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Light-Driven Proton Transfer for Cyclic and Temporal Switching of Enzymatic Nanoreactors

Silvia Moreno, Priyanka Sharan, Johanna Engelke, Hannes Gumz, Susanne Boye, Ulrich Oertel, Peng Wang, Susanta Banerjee, Rafal Klajn, Brigitte Voit, Albena Lederer, * and Dietmar Appelhans*
Supporting Information

LIGHT-DRIVEN PROTON TRANSFER FOR CYCLIC AND TEMPORAL SWITCHING OF ENZYMATIC NANOREACTORS

Silvia Moreno\textsuperscript{a}, Priyanka Sharan\textsuperscript{a,b,c}, Johanna Engelke\textsuperscript{a,b}, Hannes Gumz\textsuperscript{a,d}, Susanne Boye\textsuperscript{a}, Ulrich Oertel\textsuperscript{a}, Peng Wang\textsuperscript{a,b}, Susanta Banerjee\textsuperscript{c}, Rafal Klajn\textsuperscript{e}, Brigitte Voit\textsuperscript{a,b,d}, Albena Lederer\textsuperscript{a,b}, Dietmar Appelhans\textsuperscript{a,*}

\textsuperscript{a} Leibniz-Institut für Polymerforschung Dresden e.V., Hohe Straße 6, 01069 Dresden, Germany,
E-mail: applhans@ipfdd.de; lederer@ipfdd.de
\textsuperscript{b} Faculty of Chemistry and Food Chemistry, Technische Universität Dresden, 01062 Dresden, Germany
\textsuperscript{c} Materials Science Centre, Indian Institute of Technology Kharagpur, Kharagpur – 721302, India
\textsuperscript{d} Center for Advancing Electronics Dresden, 01062 Dresden, Germany
\textsuperscript{e} Department of Organic Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel
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**Figure S10.** Fluorescence spectra of the reagents involved in the enzymatic assay ($\lambda_{exc} = 534$ nm, $\lambda_{obs} = 583$ nm). All the reagents were dissolved in a 10 mM NaCl solution (0.01 mg/mL Resorufin, 0.2 mg/mL glucose, 0.2 mg/mL GOx, 0.2 mg/mL HRP, 0.1 mM H$_2$O$_2$, 1 mg/mL, and 0.1 mg BCP/mL Psome-GOX).

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were prepared: (i) the first sample was irradiated with a Hg lamp for 3 cycles (each cycle: 3 min irradiation followed by 5 min relaxation), (ii) the second sample was not irradiated. Then, the pH of the samples was adjusted to 5.5 and the first enzymatic reaction was carried out. After 9 min, the pH of the solutions was adjusted to 7.5. The fluorescence spectra of Resorufin were recorded after 10 min of each enzyme assay ($\lambda_{\text{exc}} = 534$ nm, $\lambda_{\text{obs}} = 585$ nm). Conditions: $C_{\text{freeMyo}} = 0.02$ mg/mL, $C_{\text{MEH}} = 1.66$ mM in MilliQ water with 5% DMSO. It is important to note that in B), the enzymatic reaction is running during irradiation, while in A) and C), the reactions are initiated after the irradiation/relaxation cycles.

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**Figure S20.** Following the enzymatic activity of A) GOx-containing nanoreactors and B) Myo-containing nanoreactors in the presence of light-triggered MEH by monitoring the production of fluorescent product Resorufin after 1-3 cycles of irradiation/relaxation of MEH, compared to a non-irradiated sample and an experiment with pH adjusted manually ($\lambda_{\text{exc}} = 534$ nm, $\lambda_{\text{obs}} = 585$ nm). Shown here are the initial studies on both Psome-GOx and Psome-Myo aimed at optimizing the process. The optimized processes are shown in Figure 6 and Figures S21–S24.

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collapsed at pH 7.5. Each experiment was carried out at least in triplicate.

**Figure S23.** Following the production of Resorufin resulting from the enzymatic activity of GOx-containing nanoreactors in the presence of HRP and light-triggered MEH. Fluorescence intensity of Resorufin is shown after 1-3 cycles of irradiation/relaxation of MEH, compared to a non-irradiated sample and an experiment with pH adjusted manually \( (\lambda_{\text{exc}} = 534 \text{ nm}, \lambda_{\text{obs}} = 585 \text{ nm}) \). Each experiment was carried out at least in triplicate.

**Figure S24.** Following the production of Resorufin resulting from the enzymatic activity of Myo-containing nanoreactors in the presence of light-triggered MEH. Fluorescence intensity of Resorufin is shown after 1-3 cycles of irradiation/relaxation of MEH, compared to a non-irradiated and an experiment with pH adjusted manually \( (\lambda_{\text{exc}} = 534 \text{ nm}, \lambda_{\text{obs}} = 585 \text{ nm}) \). Each experiment was carried out at least in triplicate.

**Figure S25.** AF4 fractograms with light scattering detector signals and A) molar masses and B) radii of gyration for different elution times for empty Psome (black), GOx-Psome (unpurified, blue), GOx-Psome (purified by HFF, red), Myo-Psome (unpurified, green), and Myo-Psome (purified by HFF, magenta).

**Figure S26.** Stability of Myo in the presence of excess of H\textsubscript{2}O\textsubscript{2}. Following the production of Resorufin as a result of enzyme activity of A) free Myo (0.1 mg/mL in 10 mM NaCl) and B) free Myo in the presence of MEH (0.1 mg/mL of Myo and 1.66 mM of MEH in 10 mM NaCl with 5 % DMSO) in the presence of H\textsubscript{2}O\textsubscript{2} \( (\lambda_{\text{exc}} = 534 \text{ nm}, \lambda_{\text{obs}} = 585 \text{ nm}) \).

**Figure S27.** Protocols of experiments involving light-triggered adaptive enzymatic nanoreactors. **Psome-GOx:** HRP and Amplex Red are added to the enzyme assay only once cycles of MEH/SP irradiation/relaxation are completed. **Psome-Myo:** Amplex Red and H\textsubscript{2}O\textsubscript{2} are added to enzyme assay before cycles of MEH/SP irradiation/relaxation are started.

10. References
1. Materials
Poly(ethylene glycol) methyl ether (MeO-PEG-OH; $M_n = 2000$ g·mol$^{-1}$; $M_w/M_n = 1.05$), 2,2'-bipyridine, 4-aminobutanol, 2-(diethylamino)ethyl methacrylate (DEAEM), methacryloylic chloride, 2-bromoisobutryl bromide, 2-aminoethanol, copper(I) bromide, aluminum oxide (neutral, activated), glucose oxidase from Aspergillus niger (GOx, essentially salt-free, lyophilized powder), Resorufin, sodium chloride, sodium hydroxide, magnesium sulfate, D-$\text{(+)}$-glucose, myoglobin from equine skeletal muscle (Myo, essentially salt-free, lyophilized powder), dimethyl sulfoxide, and horseradish peroxidase were purchased from Sigma-Aldrich. 3,4-Dimethylmaleic acid anhydride, toluene, tetrahydrofuran, ethyl acetate, and chloroform were purchased from Acros Organics. Hydrochloric acid (37%), n-hexane, and silica gel were purchased from Merck. Amplex Red was purchased from Thermo Fischer. Anhydrous 2-butanone and triethylamine were purchased from Fluka. Protonated merocyanine (MEH) was synthesized and characterized according to a previously published procedure.\[^{[1-3]}\]

2. Instrumentation
\textbf{NMR Spectroscopy.} Bruker Advance III 500 spectrometer (Bruker Biospin, Germany) was used to record $^1$H NMR (500.13 MHz) spectra using CDCl$_3$ or DMSO-$d_6$ as solvents at room temperature. The chemical shifts were referenced to the corresponding solvent signals (CDCl$_3$: $\delta = 7.26$ ppm; DMSO-$d_6$: $\delta = 2.54$ ppm) and are expressed in ppm.

\textbf{Gel Permeation Chromatography.} The molar mass distributions ($D$), weight average molecular weight ($M_w$), and number average molecular weight ($M_n$) of block copolymers were measured using SEC equipped with a MALLS detector (MiniDAWN-LS detector, Wyatt Technology, USA) and a viscosity/refractive index (RI) detector (ETA-2020, WGE Dr. Bures, Germany). The column (PL MIXED-C with a pore size of 5 $\mu$m, 300 $\times$ 7.5 mm) and the pump (HPLC pump, Agilent 1200 series) were from Agilent Technologies (USA). THF stabilized with 0.025 % BHT was used as an eluent with a flow rate of 1 mL/min.

