Differently structured polystyrene-b-poly(methacrylic acid) polymeric micelles and their possible use as a pH sensitive drug delivery system

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Motivation for the research

In 2021 I completed my Bachelor degree in Biomedical Technology. For my Bachelor's Research Project I researched the foreign body response to biodegradable polymers orchestrated by macrophages and the influence of anti-inflammatory drugs on this process. I found the whole project very interesting and wanted to research something similar in my Master Research Project. For this I contacted Prof Dr Patrizio Raffa who's research group studies new smart and sustainable polymeric products. For some publications he has worked together with the supervisor from my Bachelor's Project. Patrizio's research group is however more focused on engineering of new polymers but Patrizio himself was interested in researching possible applications of his amphiphilic polymers. So together we designed an experiment where I would test out the possibility of his polymers to be used as a pH sensitive drug delivery system.

Abstract

There is a problem with standard drug administration. Drugs enter healthy organs that should not be involved in a treatment and cause toxic effects. Polymeric micelles (PMs) show promising results for drug delivery applications due to their separated core/shell structure and ability to respond to external or internal stimuli when they are in vivo. In this study, different types of polystyrene-b-poly(methacrylic acid) (PSPMAA) pH sensitive polymers were used to prepare micelles and the possibility to use the PMs as a pH sensitive drug delivery system.

Size, surface charge and encapsulation efficiency of all micelles were determined. All micelles show good mean encapsulation efficiencies with values between 78.16% and 85.12%, mean sizes between 96.55 and 319.00 nm and mean zeta potential between -30.55 mV and -38.15 mV.

To test a possible controlled release mechanism, all of the micelles were put in solutions of pH ~7 and pH ~4 for 24 hours. Size and zeta potential was measured at the start and at the end of the release experiment for all PMs. Results show that all of the PSPMAA PMs stayed intact in the pH ~7 solution. The PSPMAA diblocks and the PSPMAA triblock stay intact both in the acidic and neutral pH solution. The other PSPMAA PMs released most of the encapsulated fluorescent probe in the more acidic environment. Comparing our results with other studies, our PMs might be suitable for biomedical applications.

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1. Introduction

A more extensive literature review can be found in the Supplementary Material under '. Literature Review'.

1.1 Problem standard drug administration

The current way of drug delivery is not optimal according to multiple scientific publications (1–3). A major problem that conventional pharmacotherapy encounters is the aspecific distribution of drugs where drugs enter healthy organs that should not be involved in the treatment and causing toxic effects (2). To maximize the response and reduce side effects, the medicine should ideally only be exposed to the tissues that contain the pharmacological target (4). The concentration of drugs in the blood stream rises quickly after administration and short after already declines (1). These issues result in a decrease in the accessibility of drugs at their intended location, which lowers the likelihood of successful treatment. Additionally, these problems may cause undesirable side effects in other parts of the body (1,2,5,6).

1.2 Polymer-based drug delivery systems

Polymer-based drug delivery systems (DDSs) are one of the most promising systems that show good results for tackling various problems with conventional medication delivery (7). Due to the large variety in polymers, polymer-based DDSs can be used for various drug delivery applications (8).

Polymeric micelles (PMs), an example of a polymer-based DDS, have drawn a lot of scientific attention as a flexible nanomedicine platform with enhanced pharmacological and effective response in the area of drug delivery (5,7). PMs show promising results for drug delivery applications due to their separated core/shell structure and ability to respond to external or internal stimuli when they are in vivo (2).

1.2.1 Polymeric micelles

About thirty years ago, it was first thought that micelles produced by amphiphilic block copolymers in aqueous solution could serve as carriers for hydrophobic drugs (9). Since then, many studies have been conducted on the use of amphiphilic block copolymers in the synthesis of PMs as possible treatment administration. A number of new block copolymers have been developed for micelle-based DDSs. As a result of these developments, more PM based DDSs are undergoing either preclinical animal trials or clinical trials in preparation for regulatory approval or have already been approved (9).

1.2.1.1 Structure

PMs are mostly formed of diblock, triblock or graft amphiphilic polymers that have both hydrophilic and hydrophobic components. Amphiphilic polymers will form amphiphilic micelles formed by hydrophobic interactions (8). In aqueous solutions amphiphilic block copolymers at a certain concentration will start to self assemble into PMs (10). The hydrophilic part of the amphiphilic block copolymer will direct outwards which forms the shell while the hydrophobic part will direct inwards and forms the core (8). The separated core/shell structure makes PMs able to capture unstable and insoluble drugs (11,12). The selection of the hydrophobic and hydrophilic segments of the copolymer can be adjusted for both site-specific delivery and controlled drug release (6).

1.2.1.2 Preparation

The technique used to prepare PMs is dependent on the properties of the block copolymer, with factors such as the method of addition, ratio of aqueous to organic materials, and copolymer concentration playing a crucial role. Hence, it is essential to optimize these factors to achieve a standard recipe that can produce PMs with suitable physicochemical and functional properties (8,13). The process of encapsulating drugs in PMs can be performed during synthesis or in a subsequent stage, depending on the technique and properties of the drugs. Directly dissolving is the simplest technique for preparation of PMs; others include dialysis, (oil-in-water) solvent evaporation, freeze drying, the nanoprecipitation method and more (7,8,13). In our experiment we used the nanoprecipitation method.

The nanoprecipitation method involves combining two miscible solvents, one of which acts as a good solvent (typically an organic solvent like ethanol, isopropanol or acetone) and the other as a non-solvent for the polymer, such as water (14). The organic solvent is then removed via dialysis or evaporation, resulting in the formation of PMs in the solution (15,16). Additional preparation methods can be found in the supplementary material.

1.2.1.3 Characterization

PMs are for instance characterized by their size, surface charge and encapsulation efficiency which will be discussed below.

The chemical composition, length of the hydrophilic and hydrophobic segments as well as the molecular weight of the block copolymer can all have an impact on the size and morphology of the resulting PMs (10,17). Most PMs have a diameter ranging within 5 and 200 nm (11,18). Above 200 nm they can still be considered PMs however this might lower the in vivo performance which is explained in the next section and more in depth in the literature review in section '1.2.1 Retention and Release' (19). Surface characteristics such as surface charge (zeta potential) have an influence on the behaviour of PMs in vivo (10). A stable system that inhibits nanoparticle aggregation is thought to consist of nanoparticles with zeta potentials greater than +30 mV or less than -30 mV (20). The most popular methods for measuring size and zeta potential of micelles is dynamic light scattering (DLS) (10).

The percentage of (model) drug that eventually successfully gets encapsulated by the PMs is called the Encapsulation Efficiency (EE). EE is calculated as a percentage of drug that gets encapsulated to the total amount of added drug. Both the characteristics of the polymer and the encapsulated (model) drug themselves can affect how well a drug is encapsulated (7).

1.2.1.4 In vivo performance

Stability in the bloodstream

As mentioned before most PMs have sizes between 10 and 200 nm (11,18). Above 200 nm they could still be considered PMs however this can lower the in vivo performance (19).

PMs might be easily eliminated from the body by biocomponent adsorption and/or complement activation (9). Proteins and other biocomponents have a harder time adhering to smaller sized particles (11). The same applies to complement activation, smaller particles are less likely to induce complement activation (21). According to La-Beck et al. nanoparticles smaller than 30 nm are not likely to induce activation of the complement system, which means that these PMs are more likely to arrive at their destined release location (22).

According to many different studies PMs with a negative surface charge have a better blood compatibility and are removed from the plasma at a lower rate compared to PMs with neutral surfaces (23)(24). Many serum proteins in the blood have a net negative charge, which explains why positively charged PMs adsorb more proteins than other PMs (25). Micelles with positive zeta potential, on the other hand, are characterised by relatively low stability in biological fluids due to non-specific protein binding and higher aggregation in vivo (7). Cell membranes of phagocytic like macrophages are negatively charged. Nandhakumar et al. discovered that positively charged NPs interact more with phagocytic cells and are therefore cleared out of the blood more easily than negatively charged NPs (25).

Cellular uptake

With smaller particles (approximately 15-30 nm) and bigger particles (about 70–240 nm), it has been demonstrated that cellular particle uptake was lower (26). According to Zhang et al. micelles with sizes between 10-100 nm are able to be taken up by cancerous cell (21). A study by Raveendran et al. showed that micelles up till 200 nm could be taken up by cancerous cells (27). So it seems that according to several studies it is possible that different PMs with sizes between 10-200 nm are able to be absorbed by cells.

Some PMs are slightly negative while others very negatively charged. The effect of surface charge on cellular uptake seems to be conflicting. According to different studies compared to PMs with neutral surfaces, PMs with a slightly negative surface charge undergo more efficient accumulation in tumour tissues (23,24). And according to Zhou et al. PMs with the most negatively charged surface showed the highest uptake by human bladder tumour cells (28). In contrast to these observations other studies describe that positively charged NPs were taken up more than negatively charged NPs due to electrostatic interactions with cell membranes (24,25). It is probable that other factors like particle size, nature of charged groups, charge density etc. have an influence on cellular uptake (25).

Burst release

Drug molecules carried by DDSs are hydrophobic and blood is a polar solvent, therefore it is anticipated that drug molecules will diffuse into micelle core and remain there during circulation (29). However in several controlled release studies, a significant initial batch of drug molecules is released right away into the medium before the release rate stabilises (30). After administration into aqueous media swelling, erosion, diffusion and dissolution processes are frequently what control the release. Although swelling, dissolving, and partitioning result in rapid release, diffusion and erosion have been identified as the main mechanisms in formulations for sustained release (31). A PM frequently features a drug concentration gradient from the edge to the core at the moment of administration. Where more drug molecules are situated in the core. As a result, when the PM is placed into the release medium drug molecules transported at the interface or in the corona of the PM will release more quickly than those carried inside the core (29,31). Burst release is the name given to this method of release (29).

