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ARTICLE

Robust Formation of Biodegradable Polymersomes by Direct Hydration

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Herein, we report a robust way for the formation of biodegradable poly(ethylene glycol)-*block*-poly(ε-caprolactone) (PEG-*b*-PCL) polymersomes, *via* direct hydration of a highly concentrated block copolymer / oligo(ethylene glycol) solution. Polymersomes with variable membrane thickness were formed under relatively mild conditions in a short time, by changing the hydrophobic block length. Plunge freezing followed by cryo transmission electron microscopy (Cryo-TEM) was utilized to visualize the morphology of newly-formed polymersomes in their native condition. An MTT cytotoxicity study showed that the as-prepared polymersomes have good biocompatibility to hCMEC/D3 brain endothelial cells. As this method does not involve the use of small molecular organic solvent, sonication or freeze-thawing steps, it can offer the opportunity to form biodegradable polymersomes on-site. The work may facilitate the bench-to-bedside translation of biodegradable polymersomes as robust drug nanocarriers.

Introduction

In selective solvents, amphiphilic block copolymers have the capacity to assemble into discrete structures such as micelles, polymersomes, and multicompartment cylinders.[1-5] Polymersomes (polymeric vesicles) can be regarded as the polymeric analogue of liposomes.[6] They can readily encapsulate water-soluble hydrophilic compounds inside of their aqueous cavities and hydrophobic molecules within their lamellar membrane.[7, 8] They have also been designed to display functionalities such as targeting groups at their surface and stimulus-responsiveness.[9-11] Based on these features, polymersomes have been considered to be highly interesting as delivery vehicles.

In order to be suitable for biomedical applications, polymersomes have to be composed of biodegradable, or at least biocompatible block copolymers.[12-14] In the recent past, a range of biodegradable polymersomes have therefore been developed.[15-22] Poly(ethylene glycol)-*block*-poly(ε-caprolactone) (PEG-*b*-PCL) is one of the most studied candidates.[19, 23-31] PCL is a semi-crystalline, hydrophobic biodegradable polymer that is FDA-approved for use in drug delivery. Although PEG is not biodegradable, it is generally considered nontoxic and it can help to prolong the blood circulation times of polymersomes.

It has been shown, however, that aqueous assembly of PEG-*b*-PCL is quite sensitive to the preparation procedure and experimental conditions.[32, 33] Currently, the conventional methods to form PEG-*b*-PCL polymersomes are the solvent displacement method[32] and thin film hydration method.[23, 33]

The widely used solvent displacement method[32] involves dissolving the block copolymer into a water-miscible organic solvent (such as tetrahydrofuran, acetonitrile or dimethyl sulfoxide). The solution is mixed slowly with water under agitation to form polymersomes. However, the organic co-solvent needs to be completely removed by dialysis or evaporation from the aqueous suspension post-assembly. Moreover, this method is not well suited for encapsulation of bioactive biomacromolecules, which may be denatured in contact with organic solvent.

For the thin film rehydration method,[33] the block copolymer is dissolved in a solvent that solvates both blocks, and the solution is slowly dried under reduced pressure to form a thin film. Then the film is rehydrated with an aqueous solution. Water diffuses into the pre-ordered lamellar film and the outermost layers bud off to form polymersomes. Freeze-thaw cycles and the addition of sonic energy should also be applied to obtain unilamellar nano-sized polymersomes. This method has manufacturing disadvantages in terms of time, cost and facilities.

Hubbell et al. described the preparation of poly(ethylene glycol)-*block*-poly(propylene sulfide) (PEG-*b*-PPS) polymersomes by a new direct hydration method. PEG-*b*-PPS diblock copolymers were blended with poly(ethylene glycol)

dimethyl ether M_w 500 Da (PEG 500 DME). Rapid dissolution of PEG 500 DME upon hydration of the mixture yielded polymersomes. This approach can also yield high encapsulation efficiencies, possibly due to the presumed intermediate sponge phase of the hydrated polymer/PEG matrix.[34] Zhong et al. showed that it is possible to make biodegradable polymersomes by the same direct hydration method as Hubbell. However, the process involved relatively high temperatures (up to 95°C) and multiple ultrasonication cycles, which limited the on-site formation of polymersomes. Moreover, no detailed results about the polymersome structures were shown.[35]

Here we present a modified direct hydration method as a robust, mild and fast way for the preparation of PEG-*b*-PCL polymersomes. The polymersome size and membrane thickness can be readily optimized. Similar to the formation of proliposomes,[36, 37] this method offers the opportunity to form biodegradable polymersomes fast and highly efficient, for example as a ready-to-use sample for drug delivery, which may facilitate the use of biodegradable polymersomes as drug nanocarriers.

