A Three-Enzyme Cascade Reaction through 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 surface coupling was achieved by click chemistry between acetylene-functionalised anchors on the surface of the polymersomes and azido functions of HRP, which were introduced by 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 was oxidised by GOx to gluconolactone in a second step. The hydrogen peroxide produced was used by HRP to oxidise 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to ABTS•+. Kinetic analysis revealed that the reaction step catalysed by HRP is the fastest in the cascade reaction.

Keywords: cascade reactions · enzyme immobilization · macromolecular chemistry · nanostructures · polymers

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 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.

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] the bilayer membranes of which 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 with 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 possible in block copolymer synthesis allows one to tune the properties of polymersomes.[11–13] A drawback of polymersome membranes is their low permea-
bility, even to water, which hampers their application as nanoreactors. To overcome this problem, the diffusion of solvent and substrate molecules has been enabled by the inclusion of channel proteins or proton pumps in the polymeric bilayer.

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-\text{-}b-\text{-}poly(\text{-}l\text{-}isocyanoalanine\text{-}(2\text{-}thio-phen\text{-}3\text{-}yl\text{-}ethyl)amide) (PS\text{-}PIAT). It is a rod–coil type of amphiphilic copolymer consisting of a rigid polyisocyanide block and a flexible polystyrene block. On dispersal in water it forms porous polymersomes that possess a relatively high degree of diffusion. Small molecules can move across their membranes, whereas larger molecules, such as proteins, cannot.

Previously we described the use of PS\text{-}PIAT polymersomes as scaffolds for the positional assembly of two different enzymes. Glucose oxidase (GOx) was encapsulated in the lumen of the polymersome, whereas horseradish 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). CalB was added separately to the polymersome dispersion. In this cascade system, 1,2,3,4-tetra\text{-}O\text{-}acetetyl\text{-}b\text{-}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, which was used to convert 2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) into ABTS$^+$. In the system described above, only two out of three enzymes were linked to the polymersome. While a variety of tools is available for the preparation of polymer/enzyme biohybrids, we have recently developed methodologies to specifically anchor an enzyme to the surface of a polymersome, 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\text{-}PIAT to introduce surface functionalities while maintaining membrane porosity, that is, 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 through 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 step. The system also features a more controlled spatial positioning of the enzymes. Herein the construction of the three-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, to be able to prepare this three-step reactor, azido groups needed to be introduced into HRP. To realise this, we applied a diazo transfer reaction directly on the amines of the lysine residues and the amino terminus of HRP. This facile diazo transfer reaction uses imidazole-1-sulfonyl azide hydrochloride (2, Scheme 1).

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**Scheme 1.** Structures of block copolymer “anchor” 1, diazo transfer reagent 2, Ru-labelling compound 3 and glucose acetate 4.

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**Figure 1.** Positional assembly of enzymes in a polymersome. A mixture of PS\text{-}PIAT and anchor 1 is lyophilised with CalB and then dissolved in THF. This mixture is then injected into an aqueous buffer containing GOx, encapsulating it in the inner compartment and subsequently trapping CalB in the polymeric bilayer. A third enzyme, HRP, is immobilised on the polymersomal perimeter through a covalent linkage to anchor 1, creating an outer shell of enzymes.

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 if it were embedded in them;[21] 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 previously been successfully incorporated in a PS-PIAT membrane.[20, 21] Our choice to embed CalB in the bilayer of the vesicles was primarily motivated by the fact that it is more hydrophobic than HRP. 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.[25, 26] 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 through 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[21] was then dissolved in THF and gently dripped into a phosphate buffer containing GOx (0.25 mg mL⁻¹). After half an hour of equilibration, non-encapsulated enzymes and THF were removed by filtration, and the resulting mixture was analysed by electron microscopy (Figure 3), 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- or multisite protein engineering replacement strategies, the level of control provided by these laborious methods is not needed for merely immobilising enzyme molecules. This led us to apply the mild and generally facile diazo transfer reaction to amines, carried out with 2 (Scheme 1) as the reagent and catalysed by Cu(II), which has been shown to be suitable for protein modification in aqueous solution. To this end, an aqueous solution of HRP, K_2CO_3, and CuSO_4 was treated with water-soluble 2 for twelve hours, after which time the enzyme was purified by 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.

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 anchor their inner aqueous compartments and 10% acetylene per enzyme is required for surface immobilisation.

Figure 4. 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).

Table 1. From these results, it appears that the efficiency of the polymersomes remained unperturbed, as is shown in Figure 3.

### 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 with GOx in their aqueous compartment and CalB in their bilayer membrane. After conjugation, the vesicles were washed by using a cutoff filter that allowed passage of unconnected enzymes while retaining intact polymersomes in the supernatant. The transmission electron micrographs shown in Figure 3 revealed the unaltered spherical morphology of the polymersomes.

