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Magnetic nanoparticles containing labeling reagents for cell surface mapping

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To People Who Believe In Science

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Figure 5.3. Sequence of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, YGR192C), isozyme 3 labeled by sulfo-NHS ester modified Fe_3O_4@SiO_2 MNPs. The exposed cell surface peptides are highlighted in green and labeled amino acid residues are marked by asterisk (*).

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Abstract

Cell surface proteins play an important role in understanding cell-cell communication, cell signaling pathways, cell division and molecular pathogenesis in various diseases. Commonly used biotinylation regents for cell surface mapping have shown some potential drawbacks such as crossing the cell membrane, difficult recovery of biotinylated proteins from streptavidin/avidin beads, interference from endogenous biotin and nonspecific nature of streptavidin. With aim to solve these problems, we introduced sulfo-*N*-hydroxysuccinimidyl (NHS) ester functionalized magnetic nanoparticles containing cleavable groups to label solvent exposed primary amine groups of proteins. Silica coated iron oxide magnetic nanoparticles (Fe_3O_4@SiO_2 MNPs) were linked to NHS ester groups via a cleavable disulfide bond. Additionally, the superparamagnetic properties of Fe_3O_4@SiO_2 MNPs facilitate efficient separation of the labeled peptides and removal of the detergent without any extra step of purification. In the last step, the disulfide bond between the labeled peptides and MNPs was cleaved to release the labeled peptides. The disulfide linked NHS ester modified Fe_3O_4@SiO_2 MNPs were tested using a small peptide, and a model protein (bovine serum albumin) followed by liquid chromatography-tandem mass spectrometry analysis (LC-MS/MS) of labeled peptides. In the next step, disulfide linked, NHS ester modified Fe_3O_4@SiO_2 MNPs (150 nm) successfully labeled the solvent exposed cell surface peptides of *Saccharomyces cerevisae*. Electron microscopic analysis confirmed the cell surface binding of NHS ester modified Fe_3O_4@SiO_2 MNPs. Mass spectrometric analysis revealed the presence of 30 unique proteins containing 56 peptides.

Another MNPs based labeling reagent was developed to target solvent exposed carboxyl acid residues of peptides and proteins. The surface of Fe_3O_4@SiO_2 MNPs was modified with free

amine groups via a disulfide bond. Solvent exposed carboxyl groups of ACTH 4-11 and BSA were labeled by using1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) chemistry. Upon cleaving the disulfide bond, labeled peptides were analyzed by LC-MS/MS.

The MNPs containing labeling reagents offers specific labeling under physiological conditions and rapid magnetic separation of labeled peptides prior to mass spectrometric analysis. The ability of large Fe_3O_4@SiO_2 MNPs to specifically attach to cell surface makes them a potential candidate to study the surface of variety of different cell types and complex proteins surrounded by lipid bilayer.

Keywords: Iron oxide nanoparticles, cell surface proteins, covalent labeling, mass spectrometry, proteomics

Chapter 1

Introduction

1.1. Chemical labeling mediated mass spectrometry based analysis of proteins and cell surface proteins

The post genomic era faces a major challenge of obtaining information about novel genes and their correlated proteins. Correlating the gene sequence with structural information of proteins is a daunting task, and is mainly performed by computational prediction methods.[1]The majority of functions of living cell are performed by proteins. Protein molecules are made of a chain of amino acids, which can be hydrophilic or hydrophobic in nature. Amino acids are organic compounds containing an amine ($-NH_2$), a carboxylic group (-COOH), and a specific side chain. The amino acid residues in proteins undergo structural and conformational changes upon interaction with other proteins or molecules. Studying the structural and conformational changes of proteins can provide clues to understand structure-function relationships.[2] Functions of proteins can be as diverse as their nature. Enzyme proteins are substrate specific and catalyze the metabolic reaction by lowering the activation energy, which results in the reaction proceeding thousands of times faster than the one without enzymes.[3,4] Cytoplasmic proteins such as microtubules and actin provide mechanical support to eurokaryotic cell structure.[5,6,7] The plasma membrane and cell surface proteins play a major role in cell-cell and cell-pathogen interactions.[8,9,10,11] It is well known that the function of proteins can be significantly affected by a minute variation in the structure.[12] In fact, the primary step in studying the function of proteins involves a thorough structural analysis of proteins. Structural information obtained about proteins could shed light on various signaling pathways, cell division, metabolism, and their role in proteins in various diseases.

Considering the role of structure of proteins in their functions, a variety of techniques were utilized to obtain structural information. Nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography are the two most common techniques used for studying high resolution and also 3D protein structure at the atomic level. However, these techniques sometimes cannot be successfully used due to sample size, amount, and complexity of protein samples.[13,14] A variety of other approaches, including chemical labeling mediated mass spectrometry based analysis, have shown potential in the analysis of peptides and proteins.[15] Chemical labeling (covalent and non-covalent) involving the use of cross linkers, which contain two functional groups separated by a cleavable bond, can create intramolecular or intermolecular bonds between amino acid chains. Subsequently, amino acids can be separated via a cleavable bond to study the modifications using mass spectrometry.

In last two decades, the analysis of proteins has been greatly influenced by the evolution of mass spectrometry instruments.[16,17,18,19,20] The sensitivity, mass accuracy, and small sample volume required for mass spectrometric analysis has facilitated the characterization and identification of complex proteins. A typical mass spectrometer is made of three main components: ionization source, mass analyzer, and detector. Mass analyzers and detectors are maintained under vacuum to avoid unnecessary interaction between ions, and to efficiently traverse ions from source to analyzer without any interference. Electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) are two commonly used soft ionization techniques for the analysis of peptides and proteins. ESI produces multiply charged ions when the sample fluid is forced through a capillary in an electric field. The presence of an electric field is required for effective spraying. Ions are further sorted according to their *m/z* ratio in the mass analyzer (linear ion trap, triple quadrupole, time of flight, orbitrap, etc).[21] Time of flight mass

analyzers differentiate the ions by measuring the time required to travel from the source to reach the detector, provided all the ions receive the same kinetic energy. Quadrupole ion traps confine the ions between four hyperbolic parallel electrodes. Two opposite electrodes are maintained at a positive magnitude of AC and DC potentials and the other two opposite rods are maintained at negative AC and DC potentials. In linear ion traps (2-dimensional), ions are confined axially and radially using electric fields applied across four planar electrodes, and these allow only a single m/z to reach the detector. Linear ion traps are known to store a larger number of ions than 3-dimensional quadrupole mass spectrometers. In 3-dimensional quadrupole mass spectrometers, the ions are stored in a 3-dimensional electric field and multiple m/z is allowed to reach to detector. Multiple stages of mass analyzers can be combined to achieve fragmentation of precursor ions in successive analyzers. Precursor ions can be fragmented in the collision cell by collision induced dissociation (CID), which involves collision of an ion with neutral gas molecules resulting in dissociation of the ion. A typical tandem mass spectrometry instrument can be coupled with a chromatographic instrument in order to separate peptides or proteins prior to mass spectrometric analysis. In a typical "bottom up" experiment, a protein is digested into small peptides by using enzymes/chemicals prior to mass spectrometric analysis. The mass of the peptide is measured by the mass spectrometer in terms of *m/z*. However, more than one peptide can have the same nominal mass, and can be differentiated on the basis of amino acid sequences in those peptides. The ion of interest can be isolated and fragmented to generate a unique set of fragmented ions. If the charge is retained on the N-terminus, the ions are categorized as *a*, *b*, and *c*, and if the charge is retained on the C terminus, then the ions are categorized as *x*, *y*, and*z*.[22,23,24] Cleavage of the amide bond by CID results in *b* and *y* ions. The types of peptide fragments observed depends on many factors such as charge of the peptide, sequence, amount, and the way

energy is introduced. Fractionated proteins are analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), and the tandem spectra of peptides are manually or computationally matched with the theoretical tandem spectra using protein databases such as SEQUEST, MASCOT, OMSSA and XTandem. The SEQUEST algorithm utilizes a cross correlation approach to find out how well the experimental fragmentation spectrum matches with the theoretical fragmentation spectrum. A cross correlation score (Xcorr) is then assigned to each matched peptide. Xcorr can be calculated by dividing the cross correlation by the average auto correlation (background). The Mascot algorithm follows a probability based scoring approach to match the experimental fragmentation spectrum with the theoretical fragmentation spectrum. Total score ($10*\log_{10}(P)$) is the absolute probability that the observed match is a random event.

1.2. Conventional tools to study cell surface proteins (CSPs)

The surface of a cell is made of proteins, lipids, and carbohydrates, which are arranged in a three-dimensional manner. Various types of proteins at the interface (including cell adhesion molecules, growth factors, hormone receptors, and transporters) play an array of roles in cell communication. Additionally, CSPs are known to be associated with cell-cell interactions, ion transport, and cell signaling. Cell surface markers serve as a signature for particular cell types which are also expressed as disease biomarkers in various illnesses such as cancer, diabetes, and Alzheimer's disease. Identification of these disease biomarkers could be an essential factor for novel vaccine and drug development. Due to the importance of CSPs, a tremendous amount of effort was poured into developing strategies to effectively isolate and identify CSPs. However, heterogeneity, hydrophobic nature, and low abundance of CSPs create obstacles in efficient isolation and characterization of CSPs.

1.2.1. Traditional approaches to study CSPs

Traditionally, CSPs have been isolated by subcellular fractionation, density centrifugation, and sequential extraction methods. The centrifugation approach involves lysis or rupturing of cells prior to centrifugation using microdissection[25], sonic disruption[26], use of glass beads, [27] mild heat treatment,[28] alkaline[28] buffers, acid buffers, ionic buffers,[29] chelating agents,[30] detergent[31], reducing agents[32], homogenization using ice cold $NaHCO_3$ buffer to pH 7.5, [33] and isotonic sucrose solution.[34,35,36,37] Anionic detergents such as sodium dodecyl sulfate (SDS) were used for solubilization and purification of membrane proteins.[38] Detergents are amphiphilic in nature which helps to dissociate proteins and lipids tightly bound in the plasma membrane. Removal of detergents prior to mass spectrometric analysis and solubilization of the cell membrane using SDS without denaturing the structure of proteins is a challenging task. Various mild, non-denaturing agents were introduced to minimize the denaturing ability of SDS.[39,40] Combination of zwitterionic detergent, Zwittergent 3–10, and the nonionic detergent, Triton X-114, showed similar activity to SDS, but minimized the denaturing nature of SDS.[41] Centrifugation, subcellular fractionation, and detergent based partitioning have improved the isolation of cell surface and plasma membrane proteins. However, these techniques still lack specificity, reproducibility, and contaminate samples from intracellular proteins.[42,43,44] Negatively charged cell surfaces can also be targeted by cationic silica beads which electrostatically interact with negatively charged CSPs. Subsequently, CSPs are cross linked to beads by anionic polymers followed by cell disintegration. The next step involves separation of CSPs from cell debris and lysates using centrifugation. In the final step, CSPs are eluted from silica beads using detergents.[45] However, contamination from intracellular proteins raises questions about the specificity of this technique.[46]

In order to improve the specificity of targeting CSPs, chemical labeling/modification of proteins coupled with mass spectrometry have been commonly used. One of the most widely used approaches for cell surface labeling is biotin-streptavidin/avidin chemistry. Various biotinylation reagents were developed to covalently label/tag the lysine, cysteine, aspartic acid, and glutamic acid residues of CSPs followed by affinity capturing of biotinylated proteins by avidin/streptavidin beads.[47]Biotinylation reagents are made of three components; a biotin moiety for capturing the biotinyated proteins with avidin/streptavidin beads, a cleavable linker to facilitate the release of biotinylated proteins from beads, and a reactive group to covalently conjugate biotinylation reagents to proteins. Due to the abundance of lysine residues in CSPs, biotinylation reagents containing *N*-hydrosuccinimidyl (NHS) moiety, which can target the primary amine groups, have been widely used.[48,49,50,51,52,53] The NHS ester group reacts with primary amines in aqueous solvents, at physiological pH and room temperature, which are the ideal conditions to study the proteins in native conditions. NHS ester containing biotinylation reagents such as NHS/sulfo-NHS-LC-biotin, NHS-sulfo-NHS-SS-biotin and NHS-PEG-biotin are commercially available.[47]Upon biotinylation of CSPs, streptavidin/avidin immobilized on agarose/magnetic beads is introduced to capture the biotinylated CSPs. Even though biotinylation reagents are widely used, they have shown some pitfalls. The negative charged sulfonate group is believed to be membrane impermeable, which is an important factor in order to label the surface exposed amine groups. Despite having a negative charge, sulfo-NHS-LC biotin can cross the cell membrane, and label the cytoplasmic proteins.[54]The small size of biotinylation reagents such as sulfo-NHS-LC biotin could be responsible for permeation through the cell membrane, thus labeling cell membrane or cytoplasmic proteins. The elution of biotinylated proteins from streptavidin/avidin solid supports is a difficult task due to the highly

stable interaction between biotin and avidin. The reactivity of streptavidin/avidin towards proteins is another potential drawback of biotinylation reagents. Masuoka et al. demonstrated nonspecific avidin binding to the cell wall of *Candida albicans* in the absence of biotin via hydrophobic or electrostatic interactions.[55] Streptavidin was also found to be nonspecifically bound to human B cell and myeloid cell surface proteins.[56] Biotin is present in various components of living cells[57,58] such as telomerase complex,[59] and is involved in various biological processes involving cell growth and the citric acid cycle. The endogenous biotin can cause contamination,[60,61]and compete with the capturing avidin/streptavidin beads by capturing endogenous biotin instead of biotin conjugated to CSPs, thus affecting the capturing efficiency. Therefore, pitfalls of all these techniques necessitate the need to develop a cell membrane impermeable, highly specific, and easily separable cell surface labeling reagent.

1.3. Magnetic nanoparticles (MNPs) for sample preparation in proteomics

Sample preparation is one of the important steps in proteomics to achieve optimum sample amount prior to LC-MS/MS analysis. Isolation of the protein of interest from complex samples can be obtained by typical gel based or gel free techniques, which could be lengthy, tedious, and complicated, resulting in loss of peptides/protein. In the last few years, MNPs have emerged as a tool to quickly and efficiently isolate proteins from complex matrices.[62,63,64,65,66] MNPs, mostly iron oxide (Fe_3O_4), have been widely chosen in the healthcare sector due to its magnetic, optical, and chemical properties.[67,68,69,70,71] The Fe_3O_4 MNPs can be synthesized by techniques such as co-precipitation, hydrothermal synthesis, thermal decomposition, flow injection analysis, and aerosol/vapor phase methods. The Fe_3O_4 MNPs can be stabilized/coated with a hydrophilic layer to improve the aqueous stability, avoid oxidation of Fe_3O_4, and provide a platform for further characterization.[72] Various coating agents such as silica,[73] polyethylene glycol, dextran, chitosan,

and polyvinyl alcohols have been employed to coat the surface of magnetic nanoparticles. Silica has emerged as a most versatile coating agent due to ease of technique and improved stability in aqueous solvents. The Fe_3O_4 MNPs can be coated with silica using Stöber[74] or a reverse microemulsion technique[75] depending on the nature of the Fe_3O_4 MNPs surface (hydrophilic/hydrophobic). The Stöber or sol gel technique is based on hydrolysis and condensation of a silica precursor. The diameter of silica coated Fe_3O_4 MNPs ($Fe_3O_4@SiO_2$ MNPs) can be conveniently controlled by changing the quantity of the silica precursor, base, and reaction time. The free hydroxyl groups on $Fe_3O_4@SiO_2$ MNPs surfaces can be further modified by primary amine, thiol, or carboxylic acid groups, which serve as an intermediate step for conjugation of proteins. Silane coupling reagents such as (3-aminopropyl) triethoxysilane (APTES) and (3-mercaptopropyl) trimethoxysilane (MPTMS) are commonly used to introduce amine and thiol groups on the surface of $SiO_2@Fe_3O_4$ MNPs. The magnetic properties of MNPs decorated with amino acid functional groups allows quick, soft, and target specific separation of peptides/proteins conjugated to MNPs. Amine/thiol coated $Fe_3O_4@SiO_2$ MNPs can be attachedto various linkers to introduce functional groups such as *N*-hydroxysuccinimidyl (NHS) ester, *N*-isothiocyanate, and pyridyl disulfide, which can be covalently conjugated to the side chain of lysine containing amine and cysteine thiol groups. An NHS ester group can be covalently conjugated to free amine groups of peptides/proteins under physiological conditions to form a strong covalent bond, which is stable under various pH and temperature conditions. Recent application of functionalized MNPs in proteomics were either focused on selective enrichment of low abundance proteins or improved digestion of proteins using enzyme immobilized MNPs.[66] Rapid isolation and identification of *N*-blocked peptides from tryptic digest proteins of HepG2 cells were achieved by isothiocyanate coupled MNPs.[76]. Low abundance proteins containing free

thiol groups of cysteine residues were also targeted by pyridyl disulfide modified MNPs from complex peptide mixtures and depleted serum samples prior to LC-MS/MS analysis.

1.4. Contribution of magnetic nanoparticles (MNPs) towards development of a cell impermeable labeling reagent

The major challenges of development of a cell impermeable labeling reagent are associated with improving the size, specificity, and separation of currently available chemical labeling regents. Biotinylation reagents can provide ambiguous results due to intracellular uptake in the cell, difficulty to elute biotinylated peptide, and non specific interaction of streptavidin towards the protein.[77]To avoid the intracellular uptake of the labeling reagent, tunable MNPs can be introduced as a part of the labeling reagent to carry an amino acid reactive group. Larger size of MNPs assures the cell surface binding, which eliminates the chances of labeling intracellular proteins. Moreover, superparamagnetic properties of MNPs provide an edge over conventional time consuming separation techniques. The surface of the MNPs can be conveniently manipulated with reactive functional groups that can assist to introduce amino acid reactive functional groups. Considering these advantages of MNPs, we have developed a series of magnetic nanoparticles based labeling reagents containing three main parts: an amino acid reactive group, a cleavable bond, and MNPs. Amino acid reactive group covalently bind with the protein of interest, MNPs quickly recover the protein bound to MNPs and cleavable bond assist specific cleavage of labeled peptides from MNPs.

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Chapter 2

Covalent labeling of amine groups in peptides and proteins with *N*-hydroxysuccinimidyl ester modified $Fe_3O_4@SiO_2$ magnetic nanoparticles containing cleavable disulfide-bond linkers

2.1 Abstract

The reaction between NHS ester and primary amine group is one of the most commonly used chemistry for bioconjugation. In this research work, we have conjugated NHS ester group on the surface superparamagnetic silica coated iron oxide magnetic nanoparticles $Fe_3O_4@SiO_2$ MNPs. Further, we report the development of a protocol to label primary amines in peptides/proteins using NHS ester modified $Fe_3O_4@SiO_2$ MNPs. The MNPs-peptide adduct can be easily isolated using a magnet and no additional purification step is required. Moreover, MNPs conjugated peptide/protein can be cleaved at the disulfide linker to remove the MNPs moieties. The labeled peptides were analyzed by using LC-MS/MS for sequence and labeled site identification. This novel approach allowed us to characterize 6 lysine residues on the solvent accessible surface of native bovine serum albumin. Low-cost synthesis, rapid and convenient magnetic separation and specificity towards primary amine groups makes NHS ester coated $Fe_3O_4@SiO_2$ MNPs a potential labeling probe to study the living cell surface peptides/proteins. Studying cell surface proteins of bacterial/fungal/mammalian cell lines will provide an important piece of information which can be utilized to study biological functions of a cell, vaccine development and drug targeting process.

2.2 Introduction

The rapidly growing field of MNPs-protein conjugation has been decorated by extensive applications in cell and tissue imaging[1], drug delivery[2], isolation and purification of peptides/proteins[3]. MNPs have been functionalized with various protein/peptide reactive groups to enrich different proteomes. Separation of magnetic MNPs 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 like chromatography, filtration, centrifugation, etc[3].

Among all types of MNPs, iron oxide (Fe_3O_4) MNPs have a long history of being used for isolation and separation of peptides/proteins[5,6]. Properties such as high magnetization values, relatively easy control over the size, and narrow particle size distributions, make iron oxide MNPs better choices in conjugating proteins/peptides for isolation/separation purposes[7]. Moreover, Fe_3O_4 MNPs can be coated with silica(Fe_3O_4@SiO_2) to facilitate further surface modifications by various functional groups[8,9]. The silica coating protects the iron oxide from oxidation and disrupts possible specific and/or non-specific interactions between iron oxides and biological molecules[10]. Usually the surface of Fe_3O_4@SiO_2 MNPs can be modified by intermediate groups such as –NH2 and –SH [9,11] before further conjugation to functional groups that interact with peptides/proteins. Such modified magnetic MNPs can be used to form adducts with peptides/proteins via covalent as well as non-covalent interactions, and covalent interactions are preferred to non-covalent interactions in adduct formation due to the stability of covalent bonds at different pH and temperatures[12]. The functional groups that can be employed to form covalent bonds with proteins/peptides usually target unmodified side chains of amino acid

residues such as –SH (cysteine), -OH (serine, tyrosine), -COOH (aspartic acid, glutamic acid) or –NH2 (lysine)[13,14]. The low abundance of cysteine allows site specific labeling in a protein[15]. These unmodified cysteine residues can be labeled using haloacetyl and alkyl halide derivatives[16], maleimides[17], aziridens[18] and thiol-disulfide exchange reagents[19]. A recent study by Palani et al demonstrated that functionalized the Fe_3O_4@SiO_2 MNPs with thiol specific conjugating groups were employed to enrich cysteinyl peptides through disulfide bond formation[6]. Carboxylic acid containing amino acids can be targeted by using diazoalkanes and diazoacetyl compounds[20,21] , carbonyldiimidazole[22] and carbodiimide[23]. Hydroxyl group containing amino acids can be targeted using epoxides and oxiranes, oxidation with periodate[24], alkyl halogens[25] and isocyanates[26]. Primary amine groups in unmodified lysine residues and N-termini can be labeled with N-hydroxysuccinimidyl ester (NHS) esters[27], isothiocyanates[5], isocyanates[28] and acyl azides[29], and the most commonly used reaction to label primary amines is through the covalent bond formation with NHS esters.

Lysine residues are three times more abundant than cysteine residues in living organisms[30] which makes them a potential better sites for protein/peptide labeling. The unmodified lysine side chain (-NH_2) forms an amide bond with NHS esters at physiological pH in aqueous environment. Qian et al synthesized a fluorous affinity reagent and utilized it to label primary amines groups in peptides/proteins via active sulfo-NHS ester group[27]. Zhao et al demonstrated the use of isothiocyanate coupled magnetic MNPs for the separation of the blocked N-terminal peptides through covalent bonding. Kerr et al demonstrated primary amine selective lanthanide metal chelating labels to improve the peptide quantitation measurements[31]. Recently, Nicolardi et al labeled primary amine group in the Cu-protein Azurin using fluorescent label ATTO 655 NHS[32]. A biotinylation agent, (sulfo) NHS-LC-biotin was used to enrich primary

amine groups in the accessible vascular endothelial proteins[33]. Along with the NHS ester, NHS-LC-biotin contains biotin which is known for strongest non-covalent interaction with avidin and streptavidin. Another biotinylation agent, (sulfo) NHS-SS-biotin which contains amine binding group, avidin interacting group and disulfide bonds for cleavage was used to enrich cell surface proteomes of chronic and acute leukemia cell lines[34]. The sodium sulfonate group present on these compounds prevents it from crossing the lipid bilayer of cell membrane thereby conjugating to extracellular proteins. However, these labeling reagents require affinity/chromatographic separation to separate the conjugated peptide/protein for further analysis which makes this approach tedious. Conjugation of these NHS ester moieties on the surface of magnetic MNPs can provide an edge over the conventional labeling reagents. NHS ester modified magnetic MNPs (Thermo Fisher Scientific, Rockford, IL) are commercially available which are commonly used for immunoprecipitation. Identification of labeled proteins and labeled sites in those proteins were not part of the application of those commercially available MNPs that label primary amine groups in protein/peptide. In order to identify the labeling site in protein/peptides, it is necessary to trim magnetic beads (bulky compared to protein/peptide) from the conjugated protein/peptides before the labeled protein/peptides can be analyzed by mass spectrometry based approaches[27].

