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A three-enzyme cascade reaction via positional assembly of enzymes in a polymersome nanoreactor

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Abstract: Porous polymersomes based on block copolymers of isocyanopeptides and styrene have been used to anchor enzymes at three different locations, namely in their lumen (Glucose Oxidase, GOx), in their bilayer membrane (Candida antarctica lipase B, CalB), and on their surface (Horseradish Peroxidase, HRP). The latter coupling was achieved via 'click' chemistry between acetylene functionalised anchors on the surface of the polymersomes and azido functions of HRP, which were introduced using a direct diazo transfer reaction to lysine residues of the enzyme. To determine the encapsulation and conjugation efficiency of the enzymes, they were decorated with metal ion labels and analysed by mass spectrometry. This revealed an almost quantitative immobilisation efficiency of HRP on the surface of the polymersomes and a more than statistical incorporation efficiency for CalB in the membrane, and for GOx in the aqueous compartment. The enzyme decorated polymersomes were studied as nanoreactors in which glucose acetate was converted by CalB to glucose, which in a second step was oxidised by GOx to gluconolactone. The produced hydrogen peroxide was used by HRP to oxidise ABTS to $ABTS^{\cdot+}$. Kinetic analysis revealed that the reaction step catalysed by HRP is the fastest in the cascade reaction.

Keywords: cascade reactions · enzyme immobilisation · macromolecular chemistry · nanomaterials · polymersomes

Introduction

Compartmentalisation is one of the techniques that cells adopt to enable a high level of control over (bio-)chemical processes, for instance the order in which enzymes react. In many cases, the compartment also serves to protect the cell from the action of its degrading contents, as is the case with lysosomes. It furthermore serves as a scaffold for the precise positional assembly of enzymes that work together in a multistep cascade reaction. Positioning of enzymes on the surface of a compartment, in its interior, its membrane, or any combination thereof can be found, for instance in mitochondria for the enzymes involved in the citric acid cycle.

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In an effort to mimic these complex enzyme systems, many studies concerning enzyme encapsulation or assembly have been reported in the literature.[1-5] The focus of this research initially was on phospholipid liposomes,[6, 7] whose bilayer membranes are rather similar to those of naturally occurring cells. However, the relative fragility of liposomes limits their potential applicability. Increased mechanical and thermodynamic stability has been achieved by preparing vesicles via layer-by-layer deposition methods.[8, 9] Compartmentalisation based on sol-gel chemistry has also been reported,[1, 3] although this approach parts with the notion of discrete vesicular objects.

Like liposomes, polymersomes are spherical aggregates that contain a bilayer architecture. They are formed by the self-assembly of amphiphilic block copolymers in an aqueous environment.[10] Their polymeric bilayer shows a greater stability, mainly due to the lower critical aggregation concentration of amphiphilic macromolecules.[10] The large chemical versatility which is possible in block copolymer synthesis allows one to tune the properties of polymersomes.[11-13] A drawback of polymersome membranes is their low permeability even to water, which hampers application as nanoreactors.[10] To overcome this problem, the diffusion of solvent and substrate molecules has been enabled by the inclusion of channel proteins[14] or proton pumps[15] in the polymeric bilayer.

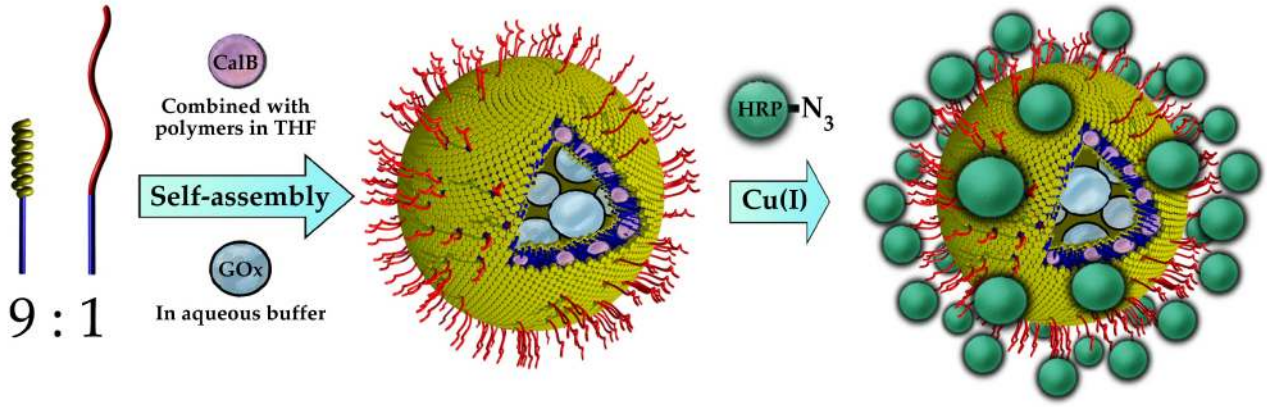
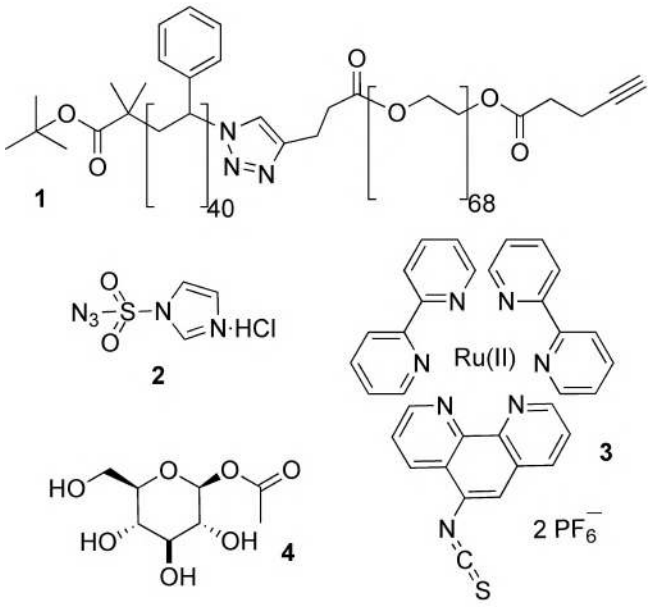


