Optimizing the radiolabeling of block copolymer micelles using ^{89}Zr

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Abstract

Polymeric micelles are increasingly used in chemotherapy. To get a greater understanding of the behaviour of these micelles is important. Nuclear imaging, such as SPECT and PET can provide relevant information about the behaviour of the micelles. However, to use these techniques, the micelles must be radiolabelled. In this thesis a chelator free method will be described to radiolabel micelles.

The goal of this thesis is to radiolabel block copolymers with Zr-89. For this purpose series of experiments were carried out. In these experiments three different concentrations $ZrCl_4$ are applied, $C_1 = 0.04mM$, $C_2 = 0.07mM$ and $C_3 = 1mM$. These experiments are conducted for two different types of polymer micelles, PCL-PEO 2800-2000 and PLA-PEO 10500-5000. With these polymers, micelles are prepared, which are used for the radiolabeling process. The fractions obtained by the SEC (size exclusion chromatography) were analysed and the data was processed. At the first concentration $C_1 = 0.04mM$, PCL-PEO 2800-2000 had an efficiency of 37,65 ± 2,48 %. PLA-PEO 10500-5000 gave a radiolabeling efficiency of 28,27 ± 1,23 %. The second concentration, $C_2 = 0.07mM$, showed a decrease in radiolabeling efficiency. For the PCL-PEO-2800-2000 polymeric micelles a radiolabeling efficiency of 23,30 ± 13,94 % was found and for the PLA-PEO 10500-5000 micelles an efficiency of 19,76 ± 1,70 was found. Lastly at the third concentration, $C_3 = 1mM$, for PCL-PEO 2800-2000 the radiolabeling efficiency was found to be 0,88 ± 0,07 %. The PLA-PEO 10500-5000 micelles gave back 2,91 ± 1,68 % as radiolabeling efficiency. A stablity test was performed at $C_1 = 0.04mM$. The PCL-PEO 2800-2000 polymeric micelles had a retention ratio of 37,57 ± 4,35 %. The PLA-PEO 10500-5000 performed better, 69,03 ± 1,27 % of the zirconium was still found to be in the micelles after being challenged by diethylenetriaminepentaacetic acid, or in short, DTPA. A stability test was also performed at $C_3 = 1mM$, but results were found that are not usable. This experiment should be done again in future research.

Contents

1 Introduction

In the last two decades, nanotechnology has shown to have a great potential in the field of medicine. An example of so called nano carriers are micelles. In chemotherapy micelles are looked upon as one of the most promising candidates for transporting drugs to a tumor. Polymeric micelles can be made from more than one polymer. In this paper, two block-copolymers are used to construct micelles. The two block copolymers are composed of hydrophilic and hydrophobic blocks. The hydrophobic polymer is used to construct the core of the micelle, while the hydrophilic polymer constructs the corona. With their core-shell structure they are used to encapsulate insoluble materials in their core, which can then be brought to the desired site in the body. This works because of the enhanced permeability and retention (EPR) effect. This effect is based on the fact that tumors need a great blood supply and therefore create their own defective blood vessel system. They also lack lymphatic drainage. Which results in accumulation of micelles, or other nanocariers, in tumor tissue.

The importance of these nanocariers can not be stressed enough. The EPR effect differs per patient, and it is important to know whether the micelles accumulate sufficiently in tumors. To see how the micelles accumulate, for example, PET (Positron Emission Tomography) imaging can be used. The performance of micelles in vivo is influenced by different factors, where the size and stability of micelles play an important role. The conventional way of radiolabeling micelles is to conjugate a chelator molecule to the micelles. However, this chelator molecule can alter the properties of these micelles, which is a major drawback. At the ARI group, located at the Reactor Institute Delft, a new chelator free method has been developed.

This thesis will start with radiolabeling four different types of micelles with ^{111}In. These experiments serve to select the most promising block copolymers. The ultimate goal of this thesis is to radiolabel micelles with Zr-89 achieving high stability. For this purpose the effect of zirconium concentration on the efficiency and stability is investigated. The stability of these radiolabled micelles will also be determined using DTPA. This is a chelator that binds well with radioactive materials. The stability will be determined by comparing the fraction of ^{89}Zr that is released from the micelles with the fraction of the ^{89}Zr that stays in the micelles after exposure to DTPA (1).

In the following chapter, the theory behind this experiment will be explained including the construction of micelles and the EPR effect. Chapter three will contain a detailed description of how the experiments were conducted. Thereafter the results will be displayed and discussed followed by a conclusion. At the end an appendix can be found that contains a part of the measured counts in the collected fractions during this work.

2 Theory

2.1 Micelles and delivery

Conventional chemotherapy is the most common therapeutic method for treating cancer. Small toxic molecules are released in the patient that interact with DNA and cause cell death. The downside of this method is that these chemotherapeutic agents are non-selective and can damage, not only cancer cells, but also healthy tissues. With the use of block copolymers, micelles can be constructed that can carry drugs with high efficiency, and specifically target cancer tissue (21).

2.1.1 Building blocks

Polymeric micelles are self-assembling nano structures, made from amphiphilic block copolymers. Amphiphilic block copolymers are polymers composed of hydrophilic and hydrophobic segments (5). The copolymers are made of two homopolymer blocks that are connected by a covalent bond (6). For example, if polymer A joined together with polymer B via a covalent bond, the block copolymer A-B will be created. In the experiments described in this paper, four different block copolymers were used. PCL-PEO (2800-2000), PEO-PLA (5000-5500), PEO-PCL(5000-6500) and PLA-PEO(10500-5000). The numbers denote the average molecular weight of the corresponding homopolymer. This is denoted, because not every molecule has the same length, thus resulting in different molecular weights (7). Poly(ethylene oxide), PEO for short, is one of the most frequently used hydrophilic blocks. Because this polymer is hydrophilic, it will construct the corona of the micelle, while poly ϵ-caprolactone (PCL) and polylactic acid (PLA) are used to construct the hydrophobic core of the micelles. The micelles are formed by self-assembly in an aqueous environment. This happens, because it is entropically favored. This self-assembly only occurs above their critical micelle concentration (CMC) to result in the formation of micelles with a core-shell structure. Because the micelles are composed of a hydrophobic core, they can encapsulate water-insoluble drugs. The hydrophilic corona helps to avoid recognition by the immune system. In figure 1 a schematic of a drug-loaded micelle can be seen.

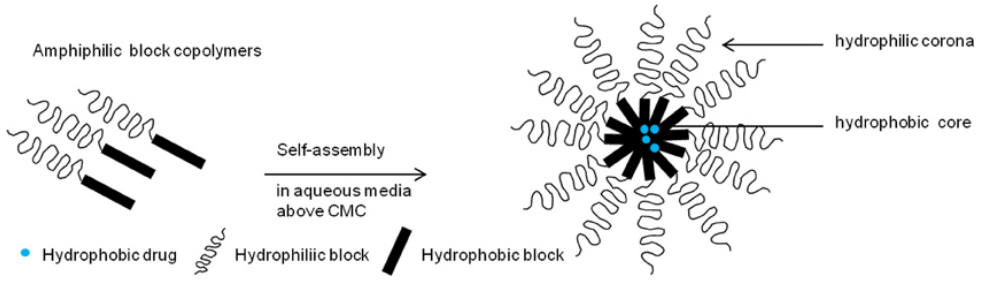

Figure 1: Formation of Drug-loaded micelle from block copolymers. Starting at the left side with block copolymers that form into micelles by self-assembly (8).

2.1.2 Passive targeting

The characteristics described above have made radiolabled micelles favourable for applications in chemotherapy. In these applications it is crucial that the micelles are brought to the desired site. This can be accomplished by active or passive targeting. Nanocarriers like micelles can accumulate in tumors due to the enhanced permeability and retention (EPR) effect. This effect is an example of passive targeting. It is based on the fact that a tumor is heavily dependent on blood supply, so it effectively creates its own abnormally formed blood vessel system. This ensures that the micelles will move to the tumor (2). This in combination with the production of vascular permeability factors, make the tumor blood vessels highly permeable. The growing tumor cells compress the lymph vessels, causing them to collapse, resulting in poor lymphatic drainage (8). Because lymphatics usually drain and filter these type of particles, this will result in an accumulation of radiolabled micelles in the tumor(2). In figure 2 a schematic of this effect is displayed (8).

