Supplementary information

Enzyme-Powered Nanomotors with Controlled Size for Biomedical Applications

Jiawei Sun, Motilal Mathesh, Wei Li, Daniela A. Wilson*

Institute for Molecules and Materials  Radboud University Heyendaalseweg 135, 6525 AJ, Nijmegen, the Netherlands

Heyendaalseweg 135, 6525 AJ, Nijmegen, the Netherlands E-mail: d.wilson@science.ru.nl
Contents
S1. Materials .................................................................................................................. 4
S2. Experimental Procedures: .......................................................................................... 4

S2.1. Synthesis of poly(ethylene glycol)-polystyrene block copolymer ......................... 4

S2.2. Measurement of catalase activity: .......................................................................... 5

S2.3. Oxygen measurements .......................................................................................... 5

S2.4. Preparation of stomatocyte nanomotors and encapsulation of enzymes and Nile Red inside the stomatocyte cavity via PEG addition method with Nile Red ..................... 6

S2.5. Penetration efficiency across vasculature model ...................................................... 6

S2.6. Cell viability assay ............................................................................................... 7

S2.7. Uptake efficiency assay by flow cytometry assay .................................................... 8

S3. Supplementary Figures and Tables ............................................................................. 8

Figure S1. Cryo-TEM of stomatocytes with different PS length. ....................................... 9

Figure S2. Reshaping the stomatocytes back into polymersomes via the solvent addition method. ............................................................................................................ 9

Figure S3. Calibration curves for SDS-PAGE and enzyme activity assay ......................... 10

Figure S4. Characteristics for particles in vitro experiments. ............................................ 11

Figure S5. Vasculature model. ....................................................................................... 12

Figure S6. The morphology of monolayer endothelial cells. The morphology was studied before and after conducting the EPR penetration experiment .................................... 13

Figure S7. Evaluation of TEER ..................................................................................... 14

Figure S9. CLSM images of membrane permeability assay ............................................ 15

Figure S10. CLSM images of membrane permeability assay ........................................... 16
S1. Materials

Unless and otherwise stated, all reagents and chemicals were purchased from commercial sources and used as received. Styrene (Sigma-Aldrich) was distilled to remove the inhibitor before polymerization. CuBr (Sigma-Aldrich) for ATRP was stirred with glacial acetic acid followed by washing with ethanol and diethyl ether and protected under Ar. Tetrahydrofuran (THF) for reaction was distilled under Argon from sodium/benzophenone. Ultra pure MilliQ water obtained from MilliQ QPOD purification system (18.2 MΩ) was used for self-assembly and quenching of polymersomes/stomatocytes. α-methoxy-ω-hydroxy poly(ethylene glycol) (M_n 2000 g/mol), L (+) ascorbic acid, magnesium sulfate, sodium bicarbonate, sodium chloride, ethylenediaminetetraacetic acid (EDTA), 1-phenyl-1-trimethylsiloxyethene, α-bromo isobutyryl bromide, chloroform-d (CDCl_3), tert-butyl α-bromo isobutyrate and N,N,N’,N’’,N’’-Pentamethyl diethylenetriamine (PMDETA) were purchased from Sigma-Aldrich. THF and anisole were obtained from Acros. MeOH, triethylamine and hydrogen peroxide (H_2O_2) was purchased from J.T. Baker. Diethyl ether (Carlo erba Reagents), 1,4-dioxane (Biosolve BV), dichloromethane (CH_2Cl_2), Nile Red (99%) and DAPI (4’,6-Diamidino-2-Phenylindole, Dilactate), Hoechst 33342 and F-actin staining from Thermo Fisher Scientific were also used.