\textbf{Hollow Fiber Filtration.} HFF was carried out using KrosFlo Research Iii System equipped with a separation module made of polyether sulfone membrane (MWCO: 500 kDa, SpectrumLabs, USA). The transmembrane pressure was 150 mbar with a flow rate of 15 mL/min.

\textbf{Dynamic Light Scattering.} DLS measurements of aqueous polymersome solutions ($\leq 1$ mg/mL) were carried out using a Zetasizer Nano-series instrument (Malvern Instruments, UK) equipped with Dispersion Technology Software (version 5.00). The measurements were carried out over a range of pH values at 20 °C. The data were collected using the NIBS (non-invasive back-scatter) method with a helium–neon laser (4 mW, $\lambda = 632.8$ nm) and a fixed angle of 173° (acquisition time = 5 s; number of acquisitions = 25; 3 measurements). The data were analyzed using Malvern Software 7.11.

\textbf{Fluorescence Spectroscopy.} Fluorescence spectra were measured on a Fluorolog 3 (Horiba JobinYvon, USA) fluorescence spectrophotometer. The main measurements conditions: $C_{\text{sample}} = 0.1$ mg/mL Psome; $\lambda_{\text{exc}} = 534$ nm.

\textbf{UV-Vis Spectroscopy.} Two different UV-Vis spectrophotometers were used: (i) Cary 6000i (Varian) and (ii) Specord 210 Plus double beam. Samples were measured at the desired wavelength range in semi-micro cuvettes (Brand GmbH).

\textbf{UV Lamp (crosslinking of Psomes).} EXFO Omnicure 1000 (Lumen Dynamics Group Inc., Canada) equipped with a high-pressure mercury lamp as the UV source was used to crosslink the polymersomes.
Hg Lamp (MEH irradiation). A 100 W Hg short-arc lamp from the LOT Quantum Design GmbH (excitation range: 320-500 nm) with constant power control, convection cooling, reflector unit, and UV-quartz condenser (F/1.3) of a 35 mm aperture was used.

Cryogenic Transmission Electron Microscopy (cryo-TEM). Cryo-TEM images were acquired on a Libra 120 microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) at an acceleration voltage of 120 kV. The samples were prepared by dropping 2 μL of polymersome solution on copper grids coated with holey carbon foil (so-called Lacey type). A piece of filter paper was used to remove excess water; the sample was then rapidly frozen in liquid ethane at −178 °C. The blotting with the filter paper and plunging into liquid ethane was done in a Leica GP device (Leica Microsystems GmbH, Wetzlar, Germany). All images were recorded in bright field at −172 °C. The diameter and membrane thickness of the polymersome were determined from cryo-TEM images by using TEM image processing software. Several polymersome particles were analyzed at pH 8. The average diameter of polymersome was calculated by analyzing 150-250 particles. The average membrane thickness was calculated by analyzing 30 particles.

Asymmetrical Flow Field Flow Fractionation (AF4). AF4 measurements were performed with an Eclipse DUALTEC system (Wyatt Technology Europe, Germany) with a 0.001 M PBS buffer (at pH 8) as carrier liquid and 0.02 % (w/v) NaN₃ to prevent the growth of bacteria and algae. The channel spacer made of poly(tetrafluoroethylene) had a thickness of 490 μm and the channel dimensions were 26.5 cm in length and 0.6–2.1 cm in width. Regenerated cellulose membranes with a molecular weight cut-off (MWCO) of 10 kDa were used as accumulation wall (Superon GmbH, Germany). Flow rates were controlled with an Agilent Technologies 1260er series isocratic pump equipped with a vacuum degasser. The detection system consisted of a MALS detector (DAWN HELEOS II, Wyatt Technology Europe, Germany) operating at a wavelength of 659 nm, a variable wavelength detector (VWD, 1260er series, Agilent Technologies, Germany), and a refractive index (RI) detector (Optilab T-rEX, Wyatt Technology Europe GmbH, Germany) operating at a wavelength of 660 nm. All injections were performed with an autosampler (1260 series, Agilent Technologies Deutschland GmbH). The data collection and calculation of molar masses and radii was performed by Astra 6.1.2.84 software (Wyatt Technologies, USA). The channel flow rate (Fc) was maintained at 1 mL·min⁻¹ for all AF4 measurements. Unless mentioned otherwise, the focus flow (Ff) was set at 1.5 mL·min⁻¹ for 6 min. The injection volume for molar mass determination was set to 200 μL and for online DLS it was increased to 400 μL. For the separation of samples, the separation starts with an isocratic step with a cross flow rate (Fc) of 2 mL·min⁻¹ for 15 min followed by a linear Fc gradient from 2 to 0.05 mL·min⁻¹ within 2 min. The last step proceeds with Fc of 0.05 mL·min⁻¹ for 25 min.

The scaling exponent was calculated using \( R_g \propto M^\nu \), in which the scaling exponent \( \nu \) is defined by the increase of the radius of gyration dependent on molar mass. Theoretical values of \( \nu \) are 0.33 for a solid sphere and 0.5 for a statistical coil in a theta solvent.

The apparent density (\( \rho_{app} \)) was calculated as \( \rho_{app} = M_w / (V_{app}(R_g)N_A) \), where \( V_{app}(R_g) \) is the apparent volume calculated by \( R_g \) and \( N_A \) is the Avogadro constant.

3. Synthesis and characterization of BCP

The block copolymer (BCP) was synthesized through atom transfer radical polymerization (ATRP)\(^{64}\) and had a methoxy end group at the hydrophilic poly(ethylene glycol) (PEG) segment, while the hydrophobic part consisted
of pH-sensitive 2-(diethylamino)ethyl methacrylate (DEAEM) and the photocrosslinker 3,4-dimethyl maleic imidobutyl methacrylate (DIMGMA). Psome were fabricated by self-assembly of BCPs, using the so-called pH switch method (more details about preparation and characterization of BCP in SI, Figure S1 and Table S1).[65] Once the Psome were formed, they were photocrosslinked (details below) to obtain robust and stable polymeric vesicles with pH-responsive membrane permeability for switching enzymatic reactions ON and OFF.[61, 62]

Experimental description

The block copolymer (BCP) was synthesized by using standard ATRP procedure as reported previously.[4-8] PEG65-Br (100 mg), 3,4-dimethylmaleimido-butylmethacrylate (246.9 mg, 20 eq), 2-(diethylamino)ethyl methacrylate (603.4 mg, 70 eq), and bipyridine (14.5 mg, 2 eq) were mixed together in ethyl methyl ketone (1.5 mL). Under the nitrogen atmosphere, CuBr (6.7 mg, 1 eq) was added. This mixture was deoxygenated by freeze–pump–thaw cycles. The mixture was then refluxed for 19 hours at 50 °C. Then, the polymerization was terminated by exposure to air during the addition of THF. The oxidized copper catalyst was removed by passing the mixture over activated neutral aluminium oxide with THF as an eluent, and the solution was filtered using a 0.2 µm filter. The final solution was concentrated by evaporating most of the solvent, followed by precipitation with cold n-hexane. Then, dialysis was carried out in acetone using membrane with a cut-off of 2000 kDa for 24 h. The composition and the number average molecular weight (Mn) of BCPs were determined with SEC-MALLS with 1H NMR spectroscopy based on the peak integrals of PEG (3.65 ppm), DEAEMA (2.65-2.78 ppm), and DIMGMA (3.52 ppm), by taking the PEG block as an internal standard. The dispersity (D) of BCPs was determined by SEC as described in the previous section. The results are shown in Figure S1 and Table S1.