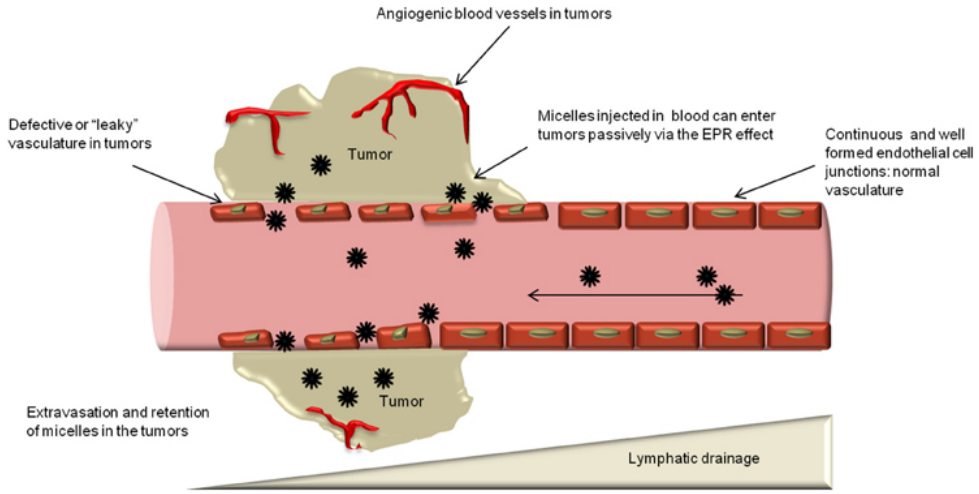

Figure 2: Schematic of the EPR effect, depicting a defective vascular system and poor lymphatic drainage, resulting in an accumulation of micelles in the tumor (8).

Unfortunately, passive targeting is not without drawbacks. Tumors can have different cut off sizes, meaning that some micelles are to big to diffuse into tumor tissue, while smaller micelles can. This and varying permeabilities can be different from tumor to tumor, but also within the same tumor there can be differences. This diversity may lead to some areas not showing the EPR effect. Only passive accumulation cannot ensure a effective drug delivery, so additional approaches like active targeting are also utilized (8).

2.1.3 Active targeting

In active targeting, peptides or antibodies are used to ensure the tumor uptake of the nanocarriers (2). However, active targeting relies of passive targeting. Cancer cells, like other cells, possess receptors and antigens. These receptors and antigens are overly-expressed on cancer cells. Active targeting uses this property by chemically modifying the surface of the polymeric micelles with targeting ligands (8). In figure 3 one can see three different types of ligands attached to micelles.

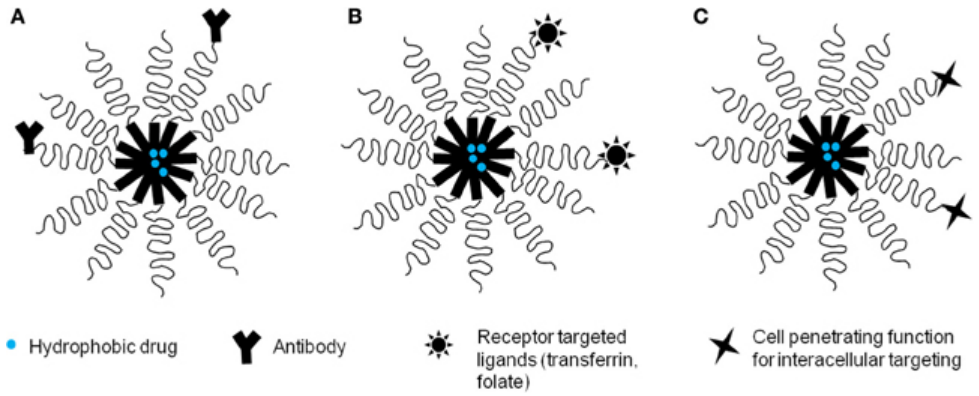

Figure 3: Schematic of micelles with different targeting ligands attached. figure 3A shows a micelle with antibodies attached. Figure 3B depicts a micelle with receptors, and finally figure 3C shows a micelle with a cell penetration function (8).

2.2 Radiolabeling micelles

2.2.1 Chelators

In general, the conventional way to radiolabel micelles is by the use of something called a chelator. This chelator, for example diethylenetriaminepentaacetic acid (DTPA), will be attached to the polymer that

makes up the hydrophilic corona, so the radioactive isotope can bind to the chelator. A big disadvantage of this method is that it requires additional synthetic steps and the chelator can alter the corona of the micelles. As a result of this, the properties of a micelle could be different, and on top of that, it could influence the in vivo performance of the micelles (1) (9).

In this paper a different approach will be used regarding the radiolabeling of micelles. A method that does not require chelators. This method is based on passive loading, where instead of the radionuclide being attached to a chelator, altering the corona of the micelles, it will be trapped inside the hydrophobic micellar core (9). This is very similar to what is seen in figure 1. Instead of a drug being trapped, it will be a radionuclide.

2.2.2 Applications: SPECT and PET imaging

Single photon emission computed tomography (SPECT) and Positron emission tomography (PET) are both imaging methods that are applied in nuclear medicine. In SPECT imaging, γ - emitting radionuclides are used that are injected into the patient. With the use of active or passive targeting as described above the radionuclides will accumulate at a specific site in the subject. A gamma camera is used, which rotates around the patient to capture data from different positions. This data is used to make a tomographic reconstruction (10). In figure 4 a schematic can be seen on how SPECT imaging works.

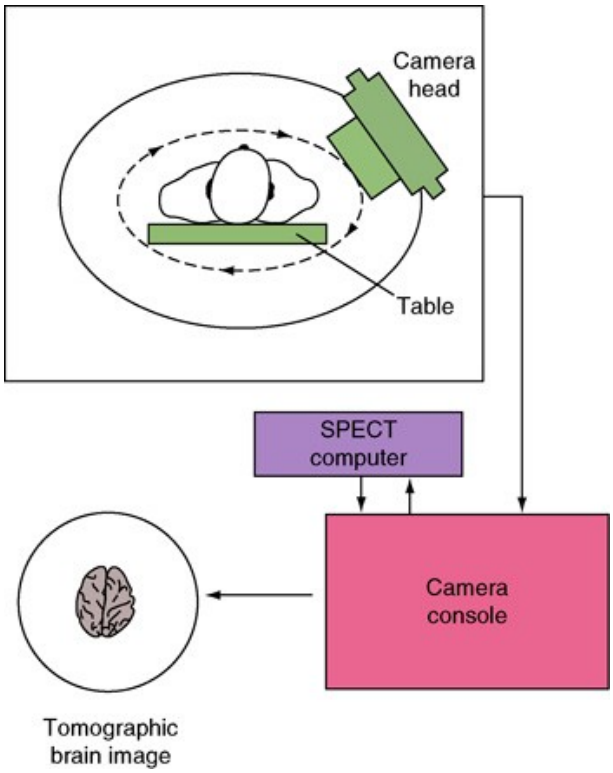

Figure 4: Schematic of SPECT imaging where one can see the camera rotating around the patient, so that a 3D image can be constructed by a computer (17).

In PET imaging, a positron emitting nuclide is administered into a subject. When the positrons are emitted they will collide with electrons in the surrounding tissue. If this happens, annihilation photons are created. A scanner detects these photons, which arrive at the detectors at 180 degrees apart from one another. A computer analyses these photons and uses them to create an image map of the subject that is being studied. The amount of radionuclides accumulated in a certain tissue affects how brightly the tissue appears on the image (11). In figure 5 a schematic drawing can be seen on how PET imaging works.

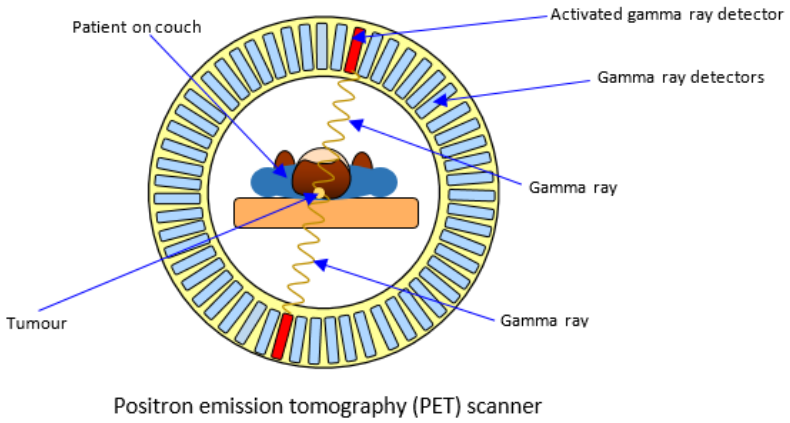

Figure 5: Schematic of PET imaging. The two red detectors are the detectors that detect an incoming gamma ray. By analysing the date over a period of time a tumor can be located by creating an image map (18).

