Supporting Information

Multifunctional gene delivery systems with targeting ligand CAGW and charge reversal function for enhanced angiogenesis

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Experimental Section

Characterization

^{1}H-NMR spectrum was recorded on an ECA-400 (400 MHz) spectrometer using deuterated chloroform ($CDCl_3$), deuterated water (D_2O), deuterated hydrochloric acid (DCl) and deuterated sodium hydroxide (NaOD) at 25 °C. Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI TOF MS) was performed on a Bruker Daltonics Inc. Autoflex Tof/TofIII MALDI-TOF mass spectrometer with DHB matrix. Transmission electron microscope (TEM) was performed on a Japanese model JEM-2100F with an accelerating voltage of 200 kV. The hydrodynamic diameter and ζ potential were detected via a Zetasizer Nano ZS (Malvern Instrument, Inc., Worcestershire, UK).

Synthesis of POSS-$(DA)_8$

The reaction was performed following the typical Schotten-Baumann procedure (Figure S1). Specifically, POSS-$(NH_3^+Cl^-)_8$ (1.5 g, 10.23 mmol $NH_3^+Cl^-$ group) was dissolved in phosphate buffer solution (PBS pH 8.5, 10 mL), and DAC (8.16g, 51.15mmol) was dissolved in anhydrous chloroform (40 mL). At 0 °C, DAC solution was slowly dropped into the PBS solution with vigorously stirring. Meanwhile, the reaction pH was kept at 8.5 by supplying 1 N sodium hydroxide (NaOH) aqueous solution. After adding, the reaction mixture was stirred overnight at RT. The organic phase was separated, washed (HCl solution, saturated NaCl solution and deionized water), dried (anhydrous Na_2SO_4), filtered, vacuum evaporated, washed (anhydrous ether) and vacuum dried to obtain crude product. Finally, the crude product was further purified through reversed-phase column chromatography of C_{18} (0.5% TFA in water/acetonitrile) to obtain POSS-$(DA)_8$. In ^{1}H NMR spectrum (Figure S2, B), the area ratio of the peak c' to peak d was 2, which proved that eight amino groups of POSS-$(NH_3^+Cl^-)_8$ were absolutely converted into amide groups by reacting with DAC. MOLDI TOF MS was performed with DHB matrix, 1887.072 $(M + Na)^+$ was found to agree with the ^{1}H NMR result (Figure S3).

Synthesis of POSS-(CG-NLS-G-TAT)$_{16}$

POSS-(DA)$_8$ (2 mg, 1.07×10^{-3} mmol), DMPA (1 mg) and TAT-G-NLS-GC (55 mg, 20.82×10^{-2} mmol) were added into a mixed solvent of THF/ultra purified water (2:1 by volume). The solution was treated by UV-light for 10 min under stirring and nitrogen atmosphere, and then dialyzed and lyophilized to obtain POSS-(CG-NLS-G-TAT)$_{16}$. In ^{1}H NMR spectrum (Figure S2, C), the signal peaks of e and f completely disappeared, meanwhile the signal peak g of TAT moiety appeared.

Synthesis of PLCA, PLCA-CAGW and PLSA-CAGW

PLL (20 mg, 1.56×10^{-1} mmol NH_2 group) and SPDP (10 mg, 3.2×10^{-2} mmol) were respectively dissolved in ultra purified water (15 mL) and methanol (2 mL), respectively. The SPDP solution was slowly added dropwise into PLL aqueous solution and the reaction mixture was continuously stirred for 4 h in dark. Afterwards, CAGW peptide (17 mg, 3.90×10^{-2} mmol) was added into the solution and continued to stir for 6 h. The reaction mixture was cooled to 0°C and adjusted to pH 8.5 using 1 N NaOH aqueous solution. Then, CA (60.90 mg, 3.90×10^{-1} mmol) was gradually added into this solution, kept pH = 8-9 by supplying NaOH aqueous solution. The reaction mixture was stirred for overnight at 0℃ after pH did not change. The solution was sequentially dialyzed (against NaOH solution with pH 8-9) and lyophilized to obtain PLCA-CAGW powder. The synthesis routes are shown in Figure S4.

PLSA-CAGW was synthesized with the same method, just using SA (39.00 mg, 3.90×10^{-1} mmol) rather than CA. PLCA was prepared with the similar method, just without the reaction procedure of PDP and CAGW. In ^{1}H NMR spectrum (Figure S5, A), the graft ratio of SPDP to the side chain of PLL was calculated to be 21% through the area ratio of signal peaks from pyridyl protons (δ = 7.2-8.3 ppm) to methine proton peaks in the PLCA main chain (δ = 4.10 ppm). After substituted by CAGW peptide, the signal peak from pyridyl protons (δ = 7.2-8.3 ppm) entirely disappeared from ^{1}H NMR spectrum (Figure S5, C and D), which illustrated that the graft ratio of CAGW peptide to PLL was equal to 21%. For CAGW peptide, the methine proton peaks from alanine and glycine residues were located at 4.0-4.2 ppm (D_2O, 25 °C). The area of methine proton peaks of PLCA-CAGW should be 1.42 times as high as that of the main

chain because of 21% graft ratio. Therefore, the conversion of primary amine from the side chain of PLL to cis-aconitic amide could be determined to be about 64% through the area ratio of methine proton peaks from the cis-aconitic moiety (δ 6.0 ppm) to the methine proton peak (δ = 4.0-4.2 ppm) (Figure S5, C). Similarly, the conversion of primary amine to succinic amide was determined to be about 76% through the area ratio of methylene proton peaks from the succinic moiety (peak m and n) to the methine proton peaks (δ = 4.0-4.2 ppm) (Figure S5, D).

Synthesis of PLL-CA

PLL-CA was synthesized through the reaction between the primary amines in PLL side chain and 2.5 times amount of CA. The conversion of these primary amine to cis-aconitic amide was determined to be about 78% through ^{1}H NMR spectrum (Figure S5, B).

Characterization

Characterization results are shown in Figure S6, S7, S8, S9 and S10.

Measurement with pH responsive hydrolysis reaction of cis-aconitic amide

The hydrolysis reaction was tracked through ^{1}H NMR spectrum. PLL-CA was chosen as the reactant model because the characteristic peak of PLL-CA could be clearly observed. PLL-CA was first dissolved in D_2O and then respectively adjusted to pH 7.4 and 5.5 by the addition of DCl. At the predetermined time (0.5h, 2h, 4h and 24h), ^{1}H NMR spectrum was performed to record the information of proton peaks. The methine proton peaks from cis-aconitic moiety obviously changed when cis-aconitic amide was hydrolyzed, because its chemical environment changed from amide (-NHCOCHC(COO^-)CH_2COO^-) to carboxylic anion ($^-$OOCCHC(COO^-)CH_2COO^-). The intensity and location change of these characteristic peaks reflected the hydrolysis. In Figure S9, the methine proton peak changed from a to a' due to the cleavage of cis-aconitic amide. At pH 7.4, peak a basically kept unchanged in the aspect of intensity and location within 4h. The peak a' with very low intensity appeared in slightly lower field in 24h. These results illustrated that cis-aconitic amide basically could not be hydrolyzed and keep stable within 24h at pH 7.4. However, at pH 5.5, peak a' was observed in lower field within 0.5h, and then peak a' became stronger, but peak a

became weak along with hydrolysis time. After 24h, peak a disappeared entirely. These results indicated that cis-aconitic amide bonds could be quickly hydrolyzed in pH 5.5 environment, even entirely hydrolyzed in 24h. By analyzing the hydrolysis under pH 7.4 and 5.5, cis-aconitic amide was confirmed to be sensitive to weak acid environment (pH = 5.5), but stable under pH = 7.4.

Preparation and characterization of binary complexes (BCPs) and ternary complexes (TCPs) with different weight ratios

In ultrasonic environment, POSS-(G-NLS-G-TAT)$_{16}$ was dissolved in pH 7.4 PBS to obtain uniform solution (0.2 mg/mL), named as POSS-(CG-NLS-G-TAT)$_{16}$ solution. The morphology of POSS-(CG-NLS-G-TAT)$_{16}$ was detected through TEM, and its ability to load and condense pZNF580 was evaluated via agarose gel electrophoresis assay. Different volumes of pZNF580 solution (50 μg mL^{-1}) was added dropwise into POSS-(CG-NLS-G-TAT)$_{16}$ solution under stirring to obtain BCPs with different w/w ratios of POSS-(CG-NLS-G-TAT)$_{16}$/pZNF580. Further, TCPs with different 5/1/w ratios (POSS-(CG-NLS-G-TAT)$_{16}$/pZNF580/PLCA-CAGW (PLCA or PLSA-CAGW) were prepared by mixing BCPs with different amounts of PLCA-CAGW, PLCA or PLSA-CAGW solution (0.15 mg/mL). The size and zeta potential of BCPs (w/w ratio = 0.5/1, 1/1, 2/1, 3/1, 4/1, 5/1) and TCPs (w/w/w ratio = 5/1/0.5, 5/1/1.25, 5/1/2.5, 5/1/5) were analyzed through a Zetasizer Nano ZS. Agarose gel electrophoresis assay was performed to determine whether pZNF580 released from TCP-CA-CAGW.

We covalently linked cationic TAT-G-NLS-GC peptide to inorganic hydrophobic octa-diallyl POSS via click chemistry to obtain 16-armed star shaped polymer POSS-(CG-NLS-G-TAT)$_{16}$. Owing to the special structure (multi-armed shape), high hydrophilic and secondary conformation of TAT-G-NLS-GC peptide, POSS-(CG-NLS-G-TAT)$_{16}$ preferred to form uniform micelles with low-aggregation in aqueous solution. POSS-(CG-NLS-G-TAT)$_{16}$ was observed to be uniform small micelles with several nanometers size when dispersed in aqueous solution (Figure S10). For BCPs with different w/w ratios, the size values were located in 100-200 nm, which was advantageous to cellular uptake. Zeta potential values increased with w/w ratio, and became positive at 2/1 ratio (Figure S7). The results of agarose gel electrophoretic assay

were shown in Figure S6. pZNF580 was bounded and fully retarded at w/w ratio of 2/1, which was consistent with zeta potential results.

Noticing that BCPs with 5/1 ratio appropriate suitable size and zeta potential value, we prepared TCPs based on BCPs (5/1 ratio) using anionic PLCA, PLCA-CAGW and PLSA-CAGW polymers. They are denoted as TCP-CA, TCP-CA-CAGW and TCP-SA-CAGW respectively. Compared with BCPs, TCP-CA-CAGW groups exhibited slightly large size (Figure S8). Besides, zeta potential values were observed to gradually decrease with increasing of 5/1/w ratio. These changes just indicated the formation of TCP-CA-CAGW, which resulted from sustained accumulation of different amounts of anionic PLCA-CAGW on the surface of cationic BCPs. By adjusting 5/1/w ratio to be 5/1/1.25, TCP-CA-CAGW possessed suitable size and almost neutral zeta potential.

Further, BCPs (5/1 ratio) were coated with anionic PLCA and PLSA-CAGW (5/1/1.25 ratio) through electrostatic interaction to obtain TCP-CA and TCP-SA-CAGW, respectively. Their size and zeta potential also showed similar tendency as TCP-CA-CAGW in size and zeta potential, demonstrating their successful preparation. At 5/1/1.25 ratio, zeta potential of TCP-SA-CAGW was higher than TCP-CA and TCP-CA-CAGW, because negative density of PLSA-CAGW was lower than PLCA-CAGW. In order to evaluate whether polyanionic PLCA-CAGW might lead to pZNF580 release or not, we performed agarose gel electrophoretic to answer this question. In Figure S6, results showed that none free pZNF580 was detected, which indicated that the coating procedure of polyanionic PLCA-CAGW didn't lead to pZNF580 release.

pH responsive zeta potential change of TCPs

Considering suitable size and zeta potential, TCPs (w/w/w ratio = 5/1/1.25) were chosen for further assay. In brief, PLCA-CAGW or PLSA-CAGW was mixed with BCPs (w/w ratio = 5/1) in acetate buffers (ABS, pH =5.5) and PBS (pH = 7.4) to obtain TCPs samples. Zeta potential of these samples was measured according to the predetermined time of 1 h, 2 h, 4 h, 7 h, 10 h, 13 h and 24 h.

Hemolysis Assay

1 mL of human blood sample was freshly obtained from a healthy human donor in Hospital of the Armed Police and centrifuged at 3000 rpm for 5 min, and the

supernatant was poured off. After washed until supernatant was clear, the packed cells were resuspended in 10 mL 0.01M PBS (pH =7.4). 20 μL of the diluted RBC suspension was added to 1 mL of nanoparticles suspension (0.5 mg/mL), and the mixture was incubated at 37 °C for 24 h. A 20 μL amount of diluted RBC suspension incubated with 1 mL of PBS or water was used as the negative or positive control, respectively. The absorbance value of the hemoglobin at 577 nm was measured with the reference wavelength of 655 nm. The percent of hemolysis was calculated as follows:

$$\text{Hemolysis}(\%) = \frac{\text{sample absorbance - negative control}}{\text{positive absorbance - negative control}} \times 100\%$$

Cell culture and transfection

HUVECs were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin solution at 37°C, 5% CO_2. When cultured to 80–90% confluence, cells were first seeded into 24-well plate at a density of 1×10^5 cells per well and cultured for 24 h. Then the cells were starved with serum-free medium for 12 h. At the time of transfection, complexes with free medium were added into each well (3 μg pZNF580 per well). After 4 h of transfection, the medium was replaced with fresh DMEM containing 10% FBS and the cells were cultured for another 24 h. The expressed green fluorescent proteins (GFPs) were observed via an inverted fluorescent microscope.

Cell viability assay

3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) powder was dissolved in 0.01 M PBS at 5 mg/mL concentration, filtered through sterilized syringe filter (0.22 μm pore diameter), and stored at 4°C. HUVECs (1×10^4 cells/well) were plated in 96-well plates and cultured in DMEM containing 10% FBS for 24 h to achieve 80% confluence. Then the medium was replaced by serum-free medium and cultured overnight. The cells were treated with appropriate concentrations of various complexes and incubated for 48 h. Then 100 μL of serum free medium with 0.5 mg/mL concentration of MTT was added followed by additional incubation of 4 h until purple color developed to detect the metabolically active cells. The medium was discarded and the cells were rinsed with PBS once to remove remaining MTT. Finally, 100 μL of DMSO was added to each well to dissolve water insoluble formazan salt. The optical

density of each well in the culture plate was measured at 490 nm wavelength. The relative cell viability was calculated as:

$$\text{relative cell viability}(\%)=\frac{OD_{490}(\text{sample})}{OD_{490}(\text{control})}\times 100\%$$

Quantitative real-time PCR

Total RNA was extracted from transfected cells using TRIzol reagent and then reverse-transcribed into cDNA using the HiFiScript cDNA Synthesis Kit. The resulting cDNAs as templates were quantified via Ultra SYBR Mixture (low ROX) on 7500 Real Time PCR system according to the manufacturer's instructions. GAPDH was used as the reference gene for normalization of target genes. The forward and reverse primer sequences were 5'-GAGGTTACTGCCTTACCCTGG-3' and 5'-ACCCAGTTCCGACTGGTTC-3' for ZNF580, 5'-AGGTGAAGGTCGGAGTCAAC-3' and 5'-CGCTCCTGGAAGATGGTGAT-3' for GAPDH, respectively. All results are expressed as the means ± SD of three independent experiments.

Western blotting

Total protein was extracted from transfected cells using RIPA lysis buffer containing 1% volume of PMSF and quantified by BCA protein assay kit. Before measuring, the protein was denatured by adding 6× SDS. All samples were separated by SDS-polyacrylamide gel electrophoresis and subjected to western blot. Membranes were incubated with rabbit anti-ZNF580 polyclonal antibody in TBST overnight and horseradish peroxidase conjugated anti-rabbit secondary antibody for 1 h. Membranes were scanned with the Tanon-5200 automatic chemiluminescence image analysis system and analyzed.

Cellular uptake and transmembrane mechanism study

Different Cy5-oligonucleotide complexes were prepared to quantitatively evaluate their cellular uptake in HUVECs by a flow cytometry (Beckman MoFlo XDP, USA). Briefly, cells (3×10^5 cells/well) were plated in 6-well plate and transfected with these Cy5-oligonucleotide complexes. After 4 h incubation, cells were centrifuged and resuspended in 300 μL PBS (pH = 7.4), then suspension was analyzed with a flow

cytometer. To investigate the pathways of cellular uptake, three endocytosis inhibitors were used, i.e., Chlorpromazine (CPZ, 30 μM), Amiloride hydrochloride (Amil, 50 μM) and Filipin Ⅲ (Filip, 5 μg mL^{-1}), to inhibit clathrin-mediated endocytosis, macropinocytosis and caveolae-mediated endocytosis, respectively. HUVECs were pretreated with each of the endocytosis inhibitors for 1h and incubated with Cy5-oligonucleotide complexes for 4h at 37 ℃. The untreated cells were used as control group. Subsequently, the cells were washed three times with 0.01 M PBS (pH =7.4) and resuspended in 300 μL PBS (pH = 7.4) after trypsinization and centrifugation. The cellular uptake was analyzed by a flow cytometry.

