# Design of Synthetic Polymer Nanoparticles that Facilitate Resolubilization and Refolding of Aggregated Positively Charged Lysozyme

Masahiko Nakamoto[†], Tadashi Nonaka[†], Kenneth J. Shea[‡], Yoshiko Miura[†], and Yu Hoshino[†*]

[†]*Department of Chemical Engineering, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan*

[‡]Department of Chemistry, University of California, Irvine, Irvine, California 92697, USA

## *SUPPORTING INFORMATION*

**Materials.**

The following chemicals and materials were obtained from commercial sources and used as received: Lysozyme and *Micrococcus lysodeikticus* cells were obtained from Sigma Aldrich. *N*,*N'*-methylenebisacrylamide (BIS), 3,3′-dithiodipropionic acid, and 1-ethyl-3-(3--dimethylaminopropyl)carbodiimide were obtained from Tokyo Chemical Industry Co., Ltd. *N*-*tert*-butylacrylamide (TBAm), sodium dodecyl sulfate (SDS), 4,4'-azobis(4-cyanovaleric acid) (V-501) and guanidium hydrochloride (GdnCl) were obtained from Wako Pure Chemical Industries, Ltd. Nanosep® centrifugal devices (MWCO: 30 kDa) was obtained from Pall Corporation. *N*-Isopropylacrylamide (NIPAm) (Wako Pure Chemical Industries, Ltd.) was recrystallized from hexanes. Dialysis tube (MWCO 12,000–14,000 Da) (Fisher bland, Inc.) was washed with water prior to use. Acrylic acid (AAc) (Wako Pure Chemical Industries) and *N*-[3-(dimethylamino)propyl] acrylamide (DMAPA) (Tokyo Chemical Industry Co., Ltd.) were purified by alumina column.Water used in this study was purified using a Direct-Q Ultrapure Water System (Merck, Ltd.).

**Preparation of NP1.**

NIPAm (80 mol %), BIS (10 mol %), AAc (10 mol %), and SDS (53.7 mg) were dissolved in 30 mL of water, resulting in a total monomer concentration of 52 mM. Next, nitrogen was bubbled through the reaction mixture for 30 min. Following the addition of V-501 (5.8 mg/0.5 mL DMSO), the polymerization was carried out at 70 °C for 3 h under a nitrogen atmosphere. The polymerized solution was purified by dialysis against an excess amount of water (changed more than 3 times a day) for 3 days. The yield of NPs was determined by measuring the weight of NPs obtained by lyophilization of 3 mL of the dialyzed solution.

**Preparation of NP2.**

NIPAm (80 mol %), BIS (10 mol %), DMAPA (10 mol %), and CTAB (67.9 mg) were dissolved in 30 mL of water, resulting in a total monomer concentration of 52 mM. Next, nitrogen was bubbled through the reaction mixture for 30 min. Following the addition of AAPD (5.6 mg/0.5 mL MilliQ), the polymerization was carried out at 70 °C for 3 h under a nitrogen atmosphere. The polymerized solution was purified by dialysis against an excess amount of water (changed more than 3 times a day) for 3 days. The yield of NPs was determined by measuring the weight of NPs obtained by lyophilization of 3 mL of the dialyzed solution.

**Preparation of NP3.**

NIPAm (20 mol %), BIS (10 mol %), TBAm (70 mol% in 1 mL methanol) and SDS (53.7 mg) were dissolved in 30 mL of water, resulting in a total monomer concentration of 52 mM. Next, nitrogen was bubbled through the reaction mixture for 30 min. Following the addition of V-501 (5.8 mg/0.5 mL DMSO), the polymerization was carried out at 70 °C for 3 h under a nitrogen atmosphere. The polymerized solution was purified by dialysis against an excess amount of water (changed more than 3 times a day) for 3 days. The yield of NPs was determined by measuring the weight of NPs obtained by lyophilization of 3 mL of the dialyzed solution.

