Electronic Supplementary Material (ESI) for Nanoscale.

Supplementary Information

Supramolecular nanomotors with "pH taxis" for active drug delivery in tumor microenvironment

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Materials

All reagents and chemicals were purchased from Sigma Aldrich and used as such unless or otherwise stated. CuBr for polymerization was purified by stirring with glacial acetic acid overnight, filtered and washed with ethanol three times followed by drying under vacuum and stored in presence of argon. Styrene was distilled to remove the inhibitor and tetrahydrofuran (THF) was distilled in argon under sodium. Dialysis bag was purchased from Spectra/Por® with MWCO of 12-14000 g/mol. Ultrapure MilliQ water was obtained from QPOD MilliQ system and used for dialysis of polymersomes, encapsulation and nanoparticle tracking analysis (NTA). Doxorubicin-HCl was obtained from Bio Connect B.V.

Instrumentation

Nuclear Magnetic resonance (NMR) characterization was carried out on Varian Inova 400 spectrometer in Chloroform ($CDCl_3$). Gel permeation chromatography (GPC) equipped with PL gel 5µm mixed D column calibrated for polystyrene (580 to 377,400 g/mol) was carried out on instrument acquired from Shimadzu with THF as eluent. Transmission electron microscopy (TEM) was carried out on JEOL TEM 1400 equipped with CCD camera at 60 kV. Samples were prepared by drop casting 6 µl of appropriately diluted

samples on a carbon coated Cu grid (200 mesh) and dried overnight at room temperature. Cryogenic TEM was also carried out with JEOL TEM 2100. All images analysis was carried out using ImageJ, available in a public domain http://fiji.sc/.[1] Malvern Zetasizer nano S was used for dynamic light scattering (DLS) measurements equipped with He-Ne laser of wavelength 633 nm. Nanoparticle tracking analysis (NTA) was carried out on NanoSight LM 10 from Malvern instruments. Leica Microsystems TSC SP8 HyVolution confocal microscope was used to visualize the cellular uptake and cytotoxicity assays.

Supplementary Methods

Synthesis of poly(ethylene glycol)-*b*-polystyrene (PEG-*b*-PS) block copolymer

Firstly, the poly (ethylene glycol)$_{44}$ macro initiator was synthesized as reported earlier.[2] In brief, poly(ethylene glycol) methyl ether (5.00 g, 2.50 mmol) was dried with toluene by co-evaporation to remove the water molecules present. THF was added to dissolve the polymer and added to a flame-dried Schlenk flask, with subsequent addition of trimethylamine (1.04 ml, 7.50 mmol). The flask was cooled to 0 °C with dropwise addition of α-bromoisobutyrl bromide (616 µl, 5.00 mmol). The solution was stirred for 24 h with slowly warming to room temperature. Once the reaction was complete, the white precipitate was filtered and the solution was evaporated to concentrate the solution (3x). The resulting polymer was precipitated in ice-cold diethyl ether (2x). The filtrate powder was vacuum dried overnight and used as such.

For synthesizing PEG-*b*-PS atom-transfer living radical polymerization (ATRP) following was used as reported earlier.[3] Schlenk tube (flame dried under vacuum) was evacuated and refilled with Ar (3x) and charged with CuBr (45 mg, 0.32 mmol). After this PMDETA (66 µL, 0.32 mmol) dissolved in anisole (0.5 mL) was added dropwise and stirred

vigorously for 15 mins followed by styrene (5 mL, 43.6 mmol) dissolved in anisole (1 mL) addition through a syringe and degassed for 15 mins. The solution was cooled to 0 °C followed by addition of PEG macro initiator (215 mg, 0.1 mmol) synthesized dissolved in anisole (1 mL) and degassed for another 15 mins. The tube was transferred to oil bath at 90 °C and the polymerization reaction was monitored continuously with ^{1}H NMR. Upon attaining the desired degree of polymerization the reaction was stopped by addition of 1-phenyl-trimethylsiloxyethene (1.91 mL, 9.28 mmol) and stirred for 2 h at room temperature. The solution was diluted with CH_2Cl_2 and extracted in presence of EDTA (65 mM) and was dried with $MgSO_4$ and concentrated. The polymer was obtained by precipitation in MeOH (3x) and dried under vacuum overnight. The polymer was obtained as a white product with Mw of 2.1 x 10^4 g/mol (^{1}H NMR in $CDCl_3$) and M_w/M_n of 1.003 (GPC).

