Fabrication and characterisation of chitosan-magnetic nanoparticles and its application for protein extraction

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ABSTRACT

Efficient extraction of proteins by removing interfering materials is necessary in proteomics, since most instruments cannot handle such contaminated sample matrices directly. In this study, chitosan-coated magnetic nanoparticles (CS-MNPs) for purification of myoglobin were successfully fabricated. First, chitosan (CS) was prepared by a deacetylation reaction during its extraction from shrimp-shell waste. Second, magnetic nanoparticles (MNPs) were synthesised, using the coprecipitation method, from aqueous Fe^{2+} and Fe^{3+} salt solutions by the addition of a base under an inert atmosphere, followed by modification of the surface of MNPs with chitosan. The morphology of the formed nanoparticles, which were about 23 nm in average diameter,was observed by transmission electron microscopy (TEM). In addition, nanoparticles were characterised using X-ray diffraction patterns (XRD), which showed the naked magnetic nanoparticles have a spinel structure and the surface modification did not result in phase change of the Fe_3O_4. The coating of MNPs was also demonstrated by scanning electron microscopy (SEM) analysis, energy dispersive analysis of X-ray spectroscopy (EDAX), andFouriertransform infrared (FT-IR)spectroscopy. The adsorption behaviour of MNPs and CS-MNPs towards myoglobin was investigated. It was found that the difference in adsorption capacity between MNPs and CS-MNPs was larger for CS-MNPs. This result makes CS-MNPs good adsorbents and attractive for using in protein extraction from biological samples.

Key words: Chitosan; Fe_3O_4 magnetic nanoparticles; coprecipitation; myoglobin; adsorption.

1. Introduction

Commonly, the process of the biological sample preparation is complicated, labour-intensive, and time consuming [1]. The main aim of the sample preparation method is to remove interfering materials and preconcentrate the proteins. As a result, the signal intensities of the

target analytes will be improved and the quantities of the target analytes can be easily measured [2, 3].

An ideal sample preparation procedure should be as simple as possible and fast. The procedure should be reproducible with high extraction recovery of the target analytes. In addition, it should require a minimum number of processing steps, minimise solvent consumption, be environmentally friendly, and have potential for use in on-line method arrangement [4, 5].

Magnetic nanoparticles (MNPs) are a type of nanoparticle that can be manipulated using a magnetic field [6]. MNPs attract more attention due to their large surface area, high magnetic properties, high coercivity, and lack of toxicity. Moreover, MNPs are rapidly prepared, have high separation efficiency, cost-effectiveness, and a simple operation process [7]. They are utilised in applications such as magnetic drug targetting, magnetic resonance imaging for clinical diagnosis, recording material, and biomedical applications [8-11].

Modification of the surface of inorganic materials with organic components has attracted considerable attention. Chitosan (CS) is becoming increasingly important among natural polymers because it is abundant, soluble in aqueous media, biocompatible in nature, biodegradable, non-toxic, and low cost [12]. Chitosan is a polysaccharide composed of β-(1,4)-2-amino-2-deoxy-D-glucose. It is a deacetylated product of chitin and is characterised by its molecular weight and the degree of acetylation [13, 14]. It is widely applied in molecular separation, electrochemical sensors and biosensors, bone substitutes and water treatment [12, 15, 16].

Using chitosan-magnetic nanoparticles as adsorbent for myoglobin has not been studied. Therefore, the present work describes preparation of chitosan and synthesis of magnetic nanoparticles, followed by coating of the fabricated material with chitosan. After synthesis, the fabricated materials were characterised using the techniques of TEM,SEM, EDAX, XRD and FT-IR spectroscopy. The performance of the fabricated material as an efficient adsorbent for standard protein, which was myoglobin, from aqueous solution was investigated. Moreover, reusability of CS-MNPs was tested.

