy group (pKₐ 11.1) could be justified by the effect of the intramolecular charge repulsion. Much more complex was the evaluation of the shifts depicted in the titration of 245. Nevertheless the strong acidity (pKₐ 3.7) of the catechol appeared reasonable considering the stabilization by resonance like in 244 together with the hydrogen bond stabilization. The second deprotonation (pKₐ 9.3) occurred at lower pH compared to 244 which suggested that the
intramolecular hydrogen bond probably accounted for this higher acidity. The next hydrogen was lost in strong basic condition ($pK_a$ 12.8) meaning that the proximity effect between the charges destabilized the conjugate base. The further deprotonation to the tetra-anionic derivative was not observed neither with NaH and BuLi in organic media. A comparison with similar reported molecules pointed out the strong acid character of 245. The first deprotonation of this probe occurred with a remarkably lower $pK_a$ value compared with different substituted analogues (e.g. tetrachloro and tetrafluoro catechols) and showed similar acidity to the dicyano and tertcyano catechols$^{274,275}$. More interesting could be the investigation of 245 as a fluorescent siderophore mimic. Siderophores are low molecular weight molecules synthesized by micro-organisms and are able to chelate with high specificity ferric ions and to release them inside the cells. Understanding the siderophores chemistry in terms of iron uptake, kinetics and complex stability is crucial for the clarification of some diseases related with bacteria or iron-overload$^{276-278}$.

Compared with 244 the PDI first deprotonation ($pK_a$ 7.3) suggested a less efficient resonance stabilization of CB by the electron-withdrawing imides probably due to the less flat aromatic system. The second hydroxy group behaved as a slight stronger acid ($pK_a$ 10.4) compared to 244 probably because of the lower charge repulsion due to the larger $\pi$-system. The resulting near-infrared absorption maximum at 760 nm, to the best of our knowledge, was unprecedented in the PDIs series$^{253}$. Afterwards we investigated the fluorescence properties (Figure 110) of these compounds and we found that the emission ($\lambda_{em} = 500$ nm) of the fully protonated 244 was comparable with the alkoxy compound$^{149}$ but the quantum yield ($\Phi_f = 1\%$) and the fluorescence lifetime ($\tau = 0.1$ ns) were much lower than the analogue ($\Phi_f = 22\%$). Nevertheless a second comparable emission peak at lower energy ($\lambda_{em} = 630$ nm) was observed in not anhydrous solvents. The excited state responsible for the red-shifted emission was populated from
the one related with higher-energy emission maximum and decayed with a longer lifetime. This process is normally referred as an excited state intramolecular proton transfer (ESIPT)\textsuperscript{279-281} probably mediated by water molecules and the red-shifted emission derived from the tautomer of 244 originated upon excitation.

A similar result with higher relative intensity of red-shifted emission peak was observed for 245 but not with PDI 246 which is justified considering that the longer distance between the imide moieties and the hydroxy substituient prevented any intramolecular proton transfer. Similar phenomena but justified as a twisted intramolecular charge transfer (TICT) were already observed for NDI based molecules\textsuperscript{282}.

The definitive assignation of the emission peaks of the mono anions 255 and 260 was problematic because of the presence in the excitation spectra of multiple species. As for the absorption spectra, the fluorescence of the deprotonated species from 245 was complicated to solve unambiguously.

Figure 110. Normalized absorption (solid) and emission (dashed) of 244 (yellow, pH 3) and 256 (blue, pH 13).

Unambiguous emission peaks were measured in strong basic condition (pH > 12) for the dianion 256 and 261. In the case of the NDI based fluorophore 256 the quantum yield remarkably increased (\(\Phi_f = 64\%\)) as compared to the
neutral 244 while the quantum yield for the PDI dianion 261 ($\phi_f = 2\%$) was similar to the one of 246. These trends indicated that the ESIPT process was responsible for the poor quantum yield and the short fluorescence lifetime of the neutral NDIs 244 and 245. The sensitivity of these probes to the pH spreads over almost all the visible range to NIR frontier. It must be pointed out that no investigation on the toxicity in cells was performed with these fluorophores. The goal of such investigation was to demonstrate the ability of this compact dyes to generate a colorful pattern to apply in biological systems and as indicator for differential chemosensing283.

Table 16. Fluorescence properties of cNDIs and cPDI

<table>
<thead>
<tr>
<th>Cpd</th>
<th>$\lambda_{em}$ (nm)</th>
<th>$\tau$ (ns)</th>
<th>$\phi_f$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>244</td>
<td>500, 630</td>
<td>0.1, 1.1</td>
<td>1</td>
</tr>
<tr>
<td>255</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>256</td>
<td>661</td>
<td>12.7</td>
<td>64</td>
</tr>
<tr>
<td>245</td>
<td>520, 620</td>
<td>0.8, 1.9</td>
<td>6</td>
</tr>
<tr>
<td>257</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>258</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>259</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>246</td>
<td>600</td>
<td>1.1</td>
<td>6</td>
</tr>
<tr>
<td>260</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>261</td>
<td>820</td>
<td>0.9</td>
<td>2</td>
</tr>
</tbody>
</table>

$^a$ Emission maxima measured in DMSO/water 4:1 at appropriate pH. $^b$ Fluorescence lifetime. $^c$ Fluorescence quantum yield.

3.5.3. Basicity of Solvents

The acid-base titrations have clearly evidenced the ability of our molecules to discriminate the pH over the whole range in aqueous media. In the same time we checked whether this remarked sensitivity could be useful for the discrimination among solvents or not (Figure 111).
As expected, the absorption maxima did not show any clear trend as a function of the Lippert solvent polarizability due to the overall absence of a molecular dipole moment due to the symmetry of the push-pull architecture. On the other hand a linear correlation was found considering the Lewis basicity as a discriminant parameter. This sensitivity toward the solvent basicity found a practical application in the detection of the DMF purity. In normal condition DMF is a colorless and odorless solvent but can slowly hydrolyze to formic acid and dimethylamine which is the typical smell of “old DMF”.

Table 17. Solvent polarizability, basicity and absorption maxima for cNDIs and cPDI

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$\Delta_{L}$ $^a$</th>
<th>$\Delta H_{BF3}^{BF3}$ ($KJ mol^{-1}$)</th>
<th>244 $\lambda_{abs}$ (nm)</th>
<th>245 $\lambda_{abs}$ (nm)</th>
<th>246 $\lambda_{abs}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>0.285</td>
<td>76.03</td>
<td>570</td>
<td>560</td>
<td>686</td>
</tr>
<tr>
<td>ACN</td>
<td>0.305</td>
<td>60.39</td>
<td>555</td>
<td>534</td>
<td>575</td>
</tr>
<tr>
<td>DCM</td>
<td>0.217</td>
<td>10.00</td>
<td>470</td>
<td>485</td>
<td>-</td>
</tr>
<tr>
<td>DMF</td>
<td>0.276</td>
<td>110.49</td>
<td>599</td>
<td>583</td>
<td>760</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.264</td>
<td>105.34</td>
<td>599</td>
<td>583</td>
<td>760</td>
</tr>
<tr>
<td>Dioxane</td>
<td>0.021</td>
<td>74.09</td>
<td>563</td>
<td>539</td>
<td>-</td>
</tr>
<tr>
<td>Ethyl</td>
<td>0.200</td>
<td>75.55</td>
<td>569</td>
<td>558</td>
<td>-</td>
</tr>
<tr>
<td>THF</td>
<td>0.209</td>
<td>90.40</td>
<td>576</td>
<td>566</td>
<td>707</td>
</tr>
</tbody>
</table>

$^a$ Lippert orientation polarizability according to the Equation 3.14. $^b$ Solvent basicity according to the $\Delta H_{0}^{BF3}$ scale$^{284}$. 

Figure 111. Absorption maxima of 244 (blue), 245 (red) and 246 (green) as function of (A) the Lippert polarity function and (B) the solvent Lewis basicity.
To prove the ability of 244 to discriminate the different basicity of DMF, we added the NDI probe in micromolar concentration to different DMF fractions coming from the distillation of solvent (Figure 112A). Dimethylamine has a boiling point much lower than DMF and should be concentrated in the first fractions giving to the solvent a more basic character. The solutions color shifted from purple to more orange-reddish color moving from the first to the last collected fractions.

Figure 112. Absorption spectra (A) of 244 (28 μM) in distilled DMF fractions: first fraction (full blue dots), last fraction (empty orange dots), commercial DMF (full black squares). Fractional absorption (B) $A_{538}/A_{610}$ of 244 in DMF fractions (black dots) compared to commercial DMF (dashed line).

A ratiometric evaluation of the two main absorption peaks at 548 and 610 nm allowed a quantitative analysis of the basicity of the fractions (Figure 112B). The commercial DMF has a $A_{548}/A_{610} = 1.77$ while first fractions of distillation are much more basic ($A_{548}/A_{610} < 1.00$). Overcoming the ratio value of 3.00 the fractions become odorless and DMF of constant purity was obtained in the last 5 fractions with a ratio value higher than 5. The results of the investigation in organic solvents have proved the sensitivity of these compounds in non-protic media. Moreover the DMF test has shown that micromolar concentration of the probe was able to discriminate among small
differences in basicity allowing potential applications of these compounds also in non-aqueous environments.

3.5.3. Boronic Esters as Core Substituents

The precedent paragraphs have shown the ability of NDIs and PDI probes to modulate their photophysical properties in response to the environmental changes. Nevertheless we wondered if our probes could be use also as a reactive building block in multicomponent architectures and in the functionalization of substrates. The catechol skeleton of 245 suggested that this molecule could be employed for the formation of boronic esters by coupling with the phenyl boronic acid (PBA). Despite the optimal conditions for the formation and the stability of the boronic esters are related to many parameters (e.g. pH, pK_a of PBA derivatives, pK_a of diols, solvent) and are still not unambiguously clarified (Scheme 8), the boronic acids were employed as biosensor for sugars, protein binding and carbohydrate transporters.

![Scheme 8](image)

Scheme 8. Schematic representation of the equilibria involved in the boronic ester formation starting from boronic acid and diol. K_{eq} (trig) and K_{eq} (tet) are the equilibrium constants of trigonal and tetrahedral form of boronic acid. Revised graphical representation from reference.
The pH range for the formation of the boronic esters with our compounds was estimated based on the pKₐ values of PBA and of our probes following the Equation 32:\(^\text{(32)}\)

\[
pH = \frac{pK_a(PBA) + pK_a(diol)}{2}
\]

The equation 32 does not consider the parameters such as steric effect, solvent, buffer, hydrogen bond which means that the values obtained can be considered just an estimation of the optimal pH. Therefore we have performed the titration with PBA at pH 8.00 following the reaction by changes in the absorption spectra. pH 8.00 was chosen as a compromise between the calculated optimal pH values (7.55 for \(244\), 6.25 for \(245\) and 8.01 for \(246\)) and the constant \(\lambda_{max}\) observed for our molecules in nearby pH range (Figure 113).

**Figure 113.** Absorption spectra of \(244\) (A) and \(245\) (B) during titration with PBA at pH 8.00 in DMSO/water 4:1.

A weak red shift \((\Delta \lambda_{abs} = 14 \text{ nm})\) was observed for \(245\) when increasing the PBA concentration (figure 88B) while the PDI \(246\), as expected, was totally insensitive to the boronic acid confirming the inability of \(246\) to form boronic esters. A different shift was observed for the hydroquinone \(244\) which was showing a quite remarkable hypsochromic shift \((\Delta \lambda_{abs} = -24 \text{ nm})\) in response to the PBA concentration.

153
Considering the initial absorption maxima of the hydroxy molecules ($\lambda_{\text{abs}}^D$), the absorption of the new species ($\lambda_{\text{abs}}^E$) and the concentration of PBA ($c_{\text{PBA}}$), the Hill analysis\(^{271}\) (Equation 33) of the experimental dose response curves (Figure 114) allowed the quantification of the dissociation constant ($K_D$) for the new species as a proof of the formation of the boronic esters.

\[
\lambda_{\text{abs}} = \lambda_{\text{abs}}^D + \frac{(\lambda_{\text{abs}}^E - \lambda_{\text{abs}}^D)}{1 + (K_D/c_{\text{PBA}})}
\]  

(33)

Figure 114. Molecular structures of the boronic ester 262 and the putative analogue 263. Absorption spectra of 244 (blue, solid) and 245 (red, dashed) and 246 (green, dotted) as function of the PBA concentration at pH 8.00 in DMSO/water 4:1.

The conversion of catechol NDI 245 into the boronic ester 262 resulted by a low dissociation constant ($K_D = 3.7 \pm 0.1 \text{ mM}$) which was in agreement with the reported data for similar compounds\(^{290-292}\). Much higher value ($K_D = 21.8 \pm$
2.3 mM) was extrapolated for the reaction of 244 with PBA. A possible explanation to the latter value might be related to the intramolecular reaction between the boronic semi-ester with the proximal carbonyl group of the imide to form 263.

