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β-Cyclodextrin-Appended Giant Amphiphile: Aggregation to Vesicle Polymersomes and Immobilisation of Enzymes

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Abstract: A giant amphiphile consisting of polystyrene end-capped with permethylated β-cyclodextrin was synthesised and found to form vesicular structures when injected as a solution in THF into water. The ability of the cyclodextrins on the surface of the polymersomes to form inclusion complexes with hydrophobic compounds was tested by carrying out a competition experiment with a fluorescent probe sensitive to the polarity of the surrounding medium. It was found that 1-adamantol can displace the fluorescent probe from the cavities of the cyclodextrin moieties of the polymersomes. The recognition of molecules by cell membranes in nature is often based on interactions with specific membrane receptors. To mimic this behaviour, the enzyme horseradish peroxidase was modified with adamantane groups through a poly(ethylene glycol) spacer and its interaction with the polymersomes was investigated. It was established that the presence of adamantane moieties on each enzyme allowed a host–guest interaction with the multifunctional surface of the polymersomes.

Keywords: amphiphiles · click reaction · cyclodextrins · enzyme immobilization · polymersomes

Introduction

There is increasing interest in self-assembled nanostructures of well-defined size and specific properties. β-Cyclodextrin (βCD) is a water-soluble receptor molecule with a hydrophobic interior,[1] which forms aggregates when appended with conventional (polymethylene) hydrophobic alkyl tails.[2] It occurred to us that βCD (diameter = 1.53 nm) might have the correct dimension to serve as a polar head group in functional amphiphiles based on polystyrene (PS),[3] which nowadays can be easily prepared by atom-transfer radical polymerisation (ATRP).[4,5] We have recently used the copper-catalysed [3+2] cycloaddition of acetylene and azide ("click" or Huisgen reaction)[6] to prepare conjugates of polymers and biomolecules and other types of compounds.[7,8] In previous studies we have also used βCD-azide derivatives as intermediates for the preparation of βCD dimers, which are excellent host molecules for different types of guests.[9,10] This click reaction, therefore, was our method of choice for the preparation of the target molecule, PS-appended permethylated βCD (**1**), from acetylene-end-capped PS (**2**)[7] and the monoazide βCD (**3**; Scheme 1).[11,12]

Vesicles of compound **1** can be used to mimic the process of recognition of biomolecules by the biological cell membrane, which is of interest to understand this phenomenon better and to be able to prepare vesicles decorated with functionalities that are specific for a certain target. As the outer and inner surfaces of these vesicles are covered with βCD receptors, they can also be used for the study of multivalent interactions, that is, the simultaneous binding of multiple ligands to one entity, which enhances binding affinity and selectivity.[13] We have previously found that polymersomes without additional functionalisation can bind enzyme molecules by non-specific, non-covalent interactions[14] or covalent bonds;[15] this allows them to be used for the preparation of nanoreactors, in which cascade reactions are performed by different enzymes. Herein we report on the non-covalent interaction of a chemically modified enzyme with the surface of multivalent polymersomes of **1**. The enzyme horseradish peroxidase (HRP; see Scheme 1 for a graphical representation) was functionalised with adamantane groups,

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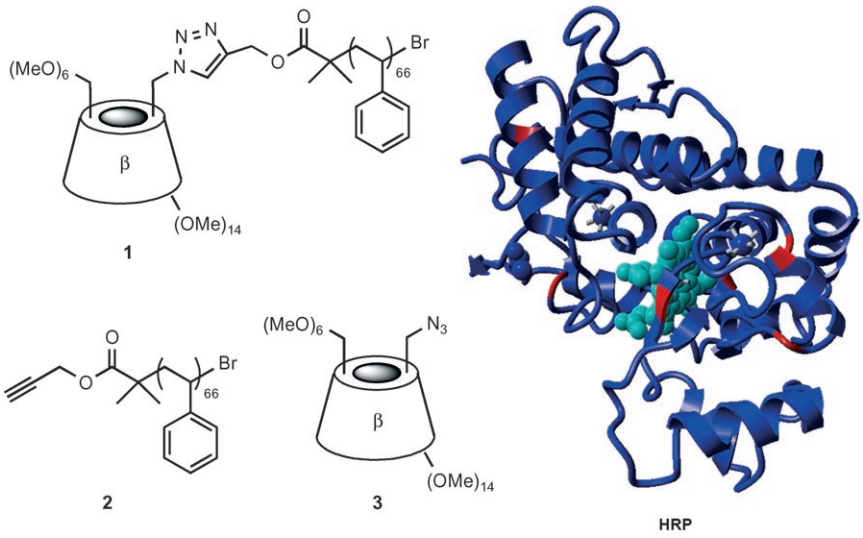


Scheme 1. Structures of **1**, **2**, **3** and the enzyme HRP. Red: lysines, light blue: haem group, dark blue: HRP tertiary structure.

