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Affinity Purification of Multifunctional Polymer Nanoparticles

Yu Hoshino,^{*,†} Walter W. Haberaecker III,[†] Takashi Kodama,[‡] Zhiyang Zeng,[†] Yoshio Okahata,[§] and Kenneth J. Shea^{*,†}

Department of Chemistry, University of California, Irvine, California 92697, Department of Mechanical Engineering, Stanford University, Stanford, California 94305, and Department of Biomolecular Engineering, Tokyo Institute of Technology, Yokohama 226-8501, Japan

Received July 4, 2010; E-mail: yhoshino@chem-eng.kyushu-u.ac.jp; kjshea@uci.edu

Abstract: 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 ($K_{\text{dapp}} \approx \text{nM}$) and a narrower affinity distribution than the average of particles before purification ($K_{\text{dapp}} > \mu\text{M}$) 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.

General procedures for the creation of synthetic materials with biomacromolecular recognition sites are of significant interest as a route to stable, robust, and mass-produced substitutes for antibodies.^{1–8} Ideally, recognition of complex biological targets, including proteins, peptides, and carbohydrates, requires multiple functional groups that contact target molecules by a combination of electrostatic, hydrogen-bonding, van der Waals, and/or hydrophobic interactions. It has been shown that copolymerization of optimized combinations and ratios of functional monomers creates synthetic polymer materials with molecular recognition sites.^{1–5} However, in contrast to antibodies whose exact sequence can be determined and cloned, polymerized materials result in heterogeneous structures with a distribution of recognition sites.^{1,3,7} This is an intrinsic property of polymers synthesized under kinetic control, in contrast to the synthetic small molecular hosts prepared by multistep reactions⁹ or by self-assembly under equilibrating conditions.¹⁰ Here we demonstrate a general procedure to purify synthetic polymer nanoparticles (NPs) with high-affinity binding sites for a target biomacromolecule from a random pool of multifunctional copolymer nanoparticles (MFNPs). These nanoparticles are approximately the size of a large protein and are “purified” on the basis of peptide affinity as in the case of antibodies, using an affinity chromatography strategy.

The concept of affinity purification of NPs was demonstrated with melittin, a 26 amino acid peptide (Figure 1a), as the target molecule. Melittin has six positive charges of which four are localized in a hydrophilic 6 amino acid sequence on the C-terminus. The remaining 20 amino acids have a high proportion of apolar residues.¹¹

For the MFNPs, we chose cross-linked *N*-isopropylacrylamide NPs (~30 nm) incorporating hydrophobic *N*-*tert*-butylacrylamide

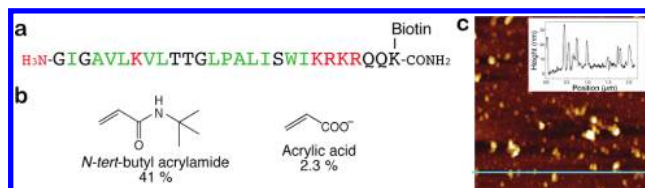


Figure 1. (a) Amino acid sequence of melittin. Hydrophobic and positive charged residues are printed in green and red respectively. (b) The chemical structures of functional monomers used for NP synthesis. (c) Solution phase AFM images of NPs. A height profile of cross section (light blue line) is shown in insert.

(TBAm) and negatively charged acrylic acid (AAc) functional monomers (Figure 1b). We have reported that NPs with this composition interacts with melittin ($K_{\text{dapp}} = 46 \mu\text{M}$) via both electrostatic and hydrophobic interactions in PBS (35 mM phosphate buffer/0.15 M NaCl, pH 7.3).⁵ However, the melittin affinity of the NPs exhibits a significant medium effect; there is only a modest affinity for melittin in low salt containing media such as water. The medium effect may be attributed to hydrophobic interactions between melittin and the NPs that are enhanced by the salting out effect of sodium phosphate and chloride ion.¹²