Intracellular distribution study

The cells were seeded at 1×10^5 cells per well into a confocal dish and cultured for 24 h. Afterward, the cells were incubated with complexes for 4 h in free serum medium to facilitate the cellular uptake. The cells were further incubated with 10% serum-containing DMEM for another 4 h and 24 h. Cells were washed with PBS twice, and added with prewarmed medium containing 0.5 mM Lyso Tracker Green to continue culture for another 1 h. Finally, the cells were washed twice with prewarmed PBS and stained with 2 μg/ mL of Hoechst 33342 at room temperature for 20 min. The cells were washed twice with PBS and observed by a confocal laser scanning microscopy (CLSM) at excitation wavelengths of 649, 504 and 350 nm for Cy5 (red), Lyso Tracker Green (green) and Hoechst 33342 (blue), respectively. The co-localization ratio (CLR) was calculated by Image-Pro Plus 6.0 software according to the following equation.

$$\text{Co-localization ratio}=\frac{\text{Number of yellow or pink pixels}}{\text{Number of yellow, red and pink pixels}}\times 100\%$$

Cell proliferation assay

At 12, 24, 48 and 72 h after transfection, cell culture medium was removed and 100 μL of free serum medium containing 10% Cell Counting Kit-8 assay (CCK-8) was added to each well of a 96-well plate. After incubation for another 4 h at 37°C in 5% CO_2, the absorbance was measured at a wavelength of 450 nm using an ELISA microplate reader (Thermo Scientific). Assays were repeated at least three times.

Cell migration assays

The wound healing assay and transwell migration assay were performed to estimate the migration capability of the transfected cells. The operations of HUVECs with the treatment of different complexes in 24-plate were described before. For wound healing assay, wound/scratch was created along the diameter of each well using a 200μL pipette tip after transfected 24h. Cell debris were removed by washing once with D-Hanks, followed by addition of fresh medium. The sites of cells were monitored and recorded by an inverted microscope at 0, 12 and 24 h. The relative recovered area at 24h was calculated using Image J software with the following equation to expose the migration ability.

$$\text{Relative recovered area (\%)}=\frac{\text{Recovered area}}{\text{Wounded area}}\times 100\%$$

For transwell migration assay, the cells transfected 24h were centrifuged, resuspended and added to the upper chambers of inserts with a density of 1×10^5 cells per well. After 6h of incubation, the migrated cells across the transwell were stained with violet crystal, photographed and counted to calculate the migration rate. By contrast, the cells without any treatment were served as a negative control.

Results

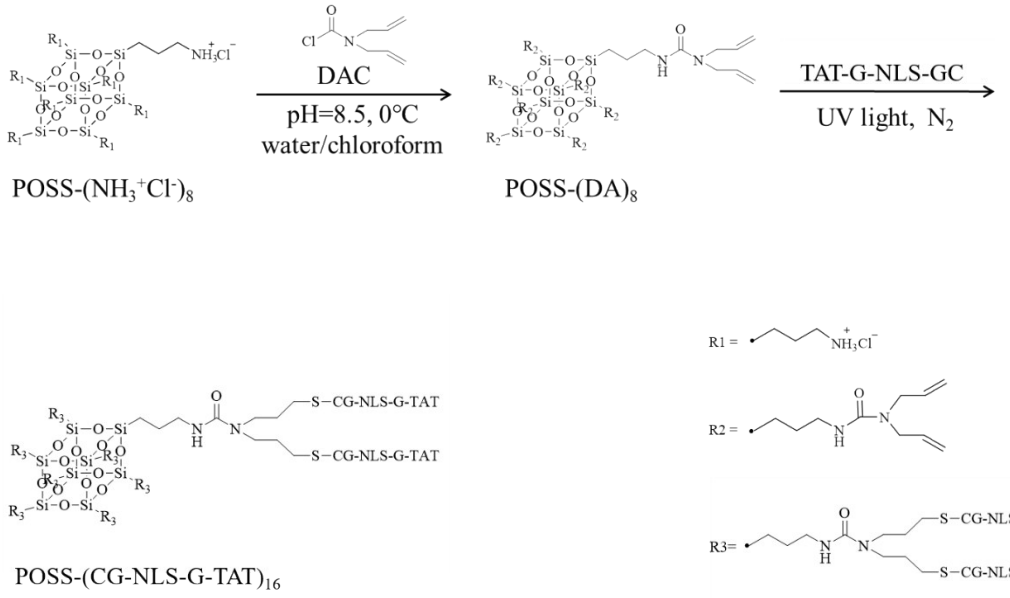

Figure S1. Synthesis of POSS-(CG-NLS-G-TAT)$_{16}$.

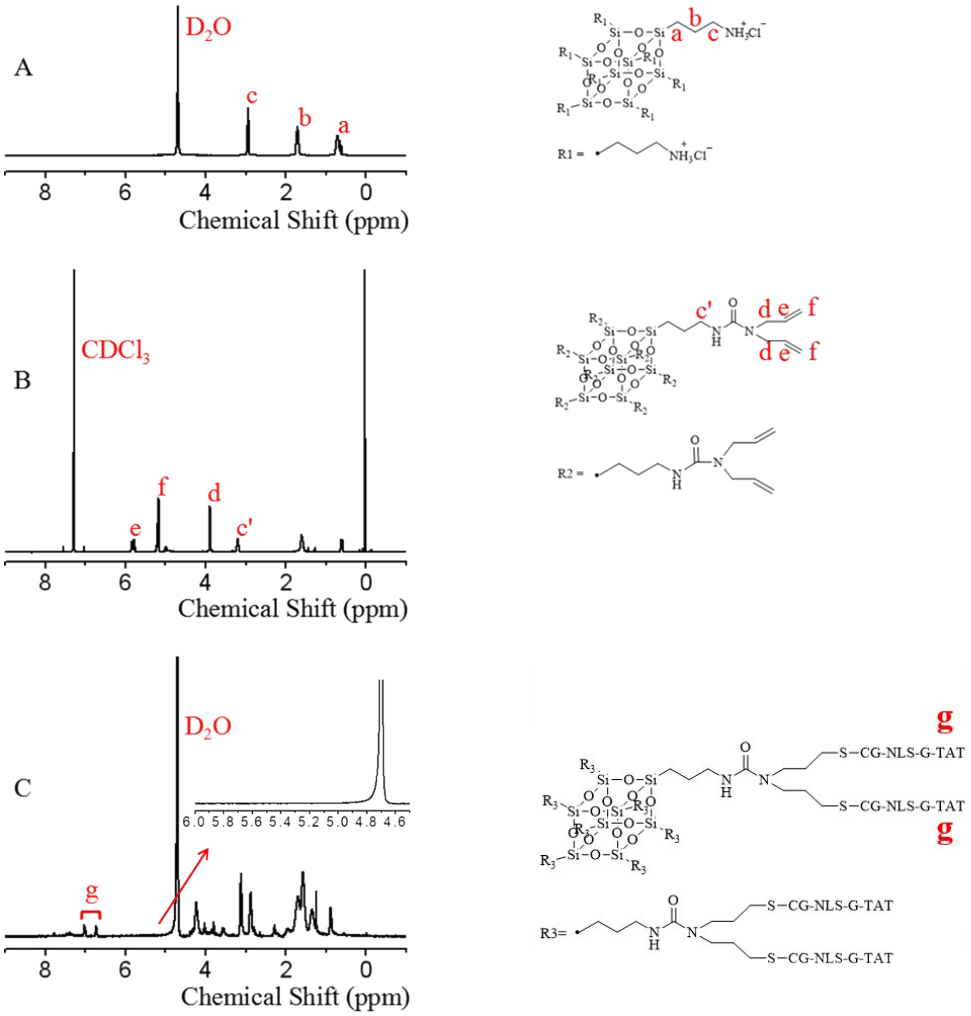

Figure S2. ^{1}H NMR spectra of (A) POSS-$(NH_3^+Cl^-)_8$ in D_2O, (B) POSS-$(DA)_8$ in $CDCl_3$ and (C) POSS-$(CG\text{-}NLS\text{-}G\text{-}TAT)_{16}$ in D_2O.

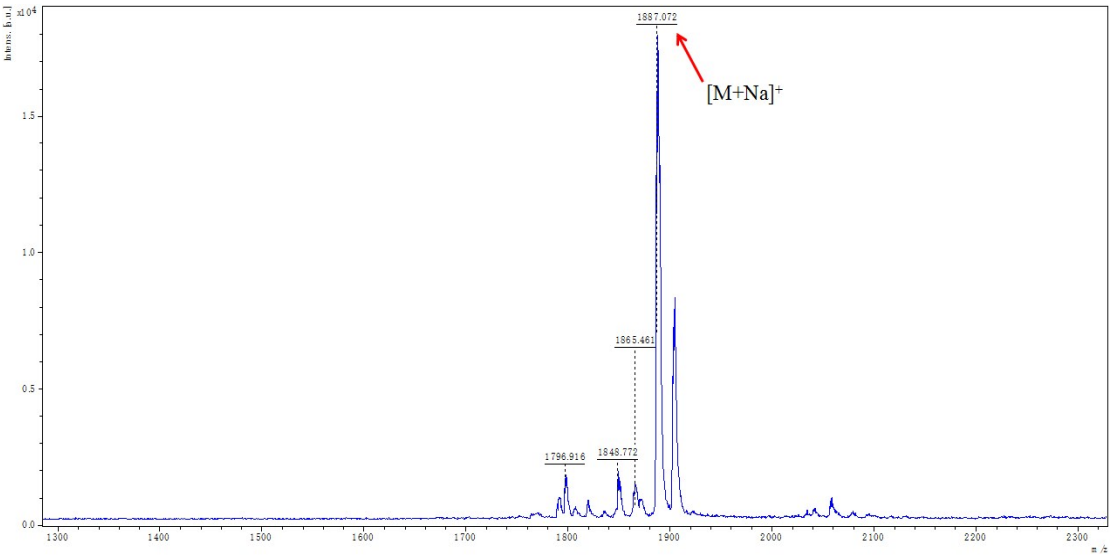

Figure S3. MALDI TOF mass spectrum of POSS-$(DA)_8$ using DHB as the matrix.

