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# Guanidinium Functionalized Superparamagnetic Silica Spheres for Selective Enrichment of Phosphopeptides and Intact Phosphoproteins from Complex Mixtures

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## ABSTRACT

Phosphorylation of protein regulates nearly all biological processes in nature. The development of enrichment technique for phosphorylated proteins is vital to systematic identification and characterization of phosphoproteins. Here, a general strategy for highly efficient capture of intact phosphorylated proteins from protein mixtures has been developed by using guanidine functionalized superparamagnetic microspheres (denoted as $Fe_3O_4$@$SiO_2$@GDN). The $Fe_3O_4$@$SiO_2$@GDN was prepared by modifying $Fe_3O_4$@$SiO_2$ with 3-guanidopropyl triethoxysilane as functionalization monomer. The resulting materials could specifically and selectively recognize phosphoproteins, and showed high binding capacities for model phosphoproteins (78.8mg $g^{-1}$ for ovalbumin (OVA) and 59.6mg $g^{-1}$ for β-Casein (β-Cas), respectively). The feasibility of the resulting material for phosphoproteins enrichment has also been demonstrated by selectively binding and capturing phosphoproteins from complex protein mixtures and real samples (milk, egg, and tissue protein extract from mouse liver), respectively. In addition, the selective enrichment of phosphopeptides has also been investigated. The proposed technique showed application potential for phosphoproteins and phosphopeptides enrichment.


**Keywords:** Guanidine; phosphoproteins enrichment; proteomic; superparamagnetic microspheres

## 1. Introduction

Phosphorylation of protein is one of the most important and dynamic post-translational modifications in nature, which regulates nearly all biological processes, including signal transduction, homeostasis, apoptosis, proliferation, transcriptional and translational regulation.[1-2] Dysregulation of protein phosphorylation has been linked to numerous serious diseases such as cancer and diabetes. Systematic identification and characterization of phosphoprotein is vital to help us understand this important modification; however, it remains a challenging task due to the relatively low abundance of phosphorylated proteins in biological samples.[3-5] Current approaches to capture phosphoproteins generally rely on the interaction of phosphate anions with metal ions, such as in immobilized metal affinity chromatography (IMAC) and in metal oxide affinity chromatography (MOAC).[6-10] Even though these techniques have been demonstrated with success to a certain extent, some disadvantages are encountered with these techniques: including poor selectivity, low adsorption efficiency and significant losses of phosphorylated proteins during the wash steps.[11-12]

Herein, we propose a general strategy for high efficient capture of intact phosphorylated proteins from protein mixtures based on interaction between phosphate groups and guanidine groups. Guanidinium group has a pKa of 13.6 and normally possesses a delocalized positive charge which is distributed over all three nitrogen atoms over a wide pH range. It has a flat and trigonal arrangement in which two protons are oriented roughly the same direction and can stabilize two parallel

hydrogen bonds. Because of strong ability to bind anions through hydrogen bonding and charge pairing interactions developing guanidinium-based artificial receptors for the purpose of anions recognition has attracted much attention. [13-14] In nature, enzymes often bind anionic substrates by using the guanidinium-containing side chain of arginine in their active sites.[15-17] On the other hand, the acid dissociation constant of phosphate groups is roughly $pKa_1$=2.12 and $pKa_2$= 7.21, respectively. The geometric arrangement of this functional group is tetrahedral about the phosphorus, in which the O-P-O bond angles are roughly 109.5$^o$ and three equivalent oxygen atoms share one or two negative charges. Thus the arrangement of phosphate group is favorable for its interaction with guanidinium group. Recently, wood et al. reported that the guanidinium group of arginine formed a more stable interaction with the phosphate group than adjacent carboxyl group from Asp or Glu. The electrostatic interaction of the arginine-phosphate exhibits "an amazing covalent-like" stability.[18-19] However, to the best of our knowledge, the approach for phosphoprotein enrichment based on the guanidinium functionalized receptor has not been exploited previously.

In this research, guanidinium functionalized superparamagentic silica spheres (denoted as $Fe_3O_4$@$SiO_2$@GDN) were prepared by using superparamagnetic $Fe_3O_4$ as core due to the facile isolation and without retaining residual magnetism after removal of the external magnetic field. The recognition properties of the resulting materials for intact phosphorylation proteins were investigated by using β-casein (β-Cas, molecular weight (MW) 24kDa, isoelectric point (pI) 4.6~5.1) and ovalbumin (OVA, MW 45kDa, pI 4.7) as model phosphoproteins, and bovine serum albumin (BSA, MW

67.0kDa, pI 4.8), hemoglobin (Hb, MW 65.0 kDa, pI 6.9 ) trypsin (Try, MW 10.5 kDa, pI 10.0), Myoglobin (Mb, MW 16.7, pI 6.99 ), Lysozyme (Lyz, MW 14.0 kDa, pI 11.0 ) and Cytochrome C (Cyc, MW 12.4 kDa, pI 9.8), as non-phosphorylated proteins, respectively. Further, the resulting material has also been evaluated for the enrichment of intact phosphoproteins from complex real samples (milk, egg, and tissue protein extract from mouse liver). The schematic diagram was shown in Figure 1.

## 2. Experimental

### 2.1 Materials and reagents

γ-Aminopropyl triethoxysilane (γ-APS), 3-(2-aminoethylamino) propyltriethoxysilane, 2-ethyl-2-thiopseudourea hydrobromide and tetraethylorthosilicate (TEOS) were all obtained from TCI (Shanghai, China). Tris(hydroxymethyl) aminomethane (Tris), 3-(N-morpholino) propanesulfonic acid (MOPS) and 4-(2-Hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) were all purchased from Alfa Aesar (Beijing, China). DL-Dithiothreitol (DTT), iodoacetamide (IAA), 3-[(3-Cholamidopropyl)dimethylammonio] propanesulfonate (CHAPS), sodium orthovanadate ($Na_3VO_4{\cdot}12H_2O$), sodium fluoride (NaF), ammonium bicarbonate, phenylmethanesulfonyl fluoride (PMSF), ethyleneglycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), glacial acetic acid, soduim acetate anhydrous were all obtained from J&K Scientific Ltd. (Beijing, China). Trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Trypsin from bovine pancreas TPCK treated and β-Cas was purchased from Sigma-Aldrich (St., Louis, MO). All other proteins were obtained from Shanghai Sangon BioTech (Shanghai, China). Fresh milk and egg were purchased from a local supermarket. Adult female C57 mice were purchased from Laboratory Animal Center of Academy of Military

Medical Sciences (Beijing, China). All aqueous solutions were prepared in doubly deionized water (DDW, 18.2 MΩ cm-1) from a Millipore water purification system (Millipore, Billerica, MA, USA). Pro-Q® Diamond phosphoprotein gel staining was obtained from life technologies corporation (Invitrogen, China).

