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# Spectroscopic investigation of luminescent biomaterials on organic semiconductors

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# Abstract

Organic semiconductor materials are emerging as highly-promising candidates for bioelectronics applications. In particular, the hydrogen-bonded organic semiconductors (HB-OSCs) family holds promise due to inherent biocompatibility, low-cost, and extremely stable performance. The starting off point of this work is the question: Can the hydrogen-bonding functional groups of HB-OSCs be directly bioconjugated with well-known biochemical moieties with the goal of creating selective and sensitive bioelectronics interfaces.

As a model system, the biotin-streptavidin "lock and key" pair was chosen. Using luminescence spectroscopy and with biofunctional luminescent probes (quantum dots or organic fluorophores), the binding behaviour of the biotin-streptavidin system was investigated when one of both components was immobilized on top of the representative HB-OSCs epindolidione and quinacridone. The efficacy of immobilization was tested by applying different washing procedures, followed by measurement of the luminescent probes. Non-hydrogen bonded organic semiconductors were evaluated for comparison. It was found that in all cases, streptavidin yields a high degree of non-specific binding on the surface, with some resistance to repeated washing steps. The biotin linker was found to enhance retention of the streptavidin, however the hydrolytic stability of the biotin linker was found to be poor, *i.e.* repeated washing steps yielded luminescence on par with non-functionalized samples. Taking advantage of the non-specific binding of streptavidin to the pigments, non-labelled protein was successfully detected and proved to be attached in a stable way using fluorescently labelled biotin.

Finally, it was found that indigo, as a low-band gap HB-OSC, quenches the luminescence of quantum-dot labelled streptavidin, demonstrating an energy transfer between the quantum dot to indigo. The finding of such an interaction opens up additional possibilities for specific detection applications.

The work presented here demonstrates the possibilities of functionalization of HB-OSCs with biomolecules by adapting standard procedures from biochemical protocols. The results herein demonstrate some of the interactions which are possible, as well as some of the accompanying obstacles.

# Kurzfassung

Organisch Halbleiter Materialien sind eine vielversprechende Gruppe an Materialien die sich für die Herstellung von Biosensoren eignen würden. Insbesondere die organischen, Wasserstoffbrücken-bildenden Pigmente (hydrogen bonding organic semiconductors, HB-OSC's) sind aufgrund ihrer inhärenten Biokompatibilität, geringen Kosten und langzeitiger Stabilität besonders geeignet. Die Grundlegende Frage, die in dieser Arbeit behandelt wird ist: Können Wasserstoffbrücken-bildende Funktionsgruppen der HB-OSC's direkt mit Biomolekülen funktionalisiert werden und damit zu selektivem Bindungsverhalten führen?

Als Grundmodel für diese Untersuchung wurde das das Biotin-Streptavidin Paar gewählt. Unter der Verwendung von Photolumineszenz-Spektroskopie und fluoreszierenden Biosonden, wurde das Bindungsverhalten des Biotin-Streptavidin Paares untersucht, wenn eine der beiden Komponenten auf dem HB-OSC immobilisiert wurde. Als Vertreter der HB-OSC's wurden Epindolidione und Quinacridone gewählt. Es wurden diverse Waschprotokolle zur Entfernung unspezifisch gebundener Biomoleküle mit anschließender Messung der Lumineszenz der Biosonden verwendet um die Stabilität der Bindung zwischen HB-OSC und Biomolekül zu untersuchen.

Zum Vergleich wurden auch organische Halbleiter getestet, die dem Kohlenstoffskelett von Epindolidione und Quinacridone ähneln, doch keine Wasserstoffbrücken bilden können. In allen Fällen wurde eine unspezifische Bindung von Streptavidin zur Oberfläche festgestellt, die sich gegenüber den Waschprotokollen als äußerst resistent erwiesen hat. Biotin als "Anker" für das Protein zu verwenden erwies sich nur bedingt als sinnvoll: einerseits wurde ein verbessertes Bindeverhalten festgestellt, doch wiederholtes Waschen führte zur Hydrolyse der Amid-Bindung zwischen Biotin und Pigment, die in Form von wesentlichen Verlusten an Lumineszenz festgestellt wurde – gewaschene Proben wiesen Lumineszenz auf par mit den unbehandelten Proben auf.

Diese unspezifische Bindung machten wir uns in späteren Experimenten zu nutzen in denen natives Protein auf die Oberfläche aufgetragen und mit fluoreszent-markiertem Biotin nachgewiesen wurde. Hierbei wurde die Bindung von Streptavidin nicht nur nachgewiesen, es wurde sogar eine vergleichsweise hohe Resistenz gegenüber den Waschprotokollen festgestellt.

Schlussendlich wurde Indigo, ein HB-OSC mit einer geringen Bandlücke, die Fluoreszenzlöschung des Quantenpunktes nachgewiesen, der als Marker auf Streptavidin fungierte. Hierbei wurde ein Energietransfer von Quantenpunkt zu Indigo festgestellt, was eine Vielzahl an Möglichkeiten für die zukünftige Verwendung als Biosensor eröffnet.

Die präsentierte Arbeit demonstriert die Biofunktionalisierung der HB-OSC's durch die Verwendung standardisierter biochemischer Methoden. Die Ergebnisse weisen auf die möglichen Interaktionen, sowie zu erwartenden Hindernisse der Methoden hin.

# Acknowledgement

For finishing this thesis, the support of many people was necessary.

First off, I would like to thank my family for supporting me, both financially as psychologically throughout my studies.

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# 1. Introduction

## 1.1 Motivation

In the course of the last years, life sciences were always presented in media as the technology of the future. The main driving force for these technologies probably lies in the health care sector, the pharmaceutical industry and food industry; no matter if it is the increase in patients with chronic diseases, legal GMO restrictions in food or pathogens on your vegetables- all of these result in a growing demand for new technologies.

This includes the demand for biosensors. Probably the most dominant example of a biosensor in everyday life is the blood-glucose sensor used daily by thousands of patients. In these, a series of immobilized enzymes in a disposable chip convert measured blood sugar into a colour response which is measured by a strictly electronic device. This seemingly simple device elegantly combines electronics and life sciences in order to increase the quality of life for people affected by diabetes.

In this thesis, the ground work for a new kind of biosensor will be presented. Cheap, readily available materials are investigated as a novel kind of substrate-coating in conjugation with the biotin-streptavidin as a model system for bio-functionalization that could be relatively easily exchanged for any system of interest.

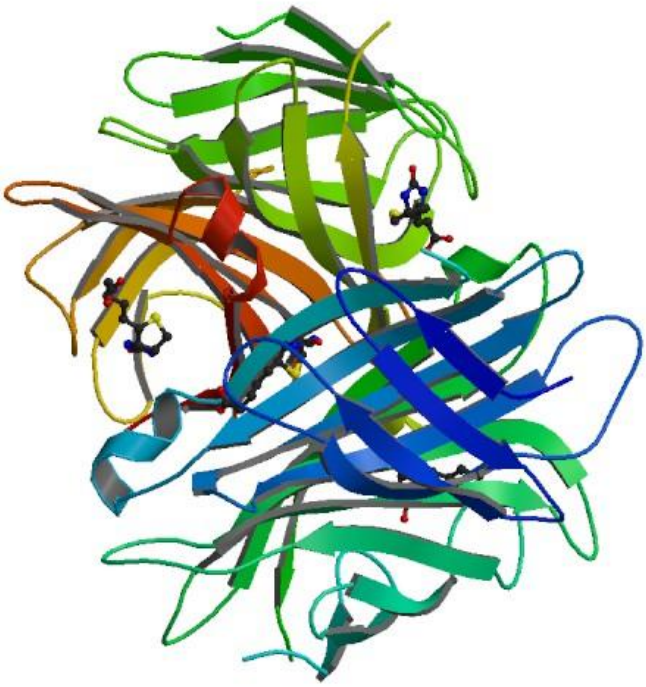

**Figure 1: 3D picture of Streptavidin with 4 bound biotins as published by Hyre *et al.* [2]**

## 1.2 Streptavidin and biotin

Discovered in 1941 by Esmond Snell and co-workers[1], the avidin-biotin binding turned out to be nature's strongest non-covalent interaction. With a dissociation constant of $10^{-14}$ M, once bound biotin (figure 1) will not be released again- in this way, the avidin-rich egg white contains an antibiotic agent preventing bacterial growth by limiting the availability of biotin.

Biotin itself plays a major role in biochemistry. Also known as vitamin B7 or vitamin H (see figure 3), biotin is an important coenzyme involved in carboxylations. An important example of its tasks is found in the Krebs cycle, where together with pyruvate carboxylase and ATP, pyruvate and one molecule of $CO_2$ are turned into oxyloacetate. Further, biotin is key to a functional fatty acid synthesis, also using inorganic $CO_2$ to elongate a carbon chain bound to coenzyme A.

The dissociation constant of avidin and its homologues can be, however, strongly altered by introducing amino acid mutations to the residues involved in binding. Mutating the binding-involved tryptophan to a phenylalanine will worsen binding by two orders of magnitude. For an illustration of Amino acids involved in biotin binding, see figure 2.

This change is but a small example of how recent developments in the toolkit of biosciences affect even long-known entities. However, this binding pair's strong binding is in general considered a great asset and has found use in many applications throughout biology.

Streptavidin, avidin's bacterial homologue, shows highly similar affinities to biotin. Both, streptavidin and avidin exhibit within the tetramer (homomer of 4 subunits) cooperative binding of biotin. The behaviour of those two pairs was studied by many groups and methods such as Yuan *et al.* using atomic force microscopy.[3] Nowadays, biotin-(strept-)avidin binding is used in various areas of research for its strong, well defined binding behaviour.

Many examples for application were reported in literature so far. For instance, Hugh *et al.*[4] used streptavidin-coated paramagnetic particles to capture and retroviral vectors. On the other hand, this pair is used in the "sandwich"-version enzyme-linked immunosorbent assay (ELISA) for a long time. An exhaustive listing of the known applications would be too long and ill-placed in this thesis an exhaustive overview of recent developments in their application can be found in the review by Dundas *et al.*[5]

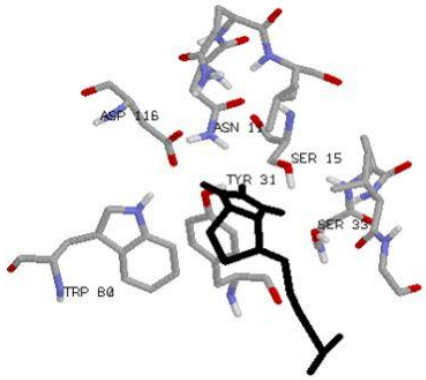


**Figure 2: Amino Acids involved in biotin binding in wild type Streptavidin as reported by Hyre *et al.* [6] The biotin molecules (black structure) ring system is hydrogen bonded to multiple amino acid residues.**

Now, I would like to point out the special case of biofunctionalized surfaces. When covering a surface of any kind with a solution containing molecules that can interact with said surface, some of these will remain on it, even after removing the solution. On a bio-modified surface, this non-specific adsorption competes with biospecific adsorption to the bio-molecule present. In our particular case, biotin was to serve as an anchor for streptavidin and was supposed to out-compete physisorption of the protein to the hydrogen-bonded organic semiconductor.

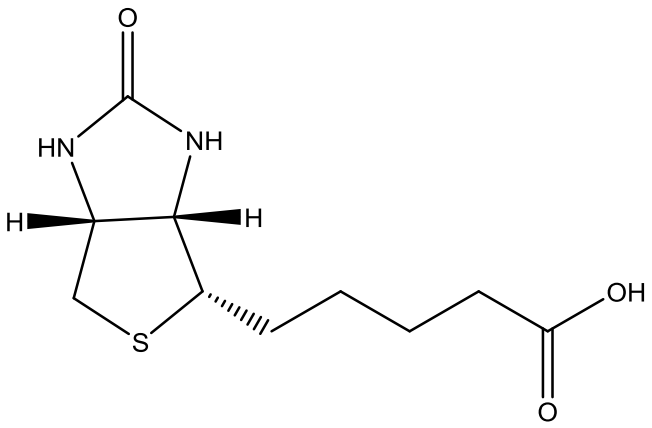


**Figure 3: Chemical structure of Biotin. The chiral ring-system serves as the molecules anchor in (strept-)avidin. For purposes of biosynthesis, the left-hand secondary amine serves as a catalyst in carboxylations.**

## 1.3 Non-covalent interactions

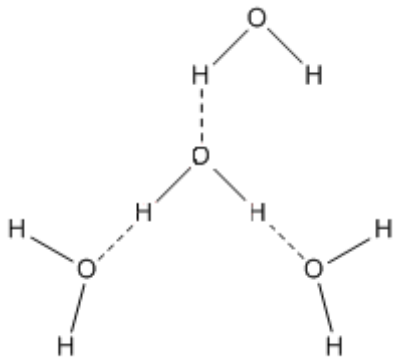


**Figure 4: Hydrogen bonds in water**

In nature, many kinds of interactions atoms are known. Apart from the covalent bond in molecules and electrostatic interaction in ions, a large variety of non-covalent interactions is known for a long time to science. One of the first people, who discovered these forces, was the Dutch scientist van der Waals, when he carried out his famous experiments on gases. When cooling down water vapour, he observed at first a clouding and later, condensation. This led him to the conclusion, that there must be an intrinsic attraction between molecules- the van-der-Waals forces.

Van-der-Waals forces include the interaction between permanent dipoles, permanent dipoles and induced dipoles, as well as between two induced dipoles. Apart from those, another molecular interaction is known, playing a much larger role in our world: hydrogen bonds. By definition, hydrogen bonds are the non-covalent interaction between a molecules hydrogen atom and an electron rich atom of another molecule (or the same in case of intramolecular interactions). Albeit this very much reminds of a van-der-Waals type interaction, the energies involved are significantly larger. In 1940, Linus Pauling[7] described the hydrogen bond as follows: "It has been recognized in recent years that under certain conditions an atom of hydrogen is attracted by rather strong forces to two atoms, instead of only one, so that it may be considered to be acting as a bond between them". In the years since this statement, many other statements on hydrogen bonds were made – strong electronegativity of the two non-hydrogen atoms involved is to be expected for instance- however, Pauling's gives a fairly good description of what is going on.

To illustrate the difference between van-der-Waals forces and H-bonds, I would like to invite you to a little Gedankenexperiment comparing water and butane. A rule of thumb, the higher molecular weight of a molecule the higher the chance of it being liquid or solid at room temperature, rather than being present in gaseous form. The molecular weights of water and butane are 18 g/mol and 58 g/mol, respectively. Judging by their weight, we would expect water to be a gas and butane to be closer to be a liquid. In reality, it is well known, it is vice versa, as a large quantity of hydrogen bonds is present in water (figure 4), whereas none can be formed within butane. Strictly speaking, these bonds make life as we know it possible.

Hydrogen bonds play also a strong role in biology, apart from giving water its known properties. As mentioned above, hydrogen bonds contribute to the folding structure of proteins, but also the highly specific base-pairing in DNA is due to exactly these bonds. The list goes on however: antigen recognition, stabilization of cartilage by water-binding[8], and many other, crucial phenomena in nature are influenced by this kind of bond.

## 1.4 Hydrogen-bonded organic semiconductors

Hydrogen-bonded semiconductors are known to humanity since biblic times. Many natural compounds such as Indigo and Tyrian purple are known to us best as the most expensive dyestuffs in antiquity. However, instead of obtaining their distinguished colour due to extensive π-conjugation, these small molecules owe it to intermolecular π-stacking while aggregated. A selection of this kind of organic semiconductor can be found in cited articles. [9, 10]

The strong attractive interactions between the organic semiconductor molecules results in a bad solubility of pigments in many solvents. In ancient times until today, in dyeing industry, pigments were reduced or oxidized in order to break this interaction and make the molecules go into solution. In the case of indigo, sodium dithionite in alkaline solution is used as the reducing agent of choice, as reported by J.R. Aspland.[11] On the other hand, if it isn't the dyeing of a coat that is your aim, but rather (long term) processing of your organic semiconductor in solution another technique is available. As long as the organic semiconductor of choice contains an amine group, such as in quinacridone or epindolidione, the amine protection mechanism using the tBOC protection group can be applied.[12] Known from peptide synthesis, the addition of Di-tert-Butyl dicarbonate (tBOC) with 4-(dimethylamino)-pyridine (DMAP) to a solution of amines will lead to their protection. In the case of other pigments, the same is valid, giving a dissolved organic semiconductor with a high yield. With a soluble molecule, many new possibilities are available. When these are no longer desired, two options for deprotection are available- either deprotection via heating when no other heat-labile species is involved. As the latter is the less work intensive procedure it will almost always be the deprotection-method of choice. Apart from their colour-properties for which they were originally used, hydrogen-bonded semiconductors exhibit varying levels of electric mobilities.[10]. Particularly of interest are the pigments Epindolidione and Quinacridone. (see figure 5, 6) Produced on a multi-ton scale, both pigments are used in inkjet printers and high-

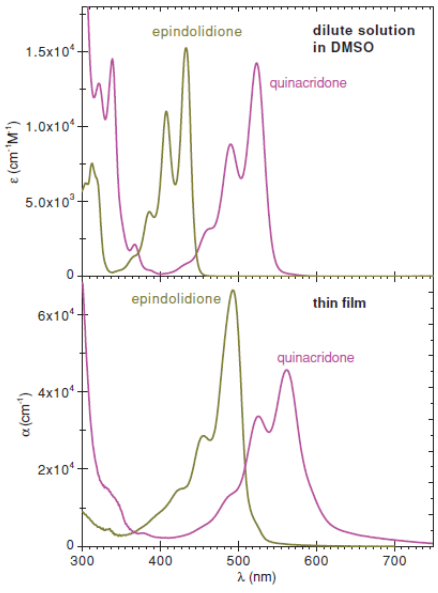


**Figure 5: UV-vis absorption spectra of Epindolidione and Quinacridone in DMSO and thin film, respectively.[13]**

performance outdoor paints and are thus cheap and easy to obtain- this combination of treats makes them interesting candidates for novel electronic devices.

