

Synthesis of zwitterionic polyelectrolyte nanogels via electrostatic-templated polymerization

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Materials

The photoinitiator 2-hydroxy-2-methyl-1-propanone (HMP), zwitterionic monomer carboxybetaine methacrylate (CBMA) and carboxybetaine acrylamide (CBAA) were purchased from TCI and used as received. Diblock copolymer PAMPS₅₀-*b*-PEO₂₃₈ with polydispersity index 1.35 was purchased from *Polymer Source* and used as the template. N, N'-Methylenebisacrylamide (MBA) was obtained from sigma-Aldrich. Deuterium oxide (D₂O) was purchased from Adamas. 3-(Trimethylsilyl) propionic-2,2,3,3-d₄ acid sodium salt (TSP) was purchased from Sigma Aldrich and used as internal standard for ¹H NMR. Cresol red (neutral), Acid Red 88 (negative) and phenosafranin (positive) were purchased from TCI. All other chemicals were of analytical grade and were used without further purification. Ultrapure water was produced with a Milli-Q water purification system.

Characterizations

¹H NMR spectra were recorded on a BRUKER AVANCE 400 spectrometer operating at 400 MHz. Reaction solution was collected and lyophilized, then the powder was dissolved in D₂O after adding NaCl to a final concentration of 3 M and the spectrum was scanned 32 times at 25 °C. UV spectra for all the samples were recorded on a SHIMADZU 1800 spectrophotometer. Fourier transform infrared spectroscopy (FT-IR) spectra were measured on a Thermo Fisher Scientific Nicolet iS20.

Transmission electronic microscopy (TEM)

TEM was performed on a JEM-1400 electron microscope operating at 100 kV. 230-mesh copper grids were coated with formvar support film followed by subsequent

coating with carbon. 10 μL of the sample solution was placed on the copper grids, and the excess solution was absorbed by lens paper. Then samples were stained by a drop of a 2% phosphotungstic acid solution onto the surface of the sample-loaded grid for 15 s, and dried at room temperature.

Light Scattering

The light scattering at a 90° angle is carried out with an ALV light scattering device, which is equipped with a 21 mW argon ion laser with a working wavelength of 632.8 nm. All samples were tested at room temperature. The CUMULANT method was used to analyze the mean apparent hydrodynamic radius (R_h), which is

$$R_h = kTq^2/6\pi\eta\Gamma$$

Where k is the Boltzmann constant, T is the absolute temperature, q is the scattering vector, η is the viscosity of the solvent, Γ is the measured average decay rate of the correlation function. The CONTIN method is used to analyze the size distribution of micelles hydrodynamic radius.

For angle-related DLS, in order to assess the angle dependency of the diffusion coefficient, six correlation functions ($D=\Gamma/q^2$) were recorded from 40° to 140° at intervals of 20° . It is known that the diffusion coefficient D of asymmetric particles decreases as the scattering vector q increases, but the diffusion coefficient D of spherical particles is independent of the scattering vector q . Where q is the scattering vector:

$$q = \left(\frac{4\pi n}{\lambda}\right) \sin\left(\frac{\theta}{2}\right)$$

Methods

Preparation of PCBMA nanogels

Typically, PEO-*b*-PAMPSNa (charge concentration 20 mM), CBMA monomer (20 mM), MBA (2 mM, 10%), and HMP (0.4 mM, 2%) were dissolved in water (2 mL) in a 10 mL Schlenk tube. The solution was adjusted to pH 2.0 using 5.0 M HCl. The tube was sealed and de-oxygenated by bubbling with nitrogen for 30 min. Then the tube was exposed to UV light for 3h. The reaction was stopped by exposure to air. Other synthesis at different salt concentrations were carried out according to the above-mentioned recipe and reaction conditions, but using different salt concentrations (20 mM, 40 mM, 60 mM, 80 mM, 100 mM). The nanogel was separated from the template by centrifuging at 8000 rpm using 4 M NaCl as eluent for ten times with an Amicon Ultra-4 centrifugal filter (molecular weight cut off = 100 kDa). The solution inside the tube was collected and dialyzed against water to remove the salt, and then used for further characterizations.

Dye loading study

Different charged dyes were titrated into the PCBMA nanogel solution (0.05 mg/mL) at pH 2.0 and pH 9.0, respectively, followed by 1-minute vortex. The scattering intensity, radius and PDI were tested by light scattering.

