Surface Charge Generation in Nanogels for Activated Cellular Uptake at Tumor-Relevant pH

*Longyu Li, Kishore Raghupathi, Conghui Yuan and S. Thayumanavan**

Department of Chemistry, University of Massachusetts, 710 N. Pleasant Street,

Amherst, MA 01003-9336, USA

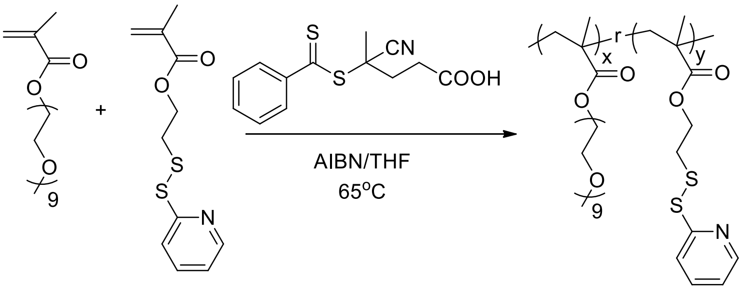
* Corresponding author: thai@chem.umass.edu

Experimental:

2,2′-dithiodipyridine, 2-mercaptoethanol, polyethylene glycol monomethylether methacrylate (MW 450 g/mol and 350 g/mol) (OEG), 2-(diisopropylamino) ethyl methacrylate (DPA), D,L-dithiothreitol (DTT), 1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate (DiI) and 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO), 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid, fluorescein isothiocyanate (FITC) and other conventional reagents were obtained from commercial sources and were used as received, unless otherwise mentioned. Polymers were synthesized using RAFT polymerization and then purified by precipitation. Pyridyl disulfide ethyl methacrylate (PDS) was prepared using a previously reported procedure.[1] ^{1}H-NMR spectra were recorded on a 400 MHz Bruker NMR spectrometer using the residual proton resonance of the solvent as the internal standard. Chemical shifts are reported in parts per million (ppm). Molecular weights of the polymers were estimated by gel permeation chromatography (GPC) using PMMA standard with a refractive index detector and THF as the elution. Dynamic light scattering (DLS) measurements and zeta potential measurements were performed using a Malvern Nanozetasizer. UV-visible absorption spectra were recorded on a Varian (model EL 01125047) spectrophotometer. The fluorescence spectra were obtained from a JASCO FP-6500 spectrofluorimeter.

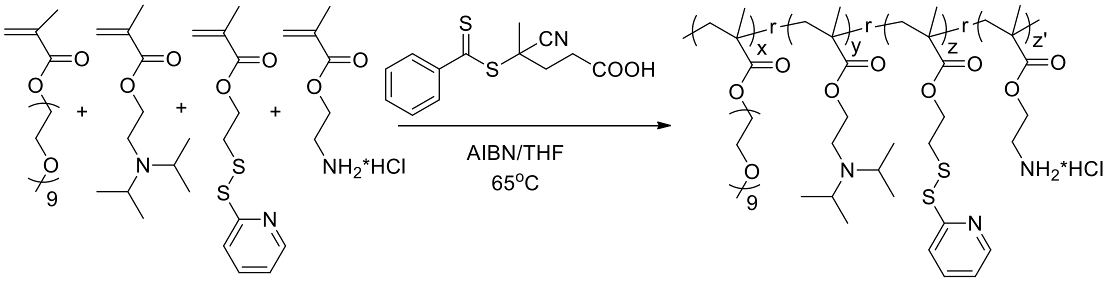
Synthesis of random copolymer P1-P4: A mixture of 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (5.9 mg, 0.021 mmol), PDS (107.4 mg, 0.421 mmol), polyethylene glycol monomethyl ether methacrylate (100 mg, 0.21 mmol), 2-(diisopropylamino) ethyl methacrylate (27mg, 0.126 mmol) and AIBN (0.7 mg, 0.00421 mmol) was dissolved in THF (0.5 mL) and degassed by performing three freeze-pump-thaw cycles. The reaction mixture was sealed and then transferred into a pre-heated oil bath at 65 °C and stirred for 10 h. To remove unreactive monomers, the resultant mixture was precipitated in cold ethyl ether (20 mL) to yield the random copolymer **P1** as a waxy solid. GPC (THF) M_n: 8.3 KDa. Đ: 1.3. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.46, 7.67, 7.10, 4.35-4.09, 3.94-3.37, 3.02, 2.62, 2.04-1.64, 1.43-0.87. Other polymers **P2-P4** were prepared by a similar method using the appropriate molar ratio of the co-monomers. The molar ratio between three monomer units was determined by integrating the methoxy protons in the polyethylene glycol unit, the aromatic protons in the pyridine and methylene protons contacted to amino groups.

