Intrinsically functionalized Silk (Bombyx Mori)

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Intrinsically functionalized Silk (*Bombyx Mori*)

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Abbreviations

EDOT: 3,4-ethylene dioxythiophene
EDOT-S: Alkoxysulfonate (3,4-ethylene dioxythiophene)
PEDOT: Poly(3,4-ethylene dioxythiophene)
PEDOT-S: Alkoxysulfonate poly(3,4-ethylene dioxythiophene)
PEDOT:PSS: Poly(3,4-ethylene dioxythiophene):poly(-stryrenesulphonic acid)
PTAA: Poly(3-thiophene acetic acid)
POWT: Poly(3[(S)-5-amino-5-carboxyl-3-oxapentyl]-2,5-thiophene ydrochloride)
6T: Sexithiophene.
ThT: Thioflavin T
TQ1: 2,3-bis-(3-octyloxyphenyl)quinoxaline-5,8-dyl-alt-thiopene-2,5-diyl
TQ1P: Poly(2,3-bis-(3-octyloxyphenyl)quinoxaline-5,8-dyl-alt-thiopene-2,5-diyl))
PL: Photoluminescence
RhB: Rhodamine B
NR: Nile Red
RH: Relative Humidity
Abstract

The goal of the thesis is to incorporate materials with either fluorescent or conductive properties in silk fibers, by feeding silkworms with a diet containing these materials. To achieve this, one would have to breed (rear) silkworms from eggs into larvae, then to feed the silkworms with this special diet containing fluorescent or conductive materials. Samples of silk were then collected either from spun cocoons or via removing the silk producing organs (silk glands) from the silkworms via dissection. The samples were then analyzed with absorbance spectrometer, spectrofluorometer or via photoluminescent measurement to determine if any materials had been incorporated into the silk fibers.

Silkworms were successfully reared from eggs up to moths, once the silkworm larvae had grown enough in size their diet were switches from their regular food (silkworm chow) to food containing conjugated molecules or polymers with fluorescent or conductive properties. A total of 14 materials were tested. One material gave a clear positive result and that was from the fluorescent compound Rhodamine B. Other fluorescent materials, Nile red and POWT yielded some results indicating their presence in the silk but the results were not conclusive. The rest of the materials all failed with being incorporated within the silk fibers; this was due to their lethality, size, lack of zwitterionic properties and such. The properties of the materials are of great importance for the uptake process, where a small zwitterionic molecule has a great change of being taken up and incorporated in the silk fibers. Whereas a big materials such as a polymer without any zwitterionic will in most cases just follow through the food in the digestive track without any uptake.
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Introduction

Goal
The goal of the master thesis is to investigate the possibility to intrinsically incorporate fluorescent or conductive materials into the silk fibers spun by silkworms (Bombyx Mori), by feeding the silkworms a modified diet containing fluorescent or conductive materials. The method of feeding silkworms with the goal of incorporating materials into the silk which the produce is a new research field with just a handful of studies. The thesis is therefore like a pilot-study to investigate the possibility of incorporating material into silk via feeding. The regular method of incorporating materials to silk fibers is coating, this can be done by submerging silk fibers in a solution of a material. The materials will with the help of either acid or heat treatment be able to bind to the silk fibers e.g. via electrostatic forces. One can also coat silk by grinding together freeze dried silk powder with a material in powder form. Both of these other two methods (submerging and grinding) have been investigated and conducted during the thesis as controls, the main method of incorporating material into the silk in the thesis have been by feeding silkworms a modified diet. To achieve this goal silkworms are reared and fed modified food containing either conductive or fluorescent materials. If proved possible it would be a greener alternative compared to regular functionalization of silk. Further improvement due to this is that you would get rid of the otherwise necessary after works which otherwise are needed to coat the silk fibers, with this also comes a commercial aspect which may lead to a more cost effective approach. Since silk is a biocompatible material it can be used for biomedical applications, to be able to produce conductive silk threads in an easy way could open up its use as a conductive wire for biomedical equipment. The second reason is to study the uptake process for incorporation, both which parameters are necessary for the materials but also how the uptake process inside the silkworm works.
Background

Silk

The most common type of silk that is used by humans comes from the silkworm *Bombyx mori* (see Figure 2). *Bombyx mori* is a domesticated silkworm and its silk fibers have very good mechanical properties, biocompatibility, low biodegradability and produces low inflammatory reaction, the silk can be seen in Figure 1. The silk has been used as sutures, base for ointment and other biomedical applications. [1]

Silk is produced by 30 000 species of spiders and by almost all species of the insect order *Lepidoptera* (roughly 113 000) which includes butterflies and moths. [2]

Silk is composed of the protein sericin acting as a protective outer layer and the protein fibroin which consists of both ordered β-sheet crystals and disordered regions (see figure 3). [3] The silk is produced in the silk gland in the silkworm, and the composition of fibroin and sericin is that of 75 wt% to 25 wt%. Fibroin consists mainly of repetitive amino acid sequences and fibroin’s structure consist of 56±5% β-folded and 13±5% α-helix structures. [4] The amino acid sequences consist of alternating hydrophilic and hydrophobic peptide sequences which allows the silk fiber to form gels or micelles via self-assembly in concentrated solutions. The hydrophobic and hydrophilic peptide sequences consist of both heavy and light polypeptides chains, with a weight of 390 kDa respectively 26kDa. The two chains are linked by disulfide bonds at the C-terminus. The fibroin protein consist mainly of three amino acids, glycine, alanine and serine in a ratio of 3:2:1, with a dominating repeat of [Gly-Ala-Fly-Ala-Gly-Ser]n blocks. These three amino acids makes up around 87% of the total weight and the remaining weight is from more bulky or polar amino acids such as, tyrosine, valine, and the acidic amino acids. [5] There exist three types of silk from the *Bombyx mori* called type I, II and III, type I is the silk solution present in the silk gland, silk II is the spun silk which has a crystalline structure of fibroin. Finally silk III exist as a crystalline form at the interface between air and water. [1] The silk from *Bombyx mori* has also been studied for the use as scaffolds for cell culturing, tissue engineering, controlled drug release, but also as biocompatible electrodes or glue. [1][7]
Silkworm rearing

Rearing
Silkworm is an insect that repeats a four-stage cycle of transformation in its life, egg-larva-pupa-moth. The moths mates and lays eggs which then closes the circle (see figure 4). Silkworms who goes through this life-cycle one time per year is called univoltine, if they go through it two times a year they are called bivoltine and any number above that is called polyvoltine. These life-cycle conditions can be affected by changing environmental parameter during egg or larval stages.

Egg hatching (incubation)
When incubating the egg (see Figure 5), the room should be illuminated roughly 16 hours á day; the humidity should be around 90% for an optimal hatching ratio, and if the humidity drops below 85% during the last stages of hatching it will increase the risk for unsuccessful hatching and death of the larvae. Temperature is another very important parameter, and excessive fluctuations will disturb the silkworm’s growth, and the temperature should be uniformly distributed. The necessary temperature for hatching is around 23-25 °C. If some eggs have developed faster than others a so called “dark incubation” method can be used during the blue-headed stage of the incubation. This will help to give a more uniform hatching of the eggs. See Figure 6 for a picture of the hatchings.
**Larvae**

The optimal temperatures and relative humidity (RH) for rearing is 26-28 °C, 90% RH for the first two instars, for the third instar 24-26 °C and 80% RH is preferred. During the fourth and fifth instar the silkworms should be reared at 20-26 °C and the humidity should be less than 75% but still above 50%. [8] See figure 7 for the rearing setup used.

As the silkworm grows in size so must their living space since they will not be able to eat probably if they live too closely packed. It is therefore necessary either to move them to a larger container or to separate them into more containers.

Silkworms eat a huge amount of food, but the only food that can sustain them is mulberry leaves. Usually you have to feed the silkworm three times a day, and the amount of the food must be suited after the size of the silkworms. The amount of food necessary to feed the silkworm from instar 1-3 is around 5% of the total volume, the fourth instar 10% and finally the fifth instar requires an astonishing 85% of the total volume of food given to the silkworm during their entire life cycle. [8]

Silkworm larvae only goal in life is to eat and grow in size, this constantly eating causes much spilling or so called “frass” (see Figure 8). The frass and left over food must be cleaned out from time to time to avoid mold related diseases. The cleaning procedure is quite simple, first you put a mesh onto top of the silkworms, then you spread food over the mesh and the silkworms will crawl up and start feeding. The mesh with the silkworms on top of it can then be lifted to another rearing space and the previous one can be cleaned. As the silkworm grow in size, different sizes of holes in the mesh will be necessary, for the first three instar meshes with 0.5 cm to 1.0 cm holes can be used after that you will need bigger holes around 3-5 cm. If the environmental conditions in the rearing space are within acceptable levels you usually only have to clean up to one time for the first instar worms, two times for the second instar and three times for the third instar. [8]

The silkworms will shed its skin four times during it life cycle since it has outgrown its previous skin, the shedding takes place between the five instars. And during the shedding the silkworms have to be kept dry and preferably not be exposed to bright light. The shedding will take up to 14 to 20 hours for the first three instars,
for instar 4 and 5 it takes 30 hours. The silkworm will be in a so called praying position prior to its shedding (see figure 9) It is important to feed the silkworm both prior and after the shedding, and if there exist inhomogeneous growth this can be mended by separating the shedding silkworms from the ones that are not shedding by using the same techniques as when cleaning or moving them one by one. With the help of meshes it is easy to separate shedding silkworms to those who are not. To get a homogenous growth it is important to wait for all the silkworm to shed their skin before feeding.[8]

After the silkworms have completed the fifth instar they will start to spin cocoons (see figure 10), and if you want cocoons of good quality it is necessary to provide the silkworms with a suitable place for spinning their cocoons, a specially made frame or box is usually for this. These kinds of boxes or frames can be made of a large range of materials such as wood, cardboard, plastic, etc. The mounting can be done in two ways, the first being that you manually lift the silkworms and place them in a mounting frame and the other one being that the mounting frame is placed on the rearing box/plate. Roughly 12 hours after mounting the silkworms will have spun a thin cocoon shape see Figure 10. Before the silkworms start to spin their cocoons fully they will discharge their faeces and urine.[8]

It takes another 60 hours until the silkworms have fully spun their cocoons see Figure 11 and four to six days later you can start to gather the cocoons since now the pupae have hardened enough. If gathered earlier there is a risk that you injure the pupae and it will bleed all over the cocoon. [8]

**Uptake of dyes**

In the wild, silkworms are feeding on mulberry leaves which contain carotenoid. The carotenoid pigments will after uptake be accumulated in the silk glands. But first the pigments are absorbed in the epithelia of the guts, transported to the hemolymph before they finally wind up in the silk gland. Due to this, wild silkworms produces cocoons with a yellow color but also the hemolymph of the silkworm will turn yellow. Even if the silk will look yellow most of the carotenoid pigments are just absorbed into the sericin and not in the fibroin fibers. Because of this the silk industry uses so called degumming solution to break down the sericin and instead collect the fibroin fibers which are white in color. Since the silkworms have been in sericulture for over 4000 years they have developed into producing white silk cocoons. An example for a
degumming solution is 5g/L anhydrous sodium carbonate, the solution is heated to 100 °C and the cocoon is immersed in this solution two times for one hour each. Before degumming the cocoon should be cut so that the pupa inside can be removed. After the degumming the silk is washed with water and then dried. After this procedure the only thing left from the silk is the fibrion fibers.[10] The silkworms used in the silk industry lack working mRNA for the carotenoid binding protein resulting in failure of transport to the hemolymph. [3]

Tansil N.C. et al has showed that Rhodamine B, 101, 116 and 110 could be intrinsically incorporated into the silk fibers of silkworms, mainly fibroin. This was done by mixing regular food, mulberry powder with luminescent substances at 0.05wt%. The silkworms were fed with this food three days after the fifth instar and they showed a color change in less than an hour. At 10 days after the fifth instar the silkworms began to spun cocoons, in the case of Rhodamine B a very bright pink colored cocoon were produced, see figure 12. The Rhodamines cocoons showed fluorescent light when lit under UV-light, see figure 12. Not just the cocoons were fluorescent under UV-light but so were the silkworms themself when feed with modified food. This was because of that the Rhodamine did also aggregate in the hemolymph and not just in the silk glands, causing the body of the silkworm to be colored and fluorescence. Confocal microscopy reveled that Rhodamine B was mainly incorporated within the fibroin fibers and not the sericin. After removing the sericin by degumming procedure the remaining fibroin fibers were still colored and luminescent. This is an important aspect since the fibroin is the commercial part of the silk.[9] The four rhodamine types that could be incorporated intrinsically had in common that they all had both carboxylic and amino groups making them zwitterionic. Due to this the dyes could be more easily taken up by the silkworm. The silkworms gut is fairly alkaline with a pH of 9.4-9.8 in the midgut and around 8.4 in the hindgut (see figure 13 for anatomical picture). This high pH will cause the carboxylic acid (COOH) to release its proton and instead it changes into its negatively charged form (COO⁻). It is hypothesized that the zwitterionic properties are of great importance for the materials transport out from the gut into the silkglands. When the dye reaches the hemolymph which has an acidic pH of 6.3-6.5 the amine groups in the dye will become positively charged due to the increased amount of hydrogens in the hemolymph. The hemolymph is an open circulatory system, which surrounds all the tissue and organs. The hemolymph acts as a transport for nutrient and oxygen, working as blood in humans. The hemolymph can also be used as a depository for nutrient and energy, and it will increase after eating and decreases during starvation. It also helps with the innate immune response.[11] This changes between negatively and positively charged side groups increased the effectiveness of the absorption and excretion of the dyes, which in turn gives them the possibility to be transferred between
different tissues inside the silkworm. Dyes that only contain amine groups and no carboxylic acid groups do not seem to be able to integrate with the silk, and they also show an increased toxicity compared to molecules with both amine and carboxylic acids groups. [12]