**2.2 Instruments**

FT-IR spectra (4000-400 cm-1) in KBr were performed on a Vector 22 spectrometer (Bruker, Germany). $^{1}$H NMR spectra were recorded with a Bruker AVIII spectrometer (Bruker, Germany). Scanning electron microscope (SEM) images were obtained with a Hitachi SU-1510 (Hitachi, Japan). Transmission electron microscope (TEM) and energy dispersive X-ray analysis (EDAX) were carried out on a JEM-2100 TEM (JEOL, Japan). The magnatic property of the ferrite microsphere was investigated with a vibrating sample magnetometer (VSM) (Quantum Design, USA). Thermo gravimetric analysis (TGA) was obtained from SDTQ600 (TA, USA) at a heating rate of 10 $^{o}$C min$^{-1}$ up to 800 $^{o}$C. X-ray photoelectron spectroscopy analysis (XPS) experiment was carried out on a XPS PHI1500VersProbe (ULVAC-PHI, Japan). Micropore size and surface area were detected on a AUTOSORB-1-MP (Quantachrome，USA). The obtained gels were observed on Imagequant TM Las4000 (GE, USA) and Gel DocTM XR (Bio-Rads, USA), respectively. All MALDI-TOF mass spectra were obtained on a Bruker FLEX$^{TM}$ time of flight mass spectrometer (Bruker, Bermen, Germany).

**2.3 Synthesis of 3-guanidopropyl triethoxysilane**

2-Ethyl-2-thiopseudourea hydrobromide (3.5 g, 18.9 mmol) was dissolved in a mixture of 3.0 mL of dimethyl sulphoxide (DMSO) and 3.5 mL of and tetrahydrofuran (THF). After magnetic stirring for 20 min, γ-APS (4.45 mL, 19 mmol) was dropped slowly at 0 $^{0}$C. The mixture was then maintained for 48 h under stirring

at 25 °C. After removal of DMSO and THF, the viscous transparent liquid was obtained, and characterized by FT-IR and $^1$H NMR, respectively. The results were shown as following: FT-IR (KBr), 3338, 3162, 2975, 2929, 2890, 1668, 1436, 1313, 1076cm$^{-1}$; $^1$H NMR (400 MHz, DMSO-$d_6$), δ = 7.095 (br.s, 4H, NH's), 3.746 (m, 6H, O-$CH_2$-C), 3.109 (m, 2H, N-$CH_2$-C), 1.563 (m, 2H, C-$CH_2$-C); 1.140 (m 9H, C-$CH_3$); 0.574( q, 2H, Si-$CH_2$-C).

### 2.4 Preparation of superparamagnetic $Fe_3O_4$@$SiO_2$ microspheres

Magnetic microspheres with an average diameter of 200 nm were synthesized by a solvothermal reduction method.[20] In detail, $FeCl_3 \cdot 6H_2O$ (2.7 g, 10 mmol) was dissolved in ethylene glycol (80.0 mL), and then anhydrous sodium acetate (7.2 g) and polyethylene glycol (2.0 mL) were also added. The mixture was first stirred with a magnetic stirring bar for 30 min, and then poured into a Teflon lined stainless-steel autoclave (100.0 mL). The autoclave was maintained at 200 °C for 8 h. After cooling to room temperature, the $Fe_3O_4$ microspheres were washed with ethanol three times, and dried in a vacuum oven at 60 °C for 6 h. Subsequently, the $Fe_3O_4$ microspheres were coated with TEOS by a modified sol-gel technique.[21] In detail, $Fe_3O_4$ microspheres (400.0 mg) were dispersed into toluene (100.0 mL) under ultrasonication. TEOS (3.0 mL) and triethylamine (3.0 mL) were then added into the mixtures in sequence. The mixtures were stirred at room temperature for 24 h. The resulting $Fe_3O_4$@$SiO_2$ microspheres were collected and washed with ethanol three times, and then dried in a vacuum oven at 60 °C for 4h.

### 2.5 Preparation of $Fe_3O_4$@$SiO_2$@GDN microspheres

The as-obtained superparamagnetic $Fe_3O_4$@$SiO_2$ microspheres (400 mg) were dispersed in 80.0 mL Tris-HCl buffer (pH 8.21, 0.1 mol L$^{-1}$), followed by the addition of 3-guanidinopropyl triethoxysilane (1.2 mL, 3.17 mmol) and TEOS (0.6 mL, 2.69

mmol). Subsequently, the mixtures were oscillated with 150 rpm $min^{-1}$ for 16 h. The products were collected and washed with deionized water several times to remove the unreacted monomers. After dried at 35 $^{o}$C for 24h, the obtained $Fe_3O_4$@$SiO_2$@GDN microspheres were dried. For comparison, amino group functionalized superparamagnetic $Fe_3O_4$@$SiO_2$ ($Fe_3O_4$@$SiO_2$@APS) microspheres were also prepared under similar condition, except that 3-aminopropyl triethoxysilanethe replaced 3-guanidopropyl triethoxysilane as functional monomer.

### 2.6 Recognition of proteins using $Fe_3O_4$@$SiO_2$@GDN microspheres

In a typical experiment, $Fe_3O_4$@$SiO_2$@GDN microspheres (10.0 mg) were added into HEPES buffer (2.0 mL, 10mM, pH 6.86) containing the target protein. When the adsorption reaction was finished, the $Fe_3O_4$@$SiO_2$@GDN microspheres were collected from the solution by an external magnetic field, and the protein concentration of the supernatant was detected. The amount of protein adsorbed onto $Fe_3O_4$@$SiO_2$@GDN microspheres was calculated from the difference of the concentration of target protein in the supernatant before and after adsorption. The adsorption capacity ($Q$, mg of protein /g of material) was calculated according to the following equation: $Q = (C_0-C_f)V/W$, where $C_0$ (mg/mL) is the initial protein concentration, $C_f$ (mg/mL) is the final protein concentration, V (mL) is the total volume of the adsorption mixture, and W (g) is the mass of material.