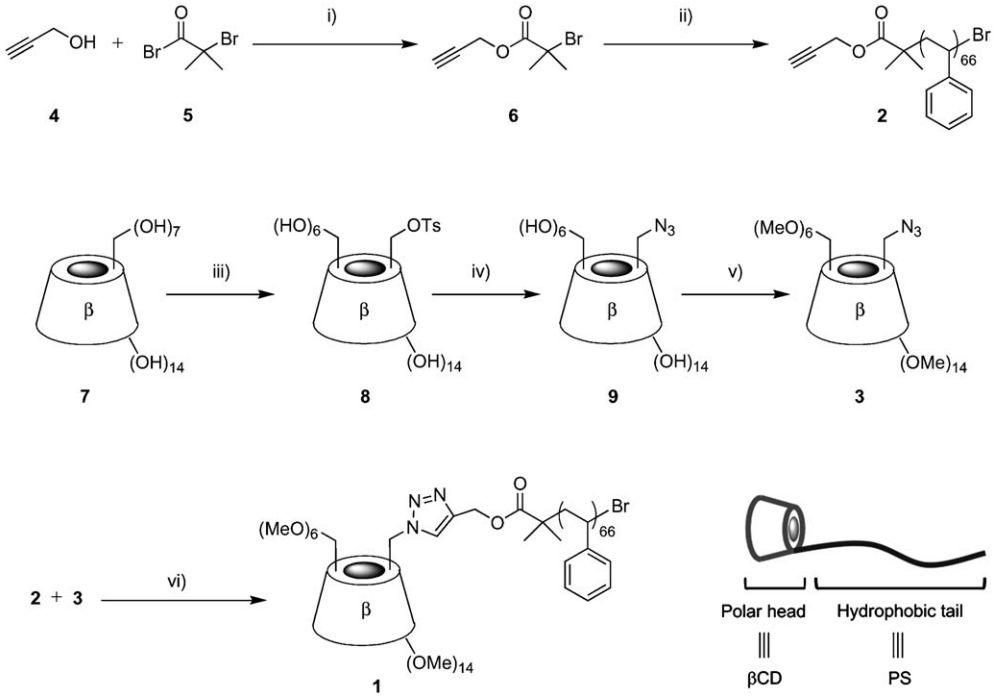
which are good guests for βCD, through a poly(ethylene glycol) (PEG) spacer. We found that HRP shows enhanced affinity for the polymersomes of **1** compared with non-functionalised HRP, and that it retains its catalytic activity when bound to the polymersome surface.

Results and Discussion

Synthesis and physical properties: Compound **2**[7] was prepared from propargyl alcohol (**4**) and bromide **5** to give **6**, which was then subjected to ATRP with styrene (Scheme 2). βCD derivative **3** was synthesised by a sequence of reactions involving monotosylation of βCD **7** on the primary side to give **8**, azide substitution to give **9** and finally a permethylation reaction.[11,12]

The product of the CuPMDTA-catalysed (PMDTA = *N*,*N*,*N*′,*N*′,*N*-pentamethyldiethylentriamine) click reaction between **2** and **3** was characterised by a variety of techniques. Infrared spectroscopy showed the disappearance of the vibrations characteristic of the azide and acetylene functional groups.

Mass spectrometry (Figure 1) and gel permeation chromatography (GPC; Figure 2) indicated that the molecular weight of the polymer had increased after the click reaction. It can be clearly observed that the molecular weight distribution of the functionalised polymer (Figure 1a) had shifted upward with respect to the starting material (Figure 1b). The average mass of **1** corresponds to the expected mass of 8450 g mol^{-1} [*M*+H] and the difference with the average mass of **2** (M_r = 7009) corresponds to the mass of **3** (M_r = 1441), which is the attached permethylated βCD.



Scheme 2. Preparation of **1** and its precursors **2** and **3**. Bottom right: schematic representation of **1**. Reagents and conditions: i) diethyl ether, pyridine, −30 °C→RT; ii) CuBr, PMDTA, anisole, 90 °C; iii) NaOH, TsCl, H_2O; iv) NaN_3, DMF, 60 °C; v) DMF, NaH, CH_3I; vi) CuBr, PMDTA, THF under N_2.

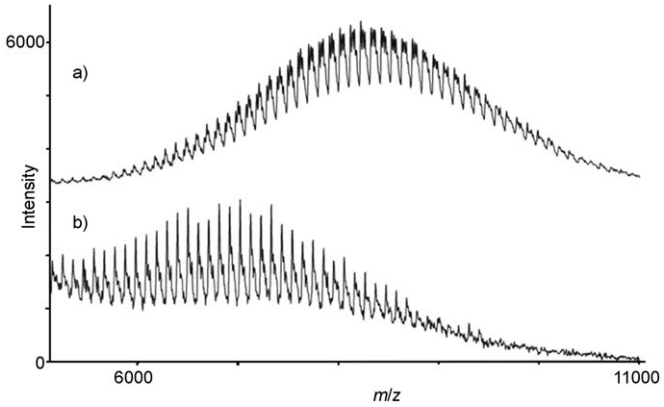


Figure 1. MALDI-TOF spectra of a) **1** and b) **2**.

In Figure 2, the GPC traces of PS-acetylene **2** and PS-βCD **1** are presented. For the latter polymer, the peak was narrower and slightly shifted to a higher molecular weight than **2**. This difference can be due in part to different interactions of the two polymers with the column, but it is consistent with the proposed formation of **1**.

