Biodegradable Hybrid Stomatocyte Nanomotors for Drug Delivery

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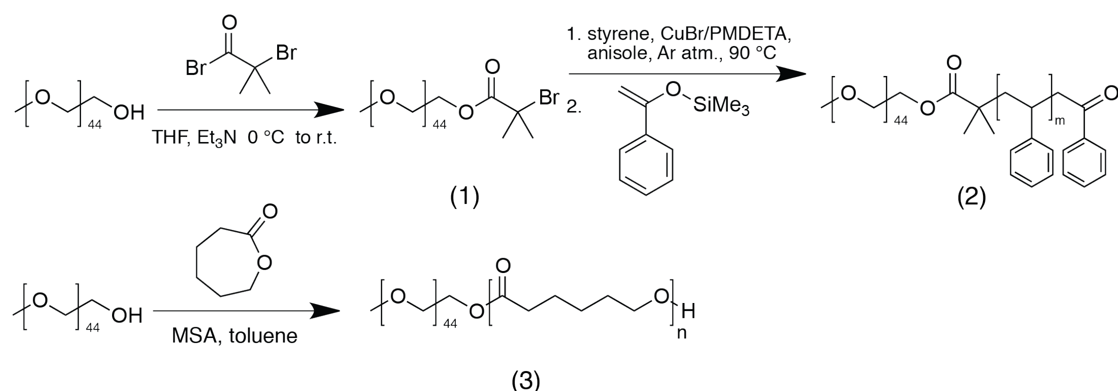
1. Materials

Unless stated otherwise, all reagents and chemicals were used without further purification. Styrene (Sigma-Aldrich) was distilled before polymerization to remove the inhibitor. *N*-isopropyl acrylamide from Sigma-Aldrich was purified by repeated recrystallization in a mixture of toluene/hexane (50:50, v/v). CuBr (Sigma-Aldrich) for ATRP was washed with acetic acid and followed by methanol (MeOH) for three times and protected under Ar. Tetrahydrofuran (THF) for reaction was distilled under Argon from sodium/benzophenone. Ultra pure MilliQ water obtained with MilliQ QPOD purification system (18.2 MΩ) was used for self-assembly and dialysis of polymersomes/stomatocytes. Spectra/Por® Dialysis Membrane MWCO: 12-14,000 g/mol was used for dialysis of polymersomes/stomatocytes. Polyvinyl pyrrolidone (PVP, Mn 10 kg/mol), poly(ethylene glycol) methyl ether (Mn 2 kg/mol), α-ω-amino-poly(ethylene glycol) (Mn 2 kg/mol), L (+) ascorbic acid, magnesium sulfate, sodium bicarbonate, potassium tetrachloroplatinate (II), sodium chloride, ethylenediaminetetraacetic acid (EDTA), 1-phenyl-1-trimethylsiloxyethene, α-bromoisobutyryl bromide, chloroform-d ($CDCl_3$), methanol-d4 (MeOD), *tert*-butyl α-bromoisobutyrate, N,N,N',N'',N''-Pentamethyldiethylenetriamine (PMDETA), Methyl Sulfonic Acid (MSA), ε-Caprolactone and dimethylformamide (DMF) were purchased from Sigma-Aldrich. THF and anisole were obtained from Acros. MeOH, triethylamine and hydrogen peroxide were purchased from J.T. Baker. Diethyl ether (Carlo erba Reagents), 1,4-dioxane (Biosolve BV), dichloromethane (CH_2Cl_2, Fisher Chemical), Doxorubicin (Dox, Bioconnect. BV) and 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindotricarbocyanine Iodide (DiR, Molecular Probes) were also used.

2. Instruments

Routine NMR spectra were recorded on a Varian Inova 400 spectrometer with $CDCl_3$ as a solvent. Malvern Zetasizer Nano NS was used for Dynamic light scattering (DLS) analysis with following settings: temperature 25 °C, He-Ne laser wavelength 633 nm and detector angle 173°. For transmission electron microscopy, a JEOL 1010 Transmission Electron Microscope with MegaView Soft Imaging camera at an acceleration voltage of 60 kV was used. Energy-dispersive X-ray element mapping was done on a Bruker Quantax EDS system with an STEM detector incorporated. Bruker D8 Advance with Cu Kα1 radiation was used for XRD measurements. DSC measurements were performed on Mettler DSC 822e (Mettler-Toledo AG, Greifensee, Switzerland). Nanoparticles tracking analysis (NTA) of stomatocytes nanomotors was performed on NanoSight NS500. *In vivo* imaging was performed on FluorVivo (INDEC BioSystems, Santa Clara, CA USA).

