Giant Amphiphiles by Cofactor Reconstitution**

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Low molecular-weight amphiphilic molecules are known to form a large variety of self-assembled structures in water, such as monolayers, micelles, vesicles, bilayers, rod- and sheetlike structures, and also helices.[1] Well-defined diblock copolymers—so-called superamphiphiles—have been shown to generate these highly ordered structures as well.[2] Recently, we reported on a new type of macromolecular surfactant, termed "giant amphiphile", in which a protein or an enzyme acts as the polar head group and a synthetic polymer as the apolar tail.[3] These biohybrid polymers differ from other protein–polymer conjugates in the sense that the protein to polymer ratio is predefined and the position of the conjugation site is precisely known. Herein we describe the construction of a giant amphiphile by direct coupling of a hydrophobic polymer chain to a redox enzyme by cofactor reconstitution. The functionalization of proteins through the reconstitution of an apoprotein with a (synthetically modified) cofactor has been demonstrated to yield a variety of proteins, many of them with interesting new properties.[4] The cofactor reconstitution method has also been applied for the controlled generation of bioactive surfaces for example, in biosensors and bio-fuel cells.[5] Here we demonstrate that the coupling of a polystyrene chain to the horseradish peroxidase (HRP) enzyme results in a giant amphiphile which forms vesicular structures in solution.

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We selected horseradish peroxidase (EC 1.11.1.7) as the biological component because it contains a cofactor (ferriprotoporphyrin IX) that can be easily modified with one or two hydrophobic chains on its carboxylic acid functional groups. Generally, the reconstitution of apo-HRP with cofactors bearing only one unmodified carboxylic acid unit leads to higher enzymatic activities compared to cofactors bearing substituents on both carboxylic acid moieties.[4e,6] Therefore, a single polymer chain end-capped with a carboxylic acid group ($M_n = 9458$, $M_w/M_n = 1.05$) was coupled to one of the carboxylic acid groups of ferriprotoporphyrin IX by a hydrophilic bis(aminoethoxy)ethane spacer (see Supporting Information for synthetic procedure).[7] The latter spacer was used to ascertain the compatibility of the polymer (**1**, Figure 1a) with the apoenzyme. The length of the spacer was chosen such that it can span the distance between the carboxylic acid moiety of the cofactor in the active site and the surface of the enzyme (approximately 10 Å, Figure 1b).

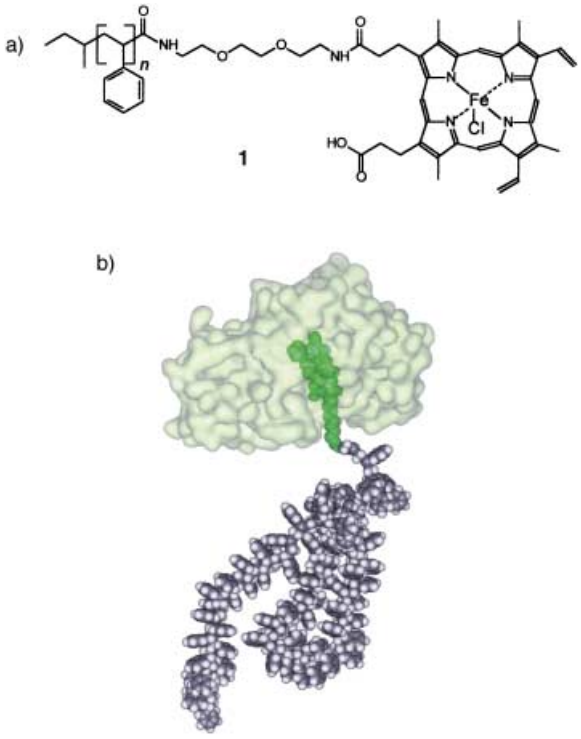

Figure 1. a) Structure of the polystyrene-modified cofactor **1**, $n = 90$; b) computer-generated model of the HRP–polystyrene-based giant amphiphile.

In a first series of experiments, cofactor reconstitution was tried by first preparing aggregates of **1**. A THF solution of the polymer (100 μL of a 0.3 mg/mL solution) was added to a phosphate buffer (1.0 mL, 20 mM potassium phosphate, pH 7.5). As expected, in the absence of the apoenzyme the resulting aggregates did not show any catalytic activity; this was determined using an 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS)/H_2O_2 assay in which apo-HRP was taken as the blank.[8,9] Both TEM (see Supporting Information) and SEM (Figure 2a) demonstrated that polymer **1** in water forms spherical aggregates with diameters of 100–1000 nm and a perforated wall structure.