Further, both these organic semiconducters show easy possibilities for modification on their amine's as it was illustrated for the tBOC protecting group- this could be used in order to tune the molecules photochemical properties or for surface modifications in actual electronic devices such as transistors.

Organic semiconductor materials are emerging as highly-promising candidates for bioelectronics applications. In particular, the hydrogen-bonded organic semiconductors (HB-OSCs) family holds promise due to inherent biocompatibility, low-cost, and extremely stable performance. The starting off point of this work is the question: Can the hydrogen-bonding functional groups of HB-OSCs be directly bioconjugated with well-known biochemical moieties with the goal of creating selective and sensitive bioelectronics interfaces.

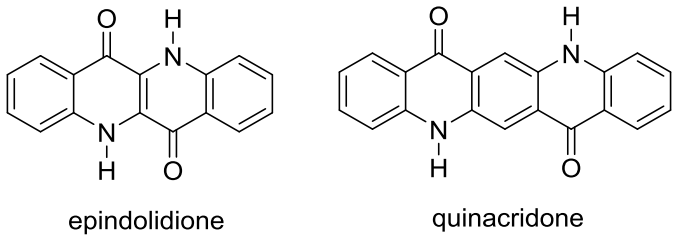


**Figure 6: Epindolidione (left) and quinacridone (right). In these hydrogen-bonded semiconductors , the amine and the carbonyl groups for hydrogen bonds towards their neighbouring molecule. One should note the particular complementarity of the molecules lower and upper part.**

As non-hydrogenbonded analogues to the aforementioned HB-OSCs, the long known organic semiconductors tetracene (see figure 7) and pentacene were used within this thesis in order to compare the surface binding-properties of biotin in the absence of hydrogen bonds to which it can covalently attach itself. Both molecules were investigated in the past, heavily, for their superb properties and possible applications, alas, turned out to lack many of these.[14]

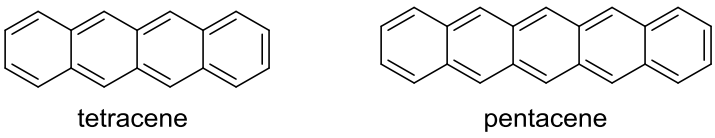


**Figure 7: Tetracene (left) and pentacene (right) are highly conjugated, well known organic semiconductors.**

## 1.5 Vacuum evaporation of organic molecules

In order to create films of organic molecules, a variety of techniques are available, where all have the pro's and con's. One such technique is vacuum evaporation.

With typical distances between source and surface being 10-20 cm, high vacuum is needed. Pressures ranging between $10^{-5}$ and $10^{-11}$ Torr are applied in order to deliver sufficient amounts of molecules to the target- for our purposes, pressures between $10^{-5}$ and $10^{-7}$ were perfectly sufficient. The only way how to control the deposition rate is the temperature at which the source is operated, which is chosen above the molecules sublimation temperature and significantly below its decomposition temperature. Deposition rates of 0.001-100 Å/s are typical where lower rates are better for control over the process, whereas higher rates avoid deposition of contaminants- in practice, a compromise is chosen with rates between 0.1 and 5 Å/s. The rate can be easily measured "*in situ*" using a quartz crystal microbalance.

The substrate temperature also plays a role in crystal-formation. As molecules on a heated substrate have more energy available, they can use it in order to move on the surface and reach a thermodynamically favoured state. Thus, by altering substrate temperature, the crystal form and size of the deposited organic molecule can be directly influenced.[15]

## 1.6 Biosensors – use and application

Biosensors have a strong impact on our society. Every day, analytical laboratories around the world use our understanding of biology and biochemicals in order to quantitatively and qualitatively analyze contents of pathogens and genetically modified organisms (GMO) in foodstuffs, our environment or (genetical) anomalies in our bodies. A large variety of biosensors with different levels of sophistication are at our disposal. Enzyme-linked Immuno Sorbed Assay (ELISA) uses antibodies and enzymes with the knowledge about their kinetics in order to create a colour response in relation to an analyte concentration. Real-time polymerase chain reaction (real-time PCR) detects specific, known DNA fragments in mixture by amplification and creation of a photoactive species. On the other hand, glucose sensors for everyday use make it possible for diabetes patients to monitor their blood sugar

levels without the need of educated personnel. Every single one of these techniques is intrinsically different, but they carry by right the tag of biosensor.

The "father of European biosensors", Frieder Scheller[16] invented his first glucose biosensor in the 1970's. Based in eastern Germany, he was very much limited by the chemicals available and his first sensor developed was based on Glucose Oxidase (GOD) cast in photogelatine by the company "ORWO Wolfen" used in colour film development which was later substituted by polyurethane glue used in shoe making. Although using such "primitive" and cheap constituents, both sensors were better and faster than the cumbersome techniques used in the west. Later, he wrote a book called "Biosensoren" (Biosensors) together with F. Schubert which was later translated and published by Elsevier- until today it is one of the most relevant books for people interested in this field.[16]

In today's blood glucose biosensors, immobilised GOD turns Glucose and oxygen into gluconol acetone and hydrogen peroxide. The peroxide can then be quantified using a peroxidase in order to turn a substrate into coloured product. Alternatively, GOD can also act directly on the substrate to form the coloured product.

While not sensors by themselves, in clinical analysis, enzyme based techniques are prevalent due to their tissue specificity. Alanine-aminotransferase is monitored to detect liver damage, creatine phosphokinase MB type to detect heart infarction, and lipase to detect pancreatitis. These are just few examples of the much number of enzymes that can be specifically detected in order to help with diagnosis. In these cases, the isolated enzyme catalyses a reaction of a substrate added- in this way, a coloured or fluorescent is created, identifying the presence and in time-resolved experiments, the amount of enzyme.[17]

As the consumption of alcohol-containing beverages and distillates is deep-rooted in our society, it is of importance to be able to detect its presence. One way to do so was presented by Patel *et al.* in a screen printed biosensor.18 Using a tandem of alcohol oxidase (AOD) and Alcohol dehydrogenase (ADH), the former enzyme turns ethanol and oxygen into peroxidase and the aldehyde while the latter enzyme recycles it back to the alcohol. Therein, the reduced form of Nicotinamide Adenine Dinucleotide (NADH) is incorporated into the sensor to serve as an electronsource for the catalytic cascade; said electrons are transported by the cascade to an electrode where they are measured amperometrically.

Apart from enzyme-base sensors, bio-specific AFM techniques were developed in recent years. The versatility of AFM, combined with the specificity of biomolecules allows for a

large variety of parameters to be measured. A biofunctionalized tip allows to detect specific modification sites[19] as well as to measure the forces that are holding together the cell wall.[20]

For the sake of completeness also natural antibiotics can have their use in biosensors. The antibiotic valinomycine was used by scientists around O. Ryba used in order to make a potassium selective electrode.[21]

Part from the sensors mentioned above, a wide variety of others are available. Next generation DNA -sequencing, Surface Plasmon Resonance, as well as transistor type biosensors- to highlight and explain them all would be only fair but over the scope of this introduction. Information on the transistor type biosensors can be found in the next sub-chapter.

## 1.7 Photoluminescence spectroscopy

When light and matter interact, only a few events are possible. Aside from scattering, the photons energy can be absorbed, exciting a molecule into an energetically higher state. In order to relax back into its ground state, it has to dispose the energy i.e. by the way of photoluminescence (PL), internal conversion (IC), conformational change, photochemical transformation or by a further transfer of said energy.

Photoluminescence can be further split up into fluorescence and phosphorescence and is an intrinsic property of many aromatic hydrocarbons, inorganic compounds such as doped glasses or crystals, and organometallic complexes. In general, fluorescence is considered to be a quick way of deexcitation with lifetimes in the range of pico- to nanoseconds, whereas phosphorescence is related to lifetimes that are as long as seconds.

In figure 8, the Perrin- Jablonski Diagram is shown. The thick lines correspond to the molecules singlet states ($S_0$, $S_1$, $S_2$) and are shown in the left-hand side opposite to the triplet states ($T_1$, $T_2$). Upon absorption, the molecule enters an excited singlet state such as wherefrom it can relax to the lower-lying levels by internal conversion (IC), by switching through vibrational (thin lines) and rotational levels (not shown). As ISC is a spin forbidden process (as the electrons spin directions has to change in order to form a triplet), this kind of process has very low abundance and takes considerably longer.

Photoluminescence is indeed a very complex process. Seemingly small changes can have large effects and are often underestimated and simplified. In organic fluorescent molecules, the colour or absorption is influenced by the degree of conjugation and substitution. An electron withdrawing group will lead to a shift of the absorption and emission spectrum where the position of said substitution will determine if it will be a red or blue shift. Further, some groups will have further impact on the photoluminescence: a hydroxyl group will lead to a line broadening, whereas a ketone or aldehyde group will probably exhibit a low lying $n \rightarrow \pi^*$ transition and thus to a low fluorescence quantum yield- if the said molecule also has a low lying $\pi \rightarrow \pi^*$ transitions, the solvents polarity will have a strong influence on its interaction with photons. Additionally, the PL of phenols and aromatic amines will exhibit a dependence on pH as protonation might affect conjugation.

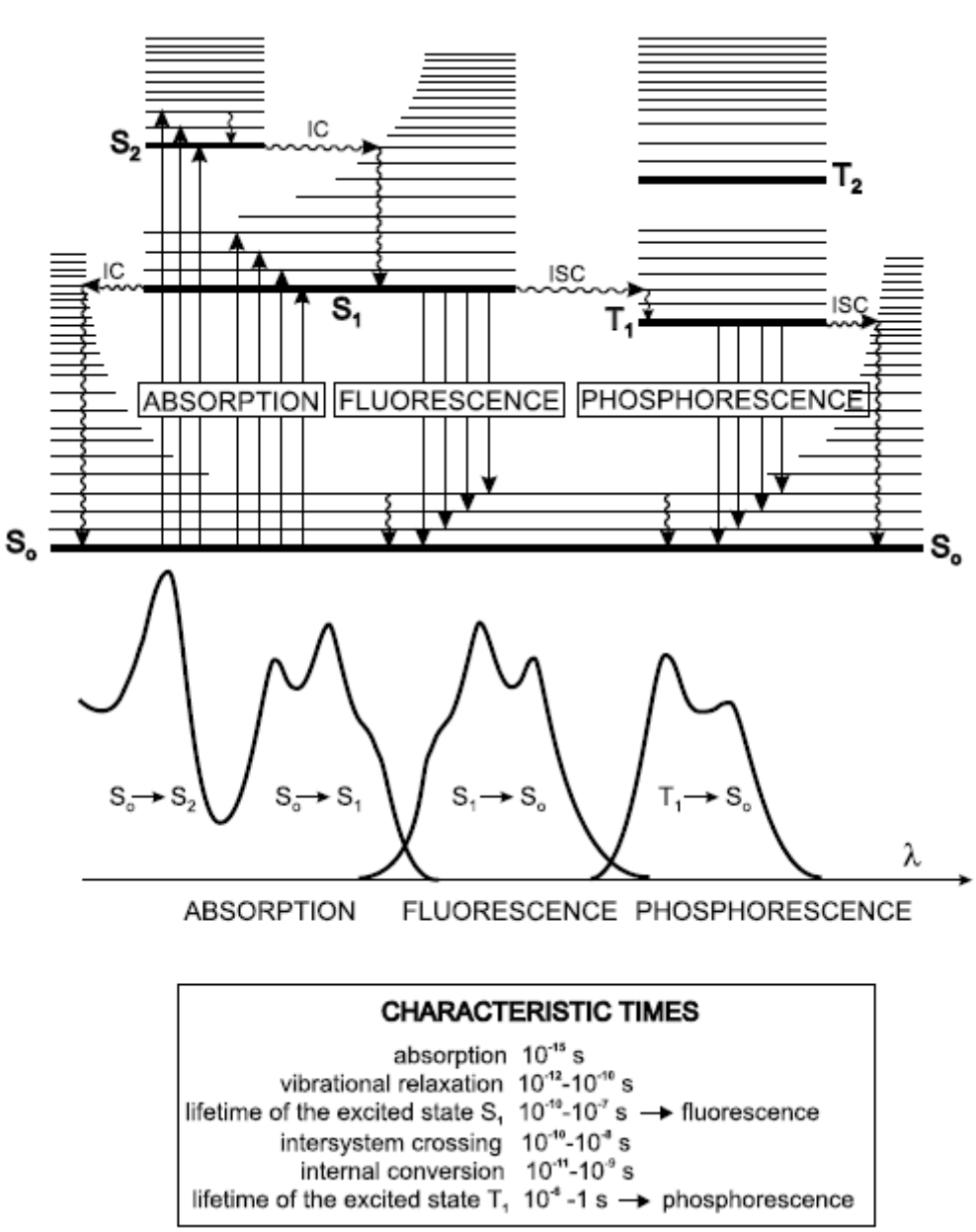


**Figure 8: Perrin - Jablonski diagram and related processes. Internal Conversion (IC) and intersystem crossing (ISC) are the only non-radiative relaxation processes shownin this graph. Relative positions of absorption and radiative deexcitations and characteristic lifetimes are shown in the lower part of the figure in order to further illustrate the possibilities.**[22]

Another way of non-radiative relaxation, the transfer of energy from one molecule to another is referred to as quenching. For this, the two molecules have to be close together either by forming a complex or, if the lifetime of the excited state doesn't allow it, just within a certain radius. In the case that one molecule's emission overlaps with the other molecules absorption

band, Förster Resonance Energy Transfer can occur- for this, both molecules have to be in a certain distance to each other.

In order to get comparative results of measured photoluminescence, quantum yields are determined by comparing the PL of the measured molecule to a luminescent "standard" of known quantum yield. The standard should be a highly fluorescent and readily available species with broad absorption and emission spectra of small overlap in order to avoid errors.

Fluorescent techniques are nowadays used in many fields of research due to various reasons such as versatility, exactness of measurement, and relative simplicity.[22]

## 1.8 Quantum dots

The field of quantum dots is comparably young with the first quantum effects experimentally discovered in 1980 by Ekimov [23] and the theoretical basis being supplied two years later by Éfros and Éfros[24].

It is important to understand, that when talking about quantum dots, one refers to quasi one-dimensional semiconductors. In the semiconductor crystal, the excited state is equivalent to the creation of an electron hole pair i.e. by the absorption of a photon. In this way, the electron is excited to the conduction band. By annihilation of said electron-hole pair, the semiconductor relaxes back to its ground state and a photon is released.

The basis of the specific properties of these nanocrystals is the quantum confinement. By varying the size of the quantum dots, the size quantization will lead to a shift in the wavelength of the released photon- the smaller the quantum dot in size, the shorter will be the resulting wavelength of the created photon.

Due to the particularly small size of the quantum dot, extremely large surface to volume ratios are encountered which effect instable, reactive particles. For this reason, Core/shell systems are used where the quantum dots (core) is engulfed in an organic layer, passivating its surface.

In this study, streptavidin- quantum dot conjugates by Invitrogen (Life Technologies) [25] were used. These CdSe - nanocrystals are encapsulated twice, first, by a layer of ZnS-semiconductor layer, and further, by a polymer film covered in multiple streptavidin proteins (see figure 9). In this way, the conjugate should specifically bind to available biotin molecules present on surfaces, thereby labelling these.

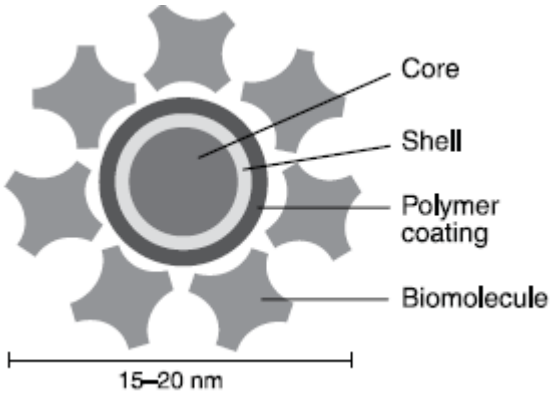


**Figure 9: Streptavidin quantum dot as described and sold by Invitrogen company. [25]**

In figure 10, the spectra of the available quantum dots can be seen. During the experiments for this thesis, solely the one with a maximum of emission at 705 nm was used. One should note the significantly high extinction coefficients of these nanocrystals.

