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Aggregation Behavior of Giant Amphiphiles Prepared by Cofactor Reconstitution

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Abstract: We report on biohybrid surfactants, termed "giant amphiphiles", in which a protein or an enzyme acts as the polar head group and a synthetic polymer as the apolar tail. It is demonstrated that the modification of horseradish peroxidase (HRP) and myoglobin (Mb) with an apolar polymer chain through the cofactor reconstitution method yields giant amphiphiles that form spherical aggregates (vesicles) in aqueous solution. Both HRP and Mb retain their original functionality when modified with a single polystyrene chain, but reconstitution has an effect on their activities. In the case of HRP the enzymatic activity decreases and for Mb the stability of the dioxygen myoglobin (oxy-Mb) complex is reduced, which is probably the result of a disturbed binding of the heme in the apo-protein or a reduced access of the substrate to the active site of the enzyme or protein.

Keywords: aggregation · amphiphiles · block copolymers · electron microscopy · vesicles

Introduction

Low-molecular-weight amphiphilic molecules are known to form a large variety of self-assembled structures in water, for example, 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 and others 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.[3,4] 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.

The modification of enzymes through the reconstitution of the corresponding apoprotein with suitably modified cofactors is an elegant way of altering the function or properties of these biomolecules.[5] The cofactor reconstitution method has also been applied for the controlled generation of bioactive surfaces, for example, in biosensors and biofuel cells.[6] Examples of cofactor-bearing proteins are glucose oxidase and heme-dependent proteins. The latter class contains a significant number of potential candidates that may be modified by the cofactor reconstitution method, as in many of them the heme group is not covalently bound to the rest of the protein. Most studies on the reconstitution of proteins with modified hemes concern the reconstitution of myoglobin (Mb, 17 kDa, Figure 1) and, albeit less frequently, hemoglobin, both of which are oxygen binding proteins.[5b,d,7–13] Metal-substituted hemes have been used to study energy-transfer processes and structural properties of the reconstituted biomacromolecules.[9] Hemes with modified vinyl and/or propionate groups have been applied to investigate oxygen-binding properties,[10] to introduce various functionalities that are helpful in the incorporation of the

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proteins into bilayers,[11] or to help enhance their catalytic properties.[5b,12,13] Very few reports have appeared on the modification of horseradish peroxidase (HRP, 43 kDa, Figure 1) by means of its cofactor.[5e,6b,14] These reports predominantly deal with the modification of the propionate groups to study the effect on the structural and catalytic properties of the enzyme.

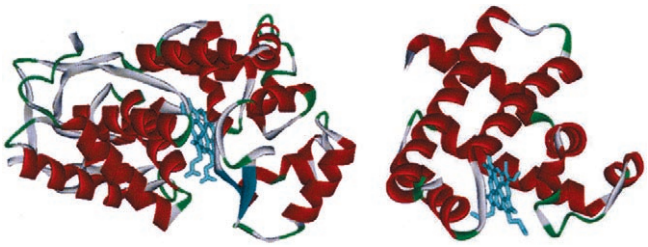


Figure 1. Crystal structures of HRP[16] (left) and Mb[17] (right) showing the positioning of the heme cofactors (blue).

The modification of a protein with large hydrophobic chains by its cofactor can lead to the formation of amphiphiles and hence aggregates of this protein in aqueous solution.[3a,b] For phospholipids and, for example, dendrimer-based diblock copolymers the structure and shape of these aggregates can be predicted.[15,2b] Aiming at a similar level of control we reasoned that the enzyme should be modified with an apolar chain of such a length that the head to tail ratio of the resulting amphiphile would be comparable to that of low-molecular-weight amphiphiles. In fact this implies that this apolar chain should be a polymer of sufficiently high molecular weight. In a previous report we demonstrated that the modification of HRP with an apolar polymer chain through the cofactor reconstitution method yields a giant amphiphile (Figure 2) that forms spherical aggregates in aqueous solution.[3c] In the present paper we extend this method to generation of Mb-based amphiphiles demonstrating the versatility of the cofactor reconstitution method. We compare the reconstitution characteristics of the two apoproteins and demonstrate the formation of vesicles from the Mb-based hybrid.

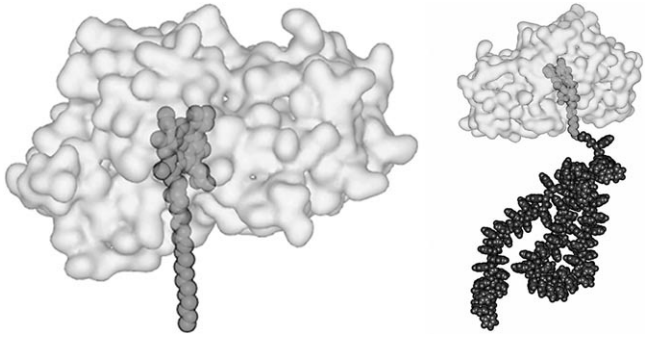


Figure 2. Left: Computer-generated model of HRP reconstituted with cofactor **2c**. Right: Computer-generated model of an HRP/**1e** giant amphiphile showing the heme group and the hydrophilic linker (10 Å) between cofactor and polymer.

Results and Discussion

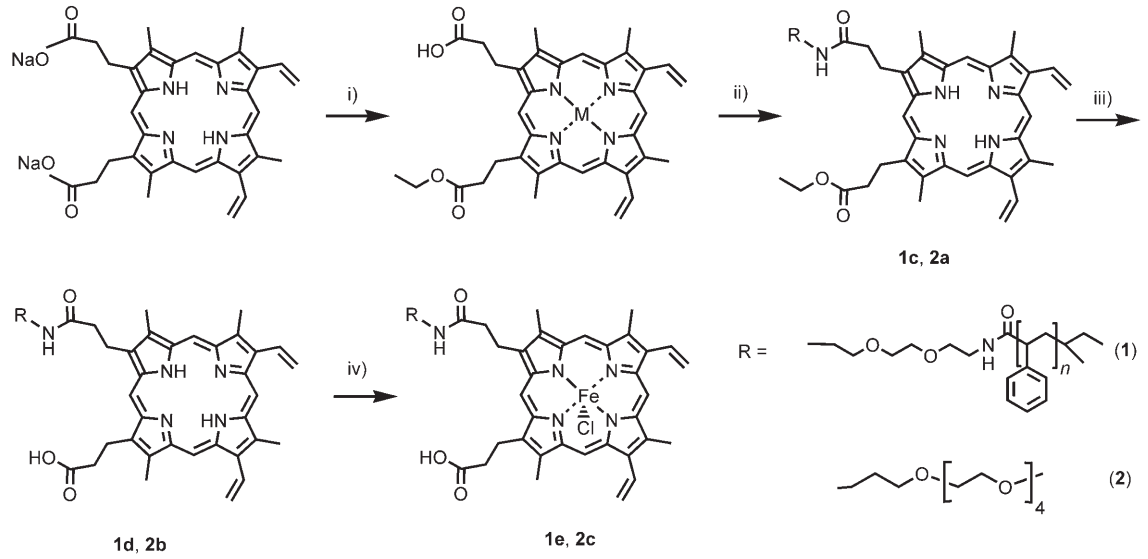
Synthesis: The heme propionates are important for the oxygen-binding properties and play a role in the correct positioning of the heme group in the active site and the stabilization of the heme–protein contact.[10g,16] For apo-HRP it has been demonstrated that the reconstitution with cofactors bearing only one modified carboxylic acid function generally leads to higher enzymatic activities relative to cofactors bearing two modified carboxylic acid functions.[5e,14b] 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 means of a hydrophilic bis(aminoethoxy)ethane spacer (**1e**) following the sequence of reactions shown in Scheme 1.[13a]

In the case of HRP 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 this enzyme (approximately 10 Å, Figure 2, left). In the case of Mb the carboxylic acid moieties of the cofactor are exposed at the surface of the protein. To investigate to what extent the ethylene oxide-based linker would interfere with the reconstitution process we also prepared cofactor **2c**, which has an oligoethylene glycol chain of 15 Å length (Figure 2, right).

Reconstitution experiments—comparison of the modified cofactors: UV/Vis measurements of apo-HRP reconstituted with **2c** in a 2.5:1 molar ratio showed spectral features similar to those of native heme inside HRP (Figure 3). The shape and λ_{max} value of the Soret band did not change with respect to apo-HRP reconstituted with native hemin. The shape and λ_{max} values of the Q-bands of the modified enzyme, however, did change, for which at present no explanation is available. The catalytic activity of the system reconstituted with **2c** (turn over frequency, TOF = 19000 hr^{-1}) was still appreciably high compared to the values obtained for native and reconstituted HRP (Table 1).