Synthesis of random copolymer P5 without DPA units:



A mixture of 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (3.0 mg, 0.02 mmol), PDS (1.25 g, 4.9 mmol), polyethylene glycol monomethyl ether methacrylate (1.0 g, 2.1 mmol) and AIBN (2.5 mg, 0.014 mmol) was dissolved in THF (5 mL) and degassed by performing three freeze-pump-thaw cycles. The reaction mixture was sealed and then transferred into a pre-heated oil bath at 65 °C and stirred for 10 h. To remove unreactive monomers, the resultant mixture was precipitated in cold ethyl ether (20 mL) to yield the random copolymer as a waxy liquid. GPC (THF) M_n: 13 kDa; PD: 1.3. ^{1}H NMR (400 MHz,$CDCl_3$) δ: 8.46, 7.68, 7.11, 4.35-4.09, 3.94-3.37, 3.03, 2.04-1.64, 1.43-0.87. The molar ratio between two monomer units was determined by integrating the methoxy protons in the polyethyleneglycol unit and the aromatic protons in the pyridine and found to be 3:7 (PEG:PDS).

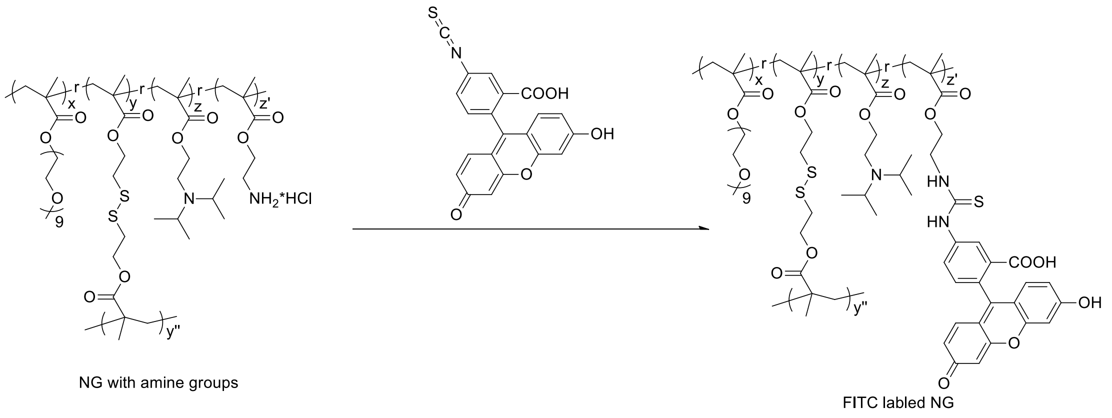
Synthesis of random copolymer with primary amine groups:



A mixture of 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (5.5 mg), PDS (75 mg), polyethylene glycol monomethyl ether methacrylate (107 mg), 2-(diisopropylamino) ethyl methacrylate (65 mg), 2-Aminoethyl methacrylate hydrochloride (4mg) and AIBN (0.6 mg) was dissolved in DMF (0.5 mL) and degassed by performing three freeze-pump-thaw cycles. The reaction mixture was sealed and then transferred into a pre-heated oil bath at 65 °C and stirred for 10 h. To remove unreactive monomers, the resultant mixture was precipitated in cold ethyl ether (20 mL) to yield the random copolymer as a waxy solid.

Encapsulation of DiI/DiO in nanogels: The polymer (10 mg) was dissolved in 1 mL water. To this solution, either a 0.02 mL of DiI stock solution (5mg/mL in acetone) or 0.04 mL DiO stock solution (2.5 mg/mL in acetone) was added into the polymer solution. The pH was adjusted by adding drops of HCl or NaOH solution (1M). The mixed solution was stirred overnight at room temperature, open to the atmosphere allowing the organic solvent to evaporate. Then a measured amount of DTT was added. After stirring for 4 hours, insoluble DiI/DiO was removed by filtration and pyridinethione was removed from the nanogel solution by extensive dialysis using a membrane with a molecular weight cutoff of 7,000 g/mol.

