Shaping Vesicles—Controlling Size and Stability by Admixture of Amphiphilic Copolymer

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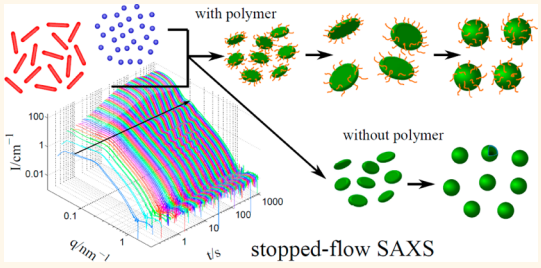
ABSTRACT



The production of structurally well-defined unilamellar vesicles and the control of their stability are of utmost importance for many of their applications but still a largely unresolved practical issue. In the present work we show that by admixing small amounts of amphiphilic copolymer to the original components of a spontaneously vesicle-forming surfactant mixture we are able to control the self-assembly process in a systematic way. For this purpose we employed a zwitanionic model system of zwitterionic TMDAO and anionic LiPFOS. As the copolymer reduces the line tension of the intermediately formed disks, this translates directly into a longer disk growth phase and formation of correspondingly larger vesicles. By this approach we are able to vary their size over a large range and produce vesicles of extremely low polydispersity. Furthermore, the temporal stability of the formed vesicles is enhanced by orders of magnitude in proportion to the concentration of copolymer added. This is achieved by exerting kinetic control that allows engineering the vesicle structure *via* a detailed knowledge of the formation pathway as obtained by highly time-resolved SAXS experiments. Synthesis of such very well-defined vesicles by the method shown should in general be applicable to catanionic or zwitanionic amphiphiles and will have far reaching consequences for controlled nanostructure formation and application of these self-assembled systems.

KEYWORDS: self-assembly · vesicles · zwitanionic surfactants · copolymer · kinetic control · morphological transition

Vesicles are highly interesting self-assembled structures not only as model systems for membranes but also as suitable delivery systems that allow encapsulating active agents either in their aqueous interior or in their hydrophobic bilayer,[1−3] thereby producing delivery vehicles that are well targeted for interacting with cell membranes.[4−7] Particularly interesting are unilamellar vesicles that form spontaneously,[8] and such formation has been well established for the case of mixtures of cationic and anionic (catanionic) surfactants,[9−12] as well as more recently for mixtures of zwitterionic and anionic (zwitanionic) surfactants.[13−16] Such investigations have also shown that the path for the formation of a given mesostructure can depend on the initial concentration of the components.[14] However, the size and polydispersity of the vesicles formed in a given surfactant mixture are difficult to predict and control and depend typically on the type of surfactants employed and potentially on the pathway of formation. This applies similarly to their stability as frequently they are only metastable and accordingly prone to subsequent aging after their formation. A conventional way for shaping vesicles is by the extrusion method, and here polydispersities down to 8% can be achieved while the size itself is controlled by the pore size employed.[17] For the case of spontaneous vesicle formation, the size may be controlled by the choice of the components in the vesicle forming surfactant mixture.[18] However, such thermodynamic control is always limited in the choice of the surfactants that can be employed and for many practical applications this reduction of choice might be restrictive.

Accordingly exerting kinetic control for the shaping of spontaneously forming vesicles would be a very interesting alternative for having structurally well-defined vesicles of variable and controlled size. Such capabiltiy is the key to successfully employing them in versatile applications and hence it is a very important task to be able to control their structural parameters in a simple way.

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Particularly interesting are spontaneously forming vesicles and one logical way for their structural control is by the admixture of small amounts of amphiphilic polymers. This approach has been taken already some while ago for the case of phospholipids[19] and has shown that in a certain limited range one is able to control stability and size of the vesicles formed. However, the outcome of structural control so far has remained rather empirical and limited in scope, and subsequent work has not improved much upon this situation.[20−22] Alternatively also the admixture of nanoparticles has been shown to allow for enhanced stability of vesicles[23] but is not really suited for controlling their size and polydispersity.

