

Epitope Discovery for a Synthetic Polymer Nanoparticle: A New Strategy for Developing a Peptide Tag

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S Supporting Information

ABSTRACT: 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.

Engineered synthetic polymer nanoparticles (NPs) with an intrinsic affinity and selectivity for target biomacromolecules are of significant interest for use in diagnostics,¹ therapeutics,² and protein purification³ and as tools to investigate biochemical processes.⁴ Recent studies show that synthetic NPs incorporating functional groups complementary to a surface domain of a target biomacromolecule can result in a high intrinsic affinity for target peptides,^{2c,5} proteins,^{2a,3,6} and polysaccharides.⁷ These materials are attractive as an inexpensive and robust alternative to affinity reagents of biological origin, including antibodies. Here we report a strategy for identifying peptides with high affinity for a synthetic polymer NP. The affinity of the NP/peptide pair is exploited for use in recombinant protein purification.

In previous studies synthetic NPs, with an intrinsic biomacromolecule affinity, were developed by a screening process from a library of NPs containing various ratios and combinations of functional groups.^{3,5–7} “Hits” with target

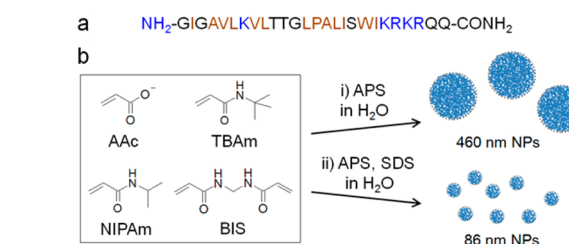


Figure 1. (a) Amino acid sequence of melittin. Positively charged and hydrophobic residues are shown in blue and brown, respectively. (b) Preparation of polymer nanoparticles.

binding affinity are subsequently fine-tuned by varying the functional group composition to improve the binding affinity.^{2c,3b} For example, we previously reported synthetic polymer NPs with high intrinsic affinity ($K_d = \mu\text{M}$ to low nM) for melittin.^{2c,5} Incorporating both negatively charged and hydrophobic functional groups into the NPs was found to be essential to create NPs with high melittin affinity.⁵ Analysis of the functional group composition of the NP and target binding affinity is a general strategy to identify the important factors to NP/biomacromolecule interactions.^{3b,5–8}

We describe an alternative strategy for creating “complementary pairs” of polymer NPs and biomacromolecules, one that involves modifying the target biomacromolecule to achieve high affinity to a specific polymer NP. Site-specific mutation, deletion, and truncation of peptides and proteins are routinely utilized by biochemists to identify essential residues for their function or to engineer their properties. Fusing peptide or protein tags with affinity for biopolymers,⁹ immobilized metal ions,¹⁰ low-molecular-weight ligands,¹¹ and proteins¹² is commonly done to assist purification of recombinant proteins.¹³ Here we draw upon these approaches to create a polymer NP/peptide tag affinity pair and demonstrate the utility of the synthetic polymer NP for protein purification. The identified NP/peptide tag pair only requires fusion of a relatively short peptide tag and inexpensive separation media. Hence, our strategy offers promise in providing a cost-effective protein purification method without requiring large fusion tags.

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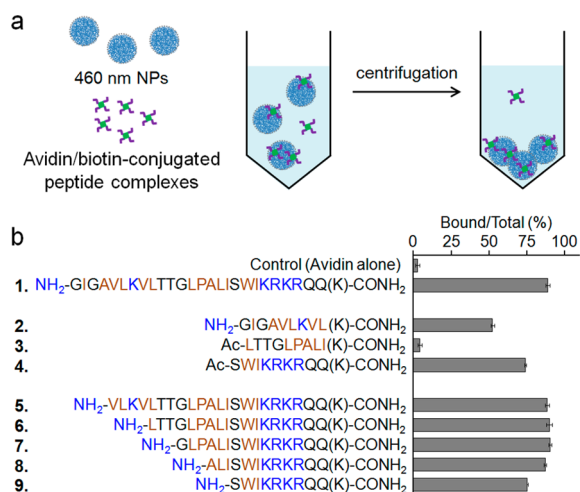


Figure 2. (a) Centrifugal sedimentation method for evaluating binding. (b) Binding of avidin/biotin-conjugated peptide complexes (4 μ M) to 460 nm NPs (3.0 mg/mL) in 35 mM sodium phosphate buffer containing 150 mM NaCl (pH 7.3). Biotin was conjugated to peptides through the C-terminal lysine.

