Supporting Information for

Antibacterial Activity of Hydrophobicity Modulated Cationic Polymers with Enzyme and pH-Responsiveness

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Experimental Section

Materials. 4-Vinyl benzyl chloride (4-VBC, 90%), myristic acid (MA, >99%), octanoic acid (OA, >99%), ethanolamine (≥99%), anhydrous *N,N*-dimethylformamide (DMF, 99.8%), pyrene (98%), Nile red (NR, ≥ 97%), furan (99%), maleic anhydride (99%), propidium iodide (PI, ≥ 99%), glutaraldehyde, urea (molecular biology grade), esterase [from porcine liver, lyophilized powder, >15 units/mg (U/mg) solid], lipase (from *Pseudomonas cepacia*, powder, >30 U/mg solid) and 2,2′-azobis(2-methylpropionitrile) (AIBN, 98%) were purchased from Sigma-Aldrich and used as received except AIBN, which was used after recrystallization from methanol. 1,4-Dioxane (99%), L-lysine monohydrochloride (99%), triethylamine (98%),

dicyclohexylcarbodiimide (DCC, 99%), di-*tert*-butyldicarbonate (DiBoc, 98%), 4-dimethylaminopyridine (DMAP, 99%), potassium carbonate (K_2CO_3, 98%), 10X phosphate buffer saline (PBS, molecular biology grade), and triton X-100 (molecular biology grade) were obtained from Sisco Research Laboratories (SRL) Pvt. Ltd., India and used without any further purification, except 1,4-dioxane which was passed through a basic alumina column just before use. Anhydrous sodium sulphate (Na_2SO_4, 99%), toluene, trifluoroacetic acid (TFA), dichloromethane (DCM), dimethyl sulfoxide (DMSO), methanol, diethyl ether, acetone, hexane (a mixture of isomers), and ethyl acetate were purchased from Merck and used as received. NMR solvents such as $CDCl_3$ (99.8% D), DMSO-d_6 (99% D), and D_2O (99% D) were purchased from Cambridge Isotope Laboratories, Inc., USA. *N,N′*-di-*tert*-butyl carbamate (Boc)-L-lysine (DBL) was prepared following a previous literature procedure.[1] 2-Dodecylsulfanylthiocarbonyl-sulfanyl-2-methylpropionic acid (DMP) was synthesized following an earlier report.[2] *Escherichia coli* (*E. coli*) DH5α was purchased from Bio Bharti Life Science Pvt. Ltd and *Bacillus subtilis* (*B. subtilis*) was gifted by Prof. Partha Pratim Datta, DBS, IISER Kolkata. *E. coli* and *B. subtilis* were cultured in the Mueller Hinton (MH) media (HiMedia Laboratories Pvt. Ltd., India) under continuous shaking (180 rpm) at 37 °C. Human intestinal cells (INT407) was procured from the National Centre for Cell Science (NCCS, Pune, India) and maintained in complete Dulbecco's Modified Eagle's medium (DMEM) (Invitrogen, Thermo Fisher Scientific), supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific), 10,000 U/mL of penicillin, and 10,000 μg/mL of streptomycin (HiMedia Laboratories Pvt. Ltd.) under 5% CO_2 at 37 °C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) and acridine orange (AO) were purchased from HiMedia Laboratories Pvt. Ltd. 96-Well tissue culture plates and dialysis membrane with molecular weight cutoff (MWCO) = 2,000 g/mol (spectra/por^6, diameter-29 mm, flat width-45 mm,

volume/length-6.4 mL/cm) were purchased from Tarsons, India and Spectrum Laboratories, USA, respectively.

Instruments used for characterization

^{1}H Nuclear magnetic resonance (NMR) spectroscopy. 500 MHz Bruker Avance III NMR and 400 MHz JEOL ECS NMR spectrometers were used for performing ^{1}H and ^{13}C NMR spectroscopic characterization of monomers and polymers using tetramethylsilane (TMS) as internal standard.

Size exclusion chromatography (SEC). The number average molar mass ($M_{n,SEC}$) and dispersity ($Đ$) of the polymers were measured using Waters SEC instrument running with 0.8 mL/min DMF eluent flow rate at 40 ºC. This instrument consists of; (a) a Waters 2414 refractive index (RI) detector, (b) one PolarGel-M guard column (50 × 7.5 mm), (c) two PolarGel-M analytical columns (300 × 7.5 mm), and (d) a Waters 1515 HPLC pump. The calibration curve was prepared by using poly(methyl methacrylate) (PMMA) standards.

UV-Vis spectroscopy. Perkin Elmer Lambda 35 spectrophotometer instrument was used to record the UV-Vis spectral data.

High-resolution mass spectrometry (HRMS). HRMS spectra of monomers are recorded in a Waters XeVO G2-XS QTof instrument.

Fluorescence spectroscopy. Emission spectra were recorded on a Horiba JobinYvon (Fluoromax-3, Xe-150 W, 250-900 nm) instrument.

Dynamic light scattering (DLS). Malvern Zetasizer Nano ZS instrument (UK) was used to measure the hydrodynamic diameter and zeta potential of all polymers. The instrument is equipped with 630 nm wavelength 4 mV helium-neon laser at a scattering angle of 173º.

Field emission scanning electron microscopy (FE-SEM). Carl Zeiss Sigma instrument (acceleration voltage 200 kV) was used for taking images of the aggregated nanoparticles.

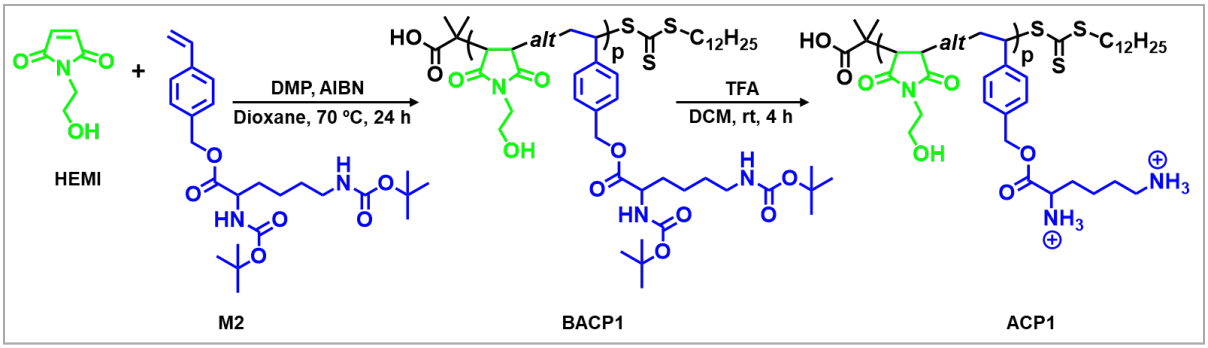
Transmission electron microscopy (TEM). JEOL JEM 2100F electron microscope (operational voltage 200 kV) was used for taking images of the aggregated nanoparticles.