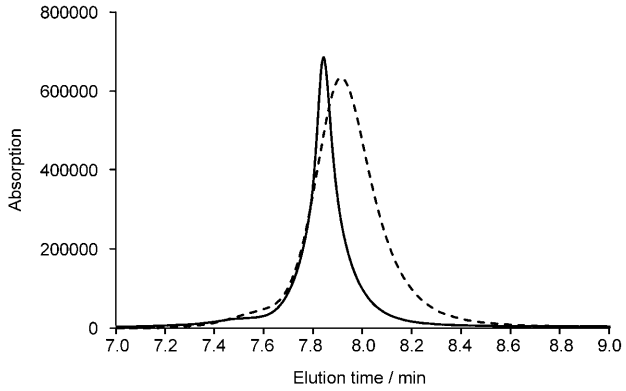


Figure 2. GPC traces of **1** (——) and **2** (-----) in THF at $\lambda = 254$ nm.

Aggregation studies: Injection of **1**, dissolved in THF, into water gave large vesicular structures (polymersomes) with a diameter of up to 10 μm. TEM micrographs (Figure 3A,B) revealed the presence of large circular structures with diameters ranging from 100 nm to 10 μm. SEM images (Figure 3C) confirmed the spherical nature of the structures.

To establish if these spherical structures were eventually completely closed or contained holes or defects, Cryo-SEM pictures were taken after six days (Figure 3D).

When the polymersomes were prepared at high temperature, some of the structures were partly opened and found to be hollow; this allowed the thickness of the outer layer to be determined (Figure 3E). This thickness (34 nm) was found to be approximately twice the length of a single extended molecule of **1** (17 nm), which suggested that the objects observed were polymersomes consisting of bilayers of molecules of **1**, presumably oriented with the apolar PS tails pointing towards each other and the polar βCD head groups

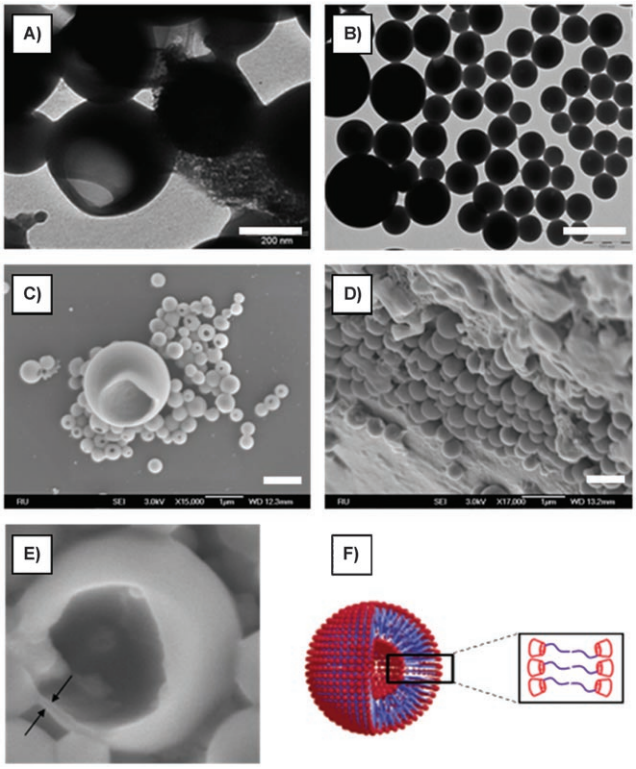


Figure 3. Electron microscope images of the aggregates formed after injecting a solution of **1** in THF into water. A, B) TEM images taken directly after injection (scale bars represent 200 and 500 nm for A and B, respectively). C) SEM image directly taken after injection. D) Cryo-SEM image taken 6 d after injection (scale bars represent 1 μm for both C and D). E) Electron micrograph indicating the thickness of the polymersomes (100 °C). F) Schematic representation of the bilayer polymersomes prepared from **1**.

facing the outer and inner aqueous compartments (Figure 3F). The polymersomes were similar in appearance to the aggregates prepared previously by us from PS-appended porphyrins,[5] but much larger than those formed from the conventional βCD amphiphiles reported to date.[2]

To verify that the βCDs are accessible for guest molecules we performed an inclusion experiment. We added propargyl dansyl (**10**), which is known to shift in its fluorescence towards lower wavelengths when interacting with a hydrophobic environment such as the interior of the CD, to a solution of polymersomes of **1**.[16] The shift, shown in Figure 4, demonstrates that the βCDs are accessible for guests. This result furthermore demonstrates that PS is not included in the βCD cavity.