Fabrication of $CaCO_3$ stomatocyte nanomotors ($CaCO_3$-sto)

Briefly, 10 mg of PEG_{44}-b-PS_{180} was dissolved in 1 ml of THF:Dioxane (4:1) mixture and stirred for 30 mins at 900 rpm. 1 ml of water was added with a syringe pump at rate of 1 mL/hr with continuous stirring, forming polymersomes. The polymersomes were transferred into a dialysis bag by tightly closing the bag and dialyzed with aqueous solution of 100 mM $CaCl_2$ solution with solution changes every 15 mins. for 1 h, 1 hour for next two hours and at the end of the day. Next day, the resulting stomatocytes were washed twice with distilled water at 10000 rpm for 10 mins. ($CaCl_2$-sto). From this, as prepared 100 µL solution was taken and aqueous Na_2CO_3 solution (0.33 M) was added at 1 mL/hr (0.33 M, 500 µL), stirred and washed twice at 10000 rpm for 10 mins and used as such for further analysis.

Fabrication of $CaCO_3$-sto with Nile Red

The stomatocytes fabrication was carried out as above, with an additional step of adding 40 µL Nile red (1 mM) during dissolving the polymer in THF:Dioxane (4:1) mixture.

DOX entrapment in $CaCO_3$-sto

The entrapment of DOX drug molecules was carried out with the same procedure as fabricating CaCO3-sto, with an extra step of adding DOX (1 mg) to Na_2CO_3 solution and stirring for 10 mins to dissolve DOX, before its addition to $CaCl_2$-sto. All the steps were carried out at 4º C inside a fridge. The as obtained samples were washed with Na_2CO_3 solution and aliquots were collected to calculate the amount of drug entrapped in $CaCO_3$-sto according to the following equation:

Entrapped DOX in $CaCO_3$-sto = $DOX_{Total} - DOX_{Wash}$

In above equation DOX_{total} is the total amount of DOX supplied for entrapment in Na_2CO_3 solution and DOX_{Wash} is the amount of DOX present in the wash. DOX content in wash was calculated by a calibration curve obtained with different concentration of DOX in Na_2CO_3 solution.

Movement analysis

The stomatocytes were diluted to appropriate concentrations under different pH conditions suitable for nanoparticle tracking analysis (Nanosight LM10). All results were analyzed using NTA 2.2 software. The speed of the nanomotors were calculated by fitting the mean square displacement (MSD) with self-diffusiophoretic model proposed by Golestanian and coworkers. The system having Brownian motion shows a linear fit of the MSD curves, while those with propelled motion shows a parabolic fit from which the speed can be extracted according to the equation $(V^2)(\Delta t^2) + (4D)\Delta t$.

Collecting of cell medium

HeLa Cells were grown in Dulbecco's modified Eagle's medium (DMEM) buffer with 10% fetal bovine serum and 100 µg/mL streptomycin for 5 days. The conditioned acidic medium was collected and used as for further experimentation.

Chemotactic studies

The chemotaxis experiments were carried out using µ-slide chemotaxis plate from Ibidi. In brief, the conditioned acidic media collected from above was loaded on the left (65 µL) and middle chamber (6 µL) of the plate. It was followed by addition of diluted samples of stomatocytes (control) and $CaCO_3$-sto (test) fabricated with Nile red in distilled water on the right side of the chamber (65 µL) and used to study the migration of particles in the pH gradient generated in the plate.

Cell Uptake of DOX encapsulated stomatocyte nanomotors

To conduct the cell uptake experiment, 300 µL of Dulbecco's modified Eagle's medium (DMEM) buffer with 5×10^4 cells/well was added to an 8-well plate (ibidi GmbH) and incubated overnight at 37 °C with 5% CO_2. The incubation buffer was then replaced by 300 µL of DMEM buffer with free DOX or DOX encapsulated stomatocyte nanomotors (Dox concentration = 5 µg/mL). After 4 hrs incubation, the cell culture medium was removed, and cells were washed with PBS three times and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. For nuclear staining, 4′,6-diamidino-2-phenylindole (DAPI) (Millipore) was added and incubated for 1 h. After the staining solution was removed, cells were washed with PBS buffer three times. The stained cells were imaged by Leica SP8 AOBS WLL confocal microscope

Cytotoxicity assay

The cytotoxicity of free DOX and DOX encapsulated stomatocyte nanomotors was evaluated using HeLa Cells. Cells were seeded into 96-well plates at a density of 5×10^3 cells per well. After 24 hrs incubation at 37°C with 5% CO_2, cells were treated with free DOX and DOX encapsulated stomatocyte nanomotors at different concentrations (0, 0.5, 1, 2.5, 5, 10 and 20 μg/mL) and incubated for another 48 hrs. After washing 3 times with PBS buffer, 100 μL of 10 times diluted Cell Counting Kit (CCK8) solution was added to each well. After the cells were incubated for 2 hrs with CCK8 solution, and plate reader was used to evaluate cell viability.