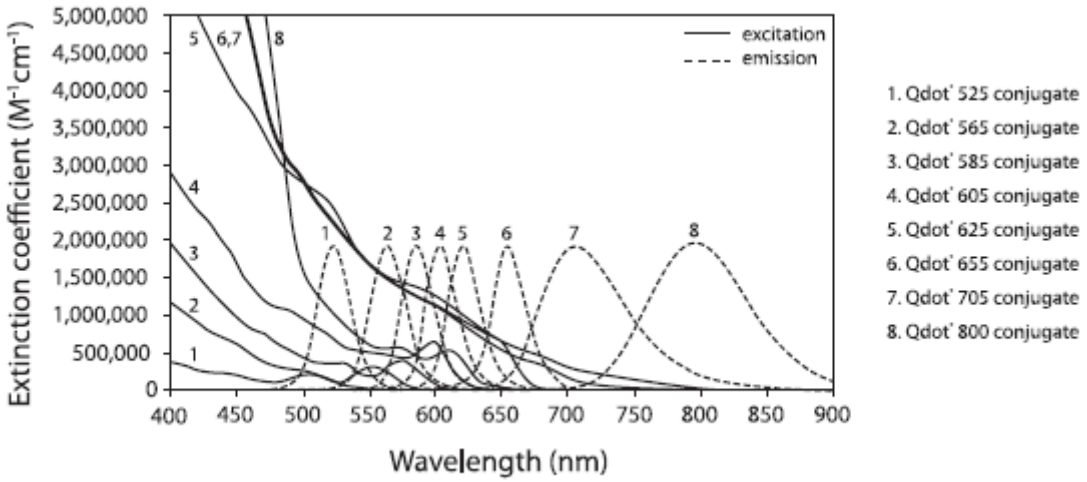


**Figure 10: Quantum-dot-streptavidin conjugate Extinction coefficients plotted against wavelength. For the experiments in this thesis, only #7 was used, with a peak of emission at 705 nm.[25]**

## 1.9 Atto dyes

Developements in the field of biochemistry induced a demand for good fluorescent markers. Since 1999, the Atto Tech company supplies researchers around the globe with such dyes that are characteristic for their height quantum yields, low photobleaching and high stability. Today, a large variety of dyes in a wide range of colours is available.

Atto dyes are small, organic molecules with a rigid structure that effects their stable fluorescence. According to the company, their dyes are mostly derivatives of the coumarin, rhodamine, carbopyronine, and oxazine fluorophores.

In this thesis, Atto 665 biotin conjugate was used a blue cationic (+1) dye emitting in the red (see figure 11). Being a relatively new compound, the company hasn't published its structural formula, yet. This small ambiguity was taken in trade for its high fluorescent yield that is needed in order to detect small quantities on a surface.[26]

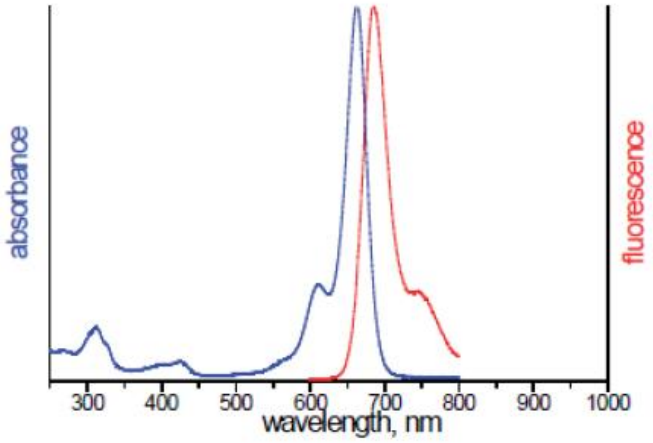


**Figure 11: Standard absorption and fluorescent emission of Atto 665 0.1 mM in neutral phosphate buffer solution.[26]**

# 2. Experimental

## 2.1 Preparation of Organic Semiconductor Films

### 2.1.1 Substrate cleaning

First, microscope glass slides were cut into the right shape and size in order to fit into the evaporation masks used later on.

After confirming they would fit the mask well, the slides were labelled by scratching and a four-step washing procedure was applied as follows:

1. sonication in room temperature (RT) acetone for 15 minutes
2. sonication in 50 °C hot isopropanol (or propan-2-ol according to IUPAC) for 15 minutes
3. sonication in 80 °C hot dilute "Hellmanex" detergent for 15 minutes
4. sonication in RT deionized water for 15 minutes

In between washing steps, the glass slides were dried using compressed, oil-free nitrogen gas and, before applying the 4$^{th}$ step, rinsing with deionized water.

### 2.1.2Substrate coating with octyl trichloro siloxane

#### *2.1.2.1 Octyl trichloro siloxane (OTS) evaporation*

After this cleaning procedure, the glass slides were dried as mentioned before and mounted in a rack for coating with octyl trichloro siloxane (OTS). Together with an uncovered flask containing 60 µL of OTS, the glass-slide containing rack was put into a glass incubation chamber and closed with the corresponding lid. Placed onto a hotplate of 120 °C, the incubation chamber was left like this overnight. In order to avoid damage to personnel and instrumentation, the whole setup was placed into a fume hood. As the OTS evaporated at the elevated temperature, the contained slides were covered in a multilayer of OTS, where the first of the layers would form a covalently attached, self-assembled monolayer (SAM). (see figure 12 for reaction)

### *2.1.2.2 Removal of physisorbed OTS*

On the next day, the lid was lifted off the incubation chamber and any excess OTS was left to evaporate in the fumehood. Aiming for the removal of any adsorbed OTS, the slides were washed in a 5 step procedure as follows:

1. rinsing with toluene for several seconds
2. rinsing with isopropanol for several seconds
3. sonication in RT toluene for 15 minutes
4. rinsing with isopropanol for several seconds
5. rinsing with 18 MΩ water for several seconds

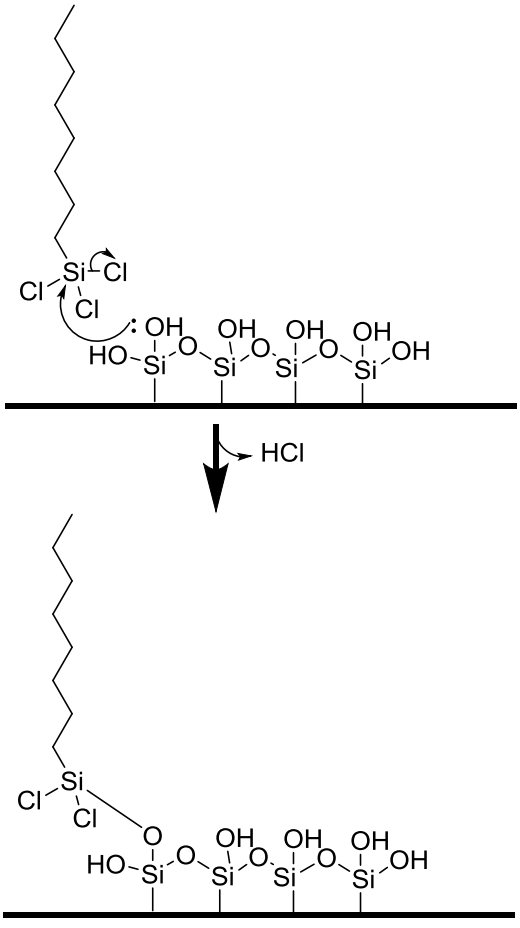


**Figure 12: OTS-functionalization of silica surface.**

As before, drying via compressed nitrogen gas was applied in between washing steps.

### 2.1.2.3 Vacuum Evaporation

Now, every glass slide was covered in a monolayer of OTS and thus ready for vacuum evaporation of the organic semiconductor (OSC). The monolayer of OTS altered the surface properties of the glass slides from polar to non-polar as a "lawn" of octyl-chains pointed towards the top of the substrate. In this way any OSC-layer on top would be safe from delamination by water.

Using an evaporation system by the "VAKSIS" (see figure 13) company, 80 nm thick layers of the respective organic semiconductors were deposited on the substrates. An average example of the parameters used can be found in table I. Detailed values for all evaporations can be found in the lab book.

**Table I: Evaporation parameters used in vacuum evaporation of the organic semiconductors**

| | Pressure /Torr | Sublimation temperature/ °C | Rate/ Å $s^{-1}$ | Thickness/ nm |
|---|---|---|---|---|
| Epindolidione | $2.6 \times 10^{-6}$ | 185-207 | 0.3-1.1 | 80 |
| Quinacridone | $1.2 \times 10^{-6}$ | 271-308 | 0.2-1.0 | 80 |
| Tetracene | $1.4 \times 10^{-6}$ | 110-130 | 0.2-0.8 | 80 |
| Pentacene | $2.1 \times 10^{-6}$ | 145-177 | 0.1-0.8 | 80 |
| Indigo | $2.0 \times 10^{-6}$ | 164-170 | 0.5-0.9 | 80 |

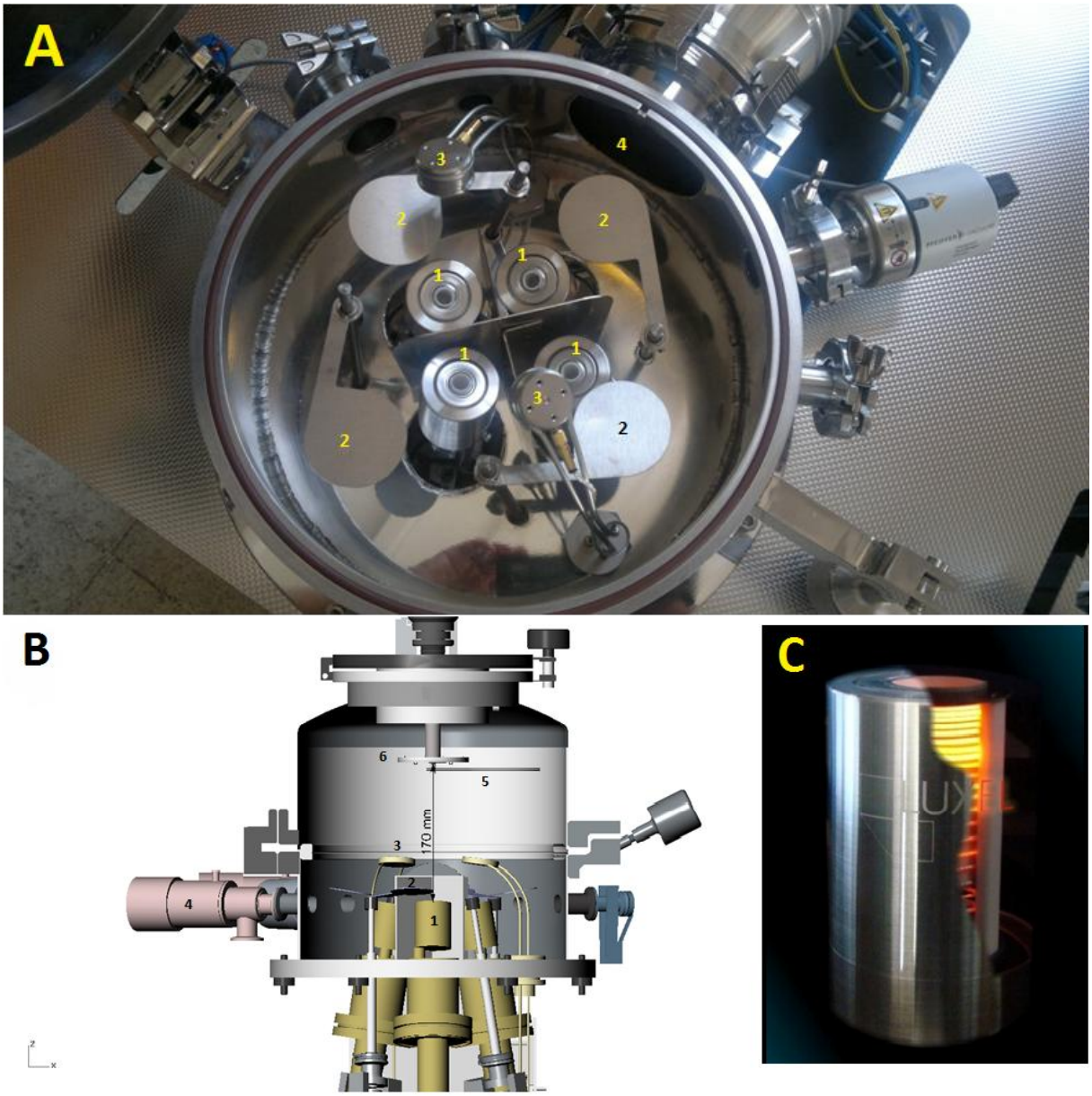


**Figure 13: A, Photograph of the vacuum chamber of the VAKSIS system for evaporation of organic molecules; B, Schematic representation of the components of the VAKSIS systems vacuum chamber; C, thermal evaporation source with isolation mantle. Components: 1. Thermal evaporation source; 2. Shutter of the evaporation source; 3. Quartz crystal microbalance; 4. To the vacuum pumps; 5. Sample-mount shutter; 6. Rotational sample-mount.**

## 2.2 Biofunctionalization: Biotinylation and application of streptavidin-quantum dot conjugate

### 2.2.1 Biotinylation

In order to make a biosensor, there is no way around the introduction of a biomolecule - in our case biotin. Although a whole set of variations for this biofunctionalization was tried out, the principle remains the same for all of them.

First, a 50 µg/mL solution of N-hydroxysucinamide- biotinate was prepared by a dilution series- as the solubility in water is rather bad, the first dilution was done using DMSO. The pH of that solution was buffered at 7.4 using 1x concentrated phosphate buffer saline (PBS). Then, the surface of the organic semiconductor film was exposed to said solution for 24 h. (see figure 14) In order to avoid drying of the biotin-solution, this was done in an air-tight container.

During the process of optimization, variations of this procedure were used, namely:

- sonication during biotin-incubation (multiple repeats of 15 minutes of sonication with incubation-times in between)
- repeated exchange of incubation solution (24h of incubation with the first solution followed by 5 times 15 minutes incubation with fresh solution)
- incubation in large volumes (15 mL) of incubation solution with the addition of 0.1 mM 4-(dimethylamino) pyridine (DMAP)
- pre-treatment of the surface with pyridine or DMAP (100 mM in water) for a 5 minutes – the organic bases were supposedly activating the surface

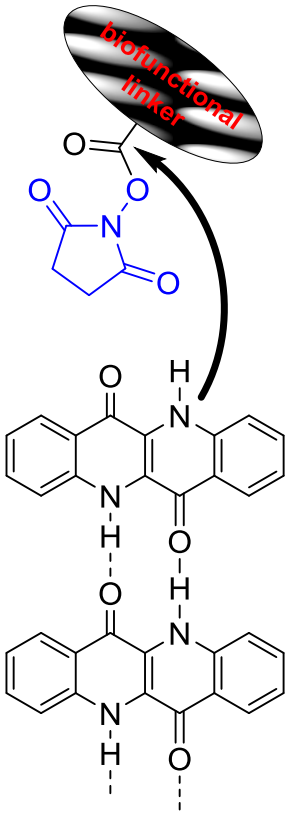


**Figure 14: Schematic of biotinylation of epindolidione surface.**

### 2.2.2 Streptavidin modification

After biotinylation, streptavidin-Qdot conjugate containing solution was prepared as suggested by the company's ("life-sciences") protocol. For this, the mother solution was centrifuged for 3 minutes at 60 000 rpm. Contrary to the suggested protocol, 2 µL of mother solution were taken out and diluted by 100 µL of PBS.

This was different from the protocol as no bovine serum albumin (BSA) was added to this solution in order to avoid unwanted effects were observed. Following the quantum dot manufacturer's incubation protocol to the letter, a 6 % solution of bovine serum albumin (BSA) in neutral buffer saline was to be used as the medium in which the Qdot-streptavidin conjugate was to be incubated with in order to avoid non-specific binding. Instead of gaining the wanted result, an effect that was termed bio-fouling was achieved. The extreme BSA content will completely inhibit streptavidin binding by this special case of competitive inhibition.

Preparing the solution without any BSA, the samples were incubated for 20 minutes with said solution. (see figure 15)

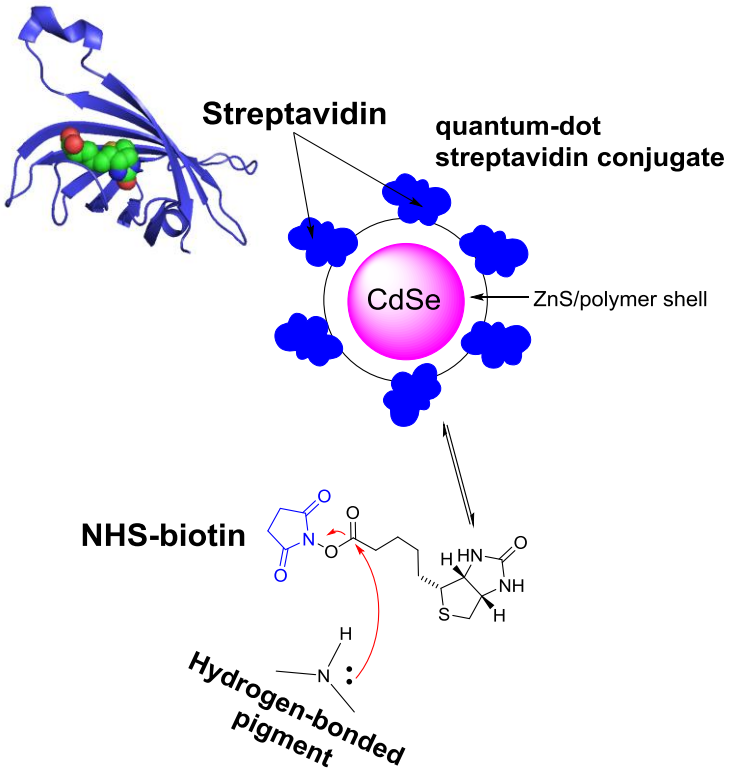


**Figure 15: Schematic of the biofunctionalization of the biotin and the surface, followed by the streptavidin-Qdot conjugate.**[27]

### 2.2.3 Washing procedures

After each measurement, the samples were exposed to a specific kind of washing procedure. This was primarily in order to remove physisorbed molecules but gave also some hint of the stability of the binding between the surface and the biomolecules.