**Coating of Silk fibers**

Poly(3,4-ethylenedioxythiophene) poly(styrenesulfonate) PEDOT:PSS is an electro conductive, hydrophilic and biocompatible material. It has been reported that soaking silk fibers in PEDOT:PSS resulted in black/blue fibers with conductivity up to 0.00117 S/cm. By adding glycerol to the already soaked silk fibers the conductivity could be increased up to 0.102 S/cm. The PEDOT:PSS was mostly surrounding the silk fibers and did not thoroughly stain the fibers. This could be demonstrated by soaking the PEDOT:PSS stained fibers in water for three day, which showed that the PEDOT:PSS came off the silk fibers. The study did also show that by incorporating glycerol to the PEDOT:PSS the fibers could be immersed in water for three days without losing the PEDOT:PSS from the fibers. Glycerol helps with the wettability and increases the water resistance helping the PEDOT:PSS to stay attached to the silk fibers. [7] Another study done by Xia Y. and Lu Y. showed a conductivity of 0.0049-0.0052 S/cm for PEDOT incorporated into silk fibroin. The PEDOT silk fibroin fibers where dark blue in color, and FTIR experiment indicated an increase in Sulfur content compared to pure silk fibroin fibers. This indicates that PEDOT has been incorporated with the fibroin fibers. They also claimed that washing the fibers 20 times only resulted in a conductivity loss of 10±2%, which is a different results compared to Tsukada S. et al study with PEDOT:PSS. [10] PEDOT-S had been successfully coated on recombinant spider silk protein (4RepCT) but also on silk from *Bombyx mori*. This was done by submerging the silk in a 90°C water solution with a conc. of 10 g/L PEDOT-S and a pH of around 2 (see figure 14 for PEDOT-S coated fibroin). The silk fiber could be coated in just 1 hour at this concentration, but at a higher pH e.g. pH 7 and ambient temperature the staining could take one to two weeks. The yielded fiber at this conditions has a dark blue color, but with a pH of 13 both the silk and the PEDOT-S would be negatively charged and if you incubated a PEDOT-S coated silk in a solution of pH 13 it will start to lose it color, due to electrostatic repulsion. There could be some other force other than electrostatic attractions that is in work when PEDOT-S is coated to silk fibers, since at pH 11 it was still possible to coat silk with PEDOT-S even if both substances should be negatively charged and repulse each other. PTAA could also be used for staining the silk fibers, and it showed similar properties as PEDOT-S. PTAA needed to be dissolved in a solution with a pH above 6 due its acetic acids pKₐ value of 4.8. [13]

**Dissolving Silk**

Silk can only be dissolved in certain types of solvents due to its large amount of hydrogen bond both intra- and intermolecular, but also because of its high crystallinity and other physiochemical properties. The hydrogen bonds help to stabilize the secondary structure of fibroin by bonding the functional groups of the peptide’s macrochain with side fragments of macromolecules. Due to that
fibroin consists of a large amount (3/4) of hydrophobic amino acids making it fairly hydrophobic and resistant against most solvents. Fibroin is insoluble in water and most organic solvents, instead of dissolving it will swell up to 30-40% of its original volume. Fibroin will dissolve in concentrated aqueous solutions of acids, and various salt solutions (organic, aqueous and aqueousorganic). [4]

Chaotropic Salts
Chaotropic salts such as CaCl₂ and LiBr has the ability to dissolve silk fibroin. To get rid of the salts the dissolved silk solution needs to be dialyzed for several days. How good the chaotropic salts will be able to dissolve fibroin depends on the concentration of the salt, but different anions and cations will have better solvency. The solvency for anions in the chaotropic salts increases in the following order, sulfate < citrate < tartrate < acetate < chloride < nitrite < bromide < iodide < thiocyanate < dichloroacetate. For cations the list looks like Ca²⁺ < Sr²⁺ < Ba²⁺ < Li⁺ < Zn²⁺. A very important aspect of the anion in the dissolving agent is its nucleophile strength. These electrophilic centers in the fibroin are mainly represented as hydroxyl-, amide- or amino groups which then are centers where the anion makes it attack. And the dissolving principle for the chaotropic salts is that the ions in the solvent interact with fibroin’s functional groups. It is assumed that the anions will make a nucleophilic attack on the functional groups of fibroin causing the intra- and intermolecular hydrogen bonds to break.

Substances
Conjugated materials especially conjugated polymers are of great interest since their capability to increase the electrical conductivity via electrochemical doping. The conjugated molecules and polymers have interesting optical and semiconducting properties which have been studied both by the academic society and the industry, in the means of developing plastic electronics and photonics. The reason for developing these kinds of devices based on organic materials is that it can be done to a reduced cost, increased mechanical flexibility, optical transparency and so on compared to regular devices made from inorganic materials. [14]

PEDOT
Poly(3,4-ethylene dioxythiophene) (PEDOT) (see Figure 15) is a highly conductive conjugated polymer, which is transparent when as a thin film, it is also much more stable than other conducting polymers. Degradation starts at 150°C and continues to degrade continually until 390°C where it completely decomposes. [15]

PEDOT is electrical conductive since it has a low band gap of around 1.5-1.6eV. When doped PEDOT’s oscillatory strength can shift from 1.5eV to under 1eV in its metallic state which means that it will require 0.5 eV less energy for switching quantum states, in this doped state it can have an electrical conductivity of up to 550 S/cm. [15]

PEDOT is biocompatible and is pro-adhesive for vast types of cells such as neurons, glia, muscle cells etc. [16]
PEDOT has been shown to have fluorescence quenching properties, Zhang Y. et al study shows that PEDOT nanoparticles has a capability to quench up to 99% of the fluorescence emission of dyes consisting of fluorescein, rhodamine or cyanane. PEDOT:PSS poly(3,4-ethylene dioxythiophene):poly(-stryrenesulphonic acid) has also been showed to exhibit quenching of luminescent light from PPV(poly-(p-phenylenevinylene). The reason for this is that there will form so called defect states in the interface between PEDOT:PSS layer and PPV. As these defect states gets filled with charge carriers it will generate a photoluminescence quenching, but there will also exist a static quenching when defect states are present. [18]

**PEDOT-S**

Poly(4-(2,3-dihydrothieno[3,4-b]-[1.4]dioxin-2-yl-methoxy)-1-butanesulfonic acid) (PEDOT-S) (see figure 16) is a water soluble, self-doped and high electrically conductive polymer. Since PEDOT is biocompatible so is PEDOT-S.[13]

**Pyrrole**

Polypyrrole (see figure 17) is a conductive polymer with biocompatible properties. It has the ability to transduce analytical signals coming from redox enzymes making it useful for biosensors. It is although most commonly used in the field of electrochemical sensors. Polypyrrole has been shown to be able to quench fluorescence emission almost completely from e.g. Rhodamine B. [19]Polypyrrole is mostly synthesized either via chemical or electrochemical reactions. Chemical synthesis is done by mixing a monomer solution of pyrrole with a strong oxidizing agent such as FeCl₃, the oxidation will then help with the polymerization of pyrrole to polypyrrole yielding a blue colored solution. [20]

**Rhodamines**

Dyes consisting of Rhodamines has high absorption coefficient, broad fluorescence in the visible range, high fluorescence quantum yield and photostability which makes them very good fluorescent probes. These dyes are fluorophores and are a derivative of xanthene along with other fluorophores such as fluorescein. Among the most used types of Rhodamines are Rhodamine 101 and Rhodamine B (see figure 18). Both of these have interesting behavior in different pH, in acidic solution these two dyes will be in a cationic form since it is carboxylic
group will be protonated. In basic solution the dyes are instead a zwitterion because of dissociation. These conformation changes induce shifts in absorption and fluorescence maxima and also a decline of the extinction coefficient. [21]

**Nile Red**

Nile red (9-diethylamino-5H-benzophenoxazine-5-one) (see figure 19) is a fluorescent compound that is quenched in aqueous environments but it will show an augmented fluorescence in hydrophobic environment. Nile red is an uncharged molecule meaning that it is more easily dissolved in hydrophobic- or organic solvents but also in lipids. Due to its uncharged state it is quite insoluble in water, it fluorescence is depending on the hydrophobicity of the environment and will increase with it. Therefore Nile red can be used as a hydrophobic probe.[23] The emission will be blue shifted when amyloid fibrils are present. Nile red will emit at 650nm in free solutions when excited at 530nm, in the present of e.g. lysosome fibrils the emission peak sits at 625nm, a blue shift of 25nm. The degree of the blue shift will depend on what type of fibril is present suggesting that the tertiary structure is of importance, insulin fibers will give a Nile red emission of 638nm, TTR (transthyretin) fibrils 616nm. [24]

**PTAA**

PTAA(see figure 20) is an anionic polythiophene derivative that can bind to amyloid fibrils. In a neutral buffer solution the carboxylic acids groups of PTAA will cause electrostatic repulsion between each other stretching out the backbone of the polymer to a more planar conformation. In neutral buffer solution the absorption peak for PTAA will be at 446nm, once it binds to e.g. native bovin insulin fibers which have β-sheet rich structure, a blue shift of the absorption peak will occur shifting it to 434nm. This is due to that the interactions between PTAA and insulin will cause the PTAA backbone to change into a more nonplanar conformation. This blue shift can also be seen for PTAA in dionized water and in interactions with positively charged peptides. This blue shift is also present in the emission where in buffer solution the emission peak sits at 556nm. Once the PTAA binds to insulin it will blue-shift the emission to 550nm with an increased intensity.[25]
6T
6T (see figure 21) is an oligomer consisting of six thiophene units. It has been studied for the use in thin film transistors, organic photovoltaic cells and also in organic light emitting diodes.[26] 6T has two emission peaks at 510 and 550nm when excited at 390nm, 6T emits polarized light strongly which can help to determine if 6T are aligned along a specific axis e.g. around a polymer/film.[27]

POWT
POWT (Poly(3-[s]-5-amino-5-carboxyl-3-oxapentyl]-2,5-thiophenylene hydrochloride) (see figure 22) is a thiophene derivative which has fluorescence properties. The fluorescence spectra from POWT depends on if it is in a free solution or if it is bound to a biomolecule such as single-stranded or double-stranded DNA. The shift in emission is thought to be because of conformal changes in the thiophene backbone of POWT switching from a planar into a nonplanar conformation or vice versa. When the positive amine group of POWT binds electrostatically with for example the positive backbone of DNA it will cause a change in conformation of POWT to more planar which in return results in a red shift due to increased conjugation length and possible aggregation with neighboring chains. In solution POWT has two emission peaks at 540nm and 670nm.[28]

Thioflavin T
Thioflavin T (figure 23) has an absorption peak at 412nm and fluorescence emission at 487nm when excited at its absorptions wavelength 412nm. Thioflavin T has the ability to bind to the β-sheet rich structure of amyloid fibrils [29] which are correlated to many diseases like Alzheimer and Parkinson to name a few, when Thioflavin T binds to the amyloid fibers it absorption peak will be red shifted by 30nm to 442nm and it will then give a strong emission at 478nm when excited at its new absorption peak at 442nm.[30] Thioflavin T can also bind to non-amyloid amorphous β-aggregates and native helical proteins. Thioflavin T has been shown to be able to increase the lifespan, reduced age-specific mortalities, slowed age-related decline in movement and improved health of *Caenorhabditis elegans*, a transparent nematode (roundworm). These properties were however possible with a dose of up to 500µM and at higher doses Thioflavin T becomes toxic and even lethal. [34]
TQ1
TQ1 (see Figure 24) is an easily synthesized polymer that is used as an electron donor for solar cell applications because of its deep HOMO (highest occupied molecular orbital) and low bandgap. It is usually paired with PCBM (Phenyl-C61— butyric acid methyl ester) which is a fullerene derivative and acting as an electron acceptor.[31]

Figure 24 Chemical structure of TQ1. [31]
Method

Silkworm rearing
The silkworm eggs and silkworm chow used was supplied by Ricks Livefood.

Rearing equipment
The environment used to breed the silkworms in was set up as seen in Figure 25 and 26. The rearing takes place in two rearing boxes with a Humidity box for controlling the humidity in both rearing boxes, all boxes is made out of Styrofoam. Rearing box 1 is used to breed and grow silkworm from eggs up to instar 4 larvae, once the silkworms morph into instar 4 they are moved to rearing box 2 instead. The temperature and humidity in rearing box 1 should be 25-28°C respectively 80-95% RH, for rearing box 2 22-25°C respectively 55-75% RH according to the supplier. To achieve this, the IR-lamp (50W EXO TERRA) is directed so that it shines more onto rearing box 1 than 2. The humidity level in both rearing boxes is controlled by the humidity box. The humidity box has a flask filled with water henceforth referred to as the humidity flask, it sits in a water bath were the temperature is controlled with an immersion heater. The lid of the humidity flask has two hoses sticking in to it; one of them lead air into the water the other one leads the humid air to both rearing boxes. By controlling the temperature and the airflow a certain relative humidity can be achieved in the rearing boxes. The hoses leading the humid air into the rearing boxes are equipped with hose clips. By tightening the hose clips the air flow in the hose is constricted leading to less humid air going in into the rearing boxes. The humidity and temperature in the rearing boxes is also affected by how much of the top is covered by a lid. So this is another way to control the temperature and humidity by selecting the airflow out of the rearing boxes.

Figure 25 Silkworm rearing setup, side view
Temperature and humidity

As mentioned the temperature was controlled by the IR-lamp and the Relative Humidity was controlled by the airflow in and out from the humidity flask and also by the temperature of the immersion heater. The temperature for both rearing boxes can be seen in figure 27 and the relative humidity level in figure 28. The humidity was not as stable as the temperature, mostly because of that the humidity flask did run dry sometimes overnight but also that the airflow from the valve was not stable and could sometimes drop or raise with time. Even so the environmental conditions could usually be fixed quite easy so that it stayed on desired conditions at least during the day. The humidity for rearing box 1 was around 80% and for rearing box 2 around 60% as can be seen in figure 28. Rearing box 1 was used for instar 1 to 3 silkworms which prefer 80-95% RH and rearing box 2 was used for instar 4-5 silkworms which prefer 60-75%. The humidity fluctuated very much because there was problems with holding a constant air flow into the humidity flask. This caused so that the humidity sometimes where either too high or too low at the beginning of the day, but it the favorable conditions were met during the day with the help of some modifications to airflow in and out of the rearing boxes. The temperature did not vary as much but there was a constant decrease as the rearing boxes got bigger or new boxes where added. The temperature was controlled by moving the IR-lamp either closer or further away from the rearing boxes. With the introduction of rearing box number 2 the temperature became a little harder to control, rearing box 1 dropped some degrees in temperature but it was still possible to rear the remaining silkworm successfully, rearing box 2 on the other hand did manage to stay in favorable conditions with a variation of +/- one degree Celsius. When rearing box 1 was removed the temperature in rearing box 2 increased as can be seen but it still managed to stay within the favorable conditions.

In the beginning there were some problems with condensation and water build up therefore a drainage system was constructed by carving a hole in the side of the rearing box to allow the excess water to escape.
Figure 27. Temperature in the both rearing boxes. The goal temperature for rearing box 1 was 24-28°C for optimal rearing of instar 1-3 silkworms. The goal temperature for rearing box 2 was 20-26°C for optimal rearing of instar 4-5 silkworms. Rearing box 2 was not implemented until day 32.