Confocal microscopy. Confocal images of the stained bacteria were done by a Leica SP8 confocal microscope (Leica Microsystems).

Optical density. Epoch2 micro-plate reader (Biotek) was used for optical density measurements of bacteria samples.

Experimental section

Synthesis of alternating copolymer without fatty acid appended maleimide (ACP1). The alternating copolymer **BACP1** (Scheme S1) was prepared using the 2-hydroxyethyl maleimide (HEMI), lysine appended styrene-based monomer (**M2**), DMP and AIBN. In a septa-sealed 20 mL glass vial, HEMI (200 mg, 1.41 mmol), **M2** (652 mg, 1.41 mmol), DMP (34.47, 0.094 mmol) and AIBN (7.71 mg, 0.047 mmol) were dissolved in 4 mL 1,4-dioxane equipped with a magnetic stirrer. Then, the vial was kept in a preheated polymerization block at 70 °C in stirring condition for 24 h after purging the polymerization reaction vial with dry N_2 for 10 min. After 24 h, the polymer was precipitated in hexane. Then, air-dried polymer was dissolved in a minimum amount of acetone and precipitated in hexane. This process was repeated 4 times, and finally, the polymer was dried under vacuum for 12 h.



Scheme S1 Synthesis of the alternating copolymer, **ACP1**.

Next, deprotection of *tert*-butyl carbamate (Boc) groups from **BACP1** polymer was carried out by TFA in DCM at room temperature (rt). In a 20 mL glass vial, 0.2 g of **BACP1** was dissolved in 2 mL DCM, and 2 mL TAF was added drop-wise to the solution. After 4 h, the solvent was removed under a rota-evaporator, dissolved in a minimum amount of methanol, and precipitated from diethyl ether. This process was repeated 3-4 times, and finally, the polymer **ACP1** was dried under vacuum for 12 h (Scheme S1).

Cell cytotoxicity assay. To access the cytotoxicity of the polymers, standard MTT assay in INT 407 cells has been performed.[3] Briefly, 1×10^4 cells/well for each well in a 96-well cell culture plate were grown in complete DMEM medium containing 10% FBS, 10,000 units/mL of penicillin and 10,000 μg/mL of streptomycin, and allowed to grow up to ~80% confluency. Next, the serial dilution of the polymer was made in a complete DMEM medium containing 10% FBS, 10,000 units/mL of penicillin and 10,000 μg/mL of streptomycin. Cells of each well were treated with polymers at different concentrations (100 μL polymer solution from each dilution) and incubated for 36-48 h at 37 ºC under 5% CO_2. For positive control of cell death, cells treated with 6M urea where we generally see 100% cell death due to the hyperosmolar stress induced by high concentration of urea causing cell cycle delay and reducing the number of cells in a concentration-dependent manner. Following incubation, cells were washed two times with 1X PBS. Then, 100 μL 0.1 mg/mL MTT solution in media was added to each well and incubated at 37 ºC under 5% CO_2 for 4 h. Finally, the metabolically formed formazan crystals from MTT reagent were dissolved in 100 μL DMSO and the absorbance was taken in Epoch 2 microplate reader at 595 nm.

Hemolysis assay. The hemotoxicity of all the polymers was checked with 5% v/v fresh chicken erythrocyte solutions. Briefly, the blood sample was collected in blood-collecting tubes coated with ethylenediaminetetraacetic acid (EDTA), which acts as an anticoagulant. Then, red blood cells (RBCs) were harvested by centrifugation at 500 rpm for 10 min, followed by

washing (2-3 times) with fresh 1X PBS. Subsequently, 5% v/v chicken erythrocyte solution was prepared in PBS and was further used to check hemotoxicity. Each polymer was tested for different concentrations between 10-1000 µg/mL. Next, 100 µL polymer solutions of different concentration was first added in a 96-well round-bottom microplate with 1X PBS as negative control and 10% triton-X 100 as positive control. Then, 100 µL of RBC solution was added to each well and incubated for 3 h at 37 ºC. The plate was centrifuged at 3000 rpm for 10 min and 100 µL supernatant was collected. The absorbance was taken in Epoch2 micro-plate reader at 540 nm. The percentage of hemolysis was calculated according to the following equation (1),

$$\% \text{ Hemolysis} = (A_{\text{sample}} - A_{\text{negative control}})/(A_{\text{positive control}} - A_{\text{negative control}}) \times 100 \quad (1)$$

Antibacterial assay. To determine the bactericidal activity of the test polymers, *E. coli* and *B. subtilis* were used in MH broth. Primary cultures of both bacteria were grown ($OD_{600} \sim 0.6$) and used as starter cultures for the antibacterial experiments. Each polymer was added at different concentrations, between 10-200 µg/mL for *E. coli* and 10-100 µg/mL for *B. subtilis*, and kept for incubation at 37 ºC at 180 rpm. The PBS buffer without polymer was kept as control (80 µL of an overnight culture of bacteria + 8 mL MH broth + buffer). Next, absorbance (~600 nm) was taken at 0 h and 16 h for both test and control, followed by % cell viability was calculated using the following equation (2),

$$\% \text{ Cell viability} = [A_{\text{test(16 h)}} - A_{\text{test(0 h)}}/A_{\text{control(16 h)}} - A_{\text{control(0 h)}}] \times 100 \quad (2)$$

Well diffusion assay. To confirm the antibacterial effect of the polymers, well diffusion assay was performed. For this, MH agar plates were prepared using 1% agar powder and MH broth. Then, wells were prepared using a sterile 2-200 µL micropipette tip. 100 µL of overnight grown *E. coli* and *B. subtilis* culture was smeared on the MH agar plate surface homogeneously. The plates were allowed to dry for 20 min. Then, 200 µL polymer solution of 4 different concentrations (20, 50, 100 and 200 µg/mL) and PBS buffer solution (control) were added to

each plate. Next, the plates were incubated at 37 ºC for 16 h. Next day, the visible diameter of the zone up to which the polymer concentrations prevent the growth of bacteria (zone of inhibition, ZOI) was measured and analyzed further.