We realised that the observed shift could be due not only to inclusion of **10** in the βCD cavities, but also to the non-specific absorption of **10** in the hydrophobic region of the PS tails in the inner part of the bilayer. To exclude this possibility we performed a competition experiment. We added a solution of 1-adamantol (**11**), which is known to have higher affinity for the cavity of methylated βCD ($4000\ M^{-1}$),[17] to polymersomes containing **10**. As shown in Figure 5, this was found to restore the original fluorescence

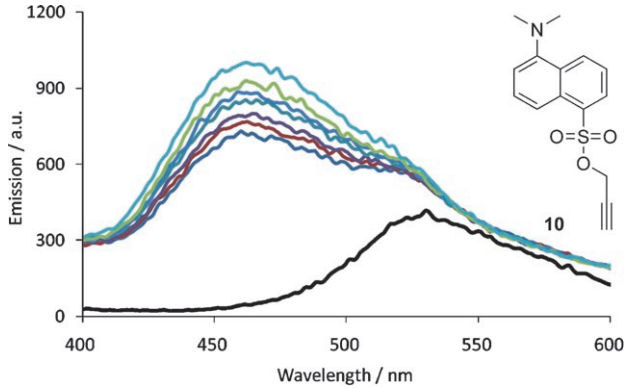


Figure 4. Fluorescence data of the inclusion of **10** in the CD cavities of the polymersomes. $\lambda_{exc}=305$ nm, emission range 400–600 nm. Black line: emission of free **10** in water (40 μM). Other traces: emission of the suspension of the polymersomes after adding different aliquots (from 1 to 10 μL) of **10**.

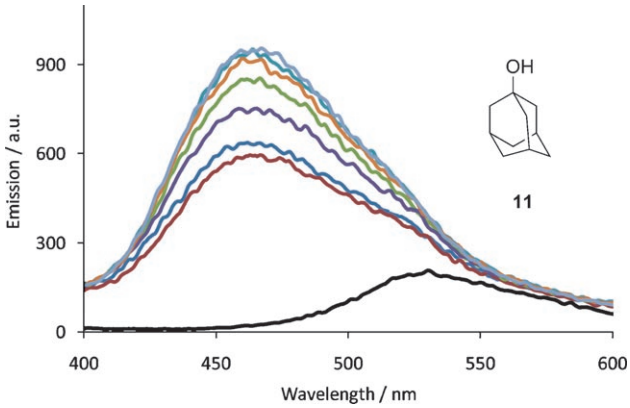


Figure 5. Fluorescence data for the exclusion of DNS with **11**. $\lambda_{exc}=$ 305 nm, emission range 400–600 nm. Black line: emission of free **10** in water (20 μM). Other traces: emission of the suspension containing polymersomes and **10** after adding different aliquots (from 1 to 15 μL) of **11**.

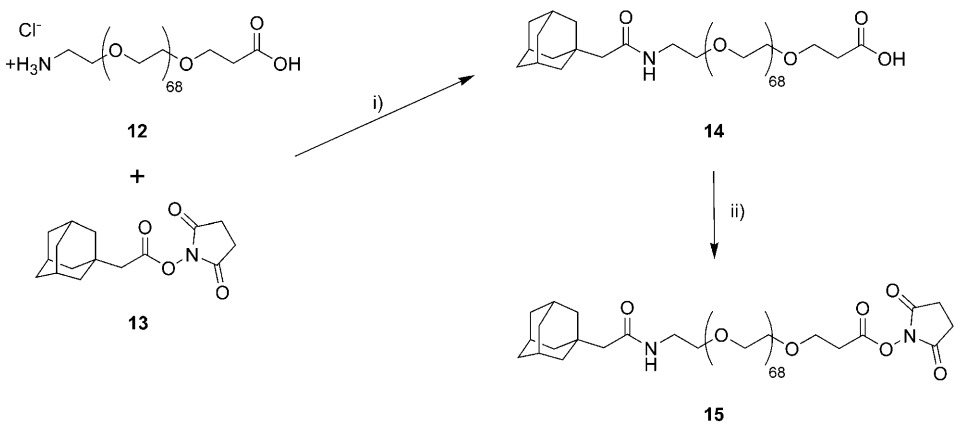
of **10** to a large extent, illustrating the formation of an inclusion complex of the βCDs of the polymersomes with **10**, which can be displaced by **11**.

Functionalisation of HRP with an adamantane–PEG spacer: The enzyme peroxidase from horseradish type VI (HRP) has a molecular mass of around 44000 and catalyses the oxidation of substrates by removal of two hydrogen atoms, for example, the oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) to a compound absorbing at 420 nm by using H_2O_2 as the oxidant. It is extensively used in conjugates in molecular biology, especially for its ability to amplify a weak signal and increase the detectability of a target molecule. It is ideal, in many respects, for these applications because it is smaller, more stable and less expensive than other popular alternatives, such as alkaline phosphatase, and itself has a characteristic absorption due to the haem group.

