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Self-Assembled Architectures from Biohybrid Triblock Copolymers

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Abstract: The synthesis and self-assembly behavior of biohybrid ABC triblock copolymers consisting of a synthetic diblock, polystyrene-*b*-polyethylene glycol (PS_m-*b*-PEG_{113}), where *m* is varied, and a hemeprotein, myoglobin (Mb) or horse radish peroxidase (HRP), is described. The synthetic diblock copolymer is first functionalized with the heme cofactor and subsequently reconstituted with the apoprotein or the apoenzyme to yield the protein-containing ABC triblock copolymer. The obtained amphiphilic block copolymers self-assemble in aqueous solution into a large variety of aggregate structures. Depending on the protein and the polystyrene block length, micellar rods, vesicles, toroids, figure eight structures, octopus structures, and spheres with a lamellar surface are formed.

Introduction

Amphiphiles are technologically important compounds and therefore have been the subject of intensive studies for many years. The classical low molecular weight structures (i.e., soaps and phospholipids) are known to form a broad range of structures when dispersed in water, for example, micelles, vesicles, and lamellar structures.[1] More recently, the self-assembly behavior of so-called super amphiphiles, that is, molecules consisting of a hydrophilic and a hydrophobic polymeric block, has been reported, and it was found that they form structures similar to those of their low molecular weight counterparts in selective solvents.[2,3] An advantage of using block copolymers is that the obtained assemblies are very stable in contrast to the assemblies of low molecular weight amphiphiles. Furthermore, by varying the length and composition of the blocks in the block copolymer, the shape of the amphiphile can be varied and hence the structure of the aggregate. In addition to the classic geometries such as micelles, vesicles, and rods, also other types of aggregates have been observed for super amphiphiles, for example, helices.[4] A new class of amphiphilic macromolecules are the biohybrid giant amphiphiles, which are composed of a protein or enzyme head group and a hydrophobic polymer tail, and different routes toward the synthesis of these compounds, for example, solid-phase synthesis and click chemistry, have been explored.[3f,5] Physical studies have revealed that these biohybrid block copolymers also self-assemble into diverse structures, while the biomolecule component remains catalytically active.

Amphiphilic triblock copolymers have received less attention than their diblock counterparts, but recent studies show that they can also assemble into complex architectures.[6] For example, a polystyrene-*b*-polyvinylpyridine-*b*-polyethyleneglycol triblock copolymer was found to form micelllar structures, which in time reorganized to yield segmented wormlike micelles.[7] Another example involves a poly(acrylic acid)-*b*-polymethylacrylate-*b*-polystyrene ABC triblock copolymer, which initially self-assembled into micellar structures in THF−water, but evaporation of the THF from the solvent mixture gave ringlike structures. Furthermore, the size and stability of the latter assemblies could be influenced by changing the valency and concentration of the counterion.[6a] In contrast to diblock copolymers, little research has been carried out on ABC triblock copolymers containing a biomacromolecular segment, and it

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would be of interest to investigate the aggregation behavior of such biomacromolecular systems and to determine whether the incorporation of the (functional) biomacromolecule leads to the same kind of self-organizational complexity.[3b,8] To our knowledge, ABC triblock copolymers containing a protein or enzyme block have not yet been reported.