Synthesis of FITC-cyt *c*

Cyt *c* were dissolved in phosphate buffered saline (PBS, pH 7.4), then FITC dissolved in DMSO was added into the above solution at a dye/protein molar ratio of 3:1. The reaction solution was stirred at room temperature for 24h in the dark. The resulting

solution was transferred to a dialysis bag and first dialyzed against PBS then against water. After the dialysis, the sample was freeze-dried and stored at -20°C before further use.

Cell culture

HeLa cells from Procell Life Science and Technology were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone) at 37°C under 5% (v/v) CO₂. The culture media were supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco) and 1% (v/v) penicillin-streptomycin (Gibco).

Intracellular delivery of cyt *c*

HeLa cells were seeded in 24-well plates at a density of 2.5×10^5 per well before cytosolic protein delivery. FITC-cyt *c* were added in nanogel solutions (0.05 mg cyt *c*/mg nanogel) with 1 min vortex. The FITC-cyt *c*@nanogel solutions were diluted with 500 μL of serum-free DMEM and incubated for 10 min at room temperature. The culture media were removed, and the cells were washed twice with PBS, before adding the FITC-cyt *c*@nanogel solutions into the wells. The cells were incubated for 4 hours, and the media were replaced by 500 μL fresh DMEM containing 10% FBS. After another 20 hours of incubation, the culture media were removed, and the cells were washed twice with PBS. The fluorescence intensity of the treated cells was observed by fluorescence microscope (Zeiss Axio Vert. A1, Germany).

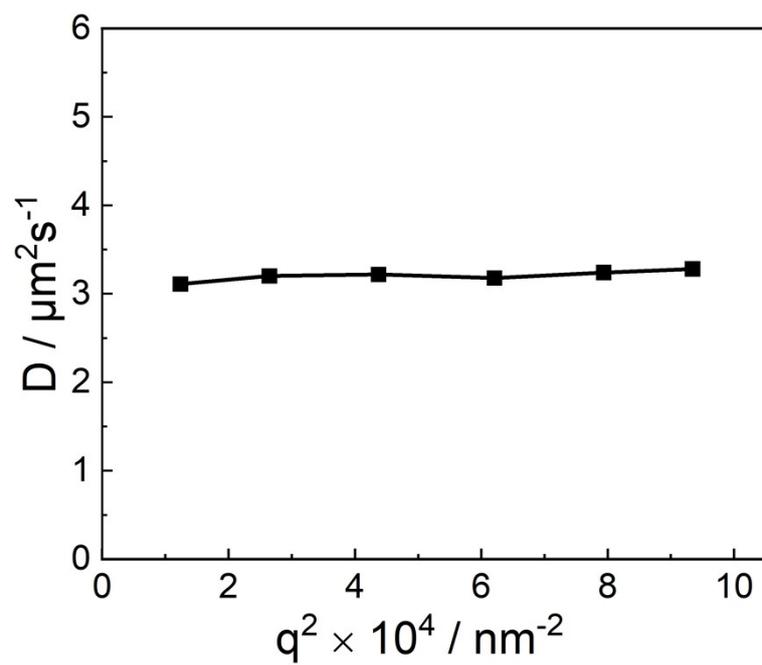


Figure S1. Angular dependence of the diffusion coefficient of PCBMA nanogels.

