Supporting Information

Synthetic Polymer Nanoparticle-Polysaccharide Interactions: A Systematic Study

Zhiyang Zeng[†], Jiten Patel[†], Shih-Hui Lee[†], Monica McCallum[†], Anuradha Tyagi[‡], Mingdi Yan[‡],

And Kenneth J. Shea[†,*]

[†]Department of Chemistry, University of California, Irvine, Irvine, CA, 92697.

[‡]Department of Chemistry, Portland State University, Portland, Oregon, 97207.

METHODS

Materials: Acrylamide, acrylic acid, (3-acrylamidopropyl)trimethylammonium chloride, 1-vinyl imidazole, dioctyl sulfosuccinate, sodium salt (AOT), ammonium persulfate (APS), N,N,N′,N′-tetramethylethylenediamine (TEMED), N-isopropylacrylamide were purchased from Sigma-Aldrich, USA. 3-Aminopropylenemethylacrylamide was purchased from Polyscience. Inc, USA. Brij30 was purchased from ACROS Organic, USA. Heparin (15 kD)was obtained from Alfa Aesar, USA and low molecular weight heparin (LMH, 5kD) from Celsus Laboratories, Inc. USA. Fondaparinux sodium was purchased from UCI medical center. All chemical were used as received. N,N′-Ethylenebisacrylamide[1] , 2-aminoethyleneacrylamide[2] and PFPA-MUTEG((1-Mercaptoundec-11-yl)tetra(ethylene glycol)-perfluorophenylazide)[3]were prepared according to literature procedures. Coatest Heparin kit was purchased from DiaPharma Group, Inc., USA. Water used in polymerization and characterization was distilled then purified using a Barnstead Nanopure Diamond TM system from Thermo Scientific. ITC experiments were performed using a Micro-Cal VP-ITC instrument

Preparation of NPs.

AAm-based NPs (Inverse Microemulsion Polymerization). A typical experiment (NP-1) was conducted as following. A monomer solution was prepared by adding acrylamide (0.45g, 6.3 mmol) and ethylene-bis-acrylamide (0.13 g, 0.77 mmol) to water (1.0 mL). Then 1.0 mL of the monomer solution was added dropwise to a deoxygenated solution containing hexanes (21.5 mL), AOT (0.8 g) and Brij 30 (1.54 g). The solution was stirred continuously during the additions. To initiate the polymerization, ammonium persulfate solution (30 μL of 10% (w/v)) and TEMED (15 μL) were added. The solution was stirred at room temperature for 2 h to assure complete polymerization. In order to remove unreacted monomers and surfactants, ethanol (40 mL) was added to precipitate the nanoparticle followed by centrifugation at 5000 RPM for 30min. The nanoparticles were washed with ethanol (4X), and resuspended into water. The suspension was dialyzed against a large excess of water (2X daily changes) for 7 days. The yield of NPs was determined by measuring the weight of a lyophilized aliquot of NP solution following dialysis. The hydrodynamic radius of the purified NPs was determined by a ZEN3600 Zetasizer (Malvern Instruments Ltd) which uses a 4mW 633 nm He-Ne laser. SPR experiments were performed on a SPR imager II (GWC Technologies, Madison, WI)

NIPAm-based NPs (Precipitation Polymerization). Recrystallized N-isopropylacrylamide (NIPAm, 54.73 mM, 309.7 mg), (3-Acrylamidopropyl) trimethylammonium chloride (ATC, 3.25 mM, 40.35 µL), N,N'-ethylenebisacrylamide (BIS, 7.02 mM, 54.1 mg) and cetrimonium bromide (CTAB, 0.055 mM, 20mg) were dissolved in 50 mL D.I. water. After completely dissolving, the solution was filtrated by filter paper and then nitrogen was bubbled through the reaction mixtures for 30 min. Following the addition of the solution of azobisisobutyronitrile (AIBN, 30 mg dissolved in 500 µL acetone), the polymerization was carried out at 60 °C for 3 h under a nitrogen atmosphere. The polymerized solutions were purified by dialysis (MWCO-12,000 to 14,000) against an excess of pure water (changed more than twice a day) for >4 days.

Size Measurements. The hydrodynamic radius of the purified NPs was determined by a ZEN3600 Zetasizer (Malvern Instruments Ltd) which uses a 4mW 633 nm He-Ne laser. Data was collected at a fixed scattering angle of 90° at 25°C. NPs (1 mg/mL) were sonicated for 5 min before each measurement. A minimum of three measurements were taken and averaged for each NP.

Isothermal Titration Calorimetry (ITC). ITC experiments were performed using a Micro-Cal VP-ITC instrument, a 0.1 mM aqueous solution of polysaccharides in 10 mM PBS was injected in equal steps of 10 µL into 1.47 mL of 1mg/mL~5mg/mL NPs solution in 10 mM PBS. The heat of dilution of the polysaccharide solutions when added to the buffer solution in the absence of NPs was also determined and subtracted from the enthalpies measured in the titration experiments. One binding sites model supplied by Microcal Inc. was used to calculate the binding constants and molecular enthalpy change of reaction from titration curve. The molar Gibbs free energy changes and entropies of reaction were calculated from the experimentally determined binding constants and enthalpies change.

