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Labeling Primary Amine Groups in Peptides and Proteins with N-Hydroxysuccinimidyl Ester Modified Fe_3O_4@SiO_2 Nanoparticles Containing Cleavable Disulfide-bond Linkers

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Abstract

The surface of superparamagnetic silica coated iron oxide (Fe_3O_4@SiO_2) nanoparticles was functionalized with a disulfide bond linked N-hydroxysuccinimidyl (NHS) ester group in order to develop a method for labeling primary amines in peptides/proteins. The nanoparticle labeled proteins/peptides formed after NHS ester reaction with the primary amine groups were isolated using a magnet without any additional purification step. Nanoparticle moieties conjugated to peptides/proteins were then trimmed by cleavage at the disulfide linker with a reducing agent. The labeled peptides were analyzed by LC-MS/MS to determine their sequences and the sites of NHS ester labeling. This novel approach allowed characterization of lysine residues on the solvent accessible surface of native bovine serum albumin. Low cost, rapid magnetic separation, and specificity towards primary amine groups make NHS ester coated Fe_3O_4@SiO_2 nanoparticles a potential labeling probe to study proteins on living cell surfaces.

Keywords

N-hydroxysuccinimidyl ester conjugation; Magnetic nanoparticles; LC-MS/MS; Cleavable primary amine group labeling reagent

INTRODUCTION

The rapidly growing field of nanoparticle (NP) conjugation to proteins has been documented with extensive applications in cell and tissue imaging,[1] drug delivery,[2] and isolation and

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The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

Materials, protocol for synthesis of iron oxide and silica coated iron oxide nanoparticles, conjugation of NHS ester to Fe_3O_4@SiO_2 NPs surface (Scheme S1), calculations for indirect quantification of NHS ester sites on the surface of Fe_3O_4@SiO_2 NPs using 1-aminopyrene (1-AP), gradient used for separation of tryptic peptides, EDX and magnetization studies of thiol coated Fe_3O_4@SiO_2 NPs, ESI-MS/MS of doubly charged ion at m/z 590 and Scheme S1 for alternative preparation of NHS ester modified Fe_3O_4@SiO_2 NPs. This material is available free of charge via the Internet at http://pubs.acs.org

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purification of peptides/proteins.[3] Functionalized by various peptide reactive groups, magnetic nanoparticles have potential for proteome enrichment because separation of magnetic nanoparticles conjugated to peptides/proteins can be simply performed in a magnetic field. Magnetic separation is a gentle process with advantages over traditional column chromatography techniques, which may disassemble large protein complexes during the separation procedure.[4] Moreover, magnetic separation is faster than other separation techniques such as chromatography, filtration and centrifugation.[3]

Amongst all types of magnetic nanoparticles, magnetite (Fe_3O_4) nanoparticles have been more often employed in the isolation and separation of peptides and proteins.[3,5] Properties such as high magnetization values, relatively easy control over particle size, and narrow particle size distributions make iron oxide nanoparticles effective for conjugating peptides and proteins for isolation/separation purposes.[6] It has been shown that Fe_3O_4 NPs can be coated with silica (to form $Fe_3O_4@SiO_2$ NPs) to facilitate further surface modifications by various functional groups.[7] The silica coating protects the iron oxide from further oxidation and disrupts possible specific and/or non-specific interactions between the iron oxide and biological molecules.[8] Usually the surfaces of $Fe_3O_4@SiO_2$ nanoparticles are first modified by intermediate groups such as $–NH_2$ and –SH before further conjugation to functional groups that react with peptides/proteins. Functionalized magnetic nanoparticles form adducts with peptides/proteins via covalent bonds as well as non-covalent interactions, and the covalent interactions are preferred over non-covalent interactions in adduct formation due to the stability of covalent bonds at different pH values and temperatures.[9] The functional groups that can be employed to form covalent bonds with peptides/proteins usually target unmodified side chains of amino acid residues such as –SH (cysteine), -OH (serine, tyrosine), -COOH (aspartic acid, glutamic acid) or $–NH_2$ (lysine).[10,11] Unmodified cysteine residues can be labeled by haloacetyl and alkyl halide derivatives,[12] maleimides,[13] aziridines [14] and thiol-disulfide exchange reagents.[15] A recent study by Palani et al. demonstrated that functionalized $Fe_3O_4@SiO_2$ nanoparticles with thiol specific conjugating groups could be employed to enrich cysteinyl peptides through disulfide bond formation with cysteine residues.[16] Carboxylic acid containing amino acids can be targeted by diazoalkanes and diazoacetyl compounds,[17,18] carbonyldiimidazoles,[19] and carbodiimides.[20] Hydroxyl group containing amino acid residues can be targeted using epoxides, oxiranes, alkyl halogens,[21] isocyanates,[22] and oxidation with periodate.[23] Primary amine groups in unmodified lysine residues and N-termini can be labeled by N-hydroxysuccinimidyl (NHS) esters[24] or sulfo-NHS esters,[25] isothiocyanates,[26] and isocyanates.[27] The most commonly used approach to label primary amines is through covalent bond formation with NHS or sulfo-NHS esters.

In living organisms lysine residues are three times more abundant than cysteine residues,[28] making primary amine groups in unmodified lysine side chains better sites for protein labeling. The unmodified lysine side chain ($–NH_2$) forms an amide bond with NHS or sulfo-NHS esters at physiological pH in aqueous environment. Qian et al. synthesized a fluorinated alkyl affinity reagent and utilized this strategy to label primary amine groups in peptides/proteins via an active sulfo-NHS ester group.[25] Zhao et al. demonstrated the use of isothiocyanate coupled magnetic nanoparticles for the separation of the unblocked N-terminal peptides through covalent bonding.[26] Kerr et al. demonstrated that primary amine selective NHS ester labels with lanthanide metal chelating moieties could be employed to improve peptide quantitation measurements.[29] Recently, Nicolardi et al. labeled primary amine groups in the copper containing protein azurin using the fluorescent label ATTO 655 NHS.[30] A popular biotinylation reagent, sulfo-NHS-LC-biotin, was used to enrich primary amine groups containing proteins in accessible vascular endothelial cells.[31] The reagent sulfo-NHS-LC-biotin contains an NHS ester connected to biotin via a spacer. The biotin moiety is known for forming among the strongest known non-covalent interactions with

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avidin and streptavidin. Another type of biotinylation reagent, sulfo-NHS-SS-biotin contains a primary amine reactive group, a biotin group, and a disulfide linker between the two. The S-S linkage allows for subsequent cleavage of the linker. Sulfo-NHS-SS-biotin was used to enrich cell surface proteomes of chronic and acute leukemia cell lines.[32] Sulfo-NHS-LC-biotin and sulfo-NHS-SS-biotin are commercially available and have been widely used in labeling peptides/proteins; the above-mentioned studies are only two examples of a variety of investigations that employed these reagents.[33–35] However, these labeling reagents have several limitations such as non-specific binding of peptides/protein to avidin[36] and poor recovery of biotin conjugated molecules in affinity chromatography.[37] Even though the sulfonate groups in sulfo-NHS-LC-biotin/sulfo-NHS-SS-biotin render these labeling reagents cell membrane impermeable, the relatively small molecular sizes of the reagents may still allow them to penetrate cell surfaces. Conjugating NHS/sulfo-NHS ester to bulky moieties such as a fluorinated alkyl group[25] or magnetic nanoparticles is expected to reduce the cell surface penetration ability of the primary amine labeling reagent. NHS ester modified magnetic nanoparticles are commercially available to label proteins mostly used for immunoprecipitation.[38] These commercially available nanoparticles for protein labeling cannot be employed in the identification of labeled sites in those proteins, because once covalently conjugated to the proteins, the nanoparticle moieties could not be cleaved from the protein moiety without comprising the structural information of the labeled sites. In order to identify the labeling sites in proteins, removal of the magnetic beads (bulky compared to most of the proteins) from the conjugated protein in an orderly manner is required prior to analyses of the labeled peptides by mass spectrometry.

