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Integration of carboxyl modified magnetic particles and aqueous two-phase extraction for selective separation of proteins

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ABSTRACT

Both of the magnetic particle adsorption and aqueous two-phase extraction (ATPE) were simple, fast and low-cost method for protein separation. Selective proteins adsorption by carboxyl modified magnetic particles was investigated according to protein isoelectric point, solution pH and ionic strength. Aqueous two-phase system of PEG/sulphate exhibited selective separation and extraction for proteins before and after magnetic adsorption. The two combination ways, magnetic adsorption followed by ATPE and ATPE followed by magnetic adsorption, for the separation of proteins mixture of lysozyme, bovine serum albumin, trypsin, cytochrome C and myloglobin were discussed and compared. The way of magnetic adsorption followed by ATPE was also applied to human serum separation.

1. Introduction

For the practical biological sample, protein-separation was difficult due to their concentration range and the tremendous heterogenity [1]. Magnetic particles have attracted more and more interest in protein separation not only due to their good biocompatible and superparamagnetic properties, but also their surface can be modified by a variety of active functional groups which play an important role in protein adsorption and affinity interactions. Some reports paid attention to the preparation of functionalized magnetic particles [2–6] and their adsorption properties to some specific proteins [7,8]. Since magnetic particles can be easily isolated from samples by using a low-cost external magnetic field without the need of complicated centrifugation steps or filtration, they have certain advantages in the rapid and selective separation of target molecule.

Aqueous two-phase system (ATPS) consists of aqueous mixtures of different water-soluble polymers or a single polymer and a specific salt. Since both of two phases in ATPS contain more than 70% water, which is typically applicable to proteins and other biological molecules [9,10]. In recent years, new ATPSs of smart polymers, which are able to change conformation by responding to a simple pH or temperature change in the environment, have been reported [11–13]. Aqueous two-phase extraction (ATPE), based on the use of PEG/salt is a simple, fast, convenient and low-cost method for selective separation and enrichment of biological molecules, which have been applied in the purification, characterization and study of biomaterials from analytical to commercial scale. Some papers described the attempts of ATPE to exploit its application in proteomics, such as the selective separation and enrichment of proteins in human saliva and plasma samples [14], the depletion of serum albumin [15], purification of human immunoglobulin G [16], the quantitative extraction of proteins in human urine [17] and model proteins separation [18].

Both magnetic separation and ATPE are simple, rapid, easily scalable and mild separation methods. The combination of magnetic particles with functional adsorption and ATPE with extraction and enrichment properties to protein is a new integrative method of liquid–solid adsorption, magnetic separation and liquid–liquid extraction, which is still in the beginning stage. The first success was achieved by Suzuki et al. [19], in which they found that the phase separation time of ATPS was shortened by the addition of the magnetic particles and protein A from recombinant *E. coli* was separated and purified with IgG-bound magnetic particles and PEG/phosphate system. Magnetic cation exchange adsorbents were introduced into a micellar ATPS by Becker et al. [20], which made the phase selectivity of lysozyme (Lyz) be effectively inverted and obtained Lyz in a high yield from the mixed solution of Lyz and ovalbumin. In our previous work [21], ATPE was used as a novel elution solution to achieve adsorbed target Lyz from imprinted polymer modified magnetic particles, which exhibits the elution, extraction and enrichment effect to adsorbed proteins. The integration of mag-

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netic particles with ATPS provided the potential application in fast separation and enrichment of proteins [22]. Up till now, there are no more related reports.

In this paper, magnetic particles adsorption integrated with ATPE were applied to proteins mixture and human serum samples. The carboxyl modified magnetic particles adsorbed proteins depending on their isoelectric points, solution pH and ionic strength. Selective extraction separation to proteins mixture by two combination ways of carboxyl modified magnetic particles adsorption and PEG/sulphate extraction were discussed and compared. The magnetic particles adsorption followed by PEG/sulphate extraction was applied to human serum.

2. Experimental

2.1. Materials

Lysozyme (Lyz), bovine serum albumin (BSA) and trypsin (Try) were purchased from Amresco (Solon, OH, USA). Cytochrome C (Cyt C) and myloglobin (Mb) were obtained from Sigma (St. Louis, MO, USA). Acrylamide (AAm), *N, N′*-methylenebisacrylamide (MBAA) and ammonium persulfate (APS) were provided by Sigma-Aldrich (Tokyo, Japan). Methacrylic acid (MAA) was supplied by Xilong Chemical Co., Ltd. (Guangdong, China). HPLC grade acetonitrile (ACN) was obtained from Fisher Chem. Alert Co. (NJ, USA). Human serum samples were supplied by Beijing Chaoyang Hospital (Beijing, China). All other reagents were of analytical grade. Deionized water was produced by a Millipore water system.