Often vesicles are nonequilibrium systems (especially for the case of multilamellar vesicles, but also unilamellar vesicles are often only metastable[24]) and therefore their structure is controlled by the pathway of formation. This formation process has been studied for the case of mixing cationic and anionic surfactants by means of time-resolved scattering experiments.[12,25−28] Typically they form *via* intermediate disk-like micelles that grow until a certain maximum size is reached and beyond this point these disks close to form unilamellar vesicles.[26−28] For the case of tetradecyldimethylamine oxide (TDMAO) as zwitterionic and various perfluorinated anionic surfactants highly time-resolved small-angle X-ray scattering (SAXS) experiments have elucidated this structural evolution in much more detail.[13,29] Immediately after mixing, very small disk-like micelles (about 4−5 nm disk radius) are formed which subsequently grow (characteristic time ∼1 s) by coalescence until beyond a certain size they become suddenly unstable and close to form rather monodisperse unilamellar vesicles. This threshold of instability is given by the ratio between the bending constants of the bilayer (κ: mean bending modulus, $\bar{\kappa}$: Gaussian modulus[30,31]) and the line tension Λ arising from the unfavorable disk rim, where the radius R_v of the formed vesicles (or alternatively their curvature c_v) is given by[13,28,29,32−35]

$$\frac{1}{c_v} = R_v = 2\frac{2\kappa + \bar{\kappa}}{\Lambda} \qquad (1)$$

For instance this idea has early on been employed by Helfrich to predict the size of vesicles generated by sonication.[34] As our kinetically controlled formation process exhibits a rather sharp transition from disks to vesicles, it leads to relatively monodisperse vesicles, which, however, often are metastable and subsequently age to yield more polydisperse vesicles.[29] This aging takes place as the initially attained curvature c_v is determined by the kinetics of the process but is not the one relevant at equilibrium that should basically depend on the bending elasticity and the entropy of dispersion, that is, one forms a metastable state here.

Of course, it is very interesting to manipulate the formation pathway in order to control vesicle size and stability in a desired way. This was exactly the aim of this work in which we employed amphiphilic copolymers to control the kinetics of the vesicle formation process, and thereby their final structure and stability. For that purpose one has to modify the formation process such as to change the ratio between bending constants and line tension in the vesicle forming amphiphilic system eq 1. This was done by adding copolymer molecules to this system, which shall preferentially be located at the disk rim. This then should lead to a reduction of the line tension, thereby shifting the transformation to larger vesicle sizes. It might be mentioned here that this idea of employing edge-active agents was pioneered by Fromherz with a comprehensive theoretical study for the case of phospholipid vesicles.[35] A larger effect on the bending elasticity by the copolymer incorporation is not expected as the effective bending modulus $2\kappa + \bar{\kappa}$ should mainly depend on the thickness of the amphiphilic layer[36,37] that should not be much affected by the presence of the copolymer, and also our experiments showed no change of the bilayer thickness.

RESULTS AND DISCUSSION

We studied the influence of the triblock ethylene oxide (EO)/propylene oxide (PO) copolymer Pluronic L35 ($EO_{11}-PO_{16}-EO_{11}$) on the vesicle formation in a zwitanionic model system composed of the surfactants lithium perfluorooctyl sulfonate (LiPFOS) and tetradecyldimethylamine oxide (TDMAO) at 50 mM total concentration. This surfactant system has been studied before and for these conditions the pure TDMAO-solution contains short cylindrical micelles with a radius of 1.9 nm and a length of 22 nm, while LiPFOS forms globular micelles which are well described as prolate ellipsoids with a rotational axis of 1.8 nm and a minor axis of 1.1 nm.[38] Upon mixing LiPFOS and TDMAO solutions around equimolar ratio unilamellar vesicles are formed in a few seconds by a spontaneous self-assembly process.[29,38]

To influence and control this formation process the amphiphilic copolymer L35 was added, where the length of the hydrophobic PO block was chosen such that it is just somewhat longer (stretched length ∼6.4 nm) than the bilayer thickness of the vesicles, in order to be able to become incorporated easily into the surfactant bilayer. This formation process was followed by ms time-resolved stopped-flow SAXS experiments where different amounts of the copolymer L35 (0−2.75 mM) were added, while keeping all other experimental parameters and the mixing ratio TDMAO:LiPFOS constant at 55:45 (chosen as here the most monodisperse vesicles are formed; see ref 38 and Supporting Information). The earliest kinetic time is given by the stopped-flow dead time of 2.3 ms and the time resolution is determined by the chosen exposure times of 5 ms. By employing sample-to-detector distances of 2 and 4 m,