Surface Plasmon Resonance (SPR). The detailed SPR protocol can be found in reference [3]. Briefly, these SPR sensors were cleaned with the piranha solution immediately before they were chemically functionalized. For SAM preparation, the total concentration of the thiol solution was kept at 4mM for PFPA-MUTEG. SPR sensors were then soaked in the thiol solution at room temperature for 3 h, followed by gentle washing in ethanol three times for 5min each, and dried with nitrogen. Heparin solution (50 µL, 1mM in water) was then pipetted to the well of the 96-well source plate according to the microarray layout designed for the experiment. The source plate was immediately placed into a robotic printer (BioOdyssey Calligrapher, Bio-Rad, Hercules, CA) for printing the designed array on functionalized SPR sensors. After printing, the SPR sensors were irradiated with a 450W medium pressure Hg lamp (Hanovia) for 5min using a 280nm long-pass optical filter. This was followed by three times washing in water for 5min each while shaking gently. Finally, the sensors were blow-dried with nitrogen. SPR experiments were performed on a SPR imager II (GWC Technologies, Madison, WI) at room temperature and at a flow rate of 100 µL/min. The experiments were carried out as follows. The array containing heparin was flushed with PH 7.4 PBS until a flat baseline was obtained. Solutions of neutral NP-1(0.01mg/mL-0.5mg/mL) in pH 7.4 PBS was introduced to the flow cell for about 30min. The running solution was then switched to the PBS buffer followed by 8M urea solution to regenerate the array surface. The sequence, PBS/NPs /PBS/urea/PBS, was then repeated to test the re-generated surface for reproducibility. The association curves and dissociation curves were fitted to afford average k_{on}, k_{off} and $k_d$by the kinetic fitting software provided by GWC Technologies.

Anticoagulant Assay. The Coatest Heparin kit was employed in this assay. Heparin binds to the protein AT-III to form a complex. This complex can inhibit the activity of FXa by binding to FXa. The residual FXa can be quantified colormetrically by a reaction with the peptide S-2222 (Bz-Ile-Glu-(g-OR)-Gly-Arg-

pNA.HCl) which liberates a dye (pNA). The color is then read photometrically at 405 nm.

1.Heparin+AT→[Heparin-AT]

2.[Heparin-AT]+FXa →[Heparin-AT-FXa]+FXa(remaining)

3.Peptide-pNA+FXa(free)→Peptide+pNA

The assay was first calibrated with 0.1 IU/mL ATIII and 0.01-0.1 IU/mL of heparin in Tris buffer (50 mM, pH=8.4)/normal plasma supplied with the kit. 100 μL of these stand solutions were incubated at 37 °C for 3min. 50 μL of FXa solution (0.71 nkat) were added into the solution and the mixture was incubated at 37 °C for another 30 seconds, at which 100 μL of S-2222 (1 mM) was added. The solution was incubated at 37 °C for exactly 3min. 150 μL of acetic acid (20% in water) was then added into the solution to stop the reaction. Transfer 100 μL the content to a cuvette and read the absorbance of the sample and the sample blank at 405 nm. For inhibition test, NPs with various concentrations were added into the standard solution with 0.1 IU/mL heparin (or low molecular heparin) and the assay was performed as described above. The inhibition percentage was calculated using the following equation:

$$\text{Inhibition\%} = \frac{\text{Intensity}_{\text{sample}} - \text{Intensity}_{\text{Blank}}}{\text{Intensity}_{\text{control}} - \text{Intensity}_{\text{Blank}}}$$

In which $\text{Intensity}_{\text{sample}}$ is the UV intensity of the solution with addition of NPs; $\text{Intensity}_{\text{control}}$ is the UV intensity of the solution without addition of NPs; $\text{Intensity}_{\text{blank}}$ is the UV intensity of the solution without addition of both heparin and NPs.

Triplicate experiments were carried out for each concentration of NPs in the inhibition test.

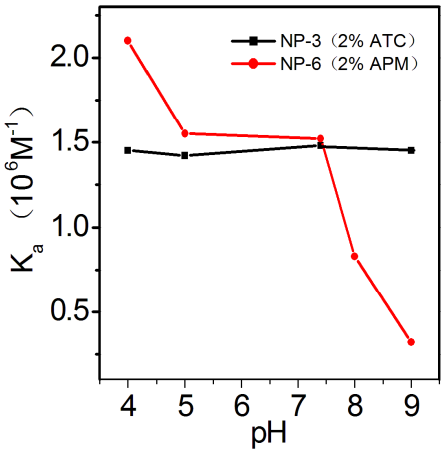

Figure 1. Affinity of NPs-heparin interaction at different pH. Two NPs were studied: NP-3 (2%ATC) (black line) and NP-6 (2%APM) (red line).

