DOI: 10.1002/anie.201406953

Biomimetic Vesicles

Self-Oscillating Vesicles: Spontaneous Cyclic Structural Changes of Synthetic Diblock Copolymers[**]

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Abstract: *A large variety of synthetic vesicles has been created for potential engineering applications and as model systems which mimic living organisms. In most cases, the structure is designed to be thermodynamically stable. However, mimicking dynamic behaviors of living vesicles still remains undeveloped. Herein, we present a synthetic vesicle which shows autonomous disintegration–reconstruction cycles without any external stimuli and which is similar to those in living organisms, such as in the nuclear envelope and synaptic vesicles. The vesicle is composed of a diblock copolymer which has a hydrophilic and a thermosensitive segment. The thermosensitive segment includes a redox moiety that acts as a catalyst for an oscillatory chemical reaction and also controls the aggregation temperature of vesicles. Furthermore, autonomous fusion of vesicles is also observed during the cycles.*

Hierarchical ordered structures and unique functions in living organisms have attracted attention not only in biology but also in physics and chemistry. In materials science, it is very important to create artificial functional materials with high efficiency, which mimic structures and functions of living organisms.[1–3] In particular, the vesicle is one of the most universal and fascinating structures because it appears in several components of living organisms, such as the eukaryotic cell membrane, the nuclear envelope, and in synaptic vesicles. One of the most important roles of vesicles is to create a closed microenvironment which is isolated from exterior regions. In biological systems, localization of molecules into selectively separated micro/nanometer-scale regions is very important because specific processes, such as metabolic, enzymatic, or polymeric reactions, can be enhanced in the encapsulated region.[4–7]

Multiple studies have investigated the fabrication of vesicles from synthetic compounds, such as phospholipids[8–10] and synthetic block copolymers.[11–15] Additionally, highly sophisticated designs have been reported, such as the imitation of living vesicles by insertion of channel proteins into a polymeric membrane,[16,17] the induction of an enzyme reaction inside the vesicles,[18] and the fabrication of multicompartment vesicles.[19,20]

The dynamics of vesicles in biological systems is another important aspect to consider. For example, it has been established that the nuclear envelope in the living cell repeats the cycle of formation and breakup of vesicles during the process of mitosis.[21] At an early stage, the nuclear envelope needs to be broken completely to allow access of the mitotic spindle to chromatin. However, the disintegrated envelope must be accurately reconstructed to give a vesicular structure that encloses the nuclear genome. Thus, rhythmic disintegration and reconstruction of the vesicular structure spontaneously occur accompanying the cycles of mitosis. The cycle of disintegration and reconstruction of synaptic vesicles also plays a crucial role in living organisms.[22,23] During the synaptic transmission cycles at the nerve terminal, synaptic vesicles are fused together with the plasma membrane to release neurotransmitters to the membrane. After fusion, the vesicles are reconstructed at the surface of the plasma membrane to recapture neurotransmitters.

Despite the importance of dynamic organization of vesicles in living organisms, the dynamics of assembly and disassembly as well as the formation mechanisms have not yet been fully understood. In biomimetic materials science, despite efforts to reproduce living systems using synthetic components, there are only a few studies highlighting the dynamic features of vesicles.[24,25]

Herein, we show that autonomous cyclic changes in the formation–fragmentation of vesicles without external stimuli occur by using a well-defined, synthetic diblock copolymer (Figure 1). To prepare such "self-oscillating vesicles", a ruthenium catalyst {Ru(bpy)$_3$} was copolymerized to the main chain of the thermosensitive poly(*N*-isopropylacrylamide) segment in the diblock copolymer. The transition-metal complex {Ru(bpy)$_3$} catalyzes the oscillating Belousov–Zhabotinsky (BZ) chemical reaction. To date, we have developed self-oscillating polymers and gels,[26–28] that undergo autonomous phase transitions, such as swelling/deswelling of gels driven by the BZ reaction.

We synthesized well-defined diblock copolymers which can form vesicles at equilibrium at a temperature above the lower critical micellization temperature, that is, the aggrega-

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[**] This work was supported in part by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists (No.262029 to R. T. and No. 11J07791 to T. U.), and Grants-in-Aid for Scientific Research from the Ministry of Education (Japan) (No. 22245037 to R. Y.). We acknowledge Dr. Aya Mizutani Akimoto and Dr. Zulma Jiménez for helpful suggestions. We also thank the Research Hub for Advanced Nano Characterization at The University of Tokyo for TEM observation.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201406953.

