Analyst

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/analyst

Analyst

RSCPublishing

ARTICLE

Selective Enrichment of Metal-binding Proteins Based on Magnetic Core/Shell Microspheres Functionalized with Metal Cations

Caiyun Fang, Lei Zhang, Xiaoqin Zhang, and Haojie Lu*

Cite this: DOI: 10.1039/x0xx00000x

Received 00th March 2015,
Accepted 00th March 2015

DOI: 10.1039/x0xx00000x

www.rsc.org/

Metal binding proteins play many important roles in a broad range of biological processes. Characterization of metal binding proteins is important for understanding their structure and biological functions, thus leading to a clear understanding of metal associated diseases. The present study is the first to investigate the effectiveness of magnetic microspheres functionalized with metal cations (Ca^{2+}, Cu^{2+}, Zn^{2+} and Fe^{3+}) as the absorbent matrix in IMAC technology to enrich metal containing/binding proteins. The putative metal binding proteins in rat liver was then globally characterized by using this strategy which was very easy to handle and could capture a number of metal binding proteins effectively. In total, 185 putative metal binding proteins were identified from rat liver including some known lower abundant and membrane-bound metal binding proteins such as Plcg1, Acsl5, etc. The identified proteins were involved in many important processes including binding, catalytic activity, translation elongation factor activity, electron carrier activity, and so on.

1 Introduction

Metals play pivotal roles in a broad range of biological processes in all living organisms. It is estimated that approximately one-third of all proteins and enzymes require a metal cofactor for functionality (thus named ‘‘metalloproteins’’), such as copper, zinc and iron to achieve their respective catalytic, regulatory and structural roles [1-2], and many diseases are also closely associated with metal metabolism disorders. Therefore, characterization of metalloproteins is important for understanding the structure and biological functions of such proteins, thus leading to a clear understanding of metal associated diseases. To this end, a variety of analytical methods have been used to analyse metalloproteome including bioinformatic approach [3], radioactive metal detection methods [4-6], colorimetric assays [7, 8], two-dimensional diagonal sodium dodecyl sulphate-polyacrylamide gel electrophoresis [9], and a combination of ICP and MALDI-MS methods [10, 11].

Immobilized metal ion affinity chromatography (IMAC) is one of the best strategies in protein pre-fractionation in which specific protein fractions can be efficiently isolated from protein mixtures by designing particular binding ligand metal interactions [12]. Hence, a large number of IMAC strategies have been developed in the case of pre-fractionate proteins for metalloproteomic investigation by immobilizing different metal ions [13], such as Zn^{2+}, Ni^{2+}, Cu^{2+} and Co^{2+} [14-16]. By using chelating Sepharose Fast Flow resin (Amersham Biosciences), She *et al.* [17] analysed Cu and Zn metalloproteomes in three human hepatoma cell lines (Hep G2, Mz-Hep-1 and SKHep-1), and Smith *et al.* [18] identified 67 copper-binding proteins in the cytoplasmic and microsomal of Hep G2 cells. Wang *et al.* [19] carried out a systematic screen for copper-binding proteins in soybean seeds by using IMAC technology. Barnett *et al.* [20] used the metal ion-charged IMAC columns (GE Healthcare, UK) to probe the major cobalt, iron, manganese, and nickel-binding proteins in the marine cyanobacterium Synechococcus sp. Heiss *et al.* [21] identified 22 Ni-interacting proteins in human B cells by IMAC based on Ni- Nitrilotriacetate (NTA)-agarose beads (Qiagen). Ge *et al.* [22] identified seven bismuth-binding proteins from Helicobacter pylori cell extracts based on the NTA derivatized resin (Roche). Millar *et al.* [23] identified 83 copper-interacting and 74 zinc-interacting proteins from Arabidopsis thaliana by IMAC. Sun *et al.* [24] prepared the Cu- and Zn-immobilized metal affinity chromatography columns by loading immobilized iminodiacetic acid (IDA, Pierce) into poly-prep columns (Bio-Rad) and analysed 232 and 16 putative Cu- and Zn-binding proteins from Streptococcus pneumonia, in which 133 proteins were present in both preparations. In these studies, agarose/sephasore beads were the typical matrix, while Ren *et al.* [25] found that the type of support medium had a major impact on selectivity and specificity of peptide selection in IMAC strategies by examining the selectivity of four different support materials with an IDA stationary phase to histidine-containing peptides.

It is necessary, therefore, to look for a more efficient matrix to study metal-binding proteins by IMAC technology.

Nanoparticles, especially magnetic composite microspheres, have been widely used in proteomic research including the enrichment of low abundant proteins/peptides, protein phosphorylation and glycolation [26, 27] due to their high surface areas and unique magnetic responsiveness. The present study uses, for the first time, magnetic core/shell microspheres functionalized with metal cations as absorbent matrix to isolate selectively putative metal-containing or metal-binding proteins by using IMAC approach. Firstly, we used transferrin as a model protein to investigate the effectiveness of this method, and found that it could be enriched effectively by the magnetic microspheres functionalized with metal ions. Then, the putative metal-binding proteins of rat liver were analysed. In total, 185 putative metal-binding proteins were identified, which were mainly binding proteins (including many known lower abundant and membrane-bound metal ion binding proteins), proteins with catalytic activity, structural molecules, transporter, and so on.

2 Experimental

2.1 Reagents and Materials

Transferrin, ammonium bicarbonate, dithiothreitol (DTT), iodoacetamide (IAA), urea, sodium dodecyl sulfate, trizma maleate, and α-cyano-4-hydroxycinnamic acid (CHCA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN, 99.9%), methanol, trifluoroacetic acid (TFA, 99.8%) and formic acid (FA) were purchased from Merck (Darmstadt, Germany). Sequencing grade modified trypsin was from Promega (Madison, WI, USA). Bradford assay reagent was obtained from Bio-Rad (Hercules, CA, USA). Imidazole was purchased from Aladdin. Glycidyl methacrylate (GMA) was obtained from Aldrich and vacuum distilled. N, N′-Methylenebisacrylamide (MBA) was bought from Fluka and recrystallized from acetone. 2, 2-Azobisisobutyronitrile (AIBN) was purchased from Sinopharm Chemical Reagents Company and recrystallized from ethanol. Deionized water used for all experiments was obtained from a Milli-Q system (Millipore, MA, USA). All other chemicals and reagents were of analytical grade and obtained from Shanghai Chemical Reagent Company.

