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Communication

Methods for production of uniform small-sized polymersome with rigid membrane

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We report a facile methodology for the formation of uniform small-sized poly(ethylene glycol)-*block*-polystyrene (PEG-*b*-PS) polymersomes, *via* extrusion and sonication methods by using organic solvent as plasticizing agent. The obtained polymersomes have diameters less than 100 nm. The size and size distribution depend on the organic solvent content and sonication time. The small-sized polymersomes are able to carry both hydrophobic and hydrophilic dyes.

Introduction

Polymer vesicles or polymersomes generated from the self-assembly of amphiphilic block copolymers in aqueous media have received significant attention for a number of applications, such as nanoreactors, *in vivo* imaging and drug delivery.[1-12] The lamellar membrane structure of polymersomes is regarded as the polymeric analogue of liposomes.[13-17] The thickness of the membrane can be controlled by adjusting the molecular weight of the amphiphilic polymers, which strongly affects their physicochemical properties, such as the mechanical stability and permeability. The thicker polymersome membrane provides the advantage of enhanced variability and improved vesicle stability.[18, 19] The polymersome structure allows water-soluble agents to be loaded into the hydrophilic inner lumen whereas hydrophobic cargos prefer to be embedded in the membrane.[20] Different types of cargos such as dyes, enzymes, as well as inorganic nanoparticles have been encapsulated into polymersomes.[21-25]

The size of the polymersomes is an important parameter that affects their applicability.[26] For example, in drug delivery, the enhanced permeability and retention effect (EPR effect) enables certain sizes of nanotransporters to accumulate selectively into tumor tissue, while small molecules diffuse unselectively into tumor as well as healthy tissue.[27] Van Hest *et al.* tested the bio-distribution of polymersomes composed of poly(ethylene glycol)-*block*-polybutadiene (PEG-*b*-PBD), with diameters between 90 nm and 250 nm. It was found that polymersomes above 120 nm were mostly cleared from the blood stream within 4 h, whereas smaller polymersomes of around 90 nm were still present even after 24 h in the blood pool, which means that small size polymersomes (SSP) are theoretically more efficient for drug delivery.[28-30] Besides in biomedical applications, small-sized vesicles can also be more effective as carriers for catalysts due to their high surface area and surface energy.[31-33]

Three methods are commonly used for the fabrication of small-sized polymersomes (SSP): direct formation, and post-formation resizing through either extrusion or sonication.[34, 35] These methods work well on polymersomes with relatively flexible membranes, such as poly(ethylene glycol)-*block*-poly(ε-caprolactone) (PEG-*b*-PCL).[34] However, polymersomes with rigid membranes are difficult to scale down below 100 nm *via* the above-mentioned post-resizing methods.

The most extensively studied rigid aggregates are assembled from polystyrene-based copolymers, such as poly(acrylic acid) -*block*-polystyrene (PAA-*b*-PS) and poly(ethylene glycol)-*block*-polystyrene (PEG-*b*-PS), due to their high stability and facile methods of characterization (*e.g.* *via* Transmission Electron Microscopy (TEM)). Taking advantage of the high glass transition temperature of PS, PEG-*b*-PS vesicles were recently found to be able to change their shape *via* a controllable shape transformation from spherical vesicles to stomatocyte shapes by adjusting the osmotic pressure over the membrane.[25, 36, 37] The key factor for performing this transition is the use of organic solvents as plasticizing agent to provide enough mobility and permeability to the PS membrane, which induces responsiveness to environmental changes.

Inspired by this work, herein, we report the use of plasticizing solvents to soften the PS membrane for resizing PEG-*b*-PS based polymersomes by extrusion into SSP with diameters less than 100 nm with narrow size distributions, for a wide range of PS lengths. The ratio between water and organic solvent proves to be crucial to induce sufficient flexibility to

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allow shape changes to occur, and to facilitate budding by the shearing forces that occur upon extrusion.

