Linköping studies in Science and Technology
Dissertation No. 1693

POLY- AND OLIGOTHIOPHENES

OPTICAL PROBES FOR MULTIMODAL FLUORESCENT ASSESSMENT OF BIOLOGICAL PROCESSES

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Linköping 2015

Cover: Fibroblasts and melanoma cells stained with a fluorescent thiophene probe called p-HTIm, DAPI and antibodies against ER, proteasomes, α -tubulin, fibronectin, or mitotracker against mitochondria.

During the course of the research underlying this thesis, Karin Magnusson was enrolled in Forum Scientium, a multidisciplinary doctoral program at Linköping University, Sweden.

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Printed by LiU-Tryck, Linköping, Sweden, 2015

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ISBN: 978-91-7685-986-5

ISSN: 0345-7524

Linköping studies in Science and Technology, Dissertation No. 1693

ABSTRACT

One interesting class of molecules in the research field of imaging biological processes is luminescent conjugated polythiophenes, LCPs. These fluorescent probes have a flexible backbone consisting of repetitive thiophene units. Due to this backbone, the probes possess unique abilities to give rise to different spectral signatures depending on their target and environment. LCPs are a polydispersed material meaning there is an uneven distribution of lengths of the probe. Recently, monodispersed chemically well-defined material denoted luminescent conjugated oligothiophenes, LCOs, with an exact number of repetitive units and distinct side-chain functionalities along the backbone has been developed. LCOs have the advantages of being smaller which leads to higher ability to cross the blood brain barrier. The synthesis of minor chemical alterations is also more simplified due to the well-defined materials.

During my doctoral studies I have used both LCPs and LCOs to study biological processes such as conformational variation of protein aggregates in prion diseases and cellular uptake in normal cells and cancer cells. The research has generally been based on the probes capability to emit light upon irradiation and the interaction with their targets has mainly been assessed through variations in fluorescence intensity, emission-and excitation profiles and fluorescence lifetime decay. These studies verified the utility of LCPs and LCOs for staining and discrimination of both prion strains and cell phenotypes. The results also demonstrated the pronounced influence minor chemical modifications have on the LCO's staining capacity.

POPULÄRVETENSKAPLIG SAMMANFATTNING

LYSANDE MOLEKYLER FÖR STUDIER AV BIOLOGISKA PROCESSER

Luminiscenta konjugerade polytiofener, LCPs, är en väldigt intressant typ av molekyl som kan användas för att studera processer i biologiska system. Strukturen hos dessa fluorescerande molekyler kan liknas vid olika element som är fästa vid en ryggrad. De olika elementen ger molekylen dess affinitet för sin målmolekyl, medan ryggraden är mest ansvarig för molekylen optiska egenskaper. Ryggraden ändrar sin struktur beroende på målmolekylen och resultatet kan avläsas med ett flertal fluorescens-metoder. Längden på ryggraden hos LCPs varierar och varje parti av en LCP består därmed av en blandning längder och storlekar. De senaste åren har Peter Nilssons forskargrupp utvecklat LCPs till molekyler med väldefinierad längd på ryggraden som då betecknas luminiscenta konjugerade oligotiofener, LCOs. Dessa mindre molekyler har större möjlighet att passera blod-hjärnbarriären samt att syntesen för små kemiska modifieringar av elementen vid ryggraden förenklas.

I den här doktorsavhandlingen beskriver jag användningen av både LCPs och LCOs för att studera strukturförändringar av proteinaggregat i prionsjukdomar och skillnader mellan normala celler och cancerceller. Forskningen har mestadels varit baserad på molekylenas förmåga att fluorescera och metoder som har använts flitigt är fluorescens-intensitet, spektrala skillnader i emission och excitation samt molekylenas fluorescens livstid. Molekylen har visat på mycket god förmåga att särskilja de olika sjukdomstillstånden och de olika celltyperna. Framförallt har väldigt små kemiska modifieringar av molekylen visat sig ge väldigt stora effekter.

ACKNOWLEDGEMENTS

Att doktorera är ett väldigt speciellt jobb. Jag har många att tacka för att jag har klarat av de här åren:

Först och främst vill jag tacka min handledare Peter Nilsson för alla bra idéer och all hjälp. Jag vet inte riktigt vad du gör, men varje gång man kommer från ditt kontor så känner man sig grymt peppad och man tror att man är en forskare av världsklass. Visst att man lätt kommer ner på jorden igen, men den där uppladdningen är så väldigt mycket värd.

Min biträdande handledare Per Hammarstöm, vars dörr alltid känns som att den är öppen, där jag alltid får konkreta och bra svar på mina funderingar och att du pressar in korrekturläsning i ditt fulla schema.

Att få ringa till Jon Jonasson eller Bertil Kågedal i tid och otid, jag har alltid känt mig välkommen med alla möjliga konstiga frågor.

Hanna som fick uppgiften att lära ingenjören om cellodling. Jag är djupt imponerad av all din kunskap och tacksam för allt du lärt mig.

Syntesarna i PetNi-gruppen – Bäck, Leffe, Matte, Katriann, Linda, Elisabet, Hamid, Bisse och gamlingarna Andreas och Timmy – Tack för alla molekyler och för att ni försökt att få mig och fatta det tuffa med organkemi.

Leffe - kontorssambon och kortbyxekungen!!! Jag hade nog aldrig pallat med dessa år utan dina hugg och skratt, gött att veta att man alltid har en fredagsbärs-kompis.

Roz, my other office-roomie, Thank you so much being so not like me – I have learned a lot!! And for taking great care of me several times in the states.

Therése, allt gött snack om allt och inget. Tack för ditt enorma stöd och lugn genom åren och för det superbra avslutet i min kappa.

Jens, utan dig så hade jag nog inte hamnat i doktorerandet. Du såg till att jag tog en kall öl ibland, för att komma tillbaka dagen efter med ny energi.

Daniel – tack för all grym hjälp med livstider och färger!

Malin – tack för trevliga pratstunder och all programmerings- och statistikhjälp.

Artur – thanks for all valuble help with cells and live-microscopy.

Stefan, Charlotte och Anette – jag är så glad att jag har varit med i Forum. Tack för att ni gör ett så grymt jobb.

Korridorskompisarna – Tack för goda Nalle-fikan med snack om det mesta, Nalle – tack för skratt och gliringar, Susanne – tack för att du bryter forskningstugget med annat tugg. Jutta för din omtänksamhet, Mikaela, Anki, Patrik, Lotta, Martin, Liza för roliga diskussioner.

Maria och Lunkan, vad kul jag hade med er i alla labrapportshögar.

Kaffeklubben med Sara – vad mycket kul vi haft på sommarkonferenser och resor med popcorn-kjolar och Marilyn, Anders, Jonas, Jonas, Katarina, Per, Robban och alla andra kaffeslukare.

En massa glada miner man möter i korridorerna som Madan – I hope to take a beer without “I have to check this gel”-break one day, Cissi, Sara, Amelie, Raul, Sofie, Alex, Maria, Patrik, Peter och Matte.

Gamlingar som jag saknar att möta i korridorerna bland annat Kanmert, Alma, Linda, Ina, Alexandra, Anna, my dear ex neighbour Abeni, Noppe, Bosse, Roger och Fredrik.

Linnea – du ger mig så mycket energi.

Alla ni på Patologen som har fått mig att känna mig välkommen in i cellodlingsvärlden, Katarina, Karin, Karin, Ida, Petra, Anna, Linda, Jakob och ni andra på plan 9.

Kerstin och Kristina – Partyhöjarna på KlinKem - Vad jag haft kul på era fester.