2.2 Preparation of Magnetic Core/Shell Microspheres Fe_3O_4/PMG (poly (N, N′-methylenebisacrylamideco- glycidyl methacrylate)) / IDA Functionalized with Metal Cations

The magnetic core/shell microspheres Fe_3O_4/PMG/IDA were prepared according to ref. [28]. Briefly, magnetic colloidal nanocrystal clusters were prepared by a modified solvothermal reaction at first. γ-methacryloxypropyltrimethoxysilane were modified to the Fe_3O_4 surface and formed abundant double bonds. Following the addition of IDA to open the epoxy ring of GMA on the surface of composite microspheres, the core/shell Fe_3O_4/PMG microspheres were synthesized via one-step distillation-precipitation polymerization of GMA in ACN, by adding MBA as cross-linker and AIBN as initiator. After being washed with H_2O, Fe_3O_4/PMG/IDA (50 mg) was incubated with 10 mL of mixture of $CaCl_2$, $CuSO_4$, $ZnCl_2$ and $FeCl_3$ solution (0.1 M) and stirred for 2 hr at room temperature (R.T.). The resultant cocktail of Fe_3O_4/PMG/IDA microspheres with different metal cations was collected, washed several times with water to remove excessive unbound metal ions and then dried in a vacuum oven at 40 °C.

2.3 Protein Extraction and Sample Preparation

Rat liver tissues were cut into small pieces and washed three times with ice-cold phosphate-buffered saline to remove blood. The tissues were dried by pledget and weighed, then homogenized on ice in lysis buffer (containing 25 mM Tris-HCl, pH8.0 and EDTA-free Protease Inhibitor Cocktail). The total lysate was centrifuged at 12 000 × g for 30 min at 4 °C and the supernatant was collected. To deplete the metal ions in proteins, the lysate was treated for 24 hr by EDTA solution with molar ratio of 1:10 at 4 °C. The protein mixtures were then dialyzed in deionized water overnight at 4 °C. To remove EDTA and metal ions, the water was changed several times and constantly stirred during dialysis. Finally, the water in sample was replaced to binding buffer in YM-3 centrifuge columns (Millipore) and the protein concentration was measured by Bradford assay using bovine serum albumin as a standard.

2.4 Selective Enrichment of Metal Containing / Binding Proteins

The microsphere cocktail of Fe_3O_4/PMG/IDA with Ca^{2+}, Cu^{2+}, Zn^{2+} and Fe^{3+} ions was first washed with lysis buffer three times. Protein mixtures in which metal ions had been removed by EDTA were then added to the magnetic microspheres together with binding buffer (25 mM Tris-HCl, pH8.0 and 5 mM imidazole). After incubating overnight at 4 °C, the protein-bound composite microspheres were separated from the remaining protein solutions by magnetic sedimentation and washed three times with binding buffer. Finally, the bound proteins were eluted from the microspheres with elution buffer (500 mM imidazole) and concentrated with YM-3 centrifuge columns. At the same time, equivalent microspheres without metal ions were used as a control in all experiments. For the model protein transferrin, the incubation was performed for 4 hrs at 4 °C and the eluate fraction was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and visualized by silver staining, while for rat liver samples, the resultant eluates were subjected to in-solution digestion as follows: the proteins were treated with 10 mM DTT at 57 °C for 30 min, alkylated with 20 mM IAA at R.T. for 1 hr in the dark and digested with trypsin at an enzyme-to-substrate ratio of 1:50 (w/w). The digestion procedure was allowed to proceed at 37 °C overnight, followed by lyophilisation. Finally, the products were analysed by LC-MS.

2.5 LC-MS Analysis and Database Search

The nano-LC MS/MS analysis was performed on a HPLC system composed of two LC-20AD nano-flow LC pumps, a SIL-20 AC auto-sampler and an LC-20AB micro-flow LC pump (Shimadzu, Tokyo, Japan) connected to a LTQ Orbitrap

mass spectrometer (Thermo Scientific, Germany). Samples were re-suspended with buffer A (5% ACN containing 0.1% FA) and injected into a CAPTRAP column (0.5 × 2 mm, MICHROM Bioresources, CA) in 4 min with a flow rate of 25 µL min-1. Subsequently, samples were separated on a Magic C18-AQ reverse phase column (100 µm id × 15 cm, Michrom Bioresources, USA) with a linear gradient from 5 to 45% buffer B (90% ACN in 1% FA) in 60 min at a flow rate of 500 nL min-1. The separated samples were introduced into the mass spectrometer via an ADVANCE 30 µm silica tip (MICHROM Bioresources, CA). The spray voltage was 1.8 kV, and capillary temperature was 180 °C. The mass spectrometer was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. Survey full-scan MS spectra with one microscan (400-1800 Da) was acquired in the Orbitrap with a mass resolution of 100,000 at m/z 400, followed by MS/MS of the eight most-intense peptide ions in the LTQ analyser. The automatic gain control (AGC) during full-MS and MS/MS was set to 1000 000 ions and 10 000, respectively. Single charge state was rejected and dynamic exclusion was used with two microscans in 10 s and 90 s exclusion duration. For MS/MS, precursor ions were activated using 35% normalized collision energy at the default activation q of 0.25 and an activation time of 30 ms.