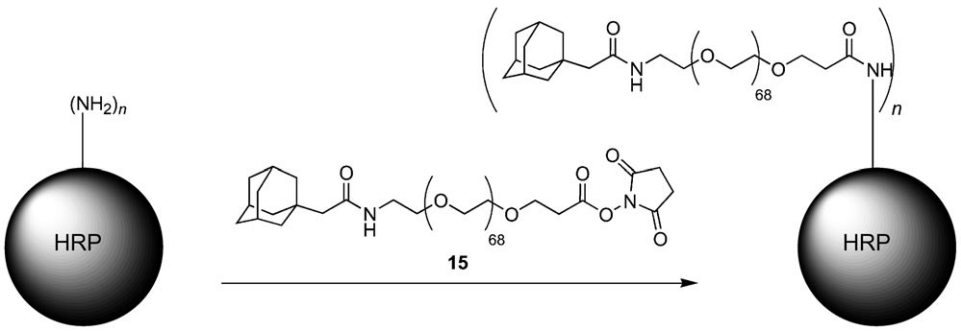
In the primary structure of HRP, six lysines are present (illustrated in Scheme 1). By analysing the protein crystal structure,[18] we found that two of the lysines are buried in the protein interior, but four are solvent accessible, and therefore, can be used to couple functional groups to the enzyme surface. In our case, we decided to modify HRP with adamantane moieties, which are good guests for βCD. Since the four lysines on the enzyme are not located in close proximity, it is necessary to have a long spacer between the enzyme and the adamantane moieties to allow the simultaneous interaction of the adamantanes with the vesicle surface. We decided to use PEG because it is very flexible and water soluble. For the synthesis of this spacer, a commercially available poly(ethylene glycol) with a terminal carboxylic acid moiety ($M_r=3000$; **12**) was coupled with **13**, followed by the activation of the carboxylic acid of PEG as an *N*-hydroxysuccinimide ester (Scheme 3).

For the functionalisation of HRP with **15** (Scheme 4), the enzyme was dissolved in acetate buffer (50 mM, pH 5.5) and a solution of **15** (25 equiv) was added in distilled THF. The reaction was gently stirred at 4 °C. After approximately 48 h, the mixture was dialysed against acetate buffer (50 mM, pH 5.5) to remove the unreacted PEG. The resulting solution was analysed by size-exclusion fast protein liquid chromatography (FPLC), using UV detection at 280 and 403 nm (absorption of the haem group of the HRP) (Figure 6, left).

It was not possible to separate the modified and native proteins by size-exclusion FPLC, probably due to the small differences in their masses and the unfavourable interaction of the PEG-adamantane tail with the column (Figure 6, left). However, sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE; Figure 6, right) clearly confirmed the formation of a new conjugate with a higher molecular weight than the native HRP. The presence of a single



Scheme 3. Synthesis of adamantyl compound **15**. Reagents and conditions: i) Et_3N, dichloromethane; ii) *N*-hydroxysuccinimide, *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDCI), CH_2Cl_2, from 0 °C to RT.



Scheme 4. Functionalisation of HRP (for detailed structure see Scheme 1) with compound **15** in acetate buffer. Reagents and conditions: NaOAc buffer pH 5.5, 4 °C, 2 d.

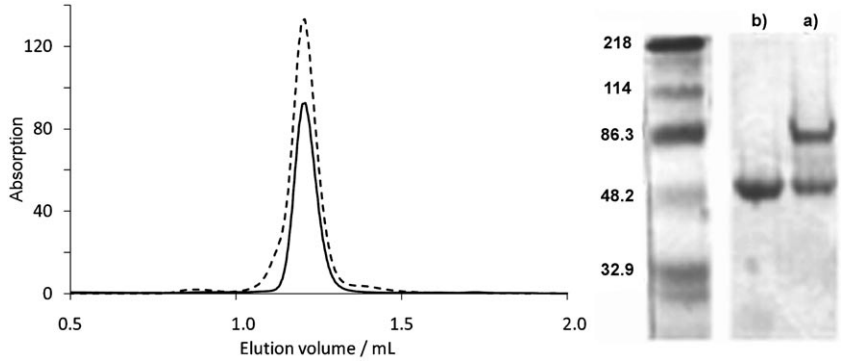


Figure 6. Left: FPLC of HRP (——) and HRP modified with compound **15** (-----) at $\lambda = 403$ nm. Right: SDS-PAGE of a) HRP modified with compound **15** after dialysis; b) HRP native (non-modified).

new band on the gel, in addition to a band due to non-modified enzyme, suggested that an equal number of PEG-adamantane tails were attached to the surface of each enzyme molecule. However, the different nature of the PEG tail interaction with the gel did not allow us to precisely quantify the molecular weight of the conjugate, and thus, the number of adamantanes attached to the HRP protein.

Interaction of modified HRP with polymersomes of 1: To investigate the presence of specific interactions of the modified HRP with the polymersomes of **1**, the following experiment was carried out: a suspension of the polymersomes in phosphate buffer was mixed with a solution of HRP functionalised with adamantane (1×10^{-5} M). In a control experiment the polymersomes were mixed with the non-modified HRP under otherwise identical conditions. After stirring for 2 h, the two suspensions were purified with several rinsing steps by using eppendorf micro test tubes equipped with filter units.

The control experiment was set up to establish when the non-functionalised HRP would be completely removed from the surface of the polymersomes, since some weak, non-specific interactions between the enzyme and the polymersomes were expected to be present. However, because of the absence of adamantanes on the enzyme in the control experiment, fewer washing steps should be required to completely remove the enzyme molecules from the surface of the polymersomes.

In Figure 7 the catalytic activity of the polymersomes functionalised with the modified enzymes after 25 rinsing steps (solid line) is reported. The HRP–polymersomes were catalytically active, whereas the absence of activity in the control shows that no enzyme is present in the polymersomes unless it is bound to them.

