Nonequilibrium Reshaping of Polymersomes via Polymer Addition

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ABSTRACT: Polymersomes are a class of artificial liposomes, assembled from amphiphilic synthetic block copolymers, holding great promise toward applications in nanomedicine. The diversity in polymersome morphological shapes and, in particular, the precise control of these shapes, which is an important aspect in drug delivery studies, remains a great challenge. This is due to a lack of general methodologies that can be applied and the inability to capture the morphologies at the nanometer scale. Here, we present a methodology that can accurately control the shape of polymersomes via the addition of polyethylene glycol (PEG) under nonequilibrium conditions. Various shapes including spheres, ellipsoids, tubes, discs, stomatocytes, nests, stomatocyte-in-stomatocytes, disc-in-discs, and large compound vesicles (LCVs) can be uniformly captured by adjusting the water content and the PEG concentration. Moreover, these shapes undergo nonequilibrium changes in time, which is reflected in their phase diagram changes. This research provides a universal tool to fabricate all shapes of polymersomes by controlling three variables: water content, PEG concentration, and time. The use of the biofriendly polymer PEG enables the application of this methodology in the field of nanomedicine.

KEYWORDS: nonequilibrium, polymersome, shape transformation, polymer addition, disc-in-disc, time-programed

Cell shape is of key importance in cell growth and cell differentiation and, as such, has been crucial in the evolution of life. For instance, it is important in the morphogenesis of tissue during the development of the living body. Furthermore, it may help a species escape from its predators. The dynamic assembly of the cytoskeleton is the result of out-of-equilibrium reactions in the cell and is accompanied by shape changes, which are needed for environmental adaptation. Out-of-equilibrium assemblies are systems that require a continuous supply energy to persist. If the energy supply stops, the system would fall apart and end up in a thermodynamic minimum state nearby. Liposomes can spontaneously exhibit various shapes, which are reminiscent of biological cell morphologies, which have inspired scientists to fabricate vesicles (synthetic liposomes) of different shapes and compositions as cell mimetic systems in order to understand the mechanism of liposomal shape change. Variations in mechanical stress, temperature, pH, or osmotic shocks and in membrane composition are capable of inducing drastic changes in liposomes, ranging from nearly spherical shapes to discocytes, stomatocytes, starfish, or pears. However, the dynamic nature of the phospholipid membrane makes these shape transitions transient due to the lack of stabilization by the cytoplasm in the cell and the fact that the cytoskeleton has glassy properties, limiting an accurate shape control.

Polymersomes assembled from amphiphilic synthetic block copolymers demonstrate enhanced membrane stiffness and provide great possibilities for tailoring both the chemical design of the membrane and its physical properties (such as stiffness, zeta-potential, and adhesiveness), which allows for good control over the shape and function of the aggregate. The shape of a polymersome is expected to affect its interaction with the cell, which is important for its flow properties, its cellular uptake, and immune regulation, as is the case for the biconcave disc-like red blood cell. Therefore, it is of great importance to be able to accurately reshape a polymersome and to understand the mechanism of the interconversions of the various shapes. Several studies have explored the possibilities to reshape polymersomes via osmotic pressure or a change in chemical structure. Block copolymers bearing liquid-crystalline side chains assembled to ellipsoids, tubes, and polyhedral shapes of polymersomes via...
RESULTS AND DISCUSSION

**Self-Assembly via Water Addition.** Polymersomes were made by first dissolving 10 mg of PEG<sub>45</sub>-b-PS<sub>230</sub> in 1 mL of THF/dioxane = 4:1 (v/v), followed by slowly adding water at a rate of 1 mL/h (Figure 1a,b). The assembled polymersomes were formed when the ratio of water and organic solvent approached 1:4 (v/v). At this point, the solvent compositions inside and outside the polymersomes are equal (i.e., the inner and outer osmotic pressures are equal), setting this as the equilibrium point. Continuous addition of water into the solution will push the self-assembled system to nonequilibrium as an osmotic pressure builds up over the membrane. The extra water addition makes the PS membrane more rigid and less permeable. As previously observed<sup>21</sup>, when the water content is 50 vol %, most of the spherical polymersomes change into rods after 4 days because the organic solvent flows out faster than water flows in, resulting in a volume decrease. The system changes back to a spherical morphology after 8 days (inflow of water releases the bending energy), indicating that the amorphous glassy nature of the PS part. The PS membrane used in our studies is composed of 230 repeating units, which limits its mobility, resulting in much longer transition time scales (i.e., the order of hours) than in the case of liposomes (seconds). This prolonged time scale gives the possibility to kinetically trap every intermediate morphology by freezing the PS membrane via quickly adding excess water. The use of PEG as a fusogen has been recently demonstrated by our group, and its ability to induce a shape change in the polymersome can already be realized with a trace amount (0.005 wt %) of this compound, compared to >15 wt % for the liposome system, which is possible because the low permeability of the membrane amplifies the osmotic pressure. In this paper, we show that the bending energy of the polymersome can be regulated by tuning the rigidity of the PS membrane, allowing good control of the nonequilibrium shape transformation and the possibility to capture various morphologies over time, leading to a complete phase diagram of the shape transformations.
nonequilibrium shape change is driven by osmotic pressure. However, the increase of water addition leads to an increase of the membrane rigidity and a decrease of the permeability, which limits the range over which the osmotic pressure can be built up. This small range indicates that only very limited morphologies (rods, discs, and stomatocytes) are accessible with this method. Furthermore, the flexibility and mobility of the polymer chains are considerably reduced, preventing spontaneous curvature from changing and membrane fusion from taking place.