The compound 262 was further subjected to a pH titration (Figure 115) to find other evidences of the presence of the boronic ester. In acid condition (\( \lambda_{abs} = 486 \) nm) was barely distinguishable from tetrahydroxy 245 while the first deprotonation (pK\(_a\) = 4.7) of the hydrated boronic ester to 264 occurred at more basic pH and was comparable with data reported in literature\(^{149}\). This shift in acidity for the first equilibrium compared to 245 was consistent with the lack of the resonance with the NDI core. The second deprotonation (pK\(_a\) = 9.4) was hampered by the charge repulsion\(^{273}\) and shifted the absorption of 265 (\( \lambda_{abs} = 544 \) nm), 24 nm blue-shifted in comparison with the putative dianion 258. Further increase of the pH was depicted by a remarkable shift in the absorption to the same value of 259 probably due to a rapid hydrolysis of the boronic ester.

![Figure 115. Absorption spectra shift (A) of 262 with increasing pH from 2 (yellow) to 12 (blue) in DMSO/water 4:1. Absorption maxima of 245 (red, dashed), 262 (orange, solid) as function of pH.](image)

The final qualitative proof of the formation of the boronic esters was found in the emission spectra. Different from 245, in acidic condition the emission of
262 showed a single maxima ($\lambda_{em} = 510$ nm) and no traces of the emission related with ESIPT (Figure 116).

Scheme 9. Schematic representation of acid/base equilibria for the boronic ester 262.

The emission spectra were observed in the pH range 3-11 but further investigations were not performed because the broad nature of the signals indicated the presence, in the same conditions, of several absorbing and emitting species. Nevertheless these probes have proved to be not only colorful indicators in solvents and in aqueous media but also to be reactive building blocks able keep their sensitivity and to modulate their photophysical properties upon remarkable modifications.

Figure 116. Normalized absorption (solid) and emission (dashed) spectra of 262 (yellow, pH 3) and absorption spectra of 264 (red, pH 7) and 265 (purple, pH 11) in DMSO/water 4:1.
3.5.5. Oxidative Imination

The oxidation-reduction enzymatic processes are the base of the cellular life and take place on a variety of different substrates such as sugars\textsuperscript{293}, lipid and hormones\textsuperscript{294}, amino acids\textsuperscript{295} and hydroquinones\textsuperscript{296}. Inspired by this huge class of substrates we investigated whether our molecules could be subjected to the oxidative processes keeping their colorful properties or not. In the same time we wondered till which point we could shift the photophysical properties of our probes by a simple modification like the aromatic core extension. The combination of these two factors prompted us to investigate the oxidative imination reaction starting from the hydroquinone 244 (Scheme 10).

Scheme 10. Schematic representation of the oxidation of 244 and \textit{in-situ} covalent capture of quinone with arylamines.

The green aromatic imines 266-271 were synthetized by the aerobic oxidation catalyzed by CAN/TEMPO\textsuperscript{297} followed by the \textit{in situ} covalent capture of the intermediate quinone with the arylamines. The reaction was followed by the absorption spectroscopy quenching aliquots of the oxidative mixtures with same amount of the amine and assuming a full conversion at constant intensity of the absorption maxima. Subsequent protonation (Figure 117) of the imines provided the reddish iminium analogues 272-277.

The quenching with aniline located the absorption of 266 ($\lambda_{\text{abs}} = 638$ nm) just 4 nm below the reported one for the tetra-amino NDI\textsuperscript{149}. The presence of
EDG in ortho or in para-position respectively in 267,268 and 270,271 did not shift the absorption spectra while the presence of the electron-withdrawing nitro group in meta-position of 269 was reflected by slight blue shift of 11 nm. The weak sensitivity to the presence of different substituents suggested a poor conjugation of the aryl moieties with the NDI core because of steric reasons.

Figure 117. Absorption spectra shift of 266 (A) with decreasing pH from 12 (blue) to 4 (red) in DMSO/water 4:1. Absorption maxima of 266 (green, solid), 267 (cyan, dashed), 268 (purple, dotted), 269 (blue, solid), 270 (magenta, dashed) and 271 (red, solid) as a function of pH.

pH titrations monitored by absorbance spectra were performed to further prove the formation of the aromatic imines, the p\(K_a\) values evaluated from the Equation 31 were in agreement with the reported data for similar aromatic compounds.

Table 18. Aromatic Imines substituents and extrapolated p\(K_a\) values

<table>
<thead>
<tr>
<th>Cpd</th>
<th>(\lambda_{abs}) (nm)</th>
<th>R(_1)</th>
<th>R(_2)</th>
<th>R(_3)</th>
<th>Cpd-H(^{+})</th>
<th>(\lambda_{abs}) (nm)</th>
<th>p(K_a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>266</td>
<td>638</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>272</td>
<td>563</td>
<td>5.9</td>
</tr>
<tr>
<td>267</td>
<td>638</td>
<td>H</td>
<td>H</td>
<td>OMe</td>
<td>273</td>
<td>561</td>
<td>6.0</td>
</tr>
<tr>
<td>268</td>
<td>638</td>
<td>H</td>
<td>H</td>
<td>NMe(_2)</td>
<td>274</td>
<td>565</td>
<td>5.9</td>
</tr>
<tr>
<td>269</td>
<td>627</td>
<td>H</td>
<td>NO(_2)</td>
<td>H</td>
<td>275</td>
<td>560</td>
<td>6.2</td>
</tr>
<tr>
<td>270</td>
<td>638</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>276</td>
<td>528</td>
<td>6.8</td>
</tr>
<tr>
<td>271</td>
<td>638</td>
<td>OMe</td>
<td>H</td>
<td>H</td>
<td>277</td>
<td>530</td>
<td>5.8</td>
</tr>
</tbody>
</table>
The protonation of the first imine was depicted by a remarkable ipsochromic shift of the absorption spectra for all the compounds. The common main broad peak in the higher energy region was related with the at least partial conversion of the electron-donor imine to the electron-acceptor iminium group.

The unsubstituted together with meta and para-substituted iminium NDIs 272-275 absorbed in a range 80 nm blue-shifted compared to the corresponding imines and showed comparable pKₐ values (5.9 < pKₐ < 6.2). A more pronounced shift (~110 nm) was observed for ortho-substituted aryl iminium ions 276 and 277 probably induced by the competing intramolecular hydrogen bonding with the vicinal substituent. In the same time the hybrid character between the weak acid phenol and the highly acidic iminium proton was reflected by a lower acidity of the NDI iminium 276 (pKₐ = 6.8). The replacement of the hydroxy group with a methoxy in 277 restored a comparable acidity with other iminium derivatives.

The oxidation and the subsequent imines formation has demonstrated, together with the formation of the boronic esters, the possibility to handle and easily functionalized these probes. Although we did not really performed these modifications for a defined target application, the above discussed results have proved how the dynamic covalent chemistry realized with these molecules could be employed to build up multicomponent systems and develop functional building blocks, sensors or probes for different applications moving for chemical biology to supramolecular chemistry.

3.6. Complete Collection of Absorption and Emission Spectra

In this paragraph the experimental complementary spectra discussed in the chapter are reported to facilitate a fast comparison with the graphs reported in the main text. The paragraph is organized following the chapter discussion. The detailed experimental procedures and conditions are reported in the chapter 5.
3.6.1. Solvatochromism

**Figure 118.** Normalized absorption (solid) and emission spectra (dotted) of 211 in, with increasingly red-shifted emission, ethyl acetate (yellow), THF (orange), dioxane (red), DCM (magenta), toluene (purple), DMSO (blue), DMF (cyan), acetone (dark green) and diethyl ether (light green).

**Figure 119.** Normalized absorption (solid) and emission spectra (dotted) of 212 in, with increasingly red-shifted emission, THF (yellow), acetone (orange), dioxane (red), toluene (magenta), diethyl ether (purple), DMSO (blue), ethyl acetate (cyan), DMF (dark green) and DCM (light green).
**Figure 120.** Normalized absorption (solid) and emission spectra (dotted) of 214 in, with increasingly red-shifted emission, ethyl acetate (yellow), toluene (orange), DCM (red), THF (magenta), acetone (purple), ACN (blue), TFE (cyan), DMF (dark green) and DMSO (light green).

**Figure 121.** Normalized absorption (solid) and emission spectra (dotted) of 215 in, with increasingly red-shifted emission, ethyl acetate (yellow), THF (orange), TFE (red), acetone (magenta), chloroform (purple), DMF (blue), DMSO (cyan), toluene (dark green) and ACN (light green).
Figure 122. Normalized absorption (solid) and emission spectra (dotted) of 216 in, with increasingly red-shifted emission, hexane (yellow), toluene (orange), ethyl acetate (red), THF (magenta), chloroform (purple), acetone (blue), DMSO (cyan), DMF (dark green) and ACN (light green).

Figure 123. Normalized absorption (solid) and emission spectra (dotted) of 217 in, with increasingly red-shifted emission, hexane (yellow), toluene (orange), diethyl ether (red), ethyl acetate (magenta), THF (purple), chloroform (blue), acetone (cyan), ACN (dark green) and DMF (light green).
Figure 124. Normalized absorption (solid) and emission spectra (dotted) of 218 in, with increasingly red-shifted emission, hexane (yellow), diethyl ether (orange), dioxane (red), ethyl acetate (magenta), acetone (purple), DCM (blue) and DMSO (dark green).

Figure 125. Normalized absorption (solid) and emission spectra (dotted) of 219 in, with increasingly red-shifted emission, hexane (yellow), diethyl ether (orange), dioxane (red), ethyl acetate (magenta), DCM (purple), acetone (blue) and DMSO (dark green).
3.6.2. Membrane Fluidity

Figure 126. Normalized emission spectra of 212 (8.1 µM, λex = 430 nm) in DOPC (A) and DPPC vesicles (B) at 25 °C (dashed, grey), heated at 55 °C (solid, gold), cooled down to 25 °C (solid, blue), heated to 55 °C (dotted, gold) and cooled to 25 °C (dotted, cyan).

Figure 127. Normalized excitation spectra of 212 (8.1 µM, λem = 600 nm) in DOPC (A) and DPPC vesicles (B) at 25 °C (dashed, grey), heated at 55 °C (solid, gold), cooled down to 25 °C (solid, blue), heated to 55 °C (dotted, gold) and cooled to 25 °C (dotted, cyan).
Figure 128. Normalized emission spectra of 214 (8.3 μM, λ_{ex} = 380 nm) in DOPC (A) and DPPC vesicles (B) at 25 °C (dashed, grey), heated at 55 °C (solid, gold), cooled down to 25 °C (solid, blue), heated to 55 °C (dotted, gold) and cooled to 25 °C (dotted, cyan).

Figure 129. Normalized excitation spectra of 214 (8.3 μM, λ_{em} = 530 nm) in DOPC (A) and DPPC vesicles (B) at 25 °C (dashed, grey), heated at 55 °C (solid, gold), cooled down to 25 °C (solid, blue), heated to 55 °C (dotted, gold) and cooled to 25 °C (dotted, cyan).
Figure 130. Normalized emission spectra of 215 (8.1 μM, λ_{ex} = 380 nm) in DOPC (A) and DPPC vesicles (B) at 25 °C (dashed, grey), heated at 55 °C (solid, gold), cooled down to 25 °C (solid, blue), heated to 55 °C (dotted, gold) and cooled to 25 °C (dotted, cyan).

Figure 131. Normalized excitation spectra of 215 (8.1 μM, λ_{em} = 530 nm) in DOPC (A) and DPPC vesicles (B) at 25 °C (dashed, grey), heated at 55 °C (solid, gold), cooled down to 25 °C (solid, blue), heated to 55 °C (dotted, gold) and cooled to 25 °C (dotted, cyan).
Figure 132. Normalized emission spectra of 218 (8.2 μM, $\lambda_{ex} = 430$ nm) in DOPC (A) and DPPC vesicles (B) at 25 °C (dashed, grey), heated at 55 °C (solid, gold), cooled down to 25 °C (solid, blue), heated to 55 °C (dotted, gold) and cooled to 25 °C (dotted, cyan).

Figure 133. Normalized excitation spectra of 218 (8.2 μM, $\lambda_{em} = 570$ nm) in DOPC (A) and DPPC vesicles (B) at 25 °C (dashed, grey), heated at 55 °C (solid, gold), cooled down to 25 °C (solid, blue), heated to 55 °C (dotted, gold) and cooled to 25 °C (dotted, cyan).
Figure 134. Normalized emission spectra of 219 (8.1 μM, λ_{ex} = 430 nm) in DOPC (A) and DPPC vesicles (B) at 25 °C (dashed, grey), heated at 55 °C (solid, gold), cooled down to 25 °C (solid, blue), heated to 55 °C (dotted, gold) and cooled to 25 °C (dotted, cyan).