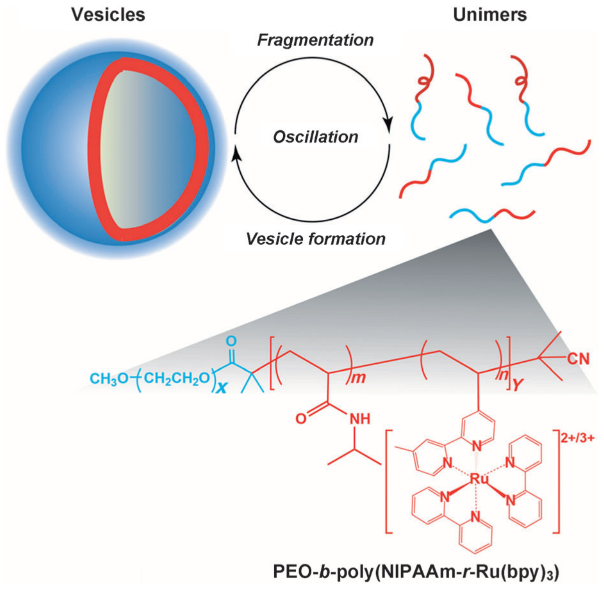

Figure 1. Representation of the formation of self-oscillating vesicles from unimers and their polymer structure.

tion temperature. The self-oscillating formation and fragmentation of vesicles without external stimuli is achieved by using the oscillatory properties of the BZ reaction. The oscillatory dynamic behavior of the vesicles, such as formation, fusion, and fragmentation without any external stimuli, was demonstrated by time-resolved dynamic light scattering measurements as well as direct observation by time-lapse bright-field optical microscopy.

The self-oscillating diblock copolymers, specifically poly(ethylene oxide)-*block*-poly(*N*-isopropylacrylamide-*random*-ruthenium(4-vinyl-4′-methyl-2,2′-bipyridine)bis(2,2′-bipyridine)bis(hexafluorophosphate)), abbreviated as (PEO-*b*-poly(NIPAAm-*r*-Ru(bpy)$_3$)), were synthesized and characterized following a reported procedure.[28] The polymer has a hydrophilic segment (PEO) as the first block and a thermoresponsive {poly(NIPAAm-*r*-Ru(bpy)$_3$)} segment as the second block, respectively. Two types of polymer, EN-1 and EN-2, were prepared, which have different molecular weights of self-oscillating segments with the same length of PEO (Table 1). Previously,[28] unimer-to-micelle oscillations were achieved for block copolymers which have a number average molecular weight $M_n = 25$ kDa and a block ratio of hydrophilic segments of 20 wt% (wt = weight). Therefore, the block ratio of hydrophilic segments for EN-2 was set to be lower than the previous study to obtain vesicles as an equilibrium structure, whereas that of EN-1 was set to be higher than EN-2.

Table 1: Characterization of polymers EN-1 and EN-2.

Name	Polymer formula[a]	M_n[a] [kDa]	MWD[b]	f_{EO} [wt%]
EN-1	PEO$_{114}$-*b*-poly(NIPAAm$_{61}$-*r*-{Ru(bpy)$_3$}$_5$)	17.7	1.27	29
EN-2	PEO$_{114}$-*b*-poly(NIPAAm$_{254}$-*r*-{Ru(bpy)$_3$}$_6$)	38.1	1.19	13

[a] Calculated by NMR spectroscopy and UV/Vis absorption spectroscopy. [b] Measured by gel permeation chromatography. MWD = molecular weight distribution. f_{EO} = weight fraction of hydrophilic segment (PEO).

Self-assembled structures of EN-1 and EN-2 polymers in the reduced state (i.e., $[Ru(bpy)_3]^{2+}$ state) were observed by transmission electron microscopy (TEM). The TEM images were recorded without staining agents, such as uranyl acetate, because the $[Ru(bpy)_3]^{2+}$ moiety incorporated in the main chain enhances the electron density contrast. The TEM image of EN-1 shows spherical micelles with a radius of several tens of nanometers, whereas EN-2 aggregates form large vesicles which have a size around 1 μm (Figures 2 a, b). However, it was still unclear from the TEM images that EN-2 formed unilamellar or multilamellar vesicles. Fluorescence microscopy (FM) and bright-field optical microscopy (OM) images were also measured in situ for EN-2 in aqueous solution (Figures 2 c and d). The image of vesicles recorded by FM clearly shows an intense green ring around the self-assembled structure, indicating the presence of a bilayer vesicle membrane where $[Ru(bpy)_3]^{2+}$ is selectively loaded.

