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COMMUNICATIONS

Published on Web 03/29/2002

Lipase Polystyrene Giant Amphiphiles

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Received December 19, 2001

Amphiphilic molecules, often also called surfactants because of their surface active properties, have been the subject of intensive studies for many years.[1] As part of our studies on the design of new building blocks for the construction of nanosized self-assembled systems we have developed a new class of amphiphilic molecules, that is, giant amphiphiles, which consist of an enzyme headgroup and a single covalently connected hydrophobic polymeric tail (Figure 1).[2]

In the case of low-molecular weight amphiphiles (e.g., phospholipids) studies have shown that the shape of the individual amphiphilic molecule very often determines the structure of the resulting aggregate.[1] Similar rules with respect to shape and structure seem to hold for the more recently introduced class of super-amphiphiles,[3] which are composed of hydrophilic−hydrophobic diblock copolymers. These block copolymers have also been shown to form highly ordered aggregates with morphologies dependent on the block lengths or the presence of inorganic salts[4] or both. In terms of both molecular volume and molecular weight the here-reported giant amphiphiles are considerably larger than their low-molecular weight and polymeric counterparts. We thought it to be of interest to investigate whether this next generation of amphiphiles would behave in the same manner as their super- and low-molecular weight analogues and also form well-defined assemblies in aqueous solution.

In the past a wide variety of polymer/enzyme adducts have been synthesized by more or less randomly coupling an enzyme to a polymeric matrix. To the best of our knowledge the synthesis of a precisely defined, nearly monodisperse enzyme−polymer hybrid, aimed at developing surfactants, has not been reported before. In the following we describe such a hybrid, derived from a lipase and polystyrene, and report on its self-assembling properties in water.

The enzyme headgroup chosen was the lipase B from *Candida antarctica* (CAL B, EC 3.1.1.3, triacylglycerol hydrolase).[5,6] This well-studied 33 kDa lipase catalyzes in aqueous solutions the hydrolysis of esters, and in anhydrous organic solvents, the reverse esterification reaction.[7] CAL B is very stable even in various organic solvents and at high temperatures. A maleimide-functionalized polystyrene of 40 repeat units (PDI = 1.04) was used as the hydrophobic tail. The specific attachment of this polymer to the enzyme was achieved by functionalizing the single disulfide bridge (Cys293-Cys311), which is exposed on the outer surface of the native lipase. The first step of the synthesis of the giant amphiphile was the complete reduction of this disulfide bridge with dithiothreitol (DTT), which was confirmed by titrating the reduced enzyme with Ellman's reagent (see Scheme 1, Supporting Information).[8−12] To study the coupling of maleimides to the resultant free thiol groups, the reduced CAL B (0.25 mM) was incubated with an excess of maleimide (0.6 mM maleimide in 20 mM phosphate buffer, pH 6.8). Titration of the product with Ellman's reagent revealed the presence of one remaining free thiol group, indicating the coupling of only one maleimide per lipase molecule (see Figure 1, Supporting Information).[13]

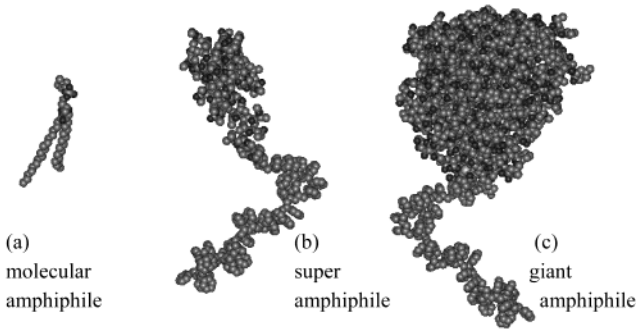

Figure 1. Computer-generated models of (a) a phospholipid representing the class of molecular amphiphiles (molecular volume ∼0.5 nm^3, molecular weight ∼1 kDa), (b) a diblock polymer of polystyrene and a polyisocyanopeptide representing the class of super-amphiphiles (molecular volume ∼6.5 nm^3, molecular weight ∼6 kDa), and (c) the lipase-polystyrene (n = 40) biohybrid representing the new class of giant amphiphiles (molecular volume ∼25 nm^3, molecular weight ∼40 kDa).

