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Polymeric Vesicles

Spontaneous Formation of Giant Unilamellar Vesicles from Microdroplets of a Polyion Complex by Thermally Induced Phase Separation**

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Vesicles are of great interest because of their potential as drug carriers, as nano- or microreactors,[1] and as model systems for living cells.[2] Coacervates are also fascinating from the viewpoint of their application in pharmaceutical microencapsulation[3] and in protein separation and purification;[4] they are also implicated in the elucidation of the origin of life and serve as a model of the condensed environment of cytosol.[5] However, the fundamental properties of coacervates are not fully understood, and the relationship between vesicles and coacervates is also unknown.[6] Some previous studies have shed light on the morphological transformation from lipid coacervates to lipid vesicles,[7] but there have been no examples of polymeric analogues reported to date. In addition, the influence of perturbations (solution compositions, temperature, etc.) on dynamic morphological changes in coacervates and vesicles has been confined to cytomimetic chemistry.[2b,c]

Herein, we report the spontaneous formation of giant unilamellar polyion complex (PIC) vesicles from micron-sized PIC coacervates that are based on biocompatible polyelectrolyte precursors. Vesicle formation is demonstrated in pure aqueous solution under thermal perturbation, which was achieved by irradiation with a focused infrared laser from an optical tweezer setup. We also describe how this morphological transformation is developed through thermally induced generation and growth of a water-rich phase inside the PIC coacervate. Additionally, the PIC coacervate acts as a "thermally driven pump" that transports water from the solution outside the vesicle to the water-rich phase within (i.e., to balloon the PIC coacervate for the formation of a PIC vesicle). Remarkably, the size of the PIC vesicles is dependent on the initial PIC coacervate size. This method of vesicle formation, which takes place in the absence of organic solvents, has great potential for the technological development of the production of microcapsules that contain biorelated compounds, as conventional microencapsulation usually requires rather toxic organic solvents.[8]

We recently developed PIC vesicles (termed "PICsomes"), which are prepared from a pair of oppositely charged diblock copolymers composed of poly(ethylene glycol) (PEG) and poly(amino acid) derivatives (Figure 1). By mixing the aqueous solutions of the components in a microtube, we easily obtained micrometer-scale polymer vesicles.[9] Based on this strategy, we discovered a preparation method for coacervate-like micrometer-sized PIC droplets. PIC microdroplets were typically prepared by mixing a solution of PEG–PAsp and a solution of a mixture of PEG–P(Asp-AP) and homo–P(Asp-AP) in an equal charge ratio in the presence of a salt (75 mM NaCl or 150 mM NaCl). PIC microdroplets were observed as dark spheres by using phase-contrast microscopy, as shown in Figure 1.

We investigated the morphological change of the PIC droplets under thermal perturbation by using optical tweezers, which can trap a microscale object that is suspended in aqueous solution at the focal plane of the objective.[10] In addition, when an infrared laser is employed as a light source, a local heating spot is generated at the focused laser point and the trapped object can be heated, as H_2O absorbs infrared light.

In fact, there have been many studies that utilize optical tweezers for local heating in order to investigate a micrometer-scale phase separation or phase transition in an oil/water system or a polymer mixture in a water system.[11] Thus, the use of optical tweezers enables the individual manipulation of a targeted PIC droplet under simultaneous thermal perturbation and real-time observation.

When the laser was introduced through the objective lens, one of the PIC droplets in the solution was trapped at the focused laser point (Figure 2a). The attractive force of the