The amino acid (aa) sequence of melittin (Figure 1a) was used as a “lead” sequence for the peptide tag in this study. However, melittin is a relatively long (26 aa) peptide with cytotoxicity and antimicrobial activity¹⁴ and thus is unsuitable as a fusion tag. Our approach was to seek an “epitope sequence” that is short and nontoxic but with sufficiently strong affinity to the NP. Polymer NPs, ~86 nm in diameter, were synthesized by pseudoprecipitation polymerization of *N*-isopropylacrylamide (NIPAm), *N*-*tert*-butylacrylamide (TBAm), acrylic acid (AAc), and *N,N'*-methylenebisacrylamide (BIS) in aqueous solution.¹⁵ NPs of ~460 nm diameter were synthesized by an identical procedure but without SDS. The melittin binding capacity of 460 and 86 nm NPs was determined to be 34 and 205 μ g/mg, respectively (Figure S2). Although the ~86 nm NP had a somewhat higher melittin capacity, both NPs were capable of efficiently capturing and neutralizing melittin.

Avidin/biotin-conjugated peptide complexes were utilized as “pseudotagged” proteins in our initial study. A series of avidin proteins derivatized with melittin-derived peptide tags were prepared by mixing solutions containing avidin and biotin-conjugated peptide tag candidates. Avidin is a tetrameric protein; thus each complex contains four identical peptide tags. After incubation for 30 min, solutions were subjected to centrifugation, and the amounts of unbound complexes in the supernatant were determined by UV absorption at 280 nm (Figure 2a). As the 460 nm NPs can be easily sedimented by centrifugation, they were utilized for rapid screening of the NP/“tagged” avidin interactions.

Figure 2b shows the percentage of the avidin/biotin-conjugated peptide complexes that are bound to the NPs. Approximately 90% of avidin/peptide complex was bound to the NPs upon attachment of peptide 1 (the entire melittin sequence), whereas avidin itself did not bind to the NPs. This result establishes that interactions between a synthetic polymer NP and a protein can be significantly strengthened by attachment of a short peptide tag to the protein. We next evaluated the binding of avidin “tagged” with peptides 2–4, truncated peptides that are composed of nine consecutive residues of the N-terminal, central, and C-terminal portions of melittin. Peptide 3 (with several hydrophobic residues but no formal charge) did not

establish NP binding affinity to the modified avidin complex. However, upon attachment of peptides 2 (with two formal positive charges) and 4 (with four positive charges), 54% and 74% of the complexes, respectively, were bound by the NPs. This observation points to the importance of positive charge for the interaction with the NPs. The avidin/peptide 4 complex showed the highest binding of the three truncated peptides. However, the NP binding affinities of peptides 2–4 were substantially lower than that of peptide 1.

We hypothesized that the lower NP binding affinity of peptide 4 compared to peptide 1 is due to the reduced number of hydrophobic residues. A previous study reported that incorporating both negatively charged (AAc) and hydrophobic monomers (TBAm) is necessary for high NP-melittin affinity.⁵ To test this hypothesis, we evaluated the NP binding affinity of a series of N-terminal truncated melittin derivatives (peptides 5–9). Our aim was to engineer the lead sequence into a peptide tag that is shorter than peptide 1 but retains sufficiently strong affinity to the NP. Approximately 90% of avidin/peptide complexes bound to the NP upon attachment of peptides 5–8. These peptides contain five or six positive charges and five or more hydrophobic residues. The shortest peptide 9 had significantly lower affinity than peptides 5–8. Also noteworthy is that peptide 9, having the same number of residues as peptide 4 but with a positively charged α -amine group at the N-terminal, produced binding affinity similar to that of peptide 4. This observation suggests that the effect of possessing an additional positive charge was balanced by a decrease in hydrophobicity. Overall the results show that high affinity for the avidin/peptide complexes can be achieved with epitope peptides containing five or more of both positively-charged and hydrophobic residues.

