Hydrogel Nanoparticles

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Polymer Nanoparticle Hydrogels with Autonomous Affinity Switching for the Protection of Proteins from Thermal Stress**

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Abstract: *We report a new material design concept for synthetic, thermally responsive poly(N-isopropylacrylamide)-based copolymer nanoparticle (NP) hydrogels, which protect proteins from thermal stress. The NP hydrogels bind and protect a target enzyme from irreversible activity loss upon exposure to heat but "autonomously" release the enzyme upon subsequent cooling of the solution. Incorporation of the optimized amount of negatively charged and hydrophobic comonomers to the NP hydrogels was key to achieve these desired functions. As the NP hydrogels do not show a strong affinity for the enzyme at room temperature, they can remain in solution without adversely affecting enzymatic activity or they can be removed by filtration to leave the enzyme in solution. The results demonstrate the promise of this approach for improving the thermal tolerance of proteins.*

The exposure of a protein solution to heat induces the transition of a folded protein to an extended, unfolded state. When the solution is cooled, some of the proteins may refold back into their native folded state, but often heating results in a loss of function, which is in part related to misfolding or aggregation of the unfolded protein chains.[1] The limited thermostability of proteins restricts the use of many protein-based reagents and drugs in locations where temperature control is not available. To date the addition of excipients[2,3] (e.g. trehalose,[4] sucrose,[5] amino acids,[6] surfactants,[7] and polymers[2,8]), encapsulation/immobilization in matrixes,[9] covalent conjugation with polymers,[10] and noncovalent complexation with inorganic[11] and organic polymer-based materials[12–15] have been reported for the protection of proteins from thermal stress. Nevertheless, each method possesses both advantages and disadvantages; thus the development of efficient and convenient protein stabilization methods remains a formidable challenge.

Among a number of approaches, the noncovalent complexation of proteins with synthetic or naturally occurring polymer-based materials[12–15] offers a simple and cost-effective way to prevent protein aggregation upon exposure to heat. Specifically, thermally responsive materials including modified polysaccharides,[13] poly(vinyl alcohol),[14] and poly(*N*-isopropylacrylamide)[15] (pNiPAm)-based polymers are of significant interest because their responsive property can be utilized for the recovery of active proteins without secondary additives. However, the previously reported materials rely on the denaturation and subsequent apparent increase in the hydrophobicity of the proteins for triggering the protein–polymer complexation.[13–15] Instead, it is more desirable if the material itself alters the affinity for target proteins in response to temperature. Herein we describe a synthetic polymer-based nanoparticle (NP) hydrogel capable of "autonomously" altering its intrinsic protein affinity at a targeted temperature below the melting temperature (T_m) of the protein and demonstrate the utility of the approach in protection of a target protein from heat.

To achieve our goal, a material must be capable of 1) binding to a protein through noncovalent interactions and protecting it from aggregation at high temperature, 2) releasing the protein at room temperature, and 3) altering its intrinsic protein affinity without relying on protein denaturation. To this end, we focused on pNiPAm-based NP hydro-