Figure 28. Relative humidity in both rearing boxes. The goal humidity for rearing box 1 was 80-90% for optimal rearing of instar 1-3 silkworms. The goal humidity for rearing box 2 was 50-75% for optimal rearing of instar 4 and 5 silkworms. Rearing box 2 was implemented day 32.
Food preparation
The food used for the silkworm is so called silkworm chow. The preparation of the food was as followed:

1. 125 gram of silkworm chow powder (Ricks Livefood) (see figure 29) was added to 340ml of hot tap water.
2. This was then stirred with a kitchen knife until all the powder had dissolved in the bowl, and a uniform consistency had been achieved.
3. A plastic cling wrap was put over the bowl; the chow was then cooked in a microwave for 4-6 minutes until it started to boil.
4. Once the chow started to boil it was taken out of the microwave and stirred around with a kitchen knife. It was yet again put in the microwave but only for 1 minute.
5. Then plastic cling wrap was pressed to the surface of the chow to retain moist and it was left to cool off.
6. Once it had cooled off, the ready silkworm chow (see figure 30) was put in a plastic bag and then stored in a fridge.

Modified food
The modified food was prepared by mixing the sought substance to be used for studying with the already prepared silkworm chow. The sought substances were usually dissolved in some liquid preferable H₂O previous to mixing to allow better dispersion in the food. The concentration of the compounds in the food ranged from 0.025-0.1 wt% with an exception of 0.5wt% in one case. The compounds that could be prepared in the way described above were Rhodamine B (NBS Biologicals 10810-B), PEDOT-S (Sigma Aldrich), PEDOT:PSS (Sigma Aldrich), PTAA, POWT, EDOT-S (Sigma Aldrich), pyrrole and Thioflavin T (Sigma Aldrich 72485-100mg).

Some substances such as TQ1, 6T (Sigma Aldrich 594687-1G) and EDOT are highly insoluble except in toxic or otherwise unsafe solvents. These substances were grinded together with NaCl forming a uniform powder which can be dissolved in water and then mixed with the silkworm chow as usual. All protocols for preparing the different types of modified food can be seen in Appendix B.
**Water content**

Since the food did dry out rather quickly and could not be eaten by the silkworm larvae, the water content in the food was investigated to see if it could be rehydrated.

The water content of the silkworm chow was measured by weighing moist food, then it was left to air dry for 24 hours and then the dry food was weighed. And the water content could be extracted by the following equation:

\[ \text{Water content (\%)} = \frac{W_{\text{wet}} - W_{\text{dry}}}{W_{\text{wet}}} \times 100 \ (\%) \]

A study of how much water would evaporate in the two different rearing boxes was conducted.

The water content test was conducted to investigate the possibly to rehydrate the food for the purpose of reusing the food, reducing the amount of wasted food.

1.05g of silkworm chow was placed in rearing box 1 and 1.03g in rearing box 2. After 24 hours in the rearing boxes there was 0.383g food left in rearing box 1 and 0.348g in rearing box 2. This yielded a water content or evaporation of 63.52% respectively 66.21% of the total weight of the food.

Around 1ml of water was added to the food dried out food to see if it could soak it up, the test conducted proved negative the food was completely dry after just 24 hours in the rearing boxes and could not absorb water efficiently. Therefore the idea of rehydrating the food was given up even if it meant that much of the food given would go to waste, instead smaller amount of food would be given at each feeding to avoid wasting to much food.

**Rearing**

The rearing was started with placing 40+ silkworm eggs (Ricks Livefood) in a plastic box which was put inside rearing box 1. The eggs were mostly black or dark in color, after four days half of the eggs had hatched. The feeding was started right away; almost no further eggs were hatched in the following days. The eggs that did not hatch had been submerged in water due to condensation at the edges of the plastic box. The newly hatched silkworms were extremely small and mostly black in color (see figure 31), after just one to two days of feeding the silkworms had at least doubled in size and become grayer in color (see figure 32). The silkworms were fed 3-4 times a day when possible due to that the food dried out quickly as stated previously, the food was applied directly on the silkworms during the first two instars, since the silkworms had a tendency to stick on to the food that they were feeding on. It was noticed that even newly hatch silkworms could crawl quite long distances but it was only noticed if there were no food present. If the silkworm is situated onto food the new food must be placed adjacent to the old one for the silkworm to be able to move onto the new food. As the silkworm grows into instar 3 and beyond they are more self-dependent and they will crawl around more to look for any fresh food and does not cling to the food as much as before.
A new batch of eggs was started almost on a weekly basis, a total of 7 batches of eggs with varying size were started.

The silkworms were moved to a new clean container if their previous one was either dirty or if they have outgrown it. The silkworms were initially moved by moving them one by one with a paint brush (see figure 33 for the paint brush). Another method used was to move old food with silkworms still on it to a new container; this method causes some problems due to that the old food will eventually start to mold. The reason to move the silkworm to a new container is to reduce the risk of molding food spreading diseases. So the amount of old food should always be kept to a minimum. A method that is usually used to move silkworms is to place a mesh over the silkworms with food on top of it, the silkworms should then be able to crawl through the holes in the mesh and onto the food. This was tested numerous of times but always with the same result that only one or two silkworms managed to crawl through and onto the food. Explanations could be that the majority of the silkworms were either happy with the old food or that they could not manage to reach the mesh and the new food situated on it. It was unfortunate that the mesh did not work since it would have been an easy method which would have saved a lot of time. As the silkworms grow they become easier to move, and they can be moved by grasping them between the thumb and index finger. Even instar 2 silkworms which are around one centimeter in length can be moved in this way if you are gentle. The use of a paint brush is also a quite reliable method but it has some drawbacks. Silkworms
produce silk throughout all the instars mainly to help them attach to the food, it is not hard to pick up a silkworm with a paint brush with some practice but it is a different story to remove them from the brush. The reasons for this is that the silkworms could be attached to a silk thread which gets wrapped up in the brush and since it is really small it is almost invisible which further increases the challenge of removing the silk and silkworm from the paint brush. The silkworms can also cling to the brush which will require that you remove them from the brush by hand.

In the beginning the silkworms were reared in two plastic boxes with lids. Condensation builds up quickly if the lids were closed even if the lids had four small holes. The main reason for this was not only the high humidity but also that the IR-lamp shinned its light right on the plastic boxes. If the lid was wrapped with aluminum foil almost no condensation occurred. So in case of any condensation the first step was to shelter with aluminum foil. The aluminum foil came in use in other ways, when the silkworm reaches instar 4 and 5 they are a lot bigger and move around more. Therefore instar 4 and 5 silkworms were reared in 55 and 90mm petri dishes (see figure 34). Around one to two silkworms were placed in each petri dish, some of the silkworms had a tendency to crawl out from their petri dish even if the lid was on since they are big enough to be able to push it away. To avoid further escapes the petri dishes were wrapped around in aluminum foil so there was 5-8cm high walls surrounding the petri dishes, the lid was put on top of the wall. This helped to keep the silkworms from escaping, there still were some silkworms that managed to crawl out but these were mainly instar 5 silkworms which sought somewhere to spin their cocoon. The silkworms seemed to cling to the aluminum foils rather than stay on the petri dish, the silkworms had some trouble to move on the petri dishes which probably was slippery to them. A way to make the surface more attractable is to rub it with sand paper but this was not tested since the silkworms still managed to move around.

To avoid that the instar 5 silkworms crawled away when they would begin to spin a cocoon it was necessary to provide them with a suitable surrounding. One way of doing this was to place the silkworm inside a toilet roll (see figure 35) preferably with some aluminum foil acting as a lid otherwise the silkworm would crawl out from it. The toilet rolls are very good since the cocoons could be more easily handled. The drawback is that you have to put the silkworms inside the toilet roll right before they start to spin. Placing the silkworm to soon will result that they will be harder to feed, since the silkworm will cling to the walls and it will become problematic to place the food somewhere where it does not eventually drop to the ground. The spilling and the food will then gather in the bottom of the toilet roll, this could be solved by laying the roll on its side but it is not such a good method because that the roll will take up more ground space but also that the food and spilling will be gathered in the roll. So one have to check for any signs that the silkworms shows before they start to spin, mainly that they will throw out all the fluids in their digestive system. Another way of providing a suitable spinning environment is to encapsulate the whole petri dish with aluminum foil. Although this method also has some drawbacks mainly that the silkworm could have problem finding a good place to spin their cocoon since they are quite picky with it, with this method
the silkworm will waste much silk since it can have a hard time spinning the cocoon’s anchor points and therefore the cocoon can become thinner than normal or altogether incomplete.

Once the silkworm has finished spinning its cocoon it will take around 10-14 days until the moth emerged from the cocoon. Both female and male moth were successfully hatched from their cocoons, female and male moths were paired together and put in petri dish with aluminum foil around it just as for the larvae. Fertilized eggs were laid and it was possible to make them hatch after a couple of days in the rearing box. Eggs were also collected and stored in the fridge.

The silk for analyzing were collected in two different ways. One way was by dissecting the silkworm prior to spinning its cocoon and extracting the silk glands from it, the other method was to gather silk from the cocoon.

**Dissection**

Silkworms were prepared for dissection by submerging them in a vinegar salt solution for at least four hours. An incision is made with a scalpel on the silkworms back from the head all along to the rear, as can be seen in figure 36. The silk glands are usually situated at both sides of the gut and can be extracted simple by pulling them out. Another method is to make an incision right behind the silkworms head perpendicular to the length of the silkworm. The silk glands can then be squeezed out from this incision. Once the silk glands has been extracted they can be pulled to form a thin fine thread, the silk gland is made of walls and a core. The core can be separate by peeling the wall off it by hand. The walls usually have a dimmer and darker color compared the core which is transparent and clear in its color.

Dissection was used to check for any evidence of any incorporation of compounds mainly by checking for any difference in color compared to how the native silk gland (control) looked; any unusual coloration of the silkworms body can also be an indication that the materials have at least passed through the gut.
Degumming silk
To get rid of the sericin from silk fiber it is necessary to degum the silk. The silk is submerged in 8M urea (Sigma Aldrich U270-9) solution with a pH 8.30. It is then heated to 65°C for 30 min. After this step the silk is washed in Milli-Q water and then air dried for 24-48 hours. This protocol was used for both silk gathered from cocoon but also for silk glands. It is important to wash the silk, since it will swell in the urea solution and if it is not washed it will increase in weight due to that urea residues are still left in and around the silk.

Spectrofluorometer
Emission spectras from solutions was measured with Fluoromax-4 Spectrofluorometer. Since the thesis was to prove if a compound had been successfully incorporated in the silk it was necessary to first degum the silk to get rid of the sericin layer and then dissolve the fibroin fibers which is the commercial and more useful part of the silk, to be able to see if the compound had bonded to the fibroin fibers. 2 ml of the sample was put in a glass cuvette with a length of 1 cm.

Spectrometer
For measuring the concentration of fibroin in silk solution but also to verify if material is bounded to the fibroin it is necessary to measure the absorbance of a given solution. The absorbance spectra was taken with a Perkin Elmer Precisely Lambda 950 UV/VIS Spectrometer, using 10mm cuvettes.

Photoluminescence setup
Photo luminescence experiments were conducted for studying the emission from the silk threads. Raw silk threads from cocoons were mounted onto microscope glass slides. Large amount of threads were mounted by adding glue onto both ends of the threads. In the case of a single silk thread it was either submerged in PDMS then heated to 65°C for 15minutes or a drop of water was added on top of the thread and finally a cover glass slide was gently pushed against it. The setup used for the photoluminescence experiments can be seen in figure 37. The glass slide with the mounted samples was secured in a sample holder, the sample was put perpendicular to the detector which is a CCD camera. The light source used for excitation was a blue laser (CW PMM-208G-VT) with a wavelength of 405nm and intensity of 4mW, it was directed onto the silk thread which then would emit light that the camera could obtain. The sample is put perpendicular to avoid the exciting light moving into the camera. Even so the silk thread does scatter light quite much so some of the exciting light comes into the camera. Therefore a filter with a cutoff of 420nm was used to remove the light from the blue laser. The experiment was done in darkness to avoid excess light sources. Prior to the experiment the camera was cooled to -60°C, the exposure time used was 5 second with a slit of 500µm for the detector.
PTAA synthesis

PTAA needed to be synthesized since it is not commercially available. The synthesis was done in-house accordingly to the procedure developed by Nilsson KPR.[29] For a more detailed view over the synthesis see Appendix D.

The synthesis protocol used to synthesize looked like:

1. 1.0738g of 3-thiopheneacetic acid was dissolved in 40mL of CHCl₃ (MERCK 602-006-004) at room temperature under N₂ atmosphere.
2. After it had dissolved 5.512g of FeCl₃ was added and the solution was still kept under N₂ atmosphere.
3. The solution was stirred for 4.5 hours yielding a black solution.
4. 10 mL acetone (Solveco 1075) was added to the solution.
5. Centrifuged at 3800rpm for 5 minutes, the red to black precipitate was saved and the supernatant was poured off.
6. 30 mL acetone was added to the precipitate and then solution was centrifuged at 3800 rpm for 5 minutes. Repeated until a clear supernatant was yielded.
7. The precipitate was dissolved in 3M NaOH (MERCK B0685762) to a total volume of 65 mL, giving a red to black solution.
8. The red to black solution was centrifuged at 3500 rpm for 5 minutes, the supernatant was saved and the precipitate discarded.
9. 15mL concentrated HCl was added to the supernatant to neutralize it yielding a red precipitate, the solution was centrifuged at 3500 rpm for 5 minutes yet again.
10. The precipitate was washed two times with water, centrifuging after each wash.
11. The washed precipitate was treated with NaOH dissolved in MeOH (Sigma Aldrich 322415-2L) for 90 minutes.
12. The solution was then concentrated with a rotary evaporator, re-dissolved in water and lyophilized overnight yielding red flakes (see figure 38).

Figure 38 PTAA-Na salt red flakes yielded from freeze drying
Dissolving silk
For studying absorbance and emission spectra it is preferred to study a solution. Therefore it was necessary to dissolve the silk samples to be able to detect if any compounds had been successfully incorporated. The dissolving agent Ajisawa’s reagent was therefore used to dissolve the silk into a solution.

Ajisawa’s reagent
Ajisawa’s reagent is a chaotrophic salt solution with the ability to dissolve proteins such as silk fibroin. It was prepared by making a CaCl$_2$:EtOH:H$_2$O solution with a molar ratio of 1:2:8. 55.5 g CaCl$_2$ (Sigma Aldrich C1016-500G) was dissolved in 72ml Milli-Q water and 58.3 ml EtOH (Solveco AB), the solution was then left to stir with a magnetic stirrer at ambient conditions.

Silk solution
A dissolved silk solution was prepared with following procedure, silk was added to Ajisawa’s reagent, it was then heated to 65-70°C and if necessary stirred with a magnetic stirrer. Different concentrations of silk solution were prepared ranging from 10g/L up to 120g/L. Where the 120 g/L took 3 days to fully dissolve in a water bath of 65-70°C and stirred with a magnetic stirrer. After being dissolved, the solution was filtered through a 2µm filter. To get rid of the CaCl$_2$ the silk solution was dialyzed with a 3500 Mw cutoff for 3 days with Milli-Q water as a buffer (see figure 39). The Milli-Q water was exchanged at least once a day. Once the dialyzing was done the remaining silk solution inside the dialyze tube was removed with a syringe.

