Self-Assembly

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Responsive Vesicles from Dynamic Covalent Surfactants**

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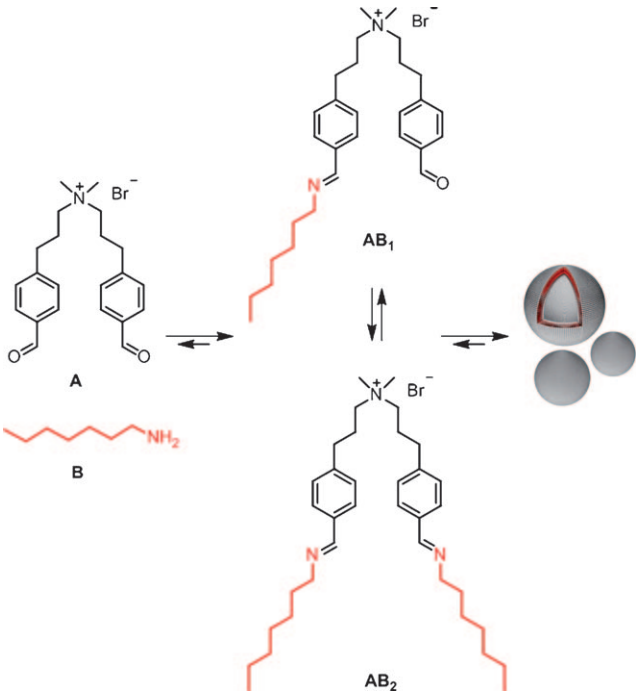
Herein we describe the use of dynamic covalent surfactants to create dynamic vesicles that are highly responsive to changes in their environment. Synthetic vesicles have drawn much attention because of their close similarity to biological cells, of which the membranes are generally composed of double-tailed phospholipid surfactants. In living systems, bilayer membranes are highly dynamic,[1] amongst others as a result of embedded proteins and carotenoids, which is essential for endo- and exocytosis, cell signaling, and cell division. In contrast, synthetic vesicles tend to be very static, especially when composed of double-chain surfactants.[2,3] A few specific exceptions exist, including dynamic systems comprised single-tailed surfactants, thus forming vesicles with critical aggregation concentrations (CAC) several orders of magnitude higher than those of their double-chain surfactant analogues. The building blocks for these systems are limited to surfactants with relatively short tails,[4,5] and vesicles are only formed within narrow pH windows.[6] Morphological transitions have been effected using surfactants that are capable of covalent structural modification in an aggregate environment. However, these transitions tend to be unidirectional, and do not allow formation from and reversal to isotropic solution.[7] The low solubility of double-chain surfactants limits spontaneous vesicle formation in water, making sonication, film hydration, and solvent injection techniques necessary to induce vesicle formation. Double-chain surfactant systems that are capable of switching reversibly between a non-aggregated state and an aggregated, vesicular state remain largely unexplored.

To change the dynamics of bilayers and vesicular assemblies, we opted to enhance the dynamics of formation and dissociation of their surfactants through the introduction of a reversible covalent bond. This should render these surfactants, and thus their assemblies, responsive to changes in their environment. The reversible and dynamic nature of the dynamic covalent bond has proven to be a powerful tool for the construction of adaptive and reversible systems[8] and already led to a variety of supramolecular aggregates like gels,[9] nanorods,[10] and micelles.[11,12]

Herein we report on the formation of dynamic vesicles, in which the responsiveness of the bilayer membrane is regulated by controlling the reversible formation of its amphiphilic surfactant constituents. For this approach, we designed a double-tailed surfactant that can form from two water-soluble, not surface-active precursors by the formation of a reversible covalent bond. As surfactant headgroup, we synthesized cationic bisaldehyde **A**, which we mixed with an apolar amine-functionalized tail **B** in water, creating cationic surfactants in situ from water-soluble precursors through the formation of reversible imine bonds. It was expected that both single-tailed $\mathbf{AB_1}$ and double-tailed $\mathbf{AB_2}$ surfactants are formed, which are in equilibrium with each other and with both precursors (Scheme 1).



