SUPPLEMENTARY INFORMATION

Programmable positive and negative chemotaxis of polymeric vesicles driven by degradation

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1. Materials and Methods

All reagents are used as received without purification unless otherwise indicated. For poly(ethylene glycol)-*b*-poly(D,L-lactide) (PEG-*b*-PLA) block copolymer synthesis, methoxy-PEG_{22}-OH (1 kDa) (Creative PEG Works), methoxy-PEG_{44}-OH (2 kDa) (Rapp Polymere), 3,6-dimethyl-1,4-dioxane-2,5-dione (D,L-lactide, Sigma-Aldrich) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, Sigma-Aldrich) were used. Dichloromethane (DCM) and toluene were dried by passing over activated alumina columns in a MBraun MB SPS800 under nitrogen and stored under argon. For the synthesis of poly(ethylene glycol)-*b*-polystyrene (PEG-*b*-PS) block copolymer, copper bromide (CuBr, Sigma-Aldrich) was washed with acetic acid (VWR International) and followed by methanol (MeOH, Thermo Fisher Scientific) for three times and stored under argon protection. Styrene (Sigma-Aldrich), anisole (Fluorochem EU) and *N,N,N',N'',N''*-pentamethyl-diethylenetriamine (PMDETA, Sigma-Aldrich) were distilled prior to use. For self-assembly and shape transformation, tetrahydrofuran (THF, VWR International), 1,4-dioxane (Biosolve), Nile red (Thermo Fisher Scientific) and PEG 2k (Sigma-Aldrich) and spectra/Por® Dialysis Membrane (molecular weight cut-off: 12-14 kDa) were used. For degradation experiments, proteinase K (Merck, 30 mAnson-U/mg), tris (hydroxymethyl) aminomethane (Merck) and dimethyl sulfone (Janssen) were used. For chemotaxis experiments, horseradish peroxidase (HRP, Sigma-Aldrich, ≥250 units/mg), hydrochloric acid (HCl, TCI Europe NV), glucose (Merck), lactic acid (LA, Thermo Fisher Scientific), Sylgard® 184 silicone elastomer kit (Dow) and trichloro(1H,1H,2H,2H-perfluorooctyl)silane (Sigma-Aldrich) were used. Ultrapure Milli-Q water (QPOD Milli-Q purification system, 18.2 MΩ) was used for the preparation of all non-deuterated aqueous solutions.

^{1}H-NMR. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were collected on a Bruker 400 MHz Avance III HD nanobay spectrometer with a BBFO probe. Tetramethylsilane ($\delta = 0.0$ ppm), the residual protons of the deuterated solvent or dimethyl sulfone ($\delta = 3.0$ ppm) were used as an internal reference. ^{1}H NMR spectra were acquired using 32 scans and a relaxation delay of 5 s. ^{1}H NMR spectra of stomatocytes with/without proteinase K in deuterated buffer were acquired using water suppression sequence. To monitor enzymatic degradation kinetics, real-time spectra were collected every 30 min over 9.5 h at 37 °C. A known amount of dimethyl sulfone (1.0 μmol) was used as internal standard for degradation quantification. ^{1}H-NMR T_1

and T_2 relaxation measurements were carried out on Bruker Avance III 500 spectrometer with a Prodigy BB cryoprobe at 37 °C. The longitudinal relaxation time, T_1, was studied by the inversion recovery method. The transverse relaxation time, T_2, was studied by the Carr-Purcell-Meiboom-Gill pulse sequence. T_1 and T_2 were obtained by nonlinear least squares fitting of a three-parameter exponential function and a mono-exponential function respectively.

GPC. Gel permeation chromatography (GPC) equipped with a PL gel 5 μm mixed D column (Polymer Laboratories) was conducted on a Shimadzu instrument with differential refractive index and UV (254 nm) detectors. The system was equilibrated at 35 °C in THF, which was used as the polymer solvent and eluent (flow rate: 1 mL/min). Polystyrene standards (molecular weight range: 266 – 59300 g/mol) were used for calibration.

DLS. Dynamic light scattering (DLS) measurements were carried out at 25 °C using Malvern Zetasizer Nano-ZS (Malvern Instruments) equipped with a He-Ne laser (633 nm, 4 mW) and Avalanche photodiode detector (173°) to evaluate the average hydrodynamic diameter (D_h) and polydispersity (PDI) of stomatocytes. DLS uses the fluctuations in scattered light intensity due to the Brownian movement of the particles to determine their size distribution derived from the Stokes-Einstein equation by assuming a hard sphere model.

Cryo-TEM. Cryogenic transmission electron microscopy (cryo-TEM) was performed using JEOL Transmission Electron Microscope 2100 with high-quality Gatan 895 ultrascan 4000 bottom mount camera (4080 × 4080 pixels) incorporated to capture the morphologies of stomatocytes. TEM grids (Quantifoil) were glow discharged by a 208 carbon coater (Cressington). 3 μL of sample was added onto the grid, blotted and vitrified through freeze plunging into liquid ethane at 100% humidity with an automatic vitrification robot, FEI Vitrobot™ Mark IV (blot time 1 s, blot force 3). Samples were loaded in a 914 High tilt cryoholder (Gatan, Munich, Germany) and inserted into a JEOL TEM 2100. Data analysis was performed using NIH-Image J.

Confocal microscopy. Confocal imaging was performed on a Leica (Wetzlar, Germany) SP8x AOBS-WLL confocal laser scanning microscopy with a 10× objective and PMT detector (553 - 800 nm) at 25 °C. Bidirectional scan direction X and a scan speed of 1000 Hz were used. The samples were loaded in a Ψ-channel microfluidic device. The analysis of a fluorescence

intensity profile was performed with NIH-Image J.

NTA. Nanoparticle tracking analysis (NTA) was performed with Nanosight LM10-HS instrument with an Marlin camera and a 60 mW blue laser illumination (405 nm). This particle-by-particle methodology produces high resolution results for the particle concentration. Typically, stomatocytes solution (1.0 μL, 0.5 mg/mL) was diluted in 1 mL water to ensure an optimized number of particles (10^7-10^9 particles/mL) for analysis. The diluted solution was injected in a sample chamber, and 3 videos of 30 s were taken for each sample. The Brownian motion of the nanoparticles was tracked at 30 frames/s.

ITC. Isothermal titration calorimetry **(**ITC) experiments were carried out on a Malvern MicroCal Auto-ITC200 instrument at 25 °C in water. Typically, PEG-*b*-PLA stomatocytes solution was loaded in the sample cell at a particle concentration of 1.2×10^{-6} mM, which was determined by NTA. LA solution was loaded in the syringe at a concentration of 0.5 mM. One titration experiment included a first injection of 0.4 μL and 20 consecutive injections of 2.0 μL. The injection interval was 200 s. The first one or two data points of titration where dilution artifacts occurred were removed before data analysis. The ITC curves were fitted and analyzed using MicroCal PEAQ-ITC Analysis software to obtain thermodynamic information on the solute-particle interaction (binding constants (K_D), reaction stoichiometry (n), enthalpy (ΔH), entropy (ΔS) and Gibbs free energy (ΔG)).

Statistical analysis. All quantitative experiments were analyzed for statistical significance between the sampled conditions. The statistical tests used (Student's *t* test for two groups, one-way analysis of variance (ANOVA) test and Tukey's honestly significant difference (HSD) post hoc test for more than two groups) are defined in the corresponding figure legends for all panels. Statistical significance is shown as follows: $0.01 < *P < 0.05$; $0.001 < **P < 0.01$; $***P < 0.001$, NS = not significant ($P > 0.05$).

