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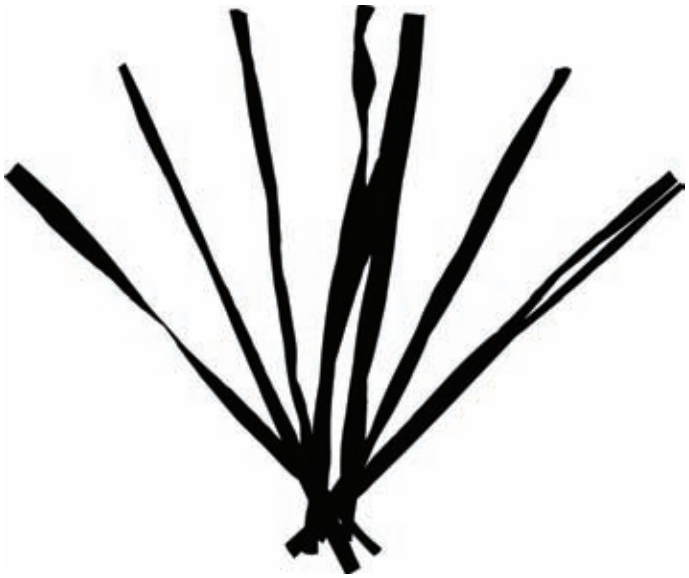
# Conjugated Polymers, Amyloid Detection and Assembly of Biomolecular Nanowires

Anna Herland



Biomolecular and Organic Electronics
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Cover: There are fiber-like objects on all scales. The cover page shows seven leaves of an Elytrigia specimen grown on the grounds of Lilla Berga Norrgård.

During the course of the research underlying this thesis, Anna Herland was enrolled in Forum Scientium, a multidisciplinary doctoral programme at Linköping University, Sweden.



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Till mina kära

# ABSTRACT

The research field of conjugated polymers has grown due to the optical and electronic properties of the material, useful in applications such as solar cells and printed electronics, but also in biosensors and for interactions with biomolecules. In this thesis conjugated polymers have been used in two related topics; to detect conformational changes in proteins and to assemble the polymers with biomolecules into nanowires.

Within biosensing, conjugated polymers have been used for detection of a wide range of biological events, such as DNA hybridization or enzymatic activity, utilizing both electronic and optical changes in the polymer. Here the focus has been to use the polymers as optical probes to discriminate between native and misfolded protein, as well as to follow the misfolding processes in vitro. The understanding and detection of protein misfolding, for example amyloid fibril formation, is a topic of growing importance. The misfolding process is strongly associated with several devastating diseases such as Alzheimer's disease, Parkinson's disease and Bovine Spongiform Encephalopathy (BSE). We have developed detection schemes for discrimination between proteins in the native or amyloid fibril state based on luminescent polythiophene derivatives. Through a synthesis strategy based on polymerization of trimer blocks rather than of monomers, polythiophene derivatives with higher optical signal specificity for amyloid-like fibrils were obtained.

Self-assembly of nanowires containing conjugated polymers is a route to generate structures of unique opto-electrical characteristics without the need for tedious topdown processes. Biomolecules can have nanowire geometries of extraordinary aspect ratio and functionalities. The DNA molecule is the most well known and exploited of these. In this thesis work the more stable amyloid fibril has been used as a template to organize conjugated polymers. Luminescent, semi-conducting, conjugated polymers have been incorporated in and assembled onto amyloid fibrils. Using luminescence quenching we have demonstrated that the conjugated material can retain the electro-activity after the incorporation process. Furthermore, the amyloid fibril/conjugated polymer hybrid structures can be organized on surfaces by the means of molecular combing and soft lithography.

In the process of generating self-assembled biomolecular nanowires functionalized with conjugated polymers, we have shown a new synthesis strategy for a water-

soluble highly conducting polythiophene derivative. This material, PEDOT-S, has shown affinity for amyloid fibrils, but can also be very useful in conventional opto-electronic polymer-based devices.

# POPULÄRVETENSKAPLIG SAMMANFATTNING

Arbetet i den här avhandlingen innefattar två områden som förefaller vitt skilda åt, nya verktyg för biokemisk/medicinsk analys och nya tillverkningsmetoder för att tillverka optiska eller elektriska komponenter på nanoskala (storlekar som är en miljondel av en millimeter). Det aktiva material som återkommer i alla uppsatser i avhandlingen är så kallade konjugerade polymer. Konjugerade polymerer är företrädesvis kolbaserade polymerer med en kemisk struktur bestående av alternerande enkel- och dubbelbindingar mellan kolatomerna. Den alternerande bindningsstrukturen ger polymererna speciella optiska och elektroniska egenskaper. De kan vara elektriskt ledande, halvledande eller isolerande, absorbera ljus av definierade våglängder och även fluorescera beroende på sitt tillstånd. Konjugerade polymerer har använts som aktivt material i solceller, lysdioder, tryckt elektronik, men även i biosensorer.

Biosensorer blir idag allt viktigare för att snabbt, tillförlitligt och enkelt kunna utföra medicinska analyser. Ju tidigare en sjukdom kan detekteras, kanske redan innan den brutit ut, desto troligare är det att den kan botas. En klass av sjukdomar som ökat de senare åren är Alzheimers och Parkinsons sjukdomar. Relaterat till dessa åkommor och även ett ökat antal andra sjukdomar, så som BSE (galna kosjukan), är att normalt förekommande proteiner får fel veckning, fel geometri. Funktionella proteiner har en definierad tredimensionell nativ struktur, men i dessa sjukdomar återfinns oftast ett speciellt protein som aggregat, amyloida plack, av långa trådar, amyloidfibrer. Den exakta relationen mellan amyloid och sjukdomsförloppen är inte helt känd. Dessa amyloidfibrer kan också bildas i provrör av flertalet proteiner. I tre av uppsatserna i den här avhandlingen använder vi konjugerade polymerer för att studera om de vanligt förekommande proteinerna insulin och lysozym är i nativt eller fibrillärt tillstånd. Genom att använda optisk spektroskopi, i absorbans eller fluorescens, kan vi avgöra om polymerna interagerar med nativt eller fibrillärt protein. Man kan uttrycka det som att polymeren ändrar färg beroende på hur proteinet ser ut. Den metoden har visat sig även fungera på vävnadsprover med amyloida plack och kan vara lovande för att bättre utreda samband mellan amyloidbildning och sjukdomstillstånd.

Ett annat område som idag utvecklas mycket snabbt är elektriska komponenter. Efterfrågan på allt snabbare och mindre strukturer i dessa komponenter är stor. De

traditionella tillverkningsmetoderna börjar dock nå en gräns för vad som är fysikaliskt möjligt att konstruera. En alternativ möjlighet, som idag är på forskningsstadiet, för att tillverka mycket små komponenter är att låta nanometerstora objekt med specifika egenskaper självmontera sig. Denna strategi kan återfinnas överallt i naturen, där enklare molekyler blir till funktionella strukturer som proteiner, vilka i sin tur bygger upp celler och till slut hela organismer. Istället för att själva, med kemisk syntes, försöka tillverka ett självmonterande system så har vi använt en naturlig struktur, amyloidfibrer. Amyloidfibrer är mycket tunna, ca 10 nanometer (ca 10000 ggr tunnare än ett mänskligt hårstrå), och kan vara 10 mikrometer, dvs. 1000 gånger längre än sin bredd . Dessutom är de, om man jämför med andra biologiska strukturer, mycket stabila. Vi har både byggt in och dekorerat amyloidfibrer med konjugerade polymer för att ge dem optisk och elektronisk funktionalitet. Efter organisering av dessa funktionaliserade fibrer på ytor har vi studerat dem framförallt med mikroskopi och optiska metoder, men även elektriska metoder. Ambitionen är att i framtiden organisera och kombinera fibrerna så att elektriska och/eller optiska komponenter kan skapas på nanoskala.

# LIST OF ARTICLES

## *Articles included in this thesis:*

**Article I**

Anna Herland, K Peter R Nilsson, Johan D M Olsson, Per Hammarström, Peter Konradsson and Olle Inganäs, *Synthesis of a regioregular zwitterionic conjugated oligoelectrolyte, usable as an optical probe for detection of amyloid fibril formation at acidic pH*, J Am Chem Soc. 2005, **127** (7): 2317-2323

**Article II**

K Peter R Nilsson, Anna Herland, Per Hammarström, and Olle Inganäs, *Conjugated polyelectrolytes: Conformation-sensitive optical probes for detection of amyloid fibril formation*, Biochemistry. 2005, **44** (10): 3718-3724

**Article III**

Andreas Åslund, Anna Herland, Per Hammarström, K Peter R Nilsson, Bengt-Harald Jonsson, Olle Inganäs and Peter Konradsson, *Studies of luminescent conjugated polythiophene derivatives - Enhanced spectral discrimination of protein conformational states*, accepted in Bioconjugate Chemistry

**Article IV**

Anna Herland, Per Björk, K Peter R Nilsson, Johan D M Olsson, Peter Åsberg, Peter Konradsson, Per Hammarström and Olle Inganäs, *Electroactive luminescent self-assembled bio-organic nanowires: Integration of semiconducting oligoelectrolytes within amyloidogenic proteins*, Advanced Materials. 2005, **17** (12): 1466-1471
Correction published due to typesetting error: Adv Mat. 17 (14): 1703

**Article V**

Anna Herland, Per Björk, P Ralph Hania, Ivan G Scheblykin and Olle Inganäs, *Alignment of a conjugated polymer onto amyloid-like protein fibrils*, Small. 2007, **3** (2): 318-325

**Article VI**

Anna Herland, Daniel Thomsson, Oleg Mirzov, Ivan G Scheblykin and Olle Inganäs, *Decoration of amyloid fibrils with luminescent conjugated polymers*, In manuscript

**Article VII**

Roger H Karlsson, Anna Herland, Mahiar Hamedi, Peter Konradsson and Olle Inganäs, *Iron Catalyzed Polymerization of Alkoxysulfonate-Functionalized EDOT gives Water-soluble PEDOT of High Conductivity*, Submitted to Chemistry of Materials

## *My contribution to the articles included in the thesis:*

**Article I**:

All experimental work, except synthesis, together with KPR Nilsson and the major part of the writing

**Article II**:

Major part of the experimental work together with KPR Nilsson and a minor part of the writing

**Article III**:

All experimental work, except synthesis, together with A Åslund and the major part of the writing together with A Åslund.

**Article IV**:

All experimental work, except synthesis, partly together with P Björk and KPR Nilsson, and the major part of the writing.

**Article V**:

All experimental work, partly together P Björk and R Hania, and the major part of the writing.

**Article VI**:

All experimental work, partly together with D Thomsson, and the major part of the writing.

**Article VII**:

All experimental work, except synthesis, together with M Hamedi and RH Karlsson. Most of the writing.

## *Related articles not included in the thesis:*

Anna Herland and Olle Inganäs, *Conjugated polymers as optical probes for protein interactions and protein conformations*, review Macromolecular Rapid Communications, DOI: 10.1002/marc.200700281

Per Björk, Anna Herland, Ivan G Scheblykin and Olle Inganäs, *Single molecular imaging and spectroscopy of conjugated polyelectrolytes decorated on stretched aligned DNA*, Nano Letters. 2005, **5** (10): 1948-1953

K Peter R Nilsson, Per Hammarström, Fredrik Ahlgren, Anna Herland, Edrun A Schnell, Mikael Lindgren, Gunilla T Westermark and Olle Inganäs, *Conjugated polyelectrolytes - Conformation-sensitive optical probes for staining and characterization of amyloid deposits*, Chembiochem. 2006, **7** (7): 1096-1104

K Peter R Nilsson, Andreas Åslund, Ina Berg, Sofie Nyström, Peter Konradsson, Anna Herland, Olle Inganäs, Frantz Stabo-Eeg, Mikael Lindgren, Gunilla T Westermark, Lars Lannfelt, Lars N G Nilsson and Per Hammarström, *Imaging Distinct Conformational States of Amyloid-beta Fibrils in Alzheimer´s Disease Using Novel Luminescent Probes*, Accepted in ACS Chemical Biology

Jimmy Wiréhn, Karin Carlsson, Anna Herland, Egon Persson, Uno Carlsson, Magdalena Svensson, and Per Hammarström, *Activity, folding, misfolding, and aggregation in vitro of the naturally occurring human tissue factor mutant R200W*, Biochemistry. 2005, **44** (18): 6755-6763

Rodrigo M Petoral, Anna Herland, Klas Broo and Kajsa Uvdal, *G-protein interactions with receptor-derived peptides chemisorbed on gold*, Langmuir. 2003, **19** (24): 10304-10309

## *Patent applications*

K Peter R Nilsson, Anna Herland, Per Hammarström, Per Björk and Olle Inganäs, *Methods using self-assembly/aggregation of biomolecules for the construction of electronic devices based on conjugated polymers*. PCT/SE/2005/001021

K Peter R Nilsson, Anna Herland, Per Hammarström, Peter Åsberg and Olle Inganäs, *Methods for determinating conformational changes and self-assembly of proteins*, PCT/SE2005/000248

# ACKNOWLEDGEMENTS - TACK

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Det här doktorsarbetet hade inte gått att utföra och definitivt inte blivit lika roligt utan en mängd samarbeten. Jag vill börja med samarbetspartners utanför Linköping.

Ivan Scheblykin and his coworkers at Chemical Physics in Lund; Daniel, Ralph and Oleg, thank you so much for introducing me in the SMS (single molecular spectroscopy if anyone thought of something else) world and spending all those hours in the dark room trying to see those little green or red threads.

Även om vi (ännu) inte lyckats få ihop allt vårt arbete till en publikation så vill jag tacka Alf Månsson och Martina Balaz, Högskolan i Kalmar. Det var verkligen intressant och spännande att lära sig hur man kan hantera motorproteiner, synd att de inte var lika förtjusta i våra polymerer. Mikael Lindgren and Frantz på NTNU Trondheim, tack för alla era ansträngningar med mina konstiga prover.

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Mahiar - Det finns så många utmaningar som jag hoppas att vi kommer möta ihop. Nu har du hjälpt mig med den här utmaningen, tack för det. Dostet daram, azizam.

Anna
Lilla Berga Norrgård
Juli 2007

# TABLE OF CONTENTS

# 1 GENERAL INTRODUCTION

This thesis is based on exploration of the chemical and optical properties of semiconducting organic polymers, π-conjugated polymers. In 1977, Heeger, McDarmid and Shirakawa demonstrated that a certain class of polymers, conjugated polymers (CPs), can be converted to a metallic conducting state if exposed to chemical dopants [1]. This discovery opened the doors to an extensive field of research and was awarded with the Nobel Prize in Chemistry (2000). The applications of CPs are numerous, but the main research developed into the use of the material as easy processable and flexible semiconductors as the active component in devices such as organic light emitting diodes (OLEDs), solar cells and transistors. It was early discovered that the optical and electronic function of these materials is highly dependent on the organization of the material, which naturally resulted in numerous structural studies and development of self-assembly processes of CPs. With new chemical modifications of CPs also water-soluble conjugated polyelectrolytes were realized. This opened the door to the combination of conjugated polymers and biological molecules, which has been explored both from the aspect of biosensing and biomolecule assisted self-assembly.