Scheme 1. Dynamic formation of imine vesicles. Imine surfactants $\mathbf{AB_1}$ and $\mathbf{AB_2}$ were prepared in situ by mixing **A** and **B** in water.

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After mixing clear solutions of the precursors in water ($[\mathbf{A}]_0 = 10$ mM, $[\mathbf{B}]_0 = 20$ mM, solution pH $= 9.5 \pm 0.2$ because of the added amine; $[\mathbf{A}]_0$ and $[\mathbf{B}]_0$ denote initial concentrations), the solution turned opalescent within minutes, indicating aggregate formation. Dynamic light scattering (DLS) experiments showed a bimodal distribution with hydrodynamic radii of (95 ± 11) nm and (5.2 ± 1.6) μm in water (Figure 2 in the Supporting Information). The aggregate morphology was investigated by confocal laser scanning microscopy (CLSM) and cryo-transmission electron microscopy (Cryo-TEM). CLSM studies showed the presence of 2–5 μm vesicles (Figure 1 a), and 50–100 nm unilamellar vesicles were found by Cryo-TEM (Figure 1 b), which is in good agreement with the results of the DLS measurements.

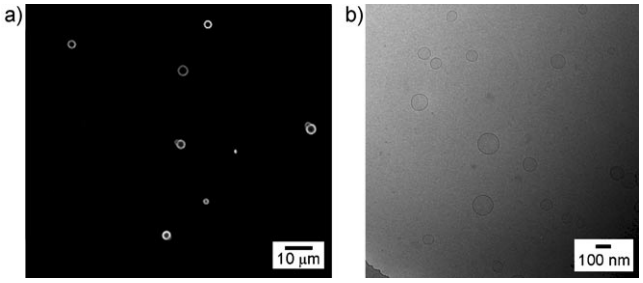

Figure 1. a) CLSM image of **A·B** in water, showing 2–5 μm vesicles. Hydrophobic Nile Red fluorescent probe (10 μM) was used for visualization of the bilayer membrane. b) Cryo-TEM shows the 50–100 nm vesicle population of **A·B**. Conditions: $[\mathbf{A}]_0 = 10$ mM, $[\mathbf{B}]_0 = 20$ mM, pH 9.5, 25 °C.

Imine formation was confirmed for an **A·B** vesicle solution ($[\mathbf{A}]_0 = 10$ mM, $[\mathbf{B}]_0 = 20$ mM; **A·B** represents an unspecified mixture of **A** and **B**) by LC-MS analysis, thus showing formation of both single- ($\mathbf{AB_1}$) and double-tailed ($\mathbf{AB_2}$) surfactants. For quantification, it was found necessary to quench the imine exchange of the equilibrated mixture by using $NaBH_4$, as such reducing **A**, $\mathbf{AB_1}$, and $\mathbf{AB_2}$ to their corresponding nondynamic alcohols and amines (see Table 1 in the Supporting Information). 45 % of **A** was converted into imine surfactants, of which the double-tailed $\mathbf{AB_2}$ was the major constituent ($\mathbf{AB_1}$: 14 %; $\mathbf{AB_2}$: 86 %; see Figure 3 in the Supporting Information). The CAC (herein defined as minimal precursor concentration needed for aggregate formation when **A** and **B** are mixed in a 1:2 ratio) of $[\mathbf{A}]_0$ was determined to be 0.4 mM using fluorescence spectroscopy with the hydrophobic Nile Red probe (Figure 4 in the Supporting Information). By measuring the surface tension a CAC of 1.7 mM was determined (Figure 5 in the Supporting Information). The fact that the CAC determined by fluorescence spectroscopy is significantly lower than the one determined by surface tension can be explained by the induction of hydrophobic interactions by the Nile Red probe.[13]

Since the vesicles are formed by reversible association of their non-amphiphilic precursors, we expected that a shift of the imine equilibrium should lead to a fast response of the vesicle bilayer. After mixing solutions of both precursors in water, vesicles were formed within a few minutes, exemplified

