Supporting Information

Tuneable Time Delay in the Burst Release from Oxidation-Sensitive Polymersomes Made by PISA

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Author Contributions

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.
Experimental part

Materials and Methods

All chemicals and solvents were purchased from Sigma-Aldrich, Carl Roth and if not mentioned, were used without further purification. The detailed preparation procedure of the block copolymers and respective polymersomes was previously reported.\textsuperscript{1} \textsuperscript{1}H-NMR was performed at room temperature on a Bruker AC 300 MHz spectrometer in D\textsubscript{2}O. ASAP-HSQC spectra were measured on a Bruker Avance III 600 MHz equipped with a triple resonance cryogenic probe in 4:1 mixture of THF-d\textsubscript{8}:D\textsubscript{2}O.\textsuperscript{2}

Size-exclusion chromatography (SEC) of the polymers was performed on an Agilent system equipped with a G1310A pump, a G7162A refractive index detector, and a set of PSS GRAM 30/1000 Å columns (Polymer Standards Service (PSS), Mainz, Germany) with N,N-dimethylacetamide (DMAc) + 0.21% LiCl as eluent at 1 mL min\textsuperscript{-1} at 40 °C. The system had been calibrated with polystyrene standards of narrow dispersity (PSS, Mainz, Germany) in a molar mass range from 374 to 1,040,000 g mol\textsuperscript{-1}.

The GOx-loaded polymersomes V3-G have been separated on a Jasco SEC system equipped with a PU-980 pump, a RI-2031 Plus refractive index detector, a UV-975 detector, and PSS SUPREMA 1000/30 Å columns connected in series, running in citrate-phosphate buffer (0.1 M, pH 7.0) at a flow rate of 1 mL min\textsuperscript{-1} at 30 °C.

DLS was performed on a ZetaSizer Nano ZS (Malvern, Herrenberg, Germany) equipped with a He–Ne laser operating at a wavelength of \(\lambda = 633\) nm. Counts were detected at an angle of 173°. The particle size was approximated as the effective diameter (Z-average) obtained by the cumulants method assuming a spherical shape. All measurements were conducted at 25 °C in semi-micro cuvettes after equilibration times of 30 s in triplicate. Every measurement included 10 runs, in which every run took 30 seconds. Apparent hydrodynamic radii were calculated using the Stokes-Einstein Equation (1):

\[
R_\theta = \frac{kT}{6\pi\eta D}
\]

\(R_\theta\) = hydrodynamic radius, \(k\) = Boltzmann constant, \(T\) = absolute temperature, \(\eta\) = viscosity of the sample, and \(D\) = apparent translational diffusion coefficient.
Degradation kinetics via in situ DLS analysis

The degradation kinetics of the vesicles in H$_2$O$_2$ solution was recorded via DLS count rate measurement. A 2 mg mL$^{-1}$ stock solution of the respective sample was prepared in PBS (0.01 M, pH 7.4). Just before starting the measurement, the same amount of 0.2 M aqueous H$_2$O$_2$ solution was added, and the mixture was filtrated through a 0.45 µm nylon syringe filter into a DLS cuvette. After sealing the cuvette with a cap, the cuvette was placed in the DLS at 37 °C and the count rate and hydrodynamic diameter were determined by cycle measurements every 5 min. Selected oxidized and quenched samples of the oxidation kinetics with different degrees of oxidation (6.5, 8.3, 12.1, 16.3, 22.7%) were analyzed to investigate the effect of pre-oxidation. The solution was purified from residual Na$_2$S$_2$O$_3$ salt by dialysis against deionized water for 3 days (4 water exchanges, MWCO: 3.5 kDa). Afterwards, the samples were incubated in 0.1 M H$_2$O$_2$, filtrated through 0.45 µm nylon filter, and the count rate and the evolution of the count rate and diameter determined by time-dependent DLS measurements.