I started my thesis work with the aim to develop biomolecule assisted self-assembly of CPs for formation of functional devices on the nanoscale. DNA, which has an inherent genetic code and extraordinary aspect ratios, has been a natural choice in many templated self-assembly processes. Together with Per Björk I started exploring combinations of CPs and DNA, but later I changed to mainly work with amyloid protein fibrils as the biomolecular material. The study of amyloid fibrils and precursors thereof is an intense field of research due to increasing knowledge of the association between these states of misfolded protein and pathogenic states such as Alzheimer's disease and spongiform encephalopathies. From the viewpoint of self-assembly the amyloid fibrils are stable and of a defined nanostructure, but variable with respect to the starting monomeric protein or peptide. In the self-assembly studies we discovered that the optical properties, in terms of absorption and emission, of certain CPs were altered when interacting with proteins in native compared to the misfolded amyloid state. These discoveries resulted in the division of my thesis work in two paths:

- The use of amyloid fibrils for self-assembly of CPs, in the semiconducting, luminescent state and the metallic state, into nanowire structures.
- The use of semiconducting, luminescent CPs as optical probes for detection of amyloid fibrillation processes.

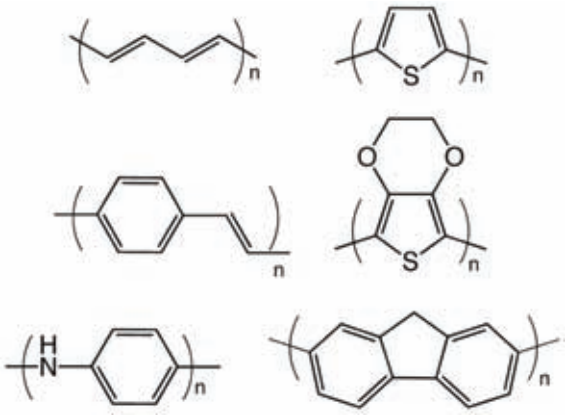
If these two paths are put in a context of future development and research, they are dealing with two very big areas, namely development of electronics and monitoring of human health. All wealthier countries in the world are facing an aging population, with an expected increase of diseases related to age, such as Alzheimer's. New and better methods for monitoring health will be a growing demand from these populations. Electronics is fundamental in the modern world and with the on-going development towards smaller and faster components, there is a need for new construction methods. Although the research is still far from industrial applications, self-assembly might be one of those methods. If possible, many consumers are most likely ready to incorporate electronic functions in many more products used in their daily life, such as parts of their homes or clothes. Conjugated polymers may give the possibility to give flexible and cheap components in such products. It is also possible that the combination of biomolecules with conjugated polymers will lead to electronic materials suitable for integration in implants to give important functions, such as possibility of stimulation or detection of biological events.

The following chapters will serve as an introduction to the publications included in this thesis, but also cover some material not yet included in publications. In Chapter 2 conjugated polymers are generally discussed, in terms of their physical properties and different classes of CPs. Chapter 3 covers the use of CPs as optical probes for detection of protein interactions and protein conformations. In chapter 4 the topic is self-assembly of nanowires and especially nanowires with biological templates and/or with CPs as a functional material. Chapter 5 is a short outlook how the themes covered in this thesis could be developed. Chapter 6 is a summary of the included publications in this thesis.

# 2 CONJUGATED POLYMERS MATERIAL PROPERTIES

## 2.1 Chemical and electronic structure

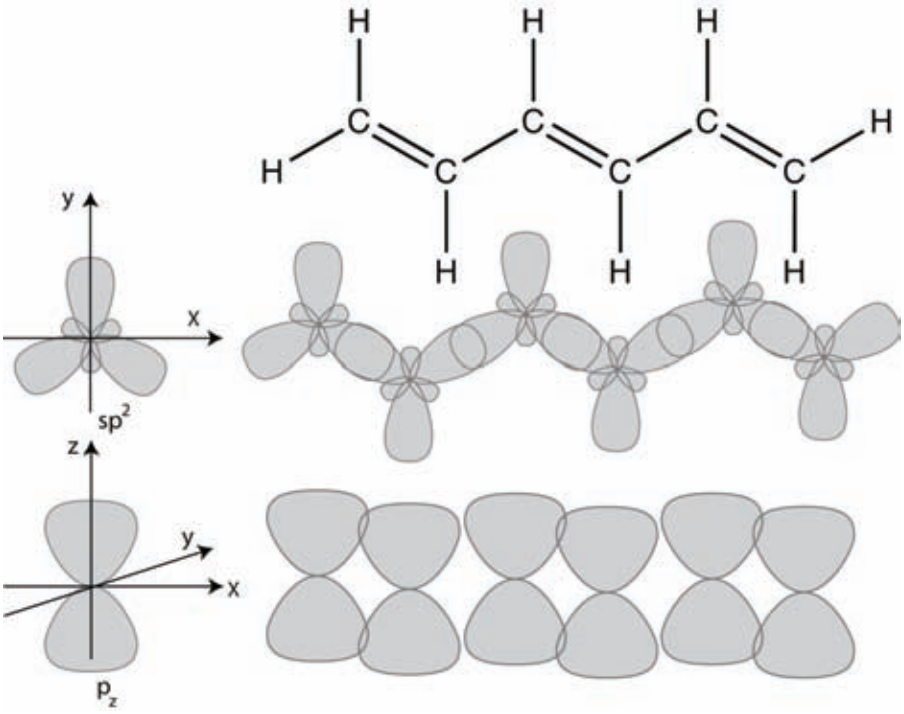
Polymers are macromolecules consisting of repeating units, mers. Natural polymers, e.g. proteins, DNA or starch are essential for our daily life, but today most of us are dependent also on synthetic polymers, such as carbon-based polyethene or polystyrene. The number of repeating units of a polymer must be so large that the addition or removal of one repeating unit has no effect of the physical properties of the material. A macromolecule consisting of fewer units is defined as an oligomer.




*2.1 Chemical structures of some of the most frequently used conjugated polymers, from left to right and top to bottom; trans-polyacetylene, polythiophene, polyparavinylene phenylene (PPV), PEDOT, polyaniline and polyfluorene.*

Conjugated polymers (CPs) are characterized by a polymer backbone consisting of alternating single and double bonds. Trans-polyacetylene (see figure 2.1), a polymer consisting only of carbon atoms connected with an alternating bond pattern, is a good model compound for illustrating the electronic structure of CPs. The carbon atoms in this configuration will be unsaturated with a $sp^2$ hybridization and one remaining $p_z$ orbital on each carbon. The $sp^2$ hybrid orbitals are organized in one plane and forming σ-bonds, with strongly localized electrons, between adjacent carbon atoms (see figure

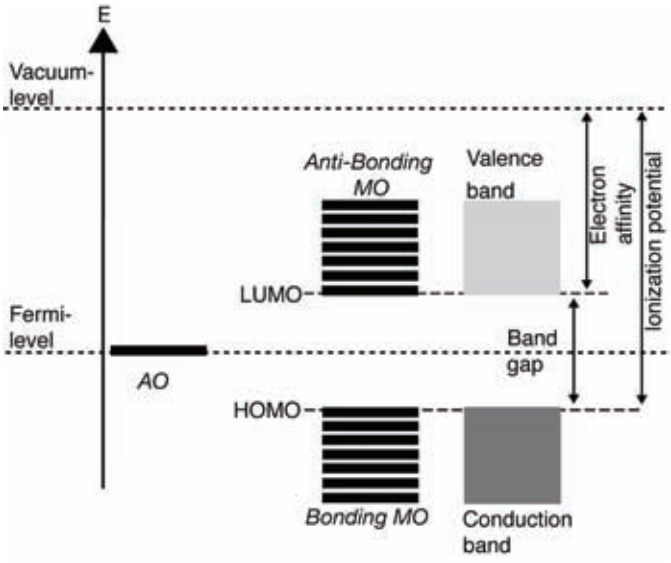
2.2). The $p_z$ orbitals have a perpendicular orientation respective to the polymer backbone. Overlapping $p_z$ orbitals constitute $\pi$-bonds between neighboring atoms, giving delocalization of $\pi$-electrons over the polymer chain. The $\pi$-bonds are considered as the main source of charge transport in conjugated systems [2]. The distance along the polymer chain, over which the electrons are delocalized, is termed the conjugation length.




*2.2 Top: The chemical structure of trans-polyacetylene. Middle: The orientation of the $sp^2$ hybrid orbitals, overlapping orbitals forming $\sigma$-bonds, with strongly localized electrons, between adjacent carbon atoms. Bottom: The $p_z$ orbitals are perpendicularly oriented relative the polymer chain. Overlapping orbitals form $\pi$-bonds. Note that the polymer chain is drawn as a dimerized structure with alternating single and double bonds.*

If the $\pi$-electrons were delocalized over a whole, long, polyacetylene molecule, giving all bonds in the chain equal length, this material would behave as a one-dimensional metal. However, the Peierls distortion theorem demonstrates that the polymer chain is more stable in a so-called dimerized state with an alternating pattern of single- and double bonds [3]. This alternating bond pattern can be interchanged with preserved ground state energy, meaning that polyacetylene has a degenerate ground state.

If the electronic structure of the polymer is described in terms of energy bands, the dimerization will give rise to the appearance of a band gap around the Fermi level (figure 2.3). The anti-bonding orbitals (π*), located higher in energy, form a conduction band, with the lowest state named LUMO (lowest unoccupied molecular orbital). The valence band is formed by the molecular orbitals with lower energy, the bonding orbitals (π), with the HOMO (highest occupied molecule orbital) as an upper energy limit. The bandgap of most conjugated polymers is within the semiconductor to insulator range, 1 – 4 eV.



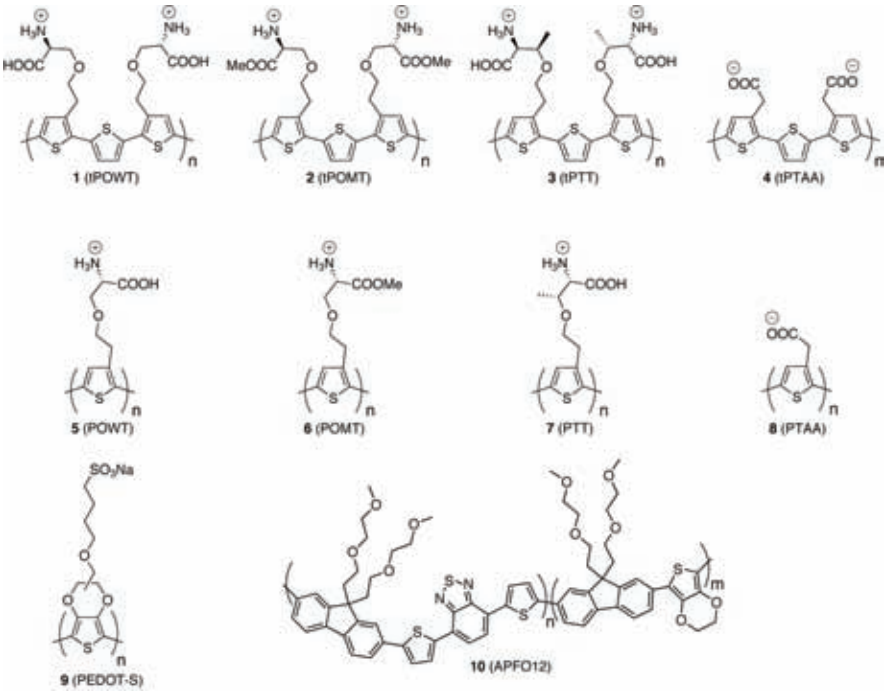

*2.3 The atomic orbitals (AO) on each bonded atom hybridize into molecular orbitals (MO). The dimerization of a polymer chain with give rise to a band gap around the Fermi level $E_F$ , separating the bonding from the antibonding MOs. Due to the high density of MOs in a conjugated polymer, the electronic structure can be described with electronic bands, the conduction band with HOMO (highest occupied molecular orbital) as the highest energy level and the valence band with LUMO (lowest unoccupied molecular orbital) as the lowest energy level. Illustrated are also the physically important energy parameters, ionization potential and electron affinity.*

Trans-polyacetylene, is the geometrically simplest conjugated polymer. Today a variety of conjugated polymers have been demonstrated, many of them with carbon ring structures as a component of the backbone (see figure 2.1). The studies presented in this thesis are based on polythiophenes. Polythiophenes have, like most conjugated polymers, a non-degenerate ground state. The ground state corresponds to the single geometry of the lowest energy, which is the aromatic form (see figure 2.6). The

quinoid structure, with an interchanged bond alternation pattern, will be of higher energy, due to a higher degree of orbital overlap. The overlapping $p_z$ orbitals of a CP will result in a planar geometry of the polymer backbone, giving a rigid material with high melting points and low solubility. Processing of these materials can be accomplished through the use of non-conjugated soluble precursor polymers, which easily, e.g. by heating, are transformed to a conjugated material. More commonly, the conjugated polymer backbone is substituted with side groups such as alkane chains to increase the solubility in organic solvents.

## 2.2 Conjugated polyelectrolytes

To enable solubility of conjugated polymers, side chain substitutions are, as mentioned above, a necessity. Solubility in polar solvents such as water can be realized through introduction of permanent ionic charges on the side chain of a conjugated polymer, giving a conjugated polyelectrolyte material. A number of conjugated polyelectrolytes have been reported in the literature, a few example are polythiophenes [4-7], polyaniline [8], polyphenylene vinylene [9], polyphenylene ethynylene [10]. In this thesis both polydisperse and well–defined regioregular polythiophene-based conjugated polelectrolytes were studied, see figure (figure 2.4).




*2.4 The conjugated polymers used most frequently during this thesis work. Compound 1-4 is so called trimer-based conjugated polyelectrolytes with a thiophene backbone. Compound 5-8 is the monomer-based analogues of 1-4.*

Water solubility can be desirable to ease the processing of CPs for electronic applications, but also opens the door for the combination of conjugated polymers and functional biomolecules, such as proteins or DNA. The ionic side chain of the conjugated polyelectrolyte can give a material sensitive to changes in pH, due to altered net charge of the polymer, and enables interaction with other charged species such as small ions or larger biomolecules. An interaction between a conjugated polymer and a biomolecule can naturally also be mainly governed by hydrophobic interactions with the polymer backbone. A number of biosensors (some examples are [11-18]) and self-assembly systems [19-22] have been realized through interactions between conjugated polymers and biomolecules, a selection of them will be further described in chapter 3 and 4.

Alternations in the polymer net charge and the interaction between the polymer and another species will affect the organization of the polymer in solution, through changed inter-chain interactions, i.e. aggregation, and/or changes of main chain (backbone) geometry. Compared to the number of studies done on conjugated

polyelectrolytes, the literature of aggregation phenomena in nonconjugated polyelectrolytes is vast and has been extensively reviewed by Dobrynin and Rubenstein [23]. A polymer backbone in a poor solvent, such as a conjugated backbone in water, tends to collapse into spherical globules. Upon increased charging of the globule, e.g. by increasing ionization of the sidechains, a critical value can be reached and the globule collapses into several smaller units. However, it is important to keep in mind that a conjugated backbone is stiffer than most non-conjugated polymer backbones. A quantitative value of the stiffness is the persistence length, which for poly-3-hexylthiophene in THFd8 was measured to 33 Å and for polystyrene to 10 Å as an example [24]. Polyelectrolytes in semidilute concentrations have even more complex behavior since avalanche condensation of counter ions can lead to phase separation of the solution into dilute and concentrated phases [23]. This complex behavior must be considered in all applications of conjugated polyelectrolytes.

We have initiated studies on the aggregation behavior of the polythiophenes used in this thesis, using analytical ultracentrifuge, dynamic light scattering (DLS) and fluorescence correlation spectroscopy (FCS). All results indicate that the polythiophenes are in clustered state in water-based solutions. Analytical ultracentrifuge showed that the monodisperse polymers tPOWT and tPTAA are in clusters of ~10 polymer chains on average, in acidic and basic conditions respectively (article III). Light scattering studies of the polydisperse POWT showed that large aggregates, diameter up to 1 μm, exist both in pure water solution and in buffered conditions (unpublished results). FCS results support DLS, diffusion times indicated that emissive aggregates of POWT in water constitute of, on average, 500 polymer chains if an average molecular weight (3400 g/mol) is assumed (unpublished results).