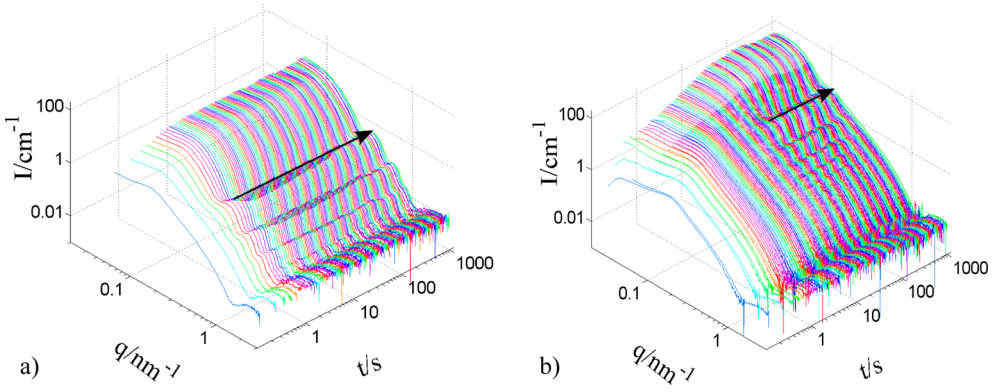

Figure 1. Structural evolution followed by SAXS. SAXS intensities as a function of time and scattering vector q for 27.5 mM TDMAO/22.5 mM LiPFOS without (a) and with 0.275 mM L35 (b).

a q-range (q is the magnitude of the scattering vector: $q = 4\pi \sin(\theta/2)/\lambda$; where θ is the scattering angle, λ is the wavelength) of 0.02−2.7 nm^{-1} was covered, which allows the us to follow size changes in the range of 2−200 nm. In Figure 1a,b, examples for such sets of scattering curves are given for the case without and with added copolymer that allow us to follow in detail the structural evolution after mixing the two surfactant solutions. As seen in Figure 1a in the absence of the copolymer, unilamellar vesicles of 10.8 nm radius and with a polydispersity index p of 0.065 are formed (for a TDMAO:LiPFOS 55:45 mixture at 50 mM total concentration) within a few seconds after mixing. However, this formation of very small, well-defined vesicles has the disadvantage that they are only short-lived and quickly age thereafter within some minutes to form much larger and more polydisperse vesicles.[38]

In all cases immediately after mixing, disk-like micelles of radius of 6−7 nm and a thickness of 3.2 nm are formed, which interact *via* electrostatic repulsion manifested by the correlation peak in the scattering curves. The initial intensity increase together with the shift of the correlation peak to lower q characterizes the disk-growth while the occurrence of a minimum and subsequent oscillations (here visible after ∼0.6 s for the case without L35 and ∼20 s for c(L35) = 0.275 mM) marks the formation of vesicles (curves for other L35 content are given in Supporting Information, Figures S1−S6). For the L35 containing sample this increase in intensity at low q occurs much longer and is much more pronounced, which indicates that here a longer growth time leads to much larger structures. For the case of 0.275 mM L35 the initially formed vesicles have a radius of 36 nm and a polydispersity index p of 0.055, which is an extremely narrow size distribution as evidenced by the well-defined oscillations in the scattering curve. On a much longer time scale the vesicles undergo an aging process that leads to a somewhat broader distribution of vesicle sizes, but p still remains rather well-defined. Generically the evolution of the scattering curves is similar, but upon copolymer addition the evolution is slower, vesicle formation (as seen by oscillations in the scattering curves around 0.2−0.4 nm^{-1}) occurs later and the resultant vesicles are larger (the minimum moves to lower q) and very well-defined with respect to their size distribution. This shift to slower transformations and larger sizes is proportional to the amount of added copolymer, as seen in the corresponding time-series of SAXS curves (Figure 5b, Supporting Information, Figures S1−S6).