Degradation kinetics by sampling and quenching

A solution of PNAM$_{25}$-b-PNAT$_{70}$ based vesicles V3 (2 mg mL$^{-1}$, 50 mL) was prepared in PBS (0.01 M, pH 7.4) and filled in a 100 mL flask. The mixture was heated to 37 °C and 50 mL of 0.2 M H$_2$O$_2$ was added under stirring. At certain time points, samples (8 mL) were taken by a precooled syringe and directly quenched by diluting with an excess of precooled Na$_2$S$_2$O$_3$ solution (10 mL, 0.15 M). Subsequently, the quenched solution was divided into two parts, the first part (~ 1 mL) was stored in the fridge at 4 °C for further DLS and cryo-TEM analysis, since freezing was expected to deform the vesicular structure and the second part for NMR analysis frozen in a -20 °C freezer to finally stop any further reactions. Afterwards, the NMR samples were dialyzed against deionized water for 3 days (4 water exchanges, MWCO: 3.5 kDa) to remove the salt, again frozen and lyophilized to obtain the pure oxidized polymer. For NMR analysis the samples were dissolved in a 4:1 mixture of THF-d$_8$ and D$_2$O and the degree of oxidation determined via HSQC-NMR (600 MHz) after phase correction by integration of the characteristic signals (Fig. S6-8, calc. 3). The measurement time was reduced by using the acceleration by sharing adjacent polarization (ASAP) pulse sequence to receive a suitable signal-to-noise ratio. The residual part of the samples was directly used for the determination of count rate and size by DLS. For selected time points near the onset of degradation, cryo-TEM was measured.
**Encapsulation of calcein**

The synthesis of PNAM$_{25}$-b-PNAT$_{70}$ based vesicles V3 was carried out as previously reported.$^{[1b]}$ PNAM$_{25}$ macro-CTA (101.9 mg of a 7.13 $\times$ 10$^{-5}$ mol g$^{-1}$ aqueous solution, 7.27 µmol, 1 eq.), NAT (80 mg, 510 µmol, 70 eq.) and VA-044 (12.1 µL of a 20 mg mL$^{-1}$ aqueous solution, 0.727 µmol, 0.1 eq.) were weighed in a 5 mL microwave vial. 1,3,5-trioxane (5 mg) was added as an internal standard and the mixture was dissolved in a 1 mM solution of calcein in ultrapure water (1.90 mL, 1.08 wt% polymer) and 1,4-dioxane (0.63 mL). After the sealing with a rubber septum, the mixture was degassed by a stream of bubbled nitrogen for 20 min under stirring. Afterwards, a sample (0.1 mL) was taken for $^1$H-NMR analysis, and the vial placed in a preheated oil bath at 70 °C. After stirring for 1 h, the vial was cooled to room temperature and opened to air. The quantitative conversion was checked by taking a sample (0.1 mL) for $^1$H-NMR analysis and the size distribution determined by SEC (50 µL). Subsequently, the vesicles were purified from free dye two times by preparative SEC (Sephadex-G75) in deionized waters. Afterwards, the concentration was gravimetrically determined by taking samples (3 × 100 µL). For the determination of the encapsulation efficiency (EE) a standard curve of calcein solutions in DMF of different concentrations was prepared by UV/Vis absorbance (490 nm) measurements. Therefore, an EE of 26% was determined. Moreover, to check the presence of free dye in solution, a 1 mg mL$^{-1}$ aqueous solution of vesicles was prepared and filtrated by an amicon ultrafiltration device (15 mL, MWCO: 3 kDa). The filtrate was collected and the amount of free calcein was determined by UV/Vis spectroscopy in DMF to be 0.014 wt%.

$$EE = \frac{[\text{Calcein}]_V - [\text{Calcein}]_{\text{free}}}{[\text{Calcein}]_0} \times 100$$  \hspace{1cm} (2)

**Calcein release study**

The kinetics of calcein release from the vesicle was investigated in time-dependent fluorescence measurements. A solution of the PNAM$_{25}$-b-PNAT$_{70}$ based vesicles V3 (2 mg mL$^{-1}$, 1 mL) with encapsulated calcein was prepared in PBS (0.01 M, pH 7.4), filled in a 2 mL quartz cuvette, and placed in the fluorescence spectrometer. Next, the cuvette was heated to 37 °C, 1 mL of an aqueous 0.2 M H$_2$O$_2$ was added under stirring and the fluorescence of calcein (Ex.: 490 nm) measured every 30 min. The emission intensity was determined by integration of the peak area from 510 to 540 nm and correlated with the incubation time. As control experiments, calcein in H$_2$O, calcein in H$_2$O$_2$, and vesicles V3 without dye in H$_2$O$_2$ were measured under the same conditions.
Encapsulation of glucose oxidase (GOx)

