Supporting Information

Feedback-Induced Temporal Control of "Breathing" Polymersomes to Create Self-Adaptive Nanoreactors

Hailong Che, Shoupeng Cao and Jan C. M. van Hest*

Bio-organic Chemistry, Eindhoven University of Technology, P.O. Box 513 (STO 3.41), 5600MB Eindhoven, The Netherlands

1. Materials

Poly(ethylene glycol) methyl ether (mPEG, M_n 2 kg/mol， Sigma, 99%), α-bromoisobutyryl bromide (Sigma, 99%), trimethylamine (Sigma, 98%), 2,2'-bipyridine (bpy, Sigma, 99%), 2-hydroxy-4-(methacryloyloxy) benzophenone (BMA, Alfa Aesar 98%), fluorescein *O*-methacrylate (FMA, Sigma, 98%), Rhodamine B isothiocyanate (Sigma, 98%), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, Sigma, 98%), urease from *Canavalia ensiformis* (Jack bean, Sigma, 50 U/mg) and horseradish peroxidase (HRP, Sigma) were used as received. *N,N*-diethylaminoethyl methacrylate (DEAEMA, Sigma, 99%) was passed through a basic alumina column to remove the inhibitor prior to use. Copper (I) bromide (CuBr, Sigma, 99.99%) was purified by stirring in acetic acid, followed by washing with acetone three times. All the solvents were used as received.

2. Instruments

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H NMR spectra were recorded on a Bruker (400MHz) spectrometer with $CDCl_3$ as the solvent and TMS as an internal standard.

size-exclusion chromatography (SEC). The molecular weights and dispersities of the polymers were characterized by a SEC system. The SEC was conducted using a Shimadzu Prominence-i SEC system with a PL gel 5 μm mixed D and mixed C column (Polymer Laboratories) with PS standard and

equipped with a Shimadzu RID-20A differential refractive index detector. THF was used as an eluent with a flow rate of 1 mL/min.

Dynamic Light Scattering (DLS). A Malvern Z90 Zetasizer equipped with a 633 nm He−Ne laser and an avalanche photodiode detector was used to characterize the hydrodynamic size of the particles. The scattering light at 90° angle was detected and used to analyze the size and distribution.

Transmission Electron Microscopy (TEM). TEM images were recorded by a FEI Tecnai 20 (type Sphera) at 200 kV. 10 μL of sample was dropped onto a carbon-coated copper grid. After removing the excess solution by blotting paper, the samples were dried at ambient conditions. For staining, a drop of phosphotungstic acid (2 %) solution was placed on the grid for 30 s.

Cryogenic transmission electron microscopy (cryo-TEM). Experiments were performed using a FEI Tecnai G2 Sphera (200 kV electron source) equipped with LaB6 filament utilizing a cryoholder or a FEI Titan (300 kV electron source) equipped with autoloader station. Samples for cryo-TEM were prepared by treating the grids (Lacey carbon coated, R2/2, Cu, 200 mesh, EM sciences) in a Cressington 208 carbon coater for 40 seconds. Then, 3 μl of the polymersome solution was pipetted on the grid and blotted in a Vitrobot MARK III at 100% humidity. The grid was blotted for 3 seconds (offset -3) and directly plunged and frozen in liquid ethane.

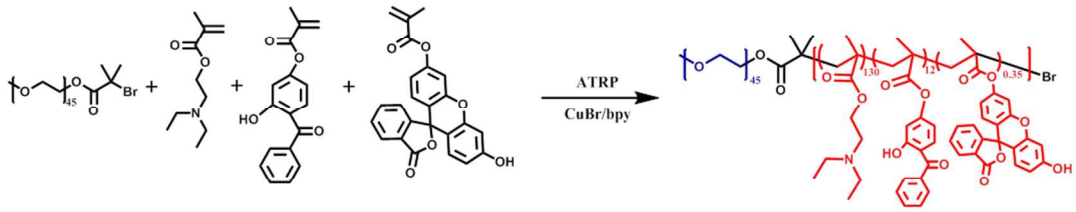
Asymmetric Flow Field-Flow Fractionation. The asymmetric flow field-flow fractionation-UV-Quels (AF4-UV-Quels) experiments were performed on a Wyatt Dualtec AF4 instrument connected to a Shimadzu LC-2030 Prominence-i system with Shimadzu LC-2030 autosampler. The AF4 was connected to a Wyatt DAWN HELEOS II light scattering detector (MALS) installed at different angles (12.9 º, 20.6 º, 29.6 º, 37.4 º, 44.8 º, 53.0 º, 61.1 º, 70.1 º, 80.1 º, 90.0 º, 99.9 º, 109.9 º, 120.1 º, 130.5 º, 149.1 º, and 157.8 º) using a laser operating at 664.5 nm and a Wyatt Optilab Rex refractive index detector. Detectors were normalized using Bovine Serum Albumin (BSA). The processing and analysis of the LS data and radius of gyration (R_g) calculations were performed on Astra 7 software (using the Berry model, which is recommended for particles of size > 50 nm). All AF4 fractionations were performed on an AF4 short channel with regenerated cellulose (RC) 10 KDa membrane (Millipore) and spacer of 350 μm.

