Electronic Supplementary Material (ESI) for Chemical Communications

Molecularly-imprinted Nanoparticles That Recognize *Naja mossambica* Cytotoxins: Binding Studies and Biological Effects

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Supplementary Information

Materials

N-isopropylacrylamide, N-t-butylacrylamide, acrylic acid, N,N'-methylenebisacrylamide, TEMED, and ammonium persulfate were purchased from Sigma-Aldrich and used without further purification. Cardiotoxins from *N. mossambica mossambica* were purchased from Sigma. Sucrose, EDTA and dialysis tubing were purchased from Fisher Scientific. Calcium chloride was purchased from Flinn Scientific. Porcine blood (containing EDTA to prevent coagulation) was purchased from Sierra Medical for Science. Pre-cast Any-kD TGX polyacrylamide gels, Laemmli sample buffer, Bio-Safe Coomassie stain, sodium dodecyl sulphate, and dithiothreitol were purchased from Bio-Rad. Purified water was purchased from Sierra Water Group.

Stock solutions

Stock solutions of monomers were 0.2 M N-t-butylacrylamide (TBAm) in ethanol and 5% N-isopropylacrylamide (NIPAm), 10% Acrylic Acid (AAc), and 2% N,N'-methylenebisacrylamide (BIS) in water. Stock solutions of 10% sodium dodecyl sulfate (SDS) were also prepared in water. These stock solutions were stored at ambient temperature. Fresh aqueous solutions of 10% ammonium persulfate (APS) were prepared as needed for each polymerization. Cardiotoxins (CTX) from *N. m. mossambica* venom were dissolved in water at a concentration of 1.25 mg/mL and stored at 4°C. The concentration of CTX solutions were confirmed by absorbance at 280 nm using the extinction coefficient ε=8980M^{-1} calculated using the molar extinction coefficients of one tryptophan, 2 tyrosines, and 4 disulfide bridges per cardiotoxin polypeptide (average molecular weight 6840.6 Daltons).

Preparation of Nanoparticles

Polymerizations were carried out on either a 2mL or a 10mL scale with a total monomer concentration of 65mM. The concentration of monomers was divided as follows: 40 mol % TBAm, 5 mol % AAc, 3 mol % BIS, 52 mol % NIPAm. Polymerization mixtures also contained 20 mM Tris Buffer, pH 8.2, 225μM SDS, 2.59mM APS, and 7.5μM cardiotoxins. The total mass of monomers in a polymerization was 7.65 mg/mL, while the mass of CTX was 51 μg/mL. 6μl of TEMED (neat) was added to each 2ml sample, 36μl to each 10mL sample. Before the addition of SDS and APS the solution of mixed monomers was deoxygenated by bubbling nitrogen through the solution for 10 minutes (2mL scale) or 15 minutes (10mL scale) [2]. Polymerization was conducted at 37°C or at ambient temperature and allowed to proceed for a minimum of four hours. The resulting nanoparticle suspensions appeared opalescent, sometimes with small quantities of visible precipitates that were removed through centrifugation (supplementary figure 1).

Supplementary Figure 1: A typical nanoparticle preparation (2 mL scale)

Characterization of Nanoparticles

Polymerization reaction products were placed in dialysis tubing (nominal MWCO 12,000-14,000), and dialyzed against 1000mL of distilled water. The dialysis water was stirred magnetically for the duration of dialysis. Dialysis was continued for five days, including 3 changes of water. The contents of the dialysis tubing were then harvested and the post-dialysis volume was estimated using a graduated vial. Typically, the volume of the polymer suspension increased by 65-120% during dialysis, resulting in a significant dilution of nanoparticles. For percent yield calculations, 1mL of the dialyzed polymer was frozen in a pre-weighed vial using dry ice. The sample was lyophilized overnight and the mass of the resulting sponge-like solid was measured (data shown in Supplementary Table 1). The hydrodynamic radii of the nanoparticles were determined using a ZEN3600 Zetasizer (Malvern Instruments Ltd), with a 4mW 633 nm He-Ne laser. Data was collected at a fixed scattering angle of 90° at 25°C. Polymers were sonicated for 5 min before each measurement. A minimum of three measurements were taken and averaged for each nanoparticle preparation.