**Shape Transformation via PEG Addition.** To explore new paths of polymersome shape change, PEG was selected for enhancing the osmotic pressure and the interaction with the polymersome membrane, as it has been demonstrated before to act as a fusogen of liposomes due to its osmophobic association effect.48 This effect demonstrates that the PEG molecules are excluded from the region adjacent to the lipid vesicle surface (exclusion layer), which induces osmotic stress onto the vesicles, resulting in membrane fusion. Based on our previous report,49 PEG with a molecular weight of 2000 was chosen as the fusogen as relatively large molecules can be excluded more effectively from the exclusion layer adjacent to the membrane surface than small molecules, which is seen as the “mechanical stress model”.49 As shown in Figure 1b, when the volume of water reached a certain value (0.3–2 mL), 200 μL of the polymersome suspension was transferred to a centrifuge tube, followed by the addition of 10 μL of an aqueous PEG2000 solution to the suspension. The shape of polymersomes immediately changed to a variety of morphologies depending on the PEG2000 concentration and water content (Figure 1c). We expect that the added PEG2000 polymer molecules in the solution will mostly stay outside of the polymersome membranes due to their inability to penetrate the thick hydrophobic PS membranes. This PEG addition procedure pushes the polymersomes even further away from the equilibrium situation when compared to the experiments in which only water is added. The morphology was fixed by quenching 200 μL of polymersome solution with 1 mL of water for transmission electron microscopy (TEM) measurement. All shapes that were captured are presented in Figure 2, together with the preparation conditions, such as water content and PEG2000 concentration that were used for the shape transformation. This information on the morphologies was used to construct a phase diagram, which is shown in Figure 3. The solid line in this figure means that only one type of shape was observed in the particular region, and the dashed line is a mixture of both shapes.

![Figure 2. TEM and cryo-TEM (insets) images of polymersome morphologies recorded after 1 min, as obtained after the addition of different concentrations of PEG2000. (a) Spherical vesicles (PEG2000 concentration, water content: 0.005 g/L, 23 vol %), (b) ellipsoids (0.02 g/L, 23 vol %), (c) tubes (0.05 g/L, 33 vol %), (d) discs (0.1 g/L, 50 vol %), (e) stomatocytes (0.5 g/L, 33 vol %), (f) nests (1 g/L, 23 vol %), (g) sto-in-stos (5 g/L, 23 vol %), (h) disc-in-discs (5 g/L, 33 vol %), (i) large compound vesicles (25 g/L, 23 vol %). The colors of the symbols match the ones in the phase diagram in Figure 3. Scale bar: black, 1 μm; white, 500 nm.](image-url)
The observed morphology changes can be categorized into two classes: (i) shape transitions with membrane fusion and (ii) transitions without membrane fusion, mainly depending on the PEG2000 concentration. When the PEG2000 concentration was above a threshold value (see the upward pointing line of the nested vesicles in the phase diagram; Figure 3, red line), the membranes started to fuse, forming nested vesicles, stomatocyte-in-stomatocytes (sto-in-stos), discocyte-in-discocytes (disc-in-discs), and large compound vesicles (LCVs) (Figure 2f–i). Below the PEG2000 threshold value, the membranes remained stable, forming ellipsoids, tubes, discs, and stomatocytes (Figure 2b–e). The different shape changes can be explained in terms of osmotic pressure and membrane permeability.