Following nuances of washing were tried out:

- rinsing for 10 s with 18 MΩ water
- sonication in 18 MΩ water for 5 minutes
- rinsing for 10 s with PBS (pH 7.4)

In this way, it was hoped, a surface similar to the one shown in the schematic in figure 16 was to be achieved.

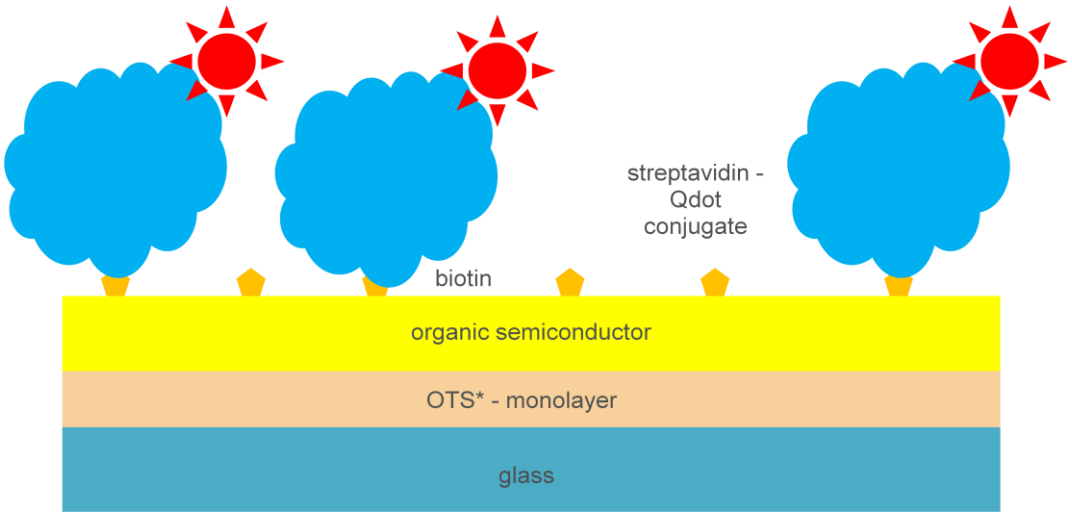


**Figure 16: Schematics of measured sample: An OTS (octadecyl trichloro sylane) monolayer covered under 80 nm of a hydrogen-bonded organic semiconductor was biotinylated and exposed to a streptavidin-quantum dot conjugate. Mind, the sizes of individual components are not in relation to each other and just serve the purpose of illustration.**

## 2.3 Inverse approach: Direct incubation of streptavidin on top of HB-OSC

Using a 1 mg/mL solution of streptavidin in PBS (pH 7.4), epindolidione and quinacridone films on OTS were incubated for 20 minutes followed by 20 minutes of "ATTO 665"-biotin conjugate, a cationic, fluorescent dye (see figure 11 for spectral characteristics).For illustration, a schematics of the prepared sample is included in figure 17.

Then, photoluminescence was measured and a washing series was carried out (each step being equal to 10 s of rinsing with PBS (pH 7.4)).

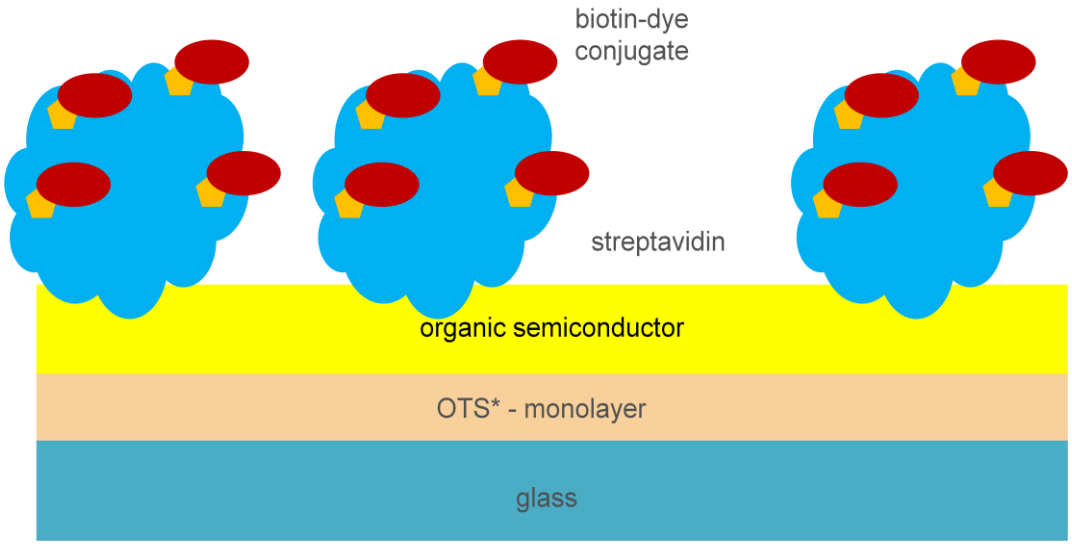


**Figure 17: Protein-first biofunctionalization. The non-specific interaction between streptavidin and organic organic semiconductoris used for immobilization, biotin-dye conjugates are used for the detection.**

## 2.4 Chemical functionalization of hydrogen-bonded organic semiconductor thin films with linker

As the stability of the direct bond between biotin and hydrogen-bonded organic semiconductor was doubted, a new way of functionalization was attempted, using a linker. The general idea was to introduce a short carbon chain with a primary amine group at its end, mimicking the desirable reactive properties found in APTES. The most straight forward way of chemical reaction would have been the use of an N-protected chloroalkylamine, i.e. chloroethylamine which is readily available at low prices. As that molecule structurally resembles parts of the chemical warfare agents LOST and N-LOST, we decided to walk a different path.

First, 1,6-dibromohexane was to be used to substitute the amine of the organic semiconductor film, yielding a monolayer of organic semiconductor modified with a bromine-capped linker. Then, using concentrated ammonia, the linker's bromine was to be substituted with a primary amine. On top of that, bio-functionalization with by biotin incubation for 24 h was carried out, followed by 20 min of streptavidin-Qdot conjugate, just as described above.

This was followed by repeated measurement of PL and washing steps using PBS (pH 7.4) for rinsing off physisorbed, fluorescently labelled biotin.

For the first reaction step, a 39 mM solution of DMAP in dichloromethane (DCM) was prepared. Then, an excess of 1,6-dibromohexane (5 % by volume) was added to the solution and the pigment-covered film mounted into a holder and was dipped into the solution. First trials showed the films stable in DCM for well over 24 hours but in the reaction mixture significantly shorter times were needed in order to prevent desorption of the film. Making an educated guess, it was concluded that this was related to the changed properties of the modified top layer. For this reason, modification of epindolidione was nigh to impossible and the reaction time of quinacridone was set to 1.5 h.

In the second step, the dry, reacted films were inserted into concentrated ammonia for 24h in order to change substitute the terminal bromine for an amine group. The reaction intended scheme can be found in figure 18. Unfortunately, this reaction wasn't going as intended and from binding behaviour the prevalent reaction is crosslinking between quinacridone molecules rather than introduction of a linker. More information on that can be found in the "results" section.

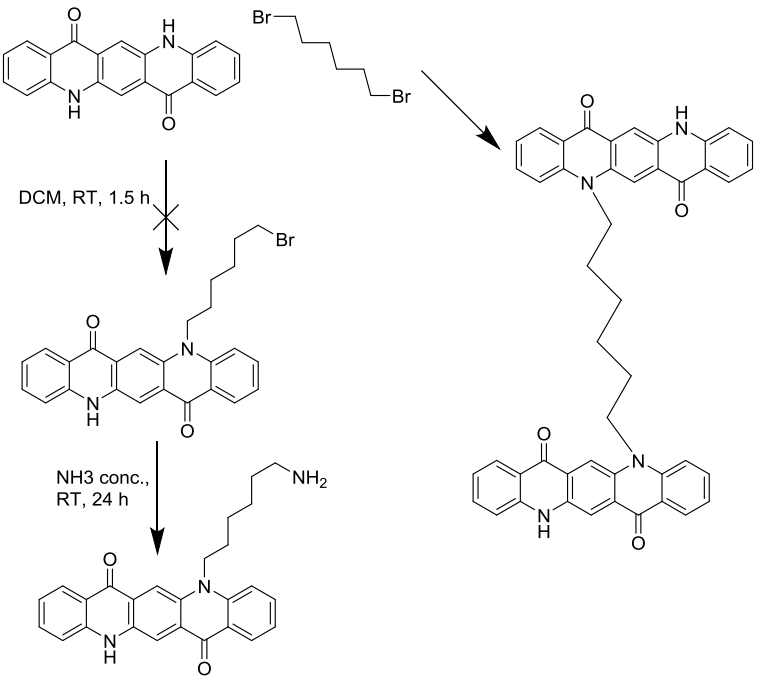


**Figure 18: Thought reaction scheme of linker introduction onto quinacridone film. The problems of this approach are further discussed in the "Results" section.**

## 2.5 Photoluminescence spectroscopy

Photoluminescence detection is a strong detection tool by which even very low concentrations of fluorescent particles can be detected- with the right equipment even single molecules can be detected![28] However, careful optimization and the use of a strongly fluorescing probe are needed.

In this thesis, as in virtually all experiments were done on thin films of OSC's absorbing and emitting in the visible light range, it was important to pick an excitation wavelength, where only the quantum dot would absorb or rather where the hydrogen bonded organic semiconductor would have a minimum in absorption.

In order to avoid false positives and fluorescence from the absorbing pigments, excitation wavelengths were chosen in a way such that they were of lower energy than at which the semiconductors absorbed.

In this way, it was possible to get a clean emission spectrum of the quantum dot, only. The respective wavelengths used can be found in the table below.

**Table II: Irradiation and emission wavelengths ranges used for different pigments in order to avoid measuring the organic semiconductor itself.**

| | $\lambda_{irradiation}$/ nm | $\lambda_{emission}$/ nm |
|---|---|---|
| Epindolidione | 630 | 660-800 |
| Quinacridone | 630 | 660-800 |
| Indigo | 500 | 630-730 |

A photograph of the fluorometer used is shown in figure 19.

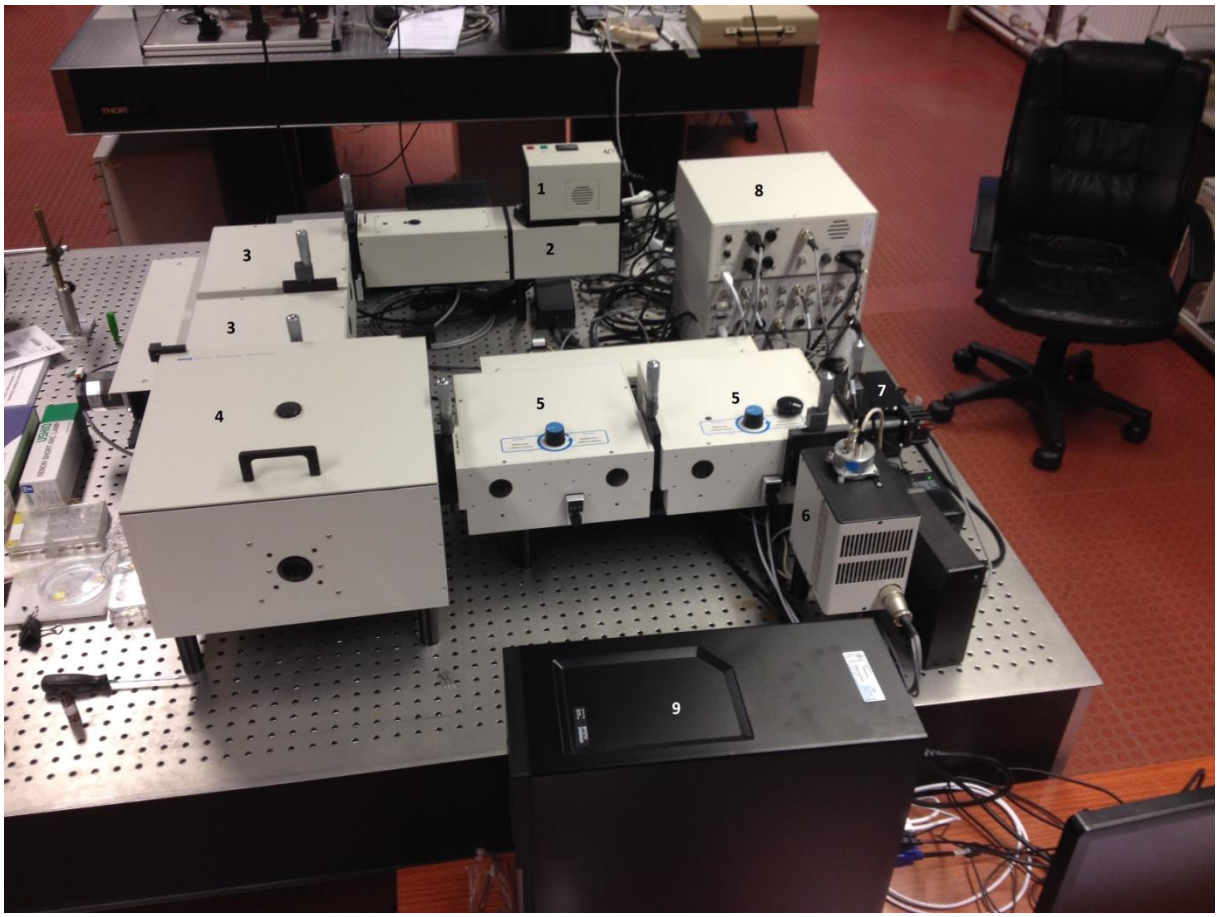


**Figure 19: Photograph of the fluorometer used for experiments in this thesis. Components: 1. Broad-band source power-supply; 2. Broad-band source; 3. Double-grating monochrometer "in"; 4.Sample compartment; 5. Double-grating monochrometer "out"; 6. Photomultiplier; 7. InGaAs detector; 8. Computer-instrument interface; 9. Computer.**

# 3. Results and discussion

At the beginning of this thesis it was the idea to use the biotin- streptavidin system on a surface of a hydrogen-bonded organic semiconductor in order to establish a proof of principle for the idea of surface modification with biospecific binding sites. Naïvely thinking, the biotin streptavidin system is the strongest non-covalent interaction in nature, so it should be an easy to determine whether our idea was valid or not.

The early experiments, however, made up for more questions rather than answers and the complexity of the problem ahead was slowly manifesting.

## 3.1 Biotinylation of pristine films

### 3.1.1 Washing with 18 MΩ water

#### *3.1.1.1 APTES versus epindolidione*

As described in the "Experimental" section, samples were prepared and further investigated using fluorescence spectroscopy. Even though a quantum dot-streptavidin conjugate was used for detection of the biotin, for simplicity it will be referred to as "streptavidin".

The results were investigated using fluorescence spectroscopy.

Primarily it was of importance to compare the behaviour of our novel, hydrogen-bonded system to a well studied surface. For this epindolidione films, biotinylated and not, were compared to equally modified monolayers of (3-aminopropyl)triethoxysilane (APTES ) on glass. The monolayers were similarly prepared as OTS films with the difference that coating was done under dry nitrogen-gas and washing procedure was limited to sonication in isopropanol and rinsing with 18 MΩ water.

The results are shown in figures 20 and 21. In both graphs, the blue line depicts the case of absence of biotin-modification, whereas the orange marks its presence. Therefore, the blue line shows the level of non-specific binding to the surface- the bigger the difference between both lines, the larger the specificity of binding.

As it can be clearly seen from the first graph, in the case that APTES is not mo shows very little to any non-specific binding and is therefore a very good system in this regard. With epindolidione, the story looks highly different. Although the presence of biotin clearly increases binding probability, nonspecific binding clearly is not little on this surface and has to be taken account for. This lead to the conclusion, that a good washing technique was needed to remove such binding but also opened the question whether this was due to the presence of many sites to form hydrogen bonds as epindolidione presents both, hydrogen-bond donors and acceptors in the form of the amine and carbonyl group.

