Protein Purification

DOI: 10.1002/ange.201107797

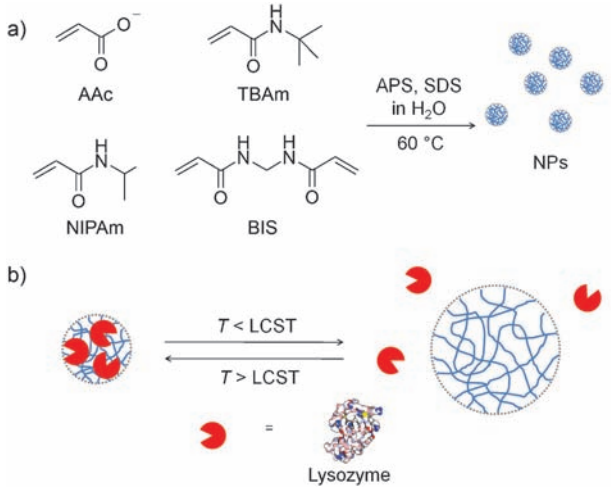
Temperature-Responsive "Catch and Release" of Proteins by using Multifunctional Polymer-Based Nanoparticles**

*Keiichi Yoshimatsu, Benjamin K. Lesel, Yusuke Yonamine, John M. Beierle, Yu Hoshino, and Kenneth J. Shea**

Synthetic nanoparticles (NPs) that have an intrinsic affinity for specific proteins are of considerable interest for their potential in biological/biomedical science and biotechnology.[1–3] In addition to their binding capability, synthetic materials offer the possibility of controlling binding affinity by external stimuli, which include light, electromagnetic radiation, and temperature,[4] and is a feature that can be used for the remote control of capture or release of target proteins in a spatiotemporally controlled manner. Herein, we report the synthesis and applications of multifunctional, polymer-based NPs that have selective protein affinity that can be modulated by external stimuli to "catch and release" the target protein.

Synthetic materials that alter their binding affinity for a target molecule in response to an external stimulus include photoresponsive synthetic receptors for recognition of ions[5] and saccharides,[6] temperature-responsive *N*-isopropylacrylamide (NIPAm) linear polymers for extraction of atrazine,[7] and temperature-responsive NIPAm-based bulk polymer hydrogels that reversibly change their affinity to target small molecules.[8] More recently, Lyon et al. and Shea and co-workers reported temperature-responsive NIPAm-based polymer NPs that can change their affinity to doxorubicin,[9] a drug molecule, and melittin, a peptide toxin,[10] respectively. However, NPs for protein targets have not been reported. Our goal was to design polymer-based NPs that possess the capability to catch and release a target protein from a complex biological sample in a reversible and temperature-responsive manner.

There are two separate, but not unrelated, requirements to create functional materials that incorporate both selective protein affinity and a trigger for the capture and release of the protein cargo. Strong affinity between designed synthetic materials and proteins has been achieved by incorporating complimentary functional groups into synthetic molecular scaffolds,[11] liner polymers,[12] dendritic polymers,[13] and synthetic NPs.[2,3,10] Based upon our experience with protein and peptide binding to synthetic polymers, we employed an approach that imparts a protein binding capability to temperature-responsive NIPAm-based NPs by incorporating functional groups that are complimentary to a target protein. Lysozyme, a 14 kDa basic protein (isoelectric point (pI) = 9.3) that is isolated from chicken-egg white, was chosen as the target protein. Acrylic acid (AAc; negatively charged monomer) and *N-tert*-butylacrylamide (TBAm; hydrophobic monomer) were selected as functional monomers to interact with the positively charged and hydrophobic residues of lysozyme. A precipitation polymerization method, previously reported by Kokufuta et al.,[14] Lyon et al.,[15] and our group,[3] was employed for the synthesis of a small library of multifunctional NPs that consist of various monomer compositions (Scheme 1a and Table 1). The NPs were synthesized by a thermally initiated free-radical copolymerization of func-



Scheme 1. a) Preparation of multifunctional polymer-based NPs. b) Illustration of temperature-responsive "catch-and-release" of a protein by multifunctional, polymer-based NPs.

Table 1: Monomer composition and hydrodynamic diameter of NPs.

Sample	Monomer content [mol%]				Hydrodynamic diameter [nm]	
	AAc	TBAm	NIPAm	BIS	in H_2O	in PBS
NP1	0	40	58	2	80	N/A[a]
NP2	5	40	53	2	86	96
NP3	10	40	48	2	89	175
NP4	5	20	73	2	99	225

[a] Not applicable as continuous increase of the measured diameter was detected, presumably as a result of the formation of aggregates.