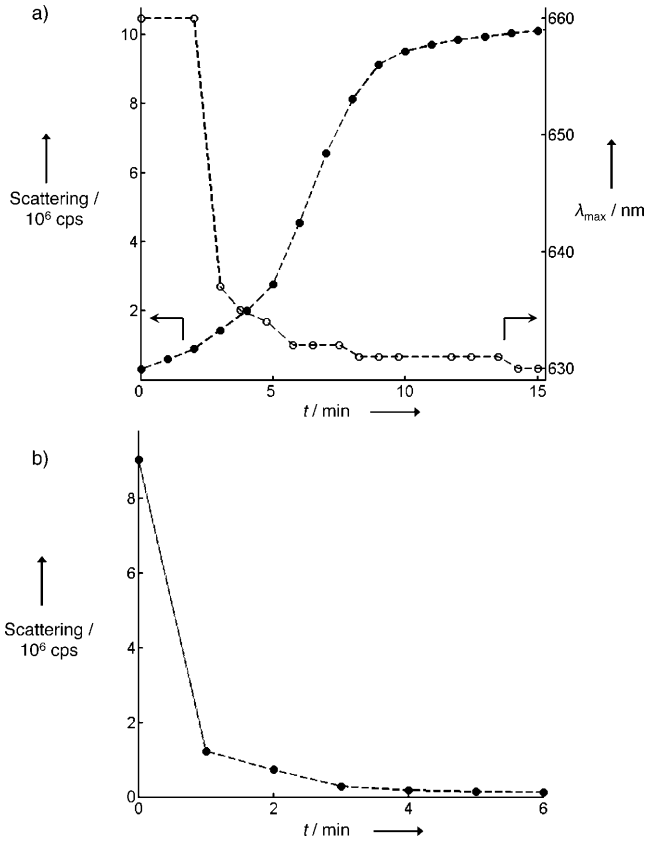

Figure 2. a) Vesicle association in time followed by DLS (●) and fluorescence spectroscopy, in which 1 μM Nile Red was used as fluorescent probe (○); precursors **A** and **B** were mixed in water, thus forming **A·B** vesicles ($[\mathbf{A}]_0 = 10$ mM, $[\mathbf{B}]_0 = 20$ mM). b) Vesicle dissociation followed in time with DLS; a vesicle solution ($[\mathbf{A}]_0 = 10$ mM, $[\mathbf{B}]_0 = 20$ mM) in water was diluted 100 times.

by a rapid increase in scattering intensity, and a concomitant blue shift of Nile Red fluorescence, indicating the formation of hydrophobic domains (Figure 2 a).

CLSM measurements showed that individual vesicles are stable for at least 50 minutes (Figure 6 in the Supporting Information). However, diluting an opalescent **A·B** vesicle solution in water 100 times ($[\mathbf{A}]_0 = 10$ mM, $[\mathbf{B}]_0 = 20$ mM) resulted in a transparent solution, thus suggesting that all vesicles had dissociated into their surfactant precursors. Dilution was followed by a rapid decrease in scattering intensity with a timescale in the order of one minute (Figure 2 b). Fluorescence emission measurements showed an emission shift from 630 nm to 660 nm; the latter corresponds to Nile Red emission in pure water and thus to the absence of hydrophobic domains.[13] A shift of the imine equilibrium towards the surfactant building blocks was confirmed by ^{1}H NMR spectroscopy. This behavior is in sharp contrast to that observed in both phospholipid and conventional double-tailed quaternary ammonium surfactant membranes, where the bilayers have a very high stability and associated slow dissociation kinetics upon dilution, related to their characteristic nano- to sub-nanomolar CACs.[2,14]

Vesicle dissociation was investigated in more detail by CLSM. We found that upon dilution otherwise stable vesicles

fuse, forming larger vesicles (Figure 3a). Then, the interfacial membrane thins to rupture, much in the same way as is observed for fusing soap bubbles. In the meantime, after fusion, the initially larger vesicle

