Electronic supporting information for:

**Intercompartmental enzymatic cascade in channel-equipped polymersome-in-polymersome architectures**

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Materials

Poly(2-methyloxazoline)\(_{12}\)–poly(dimethylsiloxane)\(_{55}\)–poly(2-methyloxazoline)\(_{12}\) (ABA) triblock copolymer and the OmpF protein channel were provided by the group of Prof. Wolfgang Meier, University of Basel, Switzerland. Polystyrene-poly(t-isoctyanoalanine(2-thiophen-3-yl-ethyl)amide) (PS-PIAT) diblock copolymer was synthesized as described previously.\(^1\) The enzymes horseradish peroxidase (HRP) and glucose oxidase (GOx), amplex red reagent, β-D-glucose, H\(_2\)O\(_2\) and sepharose 4B, were obtained from Sigma-Aldrich. Phosphate buffer saline (PBS, pH 7.4) was purchased from Invitrogen. Absolute ethanol and tetrahydrofuran (THF) were purchased from Merck. All chemicals were used as received.

Methods

Transmission electron microscopy (TEM) was performed with a Carl Zeiss Libra 120 Plus TEM, operated at 120 kV. Cryo-TEM was performed using a Titan Krios microscope (80-300kV, FEG, LN2-cooled stage for 12 grids, Ultrascan 4000 (4k CCD), Tridiem GIF (Ultrascan 1000, 2k CCD)) (FEI company, Netherlands). Dynamic light scattering (DLS) measurements were carried out using a Malvern Nano Sizer or a Brookhaven Instruments BI-200 SM, which was also used for static light scattering (SLS) measurements. Enzyme activities (UV-vis and fluorescence spectroscopy) were measured using a Tecan infinite M200Pro microplate reader.

Preparation and purification of ABA polymersomes encapsulating HRP (nanoreactors)

ABA polymersomes were prepared by the film rehydration method. Shortly, 5.0 mg of ABA polymer was dissolved in 1.0 mL of ethanol and dried slowly under a stream of nitrogen in a test tube to form a thin polymer film. Subsequently, 20 µL of 0.60 mg mL\(^{-1}\) OmpF solution was added on the polymer film and briefly vortexed to solubilize the majority of the polymer. The film was further dried under high vacuum for at least 4 hours in a desiccator. Then, 200 µL of 0.2 mg mL\(^{-1}\) HRP (ca. 4.5 µM) in PBS was added to the tube, followed by 800 µL of PBS. The mixture was gently stirred at room temperature for at least 18 h to rehydrate the film and allow for the formation of polymer vesicles. The turbid suspension was extruded through 0.45 µm and 0.20 µm PVDF syringe filters (Millipore) to obtain the vesicles. The HRP-containing ABA polymer vesicles were purified from the non-encapsulated HRP by applying them on a Sepharose 4B size-exclusion column eluting with PBS (Fig. S1). The ABA vesicles were characterized by TEM (Fig. S2) and DLS.
HRP activity measurements

A stock solution of 2.5 mg mL$^{-1}$ (9.7 mM) amplex red (10-acetyl-3,7-dihydroxyphenoxazine, Life Technologies) in DMSO was prepared and stored at -20 °C. For assay measurement, the amplex red stock solution was diluted 10 times with Milli-Q water. In a single well of a 96-well microplate (Corning), 20 µL of the ABA polymer vesicles was placed. To this, a mixture of 100 µL of PBS, 5 µL of amplex red solution (1.0 mM), and 5 µL of freshly prepared 0.03 % H$_2$O$_2$ (diluted in Milli-Q water) was added to the well. The fluorescent emission at 590 nm of resorufin product was measured immediately after mixing using a Tecan infinite M200Pro microplate reader for every 15 seconds. The concentrations of amplex red and H$_2$O$_2$ in the reaction mixture were 39 µM and 0.1 mM, respectively.

![Fig. S1 Purification of ABA polymersomes. The architecture of the polymersomes is indicated schematically in the upper left corner. (a) The polymersomes were purified from the non-encapsulated HRP and free OmpF by separation on a Sepharose 4B size-exclusion column (sample volume applied: 200 µL, fractionation range: 60 kDa-20 Mda) and collection of 200 µL fractions. To identify the fractions containing catalytically active polymersomes, the fractions were investigated by measuring the absorbance at 295 nm and assaying the peroxidase activity by amplex red. Plotting the activity and the absorbance values versus the fraction number clearly displayed overlapping peaks where the polymersomes eluted. Here enzymatic activity (as measured by the change in fluorescence emission at 590 nm minute (FI min$^{-1}$)), is plotted on the primary y-axis (yellow symbols), while absorbance is plotted on the secondary axis (blue symbols, indicated by arrows). (b) For the blank polymersomes a similar curve was seen for the absorbance, indicating the presence of polymersomes, while for the catalytic activity a slight peak was observed, probably because of the presence of a small fraction of free/absorbed HRP. The fractions showing both increased absorbance and activity were pooled, and used for the preparation of the MCPs.](image-url)
TEM measurements.
The TEM samples were prepared by dispensing 15 µL of a vesicle suspension on a holey carbon-coated copper grid followed by 30 min of incubation and removal of excess solution with a piece of filter paper. The adsorbed vesicles were then stained by dispensing and incubating 10 µL of 1% phosphotungstic acid on the copper grid for 1 min, before removal of excess solution.