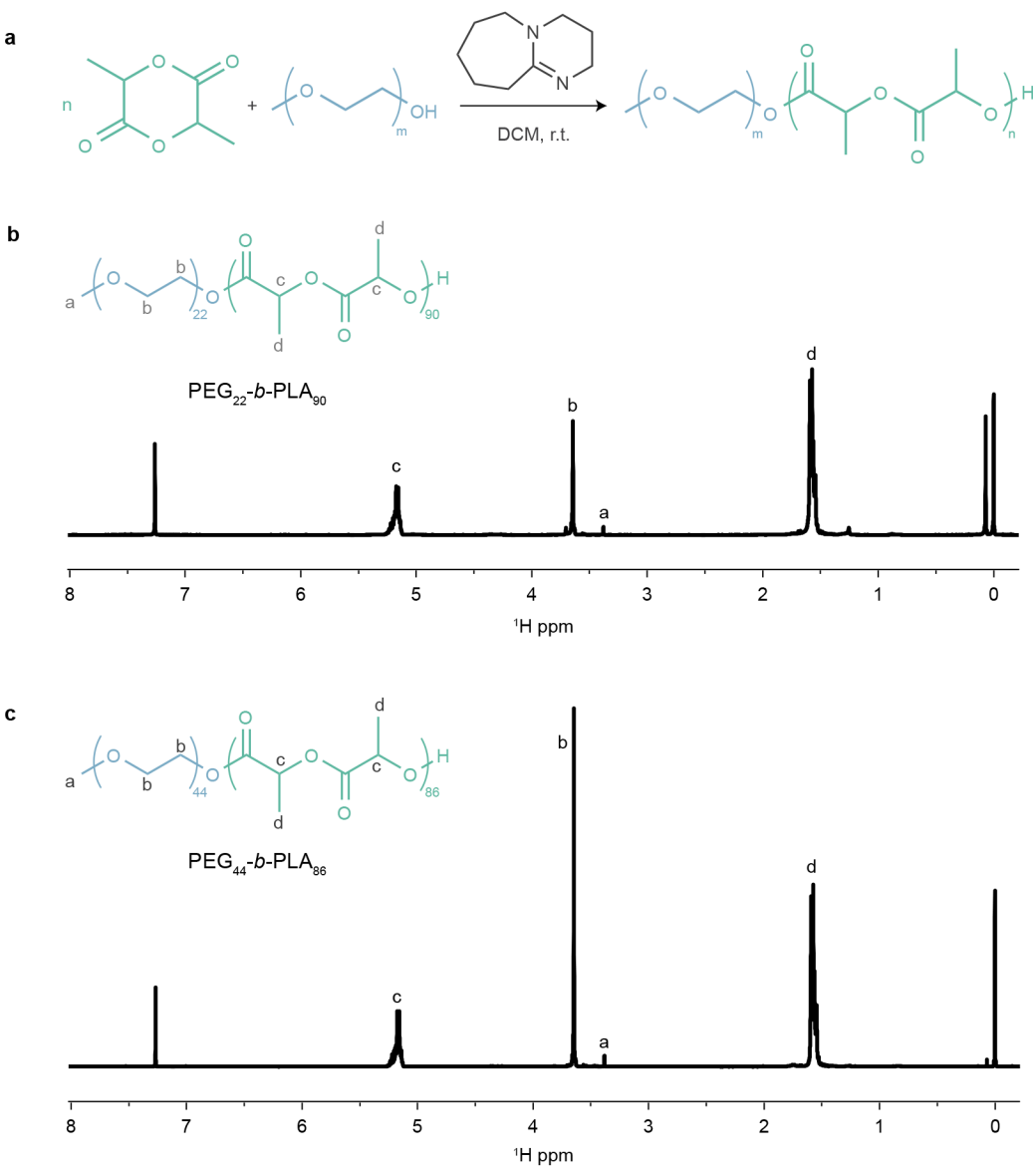
2. Synthesis

2.1 Poly(ethylene glycol)-*b*-poly(D,L-lactide) (PEG-*b*-PLA) block copolymer

PEG-*b*-PLA block copolymer was synthesized according to a previously described ring

opening polymerization method with a minor modification (**Supplementary Fig. 1a**)[1]. Polymerization was carried out under the protection of argon atmosphere. To obtain PEG_{22}-*b*-PLA_{90}, where the subscripts refer to the number average degree of polymerization, methoxy-PEG-OH 1 kDa (194 mg, 0.2 mmol) and D,L-lactide (2.6 g, 18 mmol) were mixed with dry toluene (*ca.* 50 mL) in a 250-mL round-bottom flask. Trace amounts of moisture of the reagents was removed by azeotropic distillation under reduced pressure. The dried reagents were then re-dissolved in 15 mL of dry DCM and transferred to a fresh flame-dried and nitrogen-purged Schlenk flask with a magnetic stirrer under argon. Upon adding DBU as the catalyst (15 μL, 0.1 mmol) *via* a syringe, the polymerization was proceeded for 3-4 h at room temperature. The progress of the reaction was monitored by ^{1}H-NMR until no sign of the monomer peak, which indicated the completion of polymerization. Subsequently, the solution was concentrated on a rotary evaporator and later precipitated into ice cold diethyl ether (200 mL). Product of white powder was collected by vacuum filtration followed by lyophilization. PEG_{44}-*b*-PLA_{86} was synthesized following a similar procedure by using methoxy-PEG-OH 2 kDa (388 mg, 0.2 mmol) and D,L-lactide (2.6 g, 18 mmol).

Polymer characterization: ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.25-5.15 ppm (m, PLA C***H***), 3.64 ppm (br s, PEG backbone C$\boldsymbol{H_2}$), 3.40 ppm (s, PEG C$\boldsymbol{H_3}$O), 1.65-1.55 ppm (m, PLA C$\boldsymbol{H_3}$). Copolymer composition was calculated based on methylene protons of PEG (3.64 ppm), and methine protons of PLA (5.25-5.15 ppm) (**Supplementary Fig. 1b**,**c**). $Đ_M$ (PEG_{22}-*b*-PLA_{90}) of 1.08 and $Đ_M$ (PEG_{44}-*b*-PLA_{86}) of 1.07 were determined by GPC using polystyrene as standards. NMR and GPC results confirmed the successful synthesis of PEG-*b*-PLA with high purity and monodispersity.



Supplementary Fig. 1 | Synthesis and characterization of PEG-*b*-PLA block copolymers. **a**, Synthetic route for PEG-*b*-PLA block copolymer *via* ring opening polymerization. ^{1}H NMR spectra of PEG_{22}-*b*-PLA_{90} (**b**) and PEG_{44}-*b*-PLA_{86} (**c**).