Figure 1. Positional assembly of enzymes in a polymersome. A mixture of PS-PIAT and anchor **1** is lyophilised with *Candida Antarctica* lipase B (CalB) and then dissolved in THF. This mixture is then injected into an aqueous buffer containing Glucose Oxidase (GOx), encapsulating it in the inner compartment and subsequently trapping CalB in the polymeric bilayer. A third enzyme, Horseradish Peroxidase (HRP), is immobilised on the polymersomal perimeter via covalent linkage to anchor **1**, creating an outer shell of enzymes.



Scheme 1. Structures of block copolymer 'anchor' **1**, diazo transfer reagent **2**, Ru-labelling compound **3** and glucose acetate (GAc) **4**.

A more convenient method to prepare permeable polymersomes is the use of a block copolymer that gives an intrinsically porous bilayer when self assembled. One such copolymer is polystyrene$_{40}$-*b*-poly(L-isocyanoalanine(2-thiophen-3-yl-ethyl)amide)$_{50}$ (PS-PIAT). It is a rod-coil type of amphiphilic copolymer consisting of a rigid polyisocyanide block and a flexible polystyrene block.[16-18] On dispersal in water it forms porous polymersomes that possess a relatively high degree of diffusion. Small molecules can move across their membranes while larger molecules, such as proteins, cannot.[19, 16]

In a previous paper we described the use of PS-PIAT polymersomes as scaffolds for the positional assembly of two different enzymes.[20] Glucose Oxidase (GOx) was encapsulated in the lumen of the polymersome, while Horse Radish Peroxidase (HRP) was embedded in its bilayer. The activity of these two enzymes was studied as part of a three-enzyme cascade system, which also involved the enzyme Candida antarctica lipase B (CalB). The latter enzyme was added separately to the polymersome dispersion. In this cascade system, 1,2,3,4-tetra-O-acetyl-b-glucopyranose (GAc4) was deprotected by CalB to produce free glucose, which was subsequently oxidised by GOx. This in turn produced hydrogen peroxide, which was the substrate for HRP using it to convert 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) into ABTS$^{\cdot+}$.

In the system described above, only two out of three enzymes were linked to the polymersome. Recently we have developed methodologies to anchor an enzyme to the surface of a polymersome,[21, 22] one of which is based on the 1,3-dipolar cycloaddition between an azide and an alkyne. To this end a block copolymer 'anchor' with an acetylene-functionalised hydrophilic terminus (**1**, scheme 1) was admixed with PS-PIAT to introduce surface-functionalities while maintaining membrane porosity, i.e. the ability of the polymersome to let small molecules diffuse through its membrane. We have now used this anchoring approach to develop an enzyme cascade system in which all enzymes involved are associated with one single polymersome via a controlled spatial positioning procedure, as shown in figure 1. This system therefore extends our ability to create complex cascade biomimetic systems. A practical advantage of this approach is that all catalytic enzyme species can be removed from solution by a single filtration. The system also features a more controlled spatial positioning of the enzymes. In this report the construction of the 3-enzyme polymersome reactor is described (Figure 2), together with the quantification of the efficiency of enzyme incorporation into the polymersome membrane, the lumen, and the efficiency of surface conjugation.

Finally, in order to be able to prepare this three-step reactor, azido groups needed to be introduced in HRP. To realise this, we applied a diazo transfer reaction directly on the amines of the lysine residues and the amino terminus of HRP.[23] This facile diazo transfer reaction uses imidazole-1-sulfonyl azide hydrochloride (**2**, scheme 1).[24]

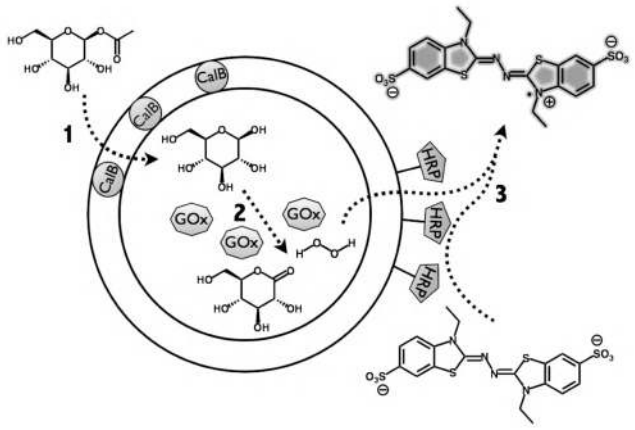


Figure 2. Schematic representation of the multistep reaction. 1: Mono-acetylated Glucose (**4**) is deprotected by CalB, which is embedded in the polymersome membrane. 2: In the inner aqueous compartment GOx oxidises glucose to gluconolactone, providing a molecule of hydrogen peroxide. 3: Hydrogen peroxide is used by HRP to convert ABTS to $ABTS^{+}$. HRP is tethered to the polymersome surface.