### 2.7 Capture of intact phosphorylated proteins from standard protein mixtures

The superparamagnetic $Fe_3O_4$@$SiO_2$@GDN microspheres (40.0 mg) were added into HEPES buffer (40.0 mL, 10 mM pH 6.86) containing a protein mixture (Cyc, Lyz, BSA, Mb and OVA, or Cyc, BSA and β-Cas), and incubated at 4 $^{0}$C for 2 h. The superparamagnetic $Fe_3O_4$@$SiO_2$@GDN microspheres were collected and washed

with 1.0 mL HEPES buffer (pH 6.86). Then 500 μL 50 vol % acetonitrile with 1.0 vol % TFA was used to elute the adsorbed proteins twice. The initial solution, washing and eluent were all analyzed by HPLC.

### 2.8 Capture of phosphopeptides from the mixture of peptides

β-Cas (1.0 mg) was first dissolved in 1.0 mL of ammonium bicarbonate solution (50 mM pH 8.0), and then digested with trypsin (enzyme/protein, 1:40 in weight ratio) at 37 °C for 16 h. Tryptic digests were diluted with HEPES (pH 6.86, 10 mM) buffer to a final concentration of 50 fmol/μL. The resulting material (6.0 mg) was put into the diluted solution (1.0 mL), and kept for 30 min. The phosphopeptides-loaded microspheres were collected by an extra magnatic field and washed two times with 2.0 mL of mixture of acetate buffer (100 mM, pH 4.0)/acetonitrile (v/v, 4:1) to remove non-specific peptides. The bound phosphopeptides were then eluted with 60 μL of 50.0% acetonitrile with 1.0% TFA. 2.0 μL of the eluent was mixed with 4.0 μL of saturated HCAA, and then 1.0 μL of mixture was added to the sample spot. Samples were analyzed by MALDI-TOF-MS after air drying.

In order to construct the complex sample, Cyc was first digested as follow: 1.24 mg of Cyc was dissolved in ammonium bicarbonate solution (500 μL, 50 mM) containning 8 M urea. The solution was kept at 37 °C for 3 h, and then 10 μL of DTT (100 mM) was added. The mixture was kept at 37°C for another 2.0 h, and then 20 μL of IAA (100 mM) was added and incubated for an additional 30 min at room temperature in the dark. The obtained solution was diluted to 5.0 mL with ammonium bicarbonate solution (50 mM) to reduce the concentration of urea. Subsequently, the protein was digested using trypsin (enzyme/protein, 1:40 in weight ratio) at 37 °C for 16 h. The digested product had a concentration of 20 pmol/μL. 40 μL of digested β-cas solution (1 pmol/μL) was mixed with 200 μL digested Cyc solution (20 pmol/μL). The mixture

was then diluted with HEPES buffer (pH 6.86, 10 mM) to a total volume of 4.0 mL, and resulted in a concentration of 10 fmol/μL and 1000 fmol/μL for digested β-Cas and digested Cyc, respectively. The resulting solution (4.0 mL) was mixed with 10.0 mg of $Fe_3O_4$@$SiO_2$@GDN for 30 min. The subsequent procedures are as same as that of phosphopeptide enrichment from β-Cas tryptic digest.

**2.9 Selective capture of intact phosphorylated proteins from complex samples**

Fat was first removed from fresh milk by centrifugation. The obtained nonfat milk was diluted 120 times with HEPES buffer (pH 6.86, 10 mM). Egg white was first separated from yolk, and then diluted 150 times with HEPES buffer (pH 6.86, 10 mM). The superparamagnetic $Fe_3O_4$@$SiO_2$@GDN microspheres (30.0 mg) were incubated with 6.0 mL of the diluted nonfat milk or the diluted egg white solution at $4^0$C for 2h. After magnetic collected, the superparamagnetic $Fe_3O_4$@$SiO_2$@GDN microspheres were washed with 1.0 mL HEPES buffer (pH 6.86) twice. Then the adsorbed proteins were removed from the materials with 1.0 mL 50 vol % acetonitrile containing 1.0 vol % TFA. The initial diluted samples, supernatant, washing and eluent were all analyzed by SDS-PAGE.

Tissue protein extract from mouse liver was prepared according to a procedure described in detail elsewhere.[22] Then the extracted liver proteins were precipitated using cold acetone, and lyophilized to dryness using a lyophilizer. 40.0 mg of liver proteins were dissolved in 40.0 mL HEPES buffer (pH 6.86, 10 mM，4 $^o$C ). The solution was centrifugated at 5000rpm/min for 5 min to remove undissolved substance. The supernatant was incubated with 35.0 mg of $Fe_3O_4$@$SiO_2$@GDN for 30 min at 4 $^o$C. Then the microspheres were washed with 2.0 mL of an acetate buffer

(100 mM, pH 4.0) /acetonitrile (v/v, 4:1) twice, and then the bound proteins were eluted by 200 μL of 50.0% acetonitrile with 1.0% TFA. The initial diluted samples, supernatant, washing and eluent were all analyzed by SDS-PAGE.

## 3. Results and Discussion

### 3.1 Preparation and characterization of $Fe_3O_4$@$SiO_2$@GDN microspheres

In this study, 3-guanidopropyl triethoxysilane was first synthesized by the reaction of 2-ethyl-2-thiopseudourea hydrobromide with γ-APS in the medium of DMSO and THF. Monodisperse magnetic $Fe_3O_4$ microspheres were prepared by a modified sol-gel process, and then coated with TEOS. The resulting magnetic $Fe_3O_4$@$SiO_2$ particles were further functionalized with a silane mixture containing 3-guanidopropyl triethoxysilane and TEOS in Tris buffer (pH 8.20), and leading to the formation of a guanidine-containing silica layer onto the surface of $Fe_3O_4$@$SiO_2$. The resulting $Fe_3O_4$@$SiO_2$@GDN microspheres were then employed to capture intact phosphorylated proteins from complex samples (Figure 1).