Results and discussion

PEG_{45}-*b*-PS_m was synthesized from a macro-initiator α-methoxy-PEG_{45}-ω-isobutyrylbromide (PEG_{45}-Br) *via* atom transfer radical polymerization (ATRP). PEG_{45}-Br was synthesized from a commercially available PEG monomethyl ether (MeO-PEO-OH, M_n = 2000) *via* an esterification of the PEG with α-bromoisobutyryl bromide at room temperature in THF. The length of PS was varied from 160-271 monomer units by tuning the reaction time (90 °C, 3-5 h), yielding four block copolymers with different block ratios. All the samples showed a narrow dispersity (Đ = 1.08-1.13) (Table 1).

Table 1. Molecular characteristics of the block copolymers

entry	M_n[a] (kg/mol)	Đ[a]	DP_n[b]	W_{PEG} (%)[c]
PEG_{45}-*b*-PS_{160}	18.7	1.08	160	10.7
PEG_{45}-*b*-PS_{206}	23.5	1.10	206	8.5
PEG_{45}-*b*-PS_{230}	26.0	1.09	230	7.7
PEG_{45}-*b*-PS_{271}	28.5	1.13	271	6.6

[a]Molecular weights and polydispersity measured by gel permeation chromatography (GPC). [b]Number average degree of polymerization was calculated from the molecular weight obtained by NMR measurements. [c]The calculated weight fraction of a PEG block in the block copolymers, based on GPC results.

PEG_{45}-*b*-PS_{206} was selected to test the suitable conditions for the fabrication of SSP. Assembly of PEG_{45}-*b*-PS_{206} was carried out by addition of water into the polymer organic solution (1 wt %, THF/dioxane=4/1 v/v). When the water content (volume) reached 18.0 %, the solution turned cloudy. At this point PEG_{45}-*b*-PS_{206} started to assemble and form non-uniform morphologies, as shown with TEM (Fig. S1a). Samples for analysis were prepared by directly adding an aliquot (50 µL) of polymersome solution at once to pure water (1 mL), thereby freezing the morphology. When the solution contained 20.0 % of water, uniform spherical polymersomes were formed, showing an average diameter of 480 nm, based on TEM and dynamic light scattering (DLS) (Fig. S1b and Fig. 1). From this point on the polymersome size did not change anymore upon increasing the water content.

After the water addition process, the polymersomes were resized by extrusion. The extrusion procedure is very simple, and consists of passing the polymersome organic solution through a filter (200 nm) for 4 times. The residual organic solvent in the solution was proposed to fulfil the role of plasticizer to enhance the flexibility and permeability of the membrane to allow the resizing procedures to function well. 20.0, 33.3 and 66.7 % water contents of the polymersome suspensions were selected for the extrusion test. When the water content was between 20.0 and 33.3%, the obtained polymersomes were highly uniform (Đ = 0.012) with sizes around 100 nm, based on DLS spectra, as shown in Fig. 1a, 1b and 1d. The size distribution was also analysed from TEM image *via* ImageJ, as shown in Fig.S2, presenting a narrow size distribution but with smaller diameter comparing with the DLS data due to drying effect. The thickness of the membrane of the small polymersomes was around 22 nm as measured from *cryo*-TEM (inset of Fig. 1b) which was similar to the thickness of polymersomes measured before extrusion; the measured values were also in agreement to our previous results[36]. Therefore the extrusion procedure did not change the bilayer arrangement of the PS chains. Taking into consideration that the calculated length of the PS_{206} chain is about 24 nm, we expect that the PS chains within the bilayer membrane are in a coiled conformation with chain-chain entanglements. When the water content increased to 66.7%, the polymersomes could pass though the filter, but the shape and size remained constant since the flexible, but more stable polymersomes were squeezed through the filter without causing any budding, as shown in Fig. 1c and 1d.