The coupling of the maleimide-functionalized polystyrene (0.05 mM, prepared from the carboxy-terminated polystyrene (n = 40) by reaction with $SOCl_2$ and then with maleimide and base, see Supporting Information) to the reduced protein (0.03 mM) was initially performed in aqueous solution (100 mM phosphate buffer, pH 6.8, containing 2 mM EDTA and 150 mM NaCl); however, only ill-defined architectures were observed by transmission electron microscopy (TEM, see Figure 3, Supporting Information).[14]

To obtain more defined structures and carry out the coupling in a more controlled manner, the reaction was carried out at the air−water interface using a Langmuir−Blodgett trough. A chloroform solution of the maleimide-funtionalized polystyrene was spread at the air−water interface, and the reduced enzyme was added to the sub-phase. The reaction was allowed to proceed for 4 h. After compression of the monolayer the pressure−surface area isotherm of the reaction mixture revealed a lift-off area of ∼23 nm^2 (Figure 2, III). When compared to the lift-off areas of the native lipase (12 nm^2, Figure 2, I) and the maleimide-functionalized polystyrene (∼5 nm^2), this increase of molecular area is in agreement with the formation of the giant amphiphile. A blank experiment using carboxy-terminated polystyrene and native CAL B was also performed to ascertain that the lift-off area increase was not due to nonspecific adsorption of the protein to the polymer monolayer. (This experiment resulted in a lift-off area of 7.5 nm^2, Figure 2, II).

A coupling reaction between nonaggregated components was subsequently tried by using a THF/water mixture (90/10 v/v). It has been previously reported that polystyrene (n = 40) remains

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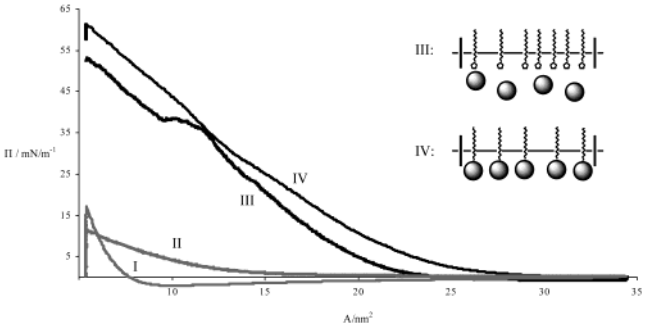

Figure 2. Surface pressure/surface area isotherms recorded in a Langmuir−Blodgett trough using a subphase containing 20 mM phosphate buffer (pH 6.8), $T = 20$ °C. (I) CAL B, (II) reaction mixture of polystyrene-maleimide and native CALB, (III) the giant amphiphile formed in the monolayer, and (IV) the giant amphiphile formed in THF/water system.

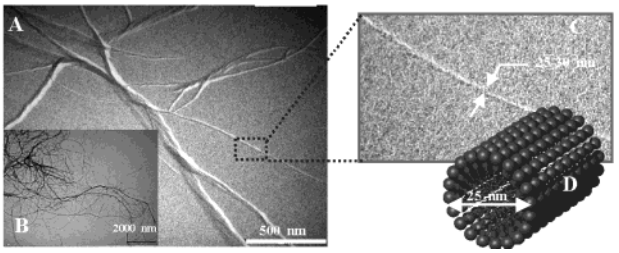

Figure 3. TEM images (Pt shadowing) of the aggregates obtained after mixing reduced CAL B in water with maleimide-terminated polystyrene, in THF/water (9/1 v/v) mixture (A, B). The insert (B) shows μm long bundles of fibers, with the smallest fiber (C) having a diameter of $\sim$ 25 nm corresponding to a micellar rod (D).