2.3 Used nuclides and their decay

2.3.1 In-111

Indium-111 is prepared by bombarding a cadmium-112 target with protons. ^{111}In has a half life time of 2.83 days, and it decays via electron capture, followed by gamma radiation to the stable cadmium-111. Electron capture happens when the nucleus of an atom absorbs an electron and converts a proton into a neutron. It has two major gamma-photon emission peaks, at 171 keV and 245 keV. In-111 is used to radiolabel for example, red and white blood cells and platelets (12). In figure 6 one can see the decay scheme for In-111.

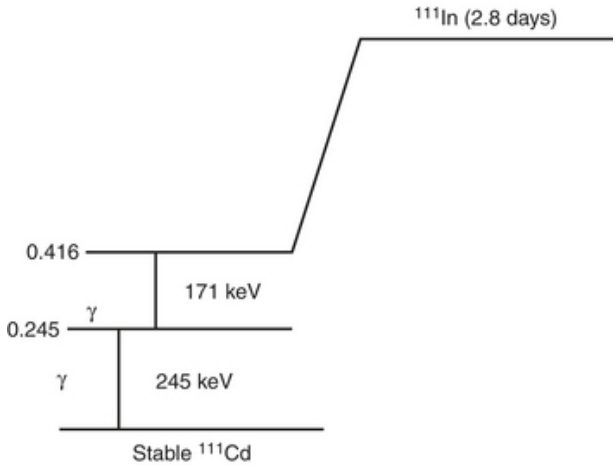

Figure 6: ^{111}In decays via electron capture to the stable cadmium-111, With a half life of 2,83 days. This figure includes gamma emission peak energies at 171 keV and 245 keV. (19).

2.3.2 Zr-89

The radioactive metal that is used during this experiment is ^{89}Zr. It has a half-life of 78.41 hours. The nuclide decays via positron emission and electron capture to ^{89m}Y with a half-life of 15.7 seconds, which in its turn decays via gamma emission to the stable ^{89}Y. This decay is depicted in figure 7.

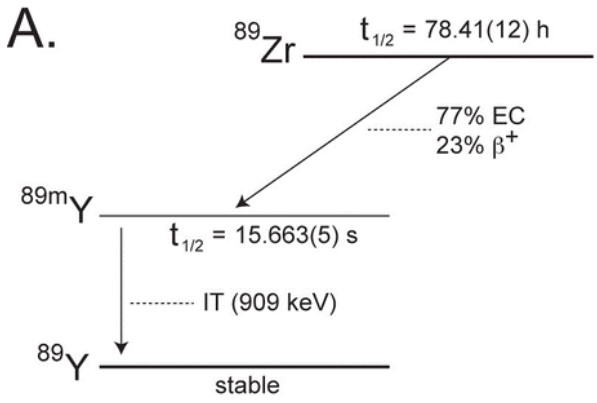

Figure 7: Decay scheme of ^{89}Zr, which decays via positron emission and electron capture to ^{89m}Y with a half-life of 15.7 seconds, which in its turn decays via gamma emission to the stable ^{89}Y (4).

Zirconium will be delivered as zirconium oxalic acid. This is dissolved in HCl so it can be used for the experiments. Due to the low positron energy (396 keV), ^{89}Zr provides high resolution PET images. And because ^{89}Zr has a half-life time of approximately three days and PET imaging relies on nanocariers with a relatively slow pharmacokinetic, which makes ^{89}Zr loaded micelles the perfect candidate for this process (3).

2.4 Analytical methods

2.4.1 Size exclusion chromatography

Size exclusion chromatography, in short SEC, is a chromatographic based method developed in 1955 (13). SEC is seen as the most efficient, and fastest method to separate molecules according to their sizes. In the SEC columns, a porous packing material is placed. A sample solution is injected into these columns. Molecules inside the sample solution with a larger volume will be eluted first due to their inability to penetrate the pores. Smaller molecules will take longer, because they can penetrate into the pores. By penetrating the pores the smaller molecules will travel a greater distance then the larger molecules (14). In figure 8 a illustration is given, showing the separation process by SEC.

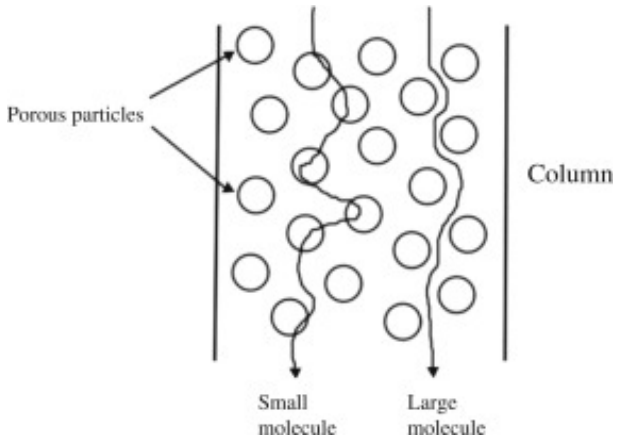

Figure 8: Schematic illustration of the separation process by size exclusion chromatography, where the small molecules take longer to be eluted due to the fact they will travel a greater distance by penetrating the pores. (14).

SEC is a low resolution separation technique. High pressure can increase the resolution, but that has a downside. A higher flow rate will result on incomplete separation, causing near simultaneous elution of molecules with different sizes. A larger column or more packing material, will result in better separation,

because the sample has to travel through more pores. With the use of a SEC column, one has to keep in mind that parameters can vary strongly (15).

2.4.2 Scintillation detector

To analyse the samples that will determine the outcome of the experiments conducted later on in this paper, A NaI scintillation detector is used. This detector makes use of a sodium iodide crystal to detect incoming gamma rays. When an incoming gamma ray hits the crystal, the crystal will emit a visible or near visible light photon. The number of produced photons by the crystal is proportional to the energy and the number of gamma rays that are absorbed. The photons emitted by the NaI crystal can be detected by a photo cathode. This photo cathode can convert the detected photons to an electric signal, completing the detector (16). In figure 9, a schematic can be seen that shows the NaI crystal absorbing radiation, and emitting photons.

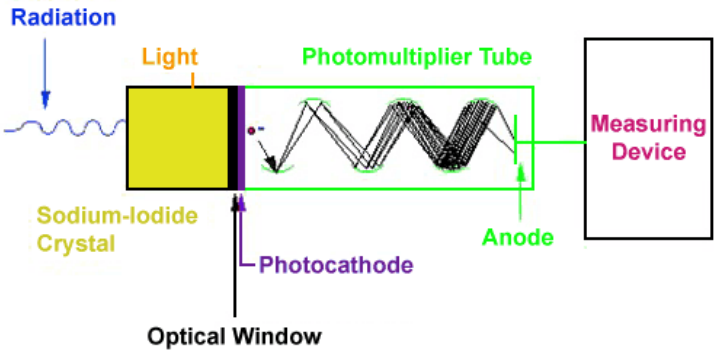

Figure 9: Schematic of the scintillation crystal detector, which can be found in a scintillation detector. The radiation hits the crystal, which will send out a photon. This will be detected by the photocathode. (20) .

3 Materials and Method

3.1 Materials

The block copolymers (PCL-PEO 2800-2000, PEO-PLA 5000-5500, PEO-PCL 5000-6500 and PLA-PEO 10500-5000) were bought from Polymer Source (Quebec, Canada). Chloroform, diethylenetriaminepentaacetic acid (DTPA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and Sephadex G-25 resins, that were used for the SEC columns, were bought from Sigma Aldrich (Zwijndrecht, the Netherlands). Zirconium chloride ($ZrCl_4$) was purchased from Alfa Aesar. HCl and acetonitrile were purchased from Central warehouse L&M. ^{111}In (specific activity: 15,5 GBq/μg) was kindly provided by Erasmus Medical Centre (Rotterdam, the Netherlands). ^{89}Zr (specific activity: 974 MBq/mL) was bought from PerkinElmer (Groningen, the Netherlands). The scintillation detector that is used in this experiment is the 2480 Wallac Wizard gamma counter, which is present at the reactor institute Delft.