3. Synthetic procedures, self-assembly and characterizations



3.1. Synthesis of α-Methoxy-poly(ethylene glycol)$_{44}$ ATRP macromolecular initiator (1)

Poly(ethylene glycol) methyl ether (5.00 g, 2.50 mmol) was dried by co-evaporation with toluene. The polymer was 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. α-bromoisobutyryl bromide

(616 mL, 5.00 mmol) was added dropwise. After addition, the resulting solution was stirred for 24h while slowly warming to room temperature. After the reaction, the white precipitate was filtered off and the solution was concentrated. The polymer was precipitated in ice-cold diethyl ether (3x). The polymer was characterized by ^{1}H-NMR in $CDCl_3$.
^{1}H-NMR (400 MHZ, $CDCl_3$) δ: 4.33 (t, $CH_2CH_2OC(O)C(CH_3)_2Br$), 3.76 (t, $CH_2CH_2OC(O)C(CH_3)_2Br$), 3.65 (br. s, PEG backbone), 3.55 (m, CH_3OCH_2), 3.38 (s, CH_3OCH_2), 1.94 (s, $C(CH_3)_2Br$) ppm.

3.2. Synthesis of poly(ethylene glycol)-*b*-polystyrene (2)

The Schlenk tube with CuBr (45 mg, 0.32 mmol) was evacuated for 15 min and refilled with Ar for three times. 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* a syringe and degased for 15 min. After cooling the mixture to 0 °C, PEG-initiator (215 mg, 0.1 mmol) dissolved in anisole (0.5 mL) was injected and the solution was degassed for another 15 min. The Schlenk tube was transferred into an oil bath at 90 °C. ^{1}H-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 reaction was terminated by cooling to room temperature after stirring for 2h. The solution was diluted with CH_2Cl_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. The polymer was characterized by ^{1}H-NMR in $CDCl_3$.
^{1}H-NMR (400 MHZ, $CDCl_3$) δ: 7.20-6.30 (br. s, PS arom.), 3.64 (br. s, PEG backbone), 3.38 (s, 3H, CH_3OCH_2), 2.30-1.20 (br. s, PS backbone), 0.90 (br. m, 6H, $C(O)C(CH_3)_2CH_2$) ppm.

3.3. Synthesis of poly(ethylene glycol)-*b*-poly(ε-caprolactone) (3)

A schlenk tube was flame dried and 400 mg (0.2 mmol) of poly-ethyleenglycol-methylether (MeO-PEG_{44}-OH) was dissolved in freshly distilled toluene. Subsequently 1.845 mL (16.6 mmol, 83 eq.) ε-Caprolactone was added. The mixture was heated to 30 °C and stirred for 30 minutes. Then 13μL MSA was added (0.2 mmol, 1 eq.) and the reaction stirred for two and a half hours at 30 °C. Thereafter the reaction was cooled to room temperature and precipitated in cold hexane. The precipitate was filtered and re-dissolved in THF and precipitated twice in cold hexane. After filtering the mixture the obtained white solid was dried under vacuum overnight.
^{1}H-NMR (400 MHZ, $CDCl_3$) δ: 4.06 (t, CH_2O PCL backbone), 3.65 (bs, PEG backbone), 3.38 (s, CH_3O), 2.31 (t, CH_2CO PCL backbone), 1.65 (m, $CH_2(CH_2)_2$ PCL backbone), 1.38 (m, $CH_2(CH_2)_2$) ppm.

3.4. Preparation of PtNPs with PVP coating

4 mL K_2PtCl_4 solution (20 mM) was added into 40 mg PVP, followed by 48 hours stirring. After that, 35 mg *L* (+) ascorbic acid in 1 mL of MilliQ water was added into the solution. The resulting solution was sonicated (VWR Ultrasonic Cleaner Model 75D) at room temperature for 1 h.