2. Experimental

2.1. Chemicals and materials

Ammonium hydroxide (NH_4OH) solution (25%), iron (II) chloride tetrahydrate ($FeCl_2.4H_2O$), iron (III) chloride hexahydrate($FeCl_3.6H_2O$) and myoglobin equine skeletal muscle were purchased from Sigma-Aldrich (Nottingham, UK) and used without further purification. Shrimp-shell waste was purchased from a local supermarket in Taif, Saudi Arabia. Sodium hydroxide (NaOH), hydrochloric acid (HCl), sodium chloride, phosphate buffered saline, and acetic acid were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Cylindrical rod magnets (40 mm diameter x 40 mm thick) for settlement of magnetic nanoparticles were purchased from Magnet Expert Ltd. (Tuxford, UK). Distilled water was employed for preparing all the solutions and reagents.

2.2. Instrumentation

A hot-plate stirrer from VWR International LLC (West Chester, PA, USA) was used. The Brunauer-Emmett-Teller (BET) model used a Surface Area and Porosity Analyser from Micromeritics Ltd. (Dunstable, UK). The transmission electron microscopy (TEM) was from JEOL Ltd. (Welwyn Garden City, UK). The scanning electron microscope, for energy dispersive X-ray spectroscopy (SEM-EDAX), was a Cambridge S360 from Cambridge Instruments (Cambridge, UK). The UV-Vis spectrophotometer came from Thermo Scientific™ GENESYS 10S (Toronto, Canada) and the X-ray diffraction (XRD) equipment from Rigaku Ltd. (Ettlingen, Germany). A WiseTherm high-temperature muffle furnace from Wisd Laboratory Instruments (Wertheim, Germany) was used. The FT-IR spectra were collected in the attenuated total reflectance (ATR) mode using a PerkinElmer RX FTIR ×2 with diamond ATR, DRIFT attachment from PerkinElmer (Buckinghamshire, UK).

2.3 Fabrication of chitosan-magnetic nanoparticles (CS-MNPs)

2.3.1. Preparation of chitosan (CS)

Shrimp-shell wastes were washed with distilled water and dried in sun for two days. After drying, the materials were ground into small pieces. Then, they were mixed with sodium hydroxide solution (40 %, w/v) in order to get a ratio of solid to alkaline solution of 1 to 15 (w/v). The temperature of reaction was kept at 100 ºC and refluxed under nitrogen atmosphere for eight hours to remove the acetyl groups from amino groups. After that, the prepared chitosan was dissolved in acetic acid solution (10 %) in order to get a ratio of solid to acidic solution of 1 to 15 (w/v). Then, the mixture was stirred at 500 rpm overnight. The mixture was centrifuged and the supernatant was moved to a beaker, Then, NaOH solution (5%) was added drop-by-drop to the supernatant to precipitate the chitosan. Finally, the prepared chitosan was washed with distilled water until the pH became neutral and it was kept in an air-tight container [17].

2.3.2. Synthesis of Fe_3O_4 nanoparticles

Magnetic nanoparticles were fabricated using the coprecipitation method. Briefly, 4.58 g of $FeCl_2.4H_2O$ and 8.93 g of $FeCl_3.6H_2O$ were dissolved under inert atmosphere in 80 mL of distilled water with vigorous stirring (1,100 rpm). The mixture was heated to 80 ºC, and then 10 mL of ammonium hydroxide (NH_4OH) solution was added to the mixture drop by drop. To ensure complete growth of nanoparticle crystals, the reaction was carried out for 30 minutes under constant stirring. After that the mixture was cooled to room temperature and the suspension was washed with distilled water. Finally, the formed magnetic nanoparticles were collected from unreacted materials using a magnet and dried in an oven at 50 °C.

2.3.3. Surface modification of MNPs with chitosan

An amount of 0.5 g of magnetic nanoparticles was added to 20 mL of chitosan prepared in acetic acid solution (2 %, w/v). The mixture was sonicated for four hours at room temperature. The CS-MNPs were collected from unreacted materials using a permanent magnet, then, the materials were washed with ethanol followed by distilled water and, finally, they were dried in an oven at 40°C for 24 hours before use.

2.4. Characterisation of the fabricated materials

The fabricated material was characterised using techniques such as TEM analysis, and SEM analysis. In addition, the compositional analysis was performed using energy dispersive analysis of X-ray spectroscopy (EDAX). For phase identification and structural analysis of the

magnetic nanoparticles, an X-ray diffraction (XRD) instrument was utilised. Fourier transform infrared spectroscopy (FT-IR) of chitosan, MNPs, and chitosan-MNPs was collected to confirm the presence of chitosan on the surface of the magnetic nanoparticles.