## *2.3 PEDOT analogues*

Since the discovery of highly conductive polyacetylene considerable effort has been put into research to develop polymer materials that are stable in the conductive state, easy processable and of reasonable cost. One of the few examples that fulfills these requirements is polyaniline and derivatives thereof [25]. However, the possible presence of benzidine units in the polymer backbone can give rise cancerogenic compounds upon degradation [26], which limits the use both in research and industrial applications. Another example of a polymer structure that fulfill these requirements is a polythiophene derivative, poly(3,4-ethylenedioxy-thiophene) also known as PEDT or PEDOT, which was developed at Bayer AG in the late 1980s [27] (see figure 2.1). Through the introduction of a dioxyethylene substitution on the

thiophene ring the undesired (α,β) and (β,β) coupling is avoided, and gives a material that is air-stable in its doped, oxidized form, due to its electron-rich character.

Today PEDOT is a commercial product of Bayer AG and one of the most well known π-conjugated polymers, thanks to the excellent electrical conductivity and electro-optic properties, as well as a good film forming properties. PEDOT is used in numerous applications, commercially in antistatic coatings in photographic films, and in research in organic devices, such as OLEDs, photovoltaic devices and printable electronics, but also in the fields of neural interconnects and biosensors [26, 28, 29].

Standard oxidative chemical or electrochemical polymerization of EDOT, 3,4-ethylene dioxythiophene, yields PEDOT as an insoluble polymer. PEDOT can have as high conductivity as 1000 S/cm when prepared with vapor phase polymerization [30]. The solubility problem was circumvented through the polymerization of EDOT in aqueous dispersions of a water-soluble polyelectrolyte, poly(styrene sulfonic acid) PSS, which gives the material PEDOT/PSS. Apart from making the PEDOT water-soluble, the PSS acts as a dopant of the conjugated polymer and gives the dispersion good film forming properties. The PEDOT/PSS is commercially available under the name Baytron P in grades of different conductivity and particle size. Through various additives the conductivity of the material can be increased $10^3$ times from the normal value of 0.8 ± 0.1 S/cm (0.03 S/cm when prepared in our lab) and surface adhesion properties can be altered [28].

Although chemically prepared PEDOT/PSS is a versatile material, e.g. for the applications *vide supra*, the dispersion has some more troublesome properties. The exact composition of PEDOT/PSS is complex to determine, making it more difficult to elucidate the cause of the physical or chemical changes of the material. PEDOT/PSS contains an excess of PSS in a dispersion of micellar structures. Upon film formation by spin coating a granular structure is obtained, with grains of doped PEDOT/PSS surrounded by a shell of PSS [28]. Additives that significantly change the conductivity of PEDOT/PSS, e.g. diethyleneglycol DEG, has been shown to alter the nanostructure of this phase separation [28]. This micellar structure might limit the possibilities to use PEDOT/PSS in nanostructures and nanofluidics. The high content of PSS makes the PEDOT/PSS acidic (pH 1.5 – 2.5) and upon increase to pH 4 the conductivity decreases 2 orders of magnitude [31]. The low pH is naturally a problem in most biological or biomedical applications, but might also cause degradation of other active materials, such as light emitting materials in OLEDs.

Due to the insoluble character of PEDOT a number of EDOT derivates have been synthesized. Reynold's group reported an alkane-substituted EDOT, possible to polymerize into a chloroform-soluble PEDOT [32], and soluble derivates of the PEDOT-like PProDOT, where the latter could demonstrate conductivities of 7 S/cm upon iodine-doping [33]. Ng *et al* synthesized a EDOT analogue with the more polar group, hydroxymethyl, giving PEDOT-methanol (PEDOT-M) [34]. The PEDOT-M was used as a starting material to form a truly water-soluble derivative of EDOT, EDOT-S (figure 4.6). EDOT-S is sodium salt of butanesulfonic acid funtionalized EDOT [35], which can be polymerized into the polyelectrolyte PEDOT-S. PEDOT–S (figure 2.4) have been electropolymerized with unsubstituted EDOT and used as cation exchange active surface [35] and with the aim to form a suitable surface for neural probes [36]. Chemical oxidative polymerization by $FeCl_3$ in chloroform of EDOT-S, which after dedoping with hydrazine and dialysis yielded a dark brown water solution, was reported by Reynold's group [37, 38]. By layer-by-layer (L-B-L) deposition of PEDOT-S and cationic polyelectrolyte poly(allylamine hydrochloride) PAH films with well reproducible electrochemical properties were generated. The L-B-L film were used as hole injecting layer in NIR emitting PLEDs with comparable properties as a spin-coated PEDOT/PSS film [39]. In paper VII we report an oxidative polymerization of EDOT-S in water to yield a fully water-soluble material; this synthesis is further discussed in chapter 4.

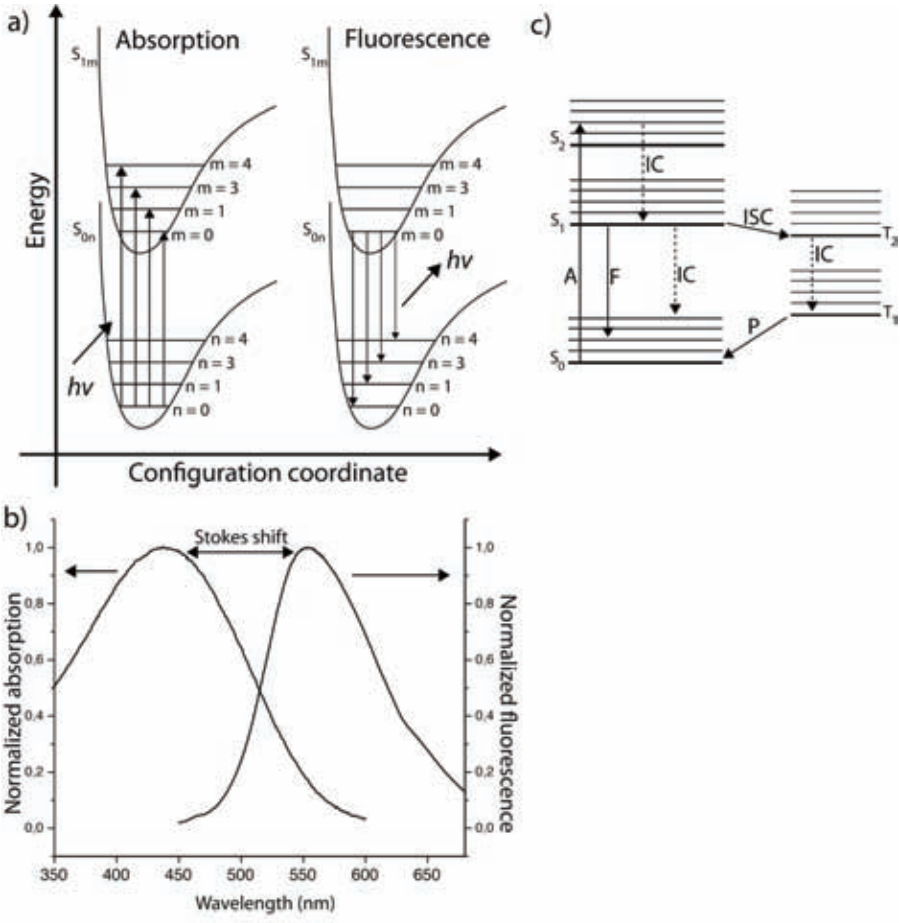
## *2.4 Optical properties; adsorption and emission*

When a conjugated molecule is exposed to photons of energy matching its band gap an absorption process can occur and an excited state (an exciton) is generated. Due to the molecular vibrations, in the C-C double bonds in a conjugated system, discrete energy levels are formed in the ground state as well as the excited state. The absorption process occurs form the lowest vibronic level of the singlet ground state $S_{00}$ to any levels $S_{1m}$ in the excited state; pictured in the Jablonski diagram, figure 2.5. An excited state relaxation occurs non-radiatively from $S_{1m}$ to $S_{10}$, through vibrations, rotations and energy translation to other molecules. A photon is emitted in a fluorescence process if the excited state is allowed to relax from the $S_{10}$ to any of the ground state levels $S_{0m}$. Through non-radiative energy dissipation the conjugated molecule returns to $S_{00}$ level. The energy difference between the absorption and emission maxima is called the Stokes shift and arises from the non-radiative transitions from $S_{1m}$-$S_{10}$ (see figure 2.5b). Conjugated polymers often exhibit a large Stokes shift due to the efficient energy migration along and in some cases in between chains, enabling emission form the lowest energy site. The lifetime of the excited state of a conjugated polymer is generally in the nanosecond regime [40].

The emission of a photon in fluorescence decay is not the only fate of the excited state. In internal conversion, which is common in conjugated polymers due to the high density of vibronic levels, the whole relaxation of the excited state occurs non-radiatively. The degree of non-radiative decay may be increased with quenchers, such as chemical defects in a polymer chain or interacting molecules in form of electron acceptors. In intersystem crossing a transition from the singlet to the triplet state occurs. Due to the spin selection rules the transition to the singlet ground state is forbidden, but it occurs with a low probability giving a weak and long-lived phosphorescence process (see figure 2.5c).

Conjugated polymers generally have broad absorption and emission spectra, which be explained by the polydispersity of the material and the variation in conjugation length, as well as different degrees of polymer inter-chain interactions. Spectral changes of conjugated polymers have been measured as a response to external stimuli, e.g. heat [41, 42], ions [5, 42] and biomolecules [4, 13, 42, 43], extensive reviews of the topic can be found in [44, 45]. In chapter 3 the use of conjugated polymers as optical probes for protein interactions and protein activity is further discussed. Shifts in the absorption and emission spectra are strongly associated with conformational changes in the polymer backbone and/or the degree of inter-chain interactions. Alternations of the torsion angle between the polymer rings, through rotation around the $\sigma$-bonds, in a conjugated polymer will affect the effective conjugation length [41, 46]. An alternation from a non-planar to planar conformation, the torsion angle approaches $180°$ (trans conformation) or less common $0°$ (cis conformation), will give a longer conjugation length, seen as a red shift in absorption and emission [42, 47]. A more planar structure increases the propensity of $\pi$-stacking of the polymer chains, which also will lead to red shifts in the optical spectra, due to the possible inter-chain energy migration. Red shifts associated with aggregation is often seen as a new vibronic structure, a distinct absorption shoulder in the longer wavelengths of the visible spectra [47, 48].

The emission intensity of a conjugated polymer is highly dependent on the degree of aggregation, a consequence of the higher probability of a non-radiative decay in the aggregated phase. Intensity differences between separated and aggregated state on one order of magnitude have been recorded [48].

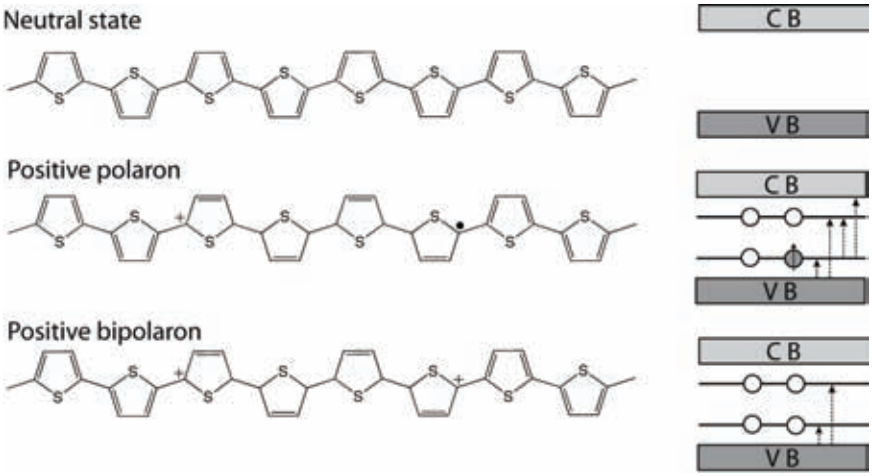



*2.5 a) Illustration of an absorption process of a photon which excites the molecule from the ground state to any of the levels in the first excited state $S_{1m}$. Non-radiative excited state relaxation brings the system to the $S_{10}$ state. A photon is emitted as fluorescence if the system relaxes radiatively from $S_{10}$ to any of $S_{0n}$. b) Absorption and fluorescence (excitation at 400 nm) spectra of PTAA (compound 8 in figure 2.4), note the large Stokes shift. c) Absorption (A) and fluorescence (F), but also non-radiative internal conversion (IC) as well as inter system crossing (ISC) to the triplet state and the spin forbidden phosphorescence (P) are illustrated.*

## 2.5 Electric properties; insulating, semiconducting or metallic?

In the pristine state conjugated polymers are semiconducting or close to insulators. Metallic conductivity of a CP can be achieved by increasing the level of positive (p-type) or negative (n-type) charge carriers in the material, a process called “doping”. Doping of CP can be accomplished through oxidation or reduction of the polymer chain either electrochemically [49] or chemically [1], using oxidizing or reducing

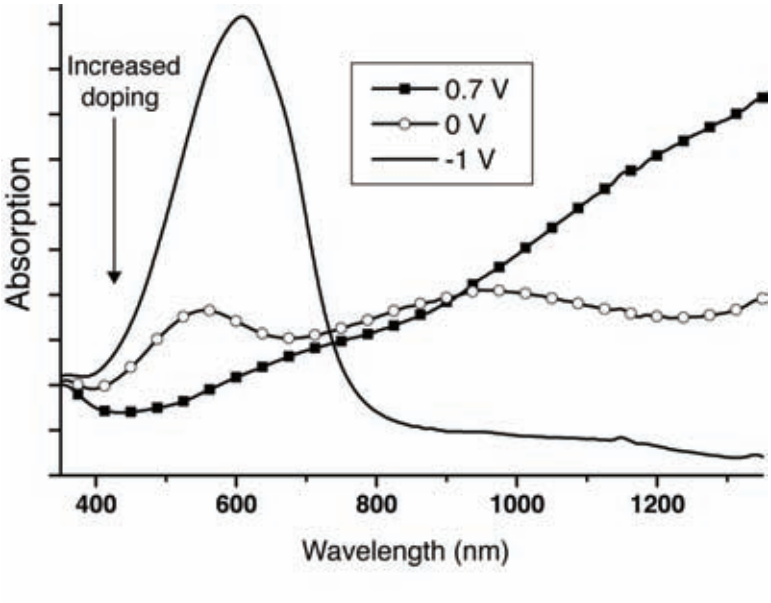
agents. The generated charge on the chain will in this processes be balanced by counter ions, making the metallic polymer into a salt [2]. Importantly, charge carriers can also be generated through light induced generation of excitons, an electron-hole pair. If followed by charge separation, this process is the basis of the generation of photoelectric currents in photovoltaic devices. Charge injection from metallic contacts into the CP without the presence of counter ions is also possible and used in organic field effect transistors.




*2.6 Chemical structures and the corresponding electronic structures of a polythiophene in the neutral and p-doped state. Note the local change in geometry, the quinoid structure, associated with the doped states. The new energy levels in the band gap are illustrated, as well as new optical transitions (dotted lines). The polaron has charge +e and spin ½, whereas the bipolaron has charge +2e and no spin.*

Oxidation (p-type doping) of a non-degenerate CP will create a polaron, a radical cation of a single electronic charge, carrying a spin [50]. The charged polaron is associated with a local change of geometry to a quinoid structure, and thereby formations of new energy levels in bandgap (see figure 2.6). Upon increased doping adjacent polarons will be unstable and form spinless doubly charged defects, bipolarons [51]. The doping-induced electronic states will facilitate conduction of charge but also give rise to new optical transitions, seen as absorption in the IR region of the spectra (see figure 2.7) [51]. Another observable optical effect of doping is the reduction of luminescence due to non-radiative quenching of singlets upon polaron interactions [52]. It is also worth commenting that electrical conductivity become three dimensional, truly metallic, only if high probability prevails that electron diffuses to a neighboring chain before traveling between defects on the single chain.