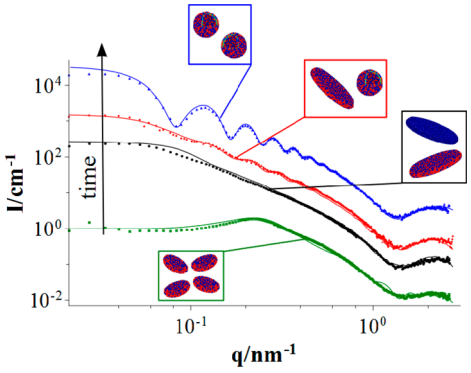

Figure 2. Examples for analysis and fits of the SAXS curves (successive curves have been multiplied by a factor 10 for better clarity) for the case of 0.275 mM L35. Curves for times and fit parameters of subsequent curves: t = 0.01 s, A = 1.0, R_{disk} = 6.4 nm; t = 10.86 s, A = 1.0, R_{disk} = 33.0 nm; t = 20.45 s, A = 0.77, R_{disk} = 35.0 nm, R_{ves} = 36 nm; t = 106.7 s, A = 0.08, R_{ves} = 36.0 nm, p = 0.07.

A detailed analysis of the scattering curves was done by decomposing the scattered intensity as the sum of the contributions from disk-like micelles ($I_{disk}(q)$) and vesicles $I_{ves}(q)$:

$$I(q) = A\Phi I_{disk}(q, R_{disk}, D) + (1 - A)\Phi I_{ves}(q, R_v, D, p_v) \quad (2)$$

where Φ is the total volume fraction of dispersed material and A is the fraction of it contained in micellar disk form. Such an analysis allows the deduction of detailed structural information and yields R_{disk}, D, R_v, and p_v, which are disk radius, bilayer thickness, vesicle radius, and polydispersity index of the vesicle radius, respectively (details for $I_{disk}(q)$ and $I_{ves}(q)$ are given in

the Supporting Information). Figure 2 shows an example for the quality of the fits for the various stages of the disk-to-vesicle transition. Despite sticking to a minimum set of free parameters, the data for the structural evolution are described very well by the model given by eq 2 and allow the deduction of the structural parameters of the aggregates and the amounts contained in the form of disks or vesicles with good precision. It should be noted that we always worked by taking into account properly the absolute scattering intensity, and our modeling is rather minimalistic by restricting ourselves to a minimal set of adjustable parameters; as we kept the bilayer thickness of disks and vesicles identical and fixed the polydispersity of the disk radius to 10% (allowing it to float freely would, of course, increase the fit quality but reduce the reliability of the other parameters, and the 10% corresponds to the average seen in these fits).

The deduced disk radius as a function of time, together with the fraction contained in the disk state are given in Figure 3 for different amounts of L35 content (the results of this analysis for the additionally measured mixing ratios is given in Supporting Information, Figures S7 and S8) and shows quantitatively the prolonged disk growth upon addition of the copolymer L35. In parallel the change of the fraction *A* quantifies that

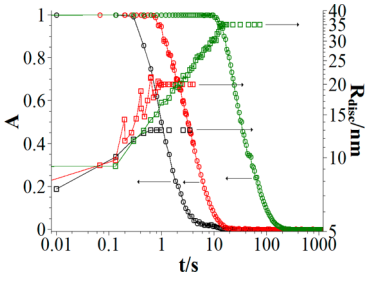

Figure 3. Time evolution of disk radius R_{disk} (open squares) and disk fraction *A* (open circles) as a function of time for three Pluronic L35 concentrations of 0 mM (black), 0.0275 mM (red), and 0.275 mM (green) (it should be noted that for long times R_{disk} was set to the maximum value achieved, as for small amounts contained in the vesicle/disk mixture an independent fitting of this parameter is not any more reliable).

the transition to vesicles occurs correspondingly later and correspondingly larger vesicles are formed. Looking at the results obtained for all samples investigated (Figure S7) it is clear that the transformation to vesicles occurs progressively slower with increasing L35 concentration. At the same time it is interesting to note that the rate of growth (and correspondingly the disk size as a function of time; see Figure S8) does not depend much on the amount of copolymer contained, except for the highest content of 2 mol % L35, where apparently some retardation of the vesicle growth is observed. The identical growth rate for lower copolymer content indicates that the growth process itself proceeds in a similar fashion, it just takes longer until the sudden instability of the disks is reached.

In summary, the vesicle formation process is well described by the scheme given in Figure 4, which summarizes schematically our findings. One always observes a growth of the disk-like micelles by fusion of disks. Beyond a certain size (given by eq 1) they become suddenly unstable, but this point of instability is shifted to much larger sizes by the presence of the copolymer that presumably attaches to the rim of the growing disks, thereby reducing their line tension Λ and making them more stable, which then leads to larger sizes eq 1. In contrast the mean bending modulus κ can be expected to be constant as the bilayer itself should be little affected by the presence of the relatively small amounts of copolymer incorporated, which means that Λ is reduced by up to a factor of 4 by the presence of the copolymer (assuming a reasonable value of 5 kT for $2\kappa + \bar{\kappa}$ a reduction of Λ from 1.0 to 0.25 kT/nm would take place).