2.2 Poly(ethylene glycol)-*b*-polystyrene (PEG-*b*-PS) block copolymer

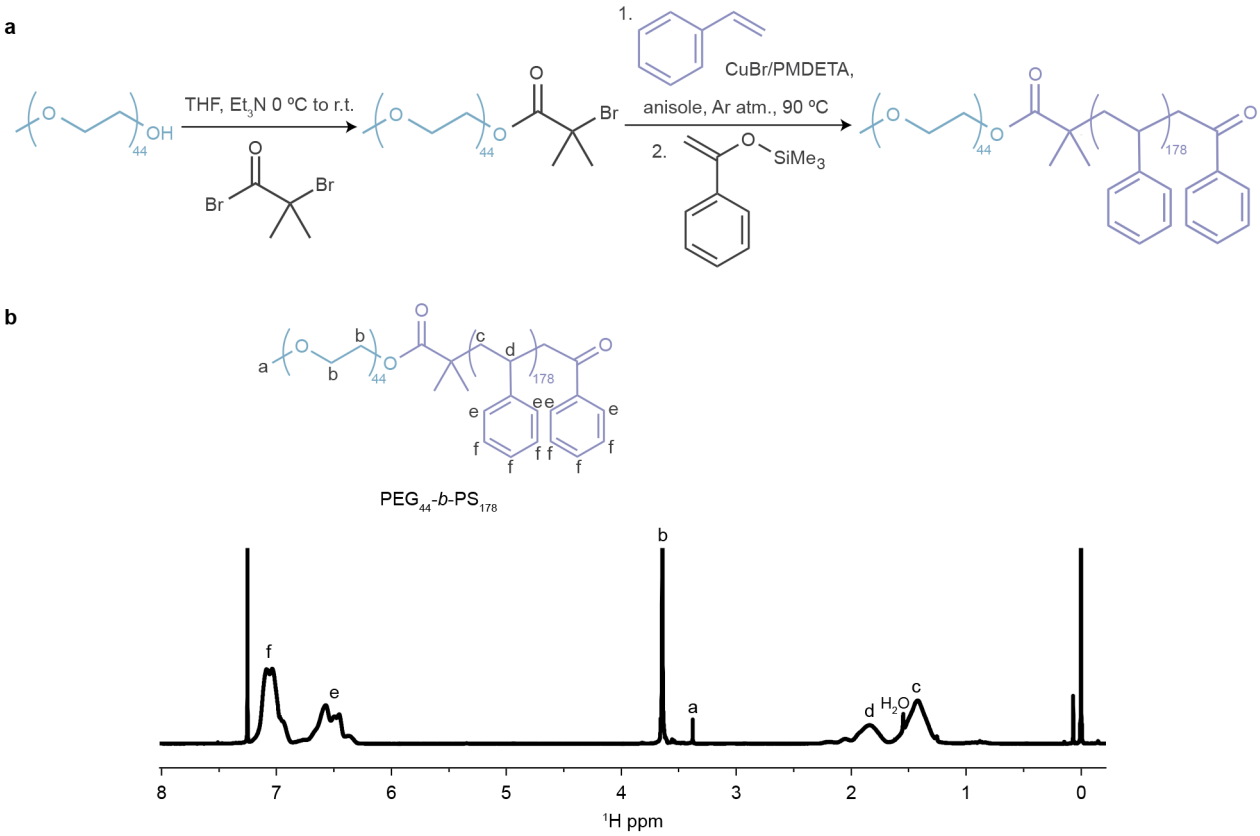
(a) PEG_{44} macroinitiator

PEG_{44} macroinitiator was synthesized according to a method reported previously[2]. Methoxy-PEG-OH 2 kDa (5.0 g, 2.5 mmol) was mixed with dry toluene (*ca.* 50 mL) in a 250-mL round-bottom flask. Trace amounts of moisture of the reagent was removed by azeotropic distillation under reduced pressure. The dried reagent was re-dissolved in 15 mL of dry THF and transferred to a fresh flame-dried and nitrogen-purged Schlenk flask with a magnetic stirrer under argon. Upon adding triethylamine (1.04 mL, 7.50 mmol), the mixture was cooled down to 0 °C, and α-bromoisobutyryl bromide (616 µL, 5.00 mmol) was injected to the Schlenk flask. The resulted solution was stirred over 24 h while slowly warming to room temperature. Next, the solution was concentrated on a rotary evaporator and later precipitated into ice cold diethyl ether (200 mL). Product was collected by vacuum filtration followed by lyophilization.

(b) PEG-b-PS block copolymer

PEG_{44}-*b*-PS_{178} was synthesized according to a previously described ATRP method with a slight modification (**Supplementary Fig. 2a**)[2]. Typically, CuBr (45 mg, 0.32 mmol) was charged in a flame-dried Schlenk flask under argon atmosphere. The Schlenk flask was then sealed with a septum and evacuated for 15 min followed by the refill of argon atmosphere. PMDETA (66 µL, 0.32 mmol) in anisole (0.5 mL) was injected to the flask, after which the mixture was stirred vigorously for 15 min with argon bubbling through the solution to remove any oxygen if present. Styrene (5 mL, 43.6 mmol) was then introduced, and the mixture was further degassed for 15 min. The mixture was cooled down to 0 °C and PEG_{44} macroinitiator (215 mg, 0.10 mmol) in anisole (1 mL) was injected to the flask. The polymerization was initiated by raising the temperature to 90 °C and the progress of polymerization was followed by ^{1}H NMR and GPC. When the desired molecular weight was obtained, 1-phenyl-trimethylsiloxyethene (1.91 mL, 9.28 mmol) was added to terminate the reaction and left to stir for 2 h. Afterwards, the mixture was diluted with DCM, extracted with EDTA solution (50 mM) twice and once with brine. The washed organic fraction was dried over $MgSO_4$ and concentrated by rotary evaporation. The concentrate was precipitated in ice cold MeOH twice, filtered and dried under vacuo overnight to obtain the product of white solid.

Polymer characterization: ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.20-6.86 ppm (m, PS arom. meta and para), 6.70-6.30 ppm (m, PS arom. ortho), 3.64 ppm (br s, PEG backbone C$\boldsymbol{H_2}$), 3.40 ppm (s, PEG, C$\boldsymbol{H_3}$O), 2.30-1.70 ppm (m, PS backbone C$\boldsymbol{H}$), 1.70-1.17 ppm (m, PS backbone C$\boldsymbol{H_2}$) (**Supplementary Fig. 2b**). $Đ_M$ of PEG-*b*-PS was determined by GPC to be 1.05.

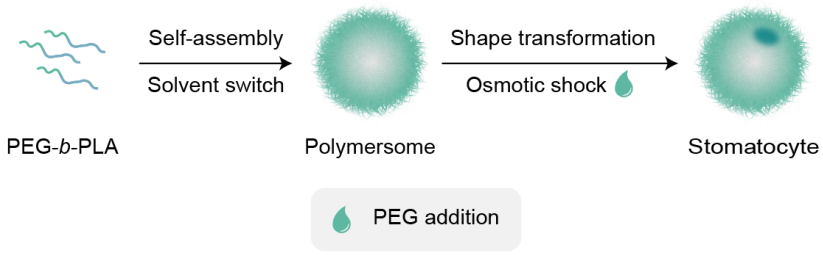


Supplementary Fig. 2 | Synthesis of PEG-*b*-PS block copolymers. a, Synthetic route for PEG-*b*-PS block copolymer *via* ATRP reaction. **b**, NMR spectra of PEG_{44}-*b*-PS_{178}.

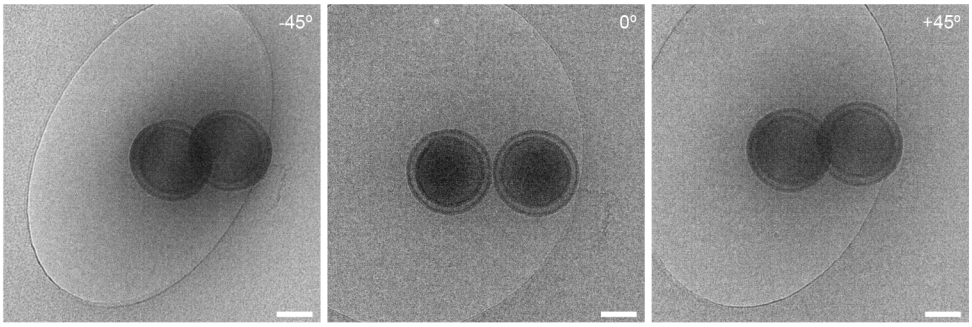
3. Preparation of PEG-*b*-PLA stomatocytes

PEG-*b*-PLA stomatocytes were prepared by combining solvent switch for self-assembly and PEG addition for shape transformation (**Supplementary Figs. 3 and 4**). Typically, a mixture of PEG_{22}-PLA_{90} (5 mg) and PEG_{44}-PLA_{86} (5 mg) were dissolved in 1 mL of organic solvent (1:4, THF: dioxane v/v) in a 15-mL glass vial with a magnetic stirrer. The vial was sealed with a rubber septum and the solution was stirred vigorously (900 rpm) for 30 min at room temperature. Water (1 mL) was added to the above solution by a syringe pump with a 5-mL syringe equipped with a steel needle at a speed of 1 mL/h to obtain polymersomes. Previously

we reported the PEG addition method to induce the shape transformation from polymersomes to stomatocytes in PEG-*b*-PS system[3]. Hereby, we adopted this method in PEG-*b*-PLA system to investigate the possibility to obtain stomatocytes for the first time. PEG aqueous solution (25 μL, 400 mg/mL) was added in polymersomes solution under a stirring speed of 900 rpm for 3 min at 4 °C. The resulted solution was dialyzed at 4 °C for 24 h against water with multiple water changes to remove all the organic solvents.



Supplementary Fig. 3 | Preparation of PEG-*b*-PLA stomatocytes. Schematic display of the fabrication of PEG-*b*-PLA stomatocytes through a combination of solvent switch and PEG addition techniques.



Supplementary Fig. 4 | Cryo-TEM images of stomatocytes made by PEG addition at different angles (-45°, 0° and 45°) demonstrating the spherical structures. Scale bars are 200 nm.