References

(1) Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B.; Tinevez, J.-Y.; White, D. J.; Hartenstein, V.; Eliceiri, K.; Tomancak, P.; Cardona, A., Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **2012,** *9*, 676-682.

(2) Peng, F.; Tu, Y.; van Hest, J. C.; Wilson, D. A., Self-Guided Supramolecular Cargo-Loaded Nanomotors with Chemotactic Behavior towards Cells. *Angew. Chem. Int. Ed.* **2015,** *54*, 11662-11665.

(3) Tu, Y.; Peng, F.; André, A. A. M.; Men, Y.; Srinivas, M.; Wilson, D. A., Biodegradable Hybrid Stomatocyte Nanomotors for Drug Delivery. *ACS Nano* **2017,** *11*, 1957-1963.

Supplementary Figures

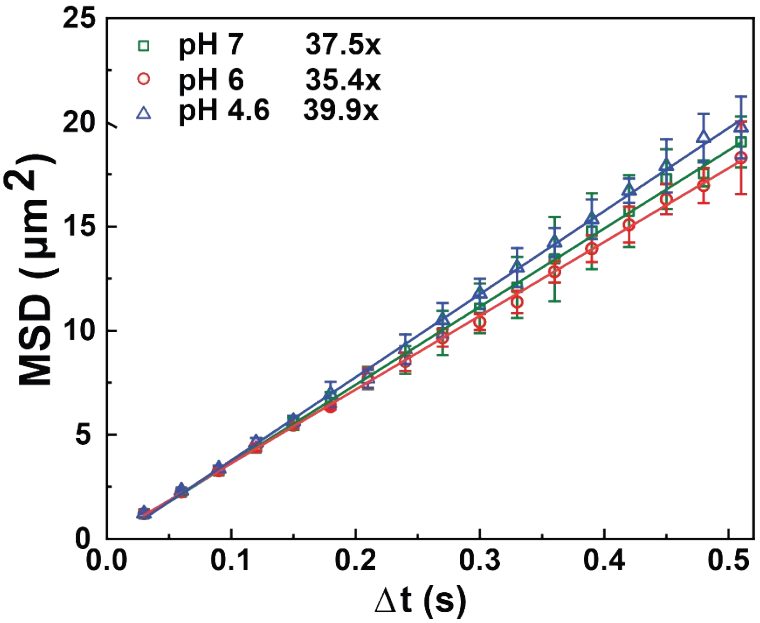

Figure S1. MSD curves for $CaCl_2$-sto under pH 5, 6 and 7 with linear fit showcasing Brownian motion.

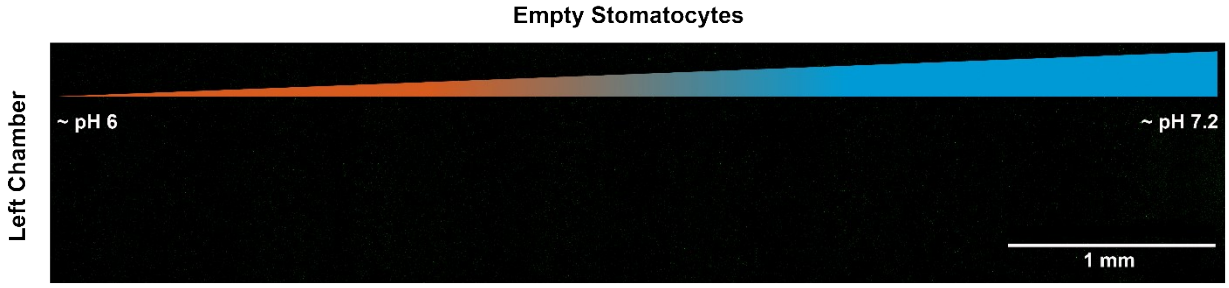

Figure S2. Panoramic view of left chamber of chemotactic plate loaded with empty stomatocytes after 1 hr showing no movement in presence of pH gradient.