4. Fabrication of empty and loaded polymersomes

Preparation of crosslinked polymersomes. Polymersomes were fabricated by the self-assembly of BCP using the so-called pH switch method. A solution of 1 mg/mL of BCP in 0.01 M HCl was prepared and stirred until the BCP was dissolved. The final solution was passed through a 0.2 µm nylon filter to remove any impurities. The self-assembly process was triggered by increasing the pH slowly to pH 8.5 through the addition of 1 M and 0.1 M NaOH. The mixture was stirred for 3 days in the dark. The solution was filtered using 0.8 µm nylon filter and crosslinked for 5 min to afford robust polymersomes.[4, 9, 10]

Glucose oxidase and myoglobin-containing polymersomes (Psome-GOx and Psome-Myo). For in-situ encapsulation of GOx and Myo, the method of Gräfe et al. and Liu et al. has been adopted.[5, 11] 13 mg of BCP was dissolved in 12 mL of 0.01 M HCl. In a separate vial, 4.8 mg of enzyme was dissolved in 1 mL of 1 mM PBS. Both solutions were filtered (0.2 µm) and then 11.5 mL of the BCP solution and 0.5 mL of the enzyme solution were mixed together to obtain a final concentration of 1 mg/mL BCP and 0.2 mg/mL enzyme. The pH was slowly increased to ≥8.5 by adding NaOH. The enzyme solution (4.8 mg/mL) was added when the pH was around 5 and it was stirred ≥8.5 for 3 days in the dark. The resulting solution was filtered using a 0.8 µm nylon filter and exposed to UV light for 5 min. The resulting solution was then purified from non-encapsulated enzyme using HFF. 11 mL of the unpurified solution (1 mg/mL) was transferred into a 50 mL cone tube attached to the hollow-fiber filtration system. The sample was diluted with a 10 mM NaCl solution to 50 mL and constantly refilled until the extraction volume was reached. The transmembrane pressure was kept at 150 mbar during the whole process until extracting a total of 150 mL.
5. Optimization of parameters and control experiments

Optimization of the polymersome crosslinking time. A 1 mg/mL polymersome solution was divided into three portions, which were crosslinked for different times (5, 10, and 15 min). For each sample, small amounts of 1 M HCl or 1 M NaOH solutions were added to reach pH 5 or 8, respectively. The cycle was repeated 5 times and the hydrodynamic diameter was measured for both acidic and basic pH by DLS. This procedure was repeated for each sample. Figure S2 and Table S2 show the corresponding results.

Dependence of the critical pH value (pH*) on the polymersome concentration. Solutions of polymersomes crosslinked for 5 min at different concentrations (0.1 mg BCP/mL, 0.25 mg BCP/mL, and 0.5 mg BCP/mL) were prepared by diluting the 1 mg BCP/mL stock solution. Then, the vesicles were titrated from basic to acidic pH by adding 0.1 M HCl while simultaneously measuring their size by DLS to determine the pH*.[10] The results are shown in Figure S12.

Stability study of enzyme-containing polymersomes (Psome-GOx and Psome-Myo) under pH cycling. Two different samples were prepared: (a) Psome-GOx in the presence of MEH, with the final concentration of GOx-Psome = 0.1 mg BCP/mL and of MEH = 1.66 mM (Vf = 8 mL, 1600 µL stock 0.5 mg/mL + 6400 µL of MEH stock (2 mM)); (b) Psome-Myo in the presence of MEH, with the final concentration of Myo-Psome = 0.1 mg BCP/mL and of MEH = 1.66 mM (Vf = 8 mL, 1600 µL stock 0.5 mg/mL + 6400 µL of MEH Stock (2 mM)). For each sample, small amounts of 1 M HCl or 1 M NaOH were added to reach pH 5.5 or 7.5, respectively. The cycle was repeated 3 times and the enzymatic activity was measured under both the acidic and basic pH.

Stock solutions: 0.2 mg/mL of HRP, 0.02 mg/mL glucose, 0.02 mg/mL Amplex Red, and 0.02 mL H2O2. For Psome-GOx samples: GOx activity at pH 7.5: 500 µL of the sample + 500 µL 10 mM PBS at pH 7.5. Then, 1 µL of glucose solution was added and kept for 5 min. Then, 1.3 µL of Amplex Red solution and 22 µL of HRP solution were added and kept for 10 min. After this time, the fluorescence spectrum of Resorufin was recorded.

GOx activity at pH 5.5: 1 µL of glucose solution was added to 500 µL of the sample and was kept for 5 min. Then, 500 µL of 10 mM PBS solution at pH 7.5 was added, followed by 1.3 µL of Amplex Red solution and 22 µL of HRP solution. After 10 min, the fluorescence spectrum of Resorufin was recorded. For Psome-Myo samples: Myo activity at pH 7.5: 500 µL of the sample + 500 µL of 10 mM PBS solution at pH 7.5. Then, 15 µL of H2O2 and 15 µL of Amplex Red solution were added. After 30 min, the fluorescence spectrum of Resorufin was recorded. Myo activity at pH 5.5: 15 µL of H2O2 and 15 µL of Amplex Red solution were added and kept for 10 min. Then, 500 µL of 10 mM PBS solution at pH 7.5 was added. After 30 min, the fluorescence spectrum of Resorufin was recorded. The results are shown in Figures S18 and S19.

Study of cyclic irradiation–relaxation MEH/SP in the absence or presence of Psome by UV-Vis absorption spectroscopy. Cycles of irradiation/relaxation were performed on a MEH solution (60 µM of MEH, 1 % DMSO, MilliQ) and a MEH solution in the presence of Psome (60 µM of MEH, 1 % DMSO, 0.004 mg BCP/mL, MilliQ).
and its UV-Vis absorption spectra were recorded. This experiment was carried out to optimize the times of irradiation and relaxation and to prove that the isomerization process is reversible and reproducible. The results are shown in Figure S3.

**pH switching by cyclic irradiation of MEH/SP for three samples in a Psomes batch solution.** Several cycles of irradiation were performed on a solution containing $C_{\text{Psomes}} = 0.1 \text{ mg BCP/mL}$ and $C_{\text{MEH}} = 1.66 \text{ mM}$ in MilliQ water with 5 % DMSO) and pH changes were monitored over time. This experiment was repeated three times, showing high reproducibility.

**Stability study of the MEH/SP system: Light-triggered switching of pH for two days.** Several cycles of irradiation were performed on a solution containing $C_{\text{MEH}} = 1.66 \text{ mM}$ in MilliQ water with 5 % DMSO; pH changes were monitored over time. The results were analyzed at 0, 24, and 48 h in order to evaluate the stability of the MEH/SP system over the time (the sample was stirred in the dark). The results are shown in Figure S8.

**Stability study of Myo in the presence of excess of H₂O₂.**

(a) **In the absence of MEH:** A solution of Myo in 10 mM NaCl (9 mL) was prepared with a final concentration of 0.1 mg/mL of Myo (900 μL of Myo solution + 8.1 mL of 10 mM NaCl solution). This solution was divided into six samples. Then, 60 μL of H$_2$O$_2$ (0.02 M) were added and kept for 0, 7.5, 15, 30, 45, and 60 min. Next, 60 μL of Amplex Red solution (0.02 mg/mL) were added, kept for 10 min, and diluted twice using a 10 mM PBS solution at pH 7.5. Then, fluorescence spectra were recorded at an excitation wavelength of 534 nm. (b) **In the presence of MEH:** A solution of Myo and MEH in 10 mM NaCl (9 mL) was prepared with a final concentration of 0.1 mg/mL of Myo and 1.66 mM of MEH in 5% DMSO (900 μL of Myo solution, 7.22 mL of MEH solution, and 880 μL of 10 mM NaCl solution). This solution was divided into six samples. Then, 60 μL of H$_2$O$_2$ (0.02 M) were added and kept for 0, 7.5, 15, 30, 45, and 60 min. Next, 60 μL of Amplex Red solution (0.02 mg/mL) were added, kept for 10 min, and diluted twice using a 10 mM PBS solution at pH 7.5. Afterwards, fluorescence spectra were recorded at an excitation wavelength of 534 nm. The results are shown in Figure S26.

6. **Study of interactions between MEH and polymersomes** (= Release of MEH from Psomes)

Stock solutions 1.85 mM of MEH (MilliQ with 5 % DMSO, irradiated for 3 min) and empty Psome (1 mg BCP/mL) were prepared following the procedures described above. Five samples of these solutions were prepared and studied ($V_t = 5$ mL): (a) Pure 1.66 mM MEH (4.5 mL MEH stock + 0.5 mL Milli Q); (b) Psome (0.1 mg BCP/mL) + 1.66 mM MEH, not irradiated (4.5 mL MEH stock + 0.5 mL empty-Psome stock); (c) Psome (0.1 mg BCP/mL) + 1.66 mM MEH, irradiated for 1 cycle (4.5 mL MEH stock + 0.5 mL empty-Psome stock; 3 min irradiation + 5 min relaxation; pH 7.15–5.43); (d) Psome (0.1 mg BCP/mL) + 1.66 mM MEH, irradiated for 2 cycles (4.5 mL MEH stock + 0.5 mL empty-Psome stock; 2 × (3 min irradiation + 5 min irradiation); pH 7.07–5.40); (e) Psome (0.1 mg BCP/mL) + 1.66 mM MEH irradiated for 3 cycles (4.5 mL MEH stock + 0.5 mL empty-Psome stock; 3 × (3 min irradiation + 5 min relaxation); pH 7.05–5.63). Subsequently, these samples were dialyzed against 10 mM NaCl (2 kDa cut-off) and analyzed by UV-Vis absorption spectroscopy ($λ = 423$ nm) after different times (0, 4, 8 and 24 h). The results are shown in Figure S6.
7. Enzymatic activity in the absence and presence of MEH by manual pH changes using different approaches

Stock solutions: 1 mg BCP/mL of Psome-GOx (encapsulation efficiency of GOx ~3-5%), 1 mg BCP/mL of Psome-Myo (encapsulation efficiency of Myo ~3%), 0.2 mg/mL of HRP solution, 0.02 mg/mL of glucose solution, 0.02 mg/mL of Amplex Red solution, 0.02 m of H₂O₂ solution, and 2.07 mM MEH (this solution should be ultrasonicated followed by irradiation by Hg lamp for 3 min to facilitate the dissolution of MEH).