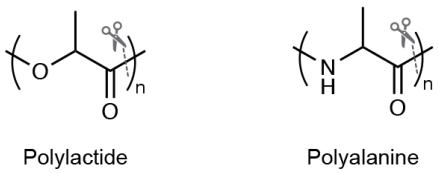
4. Degradation of PEG-*b*-PLA stomatocytes

4.1 Degradation in the absence of proteinase K (pH 5.5, pH 7.4)

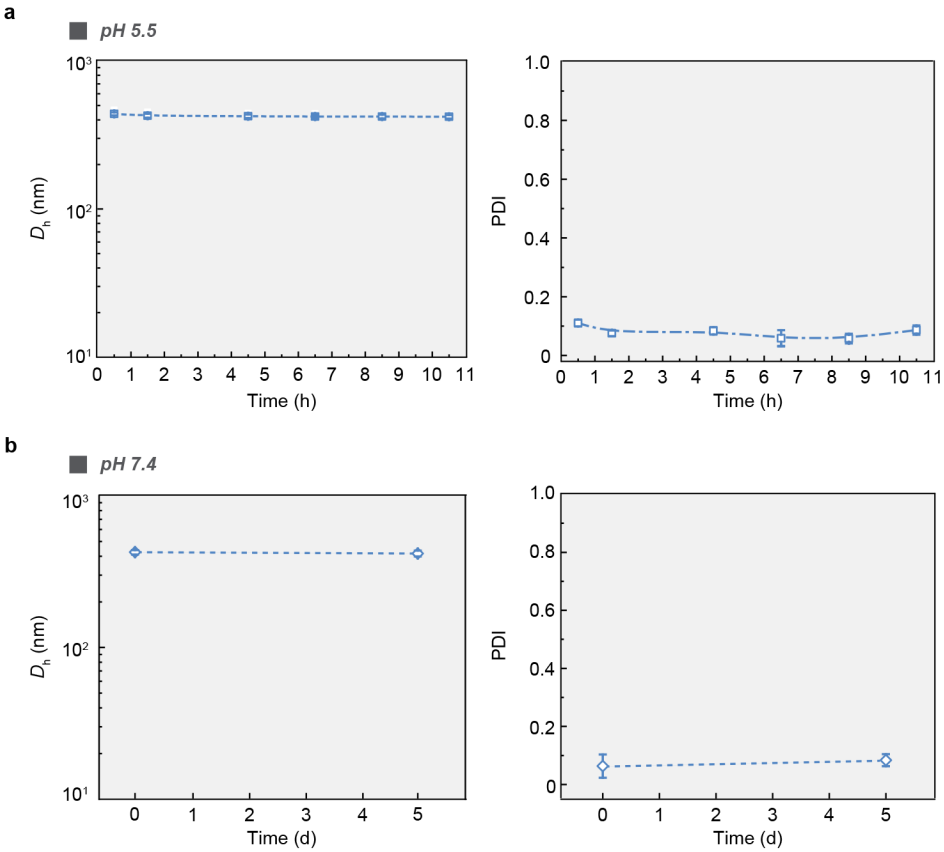
(a) DLS studies

Degradation of PEG-*b*-PLA stomatocytes (3 mL, 0.5 mg/mL) was studied in 1 mM PBS buffer at pH 5.5 and pH 7.4 for 9 h at 37 °C water bath. Aliquots were taken from the solutions at predetermined time points and were characterized by DLS (**Supplementary Fig. 6**). No

measurable disassembly in the buffer solutions (both pH 5.5 and pH 7.4) at 37 °C due to degradation was observed.



Supplementary Fig. 5 | The chemical structures of polylactide and polyalanine with the catalytically active sites highlighted.

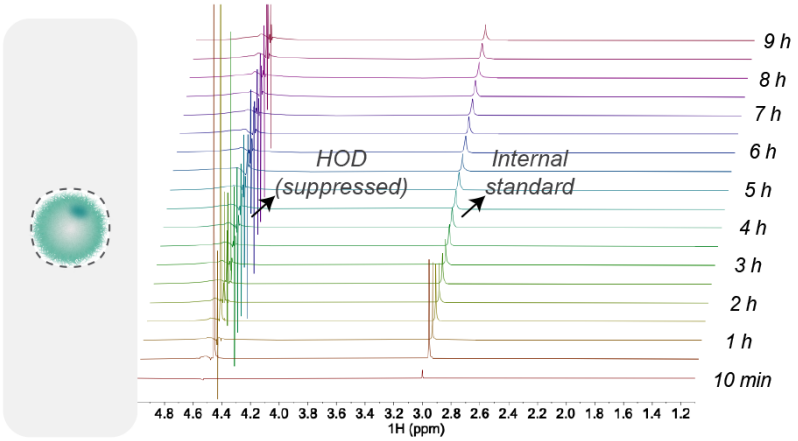


Supplementary Fig. 6 | Degradation study of PEG-*b*-PLA stomatocytes in acidic (pH 5.5) and neutral buffer (pH 7.4). D_h and PDI of stomatocytes in (**a**) acidic buffer (pH 5.5) and (**b**) neutral buffer (pH 7.4) at 37 °C as characterized by DLS respectively. The mean and standard deviation are plotted (n = 3). Lines are guided for eyes.

(b) Real-time ^{1}H NMR studies

The solution of PEG-*b*-PLA stomatocytes (0.5 mg/mL) in deuterated 1 mM PBS buffer containing 0.05% w/v NaN_3 at pH 7.4 was placed in NMR tubes and maintained at 37 °C with

spectra recorded at different time points (**Supplementary Fig. 7**). No signals related to degradation products were detected, indicating the absence of degradation products of PEG-*b*-PLA stomatocytes in the buffer media (pH 7.4).



Supplementary Fig. 7 | Real-time ^{1}H NMR spectra of PEG-*b*-PLA stomatocytes in neutral buffer (pH 7.4) at 37 °C.

4.2 Enzymatic degradation in the presence of proteinase K

(a) DLS and cryo-TEM studies

In a typical experiment, the solution of PEG-*b*-PLA stomatocytes in 1 mM PBS buffer containing 0.05% w/v NaN_3 at pH 7.4 (3 mL, 0.5 mg/mL) was placed in 15-mL vials and equilibrated at 37 °C for 0.5 h in the water bath. Proteinase K (100 μL, 727 μM in Tris-HCl buffer) was added and the solutions were vortexed and maintained at 37 °C for 10 h. Aliquots were taken from the solution at predetermined time points and were characterized by DLS. The morphological change of stomatocytes during the enzymatic hydrolysis was visualized by cryo-TEM after 0.5 h of enzyme addition.

(b) ^{1}H NMR studies

The enzymatic degradation of PEG-*b*-PLA stomatocytes was studied to determine the degradation products in NMR spectra (**Supplementary Fig. 8**):

PEG-PLA

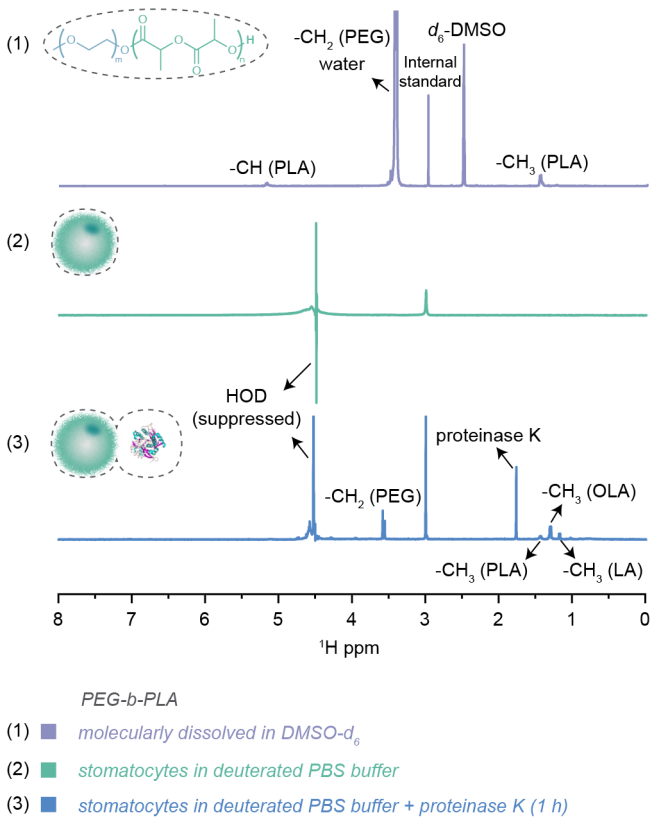
(**1**) *molecularly dissolved in DMSO-d_6*: The stomatocytes assemblies (0.6 mL, 0.5 mg/mL) prepared in water were freeze-dried and re-dissolved in DMSO-d_6 for NMR test at 25 °C.

(**2**) *stomatocytes in deuterated PBS buffer:* The stomatocytes assemblies (0.6 mL, 0.5 mg/mL)

were prepared in deuterated 1 mM PBS buffer containing 0.05% w/v NaN_3 at pH 7.4 and proton NMR spectrum was recorded at 37 °C.

(**3**) *stomatocytes in deuterated PBS buffer + proteinase K (1 h)*: The same solution as in (**2**) was prepared with the addition of proteinase K (20 μL, 727 μM in Tris-HCl buffer) for NMR test 37 °C.

