

# Nanoparticles that Distinguish Chemical and Supramolecular Contexts of Lysine for Single-Site Functionalization of Protein

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**ABSTRACT.** Lysine is one of the most abundant residues on the surface of protein and its site-selective functionalization is extremely challenging. The existing methods of functionalization rely on differential reactivities of lysine on a protein, making it impossible to label less reactive lysines selectively. We here report polymeric nanoparticles that mimic enzymes involved in the posttranslational modifications of proteins that distinguish the chemical and supramolecular contexts of a lysine and deliver the labeling reagent precisely to its  $\epsilon$  amino group. The nanoparticles are prepared through molecular imprinting of cross-linkable surfactant micelles, plus an in situ, on-micelle derivatization of the peptide template prior to the imprinting. The procedures encode the polymeric nanoparticles with all the supramolecular information needed for sequence identification and precise labeling, allowing single-site functionalization of a predetermined lysine on the target protein in a mixture.

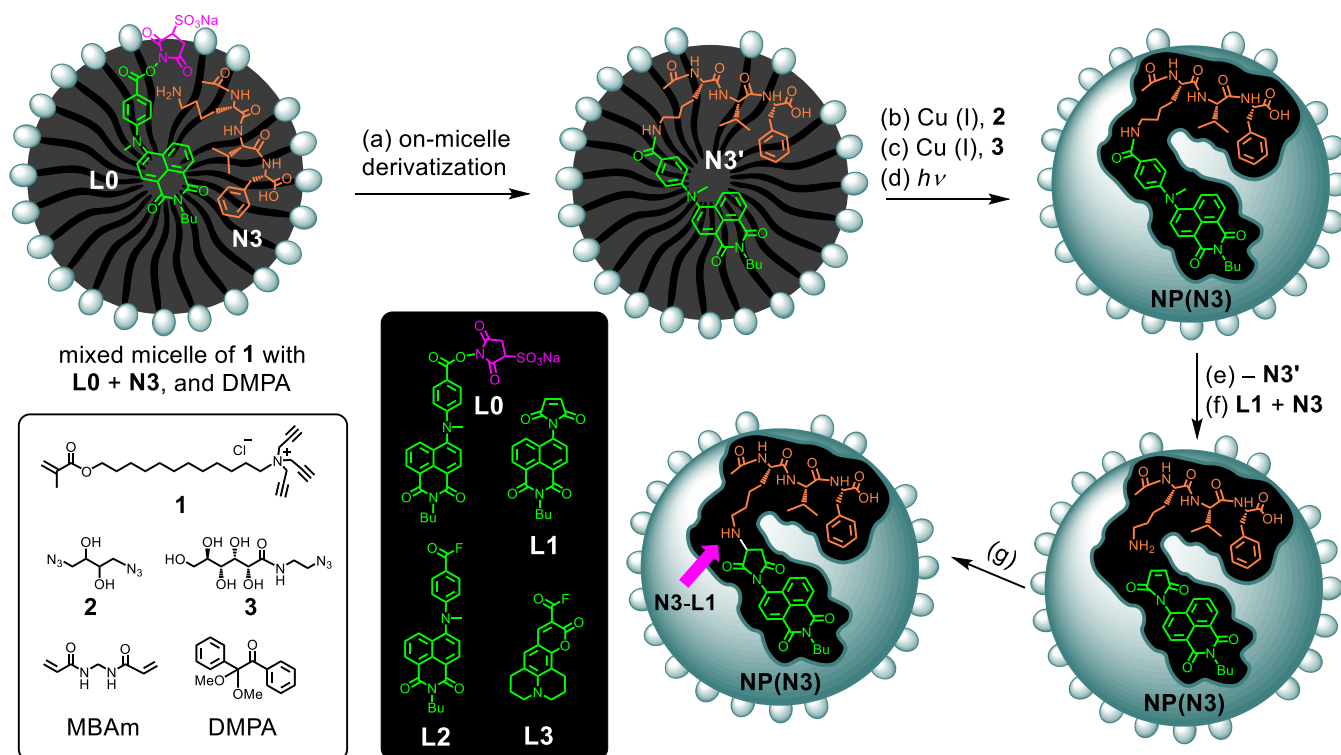
**Key words:** protein modification, binding, molecular imprinting, site selectivity, micelle