Freeze drying
The dialyzed solution was frozen with liquid nitrogen and then freeze dried for 48 hours, yielding a white powder.

Spectrophotometer analysis
To be able to determine the concentration of the fibroin solution with absorbance spectroscopy the molecular weight of fibroin and its extinction coefficient must be known.

Molecular weight of fibroin: 391593.3 g/mole. [32]
Extinction coefficient for fibroin at 280nm: 473480.[32]

With the help of Lambert-Beers law the real concentration can be obtain by using the value from the absorbance spectra. (See Appendix A)
### Table 1 Concentration of dissolved fibroin calculated from absorbance spectra

<table>
<thead>
<tr>
<th>Sample (dissolved fibroin fibers)</th>
<th>Absorbance at 280nm</th>
<th>Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 g/L</td>
<td>1,064</td>
<td>0,88</td>
</tr>
<tr>
<td>1 g/L (filtered 0.45µm)</td>
<td>1,052</td>
<td>0,87</td>
</tr>
<tr>
<td>2g/L</td>
<td>2,270</td>
<td>1,88</td>
</tr>
<tr>
<td>1g/L Dialyzed</td>
<td>0,286</td>
<td>0,24</td>
</tr>
<tr>
<td>1g/L Dialyzed + filtered (0.45 µm)</td>
<td>0.302</td>
<td>0,25</td>
</tr>
<tr>
<td>2g/L Dialyzed</td>
<td>0,669</td>
<td>0,55</td>
</tr>
</tbody>
</table>

**Regenerated silk**
Silk fibroin aggregates could be regenerated by adding 1-2ml of the dialyzed silk fibroin fibers solution to 12-14 ml of 550 g/L ammonium sulfate in a 15ml falcon tube. The falcon tubes were then centrifuged at 4000 rpms for 30min. The regenerated silk could be collected as the precipitate.

**Coating of silk**
Silk (Aurora Silk) threads were coated with fluorescent or conductive materials to act as controls for how the intrinsically incorporated silk should look like.

**Rhodamine B**
Silk fibroin threads were submerged in 0.5 and 0.05mg/ml Rhodamine B solutions, put in an oven at 65°C for 24 hours to see if the threads would be colored.

**PEDOT-S**
9mg of PEDOT-S was dissolved in 900µl of Milli-Q water to a concentration of 10g/L. Silk fibers where then submerged in the PEDOT-S solution and put in an oven at 65°C.

**Nile Red**
30.6 mg of freeze dried silk fibroin solution (120g/L) was grinded into a fine white powder. 1 mg of Nile Red powder was added to the grinded fibroin and was ground together, forming a pink/red powder. This powder was dissolved in 4ml of Milli-Q water and 100µl of this was added to 900µl of ammonium sulfate 550g/L. Once the fibroin Nile Red solution was added to the ammonium sulfate it immediately precipitated out. To separate the supernatant from the precipitate for further measurement it was necessary to centrifuge causing the precipitate to collects at the bottom and the supernatant could then easily be separated.

Some silk threads were submerged in 0.05mg/ml Nile red solution and kept at 65°C for 24 hours to see if the Nile red would be able to coat the threads.

**PTAA**
Just as for Nile Red, PTAA was also grinded together with silk fibroin powder, forming a red/pink precipitate when added to the ammonium sulfate. This could then be separated from the supernatant via centrifugation.
Silk threads were also submerged in 0.5mg/ml PTAA solutions and heated to 65°C for 24 hours yielding red to yellowish silk threads. But the colored came off during the first wash resulting in an unsuccessful coating.

**POWT**
The POWT used had been previously synthesized according to the protocol developed at BIORGEL [33] and stored in a freezer.

100µl 5mg/ml POWT was mixed with 900µl Milli-Q water. Three samples of coated silk was prepared; degummed silk was submerged in the 0.5mg/ml POWT solution and heated to 65°C, native silk was submerged in the POWT solution and heated to 65°C and degummed silk was submerged in the POWT solution and put under ambient conditions.
## Results

### Silkworm rearing

During the thesis seven batches with silkworm eggs were started and the silkworms were reared up to the fifth instar where they either were dissected or left to spin a cocoon. What each batch was fed and their end state and survival ratio can be seen in table 2.

| Original batch | Subdivided batch | Amount of eggs | Amount of silkworms | Type of food | Days required to reach cocoon state | End state | Survival ratio (%)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1,1</td>
<td>40+</td>
<td>20 (Hatched)</td>
<td>Silkworm chow</td>
<td>3 / 5 (Hatched)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2,1</td>
<td>29</td>
<td>11</td>
<td>Silkworm chow</td>
<td>11 / 27 (Hatched)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3,1</td>
<td>44</td>
<td>10 (Hatched)</td>
<td>Chow</td>
<td>5 / 20 (Hatched)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4,1</td>
<td>150</td>
<td>75 (hatched)</td>
<td>Silkworm chow</td>
<td>9 / 19 (Hatched)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5,1</td>
<td>189</td>
<td>46 (Hatched)</td>
<td>Silkworm chow</td>
<td>2 / 9 (Hatched)</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2 Information on each silkworm batch, what type of food they were given and survival ratio etc.**
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th>0.1wt%</th>
<th>Prepared for dissection (dead)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5_2</td>
<td>1</td>
<td>Silkworm chow</td>
<td>-</td>
<td>Prepared for dissection (dead)</td>
</tr>
<tr>
<td>5_3</td>
<td>1</td>
<td>Carbon</td>
<td>28</td>
<td>Cocoon</td>
</tr>
<tr>
<td>5_4</td>
<td>2</td>
<td>PTAA</td>
<td>30</td>
<td>Cocoon /Prepared for dissection</td>
</tr>
<tr>
<td>6</td>
<td>159</td>
<td>20 (Hatched)</td>
<td>Silkworm chow</td>
<td>4 / 35 (Hatched)</td>
</tr>
<tr>
<td>6_1</td>
<td>1</td>
<td>POWT 0.025-0.05wt%</td>
<td>27</td>
<td>Prepared for dissection</td>
</tr>
<tr>
<td>6_2</td>
<td>1</td>
<td>PEDOT-S+DMSO</td>
<td>24</td>
<td>Cocoon</td>
</tr>
<tr>
<td>6_2_1</td>
<td>1</td>
<td>PEDOT-S 0.05wt%+DM SO 10wt%</td>
<td>26</td>
<td>Prepared for dissection</td>
</tr>
<tr>
<td>6_3</td>
<td>1</td>
<td>PEDOT-S 0.05wt%+DM SO 10wt%</td>
<td>-</td>
<td>Prepared for dissection (dead)</td>
</tr>
<tr>
<td>6_4</td>
<td>1</td>
<td>TQ1-dimer 0.05wt%</td>
<td>27</td>
<td>Prepared for dissection</td>
</tr>
<tr>
<td>6_4_1</td>
<td>1</td>
<td>TQ1-dimer 0.05wt%</td>
<td>-</td>
<td>Prepared for dissection (dead)</td>
</tr>
<tr>
<td>6_5</td>
<td>1</td>
<td>TQ1-dimer 0.05wt%</td>
<td>27</td>
<td>Prepared for dissection</td>
</tr>
<tr>
<td>6_6</td>
<td>1</td>
<td>POWT 0.1wt%</td>
<td>33</td>
<td>Prepared for dissection</td>
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<tr>
<td>6_7</td>
<td>1</td>
<td>PEDOT-S 0.5wt%+DMSO 10%</td>
<td>31</td>
<td>Prepared for dissection</td>
</tr>
<tr>
<td>6_8</td>
<td>1</td>
<td>Nile Red 0.05wt%</td>
<td>-</td>
<td>Prepared for dissection (dead)</td>
</tr>
<tr>
<td>7</td>
<td>161</td>
<td>28 (Hatched)</td>
<td>Silkworm chow</td>
<td>12/71(Hatched)</td>
</tr>
<tr>
<td>7_1</td>
<td>1</td>
<td>TQ1-dimer 0.05wt%</td>
<td>24</td>
<td>Prepared for dissection</td>
</tr>
<tr>
<td>7_1_1</td>
<td>1</td>
<td>Silkworm chow</td>
<td>25</td>
<td>Cocoon</td>
</tr>
<tr>
<td>7_2</td>
<td>2</td>
<td>Thioflavin T</td>
<td>Preparred for dissection (dead)</td>
<td></td>
</tr>
<tr>
<td>7_3</td>
<td>1</td>
<td>PEDOT-S 0.1wt%+10%DMSO</td>
<td>32</td>
<td>Cocoon</td>
</tr>
<tr>
<td>7_4</td>
<td>2</td>
<td>Silkworm chow</td>
<td>28</td>
<td>Cocoon</td>
</tr>
<tr>
<td>7_5</td>
<td>1</td>
<td>Pyrrole 0.1wt%</td>
<td>34</td>
<td>Prepared for dissection</td>
</tr>
<tr>
<td>7_6</td>
<td>1</td>
<td>EDOT 0.1wt%</td>
<td>-</td>
<td>Prepared for dissection (dead)</td>
</tr>
<tr>
<td>7_7</td>
<td>1</td>
<td>EDOT 0.05wt%</td>
<td>Prepared for dissection (dead)</td>
<td></td>
</tr>
<tr>
<td>7_8</td>
<td>1</td>
<td>Pyrrole 0.1wt%</td>
<td>30</td>
<td>Prepared for dissection</td>
</tr>
<tr>
<td>7_9</td>
<td>1</td>
<td>POWT 0.05wt%</td>
<td>30</td>
<td>Prepared for dissection</td>
</tr>
<tr>
<td>7_10</td>
<td>1</td>
<td>EDOT-S 0.05wt%</td>
<td>40</td>
<td>Cocoon</td>
</tr>
<tr>
<td>7_11</td>
<td>1</td>
<td>EDOT-S 0.05wt%</td>
<td>40</td>
<td>Prepared for dissection</td>
</tr>
<tr>
<td>8 (Biggest from 4 and 5)</td>
<td>2</td>
<td>PEDOT-S 0.05wt%</td>
<td>31 (5) /39(4)</td>
<td>Cocoon</td>
</tr>
</tbody>
</table>
Silkworms fed modified food was placed in separate containers with one up to two silkworms in each container which can be seen in table 2. These containers are stated as subdivided batches from the original batch both because it was easier to keep track of any individual silkworm but also that the silkworm in each original batch reached the fifth instar during different point in time. Table 3 shows the survival ratio from each of the original seven batches. The survival ratio is based on the silkworms that manage to reach the cocoon spinning stage of instar 5. Two types of survival ratios are stated in the table, the first one depicting how many percent of the silkworms managed to survive from the original amount of eggs in each batch, the other survival ratio is based on the amount of hatched eggs. The survival ratio stated is rounded up. The amount of eggs and hatchling in each batch are counted by hand.

Table 3 Survival ratio of hatch and reared silkworms

<table>
<thead>
<tr>
<th>Batch</th>
<th>Total survival ratio (%)</th>
<th>Survival ratio for hatchlings (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>27</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>19</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>35</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>71</td>
</tr>
<tr>
<td>Averaged</td>
<td>7</td>
<td>27</td>
</tr>
</tbody>
</table>

As you can see in table 3, the survival ratio was very low with only a few silkworms that managed to stay alive through their whole life cycle. The hatch ratio was also quite low usually about or below 50%, the main reason for this could be that the silkworms egg were shipped from the UK during the winter and that the transport took several days. During this time the eggs would have been exposed to subzero temperature or at least drastic temperature shifts which could cause harm to the eggs. So it would be reasonably to assume that some eggs did die during the transport which in turn caused to low hatch rate. Even so the survival ratio of the hatchling was also quite low, but the last two batches had a survival rate of 35 respectively 71%. The reason for this was that most of the problems that came up during the rearing of the previous batches had been fixed, but also that rearing of silkworm is a learning process and it takes some time before you know how to treat them in the most optimal way. One of the reasons that the survival rate was so low for the first batches was that the silkworms were overfed. The silkworms were given too much food that they could not eat and since the food eventually dries out new food was put on top of the old causing stacking piles of food. This stacking in turn caused that small or weak silkworm usually got trapped in the lower layers of the food piles, not receiving any new food. The food was also given 3-4 times a day which is recommended but too much food was given in the beginning of the work. This method was corrected for the later batches, which was usually fed only 2 times a day with a smaller amount of food given each time.
Most of the different types of food did not prove directly lethal to the silkworms except Thioflavin T modified food were the silkworms died within 24 hours after eating it. EDOT was also lethal to the silkworms, but it took some days until the silkworm eventually died. The EDOT modified food was although severely less lethal compared to the Thioflavin T modified food, since it was possible to prolong the life of the silkworms by decreasing the content of EDOT from 0.1 wt% to 0.05 wt%. The silkworms did eventually die but it took a couple of more days with 0.05 wt% compared to 0.1 wt% EDOT modified food and the silkworms were far more active and energetic. Nile Red modified food also proved lethal but only to half of the silkworms which it was fed to. The procedure for mixing Nile Red into the silkworm chow required use of ethanol, and the cause of death could have been from ethanol poisoning. The ethanol was allowed to evaporate out from the food but most likely for a too short time leaving ethanol in the food. Instead it should perhaps been better to allow the food to dry out even further and then add water to moisten it up. Silkworms did also die after eating regular food, Pyrrole-, PEDOT-S + DMSO- and TQ1-dimer modified food, and it should be quite safe to assume that these deaths were due to sickness since most of the silkworms that fed on these types of food did manage to survive. Another factor that plays in the survival ratio is the wellbeing of the silkworms prior to being fed modified food, since a strong and healthy silkworm should have a better chance to be able to eat the modified food and survive.

