Nanoparticles and stem cells for drug delivery to the brain
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Chapter 6

Size dependent biodistribution and SPECT imaging of $^{111}$In-labeled polymersomes

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Abstract

Polymersomes, self assembled from the block copolymer polybutadiene-block-poly(ethylene glycol), were prepared with well-defined diameters between 90 and 250 nm. The presence of ~1% of diethylene triamine penta acetic acid on the polymersome periphery allowed to chelate radioactive $^{111}$In onto the surface and determine the biodistribution in mice as a function of both the polymersome size and poly(ethylene glycol) corona thickness (i.e. PEG molecular weight). Doubling the PEG molecular weight from 1 kg/mol to 2 kg/mol did not change the blood circulation half-life significantly. However, the size of the different polymersome samples did have a drastic effect on the blood circulation times. It was found that polymersomes of 120 nm and larger become mostly cleared from the blood within 4 hours, presumably due to recognition by the reticuloendothelial system. In contrast, smaller polymersomes of around 90 nm circulated much longer. After 24 hours more than 30 percent of the injected dose was still present in the blood pool. This sharp transition in blood circulation kinetics due to size is much more abrupt than observed for liposomes and was additionally visualized by SPECT/CT imaging. These findings should be considered in the formulation and design of polymersomes for biomedical applications. Size, much more than for liposomes, will influence the pharmacokinetics and therefore long circulating preparations should be well below 100 nm.
Introduction

Polymersomes, or polymer vesicles, are a relatively new class of nanocapsules that are formed by the self assembly of amphiphilic block copolymers in aqueous media [1]. Polymersomes can be regarded as the polymeric analogues of liposomes with a thicker, more stable and less leaky membrane. These characteristics make polymersomes an interesting class of nanocarriers for the delivery of diagnostic and therapeutic agents. For these applications it is generally desired to have long circulating particles which are not readily cleared from the blood stream by the reticuloendothelial system (RES) [2]. From liposomal in vivo studies it is known that size, next to PEGylation and charge, is a major factor that influences the blood circulation kinetics and biodistribution. Liposomes of more than 200 nm have been shown to accumulate in the spleen and liver, whereas liposomes of less than 70 nm tend to accumulate predominantly in the liver [3,4]. Liposomes with a size between 90 and 150 nm have the longest blood circulation times. The blood circulation times of liposomes can be further enhanced by the introduction of up to ten percent PEGylated phospholipids [5,6]. Polyethylene glycol (PEG) prevents the opsonisation by blood proteins and the subsequent recognition and degradation by macrophages of the reticuloendothelial system. PEGylated liposomes may exhibit blood circulation half-lives (t½) of more than 15 hours, depending on the PEG chain length, charge and size of liposomes [3,4,7]. Finally, the effect of surface charge in liposomal formulations [8] and nanocarriers [9] has been studied. Both a slightly negative and positive surface charge was reported to have a positive effect on the blood circulation kinetics. The number of reports on biodistribution of neutral polymersomes is limited and is basically restricted to the work of Discher [10,11] who used fluorescently labeled polymersomes to determine the effect of longer PEG chains and surface charge on t½ values, the work of Lee et al.[12] and the recent work by Kataoka et al. who studied the size dependence of fluorescently labeled polyelectrolyte vesicles (PICosomes) for the preferred accumulation in tumor tissue compared to healthy tissue [13]. Also near infrared dyes have been encapsulated in order to visualize tumors in mice [14,15]. Discher et al. reported that polymersomes exhibit blood circulation half-lives up to 28 hours. These high t½ values are partly due to the fact that polymersomes are fully PEGylated upon choosing PEG as the hydrophilic part of the block copolymer that constitutes the polymersome bilayer. It was furthermore shown that neutral polymersomes circulate with the highest t½ values. Finally Tsourkas et al. [16] encapsulated a Gd-based magnetic resonance imaging (MRI) contrast agent for
enhanced MRI imaging. By measuring the concentration of Gd in blood, they were also able to determine a concentration time profile.

