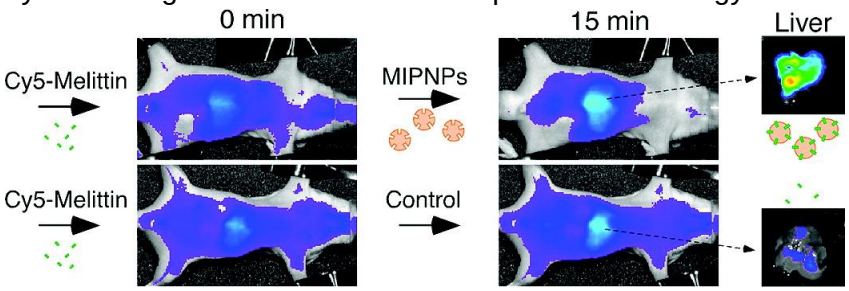
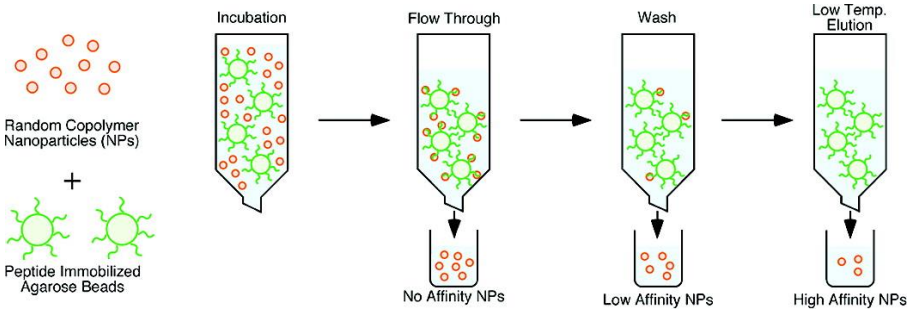
"Recognition, Neutralization and Clearance of Target Peptides in the Bloodstream of Living Mice by Molecularly Imprinted Polymer Nanoparticles: A Plastic Antibody." Yu Hoshino, Hiroyuki Koide, Takeo Urakami, Hiroaki Kanazawa, Takashi Kodama, Naoto Oku, and Kenneth J. Shea, *J. Am. Chem. Soc*. **2010**, *132*, 6644-6645.

We report that simple, synthetic organic polymer nanoparticles (NPs) can capture and clear a target peptide toxin in the bloodstream of living mice. The protein-sized polymer nanoparticles, with a binding affinity and selectivity comparable to those of natural antibodies, were prepared by combining a functional monomer optimization strategy with molecular-imprinting nanoparticle synthesis. As a result of binding and removal of melittin by NPs in vivo, the mortality and peripheral toxic symptoms due to melittin were significantly diminished. In vivo imaging of the polymer nanoparticles (or "plastic antibodies") established that the NPs accelerate clearance of the peptide from blood and accumulate in the liver. Coupled with their biocompatibility and nontoxic characteristics, plastic antibodies offer the potential for neutralizing a wide range of biomacromolecules in vivo.

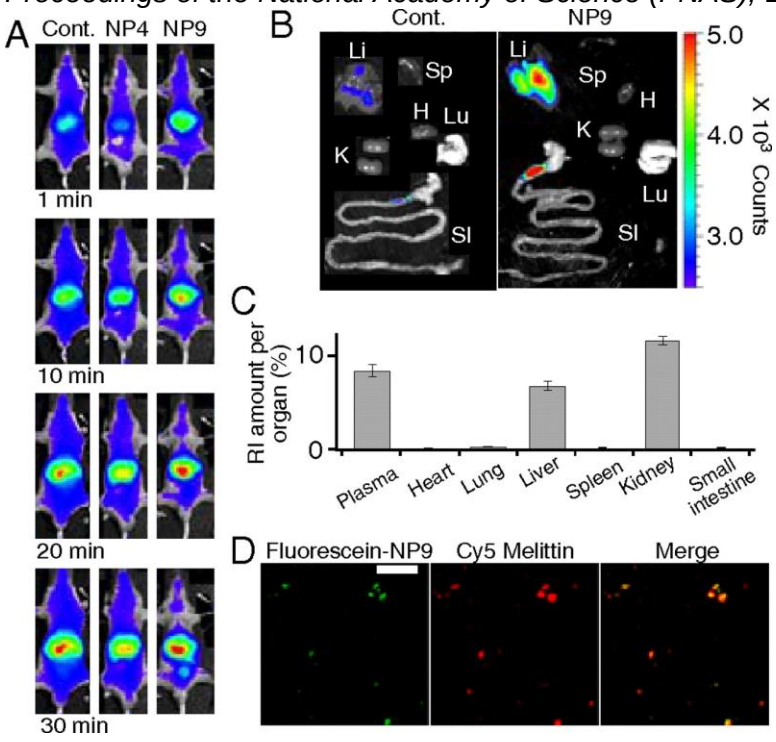


"Affinity Purification of Multifunctional Polymer Nanoparticles" Yu Hoshino, Walter W. Haberaecker III, Takashi Kodama, Zhiyang Zeng, Yoshio Okahata, Kenneth J. Shea, *J. Am. Chem. Soc*. **2010**, *132*, 13648 - 13650.