Most of the silkworms that had spun cocoons did emerge from them, the once who did not were batch 4_7, 4_3, 4_5, 4_10, 2_1, 5_1 and 7_3. Batch 4_7 which was fed 6T 0.1 wt% had not spun a complete cocoon and the spinning pattern looked rather strange compare to the others, so it safe to say that it lacked the proper protection since the cocoon was so thin that you could see through it. What is strange is that batch 4_7_1 which was also fed 6T 0.1 wt% did manage to spin a cocoon, but it was perhaps because it did spin its cocoon inside a toilet roll compared to a petri dish. Both batch 4_5 and 4_10 had small holes in their cocoons which also most probably caused a decreased protection during the final metamorphosis, killing the silkworm. The other batches that did not hatch had no visible deformities on their cocoon and they were all fed different types of food so no conclusion of what caused these silkworms to die in their cocoons could be drawn. The emerged moths were in most cases healthy and looked normal. But some of the moths had some abnormalities (See figure 40 A: Batch 6_2 moth, fed PEDOT-S + DMSO modified food. It has abnormal small wings and lack fur. B: Batch 4_2 moth (fed PEDOT-S 0.02-0.05 wt%). It has abnormally small wings and is a bit more brown/yellow colored compared to regular moths. C: Batch 4_8 moths (fed TQ1P 0.1 wt%). The two moths had developed very drastic from each other, the one in the bottom is dead in this picture probably due to its deformities. The other moth at the top managed to develop into normal healthy moth. The round white dots in the low bottom are eggs laid by one of the moths. D: Batch 4_9 (fed PEDOT:PSS 0.1 wt%). Moth closest to the bottom is a healthy specimen and the one on the top is moth with deformities. Both moths were fed and grown in the same container and both did spin their cocoons within the same day.)
40), like batch 6_2 which was fed PEDOT-S + DMSO modified food. It had small undeveloped wings and it lacked some of its fur as you can see in figure 40. Batch 4_2, fed PEDOT-S (0.02-0.05wt%) also had abnormally small wings and it was more yellow colored (see figure 40) compared to the normal white moths. But not only silkworms fed PEDOT-S had moth with abnormalities, batch 4_8 which was fed TQ1P 0.1 wt% modified food had two silkworms in it which both spun cocoons but one of the moths that emerged had the same abnormalities as stated above but the other specimen was completely healthy. Batch 4_9 which was fed PEDOT:PSS 0.1wt%, also had two silkworms and the emerged moths did show the same behavior as for batch 4_8 with one healthy and one unhealthy moth which can be seen in figure 40. This raises the question if the compounds in the modified with a certain interest in PEDOT could have any effect on the final metamorphism.

**Degumming ratio**

The silk generated from the silkworms has a protective layer of the protein sericin around it, the procedure to remove this sericin layer revealing the wanted fibroin fibers is called degumming.

**Table 4. Degumming result**

<table>
<thead>
<tr>
<th>Food / type of silk</th>
<th>Weight before (mg)</th>
<th>Weight after (mg)</th>
<th>Degumming ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow / gland</td>
<td>27.2</td>
<td>19.57</td>
<td>28</td>
</tr>
<tr>
<td>PEDOT-S / gland</td>
<td>10.62</td>
<td>8.24</td>
<td>22</td>
</tr>
<tr>
<td>PTAA / gland</td>
<td>9.72</td>
<td>7.84</td>
<td>19</td>
</tr>
<tr>
<td>Rhodamine B / gland</td>
<td>6.02</td>
<td>3.56</td>
<td>41</td>
</tr>
<tr>
<td>POWT (7_9) / gland</td>
<td>25.96</td>
<td>16.81</td>
<td>35</td>
</tr>
<tr>
<td>POWT (6_1) / gland</td>
<td>206.71</td>
<td>60.3</td>
<td>71</td>
</tr>
<tr>
<td>TQ1-dimer (6_5) / gland</td>
<td>37.87</td>
<td>29.09</td>
<td>24</td>
</tr>
<tr>
<td>Pyrrole (7_8) / gland</td>
<td>75.27</td>
<td>56.69</td>
<td>25</td>
</tr>
<tr>
<td>Nile Red (4_4) / thread</td>
<td>4.8</td>
<td>4.56</td>
<td>5</td>
</tr>
<tr>
<td>PTAA (5_4) / thread</td>
<td>155.27</td>
<td>109.42</td>
<td>30</td>
</tr>
<tr>
<td>POWT (6_6) / gland</td>
<td>105.66</td>
<td>59.73</td>
<td>44</td>
</tr>
<tr>
<td>PEDOT-S (6_3) / gland</td>
<td>48</td>
<td>34.17</td>
<td>29</td>
</tr>
<tr>
<td>Nile Red (3) / thread</td>
<td>16.93</td>
<td>16.18</td>
<td>4</td>
</tr>
<tr>
<td>POWT (6_1) / gland</td>
<td>51.21</td>
<td>35.81</td>
<td>30</td>
</tr>
</tbody>
</table>

Silk glands averaged degumming ratio 33

Silk threads averaged degumming ratio 13

The results from the degumming assay is presented in table 4, it shows that the degumming had a greater impact on the silk glands than the silk threads. Since there are only three samples of degummed threads compared to eleven gland-samples it might be unwise to jump to any drastic conclusions. But it still looks that the gland usually has a higher degree of degumming, if this might be because the urea can more easily break the hydrogen bonds or if it might be because the gland may have some contaminants still on them prior to the degumming which comes off as they degum.
Dissolving fibroin

**Absorbance Silk**

![Absorbance Silk graph](image)

Figure 41. Absorbance spectra, cuvette length 1cm. All three fibroin samples have been subtracted with the absorbance of the dissolving agent (control) Ajisawa’s reagent absorbance spectra. The peak at 280nm corresponds to the absorbance of the present protein fibroin.

The spectrophotometer analysis (see figure 41) yielded concentration of 0.88g/L for the 1g/L fibroin solution, 0.87 g/L for the filtered 1 g/L fibroin solution and 1.88 g/L for the 2 g/L fibroin solution using the extinction coefficient of 473480 M⁻¹cm⁻¹ and molecular weight of 391593.3 g/mole. The results indicate that either the extinction coefficient or molecular weight used in the calculations does not match 100% to the real fibroin samples. Another explanation is that the fibroin thread used is not completely pure or that there are some measurement errors.

**Absorbance Silk (Dialyzed)**

![Absorbance Silk (Dialyzed) graph](image)

Figure 42. Absorbance spectra, cuvette length 1cm. All fibroin samples have been subtracted with the absorbance of the water since the dialyzed fibroin solution was diluted in water. The peak at 280nm corresponds to the absorbance of the present protein fibroin. Both 20g/L samples (filtered and not filtered) have a too high concentration of protein which
mean that it will not be possible to detect the peak from the solution with the spectrophotometer without diluting the samples.

For the filtered solutions the spectrophotometer analysis (See figure 42) yielded that after dialyzing 1g/L silk fibers it reduced the concentration to 0.24 g/L. For the filtered solution 1g/L the dialyzing reduced the concentration to 0.25 g/L. The 2 g/L fibroin solution reduced its concentration to 0.55 g/L after the dialyzing step. The dialyzing shows a decrease in concentration around 3-4 times the original concentration. The cut-off in the dialyzed tube of 3500 g/mole does allow some of the protein to slip through which could explain the drastic reduction in concentration. This is examined during the dialyzing since the water will lather as a consequence of protein slipping out into the water. Another thing that contributes is that the dialyze tube itself will swell, probably because some of the water goes in and will further dilute the silk solution.

The dissolved silk fibroin solutions were studied with an emission spectrometer (Flouromax-4 Spectroflourometer). Figure 43 and 44 shows the emission spectra from the control samples with an excitation wavelength of 400nm. Two samples for each control were made, one that had been kept at ambient conditions for 48 hours prior to measurement and one that had been kept at 65°C for 48 hours, called ambient respectively heated in the figures. Both figures show that the heated sample usually gives a significant higher output compared to the ambient. The emission of the silk solutions also increases with an increased concentration and this would have to be because of that silk will emit light at around 470nm if it is excited at 400nm.[34]And as the concentration of fibroin increases in the solution so will also the emission from it. So the materials bonded to the fibers must be in such a concentration that their emission does not drown in the emission from silk fibers.

![Emission Controls](image)

**Figure 43.** Emission spectra of control samples. All samples excited at 400nm. Entrance and exit slit 5nm. Two sample for each control was tested, one that had been kept at ambient temperature for 48 hours and one that had been heated at 65°C for 48 hours. The silk solutions samples are an exception since the heating is necessary for the silk to dissolve.
Emission Controls

Figure 44. Emission spectra of control samples. All samples excited at 400nm. Entrance and exit slit 5nm. The dissolving Ajisawa’s reagent gives a higher output compared to the water spectra with 1 magnitude higher count. Two sample for each control was tested, one that had been kept at ambient temperature for 48 hours and one that had been heated at 65°C for 48 hours.

The emission spectra for the controls when exciting with 550nm (see figure 44 and 45) also share the same properties as for the once excited at 400nm. The main difference is that the dissolved fibroin solution produces a lower emission since the proteins in the silk only gets excited at with light from the range of 200-400nm.

Emission Mediums

Figure 45. Emission spectra of control samples. All samples excited at 550nm. Entrance and exit slit 5nm. Two samples for each control was tested, one that had been kept at ambient temperature for 48 hours and one that had been heated at 65°C for 48 hours.
Tested substances

The majority of the substances tested for intrinsically incorporation did not work. This was not perhaps so unexpected because there has not been much work done in the field and much of the mechanism of how the intrinsically incorporation works still lays eluded. As mentioned in the method it is thought that zwitter-ionic, hydrophobicity and self-assembly properties are all important aspect if a compound will be able to be incorporated intrinsically. [12] The absorbance spectra from the silk samples from the various batches can be seen in Appendix C. The result for the incorporation within the silk fibers for the tested substances can be seen in Table 5.

<table>
<thead>
<tr>
<th>Substances</th>
<th>Properties</th>
<th>Incorporated into silk</th>
<th>Polymer</th>
<th>Lethality</th>
<th>Hydrophilic</th>
<th>Wt% in food</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodamine B</td>
<td>Fluorescent</td>
<td>Yes</td>
<td>No</td>
<td>Toxic (at 0.05wt%)</td>
<td>Yes</td>
<td>0.025-0.05</td>
</tr>
<tr>
<td>Nile Red</td>
<td>Fluorescent</td>
<td>Inconclusive</td>
<td>No</td>
<td>Toxic (ethanol)</td>
<td>No</td>
<td>0.035-0.05</td>
</tr>
<tr>
<td>POWT</td>
<td>Fluorescent</td>
<td>Inconclusive</td>
<td>Yes</td>
<td>Harmless</td>
<td>Yes</td>
<td>0.05-0.1</td>
</tr>
<tr>
<td>PTAA</td>
<td>Fluorescent</td>
<td>No</td>
<td>Yes</td>
<td>Harmless</td>
<td>Yes</td>
<td>0.05</td>
</tr>
<tr>
<td>EDOT</td>
<td>Conductive</td>
<td>No</td>
<td>No</td>
<td>Lethal</td>
<td>No</td>
<td>0.05-0.1</td>
</tr>
<tr>
<td>EDOT-S</td>
<td>Conductive</td>
<td>No</td>
<td>No</td>
<td>Harmless</td>
<td>Yes</td>
<td>0.05</td>
</tr>
<tr>
<td>PEDOT-S</td>
<td>Conductive</td>
<td>No</td>
<td>Yes</td>
<td>Harmless</td>
<td>Yes</td>
<td>0.05</td>
</tr>
<tr>
<td>PEDOT:PSS</td>
<td>Conductive</td>
<td>No</td>
<td>Yes</td>
<td>Harmless</td>
<td>Yes</td>
<td>0.1</td>
</tr>
<tr>
<td>Pyrrole</td>
<td>Conductive</td>
<td>No</td>
<td>No</td>
<td>Harmful</td>
<td>Yes</td>
<td>0.01-0.1</td>
</tr>
<tr>
<td>Thioflavin T</td>
<td>Fluorescent</td>
<td>No</td>
<td>No</td>
<td>Lethal</td>
<td>Yes</td>
<td>0.05</td>
</tr>
<tr>
<td>6T</td>
<td>Fluorescent</td>
<td>No</td>
<td>No</td>
<td>Lethal</td>
<td>Yes</td>
<td>0.1</td>
</tr>
<tr>
<td>TQ1</td>
<td>Electron donor</td>
<td>No</td>
<td>No</td>
<td>Harmful</td>
<td>No</td>
<td>0.05</td>
</tr>
<tr>
<td>TQ1P</td>
<td>Electron donor</td>
<td>No</td>
<td>Yes</td>
<td>Harmless</td>
<td>No</td>
<td>0.1</td>
</tr>
</tbody>
</table>

As table 5 depicts only Rhodamine B yielded a clear conclusive result that the incorporation had been successful. Rhodamine B was as used as a positive control, since Tansil et. al. have already shown that RhB can be incorporated via feeding. It was shown in this thesis that it was possible to replicate the result from Tansil et. al. study. Nile Red and POWT both yielded somewhat inconclusive results, some indicating there presence in the silk fibers and some that did speak against it. Nile Red is quite similar to Rhodamine B in its structure but lacking the carboxylic acid group that RhB has. The lack of the carboxylic acid group reduces Nile Red’s zwitterionic properties which could have been one of the reason that it did not managed to produce as a clear result as RhB. In contrast to NR, POWT has zwitterionic properties due to its functional groups. But just as for NR it was hard to conclude any real results from the samples of silk that was collected and analyzed. The rest of the substances failed in the incorporation into the silk fibers. The properties of these materials are very varied, making it hard to conclude which property caused them to fail. Even so the only polymer that did produce any sign of incorporation was POWT, but since the results were inconclusive for POWT it could mean that the size of the polymer can have caused problems with their incorporation. A more in depth study of the results, including the spectra from the emission measurements can be seen in...
Appendix E. Which follows are abstracts of the results from the three most promising substances RhB, NR and POWT.

**Rhodamine B**

As previously said Rhodamine B was the only substance that managed to produce a clear result that it had been incorporated into the silk via the feeding methods. Figure 46 shows the spectra from a photo luminescent measurement of silk fibers taken from silkworms fed with Rhodamine B respectively regular unmodified diet (silkworm chow). The peak from the silk samples of RhB shows a clear and distinct peak at 570nm which is expected of RhB, this indicates that RhB has been successfully incorporated into the silk fibers since the control does not produce any peak at all. The silk produced by the silkworms fed with RhB modified diet was pink in color which also indicate that RhB has been incorporated into the silk fibers. For a more in depth study of the results on RhB see Appendix E.

![PL RhB](image)

*Figure 46 Photoluminescent measurement on silk fibers. Excitation wavelength 405nm.*
Nile Red

The results from Nile Red were inconclusive; figure 47 shows one of the emission measurements from that most strongly speak for that Nile Red could have been incorporated into the silk. Figure 47 shows a small bump from the silk sample taken from silkworm fed with Nile Red modified diet. This bump is at the range of 610-630nm which is in the range of NR’s emission peak (at 630nm) when excited at 550nm. Even if this bump is within the range of NR emission peak, the intensity of it is very small which make it hard to draw any real conclusions. What is most likely with Nile Red is that it could not travel further than to the walls of the silk glands, because it was seen that silk glands extracted via dissection form silkworms fed NR modified diet had a purple color. The purple color did come right off when washed which would indicate that most of the NR was loosely bound to the outside of the silk glands walls. There could perhaps been some small degree of NR that was incorporated into the silk fibers themselves, explaining the small bump at 630nm which was seen in emission measurements. For more results on Nile Red see Appendix E.