Little 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 with an Hb-specific protocol. For a more general approach, we chose to employ a commercially available ruthenium complex with an isothiocyanato moiety (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 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 3). 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 1, 7 nmol of the alkyn moiety became 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.
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For GOx and CalB, the incorporation efficiency was much higher than 0.1%, which would be expected based on statistical inclusion.[21] 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 glucosamine was chosen. Previously, the tetraacetate GAc4 had been used for this purpose.[21] For the present study, however, an increased solubility of the substrate in aqueous buffers was desired 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 4 (Scheme 1). Besides its greater solubility, substrate 4 is also more rapidly deprotected by CalB, which enhances the overall reaction rate of the cascade system.

The multistep nanoreactors were incubated with 4 and ABTS as described in the Experimental Section and the increase in ABTS* 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 Equation (1), which describes a two-enzyme reaction ($R^2$=0.9956; $C_p$ denotes the concentration of the final product, ABTS*; $[4]_0$ is the initial concentration of the substrate; data were fit by using the GraphPad Prism 5.0a software for Mac OS X.)

$$C_p(t) = \frac{[4]_0}{k_2-k_1} (e^{-k_1t}+k_1 e^{-k_2t})+[4]_0$$  (1)

This suggests that one of the three enzymes has an activity high enough not to influence the total kinetics. We propose that this is HRP. Its spatial position relative to GOx enables it to convert any H$_2$O$_2$ 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 because it never came into contact with this solvent. For comparison, 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 4; the progress of the reaction is dependent on the hydrolysis of 4 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.

The relatively high concentration of enzyme molecules measured by ICP–MS does not translate to 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 through a CuI-catalysed [3+2] Huisgen cycloaddition. To this end, HRP was 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-HPF molecule, and the lumen incorporates approximately 25% of the added GOx enzymes, which is more than expected for a purely 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 step. The progress curve of the reaction fits to a two-enzyme reaction model, suggesting that one of the enzymes, that is, 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 herein does not provide an inherent catalytic 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.\(^{25}\) Diazotransfer reagent 2 was prepared as described elsewhere.\(^{23}\) Deuterated chloroform (CDCl\(_3\), 99.8%), deuterated methanol (CD\(_3\)OD, 99.9%) and heavy water (D\(_2\)O, 99.9%) were purchased from Aldrich. CuSO\(_4\) (Merck); sodium ascorbate (Fluka, >99%); 4,4-diphenyl-1,10-bathophenanthroline disulfonic acid disodium salt (Sigma-Aldrich); anhydrous sodium sulfate (Fluka, 99%); K\(_2\)CO\(_3\) (Fisher); magnesium sulfate dihydrate (Fluka, 99%); Na\(_2\)N (Acros, 99%); 3 (BioChemika, Aldrich); 2,3,4,6-tetrabenzylidene-1,10-bathophenanthroline disulfonic acid di-sodium salt (Sigma-Aldrich); Cellect; acetic anhydride (Ac2O); NaHCO\(_3\), Na\(_2\)CO\(_3\), Na\(_2\)HPO\(_4\) (Merck) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Fluka, 99%) were all used as received. Charcoal-supported PD catalyst (Pd/C), HNO\(_3\), HCl, MeOH, Et\(_3\)OAc, NaCl, AgOAc, toluene, 1,4-dioxane, DMF and Et\(_2\)O were technical grade (Sigma-Aldrich). Ultrapure water was used in this study. THF (Acros, >99%) was distilled from sodium benzophenone and CH\(_2\)Cl\(_2\) (Baker) was distilled from calcium hydride and Et\(_3\)N (Baker) was purified by using a Jeol JSM T300 Scanning microscope (30 kV). Samples were prepared by placing 10 \(\mu\)L of a solution on a carbon-coated copper grid and then used as described above. TEM images of all types of polymersomes used in this study are shown in Figure 6.

**Encapsulation of enzymes in polymersomes:** For vesicles containing GOx in their lumen, 250 \(\mu\)L 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 \(\mu\)M). The remaining procedure was unchanged. For polymersomes containing CalB in their membranes, the solution containing the block-copolymers in THF was first injected into 100 \(\mu\)L of a CalB stock solution (2 mg mL\(^{-1}\), 56.1 \(\mu\)M) in THF. This dispersion was lyophilized and resuspended in THF (0.5 mL) and then used as described above. TEM images of all types of polymersomes used in this study are shown in Figure 6.