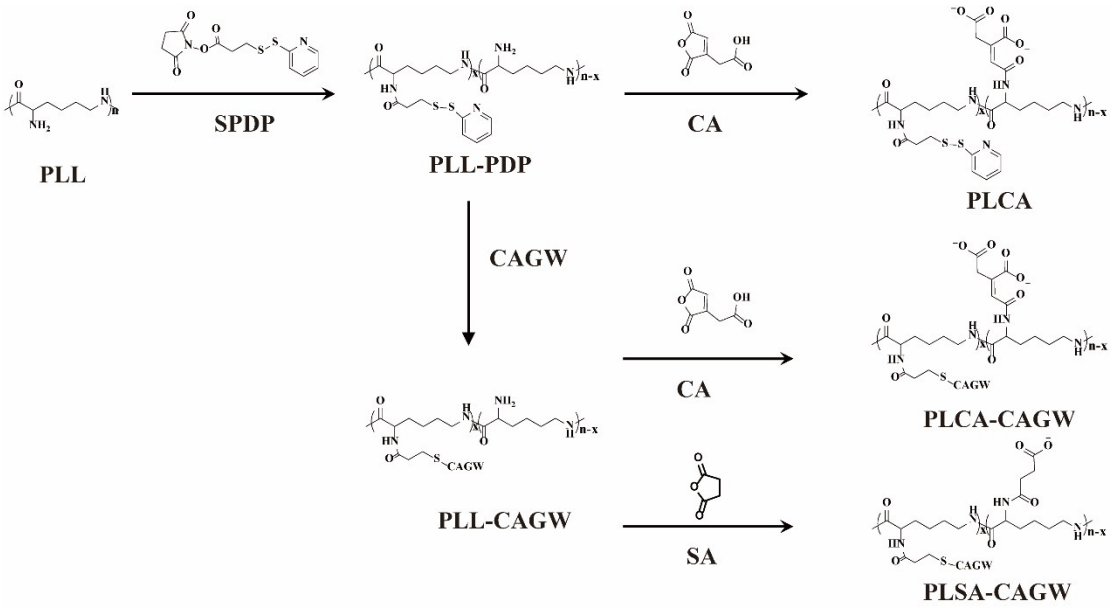

Figure S4. Synthesis routes of PLCA, PLCA-CAGW and PLSA-CAGW.

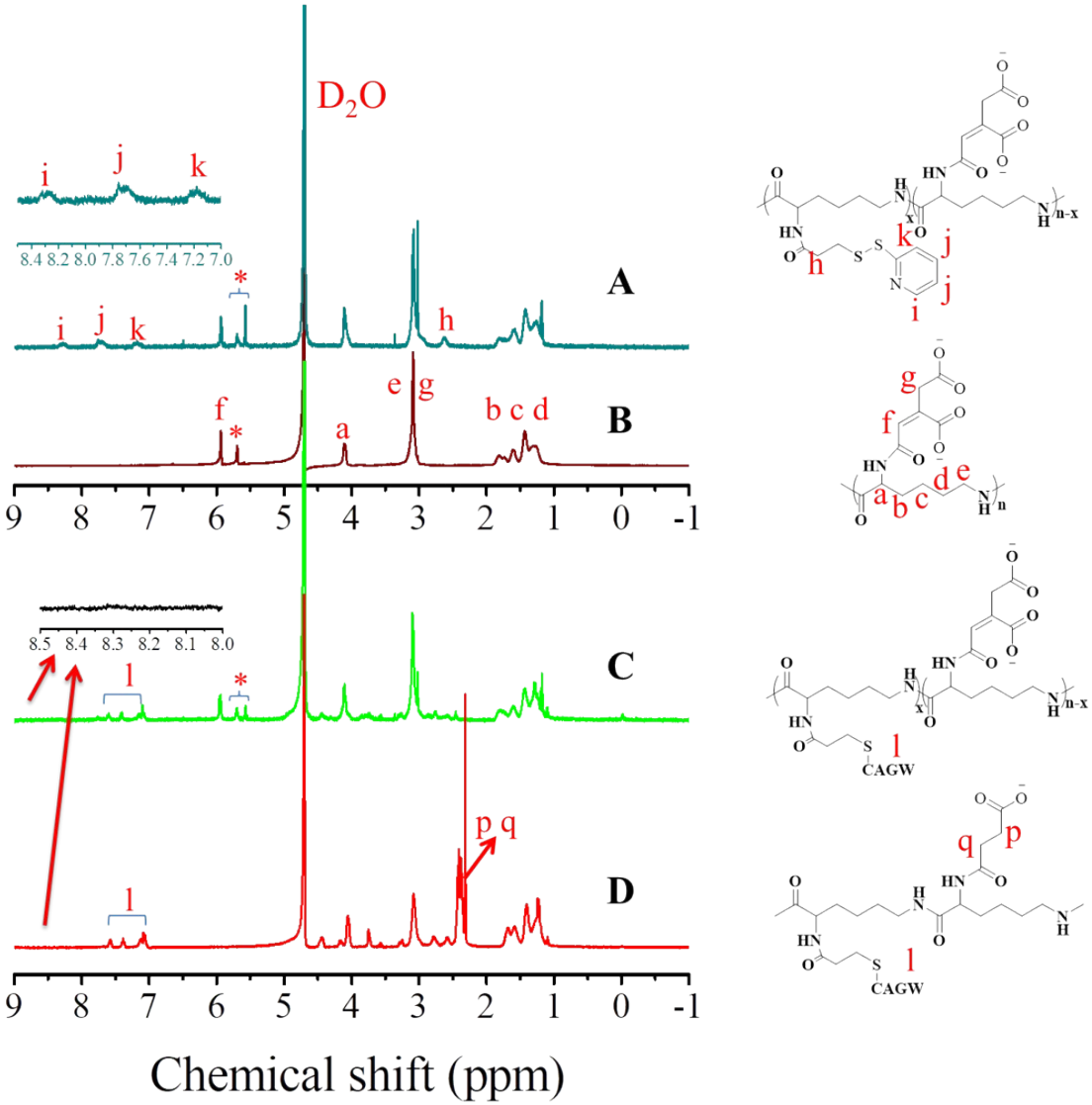

Figure S5. ^{1}H NMR spectra of polymers in D_2O. (A) PLCA, (B) PLL-CA, (C) PLCA-CAGW, (D) PLSA-CAGW. * protons arising from a decarboxylated byproduct.

