Enzymes containing porous polymersomes as nano reaction vessels for cascade reactions

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Polystyrene$_{40}$-*b*-poly(isocyanoalanine(2-thiophen-3-yl-ethyl)amide)$_{50}$ (PS-PIAT) polymersomes have the unique property of being sufficiently porous to allow diffusion of small (organic) substrates while retaining large biomolecules such as enzymes inside. Herein we report on the encapsulation and protection of glucose oxidase (GOx) and horse radish peroxidase (HRP) in PS-PIAT polymersomes and the successful employment of these functionalised nanoreactors in a cascade reaction. The demonstrated concept allows for further application in other enzymatic cascade reactions, bio-organic hybrid systems and biosensing devices.

Introduction

Cells consist of numerous different compartments that both physically and functionally separate vital biochemical processes. Communication and transport of molecules between these compartments can occur *via* an active pathway, using membrane spanning proteins, or a diffusional pathway. One approach to mimic this compartmentisation is by the use of lipid or amphiphilic block copolymer vesicles or within solgel matrices.[1–3] For biotechnology applications, polymer vesicles are superior to most of their lipid counterparts because of their higher mechanical and thermodynamical stability.[4] The drawback of this increased thermodynamic stability is that it is often more difficult for small substrate molecules, or even water, to diffuse in or out of the polymersome. A way to overcome this difficulty is by constructing stimulus-responsive membranes[5–9] that either decompose or reversibly open and close upon exposure, or by addition of channel proteins to the polymer membrane.[10–12] Another alternative, reported by Sukhorukov *et al.*, makes use of a semi-permeable polyelectrolyte membrane.[13] This membrane allows small substrate molecules to enter and leave the capsules freely whilst retaining the larger biomolecules.

Recently, we have reported on the synthesis and use of the diblock copolymer polystyrene$_{40}$-*b*-poly(isocyanoalanine(2-thiophen-3-yl-ethyl)amide)$_{50}$ (PS-PIAT, Fig. 1) as a polymersome forming amphiphile.[14] This block copolymer consists of a rigid rod polyisocyanide headgroup and a flexible polystyrene tail, making it a rod-coil type of polymer. When injected into water, PS-PIAT readily forms nanometre sized polymersomes that have the *unique* property of being sufficiently porous to enable diffusion of low molecular weight molecules, whilst retaining large biomolecules such as enzymes inside.

This porosity enables the enzyme to function as in solution, but now protected from a degrading environment, such as proteolytic

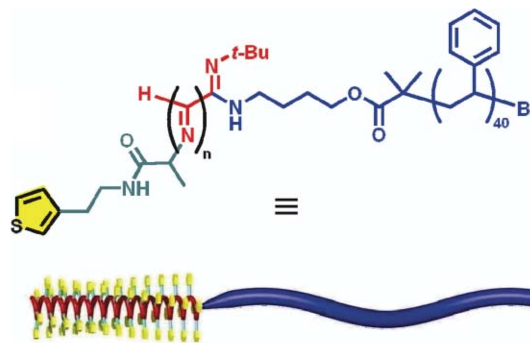

Fig. 1 Chemical and schematic structure of PS-PIAT that forms vesicular aggregates when injected into water.

enzymes and microbes, which are too large to penetrate the membrane pores. In addition, by the use of block copolymer lyophilisation followed by polymersome formation, it was demonstrated that different enzymes can be incorporated both in the central water pool and the membrane, respectively. This specific positioning enabled the efficient assembly of a cascade system in which horse radish peroxidase (HRP) and glucose oxidase (GOx) were chemically coupled within one polymersome.[15]

This report further investigates the possibility of communication between enzymes located in *different* polymersomes using the same GOx–HRP cascade reaction as mentioned above (Fig. 2). The first reaction in the cascade is the conversion of glucose by GOx into gluconolactone and H_2O_2. The formed H_2O_2 then reacts further with HRP and added 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) to form the ABTS radical cation. The cascade reaction can be easily monitored using UV spectroscopy due to a shift in the UV absorption spectrum from 340 nm to 405 nm when ABTS is oxidised to $ABTS^{\bullet +}$.