Synthesis of fluorescein-labeled DPA nanogels:



The blank DPA nanogel was made by using amine group containing polymer (10 mg/mL) using the procedures outlined above. Then excess FITC was added into the nanogel solution. After stirring for 24 hours, excess FITC was removed from the nanogel solution by extensive dialysis using a membrane with a molecular weight cutoff of 7,000 g/mol.

DLS measurement: Dynamic light scattering experiments were performed by using a digital correlator and goniometer. The light source was solid-stat laser system, operating at 514 nm. For size measurements, the nanogels in deionized water (1 mg/mL) was kept constant at 25 °C throughout the experiment. Dust was eliminated by filtering the solution through 0.45 μm polycarbonate filter. All measurements were done at a correlation time of 30 seconds. For zeta potential measurements, the pH was adjusted by adding drops of HCl or NaOH solution (1M). The nanogels solution was also kept constant at 25 °C throughout the experiment.

Dye exchange experiment: A solution of nanogel containing DiI (100 μL) was mixed with a solution of nanogel containing DiO (100 μL) in a cuvette, and then milliQ water (800 μL) was added to adjust the volume. The fluorescence spectra were recorded using the excitation wavelength of 450 nm.

DiI release experiment: A solution of nanogel containing DiI (100 μL) was mixed with a buffer solution with pH 6 or 7.4 (800 μL) in a cuvette, and then DTT stock solution (100 μL) was added. The fluorescence spectra were recorded using the excitation wavelength of 450 nm.

Confocal experiments: Confocal experiments were done using Zeiss510 META confocal laser scanning microscope. 100k HeLa cells were incubated in glass bottomed dishes, purchased from Mat tek corp, at 37 °C overnight in 5% CO_2 incubator allowing the cells stick to the bottom of dish. Then, the nutrient medium was taken out and cells were washed with 5 mL of pH 7.4 PBS buffer. In one of the confocal dish, the pH of 1 mL of nutrient media was adjusted to 6.5. Then, 100uL of 1mg/mL solution of pH sensitive nanogels were added to each of the confocal dishes maintained at pH 6.5 and at pH 7.4. The cells were incubated for 2 hrs at 37 °C and the florescence was observed at 560 nm by laser excitation at 543 nm for the DiI encapsulated nanogels and florescence was observed at 501nm by exciting at 480nm for fluorescein-attached nanogels. All images were taken using 63x oil immersion objective.

