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A New Design of Ionic Complexation and the Application for Efficient Protection of Proteins

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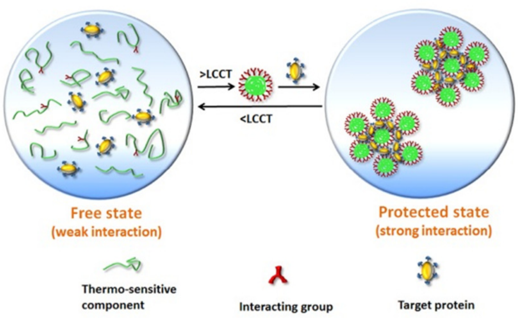
Ionic complexation is one of the most important topics in the fields of biology, physics, chemistry, and material sciences. The ionic complex normally has an upper critical complexation temperature (UCCT), i.e. the ionic complex disappears above UCCT. Herein we have for the first time demonstrated that a new ionic complexation, in contrast to the UCCT one, has a lower critical complexation temperature (LCCT), which means that the ionic complex exists above LCCT but disappears below LCCT. We have further shown that the LCCT ionic complexation can efficiently protect proteins at the denature temperature but automatically release proteins at room temperature to freely interact with the substrates. For example, 70-80% enzymatic activity was retained after heating at 70-75 °C for 60-90 min and cooling to room temperature using this strategy. Thus this new LCCT ionic complexation would provide a cost-effective approach to protecting proteins for various biomedical industries.

Ionic complexation has been extensively investigated in fields of biology, physics, chemistry and material science, as this complexation is closely related to ionic-recognition,[1] controlled drug release,[2, 3] and many biological processes.[4-6] Moreover, ionic complexation and decomplexation are essential to many important industrial applications, such as separation, adsorption and dispersion of functional species.[7-10] Intrinsically, an ionic complexation has an upper critical complexation temperature (UCCT), above which the ionic complexes decomplex into the molecularly soluble state. In this particular research, we have designed and attained a new ionic complexation between proteins and copolymers with a lower critical complexation temperature (LCCT). This ionic complex, in contrast to the UCCT one, exists at high temperatures as an assembling, but disappears/dissolves at relatively low temperatures.

The purpose of designing the novel LCCT ionic complexation is to investigate whether this complexation protects proteins cost-effectively at the temperatures at which proteins normally undergo an irreversible conformational change and lose their bioactivities.[11] Since proteins participate in most vital biological processes and have important applications in medical sciences and biotechnologies, their protection at high temperatures is important both clinically and economically. To date various protection strategies have thus been proposed to prevent proteins from denaturation at high temperatures, such as limiting protein molecules within the highly confined spaces,[12-15] and wrapping individual protein molecules through covalent[16] or noncovalent interactions with protecting matrices.[16-19] In such ways, proteins are protected through limiting their irreversible conformational changes at the denaturation temperature. However, the protecting material if co-existing in these systems severely affects the protein bioactivity after cooling to room temperature. This concern thus necessitates the removal of this protecting material. The removal process is often costly and tedious, and more complicated when the protein needs to be repeatedly protected. Therefore, we have demonstrated in this particular research that the new LCCT complexation between proteins and copolymers can efficiently protect the proteins within the well-designed polymer matrices at high temperatures, and the protected proteins are readily bioavailable after cooling to room temperature, without removing the protecting material.



Scheme 1. The schematic description of the LCCT complexation between a copolymer composed of interacting monomers and thermo-sensitive components with a counterpart protein and the subsequent smart protection of the protein.

The newly designed copolymers are mainly composed of thermo-sensitive units with a very small portion of interacting units (Scheme 1), with a lower critical solution temperature (LCST) of 30-40 ℃. The interacting unit is a charged monomer with its molar fraction being carefully controlled, and specially introduced in this system in order to interact with proteins via electrostatic interactions. The copolymer is molecularly solubilized in water at room temperature, and is thus unable to complex with the proteins because the interacting units are very sparsely distributed along the copolymer chains (usually there is one charged interacting group in several polymer chains, as discussed below). Once the temperature rises, the copolymer self-assembles into micelles with the charged interacting groups being lined up on their surface, and thus the density of interacting groups on the micelle surface is remarkably increased. Therefore the self-assembled micelles are able to form complexes with the protein and protect it at higher temperatures. Nonetheless, once the temperature decreases to 25 °C, the thermo-sensitive copolymer micelles are dissolved and dissociated, breaking the complex (e.g. decomplexation) and releasing the protein. The transition temperature is thus called lower critical complexation temperature (LCCT). This research has found that the released protein showed the activity similar to its natural one even in the presence of the thermo-sensitive copolymer, which enables the cost-effective and repeated protection of proteins.

