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Supporting Information

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Temperature-Responsive "Catch and Release" of Proteins by using Multifunctional Polymer-Based Nanoparticles**

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Supporting Information

Materials:

All chemicals were obtained from commercial sources: *N*-isopropylacrylamide (NIPAm), ammonium persulfate, Tris(hydroxymethyl)aminomethane (Tris), Glycine and Freeze-dried *Micrococcus lysodeikticus* ATCC 4698 were from Sigma-Aldrich, Inc.; Acrylic acid (AAc), and sodium dodecyl sulfate (SDS) were from Aldrich Chemical Company, Inc.; BIS was from Fluka; TBAm was from Acros Organics; Lysozyme from chicken egg white and ribonuclease A (RNase A) from bovine pancreas were from Fisher scientific, Inc.; NIPAm was recrystallized from hexane before use. Other chemicals were used as received. Nanosep membrane centrifuge filter device (MWCO: 100 kDa) was purchased from PALL corp. Precast SDS-PAGE gels (12% Mini-PROTEAN TGX™), a molecular weight marker solution (Precision plus protein standards) and Coomassie Brilliant Blue R-250 were purchased from Bio-rad Laboratories, Inc. Water used in polymerization and characterization was distilled and then purified by using a Barnstead Nanopure Diamond system.

Preparation of NPs:

NIPAm (98–(X+Y) mol%), AAc (X mol%), TBAm (Y mol%), BIS (2 mol%), and SDS (10 mg) were dissolved in water (50 mL) and the resulting solutions were filtered through a no. 2 Whatman filter paper. TBAm (Y mol%) was dissolved in ethanol (1 mL) before addition to the monomer solution, which resulted in a total monomer concentration of 65 mM. Nitrogen gas was bubbled through the reaction mixtures for 30 min. Following the addition of ammonium persulfate aqueous solution (30 mg per 50 mL), the pre-polymerization mixture was purged with nitrogen gas for 30 min and sealed under nitrogen gas. Polymerization was carried out by inserting the round bottle flask containing pre-polymerization mixture in an oil bath pre-set to 60 °C for 3 h. The polymerized solutions were purified by dialysis against an excess amount of pure water (changed more than twice a day) for 5 to 6 days.

Characterization of NPs:

The yield of NPs was determined by measuring the weight of NPs obtained after lyophilisation (table 1). Here a dilution factor due to dialysis was corrected. The hydrodynamic diameter (D_H) of NPs was determined in aqueous solutions (25 ± 0.1 °C) by dynamic light scattering (DLS) instrument equipped with a software zetasizer software Ver. 6.12 (Zetasizer Nano ZS, Malvern

Instruments Ltd) three times. The refractive index of polystyrene latex beads was used as standard. All DLS data meets quality criteria set by Malvern. The intensity-weighted average diameters of all three analyses were averaged and listed in table 1.

Lysozyme activity assay:

The measurement of lysozyme activity was performed by the previously described method. Lysozyme was dissolved at 5 µg/mL in phosphate-buffered saline (PBS; 35 mM sodium phosphate buffer/150 mM NaCl, pH 7.3). Freeze-dried *Micrococcus lysodeikticus* cells were resuspended at 125 µg/ml in PBS. The 125 µg/ml *Micrococcus lysodeikticus* cell suspension (100 µL) was added to lysozyme solution (100 µL), and cell lysis was followed at room temperature by measuring the decrease in OD at 450 nm (OD_{450}) using a NanoDrop UV-vis spectrophotometer (Thermo Scientific, Inc.) and disposable 70 µL UV-cuvette micro (Brand GmbH + Co KG). The decrease in OD_{450} for the first 1 minute was used as the measure of lysozyme activity.

Batch-mode lysozyme binding study:

Lysozyme (5 µg/mL) was preincubated with NPs (800 µg/mL) or without NPs as a control in PBS for 15 min at room temperature. Nanosep membrane centrifuge filter device (MWCO: 100 kDa) was utilized to filter off the NPs and NP-bound lysozyme for the following lysozyme activity measurements. The lysozyme activity of the filtrate was measured to estimate the concentration of lysozyme.