[*] Dr. K. Yoshimatsu, B. K. Lesel, Dr. Y. Yonamine, Dr. J. M. Beierle, Prof. K. J. Shea
Department of Chemistry, University of California, Irvine
Irvine, CA 92697 (USA)
E-mail: kjshea@uci.edu
Dr. Y. Hoshino
Department of Chemical Engineering, Kyushu University
744 Motooka, Nishi-ku, Fukuoka, Fukuoka 819-0395 (Japan)

[**] Financial support from the National Institutes of Health (GM 080506) is gratefully acknowledged. B.K.L. was a recipient of Allergan Undergraduate Research Fellowship.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201107797.

tional monomers and a cross-linking monomer (*N*,*N*′-methylene-bis(acrylamide) (BIS), 2 mol%) in aqueous solution that contained ammonium persulfate (APS) and a small amount of sodium dodecyl sulfate (SDS). After the synthesis, the NPs were purified by dialysis. Dynamic light scattering established that the solutions of NPs were monodisperse and that the NPs have hydrodynamic diameters of approximately 80–100 nm in water (Table 1).

The binding capability of NPs to lysozyme was evaluated by preincubating lysozyme (5 $\mu g\,mL^{-1}$) and NPs (800 $\mu g\,mL^{-1}$) in phosphate buffered saline ((PBS) sodium phosphate buffer (35 mM) containing NaCl (150 mM), pH 7.3). The solutions were then filtered through a Nanosep membrane filter (molecular weight cut-off (MWCO): 100 kDa, Pall Corp.) to filter the NPs and NP-bound lysozyme. The concentration of free lysozyme in the filtrate was estimated by lysozyme activity measurements by in a *Micrococcus lysodeikticus* cell suspension.[16] The results of the binding experiments are summarized in Figure 1a. A large decrease in lysozyme activity (more than 90%) was detected in the filtrate that was incubated with NP2 (AAc = 5%, TBAm = 40%) and NP3 (AAc = 10%, TBAm = 40%). These results suggest that both the hydrophobic and negatively charged groups in the NP contribute to lysozyme capture. We further investigated the in situ inhibition capability of NPs by measuring lysozyme activity in the presence of either NP2 or NP3 (800 $\mu g\,mL^{-1}$; Figure S1 in the Supporting Information). This experiment established that 800 $\mu g\,mL^{-1}$ of NP2 and NP3 inhibited 5 $\mu g\,mL^{-1}$ of lysozyme. NP2 was particularly effective at inhibiting lysozyme activity to only a few percent of the initial level. Possible explanations for this level of inhibition include blocking access of the active site of lysozyme by NP2 and/or sequestration of lysozyme into the interior of the polymer network of NP2. Inhibition by NP3 was less pronounced (50% decrease), even though the binding experiment showed that NP3 can capture the majority (more than 90%) of lysozyme from the solution. In this case, binding to NP3 apparently does not effectively prevent access of the substrate to the active site of lysozyme. It is interesting to note, however, that an increased AAc content does not result in enhanced inhibition of lysozyme activity. This result implies that the balance between hydrophobic and negatively charged groups in the NP is a critical factor in determining the binding and inhibition capability of the synthetic NPs.