**Preparation of NP4.**

NIPAm (10 mol %), BIS (10 mol %), AAc (10 mol %), TBAm (70 mol% in 1 mL methanol) and SDS (53.7 mg) were dissolved in 30 mL of water, resulting in a total monomer concentration of 52

mM. Next, nitrogen was bubbled through the reaction mixture for 30 min. Following the addition of V-501 (5.8 mg/0.5 mL DMSO), the polymerization was carried out at 70 °C for 3 h under a nitrogen atmosphere. The polymerized solution was purified by dialysis against an excess amount of water (changed more than 3 times a day) for 3 days. The yield of NPs was determined by measuring the weight of NPs obtained by lyophilization of 3 mL of the dialyzed solution.

**Preparation of NP5.**

NIPAm (10 mol %), BIS (10 mol %), DMAPA (10 mol %), TBAm (70 mol% in 1 mL methanol) and CTAB (22.6 mg) were dissolved in 30 mL of water, resulting in a total monomer concentration of 52 mM. Next, nitrogen was bubbled through the reaction mixture for 30 min. Following the addition of AAPD (5.6 mg/0.5 mL MilliQ), the polymerization was carried out at 70 °C for 3 h under a nitrogen atmosphere. The polymerized solution was purified by dialysis against an excess amount of water (changed more than 3 times a day) for 3 days. The yield of NPs was determined by measuring the weight of NPs obtained by lyophilization of 3 mL of the dialyzed solution.

**Preparation of NP6.**

NIPAm (60 mol %), BIS (10 mol %), AAc (10 mol %), TBAm (20 mol% in 1 mL methanol) and SDS (53.7 mg) were dissolved in 30 mL of water, resulting in a total monomer concentration of 52 mM. Next, nitrogen was bubbled through the reaction mixture for 30 min. Following the addition of V-501 (5.8 mg/0.5 mL DMSO), the polymerization was carried out at 70 °C for 3 h under a nitrogen atmosphere. The polymerized solution was purified by dialysis against an excess amount of water (changed more than 3 times a day) for 3 days. The yield of NPs was determined by measuring the weight of NPs obtained by lyophilization of 3 mL of the dialyzed solution.

**Preparation of NP7.**

NIPAm (30 mol %), BIS (10 mol %), AAc (10 mol %), TBAm (50 mol% in 1 mL methanol) and SDS (53.7 mg) were dissolved in 30 mL of water, resulting in a total monomer concentration of 52 mM. Next, nitrogen was bubbled through the reaction mixture for 30 min. Following the addition of V-501 (5.8 mg/0.5 mL DMSO), the polymerization was carried out at 70 °C for 3 h under a nitrogen atmosphere. The polymerized solution was purified by dialysis against an excess amount of water

(changed more than 3 times a day) for 3 days. The yield of NPs was determined by measuring the weight of NPs obtained by lyophilization of 3 mL of the dialyzed solution.

**Preparation of NP8.**

NIPAm (15 mol %), BIS (10 mol %), AAc (5 mol %), TBAm (70 mol% in 1 mL methanol) and SDS (53.7 mg ) were dissolved in 30 mL of water, resulting in a total monomer concentration of 52 mM. Next, nitrogen was bubbled through the reaction mixture for 30 min. Following the addition of V-501 (5.8 mg/0.5 mL DMSO), the polymerization was carried out at 70 °C for 3 h under a nitrogen atmosphere. The polymerized solution was purified by dialysis against an excess amount of water (changed more than 3 times a day) for 3 days. The yield of NPs was determined by measuring the weight of NPs obtained by lyophilization of 3 mL of the dialyzed solution.

**Preparation of NP9.**

BIS (10 mol %), AAc (20 mol %), TBAm (70 mol% in 1 mL methanol) and SDS (53.7 mg) were dissolved in 30 mL of water, resulting in a total monomer concentration of 52 mM. Next, nitrogen was bubbled through the reaction mixture for 30 min. Following the addition of V-501 (5.8 mg/0.5 mL DMSO), the polymerization was carried out at 70 °C for 3 h under a nitrogen atmosphere. The polymerized solution was purified by dialysis against an excess amount of water (changed more than 3 times a day) for 3 days. The yield of NPs was determined by measuring the weight of NPs obtained by lyophilization of 3 mL of the dialyzed solution.

