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Supporting Information

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Positional Assembly of Enzymes in Polymersomes Nanoreactors for Cascade Reactions

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General methods and materials

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. Succinimidyl ester disodium salts of Alexa Fluor 488 and 633 were obtained from Molecular Probes. THF was distilled over Na. The synthesis of PS-PIAT has been published elsewhere.[1] For the aggregation studies ultra pure water ($R > 18\times10^6$ Ω) was used. All other chemicals were used as received. A Perkin Elmer LS 50B fluorescence spectrophotometer was used to measure fluorescence spectra. TEM micrographs were recorded on a JEOL JEM-1010 instrument. Optical and fluorescence microscopy experiments were carried out with a Zeiss Axiovert 135 TV, fitted with a monochromatic light source for the fluorescence measurements.

General procedure for labeling enzymes with Alexa dyes

To 0.50 ml of a 1.0 g l^{-1} solution of enzyme in phosphate buffer (20 mM, pH 8.0), 50 μl of a 1.0 M sodium bicarbonate solution was added. To this solution 1.0 mg of the reactive succinimidyl ester disodium salt of Alexa 488 or Alexa 633 was added and the coupling was allowed to proceed for ~2 h at room temperature. The conjugates were purified by size-exclusion chromatography using Sephadex G-25, followed by extensive dialysis (molecular weight cutoff 7 kDa). The coupling of the dye was verified by polyacrylamide gel electrophoresis.

General procedure for the encapsulation of enzymes inside the water pool of PS-PIAT polymersomes

Into 5.0 ml of 0.10 g l^{-1} enzyme solution in phosphate buffer (20 mM, pH 7.2) 1.0 ml of a 1.0 g l^{-1} solution of PS-PIAT in THF was injected. After 30 min. of equilibrating the mixture was transferred to an eppendorf equipped with a 100 kDa cutoff filter and centrifuged to dryness. The polymersomes were redispersed in 500 μl of phosphate buffer (20 mM, pH

7.2) and centrifuged again. This step was repeated until no enzyme activity was observed in the filtrate. The resulting biohybrid was redispersed in 500 µl of phosphate buffer (20 mM, pH 7.2).

General procedure for the encapsulation of enzymes inside the polymersome membrane by lyophilization

A solution of 1.0 ml of 1.0 g l^{-1} PS-PIAT in THF was injected into 200 µl of a 100 mg l^{-1} enzyme solution in phosphate buffer (20 mM, pH 7.2), resulting in a dispersion with a PS-PIAT/enzyme molar ratio of 50:1. To this solution a tenfold excess of phosphate buffer (20 mM, pH 7.2) was added after which it was lyophilized. The resulting fluffy off-white powder was redissolved in THF and injected into phosphate buffer (20 mM, pH 7.2) having a final PS-PIAT/enzyme concentration of 500 mg l^{-1} in buffer/THF (8:5, v/v).

General procedure for the encapsulation of two different enzymes inside PS-PIAT polymersomes

From a 1.0 g l^{-1} PS-PIAT solution in THF 1.0 ml was injected into 200 µl of a 100 mg l^{-1} HRP solution in phosphate buffer (20 mM, pH 7.2). To this mixture 12 ml of ultra pure water was added and after homogenization the sample was lyophilized. The obtained off-white powder was redissolved in 1.0 ml of THF and 500 µl of this solution was injected into 2.5 ml of a 100 mg l^{-1} GOX solution in phosphate buffer (20 mM, pH 7.2). The same approach for removing non-encapsulated enzymes as described above was used.

Two-enzyme cascade reaction using enzyme-containing PS-PIAT polymersomes

Into a cuvette 500 µl of dispersion obtained from the procedure described above was transferred. Then 500 µl of a 1.8 mM ABTS solution in phosphate buffer (20 mM, pH 7.2) was added followed by the addition of 6.6 mg of β-D-glucose. The sample was well mixed using a pipette and the reaction was monitored by UV-Vis spectroscopy measuring the absorption at 420 nm.

Three-enzyme cascade reaction using enzyme-containing PS-PIAT polymersomes

From a GOX-HRP-polymersome dispersion 250 µl, obtained as described above, was transferred to a cuvette. To this 250 µl, 1.8 mM ABTS solution in phosphate buffer (20 mM, pH 7.2) was added, followed by the addition of 1.0 mg of CALB and 3.3 mg of 1,2,3,4-tetra-

O-acetyl-β-glucopyranose. The sample was well mixed with a pipette and the reaction was monitored by UV-Vis spectroscopy measuring the absorption at 420 nm.

Enzyme activity assay for CALB

From the purified PS-PIAT/CALB dispersion 500 µl was transferred to a cuvette containing 500 µl of phosphate buffer (20 mM, pH 7.2). Immediately after the addition of 1.0 ml of 1.5 µM DiFMU octanoate solution in water, the cuvette was placed in the fluorescence spectrophotometer and the increase in fluorescence was followed ($\lambda_{exc.}$ = 358 nm, $\lambda_{em.}$ = 450 nm).