Ultraviolet–visible Spectroscopy (UV/Vis). The UV absorbance spectra were recorded on a Jasco V-650 UV/Vis spectrometer at 293 K.

Fluorescence Spectroscopy. The fluorescence spectra were acquired using a PerkinElmer LS55 fluorescence spectrometer at 293 K, with 2.5 nm slit width. The excitation wavelength was set as 515 nm for all the samples.

pH Measurement. The pH change of the solution was recorded using a Mettler Toledo™ FiveEasy Plus™ FEP20 pH Meter.

3. Synthetic Procedures



Scheme S1. Synthetic route for the block polymer mPEG -*b*-P(DEAEMA$_{130}$-*co*-BMA$_{12}$-*co*-FMA$_{0.35}$) via ATRP.

Synthesis of mPEG ATRP macro-initiator

mPEG (5.00 g, 2.5 mmol) and triethylamine (TEA, 1.04 g, 7.50 mmol) were dissolved in 50 mL of THF at 0 ^{o}C, α-bromoisobutyryl bromide (616 μL, 5.00 mmol) in 10 mL of THF was slowly added dropwise for about 1 h. After addition, the mixture reacted for 36 h at room temperature. After evaporating off the THF, 15 mL toluene was added and the water-soluble triethylamine hydrochloride was removed. The filtrate was concentrated and then precipitated twice into 50 mL of ice cold diethyl ether. Finally, the product was collected and dried in a vacuum oven at 50 ^{o}C for 24 h, yielding 4.7 g white solid (yield: 94%).). ^{1}H NMR ($CDCl_3$, 400 MHz) (Figure S1): δ_H (ppm) =4.31 (s, -CH_2OOC-), 3.64 (s, -CH_2CH_2O-), 3.20 (s, -OCH_3), 1.95 (s, -OOC$(CH_3)_2$Br). SEC: M_n=2.12 kDa, M_w/ M_n=1.06.

Synthesis of block polymer mPEG45-b-P(DEAEMA$_{130}$-co-BMA$_{12}$-co-FMA$_{0.35}$) [1]

The above described macro-initiator mPEG-Br (0.426 g, 0.2 mmol), DEAEMA (5.55 g, 30 mmol), BMA (0.846 g, 3 mmol), FMA (0.04 g, 0.1 mmol), CuBr (28 mg, 0.2 mmol), bpy (36.3mg, 0.25 mmol), and 2-butanone (2 mL) were added to a round bottom flask, folflowed by degassing with nitrogen for 30 min. The reaction mixture was heated to 50 ^{o}C for 24 h with magnetic stirring. After the reaction, the resulting solution was immersed into a liquid nitrogen bath in order to stop the radical polymerization. Then the solution was diluted in 50 mL of THF and passed through a neutral alumina column twice to remove the copper catalyst. The filtrate was concentrated and then dialyzed (MWCO 3.5 kDa) against dioxane. The product was obtained by freeze drying, yielding 4.7 g product (yield: 92%). With ^{1}H NMR (Figure S2) the polymerization degree (DP) of DEAEMA and BMA was determined, which is 130 and 12, respectively. The DP of the fluorescein monomer in the block polymer was determined to be around 0.35

with UV−vis spectroscopy. The molecular weight and molecular weight distribution were determined by SEC using THF as the eluent, revealing a M_n of 29.9 kDa and M_w/ M_n of 1.08.

4. Preparation of the Rhodamine-B-labeled urease (RhB-urease)[2]

To calculate the encapsulation of urease in polymersomes, urease was labeled with Rhodamine B (RhB). In brief, RhB isothiocyanate was first dissolved in DMSO to get a 10 mg/mL stock solution. Then, 50 μL of the DMSO solution of RhB isothiocyanate was added gradually into 5 mL urease solution (20 mg enzyme/mL, pH=8, sodium carbonate, 10 mM). The reactions were performed overnight at 4°C. Labeled urease was then dialyzed against phosphate buffer (5 mM, pH = 7.4) for two days, followed by concentration *via* centrifugal filtration (MWCO = 10 kDa) and storage at 4°C for further use. RhB numbers conjugated to urease were determined by the extinction coefficients of 108,000 $M^{-1}cm^{-1}$ at 550 nm (RhB) and 20800 $M^{-1}cm^{-1}$ at 280 nm (urease). The number of conjugated RhB per urease was estimated to be 1.9.

5. Urease activity assays

Urease activity assays were performed by measuring the pH change within 10 minutes upon addition of urea. The initial pH of the urease solution (995μL) was adjusted to 4.0 by adding 1 M HCl. 5 μL Urea was added and the pH change in time was monitored with a pH meter. The relative activity of urease and RhB-urease was calculated by comparing the different slopes. Experimental conditions: 50 U/mL urease, 6 mM urea.