FE-SEM. To visualize the effect of polymers on *E. coli* and *B. subtilis* bacteria, FE-SEM was performed. Briefly, both bacteria were grown in MH broth and treated separately with **ACP2** and **ACP3** polymers. Both the bacteria grown without treatment of polymer (only PBS buffer) serve as the control for the experiment. Next, after centrifugation at 5000 rpm for 5 min, bacterial cells were washed with PBS and fixed with 2.5% glutaraldehyde for 1 h at room temperature. Next, bacterial cells were washed with PBS and sequentially dehydrated with 35, 50, 70, 95 and 100% ethanol, followed by vacuum drying. Finally, the fixed and dehydrated samples were vacuum-dried for 1 h and fixed to aluminium stubs with silver conductive paint and sputter-coated with platinum. Then, the samples were examined using FE-SEM.

Live/dead cell assay. The effect of the polymers on bacterial cells was studied by live/dead cell viability assay. As mentioned earlier, both bacteria were grown in MH broth for 4 h, treated separately with **ACP2** and **ACP3** polymers, and PBS buffer (control), followed by incubation for an additional 4 h at 37 ºC. The control and polymer-treated bacterial cells were centrifuged at 5000 rpm for 5 min, washed and resuspended with 1X PBS. 1 µL of PI dye was added to each vial of bacterial cell suspension and incubated for 5 min at room temperature, followed by washing with 1X PBS. Next, cells were stained with AO for 5 min at room temperature and washed with 1X PBS. Then, 5 µL of cell suspension was mounted on a glass slide and images were visualized in a confocal microscope using oil immersion 63X objective (NA 1.4), and the LAS-X life science microscope software was then used to acquire and process the images.

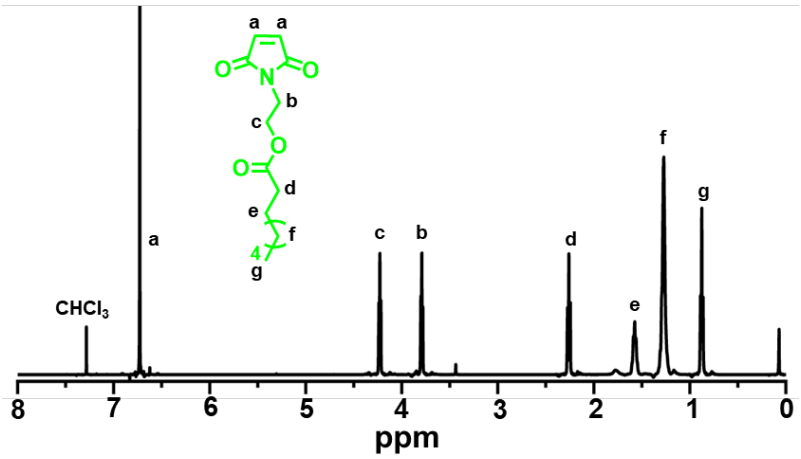


Fig. S1 ^{1}H NMR spectrum of maleimide monomer with octanoate moiety (**M1a**).

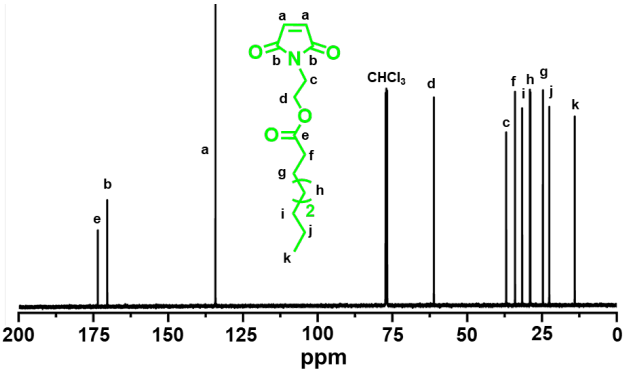


Fig. S2 ^{13}C NMR spectrum of maleimide monomer with octanoate moiety (**M1a**).

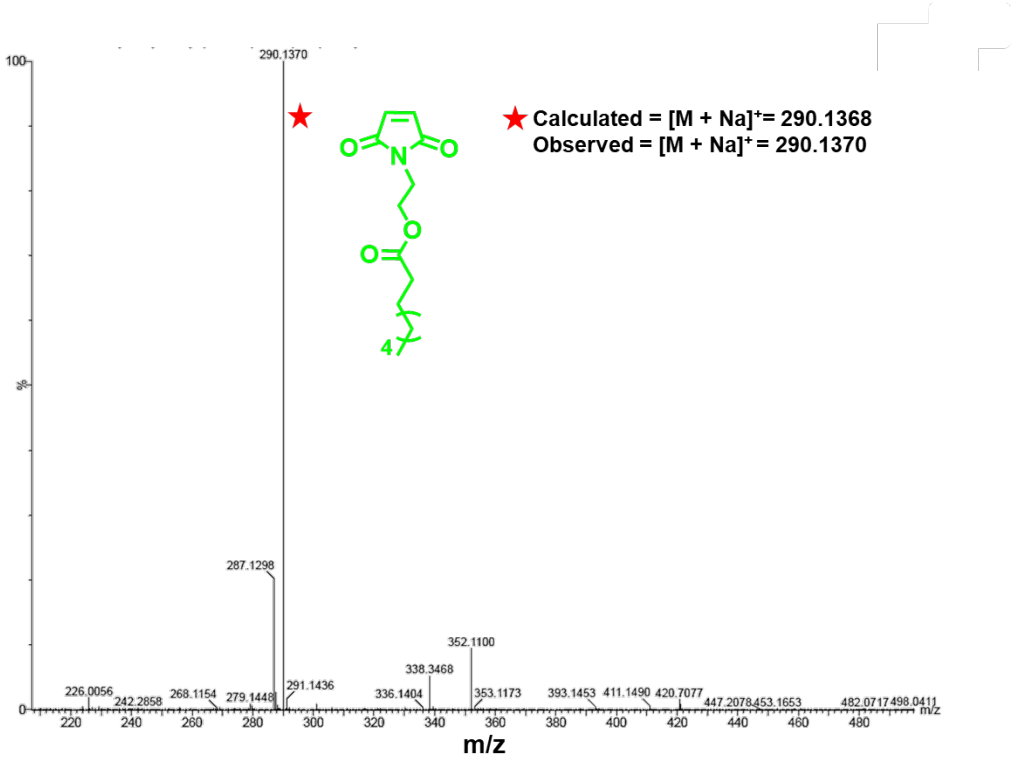


Fig. S3 HRMS of maleimide monomer with octanoate moiety (**M1a**).