In this study, we have designed NHS ester coated $Fe_3O_4@SiO_2$ NPs to label primary amine groups in peptides/proteins, not only for isolation/enrichment of the labeled peptides/ proteins, but also for identification of the labeled sites in the peptides/proteins. Iron oxide nanoparticles were selected for their magnetic properties. Silica coating of iron oxide nanoparticles was employed in order to avoid nonspecific interactions between iron oxide and peptides/proteins. Moreover, the surface of $Fe_3O_4@SiO_2$ NP was modified with primary amine/thiol groups first for further conjugation with NHS ester containing compounds. A disulfide bond was included in the linker between the NHS ester moiety and the $Fe_3O_4@SiO_2$ NPs moiety to allow easy cleavage of the linker to facilitate mass spectrometry analysis of the labeled peptides.

EXPERIMENTAL PROCEDURES

Materials

All chemicals and reagents were used as received without further purification. (Details provided in Supporting Information)

Synthesis of Superparamagnetic Iron Oxide Nanoparticles Coated by Silica ($Fe_3O_4@SiO_2$ NPs)

The iron oxide nanoparticle core was prepared by using a conventional co-precipitation method and coated with silica using a sol-gel method.[39] Details are provided in Supporting Information.

Conjugation of NHS Ester to $Fe_3O_4@SiO_2$ NPs Surface

$Fe_3O_4@SiO_2$ NPs (300 mg) were re-dispersed in a mixture of *N,N*-dimethylformamide (DMF, 30 mL) and toluene (20 mL) under ultrasonication for 5 min. Four milliliters of 3-aminopropyltriethoxysilane (APTES, 0.89 mmol) were added to the above solution and stirred for 24 h. The coated $Fe_3O_4@SiO_2$ NPs were separated by magnetic field, washed with ethanol five times, and dried under vacuum for 8 h at room temperature. Amine coated

Fe_3O_4@SiO_2 NPs were characterized by measuring the zeta potential using Möbiuζ dynamic light scattering (Wyatt Technology Corporation, Santa Barbara, CA).

In the next step, dithiobis(succinimidylpropionate) (DSP) was used to introduce the NHS ester. Amine coated Fe_3O_4@SiO_2 NPs (2 mg) were redispersed in ethanol (500 μL) and sonicated for 5 minutes. This suspension was slowly added to DSP (10 mg in 100 μL DMSO, 247.26 mM) in 5 steps (100 μL each time) and mixed well using a vortex mixer after every addition. The reaction mixture was stirred for 30 minutes at room temperature. The NHS ester coated Fe_3O_4@SiO_2 NPs were separated using a magnet, washed twice with ethanol, and dried.

Another approach was also used to prepare the same type of NHS ester coated Fe_3O_4@SiO_2 NPs. This method involved coating of Fe_3O_4@SiO_2 NPs with 3-mercaptopropyltrimethoxysilane (MPTMS) to introduce thiol groups and conjugation of thiol coated Fe_3O_4@SiO_2 NPs with NHS ester using N-succinimidyl 3-(2-pyridyldithio)-propionate (5 mg in 100 μL DMSO) (Supporting Information, Scheme 1).

Magnetization studies of amine (APTES) and thiol (MPTMS) coated Fe_3O_4@SiO_2 NPs were performed at room temperature using a vibrating sample magnetometer (MicroMag Model 3900, Princeton Measurements Corporation).

Labeling Peptide Primary Amine Groups Using NHS Ester Modified Fe_3O_4@SiO_2 Nanoparticles

A small peptide ACTH 4–11 (American Peptide Co. Sunnyvale, CA) in PBS (860 μL, pH 7.4) was added to 1 mg dried NHS ester modified Fe_3O_4@SiO_2 NPs. This suspension was stirred vigorously for 45 min. The peptide-conjugated nanoparticles were then separated using a magnet and washed three times with deionized water in order to remove any unmodified peptide. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl, 6 mM in water) was added to the peptide conjugated nanoparticles and stirred for 45 min at room temperature in order to cleave the disulfide bonds present in the linker. Nanoparticles were removed using magnetic separation and the peptide containing supernatant was saved for analysis. In order to remove excess TCEP, the supernatant was passed through a homemade micro C18 column (capacity: 6 μg peptide) and the sample (vacuum dried, resuspended in 100 μL of 5% formic acid) was analyzed by Finnigan LTQ ion trap mass spectrometer (Thermo Electron, San Jose, CA). Direct infusion flow rate was set at 1 μL/min. Electrospray capillary voltage was 2.65 kV with temperature at 200°C.

Indirect Quantification of NHS Ester Sites on the Surface of Fe_3O_4@SiO_2 NPs Using 1-Aminopyrene (1-AP)

Fluorescence spectroscopic analysis was employed to quantify the number of NHS ester groups conjugated on the surface of Fe_3O_4@SiO_2 NPs. The difference between initial and remaining 1-AP concentration before and after conjugation to nanoparticles was calculated to estimate the amount of NHS ester groups present on the surface of Fe_3O_4@SiO_2 NPs. These experiments were performed with varying amounts of NHS ester modified Fe_3O_4@SiO_2 NPs (5, 10, 15, 10 and 25 mg) to study the capture efficiency of the NPs (details provided in supplemental information).

Labeling of Primary Amine Containing Peptides of Bovine Serum Albumin (BSA) Using NHS Ester Modified Fe_3O_4@SiO_2 NPs

BSA (Sigma, St Louis MO, 10 μL, 10 mg/mL) was diluted with 85 μL of PBS and then mixed with iodoacetamide (6 μL, 200 mM) in order to fix the free thiol groups in BSA. This solution was then added to 1 mg of dried NHS ester modified Fe_3O_4@SiO_2 NPs with

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continuous stirring. The reaction was allowed to proceed for 30 min at room temperature. The protein-magnetic nanoparticle conjugates were separated from the suspension using a magnet and were washed with ethanol three times to remove any unlabeled protein. The labeled BSA was then subjected to in-solution tryptic digestion.[25] Briefly, urea (80 μL, 8.0 M) was added to the NPs linked to BSA and allowed to react for 1 h to denature the protein at 45°C. The mixture was diluted by adding ammonium bicarbonate (1 mL, 50 mM) before addition of trypsin (12 μL of 0.5 μg/μL) for digestion at 37°C for 15 h.