In order to ensure successful preparation of $Fe_3O_4$@$SiO_2$@GDN microspheres, TEM and SEM were employed to observe the morphology of $Fe_3O_4$@$SiO_2$ and $Fe_3O_4$@$SiO_2$@GDN microspheres, respectively. From Figure 2a and 2b, we observed that the core shell $Fe_3O_4$@$SiO_2$ microspheres with a 200 nm $Fe_3O_4$ core were coated with a silica layer of 8.5nm. A guanidinium-containing silica layer (20 nm) was formed onto the surface of $Fe_3O_4$@$SiO_2$ microsphere by further functionalizing with the mixture of 3-guanidopropyl triethoxysilane and TEOS (Figure 2c and 2d).

The surface composition of $Fe_3O_4$@$SiO_2$@GDN microsphere was further measured by XPS. The XPS spectrum of the resulting microspheres showed four distinct chemical species: nitrogen, carbon, oxygen, and silicon (Figure 3a). In addition, the characteristic stretching and bending vibration of guanidinium groups at 3430 and

1635 $cm^{-1}$ were clearly observed in the FT-IR spectra of the 3-guanidopropyl triethoxysilane and $Fe_3O_4$@$SiO_2$@GDN microspheres (Figure 3b) respectively, suggesting a large amount of guanidinium groups in the $Fe_3O_4$@$SiO_2$@GDN microspheres. From the results of TGA (Figure 3c), a weight loss of 27.8% occurred for $Fe_3O_4$@$SiO_2$@GDN microsphere, and 7.4% for $Fe_3O_4$@$SiO_2$. The difference can be attributed to the thermal decomposition of the guanidopropyl groups of $Fe_3O_4$@$SiO_2$@GDN microsphere. These results suggested that $Fe_3O_4$@$SiO_2$@GDN microspheres have been successfully fabricated.

The magnetization of $Fe_3O_4$ and $Fe_3O_4$@$SiO_2$@GDN were investigated by using VSM measurements (Figure 4a). The results indicated that the magnetization of $Fe_3O_4$@$SiO_2$@GDN microspheres was more than 38 EMU/g. From Figure 4a, we can conclude that these microspheres are superparamagnetic at room temperature due to the lack of magnetic hysteresis. In addition, the resulting $Fe_3O_4$@$SiO_2$@GDN microspheres are well-dispersed in water without visible aggregation (Figure 4b). In the presence of a magnetic field, however, the microspheres were completely settled to the bottom of a test bottle in 30 seconds (Figure 4c). This observation proved that the functionalized magnetic microspheres possessed a high magnetic responsiveness for use in magnetic separation.

### 3.2 Recognition property of $Fe_3O_4$@$SiO_2$@GDN microspheres

To test the recognition properties of superparamagnetic $Fe_3O_4$@$SiO_2$@GDN microspheres to phosphorylated proteins, the two classical phosphorylated proteins, β-Cas and OVA, were chosen as model proteins. The materials were incubated with HEPES buffer (pH 6.86 10 mM) of protein at 4 $^0$C. Following collection by an external magnetic field, the concentration of protein in the supernatant was measured by UV-vis at 280 nm. The amount of protein adsorbed onto materials was calculated

by the difference of concentration of protein in the supernatant before and after adsorption. From Figure 5a, we observed that capture efficiency decreased with increasing initial concentration of proteins, and the adsorption amount increased with increasing initial concentration of proteins and the maximum capacities were achieved at an initial concentration of 1.0 mg $mL^{-1}$. The adsorption capacities of the material for OVA and β-Cas were 78.8 mg $g^{-1}$ and 59.6 mg $g^{-1}$, respectively. It is worth to note that both phosphoproteins with significantly different molecular size showed high adsorption capacity. The superior phosphorylated protein adsorption property of the guanidine functionalized magnetic microspheres may result from the strong interaction between guanidine groups of the microspheres and phosphate groups of the protein.

Adsorption kinetics is one of the important parameters for understanding the protein binding mechanism and determining the potential of materials in practical application. Thus the adsorption kinetics of $Fe_3O_4$@$SiO_2$@GDN was also investigated by changing the adsorption time from 0 to 1440 min and the initial concentration of proteins were all kept constantly at 0.5 mg $mL^{-1}$. From the time course of binding (Figure 5b), the adsorption amount increased significantly in the first 60 min, reaching about 88% of the maximum binding capacity for OVA and 92% for β-Cas. The binding capacity almost has no change when the incubation time exceeds 120 min. The results demonstrated that the synthesized $Fe_3O_4$@$SiO_2$@GDN possessed a fast adsorption rate.

### 3.3 Recognition Selectivity of $Fe_3O_4$@$SiO_2$@GDN microspheres

The resulting materials have indicated higher binding capacity for chosen phosphoproteins, however, selectivity is also an important factor for its real application. Six non-phosphoproteins (including BSA, Try, Mb, Cyc, Hb and Lyz)

with different molecular size and pI were chosen as competitors, and the binding of $Fe_3O_4$@$SiO_2$@GDN microspheres towards these non-phosphoproteins were evaluated under the condition of 0.5mg $mL^{-1}$ of protein. The results indicated that the $Fe_3O_4$@$SiO_2$@GDN microspheres showed superior recognition ability towards β-Cas (49.5 mg $g^{-1}$) and OVA (56.7 mg $g^{-1}$) (Figure 6). However, the binding amount of the microspheres towards other non-phosphoproteins was in the range from 12.7 mg $g^{-1}$ (BSA) to 1.2 mg $g^{-1}$ (Cyc). Although BSA has a similar pI (4.5) value with OVA, binding capacity of $Fe_3O_4$@$SiO_2$@GDN microspheres towards BSA was significantly lower than that of OVA. Mb and Hb, which are approximately neutral under the test conditions, also showed lower binding capacities. The results demonstrated that the $Fe_3O_4$@$SiO_2$@GDN microspheres displayed specific recognition ability to phosphorylated proteins. In order to further ascertain the role of guanidinium group in phosphoproteins recognition, $Fe_3O_4$, $Fe_3O_4$@$SiO_2$ and γ-aminopropyl triethoxysilane functionalized $Fe_3O_4$@$SiO_2$ ($Fe_3O_4$@$SiO_2$@APS) were also employed to bind β-Cas and OVA, respectively. Among the three materials, $Fe_3O_4$@$SiO_2$ showed the lowest binding capacities for β-Cas (2.12 mg $g^{-1}$) and OVA (4.34 mg g-1), and $Fe_3O_4$ displayed the highest binding capacities for β-Cas (16.6 mg $g^{-1}$) and OVA (8.90 mg $g^{-1}$), which may be attributed to the interaction of unoccupied orbital of iron with the phosphate group of phosphoprotein. Although there are amine groups on the surface of $Fe_3O_4$@$SiO_2$@APS, it still showed lower binding capacities for β-Cas (8.9 mg $g^{-1}$) and OVA (5.70 mg $g^{-1}$), respectively. The superior phosphorylated protein adsorption property of the $Fe_3O_4$@$SiO_2$@GDN microspheres may result from the strong interaction between guanidine groups of the microspheres and phosphate groups of the protein. The results indicated that guanidinium group of $Fe_3O_4$@$SiO_2$@GDN microspheres played an important role in phosphoproteins recognition.