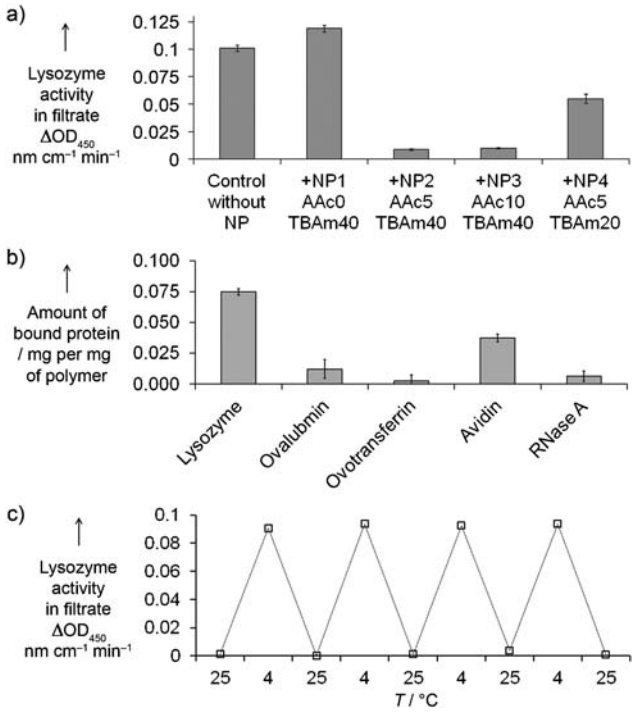


Figure 1. a) Depletion of lysozyme (5 $\mu g\,mL^{-1}$) from solutions after incubation with NPs (800 $\mu g\,mL^{-1}$) at room temperature. b) Binding selectivity of NP2 at room temp. NP2 = 2.0 $mg\,mL^{-1}$; protein conc = 200 $\mu g\,mL^{-1}$. c) Temperature-responsive "catch-and-release" of lysozyme (5 $\mu g\,mL^{-1}$) by NP2 (2.0 mg). Experiments were carried out in PBS (sodium phosphate buffer (35 mM) containing NaCl (150 mM), pH 7.3).

Based on these results, NP2 was selected for further study. The activity of lysozyme (5 $\mu g\,mL^{-1}$) was measured in the presence of various concentrations of NP2 (0–2.0 $mg\,mL^{-1}$). The 50% inhibition concentration (IC_{50}) of NP2 was determined to be 73 $\mu g\,mL^{-1}$, and near complete inhibition is achieved in the presence of 1.5 $mg\,mL^{-1}$ of NP2 (Figure S2 in the Supporting Information). Thus a 15- and 300-fold excess of NP2 (in weight) can inhibit half or nearly all, respectively, of the lysozyme in the solution. The binding selectivity of NP2 was characterized by comparing the uptake of lysozyme and four different proteins. As shown in Figure 1b, 0.075 mg and 0.037 mg of lysozyme and avidin were absorbed per mg of NP2, respectively, whereas only negligible amounts of ovalbumin and ovotransferrin were absorbed by NP2. Under the conditions of the experiment (pH 7.3), lysozyme (pI = 9.3), avidin (pI = 10.0), and RNase A (pI = 8.6) are positively charged, whereas ovalbumin (pI = 4.7) and ovotransferrin (pI = 6.1) are negatively charged. As NP2 is negatively charged at pH 7.4, the strong interaction between NP2 and the positively charged proteins (lysozyme and avidin) establishes that electrostatic interactions make an important contribution to the absorption. The greater uptake of lysozyme over avidin and RNase A might be explained by the difference in hydrophobicity of these proteins.[17] These results indicate that NP2 has a certain level of binding selectivity that arises from a combination of hydrophobic and complementary electrostatic interactions.

We next investigated the feasibility of releasing bound lysozyme from the NPs by utilizing the temperature-responsive property of NP2. Below the lowest critical solution temperature (LCST), NIPAm-TBAm copolymers are swollen, highly hydrated, and hydrophilic. They are collapsed and more hydrophobic above their LCST as a result of an entropically driven dissociation of water molecules.[18] NP2 undergoes a volume-phase transition from the collapsed "hydrophobic" state to a solvent swollen "hydrophilic" state at approximately 11 °C in PBS (Figure S3 in the Supporting Information).[10,15] As a consequence, the density of hydrophobic groups and negatively charged groups per unit volume inside the NP decreases. These changes can weaken the NP–protein interactions and trigger the release of lysozyme.

Figure 1c shows the measured lysozyme activity of the filtrate from each step of the cooling/warming cycles. As can be clearly seen, NP2 was able to catch and release lysozyme repeatedly in a temperature-responsive manner. Furthermore it was possible to recover lysozyme quantitatively by cooling the samples below the LCST. The process is reversible, as the NPs did not show significant loss of binding capability even after four cycles. Most importantly, the lysozyme activity did not decrease over the repeated catch and release cycles, which indicates that binding to NP2 does not cause denaturation of lysozyme, at least within the time scale of this study. The circular dichroism spectra of lysozyme did not significantly change before and after one cycle (Figure S4 in the Supporting Information), which supports the conclusion that catch and release does not perturb the structural integrity of lysozyme.

