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Selective adsorption of bovine hemoglobin by Ni^{2+}-functionalized silica nanoparticles

Rao Fu[a,*] & Shan Jiang[b]

[a]Northeast Electric Power University, No.169, Changchun Street, Jilin city, Jilin province, 132011, China
[b]College of Chemical Engineering, Northeast Electric Power University, Jilin city, Jilin province, China

*E-mail: furao18604322849@sina.com

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In this work, we have used Stöber method to prepare 200 nm SiO_2 particles and then immobilized metal affinity ligand iminodiacetic acid (IDA) and charged them with Ni^{2+} ions. The particles show high absorption efficiency to bovine hemoglobin and the maximum extraction amounts reached 43.4 mg/g. The particles have been applied to separate a model protein mixture of bovine hemoglobin (BHb) and bovine serum albumin (BSA). They can be separated and showed low non-specific adsorption. Then we have used these particles to extract BHb from the mixture of BHb and DNA. They have potential applications in removing abundant protein in proteomic analysis, and we hope it may become a simple, convenient, and potentially effective way to purify hemoglobin in serum.

Keywords: Iminodiacetic acid (IDA), Protein, Bovine hemoglobin (BHb)

The separation and purity of the protein is a difficult and arduous task of biological technology. There are various procedures such as precipitation, chromatography, electrophoresis, ultrafiltration that can be used for purification of proteins[1,2,3]. Different types of chromatography have become dominant due to their high-resolution power[4-7]. Immobilized metal ion affinity chromatography (IMAC) is one of the most effective methods for the adsorption of proteins[8,9] since its first introduction by Porath[10]. IMAC introduces an interesting approach for selectivity of materials based on their affinities for chelated metal ions. Proteins are assumed to interact mainly through the imidazole group of histidine, and to a lesser extent, the indoyl group of tryptophan and the thiol group cysteine[11,12,13,14]. Protein retention on IMAC matrices is affected by a wide range of variables, such as the surrounding chemical environment, the nature of the chelating group, and the metal ion specificity[15,16,17,18].

Hemoglobin (Hb) has been used as a model protein for carrying out biophysical studies to understand binding to various kinds of ligands. It plays an important role in the vascular system of animals, being a carrier of oxygen. It also aids, both directly and indirectly, the transport of carbon dioxide and regulates the pH of blood[19]. It removes hydrogen ions in the capillaries and carries them to the lungs. Besides, it is involved in many clinical diseases such as leukemia, anemia, heart disease, excessive loss of blood, etc[20]. Hb has a molecular weight of approximately 67,000 kDa and contains two a and two b subunits, each of which has one redox iron heme as its prosthetic group, the heme is located in crevices at the exterior of the subunit[21]. Hb is the major hemoprotein of red blood cell (RBC), which can reversibly bind with many kinds of endogenous and exogenous agents. With the development of biotechnology and biomedicine, the requirement for Hb is higher and higher, and it is necessary to explore a more scientific and effective method for the purifying of Hb[22,23].

In this work, we present a simple pathway to synthesize silica nanoparticles with immobilized metal affinity ligand iminodiacetic acid (IDA) charged with Ni^{2+} for fast and efficient removal of bovine hemoglobin (BHb) from the mixture of BHb, BSA and DNA, which simulated conditions of the blood. We also used SiO_2 particles charged with Fe^{3+} ions to remove the impurities of DNA in the mixture of DNA and BHb, which further purified the BHb. The particles we prepared were expected to be used for fast and efficient removal of abundant protein

BHb in bovine blood, and it may be a potentially effective way to deplete abundant protein in serum.

Materials and Methods

Materials

Ferric chloride ($FeCl_3$•$6H_2O$) was purchased from ACROS. Nickelous sulfate ($NiSO_4$•$6H_2O$) was purchased from Tianjin Bodi Chemical Company. Iminodiacetic acid (IDA), glycidoxypropyltrimethoxysilane (GLYMO), salmon sperm DNA, bovine hemoglobin (BHb), bovine serum albumin (BSA) and ethylenediaminetetraacetic acid (EDTA) were purchased from Aldrich. 1-(2-Pyridylazo)-2-naphthol (PAN) was purchased from Sinopharm Chemical Reagent Company. Anhydrous ethanol, ammonium hydroxide (25wt.%NH_3 in water), tetraethoxysilane (TEOS), Ethanol, cetyltrimethylammonium bromide (CTAB), Disodium hydrogen phosphate (Na_2HPO_4), Sodium hydroxide (NaOH) and Hydrochloric acid (HCl) were obtained from Beijing Chemical Company. Imidazole, Agarose, ethidium bromide (EB), DNA marker, acrylamide, sodium dodecyl sulfate (SDS), ammonium persulfate, tris base, Glycine, N, N, N ', N '- tetramethyl ethylenediamine (TEMED), coomassie brilliant blue, protein marker, methanol, acetic acid, and disodium hydrogen phosphate were purchased from DingGuo biological technology Art co., LTD. All reagents were of analytical grade or better. TEOS was distilled before use. Other chemicals were used without further purification. High-purity water (Pall Purelab Plus) with a resistivity of 18Mcm was used in all the experiments.

Preparation and surface modification of silica nanoparticles

Silica (SiO_2) particles were synthesized by the Stöber method[24,25,26,27] and IDA was modified on the silica particle surface by a condensation process [28]. First, IDA was reacted with 3-glycidoxypropyltrimethoxysilane (GLYMO) to form an IDA derived silicon coupling agent. Then this coupling agent was hydrolyzed and condensed with silica particles. The amount of the IDA derived silicon coupling agent was in large excess to ensure full coverage of IDA on the surface of the particles. The content of IDA on the surface of the particles was estimated by a colorimetric method[29]. 1 mg of IDA–silica particles were mixed with 300 µl $NiSO_4$ (10 µg/ml) aqueous solution for 1 h and the mixture was centrifuged to remove the silica particles. The amount of Ni^{2+} ions remained in the supernatant was determined by adding PAN and measuring the absorbance of the Ni–PAN complex solution at 570 nm. The amount of Ni^{2+} ions adsorbed on the particle surface was acquirable by subtracting the amount of Ni^{2+} ions in the supernatant from that of Ni^{2+} ions added.

Loading of Ni^{2+} ions on the IDA–silica particles was carried out by mixing 20 mg of IDA–silica particles with 50 ml of an aqueous solution of $NiSO_4$ (0.1 M). The mixture was stirred at 100 rpm for 1 h and then the resulting Ni^{2+} immobilized IDA–SiO_2 (Ni^{2+}-IDA-SiO_2) particles were collected by centrifugation and then washed by water to remove the excess Ni^{2+}. Loading of Fe^{3+} ions was carried out by using the same procedure for the immobilization of Ni^{2+} ions. It was noted that the amount of the metal ions (Ni^{2+}, Fe^{3+}) added was in large excess to ensure that the same amount of metal ions was loaded on the particle surface[26,27].

Selective removal of bovine haemoglobin from the mixtures

A binary protein mixture of BHb and BSA (200 µg/mL for BHb and BSA, respectively), a mixture of BHb and DNA (100 µg/mL for DNA and 200 µg/mL for BHb), and the mixture of BHb, BSA and DNA (100 µg/mL for DNA, 200 µg/mL for BHb and BSA, respectively) in a solution of 0.2 M phosphate-buffered saline (PBS) at pH 8.0 were mixed vigorously with 2 mg Ni^{2+}-IDA-SiO_2 nanoparticles at room temperature for 2 h, respectively. After 2 h, the particles were centrifugated and obtained the supernatant. Then the particles collected by the centrifugation were re-dispersed in 1 M solution of imidazole and the mixture was incubated with shaking at room temperature for 2 h. After centrifugation, we obtained the supernatant. The mixture before extraction by Ni^{2+}-IDA-SiO_2 and the above supernatants were measured by a UV–visible spectrophotometer, SDS-PAGE, and agarose gel electrophoresis analysis.