Compared with the ^{1}H NMR spectrum of sample (**1**), methylene (C**H**$_2$) protons of PEG chains at 3.64 ppm and methyl (C**H**$_3$) and methine (C**H**) protons of PLA chains at 1.65-1.55 ppm and 5.25-5.15 ppm were disappeared of sample (**2**), which indicates the PEG and PLA chains in self-assembled structure of stomatocytes are immobile. Followed by the addition of proteinase K after 1 h, the ^{1}H NMR spectrum of solution (**3**) showed the signals corresponding to PEG and PLA/OLA/LA, which indicates the freely-diffused degradation products of stomatocytes due to the catalytic degradation.

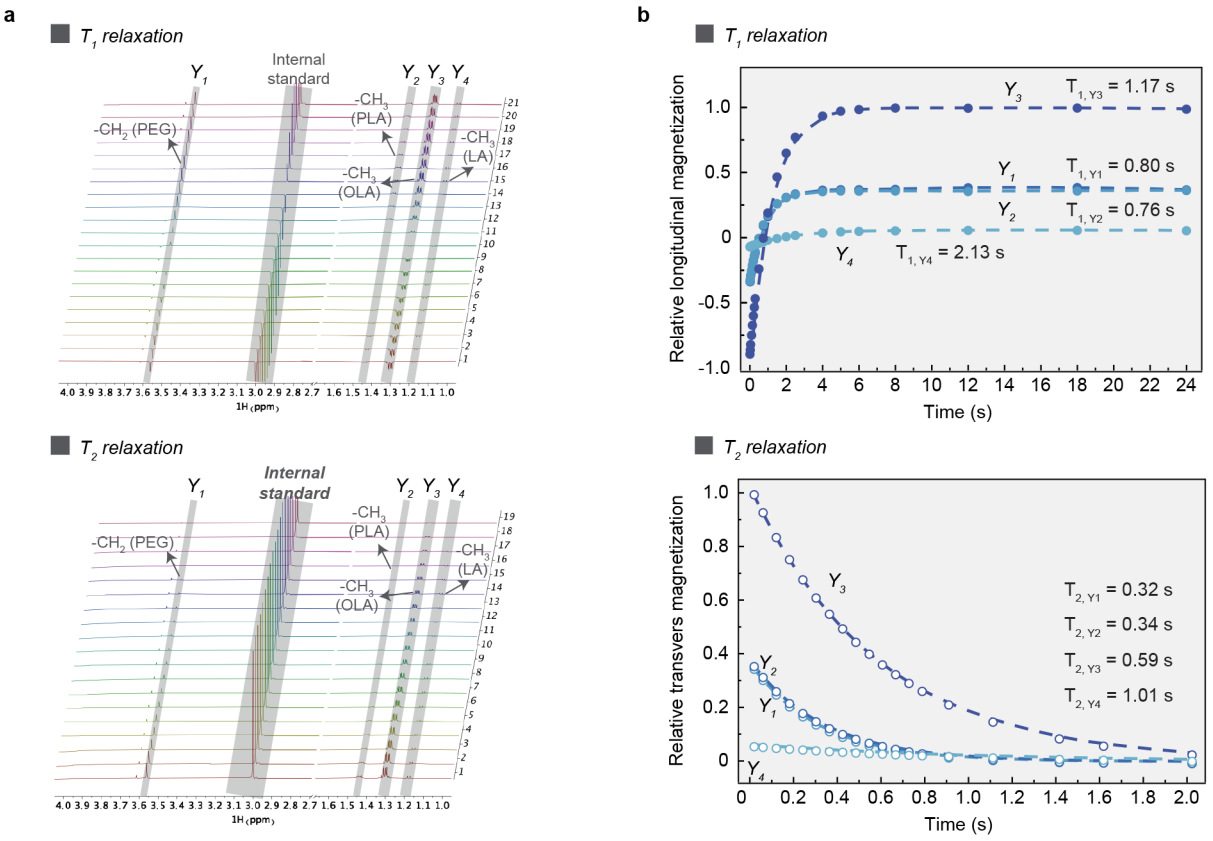


Supplementary Fig. 8 | ^{1}H NMR spectra of PEG-*b*-PLA stomatocytes in different conditions.

(c) ^{1}H NMR T_1 and T_2 relaxation studies

To further confirm the assignments of PLA/OLA/LA, the enzymatic degradation products of

PEG-*b*-PLA stomatocytes were studied by ^{1}H NMR T_1 and T_2 relaxation measurements at 37 °C (**Supplementary Fig. 9a**). All T_1 relaxation times were obtained from nonlinear least squares fitting of a three-parameter exponential function, and the T_2 relaxation times were well fitted by mono-exponential decays (**Supplementary Fig. 9b**). Shorter T_1 and T_2 relaxation times indicate fast relaxation of larger molecules. The relaxation times would therefore show an increased trend from PLA, OLA to LA. Peaks of Y_2 (1.48-1.40 ppm), Y_3 (1.34-1.27 ppm) and Y_4 (1.21-1.16 ppm) could be assigned to PLA (T_1 = 0.76 s, T_2 = 0.34 s), OLA (T_1 = 1.17 s, T_2 = 0.59 s) and LA (T_1 = 2.13 s, T_2 = 1.01 s), respectively, with peak Y_1 (3.64 ppm) of PEG (T_1 = 0.80 s, T_2 = 0.32 s) as a control.

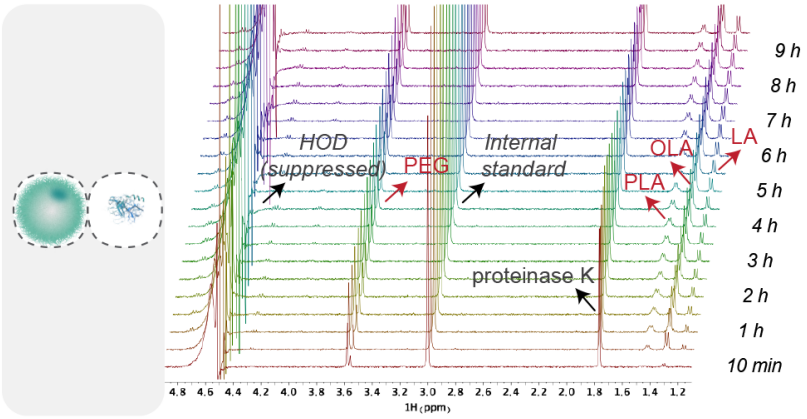


Supplementary Fig. 9 | NMR data confirm the degradation of PEG-*b*-PLA stomatocytes at 37 °C. a, ^{1}H NMR T_1 and T_2 relaxation measurements for small stomatocytes in the presence of proteinase K. Black arrows indicate the assignments for each peak. **b**, The corresponding T_1 and T_2 relaxation curves. All T_1 and T_2 relaxation times were fitted by exponential functions.

(d) Real-time ^{1}H NMR studies

Real-time ^{1}H NMR measurements were carried out with PEG-*b*-PLA stomatocytes in the

presence of proteinase K (*Stoma+proK*). Typically, the solution of PEG-*b*-PLA stomatocytes in deuterated 1 mM PBS buffer containing 0.05% w/v NaN_3 at pH 7.4 (0.6 mL, 0.5 mg/mL) with 1.0 μmol of dimethyl sulfone (δ = 3.0 ppm) as internal standard were placed in NMR tubes and equilibrated at 37 °C for 10 min. Proteinase K (20 μL, 727 μM in Tris-HCl buffer) was added to the solution and maintained at 37 °C within the NMR instrument. Real-time ^{1}H NMR spectra were collected every 30 min at 37 °C over 9.5 h (**Supplementary Fig. 10**).



Supplementary Fig. 10 | Real-time ^{1}H NMR spectra of PEG-*b*-PLA stomatocytes in neutral buffer (pH 7.4) at 37 °C in the presence of proteinase K.

(e) Percentage of degradation products

The percentage of degradation products are defined as the amount of methyl protons of degradation products in the sample of *Stoma+proK* divided by that of poly(lactic acid) in the sample of stomatocytes. For the sample of stomatocytes, equal volume of PEG-*b*-PLA stomatocytes solution was lyophilized and the as-obtained powder of assemblies was re-dissolved in DMSO-d_6, which is a good solvent to molecularly dissolve the entire block copolymer, followed by the addition of internal standard of dimethyl sulfone (1.0 μmol, δ = 3.0 ppm). The total amounts of the methyl protons were calculated as follows:

For *Stoma+proK*:

To determine the percentage of each degradation product, the integration of the methyl protons peaks of PLA (1.48-1.40 ppm), OLA (1.34-1.27 ppm) and LA (1.21-1.16 ppm) was compared to the standard, respectively. As the standard was added with a certain amount (1.0 μmol), the amount of methyl protons of each degradation product could be deduced.