**Preparation of NP10.**

BIS (10 mol %), AAc (10 mol %), TBAm (80 mol% in 1 mL methanol) and SDS (53.7 mg) were dissolved in 30 mL of water, resulting in a total monomer concentration of 52 mM. Next, nitrogen was bubbled through the reaction mixture for 30 min. Following the addition of V-501 (5.8 mg/0.5 mL DMSO), the polymerization was carried out at 70 °C for 3 h under a nitrogen atmosphere. The polymerized solution was purified by dialysis against an excess amount of water (changed more than 3 times a day) for 3 days. The yield of NPs was determined by measuring the weight of NPs obtained by lyophilization of 3 mL of the dialyzed solution.

**Preparation of fluorescent-labeled NP (NP11).**

For the fluorescent-labeled NPs, an acrylamide derivative containing fluorescein in the side chain (Fluorescein monomer) was synthesized as reported previously[21]. BIS (10 mol %), AAc (10 mol %), TBAm (70 mol% in 1 mL methanol), AFA (0.01 mol% in 0.1 mL methanol) and SDS (53.7 mg ) were dissolved in 30 mL of water, resulting in a total monomer concentration of 52 mM. Next, nitrogen was bubbled through the reaction mixture for 30 min. Following the addition of V-501 (5.8 mg/0.5 mL DMSO), the polymerization was carried out at 70 °C for 3 h under a nitrogen atmosphere. The polymerized solution was purified by dialysis against an excess amount of water (changed more than 3 times a day) for 3 days. The yield of NPs was determined by measuring the weight of NPs obtained by lyophilization of 3 mL of the dialyzed solution.

**Table S1.** Reaction condition and yield of NPs.

| | Monomaer feed ratio (mol%) | | | | | | Monomer Concentration (mM) | Surfactant | Initiator | Yield (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| | NIPAm | TBAm | AAc | DMAPA | BIS | Fluorescein monomer | | | | |
| NP1 | 80 | 0 | 10 | 0 | 10 | 0 | 52 | SDS 6.21 mM | V501 0.69 mM | 80 |
| NP2 | 80 | 0 | 0 | 10 | 10 | 0 | 52 | CTAB 6.21 mM | AAPD 0.69 mM | 80 |
| NP3 | 20 | 70 | 0 | 0 | 10 | 0 | 52 | SDS 6.21 mM | V501 0.69 mM | 81 |
| NP4 | 10 | 70 | 10 | 0 | 10 | 0 | 52 | SDS 6.21 mM | V501 0.69 mM | 94 |
| NP5 | 10 | 70 | 0 | 10 | 10 | 0 | 52 | CTAB 2.07 mM | AAPD 0.69 mM | 94 |
| NP6 | 60 | 20 | 10 | 0 | 10 | 0 | 52 | SDS 6.21 mM | V501 0.69 mM | 95 |
| NP7 | 30 | 50 | 10 | 0 | 10 | 0 | 52 | SDS 6.21 mM | V501 0.69 mM | 84 |
| NP 8 | 15 | 70 | 5 | 0 | 10 | 0 | 52 | SDS 6.21 mM | V501 0.69 mM | 91 |
| NP9 | 0 | 70 | 20 | 0 | 10 | 0 | 52 | SDS 6.21 mM | V501 0.69 mM | 89 |
| NP10 | 0 | 80 | 10 | 0 | 10 | 0 | 52 | SDS 6.21 mM | V501 0.69 mM | 83 |
| NP11 | 9.99 | 70 | 10 | 0 | 10 | 0.01 | 52 | SDS 6.21 mM | V501 0.69 mM | 90 |

**Measurement of hydrodynamic diameter and zeta potential of NPs**

Hydrodynamic diameter and polydispersity index (PDI) of NPs were monitored by dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments Ltd.) at 25 °C in 100 mM phosphate buffer (pH 7.4). Concentration of NP were 0.1 mg/mL. Zeta potential of NPs were monitored by dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments Ltd.) at 25 °C in 10 mM phosphate buffer (pH 7.4). Concentration of NP were 0.1 mg/mL.

**Preparation and characterization of unfolded lysozyme**

5 mM Lysozyme was dissolved in 6 M guanidium hydrochloride containing 100 mM tris buffer (pH 8.2) (denaturation buffer) and incubated for 3h at 37 °C. Lysozyme solution was 100 times diluted by danaturation buffer and inserted to cell (final concentration was 50 μM). Circular dichroism (CD) spectra were monitored using a Jasco spectropolarimeter (JASCO J-725G, Jasco Ltd., Tokyo, Japan). A cuvette with 0.1 mm path length was used, and the photomultiplier voltage did not exceed 500 V in the measurements. As a result, denaturation of lysozyme was confirmed (Figure S1).

