Supporting information

THE GENERATION OF STABILIZED SUPRAMOLECULAR NANORODS FROM STAR-SHAPEDPOLYGLUTAMATES

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1. MATERIALS

All reagent grade chemicals were used without any purification. Doxorubicin hydrochloride salt was purchased from Xingcheng Chempharm Co. Ltd (Zhejiang, China). Anhydrous N,N-dimethylformamide (DMF, ≥99.8% anhydrous), methanol (HPLC grade),and tetrahydrofuran anhydrous (99.8 %) were purchased from Scharlab SL (Sentmenat, Spain). Z-L-phenylalanine was purchased from Iris Biotech GmbH (Marktredwitz, Germany).NCA-Glu(OBz) was provided by PMC Isochem (Vert-le-Petit, France).

2. EXPERIMENTAL METHODS

FLUORESCENT SPECTROSCOPY

Fluorescence spectroscopy measurements were performed on an FP-6500 spectrofluorimeter (JASCO, Easton, United States) with an excitation wavelength of 550 nm and measured emission spectrum in a range of 575 to 740 nm. Polymer samples were dissolved in water or sodium chloride solution and allowed to equilibrate for 24 hours. To 800μL of each solution, 1μL of 1 mg/ml Nile Red solution in acetone was added.

SAXS

Small-Angle X-Ray Scattering (SAXS) measurements were performed at the BL11 beamline of the Alba synchrotron in Barcelona, Spain. The observed q range was 4.24×10-3 Å-1 ≤ q ≤ 3.52 $Å^{-1}$, where q is the magnitude of the scattering vector $q = (4\pi/\lambda)\sin(\theta/2)$.

All solutions were allowed to equilibrate for 24hours before measurement. Analysis and fitting were performed with ATSAS and Scatter software packages.

TEM

The transmission electron microscope (TEM) experiments were performed on the FEI Tecnai Spirit G2 with digital camera (Soft Image System, Morada, United States) and image capture software (ITEM, Osaka, Japan). The pH of TEM samples was adjusted with 0.1 M HCl or 0.1 NaOH, added in 1-5 µL quantities, and equilibrated till constant pH between additions.

cryo-TEM

Experiments were performed on the Talos Arctica (ThermoFisher Scientific) operating at 200 KV at liquid nitrogen temperature. Images were recorded with a Falcon III Direct Detector Device in linear mode with a pixel size of 1.42 A/pixel at a nominal magnification of 73000X. Samples were vitrified on a Vitrobot (ThermoFisher Scientific) from a concentration of 1mg/ml.

GPC

The GPC experiment was performed on a VISCOTEK GPCmax coupled to a VISCOTEK TDA302 detector system (Malvern, Malvern, England) - a dual LS detector (7 and 90º), viscosimeter, and RI detector. Experiment conditions: Column set: PW2500+3000+G (105/103/102 Å porosity), Flow: 0.8 ml/min, Solvent: 0.1% LiBr in DMF, Injection volume: 100µL. Temperature: 60°C.The GPC system was calibrated with well-defined poly(methyl methacrylate) (PMMA, 65kDa)standard, from Polymer Standards Service (PSS, Mainz Germany).

SANS

Small-angle neutron scattering(SANS) measurements were performed on a SANS2D instrument at the ISIS spallation source (Rutherford Appleton Laboratory, Oxfordshire, UK). The observed q range was 2×10^{-2} nm^{-1} ≤ q ≤ 4.9 nm^{-1}. All scattering data were normalized for the sample transmission, empty cell, and solvent background.

UV-Vis spectroscopy

Doxorubicin (Dox) quantification was performed in 120 mM NaCl solution at λ = 480 nm using JASCO V-630 spectrophotometer. Samples were prepared according to the following procedure:

1 ml of **F3E10**/Dox complex at molar ration 10:1 in 120 mM NaCl at concentration 10 mg/ml was transferred in 1 mL Float-A-Lyzer with membrane cut-off of 3.5 kDa. Samples were dialyzed against 120 mM NaCl for 8, 20, 32, 40, and 52 hours. After that, solutions were quantitatively transferred to 2ml tubes and lyophilized. Dry samples were redissolved in 1 ml of MilliQ water and diluted to 0.1 mg/ml with 120 mM NaCl.

Circular Dichroism Spectroscopy

Solutions were transferred to a quartz cuvette with a light path length of 0.2 mm and measured five times at room temperature with a J-1500 spectrometer (JASCO corporation, Easton, United States) under a nitrogen flow of 2.7 L·min-1.

Pharmacokinetics of Doxorubicin Release

pH-dependent Release. 3 mg of **F3E10/Dox** (10:1) was dissolved in 1 ml of either 25 mM sodium acetate/1 mM EDTA buffer (pH 5.5) or PBS buffer (pH 7.4). Samples were incubated at 37 °C. At fixed time points, the mixture was transferred to Vivaspin tubes with a membrane cut-off of 2 kDa and centrifuged at 3,200 rpm for 20 min. The permeate was collected, diluted six times, and Dox spectra collected with UV-Vis spectroscopy as described above.

Cathepsin-mediated Release. Cathepsin B mediated release was studied using an adapted method from Dubowchik et al.[1] Briefly, Cathepsin B (5U) in 60 µL of 25 mM sodium acetate/1 mM EDTA buffer (pH 5.5) was mixed with 120 µL of 30 mM DTT/15 mM EDTA and left to activate for 15 min at room temperature. 0.3 mL of 10 mg/mL **F3E10/Dox** (10:1) solution in PBS was mixed with 0.7 mL of 25 mM sodium acetate/1 mM EDTA buffer (pH 5.5) at 37 °C. This solution was added to the cathepsin B solution, and the mixture was incubated at 37 °C. Samples were collected and analyzed as described for pH-dependent release.