**Diazotransfer to HRP:** A solution of HRP in ultrapure water (200 \(\mu\)L, 2.5 mg mL\(^{-1}\)) was treated with K\(_2\)CO\(_3\) (100 \(\mu\)L of an aqueous solution, 2 mg/mL\(^{-1}\)), along with a solution of Cu\(_3\)SO\(_4\)·5H\(_2\)O in ultrapure water (10 \(\mu\)L, 10 mg mL\(^{-1}\)). The solution was gently dripped into a phosphate buffer (2.5 mL, 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 phosphate buffer (600 \(\mu\)L, 20 mM, pH 7.4) and then centrifuged again. This step was repeated six times. The resulting vesicles were redispersed in phosphate buffer (1 mL, 20 mM, pH 7.4). TEM images of all types of polymersomes used in this study are shown in Figure 6.

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

**Preparation of polymersomes:** PS-PIAT (0.5 mg) was dissolved in THF (0.5 mL) containing the appropriate wt% of anchor 1. Subsequently, it was gently dripped into a phosphate buffer (2.5 mL, 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 phosphate buffer (600 \(\mu\)L, 20 mM, pH 7.4) and then centrifuged again. This step was repeated six times. The resulting vesicles were redispersed in phosphate buffer (1 mL, 20 mM, pH 7.4). TEM images of all types of polymersomes used in this study are shown in Figure 6.

**Transmission electron microscopy (TEM):** TEM images were obtained by using a JEOL JEM 1010 microscope (60 kV) equipped with a CCD camera. Samples were prepared by placing 10 \(\mu\)L of a solution on a carbon-coated copper grid for 15 min, after which time the liquid was removed. The grid was washed with 10 \(\mu\)L ultrapure water, 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 by using a JEOL JSM T300 scanning microscope (30 kV). Samples were prepared by placing 10 \(\mu\)L of a solution on a carbon-coated copper grid for 15 min, after which time the liquid was removed. The grid was washed with 10 \(\mu\)L ultrapure water, which was subsequently removed, after which the grid was dried in vacuo. Grids were subsequently coated in 1.5 nm Pt/Au by using a BALZERS sputter machine. The structures were visualised without further treatment.

**UV spectroscopy:** UV absorption spectra were measured by using a Wallac Multilabel Counter 1420 (Victor Wallac) with 96-well microtiter 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 by using an Xseries I quadrupole machine (Thermo Fisher Scientific) with 5 mL samples containing 0.5 mg mL\(^{-1}\) AgOAc as an internal standard.
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(25 µL, 1 mg/mL). After mixing, compound 2 was added as a solution in ultrapure water (15 µL, 2 mg/mL, 1.75 equiv) and the reaction was left on a roller rack 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 ultrapure water (600 µL) and centrifuged again. This procedure was repeated for a total of five such washings. Finally, the product was redissolved in ultrapure water (200 µL). It was analysed by ES-TOF-MS: m/z: 43.2820 [M] (calcd for four transfers based on 43.178.00 found for unreacted HRP).

Conjugation of azido-HRP to polymersome surfaces: An aqueous solution of azido-functionalised HRP (33 µL, 75 µg, 2 equiv relative to I) was added to a dispersion of acetylene-functionalised polymersomes in phosphate buffer (200 µL, 20 mM, pH 7.4). Aqueous solutions of Cu²⁺SO₄·5H₂O containing sodium ascorbate (10 mM each, 33 µL) and bishethenanthroligand (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 redissolved in phosphate buffer (600 µL, 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 redissolved in phosphate buffer (200 µL, 20 mM, pH 7.4).

Synthesis of 1-O-acetyl-2,3,4,6-tetra-O-benzyl-ß-D-glucopyranose (5): 2,3,4,6-Tetra-O-benzyl-ß-D-glucopyranose (990 mg, 1.8 mmol), was dissolved in dry CH₂Cl₂ (30 mL). Et₃N (240 µL, 1.9 mmol, 1.05 equiv) was added, followed by acetic anhydride (180 µL, 1.9 mmol, 1.05 equiv). After stirring for 2 h, a further portion of Et₃N (240 µL, 1.9 mmol, 1.05 equiv) was added, followed by more Ac₂O (180 µL, 1.9 mmol, 1.05 equiv), which was left to stir for 2 h. The crude reaction mixture was washed with 1 mL aqueous HCl (3x), a 5% aqueous solution of NaHCO₃ (3 x 0.5 mL, 3.3 mL), and centrifuged to dryness. The supernatant polymer-somes that do not have CalB in their membranes, water can still hydrolyse 4. The absence of CalB can be translated into Equation (1) by reducing k₅ to a very small number relative to its original value. The following initial values were used for the fitting procedure: [4]₀ = 550, k₅ = 2 × 10⁻³ s⁻¹, k₆ = 0.002. The resulting curve, while less convincing with an R² value of 0.9962, still suggests that the overall shape of the progress curve is unaltered by the drastic reduction of k₅, indicating that CalB is not the slowest enzyme in the triad.

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