Measurement of fluorescence spectra of individual reagents. All the components were dissolved in a 10 mM NaCl solution. 0.01 mg/mL Resorufin, 0.2 mg/mL glucose, 0.02 mg/mL GOx, 0.2 mg/mL HRP, and Psome-enzyme (1 mg BCP/mL and 0.1 mg BCP/mL) were prepared and fluorescence spectra were recorded at an excitation wavelength of 534 nm. Some of the reagents were also studied at different pH values (6 and 8) (0.01 mg/mL Resorufin and 1.66 mM MEH). The results are shown in Figure S9.

pH sensitivity of Resorufin: 0.01 mg/mL of Resorufin solutions were prepared at different pH values (1 mM PBS at pH 5, 6, and 7.4). After 20 min, fluorescence spectra were recorded at an excitation wavelength of 534 nm. The results are shown in Figure S10.

Effect of irradiation on free GOx in the presence and absence of MEH: (a) In the absence of MEH: A 0.02 mg/mL solution of free GOx (6 mL) was prepared in 10 mM NaCl (120 µL of GOx solution (1 mg/mL) + 5880 µL MilliQ), the pH was adjusted to 5.5, and it was split into two samples. One of them was irradiated for 3 cycles (each cycle: 3 min irradiation followed by 5 min relaxation). Then, 3.4 µL of glucose solution (0.02 mg/mL) was added to both solutions. After 9 min, the pH was adjusted to 7.5 by diluting with 10 mM PBS twice and 4.8 µL of Amplex Red solution (0.02 mg/mL) and 16.5 µL of HRP solution (0.2 mg/mL) were added. After 10 min of HRP activity, fluorescence spectra were recorded at an excitation wavelength of 534 nm. (b) In the presence of MEH: A 0.02 mg/mL solution of GOx containing 1.66 mM MEH was prepared (V_r = 6 mL, 120 µL of GOx (1 mg/mL) + 4.8 mL of MEH (2.07 mM) + 1.080 mL of 10 mM NaCl), the pH was adjusted to 5.5, and it was split into two samples. One of them was irradiated for 3 cycles (each cycle: 3 min irradiation followed by 5 min relaxation). Then, 3.4 µL of glucose solution (0.02 mg/mL) was added to both solutions. After 9 min, the pH of both samples was adjusted to 7.5 by diluting with 10 mM PBS twice, and 4.8 µL of Amplex Red solution (0.02 mg/mL) and 16.5 µL of HRP solution (0.2 mg/mL) were added. After 10 min of HRP activity, fluorescence spectra were recorded at an excitation wavelength of 534 nm. The results are shown in Figure S14.

Effect of irradiation on free Myo in the presence and absence of MEH: (a) In the absence of MEH: A 0.02 mg/mL solution of free Myo (6 mL) was prepared in 10 mM NaCl (120 µL Myo solution (1 mg/mL) + 5880 µL of 10 mM NaCl) and split into two samples. One of them was irradiated for 3 cycles (each cycle: 3 min irradiation followed by 5 min relaxation). Then, 10 µL of H₂O₂ (0.02 m) and 10 µL of Amplex Red solution (0.02 mg/mL) were added to both solutions at pH 5.5. After 9 min, the pH of both samples was adjusted to 7.5 by diluting with a 10 mM PBS solution twice. After an additional 30 min, fluorescence spectra were recorded at an excitation wavelength of 534 nm. (b) In the absence of MEH and in the presence of Amplex Red: The concentration of Myo was 0.02 mg/mL and that of MEH was 1.66 mM (V_r = 6 mL, 120 µL of Myo solution (1 mg/mL) + 4.8 mL of MEH solution (2.07 mM) + 1.080 mL of MilliQ). The pH of the solution was adjusted to 5.5 and it was split into two samples. Then, 30 µL of H₂O₂ (0.02 m) and 30 µL of Amplex Red solution (0.02 mg/mL) were added to both solutions; one of them was kept for 10 min without irradiation and the other was irradiated for 3 cycles (each cycle: 3 min irradiation followed by 5 min relaxation). Then, the pH of both solutions was adjusted to 7.5 by
diluting with a 10 mM PBS solution twice. After 30 min, fluorescence spectra were recorded at an excitation wavelength of 534 nm. (c) In the presence of MEH: The final concentration of Myo was 0.02 mg/mL and that of MEH was 1.66 mM (V_F = 6 mL, 120 μL of Myo solution (1 mg/mL) + 4.8 mL of MEH solution (2.07 mM) + 1.080 mL of MilliQ). The pH of the solution was adjusted to pH 5.5 and it was split into two samples. One of them was irradiated for 3 cycles (each cycle: 3 min irradiation followed by 5 min relaxation). Then, 30 μL of H_2O_2 (0.02 M) and 30 μL of Amplex Red solution (0.02 mg/mL) were added to both samples. After 9 min, the pH of both samples was adjusted to 7.5 by diluting with a 10 mM PBS solution twice. After an additional 30 min, fluorescence spectra were recorded at an excitation wavelength of 534 nm. The results are shown in Figure S15.

We adopted two methods, a one-step Method 1 and a two-step Method 2. Although all the results are presented in the SI, the final experiments and all the results shown in the main text were carried out using Method 2.

**Glucose oxidase activity assay for free GOx (in the absence of MEH).** The protocol by Molecular Probes was used to probe the activity of the free enzyme (GOx) and glucose oxidase-containing polymersomes (Psome-GOx) under different conditions. In the literature, this protocol is mainly carried out in a single step.\[^{12, 13}\] For our study, the assay was modified and adapted to the Psome-GOx enzymatic nanoreactors by dividing it into two steps: (a) **Method 1 (one-step):** A 0.02 mg/mL solution of free GOx in 10 mM NaCl (4 mL) was prepared and split into two samples. For the first sample, the pH was adjusted to 5.5 and for the other – to pH 7.5. Then, 2.25 μL of glucose solution (0.02 mg/mL), 3.2 μL of Amplex Red solution (0.02 mg/mL), and 55 μL of HRP solution (0.2 mg/mL) were added to both samples. After 9 min, fluorescence spectra were recorded at pH 8. The same protocol was used to study 0.1 mg/mL Psome-GOx. (b) **Method 2 (two-step):** A 0.02 mg/mL solution of free GOx in 10 mM NaCl (4 mL) was prepared and split into two samples. For the first sample, the pH was adjusted to 5.5 and for the other – to 7.5. Then, 2.25 μL of glucose solution (0.02 mg/mL) were added to both samples. After 9 min, the pH of both solutions was adjusted to 8, and 3.2 μL of Amplex Red solution (0.02 mg/mL) and 55 μL of HRP solution (0.2 mg/mL) were added. After 5 min of final HRP activity, fluorescence spectra were recorded at an excitation wavelength of 534 nm. The results are shown in Figure S16.

**Glucose oxidase activity assay for free GOx in the presence of MEH using “pH changed manually” (Method 2).** The final concentration of GOx was 0.02 mg/mL and that of MEH was 1.66 mM (V_F = 4 mL, 80 μL of GOx solution (1 mg/mL) + 3.2 mL of MEH solution (2.07 mM) + 720 μL of 10 mM NaCl solution). This solution was split into two samples. In the first sample, the pH was adjusted to 5.5 and in the other – to 7.5. Then, 2.25 μL of glucose solution (0.02 mg/mL) was added to both samples. After 9 min, the pH of the solutions was adjusted to 7.5, and 3.2 μL of Amplex Red solution (0.02 mg/mL) and 55 μL of HRP solution (0.2 mg/mL) were added. After 5 min of HRP activity, fluorescence spectra were recorded at an excitation wavelength of 534 nm. The second step in the presence of MEH was carried out at pH 7.5 to avoid strong overlaps in the recorded spectrum. The results are shown in Figure S16.

