Model Membranes

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Chemically Locked Bicelles with High Thermal and Kinetic Stability

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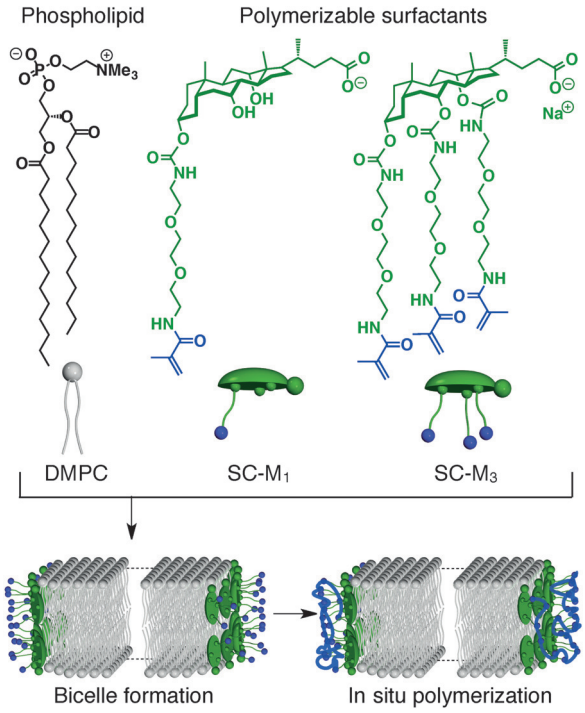
Abstract: *In situ polymerization of a bicellar mixture composed of a phospholipid and polymerizable surfactants afforded unprecedented stable bicelles. The polymerized composite showed an aligned phase over a wide thermal range (25 to > 90 °C) with excellent ^2H quadrupole splitting of the solvent signal, thus implying versatility as an alignment medium for NMR studies. Crosslinking of the surfactants also brought favorable effects on the kinetic stability and alignment morphology of the bicelles. This system could thus offer a new class of scaffolds for biomembrane models.*

Bicelles (bilayered micelles) are aqueous lipid–surfactant assemblies, in which lipid bilayer fragments are edge-stabilized with certain surfactants.[1] Since bicelles take an intermediate morphology between lipid vesicles and lipid–surfactant mixed micelles, they offer a new class of biomembrane models that combine the attractive features of both of these classic model systems. In addition, bicelles spontaneously align in magnetic fields and can therefore be used as aligned media for modern NMR techniques to obtain 3D structural information on biomolecules.[2] More recently, bicelles have been used as templates for forming composite materials through hybridization with polymers and inorganics.[3] Although various bicellar systems have been developed to date, their application has been limited owing to insufficient stability of their aligned phase. Most of the reported bicelles attain optimal alignment only under limited conditions in terms of temperature, lipid content, pH value, ionic strength, among other factors.[2e,4]

To increase the stability of the aligned phase of bicelles, a number of strategies have been proposed, including modification of the lipids and doping the lipid bilayers with cholesterol, its derivatives, or charged surfactants.[5] However, since these methods inevitably change the interior environment of lipid bilayers, an alternative approach solely based on the design of surfactant units is important. For the formation of stable bicelles, surfactants should meet two seemingly contradictory requirements. When the miscibility of the surfactant with the phospholipid is too high, their mixture is more prone to forming isotropic mixed micelles rather than bicelles. On the other hand, insufficient miscibility results in the separation of bicelles into vesicles and isotropic surfactant–phospholipid mixed micelles. Moreover, phospholipids generally show thermotropic phase transitions accompanied by drastic changes in their miscibility with surfactants,[6] which makes the design of surfactants more difficult.