2.5. Adsorption of myoglobin

A protein solution (2 mg L^{-1}) was prepared in phosphate buffer solution (20 mM, pH 5-11) and 4 mL of this solution was mixed with 20 mg of chitosan-MNPs. The mixture was shaken at 200 rpm for a certain time. After that the supernatant was separated using a magnet for 30 seconds. The change in the initial concentration of myoglobin was measured by monitoring the supernatant obtained at 280 nm using the UV-Vis spectrophotometer. Each experiment was repeated three times. The amount of myoglobin adsorbed on to chitosan-MNPs was calculated using the following equation:

$$q_e = \frac{C_o - C_e}{W} \times V[18]$$

Where q_e is the adsorption capacity (mg g^{-1}), W is the weight of the adsorbent used (g), and V is the volume of protein solution (mL), C_o and C_e are the initial and equilibrium liquid phase concentration of myoglobin, respectively (mg mL^{-1}).

2.6. Reusability of CS-MNPs

The reusability of MNPs coated with chitosan was checked by repeating the adsorption and desorption cycle of myoglobin five times using the same batch materials. The elution of protein was performed using 20 mM phosphate buffer solution (pH 5) containing 0.5 M of sodium chloride and the mixture was stirred for one hour at a speed of 200 rpm at room temperature. The preconcentrated myoglobin in elution solution was measured using the UV-Vis spectrophotometer at 280 nm. The elution ratio was found using the following expression:

$$\text{Elution ratio } (\%) = \frac{\text{amount of myoglobin eluted}}{\text{amount of myoglobin adsorbed on CS}-\text{MNPs}} \times 100[19]$$

3.Results and discussion

3.1.Preparation of CS-MNPs

Modification of the surface of magnetic nanoparticles can decrease the toxicity and undesired biological interactions [20]. Chitosan has unique characteristics such as biocompatibility, biodegradability and nontoxicity, and it has a high affinity for proteins [21]. The aim of this work was fabrication of chitosan-magnetic nanoparticles in order to use them as adsorbent for proteins. In this study, magnetic nanoparticles coated with chitosan were fabricated in two steps. The first step was preparation of chitosan from shrimp-shell waste by the deacetylation process. The second step was fabrication of naked magnetic nanoparticles using the coprecipitation method from aqueous Fe(II) and Fe(III) salt solutions by the addition of a base under inert atmosphere. After fabrication of naked magnetic nanoparticles, the magnetic nanoparticles were coated with chitosan by mixing chitosan and Fe_3O_4 aqueous slurry. The

prepared chitosan was dissolved in acetic acid since chitosan is not soluble in aqueous solution, but it is soluble in acidic solution [22].

3.2.Characterisation of CS-MNPs

The fabricated materials were characterised using techniques such as TEM,SEM, XRD,EDAX, and FT-IR spectroscopy.

3.2.1 TEM analysis

The fabricated materials were characterised using TEM analysis in order to know themorphology and the particle size of CS-MNPs. Figure 1 shows the TEM images of CS-MNPs. It can be seen that the structure of the fabricated materials is spherical in shape and has a mean diameter of 23 nm. It is known that if the MNPs have less than about 25 nm, they show super paramagnetism [21]. This means that the fabricated CS-MNPs have supermagnetic properties. However, the MNPs' interface observed by TEM lacked clarity, which could be a result of the coating with the chitosan layer. The same result was observed by other groups [23].