2.2. Apparatus

Morphological observation of the polymer particles was performed with a transmission electron microscopy (TEM; JEOL, JEM-2010, Japan). Fourier transform infrared (FT-IR) spectra of the polymer in KBr were recorded with a FT/IR spectrum BX (PerkinElmer, USA) instrument. The individual protein concentrations were determined by Shimadzu UV–vis UV-2550 spectrophotometer (Kyoto, Japan). Proteins mixture and human serum were analyzed by Shimadzu Prominence LC-20A series HPLC (Kyoto, Japan) and an Agilent C8 (250 mm × 4.6 mm, 5 μm, 300 Å) column. SDS-PAGE analysis of human serum was performed by Kaiyuan gel electrophoresis (Beijing, China) using 14.0% polyacrylamide gel.

2.3. Preparation of carboxyl modified magnetic particles

Fe_3O_4 particles were synthesized according to the literature [23]. 0.5 g Fe_3O_4 particles were dispersed in water (15 mL) by sonication, followed by the addition of AAm (1.25 g), MBAA (0.0125 g) and MAA (200 μL). The mixture was bubbled with nitrogen for 15 min and then APS (0.0725 g) was added. The polymerization occurred at 60 °C under a continuous mechanical stirring for 2 h. The resultant product was collected by an external magnetic field, followed by the immersion in 1 mol/L HCl for 48 h to remove the bare Fe_3O_4 particles. Finally, the product was washed by excess water, dried to powder in the vacuum and stored at 4 °C.

2.4. Adsorption experiments of magnetic particles and adsorption amount determination

To determine the effect of solution pH on adsorption capacity, the magnetic polymer particles 5.0 mg were first allowed to swell in 0.01 mol/L phosphate buffer saline (PBS) until equilibrium. The wet state particles then were added to 5 mL of 0.3 mg/mL protein solution (BSA, Lyz and Mb) at different pH in the range of 4.2–9.5. They were oscillated in an oscillator at 25 °C for 15 min. Following the incubation period, the particles were isolated by an external magnetic field. The residual concentration of protein in the supernatant was determined by the UV–vis spectrophotometer at 280 nm [20]. To determine the effect of ionic strength on adsorption capacity, the magnetic polymer particles 5.0 mg were first allowed to swell in 0.01 mol/L PBS until equilibrium and then mixed with 5 mL of 0.3 mg/mL protein solution (BSA, pH 4.2; Lyz, pH 7.1; Mb, pH 4.2) at different NaCl concentration in the range of 0.05–0.5 mol/L. They were oscillated in an oscillator at 25 °C for 15 min. The other processes are as described above. Bare Fe_3O_4 did also the same experiments above.

To investigate the adsorption dynamics of the magnetic particles, the magnetic polymer particles 5.0 mg were first allowed to swell in 0.01 mol/L PBS until equilibrium and then mixed with 5 mL of 0.3 mg/mL protein solution (BSA, pH 4.2; Lyz, pH 7.1; Mb, pH 4.2). They were oscillated in an oscillator at 25 °C. Following the incubation period, the particles were isolated by an external magnetic field at specific time intervals. The other processes are as described above.

The amount of adsorbed protein can be determined by the difference in concentration before and after the adsorption. The adsorption capacity (Q, mg/g) of the protein to the magnetic particles is calculated according to

$$Q = (C_0 - C_t)\,V/m$$

where C_0 and C_t are the initial protein concentration and the final protein concentration, respectively (mg/mL), V is the total volume of the adsorption mixture (mL), and m is the weight of the magnetic particles (g).

2.5. Preparation of ATPS

PEG4000 (0.1875 g) and 40% $(NH_4)\cdot_2SO_4$ (0.3125 g) were dissolved in water (0.75 g), followed by shaking and centrifugation to form two phases. The volume of two phases was obtained by a pipette. The obtained ATPS contained 15% PEG4000 and 10% $(NH_4)\cdot_2SO_4$.

