Cleavable ester linked magnetic nanoparticles for labeling of solvent exposed primary amine groups of peptides/proteins

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Short title: Ester linked nanomagnets for protein labeling

Abstract

In order to study the solvent exposed lysine residues of peptides/proteins, we previously reported disulfide linked *N*-hydrosuccinimide ester modified silica coated iron oxide magnetic nanoparticles (NHS-SS-SiO₂@Fe₃O₄ MNPs). The presence of a disulfide bond in the linker limits the use of disulfide reducing agent during protein digestion and allows unwanted disulfide formation between the MNP and protein. In the current work, the disulfide bond was replaced with a cleavable ester group to synthesize NHS ester modified SiO₂@Fe₃O₄ MNPs. Use of the cleavable ester group provides an improved method for protein labeling and allows use of disulfide reducing agents during protein digestion.

Key-words: (Ethylene glycol bis[succinimidylsuccinate]), Core-shell magnetic nanoparticles, Solvent exposed lysine residues

The labeling of proteins using chemically cleavable amino acid specific probes coupled with mass spectrometric analysis remains a major choice to study the solvent accessible surface of proteins. The amino acid specific probes can specifically label the functional group of amino acids, and a cleavable bond assists with the isolation and identification of labeled proteins [1]. Variety of cleavable amino acid specific probes targeting the primary amine groups are developed due to abundance of lysine residues on the solvent accessible surface of proteins [2], [3]. The labeling probes containing NHS/sulfo-NHS groups can covalently label the primary amine groups of lysine residues in physiological buffers and at room temperature to form a stable amide bond [3]. The disulfide linked, lysine targeting cleavable labeling reagents are commercially available and have been widely used to obtain structural information of proteins. Upon labeling, proteins are digested using reducing agents (Dithiothreitol, DTT and tris(2-carboxyethyl)phosphine), TCEP) that cleave disulfide bonds to unfold the protein structure which is required to denature the protein. Protein denaturation unfolds the protein in order to achieve the maximum efficiency for downstream processes such as tryptic digestion. The presence of disulfide bonds in the crosslinker may limit the use of reducing agents such as DTT and TCEP prior to protein digestion. Use of disulfide containing linkers is not compatible with DTT containing buffers and may be attacked by free thiol groups in complex cellular conditions. An alternative crosslinker, (Ethylene glycol bis[succinimidylsuccinate]) (EGS) could be an effective replacement for disulfide containing crosslinkers which can allow the use of disulfide cleaving reducing agents during protein digestion [4].

Upon labeling the functional groups of proteins, labeled proteins are separated and purified by using conventional electrophoretic, chromatographic or centrifugation techniques which could be time consuming, tedious, and detrimental to the structure of a protein [5]. Recently, the magnetic

nanoparticles have emerged as an effective alternative to achieve rapid and effective separation for sample preparation in proteomics [6],[7]. In our lab, we have developed disulfide linked, NHS ester modified $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs to 'label' the solvent exposed lysine residues of proteins [8] and yeast cell surface proteins [9]. The NHS/sulfo-NHS ester modified MNPs allowed labeling of lysine residues under physiological conditions at room temperature. The MNP based labeling approach offered rapid and soft magnetic separation of conjugated proteins which is advantageous over traditional protein separation techniques such as column chromatography and gel electrophoresis. However, the disulfide bond in the NHS-SS- $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs can be attacked by the reducing agents during protein digestion thereby cleaving the disulfide bond prior to the magnetic separation step. Moreover, an extra step of alkylation of free thiol groups needs to be added prior to the labeling process to protect disulfide groups in the linker. In this work, we replaced the cleavable disulfide bond with a cleavable ester bond to link NHS ester groups to the surface of $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs, and utilized ester linked, NHS ester modified MNPs to label the solvent exposed primary amine groups of proteins in its native state.

