

Electronic Supplementary Information

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Cross-linked polymersomes as nanoreactors for controlled and stabilized single and cascade enzymatic reactions

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1 Synthesis of the compounds

1.1 Synthesis of the photo cross-linker

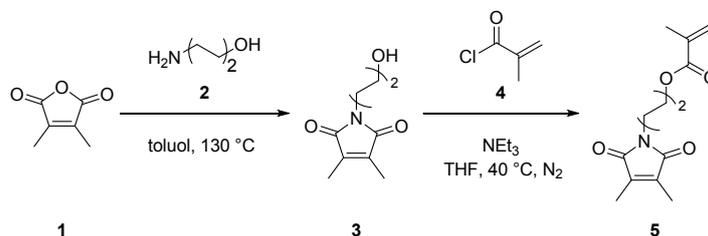


Figure 1-SI: Reaction scheme for the preparation of the cross-linker 3,4-dimethyl maleic imidobutyl methacrylate (**5**) in a two-step sequence.

Synthesis for step 1: We adopted a method by Kuckling et al.^[1] Aminobutanol (1.2 g, 14 mmol) (**2**) was dissolved in 100 mL toluene, to which maleic acid anhydride (1.71 g, 14 mmol) (**1**) was added. The mixture was kept at reflux for 2 h at a water trap. The solvent was removed by rotary evaporation at 40 °C to obtain the raw product, which was finally purified by flash chromatography (*n*-hexane/ethyl acetate, 50:50 vol-%), with yields of 89 %.

¹H - NMR: 1.51 – 1.57 (m, 3H); 1.66 (quin, $J_{\text{HH}} = 7.3$ Hz, 2H); 1.95 (s, 6H); 3.52 (t, $J_{\text{HH}} = 7.1$ Hz, 2H); 3.65 (t, $J_{\text{HH}} = 6.5$ Hz, 2H).

¹³C - NMR: 8.45 (2 CH₃); 24.99 (CH₂); 29.55 (CH₂); 37.44 (CH₂); 61.90 (CH₂); 136.93 (2 C); 172.19 (2 C).

Synthesis for step 2: We adopted a method by Abd-El-Aziz et al.^[2] Maleic imide (2 g, 10.1 mmol) (**3**) was dried under vacuum, to which THF (117 mL) was added under a nitrogen atmosphere. After the solution was cooled in ice-water, methacryloyl chloride (1.63 g, 15.6 mmol) (**4**) dissolved in THF (3 mL) was added. The reaction proceeded for 2,5 h at 40 °C before the solution was quenched into water. Then, the mixture was extracted with diethyl ether, the combined ethereal extracts were dried over anhydrous magnesium sulfate and the solvent was removed by rotary evaporation at 40 °C to get the raw product of the photo cross-linker, which was finally purified by flash chromatography (*n*-hexane/ethyl acetate/triethylamine, 74.9:24.9:0.2 Vol-%), with yields of 69%.

¹H - NMR: 1.67 – 1.69 (m, 4H); 1.94 (s, 3H); 3.53 (t, $J_{\text{HH}} = 6.6$ Hz, 2H); 4.16 (t, $J_{\text{HH}} = 6.0$ Hz, 2H); 5.55 (s, 1H); 6.09 (s, 1H).

¹³C - NMR: 8.51 (2 CH₃); 18.15 (CH₃); 25.24 (CH₂); 25.90 (CH₂); 37.35 (CH₂); 63.92 (CH₂); 125.25 (CH₂); 136.24 (C); 137.01 (2 C); 167.26 (C); 172.10 (2 C).

1.2 Synthesis of the PEG-Br macroinitiator

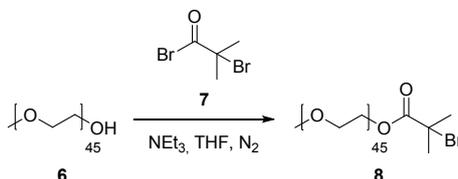


Figure 2-SI: Reaction scheme for the preparation of the PEG₄₅-Br macroinitiator (8).

We adopted a method by Armes et al.^[3] Here, MeO-PEG₄₅-OH (5.00 g, 2.5 mmol) (6) was dried at 60 °C for 1 h under vacuum. After THF (45 mL) was added under a nitrogen atmosphere, the solution was cooled down to room temperature. Subsequently, triethylamine (0.74 g 4 mmol) and 2-bromoisobutyric acid bromide (1.12 g, 4.9 mmol) (7) dissolved in THF (3 mL) were added. The reaction was carried out at room temperature for 4 d under a nitrogen atmosphere. The raw macroinitiator was precipitated in CO₂-cooled ether and re-crystallized in ethanol until a white solid was obtained. Yield: 52 %

¹H - NMR: 1.93 (s, 6 H); 3.37 (s, 3 H); 3.63 (180 H).