Database search was performed using Mascot v2.3.2 (Matrix Science, UK) as previously described against a composite database, including original and reversed protein database assuming the digestion enzyme trypsin [29]; a maximum of two missed cleavages were allowed, and full tryptic specificity required. Mass value was set as monoisotopic, and peptide charges 2+ and 3+ were taken into account. All samples were searched with oxidation (M) and carbamidomethyl (C) as variable modifications. Precursor ion mass tolerance was set to 15 ppm and product ion tolerance to 1.0 Da. The Searching database contained 7858 rat protein entries extracted from UniProt Knowledgebase (Release 2013_08) with an in-house perl script. The results were further filtered by Scaffold (Proteome Software, V4.2.0). An additional database search by X! Tandem was performed with parameters similar to Mascot except that Glu->pyro-Glu, ammonia-loss and Gln->pyro-Glu of the N-terminus were specified additionally. Peptide identifications by X! Tandem were accepted with >95.0% probability assigned by the PeptideProphet algorithm [30]. Probabilities of Mascot identifications were assigned by the Scaffold Local false discovery rate algorithm. Protein identifications with >99.0% probability by the ProteinProphet algorithm [31] and ≥ 2 unique peptides were accepted. Then, label-free quantitation for proteins eluted from metal ions - IMAC (Exp) and control (Con) was carried out by Scaffold according to identified protein' total spectra count. The proteins with ratio of Exp / Con greater than 2 were defined as metal binding proteins. If a protein was not identified in the Con group, it was also defined as metal containing / binding proteins.

2.6 Bioinformatic Analysis

Functional classification and subcellular localization analysis for the identified proteins were carried out by gene ontology (GO) (http://www.geneontology.org/), uniprot (http://www.uniprot.org/uniprot) and DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/).

3 Results and discussion

3.1 Selective Enrichment of Putative Metal Containing/Binding Proteins by Magnetic Core/Shell Microspheres Functionalized with Metal Cations

To investigate the enrichment efficiency of the magnetic microspheres to metal containing/binding proteins, transferrin was used as a model protein. Transferrin is an iron- binding protein, which can bind iron very tightly, but reversibly, and contains two specific high affinity Fe (III) binding sites. At first, 5 µg of transferrin was treated by EDTA solution to remove from it the bounded metal ions, so that it could be captured more efficiently by the magnetic nanoparticles with iron ions. The enrichment strategy was illustrated in Fig. 1.

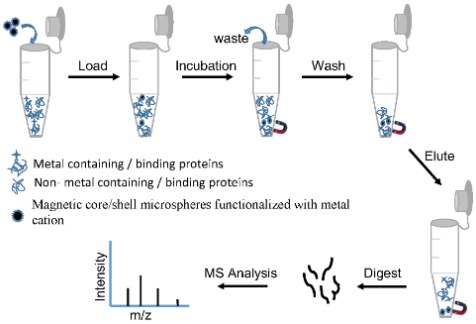


Fig.1 Flowchart of the enrichment of metal containing/binding proteins in rat liver based on magnetic core/shell microspheres functionalized with metal cations followed by LC-MS analysis.

The Fe_3O_4/PMG/IDA-Fe^{3+} microspheres was added to transferrin solutions and incubated for 4 hrs at 4 °C, before the microspheres were separated from solution by magnetic sedimentation. The proteins were eluted by 500 mM imidazole, separated by 10% SDS-PAGE and stained by silver staining. At the same time, the bare Fe_3O_4/PMG/IDA microspheres were used as control by following exactly the same procedure. As shown in Fig. 2, transferrin could be effectively captured by Fe_3O_4/PMG/IDA-Fe^{3+} microspheres (shown in the 'Exp-elute' lane), while in the control experiment, the signal was relatively very weak. Therefore, the Fe_3O_4/PMG/IDA-Fe^{3+} microspheres could be used as the IMAC matrix to enrich effectively the iron-binding proteins and very little non-specific binding was observed.

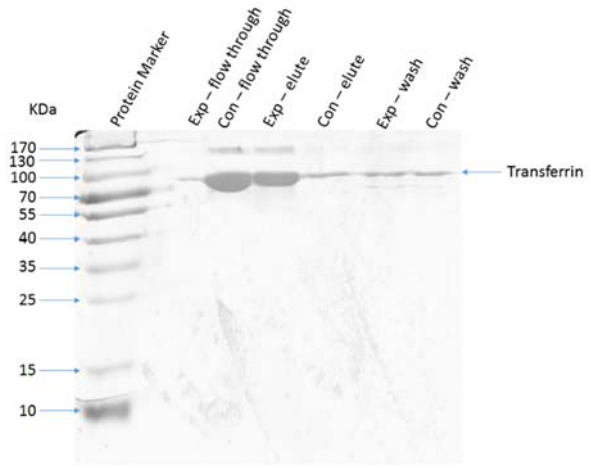


Fig.2 SDS-PAGE analysis for transferrin eluted from Fe_3O_4/PMG/IDA-Fe^{3+} microspheres (Exp: experiment group) and from bare Fe_3O_4/PMG/IDA microspheres (Con: control group). Transferrin, in which Fe^{3+} ions were delepted at first, was incubated with the magnetic microspheres and eluted with 500 mM imidazole. Then, the eluates were separated by SDS-PAGE and stained by silver staining. In Con group, transferrin existed mainly in the flow through fraction, while it was mainly in the elute fraction in Exp group.

IMAC is a promising tool for rapidly capturing proteins that are involved in metal sensing and trafficking, because the binding sites in such proteins enable facile metal exchange (i.e. binding and release) and are often located on the protein surface [2]. Compared with the regular matrix (such as agarose and sepharose) in the study of metal binding proteins by IMAC technology, nanoparticles have prominent features of high surface area, a large number of functional groups and well-defined particle sizes. Thus, it has larger adsorption capacity and a much lower limit of detection towards metal containing/binding proteins. In addition, it can make the enrichment strategy very convenient and easy to handle by using an external magnetic field, which makes composite microspheres fascinating and promising for high throughput metal binding/containing protein research.

