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Enzyme-Responsive Molecular Assembly System with Amylose-Primer Surfactants

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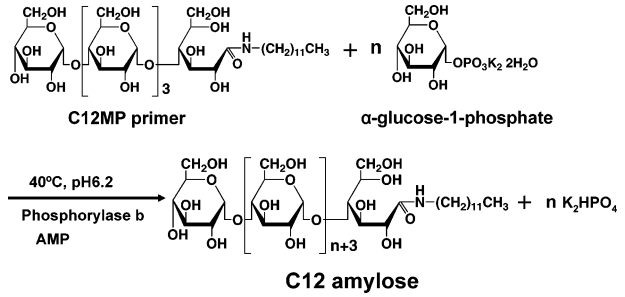
Stimuli-responsive molecular assembly systems have attracted considerable attention in the fields of biotechnology and drug delivery systems.[1] Typically, the external stimulus is heat,[2] pH,[3] light,[4] or the addition of molecules, such as glucose[5] or cyclodextrin.[6] Enzymes are also used to trigger the response of a system.[7] We report here new enzyme-responsive micellar systems in which an enzymatic reaction controls the amphiphilicity of the surfactants. The surfactants in our study consist of a short alkyl chain and a malto-oligomer as a primer which can be synthesized enzymatically.[8] In the presence of phosphorylase and α-D-glucose-1 phosphate (G1P), the elongation reaction of the saccharide chain proceeds from the non-reducing 4-OH terminus of the (α,1→4)-glucan chain. Thus, we first prepared amylose-primer surfactants (C8MP, C12MP, C16MP) where an alkyl group (C8, C12, C16) is linked to the reduced terminus of maltopentaose (MP, number of glucose units (GU) = 5). The surfactants form micelles in water, which are dissociated upon the enzymatic elongation reaction of the sugar moiety. We demonstrate here that by using this property the micelle-to-vesicle transition of mixed lipid/ primer systems can be controlled, and this presents a new method for the reconstitution of trans-membrane protein to liposome.

Amylose-primer surfactants (C8MP, C12MP, C16MP) were synthesized by reacting maltopentaose lactone and octyl-, dodecyl-, or hexadecylamine. The critical micelle concentration (CMC) of each primer, measured by surface tensiometry, was 10 mM (C8MP), 0.5 mM (C12MP), and 0.05 mM (C16MP) (Figure S1). G1P (100 mM), phosphorylase b (0.26 μM), and adenosine 5′-monophosphate sodium salt (AMP; 10 mM) were added to the primers in a Bis-tris buffer (100 mM, pH6.2) solution and were polymerized enzymatically at 40 °C (Scheme 1).

The initial rate of polymerization (v) was determined by the phosphorus analysis of orthophosphate, which is one of the products during polymerization (Figure S2). The rates of C12MP (vC12) and C8MP (vC8) above CMC (C12MP = 1.0 mM, C8MP = 20 mM) significantly increased in comparison to that of unmodified MP (vC12/vMP = 3.4, vC8/vMP = 1.6), while below CMC (C12MP = 0.1 mM, C8MP = 1.0 mM), the rates were comparable to that of MP (vC12/vMP = 1.1, vC8/vMP = 1.0). In contrast, the rate of C16MP (vC16) was much slower than that of MP above CMC (1.0 mM) (vC16/vMP = 0.28), and it was almost the same as that of MP (vC16/vMP = 1.0) around CMC (0.1 mM). Due to the limited sensitivity of this method, we could not determine the rate of C16MP below CMC (<0.05 mM). The reactivity for the alkyl primers probably does not change so much because the

Scheme 1. Enzymatic Polymerization of C12MP Primer



reaction proceeds from the non-reducing 4-OH terminus of glucan chain. The state of association of the primers may affect enzymatic polymerization.

To obtain further information about the higher reactivity in C12MP micelle, the enzymatic polymerization of C12MP primer (1.0 mM) was monitored as a function of time (Figure 1). The reaction showed two phases as a function of an increasing degree of polymerization (vC12 (Phase I) /vMP = 3.4, vC12 (Phase II) /vMP = 0.84). At the onset, polymerization proceeded rapidly, but then slowed drastically after 150 min (GU = 17). The surface tension of the reaction solution was measured as a function of polymerization under the same conditions. It also showed two phases as a function of time: an initial rapid increase and, after 150 min, a leveling-off to a value identical to that of the buffer solution in the absence of C12MP. A sample (after dialysis and freeze-drying) of C12 amylose that was polymerized for 2 h was analyzed by ^{1}H NMR, TOF MS **(**Figure S3**)** and X-ray diffraction **(**Figure S4**)**. The GU of C12 amylose was almost the same (phosphorus analysis: 16.4 ± 3.7, NMR: 16.0 ± 2.7, and MS: 15 ± 3). The MS spectrum showed that the elongated amylose chain of C12 amylose had a quite narrow distribution. In the XRD spectrum, the peak of C12 amylose showed a weak V6-I-type helix similar to the peaks characteristic of the amylose (GU = 30)/lauric acid complex.[9] We believe that in the isolated product the C12 alkyl group of C12 amylose partly forms intra- or intermolecular complexes with the amylose chain to give inclusion complexes of a helical structure.