S2. Experimental Procedures:

S2.1. Synthesis of poly(ethylene glycol)-polystyrene block copolymer: PEG-b-PS_n was synthesized according to our previous work. Briefly, Poly(ethylene glycol) methyl ether (5.00 g, 2.50 mmol) was dried by co-evaporation with toluene, and then dissolved in freshly distilled THF in a flamed-dried Schlenk flask. After adding triethylamine (1.04 mL, 7.50 mmol), the mixture was cooled to 0 °C, followed by the addition of α-bromo isobutyryl bromide (616 mL, 5.00 mmol) drop by drop. The solution was then stirred for 24 h while slowly warming to room temperature. The solution was concentrated after the white precipitate was filtered off. The
polymer was then precipitated in ice cold diethyl ether (3x) and characterized by 1H-NMR in CDCl$_3$. After synthesizing the initiator, CuBr (45 mg, 0.32 mmol) was added in the Schlenk tube and kept under vacuum for 15 min. After refilling with Ar, PMDETA (66 mL, 0.32 mmol) in anisole (0.5 mL) was added, followed by 15 min vigorously stirring. Styrene (5 mL, 43.6 mmol) in anisole (0.5 mL) was added via syringe. The solution was cooled down to 0 °C and PEG-initiator (215 mg, 0.1 mmol) was added. The Schlenk tube was transferred into an oil bath at 90 °C. 1H-NMR was used for monitoring the reaction process. Upon attainment of the required molecular weight, 1-phenyl-1- trimethylsiloxyethene (1.91 mL, 9.28 mmol) was added to quench the polymerization. The solution was diluted with CH$_2$Cl$_2$ and extracted with an aqueous EDTA solution (65 mM). The organic layer was collected and dried with MgSO$_4$ and concentrated. The polymer was obtained after precipitation in MeOH (3x), and dried under vacuum overnight and characterized by 1H-NMR in CDCl$_3$ and GPC. The amphiphilic polymer obtained, PEG$_{44}$-b-PS$_{170}$ had an number average molecular weight (M$_{n}$) of 19705 g/mol and a PDI of 1.04.

**S2.2. Measurement of catalase activity:**

For measuring the activity of encapsulated catalase, a standard curve was prepared from a 10 U mL$^{-1}$ catalase stock solution, 7 different concentrations were used in 25 μL assay volume. Catalase-containing samples were diluted to 1 x 10$^4$ in order to obtain concentrations in the standard curve concentrations. To start the reaction 25 μL of 40 μM H$_2$O$_2$ was pipetted to every well containing the standard and sample solutions. After 30 minutes of incubation at room temperature, 50 μL of a fresh mixed working solution containing HRP (0.4 U mL$^{-1}$) and Ampliflu™ Red (100 μM) was added to each well. After 5 minutes of incubation at room temperature, fluorescence intensity was measured using excitation at 550 nm and emission at 590 nm. A background correction was subtracted.
S2.3. Oxygen measurements

Oxygen measurements were performed with a clark-type oxygen sensor (Oxygraph Plus System, Hansatech Instruments). Before every set of measurements, the oxygen electrode was calibrated according to the working manual. The calibration and subsequent measurement were performed in a temperature controlled (21 °C) cylindrical chamber equipped with a magnetic stirrer (100 rpm). Temperature, sample volume and stirring speed were kept constant for all experiments. The samples were measured for 5 min. H₂O₂ with different concentrations (1 mL) was added to the system, the O₂ evolution rate was monitored when the particles was added to the chamber. Control experiments were performed by measuring O₂ level without H₂O₂.

S2.4. Preparation of stomatocyte nanomotors and encapsulation of enzymes and Nile Red inside the stomatocyte cavity via PEG addition method with Nile Red

PEG₄₄-b-PSₙ (10 mg) was dissolved in 1 mL organic solvent mixture of tetrahydrofuran (THF) and 1,4-dioxane at the ratio of 4:1 by volume with a magnetic stirring bar. After dissolving the solution for 30 min, 50 µL of Nile Red solution (dissolved in the same organic solvent) with a concentration of 1 mM was added before addition of 0.5 mL of Milli-Q water in the solution at a rate of 1 mL h⁻¹ with vigorous stirring (900 rpm). Ultra-small polymersomes were fabricated by passing 700 µL polymersome solution through a 220 nm syringe filter (screening devices) in solvent mixture of THF/Dioxane/water = 8/2/5 by volume.