### 3.4 Highly specific enrichment of phosphoproteins from standard protein mixtures

Phosphoproteins are usually present at very low concentration in complex biological samples. Thus, the separation ability of the $Fe_3O_4$@$SiO_2$@GDN microspheres for low-concentration proteins was also investigated. Here β-Cas was chosen as phosphoprotein, and its concentration was kept constant at 0.01mg $mL^{-1}$. BSA and Cyc were chosen as nonphosphoproteins, the concentration for each nonphosprotein was varied in the range of 0.01mg $mL^{-1}$ to 1.20 mg $mL^{-1}$. A series of diluted protein mixture (β-Cas, Cyc and BSA) were prepared in different mass ratio (phosphoprotein (0.01mg $mL^{-1}$)/each nonphosphoprotein) at 1:1, 1:20, 1:40 and 1:120 to simulate complex samples. The initial solution, washing solution and eluted fraction was detected by HPLC to evaluate the enrichment efficiencies of phosphorylated proteins. From Figure 7 we can observe that the peaks of BSA and Cyc are apparent in initial solution, when the mass ratio was beyond 1:20. The peaks of BSA and Cyc are also observed in washing fraction. The peak of β-Cas, which is undetectable in the initial solution and washing fraction, is significantly visible in the eluent in all cases. When the ratio is beyond 1:40, the peak of BSA is also visible in the eluent, however, the enrichment efficiencies of BSA is less than 5% in all cases. Cyc shows no significant effect to β-Cas enrichment. It is worth to note that the enrichment efficiencies of β-Cas were beyond 82.6 % and no Cyc was observed in all cases. The separation ability of the $Fe_3O_4$@$SiO_2$@GDN microspheres for another classical phosphoprotein, OVA, was also investigated in the presence of nonphosphoproteins (Lyz, BSA, Mb

and Cyc). Protein mixtures (including OVA, Lyz, Mb, Cyc and BSA) with different mass ratio (phosphoprotein (0.01 mg $mL^{-1}$)/ each nonphosphoprotein at 1:1, 1:20, 1:40 and 1:120) were obtained by varying the concentration of each nonphosphoprotein in the range of 0.01mg $mL^{-1}$ to 1.20 mg $mL^{-1}$. Figure 8 showed the results of the $Fe_3O_4$@$SiO_2$@GDN microspheres for OVA enrichment from protein mixtures. When the mass ratio is reached 1:20, the peaks of nonphosphoproteins are significantly observed in initial solution, and all of them except BSA are undetected in eluent. OVA could not be observed in initial solution, and significantly exsists in eluent in all cases. The enrichment efficiencies of OVA were beyond 50%, while Lyz, Cyc and Mb showed no effect to OVA enrichment in the studied conditions. Although BSA was observed in eluent, the enrichment efficiencies of BSA are no more than 10% in all cases. These results indicated that the resulting materials exhibited highly selective binding to phosphoproteins.

**3.5 Enrichment of phosphopeptides from the mixture of peptides**

Selective enrichment of phosphopeptides by the resulting materials was also investigated. For comparison, the β-Cas digest was directly analyzed by MS. The obtained spectrum was dominated by non-phosphopeptides, and no phosphopeptide was observed without the pretreatment procedure (Figure 9a). However, when the mixture of peptide was treated with $Fe_3O_4$@$SiO_2$@GDN microspheres, signals of phosphopeptides could be clearly observed (Figure 9b). In addition, their dephosphorylated counterparts, which may be formed during the MALDI ionization process, have also been observed in Figure 9b. The enrichment ability of the resulting

material towards phospeptides from complex samples were also investigated by a mimic biological sample, which was constructed by mixing large amounts of tryptic digests of Cyc with the tryptic digests of β-Cas (the molar ratio of Cyc to β-Cas is 100:1). The mixture of peptides was analyzed without enrichment; no phosphopeptides were detected due to the presence of high-abundance non-phosphopeptides (Figure 9c). After the enrichment, all the three phosphopeptides could be easily detected with a very clean background in the mass spectrum (Figure 9d). All these results indicated that the resulting materials also showed high selectivity to phosphopeptides.

3.6 **Highly specific enrichment of phosphoprotein from real biological samples**

Material capable of enriching phosphoproteins from real complex samples is a prerequisite for its practical application in proteomic study. Herein, milk, egg white and tissue protein extract from mouse liver were employed to further examine the selectivity of $Fe_3O_4$@$SiO_2$@GDN microspheres in the enrichment of phosphoproteins from real complex samples. The initial solution, supernatant, washing and eluent were checked by SDS-PAGE. The obtained gels were stained first with Pro-Q (Figure 10 b, d and f), which selectively stains phosphoproteins, followed by CBB staining (Figure 10 a, c and e). As shown in Figure 10a and b, more kinds of phosphoproteins were observed in eluted fraction than ininitial diluted milk sample, and eluted fraction has a much more β-cas than that of initial diluted milk sample. In addition, no protein was detected in washing solution that indicated no loss of phosphoprotein occurred during the washing steps. Similar results were also obtained in the enrichment of OVA from

egg white. In Figure 10c and d, eluted fraction had relative high concentration of OVA when compared with the initial diluted egg white mixture. In Figure 10e and f, there was little signal of phosphoprotein in initial solution, supernatant, and washing solution. However, many phosphoproteins could be found in eluent solution (Figure 10f). These results indicated the high selectivity of $Fe_3O_4$@$SiO_2$@GDN microspheres in enrichment of phosphoproteins from real biological samples. Moreover, the inherent capability of convenient enrichment by magnetic separation is the great additional advantage.