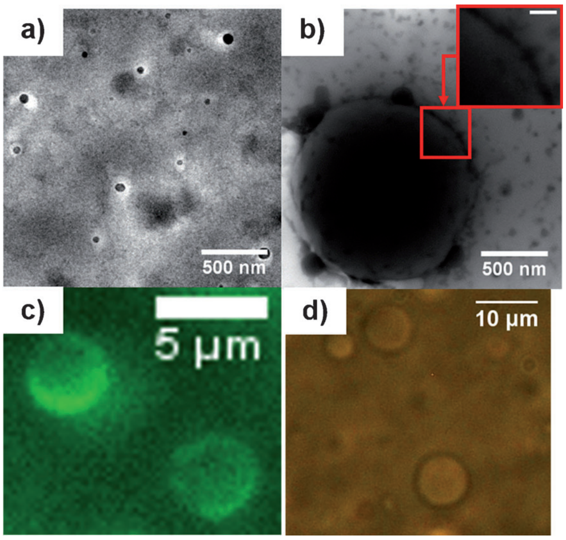

Figure 2. a, b) TEM images of self-assembled structures of EN-1 (a) and EN-2 (b). In the inset image in (b), the scale bar is 100 nm. c, d) In situ fluorescent microscopy (c) and bright-field optical microscopy (d) images of the vesicular structure in an aqueous solution of EN-2 at 26 °C.

Furthermore, dynamic light scattering (DLS) measurements were used to investigate the temperature dependence of the equilibrated hydrodynamic radius (R_h) of EN-2 in two aqueous solutions containing either reduced (PEO-*b*-poly(NIPAAm-*r*-Ru(bpy)$_3^{2+}$) or oxidized (PEO-*b*-poly(NIPAAm-*r*-Ru(bpy)$_3^{3+}$) states (Figure 3). At temperatures below the aggregation temperature, the R_h value is approximately several nanometers, irrespective of the redox state of ruthenium, which indicates that the polymers dissolve as single polymer chains (i.e., unimers). When the temperature was increased above the aggregation temperature, the R_h value abruptly increased in both redox states. Importantly, there is about a 3 °C difference between

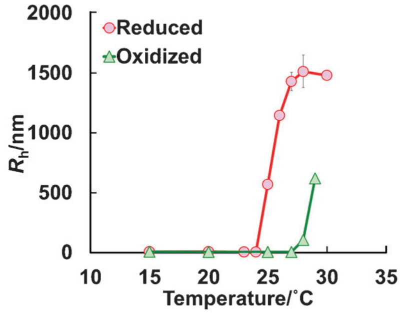

Figure 3. Temperature dependency of R_h value in an aqueous solution of EN-2 in the reduced and oxidized states. The solution contains HNO_3 (1 M), the polymer (0.1 wt%), and either $[Ce_2(SO_4)_3]$ (3 mM, for the reduced state) or $[Ce(SO_4)_2]$ (3 mM, for the oxidized state).

the aggregation temperatures of the reduced and the oxidized forms of the polymer. This is attributed to the change in hydrophilicity of the thermosensitive polymer segment with the change in the redox state of $\{Ru(bpy)_3\}$.

To cause periodic structural changes of the vesicles, we added the three reactants of the BZ reaction, specifically $NaBrO_3$, HNO_3, and malonic acid (MA), into the diblock copolymer solution at a temperature between the two aggregation temperatures. At this temperature, the polymer becomes bistable, which means that it aggregates in the reduced state and disaggregates in the oxidized state. Accompanying the periodic color change between orange and light green that is due to the oscillation of the $\{Ru(bpy)_3\}$ between the reduced and the oxidized states, rhythmic oscillation of the turbidity was observed (Figure 4a). When the color of the solution was orange, indicating the reduced state, the solution became opaque. In contrast, the solution became transparent in the oxidized state because the aggregation temperature of the oxidized polymer was higher than the experimental temperature. This result strongly implies that the diblock copolymer periodically undergoes a transition between two different states of unimers and self-assembled structures which have a large size enough to scatter light.