In this study, the negatively charged copolymer poly(N-isopropyl acrylamide (NIPAM)-co-acrylic acid (AA)) (**PNAs**) and the positively charged copolymer poly(NIPAM-co-acryloyloxyethyltrimethyl ammonium chloride (DAC)) (**PNDs**) were synthesized via radical copolymerization of NIPAM with AA and DAC, respectively.[20, 21] The molar fraction of NIPAM (MFs) was varied from 9.1% to 99.5% in PNAs and from 93% to 99.99% in PNDs, respectively (electronic supporting information (ESI) **S1**). Since copolymerization of NIPAM with AA and DAC was nearly 100% under the current conditions, the MF in the copolymer was equal to that in the feed mixture, which is the most convenient and efficient way to prepare the copolymers to attain LCCT complexation and smart protection of proteins with good repeatability (ESI **S2**). The molecular weight of as-prepared copolymers in this study was 2,300~5,800, e.g. consisting of 20-50 monomeric units. The low molecular weight copolymers were particularly prepared in this research to ensure the quick response to the temperature change. We observed that PNA with MF ≥ 45.8% and PND with MF ≥ 99.0% had a distinct LCST below 40 °C (ESI **S3**). A positively charged protein hen egg white lysozyme (PI = 11)[19] and a negatively charged protein pepsin (from porcine gastric mucosa, and PI = 1) were selected to complex with negatively charged PNAs and positively charged PNDs, respectively, to demonstrate the LCCT complexation and the smart protection of these two proteins.

The temperature-dependent complexation between the copolymer and the protein in neutral water was first examined by transmittance measurement, which is widely used to monitor complexation and decomplexation between macromolecules (ESI **S4**).[22-24] Transmittance readings of PNA/lysozyme and PND/pepsin systems were recorded during a heating/cooling cycle from 25 to 75 and then to 25 °C, as listed in Table 1.

The transmittance change of PNA/lysozyme system (Table 1) indicates that there was neither copolymer self-assembling nor complexation of PNA (MF ≥ 67.1%) with lysozyme at 25 °C. When the PNA solution was heated to 75 °C, the PNA copolymers (MF ≥ 45.8%) self-assembled into micelles, reducing the transmittance to 69-34%. In a striking contrast, the transmittance of PNA/lysozyme system (MF ≥ 45.8%) was reduced to only a few percentages at 75 °C, indicating that there was an ionic complexation between charged PNA micelles and lysozyme molecules. More interestingly, the complexation in the cases of MF = 99.0% and 99.5% was reversible as the transmittance changed back to 100% when the temperature was back to 25 °C, which is thus regarded as the LCCT complexation. In comparison, the complexation in the cases of MF = 67.1% and 81.3% was partially reversible as the transmittance was only 80-90% after cooling to 25 °C, which is thus not regarded as the LCCT complexation because there are still some PNA/lysozyme complexes existing after cooling. Note that the ionic complexation (or even precipitation) took place at room temperature in the PNA/lysozyme system with MF ≤ 45.8% even before heating (Table 1). This is because the charge density along the PNA chain is sufficiently high for its complexation with lysozyme to precipitate, similar to the normal UCCT ionic complexation.

Table 1. Transmittance (%) of the copolymer solutions (T_P) and the mixed solutions of copolymer/protein (T_{Mix}).