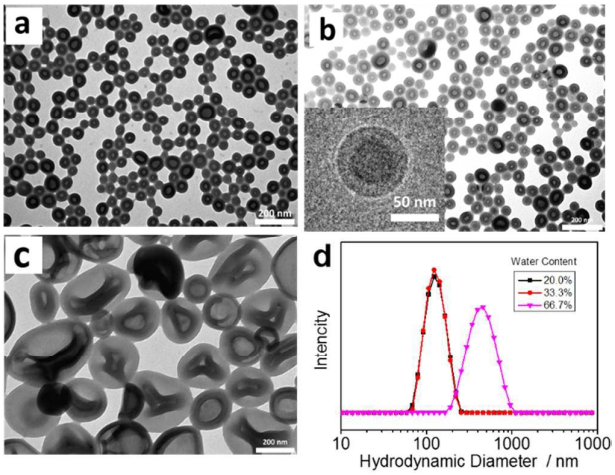

Fig. 1. DLS data (d) and TEM images of PEG_{45}-*b*-PS_{206} polymersomes after extrusion at a water content of 20.0% (a), 33.3% (b) and 66.7 % (c). Cryo-TEM image of polymersomes after extrusion at a water content of 33.3% (inset of b).

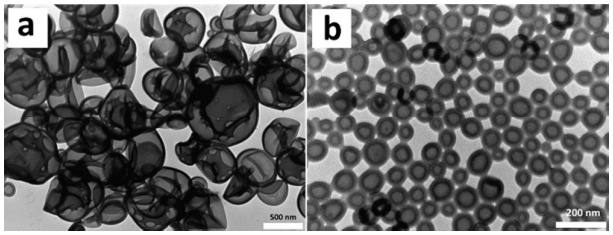

Fig.2. TEM images of PEG_{45}-*b*-PS_{206} polymersomes prepared by fast water addition (100 mL/h) before (a) and after (b) extrusion at a water content of 33.3%.

When all the organic solvent was removed *via* dialysis, polymersomes were rigidified and could not pass through the filter anymore. However, re-addition of organic solvent into the solution until 33.3 % water content made the membranes flexible again and enabled the extrusion to SSP by the same method. The obtained polymersomes displayed the same size as the vesicles after direct extrusion, as shown in Fig. S3.

In general, to obtain well-dispersed polymersomes, water is slowly added into the organic solvent to avoid random

aggregation. To demonstrate this effect, polymersomes were prepared with the same end point conditions (water content 33.3 %) except *via* different water addition rates from 1 mL/h to 100 mL/h. The morphology of the as-prepared polymersomes changed from spherical vesicles to multi-opening vesicles, as shown in Fig. 2a. Interestingly, after extrusion, the polymersomes presented the same spherical morphologies with uniform diameters around 100 nm as the ones extruded from spherical vesicles. This indicates that this extrusion method has the potential for fast fabrication of small polymersomes and to eliminate kinetic effects that originate from the fast formation process.

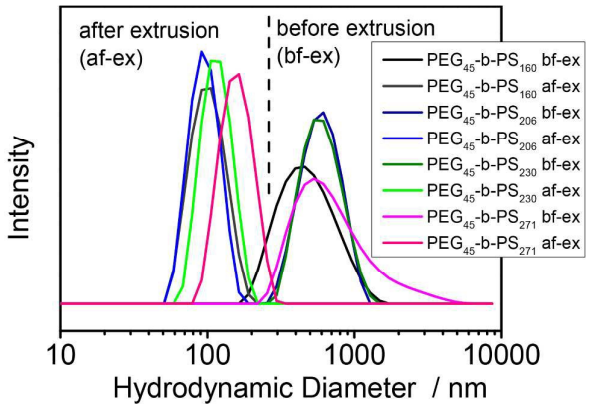

Fig.3. DLS data of polymersomes prepared from PEG_{45}-b-$PS_{160\text{-}271}$ before (bf-ex) and after (af-ex) extrusion.