Burst release increases the initial drug administration while decreasing useful lifespan of the DDS (30). This provides difficulties for the timely and site-specific administration of drugs. Moreover, it disrupts the intended release kinetics and commonly appears as a barrier for a sustained release. Moreover, the production expenses associated with the discarded portion of the medications might make a formulation financially unviable. As a result, burst release has frequently been identified as an undesirable impact, particularly for DDSs destined for prolonged release (31).

1.2.2 Stimuli-sensitive PMs

Numerous techniques have been developed to facilitate the complete release of the drugs encapsulated in a PM. One of the most effective approaches is the creation of stimuli-responsive PMs, which enable precise drug targeting and optimal release. Stimuli-responsive PMs can respond to various biological stimuli within the intracellular and extracellular environment, including pH, changes in redox potential and enzyme concentration. They can also be triggered by external stimuli, such as magnetic fields, light, temperature and ultrasound (32). Additional information on stimuli-sensitive PMs can be found in the supplementary material.

1.2.2.1 pH sensitive PMs

In a pH sensitive PM the hydrophilic part of a block copolymer usually contains an ionizable chemical group such as an amino or carboxylic group (33). At physiological pH of 7.4 these groups are deprotonated and the polymer is hydrophilic which makes the core/shell structure hold its structure in an aqueous environment. In contrast in an acidic environment the ionic groups of these polymers are protonated which makes them al positively charged. This positive charge on the backbones of all these polymers in the shell makes them repel each other and the core/shell structure opens up (1,9). pH sensitive PMs have been widely researched and will also be a focus point in this research project (4).

Sensitivity to pH changes can be effectively utilized for designing PMs that can disassemble and release their load when encountering a change in pH in the environment (3). It is well acknowledged that the pH levels vary in different parts of the human body (33). For instance in the different parts of the gastrointestinal tract, where the pH is highly acidic in the stomach (~1-2) and in the other parts more neutral (6-7) (33,34). At a more cellular level there are also variations in pH values, for instance the pH of cytosol is neutral (7.4) compared to a more acidic environment in intracellular organelles such as the Golgi apparatus (6.4), endosomes (5-6) and lysosomes (4-5) (3,33). Furthermore certain abnormal tissues like tumour tissue exhibit higher pH levels (4-5) than blood and normal tissues (7.4) due to rapid proliferation causing a deficit in oxygen and therefore an overproduction of lactic acid. The same applies to healing wounds, where after 60h into an inflammatory process a drop in pH (6.5) can be seen (4,33).

1.2.2.2 Polystyrene-b-poly(methacrylic acid) PMs

Poly(methacrylic acid) (PMAA) is a hydrophilic polymer and polyelectrolyte that is commonly used in the formation of PMs (33,35). PMAA can be linked to long hydrophobic polymers such as polystyrene (PS) (35). Strong hydrophobic polymers like PS form PMs that will be kinetically frozen in aqueous solutions and their stability and properties rely solely on the polyelectrolyte behaviour of the PMAA in the shell (36).

Every PMAA unit contains a carboxylic group that can be ionized (37). The carboxylic groups are ionised and charged at physiologic pH 7.4, which makes the polymer soluble. As explained in the previous section at acidic pH the carboxylic groups are protonated. The pH where PMAA will precipitate is around 4.6 (38). PMAA can therefore be employed as a pH-sensitive corona-forming component to facilitate precipitation of the micelle and drug release in an acidic environment (9).

1.2.3 Fluorescent probes as model drugs

Fluorescent probes have attracted a lot of attention as possible model drugs. They are appealing because of their high sensitivity and efficiency. Using fluorescent probes is cheaper than using actual drugs and fluorescent probes can be also detected by affordable equipment like UV/VIS and fluorescent spectroscopy (39).

An example of a fluorescent probe that has been used in various release studies is Coumarin-6 (C6). C6 is a very hydrophobic fluorescent probe and has been reportedly loaded into PMs (40–45).

1.2.4 Current state

There are very few pH sensitive PMs currently available for clinical use due to difficulties in either retention of the PM in the bloodstream or release of drugs at targeted site, or both. In the current research stage pH sensitive PMs are not able to achieve complete treatment of tumours (46). However some examples of pH sensitive PMs that do show potential in animal trials are shown in Table 1.

Table 1: AC: acryloyl carbonate, AP: tumor-targeting peptide, CS: chitosan, FOL: folate, F127: Pluronic F127 triblock from poly(propylene oxide) and poly(ethylene oxide), Gal: galactosylated, gPNIPAm: graft-poly(N-isopropylacrylamide), MPEG: methyl ether poly(ethylene glycol), PAE: poly(β-amino ester), P(Asp-Hyd-ADR): poly(aspartate hydrazone adriamycin), PCCS: PEGylated CS conjugates, PEG: poly(ethylene glycol), PHis: poly(hystidine), PLA: poly(D,L-lactic acid), PLLA: poly(L-lactic acid), P(TMBPEC): poly(mono-2,4,6-trimethoxybenzylidene-pentaerythritol carbonate-co-acryloyl carbonate)

Polymeric micelle	**Drug loaded**	**In vivo performance compared to traditional methods**	**Reference**
PCCS	Doxorubicin	↑ Accumulation in tumour ↓ Cytotoxicity	(47)
PHis-PEG and PLLA-PEG-FOL with F127	Doxorubicin	↓ Tumour growth	(48)
(Gal-CS-gPNIPAm)	Oridonin	↑ Accumulation in tumour ↓ Tumour growth	(49)
PHis/PEG with PLLA/PEG-FOL	Doxorubicin	↑ Accumulation in tumour ↓ Tumour growth	(50,51)
AP-PEG-PLA with MPEG-PAE	Doxorubicin	↑ Accumulation in tumour	(52)
PEG-b-P(TMBPEC-co-AC)	Paclitaxel	↑ Accumulation in tumour ↓ Tumour growth	(53)
PEG-P(Asp-Hyd-ADR)	Adriamycin	↑ Accumulation in tumour ↓ Tumour growth ↓ Cytotoxicity	(54)

A method that has been used for creating pH responsive PMs, is the use of pH sensitive polymeric blocks that can become ionized in an acidic pH environment and undergo structural changes so the drugs can be released (first 5 pH responsive polymers in Table 1). This has been discussed previously and is also the case for the PS-PMAA polymers that were used in this experiment. Another mechanism that has been used in some pH sensitive PMs is the use of linkable groups (acetals or hydrazones) that can undergo degradation when arriving at an acidic pH environment (last two pH responsive polymers in Table 1) (55).

1.3 Aim of the project

Different types of PSPMAA polymers (di/tri/4-arm-starblock) were provided (shown in Table 2). Micelles from all different PSPMAA polymers were prepared according to the nanoprecipitation method mentioned in section ‘1.2.1.2 Preparation’. Size and zeta potential measurements were performed with dynamic light scattering (DLS) to see if the different PSPMAA polymers would influence size and surface charge of the micelles. Encapsulation Efficiency (EE) was determined to see

if the different PSPMAA polymers would influence the amount of fluorescent probe that could be encapsulated in the micelles.

Table 2: The different PSPMAA polymers. PS: polystyrene, MAA: methacrylic acid

Polymer	sty (GPC)	MAA (NMR)
PSPMAA diblock 1	182	557
PSPMAA diblock 2	49	1032
PSPMAA triblock	108	625
PSPMAA 4-arm star block 1	86	995
PSPMAA 4-arm star-block 2	91	1470

This experiment was performed to see if the pH of a solution would influence release of (model) drugs out of from different PSPMAA PMs. For every different polymer the amount of probe situated in the micelles was measured in solutions with pH ~7 and pH ~4 via UV/VIS spectroscopy. Size and zeta potential were measured via DLS at the start and at the end of the release experiment for all PSPMAA PMs, to see if the micelles would show differences in size and zeta potential when put in solutions of varying pH.

2. Materials and Methods

2.1 Materials

3-(2-Benzothiazolyl)-7-(diethylamino)coumarin (Coumarin-6, >98%) was purchased from TCI chemicals. Acetone (>99%) was obtained from Honeywell Research Chemicals. Dimethyl Sulfoxide (DMSO, >99.9%) was purchased from J.T. Baker Chemicals. Acetic acid from J.T. Baker Chemicals (>99%). Sodium hydroxide from Honeywell Research Chemicals (>98%). All polymers were kindly provided by Prof. Dr. Patrizio Raffa, their synthesis is described in the publication 'Polystyrene-poly(sodium methacrylate) amphiphilic block copolymers by ATRP: Effect of structure, pH, and ionic strength on rheology of aqueous solutions' (56).

Puradisc 0.45 µm syringe filters were purchased from Whatman. A magnetic stirrer with hotplate by IKA and stirring bar were used for stirring purposes. A quartz cuvette was used for UV/VIS measurements and a disposable folded capillary cell by Malvern Panalytical was used for DLS measurement.

2.2 Methods

2.2.1 Coumarin-6 stock solution

A stock solution of coumarin-6 (C6) in DMSO (1 mg/mL) was prepared by adding 20 mg of C6 to 20 mL of DMSO. This stock solution was diluted multiple times. UV/VIS absorbance of each dilution was measured in a Agilent Cary 60 UV/VIS spectrophotometer. A calibration curve for the absorbance at 467 nm was made with the obtained results. The stock solution was stored in the freezer before further use.