¹³C - NMR: 30.73 (2 CH₃); 58.96 (C); 65.08 (CH₃); 70.53 (CH₂); 171.54 (C).

1.3 Synthesis of the poly(ethylene glycol)₄₅-*block*-poly(diethylaminoethylmethacrylate-*stat*-3,4-dimethylmaleinimidobutylmethacrylate)₉₉ (PEG₄₅-*b*-P(DEAMA-*s*-DMIBM)₉₉)

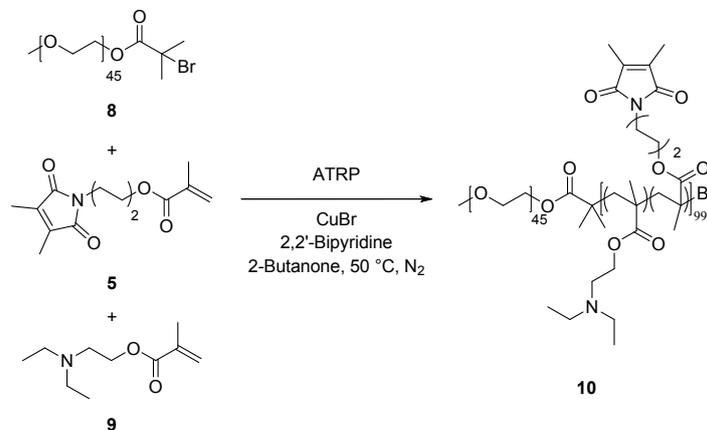


Figure 3-SI: Reaction scheme for the preparation of the final block copolymers PEG₄₅-*b*-P(DEAMA-*s*-DMIBM)₉₉ **10**.

We adopted a method by Weaver et al.^[4] PEG₄₅-Br (220 mg, 0.1 mmol) **8** and 2,2'-bipyridine (32 mg, 0.2 mmol) were mixed, and dried under vacuum. Under a nitrogen atmosphere CuBr (17 mg, 0.1 mmol) was added and the resulting mixture was dried another 30 min under vacuum. In an additional flask, diethylaminoethyl methacrylate (1.44 g, 7.77 mmol) (**9**) and the photo cross-linker (0.42 g, 1.58 mmol) **5** were dried 30 min under vacuum, to which 2-butanone (3 mL) under a nitrogen atmosphere was added. The two solutions were combined. After reacting at 50 °C for 17 h, the solution was chromatographed on a column of activated aluminium oxide with THF to remove any copper species. The raw product was washed with *n*-hexane and water before it was dried in vacuum to give a sticky polymer. Yield: 63 %

1.4 Evaluation of NMR spectra to determine polymer composition

The ratio was calculated using integrals of specific ^1H NMR signals.