3.2 Constructing micelles

In this experiment four different polymers were used. These polymers are listed below in table 1. What is also listed is the amount of corresponding solvent that was used later on to dissolve the polymers in.

Table 1: List of polymers used to construct micelles with their corresponding solvents.

Polymer	Solvent
PCL-PEO 2800-2000	200 μL chloroform
PEO-PLA 5000-5500	1 mL acetonitrile
PEO-PCL 5000-6500	200 μL chloroform
PLA-PEO 10500-5000	1 mL acetonitrile

For all of these polymers 20 mg was weighed, this was then dissolved in the correct solvent. These solutions were sonicated for 20 minutes. When the vials were finished sonicating, the polymers should be fully dissolved. If this was not the case, the solutions were sonicated for a longer period. After this process, per polymer, a glass vial was filled with 4 mL of MQ water, and a stirrer was added. MQ water was used, because it contains little to no contaminations. The polymer was added dropwise to the water, while stirring on a stirring plate without a lid. This was done to let the acetonitrile or chloroform evaporate. This solution was left overnight.

3.3 In-111 experiment

3.3.1 Radiolabeling PCL-PEO 2800-2000, PEO-PLA 5000-5500, PEO-PCL 5000-6500 and PLA-PEO 10500-5000 micelles

In this section a description is given for loading a single type of polymer micelles, as the method is the same for all types of micelles applied.
After stirring for a night, the solutions were filtered using a 450 nm cut-off filter to remove possible larger aggregates. 0.50 mL of this solution and 0.50 mL of Hepes buffer (20 mM, pH= 7,4) was added to three different vials. Once all these steps were completed and stirrers were added into the vials, the radioactive isotope could be added. In this case 40 kBq of In-111 was pipetted in each of the vials. This was then left to stir on the stirring plate for 30 minutes.
The vials had to be inserted into the Wallac gamma counter to measure the mixture's activity at that point in time. This was essential for the calculation of the total loading efficiency. Hereafter, a SEC (Size exclusion chromatography) process was performed for each individual vial. In this process the micelles were separated from the free radioactive ions present in the mixture. For each vial twenty fractions of 1 mL were collected. These fractions were again analysed by the Wallac, with a blank in front to correct for background radiation later on.

3.3.2 Radiolabeling PCL-PEO 2800-2000 and PLA-PEO 10500-5000 micelles

After the results of the last described experiment were examined, two polymers were selected to use and investigate further. PCL-PEO 2800-2000 and PLA-PEO 10500-5000 were selected. The radiolabeling was done again as described above, however different fractions were collected. The first fraction (0 to $7mL$) contained little to no activity. The second fraction (7 to $13mL$) showed the most relevant results as it contained the loaded ^{111}In-micelles. The third and last fraction (13 to $20mL$) consisted of the unloaded loose radioactive Indium-111.

A DTPA stability test was performed using these polymeric micelles. For this, the fractions from 7 to $13mL$ of all three columns were added together. This resulted in a solution that mostly contained Indium-111 loaded micelles. Then again, three times 0.50 mL of this micelle solution was taken and added to three different vials. Those vials were filled up to 1 mL with HEPES buffer. Before the DTPA was added, it was sonicated, so that it was a homogeneous mixture. To this solution $0,1mL$ of a $11mM$ DTPA mixture was added, and this was left overnight on the stirring plate. To have a zero-measurement these vials were analysed by the Wallac the next day.
This SEC process was repeated. The first fraction (0 to $7mL$) contained little to no activity. The second fraction (7 to $13mL$) showed the most relevant results as it contained the loaded In-micelles. The third and last fraction (13 to $20mL$), consisted the ^{111}In-DTPA molecules, because these molecules are smaller than the micelles, and therefore took longer to travel through the column. These fractions were analysed by the Wallac, and the data was collected, and then corrected. This data was then used to calculated the stability of these micelles.

3.4 Zr-89 experiment

3.4.1 Determining Zr^{4+} concentrations

In this experiment different amounts of Zr^{4+} were added to the 40kBq of ^{89}Zr during the loading of the micelles. CHEAQS, a program that can predict the speciation of zirconium at a given pH, was used to determine the amount of Zr^{4+} that was to be added. CHEAQS was used to determine the speciation of zirconium for which 10, 50 and 96 % occurs as Zr(OH)4 (s). $C_1 = 0.04mM$, $C_2 = 0.07mM$ and $C_3 = 1mM$ were the given concentrations respectively.

3.4.2 Radiolabeling two different types of micelles at different Zr^{4+} concentrations

To prepare the micelles 20 mg was weighed of the PCL-PEO 2800-2000 and PLA-PEO 10500-5000 polymers. The PCL-PEO 2800-2000 polymer was dissolved in 200 μL chloroform and the PLA-PEO 10500-5000 was dissolved in 1 mL of acetonitrile. These solutions were again sonicated for 20 minutes. The polymer was added drop by drop to the 4 mL water, while it was stirring on the stirring plate. This was then again to be left overnight without a lid.

In this section a description is given for loading a single type of polymer micelles, as the method is the same for both micelle mixtures.
The next day the mixture was filtered using a 450 nm cut-off filter. 0.50 mL of the micelles and 0.50 mL of HEPES buffer (20 mM, pH=7,4) were added to three vials and stirrers were added. In this case 40 kBq Zr-89 was pipetted into each of the vials. At this point, the desired $ZrCl_4$ concentration was added. For the first experiment the solution contained $C_1 = 0.04mM$ of $ZrCl_4$, in the second experiment the solution contained $C_2 = 0.07mM$ and the last experiment, the solution contained $C_3 = 1mM$ of $ZrCl_4$. This was to be left to stir on the stirring plate for 30 minutes.
To get a baseline measurement, the vials were inserted into the Wallac gamma counter to measure the mixture's activity at this point. A SEC process was performed as also described above. The fractions were collected again and analysed.

3.4.3 DTPA (diethylenetriaminepentaacetic acid) stability test

A stability test was again performed for two different concentrations, $C_1 = 0.04mM$ and $C_3 = 1mM$. The second fractions of all three columns were added together, that resulted in a solution that mostly contained zirconium loaded micelles. Then again, three times 0.50 mL of this micelle solution was added

to three different vials. Those vials were filled up to 1 mL with HEPES buffer. To this solution $0,1mL$ of the sonicated $11mM$ DTPA solution was added and it was left on the stirring plate overnight.
The SEC process was repeated, but now including the DTPA. The first fraction (0 to $7mL$) contained little to no activity. The second fraction (7 to $13mL$) showed the most relevant results as it contained the loaded Zr-micelles. The third and last fraction (13 to $20mL$), that would have consisted of loose zirconium, now consisted the Zr-DTPA molecules. The fractions were analysed by the Wallac for a last time and the retention ratio was processed in the presence of DTPA.

4 Results and discussion

4.1 Radiolabeling different micelles with In-111

First experiments were carried out with In-111 to get insight of the radiolabeling process before proceeding with Zr-89. For this purpose four different block copolymer micelles were selected. PCL-PEO 2800-2000, PEO-PLA 5000-5500, PEO-PCL 5000-6500 and PLA-PEO 10500-5000 were the selected polymers. The goal of these experiments was to see which of the micelles had the best radiolabeling efficiency, before proceeding Zr-89 radiolabeling.