Enzyme activity assay for HRP

An amount of 500 µl of purified HRP-polymersome dispersion was transferred to a cuvette. To 2.2 ml of a 0.33 µM aqueous solution of ABTS 50 µl of a 0.070 % (v/v) H_2O_2 solution in phosphate buffer (20 mM, pH 7.2) was added. From this solution 500 µl was added to a cuvette. The enzyme activity was monitored by measuring the absorption at 420 nm with UV-Vis spectroscopy.

Enzyme activity assay for GOX

To 1.0 ml of 0.14 g l^{-1} HRP in phosphate buffer (10 mM, pH 7.2) 27.4 mg ABTS was added. From this solution 10 µl was taken and mixed with 210 µl phosphate buffer (10 mM, pH 7.2), 10 µl 1 M glucose solution and 10 µl 1.0 g l^{-1} dispersion of PS-PIAT polymersomes with GOX in the water pool. With UV-Vis spectrometry the absorption at 405 nm was monitored.

For all the compartmentalized enzymes no initiation delay period, due to substrate diffusion, was observed after substrate addition. This result highlights the considerable porosity of the PS-PIAT polymersome membranes for the used substrates.

Determining the average diameter distribution of enzyme-containing polymersomes

For each enzyme a population of at least 100 polymersomes was measured from TEM micrographs in order to determine the spread and average of their diameters. The type of enzyme was of very little influence on the diameter of the polymersomes as the results were comparable for each enzyme. A representative diagram is given in Figure S1.

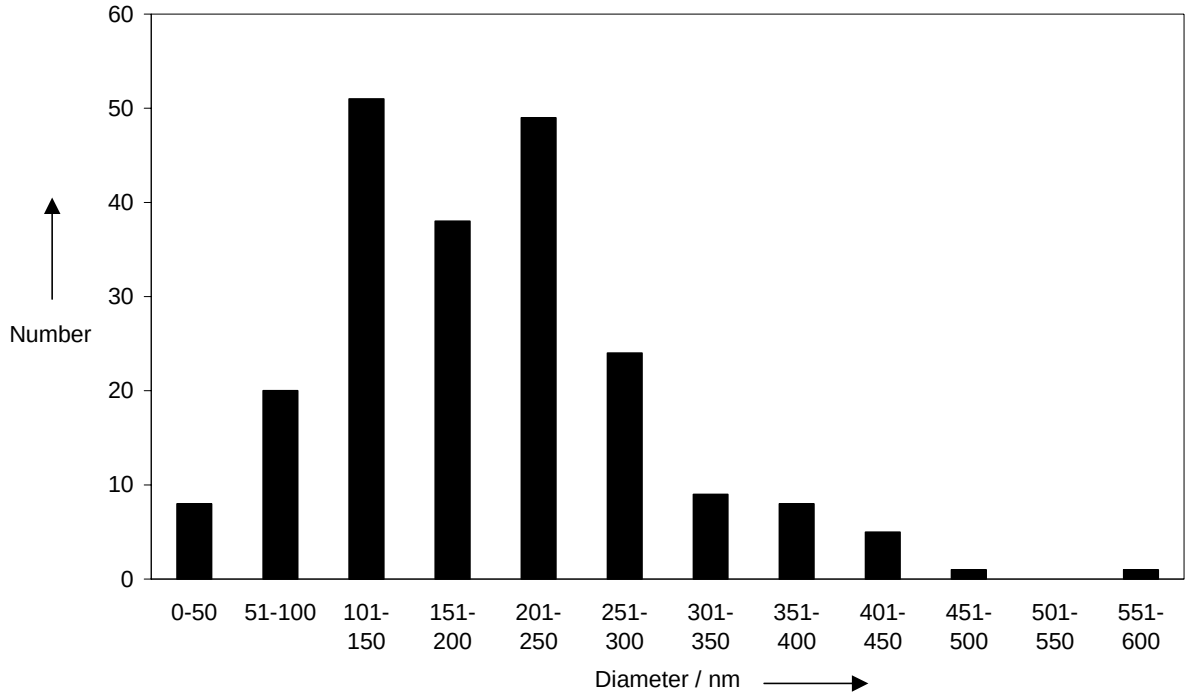


Figure S1. *Diagram of the diameter distribution of polymersomes filled with HRP.*

Reverse Positional Assembly

Interestingly, when the positional assembly of GOX and HRP was reversed, and GOX instead of HRP was located in the membrane, the resulting polymersomes were considerably less defined. TEM studies on these two types of polymersomes after filtration showed that the diameter of polymersomes with GOX in the membrane was a factor of five higher and all their membranes were ruptured (Figure S2). This lack of definition was reflected in the stability of these polymersomes. Even after 20 filtrations, enzymes were found in the filtrate, probably due to loss from fractured polymersomes. These observations were ascribed to the difference in size and polarity of the two enzymes in question. The molecular weight of HRP is 44 KDa, whereas GOX, as a dimer, is 160 KDa. It was tentatively concluded that the polymersome membrane packing was not able to accommodate the larger GOX.