Peptides 5–8 share some compositional common features with the calmodulin binding peptide tag (CBP-tag),¹⁶ a commonly used affinity tag,¹³ suggesting that these sequences may find utility upon fusion to proteins. However, as mentioned above, the cytotoxicity and antimicrobial activity of melittin may cause complications when a melittin-derived sequence is used as a fusion tag. To exploit its potential as a fusion tag, the challenge was to mitigate melittin’s toxicity without loss of NP binding affinity. Toxicity of the melittin-derived peptides was evaluated by its ability to lyse red blood cells.¹⁷ We found the red blood cell lysis activity of melittin is diminished by truncation at the N-terminus (Figure S5). Three modified (N-terminal truncated) epitope sequences were identified (6–8) as candidates. All three retained the desired NP binding affinity, but none induced red blood cell lysis, even at the highest tested concentration (300 μ M). Peptide 7, exhibiting “averaged” properties of the three sequences, was selected for further study. Plasmid vectors encoding green fluorescence protein (GFP) or glutathione-S-transferase (GST) with the sequence of peptide 7 (tMel-tag: GLPALISWIKRKRQQ) or control tag (GLSSGS) were constructed. The sequence for C-terminal His6-tag was also inserted in the plasmid vectors to allow purification of GFP constructs. The schematics of the recombinant proteins are shown in Figure 3a. The GFPs and GST were expressed in *E. coli* BL21 (DE3) strain and were purified using Ni²⁺-NTA and I-glutathione columns, respectively. Part of the purified tMel-thr-GFP (10) and tMel-thr-GST (11), containing a thrombin cleavage site between the tMel-tag and protein, was treated with thrombin to yield tag-cleaved control proteins. All obtained samples contained GFP or GST as the major component with varying amounts of contamination proteins (Figure S6).

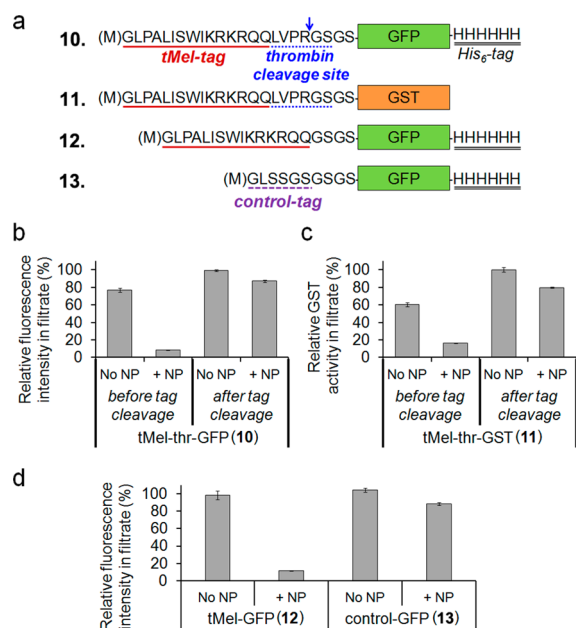


Figure 3. (a) Schematics of the expressed recombinant proteins. (b–d) Results of centrifugal filtration study. NP = 2.0 mg/mL; GFPs = 12 μ g/mL; GST = 73 μ g/mL. In 20 mM sodium phosphate buffer (pH 6.5) containing 0.1% Tween 20.