To the best of our knowledge, this is the first report on communication between enzymes located in *separate* polymersomes without the addition of artificial transport mediators in the membrane or other external stimuli. Furthermore, it is a very easy and fast

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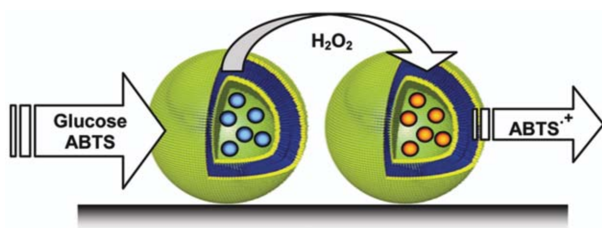


Fig. 2 Schematic representation of a cascade reaction between separate polymersomes.

procedure to encapsulate and chemically couple two different enzymes.

Results and discussion

Enzyme encapsulation

To study the interaction between the different enzymes located in different polymersomes, we encapsulated GOx and HRP separately using a standard injection method.[16] The amphiphilic block copolymer is dissolved in freshly distilled THF (1 mg mL^{-1}), followed by injection into an aqueous solution of the required enzyme (0.2 mg mL^{-1}). After 30 minutes the non-encapsulated enzyme that remains outside of the vesicle is removed by Amicon filtration. With transmission electron microscopy (TEM, Fig. 3), it was determined that the diameter of polymersomes loaded with GOx ranged from 150 to 250 nm (Table 1).

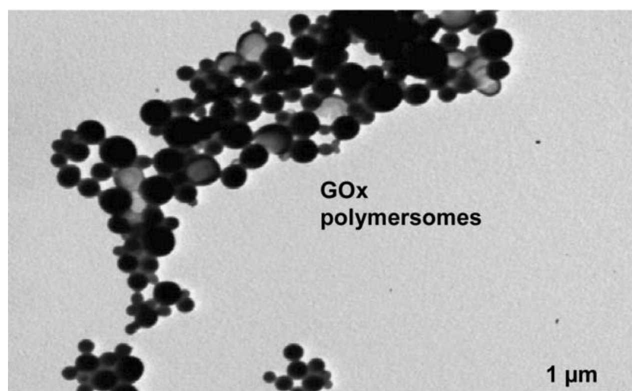
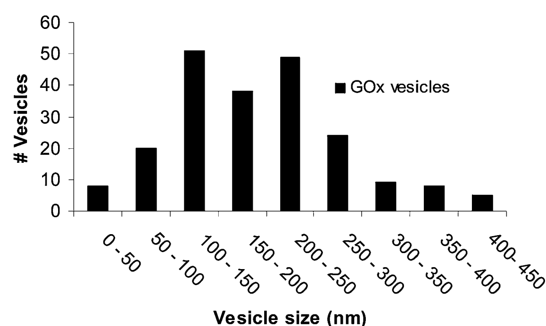


Fig. 3 Transmission electron micrographs of GOx loaded PS-PIAT polymersomes.

Table 1 Size distribution of GOx loaded polymersomes



The average diameter of polymersomes encapsulating HRP turned out to be somewhat smaller, *viz. ca.* 100 nm (Fig. 4, Table 2). We tentatively assign this size difference to the ability of the enzyme to stabilise the membrane by absorption to it. It has been previously shown that the vesicle size is dependent on the fluidity of the block copolymer.[14] Monolayer studies are currently being carried out to quantify the strength of interaction between the polymer and the enzymes.