Brudarna – Ida, Lovve, Johanna, Anna och Martina. Så mycket kul vi har haft och kommer att ha.

Love – för att du tar dig an toastandet.

Matlaget – Grymt att man kan få lagad mat och trevligt sällskap även de mest uppbokade kvällarna.

Luckan – ja tror omslaget kommer bli fasligt vackert.

Tyttorp och alla dess fyrfotade invånare – mitt andrum.

Mamma, pappa, syster med Jonas – Stöd och hjälp genom åren med allt möjligt, det hade inte funkat utan Er!!!

Björn Jättebäst! Nu vill jag ta igen tiden för resor, sovmornar och sköna häng!!!

PAPERS INCLUDED IN THE THESIS

PAPER I

Magnusson K*, Simon RA*, Sjölander D, Sigurdson CJ, Hammarström P, Nilsson KPR. Multimodal fluorescence microscopy of prion strain specific PrP deposits stained by thiophene-based amyloid ligands. *Prion*. **2014**, 8, 319-329.

PAPER II

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PAPER III

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PAPER IV

Magnusson K*, Appelqvist H*, Cieřlar-Pobuda A, Bäck M, Kågedal B, Jonasson J, Łos MJ, Nilsson KPR. An imidazole functionalized pentameric thiophene displays different staining patterns in normal and malignant cells. *Accepted in Frontiers in Chemistry, section Chemical Biology* **2015**.

**These authors contributed equally to this work.*

CONTRIBUTION TO INCLUDED PAPERS

PAPER I

Participated in planning and performing all the experiments, and was shared author of the manuscript.

PAPER II

Participated in planning and performing all the experiments, and was main author of the manuscript.

PAPER III

Participated in planning the experiments, and performed some spectroscopic experiments.

PAPER IV

Participated in planning and performing all the experiments, and was shared first author of the manuscript.

CONFERENCE CONTRIBUTIONS

Magnusson K and Nilsson KPR. Detection of prion strains. *Advances in Amyloid Sciences: From diseases to devices*, **2010**, Sandbjerg, Denmark

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PAPERS NOT INCLUDED IN THE THESIS

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ABBREVIATIONS

A β	Amyloid β
BBB	Blood brain barrier
BSE	Bovine spongiform encephalopathy
CP	Conjugated polymer
CPE	Conjugated polyelectrolyte
CWD	Chronic wasting disease
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
LCO	Luminescent conjugated oligothiophene
LCP	Luminescent conjugated polythiophene
LED	Light emitting diode
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PFA	Paraformaldehyde
PPV	Polyphenylene vinylene
PrP	Prion protein
PVC	Polyvinyl chloride
SS	Sheep scrapie
ThT	Thioflavin T
TSE	Transmissible spongiform encephalopathy

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GENERAL INTRODUCTION

The more knowledge we gain about living organisms and functions behind certain biological processes, the more we can improve the quality and standard of life. In this regard, molecular tools for imaging of biological systems are of great interest within life science research since such tools can aid in improving the understanding of life. There are many useful applications in molecular biology and fluorescent probes, for instance fluorescently labelled antibodies and fluorescent proteins, are a very vital part. Conjugated polymers and oligomers are another kind of fluorescent probe, probes that this thesis is based on. These probes have been used in attempt to distinguish different events in biological processes to gain more knowledge about certain functions.

CONJUGATED POLYMERS AND OLIGOMERS

INTRODUCTION

Conjugated polymers and oligomers possess an extensive variety of unique properties and are employed within a wide range of areas such as in light emitting diodes (LEDs), solar cell applications and chemical- and biosensors. In this thesis, the performance of these optoelectronic materials as fluorescent reporters has been evaluated and due to their fluorescent properties many interesting phenomenon have been revealed. These conjugated probes show a striking affinity for protein aggregates that are highly involved in several neurodegenerative diseases like different prion diseases and Alzheimer's disease. In addition, their chemical and photo-physical properties also make them suitable for distinguishing normal cells and cancer cells. The aim with this thesis has been to evaluate these conjugated probes for detection and characterization of prion aggregates and different cell types with the ambition of developing novel methodology for studying disease associated molecular phenomena.

CONJUGATED SYSTEMS

In conjugated materials the atoms are bound together by alternating single and double bonds. Some examples of conjugation in nature are found in hemoglobin, chlorophyll, the retinal, which detect light in the eye, and different pigments such as beta-carotene, which gives rise to a strong orange colour, see figure 1. The part of a molecule that causes it to be coloured is called chromophore and it is the conjugated system in the chromophores that makes them able to absorb and reflect light of different colours. Thus, the conjugation lends the molecule it's

optoelectronic properties and the connection between these properties and the chemical composition of conjugated systems will be discussed further below.

Figure 1. An example of a conjugated system in nature, beta-carotene, which is an orange pigment abundant in plants and fruit, for example rowan. Photo taken at Tyttorp by Karin Magnusson.

POLYMERS AND OLIGOMERS

Polymers are large molecules, macromolecules, composed of several repeating units called monomers. The word derives from the Greek language, poly = many and mer = parts. DNA, wool, and proteins are examples of natural polymers while polyester, nylon, and polyvinyl chloride (PVC) are synthetic polymers. A polymer consisting of one repetitive unit is called homopolymer. If the letter A represents this monomer, then the polymeric version would have the structure:

A copolymer consists of two or more repeating units and if the letter B represents the second unit, the structure would look like:

The length of a polymer can also be well-defined; the molecule is then called oligomer, oligo = little or few. The difference between a polymer and an oligomer is that the molecular properties of a polymer remains unchanged when removing one or a few units, whereas the properties of an oligomers alters significantly by the same treatment [1].

This combination of chemical features from both conjugated systems and polymers/oligomers impart these molecules with their unique conformation-induced optical properties.

CHEMICAL AND ELECTRONIC STRUCTURE / CONJUGATION

The fluorescent reporters used in this thesis are a particular class of organic molecules. Organic implies that a molecule is carbon-based. The carbons are connected via alternating single and double bonds and this alternation is called π -conjugation. Due to this phenomenon the electrons (π -electrons) are delocalised along the repeated monomers, which is referred to as the conjugated backbone of the molecule. The conjugated π -system is considered to be the main source of charge transport in conjugated systems [2]. Furthermore, it is due to this conjugation that the conjugated polymers have the ability to emit light, fluorescence, upon excitation and this optical property makes them extremely valuable tools within biosensor applications. The excited molecule in a conjugated system, can relax down to different levels in the ground state, resulting in emitted light of different colours, see the energy diagram in figure 2d. Figure 2 also shows the difference between a non-conjugated and a conjugated system as well as a polythiophene, a conjugated polymer with cyclic subunits upon which this thesis is based.

Figure 2. Chemical structures of different polymers and a Jablonski diagram. a) The non-conjugated polymer polyethylene, b) the conjugated polymer polyacetylene c) polythiophene and d) an energy-diagram illustrating emission of light with different colours.

CONJUGATION LENGTH

The distance an electron can move along the conjugated backbone is termed the conjugation length. The length of the backbone in polymers is most often polydispersed whereas the length of the backbone in oligomers is mostly monodispersed. Oligomers with different lengths of their backbone have different effective conjugation lengths, which leads to diversity in their electrical and optical properties. Figure 3a shows an oligothiophene called p-HTIm, where p implies *penta*, a backbone with five thiophene units and figure 3b shows h-HTIm, where h implies *hepta*, a backbone with seven thiophene units. As exemplified in figure 3a, a longer conjugation of the backbone effects the movement of electrons along the thiophene backbone and results in a more red-shifted emission spectrum with lower energy.