The reconstitution of apo-Mb with **2c** was carried out using a 1:1 apoprotein/cofactor ratio and THF as the cofactor solvent. UV/Vis spectroscopy (Figure 3) showed that also in this case the shape and λ_{max} value of the Soret band did not change. Only small changes were observed for the Q-bands (Figure 3, inset). The modified Mb still displayed oxygen binding properties similar to that of native Mb, suggesting that also in this case the heme group was correctly incorporated into the protein (Figure 4).

These results indicate that the reconstitution of the two apoproteins with a cofactor modified with an oligo(ethylene oxide) chain that has sufficient length to extend beyond the protein's surfaces results in proteins with spectral properties that are only slightly different from the spectral features of the native proteins. Importantly, both biomacromolecules still retained their function. Based on these results further studies were carried out to generate protein-based amphiphiles by modifying the oligo(ethylene oxide) chain with a hydrophobic polystyrene segment.



Scheme 1. Synthesis of modified heme cofactors. Conditions: i) P_2Cl_5, EtOH, $CHCl_3$; ii) R-NH_2, benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate, *N,N*-diisopropylethylamine, THF/DMF (1:1 v/v); iii) THF/MeOH/aqueous NaOH (12:4:1 v/v/v); iv) $FeCl_2 \cdot 4H_2O$, DMF.

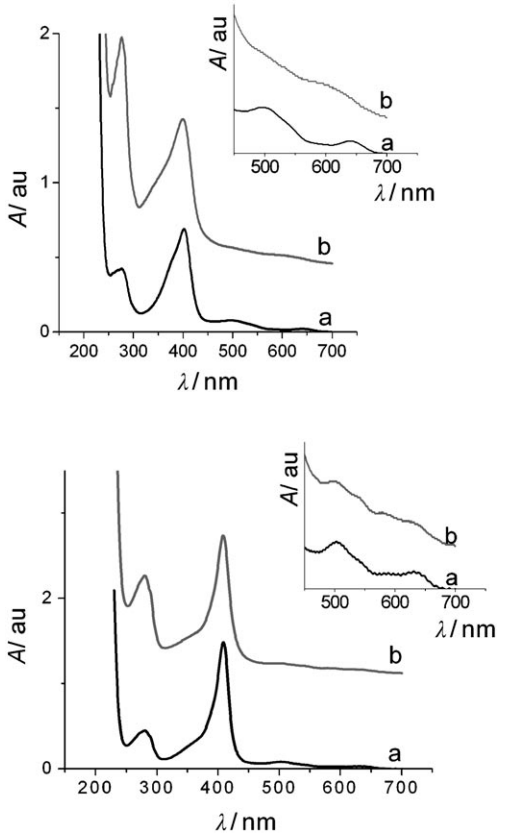


Figure 3. UV/Vis spectra recorded at 22 °C of apo-HRP (top) and apo-Mb (bottom) reconstituted with native heme (trace a) and modified cofactor **2c** (trace b). For the reconstitution of apo-HRP a 2.5:1 ratio with respect to the cofactor was used while for apo-MB a 1:1 ratio was used. Insets display the Q-band regions of the spectra.

Table 1. Enzymatic activity of modified HRP.

Reconstituted HRP[a]	TOF [h^{-1}]
Apo HRP/Heme	89000
HRP	100000
Apo HRP/**2c**	19000

[a] A 2.5-fold excess of apo-HRP was used for the reconstitutions. The TOF was calculated per cofactor for the enzymatic activity of HRP in the conversion of ABTS by hydrogen peroxide obtained at 22 °C (20 mM phosphate buffer pH 7.5).

Reconstitution with a polystyrene modified cofactor: The generation of HRP-based amphiphiles by cofactor reconstitution was first attempted starting from aggregates of **1e**. To this end a solution of the polymer in THF (0.3 mg mL^{-1}; 100 μL) was injected into phosphate buffer (20 mM potassium phosphate, pH 7.5; 1.0 mL). In the absence of HRP the resulting aggregates did not show any catalytic activity as was concluded from an experiment in which apo-HRP was taken as a reference.[17,18] Both transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (Figure 5) demonstrated that polymer **1e** forms spherical aggregates in water with diameters of 100–1000 nm and with a perforated wall structure.

The formation of such perforated spheres has not been reported before for polymeric materials.[19] For low-molecular-weight surfactants, vesicles with porous walls have been described and in these cases the observed pore structure was attributed to the relief of surface energy due to high surface charges.[20] Attempts to construct biohybrid amphiphiles by incubating the aggregates of **1e** with apo-HRP were not successful as no changes in morphology were observed and no enzymatic activity was measured. The desired amphiphile could be prepared, however, by injecting a solution of **1e** in THF into an aqueous solution containing an excess of the apoprotein.[3c] After incubation at 4 °C or 22 °C for 4 days a stable homogeneous dispersion was obtained from which the excess apoprotein was removed by dialysis. UV spectros-

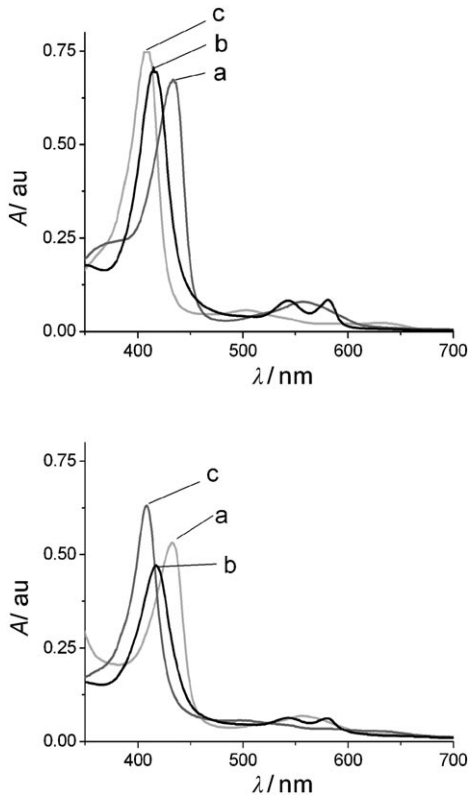


Figure 4. UV/Vis spectra of the various forms of native Mb (top) and the apo-Mb/2 hybrid (bottom) obtained a) upon treatment with sodium dithionite under anaerobic conditions (deoxy; $\lambda_{max}=433$ nm), b) upon subsequent application of oxygen (oxy; $\lambda_{max}=417$ nm), and c) upon autoxidation back to its resting state (met; $\lambda_{max}=408$ nm).

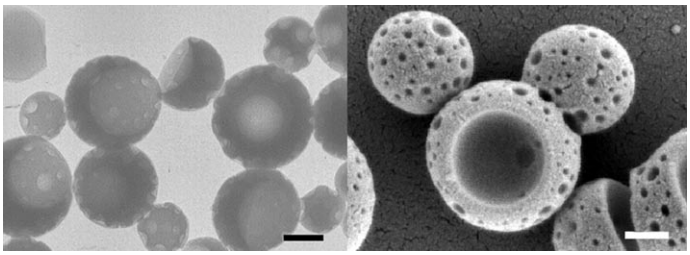


Figure 5. TEM (left) and SEM (right) micrographs of aggregates formed by **1e** in aqueous solution (20 mM potassium phosphate buffer pH 7.5). Bars represent 200 nm.

copy revealed spectral features of native heme inside HRP (Figure 6) upon incorporation of **1e** in the apoprotein. At both temperatures the reconstitution yielded a modified enzyme of which the Soret band had a λ_{max} at the same wavelength as found for the native enzyme (402 nm). The Soret band of **1e** reconstituted with apo-HRP at 22 °C was more intense than that of the enzyme reconstituted at 4 °C. The Q-bands could not be distinguished due to the turbidity of the solutions. It was observed that incubation at 22 °C yielded the highest enzymatic activity.[21] After 5 days the aggregates displayed a TOF of 360 h^{-1} in 20 mM potassium phosphate buffer at pH 7.5.[22] An electrophoretic migration

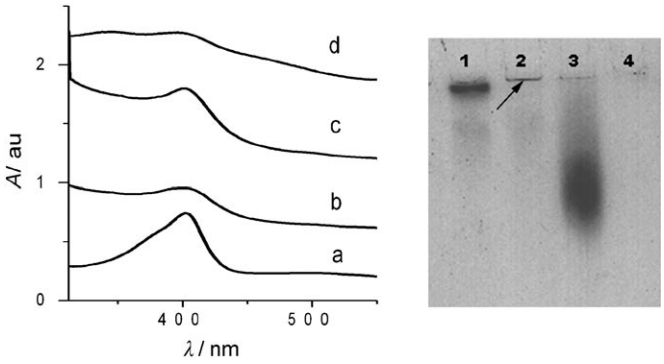


Figure 6. Left: UV/Vis spectra in the Soret band region of a) native HRP, b) apo-HRP reconstituted at 4 °C with modified cofactor **1e**, c) apo-HRP reconstituted at 22 °C with modified cofactor **1e**, and d) cofactor **1e**. Spectra were recorded in aqueous buffered solutions (pH 7.5, 20 mM potassium phosphate) at a concentration of 0.0066 mM. Right: Electrophoretic migration shift assay of 1) HRP, 2) aggregates formed by the giant amphiphiles (arrow), 3) apo-HRP, and 4) blank experiment: aggregates of **1e**.

shift assay showed that the reconstituted biohybrid formed large protein-containing aggregates that were not able to penetrate the electrophoresis gel.[23] As expected no apo-HRP was detected (Figure 6).