2.6. The combination process of magnetic adsorption and ATPE

There were two ways of combination of magnetic adsorption and ATPE. The first one is as follow: the mixture of Lyz, BSA, Mb, Try and Cyt C with same amount (3.0 mg) excepting Try (10.0 mg) was added to a 1.25 g aqueous two-phase solution above mentioned, followed by the shaking and centrifugation. Then, the resultant top and bottom phases were mixed with the carboxyl modified magnetic particles (15.0 mg), respectively. Finally, the magnetic particles were removed by an external magnetic field, and the concentrations of proteins in the supernatant of both phases were determined by HPLC.

The second way is: the carboxyl modified magnetic particles (15.0 mg) were added to a mixture of proteins (1 mL, pH 7.1, 0.01 mol/L PBS), which contained Lyz, BSA, Mb and Cyt C with the same amount (3.0 mg), and then the magnetic particles were removed by an external magnetic field. Subsequently, the supernatant was extracted by the ATPS above mentioned. The concentrations of proteins in formed top and bottom phases were determined by HPLC, respectively.

2.7. HPLC determination of proteins

Two mobile phases, (A) 80 vol% ACN and 20 vol% with 0.1 vol% trifuoroacetic acid (TFA) and (B) deionized water with 0.1 vol% TFA, were used for the linear gradient elution. The solvent flow rate was set at 1 mL/min with solvent A increasing from 37.5 to 62.5 vol% in

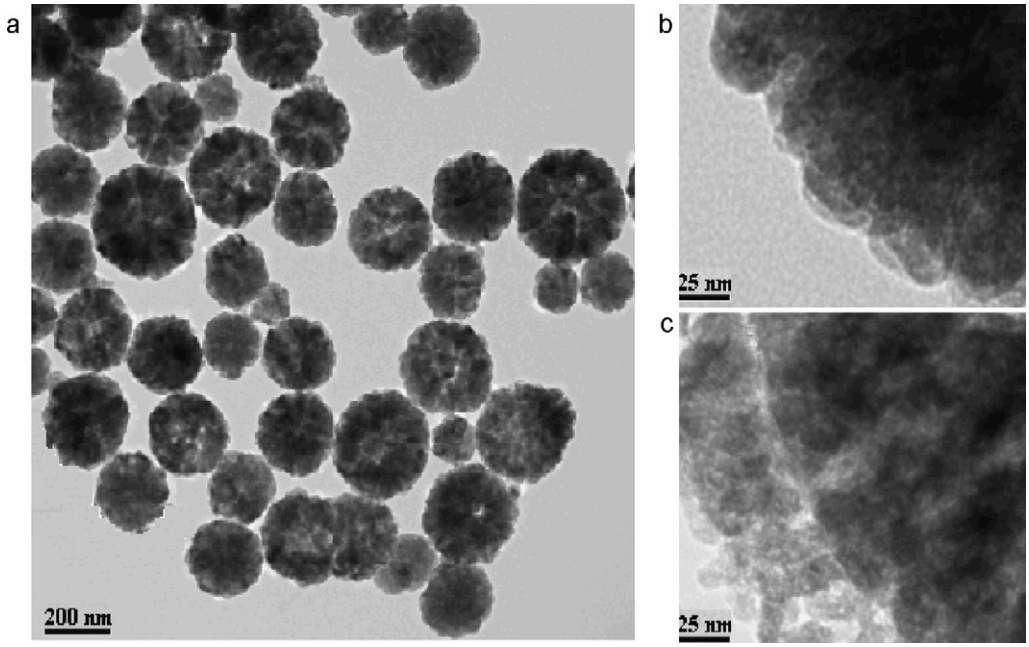

Fig. 1. TEM images of (a, b) bare Fe_3O_4 (c) and carboxyl modified magnetic particles.

20 min for proteins mixture and from 20 to 100 vol% in 80 min for real human serum. The analyte injection volume was 20 μL, and the column temperature was set at 35 °C. The samples were analyzed by a UV detector at a wavelength of 214 nm [21].

3. Results and discussion

3.1. The characterization of magnetic particles

TEM was employed to observe the morphological features of magnetic particles. From the TEM image (Fig. 1a), the bare Fe_3O_4 appeared to be spherical in shape and its diameter as estimated was about 200 nm, which was consistent with that obtained using same preparation method [23]. Comparing with Fig. 1b and c, it was clearly observed that the surface of the carboxyl modified magnetic particles had a polymer shell with defined shape and configuration with light contrast and the polymer shell had an average thickness of about 50 nm.