Fig. S2 TEM imaging of ABA and multicompartement polymersomes. ABA polymersomes encapsulating HRP and OmpF incorporated in the membrane (a) before and (b) after addition of THF. (c) Cryo TEM imaging of the same polymersomes and (d) multicompartement polymersomes. Arrows indicate the presence of inner compartments. Scale bars: a, b, c: 100 nm; d: 200 nm.

DLS and SLS measurements.
Approximately 200 µL samples of MCPs at different concentrations (62.5–500 µg mL⁻¹) were diluted in 8.0 mL of milli-Q water (filtered 5 times over 0.2 µm filters). The sample was then placed in a glass round cell and positioned inside the Brookhaven Instruments BI-200 SM. The measurement angles were set from 30° to 120° with 15° increments. A Berry plot at various concentrations was then constructed. For ABAs in presence of 10% THF, the same procedure was followed using a concentration range of 0.156–1.25 mg mL⁻¹.

Fig. S3 Berry plot of ABA polymersomes in the presence of 10% THF at various concentrations. A concentration range of 0.156–1.25 mg mL⁻¹ was used for static light scattering measurements. The plot indicates a value for the radius of gyration (R_g) of 95.8 nm, as determined from slope and intercept of the linear fit at different measurement angles. The combined light scattering experiments show the presence of swollen vesicles that nevertheless retain the vesicular state (R_g = 95.8, R_H = 95.1, \( \rho = 1.00 \)).
Preparation, purification, and characterization of multicompart ment polymersomes

The multicompart ment polymersomes (MCPs) were prepared by a sequential self-assembly method. Purified ABA polymer vesicle solution, containing HRP in the lumen and OmpF in the membrane (100 µL), was added to 400 µL of 1.0 mg mL\(^{-1}\) GOx (6.25 µM) in PBS in a glass vial. Next, 100 µL of 1.0 mg mL\(^{-1}\) PS-PIAT in THF was added drop wise to the solution and incubated for 12 hours. The suspension was filtered six times through 100 kDa Nanosep Omega centrifuge filters at 3500 rpm for 15 min (MiniSpin® plus, Eppendorf) to remove the non-encapsulated molecules. The resulting multicompart ment vesicles were redispersed in 0.5 mL of PBS. The vesicles were characterized by enzymatic activity measurements, as follows (Fig. S4).

GOx activity measurement. For measurement of GOx activity, the hydrogen peroxide produced was used for the HRP-catalyzed oxidation of amplex red. Shortly, a stock solution of 20 mg mL\(^{-1}\) β-D-glucose (2% w/v) in PBS was prepared and stored at 4°C. In a single well of a 96-well microplate (Corning), 50 µL of the MCPs was placed. An excess HRP was ensured by adding 10 µL of 1 mg mL\(^{-1}\) HRP. To this, a mixture of 50 µL of PBS, 5 µL of amplex red solution (1.0 mM) and 10 µL of stock glucose was added. The activity of the cascade reaction was measured immediately after mixing at an emission wavelength of 590 nm using a Tecan infinite M200Pro microplate reader for every 15 seconds. The concentration of glucose and amplex red were 10 mM and 43 µM, respectively.

Cryo-TEM imaging of the MCPs.

For cryo-TEM, 4 µL of sample containing nanoreactors was adsorbed onto a holey carbon-coated grid (Quantifoil, Germany). The grid was afterwards blotted with Whatman filter paper for 2 seconds and thenvitrified into the liquid ethane at -178°C using a Vitrobot (FEI company, Netherlands). Frozen grids were transferred into a Titan Krios microscope (80-300 kV, FEG, LN2-cooled stage for 12 grids, Ultrascan 4000 (4k CCD), Tridiem GIF (Ultrascan 1000, 2k CCD)) (FEI company, Netherlands).
**Dynamic and static light scattering of the MCPs.**

Light scattering experiments were performed as described before.