Cells use posttranslational modifications (PTMs) to diversify the proteome.<sup>1-4</sup> To perform this type of high precision chemistry, the enzymes involved have to recognize the chemical, supramolecular, and sometimes environmental contexts of an amino acid (AA) and then transform it while potentially a vast number of identical groups are present on the same and different proteins. If the same level of site-selectivity can be achieved chemically, researchers would not only have access to pure proteins with specific PTMs but also be able to functionalize proteins at precise positions for imaging, mechanistic studies, and/or added biological functions.<sup>5-10</sup> Site-selectivity in protein modification generally requires pre-installation of unnatural amino acids or a special peptide sequence. The biggest challenge in direct, site-selective reaction on an “off-the-shelf” protein is two-fold. First, exclusive site-selectivity is required on a highly complex biomolecule. In the literature, site selectivity in chemical-based protein modification is typically derived from differential reactivity, e.g., at the N-terminal amine,<sup>11</sup> C-terminal carboxylic acid,<sup>12</sup> and surface-exposed, rare amino acids.<sup>13-16</sup> Site-selective functionalization of surface-abundant groups at an arbitrary location is currently not possible. Second, the reaction needs to occur under mild conditions in aqueous solution at low concentrations, as proteins easily aggregate at high concentrations and often not available in large quantities.

Lysine is the most abundant amino acid on the surface of both cytoplasmic and extracellular proteins.<sup>17</sup> Making up about 5.9% of all amino acids in human proteins, it is an ideal site for functionalization given its nucleophilicity, abundance, and surface accessibility. Even though many methods for lysine functionalization have been reported in recent years,<sup>18-26</sup> the dominant strategy for selectivity depends on differential reactivity.<sup>27</sup> Not only is it difficult to achieve exclusive site selectivity in this way (due to insufficient differences in reactivity), selective labelling of less reactive lysines also is difficult or impossible. Lysine is one of the most post-translationally modified residues.<sup>28,29</sup> If the more reactive lysines happen to be involved in the biological functions of a protein that must be preserved, labelling of less reactive lysines is then required.

To perform direct, site-selective modification of a protein, one has to first recognize the targeted amino acid to define its chemical and supramolecular contexts and then deliver the labeling reagent precisely to

the functional side chain. In this way, a moderately reactive labeling agent will be able to react at the targeted site(s) due to a proximity effect,<sup>30,31</sup> while reactions at nontargeted reactive sites are avoided. The strategy is illustrated in Scheme 1, using peptide-binding nanoparticles prepared through molecular imprinting.<sup>32-40</sup> Although nanoparticles have been widely used in peptide and protein binding,<sup>41</sup> exquisite molecular recognition is required for our purpose. Toward this end, a peptide template (i.e., **N3** or the N-terminal peptide of lysozyme containing the first three residues of KVF with the N-amino group acetylated) is first solubilized in water using the micelles of **1**, along with an anionic activated ester (**L0**). Most peptides are amphiphilic in nature and thus prefer the same location of the micelle as the amphiphilic activated ester—i.e., the surfactant/water interface.<sup>42</sup> The higher effective concentrations of the two facilitate the on-micelle derivatization of the peptide, to afford **N3'** in situ (Scheme 1, step a). It should be mentioned that peptide **N3** contains a single reactive amino group (on the lysine side chain) and thus can only afford one possible amide product.



**Scheme 1.** Preparation of NP(N3) for the labeling of lysine amino side chain of Ac-KVF. The surface ligands (clicked **3**) are omitted for clarity.

The surface of the micelle of the tripropargylated surfactant **1** is covered with a layer of terminal alkynes and is readily cross-linked by diazide **2** via the highly efficient alkyne–azide cycloaddition (step b).<sup>43,44</sup> Another round of click reaction using monoazide **3** installs a layer of hydrophilic ligands on the micelle surface (step c, the ligands are omitted from the drawings in Scheme 1 for clarity). The micelles also contain 2,2-dimethoxy-2-phenyl-acetophenone (DMPA, a photoinitiator). UV irradiation initiates free radical polymerization/cross-linking of the micelle core, around the modified peptide template **N3'** (step d). Precipitation of the reaction mixture into acetone and solvent washing remove the templates (step e), to afford **NP(N3)**—i.e., the molecularly imprinted nanoparticle prepared using the derivatized **N3'** as the template to bind peptide **N3** in the labeling experiment.