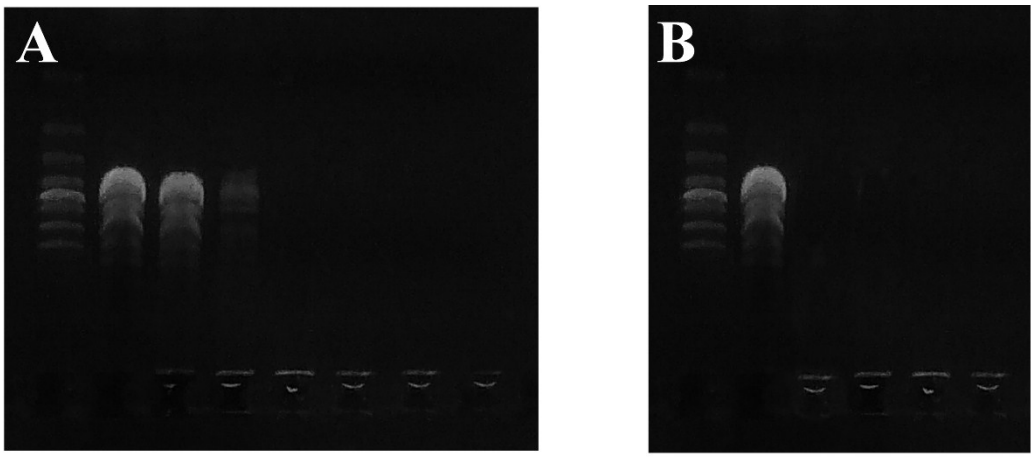

Figure S6. Agarose gel electrophoretic image of BCPs with different w/w ratios (A) and TCPs with different w/w/w ratios (B). For these two pictures, Lane 1, marker; Lane 2 naked pZNF580; Lane 3-8 of BCPs(A), POSS-(CG-NLS-G-TAT)$_{16}$/pZNF580, w/w ratios = 0.5:1, 1:1, 2:1, 3:1, 4:1 and 5:1. Lane 3-6 of TCP-CA-CAGW (B), w/w/w ratio = 5:1:0.5, 5:1:1.25, 5:1:2.5 and 5:1:5.

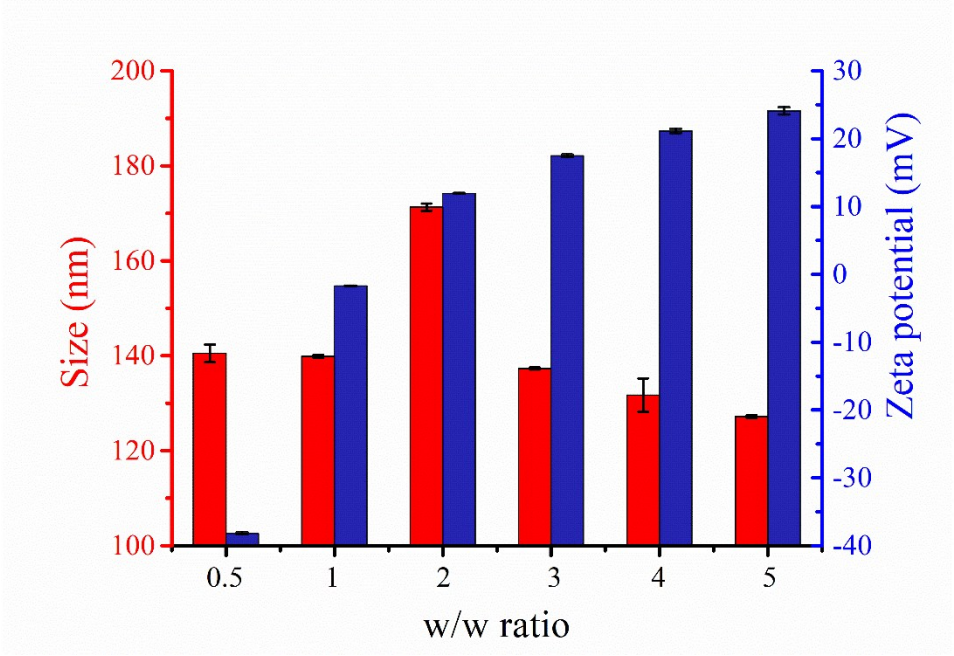

Figure S7. The size and zeta potential of BCPs with different w/w ratios of POSS-(CG-NLS-G-TAT)$_{16}$ and pZNF580.

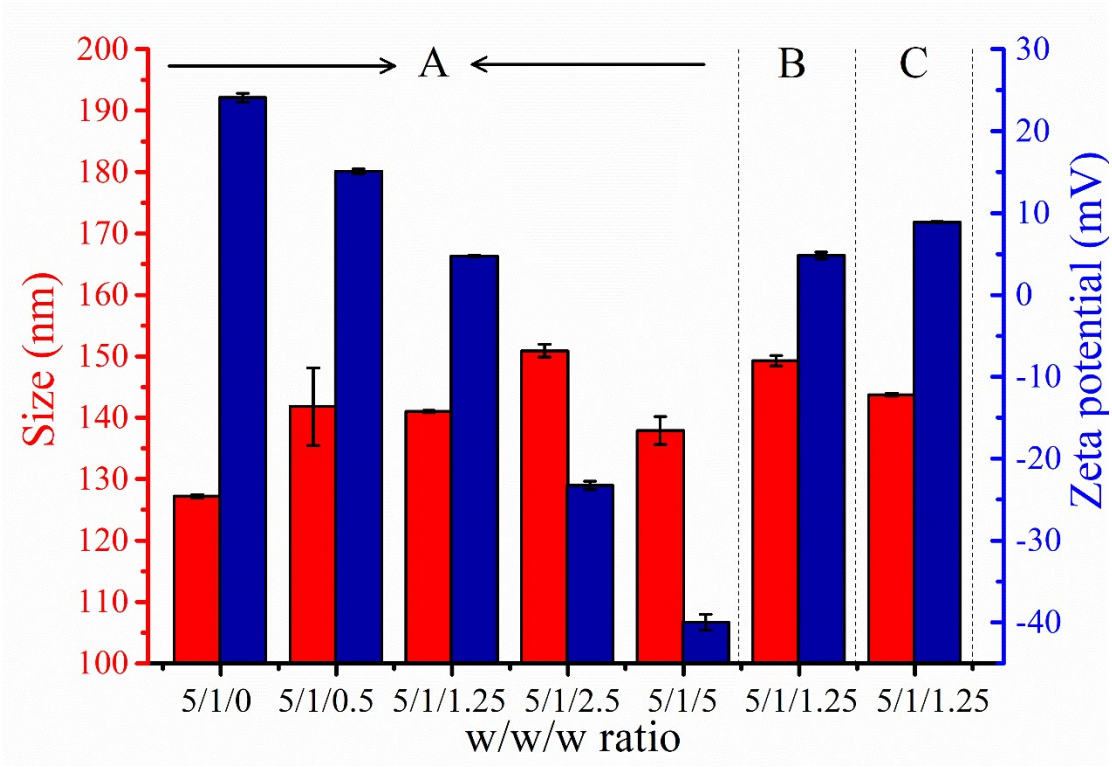

Figure S8. The size and zeta potential of TCPs with different w/w/w ratios, PLCA-CAGW (A), PLCA (B) and PLSA-CAGW (C).