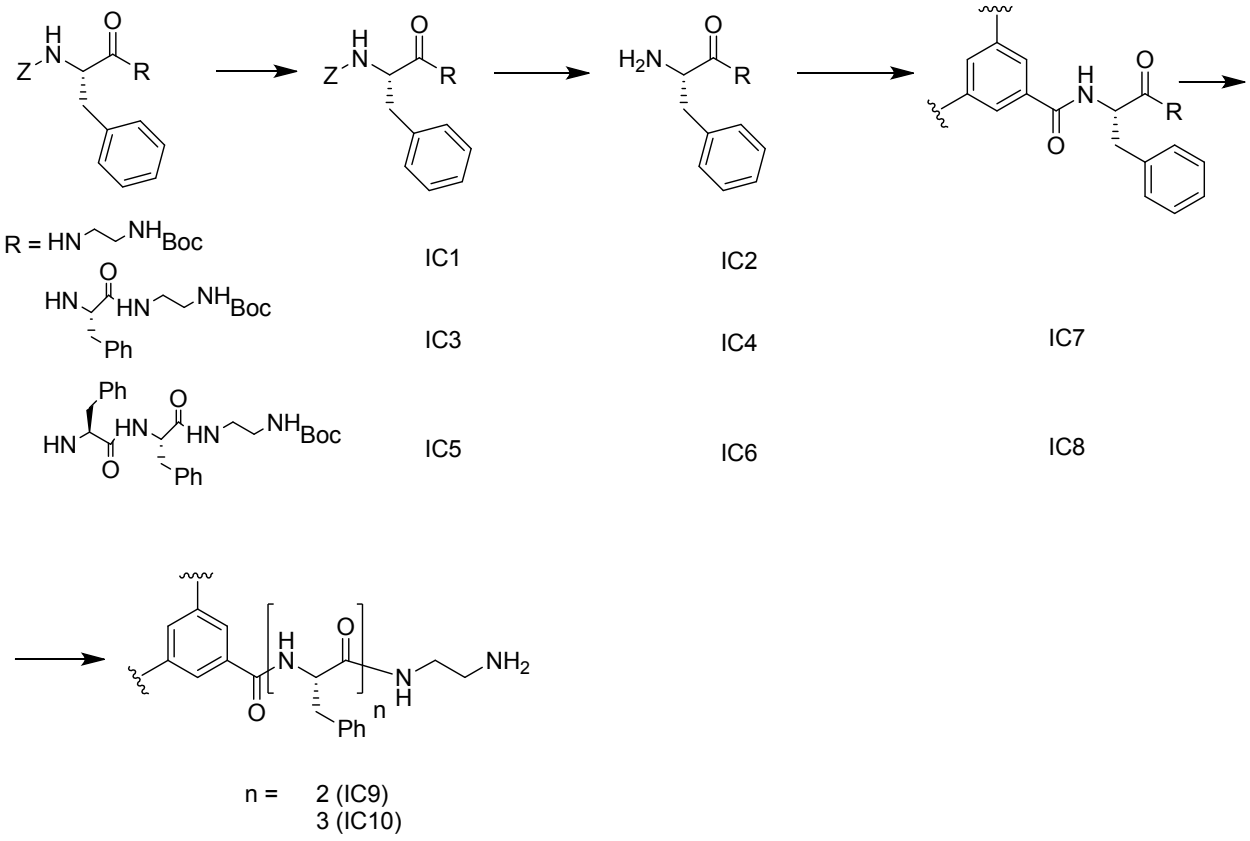
Drug Loading Efficiency Determination

In a 5 mL pear-shaped flask, 1 mL of 1 mM **F3E10** solution in 120 mM NaCl was mixed with Dox (0.1 eq) under vigorous stirring, and then the solution was left for 24 hours.

Centrifugation at 3,900 rpm for 20 min was performed to collect supernatants, and Dox content was measured on a UV-vis spectrophotometer. The drug loading efficiency was calculated as:

$$Drug\ loading\ efficiency = \frac{[Dox]_{TOTAL} - [Dox]_{FREE}}{[Dox]_{TOTAL}}$$

3. SYNTHETIC PROCEDURES



Scheme 1S. General scheme of star-shaped polypeptidic core initiator synthesis

3.1. General Synthetic Procedures for Z-protected Peptide Derivatives

In a round bottom flask with a stirrer, Z-L-phenylalanine (4.48 g, 15 mmol) and DMTMM(Cl) (4.55 g, 0.165 mmol) were dissolved in 150 mL of methanol. The solution was stirred for 10 minutes, and then corresponding amine (0.165 mmol) in 10 ml of methanol was added dropwise. The reaction was allowed to proceed overnight, and then a white precipitate was

filtered off and washed with methanol (3x50 ml). The precipitate was dried under vacuum. Yield = 90-95%.

IC-1: ^{1}H-NMR (300 MHz.DMSO-d6): δ 1.37(s, 9H), 7.1-7.37(m,10H), 2.68-3.21(m,6H),7.45(d,1H), 4.17(m,1H), 8.03(t,1H), 4.94(s,2H), 6.72(t,1H).

IC-3:^{1}H-NMR (300 MHz. DMSO-d6): δ 1.36(s,9H), 2.56-3.15(m,8H), 4.23(h,1H), 4.45(q,1H), 4.94(s,2H), 6.96(t,1H), 7.14-7.36(m,15H), 7.44(d,1H), 7.94(t,1H), 8.08(d,1H).

IC-5: ^{1}H-NMR (300 MHz. DMSO-d6): δ 1.37(s,9H), 2.60-3.10(m,10H), 4.22(dt,1H), 4.44(dt,1H), 4.53(dt,1H), 4.92(s,2H), 6.68(t,1H), 7.10-7.36(m,20H), 7.43(d,1H), 7.86(t,1H),8.03(d,1H), 8.17(d,1H).

3.2. General Procedures for the Deprotection of Z-protected Peptides

In a round bottom flask with a stirrer and nitrogen inlet and outlet, Z-protected amine, 10% (w/w) of Pd/C, and 250 mL methanol were added. The solution was purged with nitrogen for 10 min, followed by three vacuum/nitrogen cycles. A balloon containing hydrogen was connected to the system, and the suspension was stirred for 6h. The mixture was then filtered with Celite. The obtained filtrate was concentrated *in vacuo*.

Yield = 85-92%.

IC-2: ^{1}H-NMR (300 MHz. DMSO-d6): δ 1.37(s, 9H), 6.74(t, 1H), 2.86-3.12 (m,5H), 7.04-7.39(m,5H), 3.36(m,1H), 7.87(t,1H).

IC-4:^{1}H-NMR (300 MHz. DMSO-d6): δ 1.37(s, 9H), 2.71-3.22 (m,8H), 4.47(q,1H), 6.72(t,1H), 7.09-7.29(m,10H), 7.96(t,1H), 8.03(d,1H).

IC-6: ^{1}H-NMR (300 MHz. DMSO-d6): δ 1.37(s, 9H), 2.45(dd, 1H), 2.70-3.17 (m, 9H), 3.34(dd, 1H),4.43(dt,1H), 4.54(m,1H),6.70(t,1H), 7.03-7.29(m,15H), 7.90-8.10(m,2H), 8.25(d,1H).

3.3. General Procedure for IC07-08 Core Synthesis

In a round-bottom flask with a stirrer, trimesic acid (benzene-1,3,5-tricarboxylic acid) (174mg, 0.832 mmol) and DMTMM Cl (920 mg, 3.33 mmol) were dissolved in 30 ml of methanol. 3.32 mmol of the corresponding amine in 5 ml of methanol was added, and the mixture stirred for 48h. The precipitate was then filtered, washed with methanol, and dried.