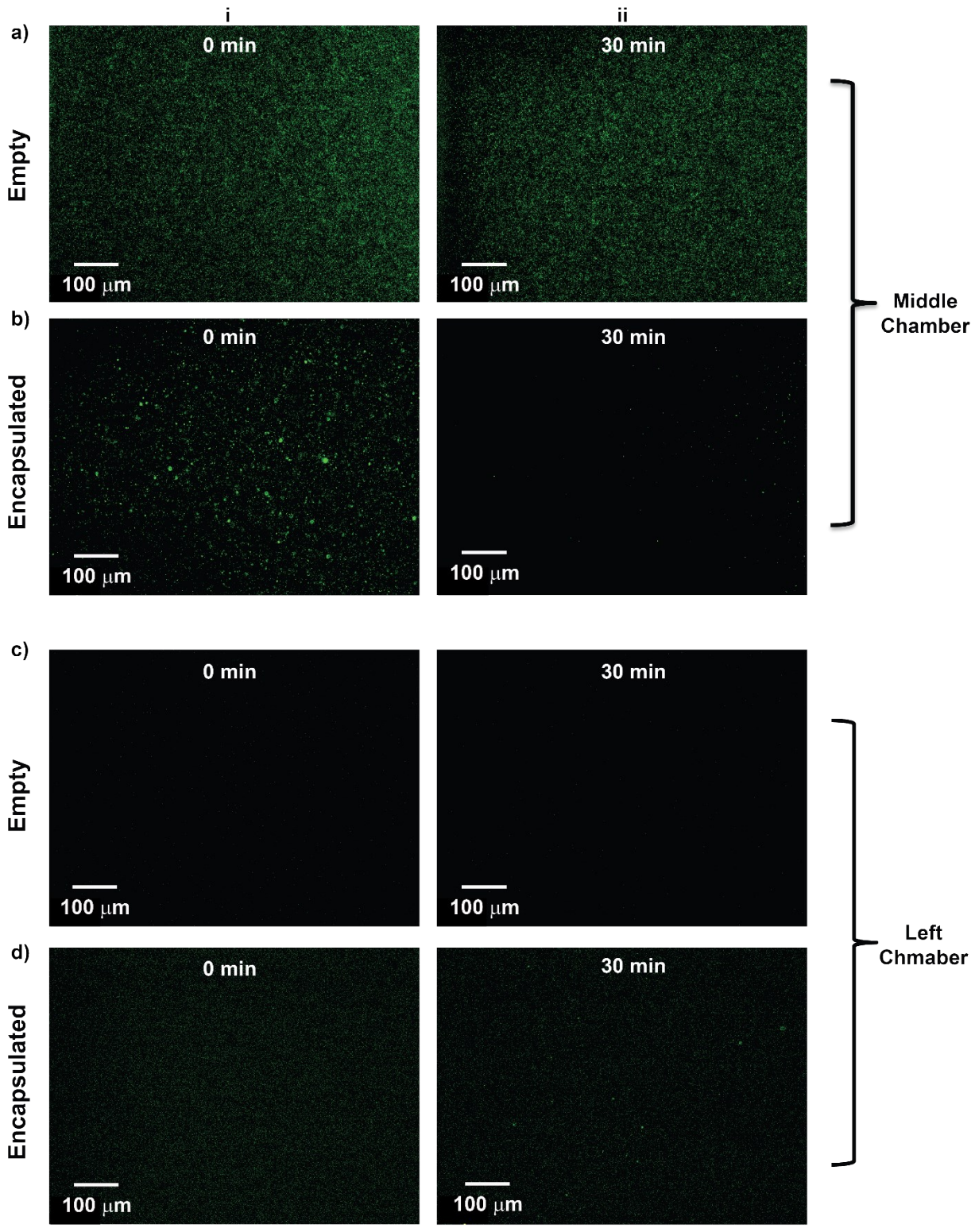

Figure S3. Fluorescence microscopy images of middle chamber with a) empty, b) $CaCO_3$-sto and left chamber with c) empty and d) $CaCO_3$-sto at i) 0 and ii) 30 mins,

respectively. Empty stomatocytes did not show any decease in particles as corresponding to $CaCO_3$-sto in middle chamber after 30 mins and left chamber showed only $CaCO_3$-sto to reach the end of left chamber after 30 mins.