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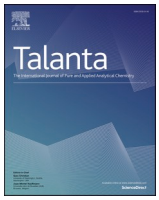

# High-efficient depletion and separation of histidine-rich proteins via $Cu^{2+}$-chelated porous polymer microspheres

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ABSTRACT

Depletion and separation of histidine-rich proteins from complicated biosamples are crucial for various downstream applications in proteome research and clinical diagnosis. Herein, porous polymer microspheres coated with polyacrylic acid (SPSDVB-PAA) were fabricated through double emulsion interfacial polymerization technique and followed by immobilization of $Cu^{2+}$ ions on the surface of SPSDVB-PAA. The as-prepared SPSDVB-PAA-Cu with uniform size and nanoscale pore structure enabled coordination interaction of $Cu^{2+}$ with histidine residues in his-rich proteins, resulting in high-performance adsorption. As metal affinity adsorbent, the SPSDVB-PAA-Cu exhibited favorable selectivity for adsorbing hemoglobin (Hb) and human serum albumin (HSA) with the maximum adsorption capacities of 152.2 and 100.7 mg $g^{-1}$. Furthermore, the polymer microspheres were used to isolate histidine-rich proteins from human whole blood and plasma, underscoring their effectiveness. The liquid chromatography tandem mass spectrometry (LC-MS/MS) results indicated that the content of 14 most abundant proteins in human plasma was depleted from 81.6 % to 30.7 % and low-abundance proteins were enriched from 18.4 % to 69.3 % after treatment with SPSDVB-PAA-Cu, illustrating potential application of SPSDVB-PAA-Cu in proteomic research.

## 1. Introduction

Histidine-rich (his-rich) proteins are a unique class of biomolecules which are closely associated with various biological activities [1]. For example, aberrant changes in the levels of his-rich proteins could cause some diseases, e.g., thrombotic disorders, pulmonary disorders, asthma, renal disease and advanced liver cirrhosis [2,3]. Within the category of these his-rich proteins, hemoglobin (Hb) and human serum albumin (HSA) are particularly prevalent in human blood and plasma which are recognized as high-abundance proteins. The detection of low-abundance protein biomarkers in complex plasma samples is challenging for analytical instruments due to interference caused by proteins of high abundance [4–6]. Consequently, selectively separate and deplete such high-abundance proteins are of great significance for proteomics analysis and identification of disease biomarkers [7].

Up to now, immobilized metal affinity chromatography has been developed as a valid method for selectively separating his-rich proteins from complicated samples, relying on the strong metal affinity interaction between transition metal ions and histidine residues in proteins [8–10]. Among them, copper ions ($Cu^{2+}$) exhibit the strongest affinity for his-rich proteins compared to other transition metal ions, such as $Ni^{2+}$, $Co^{2+}$, $Fe^{3+}$. This can be attributed to $Cu^{2+}$ not only specifically interacting with histidine sites but also binding to the peptide C-terminus [11–14]. Typically, nitrilotriacetic acid (NTA) and iminodiacetic acid (IDA) are used as chelating agent to fix metal ions on the surface of a matrix [15,16]. However, introduction of IDA and NTA leads to drawbacks such as tedious preparation process, time-consuming surface modification and relatively weak interaction [17,18]. In this context, development of immobilized metal affinity adsorbent with low-cost and high-performance for isolation of his-rich proteins is in demand.

Polymer microspheres, as innovative functional materials, have attracted widespread attention in biomedical and chemical engineering

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fields due to their exceptional resistance to acids and alkalis, adjustable size and superior mechanical properties [19–21]. Polymer microspheres with porous structure are broadly employed in biomolecule separation on account of the advantages like easy operation, large specific surface area and available internal space [22–24]. For example, hierarchical protein imprinting polymers have been prepared for specific recognition of immunoglobulin G and human serum albumin [25]. Block copolymer microspheres and membranes, equipped with nanopores, have been fabricated to isolate proteins of similar sizes [26,27]. It is an effective approach to modify functional groups on the interface of microspheres in order to enhance separation ability. For instance, Ni-NTA end-functionalized polystyrenes were applied as a nanocarrier for isolation and purification of his-tagged proteins [28]. Liu's team produced boronate affinity-based molecular imprinted polymers which exhibited great selectivity for substances with *cis*-diol structure [29]. Nevertheless, challenges such as nonspecific adsorption and prolonged separation times persist [30]. Therefore, exploitation of porous polymer microspheres with favorable separation properties has received wide attention.

In this context, heterostructure polymer microspheres featuring a negatively charged carboxylic layer and large pore size were prepared to serve as substrate materials [31]. The carboxyl groups are capable of chelating with $Cu^{2+}$ directly via electrostatic interaction of $Cu^{2+}$-$COO^-$. After immobilization of $Cu^{2+}$, the $Cu^{2+}$-chelated porous polymer microspheres, namely SPSDVB-PAA-Cu, demonstrated enhanced selectivity for his-rich proteins. This enhancement can be explained by the coordination interaction of $Cu^{2+}$ with imidazole groups in histidine residues. Finally, SPSDVB-PAA-Cu showed biological applications in isolation of Hb and HSA from human blood and consumption of high-abundant human plasma proteins which was considered as a promising adsorbent in pretreatment of biosamples.

## 2. Experimental section

### 2.1. Materials and reagents

Hemoglobin human (Hb), cytochrome *c* (Cyt-c), polyvinyl pyrrolidone (PVP, Mw~55000) and styrene (St) were purchased from Sigma-Aldrich (St. Louis, USA). Poly (vinyl alcohol) (PVA, 1788 low-viscosity) was purchased from Aladdin Bio-Chem Technology Co., Ltd. Human serum albumin (HSA) was obtained from Beijing Solarbio Science & Technology Co., Ltd. Divinyl benzene (DVB, 80 %) was derived from Macklin Biochemical Co., Ltd. Copper dinitrate ($Cu(NO_3)_2$) was obtained by Shanghai Yi En Chemical Technology Co., Ltd. Sulfuric acid ($H_2SO_4$), hydrochloric acid (HCl), acetic acid, sodium chloride (NaCl), phosphoric acid and boric acid were acquired from Damao Chemical Co., Ltd. Ethanol ($C_2H_5OH$), azodiisobutyronitrile (AIBN), sodium dodecyl sulfate (SDS), acrylic acid (AA), aluminum oxide ($Al_2O_3$), Coomassie brilliant blue G250 and imidazole were obtained from Sinopharm Chemical Reagent Co., Ltd. All reagents were used as analytical grade without further treatment except for St and DVB which were processed through $Al_2O_3$ column to eliminate inhibitor. Human blood and plasma were obtained from the Centre Hospital of Shenyang Medical College. Deionized water with 18 MΩ cm was adopted for the entire duration of the experiment.