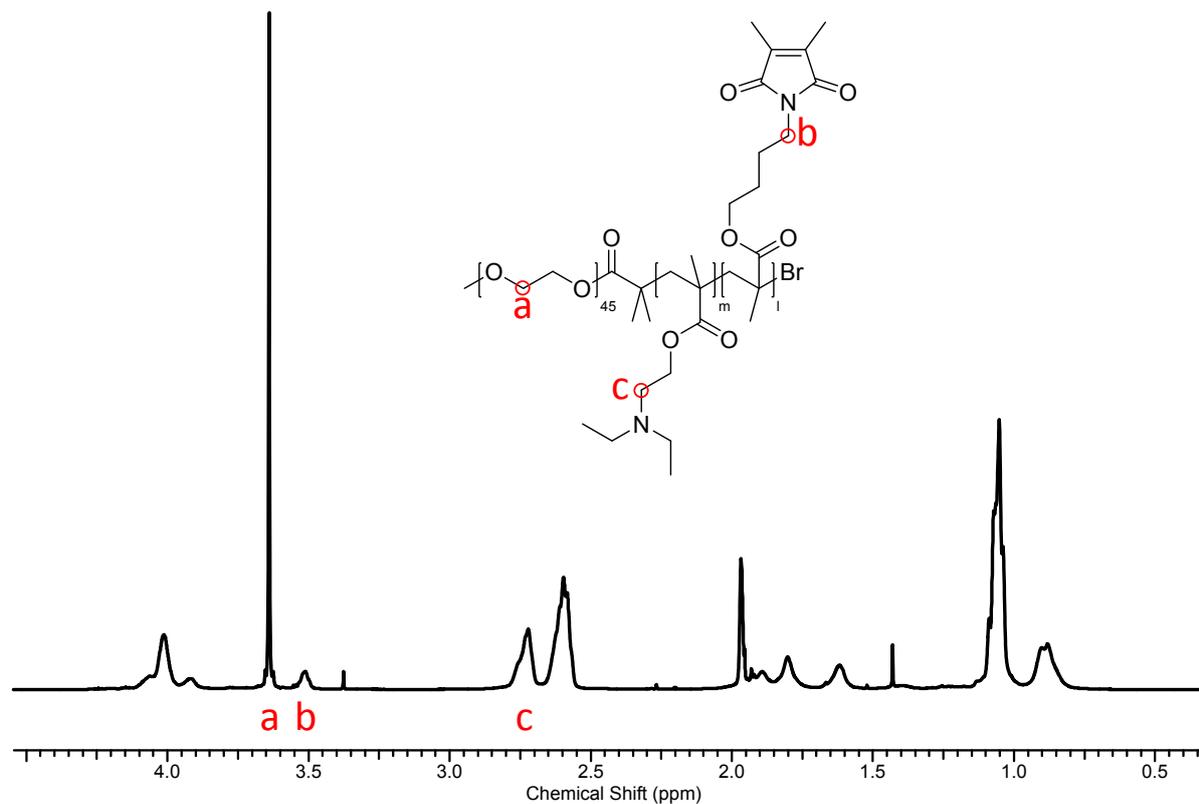


Figure 4-SI: ^1H NMR Spectrum of $\text{PEG}_{45}\text{-}b\text{-P}(\text{DEAEM-}s\text{-DMIEM})_{99}$ with labelled signals used to determine the final structure. The corresponding atoms are labelled in the polymer.

Table 1-SI: Calculations concerning the block-length ratio (hydrophilic (a) / hydrophobic (b and c)) (blr) and properties of the prepared $\text{PEG}_{45}\text{-}b\text{-P}(\text{DEAEM-}s\text{-DMIBM})_{99}$.

Integral "a"	Integral "b"	Integral "c"	blr ^[a]	Cross-linker ^[b]	M_n ^[c]	M_w/M_n ^[d]
1	0.20	0.90	1:2.2	18 mol-%	21900 g/mol	1.16

^[a] blr = block length ratio; calculated via: $\text{blr} = a : (2xb + 2xc)$; during the course of polymerization one have used 2 equivalents of DEAEM and DMIBM in total against the repeating unit in the PEG-macro initiator. ^[b] Relative amount of cross-linker polymerised within the hydrophobic block ($b/(c+b)$). ^[c] M_n is determined from signal intensities in the ^1H NMR spectra. ^[d] The distribution is determined from GPC Data using a PS calibration.

The intensity of signal “a” from the PEG part is taken as a reference, since the total amount of ethylene glycol units is known (45 units). Since PEG has a symmetric structure, the integral of peak “a” represents 4 H atoms per monomer repeating unit. In contrast, the integrals of the signals “b” and “c”, referring to PDEAEM and PDMIBM, respectively, only represent 2 H atoms each. Due to this, an integral ratio from “a” to “b+c” from 1:1 means a block-length ratio of 1:2, concerning the repeating units. The share of integral “b” within the hydrophobic block, meaning integrals “b+c”, is the amount of cross-linker polymerized into the substance.

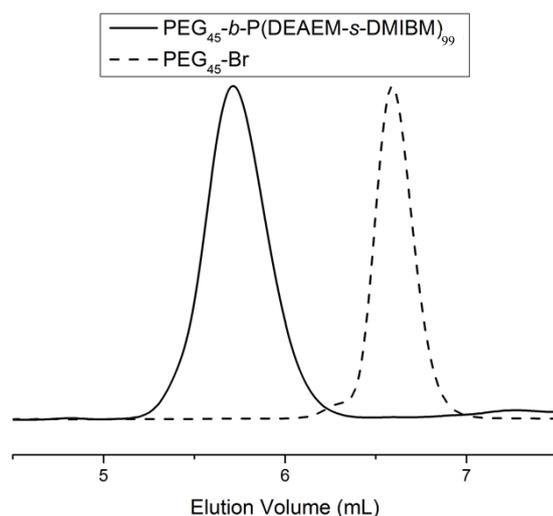


Figure 5-SI: GPC traces of the prepared $\text{PEG}_{45}\text{-}b\text{-P}(\text{DEAEM-}s\text{-DMIBM})_{99}$ copolymer and the initial $\text{PEG}_{45}\text{-Br}$ macroinitiator.