To evaluate the efficiency to capture and elute tagged proteins using the polymer NPs, a centrifugal-filtration method was employed. As the use of 460 nm NPs resulted in severe clogging of the membrane during the centrifugation, the 86 nm NPs were used for this experiment. The protein samples and the NPs (2.0 mg/mL) were mixed, incubated in a buffer solution, and passed through a centrifugal filtration device (Nanosep, Pall Corp. with molecular weight cutoff = 100 kDa) to filter NPs and NP-bound proteins. Figure 3b,c shows the fluorescence intensity and GST activity of the filtrates, respectively. We first studied how the presence or absence of tMel-tag sequence affects the binding affinity between the proteins and the NP. tMel-thr-GFP (**10**) and tMel-thr-GST (**11**) could be efficiently depleted from the solution by the NPs, whereas in the absence of NPs, significantly greater amounts of **10** and **11** passed through the filter membrane and were found in the filtrates. When tags were first cleaved from proteins by thrombin treatment and then incubated with NPs, the majority of **10** and **11** did not bind to the NPs and was found in the filtrates. These results establish that tMel-tag, a 15-aa peptide, can provide effective NP binding affinity to two distinct proteins with molecular weights (MWs) of 26–27 kDa (229–238 aa). The small decrease in fluorescence intensity or GST activity observed when the protein solutions were filtered without NPs can be attributed to adsorption of the tagged proteins onto the filter membrane. On the other hand, tMel-GFP (**12**) without a thrombin recognition sequence was found to be less “sticky” to the filter membrane while still being efficiently captured by the NPs (Figure 3d). Control-GFP (**13**) without tMel-tag or a thrombin recognition sequence passed through the filter membrane both in the presence and in the absence of NPs. The binding affinity of tMel-GFP (**12**) with NPs was also studied by incubating a fixed concentration of tMel-GFP (12 μ g/mL) with various concentrations of the NPs (Figure S7): 50% of tMel-GFPs could be captured by solutions containing 0.75 mg/mL of NPs, and ~90% (~10.8 μ g/mL) of tMel-GFP could be captured by a 120-fold excess (by weight, 1.5 mg/mL) of the NPs.

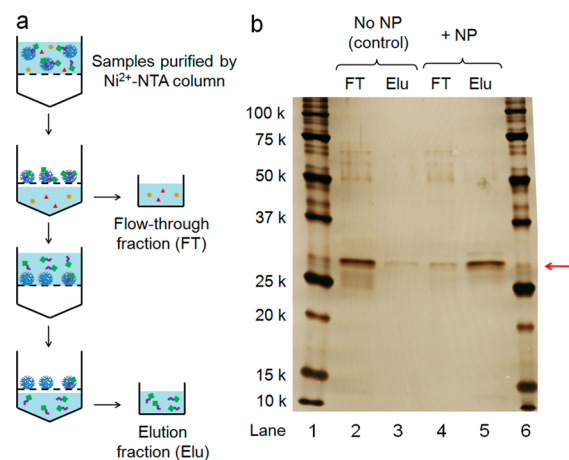


Figure 4. (a) Protein purification procedure. (b) SDS-PAGE analysis of flow-through fractions (FT) and elution fractions (Elu). Lanes 1 and 6: molecular weight markers. Lane 2: FT from the “No NP” control. Lane 3: Elu from the “No NP” control. Lane 4: FT that shows the efficient depletion of tMel-GFP by the NPs. Lane 5: Elu that contains purified tMel-GFP. The band of tMel-GFP is indicated by a red arrow.

Finally, we examined the applicability of the NP/peptide tag interaction for “polishing” protein samples that were prepurified by immobilized metal ion chromatography (IMAC). The tandem use of two affinity purification steps is an increasingly important method¹⁸ to prepare highly purified proteins¹⁹ and to isolate native protein complexes to investigate protein–protein interaction networks.²⁰ Generally, IMAC tends to copurify histidine-rich proteins with His₆-tagged proteins because the retention is based on the coordination of histidine side chains and transition metal ions (e.g., Ni²⁺, Co²⁺).²¹ The NP/tMel-tag interaction utilizes electrostatic and hydrophobic interactions and can work in a complementally manner with IMAC. In addition, the experiments using avidin/peptide complexes showed that the NPs interact strongly only with peptide tags containing more than a certain number of both hydrophobic and positively charged residues. We anticipated that the NPs can selectively capture tMel-GFP but without adsorbing the residual contamination proteins in the IMAC purified samples. The experimental design is outlined in Figure 4a. In the first capture step, prepurified samples were incubated with NPs (1.5 mg/mL) and filtered. The control experiment was carried out in the same manner but in the absence of NPs. The result of SDS-PAGE analysis of the flow-through fractions showed that tMel-GFP is efficiently captured by the NPs, while the contamination proteins with NW = 40–70 kDa did not bind and were found in the flow-through fraction (Figure 4b, lanes 2 and 4).

To elute the captured tMel-GFPs from the NPs, the effects of several solutions were screened. Solutions with high ionic strength are commonly used to elute proteins in ion-exchange chromatography. However, our initial attempt to use buffer solutions containing 150 or 250 mM NaCl did not afford efficient elution of tMel-GFPs from the NPs. Solutions containing 100 μ M or 1 mM of a cationic surfactant (cetyl trimethylammonium bromide, CTAB), used to elute chymotrypsin from functionalized gold NPs,²² were also ineffective. Instead, we found that ~70% of tMel-GFP (based on fluorescence intensity) could be recovered by incubation in 20 mM phosphate buffer (pH 6.5) containing 0.1% of Tween 20 and 250 mM guanidine hydrochloride (GuaHCl). SDS-PAGE analysis showed that highly purified tMel-GFP (>95% in band intensity, Figure 4b,

lane 5) and the contamination proteins with MW = 40–70 kDa, that occupied ~10% of the total intensity of bands in Figure 4b, lane 2, could be efficiently eliminated. In a separate experiment, we confirmed that adding 250 mM GuaHCl did not significantly affect the fluorescence intensity of tMel-GFP. This result indicates that the concentration of GuaHCl used in this study does not denature tMel-GFP. We attributed the elution of tMel-GFP to disruption of tMel-tag/NP interactions by guanidium ions and not to denaturation of the protein. In general, the presence of 250 mM GuaHCl reduces free energy differences between folded and unfolded states of proteins by 0.1–2.5 kcal/mol, and a typical total free energy change of protein folding is of the order of 5–12 kcal/mol.²³ Hence, solution containing 250 mM GuaHCl can also be used to elute many other proteins. Although elution conditions might need to be further optimized for relatively unstable proteins, the result clearly shows utility of polymer NPs to selectively isolate tagged proteins without large loss of function.

In conclusion, we demonstrate a novel epitope discovery strategy for identifying an epitope tag (short optimized 15-residue peptide tag) that exhibits high affinity for a synthetic polymer nanoparticle. The peptide sequence (tMel-tag) was fused to two recombinant proteins (GFP and GST) and the ability of the synthetic polymer NPs to effectively capture the tagged proteins was confirmed. The NP/tMel-tag interaction was used for polishing recombinant protein samples that were prepurified by IMAC. In contrast to most currently available tandem affinity tag-based protein purification systems, requiring fusion of large peptide tags and/or use of at least one costly protein-immobilized separation medium, the combination of NP/tMel-tag- and IMAC/His-tag-based systems only requires fusion of a relatively short peptide tag and inexpensive separation media. Thus, the synthetic polymer NP/short peptide tag pair can be an attractive alternative. Furthermore, since NPs have been generated with antibody-like affinity for a number of target proteins and peptides, the described strategy can result in a large number of NP/peptide combinations. We also envision that the concept of protein capturing via NP/tag interactions might be suitable for other applications, including modulating protein activity and delivering therapeutic proteins.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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