POWT

The results of POWT incorporation within the silk fibers were inconclusive. The reason that no real conclusion could be drawn was that there were indications both speaking for that POWT have been incorporated and that it had not been incorporated. Figure 48 shows the emission from silk fibers when excited at 405nm, the peak from both the samples of POWT fed silkworms and the control is at the same wavelength and no clear peak can be seen for the POWT sample. Because no clear peak is visible it speaks for that POWT have failed in its incorporation. Dissection of silkworms fed POWT revealed that the silk glands had a very faint orange color, this orange color is familiar to the orange colored which POWT has in solution. That the silk glands were orange colored could indicate that POWT had managed to be incorporated or at least perhaps as NR bound to the silk gland’s walls. But when analyzed no real evidence of POWT incorporation could be found.

Figure 47 Emission spectra. Excitation wavelength 550nm. Entrance and exit slit 5nm. Nile Red (Batch 3) conc. 8 mg/ml fibroin.
Some of the emission measurement of silk from POWT fed silkworms showed a decrease in emission when the concentration of silk in solution increased. This observation could indicate that POWT is present and shows quenching properties; it has been shown that POWT has quenching properties but that is in its doped states. The decrease in emission could also be due to measurement errors and not quenching from POWT, for a more in depth study see Appendix E.
Discussion

A problem that had to be overcome was to breed the silkworms successfully to the fifth instar. Since the thesis was started in January it were some problems with how to solve the temperature and humidity requirement necessary for rearing, even inside both the temperature and the humidity was below the required levels. The rearing problems were fixed relatively easy but with the equipment used the conditions was not perhaps the most optimal with some humidity and temperature fluctuations. Even so it was possible to rear the silkworm all the way from the egg stage up to instar 5 and eventually moths, the results of the rearing stretched beyond the initial expectations since moths did managed to hatch from their cocoon and did mate and laid fertilized eggs that later were hatched, completing the cycle. This shows that it is possible to breed silkworms in Sweden, and with the right facilities it could be further improved. But it is necessary to have an artificial environment which is a drawback. It would have been a better idea to do the rearing in the spring or summer since the weather is more habitable to the silkworms, and the necessary artificial heat and humidity sources could be kept to a minimum. Another problem that did arise was that the silkworm did take up much more space than initially thought and one should keep this in mind since the silkworms grow very fast in size. The mortality rate was quite high especially in the begin, so the space required did not grow too outrageous in size, but this is something that has to be kept in mind when doing test with silkworms.

Most of the materials tested in the thesis did not integrate into silk fibers; this was somewhat expected since the lack of previous work done in the field, meaning there were few guidelines to follow. The Rhodamine B samples produced very colorful silk threads and clear results, in agree with earlier reports. The only material other than Rhodamine B that may have been incorporated is Nile red. There seem to be some traces of Nile red in some of the samples. Even if the Nile Red only managed to coat the walls of the silk gland, it is an indication of which properties that are necessary for the uptake and transport.

Nile red is quite similar in structure to Rhodamine B, it has an amine group but instead of a carboxylic acid group it has a ketone group. Nile red is a small molecule and this would also help in the uptake process, the hydrophobicity of Nile red could also help it to pass through the epithelial tissue of the silkworm gut. It seems that the lack of a carboxylic acid group could have stopped Nile Red to fully be incorporated into the silk fibers and instead reattached in the epithelial of the silk gland walls.

That polymers and larger molecules such as PEDOT and TQ1 did not integrate is most likely due to their size, but they also lack both the amine and carboxylic acids groups that are suspected to help with the uptake. Even when fed 0.5wt% PEDOT-S food, the silk did not become coated, the silkworm’s body were also as white as before meaning that there was no PEDOT-S at all that could have passed through the gut and into the hemolymph. As mentioned before the silk gathered from the batches fed PEDOT derivatives did not show any signs at all of the suspected black/blue color that the silk should have had if it was coated. Because of this no test was done to see if the silk from the batches could conduct electricity, since it was seen as a waste of time.

The sample from batches fed POWT modified foods have given some mixed results, some of the silk glands seemed to have a faint pink color, which could come from POWT as it has a red color in solution. The emission and absorbance spectras taken have not yielded any conclusive results that would indicate the presence of any POWT in the silk thread. The odd thing though is that it looks like
a high concentration of dissolved fibroin form these batches results in a lower emission compared to lower concentrations of dissolved fibroin. It is reported that quenching can occur with POWT DNA mixes [28] and perhaps the decrease in emission could be due to quenching. But further studies have to be conducted before any conclusions can be drawn, and the decrease in emission can be due to measurement error both in the preparation of the sample and in the handling of them when measured. POWT is although the material tested that shares the most with Rhodamine B, both has an amine and carboxylic acids group which is thought to help in the uptake process. The reason that perhaps POWT did not incorporate into the silk fibers could be due to that it is a polymer and its size could be the main issue. But with the results gathered on POWT there could still be interesting to conduct further studies with higher concentration of POWT in the food to actually see if it can be incorporated.

Thioflavin T is as mentioned an amyloid probe, and it could perhaps be possible for it to bind to the β-sheet rich structure of fibroin, it is also a small molecule and it also have an amine group making it somewhat similar to Rhodamine B but even more to Nile red which seemed to have worked. It could then perhaps be possible to incorporated thioflavin T via digestion but the concentrations tested has to be considerably lower not to kill the silkworms.

Future aspect and research that can be drawn from this thesis are that a more in depth study of the required parameters for intrinsically incorporation. For example to work with a polythiophene skeleton which you can modify with different functional groups changing the physical and chemical properties of the compound. What is really important in that case is to check if the amine and carboxylic acid groups are necessary which is the hypothesis at the moment. [12] Another problem with the materials tested is most likely their size due to that they are polymers, it would be of interest then to study smaller molecule or to control the length of polymer chains. So together with testing polymers of Thiophene derivatives it could be of interest of testing monomeric or controlled length of thiophene derivatives also. There is possible to developed many different materials from thiophene with ranging properties such as conductive to fluorescence (e.g PEDOT-S respectively PTAA) making it very interesting. The hydrophobic properties also seem important according to Tansil et al. work and varying this property is another way to go in future work. The content of each sought compound in the different types of modified food was based on the previous work done by Tansil et al., but because lack of materials available. One should test a higher concentration of especially Nile Red and POWT to verify the presence of both compounds since a small response could be drowned in the emission spectra. Rhodamine B is compared to the other compounds much more colorful, so the use of 0.05-0.1 wt% content of the other materials tested are probably far too low to be able to get a clear signal from them.
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Appendix A: Lambert’s Beers Law

Lambert Beers law

\[ A = \varepsilon b c \]
\[ c = \frac{A}{\varepsilon b} \text{ mole/L} \]

Fibroin 1g/L : 1.064016 absorbance at 280nm

\[ c = \frac{1.064016}{473480 \text{ M}^{-1} \text{ cm}^{-1} \text{ m} \cdot \text{cm}^{-1}} = \frac{2.247224804 \times 10^{-6} \text{ M}}{391593.3 \text{ g/mole}} \approx 0.88 \frac{g}{L} \]

Fibroin 1g/L Filtered: 1.052417 absorbance at 280nm

\[ c = \frac{1.052417}{473480 \text{ M}^{-1} \text{ cm}^{-1} \text{ m} \cdot \text{cm}^{-1}} = \frac{2.222727465 \times 10^{-6} \text{ M}}{391593.3 \text{ g/mole}} \approx 0.87 \frac{g}{L} \]

Fibroin 2g/L: 2.269587 absorbance at 280nm

\[ c = \frac{2.269587}{473480 \text{ M}^{-1} \text{ cm}^{-1} \text{ m} \cdot \text{cm}^{-1}} = \frac{4.793416829 \times 10^{-6} \text{ M}}{391593.3 \text{ g/mole}} \approx 1.88 \frac{g}{L} \]

Fibroin 1g/L dialyzed: 0.286933 absorbance at 280nm

\[ c = \frac{0.286933}{473480 \text{ M}^{-1} \text{ cm}^{-1} \text{ m} \cdot \text{cm}^{-1}} = \frac{6.060087015 \times 10^{-7} \text{ M}}{391593.3 \text{ g/mole}} \approx 0.24 \frac{g}{L} \]

Fibroin 1g/L dialyzed filtered: 0.302169

\[ c = \frac{0.302169}{473480 \text{ M}^{-1} \text{ cm}^{-1} \text{ m} \cdot \text{cm}^{-1}} = \frac{6.38187463 \times 10^{-7} \text{ M}}{391593.3 \text{ g/mole}} \approx 0.25 \frac{g}{L} \]

Fibroin 2g/L dialyzed: 0.669168

\[ c = \frac{0.669168}{473480 \text{ M}^{-1} \text{ cm}^{-1} \text{ m} \cdot \text{cm}^{-1}} = \frac{1.4313297288 \times 10^{-6} \text{ M}}{391593.3 \text{ g/mole}} \approx 0.55 \frac{g}{L} \]
Appendix B: Preparation of Modified Food

**Rhodamine B**

Batch 1 (0.055 wt%)  
30 mg of Rhodamine B was dissolved in 2-3 ml of Milli-Q water and its was mixture together with 55 g of prepared silkworm chow yielding a purple/red colored food with 0.055 wt% Rhodamine B. The food was runny.

Batch 2 (0.05 wt%)  
25 mg of Rhodamine B was mixed together with 50 g silkworm chow with around 0.5 ml of hot tap water. Yielding food with 0.05 wt% Rhodamine B. The food was much more solid than the previous batch since of less water was used to mix. The color was also a bit less intense.

**PEDOT-S**

Batch 1 (0.02 wt%)  
2 mg of PEDOT-S was mixed together with 10 g of silkworm chow together with some water, yielding PEDOT-S 0.02 wt %. The color of the food became just slightly darker.

Batch 2 (0.02 wt%)  
2.5 mg of PEDOT-S was mixed together with 12.5 g of silkworm chow together with some water, yielding PEDOT-S 0.02 wt %.

Batch 3 (0.05 wt%)  
10mg of PEDOT-S was dissolved in 1 ml of Milli-Q water, it was then mixed together with 20g prepared silkworm chow yielding a concentration of 0.05 wt%. The color was almost the same as for the two previous batches even though that the concentration had increased.

Batch 4 (0.05 wt%)  
It was prepared in the same way as batch 3, with 20g silkworm chow.

**PEDOT-S + DMSO (Dimethyl sulfoxide)**

Batch 1 (≈0.05 wt% + 13 wt% DMSO)  
2ml DMSO was added to 15g silkworm chow and mixed together. The density of the DMSO solution is 1.1g/ml therefore 2ml results in 2.2g DMSO.

**PEDOT-PSS**

Batch 1 (0.1 wt%)  
1ml of a 1wt% PEDOT-PSS solution resulting in 10mg of PEDOT-PSS was mixed together with 10g silkworm chow, yielding food with 0.1wt% content of PEDOT-PSS.

Batch 2 (0.2 wt% + 5% DMSO)  
1 ml of PEDOT-PSS was mixed with 50µl DMSO(Dimethyl sulfoxide) and was then added to 5g of silkworm chow yielding 0.2 wt% PEDOT-PSS with 5% DMSO.

**Nile Red**

Batch 1 (0.035 wt%)  
3.5 mg of Nile Red was dissolved in some ethanol and then mixed together with 10g of silkworm chow, allowing the ethanol to evaporate before putting the food in the fridge. Yielding food with 0.025 wt% Nile Red. The color was just slightly darker than regular silkworm chow.

Batch 2 (0.05 wt%)  
2mg of Nile Red was dissolved in 1ml of EtOH plus 1 ml of Milli-Q water. It was then mixed with 4g of prepared silkworm chow, yielding 0.05wt% Nile Red.

Batch 3 (0.05wt%)  
2mg of Nile Red was dissolved in 1ml of ethanol and then was solution was then mixed together with 4g of silkworm chow.

**PTAA**

Batch 1 (0.05 wt%)  
25 mg of PTAA was dissolved in 1ml H2O mixed with 50 g silkworm chow, yielding a concentration of 0.05wt% PTAA in the silkworm chow. Even though that the PTAA solution had a clear red/yellow color, the food didn't change much in color.
TQ1P
Batch 1 (0.1 wt%)
Since TQ1P is quite insoluble, it was just spread out as evenly as possible in the food. 20.68 mg TQ1P powder was added to the 20.76 g chow and was then mixed so that the TQ1P yielding a content of 0.1 wt%.

TQ1-dimer
Batch 1 (0.05 wt%)
3.56 mg of TQ1-dimer powder was grinded with salt yielding a pink powder, 7120 mg silk worm chow was added and grinded together with the salt-TQ1-dimer powder yielding a content of 0.05 wt%.

Batch 2 (0.05 wt%)
3.25 mg TQ1-dimer was grinded with salt and 6.59 g silk worm chow just as for batch 1, yielding TQ1-dimer content of 0.049 wt%.

6T
Batch 1 (0.1 wt%)
Some mg of table salt was grinded to a fine powder, then 10 mg 6T was added and grinded together with the salt. Finally 10 g of prepared silk worm chow was added and also grinded so that the 6T powder could be evenly distributed. The content of 6T was then roughly 0.1 wt%.

Thioflavin T (0.05 wt%)
22.2 mg dissolved in 4.44 ml Milli-Q water to a concentration of 5 mg/ml. 1 ml of this solution was added to 10 g silk worm chow and was then mixed yielding 0.05 wt% Thioflavin T.

POWT
Batch 1 (0.073 wt%)
1.1 mg POWT in solution was mixed with 1.5 g silk worm chow yielding 0.073 wt%.

Batch 2 (0.025 wt%)
0.5 mg POWT in solution was mixed with 2 g silk worm chow yielding a POWT content of 0.025 wt%.

Batch 3 (0.025 wt%)
0.46 mg POWT in solution was mixed with 1.81 g silk worm chow yielding a POWT content of 0.025 wt%.

Batch 4 (0.05 wt%)
1 ml of 5 mg/ml POWT solution was mixed with 10 g silk worm chow to a concentration of 0.05 wt%.

Pyrrrole
Batch 1 (1.0 wt%)
20 µl of pyrrrole solution (density 0.967 g/ml) was added to 1 ml Milli-Q water and then mixed with 2 g silk worm chow to a concentration of 0.97 wt% pyrrrole.

Batch 2 (0.2 wt%)
20 µl of Pyrrrole solution (19.34 mg) was added to 1 ml Milli-Q water and the mixed with 10 g silk worm chow to 0.2 wt%.

EDOT
Batch 1 (0.1 wt%)
EDOT solution with a density of 1.331 g/ml (1.331 mg/µl). 3.90 µl of this solution was added to some grinded NaCl and was upon adding further grinded. 5.2 g silk worm chow was also added and grinded together yielding 0.1 wt% EDOT.