The Mb-polystyrene biohybrid amphiphiles were prepared in the same way as described for the HRP-polystyrene biohybrids, namely by injecting a solution of **1e** in THF into an aqueous solution containing an excess of the apoprotein. After 4 days of reconstitution at 22 °C the excess of apo-Mb was removed by centrifugation through a 100 kDa cut-off filter. UV/Vis spectroscopy indicated clearly that the reconstitution had yielded a modified protein for which the Soret band had a λ_{max} at 410 nm, nearly identical to the value found for the native enzyme (409 nm), indicating a successful reconstitution (Figure 7). The formation of aggregates also gave rise to an increase in scattering in the UV spectra and as a consequence the Q-bands could not be resolved.[24]

Furthermore, whereas cofactor **1e** did not display any oxygen binding in the absence of the apoprotein, the biohybrid displayed the expected oxygen binding and spectroscopic properties, indicating that the modified heme had been inserted correctly. Nevertheless, Figure 7 shows that the Soret band maxima of the deoxy and the oxy forms of the biohybrid are blue-shifted relative to those of the native deoxy-Mb (433 nm) and native *oxy*-Mb (416 nm) proteins, respectively. This suggests that in contrast to the reconstitution with **2c**, in the present case the heme environment to some extent has been disturbed.

To further investigate the oxygen binding properties of the biohybrid the autoxidation of the dioxygen myoglobin (oxy-Mb) to the ferric (met-Mb) form was monitored for both apo-Mb reconstituted with native heme and for the apo-Mb/**1e** hybrid. The oxy form of apo-Mb reconstituted with native heme displayed a half-life of 21 h under the conditions applied. As a result of the turbidity of the solution and the relatively low intensity of the Soret band, it was difficult to determine the half-life of the biohybrid oxygen

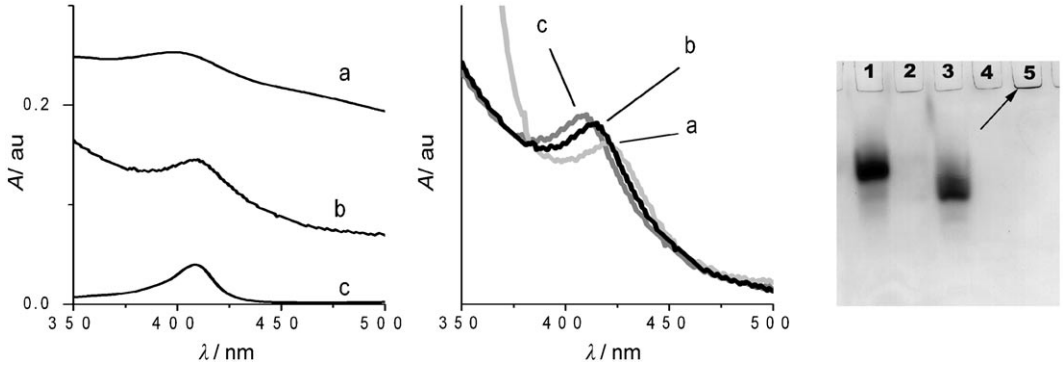


Figure 7. Left: UV/Vis spectra in the Soret band region of a) cofactor **1e**, b) apo-Mb reconstituted with modified cofactor **1e**, c) native Mb. Spectra were recorded in aqueous solution (pH 7.5, 20 mM potassium phosphate). Middle: UV/Vis spectra showing the Soret band region of the various forms of the apo-Mb/**1e** hybrid obtained a) upon treatment with sodium dithionite under anaerobic conditions (deoxy; $\lambda_{max}=420$ nm), b) upon subsequent application of oxygen (oxy; $\lambda_{max}=415$ nm), and c) upon autoxidation back to its resting state (met-Mb; $\lambda_{max}=410$ nm). Right: Electrophoretic migration shift assay of 1) apo-Mb, 2) no sample, 3) native Mb, 4) blank: aggregates of **1e**, and 5) giant amphiphiles (indicated by the arrow).

complex; however, it was evident that the oxy-Mb form was fully converted to the met-Mb form within 30 min. The observed destabilization of the oxygen complex of the biohybrid is probably the result of the modification of one of the heme propionates, which is needed to stabilize the oxy form, and the introduction of the steric bulk.[13d,25] These observations are in line with autoxidation studies on modified Mb reported in the literature, which also show a drastic decrease in the stabilization of the oxy complex.[10e,13b,d,j]

An electrophoretic migration shift assay was carried out on an aqueous buffered solution containing the apo-Mb/**1e** biohybrid. This experiment showed that the centrifuged solutions contained large protein-containing aggregates,[23] which were not able to penetrate the electrophoresis gel. As expected no apo-Mb was detected (Figure 7).

Aggregation behavior: Cryogenic scanning electron microscopy (cryo-SEM) revealed that the HRP-derived hybrid formed well-defined spherical aggregates with diameters of 60–400 nm (Figure 8). TEM indicated that in most cases these aggregates enclosed spherical objects, which mostly appeared denser (darker) than the major part of the aggregate; however, albeit to a lesser extent, lighter regions were also observed. The latter regions are tentatively assigned to the presence of an included aqueous compartment, whereas the darker regions may contain particles of un-reconstituted polystyrene-modified cofactor. Importantly, however, the main body of the aggregates appeared as a spherical object with an intermediate density. Cryo-TEM experiments also clearly demonstrated that the majority of these spherical aggregates possessed an interior with a higher electron-scattering power than the surrounding vitrified ice and that one or more dense particles were present. Inclusion experiments with the water-soluble dye 4(5)-carboxyfluorescein (not shown) revealed that the aggregates were hollow suggesting that they contain an aqueous interior,[3c] the exact nature of which needs further investigation.

Electron micrographs of solutions containing the Mb-based hybrid also revealed well-defined spherical aggregates with a slightly wider distribution of diameters ranging from 20–700 nm (Figure 9). The larger aggregates had a hollow interior and possessed a collapsed morphology typical for polymeric vesicles. The collapse is most probably due to the loss of solvent during sample preparation or to the application of high vacuum in the electron microscope. Considering

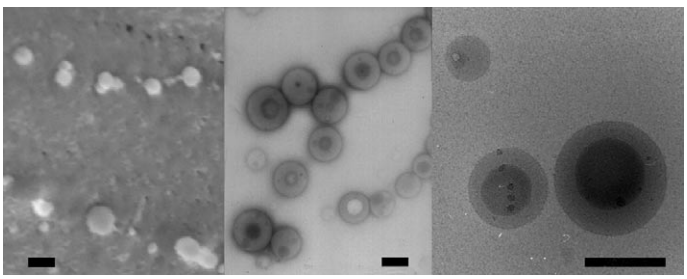


Figure 8. Electron micrographs of aggregates of biohybrids from apo-HRP and **1e** in 20 mM potassium phosphate buffer pH 7.5. Left: cryo-SEM; middle: TEM; right: cryo-TEM. Bars represent 200 nm.

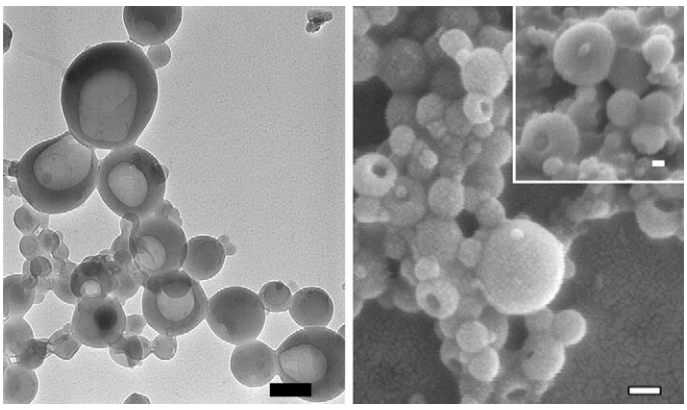


Figure 9. Left: TEM image of aggregates of biohybrids formed from apo-Mb and polymer **1e** in aqueous solution (20 mM potassium phosphate buffer pH 7.5). Right: SEM and cryo-SEM (inset) images of the same aggregates. Bars represent 100 nm.

the dimensions of the protein polymer hybrid it is likely that the observed smaller objects, with diameters between 20 and 50 nm, are micellar structures and not vesicles. As almost all aggregates appeared as strings of beads, the objects were also investigated by cryo-SEM (Figure 9, inset). These experiments confirmed the strong association of the aggregates already in solution, suggesting that a considerable amount of them was trapped in the process of fusion. Recently, Vriezema et al. described the fusion of vesicles of poly(isocyanide)-based block copolymers and reported that THF is an essential factor in the fusion process.[1] Earlier Eisenberg et al. demonstrated that the presence of THF provided the required mobility for the reorganization of polystyrene segments in block-copolymer aggregates.[26] Based on these data we propose that the fusion of the vesicles in the present case is related to the use of THF for the preparation of the protein–polymer hybrids, implying that the observed aggregates are kinetically trapped structures.