6. Formation of polymersome nanoreactors

1 mL enzyme solution (5 mg urease /mL, 2 mg HRP /mL, pH=7.4, phosphate buffer, 5 mM) was added into a 15 mL vial equipped with a magnetic stirring bar. The vial was capped with a rubber septum followed by the addition of 1 mL THF solution of block polymer (5 mg/mL) via a syringe pump with a rate of 1 mL h^{-1}. The resulting cloudy suspension was transferred to a dialysis membrane (molecular weight cutoff: 12,000-14,000 Da, flat width 25 mm). The polymersomes were dialyzed against water (1000 mL) at 4 ^oC for at least 48 h. After dialysis, the polymersome and enzymes mixture was centrifuged for 5 min at 5000 rpm *via* spin filtration over 0.22 μm membranes, the filtrate was withdrawn and fresh medium was added. This centrifugation process was repeated until no obvious

absorption at 550 nm (RhB-urease) and 403 nm (HRP's characteristic Soret band) was observed from the filtrate, which indicates that free enzymes were removed from the polymersome solution.

The enzyme incorporation efficiency was measured by determining the UV absorption of RhB-urease at 550 nm and HRP at 403 nm, respectively.[3] After removing free enzymes, 50v% DMSO was added by to the polymersome solution to destroy the polymersome structure, releasing the encapsulated enzymes. UV−vis spectra of free RhB-urease, HRP and released enzymes were recorded (Figure S8). It is clear that the disassembled polymersome mixture presents three typical absorbances: 403 nm for HRP, 485 nm for fluorescein from block copolymer, and 550 nm for RhB-labelled urease. The amount of urease and HRP encapsulated in the polymersomes was calculated, which was 25% and 14%, respectively.

7. Photo cross-linking of polymersome nanoreactors

The prepared polymersome solution was placed in the UV chamberand irradiated (350 nm) for 10 minutes.

8. Activity assay of polymersome nanoreactors

The activity of HRP-loaded polymersomes was determined by means of activity assays for HRP using ABTS as substrate. The formation of the oxidized products was monitored by UV−vis spectroscopy. The activity assay was performed at room temperature in 1 cm silica glass cuvettes by mixing 0.8 mL solution of the HRP-encapsulated polymersomes and ABTS. The enzymatic reaction was started by the addition of 50 μL H_2O_2 solution. The final concentrations for HRP, ABTS and H_2O_2 were 20 U/mL, 10 μM and 5 mM, respectively. Absorbance at 416 nm was recorded every 60 s. The relative activity of the polymersome nanoreactors was calculated by comparing the different slopes of the absorption increase in the original linear phase.

9. Chemical fuel programmed pH switch

The concentrated enzyme-filled polymersomes were first dispersed in *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES) buffer (10 mM, pH 9.0) to get a 0.8 mL polymersome dispersion (2.5 mg/mL polymer, 30 U/mL urease), at which point urease was deactivated. 5 μL concentrated HCl (1M) and aqueous urea solutions were added to the mixture, and the reaction mixture was incubated at room temperature. The final urea concentration was set as 4, 6, 8 and 10 mM. The pH change was recorded every 30 s with a Mettler Toledo™ FiveEasy Plus™ FEP20 pH Meter.

When the pH value leveled off at approximately the same level, the polymersome solutions were refueled by fresh HCl and urea solution. To study the influence of urea concentration on the programmed pH switch, the urea concentration was varied under otherwise constant conditions.

10. Chemical fuel programmed polymersome nanoreactors

Experiments were performed in 1 cm silica glass cuvettes by mixing 0.8 mL solution of the HRP-encapsulated polymersomes (2.5 mg/mL polymer, 30 U/mL urease, 10 U/mL HRP) and 5 μL ABTS (20 mM). Then, 5 μL concentrated HCl (1M) and urea was added to the mixture. The HRP-based catalytic reaction was activated by the addition of 5 μL H_2O_2 (0.8 M). Absorbance at 416 nm was recorded every 60 s. For the effect of urea concentration (4, 6, 8 and 10 mM) on product conversion, other experimental conditions were kept constant. For the self-adaptive cycle experiment, the polymersome solution was refueled by the consecutive introduction of HCl (1M) and urea. In control experiments, the free HRP-based enzymatic reaction was performed under identical conditions.

Supplementary Table 1 General method for the AFFF elution of polymersomes.

The flow conditions applied for the elution of enzymes were the following: 0.7 mL min^{-1} detector flow, 1.50 mL min^{-1} focus flow and 0.20 mL min^{-1} injection flow.

Start time (min)	End time(min)	Mode	Cross flow start (mL min^{-1})	Cross flow end (mL min^{-1})
0	1	Elution	0.50	0.50
1	2	Focus	-	-
2	3	Focus + inject	-	-
3	4	Focus	-	-
4	14	Elution	0.50	0.50
14	17	Elution	0.50	0.00
17	33	Elution	0.00	0.00
33	44	Elution + inject	0.00	0.00
44	45	Elution	0.00	0.00