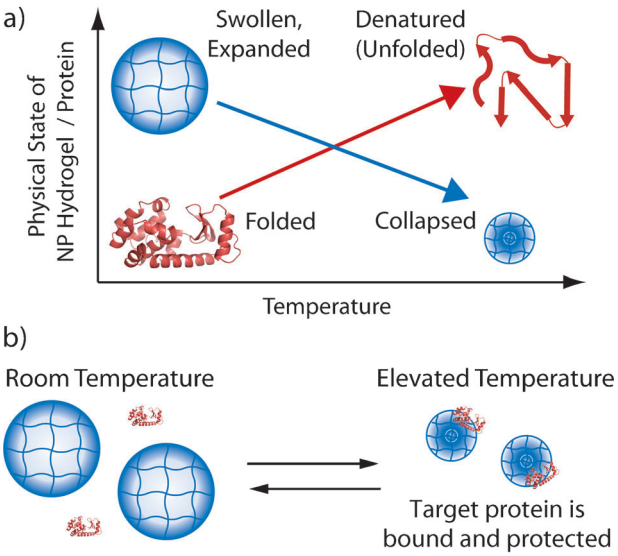

Figure 1. a) Comparison of the heat response of a protein (red) and a *N*-isopropylacrylamide (NiPAm)-based polymer NP hydrogel (blue). Proteins denature (unfold) in response to heat, whereas the NP hydrogels contract into a collapsed state. b) Illustration of the "autonomous affinity switching" property of NP hydrogels.

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gels[16] exhibiting a thermally responsive behavior that is orthogonal to proteins (Figure 1a). Heating these NP hydrogels results in a volume phase transition from an expanded, swollen state to a collapsed state above its lower critical solution temperature (LCST) due to an entropically driven dehydration of the polymer chains.[17,18] The collapsed NP hydrogels have been reported to show higher affinity for a target protein due to increased hydrophobicity and functional group density.[19] Based on these insights, we set out to synthesize and characterize NP hydrogels with the desired combination of properties.

All NP hydrogels were synthesized by pseudo-precipitation polymerization[20,21] of *N*-isopropylacrylamide (NiPAm, **1**) with comonomers (Figure 2a). Table 1 summarizes the monomer feed ratio and hydrodynamic diameter of each NP hydrogel. The feed ratio of hydrophobic *N*-*tert*-butylacrylamide (tBAm, **2**) comonomer was varied to tune both the LCST[17,21] and the protein affinity.[19,22,23] The negatively charged acrylic acid (AAc, **3**) comonomer was incorporated at a feed ratio of 5 mol% to enhance the protein affinity in the collapsed state.[19,24] Weak crosslinking by 2 mol% *N*,*N*′-methylenebisacrylamide (Bis, **4**) was employed to allow for relatively large changes in the size of the NP hydrogels upon phase transition.[25] Solution NMR spectra verified that the tBAm/NiPAm ratio in the NPs corresponded to the monomer feed ratios (Figures S1 and S2). Zeta potential measurements confirmed the incorporation of AAc into the NP hydrogels (Table S1). The LCST of **NP1**–**NP4** was determined to be 51.5 °C, 42.0 °C, 33.5 °C, and 18.5 °C by differential scanning calorimetry (DSC) in 35 mM sodium phosphate buffer (pH 7.3) (Figure S3). The LCST of NP hydrogels decreased as the hydrophobic tBAm monomer content increased, which is in good agreement with previous studies.[17,21] Dynamic light scattering (DLS) measurements confirmed that the size of the NP hydrogels decreased (by 1.8- to 2.5-fold) when the solutions were heated above the LCST (Figure 2b).

Table 1: Monomer composition and hydrodynamic diameter of NPs.

Sample	Monomer composition[a] [mol%]				Hydrodynamic diameter[b] [nm]	
	tBAm	AAc	NiPAm	BIS	in H_2O	in buffer[c]
NP1	0	5	93	2	625	550
NP2	10	5	83	2	353	332
NP3	20	5	73	2	95	242
NP4	40	5	53	2	93	132
NP5	0	0	98	2	329	330

[a] Feed ratios. [b] Measured with dynamic light scattering at 25 °C. [c] 35 mM sodium phosphate buffer (pH 7.3).

To investigate how the NP's volume phase transition influences the protein affinity, the binding of the NP hydrogels and a protein was evaluated at three different temperatures (4 °C, 25 °C, and 40 °C). Hen egg white lysozyme, a 14 kDa basic protein (isoelectric point (pI) = 9.3), was used as a target protein in this study. The procedure and results are summarized in Figure 3. NP hydrogels and lysozyme were mixed, incubated, and filtered at each temperature. A centrifugal filtration device was used to separate the NP hydrogels and the bound enzyme from free (unbound) enzyme. At 4 °C, almost all of the enzyme was found in the filtrates, indicating that **NP1**–**NP4** in the swollen state lack binding affinity for the enzyme. Although the enzyme molecules could passively diffuse into the interior of the swollen hydrogel network and be physically entrapped, such an effect was apparently negligible in our experiment. We