In this research work, Fe_3O_4 MNPs, coated with silica ($Fe_3O_4@SiO_2$) were prepared and covalently immobilized NHS ester on top of the silica layer. A disulfide bond was included in the linker between the NHS ester moiety and the $Fe_3O_4@SiO_2$ MNPs moiety. In addition to the superparamagnetic properties of the MNPs, the labeling sites of protein/peptide were also identified (by mass spectrometry based approach) during the characterization of functionalized $Fe_3O_4@SiO_2$ MNPs for primary amine labeling.

2.3 Experimental Details

Materials:

Iron (II) chloride hydrate ($FeCl_2$,$4H_2O$, 99%) was purchased from Alfa Aesar (Ward Hill, MA). Iron (III) chloride hexahydrate ($FeCl_3$,$6H_2O$, 97%), (3-aminopropyl)triethoxysilane (APTES), 3- mercaptopropyltrimethoxysilane (*MPTMS*), tetraethyl orthosilicate (TEOS), *N*,*N*-dimethylformamide (DMF), toluene, ammonium hydroxide solution, anhydrous dimethyl sulfoxide (DMSO), triethanolamine, iodoacetamide, ammonium bicarbonate and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO). Dithiobis(succinimidylpropionate) (DSP), (N-succinimidyl-3-[2-pyridyldithio]propionate) (SPDP) were purchased from Molecular Biosciences (Boulder,CO). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl) was purchased from Amresco (Solon,OH). Small peptide ACTH (4-11) was purchased from American Peptide company, Inc. (Sunnyvale,CA). Sequencing grade modified trypsin was purchased from Promega (Madison, WI). Phosphate buffered saline (PBS) was purchased from Calbiochem (Billerica,MA). Urea was purchased from Fisher Scientific (Pittsburgh, PA).

2.3.1 Synthesis of superparamagnetic iron oxide MNPs coated by silica (Fe_3O_4@SiO_2 MNPs)

The iron oxide MNPs core was prepared by using a conventional co-precipitation method[9]. $FeCl_3.6H_2O$ (1.1675 g) and $FeCl_2.4H_2O$ (0.43 g) were dissolved in deionized water (40 mL) under nitrogen with vigorous stirring at 90°C. Ammonia solution (14 M, 1.5 ml) was added to the Fe^{2+}/ Fe^{3+} solution which resulted in a color change of the reaction from orange to black immediately. Iron oxide MNPs were separated using a magnet and washed 3 times with deionized water and 3 times with ethanol.

The Fe_3O_4 MNPs were re-dispersed in a mixture of ethanol (40 mL), ultrapure water (10 mL), and ammonia solution (14 M, 0.5 mL) under ultrasonication for 5 min. Then, tetraethylorthosilicate (TEOS, 1 mL) was added to the above dispersion, and it was sonicated for another 5 min. The reaction was allowed to proceed under mechanical stirring for 8 h. The coated MNPs were separated using a magnet and washed with ethanol and DMF 3 times. The separated particles were immersed in HCl (4 N) for 20 min to dissolve the uncoated Fe_3O_4 MNPs. After separating from HCl solution, the coated MNPs were dried under vacuum overnight and divided into two batches.

2.3.2 Conjugation of NHS ester to Fe_3O_4@SiO_2 MNPs surface

The Fe_3O_4@SiO_2 MNPs (300 mg) were redispersed in a mixture of DMF (30 mL) and toluene (20 mL) under ultrasonication for 5 min. 3-aminopropyltriethoxysilane (APTES, 0.89 mM, 4 mL) was added to the above solution and stirred by using magnetic stirrer for next 24 hours. The coated Fe_3O_4@SiO_2 MNPs were separated by magnetic field and washed with ethanol five times and dried under vacuum for 8 hours. Amine coated Fe_3O_4@SiO_2 MNPs were characterized by measuring the zeta potential using dynamic light scattering (Wyatt Technology Corporation, Santa Barbara, CA) instrument.

In the next step, dithiobis(succinimidylpropionate) (DSP) was used to introduce NHS ester on the surface of amine coated Fe_3O_4@SiO_2 MNPs. Amine coated Fe_3O_4@SiO_2 MNPs (2 mg) were redispersed in ethanol (500 µL) and sonicated for 5 minutes. This suspension (500 µL) was immediately added to DSP (10 mg in 100 µL DMSO, 80 mM). The reaction mixture was stirred for 30 minutes using a thermomixer and furthers the NHS ester coated Fe_3O_4@SiO_2 MNPs were separated using a magnet, washed twice with ethanol and dried.

A similar protocol was used to conjugate thiol coated Fe_3O_4@SiO_2 MNPs with NHS ester using N-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP, 5 mg in 100 µL DMSO).Thiol coated Fe_3O_4@SiO_2 MNPs were prepared by coating with 3-mercaptopropyltrimethoxysilane (MPTMS) using the same protocol used for amine coating with APTES. Fe_3O_4@SiO_2 MNPs (300 mg) were redispersed in a mixture of DMF (30 mL) and toluene (20 mL) under ultrasonication for 5 min. To the above solution, 3-mercaptopropyltrimethoxysilane (MPTMS 0.89 mM, 4 mL) was added and the reaction mixture was stirred by using magnetic stirrer for next 24 hours. The coated Fe_3O_4@SiO_2 MNPs were separated by magnetic field and washed with ethanol five times and dried under vacuum for 8 hours. The samples were characterized by TEM-EDX. In addition to the peaks of iron, oxygen and silicon, presence of sulfur peak in the EDX spectrum confirmed the existence of MPTMS coating on the surface of Fe_3O_4@SiO_2 MNPs (Figure S1). Magnetization studies of thiol (MPTMS) coated Fe_3O_4@SiO_2 MNPs were performed at room temperature using MicroMag™ vibrating sample magnetometer (Model 3900, Princeton Measurements Corporation). Absence of hysteresis loop confirmed the superparamagnetic nature of thiol coated Fe_3O_4@SiO_2 MNPs (Figure S2).

N-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) was used to introduce NHS ester on the surface of thiol coated Fe_3O_4@SiO_2 MNPs (Scheme S1). Thiol coated Fe_3O_4@SiO_2 MNPs weretreated by TECP first to break any disulfide bond on the NP surface, and then washed 3 times using ethanol and dried under vacuum. Thiol coated Fe_3O_4@SiO_2 MNPs (2 mg) were redispersed in ethanol (500 µL) and sonicated for 5 minutes. This suspension (500 µL) was immediately added to SPDP (5 mg in 100 µL DMSO). The reaction mixture was stirred for 30 minutes using a thermomixer, and the NHS ester coated Fe_3O_4@SiO_2 MNPs were separated in magnetic field, washed twice by ethanol and dried before use.

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

2.3.3 Quantification of NHS ester conjugated on the surface of Fe_3O_4@SiO_2 MNPs by depletion approach

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

2.3.4 Labeling primary amine groups of ACTH 4-11 using NHS ester modified Fe_3O_4@SiO_2 MNPs

ACTH (60 µL, 3.3 mM in water) was diluted by adding PBS buffer (800 µL, pH=7-8) and further added to dried NHS ester modified Fe_3O_4@SiO_2 MNPs. This suspension was stirred vigorously for 45 minutes. After 45 minutes, the peptide conjugated MNPs were separated

using a magnet and washed 3 times with deionized water in order to remove any unmodified peptide. TCEP (6 mM in water) was added to the peptide conjugated MNPs and stirred for 45 minutes using a thermomixer. The MNPs were separated using a magnet and the supernatant was collected. The separated ACTH was further purified by C18 micro-column and analyzed using ESI-MS.

2.3.5 Labeling of primary amine containing peptides of BSA using NHS ester modified iron oxide MNPs

BSA (10 µL, 10 mg/mL) was diluted with 85 µL of PBS and iodoacetamide (6 µL, 200 mM) was added to the BSA solution. The BSA suspension was added to the dried NHS ester modified Fe_3O_4@SiO_2 MNPs with continuous stirring. The reaction was allowed to proceed for 45 minutes at room temperature. The protein-magnetic MNPs conjugates were separated from the suspension using a magnet and were washed with ethanol three times to remove any unreacted protein. The labeled BSA was then subjected to in-solution trypsin digestion[27]. Briefly, Urea (80 µL, 8.0 M) was added to the separated MNPs 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 trypsin (6 µg, 12 µL of 0.5 µg/µL) digestion at 37ºC for 15 h.

After 15 hours of tryptic digestion, MNPs were separated using a magnet and washed three times using water and 40% acetonitrile. TCEP (100 µL, 6 mM) was added to the to the separated iron oxide MNPs and incubated for 1 hr at room temperature. MNPs were separated using magnetic separation and supernatant was saved for the analysis. In order to remove,

excess TCEP, the supernatant was passed through ziptip (C18 column) and further, the sample was analyzed by LC-MS/MS.

2.3.6 LC-MS/MS analysis and data analysis

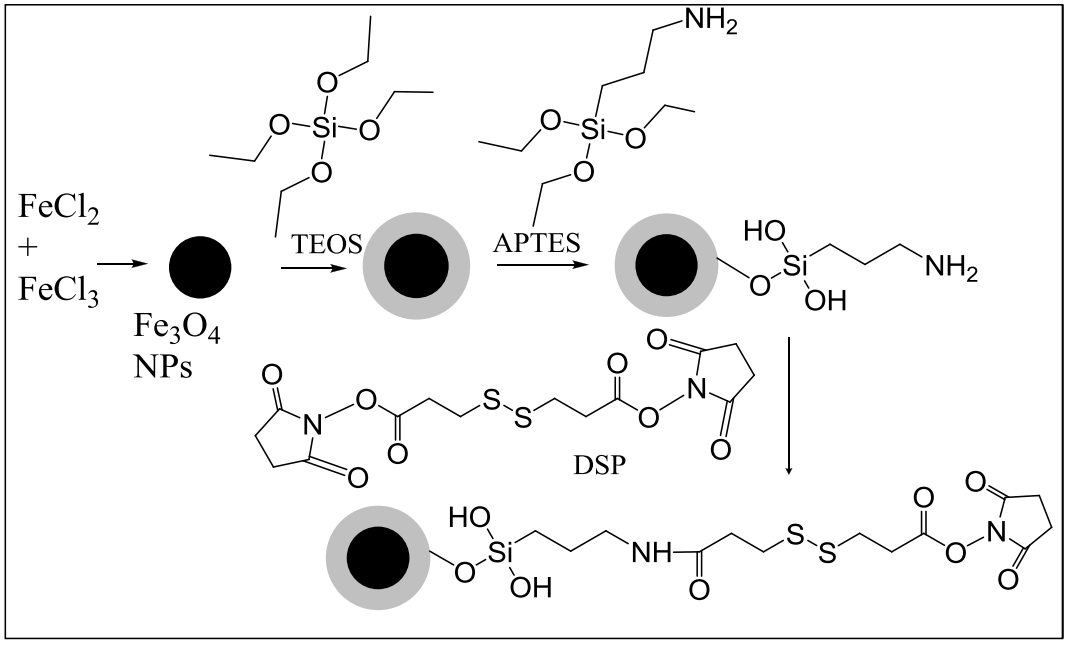
The ACTH peptide was analyzed by direct infusion methods and peptides of BSA were analyzed by performing liquid chromatography-electrospray-mass spectrometry (LC-ESI MS/MS) on a Finnigan LTQ^{TM} –ion trap mass spectrometer (Thermo Electron, San Jose, CA). BSA peptide sample (re-dissolved in 20 µL) was loaded onto a reversed phase trap column and washed with water for 5 minutes. Then the peptide mixture was eluted from the trap column and further separated by C18 reversed phase analytical column (MC-10-C18W-150MS, Micro-Tech Scientific, Vista, CA) using a 60 min gradient made of A buffer (0.1% formic acid/ 97% water/ 3% acetonitrile v/v/v) and B buffer (0.1% formic acid/ 3% formic acid/ 97% acetonitrile v/v/v) at a flow rate 1 µL/min. 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 were set to differential mass increase of 87.99 Da on lysine residues, 15.99 Da on methionine and 57.02 Da on cysteine. 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 and 3.0 for triply charged ions. MS/MS spectra of detected peptides were manually examined to ensure the quality of identification.

2.4. Results and Discussion

A variety of functional groups have been conjugated on the surface of iron oxide MNPs to make the magnetic separation more specific and selective. These literature studies inspired us to target primary amine containing peptides/proteins using NHS ester modified $Fe_3O_4@SiO_2$ MNPs**.**

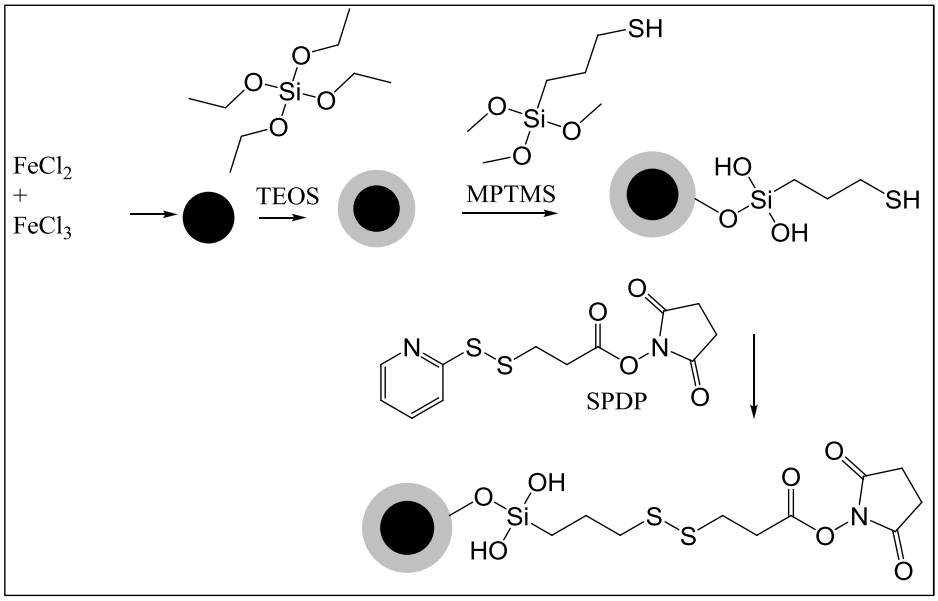
2.4.1. NHS ester conjugated $Fe_3O_4@SiO_2$ MNPs with a cleavable disulfide bond linker



Scheme 2.1: Preparation of NHS ester coated $Fe_3O_4@SiO_2$ MNPs using DSP

Superparamagnetic $Fe_3O_4@SiO_2$ MNPs were prepared by conventional co-precipitation method[9] and further coated with TEOS ($Fe_3O_4@SiO_2$) using sol-gel method[35] (Fig 1). In sol-gel process, TEOS is hydrolyzed in the presence of water and condensed on iron oxide

hydroxyl groups. This results in the formation of Fe-O-Si bond between iron and TEOS. Increasing the concentration of TEOS and degree of hydrolysis, three dimensional network via siloxane (Si-O-Si) bond is formed[35]. Silica coating was employed to protect Fe_3O_4, to avoid unnecessary interactions between the ligands and Fe_3O_4, to increase the stability of MNPs in aqueous solutions and to provide a platform for further functionalization[36]. However, our TEOS coated MNPs have shown severe aggregation which can be seen in Figure 2.1. In addition to $Fe_3O_4@SiO_2$ MNPs, we attempted to



Scheme 2.2: Alternative preparation of NHS ester modified $Fe_3O_4@SiO_2$ MNPs

conjugate NHS ester on the surface of bare Fe_3O_4 MNPs and used these MNPs to label amine groups in peptides. But, the expected peaks were not observed in MS analysis. We suspect that this might be due to the chelating ability of Fe(III) which results into formation of iron-analyte adducts[10]. The $Fe_3O_4@SiO_2$ MNPs were further coated with APTES in order to functionalize with primary amine group (scheme 2.1).

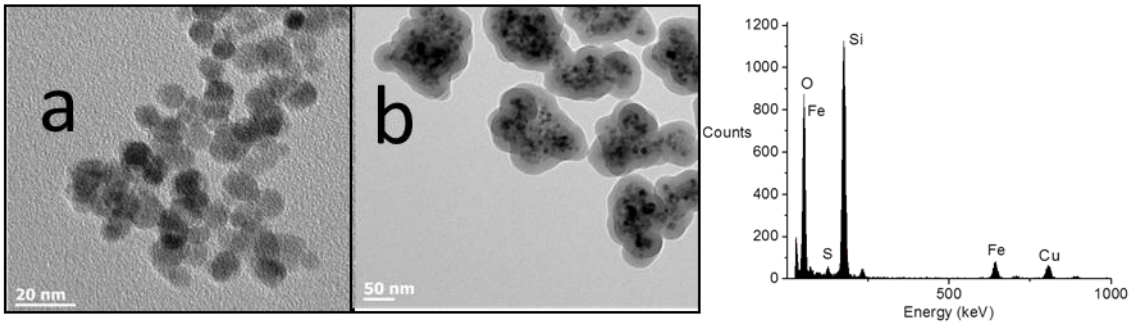


Figure 2.1: (a) TEM image of bare Fe_3O_4 MNPs synthesized by co-precipitation method and (b) $Fe_3O_4@SiO_2$ MNPscoated by sol-gel method. TEM-EDS studies of MPTES coated $Fe_3O_4@SiO_2$ MNPs (right).

The zeta potential of Fe_3O_4 MNPs and amine coated $Fe_3O_4@SiO_2$ MNPs were measured using dynamic light scattering in a phosphate buffered saline (10 mM, pH=7-8) solution which was found to be -10 and +25 respectively. After coating the $Fe_3O_4@SiO_2$ MNPs with APTES, the zeta potential became positive due to presence of amine group which protonates at neutral pH to yield a positive charge. Another set of $Fe_3O_4@SiO_2$ MNPs MNPs were coated with MPTMS to functionalize with thiol group[37] (scheme 2.2). TEM-EDS studies confirmed the presence of sulfur on the surface of $Fe_3O_4@SiO_2$ MNPs (Figure 2.1). TEM-EDS studies of uncoated $Fe_3O_4@SiO_2$ MNPs did not show any peak of sulfur.

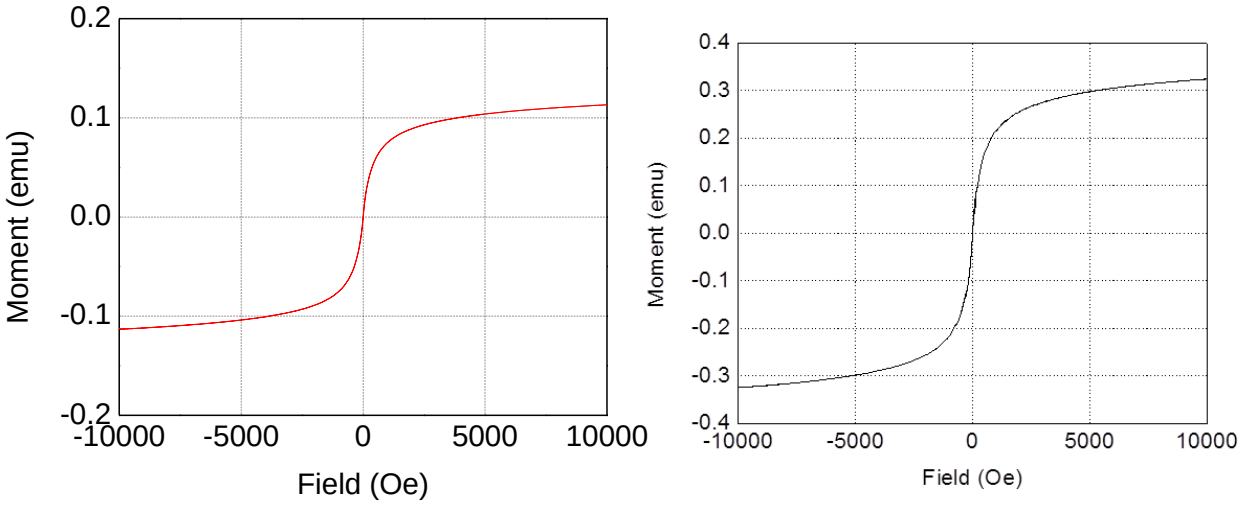


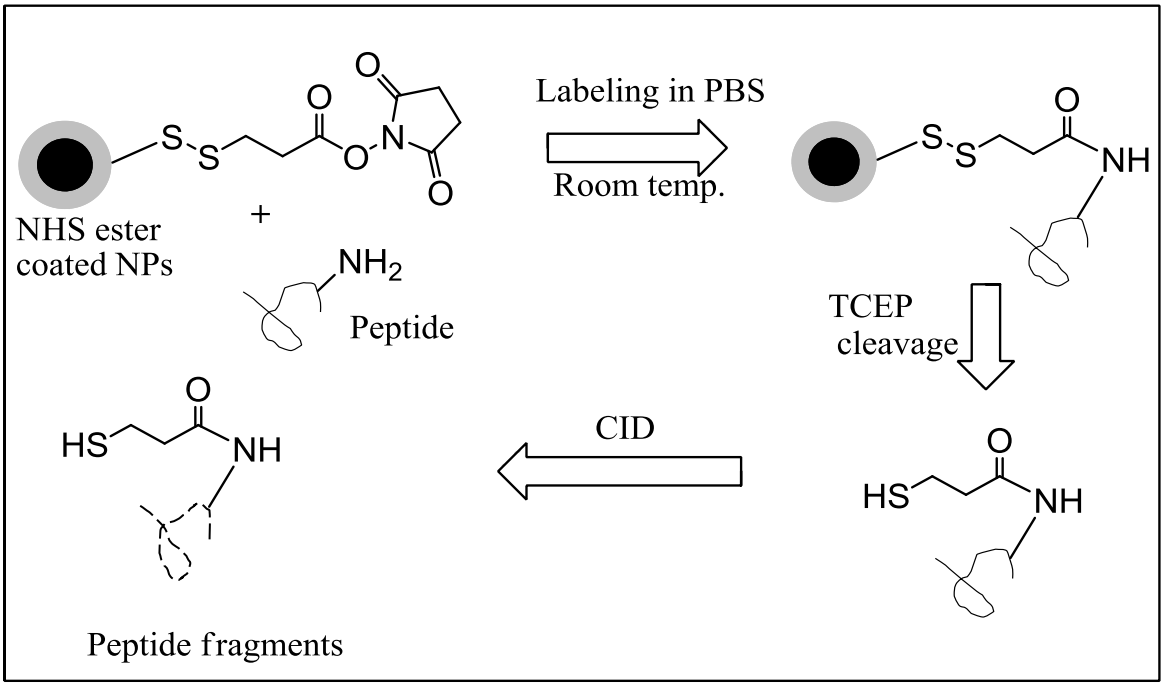
Figure 2.2: Magnetization studies of APTES (left) and MPTMS (right) coated Fe_3O_4@SiO_2 MNPs

In the next step, magnetization studies of amine and thiol coated Fe_3O_4@SiO_2 MNPs were performed using vibrating sample magnetometer (VSM) at room temperature. Absence of hysteresis loops in the magnetization curve of APTES and MPTMS coated Fe_3O_4@SiO_2 MNPs confirmed the superparamagnetic nature of the MNPs (Figure 2.2). Magnetization studies showed that coating by TEOS and APTES/MPTMS to Fe_3O_4 MNPs did not affect the superparamagnetic nature of MNPs.