## 4. Conclusions

We have demonstrated for the first time the feasibility of using guanidine functionalized $Fe_3O_4$ microspheres for high efficient capture of phosphorylated proteins and peptides in a simple, general, and inexpensive way. The $Fe_3O_4$@$SiO_2$@GDN microspheres showed the following excellent properties for the selectively capture of phosphorylated proteins and peptides. 1) The guanidine functionalized materials are dispersible in water, and can be easily collected from the mixture just by an external magnetic field. 2) The recognition ability is hardly affected by pH value of medium (data not shown). 3) The materials display excellent stability by several adsorption-desorption cycles (binding amount of protein decreased only 8.5% after six cycles.). 4) The strong specific interaction between guanidine groups and phosphate groups ensures the guanidine functionalized magnetic microspheres with high affinity and specificity to different phosphoproteins and phosphopeptides. With all these features, the guanidine functionalized

417 superparamagnetic microspheres can efficiently and selectively capture low
418 concentrations of intact phosphorylated proteins and peptides from complex mixtures.
419 It is also expected that the selective capture is not limited to proteins and peptides, but
420 can also be extended to phosphate containing biomolecules such as DNA.

421

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494 **Figure Legends**

495 **Figure 1** Schematic illustration of the material preparation and phosphoproteins
496 capture process.

497 **Figure 2** SEM and TEM characterization of $Fe_3O_4$@$SiO_2$ and
498 $Fe_3O_4$@$SiO_2$@GDN, (a), (c) SEM; (b), (d) TEM.

499 **Figure 3** Chemical analysis of $Fe_3O_4$@$SiO_2$@GDN with XPS, FT-IR, and TGA
500 techniques. a) XPS spectra of $Fe_3O_4$@$SiO_2$@GDN Surface; b) FT-IR spectra of
501 3-guanidopropyl triethoxysilane (dark line) and $Fe_3O_4$@$SiO_2$@GDN (red line); c)
502 TGA curves of $Fe_3O_4$@$SiO_2$ (dark line) and $Fe_3O_4$@$SiO_2$@GDN (red line).

503 **Figure 4** a) magnetization curve of magnetic microspheres (dark line) and
504 $Fe_3O_4$@$SiO_2$@GDN (red line), b) Solvent-dispersivity of the $Fe_3O_4$@$SiO_2$@GDN; c)
505 the collection of the $Fe_3O_4$@$SiO_2$@GDN microspheres by an external magnetic
506 field.

507 **Figure 5** a) The binding capacity of β-Cas or OVA on $Fe_3O_4$@$SiO_2$@GDN
508 microspheres; b) adsorption kinetics of β-Cas or OVA on $Fe_3O_4$@$SiO_2$@GDN
509 microsphere. Error bars represent the standard deviations, n = 3. Some error bars are
510 hidden by point markers.

511 **Figure 6** Recognition of $Fe_3O_4$@$SiO_2$@GDN towards different proteins. Error
512 bars represent the standard deviations, n = 3.

513 **Figure 7** Chromatogram of the protein mixture studied and protein binding
514 efficiencies; a) 1:1; b) 1:20; c) 1:40; d) 1:120; e) corresponding adsorption
515 efficiencies for proteins studied. The labels “1”, “2”, “3” in the chromatograms

represent the initial solution, the eluent and washing solution, respectively. Error bars represent the standard deviations, n = 3. Some error bars are hidden by point markers.

**Figure 8** Chromatogram of the protein mixture studied and protein binding efficiencies; a) 1:1; b) 1:20; c) 1:40; d) 1:120; e) Corresponding adsorption efficiencies for proteins studied. The labels "1", "2", "3" in the chromatograms represent the initial solution, the eluent and washing solution, respectively. Error bars represent the standard deviations, n = 3. Some error bars are hidden by point markers.

**Figure 9** MALDI mass spectra for selective enrichment of phosphopeptides by $Fe_3O_4$@$SiO_2$@GDN microspheres. (a) Direct analysis of tryptic digest of β-Cas; (b) phophopeptides enriched from tryptic digest of β-Cas; (c) Direct analysis of the mixture of tryptic digests of β-Cas and Cyc with ratio of 1:100; (d) phophopeptides enriched from the mixture of tryptic digests of β-Cas and Cyc with ratio of 1:100. * indicates phosphopeptides, ●indicates their dephosphorylated counterparts.

**Figure 10** SDS polyacrylamide gel electrophoresis analysis of phosphoproteins adsorbed onto the materials. S reprents supernatant fraction; W represents washing fraction; E represents eluent; M reprents marker proteins (from top to down): β-galactosidase, BSA, OVA, Lactate dehydrogenase, REase Bsp981, β-lactoglobulin, and Lyz. I represents the initial 120-fold dilution of nonfat milk in a) and b), the initial 150-fold dilution of egg white in c) and d); and tissue extract protein from mouse liver in e) and f) respectively.. The proteins were visualized with Coomassie

539 brilliant blue (figure 9a, c and e) and Pro-Q® Diamond phosphoprotein gel staining

540 (figure 9b, d and f), respectively.

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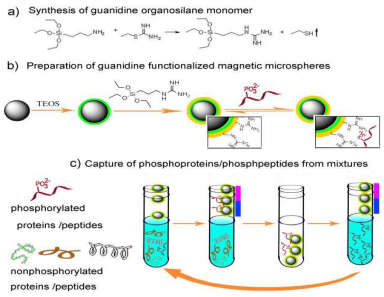
542 **TOC**

**Qiliang Deng, Jianhua Wu, Yang Chen, Zhijun Zhang, Yang Wang, Guozhen Fang, Shuo Wang*, Yukui Zhang**

Guanidinium Functionalized Superparamagnetic Silica Spheres for Selective Enrichment of Phosphopeptides and Intact Phosphoproteins from Complex Mixtures

Capture of phosphopeptides and phosphoproteins from complex mixtures has been demonstrated based on interaction between phosphate groups and guanidine groups.