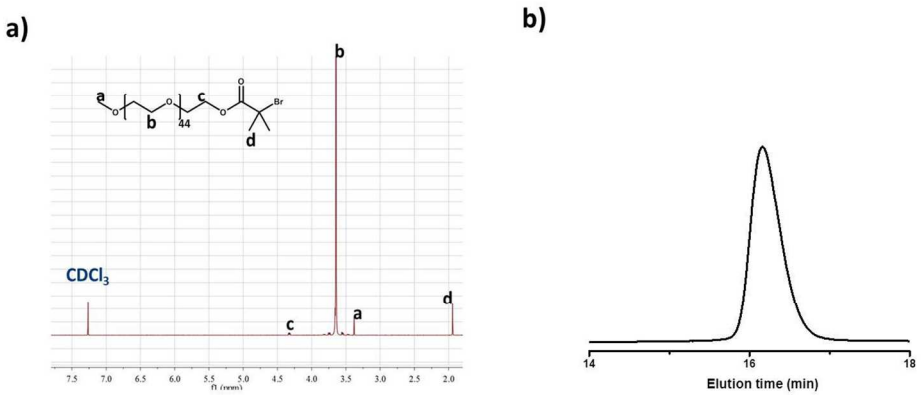


Figure S1. (a)^{1}H NMR spectrum and (b) SEC trace of $mPEG_{45}$-Br.

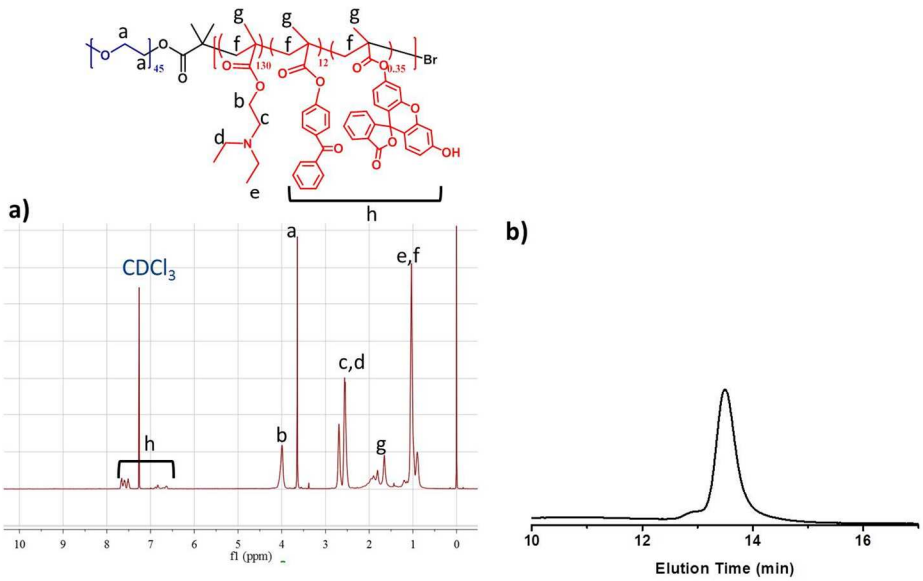


Figure S2. (a) ^{1}H NMR spectrum and (b) SEC trace of block polymer mPEG-*b*-P(DEAEMA$_{130}$-*co*-BMA$_{12}$- *co*-FMA$_{0.35}$).

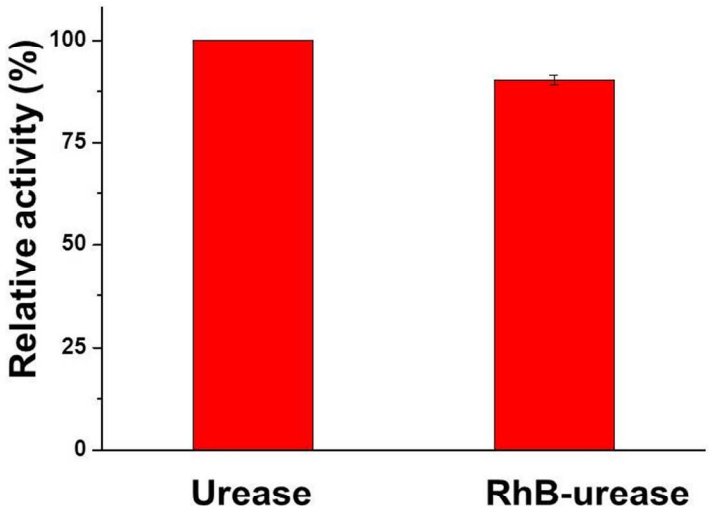


Figure S3. Relative activity of urease and RhB-urease. Experimental conditions in all cases: 50 U/mL urease, 6 mM urea. Error bars are based on standard error of mean.