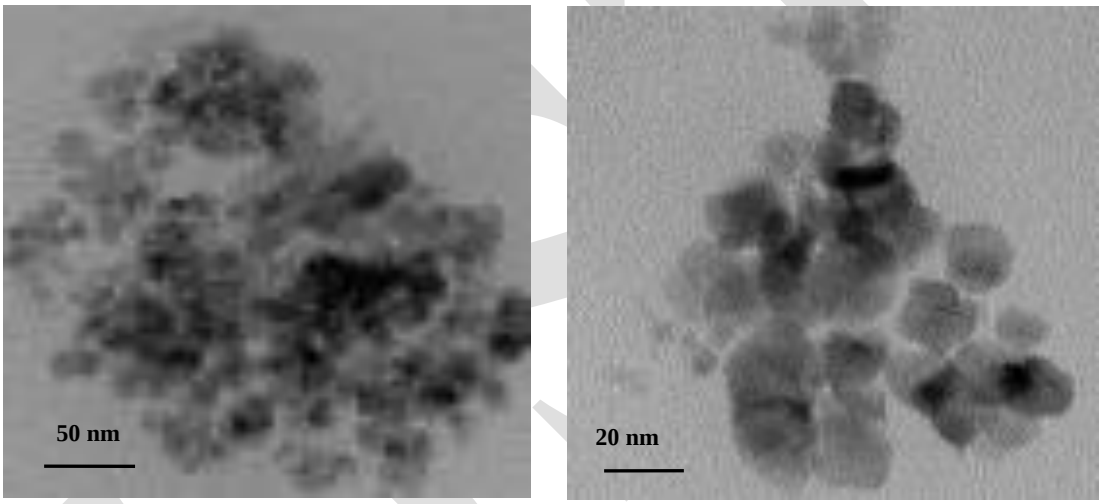

Fig. 1 TEM micrographs of magnetic nanoparticles coating with chitosan.

3.2.2. SEM analysis

The morphology and structure of chitosan and CS-MNPs were studied by SEM analysis, as can be seen in Figure 2. From the figures, it can be seen there is a huge difference in the surface morphology and roughnessbetween chitosan, Figure 2 (A), and magnetic nanoparticles coated with chitosan, Figure 2 (B). The SEM measurement showed that chitosan had a homogeneous smooth surface while CS-MNPs possessed a three-dimensional morphology and both were in the nano range.

3.2.3. XRD analysis

The XRD patterns of the magnetic nanoparticles coated with chitosan was studied. Figure 3 shows the six character peaks of Fe_3O_4 nanoparticles were observed at $2\theta = 30°$, 35°, 43°, 53°, 57°, and 62°, corresponding to (220), (311), (400), (422), (511), and (440) crystal planes of face-centered cubic Fe_3O_4, respectively [24, 25]. This result revealed that the fabricated material was pure Fe_3O_4 with a spinel structure. Moreover, this result revealed that coating magnetic nanoparticles with chitosan did not cause a change in the phase of the magnetic

nanoparticles since the characteristic peaks were observed for both naked magnetic nanoparticles, and CS-MNPs samples.

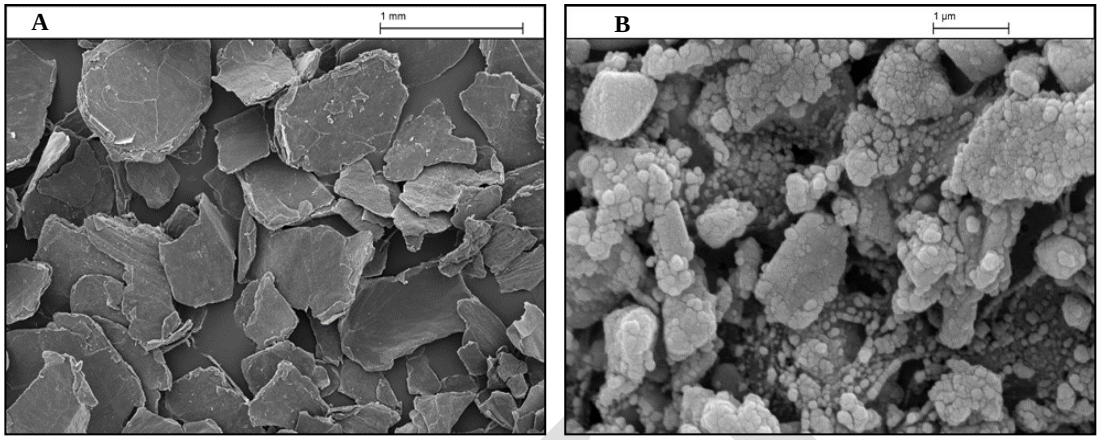

Fig. 2 SEM images of (A) chitosan, and (B) CS-MNPs.