The FT-IR spectra of the bare Fe_3O_4 and the carboxyl modified magnetic particles were shown in Fig. 2. It was notable that the carboxyl modified magnetic particles showed a new peak at 1659.13 cm^{-1} corresponding to carbonyl group of monomers used in synthesis process. The wide peak at 3388.28 cm^{-1} was attributed to the characteristic absorption peak of hydroxyl group on MAA. The IR results proved that a polymer coating with carboxyl group has been successfully grafted from bare Fe_3O_4.

3.2. Adsorption of magnetic particles to standard proteins

Prior to integrating carboxyl modified magnetic particles with PEG/sulphate system, the adsorption of magnetic particles to proteins was characterized, which relied on the proteins isoelectric point and the solution pH and ionic strength.

3.2.1. Effect of pH

Solution pH is one of the most important factors in the adsorption process, which directly affects the dissociation degree of carboxyl groups and the charged nature of proteins. The adsorption of carboxyl modified magnetic particles to proteins with different isoelectric point (BSA, pI 4.9; Mb, pI 7.0; and Lyz, pI 11.2) was investigated at solution pH 4.2–9.5. As shown in Fig. 3, for each of the three proteins, there was an apparent protein adsorption at solution pH below its isoelectric point. When solution pH to higher than protein isoelectric point, their adsorption amount on magnetic particles was gradually decreased to a constant. The adsorption phenomenon of these proteins on the magnetic particles is consistent with the electrostatic interaction mechanism of ion exchange adsorption. For example, BSA with positive charge (at pH 4.2) bound to $-COO^-$ on magnetic adsorption surface, yet it was repelled electrostatically by $-COO^-$ due to bearing negative charge (at pH 6.1), so the adsorption amount of BSA was changed with pH from 4.9–6.1 significantly. Other two proteins (Mb and Lyz) showed the same tendency. Therefore, solution pH and protein isoelectric point had a crucial effect on the adsorption of the carboxyl modified magnetic particles to protein. The bare Fe_3O_4 showed weak non-specific adsorption due to a few hydroxyl groups existed on its surface, whose adsorption capacity to three proteins changed little with the pH change.

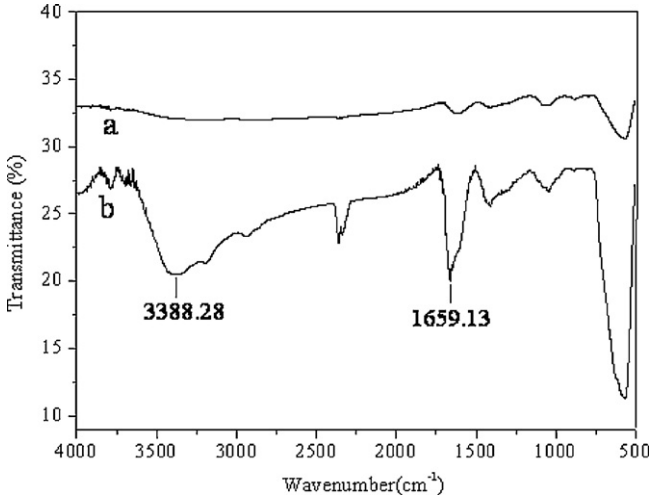

Fig. 2. FT-IR spectra of (a) bare Fe_3O_4 and (b) carboxyl modified magnetic particles.

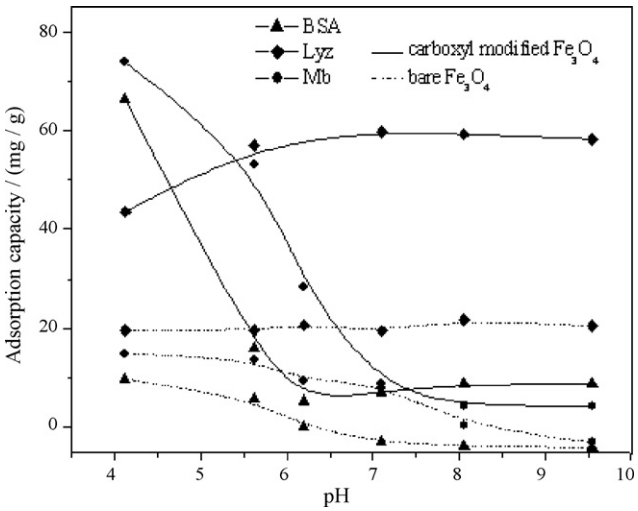

Fig. 3. Effect of pH on adsorption of proteins.