We report that multifunctional polymer nanoparticles approximately the size of a large protein can be "purified", on the basis of peptide affinity just as antibodies, using an affinity chromatography strategy. The selection process takes advantage of the thermoresponsiveness of the nanoparticles allowing "catch and release" of the target peptide by adjusting the temperature. Purified particles show much stronger affinity ($K_{d\text{app}} \approx$ nM) *and* a narrower affinity distribution than the average of particles before purification ($K_{d\text{app}} >$ μM) at room temperature but can release the peptide just by changing the temperature. We anticipate this affinity selection will be general and become an integral step for the preparation of "plastic antibodies" with near-homogeneous and tailored affinity for target biomacromolecules.

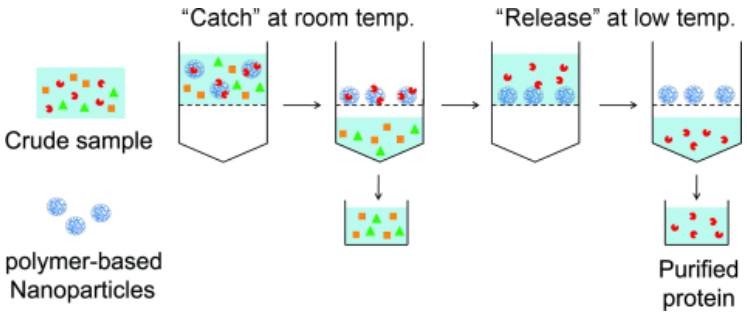


"The Rational Design of a Plastic Antidote: Synthetic Polymer Nanoparticles that Neutralize Toxic Peptides in Vivo" Yu Hoshino, Hiroyuki Koide, Keiichi Furuya, Walter W. III Haberaecker, Shih-Hui Lee, Takashi Kodama, Hiroaki Kanawaza, Naoto Oku, and Kenneth J. Shea, *Proceedings of the National Academy of Science (PNAS),* **2012**, *109*, 33-38.



Here, we report the design rationale of a plastic (synthetic hydrogel polymer) antidote for in vivo applications. Optimizing the choice and ratio of functional monomers incorporated in the NP maximized the binding affinity and capacity toward a target peptide. Biocompatibility tests of the NPs in vitro and in vivo revealed the importance of tuning surface charge and hydrophobicity to minimize NP toxicity and prevent aggregation induced by nonspecific interactions with plasma proteins. The toxin neutralization capacity of NPs in vivo showed a strong correlation with binding affinity and capacity in vitro. Furthermore, in vivo imaging experiments established the NPs accelerate clearance of the toxic peptide and eventually accumulate in macrophages in the liver. These results provide a platform to design plastic antidotes and reveal the potential and possible limitations of using synthetic polymer nanoparticles as plastic antidotes.

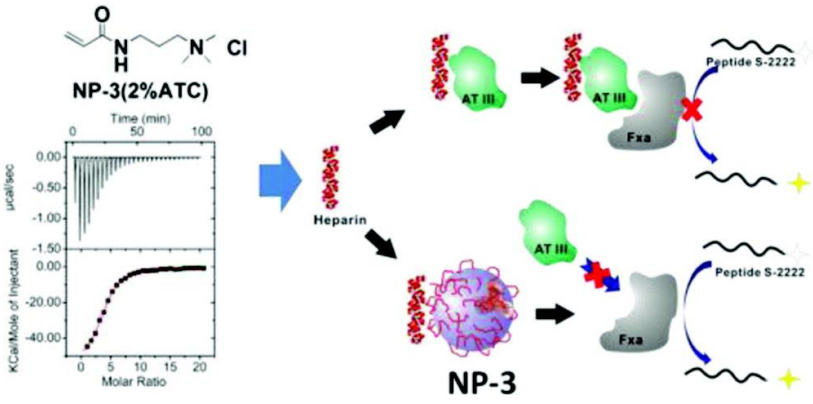
"Temperature-Responsive "Catch-and-Release" of Proteins using Multifunctional Polymer Nanoparticles", Keiichi Yoshimatsu, Benjamin K. Lesel, Yusuke Yonamine, John M. Beierle, Yu Hoshino and Kenneth J. Shea, *Angewandte Chemie Int. Ed.* , **2012**, *51*, 2405–2408.