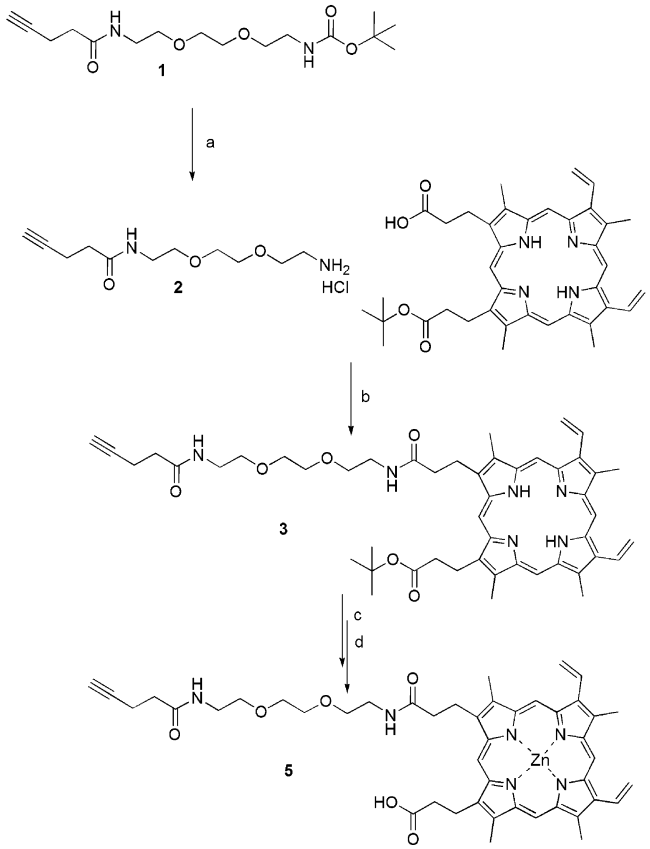
In this paper, we describe the self-assembly behavior of four biohybrid triblock copolymers, which were synthesized via direct coupling of a synthetic diblock copolymer, polystyrene-*b*-polyethylene glycol (PS_m-*b*-PEG_{113}), prepared using atom transfer radical polymerization (ATRP), to a hemeprotein by the Cu(I) catalyzed azide-alkyne [3+2] cycloaddition reaction (also called "click" reaction) and subsequent cofactor reconstitution. The latter method was selected because previous studies had revealed that the cofactor, protoporphyrinIX (PPIX), could be functionalized easily by conventional synthetic chemistry.[5a,d,9] Furthermore, the reconstitution method has been demonstrated to be applicable to a range of functional proteins, many of them with interesting properties,[9] and two biohybrid diblock copolymers have already successfully been made and studied by our group employing this procedure.[5a,d] The combination of ATRP and the click reaction has successfully been used in recent studies to attach synthetic polymers to biorelevant macromolecules.[10] As will be shown, the obtained ABC biohybrid triblock copolymers self-assemble into various structures, among which are the classical aggregates such as micelles and vesicles, but also unusual structures such as Y junctions, octopus structures, figure eight structures, and lamellae-containing spheres.

Results and Discussion

Synthesis. The triblock copolymers described in this work consist of polyethylene glycol, polystyrene, and a hemeprotein, that is, myoglobin (Mb) or horse radish peroxidase (HRP). The block copolymer polystyrene-*b*-polyethylene glycol (PS_m-*b*-PEG_n) was chosen because it is known that this macromolecule, depending on the ratio between the two different blocks, can phase separate into various structures.[4] Furthermore, the PEG block makes the polymer more water soluble, facilitating the reconstitution of the apoenzyme (see below).

Following the general synthetic strategy, monomethoxy polyethylene glycol was functionalized with an ATRP initiator, and subsequently styrene was polymerized using this initiator and PMDETA/CuBr as the ligand−metal complex (see experimental section). The initiator/monomer ratio was varied to obtain the desired block ratios (see Supporting Information for different polymers that were synthesized). After the ATRP, the terminal bromine of the block copolymers was converted into an azide function using a literature procedure.[11]

Scheme 1. Synthetic Route to Acetylene-Functionalized PPIXZn[a]



[a] (a) HCl/EtOAc, (b) py-BOP, DIPEA, THF/DMF 1:1 v/v, o.n., (c) TFA, formic acid, 6 h, (d) $Zn(OAc)_2$, THF/DMF 1:3 v/v, o.n.

The PPIX was functionalized by first protecting one of its carboxylic acid groups with a *tert*-butyl ester function.[12] Subsequently, an acetylene-functionalized spacer (**2**) was attached using standard conditions. After deprotection of the *tert*-butyl ester with trifluoroacetic acid and formic acid, zinc was inserted in the PPIX using zinc acetate in DMF/THF (see Scheme 1), yielding the acetylene derived PPIXZn **5**. The cofactor contains only one modified carboxylic acid group because it is expected that this will allow the protein or enzyme to keep its activity as compared to systems in which the cofactor bears substituents on both carboxylic acid groups.[5a,d,13] Furthermore, an ethylene glycol spacer was introduced between the acetylene function and the cofactor, to improve the compatibility of the polymer with the enzyme as is known from earlier experiments in which it was shown that reconstitution times were shorter and higher enzymatic activities were obtained when a polar spacer of at least 10 Å was used.[5a,d,14]