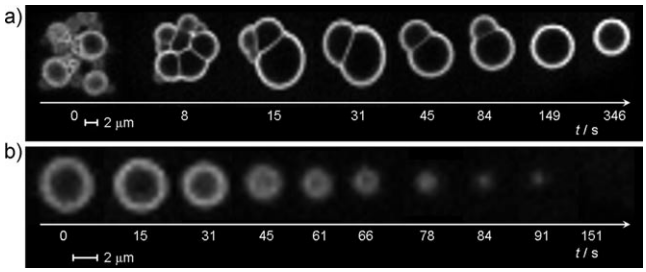

Figure 3. Vesicle dissociation followed in time with CLSM: a) adjacent vesicles fuse to form larger unilamellar vesicles which then start to shrink; b) single vesicles shrink until no longer visible. Conditions: 10 mM $[A]_0$ and 20 mM $[B]_0$, pH 9.5, 25 °C. Fluorescent probe: 10 μM Nile Red. Typically 50 μL samples were used and diluted with 90 μL 10 mM KBr solution containing 10 μM Nile Red. No significant pH shift was measured ($\Delta pH \leq 0.2$) upon dilution.

or dissolves completely, depending on the final concentration (Figure 3b). The decrease in size of the single vesicles also took place on a timescale in the order of one minute (Figure 7 in the Supporting Information), which is in good agreement with the results of the DLS measurements.

For the mechanism of vesicle dissociation, it is assumed that for vesicle-forming surfactants $\mathbf{AB_2}$, the concentration of nonaggregated surfactant in bulk is generally negligibly low and the exchange between bilayer and bulk phase is very slow (Figure 4).[2] Lowering the concentration of the surfactant precursors in the bulk surroundings by dilution drives the dissociation of the dynamic surfactants, in accordance with Le Châtelier's principle. Because of the low exchange rates

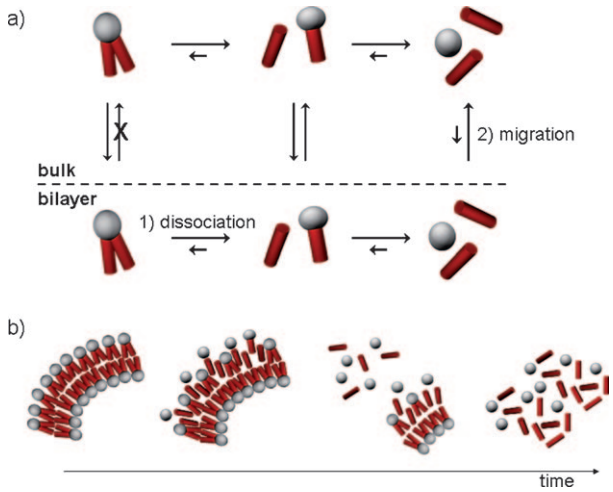

Figure 4. Proposed mechanism of vesicle dissociation upon dilution: a) surfactants in the bilayer dissociate into their precursors (1). Subsequently, the precursors migrate from the bilayer to the bulk (2); b) bilayer destabilization and shrinkage over time. Grey: cationic headgroup; red: apolar tail.

between bilayer and bulk for double-chain surfactants, it seems likely that the double-chain surfactants in the bilayer first dissociate into their precursors. Subsequently, the surfactant precursors migrate from the bilayer to the bulk solution. Because dilution initially decreases the precursor concentrations in the external bulk solution, it is likely that dilution first leads to the loss of surfactants from the outer bilayer leaflet, leading to enhanced susceptibility towards fusion between nearby vesicles. At later stages, the bilayer stability might further decrease as a result of a larger discrepancy between the outer and inner leaflet, leading to enhanced permeability and/or surfactant flip-flop, and ultimately shrinkage of the vesicles as a whole.