PNAM$_{25}$ macro-CTA (92.9 mg of a 7.82 \times 10^{-5} \text{ mol g}^{-1} \text{ aqueous solution, 7.27 \mu mol, 1 eq.}), NAT (80 mg, 510 \mu mol, 70 eq.) and VA-044 (24.2 \mu L of a 20 mg mL$^{-1}$ aqueous solution, 1.45 \mu mol, 0.1 eq.) were weighed in a 5 mL microwave vial. 1,3,5-trioxane (5 mg) was added as an internal standard and the mixture was dissolved in a solution of GOx (373 U mL$^{-1}$) in deionized water (1.68 mL) and 1,4-dioxane (0.84 mL). After sealing with a rubber septum, the mixture was degassed by a stream of bubbled nitrogen for 20 min under stirring. Afterwards, a sample (0.1 mL) was taken for $^1$H-NMR analysis, and the vial placed in a preheated oil bath at 40 °C. After stirring for 20 h, the vial was cooled to room temperature and opened to air. The quantitative conversion was checked by taking a sample (0.1 mL) for $^1$H-NMR analysis and the size distribution analyzed by SEC (50 \mu L). Subsequently, the vesicles were purified from free enzyme by preparative SEC (Sephadex-G75) in citrate-phosphate buffer (0.1 M, pH 7.0). Afterwards, the concentration was gravimetrically determined by taking samples after dialysis against deionized water and lyophilization (3 \times 100 \mu L).

Activity and concentration of GOx-loaded vesicles

The amount of encapsulated, active enzyme was assessed via amplex red (AR)-horse-radish peroxidase (HRP) assay. Therefore, a mixture of 178 \mu L of citrate-phosphate buffer (CPB, 0.1 M, pH 7.0), 1 \mu L of HRP (62.5 U mL$^{-1}$ in CPB), 10 \mu L AR (1 mg mL$^{-1}$ in DMSO), and 10 \mu L of a dispersion of GOx-loaded vesicles V3-G or empty vesicles V3 as control was prepared in a 96-well plate. Directly after starting the cascade reaction by adding 1 \mu L of glucose solution (0.2 M in CPB), the well-plate was placed in a Tecan plate-reader and the absorbance at 571 nm recorded every 10 s. To determine the GOx-concentration, a standard curve was constructed by measuring the absorbance of 10 \mu L of GOx-solutions in CPB of defined concentrations (10 to 100 \mu g mL$^{-1}$ of 373 U mg$^{-1}$). The reaction rates were determined as slopes of the linear fits and plotted against the enzyme concentration to calculate the amount of encapsulated GOx. For the sample V3-G, a loading of 0.53 wt% (373 U mg$^{-1}$) enzyme in the polymer was determined, and based on the GOx feed of 1.56 wt% an EE of 34.0% calculated.

Self-degradation assay of GOx-loaded vesicles

The degradation kinetics of the GOx-loaded vesicles V3-G in glucose solution was recorded via DLS count rate measurement. 1 mg mL$^{-1}$ stock solutions of V3-G and empty vesicles V3 as control were prepared in CPB (0.1 M, pH 7.0). Before starting the measurement, the same amount of 0.2 M glucose solution in CPB (0.1 M, pH 7.0) was added and the solutions bubbled
with compressed air for 10 min. The mixtures were filled in a cuvette, sealed with a cap, and placed in the DLS at 37 °C. The count rate and D₄ were determined by cycle measurements every 10 min.

**Cryo-TEM investigations**

The measurements were performed on a FEI Tecnai G² 20 platform with a LaB₆ filament at 200 kV acceleration voltage. For cryo-TEM measurements 8.5 µL of the solution were vitrified on Quantifoil grids using a Vitrobot Mark IV system. Liquid ethane was used as a cryogen. Samples were transferred to a Gatan 626 cryo holder and were maintained at a temperature < -175 °C during the entire process. All images were acquired with a Mega View (OSIS, Olympus Soft Imaging Systems) or an Eagle 4k CCD camera, respectively.

**Asymmetrical flow field-flow fractionation (AF4)**

AF4 measurements were performed on an AF2000 MT System from Postnova Analytics GmbH (Landsberg, Germany), equipped with a tip and focus pump (PN1130), an autosampler (PN5300), and a channel oven unit (PN4020) set to 25°C. The channel was coupled to a multiangle laser light scattering (MALLS) detector (PN3621) equipped with a 532 nm laser and measuring 21 angles, a refractive index (RI) detector (PN3150), and a UV-detector (PN3212) set to 280 nm. The channel had a trapezoidal geometry with a nominal height of 350 µm. Regenerated cellulose (RC) membrane from Postnova Analytics GmbH (10 kDa RC membrane) with a molar mass cutoff of 10 kDa was used as accumulation wall. As the mobile phase aqueous solution with 0.002 w% of NaN₃ was used. 50 µL of the sample at a concentration of 1 mg mL⁻¹ was injected with an injection flow rate of 0.2 mL min⁻¹, a focus flow rate of 0.8 mL min⁻¹, and a crossflow rate of 0.7 mL min⁻¹, resulting in a detector flow rate of 0.3 mL min⁻¹. The focusing time was 4 min before switching to elution at an exponentially decaying crossflow from 0.7 mL min⁻¹ to 0.2 mL min⁻¹ in 76.2 min. Thereafter the crossflow profile was set to decay in a linear way from 0.05 mL min⁻¹ to 0.04 mL min⁻¹ in 71 min. Before the start of the next measurement, a rinsing step was performed at 1.5 mL min⁻¹ flow of the tip pump for 20 min. After each sample measurement, a blank measurement was run which was subtracted from the data of the sample measurement for analysis. The MALLS data of the scattering angles from 20°-148° was analysed via ZIMM plot to obtain the radius of gyration (R_g) at the specified elution times.
**Table S1.** Overview of the synthesized polymers and vesicles including abbreviations, polymerization conditions, and properties.