After 15 h of tryptic digestion, nanoparticles were separated using a magnet and washed three times using water and three times using 40% acetonitrile (aq). TCEP (100 μL, 6 mM) was added to the separated iron oxide nanoparticles and incubated for 45 min at room temperature. Nanoparticles were removed using magnetic separation and the supernatant containing the peptides was saved for analysis. In order to remove excess TCEP, the supernatant was passed through a homemade micro C18 column (capacity: 6 μg peptides), and the sample was analyzed by LC-MS/MS.

LC-MS/MS Analysis and Data Analysis

Tryptic peptides of BSA were analyzed by performing liquid chromatography-electrospray-mass spectrometry (LC-ESI MS) on a Finnigan LTQ ion trap mass spectrometer (Thermo Electron, San Jose, CA). BSA peptide sample (re-dissolved in 20 μL water) was loaded onto a reversed phase trap column (flow rate 1 μL/min) and washed with water for 5 min. The peptide mixture was eluted from the trap column and further separated by a C18 reversed phase analytical column (MC-10-C18W-150MS, Micro-Tech Scientific, Vista, CA) using a 60 min gradient made of A (0.1% formic acid/ 97% water/ 3% acetonitrile v/v/v) and B (0.1% formic acid/ 3% water/ 97% acetonitrile v/v/v) at a flow rate 1 μL/min (See Table S1 for detailed gradient). Data dependent acquisition mode controlled by Xcalibur 2.2 version (Thermo Electron) was used to acquire the MS/MS data.

The acquired data were processed by Bioworks software, version 3.3 (Thermo Electron). The parameters for SEQUEST database search were set as: differential mass increase of 87.99 Da for lysine residues and unmodified N-termini, 15.99 Da for possible oxidized methionine residues and 57.02 Da for carboxyamidomethylation of cysteine residues. The number of missed cleavage sites was set to three. The search results were filtered by cross correlation score (Xcorr), i.e. 2.0 for singly charged peptide ions, 2.5 for doubly charged ions, and 3.0 for triply charged ions. MS/MS spectra of detected peptides were manually examined to ensure the quality of identification.

RESULTS AND DISCUSSION

Superparamagnetic Fe_3O_4 NPs were prepared by a conventional co-precipitation method[40] and further coated with TEOS (Fe_3O_4@SiO_2) using a sol-gel method.[39] The magnetic nanoparticles prepared by co-precipitation were spherical shaped, uniform, and in the range of 15 ± 5 nm in diameter (see Figure 1a). The layer of silica coating was employed to protect the Fe_3O_4, to avoid unnecessary interactions between the ligands and Fe_3O_4, to increase the dispersibility of nanoparticles in aqueous solution, and to provide a platform for further functionalization.[41] During the silica coating process, Fe_3O_4 nanoparticles were agglomerated, forming a cluster thereby the size of Fe_3O_4@SiO_2 nanoparticles was increased to 150 to 200 nm (see Figure 1b). Optimization on the size and shape of magnetic nanoparticles is underway and results will be described in a separate report.

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NHS Ester Conjugated Fe_3O_4@SiO_2 NPs with a Cleavable Disulfide Bond Linker

The Fe_3O_4@SiO_2 NPs were further treated by APTES to introduce primary amine groups on the NP surface (Scheme 1). The zeta potentials of Fe_3O_4 NPs and amine coated Fe_3O_4@SiO_2 NPs were measured using dynamic light scattering in a phosphate buffered saline (pH 7.4) solution, and the zeta potentials were found to be −10 ± 3 mV and +25 ± 5 mV respectively. After coating the Fe_3O_4@SiO_2 NPs with APTES, the zeta potential became positive (+25 ± 5 mV) due to the presence of the amine group, which was protonated at neutral pH to bear a positive charge. Thiol coated Fe_3O_4@SiO_2 NPs were also prepared as an intermediate, and the elemental composition was characterized by using energy dispersive spectroscopy (see Supplemental Information Figure S1). These modified particles clearly showed the presence of sulfur, indicating that MPTMS did attach to the particles.

Magnetization studies of amine and thiol coated Fe_3O_4@SiO_2 NPs were performed using a vibrating sample magnetometer (VSM) at room temperature. The absence of hysteresis loops in the magnetization curves of amine and thiol coated Fe_3O_4@SiO_2 NPs confirmed the superparamagnetic nature of the NPs (Figure 2 and Figure S2). These magnetization studies showed that coating with silica and subsequently modifying the surface with APTES or MPTMS did not affect the superparamagnetic nature of the NPs. The amine modified Fe_3O_4@SiO_2 NPs were further reacted with a homobifunctional crosslinker, DSP, through formation of an amide bond between amine group on Fe_3O_4@SiO_2 NPs and the NHS ester of DSP (Scheme 1). NHS ester modified Fe_3O_4@SiO_2 NPs can also be prepared by reacting thiol modified Fe_3O_4@SiO_2 NPs with SPDP (details provided in Supporting Information, Scheme S1).

Unmodified Fe_3O_4 NPs were also treated with APTES to introduce primary amine groups or MPTMS to introduce thiol groups on the nanoparticles surfaces.[42,43] However, we observed no evidence of DSP or SPDP conjugation to the surface of particles in these experiments. While the reason for failure of this approach is unclear, a previous study also reported similar results.[8] Since the silica coating is beneficial, no further effort was made to directly modify magnetite particles with APTES or MPTMS.

Characterization of Primary Amine Labeling by NHS Ester Conjugated Fe_3O_4@SiO_2 NPs

Reaction of NHS esters with primary amines is fast, and cross-linking by this reaction is one of the most commonly used methods in studying protein-protein interactions.[10] NHS ester modified Fe_3O_4@SiO_2 NPs were employed to label a small peptide ACTH (4–11) (M-E-H-F-R-W-G-K) in PBS (pH 7.4). Freshly prepared NHS ester modified Fe_3O_4@SiO_2 nanoparticles were immediately reacted with ACTH (4–11) in order to avoid hydrolysis of the NHS ester. The pH was maintained between 7–9 in PBS since the hydrolysis of NHS ester is pH dependent.[44]

The magnetic nanoparticles conjugated to ACTH (4–11) were cleaved by TCEP to release ACTH (4–11). In the ESI-MS spectrum of the labeled ACTH (4– 11) (Figure 3a), the singly charged ions at *m/z* 1178.47 and doubly charged ions at *m/z* 589.99 correspond to ACTH (4–11) with one primary amine modified; singly charged ions *m/z* 1266.33 and doubly charged ions at *m/z* 633.9 correspond to ACTH (4–11) with two primary amine groups modified. Modification of the peptide by labeled NPs added 87.99 Da per modified primary amine group in the peptide (Scheme 2).