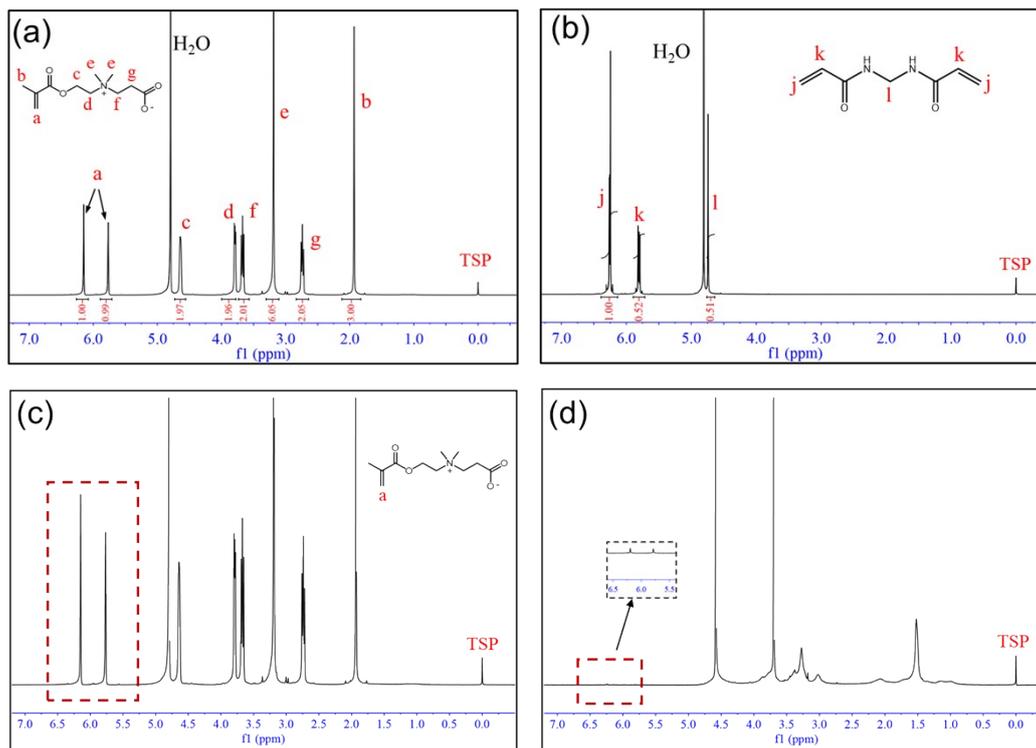


Figure S2. ^1H NMR spectra of CBMA monomer (a), MBA crosslinker (b), solution before (c) and after (d) photo-initiated polymerization for 3 h. The conversion of the monomers is calculated based on the integrals of proton resonances on the double-bonded carbons (dashed boxes) from the unreacted monomers.

As shown in Figure S2a and 2b, the chemical shift at 5.7-6.2 ppm (marked a), from the carbon-carbon double bond of CBMA, is applied to indicate CBMA monomer. The chemical shift at 6.25 ppm (marked j), from the carbon-carbon double bond of MBA, is applied to indicate MBA crosslinker. The mixture solution after polymerization was collected and lyophilized, then the powder was dissolved in D_2O for ^1H NMR measurement. TSP was added as the internal standard, and NaCl was added to the final concentration of 4 M for completely shielding electrostatic interaction. The amount of un-polymerized monomer and MBA cross-linker were determined by calculating the integral of the corresponding peak with TSP as internal standard. Based on which the monomer conversion and cross-link degree are obtained.

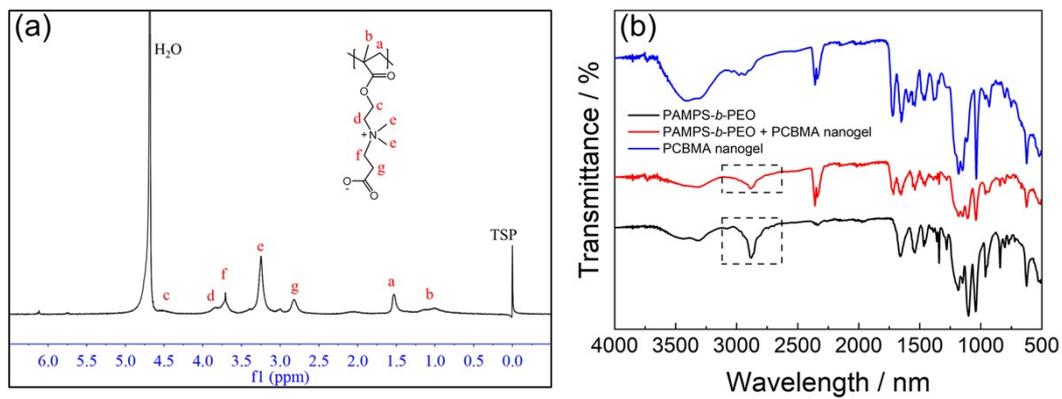


Figure S3. (a) ^1H NMR spectrum of PCBMA nanogel. (b) FT-IR spectra of PAMPS-*b*-PEO template, PCBMA nanogel before and after removing template.