One of the most quantitative methods to determine the biodistribution of drug delivery vehicles in vivo is by radio isotope labeling. This technique is often used for liposomal formulations, but has until now been hardly explored for polymersomes. There is one report on the biodistribution and radiolabeling (14C/3H) of negatively charged polymersomes [12] and one study that shows data on the encapsulation and biodistribution of a radiolabeled model drug encapsulated in polymersomes [17]. However, the effect of polymersome size on biodistribution has not been analysed before with this technique. If radiolabeling of polymersomes with a suitable isotope such as $^{111}$In would be performed, this would not only allow quantitative determination of organ distribution but also visualisation with SPECT/CT imaging, as has previously been demonstrated for 50 nm polymer micelles [18, 19].

In this paper we present a systematic study into the effect of size on the biodistribution of polymersomes via the quantitative technique of $^{111}$In radiolabeling, in combination with SPECT/CT imaging as depicted schematically in Figure 1. It is shown that polymersomes of around 90 nm in diameter have long circulation times in male Balb/C mice. Upon increasing the diameter to 120 nm and above the long circulating properties are lost and polymersomes are cleared from the blood stream within a few hours by mainly the liver and spleen. The effect on circulation time upon changing the size from 90 to 120 nm is also clearly visualized by SPECT/CT imaging.

![Figure 1. Schematic presentation of polymersome formation, $^{111}$In labeling and in vivo SPECT/CT imaging.](image-url)
Experimental procedures

Materials

Sec-butyllithium (ALDRICH 1.4M in hexane), tetrabutylammonium fluoride (TBAF) (ALDRICH, 1.0M in THF), sodium hydride (ALDRICH, 60% dispersion in mineral oil, p-isothiocyanate-benzyl diethylenetriamine penta-acetic acid (MACROCYCLICS >94%), Chelex resin (ALDRICH), were used as received. Tetrahydrofuran (THF) (ACROS ORGANICS, 99+% extra pure, stabilized with BHT) was distilled under argon from sodium/benzophenone, and triethyl amine (TEA) (BAKER) was distilled from calcium hydride under an argon atmosphere prior to use. Polymersome extrusions were performed using 200 nm filters (Acrodisc 13 mm Syringe Filter, 0.2 µm Nylon membrane) and 0.1 µm PC membrane (WHATMAN). InCl3 was purchased from Covidien, Petten, The Netherlands Instant Thin-Layer Chromatography Silica Gel impregnated glass fibre (ITLC-SG) strips were purchased from Varian. Column chromatography was carried out using silica gel (Acros (0.035-0.070 mm, pore diameter ca. 6 nm)) Note that other silica suppliers gave less satisfactory results for α-azido ω-carboxy poly(ethylene glycol).

Instrumentation

Infrared (IR) spectra were obtained using a Thermo Matson IR 300 FTIR spectrometer. Data are presented as the frequency of absorption (cm⁻¹). Proton nuclear magnetic resonance (1H NMR) spectra were recorded on a Varian Unity Inova 400 FTNMR spectrometer. Chemical shifts are expressed in parts per million (δ scale) relative to the internal standard tetramethylsilane (δ=0.00 ppm). Molecular weight distributions were measured using size exclusion chromatography (SEC) on a Shimadzu (CTO-20A) system equipped with a guard column and a PL gel 5 μm mixed D column (Polymer Laboratories) with differential refractive index and UV (λ=254 nm and λ=345nm) detection, using tetrahydrofuran (SIGMA ALDRICH chromasolv 99.9%) as an eluent at 1 mL/min and T = 35 oC. Particle size distributions were measured on a Malvern instruments Zetasizer Nano-S and zeta potentials were measured on a NanoSight NS 500 instrument.