Finally, the acetylene-functionalized PPIX (**5**) could be linked to the block copolymers by a Cu(I)-mediated click reaction in THF (see Scheme 2). Initial attempts to perform the Cu(I)-mediated click reaction, using $CuSO_4$/ascorbic acid in water/THF mixtures, were unsuccessful because of insertion of the copper into the porphyrin. Therefore, we examined the strong CuBr/PMDETA complex as the catalyst in THF at 35 °C, which

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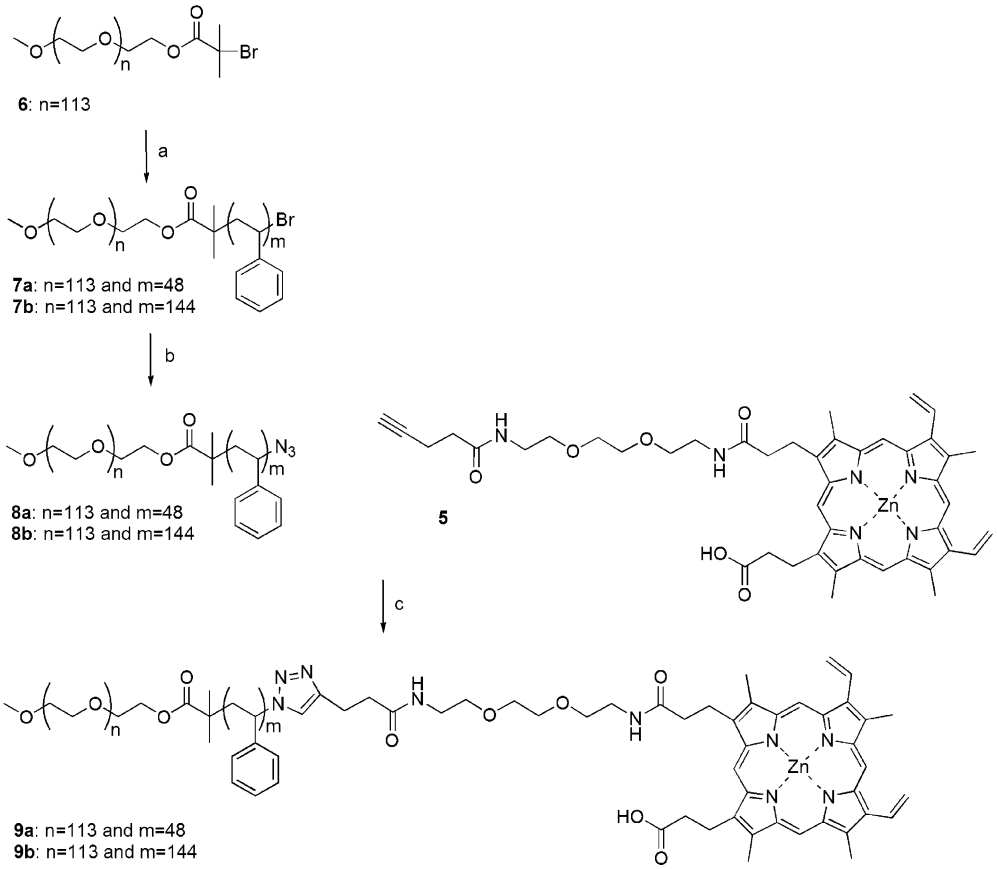
Scheme 2. Synthetic Route to PPIXZn-*b*-PS_m-*b*-PEG_n[a]



[a] (a) Styrene, PMDETA/CuBr, $T = 90$ °C, (b) $TMSN_3$, THF, o.n., (c) PMDETA/CuBr, THF, $T = 35$ °C, o.n.

turned out to be successful.[15] Using the porphyrin having Zn complexed in the core is another, preferred, way of preventing copper insertion. Gel permeation chromatography (GPC) studies monitored both at $\lambda = 418$ nm and at $\lambda = 254$ nm, the wavelengths where PPIX and polystyrene display maximum absorption intensities, respectively, showed that PPIX and the polymer elute at the same time, without notable increase in polydispersity, indicating that the blocks were connected in a highly efficient manner (see Supporting Information).[16]