3.5. Self-assembly of Stomatocyte or hybrid Stomatocyte

10 mg polymer with different ratios between PEG-*b*-PS and PEG-*b*-PCL was fully dissolved in 1 mL mixture of THF/dioxane (4:1, v/v). 1 mL of MilliQ water was slowly added into the solution by a syringe pump at a rate of 1 mL/h. After vigorous dialysis for at least 48 hours, stomatocyte and hybrid stomatocyte with different percentages of PCL was obtained. The size of hybrid stomatocyte with different PCL blending percentages was measured by DLS. (Supplementary Figure 1,2 and Supplementary Table 1)

3.6. Self-assembly of PtNPs loaded Stomatocyte or hybrid PtNPs loaded Stomatocyte

10 mg polymer with different ratios between PEG-*b*-PS and PEG-*b*-PCL was fully dissolved in 1 mL mixture of THF/dioxane (4:1, v/v). 0.35 mL of MilliQ water was slowly added by a syringe pump at a rate of 1 mL/h, followed by addition of preformed PtNPs solution (0.65 mL) also at a

rate of 1 mL/h. After dialysis for at least 48 hours, PtNPs loaded stomatocyte and hybrid PtNPs loaded stomatocyte with different percentages of PCL was obtained.

3.7. Self-assembly of PtNPs-Dox loaded Stomatocyte or hybrid PtNPs-Dox loaded Stomatocyte

10 mg polymer with different ratios between PEG-*b*-PS and PEG-*b*-PCL was fully dissolved in 1 mL mixture of THF/dioxane (4:1, v/v) before addition of 0.05 mg doxorubicin solution in DMF. 0.35 mL of MilliQ water was slowly added by a syringe pump at a rate of 1 mL/h, followed by addition of preformed PtNPs solution (0.65 mL) also at a rate of 1 mL/h. After dialysis for at least 48 hours, PtNPs-Dox loaded stomatocyte and hybrid PtNPs-Dox loaded stomatocyte with different ratios of PCL was obtained. Confocal laser scanning microscope was used to image the incorporation of Dox. (Supplementary Figure 3)

3.8. EDX measurement of hybrid Stomatocyte with 50% PEG-*b*-PCL-FITC

Energy-dispersive X-ray element mapping was performed on a Bruker Quantax EDS system with an STEM detector incorporated. FITC was linked onto polymer PEG-*b*-PCL to introduce S element for visualization. Hybrid stomatocyte samples were diluted to the appropriate concentration. Then 5 μL of diluted sample aliquot was dropped onto carbon-coated copper grid and the grid was dried overnight under room temperature. Both overview and line scans (across the membrane) of EDX measurement were performed, which can be seen in Figure 2g,h and Supplementary Figure 4.

3.9. XRD measurement of hybrid Stomatocyte with different percentages of PCL

Hybrid stomatocyte samples with different percentages of PCL blending (0%, 25%, 50%, 75%, 90%, 100% PCL) were prepared by solvent switch method. The samples were freeze-dried and measured by XRD (Bruker D8 Advance) with Cu Kα1 radiation. (Supplementary Figure 5)

3.10. DSC measurement of hybrid stomatocyte with different percentages of PCL

Hybrid stomatocyte samples with different percentages of PCL blending (0%, 25%, 50%, 75%, 90%, 100% PCL) were prepared by solvent switch method. The samples were freeze-dried for DSC measurement. DSC thermograms were recorded using the Mettler DSC 822^e (Mettler-Toledo AG, Greifensee, Switzerland). Samples (10 mg) were crimped in aluminium pans with pierced lids, equilibrated at 0 °C for 5 min and finally heated up to 100 °C at a heating rate of 2 °C/min. The measurement cell was purged with dry nitrogen gas at a flow rate of 50 mL/min during the measurements. The DSC data can be seen in Supplementary Figure 6.

3.11. SEM measurements of hybrid Stomatocyte before and after degradation

Hybrid stomatocyte samples were incubated with pH=1 citric acid-disodium phosphate buffer. On different time points, the sample was taken and dropped onto carbon-coated copper grid and the grid was dried overnight under room temperature. SEM measurements were performed to visualize the structure of hybrid stomatocyte before and after degradation. The SEM images can be seen in Figure 3a,b.