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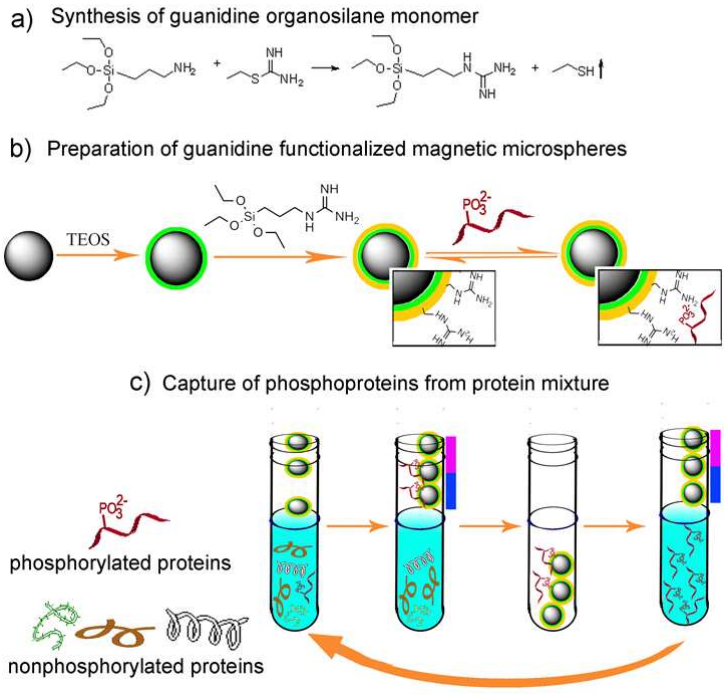
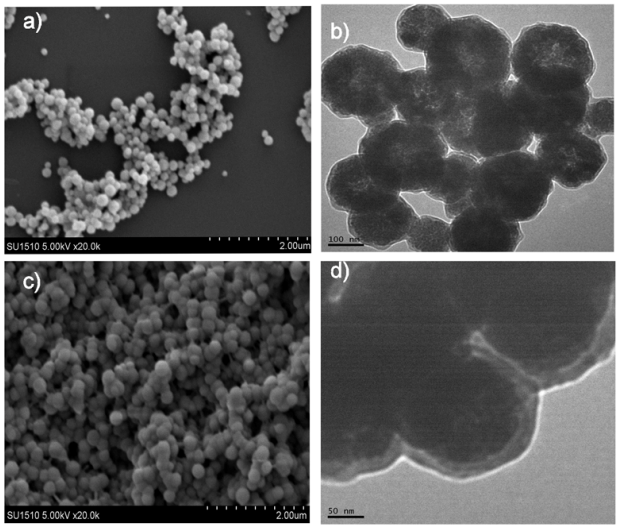



**Figure 1**

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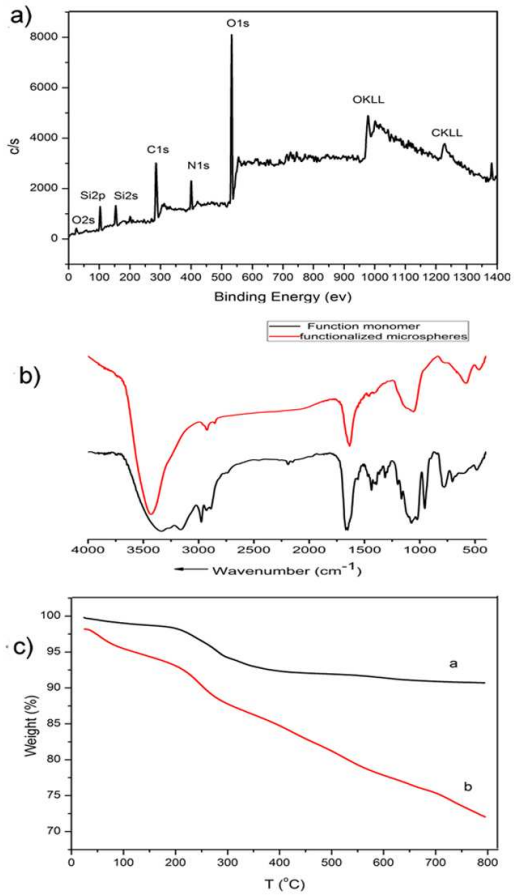


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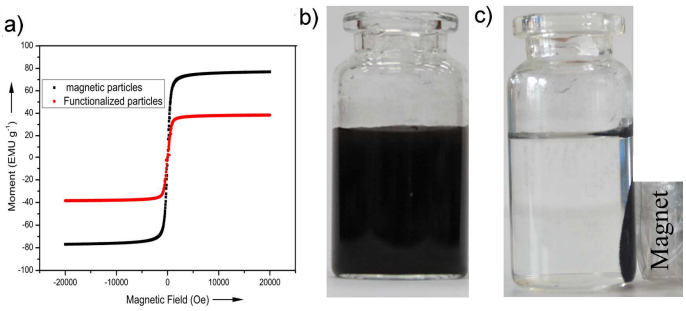


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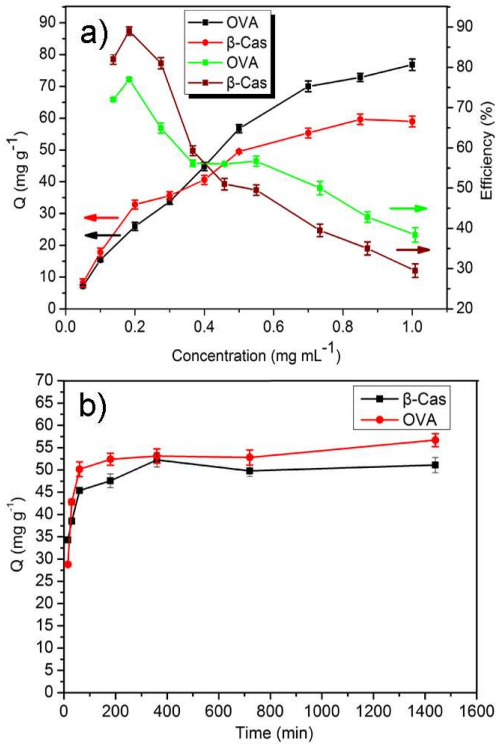


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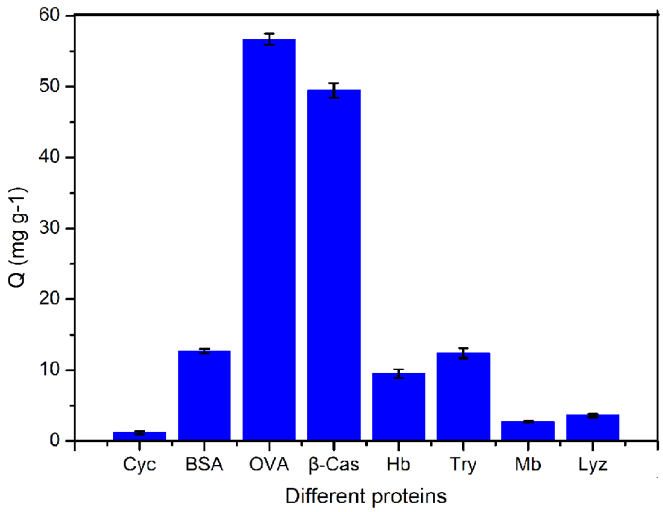