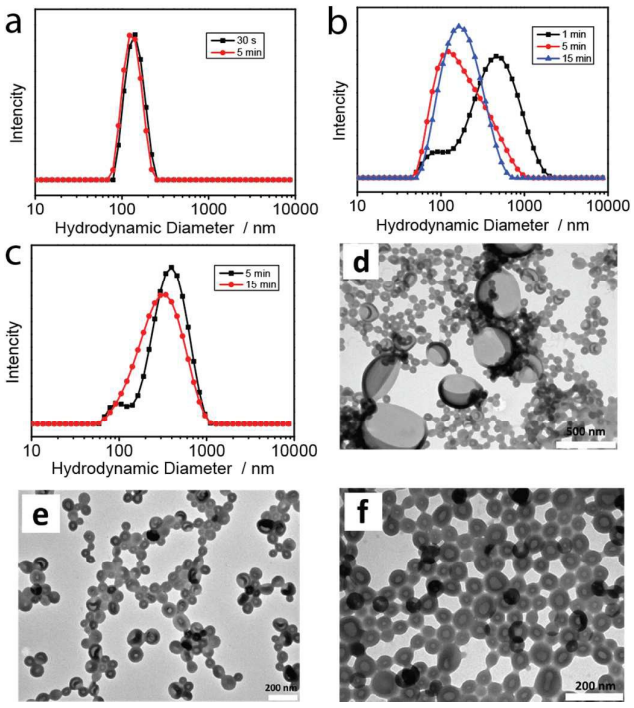

Fig. 4. DLS data and TEM images of solutions of PEG_{45}-*b*-PS_{206} polymersomes with water content of 33.3% (a), 50% (b) and 66.7% (c) after sonication at different time scale. TEM images of the polymersomes at water content of 50.0% after sonication of 1 min (d), 5 min (e) and 15 min (f).

Molecular weight is another important factor that can influence the morphology and size of the aggregates. As reported previously PEG_{45}-*b*-PS_n vesicles can only be obtained when the PS part is in a certain range of 150-280 monomer units. [36] Out of this range, other morphologies like micellar and lamellar structures will be formed. The diameter of the polymersomes increased from 400 nm to 500 nm when the PS length was increased from 160 to 271 monomer units, as shown in Fig. 3. After extrusion the diameters of all polymersomes decreased to < 100 nm with uniform size distribution, except for PEG_{45}-*b*-PS_{271} which yielded vesicles with an average diameter of about 150 nm.

Sonication is another commonly used method for transforming large vesicles into smaller ones. Based on the conditions tested from the extrusion experiments, the ratio of organic solvent and sonication time were investigated. When the water content was 33.3 %, all the large polymersomes were resized to small ones (< 100 nm) after 30 s of sonication, and a prolonged sonication time of 5 min did not change the size (Fig. 4a). At 50 % of water content sonication of 1 min could not break all the large vesicles due to increased rigidity of the membrane, and after 5 min small sized ones were only partially obtained (Fig. 4b). The size did not change with extending the sonication time to 15 min, although the vesicle population became more polydisperse as demonstrated by the TEM images (Fig. 4d-f). When the water content increased to 66.7 %, the membrane was rigid enough to resist morphological changes; even after sonication for 15 min only a small size change occurred (Fig. 4c). Thus, the best condition for fabrication of SSP *via* the sonication method is to perform the experiment at a water content around 33.3 % for 30 s. Compared with the extrusion method, sonication is easier to obtain SSP when a water content lower than 50 % can be applied.

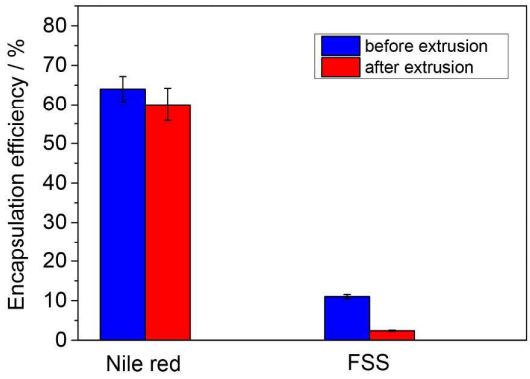

Fig.5. Encapsulation efficiency of polymersomes before (blue) and after (red) extrusion for the hydrophobic dye Nile red and hydrophilic dye FSS.