^1H - NMR: 0.75 - 0.96 (m, 2H); 0.98 - 1.11 (m, 3H); 1.69 - 1.97 (m, 3H); 1.97 - 2.07 (m, 3H); 2.28 (s, 6H); 2.51 - 2.64 (m, 4H); 2.65 - 2.79 (m, 2H); 3.39 (s, 3H); 3.65 (s, 2H); 3.70 - 3.84 (m, 2H); 3.93 - 4.12 (m, 2H).

^{13}C - NMR: 8.73 (CH_3); 12.19 (2 CH_3); 25.60 (CH_2); 30.32 (CH_3); 44.72 (C); 45.14 (CH_3); 47.67 (CH_2); 50.53 (CH_2); 50.58 (CH_2); 63.26 (CH_2); 67.49 (CH_3); 70.58 (CH_2); 137.42 (C); 177.31 (C=O).

1.5 Preparation and characterization of polymersomes

1.5.1 Preparation and cross-linking

A solution of 0.2 weight-% copolymer in acid (pH 2) water was prepared and stirred until the copolymer was dissolved. The final solution was passed through a 0.2 μm nylon filter to remove any remaining particles, including dust. Then, 1 M NaOH was added through a 0.2 μm nylon filter until pH 10 was reached. The solution was stirred for 3 d.

For the preparation of cross-linked polymersomes, a solution of polymersomes was placed in the UV chamber and was irradiated for 40 seconds.

Table 3-SI: Prepared polymersomes using the PEG₄₅-*b*-P(DEAEM-*s*-DMIBM)₉₉ copolymer, including the crosslinking time for pH stable polymersome

Polymersome size ^[a]	PDI ^[a]	Crosslinking time ^[b]
[nm]		[s]
108	0.2	40

^[a] Determined by DLS (diameter), PDI=polydispersity of polymersomes. ^[b] Crosslinking time for pH stable polymersomes.

1.5.2 Reversible swelling upon repeated changes in pH

A cross-linked polymersome solution was prepared and little amounts of 1 M HCl or 1 M NaOH were added to reach pH 4 or 8, respectively. This cycle was repeated 4 times. The result of the experiment is presented in Figure 5-SI.

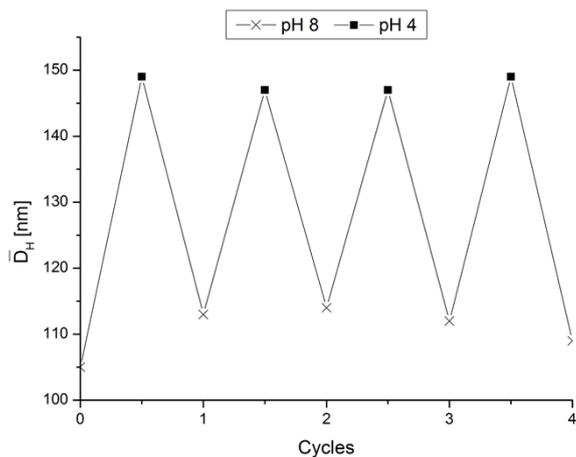


Figure 6-SI: Average hydrodynamic diameter as a function of the pH value, determined from DLS measurements. The vesicle-PDI is always at 0.2.