![Berry plot of the MCPs at various concentrations.](Fig.S5)

Fig. S5 Berry plot of the MCPs at various concentrations. A concentration range of 62.5–500 µg mL\(^{-1}\) was employed in the static light scattering measurements. Static light scattering measurements provided a radius of gyration of 163 nm (± 28), as determined from slope and intercept of the linear fit at different measurement angles, resulting in a \(\frac{R_g}{R_H}\) (\(\rho\)-value) of 1.10, indicative of the vesicular structure.

**Labeling of GOx with alexa-488.**

For confocal microscopy, flow cytometry, and estimation of the encapsulation efficiency, GOx was labeled with alexa-488 using an alexa fluor 488 protein labeling kit (Life technologies), following the protocol supplied. The labeling efficiency was calculated from the ratio of the absorbance at 280 nm (\(c_{protein} = 267,200\) M\(^{-1}\) cm\(^{-1}\)) and 495 nm (\(c_{dye} = 71,000\) M\(^{-1}\) cm\(^{-1}\)) yielding a labeling efficiency of 3.77. For (crude) estimation of the encapsulation efficiency we measured the absorbance of alexa-488 labeled GOx before and after encapsulation at 488 nm. We corrected for the scattering of the polymersomes by fitting the spectra to a first-order exponential decay function using GraphPad Prism 6.01 for Windows (Fig. S6). After correction, the relative areas before and after filtration were determined, allowing calculation of the concentration (Table S1).

![Absorption spectra of alexa-488 labeled GOx encapsulated in MCPs.](Fig.S6)

Fig. S6 Absorption spectra of alexa-488 labeled GOx encapsulated in MCPs. Spectra were acquired before and after filtration, with (black line), and without OmpF (dotted line) present in the ABA membrane. Although the absorption spectra are obscured by scattering from the polymersomes, a clear signal from the dye is observed.
### Table S1 Calculation of encapsulation efficiency.

Area of the alexa-488 absorption peak in the UV-vis spectra of MCPs, before and after filtration, and estimation of the GOx concentration. The scattering has been subtracted by fitting to an exponential decay function.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Area under the curve [AU cm]</th>
<th>Encapsulation efficiency</th>
<th>GOx concentration (mg mL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before filtration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP (+ OMPE)</td>
<td>14.7</td>
<td>NA</td>
<td>0.80</td>
</tr>
<tr>
<td>Blank MCP (no OMPE)</td>
<td>15.8</td>
<td>NA</td>
<td>0.80</td>
</tr>
</tbody>
</table>

| After filtration           |                                 |                          |                                     |
| MCP (+ OMPE)               | 7.4                             | 50.2                     | 0.40                                |
| Blank MCP (no OMPE)        | 6.0                             | 37.8                     | 0.30                                |

\(^{a}\) Not applicable

### Alexa-647 labeling of HRP.

For flow cytometry and confocal microscopy, MCPs were prepared as described above and stained with alexa-647 tyramide (Fig. S7) using a TSA (tyramide signal amplification) kit (Life technologies), essentially following the procedure supplied, except for the source of the HRP which in this case were the MCPs. After 1 h incubation, the polymersomes were dialyzed against Milli-Q overnight. For confocal microscopy, to completely remove buffer salts, which otherwise interfered with the measurements, the samples were spun down twice, at 12,000 rpm for 15 minutes, followed by resuspension in Milli-Q water.

![Fig. S7 Tyramide signal amplification. HRP-catalyzed reaction of tyramide-functional fluorescent dyes with tyrosine moieties, giving rise to self-staining. The dye depicted is alexa-488. As far as we are aware, the structure of tyramide-alexa-647 has not been reported.](image-url)
Cascade measurements.

For study of the cascade reaction in the MCPs, the same procedure was used as for measurement of the activity of GOx, except for the fact that no extra HRP was added, as this was already present in the polymersomes. For the control reaction of free GOx and single ABA polymersomes (+OmpF), a final concentration of 0.3 mg mL\(^{-1}\) GOx was chosen, which was obtained by mixing 10 µL of the ABA nanoreactors with 40 µL of 0.38 mg mL\(^{-1}\) GOx in PBS. For cascade measurements in the bulk, 40 µL of 0.38 mg mL\(^{-1}\) GOx in PBS was mixed with 10 µL of 0.05 mg mL\(^{-1}\) HRP, or an appropriate dilution, to obtain a final concentration of 0.01 mg mL\(^{-1}\) to 0.04 µg mL\(^{-1}\) HRP.