| ID   | Polymer         | $M_n$ [a) [kDa] | $D$ [b) [nm] | $D_M$ [b) [nm] | PDI [b) | EE [d) [%] |
|------|-----------------|-----------------|--------------|----------------|--------|-----------|
| V1*  | PNAM$_{25}$-b-PNAT$_{25}$ | 7.3 1.13 | 109 | 12.9 ± 1.8 | 0.16 | - |
| V1-C | PNAM$_{25}$-b-PNAT$_{50}$ | 7.2 1.08 | 131 | - | 0.18 | 5.7 |
| V2*  | PNAM$_{25}$-b-PNAT$_{50}$ | 10.5 1.08 | 88 | 17.2 ± 2.2 | 0.06 | - |
| V2-C | PNAM$_{25}$-b-PNAT$_{70}$ | 9.9 1.10 | 84 | - | 0.08 | 1.4 |
| V3*  | PNAM$_{25}$-b-PNAT$_{70}$ | 13.3 1.16 | 117 | 19.3 ± 2.3 | 0.11 | - |
| V3-C | PNAM$_{25}$-b-PNAT$_{70}$ | 13.4 1.16 | 127 | - | 0.10 | 10.9 |
| V3-G | PNAM$_{25}$-b-PNAT$_{70}$ | 12.6 1.16 | 125 | 19.2 ± 2.0 | 0.14 | 34.0 |

[a] Determined by SEC (Eluent: DMAc + 0.21 wt% LiCl, PS-calibration)  
[b] Determined by DLS measurements of the purified structures (c: 1 mg mL$^{-1}$).  
[c] Determined by graphical analysis of > 100 cryo-TEM images.  
*Samples were previously reported.  
[d] Determined by UV/Vis measurements of purified samples and by comparison of the enzymatic activity.

**Figure S1.** SEC analysis of the prepared block copolymers (Eluent: DMAc + 0.21% LiCl, PS-calibration).  
A) Samples V1-3, SEC curves were previously reported.  
B) Samples V1-3-C and V3-G. SEC analysis of vesicles. (Eluent: citrate-phosphate buffer 10 mM, pH 7.0)  
C) RI and D) UV-975 traces of GOx-loaded vesicles V3-G; empty vesicles V3 and free GOx as control.
Figure S2. Cryo-TEM images of vesicles after purification. A) Cryo-TEM of sample V1. B) Cryo-TEM of sample V2. C) Cryo-TEM of sample V3. Cryo-TEMs were previously reported.\textsuperscript{[1]}
Figure S3. Size distribution of the membrane diameter determined by graphical analysis of ≥ 100 vesicles in cryo-TEM images. A) Histogram of sample V1. B) Histogram of sample V2. C) Histogram of sample V3. D) Histogram of sample V3-G.
Figure S4. Cryo-TEM images of samples collected after different time points of the degradation kinetics. A) Sample after 350 min. B) Sample after 420 min. C) Sample after 484 min. D) Sample after 514 min.
Figure S5. A) Correlation of vesicular $T_{1/2}$, determined by fitting of the DLS degradation curves with the DP of the PNAT block. Time-dependent evolution of $D_H$ during $H_2O_2$ or glucose-induced degradation determined by DLS: B) Empty vesicles V1, V2, V3 and a mixture of V1+V2+V3 incubated in 0.1 M $H_2O_2$ (37 °C, 10 mM PBS pH 7.4). C) Partially oxidized vesicles incubated in 0.1 M $H_2O_2$ (37 °C, 10 mM PBS pH 7.4). D) GOx-loaded and empty vesicles V3-G and V3 incubated in 100 mM glucose solution (0.5 mg mL$^{-1}$, 37 °C, CPB pH 7).
Figure S6. ASAP-HSQC NMR spectra (600 MHz, THF-d₈:D₂O 4:1). A) Zoomed area with assigned signals of unoxidized control sample PNAM₂₅-b-PNAT₇₀. B) Zoomed area with assigned signals of fully oxidized control sample PNATOₓ₅₀.
**Figure S7.** ASAP-HSQC NMR spectra (600 MHz, THF-d$_8$:D$_2$O 4:1) of partially oxidized (~ 6.5%) PNAM$_{25}$-b-PNAT$_{70}$, quenched after 70 min. A) Zoomed area with assigned signals. B) Full spectra.
Figure S8. ASAP-HSQC NMR spectra (600 MHz, THF-d$_8$:D$_2$O 4:1) of partially oxidized (~ 61.7%) PNAM$_{25}$-b-PNAT$_{70}$, quenched after 534 min. A) Zoomed area with assigned signals. B) Full spectra.
Degree of oxidation determined by integration of ASAP-HSQC-NMR spectra after purification and calculated as follows (indices represent $^1$H/$^{13}$C-shifts in ppm):