Results and Discussion

Design of the three-enzyme cascade system: The objective of this study was to position enzymes at three specific locations within or on a polymersome: GOx in its lumen, CalB in its membrane and HRP on its surface (see figure 2). Since GOx is a rather large tetrameric enzyme (160 kDa), it would disrupt PS-PIAT membranes when embedded in it;[20] hence it was decided to include this enzyme inside the more spacious lumen. HRP and CalB have lower molecular weights (~43 kDa and ~33 kDa, respectively) and both enzymes have been successfully incorporated in a PS-PIAT membrane before.[19, 20] Our choice to embed CalB in the bilayer of the vesicles was primarily motivated by the fact that it is more hydrophobic than HRP. In order to enable the effective use of anchor **1**, any enzyme that was to be positioned on the surface of the polymersome needed to be functionalised with azido-moieties. To realise this, we decided to apply a diazo transfer reaction directly on the amines of the lysine residues and the amino terminus of the enzyme.[23, 24] Given our intent to modify the primary amines, the lower density of amines present in HRP as opposed to CalB would reduce the risk of polymersome aggregation due to cross-linking via bridging enzymes. Furthermore, the risk of decreasing enzymatic activity is lowered when a modified enzyme more closely resembles its native state. These considerations led us to position GOx in the lumen, CalB in the bilayer and HRP on the outer surface, necessitating the synthesis of azido-HRP.

Polymersomes loaded with two enzymes: As a first step towards the three-enzyme cascade system, polymersomes loaded with CalB in their membranes and GOx in their aqueous compartments were prepared. It was also investigated whether this system would be compatible with the presence of anchor **1**. To this end, PS-PIAT with 10 wt.-% anchor **1** was dissolved in THF and injected into an aqueous solution of CalB (2 mg mL^{-1}), after which the dispersion was immediately flash frozen in liquid nitrogen and lyophilised. The resulting CalB-polymer hybrid[20] was then dissolved in THF and gently dripped into a phosphate buffer containing 0.25 mg mL^{-1} GOx. After half an hour of equilibration, non-encapsulated enzymes and THF were removed via filtration, and the resulting mixture was analysed by electron microscopy (figure 4), revealing the presence of polymersomes with diameters ranging from 90 to 180 nm. This shows that the two enzymes and anchor **1** do not prevent the PS-PIAT polymersomes from being formed.

Preparation of azido-HRP. To expand this two-enzyme system into a three-enzyme one, HRP needed to be equipped with azide moieties. Although azido-modified amino acids can be introduced by single site or multisite protein engineering replacement strategies,[25] the level of control provided by these laborious methods is not needed for mere immobilisation of enzyme molecules. This led us to apply the mild and generally facile diazo transfer reaction to amines, carried out with imidazole-1-sulfonyl azide hydrochloride (**2**, scheme 1) as reagent and catalysed by Cu(II)[24], which has been shown to be suitable for protein modification in aqueous solution.[23] To this end, an aqueous solution of HRP, K_2CO_3 and $Cu(II)SO_4$ was treated with water-soluble **2** for twelve hours, after which the enzyme was purified using centrifugal filter devices. ESI-TOF analysis of the treated enzyme showed that an average of four amines were transformed into azides. This is a satisfactory result, given the fact that in principle only one azide per enzyme is required for surface immobilisation.

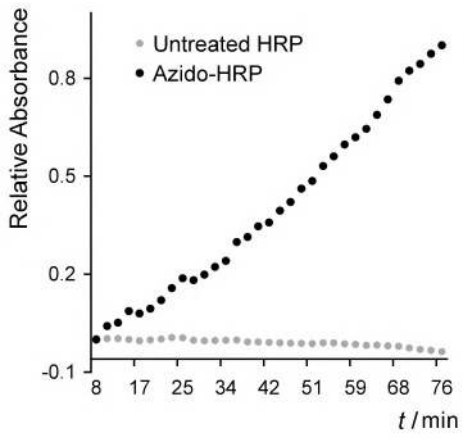


Figure 3. Progress curve for GOx polymersomes with surface-conjugated HRP. Two types of polymersomes are incubated with glucose and ABTS, and the appearance of $ABTS^{+}$ is measured as a function of time. After a short initial incubation period, polymersomes that only contain GOx do not show any increase in absorbance (grey dots). Polymersomes in which both enzymes are present do show an increasing concentration of $ABTS^{+}$ (black dots).

Subsequently, PS-PIAT polymersomes containing GOx in their inner aqueous compartments and 10% acetylene-anchor **1** in their membranes were prepared. After treatment of these aggregates with azido-HRP and Cu(I), they were washed via filtration until no HRP activity could be detected in the flow through. The resulting polymersomes, containing GOx in their lumens and HRP on their surfaces, were dispersed in phosphate buffer and studied as a two-enzyme cascade system, in which glucose is converted to gluconolactone and the resulting hydrogen peroxide used to oxidise ABTS to the coloured radical cation $ABTS^{+}$. As can be seen in figure 3, the increasing absorbance of $ABTS^{+}$ clearly indicates that the two-enzyme cascade system is working, demonstrating both the successful conjugation of HRP to the functionalised polymersome nano-reactor and the viability of more complex enzyme positioning

schemes like this. The structural integrity of the polymersomes remained unperturbed, as is shown in figure 4.

