Electronic Supplementary Material (ESI) for Polymer Chemistry.

Supporting Information for:

Polysaccharides Meet Dendrimers to Fine-tune the Stability and Release Properties of Polyion Complex Micelles

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1. Materials

PEG-[G3]-$PhCO_2Na$, AF488-PEG-[G3]-$PhCO_2Na$ and PEG-[G3]-$BnNH_2$·HCl were prepared following procedures previously described by our group.[1] CS samples were denoted as CS_x, where "x" represents the degree of polymerization (DP). A commercial CS sample (CS_{480}) was obtained from FMC BioPolymer as hydrochloride salt (Protasan Cl 113, batch number FP-110-02). The degree of acetylation (DA) of CS_{480} and two depolymerized samples (*vide infra*) was determined as 14% by ^{1}H NMR (10 mg/mL) in 2% DCl at 298 K (500 MHz) as previously described by our group.[2] ALG samples were denoted as ALG_y, where "y" represents the DP. Samples ALG_{21}, ALG_{168}, ALG_{389}, ALG_{694}, ALG_{1040}, and ALG_{2140} were obtained from DANISCO; their average molecular weight (MW) and DP were provided by the supplier. HA samples were denoted as HA_z, where "z" represents the DP. Samples HA_{71}, HA_{165} and HA_{497} were obtained from Lifecore (sodium hyaluronate); their average MW were provided by the supplier. Methoxy-poly(ethylene glycol)-block-poly(L-lysine hydrochloride) copolymer (PEG-PLL_{27}) [PEG_{5000}, MW 9300 and DP 27 by ^{1}H NMR] was purchased from Alamanda polymers.

2. General Methods

Depolymerization of CS. CS samples with DP lower than commercial CS_{480} were obtained by nitrous acid depolymerization.[3,4] Briefly, CS_{480}·HCl (500 mg) was dissolved in H_2O (50 mL) under magnetic stirring at rt. Then, 1 M $NaNO_2$ (0.25 mL) was added dropwise and stirring was continued for 3 h, before 0.12 M NaOH was added dropwise until pH 10. The resulting suspension was centrifuged, washing several times with H_2O, to afford CS_{172} (354 mg) as a white foam after lyophilization. Following the same procedure from CS_{480}·HCl (150 mg) dissolved in H_2O (15 mL) and 1 M $NaNO_2$ (0.15 mL), CS_{35}·HCl (98 mg) was obtained as a white foam after stirring overnight, followed by ultrafiltration (Amicon YM1, 1x H_2O, 2x 0.1 M HCl, 3x H_2O) and lyophilization.

Determination of the Molecular Weight of CS. The weight average molecular weight (M_W) of CS was determined by SEC-MALLS. An Iso Pump G1310A (Hewlett Packard) was connected to two PSS Novema GPC columns (10 µm, 30 Å, 8×300 mm, and 10 µm, 3000 Å, 8×300 mm). A PSS SLD7000 MALLS detector (Brookhaven Instruments Corporation) operating at 660 nm and a G1362A refractive index detector (Agilent) were connected on line. A 0.15 M NH_4OAc/0.2 M AcOH buffer (pH 4.5) was used as eluent. Polymer solutions were filtered through 0.2 µm pore size membranes before injection. Polymer concentrations were in the range 0.1 to 5 g/L, depending on DP. Refractive index increment dn/dC values were taken from the literature.[5,6,7]

Atomic Force Microscopy (AFM). PIC micelles prepared in 10 mM PB pH 7.4 were crosslinked by addition EDC (5 equiv per amino group, 100 mg/mL in H_2O). After stirring at rt overnight, reaction mixtures were dialyzed (MWCO 1 kDa) against H_2O (6 x 500 mL) to afford cross-linked PIC.

Samples for AFM imaging were prepared by depositing aqueous solutions of cross-linked PIC micelles (0.025-0.05 mg/mL) onto mica. AFM measurements were performed at an atomic force microscope Multimode Nanoscope V (Veeco) in tapping mode and processed using Vision 32, Nanoscope v7.20 and Nanoscope Analysis software. An average diameter of 53±7 nm for the CS_{35} micelles was determined by measuring the size of 25 micelles.

Cryo-Transmission Electron Microscopy (cryo-TEM). PIC micelles prepared in 10 mM PB pH 7.4 were crosslinked by addition EDC (5 equiv per amino group, 100 mg/mL in H_2O). After stirring at rt overnight, reaction mixtures were dialyzed (MWCO 1 kDa) against H_2O (6 x 500 mL) to afford cross-linked PIC.

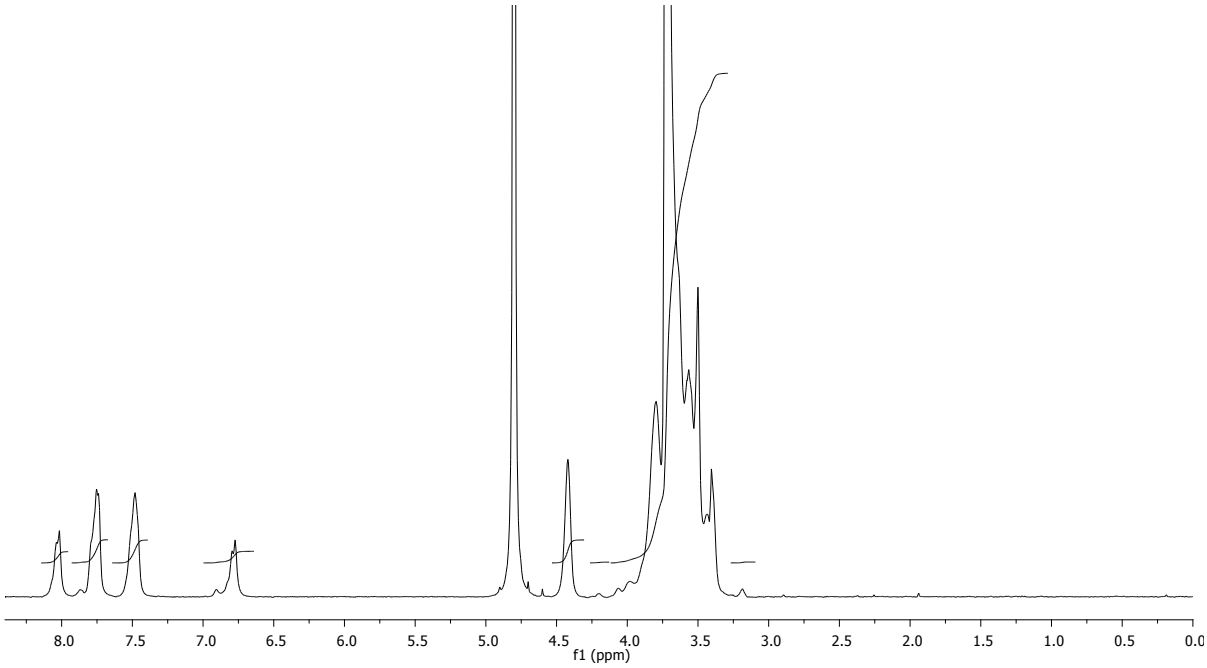
For 2D cryo-imaging, 4 μL of PIC samples (0.5 mg/mL) were applied directly onto a glow-discharged 200-mesh Quantifoil® R 2/2 holey-carbon grid and rapidly plunged into liquid ethane with the help of a Vitrobot Mark III (FEI Inc., Eindhoven, The Netherlands). Sample analysis at liquid nitrogen temperature was carried out with a JEM-2200 FS/CR (JEOL Ltd.) transmission electron microscope, using an acceleration voltage of 200 KV and defocus ranging from -1.5 to -5.0 μm. Images were taken under low-dose conditions on a 4Kx4K UltraScan 4000 CCD camera (Gatan Inc., Pleasanton, CA, USA). An in-column Omega energy filter was used in the microscope, with the energy slit width set at 30 eV, to improve the signal to noise ratio of the images. Size of PIC micelles was determined with ImageJ software (version 1.51j8) measuring the line intensity profile across the assemble. An average diameter of 24±4 nm for the CS_{35} micelles was determined by measuring the size of 25 micelles.