Aggregation experiments, in which 100 μL of a 0.3 mg/mL polymer solution in THF was injected into 1 mL of water, revealed that the PS_m-*b*-PEG_{113} block copolymers formed micelles and micellar rods depending on the length of the two polymeric blocks. When the PS block length in PS_m-*b*-PEG_{113} was increased, micellar rods were observed (Figure 1A and B). Similar observations have been made in the case of low molecular weight amphiphiles.[17] The triblock copolymers, PPIXZn-*b*-PS_m-*b*-PEG_{113} (**9a** and **9b**, see Scheme 2), were dissolved in THF (0.3 mg/mL), and 100 μL of this solution was dispersed in 1 mL of phosphate buffer (20 mM, pH 7.5). TEM studies showed the formation of assemblies resembling multicomponent vesicles (MCV's)[18] for different ratios of the PS and PEG blocks (not shown).

Reconstitution. Reconstitution experiments were carried out with both apo-Mb and apo-HRP and different PPIXZn-*b*-PS_m-*b*-PEG_{113} triblock copolymers (see Table 1 and Figure 2). It is known that apart from PPIXFe (heme) also PPIXZn can insert into Mb.[19] The PPIXZn constituted proteins do not show catalytic activity, but MbZn in contrast to native Mb is fluorescent, allowing the use of fluorescence spectroscopy, which is a more sensitive method than UV−vis spectroscopy, to monitor the progress of the reaction. The cofactor PPIXZn, however, has not been reconstituted with apo-HRP before. To check if reconstitution of PPIXZn with apo-HRP is possible, unfunctionalized PPIXZn ($\lambda_{max} = 420$ nm in phosphate buffer, pH 7.5) was treated with the apoenzyme using the same procedure as for the preparation of giant amphiphiles. After dialysis, using 3 kD tubing to remove unreconstituted PPIXZn, an absorption maximum at $\lambda = 416$ nm was observed. Excitation at $\lambda = 547$ nm gave rise to emission maxima at $\lambda = 590$ and

(15) Performing the reaction at elevated temperature gives rise to insertion of copper into the free base PPIX in agreement with earlier studies. See: de Loos, F.; Reynhout, I. C.; Cornelissen, J. J. L. M.; Rowan, A. E.; Nolte, R. J. M. *Chemm. Commun.* **2005**, 60−62.

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(20) Teale, F. W. *Biochim. Biophys. Acta* **1959**, *35*, 543. Apo-Mb was prepared according to this procedure using butanone instead of acetone.

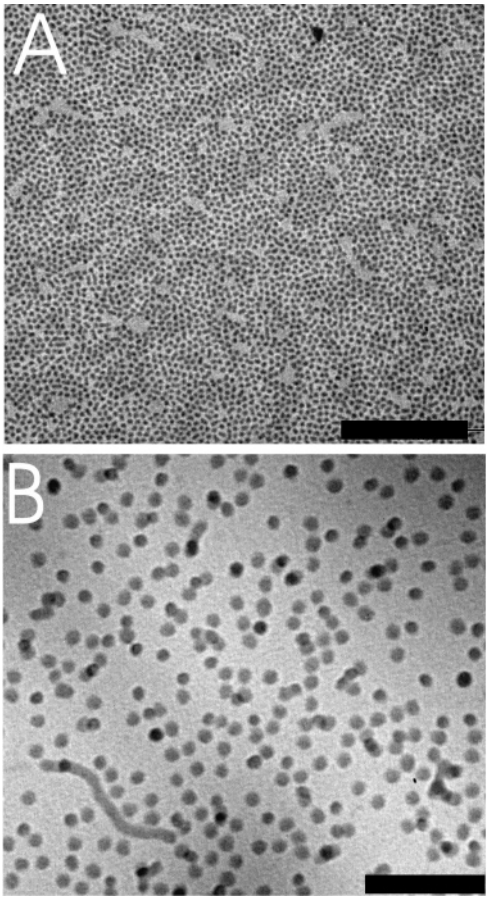

Figure 1. (A) Micellar aggregates of PEG_{113}-*b*-PS_{48}-N_3, and (B) micellar aggregates of PEG_{113}-*b*-PS_{144}-N_3. Bars represent 200 nm.