Fig. S8 Bulk cascade measurements. (a) Conversion of amplex red versus time by the GOx/HRP cascade, as measured by the increase of fluorescence at 590 nm. The concentration of GOx was fixed at 0.3 mg mL\(^{-1}\) (reflecting the GOx concentration in the MCP solution; see Fig. S7 and Table S1), while the concentration of HRP was varied between 0.01 mg mL\(^{-1}\) and 0.04 µg mL\(^{-1}\) (i.e., 100 % encapsulation efficiency or lower). For comparison, the time trace of the reaction in the presence of MCPs (orange dots), as well as that of ABA nanoreactors in the presence of free GOx (0.3 mg mL\(^{-1}\)) is also shown. (b) Plot of initial velocities of the bulk reactions measured in A versus HRP concentration. Below ca. 5 µg mL\(^{-1}\) HRP concentration, the reaction rate increases linearly with HRP concentration, indicating that HRP catalysis is the rate determining factor. Above this concentration, GOx becomes rate determining.
Scanning confocal microscopy.

Fluorescence imaging of polymersomes was carried out using a MicroTime 200 scanning confocal microscope (Pico Quant, Germany). The samples were excited by picosecond pulsed lasers emitting at 467 nm (LDH-D-C-470, PicoQuant, Germany) and 640 nm (LDH-D-C-640, PicoQuant, Berlin) controlled by a PDL 828 Sepia II laser driver (Pico Quant, Germany). Excitation light was focused on the sample to a diffraction-limited spot using a high NA objective (Olympus, Plan-Apo, NA = 1.4, 100 ×, oil immersion) mounted in a Olympus IX 71 microscope frame, and the emission was collected from the sample using the same objective and directed through a pinhole onto a Single Photon Avalanche Diode (SPAD, PDM series, Micro Photon Devices, Italy). To separate the fluorescence emission from the excitation, suitable dichroic mirrors, emission, and excitation filters were used. The samples for microscopy were prepared by drop-casting diluted vesicle solutions onto a glass cover slip (circularly shaped glass cover slides, Ø = 20 mm) for few seconds to allow for adhesion of the polymersomes to the glass surface. Excess solution was removed by a pipette. All experiments were performed in air under ambient conditions.

Fig. 59 Confocal microscopy of MCPs without OmpF. MCPs carrying HRP and GOx in the inner and outer compartment, respectively, were prepared as described in the main text. GOx was labeled with alexa-488 and imaged in the blue channel (a, b; where b is the zoom of a, as indicated by the white square). After preparation, the MCPs were incubated with alexa-647 tyramide to check for the activity of HRP, followed by imaging in the red channel. (c) No fluorescence in the red channel was observed, indicating that HRP is not accessible when OmpF is not present. Scale bars are 20 µm.
Flow cytometry.

Polymersomes (prepared as per the fluorescence microscopy experiments) were analyzed using a BD FACSCanto II flow cytometer. Alexa-488 conjugated GOx was detected using a 530 ± 15 nm (FITC) bandpass filter, while alexa-647 conjugated HRP was detected using a 660 ± 10 nm (APC) bandpass filter. Data is presented as a two dimensional dot plot between alexa-647 and alexa-488 using forward- and side-angle scatter (FSC/SSC) gating to capture the majority of the polymersomes and to exclude background from PBS. 50,000 Gated events were recorded for each set of measurements.

Fig. S10 Flow cytometry analysis of the multicomartment polymersomes. FSC/SSC dot plots of (a) PBS and (b) multicompartment polymersomes, respectively. Gating was applied (region P7) to capture the majority of the polymersomes and to exclude background from PBS. (c) Dot plot of FITC (530 ± 15 nm for detection of alexa-488) against APC (660 ± 10 nm for detection of alexa-647) for multicompartment polymersomes containing unlabeled enzymes after applying gate P7. Regions P4, P5, and P6 were selected to reflect polymersomes containing alexa-647 only, polymersomes containing alexa-488 only, and polymersomes containing both alexa-647 and alexa-488, respectively. (d) Dot plot of FITC/APC for multicompartment polymersomes containing labeled enzymes (prepared as per the method adopted for confocal microscopy) after applying gate P7. Multicomartment polymersomes containing alexa-647 and alexa-488 make up 28 % of the total polymersome population, while individual ABA polymersomes containing alexa-647 and individual PS-PIAT vesicles containing alexa-488 make up 5.7 % and 16.2 % of the total polymersome population, respectively. The remainder of the polymersome population does not contain labeled enzymes.

Table S2. Encapsulation efficiencies as calculated from Figure S10d

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Gating number</th>
<th>Fluorescence events *</th>
<th>% encapsulation (based on fluorescence counts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABA (AF647)</td>
<td>P4</td>
<td>2812</td>
<td>5.7</td>
</tr>
<tr>
<td>PS-PIAT (AF488)</td>
<td>P5</td>
<td>7991</td>
<td>16.2</td>
</tr>
<tr>
<td>MCPs (AF647+ AF488)</td>
<td>P6</td>
<td>13778</td>
<td>27.9</td>
</tr>
</tbody>
</table>

a: Total fluorescence events is 49,454

References:
