

Supplementary Information for:

Phosphorylated dendronized poly(amido amine)s as protein analogues in directing hydroxylapatite biomineralization

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0. General

Materials: Ethanolamine (EA), methyl acrylate (MA), and triethylamine (TEA) (all from Tianjin Bodi Chemical Holding Co., Ltd.), trifluoroacetic acid (TFA), 4-dimethylaminopyridine (DMAP) (both from Nanjing Tianhua Chemical Co., Ltd.), methacryloyl chloride (MAC), dimethyl phosphonate (DMP) (both from Adamas-Beta), bromotrimethylsilane (TMSiBr, from TCI), 2,2'-Azobis(2-methylpropionitrile) (AIBN, from J&K Scientific), acryloyl chloride (AC) and TritonX-100 (both from Aladdin), and fluorescein diacetate (FDA) were all used as received. N-boc-ethylenediamine (Boc-EDA)^[1], N, N-tetramethylbis(phosphonate)-2-hydroxyethyl-bis(methylene) amine^[2], and 2.5 generation PAMAM dendron with an ethanol amine core^[3] were synthesized as previously reported. All other solvents and inorganic salts were of AR grade and were used as received. All glassware used in the mineralization experiments was soaked in an H₂O/HNO₃ (65%)/H₂O₂ (1:1:1 v/v/v) solution, rinsed with ultrapure water, and finally dried with acetone.

Characterizations: ¹H NMR and ³¹P NMR (400 MHz) spectra were conducted using a Varian UNITY INOVA-400 spectrometer. Size-exclusion chromatography combined with a multi-angle laser light scattering detector (SEC-MALLS) was performed using a waters 1515 isocratic HPLC pump and a Wyatt Dawn DSP laser photometer at room temperature under a wavelength of 633 nm. DMF was used as the eluent with a flow rate of 1.0 mL/min, and the column pressure was 1200 psi. Transmit electron microscopy (TEM) was performed on a Tecnai G2 F20 S-TWIN microscope operated at 200 kV. Fluorescence images were taken on an Olympus IX71 fluorescence microscope. Scanning electron microscopy (SEM) was performed on a Hitachi S-450 microscope operated at 20 kV. X-ray diffraction (XRD) patterns were obtained with an X'Pert Pro MPD X-ray diffractometer from 5° to 60° at a rate of 4°/min, using Cu-K α radiation (λ = 0.1541 nm). Phosphorus elemental analysis was performed by molybdenum blue colorimetry^[4].

1. Syntheses

1.1. Macromonomer

2.5 generation PAMAM dendron (11.48 g, 8 mmol), dry TEA (5.5 mL, 40 mmol, 5 eq.) and DMAP (195 mg, 1.6 mmol, 0.2 eq.) were dissolved in 50 mL dry DCM in a 250 mL three-necked flask, followed by drop-wise addition of MAC (3.90 mL, 40 mmol, 5 eq., in 30 mL DCM solution) under ice-bath and N₂ protection within 1 h. The reaction mixture was then allowed to warm to room temperature and stirred for an additional 18 h. After the crystalline precipitate was filtered off, DCM was vacuum-evaporated at 30°C, followed by the addition of 70 mL saturated NaHCO₃ aqueous solution to re-dissolve the product. The solution was then extracted by DCM (3 × 100 mL). And to get the crude product, the combined organic phase was evaporated at 30°C after backwashed by saturated NaHCO₃. Purification was achieved by silica chromatography using CHCl₃/MeOH (8:1) as the eluent. Purity: >99%. Yield: 93%. ¹H NMR (CDCl₃, 400 MHz, δ, ppm): 1.97 (s, 3H, C=C-CH₃); 2.25-2.46 (m, 28H, -CH₂-CONH- and -CH₂-COOCH₃); 2.50-2.95 (m, 42H, >N-CH₂-); 3.25-3.35 (m, 12H, -CONH-CH₂-); 3.66-3.68 (s, 24H, -COO-CH₃); 4.15-4.20 (t, 2H, CO-O-CH₂-); 5.57 (s, 1H, H-CH=C-); 6.08 (s, 1H, H-CH=C-).