3.2 High-throughput Profiling of Putative Metal-Binding Proteins in Rat Liver

The liver has a wide range of functions including detoxification, protein synthesis, metabolism, etc., so hepatocytes are ideal for study of metal containing/binding proteins. We carried out the putative metal-binding proteins of rat liver by IMAC based on the magnetic core/shell microsphere cocktail functionalized with Ca^{2+}, Cu^{2+}, Zn^{2+} and Fe^{3+} ions. Metal binding proteins and metalloproteins are sensitive to various experimental conditions such as the pH and constitution of lysis buffer, and the changes in their tertiary structure often result in disturbance of the buried metal binding sites. The proteins were extracted in a state of physiological conformation by using a non-denaturing lysis buffer in our study. Additionally, to release the metal binding sites already occupied by metal ions and ensure proteins' binding capacity, the extracted proteins were firstly treated with EDTA and incubated with the cocktail of microspheres functionalized with metal cations (Ca^{2+}, Cu^{2+}, Zn^{2+} and Fe^{3+}). The eluted proteins were digested with trypsin and then subjected to LC-MS analysis.

In total, 295 proteins were identified ambiguously (Shown in Supplementary Table), including many known metal binding proteins. After analysing with label-free quantitation, 185 proteins (Table 1) were designated putative metal binding proteins from rat liver in which 85 proteins had been reported as metal binding proteins by GO, BIND and other proteomic studies [17, 18, 32]. The subcellular localization and molecular function of identified proteins were annotated by gene ontology. As shown in Fig. 3 (A), the candidates were mainly localized in cytoplasm, nucleus, mitochondrial, plasma membrane, and so on. Actually, the remaining 110 proteins, which the ratio of Exp / Con was less than 2 by label-free relative quantitation, also included 24 known metal binding proteins reported in UniProt database, such as Cytochrome P450 protein family, Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing), and so on. Maybe the unoptimized binding/elution conditions or cut-off criterion for label free quantatition caused that these proteins were detected only at low significance levels. These proteins were also listed in Table 2.

It was reported that transition metal ions were required for many aspects of mitochondrial physiology, in which copper, iron and zinc were cofactors in metalloenzymes and metalloproteins [33, 34]. In this study a range of known metal binding proteins in the mitochondrial electron transport chain, metabolism and proteolysis machinery were identified. For example, carbamoyl-phosphate synthase [ammonia], mitochondrial, which is a known calcium ion binding protein and involved in the urea cycle of ureotelic animals where the enzyme plays an important role in removing excess ammonia from the cell, was identified ambiguously. In particular, several known metal binding proteins in mitochondrion inner membrane were also detected. Cytochrome c oxidase subunit 5A mitochondrial, which contains heme moiety and can bind iron ions, lies in the mitochondrion inner membrane and is the terminal oxidase in mitochondrial electron transport. ATP synthase subunit beta and NADH dehydrogenase [ubiquinone] flavoprotein 2, which are known calcium ion binding and iron binding protein respectively, were also ambiguously identified in our experiment.

Interestingly, 24 ribosomal proteins were identified in our experiment. Among which, three proteins from 40S subunit and 10 from 60S subunit were firstly identified by proteomic techniques as putative metal-binding proteins in rat liver, which indicates that the ribosome may act as a metal ion storage site. Ribosomes are larger ribonucleoprotein complexes that mediate protein synthesis in all organisms. Zinc plays key catalytic roles in enzymes, as well as a structural role in numerous transcriptional activators and regulators. It was reported that the majority of intracellular Zn(II) reside in the ribosome. Escherichia coli 70S ribosomes tightly bound 8 equiv of Zn [35] and ribosomes from Bacillus subtilis closely bound 2.5 equiv of Zn [36]. Actually, the high percentage of ribosomal proteins

was also identified in Streptococcus pneumonia by proteomic method [24].

Notably, several membrane proteins and lower abundant proteins were identified in our study. For example, 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1 (Plcg1) was ambiguously identified, which is a known calcium binding protein and plays an important role in the regulation of intracellular signalling cascades, actin reorganization and cell migration. According to the results of protein abundance analysis (http://pax-db.org), Plcg1's abundance is 2.25 ppm (ranked bottom 25% of the rat dataset). Long-chain-fatty-acid--CoA ligase 5 (Acsl5) is another known metal binding protein and located in mitochondrion outer membrane. Acsl5 is involved in lipid metabolism and has transmembrane domain. Its abundance is 96.6 ppm (ranked top 25%) and belongs to the medium/high abundant protein. Therefore, this strategy described here for the study of metal binding proteins could be used to isolate lower abundance and membrane-bound proteins to some extent, which was probably due to the following reasons: firstly, the absorbent carriers used in this method were nanoparticles with high surface area and a large number of functional moieties, leading to a higher binding capacity and much lower limit of detection towards metal binding proteins; secondly, the metal cofactor in the metal binding proteins had been delepted by EDTA before the enrichment procedure, so the putative metal binding sites in these proteins could interact with the absorbent materials of IMAC easily.

The identified proteins were involved in multiple biochemical processes such as amino acid and protein biosynthesis, metabolism and redox homeostasis. According to their molecular functions, the identified proteins were mainly binding proteins, proteins with catalytic activity, structural molecule, transporter, proteins with electron carrier activity, etc. As shown in Fig 3 (B), 152 proteins were binding proteins among the identified proteins and the largest class. Several kinds of binding proteins were profiled including many known metal binding proteins, protein binding proteins, nucleic acid binding proteins and drug binding proteins. There were 48 known metal binding proteins reported by Uniprot, GO and BIND. For example, Parathymosin, which can mediate immune function, is a known zinc ion binding protein. Serum albumin can also bind a variety of essential and toxic metal ions, including Ca(II), Co(II), Ni(II), Cu(II), Zn(II) and Cd(II) [37]. It is believed to be the major zinc transporter in plasma [38] and has an excellent binding site for Cu(II) or Ni(II) at the N-terminus (N-terminal copper and nickel-binding motif, ATCUN) [39].