Polymere synthesis

Propyne- endcapped polybutadiene (1). A Schlenk tube was thoroughly cleaned, rinsed with butyllithium, flushed with MilliQ and oven dried over night. The Schlenk
tube was evacuated and an argon atmosphere was applied, after which 7.1 g of 1,3-butadiene was condensed at -78 °C. THF was distilled under argon over sodium/benzophenone and 10 mL was added to the flask. The polymerisation was initiated by the addition of 1.4 mL sec-butyllithium (1.4M in hexane). The mixture was allowed to heat up to -35 °C as the colour changed from pale yellow to orange. After the colour changed back to yellow the reaction was terminated by the addition of 10 mL dry THF and 400 mg trimethylsilyl propargylbromide. After all colour had disappeared 4 mL tetrabutyl ammonium fluoride (1M in THF) was added and the mixture was stirred for 1 hour. All solvents were removed and the product was dissolved in dichloromethane, after which it was filtered over a slab of silica, eluting with dichloromethane. The final product was obtained by coevaporation with 4 times 50 mL toluene to remove any traces of propargyl bromide. The product contained two molecular weight distributions as determined by size exclusion chromatography (THF): 3.7 kg/mol (60%) and 7.4 kg/mol (40%). The higher molecular weight, a polymer dimer as reported and explained by Tohyama et al.[20], is not reactive toward azides and was easily removed by silica column chromatography, after the block copolymers were formed as shown in Figures S1-S3.

a- azido ω- methoxy poly(ethylene glycol) (2a/b) general procedure. Poly(ethylene glycol) monomethyl ether (5mmol, 1 or 2 kg/mol to obtain respectively 2a or 2b) was coevaporated with benzene three times and dissolved in 250 mL dry and under argon distilled THF. The general procedure is further described for the preparation of 2a. The flask was cooled on an ice bath and air was replaced by argon before 5 mL freshly distilled triethyl amine was added. The mixture was stirred for 3 hours, after which 1.14 gram (2 eq. 10mmol) of mesyl chloride in 10 mL THF was added. The mixture was allowed to warm to room temperature and was stirred for 6 hours. All THF was removed and 100 mL methanol containing 3.25 gram (10 eq. 50mmol) sodium azide was added. The mixture was refluxed overnight after which methanol was removed and 100 mL water was added. The product was extracted with 5x 100 mL dichloromethane. The combined organic layers were dried over magnesium sulfate and DCM was removed, yielding 4.5 gram (90%) product 2a. The SEC traces of both 2a (Figure S1) and 2b (Figure S2) can be found in the electronic supplemental information. 1HNMR (2a): δ 3.37 (s, 3H, CH₃O), 3.39 (t, 2H, CH₂N₃), 3.64 (m, 90H, CH₂CH₂O). FTIR: 2098 cm⁻¹ (azide). SEC (THF): Mn (2a) = 1 kg/mol, Mw/Mn = 1.19

α- azido ω- carboxy poly(ethylene glycol). 5 g of poly(ethylene glycol) (5mmol, 1 kg/mol) was coevaporated with benzene three times and dissolved in 250 mL dry and under argon distilled THF. The flask was cooled on ice and air was replaced by argon, followed by the addition of 5 mL distilled triethyl amine. The mixture was stirred for 3
hours, after which 0.6 gram (1.1 eq. 5.1 mmol) mesyl chloride in 10 mL THF was added over 1 hour. The reaction was allowed to warm to room temperature and stirred for 6 hours. THF was removed and 100 mL methanol containing 3.25 gram (10 eq. 50 mmol) sodium azide was added. The reaction was heated to reflux overnight, whereafter methanol was removed and 100 mL water was added. The mixture of statistical products was extracted with 5x 100 mL dichloromethane. The combined organic layers were dried over MgSO₄, and dichloromethane was removed. The products were coevaporated with benzene, dissolved in 250 mL freshly distilled THF and cooled on an ice bath. An argon atmosphere was applied and 400 mg 60% sodium hydride (2 eq.) was added. After three hours 2.9 g t-butyl bromoacetate (3eq. 14 mmol) in 10 mL THF was added and the temperature was raised to 50 ºC. The reaction was allowed to proceed overnight, after which all solids were filtered off and THF was removed. The products were dissolved in 100 mL 2M HCl and heated to reflux for 5 hours. The final product was extracted with 5x 100 mL dichloromethane. The combined organic layers were dried over MgSO₄ and all solvents were removed. The product (Rf = 0.5) was purified using silica column chromatography (eluent MeOH:DCM:NH₃ =15:85:5) yielding 1.5 gram (30%) of product. TLC: Rf = 0.5, permanganate staining, eluent DCM:MeOH = 92:8. ¹HNMR: δ 3.39 (t, 2H, C₂H₃N₃), 3.64 (m, 90H, C₂H₅C₂H₂O), 4.00 (s, 2H, OCH₂COOH). FTIR: 2098 cm⁻¹ (azide), 1570 (C=O of carboxylic acid salt). SEC (THF): Mn = 1 kg/mol, Mw/Mn = 1.19