Determination of pH Values. pH values were measured with a portable pH-meter (Crison PH25) connected to a glass electrode (Crison 52 09).

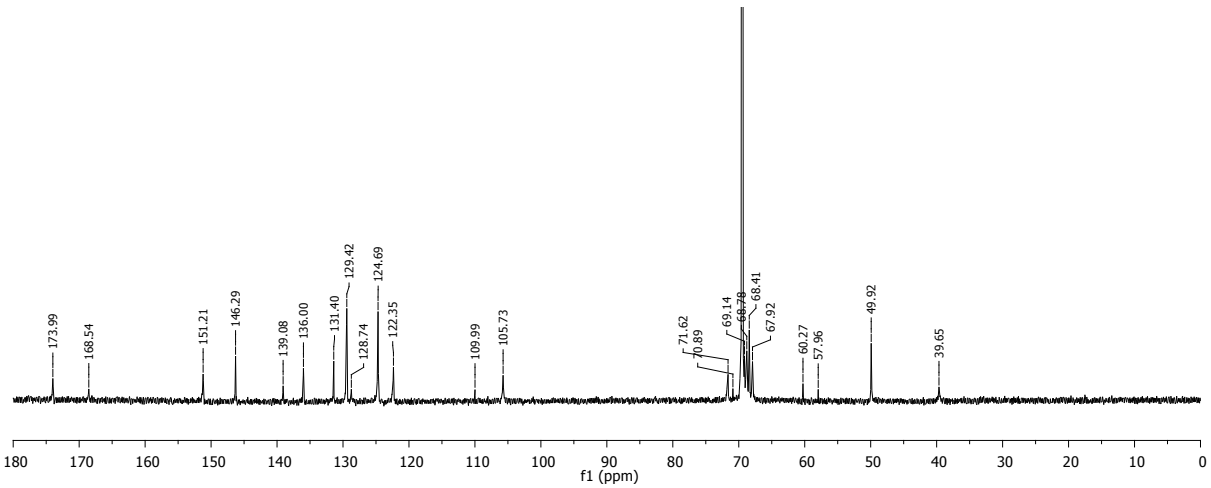
3. PEG-dendritic Block Copolymers

PEG_{5k}-[G3]-$PhCO_2Na$: ^{1}H NMR (500 MHz, D_2O) δ: 8.14-7.95 (m, 27H), 7.93-7.67 (m, 54H), 7.51 (br s, 54H), 7.01-6.65 (m, 26H), 4.44 (br s, 54H), 4.25-3.28 (m, ~879H), 3.18 (br s, 2H). ^{13}C NMR (100 MHz, D_2O) δ: 174.1, 168.5, 151.2, 146.3, 139.1, 136.0, 131.4, 129.4, 128.7, 124.7, 122.3, 109.9, 105.7, 71.6, 70.9, 69.5, 69.1, 68.8, 68.4, 67.9, 60.3, 57.9, 49.9, 39.6. IR (KBr, cm^{-1}): 3422, 2872, 1589, 1541, 1421, 1115. MALDI-TOF MS (DHB, linear mode, *m/z*): M_p 16746, M_n 17157, M_w 17331. Calcd: M_p 16918 ([M+H$^+$]), M_n 16967.

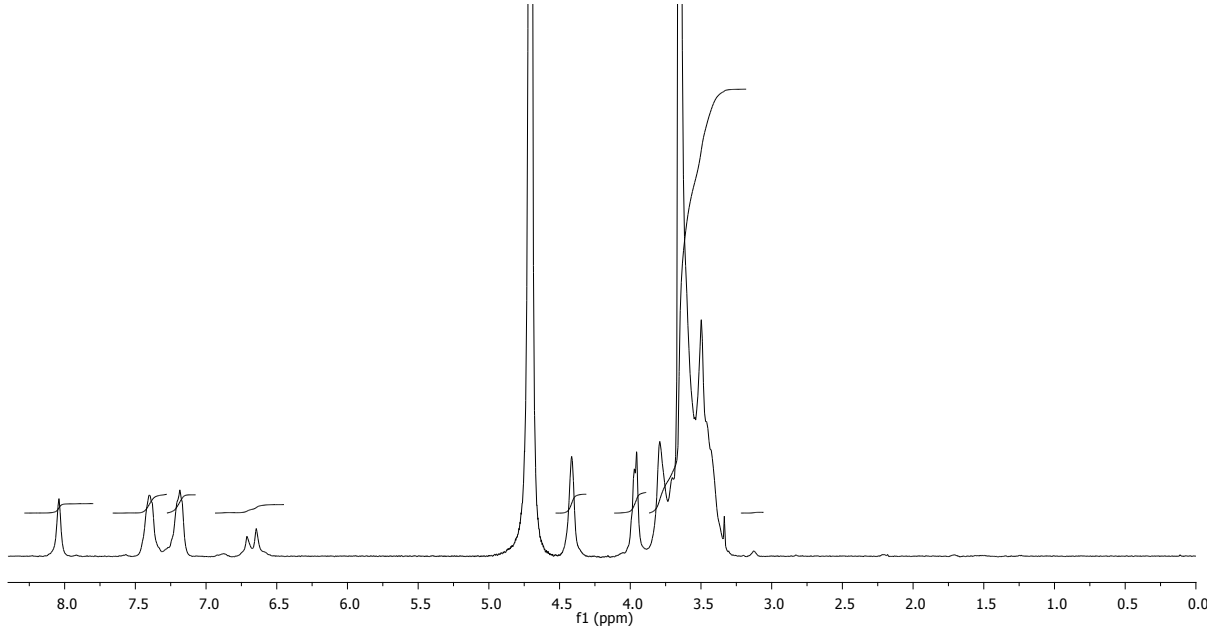
PEG_{5k}-[G3]-$BnNH_2$·HCl: ^{1}H NMR (500 MHz, D_2O) δ: 8.04 (br s, 27H), 7.40 (br s, 54H), 7.19 (br s, 54H), 6.94-6.46 (m, 26H), 4.41 (br s, 54H), 4.08-3.89 (m, 54H), 3.88-3.18 (m, ~879H), 3.12 (br s, 2H). ^{13}C NMR (126 MHz, D_2O) δ: 168.6, 158.5, 151.8, 146.7, 139.8, 132.9, 130.3, 129.8, 126.1, 122.9, 106.3, 72.4, 70.1, 69.4, 69.0, 68.6, 60.9, 58.5, 50.5, 43.1, 40.1. IR (KBr, cm^{-1}): 3417, 2878, 1111. MALDI-TOF MS (DHB, linear mode, *m/z*): M_p 16049, M_n 15832, M_w 15892. Calcd: M_p 16513 ([M+H$^+$]), M_n 16564.