Due to the reversibility of the binding, the bound HRP can still be removed by centrifugation, but it requires a higher number of washing steps than the control experiment. During the washing process, after a certain number of rinsing steps (5, 10, 17, 20, 25), the enzyme activity in the eluates of both the control experiment and the sample was tested. In Figure 7 the enzymatic activity of the eluate of the experiment (dashed line) and the control (dotted line) after the same number of filtrations (17) are presented. It can be observed that enzymatic activity is still present when using the modified HRP, whereas there are no enzyme molecules present in the control experiment. The stronger interaction of modified enzyme with polymersomes of **1** is due to the interaction of the adamantane moieties with the βCD cavities on the surface of the polymersomes.

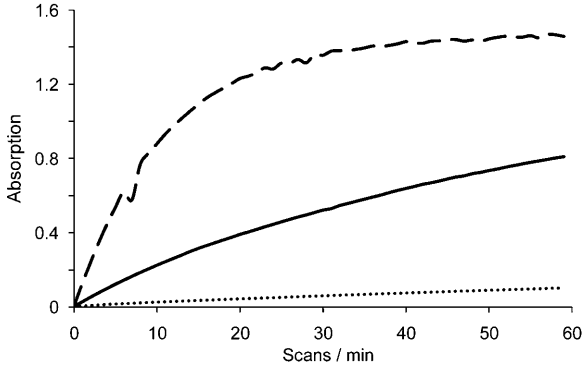


Figure 7. Activity measurements of HRP–PEG at $\lambda = 420$ nm: blank eluate after 17 rinsing steps (•••••); experimental eluate after 17 rinsing steps (– – –); activity retained in polymersomes after 25 rinsing steps (——).

Both samples from the experiment and the control were analysed by TEM to confirm the presence of the vesicles and to verify their integrity after the numerous centrifugation steps.

Conclusion

We have shown that PS-appended βCDs form well-defined vesicular structures. The solvent accessibility of the βCDs was proven by the subsequent addition of dansyl (**10**) and 1-adamantol (**11**) apolar derivatives. We subsequently utilised the host–guest interaction of βCD with adamantane to decorate the polymersomes with PEG–adamantane-functionalised HRP. Activity studies confirmed that HRP remained catalytically active and was successfully bound to the polymersomes. This opens new routes for the construction of polymersomes decorated with multiple enzymes that can act in concert.

Experimental Section

Materials: THF was purified by distillation under nitrogen from sodium/benzophenone. CH_2Cl_2 was distilled under nitrogen from CaH_2. Compounds **2** and **3** were prepared according to literature procedures.[7,11,12] All other chemicals were purchased from Aldrich, Fluka or Acros and used as received. Analytical thin-layer chromatography (TLC) was performed on Merck precoated silica gel 60 F-254 plates (layer thickness 0.25 mm) with visualisation by UV irradiation at $\lambda = 254$ nm and/or $\lambda = 366$ nm and/or staining with phosphomolybdic acid reagent or $KMnO_4$. Silica gel chromatography was performed by using Acros (0.035–0.070 mm, pore diameter ca. 6 nm) silica gel. Ultra pure water ($R > 18 \times 106\ \Omega$) was used for the aggregation studies in aqueous solutions. Dialysis membranes were purchased from Spectrum Laboratories and Dialyser Tubes (Maxi, Midi or Mini) were purchased from Novagem. Peroxidase from horseradish type VI (E.C.1.11.1.7) was purchased from Sigma.

NMR spectroscopy: ^1H and ^{13}C NMR spectra were recorded on Bruker DPX300 or Varian inova 400 spectrometers at room temperature. ^1H NMR chemical shifts are reported in ppm (δ) relative to tetramethylsilane ($\delta = 0.00$) as an internal standard when measured in $CDCl_3$, otherwise the residual solvent peak was used as a reference.

Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), dt (doublet of triplets) or m (multiplet). Broad peaks are indicated by the addition of br. Coupling constants are reported as a *J* value in Hertz (Hz). The number of protons (*n*) for a given resonance is indicated as *n*H, and is based on spectral integration values. ^{13}C NMR chemical shifts (δ) are reported in ppm relative to $CDCl_3$ ($\delta = 77.0$). Succ refers to the succinimide group.

Mass spectrometry: Electrospray LC/MS analysis was performed using a Shimadzu LC/MS 2010A system. Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) spectra were measured on a Bruker Biflex III spectrometer and samples were prepared from solutions in MeOH by using indoleacrylic acid (IAA) (20 mg mL^{-1}) as a matrix.

Infrared spectroscopy: IR spectra were recorded on an ATI Matson Genesis Series FTIR spectrometer with a fitted ATR cell. Frequencies ($\tilde{\nu}$) are given in cm^{-1}.

UV/Vis spectroscopy: UV/Vis spectra were recorded on a Varian Cary 50 spectrophotometer by using a quartz cuvette. For catalysis experiments, the UV/Vis spectra were recorded on an Elisa Ryder spectrophotometer.