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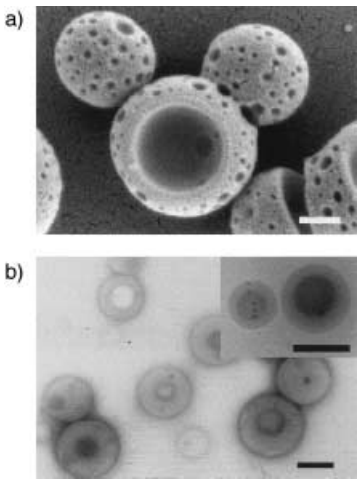

Figure 2. a) SEM micrograph of aggregates formed by **1** in aqueous solution; b) TEM and cryo-TEM (inset) images of aggregates of complexes formed between apo-HRP and **1** in aqueous solution. Scale bars represent 200 nm.

Notably, similar structures have been described for low molecular weight surfactants, but to our knowledge they are unprecedented for polymeric materials.[10,11] Attempts to construct biohybrid amphiphiles by incubating the aggregates of **1** with apo-HRP were not successful as no changes in morphology or enzymatic activity were observed.

The desired biohybrid amphiphiles could be prepared, however, by adding a THF solution of **1** to an aqueous solution containing an excess of the apoenzyme. After incubation at 4 °C for 4 days a stable homogeneous dispersion was obtained, from which the excess apoenzyme was removed by dialysis. UV/Vis spectroscopy revealed that, upon incorporation into the apoenzyme, **1** attains the spectral features of native hemin inside HRP (Figure 3). The electrophoretic migration-shift assay showed the formation of large protein-containing aggregates,[12] that were unable to penetrate the electrophoresis gel and, as expected, no apo-HRP was detected (see Supporting Information). Samples of the dialyzed solutions were transferred to carbon-coated copper grids and studied by TEM. Electron micrographs revealed that the enzyme–polymer hybrid formed vesicular aggregates with diameters of 80–400 nm (Figure 2b). In most cases these aggregates enclosed spherical objects, which were often located away from the center of the aggregates. A number of vesicles were found to contain more than one of these objects. Cryo-TEM studies showed that the aggregates in solution have a spherical nature and confirmed that the objects are only found in association with the vesicles (Figure 2b).

Although the present results are not yet conclusive, we presume that **1** in the presence of apo-HRP forms aggregates onto which the apo-HRP can be reconstituted. In this way the desired amphiphile is formed. The latter biohybrid subsequently forms vesicles, which enclose the initial aggregate. The vesicles grow from the aggregates of **1** until all apo-HRP inside the vesicles is reconstituted, leaving nonreconstituted cofactor as dark spherical objects inside or attached to the vesicles.

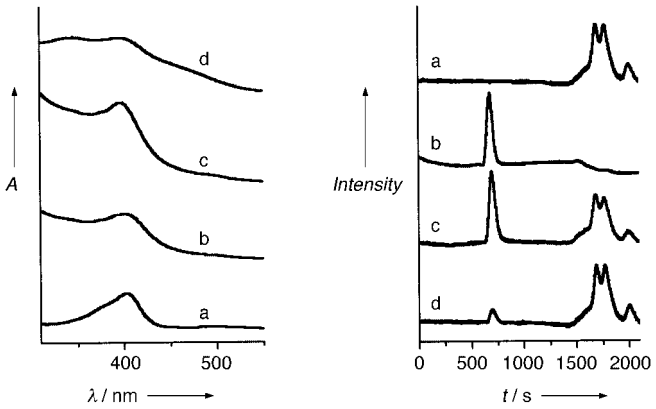

Figure 3. Left: UV/Vis spectra in the Soret-band region of a) native HRP; b) apo-HRP reconstituted at 4 °C with the modified cofactor **1**; c) apo-HRP reconstituted at 22 °C with **1**; d) cofactor **1**. Spectra were recorded in aqueous solution (pH 7.5, 20 mM potassium phosphate) at a concentration of 0.0066 mM. Right: aqueous gel permeation chromatograms of a) 4(5)-carboxyfluorescein (the commercially obtained material contains isomers, each having a different interaction with the column material); b) and c) sample of an aggregate solution prepared in the presence of 4(5)-carboxyfluorescein; d) sample of the aggregate solution after the addition of pure water to break up the aggregates. In the case of a), c), and d) the sample was excited at $\lambda_{ex} = 492$ nm and the emission of the dye was monitored at $\lambda_{em} = 516$ nm. In the case of b), for the detection of the aggregates formed by the enzyme–polymer hybrid, the UV/Vis absorption of the cofactor at 408 nm was monitored. Trace b) shows that the retention time of the water-soluble dye coincides with that of the vesicles.

