Electronic Supporting Information for publication

A New Design of Ionic Complexations and Their Application for Efficient Protection of Proteins

Qian Yang[+], Jiaojiao Wu[+], Zhi Ping Xu and Daoyong Chen***

Materials used for the present study: Absolute ethanol, n-hexane, ether, NaCl, lysozyme (from hen egg white, lyophilized, Fluka), Micrococcus lysodeikticus (Fluka), Pepsin (from porcine gastric mucosa, Aldrich), Hemoglobin (from Bovine, TCI), trichloroacetic acid (Aldrich) and [2-(Acryloyloxy) ethyl] trimethylammonium chloride solution (80 wt. % in H_2O, Aldrich) were used as received. Acrylic acid (AA, Changshu Yangyuan Chemical Co, Ltd.) was distilled under vacuum before use. N-isopropyl acrylamide (NIPAM, 98%, TCI Development Co., Ltd.) and Azodiisobutyronitrile (AIBN, 98%, Aladdin Reagent Co.) were recrystallized three times from n-hexane and ethanol, respectively.

Experimental procedures and instruments for measuring the activities of proteins and properties of the mixtures

Thermal denaturation of lysozyme. Lysozyme concentration was fixed at 0.14 mg/mL in all samples for the activity measurements. To a 5 mL of aqueous lysozyme solution in a cuvette furnitured with a plug, the copolymer (PNA) was added directly to a final concentration of 1.0 mg/mL. The mixture solution was incubated at the lysozyme denaturation temperature 75 ^{o}C. Every 30 minutes after the incubation, a portion of the solution was taken out from the cuvette, cooled and stored in an ice bath. The residual enzymatic activity was measured at 25 ^{o}C (as described in the section "lysozyme activity measurement" below) and the measurement was completed in a short time period (less than 20 minutes), so that the activity recovery that was found for lysozyme during its long time storing at room temperature is ignorable.

Lysozyme activity measurement. The enzymatic activities of lysozyme were measured on the basis of the bacteriolysis reaction with Micrococcus lysodeikticus. The aqueous suspension of the substrate at 0.24 mg/mL was freshly prepared just before use by adding Micrococcus lysodeikticus into 60 mM sodium phosphate buffer (PBS) at pH 6.2 and 25 ^{o}C. Immediately, a 50 μL aliquot of the lysozyme solution (at 0.14 mg/mL) without or with a copolymer (at 1.0 mg/mL) was added into 2 mL of the substrate suspension, and the resultant mixture was promptly put into turbidity measurements. For each system, the decrease of turbidity was monitored at 450 nm for 30 s using a UV-Vis spectrophotometer (UV-2550, Shimadzu Co., Japan) at 25^{o}C. The absorbance decay plots from 5 to 15 s were fitted to a linear equation, and the enzymatic activities were determined from the slope of the fitted line. The relative

activity was normalized to the activity of the pristine lysozyme in neutral water at 25 ^{o}C.

Thermal denaturation of pepsin. To a 5 mL of the aqueous solution of pepsin in a cuvette furnitured with a plug, the copolymer (PND) was added directly to a final concentration of 0.5 mg/mL. The mixture solution was incubated at 70^{o}C for 1 h, then cooled and stored in an ice bath.

Pepsin activity measurement. The relative activities of pepsin in water and in the respective copolymers were determined by the amount of hydrolysis of denatured hemoglobin solution produced by the enzyme after incubation for 10 min at 37 °C [1]. Typically, a denatured hemoglobin solution (pH~2.5) was prepared by dissolving 0.25 g bovine hemoglobin in 100 ml of 10 mM HCl solution. The enzyme solution (0.1 mg/mL) was prepared by dissolving 10 mg of as received pepsin in 100 mL deionized water or 0.5 mg/mL respective copolymers solutions. The assay reaction was started by the addition of 0.1 mL of enzyme solution in 2.9 mL denatured hemoglobin solution and kept the temperature at 37 °C for 10 min, and the reaction was stopped by the addition of 1 mL of TCA (7%, w/v). The resultant mixtures were then centrifuged. The pepsin activities were determined from the corresponding increase in the UV absorbance of the supernatant at 280 nm.

CD spectra measurement. The samples after the thermal denaturation treatment were diluted 7 folds and then subjected to circular dichroism (CD) measurements, using a Jasco J-715 Circular Dichroism Spectropolarimeter and a cuvette of 0.1 cm path length.

Turbidity measurement. Three miligrams of a copolymer was dissolved in 3 mL of either pure water or protein solution at room temperature in a glass cell of 2 mm path length. The transmittance of the solution was monitored at 500 nm via a UV-Vis spectrophotometer (UV-2550, Shimadzu Co., Japan) equipped with a temperature controller. Each transmittance was obtained after sufficient equilibration of the solution at that temperature.