### 2.2. Preparation of heterostructure porous polymer microspheres SPSDVB-PAA

The preparation of sulfonated polystyrene particles (SPS) and porous polymer microspheres (SPSDVB-PAA) were presented in the supplementary information according to the literature [32].

### 2.3. Preparation of $Cu^{2+}$-chelated heterostructure porous polymer microspheres SPSDVB-PAA-Cu

Typically, 0.1 g of SPSDVB-PAA was dispersed into 50 mL of deionized water uniformly under sonication, and 0.2 g of $Cu(NO_3)_2$ was dissolved in 50 mL of deionized water to form $Cu(NO_3)_2$ solution (0.4 %, w/w). Then, the $Cu(NO_3)_2$ solution was added to SPSDVB-PAA suspension to form the mixture solution with stirring for 5 h at room temperature. After rinsed with deionized water for several times by centrifuging at 10000 rpm and 5 min, the product SPSDVB-PAA-Cu was freeze-dried and stored for further use.

### 2.4. Proteins adsorption behavior of SPSDVB-PAA-Cu

Hb, HSA and Cyt-c were employed as model proteins to investigate the adsorption performance of SPSDVB-PAA-Cu. The pH values of protein solutions were adjusted with 0.04 mol $L^{-1}$ Britton-Robinson (B–R) buffer and 0.20 mol $L^{-1}$ NaOH. Typically, 2.0 mg of SPSDVB-PAA-Cu was mixed with 1.0 mL of protein solution and then oscillated for 30 min. After centrifugation at 6000 rpm for 5 min, the supernatant was adopted and measured by UV–Vis spectrophotometer at maximum absorption peak, i.e., 405 nm for Hb, 410 nm for Cyt-c and 595 nm for HSA (stained with G250 dye) to determine residual protein concentration. For the process of desorption, the SPSDVB-PAA-Cu was rinsed with pH 5.0 B–R solution (1.0 mL) and subsequently eluted with 0.5 mol $L^{-1}$ imidazole (1.0 mL) by shaking for 30 min to removing adsorbed proteins. The eluate was acquired via centrifuging at 6000 rpm for 5 min and protein concentration was measured to evaluate the recovery. Then the SPSDVB-PAA-Cu was ready to adsorb Hb again. Adsorption isotherms of proteins are described in the supplementary information.

### 2.5. Complex biological samples treatment by SPSDVB-PAA-Cu

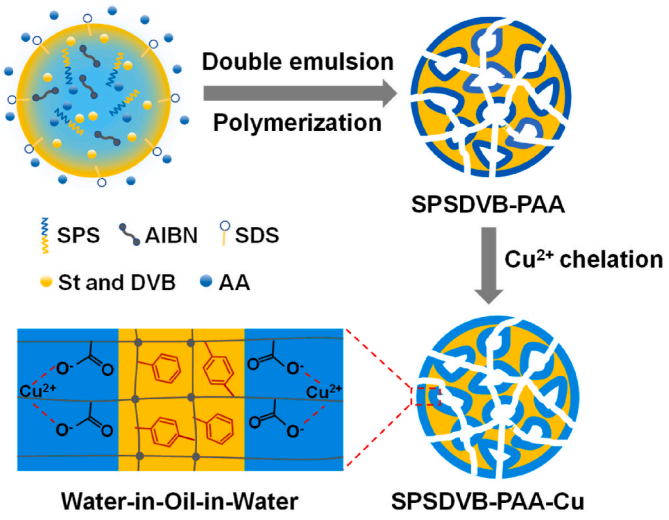
The human whole blood and plasma samples were diluted with pH 5.0 B–R. Then, 5.0 mg of SPSDVB-PAA-Cu was treated by 1.0 mL of above-mentioned biosamples. The adsorption-desorption test was carried out like previously described, with the exception that 0.5 mL of imidazole (0.5 mol $L^{-1}$) was added as eluent. The crude bio-sample, supernatant, and eluate were each set aside for analysis using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) assay, respectively. The 100-fold diluted human plasma samples and supernatant were collected for LC-MS/MS analysis, following the procedure as reported by Chen et al. [33].

## 3. Results and discussion

### 3.1. Preparation and characterizations of SPSDVB-PAA-Cu

The schematic diagram for preparation of $Cu^{2+}$-chelated porous polymer microspheres SPSDVB-PAA-Cu was exhibited in Scheme 1. The formation of a water-in-oil-in-water double emulsion system involved the combination of hydrophilic monomers (AA), hydrophobic monomers (St and DVB), amphiphilic stabilizers (SDS and SPS), and initiator (AIBN). In this system, hydrophilic monomers and SDS were dispersed in aqueous phase, while the hydrophobic monomers and SPS were dispersed in oil phase. During the interfacial polymerization process, the hydrophilic monomers (AA) underwent polymerization to form a hydrophilic polymer (PAA) coating on the exterior and interior surfaces of the hydrophobic moiety (SPSDVB). The porous structures permeated the entire particle and the porous polymer microspheres SPSDVB-PAA were synthesized. Then, positively charged $Cu^{2+}$ was immobilized on the negatively charged surface of SPSDVB-PAA via electrostatic interaction to obtain the final SPSDVB-PAA-Cu.

Surface morphology of SPS, SPSDVB-PAA and SPSDVB-PAA-Cu were monitored by scanning electron microscopy (SEM). As shown in Fig. 1a, the SPS particles reveal smooth spherical surface with a mean size of




**Scheme 1.** Illustration for the synthesis of SPSDVB-PAA-Cu.

2.11 ± 0.03 μm. After polymerization at double emulsion interface, the surface of SPSDVB-PAA particles has a large number of pores (Fig. 1b) and the size increases to an average diameter of 4.03 ± 0.11 μm (Fig. 1d). After immobilization with $Cu^{2+}$, the size of SPSDVB-PAA-Cu was measured to be 4.05 ± 0.15 μm, demonstrating $Cu^{2+}$ ions layer immobilized on the surface of SPSDVB-PAA. As illustrated in Fig. 1c, the SPSDVB-PAA-Cu particles display excellent dispersibility and dense porous structure which is advantageous for protein adsorption.

The transmission electron microscopy (TEM) images of SPSDVB-PAA and SPSDVB-PAA-Cu were presented in Fig. 2, suggesting that both are spherical structure with good dispersity. The relatively rough boundary indicates that the porous structures are distributed on the surface of microsphere. The SPSDVB-PAA-Cu has a uniform diameter of 4.06 ± 0.12 μm, agreeing well with the SEM results.