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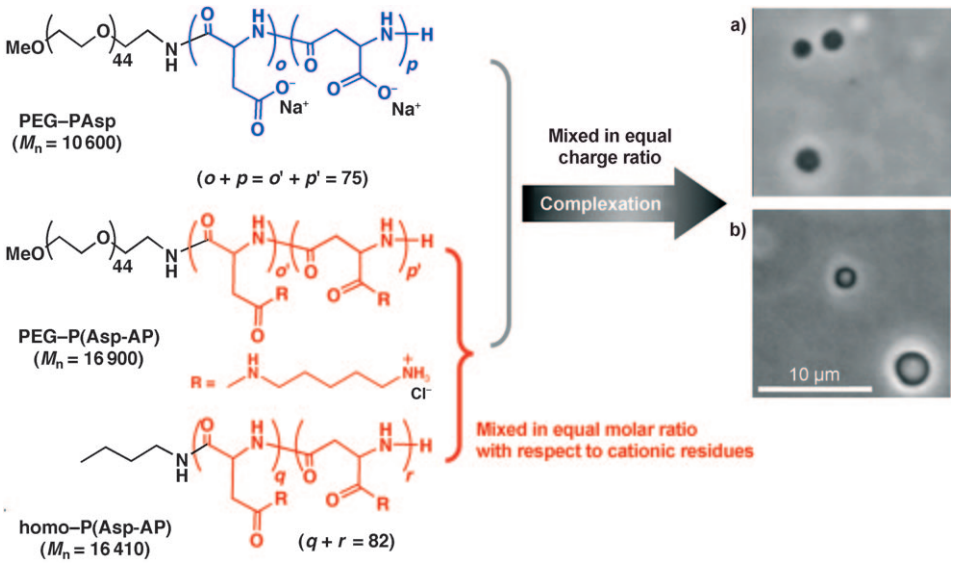

Figure 1. Preparation of PIC microdroplets: Phase-contrast micrographs of droplets in 10 mM Tris-HCl buffer (pH 7.4) with 75 mM NaCl (a) and with 150 mM NaCl (b). M_n(PEG) = 2000, degree of polymerization (DP) = 45. Asp = poly(α,β-aspartic acid) (DP = 75). P(Asp-AP) = poly([5-aminopentyl]-α,β-aspartamide). The DP value of P(Asp-AP) is 75 in PEG-P(Asp-AP) and 82 in homo–P(Asp-AP).

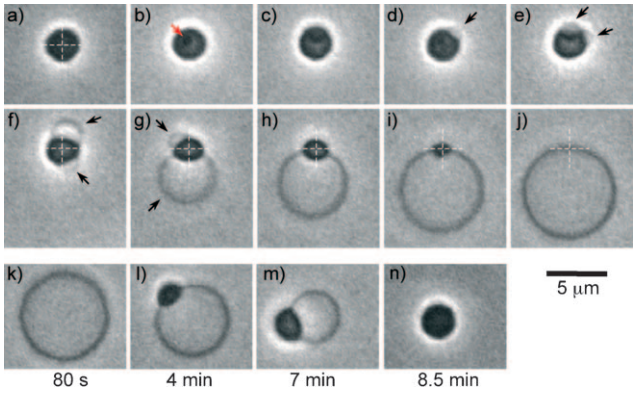

Figure 2. Time development of the morphological change of a PIC droplet during laser irradiation (top and middle row; a movie file is available in the Supporting Information) and after irradiation (bottom row) (75 mM NaCl, laser power 0.25 W). The time of image (a) is set as 0 s, and the elapsed times are 2.5 (b), 3.5 (c), 9.0 (d), 12 (e), 26 (f), 65 (g), 96 (h), 140 (i), and 165 s (j). The bottom images (k–n) show the time course of the morphological change after laser irradiation was stopped. The intersections of the two white dashed lines indicate the position of the laser spot (a, f–j). The red arrow indicates a dark spot (b) and the black arrows indicate individual vesicular compartments (d–g).

optical tweezers acts on the object, whose permittivity is greater than that of surrounding medium. Therefore, this result confirms that the suspended dark microspheres are filled with the polymer-rich phase (i.e., the PIC-rich phase) of the PIC/water system.

When the laser irradiation was continued in the presence of 75 mM NaCl, a sequence of dark spots was generated at the center of the trapped PIC droplet, that is, at the focused laser point (Figure 2b). At that point, the spot size of the focused laser was in the order of the wavelength of the laser (ca. 1 μm at most); thus, the size of the local heating point was small compared to that of the trapped PIC droplet. The generated dark spots moved away from the laser point, with their size growing slightly as they approached the surface of the PIC droplet (Figure 2c). When a dark spot reached the surface of the PIC droplet, a blister-like lump was formed (Figure 2d). Some dark spots that appeared later were fused to the blister, thereby causing the blister to grow and to form a vesicle. In some cases, the other spots became individual vesicular compartments (Figure 2e–g). Subsequently, multiple compartments fused to one another, and a single vesicle survived (Figure 2h). As the vesicle grew, the PIC droplet itself became smaller and smaller, which indicated that the polymers constituting the vesicle membrane originate from the droplet (Figure 2i). When the PIC droplet at the focused laser point (i.e., at the optically trapped point) was exhausted, vesicle formation was completed (Figure 2j). Finally, further laser irradiation did not cause any change in vesicle size. The completed spherical vesicle was trapped at the membrane, thus indicating that there was very little polymer within the vesicle. When a higher laser power was applied, the generation rate of dark spots increased and vesicle formation was completed more quickly.