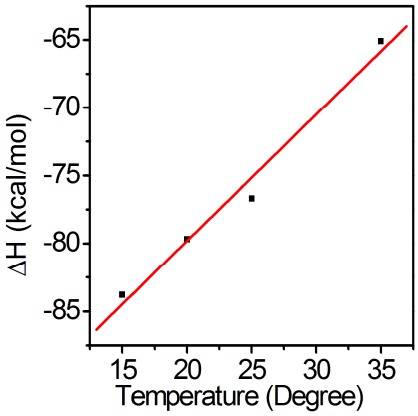

Figure 2. A plot of the enthalpy change vs temperature from the titration of heparin into solutions (10 mM potassium hydrogen phthalate, pH=4) of NP-6(2%APM) at different temperatures. The heat capacity change was calculated from the slope of the line to be 0.93 kcal/mol/deg.

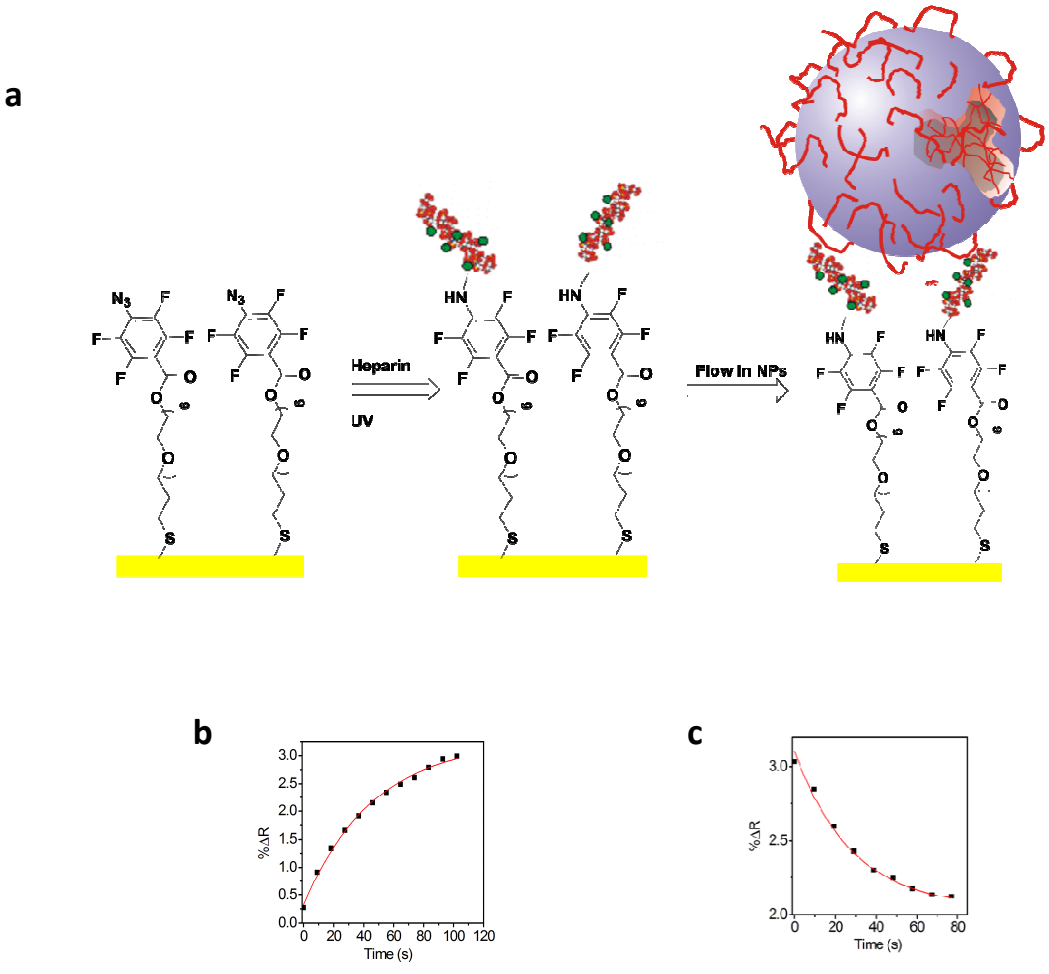

Figure 3. SPR study of the heparin-NPs interaction. a) Heparin was immobilized on the self assemble monolayer of PFPA-MUTEG under UV[3] . NP-1 was flowed into the SPR chamber.

Signals were recorded as a percentage of the SPR angle shift. b) Curve fitting of association curve. c) Curve fitting of dissociation curve.

Table 1 Composition and size of NIPAM-based NPs

Entry	NIPAm	BIS	ATC	Size
#12	90 mol%	10 mol%	-	85 nm
#13	85 mol%	10 mol%	5mol%	90 nm

Reference:

(1) Shea, K. J.; Stoddard, G. J.; Shavelle, D. M.; Wakui, F. *Macromolecules* **1990**, *23*, 4497.

(2) Timofeev, E. N.; Kochetkova, S. V.; Mirzabekov, A. D.; Florentiev, V. L. *Nucleic Acids Res.* **1996**, *24*, 3142.

(3) Tyagi, A.; Wang, X. Deng, L.; Ramström, O.; Yan, M. *Biosens. Bioelectron.* **2010**, *26*, 344.