3.2.2. Effect of ionic strength

The effect of ionic strength on protein adsorption was investigated with NaCl at concentration of 0.05–0.5 mol/L (Fig. 4). When NaCl concentration was lower than 0.1 mol/L, the magnetic particles maintained an acceptable adsorption capacity. The adsorption capacities of the magnetic particles for BSA, Mb and Lyz reduced quickly to 7%, 7% and 15% at 0.2 mol/L NaCl, respectively. When NaCl concentration was higher than 0.4 mol/L, there was nearly no adsorption, which resulted from that ionic strength destroyed the electrostatic interactions between $-COO^-$ and proteins. So, the adsorption of the magnetic particles to proteins was significantly affected by salinity and ionic strength of solution, which should be considered in real samples application. Similarly, the adsorption capacities of bare Fe_3O_4 to three proteins had no apparent change with the increase of ionic strength.

3.2.3. Adsorption rate

A binding kinetic study had been carried out for the magnetic particles to determine the adsorption rate in separation process. This is an important consideration in the practical application of the magnetic materials in a separation process [24]. The adsorption kinetics of magnetic particles for BSA, Mb and Lyz under optimized pH showed a rapid increase, achieving 92% of the equilibrium adsorption capacity for BSA and ~85% for Mb and Lyz, during the first 6 min. With longer time, the adsorption rate slowed down and eventually reached adsorption equilibrium at 10 min. So, the prepared magnetic particles were suitable for protein fast separation.

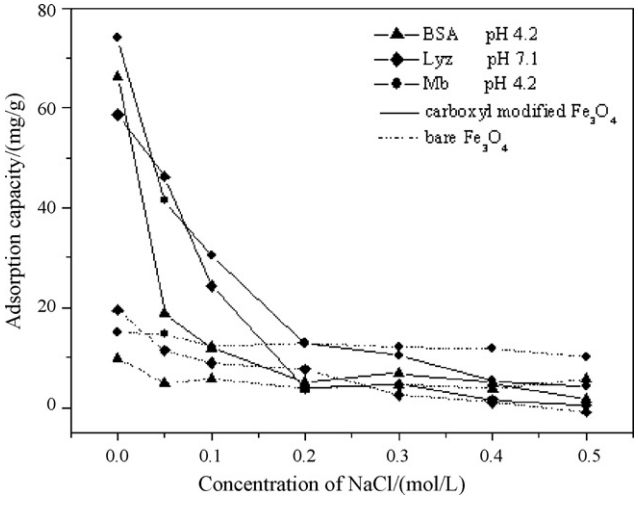

Fig. 4. Effect of ionic strength on adsorption of proteins.

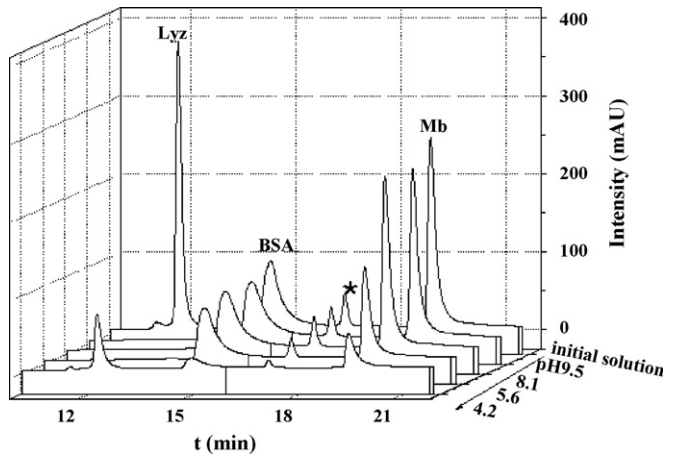

Fig. 5. Chromatograms of proteins before and after separation at different pH. The asterisk (*) denotes an impurity peak from standard Mb.

3.3. Separation of standard proteins mixture and human serum samples by magnetic particles

The adsorption of magnetic particles to the mixed proteins of BSA, Mb and Lyz showed the obvious difference, which was achieved by controlling solution pH (Fig. 5). Comparing with the initial solution, the peak of Lyz completely disappeared at pH 9.5, 8.1 and 5.6, while BSA had no apparent change at same pH. Mb peak and its impurity peak (peak*) decreased eventually with pH reduction from pH 9.5 to 5.6. However, at pH 4.2, the significant reduction of BSA and Mb indicated their adsorption at the lower pH than their pI, and their competition adsorption occupied the functional groups, which caused the adsorption decrease of Lyz. So, less amount of Lyz remained in solution at pH 4.2, which resulted in its small peak reappeared. Above results were in accordance with the expectation that proteins with pI > pH were absorbed by carboxyl group.