3.12. Drug release from hybrid Stomatocyte with 25% and 50% PCL

In vitro drug release experiments were performed under two buffer solutions containing citric acid and disodium phosphate with different pH (pH=5 and pH=7). To a 1.5 mL Eppendorf tube, 100 μL buffer and 400 μL Dox-loaded hybrid nanomotor solution with 0% PCL, 25% PCL, 50% PCl (6 mg/mL,) were added respectively and shaked for 48 hours. On different time points, the sample was centrifuged and 100 μL supernatant was taken for fluorescent measurement and replaced by 100 μL of fresh buffer solution (to ensure sink condition). The fluorescence was measured by using a black Greinder 96-well plate in a plate reader on ex/em 480/580 nm. The release of Dox from hybrid stomatocyte nanomotor can be seen in Figure 3c.

3.13. Movement analysis

Nanosight NS500 was used for the measurements of motion of hybrid stomatocytes nanomotors. The fitting of the MSD allows for calculation of the speed of the nanomotors by using the self-diffusiophoretic model proposed by Golestanian and coworkers. While a purely diffusive system would show only a linear component according to $(4D)\Delta t$ equation from which an enhanced diffusion coefficient can be extracted, our MSD curves are not linear and show a parabolic fit according to the equation $(4D)\Delta t+(v^2)(\Delta t^2)$ from which we can extract the velocity of the particles.

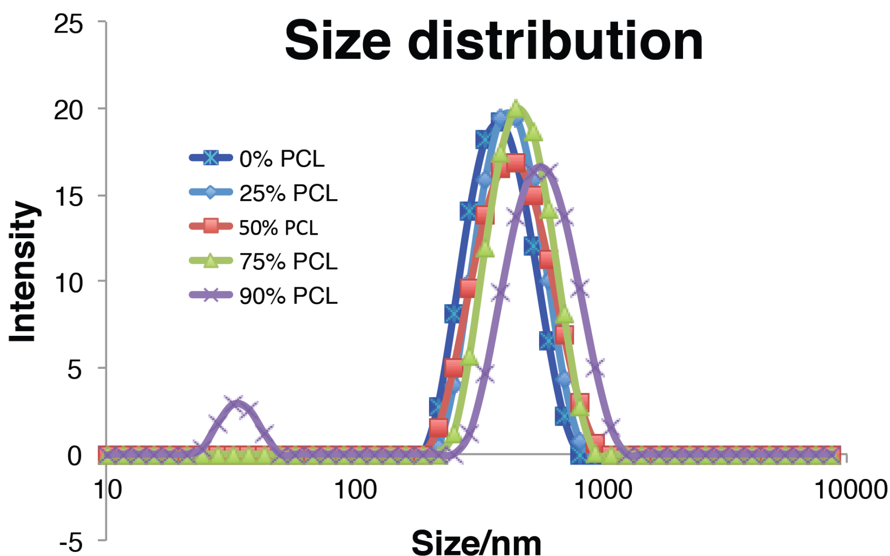
3.14. Cell uptake of hybrid Stomatocyte with 50% PCL

To an Ibidi 8-well plate, 200 μL of cell solution in complemented DMEM (4-5 X 10^5 cells/mL) was added, and incubated overnight at 37 ºC. 20 μL of hybrid nanomotor solution with 0% PCL, 25% PCL and 50% PCL, or free Dox-HCL solution (4 μg/mL) was mixed with/without 10 μL hydrogen peroxide (1.5 v/v%) with a total volume of 100 μL in DMEM. These solutions were added to the cells, and incubated for 6 hours at 37 ºC. Subsequently the cells were washed with PBS. To each well 200 μL fresh DMEM (without FBS) was added and incubated for 30 minutes at 37 ºC before measuring with confocal microscopy. Using life cell imaging, the cells were visualized and tracked the diffusiveness of Dox signal. The confocal image of cell uptake can be seen in Figure 5 and Supplementary Figure 9.