Comparison of the rate of vesicle dissociation with the rate of dissociation of a water-soluble, non-aggregating imine upon dilution shows similar time scales (Figure 8 in the Supporting Information), indicating that aggregation does not contribute significantly to the kinetic stability of the aggre-

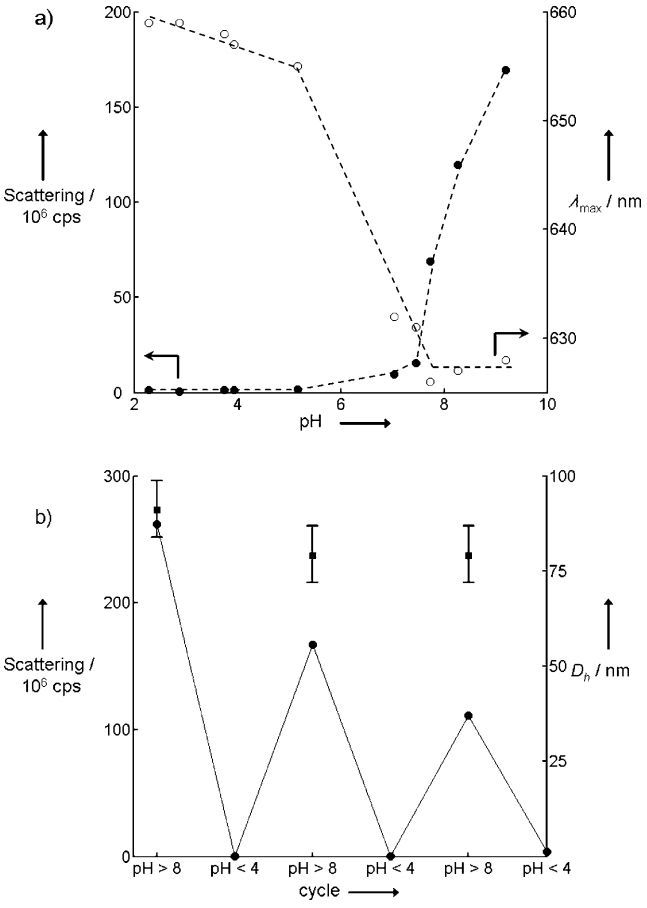

Figure 5. pH-triggered vesicle dissociation ($[A]_0 = 20$ mM, $[B]_0 = 40$ mM): a) an **A·B** vesicle solution in water (pH 9.5 ± 0.2) was acidified by addition of an aqueous HBr solution. Vesicle dissociation was studied with DLS (●) and fluorescence spectroscopy (○), in which 1 μM Nile Red was used as fluorescent probe; b) DLS (●) showed that vesicle dissociation of **A·B** is reversible going from alkaline (pH > 8) to acidic (pH < 4) pH values. The vesicle size (■), as determined by DLS methods, remained constant after each cycle. It should be noted that the scattering intensity at alkaline pH values decreased after each cycle, which is caused by dilution effects (Figure 9 in the Supporting Information).

gated imine surfactants. The similarity in time scales between imine dissociation and vesicle shrinkage and dissolution suggests that imine dissociation in the bilayer is the rate-determining step. Overall, continuous surfactant dissociation and subsequent diffusion of precursors to the bulk result in destabilization of the bilayer and shrinkage of the vesicle (Figure 4).

As an alternative means of control, vesicle response to a change in pH was investigated. We titrated a concentrated **A**·**B** vesicle solution in water with an aqueous HBr solution and found that the vesicles remained stable down to pH 7.1 ± 0.2 as observed with DLS and fluorescence spectroscopy (Figure 5a).

At pH < 4 the vesicles had completely dissociated resulting from a gradual shift of the imine association equilibrium towards its precursors as was evident from a significant decrease in scattering and a red shift of the Nile Red probe from 637 nm to around 655 nm (Figure 5a). Vesicle dissociation was entirely reversible, demonstrated by a significant increase in scattering and regeneration of the original vesicular size when the pH value of the solution was changed back to the alkaline state and vice versa (Figure 5b and Supporting Information).

In summary, we have shown how the introduction of dynamic covalent bonds in double-tailed surfactants can lead to the formation of vesicles that are highly responsive to changes in their environment. The surfactants are formed in situ from two water-soluble, not surface-active precursors by the formation of a reversible imine bond. This dynamic covalent imine bond results in vesicle assemblies with a highly dynamic bilayer. Complete vesicle dissociation is realized by dilution and change of the pH value. This study represents a new approach for accessing dynamic vesicular architectures, which are highly interesting candidates for dynamic encapsulation and release vehicles because of their ease of formation, fast dissociation, and switchable morphology.

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