The carboxyl modified magnetic particles were also applied to 50-fold diluted human serum adsorption. When solution pH was controlled at 7.1–4.2, the total protein amount of human serum in solution decreased gradually. Some of the protein bands (protein 2, 3, 4 and albumin) became light obviously, but other bands (such as protein 1) had no evident change (showed in Fig. 6). It indicated that some proteins in human serum could be adsorbed to magnetic particles through pH adjustment. Fig. 4 showed that most of albumin (pI 4.7), which accounted for more than 50% by weight of the proteins in human serum, was adsorbed to magnetic particles at pH 4.2, so high abundant proteins with pI larger than solution pH could be removed. Above results illustrated that the carboxyl modified magnetic particles can be used to partly adsorb and separate some known proteins in human serum by simply solution pH adjustment. For a complex protein sample, magnetic particles modified by carboxyl group could be used to develop fast pI-based protein separation method.

3.4. Extraction and enrichment of standard proteins in ATPS

The difference of protein partition in ATPS was determined by the nature of proteins and the physicochemical characteristics of ATPS. The protein partition coefficient K and volume ratios R of top and bottom phases [20] were used to describe the partition.

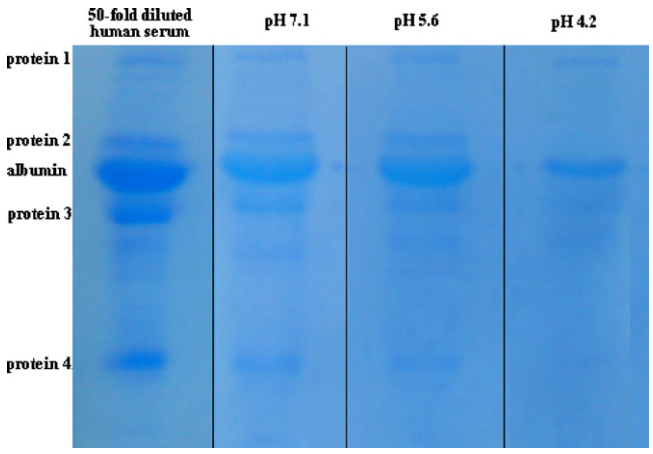

Fig. 6. SDS-PAGE analysis of human serum adsorbed by carboxyl modified magnetic particles.

K indicates the ratio of protein concentration in the top and bottom phase. For Lyz and Try, 0.103 and 0.245 values were obtained, which indicated that both Lyz and Try were distributed to two phases (Fig. 7a and b) even though the majority of Lyz was in the bottom phase. However, Cyt C, BSA and Mb were only allocated in the bottom phase (Fig. 7a), their *K* values were considered as zero since no protein peaks in top phase were detected (Fig. 7b). *R* was strongly dependent on solution pH, temperature and salt content of ATPS. In the used PEG/sulphate system (pH 7.1), *R* was 1.2, which indicated that the concentration of protein enriched in the system was roughly twice as much as the original solution without ATPS. Based on the different extraction capability of ATPS, the controllable partition of proteins in two phases was feasible [14].

3.5. Combination of magnetic adsorption and ATPE for protein mixture separation

The carboxyl modified magnetic particles separating proteins based on proteins' charged nature at certain pH. ATPE extracting proteins in two phases depended on the properties of ATPS such as surface charge, hydrophobic and hydrophilic group and hydrogen bond [22]. The combination of magnetic adsorption and ATPE showed advantages of each process and simplified separation