Batch 2 (0.05 wt%)
1.5 g of 0.1 wt% EDOT food (batch 1) was mixed with 1.5 g silk worm chow yielding roughly 0.05 wt% EDOT.

EDOT-S
Batch 1 (0.05 wt%)
3 mg of EDOT-S was dissolved in 500 µl Milli-Q water and it was then mixed with 6 g silk worm chow to 0.05 wt%.

Batch 2 (1.0 wt%)
7.02 mg EDOT-S was dissolved in 500 µl Milli-Q water and then mixed with 7.035 g silk worm chow to 1.0 wt%.

Batch 3 (0.1 wt%)
4.93 mg EDOT-S was dissolved in 500 µl Milli-Q water and then mixed with 4.9 g silk worm chow to 0.1 wt%.
Appendix C: Absorbance Spectra

The absorbance spectra is particularly useful to study the amount of protein in a solution since that protein has a clear absorbance peak at 280nm see figure 49. There is no peaks from the materials which the silkworms have been fed, meaning that either it has failed to integrate with the fibroin or that the concentration of it is too low. For example the Rhodamine B sample from batch 2_2 comes from a clear pink silk thread but no peak from the Rhodamine B can be seen although it should have a peak at 540nm but one does also see that the concentration of the fibroin is very low which could explain the lack of the Rhodmaine B peak. The same speaks for the rest of the samples tested, the concentration of the dissolved fibroin solution must be higher to get a detectable concentration of any material that coats the fibroin.

![Absorbance Spectra](image)

Figure 49. Absorbance Spectra. Cuvette length 1cm. All samples show some presence of fibroin protein corresponding to the peak at 280nm. No other peak can be seen that could further prove the presence of the sought materials which the silkworms were fed.
Appendix D: PTAA synthesis

Batch 1

9.035g NaOH was dissolved in 75ml of deionized water to a concentration of 3 Molar. See calculations below.

\[ n = c \times v \]

\[ n = 3 \text{ moles/L} \times 75 \times 10^{-3} \text{ L} = 0.225 \text{ moles} \]

\[ m = M \times n \]

\[ m = 40 \text{g/mole} \times 0.225 \text{ mole} = 9.00 \text{g} \]

1.07213g of 3-thiopheneacetic acid was dissolved in 40mL CHCl₃, and then 5.52g of FeCl₃ was added and was left for 5.5 hours in N₂ atmosphere allowing the polymerization to start. The yielded black solution was poured over into two 50ml falcon tubes and diluted with 5ml acetone in each. The two solutions where centrifuged at 3800 rpm for 5 min. Then the supernatant was discarded and the precipitate at the bottom was kept and dissolved in 30ml of acetone (See figure 50). It was then vortexed and centrifuged as before at 3800 rpm for 5 min. This step was repeated nine times, resulting in a clear supernatant. (See figure 51) The supernatant was poured out and the precipitate was stored overnight in a fridge. The next day 32 ml of 3M NaOH was added to the two falcon tubes with precipitate. They were then vortexed and centrifuged at 3500 rpm for 5min. The yielded supernatant had a dark red color and the precipitate was black. The supernatant was poured into two new 50ml falcon tubes and the precipitate was discarded. The supernatant was neutralized with 7ml of HCl (37%) in each falcon tube, vortexed and centrifuged at 3500 rpm for 5 min. The reason for dissolving the precipitate in NaOH and then neutralizing it is to further purify the sample. From this purification a black precipitate was yielded in one of the falcon tubes and the other sample formed a dark red solution with no traces of any precipitate. The sample with precipitate was washed two times with Milli-Q water (18.2 MΩcm), the solution was centrifuged between each wash. The water was poured out and the precipitate was stored in the fridge for 23 hours. A 1M NaOH in MeOH solution was prepared by dissolving 1.6g of NaOH pure pellets in 40 ml of MeOH.
The precipitate was dissolved in 5ml of MeOH and then 7.775 ml of 1M NaOH dissolved in MeOH was added and stirred for 120 minutes to allow PTAA-Na salt to form. Upon adding the NaOH you could clearly see a change in color from dark red to a light red yellowish color. The precipitate had not fully dissolved and therefore the solution was filtrated through a 0.45µm filter and a yellow solution was obtained. This solution was filtrated to a 50ml round-bottomed flask. The flask was connected to the rotary evaporator with a vacuum generated from a water-pump, and after 30min in the rotary evaporator no clear signs of evaporation could be seen. After heating the water bath to 40°C the evaporation started to take up speed, after 15 min the solution had turned to a more reddish color. After the rotary evaporation the clear red PTAA-Na solution was stored in the fridge over the weekend.

The PTAA-Na solution was frozen with liquid nitrogen. It was then lyophilized for 48 hours.

The yielded product from the synthesis were red flakes of PTAA-Na salt but there were also some white flakes which were placed higher up on the walls of the falcon tube after the freeze-drying step. These white flakes are probably due to excess NaOH. Dissolving the freeze-dried product in water yielded a red solution, see figure 52.

Batch 2

For batch 2 the same protocol as for batch 1 was used, the differences are that batch 2 required a total of 21 washing steps previous to adding NaOH. Another thing that differs is that when adding NaOH (1M) in MeOH the solution was only stirred for 90 minutes and it was not filtered either.

Method 1 yielded a red powder after concentration and the freeze drying yielded red flakes and no white flakes as compared to batch 1. Once these red flakes were dissolved in water a yellow solution was yielded. Batch 2 yielded much more product compared to batch 1.

Two different methods were tested to concentrate the polymer. Method 1: Rotary evaporation using a vacuum pump instead of a water-pump compared to batch 1. Method 2: PTAA-solution was mixed with tetrahydrofuran, and then centrifuged with a table top centrifuge yielding a yellow supernatant and black/red precipitate. The supernatant was poured off and the pellet was washed with tetrahydrofuran and blown dry with air. The washed pellet was finally dissolved in Milli-Q water yielding an orange to red solution.

Method 1 yielded red PTAA-Na salt on the edges of the flask, to obtain the salt it was necessary to dissolve it in water first, it could then be freeze dried forming the salt in powder form. Freeze-drying of the product from method 1 yielded red flakes as expected, and a stock solution of 1g/L PTAA Na-salt was prepared by dissolving 10mg PTAA Na-salt in 10 ml of Milli-Q water.
Appendix E: Tested substances

Rhodamine B

Feeding

Rhodamine B was mixed in the food, resulting in a red colored food. Only after a couple of hours after feeding, silkworms did turn pink. The silkworms did turn pinker in color as they ate more and more food. One thing that was noticed was that silkworms fed with 0.05 wt% Rhodamine B food was inactive and did not move around as much as the other silkworms. They also had a hard time to stand up on their legs and laid most of the time on their side. The silkworms did also failed in their attempts to spin a complete cocoon, these problems were not noticed in the control silkworms indicating that Rhodamine B caused these observed problems. Another silkworm fed with 0.05 wt% Rhodamine B but also with regular silkworm chow did not any of these behaviors. The effective concentration for this silkworm fed with both types of food is more like 0.025wt% and since it also was able to produce pink colored silk would mean that the disrupted behavior with the silkworms fed 005wt% RhB comes from the higher concentration. Silk produced from silkworm that had ate any degree of RhB modified food was pink in color which was expected from the red colored of RhB, see figure 54.

That the silkworm showed this inactive behavior was unexpected at first but it is not hard to assume that as the Rhodamine B changes charge as it goes from one environment to another e.g from the gut to the hemolymph due to shifts in pH, and as Rhodamine B changes charge it could perhaps disturb ion-pumps or other biological functions inside the silkworm.. One thing that could be concluded is that as the concentration of Rhodamine B increased it causes further and further disturbance in the silkworm behavior, an even higher concentration would most likely prove lethal.
Emission measurements

Figure 55 shows the photoluminescence measurement from batch 2_2 which was fed Rhodamine B modified food, with pristine silk sample as a control. The Rhodamine B sample show a clear and distinctive peak at 575nm, the intensity of the emission from the sample is so large that the control sample from pristine silk looks like a straight line. This is a clear result that the incorporation of rhodamine B has worked and it was expected since it has already been proven that it does work, it can therefore be used as a reference for the other materials that was tested.

![PL RhB](image)

**Figure 55** Photoluminescence measurement, excitation wavelength 405nm. All samples gathered from native spun silk.

Figure 56 shows the emission spectra of control samples from the fibroin dissolving agent Ajisawa’s reagent and Rhodamine B dissolved in the Ajisawa’s reagent to see how the peak from it would look in the dissolving medium. What is unexpected is that the Rhodamine B controls produce a double peak, the reason for this is unclear but could be due to that the sample is not 100% pure and there are some impurities that produces the double peak. But that the both peaks are similar in height raises further questions since impurities present would be in a much smaller concentration and therefore the emission form them would also be smaller. Another explanation is that Rhodamine B is dissolved in Ajisawa’s reagent which contains CaCl₂, water and ethanol and these compounds could perhaps influence the emission in some way.

The sample from batch 1_1 shows a clear emission from Rhodamine B see figure 57. If compared to the emission from the control of Ajisawa’s reagent and dissolved fibroin it is several magnitudes higher. The concentration of fibroin is around 7 times lower than the native controls which then speak for that the emission seen in the peak comes mainly from Rhodamine B and not fibroin. If one compares to the Rhodamine B control in figure 52 it is obvious that the Rhodamine B coated fibroin
in figure 57 only has one peak. It could be so that the possible contamination seen in figure 52 from Rhodamine B does not bind to the fibroin which would explain why it isn’t seen in the silk sample.

**Figure 56** Emission spectra from control of Rhodamine B and the fibroin dissolving agent Ajisawa’s reagent. Excitation wavelength 550nm. Both controls were tested in two different conditions one that had been stored at ambient temperature (ambient) for 2 days and the other one that had been stored at 65°C for 2 days (heated). The controls that had been heated has a higher emission compared to the ambient controls. Conc. of Rhodamine B control is 0.05mg/ml

**Figure 57** Emission spectra. Excitation Wavelength 540nm. Entrance and exit slit 5nm. Rhodamine B (Batch 1_1) fibroin concentration 2mg/ml. Even thou the pristine sample produces a slight peak when excited at 540nm it is far less than the Rhodamine B sample even if the difference in fibroin concentration is about 7 times. Which then speaks fro that the emission from the Rhodamine B sample actually comes from Rhodamine B.

The emission form the silk of batch 2_2 which also was fed Rhodamine B modified food can be seen in figure 58, this is another result which shows the clear Rhodamine B peak, although this time the sample was excited at 500nm. The concentration of the fibroin in the Rhodamine B sample was roughly 4 times greater than the native control. But the emission pattern from the two samples differs a lot meaning that the emission peak seen in the Rhodamine B sample comes from...
Rhodamine B and not only from the dissolved fibroin.

**Figure 58 Emission spectra.** Excitation wavelength 500nm. Entrance and exit slit 5nm. Rhodamine B (Batch 2_2) conc. 1mg/ml fibroin. Pristine (Batch 5_2) conc. 0.23 mg/ml fibroin.

**Submerging**

It was possible to coat silk fibroin thread by submerging them in 0.05mg/ml Rhodamine B solution heating to 65°C for 24 hours. The yielded thread can be seen in figure 53 and shared to same intense color as the silk produce by the silkworms.

**Nile Red**

**Feeding**

Silkworms fed Nile Red modified food did produce colored silk in some cases, it seemed that the Nile Red did stick loosely to the walls of the silk gland and did come off upon washing with water. Figure 59 shows silk from batch 4_4 which appears pink in color. This could be due to a contamination considered that batch 4_4 was stored in the same vinegar solution before dissection as batch 1 which was fed Rhodamine B. Since three other silkworm which was also fed 0.05 wt% Nile Red did not produce any colored silk it is highly likely that the result from batch 4_4 could be because of contamination. Silkworms fed with a higher concentration than 0.05wt% Nile Red in the food did produce a purple colored silk, but this color came right off upon washing must likely due to Nile Red high hydrophobic properties.
Emission measurement

Figure 60 shows control samples of Nile red dissolved in ajisawa’s reagent (conc. 0.05mg/ml) and it is obvious that the Nile red peaks at 660nm in the medium when excited at 550nm. Figure 60 shows photoluminescence measurement on native silk spun by silkworm fed Nile red modified food. The only sample that gives a clear emission peak is from batch 4_4, but it is far blue-shifted compared to the control seen in figure 60, not even the sample from batch 2_1 which was also fed Nile red did not show any emission peak other than from the silk itself. As figure 62 and 63 demonstrates the peak from batch 4_4 correlates better with the peak from Rhodamine B but as previously mentioned there is a chance of contamination from Rhodamine B. Figure 62 and 63 speak for that this is the case but the red shift between the presumed Nile red peak and the Rhodamine B is odd if it the Nile red would only come from Rhodamine B contamination. If the peak from the batch 4_4 (Nile red) comes from contamination it still shows a difference emission pattern with the red shift and also that there seems to be a small bump in the end of the right slope of the peak from batch 4_4. This bump is not as visible in the Rhodamine B sample from batch 2_2 which could speak for that there might yet be some Nile red in the sample from batch 4_4. The bump sits at around 630nm which is at least closer to the emission peak from the control sample of Nile red seen in figure 60 which peaks at 660nm. The emission peak from the sample from batch 3_1 (see figure 64) which also was fed Nile Red shows a small but yet distinctive peak at 610-630nm when excited at 550nm, this result indicate that there could be some Nile Red present in the silk, but the amount is very low. The peak seen in figure 64 correlates with the bump from the batch 4_4 sample, but why this peak is absent from batch 2_1 (see figure 61) is left unknown. It seems that if the peak originates from Nile red the amount of it seems fairly low since of the low intensity of the peak, but since the results are not all uniformly clear it is hard to say for certain if the incorporation of Nile red worked.

![Emission Nile red controls](image)

Figure 60 Emission spectra. Excitation wavelength 550nm. The concentration of Nile Red in the controls are 0.05mg/ml dissolved in Ajisawa“s reagent.
Figure 61 Photoluminescence measurement of Nile Red samples from batch 2_1 and 4_4, with pristine silk as a control. Excitation wavelength 405nm.

Figure 62 Photoluminescence measurement, exciting wavelength 405nm. Filter 420nm, exposure time 5 seconds. Comparison between Batch 2_2 and 4_4, batch 4_4 is slightly red shifted.
**Coating via grinding**

What speaks for that the peak seen in figure 64 actually comes from Nile red is that it was possible to coat fibroin with Nile red via grinding, this will generate a Nile red peak at around 620nm as seen in figure 66. This correlates well with the peak from batch 3_1, but there is a clear difference in emission intensity which would mean that a very small amount of Nile red has coated the fibroin from sample 3_1.