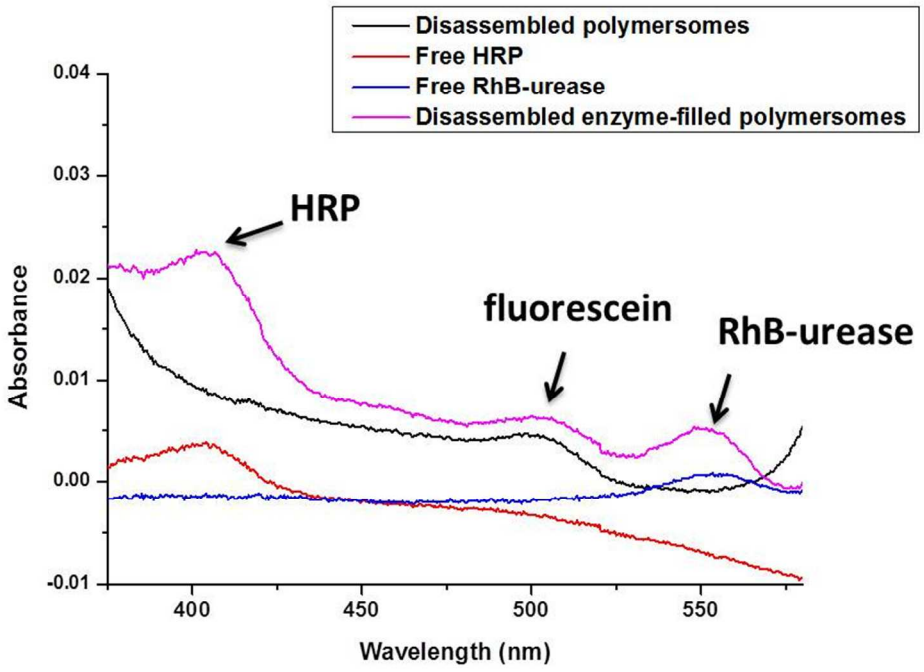


Figure S4. UV−vis spectra of disassembled polymersomes, free RhB-urease, HRP and disassembled enzyme-filled polymersomes.

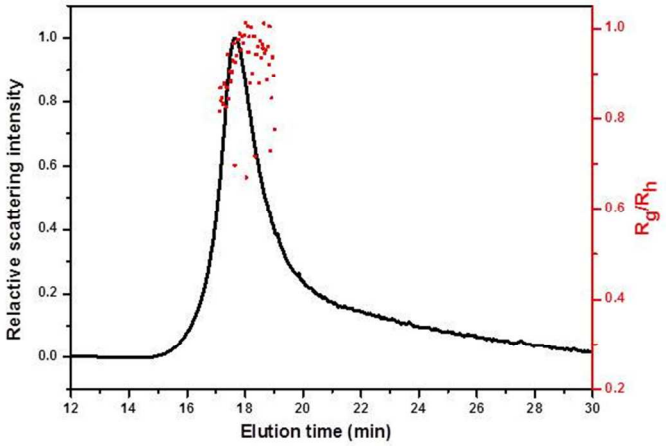


Figure S5. AFFF chromatogram of polymersomes (black line), and radii of gyration (R_g) divided by the hydrodynamic radii (R_h) over the entire size distribution peak of polymersomes (red dots). The R_g/R_h ratio value is around 0.9 which is close to 1.0, and indicates that the predominant shape is that of vesicles.

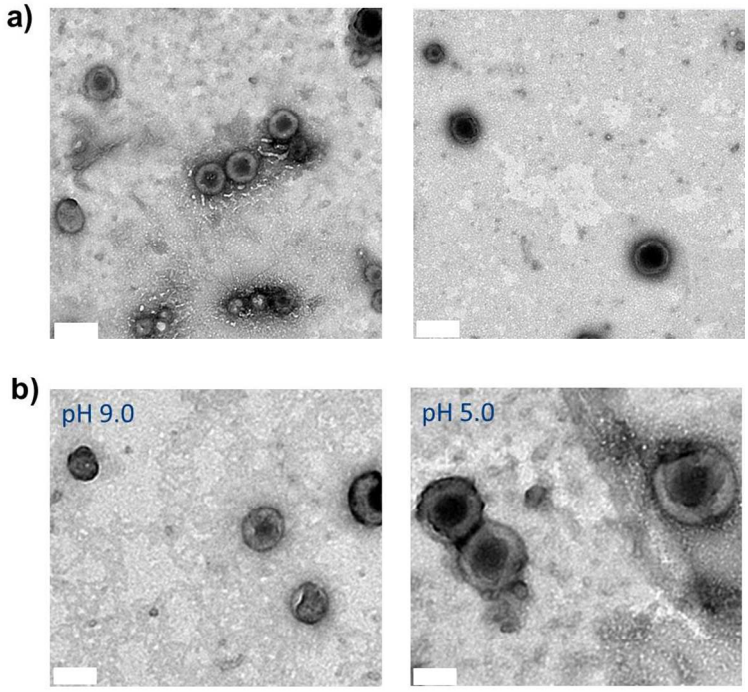


Figure S6. Dry-TEM images of polymersomes at (a) different grid areas (b) pH 9.0 (left) and pH 5.0 (right).The samples are stained with phosphotungstic acid (2 %). All scales bars are 100 nm.