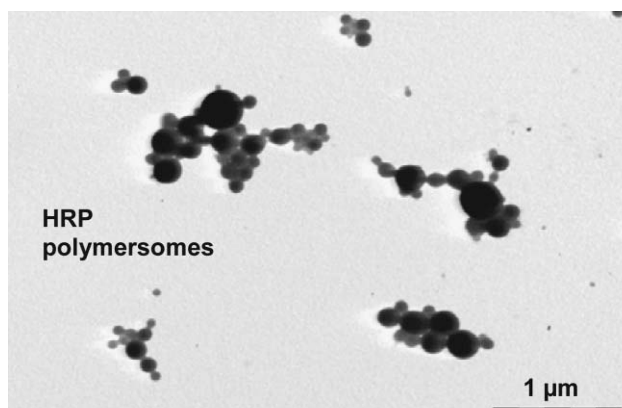
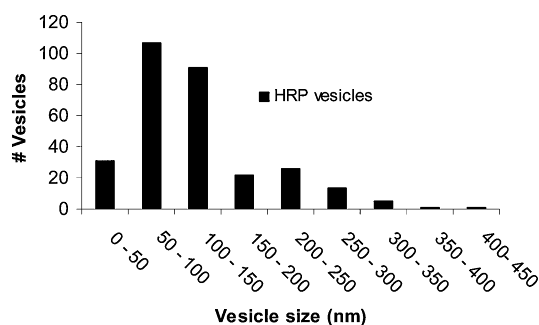


Fig. 4 Transmission electron micrograph of HRP loaded PS-PIAT polymersomes.

Table 2 Size distribution of HRP loaded polymersomes



Activity of encapsulated enzymes

Initially, the activity of the two enzymes was measured individually. To determine the activity of encapsulated GOx, glucose, HRP and ABTS were added (in excess) to the outer environment of the polymersomes. In the case of encapsulated HRP, we added H_2O_2 and ABTS to the outside of the polymersomes. Both GOx- and HRP-filled polymersomes catalysed the formation of ABTS$^{\bullet+}$, thereby showing that the encapsulated enzymes retain their activity whilst inside the polymersomes (see Fig. 5 and 6). Furthermore, this result indicates that in the case of encapsulated GOx, H_2O_2 readily diffuses out of the polymersome and reacts in a cascade fashion with free HRP in solution.

In addition, it is noteworthy that ABTS is also capable of permeating the membrane, which is a prerequisite in order to detect the activity of encapsulated HRP. In order to exclude the possibility that the observed HRP activity is due to HRP adhered to the outside of the polymersome (even after filtration and

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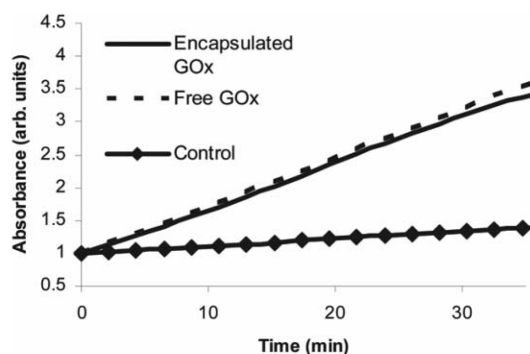

Fig. 5 Absorption of ABTS$^{\bullet+}$ at 405 nm plotted against time for free (dotted line) and encapsulated (bold line) GOx.

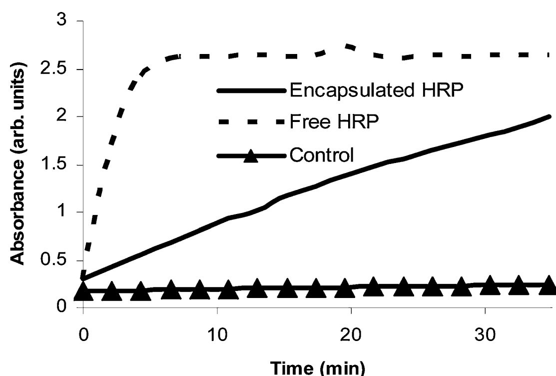

Fig. 6 Absorption of ABTS$^{\bullet+}$ at 405 nm plotted against time for free (dotted line) and encapsulated (bold line) HRP. The control experiment in the case of HRP is further explained in the text.