One of the most important roles of metal species in biological organisms is acting as cofactors of diverse enzymes. In this study, 113 identified proteins had catalytic activity, mainly including oxidoreductase activity, transferase activity, hydrolase activity, dehydrogenase activity, GTPase activity and kinase activity. Peroxiredoxins, a class of thioredoxin-dependent peroxide reductase, are multifunctional thiol-specific antioxidant proteins that are highly abundant in the cytoplasm of mammalian cells where they regulate antioxidant defence, tumour suppression and control hydrogen peroxide signalling. Here, three members of peroxiredoxins (peroxiredoxin-1, 2 and 6) were identified, which were reported as metal binding proteins in UniProt database and previous proteomic literatures [17, 18, 32]. Protein disulfide-isomerase can catalyse the formation, breakage and rearrangement of disulfide bonds; four members of this protein (protein disulfide-isomerase, protein disulfide-isomerase A3, A4 and A6) were ambiguously identified in our study.

However, it should be noted that cytosolic proteins were still the largest group in our dataset such as cytoplasmic aconitate hydratase, isocitrate dehydrogenase [NADP] cytoplasmic and superoxide dismutase [Cu-Zn], which were known metal binding proteins. In addition, according to the results of protein abundance analysis by http://pax-db.org, the identified proteins were mainly high abundance proteins (ranked from above 25% to above 5%). It was mainly because: 1) we used the moderate lysis buffer at almost physiological environment without detergents such as urea and SDS to keep the proteins in a state of physiological conformation and can still bind metal ions; but in such buffer conditions, it was usually ideal for the identification of higher abundant and more hydrophilic proteins; 2) the metal-binding sites in many proteins, for example metalloenzymes, are often deeply buried in the interior of the protein, so they were not accessible for the EDTA treatment and interacting with the immobilized metal ions; 3) the wide dynamic range of relative protein expression also made it difficult to detect those proteins expressed at low levels. Thus, combined with the enrichment strategy, improvement in techniques for isolation of low abundant proteins and more hydrophobic proteins may further enhance the inclusiveness of putative metal-binding proteins. Furthermore, it should be mentioned that this method can only study those proteins with metal binding capacity in vitro and can't provide information on in vivo metal-protein interactions exactly, which is also the common limition of available IMAC methods. So further experimental evidence should be required to confirm whether a protein is a genuine metal binding protein in vivo or not.

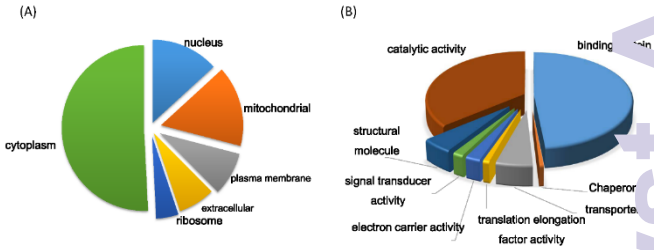


Fig.3 Results of large-scale protein annotation through Gene ontology. (A) protein subcellular localization analysis; B) protei molecular and functional analysis.

4 Conclusions

In summary, we first tested the effectiveness of magnetic core/shell microspheres functionalized with metal ions (Ca^{2+},

Cu^{2+}, Zn^{2+} and Fe^{3+}) to enrich metal containing/binding proteins, and then globally characterized the putative metal-binding proteins of rat liver by using this strategy. It was very easy to handle and capture a number of putative metal binding proteins effectively, although only those proteins with metal binding capacity in vitro can be studied by this method. In total, 185 proteins were identified as putative metal binding proteins from rat liver including some known lower abundant and membrane-bound metal binding proteins such as Plcg1, Acsl5, etc. The identified proteins were involved in many important processes including binding, catalytic activity, translation elongation factor activity and electron carrier activity. Therefore, our results indicated that the magnetic microspheres with metal ions was an ideal absorbent matrix in IMAC technology to capture proteins with metal binding capacity in vitro, and the enhanced selective recognition for putative metal-binding proteins would facilitate a complementary identification of the under-explored metal binding proteins and provide new insight into the dynamics of metal binding proteins. In parallel, it is also a relatively flexible method and can be used to study both the specific putative metal-binding proteins and its binding peptides. If a specific kind of metal ions, e.g. zinc cations, were immobilized onto the microspheres, the zinc-binding proteins could be effectively captured in vitro. If protein mixture digests were incubated with the microspheres, the zinc binding peptides could be analyzed.

Acknowledgements

The work was supported by NST (2012CB910103), NSF (21205018 and 21025519), Shanghai Pujiang Program (No. 13PJD003), and the Project-sponsored by SRF for ROCS, SEM. The authors have declared no conflict of interest.

Notes and references

Department of Chemistry and Institutes of Biomedical Sciences, Fudan University, Shanghai 200433, China.
* Corresponding author: Lu Haojie, Email: luhaojie@fudan.edu.cn.