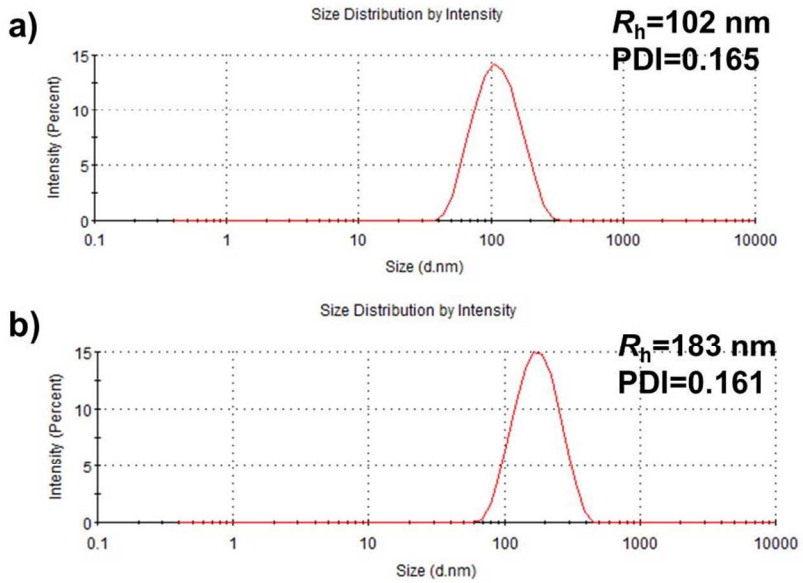


Figure S7. DLS analysis of the polymersomes at pH 9.0 (a) and 5.0 (b), respectively.

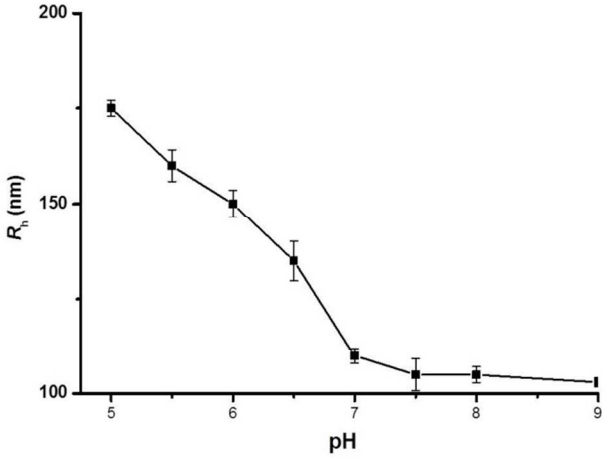


Figure S8. Correlation between the pH and R_h of the polymersomes in an aqueous solution. Error bars are based on standard error of mean

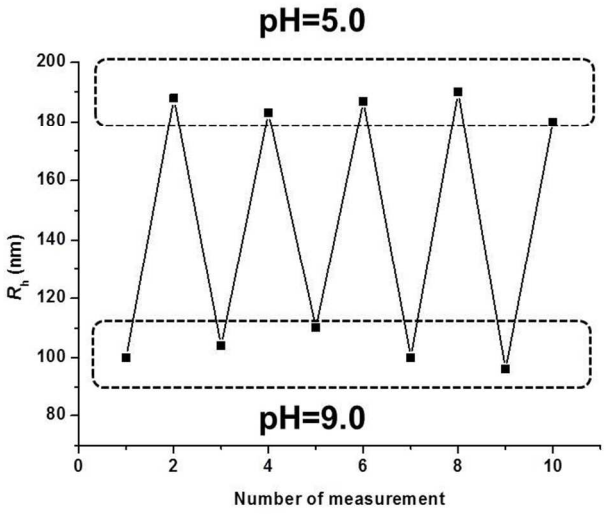


Figure S9. DLS data showing multiple-run reversibility of the polymersome diameter between pH 9.0 and pH 5.0.

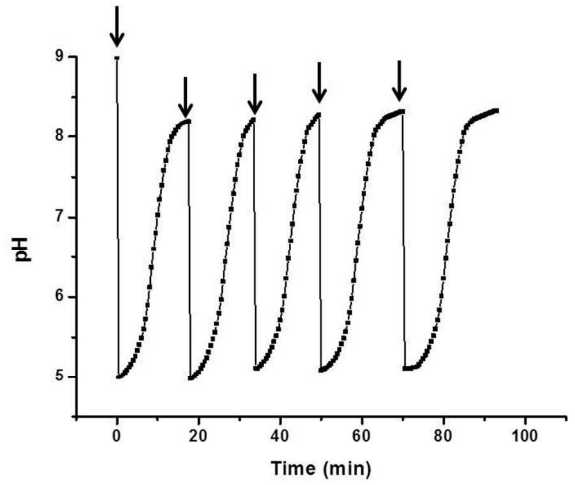


Figure S10. Reversible pH changes of the enzyme-filled polymersomes in time following repeated additions of chemical fuels. 5 μL concentrated HCl (1M) and urea aqueous solutions were added to 0.8 mL polymersome (2.5 mg/mL polymer, 30 U/mL urease).The final urea concentration was 6 mM.