**NP(N3)** is designed to bind both **N3** and a labeling reagent such as **L1** that resembles the green-colored substructure of the derivatized **N3'** in size, shape, and hydrophobicity. Also, the binding is expected to position the  $\epsilon$  amino group of the lysine of **N3** right next to the maleimide electrophile of the bound **L1** (step f), greatly facilitating the conjugation of the two to afford **N3-L1** in situ (step g).

Consistent with successful imprinting, **NP(N3)** binds its original templating peptide **N3** with a large binding constant of  $K_a = 119.0 \times 10^4 \text{ M}^{-1}$  in 10 mM HEPES buffer (Table 1, entry 1). Not only so, it also binds the parent protein strongly. The decrease in the binding constant for lysozyme (by less than 2-fold) is understandable, given that some steric clashing could be encountered in the binding of the much larger protein guest. A strong binding by the nanoparticle is critical to our proposed protein functionalization because the labeling reaction needs to occur at low concentrations of the protein. A  $K_a$  value of  $69.7 \times 10^4 \text{ M}^{-1}$  (Table 1, entry 2) translates to a dissociation constant of 1.4  $\mu\text{M}$ , sufficient if the reaction is performed at micromolar concentrations (*vide infra*). Note that nonimprinted nanoparticles (NINPs) prepared without any peptide templates display a much weaker binding (entry 15), indicating that successful molecular imprinting has been achieved.

**Table 1.** Binding properties of NP receptors for lysozyme peptides and the parent protein determined by isothermal titration calorimetry (ITC).<sup>a</sup>

| entry | NP | guest | $K_a$ | $-\Delta G$ (kcal/mol) |
|-------|----|-------|-------|------------------------|
|-------|----|-------|-------|------------------------|

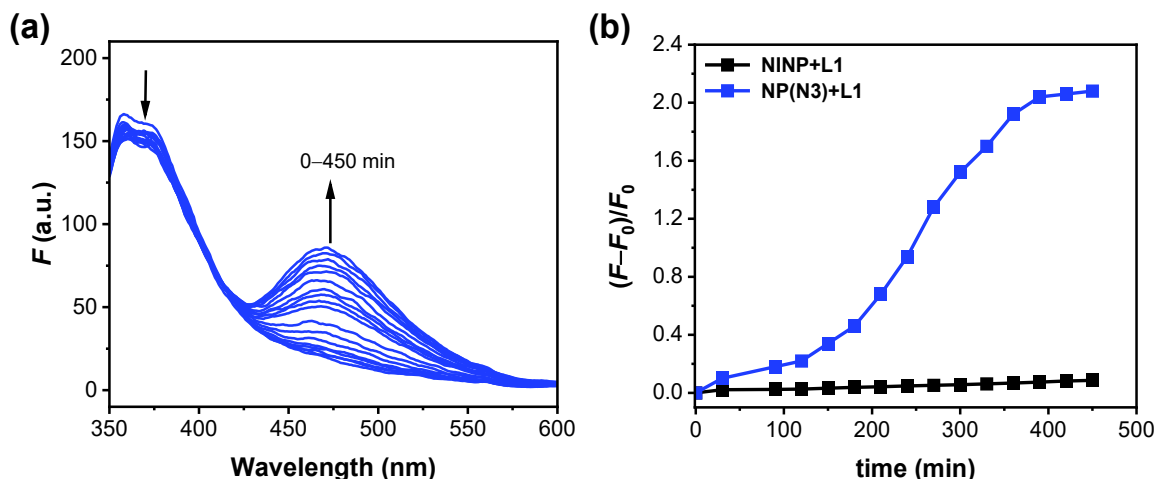
| $(\times 10^4 \text{ M}^{-1})$ |                |            |                  |      |
|--------------------------------|----------------|------------|------------------|------|
| 1                              | <b>NP(N3)</b>  | <b>N3</b>  | 119.0 $\pm$ 13.0 | 8.29 |
| 2                              | <b>NP(N3)</b>  | lysozyme   | 69.7 $\pm$ 10.0  | 7.97 |
| 3                              | <b>NP(N4)</b>  | <b>N4</b>  | 129.0 $\pm$ 6.8  | 8.34 |
| 4                              | <b>NP(N4)</b>  | lysozyme   | 75.0 $\pm$ 12.7  | 8.01 |
| 5                              | <b>NP(N5)</b>  | <b>N5</b>  | 143.0 $\pm$ 10.5 | 8.40 |
| 6                              | <b>NP(N5)</b>  | lysozyme   | 94.4 $\pm$ 9.3   | 8.15 |
| 7                              | <b>NP(N6)</b>  | <b>N6</b>  | 156.0 $\pm$ 14.4 | 8.45 |
| 8                              | <b>NP(N6)</b>  | lysozyme   | 110.0 $\pm$ 11.2 | 8.24 |
| 9                              | <b>NP(N7)</b>  | <b>N7</b>  | 178.0 $\pm$ 17.1 | 8.53 |
| 10                             | <b>NP(N7)</b>  | lysozyme   | 123.0 $\pm$ 12.9 | 8.31 |
| 11                             | <b>NP(N8)</b>  | <b>N8</b>  | 224.0 $\pm$ 55.7 | 8.66 |
| 12                             | <b>NP(N8)</b>  | lysozyme   | 141.0 $\pm$ 14.4 | 8.39 |
| 13                             | <b>NP(C10)</b> | <b>C10</b> | 235.0 $\pm$ 39.0 | 8.69 |
| 14                             | <b>NP(C10)</b> | lysozyme   | 133.0 $\pm$ 31.7 | 8.35 |
| 15                             | <b>NINP</b>    | <b>N8</b>  | <1.1             | --   |