From the SEM micrographs the thickness of the wall segment of the Mb-derived aggregates was determined to be 12 ± 4 nm (Figure 10). Assuming that the observed aggregates are bilayer vesicles, it is possible, based on the dimensions of Mb ($3.6 \times 3.4 \times 2.1$ nm), to describe the bilayer as being composed of three layers, one containing the polystyrene chains (located in the interior of the bilayer) and two flanking layers each containing protein molecules (Figure 10).[27] The volumes of the respective layers were calculated for a vesicle of average diameter of 90 nm taking into account the volumes of the protein (21 nm^3) and the polymer (14 nm^3). These volumes amounted to 5.5×10^4 nm^3 and 8.5×10^4 nm^3 for the protein layers and to 8.8×10^4 nm^3 for the polystyrene layer. From these data it can be derived that the bilayer is composed of a total of 6700 Mb molecules and 6300 polystyrene chains. In this calculation again only the volume of the protein but not its shape was considered. Considering the error (ca. 35%) in determining the thickness of the bilayer the obtained numbers are in good agreement with the proposed model.

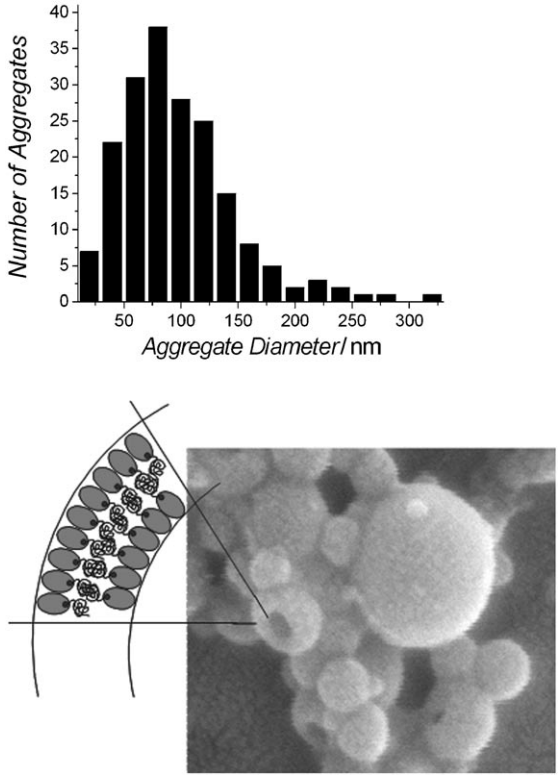


Figure 10. Top: diameter distribution of the vesicle aggregates formed by the apo-Mb/**1e** hybrid in aqueous solution. Bottom: SEM image of a vesicle aggregate and schematic representation of a bilayer segment.

Conclusions

In this paper we have shown that a macromolecular chain can be coupled to a protein by means of the cofactor reconstitution method to give a giant amphiphile. The suitability of the method was demonstrated for an enzyme (HRP) and for an oxygen-binding protein (Mb). In both cases the formed giant amphiphiles were found to generate well-defined spherical aggregates in aqueous solution. Although both HRP and Mb retained their original functionality when modified with a single polystyrene chain, reconstitution had an appreciable effect on their activity. In the case of HRP, the enzymatic activity decreased and for Mb the stability of the oxy complex was reduced. Although the reconstitution with the oligo(ethylene oxide)-modified cofactor **2c** only has a small effect on the activity of the resulting hybrid, the introduction of a polystyrene chain seems to result in a somewhat modified binding of the heme in the apoprotein. Several factors may be responsible for this behavior. First, there may be an unfavorable interaction of the protein with the polystyrene chain. Furthermore, the assembly of the hybrid molecules in the aggregates may lead to disturbance of the protein's three-dimensional structure. In addition, both proteins have their substrate access channels located near the site of attachment of the polystyrene chains, which means that these channels will be partly shielded from the aqueous solution. However, at present we cannot exclude that the residual activity predominantly arises from the smaller (micellar) aggregates, which would have a more dynamic structure than the larger vesicles.

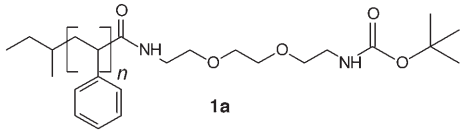
Israelachvili's theory predicts that upon changing the ratio of the cross sections of the head groups and tails of phospholipids different types of aggregates are formed.[15] This model was also found to apply for dendrimer-based diblock copolymers.[2b] Although one has to realize that in the present case the observed aggregates probably are kinetically trapped structures, it is of interest to investigate the influence of the length of the hydrophobic (polymer) tail on the type of aggregate that is generated by these biohybrids. These studies are currently being carried out.

Experimental Section

Materials: (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP), (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (py-BOP), protoporphyrin IX disodium salt (PP IX disodium salt), horseradish peroxidase, myoglobin, and ferrous chloride tetrahydrate were obtained from Sigma–Aldrich. 2,2′-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) was obtained from Fluka. Carboxy-terminated polystyrene ($M_n = 9458$, $M_w/M_n = 1.05$) was generously donated by Dr. J. J. L. M. Cornelissen.[28] {2-[2-(2-Amino-ethoxy)ethoxy]ethyl}carbamic acid *tert*-butyl ester and protoporphyrin IX monoethylester were synthesized according to literature procedures.[2,3,29]

Synthesis: All syntheses were carried out under argon atmosphere. All solvents were distilled prior to use.

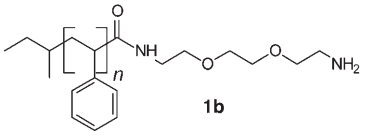
Synthesis of 1a: Carboxy-terminated polystyrene (3.0 g, 0.32 mmol), {2-[2-(2-aminoethoxy)ethoxy]ethyl}carbamic acid *tert*-butyl ester (87 mg,



1a

0.35 mmol) and BOP (164 mg, 0.37 mmol) were dissolved in a mixture of THF (20 mL) and DMF (20 mL). Diisopropylethylamine (0.22 mL, 1.2 mmol) was added and the reaction mixture was stirred for 16 h at room temperature. The solvent was evaporated in vacuo and the residue was dissolved in diethyl ether. The organic layer was extracted with aqueous saturated $NaHCO_3$ solution, water, and aqueous 0.01 N HCl solution, and dried over $MgSO_4$. The crude product was further purified by column chromatography (CH_2Cl_2 followed by MeOH/CH_2Cl_2; 1:99 v/v). Yield 2.4 g (0.25 mmol, 78%); ^{1}H NMR (400 MHz, $CDCl_3$, 22 °C, TMS): $\delta = 7.32$–6.25 (br, 445 H; -$CH_2CH(Ph)$-), 3.43–3.23 (br, 9 H; -CH_2CH(Ph)-(C=O)-, -$CH_2OCH_2CH_2OCH_2$-), 3.23–3.16 (br, 4 H; -NHCH_2-), 2.21–0.78 (br, 269 H; $CH_3CH_2CH(CH_3)(CH_2CH(Ph)_{n-1}CH_2CH(Ph)(C=O)$-), 1.48 (s, 9 H; $(CH_3)_3CO$-), 0.78–0.54 ppm (br, 6 H; $CH_3CH_2CH(CH_3)$-$(CH_2CH(Ph)_n(C=O)$-); ^{13}C NMR (100 MHz, $CDCl_3$, 22 °C, TMS): $\delta =$ 172.8, 155.9, 145.7–145.1, 128.3–125.5, 70.5–69.8, 51.0, 46.7–40.1, 31.2, 30.4–28.9, 28.7, 20.2–18.6, 11.5–11.0 ppm; MS (MALDI): $M_n = 9506$, $M_w/M_n = 1.02$.