The degree of order of the material is out-most important for inter-chain transport processes and thus conductivity [2].




*2.7 Electrochromic spectra of PEDOT-S, where the reduced state (-1V) has a clear π-π transition in the visible range. Upon p-type doping the new electronic states are clearly seen as absorption in the IR region of the spectra.*

# 3 CPS AS OPTICAL PROBES FOR PROTEIN INTERACTIONS AND CONFORMATIONS

## *3.1 Introduction*

Development of biosensors is an intensive field of research for academic and corporate research groups worldwide, and the market for biosensors has seen a good growth in the latest years. The value of the total global biosensor market varies depending on the definition of biosensor; but the market has been estimated to 10.8 billion USD 2007 with a growth rate of 10.4 % [53]. It should however be emphasized that simple disposable blood glucose testing kits make up 85 % of the market and that many predicted applications within environmental, industrial, security and other sectors have yet failed to be realized [53]. Some more advanced biosensors, such as the DNA chips, have entered that market successfully. The sequencing of the human genome combined with increased knowledge of disease-associated mutations has made the DNA chips useful in some cases of diagnostics. However many diseases do not have a specific genetic signature, but rather a change in protein or peptide expression. The possibility to study organisms at the gene level has resulted in an increasing need for new methods aiming at the protein expression level, within the research field of proteomics. Proteomics includes not only the identification and quantification of proteins, but also the determination of their localization, modifications, interactions, activities, or, ultimately, their function [54]. One of the big challenges within the biosensor area is to develop reliable and sensitive methods to study proteins from these different aspects. Proteins are however much more complex and sensitive than oligonucleotides and commercial multiparallel protein chips have not yet reached extensive use. In this thesis the focus has been on detection of misfolding and conformational changes in proteins. During the latest decades misfolding has been increasingly recognized as associated to diseases, often called amyloidoses and prion-associated diseases.

There has been very interesting demonstrations of CPs in the field of biosensing and biomedicine, with the CP as a sensing element or as actuator. CP sensors have been realized with a number of different detection schemes. Conductometric sensors, where the response is a change in conductivity in the CP material upon interaction

with the analyte, is natural choice when working with electrically conducting polymers [55, 56]. A high sensitivity can be achieved since a small amount of charge injection can give rise to changes in conductivity by multiple orders of magnitude [57]. Potentiometric sensing is another electrical approach, which relays on an analyte-induced alternation of the electrochemical potential of the system. The reversibility of the redox processes in CPs makes potentiometric sensing possible. This chapter will however not cover biosensing using CPs in an electrical mode, but focus on the use of CPs as optical probes for biosensing, especially as probes of proteins in terms of protein interactions, protein activity and protein conformation changes.

The main advantage of using a polymeric optical probe compared to small molecules in biosensing is the possibility of multiple interactions and a collective response which enhances the sensor signal [14]. Zhou *et al* described a collective response along a polymer chain as "wiring receptors in series" or the molecular wire approach [45, 58, 59] (see figure 3.1). A CP can show superior sensitivity compared to a small molecule receptor due to the delocalized electron structure, which facilitates efficient energy transport over long distances.

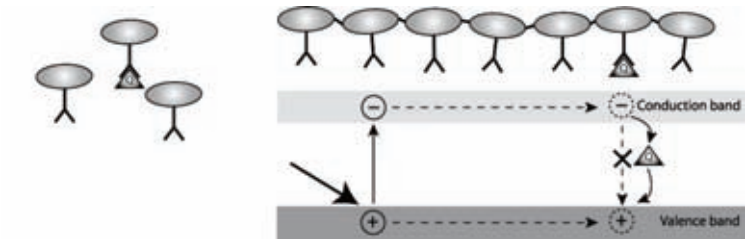



*Figure 3.1. To the left quenching of isolated fluorophores is illustrated, where only those molecules with an associated quencher will be non-emissive. To the right the molecular wire is illustrated. The quencher occupies a fraction of the receptor sites, but due to the efficient funneling of the exciton to the site of lowest energy, induced by the quencher, a complete quenching of the whole polymer chain can occur. Copyright Wiley-VCH. Reproduced with permission [60].*

The two explored sensor schemes, when using conjugated polymers as optical probes, are colorimetric detection and various changes in fluorescence. Colorimetric detection utilizes changes in absorption of the sensor material. The sensitivity of the bandgap in a CP to changes in polymer conformation offers a route for colorimetric detection. Fluorescence is a well-established method in the field of biosensing. The method has a high inherent sensitivity as well as versatility in the detection of the

signal, including changes in intensity, wavelength (in emission and excitation), energy transfer and lifetimes. Generally speaking fluorescence can be turned off in the sensing event (quenching or turn-off sensing), the sensing event can turn on the fluorescence (turn on sensing) or the wavelength of the fluorescence can be changed upon sensing (ratiometric sensing) [61]. Fluorescence detection schemes based on conjugated polymers have the possibility of significant signal amplification due to the efficient energy migration in the material.

## *3.2 Colorimetric detection*

In 1993 the first example of biosensing using a CP was demonstrated with a colorimetric detection of an receptor-ligand recognition event [13] (figure 3.2). Polydiacetylene functionalized with an analogue to sialic acid as a side chain could in a bilayer geometry undergo a clearly visible color change, from blue to red, upon interaction with the influenza virus hemagglutinin. The color change was attributed to a decreased conjugation length in the polymer caused by conformation changes in the polymer backbone. This conformation change is due to a changed degree of order of fatty acids in the bilayer assembly. This sensing principle was later developed into membranes and vesicles of the same polymer [62-64]. Specifically functionalized polydiacetylene has also been demonstrated to detect the proteins cholera toxin [65], phospholipase A [66, 67] and the binding of glucose to hexokinase [68]. More recently, solution-based colorimetric detection principles using glyco-substituted polythiophenes interacting with E.coli, lectins and influenza virus [12] and sugar-substitued poly(phenylenevinylene) detecting lectin were demonstrated [69] (see figure 3.2).

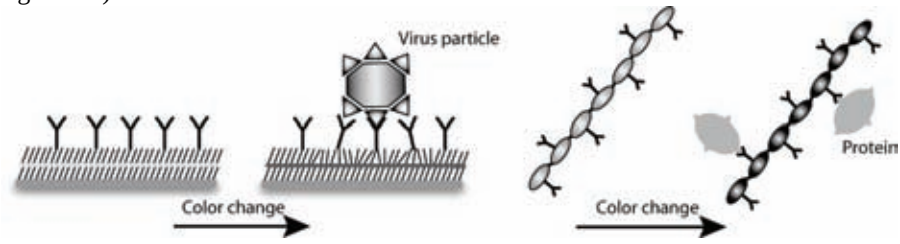



*Figure 3.2 To the left the assay by Charych et al is shown [13]. The immobilized polydiacetylene layer undergoes a color change when the attached glucose groups interact with virus particles. To the right a CP covalently modified with groups that can interact specifically with a protein is illustrated. When the protein interacts with the CP a color change can be detected [12, 43, 69-72]. Copyright Wiley-VCH. Reproduced with permission [60].*

Apart from glycosubstituents, a favorable covalent modification of a CP for sensor applications is the attachment of biotin as a side chain. The binding between biotin and avidin or streptavidin is one of the strongest biological non-covalent interactions known. Pande *et al* showed the first CP modified with biotin in a supramolecular assembly together with a phosphatase [73]. Faid and Leclerc took the biotin modification one step further and developed a polythiophene for colorimetric measurements based on a chromic shift, from violet to yellow, upon binding of streptavidin (figure 3.2) [43]. The authors claim that the chromic shift can be due to a planar to nonplanar twisting of the polymer chains which increases the energy of the π-π transition. Similarly Mouffouk *et al* could also demonstrate how avidin binding events in a biotin-functionalized polythiophene resulted in blueshift in the absorption spectra and could also be detected as a conductivity change in a polymer film [72]. Likewise as the previous study the authors attribute the effects to increased inter-ring torsion in the polymer backbone, but also points out decreased inter-polymer π-stacking as a likely cause. Detection of antigen-antibody interactions is highly interesting in biosensing applications. Englebienne *et al* synthesized a bioconjugate in form of covalent attachment of anionic polythiophenes to antigens, the proteins h-CRP and h-SA, and could colorimetrically detect the binding of antibodies to these [70, 71]. The color changes were in this study explained by a local pH change, caused by the binding of the antigen to the antibody.

## *3.3 Superquenching*

The sensitivity of the reported colorimetric approaches is not enough for most relevant biological assays. Fluorescence-based sensing has an intrinsically higher sensitivity. The first fluorescence-based CP sensing was shown by Zhou and Swager, where they showed an efficient quenching of poly(phenyleneethynylene) (PPE) upon interaction with paraquat [58, 59] (see figure 3.1). It was clearly shown that the sensitivity of the polymer system was enhanced compared to quenching of small molecular compounds. The Stern-Volmer equation was used for quantification of quenching:

$$K_{SV} = \{(F_0 / F) - 1\} / [Q]$$

[Q] is the quencher concentration, $K_{SV}$ is the Stern-Volmer constant, $F_0$ is the fluorescence intensity in absence of quencher and F is the fluorescence intensity at [Q]. Quenching can be generalized to be either static or dynamic. Static quenching is associated with a binding of the quencher to a fluorophore in the ground state, prior to excitation of the system. In dynamic quenching the quenching occurs upon collision

between the quencher and the fluorophore in an excited state. In this quenching process the lifetime of the fluorophore will be dependent on the quencher concentration, which is not the case in static quenching. [58]

This phenomena, which has been attributed as amplified quenching or superquenching, has been explored by several groups from different perspectives; for small cationic sensing [74-76], anionic sensing [77], detection of saccarides [78], hydrogen peroxide [79] and well as DNA hybridization [80, 81]. Other studies which have the potential to influence sensing via superquenching is enhanced superquenching using gold nanoparticles [82], the influence of detergents on quenching [83] and quenching of CPs immobilized on microspheres [10]. The term superquenching originates from the observation that the binding of analyte molecules to a small number of receptor sites can lead to complete emission quenching, due to the efficient energy migration in the CP material. Whitten's group was the first to report the exploration of the superquenching effect for biosensing purposes [14]. By adding the small molecule methyl viologen [$MV^{2+}$] to a water-soluble polyanionic conjugated polymer [poly(2-methoxy-5-propyloxy sulfonate phenylene vinylene (MPS-PPV)] a very efficient quenching could be achieved, $K_{sv} \approx 10^7$. One [$MV^{2+}$] molecule could in this case quench 1000 repeat units, approximately equivalent of one whole polymer chain. The detection of a protein-binding event was done using a biotinylated methyl viologen (B-MV) (see figure 3.3). B-MV quenched the emission of MPS-PPV although with lower efficiency than [$MV^{2+}$]. However upon addition of avidin a reversal of the B-MV quenching was seen, due to the strong affinity between biotin and avidin. A more versatile approach of biosensing using superquenching in CPs is the detection of antigen-antibody interactions shown by Heeger *et al* [84]. Quenching of a PPV derivative (MBL-PPV, poly-[lithium 5-methoxy-2-(4-sulfobutoxy)-1, 4-phenylenevinylene] of DPN (2, 4-dinitrophenol) and unquenching on addition of anti-DPN IgG were observed in this study. For the unquenching to be specific the CP has to be complexed with a cationic polymer, rendering a charge neutral complex. An electron transfer protein can be utilized as direct quencher of CP fluorescence with high efficiency [85, 86].

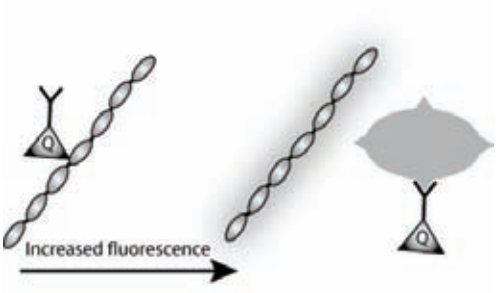



*Figure 3.3. Illustration of the superquenching assay used by Whitten et al [14] and Heeger et al [84]. A quencher labeled with a specific group interacts with the CP chain. Upon addition of a protein, streptavidin [14] or antibody [84] the quencher is removed from the vicinity of the chain and a fluorescence increase can be seen. Copyright Wiley-VCH. Reproduced with permission [60].*

The superquenching phenomenon was utilized in interesting approaches in assays of enzymatic activity in three reports in 2004 [17, 87, 88]. Pinto and Schanze used anionic PPE to formulate both a turn off and a turn on fluorescence assay for protease activity [17]. The assays are based on electrostatic interactions between one sulfonated and one carboxylated PPE and quencher labeled enzyme substrates in form of cationic peptides. The turn on sensor detected enzymatic activity at the very low thrombin concentration of 2.7 nM after 100s (and 50 pM after 50 min) incubation. $K_{cat}$ is $38s^{-1}$ under similar conditions with the same substrate [89]. The enzymatic activity resulted in the cleavage of the quencher p-nitroaniline and thereby significantly decreasing the association to the polymer, see the schematic illustration 3.4. The turn off sensor relays on the enzymatic activity of the protease papain that converts an inactive rhodamine quencher to an active quencher (see figure 3.4). The CP gives a signal enhancement of six to ten times compared to a pure rhodamine based assay. Whitten's group reported a CP based sensitive protease activity sensor [87]. Similarly to the approach of Pinto and Schanze, a quencher is covalently attached to the enzymatic peptide substrate, but the association of the quencher to the CP, a PPE, occurs via biotin and biotin binding proteins. The quenching assay is demonstrated for three proteases, both with CP in solution and coated on microspheres (see figure 3.5). This assay has to be carried out in a two-step process, where the cleavage of the peptide occurs prior to the addition of the CP or the CP-coated microspheres. To enhance the sensitivity of the assay a phycoerythrin chromophore is associated to the CP. An efficient energy transfer from the excited PPE to phycoerythrin can occur followed by emission with a narrow wavelength distribution. The detection of enzymatic activity could occur at the 13.7 nM already after 5 min, with the relatively slow BSEC enzyme ($K_{cat}$ 0.02 $sec^{-1}$). The detection of other enzymatic activities in

terms of phosphatases and kinases has also been demonstrated using the superquenching phenomena [88]. The detection is based on the fact that $Ga^{3+}$ associated PPE coated on positively charged microspheres can complex to phosphorylated peptides and proteins (see figure 3.6). Peptide substrates labeled with quenchers can be used in a direct assay and unlabelled proteins and peptides can be used in a competitive assay with a labeled tracer peptide. This assay showed sensitivity similar or superior to commercial assay kits [88, 90]. A somewhat less versatile approach, but with possibilities of higher specificity, is covalent attachment of the enzymatic substrate to the CP. Swager's group synthesized a PPE with part of the sidechains constituted of a fluorescence-quenching 14-mer peptide. Upon enzymatic cleavage of the peptide the fluorescence increases one order of magnitude [18].