As a conceptually novel approach, we report the stabilization of bicelles through in situ crosslinking of surfactants (Scheme 1). In bicellar assemblies, surfactants are considered to be localized at the edge of the lipid bilayer fragments.[7] If the in situ crosslinking of surfactants proceeds without serious alteration of the overall structure of bicelles, the resultant polymeric networks of surfactants should show enhanced affinity for the edges of lipid bilayers owing to multivalency effects, which would lead to thermodynamic and kinetic stabilization of the bicelles. Indeed, similar strategies have been successfully applied for the stabilization of other membrane models, such as vesicles.[8]



Scheme 1. Structure of the polymerized bicelles composed of a phospholipid (DMPC) and polymerizable surfactants (SC-M_1 and SC-M_3).

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To demonstrate the validity of this concept, we developed two types of polymerizable surfactants (SC-M_1 and SC-M_3; Scheme 1) derived from sodium cholate (SC). SC a representative surfactant for the formation of bicelles and possesses a quasi-planar rigid steroid skeleton with hydrophilic and hydrophobic faces residing back-to-back.[9] This characteristic two-faced structure provides SC with unique surface-active properties and enables efficient dispersal of various hydrophobic materials, including lipid bilayers, in aqueous media. By replacing the hydroxy group(s) of SC with hydrophilic chain(s) with a polymerizable terminus, the mono- and trifunctional monomers SC-M_1 and SC-M_3, respectively, were obtained.

The phase behavior of aqueous mixtures of the polymerizable surfactants (SC-M_1, SC-M_3) and 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) was investigated in D_2O through measurement of the ^2H quadrupole splitting (Figure 1 a) and ^{31}P NMR with ^1H decoupling of 6 kHz (Figure 1 b). Typically, we tested a sample with a molar ratio of DMPC/SC-M_1/SC-M_3 = 80:19:1 (10 % *w*/*v* total lipid content, in D_2O containing 100 mM KCl). At temperatures lower than the phase transition of DMPC (24 °C), where the phospholipid changes from the liquid-crystalline to the gel state, the mixture showed a nonaligned phase (Figure 1 a (i)). In the gel state, DMPC was highly miscible with the surfactants (SC-M_1 and SC-M_3), so the mixture formed isotropic mixed micelles, giving a ^{31}P NMR signal at 0 ppm (Figure 1 b (i)). When the mixture was heated in the range 25–40 °C, an obvious ^2H quadrupole splitting was observed (Figure 1 a (ii–iv)), thus indicating the formation of an aligned phase. In the ^{31}P NMR spectra, the signal at 0 ppm disappeared, while a new signal emerged at around −9 ppm, the chemical shift of which is in good agreement with magnetically aligned phospholipid bilayers with their normal perpendicular to the magnetic field (Figure 1 b (ii–iv)).[10] However, when the system was heated to more than 45 °C, where the miscibility of the phospholipids and surfactants is insufficient to maintain the homogeneity, the bicellar assemblies separated into vesicles and isotropic mixed micelles (Figure 1 b (v–vii)), which abruptly terminated the alignment ability (Figure 1 a (v–vii)).

With the expectation of stabilizing the bicelles, in situ radical copolymerization of the surfactants SC-M_1 and SC-M_3 was performed at 40 °C, which is the optimal temperature for the mixture to form alignable bicelles. Upon treating the bicellar mixture with potassium peroxodisulfate ($K_2S_2O_8$) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (1 mol % and 150 mol %, respectively, with respect to the acrylamide units), the acrylamides in the surfactants were quantitatively consumed within 15 min, as unambiguously confirmed by ^1H NMR measurement (Figure S1 in the Supporting Information). After completion of the polymerization and subsequent aging, the system changed to a viscous translucent fluid.