**Myoglobin activity assay for free Myo (in the absence of MEH).** The protocol by Molecular Probes was used to study the activity of free enzyme and enzyme encapsulated within polymersomes under different conditions. In the literature, this protocol is mainly carried out in a single step.\[^{12, 13}\] For our study, the assay was modified and adapted to the enzymatic Psome-Myo nanoreactors by dividing it into two steps: (a) **Method 1 (one step):** A 0.02 mg/mL solution of free Myo in 10 mM NaCl (6 mL; 120 μL of Myo solution (1 mg/mL) + 5880 μL of 10 mM NaCl) was prepared and split into two samples. For the first sample, the pH was adjusted at pH 5.5 and for the
other – to 7.5. Then, 10 μL of H$_2$O$_2$ solution (0.02 M) and 10 μL of Amplex Red solution (0.02 mg/mL) were added to both samples. After 9 min, both solutions were diluted twice with a 10 mM PBS solution while the pH was kept constant. After an additional 30 min, fluorescence spectra were recorded at an excitation wavelength of 534 nm.

(b) **Method 2 (two steps):** A 0.02 mg/mL solution of free Myo in 10 mM NaCl (6 mL) (120 μL of Myo solution (1 mg/mL) + 5880 μL of 10 mM NaCl) was prepared and split into two samples. For the first sample, the pH was adjusted to 5.5 and for the other – to 7.5. Then, 10 μL of H$_2$O$_2$ solution (0.02 M) and 10 μL of Amplex Red solution (0.02 mg/mL) were added to both samples. After 9 min, the pH of the solutions was adjusted to 7.5 by diluting with a 10 mM PBS solution twice. After an additional 30 min, fluorescence spectra were recorded at an excitation wavelength of 534 nm. The results are shown in **Figure S17**.

**Myoglobin activity assay for free Myo in the presence of MEH using “pH changed manually” (Method 2).** The protocol by Molecular Probes was used to study the activity of the free enzyme and glucose oxidase in-situ encapsulated by polymersomes under different conditions. In the literature, this protocol is mainly carried out in a single step.\(^{12, 13}\) For our study, the assay was modified and adapted to the enzymatic Psome-GOx nanoreactors by dividing it into two steps. A 0.1 mg/mL solution of Psome-GOx (4 mL) was prepared in a 10 mM NaCl solution and was split into two samples. For the first sample, the pH was adjusted to 5.5 and for the other – to 7.5. Then, 2.25 μL of glucose solution (0.02 mg/mL) was added to both solutions to carry out enzyme assay within a total for 9 min. Then, the pH of the solutions was adjusted to 7.5, and 3.2 μL of Amplex Red solution (0.02 mg/mL) and 55 μL of HRP solution (0.2 mg/mL) were added. After 10 min of final HRP activity, fluorescence spectra were recorded at an excitation wavelength of 534 nm.

**Myoglobin activity assay for Psome-Myo (in the absence of MEH).** The protocol by Molecular Probes was used to study the activity of the free enzyme and enzyme in-situ encapsulated by polymersomes under different conditions. In the literature, this protocol is mainly carried out in a single step.\(^{12, 13}\) For our study, the assay was modified and adapted to the enzymatic Psome-Myo nanoreactors by dividing it into two steps. A 0.1 mg/mL solution of Psome-Myo (6 mL) was prepared in a 10 mM NaCl solution and was split into two samples. For the first sample, the pH was adjusted to 5.5 and for the other – to 7.5. Then, 10 μL of H$_2$O$_2$ solution (0.02 M) and 10 μL of Amplex Red solution (0.02 mg/mL) were added to both solutions to initiate and to carry out enzyme assay in total for 9 min. Then, the pH of the solutions was adjusted to 7.5 by diluting it with a 10 mM PBS solution twice. After 30 min, fluorescence spectra were recorded at an excitation wavelength of 534 nm.

**Glucose oxidase activity assay for Psome-GOx in the presence of MEH using “pH manually changed”.** MEH solution (C$_{\text{Stock}}$ (MEH) = 2.07 mM) was prepared with ultrasonication followed by irradiation by Hg lamp for 3 min to facilitate the dissolution of MEH. Then, using this stock solution and the stock solution of Psome-GOx (prepared from 1 mg BCP/mL with 0.2 mg GOx/mL), a 4 mL solution of Psome-GOx and MEH was prepared in 10 mM NaCl to afford a final concentration of 0.1 mg/mL of Psome-GOx and 1.66 mM of MEH in water with

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5 % DMSO. This solution was divided into two samples. For the first sample, the pH was adjusted to 7.5 and for the other to 5.5. Then, we added 2.25 μL of a 0.02 mg/mL glucose solution and waited for 9 min for the final determination of GOx activity. Then, for the second step, the pH of the solutions was adjusted to 7.5, and 3.2 μL of Amplex Red solution (0.02 mg/mL) and 55 μL of HRP solution (0.2 mg/mL) were added to both samples. After 10 min of HRP activity at pH 7.5, fluorescence spectra were recorded at an excitation wavelength of 534 nm.

Myoglobin activity assay for Psome-Myo in the presence of MEH using “pH manually changed”. MEH solution (C_stock (MEH) = 2.07 mM) was prepared with ultrasonication followed by irradiation by Hg lamp for 3 min to facilitate the dissolution of MEH. Then, using this stock solution and the stock solution of Psome-Myo (prepared from 1 mg BCP/mL with 0.2 mg Myo/mL), a 4 mL solution of Psome-Myo and MEH was prepared in 10 mM NaCl to afford a final concentration of 0.1 mg/mL of Psome-Myo and 1.66 mM of MEH in water with 5 % DMSO. This solution was divided into two samples. For the first sample, the pH was adjusted to 7.5 and for the other to 5.5. Then, 60 μL of H₂O₂ solution (0.02 mM) and 60 μL of Amplex Red solution (0.02 mg/mL) were added. After 10 min, the pH of the solutions was adjusted to 7.5 by diluting with a 10 mM PBS solution twice. After 30 min, fluorescence spectra were recorded at an excitation wavelength of 534 nm.

8. Additional studies on possible side reactions in the Psome-enzyme systems and further characterization of these systems

- Photooxidation of Amplex Red. This process has been reported in the literature\textsuperscript{14,15} and we took it into account in the case of Psome-Myo nanoreactor study, which involved irradiation of Amplex Red. Higher fluorescence intensities could be obtained due to photooxidation to Resorufin, as opposed to the enzymatic activity of Myo. Thus, photooxidation could mask the desired reaction triggered by Psome-Myo. However, photooxidation of Amplex Red does not occur under our conditions. To verify this, two samples were prepared with free Myo, H₂O₂, and Amplex Red, one of which was irradiated (9 min). The obtained result (Figure S15B) was practically the same as in Figure S15A, where only the free enzyme was irradiated. These experiments allowed us to conclude that irradiation affects Myo activity only slightly.

- Decomposition of Myo in the presence of H₂O₂. It is well known that excess of H₂O₂ can influence the activity of myoglobin; in our experiments, however, the concentration of H₂O₂ is very low (Figure 6) and the amount H₂O₂ crossing the swollen membrane under light irradiation (ON state, Figure 4) is negligible. To validate this point, let us consider the data in Figure 6B. If Myo inside Psome-Myo was decomposed, the enzymatic activity would be lower, which is not the case. The enzymatic activity was purposefully lowered in the experiments shown in Figure S26, where a large excess of H₂O₂ was used.

- Spontaneous decomposition of H₂O₂. Decomposition of H₂O₂ under light irradiation has been reported.\textsuperscript{14,15} The activity of free Myo in the presence of excess H₂O₂ with and without MEH after different incubations times and light irradiation conditions was studied. In Figure S26, the desired Myo activity can be seen on the same level, but it is reduced after 30 and 60 min with and without MEH. However, in the case of Psome-Myo for Amplex Red conversion by Myo (Figure 6B), a lower concentration of H₂O₂ used is well suited for carrying out time-dependent experiments with increasing Amplex Red conversion using Psome-Myo (Figure 6B).

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- **Release of MEH from Psomes** (considering pH-dependent Polymersome-MEH interaction). Here, we considered the possible interactions between vesicle membrane and MEH. Such interactions could originate from the negatively charged sulfonate group of MEH and the positively charged ammonium groups within the membrane (due to partial protonation of the pH-sensitive poly-DEAMA block). To verify this possibility, a simple release study was carried out using UV-Vis absorption spectroscopy. Using the established conditions (Figure 2B; C_{Psomes} = 0.1 mg BCP/mL, C_{MEH} = 1.66 mM in MilliQ water with 5 % DMSO), five samples were investigated: MEH solution was dialyzed without irradiation for 24 h in the dark; MEH solution in the presence of Psomes was dialyzed without irradiation for 24 h in the dark; three MEH solutions in the presence of Psomes were irradiated and thermally relaxed for one, two, and three cycles, followed by dialysis for 24 h in the dark. UV-Vis absorption spectra were recorded at different time points. Results of this experiment are summarized in Figure S6. For all samples, most (75-90%) MEH was released within 4 h; further release was observed within an additional 20 h. One has to keep in mind that the polymersome concentration was rather low, which limits the amount of MEH capable of interacting with Psomes. Nevertheless, it is clear the presence of Psomes slows down MEH release, with the degree of deceleration increasing with the number of irradiation–relaxation cycles. These results corroborate the presence of weak noncovalent interactions between both components and confirm the cryo-TEM results showing that the presence of MEH densifies the membrane of native and cationic Psomes (i.e., integration of MEH within membrane or its attachment to outer membrane surface) (Figure 2C).