2.2.2 Polymeric micelles

50 mg of polymer was weighed and added together with a stirring bar to a 20 mL vial. To this vial 5 mL of the C6 stock solution was added. This was left to stir at 600 rpm at room temperature (25 °C)

until the polymer was completely dissolved. After dissolution the polymer/C6 solution was added to a 50 mL centrifugation tube. 45 mL of acetone was added to precipitate the polymer and C6. This was centrifuged for 15 min at 4000 rpm in a centrifuge. The supernatant was added to a 100 mL round bottom flask. The supernatant containing acetone, DMSO and unencapsulated C6 was added to a rotary evaporator to evaporate all of the acetone for 30 minutes at 556 bar. UV/VIS absorbance of the DMSO and C6 solution was measured with UV/VIS spectroscopy to determine the Encapsulation Efficiency. The precipitated polymer and C6 were added to a 20 mL vial and a stirring bar and 5 mL of basic water (10^{-5}M NaOH aqueous solution) was added to this vial. This was left to stir overnight to form the micelles. The micelle suspension was diluted ten times and size and zeta potential was measured at 25°C in a Malvern Panalytical Zetasizer Ultra DLS instrument.

This procedure was used for the preparation of micelles from all polymers. The preparation of the micelles was performed in duplicate for every polymer.

2.2.3 Preparation of solutions

To make a 10^{-5}M pH ~7 solution a small amount of sodium hydroxide was added to MQ water. A small amount of acetic acid was added to MQ water to make a 10^{-4}M aqueous solution of pH ~4. For each solution a pH strip was used to estimate pH values.

2.2.4 Release experiment

1 mL of the micelle suspension was added to 20 mL of different aqueous solution with varying pH values (7 and 4). Temperature was set at 37°C and the solution was stirred at 300 rpm. At different time stamps (0, 0.5, 1, 2, 4, 6 and 24 hours) 1 mL of the solution was taken. UV/VIS absorbance of each sample was measured with UV/VIS spectroscopy. After UV/VIS absorbance measurements the 1 mL sample with micelles was added back into the solution containing the bulk of the micelles. Size and zeta potential of the micelles were measured only at the start and end (0 and 24 hours) of the experiment at 37°C in a Malvern Panalytics Zetasizer Ultra DLS machine. This release experiment was done in duplicate for every polymer in both solutions with different pH.

2.2.5 Statistical analysis

2.2.5.1 Micelles

The results of the produced micelles are expressed as the mean +/- standard deviation and were statistically compared by a two-sample t-test. As a null hypothesis we state that the different measurements do not have a significant difference and an alpha-value of 0.05 was used.

2.2.5.2 Release UV/VIS absorbance

For the release experiment statistical analysis was performed using Welch t-test to look for significant differences in release in solutions with different pH value. An alpha-value of 0.05 was used, the null hypothesis stated that the different pH solutions show no significant difference.

2.2.5.3 Release size and zeta potential

For the size and zeta potential results one-way ANOVA was used to pairwise compare the significant differences between the release in the solutions with varying pH. Again an alpha value of 0.05 was used and the null hypothesis stated that the different pH solutions would not lead to differences in size and zeta potential during the release experiment.

3. Results

3.1 Calibration curve

To determine the Encapsulation Efficiency a calibration curve was produced. A stock solution of C6 in DMSO (1 mg/mL) was diluted multiple times, UV/VIS absorbance of each dilution was measured in a Agilent Cary 60 UV/VIS spectrophotometer. A calibration curve for the absorbance at 467 nm was made with the obtained results. The absorbance was plotted against the concentration and a trendline through these points was produced with a R^2 value of 0.9954. The graph can be seen in Figure 1 and the full UV/VIS absorbance spectrum can be found in Appendix 1.

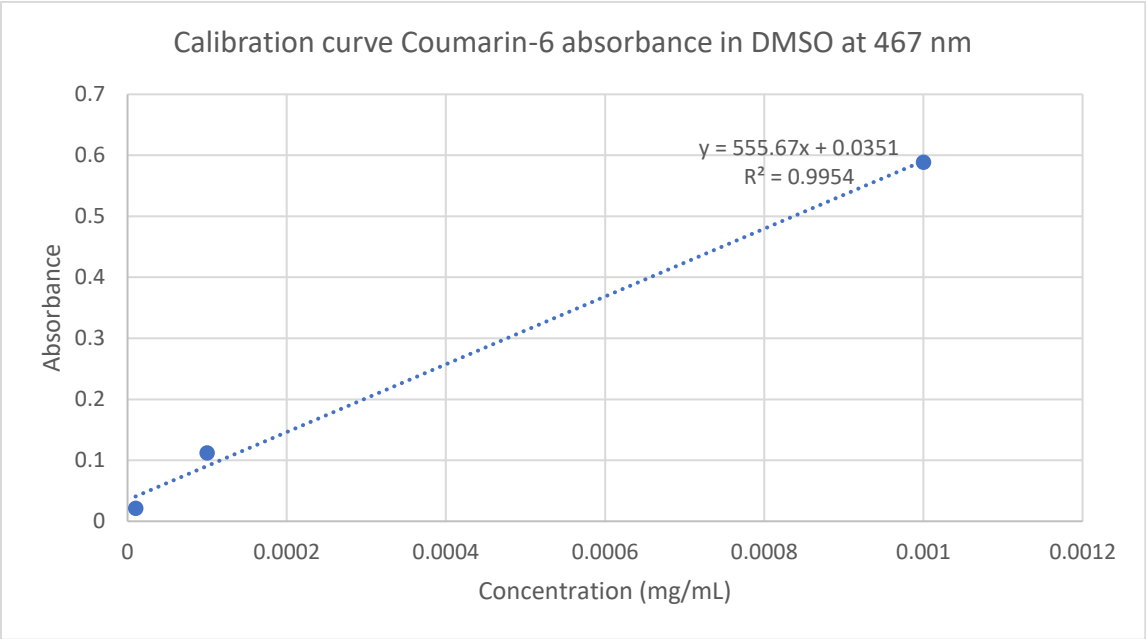

Figure 1: Calibration curve of fluorescent probe Coumarin-6 in dimethyl sulfoxide.

3.2 Micelles

3.2.1 Encapsulation Efficiency

The Encapsulation Efficiency (EE) was determined to see if the different polymers would have an effect on the amount of fluorescent probe that could be encapsulated by their micelles. By using the equation from the calibration curve in Figure 1, the Encapsulation Efficiency (EE) of the micelles of each polymer was determined. Results of the of the one-way ANOVA test within each group and between groups are shown in Appendices 7 and 8.

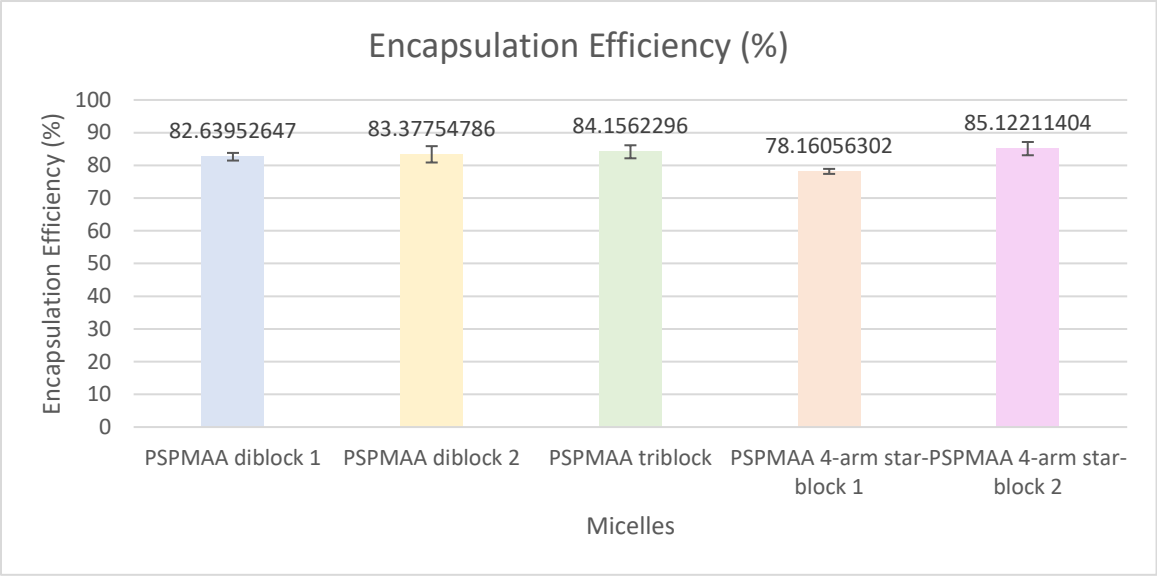

Figure 2: The average encapsulation efficiency of the micelles shown per polymer in percentages with the standard deviation shown in error bars. PS: Polystyrene, MAA: methacrylic acid.

Overall it can be seen that the EE are quite similar for the different polymers and their micelles. The lowest EE was seen in the micelles made out of the PSPMAA 4-arm star-block 1 polymer with a value of 78.16%. Intermediate encapsulation efficiencies were seen in micelles made of PSPMAA diblock 1, PSPMAA diblock 2 and PSPMAA triblock with respective values 82.64%, 83.38% and 84.16%. The micelles with the highest EE were made out of the PSPMAA 4-arm star-block 2 polymer with a value of 85.12%.
The one-way ANOVA test within the groups had a p-value of 0.52 and between groups 0.20, which implies no significant difference between the different tries in producing the micelles.