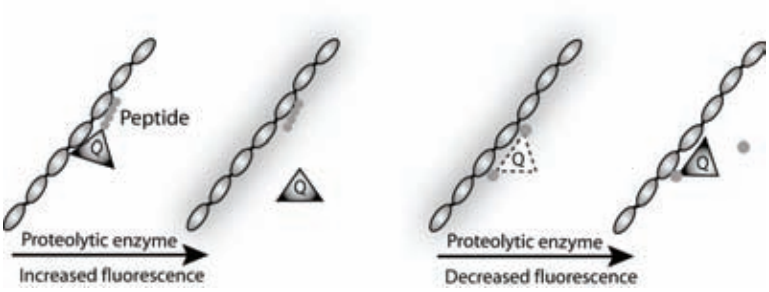



*Figure 3.4 The protease assay demonstrated by Pinto and Schanze [17]. To the right a quencher labeled peptide is electrostatically associated to the CP chain. Upon addition of a proteolytic enzyme, the peptide is cleaved and the quencher dissociates, whereupon the fluorescence increases. To the right a "caged" quencher is electrostatically associated to the CP chain. Upon addition of a proteolytic enzyme one of the peptide chains of the "caged" quencher is cleaved and dissociates. The quencher is now active and the fluorescence decreases. Copyright Wiley-VCH. Reproduced with permission [60].*

A serious drawback of superquenching assays is that, when used in complex media, compounds that autofluoresce or unquench/quench fluorescence nonspecifically can give false positive or negative signals. In an superquenching kinas/phosphatase assay tested for applications in high throughput screening it was shown that 24 of 84 tested kinas/phosphatase inhibitors quench the CP unspecifically [90]. Several groups report that CPs can interact unspecifically with proteins to give both enhanced and decreased fluorescence [84, 91, 92]. Enhanced fluorescence is thought to originate from proteins breaking up complexes of polymers chains in a detergent like manner; whereas protein induced quenching is often attributed to electrostatic interactions. The unspecific interactions between CP and proteins has also be utilized in sensor assays, as demonstrated in a protease assay by Zhang *et al* [93]. The interaction between a

protein (BSA) and the anionic $PPESO_3$ leads to a blueshifted fluorescence with increased intensity. The enzymatic digestion of the protein resulted in redshifted fluorescence and decreased intensity.

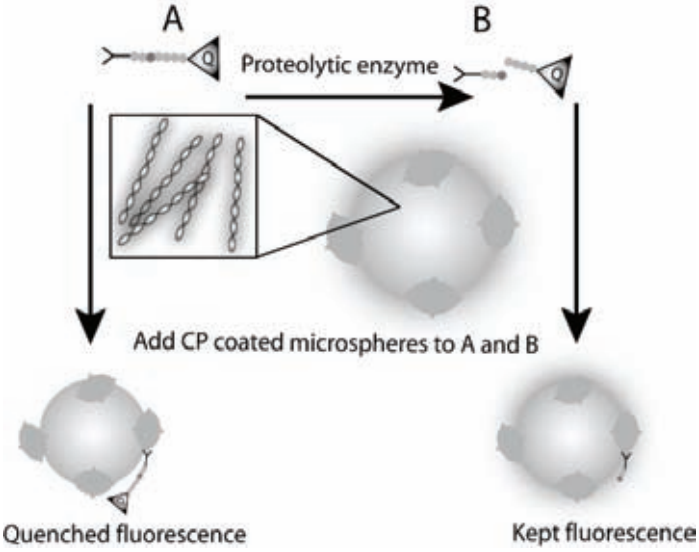



*Figure 3.5. The protease assay demonstrated by Whitten et al [87]. A peptide is labeled both with a quencher and biotin. In path A streptavidin (or other biotin binding protein) labeled microspheres coated with CP are added to the peptide. The biotin interacts with the streptavidin and quenching occurs. In path a proteolytic enzyme has digested B the peptide. Upon addition of the microspheres the fluorescence is retained. Copyright Wiley-VCH. Reproduced with permission [60].*

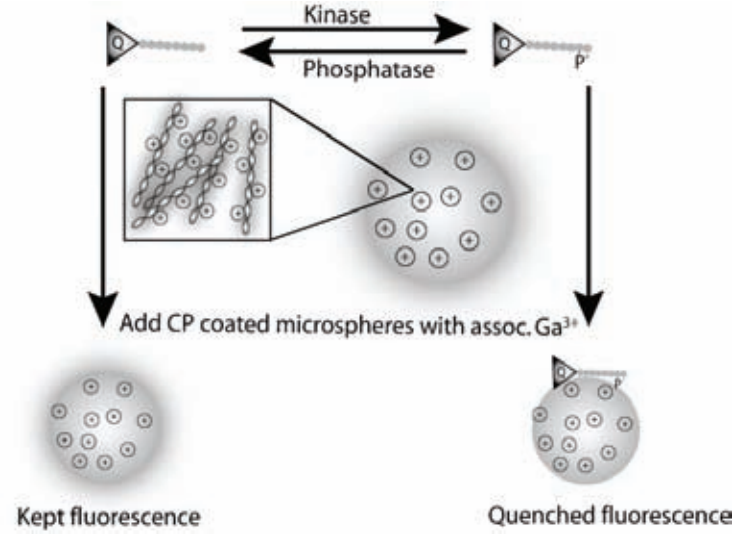



*Figure 3.6 An illustration of the kinase/phosphatase assay demonstrated by Rininsland et al [88]. The assay uses the fact that phosphorylated peptides interact*

*with $Ga^{3+}$ ions. Microspheres are coated with a CP, which electrostatically interacts with $Ga^{3+}$. If a quencher labeled peptide is phosphorylated by a kinase the peptide and thereby the quencher associates to the sphere and the fluorescence is quenched. If the peptide is dephosporylated the spheres will keep the fluorescence. Copyright Wiley-VCH. Reproduced with permission [60].*

## 3.4 Light harvesting

Another set of approaches, where the efficient energy migration in CPs is utilized are the so-called light harvesting methods, where an optical amplification is achieved through Förster transfer to a fluorophore. This detection scheme was first applied to a specific DNA detection assay, where an electrostatic interaction between the DNA strand and CPE gave an efficient Förster transfer to a fluorophore labeled PNA strand if hybridization had occurred [94-96]. The demonstration of proteins in a light harvesting assay was first done by Wang and Bazan [97]. The recognition event by the protein/polypeptide Tat-C of a RNA sequence was detected through the energy transfer from a cationic CP to fluorescein (see figure 3.7). Crucial for this detection assay is that the protein-RNA complex has a negative net charge to ensure electrostatic attraction with the CP. Zheng *et al* showed that an efficient energy transfer between a biotinylated PPE and fluorophores covalently attached to streptavidin can occur [98]. Interestingly, the study revealed that dyes with lower spectral overlap with the CP can show increased energy transfer, probably due to orbital interactions between the dye and the CP. The light harvesting approach has the potential of being more specific and less sensitive to nonspecific interactions. Two studies report how the detection kinase/phosphatase assay, showed by Whitten's group, can be more sensitive and selective using peptide substrates labeled with dyes to which energy transfer can occur [88, 90, 99, 100]. The specific signal in light harvesting is associated with quenching of the polymer fluorescence as well as enhancement of the dye emission making it both a turn off and turn on sensor.

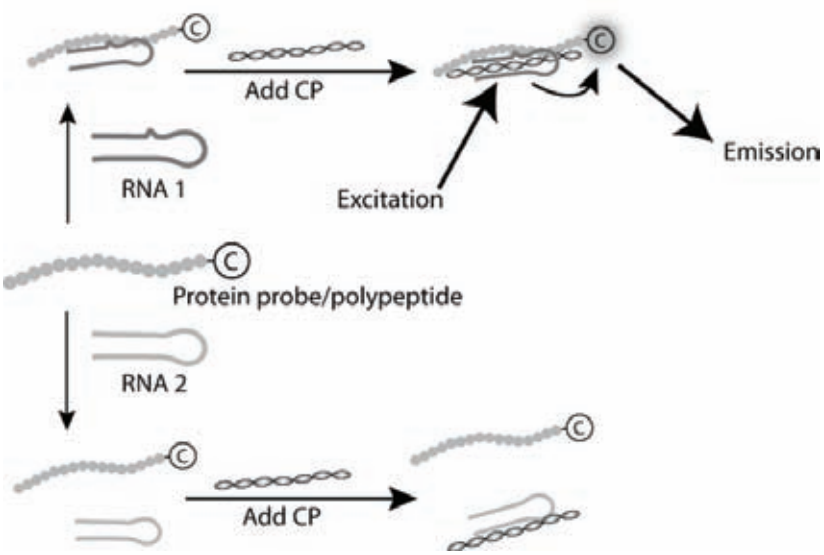



*Figure 3.7 An illustration of the RNA detection assay based on a chromophore labeled protein/polypeptide and light harvesting CP demonstrated by Wang and Bazan [97]. The upper pathway starts with a specific binding of the RNA1 sequence to the protein. A cationic CP can electrostatically interact with the protein/RNA complex, which brings the CP in proximity of the chromophore. Upon excitation of the CP an efficient energy transfer (FRET) occurs from the polymer to the chromophore. In the lower pathway a non-binding RNA is added to the protein. The addition of a cationic CP gives a complex with the RNA, whereas the protein remains separate and upon excitation of the CP no energy transfer can occur to the chromophore. Essential for a functional assay is that the protein/RNA complex has a negative net charge. Copyright Wiley-VCH. Reproduced with permission [60].*

## 3.5 Conformational changes and superlightning

By utilizing chromic changes both in fluorescence and absorbance, of poly- and oligothiophenes with charged sidechains, a number of sensitive sensor schemes have been demonstrated, first DNA hybridization [4, 15, 16] followed by detection of protein [101], as well as peptide interactions [102] and work within this thesis on protein conformational changes [102-105], paper III.

The chromic changes of a cationic poly(3-alkoxy-4-methyl-thiophene), when complexed to ssDNA or dsDNA are attributed to conformation changes in the conjugated backbone [4, 15]. This sensor scheme has been applied to specific protein detection with the help of the artificial nucleic acid ligands called aptamers [101] (see figure 3.8). Upon ratiometric complexation with the aptamer, which is an ssDNA sequence, the polythiophene forms red-violet aggregates, which differs significally in

absorption and fluorescence compared to the pure CP. The specific binding of a protein, α-thrombin, to the CP/aptamer complex results in blueshift in the absorption spectrum of the polymer and an intensity increase in the fluorescence (see figure 3.8). The changes in optical properties are attributed to changes in polymer aggregation and conformation, governed by the change of the aptamer structure, from unfolded to folded, in the protein recognition event. The detection limit of this system is 10pM.

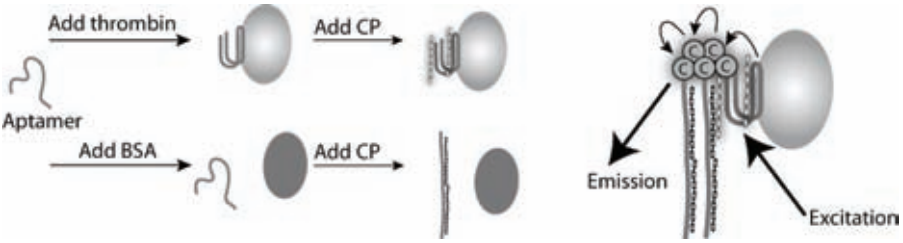



*Figure 3.8 Left) An illustration of a thrombin detection assay utilizing a ssDNA aptamer probe and conformational changes in a cationic CP[101]. In the upper pathway the aptamer binds specifically to thrombin with a quadruplex structure. A complex between a cationic CP and thrombin/aptamer gives increased fluorescence intensity and blue shifted adsorption. The lower pathway shows the addition of an unspecific protein, BSA. The cationic CP forms a complex with the aptamer and gives more quenched fluorescence and red shifted adsorption. Right) An illustration of the complex between chromophore labeled aptamers and CP used in a surface based thrombin detection chip [11]. The binding of the thrombin gives a fraction of the aptamer a quadruplex structure and the complexed CP chains an increased fluorescence. An ultrafast energy transfer, superlightning, can occur and one CP donor can excite multiple acceptors, chromophores. Copyright Wiley-VCH. Reproduced with permission [60].*

The aptamer/CP complex for protein detection has by the same group been proven to work at surfaces, for biochip applications [11]. To enhance the fluorescence signal the aptamer is labeled with a chromophore acceptor, to use the so-called superlightning effect, which as been more extensively studied in a DNA hybridization sensor [106-108]. The binding of a protein to the aggregated aptamer/CP leads to conditions, which allow the CP to work as a donor with an efficient resonance energy transfer in the ultrafast regime to the chromophore. The outcome is that one donor can excite a large number of acceptors (see figure 3.8). The consequence of this "superlightning" phenomenon is that the binding of few analytes, in this case proteins, to a polymer/aptamer aggregate gives a remarkable increase in the emission from the chromophores. This surface based system has shown a detection limit in the pM range but with a very low sample load ($1.5 \times 10^7$ molecules in 0.4 μl.) The specificity

of these chips has so far only been shown in pure samples of one protein and not in more complex media, such as serum or whole blood.

The chromic changes of a zwitterionic poly(thiophene) (POWT figure 2.4) were used in detection of conformational changes, from random coil to a four-helix bundle, in synthetic peptides [102]. Differences in spectral characteristics could be seen if the CP was interacting with a positively or negatively charged peptide in random coil or the helical assembly formed by the two peptides. Interestingly, the induced circular dichroism (CD) of the CP was increased upon interaction with the four-helix bundle, which was attributed to an increased twist of the CP backbone. However, with the new results on aggregation behavior of POWT, mentioned in chapter 2, differences in aggregation as the cause for change in the CD signal must be considered. Induced CD signals in polythiophenes as a consequence of aggregation is discussed by Langeveld-Voss *et al* [48]. The same CP could monitor the conformational changes in a larger system, the calcium binding protein calmodulin [105]. The authors suggest that the CP interacts with calmodulin through electrostatic and hydrogen bonding to give planarized and aggregated polymer chains. When the complex is exposed to $Ca^{2+}$ the protein undergoes a major conformational change detected through blue shift in absorption and emission, recognized as a more non-planar backbone and separated polymer chains (see figure 3.9). The $Ca^{2+}$ induced change in the protein enables a specific interaction of a secondary protein Calcineurin, which in this study can be followed by the changes in optical properties of the same CP.

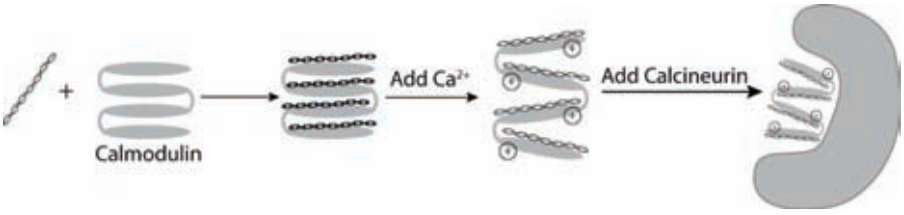



*Figure 3.9 An illustration of an assay for calcium induced conformaional changes in calmodulin and detection of calcineurin shown by Nilsson and Inganäs [105]. A zwitterionic CP, POWT, forms a complex with the protein calmodulin, which gives a red shifted adsorption and a quenched and red shifted fluorescence of the CP. The addition of $Ca^{2+}$ results in a conformational change of calmodulin, which can be monitored as a blue shift in adsorption and fluorescence and increased fluorescence intensity. The calcium-activated calmodulin can bind specifically to the protein calcineurin, which can be seen as an increased ratio of the emission wavelengths 540nm/670nm. Copyright Wiley-VCH. Reproduced with permission [60].*

We have shown how thiophene based CPs can been used as optical probes to monitor a protein misfolding process, the conversion of native proteins to an amyloid fibrillar state. A further description of the amyloid fibril formation can be found in chapter 4. It is well established that such conformational changes in proteins can be associated to pathogenic states, such as Alzheimer's disease, amyloidoses, and transmissible spongiform encephalopathy [109, 110]. An anionic polythiophene, poly(thiophene acetic acid), demonstrate a visible chromic shift , from yellow to orange, when interacting with native insulin or lysozyme compared to amyloid fibrils of the same protein [104]. The changes in emission of the CP were used to follow the kinetics of an amyloid fibril formation process. A study of the polarization of the emitted light from single fibril or bundles of fibrils decorated with the CP concludes that the CP is oriented with the polymer chain axis parallel to the fibril axis [111]. A regioregular well-defined, so-called trimer-based, polythiophene, with an amino acid derivate as side chain, show a distinct change in fluorescence emission when interacting with insulin amyloid fibrils [103]. Importantly, the emission spectrum is virtually unaltered from a buffer environment to a buffer environment with the native protein present. This detection scheme offers an attractively low background signal, if small traces of misfolded protein are found in presence of native protein. A larger comparing study between eight conjugated polythiophene derivates (LCPs) and their discrimination of a protein (insulin) in the native or amyloid-like fibrillar state is presented in paper III. Compared to their monomer-based analogues, trimer-based CPs showed significantly better optical signal specificity for amyloid-like fibrils, seen from increased quantum yield and spectral shifts.