2.4.2. Characterization of primary amine labeling by NHS 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.[13] NHS

ester modified $Fe_3O_4@SiO_2$ MNPs were employed to label a small peptide ACTH (4-11) (M-E-H-F-R-W-G-K) in PBS (10 mM, pH = 7-8). Freshly prepared NHS ester modified $Fe_3O_4@SiO_2$ MNPs 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.[19]



Scheme 2.3: Peptide labeling using NHS ester coated $Fe_3O_4@SiO_2$ MNPs prepared by using DSP

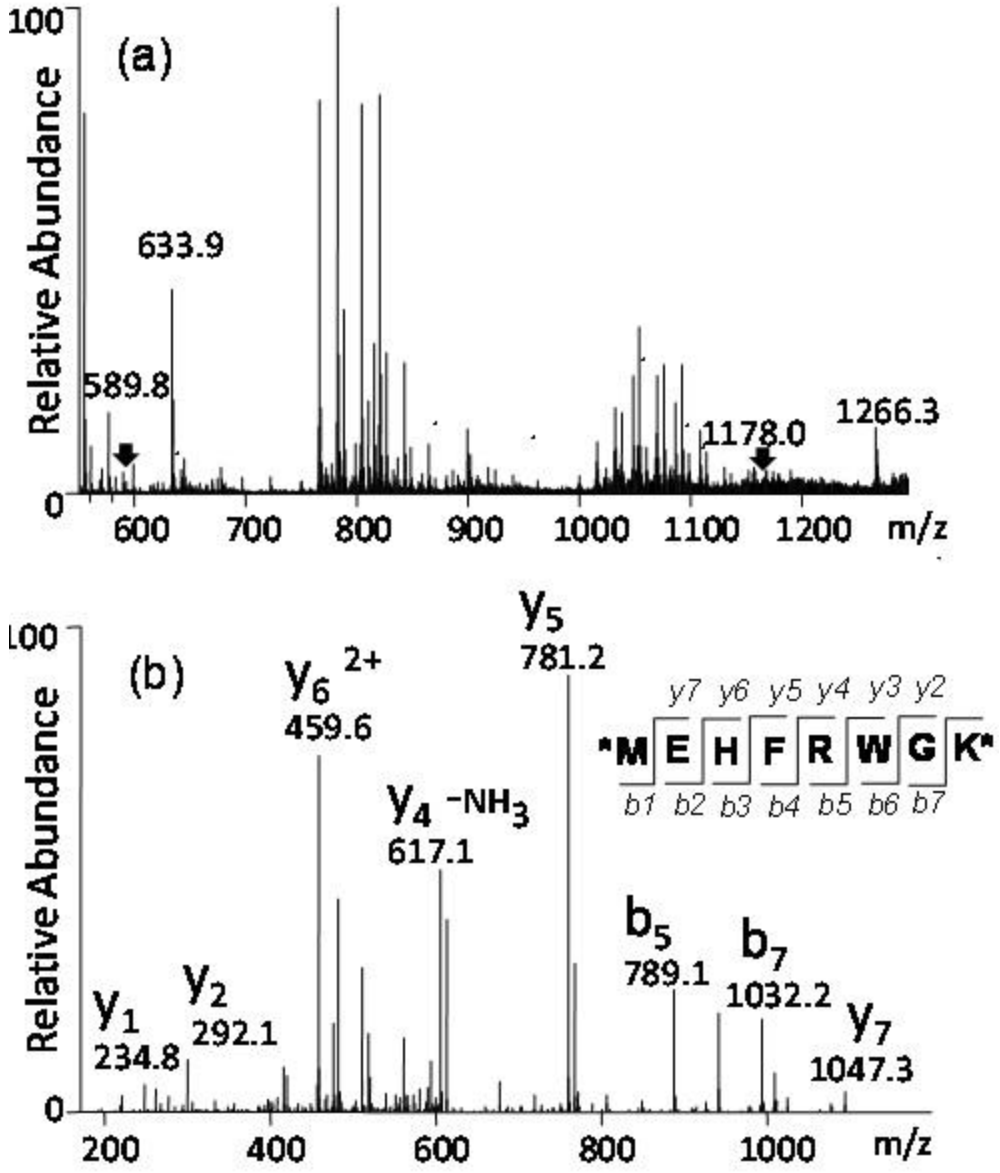


Figure 2.3: ESI-MS analysis of peptide ACTH (4-11) labeled by NHS ester modified Fe_3O_4@SiO_2 MNPs and then treated with TCEP to cleave disulfide bond. (a) In this mass spectrum, singly ion charged at *m/z* 1178.0 corresponds to ACTH (4-11) with one primary amine group modified; doubly charged ion at *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.

The magnetic MNPs conjugated to ACTH were cleaved by TCEP to release labeled ACTH. In the ESI-MS spectrum of the labeled ACTH (Figure 1.3a), the singly charged ions at *m/z* 1178.47 and doubly charged ions at *m/z* 589.99 correspond to ACTH with one primary amine modified; singly charged ions *m/z* 1266.33 and doubly charged ions at *m/z* 633.9 correspond to ACTH with two primary amine groups modified. Modification of the peptide by labeled MNPs added 87.99 Da per modified primary amine group in the peptide (Scheme 2.3). Tandem mass (MS/MS) spectrometry was performed on labeled ACTH to identify the sequence and modification site(s). Upon collision induced dissociation (CID), the labeled ACTH precursor ion at *m/z* 633.9 yielded a series of fragment ions (Figure 2.3b) 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 2.3b) matched the b and y ions of labeled ACTH (with both the peptide N-terminus and lysine side chain modified) predicted by MS-product program [38]. 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 ions, including those ACTH modified by NHS ester conjugated Fe_3O_4@SiO_2 MNPs prepared according to Scheme 2.2. SPDP coated Fe_3O_4@SiO_2 MNPs (Scheme 2.2) are structurally similar to NHS ester coated Fe_3O_4@SiO_2 MNPs prepared by Scheme 1.1, and were used to label amine groups in ACTH 4-11 and BSA according to the same protocol as mentioned in the materials and methods section. ESI-MS/MS of peptide ACTH (4-11) labeled by NHS ester

modified Fe_3O_4@SiO_2 MNPs. A doubly charged ion at m/z 590 (the peptide with one primary amine group modified) was isolated and fragmented to generate MS/MS spectrum (Figure 2.4). Sequence of peptide was confirmed by matching the *b* and *y* ions with the mass spectral signals. Gradient used in the elution of peptides from reversed phase LC column for electrospray mass spectrometry analysis

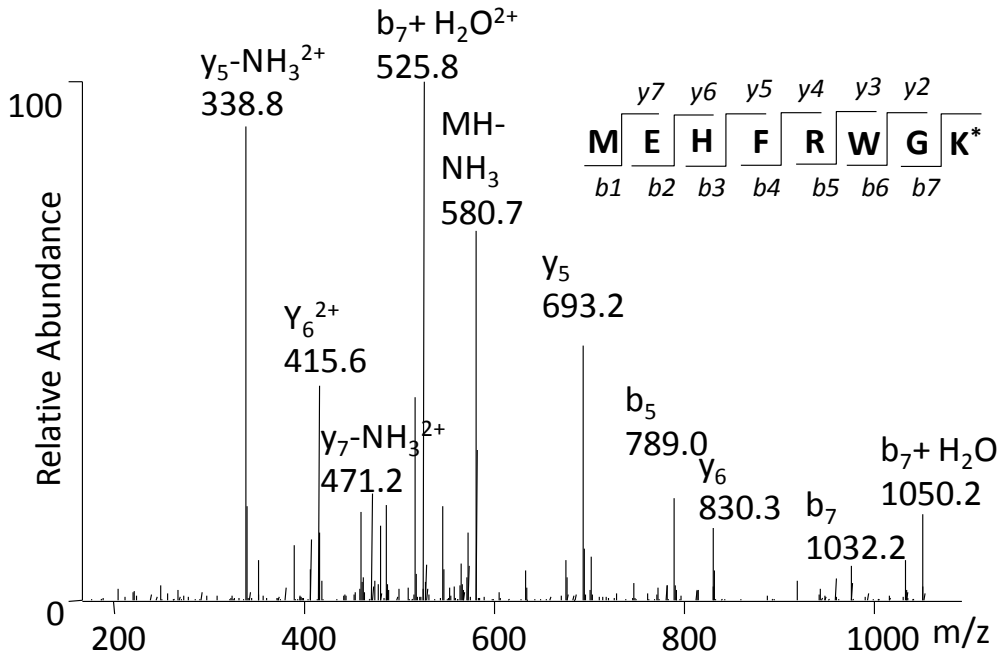


Figure 2.4: ESI-MS/MS of peptide ACTH (4-11) labeled by NHS ester modified Fe_3O_4@SiO_2 MNPs and then treated with TCEP to cleave disulfide bond. A doubly charged ion at m/z 590 (correspond to ACTH with one primary amine modified and doubly charged) was isolated and fragmented using CID to generate MS/MS spectrum. Sequence of the peptide was confirmed by matching the *b* and *y* ions with detected *m/z* values.

The concept (Scheme 2.3) of labeling primary amine groups by NHS ester conjugated Fe_3O_4@SiO_2 MNPs (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).

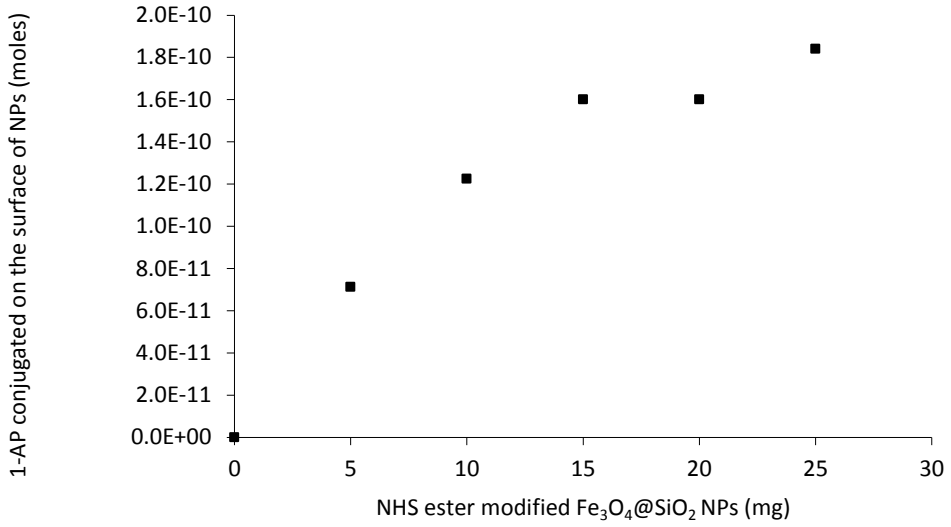


Figure 2.5: Indirect fluorometric quantitative analysis of 1-AP conjugated on the surface of NHS ester modified Fe_3O_4@SiO_2 MNPs (5, 10, 15, 20 and 25 mg). The difference between the amount of 1-AP before and after reaction with NHS ester modified Fe_3O_4@SiO_2 MNPs was calculated to determine the amount of 1-AP conjugated on the surface.

The amount of 1-AP conjugated on the surface NHS ester modified Fe_3O_4@SiO_2 MNPs (5, 10, 15, 10 and 25 mg) is shown in Figure 2.5. Even though the relationship between the amounts of NHS ester modified Fe_3O_4@SiO_2 MNPs and amount of 1-AP conjugated was completely linear, the amount of 1-AP conjugated was well correlated with the amount of

Fe_3O_4@SiO_2 MNPs. Based on this data set, and using an assumed average particle size of 150 nm, there were 44 ± 12 available binding sites per particle.

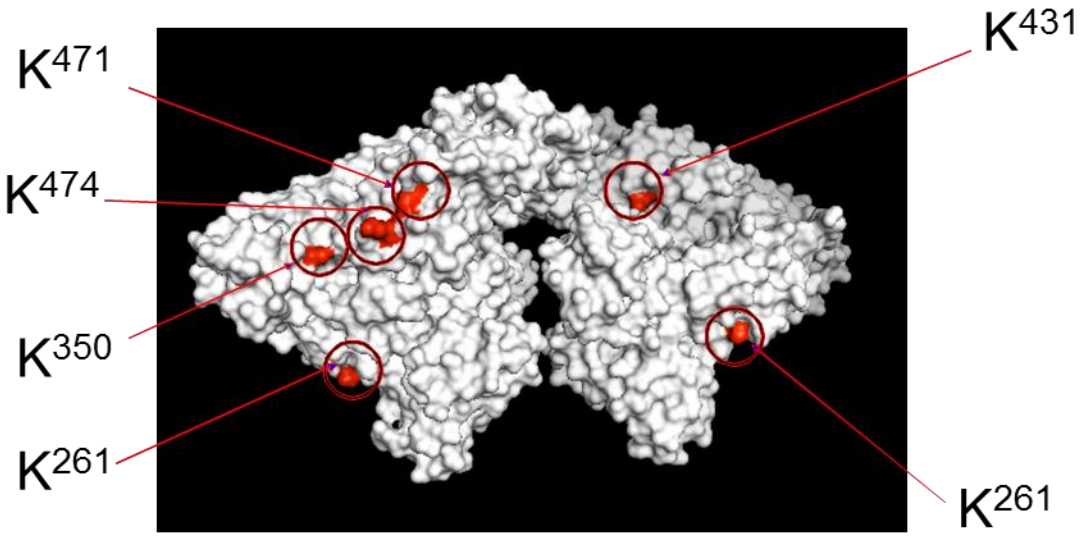


Figure 2.6: Homo dimer of mature bovine serum albumin (PDB 3v03) showing lysine residues (highlighted in red) labeled by NHS ester modified Fe_3O_4@SiO_2 MNPs.

NHS ester modified Fe_3O_4@SiO_2 MNPs 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.

Labeled lysine can be found in the middle of the peptide since trypsin does not cleave the protein at labeled lysine (Figure 2.6). A monomer of BSA contains 58 lysine residues however we were able to label only 6 residues present on the surface. This can be attributed to the size and aggregation of Fe_3O_4@SiO_2 MNPs. In addition, lack of monodispersity in MNPs is one of the reasons for not gaining access to every lysine present in BSA.

Position of amino acid residues in the sequence in mature BSA[a]	**Peptide sequence [b]**	**Xcorr[c]**
65-76	SLHTLFGDELC$^{75\text{-}91}$K	3.64
82-93	ETYGDMADC$^{90\text{-}101}$C$^{91\text{-}75}$EK	2.93
82-98	ETYGDMADC$^{90\text{-}101}$C$^{91\text{-}75}$EKQEPER	3.36
160-173	YNGVFQEC$^{167\text{-}176}$C$^{168\text{-}123}$QAEDK	3.81
257-273	***ADLAK*YIC$^{264\text{-}278}$DNQDTISSK***	***4.09***
262-273	YIC$^{264\text{-}278}$DNQDTISSK	4.08
348-362	***LAK*EYEATLEEC$^{359\text{-}368}$C$^{360\text{-}315}$AK***	***4.36***
351-362	EYEATLEEC$^{359\text{-}368}$C$^{360\text{-}315}$AK	2.65
363-375	DDPHAC$^{368\text{-}359}$YSTVFDK	2.93
397-409	LGEYGFQNALIVR	3.6
413-427	***K*VPQVSTPTLVEVSR***	***3.8***
428-444	***SLGK*VGTRC$^{436\text{-}447}$C$^{437\text{-}391}$TKPESER***	***3.61***
445-458	MPC$^{447\text{-}436}$TEDYLSLILNR	4.54
466-483	***TPVSEK*VTKC$^{475\text{-}486}$C$^{476\text{-}460}$TESLVNR***	***4.92***
466-483	***TPVSEKVTK*C$^{475\text{-}486}$C$^{476\text{-}460}$TESLVNR***	***3.95***
472-483	VTKC$^{475\text{-}486}$C$^{476\text{-}460}$TESLVNR	3.85
484-499	RPC$^{486\text{-}475}$FSALTPDETYVPK	4.16
505-520	LFTFHADIC$^{513\text{-}558}$TLPDTEK	4.2
509-520	HADIC$^{513\text{-}558}$TLPDTEK	2.72
Superscript: Cysteine residues in the peptides were part of a disulfide bond which was cleaved in the last step of reduction by TCEP. These disulfide bond linkages were mentioned in a superscript, e.g. C75-91 indicate that C75 (in SLHTL...) form disulfide bond with C91(in ETYGD…). *lysine residues modified by NHS ester tag. The peptides		

highlighted in bold/italic contain at least one labeled lysine residue. [a]Position of amino acid residues in the sequence of matured BSA. [b]Peptide sequence in matured BSA with positions of cysteine residues. [c] Cross-correlation score provided by SEQUEST algorithm

Table 2.1: Identified tryptic peptides derived from BSA labeled by NHS ester modified $Fe_3O_4@SiO_2$ MNPs

The another reason for not being able to label all the lysine was the exclusion of one of important step in the trypsin digestion which involves the use of a reducing agent, DTT to break the disulfide bonds in the protein to unfold the protein and make it available for digestion. However, the presence of disulfide bond on the spacer arm of NHS ester coated $Fe_3O_4@SiO_2$ MNPs did not allow us the use of DTT to unfold protein. The use of DTT will have cleaved the disulfide bonds on the spacer arm thereby isolating magnetic MNPs from NHS ester group before peptide enrichment. The addition of this step would have helped NHS ester coated $Fe_3O_4@SiO_2$ MNPs to gain access to more number of lysine residues.

In addition to labeled peptide, several unlabeled peptide were found in the LC-MS/MS analysis. In order to find the source of these peptides, the cysteine residues present in unlabeled peptides were numbered. The positions of the cysteine residues and disulfide bonds were cross-checked with the putative BSA sequence studied by Brown at al[39]. All the cysteine residues found in labeled and unlabeled peptide were part of a disulfide bonds present in BSA (Table 1.1) which shows that the peptides conjugated to MNPs were linked to other peptides though disulfide bond. In the first step of tryptic digestion, cysteine residues were preserved by adding iodoacetamide in order to protect the disulfide bond present on the crosslinker. This precaution has been taken because cysteine residues in BSA might break the disulfide bond in the crosslinker through which NHS ester is attached to $Fe_3O_4@SiO_2$ MNPs. In the last step, when TCEP was employed to cleave disulfide bonds in the crosslinker, disulfide bonds between labeled and unlabeled peptide were cleaved thereby releasing cysteine residues. These cysteine

residues can be observed in both labeled and unlabeled peptide (Table 1). Table 1 shows the number of amino acid sequence residues in mature BSA and positions of disulfide bonds in putative BSA.

2.5. Conclusion

We have demonstrated the specificity of NHS-ester modified Fe_3O_4@SiO_2 MNPs towards labeling primary amine containing peptides and proteins. Along with a small peptide, these MNPs can be used to label primary amines in a complex protein sample. The simplicity of the synthesis and functionalization of NHS ester modified Fe_3O_4@SiO_2 MNPs, effectiveness for the labeling of primary amine containing peptides/proteins and rapid, inexpensive protocol makes them a better approach in the field of MNPs-protein bioconjugation.

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Chapter 3

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

3.1 Abstract

Chemical labeling approaches used for protein footprinting utilizes widespread use of disulfide bond containing crosslinkers. 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 thiol groups of proteins and the linker. Previously, we reported disulfide linked *N*-hydrosuccinimide ester modified silica coated iron oxide magnetic nanoparticles (NHS-SS-Fe_3O_4@SiO_2 MNPs) to study the solvent exposed lysine residues of peptides/proteins. The disulfide bond in NHS-SS- Fe_3O_4@SiO_2 MNPs restricted the use of reducing reagent based protein digestion and formed uncessary disulfide bond between labeled and unlabled peptides. In the current work, the disulfide bond was replaced with a cleavable ester group to synthesize NHS ester modified SiO_2@Fe_3O_4 MNPs. The surface exposed amine groups of BSA and β-lactoglobulin were labeled using ester linked, NHS ester modified Fe_3O_4@SiO_2 MNPs under physiological conditions at room temperature. Protein-MNPs conjugates were further Use of the

cleavable ester group provide an improved method for protein labeling and allow use of disulfide reducing agents during protein digestion.

3.2 Introduction

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, lysines 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 crosslinkermay 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 SiO_2@Fe_3O_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-Fe_3O_4@SiO_2 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 Fe_3O_4@SiO_2 MNPs, and utilized ester linked, NHS ester modified MNPs to label the solvent exposed primary amine groups of proteins in its native state.

3.3 Experimental Details

3.3.1 Materials

Iron (II) Oxide (FeO(OH), Oleic Acid, dimethyl sulfoxide (DMSO, anhydrous), (3-Aminopropyl)triethoxysilane (APTES, 95%), tetraethylorthosilicate (TEOS, 99%), Igepal CO-520, cyclohexane, dansylcadaverine, ≥97%, hydroxylamine hydrochloride and bovine serum

albumin (BSA) and β-lactoglobulinwere purchased from Sigma-aldrich (St. Louis, MO). 1-Octadecene was purchased from Alfa Aesar (Ward Hill, MA). Ethylene glycolbis(succinimidylsuccinate) (EGS, +99%) was purchased from ProteoChem (Loves Park, IL). Phosphate buffered saline (PBS) was purchased from Calbiochem (Billerica,MA). Dithiothreitol and iodoacetamide were purchased from Piercenet, Thermo Scientific (Rockford, IL). ACTH (4-11) was purchased from American Peptide Company (Sunnyvale,CA). Ethanol was purchased from Pharmco-AAPER (Brookefield,CT). Nanopure deionized and distilled water water (18.2 MΩ) was used for all experiments.

3.3.2. Synthesis of Fe_3O_4 MNPs by thermal decomposition of carboxylic salts

Briefly, FeO(OH), oleic acid, and octadecene were mixed in a three neck flask and heated at 320˚C for 60 minutes. The color of solution was turned from black to brown indicating the formation of nanocrystals. The Fe_3O_4 MNPs were separated using a magnet, and washed with ethanol several times. The Fe_3O_4 MNPs were air dried at room temperature and stored in toluene.

3.3.3 Silica coating of Fe_3O_4 MNPs by reverse micro-emulsion approach (Scheme S1)

The Fe_3O_4 MNPs (400 µL of 10 mg/mL) were dissolved in cyclohexane (4 mL) and Igepal-CO-520 (0.247 g) then sonicated for 15 min. The suspension was further mixed with tetraethyorthosilicate (25 µL) and sonicated again for 10 min. In the last step, ammonium hydroxide (50 µL) was added and sonicated for 15 min. The suspension was stirred using a magnetic stirrer at room temperature for 24 h. The SiO_2@Fe_3O_4 MNPs were magnetically recovered, washed using ethanol several times, and dried at room temperature.

3.3.4 Functionalization of Fe_3O_4@SiO_2 MNPs with amine groups (Scheme S1)

The SiO_2@Fe_3O_4 MNPs (10 mg) were resuspended in ethanol (10 ml) followed by 20 min of sonication. In the next step, APTES (95%, 100 µL) was added dropwise and the mixture was

mechanically stirred at room temperature for 24 h. Amine modified Fe_3O_4 SiO_2@Fe_3O_4 MNPs were magnetically separated, washed several times with ethanol, and air dried at room temperature.

3.3.5 Synthesis of cleavable linked, NHS ester modified Fe_3O_4@SiO_2 MNPs (Scheme S1)

Amine modified Fe_3O_4@SiO_2 MNPs (1 mg) were mixed with ethanol (100 μL), followed by sonication for 10 min. EGS (12 mg in μL DMSO, 100 final conc. 0.13 M) was added dropwise to the solution of amine modified SiO_2@Fe_3O_4 MNPs and allowed to react for 20 min at room temp. EGS modified Fe_3O_4@SiO_2 MNPs were recovered by magnetic separation, washed with ethanol, and dried under vacuum.

3.3.6 Labeling amine groups of ACTH (4-11) using cleavable ester linked, NHS ester modified Fe_3O_4@SiO_2 MNPs

Labeling of BSA and β-lactoglobulin was performed by following a protocol as reported earlier [10] with minor modifications. Protein sample (BSA or β-lactoglobulin, 10 μL, 10 mg/mL) solution was mixed with ester cleavable, NHS ester modified SiO_2@Fe_3O_4 MNPs followed by addition of PBS, pH=7.4 (190 μL). The mixture was allowed to stir at room temperature for 40 min. Protein conjugated SiO_2@Fe_3O_4 MNPs were magnetically separated and washed with water (6X). The unreacted NHS ester groups on the surface of SiO_2@Fe_3O_4 MNPs were quenched by reacting with Tris-HCl (100 μL of 50 mM) for 15 min followed by washing with water (3X). Protein conjugated SiO_2@Fe_3O_4 MNPs were incubated with urea (8M, aq. 80μL) and DTT (5μL of 200 mM) at 45˚C for 1 hr. Free thiol groups were alkylated with iodoacetamide (10 μL of 200 mM) for 1 hr in the dark. Trypsin was added (6 μg) with ammonium bicarbonate (1 mL of 50 mM) followed by digestion for 15 hr at 37˚C. The tryptic peptide conjugated SiO_2@Fe_3O_4 MNPs were magnetically separated and washed with water (3X), andwater:ACN (30:70, 6X). Labeled

tryptic peptides were isolated from SiO_2@Fe_3O_4 MNPs by cleaving the ester bond using hydroxylamine (200 μL of 2M, pH=8.5) for 4 hr at 37˚C. The SiO_2@Fe_3O_4 MNPs were magnetically separated, and the supernatant was saved for further analysis.