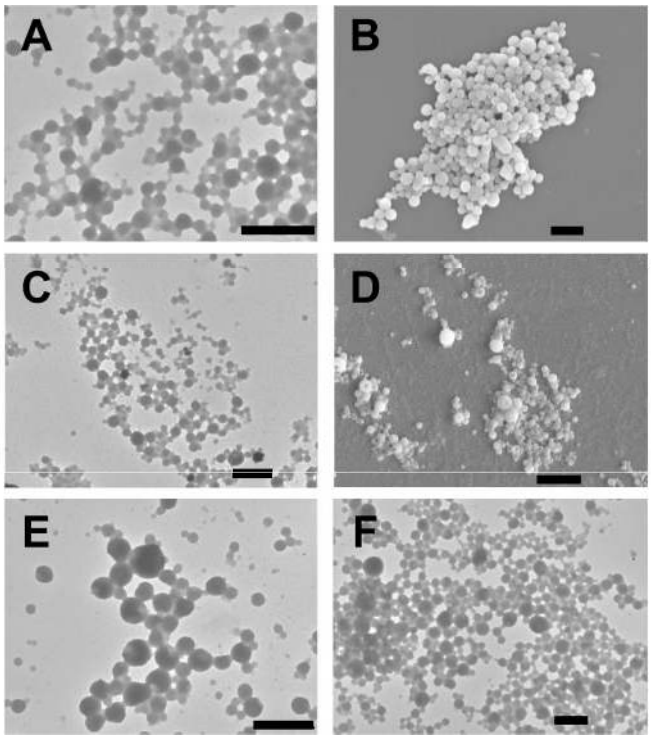


Figure 4. Electron micrographs of biohybrid polymersomes. All micrographs shown are of PS-PIAT vesicles containing an admixed 10 wt.-% of **1**. The scale bar represents 500 nm. Their enzymatic content is as follows: a. GOx in lumen (TEM); b. GOx in lumen, HRP on surface (SEM); c. GOx in lumen, CalB in membrane, HRP on surface (TEM); d. GOx in lumen, CalB in membrane, HRP on surface (SEM); e. **3**-labelled GOx in lumen (TEM); f. **3**-labelled CalB in membrane.

Polymersomes loaded with three enzymes: In a next step, azido-HRP was conjugated to the acetylene anchors that were part of the above mentioned two-enzyme polymersomes carrying GOx in their aqueous compartment and CalB in their bilayer membrane. After conjugation, the vesicles were washed using a cutoff filter that allowed passage of unconnected enzymes while retaining intact polymersomes in the supernatant. The transmission electron micrographs shown in figure 4 revealed an unaltered spherical morphology of the polymersomes.

Not much has previously been reported on the actual efficiency of these incorporation processes. Rameez et al. determined the efficiency of haemoglobin (Hb) encapsulation in the polymersome lumen by lysis of the vesicles, followed by UV-Vis detection of released Hb, using an Hb-specific protocol.[26] For a more general approach, we chose to employ a commercially available ruthenium complex with an isothiocyanato-moiety (compound **3**), which is easily covalently linked to any molecules containing free amines, as most proteins do in their lysine residues or *N*-termini. After encapsulation or immobilisation, the use of inductively coupled plasma - mass spectrometry (ICP-MS), allowed for the quantitative detection of ruthenium, and hence of proteins.

Each of the three different enzymes was labelled and separately incorporated into the polymersomes or conjugated to them in the same way as shown above for the unlabelled enzymes (polymersomes containing the labelled enzymes are shown in figure 4). Results for CalB and GOx incorporation, as well as HRP-conjugation efficiency, are given in table 1. From these results it appears that the efficiency of conjugating azido-HRP to the polymersome surface is very high. By applying 10 wt.-% of the anchor compound **1**, 7 nmol of alkyne moieties become incorporated in the periphery of the polymersome. Assuming an equal distribution of the anchor molecules over both membrane layers, it can be calculated that around 3.5 nmol of acetylene functions are present on the polymersome outer surface. The average value, as determined by ICP-MS, of 3.2 nmol HRP after conjugation implies that more than 90% of all available acetylene sites were occupied by HRP.

Table 1. Encapsulation and conjugation efficiencies of enzymes in PS-PIAT polymersomes.

Labelled Enzyme	Location	Amount Used	Retained in Biohybrid	Efficiency
CalB	In polymeric bilayer	5.61 nmol	0.80 - 1.13 nmol	17.2% ± 2.96%
GOx	In lumen	3.91 nmol	0.90 - 1.06 nmol	25.0% ± 2.09%
HRP	Attached to surface	12.50 nmol	2.77 - 3.61 nmol	92% of theoretical maximum[a]

[a] Measured: 25.5% ± 3.37% - The theoretical maximum for HRP is dictated by the amount of acetylenes introduced via anchor **1**.

For GOx and CalB, the incorporation efficiency was much higher than the 0.1% that would be expected based on statistical inclusion.[20] This implies a mechanism of polymersome formation that is somehow influenced by the presence of the enzymes, perhaps through nucleation of block-copolymer aggregates around transient protein aggregates.

Three-enzyme cascade catalysis: As a substrate for the three-enzyme cascade reaction of CalB, GOx and HRP, an acetate-protected glucose was chosen. Previously, the tetra-acetate GAc4 had been used for this purpose.[20] For the present study, however, an increased solubility of the substrate in aqueous buffers was desired, in order to achieve a more homogeneous reaction mixture. The orthogonally protected 2,3,4,6-tetra-O-benzyl-D-Glucopyranose was acetylated at its anomeric position and subsequently hydrogenated to produce GAc (**4**, scheme 1). Besides its greater solubility, GAc is also more rapidly deprotected by CalB, which enhances the overall reaction rate of the cascade system.

The multistep nanoreactors were incubated with GAc (**4**) and ABTS as described in the experimental section and the increase in $ABTS^{\cdot+}$ concentration as a function of time was measured by UV-vis spectroscopy. The results are presented in figure 5. The curve is S-shaped and the first two thirds of the data points can be fitted to the equation shown in figure 6, which describes a two-enzyme reaction (R^2 value = 0.9956; Data were fit using GraphPad Prism 5.0a for Mac OS X.)