Tandem mass (MS/MS) spectrometry was performed on labeled ACTH (4–11) to identify the sequence and modification site(s). Upon collision induced dissociation (CID), the labeled ACTH (4–11) precursor ion at m/z 633.9 yielded a series of fragment ions (Figure

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3b) that corresponded to cleavages at peptide bonds in the peptide, i.e. *b* and *y* ions shown in Figure 3b inset. Major peaks in the MS/MS spectrum (Figure 3b) matched the *b* and *y* ions of labeled ACTH (4–11) (with both the peptide N-terminus and lysine side chain modified) predicted by MS-product program[45]. This result confirmed that the peptide precursor ion at m/z 633.9 was the doubly charged ACTH (4–11) with modified N-terminus and modified lysine side chain. Similar tandem mass spectrometry experiments were also performed on other labeled ACTH (4–11) ions, including those ACTH (4– 11) modified by NHS ester conjugated Fe_3O_4@SiO_2 NPs prepared according to Scheme S1 (see Supporting Information). The concept (Scheme 2) of labeling primary amine groups by NHS ester conjugated Fe_3O_4@SiO_2 NPs (with disulfide bond linker) was proven to be a novel and effective approach to isolate and analyze primary amine group-containing biomolecules (especially proteins and peptides).

The amount of 1-AP conjugated on the surface NHS ester modified Fe_3O_4@SiO_2 NPs (5, 10, 15, 10 and 25 mg) is shown in Figure 4. Even though the relationship between the amounts of NHS ester modified Fe_3O_4@SiO_2 NPs and amount of 1-AP conjugated was not completely linear, the amount of 1-AP conjugated was well correlated with the amount of Fe_3O_4@SiO_2 NPs. Based on this data set, and using an assumed average particle size of 150 nm, there were 44 ± 12 available reactive sites per particle.

Protein Labeling by NHS Ester Conjugated Fe_3O_4@SiO_2 NPs

NHS ester modified Fe_3O_4@SiO_2 NPs were used to label primary amine groups in bovine serum albumin (BSA, GenBank accession no: CAA76847.1, gi: 3336842, PDB 3v03). The conjugation reaction was performed in water at room temperature, under which the native structure of BSA was maintained. Six lysine residues (out of 58 in a BSA monomer) were labeled according to detected peptides listed in Table 1 and these labeled lysine residues were located on the solvent accessible surface of BSA (Figure 5)[46].

Once a lysine residue is labeled, it is no longer a site for proteolytic cleavage by trypsin. Consequently, labeled lysine residues are usually found in the middle of the peptide sequences and not at the C-terminus as is the case for unlabeled lysine groups. For example, K^{261} in ADLAK*YI$C^{264–278}$DNQDTISSK (cysteine 264 linked to cysteine 278 via disulfide bond) and K^{350} in LAK*EYEATLEE$C^{359–368}$$C^{360–315}$AK were labeled and did not appear at the C-terminus of the peptide (Table 1). In addition to labeled peptides, several unlabeled peptides were also detected in the LC-MS/MS analysis, e.g. ETYGDMAD$C^{90–101}$$C^{91–75}$EK, DDPHA$C^{368–359}$YSTVFDK, and K in these peptides were not labeled. These peptides were linked to labeled peptide through disulfide bonds and were released upon cleavage by TCEP. Some of the unlabeled lysine residues are on the surface of BSA as well, e.g. K^{93}, but they may not be accessible to Fe_3O_4@SiO_2 NPs because of the large size of the NPs or because the neighboring K could have be labeled and subsequently block the accessibility of another incoming nanoparticle. The presence of the disulfide bonds allow the unlabeled peptides to be co-separated during magnetic separation. After cleavage of disulfides with TCEP, the unlabeled peptides were then released and observed by mass spectral analysis. Other lysine residues were buried inside the structure of BSA due to its native state and were not accessible to NHS ester coated Fe_3O_4@SiO_2 NPs. Cleavage of disulfide bonds in BSA would allow the protein to be partially unfolded to facilitate more tryptic cleavage. However, in order to protect the disulfide bond linker in the NHS ester coated Fe_3O_4@SiO_2 NPs, reducing reagents such and DTT or TECP could not be employed to cleave disulfide bonds in the protein prior to magnetic separation.

Most of the peptides identified after magnetic labeling contained at least one cysteine residue. BSA is a well studied protein, and positions of the disulfide bonds can be found in the literature.[47] According to the cysteine residues and positions of disulfide bonds

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numbered by Brown et al.[47] in the structure of matured BSA, all the cysteine residues found in labeled and unlabeled peptides characterized in our study participated in disulfide bond formation (Table 1). Some of the labeled peptides were linked to unlabeled peptides though a disulfide linkage. In order to separate the Fe_3O_4@SiO_2 NPs from tryptic peptides, TCEP was employed to cleave disulfide bonds in the linker between the NPs and the labeled peptides. During this process, disulfide bonds between labeled and unlabeled peptides were also cleaved thereby releasing cysteine residues. This was evident by the fact that TPVSEKVTK*$C^{475–486}C^{476–460}$TESLVNR and RP$C^{486–475}$FSALTPDETYVPK were linked through disulfide bond (C475-C486). In addition, one unlabeled peptide without disulfide linkages was also detected (LGEYGFQNALIVR), and it was likely retained during magnetic separation because of strong non covalent interactions within the protein tertiary structure. Before tryptic digestion, unmodified cysteine residues (if there were any in BSA) were alkylated by adding iodoacetamide in order to protect the disulfide bond present in the linker of the NHS ester conjugated Fe_3O_4@SiO_2 NPs. Thiol groups of the cysteine residues modified by iodoacetamide will add 57.02 Da on cysteine residues, but such modification was not observed in the mass spectra collected in this study. In addition to positions of lysine residues (labeled and unlabeled), the position of cysteine residues were also obtained, and the possibility of such cysteine residues to be part of disulfide bond provided further structural information on disulfide bonds of the protein (Table 1).

All of these results indicate that NHS ester coated Fe_3O_4@SiO_2 NPs are promising labeling reagents to study the surface of proteins containing primary amines. However, the aggregation of nanoparticles after silica coating resulted in nanoparticles of great heterogeneity in size, and subsequently affected the effectiveness of this protein/peptide labeling approach. Additionally, the total number of reactive sites of nanoparticles can be affected by batch-to-batch reproducibility, affecting the number of available amine groups for NHS ester reaction. However, this issue might be minimized by increasing the length of spacer (such as incorporation of polyethylene glycol) between the nanoparticles and disulfide bond to distance the NHS ester group from the amine groups. This approach may also be improved by including a fluorophore between the NHS groups and the disulfide bond, which can be used for imaging purposes. Thus, this novel protein/peptide labeling approach should be improved by using NPs well controlled in size and number of NHS ester groups per particle.