2 Enclosure of enzymes in polymersomes

2.1 Control experiment of the cascade reaction

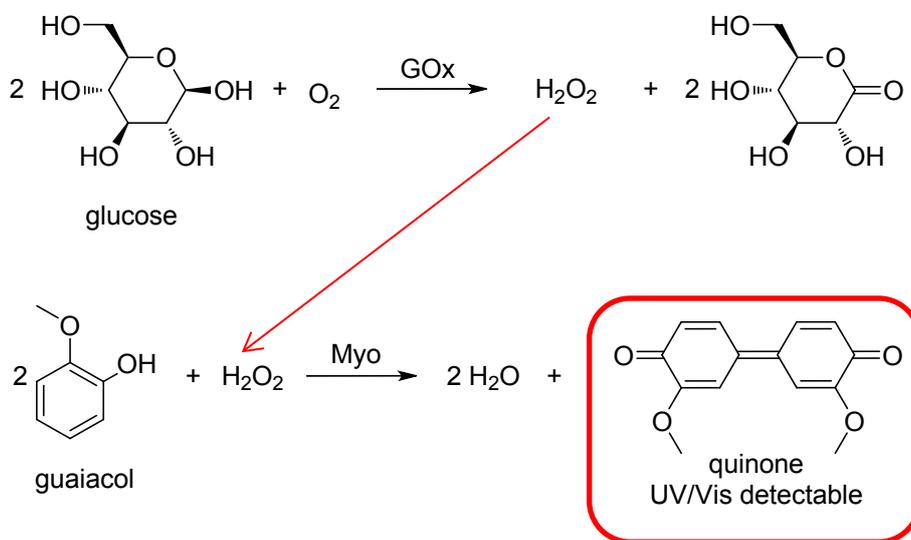


Figure 7-SI: Enzymatic cascade reaction for glucose oxidase (GOx) and Myoglobin (Myo).

Stock solutions of GOx (0.1 mg/mL in phosphate buffer 0.1 M pH 6 or 8, respectively), Myo (1 mg/mL in phosphate buffer 0.1 M pH 6 or 8, respectively), glucose (1 M in phosphate buffer 0.1 M pH 7.4), and guaiacol (0.1 M in phosphate buffer 0.1 m pH 7.4) were prepared.

To a sample of GOx (250 μ L), Myo (50 μ L), guaiacol (8 μ L) and glucose (8 μ L) were added. After addition, the UV-Vis measurement started at 470 nm. In case that one compound is missing, it was replaced by phosphate buffer (0.1 M pH 6 or 8, respectively).

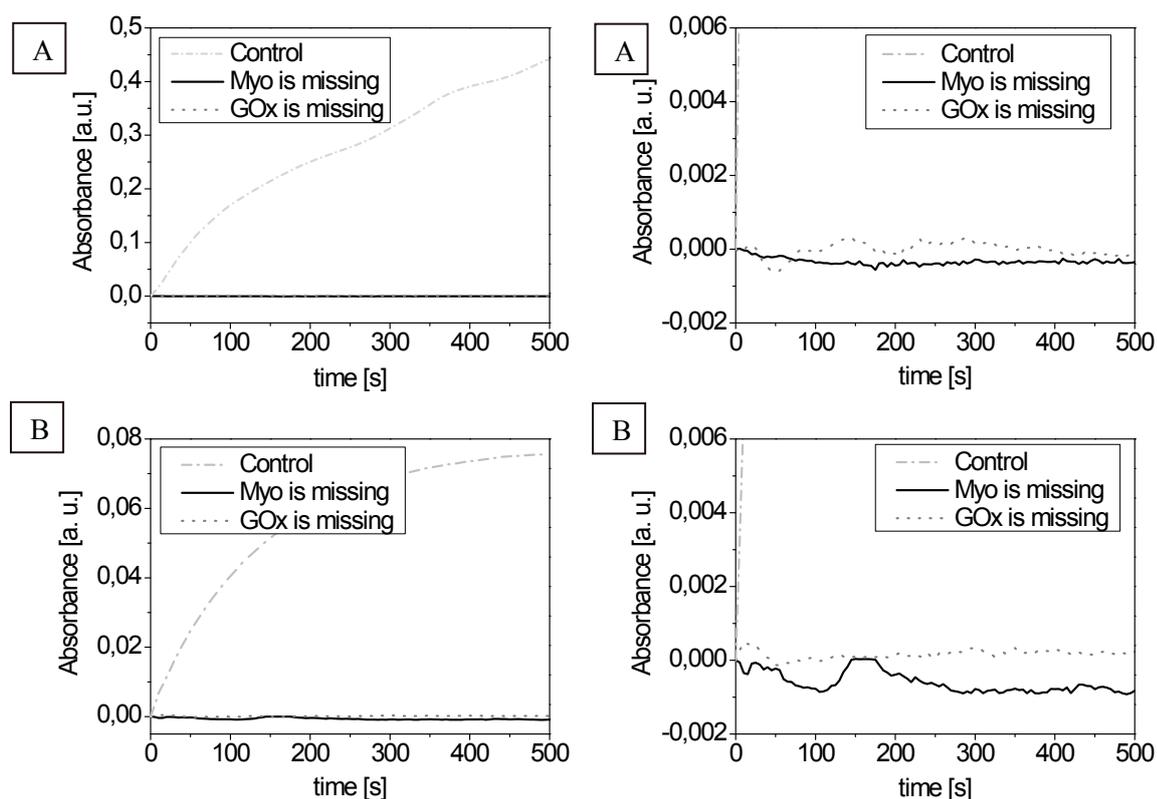


Figure 8-SI: Absorbance at 470 nm as function of time at pH 6 (A) and 8 (B). Left and right differ in the axis scale. In case that all compounds are present (Control), an increase in absorbance is detectable.