Catch me if you can: Multifunctional, polymer-based nanoparticles that are capable of temperature-responsive "catch-and-release" of a target protein have been synthesized. The process is reversible and does not denature the proteins. An optimized combination of functional monomers imparts binding selectivity toward a target protein over other proteins.

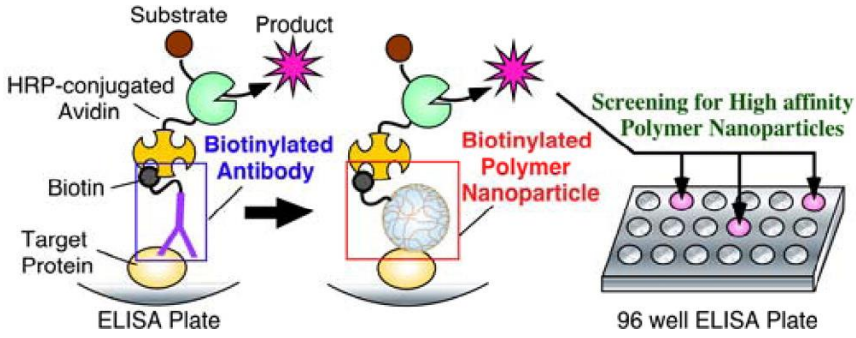
"Synthetic Polymer Nanoparticle-Polysaccharide Interactions: A Systematic Study" Zhiyang Zeng, Jiten Patel, Shih-Hui Le1, Monica McCallum, Anu Tyagi, Mingdi Yan, Kenneth J. Shea, *Journal of the American Chemical Society*, **2012**, *134*, 2681 - 2690.

The interaction between synthetic polymer nanoparticles (NPs) and biomacromolecules (e.g., proteins, lipids, and polysaccharides) can profoundly influence the NPs fate and function. Polysaccharides (e.g., heparin/heparin sulfate) are a key component of cell surfaces and the extracelluar matrix and play critical roles in many biological processes. We report a systematic investigation of the interaction between synthetic polymer nanoparticles and polysaccharides by ITC, SPR, and an anticoagulant assay to provide guidelines to engineer nanoparticles for biomedical applications. The interaction between acrylamide nanoparticles (~30 nm) and heparin is mainly enthalpy driven with submicromolar affinity. Hydrogen bonding, ionic interactions, and dehydration of polar groups are identified to be key contributions to the affinity. It has been found that high charge density and cross-linking of the NP can contribute to high affinity. The affinity and binding capacity of heparin can be significantly diminished by an increase in salt concentration while only slightly decreased with an increase of temperature. A striking difference in binding thermodynamics has been observed when the main component of a polymer nanoparticle is changed from acrylamide (enthalpy driven) to *N*-isopropylacryalmide (entropy driven). This change in thermodynamics leads to different responses of these two types of polymer NPs to salt concentration and temperature. Select synthetic polymer nanoparticles have also been shown to inhibit protein–heparin interactions and thus offer the potential for therapeutic applications.



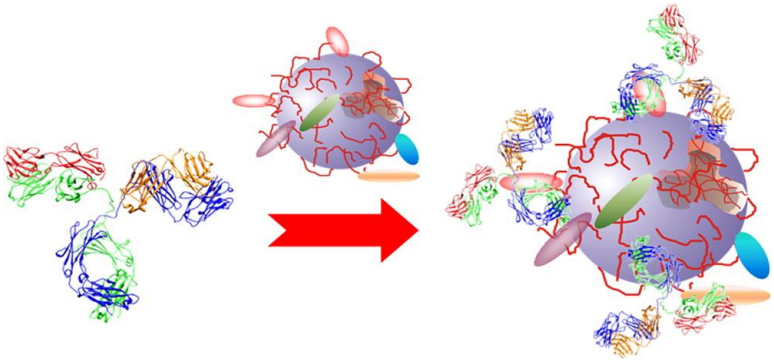
"An ELISA-mimic screen for synthetic polymer nanoparticles with high affinity to target proteins" Yusuke Yonamine, Yu Hoshino and Kenneth J. Shea. *Biomacromolecules*, **2012**, *13*, 2952–2957.

In this study, we modified an immunological assay (enzyme-linked immunosorbent assay: ELISA) into a high-throughput screening method to select nanoparticles with high affinity to target proteins. Histone and fibrinogen were chosen as target proteins to demonstrate this concept. The selection process utilized a biotinylated NP library constructed with combinations of functional monomers. The screen identified NPs with distinctive functional group compositions that exhibited high affinity to either histone or fibrinogen. The variation of protein



affinity with changes in the nature and amount of functional groups in the NP provided chemical insight into the principle determinants of protein-NP binding. The NP affinity was semiquantified using the ELISA-mimic assay by varying the NP concentrations. The screening results were found to correlate with solution-based assay results. This screening system utilizing a biotinylated NP is a general approach to optimize functional monomer compositions and can be used to rapidly search for synthetic polymers with high (or low) affinity for target biological macromolecules.