<sup>a</sup> Titrations were performed at 298K in 10 mM HEPES buffer (pH=7.5) in triplicates, with the indicated errors. All the N-terminal peptides (**N3–N8**) have the N-terminal amino group acetylated.

The N-terminal peptides in Table 1 contain up to 8 residues (KVFGRC<sub>EL</sub>). Our binding data shows that the binding of **N8** by **NP(N8)** is about twice as strong as the binding of **N3** by **NP(N3)**, with the binding free energy only differing by about 0.37 kcal/mol. Hence, the first three residues represent the major contributor to the binding. A possible reason for this result could be the clustering of two hydrophobic amino acids (VF) at AA2 and AA3. Effective molecular imprinting of a template requires its inclusion in the micelle. Hydrophobic residues, especially close by, can help KVF enter the micelle more easily and be imprinted more effectively. The remaining residues in **N8**, especially GRCE, do not

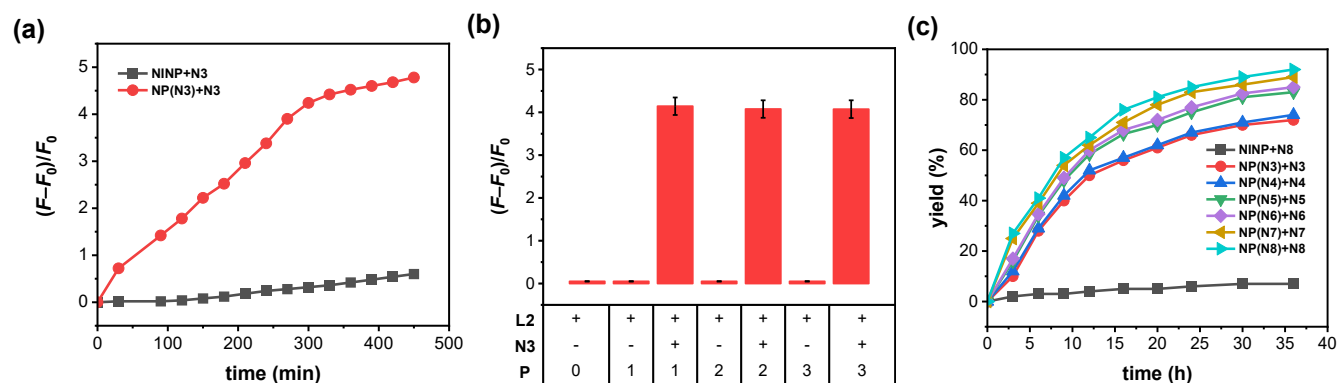
have a notable hydrophobic side chain, which could be the reason why they do not contribute as much to the overall binding. Importantly, the nanoparticles display strong binding toward their targeted (N- or C-terminal) peptides, as well as to the parent protein.