2.2 Pure enzyme activity at pH 6 and 8 with or without UV-irradiation

Stock solutions of guaiacol (0.1 M in phosphate buffer 0.1 M pH 7.4), ABTS (0.1 M in phosphate buffer pH 7.4), glucose (1 M in phosphate buffer 0.1 M pH 7.4) and H₂O₂ (1 M in phosphate buffer 0.1 M pH 7.4) were prepared. To investigate the influence of UV-irradiation, 2 mL of an enzyme solution was irradiated 40 s in the UV chamber. The investigations were performed at different pH values by dissolving the enzymes in phosphate buffer of pH 6 and 8.

Myoglobin (Myo): To a Myo sample (300 μL), guaiacol (8 μL) and H_2O_2 (8 μL) were added. After preparation, the UV monitoring at 470 nm was started and data points were recorded every second. The activity was determined after 300 seconds.

Horseradish Peroxidase (HRP): To a HRP sample (300 μL) ABTS (8 μL) and H_2O_2 (8 μL) were added. After preparation, the UV monitoring at 405 nm was started and data points were recorded every second. The activity was determined after 100 seconds.

Glucose Oxidase (GOx): Stock solutions of myoglobin (1 mg/mL in phosphate buffer 0.1 M pH 6 and 8) were prepared. To a GOx sample (250 μL), Myo (50 μL), guaiacol (8 μL) and H_2O_2 (8 μL) were added. After preparation, the UV monitoring at 470 nm was started and data points were recorded every second. The activity was determined after 200 seconds.

2.3 Preparation of cross-linked and non-cross-linked enzyme-filled polymersomes

10 mg of Polymer was dissolved in 2 ml water at pH 1.5, while 1 mg of the enzyme was dissolved in 4 ml of 0.1 M PBS (pH 7.4). Both solutions were combined and the pH adjusted to pH 8 by adding 0.5 M NaOH slowly. The solution was stirred for 3 d. To receive cross-linked enzyme-filled polymersomes, 2 mL of the solution was irradiated 40 s in the UV chamber. The solutions were purified using the hollow fibre filtration (paragraph 2.6).

Myo: An aliquot of 300 μL was used for the following experiments. 3 aliquots were taken at pH 8, while 3 were taken after a pH switch to pH 6 and three again after changing the pH value back to 8. In Total, 9 aliquots were taken, 3 at each pH value. For an activity experiment, the sample was treated with guaiacol (8 μL) and H_2O_2 (8 μL). The sample was stirred for 1 minute, and the UV monitoring at 470 nm started subsequently. Data points were recorded every second. The activity was determined after 400 seconds.

HRP: An aliquot of 300 μL was used for the following experiments. 3 aliquots were taken at pH 8, while 3 were taken after a pH switch to pH 6 and three again after changing the pH value back to 8. In Total, 9 aliquots were taken, 3 at each pH value. For an activity experiment, the sample was treated with ABTS (8 μL) and H_2O_2 (8 μL). The sample was stirred for 1 minute, and the UV monitoring at 405 nm started subsequently. Data points were recorded every second. The activity was determined after 400 seconds.

GOx: An aliquot of 250 μL was used for the following experiments. 3 aliquots were taken at pH 8, while 3 were taken after a pH switch to pH 6 and three again after changing the pH value back to 8. In Total, 9 aliquots were taken, 3 at each pH value. For an activity experiment, the sample was treated with Myo (50 μL), guaiacol (8 μL) and H_2O_2 (8 μL). The sample was stirred for 1 minute, and the UV monitoring at 470 nm started subsequently. Data points were recorded every second. The activity was determined after 500 seconds.

2.4 Reaction sequence within cross-linked GOx and Myo-filled polymersomes

Preparation: 10 mg of Polymer was dissolved in 2 ml water at pH 1.5, while 4 mg GOX and 1 mg Myoglobin were dissolved in phosphate buffer (4 ml, 0.1 M pH 7.4). The solutions were combined and the pH adjusted to 8 by adding 0.5 M NaOH slowly. The solution was stirred for 3 days. Afterwards, the solution was irradiated for 40 seconds in the UV-chamber. The resulting solutions were combined and subsequently cleaned from non-enclosed enzyme using the hollow fibre filtration.