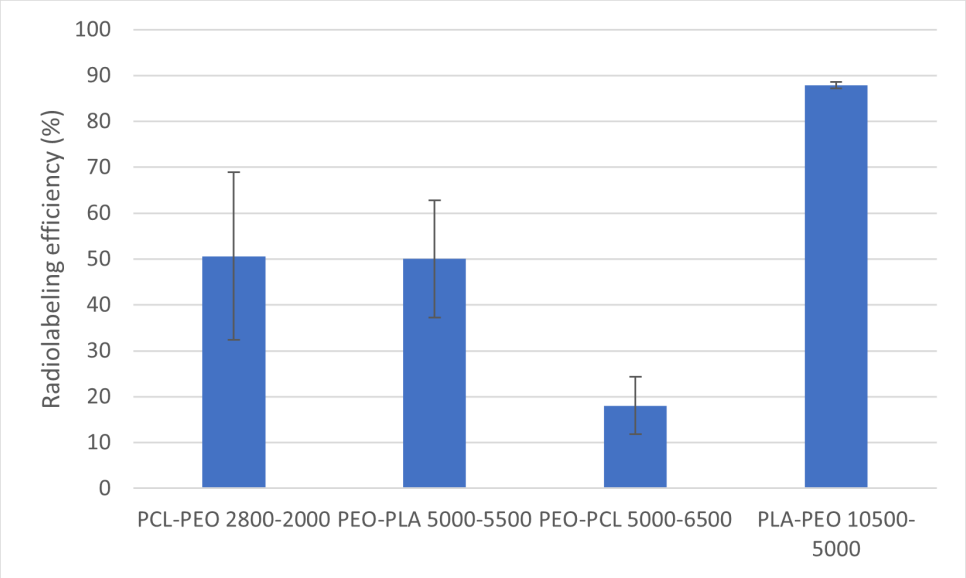

Figure 10: Radiolabeling efficiency of In-111 for four different polymer micelles, carried out in 4 different experiments. Each experiment is executed in triplo, so a standard deviation can be obtained. The polymer concentration for PCL micelles was 4,76 mg/mL before filtration with a 450 nm cutoff filter, and for PLA micelles it was 4,00 mg/mL before filtration with a 450 nm cutoff filter. A total of 40kBq was added per experiment, with a radiolabeling time of 30 min. (pH = 7)

In figure 10 the results for the first four experiments are displayed. As can be seen in the graph, PCL-PEO 2800-2000 and PLA-PEO 10500-5000 are the most promising candidates. The use of these polymers resulted in an efficiency of 50,60 ± 18,26 % and 87,91 ± 0,71 % respectively. The lowest radiolabeling efficiency of PEO-PCL 5000-6500, can be explained by the fact that a mistake was made during the process of making the micelles. The lid was on the vial when it was stirring overnight. As a result of this the chloroform did not evaporate sufficiently and thus resultated in a bad batch of micelles. When the results are compared with the results found by Lui et al. (1), one can see that for the PCL-PEO 2800-2000 and PEO-PLA 5000-5500 micelles the radiolabeling efficiency should be higher. In that paper a radiolabeling efficiency of 69,7 ± 1,49 % was found for PLA micelles, and a radiolabeling efficiency of 83,75 ± 1,49 % was found for PCL-6500 micelles. The PEO-PLA 5000-5500 polymer, which resulted in a loading efficiency of 50,03 ± 12,73 %, is around 20 % lower than expected. The fact that the PCL-PEO 2800-2000 and the PEO-PLA 5000-5500 micelles are under performing is probably a consequence of not adding the polymer mixture to the water drop by drop due to a mistake during the addition of the polymer to the water. This probably resulted in the formation of large polymer clusters and worm-like micelles. These large micelles were filtered out the day after, resulting in fewer micelles than expected, and a lower radiolabeling efficiency. Only the PLA-PEO 10500-5000 showed similar results to what was found by Lui et al. (1). Based on these results two polymers were selected to work with during the rest of the experiment. PCL-PEO 2800-2000 and PLA-PEO 10500-5000 were chosen because these had the most promising results.

4.2 DTPA stability test using selected polymer micelles

In figure 11 the results of another radiolabeling experiment can be seen. This time the experiment was conducted for the two selected polymers only. On the y-axis the average loading efficiency can be seen in percentages. For the average radiolabeling efficiency of PCL-PEO 2800-2000, 78,96 ± 3,62 % was found. The experiment using the PLA-PEO 10500-5000 polymer resulted in an efficiency of 76,47 ± 8,38 %. The loading efficiency that was found in these experiments is in line with what was found by Lui et al. (1), and thus what was expected.

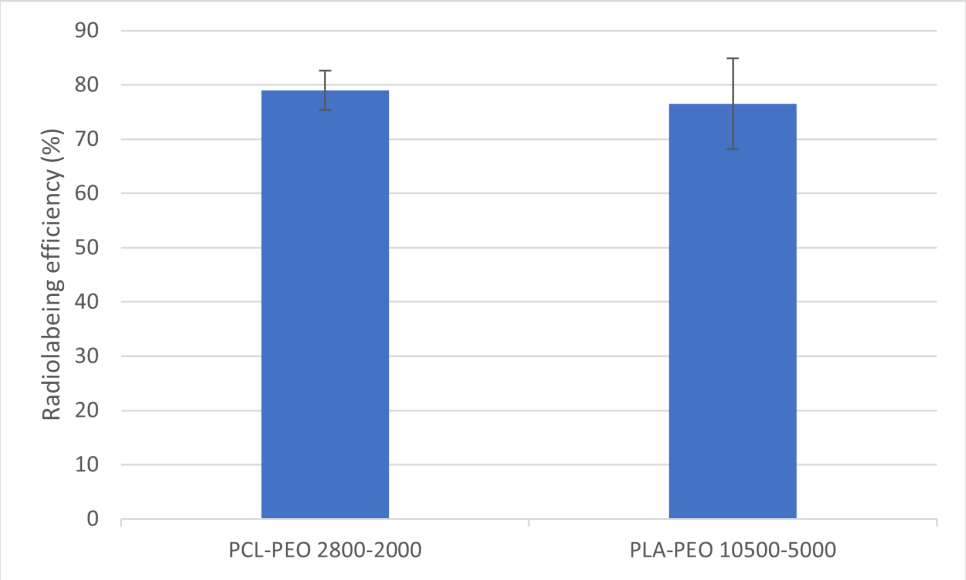

Figure 11: Radiolabeling efficiency of In-111 for two selected polymers. The polymer concentration for PCL-PEO 2800-2000 micelles was 4,76 mg/mL before filtration, and for PLA-PEO 10500-5000 micelles it was 4,00 mg/mL before filtration. A total of 40kBq was added per experiment, with a radiolabeling time of 30 min. Each experiment was performed in triplo. The depicted values show an average including a standard deviation(pH = 7).

The loaded ^{111}In-micelles were challenged by DTPA. In figure 12 the calculated retention ratio for Indium-111 in the micelles for the two used polymers can be found. The results show that the PCL-PEO 2800-2000 micelles were significantly more stable than the PLA-PEO 10500-5000 micelles. After the SEC, 34,93 ± 2,98 % and 22,35 ± 12,71 % of the In-111 isotopes were still inside the core of a micelle. These numbers are low if they are compared to a similar experiment conducted by Lui et al. (1) for her thesis. Those results showed that just 7,28 ± 1,35 % of the In-111 was removed from PCL micelles, and 12,88 ± 1,52 % of the In-111 was removed from the PLA micelles.
The low stability could be a consequence of the pH not being optimal. In this thesis the pH was measured to be 7. Huanhuan et al. found a pH of 7,4. This difference could have resulted in the In-111 not being in the optimal chemical form, and was easily removed from the micelles.

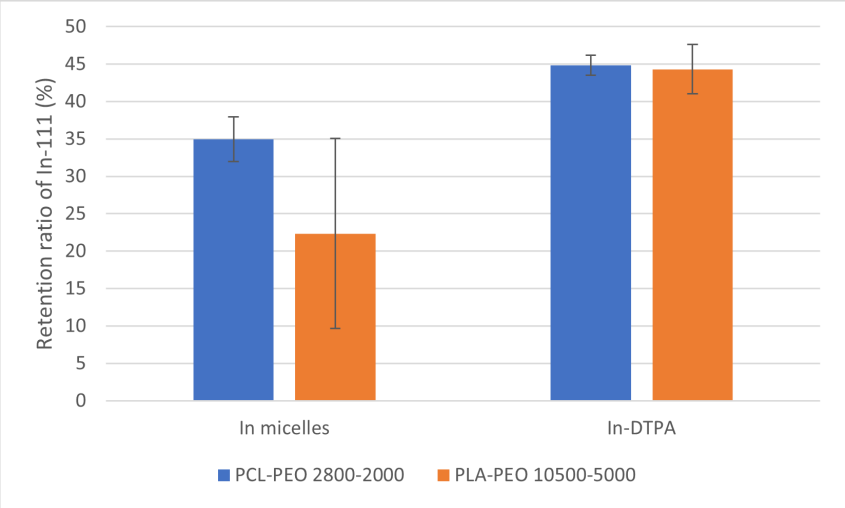

Figure 12: Stability of the In-111 radiolabled PCL-PEO 2800-2000 and PLA-PEO 10500-5000 micelles, after being challenged with DTPA for 12h (concentration: 11mM) (pH = 7).