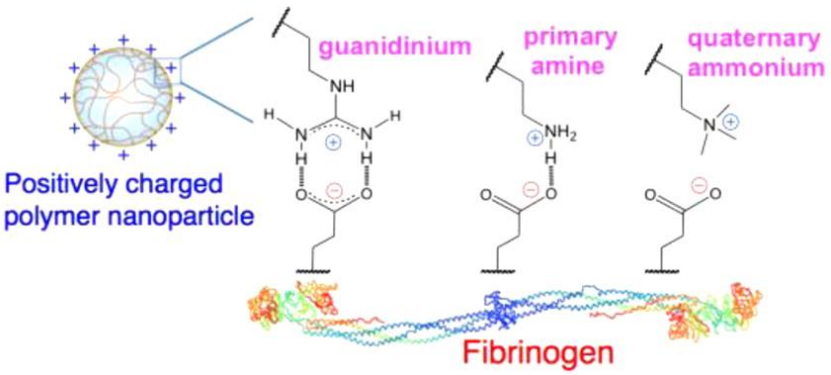
"Engineered Synthetic Polymer Nanoparticles as IgG Affinity Ligands".
Shih-Hui Lee, Yu Hoshino, Arlo Randall, Zhiyang Zeng, Piere Baldi, Ruey-an Doong and Kenneth. J. Shea, *Journal of the American Chemical Society*, **2012**, *134*, 15765-15772.
In this study, we report a lightly cross-linked (2%) *N*-isopropyl acrylamide (NIPAm) synthetic polymer NP (50–65 nm) incorporating hydrophobic and carboxylate groups that binds with high affinity to the Fc fragment of IgG. The affinity and amount of NP bound to IgG is pH dependent.



The hydrogel NP inhibits protein A binding to the Fc domain at pH 5.5, but not at pH 7.3. A bioinformatics study was used to identify potential NP–protein interaction sites. Candidates include a NP binding domain that overlaps with the protein A–Fc binding domain at pH 5.5. The computational analysis supports the inhibition experimental results and is attributed to the difference in the charged state of histidine residues. Affinity of the NP (3.5–8.5 nM) to the Fc domain at pH 5.5 is comparable to protein A at pH 7. These results establish that engineered synthetic polymer NPs can be formulated with an intrinsic affinity to a specific domain of a large biomacromolecule.

"The Polymer Nanoparticle-Protein Interface. Evaluation of the Contribution of Positively Charged Functional Groups to Protein Affinity" Yusuke Yonamine, Keiichi Yoshimatsu, Shih-Hui Lee, Yu Hoshino, Yoshio Okahata and Kenneth J. Shea, *ACS Applied Materials & Interfaces*, **2013**, *5*, 374–379.

Cationic-functionalized polymer nanoparticles (NPs) show strikingly distinct affinities to proteins

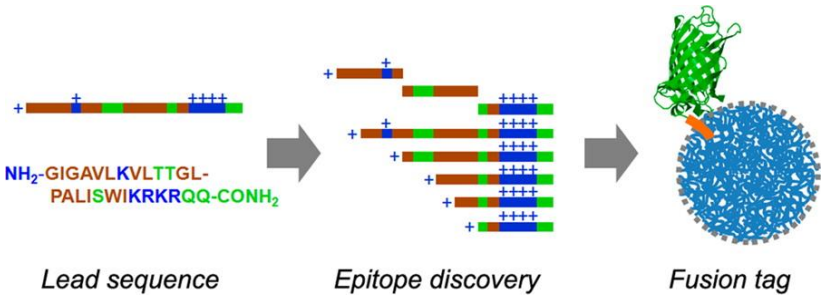


depending on the nature of the cationic functional group. *N*-Isopropylacrylamide (NIPAm) polymer NPs incorporating three types of positively charged functional groups (guanidinium, primary amino, and quaternary ammonium groups) were prepared by precipitation polymerization. The affinities to fibrinogen, a protein with an isoelectric point (pI) of 5.5, were compared using UV–vis

spectrometry and a quartz crystal microbalance (QCM). Guanidinium-containing NPs showed the highest affinity to fibrinogen. The observation is attributed to strong, specific interactions with carboxylate groups on the protein surface. The affinity of the positively charged NPs to proteins with a range of pIs revealed that protein-NP affinity is due to a combination of ionic, hydrogen bonding, and hydrophobic interactions. Protein affinity can be modulated by varying the composition of these functional monomers in the acrylamide NPs. Engineered NPs containing the guanidinium group with hydrophobic and hydrogen bonding functional groups were used in an affinity precipitation for the selective separation of fibrinogen from a plasma protein mixture. Circular dichroism (CD) revealed that the protein was not denatured in the process of binding or release.