## *3.6 Staining with CP*

Very few of the detection schemes mentioned in this chapter have been demonstrated in more complex biological environments with several interfering species, such as detection in blood, urine etc, or for detection/discrimination of microbes. However, the first biodetection application shown with CP was viral sensing [13]. The same group later showed glycosubstitued polythiophenes, which underwent colorimetric changes when interacting with E.coli bacteria and influenza virus [12]. More lately some interesting publications have shown the use of CP for staining purposes of bacteria [112, 113], amyloid deposits [114, 115] and cellular structures [116]. In these applications several biological constructs, such as proteins, carbohydrates, lipids, nucleic acids and combinations thereof, can be involved in the CP interactions.

Disney *et al* synthesized a PPE functionalized with the carbohydrate mannose and showed a selective binding of this polymer to the mannose-binding lectin concanavalin and to the bacteria E. coli, through the mannose receptors located on the bacterial pili [112]. The detection specificity was clearly illustrated, since the polymer does not bind to a modified bacterial strain, not expressing the mannose receptors. Importantly, the study demonstrates that the interaction with concanavalin and the bacteria is enhanced due to multivalent interactions with several functionalized side chains along the CP, thus clearly motivating the use of a polymer for staining purposes. Lu *et al* demonstrated that a cationic PPE could coat and possibly penetrate the cell wall of E. Coli and spores of Bacillus anthracis [113]. More interestingly the study also demonstrated a biocidal activity, with up to 40 % reduction in population when the coated microbes were exposed to white light. The authors claim that interfacial generation of singlet oxygen might be a likely explanation of the biocidal activity and speculates that CPs with higher intersystem crossing efficiency could be more effective biocidal agents. The mechanisms behind the bacterial coating and the possible penetration of the bacterial cell wall are however not discussed. Mammalian cells exhibit an even higher complexity than microbes. A recent study by Björk *et al* demonstrates how polythiophenes can stain chromatin, cytoplasmatic and nuclear vesicles and cytoskeleton components in cultured cells [116]. Upon staining at low pH with two CP with cationic side chains, clear visualization of chromatin in cell nuclei and chromosomes was achieved, indicating an electrostatic complex between the positively charged polyelectrolyte and negatively charged DNA. The two cationic and an anionic polythiophene showed staining of vesicles in the cytoplasma, which the authors discuss is due to an interaction with the polymer backbone and some unknown intracellular structure. The emission characteristics of the cationic polythiophenes are clearly different when staining chromatin compared to the vesicles, attributed to different backbone conformation and state of aggregation of the CP. This study also shows that the incubation of an anionic CP with live cells leads to staining of cytoskeleton components and no decreased survival of the cells. Even higher in complexity are studies of multicellular organisms and tissue samples. Conjugated polythiophenes have been demonstrated as selective for staining of amyloid deposits in *ex vivo* tissue samples. By varying the staining conditions amyloid deposits of amyloid light chain, islet amyloid polypeptide and Alzheimer's beta peptide could be detected using cationic as well as anionic CP [114, 115] (See figure 3.10). We suggest that the interaction between the CP and the amyloid deposit originates from the hydrophobic and highly repetitive structure of both species. The study also emphasize that the emission properties of amyloid deposits of the same proteins in various tissue exhibit different emission properties when stained with CP.

This phenomenon is suggested to originate from the different conformation of the same protein in the different plaques, which gives the polymer backbone different states of conformation. Different degree of polymer inter-chain interactions in different plaques is another plausible explanation.

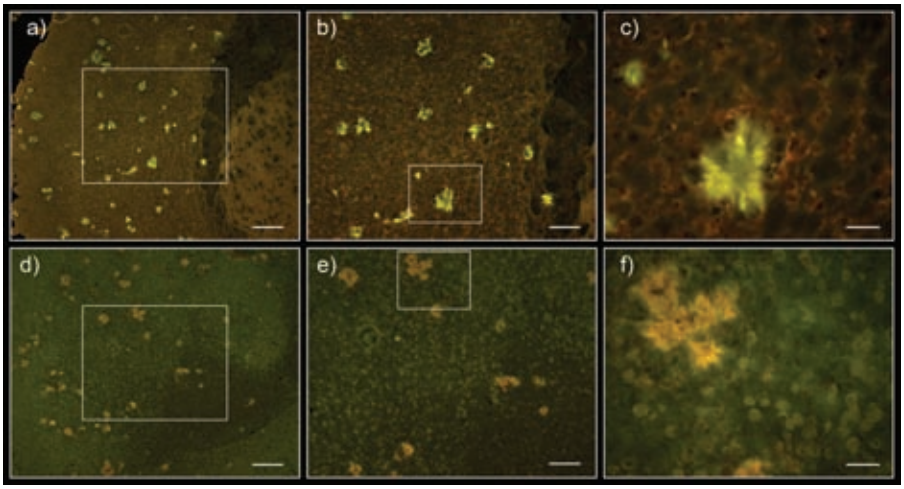



*Figure 3.10 Fluorescence micrographs of 5 μm serial sections of stained amyloid plaque and cerebrovascular amyloid in brain sections from 18 month tg-APPSwe mouse. Large amounts of bulky plaques appear throughout the cortex. a) – c) tPTAA staining in 100 mM Na-Carbonate pH10 d)-f) PTAA staining in 100 mM Na-Carbonate pH10. The trimer-based probe (tPTAA) gives as slightly higher contrast compared to the monomer-based analogue (PTAA). Scale bars represent a) & d) 300 μm, b) & e) 150 μm, c) & f) 40 μm*

## 3.7 Conclusions and outlook of biosensing using CP

This chapter contains numerous examples that demonstrate the possibility to use conjugated polymers for interactions with protein and detection of protein interactions with other species. If these sensing schemes are to be applied for biodetection purposes generally, there are high demands to be fulfilled. Compared to established methods, a CP based method, as well as any new detection method, has to improve one or several of the following sensing features; ease of use, price, speed, sensitivity, specificity and toxicity.

If the CPs used for protein biodetection are compared, they all have ionic sidechains, but can apart from this feature be divided in two groups. Group 1 is polymers that have a covalent side chain functionalization, which is known to specifically interact with a second species, such as biotin/biotin binding proteins (See

image 3.2). Group 2 are polymers, which have an ionic side chain not known to interact specifically with the species to be detected but sensing is possible through two paths, A and B. In path 2A, polymers exhibit a strong noncovalent interaction with a recognizing species that interact specifically with the analyte, such as RNA-binding protein and RNA. In path 2B, which was used in this thesis work, the polymer has under certain conditions a strong selectivity for a certain protein or protein structure, such as for staining of amyloid deposits in tissue. Considering specificity, polymers in group 1 should have high specificity, but this does not necessarily give a strong sensing signal. The major drawback of group 1 polymers is the lack of versatility, where every application needs a special synthesis, which would lead to a higher cost of the sensor. Group 2A can have high specificity due to the recognizing species, combined with the advantage of being more versatile and through this hopefully more cost efficient. However the interaction with the recognizing species is potentially sensitive and often requires a controlled sensing environment. Group 2B has shown a high specificity, but can suffer from the same or even higher sensitivity to changes in the experimental conditions as 2A. Importantly all polymers have an inherent propensity of multivalent interactions due to the repeating structure, which should be put in consideration when designing new detection schemes using CPs.

Apart from this difference in the molecular interaction between the CP and the analyte there are different sensing principles; colorimetric and ratiometric sensing with changes in absorbance and fluorescence respectively, superquenching, light harvesting and superlightning. The colorimetric and ratiometric sensing has the obvious limitation, that colored compounds, or autofluorescence from the analyte, can lead to alternations in the sensing signal. The signal in this class of CP-based sensors can often be strong, with large wavelength shifts in absorbance and or fluorescence. The cause of these shifts is changes in the polymer backbone conformation and/or state of aggregation. In the superlightning approach the aggregation or micellation between a CP and a labeled DNA probe is fundamental for the function of the technique [101, 106, 108]. A light scattering study of DNA hybridization shows a complex size behavior of the CP/DNA micelles upon different degrees of hybridization [106]. The correlation between the biosensing mechanism and the degree of aggregation in CPs has, with the exception of superlightning, not been well studied. The cationic polythiophene used in the superlightning approach has a blue shifted fluorescence of high intensity and low light scattering intensity, from which the authors conclude it to be highly water soluble [106]. Several anionic CPs have shown optical characteristics indicating aggregation in pure water solutions. Tan *et al*

concluded from adsorption and fluorescence studies that the PPE-$SO_3$ is aggregated in water solution, but appears in a monomeric state in the better solvent methanol [117]. Swager´s group synthesized a PPE substituted with carboxylterminated poly(ethylene glycol), to increase water solubility and decrease aggregation, but could only achieve this in alkaline solutions or with addition of surfactants [18]. The anionic MPS-PPV used for detection of avidin in a superquenching assay shows aggregation features at millimolar concentration [14]. Carboxylated polythiophenes have demonstrated solubility in alkaline solutions [5, 118], but also in pure water [119]. An interesting observation of the aggregation behavior of a carboxylated fluorene containing copolymer was made by Wang *et al*; in acidic water solution dynamic light scattering shows CP aggregation, which was connected with increased fluorescence intensity [120]. The increased fluorescence was suggested to be a result of water shielding within the aggregates. The light scattering and FCS studies discussed above showed that the polythiophenes used in this thesis work also are prone to aggregate in water solutions. As discussed in chapter 2 the aggregation phenomena in polyelectrolytes solutions are very complex. From the references above and literature about aggregation in nonconjugated polyelectrolytes [23] it can be concluded that use of aggregation in the sensing principle can be a way to increase collective responses in CP, but has the problems of being hard to predict and is sensitive to environmental changes. Superquenching has the problem of false positive signals if other quenchers are present in the sensing environment, and false negative signals in presence of compounds with emission at the same wavelengths as the polymer. Light harvesting and super lightning can be less sensitive to false signals if the emitted detected signal is chosen not to interfere with fluorescence from commonly occurring species in the sample.

The demand for protein analyses has the potential to grow significantly and would profit greatly from versatile and sensitive optical sensing. Conjugated polymers, if used in properly designed sensor schemes, offers the possibility of multivalent interactions and collective optical responses. Hopefully CPs will be realized in chip based sensing applications for simple and fast multiple protein analyses, not only for protein detection but also for protein quantification.

# 4 SELF-ASSEMBLED NANOWIRES OF CPS AND BIOLOGICAL MACROMOLECULES

## *4.1 Self-assembly and nanowires – overview*

All known living systems constitute of small building blocks, hierarchically assembled into functional superstructures. Three examples of increasing complexity are a protein, a living cell and a human being. These three "components" are formed in an autonomous assembly process, which occurs without human intervention, called *self-assembly*. The term self-assembly is used in numerous fields of science, with varying definitions. Whitesides and Grzybowski limited self-assembly to processes that involve preexisting components, are reversible and can be influenced by proper design of the components [121]. In this thesis some examples of static self-assembly processes are studied, where the formation process might consume energy, but once formed the object is in a stable equilibrium state. Dynamic self-assembly processes, which are not as well understood as the static, are based on interactions that require dissipation of energy to exist [121]. One well-known example of dynamic self-assembly is the dynamic cytoskeleton structures in the cell. Self-assembly started from the chemists view of interest on molecular scales, but has spread to material science and nanofabrication in the form of so-called bottom-up processes. Top-down processes (the opposite of bottom-up), which are extensively used in microfabrication, rely on external tools to assemble, shape and modify materials into a desired structure and function. The bottom-up approach, relying on self-assembly, has the potential to generate very dense functional systems, from the macro to the nanoscale, with lower energy consumption than conventional top-down systems. Self-assembly/bottom-up systems have been studied for the formation of new functional materials and devices both using natural building blocks, such as DNA [122] or proteins [123], but also with synthetic molecules [124]. It is important to point out that self-assembly is very difficult to control into higher levels of hierarchy and no systems of high complexity have been demonstrated.

One step on the way to understand and control a self-assembly process is to understand the interactions involved. In molecular self-assembly non-covalent

interactions dominate; van der Waals, electrostatic and hydrophobic interactions, as well as aromatic π-stacking and hydrogen and coordination bonds, govern the process [121, 125, 126]. Bottom-up processes often involve self-assembly of larger components, such as wires or dots on the meso- or macroscale. Here other kind of interactions can have significant influence, for example the influence of gravitational attraction, external electromagnetic fields, as well as magnetic, capillary, and entropic forces must be considered [121].

A geometry of special interest in the field of self-assembly is more or less one-dimensional structures; wires, threads or string like objects, often termed nanowires (NWs). With knowledge from the IC industry it is easy to imagine how organized nanowires with different or variable physical features, such as insulating, semiconducting and metallic properties, can be assembled into a device or a logic system. Two well-studied classes of NWs are semiconducting inorganic nanowires [127, 128] and carbon nanotubes [129]. These materials can have excellent optical, electrical and structural properties, but are normally very non-dynamic and stiff materials. Natural biomolecular nanowires, which will be further discussed in the section below, and nanowires or tubes assembled from synthetic molecules, are dynamic structures. Apart from the possibility to functionalize these structures, with for example semi-conducting materials, the inherent properties of molecular recognition and reversible assembly/disassembly can offer new routes to design logic systems.