This formation mechanism controls the structure of the formed vesicles and Figure 5a gives the scattering curves of freshly formed vesicles, *i.e.*, after complete transformation from disks to vesicles, for various content of copolymer in the surfactant mixture. With increasing copolymer content the characteristic minimum of the scattering intensity moves to lower *q*, thereby showing the corresponding increase in size. The vesicle radii R_v and polydispersity index *p* obtained by the quantitative analysis for the same surfactant

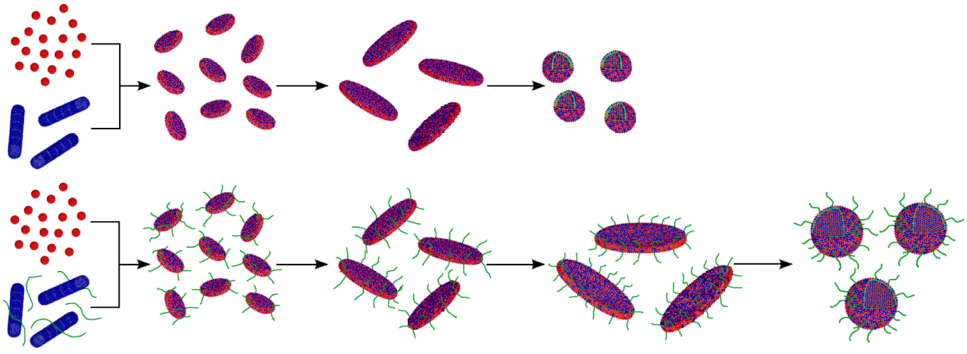

Figure 4. Mechanism of the formation of monodisperse vesicles for the case of the pure surfactant system (top) and for addition of amphiphilic copolymer (bottom).

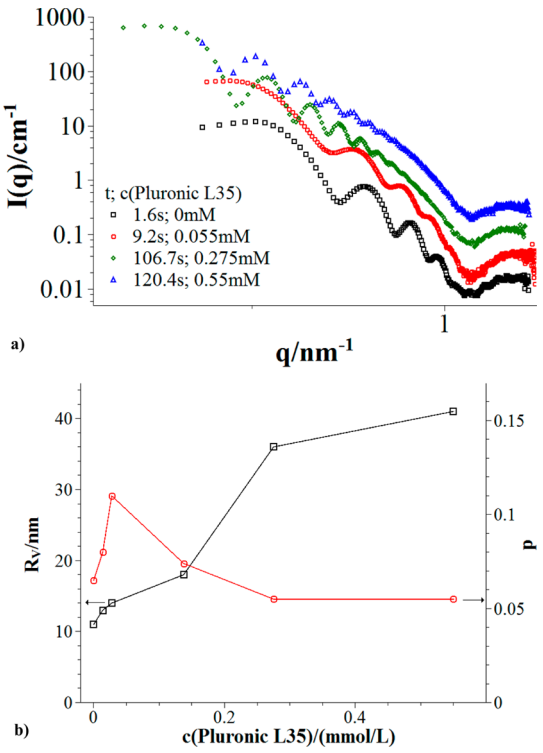

Figure 5. (a) SAXS intensities $I(q)$ as a function of the scattering vector q of nascent vesicles for different polymer contents. (b) vesicle radius R_v (open squares) and polydispersity index p (open circles) for the initially formed vesicles as a function of copolymer L35 concentration in samples composed of 27.5 mM TDMAO/22.5 mM LiPFOS (25 °C).