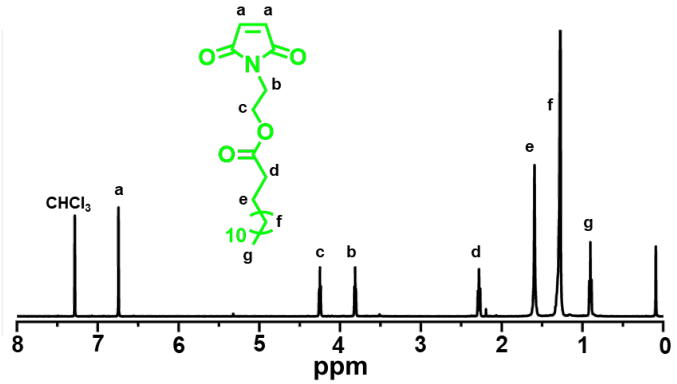


Fig. S4 ^{1}H NMR spectrum of maleimide monomer with myristate moiety (**M1b**).

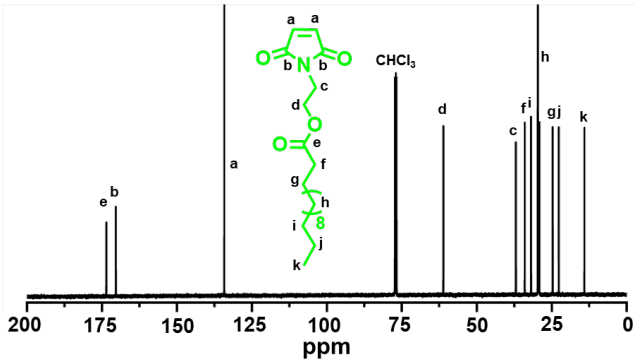


Fig. S5 ^{13}C NMR spectrum of maleimide monomer with myristate moiety (**M1b**).

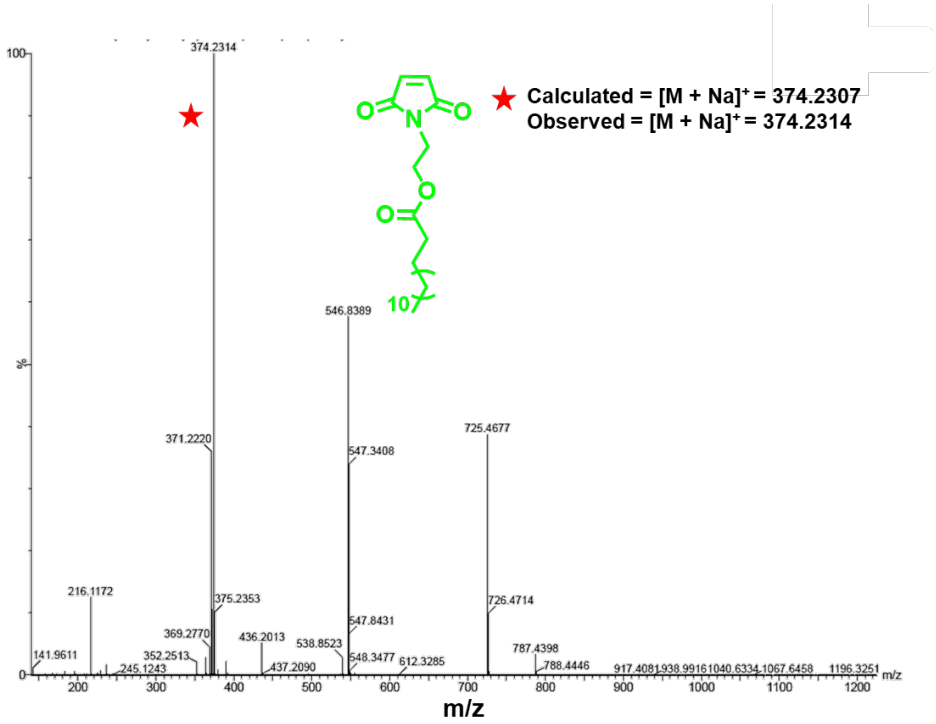


Fig. S6 HRMS of maleimide monomer with myristate moiety (**M1b**).

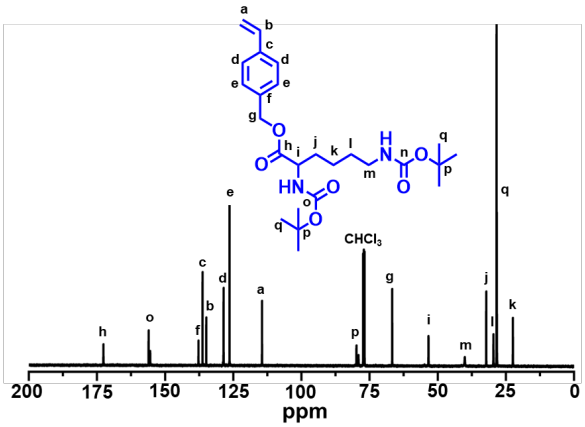


Fig. S7 ^{13}C NMR spectrum of lysine appended styrenic monomer (**M2**).