α-azido ω-amino poly(ethylene glycol) (3). 1 g (1 mmol ) of α-azido ω-carboxy poly(ethylene glycol) was dissolved in 50 mL methanol and 5 drops of concentrated sulphuric acid were added. The mixture was heated under reflux overnight, after which methanol was removed. The product was dissolved in 50 mL water and extracted with 5 times 50 mL dichloromethane. The organic layer was dried over MgSO₄ and all solvents were removed. TLC: Rf = 0.65, permanganate staining, eluent DCM:MeOH = 92:8. Indicative ¹HNMR shifts: δ 4.17 (s, 2H, OCH₂COOMe), 3.75 (s, 3H, COOCH₃) and IR: 1748 cm⁻¹ (carbonyl/ester)

The product was dissolved in 50 mL methanol and 0.5 mL 1,2-diamino ethane was added (large excess). The mixture was heated under reflux for 48 hours, after which all methanol was removed. The product was dissolved in 50 mL 1M. hydrochloric acid and extracted with 5 times 50 mL dichloromethane. The combined organic layers were dried over MgSO₄ and all solvents were removed to yield 800 mg product (80%). TLC: Rf = 0.55, permanganate and nynhydrin staining, eluent DCM:MeOH = 92:8. ¹HNMR: δ 2.99 (t, 2H CH₂CH₂NH₂), 3.39 (t, 2H, CH₂N₃), 3.64 (m, 90H, CH₂CH₂O), 4.01 (s, 2H, OCH₂CONH). FTIR: 2098 cm⁻¹ (azide), 1696 cm⁻¹ (amide). SEC (THF): Mn = 1 kg/mol, Mw/Mn = 1.24.
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**Polybutadiene-block-poly(ethylene glycol) general procedure (4a/b, 5).** Poly(ethylene glycol) 2a, 2b or 3 (2a, 3: 80 mg 0.08mmol, 2b: 160 mg 0.08mmol) and 1 (800 mg, 2.6 eq. 0.21mmol) were dissolved in 10 mL dry tetrahydrofuran under an argon atmosphere. The general procedure is further described for the coupling of 2a and 1. The temperature was raised to 55 °C and 30 mg CuBr and 70 mg PMDETA were added. The reaction was allowed to proceed for 12 hours after which all solvents were removed. The crude product was redissolved in 50 mL dichloromethane and washed three times with 25 mL 0.33 M EDTA. The organic layer was dried over MgSO₄ and poured on a short silica column, which was eluted with dichloromethane. After all non-reacted polybutadiene was flushed off the product was eluted with 8 v% methanol in dichloromethane. After removal of all solvents 250 mg of the product was obtained in 60% yield (4a). The product was analyzed by size exclusion chromatography, showing a single size distribution (PDI = 1.14) with a shift toward higher hydrodynamic volume compared to polybutadiene. ¹NMR: δ 1.16 (m, 134H, CH₂CH), 2.11 (m, 67H, CH₂C), 3.37 (s, 3H, CH₃O), 3.64 (m, 90H, CH₂CH₂O), 4.94 (m, 134H, CHC), 5.45 (m, 67H, CHCH₂). Mn was determined by NMR (4a. 4.7 kg/mol, 4b. 5.7 kg/mol, and 5. 4.7 kg/mol) All SEC traces are shown in the electronic supplemental information Figures S1 (4a), S2 (4b) and S4 (5).