3.2.2 Size

To see whether the different polymers structure would have an effect on the formed micelles, the mean micellar size was determined for each polymer Figure 3 by DLS measurements. Results of the one-way ANOVA tests are shown in Appendices 9 and 10.

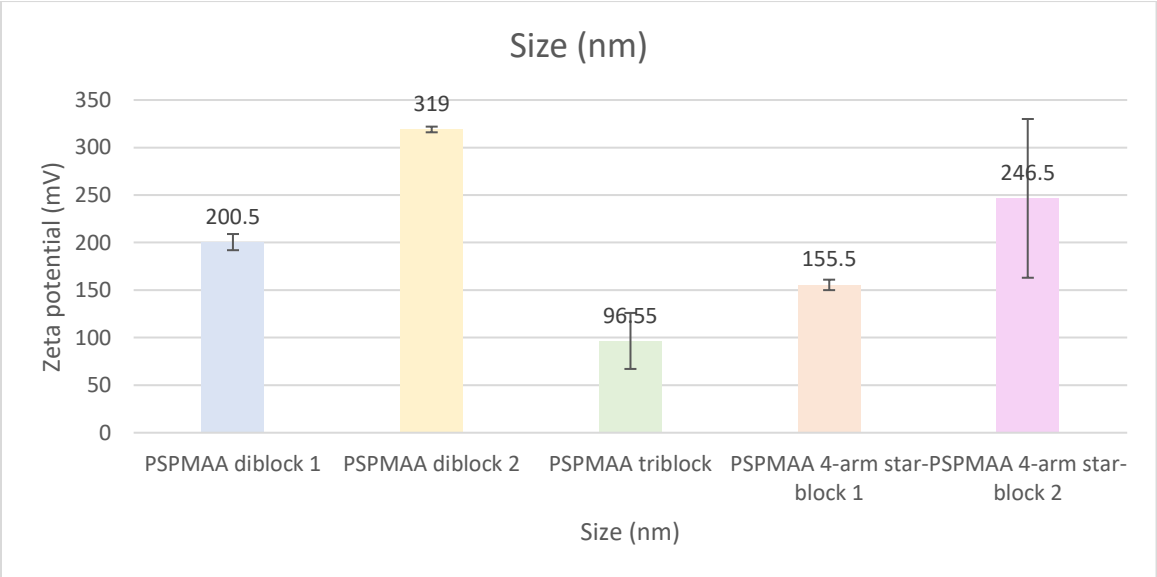

Figure 3: The average size of micelles shown per polymer in nanometres with the standard deviation shown in error bars. PS: Polystyrene, MAA: methacrylic acid.

As seen in Figure 3 the micelles with the biggest mean size are micelles made of the PSPMAA diblock 2 polymer with a means size of 319.00 nm. The results show that micelles made of the PSPMAA triblock make the smallest micelles out of all with a mean size of 96.55 nm. Intermediate micelles sizes can be seen from polymers PSPMAA 4-arm star-block 2, PSPMAA diblock 1 and PSPMAA triblock respectively 245.50, 200.50 and 155.50 nm big.
Standard deviations in all groups are very small except for the PSPMA 4-arm star-block 2 and the PSPMA triblock micelles, this will be covered in the discussion. The one-way ANOVA test within the groups had a p-value of 0.46 and between groups 0.06, which implies no significant difference between the different tries in producing the micelles.

3.2.3 Zeta potential

Zeta potential was measured to see whether the different polymers would have micelles with amount of charges at the surface. The mean zeta potential of the micelles is shown per different polymer in Figure 4 with the standard deviation shown in error bars. Statistical analysis results of the one-way ANOVA test within each group and between groups can be seen in Appendices 11 and 12.

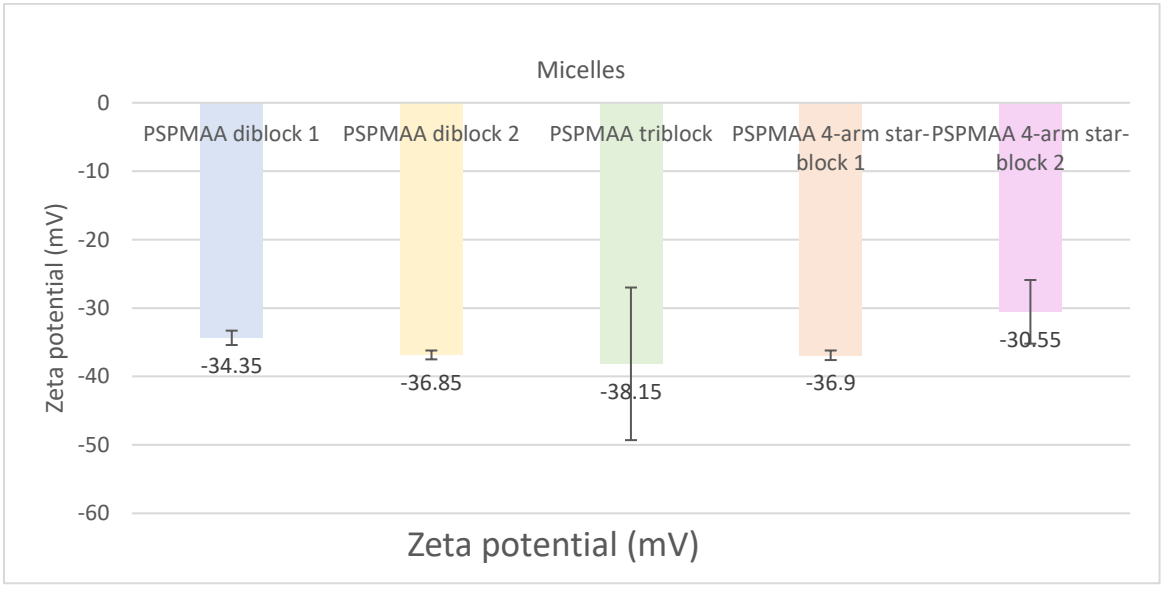

Figure 5: The average zeta potential of the micelles shown per polymer in millivolts with the standard deviation shown in error bars. PS: Polystyrene, MAA: methacrylic acid.

The zeta potentials of the different polymeric micelles look quite similar. Where intermediate zeta potentials were found in micelles made of PSPMAA diblock 1 (-34.35 mV), PSPMAA diblock 2 (-36.85 mV) and PSPMAA 4-arm star-block 1 polymers (-36.90 mV). The least negative zeta potential was seen in micelles made of the PSPMAA 4-arm star-block 2 with a value of -30.55 mV. The most negative zeta potential was seen in PSPMAA triblock micelles with a value of -38.15 mV. However both of these do have some high error bars, which will be discussed in the discussion.

The one-way ANOVA test within the groups had a p-value of 0.18 and between groups 0.77, which implies no significant difference between the different tries in producing the micelles.

3.3 UV/VIS absorbance

3.3.1 Polymers

To visualize the differences in UV/VIS spectra between the polymer (without fluorescent probe), micelles and the fluorescent probe C6 and their spectra are shown in Figure 6 and Appendices 2-6.

3.3.1.1 PSPMAA diblock 1

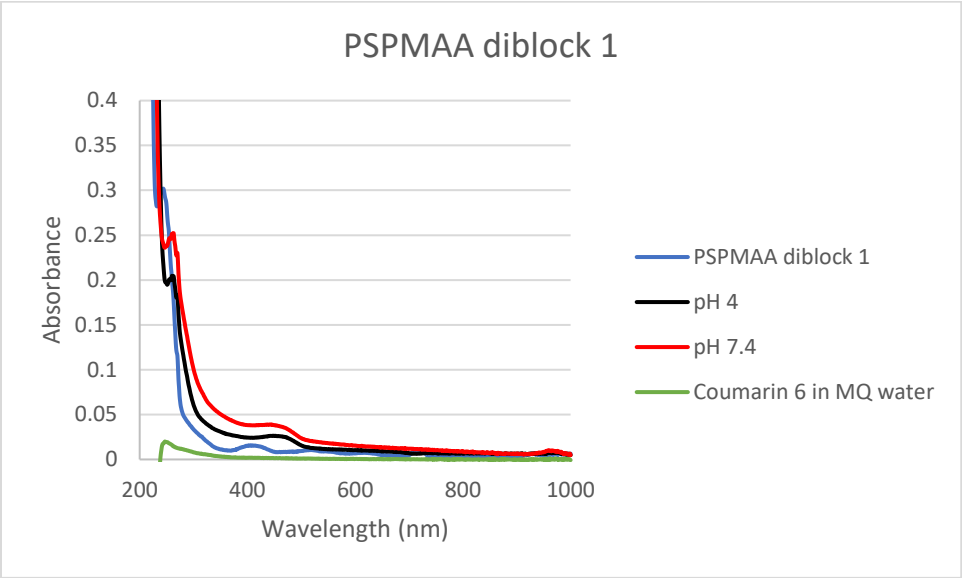

Figure 6: The UV/VIS absorbance PSPMAA diblock 2 polymer without the fluorescent probe in basic water shown in blue. The UV/VIS absorbance of the micelles in black (pH ~4) and red (pH ~7). The UV/VIS absorbance of Coumarin-6 in MQ water in green.

3.3.2 Release experiment

To see if the different polystyrene-polymethacrylate micelles would release the encapsulated fluorescent probe a release quantification was performed. For every different polymer the amount of probe situated in the micelles was measured in solutions with pH ~7 and pH ~4. The amount of release is expressed in percentages with time stamp 0 set as 100%. The fluorescent probe C6 does not show absorbance in the release medium. So it was expected that gradually less absorbance would be measured due to some release of the fluorescent probe. The concentration of polymer micelles was 0.1 mg/mL in the release medium.