Figure 135. Normalized excitation spectra of 219 (8.1 μM, λ_{em} = 570 nm) in DOPC (A) and DPPC vesicles (B) at 25 °C (dashed, grey), heated at 55 °C (solid, gold), cooled down to 25 °C (solid, blue), heated to 55 °C (dotted, gold) and cooled to 25 °C (dotted, cyan).
3.6.3. Membrane Potential

Figure 136. Normalized emission spectra of 214 (A, 8.3 μM, $\lambda_{ex} = 380$ nm) and 215 (B, 8.1 μM, $\lambda_{ex} = 380$ nm) in EYPC vesicles at 0 (solid, gold) and -180 mV (dashed, blue).

Figure 137. Normalized excitation spectra of 214 (A, 8.3 μM, $\lambda_{em} = 530$ nm) and 215 (B, 8.1 μM, $\lambda_{em} = 530$ nm) in EYPC vesicles at 0 (solid, gold) and -180 mV (dashed, blue).
Figure 138. Normalized emission spectra of 218 (A, 8.2 μM, λ<sub>ex</sub> = 430 nm) and 219 (B, 8.1 μM, λ<sub>ex</sub> = 430 nm) in EYPC vesicles at 0 (solid, gold) and -180 mV (dashed, blue).

Figure 139. Normalized excitation spectra of 218 (A, 8.2 μM, λ<sub>em</sub> = 570 nm) and 219 (B, 8.1 μM, λ<sub>em</sub> = 570 nm) in EYPC vesicles at 0 (solid, gold) and -180 mV (dashed, blue).
3.6.4. pH Dependence

**Figure 140.** Absorption spectra shift of 245 with increasing pH from 3 (yellow) to 12 (blue) in DMSO/water 4:1

**Figure 141.** Absorption spectra shift of 246 with increasing pH from 3 (red) to 12 (green) in DMSO/water 4:1
3.6.5. Deconvoluted Spectra

Figure 142. Deconvoluted HypSpec spectra of 244, 255 and 256 in DMSO/water 4:1 from experimental data

Figure 143. Deconvoluted HypSpec spectra of 246, 260 and 261 in DMSO/water 4:1 from experimental data
3.6.6. Emission Spectra

**Figure 144.** Normalized absorption (solid) and emission (dashed) spectra of 245 (yellow, pH 2) and absorption spectra of 258 (magenta, pH 9) and 259 (blue, pH 14) in DMSO/water 4:1.

**Figure 145.** Normalized absorption (solid) and emission (dashed) spectra of 246 (red, pH 5) and 261 (green, pH 12) in DMSO/water 4:1.
3.6.7. Spectra of Imines

**Figure 146.** Absorption spectra shift of 267 (A), 268 (B), 269 (C), 270 (D) and 271 (E) with decreasing pH from 12 (blue) to 4 (red) in DMSO/water 4:1
CHAPTER 4

PERSPECTIVES

4.1. General

The intriguing results obtained with the oligothiophene probes focus the attention of this chapter on the possible modifications of these probes and will be discussed in details. On the other hand, the NDI and PDI chromophores were investigated in detail and the possible next steps could be focused on the evaluation of the toxicity in living systems and on the improvement of the solubility in aqueous media.

The insertion of linear hydrocarbon chains at one imide side of PDI 278 and NDI 279 with a concomitant presence of a hydrophilic moiety on the other side of the probes should provide amphiphilicity to the fluorophores. A multiple charged head would be ideal to assure solubility in aqueous media to the more hydrophobic scaffold of 278. In parallel, the location of 279 in the membrane bilayers should provide a probe able to discriminate the pH processes that occurs in the proximity of the membranes. Moreover, dynamic functionalization(s) on the imide(s) 280 and 281 should allow the connections (Figure 147), without perturbing the photophysical properties, between fluorophores and many biological substrates like lipids, proteins, amino acids, antibodies allowing the visualization of the pH dependent biological processes. In any case, the toxicity investigation is a key point which needs to be filled before any further functionalization and application in biological systems.
4.2. Planarizable Push-Pull Oligothiophenes

As described in the chapter 3 many parameters play a role in the sensitivity of these probes to membranes viscosity and transmembrane potential. The following discussion is based on the described results in LUVs which have shown a better discrimination with the cyanovinyl acceptor as compared to the hydrazine moieties.

As a consequence the number of the possible modifications is endless and to really gain sensitivity they should be justified in order to optimize the balance between the synthetic efforts and the potential results. For this reason some possible modifications are described with the potential improvements in terms of sensitivity.
4.2.1. Length

The transmembrane potential results have shown a complete loss in sensitivity when moving from the tetramers to the trimer molecules. These results suggest that the length of the probes is an important factor to consider and investigate towards sensitivity improvements based on this phenomenon. Longer analogue compounds like 282-284 (Figure 148) could clarify if this loss in the responsiveness was related strictly with the weaker electronical properties of the probes or if the overall length is really a key factor in the detection of $\Delta \psi$.

![Figure 148. Schematic representations of the longer backbone analogues of the investigated probes](image)

Contrary the insertion of one or more thiophene units should not improve the sensitivity towards the membranes viscosity but it could decrease the observed shifts in the excitation spectra because of the more pronounced planarity gained by a longer $\pi$-system\textsuperscript{55}. A comparison between the present results and the response given by 282 could support or drop further extensions of the thiophene system and move the attention to other modifications. In general, the investigation of longer analogues is not expected to increase the
sensitivity of the fluorescence spectra because of the pronounced flat character of the excited state.

4.2.2. β-Substituents

The investigation in vesicles of our probes clearly showed, both in the fluidity and in the transmembrane cases, that the twisted ground state is more sensitive to the environmental changes as compared to the more planar excited state. Moreover the slight increases of the fluorescence intensities of 211 and 212 in the transmembrane potential experiment could be related with a not perfect planarization of the $S_1$ in membranes. As a consequence it appears evident that disturbing the planarization in the excited state could be reflected by changes of fluorescence quantum yield and probably also by emission shifts.

![Schematic representations of different β-substituted analogues of tested probes.](image)

Figure 149. Schematic representations of different β-substituted analogues of tested probes.

Analogues 285-287 of the tested probes with substituents in different positions or increased number of β-substituents or finally with bulkier groups will force the molecules in a more deplanarized conformation\(^5\) (Figure 149). A more drastic approach would change the overall structure of the probes to a more rigid molecule 288 with only one possible deplanarization center. Such molecule in which the donor and the acceptor groups are located on different
emi-bridges, would work like an on-off system. In the flat conformation the overall conjugation is double as compared to the fully deplanarized one and also the interaction between the donor and acceptor moieties will be completely different in the two cases.

A more deplanarized ground state would be reflected by larger shifts of the excitation spectra upon the induced planarization by the membrane phase or by the dipole interaction with the membrane potential. In the same time a more twisted excited state in the liquid phase or without $\Delta \psi$ could respond with lower fluorescence intensity and/or ipsochromic shifts of the emission peak. As for the $\pi$-system length also here a comparison between the known results and the one from 285 and 286 will clearly demonstrate the role and the importance of the deplanarization in the detection of the membrane fluidity and potential.

4.2.3. Dipole Moment

The molecular dipole moment seems to play a key role in the bilayer membrane potentials discrimination while no remarkable effects of the electronic properties are present in the visualization of membranes fluidity. Based on this observation stronger dipole moments could provide a better shift in the excitation spectra and more pronounced fluorescence intensity changes related to the increased electric potential. The EWG screening has already shown that the cyanovinyl moiety is a strong and stable electron acceptor in this system and provided better results as compared to other withdrawing groups.
Similar to the investigation done for the EWG, a screening of similar probes bearing different donor moieties (Figure 150) could provide interesting results in terms of electronic properties. The presence of an EDOT unit as in 289 or an amine like in 290 and 291 as EDG will increase the strength of the push-pull systems and probably give a better sensitivity. Nevertheless, the synthesis of the amino oligothiophenes could be problematic because of the low stability of some intermediates while EDOT analogues as 289 are possible to synthesize in a straightforward manner. Based on the results of this new compound, it would be easier to evaluate the effect of the donor groups on the responsiveness of the probes in membranes.

These could be the guidelines to improve the discrimination among membrane phases and the transmembrane potentials. The three ways were described separately but they should not be considered independent. The sensitivity complicated systems like the membrane bilayers certainly comes from the contribution of all these three aspects and maybe others that are not clear at this time. The results described in this work and the straightforward ways for the synthesis of many analogues clearly support the investigation of this class of compounds as membrane probes.
CHAPTER 5

EXPERIMENTAL SECTION

5.1. General

5.1.1. Reagents, Solvents and Equipment

Reagents for synthesis, if not otherwise specified, were purchased from Aldrich, Fluka and Acros. Amino acid derivatives and HBTU (O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate were purchased from Novabiochem. Thiophene, NIS (N-Iodosuccinimide) and CsF were from Apollo Scientific. Egg yolk phosphatidylcholine (EYPC), 1,2-Dioleoyl-sn-glycerol-3-phosphatidylcholine (DOPC) and 1,2-Dipalmitoyl-sn-glycerol-3-phosphatidylcholine (DPPC) were purchased from Avanti Polar Lipids. Acid, base, were purchased from Aldrich and Fluka. Buffers, salts, Rhodamine 6G, IR-140, valinomycin and mellitin of best grade available were purchased from Fluka or Aldrich and used as received.

Dry TEA and DMF used in synthesis were distilled over CaH₂. Organic solvents such acetonitrile, dichloromethane, diethyl ether, tetrahydrofurane and toluene were purified by solvent purification system from Solv-tek. Other solvents used for synthesis and experiment were HPLC grade purity. Buffer solutions were prepared with bidistilled water.
Solvent were evaporated using R-200 Rotavapor from Buchi equipped with a vacuum controller PVK 610 from MLT Labortechnik AG or with Vacuum MZ 2C model and heating bath from Buchi.

All glassware used for synthesis and experiments was washed with soap solution, water (and bidistilled water for LUVs formation and experiment), acetone and dried in an oven to ensure dryness. Glassware used in anhydrous and inert atmosphere was cooled down to room temperature by argon flux. Molecular sieves kept in the oven at least 48 h before using.

5.1.2. Equipment for Characterization

Column chromatography was carried out on silica gel 60 (Fluka, 40-63 μm). Analytical thin layer chromatography (TLC) was performed on aluminum sheets (20 x 20 cm) covered with silica gel 60 (Fluka, 0.2 μm).

UV-Vis spectra were recorded on a JASCO V-650 spectrophotometer or on a Cary 50 spectrophotometer equipped with a stirrer and a temperature controller (25 ± 0.1 °C) using standard 1 cm pathlength quartz cell and are reported as a maxima absorption wavelength λ in nm or wavenumber ν in cm⁻¹ (extinction coefficient ε in M⁻¹ cm⁻¹).

Melting points (Mp) were measured on heating table equipped with microscope from Reichert (Austria) and are given in Celsius degree °C.

Fourier transform infrared (FT-IR) spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer (ATR, Golden Gate) at room temperature. Intensities of the vibrational band are reported as wavenumber ν in cm⁻¹ with band intensities indicated as s (strong), m (medium), w (weak) and br (broad).

¹H and ¹³C spectra were recorded (as indicated) either on a Bruker 300 MHz, 400 MHz or 500 MHz spectrometer and were processed by Mestrenova software. Chemical shift δ are reported in ppm downfield relative to TMS (δ =
0) internal reference in the solvents. Spin multiplicities are reported as singlet (s), doublet (d), triplet (t), quartet (q) and quintet (quint) with coupling constants \( J \) given in Hz or multiplet (m). \(^1\)H and \(^{13}\)C resonances were assigned with the aid of additional 1D and 2D NMR spectra (H,H-COSY, DEPT 135, HSQC and HMBC).

ESI-MS (Electron Spray Ionization Mass Spectroscopy) were performed on a Finnigan MAT SSQ 7000 instrument or an ESI API 150EX and are reported as mass-per-charge ratio \( m/z \) (intensity in \%, [assignment]).

ESI-HRMS (Electron Spray Ionization High Resolution Mass Spectroscopy) and EI-HRMS (Electronic Impact High Resolution Mass Spectroscopy) were performed, respectively, on a QSTAR Pulsar (AB/MDS Sciex) and DFS-Thermofischer and are reported as mass-per-charge ratio \( m/z \) calculated and observed.

5.1.3. Equipment used During Experiments

Fluorescence spectra were recorded on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon GmbH) or a Fluoromax-4 spectrofluorometer (Horiba Scientific) equipped with a stirrer and temperature controller (25 ± 0.1 °C) using standard 1 cm pathlength glass cell. Fluorescence spectra were corrected using instrument-supplied correction factors. Jobin Yvon FluoroLog 3 spectrofluorometer was used to cover the spectral region up to 950 nm and fluorescence spectra were corrected for the wavelength-dependent sensitivity of the detection.