Sample	25 °C Before heating		75 °C		25 °C After heating	
	T_P	T_{Mix}	T_P	T_{Mix}	T_P	T_{Mix}
PNA						
MF9.1%	100	--[a]	100	--[a]	100	--[a]
MF22.0%	100	--[a]	100	--[a]	100	--[a]
MF45.8%	100	44	69	14	100	43
MF67.1%	100	100	42	2[a]	100	92[a]
MF81.3%	100	100	39	2[a]	100	85[a]
MF99.0%	100	100	36	5	100	100
MF99.5%	100	100	34	1	100	100
PND						
MF93.0%	100	100	99	93	100	98
MF99.0%	100	100	36	15	100	89
MF99.5%	100	100	16	3	100	91
MF99.7%	100	100	11	3	100	95
MF99.9%	100	100	26	19	100	100
MF99.99%	100	100	14	14	100	100

[a]Precipitates formed in the solutions. The transmittances of aqueous lysozyme (0.14 mg/mL) solution and aqueous pepsin (0.1 mg/mL) solution at both the temperatures were set 100%.

The LCCT complexation in the PND/pepsin system occurred only in the case of MF = 99.9%. In this special case, the ionic complexation did not occur at 25 °C, but took place upon heating to 75 °C, and fully disappeared when the system was cooled to 25 °C (Table 1). At MF < 99.9%, the complexation was also heating-enhanced but only partially reversible as the transmittances after cooling were less than 100% (Table 1). Very particularly, the transmittance of the PND/pepsin system was the same as that of the copolymer solution at 75 °C with MF = 99.99%, revealing that no PND/pepsin complexation occurred in this system. This is because the number of the interacting units is too few to form a stable PND/pepsin ionic complex even though the interaction is enhanced at the high temperature.

The temperature-dependent zeta potential of copolymer solutions suggests the mechanism for the LCCT ionic complexation. The zeta potential of the PNA solution (Fig. 1a) was remarkably dependent on the temperature. When the temperature was below ~35 °C, the copolymers were all molecularly solubilized and the absolute value of zeta potentials was relatively low and unchanged. At temperatures above ~35 °C, the copolymer chains assembled into micelles, and the charged COO^- groups were concentrated on the surface, resulting in a much more negative zeta potential, particularly in the case of MF = 99.0% and 99.5%. The temperature-dependence of the zeta potential confirms that it is the concentrated COO^- groups that complex with lysozyme to form ionic complexation at the higher temperature, as further explained below.

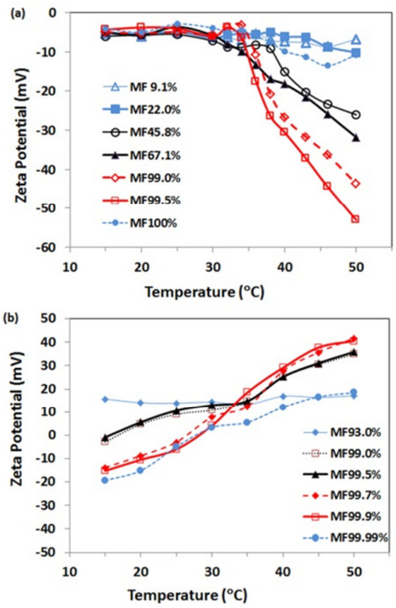


Fig. 1. Temperature-dependent zeta potentials of the PNAs (a) and PNDs (b) in solution with the concentration of 1.0 mg/mL.

At lower temperatures and MF ≥ 67.1%, PNA is molecularly solubilized, and cannot form any stable ionic complexes with lysozyme. When the temperature rises to over ~35 °C, thermo-sensitive PNA self-assembles into micelles, during which COO- groups are concentrated on the micelle surface so as to complex with lysozyme. Note that the LCCT ionic complexation only occurs with MF = 99.0% and 99.5%, largely attributed to the effect of concentrated interacting groups (-COO^- groups) on the surface. Suppose each PNA molecule has 30 monomer units on average, each PNA molecule thus carries 0.15 negatively charged –COO^- groups if MF = 99.5%. This means that there is only one –COO^- group in 6-7 PNA chains (Scheme 1). Therefore, these PNA chains self-assemble into closely packed micelles upon heating in such a way that most –COO^- groups are lined up on the micelle surface, leading to a high surface charge density and ionically complexing with proteins. Cooling down de-assembles the micelles, decomplexes the ionic PNA/lysozyme complexes, and releases the protein. In contrast, if MF = 80%, each PNA molecule has about six negatively charged –COO^- groups, which cannot be all arranged on the micelle surface. Therefore, inclusion of –COO^- groups within micelles substantially affects the self-assembling upon heating, leading to loosely packed micelles with a lower density of –COO^- groups on the surface, which is not sufficient for ionic complexation. This reasoning is supported by the fact that the zeta potential of PNA solutions with MF = 99.5% and 99.0% is more negative at temperatures above 40 °C (Fig. 1a).