- **Light-driven proton transfer: Diameters of Psome-GOx after 1, 2, and 3 irradiation/relaxation cycles.** We additionally determined the hydrodynamic diameters (D_h) of samples “1 cycle of irradiation”, “2 cycles of irradiation” and “3 cycles of irradiation” following the enzymatic assay (Table S7). For Psome-GOx in the shrunken state (i.e., OFF state) at pH = 7–7.2, D_h are in the range 173–192 nm, in contrast to 107 nm for Psome-GOx before the enzyme assay. In a detailed DLS study, using variation of the parameters (Table S3-S6) taken from Table S7, we detected the formation of aggregates with a low polydispersity (0.156–0.174) when NaCl and MEH were combined in one system (D_h = 164–182 nm). When a high concentration of NaCl or MEH with or without irradiation were used separately, no large aggregates were observed (D_h < 113 nm). It is important to emphasize that the presence of the aggregates does not affect the function of the designed nanoreactor Psome-GOx (Figure 6).

- **Fluorescence interference of the reaction product Resorufin.** We verified that there is no fluorescence interference of the reaction product Resorufin (fluorescence in the range 550–700 nm with λ_{max} = 583 nm) with the other components of the system during the enzyme assay in the absence of MEH (Figure S9). Both MEH (Figure S11) and Resorufin (Figure S10) feature pH-dependent fluorescence. Resorufin exhibits the highest fluorescence intensity at pH 7.5. The fluorescence of MEH overlaps with the Resorufin signal, but it is very low at pH 7.5. MEH quenches Resorufin’s fluorescence, especially at the lower pH of 5.5. It is important to note that this overlap hampers quantitative studies.

- **Two different enzymatic mechanisms for Psome-GOx and Psome-Myo.** The diffusion of the substrates inside the enzymatic nanoreactors is essential for the enzymatic conversion in ON state (under irradiation). Psome-Myo catalyzes a monoienzymatic reaction, while Psome-GOx – a bienzymatic cascade. A complete overview of each step of both experiments is presented in Figure S27.
*Psome-GOx nanoreactor:* (1) Diffusion of glucose to the polymersome’s lumen in the ON state, (2) Generation of H₂O₂ and its diffusion to the outside; steps 1 and 2 take place during the irradiation cycles; once the cycles are completed and the system is switched to the OFF state, (3) HRP and Amplex Red are added in excess, and (4) production of Resorufin is followed after 10, 20, 30, and 60 min.

*Psome-Myo-nanoreactor:* (1) Diffusion of Amplex Red and H₂O₂ to the polymersome’s lumen in the ON state; (2) Production of Resorufin inside the polymersome. Steps 1 and 2 take place during the irradiation cycles; once the cycles are completed and the system is switched to the OFF state, (3) Production of more Resorufin in the lumen is followed within the next 30, 60, 90, and 120 min. In this case, the diffusion of Amplex Red to the lumen in step 1 is crucial. The conversion of Amplex Red is slow due to this limiting step and the low concentration of encapsulated Myo (Figure S27).

The Myo-catalyzed reaction inside Psome-Myo is slower compared to GOx-catalyzed reaction outside Psome-GOx. This is due to (i) a lower enzyme activity (the activity of Myo is much lower than that of HRP activity; Figures S14 and S15); (ii) a lower encapsulation efficiency for Myo (1-2 %) compared with GOx (3-5 %); (iii) the mandatory diffusion process, wherein Amplex Red must cross the membrane to reach the polymersome’s lumen in the case of Psome-Myo; this is the rate-limiting step, which can only occur when the polymersome’s membrane is swollen (ON state). In contrast, for Psome-GOx, H₂O₂ must diffuse outside the Psome, where HRP and Amplex Red are added for the final step of Resorufin production (Figure S27).

It is also important to mention that in both cases, no release of the enzyme from the nanoreactors was observed and the irradiation did not show any noticeable effect on the stability of the polymersomes or the activity of the enzyme (Figures S18 and S19).

Possible side effects, such as enzyme degradation (in the presence of MEH and/or under irradiation), spontaneous decomposition of H₂O₂,¹⁴,¹⁵ and photooxidation of Amplex Red,¹⁶,¹⁷ were taken into account. Several control experiments were carried out and are presented in the SI. These experiments did not show any significant deleterious effects on the performance of the enzymatic nanoreactors (Figure 6).

**- Indication for carrying out light-driven experiments.** For the light-driven experiments to produce repeatable pH change, a high-intensity lamp was required and the reversibility depended on the distance between the lamp and the sample and the dissolution state of MEH. Heating of the solution should be avoided as it might affect the stability of enzyme-loaded Psomes. Therefore, it was very important to design and optimize the irradiation and relaxation time in such a way that the desired effect of reversibly switchable pH change is achieved. Careful control of these parameters leads to high reversibility and fast temporal cycling.
9. Additional figures and tables

**Table S1.** Specifications of block copolymers synthesized by ATRP.

| Code | Polymer chemical composition | $M_w$ (g/mol)$^a$ | $M_n$ (g/mol)$^a$ | $D (M_w/M_n)^a$ | $M_p$ (estimated by NMR)$^b$ |
|------|-----------------------------|------------------|------------------|-----------------|-----------------|
| BCP1 | PEG$_{45}$-b-(DEAEMA$_{82}$-s-DMIBM$_{20}$) | 24000 | 19400 | 1.23 | 22580 |
| BCP2 | PEG$_{45}$-b-(DEAEMA$_{78}$-s-DMIBM$_{22}$) | 22200 | 17100 | 1.29 | 22430 |

$^a$Molecular weight distribution determined by SEC-MALLS. $^b$Molecular weight calculated by $^1$H NMR.

**Table S2.** Hydrodynamic radii of crosslinked polymersomes at pH 5 and pH 8.6 studied by DLS in MilliQ.

| Time of crosslinking | pH 5 | pH 8.6 |
|---------------------|------|--------|
| 90 s                | 77.2 | 51.7   |
| 3 min               | 65.7 | 52.3   |
| 5 min               | 62.3 | 52.5   |
| 10 min              | 60.1 | 52.7   |

**Table S3.** Hydrodynamic diameters of crosslinked polymersomes in the presence of high concentration of NaCl studied as a function of time by DLS ($C_{pomes} = 0.1 \text{ mg BCP/mL}$, 150 mM NaCl in MilliQ).

| Time (h) | Diameter (nm) | PDI |
|----------|---------------|-----|
| 0        | 88.3          | 0.157 |
| 1        | 97.0          | 0.220 |
| 2        | 90.6          | 0.162 |
| 3        | 94.6          | 0.181 |
Table S4. Hydrodynamic diameters of crosslinked polymersomes at pH 7.2 in the presence of MEH studied as a function of time by DLS ($C_{\text{Psome}} = 0.1 \text{ mg BCP/mL, } C_{\text{MEH}} = 1.66 \text{ mM, MilliQ and 5% DMSO}$).

| Time (min) | Diameter (nm) | PDI   |
|-----------|---------------|-------|
| 0         | 108.1         | 0.149 |
| 8 min     | 110.3         | 0.149 |
| 16 min    | 109.7         | 0.165 |
| 24 min    | 110.4         | 0.153 |
| 32 min    | 110.9         | 0.157 |
| 60 min    | 110.3         | 0.160 |
| 120 min   | 110.0         | 0.159 |
| 180 min   | 110.5         | 0.155 |

Table S5. Swelling-shrinking of crosslinked polymersomes in the presence of MEH; $D_h$ determined by DLS ($C_{\text{Psome}} = 0.1 \text{ mg BCP/mL, } C_{\text{MEH}} = 1.66 \text{ mM, MilliQ and 5% DMSO}$). The pH was adjusted manually.

| Cycle/pH               | Diameter (nm) | PDI   |
|------------------------|---------------|-------|
| 0 (pH 7.2)             | 108.1         | 0.149 |
| 0.5 cycle (pH 5.5)     | 135.4         | 0.124 |
| 1 cycle (pH 7.2)       | 164.5         | 0.151 |
| 1.5 cycles (pH 5.5)    | 168.9         | 0.160 |
| 2 cycles (pH 7.2)      | 166.5         | 0.146 |
| 2.5 cycles (pH 5.5)    | 175.8         | 0.158 |
| 3 cycles (pH 7.2)      | 181.6         | 0.167 |