Zeta potential measurement. Zeta potentials of respective copolymer solutions at different temperatures were obtained with Zetasizer Nano from Malvern Instruments, England, equipped with a clear disposable zeta cell. In each solution, the concentrations of the copolymer and NaCl were 1.0 mg/mL and 5 μmol/mL, respectively. Each zeta potential was recorded after sufficient equilibration of the solution at that temperature.

S1. Preparation of poly (N-isopropyl acrylamide (NIPAM)-co-acrylic acid (AA)) (PNAs) and poly (NIPAM-co-acryloyloxyethyltrimethyl ammonium chloride (DAC)) (PNDs)

The PNAs and PNDs were synthesized via radical copolymerizations of N-isopropyl acrylamide (NIPAM) with acrylic acid (**AA**) and **NIPAM** with acryloyloxyethyltrimethyl ammonium chloride (**DAC**) in absolute ethanol, respectively. Both copolymerizations were initiated by AIBN. In a typical copolymerizaion for PNA preparation, 1.315 g (10 mmol) NIPAM, 0.686 mL (10

mmol) AA and 0.0164 g (0.01 mmol) AIBN were mixed in 50 mL absolute ethanol in a three-neck round-bottom flask, which was subsequently purged with Ar gas to eliminate residual O_2 in the system. Then, the flask was sealed and the polymerization was conducted at 65 ^oC for 18 h. We have found that 18 hours was long enough for complete polymerization (100%). Other PNAs were prepared by changing the molar ratio of NIPAM:AA in the feed mixture. PNDs were prepared similarly under the same conditions except that the AA was replaced by DAC.

S2. Explaining advantages of the 100% conversion method to prepare the copolymers at very high molar factions of NIPAM (MFs)

It is confirmed that LCCT complexation and the smart protection can be obtained only when the molar fractions of NIPAM (MFs) are very high (close to 100%). For example, in the system of PNA/lysozyme, the MF has to be as high as 99.5% to realize LCCT complexation and the smart protection of lysozyme; in the PND/pepsin system, the very high MF (99.9%) is required for LCCT complexation and the smart protection. In the present study, the copolymer at a very high MF was prepared by polymerizing the monomer mixture to 100% conversion. Therefore, the MF in the copolymer was equal to the molar fraction of NIPAM in the feed monomer mixture. We have confirmed that the 100% conversion method can be well repeated in preparation of copolymers, LCCT ionic complexation and the smart protections.

As the average molecular weight of the copolymers used in the present study were 2300-5800, so the degree of polymerization was about 30 on average and the as-prepared copolymer at a very high MF should contain a large fraction of PNIPAM homopolymer. For example, in the case of PND with MF of 99.9%, there was about one DAC unit in every 33 polymer chains (i.e., 1 DAC unit in 999 NIPAM units). *As result, in the sample prepared using the 100% conversion method, the fraction of the polymer chains containing multiple DAC units in each chain should be extremely low.* This is significant because the polymer chains containing multiple interacting units in each chain may form the stable complexes with the proteins that will not decomplex at 25 ^oC after the heating treatment (Table 1), and thus decrease the activity remarkably (Figure 2A). This is because multiple interacting units in the same polymer chain may considerably enhance the stability of its complexation with protein. It is well known that the A-A/D-D complex is much more stable than the A/D complex (A-A or D-D represents the molecule with two interacting units, while D and A denotes the molecule with only one interacting unit; D and A can interact noncovalently with each other).

Therefore, at a very high MF and a high temperature the micelle particles interacting with the protein are composed of a large amount of PNIPAM homopolymers and a small amount of the AA or DAC functionalized PNIPAM chains. At 25^oC after the heating treatment, the particles dissociate into individual polymer chains. Since single mono-functionalized PNIPAM chain cannot form stable complex with the protein, the protected protein is automatically released out.

S3. Determination of LCSTs for the copolymers

The lower critical solution temperatures (LCSTs) of the copolymers were determined by transmittance measurements using a UV-Vis spectrophotometer (UV-2550, Shimadzu Co., Japan) equipped with a temperature controller. Typically, copolymer aqueous solution at 1.0 mg/mL was poured into a 1.0 cm path length cuvette at 25 ^{o}C, and then the transmittance of solution at different temperatures were measured at 500 nm (the heating rate is 0.2 ^{o}C/min, and the temperature range for the measurements was 25-60 ^{o}C). According to our repeated experiments, PNA with MF $\geq$ 45.8% or PND with MF $\geq$ 99% had a distinct LCST below 40 ^{o}C.

S4. Reliability of the transmittance method for detecting the complexation and decomplexation:

Complexation among macromolecules certainly leads to the complexes with a much larger mass. Except that the concentration is extremely low, the complexation between two macromolecules will greatly increase the scattering intensity and remarkably decrease the transmittance. The scattering intensity is proportional to the square of the complex mass.