The error bars represent standard deviation of three independent measurements.

Lysozyme inhibition study:

Lysozyme (5 µg/mL) was preincubated with various concentrations of NP2 or without NPs as a control in PBS (100 µL) for 15 min at room temperature. V_0, the initial *Micrococcus lysodeikticus* cell lysis velocity of lysozyme in absence of NPs, and V, that of lysozyme in presence of NPs, were measured by the method described in above. The relative activity of the mixture was calculated by the equation:

$$\text{Relative activity} = V / V_0$$

The error bars represent standard deviation of three independent measurements.

Batch-mode binding selectivity study:

Each protein (Ovalbumin, ovotransferrin, lysozyme, avidin, RNase A; 0.2 mg/mL) were preincubated with NPs (2.0 mg/mL) or without NPs as a control in 200 µL of PBS for 15 min at room temperature. Nanosep membrane centrifuge filter device (MWCO: 100 kDa) was utilized to filter off the NPs and NP-bound proteins. To estimate initial protein concentration (I) and the concentration of unbound protein (U) in the filtrates, respectively, ABS at 280 nm of the filtrate was measured using a NanoDrop UV-vis spectrophotometer (Thermo Scientific, Inc.) and disposable 70 µL UV-cuvette micro (Brand GmbH + Co KG). The amount of bound proteins was calculated by the equation:

$$\text{The amount of bound protein} = I - U$$

The error bars represent standard deviation of three independent measurements.

Determination of phase transition temperature of NPs.

The lower critical solution temperature (LCST) or volume phase transition temperature of NP2 in water was studied using DLS measurements. Figure S3 shows the hydrodynamic diameters (D_H) of NP2 as a function of temperature in water. Before measurements were taken, the solutions were incubated at each temperature for 25 min to achieve thermal equilibration. Each point represents average of five measurements.

Repeated "catch-and-release" of lysozyme by NP2:

Step 1. "Catch" at room temperature: Lysozyme (5 µg/mL) was incubated with NP2 (2.0 mg/mL) in 2.0 mL of PBS at room temperature for 30 min. After incubation, 200 µL of the mixture was sampled using a pipette and was centrifuged in Nanosep membrane filter device (MWCO: 100 kDa) to filtrate off NPs and NP-bound lysozyme. The filtrate was kept at room temperature until the lysozyme activity is measured in step 3.

Step 2. "Release" or *cold elution* at 1 °C: The lysozyme/NP mixture was cooled down by incubating in a water bath set at 1 °C for 30 min. After incubation, 200 µL of the mixture was sampled using a pipette and was centrifuged in Nanosep membrane filter device (MWCO: 100 kDa) at 2-4 °C. The filtrate was warmed up to room temperature and was kept at room temperature until the lysozyme activity is measured in step 3.

Step 3. After the "catch-and-release" cycles (as described in step 1 and 2) were repeated 4 times in total, the lysozyme activity of the filtrate was measured by the method described in above.

Circular dichroism (CD) spectrum measurement:

Lysozyme (200 µg/mL) was incubated with NP2 (2.5 mg/mL) in 400 µL of PBS at room temperature for 30 min. After incubation, the mixture was centrifuged in Nanosep membrane filter device (MWCO: 100 kDa). After all the solution is passed through the membrane, 400 µL of PBS was added onto the membrane filter unit and the sample was cooled down by incubating in a water bath set at 1 °C for 30 min. The solution was passed through the filter membrane device by centrifugation at 2-4 °C and the CD spectrum of recovered lysozyme was measured using J-810 CD spectropolarimeter (JASCO, inc.).

"Catch-and-release" purification of lysozyme from chicken egg white:

Step 1. Preparation of an egg white sample: The chicken egg whites separated carefully without disturbing the egg yorks were diluted 50-fold with PBS in a polypropylene tube. To ensure homogeneous mixing, the tube was placed in ultrasound sonication bath for 30 min at room temperature. The diluted egg white solution was filtered using Nanosep membrane filter device and was stored at 4 °C until use.