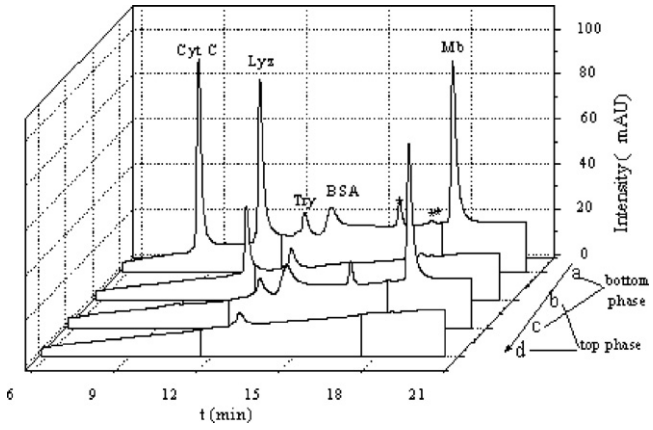

Fig. 7. Chromatograms for separation of five standard proteins using the combination of PEG/sulphate extraction followed by carboxyl modified magnetic particles adsorption. (a) 40-fold diluted bottom phase; (b) 25-fold diluted top phase; (c) 40-fold diluted bottom phase after adsorption by magnetic particles; (d) 25-fold diluted top phase after adsorption by magnetic particles. The asterisk (*) denotes an impurity peak from standard Mb and two asterisks (**) denote an impurity peak from other standard proteins.

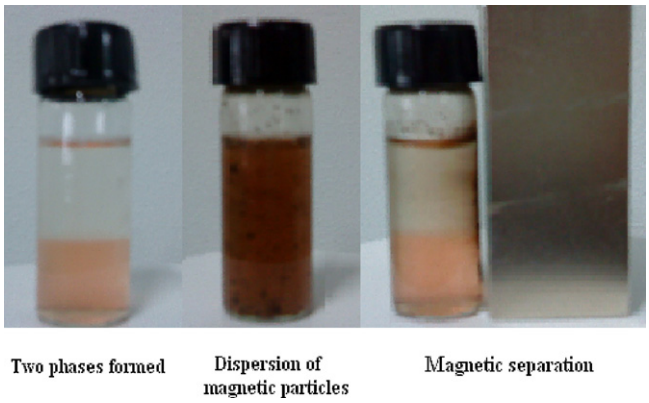

Fig. 8. The schematic process of ATPE followed by magnetic particles adsorption. The red-colored protein is used in order to obviously observe two phases formed.

process. Fig. 8 figured the combination process with liquid–liquid extraction, liquid–solid adsorption and magnetic separation.

3.5.1. ATPE followed by magnetic particles adsorption

The mixture of five proteins of Lyz, BSA, Mb, Cyt C (pI 10.2) and Try (pI 10.8) was first extracted by PEG-sulphate at pH 7.1, and then the magnetic particles were added into the two phases, respectively. After APTE, Cyt C, BSA and Mb only existed in the bottom phase (Fig. 7a), while Lyz and Try were in both phases with different partition (comparing Fig. 7a and b). Since the average concentration of ammonium sulphate in ATPS was roughly 0.75 mol/L, the high ionic strength of salt would affect the adsorption, so the top (PEG-rich) phase was 25-fold diluted and the bottom (salt-rich) phase was 40-fold diluted before the magnetic particles were added. Comparing Fig. 7a with Fig. 7c, Cyt C and Lyz peaks disappeared in bottom phase after adsorption, and Try, BSA and Mb peaks kept no change. Comparing Fig. 7b with Fig. 7d, Lyz disappeared in top phase after adsorption, and Try was adsorbed less, which was consistent with the results of bottom phase. In addition, the impurities from standard Mb and other proteins (marked with * and **) as unknown proteins also presented different partition and adsorption. Above results showed that the carboxyl modified magnetic particles adsorbed different proteins in both phases according to proteins nature. Therefore, proteins could be separated quickly from solution under magnetic field. The combination of ATPE followed by magnetic adsorption made five mixed proteins preliminarily separated into different groups according to their partition and adsorption properties. Due to the higher salt concentration in PEG/sulphate system, both phases needed to be diluted before magnetic adsorption, and the bottom phase as salt-rich phase required a higher dilution ratio. Moreover, we found the combination of ATPE followed by magnetic adsorption was helpful to inhibit BSA digestion caused by Try (mentioned in next).