The FT-IR spectrum of SPSDVB-PAA-Cu was displayed in Fig. S1. The typical peaks appeared at 2922 $cm^{-1}$ and 2850 $cm^{-1}$ are due to characteristic bands of $–CH_2$-, and the peaks at 3080, 3052 and 3023 $cm^{-1}$ are ascribed to stretching vibration of –CH = in benzene ring. The bending vibration bands of C═C in aromatic ring are observed at 1600 $cm^{-1}$, 1487 $cm^{-1}$, and 1446 $cm^{-1}$. The characteristic peak at 1696 $cm^{-1}$ is assigned to stretching vibration of C═O in polyacrylic acid (PAA) [34]. These adsorption peaks represent chemical structure of SPSDVB-PAA-Cu, indicating that the polymer microspheres consist of poly St, poly DVB and PAA.

The chemical structure and elemental composition of SPSDVB-PAA-Cu were measured via X-ray photoelectron spectroscopy (XPS). As shown in Fig. 3a, the distinct peaks of C 1s, O 1s and Cu 2p are observed in full-curve of SPSDVB-PAA-Cu, indicating the existence of C, O, and Cu elements derived from poly (St-DVB), PAA and $Cu^{2+}$, respectively. The elemental contents of C, O and Cu in SPSDVB-PAA-Cu were measured to be 83.29 %, 15.45 % and 1.26 %. In Fig. 3b, the high resolution XPS spectrum of C 1s is divided into three characteristic peaks ascribed to C–C and C═C bonds (284.9 eV) in benzene ring, and C–O (286.1eV) and C═O (289.0eV) in PAA [35]. In O 1s XPS spectrum (Fig. 3c), three types of peaks at 531.4, 532.1 and 533.0 eV are obtained corresponding to C═O, Cu–O and C–O, respectively, agreeing well with C 1s XPS spectrum [36]. As shown in Fig. 3d, the Cu 2p spectrum exhibited binding energies at 934.5 and 954.0 eV which fit well with Cu $2p_{3/2}$ and Cu $2p_{1/2}$ [37]. The XPS results further indicated that the SPSDVB-PAA was successfully prepared, and copper ions were effectively immobilized on the surface of polymer microspheres.

The specific surface area and the pore size distribution of SPSDVB-PAA-Cu were obtained by mercury intrusion porosimetry (MIP). The MIP surface area, intrusion volume and porosity were determined to be 16.3 $m^2\ g^{-1}$, 1.05 mL $g^{-1}$ and 49.1 %, respectively. The high porosity and pore volume suggested dense macropores penetrating throughout the entire microspheres (as shown in Scheme 1). As shown in Fig. S2, the

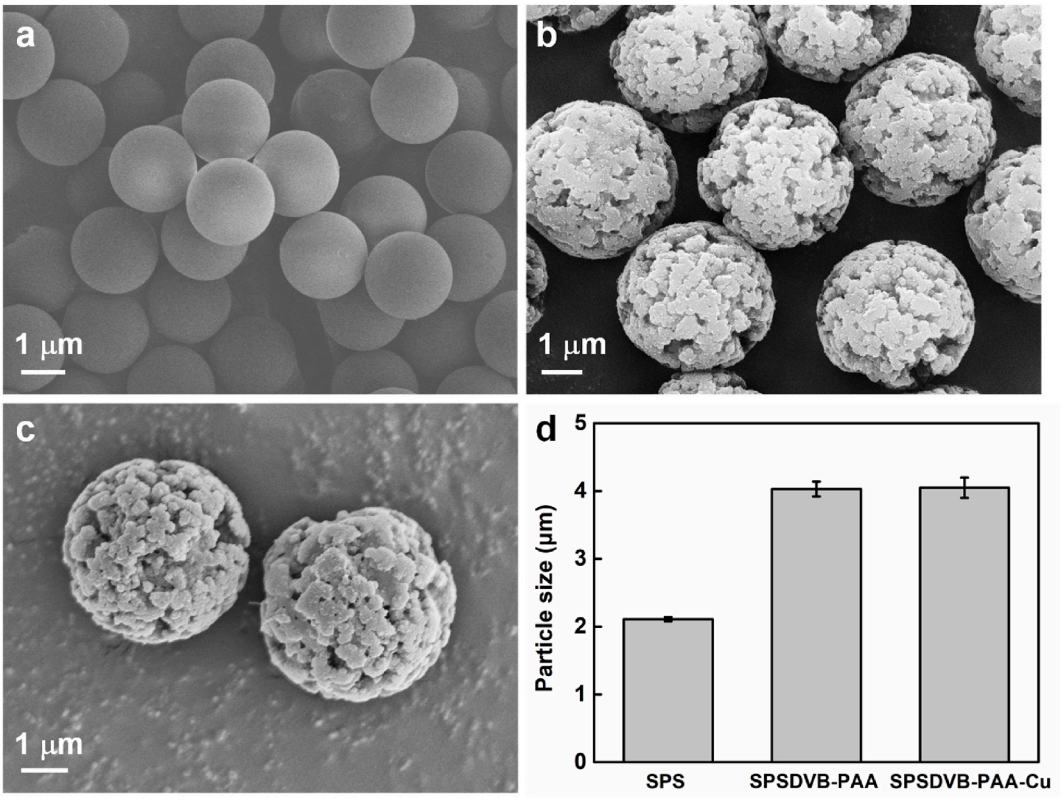



**Fig. 1.** SEM images of (a) SPS, (b) SPSDVB-PAA and (c) SPSDVB-PAA-Cu; (d) Size dimension of SPS, SPSDVB-PAA and SPSDVB-PAA-Cu particles.