Step 2. "Catch" at room temperature: The egg white sample (prepared in step 1.) was incubated with 2.0 mg/mL of NP2 (2.0 mg/mL) in 400 µL of PBS at room temperature for 30 min. After incubation, the mixture was centrifuged in Nanosep membrane centrifugal filter device, until all the solution is passed through the membrane. The filtrate (*flow through* fraction) was collected and was analyzed by SDS-PAGE.

Step 3. "Release" or cold elution at 1 °C: To recover the lysozyme from the NPs retained on the membrane, 300 µL of PBS was added to the membrane filter unit and the sample was cooled down by incubating in a water bath set at 1 °C for 30 min. The solution was passed through membrane filter using centrifugation at 2-4 °C and the filtrate was collected and was analyzed by SDS-PAGE.

SDS-PAGE analysis:

The filtrate from each purification step (7.5 µL) was mixed with a sample preparation solution (7.5 µL; water (68 vol%), glycerol (20 vol%), 2-mercaptoethanol (12 vol%), Tris (66.7 mM), SDS (40 mg/mL), bromophenol blue (0.04%, w/v)) and heated at 90 °C for 5-10 min. 10 µL of premixed samples or 7.5 µL of the molecular weight marker solution were loaded into 12% SDS-PAGE gel immersed in a running buffer (aqueous solution containing 25 mM Tris, 192 mM Glycine and 0.1% SDS), separated by electrophoresis, and were stained with Coomassie Brilliant Blue R-250.

Experimentals:

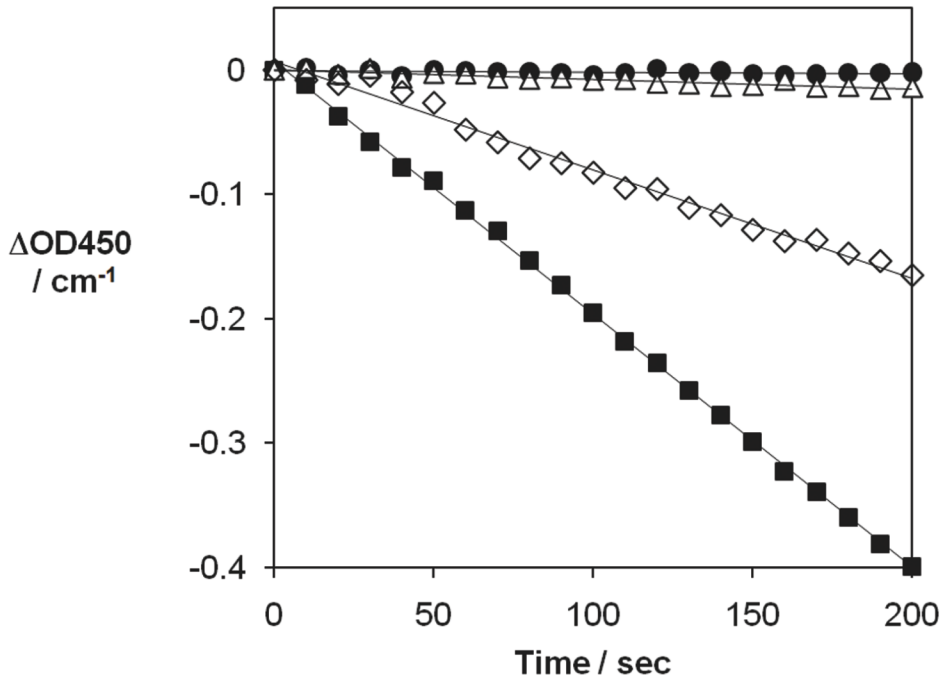

Figure S1. *Micrococcus lysodeikticus* cell lysis kinetics in absence of lysozyme (filled circle), presence of lysozyme (filled square), lysozyme + NP2 (open triangle) and lysozyme + NP3 (open diamond). Measured in PBS (35 mM sodium-phosphate buffer containing 150 mM NaCl, pH 7.3) at room temperature. Lysozyme concentration: 5 µg/mL. NP concentration: 800 µg/mL. A clear reduction in lysozyme activity is seen when NP2 and NP3 are present.