As compared to other carrier systems like micelles and liposomes, polymersomes exhibit enhanced performance for encapsulation and delivery of hydrophobic and hydrophilic drugs. [38-40] In this study, it is important to investigate the capability of loading cargos before and after the size change. Nile red and fluorescein sodium salt (FSS) were employed as

models of hydrophobic and hydrophilic drugs respectively. The two types of dyes were initially mixed with the PEG-*b*-PS solution and encapsulated in the polymersomes during the polymersome formation process. The SSP were obtained *via* the extrusion method at a water contents of 33 %. Free dyes in the solvent were removed *via* dialysis against water. Encapsulation of the dyes did not affect the obtained SSP with regard to size and shape, based on TEM images (Fig. S4).

In general during the extrusion process dyes in the vesicles will be partially released to the environment. To investigate the amount of dye released, we freeze-dried the large and small sized polymersomes loaded with dyes, weighted the same amount of the dry polymer-dye powder, and dissolved it in equal volumes of the organic solvents (Nile red in THF/dioxane=4:1 v/v; FSS in DMF/water=19:1 v/v). Fluorescence spectroscopy was employed to calculate the encapsulation efficiency by determining the intensity change after extrusion. As shown in Fig. 5a, the encapsulation efficiency of Nile red for large sized polymersome was 64 %, and decreased to 60 % after extrusion to small size. This is because the hydrophobic Nile red mainly stays in the hydrophobic region (PS membrane). During dialysis some released Nile red in the solvent mixture was possibly reincorporated into the membrane, which contributed to a high encapsulation value. On the contrary, hydrophilic FSS was encapsulated with relatively low efficiency (11 %), and a large fraction of the hydrophilic dye was lost (to 2.4 %) during the process of resizing (Fig. 5b). This large decrease (about 5 times) in the encapsulation efficiency is most probably caused by the volume shrinkage after resizing, as calculated and shown in the SI. These results demonstrate that the SSP fabricated by this method have the potential to carry in particular hydrophobic cargos, and that in case of hydrophilic compounds the extrusion has to be performed in aqueous solution containing the molecules to be encapsulated.

Experimental

Materials and Methods. All reagents and chemicals were purchased from commercial sources and used as received. MilliQ-water (18.1 MΩ) was used throughout the experiments. NMR spectra were performed on a Varian Inova 400 spectrometer with $CDCl_3$ as a solvent. Molecular weights of the block copolymers were measured on a Shimadzu Prominence GPC system equipped with a PL gel 5 µm mixed D column (Polymer Laboratories) and differential refractive index and UV (254 nm) detectors. THF was used as an eluent with a flow rate of 1 mL/min. Dynamic light scattering (DLS) experiments were carried out on a Malvern Zetasizer Nano S equipped with a He-Ne (633 nm, 4 mW) laser and an Avalanche photodiode detector at an angle of 173 °. Dispersion Technology Software (Malvern Instruments) was employed for DLS measurements. Transmission electron microscopy (TEM) samples were prepared in the following way: a solution of sample (6 µL) was air-dried on a carbon-coated Cu TEM grid (200 mesh). A TEM JEOL 1010 microscope at an acceleration voltage of 60 kV was used to perform the measurements. Sonicator VWR USC300TH was used for the sonication epxeriments at room temperature. The fluorescence measurements were carried out on a Perkin-Elmer (LS-55) luminescence spectrophotometer.

Polymerization of PEG-b-PS. Poly(ethylene glycol) macroinitiators and block copolymers, poly(ethylene glycol)-*b*-polystyrene (PEG-*b*-PS) were synthesized via ATRP by a method reported previously[41]. All block copolymers were characterized by ^{1}H NMR and GPC to evaluate the molecular weight and the size distribution, as shown in Table 1.