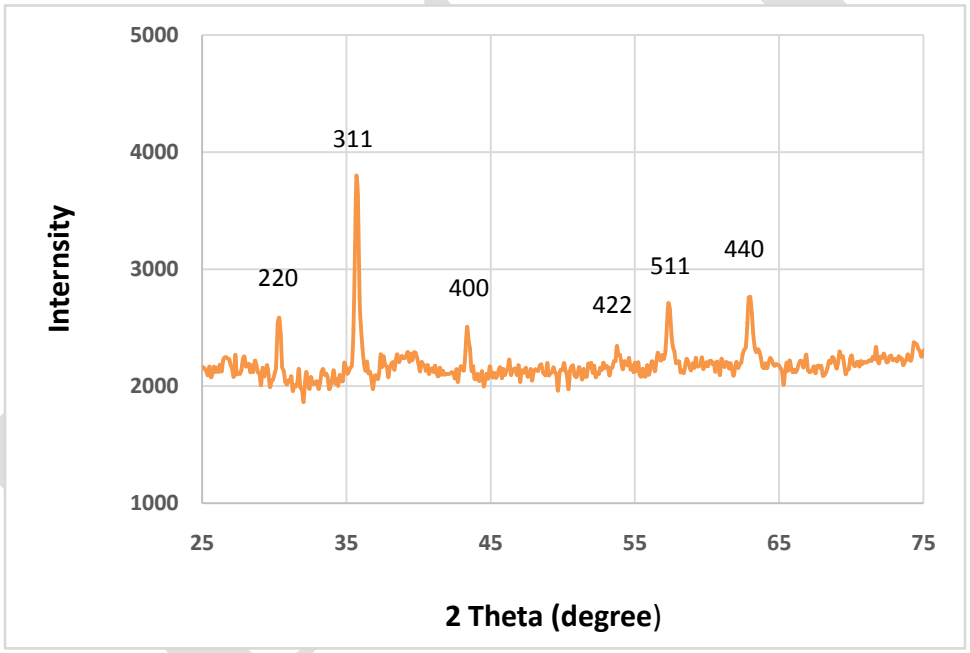

Fig. 3. XRD patterns for the chitosan-magnetic nanoparticles.

3.2.4. EDAX analysis

EDAX analysis was utilised for the elemental analysis of chitosan, naked magnetic nanoparticles and magnetic nanoparticles coated with chitosan. Figure 4 shows the EDAX spectra of the three samples that showed the energy levels of X-rays received by the EDAX detector.