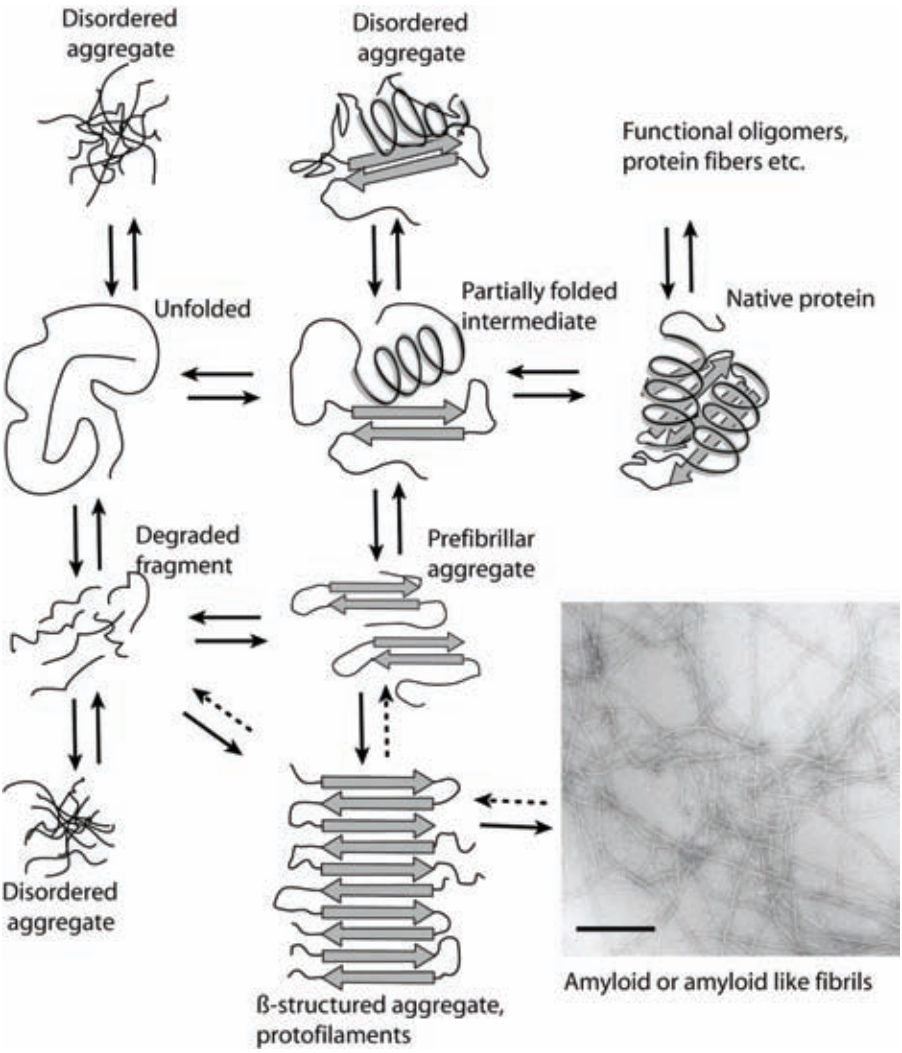
## *4.2 Biomolecular nanowires and assembly thereof*

The most well-known biomolecular nanowire is deoxyribonucleic acid, DNA. The aspect ratio of DNA is extra-ordinary, since the diameter is 1 nm and 2 nm of single- and double-stranded DNA respectively and single DNA strands of several tens of μm can be handled. The functionality of DNA as a nanowire is also one-of-a-kind with the inherent property of the genetic code, the Watson-Crick base pairs [130]. Furthermore, DNA is a relatively chemically robust molecule and its extensive use in molecular biology has lead to well-developed large-scale synthesis methods of DNA [123]. DNA has not only been used as a wire, but through design and synthesis the base-pairing have been utilized in advanced structures 2D and 3D structures [122, 126, 131-133].

Proteins and peptides is another class of biomolecular material that can be used to generate nanowires, often called fibrillar or filamentous proteins or protein fibers [126, 132, 133]. In contrast to DNA the assembly of protein fibers normally occurs from

building blocks in form of proteins or peptide monomers. A large heterogeneity and amount of monomers is available from pure synthetic approaches as well as over-expression of recombinant protein variants. The extra-cellular matrix protein collagen, the cytoskeleton proteins actin and tubilin, the motor protein myosin, silk proteins and filamentous phage coat proteins all have interesting inherent properties in their fiber form [123, 133]. In this thesis the focus has been protein amyloid fibrils. When exposed to destabilizing conditions some proteins and peptides can aggregate into well ordered, fibrillar assemblies, amyloid fibrils [134, 135]. The fibril formation can be generalized to a nucleation-dependent process, with the re-arrangement of the native protein structure to form a nuclei, followed by extension of fibrils from these (see figure 4.1) [136, 137]. Recent studies have shown that the ability to form amyloid fibrils is a common and perhaps generic property of all polypeptide chains [136, 138]. Apart from that the fibrillation process leads to loss of the original biological function, amyloid fibrils and precursors thereof are being increasingly recognized as associated to disease states, including Alzheimer´s disease and spongiform encephalopathies [109, 134, 136, 139, 140]. However, a number of reports have also demonstrated the importance of amyloid-like architectures in biological systems [132, 141]. As a biomolecular nanowire the amyloid fibril has an extreme stability and the potential to expose functional groups. Although no structural or sequential homology of amyloidogenic proteins, the amyloid fibril structure appears generic, showing linear filaments with a diameter of approximately 10 nm [142] for mature fibrils and lengths up to 10 μm (figure 4.1). Fiber diffraction studies have revealed a cross-β structure as characteristic of amyloid fibrils, i.e. the β-strands are perpendicular to the fibril axes forming axially aligned extended β-sheets [135].

Electrical conductivity is an often desired of property a nanowire. The degree of inherent conductivity of DNA is a matter of debate, depending on experimental conditions, insulating, semiconducting as well as conducting properties can be measured [143]. When it comes to natural protein- and peptide-based nanowires only insulating properties are to expect.

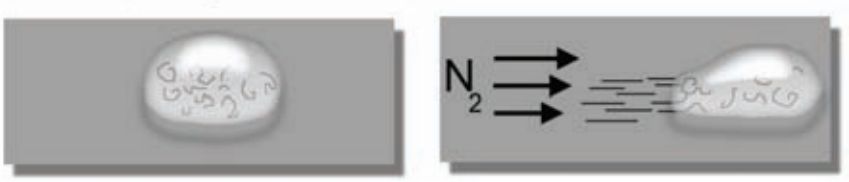



*4.1 Schematic illustration of various aggregation paths of a protein. The scale represents 200 nm.*

To achieve good conductivity in both DNA and protein fibers several examples of metallization have been demonstrated [123, 144-147]. Metallization is associated with significant increase of the wire diameter, 10 to 50 times for DNA [148], and at least 5 times for amyloid fibrils [147].

Organization is fundamental to utilize the functionality of any nanowire, biomolecular as well as inorganic. The two most common organization principles of biomolecular nanowires utilize a bulk liquid flow [144], or a moving air-water

interface (molecular combing) [148, 149] (figure 4.2). The stretching of DNA using the moving air-water interface has been done using a number of surface modifications, which should favor an interaction strong enough to partially attach the DNA strand and allow elongation perpendicular to the moving air-water interface [148]. More stiff structures such as inorganic nanowires [150, 151] and carbon nanotubes [152], as well as amyloid fibrils [21] have also been aligned using liquid flow and moving air-liquid interfaces.



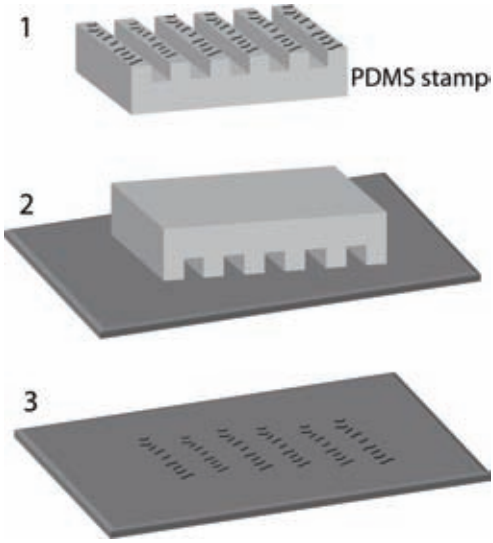

*4.2 Schematic illustration of molecular combing (moving air-water interface). (Left) A droplet of liquid with wire-like molecules in a more or less coiled geometry is deposited on a surface. The molecules have a weak affinity to the surface. (Right) The droplet is moved along the surface, e.g. using a directed gas stream. The molecules are elongated perpendicularly to the moving air-water interface and left stretched on the surface.*

Soft lithography, the use of elastomeric stamps for printing molecules, was first reviewed by Xia and Whitesides in 1998 [153]. Alignment of nanowires on elastomeric stamps followed by transfer from the stamp to a solid substrate, called transfer printing, have been demonstrated for carbon nanotubes [124], DNA [20] and amyloid fibrils [21] (see figure 4.3). Transfer printing, especially combined with patterned stamps, increases the variety of possible geometries and substrates for nanowire organization. In addition the printing enable sequential deposition of multiple layers of wires and thereby more complex and interesting systems [124].

## *4.3 Nanowires containing CPs*

In conjugated polymers the supramolecular order is crucial for the performance in opto-electronic processes. Electronic transport is strongly affected by different degrees of order and in light emitting applications the efficiency will be dependent on the spatial arrangement of individual chromophore segments [154]. In a polymer film, the deposition method, but also the molecular weight, as well as the nature and position of a side chain will affect the material properties. An illustrating example is that a high molecular weight highly regioregular poly(3-hexylthiophene), forming crystalline films, can have 6 orders of magnitude higher charge mobility than a regiorandom, non-crystalline variant of the polymer [154]. “Classical” polymers have

an amorphous character and large weight distributions that normally prevents the formation of well-defined structures by conventional solvent-based deposition techniques [154].




*4.3 Schematic illustrator of transfer printing. 1) The molecules to be printed are deposited on the elastomeric PDMS stamp, e.g. with molecular combing. 2) The stamp is put in conformal contact with the substrate for a short period of time (seconds to minutes). 3) The stamp is carefully removed and the molecules are left on the substrate.*

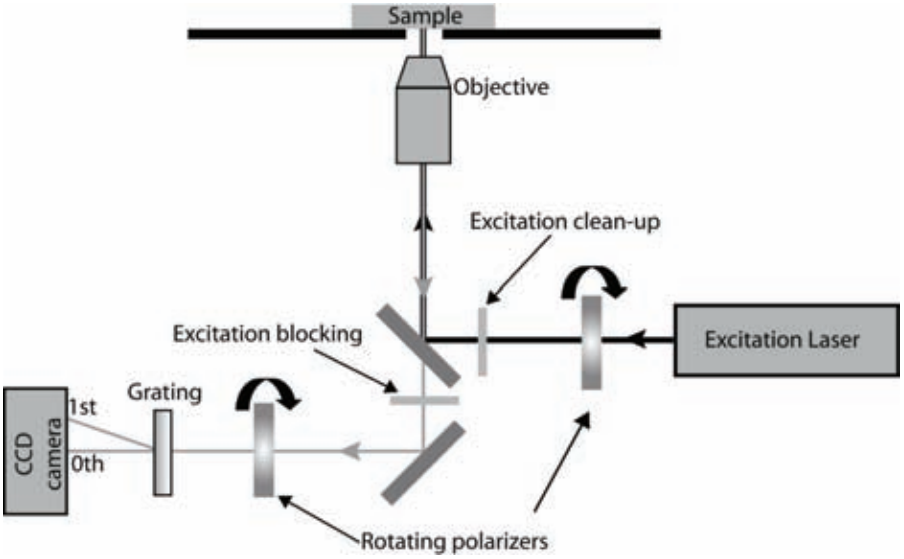
Most conjugated polymers can form inter-chain interactions through π-stacking, an interaction that can utilized for the formation of one-dimensional objects, nanowires [125]. The most commonly used techniques for form pure polymer nanowires are template synthesis, electrospinning, self-assembly, chiral reactions and interfacial polymerization (reviewed in [154, 155]). Template synthesis of nanowires is achieved by letting the formation, often electropolymerization, of the conjugated material be constrained by a porous structure, i.e. a membrane [156-158]. The method is versatile since many CPs can be electropolymerized, but dissolving the template while keeping separated nanowires of good quality is a difficult task. Electrospinning is very limited to which polymers can be used, but polythiophene and polyaniline, as well as hollow polyvinylpyrrilidone nanowires with conjugated polymer within has been demonstrated [159, 160]. In nanowires of polyaniline/poly(ethylene oxide) formed with electrospinning, it was shown that wires with a diameter less than 15 nm were insulating, an example of the problems that can arise when going to nanodimensions [155]. Plausible explanations for this behavior are that the small diameter makes

complete de-doping in air possible or that the diameter is smaller than the grain size of the materials. In self-assembly of conjugated polymer into defined nanostructures control of the deposition conditions is crucial when going from solution to solid state. Normally the assembly is dependent on low surface interaction strength [154]. Thiophene derivates, as well as PPE, poly(aniline), poly(indenofluorene)s and poly(fluorene) have been shown to form nanowire geometries. Typically it can be seen that the polymer main chain axis is perpendicular to fibrillar axis, which makes it plausible that π-stacking is the interaction governing the fibril formation. In very few of the reported nanowires formed from semiconducting conjugated polymers mobility measurements are reported, but recently Cho *et al*, demonstrated that a network of P3HT nanowires showed one order of magnitude higher field effect mobility compared to plain film [161]. Bjornholm *et al* used the collapse of Langmuir-Blodgett films to form nanowires of a amphiphilic polythiophene and could measure a conductivity of 40 S/cm after iodine doping [162]. A route to better control of the self-assembly processes in pure polymer systems, is the quite advanced synthesis of rod-coil block copolymer, containing segments of well-known conjugated polymers [163, 164].

Another way to control the assembly of CP is coating on or incorporation in an already existing template, such as a biomolecular wire. DNA has been the most commonly used biomolecular template onto which conjugated polymers have been polymerized. Both Ma *et al* and Nickels *et al* demonstrated the formation of polyaniline coated DNA, formed through oxidative polymerization of aniline monomers on surface immobilized DNA [165, 166]. Both reports concluded that the polyaniline was in an undoped state and Ma *et al* showed that how proton induced doping-dedoping could change the conductance significantly. Also polypyrrole has been polymerized onto DNA, importantly, surface immobilization of the nanowire has been demonstrated after the polymerization [167]. Another interesting biomolecular template is the tobacco-mosaic-virus, which can form 30 times longer nanowires if the assembly of the virus is supported by a polymerization of polyaniline [168].

We have worked with the assembly of water soluble conjugated polyelectrolytes onto DNA and amyloid fibrils [19-21], as well as incorporation of the polyelectrolytes into amyloid fibrils [22]. In paper VI we also demonstrate that water insoluble conjugated polymers can be assembled onto amyloid fibrils. The assembly processes was done in solution and followed by deposition and organization on surfaces using molecular combing and transfer printing. The CPs in these reports are in the

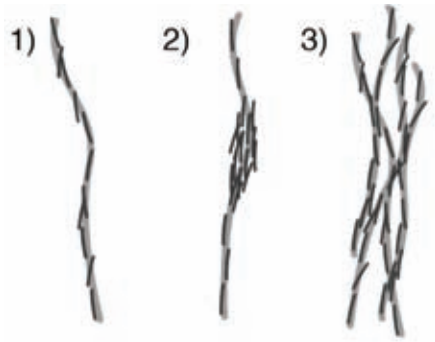
luminescent semiconducing state, making optical studies a good route to understand the structure of the wires. The conductivity of the water soluble conjugated polyelectrolytes is very low, also when measured in pure polymer films, and attempts to measure the field effect mobility has failed. However, the materials exhibit electro-activity shown with electrochemical emission quenching of the pure polymer POWT [169] and also on hybrid material formed by incorporation of the trimer-based tPOWT into amyloid fibrils [22].




*4.4 Schematic illustration of the SMS-setup used for recording emission of single or few emitting molecules. Note that both the $0^{th}$ and $1^{st}$ order diffraction from the grating hit the CCD, giving the image and spectrum respectively.*

The organization of the biomolecular nanowires decorated with conjugated polyelectrolytes on surfaces enables studies of single objects using single molecule spectroscopy (SMS) techniques. SMS is based on a fluorescence microscope where the emitted light from a sample pass through a diffraction grating and the zeroth order, the image, and the first order diffraction hit a very sensitive camera (see image 4.4). With this technique spectra of single fluorophores, and in our case, spectra of parts of wires can be obtained. On DNA decorated with POMT a correlation between emission intensity and peak maximum could be seen, where brighter objects were more red-shifted [19]. The brighter objects might be composed of larger assemblies of close-packed polymer chains, where energy transfer to low energy sites is possible. From very weakly emissive spots on DNA strands with discontinuous coating an

on/off phenomena, so-called blinking, could be seen. From the occurrence of blinking it can be concluded that the weak emissive polymer clusters, or single polymer chains, are smaller than the 10 nanometers constituting the upper limit of fluorescence energy transfer from a fluorescence quencher in conjugated polymers. Insulin amyloid fibrils coated with the anionic CP show no clear correlation between emission intensity and spectral maximum [21]. Brighter emissive parts of fibrils might be bundles where the local concentration of polymer chains is high enough to give higher intensity, but with too weak polymer inter-chain interactions to facilitate transport of excited states to energy sites (see figure 4.5). In our published report continuously coated, non-blinking, fibrils are reported. In later studies with discontinuously coated fibrils, very weakly emissive spots demonstrated blinking phenomena.