Inclusion experiments were carried out to establish further the vesicular nature of the aggregates. These experiments showed that it is possible to include a water-soluble dye (4(5)-carboxyfluorescein) in the aggregates, which is released upon rupture of the aggregates through osmotic pressure induced by the addition of pure water to the buffered aggregate dispersion (Figure 3).

The enzymatic activity of the HRP–polystyrene–**1** aggregates was tested, but no enzymatic activity could be observed when the enzyme was reconstituted with **1** at 4 °C. However, when the reconstitution of apo-HRP with **1** was carried out at 22 °C the enzyme–polymer hybrid surprisingly regained activity. After 5 days the aggregates displayed a turn-over frequency (TOF) of 360 h^{-1} in 20 mM potassium phosphate buffer at pH 7.5.[13] Longer reconstitution times resulted in a decrease in activity, probably caused by thermal denaturation. Under the same conditions for HRP, a TOF of 76000 h^{-1} was found after 5 days.

In summary, we have shown for the first time that the modification of an enzyme with a macromolecular chain by the cofactor reconstitution method yields giant amphiphiles that can form aggregates in aqueous media, and which still retain some catalytic activity.

Experimental Section

Reconstitutions were carried out by adding 1.0 mL of a THF solution of the modified cofactor **1** by syringe into 10.0 mL of an aqueous phosphate-buffered solution (20 mM, pH 7.5) containing a 2.5-fold excess of the apoenzyme. After 4 days, the excess of apoenzyme was removed by dialysis using a membrane with a 300 kDa cut-off, which allows the apoenzyme

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(44 kDa) and the individual heme cofactors (10 kDa) to pass through, while retaining any aggregates formed by the enzyme–polymer hybrid.

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[13] When **1** was incubated with glucose oxidase (GOx) or acylase, two non-heme enzymes containing several lysine and histidine residues on their periphery, no peroxidase activity was observed. This implies that the enzymatic activity of **1** incubated with apo-HRP is not caused by the interaction of **1** with amino acid residues on the apo-HRP periphery.

Active Immunization with a Glycolipid Transition State Analogue Protects against Endotoxic Shock**

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Septic shock is one of the ten leading causes of both infant and adult mortality in the United States and, according to the Centers for Disease Control and Prevention, directly resulted in over 30000 deaths in 1999 alone. Lipopolysaccharide (LPS) released from the bacterial membrane after bacteriolysis is responsible for many of the toxic effects associated with gram-negative bacterial septic shock.[1] Lipid A (**1**; the *E. coli* structural form is shown in Figure 1) is the main toxic determinant of LPS and is known to stimulate host macrophages to secrete increased amounts of various cytokines, which include tumor necrosis factor-α (TNF-α), interleukin-1β, and interleukin-6.[2] Immunomodulation of this inflammatory cascade has been suggested as a crucial but inadequately addressed element in the treatment of sepsis.[3] While passive immunization with monoclonal antibodies directed against components of the inflammatory cascade or LPS itself[4] have shown promise at the research level, these strategies have to date been ineffective in extensive clinical trials.[5]

As a primary step in a strategy that may ultimately lead to a new immunomodulatory treatment for septic shock, we report that active immunization with keyhole limpet hemocyanin (KLH) glycoconjugates of novel bisphosphonate analogues of lipid X **2a**,**b** (see Figure 1) offers significant in vivo protection against a sublethal lipid A challenge.

Raetz[6] has shown that the biological effects of the *E. coli* lipid A (**1**) require the presence of several key structural features: both phosphate groups, the glucosamine disaccharide, and all the fatty acyl chains, especially the 2′-lauroyl and 3′-myristoyl acyloxy residues (R′ and R of **1** in Figure 1).

Our designed lipid A mimics (**2a**,**b**, Figure 1) incorporate the following features: 1) a glucosamine 4′-phosphate β-*O*-butyl lipid X saccharide analogue of lipid A; 2) truncated 2′- and 3′-acyloxychains that contain both the lipid A *R* stereo-

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