Results and discussion

The commercially available PEG monomethyl ether (MeO - PEO-OH, M_n = 2,000) was used as a macroinitiator to synthesize PEG-*b*-PCL. Normally, the preparation of biodegradable polyesters involves the use of metal catalysts such as tin 2-ethylhexanoate (stannous(II) octanoate).[38] For *in vivo* applications, the use of non-metallic catalysts is highly desired in order to minimize the potential toxicity effects.[39] We therefore chose methanesulfonic acid (MSA) as catalyst to produce PEG-*b*-PCL in a well-controlled way.[40] Polymerizations were conducted under mild conditions (30 °C, 2.5-3.5 h), yielding five block copolymers with different block ratios (Table 1).

Table 1. Polymers used in this study.

Sample code[a]	*PDI*[b]	f_{PEG}[c]
$PEG_{2K}PCL_{1.8K}$	1.04	0.53
$PEG_{2K}PCL_{4.3K}$	1.08	0.32
$PEG_{2K}PCL_{8.5K}$	1.14	0.19
$PEG_{2K}PCL_{9.5K}$	1.11	0.17
$PEG_{2K}PCL_{11K}$	1.11	0.15

[a] ^{1}H NMR spectroscopy was utilized to characterize the number-average molecular weight of the PEG-*b*-PCL copolymer. [b] GPC was employed to characterize the molecular weight distribution (PDI). [c] Volume fractions f_{PEG} were calculated from the melt densities of the two blocks, 1.13 g/cm^3 for PEG and 1.14 g/cm^3 for PCL.

The assembly of $PEG_{2K}PCL_{4.3K}$ and $PEG_{2K}PCL_{8.5K}$ were first based on the widely studied solvent displacement method and thin film rehydration method. For the solvent displacement method, micelles of 20 nm size were found for $PEG_{2K}PCL_{4.3K}$ (Fig. 1a) whereas $PEG_{2K}PCL_{8.5K}$ resulted in micelles of 30 nm size (Fig. 1b). No polymersome structures could be found from either of these two polymers. *Via* the thin film rehydration method, $PEG_{2K}PCL_{4.3K}$ resulted in micelles of 55 nm size (Fig. 1c). For $PEG_{2K}PCL_{8.5K}$, this method resulted in a mixture of micelles and polymersomes (Fig. 1d).

The presence of tens or hundreds of solvophobic ε-caprolactone repeat units in each chain may result in kinetically trapped non-equilibrium states after assembly in aqueous environment. The final assembly structures can be sensitive to the processing route taken. The kinetically trapped morphologies derived from the local arrangement of polymer chains are sensitive to the experimental conditions, such as the order and rate of addition of compounds into the system, their concentrations used, as well as the applied stirring conditions. This resulted in the inconsistencies in the reports about the formation of PEG-*b*-PCL polymersomes.[32, 33]

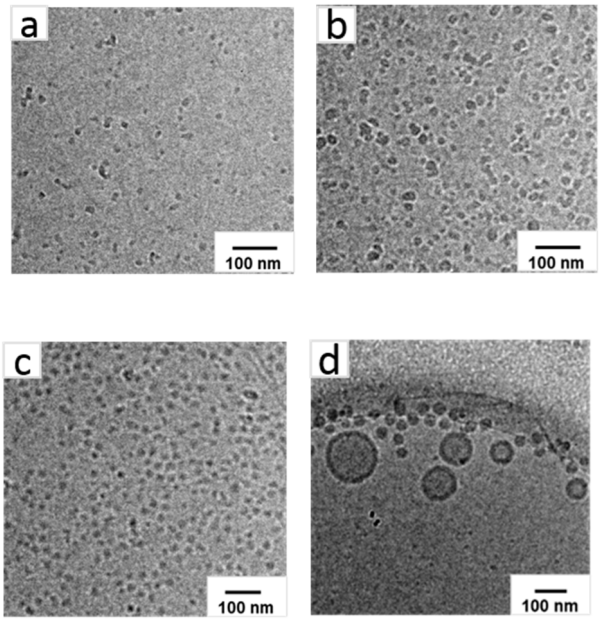

Figure 1. Representative cryo-TEM images of assembled structures from the solvent displacement method a. $PEG_{2K}PCL_{4.3K}$ b. $PEG_{2K}PCL_{8.5K}$; and from the film hydration method c. $PEG_{2K}PCL_{4.3K}$ and d. $PEG_{2K}PCL_{8.5K}$.