CONCLUSION

Primary amine reactive NHS ester groups were conjugated on the surface of Fe_3O_4@SiO_2 NPs through covalent bond formation. The novel NHS ester coated Fe_3O_4@SiO_2 NPs were successfully applied to label peptide/protein samples at room temperature and in aqueous environment, conditions under which the native structures of protein/peptide were preserved. Primary amine groups (mostly unmodified lysine side chains) on the solvent accessible surface of protein BSA were the targets of labeling by NHS ester coated Fe_3O_4@SiO_2 NPs, and the labeled sites were determined by LC-MS/MS based proteomic analysis. Separation by magnetic field facilitated specific enrichment of magnetic nanoparticle labeled proteins/peptides. It was confirmed in this study that lysine residues labeled by NHS ester coated Fe_3O_4@SiO_2 NPs were exposed on the surface of native BSA, and such results implied that the overall approach developed in this study could be a useful tool in investigating the exposed portion of proteins and even intact cells. The reaction of labeling primary amine groups by NHS ester coated Fe_3O_4@SiO_2 NPs could be completed in less than two hours, suggesting that the reactivity of NHS ester moiety in the nanoparticle labeling reagent is comparable to that of the same moiety in other primary amine labeling reagents such as sulfo-NHS-SS-biotin. The simplicity in synthesis and functionalization of the labeling reagent, effectiveness of labeling of primary amine containing peptides/proteins,

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easy magnetic separation, and cleavable disulfide bond in the linker make labeling by NHS ester modified Fe_3O_4@SiO_2 NPs a useful approach in the field of nanoparticles-protein bioconjugation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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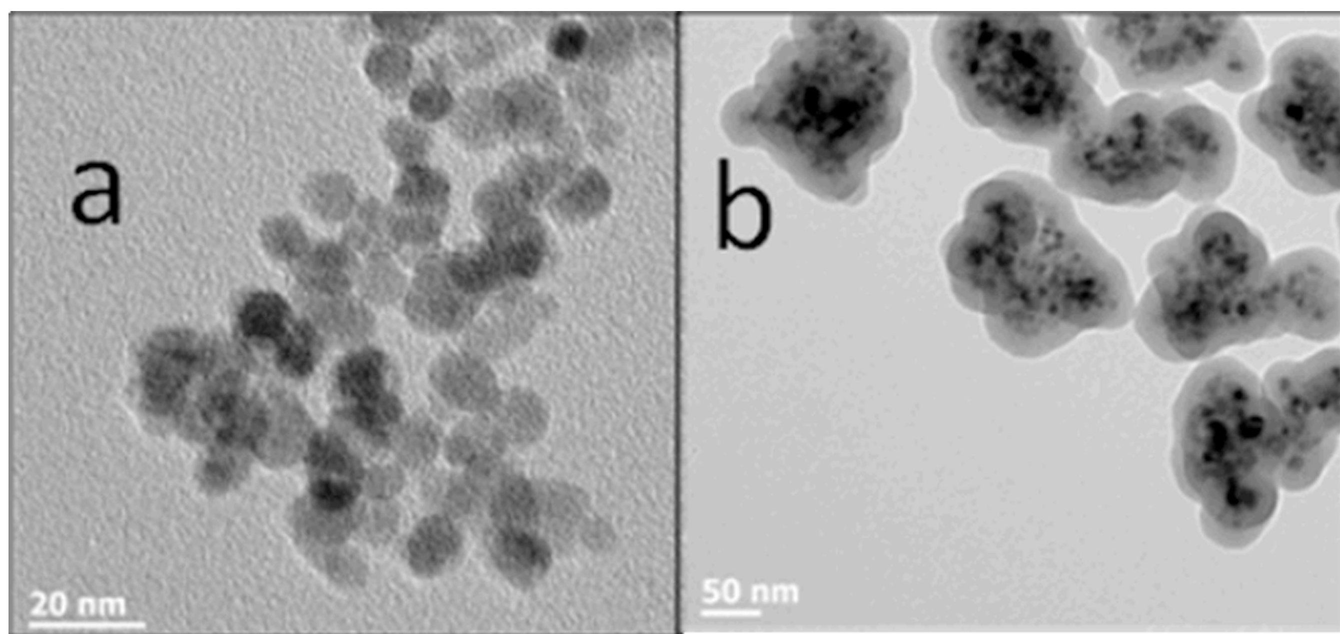

Figure 1.
(a) TEM image of bare Fe_3O_4 NPs synthesized by co-precipitation method and (b) Fe_3O_4@SiO_2 NPs coated by sol-gel method.

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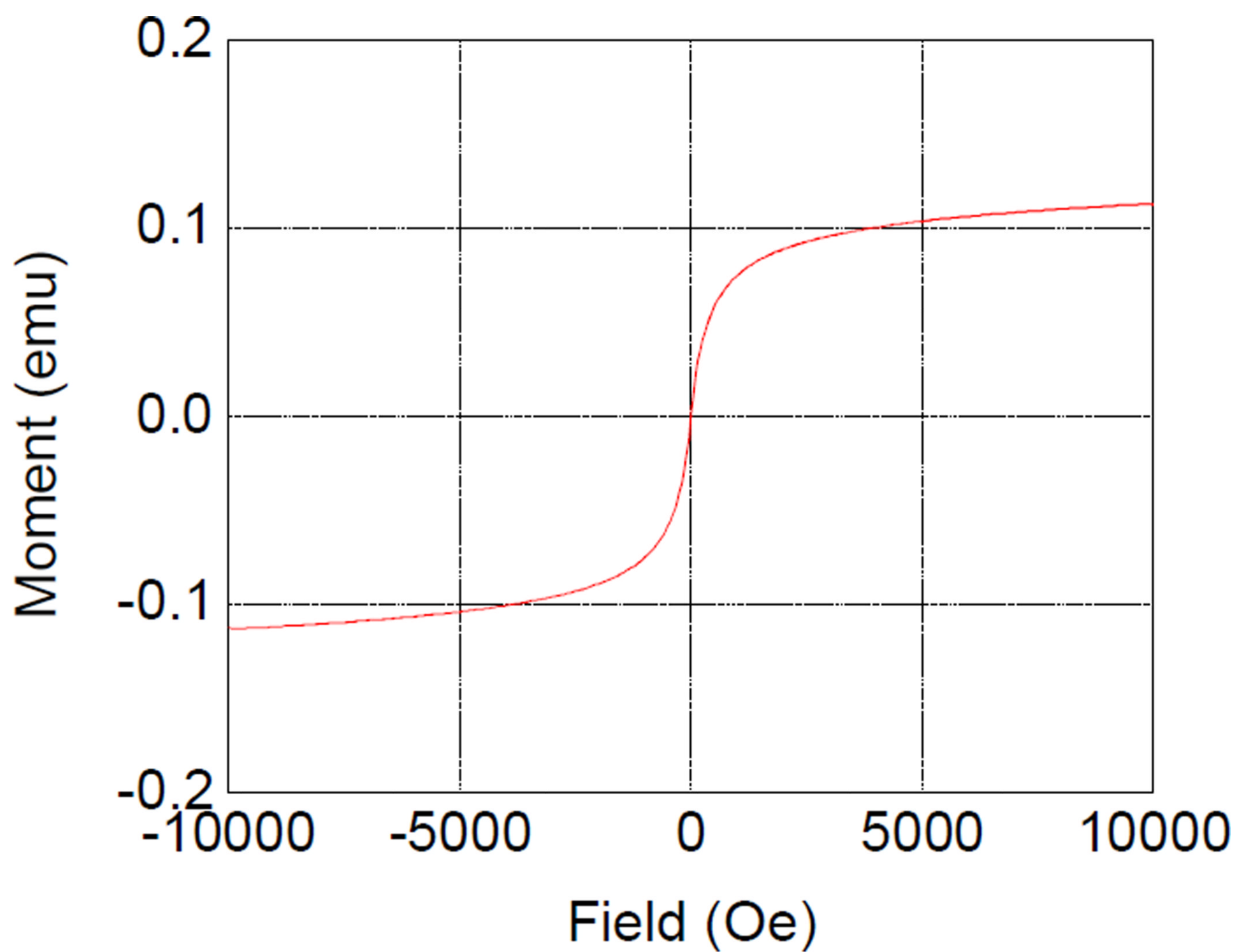