Characterizations

UV-visible absorption spectra were collected with a Varian Cary-100 scan UV-visible spectrophotometer. Dynamic light scattering (DLS) measurements were performed using a particle size analyzer (BI-90 Plus, Brookhaven Instruments) with a scattering angle of 90°. Transmission electron microscope (TEM) images were observed using a Hitachi H-8100IV electron microscope at 200 kV by using a carbon-coated copper grid as substrates. Samples were prepared by drop casting of the colloidal solution (the aliquots were taken during the course of a reaction) onto a Formvar-coated copper

grid. DNA and protein electrophoresis apparatuses were both of DYY-6-c model electrophoresis apparatus. All the experiments were carried out at room temperature (25 ± 2°C).

Results and Discussion

Typical TEM images of Ni^{2+} immobilized IDA–SiO_2 (Ni^{2+}-IDA-SiO_2) particles are shown in Fig. 1. The dispersity and diameter of IDA–silica nanoparticles showed little change compared with SiO_2 nanoparticles, indicates that a silicon coupling agent had little influence on the morphology of SiO_2 nanoparticles.

The IDA–silica nanoparticles were used to load Ni^{2+} ions onto the surface of particles. The amount of Ni^{2+} ions remained in the supernatant was determined by adding PAN and measuring the absorbance of the Ni–PAN complex solution at 570 nm[27], as shown in Fig. 2. The amount of Ni^{2+} ions adsorbed on the particle surface was acquirable by subtracting the amount of Ni^{2+} ions in the supernatant from that of Ni^{2+} ions added. We used absorption at 570 nm as the vertical and the concentration of Ni^{2+} ions as the abscissa to draw the standard curve (Fig. 3). Assuming that each IDA is coordinating with one Ni^{2+} ion, the content of IDA on the surface of IDA–SiO_2 particle was determined to be 38.6 μmol/g. The removal of BHb by Ni^{2+}-IDA-SiO_2 particles from mixtures such as BSA and BHb, DNA and BHb, DNA, BSA and BHb were investigated. The key to protein extraction is the relationship between pH and the isoelectric point of the protein. We can control the pH of the solution and use the difference of protein IEP to separate or purify proteins by coordinating with the particle surface. Considering bovine serum albumin (BSA) and bovine hemoglobin (BHb) in phosphate solution of pH 8.0 as an example, the mixture before extraction by Ni^{2+}-IDA-SiO_2 was as supernatant A, the supernatant after extraction by Ni^{2+}-IDA-SiO_2 was as B and the supernatant of desorption by imidazole was as C. We measured the

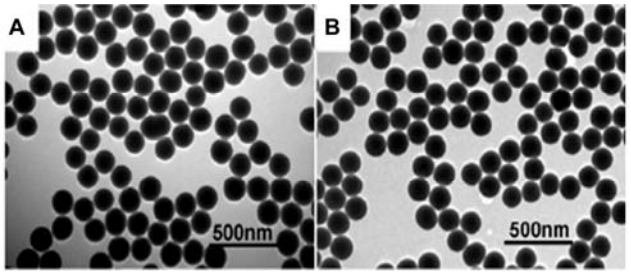

Fig. 1 — TEM images of (A) sillia nanospheres and (B) IDA-SiO_2 nanospheres (the content of IDA: 38.6μmol/g)

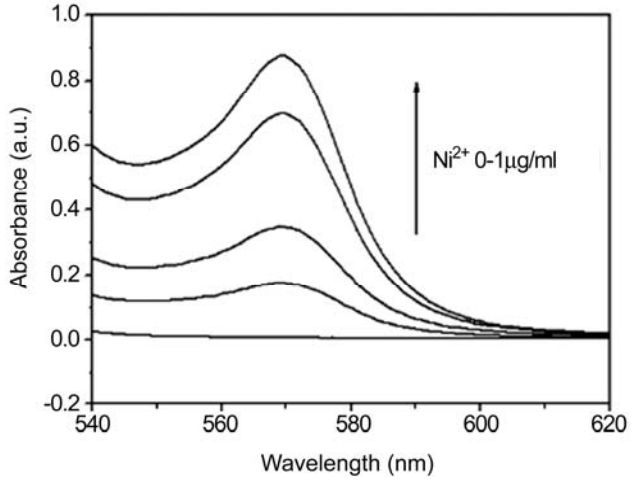

Fig. 2 — Absorption spectra of Ni^{2+}-PAN at different Ni^{2+} concentrations

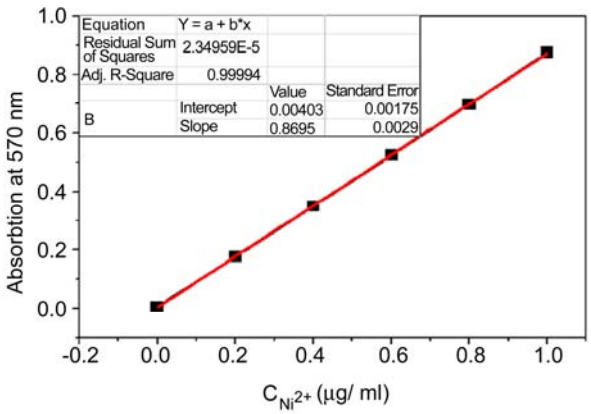

Fig. 3 — Standard curve of 570 nm absorption and Ni^{2+}concentration

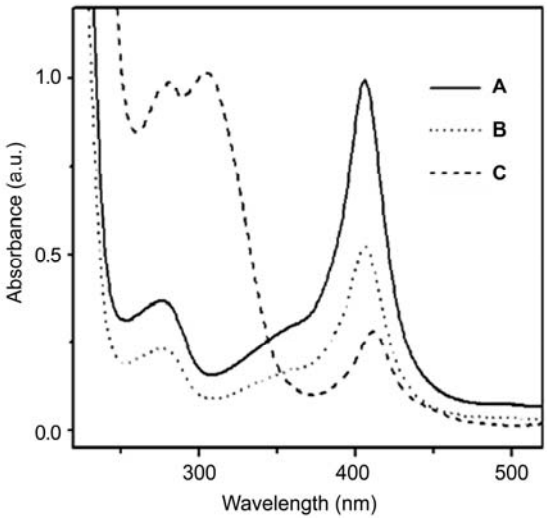

Fig. 4 — Absorption spectra of the mixture of BSA and BHb before (A) and after (B) extraction by Ni^{2+}-IDA-SiO_2 and (C) desorption by imidazole

absorption spectra of them, as shown in Fig. 4. The absorbance of BHb at 406 nm decreased obviously, and a strong absorption peak was also emerged in the supernatant of desorption by imidazole. But as is known, both of the two proteins have absorption peak at 280 nm, while it was covered by the absorption peak of imidazole, we can't judge whether BSA has existed in the adsorption solution. So, the supernatants of A, B and C were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the results are shown in Fig. 5.

From the SDS-PAGE analysis, it could be seen that only the BHb band faded (lanes b) in comparison with BSA after treatment with Ni^{2+}-IDA-SiO_2 particles. The desorption solution by imidazole only showed the BHb band (lanes c) which demonstrated that the Ni^{2+}-IDA-SiO_2 nanoparticles displayed specific absorption to BHb at pH = 8.0. The adsorption of protein onto the immobilized metal affinity adsorbents is ascribed to specific interactions. The specific interaction occurred between the exposed histidine residues on the protein surface and the immobilized metal ions. Proteins bind to immobilized Ni^{2+} chelated largely through the coordination of histidine residues to metal ions and the binding is more likely to be affected by the number of histidine residues in the protein and their accessibility[30]. The number of accessible histidine residues on BHb was 24, while the histidine residues number of BSA was only two. The Ni^{2+}-IDA-SiO_2 nanoparticles would assume a more negative character at pH 8.0. Also, the negatively charged amino acid residues on BHb (pI = 7.5) and BSA (pI = 4.8) are unlikely to be directly involved in the interaction with Ni^{2+}-IDA-SiO_2 nanoparticles. In this case, the BHb was most removed by the particles, while BSA was little removed. The results fully indicated the efficient binding of BHb on the Ni^{2+}-IDA-SiO_2 nanoparticles. The removal of BHb by Ni^{2+}-IDA-SiO_2 particles from mixtures of BSA, DNA, and BHb was investigated. We measured the absorption spectra of the mixture, as shown in Fig. 6. The absorbance of BHb at 406 nm decreased obviously after extraction (Fig. 6B), which was also existed in desorption by imidazole (Fig. 6C). While the absorbance of DNA at 260 nm in desorption by imidazole declined slightly (B), but as was seen, the absorption peak was covered by the adsorption peak of imidazole, we can't judge whether DNA has existed in the adsorption solution.