**Conjugation of Diethylene Triamine Penta Acetic acid (DTPA) (6).** 110 mg amine end-functional polybutadiene-block-poly(ethylene glycol) (23 µmol) was dissolved in 10 mL THF. To the solution, 14 mg (0.95 eq. 22 µmol) p-isothiocyanate-benzyl diethylene-triamine-penta-acetic-acid and 5 mL triethylamine were added. The mixture was stirred at room temperature for 48 hours, after which all solvents were removed. The products were dissolved in 5 mL THF and cooled on ice for 6 hours, after which the solution was filtered through a 200 nm syringe filter. The product was obtained by removing all THF. The DTPA end groups were not quantified, yet their presence was confirmed by FTIR and ¹¹¹In test labeling. FTIR: 1730 cm⁻¹ (carboxylic acid). SEC (THF): Mw/Mn = 1.26, Mw (theoretical) = 5.3 kg/mol.

**Polymersome formation (general)**

9.9 mg 4a (2.1 µmol) or 4b (1.7 µmol) and 0.1 mg (0.02 µmol) diethylene-triamine-penta-acetic-acid functional polybutadiene-block-poly(ethylene glycol) 6 were dissolved in 200 µL THF. Polymersomes were formed by the slow addition of 0.6 mL 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer of pH 5.5. The samples were passed three times through a 200 nm syringe filter to yield polymersomes of ~250 nm. Polymersomes of ~120 nm were obtained by multiple extrusion through 100 nm filters (one pass will yield ~160 nm polymersomes) and polymersomes of less than 100 nm...
Polymersome size

nm were obtained by treating the sample with ultrasonic sound waves (48 kHz, 200W) for 30 minutes at 35 ºC. Samples were washed and concentrated to 200 µL by means of a spin column (100 kD MWCO, 3000 rpm). Note that for the biodistribution samples a stock solution containing 9.9 mg 4a and 0.1 mg 6 per 200 µL THF was prepared to prevent variation in concentration and ratio of polymers.

\textit{\textsuperscript{111}In labelling}

To 30 µL polymersomes, 150 µL 0.1 M. 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 5.5) and 15 MBq \textsuperscript{111}InCl\textsubscript{3} were added. The labeling mixture was incubated at room temperature for 30 minutes. Labeling efficiency was analyzed by Instant Thin-Layer Chromatography Silica Gel impregnated glass fibre (ITLC-SG), developed in 0.1 M NH\textsubscript{4}Ac (pH 5.5)/0.1 M EDTA (1:1, v/v). Samples with a labeling efficiency of more than 95 percent were used without purification and diluted with PBS buffer prior injection. Samples with a coupling efficiency of less than 95 percent were purified over a PD10 desalting column and diluted with PBS.

\textit{Blood plasma stability}

To study the stability of polymersome preparations, 20 µL of radiolabeled polymersomes was incubated in 500 µL blood serum for 4 and 24 hours at 37 ºC. Association of \textsuperscript{111}In with the polymersomes was analyzed by ITLC-SG, developed in 0.1 M NH\textsubscript{4}Ac (pH 5.5)/0.1 M EDTA (1:1, v/v).

\textit{Biodistribution studies}

Biodistribution was analyzed in male BALB/c mice (6 weeks of age, 20-23 g per animal). The animals were divided in 8 groups of 4 animals and injected in their tail vein with 250 kBq (0.2 mL or 0.42 mg/kg) of \textsuperscript{111}In-labeled polymersome preparation. Mice were sacrificed by CO\textsubscript{2} inhalation 4 or 24 h postinjection (p.i.), a blood sample was drawn, and tissues of interest were dissected, weighed, and counted in a gamma-counter along with a standard of the injected activity to allow calculation of the injected dose per gram tissue (% ID/g). Animal experiments were approved by the local animal welfare committee and carried out according to national regulations.
Two animals were selected for SPECT/CT imaging and injected with 15 MBq (0.2 mL, 25.2 mg/kg) $^{111}$In-polymersomes in the tail vein. The animals were sacrificed by CO$_2$ inhalation and scanned with the U-SPECT-II (MILabs) 4 hours postinjection.

**Results and Discussion**

The amphiphilic block copolymer used in this study was polybutadiene-$block$-poly(ethylene glycol) (PBd-PEG) because of its well-known biocompatibility and glass transition temperature ($T_g$) well below room temperature. Because of this low $T_g$ the membrane of polymersomes formed from PBd-PEG remains fluidic which allows good control over the size via standard sizing techniques such as extrusion.