Supplementary Table 1: Gravimetric Data from Polymerization attempts

Batch #	1	2	3	4
Polymerization volume	2 mL	2 x 2 mL	10	10
Predialysis monomer concentration	7.65 mg/mL	7.65 mg/mL	7.65 mg/mL	7.65 mg/mL
Predialysis monomer mass	15.30 mg	30.60 mg	76.5 mg	76.5 mg
Post-dialysis volume	4.5 mL	7.25 mL	14.0 mL	11.5 mL
Post-dialysis polymer concentration (assayed by lyophilization of 1 mL of sample)	2.5 mg/mL	2.9 mg/mL	3.7 mg/mL	3.8 mg/mL
Total mass of polymer	11.25 mg	21.0 mg	51.8 mg	43.7 mg
Percent yield	74%	69%	68%	57%

Preparation of Erythrocytes

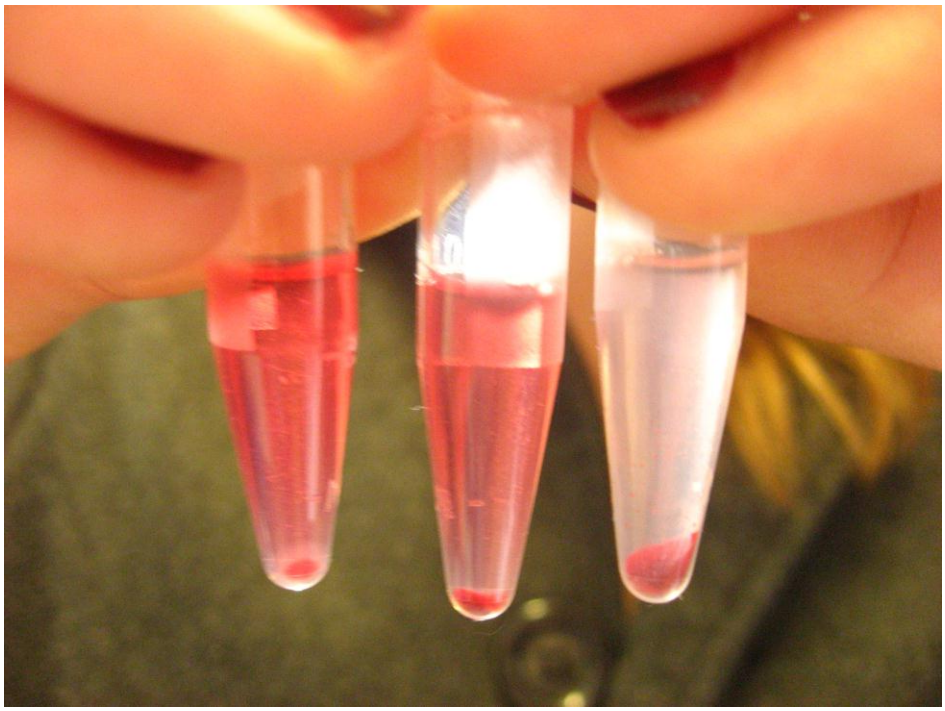
Aliquots of pig blood were centrifuged at 1500*g* in 15mL conical centrifuge tubes for approximately twelve minutes, the plasma and white blood cells were removed by aspiration, and the red cells were resuspended in 0.01M sodium phosphate, 1mM EDTA, 0.3M sucrose solution, pH 7.30 (solution A) [1]. This process was repeated once. Erythrocyte suspensions were stored at 4°C and were used in experiments within 12 days of harvest.

Hemolytic Assays

The concentration of red blood cells used for hemolytic assays was $4.4x10^{11}$ cells/L, based on the reported concentration of erythrocytes in whole blood as $5x10^{12}$ cells/L [3]. The concentration of CTX used in the assays was 3.25 micromolar. Nanoparticle stock solution concentrations were approximately 3 mg/mL.

Varying concentrations of nanoparticles suspended in isotonic phosphate-buffered sucrose (solution A) (510 µL) were preheated to 37˚C prior to the addition of cardiotoxins (10. µL of 180 µM CTX) and erythrocytes in solution A (50 µL). Reaction mixtures were incubated in 1.5mL microcentrifuge tubes at 37˚C. After 30-minutes incubation, the suspensions were centrifuged for two minutes at 13,000 rpm to pellet the remaining erythrocytes. Wavelength scans of the supernatant were taken between 350nm and 550nm

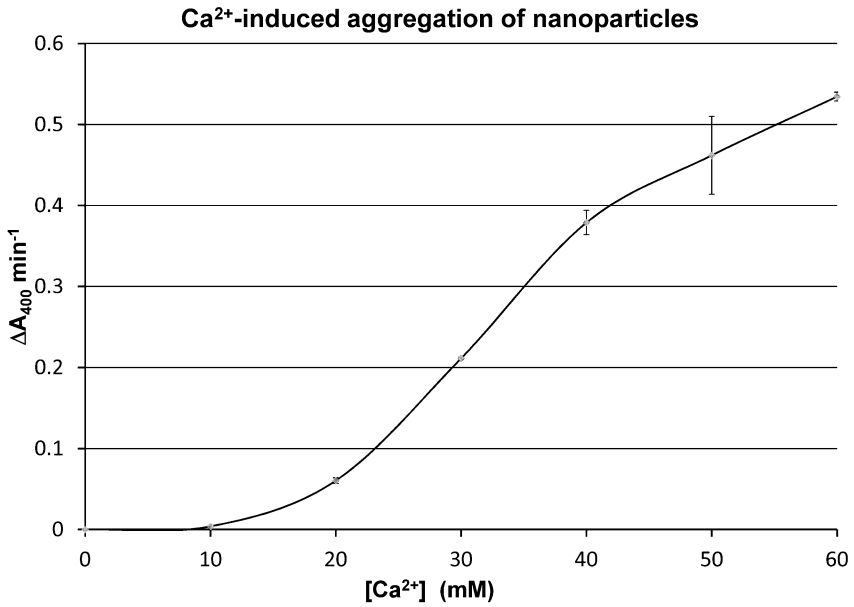
in a 2mm pathlength optical glass cuvette, and the absorbance peak at 414nm was used to determine the percent hemolysis. The relative hemolysis caused by CTX proteins was determined by subtracting out absorbance at 414 nm due to spontaneous hemolysis (determined by running a control of red blood cells in solution A) and the absorbance at 414 nm due to nanoparticle light scattering. 100% hemolysis was determined by running a control of red blood cells in distilled water. A photograph of typical results from a hemolysis experiment is shown in Supplementary Figure 2.