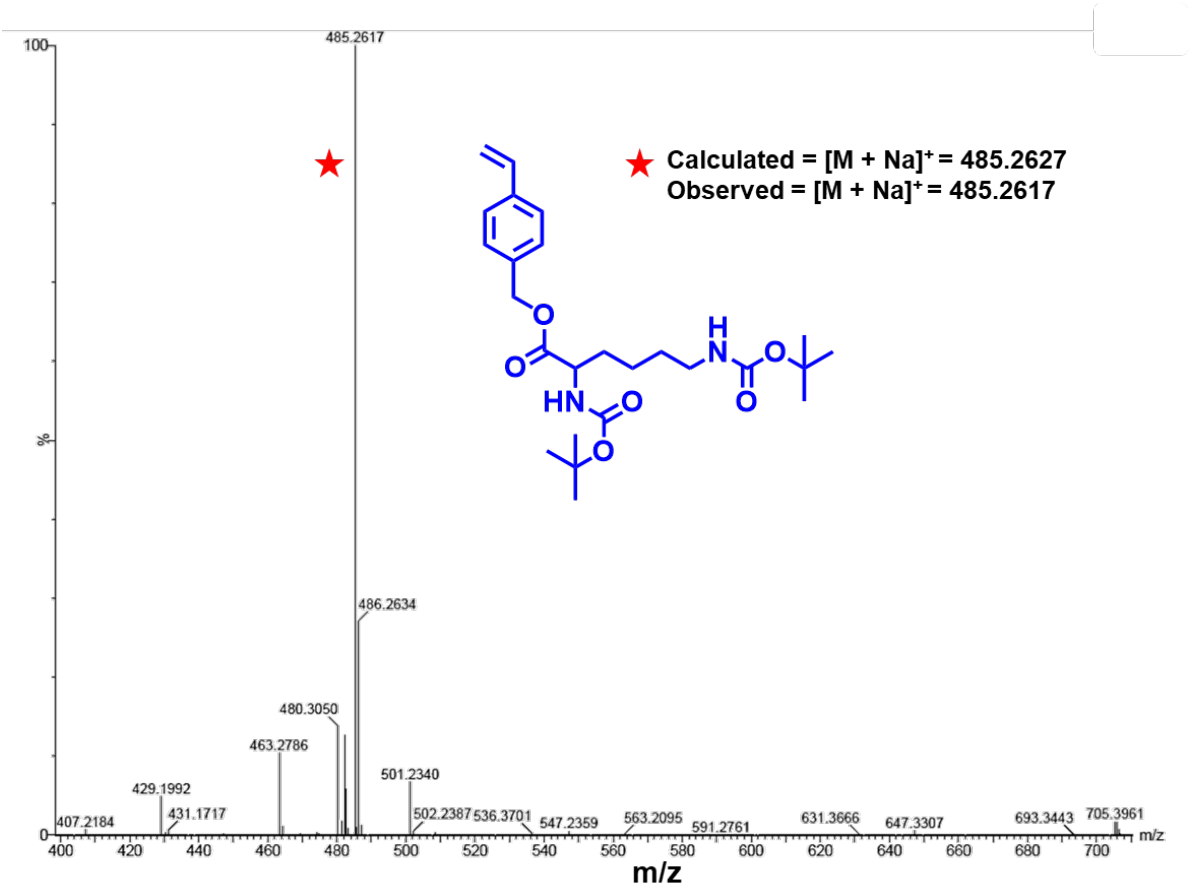


Fig. S8 HRMS of lysine appended styrenic monomer (**M2**).

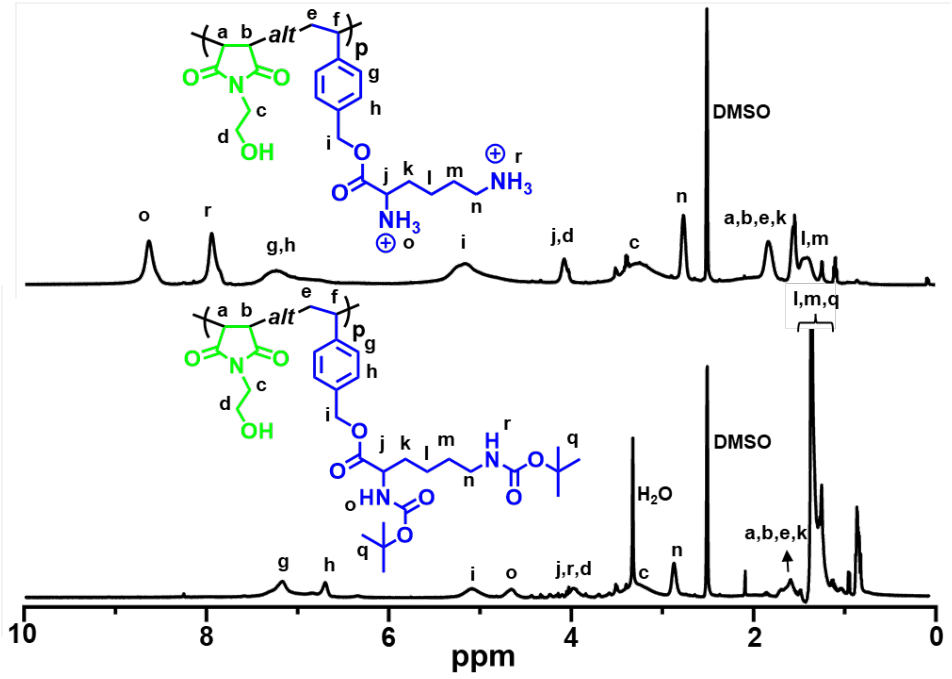


Fig. S9 ^{1}H NMR spectra of **BACP1** and **ACP1**.