Table 1. Amphiphilic Triblock Copolymers

apo protein	polymer	triblock copolymer
apo-Mb	PPIXZn-*b*-PS_{48}-*b*-PEG_{113}	MbZn-*b*-PS_{48}-*b*-PEG_{113}
apo-Mb	PPIXZn-*b*-PS_{144}-*b*-PEG_{113}	MbZn-*b*-PS_{144}-*b*-PEG_{113}
apo-HRP	PPIXZn-*b*-PS_{48}-*b*-PEG_{113}	HRPZn-*b*-PS_{48}-*b*-PEG_{113}
apo-HRP	PPIXZn-*b*-PS_{144}-*b*-PEG_{113}	HRPZn-*b*-PS_{144}-*b*-PEG_{113}

$\lambda = 630$ nm. Both observations point to a successful reconstitution of PPIXZn in the apo-HRP.

The reconstitution of the polymers PPIXZn-*b*-PS_m-*b*-PEG_{113} with apoprotein was carried out as described by Boerakker et al. using slightly different conditions.[5a,d] Instead of the mixture of polymer and apoenzyme being stirred in a glass vial, the reconstitution mixture was gently shaken in a plastic tube. This prevents absorption of the protein to the glass wall, reducing the chance of denaturation. Earlier experiments had revealed that upon reconstitution of PS-*b*-PPIX with apoprotein firm stirring was needed, otherwise the polymer would precipitate from solution and reconstitution would not occur. During the process of reconstitution with the apoenzyme, stable aggregates are gradually obtained. In the case of PPIXZn-*b*-PS_m-*b*-PEG_{113}, the PEG part is water soluble, and, as a consequence, no stirring is needed and the triblock copolymer will stay in solution. After 4 days of incubation, the reaction mixture was dialyzed against a phosphate buffer (20 mM, pH7.5) using 100 kDa dialysis tubing to allow the apo-enzymes and non-reconstituted polymers to pass the membrane, while the amphiphilic triblock copolymers remain inside.

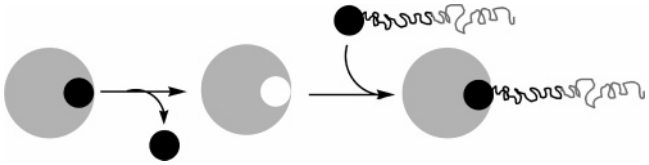

Figure 2. Schematic representation of the reconstitution method. In the first step, the cofactor is removed by extraction at pH = 2. In the second step, the modified cofactor is added at pH = 7.5 to obtain the giant amphiphile.[20]

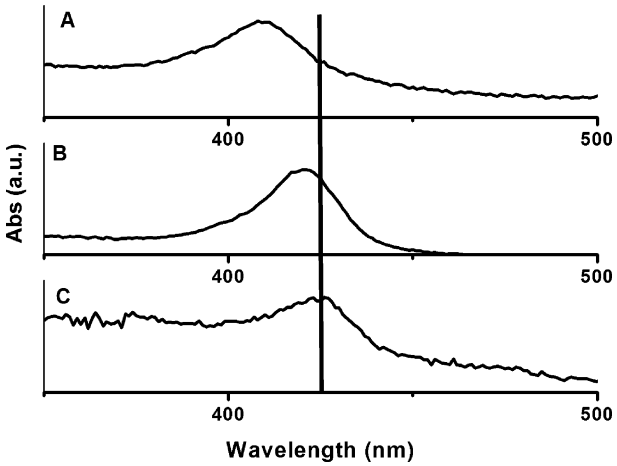

Figure 3. UV−vis spectra of (A) Mb with $\lambda_{max} = 409$ nm, (B) PPIXZn-*b*-PS_{48}-*b*-PEG_{113} with $\lambda_{max} = 420$ nm, and (C) MbZn-*b*-PS_{48}-*b*-PEG_{113} with $\lambda_{max} = 426$ nm. All spectra are recorded in a pH = 7.5 20 mM phosphate buffer. The UV−vis spectra of **9b** and MbZn-*b*-PS_{144}-*b*-PEG_{113} are identical to Figure 3B and C, respectively.

UV−vis spectra of the Mb-containing macromolecules are shown in Figure 3. After reconstitution with apo-Mb and purification, a shift of 6 nm is observed with respect to **9a** and **9b**, from $\lambda = 420$ nm to $\lambda = 426$ nm. This latter value is near the maximum absorption observed for MbZn.[21] Excitation of the suspension at $\lambda = 553$ nm caused an emission at $\lambda = 593$ nm, which is in agreement with literature data for MbZn.[21] Usually a second emission band is observed for this compound; however, this signal was not found in the present case due to scattering of the light by the suspension.