The effective conjugation length of backbone is also related to the conformation of thiophene backbone and conjugated thiophenes can adopt many different conformations due to twisting of the backbone, see figure 3c, all depending on their target and their environment. The conformation of the backbone highly affects the movement of electrons along the backbone, which in turn leads to alterations of the optical properties. A planar conformation increases the conjugation length allowing for movement of electrons along the backbone whereas a more twisted shape decreases the conjugation length hindering the free movement of electrons. The planarization of the backbone results in a red-shifted emission spectrum with lower energy and the more twisted conformation leads to a blue-shifted emission spectrum with higher energy. Aggregation of the probes also alters the optical properties into a red-shifted emission spectrum as well as a decrease in intensity of the fluorescence, due to stacking of adjacent thiophene backbones, see figure 3d.

Figure 3. Chemical structures of thiophene probes with different conjugation lengths and schematic presentations of the emission spectral differences. a) The pentamer p-HTIm, b) the heptamer h-HTIm, c) p-HTIm with twisted backbone and d) aggregated form of p-HTIm.

CHEMICAL ALTERATIONS

Solubility is very important for molecules in biochemical applications. For instance, fluorescent probes for live cell staining needs to be soluble under physiological conditions. As long as a polythiophene is in an unsubstituted form as in figure 2c, it is not typically soluble in polar solvents. However, the solubility, as well as the biocompatibility, of a polythiophene increases dramatically when adding ionic substituents along the repetitive thiophene backbone. These side-chains can be of great variability; they can be anionic, cationic, zwitterionic or without charge (as the side-chains in figure 3). The solubility increases most often with increasing charge. Furthermore, improvements in synthetic methods have offered the possibility to

functionalize the well-defined oligothiophene at distinct positions along the backbone [3], [4]. Figure 4a shows positions for possible alterations as R_x .

The chemical nature of the side-chain functionalities has also been shown to be imperative for achieving poly- and oligothiophenes selective for a specific target. Even very small side-group modifications can greatly impact interactions between the thiophene probe and the intended target; the target can be of a completely other character before and after minor modifications. A common side-group is the carboxyl group shown in figure 4b.

Alterations of the side-chains on the ends of the thiophene backbone have also prominent influence on the oligothiophene's optical properties when binding their target [4]. Figure 4c shows a thiophene probe with carboxyl groups extending the thiophene backbone, in comparison with the thiophenes in figure 3 and 4b, which have hydrogen as end-groups. It is also possible to add other end-groups, both symmetrically on both ends and asymmetrically as in figure 4d, where a porphyrin, a group that will be discussed further on, is attached only to one side.

Replacing one of the thiophene units within the backbone as in figure 4e can also alter the conjugation length and the properties of the probe. Substitution of a thiophene moiety with a selenophene or a phenylene units [5] alters the conformational freedom and thereby affect the conjugation length. Thus, these chemical modifications of the conjugated backbone can efficiently be utilized to tune the fluorescent properties of the probes.

Figure 4. Chemical structures of thiophene probes with different chemical alterations. a) general structure showing possible alteration probes positions as R_i ; b) probe with carboxyl groups as side-groups, p-HTAA, c) probe with carboxyl groups as side-groups and as end-groups, p-FTAA, d) probe with different end-groups; porphyrin and carboxyl group, p-FTAA-porph and e) probe with a phenyl-group in the thiophene backbone, p-FTAA-Ph.

These chemical variations mentioned above might seem small but they have great impact on the probe's properties and it opens up an enormous area of possible applications within biological detection. Due to the affinity for different biomolecular targets and the fact that the molecular interaction can be comprehended in a measurable signal, conjugated thiophenes have been utilized for a variety of applications within the biosensor field.

CONJUGATED BIOSENSORS – FROM POLYDISPERSED TO MONODISPERSED MATERIAL

CONJUGATED POLYMERS, CPs, A HISTORICAL PERSPECTIVE OF CONJUGATED BIOSENSORS

INTRODUCTION AND APPLICATIONS

It was discovered in the 1970's that the conjugated polymer (CP) polyacetylene can become almost as conductive as metal [6]. Due to the conjugated system, the electron movement and charge transport in CPs make them suitable as semiconductors or conductors. The combination of the optical and electrical conductivity, as well as the processing advantages and the mechanical properties of polymers make the CPs a very valuable class of molecules. CPs can be used in a wide range of applications including light emitting diodes (LEDs) [7] and solar cells [8]. Examples of CPs are polyphenylene vinylene (PPV), polyaniline and polythiophene. However, for applications within chemical-and biosensors, some modifications of the chemical structure are required.

CONJUGATED POLYELECTROLYTES, CPEs

INTRODUCTION

As mentioned above, solubility under physiological conditions is essential for fluorescent probes within biochemical applications. Water soluble CPs can be achieved by adding charged side-chains to the conjugated backbone of the polymer. The polymer is then developed into an electrolyte denoted conjugated polyelectrolyte, CPE [9]. Heeger and co-workers initiated research on CPEs in

1987 [10]. CPEs can be synthesized from many types of polymers including the CPs mentioned above, PPV [11], polyaniline [12], and polythiophene [13]. Figure 5 shows examples of CPEs.

Figure 5. Chemical structures of two CPEs.

APPLICATIONS

An important application for CPEs is likewise to CPs also solar cells but this is not anything this thesis will focus on. The addition of side-chains opened up the opportunity to apply CPEs into research involving chemical- and biosensors and the first biological application was reported in 1993 [14]. Both specific interactions like biotin-avidin [15] and antibody-antigen [16] can be studied, as well as unspecific interactions like electrostatic [17] and hydrophobic bindings [18]. Targets like small ions, small biomolecules, proteins, enzyme activity and DNA have been explored [19].

CPEs AS FLUORESCENT REPORTERS

Bin Liu (National University of Singapore, Singapore) has studied grafted CPEs and shown their function as self-assembled highly fluorescent nanoparticles that have remarkable properties for cell imaging [20]-[22]. To date, the group has now synthesized CPs exhibiting excellent properties for photothermal therapy [23] and oligomeric material for photodynamic therapy [24].

The Shu Wang group (University of Chinese Academy of Science, China) has also shown interesting results regarding fluorescent conjugated polymer nanoparticles and their ability for cell imaging [25], [26]. In addition they have shown very interesting results with a conjugated polythiophene where the probe clearly can distinguish living and apoptotic cancer cells [27].

DETECTION MECHANISMS

CPEs possess unique structural and optical properties, which makes them very interesting tools for optical sensing. As mentioned above, the charged side-chains provide the probe water solubility and ability for sensing in biological assays where aqueous media is required. The charges also provide for interactions with ionic species. Another advantage is their amphiphilic character; due to the hydrophobic backbone and the hydrophilic side-chains, CPEs are able to aggregate in aqueous solutions and some CPEs can form supramolecular complexes [28]. This amphiphilic character allows for both electrostatic and hydrophobic interactions with different analytes. CPEs provide an exceptionally straightforward and simple implementation, whereas many other fluorescent assays rely on externally applied reporter systems, for example fluorophore labelled antibodies. The probes are also very sensitive, as very small external stimuli are necessary due to the sensitive and collective responses of the CPEs because of their ability of amplified quenching effect and conformational rearrangement. Detection at as low as the zeptomolar range has been published [29].

The sensing mechanisms can be divided into three different categories; amplified quenching mechanism, fluorescence resonance energy transfer (FRET) and changes in conformation [19], which are all described in figure 6. Amplified quenching and sensing mechanisms with FRET are related to and based on very similar principles, whereas altered photophysical properties are more exceptional. Combinations of these methods can be utilized to improve the detection limits and results.