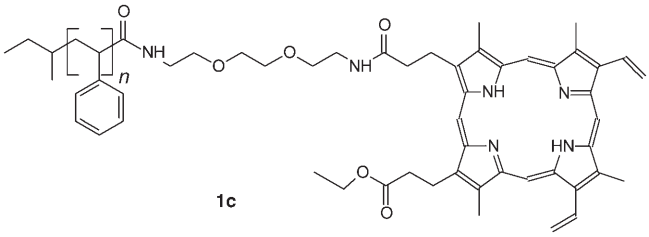
Synthesis of 1b: Compound **1a** (2.1 g, 0.22 mmol) was dissolved in CH_2Cl_2 (50 mL). Trifluoroacetic acid (2.4 mL, 31 mmol) was added and the mixture was stirred for 4 h at room temperature. The reaction mix-



1b

ture was concentrated in vacuo and the residue dissolved in $CHCl_3$. The organic layer was extracted with aqueous 5% NH_3 solution, aqueous saturated NaCl solution, and water, and then dried over $MgSO_4$: Yield 2.1 g (0.22 mmol, 99%); ^{1}H NMR (400 MHz, $CDCl_3$, 22 °C, TMS): $\delta =$ 7.32–6.25 (br, 445 H; -$CH_2CH(Ph)$-), 3.43–3.23 (br; 9 H; -CH_2CH(Ph)(C=O)-, -$CH_2OCH_2CH_2OCH_2$-), 3.23–3.16 (br, 2 H; -(C=O)NHCH_2-), 2.80–2.71 (br, 2 H; CH_2NH_2), 2.21–0.78 (br, 269 H; $CH_3CH_2CH(CH_3)$-$(CH_2CH(Ph)_{n-1}CH_2CH(Ph)(C=O)$-), 0.78–0.56 ppm (br, 6 H; $CH_3CH_2CH(CH_3)(CH_2CH(Ph)_n(C=O)$-); ^{13}C NMR (100 MHz, $CDCl_3$, 22 °C, TMS): $\delta = 172.8$, 145.8–145.3, 128.4–125.6, 70.5–69.7, 51.0, 46.7–40.1, 31.2, 30.4–28.9, 20.2–18.6, 11.5–11.0 ppm.

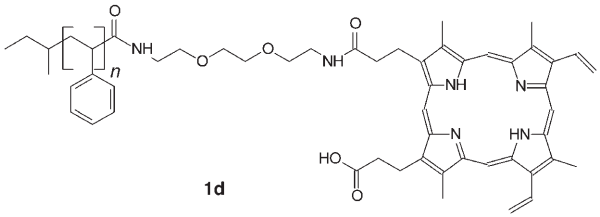
Synthesis of 1c: Compound **1b** (247 mg, 0.026 mmol) was dissolved in a mixture of THF (7 mL) and DMF (7 mL). Protoporphyrin IX monoethyl ester (20.5 mg, 0.035 mmol), py-BOP (19.8 mg, 0.038 mmol) and diisopro-



1c

pylethylamine (0.024 mL, 0.13 mmol) were added. After stirring for 16 h at room temperature the solution was concentrated in vacuo. The residue was dissolved in diethyl ether and the organic layer was extracted with an aqueous saturated $NaHCO_3$ solution, water, and an aqueous HCl (0.01 N) solution, and was subsequently dried over $MgSO_4$. The crude product was purified by column chromatography (EtOAc/hexane, 3/1 v/v). Yield 161 mg (0.016 mmol, 63%); ^{1}H NMR (400 MHz, $CDCl_3$, 22 °C, TMS): $\delta = 10.1$ (m, 4 H; CH), 8.3 (m; 2 H; -CH=CH_2), 7.3–6.3 (br, 445 H; -$CH_2CH(Ph)$-), 6.1 (m, 4 H; -CH=CH_2), 4.4 (t, $J = 6$ Hz, 4 H; -CH_2CH_2-(C=O)-), 4.0 (q, $J = 6$ Hz, 2 H; CH_2CH_3), 3.6 (s; 12 H; CH_3), 3.3 (t, $J =$ 6 Hz, 4 H; -CH_2CH_2(C=O)-), 3.18–3.02 (br, 13 H; -CH_2CH(Ph)(C=O)-, -$CH_2OCH_2CH_2OCH_2$-, -NHCH_2-), 2.21–0.78 (br, 269 H; CH_3CH_2CH-$(CH_3)(CH_2CH(Ph)_{n-1}CH_2CH(Ph)(C=O)$-), 1.0 (t, $J = 6$ Hz, 3 H; CH_2CH_3), 0.78–0.56 ppm (br, 6 H; $CH_3CH_2CH(CH_3)(CH_2CH(Ph)_n(C=$ O)-); ^{13}C NMR (125 MHz, $CDCl_3$, 22 °C, TMS): $\delta = 173.9$, 172.9, 172.6, 145.8–145.3, 130.4, 128.4–125.6, 121.2, 98.2, 97.6, 97.4, 97.0, 69.5, 69.0, 68.8, 68.4, 60.9, 50.9, 46.8–40.1, 39.0, 37.2, 31.2, 30.4–28.9, 22.3, 22.2, 20.2–18.6, 14.5, 11.5–11.0 ppm; UV/Vis (MeOH/$CHCl_3$, 1:10 v/v): $\lambda_{max} = 405$, 505, 541, 571, 606, 668 nm; MS (MALDI): $M_n = 10020$, $M_w/M_n = 1.01$.

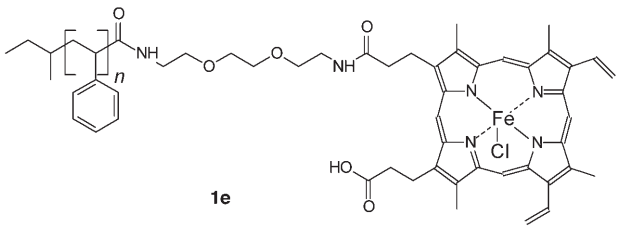
Synthesis of 1d: Compound **1c** (151 mg, 0.015 mmol) was dissolved in THF (4 mL). MeOH (1.4 mL) and aqueous NaOH solution (0.5 N, 0.33 mL) were added. The reaction mixture was stirred for 23 h. Water



1d

(4 mL) was added and the pH was adjusted to 2 with aqueous HCl solution (1.0 N, 1.5 mL). $CHCl_3$ was added and the organic layer was extracted with water and dried over Na_2SO_4. Yield 140 mg (0.014 mmol, 98%); ^{1}H NMR (400 MHz, $CDCl_3$, 22 °C, TMS): $\delta = 10.0$ (m, 4 H; CH), 8.20 (m; 2 H; -CH=CH_2), 7.4–6.3 (br, 445 H; -$CH_2CH(Ph)$-), 6.21 (m, 4 H; -CH=CH_2), 4.40 (br, 2 H; -CH_2CH_2(C=O)OH), 4.20 (br, 2 H; -CH_2CH_2(C=O)NH-), 3.61 (s; 12 H; CH_3), 3.58 (br, 2 H; -CH_2CH_2(C=O)OH), 3.42 (br, 2 H; -CH_2CH_2(C=O)NH-), 3.18–3.02 (br, 13 H; -CH_2CH(Ph)(C=O)-, -$CH_2OCH_2CH_2OCH_2$-, -NHCH_2-), 2.37–0.78 (br, 269 H; CH_3CH_2CH-$(CH_3)(CH_2CH(Ph)_{n-1}CH_2CH(Ph)(C=O)$-), 0.78–0.54 ppm (br, 6 H; $CH_3CH_2CH(CH_3)(CH_2CH(Ph)_n(C=O)$-); UV/Vis (MeOH/$CHCl_3$, 1:10 v/v): $\lambda_{max} = 405$, 504, 538, 577, 632, 669 nm.

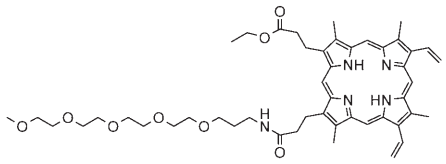
Synthesis of 1e: Compound **1d** (51 mg, 0.0051 mmol) and $FeCl_2 \cdot 4H_2O$ (11 mg, 0.054 mmol) were dissolved in a mixture of THF (3.7 mL) and DMF (11.1 mL) and stirred for 17 h at 65 °C. Air was bubbled through for 15 min after which the solvent was evaporated in vacuo. The residue was dissolved in $CHCl_3$ and the organic layer was extracted with aqueous HCl solution (0.001 N) until the aqueous layer was colorless. The organic



1e

layer was washed with aqueous saturated NaCl solution and water, and dried over $MgSO_4$. The crude product was purified by column chromatography (MeOH/$CHCl_3$, 10/90 v/v). Yield 39 mg (0.0039 mmol, 76%); UV/Vis (MeOH/$CHCl_3$, 1:10 v/v): $\lambda_{max} = 400$, 488, 587 nm.