Of particular interest is that the polymerized mixture showed an aligned phase over an unprecedentedly wide temperature range (Figure 1 c, d). According to the ^{31}P NMR spectra, the system was composed solely of aligned bilayers from 25 °C to over 90 °C (Figure 1 d (ii–vii)), where the signals of isotropic mixed micelles or vesicles were not detected at all (Figure 1 d (ii–vii)). Before the present work, there have been only two bicellar systems that show an aligned phase at temperatures higher than 65 °C, which were reported very recently.[5f,g] In addition, even when the system was cooled to 20 °C, where DMPC is known to readily miscible with surfactants owing to the transition to a gel state, our bicellar mixture did not change to isotropic mixed micelles (Figure 1 d (i)). Most likely, the covalently crosslinked networks of the surfactants were able to strongly adhere to the bilayer

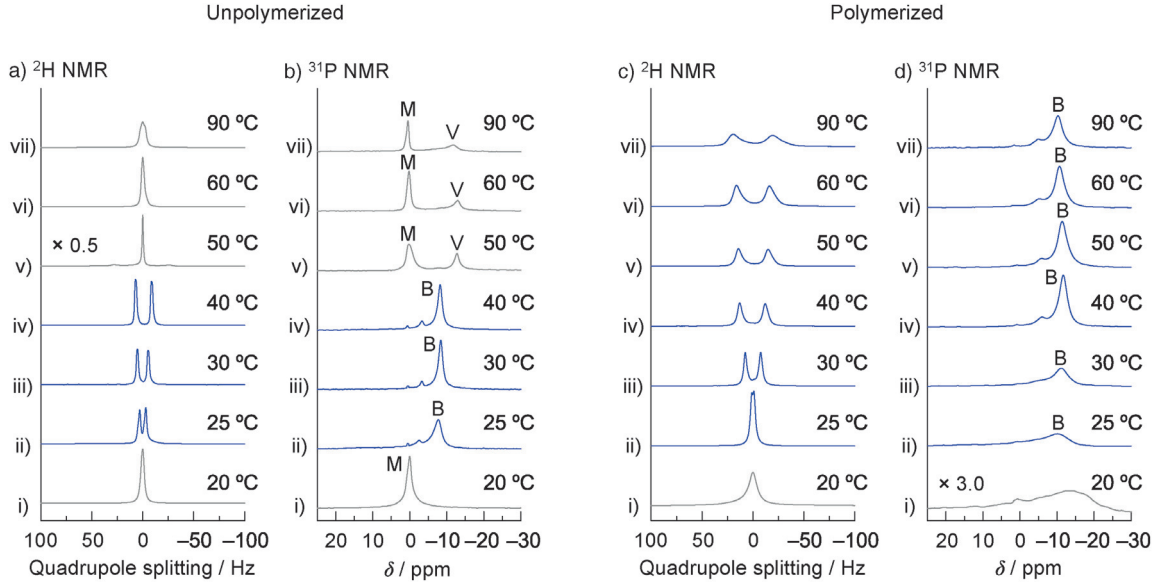

Figure 1. Variable-temperature NMR spectra of bicellar mixtures composed of DMPC and polymerizable surfactants (SC-M_1 and SC-M_3) with a molar ratio of DMPC/SC-M_1/SC-M_3 = 80:19:1 in D_2O containing 100 mM KCl (total lipid content, 10 % *w*/*v*). a, b) Before polymerization. c, d) After polymerization. ^2H quadrupole splitting (a, c) and ^{31}P NMR spectra with ^1H decoupling of 6 kHz (b, d) were measured. In the ^{31}P NMR spectra, M, B, and V indicate signals assignable to micelles, bicelles, and vesicles, respectively.

edges, which prevented drastic structural reorganization of the lipid bilayers. Although the polymerized mixture did not align at temperatures lower than 25 °C (Figure 1c (i)), the alignment perfectly recovered when the mixture was heated to above 25 °C. Upon repeating the temperature change across 25 °C, the phase transition took place without any degradation.