IC7. ^{1}H-NMR (300 MHz. DMSO-d6): δ 1.37(s, 9H),2.69-3.14 (m, 7H), 3.28 (dd, 1H), 4.48 (did, 1H), 4.76 (did, 1H), 7.06-7.34 (m, 10H), 8.23 (t, 1H), 8.29 (t, 1H), 8.31 (s, 1H), 8.86 (d, 1H).

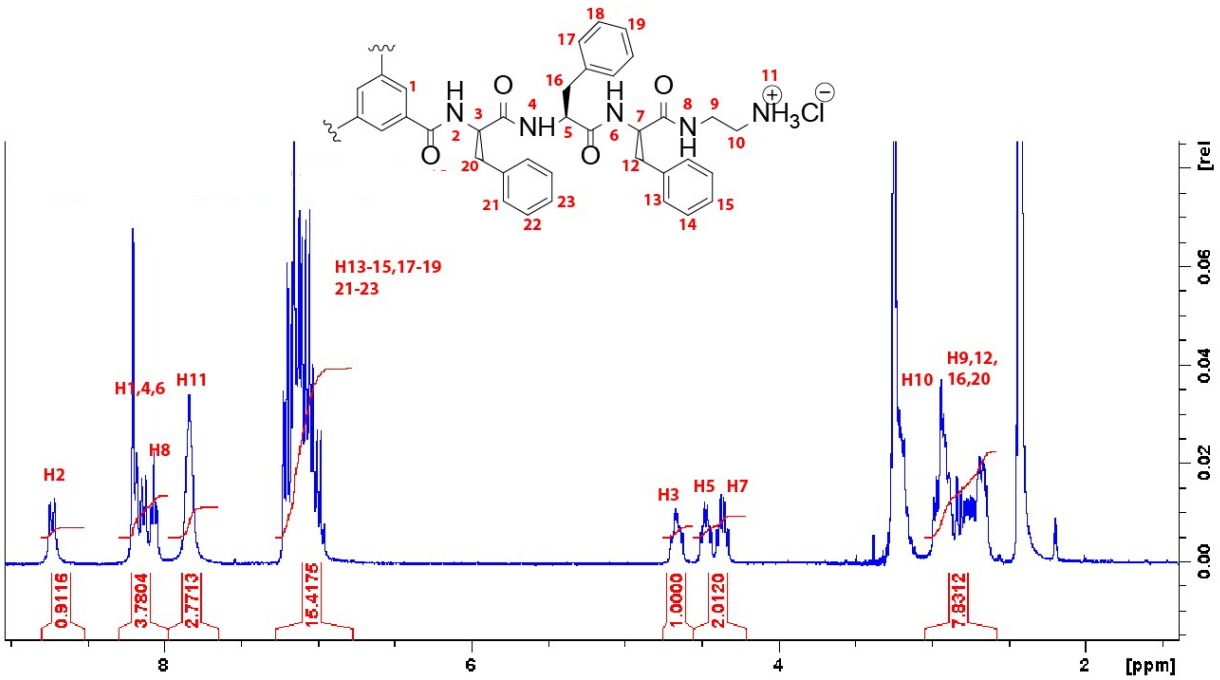
IC8. ^{1}H-NMR (300 MHz. DMSO-d6): δ 1.36 (s, 9H),2.70-3.17 (m, 10H), 4.45 (dt, 1H), 4.57 (dt, 1H), 4.75 (did, 1H),6.68 (t, 1H)6.98-7.33 (m, 15H), 7.88 (t, 1H), 8.57 (d, 1H), 8.18 (s, 1H,), 8.67 (d, 1H).

3.4. General Procedures for IC09-10 Initiator Synthesis

In a round-bottom flask with a stirrer, 1g of compound **IC07** or **IC08** were suspended in 10 ml of methanol. 10 ml of 4M hydrogen chloride in dioxane was added and the solution allowed to react for four hours. Reaction completion was monitored by NMR. The mixture was precipitated in diethyl ether (100 ml), washed with the same solvent (2x200 ml), and air-dried. Compound**IC10** (**F3)** was isolated as a hydrochloride salt during this step.

The compound was dissolved in water, and 2M NaOH was added till pH 9.0, and the mixture was stirred for 30 min. The precipitate was filtered, washed with water until pH 5.5-6.0, and freeze-dried.

IC9. ^{1}H-NMR (300 MHz. DMSO-d6):2.47 (dd, 1H), 2.8-3.1 (m, 7H), 4.49 (did, 1H), 4.74 (did, 1H), 7.08-7.31 (m, 10H), 7.81 (t, 1H), 8.2 (t, 1H), 8.23 (s, 1H), 8.77 (d, 1H).