For sample of stomatocytes:

The integration of the methyl protons peak at 1.52-1.40 ppm which belongs to the PLA of stomatocytes was compared to the standard (**Supplementary Fig. 8**, sample 1). The total

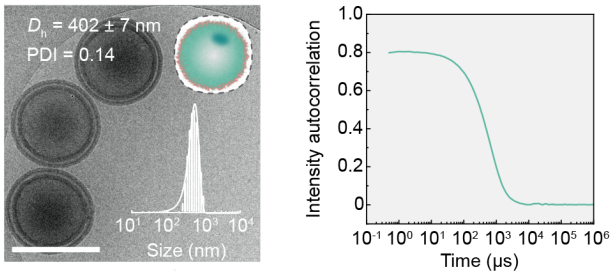
amount of methyl protons of PLA in stomatocytes could be calculated and set as 100%. By normalizing the amount of methyl protons of each degradation product to the total amount of PLA methyl protons of stomatocytes (set as 100%), the percentage of degradation products could be obtained.

5. Preparation of fluorescence probing stomatocytes

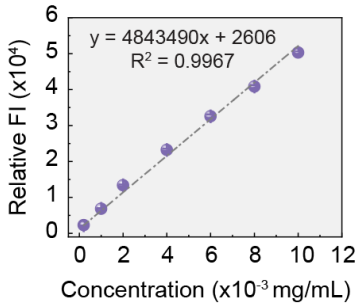
(a) Formation of fluorescence probing PEG-b-PLA stomatocytes

In a model experiments, the fluorescence probing PEG-*b*-PLA stomatocytes were obtained by following the same self-assembly and shape transformation procedures as above, except for adding Nile red in THF solution (10 μL, 10 mg/mL) to the initial 1 mL organic solution of polymer. The assembly procedure was protected from light by aluminum foil. PEG-*b*-PLA stomatocytes/Nile red solution (0.5 mg/mL) was purified by size exclusion chromatography with Sephadex G-25 to remove the excess dye and stored at 4 °C in darkness.

The morphology, size, size distribution and intensity autocorrelation of fluorescence probing PEG-*b*-PLA stomatocytes were determined by cryo-TEM and DLS (**Supplementary Fig. 11**). The concentration of Nile red of PEG-*b*-PLA stomatocytes was determined to be 2.5 μg/mL from the calibration curve in **Supplementary Fig. 12**. Compared with the results of empty stomatocytes, the encapsulation of negligible amounts of Nile red had trivial influence on the assembly of PEG-*b*-PLA stomatocytes in size, distribution and morphology.



Supplementary Fig. 11 | Characterization of fluorescence probing PEG-*b*-PLA stomatocytes. Cryo-TEM image and intensity autocorrelation curve of Nile red-loaded PEG-*b*-PLA stomatocytes. Inset: D_h and PDI measured by DLS. The numerical data are presented as mean ± standard deviation (n = 3). Scale bar is 500 nm.

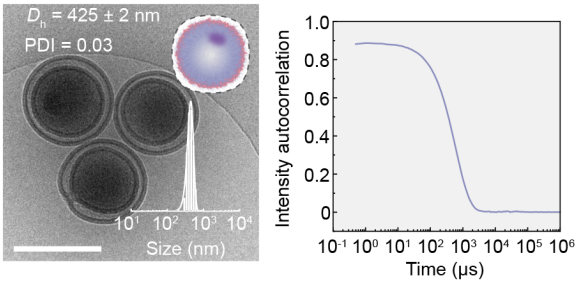


Supplementary Fig. 12 | Calibration curve to determine of the amount of Nile red in fluorescence probing stomatocytes.

(b) Formation of fluorescence probing PEG-b-PS stomatocytes as a control

The fluorescence probing PEG-*b*-PS stomatocytes were fabricated as a control. Typically, PEG_{44}-*b*-PS_{178} (10 mg) was dissolved in 1 mL of organic solvent (4:1, THF: dioxane v/v) in a 15-mL glass vial with a magnetic stirrer. Nile red in THF solution (10 μL, 10 mg/mL) was added into the vial which was further closed with a rubber septum and covered by aluminum foil. The solution was stirred vigorously (at 900 rpm) for 30 min at room temperature. Water (1 mL) was added to the above solution by a syringe pump with a 5-mL syringe equipped with a steel needle at a speed of 1 mL/h. PEG aqueous solution (20 μL, 100 mg/mL) was added into the generated cloudy solution under a stirring speed of 900 rpm for 3 min. Afterwards, the resulted solution was dialyzed to remove organic solvents. PEG-*b*-PS stomatocytes/Nile red solution (0.5 mg/mL) was purified by SEC using Sephadex G-25 to remove the excess dye and stored at 4 °C in darkness.

The morphology, size, size distribution and intensity autocorrelation of fluorescence probing PEG-*b*-PS stomatocytes were determined by cryo-TEM and DLS (**Supplementary Fig. 13**). The concentration of Nile red of PEG-*b*-PS stomatocytes was determined to be 2.5 μg/mL from the calibration curve in **Supplementary Fig. 12**.

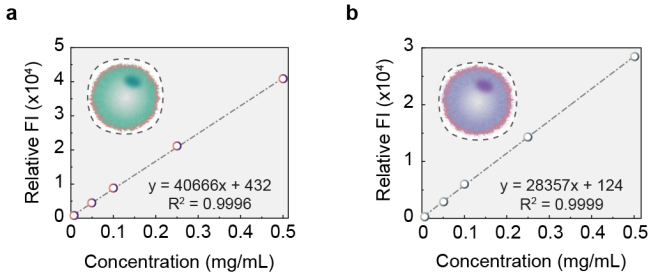


Supplementary Fig. 13 | Characterization of fluorescence probing PEG-*b*-PS

stomatocytes. Cryo-TEM image and intensity autocorrelation curve of Nile red-loaded PEG-*b*-PS stomatocytes. Inset: D_h and PDI measured by DLS. The numerical data are presented as mean ± standard deviations (n = 3). Scale bar is 500 nm.

(c) Calibration between fluorescence intensity and concentration

Both fluorescence probing stomatocytes stock solutions in water were prepared and kept at 4 °C in darkness. Series of solutions were prepared by diluting the stock solutions in water. The fluorescence intensity was measured by using a Tecan Spark 200 plate reader (λ_{ex} = 553 nm, λ_{em} = 638 nm). A linear relationship was observed between fluorescence intensity and concentration in the range of 0-0.5 mg/mL in **Supplementary Fig. 14**. During all chemotactic experiments, we chose the concentration of stomatocytes at 0.5 mg/mL within the linear range. Therefore, the normalized fluorescence intensity profiles can represent the normalized concentration profiles.



Supplementary Fig. 14 | Calibration between fluorescence intensity and concentration. (**a**) PEG-*b*-PLA stomatocytes, (**b**) PEG-*b*-PS stomatocytes.

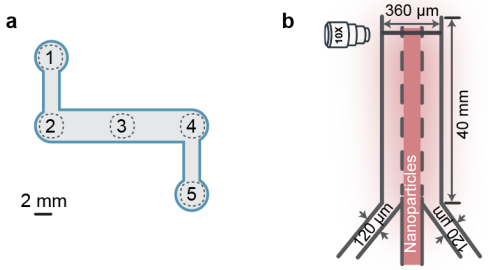
6. Chemotaxis studies of PEG-*b*-PLA stomatocytes

6.1 Positive chemotaxis

(a) Chemotaxis study in the glass flow device

A glass flow device with dimensions of 22 mm (length) × 4 mm (width) × 5.5 mm (depth) as designed by us previously[4] was used to evaluate chemotaxis under a Leica DMIRE2 widefield fluorescence microscopy (**Supplementary Fig. 15a**). The glass channel was first filled with 400 μL of water. For the experimental group, immediately after the addition of 5 μL of

proteinase K (1.9 mM) to position 1, 5 μL of PEG-*b*-PLA/Nile red stomatocytes (0.5 mg/mL) was gently added to position 5. For the control group, 5 μL of proteinase K was replaced by 5 μL of water. To visualize the displacement of particles, the front line of fluorescent stomatocytes was imaged after 2 h (λ_{ex} = 553 nm, λ_{em} = 638 nm). The moving displacement (*d*) was defined as the distances from the input point to the front line of fluorescent stomatocytes.