4.3 Loading efficiency Zirconium-89

4.3.1 Determining the speciation of zirconium

To determine the concentrations at which to examine the radiolabeling efficiency, CHEAQS was used. The speciation of zirconium species at relevant concentrations are displayed in figure 13 below. In this figure, there are three interesting intervals to examine. The first interval is when there is more Zr(OH)4 (aq) than Zr(OH)4 (s). The second interval is when the amount of Zr(OH)4 (aq) is approximately equal to the amount of Zr(OH)4 (s). In last and third interval the amount of Zr(OH)4 (s) is higher than the amount of Zr(OH)4 (aq). From these three intervals, three concentrations were selected, namely $C_1 = 0,04mM$, $C_2 = 0,07mM$, and $C_3 = 1mM$.

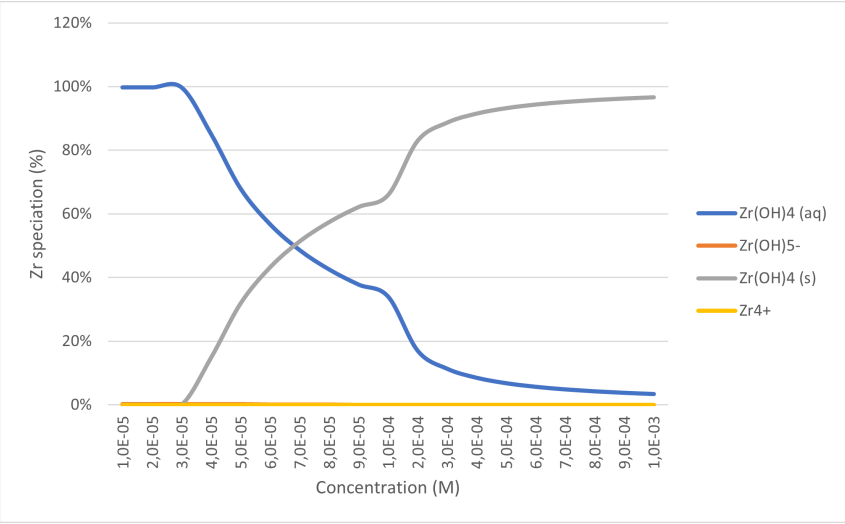

Figure 13: Zirconium speciation for varying concentrations in equilibrium at pH = 7. This figure is based on data calculated by CHEAQS.

4.3.2 Radiolabeling micelles with zirconium-89 at different concentrations

In figure 14 the loading efficiencies are depicted for three different Zr^{4+} concentrations. From left to right the concentrations are given by; $C_1 = 0,04mM$, $C_2 = 0,07mM$, and $C_3 = 1mM$. The green bar shows

the loading efficiency for the PCL-PEO 2800-2000 polymer and the yellow bar the PLA-PEO 10500-5000 polymer.

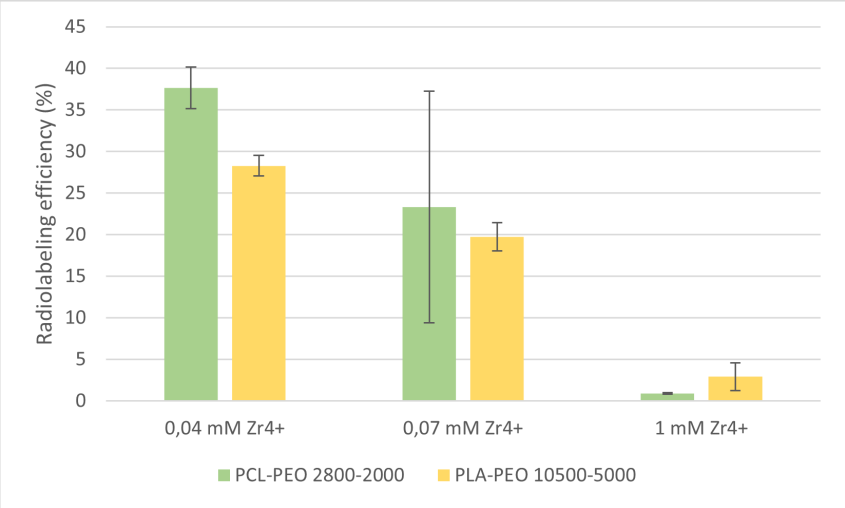

Figure 14: Loading efficiency of Zr-89 in PCL-PEO 2800-2000 and PLA-PEO 10500-5000 at various concentrations of $ZrCl_4$. The polymer concentration for PCL-PEO 2800-2000 micelles was 4,76 mg/mL before filtration with a 450 nm cutoff filter, and for PLA-PEO 10500-5000 micelles it was 4,00 mg/mL before filtration with a 450 nm cutoff filter. A total of 40kBq was added per experiment, with a radiolabeling time of 30 min. (pH = 7)

In this experiment the PCL-PEO 2800-2000 performed best with an average radiolabeling efficiency of 37,65 ± 2,48 % at a concentration of $C_1 = 0,04mM$. This is then followed by the PLA-PEO 10500-5000 polymer at the same concentration. For that type of micelles 28,27 ± 1,23 % was found. At the next concentration $C_2 = 0,07mM$ two lower efficiencies were found, 23,30 ± 13,94 % for the PCL-PEO 2800-2000 micelles and 19,76 ± 1,70 % for the PLA-PEO 10500-5000 micelles. Finally the last concentration $C_3 = 1mM$, at this concentration 96,5 % of the zirconium is in a solid state. The lowest radiolabeling efficiency was found. For PCL-PEO 2800-2000 0,88 ± 0,07 % was found and for the PLA-PEO 10500-5000 micelles a value of 2,91 ± 1,68 % was found.

The loading efficiency decreases each time the concentration is increased. This can be explained by the fact that when the concentration is increased, more zirconium was present in the mixture. However, the amount of micelles stays approximately the same, and the amount of loaded zirconium-89 does not change a lot. So there was increasingly more zirconium present in the mixture, but the same amount of micelles, resulting in a decreasing loading efficiency. Another explanation comes to mind when the results of the measurement at C_3 are examined (see appendix). In table 6 can be seen that in vial 1.1 the Wallac counted close to half a million decays. If table 7 is looked upon and the corresponding fractions of vial 1.1 (1.1.1) (1.1.2) (1.1.3) are investigated it can be seen that the Wallac counts a total of 4500 decays. That is approximately one percent of the total counts before the SEC. This indicates that the precipitated ^{89}Zr was probably left behind in the glass vials as it was added to the SEC column. Or it could have been added to the column but ended up sticking to the tube or another part, resulting the particles coming out when the column was being cleaned. The spread of the 0.07 mM PCL-PEO 2800-2000 bar is a consequence of this. In table 4 (see appendix), one can see that the residue left in the third vial is significantly higher than in the other two vials. If table 5 (see appendix) is examined, one sees that the activity measured in the fractions that should contain the loaded micelles and the free activity (1.3.2 and 1.3.3 respectively), is also lower than their counterparts from the fractions caught from the other columns (1.1.2)(1.1.3)(1.2.2) and (1.2.3). Because the fraction that should contain the radiolabled micelles has less counts, it will result in lower radiolabeling efficiency than the other two fractions obtained from the in triplo executed experiment, and thus resulting in a large standard deviation.

In a paper made by Lui et al. (1) a similar experiment was conducted. In that experiment PCL-

6500 micelles are loaded with ^{89}Zr in the presence of stable Zr^{4+}. The concentrations that were used were lower than the concentrations used in this experiment, but a downward trend can also be seen. At a concentration of 500 nM she established a radiolabeling efficiency of just under forty percent. That number is close to the radiolabeling efficiency measured in this experiment at a concentration of 40 μM. So even though the concentration is higher in this experiment, a similar result is found. This can be an indication of the downward trend coming to a halt for a certain interval of concentrations before continuing this trend downwards.

4.4 Stability test zirconium-89 at a concentration C_1 = 0,04 mM

The zirconium-89 loaded micelles that were obtained were challenged by DTPA. In figure 15 the retention ratios are displayed. For the PCL-PEO 2800-2000 polymer micelles, there was found that 37,57 ± 4,35 % of the zirconium stays inside the micelles and 48,17 ± 5,74 % was removed from the micelles. The PLA-PEO 10500-5000 polymer micelles did perform a lot better. 69,03 ± 1,27 % of the zirconium was still found to be inside of the micelles, while 27,65 ± 2,90 % was bonded to DTPA.