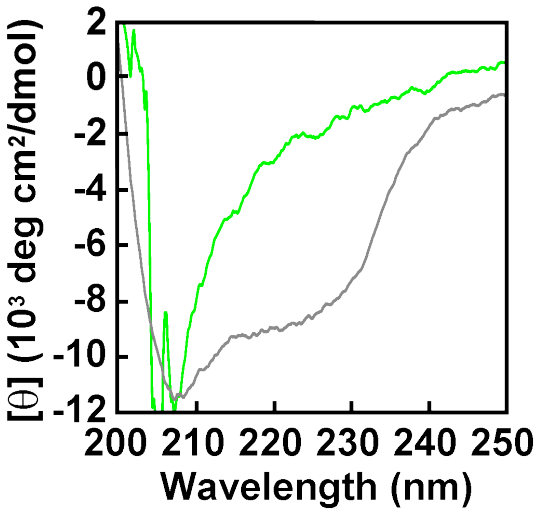


Figure. S1. CD spectrum of native lysozyme (**gray**) and unfolded lysozyme (**green**).

**Preparation and characterization of aggregated lysozyme**

Aggregated lysozyme was prepared by 100 times diluting 5 mM denatured lysozyme by 100 mM phosphate buffer (pH 7.4). Immediately, solution was turned to opaque (Figure S2). The size of aggregates increased as a function of time after dilution (Figure S2a, b and c). In contrast, native lysozyme solution was completely transparent (Figure S2d). Hydrodynamic diameter of aggregated lysozyme was observed by dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments Ltd.). After 1h incubation from dilution, micro meter aggregates was confirmed (Figure S3a). In contrast hydrodynamic diameter of native lysozyme in 1% denaturation buffer containing 100 mM phosphate buffer (pH 7.4) was 4.5 nm (Figure S3b). After 1h pre-incubation (25 °C), the diluted solution was filtered by centrifugal ultrafiltration (MWCO: 30 kDa, 13.5 kG, for 15 min at 25 °C) (Nanosep® centrifugal devices, Pall Corp.). It was confirmed that almost all (>90%) lysozyme was trapped by the filter (Figure S4).

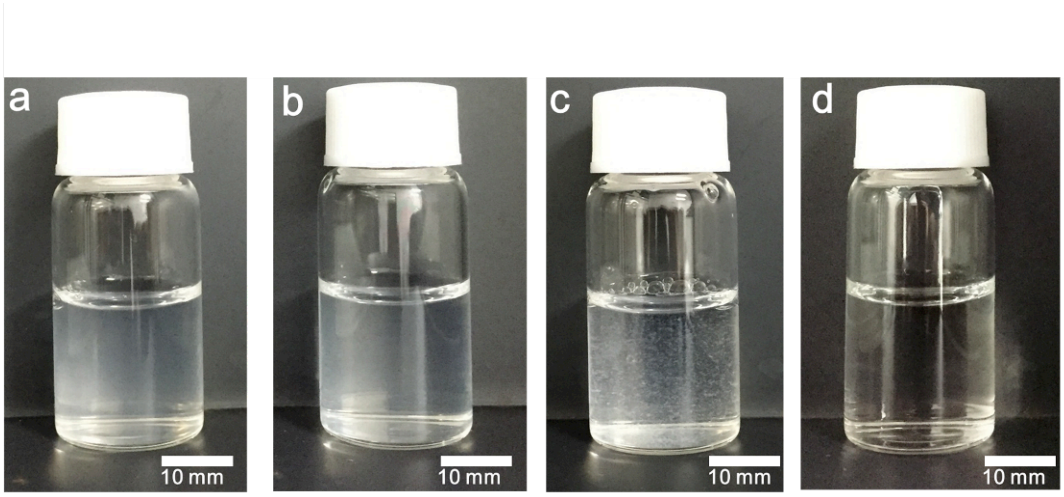


Figure. S2. Photographs of aggregated lysozyme (**a**) several min, (**b**) 3h, and (**c**) 5h after 100 times dilution of 5 mM denatured lysozyme and (**d**) 50 μM native lysozyme solution.