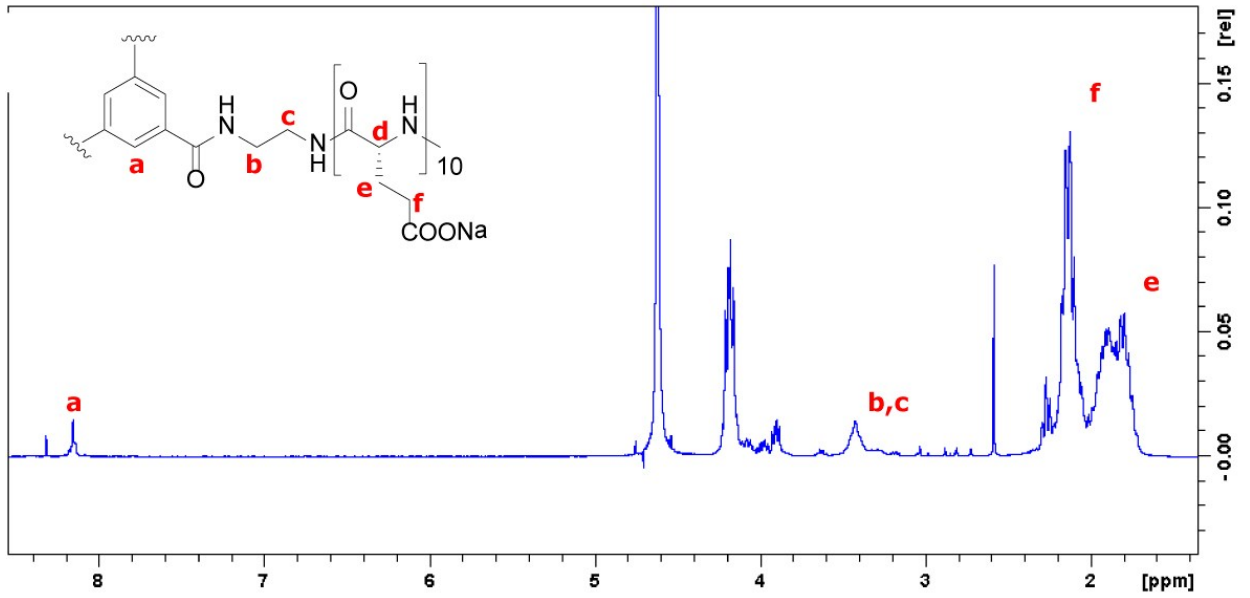


IC10 (F3). ^{1}H-NMR (300 MHz. DMSO-d6): 2.68-3.08 (m, 8H), 3.18-3.33 (m, 2H), 4.44 (did, 1H), 4.56 (did, 1H),4.76 (did, 1H),7.00-7.33 (m, 15H), 7.77 (br.t., 3H), 8.15-8.33 (m, 3H).

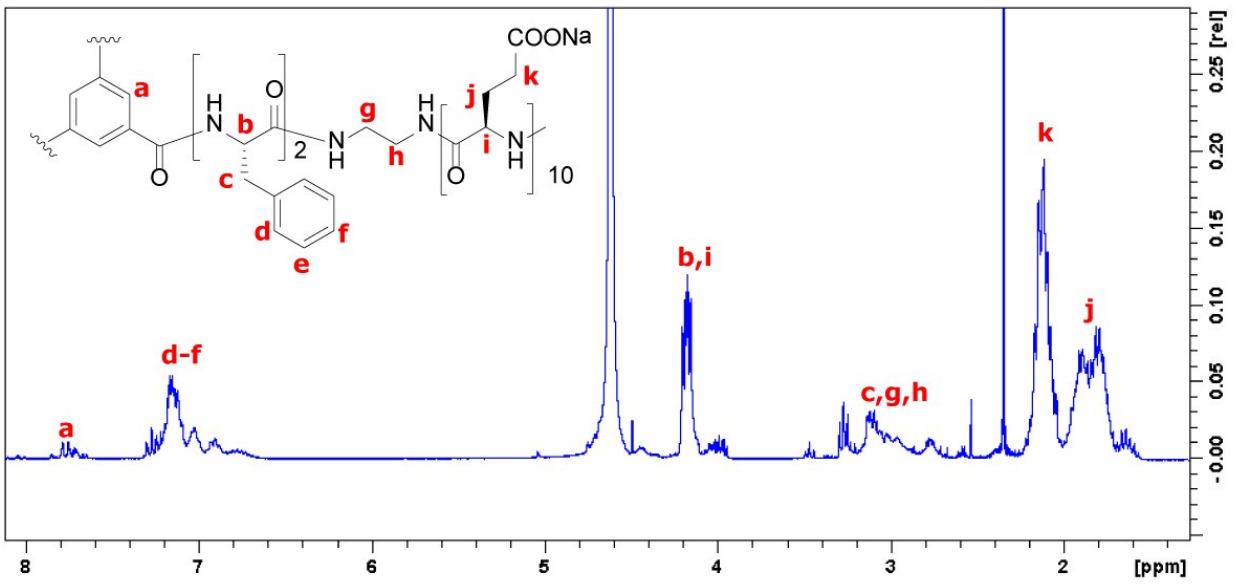
3.5. General Procedures for Polymerization

NCA-Glu-OBz (3g, 11.5 mmol) was transferred into a Schlenk tube fitted with a stirrer and a stopper. The tube was purged with three vacuum/nitrogen cycles, and 6 ml of anhydrous DMF was added. The initiator (0.383 mmol, 0.255 mmol, or 0.153 mmol) was added, and the mixture was left stirring for three days in an inert atmosphere. 6 ml of THF was then added and the mixture was precipitated in cold diethyl ether (100 ml), filtered, and washed with the same solvent (2x100 ml).

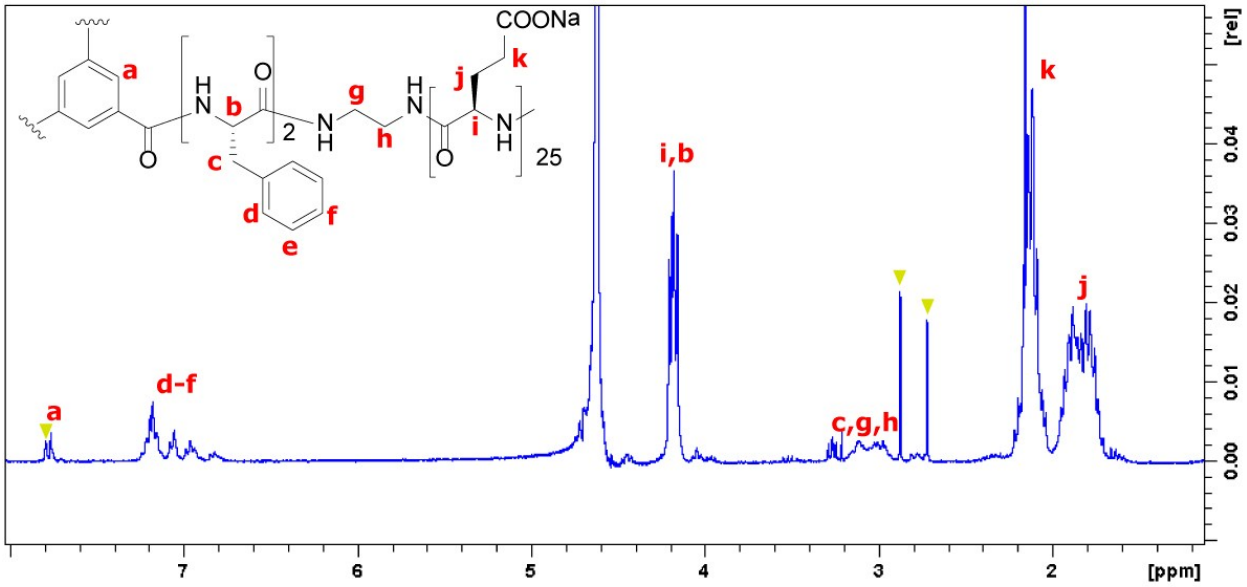
The corresponding protected star-PGA was transferred to a round-bottom flask and dissolved in 25 ml of TFA. 48 % HBr (2.5 ml) was added and the mixture allowed to react for 5-12 hours. The mixture was precipitated in diethyl ether (200 ml), filtered, and washed with the same solvent (2x200 ml). The polymer was further purified by acidification of the corresponding basic solution (pH 9) with 6M HCl followed by washing with water, a second basification with saturated sodium bicarbonate solution, dialysis and further lyophilization.