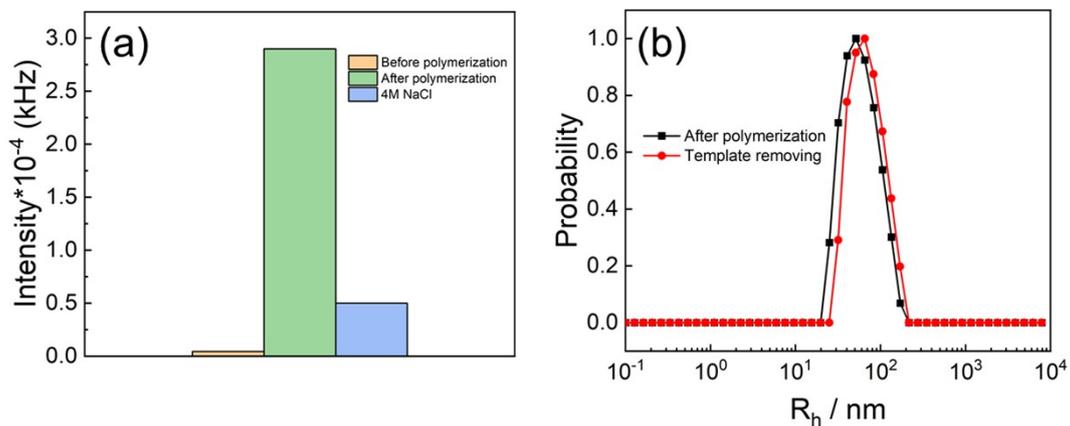


Figure S4. (a) Light scattering intensity of reaction solution before polymerization, after polymerization, and with added 4M NaCl. (b) Size distribution of PCBAA nanogels before and after template separation. (Nanogel synthesis was carried out at pH 2, charge mixing ratio 1:1, cross-linker 10% and 20 mM NaCl.)