Figure 2.
Magnetization studies of APTES coated Fe_3O_4@SiO_2 NPs

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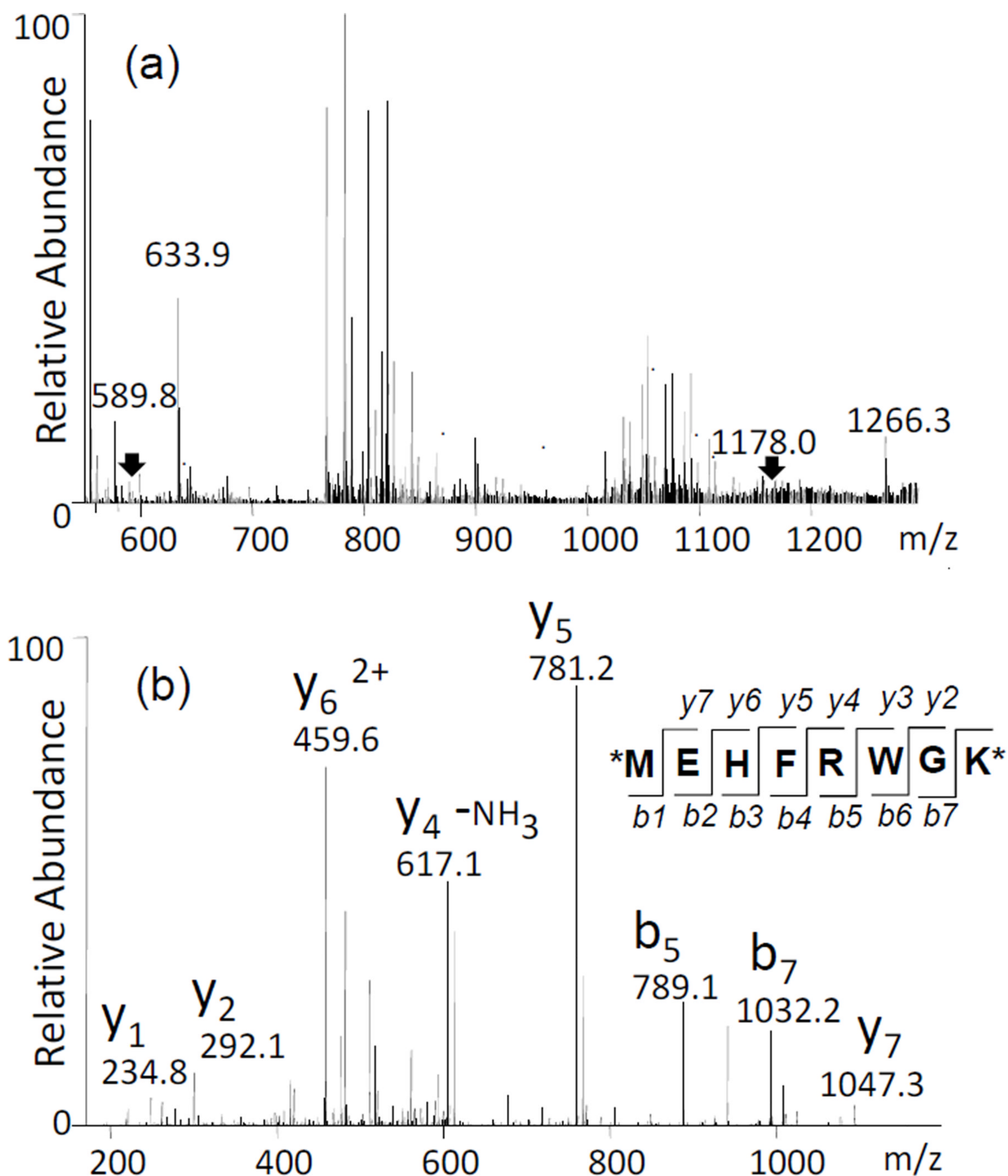

Figure 3.

ESI-MS analysis of peptide ACTH (4–11) labeled by NHS ester modified Fe_3O_4@SiO_2 NPs and then treated with TCEP to cleave disulfide bond. (a) In this mass spectrum, singly ion charged at *m/z* 1178.0 corresponds to ACTH (4–11) with one primary amine group modified; doubly charged ion at *m/z* 589.8 corresponds to ACTH 4–11 with one primary amine group modified; and singly charged ion at *m/z* 1266.3 and doubly charged ion at *m/z* 633.9 correspond to ACTH with both primary amine groups modified. (b) ESI-MS/MS of doubly charged peptide ACTH (4–11) ion at m/z 633.9. Sequence of peptide was confirmed by matching the *b* and *y* ions with detected m/z values.

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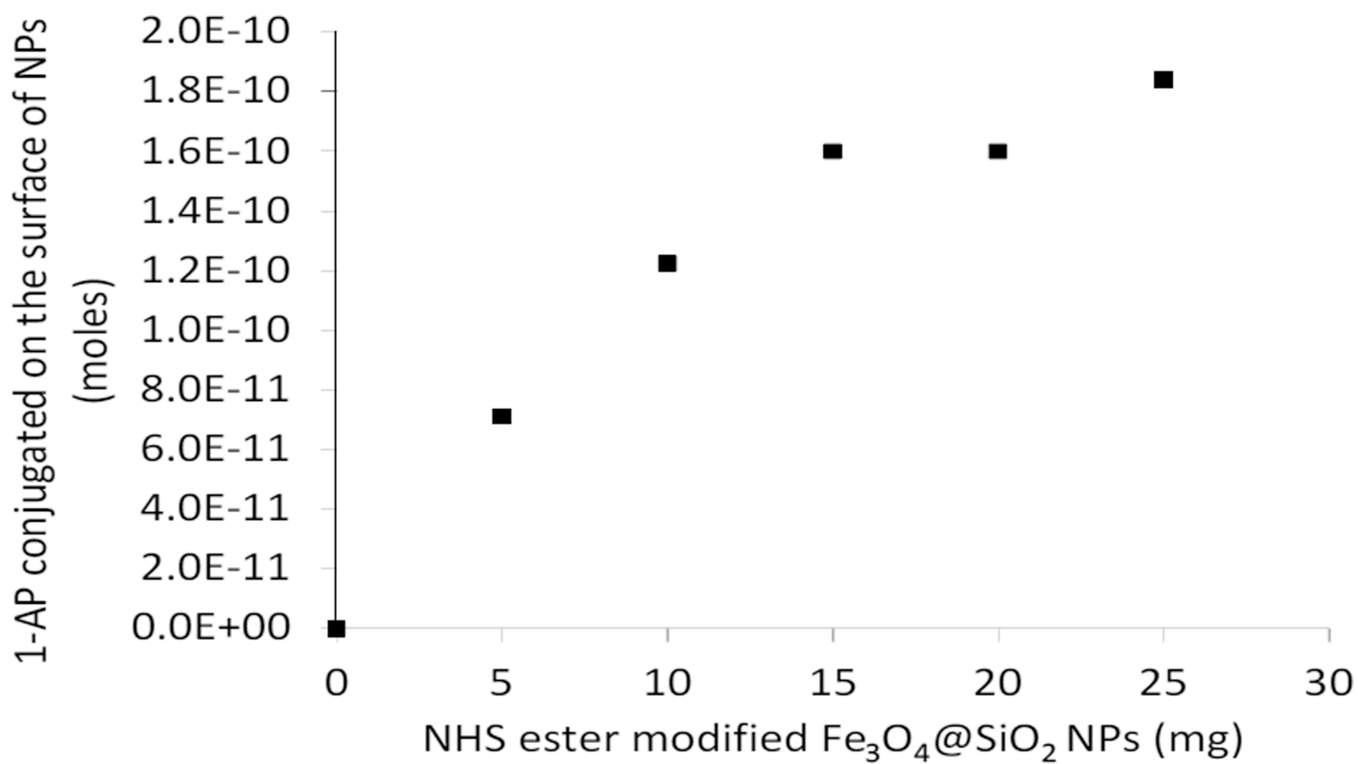