Autoclavable micropipettes from Nichiryo (Japan), Socorex (Switzerland), Vaudoux-Eppendorf (Switzerland) calibrated for volumes 0-10 \( \mu \)l, 20-200 \( \mu \)l and 100-1000 \( \mu \)l were used to prepare and transfer solutions.

Electrochemical measurements were performed on an Electrochemical Analyzer with Picoamp booster and a Faraday cage (CH Instrument 660C).
pH values were measured with a Consort C832 multi-parameter analyzer equipped with a VWR glass membrane pH electrode calibrated with Titrisol solution from Merck at pH 4.00 and 7.00.

Large Unilamellar Vesicles (LUVs) were prepared by extrusion with a Mini-Extruder from Avanti Polar Lipids (poor size 100 nm) equipped with a heating plate.

5.2. Synthesis

5.2.1. Push-Pull Quaterthiophenes

\[
\text{Amino(2-hydrazinyl-2-oxoethyl)amino} \quad \text{methaniminium (240). This compound was synthetized in four steps following previous reported procedure}^{171}.
\]

\[
\text{2-Hydrazinyl-2-oxoacetate (241). This compound was synthetized in three steps following previous reported procedure}^{179}.
\]

\[
\text{Amino(2-(2-aminooxy)acetamido) ethyl)amino} \quad \text{methaniminium (242). This compound was synthetized in four steps following previous reported procedure}^{178}.
\]
Methyl 4-methyl-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)thiophene-2-carboxylate (227).

This compound was synthesized in two steps following previous reported procedure.\textsuperscript{123,124}

2-Iodo-5-methoxythiophene (221). To a solution of 220 (2.00 g, 17.51 mmol) in DCM and AcOH (60 ml, 1:1) at 0 °C, NIS (3.93 g, 17.51 mmol) was added in three portions over a period of 1.5 h. Then the reaction was stirred at rt for 3 h. Water (20 ml) was added to the reaction mixture and the acid was neutralized with a saturated solution of NaHCO₃. The mixture was extracted with DCM (4 x 200 ml). The organic layer was dried over MgSO₄ and the solvent evaporated in vacuo. Silica gel column chromatography (petroleum ether) of the residue gave 221 (3.74 g, 89%) as a pale yellow oil. The physical and spectroscopic data were identical to those reported in reference.\textsuperscript{209}

5'-Methoxy-3-methyl-2,2'-bithiophene (223). To a solution of 221 (1.71 g, 7.12 mmol) and 222 (1.86 g, 8.29 mmol) in freshly distilled DMF (40 ml) under an Ar atmosphere, CsF (3.64 g, 23.96 mmol) and Pd(PPh₃)₄ (0.82 g, 0.71 mmol) were added, then the mixture was stirred at 80 °C overnight. The mixture was cooled down to rt, water (40 ml) was added and the mixture was extracted with diethyl ether (5 x 200 ml). The organic layer was dried over MgSO₄ and the solvent evaporated in vacuo. Silica gel column chromatography (petroleum ether) of the residue gave 223 (1.07 g, 72%) as a pale yellow oil. \( R_f \) (petroleum ether): 0.4; IR (neat): 2934 (w), 1532 (s), 1490 (s), 1418 (m), 1205 (s), 1047 (m), 993 (m), 767 (s), 703 (s), 616 (s); \(^1\)H NMR (400 MHz, CDCl₃): 7.08 (d, \(^3\)J (H,H) =
5.2 Hz, 1H), 6.84 (d, \( \delta (H,H) = 5.2 \text{ Hz}, 1\text{H} \)), 6.73 (d, \( \delta (H,H) = 3.6 \text{ Hz}, 1\text{H} \)),
6.16 (d, \( \delta (H,H) = 3.6 \text{ Hz}, 1\text{H} \)), 3.91 (s, 3H), 2.34 (s, 3H); \(^{13}\text{C NMR} (100 \text{ MHz},
\text{CDCl}_3): 166.3 (s), 133.3 (s), 131.5 (s), 131.2 (d), 123.5 (d), 122.78 (s), 122.71 (d), 104.2 (d), 60.3 (q), 15.2 (q); MS (ESI, DCM/MeOH 1:1): 214.4 (100, [M+H])
\(^{+}\); HRMS (ESI, +ve) calcd for C\(_{10}\)H\(_9\)O\(_2\)S\(_2\): 311.0245, found: 311.0264.

**5-Iodo-5'-methoxy-3-methyl-2,2'-bithiophene (224).**

To a solution of 223 (0.50 g, 2.37 mmol) in DCM and AcOH (40 ml, 1:1) at 0 °C, NIS (0.54 g, 2.37 mmol)
was added in three portions over a period of 1.5 h. Then the reaction was stirred at rt for 2 h. Water (20 ml) was added to the reaction mixture and the acid was neutralized with a saturated solution of NaHCO\(_3\). The mixture was extracted with DCM (4 x 100 ml). The organic layer was dried over MgSO\(_4\) and the solvent evaporated in vacuo. Silica gel column chromatography (petroleum ether) of the residue gave 224 (0.70 g, 88%) as a pale yellow oil. \( R_f \) (petroleum ether): 0.4; IR (neat): 2930 (w), 1528 (s), 1485 (s), 1412 (m), 1200 (m), 1051 (m), 992 (m), 765 (s); \(^1\text{H NMR} (400 \text{ MHz}, \text{CDCl}_3): 6.99 (s, 1H), 6.68 (d, \( \delta (H,H) = 3.6 \text{ Hz}, 1\text{H} \)), 6.15 (d, \( \delta (H,H) = 3.6 \text{ Hz}, 1\text{H} \)), 3.90 (s, 3H), 2.29 (s, 3H); \(^{13}\text{C NMR} (100 \text{ MHz}, \text{CDCl}_3): 166.9 (s), 140.8 (d), 137.6 (s), 135.2 (s), 124.1 (d), 121.3 (s), 104.3 (d), 70.1 (s), 60.4 (q), 15.2 (q); MS (ESI, DCM/MeOH 1:1): 337 (80, [M+H])
\(^{+}\); HRMS (EI, +ve) calcd for C\(_{10}\)H\(_9\)O\(_2\)S\(_2\): 335.9134, found: 335.9136.

**5''-Methoxy-3,4'-dimethyl-2,2':5',2''-terthiophene (225).**

To a solution of 224 (0.50 g, 1.48 mmol) and 222 (0.40 g, 1.78 mmol) in freshly distilled DMF (15 ml) under an Ar atmosphere, CsF (0.67 g, 4.44
mmol) and Pd(PPh₃)₄ (0.17 g, 0.15 mmol) were added then the mixture was stirred at 80 °C overnight. The mixture was cooled down to rt, water (40 ml) was added and the mixture was extracted with diethyl ether (4 x 200 ml). The organic layer was dried over MgSO₄ and the solvent evaporated in vacuo. Silica gel column chromatography (ethyl acetate/petroleum ether 1:99) of the residue gave 225 (0.32 g, 71%) as a yellow oil. Rₜ(ethyl acetate/petroleum ether 1:99): 0.4; IR (neat): 2931 (w), 1527 (s), 1490 (s), 1425 (m), 1200 (m), 1050 (m), 992 (m), 827 (m), 766 (m), 615 (m); ¹H NMR (400 MHz, CDCl₃): 7.11 (d, ³J(H,H) = 5.2 Hz, 1H), 6.89 (s, 1H), 6.76 (d, ³J(H,H) = 5.2 Hz, 1H), 6.76 (d, ³J(H,H) = 4.0 Hz, 1H), 6.18 (d, ³J(H,H) = 4.0 Hz, 1H), 3.92 (s, 3H), 2.40 (s, 3H), 2.35 (s, 3H); ¹³C NMR (100 MHz, CDCl₃): 166.4 (s), 133.9 (s), 133.5 (s), 133.3 (s), 131.5 (d), 131.3 (s), 131.1 (s), 129.5 (d), 123.4 (d), 123.3 (d), 122.5 (s), 104.3 (d), 60.4 (q), 15.5 (q), 15.3 (q); MS (ESI, DCM/MeOH 1:1): 307 (100, [M+H]+). HRMS (ESI, +ve) calcd for C₁₅H₁₁O₃S: 306.0201, found: 306.0197.

5-Iodo-5''-methoxy-3,4'-dimethyl-2,2':5',2''-terthiophene (226). To a solution of 225 (200 mg, 0.65 mmol) in DCM and AcOH (15 ml, 3:1) at 0 °C, NIS (102 mg, 0.46 mmol) was added in three portions over a period of 1.5 h. Then the reaction was stirred at rt for 1 h. Water (15 ml) was added to the reaction mixture and the acid was neutralized with a saturated solution of NaHCO₃. The mixture was extracted with DCM (4 x 100 ml). The organic layer was dried over MgSO₄ and the solvent evaporated in vacuo. Silica gel column chromatography (DCM/petroleum ether 1:9) of the residue gave pure 226 (127 mg, 45%, conversion yield 73%) as a yellow oil together with 225 (56 mg). Rₜ(DCM/petroleum ether 1:9): 0.5; IR (neat): 2927 (w); 1527 (s), 1490 (s), 1425 (s), 1254 (m), 1200 (s), 1051 (m), 993 (m), 827 (m), 766 (m), 704 (m), 615 (m); ¹H NMR (400 MHz, CDCl₃): 7.01 (s, 1H), 6.82 (s, 1H), 6.75 (d, ³J(H,H) = 4.0 Hz, 1H), 6.17 (d, ³J(H,H) = 4.0 Hz, 1H), 3.91 (s, 3H); 2.35
(s, 3H), 2.33 (s, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$): 166.6 (s), 141.2 (d), 137.1 (s), 135.6 (s), 133.55 (s), 133.52 (s), 132.0 (s), 129.9 (d), 123.6 (d), 122.3 (s), 104.4 (d), 70.9 (s), 60.4 (q), 15.4 (q), 15.2 (q); MS (ESI, MeOH 1:1): 433 (90, [M+H]$^+$), 306 (100, [M-I]$^-$).

Methyl 5'''-methoxy-3,4',4''-trimethyl-[2,2':5',2''-5'',2''']-quaterthiophene-5-carboxylate (217). To a solution of 226 (157 mg, 0.36 mmol) and 227 (123 mg, 0.44 mmol) in freshly distilled DMF (15 ml) under an Ar atmosphere, CsF (165 mg, 1.09 mmol) and Pd(PPh$_3$)$_4$ (42 mg, 0.04 mmol) were added then the mixture was stirred at 80 °C overnight. The mixture was cooled down to rt, water (25 ml) was added and the mixture was extracted with diethyl ether (4 x 200 ml). The organic layer was dried over MgSO$_4$ and the solvent evaporated in vacuo. Silica gel column chromatography (acetone/petroleum ether 5:95) of the residue gave 217 (112 mg, 67%) as a pale orange solid. R$_f$(acetone/petroleum ether 5:95): 0.3; Mp: 127-128 °C; IR (neat): 2953 (w), 1700 (s), 1538 (m), 1429 (m), 1243 (s), 1191 (m), 1075 (m), 997 (m), 748 (m), 614 (w); $^1$H NMR (500 MHz, CDCl$_3$): 7.56 (s, 1H), 7.02 (s, 1H), 6.94 (s, 1H), 6.78 (d, $^3$J (H,H) = 4.0 Hz, 1H), 6.19 (d, $^3$J (H,H) = 4.0 Hz, 1H), 3.93 (s, 3H), 3.88 (s, 3H), 2.42 (s, 6H), 2.36 (s, 3H); $^{13}$C NMR (125 MHz, CDCl$_3$): 166.7 (s), 162.7 (s), 138.5 (s), 137.7 (d), 134.38 (s), 134.36 (s), 133.7 (s), 132.7 (s), 132.64 (s), 132.60 (s), 132.0 (s), 130.9 (d), 129.8 (d), 129.4 (s), 123.6 (d), 122.4 (s), 104.5 (d), 60.4 (q), 52.3 (q), 15.8 (q), 15.7 (q), 15.4 (q); MS (ESI, chloroform/MeOH 1:1): 461 (100, [M+H]$^+$); HRMS (EI, +ve) calcd for C$_{22}$H$_{30}$O$_3$S$_4$: 460.0290, found: 460.0299.
(5'''''-methoxy-3,4',4''''-trimethyl-[2,2':5',2'''';5'',2'''']-quaterthiophen]-5-yl)methanol (229). To a solution of 217 (150 mg, 0.32 mmol) in DCM (10 ml) at -78 °C under an Ar atmosphere, DIBAL (860 µl, 1 M in toluene) was added dropwise over a period of 10 minutes. The mixture was stirred for 2 h then MeOH (5 ml) and brine solution (5 ml) were added before warming the mixture up to rt. The solution was extracted with DCM (3 x 25 ml). The organic layer was washed over MgSO₄ and the solvent evaporated in vacuo. Silica gel column chromatography (DCM) of the residue gave 229 (99 mg, 70%) as a dark yellow solid. Rₜ (DCM): 0.2; Mp: 130-131 °C; IR (neat): 3307 (m), 2917 (m), 2917 (m), 1526 (s), 1526 (s), 1258 (m), 1200 (m), 1146 (m), 1055 (w), 1008 (s), 929 (w), 848 (m), 814 (s), 759 (s), 610 (s); ¹H NMR (400 MHz, CDCl₃): 6.91 (s, 1H), 6.89 (s, 1H), 6.79 (s, 1H), 6.77 (d, ³J (H,H) = 4.0 Hz, 1H), 6.18 (d, ³J (H,H) = 4.0 Hz, 1H), 4.75 (d, ³J (H,H) = 6.0 Hz, 2H), 3.92 (s, 3H), 2.40 (s, 3H), 2.37 (s, 3H), 2.35 (s, 3H), 1.78 (t, ³J (H,H) = 6.0 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃): 166.5(s), 141.1 (s), 134.2 (s), 133.8 (2 x s), 133.6 (s), 133.1 (s), 131.5 (s), 131.2 (s), 131.0 (s), 130.1 (d), 129.8 (d), 129.4 (d), 123.4 (d), 122.5 (s), 104.4 (d), 60.4 (q), 60.2 (t), 15.8 (q), 15.7 (q), 15.4 (q); MS (ESI, chloroform/MeOH 1:1): 433 (50, [M+H]⁺), 415 (100, [M-OH]⁺); HRMS (EI, +ve) calcd for C₂₁H₂₀O₂S₂: 432.0341, found: 432.0352.