NPs were synthesized by a free radical copolymerization. To quantify the concentration of the NPs by fluorescence and to monitor the hydration of polymer chains by the solvatochromic shift of λ_{em} , *N*-[2-[[[5-(dimethylamino)-1-naphthalenyl]sulfonyl]-amino]ethyl]-2-methyl-2-propenamide was synthesized and incorporated (1 mol %) into the NPs (Supporting Information).¹³ The concentrations of total monomer, cross-linker, and surfactant (sodium dodecyl sulfate) were optimized to adjust the size of the NPs to approximately 30 nm.^{5,8,14} Incorporation of the *tert*-butyl (41 mol %) and carboxylic acid (2.3 mol %) group in the MFNPs was quantified by ¹H NMR and inverse-gated ¹³C NMR utilizing ¹³C-enriched AAc (Supporting Information). NP size was determined by DLS and AFM (Figure 1c).

The protocol for affinity sorting is shown in Figure 2a. To sort MFNPs on the basis of melittin affinity, we immobilized melittin on avidin agarose beads thru an avidin–biotin interaction (Supporting Information). NPs are first incubated with melittin-agarose beads for 2 h at 25 °C. Here, 100 mM phosphate buffer was used as the NP adsorption media since interactions between melittin and NPs in water were too weak to isolate sufficient amounts of NPs for study (Supporting Figure 1). The supernatant was then separated from the beads, and the concentration of NPs remaining in the supernatant was quantified by fluorescence. When NPs (12 $\mu\text{g mL}^{-1}$) were incubated with different volumes of melittin-immobilized beads, the amount of NPs that were captured by the beads depended on the volume of melittin beads when the volume of beads was less than ~20 $\mu\text{L mL}^{-1}$. However, when more than ~20 $\mu\text{L mL}^{-1}$ of beads were incubated, no further capture of NPs was

[†] University of California.[‡] Stanford University.[§] Tokyo Institute of Technology.

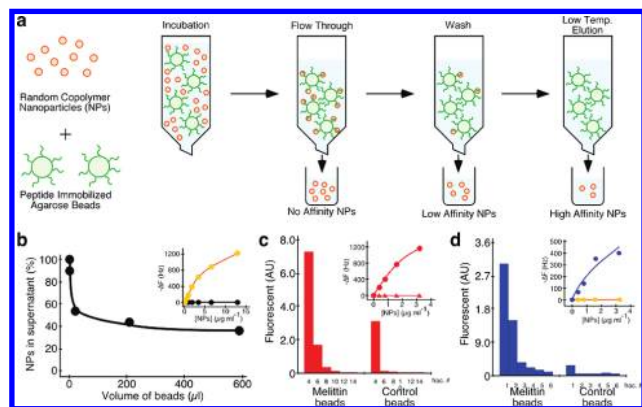


Figure 2. (a) Protocol for affinity sorting. Randomly copolymerized NPs are incubated with melittin immobilized agarose beads in 100 mM phosphate buffer (pH 7.4) at 25 °C. Ratio of NPs remaining in solution after incubation with different volumes of melittin immobilized beads is plotted in (b). Interaction (QCM) between melittin and NPs before incubation (yellow) and remaining after incubation (gray) is shown in (b) insert. NPs on the beads were first washed with water at 25 °C and then eluted with cold water (1 °C). Each cycle (12 h incubation) was repeated until no further elution of NPs was observed. Fluorescent intensity of NPs in each fraction from washing (red) and cold elution (blue) cycles is plotted in (c) and (d) respectively. Interaction between melittin and wash fraction in 100 mM phosphate buffer (red circles) and in water (red triangles) at 25 °C is shown in (c) insert. Interaction between melittin and NPs before incubation (yellow) and cold elution (blue) in water at 25 °C is shown in (d) insert.

observed; approximately 40% of the NPs remained in the supernatant (Figure 2b). The interaction between melittin and NPs *before incubation* or the *unbound fraction* remaining in the supernatant *after incubation* was analyzed by 27-MHz QCM in 100 mM phosphate buffer.^{5,8,15} The QCM results showed that MFNPs in the *unbound fraction* did not interact with melittin, although the MFNPs *before incubation* showed a modest interaction with melittin under the same conditions (Figure 2b insert). This indicates the as-synthesized MFNPs are comprised of a heterogeneous mixture of particles, of which some have melittin affinity under the binding conditions (100 mM phosphate) and others do not.