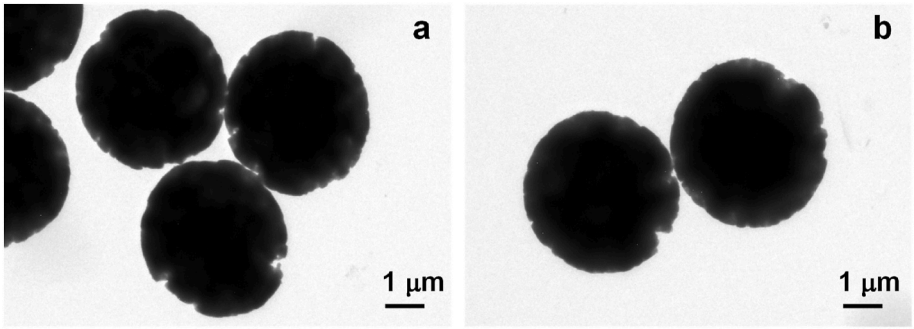



**Fig. 2.** TEM images of (a) SPSDVB-PAA and (b) SPSDVB-PAA-Cu.

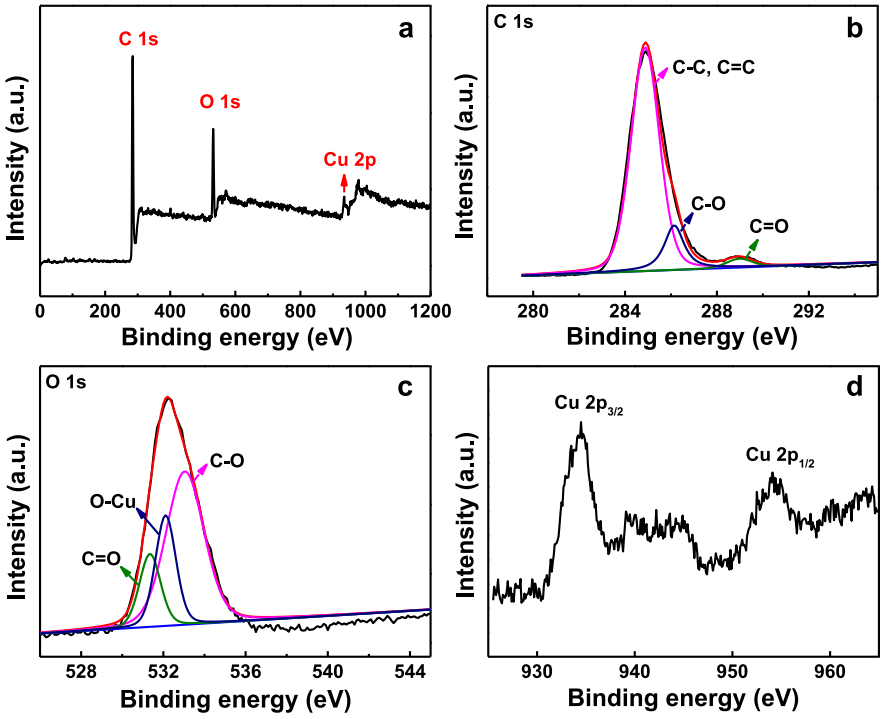



**Fig. 3.** (a) XPS full curve and high resolution XPS spectra of (b) C 1s, (c) O 1s and (d) Cu 2p of SPSDVB-PAA-Cu.

pore size distribution of SPSDVB-PAA-Cu has two peaks at around 800–1600 nm and 60–120 nm, which indicating the large pores are not uniform in size. The macroporous structures facilitate penetration and adsorption of proteins species on the SPSDVB-PAA-Cu microspheres.

### 3.2. Adsorption performance of SPSDVB-PAA-Cu to proteins

Three proteins with different amounts of histidine residues, e.g., Hb, HSA and Cyt-c were selected as model proteins in order to investigate adsorption selectivity of SPSDVB-PAA-Cu towards his-rich proteins. As shown in Fig. 4a, SPSDVB-PAA-Cu exhibited high adsorption efficiencies (above 80 %) for Hb at a wide pH range of 4.0–9.0 and the maximum is 95.6 % at pH 5.0. The driving force of protein adsorption is mainly depended on strong metal coordination interaction between immobilized $Cu^{2+}$ in SPSDVB-PAA-Cu microspheres and a large number of histidine residues in Hb [38]. For HSA, the highest adsorption efficiency is obtained at pH 5.0 which is close to the isoelectric point of HSA (pI = 4.7). In this case, the structure of HSA becomes loose, hydrophobic core unfolds and more histidine residues are exposed on the surface which are combined with $Cu^{2+}$ through metal affinity interaction, leading to the high adsorption efficiency. When pH deviates from pI, the peptide chains of HSA are curled, the exposed histidine residues reduced, causing weak metal affinity interaction and the deterioration of adsorption efficiencies [39]. For Cyt-c (pI = 10.6), the adsorption efficiencies increase gradually as pH approaches pI which exhibits similar adsorption behavior to HSA. Hence, the maximum adsorption efficiencies of Hb and HSA can be achieved at pH 5.0, simultaneously.

The adsorption capacities (Q) of SPSDVB-PAA-Cu for Hb, HSA and Cyt-c were investigated at room temperature and the results were shown in Fig. 4b. At pH 5.0, the adsorption capacities of Hb and HSA are quickly increased and reach equilibrium at 800 μg $mL^{-1}$ of protein concentration, while the adsorption capacities of Cyt-c are almost constant from 100 to 400 μg $mL^{-1}$. As his-rich proteins, Hb and HSA contain 24 and 16 of exposed histidine residues, respectively, whereas 1 for Cyt-c, resulting in different adsorption capacities [40,41]. It is apparent that the metal affinity interaction of SPSDVB-PAA-Cu and proteins is highly specific and the strength is associated with the number of accessible histidine residues. As a comparison, the SPSDVB-PAA microspheres without $Cu^{2+}$ were performed the same adsorption experiment as SPSDVB-PAA-Cu microspheres. As shown in Fig. 4c, SPSDVB-PAA has much lower adsorption capacities for Hb, HSA and Cyt-c, suggesting that metal-affinity interaction play a vital role in protein adsorption via