Two-Sample t-tests Assuming Unequal Variances were performed for the different polymeric micelles to see if there was a significant difference between the release of the probe in different pH solutions. ANOVA results can be found in Appendices 13-17.
Overall it can be seen that the final the amount of probe situated in the micelles was lower in the pH ~4 solution than in the pH ~7 solution for all polymeric micelles except for the micelles made out of the PSPMAA triblock and the PSPMAA 4-arm star-block 2 polymers. The least amount of probe situated in the micelles was seen in the PSPMAA 4-arm star-block 1 micelles in pH ~4 solution (93.64%). The highest difference in the amount of probe in micelles in different pH solutions was seen by the PSPMAA diblock 1 micelles (29.47% difference).
Unexpectedly almost in all the experiments, the amount of measured probe situated in the micelles ended up being higher than 100%, this will be addressed in the discussion.

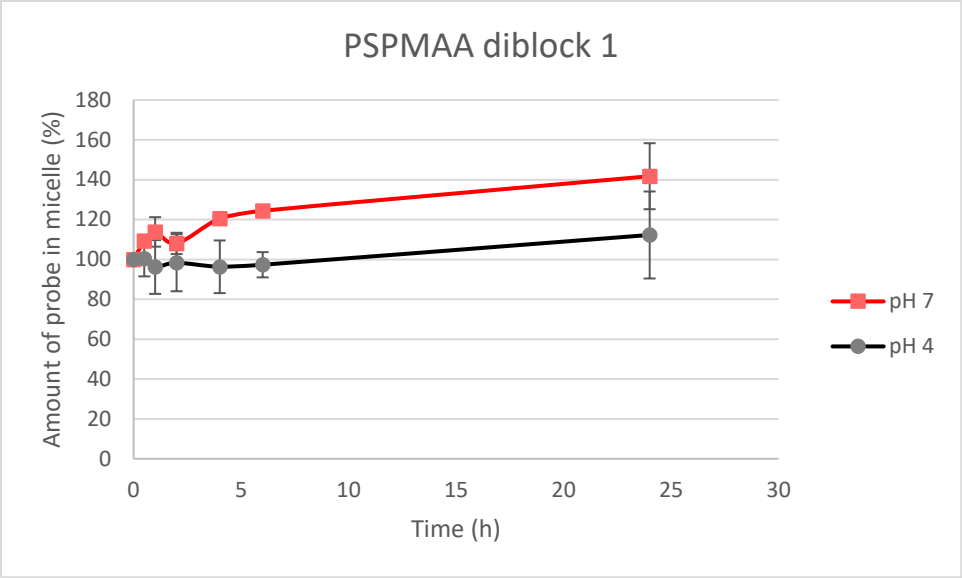

Figure 7: The average amount of probe in the PSPMAA diblock 1 micelles at different pH values shown in percentage with the standard deviation shown in error bars.

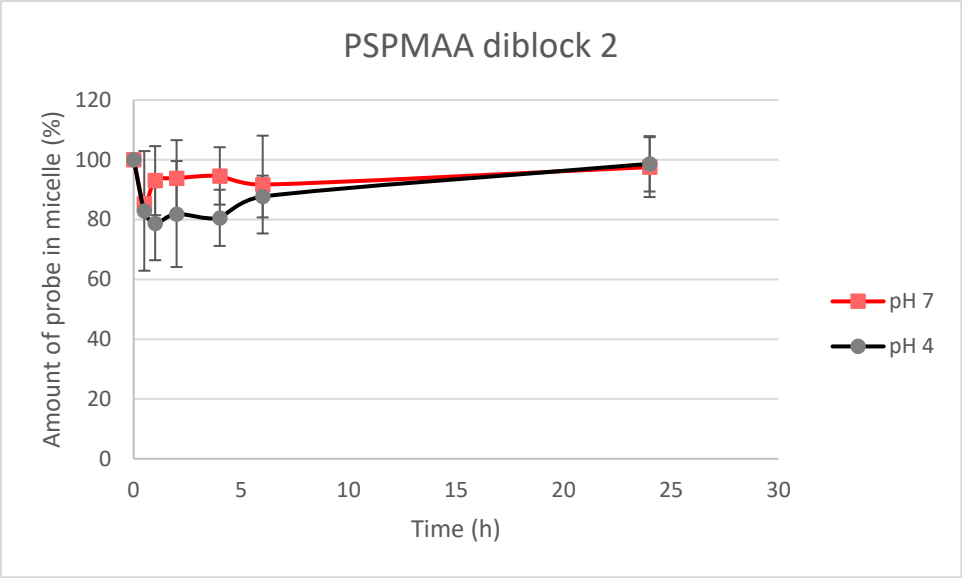

Figure 8: The average amount of probe in PSPMAA diblock 2 micelles at different pH values shown in percentage with the standard deviation shown in error bars.

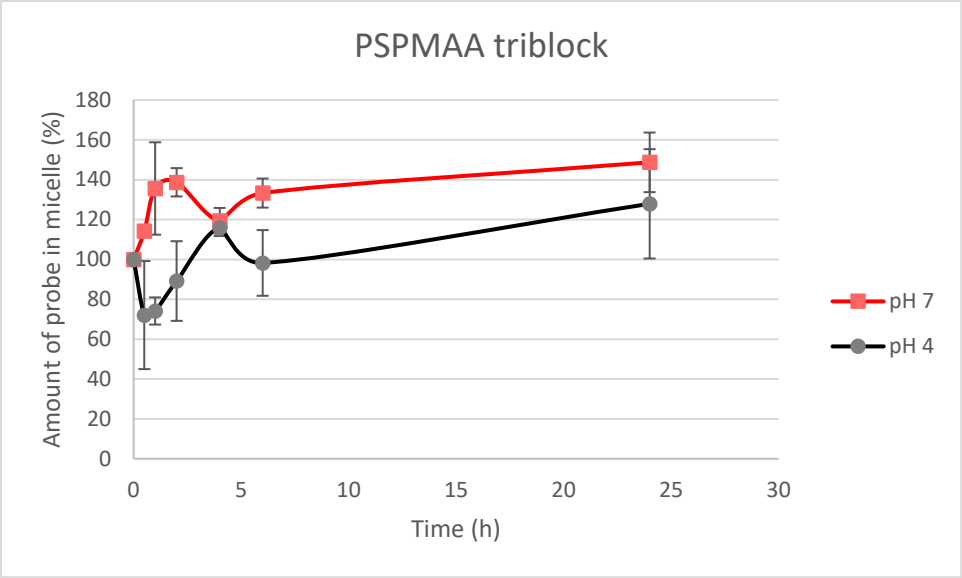

Figure 9: The average amount of probe in the PSPMAA triblock micelles at different pH values shown in percentage with the standard deviation shown in error bars.

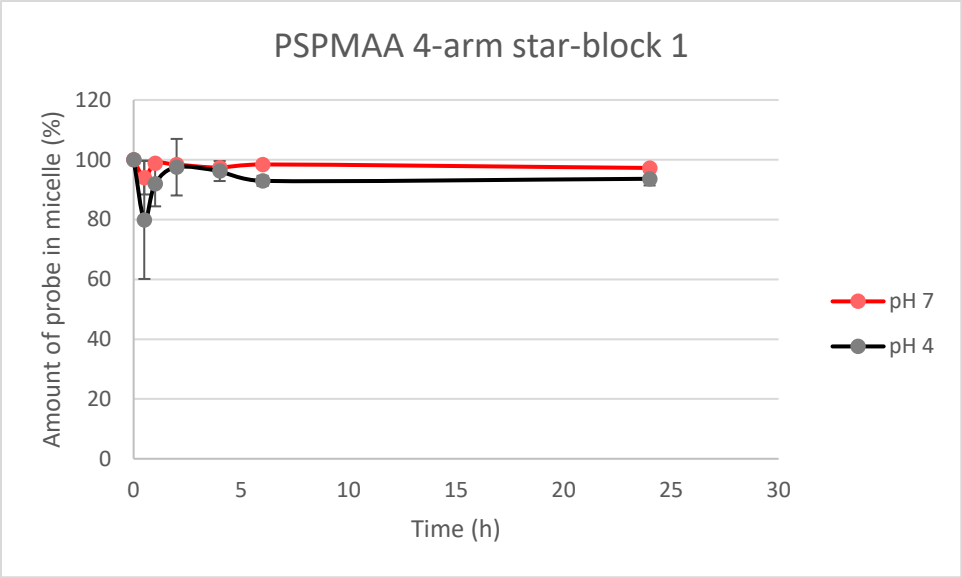

Figure 10: The average amount of probe in the PSPMAA 4-arm star-block 1 micelles at different pH values shown in percentage with the standard deviation shown in error bars.