Supplementary Figure 2: Inhibition of CTX-induced hemolysis by varying concentrations of molecularly imprinted nanoparticles. Increasing concentrations of nanoparticles were incubated with pig erythrocytes in the presence of 3.1 µM CTX. The three tubes contain approximately 0.6, 1.2 and 2.4 mg/mL of nanoparticles respectively. The degree of hemolysis is evident in the intensity of red color (due to hemoglobin) present in the supernatant. Additional evidence of erythrocyte preservation is evident in the cell pellets—the tube on the left shows only a small fraction of intact red cells (with a significant fraction of red-cell ghost membranes), while the tube on the right shows a healthy red cell pellet..

Calcium Ion Aggregation Studies

In a semi-micro quartz cuvette, a 1.0 mL reaction mixture was prepared containing 20 mM Tris buffer (pH 8.2), 450 micrograms of molecularly imprinted nanoparticles, and varying concentrations of calcium chloride (added as a 1.0 M stock solution). After the addition of $CaCl_2$, nanoparticle aggregation was monitored by taking absorbance readings at 400 nm every 2 seconds. Initial rates of ΔA400 per minute were calculated from the first 10 seconds of each kinetic run. The data shown in supplementary figure 3 is the average of two separate trials, with error bars spanning the high and low data points collected.



Supplementary Figure 3: Calcium ion-induced aggregation of nanoparticles. Calcium ions are shown to be capable of inducing aggregation of molecularly imprinted nanoparticles, resulting in increased absorbance at 400 nm. The relatively high concentration of Ca^{2+} required to induce aggregation demonstrates that these nanoparticles bind calcium ions with low affinity.

CTX aggregation studies

In a microscale quartz cuvette, a 400 microliter reaction mixture was prepared, containing 20 mM Tris buffer (pH 8.2) and 180 micrograms of molecularly imprinted nanoparticles. Following an initial absorbance measurement at 400 nm, varying concentrations of CTX were added in minimal volumes to initiate aggregation. Nanoparticle aggregation was allowed to proceed for 2.5-minutes at ambient temperature and a subsequent absorbance measurement at 400 nm was taken. The ΔA400 was plotted against CTX concentration to determine a dose-response curve.

Protein-binding Competition Assays

400-microliter volumes containing 20 mM Tris buffer (pH 8.0), 160 micrograms molecularly imprinted nanoparticles, and binary mixtures of proteins (lysozyme + CTX, myoglobin + CTX or lysozyme + myoglobin) were prepared in microcentrifuge tubes. Each protein in the mixture was present at a concentration of 67 µg/mL (27 µg per 400 µL reaction). Following a 5-minute incubation at ambient temperature, protein-nanoparticle aggregates were collected by centrifugation at 13k rpm for 1 minute. The supernatant in each tube was carefully removed using a Pasteur pipet, and the pellets were then washed with 200 microliters of 100 mM Tris buffer (pH 8.0). Following another 1-minute spin at 13,000 rpm, the supernatant was again removed using a Pasteur pipet. The remaining pellets were then resuspended in Laemmli sample buffer. Dithiothreitol was added to a final concentration of 0.1 M, and the samples were denatured by heating at 95°C for 5 minutes. Samples were subsequently loaded on an Any-kD pre-cast Biorad TGX polyacrylamide gel, and stained using Bio-Rad Biosafe Coomassie Stain.

The Advanced Chemical Research Program

The work described in this paper was performed by high school students enrolled in the Advanced Chemical Research Program at Laguna Beach High School. This program is offered to scientifically talented 12th grade students. The first semester of the course trains students in numerous advanced techniques such as UV-Vis spectroscopy, TLC, organic extractions, NMR, Fluorescence, Gel filtration, PAGE, and Mass Spectrometry. The second semester of the course provides students with the opportunity to pursue original research projects in teams of 3-4 students. Safety training is included throughout the year. The program has been described in detail in the *Journal of Chemical Education* [4].

References

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