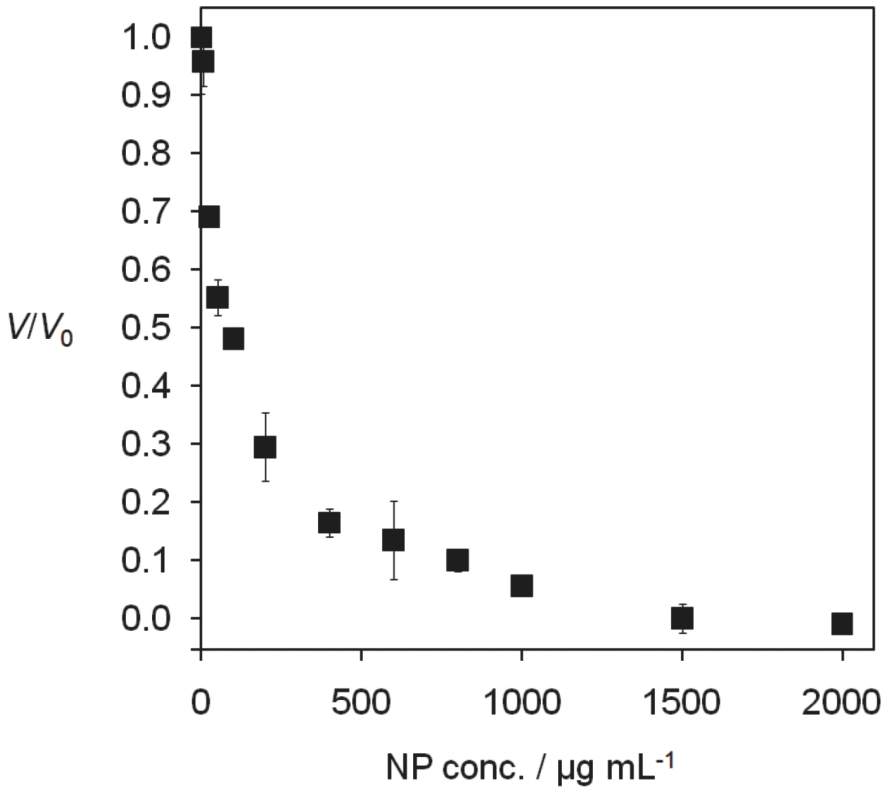

Figure S2. Inhibition of lysozyme (5 µg/mL) in presence of various conc. of NP2. V/V_0 is a ratio of V, the initial *Micrococcus lysodeikticus* cell lysis velocity of lysozyme in presence of NPs to V_0, that in absence of NPs. Measured in PBS (35 mM sodium-phosphate buffer containing 150 mM NaCl, pH 7.3) at room temperature.