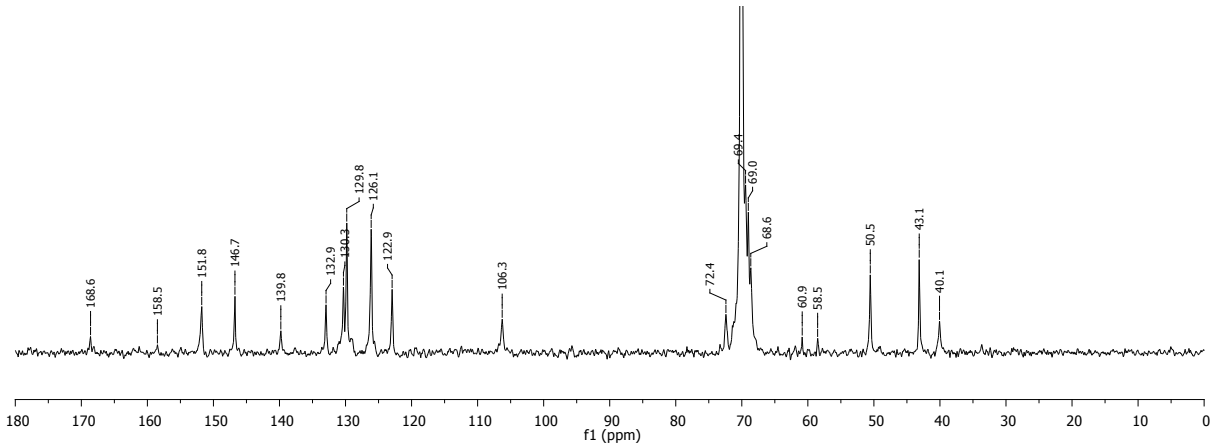
^{1}H NMR spectrum (500 MHz, D_2O) of PEG-[G3]-PhCO_2Na



^{13}C NMR spectrum (100 MHz, D_2O) of PEG-[G3]-PhCO_2Na



^{1}H NMR spectrum (500 MHz, D_2O) of PEG-[G3]-BnNH_2·HCl



^{13}C NMR spectrum (126 MHz, D_2O) of PEG-[G3]-BnNH_2·HCl

4. Preparation of PIC Micelles

Solutions of polysaccharides and block copolymers were aged at rt overnight before preparation of PIC.

CS-based Micelles. PEG-[G3]-$PhCO_2Na$ (2.81 mg/mL) was dissolved in 10 mM Na_2HPO_4/0.1 M NaOH (10% v/v) to give a solution of pH≈12. CS (0.5 mg/mL) was dissolved in 10 mM NaH_2PO_4 and the pH of the solution was adjusted to 3.1-3.3 by addition of 0.12 M HCl (approximately 50 µL HCl to 4 mL of CS solution). Both solutions of polymers were filtered through 0.45 µm nylon filters immediately before PIC formation. Micelles were prepared by mixing the above solutions in a 1:2 volume ratio (stoichiometric carboxylate to amine ratio, micelle concentration 1.28 mg/mL). The final pH of the PIC micelle solution was 7.2-7.4. Solutions were aged overnight at rt, under vigorous stirring before analysis.

ALG-based Micelles. PEG-[G3]-$BnNH_2$·HCl (1.47 mg/mL) was dissolved in 10 mM NaH_2PO_4. ALG (0.9 mg/mL) was dissolved in 10 mM Na_2HPO_4/0.1 M NaOH (12% v/v). Both solutions were filtered through 0.45 µm nylon filters immediately before PIC formation. Micelles were prepared by mixing these solutions in a 2:1 ratio (stoichiometric amine to carboxylate ratio, micelle concentration: 1.10 mg/mL). The final pH of the PIC micelle solution was 7.2-7.4. Solutions were aged for 3 h at rt, under vigorous stirring before analysis. Same experimental conditions were applied for the preparation of control linear PIC micelles from a solution of PEG-PLL_{27} in 10 mM NaH_2PO_4 (0.82 mg/mL).

HA-based Micelles. PEG-[G3]-$BnNH_2$·HCl (1.12 mg/mL) was dissolved in 10 mM NaH_2PO_4. HA (1.40 mg/mL) was dissolved in 10 mM Na_2HPO_4/0.1 M NaOH (12% v/v). Both solutions were filtered through 0.45 µm nylon filters immediately before PIC formation. Micelles were prepared by mixing these solutions in a 2:1 ratio (stoichiometric amine to carboxylate ratio, micelle concentration: 1.16 mg/mL). The final pH of the PIC micelle solution was 7.2-7.4. Solutions were aged for 3 h at rt, under vigorous stirring before analysis.

Fluorescently Labeled (Alexa Fluor 488) CS-based PIC Micelles. Alexa Fluor 488-labeled CS-PIC micelles were prepared from CS_{35} and a mixture of PEG-[G3]-$PhCO_2Na$ and AF488-PEG-[G3]-$PhCO_2Na$[1] (9:1 molar ratio) following the above procedure for CS-PIC micelles.

Fluorescently Labeled (FITC) ALG-based PIC Micelles. PIC micelles from PEG-[G3]-$BnNH_2$·HCl and ALG_{21} were prepared as described above. Then, a solution of FITC (16 µL, 5 mol% per amine group, 1 mg/mL in PBS pH 7.4) was added to 1 mL of micelles. The resulting mixture was stirred at rt overnight. Unbounded FITC was removed by extensive dialysis (Spectra/Por® 6, MWCO 1 KDa) against PBS pH 7.4 to give FITC-labeled ALG-PIC micelles.