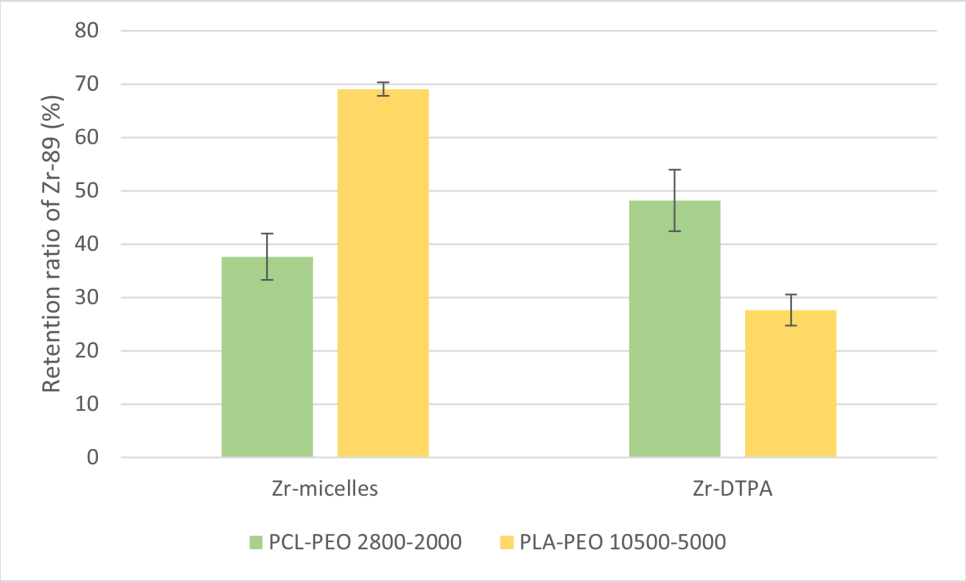

Figure 15: Stability of the zirconium loaded micelles, after being exposed for 12 hours to $0,1mL$ *of a 11 mM DTPA solution at a concentration of* C_1 *= 0,04 mM. (pH = 7)*

The results of the PCL-PEO polymer comply with results found by Lui et al. (1). 37,57 ± 4,35 % of the zirconium was found inside the micelles after being challenged by DTPA. What can be seen in figure 15 is that the PLA-PEO 10500-5000 micelles have a higher stability according to these results. 69,03 ± 1,27 % of the loaded zirconium was still in the micelles after being challenged by DTPA.
However, as this was the last conducted experiment, it could be that the SEC columns were not being packed well enough. If this was the case, the ^{89}Zr-DTPA molecules could have been coming out of the columns earlier than expected, and ended up in the second fraction that was captured. This could have resulted in a measurement of more counts in this fraction that in theory should contain mostly micelles.

4.5 Stability test zirconium-89 at concentration C_3 = 1 mM

A stability test was also performed for loaded micelles obtained at a concentration of C_3 = 1 mM. In figure 16 the results of the stability test can be seen. What is immediately noticeable is that the percentage of zirconium, that binds to a DTPA molecule, for PCL-PEO 2800-2000 is 196,76 ± 138,79

%. Which is of course impossible. One can also note that the percentages of the green and yellow bars together, do not add up to a hundred %. These are all indications that something went wrong during this experiment.

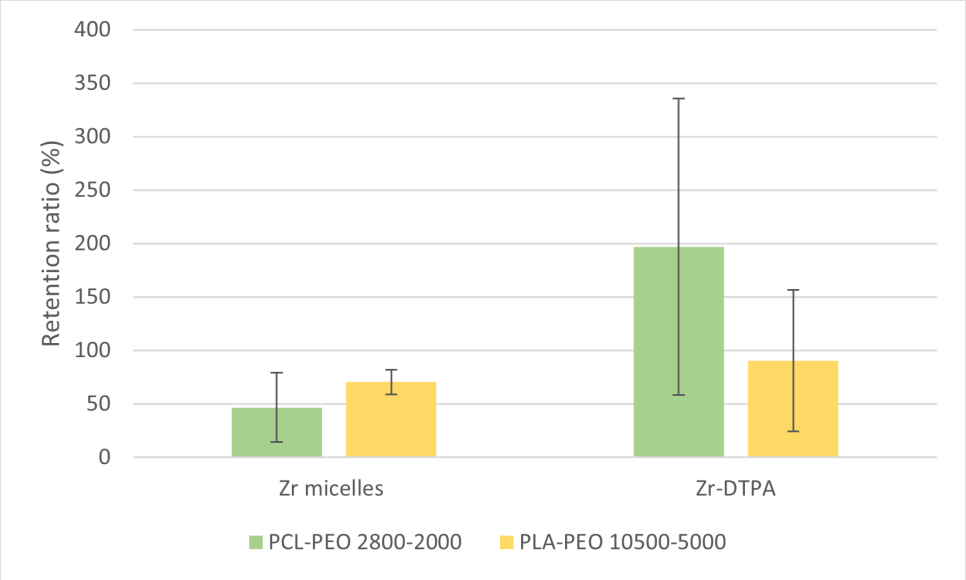

Figure 16: Stability of the Zirconium loaded micelles, after being exposed for 12 hours to $0,1mL$ *of a 11 mM DTPA solution at a concentration of* $C_3 = 1$ *mM. (pH = 7)*

In table 2 below one can see the results of one of the measurements that contributed to the PCL-PEO 2800-2000 results. Particular is that the number of counts in the base-line measurement, is way less than the number of counts measured in the fraction, that should contain the Zr-DTPA molecules. This results in the strange percentages displayed in figure 16. There are several circumstances that could have caused this. After the experiment to calculate the loading efficiency was completed, the the second fractions (7 to $13mL$) from the in triplo executed experiment were added together. It is possible that during the addition of the three vials containing micelles, a part of the activity that was inside the vials, was unfortunately left behind. This however, would only explain the low counts measured during the base line measurement. What could be a possible explanation for the relatively high measured counts in the third fraction, is that the day before this measurement, the columns were used for determining the loading efficiency at $C_3 = 1\ mM$. It could be that when the active solution was added to the columns, some precipitates stuck to the top of the columns. Most of them would have been washed out when the columns were cleaned using a 1 M HCl solution, but some could have stayed behind. If this is the case, and the remaining precipitates came out during the SEC process of the DTPA stability test, it would make sense that such a high number of decays were detected in the third fraction, as that was the fraction where the Zr-DTPA molecules were collected.

Table 2: Result of a single DTPA stability test at a concentration of $C_3 = 1mM$.

	Measured counts per second
Base line measurement	623,551
Fraction 1	6,823
Fraction 2	436,110
Fraction 3	2077,179

Table 3 shows the average of decays counted in the fractions. One can see that the third fraction contains a lot more on average than the second fraction. Also, the sum of the fractions is significantly higher than what is measured in the baseline measurements. This makes the explanation given above possible. As this is also a bigger difference than is seen in, for example, the stability test implemented at a concentration of $C_1 = 0,04mM$.

Table 3: Average of the fractions captured during the DTPA stability test at a concentration of $C_3 = 1mM$

	Average counts
baseline measurements	2078,455
Fraction 1	12,546
Fraction 2	990,358
Fraction 3	1978,679

5 Conclusions and recommendations

5.1 Indium-111 experiments

In the first set of experiments, four different polymers were used to construct micelles. These micelles were radiolabled with In-111 and their loading efficiency was determined. PLA-PEO 10500-5000 had the best results with a loading efficiency of 87,91 ± 0,71 %. This is followed by PCL-PEO 2800-2000, which resulted in a radiolabeling efficiency of 50,60 ± 18,26 %. These two polymers resulted in the highest average loading efficiency compared to PEO-PLA 5000-5500 and PEO-PCL 5000-6500. They resultated in 50,02 ± 12,74 % and 18,04 ± 6,29 % respectively. PLA-PEO 10500-5000 and PCL-PEO 2800-2000 were selected to work with in the following experiments because they had the best loading efficiency.