Figure 1 shows the fluorescence spectra of **L1** in the presence of **N3** and **NP(N3)** at room temperature over time. A distinctive new emission peak is observed at 468 nm (Figure 1a) that becomes more intensive over time (Figure 1b, blue curve).



**Figure 1.** (a) Fluorescence spectra of **L1** (40  $\mu$ M) in the presence of 40  $\mu$ M **NP(N3)** and 20  $\mu$ M of **N3** in water at room temperature over 450 min.  $\lambda_{\text{ex}} = 330$  nm. (b) Changes in the relative fluorescence intensity at 468 nm (blue line) for the reaction between 40  $\mu$ M of **L1** and 20  $\mu$ M of **N3** in the presence of 40  $\mu$ M of **NP(N3)**. The control experiment (red line) involves 40  $\mu$ M of **NINP** in place of **NP(N3)**.

The distinctive increase in fluorescence also occurs when an acid fluoride labeling reagent (**L2**) is mixed with **N3** and **NP(N3)** (Figure 2a). **L2** matches the green substructure of **N3'** even more than **L1**; its reaction thus is fully expected. Importantly, other lysine-containing tripeptides (AAK, AKK, and/or KKK) alone or together do not give the new peak upon mixing with **N3** and **NP(N3)** (Figure 2b). In addition, the large increase in fluorescence only appears when **N3** is in the solution, whether or not the other lysine-containing peptides are present. This is in full agreement with our proposed molecular recognition-based peptide derivatization.