5''''-methoxy-3,4',4''''-trimethyl-[2,2':5',2'''';5'',2'''']-quaterthiophene]-5-carbaldehyde (216). To a solution of 229 (90 mg, 0.21 mmol) in DCM (5 ml), MnO₂ (40 mg, 0.46 mmol) were added. The mixture was stirred at rt for 15 minutes then the dark inorganic solid was filtered off and the filtrate was concentrated in vacuo. Silica gel column chromatography (DCM) of the residue gave 216 (64 mg,
71%) as an orange solid. \( R_f \) (DCM): 0.5; Mp: 124-125 °C; IR (neat): 2922 (w), 1643 (s), 1535 (m), 1481 (m), 1447 (m), 1424 (m), 1224 (m), 1149 (m), 997 (m), 822 (m), 777 (m), 670 (m); \(^1\)H NMR (400 MHz, CDCl\(_3\)): 9.80 (s, 1H), 7.53 (s, 1H), 7.10 (s, 1H), 6.96 (s, 1H), 6.78 (d, \(^3\)J (H,H) = 4.0 Hz, 1H), 6.19 (d, \(^3\)J (H,H) = 4.0 Hz, 1H), 3.93 (s, 3H), 2.47 (s, 3H), 2.36 (s, 3H); \(^13\)C NMR (100 MHz, CDCl\(_3\)): 182.5 (d), 166.7 (s), 141.8 (s), 140.6 (d), 139.6 (s), 134.7 (s), 133.7 (s), 132.4 (2 x s), 132.3 (s), 131.6 (d), 130.0 (s), 123.7 (d), 122.3 (s), 105.5 (d), 60.4 (q), 16.1 (q), 15.8 (q), 15.4 (q); MS (ESI, DCM/MeOH 1:1): 431 (100, [M+H\(^+\)], 416 (45, [M-CH\(_3\)]\(^+\)); HRMS (EI, +ve) calcd for C\(_{31}\)H\(_{19}\)O\(_2\)S\(_4\): 430.0177, found: 430.0177.

\[(E)-2\text{-cyano-3-(5''-methoxy-3',4',4''-trimethyl-2,2':5',2''-quaterthiophen}-5-yl)\text{acrylic acid (231).}\] To a solution of 216 (55 mg, 0.13 mmol) and cyanoacetic acid 230 (22 mg, 0.26 mmol) in ACN (10 ml) under an Ar atmosphere, piperidine (10 \(\mu\)l, 73 \(\mu\)mol) was added and the mixture was refluxed for 4 h. Then the solvent was evaporated in vacuo. Silica gel column chromatography (AcOH/MeOH/DCM 1:10:89) of the residue gave 231 (40 mg, 63%) as a black solid. \( R_f \) (AcOH/MeOH/DCM 1:10:89): 0.3; Mp: > 230 °C; IR (neat): 2926 (m, br), 2277 (w), 2218 (w), 1718 (m), 1666 (s), 1563 (m), 1516 (w), 1484 (w), 1414 (s), 1381 (s), 1258 (m), 1214 (m), 1172 (s), 1059 (w), 984 (w), 876 (w), 819 (w), 770(w), 650 (w); \(^1\)H NMR (500 MHz, DMSO-d\(_6\)): 8.34 (s, 1H), 7.81 (s, 1H), 7.38 (s, 1H), 7.22 (s, 1H), 6.94 (d, \(^3\)J (H,H) = 4.0 Hz, 1H), 6.39 (d, \(^3\)J (H,H) = 4.0 Hz, 1H), 3.92 (s, 3H), 2.44 (s, 3H), 2.42 (s, 3H), 2.34 (s, 3H); \(^13\)C NMR (125 MHz, DMSO-d\(_6\)): 165.9 (s), 163.4 (s), 145.1 (d), 143.7 (d), 139.2 (s), 135.0 (s), 134.8 (s), 133.7 (s), 132.4 (s), 132.3 (s), 132.1 (d), 131.4 (s), 131.3 (s), 131.1 (s), 130.4 (d), 124.2 (d), 120.8 (s), 117.0 (s), 116.8 (s), 104.9 (d), 60.4 (q), 15.34 (q), 15.32 (q), 15.0 (q); MS (ESI, chloroform/MeOH
1:1 + 1% AcOH): 498 (100, [M+H]+). HRMS (ESI, +ve) calcd for C_{23}H_{36}N_{10}O_{3}S_{4}Na: 520.0140, found: 520.0141.

(E)-2-cyano-N-(2,2-dimethoxyethyl)-3-(5''-methoxy-3',4',4''-trimethyl-[2,2':5',2'':5'',2''']-quaterthiophen]-5-yl)acrylamide (213). To a solution of 231 (23 mg, 46 μmol) and HBTU (44 mg, 0.16 mmol) in freshly distilled DMF (5 ml) under an Ar atmosphere, distilled TEA (16 μl, 0.11 mmol) was added and the mixture was stirred for 5 minutes at rt. Then 2,2-dimethoxyethylamine 232 (0.1 ml, 0.92 mmol) was added and the mixture was stirred for 1.5 h. Then the solvent was evaporated in vacuo. Silica gel column chromatography (DCM) of the residue gave 213 (23 mg, 85%) as a dark red solid. R_{f} (acetone/DCM 5:95): 0.4; Mp: 177-178 °C; IR (neat): 3350 (w), 2924 (m), 2202 (w), 1661 (m), 1574 (s), 1518 (s), 1424 (s), 1375 (m), 1256 (s), 1198 (s), 1128 (m), 1058 (m), 970 (m), 817 (m), 762 (m), 717 (m), 615 (m); \textsuperscript{1}H NMR (400 MHz, CDCl_{3}): 8.25 (s, 1H), 7.46 (s, 1H), 7.14 (s, 1H), 6.96 (s, 1H), 6.78 (d, \textit{J}(H,H) = 4.0 Hz, 1H), 6.42 (m, 1H), 6.18 (d, \textit{J}(H,H) = 4.0 Hz, 1H), 4.46 (m, 1H), 3.92 (s, 3H), 3.56 (m, 2H), 3.44 (s, 6H), 2.45 (s, 3H), 2.42 (s, 3H), 2.36 (s, 3H); \textsuperscript{13}C NMR (100 MHz, CDCl_{3}): 166.7 (s), 161.2 (s), 144.3 (d), 141.7 (d), 141.2 (s), 134.8 (s), 134.6 (s), 133.9 (s), 133.7 (s), 132.6 (s), 132.4 (2 x s), 132.1 (s), 131.8 (d), 129.9 (d), 123.7 (d), 122.3 (s), 117.4 (s), 104.5 (d), 102.5 (d), 98.7 (s), 60.4 (q), 54.7 (2 x q), 42.0 (t), 15.9 (q), 15.8 (q), 15.4 (q); MS (ESI, chloroform/MeOH 1:1): 585 (60, [M+H]+), 553 (100, [M-OCH_{3}]^{+}); HRMS (EI, +ve) calcd for C_{23}H_{36}N_{10}O_{3}S_{4}: 584.0926, found: 584.0947.
[2,2':5',2''-quaterthiophen]-5-yl)-N-(2-oxoethyl)acrylamide (233).

To a solution of 213 (10 mg, 0.02 mmol) in DCM (3 ml), TsOH·H₂O (1 mg, 5.26 µmol) was added and the mixture was stirred at rt overnight. Then the solvent was evaporated in vacuo. Silica gel column chromatography (acetone/DCM 1:9) of the residue gave 233 (5 mg, 54%) as a dark red solid. Rᵣ (acetone/DCM 1:9): 0.4; Mp: 192-193 °C; IR (neat): 3407 (w), 2922 (w), 2203 (w), 1729 (w), 1658 (m), 1574 (s), 1518 (m), 1425 (s), 1376 (m), 1259 (m), 1174 (m), 1059 (m), 979 (m), 819 (m), 694 (w), 614 (m); ¹H NMR (400 MHz, CDCl₃): 9.72 (s, 1H), 8.26 (s, 1H), 7.49 (s, 1H), 7.16 (s, 1H), 6.97 (s, 1H), 6.88 (t, ³J (H,H) = 4.8 Hz, 1H), 6.79 (d, ³J (H,H) = 4.0 Hz, 1H), 6.19 (d, ³J (H,H) = 4.0 Hz, 1H), 4.34 (d, ³J (H,H) = 4.8 Hz, 2H), 3.93 (s, 3H), 2.46 (s, 3H), 2.43 (s, 3H), 2.37 (s, 3H); ¹³C NMR (125 MHz, CDCl₃): 195.4 (d), 166.8 (s), 161.4 (s), 144.7 (d), 142.0 (d), 141.8 (s), 135.0 (s), 134.70 (s), 134.68 (s), 134.2 (s), 133.8 (s), 132.53 (s), 132.50 (s), 132.4 (s), 132.0 (d), 130.0 (d), 123.7 (d), 122.3 (s), 117.2 (s), 104.5 (d), 97.8 (s), 60.5 (q), 50.9 (t), 15.9 (q), 15.8 (q), 15.4 (q); MS (ESI, chloroform/MeOH 1:1): 539 (100, [M+H]+); HRMS (ESI, +ve) calcd for C₂₆H₂₃N₂O₅S₄: 539.0586, found: 539.0569.

5.2.2. Push-Pull Terthiophenes

Methyl 5''-methoxy-3,4'-dimethyl-[2,2':5',2''-terthiophene]-5-carboxylate (234). To a solution of 224 (611 mg, 1.82 mmol) and 227 (615 mg, 2.18 mmol) in freshly distilled DMF (25 ml) under an Ar atmosphere, CsF (830 mg, 5.46 mmol) and Pd(PPh₃)₄ (210
mg, 0.18 mmol) were added then the mixture was stirred at 80 °C overnight. The mixture was cooled down to rt, water (25 ml) was added and the mixture was extracted with diethyl ether (4 x 300 ml). The organic layer was dried over MgSO₄ and the solvent evaporated in vacuo. Silica gel column chromatography (DCM) of the residue gave 234 (430 mg, 65%) as a pale dark yellow solid. Rᵢ (DCM): 0.4; Mp: 95-96 °C; IR (neat): 3058 (w), 2952 (w), 1701 (s), 1543 (m), 1444 (m), 1244 (s), 1199 (s), 1077 (m), 982 (m), 748 (m), 615 (w); ¹H NMR (400 MHz, CDCl₃): 7.57 (s, 1H), 7.01 (s, 1H), 6.80 (d, ³J (H,H) = 4.0 Hz, 1H), 6.19 (d, ³J (H,H) = 4.0 Hz, 1H), 3.93 (s, 3H), 2.41 (s, 3H), 2.37 (s, 3H); MS (ESI, chloroform/MeOH 1:1): 365 (100, [M+H]+); HRMS (ESI, +ve) calcd for C₁₇H₁₇O₄S₃: 365.3340 found: 365.0332.