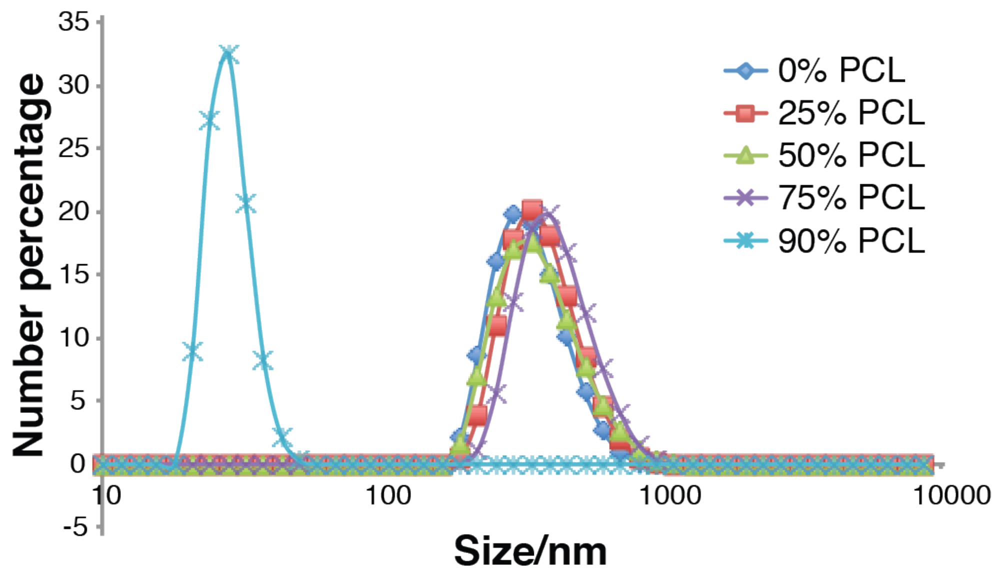
3.15.In vivo experiment

5 mg PEG-*b*-PS and 5 mg PEG-*b*-PCL were fully dissolved in 1 mL mixture of THF/dioxane (4:1, v/v). After addition of 20 μL DiR solution (1mg/mL in DMF), 1 mL of MilliQ water was slowly added at a rate of 1 mL/h. After vigorous dialysis for at least 48 hours, DiR-loaded hybrid stomatocyte with 50% PCL was obtained. The sample (300 μL) was injected intravenously (caudal vein). *In vivo* images of the mouse respectively at day 3 and day 7 were obtained on a FluorVivo 300 (INDEC BioSystems, Santa Clara, CA USA). (Supplementary Figure 10)