Table S6. Hydrodynamic diameters of crosslinked polymersomes in the presence of MEH studied by DLS after several cycles ($C_{\text{Psome}} = 0.1 \text{ mg BCP/mL, } C_{\text{MEH}} = 1.66 \text{ mM, MilliQ with 5% DMSO}$). Each cycle = 3 min irradiation followed by 5 min thermal relaxation (in the dark).

| Time (h) | Diameter (nm) | PDI   |
|----------|---------------|-------|
| 0 (pH 7.2) | 108.1     | 0.149 |
| 1 cycle  | 108.5         | 0.148 |
| 2 cycles | 113.5         | 0.142 |
| 3 cycles | 113.2         | 0.147 |
**Table S7.** Determination of the pH values [initial pH value (high), after irradiation (low), and after relaxation (high)] for bienzymatic reaction of GOx and HRP in Figure 6 and hydrodynamic diameter (D<sub>h</sub>) of polymersomes after each experiment series. Conditions: C<sub>Psome-GOx</sub> = 0.1 mg BCP/mL + 0.02 mg GOx/mL, C<sub>MEH</sub> = 1.66 mM in MilliQ water with 5% DMSO.

| Sample | pH<sup>a</sup> | D<sub>h</sub><sup>b</sup> (nm) |
|--------|----------------|-------------------------|
|        | Cycle 1        | Cycle 2                 | Cycle 3                  |
|        | start          | after h<sup>v</sup>      | after relax.              | after h<sup>v</sup> | after relax. | after h<sup>v</sup> |
| 1 cycle| 7.12           | 5.54                    | -                        | -                    | -            | -                   |
| 2 cycles| 7.09           | 5.46                    | 7.07                     | 5.57                 | -            | -                   |
| 3 cycles| 7.09           | 5.45                    | 7.08                     | 5.51                 | 7.07         | 5.72                |

<sup>a</sup> relax. = thermal relaxation in dark; <sup>b</sup> D<sub>h</sub> = hydrodynamic diameter; for non-irradiated Psome, D<sub>h</sub> = 107 nm (PDI = 0.260); PDI for irradiated samples in the range 0.156–0.174.
Figure S1. $^1$H NMR spectrum of poly(ethylene glycol)$_{45}$-block-poly((diethylamino)ethyl-methacrylate-stat-3,4-dimethylmaleimidobutyl methacrylate) (PEG$_{45}$-b-P(DEAMA-s-DMIBM)$_{100}$) block copolymer.

Figure S2. A) Hydrodynamic diameter of the crosslinked polymersomes (Empty-Psome) ($C_{\text{Psomes}} = 1$ mg BCP/mL, crosslinking time = 5 min) at pH 5 and pH 8 determined by DLS. B) Cryo-TEM image of Psomes ($C_{\text{Psomes}} = 0.5$ mg BCP/mL, crosslinking time = 5 min in the presence of 1 mM of MEH) at pH 8. Diameter $92.8 \pm 19$ nm; membrane thickness $14.5 \pm 2.3$ nm. The average of polymersome diameter was calculated by analyzing 50 particles. The average of membrane thickness was calculated by analyzing 20 particles.
Figure S3. Diameter and membrane thickness of the empty, crosslinked Psome in the presence of MEH, (i) non-irradiated and after (ii) 3 min of irradiation (no relaxation) and (iii) 3 min irradiation followed by 5 min relaxation (C_{Psomes} = 0.1 mg BCP/mL, crosslinking time = 5 min, 1.66 mM MEH) studied by cryo-TEM. The average Psome diameter was calculated by analyzing 150–250 particles. The average of membrane thickness was calculated by analyzing 30 particles.

| Condition                                      | Diameter (nm) | Thickness (nm) |
|------------------------------------------------|---------------|----------------|
| Non irradiated, no relaxation (Empty-Psome)   | 91.7 ± 19.0   | 27.7 ± 8.5     |
| Irradiated, no relaxation (Empty-Psome)       | 105.6 ± 27.0  | 26.7 ± 6.8     |
| Irradiated, after 5 min relaxation (Empty-Psome) | 89.7 ± 32.7   | 27.9 ± 5.4     |

Figure S4. Time-resolved UV-Vis absorption spectra accompanying A) irradiation of MEH (λ_{ex} = 436 nm), and B) relaxation to the initial state. C_{MEH} = 60 µM (MilliQ water with 5% DMSO).
Figure S5. UV-Vis absorption study on the cyclic irradiation-relaxation of A) MEH/SP (λ = 423 nm) (C_{MEH} = 60 μM, MilliQ water with 1% DMSO). B) MEH/SP in the presence of polymersomes (C_{MEH} = 60 μM, MilliQ water with 1% DMSO, C_{Psomes} = 0.004 mg BCP/mL, λ_{max} = 423 nm).

Figure S6. Study of the interactions between MEH and Psomes by following the release of MEH from Psomes using UV-Vis absorption (λ = 423 nm) over 24 h under different conditions.
Figure S7. $^1$H NMR spectrum of protonated merocyanine (MEH) used in all experiments in this study.
Figure S8. Studying the stability of MEH/SP. Light-triggered conversion between MEH and SP as a reversible pH switch ($C_{MEH} = 1 \text{ mM in MilliQ water with 5 }\% \text{ DMSO}$). A) Freshly prepared sample, B) after 24 h and C) after 48 h under stirring in the dark. Irradiation time = 3 min; relaxation time (in the dark) = 5 min.
Figure S9. Fluorescence spectra of the reagents involved in the enzymatic assay ($\lambda_{\text{exc}} = 534 \text{ nm}$, $\lambda_{\text{obs}} = 583 \text{ nm}$). All the reagents were dissolved in a 10 mM NaCl solution (0.01 mg/mL Resorufin, 0.2 mg/mL glucose, 0.2 mg/mL GOx, 0.2 mg/mL HRP, 0.1 M H$_2$O$_2$, 1 mg/mL, and 0.1 mg BCP/mL Psome-GOx).

Figure S10. pH dependence study of Resorufin by fluorescence spectroscopy ($\lambda_{\text{exc}} = 534 \text{ nm}$, $\lambda_{\text{obs}} = 583 \text{ nm}$). A) 0.01 mg/mL Resorufin solutions were prepared at pH 8, 7.5, 7, 6.5, 6, and 5.5 and were incubated for 20 min before the spectra were recorded ($\lambda_{\text{exc}} = 534 \text{ nm}$, $\lambda_{\text{obs}} = 583 \text{ nm}$). B) 0.01 mg/mL Resorufin solutions were prepared at pH 8, 7.5, 7, 6.5, 6, and 5.5 and were incubated for 20 min. Then, the pH was shifted to pH 7.4 for all samples and the spectra were recorded (0.001 mg/mL Resorufin; $\lambda_{\text{exc}} = 534 \text{ nm}$, $\lambda_{\text{obs}} = 583 \text{ nm}$).
Figure S11. A) Changes in the UV-Vis absorption spectra accompanying acid-base titration of MEH (C$_{\text{MEH}}$ = 60 µM in MilliQ with 1% DMSO). B) Fluorescence spectra of MEH at different pH values (C$_{\text{MEH}}$ = 1.66 mM in 1 mM PBS with 5% DMSO). C) Fluorescence spectra of Resorufin in the presence of MEH at different pH values (C$_{\text{MEH}}$ = 1.66 mM + 0.001 mg/mL Resorufin in 1 mM PBS with 5% DMSO). D) Fluorescence spectra of 0.001 mg/mL of Resorufin or Amplex Red at pH 7.5 and a mixture of both compounds with 1.66 mM MEH at pH 7.5. E) Fluorescence spectra of 0.001 mg/mL Resorufin and 0.001 mg/mL of Amplex Red (higher slit was used), both at pH 7.5 with 1.66 mM MEH.
**Figure S12.** Influence of MEH (non-irradiated) on pH-dependent swelling of Psome-enzyme and their pH* value compared to pH* of empty Psome with and without MEH measured by DLS.