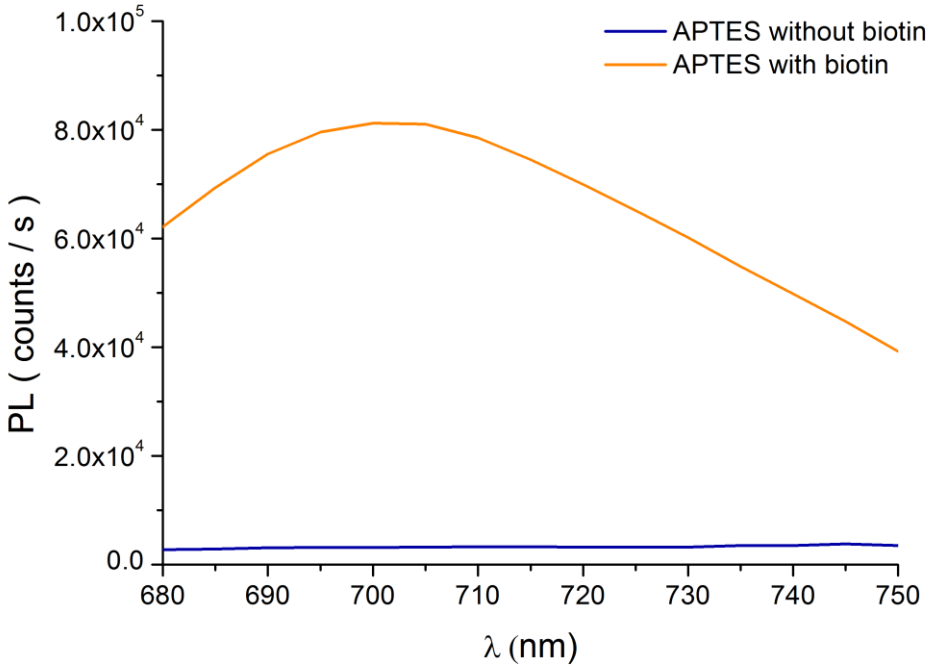


**Figure 20: Fluorescence of quantum dot on APTES self-assembled monolayer.**

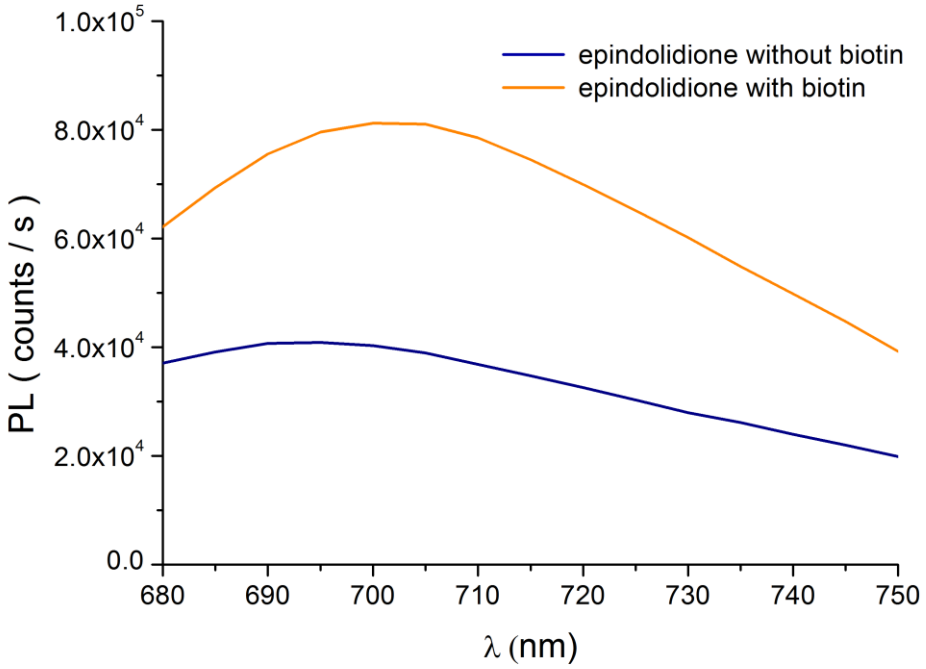


**Figure 21: Fluorescence of quantum dot on the surface of 80 nm of epindolidione.**

In order to determine the effect of the presence or absence of hydrogen bonds on a surface towards binding-behaviour a system as similar to our hydrogen-bonded semiconductors, epindolidione and quinacridone (figure 6), was needed. For this reason, the organic semiconductors tetracene and pentacene (figure 7) were used to prepare samples for comparison in biotinylated and non-biotinylated form.

### 3.1.1.2 Comparison of HB-OSCs versus non-hydrogen bonded OSCs

In the figures 22 and 23, the effect of washing-off the streptavidin-Qdot conjugate off the respective surface can be seen. First, I would to point out that between the non-modified surfaces, no to very little difference in measured intensity can be seen which suggests that the non-specific binding of protein towards the surface is not related to the presence or absence of hydrogen-bonds but rather to van-der-Waals forces. Since it is known that epindolidione and quinacridone both form a polycrystalline structure with lots of crevices[13] and thus a lot of surface, proteins have an easy way how to stick onto the pigment. As similar results were obtained for the "bare" structures, it might suggest a similar structure being present.

Now, focusing on the bio-modified surfaces, one thing quickly becomes apparent: there is no difference if biotin was applied or not. The presence of hydrogen-bonds is clearly prerequisite for biotin to stick on the surface and positively influence the binding behaviour of the applied protein.

This can be seen in figure 22 and 23 when focusing on the blue rectangles. At the beginning of the washing series, PL values double of the non-modified surfaces were obtained. However, when washing the samples further, this decreased in both cases. From this, the conclusion lies near that biotin, our anchor molecule first binds to the protein in one way or another, but is washed away together with the molecule.

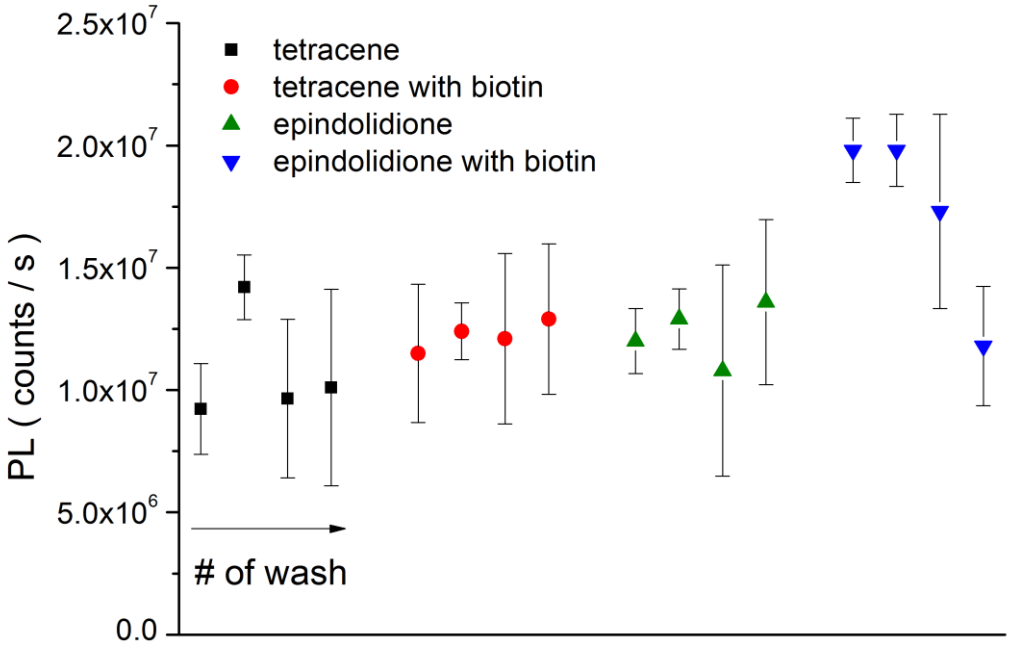


**Figure 22: Integrals (630-800 nm) of fluorescence spectra of quantum dot on top of tetracene and epindolidione. The results of repeated washing are grouped by surface investigated- results within a group are ordered with increasing number of repetition from left to right. The error bars correspond to 1 standard deviation.**

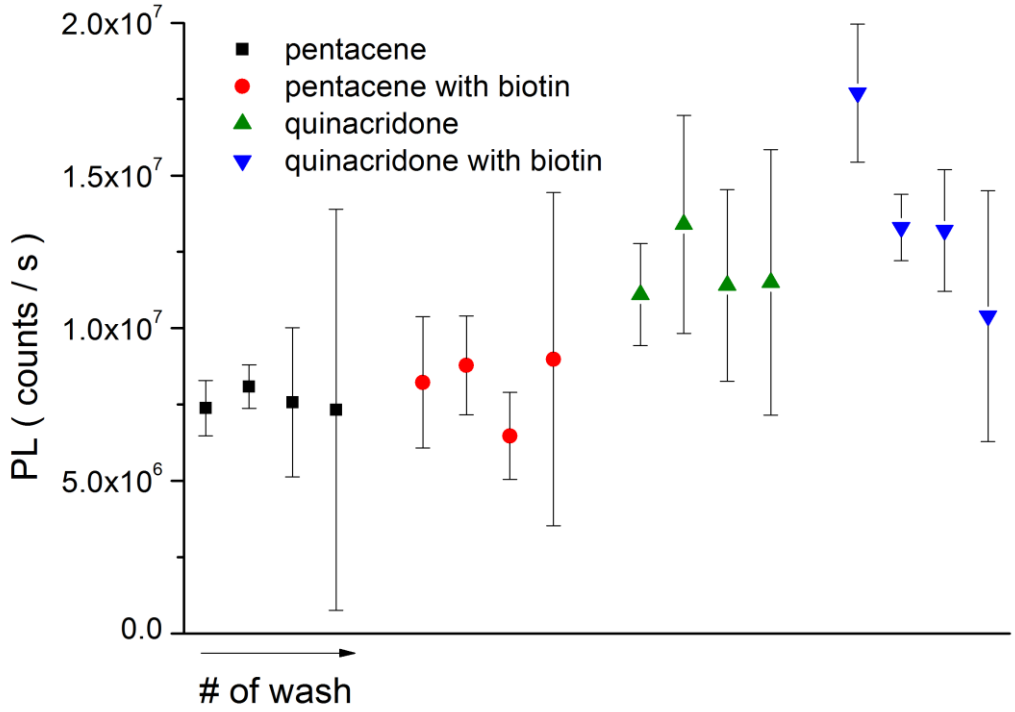


**Figure 23: Integrals (660-800 nm) of fluorescence spectra of quantum dot on top of pentacene and quinacridone. The results of repeated washing are grouped by surface investigated- results within a group are ordered with increasing number of repetition from left to right. The error bars correspond to 1 standard deviation.**

### 3.1.1.3 Increasing the biotinylation efficiency

#### *3.1.1.3.1 Pyridine pre-treatment*

Now, the question remained how it is possible to break the covalent bond between the linker and the surface. Carrying out the biotinylation reaction of epindolidione out in solution, the result was obtained, that the best efficiency was obtained when an organic base such as pyridine was added to the reaction, following a similar logic as Glowacki *et al.* with the protection of indigo[12].

For this reason, a trial was done of an epindolidione film. Before applying the biotin solution, the organic semiconductor was covered in a droplet of 100 mM pyridine solution in water. Then the same procedure as before was applied. (see figure 24)

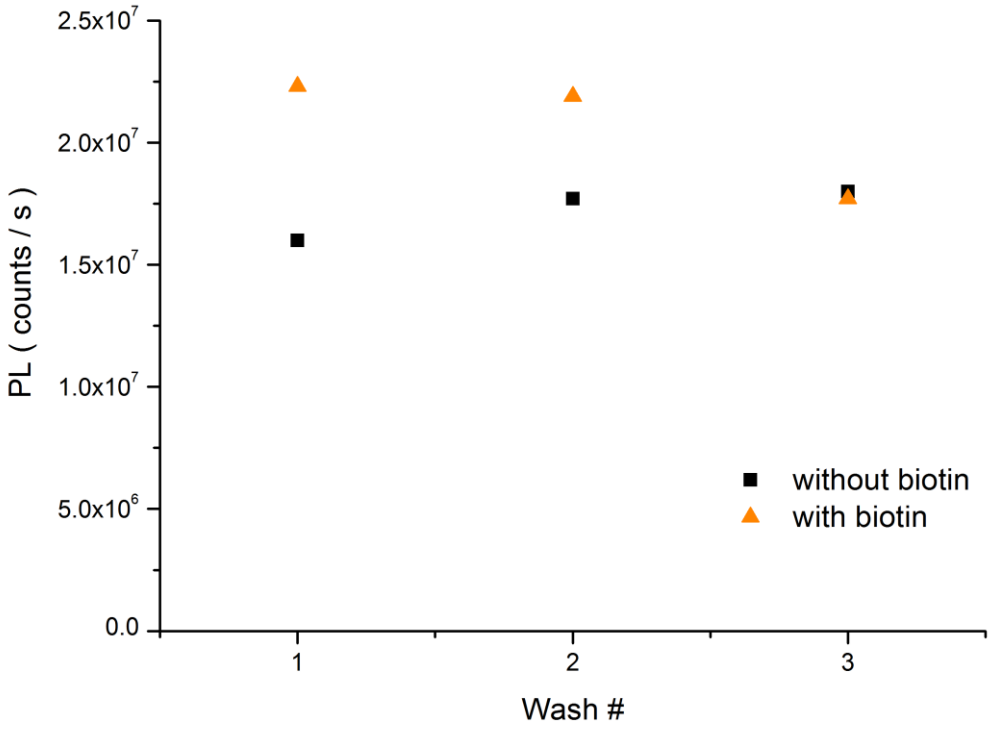


**Figure 24: Integrals of fluorescence spectra (630-800 nm) of quantum dot on pyridine pre-treated epindolidione.**

#### *3.1.1.3.2 Comparison of DMAP pre-treatment against sonication during biotin-incubation*

Observing the results in figure 22, a similar pattern can be seen as before- after a slightly stronger PL in the beginning, repeated washing with water lead to equal intensities on both kinds of sample. Therefore, the need for a more efficient catalyst than pyridine was at hand. Thus, DMAP, 4-(dimethylamino) pyridine, was chosen as a base- the electron pushing amino-group was hoped to deliver the wanted effect of increased reactivity.

In order to investigate another suspicion we had, this was compared with the result of sonication during the annealing procedure. To elaborate, biotin (figure 3) is also capable of forming hydrogen-bonds, having both donors and acceptors incorporated in its ring system. For this reason it was believed, that hydrogen bonding towards the surface was not out of question. A schematic of this can be seen in figure 25.

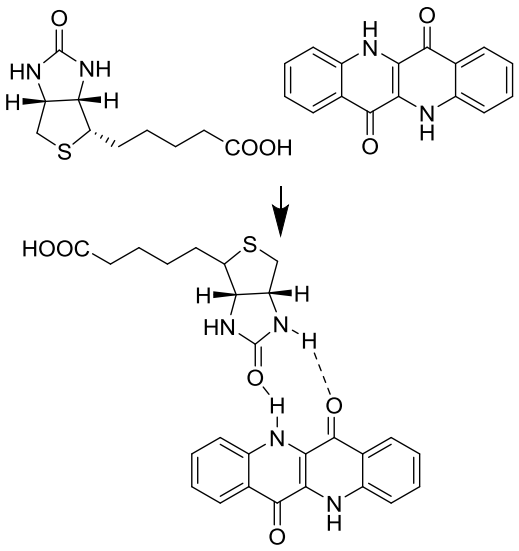


**Figure 25: Schematic representation of biotin hydrogen bonding with epindolidione.**

The samples were prepared just as described before using sonication and DMAP pre-treatment and investigated by fluorescence spectroscopy. The results of this are seen in figure 26 and 27, for epindolidione and quinacridone, respectively.

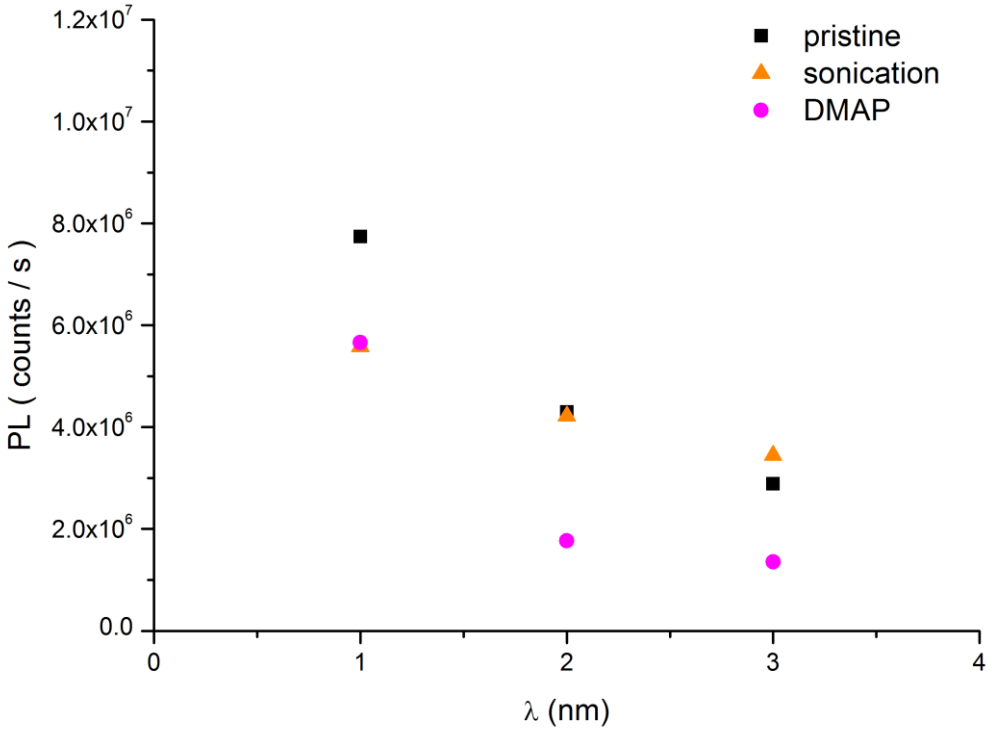


**Figure 26: Integrals (630-800 nm) of fluorescence spectra of quantum dot on epindolidione. Pristine denotes the fluorescence of non-biotinylated samples, sonication and DMAP the modification of the biotinylation treatment.**

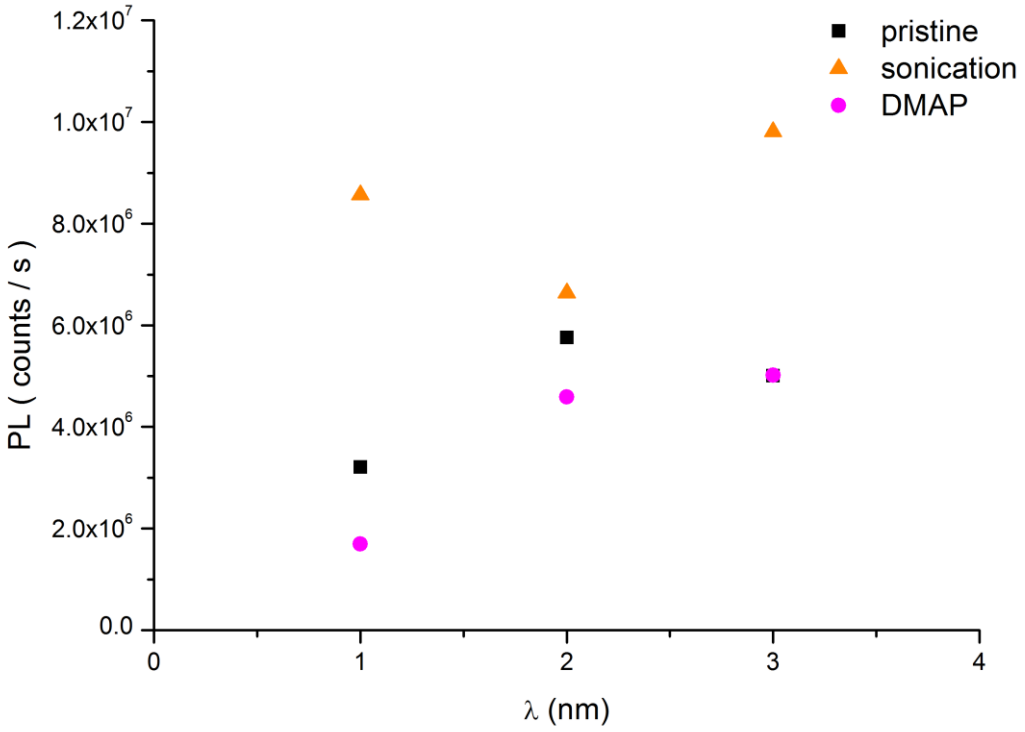


**Figure 27: Integrals (660-800 nm) of fluorescence spectra of quantum dot on quinacridone. Pristine denotes the fluorescence of non-biotinylated samples, sonication and DMAP the modification of the biotinylation treatment.**

From in these spectra, one thing becomes apparent. First, there is no real trend to be observed for quinacridone samples, while sonication seems to be the best way. This is opposed to the results of epindolidione where there is a decrease in emission visible, that end up in a similar intensity after the third round of washing. Pre-treatment with DMAP made the impression of being the worst of these ways of modification, which was surprising and contradicting when compared to the result of incubation with pyridine.