This suggests that one of the three enzymes has an activity high enough not to influence the total kinetics. We propose this is HRP. Its spatial position relative to GOx enables it to convert any H_2O_2 molecule relatively quickly after its generation, eliminating HRP from the rate equation. Its method of immobilisation also renders it the enzyme that is least hindered by its crowded environment. Furthermore, it is also the enzyme that has not been suffering from the effects of THF since it never came into contact with this solvent. For comparison, also a reaction with a polymersome system lacking

CalB in the membrane was performed (figure 5). These polymersomes do not have the ability to catalytically deprotect GAc; the progress of the reaction is dependent on the hydrolysis of GAc by water. As can be seen in figure 5, this system displays a significantly lower rate of reaction. The similarity between the curves for the two-enzyme and the three-enzyme system shows that CalB is not the fastest of the three enzymes, again pointing towards HRP as taking this role. Unfortunately, our analysis using equation 1 does not allow us to determine the slowest step of the three enzyme cascade reaction. Measurement of a solution that was filtered to remove the polymersomes showed no conversion of ABTS, indicating that all activity that was measured resulted from enzymes associated with the polymersomes.

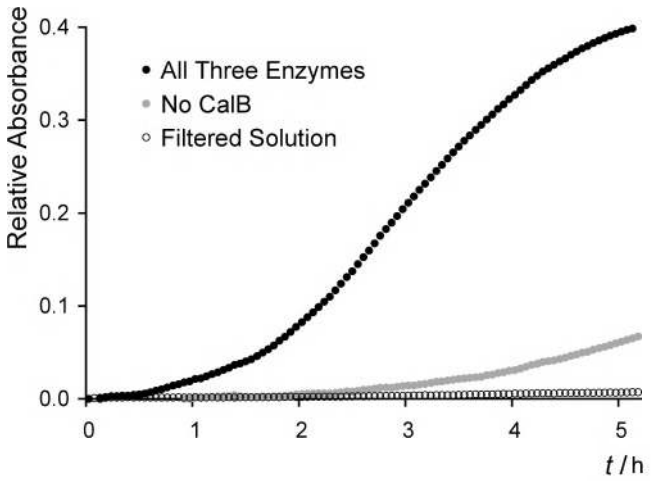


Figure 5. Progress curve for the three-enzyme cascade reaction (see figure 2). The black dotted line shows the increasing concentration of the cascade's final product, $ABTS^{+}$. After filtration of the solution to remove polymersomes, no enzymatic activity could be detected anymore (open dots). The system that lacked CalB still showed activity due to chemical hydrolysis of **4** (grey dots).

$$C_p(t) = \frac{[GAc]_0}{k_2 - k_1}\left(-k_2 e^{-k_1 t} + k_1 e^{-k_2 t}\right) + [GAc]_0$$

Figure 6. Rate equation for a two-enzyme cascade reaction system. C_p denotes the concentration of the final product, $ABTS^{+}$; $[GAc]_0$ is the initial concentration of the substrate (**4**).

The relatively high concentration of enzyme molecules measured by ICP-MS does not translate in a higher total activity. A possible explanation is that diffusion of substrate or product through the membrane is rate-limiting, or that the confinement of the enzyme molecules in a small space is detrimental to their activity. The temporary exposure to THF that some enzymes undergo may also adversely influence their activity. Further studies are required to obtain more information regarding this issue.

Conclusion

We have constructed biohybrid polymersome nanoreactors in which three different enzymes are spatially positioned, and precisely ordered. These enzymes were incorporated in the membrane (CalB), encapsulated in the inner aqueous compartment (GOx), and attached to the surface of the polymersome (HRP.) The conjugation of HRP to the polymersomes was realised via a Cu(I)-catalysed [3+2] Huisgen cycloaddition. To this end the HRP had been provided with azide functions following a diazo transfer reaction on lysine residues or the N-terminus, carried out in aqueous buffer. The vesicular morphology of the resulting three-step nanoreactor is unaffected by this decoration. More than 90% of the available handles on the polymersome surface are occupied by an azido-HRP molecule, and the lumen incorporates approximately 25% of the added GOx enzymes, which is more than expected for a pure statistical encapsulation process. The CalB enzymes are incorporated in the bilayer membrane with an efficiency of 17%. The nanoreactor is capable of performing a three-step cascade reaction and can be removed from the solution by a single filtration. The progress curve of the reaction fits to a two-enzyme reaction model, suggesting that one of the enzymes, i. e. HRP, does not influence the overall kinetics, probably as a result of its location on the surface of the polymersome. Although the macromolecular assembly of the model enzyme cascade reaction presented here does not provide an inherent catalytical advantage over a mixture of the same enzymes when freely dissolved, it clearly illustrates the viability of advanced enzyme positioning in polymersomes.