elaborate washing steps), we prepared non-filled polymersomes to which HRP was added. This sample was left to stand for two days at 4 °C and then filtered in the same manner as in the encapsulation experiments. The same ABTS-assay as used normally was employed to determine the activity of the filtered polymersomes, revealing no activity (bold triangled line in Fig. 6) and it is therefore safe to conclude that only enzyme activity on the *inside* of the polymersome is measured.

From the graph in Fig. 6, it can be seen that encapsulated HRP has a lower activity than free HRP with the same (estimated) concentration. We believe that this is due to partial denaturation of HRP by THF which is needed for the formation of the polymersomes.

Prolonged enzyme activity inside polymersomes

It is known from the literature that enzymes encapsulated in polymersomes retain their activity for longer periods of time due to protection from their potentially harmful environment.[17] To prove that encapsulated proteins also retain their activity for longer periods of time inside PS-PIAT polymersomes, we compared the activity decrease of free GOx and HRP stored under the same conditions as encapsulated GOx and HRP. The activities of both free and encapsulated enzymes were measured over a period of two weeks. The results are depicted in Table 3.

Table 3 Decrease of enzymatic activity over time for encapsulated and free GOx and HRP. The relative activities are given in percentages

	$t = 0$	$t = 15$ days
Free GOx	100%	59%
Encaps. GOx	100%	98%
Free HRP	100%	19%
Encaps. HRP	100%	47%

The above results indicate that enzymes can be successfully encapsulated, resulting in protection and retained activity over longer periods of time for both GOx and HRP in the water pool of PS-PIAT polymersomes.

These experiments support the idea that ABTS and the charged radical cation can readily diffuse in and out of the polymersomes, whilst large biomolecules such as enzymes cannot. Attempts to determine the exact molecular weight cutoff of our polymersome membrane using standard leaching techniques were unsuccessful.[18]

Cascade reaction between two polymersomes and protection from proteolytic proteins

As mentioned before, enzymes encapsulated in polymersomes are protected from potentially harmful proteolytic enzymes and microbes. This accounts partially for the fact that their activity is retained for longer periods of time than the activity of free enzyme in solution.[19] Since the PS-PIAT polymersomes possess an intrinsically porous membrane, we investigated if GOx and HRP are indeed protected from proteolytic enzymes, by intentionally adding proteases to the cascade system depicted in Fig. 2. Subtilisin A was used for this purpose, since it is perfectly capable of denaturing enzymes such as GOx and HRP, which was confirmed by SDS-gel. We also put the enzymes protected inside the PS-PIAT polymersomes on the gel, but this gave no results because the polymersomes did not denature under these conditions. We could therefore only check the enzymatic activity after protease addition using the ABTS assay.

To build the coupled system depicted in Fig. 2, polymersomes containing GOx and polymersomes containing HRP were mixed in a 1 : 1 (v/v%) ratio. Assuming that the enzyme concentration inside both polymersomes is equal to the initial enzyme concentration in solution, the effective GOx : HRP ratio was calculated to be 1 : 2.6 (in active units U). Subsequent addition of an excess amount of ABTS and glucose to the mixture resulted in clear formation of the ABTS radical cation, confirming the occurrence of a cascade reaction between the two polymersomes (bold line, Fig. 7). Even after addition of subtilisin A, the cascade reaction was still proceeding, proving that the encapsulated proteins are protected inside the PS-PIAT polymersomes (bold dotted line, Fig. 7).[20] As a control, the three enzymes (GOx, HRP and SubA) were mixed together and an excess amount of ABTS and glucose was added. This did not result in formation of the ABTS radical cation as can be seen from the bold squared line depicted in Fig. 7.