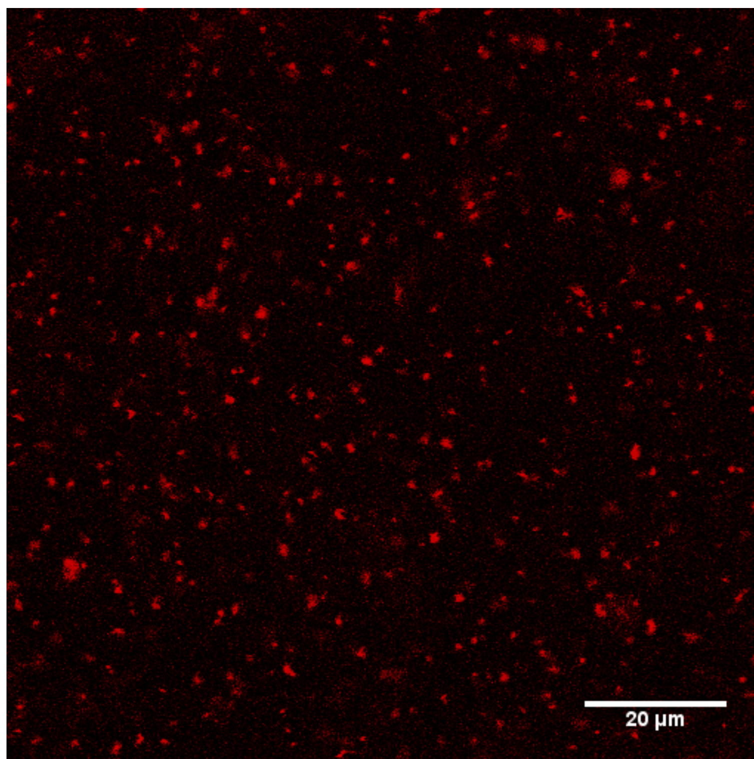
4. Supplementary Figures and Tables



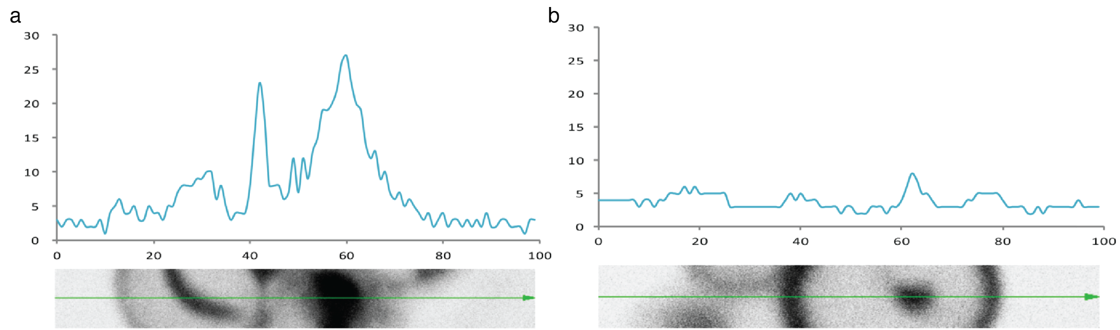
Supplementary Figure 1. Intensity size of stomatocyte and hybrid stomatocyte.



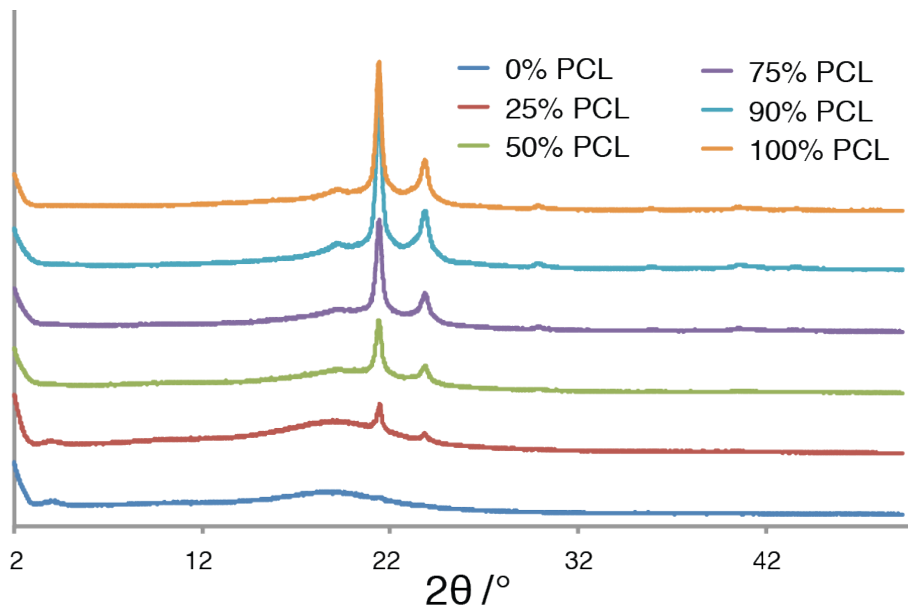
Supplementary Figure 2. Number size of stomatocyte and hybrid stomatocyte.



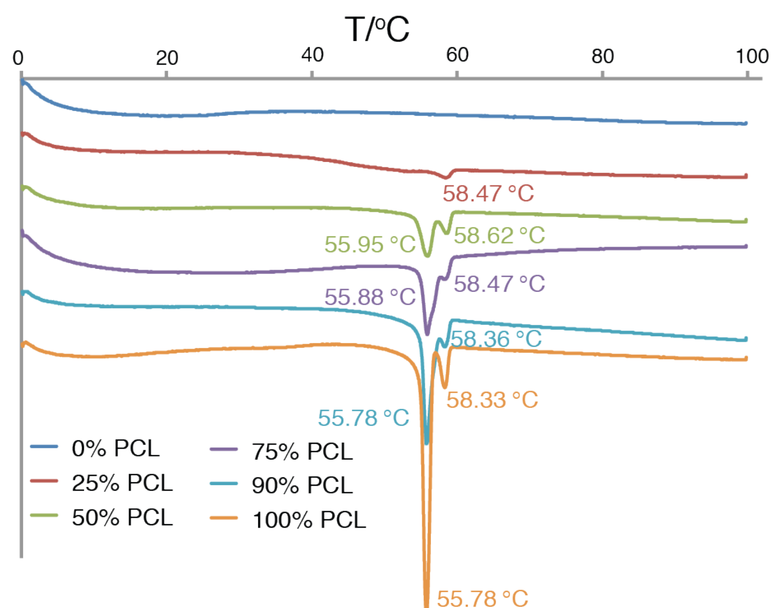
Supplementary Figure 3. Confocal images of PtNPs-encapsulated fluorescent Dox-loaded hybrid stomatocyte. The size looks bigger than expected due to the Brownian motion during the measurement.