(5''-methoxy-3,4''-dimethyl-[2,2':5',2''-terthiophen]-5-yl)methanol (235). To a solution of 234 (200 mg, 0.60 mmol) in DCM (15 ml) at -78 °C under an Ar atmosphere, DIBAL (1.1 ml, 1 M in toluene) was added dropwise over a period of 10 minutes. The mixture was stirred for 2 h then MeOH (7 ml) and brine solution (7 ml) were added before warming the mixture up to rt. The solution was extracted with DCM (3 x 40 ml). The organic layer was dried over MgSO₄ and the solvent evaporated in vacuo. Silica gel column chromatography (DCM) of the residue gave 235 (190 mg, 94%) as a yellow solid. Rᵢ (DCM): 0.2; ¹H NMR (400MHz, CDCl₃): 6.88 (s, 1H), 6.78 (s, 1H), 6.77 (d, ³J (H,H) = 4.0 Hz, 1H), 6.19 (d, ³J (H,H) = 4.0 Hz, 1H), 4.73 (s, 2H), 3.92 (s, 3H), 2.36 (s, 3H), 2.35 (s, 3H).
5''-methoxy-3,4'-dimethyl-[2,2':5',2''-terthiophene]-5-carbaldehyde (236). To a solution of 235 (200 mg, 0.60 mmol) in DCM (15 ml), MnO₂ (150 mg, 1.73 mmol) were added. The mixture was stirred at rt for 20 minutes then the dark inorganic solid was filtered off and the filtrate was concentrated in vacuo. Silica gel column chromatography (DCM) of the residue gave 236 (147 mg, 73%) as an orange solid. Rᵣ (DCM): 0.5; Mp: 82-83 °C; IR (neat): 2925 (w), 2802 (w), 1644 (s), 1539 (m), 1483 (m), 1442 (m), 1356 (m), 1244 (m), 1157 (m), 988 (m), 851 (m), 763 (m), 673 (m); ¹H NMR (400 MHz, CDCl₃): 9.80 (s, 1H), 7.52 (s, 1H), 7.08 (s, 1H), 6.36 (d, ³J(H,H) = 4.0 Hz, 1H), 6.19 (d, ³J(H,H) = 4.0 Hz, 1H), 3.93 (s, 3H), 2.45 (s, 3H), 2.37 (s, 3H); MS (ESI, chloroform/MeOH 1:1): 335 (100, [M+H]+), 320 (24, [M-CH₃+H]+); HRMS (ESI, +ve) calcd for C₁₆H₁₅O₂S: 335.0228 found: 335.0233.

(E)-2-cyano-3-(5''-methoxy-3,4'-dimethyl-[2,2':5',2''-terthiophen]-5-y)acrylic acid (237). To a solution of 236 (108 mg, 0.32 mmol) and cyanoacetic acid 230 (50 mg, 0.59 mmol) in ACN (20 ml) under an Ar atmosphere, piperidine (30 μl, 0.2 mmol) was added and the mixture was refluxed for 3 h. Then the solvent was evaporated in vacuo. Silica gel column chromatography (AcOH/MeOH/DCM 1:10:89) of the residue gave 237 (104 mg, 81%) as a black solid. Rᵣ (AcOH/MeOH/DCM 1:10:89): 0.3; Mp: > 230 °C; IR (neat): 2918 (m, br), 2212 (w), 1678 (s), 1551 (m), 1421 (w), 1414 (s), 1354 (s), 1266 (m), 1197 (m), 1166 (s), 1060 (w), 988 (w), 847 (w), 748 (w); ¹H NMR (400 MHz, DMSO-d₆): 8.28 (s, 1H), 7.75 (s, 1H), 7.29 (s, 1H), 6.96 (d, ³J(H,H) = 4.0 Hz, 1H), 6.36 (d, ³J(H,H) = 4.0 Hz, 1H), 3.88 (s, 3H), 2.37 (s, 3H), 2.32 (s, 3H); MS (ESI, chloroform/MeOH 1:1 + 1% AcOH): 402 (100, [M+H]+). HRMS (ESI, +ve) calcd for C₁₉H₁₆N₁O₂S₃: 402.0286 found: 402.0284.
(E)-2-cyano-N-(2,2-dimethoxyethyl)-3-(5''-methoxy-3,4'-dimethyl-[2,2':5',2''-terthiophen]-5-yl)acrylamide (238). To a solution of 237 (50 mg, 0.12 mmol) and HBTU (142 mg, 0.37 mmol) in freshly distilled DMF (10 ml) under an Ar atmosphere, distilled TEA (53 μl, 0.37 mmol) was added and the mixture was stirred for 5 minutes at rt. Then 2,2-dimethoxyethamine 232 (270 μl, 2.49 mmol) was added and the mixture was stirred for 1.5 h. Then the solvent was evaporated in vacuo. Silica gel column chromatography (DCM) of the residue gave 238 (39 mg, 63%) as a dark red solid. Rf (DCM): 0.4; Mp: 149-150 °C; IR (neat): 3359 (w), 2930 (w), 1659 (m), 1570 (s), 1524 (s), 1424 (s), 1372 (m), 1255 (m), 1120 (m), 1054 (m), 994 (m), 817 (m), 763 (m), 617 (m); 1H NMR (400 MHz, CDCl3): 8.24 (s, 1H), 7.45 (s, 1H), 7.11 (s, 1H), 6.81 (d, 3J (H,H) = 4.0 Hz, 1H), 6.42 (m, 1H), 6.19 (d, 3J (H,H) = 4.0 Hz, 1H), 4.46 (m, 1H), 3.93 (s, 3H), 3.56 (m, 2H), 3.43 (s, 6H), 2.43 (s, 3H), 2.36 (s, 3H); MS (ESI, chloroform/MeOH 1:1): 489 (60, [M+H]+), 457 (100, [M-OCH3]+); HRMS (ESI, +ve) calcd for C23H28N2O3S: 489.0970 found: 489.0974.

(E)-2-cyano-3-(5''-methoxy-3,4'-dimethyl-[2,2':5',2''-terthiophen]-5-yl)-N-(2-oxoethyl)acrylamide (239). To a solution of 238 (10 mg, 0.02 mmol) in DCM (4 ml), TsOH·H2O (1 mg, 5.26 μmol) was added and the mixture was stirred at rt overnight. Then the solvent was evaporated in vacuo. Silica gel column chromatography (acetone/DCM 1:9) of the residue gave 239 (8 mg, 84%) as a dark red solid. Rf (acetone/DCM 1:9): 0.4; 1H NMR (400 MHz, CDCl3): 9.73 (s, 1H), 8.26 (s, 1H), 7.50 (s, 1H), 7.14 (s, 1H), 6.88 (t, 3J (H,H) = 4.8 Hz, 1H),
6.84 (d, $^3J (H,H) = 4.0$ Hz, 1H), 6.22 (d, $^3J (H,H) = 4.0$ Hz, 1H), 4.34 (d, $^3J (H,H) = 5.2$ Hz, 2H), 3.94 (s, 3H), 2.46 (s, 3H), 2.39 (s, 3H).

5.2.3. Push–Pull Oligothiophene Amphiphiles

Amphiphiles 211, 212, 214, 215, 218, 219 and 254 were prepared in-situ by covalent capture as oximes or hydrazones. This strategy has been used extensively for the preparation of otherwise difficult to access products with extreme physical properties that complicate standard purification and characterization. Following optimized procedures, the products could thus be used without further purifications.

(9E,14E)-1-amino-14-cyano-15-(5''-methoxy-3,4',4''-trimethyl-[2,2':5',2''-quaterthiophen]-5-yl)-6,13-dioxo-8-oxa-2,5,9,12-tetraazapentadeca-9,14-dien-1-iminium (211). To a solution of 233 (1 mg, 1.8 μmol) in DMF (2 ml) at rt and silica gel column chromatography (AcOH/MeOH/DCM 1:10:89) of the residue gave 211 (1.3 mg, quant) as a red solid. Rf (AcOH/MeOH/DCM 1:10:89): 0.2; MS (ESI, chloroform/MeOH 1:1 + 1% AcOH): 696 (100, [M+H]+). HRMS (ESI, +ve) calcd for C$_{31}$H$_{34}$N$_7$O$_4$S$_6$: 696.1549, found: 696.1525.
(5''-methoxy-3,4',4''-trimethyl-[2,2':5',2'':5'',2'''-quaterthiophen]-5-yl)acrylamido(ethylidene)amino)oxyacetate (212). To a solution of 233 (1 mg, 1.8 μmol) in DMSO (100 μl), 243 as hemihydrochloric salt (0.7 mg, 5.4 μmol) in DMSO (100 μl), AcOH (1 μl) was added and the mixture was stirred at 60 °C overnight. The mixture was diluted with DCM (2 ml) at rt and silica gel column chromatography (AcOH/MeOH/DCM 1:10:89) of the residue gave 212 (1.1 mg, quant) as a red solid. Rf (AcOH/MeOH/DCM 1:10:89): 0.4.

[2,2':5',2'':5'',2'''-quaterthiophen]-5-yl)methylene)hydrazinyl)-2-oxoethyl)amino) methaniminium (214). To a solution of 216 (1 mg, 2.3 μmol) in DMSO (100 μl), 240 (0.8 mg, 4.6 μmol) in DMSO (100 μl), AcOH (1 μl) was added and the mixture was stirred at 60 °C for 3 h. The mixture was diluted with DCM (2 ml) at rt and silica gel column chromatography (AcOH/MeOH/DCM 1:10:89) of the residue gave 214 (1 mg, 80%) as a dark orange solid. Rf (AcOH/MeOH/DCM 1:10:89): 0.2; MS (ESI, chloroform/MeOH 1:1 + 1% AcOH): 545 (100, [M+H]+). HRMS (ESI, +ve) calcd for C23H28N4O3S4: 544.0963, found: 544.0970.
quaterthiophen]-5-yl)methylene)hydrazinyl]-2-oxacetate (215). To a solution of 216 (1 mg, 2.3 µmol) in DMSO (100 µl), 241 as mono hydrochloric salt (2.6 mg, 18.6 µmol) in DMSO (100 µl), AcOH (1 µl) was added and the mixture was stirred at 60 °C overnight. The mixture was diluted with DCM (5 ml) at rt and washed with HCl 1 M solution (2 x 10 ml) and brine (3 x 10 ml). The organic layer was dried over MgSO₄ and the solvent evaporated *in vacuo* to yield 215 (1.2 mg, quant) as a dark orange solid.

(9E,14E)-1-amino-14-cyano-15-(5''-methoxy-3-methyl-[2,2':5',2'']-terthiophen]-5-yl)-6,13-dioxo-8-oxa-2,5,9,12-tetraazapentadeca-9,14-dien-1-iminium (218). To a solution of 239 (1 mg, 2.3 µmol) and 242 as mono hydrochloric salt (1.8 mg, 8.6 µmol) in DMSO (100 µl), AcOH (1 µl) was added and the mixture was stirred at 60 °C overnight. The mixture was diluted with DCM (2 ml) at rt and silica gel column chromatography (AcOH/MeOH/DCM 1:10:89) of the residue gave 218 (1.3 mg, quant) as a red solid. *R*ₜ(AcOH/MeOH/DCM 1:10:89): 0.2;

2-(((E)-(2-((E)-2-cyano-3-(5''-methoxy-3,4'')-dimethyl-[2,2':5',2'']-terthiophen]-5-yl)acrylamido)ethylidene)amino)oxy) acetate (219). To a solution of 239 (1 mg, 2.3 µmol) in DMSO (100 µl), 243 as
hemihydrochloric salt (1.0 mg, 7.7 μmol) in DMSO (100 μl), AcOH (1 μl) was added and the mixture was stirred at 60 °C overnight. The mixture was diluted with DCM (2 ml) at rt and silica gel column chromatography (AcOH/MeOH/DCM 1:10:89) of the residue gave 219 (1.1 mg, quant) as a red solid. \( R_f \) (AcOH/MeOH/DCM 1:10:89): 0.4.

5.2.4. PDI Fluorophores and Amphiphiles

5,12-dibromo-2,9-dicyclohexylanthra[2,1,9-def:6,5,10-d'e'f']diisoquinoline-1,3,8,10(2H,9H)-tetrone (248).

This compound was synthesized in two steps following previous reported procedure\(^{264}\).

2,9-dicyclohexyl-5,12-dithoxyanthra[2,1,9-def:6,5,10-d'e'f']diisoquinoline-1,3,8,10(2H,9H)-tetrone (250).

To a solution of NaH (24 mg, 1.00 mmol) in ethanol (10 ml), 248 (100 mg, 0.14 mmol) was added. The solution was refluxed at 80 °C for 18 h. The solution was allowed to cool down to rt and the solvent was removed in vacuo. Silica gel column chromatography of the residue (DCM) gave 250 (40 mg, 45%) as a red powder. \( R_f \) (DCM): 0.3; Mp: > 220 °C; IR (neat): 2924 (w), 2854 (w), 1694 (s), 1644 (s), 1596 (w), 1414 (w), 1245 (s); \(^1\)H NMR (400 MHz, CDCl\(_3\)): 9.65 (d, \(^3\)J (H,H) = 8.4 Hz, 2H), 8.56 (d, \(^3\)J (H,H) = 8.4 Hz, 2H),
8.48 (s, 2H), 5.12-5.02 (m, 2H), 4.57 (q, $^3J$(H,H) = 6.8 Hz, 4H), 2.65-2.54 (m, 4H); 1.97-1.89 (m, 6H), 1.83-1.76 (m, 4H), 1.73 (t, $^3J$(H,H) = 6.8 Hz, 6H), 1.53-1.45 (m, 6H); MS (ESI, +ve, DCM/MeOH 10:1): 643 (100, [M+H]⁺), 561 (60, [M - Cyclohexylamine]⁺).