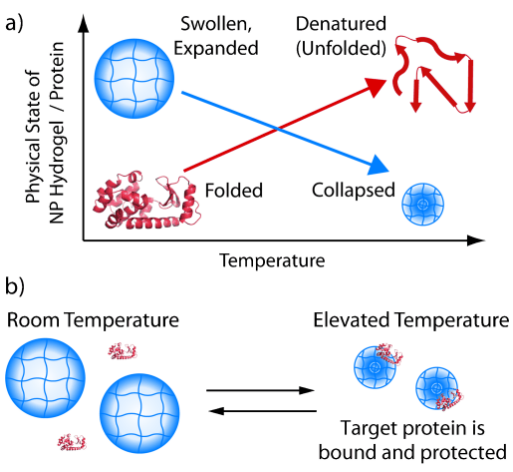
"Epitope Discovery for a Synthetic Polymer Nanoparticle: A New Strategy for Developing a Peptide Tag" Keiichi Yoshimatsu, Tomohiko Yamazaki, Yu Hoshino, Paul E. Rose, Linda F. Epstein, Les P. Miranda, Philip Tagari, John M. Beierle, Yusuke Yonamine, and Kenneth J. Shea, *Journal of the American Chemical Society*, **2014,** *136***,** 1194 - 1197.

We describe a novel epitope discovery strategy for creating an affinity agent/peptide tag pair. A synthetic polymer nanoparticle (NP) was used as the "bait" to catch an affinity peptide tag. Biotinylated peptide tag candidates of varied sequence and length were attached to an avidin platform and screened for affinity against the polymer NP. NP affinity for the avidin/peptide tag complexes was used to provide insight into factors that contribute NP/tag binding. The identified epitope sequence with an optimized length (tMel-tag) was fused to two recombinant proteins. The tagged proteins exhibited higher NP affinity than proteins without tags. The results establish that a fusion peptide tag consisting of optimized 15 amino acid residues can provide strong affinity to an abiotic polymer NP. The affinity and selectivity of NP/tMel-tag interactions were exploited for protein purification in conjunction with immobilized metal ion/His6-tag interactions to prepare highly purified recombinant proteins. This strategy makes available inexpensive, abiotic synthetic polymers as affinity agents for peptide tags and provides alternatives for important applications where more costly affinity agents are used.



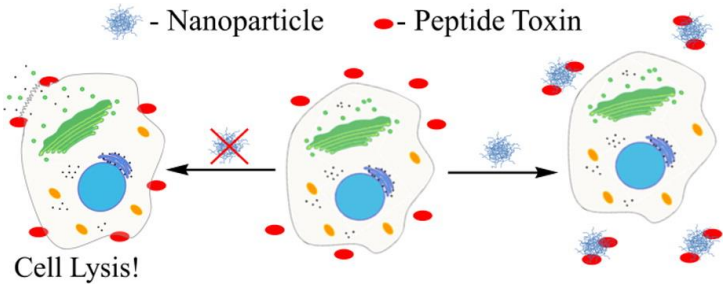
"Polymer Nanoparticle Hydrogels with Autonomous Affinity Switching for Protection of Proteins from Thermal Stress" John M. Beierle, Keiichi Yoshimatsu, Beverly Chou, Benjamin K. Lesel, Kenneth J. Shea, *Angewandte Chemie Int. Ed.*, **2014**, *53*, 9275-9279.

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.

"Engineering Nanoparticle Anti-toxins Utilizing Aromatic Interactions" Adam Weisman, Yingyao Allie Chen, Yu Hoshino and Kenneth Shea, *Biomacromolecules*, **2014**, *15*, 3290-3295. Methicillin resistant *Staphylococcus aureus* (MRSA) is a highly virulent bacterium capable of inflicting severe infections. This pathogen has a long history of developing resistance to



antibacterial drugs, and many phenotypes are capable of disabling the host immune response by releasing peptide and protein toxins with the capacity to lyse human polymorphonuclear neutrophils. The peptide phenol-soluble modulin α3 (PSMα3) has been identified as an important toxin released by the most virulent strains of MRSA. A library of polymer nonaparticles was synthesized by precipitation polymerization and screened for their ability to bind and neutralize this toxin. To generate high affinity, monomers were chosen to compliment the functional groups of PSMα3. Nanoparticles incorporating aromatic monomers provided a high affinity for the peptide and were effective at neutralizing its toxicity in vitro.