The magnitude of ^{2}H quadrupole splitting of the solvent signal is a reliable probe for evaluating the ability of bicelles to align the surrounding molecules, which is crucial for NMR structural studies of biomolecules through observing residual dipole couplings and chemical shift anisotropies.[1] In the case of common bicelles, the splitting is strongly dependent on temperature and is at a satisfactory level only within a narrow temperature range. Indeed, our bicellar mixture before polymerization showed meaningful ^{2}H quadrupole splitting (ca. 15 Hz) only within the thermal range 40–45 °C (Figure S2 (i)). In sharp contrast, the polymerized bicellar mixture achieved 15 Hz splitting even at 30 °C. Moreover, the splitting increased up to 40 Hz as the temperature was raised to 90 °C (Figure S2 (ii)). Thus, our polymerized bicellar mixture serves as a useful alignable medium from 30 °C to more than 90 °C, which implies potential application for investigating the structure and dynamics of various proteins, including those that are thermally unstable and thermophilic, at their biologically relevant temperatures. It should also be mentioned that the present system has room for further improvement through alteration of the composition of the phospholipid bilayer parts, such as doping with cholesterol derivatives and the use of other phospholipids.[5]

In order to characterize our unpolymerized and polymerized bicelles further, ^{31}P NMR spectra were measured with higher ^{1}H decoupling (42 kHz; Figure S3). Consistent with the data with lower ^{1}H decoupling (6 kHz; Figure 1b,d), the unpolymerized mixture exhibited an aligned phase only within a narrow thermal range (Figure S3a), while the polymerized mixture showed a characteristic signal of aligned bicelles over a wide thermal range from 25 °C to more than 90 °C (Figure S3b). In terms of peak shape and chemical shift, the signal of the polymerized mixture was minimally dependent on temperature. Closer analysis of the data revealed that the signal of the polymerized mixture was gradually broadened and shifted upfield with increasing temperature. Based on these data, the bilayer order parameters of the bicelles were evaluated (Figure S4).[7a,11] Over the entire region from 25 to more than 90 °C, the polymerized bicelles exhibited bilayer order parameter values ($S_{bicelle} = 0.68$–0.80) comparable to typical bicelles. In addition, the polymerized bicelles always showed higher order parameter values than the unpolymerized bicelles ($S_{bicelle} = 0.48$–0.64).

For the stabilization of polymerized bicelles, the multifunctional monomer SC-M_3 was found to play a crucial role. As a control experiment, the same in situ polymerization was conducted by using an SC-M_3-free mixture (molar ratio DMPC/SC-M_1 = 80:20). Before in situ polymerization, the SC-M_3-free system formed an aligned phase slightly more stable than that of the SC-M_3-doped mixture (DMPC/SC-M_1/SC-M_3 = 80:19:1). When the in situ polymerization of the SC-M_3-free bicelles was conducted at 40 °C, the resultant mixture again exhibited an aligned phase with an expanded thermal range (27–90 °C). However, over the whole thermal range, a considerable amount of isotropic mixed micelles coexisted in the system, as confirmed by ^{31}P NMR (Figure S5). Most likely, the crosslinked networks of SC-M_1 and SC-M_3 were able to "freeze" the structure of bicelle templates more efficiently than the simple linear polymers of SC-M_1.

Our in situ crosslinking method was applicable to bicellar mixtures with various average sizes. In general, the size of the bicelles is tunable by changing the molar ratio of phospholipids and surfactants, where the size becomes smaller as the ratio of surfactants is increased.[9b,12] For example, a mixture with a higher content of surfactants (molar ratio DMPC/SC-M_1/SC-M_3 = 33:64:3) formed small fast-tumbling bicelles (Figure S6 (i)). When this mixture was treated with the reagents for radical polymerization, the in situ crosslinking of the surfactants again proceeded smoothly without serious structural alteration of aggregates to afford smaller polymerized bicelles (Figure S6 (ii)).