9-cyclohexyl-5,12-diethoxy-1H-
isochromeno[6',5',4':10,5,6]anthra[2,1,9-
def]isoquinoline-1,3,8,10(9H)-teträone
(251). To a solution of KOH (215 mg, 3.83 mmol) in iPrOH (15 ml), 250 (45 mg, 0.07 mmol) was added. The mixture was refluxed at 85 °C for 4 h. The solution was poured in AcOH (20 ml) and allowed to cool down to rt. The solvent was removed in vacuo overnight. The residue was suspended in DMAc (10 ml) and cyclohexylamine (10 ml, 0.09 mmol) was added. The mixture was refluxed at 120 °C for 3 h. The solvent was evaporated in vacuo. Silica gel column chromatography (DCM/MeOH 97:3) of the residue gave 251 (14 mg, 35%) as a dark violet powder. $R_t$ (DCM/MeOH 97:3): 0.4; Mp: > 220 °C; IR (neat): 2933 (w), 2853 (w), 1770 (w), 1730 (w), 1696 (w), 1653 (w), 1598 (w), 1260 (w), 1020 (s); $^1$H NMR (400 MHz, CDCl₃): 9.69 (d, $^3J$(H,H) = 8.4 Hz, 1H), 9.68 (d, $^3J$(H,H) = 8.4 Hz, 1H), 8.61 (d, $^3J$(H,H) = 8.4 Hz, 1H), 8.60 (d, $^3J$(H,H) = 8.4 Hz, 1H), 8.51 (s, 1H), 8.47 (s, 1H), 5.12-5.00 (m, 1H), 4.60 (q, $^3J$(H,H) = 6.8 Hz, 2H), 2.64-2.53 (m, 2H), 1.97-1.89 (m, 2H), 1.83-1.77 (m, 3H), 1.75 (t, $^3J$(H,H) = 6.8 Hz, 6H), 1.53-1.46 (m, 3H); MS (ESI, +ve, DCM/MeOH 10:1): 562 (100, [M+H]⁺).
2-cyclohexyl-5,12-diehtoxyanthra[2,1,9-def:6,5,10-d'e'f']diisoquinoline-1,3,8,10(2H,9H)-tetraone (252). To a solution of 251 (33 mg, 0.06 mmol) in DMF (4 ml), urea (141 mg, 2.35 mmol) was added. The mixture was refluxed at 130 °C for 14 h. The solution was allowed to cool down to rt and the solvent was evaporated in vacuo. Silica gel column chromatography (DCM/MeOH 99:1) of the residue gave 252 (24 mg, 72%) as a dark red-violet powder. $R_t$ (DCM/MeOH 99:1): 0.2; Mp: > 220 °C; IR (neat): 3342 (w), 2922 (s), 2852 (w), 1702 (w), 1462 (w), 1273 (w); $^1$H NMR (400 MHz, CDCl$_3$/CD$_3$OD 4:1): 9.51 (d, $^3$J (H,H) = 8.4 Hz, 1H), 9.50 (d, $^3$J (H,H) = 8.4 Hz, 1H), 8.83 (d, $^3$J (H,H) = 8.4 Hz, 1H), 8.39 (d, $^3$J (H,H) = 8.4 Hz, 1H), 8.33 (s, 1H), 8.32-8.31 (m, 1H), 8.27 (s, 1H), 4.99-4.93 (m, 1H), 4.47 (q, $^3$J (H,H) = 6.8 Hz, 4H), 2.58-2.47 (m, 2H), 1.95-1.84 (m, 2H), 1.78-1.71 (m, 3H), 1.65 (t, $^3$J (H,H) = 6.8 Hz, 6H), 1.54-1.50 (m, 3H); MS (ESI, +ve, DCM/MeOH 10:1): 561 (100, [M+H]$^+$.)

4-(9-cyclohexyl-5,12-diehtoxy-1,3,8,10-tetraoxo-9,10-dihydroanthra[2,1,9-def:6,5,10-d'e'f']diisoquinolin-2(1H,3H,8H)-yl)benzaldehyde (247). To a solution of 252 (24 mg, 0.04 mmol), 4-formylphenylboronic acid 253 (19.3 mg, 0.13 mmol) and Cu(OAc)$_2$ (15.6 mg, 0.08 mmol) in chloroform (4 ml) containing 4 Å molecular sieves under nitrogen atmosphere, TEA (18 µl, 0.13 mmol) were added. The reaction mixture was purged with oxygen and then stirred at 40 °C for 16 h. The
reaction mixture was then evaporated to dryness and the residue was purified by silica gel column chromatography (acetone/DCM 5:95) to give 247 (18 mg, 65%) as a dark red powder. Mp: > 220 °C; Rf (acetone/DCM 5:95): 0.5; IR (neat): 2922 (s), 2852 (w), 1691 (s), 1649 (s), 1596 (s), 1414 (w), 1335 (w), 1260 (s), 1017(s); 1H NMR (400 MHz, CDCl3): 10.18 (s, 1H), 9.71 (d, 3J (H,H) = 8.4 Hz, 1H), 8.52 (s, 1H), 8.48 (s, 1H), 8.12 (d, 3J (H,H) = 8.4 Hz, 2H), 7.59 (d, 3J (H,H) = 8.4 Hz, 2H), 5.09-5.00 (m, 1H), 4.58 (q, 3J (H,H) = 6.8 Hz, 4H), 2.64-2.54 (m, 2H), 1.98-1.90 (m, 2H), 1.84-1.77 (m, 3H), 1.74 (t, 3J (H,H) = 6.8 Hz, 6H), 1.53-1.49 (m, 3H); MS (ESI, +ve, DCM/MeOH 10:1): 665 (100, [M+H]+).

(E)-amino(2-(2-(4-(9-cyclohexyl-5,12-diethoxy-1,3,8,10-tetraoxo-9,10-dihydroanthra[2,1,9-def:6,5,10-d’e’f’]diisoquinolin-2(1H,3H,8H)-yl)benzylidene)hydrazinyl)-2-oxoethyl)amino)methaniminium (254). To a solution of 247 (2 mg, 3.0 µmol) in DMSO (1 ml), 240 (0.5 mg, 3.0 µmol) in DMSO (100 µl), AcOH (1 µl) was added and the mixture was stirred at 60 °C for 1 h. The solvent was removed in vacuo to yield 254 (2.4 mg, quant) as a red solid. MS (ESI, chloroform/MeOH 1:1 + 1% AcOH): 778 (100, [M+H]+).
5,12-bis(allyloxy)-2,9-dicyclohexylanthra[2,1,9-de:f:6,5,10-d'e'f']diisoquinoline-1,3,8,10(2H,9H)-tetraone (249).

To a solution of 248 (100 mg, 0.14 mmol) in dry DMF (10 ml), AllylONa (300 μl of 2.5 M solution of NaH in allylalcohol) was added at rt. The mixture was refluxed to 100°C for 1 h. The solution was allowed to cool down to rt and 30 ml of H2O were added. The resulting suspension was diluted with DCM (30 ml). The organic layer was washed with brine (2 x 100 ml), H2O (1 x 100 ml), dried over Na2SO4 and concentrated in vacuo. Silica gel column chromatography (DCM) of the residue gave 249 (53 mg, 55%) as a dark red powder. Rf (DCM): 0.3; Mp: > 220 °C; IR (neat): 2924 (w), 2854 (w), 1692 (s), 1649 (s), 1596 (s), 1411 (w), 1332 (s), 1271 (s); 1H NMR (500 MHz, CDCl3): 9.61 (d, 1J (H,H) = 8.4 Hz, 2H), 8.57 (d, 1J (H,H) = 8.4 Hz, 2H), 8.27 (s, 2H), 6.26 (ddt, 1J (H,H) = 17.1, 10.6, 5.3 Hz, 2H), 5.62 (dd, 3J (H,H) = 17.1, 1.1 Hz, 2H), 5.48 (dd, 3J (H,H) = 10.5, 1.1 Hz, 2H), 5.15 - 4.99 (m, 6H), 2.59 (qd, 1J (H,H) = 12.3, 3.2 Hz, 4H), 1.94 (d, 3J (H,H) = 12.3, 4H), 1.88-1.65 (m, 6H), 1.51-1.32 (m, 6H); 13C NMR (100 MHz, CDCl3): 164.0 (s), 163.9 (s), 156.3 (s), 133.7 (s), 132.0 (d), 129.3 (d), 128.8 (d), 123.9 (s), 123.9 (s), 122.0 (s), 121.8 (s), 119.4 (t), 118.0 (d), 70.9 (t), 54.0 (d), 29.7 (t), 29.1 (t), 26.6 (t), 25.5 (t); MS (ESI, DCM): 667 (100, [M + H]+); HRMS (ESI, +ve) calcd for C42H39N6O6N2+: 667.28026, found: 667.27953.
2,9-dicyclohexyl-5,12-dihydroxyanthra[2,1,9-de:6,5,10-d'e'l']disoquinoline-1,3,8,10(2H,9H)-tetrone (246).

To a solution of 249 (100 mg, 0.15 mmol) and Pd(PPh₃)₄ (6 mg, 5 µmol) in DCM (40 ml), phenylsilane (50 µl, 0.40 mmol) was added. The solution was stirred at rt for 1 h. The solvent was removed in vacuo and the residue was washed with petroleum ether (12 x 10 ml) and diethyl ether (12 x 10 ml). The solid was dried overnight in vacuo to give 246 (70 mg, 79%) as a dark violet powder. Mp: > 220 °C; IR (neat): 3271 (w), 2925 (w), 2854 (w), 1688 (s), 1641 (s), 1582 (s), 1029 (w); ¹H NMR (500 MHz, DMSO-d₆): 9.63 (d, ³J (H,H) = 8.4 Hz, 2H), 8.33 (d, ³J (H,H) = 8.4 Hz, 2H), 8.28 (s, 2H), 4.89 (tt, ³J (H,H) = 12.3, 3.7 Hz, 2H), 2.44-2.36 (m, 4H), 1.86 (d, ³J (H,H) = 12.3 Hz, 4H), 1.74-1.69 (m, 6H), 1.43-1.32 (m, 3H), 1.24 (dd, ³J (H,H) = 13.5, 9.6 Hz, 3H); ¹³C NMR (100 MHz, DMSO-d₆): 163.3 (s), 163.0 (s), 133.7 (s), 131.47 (s), 128.8 (q), 128.7 (q), 128.4 (q), 127.7 (d), 127.3 (d), 122.9 (q), 122.5 (q), 122.1 (d), 120.8 (q), 117.9 (s), 52.8 (d), 28.6 (t), 26.1 (t), 25.2 (t); MS (ESI, DCM/MeOH 1:1): 585 (100, [M - H]).

5.3. Methods

5.3.1. Solvatochromism

In a typical experiment, aliquots (20 µl) of a solution of compound 211 (0.8 mM, DCM/MeOH 9:1) were diluted with air saturated solvents (2 ml, Table 3.2). The solutions were stirred at 25 ± 0.1 °C for 5 minutes before the spectra
acquisition. An addition of TEA (20 μl) was performed for compounds 212 and 215 to achieve complete deprotonation.

5.3.2. pH Dependence

In a typical experiment, aliquots (10 μl) of NaOH (0.05 M) and HCl (0.04 M) were added to a solution of 244 in DMSO (2 ml, 0.04 mM) and water (0.5 ml, 1 mM TEOA pH 7.0). The solutions were stirred at 25 ± 0.1 °C for 5 minutes before measuring the pH by direct insertion of the electrode in the solution. Finally, at stable pH (± 0.01), absorption spectra were recorded after each addition of acid or base. The absorption maxima (λ_{max}) were plotted in function of measured pH and the pKₐ values were determined by fitting the data with Hill equation²⁷¹ (Equation 31).

5.3.3. Fluorescence Quantum Yields

The sample concentration was set in order to have absorption values below 0.1 at the absorption maximum (λ_{abs}). The fluorescence quantum yields (Φ_f) were evaluated based on external standards Rhodamine 6G¹⁸⁵ and IR-140¹⁸⁶, by using the Equation 1¹⁸⁴.

5.3.4. Sensing of DMF Purity

Commercial available DMF (150 ml) was distilled at 165-170°C and collected in 20 consecutive fractions (5 ml). Each fraction was rapidly cooled down in a water bath. A solution of 244 in DCM (200 μl, 0.38 mM) was added
to each fraction (2.5 ml) and the solutions were stirred at 25 ± 0.1 °C for 5 minutes before the spectra acquisition. The ratio \( F \) between the absorption at 538 and 610 nm of 244 was calculated and plotted for each fraction.