monodispersed in THF solutions with up to 10% water content.[15] The reaction was performed in a THF solution of maleimide−polystyrene (0.03 mM) to which the reduced enzyme in water (0.015 mM, 100 mM phosphate buffer, pH 6.8, 2 mM EDTA, 150 mM NaCl) was added to give a final water content of 8%. The reaction was allowed to proceed overnight, and then additional water was slowly added over a period of $\sim$2 h.[16] TEM studies of the resulting reaction mixture revealed the presence of well-defined μm-long fibers (Figure 3A,B). Closer examination of these fibers showed that they were built up from bundles of rods, with the smallest rod having a diameter between 25 and 30 nm (Figure 3C). The size of the smallest rod corresponds closely to the predicted diameter of a micellar architecture formed by the self-assembly of the giant amphiphile (Figure 3D).[17] It is remarkable that these giant amphiphiles exhibit self-assembling properties similar to those of their low-molecular weight counterparts.

These giant amphiphiles were also spread at the air−water interface of a Langmuir−Blodgett trough. The $\sim$28 nm^2 lift-off area measured after compression of the monolayer (Figure 2, IV), further verifies the formation of the biohybrids.

Preliminary catalytic studies were carried out on the self-assembled fibers using the pro-fluorescent substrate 6,8-difluoro-4-methylumbelliferyl octanoate (DiFMU-octanoate). The activities of the native lipase and the reduced lipase were also determined for comparison (see Supporting Information). The lipase retained $\sim$70% of its initial activity after the disulfide reduction step. In contrast, the fibers exhibited only 6−7% of this activity. This decrease in activity can probably be ascribed to destabilization of the active conformation of the enzyme due to the presence of the hydrophobic polystyrene tail, which is attached without the help of a hydrophilic spacer. In addition, upon aggregation of the giant amphiphiles from fibers to bundles of fibers, fewer active sites of the enzyme molecules will remain accessible to substrate molecules.[18]

In conclusion, we have shown that monodispersed and precisely defined giant amphiphiles consisting of an enzyme covalently connected to a single hydrophobic polymeric tail can be prepared and induced to self-assemble in a fashion similar to that of low-molecular weight amphiphiles. Further studies are directed at investigating the influence of different polymer tails (i.e., polystyrenes of different lengths with different hydrophilic spacer groups for attachment to the enzyme) upon the activity and the self-assembling behavior of the giant amphiphiles.

Acknowledgment. This research was supported by the EC TMR Sisitomas and Smarton programs. We thank Dr. J. J. L. M. Cornelissen and Dr. N. A. J. M. Sommerdijk for initial discussions and Novo Nordisk A/S for the generous donation of the lipase.

Supporting Information Available: Experimental Section (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) Thionitrobenzoate (TNB^-), which is formed upon reaction of Ellman's reagent with a sulfhydryl group, has an intense absorption band at 412 nm ($\epsilon = 14150\ M^{-1}\ cm^{-1}$) which allowed an accurate detection of the free sulfhydryls after the reduction step (see Supporting Information).
(13) The addition of only one maleimide is thought to be the result of steric hindrance.
(14) Unlike in the case of low-molecular weight amphiphiles where sonification is often used to aid self-aggregation, this approach could not be used for these molecules, since sonification destroys the enzyme.
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(16) The formation of the giant amphiphile was confirmed by SEC chromatography, electrophoresis, and UV titrations (see Supporting Information).
(17) Calculations using the Israellachvilli's rules predict for the assembly a spherical micellar architecture ($P = 0.04$). These calculations, however, do not allow one to discriminate between spherical and rodlike micelles because the precise volume of the polystyrene tail and the diameter of the enzyme group are not known.
(18) According to simple calculations, if a hexagonal packing is assumed, seven rods when assembled have only 40% of their surface area accessible to substrate molecules. If larger assemblies are formed, this value drops further. Hence, it is not unreasonable to assume that upon aggregation the catalytic activity drops considerably.

JA017809B