Formation Efficiency of PIC Micelles. The formation efficiency of PIC micelles was assessed by quantifying the amount of free PEG-[G3]-$BnNH_2$·HCl in solutions of micelles prepared as described above with ALG_{21}, ALG_{168}, and ALG_{389}. To this end, any free PEG-[G3]-$BnNH_2$·HCl in the solution of micelles (300 µL in 10 mM PB 7.4, 150 mM NaCl) was separated by ultracentrifugation (308000 RCF, 15 °C, 1 h). A solution of PEG-[G3]-$BnNH_2$·HCl (300 µL in 10 mM PB 7.4, 150 mM NaCl, 0.98 mg/mL) was submitted to identical ultracentrifugation conditions as control to rule out precipitation of the block copolymer during the process. Afterwards, the concentration of free PEG-[G3]-$BnNH_2$·HCl in the micelles and control solution was determined by measuring their UV absorbance at 251 nm (NanoDrop 1000) and comparing to a calibration curve made from the absorbance of freshly prepared solutions of PEG-[G3]-$BnNH_2$·HCl of known concentrations. It was

revealed that 100% of PEG-[G3]-$BnNH_2$·HCl control remains in solution after ultracentrifugation, confirming the validity of the method. When applied to the PIC micelle supernatants derived from ALG_{21}, ALG_{168}, and ALG_{389}, a 4.8, 5.6, and 5.1% (±1.0%) of free PEG-[G3]-$BnNH_2$·HCl were detected, respectively, which accounts for an efficiency of micelle formation *ca.* 94%.

5. Dynamic Light Scattering (DLS) and Stability of PIC Micelles

DLS measurements were performed on a Malvern Nano ZS (Malvern Instruments, U.K.), operating at 633 nm with a 173º scattering angle, at 25 or 37 ºC. DLS mean diameters were obtained from the volume particle size distribution provided by Malvern Zetasizer Software. DLS histograms were obtained from the volume and intensity particle size distributions.

PIC micelles prepared following the general procedures shown above were left at rt for 3 h (ALG, HA) or overnight (CS) before DLS measurements ("upon formation"; 10 mM PB, pH 7.4, 25 °C). Then, to assess their stability towards ionic strength, micelles were supplemented with 150 mM NaCl and DLS recorded (at 25 °C) after 1 h at rt. The stability of these micelles towards simulated physiological conditions (10 mM PB, pH 7.4, 150 mM NaCl) was analyzed after heating for at least 24 h at 37 °C (DLS at 37 °C).

General note on PIC micelles. Filtering of PIC micelles before DLS measurements was avoided to prevent removal of large aggregates that could obscure the analysis of micelle formation and stability.

CS PIC micelles:

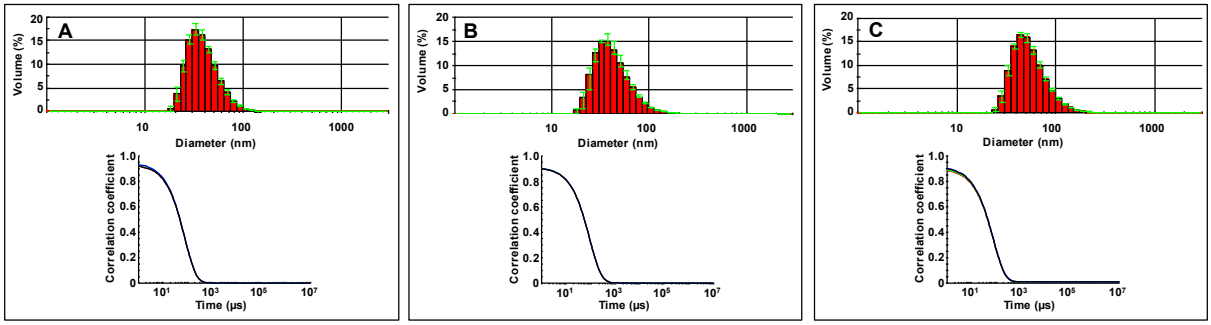


Figure S1. PIC micelles prepared from PEG-[G3]-$PhCO_2Na$ and CS_{35} in 10 mM PB, pH 7.4, 25 ºC. DLS histograms and correlation functions (25 ºC) recorded upon formation (A) and 1 h after the addition of 150 mM NaCl (B). DLS histogram and correlation function (37 ºC) of the micelles in 150 mM NaCl after heating at 37 ºC for 24 h (C).

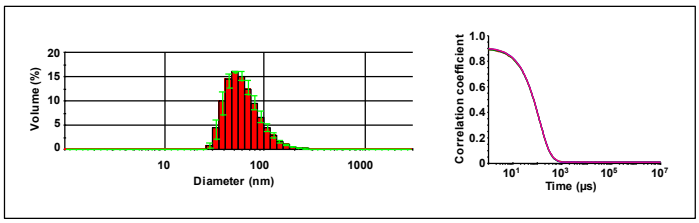


Figure S2. PIC micelles prepared from PEG-[G3]-$PhCO_2Na$ and CS_{35} in 10 mM PB, pH 7.4, 25 ºC. DLS histogram and correlation function (25 ºC) recorded after freeze-drying and resuspension in H_2O.

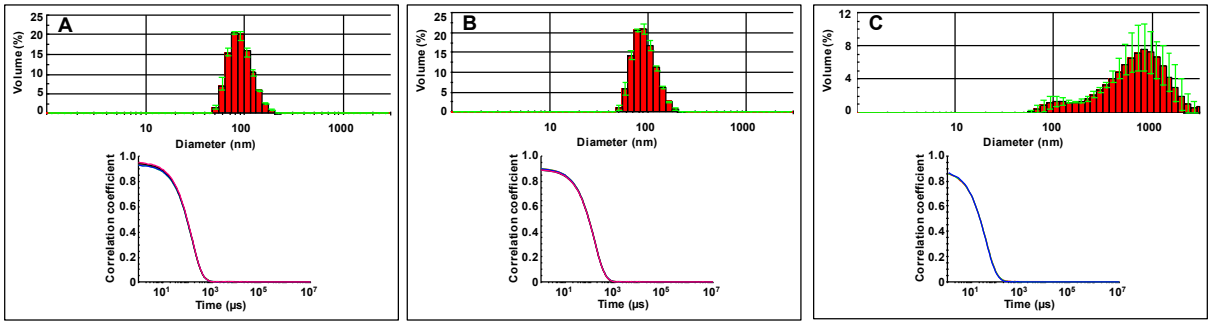


Figure S3. PIC micelles prepared from PEG-[G3]-$PhCO_2Na$ and CS_{172} in 10 mM PB, pH 7.4, 25 ºC. DLS histograms and correlation functions (25 ºC) recorded upon formation (A) and 1 h after the addition of 150 mM NaCl (B). DLS histogram and correlation function (37 ºC) of the micelles in 150 mM NaCl after heating at 37 ºC for 24 h (C).