Finally, we evaluated the applicability of NP2 for purification of lysozyme from chicken-egg whites. The procedure of catch and release purification is outlined in Figure 2a. The egg white (diluted 50 times in PBS) and NP2 (2.5 mg mL^{-1}) were incubated for 30 min at room temperature. The mixture was then passed through a filter membrane (MWCO: 100 kDa) at room temperature. A control experiment was carried out under identical conditions except for the omission of NPs. The proteins in flow-through fractions were analyzed by SDS-PAGE (Figure 2b, lanes 2 and 7). Whereas ovalbumin (45 kDa), ovotransferrin (77 kDa) and ovomucoid (28 kDa) could be detected in both filtrates, lysozyme (14 kDa) was only detected in the filtrate of the control experiment. This result indicates that NP2 is capable of selectively capturing lysozyme over other major egg-white proteins. Subsequently, the same membrane filter unit was filled with a fresh solution of PBS and incubated for 30 min at 1 °C to elute lysozyme from the NPs that were retained on the membrane. Lysozyme was detected in the cold-elution fraction from the NP2, whereas none was detected in the control (Figure 2b, lanes 3 and 8). Although a trace amount of ovalbumin was also detected in the cold-elution fraction from NP2, the purity of the sample could be easily improved after an extra cycle of catch and release purification (Figure 2b, lane 5). The amount of recovered lysozyme (based on enzymatic activity) after the first and second cycles was estimated to be 84% and 79% respectively. The specific activity of purified lysozyme was 41579 units mg^{-1}, which is comparable to that of a commercially available product.

In conclusion, we have demonstrated multifunctional, polymer-based NPs as temperature-responsive catch and release machines for sequestering target proteins. NIPAm-based NPs that contain various combinations of hydrophobic and negatively charged monomers were screened for in situ lysozyme affinity and inhibition capability. NPs with an optimized composition selectively capture lysozyme without denaturation. The protein can be subsequently released from the NP by simply cooling to 1 °C. These results suggest that designed multifunctional NPs have the potential for the spatiotemporally controlled capture and release of proteins. This property and the NPs inherent selectivity were successfully applied for catch and release purification of lysozyme from chicken-egg whites. As NPs with similar composition can selectively capture or release target peptides[10] and proteins in a spatiotemporally controlled manner, we envision that this concept can find utility in a number of areas in biological/biomedical science and biotechnology.

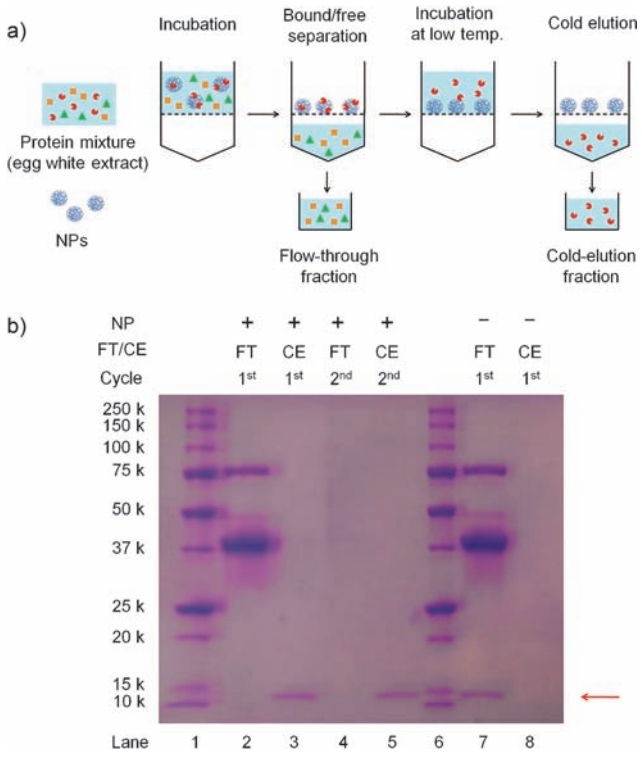


Figure 2. a) Schematic of "catch-and-release" lysozyme purification from egg whites. b) SDS-PAGE analysis of flow-through fraction (FT) and cold-elution fraction (CE) after Coomassie Brilliant Blue R-250 staining. Lanes 1 and 6: molecular weight markers. Lane 2: FT from first cycle. Lane 3: CE from first cycle. Lane 4: FT from second cycle. Lane 5: CE fraction from second cycle. Lane 7: FT from the control. Lane 8: CE from the control.

Received: November 5, 2011
Revised: January 10, 2011
Published online: January 27, 2012

Keywords: molecular recognition · nanoparticles · phase transitions · polymers · proteins

[1] R. Duncan, *Nat. Rev. Drug Discovery* **2003**, *2*, 347–360; T. Cedervall, I. Lynch, M. Foy, T. Berggard, S. C. Donnelly, G. Cagney, S. Linse, K. A. Dawson, *Angew. Chem.* **2007**, *119*, 5856–5858; *Angew. Chem. Int. Ed.* **2007**, *46*, 5754–5756; S. Linse, C. Cabaleiro-Lago, W. F. Xue, I. Lynch, S. Lindman, E. Thulin, S. E. Radford, K. A. Dawson, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 8691–8696; Y. Hoshino, H. Koide, T. Urakami, H. Kanazawa, T. Kodama, N. Oku, K. J. Shea, *J. Am. Chem. Soc.* **2010**, *132*, 6644–6645; Y. Hoshino, K. J. Shea, *J. Mater. Chem.* **2011**, *21*, 3517–3521.