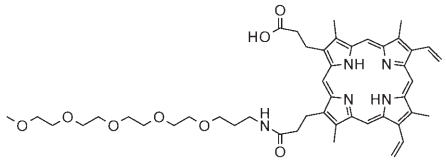
Synthesis of 2a: This compound was prepared using the same procedure as described for compound **1c**. Quantities: protoporphyrin IX monoethyl ester (47 mg, 0.08 mmol), py-BOP (45 mg, 0.09 mmol), DIEA (0.13 mL),



2a

(4,7,10,13,16-pentaoxaheptadecyl)amine HCl salt (21.4 mg, 0.07 mmol). The product was purified by column chromatography (silica gel, MeOH/$CHCl_3$, 1/99 v/v). Yield: 56.8 mg (95%) as a purple powder; ^{1}H NMR (300 MHz, $CDCl_3$, 22°C, TMS): $\delta = 10.1$ (m, 4H; CH), 8.3 (m, 2H; -C*H*=CH_2), 6.3 (m, 4H; -CH=C*H*$_2$), 4.4 (t, $J = 6$ Hz, 4H; -C*H*$_2$CH$_2$(C=O)-), 4.0 (q, $J = 6$ Hz, 2H; -OC*H*$_2$CH$_3$), 3.6 (s, 12H; CH_3), 3.3–2.5 (m, 27H; -NHC*H*$_2$CH$_2$C*H*$_2$(OC*H*$_2$CH$_2$)$_4$OC*H*$_3$, -CH$_2$C*H*$_2$(C=O)O-), -CH$_2$C*H*$_2$(C=O)NH-), 1.85 (m, 2H; -(C=O)NHCH$_2$C*H*$_2$-), 1.0 ppm (t, $J = 6$ Hz, 3H; -CH$_2$C*H*$_3$); ^{13}C NMR (100 MHz, $CDCl_3$, 22°C, TMS): $\delta = 173.7$, 172.5, 130.1, 120.2, 97.2–95.7, 71.9, 70.6–70.2, 69.4, 60.6, 59.0, 42.6, 40.0, 36.9, 31.2, 22.9, 21.5, 13.8, 11.5, 11.4, 11.3, 11.2 ppm; IR(neat): $\tilde{\nu} = 3312$ (N–H), 2922, 2865 (C–H), 1730 (C=O, ester), 1644, 1546 cm^{-1} (C=O, amide); UV/Vis (MeOH/$CHCl_3$, 1:10 v/v): $\lambda = 404$, 505, 541, 574, 628 nm; MS (MALDI): m/z: calcd for $[M+H]^+$: 838.5; found: 838.4; elemental analysis calcd (%) for $C_{46}H_{63}N_5O_8$: C 68.79, H 7.58, N 8.36; found: C 68.68, H 7.64, N 8.31.

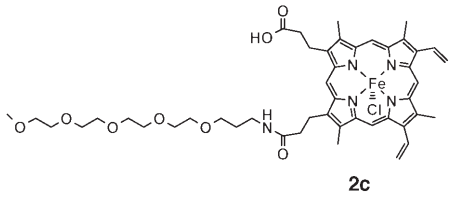
Synthesis of 2b: This compound was prepared by using the same procedure described for compound **1d**. The product was dried over magnesium sulfate. Starting quantities of **2a**: 55 mg, 0.07 mmol. Yield: 52 mg (98%)



2b

as a purple powder. ^{1}H NMR (400 MHz, $CDCl_3$ with one drop of MeOD, 22°C, TMS): $\delta = 10.0$ (m, 4H; CH), 8.2 (m; 2H; -C*H*=CH$_2$), 6.2 (m, 4H; -CH=C*H*$_2$), 4.3 (br, 2H; -C*H*$_2$CH$_2$(C=O)OH), 4.2 (br, 2H; -C*H*$_2$CH$_2$(C=O)NH-), 3.6 (m, 12H; CH_3), 3.3–2.6 (m, 27H; -NHC*H*$_2$CH$_2$C*H*$_2$-(OC*H*$_2$CH$_2$)$_4$OC*H*$_3$, -CH$_2$C*H*$_2$(C=O)O-), -CH$_2$C*H*$_2$(C=O)NH-), 1.8 ppm (m, 2H; -(C=O)NHCH$_2$C*H*$_2$-); ^{13}C NMR (100 MHz, $CDCl_3$, 22°C, TMS): $\delta = 178.2$, 172.9, 130.2–129.9, 120.4–119.9, 96.8–96.2, 71.7, 70.6–70.1, 69.4, 59.0, 42.5, 40.0, 39.5, 31.3, 22.7, 22.6, 11.4–11.1 ppm; IR(neat): $\tilde{\nu} = 3311$ (N–H), 2919, 2857 (C–H), 1707 (C=O, acid), 1643, 1551 cm^{-1} (C=O, amide); UV/Vis (MeOH/$CHCl_3$, 1:10 v/v): $\lambda = 405$, 506, 541, 574, 628 nm; MS (MALDI): m/z: calcd for $[M+H]^+$: 810.4; found: 810.3; elemental analysis calcd (%) for $C_{46}H_{59}N_5O_8$: C 68.21, H 7.34, N 8.65; found: C 68.13, H 7.39, N 8.62.

Synthesis of 2c: This compound was prepared using the same procedure described for compound **1e**. Quantities: **2b** (21 mg, 0.03 mmol), $FeCl_2.4H_2O$ (57 mg, 0.29 mmol). Yield: 23 mg (97%) as a purple powder.



2c

IR(neat): $\tilde{\nu} = 3313$ (N–H), 2919, 2853 (C–H), 1723 (C=O, acid), 1637, 1553 cm^{-1} (C=O, amide); UV/Vis (MeOH/$CHCl_3$, 1:10 v/v): $\lambda = 391$, 509, 640 nm; MS (MALDI): m/z calcd for $[M-Cl]^+$: 863.4; found: 863.2; elemental analysis calcd (%) for $C_{46}H_{57}ClFeN_5O_8$: C 61.44, H 6.39, N 7.79; found: C 61.38, H 6.44, N 7.75.

Preparation of apoproteins: Apo-HRP and apo-Mb were prepared by a literature procedure.[30]

Reconstitution experiments: Since the reconstitution of apo-HRP with the modified cofactors was performed with THF as the solvent for the cofactor, reconstitution experiments with native heme and apo-Mb were also carried out with THF as the cosolvent. The results were compared with the commonly used procedure, which makes use of aqueous 0.1 M sodium hydroxide solution.[30] To this end apo-Mb was reconstituted with native heme by using both THF and aqueous 0.1 M sodium hydroxide solution as the cofactor solvents. No significant differences were observed between the two reconstituted Mb's by UV/Vis spectroscopy. Hence THF was used for the further reconstitution experiments in order to facilitate the comparison with HRP.

Reconstitutions were carried out at 4°C or at 22°C by injecting a solution of modified cofactor **1e** (0.44 mg, 4.4×10^{-5} mmol) in THF (1.0 mL) into an aqueous phosphate-buffered solution (10.0 mL, 20 mM, pH 7.5) containing a 2.5-fold excess of the apoprotein (1.1×10^{-4} mmol). After 4 days the excess of apoprotein was removed by dialysis using a membrane with a 300 kDa cut-off, which allows the apoprotein (44 kDa and 17 kDa) and the individual heme cofactors (10 kDa) to pass, while retaining any aggregates formed by the protein–polymer hybrid. Reconstitutions with modified cofactor **2c** were done by adding a solution of **2c** in THF to a buffered aqueous solution containing an equimolar amount of apo-Mb or a 2.5-fold excess of apo-HRP (potassium phosphate, 20 mM, pH 7.5).

TEM and SEM: Aggregates of **1e** were prepared by injecting a solution of the polymer in THF (100 μL, 0.3 mg mL^{-1}) into phosphate buffer (1.0 mL, 20 mM potassium phosphate, pH 7.5) and subsequently studied on unstained carbon-coated copper grids by TEM (JEM-2000FX) at 80 kV and SEM (Jeol 6330 FESEM, after coverage with Au) at 3.0 kV.

Cryo-SEM: For cryo-SEM studies, a sample of the aggregate dispersion was supported for freezing between two hollow rivets end-to-end. The rivet pair, held with fine forceps, was then plunged rapidly into nitrogen-slush, transferred to liquid nitrogen, inserted into a rivet-clamping holder, and transferred to the specimen of an Oxford Alto 2500 cryo transfer system. On the preparation coldstage (−120°C) the upper unclamped rivet was knocked off the clamped one by using a cold knife. This exposed a fractured face of the frozen material within the clamped rivet. Subsequently the surface was etched at −90°C for 5 min and finally covered with Au/Pt (1.5 nm) by using a Denton microwave unit and studied at a Jeol 6330 FESEM at −140°C.