Spherical shapes (Figure 2a) are stable and sustained in the region of the phase diagram, where the PEG2000 concentration remains below 0.01 g/L and the water content is low (the situation of a highly permeable membrane) because such PEG concentrations generate very small osmotic pressures, which can easily equilibrate due to the high permeability of the membrane. Spherical shapes are also found at high PEG2000 concentrations and high water contents (the situation of a low permeable membrane) when the rigid membrane prevents water and organic solvent to leak out. A slight increase of the PEG2000 concentration at low water content leads to an increase in the osmotic pressure, resulting in a shape transformation from sphere to ellipsoid. The ellipsoid shape will gradually elongate to a tube by continuous increasing the osmotic pressure and water content. In the region of high organic solvent content (leading to a membrane with high permeability and low rigidity), the osmotic pressure is high enough to increase the curvature, meanwhile pushing much more organic solvent out, resulting in a shape transformation to stomatocytes. In the region of low organic solvent, the shape changes to a disc as it requires a higher bending energy for the transformation to a stomatocyte, whereas only a small volume of organic solvent is available to leak out. As can be seen in Figure 3, stomatocytes are obtained over a large region and are well under control as they are situated at the lowest energy state. Moreover, we found that the size of the opening of stomatocytes can be manipulated by adjusting the water content. As shown in Figure S1, the average diameters of the open mouth of the stomatocytes decreased from 414 to 177 nm and further to 21 nm when the water content fell from 60 to 50 vol % and then to 33.3 vol %.

Nestled shape structures were formed when, with the assistance of the PEG2000, a larger reduction of the inner volume was possible, coupled with a fusion of the mouth. During this fusion process, a part of the PEG2000 solution would be entrapped inside the nested shape, leading to an increase in the organic solvent content. Higher osmotic pressure will push the organic solvent out for the second time, forming a disc-in-disc shaped structure. As demonstrated in the phase diagram, this disc-in-disc shape possesses a high bending energy, hence, capture of such morphologies is only possible when the membrane is relatively rigid. When the membrane is too flexible (water content 23 vol %), these disc and disc-in-disc shapes are not obtainable; instead, stomatocytes and sto-in-sto shapes are formed directly. If the concentration of PEG2000 is greater than 10 g/L, the polymersome corona cannot be stabilized by electrostatic and steric effects, leading to membrane fusion and to large compound vesicles. Shorter PS chain (PEG45-PS190) polymersome transformed to similar shapes as PEG45-PS230 at the same concentration (Figure S3), but the PEG45-b-PS160 polymersome membrane is too flexible to be fused to irregular shapes even at low PEG2000 concentrations (Figure S2).

Equilibration after PEG Addition. The process after PEG addition continuously induces a shape change until equilibrium is reached (Figure 1d). This shape change mainly involves three processes. First, after the first balancing of the osmotic pressure, the polymersome will reinflate by the simultaneous inflow of water and organic solvent to decrease the bending energy, while maintaining the osmotic balance. Second, PEG

![Figure 3. Phase diagram of polymersome morphologies before equilibrium. The points in the picture correspond to the images in Figure 2. The solid line means that only one shape is observed in the region; the dashed line means a mixture of two shapes in that area. The red line is defined as being the threshold concentration line for fusion.](image-url)
molecules diffusing in and out of the polymersome will continuously assist in the fusing/aggregation of the polymersomes. Third, the polymer chains will rearrange in order to release the bending energy. These three factors synergistically work on the self-assembled system through a nonequilibrium process via different morphologies to adapt to the minimum energy path. Figure 4a,b presents the phase diagram change after 2 days and 30 days. At low water content, the membrane is flexible and slightly permeable to water, allowing inflow of both water and organic solvent, resulting in the shape transformation from long/narrow tubes (~5 μm long) to shorter/wider tubes (~2 μm long). The shorter/wider tubes cannot transform back to spheres because they reached the kinetic equilibrium state (Figure 4c). As the shape change from sphere to disc is discontinuous, the excess energy generated from the osmotic pressure is stored in the PS membrane via polymer chain tension. This tension is then slowly alleviated in time by chain rearrangements, which can also be seen as bending energy. Thus, a high concentration of PEG creates a high osmotic pressure, which flattens the polymersome to a disc shape. The extra bending energy can be slowly released via a shape change to stomatocyte (Figure 4d). In the same context, but when PEG is present in high concentration and the membrane is plasticized by the large amount of organic solvent, all morphologies, such as sto-in-sto, disc-in-disc, and nest shapes, equilibrate to large composite vesicles, due to the interaction of PEG with the membrane of the polymersome (Figure 4f). Smaller PEG concentrations and a highly flexible membrane will push the stomatocytes to form multiopened stomatocytes (Figure 4e). Furthermore, once the membranes are fused together, it is impossible at this condition to separate them back to polymersomes again, which would need the system to overcome extremely high surface energies.