An aliquot of 300 μL was used for the following experiments. 3 aliquots were taken at pH 8, while 3 were taken after a pH switch to pH 6 and three again after changing the pH value back to 8. In Total, 9 aliquots were taken, 3 at each pH value. For an activity experiment, the sample was treated with guaiacol (8 μL) and H_2O_2 (8 μL). The sample was stirred for 5 minute, and the UV monitoring at 470 nm started subsequently. Data points were recorded every second.

2.5 Reaction sequence between two cross-linked polymersomes

Preparation of GOx-filled cross-linked polymersomes: 10 mg of Polymer was dissolved in water (2 mL pH 1.5), while 4 mg GOx was dissolved in phosphate buffer (4mL, 0.1 M pH 7.4). The solutions were combined and the pH adjusted to 8 by adding 0.5 M NaOH slowly. The solution was stirred for 3 d. Afterwards, the solution was irradiated for 40 s in the UV-chamber and purified by the hollow fibre filtration.

Preparation of Myoglobin-filled cross-linked polymersomes: 10 mg of Polymer was dissolved in water (2 mL pH 1.5), while 1 mg Myo was dissolved in phosphate buffer (4mL, 0.1 M pH 7.4). The solutions were combined and the pH adjusted to 8 by adding 0.5 M NaOH slowly. The solution was stirred for 3 d. Afterwards, the solution was irradiated for 40 s in the UV-chamber and purified by the hollow fibre filtration.

The prepared solutions of Myo- and GOx-filled cross-linked polymersomes were combined in a ratio of 1:1. An aliquot of 300 μL was used for the following experiments. 3 aliquots were taken at pH 8, while 3 were taken after a pH switch to pH 6 and three again after changing the pH value back to 8. In Total, 9 aliquots were taken, 3 at each pH value. For an activity experiment, the sample was treated with Guaiacol (8 μL) and Glucose (8 μL). The sample was stirred for 5 minute and the UV monitoring at 470 nm started afterwards, data points were recorded every second.

2.6 Purification of enzyme-filled polymersomes with the hollow fibre filtration

6 ml of the unpurified solution was transferred into a 50 ml cone tube, which attached to the hollow fibre filtration system. The sample was diluted with phosphate buffer (0.1 M pH 8) to 30 mL and constantly refilled until the extraction volume was reached. A summary of the parameters during the extraction process is given in table 3-SI.

Table 4-SI: Extraction volume and transmembrane pressure for the separation of non-encapsulated enzymes and the enzyme-filled polymersomes

Enzyme	Extraction volume [ml]	Transmembrane pressure [mbar]
Myoglobin	70	180
Horseradish peroxidase	200	180
Glucose Oxidase	150	200

2.7 DLS measurements of non cross-linked Myo-filled polymersomes

Non cross-linked Myo-filled polymersomes were received analog to paragraph 2.3 (preparation) and 2.6 (purification).

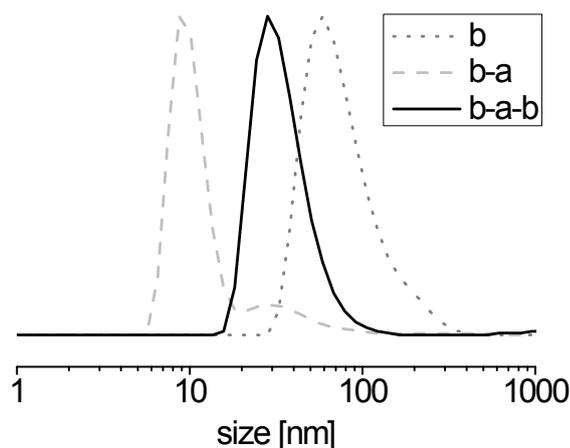


Figure 9-SI: Size distribution of non cross-linked Myo-filled polymersomes at a basic (b, pH 8), then at an acidic (b-a, pH 6), and again at a basic (b-a-b, pH 8) pH value.

2.8 Determination of the encapsulation efficiency

The calibration curve was plotted by determination of the change in absorption of different mass concentrations of myoglobin (mass concentrations of 0.36364, 0.30303, 0.24242, 0.18182, 0.12121, 0.06061 mg/mL in phosphate buffer 0.1 M pH 6). Stock solutions of guaiacol (0.1 M in phosphate buffer 0.1 M pH 7.4) and H₂O₂ (1 M in phosphate buffer 0.1 M pH 7.4) were prepared.