3.3.7 Mass spectrometric analysis of labeled ACTH (4-11), BSA and β-lactoglobulin

A chip consisting of a 160 nL enrichment column and a 150 mm analytical column packed with C18, 5 m beads with 300˚A pores was utilized for chromatographic separation. The sample (2 μL) was transferred to the enrichment column via the capillary pump operating at a flow rate of 4 μL/min. The nano pump was operated at a flow rate of 600 nL/min. The MS source was operated at 300˚C with 5 L/min N_2 flow and a fragmentor voltage of 175 V. N_2 was used as the collision gas, and the collision energy varied as a function of mass and charge using a slope of 3.7V/100 Da and an offset of 2.5 V. Both the quad and TOF were operated in the positive ion mode. The calibration standards contained reference compounds of 322.048121 and 1,221.990637 Da which were continually leaked into the source for mass calibration. LC chromatograms and mass spectra were analyzed using Mass-Hunter software (Version B.0301; Agilent Technologies).

3.4 Results and Discussion

In this study, the Fe_3O_4 MNPs were prepared by a thermal decomposition method as reported earlier [11]. The Fe_3O_4 NPs were characterized by TEM and XRD (Figure 3.1). The Fe_3O_4 MNPs were spherical, uniform, and average diameter was found be ~10 nm (Figure 3.1). The core shell Fe_3O_4@SiO_2 MNPs were prepared by coating Fe_3O_4 MNPs with silica by using a reverse microemulsion technique [12]. TEM characterization confirmed the spherical and core shell nature of Fe_3O_4@SiO_2 MNPs. The average diameter of Fe_3O_4@SiO_2 MNPs was ~35 nm and showed stability in aqueous solvents (Figure 3.1). The SiO_2@Fe_3O_4 MNPs were further modified with amine groups using (3-aminopropyl)triethoxysilane (APTES) [7]. Upon addition of amine groups

onto the surface of Fe_3O_4@SiO_2 MNPs, the observed zeta potential changed from an original negative value (-9 ± 5 mV) to a positive value (+23 ±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 Fe_3O_4@SiO_2 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.

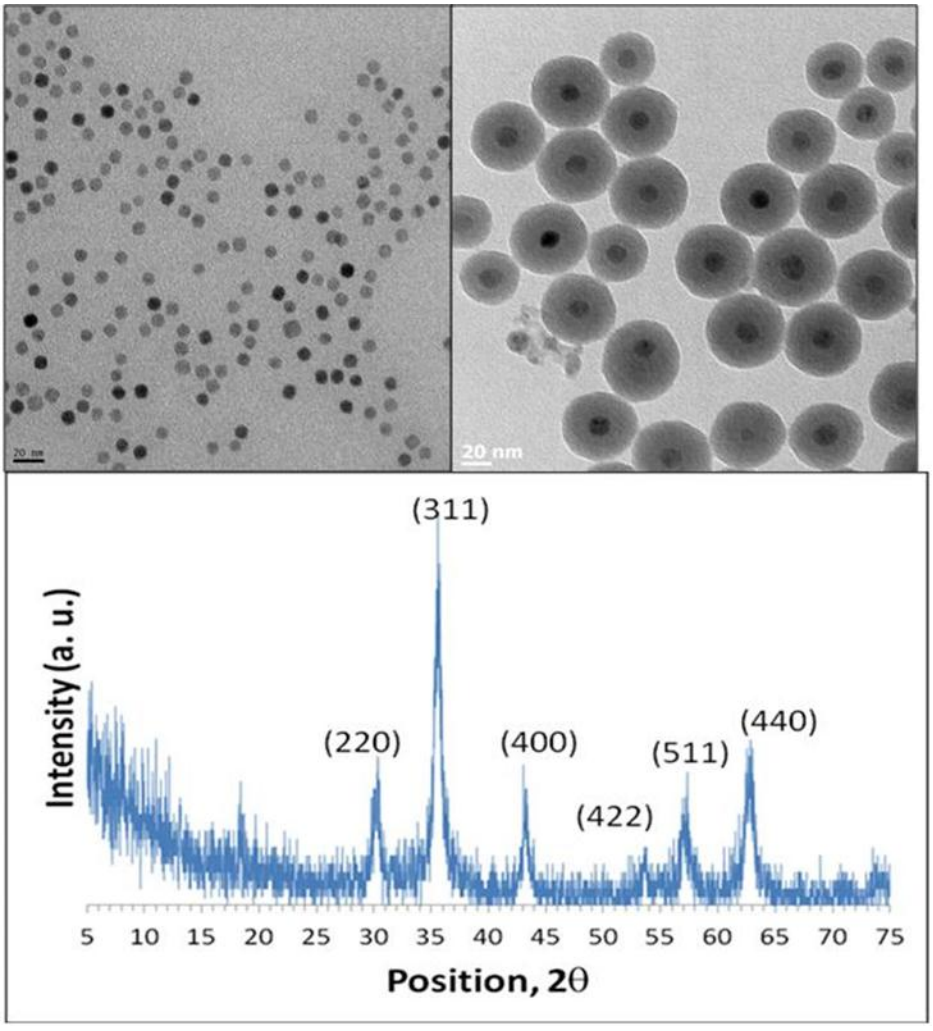
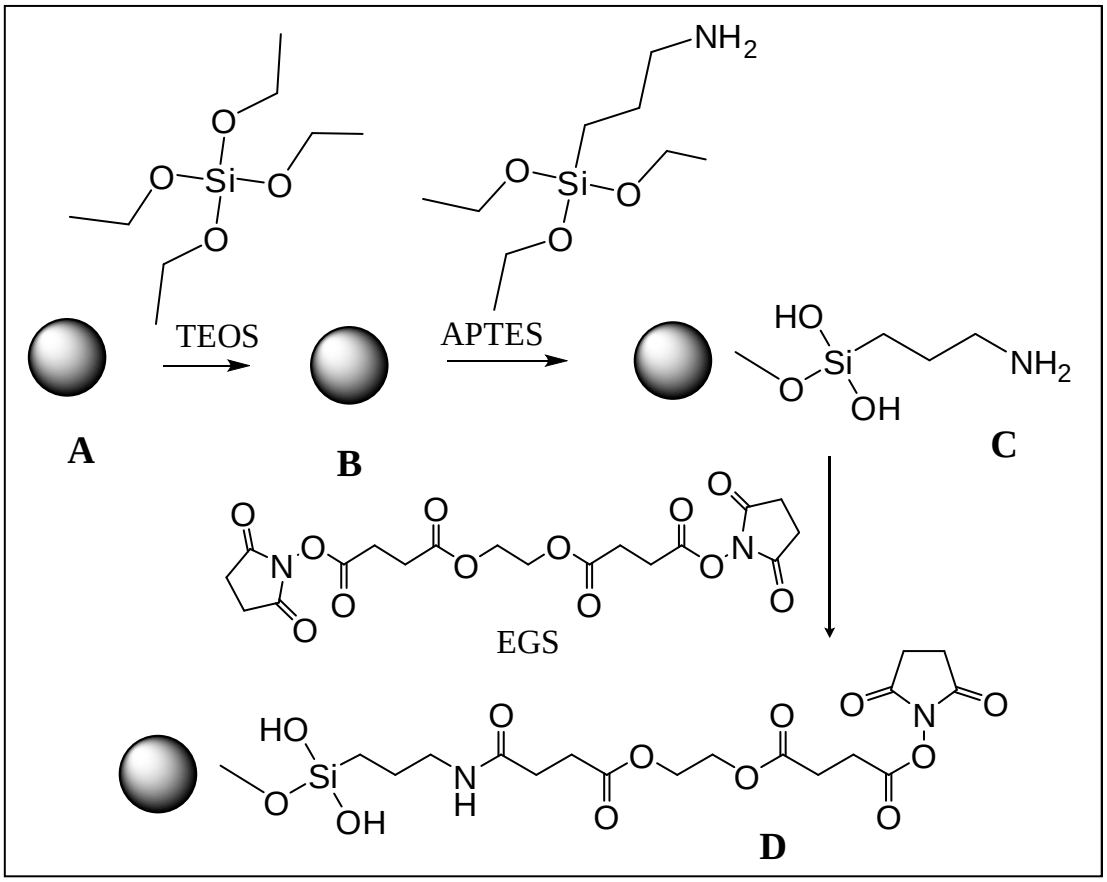


Figure 3.1: TEM analysis of Fe_3O_4 MNPs (~10 nm, top left) and (B) Fe_3O_4@SiO_2 MNPs (~35 nm, top right). The Fe_3O_4 and Fe_3O_4@SiO_2 MNPs were found to be spherical, uniform and

stable in aqueous solvents. The XRD of Fe_3O_4 MNPs (bottom) was found to be a match for Fe_3O_4 index.



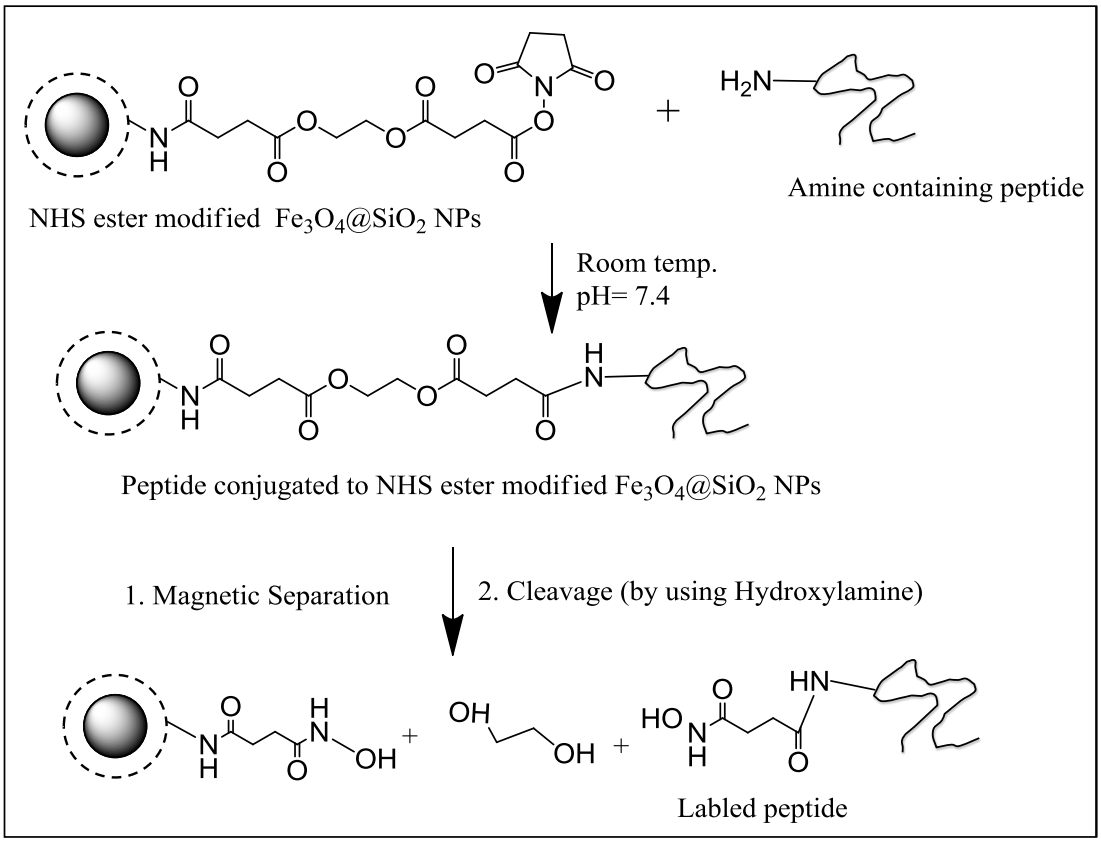
Scheme 3.1: Synthesis of ester linked, NHS ester modified $Fe_3O_4@SiO_2$ MNPs.

A homobifuntional crosslinker (ethylene glycol bis[succinimidylsuccinate], EGS) was utilized for conjugation of NHS ester groups on the surface of amine modified $Fe_3O_4@SiO_2$ MNPs (Scheme 3.1). In order to avoid crosslinking between MNPs, a high concentration of EGS was maintained during the coupling reaction. The amine modified $Fe_3O_4@SiO_2$ MNPs were

slowly added to a DMSO solution of EGS and allowed to react for 20 min. The NHS ester modified Fe_3O_4@SiO_2 MNPs were magnetically separated, washed with ethanol three times, and dried under vacuum. A depletion approach was used to quantify the active NHS ester groups on the surface of Fe_3O_4@SiO_2 MNPs. An amine containing flurophore, dansylcadaverine (excitation: 335 nm and emission: 513 nm) was conjugated to NHS ester modified SiO_2@Fe_3O_4 MNPs, and the remaining amount of dansylcadaverine was fluorimetrically measured to determine the amount of dansylcadaverine conjugated to Fe_3O_4@SiO_2 MNPs. The amount of dansylcadaverine conjugated to Fe_3O_4@SiO_2 MNPs corresponds to active NHS ester groups on the surface of Fe_3O_4@SiO_2 MNPs. These studies indicated 694 available NHS ester binding sites per 1 mg of Fe_3O_4@SiO_2 MNPs.

In order to investigate the ability of ester linked, NHS ester modified Fe_3O_4@SiO_2 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 Fe_3O_4@SiO_2 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 h 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.



Scheme 3.2: Labeling of amine containing peptides using cleavable ester linked, NHS ester modified Fe_3O_4@SiO_2 MNPs.

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. (See supporting information) 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 Fe_3O_4@SiO_2 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 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.

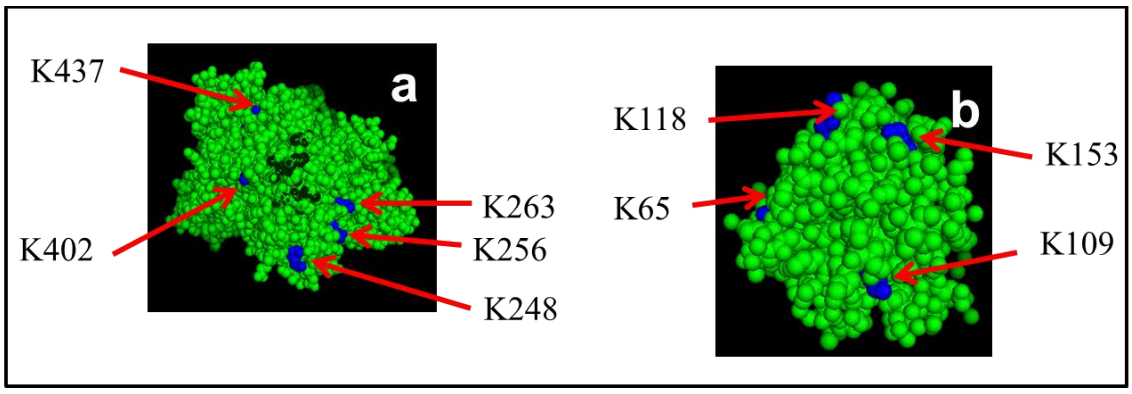


Figure 3.2: Labeled lysine residues (blue) of (a) BSA and (b) βlactoglobulin (left bottom) with ester linked, NHS ester modified Fe_3O_4@SiO_2 MNPs.

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. 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.

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

Table 3.1: List of peptides with labeled lysine residues (represented k).

Ester linked, NHS ester modified Fe_3O_4@SiO_2 MNPs successfully labeled the lysine residues located on solvent accessible surfaces of BSA and β lactoglobulin (Figure 3.2). NHS ester modified Fe_3O_4@SiO_2 MNPs labeled four lysine residues in β lactoglobulin (contains 16 lysine residues) and six lysine residues in BSA (contains 58 lysine residues) (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. (Table 3.1) In previous work, the captured peptides were released from the nanoparticles by cleaving the disulfide linker (NHS-SS-Fe_3O_4@SiO_2 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.

3.5. Conclusion

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.

3.6. Acknowledgements

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3.7. References

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Chapter 4

Mapping solvent exposed carboxylic acid residues of proteins using disulfide linked amine modified $Fe_3O_4@SiO_2$ MNPs

4.1. Abstract

In this work, we have developed a magnetic nanoparticles (MNPs) based approach to label solvent exposed glutamic and aspartic acid residues of proteins. The surface of silica coated iron oxide magnetic nanoparticles ($Fe_3O_4@SiO_2$ MNPs) was modified with primary amine groups via a disulfide bond (NH_2-SS-$Fe_3O_4@SiO_2$ MNPs). The carboxylic acid residues of proteins were labeled by NH_2-SS-$Fe_3O_4@SiO_2$ MNPs by using water soluble 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) coupling chemistry. The protein-MNPs conjugate was separated by a magnet followed by cleavage of the disulfide bond, which introduced a mass shift of 59.14 Da on the aspartic and glutamic acid residues of the corresponding protein. The LC-MS/MS analysis of labeled peptides revealed labeling of one aspartic acid and five glutamic acid residues located on the solvent accessible surface of BSA. The superparamagnetic properties of NH_2-SS-$Fe_3O_4@SiO_2$ MNPs offered easy, rapid, and clean magnetic separation of labeled proteins. The large size of MNPs assured the labeling of solvent exposed carboxyl groups without any unnecessary labeling of internal carboxyl groups of proteins. The NH_2-SS-$Fe_3O_4@SiO_2$ MNPs demonstrated the ability to specifically target surface

exposed carboxyl groups of proteins, and can be utilized to study the surface of complex lipoproteins.

4.2. Introduction

Structural analysis of the solvent exposed surface of proteins provides important information about protein-protein interactions, protein-DNA interactions, and protein solubility. The surface exposed amino acids contribute toward solubility of proteins. The information about the surface of proteins can be utilized for crystallization of membrane proteins, and treatment of human disease. Moreover, studying the solvent accessible surface of proteins can also help to predict the secondary structure of proteins.[1,2] Reactive functional groups of amino acids can be labeled by using chemical reagents, and the labeled peptides or proteins can be analyzed using mass spectrometry to determine the identification of the labeled site. Commonly targeted functional groups include amine groups of lysine residues, carboxylic acid groups of aspartic acid, and glutamic acid residues, thiol groups of cysteine residues, and hydroxyl groups of tyrosine and serine residues. The amino acids can be labeled through covalent or non-covalent interactions; however, covalent interactions are preferred due to their stability under different pH, temperature, and solvent conditions.

Acidic amino acids such as glutamic acid and aspartic acid are localized on the surface of proteins, and the identification of these amino acid sites can help to study protein-protein interactions in complex systems.[3] Trevino et al demonstrated the favorable contribution of solvent exposed aspartic acid, glutamic acid, and serine towards the solubility of ribonuclease compared to hydrophilic amino acids.[4] Carbodidimides such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) have been commonly used to modify carboxylic acid containing amino acids. EDC is a zero length cross linker which activates the carboxyl group of

proteins. The activated group is further attacked by an amine containing nucleophile forming a covalent bond between the amine and carboxyl group. EDC reacts with carboxyl groups to form an *O*-acylurea intermediate, which further forms an amide bond with an amine group. The *O*-acylurea intermediate formed after activation of the carboxyl group can be hydrolyzed in aqueous solvent thereby affecting the yield of reaction. In order to solve this problem, NHS or sulfo-NHS can be added to the reaction in order to react with unstable *O*-acylurea and form a more stable amine reactive NHS/sulfo- NHS ester.[5] The reaction between NHS or sulfo-NHS esters and amine groups can be achieved at physiological pH and room temperature helping to preserve the native structure of proteins. Geren et al. used EDC to modify carboxylic acid groups of adrenodoxin and identified glutamic and aspartic acid residues of bovine adrenodoxin by HPLC peptide mapping. [6] EDC has been used in conjunction with amine containing nucleophiles such as glycinamide [7], glycine ethyl ester (GEE)[8,9,10] glycine methyl ester[11, 12], and methylamine or taurine[13] to modify and identify the carboxyl groups of proteins. The active and inactive forms of Ras proteins were compared by modifying the carboxyl groups of the Ras protein by EDC/glycinamide chemistry under mild conditions.[14] The carboxyl groups of aspartate and glutamate of a transmembrane receptor, Her4, were modified by EDC/GEE to study the dimerization of the Her4 protein. This carboxylic footprinting approach detected 37 glutamate and aspartate residues.[15] Recently, Zhang et al used EDC/GEE chemistry to modify solvent accessible aspartic and glutamic acid residues of calmodulin protein. The EDC/GEE modification (reaction time: 90 seconds) did not perturb the secondary structure of calmodulin as verified by circular dichroism studies.[16] A similar approach was recently employed to study the dimerization and phosphorylation on Her2 and Her3 kinase domains.[17]

Carbodiimides can also be combined with affinity based biotinylated reagents such as biotin-polyethylene glycol (PEG)-amine which are commercially available for modification of aspartate and glutamate of proteins. [18,19] Biotinylation reagents are made of biotin, a cleavable linker, and an amino acid reactive group. Upon modification of proteins, biotinylated proteins are captured by using avidin/streptavidin immobilized polymer beads, magnetic beads, or well plates owing to the strongest non-covalent interaction between biotin and avidin/streptavidin. Biotin-PEG-amine can be covalently conjugated to aspartate and glutamate by using EDC as a crosslinker followed by affinity capture using avidin/streptavidin resin beads. The major disadvantage of biotinylated reagents is the difficulty in elution of biotinylated proteins from avidin/streptavidin beads due to the stronger complex formed between biotin and avidin/streptavidin resin beads.[20]

Magnetic nanoparticles (MNPs) have been widely employed for sample preparation in proteomics due to simple and rapid separation of MNPs, high surface to volume ratio, and easy functionalization strategies.[21] MNPs can be conveniently functionalized with various amino acid reactive groups which increase the specificity of these MNPs towards amino acids. For biological applications, iron oxide MNPs (Fe_3O_4) are preferred among all types of magnetic nanoparticles due to low toxicity, controlled synthesis, and superparamagnetic properties. The Fe_3O_4 MNPs can be stabilized by a protective silica or polyethylene glycol layer to avoid oxidation of Fe_3O_4 MNPs, provide a platform for further functionalization, and minimize unnecessary interaction between Fe_3O_4 and biomolecules. Silica coated Fe_3O_4 MNPs can further be functionalized with amine, thiol, or carboxylic acid groups which act as intermediates for functionalization with amino acid reactive groups. The amino acid reactive groups can be conjugated to the surface of MNPs to direct them towards the amino acids of interest.[22,23]

Magnetic separation can provide gentle separation of proteins conjugated to MNPs compared to column chromatography which can be detrimental to proteins. Magnetic separation is inexpensive compared to chromatographic instruments, and is quicker than filtration and centrifugation.[24] A variety of functional groups including sulfo-NHS ester[25], *N*-isothiocyanate[26], and pyridyl disulfide [27] were immobilized on the surface of MNPs for enrichment of proteins from a complex mixture. Even though the abovementioned studies discuss amino acid mediated, covalent conjugation of magnetic nanoparticles to peptides/proteins, the focus of studies was on the enrichment of peptides.

Previously, we reported the synthesis of disulfide linked NHS ester modified $Fe_3O_4@SiO_2$ MNPs to covalently label the solvent exposed amine groups of peptides/proteins.[28] In this work, we have modified the surface of thiol coated magnetic nanoparticles with a linker containing an amine group via a disulfide bond. Disulfide linked amine modified magnetic nanoparticles were used to label surface exposed carboxyl groups of bovine serum albumin (BSA) using EDC mediated conjugation chemistry. The superparamagnetic properties of iron oxide allowed the simple and quick separation of nanoparticle-protein conjugates. Easy cleavage of the disulfide bonds left a tag on aspartic and glutamic acid residues, and the labeled sites were characterized by mass spectrometry.