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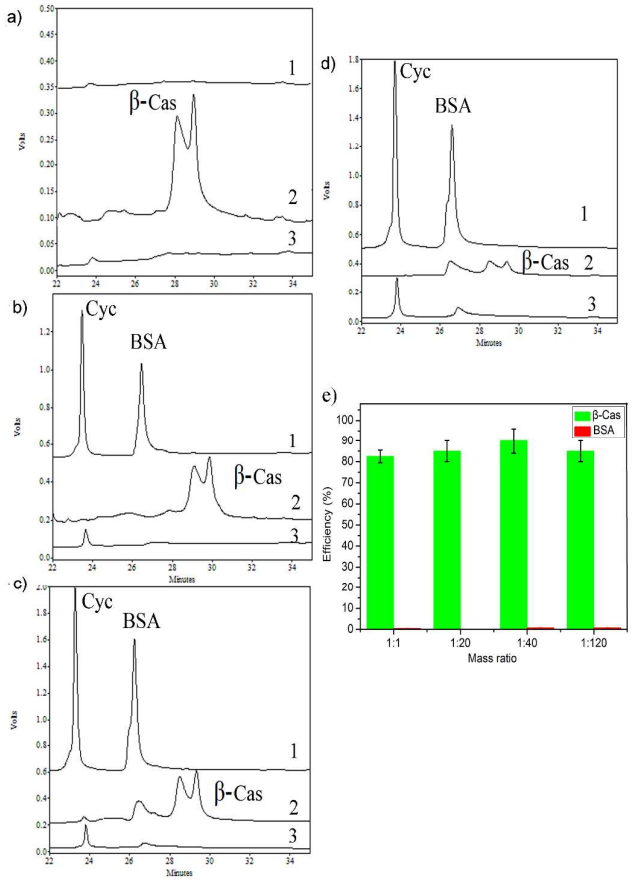


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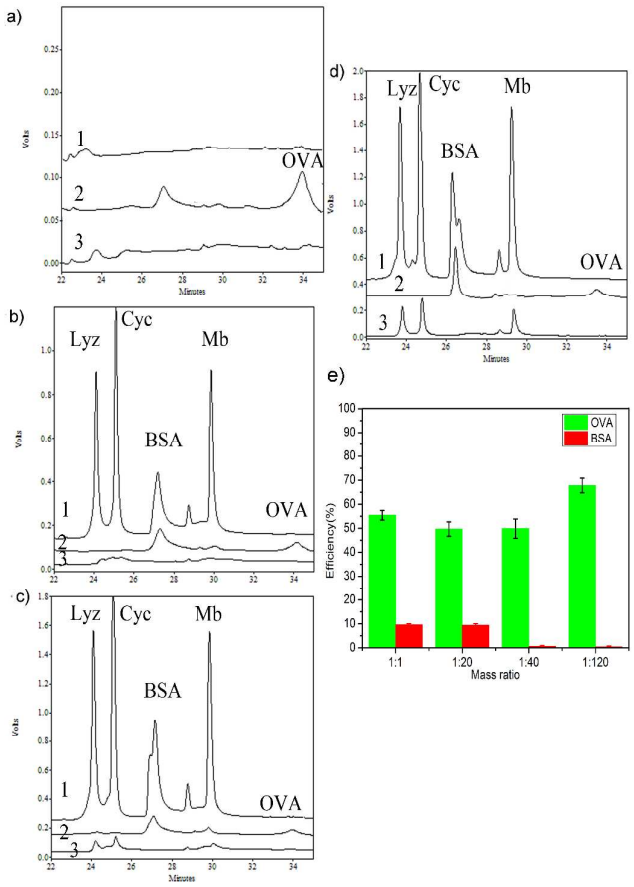


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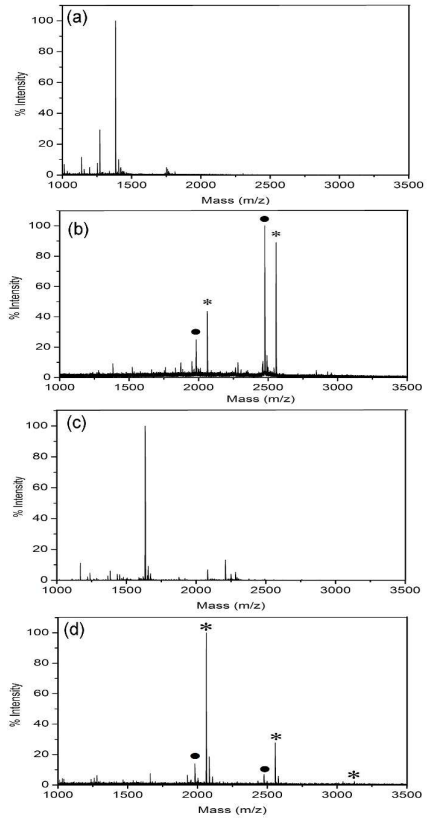


**Figure 9**

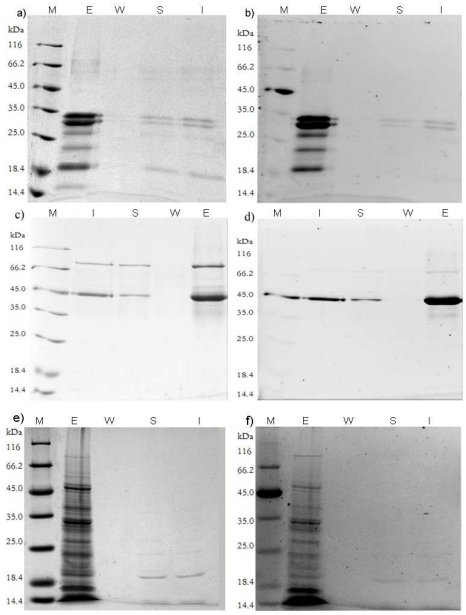


**Figure 10**