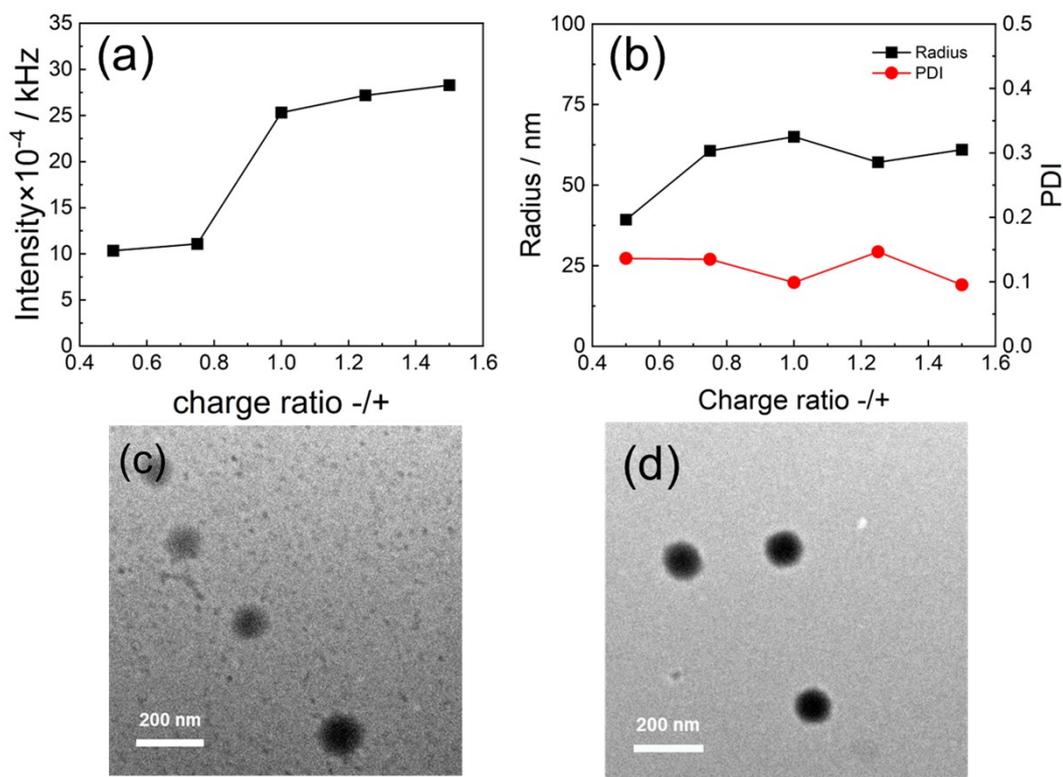


Figure S5. Light scattering intensity (a), hydrodynamic radius and PDI (b) of PCBMA nanogels prepared at different charge ratio ($-/+$). TEM images of PCBMA nanogels prepared at charge ratios of 0.75 (c) and 1.25 (d). (Nanogel synthesis was carried out at pH 2, cross-linker 10% and 20 mM NaCl.)

The light scattering intensity increased with increasing $-/+$ charge ratio and reached a (nearly) plateau at the charge ratio of 1/1 (Figure S5a). The size of the nanogels hardly change when the $-/+$ ratio above 0.75 (Figure S5b). The PDI results and TEM images indicated that the obtained nanogels featured narrow size distribution and spherical morphology independent on the mixing charge ratio (Figure S5c, S5d). The above results suggest that the stoichiometric charge ratio is the preferred mixing ratio for fabrication of PIC micelles and nanogels based on ETP method.

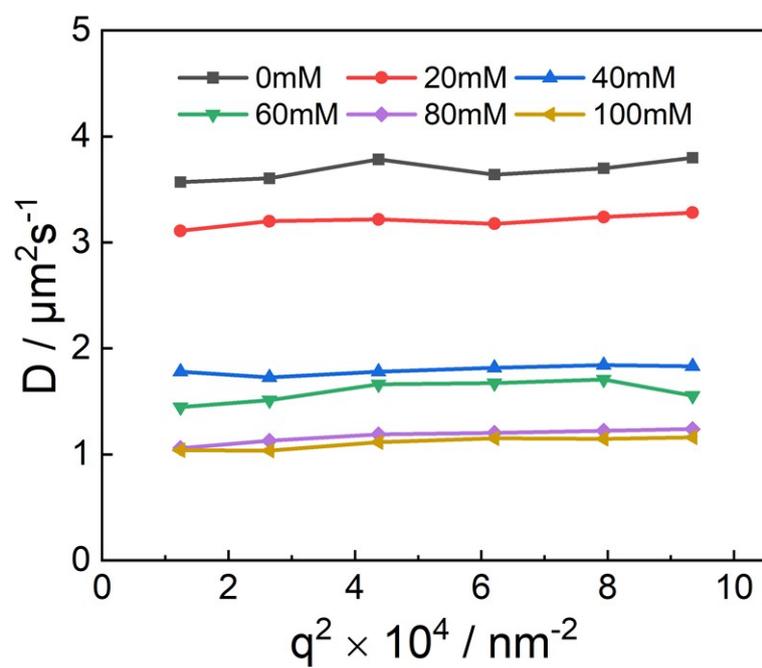


Figure S6. Diffusion coefficient of PCBMA nanogels prepared at different salt concentration.

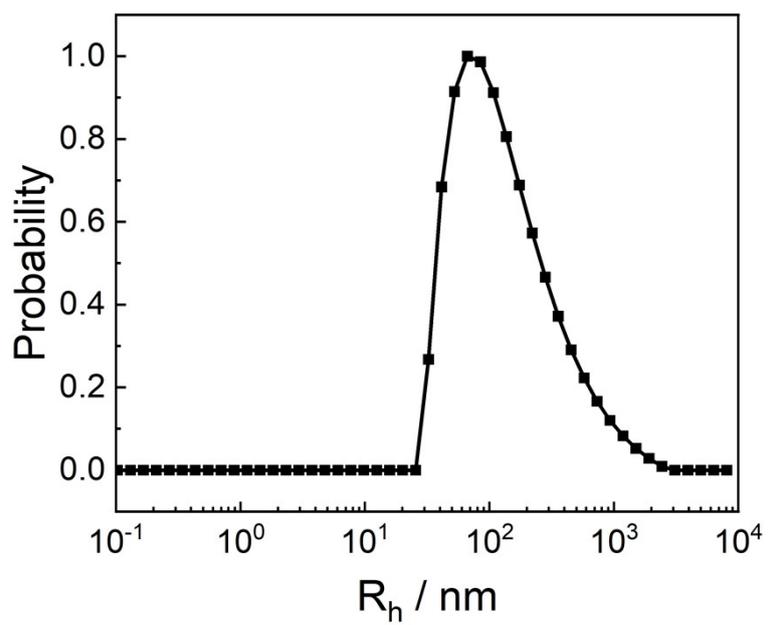


Figure S7. Size and size distribution of PCBMA nanogels prepared at 120 mM NaCl concentration.

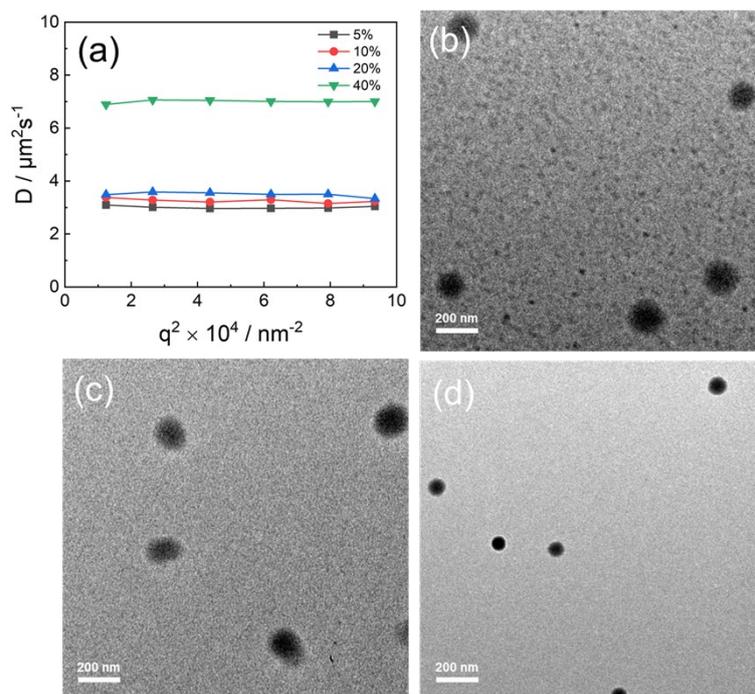


Figure S8. (a) Angular dependence of the diffusion coefficient of PCBMA nanogels prepared at different cross-linker fractions. TEM images of phosphotungstic acid-stained PCBMA nanogels prepared at different cross-linker fractions [(a) 5%, (b) 20%, and (c) 40%].

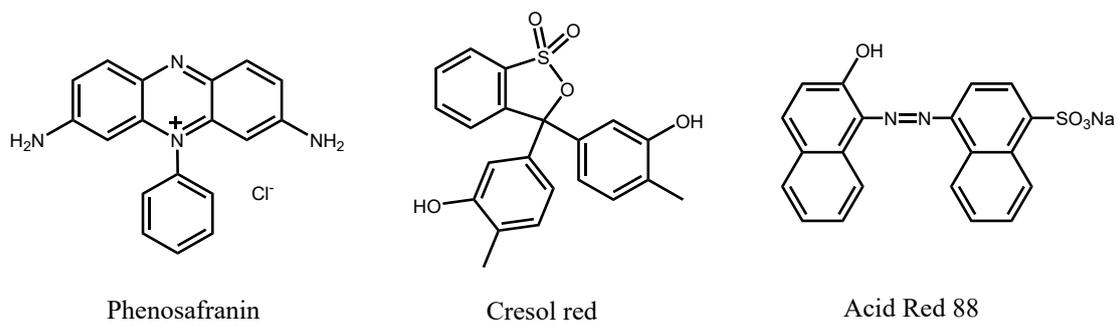


Figure S9. Chemical structures of different charged dyes used in this study.