So, the supernatants of A, B and C were analyzed by both SDS-PAGE and agarose gel electrophoresis. The results were shown in Figs 7 and 8. From the

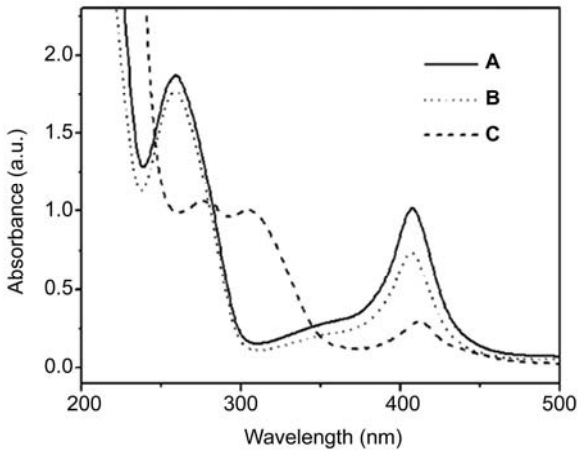

Fig. 6 — Absorption spectra of the mixture of DNA and BHb (A) before and (B) after extraction by Ni^{2+}-IDA-SiO_2 and (C) desorption by imidazole

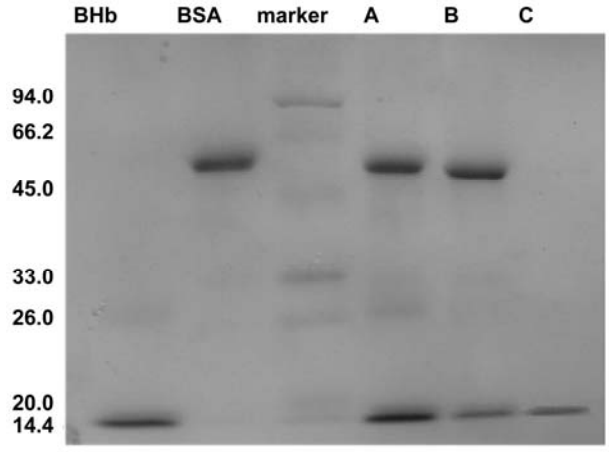

Fig. 5 — Protein gel electrophoresis of mixture of BSA and BHb (A) before and (B) after extraction by Ni^{2+}-IDA-SiO_2 and (C) desorption by imidazole

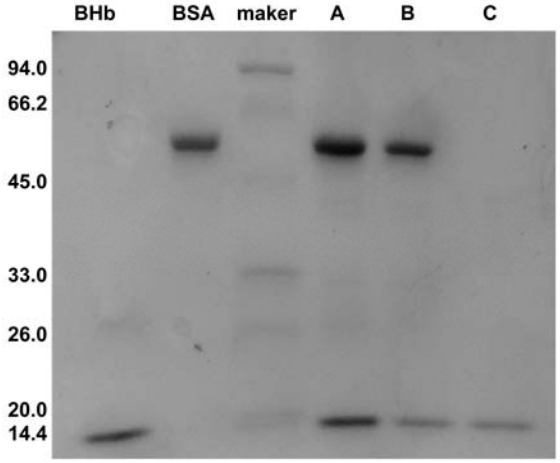

Fig. 7 — Protein gel electrophoresis of the mixture of DNA and BHb (A) before and (B) after extraction by Ni^{2+}-IDA-SiO_2 and (C) desorption by imidazole

SDS-PAGE analysis, it could be seen that all of the supernatants of A, B and C showed the BHb band which demonstrated that the Ni^{2+}-IDA-SiO_2 nanoparticles can adsorb BHb from the mixture. While only the BHb band faded (lanes B) in comparison with BSA after treatment with Ni^{2+}-IDA-SiO_2 particles.