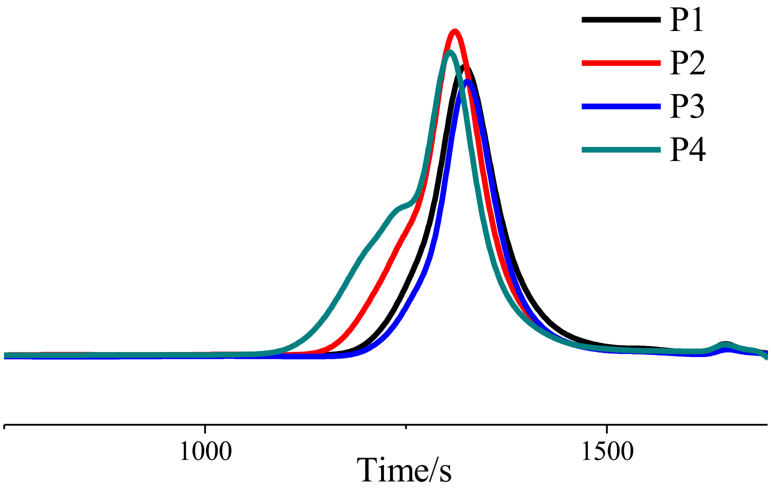


Figure S1 GPC results for P1-P4

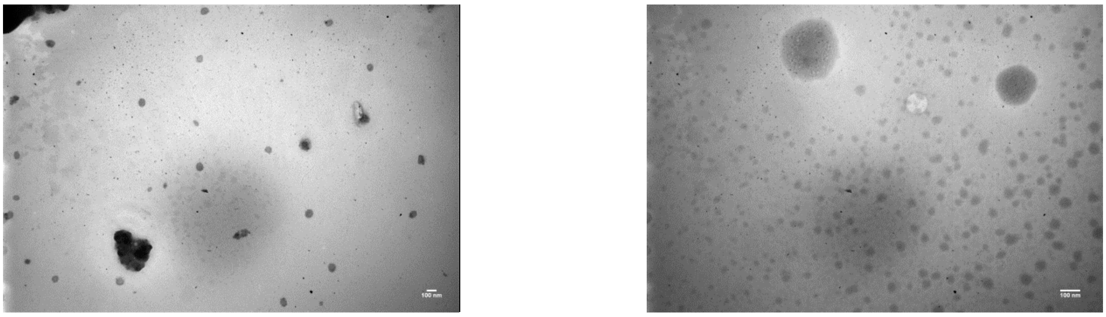


Figure S2. TEM of one DPA nanogel at pH 7.4 (left) and pH 6.5 (right) (Scale bar 100nm)

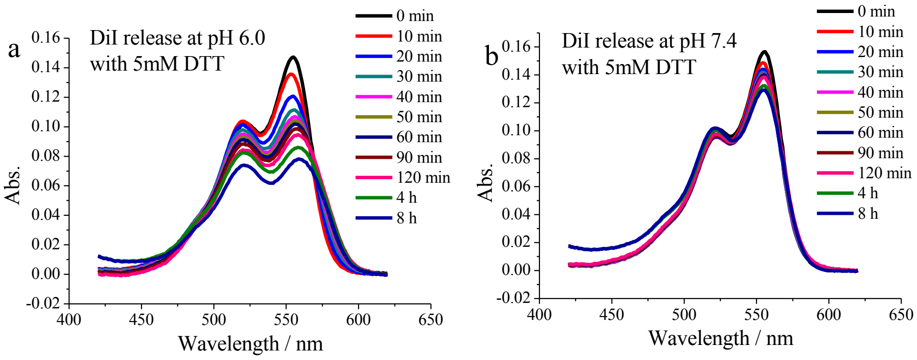


Figure S3. Absorption spectra for DiI release experiment at a) pH 6.0 with 5 mM DTT and b) pH 7.4 with 5mM DTT

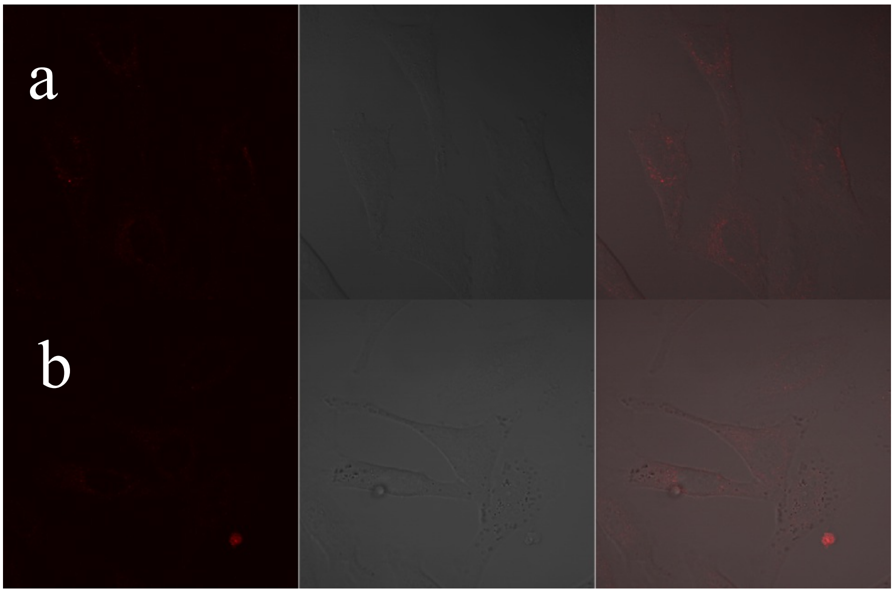


Figure S4. (a) and (b) are confocal images of the control nanogel **NG5** (without DPA units) loaded with DiI at pH 7.4 and 6.5 respectively after incubation with HeLa cells for 2 hours. The nanogels used were made from polymer without DPA units. Cells were imaged using a 63 × oil-immersion objective. Within each image set, left panel is the red channel that shows DiI emission; middle panel is the DIC image, and right panel is an overlap of both images.

References

(1) Ghosh, S.; Basu, S.; Thayumanavan, S. *Macromolecules* **2006**, *39*, 5595-5597.