Conclusion and discussion

We have shown that polymersomes prepared from polystyrene$_{40}$-*b*-poly(isocyanoalanine(2-thiophen-3-yl-ethyl)amide)$_{50}$ (PS-PIAT)

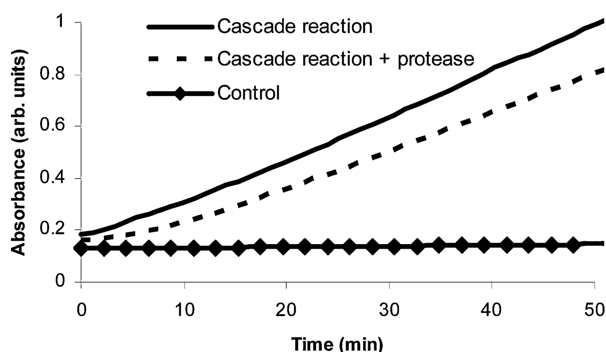

Fig. 7 Absorption of $ABTS^{\bullet+}$ at 405 nm as a function of time for the cascade reaction depicted in Fig. 2. The control experiment is further explained in the text.

can encapsulate GOx and HRP, while preserving the activity of the enzyme inside. The encapsulation procedure is easy and reliable; the encapsulated enzyme is protected, allowing its enzymatic activity to be retained. The unique porous membrane of the PS-PIAT polymersomes enables the enzyme to function as in solution and there are no additional steps necessary to assure substrate transport, *e.g.* by modifying the polymer membrane. The encapsulation has enabled us to chemically couple the two enzymes in a cascade reaction, by simply mixing the two polymersome suspensions together. To the best of our knowledge, this is the first report of such a reaction involving enzymes located in separate polymersomes without the need of artificial transport mediators in the membrane.

We are currently investigating the possibilities of implementing PS-PIAT polymersomes in other enzymatic cascade reactions to further demonstrate the strength and versatility of our system. The above described concept allows for construction of hybrid systems and one exciting possibility is the use of the combined GOx–HRP system in asymmetric organic synthesis.[21] Another application, which we are currently investigating, is the implementation of PS-PIAT polymersomes in biosensing and biofuel devices.

Experimental section

All enzyme encapsulation experiments were carried out using freshly distilled THF and 20 mM phosphate buffer at pH 7.2. Glucose oxidase from *Aspergillus niger* (E.C. 1.1.3.4), horse radish peroxidase from *Amoracia rusticana* (E.C. 1.11.1.7) and 2,2′-azine-bis-(3-ethylbenzthiazoline-6-sulfonic acid) were obtained from Sigma-Aldrich and used without further purification. PS-PIAT was synthesised as reported earlier.[22] Enzyme loaded polymersomes were filtered using Amicon micropore filters with a 100 kDa molecular weight cutoff. All enzymatic activities were measured at 405 nm using a Victor Wallac 1420 multiplate reader. Transmission electron microscopy was measured on a JEOL 1010, using carbon coated copper grids.

Enzyme encapsulation

A solution of PS-PIAT in THF (1 mg mL^{-1}) was slowly injected into an enzyme containing buffered solution (0.2 mg mL^{-1}), resulting in a turbid mixture with a final THF–H_2O ratio of 1 : 6.

The mixture was allowed to aggregate for 30 minutes at room temperature before removing non-encapsulated enzymes by filtration. The residue was thoroughly washed with phosphate buffer until no enzymatic activity could be detected in the filtrate. The residue was then resuspended in phosphate buffer.

Enzymatic activity measurement

GOx and HRP activity was assayed using ABTS. Stock solutions of ABTS (50 mM), glucose (1 M) and H_2O_2 (0.07%) were prepared freshly prior to measurement. Activity measurements were carried out in 96-well plates (Greiner bio-one) in a total end volume of 300 μL per well.

Acknowledgements

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