1 J. P. Barnett, D. J. Scanlan and C. A. Blindauer, *Anal. Bioanal. Chem.* 2012, **402**, 3311-3322.
2 K. J. Waldron, J. C. Rutherford, D. Ford and N. J. Robinson, *Nature* 2009, **460**, 823-830.
3 C. Andreini, I. Bertini and A. Rosato, *Acc. Chem. Res.* 2009, **42**, 1471-1479.
4 A. Katayama, A. Tsujii, A. Wada, T. Nishino and A. Ishihama, *Eur. J. Biochem.* 2002, **269**, 2403-2413.
5 A. M. Sevcenco, G. C. Krijger, M. W. Pinkse, P. D. Verhaert, W. R. Hagen and P. L. Hagedoorn, *J. Biol. Inorg. Chem.* 2009, **14**, 631-640.
6 A. M. Sevcenco, W. R. Hagen and P. L. Hagedoorn, *Chem. Biodivers*, 2012, **9**, 1967-1980.
7 M. Ferrer, O. V. Golyshina, A. Beloqui, P. N. Golyshin, K. N. Timmis, *Nature* 2007, **445**, 91-94.
8 M. Högbom, U. B. Ericsson, R. Lam, H. M. Bakali, E. Kuznetsova, P. Nordlund, D. B. Zamble, *Mol. Cell. Proteomics* 2005, **4**, 827-834.
9 V. L. Herald, J. L. Heazlewood, D. A. Day and A. H. Millar, *FEBS Letters* 2003, **537**, 96-100.
10 S. B. Jayasinghe and J. A. Caruso, *Int. J. Mass Spectrom.* 2013, **3**[illegible] 33-36.
11 S. B. Jayasinghe and J. A. Caruso, *Int. J. Mass Spectrom.* 2011, **30**[illegible] 16-27.
12 R. C. Cheung, J. H. Wong and T. B. Ng, *Appl. Microbiol. Biotechnol.* 2012, **96**, 1411-1420.
13 W. Shi and M. R. Chance, Cell. *Mol. Life Sci.* 2008, **65**, 3040-3048
14 P. Jungblut, H. Baumeister and J. Klose, *Electrophoresis* 1993, **1**[illegible] 638-643.
15 X. Sun, R. Ge, J. F. Chiu, H. Sun and Q. Y. He, *Met. Based Drugs* 2008, **2008**, 289490.
16 E. Zatloukalová and Z. Kucerová, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2004, **808**, 99-103.
17 Y. M. She, S. Narindrasorasak, S. Y. Yang, N. Spitale, E. A. Robe[illegible]s and B. Sarkar, *Mol. Cell. Proteomics* 2003, **2**, 1306-1318.
18 S. D. Smith, Y. M. She, E. A. Roberts and B. Sarkar, *J. Proteome R*[illegible] 2004, **3**, 834-840.
19 Y. Wang, H. Li, Y. F. Qiu, N. Li, W. H. Sun, Z. H. Shan, *Plant Biosystems* 2014, **148**, 88-95.
20 J. P. Barnett, D. J. Scanlan, C. A. Blindauer, *Anal. Bioanal. C*[illegible] 2012, **402**, 3371-3377.
21 K. Heiss, C. Junkes, N. Guerreiro, M. Swamy, M. M. Camacho-Carvajal, W. W. Schamel, I. D. Haidl, D. Wild, H. U. Weltzien, H. J. Thierse, *Proteomics* 2005, **5**, 3614-3622.
22 R. Ge, X. Sun, Q. Gu, R. M. Watt, J. A. Tanner, B. C. Wong, H. [illegible] Xia, J. D. Huang, Q. Y. He, H. Sun, *J. Biol. Inorg. Chem.* 2007, **12**[illegible] 831-842.
23 A. H. Millar, Y. F. Tan, N. O'Toole and N. L. Taylor, *Plant Physiol.* 2010, **152**, 747-761.
24 X. S. Sun, C. L. Xiao, R. G. Ge, X. F. Yin, H. Li, N. Li, X. Y. Yang, Y. Zhu, X. He and Q. Y. He, *Proteomics* 2011, **11**, 3288-3298.
25 D. Ren, N. A. Penner, B. E. Slentz, H. D. Inerowicz, M. Rybalko and F. E. Regnier, *J. Chromatogr. A* 2004, **1031**, 87-92.
26 W. F. Ma, Y. Zhang, L. L. Li, Y. T. Zhang, M. Yu, J. Guo, H. J. Lu and C. C. Wang, *Adv Funct Mater.* 2013, **23**, 107-115.
27 Y. Zhang, M. Kuang, L. J. Zhang, P. Y. Yang, H. J. Lu, *Anal. Chem.* 2013, **85**, 5535-5541.
28 Y. T. Zhang, Y. K. Yang, W. F. Ma, J. Guo, Y. Lin and C. C. W[illegible] *ACS Appl. Mater. Interfaces* 2013, **5**, 2626-2633.
29 S. J. Cui, L. L. Xu, T. Zhang, M. Xu, J. Yao, C. Y. Fang, Z. Feng, P. Y. Yang, W. Hu and F. Liu, *J. Proteomics* 2013, **84**, 158-175.
30 A. Keller, A. I. Nesvizhskii, E. Kolker, R. Aebersold, *Anal. Chem.* 2002, **74**, 5383-5392.
31 A. I. Nesvizhskii, A. Keller, E. Kolker and R. Aebersold, *Anal. Chem.* 2003, **75**, 4646-4658.
32 P. P. Kulkarni, Y. M. She, S. D. Smith, E. A. Roberts and B. Sarkar, *Chem. Eur. J.* 2006, **12**, 2410-2422.
33 F. Pierrel, P. A. Cobine and D. R. Winge, *BioMetals* 2007, **20**, 67[illegible] 682.
34 R. Lill, and G. Kispal, *Res. Microbiol.* 2001, **152**, 331-340.
35 M.P. Hensley, D.L. Tierney, and M.W. Crowder, *Biochemistry* 2011, **50**, 9937-9939.

36 S.E. Gabriel, and J.D. Helmann, *J. Bacteriol.* 2009, **191**, 6116-6122.
37 J. Lu, A.J. Stewart, P.J. Sadler, T.J.T. Pinheiro, and C.A. Blindauer. *Biochem. Soc. Trans.* 2008, **36**, 1317-1321.
38 R.J. Cousins, *Clin. Physiol. Biochem.* 1986, **4**, 20-30.
39 C. Harford, and B. Sarkar, *Acc. Chem. Res.* 1997, **30**, 123-130.