Preparation of Polymersomes. Modified from our former literature report[36], a typical procedure is described: PEG_{45}-b-PS_{206} (10 mg) was dissolved in a solvent mixture of tetrahydrofuran (THF) and 1,4-dioxane (dioxane) (1 mL, 4:1 by volume) in a 15 mL capped vial with a magnetic stirrer. After dissolving the solution for 1 h at room temperature, a syringe pump equipped with a syringe with a needle was calibrated to deliver water with a speed of 1 mL/h. The needle from the syringe was inserted into the vial of which the cap was replaced by a rubber septum. 3 mL of water was pumped into the organic solution with vigorous stirring (900 rpm). When finishing the water addition, 50 µL of the suspension was dropped at once into 1 mL of pure water with stirring, which ensured a rapid quenching of the PS domain within the bilayer of the polymersomes. DLS and TEM were employed to characterize the size and morphology of the polymersomes (average diameter of 480 nm with a moderate size distribution (Polydispersity: 0.09)).

Preparation of SSP by the Extrusion Method. The procedure was the same as describe above for the preparation of polymersomes, with the difference that water was only added until a desired amount. The polymersome suspension was transferred to a syringe (3 mL) and was passed through a filter (Acrodisc® 13 mm syringe filter with 0.2 µm nylon membrane) for 4 times.

Polymersomes (10 mg) with rigid membranes were plasticized by the addition of organic solvent (THF/dioxane=4/1 v/v, 1 mL) to an aqueous suspension (0.5 mL), which was passed through the membrane for 4 times.

Preparation of SSP by the Sonication Method. The polymersome (10 mg) aqueous suspensions (1.5 mL) with certain percentage of organic solvent (33.3%, 50% and 66.7%) were put into a 5 mL glass vial and were sonicated for 30s, 1, 5 or 15 minutes in a sonicator bath at 20 ^{o}C.

Preparation of polymersomes encapsulated with dyes. Nile red and FSS encapsulation: the procedures are the same as for the preparation of polymersomes, except that either Nile red (10 µL of 500 µg/mL in acetone) or FSS (10 µL of 1 mg/mL in DMF/H_2O=19:1) were mixed in the polymer solution before water was added. Small sized polymersomes were fabricated at a water content of 33 vol.%. After finishing addition of water, the suspensions were transferred to a dialysis tube (12000-14000Da), and dialyzed against pure water for 7 d with frequently changing water.

Encapsulation efficiency measurement. The pure samples after dialysis were freeze-dried under high vacuum for 1 d. 2 mg of the dried polymersomes encapsulated with Nile red and FSS were dissolved in 500 µL THF/dioxane=4:1 and 500 µL

DMF/water=19:1, respectively. As a control solutions were prepared by mixing 2 μL of Nile red (500 μg/mL) or FSS (1 mg/mL) solution, with 500 μL THF/dioxane=4:1 or 500 μL DMF/water=19:1. 100 μL of each sample was transferred to a fluorescence cuvette for fluorescence intensity measurements (Nile red λex = 490 nm, λem = 596 nm; FSS λex=480 nm λem=530 nm). The encapsulation efficiency (EE) was calculated by equation below.

EE%=Intensity of the sample/Intensity of the control × 100%

Conclusions

In summary, we have developed a facile and fast method to prepare uniform small sized (< 100 nm) polymersomes with rigid membranes, using PEG_{45}-*b*-PS_n as an example. Inspired by our previous work in which organic solvents were used as plasticising agent for the PS membrane, extrusion and sonication methods were shown to be able to resize flexible large polymersomes to uniform small-sized (< 100 nm) ones. Water content and PS length were found to influence the size and distribution of the polymersomes. These methods also worked well on ill-defined large polymersomes, which were resized, into monodisperse spherical vesicles demonstrating thus the robustness of the procedure. Moreover, both hydrophobic and hydrophilic dyes could be loaded in the SSP, proving a potential application in drug delivery.

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