For this reason, the experiment was repeated- with even more surprising results, as seen in figures 28 and 29.

In figure 28, epindolidione seems to be at first very susceptible to DMAP pre-treatment but this rapidly changes when more water is applied. The rest doesn't seem to change over time.

In figure 29, as opposed to the result from the previous measurement, DMAP pre-treatments seemed to be distinguished among the treatments, showing best photoluminescence and thus best retention of the protein. And also here, no big influence of washing was observed for

neither of the other samples, be it the ones treated with sonication or the pristine ones. This was indeed very confusing as the results contradicted each other.

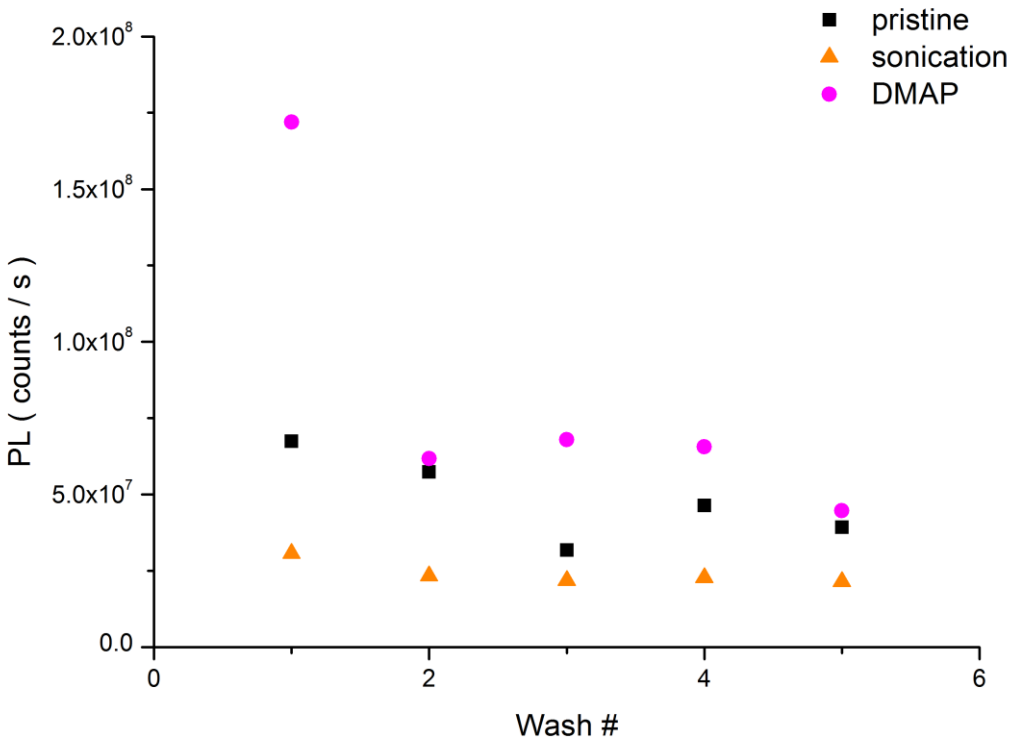


**Figure 28: Integrals (630-800 nm) of fluorescence spectra of quantum dot on epindolidione. Pristine denotes the fluorescence of non-biotinylated samples, sonication and DMAP the modification of the biotinylation treatment.**

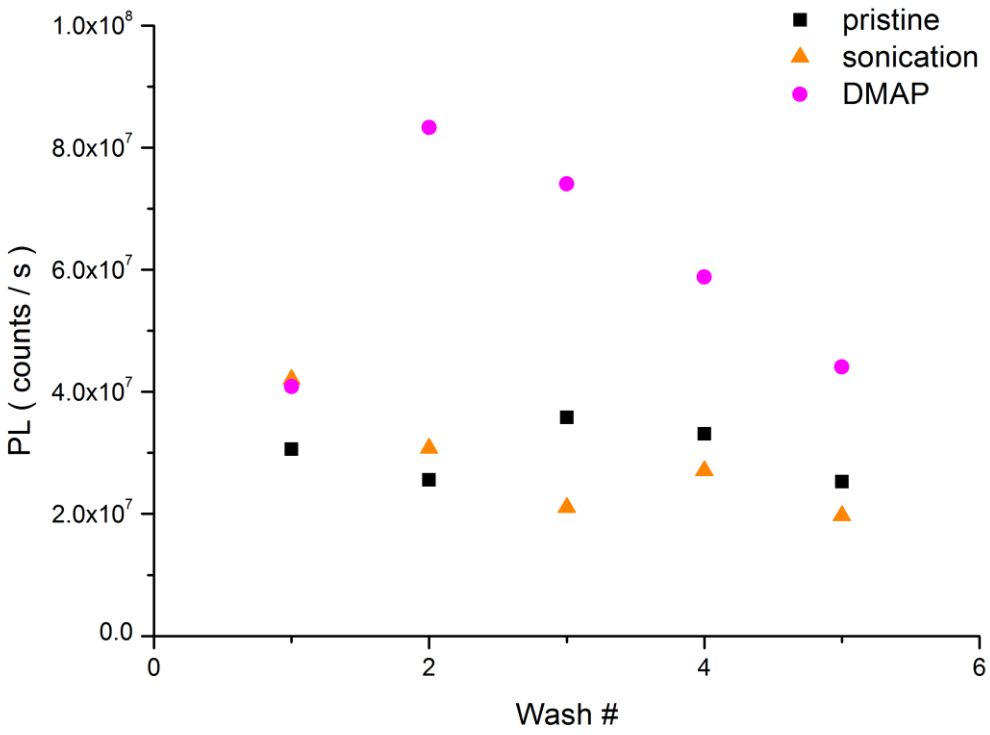


**Figure 29: Integrals (660-800 nm) of fluorescence spectra of quantum dot on quinacridone. Pristine denotes the fluorescence of non-biotinylated samples, sonication and DMAP the modification of the biotinylation treatment.**

#### *3.1.1.3.3 Comparison of base pre-treatment against direct biotinylation*

The result of this can be seen in figure 30 and 31. From the former of both graphs one can see in the case of an unmodified surface an overall stagnation of fluorescence contrary to repeated rinsing with highly pure water, opposing a slow removal of bound streptavidin-conjugate in the case of regular biotinylation. Application of base worsened results.

Judging from the latter graph, slight washing-off of protein can be seen in all cases except for pyridine application which showed the worst protein retention from the beginning to the end. Both graphs suggest that the effort of applying biotin in order to get a specific binding is an effort equal to fighting windmills.

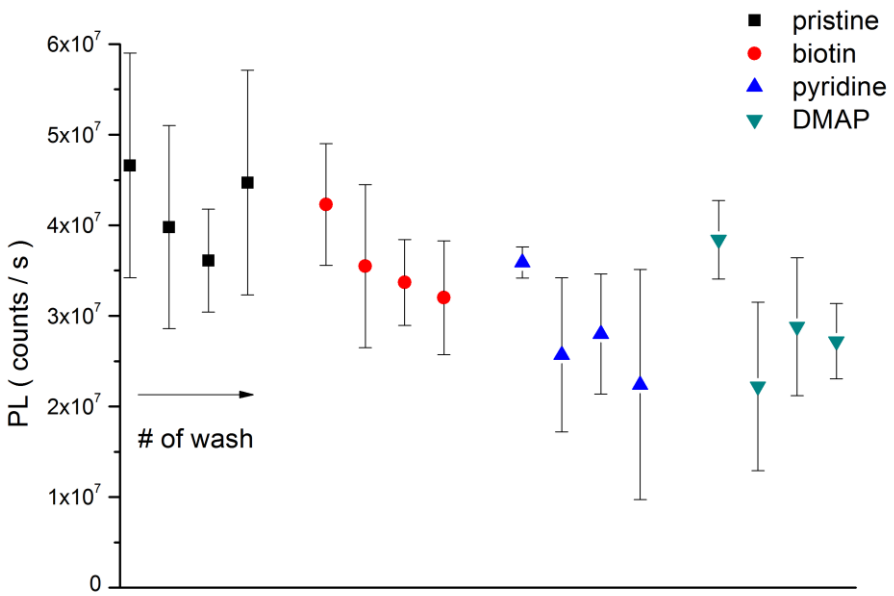


**Figure 30: Integral averages (630-800 nm) of quantum dots on epindolidione. Results of repeated washing are grouped by surface modification investigated- results within a group are ordered with increasing number of repetition from left to right. The error bars correspond to 1 standard deviation.**

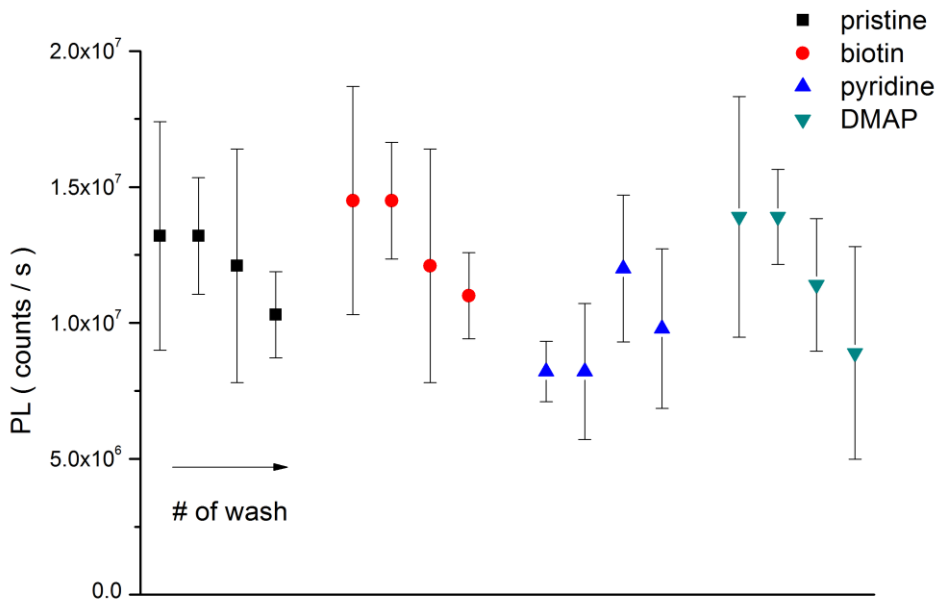


**Figure 31: Integral averages (660-800 nm) of quantum dots on quinacridone. Results of repeated washing are grouped by surface modification investigated- results within a group are ordered with increasing number of repetition from left to right. The error bars correspond to 1 standard deviation.**

#### *3.1.1.3.4 Repeated biotinylation*

From the results above it was clear, that biotinylation was the weak link in the modification procedure. As a last effort before discontinuing this kind of experiment, an effort was made to incubate the surface repeatedly, for short intervals, with biotin.

As it can be seen from figure 32, repeated incubation with biospecific linker did not lead to a better binding, but quite to the contrary worsened the binding properties compared to pristine samples.

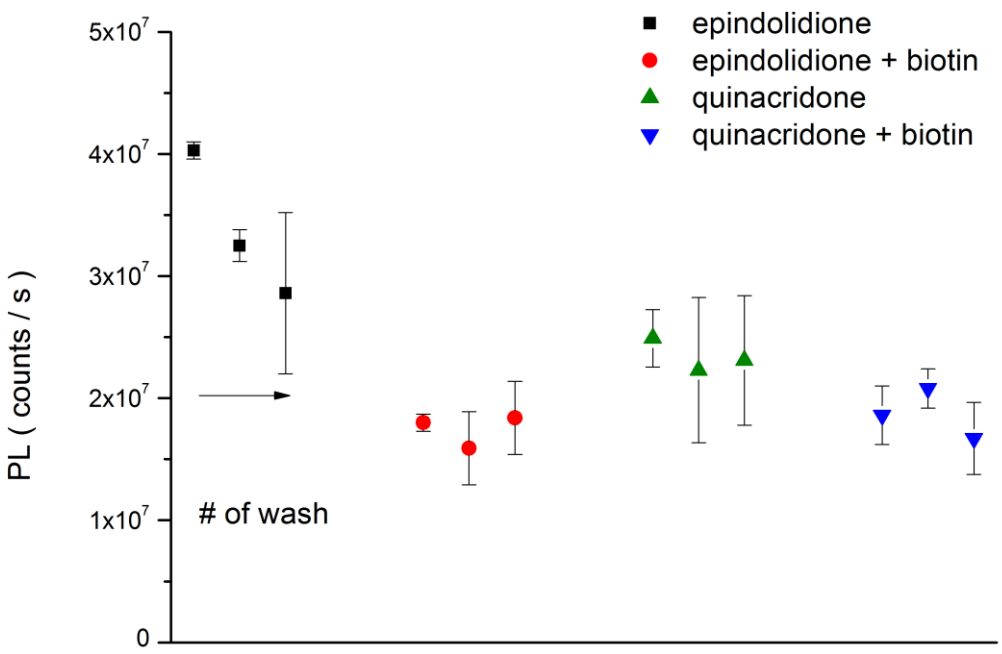


**Figure 32: Integral averages (660-800 nm) of quantum dots on epindolidione and quinacridone. Results of repeated washing are grouped by surface modification investigated- results within a group are ordered with increasing number of repetition from left to right. The error bars correspond to 1 standard deviation.**

### 3.1.2 Washing with PBS

#### *3.1.2.1 Comparison of APTES against HB-OSCs*

During and after the presentation of parts of this work, outside our institute, crucial advice concerning the use of 18 MΩ water was received. Although very pure and ideal to prevent pollution during rinsing, the very lack of impurity serves said water susceptible to extreme pH change from dissolved ambient carbon dioxide. As proteins are extremely sensitive to pH changes, a different solvent was advised to be used.

Further, the amide bond connecting biotin to the respective organic semiconductor was supposedly not stable towards hydrolysis due to the ring systems conjugation.

For both reasons, all the relevant experiments were to be repeated using phosphate buffer saline (PBS) at a pH of 7.4 as the new washing solution.

First of all, APTES was again compared to epindolidione and quinacridone, as shown in figure 33.