CONCLUSION

In summary, we have demonstrated a universal methodology for reshaping nanometer-scale polymersomes into various new morphologies in a controllable manner via PEG-guided nonequilibrium self-assembly. Furthermore, we have constructed the complete phase diagrams of all transitions. Each of the produced morphologies is highly uniform, and the structures are fully reproducible, including the stomatocyte openings, which can be strictly controlled. The latter is of great importance and can be used as a soft method for encapsulation purposes (e.g., of drug molecules). All structures started from a spherical morphology and transformed to various shapes via different routes whose mechanisms have been investigated. Low concentrations of PEG2000 below the threshold concentration (red line in Figure 3) induce only osmotic pressure over the membrane, resulting in the transition from the spherical shape to ellipsoid, tube, disc, and stomatocyte. Above the threshold concentration, PEG2000 not only creates a high osmotic pressure but also acts as a fusogen of the membrane due to the effect of osmophobic association, which assists the shape change to nest, sto-in-sto, disc-in-disc, and LCV. The rigidity of the membrane, which is tuned by the ratio of water and organic solvent regulates the path of the shape changes and the kinetics, allowing each shape during the transformation to be captured in time spans varying from hours to days. We demonstrated that all of these shape transformation procedures are kinetic traps and nonequilibrium at short time scales. The kinetic pathway strongly

Figure 4. Morphology changes of polymersomes after equilibration. The phase diagrams of polymersome morphologies after equilibration for 2 days (a) and 30 days (b). At selected conditions, which are the same as in Figure 2, the long tubes (c), discs (d), stomatocytes (e), and nests (f) change to short/wide tubes, multiopened stomatocytes, and LCVs, after equilibration times of 2 days and 30 days. The colors match the labels in Figures 2 and 3. Scale bar: 1 μm.
depends on parameters such as the organic solvent (THF and dioxane) type, ratio, and water content, as well as the added PEG2000. These differently shaped polymersomes are promising candidates for applications as nanocontainers or nanomachines. As we already demonstrated, the nonbiodegradable PS could be replaced by other biodegradable polymers such as poly(D,L-lactide), which exhibits a similar glassy behavior at room temperature as PS, offering opportunities for the practical application of the aggregates in drug delivery.

EXPERIMENTAL METHODS

Materials. All reagents and chemicals were purchased from Sigma-Aldrich and used as received. Milli-Q water (18.1 MΩ) was used throughout the experiments. The molecular weights of the block copolymers were measured with 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. NMR spectra were performed on a Varian Inova 400 spectrometer with CDCl3 as a solvent. Transmission electron microscopy samples were prepared in the following way: a solution of a 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. A sonicator VWR USC300TH was used for the sonication experiments at room temperature. A JEOL 2100 cryo-transmission electron microscope was used for a detailed characterization. PEG-b-PS was synthesized as described in our previous paper.31

Preparation of Polymersomes. The procedure as described in our previous literature report was used.22 A typical procedure is as follows: PEG45-b-PS30 (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 the compounds were dissolved for 1 h at room temperature, a syringe pump equipped with a syringe with a needle was calibrated to deliver compounds at a speed of 1 mL/h. The needle from the syringe was inserted into the vial of which the cap was replaced with a rubber septum. An amount of 0.3 mL of water was pumped into the organic solution with vigorous stirring (900 rpm). After the water addition was finished, 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.

PEG Addition Methodology. A polymersome solution (200 μL, 0.8 g/L) in a mixture of THF/dioxane water = 10:3 (or 10:5, 10:10, 10:20, 10:30 and 10:40) by volume was loaded in a 1.5 mL Eppendorf centrifugation tube. An amount of 10 μL (or another amount) of a PEG2000 aqueous solution (1, 10, 100 g/L) was added to the polymersome suspension (200 μL) with a frequency of 1200. After 1 min, 20 μL of the polymersome suspension was taken out to be quenched by 1 mL of ultrapure water.

Equilibrium. The polymersome suspension was left in the shaker while the temperature was kept constant (20 °C). At the desired time, 20 μL of the polymersome suspension was quenched into 1 mL of water located in a 1.5 mL centrifuge tube. The tube was centrifuged at 10,000 rpm for 5 min to remove the added PEG. The polymersomes aggregated at the bottom of the tube were dispersed by addition of 1 mL of water, then one drop of the suspension was taken to prepare the TEM sample.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.9b04740.

Materials and instrumentation; synthesis methods of PEG-b-PS and polymersomes, as well shape transformation via PEG addition methodology; TEM images of stomatocytes with various mouth openings; shape change of polymersomes assembled from different PS lengths (PDF)

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Author Contributions

Y.M. and W.L. contributed equally. Y.M. and D.A.W. designed the experiments and wrote the manuscript. Y.M., W.L., Y.T., and F.P. performed the experiments and analyzed the results. G.J.A.J. performed the cryo-transmission electron microscope measurements. Y.M., W.L., Y.T., F.P., R.J.M.N., and D.A.W. interpreted the results.

Notes

The authors declare no competing financial interest.

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