As an additional benefit of crosslinking the surfactants, our polymerized bicelles showed notable kinetic stability. This is quite unlike the case of common bicelles, which are generally under rapid equilibrium of fission–fusion events. To detect the kinetics of bilayer fission and fusion, two samples of bicelles with different size distribution were prepared and then mixed (Figure 2a). The smaller polymerized bicelles, prepared from a mixture with a higher content of surfactants (molar ratio DMPC/SC-M_1/SC-M_3 = 33:64:3), showed a ^{31}P NMR signal at around 0 ppm (Figure 2c, (ii)), which could be easily distinguished from the ^{31}P NMR signal of the

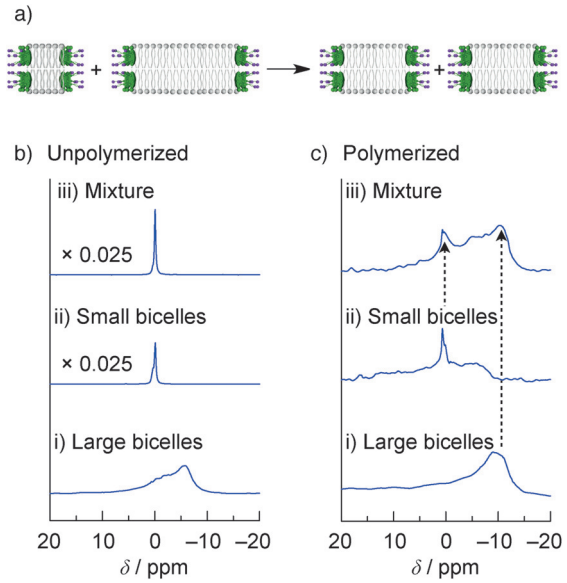

Figure 2. Kinetic studies of bilayer fission–fusion process of the bicelles. a) Schematic representation of the relaxation of bicelles to their equilibrium size. b, c) ^{31}P NMR spectra of unpolymerized (b) and polymerized (c) bicellar mixtures in D_2O containing 100 mM KCl at 40 °C. i) mixture with a molar ratio of DMPC/SC-M_1/SC-M_3 = 80:19:1 (total lipid content, 10% *w*/*v*), ii) mixture with a molar ratio of DMPC/SC-M_1/SC-M_3 = 33:64:3 in D_2O containing 100 mM KCl (total lipid content, 5% *w*/*v*), and iii) mixture of (i) and (ii) with a volume ratio of 3:5.

larger polymerized bicelles (DMPC/SC-M_1/SC-M_3 = 80:19:1; Figure 2c (i)). Upon mixing the polymerized samples of the smaller and larger bicelles, their ^{31}P NMR signals were separately observed at their original chemical-shift regions (Figure 2c (iii)). In sharp contrast, when the unpolymerized samples of the small (Figure 2b (ii)) and large (Figure 2b (i)) bicelles were mixed in the same manner, only a unimodal signal was observed in the ^{31}P NMR spectrum (Figure 2b (iii)), thus suggesting that the relaxation of bicelles to their equilibrium size was completed just after the mixing of the samples. These observations clearly indicate that the fusion and fission of bicelles was significantly suppressed by crosslinking of the surfactants. Considering this enhanced kinetic stability, these polymerized bicelles could offer useful scaffolds for model membranes with complicated substructures, including lipid-raft domains.