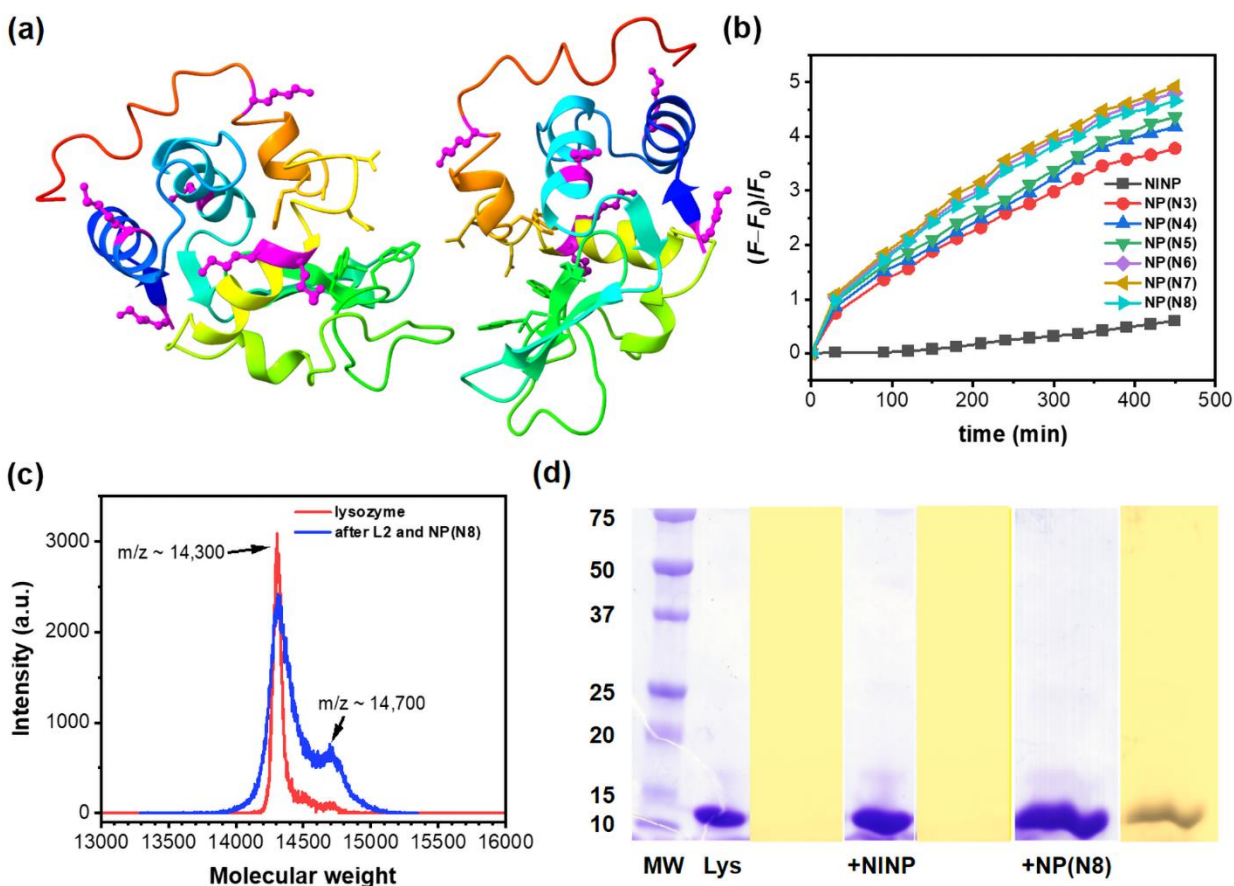


**Figure 2.** (a) Changes in the relative fluorescence intensity of 40  $\mu$ M of **L2** upon incubation with 20  $\mu$ M of **N3** and 40  $\mu$ M of **NP(N3)** or 40  $\mu$ M of **NINP** over time at room temperature. (b) Changes in the relative fluorescence intensity of 40  $\mu$ M of **L2** after 450 min under various labeling conditions. The row with the **N3** heading indicates the absence (–) or the presence (+) of the **N3** tripeptide Ac-KVF for the desired lysine functionalization. The row with the **P** heading indicates the absence (0) or the presence of 1 (AAK), 2 (AAK+AKK), or 3 (AAK+AKK+KKK) competing lysine-containing tripeptides in the labeling experiments. (c) Conversion yields in the labeling experiments using 40  $\mu$ M of **L2** and 20  $\mu$ M of **N3–N8** in the presence of 40  $\mu$ M of the corresponding **NP** hosts as a function of time at room temperature. The reaction yields were determined by LC-MS using calibration curves generated from authentic samples of the peptides.

To further confirm the nanoparticle-induced reaction, we determined the conversion yields of **N3** over a period of 36 h using LC-MS analysis (Figure 2c, red curve). Similar reactions happened with all the other N-terminal peptides (**N4–N8**) using their corresponding nanoparticle receptors. The yields are generally higher with an increase in the length of the peptide template, a result expected for binding-promoted chemical reactions. In contrast, negligible conversion of the peptide occurs in the presence of **NINP** (black curve), confirming the importance of molecular recognition in the reaction. Since the fluorescence increase in Figure 2a occurs in a similar time frame as the labeling reaction (Figure 2c), the stronger emission should come from the labeled product. Although both the labeling reagent and the labeled peptide contain the naphthaleneimide fluorophore, the labeling reaction happens at a site

electronically coupled to the naphthaleneimide and thus is expected to influence its emission. In addition, during the labeling reaction, the labeled peptide is expected to occupy the imprinted pocket better than the unlabeled peptide plus **L2**. Thus, the microenvironment around the fluorophore would also change subtly during the reaction, which could be another contributor to the emission change. Note that, **L1** and **L2** undergo almost no change in fluorescence (Figures 1b and 2a, black curve) in the control experiments using NINPs instead of **NP(N3)**.