The desorption solution by imidazole showed no band of the BSA (lanes C) which demonstrated that the Ni^{2+}-IDA-SiO_2 nanoparticles displayed no absorption to BSA at pH = 8.0. Fig. 8 shows that there was no DNA band in the desorption solution by imidazole, which indicated that the Ni^{2+}-IDA-SiO_2 nanoparticles displayed no absorption to DNA in the mixture of BHb and DNA at pH 8.0. BHb bounded to immobilized Ni^{2+} chelated largely through the coordination of histidine residues to metal ions while DNA has the little coordinate capacity with Ni^{2+} ions and both DNA and Ni^{2+}-IDA-SiO_2 nanoparticles were negatively charged at pH 8.0, DNA was unlikely to be adsorbed on to Ni^{2+}-IDA-SiO_2 nanoparticles through Electrostatic interaction. So, the Ni^{2+}-IDA-SiO_2 nanoparticles displayed specific absorption to BHb in the mixture of BHb, BSA, and DNA at pH 8.0.

We also used Ni^{2+}-IDA-SiO_2 particles to investigate the adsorption behavior to the mixture of DNA and BHb. BHb with a concentration of 100 μg/mL and DNA of 25 μg/mL were mixed and adjusted pH value to 4.0, which was defined as supernatant 1. First, the supernatant was mixed vigorously with 2 mg Fe^{3+}–IDA–SiO_2 nanoparticles at room temperature for 2 h respectively. After 2 h, the particles were centrifugation to obtain supernatant 2. Then the particles collected by the centrifugation were re-dispersed in solutions of pH 7.0 and 9.0, respectively and the mixture was incubated with shaking at room temperature for 2 h. After centrifugation, we obtained the supernatant 3 and 4. The above supernatants were measured by a UV–visible spectrophotometer, SDS-PAGE, and agarose gel electrophoresis analyses are shown in Figs 9, 10 and 11, respectively.

Combined the results of Figs. 9, 10 and 11, we can see, both parts of BHb and DNA was adsorbed on the particle surface when pH 4.0; when pH adjusted to 7.0, both BHb and DNA were adsorbed on the particle

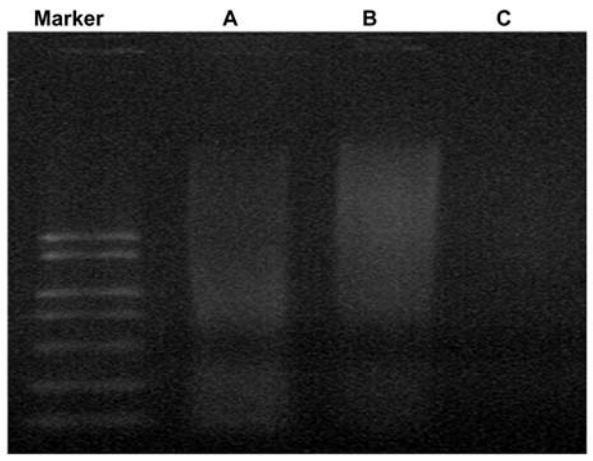

Fig. 8 — Agarose gel electrophoresis of the mixture of DNA and BHb (A) before and (B) after extraction by Ni^{2+}-IDA-SiO_2 and (C) desorption by imidazole

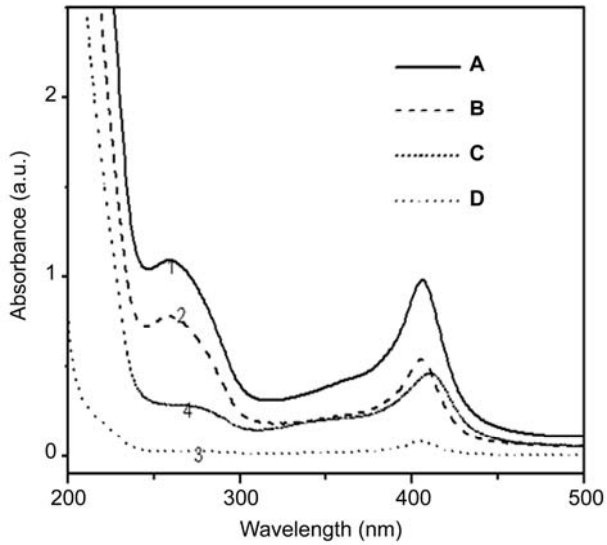

Fig. 9 — Absorption spectra of BHb (A) before and (B-D) after extracted by Fe^{3+}-IDA-SiO_2 particles at different pH (b-pH 4.0; c-pH 7.0; d-pH 10.0)