When irradiation was stopped, the vesicle maintained its morphology for a 1–2 minutes, after which time the membrane fluctuated and the vesicle shrunk slightly (Figure 2k). Then, a dark domain, which seemed to be a reformed PIC droplet, appeared on the surface of the vesicle, and the membrane fluctuation reduced (Figure 2l). Subsequently, the dark domain enlarged, while the vesicle underwent gradual shrinkage (Figure 2m). Finally, a PIC droplet of the same size as that before laser irradiation was reformed (Figure 2n). This morphological transition between the PIC droplet and the vesicle was reversible and repeatable (data not shown), thereby supporting the concept that the whole process is controlled by switching the thermal perturbation on and off.

As shown in Figure 2, phase contrast microscopy images showed a continuous generation of dark spots in a PIC microdroplet during laser irradiation. The dark spots are attributed to a lower permittivity phase because they moved away from the focused laser point. Thus, the dark spots should be a water-rich phase in the PIC droplet, that is, in the surrounding polymer-rich phase. A similar phenomenon has been reported in which water microdroplets were generated and moved away from the laser focus when the oil-rich phase of an oil/water system was irradiated by the focused laser.[11c] It is interesting to understand how the water-rich phase is

formed inside the PIC droplet. In our system, this microscale phase separation is caused by thermal perturbation by the focused infrared laser. In fact, when we employed D_2O as a solvent (which absorbs less than 10% of 1066 nm wavelength light than H_2O[11a]), dark spots, (i.e., a water-rich phase) were not generated in the PIC droplets (see the Supporting Information). Consequently, this formation process of the giant vesicle from the PIC droplet by thermal perturbation can be explained as follows:

1) Nucleation and growth of the water-rich phase in the PIC droplet occurs when thermal perturbation is applied, and the generated water-rich phase moves to the surface because of its low refractivity. In this case, we assume that the PEG section of the copolymers tends to extend toward the water-rich phase because of its hydrophilicity (Figure 3a).

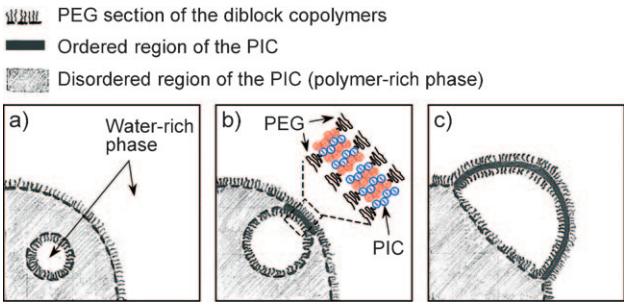

Figure 3. Proposed mechanism for the formation of a PIC membrane.

2) The unilamellar PIC membrane is self-assembled because of the proximity of the generated water-rich phase and the outer water-rich phase (Figure 3b). The PIC membrane consists of a dehydrated and ordered PIC complex layer, which is rather hydrophobic and sandwiched between hydrophilic PEG layers (Figure 3b, inset).

3) The blister-like morphology is formed by the PIC membrane that keeps the inner water-rich phase separate from the outer water-rich phase (Figure 3c). The blister grows because of the supply of the water-rich phase, which is generated sequentially at the vicinity of the laser irradiation point of the PIC droplet. When multiple blisters are formed on the droplet, the blisters fuse with each other to reduce surface tension. During growth of the blister, the polymer-rich phase (i.e., the optically trapped PIC droplet) receives water from the surrounding water-rich phase to maintain its volume fraction while it supplies water to the inner water-rich phase, that is, the PIC droplet works as a "thermally driven pump" that supplies water from the outer phase to the inner phase.