In this study, the Fe_3O_4 MNPs were prepared by a thermal decomposition method as reported earlier [10]. The Fe_3O_4 NPs were characterized by TEM and XRD (Fig 1A and Fig 1 in Ref. [13]). The Fe_3O_4 MNPs were spherical, uniform, and average diameter was found be ~10 nm (Fig 1A). The core shell $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs were prepared by coating Fe_3O_4 MNPs with silica by using a reverse microemulsion technique [11]. TEM characterization confirmed the spherical and core shell nature of $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs. The average diameter of $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs was ~35 nm and showed stability in aqueous solvents (Fig. 1B). The $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs were further modified with amine groups using (3-aminopropyl)triethoxysilane (APTES) [12]. Upon addition of amine groups onto the surface of $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs, the observed zeta

potential changed from an original negative value (-9 ± 5 mV) to a positive value ($+23 \pm 5$ mV) as observed by DLS analysis (Wyatt Technology Corporation, Santa Barbara, CA), confirming the attachment of amine groups to the surfaces. The superparamagnetic nature of amine coated $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs was studied by using a vibrating sample magnetometer (Micro-Mag Model 3900, Princeton Measurements Corporation). Absence of magnetic hysteresis indicated that the coating on the iron oxide core did not affect the superparamagnetic properties (data not shown). A homobifunctional crosslinker, (ethylene glycol bis[succinimidylsuccinate], EGS) was utilized for conjugation of NHS ester groups on the surface of amine modified $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs (Scheme 1 and Fig 2 in Ref. [13]). In order to avoid crosslinking between MNPs, a high concentration of EGS was maintained during the coupling reaction. The amine modified $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs were slowly added to a DMSO solution of EGS and allowed to react for 20 min. The NHS ester modified $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs were magnetically separated, washed with ethanol three times, and dried under vacuum (Scheme 1 in Ref [13]). A depletion approach was used to quantify the active NHS ester groups on the surface of $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs. An amine containing fluorephore, dansylcadaverine (excitation: 335 nm and emission: 513 nm) was conjugated to NHS ester modified $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs, and the remaining amount of dansylcadaverine was fluorimetrically measured to determine the amount of dansylcadaverine conjugated to $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs (Fig 4 in Ref. [13]). The amount of dansylcadaverine conjugated to $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs corresponds to active NHS ester groups on the surface of $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs. These studies indicated 694 available NHS ester binding sites per 1 mg of $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs(Section 2.6 in Ref. [13]).

In order to investigate the ability of ester linked, NHS ester modified $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs to label amine groups, a small peptide, ACTH (4-11) containing two amine groups (side chain of

lysine residue and *N*-terminus) was chosen. The NHS ester modified SiO₂@Fe₃O₄ MNPs (0.5 mg) were mixed with ACTH 4-11 (10 µg in 100 µL PBS, pH=7.4) at room temperature and physiological pH for 40 min. Peptide conjugated MNPs were magnetically separated and washed several times using magnetic separation. The ester bond was subsequently cleaved by subjecting it to hydroxylamine treatment for 4 hrs at 37°C. (Scheme 1) EGS contains two ester sites that can be cleaved under basic conditions using hydroxylamine to release ethylene glycol (Scheme 1). After cleaving the ester bond, the MNPs were separated by using a magnet, and the supernatant was passed through a C18 zip tip column (EMD millipore, Billerica, MA) and dried. The dried material was resuspended in a mixture of ACN (3%) and formic acid (5%) (100 µl) and analyzed with LC/MS/MS using an Agilent 1200 LC system, an Agilent Chip Cube interface, and an Agilent 6520 Q-TOF tandem mass spectrometer (Agilent Technologies, Santa Clara, CA). The NHS ester labeling reaction introduced a mass shift of +115.09 Da for modified primary amine groups of ACTH 4-11. Mass spectrometric analysis revealed the presence of peptides containing two labeled amine groups (lysine residues and *N*-terminus) or peptides containing only one labeled amine group (either lysine residues or *N*-terminus). Doubly charged as well as triply charged peptides of both the combinations were also found. (Fig 3 of Ref. [13]) The *b* and *y* ions fragmented during CID were matched with the theoretically calculated *b* and *y* ions. Presence of series of *b* and *y* ions in the mass spectra confirmed the amine label on the peptide.