The concept of amplified quenching associated with CPs was first published by Swager and co-workers in 1995 [30] and is now also associated with CPEs [31]. Fluorescence quenching implies decrease in fluorescence due to energy transfer between two molecules, which can occur by different mechanisms [32]. Dynamic quenching occurs when the fluorophore (in its excited state) collides with a so-called quencher. Static quenching occurs when fluorophores form non-fluorescent complexes with quenchers and this process is not dependent on diffusions or molecular collisions. Oxygen, halogens and amines are some examples of molecules that can act as quenchers. It is shown that the amplification increases with polymer chain length and in phenomenon known as superquenching. Quenching mechanism is widely used for detection of different ions [33], [34] as for example in a pH sensor.

FRET implies energy transfer from a donor fluorophore to an acceptor fluorophore but in contrast to quenching, the acceptor emits light. FRET occurs when the emission spectrum of the donor overlaps with the absorption spectrum of the acceptor and is also dependent on the distance between the two molecules [32]. CPEs are particularly excellent donors [19]. FRET can in many cases be used in DNA sensing [35]-[37]

The most advantageous mechanism is the ability to change photophysical properties of the CPE as a result of conformational changes of the conjugated backbone [19]. Depending on target and environment, CPEs can undergo structural changes that affect their photophysical properties and can be visualized as altered absorption and/or emission spectra. This conformational flexibility of the conjugated backbone is based on non-covalently interactions with the biomolecule of interest and is used, for example, in protein sensing applications [38], [39].

Figure 6. Schematic images of different detection mechanisms; quenching, FRET and conformational alteration.

Polythiophenes, the probes that are used extensively in this thesis, have great ability to undergo conformational alterations and are widely used to detect conformational changes in proteins [38], [40], synthetic peptides [41], [42] as well as for DNA detection [43]. It is this conformational induced optical phenomena that is utilized for the studies in this thesis and these thiophene-based fluorescent reporters will be discussed in the following sections.

LUMINESCENT CONJUGATED POLYTHIOPHENES, LCPs

INTRODUCTION

Luminescent conjugated polythiophenes (LCPs) are a kind of CPE with thiophenes as the repeated unit in the conjugated backbone, see the chemical structure in figure 7. Thiophene is a heterocyclic aromatic compound with the formula C_5H_4S and the name derives from the Greek language, theion = sulphur and phaino = shining. Thiophene was first discovered in 1892 as an impurity in benzene [44] and it has similar properties as the benzene compounds, for instance in boiling point and smell. The structure can be found in petroleum or coal and is very well known in therapeutic applications [45].

Figure 7. Chemical structure of thiophene.

LCPs AS FLUORESCENT REPORTERS

Mario Leclerc's group (Université Laval, Canada) utilizes LCPs for optical detection of DNA samples and have reported very simple and rapid detection of nucleic acids with a positively charged polythiophene [29], [46]. This hybrid DNA/polythiophene complex has been useful in the detection of ions, proteins and enantiomers [47]. Another very successful research group in the area of conjugated thiophenes is lead by Olle Inganäs (Linköping University, Sweden). Peter Nilsson and others in Inganäs' group initiated the story of using LCPs in biological applications in Linköping by combining LCPs that originally were synthesized for solar cell applications with biomolecules. The initial LCP was POWT [13], a zwitterionic probe that was able to detect peptide conformational changes from random coil to a four-helix bundle [41]. POWT has also shown to be able to follow DNA hybridisation [43], conformational changes of calmodulin upon binding to calcium [48] and is also capable of staining fixed human fibroblasts [49]. POMT was the second original LCP [50] and the third one, which has been used extensively, is PTAA [51]. The chemical structures of the three original LCPs are shown in figure 8.

Figure 8. Chemical structures of some LCPs.

As previously mentioned, these LCPs were originally synthesized for solar cell applications but serendipitously these thiophene based fluorescent probes were found useful as ligands for disease-associated events such as protein aggregation in prion diseases and cellular alterations in cancer. To better understand the interactions between the thiophene probes and these diseases, the diseases will first be described briefly.

PROTEIN AGGREGATION DISEASES

Proteins are extremely important components in living organisms and are involved in virtually all machineries and processes in the cell. To be able to perform, it is extremely essential that the proteins maintain their correct fold although this is not always the case. The proteins can become misfolded and develop aggregates denoted amyloid that are involved in different protein aggregation diseases like prion diseases (bovine spongiform encephalopathy (BSE, mad cow disease), scrapie, chronic wasting disease (CWD), kuru, and Creutzfeldt-Jacob disease), Alzheimer's, Parkinson's and Huntington's diseases as well as Type II diabetes and systemic amyloidosis.

The aggregation pathway is often described as a nucleation-dependent polymerization mechanism where native monomers misfold into β -sheets that form oligomers and prefibrillar aggregates, which develop into protofibrils and then eventually amyloid fibrils [52], see figure 9. This aggregation process can be very time consuming which is not preferable in research. It has been shown that the process can be accelerated by seeding with preformed amyloid fibrils where a small aggregate seed acts as a template [53], resulting in a shift to the left of the schematic kinetic curve in figure 9. The complex formation of amyloid fibrils involves multiple pathways and the process just described only serves as a model.

Figure 9. General scheme of amyloid fibril formation together with a schematic kinetic curve of the growth.

The protein aggregation diseases that this thesis will focus on are prion diseases. These fatal progressive neurodegenerative diseases, also known as transmissible spongiform encephalopathy (TSE), can affect both humans and animals [54]. The diseases break out when the normal cellular prion protein, PrP^{C} , (C from cellular or common) is converted into the disease-linked form PrP^{Sc} (Sc from Scrapie) [55], [56]. PrP^{C} is found in several locations yet its normal function is not fully understood, whereas the aggregated form, PrP^{Sc} , forms plaques in the brain. Even though the primary structure of the protein is identical, the plaques can vary in conformation and this is believed to give rise to distinct prion strains [57], [58]. Conventional amyloid agents cannot distinguish different strains whereas this strain phenomenon is not a difficulty for thiophene probes [59]. Prion research using thiophenes is continued in this thesis, involving two different prion strains, Sheep Scrapie (SS) and Chronic Wasting Disease (CWD).

SS was reported as early as in the 18th century [60] and is affecting, as the name implies, sheep but also goats. The central nervous system is affected and a very obvious symptom is alteration in the fleece. CWD is a disease affecting mule deer, white-tail deer and Rocky Mountain Elk [61]. CWD has been observed since the 1960's and symptoms are weight loss and behaviour alterations [62].

It has now been revealed that development of several other neurodegenerative diseases such as Alzheimer's and Parkinson's disease are highly affected by seeding injections through a prion-like mechanism [63]. Furthermore, heterogeneous populations of protein aggregates similar to prion strains have also been observed in these neurodegenerative diseases [64], [65]. It was recently shown that seeded growth of brain extract from two Alzheimer's patients with distinct clinical

histories resulted in different structures of the fibrils and two different strains of α -synuclein have been discovered in Parkinson's disease. Prion-like seeding is not limited to only neurodegenerative disorders; it can even be involved in cancer where the tumour protein p53 can aggregate into amyloid-like assemblies of the protein [66], [67]. The assemblies seed further aggregation which probably leads to lost ability to suppress cell proliferation leading to malignant growth.

CANCER

Cancer is one of the leading causes of mortality worldwide. The dreadful disease implies cell division without stopping and the process can start almost anywhere in the body. When normal cells become damaged or grow old, they die via a process known as programmed cell death or, apoptosis. Cancer cells, on the other hand do not undergo this programmed cell death. The damaged cells continue to divide and form growths, tumours, which can spread to surrounding tissue. The cancerous malignant tumours can cause severe damage to different body functions, which can lead to death.