1.2. Polymerization

DPG2.5

Polymerization of the above macromonomer was done in the bulk. The macromonomer (5 g) and AIBN (25 mg, 0.5 wt %) were dissolved in DCM, and were then vacuum-evaporated under low temperature until complete removal of the solvent. The reaction mixture was heated to 90°C, magnetic stirring become difficult after 20 min due to increasing viscosity, but the reaction was allowed to kept for a total of 2 h. When cooled to room temperature, the mixture was gradually dissolved in methanol and precipitated twice in cold diethyl ether. The precipitant was vacuum dried and gave DPG2.5 as yellowish foam. Yield: 69%. ¹H NMR (CD₃OD, 400 MHz, δ, ppm): 1.24 (s, mainchain -CH₃); 1.94 (m, mainchain -CH₂-); 2.30-2.50 (m, -CH₂-CONH- and -CH₂-COOCH₃); 2.50-3.00 (m, >N-CH₂-); 3.20-3.40 (m, -CONH-CH₂-); 3.65-3.80 (s, -COO-CH₃); 4.15-4.30 (m, CO-O-CH₂-). SEC-MALLS: M_w = 175, 000 g/mol, PDI = 1.55, the refractive-index increment (dn/dc) is 0.094 mL/g detected at 633 nm.

1.3. Higher-generation dendronized PAMAMs (DPs)

Boc-DPG3.0

A methanol solution of DPG2.5 (1.88 g, 1.25 mmol, 20 wt%) was drop-wisely added into a 70 wt% Boc-EDA (16 g, 100 mmol, 10 eq. to methyl ester groups) methanol solution under ice-bath and nitrogen protection. The solution was magnetically stirred at 5°C for 8 d, concentrated and precipitated twice in large excess of cold diethyl ether. The precipitant was vacuum dried to obtain Boc-DPG3.0 as yellowish foam. Yield: 94%. ¹H NMR (CD₃OD, 400 MHz, δ, ppm): 1.28 (m, mainchain -CH₃); 1.42-1.44 (s, -NHCOO-C(CH₃)₃); 1.94 (m, mainchain -CH₂-); 2.35-2.55 (br, -CH₂-CONH-); 2.80-2.95 (br, >N-CH₂-); 3.10-3.25 (br, -CONH-CH₂-CH₂N< and (CH₃)₃C-O-CO-CH₂-); 3.45-3.60 (br, (CH₃)₃C-O-CO-CH₂-).

DPG3.0

De-protection of the surface amino groups of Boc-DPG3.0 (2.91 g, 1.15 mmol) was done in methanol solution (20 wt%), in which an excess of TFA (5 equivalents to Boc groups) was added. The mixture was vigorously stirred at 50°C for 1 d to fully hydrolyze the Boc groups. After that, the solvent was evaporated and the residue was precipitated twice in cold diethyl ether. The precipitant was vacuum dried to obtain DPG3.0 as yellowish foam. Yield: 93%. Degree of hydrolysis: >99%. ¹H NMR (CD₃OD, 400 MHz, δ, ppm): 1.18-1.21 (br, mainchain -CH₃); 1.94-1.99 (br, mainchain -CH₂-); 2.83-2.94 (br, -CH₂-CONH-); 3.10-3.27 (br, >N-CH₂-); 3.49-3.66 (br, -NHCH₂-CH₂-NH₂); 3.66-3.81 (br, -CONH-CH₂-); 3.96 (br, -CO-O-CH₂-).

DPG3.5

A methanol solution of DPG3.0 (1.73 g, 1 mmol, 20 wt%) was added into MA (3.60 mL, 40 mmol, 5 eq. to amino groups, 50 wt% in methanol) under ice-bath and N₂ protection. The mixed solution was magnetically stirred at 40°C for 3 d, and was then evaporated and vacuum dried to obtain DPG3.5 as yellowish foam. Yield: 91%. ¹H NMR (CD₃OD, 400 MHz, δ, ppm): 1.15-1.19 (br, mainchain -CH₃); 1.95-1.99 (br, mainchain -CH₂-); 2.71-2.78 (br, -CH₂-CONH-); 2.80-2.88 (br, -CH₂-COOCH₃); 3.28-3.32 (br, >N-CH₂-); 3.45-3.54 (br, -CONH-CH₂-); 3.72-3.76 (br, -CO-O-CH₃).

1.4. Carboxylated DPs

Half-generation dendronized PAMAMs in grams were dissolved in methanol solution of NaOH (1.5 equivalents to ester groups), the solution was refluxed at 45°C for 2 days. Methanol was evaporated and the residue was purified by ultrafiltration (after pH was adjusted to approaching neutral). Sodium salts of carboxylated dendronized PAMAMs were obtained via lyophilization.

Carboxylated DPG2.5 (DPG2.5-COOH)

Yield: 75%. ¹H NMR (CD₃OD, 400 MHz, δ, ppm): 1.05-1.22 (br, mainchain -CH₃); 1.95-2.05 (br, mainchain -CH₂-); 2.50-2.70 (br, -CH₂-CONH- and -CH₂-COONa); 3.15-3.40 (br, >N-CH₂-); 3.50-3.70 (m, -CONH-CH₂-).