4.3. Materials and Methods

Iron (II) Oxide (FeO(OH)), oleic acid, dimethyl sulfoxide (DMSO, anhydrous), (3-aminopropyl)triethoxysilane (APTES, 95%), tetraethylorthosilicate (TEOS, 99%), Igepal CO-520, cyclohexane, dansylcadaverine (≥97%), hydroxylamine hydrochloride, fluorescein isothiocyanate (FITC, 90%), bovine serum albumin (BSA), and β-lactoglobulin were purchased from Sigma-Aldrich (St. Louis, MO). 1- Octadecene was purchased from Alfa Aesar (Ward Hill,

MA). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC-HCl,+99%), and tris(2-Carboxyethyl)phosphine hydrochloride (TCEP-HCl) were purchased from ProteoChem (Loves Park, IL). Phosphate buffered saline (PBS) and 2-(*N*-morpholino)ethanesulfonic acid (MES) was purchased from Calbiochem (Billerica,MA). Dithiothreitol and iodoacetamide were purchased from Piercenet, Thermo Scientific (Rockford, IL). ACTH (4-11) was purchased from American Peptide Company (Sunnyvale,CA). Ethanol was purchased from Pharmco-AAPER (Brookefield,CT). Nanopure deionized and distilled water (18 MΩ) was used for all experiments.

4.3.1. Synthesis of thiol modified silica coated iron oxide nanoparticles

Fe_3O_4 and Fe_3O_4@SiO_2 MNPs were prepared as reported in literature.[29] Briefly, FeO(OH), oleic acid, and octadecene were mixed in a three neck flask and heated at 320˚C for 60 minutes. The color of solution turned from black to brown indicating the formation of nanocrystals. The Fe_3O_4 MNPs were separated using a magnet, and washed with ethanol several times. The Fe_3O_4 MNPs were air dried at room temperature and stored in toluene. The Fe_3O_4 MNPs (400 µL of 10 mg/mL of toluene), cyclohexane (4 mL), and Igepal-CO-520 (0.247 g) were mixed and sonicated for 15 minutes. Tetraethyorthosilicate (25 µL) was added and sonicated further for 10 minutes followed by the addition of ammonium hydroxide (50 µL, 28 % w/v) with sonication for 15 minutes. This solution was stirred using a magnetic stirrer at room temperature for 24 h. The Fe_3O_4@SiO_2 MNPs were magnetically separated and washed with ethanol several times followed by air drying at room temperature. Surface modification of Fe_3O_4@SiO_2 MNPs with thiol was performed using a method reported in the literature.[30] The Fe_3O_4@SiO_2 MNPs (100 mg) were resuspended in ethanol (80 mL of ethanol and 20 mL of water), and sonicated for 15 minutes. MPTMS (1mL) was added to the stirring suspension of Fe_3O_4@SiO_2 MNPs, and stirred for 12 h

at room temperature. The thiol coated Fe_3O_4@SiO_2 MNPs were magnetically separated, washed, and dried at room temperature.

4.3.2. Synthesis of disulfide linked amine modified Fe_3O_4@SiO_2 MNPs

Thiol coated Fe_3O_4@SiO_2 MNPs (1 mg in 100 μL water) were pretreated with TCEP (0.1 mM, 300 μL of water) for 60 minutes, and magnetically washed several times with water and acetonitrile to remove unreacted TCEP. Thiol coated Fe_3O_4@SiO_2 MNPs (1 mg) were resuspended in PBS (pH=7.4, 1 mL). Pyridine dithioethylamine hydrochloride (6.9 mg in 100 uL PBS) was added to thiol coated Fe_3O_4@SiO_2 MNPs followed by shaking for 30 minutes. The resulting disulfide linked amine coated Fe_3O_4@SiO_2 MNPs (NH_2-SS-Fe_3O_4@SiO_2 MNPs) were magnetically separated and washed with water three times.

Rhodamine Red™-X, succinimidyl ester dye (10 μL of 1 mg/mL of methanol) was conjugated to amine modified Fe_3O_4@SiO_2 MNPs (1 mg) followed by fluorescence microscopic imaging to confirm the presence of amine groups on the surface of Fe_3O_4@SiO_2 MNPs. Images were acquired using an Olympus IX 71 inverted fluorescence microscope with a high performance 16-bit resolution, back illuminated CCD camera (Roper Scientific), and a 100 W Hg lamp.

4.3.3. Quantification of amine groups on the surface of Fe_3O_4@SiO_2 MNPs by 'depletion' approach

The NH_2-SS-Fe_3O_4@SiO_2 MNPs were treated with iodoactamide to fix unreacted thiol groups that may not have been modified with amine groups. Fluorescein isothiocyanate (FITC) (40 μM, 700 μL of ethanol) was separately conjugated to NH_2-SS-Fe_3O_4@SiO_2 MNPs (0.5 mg) for 2 h at room temperature in the dark. The FITC conjugated NH_2-SS-Fe_3O_4@SiO_2 MNPs were magnetically separated and the fluorescence of the supernatant was measured using a Cary

Eclipse fluorescence spectrophotometer (Agilent, Santa Clara, CA). The difference between initial and final amounts of FITC provided an estimate of quantity of FITC conjugated to NH_2-SS-Fe_3O_4@SiO_2 MNPs.

4.3.4. Labeling carboxyl groups of ACTH (4-11) using NH_2-SS- Fe_3O_4@SiO_2 MNPs

The NH_2-SS-Fe_3O_4@SiO_2 MNPs (5 mg) were dispersed in MES/NaOH buffer (pH=7.4, 185 µL). ACTH (4-11, 5 µL of 2.3 mM in water) was added to NH_2-SS-Fe_3O_4@SiO_2 MNPs followed by the addition of EDC (10 µL of 1 M MES), and the reaction was allowed to mix on the vortex stirrer for 2 h. The peptide conjugated MNPs were magnetically separated and washed with water, acetonitrile (ACN, 20%), ACN (50%), and ACN (70%). Peptide conjugated MNPs were further subjected to TCEP (150 µL of 20 mM in water) reduction for 1 h to release the labeled peptide from MNPs. After 1 hr, the supernatant was collected by magnetic separation and dried under a vacuum. The labeled peptides were further purified by using a Pierce™ C18 spin column (Thermo Fisher Scientific, Grand Island, NY) and analyzed by LC-MS/MS.

4.3.5. Labeling of aspartic acid and glutamic acid residues of BSA using NH_2-SS-Fe_3O_4@SiO_2 MNPs

Bovine serum albumin (10 µL of 1mg/1mL in PBS) was reacted with iodoacetamide (10 µL of 200 mM in water) in the dark for 1 h, followed by addition of NH_2-SS- Fe_3O_4@SiO_2 MNPs (15 mg in 360 µL of MES, pH = 7.4). After thoroughly mixing, EDC (20 µL of 1M) was added to the BSA and MNPs mixture. The reaction was allowed to proceed for 2 h at room temperature. The MNPs-BSA conjugates were magnetically separated and washed with water three times, and then acetonitrile (70%) three times. The MNPs-BSA conjugates were further subjected to tryptic digestion. Briefly, the MNPs conjugated to BSA were denatured by reacting with urea (100 µL, 8M) at 45˚C for 1 hr. Trypsin (6 µg) was added to the denatured BSA, and the solution was

diluted by adding aqueous ammonium bicarbonate (1 mL, 50 mM). The reaction was allowed to proceed for 12 h at 37˚C.

After 12 h, the MNPs were separated using a magnet, and thoroughly washed three times with water, three times with aqueous acetonitrile (20%), and three times aqueous acetonitrile (70%). Modified peptides were cleaved from nanoparticles by reacting with TCEP (400 μL of 20 mM) for 1 h at room temperature. The MNPs were magnetically separated then washed, and the supernatant containing peptides were saved for mass spectrometric analysis. The supernatant was obtained by washing the MNPs three times with water, three times with aqueous acetonitrile (20%), and three times with aqueous acetonitrile (70%) to elute the peptides from MNPs. All the washings were combined and dried under a vacuum. The labeled tryptic peptides were further purified by using a Pierce^{TM} C18 spin column (Thermo Fisher Scientific, Grand Island, NY) and analyzed by LC-MS/MS.

4.3.6. LC-MS/MS analysis of carboxyl modified BSA peptides

Labeled tryptic peptides were resuspended in an aqueous mixture (50 μL) containing ACN (3%) and formic acid (5%). Samples were analyzed 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, USA). A chip consisting of a 160 nL enrichment column and a 150 mm analytical column packed with C18, (5 micron beads with 300 Å pores) was utilized for chromatographic separation. The MS source was operated at 300˚C with 5 L/min N_2 flow and a fragmentor voltage of 175 V. N_2 was used as the collision gas, and the collision energy varied as a function of mass and charge using a slope of 3.7V/100 Da and an offset of 2.5 V. Positive ion mode was applied for the operation of quad and TOF. Calibration reference compounds of 322.048121 and 1,221.990637 Da were continually released into the source for mass calibration.

An initial MS scan was performed from m/z 300 to 1600, and up to three multiple charged ions were automatically selected for MS/MS analysis. Following the initial run, a second injection was made excluding ions previously targeted in the MS/MS analysis. LC chromatograms and mass spectra were analyzed using Mass-Hunter software (Version B.0301; Agilent Technologies). Data files were transferred to an Agilent work station equipped with Spectrum Mill software (Agilent Technologies) for peptide sequencing and protein identification.

4.4. Results and discussion

We have synthesized Fe_3O_4 MNPs by thermal decomposition of iron carboxylate salts as reported in literature. The thermal decomposition approach was simple, raid, and produced high quality Fe_3O_4 MNPs. Transmission electron microscope (TEM) image analysis confirmed the spherical shape, uniform nature, and size (8-10 nm in diameter) of Fe_3O_4 MNPs. (Figure 4.1a)

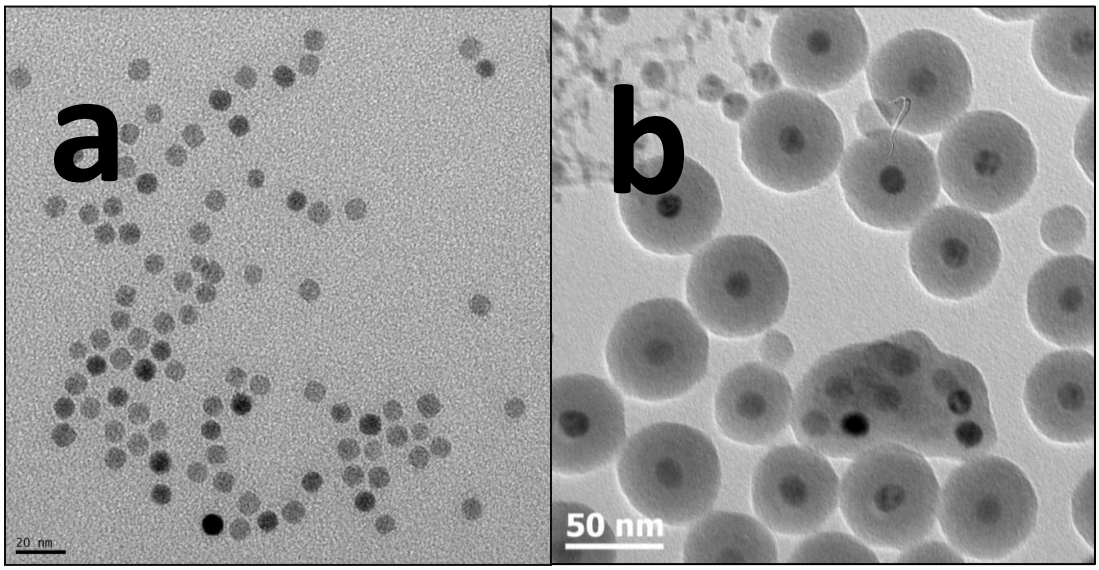


Figure 4.1: (a) TEM images of bare Fe_3O_4 MNPs synthesized by thermal decomposition method and (b) Fe_3O_4@SiO_2 MNPs coated by reverse microemulsion method.

The Fe_3O_4@SiO_2 MNPs were prepared by reverse microemulsion method which utilized surfactant to convert the hydrophobic surface of Fe_3O_4 to hydrophilic surface. The Fe_3O_4@SiO_2

MNPs were nearly uniform, spherical, 40-55 nm in diameter, and well dispersible in aqueous solvents. (Figure 4.1b) Energy dispersive spectroscopy (EDX) analysis confirmed the presence of silica layer on the surface of Fe_3O_4@SiO_2 MNPs (Figure 4.2). The silica coating was mainly introduced to minimize the nonspecific interaction between Fe_3O_4 and proteins, increase the stability of MNPs in aqueous solvents, and provide a platform for further functionalization. Efforts were made to prepare uniform Fe_3O_4@SiO_2 MNPs to provide better quantitative control over the loading capacity of MNPs.

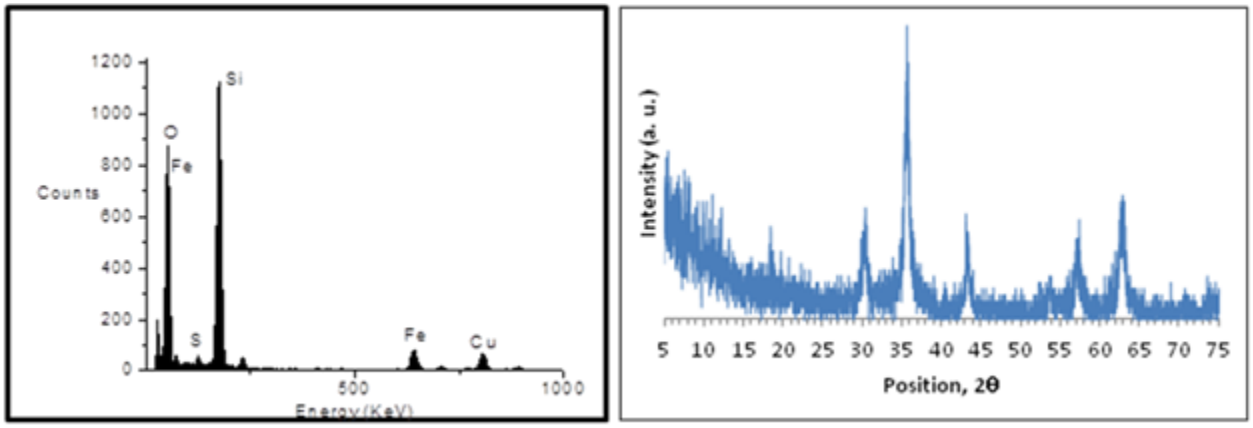


Figure 4.2: EDX analysis of thiol coated Fe_3O_4@SiO_2 MNPs (left) and XRD spectra of Fe_3O_4 MNPs.

4.4.1. Disulfide linked amine modified silica coated iron oxide nanoparticles (NH_2-SS-Fe_3O_4@SiO_2 MNPs)

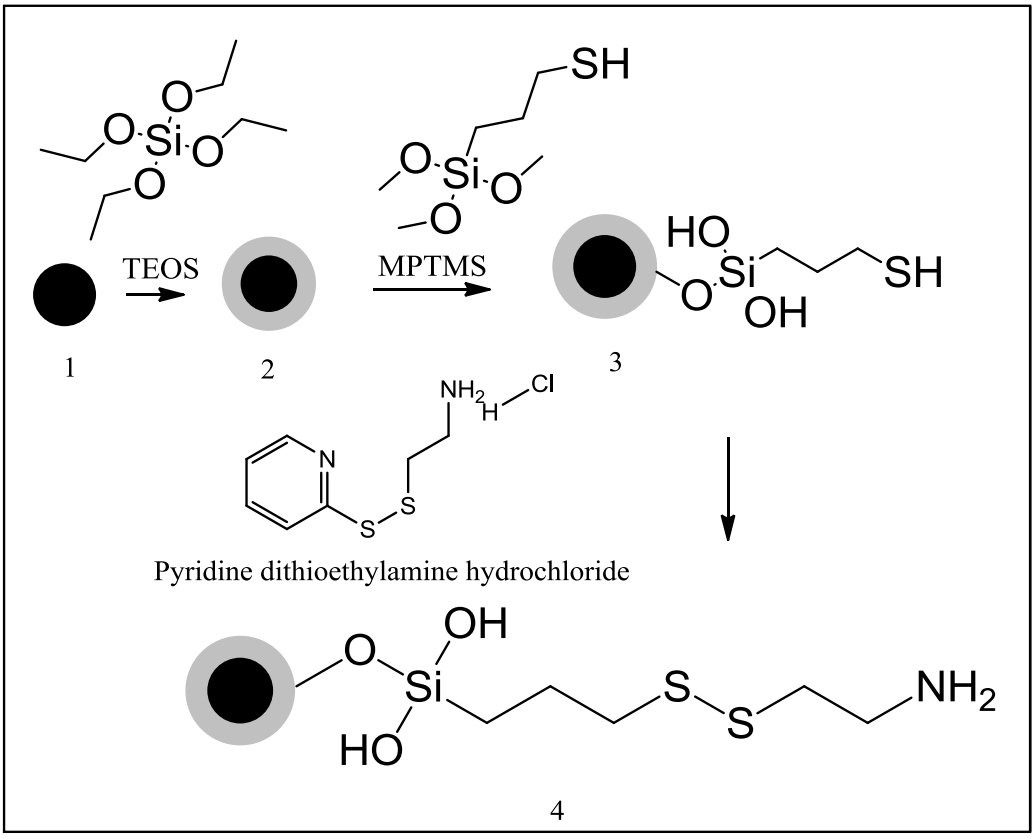
The Fe_3O_4@SiO_2 MNPs were coated with thiol groups by using MPTMS (Scheme 4.1). The presence of thiol groups was confirmed by TEM-EDX which showed a peak for sulfur (Figure 4.2). Thiol coated Fe_3O_4@SiO_2 MNPs were treated with TCEP to cleave unnecessary disulfide bonds formed between free thiol groups on the surface of Fe_3O_4@SiO_2 MNPs. Upon TCEP treatment, thiol coated Fe_3O_4@SiO_2 MNPs were thoroughly washed with water and acetonitrile to ensure complete removal of TCEP, which could be detrimental for further

disulfide bond formation with pyridine dithioethylamine hydrochloride. The NH_2-SS-Fe_3O_4@SiO_2 MNPs were prepared by reacting the pyridyl disulfide group of pyridine dithioethylamine hydrochloride with thiol groups on the surface of Fe_3O_4@SiO_2 MNPs via disulfide bond formation (Scheme 4.1).

The confirmation of reaction completion between thiol coated MNPs and pyridine dithioethylamine was obtained by measuring the absorbance of a byproduct pyridine-2-thione at 343 nm. The amine groups on the surface of Fe_3O_4@SiO_2 MNPs were characterized by measuring the zeta potential by dynamic light scattering in phosphate buffered saline (10 mM, pH 7.4). The zeta potential of thiol coated MNPs was found to be -9 ± 5 mV, which turned positive **(**+23 ±5 mV) upon amine modification due to protonation of amine groups at pH 7.4.

The presence of amine groups on the surface of Fe_3O_4@SiO_2 MNPs was characterized using amine reactive dye, Rhodamine Red™-X, succinimidyl ester (Ex: 560 nm and Em: 580 nm). The red fluorescence of Rhodamine Red conjugated NH_2-SS-Fe_3O_4@SiO_2 MNPs can be seen in fluorescence images (Figure 4.3b), whereas a blank (NH_2-SS-Fe_3O_4@SiO_2 MNPs) did not show any fluorescence (Figure 4.3a).

An amine reactive flurophore, FITC, was utilized for quantification of amine groups on the surface of Fe_3O_4@SiO_2 MNPs. FITC can also react with unmodified thiol groups of NH_2-SS-Fe_3O_4@SiO_2 MNPs. The unreacted thiol groups on the surface of NH_2-SS-Fe_3O_4@SiO_2 MNPs were fixed using iodoacetamide to avoid unnecessary interaction between FITC and thiol groups. The depletion approach involved the measurement of the depleted amount of FITC after conjugation to NH_2-SS-Fe_3O_4@SiO_2 MNPs (Figure 4.3).



Scheme 4.1: Protocol for synthesis of disulfide linked, amine modified Fe_3O_4@SiO_2 MNPs (NH_2-SS-Fe_3O_4@SiO_2 MNPs)

The difference between the initial amount of FITC used for conjugation and final amount of FITC remaining after conjugation provided an estimate of FITC conjugated on the surface of NH_2-SS-Fe_3O_4@SiO_2 MNPs, which was similar to active amine groups of NH_2-SS-Fe_3O_4@SiO_2

MNPs. The NH_2-SS-Fe_3O_4@SiO_2 MNPs (0.5 mg) were found to be loaded with ~13,000 free amine groups.

4.4.2. Labeling carboxylic acid groups of a peptide using NH_2-SS-Fe_3O_4@SiO_2 MNPs

The amine containing nucleophiles can modify free carboxyl groups of peptides using an EDC linker at room temperature and at pH 7-8. The ability of NH_2-SS-Fe_3O_4@SiO_2 MNPs to label carboxyl groups was verified by using a small peptide, ACTH (4-11) (M-E-H-F-R-W-G-K), in MES/NaOH (10 mM, pH = 7-8). Freshly prepared NH_2-SS-Fe_3O_4@SiO_2 MNPs were utilized for labeling reaction (Scheme 2).

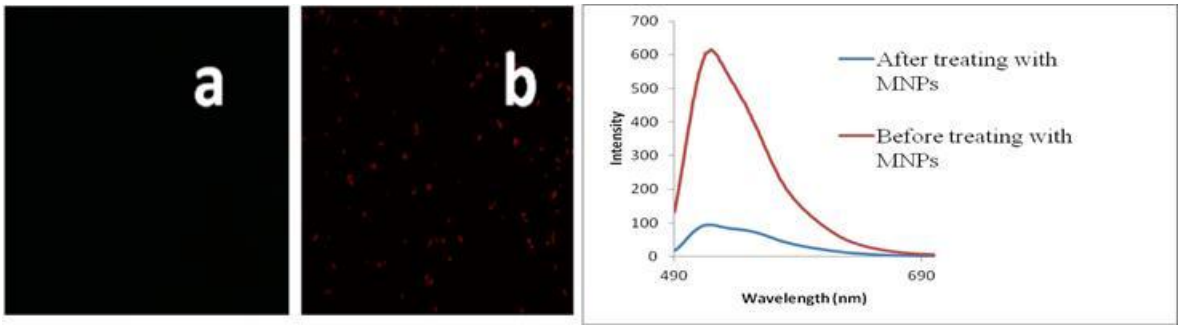
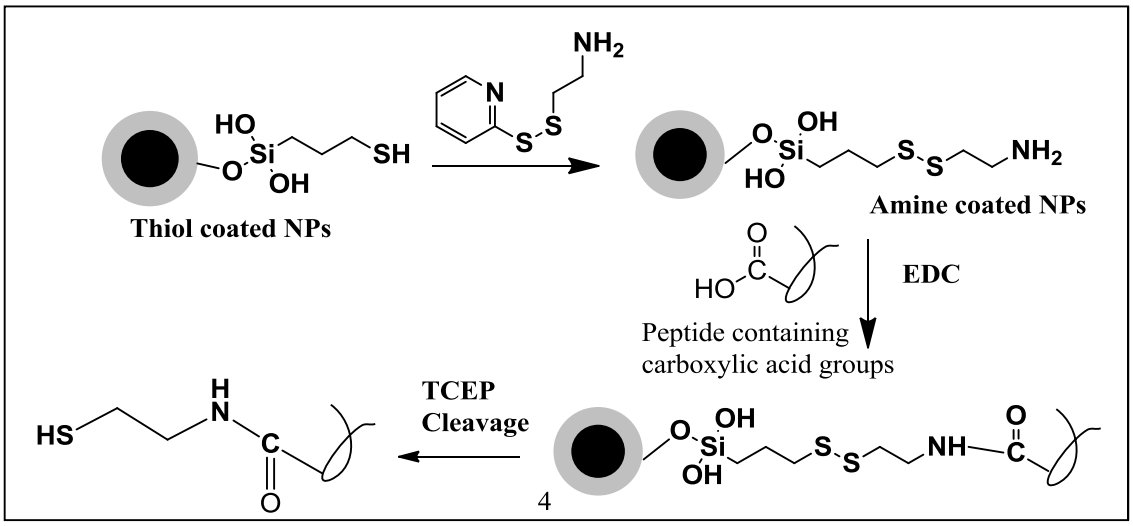


Figure 4.3: Fluorescence microscopic images of (a) amine modified Fe_3O_4@SiO_2 MNPs (b) amine modified Fe_3O_4@SiO_2 MNPs reacted with Rhodamine Red™-X, succinimidyl ester (Ex: 560 nm and Em: 580 nm). Quantitative analysis of amine groups on the surface of NH_2-SS-Fe_3O_4@SiO_2 MNPs (0.5 mg) by depletion approach.