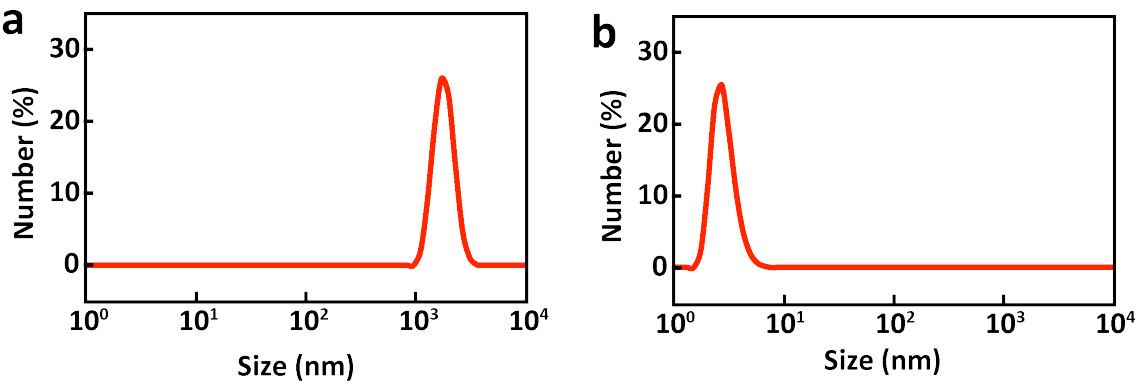


Figure. S3. Hydrodynamic diameter distribution of (**a**) aggregated lysozyme and (**b**) native lysozyme. Total amount of lysozyme were 50 μM in both measurement.

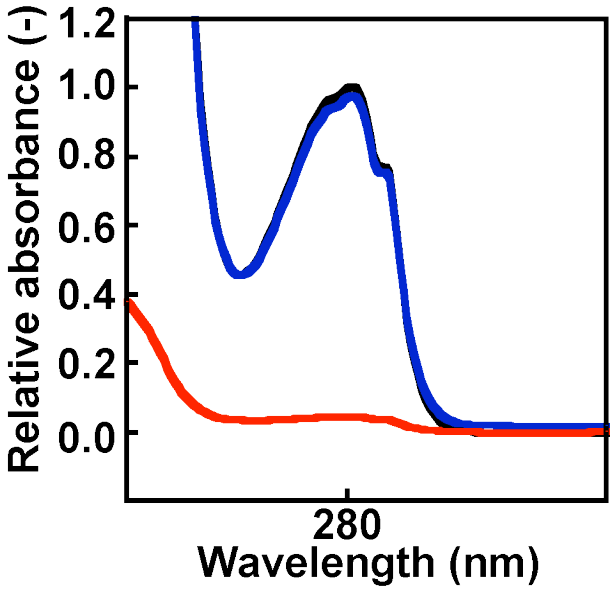


Figure. S4. Relative absorbance of 50 μM native lysozyme that passed through the filter (**blue**) and diluted solution of denatured lysozyme (**red**). Absorbance were normalized by absorbance of 50 μM native lysozyme without filtration (**black**).

[

**Effect of concentration of lysozyme in dilution process on recovery yield**

Aggregated lysozyme were prepared by 100 times diluting of 0.3-10 mM unfolded lysozyme by 100 mM phosphate buffer (pH 7.4). After 1 h incubation (25 °C), the diluted solution was filtered by centrifugal ultrafiltration (MWCO: 30 kDa, 13.5 kG, for 15 min at 25 °C) (Nanosep® centrifugal devices, Pall Corp.). Effect of concentration of lysozyme in dilution process on recovery yield is shown in Figure S5a. Fraction of lysozyme that passed through the filter increased with decreasing of lysozyme concentration in dilution process.