To a sample of myoglobin (300 μ L) both substrates guaiacol (8 μ L) and H₂O₂ (8 μ L) were added. After addition, the UV-Vis measurement started at 470 nm. Change in absorbance was calculated after 5 minutes.

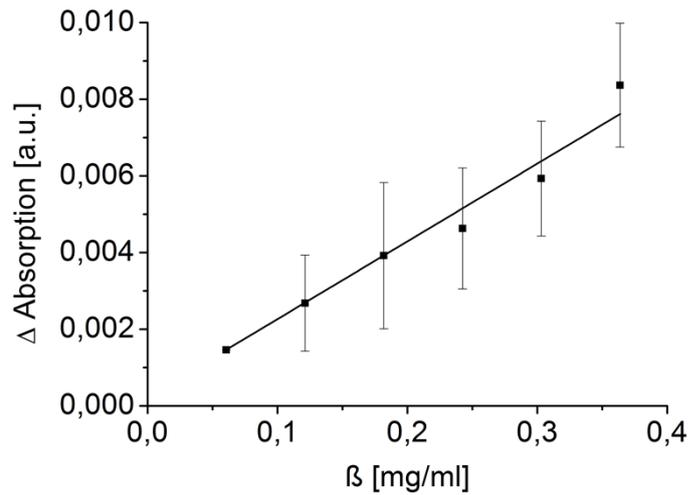


Figure 10-SI: Change in absorption as a function of mass concentrations of myoglobin in 5 minutes. Regression equation $f(x) = 0,0001088550x + 0,0004574500$, $r^2 = 0,967$

The preparation of cross-linked and non cross-linked Myo-filled polymersomes was performed analog to paragraph 2.3. Change in absorbance was calculated after 5 minutes at pH 6. In total, three measurements were conducted and the statistical average was used for the discussion.

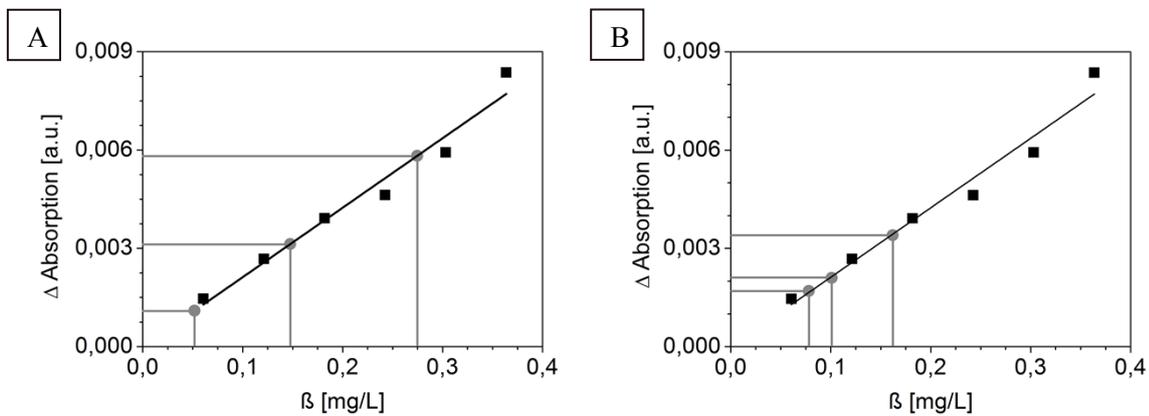


Figure 11-SI: Determination of the encapsulation efficiency of non cross-linked (A) and cross-linked (B) Myo-filled polymersomes at pH 6.

Table 5-SI: Determination of the encapsulation efficiency

system	Δ Absorbance [a.u.]	calculated β [mg/L]	encapsulated amount of Myo [mg] ^[a]	encapsulation efficiency [%]
cross-linked Myo- filled	0,002146	0,101263	0,003038	0,30
	0,003440	0,162287	0,004869	0,49
	0,001646	0,077684	0,002331	0,23
non cross-linked Myo-filled	0,003128	0,147573	0,004427	0,47
	0,001096	0,051747	0,001552	0,15
	0,005823	0,274665	0,008240	0,82

^[a] Volume of the Myo-filled polymersome solution after cleaning process was typically 30 mL

3 References

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