One protein that has been studied extensively according to cancer is the tumour suppressing p53 protein, which has generated more than fifty thousand publications [68]. p53 is involved in several anti-cancer functions such as cell growth regulation, division, survival and programmed cell death [69]. Due to mutations in the gene encoding p53 (*TP53*) or alterations in the status of p53 modulators is the activity of p53 compromised in all cancer types and lost p53 function is involved in more than 50% of all human cancers [67]. DNA damage and hyperproliferation are common stress stimuli leading to mutant p53 and cancer aggression.

The cellular processes in normal and cancer cells differ to a high extent and thiophene probes have earlier shown to be able to distinguish these cell types [49]. The continuation of this research is described in this thesis.

CONJUGATED THIOPHENE PROBES IN COMPARISON WITH OTHER DETECTION REPORTERS

The first agent to be used for amyloid detection is called Congo red [70], and is still widely in use. Thioflavin T (ThT) is the name of another agent, mainly used for protein fibrillation studies *in vitro* [71]. A disadvantage with Congo red, ThT and their derivatives is their rigid backbones; hence their ability in distinguishing

different amyloid deposits is limited. Congo red's inability for crossing the blood brain barrier (BBB) is also a drawback. The backbone of the thiophene probes, on the other hand, is flexible and as mentioned earlier, these probes are exceptional tools for distinguishing amyloid deposits. A number of well-defined thiophene probes possess the ability to cross the BBB. A criterion for detection using thiophene probes is the requirement of a fluorescence microscope, which is not needed for Congo red analysis. As seen in figure 10, all three reporters show nice images of prion aggregates.

Figure 10. Images and chemical structures of different detection reporters staining mCWD plaque; the LCP PTAA, Thioflavin T, ThT and Congo red. Reprinted with permission from Nature publishing group [59].

A great advantage with thiophene probes is their resistance to photobleaching whereas other probes for cellular detection might lack this essential property. Moreover, the thiophenes do not need any external reporters for detection and the implementation is extremely simple and straightforward. In addition to the thiophene probes, DAPI and mitotracker are two examples of directly bound reporters, both used in this thesis, see figure 11. Another extremely valuable fluorescent detector is the green fluorescent protein, GFP [72], a system where the GFP gene is used as an expression reporter for localization of proteins in living organisms. However, the GFP system is not used in this thesis. The lack of external reporter systems reduces the time required for the experiment as well as the risk of inaccuracies. Other systems such as fluorophore-labelled antibodies are more time consuming and require more precise administration. The small size of the thiophene probes compared to antibodies for example, improves the accuracy in colocalization studies. Small molecules become more important as techniques like super-resolution imaging is developed where the resolution is extremely high [73].

Figure 11. Fluorescence images of human fibroblasts stained with different detection reporters.

LCPs AS FLUORESCENT REPORTERS FOR PROTEIN AGGREGATES AND CELLS

In 2005, Peter Nilsson and co-workers showed that the negatively charged LCP called PTAA can be used for detecting the formation of amyloid fibrils *in vitro* [38]. As previously mentioned, formation of amyloid fibrils is seen in diseases like Alzheimer's. PTAA binds to both the native form of the proteins and to the amyloid fibrillar form. Due to conformational changes in the thiophene backbone upon binding to the different forms, resulting in spectral shifts, PTAA is fully capable of distinguishing the two forms of the proteins.

Chemical alterations of the monomer-based LCPs into trimer-based polydispersed probes enhanced the discrimination between the amyloid fibrillar form of the protein and the native form [40], [74]. These results inspired efforts to modify the molecules to enhance the selectivity and improve their properties as diagnostic tools for protein aggregation diseases.

LCPs have now demonstrated the ability to monitor fibril formation *in vitro* and by persistent research, the probes were also evaluated as conformation-sensitive optical tools in imaging of more complex systems as tissue samples [39], [75]. The results showed that the LCPs are excellent tools in discriminating amyloid, even in tissue.

LCPs have also been used in research involving prion diseases. PTAA showed excellent separation in emission spectra of different prion strains [59]. An additional probe was introduced, PTMI, a positively charged polythiophene with a functional group including methyl imidazole. PTMI also showed selectively binding to prion aggregates, however the spectral discrimination was not as substantial as with PTAA. Prion strain interactions were further investigated [76] showing that prion strain interactions are highly selective. Emission spectral analysis of PTAA discriminated the strains from each other and in addition, hybrid aggregates developed from two separated strains, were also detected.

Paper I is a subsequent work developing the strain separation methodology by evaluating additional thiohene probes. The ability of PTAA for strain separation was compared to several oligothiophenes - probes with a well-defined length of their backbone, which will be discussed later in more detail. In addition to emission spectral analysis, we also examined the samples with excitation spectra and with fluorescence lifetime imaging microscopy.

In an innovative work LCPs have been employed as fluorescent probes in complex systems as in staining of both fixed and live cells [49]. Anionic, cationic and zwitterionic polythiophene probes targeted probable lysosome-related components in the cells. Fascinating results showed staining of live human fibroblasts and live malignant cells with the anionic PTAA, indicating distinct differences in staining pattern due to cell type.

In **Paper II** we investigated live cell staining with PTAA more in detail. Staining patterns of diverse cell lines, both normal and malignant, were studied.

LCPs have shown great specificity towards protein aggregates and even in vital cell staining. The next step was to use the probes in more complex environments such as live animals. When utilizing a probe in living systems there are some pre-existing requirements; the probe needs to be small enough to be able to cross the BBB and it needs to be stable under physiological conditions. Drawbacks with LCPs are their size and some of them require acidic or alkaline buffer systems. Thus, the design of LCPs was modified in an attempt to fulfil the new requirements.

LUMINESCENT CONJUGATED OLIGOTHIOPHENES, LCOs

INTRODUCTION

The new thiophene probes were designed to have a well-defined length of their conjugated backbone and were termed luminescent conjugated oligothiophenes, LCOs. In contrast to a batch of LCPs where there is a heterogeneous distribution of repetitive thiophenes in the backbone, all the molecules in an LCO batch have the same the backbone length, see figure 12. By going from polydispersed material to monodispersed material, the aim was to improve the possibilities to cross the BBB and the selectivity for the target in mind. The more well-defined synthesis also opens up the opportunity for more specific small chemical modifications, a topic that started when comparing polydispersed monomers to polydispersed trimers [74].

Figure 12. Schematic images of the difference between a batch of LCPs and LCOs a) the heterogeneous distribution of lengths for the LCP PTAA and b) the homogeneous LCO h-FTAA having all the same length of the thiophene backbone.

LCOs AS FLUORESCENT REPORTERS

Giovanna Barbarella group (University of Bologna, Italy) has succeeded in developing oligothiophenes for stem cell imaging [77] and has also shown that selected oligothiophenes have the capability to distinguish protein types inside living cells [78]-[80]. It is most likely that Barbarellas thiophene probes, due to a succinimidyl ester group, covalently binds to amino groups of the intracellular protein target.

THE EFFECT OF MINOR CHEMICAL MODIFICATIONS OF LCOs

In the first paper with LCOs from Linköping the anionic p-FTAA was introduced together with the decarboxylated p-HTAA and the methylated p-FTAM [3]. The LCOs were designed with five thiophene units in their conjugated backbone, therefor the “p” which stands for penta. All three probes showed specific staining of protein aggregates in brain tissue slides and p-FTAA and p-HTAA readily crossed the BBB. In addition, p-FTAA was able to detect the early formed, presumably toxic species in the amyloid fibrillation pathway, a phenomenon not seen with previously reported LCPs. p-FTAA also distinguished the two hallmarks for Alzheimer’s disease namely Amyloid β ($A\beta$) plaques and neurofibrillary tangles. The different results from the three probes highlights the great impact of small structural chemical modifications on the properties of the probes. The more precise chemistry behind these well-defined probes opens up the opportunity for the LCOs to develop into unique fluorescent reporters.

The impact of small modifications in the design of backbone length and different end-groups was further investigated [4]. The results showed that LCOs consisting of five to seven thiophene units with carboxyl groups as end-groups were required to spectrally distinguish the hallmarks of Alzheimer’s disease.

Chemical modifications embracing substitution in the thiophene backbone has also been evaluated for probable enhancement in spectral discrimination of $A\beta$ plaques and neurofibrillary tangles [5]. By replacing one thiophene unit with a selenophene or a phenyl ring the spectral separation was reduced. The insertions make the backbones more rigid; hence, the conformational freedom of the backbone is very essential for discrimination of the hallmarks of Alzheimer’s disease.

All these novel well-defined probes opens up the opportunities to find a highly selective probe for discrimination of normal cells and cancer cells. In **Paper III** we examined a library of LCOs for stainability of a variety of cell types, both normal

cells and cancer cells. The well-defined versions of the polydispersed PTMI, the probe with imidazole functionality along the thiophene backbone, showed extremely interesting results and this study verified that the chemical nature of the side-chain functionalities was influencing the probes performance as staining reagents. As seen in figure 13, probes with similar chemical structures have diverse affinities.

Figure 13. Effect of minor chemical modifications. a) mCWD aggregate stained with h-FTAA b) Human fibroblast stained with another heptamer, h-HTMI.

Paper IV is a subsequent work evaluating and examining one of the LCOs with imidazole functionality. The focus is directed to the details in the staining patterns in normal and malignant cells.

The development within probe design lead to modifying the end-groups asymmetrically and combine thiophenes with other interesting ligands. Porphyrin is an interesting group with heterocyclic and aromatic properties, often used as photosensitizer in photodynamic therapy (PDT) [81], [82]. PDT uses photosensitizers in combination with light as a noninvasive method to kill cancer cells. The multimodal oligothiophene-porphyrin ligand p-FTAA-porph has shown enhancement in spectral assignment of A β deposits in tissue sections [83].

METHODOLOGY

PROBE SYNTHESIS

Bithiophenes was synthesized as early as 1890s [84] but synthesis of polythiophenes was not reported until 1980's [85]. Today it is possible to synthesize thiophene probes with a well-defined length, oligothiophenes. Thiophenes can be put together with the so called Suzuki-coupling [86], [87], where a carbon-carbon single bond is formed by a boronic acid with a halide using a palladium catalyst and a base. In the synthesis of polythiophenes, polymerisation is used which implies adding continuously repeating units to a growing chain. The extension will continue until there are no more units left to react or the reaction has reached its equilibrium. The lengths of the polythiophenes are therefore varying. The oligothiophenes on the other hand, are built by adding the required units one by one. Time-consuming purifications are needed between the additions but the synthesis results in a thiophene probe with a well-defined length, see the different routes of synthesis in figure 14.

Figure 14. Synthetic route to a) the polythiophene PTAA and b) the oligothiophene p-HTAA.

PROBE STAINING

The staining protocol for the thiophene probes is very uncomplicated and straightforward. The probe is dissolved in deionized water to a concentration of 1.5 mM, then diluted to μM -range in cell culture medium or buffer and then added to the sample for 30 minutes. Superfluous probe is washed away and depending on the experiment, the sample is put back into cell culture medium or mounted on a glass slide for analysis. As seen in figure 15, the procedure is very simple having for example antibody staining in mind, which is much more time consuming.

Figure 15. Experimental setup for probe staining.

TISSUE

By using tissue as a model system for our thiophene probes, we can study the performance of the probe at a higher cellular hierarchy level.

Even though tissue is not living material, it is a very profitable system in research. Tissue is the intermediate between cells and the structure of a whole organism. Differences both on a cellular level as well as on an extracellular level, the so-called extracellular matrix can be studied.

The tissue in this thesis origin from brain in murine PrP overexpressing tga20 transgenic mice [88]. The mice were intracerebrally inoculated with brain homogenates derived from Suffolk sheep from Colorado who were naturally infected with SS or from a mule deer naturally infected with CWD, also from Colorado. Brain homogenates from diseased tga20 mice were passaged several times into tga20 mice. The tissues studied here are the 4th passage for SS and the 6th for CWD.

CELLS

Cells are an extremely interesting system for studying the implementation of the different thiophene probes. The cells can be very selective of what kind of molecules that are allowed to enter through the plasma membrane, and also what molecules are allowed to stay once entered. A small charge, a variation of size or a different combination of bindings in a molecule can make enormous alteration of cellular uptake. Cell culturing has been used in research for many years, there were researchers who were able to grow animal cells *in vitro* already in the beginning of the twentieth century [89].

The cell is a building block for all living organisms, and the number of cells in a human being is 10^{12} - 10^{16} [90]. The cell consists of many different organelles and compartments [91]. The structures and compartments we found likely to be associated to the thiophene probes are fibronectin, Golgi, lysosomes and mitochondria. Fibronectin is an extracellular large protein, which is important for many interactions between the cells and the extracellular matrix. Thereby it helps cells to attach to the matrix as well as guide cell movements in developing tissues. The Golgi apparatus is a collection, sorting and dispatching station for products

from the endoplasmic reticulum (ER). Golgi also plays an important role in carbohydrate synthesis. Lysosomes are acidic organelles that are responsible for the intracellular digestion of macromolecules. The acidic interior with a pH of about 4.5-5.0 is required for the optimal activity of cleavage and degradation. Mitochondria are organelles responsible for most of the ATP production in eukaryotic cells. In some cells they seem to be associated with microtubules whereas in other cells they can form long filaments or chains. Figure 16 shows human fibroblasts stained with DAPI and antibodies against the interesting compartments or mitotracker staining mitochondria.

Figure 16. Fluorescence images of different compartments in cells.

Cells used in this thesis are mainly human fibroblasts (MRC-5 and AG01518), a very common cell type in connective tissue, and malignant melanoma cells (SK-MEL-28). Other cells that have been used are primary human melanocytes and cells from breast cancer (MDA-MB-231), cervical cancer (HeLa), colon cancer (HCT-116) and neuroblastoma (SH-SY5Y). The cells were cultured in humidified air with 5% CO₂ at 37°C.

IMMUNOCYTOCHEMISTRY

Immunocytochemistry is used in this thesis for comparing the location of the thiophene probes to known compartments in the cell.

Immunocytochemistry is a technique for detection of antigens in cells by using antibodies. Antibodies have been used as cytochemical agents since 1940's [92]. A two-step immunocytochemistry reaction involving a primary antibody and a fluorescently labelled secondary antibody is preferred for achieving high sensitivity.

The cells are first fixed in 4% paraformaldehyde (PFA) for crosslinking macromolecules and preserving the cell morphology. After fixation an incubation buffer is added to the cells, containing saponin for permeabilisation and bovine serum albumin to prevent unspecific interactions. The unlabelled primary antibody directed towards the compartment of interest is added for two hours followed by incubation with the secondary antibody for one hour. The secondary antibody is conjugated to a fluorescent marker that can be visualised by using fluorescence microscopy. It is incredibly essential to use a fluorescent marker with an emission spectrum that is well separated from the emission spectrum of the thiophene probe. Alexa Fluor 594 is used in this thesis; the probes' emission spectra are located at lower wavelengths.