Scheme 4.2: Overview of the labeling of free carboxylic acid groups of a peptide using NH_2-SS-Fe_3O_4@SiO_2 MNPs

The peptide conjugated MNPs were magnetically separated, washed, and subjected to TCEP digestion to cleave the disulfide bond. ACTH (4-11) contain two free carboxyl groups (glutamic acid and lysine residue at C-terminus) that can be modified by NH_2-SS-Fe_3O_4@SiO_2 MNPs. Mass spectrometric analysis revealed the presence of peptides containing one or two labeled carboxyl groups. The NH_2-SS- Fe_3O_4@SiO_2 MNPs introduced a mass shift of 59.14 Da on the free carboxylic group.

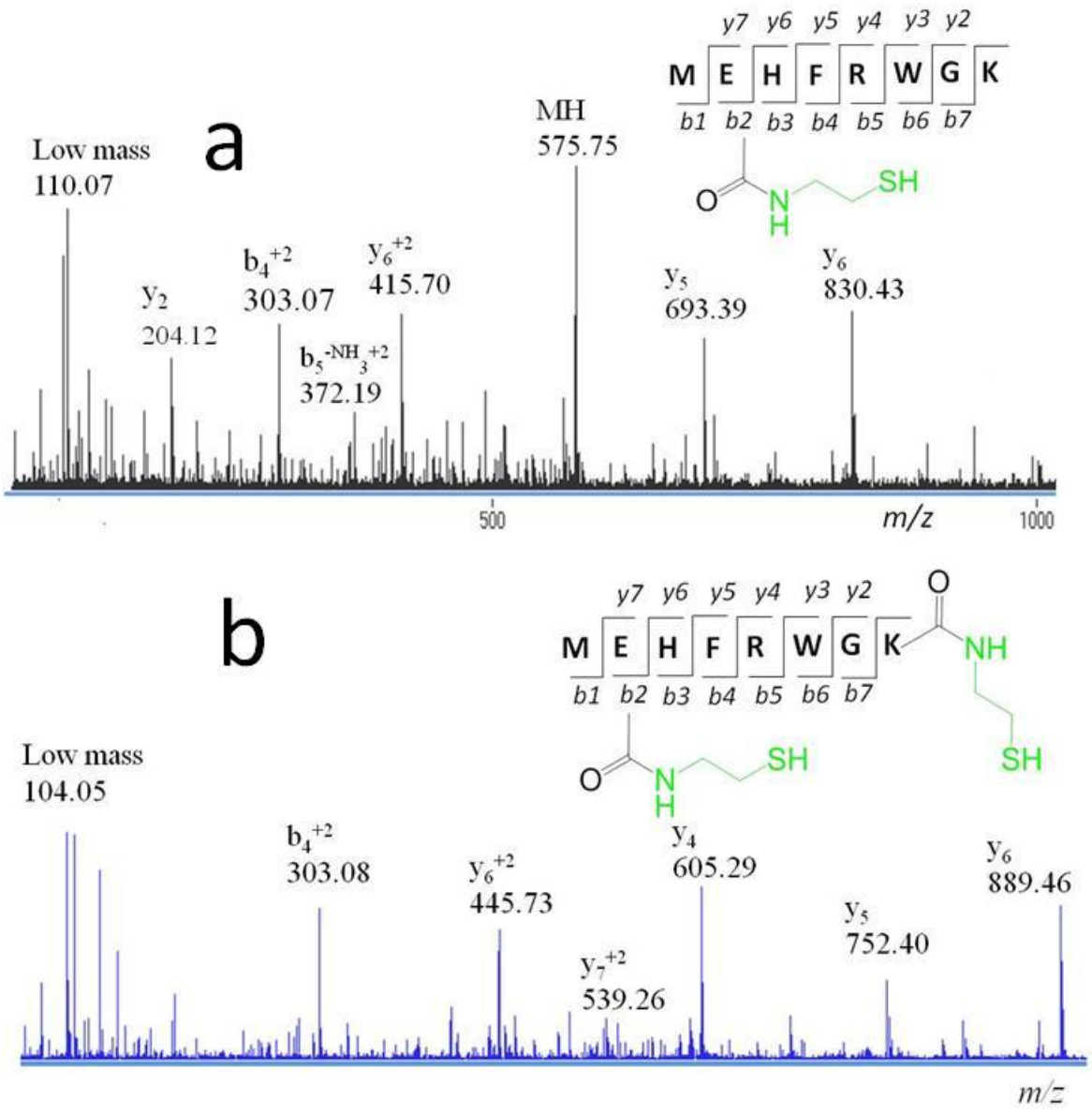


Figure 4.4: ESI-MS analysis of peptide ACTH (4-11) labeled by amine modified Fe_3O_4@SiO_2 MNPs and then treated with TCEP to cleave disulfide bond. (a) MS/MS of doubly charged ACTH (4-11) with one carboxyl group modified (*m/z* = 575.75 Da) with NH_2-SS-Fe_3O_4@SiO_2 MNPs (b) MS/MS of doubly charged peptide ACTH (4-11) ion with both carboxyl groups modified (*m/z* = 604.78 Da) with NH_2-SS-Fe_3O_4@SiO_2 MNPs. Sequence of peptide was confirmed by matching the *b* and *y* ions with detected m/z values.

Thus, ACTH (4-11) with one labeled carboxyl group yielded a singly charged ion of 1148.54 Da and a doubly charged ion of 575.27 Da. ACTH (4-11) with two labeled carboxyl groups yielded a singly charged ion of 1207.56 Da and a doubly charged ion of 604.78 Da. Tandem mass spectrometry of 575.27 Da (Figure. 4a) and 604.78 Da (Figure. 4b) produced a series of b and y ions, which were matched with theoretically calculated b and y ions. All the major peaks in the MS/MS spectra were matched with the theoretical values, confirming the label on the carboxylic acid groups of ACTH (4-11). Thus, the NH_2-SS-Fe_3O_4@SiO_2 MNPs were able to label the carboxyl groups of a peptide at room temperature and pH 7.4, while offering quick magnetic separation following the release of labeled peptides for further characterization.

4.4.3. Characterization of labeled carboxylic acid groups of proteins with disulfide linked amine modified Fe_3O_4@SiO_2 MNPs

Bovine serum albumin was used to test the ability of NH_2-SS-Fe_3O_4@SiO_2 MNPs to label the solvent exposed glutamic acid (E) and aspartic acid (D) residues of a protein. BSA was chosen due to its known crystallographic structure and amino acid sequence. The labeling reaction was performed at room temperature and pH 7-8 to preserve the native structure of BSA. BSA is a serum albumin protein which contains 607 amino acids. BSA contains a total of 59 aspartic acid and 40 glutamic acid. The NH_2-SS-Fe_3O_4@SiO_2 MNPs were able to label five residues of aspartic acid and one residue of glutamic acid on the solvent exposed surface of BSA (Figure 6, Table 1). The large size of NH_2-SS-Fe_3O_4@SiO_2 MNPs may not be able to access all of the carboxyl groups on the surface of BSA.

Position of labeled amino acid residue of mature BSA	Sequence of peptide	Score

E424	(K)VPQVSTPTLVeVSR(S)	17.15
D111	(K)DDSPdLPK(L)	19.38
E140	(K)YLYeIAR(R)	12.07
E229	(K)AEFVeVTK(L)	13.66
E382	(K)HLVDePQNLIK(Q)	8.7
E310	(K)DAIPENLPPLTADFAeDK(D)	14.69

Table 4.1: List of labeled tryptic peptides of BSA by NH_2-SS-Fe_3O_4@SiO_2 MNPs.

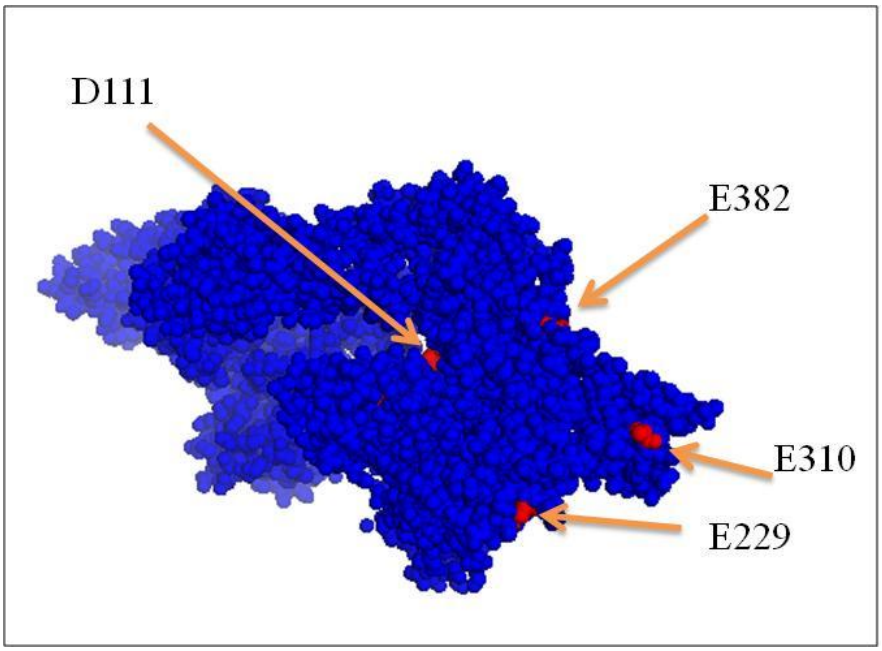


Figure 4.5: Labeled aspartic acid (D) and glutamic acid (D) residues (highlighted in red) of the solvent accessible surface of BSA.

Moreover, the postlabeling protocol excludes the use of dithiothreitol (DTT), which is used to unfold the protein by cleaving the disulfide bonds prior to the trypsin digestion process. The disulfide bond present in NH_2-SS-Fe_3O_4@SiO_2 MNPs can be cleaved by DTT resulting in premature release of labels from MNPs.

4.5. Conclusion

The NH_2-SS-Fe_3O_4@SiO_2 MNPs were synthesized, characterized, and tested to label the free carboxyl groups of peptides and proteins. Free carboxyl groups of peptides and proteins were modified at room temperature and physiological pH. Incorporation of disulfide bonds between the amine group and MNPs allowed specific cleavage of labeled peptides. Magnetic properties of NH_2-SS-Fe_3O_4@SiO_2 MNPs allowed rapid and efficient removal of labeled peptides from the solution. Simplicity of labeling and rapid magnetic recovery of labeled peptides make NH_2-SS-Fe_3O_4@SiO_2 MNPs an effective approach to study solvent exposed surfaces of complex proteins.

4.6. Acknowledgements

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Chapter 5

Labeling live *S. cerevisae* by surface-targeted magnetic nanoparticles reagents for exposed peptide segment mapping

5.1. Abstract

Labeling solvent exposed lysine residues for separation of proteins is an important approach in cell surface proteomics. The ability of labeling reagent to cross the cell membrane often raises questions on the location of labeled proteins. Despite the many advantages offered by affinity based biotinylation reagents, contamination by endogenous biotin and streptavidin, and difficulty in eluting labeled peptides from streptavidin beads could interfere with the labeling and recovery of labeled peptides. We utilized sulfo-*N*-hydroxysuccinimidyl (NHS) ester modified core shell silica coated iron oxide magnetic nanoparticles (Fe_3O_4@SiO_2) MNPs to label the solvent exposed lysine residues of living cell surface of *Saccharomyces cerevisae*. TEM analysis provided the confirmation of cell membrane impermeable nature of sulfo-NHS ester modified Fe_3O_4@SiO_2 MNPs due to the large particle diameter (~ 150 nm). Solvent exposed lysine residues were labeled under physiological conditions to preserve the native structure of cell surface proteins of living cells. Magnetic nanoparticles based separation assisted in post labeling procedures including rapid removal of detergents prior to LC-MS/MS analysis. The labeled peptides were recovered easily without using any harsh conditions by cleaving disulfide bond between MNPs and labeled peptides. LC-MS/MS analysis allows identification of.labeled peptide segments on the solvent accessible surface of *S. cereveisae*. Impermeable nature, quick magnetic separation and simple elution of labeled peptides makes this MNPs based labeling reagent an ideal choice to study the living cell surface.

5.2. Introduction

Yeast cell wall/surface proteins (CSPs) play crucial roles in cell surface mediated communications that involve processes such as protein-protein interaction, signal transduction and ion transportation across the cell membrane.[1] In order to isolate CSPs, cell walls are usually

disintegrated first by using glass beads, [2] mild heat treatment,[3] alkaline[3] or acid buffers, ionic buffers,[4] chelating agents,[5] detergent[6] or reducing agents.[7] However, CSPs prepared by using these traditional cell disruption techniques can be contaminated by proteins from other cell compartments such as cytoplasm and organelles. [8,9,10] Thus, the unambiguous identification of CSPs due to contaminations still remains a major challenge in studying the exposed CSPs. Alternative approaches to study CSPs involve strategies of protein labeling with chemical compounds followed by mass spectrometric characterization of the chemically labeled CSPs. In the past two decades, cell surface protein studies heavily relied upon biotinylation regents which could be captured by avidin/strptavidin beads/column for affinity separation/purification.

Various biotinylation reagents were developed to covalently label/tag lysine, cysteine, aspartic acid and glutamic acid residues of CSPs followed by affinity capturing of biotinylated proteins by avidin/streptavidin beads.[11] Biotinylation reagents are usually comprised of two key components: a biotin moiety for affinity captures of the labeled proteins and a protein/peptide reactive group; an optional cleavable linker between the two moieties may also be included to to facilitate the release of biotinylated proteins after affinity separation/purification. Due to the abundance of lysine residue in CSPs[12], the biotinylation regents containing *N*-hydroxysuccinimide (NHS) (or sulfo N-hydroxysuccinimide, sulfo-NHS) ester moiety have been widely used to label proteins/peptides through the primary amine groups. The NHS/sulfo-NHS ester groups react with primary amines in aqueous solvents, at physiological pH, and at or below room temperature, conditions under which the native protein structures can be preserved during the reaction. [13] NHS ester containing biotinylation regents such as NHS/sulfo-NHS LC-biotin, NHS-sulfo NHS-SS-biotin and NHS-PEG-biotin are commercially available,[11] and the labeling reagents usually come with kits that include streptavidin/avidin immobilized on resin/magnetic

beads for the capture of biotinylated CSPs. Since NHS esters are less soluble in aqueous solvents, the water soluble sulfo-NHS ester containing biotin compounds are mostly employed for labeling of exposed CSPs. Moreover, the negative charged sulfonate group is membrane impermeable, and it helps to limit the labeling reaction within the surface exposed amine groups. The size of biotinylation regents is another crucial factor in labeling cell surface proteins. It has been reported that the small biotinylation reagents such as sulfo-NHS-LC biotin could still occasionally cross the cell membrane and label the cytoplasmic proteins [14] Up to 15% of cytosolic proteins were found in the list of integral plasma proteins identified by biotinylation reagent.[15] The non-specific binding between streptavidin/avidin and proteins is another potential drawback in the application of biotinylation reagents. Masuoka et al. demostrated nonspecific binding of avidin to the cell wall of *Candida albicans* via hydrophobic or electrostatic interactions.[16] In another occasion streptavidin was also found to be nonspecifically bound to human B cell and myleiod cell surface proteins.[17] Biotin is present in various types of living cells[18,19] involved in various biological processes such as cell growth, citric acid cycle and telomerase complex formation [20]. The endogenous biotin is a source of cause contamination[21,22,22] and competes with biotin conjugated to CSPs in binding avidin/streptavidin beads for affinity based separation/purification.

Effective isolation/separation of the labeled CSPs without affecting protein structure is another challenge in the labeling approaches to study CSPs. The elution of biotinylated proteins from avidin beads is difficult due to the highly stable interaction between biotin and avidin. Some research studies have discussed use of harsh conditions such as boiling in high salt conditions or use of EDTA at 94˚C for several minutes to break the noncovalent interaction between streptavidin immobilized on resin beads and biotin, [23] and these harsh conditions were detrimental to the integrity of CSPs. Some streptavidin/monomeric avidin molecules have (CaptAvidin from Molecular Probes, Immobilized Monomeric Avidin, Pierce)lower binding to biotin, so biotinylated proteins can be eluted such material under mild conditions but at high pH. However, the reduced affinity between streptavidin/avidin can affect effectiveness of biotinylated peptides capture, and the high pH may induce structural change in CSPs. A cleavable linker between the biotin moiety and the protein/peptide reactive group could help to release the biotinylated proteins/peptides from immobilized avidin after affinity separation. The most widely applied disulfide bond linker can be found in commercially available reagents such as sulfo-NHS-SS-biotin (Pierce..) An alternative photocleavable linker, 1-2(nitrophenyl)-ethyl moiety (labile upon exposure to light of wavelength 300-360 nm) is employed in sulfo-NHS-PC-LC-Biotin (Pierce). Elia et al introduced cleavable vicinal diol group between biotin and NHS ester. Vicinal diol group can be cleaved under mild conditions by using oxidants such as sodium metaperiodate. It should be noted that oxidative cleavage of linker can cause unwanted oxidation of amino acid side chains in labeled proteins/peptides.

Cleavable linkers/spacers were also included in protein/ labeling reagents with affinity moeities other than biotin. For example, an acid cleavable tertiary carbamate linker was included in the “fluorous” tag developed by Qian et al [24]. Sulfo- (Ethylene glycol

bis[sulfosuccinimidylsuccinate]) (EGS) is an amine reactive cross linker which contains two NHS esters groups separated by a carbonyl group that is cleavable by using reducing agents such as hydroxylamine; hydroxylamine can also cleave aspragine-glycine bond. The strong interaction between cucurbit[7]uril (CB[7]) and ferrocence derivatives was utilized in a labeling reagent to capture plasma membrane proteins. The carboxyl containing ferrocence derivatives can label free amines of membrane proteins using EDC chemistry, and the 'ferrocenylated' proteins can be further captured by using cucurbit[7]uril (CB[7]) conjugated sepharose beads. It cannot be ruled out that ferrocence derivatives may be able to cross the cell membrane.[25]

Once conjugated by amino acid reactive groups, magnetic nanoparticles can be utilized to label proteins/peptides. The superparamagnetic properties, tunable size and simple surface-functionalization strategies have made iron oxide (Fe_3O_4) nanoparticles (MNPs) attractive tools in sample preparation of proteins for further analysis. The Fe_3O_4 MNPs can be coated with a protective silica layer to improve the aqueous stability, improve functionalization and reduce the non-specific interaction between Fe_3O_4 and biological molecules. The silica coated iron oxide ($Fe_3O_4@SiO_2$) MNPs can further be modified by amine, thiol or carboxylic acid groups which serve as intermediates for immobilization of protein/peptide reactive group on MNP surface. We recently reported a, NHS ester modified $Fe_3O_4@SiO_2$ MNPs (through a disulfide bond linker) to label primary amine groups on the solvent exposed surface of proteins.[26] In this work, NHS/sulfo-NHS ester modified $Fe_3O_4@SiO_2$ MNPs will be employed to study the cell surface exposed proteins of *S. cerevisae.* The cell wall/cell surface proteins of *S. cerevisae* have been extensively studied by biotinylation of lysine[27,28,29] and cysteine residues[30] coupled with mass spectrometric characterization. True cell surface localization of proteins identified by biotinylation/mass spectrometry analysis could not be assigned confidently due to the fact that

biotinylation reagents might penetrate cell surface. Unique properties of magnetic nanoparticle labeling reagents compared to other labeling reagents in the same category provide opportunities to profile cell surface proteins with the labeled sites as the exposed portions of such proteins.

5.3. Experimental Materials

5.3.1. Materials:

Ferric (III) chlroride hexahydrate, ($FeCl_3$, 99%), dimethyl sulfoxide (DMSO, anhydrous), (3-mercaptopropyl)trimethoxysilane (MPTMS, 99%), glucose and tetraethylorthosilicate (TEOS, 99%) were purchased from Sigma-aldrich (St. Louis, MO). Trisodium citrate, anhydrous, 99%, and sodium acetate, anhydrous, 99% were purchased from Alfa Aesar (Ward Hill, MA). Sodium citrate, dihydrate was purchased from J. T. Baker Chemicals (Center Valley, PA). Ammonium hydroxide, was purchased from EMD Millipore (Billerica, MA). Ethylene glycol and ammonium sulfate was purchased from VWR internationals (Radnor, PA). Sulfo- succinimidyl 6-(3'- [2-pyridyldithio] -propionamido)hexanoate (sulfo-LC-SPDP, +99%) and Sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate were purchased from Piercenet (Rockford,IL,USA). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl) was purchased from Amresco (Solon, OH). Phosphate buffered saline (PBS) was purchased from Calbiochem (Billerica,MA). Ethanol was purchased from Pharmco-AAPER (Brookefield,CT). Nanopure deionized and distilled water water (18.2 MΩ) was used for all experiments.

5.3.2. Cell culture

S. cerevisae cells (Y9763) were grown in GYEP media (Difco) (~25 ml) at 37˚C and incubated for 2 days, changing media every 24 h to the log phase (10^8 cells). Cells were collected by centrifugation at 3˚C and washed thrice with PBS (pH= 7.5). Cells were further respuspended in PBS (pH 7.5, 300 ul) before use in labeling reaction for imaging purposes

The same *S. cerevisae* cells (Y9763) were also grown in YNB media which was composed of yeast nitrogen base (without amino acids and without ammonium sulphate, 0.17%), glucose (2%) and ammonium sulfate (0.5%) in nanopure water (100 ml). The cells were grown in YNB media (~25 ml) at 37˚C for five days, transferring to fresh media every after 26 h. The cells were harvested in log phase (total number of cells = 1.2×10^{11}), centrifuged at 3˚C and washed thrice using PBS. Cells were further resupended in PBS (400 µl) and further reacted with sulfo-NHS ester modified $Fe_3O_4@SiO_2$ MNPs for surface protein profile.

5.3.3. Labeling *S. cerevisae* using sulfo-NHS modified $Fe_3O_4@SiO_2$ MNPs

The sulfo-NHS ester modified $Fe_3O_4@SiO_2$ MNPs were re-dissolved in ethanol (240 µl) and divided into 12 similar parts (20 ul each) using three 48 well plates. Only four wells of each plate were utilized. All three well plates containing sulfo NHS ester modified $Fe_3O_4@SiO_2$ MNPs were transferred to a desiccator connected to vacuum to remove the ethanol from MNPs suspension.

S. cerevisiae cells grown in yeast nitrogen base (YNB) media were utilized for labeling purpose. The cells were collected and washed with PBS containing IA (0.5 mM) for 3 times and counted using haemocytometer. (Approximately 1.2×10^{11} in 400 µL PBS). The sulfo-NHS ester modified Fe_3O_4 @ SiO_2 MNPs were mixed with cells by following a special mixing method reported earlier. Briefly, cells were mixed with sulfo-NHS ester modified $Fe_3O_4@SiO_2$ MNPs in a three step method: Cells were divided into four parts and added to the four wells of first well plate containing sulfo-NHS ester modified Fe_3O_4 @ SiO_2 MNPs. The mixture was allowed to mix on a shaker in cold room at 3˚C for 30 minutes. The suspension containing cells and sulfo NHS ester modified Fe_3O_4 @ SiO_2 MNPs was transferred to the four wells of second well plate and allowed to react for 30 minutes. Similar step was repeated for third well plate and after 30

minutes, all the four parts were combined in a centrifuge tube (1.5 ml). Excess sulfo NHS ester groups were quenched by adding tris-HCl (300 µl, 1M) for 20 minutes at room temperature, centrifuged and the supernatant containing PBS and tris-HCl was removed.

5.3.4. TEM of *S cerevisae* treated with sulfo NHS ester modified Fe_3O_4 @ SiO_2 MNPs

Thiol coated Fe_3O_4 @ SiO_2 MNPs-100 nm in diameter (5 mg) were redispersed in ethanol (500 ul) and treated with sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) in anhydrous DMSO (50 ul) (10mg/ml final concentration of sulfo-SMCC) to functionalize with sulfo-NHS ester group. The reaction was allowed to proceed at room temperature for 90 minutes. Sulfo-NHS ester modified Fe_3O_4@SiO_2 MNPs were washed with ethanol several times and further vaccum dried to remove ethanol. *S. cerevisae* (Y9763) were grown in glucose, yeast, and peptone (GYEP) media for 24 h. The cells were collected, washed with PBS and counted using haemocytometer. *S cerevisae* (10^8 cells) were treated with sulfo NHS ester modified Fe_3O_4@SiO_2 MNPs for 90 minutes in PBS (pH= 7.4) at 3˚C. The similar protocol was repeated with Fe_3O_4@SiO_2 MNPs-20 nm in diameter.