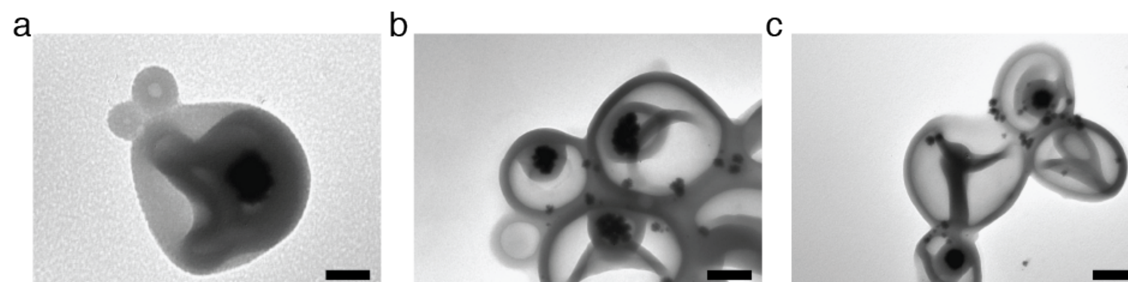
Supplementary Figure 4. EDX line scans of S element using STEM mode across the membrane (the green arrow) of hybrid stomatocyte with 50% PEG-*b*-PCL-FITC. The TEM images on the bottom are the structure of measured vesicles. From the Fig.S4a, S element is not distributed evenly on the structure of vesicles with 50% PEG-*b*-PCL-FITC, which also indicating domain formation of hybrid stomatocyte when 50% PCL was mixed.



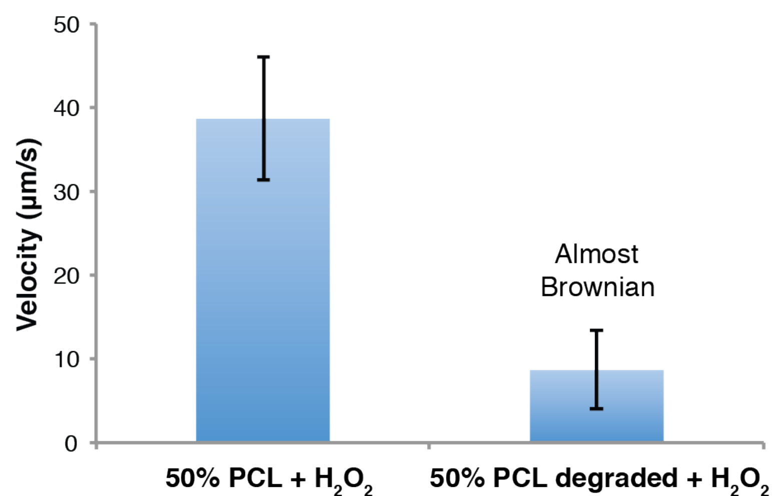
Supplementary Figure 5. XRD of hybrid stomatocyte with different percentage of PCL blending.