In the second set of experiments, the two polymers that were selected were used again in a radiolabeling experiment. The results were better for the PCL-PEO 2800-2000 polymer micelles, and gave back a radiolabeling efficiency of 78,96 ± 3,62 %. The use of PLA-PEO 10500-5000 resulted in a loading efficiency of 76,47 ± 8,38 %. These results are in line with what is found by Lui et al. (1) in her research, and show that with the use of these polymers, one can achieve a high loading efficiency using In-111 without the use of chelators.
A stability test was conducted during this experiment. The micelles were not stable enough for in vivo purposes according to this study. However the literature shows more promising results, so the stability of these micelles should be investigated further.

5.2 Zr-89 experiments

The zirconium experiments were conducted for two different micelles, PCL-PEO 2800-2000 and PLA-PEO 10500-5000. These micelles were radiolabeled in the presence of three different Zr^{4+} concentrations. From figure 14 can be concluded that the increase of Zr^{4+} atoms in the mixture does not promote the loading efficiency of the zirconium into the micelles. Besides the PCL-PEO 2800-2000 polymer had better results on average regarding the loading efficiency. It resulted in a loading efficiency of 37,65 ± 2,48 % at a concentration of $C_1 = 0,04mM$. At the next concentration $C_2 = 0,07mM$ a lower efficiency was found, 23,30 ± 13,94 % for the PCL-PEO 2800-2000 micelles. At the third concentration $C_3 = 1mM$, the lowest radiolabeling efficiency was found, namely 0,88 ± 0,07 %. The PLA-PEO 10500-5000 polymer micelles resulted in a radiolabeling efficiency of 28,27 ± 1,23 % at $C_1 = 0,04mM$, 19,76 ± 1,70 % at $C_2 = 0,07mM$, and 0,88 ± 0,07 % at $C_3 = 1mM$.

The retention ratio in the presence of DTPA has been calculated for the two types of micelles for a Zr^{4+} concentration of $C_1 = 0,04\ mM$. For PCL-PEO 2800-2000 a retention ratio of 37,57 ± 4,35 % was found and for PLA-PEO 10500-5000 a retention ratio of 69,03 ± 1,27 %. What is interesting to see is that although the PLA-PEO 10500-5000 polymer had a worse loading efficiency, it results in a significantly better retention ratio than the PCL-PEO 2800-2000 polymer micelles at this concentration. As the result for PCL-PEO 2800-2000 is similar to results found in previous studies, there is no further investigation necessary. Regarding the PLA-PEO 10500-5000 polymer micelles, the stability that is found is high compared to the results found in previous studies. This would be interesting to investigate further, to see if the PLA-PEO 10500-5000 polymer micelles do really have a higher stability on average.

In the previous section the results for the DTPA stability test at a Zr^{4+} concentration of $C_3 = 1mM$ are displayed. The results showed multiple strange features like 196,76 ± 138,79 % of the Zirconium binding to DTPA. These results are not reliable and this experiment must be done again. Before this experiment is conducted, the SEC columns need to be cleaned extensively, as it is suspected that left precipitates is part of the cause.

References

[1] Liu, H. (2021) Application of poly micelles combined with ionizing radiation in cancer treatment, Ridderprint.

[2] Laan, A. C., Santini, C., Jennings, L., de Jong, M., Bernsen, M. R., Denkova, A. G. (2016). Radiolabeling polymeric micelles for in vivo evaluation: a novel, fast, and facile method. EJNMMI research, 6(1), 12. https://doi.org/10.1186/s13550-016-0167-x

[3] van de Watering, F. C., Rijpkema, M., Perk, L., Brinkmann, U., Oyen, W. J., Boerman, O. C. (2014). Zirconium-89 labeled antibodies: a new tool for molecular imaging in cancer patients. BioMed research international, 2014, 203601. https://doi.org/10.1155/2014/203601

[4] Zeglis, B. and Lewis, J. (2015) The Bioconjugation and Radiosynthesis of 89Zr-DFO-labeled Antibodies https://www.researchgate.net/figure/A-A-simplified-decay-scheme-and-B-some-salient-decay-characteristics-of-89-Zr$_fig1_2$73148426

[5] Bas, S. Soucek, M.D. (2006) Synthesis, characterization and properties of amphiphilic block copolymers of 2-hydroxyethyl methacrylate and polydimenthylsiloxane prepared by atom transfer radical polymerization. https://www.nature.com

[6] Hu, X. Xiong, S. (2022) Fabrication of Nanodevices Through Block Copolymer Self-Assembly. https://doi.org/10.3389/fnano.2022.762996

[7] Biopharma PEG, (2019), The Difference Between Monodisperse and Polydisperse Polymers. https://www.biochempeg.com/article/61.html

[8] Aditi M. Jhaveri, Vladimir P. Torchilin (2014) Multifuncional polymeric micelles for delivery of drugs and siRNA. https://doi.org/10.3389/fphar.2014.00077

[9] Laan, A.C., Santini, C., Jennings, L. et al. Radiolabeling polymeric micelles for in vivo evaluation: a novel, fast, and facile method. EJNMMI Res 6, 12 (2016). https://doi.org/10.1186/s13550-016-0167-x

[10] Chakravarty R, Hong H, Cai W. Image-Guided Drug Delivery with Single-Photon Emission Computed Tomography: A Review of Literature. Curr Drug Targets. 2015;16(6):592-609. doi: 10.2174/1389450115666140902125657. PMID: 25182469; PMCID: PMC4346511.

[11] Hopkins, J. medicine, Positron Emission Tomography (PET). https://www.hopkinsmedicine.org/health/treatment-tests-and-therapies/positron-emission-tomography-pet

[12] Davey, R.J. , AuBuchon, J.P. in Blood Banking and Transfusion Medicine (Second Edition), 2007

[13] Sorci, M. , Belfort, G. in Bio-nanoimaging, 2014

[14] Deb, P.K., Tekade, R.K. in Basic Fundamentals of Drug Delivery, 2019

[15] Boone, C. Adamec, J. in Proteomic Profiling and Analytical Chemistry (Second Edition), 2016

[16] Kramar, U. in Encyclopedia of Spectroscopy and Spectrometry, 1999

[17] Cowan, R.L., Kessler, R. (2010). SPECT Imaging. In: Stolerman, I.P. (eds) Encyclopedia of Psychopharmacology. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-540-68706-1$_5$2

[18] Schoolphysics (2022). Positron emission tomography (PET) scans https://www.schoolphysics.co.uk/age16-19/Medical%20physics/text/PET%20scans/index.html

[19] iKnowlegde (2015) ,Emergency Nuclear Radiology, https://clinicalgate.com/emergency-nuclear-radiology/

[20] Equipco, https://www.equipcoservices.com/support/tutorials/introduction-to-radiation-monitors/

[21] Avramović N, Mandić B, Savić-Radojević A, Simić T. Polymeric Nanocarriers of Drug Delivery Systems in Cancer Therapy. Pharmaceutics. 2020 Mar 25;12(4):298. doi: 10.3390/pharmaceutics12040298. PMID: 32218326; PMCID: PMC7238125.

Appendix

Relevant value's of C_2 measurement

Table 4: Number of decays the wallac measured of the same vial before and after the sample was added to the column

Vial	Counts (CPM)	Counts in residue (CPM)
1.1	1233927,992	49454,11901
1.2	1211845,453	56196,64868
1.3	1226362,399	78619,53927

Table 5: Number of decays measured in the fractions captured during the SEC

Vial	Corrected for decay (CPM)
1.1.1	-6,791592378
1.1.2	337844,8481
1.1.3	33453,21656
1.2.1	12,11095926
1.2.2	362820,0421
1.2.3	13436,14904
1.3.1	7,301035474
1.3.2	154238,3459
1.3.3	2406,803816

Relevant value's of C_3 measurement

Table 6: Measurement of vials before SEC

Vial	Corrected for decay (CPM)
blanco	
1.1	412800,7831
1.2	407135,3473
1.3	392931,7931
blanco	
2.1	350353,4794
2.2	277525,711
2.3	1398850,595

Table 7: Number of counts after the SEC was performed

Vial	Corrected for decay (CPM)
1.1.1	38,60363165
1.1.2	3803,167092
1.1.3	455,767065
1.2.1	49,57843992
1.2.2	3788,59178
1.2.3	721,0801449
1.3.1	45,83319733
1.3.2	3139,60611
1.3.3	357,0334219
2.1.1	62,97502846
2.1.2	7257,119118
2.1.3	257,7033483
2.2.1	11,83397346
2.2.2	13468,62411
2.2.3	736,4550852
2.3.1	60,20072868
2.3.2	25593,74619
2.3.3	1234,437034