4) The microphase separation and formation of the PIC membrane progress until the PIC droplet is completely exhausted at the focused laser point, and, finally, formation of the single spherical PIC vesicle is complete. Instead of a "pump," an ordered and rather hydrophobic PIC membrane remains at the focused laser point; thus, the completed vesicle maintains its size.

We propose that the formation of the unilamellar PIC vesicle occurs through the formation process shown in Figure 3. We plotted various initial volumes of PIC droplets against their final surface areas of the formed vesicles, and confirmed that the points lie roughly on a straight line that is obtained by a least-squares method (see the Supporting Information), that is, the final size of the PIC vesicle is dependent on the initial size of the PIC coacervate. Strikingly, this result clearly supports the observation that the formed PIC membrane has a uniform thickness regardless of the initial size of the PIC droplet. The estimated average thickness of the membrane was approximately 60 nm, which is calculated as the slope of the regression line. This value indicates that the vesicles have a unilamellar structure, when it is considered that contour lengths of the block copolymers should be of the same order,[12] and that the size of the PIC micelle prepared from PEG–PAsp and PEG–P(Asp-AP) and the micelle prepared from PEG–PAsp and the mixture of PEG–P(Asp-AP)/homo–P(Asp-AP) are approximately 40 nm and 50 nm, respectively (see the Supporting Information).

It should be noted that the stability of the PIC membrane depends on the solution conditions. When the salt content is higher (e.g., 150 mM NaCl), the generated blisters break easily before sufficient growth has occurred, and a spherical vesicle does not completely form (see the Supporting Information). This phenomenon can be explained by the electrostatic interaction between polyelectrolytes in a PIC membrane being reduced, which results in membrane instability. Considering the phenomenon in the presence of 75 mM NaCl, where the formed vesicle could not be maintained without continuous thermal perturbation, solution conditions (ionic strength, temperature, etc.) or polymer properties (molecular weight, structure of side chains, etc.) that reduce the surface tension should be optimized in order to stabilize the PIC vesicle after thermal perturbation.

In conclusion, we have elucidated the formation process of giant unilamellar polymer vesicles that were formed spontaneously from a droplet of PIC by thermal perturbation. The size of the resulting vesicles is determined on the basis of the initial droplet size. The microphase separation within the PIC droplet and the formation of the hydrophobic ordered PIC layer are the key mechanisms in vesicle formation. The use of PIC droplets as precursors for mass production of unilamellar vesicles has great potential for encapsulating therapeutic, cosmetic, and nutritional compounds. The combination of the mature technology of droplet formation with microfluidics can stabilize the vesicle and efficiently load cargoes.

Experimental Section

Materials: Detailed information about materials and the synthetic procedure for homo–P(Asp-AP) are provided in the Supporting Information.

Preparation of PIC droplets: All the polymers were dissolved separately in tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer containing a certain concentration of NaCl. The final composition of each of the polymer solutions was as follows: polymer (0.5 mg mL^{-1}), Tris-HCl (10 mM, pH 7.4), and NaCl (75 mM), and polymer (0.8 mg mL^{-1}), Tris-HCl (10 mM, pH 7.4), and NaCl (150 mM). Solutions of PEG–P(Asp-AP) and homo–P(Asp-AP) were blended in an equimolar ratio with respect to cationic residues.

The resulting mixture was then added to the PEG–PAsp solution in an equal charge ratio, followed by vortex mixing for 30 s, to produce the PIC microdroplets as a slightly turbid solution. The concentrations of each polymer in the final mixtures were 9.4 μM PEG–P(Asp-AP), 8.6 μM homo–P(Asp-AP), and 19 μM PEG–PAsp for the 75 mM NaCl system, and 15 μM PEG–P(Asp-AP), 14 μM homo–P(Asp-AP), and 30 μM PEG–PAsp for the 150 mM NaCl system. The PIC droplet solution was confined between cover slips, sealed to prevent evaporation, and observed. All experiments were carried out at RT ((297 ± 2) K).

Instruments: Optical microscopic observation was performed using a phase-contrast microscope (IX-71, OLYMPUS). A linearly polarized ytterbium fiber laser (CW, $\lambda = 1066$ nm; IPG Laser GmbH) was employed as a light source for the optical tweezers. The laser was driven at 0.2–0.4 W, the PIC was irradiated through an oil-immersed objective lens (100×, Ph3, numerical aperture 1.35).