Experimental Section

Materials. PS-b-PEG-acetylene **1** was prepared as previously described.[21] Diazo transfer reagent **2** was prepared as described elsewhere.[23] Deuterated chloroform ($CDCl_3$, 99.8%), deuterated methanol (CD_3OD, 99.8%) and heavy water (D_2O, 99.9%) were purchased from Aldrich, $CuSO_4$ (Merck), sodium ascorbate (Fluka, >99%), 4,7-diphenyl-1,10-bathophenanthroline disulfonic acid disodium salt (ligand) (Sigma-Aldrich), anhydrous sodium sulphate (Fluka, 99%), K_2CO_3 (Fisher), magnesium sulfate dihydrate (Fluka, 99%), NaN_3 (Acros, 99%), Bis(2,2'-bipyridine)-(5-isothiocyanato-phenanthroline)ruthenium bis(hexafluorophosphate) (**3**) (BioChemika, Aldrich), 2,3,4,6-tetra-O-benzyl-D-Glucopyranose (Aldrich), celite, acetic anhydride (Ac2O), $NaHCO_3$, Na_2CO_3 and Na_2HPO_4 (Merck) and 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Fluka, 99%) were all used as received. Charcoal-supported Pd-catalyst (Pd/C), nitric acid (HNO_3), hydrochloric acid (HCl), methanol (MeOH), ethyl acetate (EtOAc), sodium chloride (NaCl), silver acetate (AgOAc), toluene, 1, 4-dioxane, dimethylformamide (DMF) and diethyl ether (Et_2O) were of technical grade (Baker) and used as received. Styrene (Aldrich) was distilled before use. Tetrahydrofuran (THF) (Acros, 99+%) was distilled from sodium and benzophenone and CH_2Cl_2 (Baker) was distilled from calcium hydride and triethylamine (Et_3N) (Baker) was distilled from CaCl prior to use. Ultrapure water was purified using a WaterPro PS polisher (Labconco, Kansas City, MO) set to 18.2 MΩ/cm.

Candida antarctica Lipase B, recombinant from *Aspergillus oryzae* (E.C. 3.1.1.3), horseradish peroxidase (E.C. 1.11.1.7) type VI, and glucose oxidase (E.C. 1.1.3.4) type X-S from *Aspergillus niger* were purchased from Sigma (BioChemika). PS_{40}-$PIAT_{50}$ was purchased from Encapson B.V. (Nijmegen, The Netherlands).

^{1}H NMR spectroscopy: spectra were recorded on a Varian inova400 instrument at room temperature. ^{1}H NMR spectra are reported in ppm (δ) relative to tetramethylsilane (δ=0.00) when measured in $CDCl_3$. In CD_3OD the solvent residual peak is used as a reference. Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), m (multiplet), or b (broad). The number of protons (*n*) for a given resonance is indicated as *n*H, and is based on spectral integration values.

Transmission electron microscopy (TEM): TEM images were obtained using a JEOL JEM 1010 microscope (60 kV) equipped with a CCD camera. Samples were prepared by placing 10 µL of a solution on a carbon-coated copper grid for 15 min., after which the liquid was removed. The grid was washed by placing 10 µL MQ on it, which was subsequently removed, after which the grid was dried in vacuo. The structures were visualised without further treatment.

Scanning electron microscopy (SEM): SEM images were obtained using a JEOL JSM T300 Scanning microscope (30 kV). Samples were prepared by placing 10 µL of a solution on a carbon-coated copper grid for 15 min., after which the liquid was removed. The grid was washed by placing 10 µL MQ on it, which was subsequently removed, after which the grid was dried in vacuo. Grids were subsequently coated in 1.5 nm Pt/Au using a BALZERS sputter machine. The structures were visualised without further treatment.

UV spectroscopy: UV absorption was measured using a Wallac Multilabel Counter 1420 (Victor Wallac) using 96-well micro titer plates. All samples were freshly prepared and measurements were started immediately after mixing.

Inductively coupled plasma - mass spectrometry (ICP-MS): ICP-MS measurements were performed using an Xseries I quadropole machine (Thermo Fisher Scientific) using 5 mL samples containing 0.5 mg mL^{-1} AgOAc as an internal standard.

Mass spectrometry: ESI-TOF measurements were performed using an AccuTOF-CS (Jeol). Samples were prepared in MQ containing 0.5% (vol.) formic acid with a final concentration of 2 mg mL^{-1}. ESI-ion trap spectra were obtained using an LCQ advantage max (Thermo Finnigan, Thermo scientific) on samples in MeOH with a final concentration of 1 mg mL^{-1}.

Preparation of polymersomes: PS-PIAT (0.5 mg) was dissolved in tetrahydrofuran (THF, 0.5 mL) containing the appropriate wt.-% of anchor **1**. Subsequently, it was gently dripped into 2.5 mL of a phosphate buffer (20 mM, pH 7.4) and left to self-assemble for 30 min. The suspension was then transferred to an Amicon Ultra Free-MC centrifugal filter with a cutoff of 100 kDa and centrifuged to dryness. The polymersomes were redispersed in 600 µL of a phosphate buffer (20mM, pH 7.4) and then centrifuged again. This step was repeated six times. The resulting vesicles were redispersed in 1mL phosphate buffer (20 mM, pH 7.4). TEM images of all types of polymersomes used in this study are shown in figure 7.

Encapsulation of enzymes in polymersomes: For vesicles containing GOx in their lumen, 250 µL of the buffer was replaced by an equal volume of a GOx stock solution in the same buffer (2.5 mg mL^{-1}, 15.6 µM). The further procedure was unchanged. For polymersomes containing CalB in their membranes, the THF solution containing the block-copolymers was first injected into 100 µL of a CalB stock solution (2 mg mL^{-1}, 56.1 µM) in ultrapure water (MQ). This dispersion was lyophilised and redissolved in THF (0.5mL) and then used as described above. TEM images of all types of polymersomes used in this study are shown in figure 7.