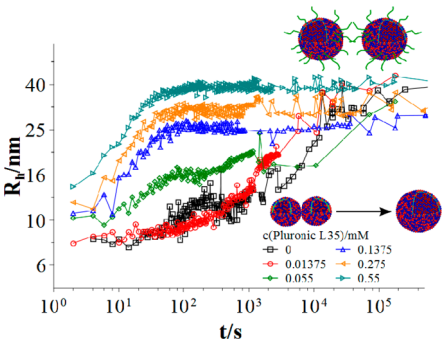

Figure 6. Long time evolution of the vesicles-aging. Hydrodynamic radius R_h obtained from DLS as a function of time for various amounts of added L35 (always TDMAO/LiPFOS = 55:45; c_{tot} = 50 mM). For short times and samples with a polymer concentration larger than 0.055 mM one observes the initial growth of disk-like micelles prior to vesicle formation.

mixture as a function of the amount of added copolymer L35 are given in Figure 5b. It shows very nicely how the radius of the nascent vesicles increases systematically from 11 nm for the pure surfactant mixture up to ~41 nm for a content of 0.55 mM L35. At the same time for the copolymer containing vesicles p is typically around 0.05−0.07, which means that here always vesicles with a very narrow size distribution are formed. Accordingly by adding small amounts of copolymer (0−0.55 mM; compared to 50 mM surfactant) we can control the size of the vesicles over a large size range in a reliable fashion thereby forming very monodisperse unilamellar vesicles. For the addition of very small amounts of L35 an unexpected maximum of p is observed, which might be related to the fact that here only on the order of about one polymer molecule per initially formed disk is contained and thereby the growth process might not proceed in a very homogeneous fashion.

Yet the main shortcoming of the structural definition of the initially investigated zwitanionic surfactant mixture was its subsequent, relatively rapid aging which yielded much less well-defined vesicles after some minutes. This is a general shortcoming of unilamellar vesicles as for most cationic vesicle systems studied so far one observes similar aging processes.[39] However, an investigation of the long-time behavior of the formed vesicles by means of DLS (dynamic light scattering) measurements shows that the subsequent aging can be modified to a large extent by the copolymer addition, becoming much slower with increasing copolymer content (Figure 6). This effect of enhanced stability is very pronounced already for the addition of very small amounts (~0.05 mM L35, *i.e.*, one copolymer molecule per 1000 surfactant molecules or equivalently 50 copolymer molecules per vesicle) by enlarging the stability time drastically from 20 s to more than several days, that is, by a factor of 10000. For samples with c(L35) > 0.1 mM the aging was even suppressed effectively over the whole maximum observation period of about 40 days, which means that by the copolymer addition short-lived well-defined vesicles have been transformed to long-time stable self-assembled colloidal entities. This is certainly a very striking effect and may be related to a substantial steric stabilization induced by the presence of the copolymer in the vesicle bilayers. However, a further reason for this much prolonged stability of the copolymer containing vesicles might be that these larger vesicles are closer to their preferred spontaneous curvature and therefore already much closer to a situation favored by the bending energy.[40]

CONCLUSION

In this work we studied the effect of added amphiphilic copolymers on the spontaneous formation of well-defined vesicles by state-of-the-art stopped-flow SAXS experiments. The quantitative analysis shows that after mixing, small disk-like micelles are always formed, that grow subsequently in size until they reach a critical size, determined by the ratio of bending constant and line tension, beyond which they become unstable and are swiftly transformed to unilamellar vesicles. By admixing small amounts of amphiphilic copolymer (on the order of one copolymer molecule per 1000 surfactant molecules) we can modify the

formation process in a desired way. The incorporation of the block copolymer into the rim of the intermediately formed disk-like micelles stabilizes them by lowering the line tension. This leads to a prolonged growth time that is proportional to the amount of copolymer contained. Subsequently, very monodisperse vesicles are formed, whose size is proportional to the copolymer content. Particularly striking in that context is the extremely low polydispersity of spontaneously formed vesicles as it that has not been observed before. More importantly, the added copolymer also stabilizes the unilamellar vesicles (which are formed under kinetic control) against subsequent aging, thereby increasing the characteristic time of aging that takes place in some minutes for the vesicles without copolymer by at least 4−5 orders of magnitude and for higher copolymer content even beyond our maximum observation window of 50 days. This drastic increase in stability might simply be due to kinetic stabilization of the vesicles by the presence of the copolymer but is potentially also related to the fact that these larger vesicles are closer to their preferred spontaneous curvature.[40]

This much higher stability means that instead of having these very well-defined vesicles formed by kinetic control for some seconds we can retain them now for days or even many weeks. This completely changes their potential for applications as for most of them, such as delivery vehicles or as templates for fabricating nanostructured materials, colloidal stability for at least several hours is essential. In the past, vesicles have been employed as templates for the formation of hollow silica nanoparticles,[41,42] but that has necessarily been done on much more polydisperse vesicles, which could now be changed in future experiments.