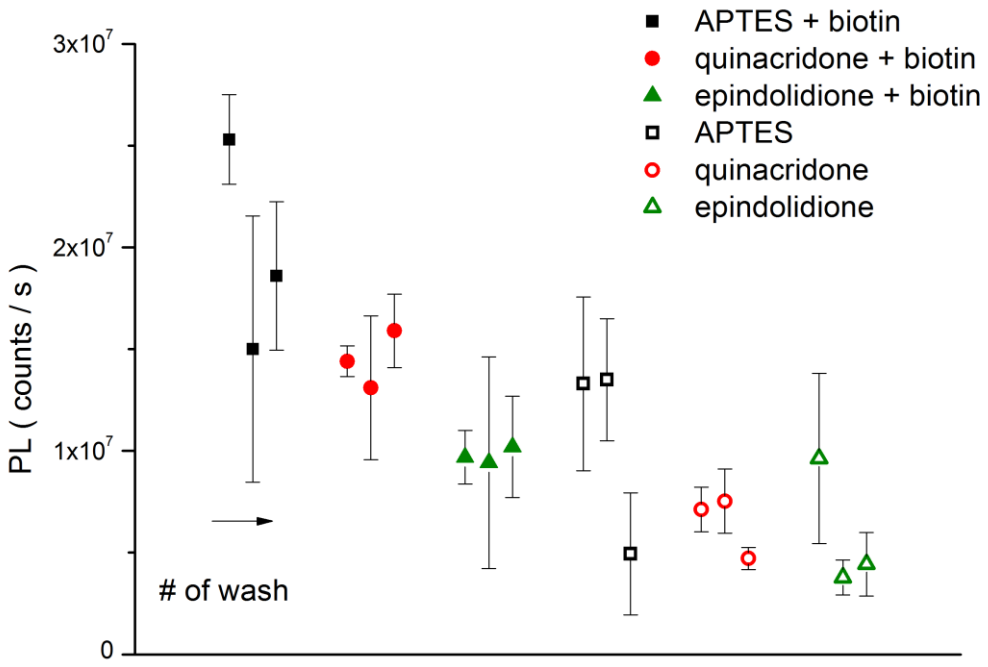


**Figure 33: Integral averages (660-800 nm) of quantum dots on APTES, epindolidione, and quinacridone. Results of repeated washing are grouped by surface modification investigated- results within a group are ordered with increasing number of repetition from left to right. The error bars correspond to 1 standard deviation.**

Contrary to previous results, a perfect washing of non-specifically bound protein off APTES was not possible in this case- this was the same even during repetition. On the other hand, the impression is that streptavidin on hydrogen-bonded pigments shows more specific than non-specific binding. Note, that although it was not possible to wash away the protein off the biotinylated surfaces, the same was true for the non-biotinylated ones, so overall it was not possible to improve the results by applying a buffer system.

### *3.1.2.2 Increasing the biotinylation efficiency*

*3.1.2.2.1 Comparison of base pre-treatment against direct biotinylation*

Now, base pre-treatment was investigated in the same way, using the buffer system for washing. The resulting photoluminescent spectra of the probe can be seen below in figure 34 and 35.

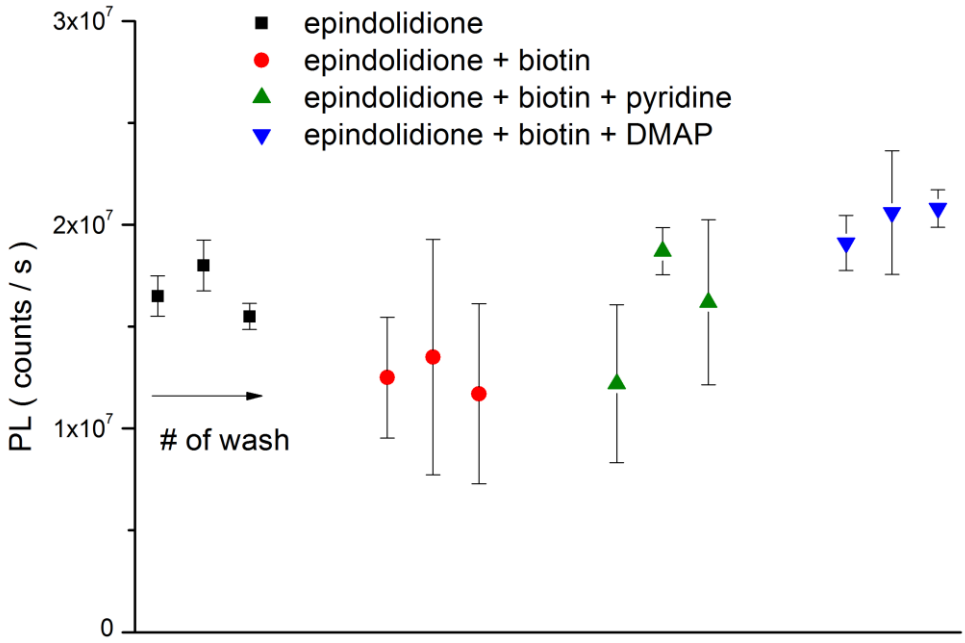


**Figure 34: Integral averages (660-800 nm) of quantum dots on epindolidione. Results of repeated washing are grouped by surface modification investigated- results within a group are ordered with increasing number of repetition from left to right. The error bars correspond to 1 standard deviation.**

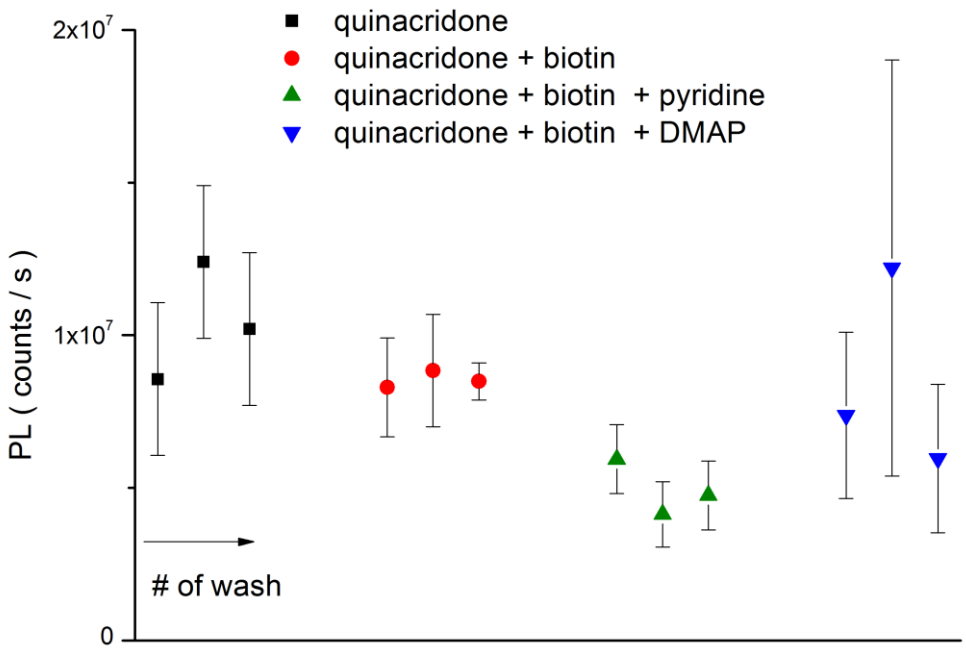


**Figure 35: Integral averages (660-800 nm) of quantum dots on quinacridone. Results of repeated washing are grouped by surface modification investigated- results within a group are ordered with increasing number of repetition from left to right. The error bars correspond to 1 standard deviation.**

From both figures above, similar results were obtained as from the comparison to APTES. Although, on first glance different from the results obtained when washing with pure water no improvement to the previous washing procedure could be found. From this it is evident, that although being mild enough to keep the biotin-organic semiconductor bond from hydrolysing, it is also too mild to wash-off the non-specifically bound protein.

## 3.2 New approaches

### 3.2.1 Introduction of linker a linker between HB-OSC and biotin

As stated above, there is a substantial problem with using biotin directly linked to the pigments because of the bonds instability towards hydrolysis. For that reason it was deemed important to use a linker between organic semiconductor and biotin.

The reaction can be found in the "Methods and Experiment" section. For reasons stated there, only photoluminescent measurements on top of quinacridone were possible.

All samples were incubated with biotin and streptavidin-Qdot conjugate; washing was done using PBS pH 7.4.The results of this experiment can be seen in figure 36.

As one can clearly see from the figure, there is a 3-4 fold difference between the emission of linker-modified surface and samples without linker in advantage for the samples lacking any linker. This was twice surprising: once, because there was a decrease in fluorescence where an increase was expected, twice because the difference to simply non-modified samples was larger than in most previous experiments, where this was hoped for.

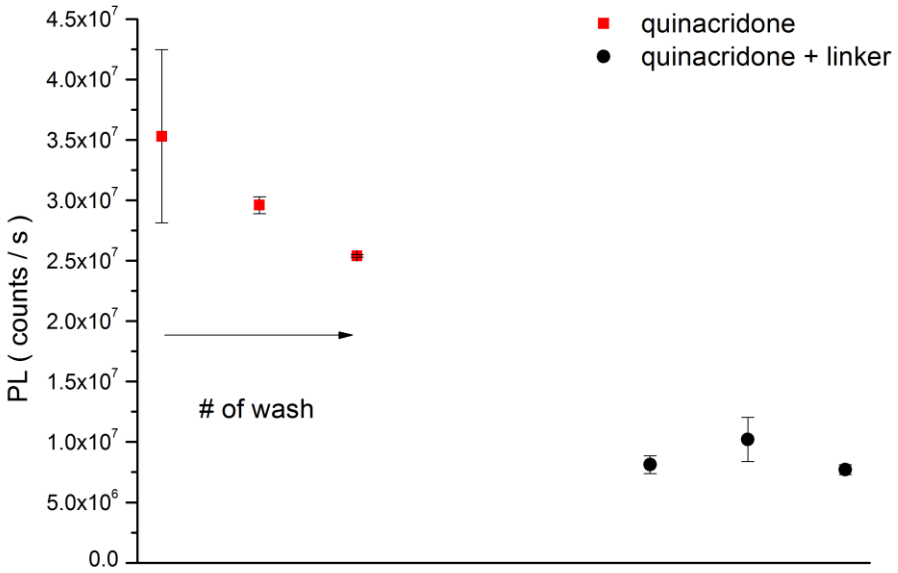


**Figure 36: Integral averages (660-800 nm) of quantum dots on quinacridone, with and without linker. Results of repeated washing are grouped by surface modification investigated- results within a group are ordered with increasing number of repetition from left to right. The error bars correspond to 1 standard deviation.**

As we knew by then, non-specific binding has a strong effect in these experiments, it was assumed, that there was simply too little quinacridone to which the protein could attach itself. For this reason profilometry measurements were done: on average, 58 out of 80 nm remained after reaction. So we can say that there was indeed material lost, but there should be still enough material left for protein-retention unless dramatic changes occurred to the surface-structure i.e. due recrystallization into larger crystals. Since this was not likely, it seemed probable that this was due to chemical surface modification, namely linking two quinacridone molecules. This would also make a lot of sense since the linkers leaving groups were equal and after reaction the local concentration of the alkylbromide is effectively elevated in the vicinity of the reaction site. For illustration of said reaction model, the crystal structure of quinacridone was included in figure 37.

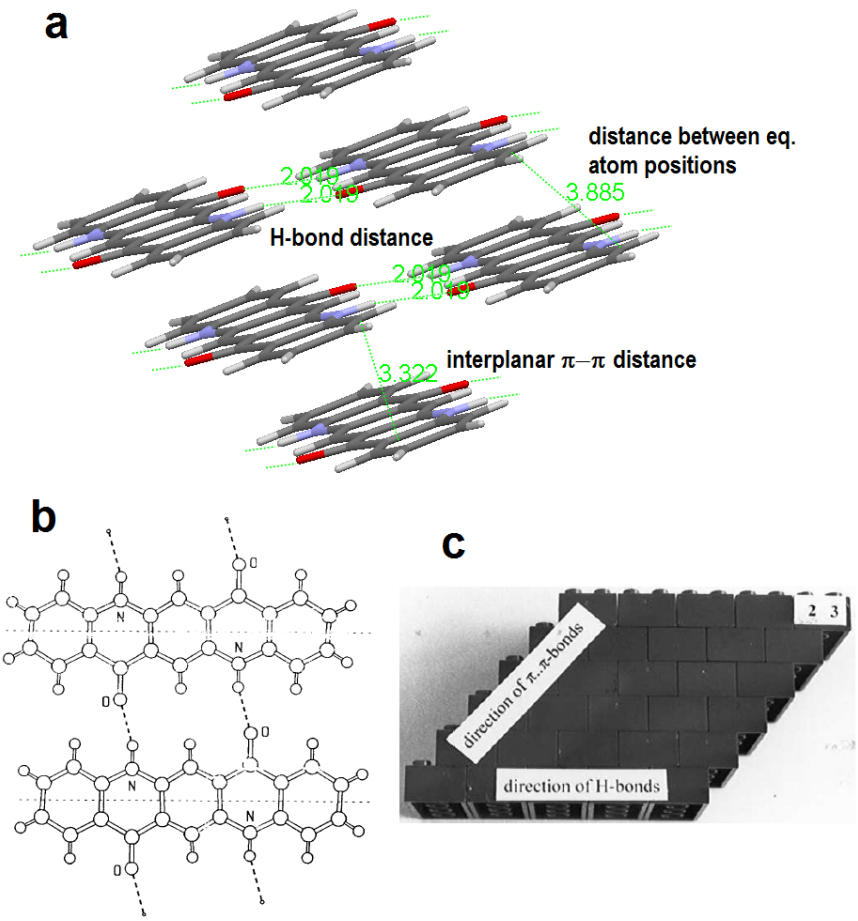


**Figure 37: Quinacridone crystal structure b-phase. a, distances between π-stacking and hydrogen bonding; b, hydrogen bond direction between individual molecules ; c, simplified depiction of crystal structure using bricks.[13]**

### 3.2.2 Inverse approach: Direct streptavidin modification on top of HB-OSCs

So far, non-specific binding was a huge problem. It was not only happening on all kinds of surfaces used so far, but was also an issue to remove. Washing techniques failed for being too mild in general or too harsh for the biotin-organic-semiconductor bond. So instead of trying to overcome this, which seemed a fight lost, issue was approached from the other way around- attaching streptavidin, without any quantum dot, to the surface of the organic semiconductor layer by non-covalent interactions and detect its presence by a fluorescently-labelled biotin.

That this would work was supported by older results from the time when washing procedures with 18 MΩ water were used. Back then, following the quantum dot manufacturer's incubation protocol to the letter, BSA was added to the Qdot-streptavidin conjugate containing solution. Instead of obtaining the minimalization of non-specifically bound streptavidin, results shown in figure 38 was obtained.

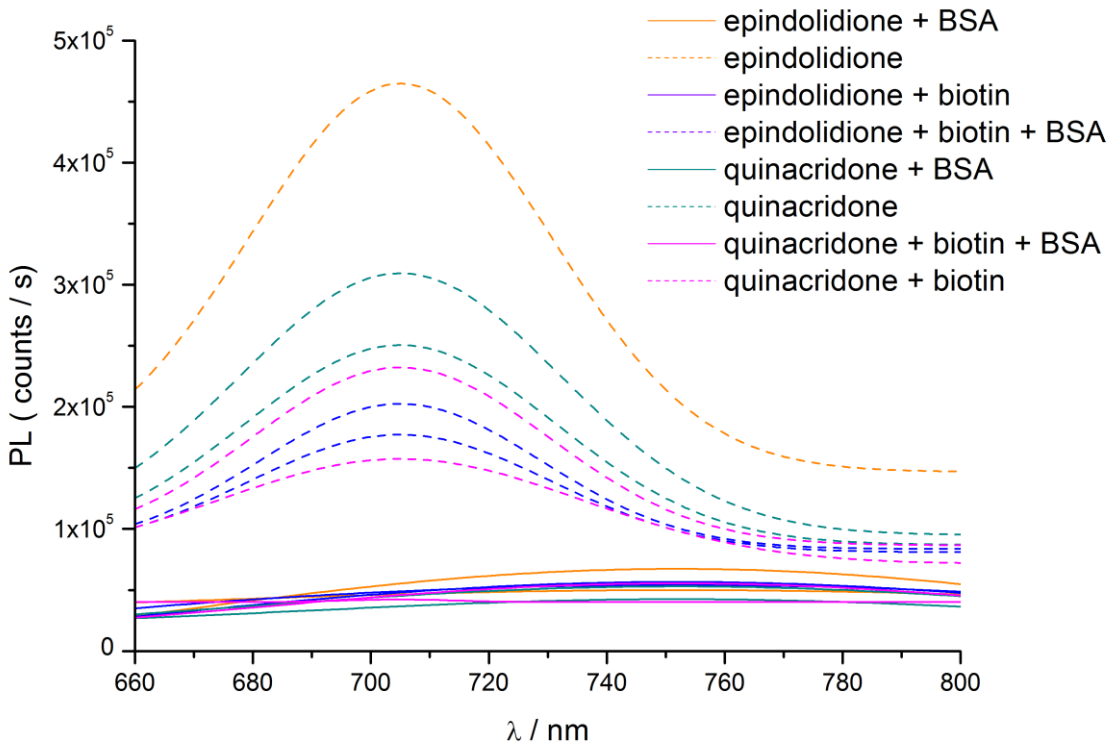


**Figure 38: Photoluminescence of quantum dots incubated with and without BSA on top of hydrogen-bonded pigments. Excitation wavelength was chosen to be 630 nm. The dashed lines refer to incubation without, solid lines to incubation with BSA. The long rise with a maximum around 760 nm is supposedly related to weak fluorescence emerging from the pigments.**

Figure 38 suggests two things: First, the protocol without any BSA is in our case the better one, second, BSA perfectly sticks to all the surface area available and sterically hinders the fluorescent probe to bind itself. Thus, we can speak of biofouling of the surface- and that was exactly what we wanted in this case.

Washing procedures were executed using PBS- the washing steps correspond to 10, 15, and 20 s, in chronologic order.