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[1] a) D. E. Discher, A. Eisenberg, *Science* **2002**, *297*, 967; b) M. Antonietti, S. Förster, *Adv. Mater.* **2003**, *15*, 1323; c) V. P. Torchilin, *Nat. Rev. Drug Discovery* **2005**, *4*, 145.
[2] a) S. S. Mansy, J. P. Schrum, M. Krishnamurthy, S. Tobé, D. A. Treco, J. W. Szostak, *Nature* **2008**, *454*, 122; b) Y. Zhou, D. Yan, *J. Am. Chem. Soc.* **2005**, *127*, 10468; c) Y. Zhou, D. Yan, *Angew. Chem.* **2005**, *117*, 3287; *Angew. Chem. Int. Ed.* **2005**, *44*, 3223.
[3] a) K.-M. Jin, Y.-H. Kim, *J. Controlled Release* **2008**, *127*, 249; b) W. Dong, R. Bodmeier, *Int. J. Pharm.* **2006**, *326*, 128.
[4] a) J. Xia, P. L. Dubin in *Macromolecular Complexes in Chemistry and Biology* (Eds.: P. L. Dubin, J. Bock, R. Davis, D. N. Schulz, C. Thies), Springer, Berlin, **1994**, p. 247; b) C. L. Cooper, P. L. Dubin, A. B. Kayitmazer, S. Turksen, *Curr. Opin. Colloid Interface Sci.* **2005**, *10*, 52; c) M. Jahanshahi, S. Williams, A. Lyddiatt, S. A. Shojaosadati, *IEE Proc.-Nanobiotechnol.* **2004**, *151*, 176.
[5] A. P. Minton, *Biophys. J.* **2005**, *88*, 971.
[6] T. Imura, H. Yanagishita, D. Kitamoto, *J. Am. Chem. Soc.* **2004**, *126*, 10804.
[7] a) S. Batzri, E. D. Korn, *Biochim. Biophys. Acta Biomembr.* **1973**, *298*, 1015; b) F. Ishii, A. Takamura, Y. Ishigami, *Langmuir* **1995**, *11*, 483; c) F. Meng, C. Hiemstra, G. H. M. Engbers, J. Feijen, *Macromolecules* **2003**, *36*, 3004.
[8] a) H. N. Yow, A. F. Routh, *Soft Matter* **2006**, *2*, 940; b) R. K. Shah, J.-W. Kim, J. J. Agresti, D. A. Weitz, L.-Y. Chu, *Soft Matter* **2008**, *4*, 2303.
[9] a) A. Koide, A. Kishimura, K. Osada, W.-D. Jang, Y. Yamasaki, K. Kataoka, *J. Am. Chem. Soc.* **2006**, *128*, 5988; b) A. Kishimura, A. Koide, K. Osada, Y. Yamasaki, K. Kataoka, *Angew. Chem.* **2007**, *119*, 6197; *Angew. Chem. Int. Ed.* **2007**, *46*, 6085; c) A. Kishimura, S. Liamusuwan, H. Matsuda, K. Osada, Y. Yamasaki, K. Kataoka, *Soft Matter* **2009**, *5*, 529.
[10] A. Ashkin, *IEEE J. Sel. Top. Quantum Electron.* **2000**, *6*, 841.
[11] a) M. Ishikawa, H. Misawa, N. Kitamura, R. Fujita, H. Masuhara, *Bull. Chem. Soc. Jpn.* **1996**, *118*, 11968; b) N. F. Bunkin, A. V. Lobeyev, *Colloids Surf. A* **1997**, *129*, 33; c) S. Mukai, N. Magome, H. Kitahata, K. Yoshikawa, *Appl. Phys. Lett.* **2003**, *83*, 2557; d) N. Kitamura, M. Yamada, S. Ishizaka, K. Konno, *Anal. Chem.* **2005**, *77*, 6055.
[12] a) A. Harada, K. Kataoka, *Science* **1999**, *283*, 65; b) A. Harada, K. Kataoka, *Macromolecules* **2003**, *36*, 4995.