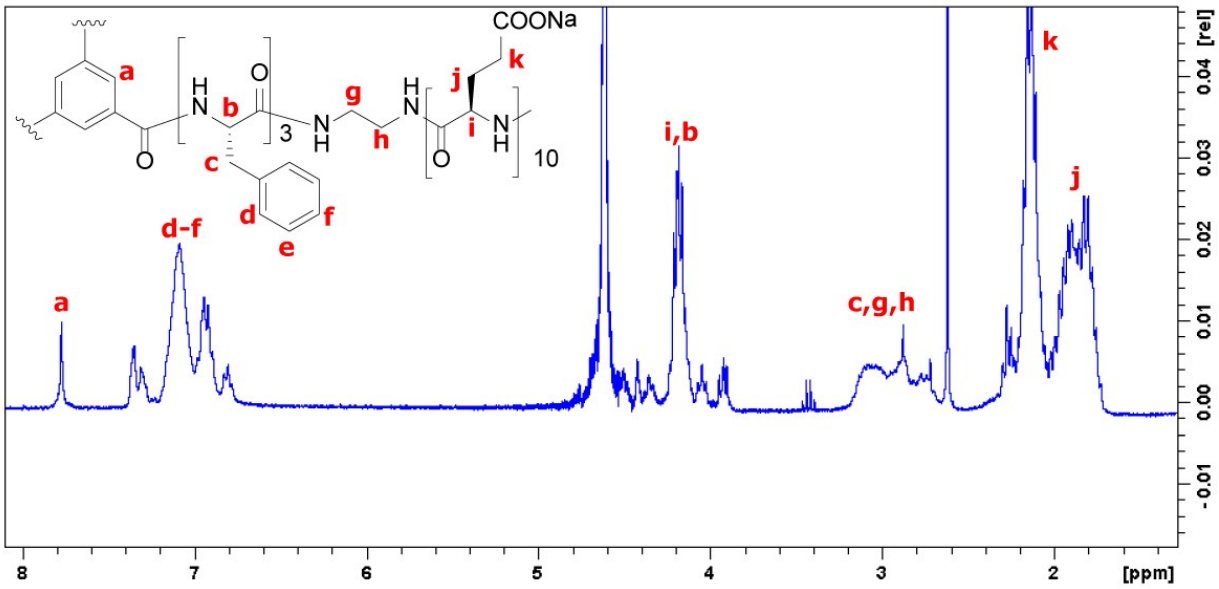
F0E10. *^{1}H-NMR (300 MHz. D2O)*: 1.68-2.27 (m, 168H), 3.42 (m, 12H), 4.18 (m, 40H), 8.15 (br. s, 3H).



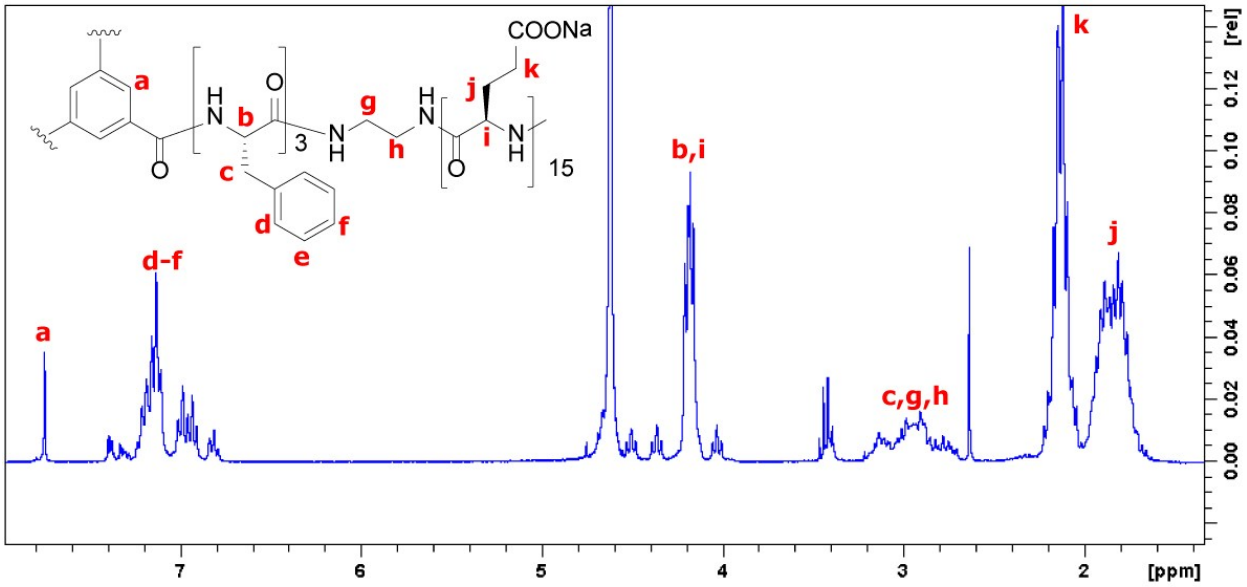
F2E10. *^{1}H-NMR (300 MHz. D2O):* 1.54-2.22 (m, 141H), 2.66-3.31 (m, 38H), 4.19 (m, 34H), 6.64-7.39 (m, 45H), 7.76 (br. s, 3H).



F2E25. *^{1}H-NMR (300 MHz. D2O):* 1.51-2.25 (m, 350H), 2.66-3.31 (m, 24H), 4.19 (m, 85H), 6.64-7.39 (m, 30H), 7.76 (br. s, 3H). (Yellow triangle - DMF peaks).



F3E10. *^{1}H-NMR (300 MHz. D2O):* 1.69-2.38 (m, 130H), 2.66-3.31 (m, 30H), 4.19 (m, 31H), 6.64-7.39 (m, 45H), 7.76 (br. s, 3H).



F3E15. *^{1}H-NMR (300 MHz. D2O):* 1.57-2.24 (m, 204H), 2.66-3.31 (m, 30H), 4.19 (m, 47H), 6.64-7.39 (m, 45H), 7.76 (br. s, 3H).