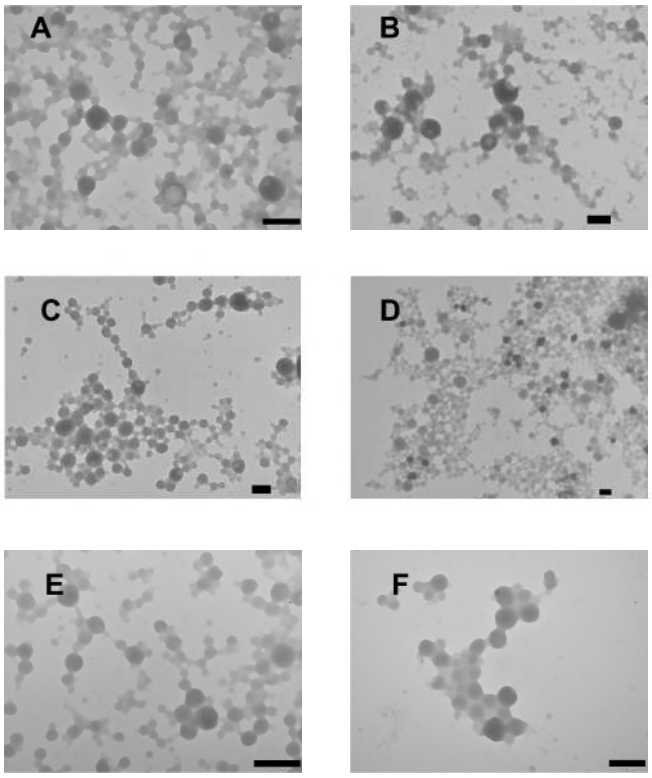


Figure 7. Transmission Electron Micrographs of polymersomes. All micrographs are TEM images of PS-PIAT polymersomes containing 10 wt.-% of anchor **1**. The black scale bar denotes 200 nm. A., C. and E., are polymersomes containing GOx in their lumen and CalB in their membranes. B., D. and F., are the same polymersomes, but now with HRP conjugated to their surfaces. In A. and B., HRP is Ru-labelled using **3**. in C. and D. CalB is Ru-labelled using **3**. In E. and F. GOx is Ru-labelled using **3**.

Diazo transfer to HRP: A solution of HRP in MQ (200 µL, 2.5 mg mL^{-1}) was treated with K_2CO_3 (100 µL of an aqueous solution, 2 mg mL^{-1}), along with 25 µL of a $Cu(II)SO_4 \cdot 5H_2O$ solution in MQ (1 mg mL^{-1}). After mixing, **2** was added as a solution in MQ (15 µL, 2 mg mL^{-1}, 1.75 equiv.) and the reaction was left on a roller bank overnight. The reaction mixture was transferred to an Amicon UltraFree-MC centrifugal filter with a 3 kDa cutoff and centrifuged to dryness. The supernatant was redissolved in 600 µL MQ and centrifuged again. This procedure was repeated for a total of five such washings. Finally, the product was redissolved in 200 µL MQ. It was analysed by ESI-TOF. MS: *m/z* 43282.00 (M, calc. (for four transfers based on 43178.00 found for unreacted HRP): 43281.96).

Conjugation of azido-HRP to polymersome surfaces: To a dispersion of acetylene-functionalised polymersomes in 200 µL phosphate buffer (20 mM, pH 7.4) an aqueous solution of azido-functionalised HRP (33 µL, 75 µM, 2 equiv. relative to **1**) was added. Aqueous solutions of $Cu(II)SO_4 \cdot 5H_2O$ containing sodium ascorbate (10 mM each, 33 µL) and bathophenanthroline ligand (10 mM, 33 µL) were pre-mixed and subsequently added to the dispersion, which was left at 4°C for 60 h. The mixture was then transferred to an Amicon UltraFree-MC centrifugal filter with a 100 kDa cutoff and centrifuged to dryness. The supernatant polymersomes were redispersed in 600 µL phosphate buffer (20 mM, pH 7.4) and centrifuged again. This step was repeated until no enzyme activity could be detected in the filtrate. The resulting biohybrid was redispersed in 200 µL phosphate buffer (20 mM, pH 7.4).

Synthesis of 1-O-acetyl-2,3,4,6-tetra-O-benzyl-D-glucopyranose (5): 2,3,4,6-Tetra-O-benzyl-D-glucopyranose (990 mg, 1.8 mmol), was dissolved in 30 mL dry CH_2Cl_2. Triethylamine (Et_3N), 240 µL (1.9 mmol, 1.05 equiv.) was added, followed by 180 µL (1.9 mmol, 1.05 equiv.) acetic anhydride (Ac_2O). After 2 h. of stirring, a further 240 µL Et_3N (1.9 mmol, 1.05 equiv.) was added, followed by 180 µL Ac_2O (1.9 mmol, 1.05 equiv.), which was left to stir for 2 h. The crude reaction was washed with 3x aqueous 1M HCl, 2x aqueous 5% $NaHCO_3$, ultrapure water, and brine. Pure 1-O-acetyl-2,3,4,6-tetra-O-benzyl-D-glucopyranose (860 mg, 82%) was obtained after flash chromatography (CH_2Cl_2). ^1H NMR (400 MHz, $CDCl_3$): δ=2.05 (s, 3H, Ac), 3.59 (m, 2H, C^6H_2), 3.73 (m, 4H, C^2H, C^3H, C^4H, C^5H), 4.50-4.91 (m, 8H, benzylic), 5.60 (d, 1H, anomeric), 7.14-7.32 (m, 20H, Ar). MS: *m/z* 605.30 (M + Na, calc. 605.25).