**Effect of further dilution of aggregated lysozyme on recovery yield**

Aggregated lysozyme were prepared by 100 times diluting of 5 mM unfolded lysozyme by 100 mM phosphate buffer (pH 7.4). After 1 h pre-incubation at 25 °C, aggregated lysozyme was further 2-5 times diluted by 100 mM phosphate buffer (pH 7.4) and the solution was incubated for 5 h at 25 °C. The diluted solution was filtered by centrifugal ultrafiltration (MWCO: 30 kDa, 13.5 kG, for 15 min at 25 °C) (Nanosep® centrifugal devices, Pall Corp.). Effect of dilution ratio on recovery yield is shown in Figure S5b. Fraction of lysozyme that passed through the filter increased with further dilution of aggregated lysozyme.

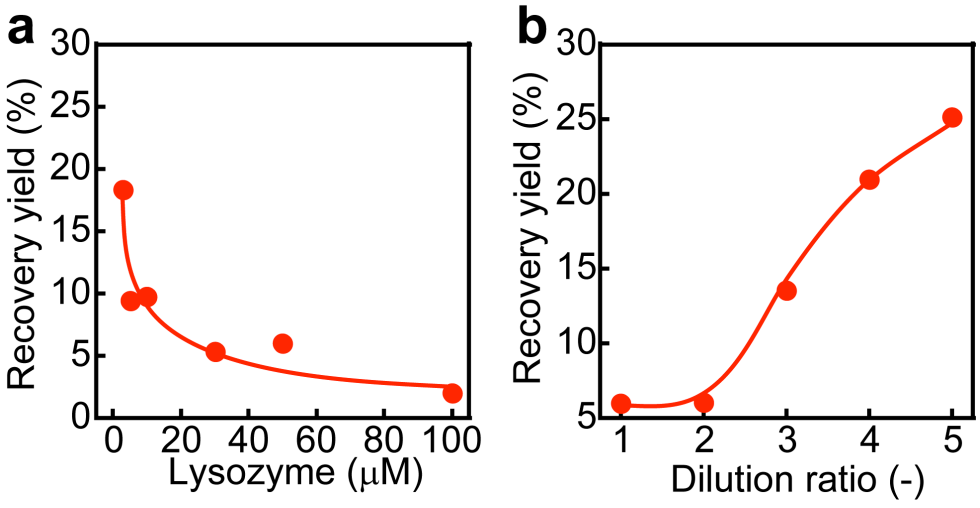


**Figure S5.** Recovery yield of lysozyme (**a**) after dilution of 0.3-10 mM of unfolded lysozyme as a function of final concentration of lysozyme in solution and (**b**) after dilution of 50 μM of aggregated lysozyme as a function of dilution ratio.

**Evaluation of NPs leakage amount in filtration process by utilizing fluorescence labelled NP11**

To evaluate leakage of NP in filtration experiment, fluorescence labelled NP (**NP 11**) was prepared (Table S1). **NP11** was added to 100 mM phosphate buffer (pH = 7.4) (final concentration was 50 μg/mL) and incubated for 1h at 25 °C After filtration, fluorescence intensity of solution that pass the filter was measured by using fluorescent spectrometer (JASCO, Tokyo, Japan) (excitation wavelength and emission wavelength were λ =500 nm and λ =520 nm, respectively.). Fluorescence intensity of filtered solution was less than that of **NP11** solution at 0.5 μg/mL. This result revealed that leakage of NP was less than 0.1% in filtration experiment (Figure S6).