### 5.3.5. Boronic Esters as Core Substituents

In a typical experiment, aliquots (10 or 20 µl, 7.0 mM in DMSO) of phenylboronic acid were added to a solution of 245 (0.80 mg, 1.4 µmol) in 4:1 DMSO/H2O (2 ml, 1mM Tris pH 8.00) and the solutions were stirred at 25 ± 0.1 °C for 5 minutes before checking the pH by direct insertion of the electrode in the solution. At stable pH (± 0.01), absorption spectra were recorded after each addition of boronic acid. The absorption maxima (\( \lambda_{\text{max}} \)) were plotted in function of phenylboronic acid concentration in solution and the \( K_D \) values were determined by fitting the data with Hill equation \(^{271} \) (Equation 33). pH profiles of boronic esters were measured and analysed as described before.

### 5.3.6. Oxidative Imination

In a typical experiment, to a solution of 244 (5.0 mg, 9 µmol) in dry ACN (4 ml), CAN (1.2 mg, 2 µmol) and TEMPO (0.7 mg, 4 µmol) were added at rt. The mixture was refluxed at 100 °C in O2 atmosphere. The reaction was followed by UV-Vis measurements of aliquots (200 µl) of the boiling mixture quenched with a solution of aniline (0.5 ml, 5.5 mmol) in 4:1 DMSO/H2O (2.5 ml, 1 mM TEOA pH 7). Constant intensity and shape of the peak at 638 nm suggested that the reaction was completed after 2 h. The red shifted absorption at 638 nm, compared with the absorption of 244 at the same pH, was consistent
with the formation of iminoquinone 266. pH profiles of aromatic imines were measured and analysed as described before.

5.3.7. Cyclic Voltammetry

Cyclic voltammograms of compounds 213, 216 and 217 were determined using cyclic voltammetry (scan rate 100 mV/s, supporting electrolyte: 0.1 M Bu4NPF6, working electrode: glassy carbon, counter electrode: Pt wire, reference electrode: SCE, solvent: DCM). The HOMO, LUMO energies corrected by Fe/Fe⁺ and the energy gap were calculated according to the Equations 25-28.

5.4. Studies in Lipid Bilayer Membranes

5.4.1. DOPC LUVs

A thin lipid film was prepared by evaporating a solution of DOPC (25.0 mg, 0.03 mmol) in MeOH/CHCl₃ 1:1 (2.0 ml) on a rotary evaporator (rt) and then overnight in vacuo. The resulting film was hydrated with 1.0 ml buffer (10 mM Tris, 100 mM NaCl, pH 7.4) for 30 min at rt, subjected to freeze-thaw cycles (7×, liquid N₂, 40 °C water bath) and extrusions (17×) through a polycarbonate membrane (pore size, 100 nm). Final conditions: ~32 mM DOPC; 10 mM Tris, 100 mM NaCl, pH 7.4. The vesicles were used within the week of preparation.
5.4.2. DPPC LUVs

A thin lipid film was prepared by evaporating a solution of DPPC (22.5 mg, 0.03 mmol) in MeOH/CHCl$_3$ 1:1 (2.0 ml) on a rotary evaporator (rt) and then overnight in vacuo. The resulting film was hydrated with 1.0 ml buffer (10 mM Tris, 100 mM NaCl, pH 7.4) for 30 min at 55 °C, subjected to freeze-thaw cycles (7×, liquid N$_2$, 55 °C water bath) and extrusions (21× at 55 °C) through a polycarbonate membrane (pore size, 100 nm). Final conditions: ~31 mM DPPC; 10 mM Tris, 100 mM NaCl, pH 7.4. The vesicles were used within the week of preparation.

5.4.3. EYPC LUVs (KCl)

A thin lipid film was prepared by evaporating a solution of EYPC (25 mg, 0.03 mmol) in MeOH/CHCl$_3$ 1:1 (2.0 ml) on a rotary evaporator (40 °C) and then overnight in vacuo. The resulting film was hydrated with 1 ml buffer (100 mM KCl, 10 mM Tris, pH 7.0) for 30 min at 37 °C, subjected to freeze-thaw cycles (7×, liquid N$_2$, 37 °C water bath) and extrusions (15×) through a polycarbonate membrane (pore size, 100 nm). Extravesicular components were removed by size exclusion chromatography (Sephadex G-50, Sigma-Aldrich) with 100 mM NaCl, 10 mM Tris, pH 7.0. The collected fractions were diluted with buffer to 6 ml. Final conditions: ~2.5 mM EYPC; inside: 100 mM KCl, 10 mM Tris, pH 7.0; outside: 100 mM NaCl, 10 mM Tris, pH 7.0. The vesicles were used within the week of preparation.
5.4.4. Temperature Dependence (DPPC, DOPC)

In a typical procedure, to a 1900 µl gently stirred, thermostated buffer (25 ± 0.1 °C, 10 mM Tris, 100 mM NaCl, pH 7.4) in a glass cuvette, DOPC LUVs (100 µl) and 211 (20 µl, 0.8 mM in DMSO) were added. The solution was stirred at 25 ± 0.1 °C for 30 minutes before the spectra acquisition (λ<sub>ex</sub> = 430 nm, λ<sub>em</sub> = 600 nm). The temperature was then raised to 55 ± 0.1 °C and the solution was kept at this temperature for 45 minutes before the spectra acquisition. Then the temperature was lowered down to 25 ± 0.1 °C and the spectra were acquired after 45 minutes. The temperature cycle was repeated a second time. The same procedure was applied for DPPC LUVs (100 µl).

5.4.5. Membrane Potential Calibration

Adapted from established procedures, 200 µl EYPC LUVs (KCl) and safranin O (16 µl, 60 µM) were added to 1684 µl gently stirred, thermostated buffer (according to the Table 14, 25 ± 0.1 °C, 100 mM MCl, 10 mM Hepes, pH 7.0) in a disposable plastic cuvette. The time-dependent change in fluorescence of safranin O (λ<sub>em</sub> = 581 nm; λ<sub>exc</sub> = 522 nm) was monitored during the addition of valinomycin (20 µl, 100 µM DMSO solution) at t = 50 s. The fluorescence intensity (I) increased as the membrane potential was built up to the expected membrane potential, which was reached after 250 s. At the end of the experiment, melittin (40 µl, 100 µM in water) was added at t = 300 s. The fluorescence intensities were normalized (Equation 3.30) and plotted against the membrane potential calculated by the Nernst equation (Equation 29).
5.4.6. Dependence on Membrane Potential (EYPC)

In a typical experiment, to a gently stirred, thermostated buffer (1700 µl, 25 ± 0.1 °C, 100 mM MCI (Na/K ratio: 1024:1), 10 mM Tris, pH 7.0) in a glass cuvette, EYPC LUVs (KCl) (200 µl) and 211 (20 µl, 0.8 mM in DMSO) were added. After 4 min the emission and excitation spectra were acquired (λ<sub>ex</sub> = 430 nm, λ<sub>em</sub> = 600 nm). Valinomycin (20 µl, 100 µM in DMSO) was added at t = 6 min. Once the theoretical membrane potential was established at t = 10 min the emission and excitation spectra were acquired. The membrane potential was then destroyed by the addition of melittin (40 µl, 100 µM in water) at t = 15 min and the spectra were acquired to verify the reversibility of the red shift, which was confirmed in every case.

5.4.6. Time-Dependent Fluorescence in Polarized Vesicles

In a typical experiment, to 1700 µl gently stirred, thermostated buffer (25 ± 0.1 °C, 100 mM MCI (Na/K ratio: 1024:1), 10 mM Tris, pH 7.0) in a glass cuvette, 200 µl EYPC LUVs (KCl) and 211 (10 µl, 0.8 mM in DMSO) were added. The time-dependent change in fluorescence intensity (λ<sub>em</sub> = 630 nm, λ<sub>exc</sub> = 440 nm) was monitored during the addition of valinomycin (20 µl, 100 µM in DMSO, at t = 50 s) and melittin (40 µl, 100 µM in water, at t = 125 s). The fluorescence traces were normalized according to the Equation 30.
### 5.5. Abbreviations

#### 5.5.1. Analytics

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>$^1$H-NMR</td>
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<tr>
<td>COSY</td>
<td>Correlation Spectroscopy</td>
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<td>DEPT</td>
<td>Distortionless enhancement by polarization transfer</td>
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<td>Electron Impact Ionization</td>
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<tr>
<td>SCE</td>
<td>Saturated calomel electrode</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Vis</td>
<td>Visible</td>
</tr>
<tr>
<td>$z$</td>
<td>Charge</td>
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</table>

#### 5.5.2. Solvents

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DMAc</td>
<td>$N,N$-Dimethylacetamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
</tbody>
</table>
DMF \( N,N\text{-Dimethylformamide} \)
MeOH Methanol
iPrOH Isopropanol
TEA Triethylamine
TEAO Triethanolamine
TFA Trifluoroacetic acid
TFE 2,2,2-Trifluoroethanol
THF Tetrahydrofuran

5.5.3. Chemicals and Reagents

Fe Ferrocene
HBTU \( O\text{-}(\text{Benzotriazol-1-yl})-N,N,N',N'\text{-tetramethyluronium} \)
CAN Cerium (IV) ammonium nitrate
TEMPO 2,2,6,6-Tetramethylpiperidinoxy
DIBAL Diisobutylaluminum hydride
DOPC 1,2-dioleoyl-sn-glycero-3-phosphocholine
DPPC 1,2-dipalmitoyl-sn-glycero-3-phosphocholine
EYPC Egg yolk phosphatidylcholine
NIS \( N\text{-Iodosuccinimide} \)
PBA Phenyl boronic acid
Tris Tris(hydroxymethyl)aminomethane
TsOH•H2O \( p\text{-Toluenesulfonic acid monohydrate} \)
Hepes 4-(2-Hydroxyethyl)piperazine

5.5.4. Symbols and Other Abbreviations

1D One-dimensional
2D Two-dimensional
\( \delta \) Chemical shift
\( \Delta \) Difference
\( \Delta \psi \) Transmembrane potential
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>$\varepsilon_1$</td>
<td>Relative permittivity</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>Wavelength</td>
</tr>
<tr>
<td>$\mu$</td>
<td>Dipole moment</td>
</tr>
<tr>
<td>$\mu\text{m}$</td>
<td>Micrometer</td>
</tr>
<tr>
<td>$\mu\text{M}$</td>
<td>Micromolar concentration</td>
</tr>
<tr>
<td>$\nu$</td>
<td>Frequency</td>
</tr>
<tr>
<td>$\tau$</td>
<td>Fluorescence lifetime</td>
</tr>
<tr>
<td>$\Phi$</td>
<td>Fluorescence quantum yield</td>
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<tr>
<td>$\psi_s$</td>
<td>Membrane surface potential</td>
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<tr>
<td>$\psi_d$</td>
<td>Membrane dipole potential</td>
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<tr>
<td>$\AA$</td>
<td>Angstrom</td>
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<tr>
<td>$a$</td>
<td>Onsager radius</td>
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<td>abs</td>
<td>Absorbance</td>
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<tr>
<td>c</td>
<td>concentration</td>
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<tr>
<td>$c$</td>
<td>Light speed in vacuum</td>
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<tr>
<td>CT</td>
<td>Charge-transfer</td>
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<tr>
<td>CV</td>
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<tr>
<td>D</td>
<td>Debye</td>
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<tr>
<td>e or $S_1$</td>
<td>First excited state</td>
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<tr>
<td>E</td>
<td>Energy</td>
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<tr>
<td>EDG</td>
<td>Electron-donating group</td>
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<tr>
<td>em</td>
<td>Emission</td>
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<tr>
<td>EWG</td>
<td>Electron-withdrawing group</td>
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<td>ex</td>
<td>Excitation</td>
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<td>f</td>
<td>Solvent polarizability</td>
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<tr>
<td>g or $S_0$</td>
<td>Ground state</td>
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<td>GUVs</td>
<td>Giant unilamellar vesicles</td>
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<td>$h$</td>
<td>Plank constant</td>
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<tr>
<td>H</td>
<td>Enthalpy</td>
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<tr>
<td>HOMO</td>
<td>Highest occupied molecular orbital</td>
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<tr>
<td>I</td>
<td>Integrated intensity</td>
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<tr>
<td>$K_a$</td>
<td>Acid dissociation constant</td>
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<tr>
<td>$K_D$</td>
<td>Dissociation constant</td>
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<td>J</td>
<td>Coupling constant</td>
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<tr>
<td>$L_{\alpha}$</td>
<td>Liquid-disordered phase</td>
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<td>$L_\beta$ or $L_{\beta}$</td>
<td>Solid-ordered phase</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>LUMO</td>
<td>Lowest unoccupied molecular orbital</td>
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<td>Refractive index</td>
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<td>Electric field</td>
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<td>Reduction</td>
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<td>Room temperature</td>
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<td>Standard</td>
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<tr>
<td>T\textsubscript{m}</td>
<td>Lamellar chain melting temperature</td>
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