The assembly process was then changed to the modified direct hydration procedures for the formation of polymersomes[34] and the formation of liposomes out of liquid proliposomes.[37] Block copolymers were first dissolved at high concentration in low molecular weight PEG. The melting point for PCL is around 60°C, therefore PEG-*b*-PCL was initially mixed with PEG550 at 60°C for 15 min to obtain a homogeneous solution. Water was then added gradually to exchange and dilute the low molecular weight PEG, which resulted in dispersing the formulation into free polymersomes in solution.

The detailed structures of the aggregates were studied by cryo-TEM. The adopted morphologies could be ascribed to

spherical micelles, polymersomes and macroscopic precipitates in response to different f_{PEG}. At high relative block lengths of PEG ($PEG_{2K}PCL_{1.8K}$, Fig. 2a), spherical micelles were formed that avoid overcrowding of the PEG chains. As the relative PCL content increased ($PEG_{2K}PCL_{4.3K}$ Fig. 2b, $PEG_{2K}PCL_{8.5K}$ Fig. 2c and $PEG_{2K}PCL_{9.5K}$ Fig. 2d), the crowding of the PEG chains decreased and polymersomes were formed. It can be seen that unilamellar polymersomes with narrow size distribution were prepared. The sizes observed with cryo-TEM (around 100 nm) were comparable with DLS results (Supporting Information Fig. S1). With low relative block lengths of PEG ($PEG_{2K}PCL_{11K}$), no stable aggregated structures were formed and precipitation occurred.[32, 41]

Due to the contrast provided by the hydrophobic core of the polymersome membrane, the membrane thickness could be directly measured from the Cryo-TEM images. A correlation was observed, as expected, between membrane thickness and molecular weight of the hydrophobic block (10 nm for PCL 4,300, 20 nm for PCL 8,500 and 25 nm for PCL 9,500). The membrane core thickness of polymersomes is considerably thicker than in the liposome system (about 5 nm) indicating better structural stability of the polymersomes compared to liposomes.[42] The adjustable thickness of the polymersome membrane affords the opportunity to tune the degradation time and control the release of encapsulates. The polymersome morphology adopted was found to be not sensitive to the concentration of initial PEG-*b*-PCL in PEG 550 (90 mg/mL to 500 mg/mL).

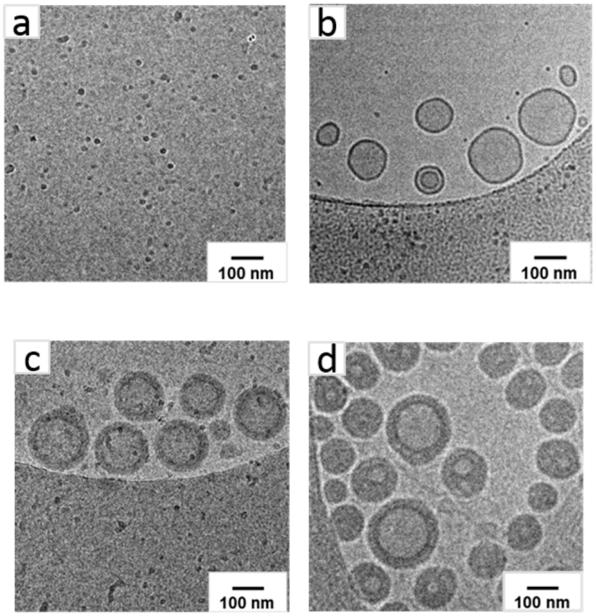

Figure 2. Representative cryo-TEM images of discrete structures for PEG-*b*-PCL by the direct hydration method. a. $PEG_{2K}PCL_{1.8K}$; b. $PEG_{2K}PCL_{4.3K}$; c. $PEG_{2K}PCL_{8.5K}$; d. $PEG_{2K}PCL_{9.5K}$.