FLUORESCENCE

Due to the conjugated system in the thiophene probes the probes are capable of emitting light upon irradiation. And since the thiophenes can twist or become more planar resulting in alterations in emitted light, the emission and excitation spectrum as well as the lifetime give information about the target and the environment of the thiophene.

Fluorescence is a process where molecules emit light from electronically excited states, see the Jablonski diagram in figure 17 [32]. The molecule is hit by a photon that excites electrons via absorption to a higher energy level, the excited state, at a time-range of 10^{-15} s [32]. To receive an excitation spectrum the emission light is held constant and the excitation light is scanned through different wavelengths. The average time for the electrons in the excited state prior to return to the ground state can be measured in 10^{-8} s [32] and is called the molecule's fluorescence lifetime. The lifetime determines the available time for the molecule to interact with its environment and the images generated with Fluorescence Lifetime Microscopy (FLIM) are based on the decay time rather than emission intensity. The relaxation of the molecule results in emission of a photon, which leads to production of light. This emitted light can be collected in emission spectra, which is acquired by keeping the excitation light at a fixed wavelength and detect the intensity of light emitted at longer wavelengths.

Figure 17. Jablonski diagram illustrating the processes of excitation, absorption, FLIM and emission.

MICROSCOPY

The fluorescence from the thiophene probes shows remarkable patterns, of which the details are suitably studied with fluorescence microscopy, and the confocal unit improves the optical resolution.

Confocal microscopy was invented in late 1950's [93] and is the most central instrument in these projects. Here the light goes through a pinhole and only the light in focus reaches the detector. Most of the work is done with an inverted LSM780 Zeiss confocal laser scanning microscope. Due to a 32 channel QUASAR GaAsP spectral detector and a tunable In Tune laser (488-640 nm) emission and excitation spectra can be recorded. The system is equipped with a modular FLIM system from Becker and Hickl resulting in ability of fluorescence lifetime studies.

FLOW CYTOMETRY

The technique is used to gain information about the thiophenes in a larger scale and not as detailed as with microscopy.

Flow cytometry is also based on fluorescence and has been used since 1960s [94]. Stained cells in suspension pass a laser, which detects the fluorescence intensity of the cells, see figure 18. The instrument used in this thesis is Gallios flow cytometer from Beckman Coulter.

Figure 18. Illustration of flow cytometry.

CELL VIABILITY DETECTION

The possible toxic effect of the thiophene probes is of high interest, so detection of cell viability and cell death is important.

Cell viability of cell cultures has been analysed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay since the beginning of 1980's [95]. The method is based on the metabolically active cell's capability to convert MTT to formazan, of which the quantity can be detected with a spectrophotometer.

Another method to detect cell death is to study cell morphology with light microscopy. Healthy cells are outstretched and if the cell density and/or the cell volume are reduced, there is a clear sign of unhealthiness.

STATISTICS

To be able to tell differences between spectra, the software R can be used for calculations [96]. Each spectrum is rendered as a vector and the Euclidean distance is calculated as the square distance between two vectors. The Euclidean distances

are then used to build a hierarchy of clusters, where clusters based on similarity are built. The relations of individual spectra can be presented both in dendrograms and in heat maps. An advantage with this method is that comparison involves the complete spectrum, not only the intensity at certain wavelengths.

AIM

The general aim with this thesis has been to evaluate fluorescent thiophene probes with the attempt to find probes with ability to distinguish different events in biological systems. The thesis has followed the development from polythiophenes to oligothiophenes and has evaluated their different properties as fluorescent staining agents for biological processes. Tissues and cultured cells have been used as model systems for detection and characterization of prion diseases and different cell types including cancer cells.

SUMMARY OF PAPERS

PAPER I

Multimodal fluorescence microscopy of prion strain specific PrP deposits stained by thiophene-based amyloid ligands

As mentioned earlier, the polydispersed and anionic LCP PTAA has shown to be able to distinguish prion strains due to dissimilar emission spectra [59], [76]. Paper I followed up this story by using three additional LCOs and two additional methods for strain separation studies.

In this study, we examined brain sections of mice inoculated with two different prion strains, mouse adapted Sheep Scrapie (mSS) or mouse adapted Chronic Wasting Disease (mCWD). We stained the sections with the polydispersed LCP PTAA or with either of the chemically well-defined LCOs p-FTAA, p-KTAA or h-FTAA, see the structures in figure 19. The sections were then examined with fluorescence microscopy and emission and excitation spectra were collected. All four thiophene probes provided both emission and excitation spectral differentiation, however PTAA was identified as the most effective compound. As a supplementary approach, Euclidean distance was utilized for the comparison, providing numbers of the disparity. The reason for the efficiency of strain discrimination with PTAA can be its polydispersity and length of the thiophene backbone. The well-defined LCOs might lack this ability to undergo the same prominent alteration.

Figure 19. Chemical structures of the thiophene probes included in Paper I; PTAA, p-FTAA, p-KTAA and h-FTAA

In addition, the altered conformation of the probes was observed with the more sensitive method Fluorescence Lifetime Imaging Microscopy (FLIM), where the fluorescence decay after excitation of the probe is monitored, see figure 20. In contrast to the emission-and excitation profiles, the chemically well-defined LCOs displayed the most efficient fluorescence differentiations of the prion strains and the polydispersity seemed to become a drawback.

Figure 20. Colour coded fluorescence lifetime images of prion deposits in brain tissue stained with LCP or LCOs.

In conclusion, we distinguished two prion strains with four thiophene ligands by using multimodal fluorescence microscopy; emission, excitation and FLIM.

PAPER II

Differential vital staining of normal fibroblasts and melanoma cells by an anionic conjugated polyelectrolyte

Björk and co-workers used the polydispersed anionic PTAA for vital staining of human fibroblasts and melanoma cells with results indicating differential staining due to cell type [49]. In Paper II, we continued the investigation of the behaviour of PTAA in normal cells and in malignant cells in more detail. PTAA was for long the only thiophene probe we were able to visualize after live staining of cells.

The LCP PTAA was used for vital staining of two normal (fibroblasts and melanocytes) and five malignant cell lines (melanoma, cervical cancer, breast cancer, colon cancer and neuroblastoma), see figure 21. The staining pattern was examined with confocal microscopy and the result was striking; PTAA displayed a peripheral punctated pattern in the normal cells whereas the dotted pattern was concentrated in a one-sided perinuclear localization in the malignant cells.

Figure 21. Fluorescence images of PTAA staining of normal cells (b-c) and cancer cells d-h). a) Chemical structure of PTAA, b) human fibroblasts, c) melanocytes, d) melanoma, e) cervical cancer, f) breast cancer, g) colon cancer and h) neuroblastoma cells stained with PTAA (green) and DAPI (blue).

To assess the cellular targets for PTAA, the PTAA pattern was compared to staining patterns of known organelles obtained mainly by using different antibodies. The costaining experiments revealed fibronectin as a potential target directly after staining, see figure 22. 24 h after the staining procedure PTAA

seemed to have relocated from fibronectin to a lysosome-associated location in the cells. These experiments were only possible to perform on the fibroblasts since the permeabilization buffer necessary for consistent antibody staining demolished the PTAA pattern in melanoma cells. This is yet another pronounced discrimination of the two cell lines.

Figure 22. Fluorescence images of human fibroblasts costained with PTAA (green) and a) fibronectin or j) lysosomes (red).

PTAA showed no toxic effect to fibroblasts or to melanoma cells until irradiation. Excitation of light with a certain wavelength to live cells just recently stained, made the cells shrink and show apoptotic morphology. An extremely interesting finding was that irradiation of cells stained 24 h earlier showed no toxic effect, indicating that the cellular location of the probe was determining the photo-induced toxicity.