**Figure S13.** Volume-dependent distribution of hydrodynamic diameters of the free enzymes (C_{enzyme} = 1 mg/mL in 1 mM PBS, following filtration through a 0.1 µm filter) studied by DLS.
**Figure S14.** Effect of irradiation on the activity of free GOx. Enzymatic activity of (A) free GOx and (B) free GOx in the presence of MEH before irradiation and after 3 cycles of irradiation/relaxation. In all cases, two samples were prepared: (i) the first sample was irradiated with a Hg lamp for 3 cycles (each cycle: 3 min irradiation followed by 5 min relaxation), (ii) the second sample was not irradiated. Then, the pH of the samples was adjusted to 5.5 and the first enzymatic reaction was carried out. After 9 min, the pH was adjusted to 7.5 and the second enzymatic reaction was carried out. The fluorescence spectra of Resorufin were recorded after 10 min of each enzyme assay ($\lambda_{\text{exc}} = 534$ nm, $\lambda_{\text{obs}} = 585$ nm). Conditions: $C_{\text{GOx}} = 0.02$ mg/mL, $C_{\text{MEH}} = 1.66$ mM in MilliQ water with 5 % DMSO.

**Figure S15.** Effect of irradiation on Amplex Red and the activity of free Myo. Enzymatic activity of (A) free Myo and (B) free Myo in the presence of Amplex Red and H$_2$O$_2$ and (C) free Myo in the presence of MEH after 3 cycles of irradiation/relaxation. In all cases, two samples were prepared: (i) the first sample was irradiated with a Hg lamp for 3 cycles (each cycle: 3 min irradiation followed by 5 min relaxation), (ii) the second sample was not irradiated. Then, the pH of the samples was adjusted to 5.5 and the first enzymatic reaction was carried out. After 9 min, the pH of the solutions was adjusted to 7.5. The fluorescence spectra of Resorufin were recorded after 10 min of each enzyme assay ($\lambda_{\text{exc}} = 534$ nm, $\lambda_{\text{obs}} = 585$ nm). Conditions: $C_{\text{Myo}} = 0.02$ mg/mL, $C_{\text{MEH}} = 1.66$ mM in MilliQ water with 5 % DMSO. It is important to note that in B), the enzymatic reaction is running during irradiation, while in A) and C), the reactions are initiated after the irradiation/relaxation cycles.
Figure S16. Optimizing the conditions of the enzymatic assay of free glucose oxidase (GOx) for subsequent light-driven proton transfer experiments. Enzymatic assay of free GOx in the absence or presence of MEH using two different approaches, with pH adjusted manually studied by fluorescence spectroscopy. Conditions: $C_{\text{freeGOx}} = 0.02 \, \text{mg/mL}$, $C_{\text{MEH}} = 1.66 \, \text{mM}$ in MilliQ water with 5 % DMSO ($\lambda_{\text{exc}} = 534 \, \text{nm}$, $\lambda_{\text{obs}} = 585 \, \text{nm}$).
**Figure S17.** Optimizing the conditions of the enzymatic assay of free myoglobin (Myo) for light-driven proton transfer. Enzymatic assay of free Myo in the absence or presence of MEH using two different approaches, with pH adjusted manually, studied by fluorescence spectroscopy. Conditions: $C_{\text{free Myo}} = 0.02 \, \text{mg/mL}$, $C_{\text{MEH}} = 1.66 \, \text{mM}$ in MilliQ water with 5% DMSO ($\lambda_{\text{exc}} = 534 \, \text{nm}, \lambda_{\text{obs}} = 585 \, \text{nm}$).

**Figure S18.** Studying the release of the enzyme (GOx) from GOx-containing polymersomes (Psome-GOx). Psome-GOx activity in the presence of MEH at pH 7.5 and pH 5.5 studied by following the amount of fluorescent product Resorufin (0.1 mg BCP/mL + 1.66 mM MEH; $\lambda_{\text{exc}} = 534 \, \text{nm}, \lambda_{\text{obs}} = 585 \, \text{nm}$). Each experiment was carried out at least in triplicate.
Figure S19. Studying the release of the enzyme (Myo) from Myo-containing polymersomes (Psome-Myo). Psome-Myo activity in the presence of MEH at pH 7.5 and pH 5.5 studied by following the amount of fluorescent product Resorufin (0.1 mg BCP/mL + 1.66 mM MEH; $\lambda_{\text{exc}} = 534$ nm, $\lambda_{\text{obs}} = 585$ nm). Each experiment was carried out at least in triplicate.

Figure S20. Following the enzymatic activity of A) GOx-containing nanoreactors and B) Myo-containing nanoreactors in the presence of light-triggered MEH by monitoring the production of fluorescent product Resorufin after 1-3 cycles of irradiation/relaxation of MEH, compared to a non-irradiated sample and an experiment with pH adjusted manually ($\lambda_{\text{exc}} = 534$ nm, $\lambda_{\text{obs}} = 585$ nm). Shown here are the initial studies on both Psome-GOx and Psome-Myo aimed at optimizing the process. The optimized processes are shown in Figure 6 and Figures S21–S24.
**Figure S21.** Following the production of Resorufin resulting from the enzymatic activity of GOx-containing nanoreactors with in the presence of HRP and light-triggered MEH. Fluorescence intensity of Resorufin is shown after 1-3 cycles of irradiation/relaxation of MEH, compared to a non-irradiated sample and an experiment with pH adjusted manually ($\lambda_{\text{exc}} = 534 \text{ nm}$, $\lambda_{\text{obs}} = 585 \text{ nm}$). Data validated after 10 min of assay run, when the polymersome membrane was collapsed at pH 7.5. Each experiment was carried out at least in triplicate.

**Figure S22.** Following the production of Resorufin resulting from the enzymatic activity of Myo-containing nanoreactors in the presence of light-triggered MEH. Fluorescence intensity of Resorufin is shown after 1-3 cycles of irradiation/relaxation of MEH, compared to a non-irradiated sample and an experiment with pH adjusted manually ($\lambda_{\text{exc}} = 534 \text{ nm}$, $\lambda_{\text{obs}} = 585 \text{ nm}$). Data validated after 10 min of assay run, when the polymersome membrane was collapsed at pH 7.5. Each experiment was carried out at least in triplicate.
**Figure S23.** Following the production of Resorufin resulting from the enzymatic activity of GOx-containing nanoreactors in the presence of HRP and light-triggered MEH. Fluorescence intensity of Resorufin is shown after 1-3 cycles of irradiation/relaxation of MEH, compared to a non-irradiated sample and an experiment with pH adjusted manually ($\lambda_{\text{exc}} = 534$ nm, $\lambda_{\text{obs}} = 585$ nm). Each experiment was carried out at least in triplicate.

**Figure S24.** Following the production of Resorufin resulting from the enzymatic activity of Myo-containing nanoreactors in the presence of light-triggered MEH. Fluorescence intensity of Resorufin is shown after 1-3 cycles of irradiation/relaxation of MEH, compared to a non-irradiated and an experiment with pH adjusted manually ($\lambda_{\text{exc}} = 534$ nm, $\lambda_{\text{obs}} = 585$ nm). Each experiment was carried out at least in triplicate.
Figure S25. AF4 fractograms with light scattering detector signals and A) molar masses and B) radii of gyration for different elution times for empty Psome (black), GOx-Psome (unpurified, blue), GOx-Psome (purified by HFF, red), Myo-Psome (unpurified, green), and Myo-Psome (purified by HFF, magenta).

Figure S26. Stability of Myo in the presence of excess of H$_2$O$_2$. Following the production of Resorufin as a result of enzyme activity of A) free Myo (0.1 mg/mL in 10 mM NaCl) and B) free Myo in the presence of MEH (0.1 mg/mL of Myo and 1.66 mM of MEH in 10 mM NaCl with 5 % DMSO) in the presence of H$_2$O$_2$ ($\lambda_{exc} = 534$ nm, $\lambda_{obs} = 585$ nm).
Nanoreactor Psome-GOx
Mechanism through Amplex Red conversion outside in OFF state

1. Glucose diffusion to the lumen in "ON state"
2. Generation of H$_2$O$_2$ + H$_2$O$_2$ diffusion to outside
3. Addition of HRP and Amplex Red outside in "OFF state"
4. Production of Resorufin for 10, 20, 30 and 60 min in "OFF state" by H$_2$O$_2$ conversion outside

Nanoreactor Psome-Myo
Mechanism through Amplex Red conversion inside Psome lumen in OFF state

1. Amplex Red + H$_2$O$_2$ diffusion to the lumen in "ON state"
2. Production of Resorufin inside
3. Production of additional Resorufin in the lumen for 30, 60, 90 and 120 min in "OFF state"

Figure S27. Protocols of experiments involving light-triggered adaptive enzymatic nanoreactors. **Psome-GOx:** HRP and Amplex Red are added to the enzyme assay only once cycles of MEH/SP irradiation/relaxation are completed. **Psome-Myo:** Amplex Red and H$_2$O$_2$ are added to enzyme assay before cycles of MEH/SP irradiation/relaxation are started.

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