This post-coating was done by grinding freeze dried fibroin powder with Nile red forming a light purple powder. As described in the method segment the dissolved powder was precipitated by adding it to ammonium sulfate. The fluorescence and absorbance of this precipitate was then analyzed. Figure 65 shows the absorbance spectra, the stock solution shows a peak between 450-
600nm which comes from Nile Red. The supernatant shows no signs of any Nile Red but there is some protein in it which perhaps is not so strange, since some small fibroin fragment could be left in the supernatant. The precipitate shows that the amount of fibroin is around the same as the diluted stock solution which would be expected since most of the fibroin should precipitate out in the ammoniumsulfate. The Nile Red peak cannot be seen and could be of big aggregate of precipitate which gives a high absorbance which drowns the Nile Red peak, but since there are not any clear Nile Red peak in the supernatant it would be safe to assume that the Nile Red still sticks with the fibroin both since of the emission spectra but also that the precipitate has a clear red color.

Figure 66 shows the emission spectra using an excitation wavelength of 550nm, the spectra clearly shows that the Nile red has coated the fibroin since the emission is much larger in the precipitate than it is in the supernatant. An explanation for the higher emission from the precipitate compared to the stock solution is because in the stock solution you have a fully dissolved Nile red coated fibroin, but the precipitate was impossible to redissolve in water therefore containing big aggregates that is the culprit of the increased emission. This can also be seen in figure 65 which shows the absorbance spectra of the precipitate, stock solution and supernatant, it is also obvious here that the aggregation of the precipitate distorts the signal. In the stock solution the peak from Nile Red at 500nm is clearly seen but in the sample with precipitate it becomes harder to tell if any Nile Red is present from the absorbance spectra alone. The concentration of fibroin in both the stock and precipitate solutions are almost the same which would be expected if all the fibroin does precipitate out as it mixes with the ammonium sulfate. The supernatant does not show any signs of Nile Red but perhaps a little degree of fibroin.

**Absorbance Nile red**

![Absorbance Nile red](image)

*Figure 65. Absorbance spectra of fibroin coated with Nile red. All samples diluted 10 times.*
Submerging

It was possible to coat fibroin threads in a 0.05mg/ml Nile red solution at 65°C for 24 hours. It yielded pink to purple silk thread as can be seen in figure 67. It is possible to coat silk with Nile Red via post-methods such as grinding and submerging in solution. But if it is possible to incorporate Nile Red into the silk fiber via feeding is still unclear, some results point to its possibility but the majority of the results give inconclusive results. The case seems to be that the Nile Red can travel to the walls of the silk gland which was seen with the higher concentrations of Nile Red but degree of Nile Red in the silk fiber is extremely small or absent.

POWT

Feeding

The silkworm themselves have not shown any color change but especially the silk glands had a faint pink color, perhaps not covering the silk completely uniformly but at least in spots. The color became much clearer for batch 6_1 after degumming and air drying its silk glands see figure 68.

Emission measurement

Figure 69 shows photoluminescence measurement taken with an excitation wavelength of 405nm from a sample of batch 6_1 silk which was fed POWT modified food. Even if the signals from the POWT sample produces a higher emission than the pristine control it still share the same emission wavelength, the increased and more visible peak could be because of measurement problems. These
problems come from that it is hard to excite the same amount of silk thread from sample to sample with the PL-equipment. It is quite plausible that the emission peaks from the pristine control sample comes from a smaller amount of silk which will result in a smaller emission at 480-490nm from the silk fibroin. Even so the emission from POWT should sit at around 540nm (see figure 70) compared to how it looks in figure 69.

![PL POWT](image)

**Figure 69** Photoluminescence measurement, excitation wavelength 405nm.

![Emission POWT controls](image)

**Figure 70** Emission spectra, excitation wavelength 400nm.
Figure 71, 72 and 73 shows another interesting phenomena, as the concentration of the POWT increases the emission decreases, if this is because of quenching is hard to tell. POWT has the ability to quench when it becomes doped but this should not be the case here. The only explanation given is either that the samples have been swapped or that POWT is present in a doped state which causes quenching but that seem highly unlikely. The measurement cannot prove that any POWT is present either since it peak at 560-570nm is absent, but why a higher concentration of dissolved silk fiber would result in a decreased emission pattern if not any other material is present remains elusive.

**Figure 71** Emission spectra. Excitation wavelength of 350nm. Entrance and exit slit 5nm. Fibroin conc. Pristine: 3.3mg/ml, POWT (Batch 6_1) : 12.6mg/ml POWT (Batch 7_9): 6,3 mg/ml.

**Figure 72** Emission spectra. Excitation wavelength of 400nm. Entrance and exit slit 5nm. Fibroin conc. POWT (Batch 6_1) : 12.6mg/ml POWT (Batch 7_9): 6,3 mg/ml
PTAA

The silk produced from silkworm fed PTAA modified food (0.05wt%) didn’t show any visual signs of any PTAA present. Figure 74 shows the emission from batch 4_6 silk, taken with the photoluminescence setup mentioned before with an excitation source of 405nm. Just as for the POWT sample(batch 6_1) the sample from batch 4_6 (PTAA) shows a peak at 480nm and the morphology is quite similar to the pristine sample. When looking at figure 74 which show the emission spectra from a control sample of PTAA, one can see that the emission peak from PTAA has its maximum at 470nm when excited at 400nm (see figure 75). Fibroin also has an emission peak at around the same wavelength when excited at 400nm (see figure 76), this will mean that it becomes very hard to get any conclusive results about PTAA presence in the silk. The only indicator that can be given is if the signal is significantly higher than for the pristine silk control, and for the batch 4_6 sample it is impossible to determine anything. The results from fluorescence measurement on dissolved fibroin from silkworms fed PTAA modified food did not give any conclusive results either, see figure 76. The PTAA sample (5_4) and the Pristine sample both share a similar concentration of dissolved fibroin, 3.9mg/ml respectively 3.3mg/ml. Both also show as similar emission spectrum the reason the pristine solution has a higher output could either be because of measurement fault in both in weighing the silk fibers but also in the volume of dissolving agent. It also comes down to how good the degumming step went, how much sericin is still left which would interfere with the concentration but also that the fibroin in the silk tends to swell when washed and even after air drying there could be some water still trapped in the silk. The figure shows that there is not any evidence that PTAA have successfully coated the fibroin, and even so it would be hard to tell since both PTAA and fibroin have their emission peaks at the same interval. One would expect that if PTAA had managed to coat the fibroin the output would be even higher which it is not in the case here.
Figure 74 Photoluminescence measurement, excitation wavelength 405nm.

Figure 75 Emission Spectra, excitation wavelength 400nm.
Figure 76 Excitation wavelength of 350nm. Fibroin conc. Pristine: 3.3mg/ml, PTAA(5_4): 3.9 mg/ml.

Just as for the Nile Red coated fibroin, PTAA powder was grinded together with fibroin powder and precipitated out in ammonium sulfate. The emission spectra (see figure 77) shows that the main amount of PTAA is still left in the precipitate but compared to Nile Red the supernatant contains more of the fluorescent molecule. PTAA is a water soluble molecule and therefore some of the PTAA will dissolve in the water instead of being coated to the fibroin since both has a very similar pI and the electrostatic forces will not help them to bind to each other.

Figure 77. Emission spectra of fibroin coated with PTAA by grinding. Excitation wavelength 400nm.
The absorbance spectra for the PTAA grinded silk can be seen in figure 78 and it does show that the precipitate contains both fibroin (peak at 280nm) and PTAA (peak at 400nm). A difference compared to the Nile Red samples is that for PTAA the precipitate does not have a fibroin concentration which is similar to the stock solutions instead it is drastically smaller in the precipitate. The big aggregates in the precipitate sample produce an overall higher background absorbance, which is easily seen from wavelength 550nm and above. Figure 79 shows the PTAA peak for all the samples but normalized so that the morphology can be compared. Both the precipitate and stock solution samples which both contains PTAA coated silk shows similar and overlapping PTAA peak, both samples also shows that they is a blue-shift compared to PTAA dissolved in H₂O and ammonium sulfate. This could be an indication that PTAA have managed to coat and bind to the fibroin via grinding since there is a blue-shift in absorbance, which is the case when insulin is added to PTAA solution.[25] The supernatant clearly shows that it does not contain any amount of PTAA at all since there is no peak.

**Absorbance PTAA**

![Absorbance Spectra](image)

Figure 78. Absorbance Spectra, cuvette length 1cm.
EDOT derivatives

EDOT

EDOT proved lethal to the silkworms, they died after a couple of feeding, where the once fed 0.05wt% managed to survive for a couple more days compared to the once fed 0.1wt%. Because of the lethality of EDOT no further test was conducted. The dissection did not yield any conclusive results, the silkworm’s body was a bit more fragile compared with the control silkworms but this was mostly due to that decomposition had begun. Since the silkworms died early their silk glands had not grown much in size making them small, fragile and very hard to handle. The silk glands were yellowish in color and transparent, but they did not differ too much from glands taken from control silkworms which usually are either completely transparent or yellow in color.

EDOT-S

EDOT-S was easier than EDOT to handle due the water solubility. The silkworm was also more active and eating more of the food compared to the ones fed EDOT. One of the silkworms fed EDOT-S died and the other one started to spin a cocoon but was not able to complete it (see figure 80), suggesting that the EDOT-S could have affected it in some way. The silk glands gathered from dissection were (see figure 81) similar to glands gathered from controls.

Figure 80 Picture of silkworm fed EDOT-S 0.05wt% that have failed to spin a complete cocoon.
PEDOT derivatives

**PEDOT-S**

PEDOT-S modified food was not incorporated into the silk. Dissection did not indicate where it may have ended up. The only difference, compared to the control, is that the silkworms fed PEDOT-S produced a darker spilling, which indicates that the PEDOT-S pass right through the gut without any uptake. No part of the silkworm’s body contained any blue color which is associated with PEDOT-S. The silkworms did not have any problem eating the PEDOT-S modified food and they produced normal cocoons. Since there was not any visible proof that PEDOT-S had coated the silk it was decided to test if DMSO could help in the transport.

**PEDOT-S + DMSO**

To see if DMSO could help with the transport of PEDOT-S out of the gut of the silkworm it was mixed into the food at various concentrations. Two silkworms were fed modified food containing 0.05wt% PEDOT-S and around 10-15wt% DMSO. Where one of the silkworms did die after 4 days of feeding, the one that did not die managed to reach the spinning state and they were examined via dissection. The silk glands did not differ from the control and no signs of any blue or even dark brown color could be seen. One silkworm was also fed modified food containing 0.1wt% PEDOT-S dissolved in 10% DMSO. The silkworm managed to spin a cocoon but there was no sign that PEDOT-S had coated the silk. Finally, one silkworm was fed modified food with a content of 0.5wt% PEDOT-S dissolved in 10% DMSO solution. The food was pitch black in color see figure 82. Even though the food contained such high amount of PEDOT-S neither was the silkworms body nor glands colored in any way when inspecting via dissection.

**PEDOT:PSS + DMSO**

Silkworms were also fed food containing PEDOT:PSS with some DMSO, and it did not give any different results just as all the PEDOT-S samples tested, therefore it was decided that no further test would be conducted with PEDOT:PSS.
Pyrrole

Pyrrole has the property that it will turn from colorless into blue as it becomes oxidized. [20] Silkworms were fed chow containing pyrrole monomers to see if it would produce blue colored silk. The silk that were extracted did not show any sign of any oxidized pyrrole into polypyrrole and even after submerging the silk in ammoniumpersulphate, which is a strong oxidizing agent, the silk was still white colored meaning that the incorporation of pyrrole had failed.

Thioflavin T

Thioflavin T was mixed into the food to a concentration of 0.05 wt%. Two silkworms were fed it. But after just one day of eating Thioflavin T both silkworms were dead. This was not clear until the following day when they had started to turn black because of decomposition. The two silkworms were submerged in a vinegar salt solution to preserve them for dissection. The reason for that the silkworms was not submerged the day before was that the two silkworms were laying on their side, like the silkworms which was feed rhodamine B and since both RhB and thioflavin T is quite similar in their molecular structure it was assumed that they could show the same behavior. The difference compared to the silkworms fed with rhodamine B was that they survived even if they were inactive most of the time. Upon dissection of the silkworms fed with thioflavin T the silk glands looked like regular silk glands, with a slightly yellow transparent color. The silkworms showed dark black spots on their body which were hard and most likely due to coagulated blood.

Most of the substances tested were not lethal to the silkworms. Thioflavin T was the most lethal substance, even though it is thought to have lifespan elongating properties. The silkworms died because that at a high enough concentration Thioflavin T is toxic. [30] It would be necessary to test even lower concentration but this was not tested because of a limited supply of silkworms.

6T

One silkworm was fed 6T modified food. Since 6T is a highly hydrophobic material that is quite insoluble, it was grinded together with salt and then mixed into the food. The silkworm fed the 6T modified food did not show any color changes and it lived and moved similar to the controls, and managed to spin a normal looking cocoon. The color of the cocoon was white similar to the controls. So there was no clear evidence that 6T had been taken up by the silkworm (see figure 83), there is no clear difference in the emission spectra from the 6T sample compared to the control sample. 6T is another material that failed to incorporate into the silk.
Figure 83 Photoluminescence measurement, excitation wavelength 405nm.

TQ1 derivatives

TQ1P

The silkworms were fed TQ1P modified food, the TQ1P was spread out as evenly as possible into the food but it still were quite big pieces of the dried powder of TQ1P which could have been too big for the silkworm to digest. TQ1 is a big molecule compared to the other tested and in its polymer form TQ1P it becomes even larger, and since there silkworms produced regular white colored cocoon a smaller variant of TQ1 was tested mainly TQ1-dimer.

TQ1-dimer

Since TQ1P is a quite large polymer, TQ1-dimer was tested because of its smaller size. TQ1-dimer was also more evenly spread into the food by grinding it with salt. TQ1-dimer modified food did not produce any conclusive results in a first glance; all the silk glands were normal (white/yellow) in color and no signs of any red or pink silk which would be expected since the TQ1 powder is pink/light red colored. The results from dissecting the silkworms was a bit varied, the first one dissected (6_5) did not show any color change in either the silk core or walls but there was some red color in the rear entrails of the silkworm probably suggesting that the TQ1-dimer does not pass out from the gut but instead collects in the rectum of the silkworm (see figure 84). Batch 7_1 did not produce any color change either but the silk gland was extremely hard and brittle even inside the silkworms body just like for the Rhodamine B
fed silkworms. Batch 6_4 did spin after only one day upon feeding TQ1-dimer modified food, and it only showed that the silk was fragile compared to pristine silk. Batch 6_4_1 died after eating TQ1-dimer modified food for four days, the dissection could not produce any conclusive results if TQ1-dimer killed it since there was no clear build up that could be seen. The silk glands were very small and fragile so it could have been that the silkworm was sick.

No clear evidence could be found that TQ1-dimer had managed to coat the silk.