Fluorescence spectroscopy: Fluorescence spectra were measured on a Perkin–Elmer LS 55 fluorescence spectrophotometer by using a 50 μL quartz cuvette.

TEM: TEM images were obtained by using a JEOL JEM 1010 microscope (60 kV) equipped with a CCD camera. Samples were prepared by pouring a drop of the aggregate suspension onto a carbon-coated copper grid, which was allowed to dry in air. The structures were visualised without further treatment. The aggregate solution was prepared by desolving amphiphile **1** (1 mg) in distilled THF (1 mL). The resulting solution was then slowly injected into ultrapure (Millipore) water (1 mL). The sample was shaken by hand to obtain a homogeneous mixture. Measurements of the sealed sample were done after 5 min, 1 d, 3 d and 6 d.

SEM: SEM was performed on a JEOL JSM-6330F instrument by using the same samples as prepared for TEM. Before measurement a 1.5 nm layer of Pd/Au was sputtered on the grids with a Cressington 208 HR sputter coater fitted with a Cressington layer-thickness controller.

GPC: Molecular weight distributions were measured with a Shimadzu size-exclusion column equipped with a guard column and a PL gel 5 μm mixed D column (Polymer Laboratories) with differential refractive index and UV ($\lambda = 254$ nm and $\lambda = 330$ nm) detection using THF as an eluent (1 mL min^{-1} at 35 °C). PS standards were used for calibration.

Compound 1: Nitrogen was bubbled through distilled THF for 1 h prior to use. Compounds **2** (0.05 g, 0.03 mmol) and **3** (0.24 g, 0.03 mmol) were dissolved in THF (3 mL) under nitrogen. A solution of CuBr (0.005 g, 0.03 mmol) and PMDTA (0.007 mL, 0.03 mmol) in THF (2 mL) was made free of air by bubbling N_2 through it for 30 min and it was added to the solution of **2** and **3**. The mixture was stirred under a nitrogen atmosphere for 48 h. The solvent was removed by evaporation and the solid obtained was purified by column chromatography ($CHCl_3 \rightarrow CHCl_3$/MeOH 4:1 v/v). Product **1** was obtained as a white solid (0.12 g, 41 %). ^1H NMR (400 MHz, $CDCl_3$): $\delta = 1.25$–2.10 (br m; aliphatic protons of the PS tail) 3.15–4.00 (m, 118H), 7.09 ppm (br m; aromatic protons of the PS tail); IR: $\tilde{\nu} = 3023$, 3053, 3088 (CH_2); 2841, 2919 (CH); 1938, 1873, 1804, 1739 (C=O); 1600 (C=C); 1160 (O-Me); 693, 771 cm^{-1} (monosubstituted benzenes); the vibration of the N_3 group of compound **3** had disappeared in compound **1**.

1-[2-(1-Adamantyl)acetoxy]succinimide (13): 1-Adamantylacetic acid (0.30 g, 1.5 mmol) and *N*-hydroxysuccinimide (0.19 g, 1.6 mmol) were dissolved in distilled CH_2Cl_2 under a nitrogen atmosphere. The mixture was cooled to 0 °C and EDCI (0.33 g, 1.7 mmol) was added. The mixture was stirred under a nitrogen atmosphere for 20 h. The solvent was removed and the solid was redissolved in EtOAc and extracted with NH_4Cl (2×), dried over anhydrous sodium sulfate, filtered and evaporated to dryness. Product **13** was obtained as a white solid (0.38 g, 87 %). M.p. 134 °C; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 1.67$–1.74 (d, 12H), 2.01 (s, 3H), 2.33 (s, 2H), 2.82 ppm (s, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 169.2$ (CO succ), 166.2 (CO), 45.3 (adamantane-CH_2-CO), 41.9, 36.5 (CH_2 from adamantane), 33.1 (quaternary C), 28.5 (CH from adamantane), 25.6 ppm (CH_2 succ).

3-{2-[2-(1-Adamantyl)acetamido]ethyl[octahexaconta(oxyethylene)]}propanoic acid (14): Compound **12** (0.20 g, 0.06 mmol) was dissolved in distilled CH_2Cl_2 (20 mL) under a N_2 atmosphere followed by the addition of triethylamine (19 μL, 0.13 mmol). The mixture was stirred for 10 min and compound **13** (0.03 g, 0.10 mmol) was added. The reaction was stirred for 20 h and the resulting mixture extracted with a saturated aqueous solution of $NaHCO_3$ (2×) and HCl (1 M), dried over anhydrous sodium sulfate, filtered and evaporated to dryness. The product was precipitated in Et_2O and filtered. The solid was dissolved in $CHCl_3$. The solvent was removed in vacuo and the product was dried under high vacuum to give compound **14** as a white powder (0.18 g, 50 %). ^1H NMR (300 MHz, $CDCl_3$): $\delta = 3.35$–3.85 (br m, 276H), 2.58 (t, 2H), 1.95 (s, 3H), 1.92 (s, 2H), 1.57–1.70 ppm (m, 12H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 172.4$ (CO), 170.5 (CO), 70.0 (CH_2 repeating unit), 66.2, 65.3, (CH_2), 51.0 (adamantane-CH_2-CO), 42.1, 36.3 (CH_2 from adamantane), 28.1 (CH from adamantane), 38.5 (O-CH_2), 36.3 (CH_2-COOH), 32.2 ppm (quaternary C).