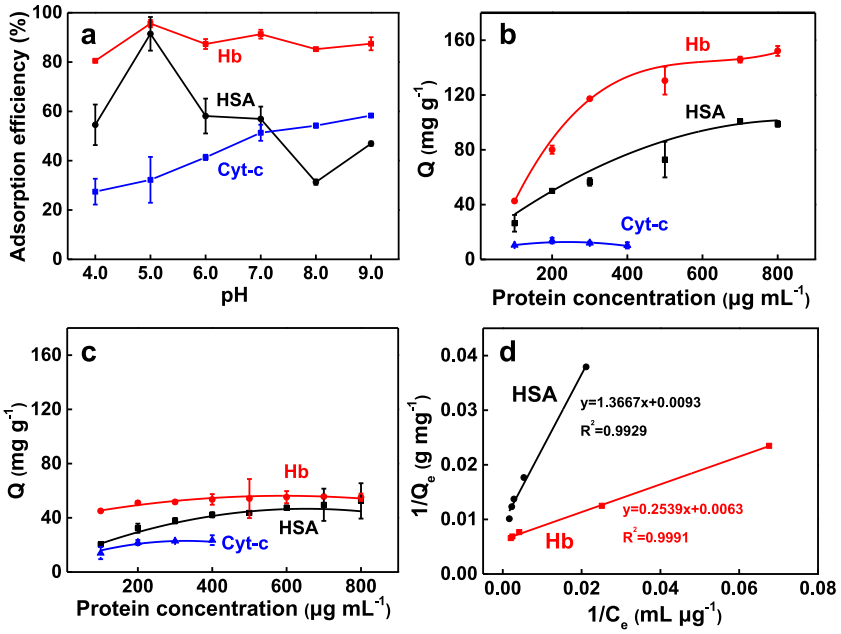



**Fig. 4.** (a) pH-dependent adsorption efficiencies of Hb, HSA and Cyt-c using SPSDVB-PAA-Cu. Protein solution: 1.0 mL, 100 μg $mL^{-1}$; sorbent quantity: 2.0 mg; adsorption time: 30 min. Adsorption capacities of Hb, HSA and Cyt-c on (b) SPSDVB-PAA-Cu and (c) SPSDVB-PAA, respectively. Protein solution: 1.0 mL, pH 5.0 B–R, 100–800 μg $mL^{-1}$; sorbent quantity: 2.0 mg; adsorption time: 30 min. (d) The fitting curves of *Langmuir* model for Hb and HSA adsorbed on SPSDVB-PAA-Cu.

immobilizing $Cu^{2+}$ on the surface of SPSDVB-PAA.

The *Langmuir* model was applied to evaluate adsorption isotherms of Hb and HSA on SPSDVB-PAA-Cu. As shown in Fig. 4d, the isotherms of Hb and HSA were fitted well with *Langmuir* model, illustrating monolayer adsorption and chemisorption on SPSDVB-PAA-Cu [8]. The theoretical maximum adsorption capacities were calculated to be 158.7 mg $g^{-1}$ for Hb and 107.5 mg $g^{-1}$ for HSA, which consistent with the experimental maximum adsorption capacities of 152.2 mg $g^{-1}$ for Hb and 100.7 mg $g^{-1}$ for HSA. Comparisons of the adsorption properties for his-rich proteins by the adsorbent proposed in this work with those previously reported were shown in Table S1. The SPSDVB-PAA-Cu exhibited a higher adsorption capacity for Hb along with shorter adsorption time than most of those adsorbents. Considering simple and cost-efficient preparation of the polymer microspheres, the SPSDVB-PAA-Cu has the superiority for separation of his-rich proteins.

The adsorbed proteins should be recovered by stripping agent. Considering the strong metal affinity force between $Cu^{2+}$ and his-rich proteins, imidazole can be employed as a competitive reagent to bind with $Cu^{2+}$, causing protein molecules stripping from the surface of SPSDVB-PAA-Cu. The experimental results indicated that using 0.5 mol $L^{-1}$ imidazole can yield favorable recovery rates for Hb and HSA, with elution efficiencies reaching up to 70 %.

The reusability and stability of the adsorbent are crucial for practical applications. Therefore, the recyclability of SPSDVB-PAA-Cu was evaluated by five-cycle adsorption-elution processes and the results were shown in Fig. 5a. The SPSDVB-PAA-Cu maintained high adsorption efficiencies of up to 80 % for Hb after five adsorption-elution cycles, demonstrating the favorable reusability of SPSDVB-PAA-Cu for his-rich proteins separation. To access the stability of SPSDVB-PAA-Cu microspheres after adsorption-elution process, the leaching amounts of $Cu^{2+}$ from the adsorbent to eluent were determined by ICP-MS analysis and the results showed that there are 0.15 μg/mL and 0.04 μg/mL of $Cu^{2+}$ in the 1st and 5th eluent, respectively. Furthermore, the structure and morphology of SPSDVB-PAA, SPSDVB-PAA-Cu and SPSDVB-PAA-Cu after elution were characterized by X-ray diffraction (XRD), SEM and TEM. The XRD patterns in Fig. 5b showed that a wide diffraction peak at $2\theta = 20^{\circ}$ was observed without characteristic peaks, indicating that the SPSDVB-PAA, SPSDVB-PAA-Cu and SPSDVB-PAA-Cu after elution are amorphous structure. The SEM and TEM images of SPSDVB-PAA-Cu after 1st and 5th elution in Fig. S3 exhibited porous microsphere

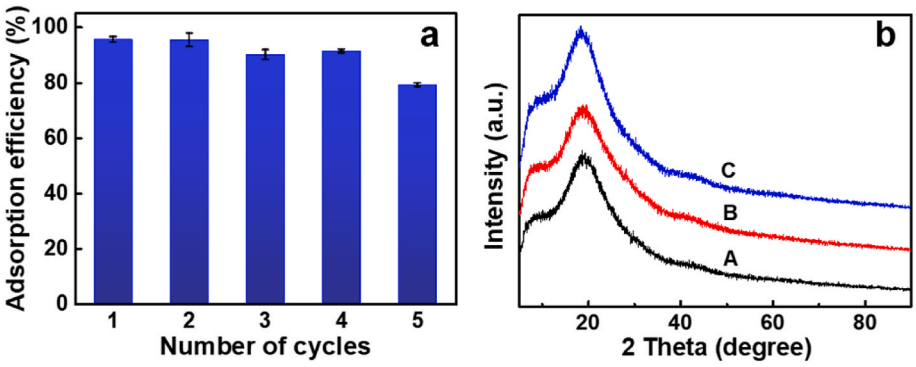



**Fig. 5.** (a) Adsorption efficiencies of Hb by SPSDVB-PAA-Cu over five cycles of reuse. (b) XRD patterns of (A) SPSDVB-PAA, (B) SPSDVB-PAA-Cu and (C) SPSDVB-PAA-Cu after elution.

structures with uniform sizes corresponding to the morphology shown in Fig. 1b and c and 2, which demonstrating the SPSDVB-PAA-Cu ha excellent stability after five adsorption-elution cycles and the low loss rate of $Cu^{2+}$ has no influence on the adsorption-elution experiment.