3.5.2. Magnetic adsorption followed by ATPE

Reversely, the second way is the magnetic adsorption followed by ATPE. Here, four proteins of Lyz, BSA, Mb and Cyt C were used and Try was excluded since it made BSA digestion. When magnetic particles were added into the proteins mixture, Cyt C and Lyz were adsorbed completely and BSA and Mb remained in solution. Then adding the mixture of PEG and sulphate into the remained solution, the top and bottom phase were formed. BSA and Mb were fully allocated in the bottom phase, and their concentrations were significantly higher than the original solution. It indicated that Cyt C and Lyz could be separated by magnetic particles adsorption directly, and the remained BSA and Mb were enriched in bottom by ATPE. So ATPS made proteins partition and enrichment after magnetic adsorption. On the other hand, when adding the mixture of PEG

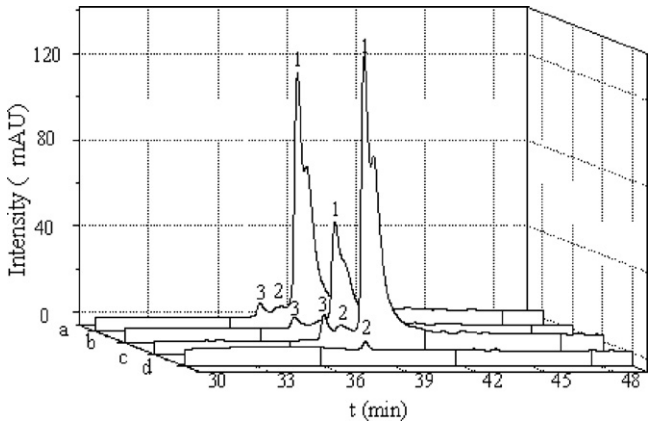

Fig. 9. Chromatograms for separation of human serum using the combination of carboxyl modified magnetic particles adsorption followed by PEG/sulphate extraction. (a) 50-fold diluted human serum; (b) 50-fold diluted human serum after adsorption by magnetic particles; (c) bottom phase; (d) top phase. (1) high abundant protein mixture; (2) unknown proteins; (3) unknown proteins.

and sulphate to magnetic particles, the adsorbed proteins would be eluted out and enriched. Here, ATPS was as elution solvent, which had been reported in our previous work [21].

Compared the two ways, both of them could separate and enrich target protein from mixture by adsorption and extraction. However, the way of magnetic adsorption followed by ATPE was more convenient to use without the dilution process.

3.5.3. Application for human serum samples separation

The magnetic adsorption followed by ATPE was applied to human serum samples. After adsorption by carboxyl modified magnetic particles (pH 4.2), the high abundance proteins (peak 1) in human serum decreased significantly and other high abundance proteins (peak 2 and 3) almost kept no change (comparing Fig. 9a and b). Here, the high abundant albumin (pI 4.7) was included in peak 1 as the standard albumin had the same retention time. Since, albumin with net positively charge at pH 4.2 could be adsorbed to carboxyl on magnetic particles, it could be removed under an extra magnetic field. According to the decrease of the peak area (peak 1) after magnetic particles adsorption, the adsorption amount of proteins could reach 35%. Subsequently, the remained human serum solution was extracted by PEG/sulphate, peak 1 and 3 mainly distributed in bottom phase (Fig. 9c), and their peaks were nearly double (comparing with Fig. 9b). It indicated that enrichment factor of PEG/sulphate system was nearly two, which can be improved by using optimized ATPS. Meanwhile, proteins (such as peak 2) were also enriched and distributed in both bottom and top phases, which were separated with other remained proteins (peak 1 and 3) and distributed in top phase (Fig. 9d). Therefore, after the removal of most of high abundant albumin by magnetic separation, PEG/sulphate re-partitioned the remained proteins and showed enrichment and separation capability.

Magnetic separation integrated with ATPE realized the rapid separation of high abundance proteins from human serum samples and the enrichment of remained proteins, which has a potential application in practical complex samples. However, due to the universality of co-adsorption, the co-adsorption of unknown proteins in magnetic adsorption process is not clear, which need to be studied further. Following above experiment results of standard protein mixture separation, the solution pH regulation based on proteins pI is one way to minimize co-adsorption.

4. Conclusions

Proteins could be separated from mixture based on electrostatic adsorption by the use of carboxyl modified magnetic particles and simple solution pH regulation. ATPE exhibited selective separation for proteins before and after magnetic adsorption. The combination of functional magnetic particles and ATPE not only had the virtue of selective adsorption and rapid magnetic separation, but also could selectively separate and enrich protein. Moreover, due to species diversity of functional magnetic particles and types diversity of ATPS, the combination of magnetic adsorption and ATPE could be extended to more application in complex samples.

Both magnetic adsorption and ATPE are simple and fast separation method. Their combination involves in the process of liquid–solid adsorption, liquid–liquid extraction and magnetic separation, which might be very promising in large-scale protein separation.

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