1 mg catalase was dissolved in 50 µL water, and this 50 µL solution was added into the polymersome solution or ultra-small polymersome solution. After stirring for 2 min, 10 µL or 50 µL of PEG2000 solution (100 mg/mL) was added and the solutions were quenched as described. The organic solvent and non-encapsulated enzymes in the system was washed by centrifugation.

S2.5. Penetration efficiency across vasculature model
Pulmonary artery endothelial cells (PAEC) were seeded on transwell inserts at 50,000 cells/filter and incubated for 48 h, after a monolayer cell was formed, $8 \times 10^9$ particles were added and incubated for 2 h. Transepithelial/transendothelial electrical resistance (TEER) values ($\Omega/cm^2$) were measured before conducting the experiment to prove the formation of monolayered cells and after the experiment to study the influence of motors on the monolayered cells, via chopstick electrode recording with an EVOM2 apparatus (World Precision Instruments). Resistance values are reported after correcting it with the resistance from cell-free inserts measured alone. For each experiment, 3 replicates were performed. After incubation, the media was collected from each chamber (both bottom and upper side). Collected particles were centrifuged, resuspended in PBS, and transferred on a 96-well plate. The fluorescence intensity of Nile red was checked by plate reader.

The cell morphology was also studied before and after conducting the experiment, the cell nucleus was dyed with Hoechst 33342 for 10 min, and the cells morphology was observed by F-actin staining. After the staining solution was removed, cells were washed with PBS buffer for 3 times, the stained cells were imaged by SP8× confocal microscope.

S2.6. Cell viability assay

HeLa Cells were transferred to 96-well plates using a standard trypsin-based technique with a final concentration of $5 \times 10^4$ cells per mL. After 24 h incubation, ultra-small stomatocyte polymersomes (USSP) (a) and ultra-small stomatocyte nanomotors (USSN) were added with cell culture medium at different concentrations ($0, 0.02, 0.05, 0.1$ and $0.25$ mg mL$^{-1}$) and incubated for another 24 h. After washing 3 times with PBS buffer, 100 µL of 10 times diluted Cell Counting Kit solution was added to each well. After the cells were incubated for 2 h, plate reader was used to evaluate cell viability in the presence of different samples.

The cell viability assay were also conducted by CLSM. Briefly, 200 µL of Dulbecco’s modified Eagle’s medium (DMEM) buffer with $1 \times 10^5$ cells was added to an 8-well plate (ibidi GmbH) and incubated
overnight at 37 °C. The incubation buffer was then replaced by 200 µL DMEM buffer with small stomatocyte polymersomes (SSP), ultra-small stomatocyte polymersomes (USSP), small stomatocyte nanomotors (SSN) and ultra-small stomatocyte nanomotors (USSN) for 3.5 h (particle concentration 2×10^10 particles/mL) in the presence/absence of 2 mM H_2O_2. Cell nucleus were dyed with Hoechst for 10 minutes and propidium iodide was used for detecting the permeability of cell membrane. After the staining solution was removed, cells were washed with PBS buffer for 3 times and the stained cells were imaged by SP8× confocal microscope.

**S2.7. Uptake efficiency assay by flow cytometry assay**

The uptake efficacy of SSN and USSN with and without H_2O_2 towards Hela cells were measured by flow cytometry. Briefly, SSN and USSN (2×10^10 particles/mL) were incubated with Hela cells for 3.5 h. Non internalized stomatocytes were removed by washing cells with PBS for three times, cells were collected and washed for three times. The fluorescent signal was detected by flow cytometry.

**S3. Supplementary Figures and Tables**
Figure S1. Cryo-TEM of stomatocytes with different PS length. The thickness of the membrane was measured.