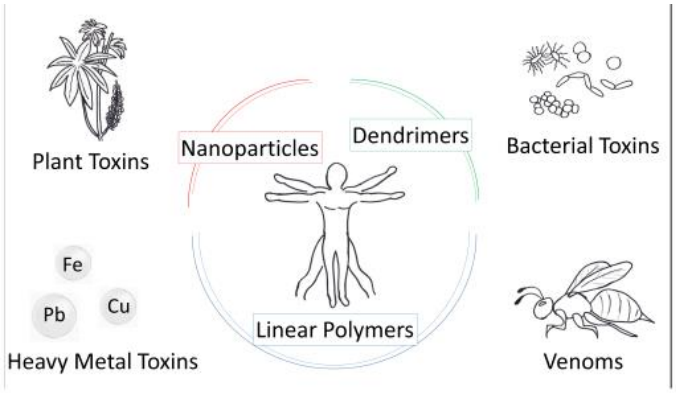
"Preparation and evaluation protocol for abiotic polymer nanoparticles for sequestration and neutralization of a target peptide toxin" Keiichi Yoshimatsu, Hiroyuki Koide , Yu Hoshino & Kenneth J. Shea, *Nature Protocols*, **2015**, *10*, 595–604.

Synthetic polymer nanoparticles (NPs) with intrinsic affinity for target biomacromolecules hold great promise in the development of novel tools for biological and biomedical research. We describe the design and synthesis of abiotic, synthetic polymer NPs with high intrinsic affinity for a peptide toxin melittin. This protocol describes a

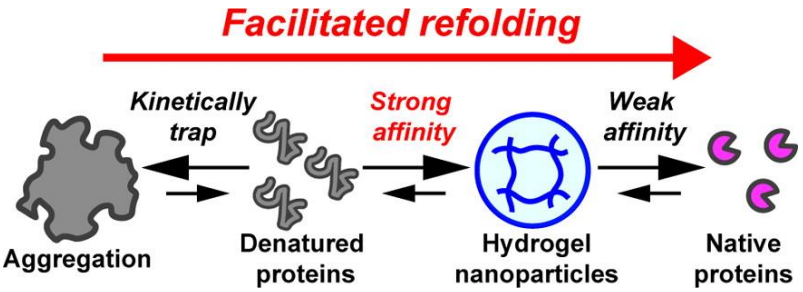
step-by-step procedure for the preparation and evaluation of synthetic polymer NPs for sequestration and neutralization of the target peptide toxin. The polymer NPs can be synthesized in a one-step polymerization reaction using commercially available reagents. The polymerization reaction for the synthesis of polymer NPs takes several hours, and the total protocol including subsequent purification and characterization by dynamic light scattering, NMR and toxicity neutralization assays takes 1–2 weeks in total.

"Polymeric antidotes for toxin sequestration" Adam Weisman, Beverly Chou, Jeffrey O'Brien, Kenneth J. Shea, *Advanced Drug Delivery Reviews*, **2015**, *90*, 81-100.

Toxins delivered by envenomation, secreted by microorganisms, or unintentionally ingested can pose an immediate threat to life. Rapid intervention coupled with the appropriate antidote is required to mitigate the threat. Many antidotes are biological products and their cost, methods of production, potential for eliciting immunogenic responses, the time needed to generate them, and stability issues contribute to their limited availability and effectiveness. These factors exacerbate a world-wide challenge for providing treatment. In this review we evaluate a number of polymer constructs that may serve as alternative antidotes. The range of toxins investigated includes those from sources such as plants, animals and bacteria. The development of polymeric heavy metal sequestrants for use as antidotes to heavy metal poisoning faces similar challenges, thus recent findings in this area have also been included. Two general strategies have emerged for the development of polymeric antidotes. In one, the polymer acts as a scaffold for the presentation of ligands with a known affinity for the toxin. A second strategy is to generate polymers with an intrinsic affinity, and in some cases selectivity, to a range of toxins. Importantly, *in vivo* efficacy has been demonstrated for each of these strategies, which suggests that these approaches hold promise as an alternative to biological or small molecule based treatments.