ALG PIC micelles:

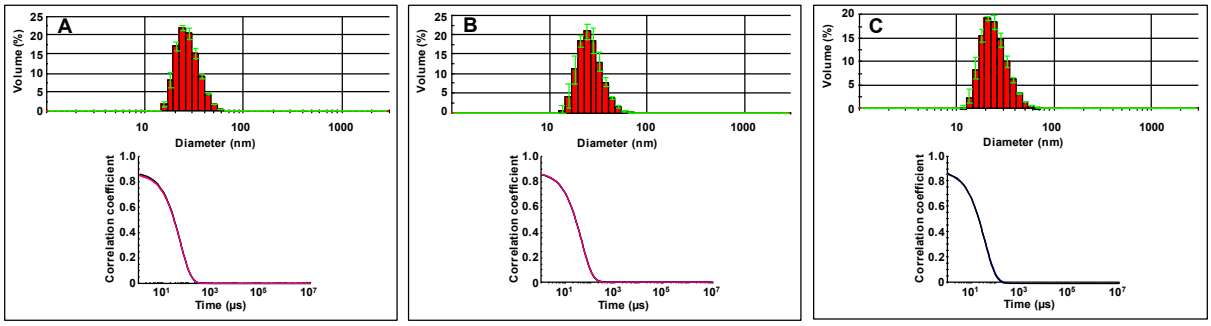


Figure S4. PIC micelles prepared from PEG-[G3]-$BnNH_2$·HCl and ALG_{21} in 10 mM PB, pH 7.4, 25 ºC. DLS histograms and correlation functions (25 ºC) recorded upon formation (A) and 1 h after the addition of 150 mM NaCl (B). DLS histogram and correlation function (37 ºC) of the micelles in 150 mM NaCl after heating at 37 ºC for 24 h (C).

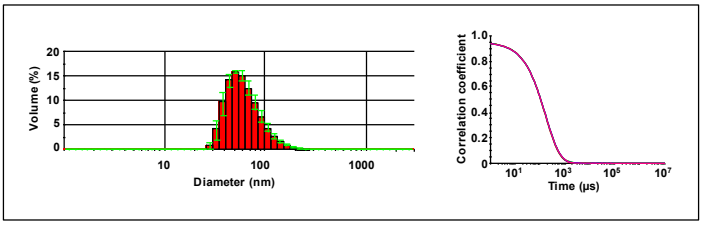


Figure S5. PIC micelles prepared from PEG-[G3]-$BnNH_2$·HCl and ALG_{21} in 10 mM PB, pH 7.4, 25 ºC. DLS histogram and correlation function (25 ºC) recorded after freeze-drying and resuspension in H_2O.

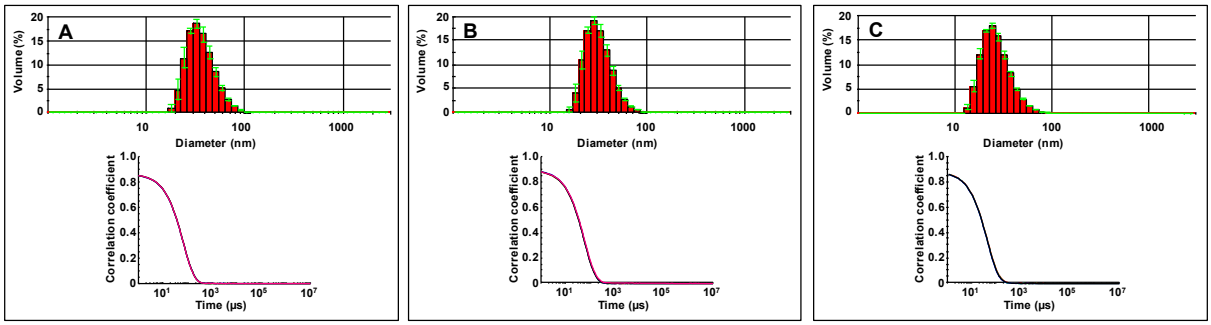


Figure S6. PIC micelles prepared from PEG-[G3]-$BnNH_2$·HCl and ALG_{168} in 10 mM PB, pH 7.4, 25 ºC. DLS histograms and correlation functions (25 ºC) recorded upon formation (A) and 1 h after the addition of 150 mM NaCl (B). DLS histogram and correlation function (37 ºC) of the micelles in 150 mM NaCl after heating at 37 ºC for 24 h (C).

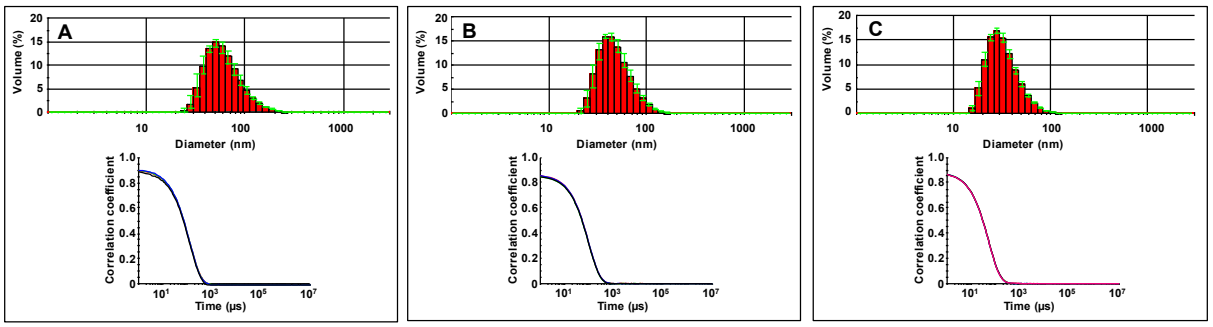


Figure S7. PIC micelles prepared from PEG-[G3]-BnNH$_2$·HCl and ALG$_{389}$ in 10 mM PB, pH 7.4, 25 ºC. DLS histograms and correlation functions (25 ºC) recorded upon formation (A) and 1 h after the addition of 150 mM NaCl (B). DLS histogram and correlation function (37 ºC) of the micelles in 150 mM NaCl after heating at 37 ºC for 24 h (C).

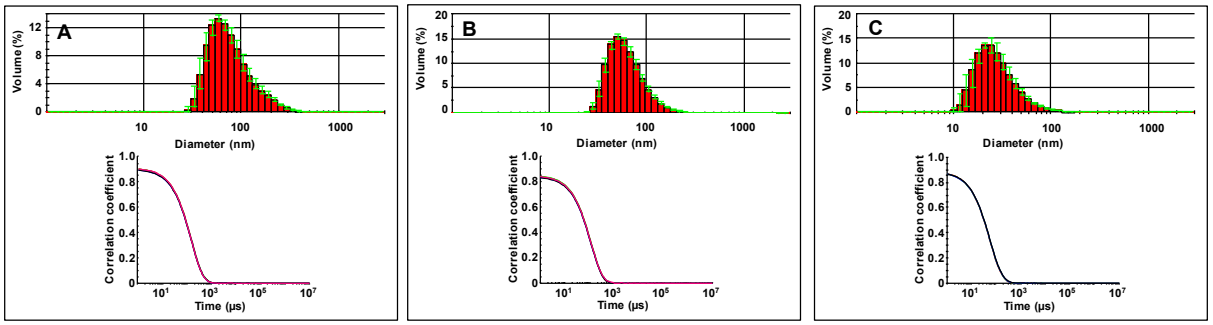


Figure S8. PIC micelles prepared from PEG-[G3]-BnNH$_2$·HCl and ALG$_{694}$ in 10 mM PB, pH 7.4, 25 ºC. DLS histograms and correlation functions (25 ºC) recorded upon formation (A) and 1 h after the addition of 150 mM NaCl (B). DLS histogram and correlation function (37 ºC) of the micelles in 150 mM NaCl after heating at 37 ºC for 24 h (C).