Figure 4.

Indirect fluorometric quantitative analysis of 1-AP conjugated on the surface of NHS ester modified Fe_3O_4@SiO_2 NPs (5, 10, 15, 20 and 25 mg). The difference between the amount of 1-AP before and after reaction with NHS ester modified Fe_3O_4@SiO_2 NPs was calculated to determine the amount of 1-AP conjugated on the surface.

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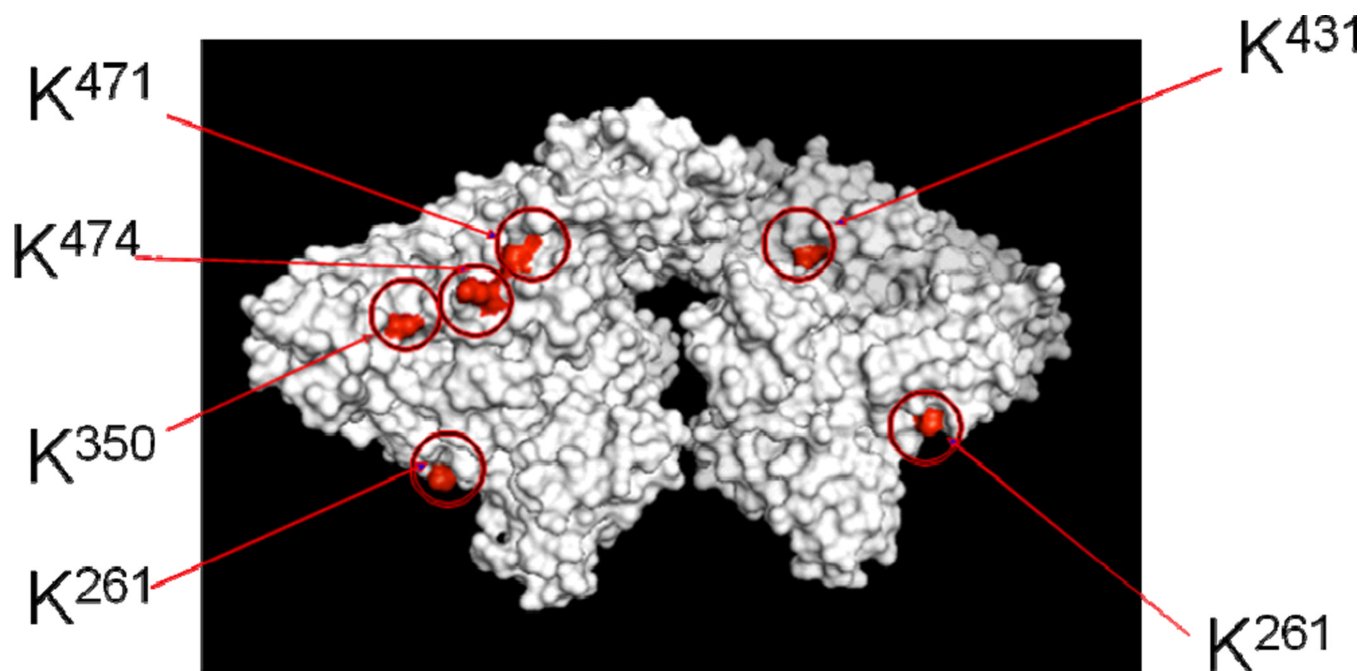

Figure 5.
Homo dimer of matured bovine serum albumin (PDB 3v03) showing lysine residues (highlighted in red) labeled by NHS ester modified Fe_3O_4@SiO_2 NPs.

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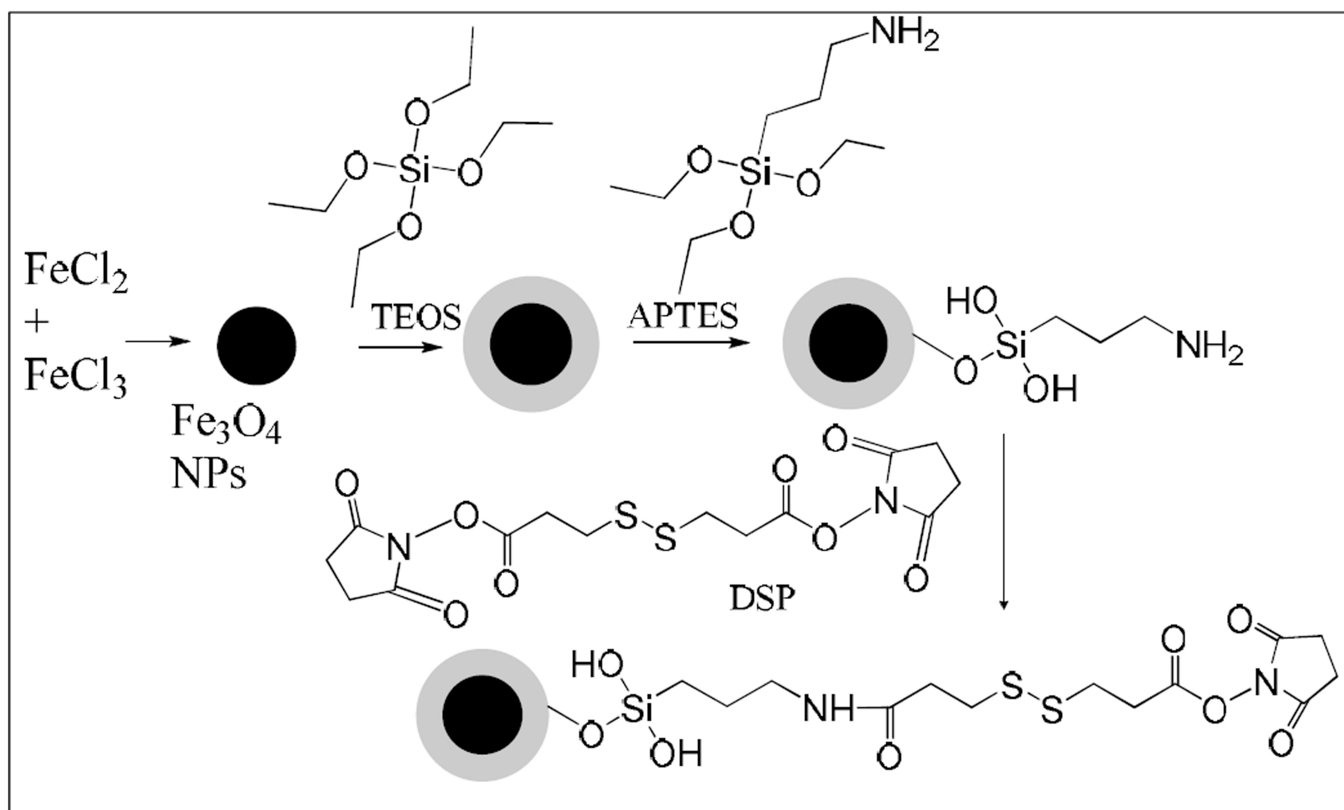