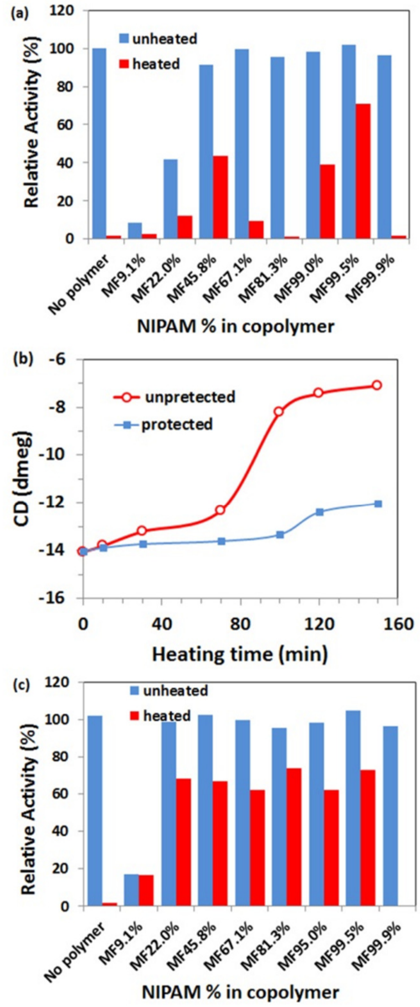


Fig. 2. (a) The relative lysozyme activity at 25 °C in water and in PNA mixtures before and after heating at 75 °C for 90 min. (b) The change of ellipticity at 222 nm in CD spectra of unprotected and protected lysozyme (PNA with MF = 99.5%) during heating at 75 °C. (c) The relative lysozyme activities measured after adjusting pH of unheated and heated solutions to 2.0. Lysozyme concentration: 0.14 mg/mL; PNA concentration: 1.0 mg/mL in the mixture. The pH adjustment was conducted at 25 °C after heating treatment at 75 °C for 90 min.

Similarly, the temperature dependence of the zeta potential of the PND solution with MF ≥ 93% (Fig. 1b) also accounts for the heating-enhanced complexation between PND and pepsin. PND chains assemble into micelles upon heating and thus the positively charged $–NH_3^+$ groups are concentrated on the surface, which allows complexation with negatively charged pepsin. Note that the zeta potential of PND with MF of 99.99% was only 15 mV at 50 °C, which seems too low for PND to ionicially complex with pepsin, consistent with the observation via monitoring the transmittance (Table 1).

Such an LCCT ionic complexation can efficiently protect proteins upon heating. In this test, we firstly measured the activity of lysozyme in neutral water using *Micrococcus lysodeikticus* as the substrate and regarded it as 100%. Then we determined the relative activity of lysozyme in the PNA solution before and after heating at 75 °C for 90 min. As shown in Fig. 2a, the relative activity of lysozyme in neutral water after heating treatment was only 1.3%. In sharp contrast, the relative activity with PNA protection was up to 71.0% after heating, depending on the MF value (Fig. 2a). The highest activity retention after heating treatment was achieved with MF of 99.5%, which is a result from the LCCT nature of the lysozyme/PNA complexes and the protection by the copolymer micelles. Since the activity was measured directly without removing PNA, this protein protection strategy is more cost-effective and convenient.