Lysozyme contains 6 lysines (Figure 3a), all exposed to solvent and reactive in typical chemical derivatizations.<sup>45</sup> Encouragingly, the distinctive increase in fluorescence observed in peptides is also observed when **L2** is incubated with lysozyme in the presence of **NP(N3)**–**NP(N8)** (Figure 3b). After 24 h at room temperature, MALDI MS analysis shows a new peak ca. 400 m/z higher than the parent peak (Figure 3c). A single label of **L2** would add 385 Dalton to the molecular weight of the protein.



**Figure 3.** (a) Crystal structure of lysozyme viewed from two angles (PDB ID: 1HEW). Molecular graphics was created using UCSF Chimera. The peptide chain is colored from blue (N-terminus) through the

rainbow spectrum to red (C-terminus). Lysines side chains are shown in the ball-and-stick models and colored in magenta. **(b)** Changes in the relative fluorescence intensity for the reaction between 40  $\mu$ M of **L2** and 20  $\mu$ M of lysozyme in the presence of 40  $\mu$ M of **NP(N3)–NP(N8)** as a function of time at room temperature. NINP was used as the control. **(c)** MALDI Mass spectrum of lysozyme before (red) and after reaction (blue) with 40  $\mu$ M of **L2** and 20  $\mu$ M of lysozyme in the presence of 40  $\mu$ M **NP(N8)** in HEPES buffer (pH 7.5) after 24 h at room temperature. **(d)** SDS-PAGE gel of lysozyme before and after reaction with **L2** in the presence of NINP and **NP(N8)**, respectively, in HEPES buffer (pH 7.5) after 24 h at room temperature. The yellow panels show the gel bands under a Typhoon Alexa Fluor-488 scanner.

To confirm the fluorescent labeling, we performed SDS-PAGE on lysozyme before and after the treatment with **L2** and **NP(N8)** (Figure 3d). The gel shows nearly identical bands of protein after the treatment, indicating that the molecular weight of the protein undergoes little change upon the labeling. Importantly, fluorescent detection in the green channel reveals a fluorescent fraction in the front end of the protein band, indicating both (nonfluorescent) lysozyme and the labelled, fluorescent protein are present and the latter has a slightly higher molecular weight. This result is in full agreement with the MALDI results in Figure 3c. Significantly, this fluorescent band is absent when **L2** is used in combination NINP, indicating that the protein labeling also relies on molecular recognition of the imprinted nanoparticle.

To identify the site of labeling on lysozyme, we performed protein digestion using endoproteinase Glu-C, which cleaves the C-terminal side of either glutamic or aspartic acid. The digested sample was then analyzed using LC-MS/MS-based protein sequencing, which unequivocally shows that the lysine at the N-terminus was labelled (Figure S12). The LC-MS/MS analysis only indicates that K1 is labeled. Since the N-terminal lysine contains two amino groups, there is a possibility that the labeling could occur at the N-terminal amine instead of the  $\epsilon$  amino group. Nonetheless, given that the nanoparticle is designed to position the labeling reagent to the  $\epsilon$  amino group (Figures 1 & 2 and discussion) and that

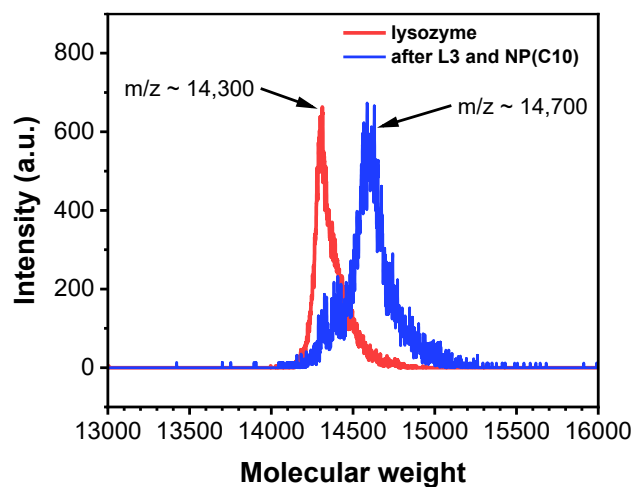
the  $\epsilon$  amino group of K116 can be labelled efficiently (Figure 4 and discussion, *vide infra*), K1 is most likely labeled at the  $\epsilon$  amino group.