4. SUPPLEMENTARY FIGURES

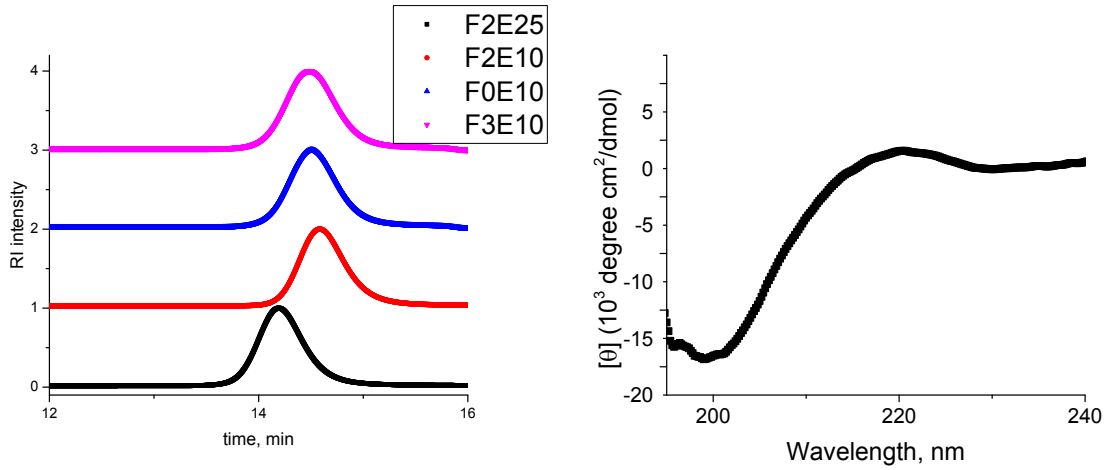


Figure S1. GPC chromatograms of compounds F0E10 (black), F2E10 (blue), F3E10 (red), and F2E25 (green) before deprotection (right) and CD spectrum of F3E10 at pH 7.4 (right).

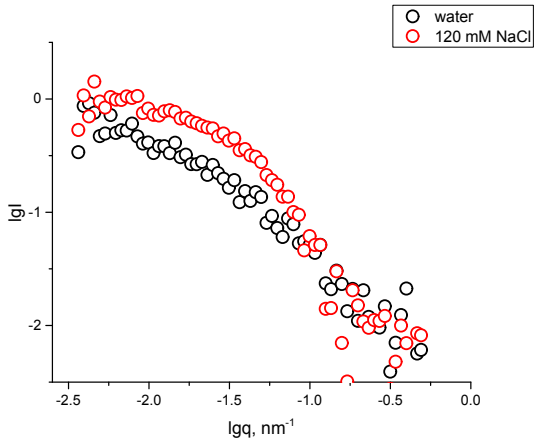


Figure S2. SANS data of **F3E10** in water and 120 mM NaCl

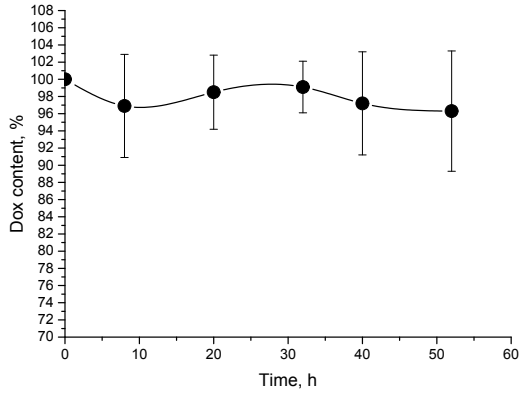


Fig. S3. Content of Dox in **F3E10**/Dox complexes during dialysis for various periods of time. Dox content is represented $100*c(Dox)_t/c(Dox)_0$ with mean ± SD (n=3).

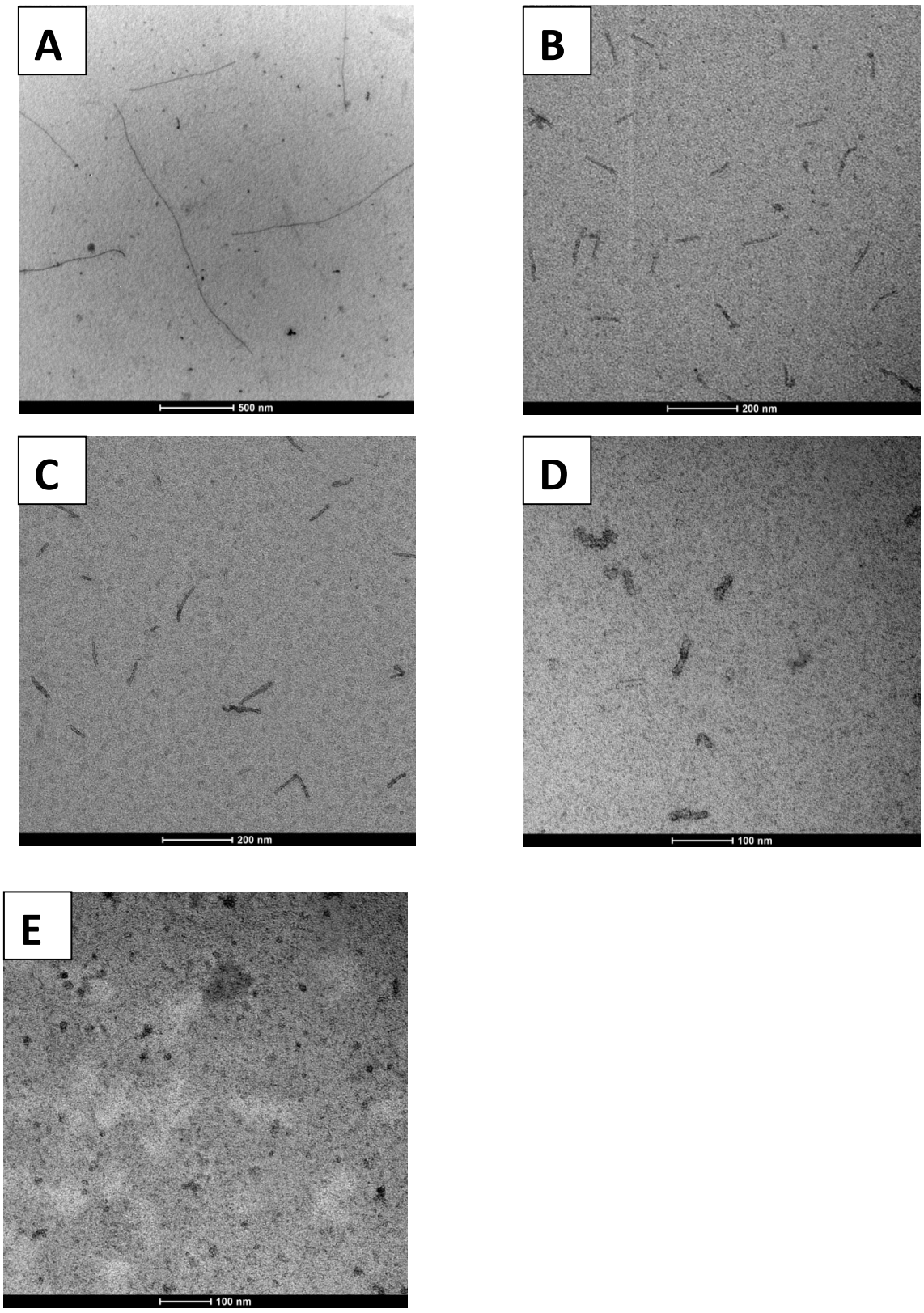


Fig. S4. TEM image of **F3E10/Dox** complexes prepared at pH 5.5 (A), 6.5 (B), 7.4 (C), and 8.5 (D) and (E) TEM image of **F3E10** (samples in 120 mM NaCl at 1mg/ml, uranyl acetate staining) as control