Since the hydrophilicity of the C12MP molecules increases with an increase in the degree of polymerization, it is expected that the CMC of the product increases as well, and that eventually the micelle will dissociate. This dissociation occurs after the CMC reaches a value of 1.0 mM in the condition in Figure 1. These data suggest that the reaction in C12MP micelles proceeds very quickly due to the local concentration effect of the substrate (primer) on the enzymatic reaction. Although the reason for low reactivity of C16MP miceller system is not yet clear, one plausible explanation is the slow dynamics in the escape of primers from the micellar

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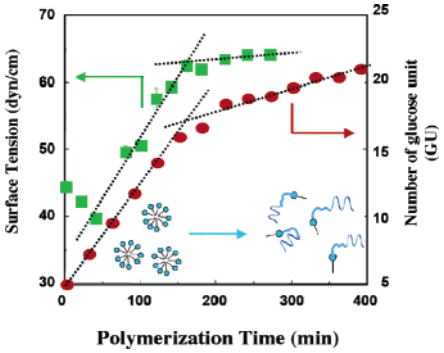

Figure 1. Degree of polymerization and surface tension of C12MP (1.0 mM) as a function of time. Enzymatic polymerization was performed in the presence of G1P (100 mM), phosphorylase b (0.26 μM), and AMP (10 mM) at 40 °C, pH 6.2.

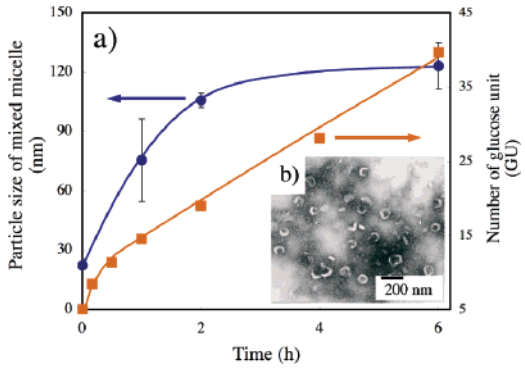

Figure 2. (a) Time course of the change in diameter of DPPC/C12MP liposome by enzymatic polymerization. (b) TEM images of DPPC liposome formed by 6 h of enzymatic polymerization of C12MP. Enzymatic polymerization was performed in the presence of G1P (100 mM), phosphorylase b (1.3 μM) and AMP (10 mM) at 40 °C, pH 6.2.

surface to react with the enzyme because of the higher hydrophobicity of C16MP.

We investigated how the micelle-to-vesicle transition in phospholipid-primer surfactant systems can be controlled by an enzymatic stimulus. A mixed micelle of L-α-dipalmytoyl phosphatidylcholine (DPPC) and C12MP had a diameter of 21.1 nm by DLS. Upon enzymatic treatment of the mixed-micelle solution, the size of the assemblies increased as a function of the duration of polymerization (Figure 2a). After 6 h of polymerization (GU = 40), the mixture contained liposomes (123.1 ± 11.8 nm) with a narrow size distribution (Figure 2, Figure S4). Approximately 99% of the C12 amylose was removed from the liposome fraction. An increase in the hydrophilicity of the C12 amylose surfactant via polymerization induced release of the surfactant from the mixed micelle, resulting in DPPC liposomes. This new type of micelle−vesicle transition system can be used in the purification of transmembrane proteins, as described below.

Sugar surfactants such as octylmaltose are commonly used as solubilizers of membrane proteins.[10,11] Generally, membrane proteins are reconstituted into liposomes after first being solubilized in the surfactant micelle. In this process, the surfactant is eliminated from mixed micelles of surfactant/lipid/membrane protein by dialysis, hydrophobic adsorption, or chromatography,[11] and finally liposome-reconstituted membrane protein is obtained. This process is time-consuming, and the yield of functional reconstituted membrane protein is sometimes low. Micelle−vesicle transition systems caused by enzymatic polymerization can be effective for the reconstitution and refolding of membrane proteins. We demonstrate this possibility in the case of bacteriorhodopsin (BR), a model membrane protein. BR was solubilized in C12MP micelle, and the BR/C12MP mixed solution was added to a C12MP/DPPC mixed-micelle solution. Subsequently, the enzyme was added to this solution, and after 6 h the mixtures consisted of liposomes with a hydrodynamic radius of 143.4 ± 7.0 nm. To assess the proton pump activity of BR in reconstituted liposomes,[12] the solution was illuminated with a 480-W lamp focused on a 435-nm long path filter, and pumping was monitored using a pH electrode. The BR in the reconstituted liposomes showed a greater light-induced H^+ influx and light responsiveness than the mixed-micelle system (Figure S5). In the post-genome era, molecular chaperone technology is important in protein science, such as in the regeneration of proteins from protein aggregations such as inclusion bodies,[6] and to control the folding or effective reconstruction of a membrane proteins to liposome. Enzyme-responsive micelle−vesicle transition systems offer a new option for the folding and reconstitution of membrane proteins to liposomes.

In conclusion, the association of amylose primer surfactants was controlled by changing the amphiphilicity with a chain-elongation reaction triggered by the addition of phosphorylase. The enzymatic reaction occurs under mild conditions. It is also important to remember that phosphorylase b is activated in the presence of AMP, which can initiate polymerization. Thus, C12MP micelles can be viewed as AMP-responsive systems. These new enzyme-reactive surfactants may be widely applicable not only in colloid chemistry but also in biotechnology such as protein science.

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Supporting Information Available: Full experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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