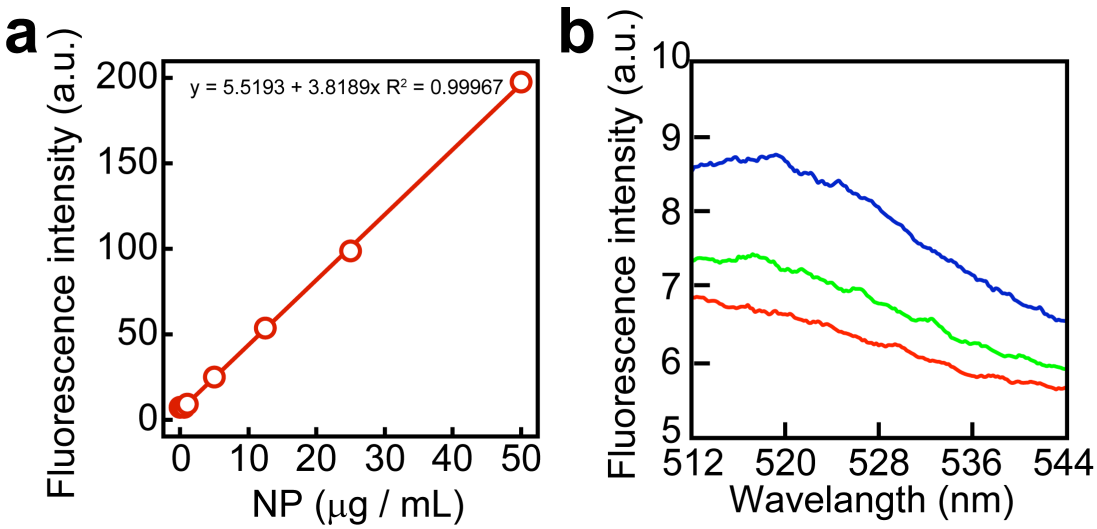


**Figure S6.** (**a**) Standard curve of fluorescence intensity as a function of **NP11** concentration. (**b**) Fluorescence intensity of **NP10**, concentration were 1 μg / mL (**blue**), 0.5 μg / mL (**green**) and fluorescence intensity of solution that passed filter (**red**).

**Confirmation of secondary structure of lysozyme re-solubilized by NP4**

CD spectra of lysozyme that resolubilized by **NP4** was monitored using a Jasco spectropolarimeter (JASCO J-725G, Jasco Ltd., Tokyo, Japan). A cuvette with 0.1 mm path length was used, and the photomultiplier voltage did not exceed 500 V in the measurements (Figure S7). Resolubilized lysozyme showed exactly the same spectrum as native lysozyme, indicating that the resolubilized lysozyme has native higher structure.

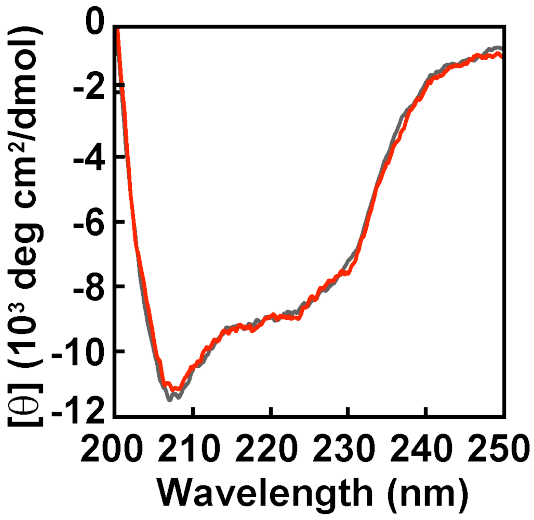


Figure. S7. CD spectrum of native lysozyme (**gray**) and resolubilized lysozyme by **NP4** (**red**).

**Evaluation of enzymatic activity of lysozyme re-solubilized by NP4**

The measurement of lysozyme activity was performed by the previously described method[19]. Freeze-dried *Micrococcus lysodeikticus* cells were resuspended at 200 μg/ml in 100 mM phosphate buffer (pH 7.4). Lysozyme solution (50 μL) was added to *Micrococcus lysodeikticus* cell suspension (1950 μL), and cell lysis was followed at 25°C by measuring the decrease in OD at 450 nm (OD450) with steering at 350 rpm using a UV-vis spectra were recorded using an Agilent 8453 (Agilent Technologies, USA) spectrophotometer. The decrease in OD450 for the first 1 minute was used as the measure of lysozyme activity. Relative enzymatic activity was defined as slope for first 1 min of sample divided by slope for first 1 min of 50 μM lysozyme. Relative activity of resolubilized lysozyme was 78±11%, showing almost same percentage with recovery yield of **NP4** (Figure S8).