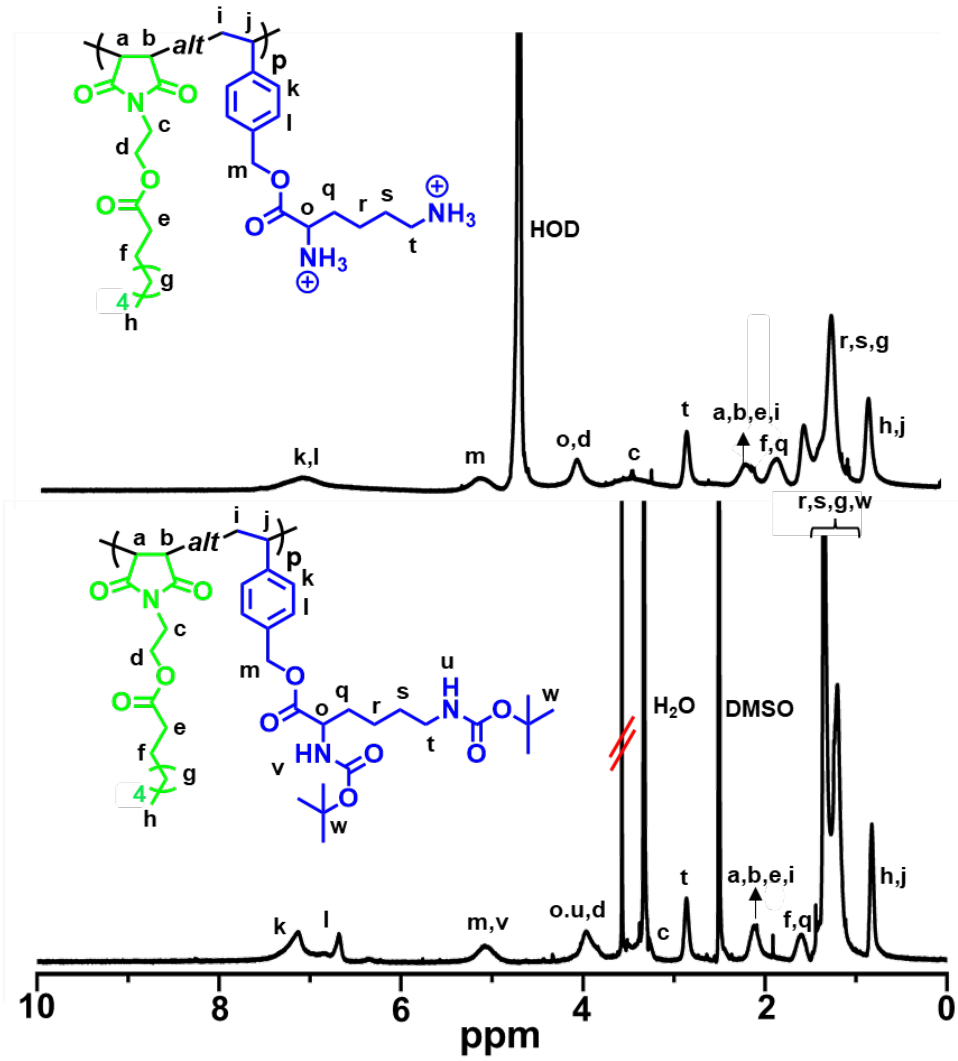


Fig. S10 ^{1}H NMR spectra of **BACP2** and **ACP2**.

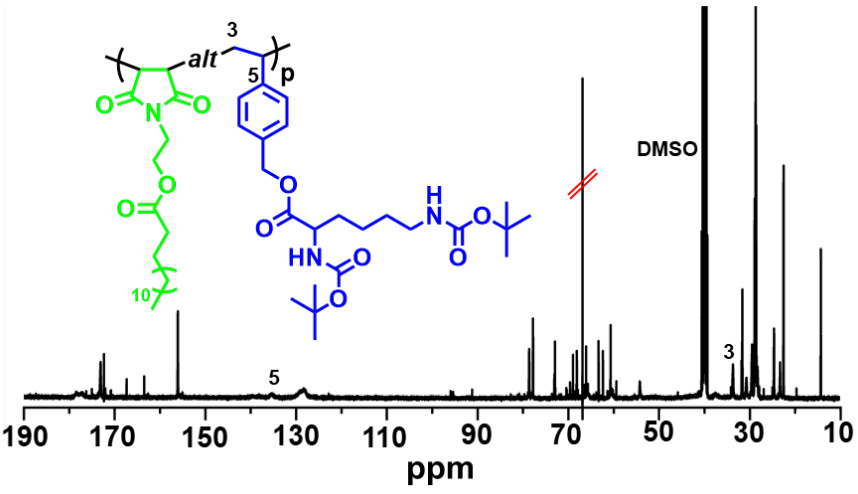


Fig. S11 ^{13}C NMR spectrum of **BACP3**.

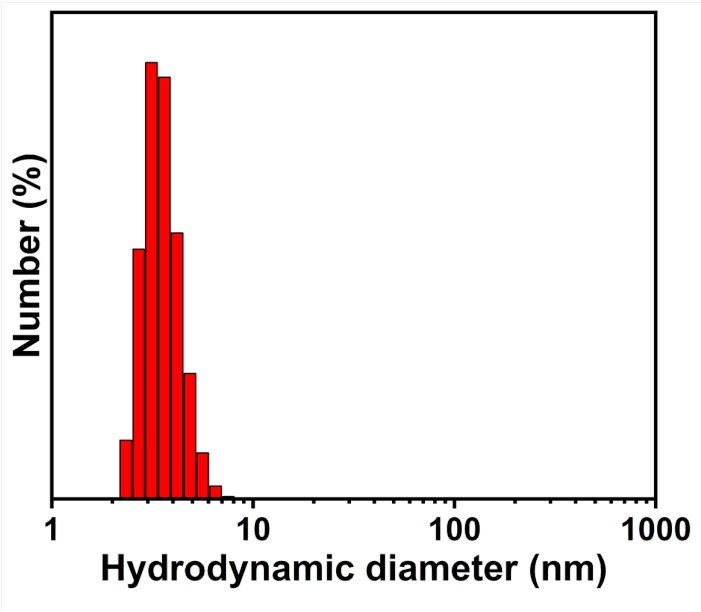


Fig. S12 Hydrodynamic diameter of **ACP1**.