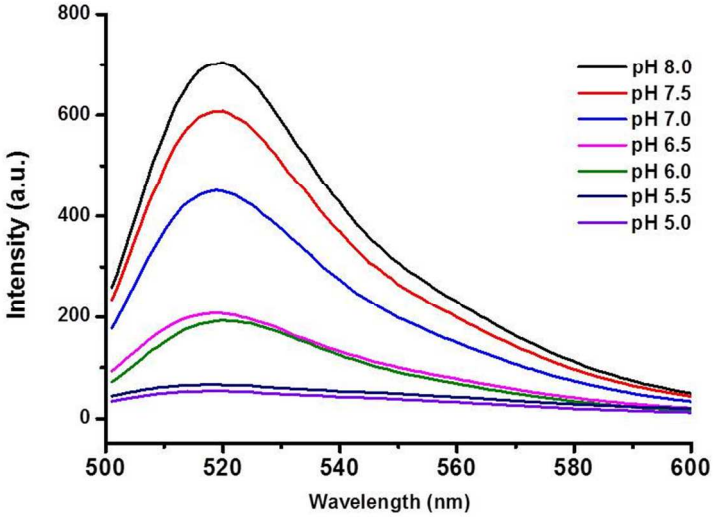


Figure S11.The fluorescence responses of the polymersomes to different pH.

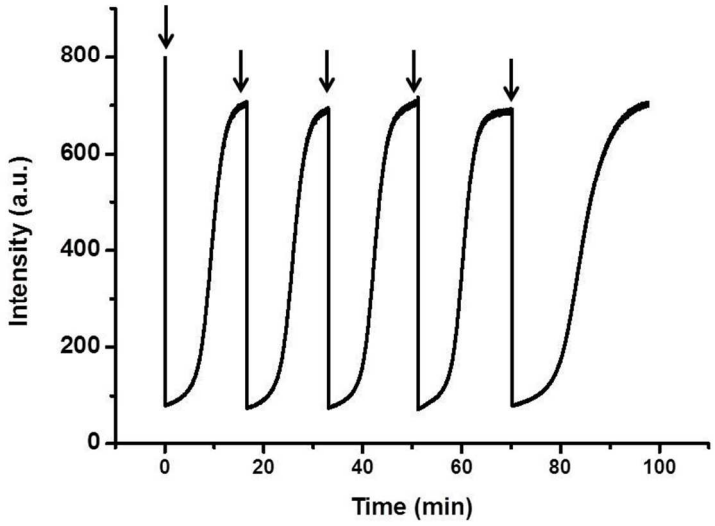


Figure S12. Reversible fluorescence changes of enzyme-filled polymersomes in time following repeated additions of chemical fuels. 5 μL concentrated HCl (1M) and urea aqueous solutions were added to 0.8 mL polymersome (2.5 mg/mL polymer, 30 U/mL urease).The final urea concentration was 6 mM.

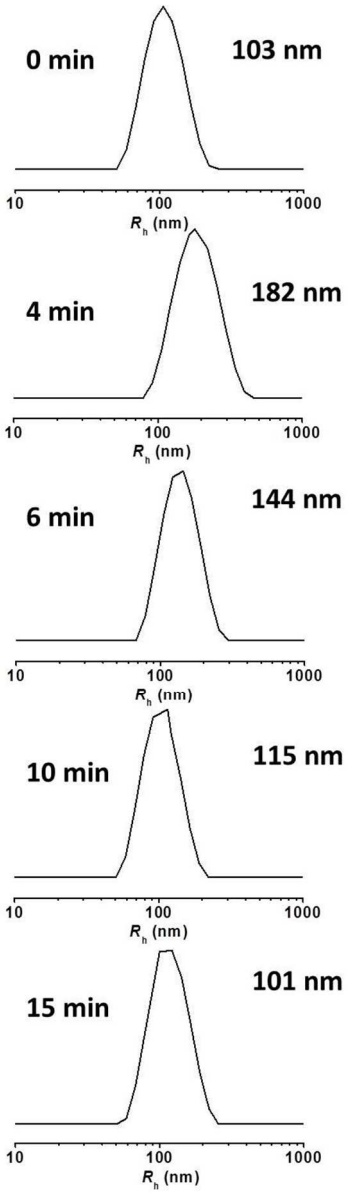


Figure S13. Size distribution changes of enzyme-filled polymersomes in time upon addition of chemical fuels. 5 μL concentrated HCl (1M) and urea aqueous solutions were added to 0.8 mL polymersome (2.5 mg/mL polymer, 30 U/mL urease).The final urea concentration was 6 mM.

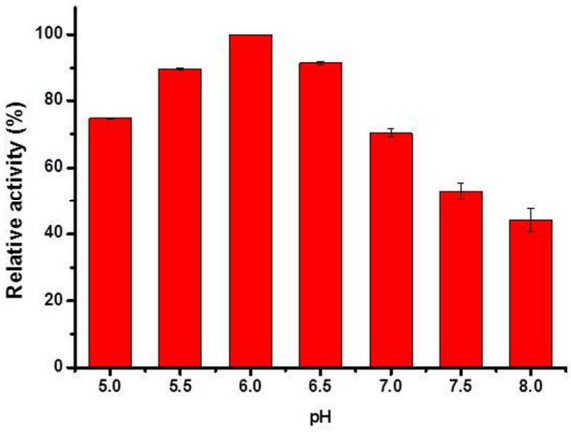


Figure S14.Relative enzymatic activity as a function of pH for free HRP at room temperature using ABTS as a substrate. Error bars are based on standard error of mean.