Carboxylated DPG3.5 (DPG3.5-COOH)

Yield: 78%. ¹H NMR (CD₃OD, 400 MHz, δ, ppm): 1.00-1.15 (br, mainchain -CH₃); 1.90-2.00 (br, mainchain -CH₂-); 2.30-2.40 (br, -CH₂-CONH- and -CH₂-COONa); 2.55-2.80 (br, >N-CH₂-); 3.30-3.40 (m, -CONH-CH₂-).

1.5. Phosphorylated DPs

1.5.1. Phosphorylation via sodium trimetaphosphate (STMP)

Tri-phosphates terminated DPG3.0 (DPG3.0-P₃)

Solid sodium trimetaphosphate (12.24 g, 40 mmol, 10 eq. to amino groups) was added directly into a basic aqueous solution of DPG3.0 (0.86 g, 0.5 mmol, pH adjusted to 12), and the solution was stirred under room temperature for 2 d. The product was obtained by ultrafiltration of the solution, followed by lyophilization. Yield: 69%. ¹H NMR (DMSO-*d*₆, 400 MHz, δ, ppm): 1.23-1.27 (br, mainchain -CH₃); 1.95-

2.02 (br, mainchain $-\underline{\text{CH}_2}-$); 2.85-3.02 (br, $-\underline{\text{CH}_2}-\text{CONH}-$); 3.15-3.35 (br, $>\text{N}-\underline{\text{CH}_2}-$); 3.53-3.88 (br, $-\text{NHCH}_2-\underline{\text{CH}_2}-\text{PO}-$ and $-\text{CONH}-\underline{\text{CH}_2}-$); 3.99 (br, $-\text{CO}-\text{O}-\underline{\text{CH}_2}-$). ^{31}P NMR (DMSO- d_6 , 400 MHz, δ , ppm): -0.36 ($-\text{NH}-\underline{\text{P}}-\text{O}-\text{P}-\text{O}-\underline{\text{P}}-$); -5.02 ($-\text{NH}-\underline{\text{P}}-\text{O}-\text{P}-\text{O}-\underline{\text{P}}-$); -20.45 ($-\text{NH}-\underline{\text{P}}-\text{O}-\underline{\text{P}}-\text{O}-\underline{\text{P}}-$). Phosphorus elemental analysis: found 17.35 wt% (theoretical value 20.59 wt%, calcd for $\text{C}_{76}\text{H}_{143}\text{O}_{88}\text{N}_{29}\text{P}_{24}$).

1.5.2. Phosphorylation via bis-phosphonate bearing molecules

Ethyl N,N-tetramethylbis(phosphonate)-bis(methylene) amine acrylate

N, N-tetramethylbis(phosphonate)-2-hydroxyethyl-bis(methylene) amine (9.15 g, 30 mmol), dry TEA (20.8 mL, 150 mmol, 5 eq.) and DMAP (0.73 g, 6 mmol, 0.2 eq.) were dissolved in 50 mL dry DCM, and a solution of AC (7.25 mL, 90 mmol, 3 eq., in 30 mL DCM) was drop-wisely added under ice-bath and N_2 protection within 1 h. The reaction mixture was then allowed to warm to room temperature and stirred for an additional 18 h. After the crystalline precipitate was filtered off, DCM was vacuum-evaporated at 30°C, followed by the addition of 150 mL saturated NaHCO_3 aqueous solution to re-dissolve the product. The solution was then extracted by DCM (3×100 mL). The combined organic phase was backwashed by saturated NaHCO_3 and then vacuum-evaporated at 30°C to get the final product. Purity: >95%. Yield: 93%. ^1H NMR (CDCl_3 , 400 MHz, δ , ppm): 3.00 (m, 2H, $-\text{OCH}_2-\underline{\text{CH}_2}-\text{N}<$); 3.10 (s, 4H, $\text{N}-\underline{\text{CH}_2}-\text{PO}-(\text{OCH}_3)_2$); 3.63 (s, 12H, $\text{PO}-(\text{OCH}_3)_2$); 4.14 (m, 2H, $-\text{OCH}_2\text{CH}_2\text{N}<$); 5.70 (m, 1H, $\text{H}-\underline{\text{CH}}=\text{CH}-\text{COO}-$); 5.98 (m, 1H, $\text{H}-\text{CH}=\underline{\text{CH}}-\text{COO}-$); 6.25 (m, 1H, $\text{H}-\underline{\text{CH}}=\text{CH}-\text{COO}-$).