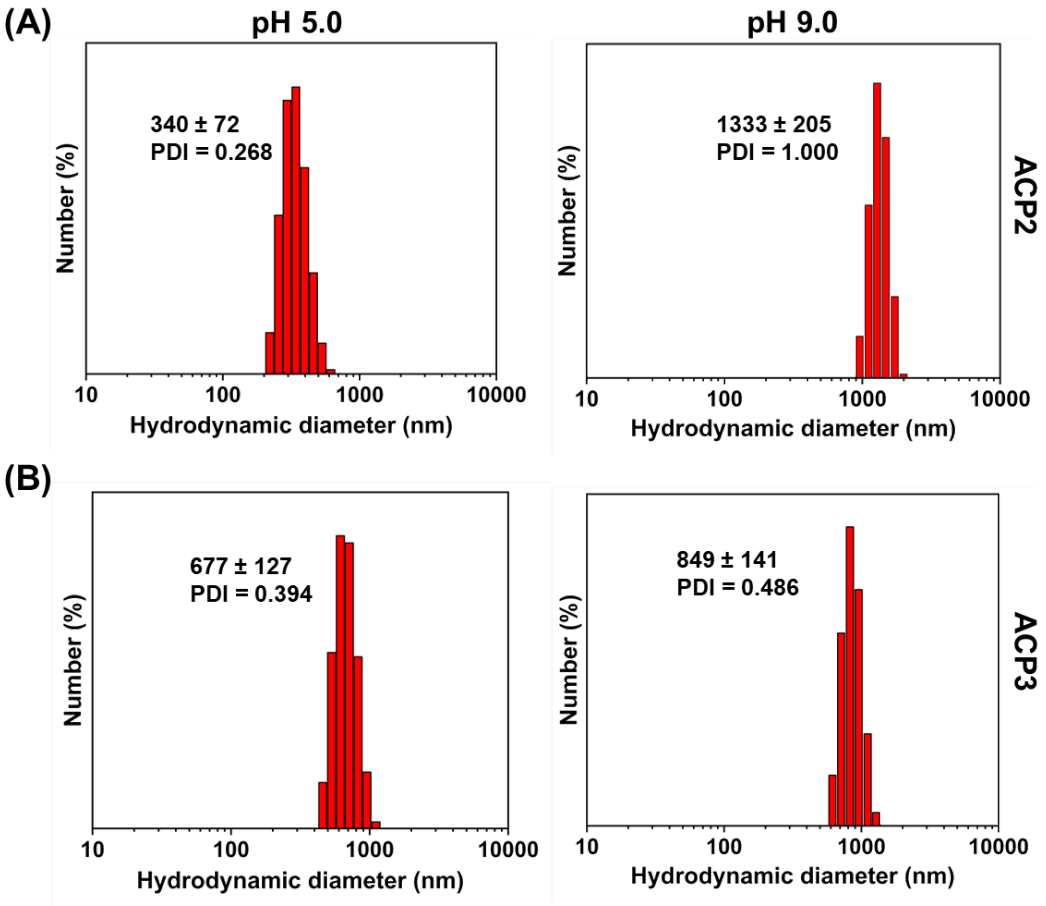


Fig. S13 Hydrodynamic diameter of (A) **ACP2** and (B) **ACP3** at two different pH (5.0 and 9.0).

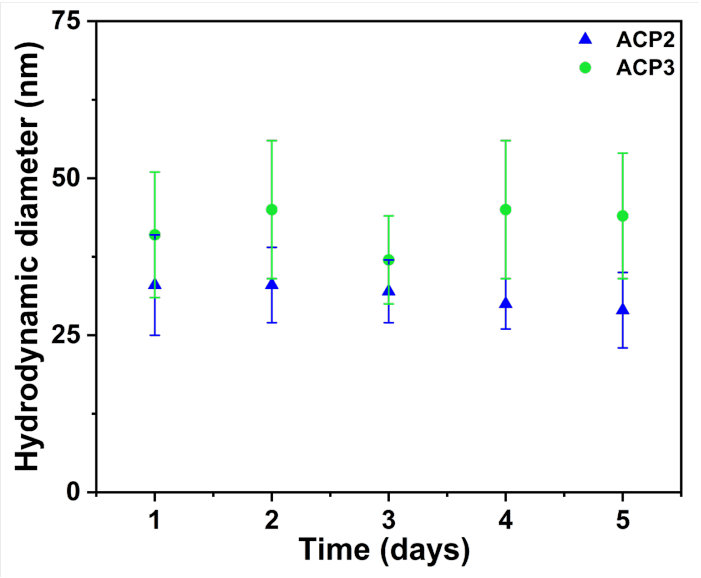


Fig. S14 The stability of the **ACP2** and **ACP3** polymer nanoaggregates in 1X PBS (pH 7.4) for 5 days.

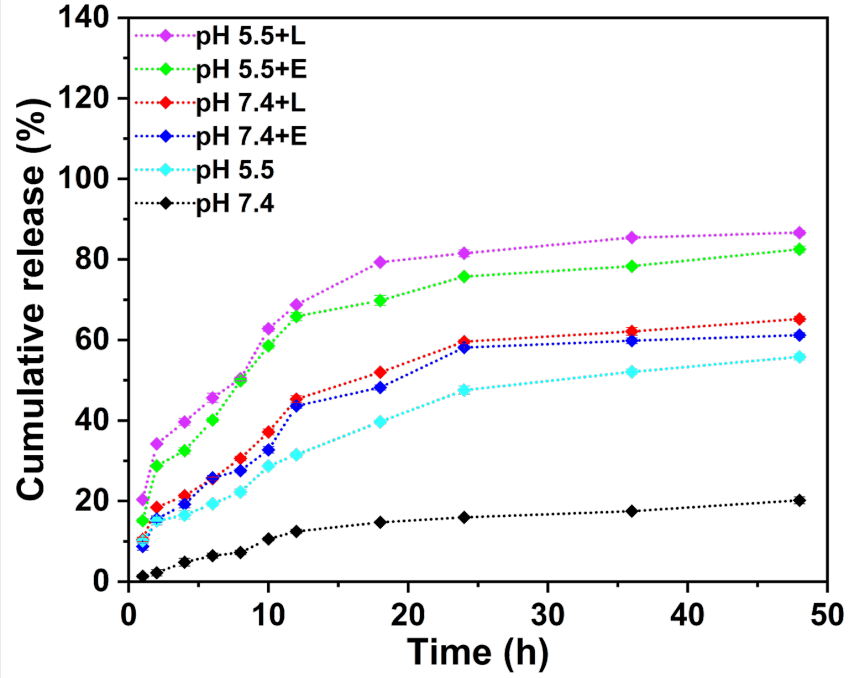


Fig. S15 Time-dependent cumulative release plot of NR from the interior of **ACP2** nanoaggregates in the absence/presence of enzyme and pH.

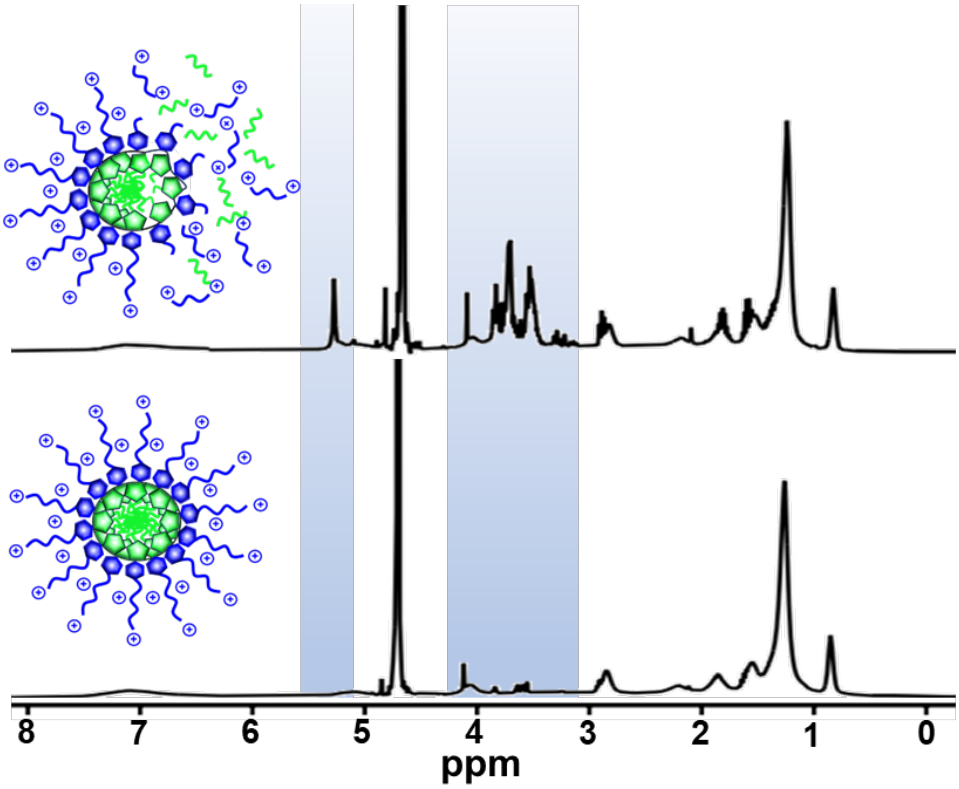


Fig. S16 ^{1}H NMR spectra of degradation of **ACP3** nanoaggregates before (lower line) and after (upper line) treatment with lipase at pH 5.0 in D_2O.