### 3.3. Isolation of his-rich proteins from complicated biosamples by SPSDVB-PAA-Cu

The practical application of SPSDVB-PAA-Cu was studied by selective isolation of his-rich proteins from human blood and plasma samples. As shown in Fig. 6, 300-fold diluted human blood biosample exhibited several major protein bands in lane 2 assigned to human serum albumin (HSA) at 66.4 kDa and hemoglobin (Hb) at around 14.3 kDa. After treated by SPSDVB-PAA-Cu, the band of HSA almost disappears and Hb band becomes weak in lane 3, indicating that his-rich proteins can be selectively captured by SPSDVB-PAA-Cu. After eluting with 0.5 mol $L^{-1}$ imidazole, the bands of HSA and Hb reappear in lane 4, according with standard Hb and HSA bands (lane 5). The SDS-PAGE analysis illustrates SPSDVB-PAA-Cu as a sorbent achieved the isolation of his-rich proteins from human whole blood.

In addition, 300-fold diluted human plasma sample was further applied to verify the practicability of SPSDVB-PAA-Cu in complex biological sample. As shown in Fig. 7, the protein bands of plasma sample in lane 2 are almost identical to blood sample except for the absence of hemoglobin. After treatment with SPSDVB-PAA-Cu, the HSA band disappears in the supernatant (lane 3) and reappears in the eluate (lane 4), which existed in the same position as standard HSA band (lane 5). The results demonstrated that SPSDVB-PAA-Cu is effectively feasible for selectively separating HSA from human plasma.

To evaluate the efficiency of depleting high-abundant proteins from human plasma, the crude plasma sample and the supernatant after treated with SPSDVB-PAA-Cu were digested and analysed by LC-MS/MS. The 14 most abundant proteins in human plasma, e.g. albumin (HSA), transferrin, apolipoprotein A-I, alpha-2-macroglobulin, alpha-1-antitrypsin, haptoglobin, IgHA, IgHG, Apolipoprotein B, IgHM, hemopexin, alpha-1-acid glycoprotein, vitamin D-binding protein and histidine-rich glycoprotein are represented in Fig. 8a and Table S2, accounting for 81.6 % of the total protein content. After treatment by SPSDVB-PAA-Cu, the content of serum albumin was reduced from 41.1 % to 6.1 % and other low-abundance proteins were increased from 18.4 % to 69.3 % as shown in Fig. 8b, indicating that the removal of high abundance proteins and enrichment of low abundance proteins were

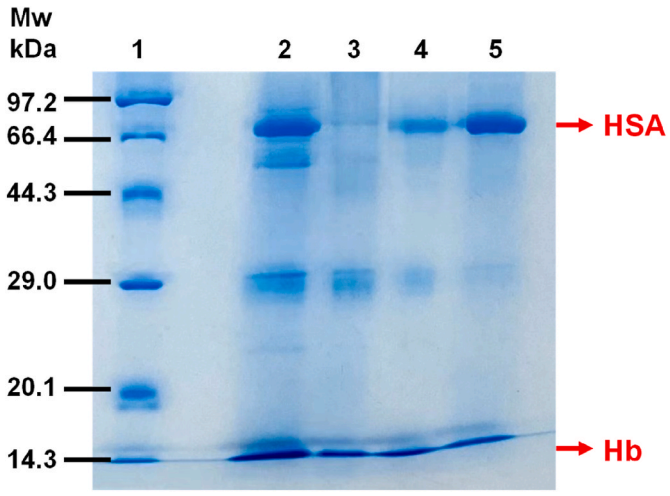



**Fig. 6.** SDS-PAGE analysis results. Lane 1: protein markers; Lane 2: 300-fold diluted human blood; Lane 3: the supernatant of blood sample after treatment with SPSDVB-PAA-Cu; Lane 4: the recovered proteins eluted with 0.5 mol $L^{-1}$ imidazole; Lane 5: 100 μg $mL^{-1}$ of Hb and HSA standard solution.

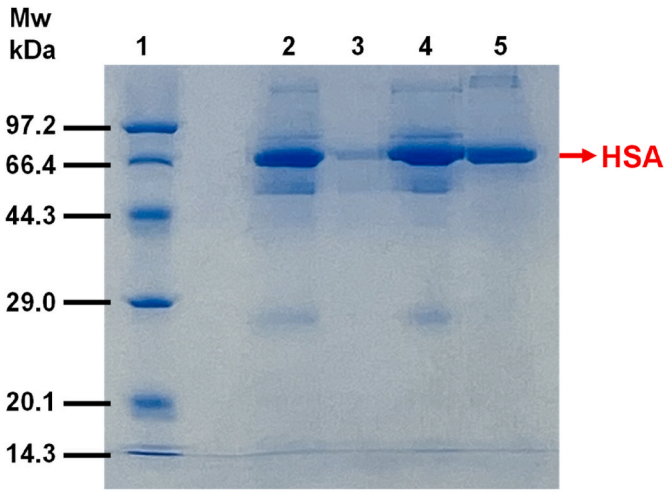



**Fig. 7.** SDS-PAGE analysis results. Lane 1: protein markers; Lane 2: 300-fold diluted human plasma; Lane 3: supernatant after treatment with SPSDVB-PAA-Cu; Lane 4: the recovered proteins eluted with 0.5 mol $L^{-1}$ imidazole; Lane 5: 100 μg $mL^{-1}$ of HSA standard solution.

achieved. In addition, a number of new protein species were enriched to a certain extent. The purification and enrichment of these low abundance proteins, most of which are biomarkers, are essential for a comprehensive understanding of their biological functions. For example, Zinc finger proteins play an important role in the regulation of gene expression, cell differentiation, embryonic development and other life processes. Ribonuclease 4 could improve the cellular immune function of tumor patients and inhibit the growth of tumor. Human platelet factor 4 variant is a potent inhibitor of angiogenesis. Hence, the SPSDVB-PAA-Cu has been demonstrated to be an effective absorbent for depletion of high-abundant plasma proteins in order to enhance the identification of low abundance proteins.

## 4. Conclusion

To conclude, we fabricated porous polymer microspheres (SPSDVB-PAA) by means of double emulsion interfacial polymerization process, followed by chelation with $Cu^{2+}$. The as-prepared SPSDVB-PAA-Cu with good dispersion, dense and large porous structure ensured high performance adsorption of his-rich proteins. Owing to the strong metal coordination interaction between $Cu^{2+}$ and histidine residues, the SPSDVB-PAA-Cu exhibited favorable adsorption selectivity to Hb and HSA. This enabled the effective separation and enrichment of his-rich proteins from real biological samples, including human blood and plasma. Furthermore, LC-MS/MS analysis confirmed that SPSDVB-PAA-Cu was effective in depleting high abundance proteins and enriching low abundance proteins from human plasma. Taken together, a novel metal-affinity sorbent was developed for his-rich proteins separation and showed promising potential for applications in biomedical field.