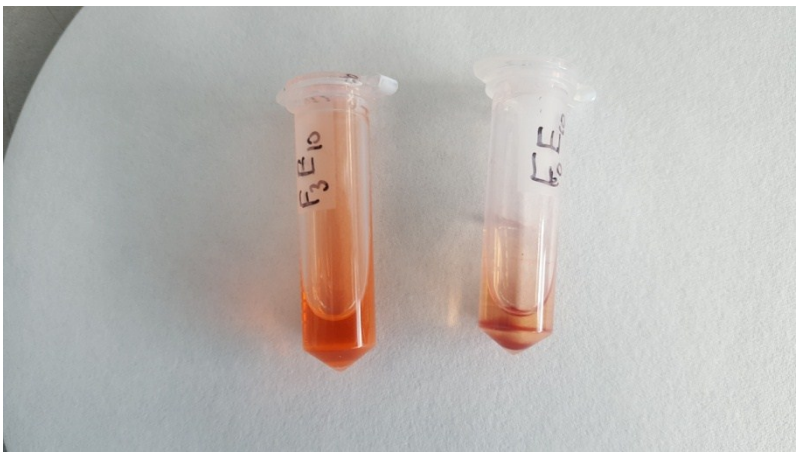


Figure S5. Solutions of **F3E10** and **F0E10**, 1 min after the addition of Dox. The presence of a precipitate in the case of **F0E10** can be seen clearly.

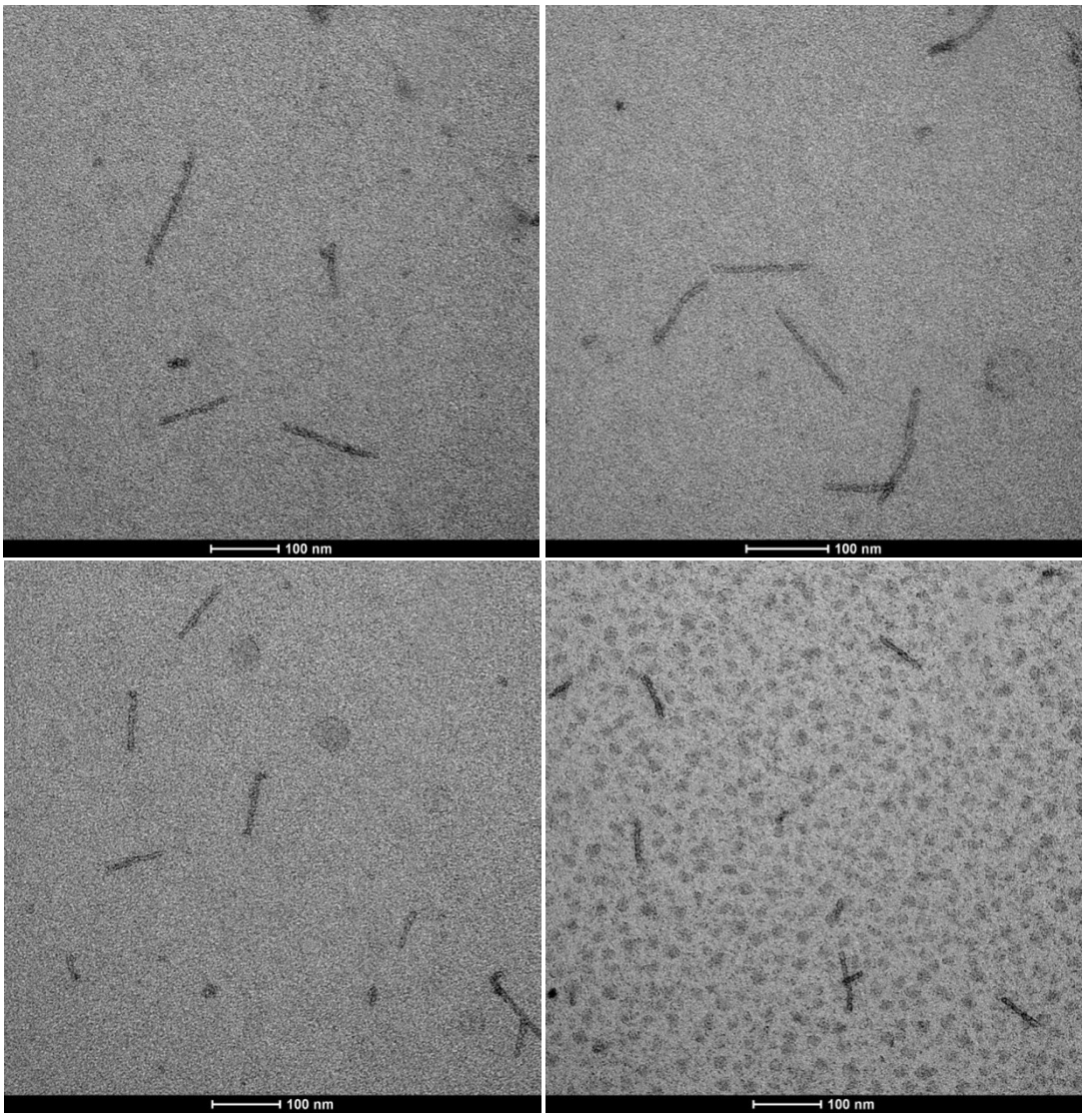


Figure S6. TEM images of four different batches of **F3E10/Dox** to check batch-to-batch reproducibility of the implemented synthetic procedure (samples in 120 mM NaCl at 1mg/ml, uranyl acetate staining).