Ethyl N,N-bis(phosphonate)-bis(methylene) amine acrylate

TMSiBr (52.8 mL, 400 mmol, 5 eq. to phosphonate groups) was drop-wisely added into an anhydrous DCM solution of ethyl N,N-tetramethylbis(phosphonate)-bis(methylene) amine acrylate (7.18 g, 20 mmol), the mixture was stirred at room temperature overnight. Afterwards, an excess of methanol was added into the silylated polymer and the mixture was stirred for another 12 h. Then the product was vacuum-evaporated at 30°C and purified by silica chromatography using $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1:1) as the eluent to obtain the final product as a yellow powder. Yield: 73%. ^1H NMR (DMSO- d_6 , 400 MHz, δ , ppm): 3.30 (m, 2H, $-\text{OCH}_2-\underline{\text{CH}_2}-\text{N}<$); 3.65 (d, 4H, $\text{N}-\underline{\text{CH}_2}-\text{PO}-(\text{OH})_2$); 4.57 (m, 2H, $-\text{OCH}_2\text{CH}_2\text{N}<$); 5.99 (m, 1H, $\text{H}-\underline{\text{CH}}=\text{CH}-\text{COO}-$); 6.20 (m, 1H, $\text{H}-\text{CH}=\underline{\text{CH}}-\text{COO}-$); 6.45 (m, 1H, $\text{H}-\underline{\text{CH}}=\text{CH}-\text{COO}-$).

Bis-phosphonates terminated DPG3.0 (DPG3.0- P_2)

A methanol solution of DPG3.0 (0.86 g, 0.5 mmol, 20 wt%) was added into ethyl N,N-bis(phosphonate)-bis(methylene) amine acrylate (6.06 g, 20 mmol, 5 eq. to amino groups, 50 wt% in methanol) under ice-bath and N_2 protection. The solution was magnetically stirred at 40°C for 3 d, methanol was evaporated and the residue was purified by ultrafiltration. Further lyophilization yielded the yellowish foam product. Yield: 91%. ^1H NMR (DMSO- d_6 , 400 MHz, δ , ppm): 1.14-1.23 (br, mainchain $-\underline{\text{CH}_3}$); 1.80-1.87 (br, mainchain $-\underline{\text{CH}_2}-$); 3.01- 3.13 (br, $-\underline{\text{CH}_2}-\text{CONH}-$ and $-\underline{\text{CH}_2}-\text{COO}-$); 3.20-3.30 (m, $-\text{N}-\underline{\text{CH}_2}-\text{PO}-(\text{OH})_2$); 3.38-3.60 (br, $>\text{N}-\underline{\text{CH}_2}-$); 3.63-3.80 (br, $-\text{CONH}-\underline{\text{CH}_2}-$); 4.16-4.26 (br, $-\text{COO}-\underline{\text{CH}_2}-$); 5.9-7.3 (br, $-\text{PO}-(\text{OH})_2$); 7.85-8.25 (m, $-\text{CONH}-$). ^{31}P NMR (DMSO- d_6 , 400 MHz, δ , ppm): 20.79 ($-\text{N}-\text{CH}_2-\underline{\text{PO}}-(\text{OH})_2$). Phosphorus elemental analysis: found 13.78 wt% (theoretical value 15.08 wt%, calcd for

C₁₈₈H₃₉₁O₁₄₄N₄₅P₃₂).

2. Mineralization

2.1. Mineralization of DPs on nano-scale

Firstly, 1 mg/mL aqueous solution of each polymer (containing 30 mM HEPES and 1% penicillin/streptomycin, the pH value was adjusted to 7.0) was prepared. The solution was magnetically stirred for at least 1 h to fully dissolve the polymer before it was treated by microfiltration. After that, traces of concentrated CaCl₂ and K₂HPO₄ aqueous solution was added in succession to make sure the final concentrations of Ca²⁺ and PO₄³⁻ reached 5 mM and 3 mM, respectively. The mixed solution was transferred into polyethylene tubes, sealed and shaken, then incubated in a 37°C water bath for 7 days.

2.2. Fibrillation and mineralization of DPs on micro-scale

5 mg/mL aqueous solutions of DPs (containing 5 mM Ca²⁺, 30 mM HEPES and 1% penicillin/streptomycin, the pH value was adjusted to 7.0) were prepared. The samples were gently stirred at 37°C for 24 h to induce fibrillation and mature the microfibers, millimeters long microfibers were generated. After that, traces of concentrated CaCl₂ and K₂HPO₄ aqueous solution was added in succession to make sure the final concentrations of Ca²⁺ and PO₄³⁻ reached 10 mM and 6 mM, respectively. The mixed solution was transferred into polyethylene tubes, sealed and shaken, then incubated in a 37°C water bath for 7 days.