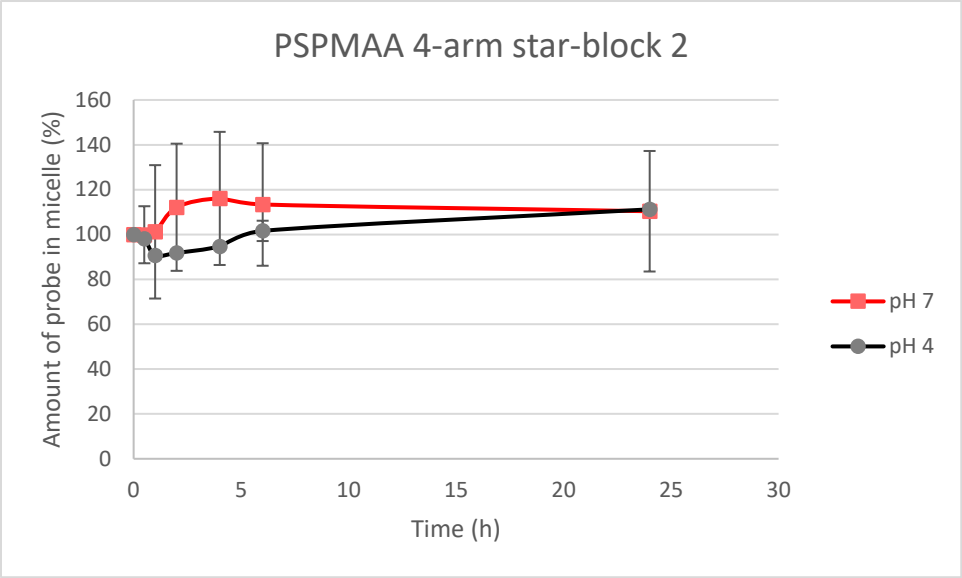

Figure 11: The average amount of probe in the PSPMAA 4-arm star-block 2 micelles at different pH values shown in percentage with the standard deviation shown in error bars.

Final amount of probe situated in the PSPMAA diblock 1 micelles in pH ~7 solution was 141.74% and in pH ~4 solution 112.27%. For PSPMAA triblock micelles this was 148.70% at pH ~7 and at pH ~4 127.89%. For PSPMAA 4-arm star-block 1 micelles this was 97.26% in the pH ~7 solution and 93.64% in the pH ~4 solution. It seems that for these PMs at all time stamps there was a higher amount of probe situated in the micelles in pH ~7 solution than in pH ~4 solution.

This was not the case for PSPMAA diblock 2 and PSPMAA 4-arm star-block 2 PMs. The final amount of probe situated in the PSPMAA diblock 2 micelles in pH ~7 solution was 97.54% while in pH ~4 solution it was 98.64%. For the 4-arm star-block 2 final amount of probe in the micelles in pH ~7 solution was 110.37% and in pH ~4 solution 111.22%. At all other time stamps however the amount of probe in these micelles was higher in the pH ~7 solution than the pH ~4 solution.

In case of PSPMAA diblock 2, PSPMAA triblock and 4-arm star-block 1 a 'burst release' was seen, however at longer times the amount of probe in the micelles appears to invert trend, which is hard to explain. This will be discussed in the next section.

The t-test results of PSPMAA diblock 1 showed a p-value of 0.017205 and PSPMAA triblock t-test showed a p-value of 0.01029, which means that there is a significant difference in amount of probe in micelles in different pH solutions for these PMs.

For PSPMAA diblock 2, PSPMAA 4-arm star-block 1 and PSPMAA 4-arm star-block 2 the p-value of the t-tests were respectively 0.115744, 0.115753, and 0.847753, which means no significant difference in amount of probe in these PMs at different pH values.

Also standard deviations were sometimes high and overlap for certain measurements, this will be addressed in the discussion.

3.3.3 Release size and zeta potential

To see if the micelles would show differences in size and zeta potential when put in solutions of varying pH, the size and zeta potential was measured at the start and end of the release experiment for every different polymeric micelle. One-way ANOVA tests (Appendices 18-27) were performed to

the size and zeta potential measurements to determine if there is a significant difference in this between the micelles in solutions of varying pH.

Overall to all different polymeric micelles applies that their size was bigger and their zeta potential was more negative in pH ~7 solution both at the start and end of the release experiment. The results are all shown in Table 3 and afterwards discussed per polymer.

	PSPMAA diblock 1		**PSPMAA diblock 2**	
	pH 4	**pH 7**	**pH 4**	**pH 7**
Size start (nm)	94.00 (± 11.00)	184.00 (± 4.85)	119.24 (± 111.77)	306.00 (± 3.50)
Size end (nm)	98.15 (± 4.85)	216.00 (± 14.00)	4.01 (± 3.46)	299.50 (± 14.50)
Zeta start (mV)	-25.35 (± 15.15)	-40.50 (± 15.89)	-19.04 (± 16.17)	-39.85 (± 0.93)
Zeta end (mV)	-23.92 (± 15.95)	-40.15 (± 0.35)	-1.95 (± 0.925)	-38.00 (± 4.00)
	PSPMAA triblock		**PSPMAA 4-arm star-block 1**	
	pH 4	**pH 7**	**pH 4**	**pH 7**
Size start (nm)	58.045 (± 50.96)	120.00 (± 58.97)	143.50 (± 17.50)	238.50 (± 48.50)
Size end (nm)	67.03 (± 58.97)	159.00 (± 33.00)	54.45 (± 48.56)	216.00 (± 36.00)
Zeta start (mV)	-12.20 (± 9.51)	-26.80 (± 13.00)	-19.39 (± 13.41)	-42.25 (± 16.34)
Zeta end (mV)	-13.72 (± 12.99)	-29.7 (± 3.00)	-18.87 (± 16.34)	-43.65 (± 1.45)
	PSPMAA 4-arm star-block 2			
	pH 4	**pH 7**		
Size start (nm)	48.49 (± 40.72)	231.00 (± 3.80)		
Size end (nm)	10.31 (± 3.49)	246.50 (± 25.50)		
Zeta start (mV)	-2.34 (± 0.25)	-35.00 (± 3.19)		
Zeta end (mV)	-8.12 (± 3.19)	-35.65 (± 1.85)		

Table 3: The average size and zeta potential of all of the micelles in solutions of varying pH, measured at the start and end of the release experiment. Size measurements shown in nanometres and zeta potential measurements shown in millivolts with the standard deviation shown within parenthesis.

3.3.3.1 PSPMAA diblock 1

The PSPMAA diblock 1 micelles show a slight increase in size during the release experiment in both the pH ~4 (94.00 nm → 98.15 nm) and pH ~7 (184.00 nm → 216.00 nm) solution. Sizes of the micelles were a lot smaller in de pH ~4 solution than in the pH ~7 solution. The standard deviations of size of the micelles in different pH do not overlap which means there is a significant difference.

The zeta potential of the micelles is less negative for the micelles in the pH ~4 solution than in the pH ~7 solution. The zeta potential became slightly less negative during the release experiment of the micelles in pH ~7 solution (-40.5 mV → -40.15 mV) and in pH ~4 solution (-25.35 mV → -23.915 mV). The standard deviations of the zeta potential of the micelles in different pH do overlap which means no significant difference.

3.3.3.2 PSPMAA diblock 2

Micelles made out of the PSPMAA diblock 2 polymer showed almost no shrinkage when in solution of pH ~7 (306.00 nm → 299.5 nm). The same micelles did show shrinkage in the more acidic solution (119.24 nm → 4.01 nm). The standard deviations of size of the micelles in different pH do not overlap which means there is a significant difference.

The zeta potential of the micelles in solution of neutral pH stayed quite the same (-39.85 mV → -38.00 mV). In the pH ~4 solution the micellar charge became less negative (-19.04 mV → -1.95 mV).

The standard deviations of the zeta potential of the micelles in different pH do overlap which means no significant difference.

3.3.3.3 PSPMAA triblock

Micelles made of the polymer PSPMAA triblock showed an increase in size during the release experiment both for the pH ~4 solution (58.05 nm → 67.03 nm) and pH ~7 solution (120 → 159 nm). The standard deviations of size of the micelles in different pH do overlap which means there is no significant difference.

The zeta potential of the micelles also became more negative in both the pH ~4 solution (-12.20 mV → -13.72 mV) and pH ~7 solution (-26.80 mV → -29.70 mV). The standard deviations of the zeta potential of the micelles in different pH do overlap which means no significant difference.

3.3.3.4 PSPMAA 4-arm star-block 1

The PSPMAA 4-arm star-block 1 micelles stayed almost the same size when immersed in the solution of pH ~7. In the pH ~4 solution the micelles shrunk in size during the release experiment (143.50 nm → 54.45 nm). The standard deviations of size of the micelles in different pH do not overlap which means there is a significant difference.

The zeta potential was less negative for the micelles in pH ~4 solution than in the pH ~7 solution. The zeta potential of the micelles in pH ~7 solution stayed quite the same during the release experiment (-42.25 mV → -43.65 mV). The same applies to the zeta potential of the micelles in pH ~4 solution (-19.39 mV → -18.87 mV). The standard deviations of the zeta potential of the micelles in different pH at the start of the experiment do overlap which means no significant difference. However the standard deviations at the end of the experiment do not overlap so there is a significant difference there.

3.3.3.5 PSPMAA 4-arm star-block 2

PSPMAA 4-arm star-block 2 micelles showed a slight increase in size when immersed in the solution with neutral pH (231.00 nm → 246.50 nm). In the more acidic solutions the micelles shrunk in size (48.49 nm → 10.31 nm). The standard deviations of size of the micelles in different pH do not overlap which means there is a significant difference.

The zeta potential of the micelles became more negative in both the pH ~4 solution (-2.34 mV → -8.14 mV) and the pH ~7 solution (-35.00 mV → -35.65 mV). The standard deviations of the zeta potential of the micelles in different pH do not overlap which means there is a significant difference.

4. Discussion

4.1 Micelles

All micelles show decent mean encapsulation efficiencies with values between 78.16% and 85.12%, mean sizes between 96.55 and 319.00 nm and mean zeta potential between -30.55 mV and -38.15 mV.

4.1.1 Influence of PS and PMAA segments length on micelles properties

The different polymers all are block copolymers made of polystyrene and poly(methacrylic acid). The difference between them is the amount of repeating units of styrene and methacrylic acid each block copolymer consists of, as well as the structure (diblock, triblock, or star block). The different

polymers and the amount of styrene and methacrylic acid repeating units are shown in Table 2 in section '1.3 Experiment explanation'.