Synthesis of 1-O-acetyl-D-glucopyranose (4, GAc). Compound **5** (500 mg, 0.86 mmol) was dissolved in 25 mL MeOH / EtOAc (2:1, v/v). To this solution, 10 mg of charcoal-supported Pd-catalyst was added. The solution was shaken under 3 bar H_2 pressure for 90 min using a Parr apparatus. The Pd/C was removed by filtration over celite and the solution was concentrated to yield 1-O-Acetyl-D-Glucopyranose as a clear, waxy solid (176 mg, 92%). ^1H NMR (400 MHz, CD_3OD): δ=2.02 (s, 3H, Ac), 3.04 (b, 2H, C^3H and C^5H), 3.13 (b, 2H, C^6H_2), 3.38 (m, 1H, C^2H), 3.58 (m, 1H, C^5H), 4.55 (t, 1H, CH^2OH), 4.99 (b, 1H, C^2HOH), 5.08 (b, 1H, C^3HOH), 5.25 (b, 1H, C^4HOH), 5.29 (d, 1H, anomeric H). MS: *m/z* 245.05 (M + Na, calc. 245.06).

Activity assay for biohybrid polymersomes: A stock solution of 1-O-acetyl-D-glucopyranose **4** (1 M in 20 mM phosphate buffer, pH 7.4) was freshly prepared before each series of measurements, as was an ABTS stock solution (4 mM in 20 mM phosphate buffer, pH 7.4). In a single well of a 96-well micro titer plate a dispersion of polymersomes (100 µL) or an aliquot of control solution (100 µL) was placed, followed by the glucose acetate (40 µL) and the ABTS (20 µL) stock solutions. The monitoring of the formation of the radical cation of ABTS via its absorption at 405 nm was started immediately after mixing.

Ru-labelling of enzymes: To a weighed quantity of bis(2,2'-bipyridine)-(5-isothiocyanato-phenanthroline)ruthenium bis(hexafluorophosphate) (**3**) an aqueous solution of the desired enzyme in MQ was added in such an amount that 0.5 equiv. of the metal complex were present for every amine in the protein, counting only its lysine residues and N-terminus. Then, 10 vol.-% of an aqueous Na_2CO_3 solution in MQ (1 mg mL^{-1}) was added and the reaction mixture was left at 4°C for 14 h. Hereafter, it was filtered using an Amicon UltraFree-MC centrifugal filter with a 3 kDa cutoff. The supernatant was redissolved in 600 µL MQ and centrifuged again. This procedure was repeated for a total of five such washings. Finally, the product was redissolved in an aliquot of MQ equal to that of the enzymatic solution initially used. Reactions were verified via inductively coupled plasma mass spectrometry (ICP-MS). Labelled azido-HRP was reacted with 0.1 equivalents of **3** prior to a diazo transfer as described above.

ICP-MS analysis of biohybrid polymersomes: Dispersions of polymersomes in MQ containing Ru-labelled enzymes were lyophilised. The dry vesicles were then destructed in concentrated nitric acid (0.5 mL) at 80°C for 3 h. The samples were cooled to room temperature and silver acetate (AgOAc) was added as an internal standard (2 mg mL^{-1} in MQ, 1.25 mL). The total volume of each sample was then brought to 5.0 mL using MQ prior to measurement. Resulting ppm values were expressed as molarities by standardising Ru-counts on Ag-counts and comparing these results to samples containing known amounts of labelled enzyme.

Curve fitting: Curves were fit using Prism 5.0a for Mac OS X. All fits were least-squares fits using one thousand iterations. For equation 1, initial values were set as follows: $[GAc]_0 = 550$, $k_1 = 0.02$, $k_2 = 0.002$; Only the first two-thirds of data points were fit, leading to a curve with an R^2 value of 0.9956. The last forty percent of data points could be fit to a sigmoidal curve of the shape $C_p(t)=[GAc]_0 \cdot t^h/(Km + t^h)$ where

Km represents the Michaelis-Menten constant. This led to a curve with an R^2 value of 0.9997, suggesting that the decay of $ABTS^{+}$ is responsible for the departure from equation 1. In the measurement of polymersomes that do not have CalB in their membranes, water can still hydrolyse GAc. The absence of CalB can be translated into equation 1 by reducing k_1 to a very small number compared to its original value. The following initial values were used for the fitting procedure: $[GAc]_0 = 550$, $k_1 = 2 \cdot 10^{-9}$, $k_2 = 0.002$. The resulting curve, while less convincing with an R^2 value of 0.9682, still suggests that the overall shape of the progress curve is unaltered by the drastic reduction of k_1, indicating that CalB is not the slowest enzyme in the triad.

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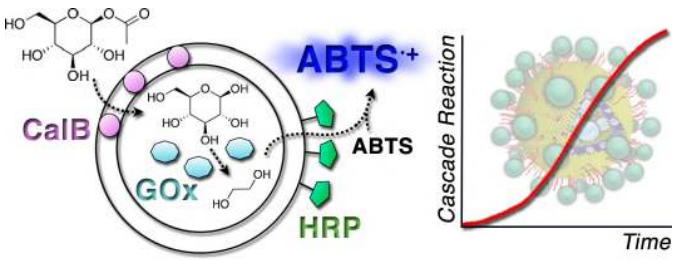
Polymersomes

*Stijn F. M. van Dongen, Madhavan Nallani, Jeroen J. L. M. Cornelissen, Roeland J. M. Nolte, Jan C. M. van Hest**

A three-enzyme cascade reaction via positional assembly of enzymes in a polymersome nanoreactor



A porous polymersome was equipped with three different enzymes at three spatially separate locations: CalB in its lumen, GOx in its bilayer membrane, and HRP conjugated to its surface.

The encapsulation efficiency of the enzymes was determined, and their activity as part of a three-enzyme cascade reaction was investigated.