The lysozyme protection by PNA copolymer micelles with MF of 99.5% has also been confirmed by circular dicroism (CD) spectra after heat treatment at 75 °C for up to 150 min (Fig. 2b). Note that the ellipticity at 222 nm is proportional to the denaturation fraction of lysozyme in as-heated samples.[25] As shown in Fig. 2b, the ellipticity of both protected and unprotected lysozyme before heating was the same (-14 dmeg). Clearly, the ellipticity of unprotected lysozyme increased to -8 dmeg after heating at 75 °C for 150 min, suggesting collapse of the α-helix and denaturation of lysozyme.[25] However, the ellipticity of lysozyme in the PNA mixture with MF of 99.5% just slightly increased to -13 dmeg upon the same heat-treatment, demonstrating that lysozyme is well protected by PNA during heating. Furthermore, lysozyme can be repeatedly protected by PNA through multiple heating/cooling cycles, with about 60% activity retained after each cycle (ESI **S5**).

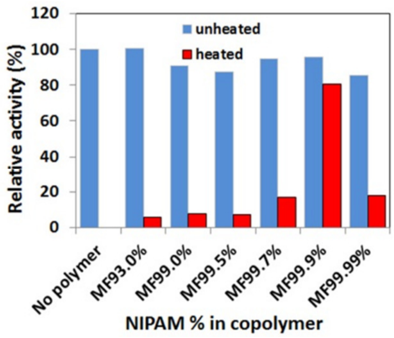


Fig. 3. The relative activity of pepsin at 25 °C in water and in PND solutions before and after heating at 70 °C for 60 min.

As can be also seen in Fig. 2a, the relative activity of lysozyme in the other PNA mixtures after heating treatment was considerably lower. Very interestingly, the relative activity in these PNA mixtures after heating treatment could be recovered to 60-70% when the pH of heated PNA mixtures was adjusted to 2.0. This observation suggests that lysozyme was actually protected in the PNA mixture efficiently with MF of 22%-99% at higher temperature, but not bioavailable at 25 °C. This is because the PNA-protein complexation is ionic, e.g. between COO- groups of PNAs and positively charged lysozyme. Since adjusting pH to 2.0 protonates all COO- groups to COOH, the ionic interactions are largely weakened and then the protected lysozyme released for biological action. Obviously, these ionic complexations are not LCCT type. Only the PNA/lysozyme complexation at MF of 99.5% is a true LCCT complexation (ESI **S6**) as the relative activity of heated lysozyme was not affected by pH adjustment (71.0%, Fig. 2c). Some more descriptions and explanations for the activity issue are included in ESI **S7**.

It is known that many proteins are negatively charged. One example is pepsin, which is selected as another model to complex with the positively charged PND. The protection efficiency of pepsin by PNDs was also evaluated by measuring its relative activity before and after heating treatment at 70 °C for 60 min, using haemoglobin as the substrate. The activity of heated samples was also directly measured without removing the copolymer. As exhibited in Fig. 3, the efficient protection was achieved with MF of 99.9% (ESI **S2**) as the activity after heat-treatment was as high as 80%. However, pepsin protection in all other cases was not efficient, with the relative activity being only 10-20%. This sharp contrast has again demonstrated that the efficient protection of pepsin requires a real LCCT complexation between PND and pepsin, just because only LCCT complexation can largely restrict the pepsin conformation change at 70 °C and fully release pepsin at 25 °C. The efficient protection of pepsin by PND at MF of 99.9% has also been confirmed by the ellipticity change in 210–220 nm in CD spectra of protected and unprotected pepsin upon heating at 70 ℃ for 180 min (data not shown).

Conclusions

In conclusion, we have designed particular thermo-sensitive copolymers that can form the LCCT ionic complexation with proteins. The LCCT ionic complex just exists at a temperature higher than LCCT, on the contrary to the conventional ionic complex that normally has a UCCT. We have further demonstrated that when the ionic complexation between the protein and copolymer micelles is a real LCCT one, the protein can be well protected at higher temperatures, and fully released at room temperature with the activity being not substantially affected by the copolymer presence. This new concept and the new protection strategy would provide a more efficient and cost-effective approach to protecting proteins for various biomedical industries.

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Notes and references

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† Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.
Electronic Supplementary Information (ESI) available: Experimental details, the characterization of P(NIPAM-co-AA)s, the repeated protection and release of lysozyme using the copolymer in several heating/cooling cycles between 25 and 75 °C and some explanations are included in supporting information. See DOI: 10.1039/c000000x/

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