1-(3-{2-[2-(1-Adamantyl)acetamido]ethyl[octahexaconta(oxyethylene)]}-propoxy) succinimide (15): Compound **14** (0.18 g, 0.06 mmol) and *N*-hy-

droxysuccinimide (0.008 g, 0.07 mmol) were dissolved in distilled CH_2Cl_2 (15 mL) under a N_2 atmosphere. The mixture was cooled to 0 °C and EDCI (0.02 g, 0.12 mmol) was added. The reaction was stirred for 24 h and the resulting mixture was extracted with HCl (0.5 M), dried over anhydrous sodium sulfate, filtered and evaporated to dryness. The product was precipitated in Et_2O and the solid was dissolved in $CHCl_3$. The solvent was removed in vacuo and the product was dried under high vacuum to give compound **15** as a white powder (0.15 g, 80 %). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.38$–3.87 (br m, 276 H), 2.89 (t, 2 H), 2.83 (s, 4 H), 1.98 (s, 3 H), 1.92 (s, 2 H), 1.57–1.70 ppm (m, 12 H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 170.9$ (CO), 168.8 (CO succ.), 166.6 (CO), 70.4 (CH_2 repeating unit), 65.7, 65.6, (CH_2), 51.5 (adamantane-CH_2-CO), 42.5, 36.7 (CH_2 from adamantane), 29.5 (CH from adamantane), 38.8, 32.0 (CH_2), 32.6 (quaternary C), 25.5 ppm (CH_2 succ).

Binding of 10 in polymersomes of 1: A suspension of the polymersomes of **1** (0.5 mg mL^{-1}) was prepared as described above and kept for 6 d in a sealed tube to allow them to close completely. Before any fluorescence measurements were recorded, the vesicles were dialysed against ultrapure water for one night to remove THF. In the inclusion experiment, different aliquots of a stock solution of **10** (1 mM in THF) were added until a final concentration of 40 μM was reached. The mixture was followed in time by fluorescence spectroscopy. The competition experiment was carried out after 18 h by using the suspension containing polymersomes and **10** mentioned before. Since the measured emission was off the scale, the suspension was diluted to half the concentration and then different aliquots of a stock solution of **11** (2 mM in THF/H_2O 1:1) were added. Fluorescence measurements were then performed.

Functionalisation of HRP with the adamantane–PEG derivative: HRP (1 mg) was dissolved in NaOAc buffer (160 mL, 50 mM, pH 5.5) followed by the addition of a solution of **15** (1 mg) in distilled THF (30 μL). The reaction was stirred for 48 h at 4 °C and dialysed by using dialysis tubes (MWCO 12–14 KDa) against NaOAc buffer (50 mM, pH 5.5) for 24 h, changing the buffer three times to remove the unreacted PEG. The solution obtained from dialysis (0.14 mM) was analysed and purified by FPLC. Injections of aliquots (20 μL) of the samples in the FPLC column (Superdex 200) at room temperature were monitored by UV detection at 280 and 403 nm. Fractions (60 μL) were collected in well plates. SDS-PAGE was performed by using a 10 % polyacrylamide gel containing 1 % SDS. The samples were neither heated nor treated with β-mercaptoethanol before loading onto the gel. The concentration of the samples was 0.09 mM both for native HRP and for HRP modified with the adamantane–PEG derivative.

Reaction between HRP modified with 15 and polymersomes of 1: A suspension of the polymersomes of **1** (0.5 mg mL^{-1}) was prepared as described above and kept for 6 d in a sealed tube to allow them to close completely. A volume (500 μL) of this suspension was dialysed against phosphate buffer (20 mM, 100 mM NaCl, pH 7.2) by using dialysis bags (MWCO 12–14 KDa). Only 300 μL of the resulting suspension were mixed for 2 h at room temperature with a solution of HRP functionalised with compound **15** (200 μL, 1×10^{-5} M).

A control experiment was performed using the non-modified HRP, under the same reaction conditions.

Free enzyme molecules were removed from the reaction mixture by filtration using an eppendorf micro test tube fitted with a filter unit with pores of 0.1 μm. The eppendorf micro test tube was centrifuged at 3000 rpm for 5 min and fresh buffer solution was added before the tube was again subjected to centrifugation. This sequence was repeated 15–25 times after which time the eluate of the control experiment no longer showed any enzymatic activity. The sample remaining on the filter was redispersed in buffer solution (300 μL) and catalysis experiments were performed. A sample (10 μL) prepared by the procedure described above was added to a well plate (250 μL) followed by the subsequent addition of phosphate buffer (220 μL, 20 mM, pH 7.2), an aqueous solution of ABTS (10 μL, 0.05 M) and an aqueous solution of H_2O_2 (10 μL, 7 %). The sample was mixed thoroughly by using a pipette and the progress of the reaction was monitored by UV/Vis spectroscopy, measuring the absorption at 420 nm.

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