The cells conjugated to sulfo NHS ester modified Fe_3O_4 @ SiO_2 MNPs were manually fixed for TEM analysis. The cells-MNPs mixture was incubated with glutaraldehyde (3%) solution over night and centrifuged to remove supernatent. The s-collidine buffer was further added to the mixture, mixed with a pipette and incubated for 5 minutes followed by repetition of same procedure one more time. The mixture was centrifuged for 3 minutes, supernatent was removed and incubated with s-collidine (5 ml), osmium tetroxide (2 ml) and pinch of sucrose. After 5 minutes, supernatent was removed by centrifugation for 3 minutes and s-collidine buffer was added and incubated for 5 minutes. The same step was repeated one more time. The mixture was further centrifuged for 3 minutes; supernatant was poured off and incubated with ethyl

alcohol (50%). The same steps were repeated with ethanol (70 % and 95 %) with 5 minutes incubation and ethyl alcohol (100%) with 15 minutes incubation for two times. After 15 minutes, the supernatant was poured off using centrifugation and the mixture was incubated for 15 minutes with absolute ethyl alcohol: firm spur in 1:1 (200 µL:200 µL), 3:1 (300 µL: 100 µL), 1:1 (200 µL:200 µL), 1:3(100 µL:300 µL) and 100 % firm spur for three times. The mixture was centrifuged for 3 minutes while changing the ratio of ethyl alcohol and firm spur. The mixture was centrifuged for 5 minutes, supernatant was removed and the sediment was added to the embedding capsule. Hard spur (100%) was added to the capsule and slowly mixed. The capsule was placed in an oven at 80-85˚C overnight. TEM samples for cells conjugated to Fe_3O_4 @ SiO_2 MNPs-20 nm in diameter were prepared by using thermal decomposition of carboxylate salts. Briefly, FeO(OH) (2 mmol), oleic acid (8 mmol) and 1-octadecene (5 gms) were heated at 320˚C for 60 minutes. The magnetic nanoparticles were separated by magnet and washed several times using magnetic separation. To prepare Fe_3O_4@ SiO_2 MNPs-20 nm, the magnetic nanoparticles were further coated with silica using reverse microemulsion method as described earlier. The Fe_3O_4 nanoparticles (400 µL, 10 mg/mL) were mixed with cyclohexane (4 mL) and Igepal CO-520 (0.247 gm) followed by sonication for 15 minutes. Tetraethylorthoslicate (25 µL) was added to the solution and sonicated for 10 minutes. In the last step, ammonium hydroxide (50 µL, 30%) was added, sonicated for 15 minutes and mixture was stirred overnight at room temperature. The Fe_3O_4@ SiO_2 MNPs-20 nm were magnetically recovered and washed with ethanol several times using magnetic separation.

5.3.5. LC-MS/MS analysis of cell surface peptides labeled by sulfo-NHS ester modified Fe_3O_4@SiO_2 MNPs

The cells conjugated to Fe_3O_4 @ SiO_2 MNPs were disintegrated by using a mixture of

methanol (400 µl), SDS in methanol (2%), PRI cocktail (5 µl), with IA (25 µl, 200 mM. The mixture was vortexed at high speed and incubated at 60°C for 10 minutes. The CSPs conjugated to Fe_3O_4@ SiO_2 MNPs were separated by using magnetic separation. The CSPs conjugated to Fe_3O_4@SiO_2 MNPs were denatured by using 60% methanol (400 µL) with PRI cocktail (5 µL) and high concentration of IA (25 µL, 200 mM) and allowed it to react for 15 minutes. The CSPs conjugated Fe_3O_4 @ SiO_2 MNPs were separated by a magnet and washed six times using methanol to remove PRI cocktail. Further, the CSPs on the surface of Fe_3O_4@SiO_2 MNPs were subjected to tryptic digestion by adding Trypsin (20 µg) in methanol:ammonium bicarbonate (60:40) (1mL) along with IA (6 µL, 200 mM) and allowed it to react overnight at 37˚C. Tryptic peptides conjugated to Fe_3O_4@SiO_2 MNPs were separated using magnetic separation and washed with (2X), and 70% ACN (5X) to remove any nonspecifically bound peptides to the surface of Fe_3O_4@SiO_2 MNPs. The modified cell surface peptides were isolated from Fe_3O_4@SiO_2 MNPs by cleaving a disulfide using TCEP (20mM, 400 µL) for 60 minutes. The Fe_3O_4@SiO_2 MNPs were separated by a magnet and supernatant was kept for further treatment. The Fe_3O_4@SiO_2 MNPs were washed using water (3X), Acetonitrile (20%, 3X), Acetonitrile (50%, 3X) and Acetonitrile (70%, 3X) to ensure complete removal of modified peptides. Excessive TCEP was removed by using C18 spin columns.

Labeled peptides were characterized by liquid chromatography electrospray-mass spectrometry (LC-ESI MS) on a Thermo LTQ Orbitrap mass spectrometer. (Thermo Electron, San Jose,CA). Dried peptides sample were dissolved in 30 µl of water and loaded on reversed phase trap column (flow rate 2ul/min) followed by washing with water for 5 minutes. The peptide mixtures were further separated on 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 0.2 μL/min. The mass spectrometer was operated in data dependent acquisition mode.

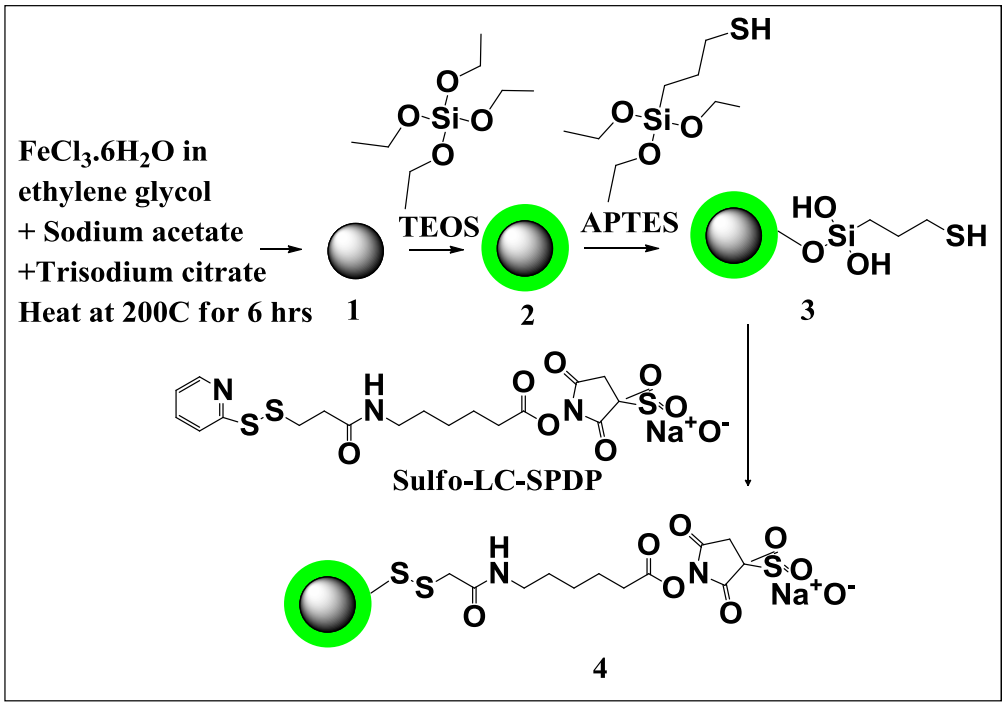
5.3.6. Database search for protein identification

The MS/MS spectra were processed by PEAKS (Bioinformatics Solutions Inc, Ontario1, Canada) Default search parameters were employed in addition to these settings: differential mass increase of 201.28 Da for lysine residues and unmodified N-termini, 15.99 Da for possible oxidized methionine residues, and 7.02 Da for carboxyamidomethylation of cysteine residues. The number of missed cleavage sites was set to three. The MS/MS spectra of detected peptides were manually examined for presence of series of b and y ions to ensure the quality of identification. Database search was performed by using saccharomyces genome database.[31,32]

5.4. Results and Discussion

5.4.1. Sulfo-NHS ester modified Fe_3O_4@SiO_2 MNPs

Synthesis of sulfo-NHS ester modified Fe_3O_4@SiO_2 MNPs consisted of four steps; i) Synthesis of Fe_3O_4 MNPs ii) coating the surface of Fe_3O_4 MNPs with silica to prepare Fe_3O_4@SiO_2 MNPs , iii) Surface modification of Fe_3O_4@SiO_2 MNPs with thiol groups and iv) conjugation of sulfo-NHS ester group to the thiol coated Fe_3O_4@SiO_2 MNPs (scheme 5.1).



Scheme 5.1: Synthesis of sulfo-NHS ester modified Fe_3O_4@SiO_2 MNPs.

The Fe_3O_4 MNPs prepared were prepared by hydrothermal synthesis by heating iron precursors at 200˚C. The Fe_3O_4 MNPs were found to be stable in aqueous solvents and 80 nm in diameter. The Fe_3O_4 MNPs were further capped with citrate layer followed by silica coating to form Fe_3O_4@SiO_2 MNPs. TEM characterization of Fe_3O_4@SiO_2 MNPs showed that the MNPs were spherical, monodispersed, uniform, stable in aqueous solvents and ~150 nm in diameter. The Fe_3O_4 @ SiO_2 MNPs were further modified by thiol groups using thiol containing silane coupling reagent. TEM-EDX showed a peak of sulfur which confirmed the presence of thiols on the surface of Fe_3O_4@SiO_2 MNPs. The next step involved conjugation of sulfo-LC SPDP to the thiol groups on the surface of Fe_3O_4 @ SiO_2 MNPs via disulfide exchange reaction.

The successful conjugation of sulfo-NHS ester group via disulfide bond formation was determined by conjugating an amine containing fluorpohore i.e. dansylcadaverine to sulfo-NHS ester modified Fe_3O_4@SiO_2 MNPs. The green fluorescence of dansylcadaverine modified MNPs

was detected by using fluorescence microscopy which confirmed the presence of sulfo-NHS ester group. The superparamgnetic nature of Fe_3O_4, $Fe_3O_4@SiO_2$ MNPs and sulfo-NHS ester modified $Fe_3O_4@SiO_2$ MNPs was confirmed by performing magnetization studies using vibrating sample magnetometer. The absence of hysteresis loop confirmed the superparamagnetic nature of $Fe_3O_4@SiO_2$ MNPs and sulfo-NHS ester modified $Fe_3O_4@SiO_2$ MNPs.

5.4.2. Sulfo-NHS ester modified $Fe_3O_4@SiO_2$ MNPs do not penetrate cell surface of *S cerevisae*

The strategy used in this study involves development of impermeable and selective labeling reagent to target amine group containing CSPs of the living yeast cells. It was required to show the impermeable nature of labeling reagent to confirm the cell surface binding to the cell surface and inhibition of internalization of sulfo NHS modified $Fe_3O_4@SiO_2$ MNPs. Electron microscopic investigations were carried out on MNPs conjugated to *S. cerevisae* cells to prove the impermeable nature of MNPs. In order to use healthy growing cells for labeling experiment, *S. cerevisae* cells were chosen in the log phase. The growth curve for *S. cerevisae* in GYEP and YNB media was created. *S cerevisae* cells were found to be growing faster in GYEP media (10^8 cells in ~24 h) than YNB media (10^8 cells in ~30 h). Cells grown in GYEP media were chosen for imaging purpose. Sulfo NHS ester modified $Fe_3O_4@SiO_2$ MNPs were prepared by using non-cleavable linker, sulfo SMCC. Sulfo NHS modified $Fe_3O_4@ SiO_2$ MNPs (5 mg) were used to label the cell surface of *S. cerevisae* (10^8 cells) at 3˚C and at physiological pH to preserve the native structure of CSPs. The sulfo-NHS ester modified Fe_3O_4 $@SiO_2$ MNPs (~150 nm) conjugated to the cell surface were manually embedded in resin matrix and imaged by using transmission electron microscopy (TEM). (Fig) Meticulous analysis of TEM grid showed the cell

surface cell surface conjugation of sulfo-NHS ester modified Fe_3O_4@SiO_2 MNPs without any internalization of MNPs by cells (Fig 5.1). TEM grid only showed a portion of sample so, the possibility of cells labeled with more NHS ester modified Fe_3O_4@SiO_2 MNPs cannot be denied. Bright white spots were found on the TEM grid which appeared due to time dependent damage caused by the intensity of electron beam.

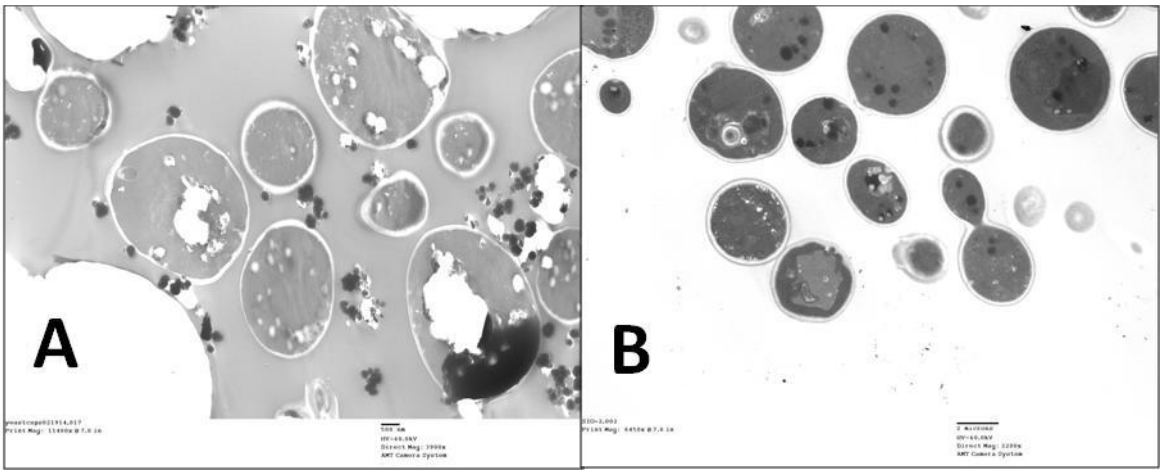


Figure 5.1: TEM analysis of (A) sulfo-NHS ester modified Fe_3O_4@SiO_2 MNPs-150 nm and (B) sulfo-NHS ester modified Fe_3O_4@SiO_2 MNPs-20 nm conjugated to the living cell surface of *S. cerevisae*. Cells were grown in GYEP media and sulfo NHS ester modified Fe_3O_4@SiO_2 MNPs were prepared by conjugating a non-cleavable linker, sulfo-SMCC to the surface of thiol coated Fe_3O_4@SiO_2 MNPs. The larger MNPs (~150 nm) were found to be localized on the surface of *S. cerevisae* whereas the small MNPs (20 nm) were easily ingested by the cells.

In another experiment, sulfo-NHS ester group was conjugated to the surface of small Fe_3O_4@SiO_2 MNPs (20 nm) in a similar manner, and tested for cell impermeability using TEM. The negatively charged small Fe_3O_4@SiO_2 MNPs has presence inside the cell of *S. cerevisae*. Thus, it was found that the negatively charged larger sulfo NHS ester Fe_3O_4@SiO_2 modified

MNPs were efficiently localized on the cell surface whereas small Fe_3O_4@SiO_2 MNPs were found inside the cell either by size dependent intracellular uptake or endocytosis.

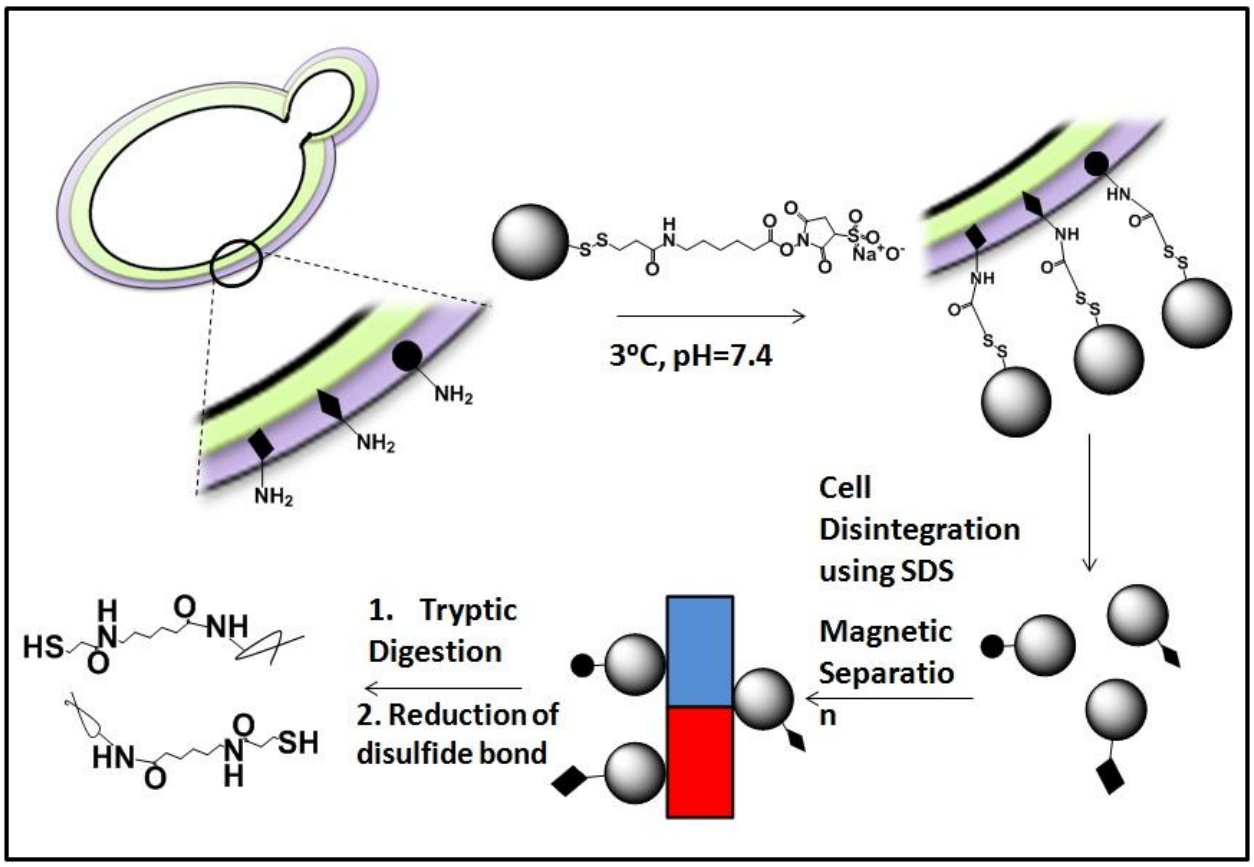
The most promising approach to study CSPs relies upon labeling of CSPs by chemical regents followed by identification of labeled peptides using mass spectrometry. An ideal labeling reagent should be able to label the CSPs at physiological pH and room temperature or below room temperature to preserve the native structure of CSPs. The labeling reagent should not be able to cross the cell membrane in order to avoid any labeling of intracellular/cytoplasmic proteins. Additionally, the labeled proteins should be easily separated for further characterization to identify the labeled sites. Cell impermeable biotinylation reagents such as sulfo-NHS LC biotin, and sulfo NHS SS biotin have been used to label the lysine residues of the cell surface and these lysine residues were claimed to be located on the cell surface. These claims were based on the fact that negative charge of sulfo NHS ester group restricts the internalization of biotinylation compounds in the cell. However, the observation by Yu et al shed light on the permeation of these sulfo-biotinylation regents in rat kidney perfused inner medullary collecting ducts at even low temperature, (2˚C) which is supposed to inhibit endocytosis.[14] The mechanism of internalization of negatively charged chemical reagents was not studied and still remains a topic of further investigation. We suspected that the internalization of sulfo- biotinylation reagents might be either due to the small size of reagents which can permeate thorough the cell membrane or endocytotsis. Considering this fact, we increased the size of the labeling reagent by conjugating the amine reactive linker to the surface of Fe_3O_4@SiO_2 MNPs via a disulfide bond. For labeling purpose, *S. cerevisae* cells grown in YNB media were instead of GYEP media since the presence of yeast proteins in GYEP media may interfere with labeling process. The diameter of Fe_3O_4@SiO_2 MNPs was ~150 nm which inhibited the intracellular uptake and assisted the cell

surface conjugation to the cell surface of *S. cervisae* cells (2-3 μm in diameter, Figure 5.2) To the best of our knowledge, the visual proof for impermeable nature of cell surface labeling reagent have not been shown in literature and we are the first to provide images of cell surface conjugation by MNPs containing labeling reagent or any cell surface labeling reagent. The polymerization step prior to imaging could cause agglomeration of sulfo NHS ester modified $Fe_3O_4@SiO_2$ MNPs resulting into increased weight of the NPs aggregates. During manual slicing of cells conjugated to NPs embedded in the polymer, the heavy NPs were precipitated and increased the thickness resulting into difficulty in thin slicing of the wax samples. Well dispersed individual or less aggregated MNPs embedded in wax were efficiently sliced and appeared on TEM grid (Fig. 5.1). We also found out that the small $Fe_3O_4@SiO_2$ MNPs (~20 nm) despite being modified with negatively charged sulfo NHS ester group were quickly internalized by the yeast cells. The negatively charged cell membrane should not favor the uptake of negatively charged NPs. Even though charge on the surface of MNPs play an important role in internalization, size of NPs still remains a major factor for cell membrane-NPs interactions. Due to the internalization issue, small sized $Fe_3O_4@SiO_2$ MNPs were not considered for cell surface protein identification studies.

5.4.3. Cell surface labeling of solvent exposed amine groups using sulfo NHS ester modified $Fe_3O_4@SiO_2$ MNPs

Cell surface labeling with sulfo NHS ester modified $Fe_3O_4@SiO_2$ MNPs involved following main steps, a) Modification of amine groups on living *S. cerevisae* cells using sulfo-NHS ester modified $Fe_3O_4@SiO_2$ MNPs, b) SDS- lysis of cells conjugated to MNPs, c) magnetic separation of CSPs conjugated to $Fe_3O_4@SiO_2$ MNPs d) tryptic digestion of CSPs conjugated to $Fe_3O_4@SiO_2$ MNPs e) reduction of disulfide bond followed by magnetic separation to elute

labeled peptides (scheme 5.2). The quantity of sulfo-NHS ester modified Fe_3O_4@ iO_2 MNPs required to label the cells were determined by performing the accurate quantitative analysis of NHS ester groups on the surface of MNPs and found to be 1.4×10^{15} per mg of NHS ester modified Fe_3O_4@SiO_2 MNPs. *S cerevisae* were grown in YNB media and collected in log phase to ensure healthy growing cells. *S cerevisae* (1.2×10^{11}) were mixed with sulfo NHS ester modified Fe_3O_4@SiO_2 MNPs at 3˚C in PBS (pH= 7.5) to preserve the native structure of cells. The reaction was carried out by following special 'combination' approach which improved the efficiency of conjugation of NHS ester groups with proteins. Iodoacetamide was added throughout the labeling reaction in order to protect the disulfide bonds between the sulfo-NHS ester group and MNPs. Upon labeling the surface exposed amine groups, the remaining sulfo-NHS ester groups on the surface of Fe_3O_4@SiO_2 MNPs were fixed by adding tris HCl. This precaution was taken to avoid unnecessary labeling of proteins during cell lysis and further protein digestion steps. MNPs conjugated to CSPs along with intact cells were further lysed by using SDS and methanol. The mixture of lysed cells and MNPs conjugated to CSPs were further separated by quick magnetic separation. Magnetic separation allowed quick removal of cell debris and unnecessary proteins adsorbed on the surface of Fe_3O_4@SiO_2 MNPs. The MNPs–CSPs adduct can be washed several times in a few minutes due to the high magnetization of Fe_3O_4@SiO_2 MNPs. After tryptic digestion of CSPs attached to MNPs, magnetic separation was performed to separate labeled tryptic peptides conjugated to MNPs.