We studied the corresponding cryo-TEM images of the samples also after removing of PEG 550 by dialysis. The analysed structures did not show large morphological changes that could have been caused by the dialysis process (Supporting Information Fig. S2).

The processing temperature for the water addition step was then lowered to more physiologically relevant conditions. As low as 37°C, polymersomes were still formed efficiently for $PEG_{2K}PCL_{4.3K}$ (Fig. 3a); while below 30°C, macroscopic precipitates were obtained. The temperature range allows for the encapsulation of heat-sensitive bioactive biomacromolecules without denaturation. Fig. 3b shows the dependence of viscosity for PEG 550 and $PEG_{2K}PCL_{4.3K}$ -PEG 550 solutions at different temperatures. At temperatures below 30°C, the viscosity of the polymer solution increases significantly. The assembly process to polymersome formation was strongly suppressed due to the change in mobility of the polymer chains.

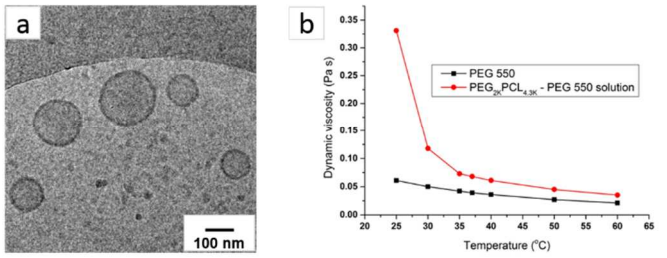

Figure 3. a. Representative cryo-TEM images of $PEG_{2K}PCL_{4.3K}$ formed at a processing temperature of 37 °C. b. Specific viscosity *vs* temperature for PEG 550 and $PEG_{2K}PCL_{4.3K}$ -PEG 550 solutions.

We demonstrated that the ratio of hydrophilic to hydrophobic blocks in PEG-*b*-PCL copolymers determines the shape of the self-assembly structures. The possibility of forming polymersomes from PEG-*b*-PCL block copolymers with binary components[43, 44] was also examined. The use of blending to direct aggregate shape is well known in biological systems, for example local composition fluctuations of different lipid molecules in the cell membrane can lead to membrane curvature to assist shape-driven processes such as budding and endosome production.[43, 45] Fig. 4 shows that polymersomes were readily obtained by the direct hydration method from a 1:1 ratio $PEG_{2K}PCL_{1.8K}$: $PEG_{2K}PCL_{4.3K}$ (Fig. 4a) and 1:1 ratio $PEG_{2K}PCL_{1.8K}$: $PEG_{2K}PCL_{8.5K}$ (Fig. 4b). The robust formation

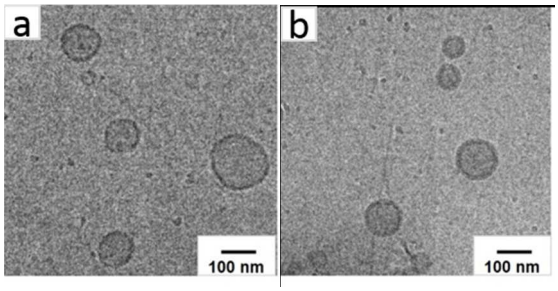

Figure 4. Representative cryo-TEM images of polymersomes formed out of binary mixtures of PEG-*b*-PCL by the direct hydration method. a. 1:1 ratio $PEG_{2K}PCL_{1.8K}$: $PEG_{2K}PCL_{4.3K}$; b. 1:1 ratio $PEG_{2K}PCL_{1.8K}$: $PEG_{2K}PCL_{8.5K}$.

of polymersomes in the right block ratio indicates that molecular weight distribution has little influence on the polymersome formation, which is consistent with literature.[33, 43]

The biocompatibility of the as-prepared polymersomes emulsion (initial copolymer concentration: 9 mg/mL and initial PEG 550 concentration: 90 mg/mL) was evaluated using an MTT- cytotoxicity assay. As shown in Fig. 5, the viability of hCMEC/D3 brain endothelial cells was not affected up to 200 μg/mL concentration indicating that the as-prepared polymersome emulsion can directly be used for further biomedical applications such as drug delivery.