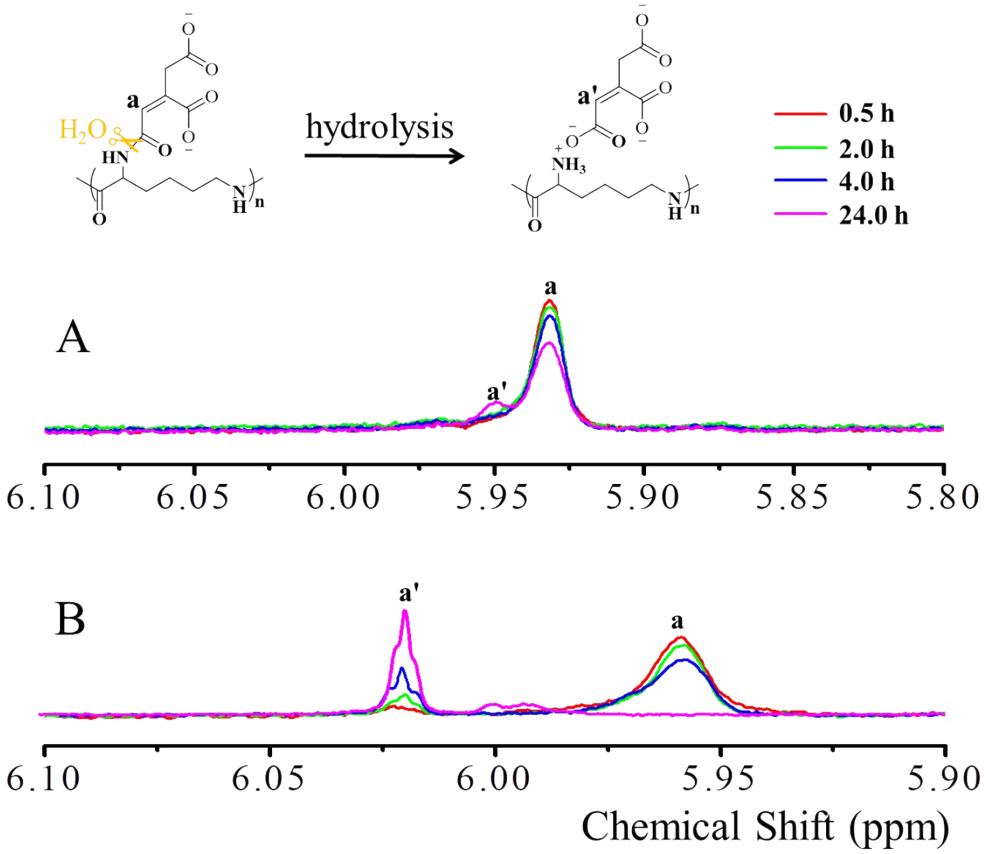

Figure S9. The pH responsive hydrolysis reaction of PLL-CA at pH 7.4 (A) and pH 5.5 (B) recorded by ^{1}H NMR spectrometer.

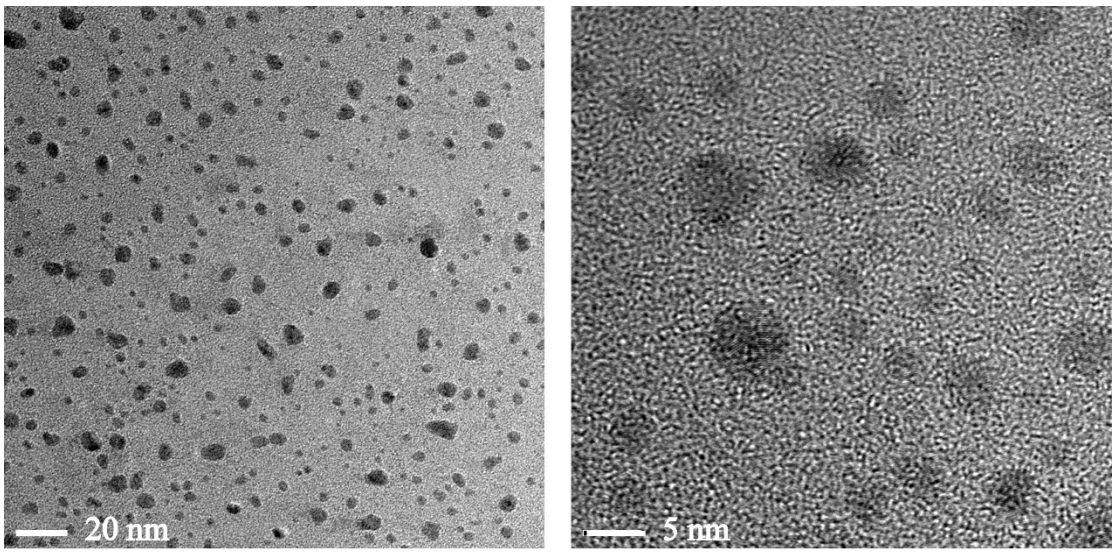

Figure S10. Morphology of POSS-(G-NLS-G-TAT)$_{16}$ dispersed in water with different magnification detected by TEM.