To further analyze the oscillating behavior of the diblock copolymer solution, time-resolved DLS measurements were employed. In the measurements, the averaged scattering intensity and autocorrelation functions were collected at 2 second intervals and the data were theoretically analyzed to calculate R_h values (Supporting Information). Figure 4b shows the time evolution of the normalized scattering intensity and the R_h values for the diblock copolymer solution (0.1 wt%) which contains an appropriate concentration of BZ substrates at a bistable temperature (25 °C). Periodic changes in scattering intensity with a large amplitude were obtained (blue plots in Figure 4b), which suggests that oscillation occurs between two states; large aggregates with a high light scattering ability and small particles with a low scattering ability. From the oscillating waveform (Figure 4c), it is clear that a decrease in scattering intensity occurred faster than the increase in intensity. This can be explained by the kinetics of the BZ reaction, where the process of autocatalytic oxidation from $[Ru(bpy)_3]^{2+}$ to $[Ru(bpy)_3]^{3+}$ occurs more rapidly than the opposite reduction process. Another possible explanation may be the difference in kinetics between vesicle association and dissociation. Dissociation of the assembled structures proceeds more rapidly than association of the reduced diblock copolymer molecules, as to form the assembled structures, a polymer molecule is required to approach another in a specific configuration.

The red plots in Figure 4c reveal that R_h values also periodically oscillated with an amplitude over 100 nm. This suggests that structural changes occur between vesicles and its fragmented states (i.e., unimers). The gradual increase of R_h values and the baseline of scattering intensity with increasing time, that is, after repeated cycles (Figure 4b), may be attributed to the growth of vesicles which cannot be easily broken down, even when the diblock copolymer is in the hydrophilic oxidized state. During the oscillations, the maximum R_h value is smaller than the equilibrium R_h value which is on the micrometer scale (see Figure 3). A possible reason for this difference is that the oscillation period is too short for vesicles to increase in size sufficiently to form the equilibrated size.

Finally, in situ real-time observation of the self-oscillating behaviors of vesicles was carried out by using bright-field OM with a time-lapse mode. The diblock copolymer solution, under BZ reaction conditions, was spread onto a glass slide and directly observed. The propagation of a “chemical wave” was observed, indicat-

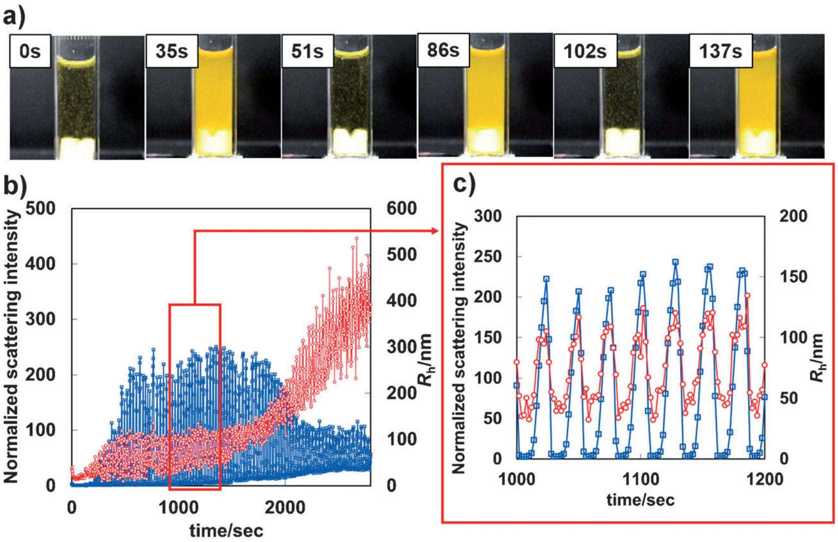

Figure 4. a) Periodic changes in appearance of an aqueous solution of EN-2 during the BZ reaction under stirring. The white object at the bottom of the cuvette is a magnetic stirrer bar. b) Time evolution of the normalized scattering intensity (blue plots) and R_h values (red plots) of the aqueous solution of EN-2 during the BZ reaction. c) Expanded portion of the oscillation. BZ reaction conditions: polymer (0.1 wt%), HNO_3 (0.3 M), $NaBrO_3$ (0.4 M), and MA (0.025 M) at 25 °C.