As our approach is based on a general concept for employing amphiphilic copolymers during the formation process, this finding should be generally applicable to catanionic and zwitanionic surfactant systems, hence to a large class of relevant systems. Therefore this novel path of forming and shaping vesicles is expected to be of fundamental impact on many future applications where such vesicles of well-defined size and structural properties are required.

EXPERIMENTAL SECTION

Sample Preparation. Lithium perfluorooctyl sulfonate (LiPFOS, $C_8F_{17}SO_3Li$, M_w = 506.06 g/mol, cmc = 6.3 mM[43]) was from TCI Europe (purity >96%) and used without further purification. Tetradecyldimethylamine oxide (TDMAO, $C_{14}H_{29}N(CH_3)_2O$, M_w = 257.46 g/mol, cmc = 0.12 mM[44]) was obtained by freeze-drying Ammonyx M (gift from Stepan Europe). The water content of the freeze-dried TDMAO was determined by Karl Fischer titration with a Titrando 836 (Metrohm AG) to be <1 wt %. Pluronic L35 ($EO_{11}-PO_{16}-EO_{11}$) was a gift from BASF. The surfactant stock solutions and surfactant−polymer stock solutions were prepared by dissolving the solid surfactants and the polymer in doubly filtered Millipore water.

Stopped-Flow Small-Angle X-ray Scattering (SAXS) Experiments. In each run the mixtures were prepared in a stopped-flow device (BioLogic SFM-400) from a premixed 50 mM TDMAO/L35 solution to which a 50 mM LiPFOS solution was admixed in a ratio 55:45. The total mixing volume was 600 μL and the flow rate was 6 mL/s (dead volume: 50 μL). Measurements were started simultaneously with the mixing procedure so that the first acquisition was performed during mixing, which allowed a check on the reproducibility of mixing. All kinetic times are calculated with respect to the time when the hard stop was closed (the kinetic or aging time then increases from the dead time of 2.3 ms). Structural changes were followed by highly time-resolved SAXS on ID02 at the ESRF (Grenoble, France)[45] for a total time of up to 20 min. Two instrumental set-ups were used with sample−detector distances of 2 and 4 m and a wavelength of 1 Å, thereby covering a q-range of 0.02−2.7 nm^{-1}. To ensure reproducibility of the experiment and to achieve good time-resolution of the process the scattering of every mixture was monitored over a time range of 20 min with low time resolution and the mixing was monitored again over a shorter time range of a few seconds with high time resolution. SAXS-data were recorded using a high sensitivity fiber optically coupled FReLoN (fast read low noise) Kodak CCD detector, with a nominal dynamic range of 16 bit, in 4 × 4 binning (resolution 512 × 512) at a read out rate of 5 frames/sec and with exposure times of 5 or 10 ms. All scattering patterns were corrected for the CCD dark counts and detector response function and normalized to an absolute intensity scale. The azimuthal averages of the normalized images were calculated using BerSANS,[46] and the background scattering from the solvent water was subtracted by an in-house developed Matlab-application. The data analysis was done with the new developed Matlab based tool SASET for analyzing series of small angle scattering data.

Dynamic Light Scattering (DLS). DLS measurements with a high time resolution (2s) (up to t_{max} = 30 min) were performed in a combined stopped-flow-DLS apparatus. Here a diode pumped Nd:YAG laser with a wavelength of 532 nm (20mW, Compass 215M-20, Coherent) was used, the scattered light was collimated by a combination of a fiber collimator (60FC, Schäfter + Kirchhoff) and a single mode glass fiber cable (SMC, Schäfter + Kirchhoff) and then recorded by a single photon counting module (Count-250C-FC, Laser Components). We used the Flex02-1D digital correlator to process the signal. Measurements over longer time periods were performed at 90° scattering angle with an ALV/CGS-3 goniometer system with a 22 mW He−Ne laser (λ = 632.8 nm) and employing a pseudo-cross-correlation. The samples were prepared 30 s before the first measurement by mixing adequately chosen volumes of two surfactant stock solutions and homogenized by vigorous shaking. The samples were thermostatted at 25 °C in a toluene bath during the measurements.

Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: Details of the analysis of the scattering data, additional scattering curves (Figures S1−S6), and the results of their analysis (Figures S7−S8). This material is available free of charge *via* the Internet at http://pubs.acs.org.

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