When the polymers **9a** and **9b** were reconstituted with apo-HRP, a small shift of 4 nm was observed after purification, from $\lambda = 420$ nm to $\lambda = 416$ nm, which is in agreement with the results described above for the reconstitution of PPIXZn (Figure 4). Fluorescence experiments were also in agreement with the reference compound; excitation at $\lambda = 547$ nm gave an emission at $\lambda = 590$ nm.

An electrophoresis migration-shift essay was carried out as final proof of the synthesis and purity of the amphiphilic ABC triblock copolymers (Table 2). Using sodium docecyl sulfonate polyacryl amide gel electrophoresis (SDS PAGE), the proteins are completely denaturated, and the interaction between the cofactor and the enzyme is lost. In all cases, a band, corresponding to the mass of Mb or HRP, was observed for both the native proteins and the giant amphiphiles (see Supporting Information), which is expected, because the polymers are no longer coordinated to the proteins after the denaturation step. With PAGE electrophoreses (i.e., using nondenaturating condi-

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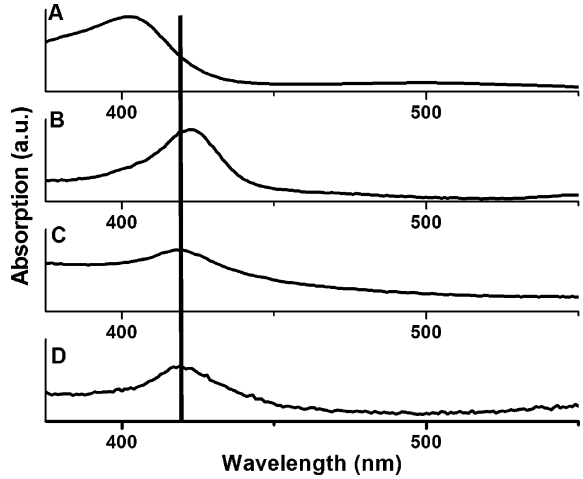

Figure 4. UV−vis spectra of (A) HRP with $\lambda_{max} = 401$ nm, (B) PPIXZn-*b*-PS_{48}-*b*-PEG_{113} with $\lambda_{max} = 420$ nm, (C) Blanc, that is, HRPZn with $\lambda_{max} = 416$ nm, and (D) HRPZn-*b*-PS_{48}-*b*-PEG_{113} with $\lambda_{max} = 416$ nm. All spectra are recorded in a pH = 7.5 20 mM phosphate buffer. The UV−vis spectra of **9b** and HRPZn-*b*-PS_{144}-*b*-PEG_{113} are identical to those in Figure 4B and D, respectively.

Table 2. Summary of the Gel-Electrophorese Studies

	apo proteins		bioconjugate block copolymers	
	Mb	HRP	Mb	HRP
SDS PAGE	visible[a]	visible[a]	visible[a]	visible[a]
PAGE	visible[a]	visible[a]	not visible[b]	not visible[b]

[a] Visible bands penetrating the gel were observed. [b] No bands penetrating the gel could be observed (see Supporting Information).

tions), proteins can be separated on the basis of their three-dimensional structures. The protein−cofactor interaction remains intact, and a difference is therefore expected between the native protein and the biohybrid macromolecule. Bands corresponding to the different proteins migrate in the electrophoresis, whereas, in line with earlier observations, the giant amphiphiles are too big to penetrate into the gel.[5a,d] The absence of protein in the latter cases furthermore indicates that all HRP and Mb is conjugated to the block copolymer, in combination with the spectroscopic data pointing to the successful formation of the giant triblock copolymers.