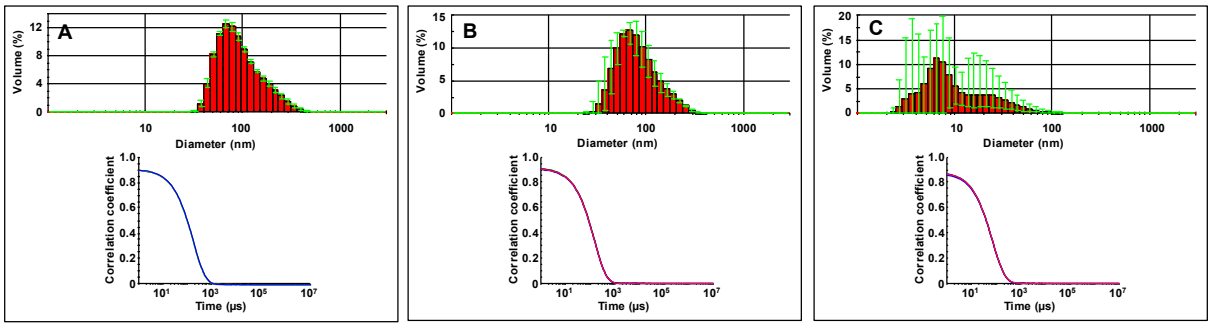


Figure S9. PIC micelles prepared from PEG-[G3]-BnNH$_2$·HCl and ALG$_{1040}$ in 10 mM PB, pH 7.4, 25 ºC. DLS histograms and correlation functions (25 ºC) recorded upon formation (A) and 1 h after the addition of 150 mM NaCl (B). DLS histogram and correlation function (37 ºC) of the micelles in 150 mM NaCl after heating at 37 ºC for 24 h (C).

Control linear PEG-PLL$_{27}$/ALG PIC micelles:

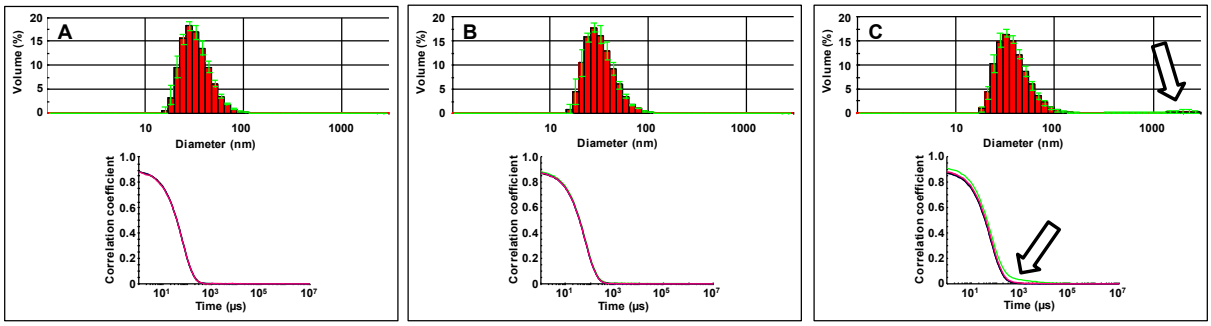


Figure S10. PIC micelles prepared from PEG-PLL$_{27}$ and ALG$_{21}$ in 10 mM PB, pH 7.4, 25 ºC. DLS histograms and correlation functions (25 ºC) recorded upon formation (A) and 1 h after the addition of 150 mM NaCl (B). DLS histogram and correlation function (37 ºC) of the micelles in 150 mM NaCl after heating at 37 ºC for 24 h (C). Arrows denote incipient aggregation.

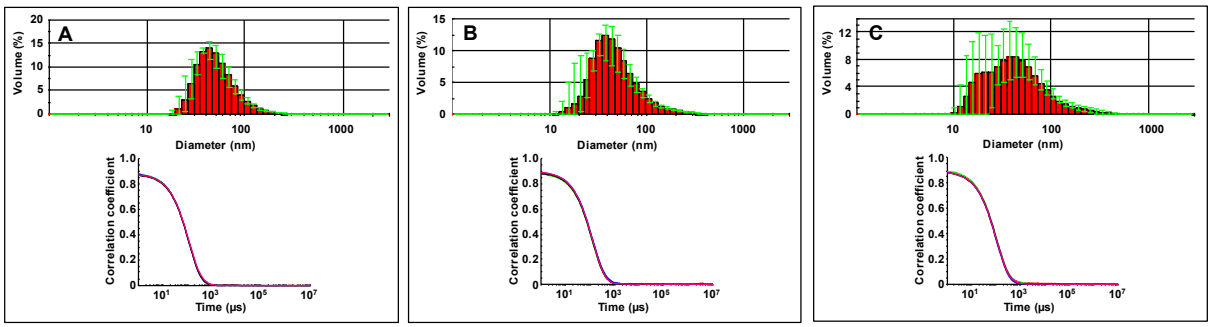


Figure S11. PIC micelles prepared from PEG-PLL$_{27}$ and ALG$_{168}$ in 10 mM PB, pH 7.4, 25 ºC. DLS histograms and correlation functions (25 ºC) recorded upon formation (A) and 1 h after the addition of 150 mM NaCl (B). DLS histogram and correlation function (37 ºC) of the micelles in 150 mM NaCl after heating at 37 ºC for 30 min (C).

HA PIC micelles:

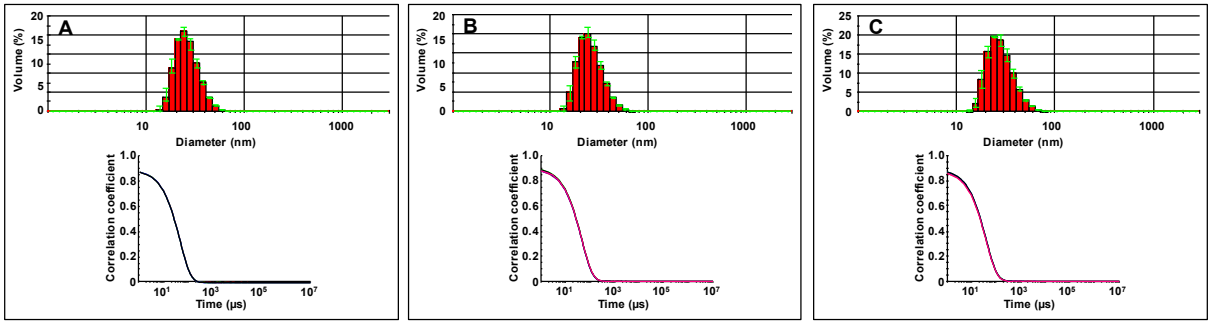


Figure S12. PIC micelles prepared from PEG-[G3]-$BnNH_2$·HCl and HA_{71} in 10 mM PB, pH 7.4, 25 ºC. DLS histograms and correlation functions (25 ºC) recorded upon formation (A) and 1 h after the addition of 150 mM NaCl (B). DLS histogram and correlation function (37 ºC) of the micelles in 150 mM NaCl after heating at 37 ºC for 24 h (C).