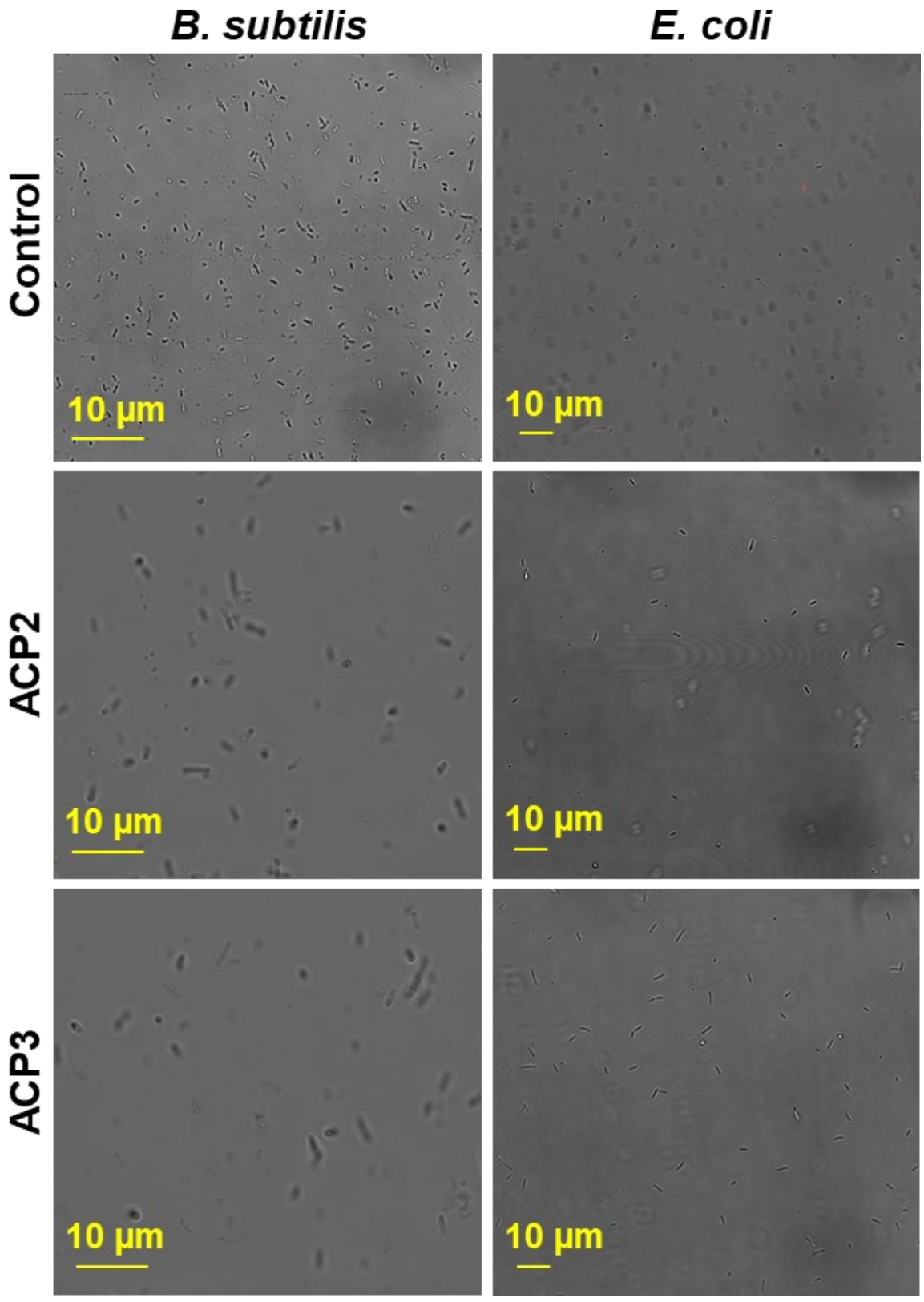


Fig. S17 Bright field images of the *B. subtilis* and *E. coli* before (control) and after treatment with **ACP2** and **ACP3**.

Table S1 Hemolysis, cell viability to healthy cells and minimum inhibitory concentration (MIC) results of two polymers against *B. subtilis* and *E. coli*.

Polymer	HC_{50} (μg/mL)[a]	MIC_{50} (μg/mL)[b]		Selectivity index[c]		EC_{50} (μg/mL)[d]
		B. subtilis	*E. coli*	*B. subtilis*	*E. coli*	
ACP2	> 10^3	26.51	20.37	>38	>50	>500
ACP3	> 10^3	11.62	23.23	>86	>43	>500

[a]HC_{50} (polymer concentration at which 50% treated erythrocytes remain viable) and [d]EC_{50} (polymer concentration at which 50% treated cells are safe) values were calculated using standard hemolysis assay and MTT assay (INT 407 cell lines) fitting curve, respectively. [b]MIC_{50} (polymer concentration that inhibits 50% growth of bacteria)) values were determined in MH broth against *B. subtilis* and *E. coli*. [c]Selectivity index was determined from the ratio of HC_{50} and MIC_{50}.

Table S2 Zeta potential values of **ACP1-ACP3** polymers in pH 5.0 and pH 9.0 solutions.

Polymer	Zeta potential (ξ)	
	pH 5.0	pH 9.0
ACP1	+29.4	+6.59
ACP2	+46.9	+1.70
ACP3	+40.4	+2.13

References

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2. J. T. Lai, D. Filla and R. Shea, *Macromolecules*, 2002, **35**, 6754−6756.

3. R. Ghosh, M. Malhotra, R. R. M. Sathe and M. Jayakannan, *Biomacromolecules*, 2020, **21**, 2896–2912.