[2] V. M. Rotello, N. O. Fischer, C. M. McIntosh, J. M. Simard, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 5018–5023; V. M. Rotello, C. C. You, A. Verma, *Soft Matter* **2006**, *2*, 190–204.

[3] Y. Hoshino, T. Urakami, T. Kodama, H. Koide, N. Oku, Y. Okahata, K. J. Shea, *Small* **2009**, *5*, 1562–1568.

[4] M. A. C. Stuart, W. T. S. Huck, J. Genzer, M. Muller, C. Ober, M. Stamm, G. B. Sukhorukov, I. Szleifer, V. V. Tsukruk, M. Urban, F. Winnik, S. Zauscher, I. Luzinov, S. Minko, *Nat. Mater.* **2010**, *9*, 101 – 113.
[5] S. Shinkai, T. Ogawa, T. Nakaji, Y. Kusano, O. Nanabe, *Tetrahedron Lett.* **1979**, *20*, 4569 – 4572; S. Shinkai, T. Nakaji, Y. Nishida, T. Ogawa, O. Manabe, *J. Am. Chem. Soc.* **1980**, *102*, 5860 – 5865; S. Shinkai, T. Nakaji, T. Ogawa, K. Shigematsu, O. Manabe, *J. Am. Chem. Soc.* **1981**, *103*, 111 – 115.
[6] H. Shinmori, M. Takeuchi, S. Shinkai, *J. Chem. Soc. Perkin Trans. 2* **1996**, 1 – 3; S. Shinkai, H. Shinmori, M. Takeuchi, *J. Chem. Soc. Perkin Trans. 2* **1998**, 847 – 852.
[7] S. O. Gonzalez, S. Furyk, C. M. Li, S. E. Tichy, D. E. Bergbreiter, E. E. Simanek, *J. Polym. Sci. Part A* **2004**, *42*, 6309 – 6317.
[8] T. Oya, T. Enoki, A. Y. Grosberg, S. Masamune, T. Sakiyama, Y. Takeoka, K. Tanaka, G. Q. Wang, Y. Yilmaz, M. S. Feld, R. Dasari, T. Tanaka, *Science* **1999**, *286*, 1543 – 1545.
[9] L. A. Lyon, M. J. Serpe, K. A. Yarmey, C. M. Nolan, *Biomacromolecules* **2005**, *6*, 408 – 413.
[10] Y. Hoshino, W. W. Haberaecker III, T. Kodama, Z. Zeng, Y. Okahata, K. J. Shea, *J. Am. Chem. Soc.* **2010**, *132*, 13648 – 13650.
[11] H. Yin, A. D. Hamilton, *Angew. Chem.* **2005**, *117*, 4200 – 4235; *Angew. Chem. Int. Ed.* **2005**, *44*, 4130 – 4163.
[12] S. J. Koch, C. Renner, X. L. Xie, T. Schrader, *Angew. Chem.* **2006**, *118*, 6500 – 6503; *Angew. Chem. Int. Ed.* **2006**, *45*, 6352 – 6355.
[13] R. Haag, J. Dernedde, A. Rausch, M. Weinhart, S. Enders, R. Tauber, K. Licha, M. Schirner, U. Zugel, A. von Bonin, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 19679 – 19684.
[14] E. Kokufuta, S. Ito, K. Ogawa, H. Suzuki, B. L. Wang, R. Yoshida, *Langmuir* **1999**, *15*, 4289 – 4294.
[15] L. A. Lyon, J. D. Debord, *Langmuir* **2003**, *19*, 7662 – 7664.
[16] D. Shugar, *Biochim. Biophys. Acta* **1952**, *8*, 302 – 309.
[17] T. D. Durance, S. Nakai, *J. Food Sci.* **1988**, *53*, 1096 – 1102; S. Ghose, T. M. McNerney, B. Hubbard, *Biotechnol. Bioeng.* **2004**, *87*, 413 – 423.
[18] H. G. Schild, *Prog. Polym. Sci.* **1992**, *17*, 163 – 249.