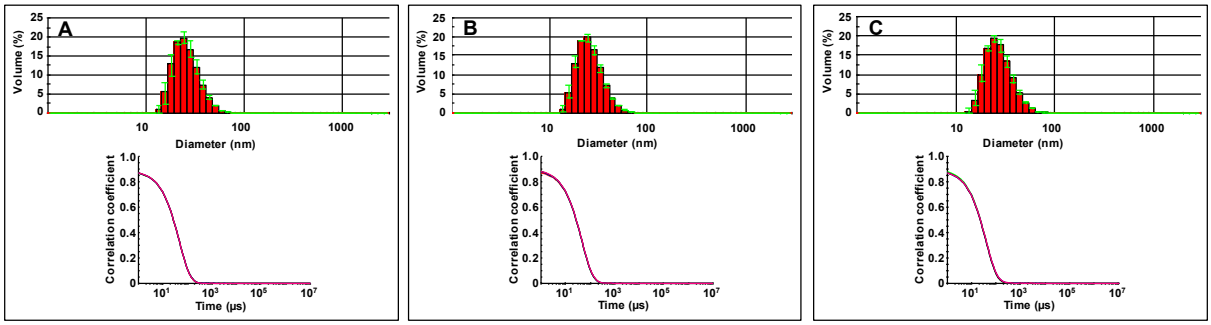


Figure S13. PIC micelles prepared from PEG-[G3]-$BnNH_2$·HCl and HA_{165} in 10 mM PB, pH 7.4, 25 ºC. DLS histograms and correlation functions (25 ºC) recorded upon formation (A) and 1 h after the addition of 150 mM NaCl (B). DLS histogram and correlation function (37 ºC) of the micelles in 150 mM NaCl after heating at 37 ºC for 24 h (C).

6. Z-Potentials

Z-potential values of PIC micelles were obtained by laser doppler anemometry (LDA), measuring the mean electrophoretic mobility (Malvern Zetasizer Nano ZS, Malvern Instruments). Measurements were performed in 10 mM PB pH 7.4 (Hückel approximation). PIC micelles revealed values close to zero: CS_{35} (-0.78 mV), ALG_{21} (-1.38 mV) and HA_{71} (-1.92 mV).

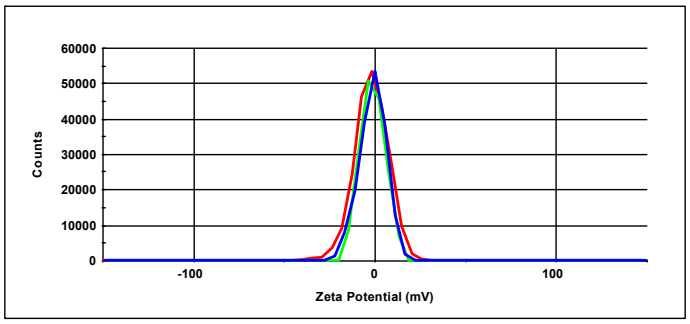


Figure S14. Z-potential of PIC micelles prepared from PEG-[G3]-$PhCO_2Na$ and CS_{35} in 10 mM PB, pH 7.4.

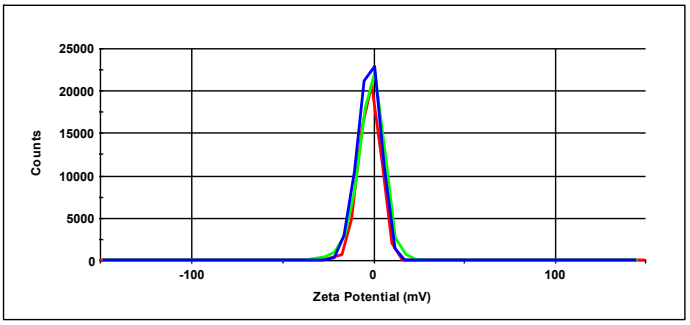


Figure S15. Z-potential of PIC micelles prepared from PEG-[G3]-$BnNH_2 \cdot HCl$ and ALG_{21} in 10 mM PB, pH 7.4.

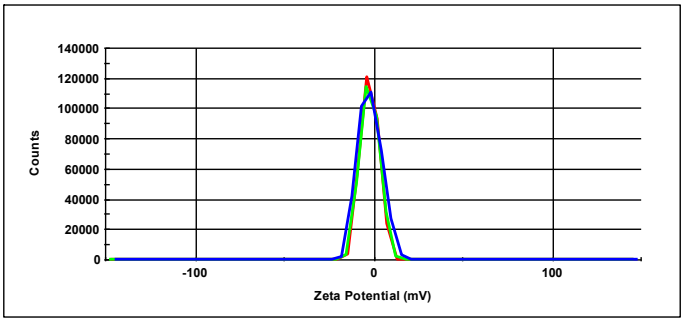


Figure S16. Z-potential of PIC micelles prepared from PEG-[G3]-$BnNH_2 \cdot HCl$ and HA_{71} in 10 mM PB, pH 7.4.

7. Doxorubicin (DOX) Loaded PIC Micelles

Encapsulation efficiency (EE) describes the fraction of drug incorporated into a micelle compared to the total amount of drug used in its preparation (EE = weight of drug in micelle × 100/weight of feeding drug).

Drug loading (DL) refers to the mass fraction of a micelle that is composed of the drug (DL = weight of drug in micelle × 100/weight of drug-loaded micelle).

Preparation of DOX-loaded PIC micelles. PIC micelles from PEG-[G3]-$BnNH_2$·HCl and ALG_{21}, ALG_{168}, and ALG_{389} were prepared as described above. Then, an aqueous solution of DOX (1/3 of PIC micelle volume, 1.0 mg/mL in H_2O) was added to the solution of micelles and the resulting mixture was stirred at rt overnight. Unloaded DOX was removed by dialysis (Spectra/Por® 6, MWCO 1 KDa) against 200 mL PBS pH 7.4 for 12 h in the dark at 37 ºC. To determine the EE and DL of DOX, aliquots of the buffer solution were taken at the end of the dialysis (3 × 150 μL) and placed in a 96-well microplate. Then, 500 mM acetate pH 4 buffer (50 μL) was added to each well and the fluorescence of the samples was measured at a microplate reader (exc. 485±20 nm, em. 535±20 nm; Tecan Infinite F200 PRO). The concentration of unloaded DOX in solution was determined by comparison with a standard calibration curve made from the fluorescence emission of fresh solutions of DOX of known concentration prepared under identical conditions. EE of 56, 54 and 50% and DL of 17, 16, and 15% were obtained for the ALG_{21}, ALG_{168}, and ALG_{389} micelles, respectively.