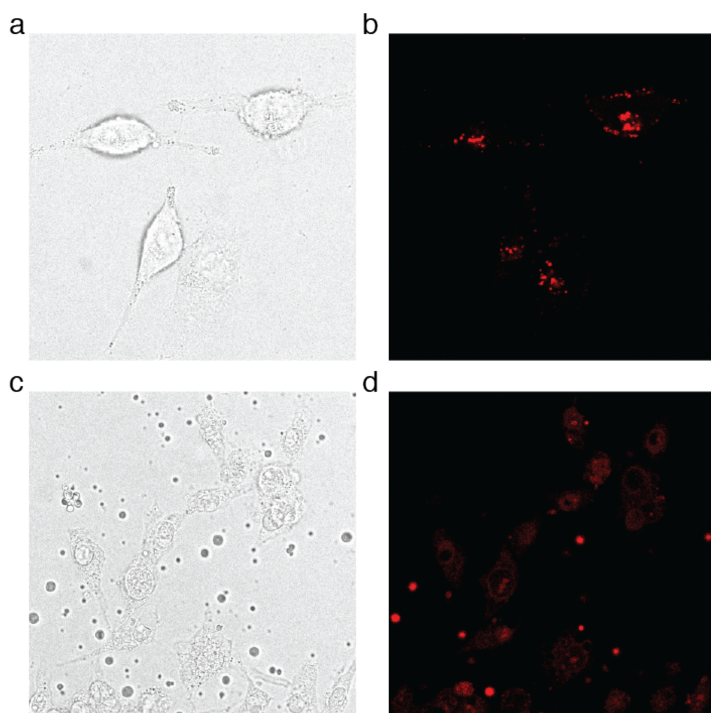
Supplementary Figure 6. DSC of hybrid stomatocyte with different percentage of PCL blending.



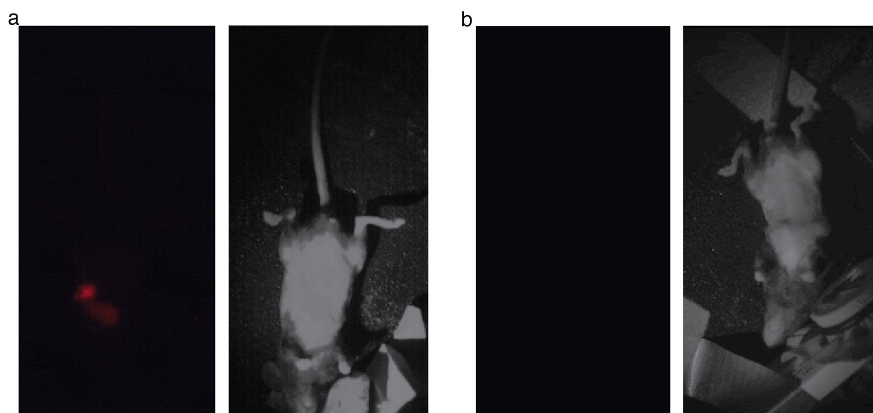
Supplementary Figure 7. TEM of hybrid stomatocyte with different percentage of PCL blending. (a) TEM of stomatocyte nanomotor without PCL; (b) TEM of stomatocyte nanomotor with 25% PCL; (c) TEM of stomatocyte nanomotor with 50% PCL. Scale bar: 100 nm.



Supplementary Figure 8. Velocity of hybrid nanomotor with 50% PCL before and after degradation in presence of hydrogen peroxide. After degradation of PCL in acidic condition, the motion behavior of biodegradable hybrid stomatocyte nanomotor was studied, which was almost Brownian motion. Directional motion was fitted using the equation $(4D)\Delta t+(v^2)(\Delta t^2)$.



Supplementary Figure 9. Cell uptake of Dox from hybrid stomatocyte nanomotor without hydrogen peroxide. (a) bright field images of cells after incubating with Dox-loaded stomatocytes nanomotors (without PEG-*b*-PCL) without hydrogen peroxide; (b) Confocal images of cells after exposure to Dox-loaded stomatocytes nanomotors without PEG-*b*-PCL; (c) bright field images of cells after incubating with Dox-loaded stomatocytes nanomotors (50% PEG-*b*-PCL) without hydrogen peroxide; (d) Confocal images of cells after exposure to Dox-loaded stomatocytes nanomotors with 50% PEG-*b*-PCL. Red dots in figure d indicated the dead cells.



Supplementary Figure 10. *In vivo* images of the mouse injected with DiR-loaded hybrid stomatocyte with 50% PCL. (a) *in vivo* image of the mouse at day 3; (b) *in vivo* image of the mouse at day 7. The *in vivo* experiment demonstrated the biodegradability of hybrid stomatocyte nanomotors, as the fluorescence is no longer detectable by day 7.

Supplementary Table 1. Size and PDI of hybrid stomatocytes with different ratios of PCL

Group	Size/nm	PDI
0% PCL	376.1	0.076
25% PCL	410.2	0.084
50% PCL	401.1	0.149
75% PCL	402.3	0.224
90% PCL	369.9	0.542