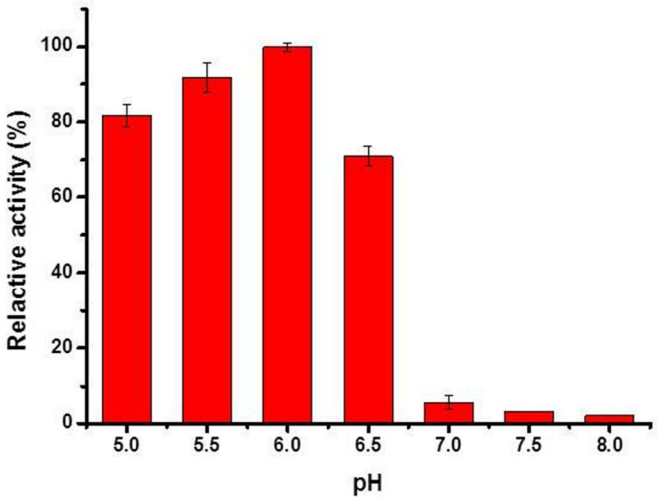


Figure S15. Relative enzymatic activity as a function of pH for HRP encapsulated in polymersome nanoreactors at room temperature using ABTS as a substrate.

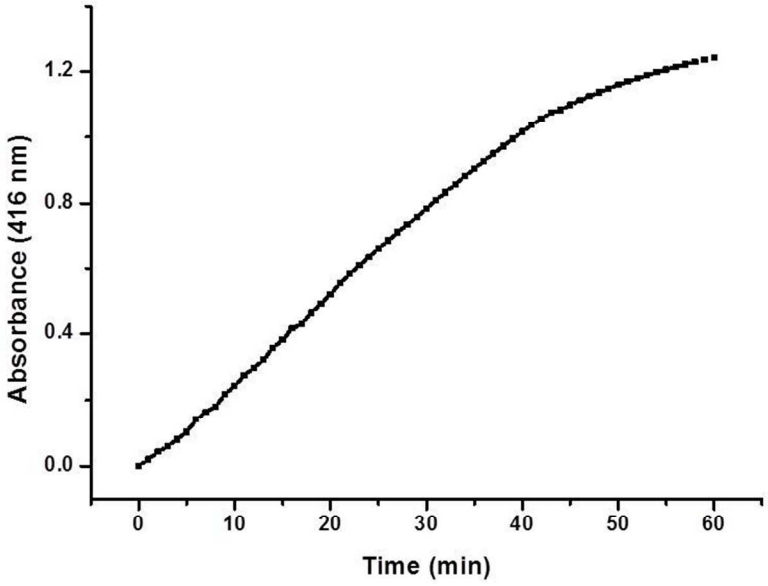


Figure S16. Free HRP activity assay with ABTS as substrate at pH 6.0 (HRP: 10 U/mL; ABTS: 8 mM; H_2O_2: 5 mM). As the free HRP demonstrates highest activity at pH 6.0, the activity assay was conducted at this pH value for the comparison with HRP encapsulated in polymersome nanoreactors.

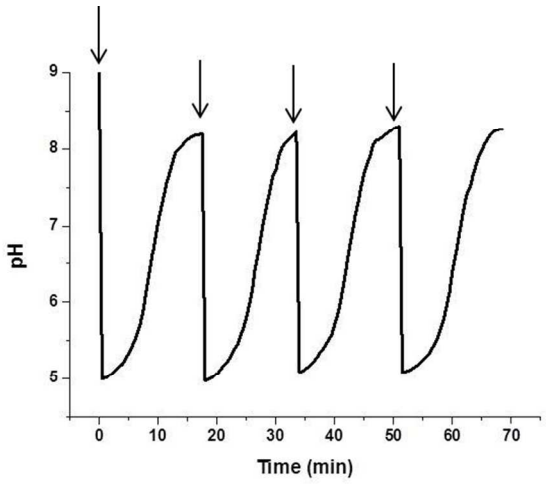


Figure S17. Reversible pH changes of the enzyme-filled polymersomes (2.5 mg/mL polymer, 30 U/mL urease) in the presence of ABTS. The final urea concentration was 6 mM. Experimental conditions: HRP: 10 U/mL; ABTS: 8 mM; H_2O_2: 5 mM.

References

(1) Yassin, M. A.; Appelhans, D.; Mendes, R. G.; Rümmeli, M. H.; Voit, B. *Chem. Eur. J.* **2012**, *18*, 12227.

(2) Liu, Y.; Du, J.; Yan, M.; Lau, M. Y.; Hu, J.; Han, H.; Yang, O. O.; Liang, S.; Wei, W.; Wang, H. *Nat. Nanotechnol.* **2013**, *8*, 187.

(3) Spulber, M.; Najer, A.; Winkelbach, K.; Glaied, O.; Waser, M.; Pieles, U.; Meier, W.; Bruns, N. *J. Am. Chem. Soc.* **2013**, *135*, 9204.