4.1.1.1 Size

Micellar sizes were high in case of micelles from PSPMAA diblock 2 and PSPMAA 4-arm star-block 2 (see Figure 3). The thing that these polymers have in common is that they have longer poly(methacrylic acid) blocks than the other polymers (Table 2). It is logical that longer polymers lead to bigger micelles. But also by increasing the hydrophilic block length or decreasing the hydrophobic block length the water absorption of the copolymers is increased, which also leads to bigger micelles (57).

Also PSPMAA 4-arm star-block 1 contains many MAA segments, however micelles of this polymer were a bit smaller. This is however logical because the MAA segments are divided over 4 'arms', so the PMAA blocks are 4 times shorter which leads to a more compact polymer structure and smaller micelles.

This also explains why micelles of the PSPMAA triblock polymer were smaller. By dividing the amount of MAA segments with the amount of PMAA blocks in the polymer, it seems that this triblock polymer has the smallest PMAA blocks out of all the five polymers.

4.1.1.2 Encapsulation Efficiency (EE)

The UV/VIS absorbance of the supernatant from each micelle (unencapsulated fluorescent probe) was measured, concentration could then be found via the calibration curve and afterwards EE could be calculated. EE of the micelles were quite similar for PSPMAA diblock 1, PSPMAA diblock 2, PSPMAA triblock and PSPMAA 4-arm star-block 1 (82.64%-85.12%). Only PSPMAA 4-arm star-block 1 micelles had a smaller EE of 78.16%.

Unfortunately not a lot of research has been done on PSPMAA polymeric micelles and EE. However we can compare it to EE values of research on different PMs. For instance Ozturk et al. prepared poly(ε-caprolactone)-poly(N-vinylcaprolactam-co-N-vinylpyrrolidone) PMs with the anti-inflammatory drug indomethacin encapsulated and the PMs showed EE values between 68.5 and 75.1% (58). Another example is by Yang et al. where they made poly(ethylene glycol)-poly(ε-caprolactone)-poly (trimethylene carbonate) micelles that encapsulated curcumin (a new anti-tumour drug) and showed EE values of ~96% (59). Another example of a study is micelles made of different types of (e-caprolactone)-b-poly(2-(diethyl- amino)ethyl methacrylate)-b-poly(poly(ethylene glycol) methyl ether methacrylate) copolymers where the maximum EE value was only 60% (60). So the EE values of our PMs are similar or higher than micelles from other studies.

It is interesting to see that EE of both diblock polymers are quite similar while the PS PMAA segments differ. According to different studies increasing the core hydrophobicity of micelles increases the capacity of the micelles to carry drugs (29,57). Longer hydrophobic chains mean larger hydrophobic cores and the ability for more hydrophobic drugs to be encapsulated in the core (11,12). This does not apply to the EE results. PSPMAA diblock 2 has longer PMAA chains and it might be possible that EE is high because there is also some fluorescent probe encapsulated in the corona of these micelles. It is for instance possible for drugs to be encapsulated in the shell structure of PMs, however this usually applies to drugs with intermediate polarity (55). Stepanek et al. showed in a study that the hydrophobic properties of PMAA are more pronounced towards the inner shell and this forms a very dense hydrophobic layer around the PS core (36). This makes it able to solubilize compounds not only in the hydrophobic PS core but also in the dense hydrophobic layer around the core (61). This could also be an explanation for our results.

If we look at both the PSPMAA 4-arm star-block micelles there is a difference in the EE. PSPMAA 4-arm star block 1 micelles have less (78.16%) probe encapsulated than PSPMAA 4-arm star-block 2 micelles (85.12%). These polymers are quite similar in amount of styrene/methacrylic acid and in structure, however PSPMAA 4-arm star-block 1 does have smaller PSPMAA blocks. This observation could substantiate what we mentioned in the previous paragraph, that it is also possible to encapsulate the probe in the hydrophilic corona. Further investigation would be needed to conclude these claims. For instance by looking at the micelles with fluorescence microscopy to see where the fluorescence is prominent in the micelles.

4.1.1.3 Zeta potential

Micellar surface charge values, expressed as zeta potential, were all within -30.55 mV and -38.15 mV. This negative charge can be explained by the anionic carboxyl groups from the PMAA that are distributed at the surface of the micelles (62).

Phan et al. looked at the correlation between amphiphilic balance, size and zeta potential. They found that smaller PMs resulted in more negative zeta potentials. There was also a relation between hydrophilic block length and zeta potential, where the polymer with the lowest percentage of hydrophilic block lead to PMs with the most negative zeta potential. Phan et al. speculate that the shorter hydrophilic blocks lead to a denser sort of 'brush' on the PM surface and therefore enhanced charge/surface area properties (63).

There is unfortunately not a pattern seen in our measurements in amount of styrene/methacrylic acid segments and zeta potential. There is also not a pattern seen in size and zeta potential in our results. So something else outside the scope of our study is influencing zeta potential.

4.1.2 Statistical Analysis

First the statistical analysis for the micelles will be discussed. For every polymer the whole process of preparation of the micelles was performed twice. ANOVA tests of the EE, size and zeta potential all had p-values higher than 0.05. So the different tries in production did not influence the micellar EE, size and zeta potential, which is positive.

The standard deviation of the micellar size from PSPMAA 4-arm star-block 2 micelles has a high value. While dissolving the polymer in DMSO, the PSPMAA 4-arm star-block 2 polymer dissolved better in the second production than the first. The PSPMAA 4-arm star-block 2 polymer did not dissolve well which could explain the bigger sized micelles in the second production. This could explain the higher standard deviation in this group.

The zeta potential measurements show high standard deviation in case of the PSPMAA 4-arm star-block 2 and PSPMAA triblock micelles. In both cases the smaller sized micelles also had less negative surface charges. For PSPMAA 4-arm star-block 2 the same reason illustrated in the previous paragraph could be applied for the high standard deviation in zeta potential. No abnormal observations were made during the production of the PSPMAA triblock micelles. It could be that there are some unknown impurities in the micellar solution that affected the size and zeta potential of these micelles.

However, it has to be kept in mind that the experiment was only performed in duplicate, statistical tests probably do not show a lot of certainty.

4.2 Release experiment

4.2.1 UV/VIS absorbance measurements

As can be seen in Figure 6 the fluorescent probe in the micelles show UV/VIS absorbance. When immersed in an aqueous solution we expected the micelles would open up and release the encapsulated probe. The fluorescent probe C6 shows negligible UV/VIS absorbance in water. Therefore we imagined during the release experiment the intensity of the UV/VIS absorbance of the probe in the micelles would decrease. Furthermore we expected that in an acidic solution the micelles would release more or release faster. However, as it will be shown, the results from the UV/VIS absorbance measurements did not support this hypothesis.

For instance, this is shown in the UV/VIS absorbance measurements from the PSPMAA diblock 2 micelles (Figure 8): the expected drop in UV/VIS absorbance intensity can be seen at the beginning, where there is also a sharper decrease of intensity in case of pH ~4 than pH ~7. This is consistent with the hypothesis (xxx), however, at longer release times an increase in intensity is recorded, even surpassing the initial value, and in case of these micelles there is also almost no difference in UV/VIS absorbance intensity between the two solutions of different pH. The same pattern in UV/VIS absorbance measurements can be seen in all different polymeric micelles that we studied. Possible explanations for this unexpected behaviour are discussed in the paragraph 'Change in UV/VIS absorbance intensity of C6 due to protonation'.

4.2.1.1 Statistical Analysis

The t-tests on the UV/VIS absorbance measurements of the release part of the experiment show that there is not a significant difference in amount of probe situated in the PSPMAA 4-arm star-block 1, PSPMAA 4-arm star-block 2 and PSPMAA diblock 2 micelles in different pH solutions. T-tests do suggest that there is a significant difference in amount of probe situated in the micelles at different pH for PSPMAA diblock 1 and PSPMAA triblock micelles, however the standard deviations sometimes overlap which might suggest otherwise.

Also here it has to be noted that the experiment was only performed in duplicate, so statistical tests do not show a lot of certainty.

4.2.1.3 Change in UV/VIS absorbance intensity of Coumarin-6 due to protonation

Bečić et al. did a study on different 4-hydroxycoumarin derivatives and their UV/VIS absorbance spectra in solutions with different pH values. It was found that protonation of the solvents (chloroform and acetonitrile) causes a change in absorption spectrum of the coumarin derivative. As protonated forms contain more energy than their unprotonated counterparts, the absorption maxima are batochromically shifted. Besides a shift in maximum wavelength results of their study also show there was an increase in intensity of the UV/VIS absorbance of the coumarin derivative in the more acidic solution (with similar concentrations of probe) (64). A follow-up study on the UV/VIS absorbance of C6 in solutions of varying pH should be done to confirm this.

In our case, we expect that the fluorescent probe is mostly situated within the hydrophobic core. For the probe to be influenced by the solvent would mean that there is some way that the solvent is able to reach into the core of the micelle. To compare this with our results, as mentioned above first a drop in UV/VIS absorbance intensity can be seen and quickly after there is an increase in intensity. It could be that it takes some time for the solvent to enter the polymeric micelle and therefore only after an hour an increase in intensity can be seen. The first drop in intensity could then still be caused by opening/disruption of the micelle because by then the solvent might have not reached the hydrophobic core yet and caused a change in UV/VIS absorbance intensity.

So unfortunately it seems that our designed methods are not suitable for these type of release experiments. Possible adjustments to the methods are explained in section '6. Limitations and future recommendations'.