Figure S2. Reshaping the stomatocytes back into polymersomes via the solvent addition method. (a) TEM and (e) Cryo-TEM of stomatocytes with normal stomach obtained after the addition of 150 μL of organic solvent (THF/dioxane, 80:20 v/v); (b) TEM and (f) Cryo-TEM
of stomatocytes with smaller stomach obtained after the addition of 300 μL of organic solvent (THF/dioxane, 80:20 v/v); (c) TEM and (g) Cryo-TEM reshaped stomatocytes obtained after the addition of 450 μL of solvent (THF/dioxane, 80:20 v/v); (d) TEM and (h) Cryo-TEM of reshaped polymersomes after the addition of 600 μL of solvent (THF/dioxane, 80:20 v/v). scale bars a, b, c, d 200 nm; c, f, g, h 150 nm.

Figure S3. Calibration curves for SDS-PAGE and enzyme activity assay. (a) calibration curve of catalase from SDS-PAGE; (b) intensity profile of SDS-PAGE lanes; (c) calibration curve from Amplex red assay.
Figure S4. Characteristics for particles in vitro experiments. (a, c) Size distribution of SSN and USSN; (b, d) TEM image of SSN and USSN.

| Sample | Average Size | PDI       |
|--------|--------------|-----------|
| SSN    | 392.40 ± 7.81| 0.078 ± 0.022|
| USSN   | 150.46 ± 1.16| 0.047 ± 0.017|

Figure S5. Vasculature model. (a) Illustration of transwell inserts EPR model; (b) penetration ratio of different nanomotors with or without H₂O₂.
Figure S6. The morphology of monolayer endothelial cells. The morphology was studied before and after conducting the EPR penetration experiment. The cell nucleus was dyed with Hoechst 33342 and the cells were observed by F-actin staining.
Figure S7. Evaluation of TEER. (A) monolayer endothelial cells before incubation; (B) monolayer endothelial cells after incubating with SSN; (C) monolayer endothelial cells after incubating with USSN; (D) monolayer endothelial cells after incubating with SSN + H$_2$O$_2$; (E) monolayer endothelial cells after incubating with USSN + H$_2$O$_2$.

Figure S8. Cytotoxicity evaluation, USSP (a) and USSN (b) by measuring the cell viability of Hela cells through Cell Counting Kit 8 (CCK 8) assay.
Figure S9. CLSM images of membrane permeability assay. HeLa cells incubated with SSP, USSP, SSN and USSN for 3.5 h. Cell nucleus were dyed with Hoechst for 10 minutes and propidium iodide was used for detecting the permeability of cell membrane, scale bar represents 40 µm.
Figure S10. CLSM images of membrane permeability assay. HeLa cells incubated with SSP, USSP, SSN and USSN for 3.5 h. Cell nucleus were dyed with Hoechst for ten minutes and propidium iodide was used for detecting the permeability of cell membrane, scale bar represents 40 µm.
Figure S11. Cellular uptake of nanomotors measured by flow cytometry. HeLa cells incubated with small SSN with (b) or without H$_2$O$_2$ (a) and USSN with (d) or without H$_2$O$_2$ (c) for 3.5 h; (e) cell fluorescence; (f) overlay of all samples.

S4.3D Videos for cell uptake.
Video S1 . 3D structure of cell uptake for USSN with H\textsubscript{2}O\textsubscript{2}.
Video S2 . 3D structure of cell uptake for USSN without H\textsubscript{2}O\textsubscript{2}.

REFERENCES

1. Chirino, A. J.; Ary, M. L.; Marshall, S. A. Minimizing the Immunogenicity of Protein Therapeutics. Drug Discov. Today 2004, 9, 82-90.
2. Daniels, B. P.; Cruz-Orengo, L.; Pasieka, T. J.; Couraud, P.-O.; Romero, I. A.; Weksler, B.; Cooper, J. A.; Doering, T. L.; Klein, R. S. Immortalized Human Cerebral Microvascular Endothelial Cells Maintain the Properties of Primary Cells in an in Vitro Model of Immune Migration across the Blood Brain Barrier. J. Neurosci. Methods 2013, 212, 173-179.