The general synthetic route towards the three block copolymers used in this study is depicted in Scheme 1. As can be seen from this Scheme we adopted a modular approach based on the copper-catalyzed [2+3] cycloaddition reaction [21]. One of the main advantages of this modular approach is the possibility to vary the molecular weight of PEG between 1 and 2 kg/mol, while keeping the molecular weight (distribution) of PBd exactly constant at 3.7 kg/mol. Polybutadiene was synthesised by means of anionic polymerisation. The reaction was initiated with sec-butyllithium at -35 ºC. After all monomer was consumed, the living polymer was endcapped with 3-bromo-1-(trimethylsilyl)-1-propyne [22]. Deprotection with tetrabutyl ammonium fluoride yielded the alkyne-functional polybutadiene 1. Poly(ethylene glycol) monomethyl ether was purchased with a molecular weight of either 1 or 2 kg/mol. The introduction of an azide was straight forward by mesylation and azidation as published elsewhere [23]. PBd and PEG were coupled in THF at 60 ºC by the addition of copper bromide and PMDETA as ligand. This reaction yielded the non functional block copolymers PBd-PEG with a molecular weight of approximately 4.7 kg/mol ($4a$) or 5.7 kg/mol ($4b$) depending on the PEG molecular weight. These inert block copolymers served as the basic building blocks for polymersome formation.

To allow radiolabeling of polymersomes with $^{111}$In a third block copolymer was synthesised with diethylene triamine penta acetic acid (DTPA) as chelating end group. Polybutadiene was coupled to $\alpha$-amino-$\omega$-azido-poly(ethylene glycol) via the same click approach as described above to obtain amine end-functional PBd-PEG (5) with a molecular weight of 4.7 kg/mol (PEG molecular weight of 1 kg/mol). DTPA is a metal chelating agent that coordinates well to bi- and trivalent metals. Copper, as used in the coupling reaction of PBd and PEG, can also coordinate strongly to DTPA. To prevent undesired occupation of DTPA by copper, all traces of copper in the different PBd-PEG analogues were removed by treating the polymers with Chelex resin in THF (as
confirmed by ICP-MS). Finally p-benzyl-isothiocyanate DTPA was introduced in block copolymer 5 by reacting the amine end group and isothiocyanate in THF with triethylamine as base to form polymer 6. Polymersomes were formed by slowly diluting a solution of 4a:6 or 4b:6 = 99:1 (w:w) in THF with 2-(N-morpholino)ethanesulfonic acid (MES) buffer with a pH of 5.5. After the addition of MES buffer the samples were extruded through 0.2 µm syringe filters to yield polymersomes of around 250 nm diameter. To size the polymersomes even further down the samples were either extruded through 100 nm filters (to yield ~120 nm polymersomes) or treated with ultrasonic sound waves at 35 ºC (to yield ~90 nm polymersomes). After resizing, the samples were washed with MES buffer and concentrated. It should be noted that extrusion through 100 nm membranes yielded the narrowest particle size distribution, whereas ultrasound waves and extrusion through syringe filters gave broader distributions. All particle polydispersities were found to be below 0.15, which is comparable to results often encountered for liposomal formulations (See Figures S5 and S6 for the full DLS curves). The characteristics of the polymersomes are summarized in Table 1, as is the $^{111}$In labeling efficiency and blood plasma stability of the different samples which will be discussed next.
Scheme 1: Overview of polymers and the reaction path towards these compounds. Exact details on the synthesis can be found in the experimental section of the article. i) anionic polymerisation of 1,3 butadiene followed by endcapping and deprotection ii) azidation with MsCl followed by NaN₃ iii) coupling of polymers via copper catalysed [2+3] cycloaddition iv) NaH, t-butyl bromoacetate in THF, followed by deprotection in 1M HCl v) esterification in MeOH/H₂SO₄ vi) nucleophilic acyl substitution by 1,2 diamino ethane in MeOH vii) amine coupling of p-SCN-Bn-DTPA.
The Zeta potential was measured on a NanoSight NS500 in tap water with an applied voltage of 24. Polymersomes (250 nm) formed from only polymer 4a, i.e. no DTPA chelated to the surface, have a zeta potential of -6.32 mV.