4.2.2 Size and zeta potential during release measurements

Size and zeta potentials were measured to see if the solutions with different pH values would have an influence on the size and zeta potential of the different micelles.

4.2.2.1 Comparing size and surface charge between micelles at different pH

First the size measurements will be discussed. To all different polymeric micelles applies that their size was larger in pH ~7 solution than pH ~4 solution both at the start and end of the release experiment. The micelles shrink when they are in an acidic solvent. This can be compared to what certain pH responsive hydrogels are able to do, see Figure 12 for a schematical representation. For instance Chatterjee et al. produced a hydrogel network made of PAA and it was used as a pH-responsive hydrogel system that shrunk while in acidic pH (65).

Micellar surface charge became less negative as the pH decreased. This is inline with other research (23). As the pH drops, the carboxyl groups of the PMAA all become protonated and lead to a less negative surface charge (23).

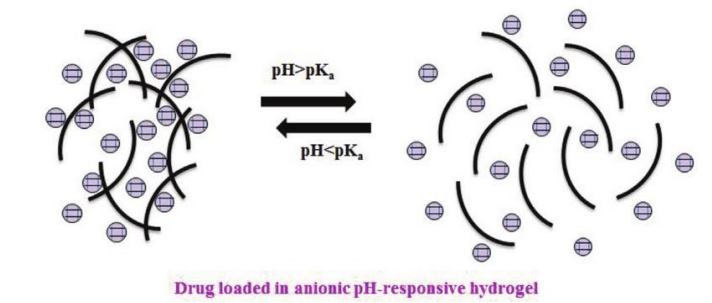

Figure 12: Schematic illustration of a pH-responsive hydrogel (65).

4.2.2.2 Size and zeta potential before and after release experiment

As mentioned in the introduction most PMs have sizes between 10 and 200 nm (11,18). Therefore the assumption was made that particles above 10 nm can be classified as micelles.

Both PSPMAA diblock 1 and PSPMAA triblock micelles did not shrink in size but expanded a little during the release experiment. PSPMAA diblock 1 micelles expanded 4% and polymer PSPMAA triblock micelles 17% in size after 24 hours. At the end of the release experiment particles were sized 98.15 nm (PSPMAA diblock 1) and 67.03 nm (PSPMAA triblock) in pH ~4 solution. Which means that there are still some micelles intact after the release. The interesting thing is that these two polymers have the lowest amount of PMAA segments. The PMAA chains of these polymers are shorter than the other polymers. PSPMAA diblock 1 and PSPMAA triblock micelles have less carboxyl groups in and it might be that this causes them to be less affected by pH. The hydrophilic chains do not repel each other as much and therefore open up less than polymers with longer hydrophilic chains like PSPMAA diblock 2, PSPMAA 4-arm star-block 1 and PSPMAA 4-arm star-block 2.

This is not the case for micelles of the other three polymers which shrunk during the release experiment. PSPMAA 4-arm star-block 1 also shrunk almost three times in size during the release experiment in the pH ~4 solution. Measured PSPMAA 4-arm star-block 1 particles after 24 hours in pH ~4 solution were 54.45 nm. PSPMAA 4-arm star-block 1 micelles are thus still intact after 24 hours. For PSPMAA 4-arm star-block 1 it is therefore difficult purely looking at the size

measurements to tell if all of the encapsulated probe has been released. It has however shrunk 3 times in size, so an assumption is made that a huge portion of the probe has been released.

PSPMAA diblock 2 and PSPMAA 4-arm star-block 2 micelles were both sized <~10 nm after 24 hours. These particles are so small that it could be that there are no intact micelles anymore in the solution. With polymer PSPMAA diblock 2 and PSPMAA 4-arm star-block 2 we can safely say that most of the encapsulated fluorescent probe has been released.

Results from the zeta potential measurements before and after the release almost all show small changes. There is not a clear pattern to see, some become slightly less negative and some slightly more negative. Only the PSPMAA diblock 2 and PSPMAA 4-arm star-block 2 micelles show a different result. The PSPMAA diblock 2 micelles in pH ~4 solution became a lot less negative after 24 hours of the release experiment (-19.04 mV → -1.95 mV). PSPMAA 4-arm star-block 2 micelles in pH ~4 solution became a lot more negative (-2.34 mV → -8.14 mV). It is not clear what is influencing the zeta potential during the release experiment. It might be that there is some interaction with released probe that influences the zeta potential but there is not been done a lot of research in this field. Furthermore we also have to mention here that this results could also be a coincidence due to the small number of tests.

4.2.2.3 Statistical Analysis

The only micelles that seem to have no significant difference in size in the different pH solutions are the PSPMAA triblock micelles. This is logical because there are still some intact PSPMAA triblock micelles present in the pH ~4 solution so the difference in size is already smaller than some other (PSPMAA diblock 2, PSPMAA 4-arm star block 1 and PSPMAA 4-arm star block 2) micelles. Only PSPMAA diblock 1 micelles do seem to have a significant difference in size in different pH solutions while there probably are still some intact micelles present.

Looking at the zeta potential during release experiments, only 6 and 8 micelles show significant differences. These are also the micelles that show the most differences in size and zeta potential between the two pH solutions. This substantiates our speculations in the previous section, there is a difference between the two pH solutions.

Also here it has to be noted that the experiment was only performed in duplicate, so statistical tests do not show a lot of certainty.

4.2.2.4 Probable in vivo performance looking at size and zeta potential

As mentioned in the introduction section the size and zeta potential of micelles influences their stability in the bloodstream and ability to be taken up by cells.

Stability in the bloodstream

The pH ~7 solution was chosen to mimic the pH of blood (pH 7.4). All of our produced micelles either stayed almost the same size or slightly expanded in size. So micelles were still intact after 24 hours in pH ~7, which is positive because it shows they are stable in a solution that has a pH value that is comparable to blood.

In pH ~7 solution our micelles had a surface charge between -26.8 mV and -42.25 mV. As mentioned in the section '1.2.1.3 Characterization' stability of micelles also depends on their surface charge or zeta potential. As mentioned in the introduction section stable PMs show surface charges greater than +30 mV or less than -30 mV and positively charged PMs are cleared out of the blood more easily.

Comparing our results with other researches that are also mentioned in the section '1.2.1.4 In vivo performance', our micelles might show good stability in the bloodstream.

Cellular uptake

The pH ~4 solution was chosen to mimic a cancerous cellular environment. As mentioned in the introduction section PMs with sizes between 10-200 nm are able to be absorbed by cells. At the start of the release experiment our micelles were sized between 48.49 and 143.50 nm in the pH ~4 solution. This means that they all fall under the criterium of having a size smaller than 200 nm.

Zeta potential measurements of our micelles were in a range of -2.34 to -25.35 mV. As mentioned in the introduction section different researches show different results regarding to zeta potential and cell uptake.

It is thus difficult to conclude if our produced micelles are able to be taken up by targeted cells easily. Size measurements show promising results. A follow up study on cellular uptake should be done to give a more clear view.

Burst release

As mentioned in the introduction section an unwanted effect that most PMs seem to have is burst release. Looking at the results of the UV/VIS absorbance measurements some polymeric micelles show an initial quick decrease in amount of fluorescent probe situated in the micelles. For instance, Figure 8 shows that for micelles of polymer PSPMAA diblock 2, more than 20% decrease in UV/VIS absorbance intensity withing the first half hour. An explanation to this could be that there are some C6 molecules situated more in the shell of the PM and are released in a thus called 'burst release' manner.

As mentioned previously this is an undesired effect, especially for DDSs that are designed for a prolonged release. More tests should be done to substantiate this claim.

5. Conclusion

All micelles show good mean encapsulation efficiencies with values between 78.16% and 85.12%, mean sizes between 96.55 and 319.00 nm and mean zeta potential between -30.55 mV and -38.15 mV.

The size measurements from the release part show that all of the PMs are able to stay intact in pH ~7 solution. PSPMAA diblock 1 and PSPMAA triblock micelles stay intact both in the acidic and neutral pH solution. With PSPMAA diblock 2 and PSPMAA 4-arm star-block 2 micelles it can be stated that most of the encapsulated fluorescent probe has been released. For PSPMAA 4-arm star-block 1 micelles we assume that a huge portion of the probe has been released. All of our PMs thus show stability in pH ~7 solution and some PMs are able to open up and release their load at an acidic pH, which is very positive. Comparing our size results with other researches, our micelles might show good stability in the bloodstream and could possibly be taken up by cells.

However, there are limitations to this study and further research is recommended to substantiate our claims which can be found in the next section '6. Limitations and future recommendations'.

6. Limitations and future recommendations

As we mentioned before, the experiment was only performed in duplicate due to limited time. Statistical tests therefore do not provide any substantial conclusions. To conclude if our claims are valid the experiment should be done multiple times more.

Unfortunately by looking at the results from the release experiment it seems that our designed methods for the UV/VIS absorbance measurements do not work for our micelles and fluorescent probe. There are other possibilities to measure release that could be used instead of our methods. For instance by measuring released C6 in solution by either using fluorescence spectroscopy or adding a small amount of solvent that is suitable for C6 and measuring UV/VIS spectroscopy.

We concluded that purely by looking at the size and zeta potential measurements from the release experiment that our micelles might show good stability in the bloodstream and could be taken up by certain target cells. However a follow up study on cellular uptake should be done to confirm our suspicions. Furthermore, a cytotoxicity study has to be performed to conclude suitable biomedical applications.

Certain claims were made that it might be possible that the fluorescent probe could also be situated in other places than the hydrophobic core. Additional research would be required to substantiate these claims. This could be done by using fluorescence microscopy and observing the regions in the micelles where fluorescence is most noticeable.

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