Aggregation Studies. To study the aggregation behavior of the different biohybrid triblock copolymers, electron microscopy (EM) experiments were carried out. After 4 days of incubation, the giant amphiphiles were dialyzed against a phosphate buffer to remove apoprotein and THF (see above). After dialysis, the water content is 100%, and the assemblies of the amphiphiles become kinetically trapped because of the glassy nature of the PS chains. Transmission electron microscopy (TEM) investigations (Figure 5, left) showed that the aggregates of HRPZn-*b*-PS_{48}-*b*-PEG_{113} are vesicular in nature and polydisperse in size. These types of structures have been reported for synthetic amphiphilic ABA triblock copolymers by Zhu et al.,[22] who suggested that, by varying the time of aggregation, that is, the reconstitution time in our case, and the concentration of the triblock copolymers, control over the vesicle size can be obtained. However, in our case, a shorter reconstitution time resulted in a lower yield of the biohybrid triblock copolymer,

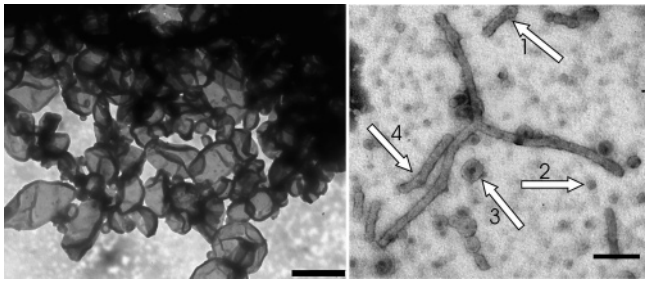

Figure 5. Transmission electron microscopy (TEM) pictures of vesicular aggregates of HRPZn-*b*-PS_{48}-*b*-PEG_{113} (left) and aggregates of HRPZn-*b*-PS_{144}-*b*-PEG_{113} (right). Arrows: 1, junction between micelle and micellar rod; 2, micelle; 3, toroid; and 4, junction between two micellar rods. Bars represent 200 nm.

and longer reconstitution time led to denaturation of the protein because the protein is in contact longer with THF.[5a] At this moment, it is not clear how the different blocks of the triblock copolymers are situated inside the vesicles. Likely the polystyrene is in the middle of the vesicle membrane, but whether the PEG or HRP block is on the outside of the aggregate or whether they are mixed, in which case the polystyrene block forms a hairpin, is not known at the moment. In the latter case, both hydrophilic segments reside at the aggregate surface, providing a more curved interface potentially leading to a cylindrical micelle. Comparable behavior for ABA triblock copolymers is well known.[23] More experiments, however, have to be carried out to get a detailed picture of the aggregate structure.

Increasing the polystyrene length with respect to the PEG block was found to change the aggregation behavior significantly (Figure 5, right). Instead of large polydisperse vesicular structures, micellar aggregates were observed for HRPZn-*b*-PS_{144}-*b*-PEG_{113}. The increase in length of the PS segment will also make it easier to form the hairpin formation, suggested above. There are multiple morphologies present presumably because the aggregates are kinetically frozen after removal of the cosolvent (i.e., THF). As a consequence of this process, also so-called intermediate assemblies can be observed by TEM. Multiple morphologies may also occur if the system is under equilibrium condition and in the boundary regime between single morphology domains of the phase diagram.

Among the more common micellar structures such as micellar rods and spherical micelles, Y-shaped structures were observed. The formation of these structures probably results from the collapse of cylindrical micelles both through end-to-end connection and by connection to the midsection of a neighboring cylinder (or itself).[6a] Arrow 1 in Figure 5 indicates where a micellar sphere is connected to a micellar rod, which would strengthen this conclusion. The rod-to-sphere transition as postulated by Burke and Eisenberg[24] is an alternative mechanism that can explain these observations. Arrow 3 shows that besides micellar structures also toroidal structures are formed.

The formation of micellar structures by ABC triblock copolymers has been predicted by Wang et al. using the self-consistent field theory (SCFT); however, in this case, the hydrophilic part was relatively short and the other two blocks

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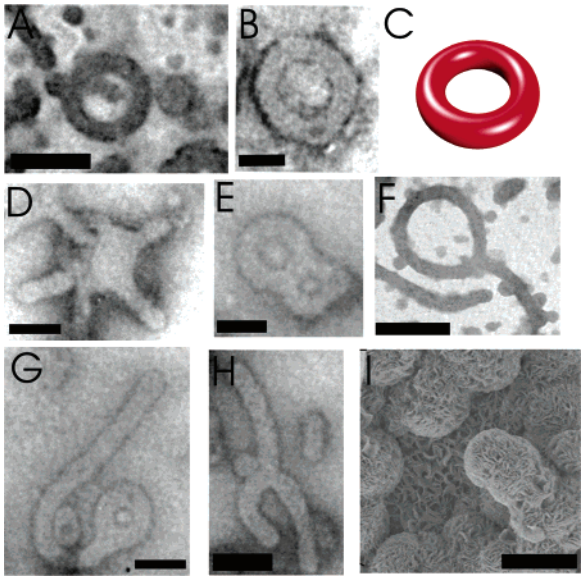