Cryo-TEM: Thin films of the aggregate dispersion were prepared in the Vitrobot (PC controlled vitrification robot, patent applied, Frederik et al 2002, patent licensed to FEI) at room temperature and a relative humidi-

ty >95%.[31] In the preparation chamber of the Vitrobot a 3 microliter sample was applied on a Quantifoil grid (R 2/2, Quantifoil Micro Tools GmbH; freshly glow discharged just prior to use), excess liquid was blotted away, and the thin film thus formed was shot (acceleration about 3 g) into melting ethane. The vitrified film was transferred to a cryoholder (Gatan 626) and observed at −170°C in a Philips CM 12 microscope operating at 120 kV. Micrographs were taken at low-dose conditions.

Electrophoretic migration shift assay: A slightly modified literature procedure was used to perform PAGE in 0.75 mm thick slab gels.[23] No stacking gels were used. Separating gels contained 10% acrylamide and 0.27% bis-acrylamide in 1.5 M Tris-HCl (pH 8.8). Aqueous 25 mM Tris-HCl, 192 mM glycine (pH 8.3) solution was used as the migration buffer. Gels were stained with Coomassie brilliant blue.

Inclusion experiments: For these experiments we selected the fluorescent dye 4(5)-carboxyfluorescein as it is negatively charged and is thought to have little interaction with the negatively charged enzyme. To exclude any effect of the dye on the reconstitution process, we first investigated the reconstitution of apo-HRP with its natural heme cofactor in the presence of several concentrations of 4(5)-carboxyfluorescein. The concentrations of dye used were 1.1×10^{-8}, 1.1×10^{-7}, and 1.1×10^{-6} M, the last concentration having an absorption of 0.1 as determined with UV spectroscopy. In all cases we observed a lower activity for the HRP reconstituted in the presence of dye relative to the blank (apo-HRP reconstituted with its native cofactor in the absence of the dye). For the lowest concentrations of dye the enzymatic activity was 62.5% of the blank. For the 1.1×10^{-6} M dye solution 45% of enzymatic activity was regained. Subsequently apo-HRP was reconstituted with **1e** as described above in the presence of 4(5)-carboxyfluorescein (1.1×10^{-6} M). After 4 days the solution was dialyzed (300 kDa MWCO, Spectra/Por) against phosphate buffer solution (20 mM, pH 7.5) and the decrease in 4(5)-carboxyfluorescein concentration was monitored using fluorescence spectroscopy ($\lambda_{ex}=492$ nm, $\lambda_{em}=516$ nm). Next, samples of the remaining aggregate dispersion were analyzed by aqueous gel permeation chromatography (GPC) using a Superdex 200 PC 3.2/30 (Amersham Pharmacia Biotech) column. To release the dye from the aggregates a sample of the aggregate dispersion was diluted with pure water (9 volumes) and analyzed by aqueous GPC upon standing for 5 min.

Enzymatic activity assays: Enzymatic activities were studied by using an ABTS/H_2O_2 assay. An aqueous H_2O_2 solution (0.05 mL, 0.07%) was added to a solution of ABTS (2.2 mL, 3.3×10^{-4} M). Finally, the respective reconstitution mixtures (0.05 mL) were added, upon which the enzymatic activity was monitored for 60 s by using UV/Vis spectroscopy ($\lambda_{max}=420$ nm). The enzymatic activities of apo-HRP at 4°C and at 22°C monitored during the time span of the reconstitution were taken as blanks. The enzymatic activity of HRP was monitored in the same way.

Oxygen binding studies: A solution of (modified) Mb (0.00015 mM) in aqueous potassium phosphate buffered solution (2 mL, 20 mM, pH 7.5, 2 mL) was placed in a cell equipped with an injectable screw cap, degassed under vacuum for 1 h, and brought to atmospheric pressure with argon. Subsequently, an aqueous sodium dithionite solution (20 μL, 0.011 M) was added and the solution was shaken for 10 s. Oxygen was passed through the solution and the solution was exposed to air and left to stand for autoxidation. During the experiments the cell was kept at 25°C. Changes were monitored by UV/Vis spectroscopy.

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[1] M. C. Feiters, R. J. M. Nolte in *Advances in Supramolecular Chemistry, Vol. 6* (Ed.: G. W. Gokel), JAI, Stamford, **2000**, pp. 41–156.

[2] a) I. Gitsov, K. L. Wooley, J. M. J. Fréchet, *Angew. Chem.* **1992**, *104*, 1282; *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 1200; b) J. C. M. v. Hest, M. W. P. L. Baars, D. A. P. Delnoye, M. H. P. v. Genderen, E. W. Meijer, *Science* **1995**, *268*, 1592; c) J. P. Spatz, S. Mößmer, M. Möller, *Angew. Chem.* **1996**, *108*, 1673; *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 1510; d) J. J. L. M. Cornelissen, M. Fischer, N. A. J. M. Sommerdijk, R. J. M. Nolte, *Science* **1998**, *280*, 1427; e) D. M. Vriezema, J. Hoogboom, K. Velonia, K. Takazawa, P. C. M. Chistianen, J. C. Maan, A. E. Rowan, R. J. M. Nolte, *Angew. Chem.* **2003**, *115*, 796; *Angew. Chem. Int. Ed.* **2003**, *42*, 772.

[3] a) J. M. Hannink, J. J. L. M. Cornelissen, J. A. Farrera, P. Foubert, F. C. De Schryver, N. A. J. M. Sommerdijk, R. J. M. Nolte, *Angew. Chem.* **2001**, *113*, 4868; *Angew. Chem. Int. Ed.* **2001**, *40*, 4732; b) K. Velonia, A. E. Rowan, R. J. M. Nolte, *J. Am. Chem. Soc.* **2002**, *124*, 4224; c) M. J. Boerakker, J. M. Hannink, P. H. H. Bomans, P. M. Frederik, R. J. M. Nolte, E. M. Meijer, N. A. J. M. Sommerdijk, *Angew. Chem.* **2002**, *114*, 4413; *Angew. Chem. Int. Ed.* **2002**, *41*, 4239.

[4] K. L. Heredia, D. Bontempo, T. Ly, J. T. Byers, S. Halstenberg, H. D. Maynard, *J. Am. Chem. Soc.* **2005**, *127*, 16955.

[5] a) For a review on chemical modifications of proteins by the cofactor reconstitution method, see: I. Hamachi, S. Shinkai, *Eur. J. Org. Chem.* **1999**, 539; more recent reports: b) T. Hayashi, Y. Hitomi, T. Ando, T. Mizutani, Y. Hisaeda, S. Kitagawa, H. Ogoshi, *J. Am. Chem. Soc.* **1999**, *121*, 7747; c) K. Kobayashi, T. Nagamune, T. Furuno, H. Sasabe, *Bull. Chem. Soc. Jpn.* **1999**, *72*, 691; d) I. Hamachi, H. Takashima, Y.-Z. Hu, S. Shinkai, S. Oishi, *Chem. Commun.* **2000**, 1127; e) A. D. Ryabov, V. N. Goral, L. Gorton, E. Csöregi, *Chem. Eur. J.* **1999**, *5*, 961; f) A. Riklin, E. Katz, I. Willner, A. Stocker, A. F. Bückmann, *Nature* **1995**, *376*, 672; g) R. Blonder, E. Katz, I. Willner, V. Wray, A. F. Bückmann, *J. Am. Chem. Soc.* **1997**, *119*, 11747.

[6] a) For a review containing several examples of surface modification by the cofactor reconstitution method, see: I. Willner, E. Katz, *Angew. Chem.* **2000**, *112*, 1230; *Angew. Chem. Int. Ed.* **2000**, *39*, 1180; b) H. Zimmermann, A. Lindgren, W. Schuhmann, L. Gorton, *Chem. Eur. J.* **2000**, *6*, 592; c) E. Katz, A. F. Bückmann, I. Willner, *J. Am. Chem. Soc.* **2001**, *123*, 10752.

[7] a) A. Suzuki, K. Okuda, K. Kawagoe, Y. Aoyama, H. Ogoshi, *Chem. Lett.* **1985**, 1169; b) T. Hayashi, Y. Hitomi, H. Ogoshi, *J. Am. Chem. Soc.* **1995**, *117*, 11606.

[8] G. N. Phillips, Jr. in *Handbook of Metalloproteins, Vol. 1* (Eds.: A. Messerschmidt, R. Huber, T. Poulos, K. Wieghardt), Wiley, Chichester, **2001**, pp. 5–15, and references therein.