Table 1: Overview of polymersome characteristics. The Zeta potential was measured on a NanoSight NS500 in tap water with an applied voltage of 24. Polymersomes (250 nm) formed from only polymer 4a, i.e. no DTPA chelated to the surface, have a zeta potential of -6.32 mV.

<table>
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<tr>
<th>ID</th>
<th>size (nm)</th>
<th>PDI</th>
<th>$M_\text{w}$ PEG (kg/mol)</th>
<th>$\zeta$ potential</th>
<th>$^{111}$In labeling efficiency</th>
<th>Specific activity (MBq/mg)</th>
<th>Plasma stability 4h</th>
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$^{111}$In labeling was performed by adding 15 MBq $^{111}$InCl$_3$ to 30 µL of polymersome solution. Samples that labeled with an efficiency of more than 95 percent were used without further purification, and were diluted with PBS to 1.25 MBq per mL. The one sample that had a labeling efficiency of only 60 percent (2k90) was purified over a PD10 desalting column after which it was also diluted with PBS. The stability of the $^{111}$In-labeled preparations was tested by adding 20 µL of each sample to 500 µL human blood serum. After 24 hours about 80 percent of the Indium-111 radioactivity was still associated with the polymersomes as summarized in Table 1. These results show that these polymersomes of well-defined size allow for stable radio isotope labeling for in vivo applications.

After establishing polymersome synthesis and evaluation of the radiolabeling stability we studied the biodistribution as function of size and peripheral PEG thickness. As might be expected from similar studies based on liposomes, larger particles will influence the circulation kinetics negatively. In order to study if a similar trend is also valid for polymersomes, we injected male Balb/c mice with 250 kBq (0.2 mL or 0.42 mg/kg) of $^{111}$In-labeled polymersome solution in the tail vein. Each group contained four animals which were sacrificed after 4 or 24 h. Tissues of interest were collected, weighed and counted for radioactivity. The tissue distribution is depicted in Figure 2.

Upon looking at the blood levels a clear effect of polymersome size can be recognized immediately. Vesicles of 90 nm circulate much longer than vesicles of 120 nm and larger. After 4 hours more than 50 percent (based on 1.5 g blood per animal) of the 90 nm polymersomes were still present in the blood pool, which after 24 hours was reduced to 25 percent (based on a blood volume assumption of 1.5 g per animal). This allows us to estimate the blood half live of these 90 nm polymersomes to be around 20 hours, a number comparable to blood circulation half lives as reported by Photos et al [10]. Upon increasing the particle size both the liver and spleen accumulation became higher, an effect that is more abrupt for the liver than for the spleen. The abundance of
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90 nm polymersomes in lungs was relatively high, which most likely is caused by high blood levels in lung tissue. These data show that – similarly to liposomes – smaller polymersomes circulate longer. However there is a major difference in the correlation between size and blood circulation kinetics of these polymersomes and liposomes. For polymersomes the transition from long-circulating to short-circulating, upon increasing the size, is much more abrupt than for liposomes. A possible explanation might be found in the more rigid [1] structure of polymersomes which results in less flexibility. This feature could be of practical use since it could have a beneficial effect on tumor accumulation via the enhanced permeability and retention (EPR) effect [13,24].

**Figure 2.** Biodistribution of differently sized 111In-labeled polymersomes in Balb/C mice (n=4/group). Left: 4 h p.i., right: 24 h p.i.
In order to investigate the effect of the PEG corona on biodistribution two polymersome samples were included in these experiments with an average diameter of ~90 nm, but with different PEG lengths of 1 and 2 kg/mol (Table 1 and Figure 2). Although the sample with the thicker PEG corona seemed to circulate longer in the blood stream and showed lower liver and spleen accumulation this effect is not significant, especially because there is also a small size difference between both samples.

A powerful method of imaging organs/tissue particle distributions in living animals (and humans) is by Single Photon Emission Computed Tomography (SPECT) imaging. SPECT/CT imaging is a tool often used in nuclear medicine to obtain three dimensional images in a non-invasive manner. The polymersomes as described in this paper have a high enough specific activity to image them by this technique. Typically for imaging of mice 15 MBq of activity is needed, an amount easily accessible via the route described herein.