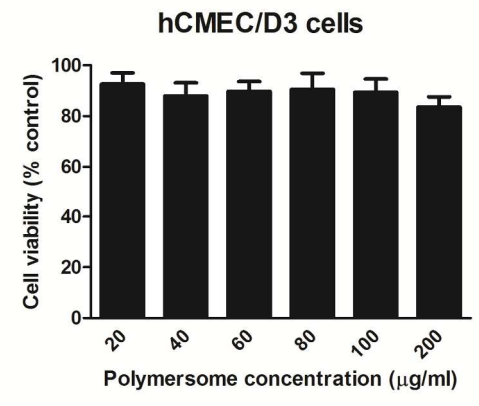

Figure 5. Relative cell viability of hCMEC/D3 cells against as-prepared $PEG_{2K}PCL_{4.3K}$ polymersomes emulsion after incubation for 21 h at different concentrations. Each value represents the mean ± S.D. of three independent experiments performed in triplicate.

Experimental

Materials

Unless stated otherwise, all chemicals were used without further purification. THF was distilled under Ar from sodium/benzophenone. The water utilized in the self-assembly of polymersomes was double deionized with a *Labconco Water Pro PS* purification system (18.2 MΩ). ε-Caprolactone (ε-CL, 99%, Alfa Aesar) was stirred over CaH_2 for 24 h at room temperature and then it was distilled at reduced pressure under nitrogen. α-Methoxy-poly(ethylene glycol)$_{44}$-ω-hydroxide (MeO–PEG_{44}–OH, M_n = 2,000 g/mol) was dried by co-evaporation with anhydrous toluene using a rotary evaporator. Methane sulfonic acid (MSA) and Amberlyst® A21 were purchased from Sigma-Aldrich and were used without further purification. Spectra/Pore dialysis membranes (MWCO 12, 000-14, 000) were purchased from Spectrum Laboratories.

Synthesis of poly(ethylene glycol)-*block*-poly(ε-caprolactone) PEG-*b*-PCL

Dry MeO–PEG_{44}–OH was added to a Schlenk flask as a solution in dry toluene. The desired amount of CL was then added and the resulting solution was equilibrated at 30°C for 10 min. MSA (1:1molar ratio with MeO–PEG_{44}–OH) was then added and the reaction mixture was stirred at 30 °C for 2.5 h. After cooling to room temperature, the mixture was treated with Amberlyst® A21 in order to remove the catalyst. The resin was removed by filtration and the product was precipitated in excess cold hexane. The crude product was dissolved in THF and precipitated in excess cold hexane again twice.

Assemblies of PEG-*b*-PCL

Direct hydration method

Modified literature procedure:[34] 10 mg of PEG-*b*-PCL and 100 mg of PEG 550 were weighed into a 1.5 mL centrifuge tube, heated at 60°C, and stirred with 300 rpm speed for 20 min. After the sample solution was cooled to the desired temperature, 100 μL of water was added and stirred for 30 mins. Then 200 and 700μL of water were added, with mixing after each addition. The polymersome emulsion (polymer concentration: 9 mg/mL) was passed through a filter (Acrodisc® 13 mm syringe filter with 0.2 μm nylon membrane) after preparation. Unless stated otherwise, the measurements in the manuscript were based on this concentration.

Solvent displacement method

Modified literature procedure:[46] 10 mg PEG-*b*-PCL was dissolved in 1 mL distilled THF in a 15 mL vial charged with a magnetic stirring bar. The vial was capped with a rubber septum and the solution was stirred for at least 30 min at room temperature. Then, ultrapure water (3 mL) was delivered to the polymer solution *via* a syringe pump at a rate of 1 mL h^{-1} while stirring the solution vigorously. The turbid suspension was transferred into a dialysis membrane which was swollen in the dialysis medium for about 30 min. The mixtures were dialysed against water (700 mL) for 24 h, while replenishing the water two times.

Film hydration method

Modified literature procedure:[47] In brief, 120 mg PEG-*b*-PCL copolymer was dissolved in 4 mL chloroform and put into a round bottom flask. Then, the solvent was removed by rotary evaporation under reduced pressure to form a thin film. Any trace of residual solvent was then evaporated under nitrogen. The dried film was hydrated with 10 mL water under vigorous stirring. Upon hydration, polymersome self-assembly was further promoted at 60 °C for 4 h.