The ability of ester linked, NHS ester modified SiO₂@Fe₃O₄ MNPs to label solvent exposed lysine residues of proteins was tested with two model proteins: bovine serum albumin (BSA, PDB: 3v03) and β lactoglobulin (PDB: 2Q2M). The proteins were chosen due to availability of crystal structure of bovine serum albumin and β-lactoglobulin. The presence of

primary amine groups on the surface of these model proteins made them a perfect candidate to perform proof of concept studies with cleavable, ester linked, NHS ester modified $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs. The labeling reaction was performed in a similar way as with the peptides. After conjugating the proteins to MNPs, the remaining NHS ester groups were quenched by adding tris-HCl. A quenching step was required to avoid any unnecessary labeling of digested peptides. The MNP-protein adducts were magnetically separated, washed and denatured using urea and DTT followed by treatment with iodoacetamide to alkylate free thiol groups. The denatured protein conjugated to MNPs was further subjected to tryptic digestion. The tryptic peptides were isolated from MNPs by cleaving the ester group followed by magnetic removal of the MNPs. (Section 2.4 of Ref. [13]) The supernatant containing labeled peptides was desalted using C18 zip-tip column (EMD millipore, Billerica, MA) and dried. Upon mass spectrometric analysis, data files were processed by using an Agilent workstation equipped with Spectrum Mill software (Agilent Technologies, Santa Clara, CA) for peptide sequencing and protein identification. The parameters for database search were set as follows: differential mass increase of 115.09 Da for lysine residues and unmodified N-termini, and 15.9 Da for possible oxidized methionine residues. The number of missed cleavage sites was set to three. Ester linked, NHS ester modified $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs successfully labeled the lysine residues located on solvent accessible surfaces of BSA and β lactoglobulin (Fig 2). NHS ester modified $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs labeled four lysine residues in β lactoglobulin (contains 16 lysine residues) and six lysine residues in BSA (contains 58 lysine residues) (Fig. 2, Table 1). All the labeled lysine residues were found in the middle of the cleaved peptide sequence since labeled lysine residues are not recognized as a cleavage site by trypsin. In previous work, the captured peptides were released from the nanoparticles by cleaving the disulfide linker (NHS-SS- $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs) using TCEP.

Because the disulfide cleaving agent (e.g. TCEP) cannot be used until this last step, disulfide bonds in the protein could not be cleaved prior to trypsinization, which is often an important step for protein digestion. Consequently, disulfide containing proteins might be difficult to analyze using the disulfide-based MNPs approach. This limitation was overcome in the current work by replacement of the disulfide linker with an ester linker.

To conclude, cleavable ester linked, NHS ester modified MNPs were successfully used to label solvent exposed amine groups of BSA and β -lactoglobulin. The presence of the ester bond allowed digestion of MNPs-conjugated proteins in the presence of disulfide bond reducing agent, DTT. Moreover, protein digestion can be more effectively carried out since disulfide cleavage and protection with iodoacetamide allow better access by the proteolytic enzyme. Replacing the disulfide linker with an ester linker makes the new labeling reagent a promising tool for cellular systems that require digestion in the presence of reducing agents such as DTT or TCEP. The work presented here provides opportunities for future studies of solvent exposed amine residues of complex and heterogeneous cell surface/cell wall proteins and lipoproteins using cleavable ester linked NHS ester modified $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs. These MNPs can also be applied to study complex protein mixtures.

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Figure legends

Fig 1: TEM analysis of (A) Fe_3O_4 MNPs (~10 nm) and (B) $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs (~35 nm). The Fe_3O_4 and $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs were found to be spherical, uniform and stable in aqueous solvents.

Fig 2: Labeled lysine residues (blue) of BSA (left top) and β lactoglobulin (left bottom) with ester linked, NHS ester modified $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs.

Scheme 1: Labeling of amine containing peptides using cleavable ester linked, NHS ester modified $\text{SiO}_2@\text{Fe}_3\text{O}_4$ MNPs.

Table 1: List of tryptic peptides with labeled lysine residues (represented k).

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Figure 1

Figure 2

Table 1

Name of a protein	Labeled peptides	Sequence of labeled peptides	Score
BSA			
	452-459	(R)SLGkVGTR(C)	69.9
	257-266	(K)LVTDLTkVHK(E)	61.1
	400-413	(K)LkHLVDEPQNLIK(Q)	62
	242-256	(R)LSQKFPkAEFVEVTK(L)	64.7
	249-263	(K)AEFVEVTKLVTDLTk(V)	65.6
	437-451	(R)kVPQVSTPTLVEVSR(S)	91.1
β lactoglobulin			
	59-78	(R)VYVEELkPTPEGDLEILLQK(W)	86.9
	110-119	(K)VLVLDTDYkK(Y)	75.3
	102-118	(K)IDALNENkVLVLDTDYK(K)	62.3
	143-156	(R)TPEVDDEALEkFDK(A)	69.4