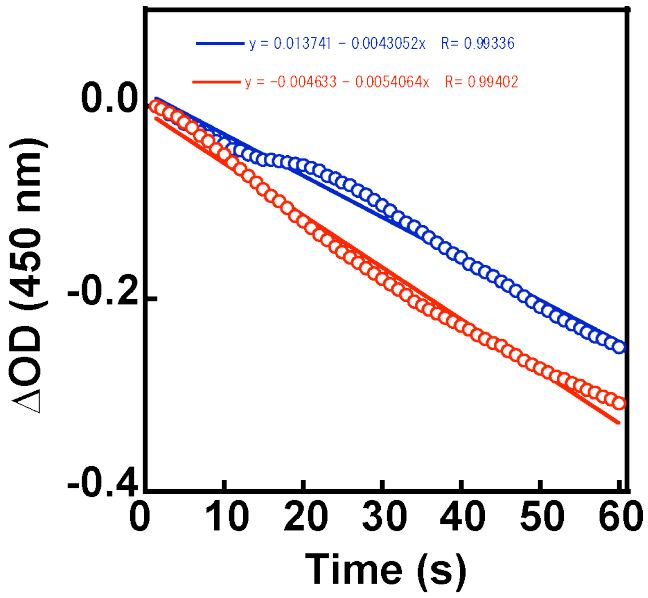


Figure. S8. Typical result of enzymatic activity measurement of resolubilized lysozyme (**blue**) and 50 μM of native lysozyme (**red**).

**QCM measurement**

A 27 MHz quartz crystal microbalance (QCM) system (Affinix Q4, Ulvac Inc.) was used to monitor and quantify interactions between the NPs and denatured/native lysozyme. All QCM experiment in this study were conducted at 25 °C.

**Immobilization of lysozyme on the surface of the QCM sensor**

Lysozyme was immobilized on the QCM electrode via an amine coupling procedure. Gold electrodes were cleaned twice with piranha solution (fresh mixture of $H_2O_2$ (aq) and $H_2SO_4$; 30% $H_2O_2$ (aq)/$H_2SO_4$ = 1:3 (v/v)) for 10 min. Next, 3,3'-Dithiodipropionic acid (0.2 mL, 1 mM in water) was loaded into the QCM cells and then incubated for more than 30 min (100 mM 3,3'-Dithiodipropionic acid was dissolved in ethanol and 100 times diluted by water.) . The resulting cells were washed with pure water and then the carboxylic acid groups on the gold surface were activated for 30 min by loading 0.1 mL of a 1:1 v/v aqueous solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (100 mg/mL) and *N*-hydroxysuccinimide (100 mg/mL) to form *N*-hydroxysuccinimidyl (NHS) esters. After rinsing the activated cells with water several times, 100 mM phosphate buffer (pH 7.4) was added to the cells and the cells were set on the QCM system. After stabilization of the oscillation frequency, lysozyme (concentration in the cell after injection: 50 μM) was injected on the activated QCM electrodes. Significant frequency change was not observed after the second injection of lysozyme (concentration in the cell after injection: 100 μM), indicating that lysozyme was immobilized only on the surface of the sensor and not on lysozyme.

**Immobilization of NPs on the QCM sensor**

After rinsing the lysozyme-immobilized cells to remove the non-immobilized lysozyme using the 100 mM phosphate buffer, the cells were filled with the buffer (500 μL) and set on the QCM system. After stabilization of the oscillation frequency, NPs (concentration in the cell after injection: 100 μg/mL) was repeatedly injected into the cells until saturation of the frequency change was achieved.

**Observation of denatured lysozyme binding on NPs on the surface of the gold sensor on QCM cells**

After rinsing the **NP**-immobilized cells to remove the non-immobilized NP by 100 mM phosphate buffer (pH 7.4), the cells were filled with phosphate buffer and set on the QCM system. Next, various concentrations of denatured lysozyme (concentration in the QCM cells: 10 to 500 nM) were injected (injected volume were uniformed at 5 μL) into the cells and the frequency change was monitored. Apparent association equilibrium constant ($K_a$) was estimated by assuming Langmuir typed binding.

**Observation of native lysozyme binding on NPs on the surface of the gold sensor on QCM cells**

After rinsing the NPs-immobilized cells to remove the non-immobilized NPs by 1% denaturation buffer containing 100 mM phosphate buffer (pH 7.4), the cells were filled with 1% denaturation buffer containing phosphate buffer and set on the QCM system. Next, various concentrations of native lysozyme (concentration in the QCM cells: 0.5 to 5 μM) were injected into the cells and the frequency change was monitored. Apparent association equilibrium constant ($K_a$) was estimated by assuming Langmuir typed binding.