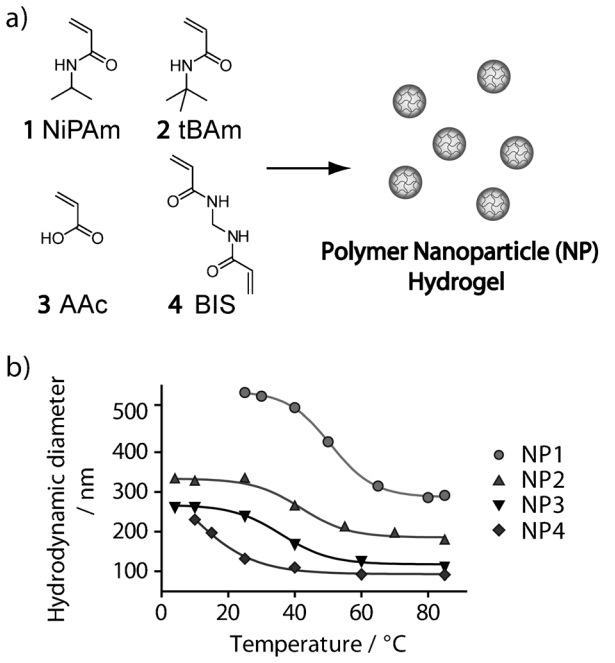

Figure 2. a) Synthesis of thermally responsive polymer nanoparticle (NP) hydrogels. b) Hydrodynamic diameters of NP hydrogels at different temperatures in 35 mM sodium phosphate buffer (pH 7.3). Measured with dynamic light scattering (DLS).

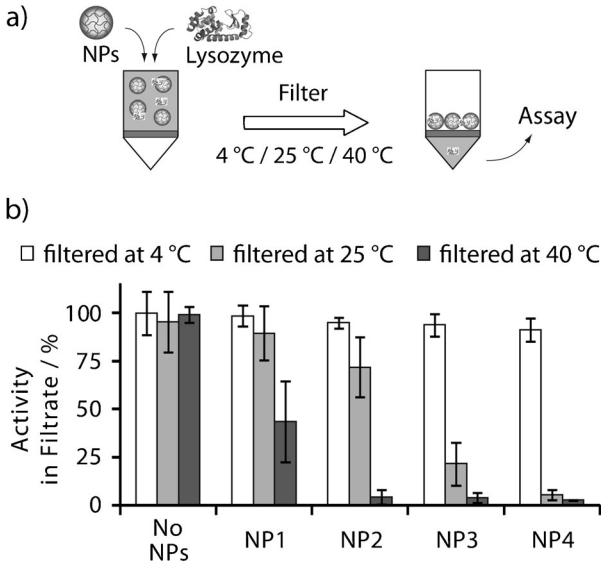

Figure 3. a) Procedure for studying the binding of lysozyme (32 μg mL^{-1}) to polymer NP hydrogels (2.0 mg mL^{-1}) in 35 mM sodium phosphate buffer (pH 7.3). b) The activity in the filtrates was measured at room temperature with the *Micrococcus lysodeikticus* cell lysis assay.

attributed this to the relatively low concentration (0.2% w/v) of the NP hydrogels and the intrinsically low affinity of the swollen NPs for the protein. At 25 °C **NP1** and **NP2** absorbed a small portion of the enzyme and **NP3** and **NP4** bound > 80% of the enzyme. At 40 °C, all the NP hydrogels were at least partly collapsed and **NP1** adsorbed approximately half of the enzyme, whereas **NP2**–**NP4** adsorbed more than 90% of the enzyme. Control experiments without NPs (No NPs) confirmed that the incubation and filtration at 4 °C, 25 °C, or 40 °C does not affect the activity of the enzyme. Overall, the physical state (swollen or collapsed) of the NP hydrogels correlated well with the NP–enzyme affinity. These results demonstrate the possibility to tune the NP hydrogels with regard to the temperature at which they begin to alter their intrinsic protein affinity.