3. Cell experiments

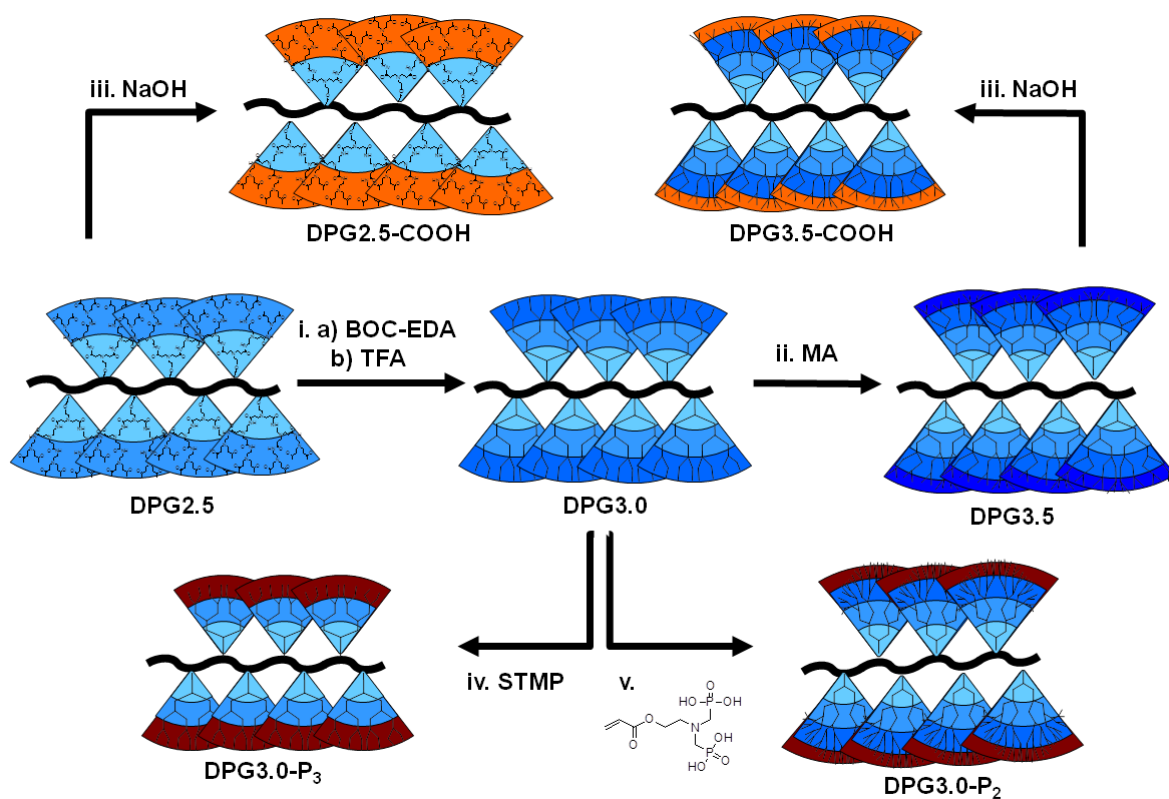
3.1. BMSCs cultivation on DP/HA complexes films

BMSCs were harvested from SD rats weighing about 100 g. Briefly, the femura and fabias of SD rats were peeled off and cleaned, and the bone marrow was flushed out via culture media. Afterwards, the bone marrow cells were planted in cell culture flasks containing α -MEM supplemented with 10% FBS at 37°C in a humidity atmosphere with 5% CO₂. The culture media was changed every other day to remove the non-adherent cells.

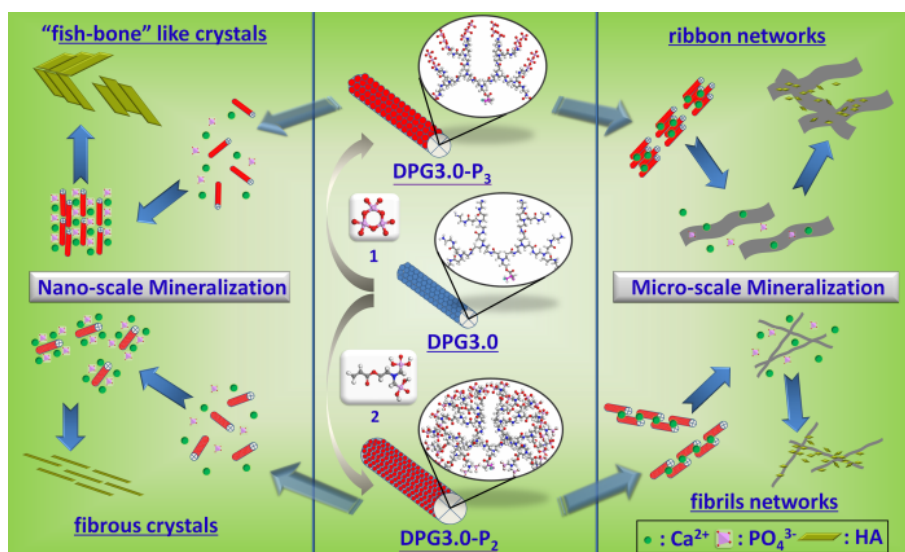
A fixed volume of suspensions of DP/HA complexes were spread on glass slides and dried, making sure that the complexes fully covered the glass surface. All samples were sterilized by steam autoclaving at 120°C for 30 min. Afterwards, BMSCs were seeded on these glass slides at a density of 6000/cm² and were cultured in α -MEM supplemented with 10% FBS for 7 days. The culture media was changed every two days.