Cell culture

Human cerebral microvascular endothelial hCMEC/D3 cells were maintained in 25 cm^2 flasks precoated with 150 μg/ml rat tail collagen type-I (Trevigen #3440-100-01) in endothelial basal medium-2 (EBM-2, Lonza #CC-3156) supplemented with 1 ng/ml human basic fibroblast growth factor (Peprotech #100-18B), 5 μg/ml ascorbic acid (Sigma-Aldrich #A4544), 1.4 μM hydrocortisone (Sigma-Aldrich #H-0135), 10mM HEPES (Gibco #15630-056), 1% (v/v) chemically defined lipid concentrate (Gibco #11905-031), 5% (v/v) foetal bovine serum, 100 units/ml of penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

Cell viability assay

The viability of hCMEC/D3 cells after exposure to increasing concentrations of polymersomes emulsion was evaluated by performing a 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT, Sigma-Aldrich #M2128) assay. 5 x 10^4 hCMEC/D3 cells/cm^2 were seeded in 96-wells plates precoated with 150 µg/ml rat tail collagen type-1 and grown for 6 days, respectively. The medium of hCMEC/D3 cells was replaced every other day. Cells were treated in triplicate with 20-200 µg/ml of polymersomes emulsion, diluted in EBM-2 (final volume of 150 µl), for 21 hours. Untreated cells in EBM-2 were used as a control. During the final 3 hours of incubation, cells were exposed to 15 µl MTT solution (5 mg/ml in PBS). Thereafter, the medium was removed and formazan crystals were dissolved in 100 µl of DMSO. Upon complete solubilization of the crystals, the optical density of each well was measured using a microplate spectrophotometer (Bio-Tek Instruments) at 520 nm.

Instrumentation

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Inova 400 (400 MHz for 1H) spectrometer. Molecular weights of the block copolymers were measured on a Shimadzu size exclusion column equipped with a guard column, a Polymer Laboratories gel 5 µm mixed D column and differential refractive index (RI) and UV (λ = 254 nm) detection. The system was eluted with tetrahydrofuran (THF, analysis grade) using a flow rate of 1 mL min^{-1} at 35 °C. The calibration was performed with polystyrene standards. DLS measurements were carried out on a Malvern Instruments Nano-ZS zetasizer. Dynamic viscosity was measured by a TA Instruments DHR-2 rheometer.

Cryogenic transmission electron microscopy (cryo-TEM)

For plunge freezing, polymersomes were taken directly at room temperature from the hydrated mixture for processing without further centrifugation. A 3 µl droplet of various mixture samples was applied to a glow-discharge R2/2 quantifoil copper grid (Quantifoil Micro Tools, Jena, Germany) mounted in an environmentally controlled chamber at 100% humidity, blotted and frozen in vitreous ice by plunging into liquid ethane using the Vitrobot (FEI, Eindhoven, The Netherlands). Grids were transferred to a Gatan model 914 cryoholder (Gatan, Pleasanton, CA) under liquid nitrogen and inserted into a Jeol 2100 transmission electron microscope (Jeol, Tokyo, Japan) operating at 200 kV. The vitreous state of the preparation was confirmed by electron diffraction. Low-dose images, with exposures between 20 and 30 electrons per $Å^2$ and under-focus values of 5 µm were recorded with a 4096×4096 pixel CCD camera (Gatan) at 40.000× magnification.

Conclusions

We have developed a robust and fast method to form nano-sized biodegradable polymersomes. The assembly of a range of PEG-*b*-PCL block copolymers by a modified direct hydration method was examined. By dissolving the appropriate f_{PEG} block copolymer in PEG 550 at relatively high concentration, the solution being hydrated upon addition of an aqueous solution, we were able to drive rapid formation of highly concentrated polymersomes under mild conditions. This method was generalized to produce high-quality polymersomes with well-controlled size and membrane thickness. It does not involve the use of small molecular organic solvent, sonication or freeze-thawing steps. These unique features, in addition to non-cytotoxicity suggest that this approach holds great potential to scale up the production of biodegradable polymersomes for clinical tests.

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Notes and references

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