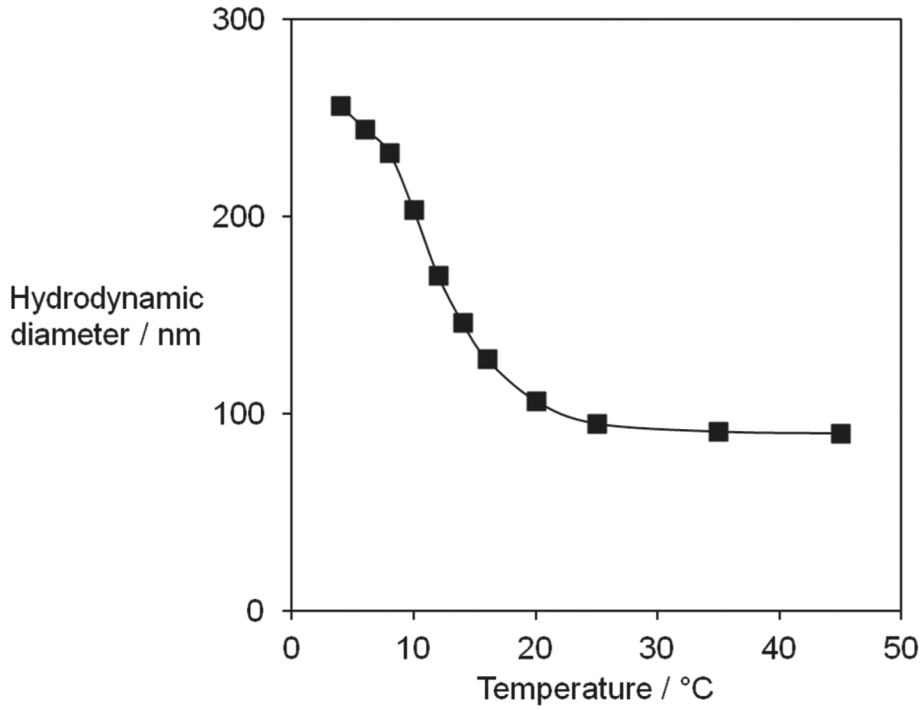

Figure S3. Volume phase transition curves for NP2 (50 μg/mL) in PBS (35 mM sodium-phosphate buffer containing 150 mM NaCl, pH 7.3). Hydrodynamic diameters of NP2 were determined by dynamic light scattering (DLS) measurements. Before measurements were taken, the solutions were incubated at each temperature for 25 min to achieve thermal equilibration. Each point represents average of five measurements. As shown, hydrodynamic diameter rapidly increased at temperatures around 11 °C.

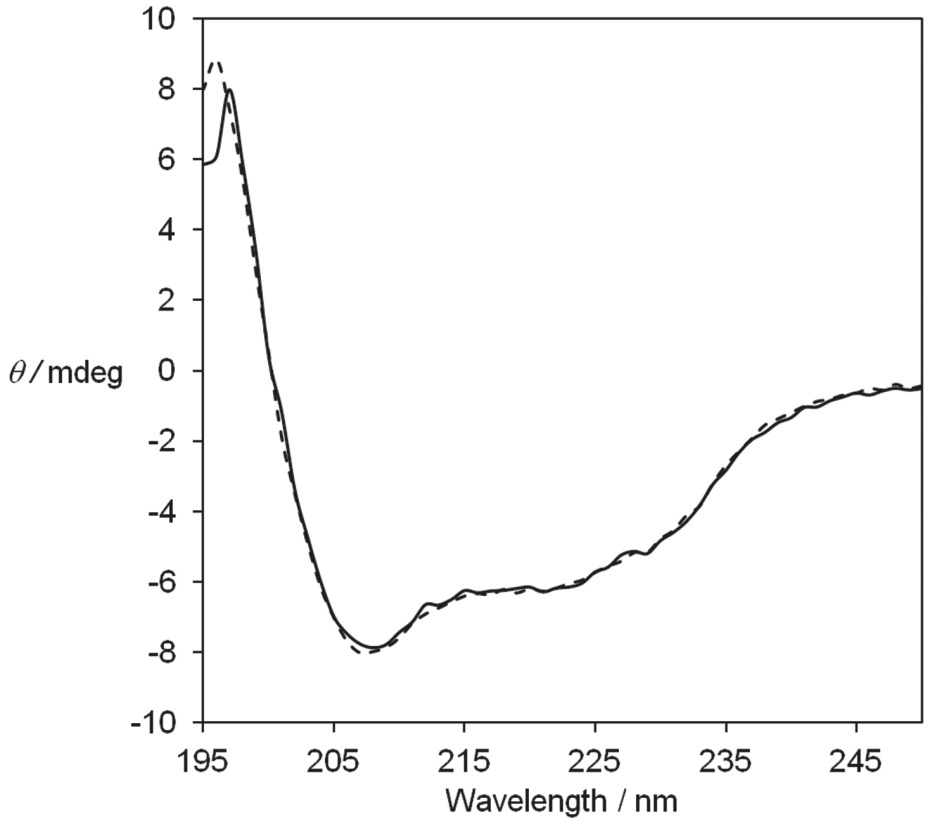

Figure S4. Circular dichroism spectra of lysozyme (4 μM) before (dashed line) and after (solid line) the "catch-and-release" by NP2 in PBS (35 mM sodium-phosphate buffer containing 150 mM NaCl, pH 7.3) at room temperature.