The protein labeling was performed under physiological conditions, at which the lysine is expected to be protonated and not nucleophilic. However, the microenvironment around an acid or base is known to strongly impact its acidity/basicity, in both proteins<sup>46</sup> and synthetic host.<sup>47-49</sup> The ammonium side chain of a lysine, for example, has a  $pK_a$  of 10.6 in water but shifted to 5.6 in the active site of acetoacetate decarboxylase. There are two main strategies used by proteins to shift the  $pK_a$  of a functional group. A hydrophobic microenvironment favors the acid/base in the neutral form relative to the charged form that is better solvated in water.<sup>50</sup> In addition, positive charges nearby through electrostatic interactions help the deprotonation of an acid.<sup>51</sup> Both factors are present in our cross-linked cationic micelles and favor the unprotonated neutral form of lysine, helpful to the nucleophilic attack of the labeling agent.

The single-site functionalization of lysozyme could be performed in a protein mixture. Figure S13 in the Supporting Information shows that only lysozyme is labeled when an equimolar mixture of lysozyme, cytochrome C, bovine serum albumin (BSA), ovalbumin (OVA), horse radish peroxidase (HRP),  $\alpha$ -amylase, and  $\beta$ -galactosidase is incubated with **L2** and **NP(N8)** for 24 h. Clearly, despite the numerous lysines present in the mixture, the nanoparticle is able to direct the labeling reagent precisely to its protein target at a predetermined site.

In forming the template, **L0** was chosen for its amphiphilicity so that it could better react with the amphiphilic peptide template (Scheme 1). A different labeling agent (**L1** or **L2**) has to be used for the protein modification. One ideally would like to use a single labeling reagent for both the peptide derivatization and protein labeling, to simplify the synthesis of the reagents and preparation of the nanoparticles. To explore this possibility, we employed a hydrophobic coumarin derivative **L3** in both steps, this time using the acetylated C-terminal peptide **C10** (Ac-KGTDVQAWIR) as the template. To

our delight, the majority the lysozyme after 24 h at 37 °C undergoes the desired modification (Figure 4), with the LC-MS/MS analysis showing a single site labeling at K116 near the C-terminal (Figure S14).



**Figure 4.** MALDI Mass spectrum of lysozyme before (red) and after reaction (blue) with 40  $\mu$ M of **L3** and 20  $\mu$ M of lysozyme in the presence of 40  $\mu$ M **NP(C10)** in HEPES buffer (pH 7.5) after 24 h at 37 °C.

In summary, although lysine represents an attractive target for protein modification, its abundance and surface accessibility also make its single site reaction enormously challenging.<sup>27</sup> This work illustrates a biomimetic method to overcome the difficulties. Using molecularly imprinted nanoparticles that act as both the homing device and the delivery vehicle (Scheme 1), we demonstrate that single-site lysine functionalization is not only possible but also can occur at predetermined sites with good yields (Figure 4). The site selectivity is derived from the extraordinary molecular recognition of these imprinted nanoparticles, distinguishing the addition,<sup>52</sup> removal,<sup>52</sup> and shift<sup>38</sup> of a single methyl (or methylene) group in the guest during binding. The best labeling site should be a lysine in an unstructured region of the targeted protein, such as the N/C-terminal or a flexible loop/linker, given that the our nanoparticle is imprinted for an unstructured short peptide template. The kinetics also has room for improvement. The attractiveness of the method is the facile preparation of the nanoparticles (through a one-pot reaction at

room temperature using commercially available peptides derivatized in situ). The entire preparation and purification take less than 3 days.

Single site-selective functionalization of protein opens up unlimited possibilities for protein manipulation and studies important to both fundamental research and biotechnology.<sup>5-10</sup> The strategy utilized in this work should be general and potentially could be applied to other residues as well. As long as the nanoparticles (or other receptors) can bind both a specific sequence of the peptide on the protein and the labeling reagent strongly and position the two within a close distance, a high level of selectivity is expected.

## **ASSOCIATED CONTENT**

### **Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website.

Synthetic procedures, characterization of compounds and materials, ITC binding curves, additional tables and figures, and NMR data.

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#### **Notes**

The authors declare no competing interest.

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