Supplementary Fig. 15 | Chemotactic devices. (**a**) The glass flow device design and (**b**) Ψ-channel microfluidic device design.

(b) Fabrication of microfluidic device

Due to the perfect light transmittance, strong chemical inertness, non-toxic, low cost and easiness in modification or processing, polydimethylsiloxane (PDMS)[5] was chosen to fabricate a Ψ-channel microfluidic device with dimensions of 4 cm (length) × 360 μm (width) × 100 μm (depth) (**Supplementary Fig. 15b**). PDMS (Sylgard® 184) elastomer was prepared by mixing prepolymer and crosslinking agent (10:1 w/w) and poured onto the silicon master followed by 3-hour degassing under vacuum. The PDMS was cured at 65 °C for 3 h, washed with isopropanol and blow dried with nitrogen. After the oxygen plasma treatment, the PDMS was bonded to a clean glass slide (VWR). The channels were coated with trichloro(1H,1H,2H,2H-perfluorooctyl)silane and the device was baked at 110 °C overnight. The fluid was injected inside PDMS channels using syringe pumps through polyethylene tubes (VWR, Internal diameter 0.5 mm).

(c) Chemotaxis study in the Ψ-channel microfluidic device

Quantitative assessment of chemotaxis was performed with SP8× AOBS-WLL confocal laser scanning microscopy with a 10× objective. In a typical experiment, water, PEG-*b*-PLA/Nile red stomatocytes (0.5 mg/mL) and proteinase K solution (23 μM) were flowed through left, middle and right channel, respectively. Stable linear gradients perpendicular to the flow direction are generated by mixing laminar flow inside device under low Reynolds number

conditions. Due to laminar flow, the effective width of each flow channel is 120 μm. We chose the flow rate of 50 μL/h, corresponding to an average laminar flow of 1157 μm/s as calculated from the volumetric flow rate and cross-sectional area of the device. The design of the device ensures enough gradient stability and interaction time between stomatocytes and the objective solutes[6]. Region of interest is selected at a distance of 38 mm downstream from the inlets across the width of the device, where interaction time of stomatocytes and objective solutes is 32.8 s. After reaching a stable flow, the fluorescence intensity profiles at the region of interest were recorded for about 6 min (667 frames, 0.518 s/frame). The stack-averaged fluorescence intensity (I) was normalized as follows:

$$I_{Normalized} = \frac{I - I_{min}}{I_{max} - I_{min}}$$

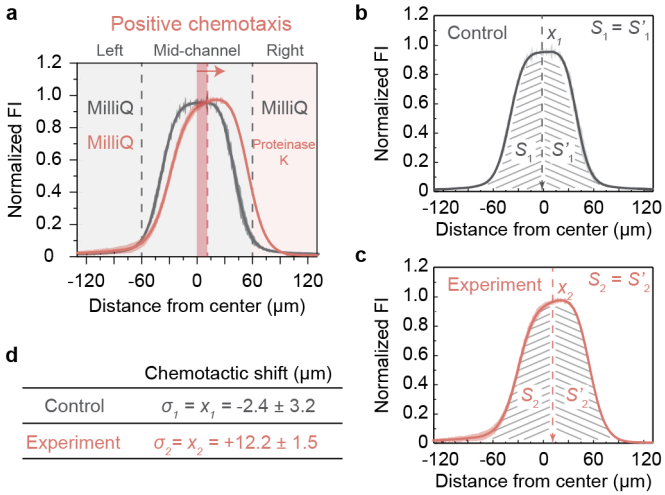
Where $I_{\mathrm{Normalized}}$ is the normalized intensity of stomatocytes, I_{max} and I_{min} are maximum and minimum intensities across the channel width under consideration (see the next paragraph *(d)*).

(d) Calculation of chemotactic shift in the Ψ-channel microfluidic device

Due to the adsorption of fluorescent particles near the channel walls, intensity profile was plotted as a function of distance from center except for 50 μm next to both sidewalls (x = 0 represents the center of the device, x ranges from -130 to 130 μm) from each experiment. To better define the chemotactic shift, we tried to find the area bisector (x) for each profile which was used to represent the main population of stomatocytes. The calculation of chemotactic shift included the following steps:

1) Measuring the area under the plots of normalized fluorescence intensity *vs* distance for the control (grey curve) and the experiment (red curve) analyzed by Origin as shown in **Supplementary Fig. 16a**.

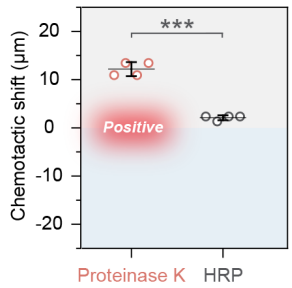
2) Finding the area bisector (x) which can equally separate the area under each plot. In particular, for the control, the area bisector x_1 makes $S_1 = S_1'$ (**Supplementary Fig. 16b**); for the experiment, the area bisector x_2 makes $S_2 = S_2'$ (**Supplementary Fig. 16c**). The chemotactic shift (σ) was measured perpendicular to the flow direction, and was defined as $\sigma = x$ (μm) (**Supplementary Fig. 16d**).



Supplementary Fig. 16 | An example of the calculation of chemotactic shift. a, fluorescence intensity profiles of PEG-*b*-PLA stomatocytes in water (control) and in a gradient of proteinase K by using the Ψ-channel microfluidic device (n = 4). The mean and standard deviations are plotted. A red arrow indicates that PEG-*b*-PLA stomatocytes move toward proteinase K. The red bar indicates the chemotactic shift. **b**, the calculation of the area bisector (x_1) for the control. **c**, the calculation of the area bisector (x_2) for the experiment. **d**, quantification of the chemotactic shift (σ).

(e) Chemotaxis using HRP as control

HRP, an enzyme which is unable to catalyze the degradation, was used as a control experiment. Water, PEG-*b*-PLA/Nile red stomatocytes solution (0.5 mg/mL) and HRP solution (23 μM) were flowed through left, middle and right channel, respectively (**Supplementary Fig. 15b**). The fluorescence at the region of interest were recorded for about 6 min and stack-averaged fluorescence intensity was then obtained with NIH-Image J, followed by the plotting of the intensity profile as described previously.



Supplementary Fig. 17 | Confirming catalysis-induced positive chemotaxis. Comparison of the quantification of chemotactic shift in response to a gradient of HRP or proteinase K (n = 4). The mean and standard deviations are plotted. Statistical analysis: unpaired two-sided Student's *t* test, ***$P < 0.001$.

6.2 Negative chemotaxis

(a) Chemotaxis study in the glass flow device

Immediately after the addition of 5 μL of LA (1M) to position 1, 5 μL of PEG-*b*-PLA/Nile red stomatocytes (0.5 mg/mL) was gently added to position 5 (**Supplementary Fig. 15a**). To visualize the displacement of particles, the front line of fluorescent stomatocytes was imaged after 2 h (λ_{ex} = 553 nm, λ_{em} = 638 nm). The moving displacement (*d*) was defined as the distances from the input point to the front line of fluorescent stomatocytes.

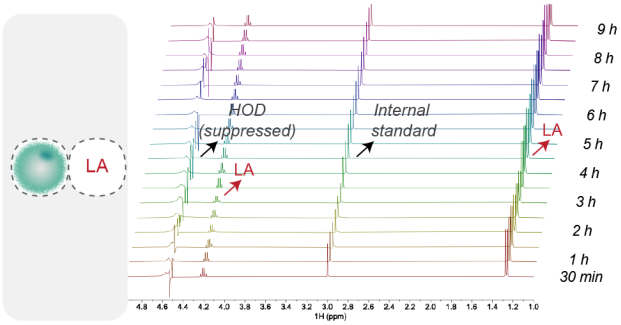
(b) Chemotaxis study in the Ψ-channel microfluidic device

Water, PEG-*b*-PLA/Nile red stomatocytes (0.5 mg/mL) and LA solution (10 mM, pH 3.1) were flowed through left, middle and right channel, respectively (**Supplementary Fig. 15b**). The fluorescence at the region of interest were recorded for about 6 min and stack-averaged fluorescence intensity was then obtained with NIH-Image J, followed by the plotting of the intensity profile as described previously.