Scheme 5.2: Protocol for labeling solvent exposed amine groups of S.cerevisae using sulfo NHS ester modified Fe_3O_4@SiO_2 MNPs.

In the last step, labeled peptides were released from MNPs by cleaving the disulfide bond between MNPs and peptides, magnetically separated and supernatants were analyzed by using LC-MS/MS. A mass shift of 201.08 Da was incorporated on amine groups after labeling by the sulfo NHS ester modified Fe_3O_4@SiO_2 MNPs. The LC-MS/MS analysis of labeled peptides was assigned to 30 unique proteins containing 56 peptides. The proteins were identified by comparing with saccharomyces genome database. Of the 28 proteins identified in database search, 4 proteins are classified as cell wall proteins and 9 as plasma membrane proteins, 17 proteins belongs to mitochondrion proteins, 12 membrane proteins, 6 mitochondrial envelope, 6 nucleus, 5 ribosomal and 4 vacuole proteins. (Figure 5.2)

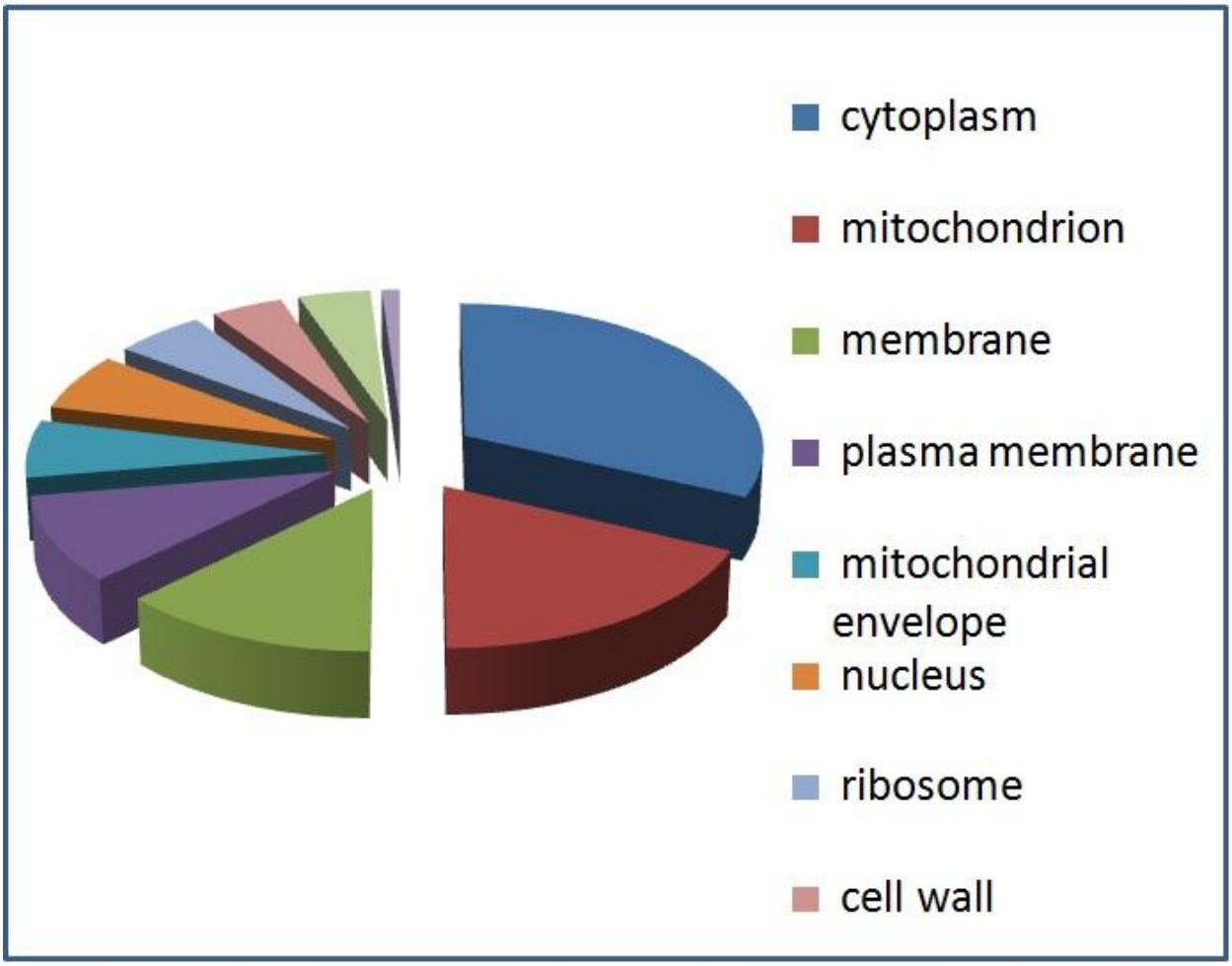


Figure 5.2: Pie chart of the surface exposed labeled proteins of *S.cerevisae* using sulfo-NHS ester modified Fe_3O_4@SiO_2 MNPs.

The peptides contained the labeled lysine residues (K) at the N-terminus and/or in the middle of the sequence. (Table 5.1) In addition to lysine, sulfo-NHS ester modified Fe_3O_4@SiO_2 MNPs labeled the hydroxyl containing amino acids such as serine and tyrosine.

Various reports in literature utilized the labeling based approaches for identification of cell surface proteins.[33,34] Despite the use of biotinylation or other labeling reagents to label primary amine groups of cell surface or membrane peptides, major emphasis was placed on identification of isolated proteins (presumably CSPs) rather that the labeled sites. The sequence of labeled peptide with labeled site(s) was rarely reported. Identification of cell surface peptides containing labeled amino acid residues could be a point of attachment with other cells. To the best of our knowledge, the first study which identified the labeled lysine residues was reported by biotinylation reagent (sulfo-NHS-LC-biotin) was employed for labeling and identification of labeled lysine residues of surface plasma membrane proteins of melanoma cells. Biotinylation in combination with isotope labeling to identified and quantified 100 membrane and membrane associated proteins [35]

Compared to biotin-streptavidin strategy, no additional step is required for separation of labeled peptides which eliminated the problem difficult elution of labeled peptides from solid supports. Of all the amine reactive groups, the sulfo-NHS/NHS group was chosen due to its high selectivity and ability to covalently react with amine groups at physiological pH and room temperature. Sulfo-NHS ester group provided negative charge to the MNPs which served as an additional factor to inhibit intracellular uptake of MNPs. The NHS/sulfo-NHS react have shown some reactivity towards hydroxyl groups of tyrosine, serine and threonine residues but the efficiency of reaction at physiological pH is significantly lower than the reaction with amine groups. [36]. The modification of serine, tyrosine and threonine results into identical mass shift which can be identified in PEAKS PTM function.

After labeling the amine groups of CSPs, the next step involved extraction of labeled proteins from the cells which can be performed by using a detergent or reducing agents. *S. cerevisae* cells are protected by rigid cell wall which are difficult to disintegrate. The common approach to break the cell wall involves use of detergents such as SDS which can solubilize lipids embedded in cell membrane. Use of detergents also reduces nonspecific interaction between proteins and loss of proteins due to adsorption to surfaces. The SDS-polyacrylamide gel electrophoresis (PAGE) technique have been used for the isolation of cell wall/surface proteins from *S. cerevisae* because the detergent can be removed during the process. Presence of detergents could be detrimental to various downstream processes such as purification by chromatography and electrophoresis , and it can suppress the ionization of analyte[37] by introducing interference from background and adduct formation and affect the chromatographic resolution. Conventional methods to remove detergents such as dialysis, ion exchange chromatography or precipitation can be time consuming, tedious and require large amount of proteins. Magnetically separable sulfo-NHS ester $Fe_3O_4@SiO_2$ MNPs can offer quick removal of SDS from proteins conjugated to MNPs. It usually takes less than a minute to achieve magnetic separation and this allows to wash the sample repetitively in short period of time. The SDS based disruption of cells conjugated to MNPs released various proteins from other cell compartments but such proteins can be removed with SDS by using a magnet. Thus, the separation of these unlabeled cellular proteins was way quicker than conventional protein separation techniques.

5.4.4. Sulfo-NHS ester $Fe_3O_4@SiO_2$ MNPs label solvent exposed lysine containing peptides of CSPs

According to LC-MS/MS analysis, the solvent exposed amine group were found at the N-terminus of protein or in the middle of the sequence of a peptide. (Table 1) Because sulfo-NHS

ester modified Fe_3O_4@SiO_2 MNPs cannot cross the cell membrane, all the exposed lysine residues were modified irrespective of their position in proteins i.e. N-terminus or middle of the sequence. Since trypsin cleave only unmodified lysine and arginine residues, the labeled lysine cannot be identified as a cleavage site by trypsin. Few tryptic peptides containing cysteine residues were found in the database search. These cysteine residues might have been created

Number	Accession Number	Protein	Peptides	-10logP
1	YGR192C	Triose-phosphate Hydrogenase	T(+201.28)VDGPSHK(+201.28)DWR	20.63
2	YHR174W	ENO2	LAK(+201.28)LNQLLR	19.01
			WLTGVELADMYHSLMK(+201.28)R	77.82
			RGNPTVEVELTTEK(+201.28)GVFR	65.95
			S(+201.28)IVPSGASTGVHEALEMR	79.45
			RIGSEVYHNLK(+201.28)SLTK(+201.28)	22.26
3	YLR259C	Hsp60	ASLLK(+201.28)GVETLAEAVAATLGPK	70.76
			GVETLAEAVAATLGPK(+201.28)GR	41.43
			K.GSIDITTTNSYEK(+201.28)EK.L	45.39
			R.PAK(+201.28)QIIENAGEEGSVIIGK.L	36.77
4	YJR121W	ATP synthase	T(+201.28)PQGK(+201.28)LVLEVAQHLGENTVR	53.74
5	YCR012W	3-PhosphoGlycerate Kinase	YSLAPVAK(+201.28)ELQSLLGK	62.04
6	YDR382W	Ribosomal Protein P2 Beta	M(+201.28)KYLAAYLLLVQGGNAAPSAADIK	87.46
7	YIL094C	LYSine requiring, Homo-isocitrate dehydrogenase	S(+201.28)TALMLEFLGHNEAAQDIYK	85.9
8	YOL121C	Ribosomal Protein of the Small subunit 40S	K(+201.28)VLQALEK(+201.28)IGIVEISPK	75.03
9	YGR254W	ENO1	LAK(+201.28)LNQLLR	19.01
			R.GNPTVEVELTTEK(+201.28)GVFR	65.95
			R.S(+201.28)IVPSGASTGVHEALEMR	79.45

			K.AVDDFLISLDGTANK(+201.28)SK	57.23
10	YBL099W	ATP synthase	S(+201.28)VHEPVQTGLK(+201.28)AVDALVPIGR	73.87
11	YAL003W	Elongation Factor Beta	AIEMEGLTWGAHQFIPIGFGIK(+201.28)K	67.38
12	YOR167C	Ribosomal Protein of the Small subunit 40S	TPVTLAK(+201.28)VIK(+201.28)VLGR	61.01
13	YDR155C	Cyclosporin A-sensitive Proline Rotamase	HVVFGEVVDGYDIVKK(+201.28)VESLGSPSGATK	50.26
14	YHR193C	Enhancer of Gal4 DNA binding, Alpha subunit of the nascent polypeptide-associated complex (NAC)	ELIGK(+201.28)LGLK(+201.28)QIPGIIR	30.71
15	YOL039W	Ribosomal Protein P2 Alpha	M(+201.28)KYLAAYLLLNAAGNTPDATK	20.61
16	YGR192C	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), isozyme 3	RYAGEVSHDDK(+201.28)HIIVDGK	38.91
			KT(+201.28)VDGPSHK(+201.28)DWR	31.43
			RTASGNIIPSSTGAAK(+201.28)AVGK	49.43
			KVLPELQGK(+201.28)LTGMAFR	55.38
17	YJR009C	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), isozyme 2	KHIIVDGHK(+201.28)IATFQER	26.26
			KT(+201.28)VDGPSHK(+201.28)DWR	31.43
			RTASGNIIPSSTGAAK(+201.28)AVGK	49.43
18	YJL052W	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), isozyme 1	K.T(+201.28)VDGPSHK(+201.28)DWR.G	31.43
			K.TASGNIIPSSTGAAK(+201.28)AVGK(+20	40.6

			1.28)VLPELQGK.V	
			K.VLPELQGK(+201.28)LTGMAFR.V	55.38
			R.TASGNIIPSSTGAAK(+201.28)AVGK.V	49.43
19	YDR382W	Ribosomal protein P2 beta	K.GSLEEIIAEGQK(+201.28)K.F	54.72
			R.INELLSSLEGK(+201.28)GSLEEIIAEGQK.K	43.87
			M(+201.28)KYLAAYLLLVQGGNAAPSAADIK.A	68.89
20	YLR044C	Pyruvate DeCarboxylase	R.VATTGEWDK(+201.28)LTQDK.S	44.53
21	YKL060C	Fructose 1,6-bisphosphate aldolase	R.K(+201.28)(+201.28)TGVIVGEDVHNLFTYAK.E	42.27
22	YER091C	Cobalamin-independent methionine synthase	R.APEQFDEVVAAIGNK(+201.28)QTLSVGIVDGR.N	47.2
23	YJR104C	Cytosolic copper-zinc superoxide dismutase	R.S(+201.28)VVIHAGQDDLGK.G	45.63
24	YOR020C	Heat Shock Protein	K.T(+201.28)ASGLYLPEK.N	37.56
			K.T(+201.28)ASGLYLPEK(+201.28)NVEK.L	26.85
25	YJR045C	Hsp70 family ATPase, Stress-Seventy subfamily C	R.FK(+201.28)TETGIDLENDR.M	31.28
			K.IVK(+201.28)HSNGDAWVEAR.G	24
26	YDR226W	Adenylate Kinase	R.SQIAK(+201.28)GTQLGLEAK.K	49.68
27	YGR209C	Cytoplasmic thioredoxin isoenzyme	R.VVGANPAAIK(+201.28)QAIASNV	35.19
28	YOR167C/ YLR264W	Ribosomal Protein of the Small subunit	K.TPVTLAK(+201.28)VIK(+201.28)VLGR.T	20.05

Table 5.1: List of cell surface proteins along with corresponding exposed cell surface peptides of *S. cerevisae* containing labeled lysine residues by sulfo-NHS ester modified Fe_3O_4@SiO_2 MNPs. The labeled lysine residues are represented by **K.** In addition to lysine, tyrosine, serine and C-

terminus of proteins were also labeled by sulfo-NHS ester tag. Certain peptides containing cysteine residues are shown the list which might have been produced from cleavage of disulfide bond in the final step of isolation of labeled cell surface peptides from MNPs.[38] The sulfo NHS ester modified NPs labeled the part of proteins i.e. which were exposed to the cell surface environment (Figure 5.3). To the best of our knowledge, this type of information has not been reported earlier. We were able to identify 56 cell surface peptides associated with 30 proteins located on the solvent exposed part of *S. cerevisae*. We found SSA1, TDH1, TDH2, and TDH3 which are associated with cell wall, plasma membrane, membrane proteins, mitochondrion and cytoplasm. Few mitochondrial proteins were detected on the cell surface of yeast. These proteins were detected by matching the sequence of tryptic peptides. The proteins detected on the cell surface might belong to the same family of mitochondrial proteins that share the similar peptide sequence which can be verified by BLAST search. [39]

```
1    MVRVAINGFG  RIGRLVMRIA  LSRPNVEVVA  LNDPFITNDY  AAYMFKYDST  HGRYAGEVSH
61   DDK*HIIVDGK KIATYQERDP  ANLPWGSSNV DIAIDSTGVF  KELDTAQKHI  DAGAKKVVIT
121  APSSTAPMFV  MGVNEEKYTS  DLKIVSNASC  TTNCLAPLAK  VINDAFGIEE  GLMTTVHSLT
181  ATQKT*VDGPS HKDWRGGRTA  SGNIIPSSTG  AAK*AVGKVLP ELQGK*LTGMA FRVPTVDVSV
241  VDLTVKLNKE  TTYDEIKKVV  KAAAEGKLKG VLGYTEDAVV  SSDFLGDSHS  SIFDASAGIQ
301  LSPKFVKLVS  WYDNEYGYST RVVDLVEHVA  KA
```

Figure 5.3: Sequence of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, YGR192C), isozyme 3 labeled by sulfo-NHS ester modified Fe_3O_4@SiO_2 MNPs. The exposed cell surface peptides are highlighted in green and labeled amino acid residues are marked by asterisk (*).

Experimental evidences accumulated over the last several years have shown that various glycolytic and cytosolic proteins can be transported to the cell surface in yeast cells systems such as *S. cerevisae* and *C. albicans*. These CSPs proteins may or may not contain *N*-teminal signal peptides which drive them to the cell surface. We found various multiple location proteins such as glycerladehyde phosphate-3-dehydrogenase (GADPH, isozyme 1-3), and stress seventy

subfamily A (HSP 70) in the SGD search. Glycerladehyde phosphate-3-dehydrogenase (GADPH, isozyme 1-3) is a classic glycolytic enzyme which conduct oxidative phosphorylation of glycerladehyde-3-phosphate to 1,3-diphosphate. However, GADPH can be commonly found in multiple cellular locations including plasma membrane, membrane and cell wall. Numerous reports can be found in literature, which provides immune assay proof for the presence of GADPH on the cell surface. [40] The GADPH located on the yeast cell surface (*Candida albicans*) participate in host attachment by binding with plasminogen, laminin and fibrolectin. [41],[42]Heat shock protein 70 (HSP 70) is an intracellular chaperone protein involved in intracellular protein refolding. Several studies have provided evidence of presence of HSP 70 on the cell surface, but the function of HSP70 on the cell surface still remains unknown. HSP 70 was found to be extended through cell wall to plasma membrane which shows the possibility of serving as a cell receptor or part of translocation complex.[43] Enolase is another protein which lack N-terminal signal peptide but can be abundantly found on the yeast cell surface. [44],[45] Enolase found on the surface of *C. albicans* binds with plasminogen which might be related with invasion of microvascular endothelial cells. [46]

5.5. Conclusion

We have presented sulfo-NHS ester modified MNPs to label the solvent exposed lysine residues on the living cell surface of *S cerevisae*. The large size of MNPS prevents the intracellular uptake of the labeling moiety, which is a major challenge in the development of a cell surface labeling reagent. The impermeable nature of MNPs (80 nm or larger) assisted in selective labeling of solvent exposed lysine residues as verified by electron microscopic imaging. Owing to NHS ester-amine chemistry, the labeling reaction was carried out at physiological conditions thereby maintaining the native structure of CSPs. Furthermore, magnetic properties of

MNPs allowed easy and quick removal of SDS after cell disintegration. The elution of labeled cell surface peptides from MNPs can be performed in aqueous solvents, at room temperature and without any harsh conditions. Sulfo NHS ester modified MNPs successfully labeled solvent accessible lysine residues on cell surface. The sulfo NHS ester modified MNPs can be potentially used to study the cell surface of living cell due to its impermeability, aqueous compatibility and ability to easily elute captured peptides.

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Summary and future work

Cell surface proteomics studies attract researchers due to the role of CSPs in cell communication and cell-pathogen interactions, which can provide potential targets for drug as well as vaccine development. Cell surface labeling using small molecules such as biotinylation reagents are widely used to map the cell surface. However, the ability of biotinylation reagents to cross the cell membrane can provide misleading information related to the position of CSPs. In this work, we have developed labeling reagents containing $Fe_3O_4@SiO_2$ MNPs to target surface exposed lysine, aspartic acid and glutamic acid residues of protein and CSPs.

To develop a membrane impermeable labeling reagent, we exploited the larger size of $Fe_3O_4@SiO_2$ MNPs. Amine reactive NHS ester groups were conjugated to the surface of Fe3O4@SiO2 MNPs via a disulfide bond. Surface exposed primary amine groups were targeted using NHS ester modified $Fe_3O_4@SiO_2$ MNPs under physiological conditions. The disulfide bond present in the labeling reagent provided control over specific release of labeled peptides from MNPs prior to mass spectrometric analysis. Initially the efficiency of NHS ester modified $Fe_3O_4@SiO_2$ MNPs was tested with a small peptide and a protein. Upon conformation with model proteins, the surface exposed amine residues of CSPs of *S. Cerevisae* were labeled using sulfo-NHS ester modified $Fe_3O_4@SiO_2$ MNPs. Mass spectrometric results showed presence of 30 surface proteins which were labeled by NHS ester groups on $Fe_3O_4@SiO_2$ MNPs. Additionally, MNPs allowed quick removal of detergent, which is use to dissolve cell membrane upon cell labeling. Removal of detergents is a time consuming process and usually performed by dialysis, chromatography or electrophoresis.

A disulfide bond present in the linker can interfere with protein digestion and form unwanted disulfide bonds between the thiol groups of proteins and the linker. To avoid this

problem, disulfide bond in the linker was replaced with cleavable ester groups and ester linked, NHS ester modified Fe_3O_4@SiO_2 MNPs were synthesized. Surface exposed amine groups of peptides and proteins were labeled by cleavable ester linked, NHS ester modified Fe_3O_4@SiO_2 MNPs and analyzed by LC-MS/MS.

In addition to lysine residues, aspartic acid and glutamic acid residues are abundantly available on the surface of proteins and CSPs, which can be used to map the surface of proteins and CSPs. Disulfide linked, amine modified Fe_3O_4@SiO_2 MNPs were developed to target the free carboxyl acid groups of aspartic acid and glutamic acid residues using EDC linker. Labeling carboxyl groups of carboxyl groups of peptides and proteins under physiological conditions was performed using disulfide linked, amine modified Fe_3O_4@SiO_2 MNPs. The disulfide linked, amine modified Fe_3O_4@SiO_2 MNPs successfully labeled the surface exposed carboxyl groups of peptides and proteins.

Due to the ability of sulfo-NHS ester modified Fe_3O_4@SiO_2 MNPs to efficiently target the primary amine groups of cells surface, they can be employed to a study the cell surface of a variety of cells. We intend to continue this research by targeting the surface of cancer cell lines such as HeLa and breast cancer cell line (SkBr3). The size of Fe_3O_4@SiO_2 MNPs will be manipulated in order to avoid intracellular uptake of sulfo-NHS ester modified Fe_3O_4@SiO_2 MNPs. The labeled lysine residues on the surface of cancer cell lines could shed light on the cell-cell interaction and can be utilized to develop a potential drug targets.

The sulfo NHS ester modified Fe_3O_4@SiO_2 MNPs can also be utilized to map the surface of antibodies, which are commonly used to target the cancer cells *in vitro* and *in vivo*. In biomaterials science, antibody conjugated nanoparticles are increasingly used to for diagnosis and treatment of various illnesses. It is important to understand the surface exposed functional

groups of antibodies to choose proper conjugation strategy and achieve efficient conjugation to nanoparticles. Sulfo NHS ester modified Fe_3O_4@SiO_2 MNPs can detect surface exposed lysine residues of antibodies, which can also help to probe antibody-antigen interactions.

Structural analysis of apolipoprotein B-100 (apo B-100) in low density lipoproteins (LDL) is hampered by the larger size and hydrophobic nature of apoB-100. The solvent accessible surface of apoB-100 in native LDL can provide important information about its interaction with LDL receptors. We propose to use disulfide linked, amine modified Fe_3O_4@SiO_2 MNPs to label surface exposed aspartic and glutamic acid residues of apo B-100.

VITA

Ujwal S. Patil was born in Jalgaon, in the state of Maharashtra, India. He was admitted into the Bachelor of Pharmacy program at Sant Gadage Baba Amravati University, Amravati, India. He graduated in May 2007 and joined Master of Science (MS) program in pharmaceutical sciences at Idaho State University (ISU), Pocatello, Idaho, USA. He obtained MS degree from ISU in May 2010. In August 2010, he was admitted to the graduate program at Department of Chemistry, University of New Orleans, New Orleans, Louisiana, USA. He worked in Dr. Matthew A. Tarr's research group for pursuing a Ph.D. in Bioanalytical Chemistry.