Table 1. Identification of metal-binding proteins in rat liver

No	Accession Number	Protein Name	No	Accession Number	Protein Name
1	P62260	14-3-3 protein epsilon	94	P08010	Glutathione S-transferase Mu 2
2	P68255	14-3-3 protein theta	95	O35077	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic
3	P63102	14-3-3 protein zeta/delta	96	Q5I0P2	Glycine cleavage system H protein, mitochondrial
4	P11915	Non-specific lipid-transfer protein	97	P13255	Glycine N-methyltransferase
5	P10686	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1	98	P09811	Glycogen phosphorylase, liver form
6	P23457	3-alpha-hydroxysteroid dehydrogenase	99	P23785	Granulins
7	P46953	3-hydroxyanthranilate 3,4-dioxygenase	100	Q5HZY2	GTP-binding protein SAR1b
8	P97532	3-mercaptopyruvate sulfurtransferase	101	P06866	Haptoglobin
9	P24008	3-oxo-5-alpha-steroid 4-dehydrogenase 1	102	P55063	Heat shock 70 kDa protein 1-like
10	P63324	40S ribosomal protein S12	103	P63018	Heat shock cognate 71 kDa protein
11	P62703	40S ribosomal protein S4, X isoform	104	P82995	Heat shock protein HSP 90-alpha
12	P38983	40S ribosomal protein SA	105	P34058	Heat shock protein HSP 90-beta
13	P50554	4-aminobutyrate aminotransferase, mitochondrial	106	P01946	Hemoglobin subunit alpha-1/2
14	P32755	4-hydroxyphenylpyruvate dioxygenase	107	P02091	Hemoglobin subunit beta-1
15	Q9JLJ3	4-trimethylaminobutyraldehyde dehydrogenase	108	Q794E4	Heterogeneous nuclear ribonucleoprotein F
16	P19945	60S acidic ribosomal protein P0	109	P61980	Heterogeneous nuclear ribonucleoprotein K
17	P19944	60S acidic ribosomal protein P1	110	A9UMV8	Histone H2A.J
18	P02401	60S acidic ribosomal protein P2	111	Q00715	Histone H2B type 1
19	P23358	60S ribosomal protein L12	112	P84245	Histone H3.3
20	P84100	60S ribosomal protein L19	113	P62804	Histone H4
21	P62890	60S ribosomal protein L30	114	P22791	Hydroxymethylglutaryl-CoA synthase, mitochondrial
22	P62902	60S ribosomal protein L31	115	P27605	Hypoxanthine-guanine phosphoribosyltransferase
23	P50878	60S ribosomal protein L4	116	Q64632	Integrin beta-4
24	P21533	60S ribosomal protein L6	117	Q10758	Keratin, type II cytoskeletal 8
25	P05426	60S ribosomal protein L7	118	Q5M875	17-beta-hydroxysteroid dehydrogenase 13
26	P85968	6-phosphogluconate dehydrogenase, decarboxylating	119	P41562	Isocitrate dehydrogenase [NAD], cytoplasmic
27	P06761	78 kDa glucose-regulated protein	120	Q64573	Liver carboxylesterase 4
28	P49911	Acidic leucine-rich nuclear phosphoprotein 32 family member A	121	Q5SGE0	Leucine-rich PPR motif-containing protein, mitochondrial
29	P60711	Actin, cytoplasmic 1	122	P04642	L-lactate dehydrogenase A chain
30	Q499N5	Acyl-CoA synthetase family member 2, mitochondrial	123	P18163	Long-chain-fatty-acid--CoA ligase 1
31	Q64640	Adenosine kinase	124	O88813	Long-chain-fatty-acid--CoA ligase 5
32	P29410	Adenylate kinase 2, mitochondrial	125	Q920P0	L-xylulose reductase
33	P13601	Aldehyde dehydrogenase, cytosolic 1	126	P30904	Macrophage migration inhibitory factor
34	P11884	Aldehyde dehydrogenase, mitochondrial	127	P70580	Membrane-associated progesterone receptor component 1
35	P24090	Alpha-2-HS-glycoprotein	128	P57113	Maleylacetoacetate isomerase
36	Q9Z1P2	Alpha-actinin-1	129	O88989	Malate dehydrogenase, cytoplasmic
37	Q64057	Alpha-aminoadipic semialdehyde dehydrogenase	130	Q02253	Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial
38	P04764	Alpha-enolase	131	P08011	Microsomal glutathione S-transferase 1
39	P70473	Alpha-methylacyl-CoA racemase	132	Q03626	Murinoglobulin-1
40	P21396	Amine oxidase [flavin-containing] A	133	Q64119	Myosin light polypeptide 6
41	P02650	Apolipoprotein E	134	P13832	Myosin regulatory light chain RLC-A
42	P07824	Arginase-1	135	P04182	Ornithine aminotransferase, mitochondrial