After investigating the thermally responsive lysozyme "affinity switching" of **NP1**–**NP4**, we evaluated the ability of the NP hydrogels to protect the enzyme from thermal stress. As outlined in Figure 4a, solutions containing the NP hydrogels and lysozyme were incubated at 85 °C for 30 min, then cooled in an ice water bath for 30 min. The residual enzymatic activity was then measured at room temperature. When the enzyme alone was subjected to heat, the activity decreased to 25%. In contrast, the enzyme retained 80% of its activity in the presence of **NP1** or **NP2** (Figure 4b). **NP3** also protected the enzyme although the observed activity was lower than that with **NP1** or **NP2**. The activity of the solution containing **NP4** was comparable to the control experiment without NPs. Because **NP4** binds and inhibits lysozyme at room temperature (Figure S4), the experiment was repeated with an additional filtration step at 4 °C after heating. The result also indicated that **NP4** does not protect the enzyme (Figure S5), suggesting that the incorporation of 40 mol% of the hydrophobic comonomer results in a loss of performance. **NP5** (0 mol% AAc and 0 mol% tBAm) also did not prevent activity loss of the enzyme.

Figure 4c shows the result when a fixed amount (2.0 mg mL^{-1}) of **NP1** or **NP2** and different concentrations of the enzyme were mixed and subjected to heat. The protective effect of **NP1** and **NP2** gradually diminished as the protein concentration increased. Meanwhile, it was worth noting that **NP2** (5 mol% AAc and 10 mol% tBAm) showed a greater efficiency than **NP1** (5 mol% AAc and 0 mol% tBAm) at an enzyme concentration of 50 μg mL^{-1} or higher, whereas the effect of both NPs was comparable at low enzyme concentrations. We speculate that the incorporation of 10 mol% of tBAm results in an optimization of the LCST and/or the binding capacity of **NP2**. Figure 5 shows residual activity of the **NP2**–lysozyme solution that was subjected to repeated heating–cooling cycles. It was shown that the lysozyme could retain about 30% of its initial activity in the presence of **NP2** even after four heating–cooling cycles, whereas the enzyme lost nearly 95% of its activity after three cycles in the absence of NP hydrogels. This data indicates that **NP2** continues to protect the enzyme over repeated heating–cooling cycles, which proves the robustness of the system.

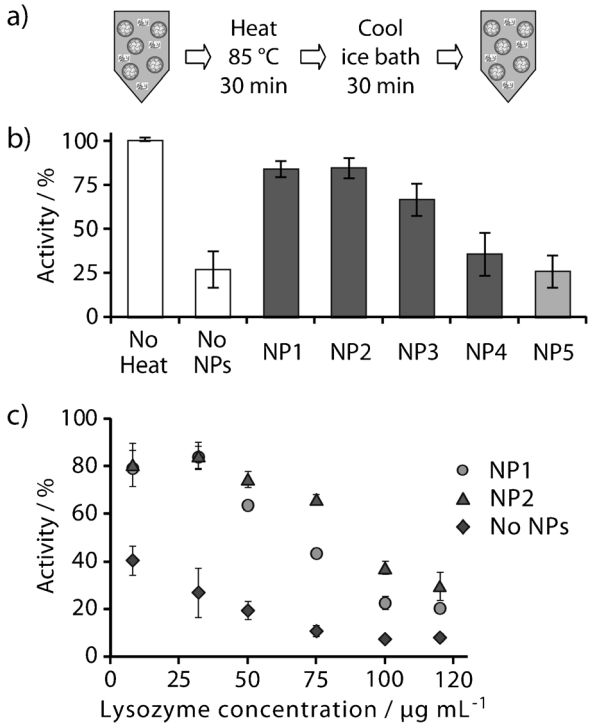

Figure 4. a) Thermal inactivation experiment. b) Effect of polymer NP hydrogels (2.0 mg mL^{-1}) on the activity of lysozyme after heating. c) Effect of **NP1** and **NP2** (2.0 mg mL^{-1}) on the activity of different concentrations of lysozyme after heating.