In conclusion, we demonstrated a discrimination of normal cells and cancer cells with the polydispersed thiophene PTAA.

PAPER III

Cell type related differences in staining with pentameric thiophene derivates

Development of LCPs into LCOs opened up the opportunity for screening a library of chemically well-defined thiophene probes and examining their stainability of a various cell types, including cancer cells. All eight thiophene probes were designed with pentameric backbone with different varieties of the side-chains, see the structures in figure 23. This study was aiming for a more quantitative approach; therefore flow cytometry was used as the main technique.

Figure 23. Chemical structures of the pentameric thiophene probes studied in Paper III.

The results revealed several promising candidates that showed strong or moderate staining capacity due to cell type, see table 6-1. It was only p-HTEA that stained cancer cells to the same extent as normal cells. The rest of the probes showed higher fluorescence in cancer cells. The most interesting results were obtained by thiophenes with diverse imidazoles as side-chains.

Table 1. Stainability of different cell lines stained with different LCOs.

Name	Side chain	SKBR-3	MDA-MB-468	MCF7	Hela	HCT116	DU-145	HEK	PC-3	K562	Fibroblasts
p-HTAA	Anionic (-COOH)	5,92	9,02	11,43	23,21	12,47	7,66	0,07	0,34	5,82	0,09
p-HTEA	Cationic (-NH ₃)	100,00	100,00	100,00	100,00	100,00	100,00	100,00	100,00	100,00	99,30
p-HTMI	Methylated imidazole	21,63	72,46	27,45	26,34	11,83	64,93	79,38	74,05	29,20	0,17
p-HTA-Histamine	Histamine	32,67	49,30	6,94	2,40	14,54	25,45	0,77	8,30	59,64	0,00
p-HTIm	Imidazole	98,00	99,00	95,96	99,00	98,18	98,21	99,90	99,14	99,40	22,24
p-HTA-Tyr	Tyrosine	0,23	0,25	0,00	0,28	0,24	0,12	0,05	0,08	0,00	0,14
p-HTA-Arg	Arginine	0,36	0,00	0,00	0,07	0,00	0,00	0,00	0,06	0,00	0,09
p-HTA-Glu	Glutamate	1,94	0,00	0,00	0,39	0,12	0,00	0,07	0,15	0,00	0,00

p-HTIm, an LCO functionalized with imidazole, showed very strong fluorescence in the cancer cells whereas the intensity difference to normal cells was rather large. The staining of p-HTMI, which in addition to the imidazole in p-HTIm also is functionalized with a methyl group, was significantly increased in cells with inactivation of the p53 gene. The other tested probes did not show similar result, indicating that the molecular target of p-HTMI is some cellular component regulated by p53.

To conclude, the LCOs are promising tools to distinguish normal cells and cancer cells, particularly the probes with imidazole functionalities. Very small chemical modifications eventuated in great effects where only one of the imidazole functionalities were affected by the p53 status.

PAPER IV

An imidazole functionalized pentameric thiophene displays different staining patterns in normal and malignant cells

The interesting results of the probes with imidazole functionalities in Paper III opened up the curiosity for further studies of the staining pattern. Paper IV is focused on the details of the staining pattern of p-HTIm.

The pentameric thiophene probe p-HTIm was used for staining of two normal (fibroblasts and melanocytes) and five cancer cell lines (melanoma, cervical cancer, breast cancer, colon cancer and neuroblastoma), even though the focus was at fibroblasts and melanoma cells. Background staining was clearly reduced compared to the staining with PTAA in Paper I, and the difference in staining pattern between the cell types was still remarkable, see figure 24. The staining in normal cells was located in a peripheral punctated pattern and partly co-localized with lysosomes in the fibroblasts, see figure 25. In contrast, the pattern was seen in smaller structures and located in a one-sided perinuclear pattern in cancer cells, and associated with Golgi in melanoma cells. The different locations were, in addition to confocal microscopy, also verified with FLIM where it was clear that the fluorescence life-time decay of p-HTIm was different in the two cell lines.

Figure 24. Cells stained with p-HTIm. a) Chemical structure of p-HTIm. Fluorescence images of b) normal cells: human fibroblasts and melanocytes and c) cancer cells: melanoma, breast cancer, cervical cancer, colon cancer and neuroblastoma cells stained with PTAA (green) and DAPI (blue).

Figure 25. Fluorescence images of costaining experiments of p-HTIm and antibodies against Golgi and lysosomes in human fibroblasts and melanoma cells.

The uptake of p-HTIm was temperature dependent which was clearly seen as significantly lower fluorescence intensity when performing the experiments at lower temperature. The really fascinating result was the decrease in fluorescence staining of chemically related analogues to p-HTIm, see the structures in figure 26. Minor chemical modifications of p-HTIm almost extinguished the ability of the probe to stain the cells.

A

Figure 26. Chemical structures of analogues to p-HTIm.

In conclusion, we confirmed p-HTIm's ability to distinguish normal cells and cancer cells and also verified the great impact of minor chemical modifications of the imidazole side-chain functionalities on the cell stainability of p-HTIm.

CONCLUSIONS

The unique conjugated thiophene probes have shown remarkably interesting results in their ability of multimodal fluorescence assessment of biological systems. Polythiophenes have earlier shown to be useful in detection and distinguishing protein aggregates and cell types, whereas the novel oligothiophenes seem to possess even more improved properties. The well-defined probes seem to bind more specifically with less background staining. More exact synthesis of minor chemical modifications is also highly improved which leads to very interesting studies of the probes' altered properties.

More specifically both the LCP PTAA and the LCOs p-FTAA, p-KTAA and h-FTAA have shown very good separation of prion strains using multimodal fluorescence assessments (Paper I).

PTAA was also for many years the only thiophene probe in our library able to show fluorescence staining of living cells (Paper II). The unique finding was the different staining patterns in normal cells compared to cancer cells.

In the expanded library of well-defined probes some interesting LCOs for vital cell staining were found (Paper III). Even here, the stainability was different due to cell type for some of the LCOs. The most interesting probes were LCOs with diverse imidazole motifs along the backbone; p-HTIm and p-HTMI.

The different staining of normal cells and cancer cells by p-HTIm was confirmed and investigated further (Paper IV), where the major differences in stainability caused by minor chemical modifications was striking. p-HTIm showed exceptional strong fluorescence compared to its analogues.

FUTURE PERSPECTIVES

The library of thiophene probes is constantly expanding and improved. Further chemical improvement of the probes will hopefully lead to higher quality of staining and enhanced insight in disease progression. The thiophene probes have proven to be excellent detection markers of protein aggregation processes, and since aggregation now also seems to be enrolled in cancer, the probes might have a bright future within cancer diagnostics.

Porphyrins, a therapeutically interesting group, have recently successfully been coupled to thiophene probes and shown staining of protein aggregates [83]. Experiments with porphyrins added to thiophenes with imidazole motifs as cellular staining agent are ongoing in our laboratory. Porphyrins can be used as photosensitizer in photodynamic therapy and the synthesis can be done to improve selectivity for cancer cells. The thiophenes can also be designed to improve selectivity as well as be used as killing agent upon irradiation. By combining the features of porphyrins and thiophenes, an excellent therapeutic agent might be developed.

In order to find the specific target of the thiophene probes, other synthetic routes might be required. An azid-functionalized thiophene that can be utilized in click-chemistry has been synthesized [97]. If similar functionality can be added to the LCOs with imidazole motifs, the detected target can hopefully be isolated and characterized. The knowledge of the target might clarify both the difference in cell types and improvement in the design of the probes.

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