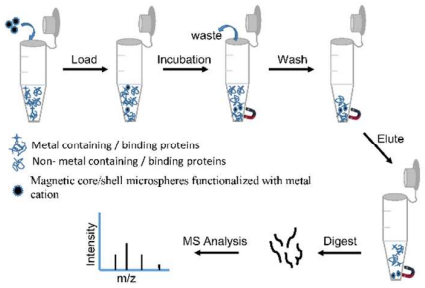
43	Q9QZH8	Arylacetamide deacetylase	136	Q8CGU6	Nicastrin
44	P15999	ATP synthase subunit alpha, mitochondrial	137	P19234	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial
45	P19511	ATP synthase subunit b, mitochondrial	138	Q63083	Nucleobindin-1
46	P10719	ATP synthase subunit beta, mitochondrial	139	Q63276	Bile acid-CoA:amino acid N-acyltransferase
47	P31399	ATP synthase subunit d, mitochondrial	140	P19804	Nucleoside diphosphate kinase B
48	P35434	ATP synthase subunit delta, mitochondrial	141	P07756	Carbamoyl-phosphate synthase [ammonia], mitochondrial
49	P35435	ATP synthase subunit gamma, mitochondrial	142	P00481	Ornithine carbamoyltransferase, mitochondrial
50	Q06647	ATP synthase subunit O, mitochondrial	143	O09171	Betaine--homocysteine S-methyltransferase 1
51	P10111	Peptidyl-prolyl cis-trans isomerase A	144	P04550	Parathymosin
52	Q03248	Beta-ureidopropionase	145	Q63716	Peroxiredoxin-1
53	Q5I0J9	Putative L-aspartate dehydrogenase	146	P35704	Peroxiredoxin-2
54	Q9ES38	Bile acyl-CoA synthetase	147	O35244	Peroxiredoxin-6
55	P62161	Calmodulin	148	P04176	Phenylalanine-4-hydroxylase
56	P97571	Calpain-1 catalytic subunit	149	P31044	Phosphatidylethanolamine-binding protein 1
57	P18418	Calreticulin	150	Q9ES21	Phosphatidylinositide phosphatase SAC1
58	P13383	Nucleolin	151	Q8CFN2	Cell division control protein 42 homolog
59	P04762	Catalase	152	P11598	Protein disulfide-isomerase A3
60	P22734	Catechol O-methyltransferase	153	P38659	Protein disulfide-isomerase A4
61	P04785	Protein disulfide-isomerase	154	Q63081	Protein disulfide-isomerase A6
62	Q6UPE0	Choline dehydrogenase, mitochondrial	155	P61459	Pterin-4-alpha-carbinolamine dehydratase
63	P00173	Cytochrome b5	156	P85973	Purine nucleoside phosphorylase
64	P11240	Cytochrome c oxidase subunit 5A, mitochondrial	157	P12007	Isovaleryl-CoA dehydrogenase, mitochondrial
65	P10633	Cytochrome P450 2D1	158	Q03336	Regucalcin
66	Q63270	Cytoplasmic aconitate hydratase	159	P50169	Retinol dehydrogenase 3
67	Q68FS4	Cytosol aminopeptidase	160	P02696	Retinol-binding protein 1
68	P55159	Serum paraoxonase/arylesterase 1	161	Q64380	Sarcosine dehydrogenase, mitochondrial
69	P16086	Spectrin alpha chain, non-erythrocytic 1	162	Q64428	Trifunctional enzyme subunit alpha, mitochondrial
70	P80254	D-dopachrome decarboxylase	163	P02770	Serum albumin
71	P0C2X9	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	164	P28037	Cytosolic 10-formyltetrahydrofolate dehydrogenase
72	P06214	Delta-aminolevulinic acid dehydratase	165	Q68FP2	Serum paraoxonase/lactonase 3
73	Q6P6R2	Dihydrolipoyl dehydrogenase, mitochondrial	166	O35412	Signal-induced proliferation-associated 1-like protein 1
74	Q63150	Dihydropyrimidinase	167	P48721	Stress-70 protein, mitochondrial
75	Q5RKL4	Dimethylglycine dehydrogenase	168	P27867	Sorbitol dehydrogenase
76	P29147	D-beta-hydroxybutyrate dehydrogenase, mitochondrial	169	Q641Y0	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit
77	P25235	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2	170	Q68FT5	S-methylmethionine--homocysteine S-methyltransferase BHMT2
78	P62630	Elongation factor 1-alpha 1	171	Q3T1J1	Eukaryotic translation initiation factor 5A-1
79	Q68FR6	Elongation factor 1-gamma	172	P10960	Sulfated glycoprotein 1
80	P14604	Enoyl-CoA hydratase, mitochondrial	173	P17988	Sulfotransferase 1A1
81	P07687	Epoxide hydrolase 1	174	P07632	Superoxide dismutase [Cu-Zn]
82	Q4KM77	Etoposide-induced protein 2.4 homolog	175	Q920L2	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial
83	Q68FQ0	T-complex protein 1 subunit epsilon	176	P11232	Thioredoxin
84	P05369	Farnesyl pyrophosphate synthase	177	P46462	Transitional endoplasmic reticulum ATPase
85	P45953	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial	178	P32089	Tricarboxylate transport protein, mitochondrial
86	P02692	Fatty acid-binding protein, liver	179	Q8VIF7	Selenium-binding protein 1
87	P30839	Fatty aldehyde dehydrogenase	180	P48500	Triosephosphate isomerase
88	P97612	Fatty-acid amide hydrolase 1	181	Q63610	Tropomyosin alpha-3 chain
89	P00884	Fructose-bisphosphate aldolase B	182	Q6P9T8	Tubulin beta-4B chain
90	P25093	Fumarylacetoacetase	183	O70199	UDP-glucose 6-dehydrogenase
91	P09606	Glutamine synthetase	184	P36511	UDP-glucuronosyltransferase 2B15
92	P04041	Glutathione peroxidase 1	185	P55053	Fatty acid-binding protein, epidermal
93	P00502	Glutathione S-transferase alpha-1			

Analyst RSCPublishing

ARTICLE

Table 2. Summary of known metal binding proteins detected at low significance levels

Accession No.	Protein Description	Accession No.	Protein Description
P06757	Alcohol dehydrogenase 1	P05179	Cytochrome P450 2C7
P12711	Alcohol dehydrogenase class-3	P12939	Cytochrome P450 2D10
P16638	ATP-citrate synthase	P33274	Cytochrome P450 4F1
Q4KLZ6	Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing)	Q6UPE1	Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial
O35826	Bifunctional UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase	P12785	Fatty acid synthase
P15709	Bile salt sulfotransferase	P04905	Glutathione S-transferase Mu 1
P14141	Carbonic anhydrase 3	P56574	Isocitrate dehydrogenase [NADP], mitochondrial
Q68FY0	Cytochrome b-c1 complex subunit 1, mitochondrial	Q62902	Protein ERGIC-53
P04799	Cytochrome P450 1A2	P52873	Pyruvate carboxylase, mitochondrial
P11711	Cytochrome P450 2A1	P12928	Pyruvate kinase PKLR
P11510	Cytochrome P450 2C12, female-specific	P13444	S-adenosylmethionine synthase isoform type-1
P05178	Cytochrome P450 2C6	P50137	Transketolase



An new method based on magnetic microspheres with metal ions was developed and applied to characterize putative metal binding proteins.