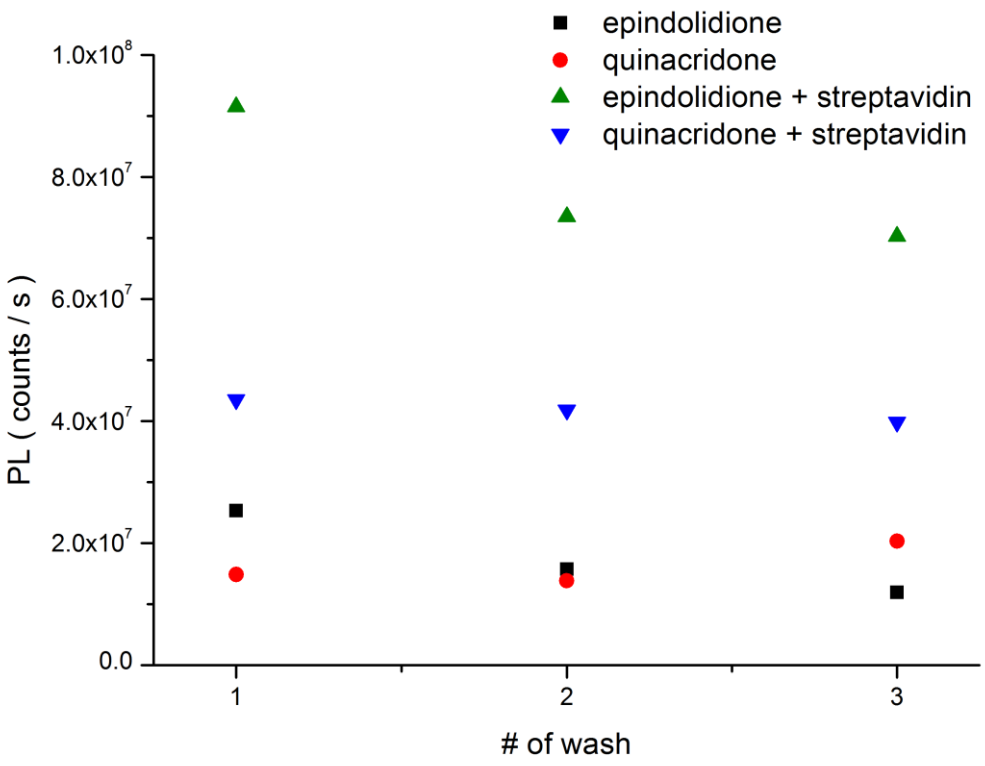


**Figure 39: Integral averages (678-720 nm) of ATTO 665 emission on epindolidione and quinacridone. Presence of streptavidin was a clear advantage for dye retention and was according to expectations. The excitation wavelength was fixed to 663 nm and an appropriate long pass filter was used.**

As seen in figure 39, conditions for washing away the dye were relatively mild, none the less present, which can be especially well seen in samples of streptavidin on epindolidione. Non-modified surfaces do show about 2-5 times less emission as compared to biofunctionalized samples- residual fluorescence can be explained by non-specific binding of dye molecules bound on the relatively large surface area. This binding, be it by the biotins ring-system forming hydrogen bonds or by the dye molecule itself, was so strong, that washing procedures with PBS were not sufficient for its total removal.

### 3.2.3 Energy transfer between hydrogen-bonded organic semiconductor and Qdot

Now, that the question of binding was addressed, the question of optical interaction remained. In principle, as pigments interact with light just as the quantum dot bound to it, it was of interest to investigate if there was any interaction between those two.

For this reason, samples as described before were prepared where the hydrogen bonded organic semiconductor was indigo. Compared to epindolidione and quinacridone, indigo is a much smaller molecule forming intra-molecular hydrogen bonds, instead of inter-molecular ones. Further, no strong fluorescent emission is known. Since it was not the aim to observe binding but rather to compare emission, neither biotin nor any washing were involved in the preparation- the conjugate containing solution was left to dry on the surface.

First, the ready samples were subject to UV-vis spectroscopy in order to measure their light absorption, as shown in figure 40.

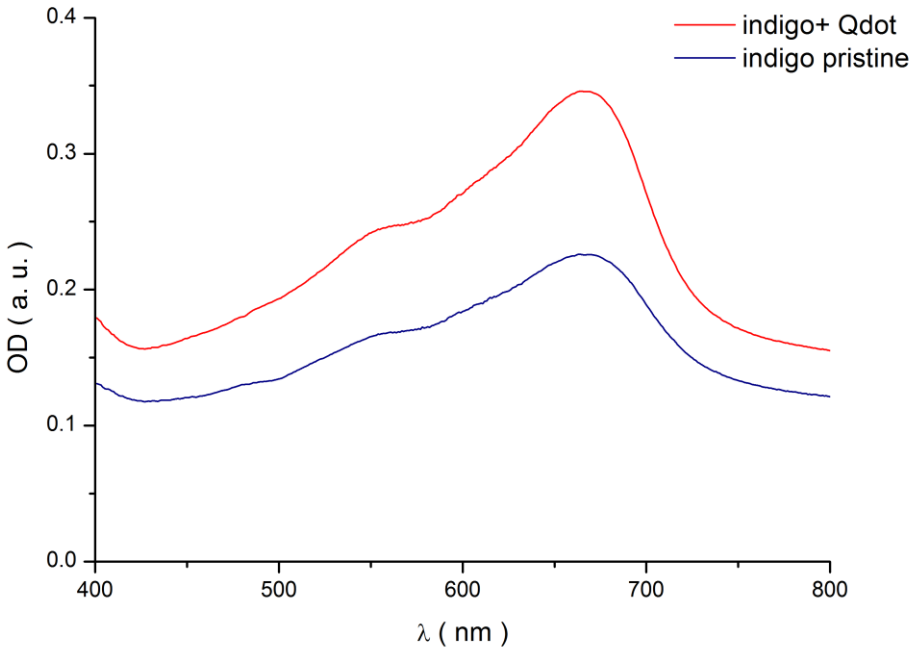


**Figure 40: UV-vis absorption spectrum of indigo film with and without quantum dot.**

From the spectrum it is clear, that there truly were enough quantum dots on the in order to increase absorption of the sample. Also, there was not only a general increase, which might be argued to be due to a bad baseline- the increase in fact corresponds to an overlap with the

quantum dots spectrum as the absorption increases towards the blue (the Qdot's spectra can be seen in figure 10).

Now, that a true difference in absorption was seen and thereby the presence of quantum dot on the sample was proven, fluorescent spectra were measured as shown in figure 41.

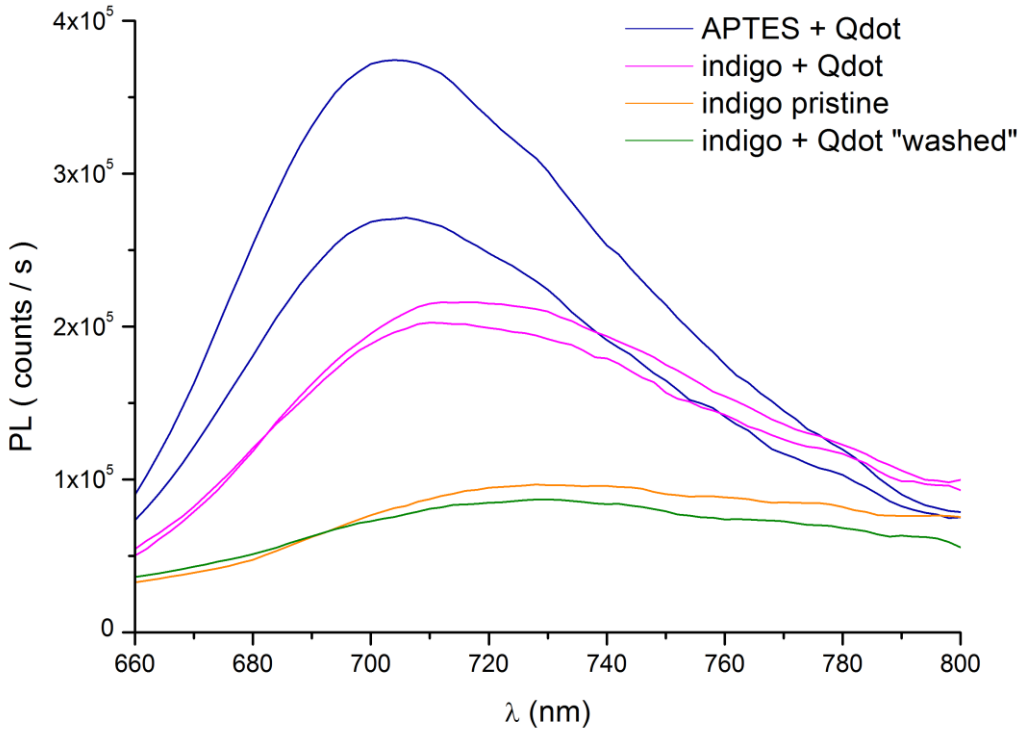


**Figure 41: Emission spectra of indigo (80 nm) surfaces with and without quantum dot compared to APTES with quantum dot. Excitation wavelength was chosen 630 nm such that both, organic semiconductorand quantum dot were excited.**

The logic behind this experiment was quite simple. If both, indigo with and without protein-conjugate were emitting equally, there was no energy transfer between both molecules, otherwise there was.

As it can be seen from the spectrum, there indeed was less fluorescence and a slight bathochromic shift on indigo as compared to APTES. This emission was even less for pristine indigo, as expected. A 2-fold decrease in emission might be considered to be very small; however, one has to take into account, that drying the solution left a macroscopic film behind, visibly thicker than the organic semiconductor layer. This film contains a variety of salts and organic molecules were previously part of the solution and now form a barrier between Qdots and the pigment, the distance making energy transfer impossible.

As a last thing, I would like to point out, that after washing the samples a total removal of the quantum dots off the indigo was possible. This is somehow surprising since this wasn't the case even for tetracene- and pentacene-covered surfaces but might be explained by the strong intra-molecular hydrogen-bonding, keeping each molecule to itself rather than strongly interacting with its surroundings.

# 4. Conclusion

A series washing experiments with many variations was carried out in order to establish a viable washing technique for the of biotinylated hydrogen-bonded pigments epindolidione and quinacridone. Contrary to expectations, statistical analysis of this, washing with 18 MΩ water did not lead to results hoped for and non-specifically bound streptavidin still remained the prevalent feature. In the course of these experiments it was realized that although being highly pure, 18 MΩ water is not suited for washing procedures as ambient $CO_2$ will strongly affect the solutions pH. This in turn, will affect covalent bond between the biotin and organic semiconductor as it proved to be very instable towards hydrolysis.

For this reason, washing techniques using pH 7.4 PBS were tried out- the acidity of this solution being ideal for the protein to stay in its native, functional form. In that case, the biotin bond towards the organic semiconductor remained stable, but so did the non-specifically bound streptavidin. This is an issue and washing procedures should be repeated in future using a buffer of lower pH in order to achieve a compromise between hydrolysis of the chemical bond and washing efficiency.

The washing procedures were also applied for APTES-covered samples, where the biotin bond proved to be more stable and less non-specific binding was observed in the case of washing with 18 MΩ water. Also in this case, washing with PBS proved to be less efficient.

Further, a comparison of epindolidione and quinacridone to tetracene and pentacene was carried out. Biotinylation of hydrogen-bonded pigments helped only slightly to improve binding and the bond soon succumbed to hydrolysis. Again, non-specific binding of similar scale was observed in all the species which has to be put down to the surface topography- this might turn out to be an important finding, as in the case of tetracene and pentacene no hydrogen bonds can be formed but still a similar intensity of photoluminescence was found. This indicates that the proteins interaction with the surface is mainly due to van-der-Waals forces, rather than due to hydrogen bonds. It would be of interest to investigate this binding behaviour with proteins different in size and biological function.

By serendipity, a fluorescent polymorph of epindolidione and quinacridone was discovered. Recrystallization of the pigments by sonication in 18 MΩ water at room temperature turned out to be possible and also helped to wash away any bound protein- the growth of fluorescence, however, caused troubles for the main focus of this investigation as it appeared within the measurement window of the experiment. The recrystallization of these pigments

was not further investigated as it would have been beyond scope and bearings of this thesis and will be subject of further research.

Attempting an introduction of a linker between biotin and the organic semiconductor turned out to be more troublesome than expected. The solubility of both pigments in organic solvents turned out to be a huge problem, especially for the already modified molecules. Further, using 1,6-dibromohexane as a starting material for the linker, a deactivation of the surface was observed as compared to surfaces without any "linker". Altering this reaction using a shorter linker or an alkyl halide with two different halides attached to it to have different reactivities of both ends would be an option.

For this reaction, however, an appropriate solvent should be found that wouldn't dissolve the organic semiconductor during reaction. Exactly this dissolving of the semiconductor during synthesis might turn out to be a major caveat.

The chain length of said linker might prove also problematic for sensitivity of a future biodetector. Supposing implication of a transistor structure, the increased distance between the semiconductor and the molecule that is to be detected might turn out to be for the detectors sensitivity.

Using an inverse approach, non-labelled streptavidin was applied first to the organic semiconductor and was only then detected by the Atto665-biotin conjugate. Indeed, a good retention of the protein was observed and might be of use for further research of biodetectors as similar behaviour was observed for BSA. As both proteins are globular proteins that are at home in solution, it might be interesting to investigate whether the same would be possible for structural proteins or the extracellular part of membrane proteins. This might lead to interesting applications for *in-vitro* cell cultures.

At last, a photo-interaction study between indigo and the streptavidin-bound quantum dots was done. Even though only a relatively small decrease in emission was observed, it is believed that the intensity of residual photoluminescence is rather related to large distance between quantum dot and organic semiconductor rather due to inefficient interaction. Further, washing with water and PBS showed both very strong effect on the protein and it was possible to obtain pristine films by short washing times on the scale of seconds. Compared to epindolidione and quinacridone, the interaction turned out as being very weak and is thought to be related to intra-molecular hydrogen-bonds formed by indigo.´

The immobilization of biotin, however, turned out to be more problematic as the biotin-pigment amide bond appears to be weak against hydrolysis during washing procedures. On the other hand, this doesn't suggest that small molecules cannot be immobilized at all since this might be related to an intrinsic property of biotin. For that reason, different sets biosystems should be investigated to establish a deeper understanding of the underlying interactions.

Overall, a lot of knowledge and experience was obtained concerning bio-functionalization of hydrogen-bonded pigments and it seems possible that future application for biosensors is indeed plausible, especially for the detection of proteins by their non-specific immobilisation and identification using fluorescent probes.

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# 6. **Curriculum vitae**

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| 10/2012- now | Study of master curriculum „Joint Master Programme: Biological Chemistry“ at Johannes Kepler Universität (Linz, Austria) and University of South Bohemia (South Bohemia, Czech Republic) |
| 10/2009-6/2012 | Study of bachelor curriculum „Joint Master Programme: Biological Chemistry“ at Johannes Kepler Universität (Linz, Austria) and University of South Bohemia (South Bohemia, Czech Republic) |
| 10/2004-6/2008 | „Billinguales Oberstufenrealgymnasium Komensky“, Vienna (with emphasis on mathematics and descriptive geometry) |

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| | |
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| 9/2014-now | Master student at Linz Institute of Organic Solar Cells |
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| 7/2013-8/2013 | Linz Institute of Organic Solar Cells |
| 9/2012-1/2013 | Internship at the Institute of Nanobiology and structural biology (Czech academy of sciences) and at the Institute of Physical Chemistry (Czech academy of sciences) |
| 9/2011 | Bachelorthesis ($2^{nd}$ thesis) at Institute of Inorganic Chemistry (Center for Nanobionics and Photochemical Sciences) |
| 8/2011 | Internship a the "Austrian Agency for Health and Food Safety (AGES)" (Center of Competence: Biochemistry) |
| 5/2010-7/2011 | Bachelorthesis at the Laboratory of Nematology, Institute of Parasitology (Czech academy of sciences) |
| 12/2010-4/2011 | Internship at the Laboratory of Nematology, Institute of Parasitology (Czech academy of sciences) |
| 8/2010 | Internship a " Austrian Agency for Health and Food Safety (AGES)" (Institute of Plant Variety) |
| 10/2008- 3/2009 | Compulsory basic military service including posting to the Austrian-Slowakian border (medic- training completed) |
| 10/2006-8/2008 | Journalist and moderator at the Austrian Broadcasting Corporation (ORF) Radio 1476 (for the weekly broadcasting of the Czech minority programme "Rádio Zvídavý Mikrofon") |
| 9/2008 | Internship at "Karasek Wietrzyk Rechtsanwälte" (law firm with focus on business law) |
| 8/2007 | Internship at "Karasek Wietrzyk Rechtsanwälte" (law firm with focus on business law) |

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|---|---|
| 8/2006 | Internship at “Karasek Wietrzyk Rechtsanwälte“ (law firm with focus on business law) |

## LANGUAGES

| | |
|---|---|
| German | (mother tongue) |
| Czech | mother tongue) |
| English | (fluent in verbal and written communication; work and study language) |
| French | (A2; 3 years at school and several stays at France and Belgium) |
| Russian | (A1/A2, refreshed at a course at JKU) |
| Slowak | passive knowledge |
| Japanese | (A1, including scripts Hiragana and Katakana) |

## ADDITIONAL SKILLS

Driving licence (B)

Passive knowledge of programming languages Java and Perl

Social skill due to army service as a medic, journalistic work, and several internships

## SCIENTIFIC CONTRIBUTIONS

Poster presentation at BioEl 2014 International Winterschool on Bioelectronics – Kirchberg in Tirol, Austria, February 22$^{nd}$ – March 1$^{st}$ 2014

Poster presentation at BioEl 2015 International Winterschool on Bioelectronics – Kirchberg in Tirol, Austria, February 28$^{th}$ – March 7th 2014

Bioconjugation of hydrogen-bonded organic semiconductors (submitted)

Farka D, Scharber M, Głowacki ED, Sariciftci NS; Reversible Photochemical Isomerization of *N*,*N*’-di(*t*-butoxycarbonyl)indigos; (submitted)