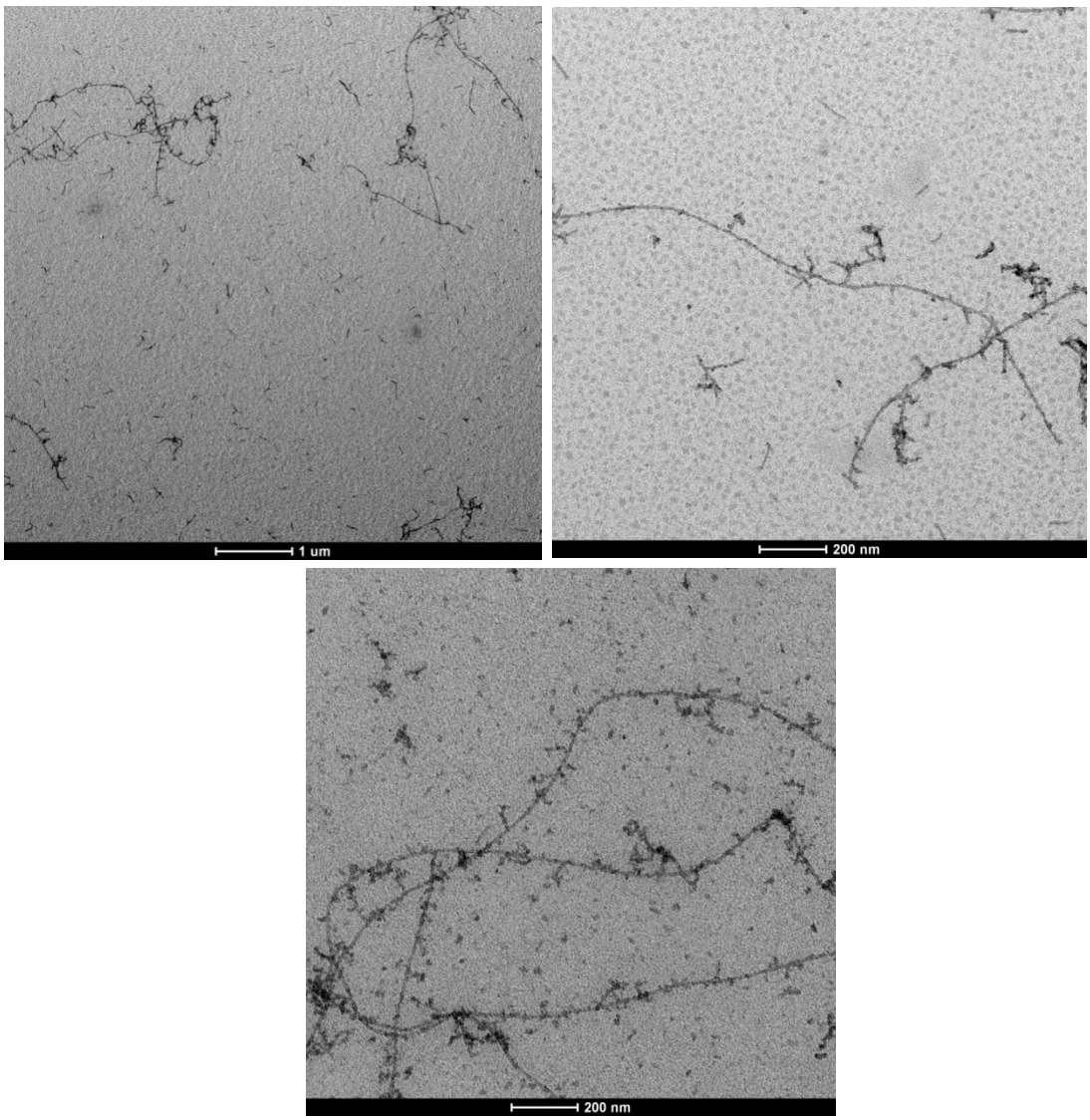


Figure S7. TEM images of **F3E10/Dox** particles obtained with non-optimal preparation strategy (samples in 120 mM NaCl at 1mg/ml, uranyl acetate staining)

5. HYPOTHETICAL MODEL OF SELF-ASSEMBLY

Existing models of the self-assembly of BTA-based molecules include a twist of the ring, as shown in **Figure S8A,** with the centers of the BTA cores situated on the z-axis. Repulsions between corresponding donors and acceptors are balanced by the formed hydrogen bonds. This model is sufficient to explain the appearance of chirality in the systems and explains the majority of BTA-based low molecular weight systems well.

We hypothesized that our systems assemble into a helical structure according to the molecule twist mechanism, as shown in **Figure S8B**. We hypothesized that π-π stacking interactions are more energetically favorable in the case of offset stacking than for sandwich stacking. Therefore, the system of directional hydrogen and aromatic stacking interactions will not be disrupted. However, in the center of the molecule, BTA rings will be shifted one against the other, so that interactions will occur through amide-πstacking and π-π stacking.

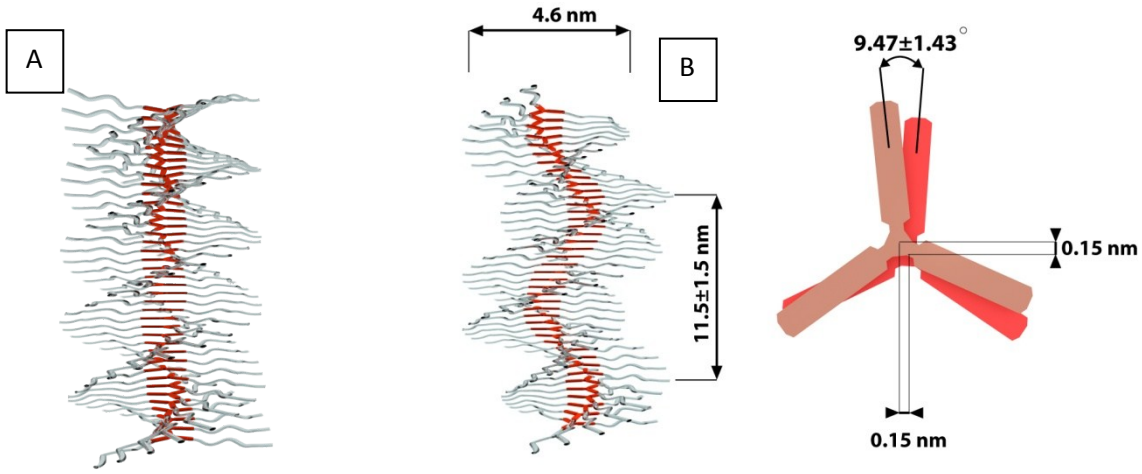


Figure S8. The existing model of BTA stacking[17] (**A**) and the proposed model of **F3E10** assembly (**B**).