$$
\text{Degree of oxidation} = \frac{\text{Area}_{2.7,32.5} + \text{Area}_{3.1,36.3} + \text{Area}_{3.4,32.6}}{\text{Area}_{2.7,32.5} + \text{Area}_{3.1,36.3} + \text{Area}_{3.4,32.6} + \text{Area}_{1.7,26.1}} \times 100
$$

Figure S9. Evolution of the degree of oxidation with the incubation time of PNAM$_{25}$-b-PNAT$_{70}$ V3 in 0.1 M H$_2$O$_2$ (1 mg mL$^{-1}$, PBS pH 7.4, 37 °C). *Samples not completely dissolved.

Table S2. Comparison of partially oxidized vesicles before and after long-term storage.

| Degree of oxidation$^{a)}$ [%] | $D_h^{b)}$ [nm] | $PDF^{b)}$ | $D_h^{c)}$ [nm] | $PDF^{c)}$ |
|-----------------------------|--------|------|--------|------|
| 0                           | 117.4  | 0.11 | -      | -    |
| 6.5                         | 118.3  | 0.09 | 122.7  | 0.12 |
| 8.3                         | 117.2  | 0.17 | 122.1  | 0.12 |
| 12.1                        | 116.5  | 0.07 | 123.2  | 0.08 |
| 16.3                        | 113.7  | 0.08 | 123.8  | 0.02 |
| 22.7                        | 116.4  | 0.08 | 118.6  | 0.09 |

[a] Determined by NMR of quenched samples. [b] Determined by DLS measurements of the purified structures (c: 1 mg mL$^{-1}$). [c] Determined after storage for ≥ 6 months.
Figure S10. Intensity-averaged size distributions of partially oxidized vesicles measured directly after quenching and storage for ≥ 6 months. A) Vesicles based on PNAM25-\(b\)-P(NAT65-\(co\)-NATOx3). B) Vesicles based on PNAM25-\(b\)-P(NAT64-\(co\)-NATOx6). C) Vesicles based on PNAM25-\(b\)-P(NAT61-\(co\)-NATOx9). D) Vesicles based on PNAM25-\(b\)-P(NAT59-\(co\)-NATOx11). E) Vesicles based on PNAM25-\(b\)-P(NAT54-\(co\)-NATOx16).
**Figure S11.** Cryo-TEM images of vesicles after purification. Cryo-TEM of calcein-loaded vesicles V3-C. A) 200 nm magnification. B) 100 nm magnification. Cryo-TEM of GOx-loaded vesicles V3-G. C) 200 nm magnification. D) 500 nm magnification.
Figure S12. Asymmetric flow-field flow fractionation (AF4) analysis of vesicles. Elugrams with RI, MALLS 90° and UV/Vis (490 nm) detection: A) Calcein-loaded vesicles V3-C, C) GOx-loaded vesicles V3-G, E) Empty vesicles V3. Distribution of R₇ calculated by ZIMM plot based on the MALLS detection: B) Calcein-loaded vesicles V3-C, D) GOx-loaded vesicles V3-G, F) Empty vesicles V3.
Figure S13. A) Reaction scheme of the GOx- and HRP catalyzed conversion of amplex red to resorufin. B) Increase in absorbance of resorufin in solutions containing amplex red and varying GOx concentrations. C) Standard curve constructed by linear fitting of the reaction rates of GOx solutions of defined concentration. D) Standard curve constructed by linear fitting of the absorbance (490 nm) of calcein solutions in DMF. E) Overlay of the fluorescence emission spectra of pure calcein and calcein-loaded vesicles V1-3-C (Ex.: 493 nm, Em.: 500-650 nm, 2 mg mL⁻¹ in 0.1 M CPB pH 7.0 or DMF).
References

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