## CRediT authorship contribution statement

**Qing Chen:** Writing – review & editing, Methodology, Conceptualization. **Lijie Zhang:** Validation, Investigation. **Yang Zhang:** Methodology, Data curation. **Jiajun Shen:** Formal analysis. **Dandan Zhang:** Resources, Data curation. **Mengmeng Wang:** Writing – original draft, Supervision, Resources, Funding acquisition.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

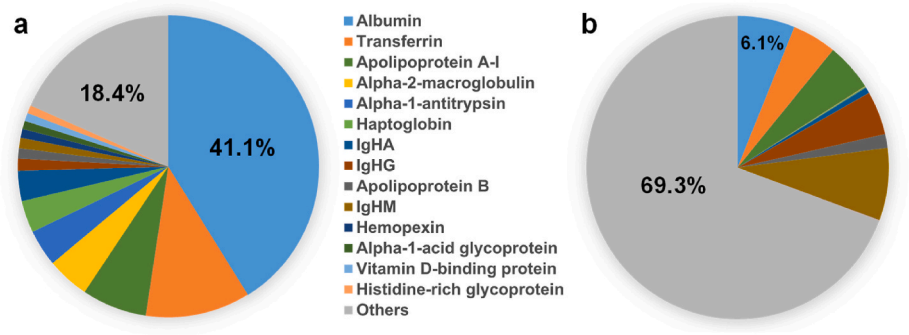



**Fig. 8.** The circular distribution of (a) top 14 high-abundant proteins in crude human plasma and (b) supernatant after treatment with SPSDVB-PAA-Cu.

the work reported in this paper.

## Data availability

No data was used for the research described in the article.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2024.126337.

## References

[1] K. Du, X. Liu, S. Li, L. Qiao, H. Ai, Synthesis of $Cu^{2+}$ chelated cellulose/magnetic hydroxyapatite particles hybrid beads and their potential for high specific adsorption of histidine-rich proteins, ACS Sustainable Chem. Eng. 6 (2018) 11578–11586.
[2] A.L. Jones, M.D. Hulett, C.R. Parish, Histidine-rich glycoprotein: a novel adaptor protein in plasma that modulates the immune, vascular and coagulation systems, Immunol. Cell Biol. 83 (2005) 106–118.
[3] S. Shi, W. Zhang, H. Wu, Y. Li, X. Ren, M. Li, J. Liu, J. Sun, T. Yue, J. Wang, In situ cascade derivation toward a hierarchical layered double hydroxide magnetic absorbent for high-performance protein separation, ACS Sustainable Chem. Eng. 8 (2020) 4966–4974.
[4] R. Gao, X. Cui, Y. Hao, G. He, M. Zhang, Y. Tang, Preparation of $Cu^{2+}$-mediated magnetic imprinted polymers for the selective sorption of bovine hemoglobin, Talanta 150 (2016) 46–53.
[5] S. Surinova, R. Schiess, R. Hüttenhain, F. Cerciello, B. Wollscheid, R. Aebersold, On the development of plasma protein biomarkers, J. Proteome Res. 10 (2011) 5–16.
[6] S. Fekete, A. Beck, J.L. Veuthey, D. Guillarme, Ion-exchange chromatography for the characterization of biopharmaceuticals, J. Pharm. Biomed. Anal. 113 (2015) 43–55.
[7] L. Pringels, V. Broeckx, K. Boonen, B. Landuyt, L. Schoofs, Abundant plasma protein depletion using ammonium sulfate precipitation and Protein A affinity chromatography, J. Chromatogr. B 1089 (2018) 43–59.
[8] Q. Xu, W. Jiang, F. Bu, Z.F. Wang, Y. Jiang, Magnetic dendritic polymer nanospheres for high-performance separation of histidine-rich proteins, ACS Appl. Mater. Interfaces 15 (2023) 30837–30848.
[9] V. Riguero, R. Clifford, M. Dawley, M. Dickson, B. Gastfriend, C. Thompson, S. C. Wang, E. O'Connor, Immobilized metal affinity chromatography optimization for poly-histidine tagged proteins, J. Chromatogr. A 1629 (2020) 461505.
[10] L. Shi, Y. Tang, Y. Hao, G. He, R. Gao, X. Tang, Selective adsorption of protein by a high-efficiency $Cu^{2+}$-cooperated magnetic imprinted nanomaterial, J. Separ. Sci. 39 (2016) 2876–2883.
[11] P. Hu, J.A. Loo, Gas-phase coordination properties of $Zn^{2+}$, $Cu^{2+}$, $Ni^{2+}$, and $Co^{2+}$ with histidine-containing peptides, J. Am. Chem. Soc. 117 (1995) 11314–11319.
[12] M. Ge, J. Zhang, Z. Gai, R. Fan, S. Hu, G. Liu, Y. Cao, X. Du, Y. Shen, Synthesis of magnetic $Fe_3O_4$@PS-ANTA-$M^{2+}$ (M=Ni, Co, Cu and Zn) nanospheres for specific isolation of histidine-tagged proteins, Chem. Eng. J. 404 (2021) 126427.
[13] Y. Niu, Y. Tang, R. Gao, X. Chen, Y. Wang, Y. Gao, S. Zhang, S. Hussain, Y. Hao, S. Wang, One-step synthesis of Sustainable montmorillonite-supported, copper-doped magnetic nanoparticles for highly specific separation of his-rich proteins, ACS Sustainable Chem. Eng. 10 (2022) 5341–5351.
[14] Q.L. He, B.X. Jia, Z.R. Luo, Z. Zhang, Y.F. Feng, B. Zhou, Construction and comparison of Prussian blue analogs nanomaterials with tailorable coordination metal ions as novel adsorbents for efficient structure-selective protein adsorption, Microchem. J. 191 (2023) 108832.
[15] W. Wang, F. Zhou, X. Cheng, Z. Su, H. Guo, High-efficiency $Ni^{2+}$-NTA/PAA magnetic beads with specific separation on His-tagged protein, IET Nanobiotechnol. 14 (2019) 67–72.
[16] Q. Ni, B. Chen, S. Dong, L. Tian, Q. Bai, Preparation of core-shell structure $Fe_3O_4$@$SiO_2$ superparamagnetic microspheres immobilized with iminodiacetic acid as immobilized metal ion affinity adsorbents for His-tag protein purification, Biomed. Chromatogr. 30 (2016) 566–573.
[17] M.M. Wang, S. Chen, Y.L. Yu, J.H. Wang, Novel $Ti^{4+}$-chelated polyoxometalate/polydopamine composite microspheres for highly selective isolation and enrichment of phosphoproteins, ACS Appl. Mater. Interfaces 11 (2019) 37471–37478.
[18] W. Ma, Y. Zhang, L. Li, Y. Zhang, M. Yu, J. Guo, H. Lu, C. Wang, $Ti^{4+}$-Immobilized magnetic composite microspheres for highly selective enrichment of phosphopeptides, Adv. Funct. Mater. 23 (2013) 107–115.
[19] M.F. Jimenez-Solomon, Q. Song, K.E. Jelfs, M. Munoz-Ibanez, A.G. Livingston, Polymer nanofilms with enhanced microporosity by interfacial polymerization, Nat. Mater. 15 (2016) 760–767.
[20] J.B. Fan, C. Huang, L. Jiang, S. Wang, Nanoporous microspheres: from controllable synthesis to healthcare applications, J. Mater. Chem. B 1 (2013) 2222–2235.
[21] Q. Gao, C. Wang, H. Liu, Y. Chen, Z. Tong, Dual nanocomposite multihollow polymer microspheres prepared by suspension polymerization based on a multiple pickering emulsion, Polym. Chem. 1 (2010) 75–77.
[22] T. Jin, Z. Xiong, X. Zhu, N. Mehio, Y. Chen, J. Hu, W. Zhang, H. Zou, H. Liu, S. Dai, Template-free synthesis of mesoporous polymers for highly selective enrichment of glycopeptides, ACS Macro Lett. 4 (2015) 570–574.
[23] J. Kim, Y. Piao, N. Lee, Y.I. Park, I.H. Lee, J.H. Lee, S.R. Paik, T. Hyeon, Magnetic nanocomposite spheres decorated with NiO nanoparticles for a magnetically recyclable protein separation system, Adv. Mater. 22 (2010) 57–60.
[24] Q. Xiao, K. Zhou, C. Chen, M. Jiang, Y. Zhang, H. Luo, D. Zhang, Hollow and porous hydroxyapatite microspheres prepared with an O/W emulsion by spray freezing method, Mater. Sci. Eng., C 69 (2016) 1068–1074.
[25] A. Nematollahzadeh, W. Sun, C.S. Aureliano, D. Lutkemeyer, J. Stute, M. J. Abdekhodaie, A. Shojaei, B. Sellergren, High-capacity hierarchically imprinted polymer beads for protein recognition and capture, Angew. Chem. Int. Ed. 50 (2011) 495–498.
[26] H. Yu, X. Qiu, S.P. Nunes, K.V. Peinemann, Biomimetic block copolymer particles with gated nanopores and ultrahigh protein sorption capacity, Nat. Commun. 5 (2014) 4110.
[27] X. Qiu, H. Yu, M. Karunakaran, N. Pradeep, S.P. Nunes, K.V. Peinemann, Selective separation of similarly sized proteins with tunable nanoporous block copolymer membranes, ACS Nano 7 (2013) 768–776.
[28] M.A. Kadir, S.J. Kim, E.J. Ha, H.Y. Cho, B.S. Kim, D. Choi, S.G. Lee, B.G. Kim, S. W. Kim, H.j. Paik, Encapsulation of nanoparticles using nitrilotriacetic acid end-functionalized polystyrenes and their application for the separation of proteins, Adv. Func. Mater. 22 (2012) 4032–4037.
[29] Z. Liu, H. He, Synthesis and applications of boronate affinity materials: from class selectivity to biomimetic specificity, Acc. Chem. Res. 50 (2017) 2185–2193.
[30] Y. Song, H. Bao, X. Shen, X. Li, X. Liang, S. Wang, Emerging nanoporous materials for biomolecule separation, Adv. Func. Mater. 32 (2022) 2113153.
[31] Y. Song, J.B. Fan, X. Li, X. Liang, S. Wang, pH-Regulated heterostructure porous particles enable similarly sized protein separation, Adv. Mater. 31 (2019) e1900391.
[32] Y. Song, X. Li, J.B. Fan, H. Kang, X. Zhang, C. Chen, X. Liang, S. Wang, Interfacially polymerized particles with heterostructured nanopores for glycopeptide separation, Adv. Mater. 30 (2018) e1803299.
[33] X. Wu, Q. Mao, Y. Hao, J. Yang, X. Zhang, Z. Chi, G. Liu, M. Wang, Q. Chen, X. Chen, Isolation of cytochrome c for proteomics with lindqvist-type polyiodate modified metal organic framework, J. Chromatogr. A 1693 (2023) 463869.