S5. The relative activities of lysozyme after different heating/cooling cycles:

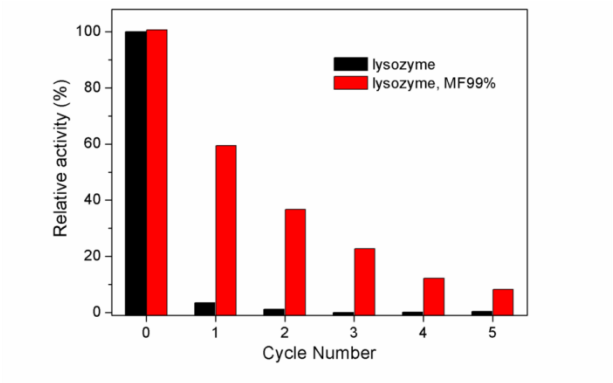

Figure S1. Relative activity of lysozyme at 25 ^{o}C of unprotected (black) and protected lysozyme with PNA with MF of 99% (red) after different heating/cooling cycles. The concentration of lysozyme and PND in the system was 0.14 and 1.0 mg/mL, respectively. In a heating-cooling cycle, *the sample was heated at 75 ^{o}C for 60 min* and then cooled in an ice bath for 20 min. The lysozyme activity was measured at 25 ^{o}C. In general, about 60-70% activity was maintained after each cycle in the protected cases while less than 5% was found in the unprotected cases after each cycle.

S6. Explaining why the ionic complexation with MF of 99.0% is not a true LCCT complexation:

In our repeated experiments, even using PNAs of different synthesis batches, the relative activity of lysozyme at MF of 99.5% is remarkably larger than that at MF of 99.0% (Figure 1a in the main text). As mentioned before, the MFs of NIPAM in

PNAs are the molar fractions of NIPAM in the feed monomer mixtures. Note that the AA/NIPAM molar ratio in the feed for preparing the copolymer at MF of 99.0% is two times that in the feed for preparing the copolymer at 99.5%. It is known that a random copolymer prepared by a radical copolymerization is polydisperse in both the chain composition and the molecular weight. Therefore, the possibility that there are some copolymer chains with multiple interacting groups in the copolymer at MF of 99.0% is remarkably higher than that in the copolymer at MF of 99.5%. We believe that, at MF of 99.0%, some of the copolymer chains with multiple interacting groups still adhere to lysozyme. The adhering has little effect on the transmittance (since the transmittance of the lysozyme/PNA system with MF of 99% after cooling to 25 ^{o}C is 100% (Table 1 in the main text)), but prohibits the contact between lysozyme and the large substrate and thus decrease the relative activity. This is confirmed by the fact that the relative activity at 99% is remarkably increased after adjusting the pH to 2.0 (see also the main text).

S7. More description of the relative activities shown in Figure 2a.

The relative activities of lysozyme in water and in PNA solutions before (RABs) and after (RAAs) heat treatment at 75 ^{o}C for 90 min were measured at 25 ^{o}C using Micrococcus lysodeikticus as the substrate (Figure 2a). In the cases with MFs of 9.1% and 22.0%, RABs decreased remarkably to 9% and 42%, respectively, due to strong PNA-protein interactions. However, when MFs are larger than 67.1%, RABs are higher than 95%. The RABs at different MFs suggest that PNAs with MFs $\leq$ 22% strongly complex with while those with MFs $\geq$ 67.1% have no remarkable interaction with protein at 25 ^{o}C. The strong copolymer/lysozyme interaction inhibits the contact between lysozyme and the substrate, thus decreasing the activity. PNA with MF of 45.8% has a certain interaction with lysozyme, leading to the small decrease in RAB to 90%. The protection capability of a copolymer was evaluated by lysozyme RAA with the copolymer. As indicated in Figure 1A, RAA of pure lysozyme was 1.3%, and that protected with PNA with MFs of 9.1%, 22.0%, 67.1%, 81.3%, and 99.9% were all less than 13%. Differently, RAAs protected with MFs of 45.8%, 99.0% and 99.5% were higher than 40% (Figure 1a). In particular, the RAA was 71% with MF of 99.5%, among the highest activities maintained through protection of existing methods. It is more significant that the existence of PNA with MF of 99.5% does not affect the lysozyme activity so that repeat protection can be conducted very conveniently. PNA with MFs of 67.1% and 81.3% precipitated with proteins at 75 ^{o}C and the precipitates was remained at 25 ^{o}C, reducing the RAAs in these two cases. PNA with MF of 99.9% has too few interacting groups to complex with lysozyme to protect the protein.

References

[1] H. Bisswanger, *Practical enzymology* **2004**, 140–141.