Scheme 1.
Preparation of NHS Ester Coated $Fe_3O_4@SiO_2$ NPs Using DSP

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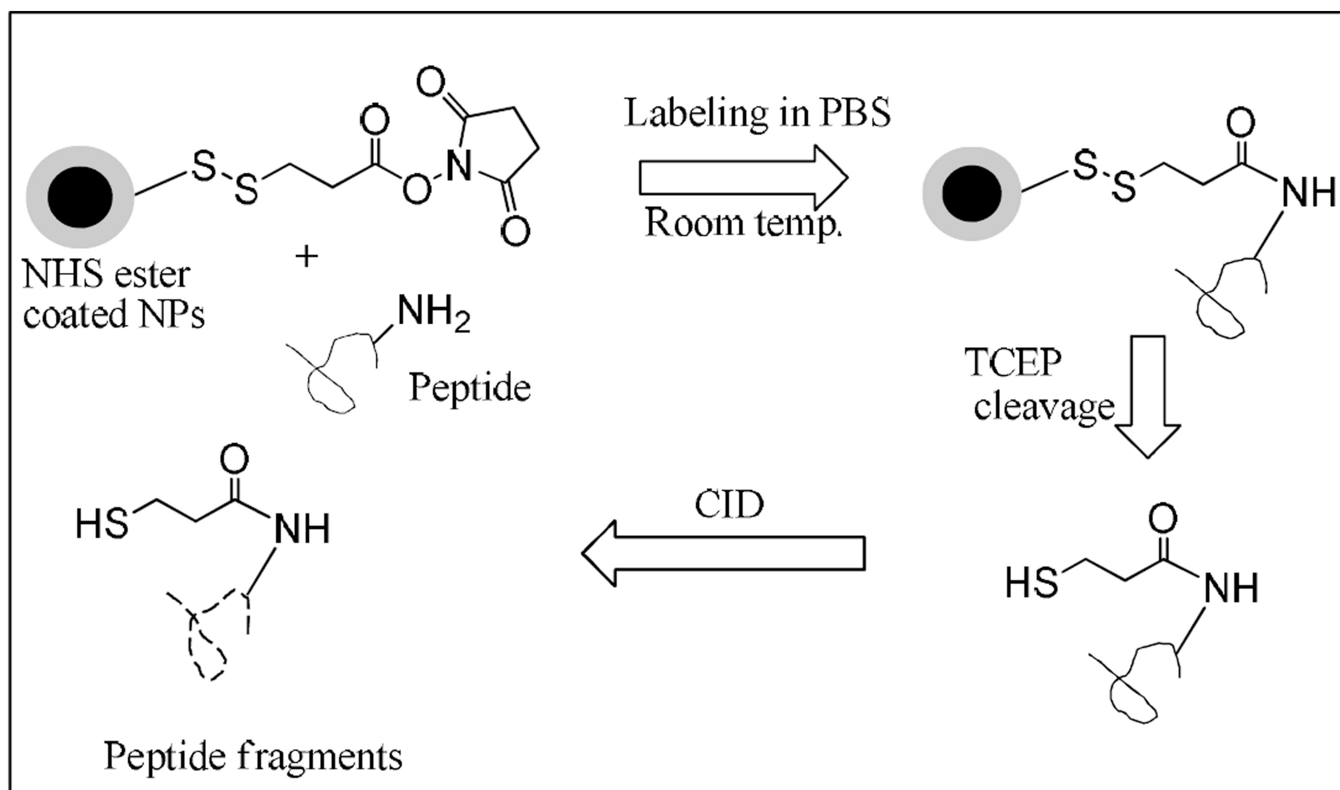

Scheme 2.
Peptide Labeling Using NHS Ester Coated Fe_3O_4@SiO_2 NPs

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Table 1

Identified Tryptic Peptides Derived from BSA Labeled by NHS Ester Modified Fe_3O_4@SiO_2 NPs[a]

Position of amino acid residues in the sequence in mature BSA[b]	Peptide sequence[c]	Xcorr[d]
65–76	SLHTLFGDELC$^{75–91}$K	3.64
82–93	ETYGDMADC$^{90–101}$C$^{91–75}$EK	2.93
82–98	ETYGDMADC$^{90–101}$C$^{91–75}$EKQEPER	3.36
160–173	YNGVFQEC$^{167–176}$C$^{168–123}$QAEDK	3.81
257–273	***ADLAK*YIC$^{264–278}$DNQDTISSK***	***4.09***
262–273	YIC$^{264–278}$DNQDTISSK	4.08
348–362	***LAK*EYEATLEEC$^{359–368}$C$^{360–315}$AK***	***4.36***
351–362	EYEATLEEC$^{359–368}$C$^{360–315}$AK	2.65
363–375	DDPHAC$^{368–359}$YSTVFDK	2.93
397–409	LGEYGFQNALIVR	3.6
413–427	***K*VPQVSTPTLVEVSR***	***3.8***
428–444	***SLGK*VGTRC$^{436–447}$C$^{437–391}$TKPESER***	***3.61***
445–458	MPC$^{447–436}$TEDYLSLILNR	4.54
466–483	***TPVSEK*VTKC$^{475–486}$C$^{476–460}$TESLVNR***	***4.92***
466–483	***TPVSEKVTK*C$^{475–486}$C$^{476–460}$TESLVNR***	***3.95***
472–483	VTKC$^{475–486}$C$^{476–460}$TESLVNR	3.85
484–499	RPC$^{486–475}$FSALTPDETYVPK	4.16
505–520	LFTFHADIC$^{513–558}$TLPDTEK	4.2
509–520	HADIC$^{513–558}$TLPDTEK	2.72

[a] Superscript numbers refer to the cysteine residues in the peptides that were part of disulfide bond which was cleaved in the last step of reduction by TCEP. These disulfide bond linkages are mentioned in a superscript; e.g., C75-91 indicate that C75 (in SLHTL…) form disulfide bond with C91(in ETYGD…). K* represents lysine residues modified by NHS ester tag. The peptides highlighted in bold/italic contain at least one labeled lysine residue.

[b] Position of amino acid residues in the sequence of matured BSA.

[c] Peptide sequence in matured BSA with positions of cysteine residues.

[d] Cross-correlation score provided by SEQUEST algorithm.