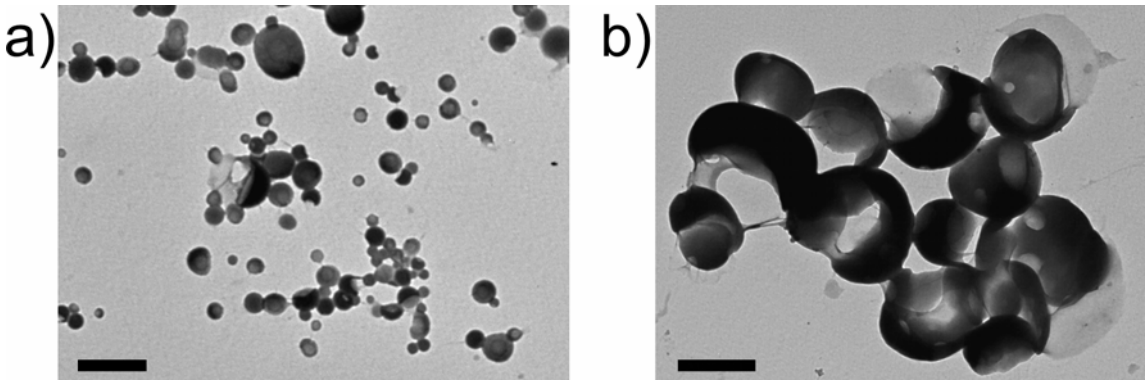
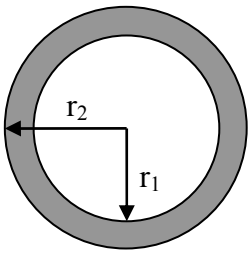


Figure S2. *TEM micrographs of polymersomes after filtration. a) Polymersomes with HRP in the membrane and GOX in the water pool, b) polymersomes with GOX in the membrane and HRP in the water pool. The scale bars represent 500 nm.*

Calculation of encapsulated Enzymes number

With the following calculation the total number of GOX enzymes in the water pool and HRP enzymes in the membrane of PS-PIAT polymersomes were determined.

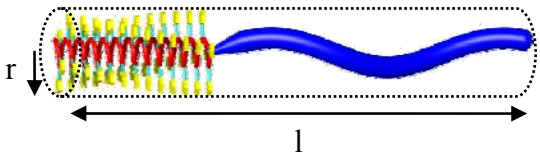
PS-PIAT polymersome



$r_1 = 220$ nm

$r_2 = 250$ nm

PS-PIAT molecule



r = 1.1 nm

l = 15 nm

Formula to calculate the volume of a sphere: $V = 4/3*\pi*r^3$

Formula to calculate the volume of a cylinder: $V = 2*\pi*r^2$

$V_{water\ pool} = 4.5*10^{-20}\ m^3$

$V_{polymersome} = V_2 - V_1 = 2.1*10^{-20}\ m^3$

$V_{PS\text{-}PIAT} = 5.4*10^{-26}\ m^3$

$N_{PS\text{-}PIAT\ per\ polymersome} = V_{polymersome}*(V_{PS\text{-}PIAT})^{-1} = 39*10^4$

$M.w._{PS\text{-}PIAT} = 1.1*10^4\ g.mol^{-1}$

$M.w._{GOX} = 1.6*10^5\ g.mol^{-1}$

$M.w._{HRP} = 4.4*10^4\ g.mol^{-1}$

Weight	in mg	in mol	in molecules
PS-PIAT	0.50	$4.5*10^{-8}$	$2.7*10^{16}$
GOX	0.25	$1.6*10^{-9}$	$9.4*10^{14}$
HRP	0.020	$4.5*10^{-10}$	$2.7*10^{14}$

$N_{polymersomes} = N_{PS\text{-}PIAT\ weigh\ in}/N_{PS\text{-}PIAT\ per\ polymersome} = 7.1*10^{10}$

$V_{Total\ inside\ polymersomes} = N_{polymersomes}*V_{polymersome} = 3.2\ \mu l$

$V_{total} = 3.0$ ml

Volume fraction = $1.1*10^{-3}$

Enzyme	$N_{total\ of\ enzyme\ in\ vesicles}$	[enzyme in vesicles] (in M)	$N_{total\ of\ enzyme\ per\ vesicles}$
GOX	$9.9*10^{11}$	$1.6*10^{-12}$	14
HRP	$2.9*10^{11}$	$4.8*10^{-13}$	4.1

References

[1] D. M. Vriezema, A. Kros, R. de Gelder, J. J. L. M. Cornelissen, A. E. Rowan, R. J. M. Nolte, *Macromolecules* **2004**, *37*, 4736.