ing the progress of the BZ reaction (Figure S2 in the Supporting Information). The images provide confirmation that a rhythmical self-oscillation between association and dissociation of vesicles occurs that is coupled with the travelling waves (see movie S1 in the Supporting Information). The transition from dissociation to association of vesicles on a micrometer scale during the BZ reaction is shown in Figures 5 a–d. In the association process, each vesicle exhibited Brownian motion and gradually increased their size. On the other hand, vesicles quickly disappeared within a few seconds in the fragmentation process. This observation is consistent with the result from the time-resolved DLS measurements that the fragmentation process occurs more rapidly than the vesicle formation process. Note, the observation that vesicles disappear within a few seconds does not necessarily imply that vesicles are broken up into individual polymer chains in the same time scale, because the space resolution of OM is not high enough to observe the polymer chains.

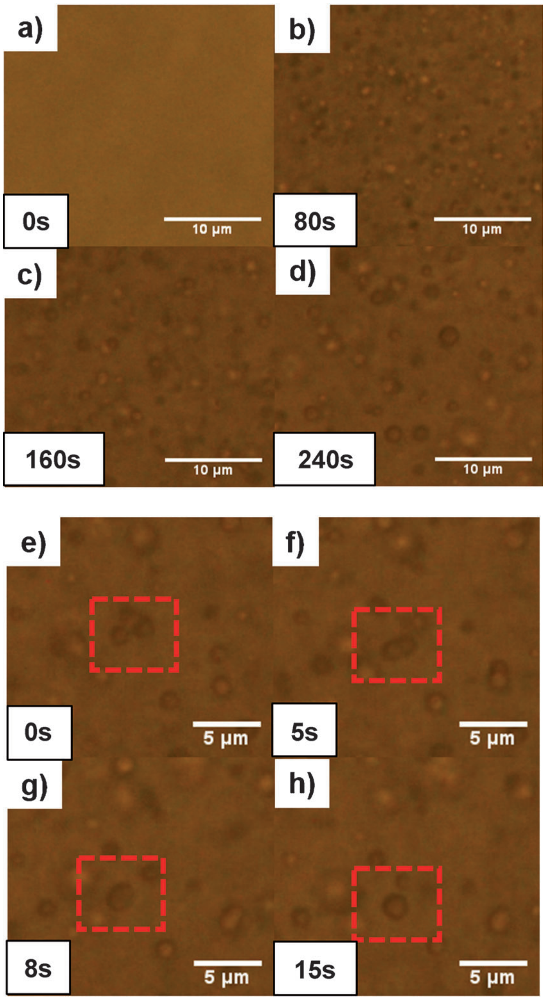

Figure 5. Bright-field optical microscopy images recorded at different times showing a–d) the transition from fragmented states (unimers) to vesicles (scale bar = 10 μm), and e–h) the fusion process of two independent vesicles to form a larger vesicle. Conditions: polymer (1.5 wt%), HNO_3 (0.7 M), $NaBrO_3$ (0.2 M), and MA (0.025 M) at 26 °C.

During the process of vesicle growth accompanying the reduction of $[Ru(bpy)_3]^{3+}$, the autonomous fusion of two vesicles to form a larger vesicle was also observed, similar to biological processes occurring at the cell membrane (see movie S2 in the Supporting Information).[29] A series of bright-field OM images which record this fusion process is shown in Figure 5 e–h. Firstly, two independent vesicles collided with each other, and then they deformed and partially fused (Figures 5 e–g). Finally, the partially fused vesicles grew to form a larger vesicle (Figure 5 h).

Although micelle-to-vesicle oscillations of fatty acid solutions coupled with a pH-oscillator in a continuously stirred tank reactor have been described,[24] this is the first time that autonomous cycling between the formation and fragmentation of artificial vesicles composed of synthetic block copolymers has been realized and directly observed. As a model of vesicles in living organisms that show dynamic behavior, the self-oscillating vesicles reported herein will provide novel concepts and biomimetic materials. Furthermore, the periodic changes between higher order structure formation and fragmentation of the vesicle has great potential for a large variety of engineering applications, for example, an autonomous encapsulation and release of selected agents such as synaptic vesicles. Additionally, as it has been shown that encapsulation of BZ substrates in lipid vesicles allowed chemical communication across the membrane,[25] it would be interesting to study the effect of coupling between chemical communication and the structural oscillations studied herein.

Received: July 7, 2014
Revised: August 13, 2014

Keywords: biomimetics · block copolymers · oscillating structures · ruthenium · vesicles

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