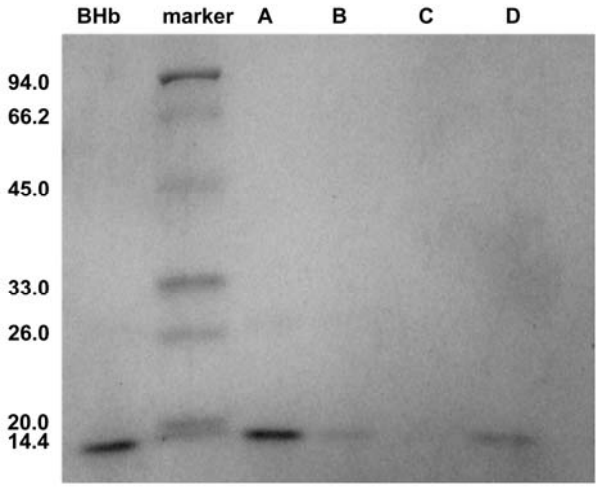

Fig. 10 — Protein gel electrophoresis of BHb and DNA before (A) and (B-D) after extracted by Fe^{3+}-IDA-SiO_2 particles at different pH (b-pH 4.0; c-pH 7.0; d-pH 10.0)

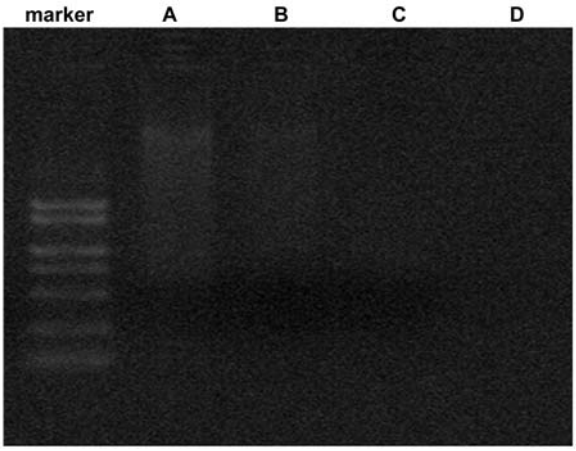

Fig. 11 — Agarose gel electrophoresis of BHb and DNA (A) before and (B-D) after extracted by Fe^{3+}-IDA-SiO_2 particles at different pH (b-pH 4.0; c-pH 7.0; d-pH 10.0)

surface entirely; when pH was 10.0, DNA had nearly no desorption, but BHb desorbed to the solution partly. It was demonstrated that we can separate BHb and DNA by adjusting pH values. As we have known[3], the best condition for adsorption of DNA by Fe^{3+}–IDA–SiO_2 particles was pH 4.0. At this pH value, the charge of BHb whose IEP is higher was in contrast to the particle surface and didn't compete with DNA, so DNA could be adsorbed on the particle surface. For the mixture of BHb and DNA, when pH was 7.0, the charge on BHb decreased and was adsorbed on the particle surface; when pH was higher to 10.0, BHb was negative charged and the adsorbed BHb was released to solution due to the electrostatic repulsion between the particle surface and BHb. While DNA could be still absorbed effectively on the surface of particles via direct electrostatic interaction due to the salt in the solution left by adjusting pH values. So we can acquire BHb with higher purity through adjusting pH values to extract a little DNA in the mixture by Fe^{3+}–IDA–SiO_2 nanoparticles.

Conclusions

In summary, we present a simple method to synthesize SiO_2 nanoparticles with immobilized IDA charged with Ni^{2+} (Fe^{3+}) ions. Ni^{2+}-IDA-SiO_2 nanoparticles showed specific absorption to BHb in different mixtures of such as BHb and BSA, BHb and DNA, BHb, BSA and DNA at pH 8.0. Fe^{3+}–IDA–SiO_2 nanoparticles extract a little DNA in the mixture of DNA and BHb, which can obtain a higher purity of BHb. We hope it may become a simple, convenient, and potentially effective way to purify haemoglobin in serum.

Conflict of interest

The authors declare no conflict of interests in this study.

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