Figure 6. Different aggregates of Mb-containing giant triblock copolymers. TEM images of aggregates of MbZn-*b*-PS_{144}-*b*-PEG_{113} with (A,B) toroids, (C) schematic figure of a toroid, (D) octopi, (E) figure eights, and (F, G, and H[6a]) micellar aggregates. (I) Scanning EM (SEM) images of aggregates of MbZn-*b*-PS_{48}-*b*-PEG_{113} that form spherical aggregates consisting of lamellae. Bars represent 100 nm for Figure (A−H) and 500 nm for Figure I.

were hydrophobic.[25] Detailed studies are currently being carried out to see whether the number of formed aggregates can be decreased.

Micellar structures were also formed by MbZn-*b*-PS_{144}-*b*-PEG_{113}, but in this case a larger number of other types of aggregates was observed as well (Figure 6), for example, figure eight structures, octopus structures, dumbbells, toroids, etc. Pochan et al. also observed a large variety of structures, but by varying the conditions they could tune the aggregation behavior in such a way that, for example, only ringlike aggregates were obtained.[6a] Again, it is postulated that the formation of these aggregates proceeds via the mechanisms described above and that the aggregates are kinetically trapped or under equilibrium conditions in the boundary regime between single morphology domains.

When the PS part was shortened with respect to the other two blocks, the aggregation behavior changed dramatically. Figure 6I shows that the triblock copolymer now formed spherical aggregates, which possessed lamellae-like structures, which have not been observed before for polymeric amphiphiles. These structures can also be found in the background of the pictures. Energy-dispersive X-ray (EDX) analysis of these aggregates revealed that they were mainly made up of C, N, H-based material and that no salt was present that could otherwise explain the lamellae-like structures. The assemblies formed by MbZn-*b*-PS_{48}-*b*-PEG_{113} were found to be stable in time and could be reproduced using different batches of the material. At this moment, it is not clear how these aggregates are formed, and more research has to be carried out to obtain insight into the formation mechanism of these assemblies.

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Conclusion

We have prepared new macromolecular architectures, which are the first examples of biohybrid ABC triblock copolymers consisting of a protein and a synthetic diblock copolymer. With respect to the synthesis of these amphiphilic biohybrid macromolecules, a strategy combining controlled radical polymerization, "click" chemistry, and cofactor reconstitution has been used. This allowed the preparation of a variety of (tri)block copolymers, which were found to form complex and unusual nanometer-sized architectures by processes of self-organization. The HRP- and Mb-containing copolymers self-assembled into vesicles, micellar structures, or lamellae-containing spheres. From earlier experiments, it was known that amphiphilic diblock copolymers having a hydrophobic polystyrene segment and a myoglobin or HRP headgroup can generate spherical aggregates, presumably vesicles.[4a,d] Addition of a third (hydrophilic) segment to this biohybrid diblock copolymer apparently significantly changes the self-organization behavior of the macromolecular building blocks. The micellar aggregates formed by MbZn-*b*-PS_{144}-*b*-PEG_{113} and HRPZn-*b*-PS_{144}-*b*-PEG_{113} are not unexpected and have been observed before for synthetic ABC triblock copolymers.[5a] The Y-junctions and toroids are quite rare and are probably the result of a fusion process involving two micellar rods. The large number of different aggregates observed in one sample is unusual and may be explained by the fact that the aggregates become kinetically trapped after dialysis because of the glassy nature of polystyrene at ambient temperature. Another explanation is that the system is under equilibrium but situated in the coexisting region between two single morphology regimes. The precise orientation of the different blocks in the formed aggregates is currently under investigation. The present new macromolecular architectures open many possibilities for applications, for instance, as catalysts allowing cascade reactions to be performed if different types of enzymes are attached to the block copolymer structures. Work along this line is in progress.

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Supporting Information Available: Experimental data of the synthesis and characterization of the different compounds. GPC traces of the click reaction between **8** and **9** and the blanco experiments. DSC measurements of different block copolymers. Physical characteristics of different block copolymers. This material is available free of charge via the Internet at http://pubs.acs.org.

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