Last, small-angle X-ray scattering (SAXS) profiles of the polymerized bicelles were measured and they revealed the significant influence of the crosslinking of surfactants on the alignment morphology of bicelles. As shown in Figure 3a, both the unpolymerized (i) and polymerized (ii) mixture showed a broad peak between $q = 0.8$ and 2.5 nm^{-1}, which is attributable to the scattering of single lipid bilayers with a thick of approximately 4 nm.[13] Interestingly, the polymerized mixture showed a strong sharp Bragg peak at $q = 0.3$ nm with a d-spacing of 20 nm (Figure 3a (ii)). In the 2D SAXS pattern of an anisotropically oriented sample held in a thin capillary to induce macroscopic alignment by the anisotropic container-shape effect, the broad scattering ($q = 0.8$ and 2.5 nm^{-1}) and the Bragg peak ($q = 0.3$ nm) were observed in the regions with similar azimuthal angle (Figure 3c,d). This observation unambiguously indicates smectic-type orientation, in which bicelles are cofacially aligned along the normal vector of bilayers (Figure 3b, upper model). According to the half-width of the Bragg peak at $q = 0.3$ nm, the averaged number of spatially correlated bicelle sheets in the stack was estimated to be 13.[14] Contrary to this, the unpolymerized mixture showed a weak diffraction with an interlayer d-spacing of 6.2 nm (Figure 3a (i)), which is attributable to close stacking of the bicelles (Figure 3b, lower model), which is observed for common bicelles.[14b,15] Overall, our polymerized bicelles assembled in an unprecedented structure in terms of population of ordered bilayers, persistent length of alignment, and length of periodicity. Such unique alignment morphology is most likely the origin of the exceptional thermal stability and alignment-inducing ability of our polymerized bicelles. Also noteworthy is the distance between the polymerized bicelles (20 nm), which is five times as large as the thickness of single lipid bilayers (about 4 nm). This fact indicates that lipid bilayers in the polymerizable mixture are in a well-hydrated state (Figure 3b, upper model), which is expected to provide more realistic biomembrane models compared with densely stacked multilamellar vesicles.

In conclusion, we succeeded in stabilizing phospholipid bicelles through the in situ polymerization of the surfactants. The polymerized bicellar mixture showed an aligned phase over an unprecedentedly wide temperature range (from 25 to > 90 °C) with large ^{2}H quadrupole splitting of the solvent D_2O signal, which suggests utility as an alignment medium for NMR measurements. In addition, the chemical locking of the surfactants improved the kinetic stability and alignment morphology of the resultant fragmented lipid bilayers, which means that this approach could offer a new class of scaffolds for biomembrane models.

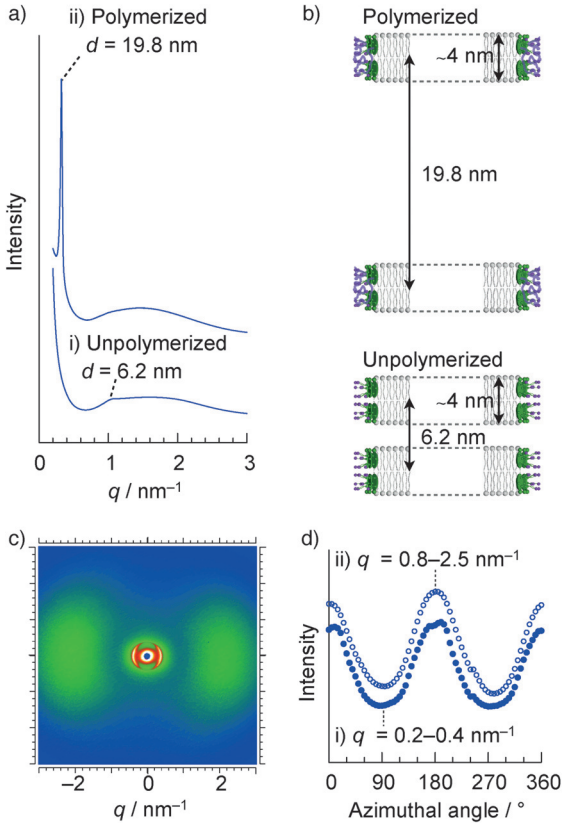

Figure 3. Alignment morphology of the bicelles. a) SAXS profile of unpolymerized (i) and polymerized (ii) bicellar mixtures with a molar ratio of DMPC/SC-M_1/SC-M_3 = 80:19:1 in D_2O containing 100 mM KCl (total lipid content, 10% *w*/*v*) at 40 °C. b) Schematic representation of the long- (upper model) and short- (lower model) range ordering of stacked bicelles. c) 2D SAXS image of the polymerized bicellar mixture macroscopically aligned in a thin capillary. d) Plotting of total scattering intensities in the 2D SAXS of the aligned sample at $q = 0.2$–0.4 (i) and $q = 0.8$–2.5 nm^{-1} (ii) as a function of the azimuthal angle.

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