3.2. BMSCs morphology and proliferation

In order to study cell morphology, fluorescence images of BMSCs were taken after the samples were stained by FDA for 5 min. Cell counting kit-8 (CCK-8) assay was done to analyze BMSC proliferation, the optical densities (OD) values of the CCK-8 containing culture media at 450 nm were measured after cultivating for 1 h at 37°C. Bicinchoninic acid (BCA) protein assay kit was measured at 562 nm to determine the total protein in cell lysates, and the cells were lysed by 2% TritonX-100.



Scheme S1 Schematic illustration of the syntheses of dendronized PAMAMs (DPs). Conditions: i) a) BOC-EDA, methanol, r.t., 8 d; b) TFA, methanol, 50°C, 1 d; ii) MA, methanol, 40°C, 3 d; iii) NaOH, methanol, 45°C, 2 d; iv) sodium trimetaphosphate (STMP), water, r.t., 2 d; v) Ethyl N,N-bis(phosphonate)-bis(methylene) amine acrylate, methanol, 40°C, 3 d.



Scheme S2 Phosphorylation of dendronized PAMAMs and their directed HA biomineralization on both nano- and micro-scales.

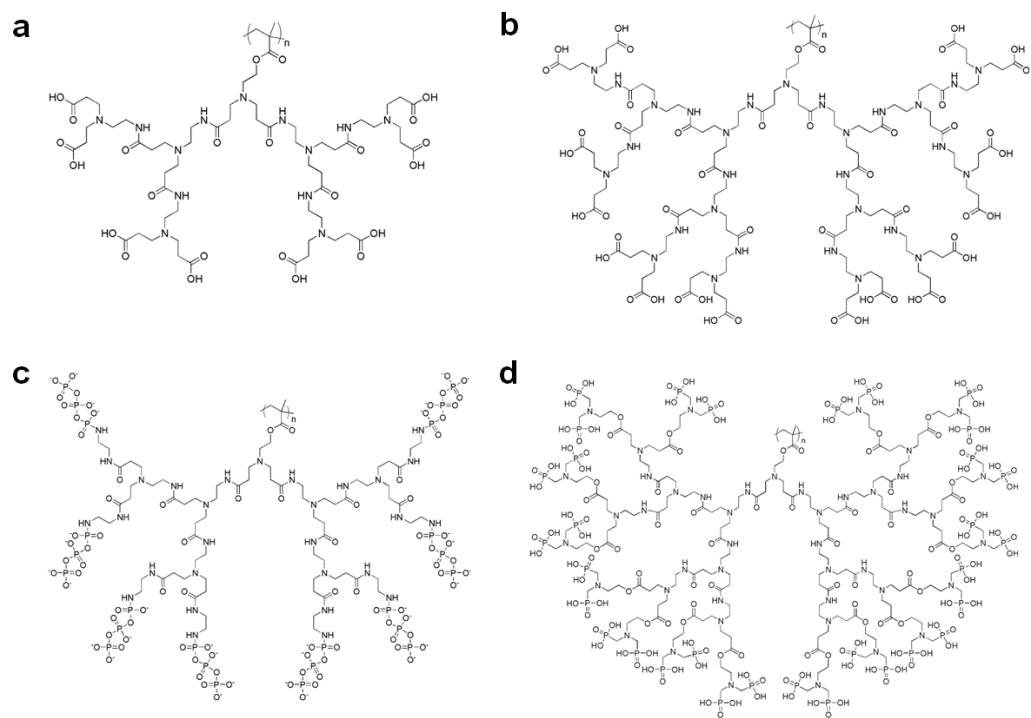


Fig. S1 Chemical structures of (a) DPG2.5-COOH, (b) DPG3.5-COOH, (c) DPG3.0-P₃, and (d) DPG3.0-P₂.