(c) Investigation of autocatalysis by real-time ^{1}H NMR study

Typically, the solution of PEG-*b*-PLA stomatocytes in deuterated 1 mM PBS buffer containing 0.05% w/v NaN_3 at pH 7.4 (0.6 mL, 0.5 mg/mL) with 1.0 μmol of dimethyl sulfone as internal standard were placed in NMR tubes and equilibrated at 37 °C for 10 min. 20 μL deuterated LA solution (final concentration,10 mM) was added to above solution and maintained at 37 °C

within the NMR instrument. Real-time ^{1}H NMR spectra were collected every 30 min over *ca.* 9 h at 37 °C (**Supplementary Fig. 18**).

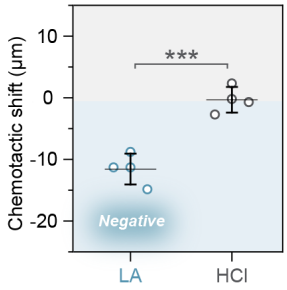


Supplementary Fig. 18 | Real-time ^{1}H NMR spectra of PEG-*b*-PLA stomatocytes at 37 °C in the presence of LA.

6.3 Ruling out alternative mechanisms behind negative chemotaxis

(a) pH effect

Water, PEG-*b*-PLA/Nile red stomatocytes solution (0.5 mg/mL) and HCl solution (pH 3.1) were flowed through left, middle and right channel, respectively. The fluorescence at the region of interest were recorded for about 6 min and stack-averaged fluorescence intensity was then obtained with NIH-Image J, followed by the plotting of the intensity profile as described previously.



Supplementary Fig. 19 | Ruling out the pH effect on LA-induced negative chemotaxis. Comparison of the quantification of chemotactic shift in response to a gradient of HCl or LA (n = 4). The mean and standard deviations are plotted. Statistical analysis: unpaired two-sided Student's *t* test, ***$P < 0.001$.

(b) Diffusiophoresis mechanism

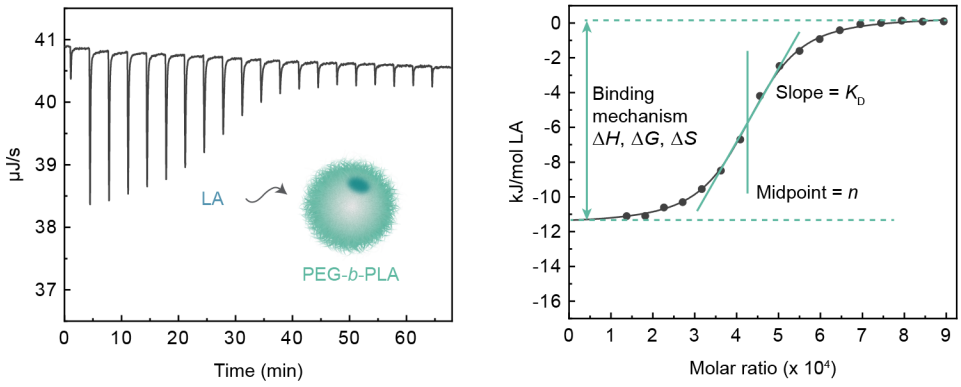
LA (pK_a 3.86) would exist in ~85% in the unionized form (LA) and ~15% in the ionized form (Lactate^{-}) under the condition applied (10 mM, pH 3.1). To consider non-electrolyte

diffusiophoresis and electrolyte diffusiophoresis, the percentage of each species is calculated from the Henderson-Hasselback equation as below:

$$pH = pK_a + log\frac{[Lactate^-]}{[LA]} \quad (1)$$

where [Lactate$^-$] is the concentration of ionized Lactate$^-$, and [LA] is the concentration of unionized LA. Since LA has a pK_a value of 3.86, when pH is 3.1, the proportion of ionized Lactate$^-$ to unionized LA would be 0.18 according to equation (1). Thus, there is a ~85% proportion of unionized LA, a ~15% proportion of ionized Lactate$^-$ (with an equal amount of H^+). Unionized LA contributes to non-electrolyte diffusiophoresis, and ionized Lactate$^-$ and H^+ contribute to electrolyte diffusiophoresis.

The interaction between stomatocytes and LA was studied with ITC (**Supplementary Fig. 20**).



Sample	K_D (nM)	n (x 10^4)	ΔH (kJ/mol)	$T\Delta S$ (kJ/mol)	ΔG (kJ/mol)
PEG-*b*-PLA stomatocytes + LA	941	4.17	-23.8	10.6	-34.4

Supplementary Fig. 20 | ITC analysis of the interaction between PEG-*b*-PLA stomatocytes and LA. 0.5 mM LA solution was injected into PEG-*b*-PLA stomatocytes solution at a particle concentration of 1.2 × 10^{-6} mM for ITC measurement. Binding constants (K_D), reaction stoichiometry (n), enthalpy (ΔH), entropy (ΔS) and Gibbs free energy (ΔG) are listed in the table.

(c) Osmophoresis mechanism

Water, PEG-*b*-PLA/Nile red stomatocytes solution (0.5 mg/mL) and glucose solution (10 mM) were flowed through left, middle and right channel, respectively. The fluorescence at the region of interest were recorded for about 6 min and stack-averaged fluorescence intensity was then obtained with NIH-Image J, followed by the plotting of the intensity profile as described

previously.

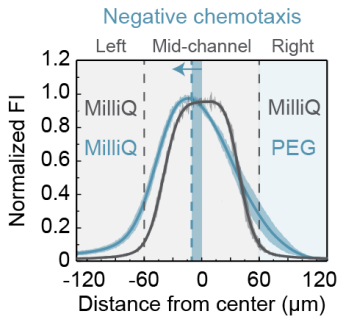
(d) Density-driven mechanism

Water, PEG-*b*-PS/Nile red stomatocytes solution (0.5 mg/mL) and LA solution (10 mM) were flowed through left, middle and right channel, respectively. The fluorescence at the region of interest were recorded for about 6 min and stack-averaged fluorescence intensity was then obtained with NIH-Image J, followed by the plotting of the intensity profile as described previously.

6.4 Validation of degradation-driven mechanism

(a) Negative chemotaxis induced by PEG

Water, PEG-*b*-PLA/Nile red stomatocytes solution (0.5 mg/mL) and PEG solution (10 mM) were flowed through left, middle and right channel, respectively. The fluorescence at the region of interest were recorded for about 6 min and stack-averaged fluorescence intensity was then obtained with NIH-Image J, followed by the plotting of the intensity profile as described previously.



Supplementary Fig. 21 | Negative chemotaxis of PEG-*b*-PLA stomatocytes in the presence of a gradient of PEG. Fluorescence intensity profiles of PEG-*b*-PLA stomatocytes in water (control) and in a gradient of PEG by using the Ψ-channel microfluidic device (n = 4). The mean and standard deviations are plotted. A blue arrow indicates that PEG-*b*-PLA stomatocytes move away from PEG. The blue bar indicates the chemotactic shift.

(b) Chemotaxis using PEG-b-PS stomatocytes as control

Water, PEG-*b*-PS/Nile red stomatocytes solution (0.5 mg/mL) and PEG solution (10 mM) were flowed through left, middle and right channel, respectively. The fluorescence at the region of interest were recorded for about 6 min and stack-averaged fluorescence intensity was then

obtained with NIH-Image J, followed by the plotting of the intensity profile as described previously.

References

1. Toebes, B.J., Cao, F. & Wilson, D.A. Spatial control over catalyst positioning on biodegradable polymeric nanomotors. *Nat. Commun.* **10**, 5308 (2019).
2. Tu, Y. et al. Self-propelled supramolecular nanomotors with temperature-responsive speed regulation. *Nat. Chem.* **9**, 480 (2016).
3. Men, Y., Li, W., Janssen, G.-J., Rikken, R.S.M. & Wilson, D.A. Stomatocyte in Stomatocyte: A New Shape of Polymersome Induced via Chemical-Addition Methodology. *Nano Letters* **18**, 2081-2085 (2018).
4. Peng, F., Tu, Y., van Hest, J.C.M. & Wilson, D.A. Self-guided supramolecular cargo-loaded nanomotors with chemotactic behavior towards cells. *Angew. Chem. Int. Ed.* **54**, 11662-11665 (2015).
5. Duffy, D.C., McDonald, J.C., Schueller, O.J.A. & Whitesides, G.M. Rapid Prototyping of Microfluidic Systems in Poly(dimethylsiloxane). *Anal. Chem.* **70**, 4974-4984 (1998).
6. Somasundar, A. et al. Positive and negative chemotaxis of enzyme-coated liposome motors. *Nat. Nanotechnol.* **14**, 1129–1134 (2019).