[9] a) J. J. Leonard, T. Yonetani, J. B. Callis, *Biochemistry* **1974**, *13*, 1460; b) A. W. Axup, M. Albin, S. L. Mayo, R. J. Crutchley, H. B. Gray, *J. Am. Chem. Soc.* **1988**, *110*, 435; c) E. Zahavy, I. Willner, *J. Am. Chem. Soc.* **1996**, *118*, 12499; d) T. Yonetani, T. Asakura, *J. Biol. Chem.* **1969**, *244*, 4580; e) J. A. Cowan, H. B. Gray, *Inorg. Chem.* **1989**, *28*, 2074; f) S. Aono, S. Nemoto, A. Ohtaka, I. Okura, *J. Mol. Catal. A* **1995**, *95*, 193.

[10] a) M. Sono, T. Asakura, *J. Biol. Chem.* **1975**, *250*, 5227; b) H. Ogoshi, K. Kawabe, S. Mitachi, Z.-I. Yoshida, K. Imai, I. Tyuma, *Biochim. Biophys. Acta* **1979**, *581*, 266; c) E. D. Coulter, M. Sono, C. K. Chang, O. Lopez, J. H. Dawson, *Inorg. Chim. Acta* **1995**, *240*, 603; d) S. Neya, N. Funasaki, N. Igarashi, A. Ikezaki, T. Sato, K. Imai, N. Tanaka, *Biochemistry* **1998**, *37*, 5487; e) T. Hayashi, T. Takimura, Y. Aoyama, Y. Hitomi, A. Suzuki, H. Ogoshi, *Inorg. Chim. Acta* **1998**, *275–276*, 159; f) E. Monzani, G. Alzuet, L. Casella, C. Redaelli, C. Bassani, A. M. Sanangelantoni, M. Gulotti, L. De Gioia, L. Santagostini, F. Chillemi, *Biochemistry* **2000**, *39*, 9571; g) T. Hayashi, T. Matsuo, Y. Hitomi, K. Okawa, A. Suzuki, Y. Shiro, T. Iizuka, Y. Hisaeda, H. Ogoshi, *J. Inorg. Biochem.* **2002**, *91*, 94.

[11] I. Hamachi, K. Nakamura, A. Fujita, T. Kunitake, *J. Am. Chem. Soc.* **1993**, *115*, 4966.

[12] T. Hayashi, Y. Hitomi, H. Ogoshi, *J. Am. Chem. Soc.* **1998**, *120*, 4910.
[13] a) I. Hamachi, T. Matsugi, K. Wakigawa, S. Shinkai, *Inorg. Chem.* **1998**, *37*, 1592; b) I. Hamachi, Y. Tajiri, S. Shinkai, *J. Am. Chem. Soc.* **1994**, *116*, 7437; c) I. Hamachi, T. Matsugi, S. Tanaka, S. Shinkai, *Bull. Chem. Soc. Jpn.* **1996**, *69*, 1657; d) I. Hamachi, Y. Tajiri, T. Nagase, S. Shinkai, *Chem. Eur. J.* **1997**, *3*, 1025; e) I. Hamachi, S. Tanaka, S. Shinkai, *J. Am. Chem. Soc.* **1993**, *115*, 10458; f) I. Hamachi, K. Nomoto, S. Tanaka, Y, Tajiri, S. Shinkai, *Chem. Lett.* **1994**, 1139; g) H. Murakami, Y. Okusa, S. Kiyonaka, I. Hamachi, S. Shinkai, N. Nakashima, *Chem. Lett.* **2000**, 46; h) Y.-Z. Hu, H. Takashima, S. Tsukjij, S. Shinkai, T. Nagamune, S. Oishi, I. Hamachi, *Chem. Eur. J.* **2000**, *6*, 1907; i) I. Hamachi, S. Tanaka, S. Tsukjij, S. Shinkai, S. Oishi, *Inorg. Chem.* **1998**, *37*, 4380; j) Y.-Z. Hu, S. Tsukiji, S. Shinkai, S. Oishi, I. Hamachi, *J. Am. Chem. Soc.* **2000**, *122*, 241; k) I. Hamachi, S. Tsujiki, S. Shinkai, S. Oishi, *J. Am. Chem. Soc.* **1999**, *121*, 5500; l) Y. Hitomi, T. Hayashi, K. Wada, T. Mizutani, Y. Hisaeda, H. Ogoshi, *Angew. Chem.* **2001**, *113*, 1132; *Angew. Chem. Int. Ed.* **2001**, *40*, 1098; m) T. Hayashi, Y. Hisaeda, *Acc. Chem. Res.* **2002**, *35*, 35.
[14] a) A. C. Maehly, *Nature* **1961**, *192*, 630; b) M. Tamura, T. Asakura, T. Yonetani, *Biochim. Biophys. Acta* **1972**, *268*, 292; c) R. K. DiNello, D. H. Dolphin, *J. Biol. Chem.* **1981**, *256*, 6903; d) S. Adak, R. K. Banerjee, *Biochem. J.* **1998**, *334*, 51; e) R. Makino, I. Yamazaki, *J. Biochem.* **1972**, *72*, 655.
[15] a) J. N. Israelachvili, D. J. Mitchell, B. W. Winham, *J. Chem. Soc. Faraday Trans. 2* **1976**, 1525; b) J. N. Israelachvili, S. Marcelja, R. G. Horn, *Q. Rev. Biophys.* **1980**, *13*, 121.
[16] a) S. J. Smerdon, S. Krzywda, A. J. Wilkinson, R. E. Brantley, Jr., T. E. Carver, M. S. Hargrove, J. S. Olson, *Biochemistry* **1993**, *32*, 5132; b) G. N. La Mar, S. D. Emerson, J. T. J. Lecomte, U. Pande, K. M. Smith, G. W. Craig, L. A. Kehres, *J. Am. Chem. Soc.* **1986**, *108*, 5568; c) J. B. Hauksson, G. N. La Mar, R. K. Pandey, I. N. Rezzano, K. M. Smith, *J. Am. Chem. Soc.* **1990**, *112*, 6198.
[17] F. W. Teale, *Biochim. Biophys. Acta* **1959**, *35*, 543; apo-HRP was prepared according to this procedure by using butanone instead of acetone.
[18] N. P. Groome, *J. Clin. Chem. Clin. Biochem.* **1980**, *18*, 345.
[19] a) H. Hoffmann, C. Thunig, U. Munkert, H. Meyer, W. Richter, *Langmuir* **1992**, *8*, 2629; b) M. Almgren, *Biochim. Biophys. Acta* **2000**, *1508*, 146, and references therein.
[20] M. Dubois, B. Demé, T. Gulik-Krzywicki, J.-C. Dedieu, C. Vautrin, S. Désert, E. Perez, T. Zemb, *Nature* **2001**, *411*, 672.
[21] Longer reconstitution times resulted in a decrease in activity. When the reconstitution was performed at 4 °C no enzymatic activity was observed. Also the use of other cofactor solvents such as DMF did not result in an increase of enzymatic activity, rather a precipitate indicative of denaturation was observed after 4 days.
[22] When **1e** was incubated with glucose oxidase (GOx) and 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 **1e** incubated with apo-HRP is not caused by the interaction of **1e** with amino acid residues on the apo-HRP periphery.
[23] M. Arnold, P. Ringler, A. Brisson, *Biochim. Biophys. Acta* **1995**, *1233*, 198.
[24] The scattering may be also partially caused by the presence of some denaturated protein.
[25] T. Hayashi, Y. Hitomi, A. Suzuki, T. Takamura, H. Ogoshi, *Chem. Lett.* **1995**, 911.
[26] A. A. Choucair, A. H. Kycia, A. Eisenberg, *Langmuir* **2003**, *19*, 1001.
[27] S. V. Evans, G. D. Brayer, *J. Mol. Biol.* **1990**, *213*, 885.
[28] Carboxy-terminated polystyrene was synthesized according to a literature procedure: J. C. M. v. Hest, D. A. P. Delnoye, M. W. P. L. Baars, C. Elissen-Román, M. H. P. v. Genderen, E. W. Meijer, *Chem. Eur. J.* **1996**, *2*, 1616–1626.
[29] a) R. Zuckermann, E. Martin, D. Spellmeyer, G. Stauber, K. Shoemaker, J. Kerr, G. Figliozzi, D. Goff, M. Siani, R. Simon, S. Banville, E. Brown, L. Wang, L. Richter, W. Moos, *J. Med. Chem.* **1994**, *37*, 2678–2685; b) E. Tsuchida, H. Nishide, Y. Sato, M. Kaneda, *Bull. Chem. Soc. Jpn.* **1982**, *55*, 1890–1895.
[30] T. Asakura, *Methods Enzymol.* **1978**, 447.
[31] P. M. Frederik, D. H. W. Hubert, *Methods Enzymol.* **2005**, *391*, 431–448.

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