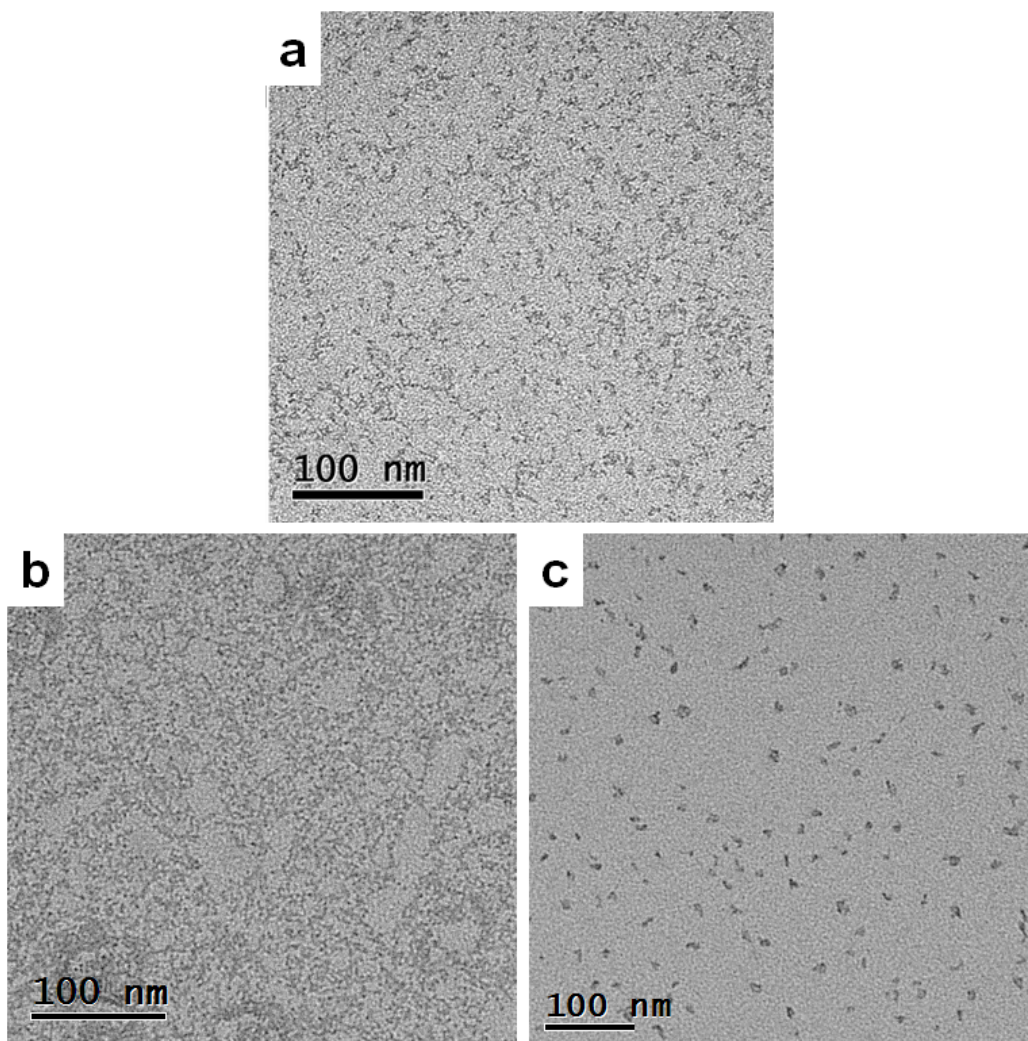


Fig. S2 TEM images of (a) DPG3.5-COOH, (b) DPG3.0-P₃ and (c) DPG3.0-P₂ molecules.

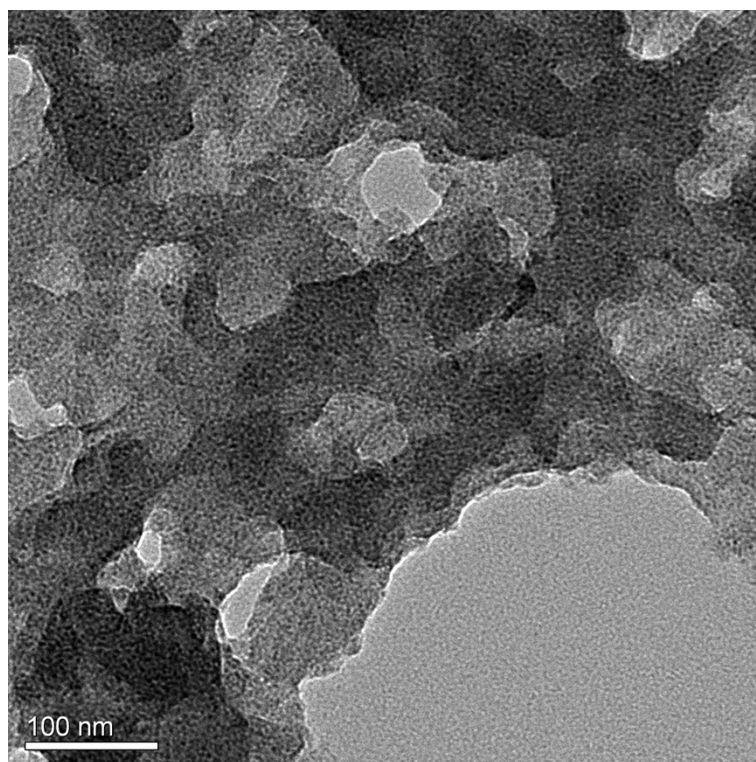


Fig. S4 DPG2.5-COOH/CaP formed only amorphous aggregates after 7 days' incubation.