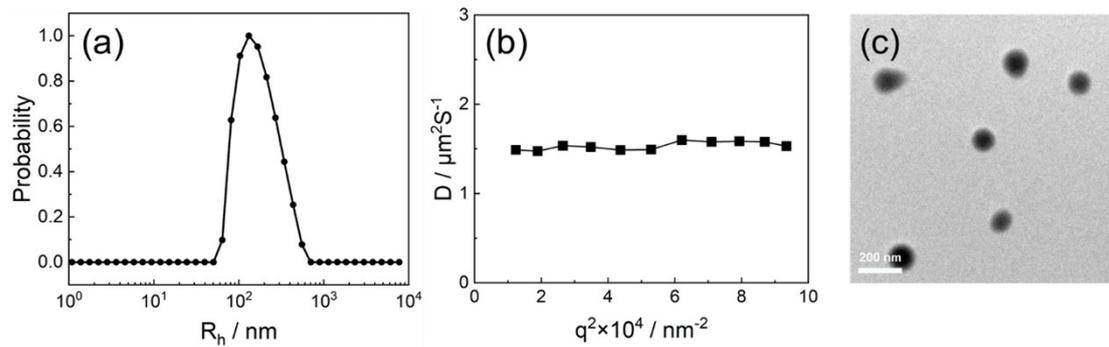


Figure S10. Size distribution (a), angular dependence of the diffusion coefficient (b) and TEM image (c) of PCBMA nanogel at pH 9. (Nanogel synthesis was carried out at pH 2, charge mixing ratio 1:1, cross-linker 10% and 20 mM NaCl.)

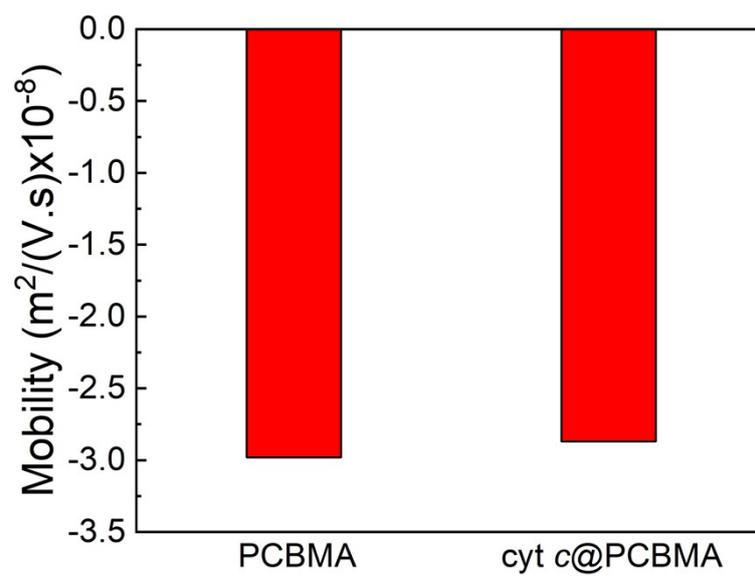


Figure S11. Electrophoretic mobility of PCBMA nanogel and cyt *c*@PCBMA nanogel.

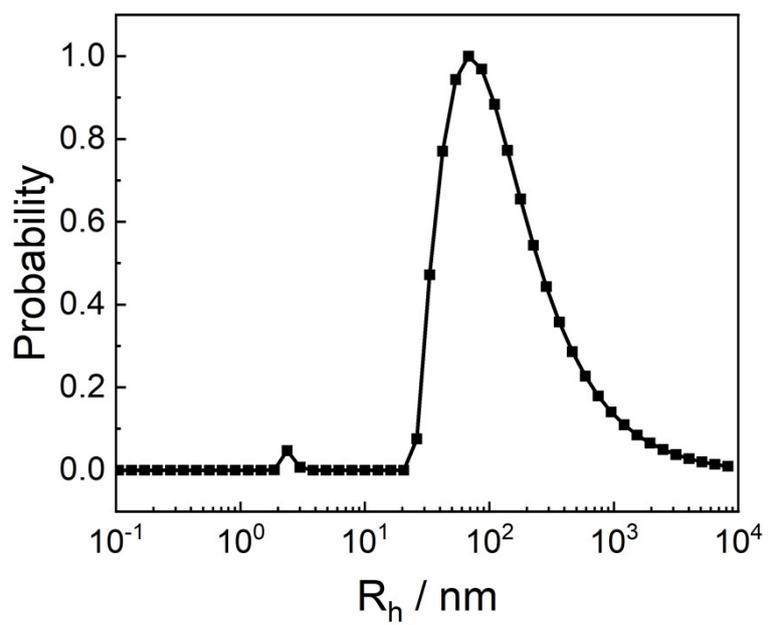


Figure S12. Size and size distribution of PCBMA nanogels after loading excess cyt *c*.