[34] M.M. Wang, S. Chen, Y.L. Yu, J.H. Wang, Polyoxometalate-functionalized macroporous microspheres for selective separation/enrichment of glycoproteins, Chem. Commun. 56 (2020) 9870–9873.
[35] Y. Liang, J. Liu, L. Wang, Y. Wan, J. Shen, Q. Bai, Metal affinity-carboxymethyl cellulose functionalized magnetic graphene composite for highly selective isolation of histidine-rich proteins, Talanta 195 (2019) 381–389.
[36] M.M. Wang, S. Chen, D.D. Zhang, Y.L. Yu, J.H. Wang, Immobilization of a Ce(IV)-substituted polyoxometalate on ethylenediamine-functionalized graphene oxide for selective extraction of phosphoproteins, Microchim. Acta 185 (2018) 553.
[37] P.F. Guo, D.D. Zhang, Z.Y. Guo, M.L. Chen, J.H. Wang, Copper-decorated titanate nanosheets: novel homogeneous monolayers with a superior capacity for selective isolation of hemoglobin, ACS Appl. Mater. Interfaces 9 (2017) 28273–28280.
[38] Y. Zhang, L.G. Xing, X.W. Chen, J.H. Wang, Nano copper oxide-incorporated mesoporous carbon composite as multimode adsorbent for selective isolation of hemoglobin, ACS Appl. Mater. Interfaces 7 (2015) 5116–5123.
[39] Z.Y. Guo, Y. Zhang, D.D. Zhang, Y. Shu, X.W. Chen, J.H. Wang, Magnetic nanospheres encapsulated by mesoporous copper oxide shell for selective isolation of hemoglobin, ACS Appl. Mater. Interfaces 8 (2016) 29734–29741.
[40] Q. Chen, M.M. Wang, X. Hu, X.W. Chen, J.H. Wang, An octamolybdate-metal organic framework hybrid for the efficient adsorption of histidine-rich proteins, J. Mater. Chem. B 4 (2016) 6812–6819.
[41] G.E. Wuenschell, E. Naranjo, E.H. Arnold, Aqueous two-phase metal affinity extraction of heme proteins, Bioprocess Eng. 5 (1990) 199–202.