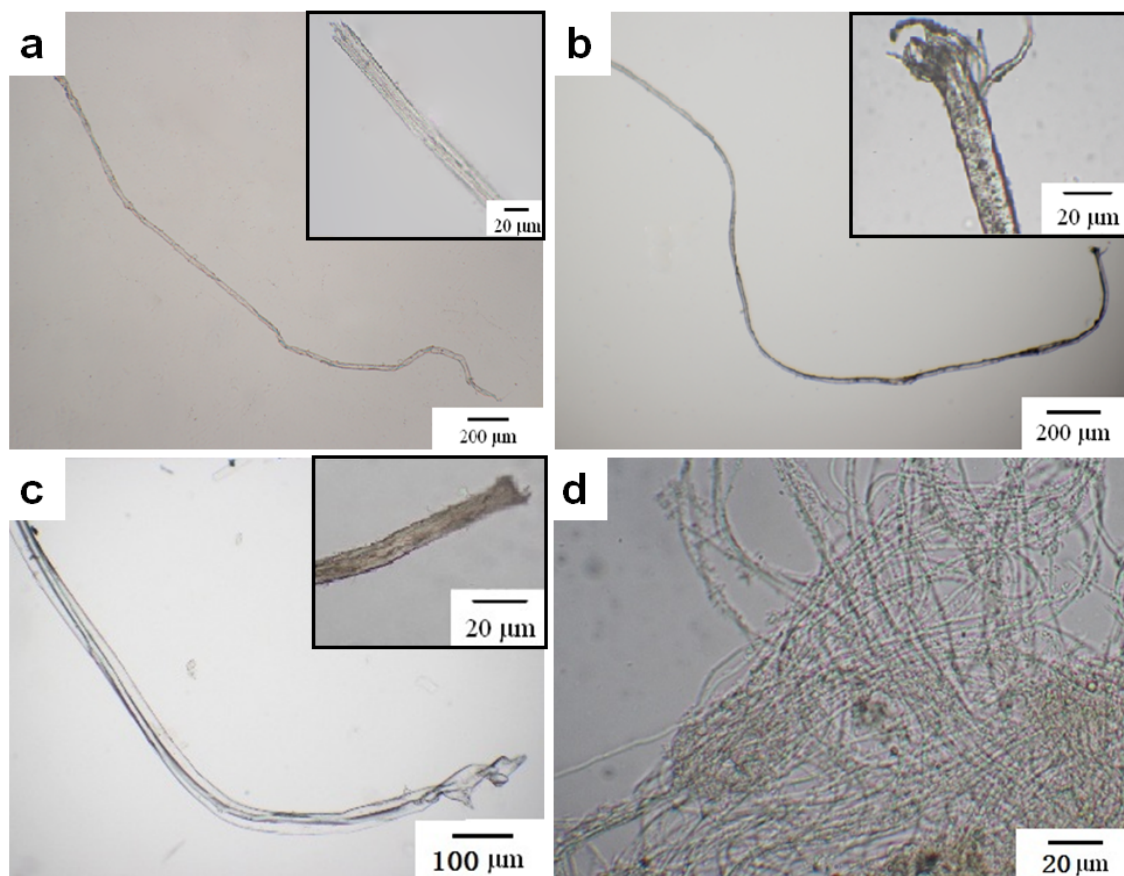


Fig. S5 Optical microscopy images of microfibers formed by (a) DPG2.5-COOH, (b) DPG3.5-COOH, (c) DPG3.0-P₃ and (d) DPG3.0-P₂. Insets show enlarged images.

Table S1 Morphology of DP/CaP complexes on nano-scale.

Sample	Length*	Width*	Aspect ratio
DPG2.5-COOH/CaP	-	-	-
DPG3.5-COOH/CaP	319.1 ± 53.7	27.9 ± 3.2	11.43
DPG3.0-P3/CaP	262.1 ± 33.6	24.4 ± 2.1	10.74
DPG3.0-P2/CaP	176.5 ± 37.3	17.7 ± 1.9	9.97

*Means ± standard error of mean for n = 50.

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