*4.5 Schematic illustrations of amyloid fibril decorated with a CP. 1) A single decorated fiber. 2) and 3) picture situations that could lead to stronger emission from a spot. In 2) the polymer chains are aggregated and energy migration should lead to a redshifted emission. In 3) the emission will be enhanced, but the polymer chains are not enough aggregated to facilitate an efficient energy migration.*

To study the anisotropy of fluorescence of a CP on aligned wire-like complexes, we used circularly polarized light to excite the molecules and registered the emitted light through a rotating polarizer. From PTAA decorated on amyloid fibrils an anisotropy ratio (intensity with the polarizer parallel to the fibrillar axis divided with intensity perpendicular to the axis) varying from 17, on weakly emitting objects, to 2.5 of bright objects, could be recorded. The anisotropy shows that the preferential orientation of the polymer chain axis is along the fibrillar axis. However, based on comparisons with earlier studies, where photoluminescence anisotropies G of 60 and 40, in MEH-PPV and poly(3-octylthiophene) were measured [170, 171], we can conclude that the polymer chains are far from fully aligned on the fibrils. In paper VI we shown that also the water insoluble APFO12 can decorate amyloid fibrils with the

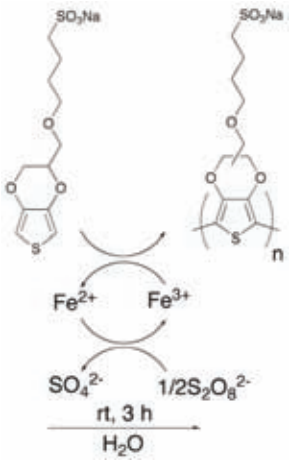
polymer chain axis along the fibrillar axis. However measurements of the polarization anisotropy in both absorption and emission showed higher G-values than for PTAA, significant of more well oriented absorbing and emitting dipoles. Also on DNA water soluble CPs assemble with the polymer chain axis aligned along the axis of the biomolecular nanowire, although we have not measured as high anisotropy ratios as on amyloid fibrils (unpublished results).

## *4.4 Nanowires with PEDOT-analogues*

PEDOT has, as mentioned in Chapter 2, unique electrical properties compared to other CPs, which makes it to a natural choice as a material desirable to integrate into nanowire structures. Both soft and hard templates have been used to synthesize wire-like structures in PEDOT [172-174]. Another very interesting approach is the molecular combing of PEDOT/PSS shown in two publications by Ito's group [175, 176]. Using a receding air-water interface on a silicon oxide surface nanowires of a few nanometers height and lengths up to 1.3 μm were formed. Conductivities similar to PEDOT/PSS films could be measured.

The focus in this thesis work has been assembly of conjugated polymers coordinated by biomolecules. Using the water-soluble dispersion Baytron P, containing PEDOT in an excess of PSS, it is difficult establishing conditions where an interaction between PEDOT and a biomolecule is plausible. Interactions between PSS and a biomolecule could result in phase separation of Baytron P. The relatively large particle size of the dispersion will also make an even coating of a wirelike template unlikely. Polymerization of PEDOT onto functional biomolecules is limited by the poor water-solubility of EDOT. We have been able to form dispersions of EDOT in high concentrations of DNA (30 mg/ml in $H_20$). For polymerization Fe(TOs) (iron tosylate) was added to the dispersion, which gave partial precipitation of the DNA. Cross-linking between Fe and DNA is likely to occur. After 10 hours of polymerization a dark blue gel-like material was formed and was precipitated with MetOH. The material could not form a homogenous solution in water, but rather consists of aggregates that precipitate with time. The conductivity of the DNA-PEDOT in a film formed from the dispersion was estimated to be roughly 10 % of that of Baytron P using 2 point probe measurements. A polymerization of EDOT in presence of DNA in dilute conditions, similar to the approach Houlton *et al* took with polypyrrole, [167] would be very interesting and is being evaluated in our lab. Recently we have found a new polymerization route of PEDOT- S (paper VII). Catalytic amounts of $FeCl_3$ are used and the $Fe^{3+}$ ion is regenerated with $Na_2S_2O_8$, which after dialysis gives a dark blue, fully water soluble material (see figure 4.6).

This PEDOT-S is a fully water-soluble material with a conductivity of 1.1 S/cm in spin-coated films. The solubility of this one-species PEDOT material facilitates nanowire assembly with biomolecular templates. Amyloid fibrils have multiple charges, due to the repetitive molecular structure, enabling strong interactions with polyelectrolytes [177]. A natural polyelectrolyte often found in amyloid deposits in tissue is heparan sulfate, which as well as heparin have been found to interact strongly with amyloid fibrils *in vitro* [177]. Both these polyelectrolytes contain sulfate groups, similar to the sulfonate groups found on PEDOT-S. Interaction between amyloid fibrils and PEDOT-S can in solution be seen as a visible co-sedimentation of the species to a gel-like lightly blue material. The precipitate and supernatant were drop-casted on surfaces into irregular films and the conductivity was determined with 2 point probe measurements. Taking 220 μM of PEDOT-S and 160 μM of insulin amyloid fibrils (concentration is given on monomer basis) the precipitate shows ~100 times higher conductivity than the supernatant. This value, together with the clearly visible blue color of the precipitate, shows that PEDOT-S is concentrated on the fibrils. The complex of PEDOT-S/fibrils gives a higher conductivity than the supernatant, although the bare fibrils are insulating. As a reference PEDOT-S at this concentration in water will not precipitate.




*4.6 Polymerization of EDOT-S in water with catalytic amounts of iron and excess of persulfate ions.*

# 5 FUTURE OUTLOOK

## 5.1 Protein detection

The detection schemes for protein misfolding and tissue staining using polythiophenes could most likely be improved with further development of assay conditions and design of new polymers. New side chains substitutions as well as backbone components should be introduced to elucidate the physicochemical origin of the specificity for the amyloid structure. New backbone components might also contribute to sensing polymers with higher quantum yield. Interesting side chain substitution would be sulfate or sulfonate groups, due to possible interaction with amyloid fibrils as discussed in chapter 4.

A route to take the polythiophene-based detection of misfolding to more commercial applications is to develop surface based techniques. The polythiophenes have shown strong interactions with amyloid fibrils, indicating that they can be used as capturing agents for amyloid in homogenized tissue. Today some test kits for BSE in cattle have polyelectrolyte capturing agents and the detection is made with antibodies and proteinase K digestion. Conjugated polymers could have the possibility to be used as both as capturing agents and reporters, if the optical properties of the polymer change in the capturing event.

In further development of conjugated polymers for biosensing it is important to understand and predict the behavior of conjugated polymers in water solutions and in surface interactions. A clearer picture of the connection between changes in optical properties, such as absorption, emission or chirality, and aggregation or conformational changes of the polymer chains is needed. If this could be achieved better detection schemes for more delicate events, such as protein-protein interactions, might be designed.

## 5.2 Nanowire assembly

Using biomolecules to organize conjugated polymers is an interesting area of research. Self-organization of wire-like objects into devices for logical functions is a truly challenging task and in this thesis only very small steps have been taken on the way to that goal. A smaller goal is to integrate conjugated polymers coordinated by

biomolecules into bulk and film devices, such as organic transistors or photovoltaic devices. The importance of the polymer chain organization in electrical and opto-electrical applications has been discussed in earlier chapters. In certain devices the coordination of two species relative to each other is also considered crucial for good performance. One example is the PCBM and the conjugated polymer in an organic bulk heterojunction solar cell. The specific interactions of biomolecules can be one way to govern organization.

In the process of forming conducting nanowires out of biomolecular templates, the synthesis of PEDOT-S was a break-through. The amyloid fibrils combined with PEDOT-S are currently studied with scanning probe methods to image the conductivity on the single wire level. Further development of water-soluble PEDOT-analogues, is interesting for combinations with other biomolecular nanowires, such as DNA. Furthermore combinations with semiconducting and conducting polymers on the same or separate nanowires are of interest to construct more complex devices. Geometries of interest on the same nanowire are both co-axially coated layers of different active materials and segments of different ative materials along the wire. A route to achieve the later could be to use specific biological interactions, such as DNA hybridization to position the active materials relative to each other. In combinations of separate wires of the same or different conjugated polymers, good control of the contact between the wires will be crucial. Based on fibers with micrometer sized diameters coated with PEDOT-PSS, Hamedi *et al* demonstrated the very interesting concept of WECTs, wire electrochemical transistors [178]. Crossings of wires were immersed in a solid polymer electrolyte and by applying a gate voltage on one wire the other will be de-doped, yielding a transistor with characteristics similar to a p-type depletion mode CMOS-FET. Through the combinations of crossings with WECT-function, ohmic contact and insulating crossings logic functions such as inverters and multiplexers were constructed. Furthermore the authors have demonstrated the stability of the WECT, in terms of horizontal displacement of the wires as well as the amount and shape of the electrolyte. It would be interesting to take the concept of the fiber-based logic functions demonstrated by Hamedi *et al* and realize similar components on a scale 1000 times smaller, by using biological nanowires coated with conjugated polymer.

# 6 SUMMARY OF PAPERS

Papers I-III include studies of polythiophene derivates as optical probes for amyloid fibrils. In paper IV-VI amyloid fibrils are used as biological nanowires. Studies of the physical properties of conjugated polymers associated to these wires are described. In paper VII the synthesis of a water-soluble conducting polymer and characterization of the optical and electrical properties is reported. This polymer has the potential to interact well with amyloid fibrils.

## *Paper I – Demonstration of a zwitterionic conjugated oligoelectrolyte as an optical probe for amyloid fibril formation*

Formation of amyloid *in vivo* is associated with several states of disease. A number of proteins and peptides are known to form amyloid fibrils *in vitro*, with loss of the original protein function as a consequence. In paper I insulin and lysozyme were chosen as model systems for amyloid fibril formation. We reported the synthesis of a zwitter-ionic, monodisperse, regioregular, polythiophene derivative, based on a trimer-block of thiophenes. This CP, called PONT and later renamed tPOWT, was demonstrated as an optical probe, in fluorescence, for discrimination between native and fibrillated protein. The assay to detect fibrils was performed at the low pH where the model proteins were fibrillated. By using a ratio between two emission wavelengths of the CP the kinetics of the amyloid fibril formation could be followed. The fibrillation was verified with transmission electron microscope and circular dichroism (CD).

## *Paper II – Demonstration of a anionic conjugated polyelectrolyte as an optical probe for amyloid fibril formation*

In paper II another CP is demonstrated as an optical probe for amyloid fibril formation using the same model proteins as in paper I. The CP is an anionic polythiophene derivate, PTAA, used at pH 7. By monitoring the optical signals of the CP in absorption and emission we could follow the fibril formation kinetics. The fibrillation was verified with CD and Congo Red, a known probe for amyloid fibril formation. An important difference from the performance of tPOWT is that for PTAA the spectral characteristics are different in the three cases buffer, interacting with

native protein and fibrillated protein. For tPOWT the spectra in buffer and interacting with native insulin are virtually indistinguishable.

### *Paper III – Comparison between eight conjugated polymers for discrimination of the native and amyloid fibril state*

Based on the discovery that polythiophene derivatives can discriminate between protein in the native and fibril state and that these CPs can be used for staining of amyloid in tissue samples [115], a comparing study of eight CPs was initiated. Four of these CP are based on monomeric thiophene units with anionic, cationic or zwitter-ionic sidechain. The other four are the trimer-based analogues of the monomer-based polymers. Based on fluorescence studies we demonstrated that the trimer-based CPs had higher optical signal specificity for fibrils compared to the monomer-based analogues. Two CPs were studied more deeply; tPOWT and tPTAA, the trimer-based version of the polymer in paper II. Titration experiments showed that these CPs can detect less than two percent of fibrils in presence of native protein. Furthermore analytical ultracentrifugation showed that these two CPs are in a state of small clusters in aqueous solution.

### *Paper IV – Integration of an electroactive conjugated polymer in a biological nanowire*

Amyloid fibrils are highly stable compared to other biological nanowires, which make them an interesting choice for assembly of nanowires with opto-electrical functionality. In paper I-III we demonstrated the interaction between amyloid fibrils and polythiophene derivatives with charged side chains. In paper IV we let CPs be present in a solution during fibrillation. From microscopy and fluorescence spectroscopy studies we could conclude that the CPs were incorporated into the fibril structure. This result was surprising since interacting species often intervene with fibrillation and the CPs are degraded if incubated without protein. However upon fibrillation for longer time bundled fibrils were generated in a geometry not seen without the CP. The electro-activity of these bundles were shown with electrochemical quenching, were doping of the polymer resulted in loss of fluorescence.

### *Paper V – Decoration of water-soluble conjugated polymer onto a biological nanowire*

The interaction between fibrils and PTAA was shown in paper II. To study the decoration of PTAA onto fibrils on the single fibril level it is crucial to be able to

deposit the fibrils in a controlled manner. Molecular combing, where molecules are stretched and aligned perpendicular to a receding air-water interface, has been applied to e.g. DNA and carbon nanotubes. Molecular combing was used in paper V to align PTAA complexed with insulin fibrils on hydrophobic surfaces. The decorated fibrils are also aligned on patterned PDMS (silicon rubber) stamps and printed onto surfaces. The emission of the aligned fibrils was studied with single molecular spectroscopy (SMS) techniques. The emission from the fibrils on surfaces was red-shifted compared to solution, but no clear correlation between intensity and peak wavelength could be found. To elucidate the organization of PTAA chains on the fibrils, a rotating polarizer was introduced in the beam of the emission and the modulation was recorded. In CPs, the emissive dipoles are preferentially aligned along the polymer chain axis. For the fibrils decorated with PTAA an Imax/Imin in the range 4 – 17, with an average of 7, was measured. The maximum was recorded with the polarizer parallel to fibril axis, indicating a main orientation of the polymer chains along the fibrillar axis.

### *Paper VI – Decoration of the biological nanowire with a water-insoluble polymer*

Amyloid fibrils are relatively stable in aqueous solution and dry in air. However, most well performing CPs for device applications are only soluble in organic solvents. Highly non-polar organic solvents are not compatible with native proteins and even amyloid fibrils will be partially aggregated or degraded upon exposure. THF is an organic solvent, which is miscible with water and dissolves CPs with polar side chains. In paper VI we show the decoration of fibrils with an alternating polyfluorene, APFO 12. With SMS techniques and rotating polarizers in both emission and excitation we could conclude that the modulation is higher than with PTAA, Imax/Imin on average ~10.5 for emission. Also this polymer is organized with the polymer chains oriented along the fibril.

### *Paper VII – Synthesis and characterization of a water-soluble conducting polymer*

In the above mentioned papers the conjugated polymers are in semiconducting, luminescent state. PEDOT-PSS is a commercially available dispersion of a CP that is conducting in the pristine state. For interaction with biological nanowires and patterning on the nanoscale a conducting mono-component system, preferable water soluble, desirable. With this ambition synthesis of PEDOT analogues with charged side chains was initiated. In paper VII we report a new polymerization route of the

monomer EDOT-S to yield PEDOT-S, a PEDOT analogue with a butanesulfonic side chain substitution. The polymerization was made with iron chloride in catalytic amount and regeneration of $Fe3^{+}$ with sodium persulfate. The resulting polymer has good water solubility and high conductivity (1.1 S/cm) in the pristine state. Furthermore we the material demonstrates good electrochromic contrast ~48% at $\lambda_{max}$, which show potential for use passive display application.

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