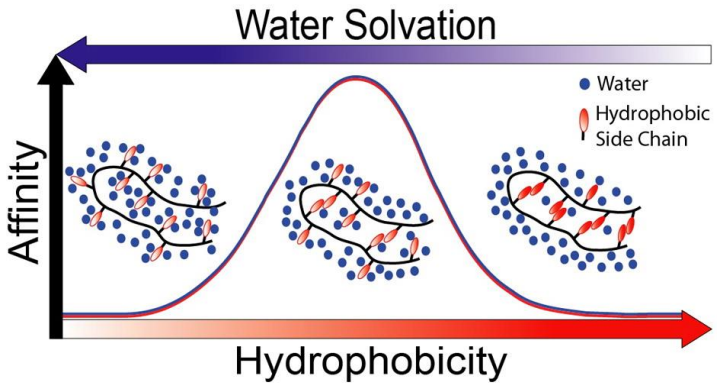
"Design of Synthetic Polymer Nanoparticles that Facilitate Resolubilization and Refolding of Aggregated Proteins, Masahiko Nakamoto, Tadashi Nonaka, Kenneth J. Shea, Yoshiko Miura, and Yu Hoshino, *Journal of the American Chemical Society*, **2016,** *136*, 4282 – 4285.
Designed polymer hydrogel nanoparticles (NPs) capable of facilitating resolubilization and refolding of an aggregated protein, positively charged lysozyme, are prepared. NPs designed to interact strongly with denatured lysozyme and relatively weakly with native lysozyme, facilitated resolubilization and refolding of aggregated lysozyme. Such NPs could be prepared by copolymerizing optimized combinations and populations of functional monomers. The refolded lysozyme showed native conformation and enzymatic activity. Eleven grams of



aggregated protein was refolded by 1 g of NPs. However, NPs having low affinity to denatured lysozyme and NPs having high affinity to both denatured and native lysozyme showed relatively low facilitation activity. Our results suggest a potential strategy for the design of artificial chaperones with high facilitating activity.

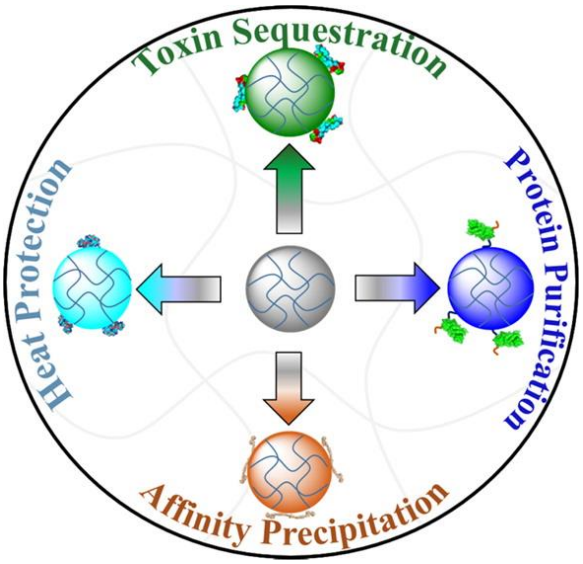
"Tuning Hydrophobicity in an Abiotic Affinity Reagent. Polymer Hydrogel Affinity Reagents for Molecules with Lipid-like Domains." Beverly Chou, Peter Mirau, Tian Jian, Szu-Wen Wang, and Kenneth J. Shea, *Biomacromolecules*, **2016**, *17*, 1860–1868.

Hydrophobic interactions often dominate the associative forces between biomacromolecules. A synthetic affinity reagent must be able to exploit and optimize these interactions. We describe synthesis of abiotic affinity reagents that sequester biomacromolecules with lipid-like domains. NIPAm-based copolymer nanoparticles (NPs) containing C4–C8 hydrophobic groups were evaluated for their affinity for lipopolysaccharides (LPS), the lipophilic component of the outer membrane of Gram-negative bacteria. Optimal affinity was found for NPs incorporating a linear C4 hydrocarbon group. 1D and 2D ^{1}H NMR studies revealed that in water, the longer chain (C6 and C8) alkyl groups in the hydrogel NPs were engaged in intrachain association, rendering them less available to interact with LPS.



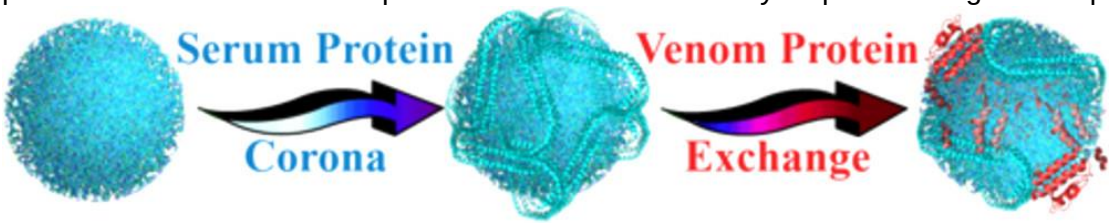
Optimal LPS–NP interaction requires maximizing hydrophobicity, while avoiding side chain aggregation. Polymer compositions with high LPS binding were grafted onto agarose beads and evaluated for LPS clearance from solution; samples containing linear C4 groups also showed the highest LPS clearance capacity.