In order to illustrate by SPECT/CT the abrupt transition from long circulating to fast clearance upon increasing the polymersome size, we prepared samples of 90 nm polymersomes and 120 nm polymersomes with 15 MBq of $^{111}$In. The procedure was analogous as described above, only the dilution factor with PBS was adjusted to end up with a sample of 75 MBq per mL. Each animal was injected intravenously with 15 MBq (0.2 mL, 25.2 mg/kg) $^{111}$In-polymersomes in the tail vein and was scanned after 4 hours. The resulting images are depicted in Figure 3. The top panel of Figure 3 (a-c) shows the results for the long circulating polymersomes of 90 nm with a PEG molecular weight of 1 kg/mol. As visualized by radioactivity in the heart and lungs, the polymersomes are still present in the blood circulation. The lower panel of Figure 3 (d-f) shows 4 h post-injection scans of the 120 nm polymersomes. All the radioactivity is present in liver and spleen. This is in agreement with the biodistribution data as discussed above. These scans again show the strong size dependence of polymersomes with regard to blood circulation kinetics.
Conclusions

We have demonstrated that for polymersomes, size is an important factor in the blood circulation kinetics. Polymersomes of 120 nm and larger are readily cleared from the blood, whereas smaller polymersomes of approximately 90 nm are long circulating with an estimated blood half life of 24 hours. We have shown that polymersomes containing 1 percent of a DTPA end-functional amphiphilic block copolymer of polybutadiene-block-poly(ethylene glycol) can be prepared with a sufficient specific activity for SPECT/CT imaging. The effect of size on the biodistribution could therefore also be illustrated by this technique to confirm that 90 nm polymersomes are long circulating whereas 120 nm polymersomes readily accumulate in the liver and spleen. The effect of increasing the PEG molecular weight from 1 to 2 kg/mol was not found to be significant in this case. These findings should be taken into account upon designing polymersome formulations for imaging or drug delivery purposes.
Polymersome size, much more than for liposomes, will influence the circulation kinetics of polymersomes.

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References

Chapter 6


Supplementary information to Chapter 6

**Figure S1**: SEC (THF) traces of alkyne –endcapped polybutadiene after anionic polymerization (solid line, 1), α-azido ω-methoxy poly(ethylene glycol) (short dashed line, 2a) and the block copolymer polybutadiene-b-poly(ethylene glycol) (long dashed line, 4a).

**Figure S2**: SEC (THF) traces of alkyne –endcapped polybutadiene after anionic polymerization (solid line, 1), α-azido ω-methoxy poly(ethylene glycol) (short dashed line, 2b) and the block copolymer polybutadiene-b-poly(ethylene glycol) (long dashed line, 4b).
Figure S3: SEC (THF) traces: The dimeric compound present in alkyne-endcapped polybutadiene (solid line smallest peak, 1) is not reactive towards azides and therefore will not form a block copolymer with \(\alpha\)-azido \(\omega\)-methoxy poly(ethylene glycol). After the block copolymer is formed the dimer and excess of 1 are easily removed by silica column chromatography. When eluting in dichloromethane, polybutadiene and its dimer have an Rf value of 1, whereas the desired block copolymer (4a/4b) has an Rf value of 0. The dichloromethane fraction mainly contains the dimer plus the excess of polybutadiene as shown in the dashed trace. Block copolymers 4a/4b are eluted in 8 v% methanol in dichloromethane; their SEC traces are shown in figures S1 and S2.

Figure S4: SEC (THF) trace of amine end-functional polybutadiene-b-poly(ethylene glycol) (5).
**Figure S5**: DLS intensity size distributions of sample 2k90nm (top) and 1k 90nm (bottom) as measured on a Malvern Nano S machine. Both samples were resized by treatment with ultrasound (48 kHz, 200 W, 35 degrees Celsius).
Figure S6: DLS intensity size distributions of sample 1k120nm (top) and 1k 250nm (bottom) as measured on a Malvern Nano S machine. Both samples were resized by means of extrusion through respectively 200 and 100 nm membranes.