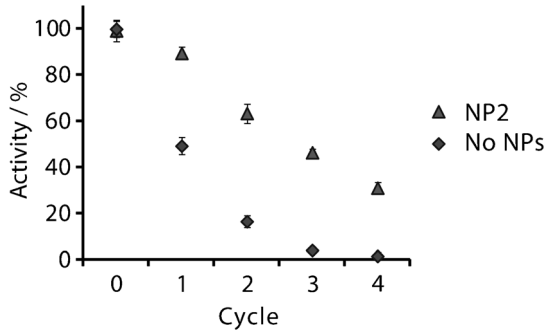

Figure 5. Ability of **NP2** (2.0 mg mL^{-1}) to stabilize lysozyme (32 μg mL^{-1}) over four heating–cooling cycles [85 °C (for 20 min) to RT (for 20 min)]. The measured activities were normalized against an experiment with pure lysozyme that did not involve NPs and was not subjected to heat (No NPs, cycle 0), which was treated as 100%.

Finally we conducted studies to investigate the enzyme protection mechanism. The addition of **NP2** after the protein had been heated (85 °C for 30 min) did not lead to a recovery of the enzymatic activity, indicating that **NP2** does not affect refolding. Our attempt to measure circular dichroism (CD) spectra of lysozyme at high temperature was hindered by the strong light scattering of the collapsed NP hydrogels. Alternatively, differential scanning calorimetry (DSC) was used to analyze the melting temperature (T_m) of the protein. Samples containing 80 μg mL^{-1} lysozyme either alone or in the presence of **NP2** (2.0 mg mL^{-1}) were tested (Figure S6). A slight shift in the T_m of the protein in the presence of **NP2** was observed; the T_m was reduced from 64.2 °C to 62.5 °C.

Whereas the T_m of the lysozyme can be affected by pH, buffer solutions with or without **NP2** showed no greater than a 0.03 pH unit shift at room temperature, 50 °C, and 70 °C. Because the lysozyme is bound to **NP2** above 40 °C, we interpreted this shift of the T_m as the result of a slight destabilization of the folded protein upon association with **NP2**. This was counter-intuitive because the **NP2**–protein association ultimately resulted in the protection of enzyme at elevated temperature. Although further investigations will be needed to fully understand this observation, we tentatively hypothesize that the origin of the enzymatic activity preservation arises from the isolation of the "bound enzyme", which prevents the aggregation and an irreversible loss of function. This effect outweighs the slightly negative influence of the NP association on protein folding.

In summary, we have described a new material design concept for a polymer NP hydrogel that displays both autonomous switching of the affinity for a target protein and the ability to protect the protein from thermal stress. In the presence of the optimized NP hydrogels, lysozyme retained over 80 % of its activity after it had been heated at 85 °C for 30 min. We hypothesize that the NP hydrogels form a complex with lysozyme below its T_m, which prevents protein aggregation at elevated temperature, thereby mimicking the action of a "passive aggregation inhibitor" type of heat shock protein.[26] Since NP hydrogels with affinity for a number of proteins[22,27] can be created from combinations of functional monomers, the described approach should be applicable to other target proteins. As each protein has a different T_m and surface properties, the optimal monomer composition may differ in each case. However, we believe that the presented insights, particularly the importance of the hydrophobic comonomer content and the LCST, provide a useful basis for the protection of proteins with a range of different characteristics. We also envision that a similar material design concept may be applied in areas that include the refolding of chemically denatured[28] proteins and the protein production by a cell-free protein synthesis system.[29]

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