"Tuning the Protein Corona of Hydrogel Nanoparticles. The Synthesis of Abiotic Biomacromolecule Affinity Reagents." *Jeffrey O'Brien, Kenneth J. Shea, Accounts of Chemical Research*, **2016**, *49*, 1200−1210.
Nanomaterials, when introduced into a complex, protein-rich environment, rapidly acquire a protein corona.
In this Account, we demonstrate that small libraries of synthetic polymer NPs incorporating a diverse pool of functional monomers can be screened for candidates with high affinity and selectivity to targeted biomacromolecules. Through directed synthetic evolution of NP compositions, one can tailor the protein corona to create synthetic organic hydrogel polymer NPs with high affinity and specificity to peptide toxins, enzymes, and other functional proteins, as well as to specific domains of large proteins. In addition, many NIPAm NPs undergo a change in morphology as a function of temperature. This transformation often correlates with a significant change in NP–biomacromolecule affinity, resulting in a *temperature-dependent* protein



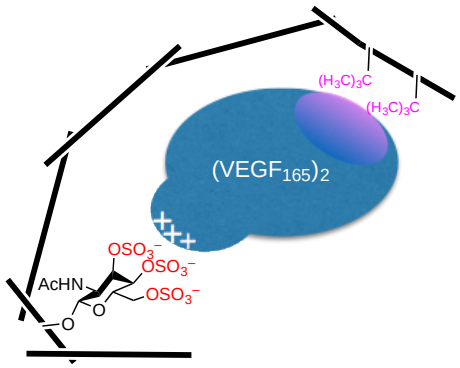
corona. This temperature dependence has been used to develop NP hydrogels with autonomous affinity switching for the protection of proteins from thermal stress and as a method of biomacromolecule purification through a selective thermally induced catch and release. In addition to temperature, changes in pH or buffer can also alter a NP protein corona composition, a property that has been exploited for protein purification. Finally, synthetic polymer nanoparticles with low nanomolar affinity for a peptide toxin were shown to capture and neutralize the toxin in the bloodstream of living mice.

"Engineering the Protein Corona of a synthetic polymer nanoparticle for Broad-Spectrum Sequestration and Neutralization of Venomous Biomacromolecules"
Jeffrey O'Brien, Shih-Hui Lee, Shunsuke Onogi, Kenneth J. Shea, *Journal of the American Chemical Society, **2016,** 138 16604 - 16607*.
Biochemical diversity of venom extracts often occurs within a small number of shared protein families. Developing a sequestrant capable of broad-spectrum neutralization across various protein isoforms within these protein families is a necessary step in creating broad-spectrum



antivenom. Using directed synthetic evolution to optimize a nanoparticle (NP) formulation capable of sequestering and neutralizing venomous phospholipase A_2 (PLA_2), we demonstrate that broad-spectrum neutralization and sequestration of venomous biomacromolecules is possible via a single optimized NP formulation. Furthermore, this optimized NP showed selectivity for venomous PLA_2 over abundant serum proteins, was not cytotoxic, and showed substantially long dissociation rates from PLA_2. These findings suggest that it may show efficacy as an in vivo venom sequestrant and may serve as a generalized lipid-mediated toxin sequestrant.

"Synthetic Polymer Protein Affinity Reagents. A Polymer Nanoparticle with Engineered Affinity for a Vascular Endothelial Growth Factor ($VEGF_{165}$), Hiroyuki Koide, Keiichi Yoshimatsu, Yu Hoshino, Shih-Hui Lee, Ai Okajima, Saki Ariizumi, Yudai Narita, Yusuke Yonamine, Adam C. Weisman, Yuri Nishimura, Naoto Oku1, Yoshiko Miura, and Kenneth J. Shea, *Nature Chemistry*, **2017**, *9*, 715 – 722.



Protein affinity reagents (most commonly antibodies) are widely used in basic research, diagnostics and separations and for clinical applications, the most common of which are antibodies. However, they often suffer from high cost, and difficulties in their development, production and storage. Here we show that a synthetic polymer nanoparticle (NP) can be engineered to have many of the functions of a protein affinity reagent. Polymer NPs with nM affinity to a key vascular endothelial growth factor (VEGF165) inhibit binding of the signalling protein to its receptor VEGFR-2, preventing receptor phosphorylation and downstream VEGF165-dependent endothelial cell migration and invasion into the extracellular matrix. In addition, the NPs inhibit VEGF-mediated

new blood vessel formation in Matrigel plugs in vivo. Importantly, the non-toxic NPs were not found to exhibit off-target activity. These results support the assertion that synthetic polymers offer a new paradigm in the search for abiotic protein affinity reagents by providing many of the functions of their protein counterparts.

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