Melittin immobilized beads that were incubated with NPs (120 $\mu\text{L mL}^{-1}$) were washed extensively (12 h/cycle) with pure water at 25 °C. During the first few cycles, quantities of NPs were washed from the beads. However, the concentration of NPs diminished with every washing cycle and no NPs were detected after the eighth cycle. QCM revealed that the NPs from this *wash fraction* rebound with melittin in 100 mM phosphate buffer but little interaction was observed in water (Figure 2c insert), indicating NPs that recognize melittin in 100 mM phosphate buffer but not in water were removed from the beads in this washing step (Figure 2c).

In the general protocol for affinity purification of proteins, isolated molecules on the solid affinity support can be released by selective elution induced by either pH and/or salt gradients or by adding an excess of competing ligand.¹⁶ In this study, we capitalized on the temperature-induced phase change of the NIPAm based MFNPs.^{2,5,14} Our NPs undergo a thermal phase transition at ~ 10 °C (Supporting Information, Supporting Figure 2).¹⁴ Above the transition temperature, where they are synthesized, and where affinity (adsorption) studies are carried out, they have a collapsed structure, with some containing melittin affinity sites. Below the transition temperature they are solvent swollen and little affinity to melittin was observed.⁵

When beads washed at 25 °C were incubated at 1 °C in water for 12 h, significant amounts of NPs were released from the melittin-immobilized beads (Figure 2d). In contrast, relatively few NPs were

eluted from agarose beads that were not functionalized with melittin (control beads). A QCM experiment revealed that the NPs that were released by this *cold elution* were found to strongly interact with melittin at 25 °C in pure water ($K_{\text{dapp}} = 0.66\text{--}2.3$ nM, Figure 2d insert). This affinity is comparable to a typical antibody–antigen interaction. In addition, the affinity switching was reversible through 5 cycles of cooling (1 °C) and warming (25 °C). *Cold elution* was observed from melittin-immobilized beads for the first few elution cycles (12 h/cycle). The amount of NPs released after the third elution cycle however was dramatically lower, and no NPs were detected after the sixth elution cycle (Figure 2d). This establishes that the affinity for melittin could be dramatically lowered by cooling to 1 °C resulting in their release from the agarose beads. The yield of NPs in the *cold elution step* is several percent, but importantly they showed a significantly stronger affinity for melittin than the NPs *before affinity sorting*. (Note: the interaction measured by QCM in water was not observed *before sorting* (Figure 2d insert).) To compare the affinity distribution of NPs in solution *before sorting* and those released by *cold elution*, each batch of NPs (240 ng mL^{-1}) was incubated with melittin immobilized beads in water at 25 °C overnight. Although only a portion of the NPs were captured from the NPs *before sorting*, all of the NPs in the *cold elution* were recaptured by melittin-immobilized beads (Supporting Figure 3). This result indicates that the affinity distribution of the NPs released by *cold elution* is narrower than the NPs *before sorting*.

From these results, we conclude that synthetic polymer nanoparticles with a high affinity for the peptide melittin can be “purified” from a random pool of multifunctional copolymer nanoparticles by an affinity chromatography strategy. Each fraction isolated during the affinity sorting process shows a different affinity for the target peptide. The “selected” NPs have a much stronger and narrower affinity distribution than the materials before purification. We anticipate this affinity purification is applicable for most nanosize materials for molecular recognition including molecularly imprinted NPs⁸ and functionalized inorganic NPs¹⁷ and will become an integral step for the preparation of “plastic antibodies” with a near-homogeneous and tailor-made affinity for target molecules.

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Supporting Information Available: Experimental procedures and supporting data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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