Fluorescence spectra of DOX-loaded micelles in PBS (samples diluted 20× with H_2O) were recorded in a NanoDrop 3300 fluorospectrometer (exc. 470±10 nm).

In vitro Release Study of DOX. DOX-loaded PIC micelles derived from PEG-[G3]-$BnNH_2$·HCl and ALG_{21}, ALG_{168}, and ALG_{389} were prepared and dialyzed as described above (Spectra/Por® 6, MWCO 1 KDa; 800 μL of micelles against 200 mL PBS pH 7.4, 37 °C, 12 h in the dark). Then, dialysis bags were moved to 50 mM acetate buffer pH 5.0, 100 mM NaCl (200 mL) and dialysis continued at 37 °C in the dark. At fixed times, aliquots (3 × 150 μL) were taken and placed in 96-well microplates. Then, 500 mM acetate pH 4 buffer (50 μL) was added to each well and the fluorescence of the samples was measured in a microplate reader (exc. 485±20 nm, em. 535±20 nm; Tecan Infinite F200 PRO). The amount of released DOX was calculated by comparison with a standard calibration curve made from the fluorescence emission of fresh solutions of DOX of known concentration, prepared under identical conditions.

Stability of DOX-loaded PIC Micelles in Serum. DOX-loaded PIC micelles derived from PEG-[G3]-$BnNH_2$·HCl and ALG_{21}, ALG_{168}, and ALG_{389} were prepared as described above. After unloaded DOX was removed by dialysis (Spectra/Por® 6, MWCO 1 KDa) against PBS pH 7.4 (12 h, 37 °C) in the dark, DOX-loaded micelles were incubated in fetal bovine serum (FBS) at 37 °C in the dark for 2, 12, and 24 h (volume ratio micelle solution:FBS, 1:9). Then, the mixtures were filtered through centrifugal filters (Amicon Ultra-4, MWCO 10 KDa, 6000 rpm, 60 min, 15 °C). Aliquots of the filtered solution (3 × 150 μL) were taken and placed in a 96-well microplate. Then, 500 mM acetate pH 4 buffer (50 μL) was added to each well and the fluorescence of the samples was measured in a microplate reader (exc. 485±20 nm, em. 535±20 nm; Tecan Infinite F200 PRO). Released DOX was calculated by comparing the fluorescence of the samples with a calibration curve made with solutions of known concentrations of DOX processed in the same way as the micelles.

Table S1. Percentage of DOX released from PIC micelles incubated in 90% FBS at 37 °C.

PIC Micelle	Time (h)		
	2	**12**	**24**
PEG-[G3]-$BnNH_2$/ALG_{21}	5±1	7±1	10±1
PEG-[G3]-$BnNH_2$/ALG_{168}	8±1	12±2	15±2
PEG-[G3]-$BnNH_2$/ALG_{389}	10±2	16±2	22±3

8. Cell Studies

Cell Cultures. Human adenocarcinoma alveolar basal epithelial (A549) cells, obtained from the European Collection of Authenticated Cell Cultures (ECACC), were cultured at 37 °C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) with high glucose, containing 10% fetal bovine serum (FBS) and supplemented with 50 U/mL penicillin and 50 U/mL streptomycin. All cell experiments were performed with this modified DMEM containing 10% FBS and simply referred in the text as "medium".

Cytotoxicity Assays. A549 cells were seeded in a 96-well microplate (4000 cells/well) in 100 µL of DMEM supplemented with 10% FBS and maintained at 37 ºC in a 5% CO_2 atmosphere for 24 h. Then, growth medium was replaced by fresh medium containing different concentrations of micelles and incubated for selected periods of time. Afterwards, 10 µL of a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution (5 mg/mL in PBS) were added to each plate and incubated for 4 h, before 100 µL of solubilizing solution (10% SDS in 0.01 M HCl) were added and the resulting mixtures incubated overnight. Absorbance was read at 570±10 nm in a microplate reader (Tecan Infinite F200 PRO) and the relative cell viability was calculated according to: $(A_{sample}\text{-}A_{blank}/A_{control}\text{-}A_{blank}) \times 100$, where A_{sample} corresponds to the absorbance of well with cells treated with a determined sample, A_{blank} to the absorbance of well without cells, and $A_{control}$ to the absorbance of well with untreated cells.

Flow Cytometry. A549 cells were seeded in a 96-well microplate (17000 cells/well) in 150 µL of DMEM supplemented with 10% FBS and maintained at 37 ºC in a 5% CO_2 atmosphere for 24 h. Then, growth medium was replaced by fresh medium containing DOX-loaded micelles and incubated at 37 ºC for 1 h. Cells were rinsed twice with PBS and trypsinized. Afterwards, DMEM supplemented with 2% FBS and 5 mM EDTA was added before analysis by flow cytometry (Guava® easyCyte BG HT Instrument). DOX fluorescence was detected by excitation with blue laser (488 nm), and emission detection in yellow channel (583 ± 26 nm).

Cellular Uptake Studies. A549 cells were seeded onto coverslips in a 12-well microplate and incubated overnight in 1 mL of DMEM supplemented with 10% FBS. Then, medium was replaced by solutions of PIC micelles [(i) 100 µL of fluorescently labeled (AF488-CS-PIC or FITC-ALG-PIC) micelles in 900 µL of medium; (ii) 25 µL of DOX-loaded ALG-PIC micelles or control free DOX in 975 µL of medium) and incubated at 37 ºC for a given time. Afterwards, medium was removed, cells were washed twice with PBS, and fresh medium (1 mL) was added before cells were incubated again for a given time. Half an hour before observation, cellular nuclei were stained by adding 10 µL of 2 µM Hoechst 33258 solution to each well, and acidic organelles stained by adding 10 µL of 5 µM LysoTracker Red (for PIC micelles labeled with Alexa Fluor 488 or FITC) or 10 µL of 100 µM LysoTracker Green (for free DOX and DOX-loaded PIC micelles). Immediately before observation, medium was removed and cells were washed twice with PBS before fresh medium was added. Intracellular distributions were observed by fluorescence microscopy using an Olympus BX-51 microscope equipped with an Olympus DP-71 camera.

The parameters of the fluorescent channels are the following ones. Blue channel: ultraviolet excitation U-MWU2: excitation filter 360-370 nm, emission filter 420 nm and dichromatic mirror 400 nm. Green channel: blue excitation U-MWB2: excitation filter 460-490 nm, emission filter 520 nm and dichromatic mirror 500 nm. Red channel: green excitation UMNG2: excitation filter 530-550 nm, emission filter 590 nm and dichromatic mirror 570 nm. Images were processed with Adobe Photoshop software.

9. References

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