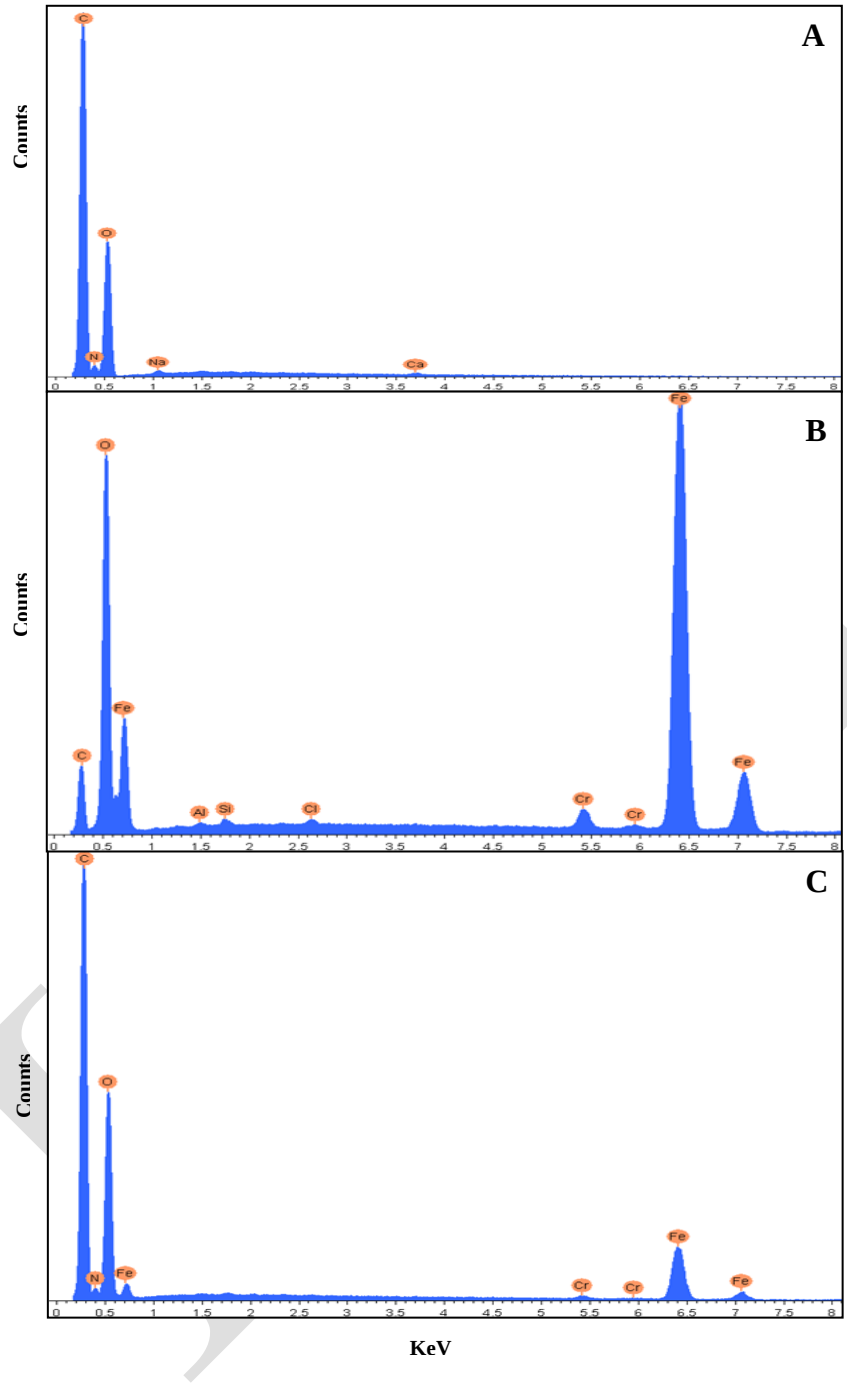

Fig. 4 EDAX images of (A) chitosan, and (B) naked MNPs, and (C) CS-MNPs.

The EDAX spectrum of chitosan, Figure 4 (A), shows the higher peaks in the chitosan spectrum are carbon (C) and oxygen (O), which are the main contents of this natural polymer. In terms of the EDAX spectrum of the MNPs sample, Figure 4 (B), the spectrum shows strong peaks for Fe and O, which are the composition components of Fe_3O_4 by co-precipitation synthesis. Figure 4(C) shows that different compositions were recorded for the CS-MNPs sample. Coating of MNPs with chitosan was confirmed by the increase in the peak intensity of

carbon. It is important to mention that the low content of iron (Fe) and the high content of carbon (C) are attributed to EDAX realised on the surface of the grafting material [15].

3.2.5. FT-IR analysis

Figure 5 shows the infrared spectra of (A) naked MNPs, (B) MNPs coated with chitosan, and (C) pure chitosan. In the spectrum of MNPs, Figure 5 (A), a strong peak at 559 cm^{-1} is attributed to the Fe-O bond vibration of Fe_3O_4[26, 27]. Figure 5 (C) shows the stretching vibration band at 3266 cm^{-1},which was related to the amino (NH_2) and hydroxyl (OH) groups. The absorption band at 2878 cm^{-1} was attributed to C-H stretching vibrations. The bands at 1621 and 1552 cm^{-1} represented the C=O stretching vibration of amide and the bending vibration of NH_2 groups, respectively. The band at 1375 cm^{-1} is attributed to C-N stretching vibrations and a group of bands from 1150 to 850 cm^{-1} corresponded to stretching vibrations of C-O-C and C-O [28]. The main characteristic absorption bandsfor chitosan were observed in the CM-MNPs, Figure 5 (B). These results confirmedthe coating of chitosan on the surface of the magnetic nanoparticles.

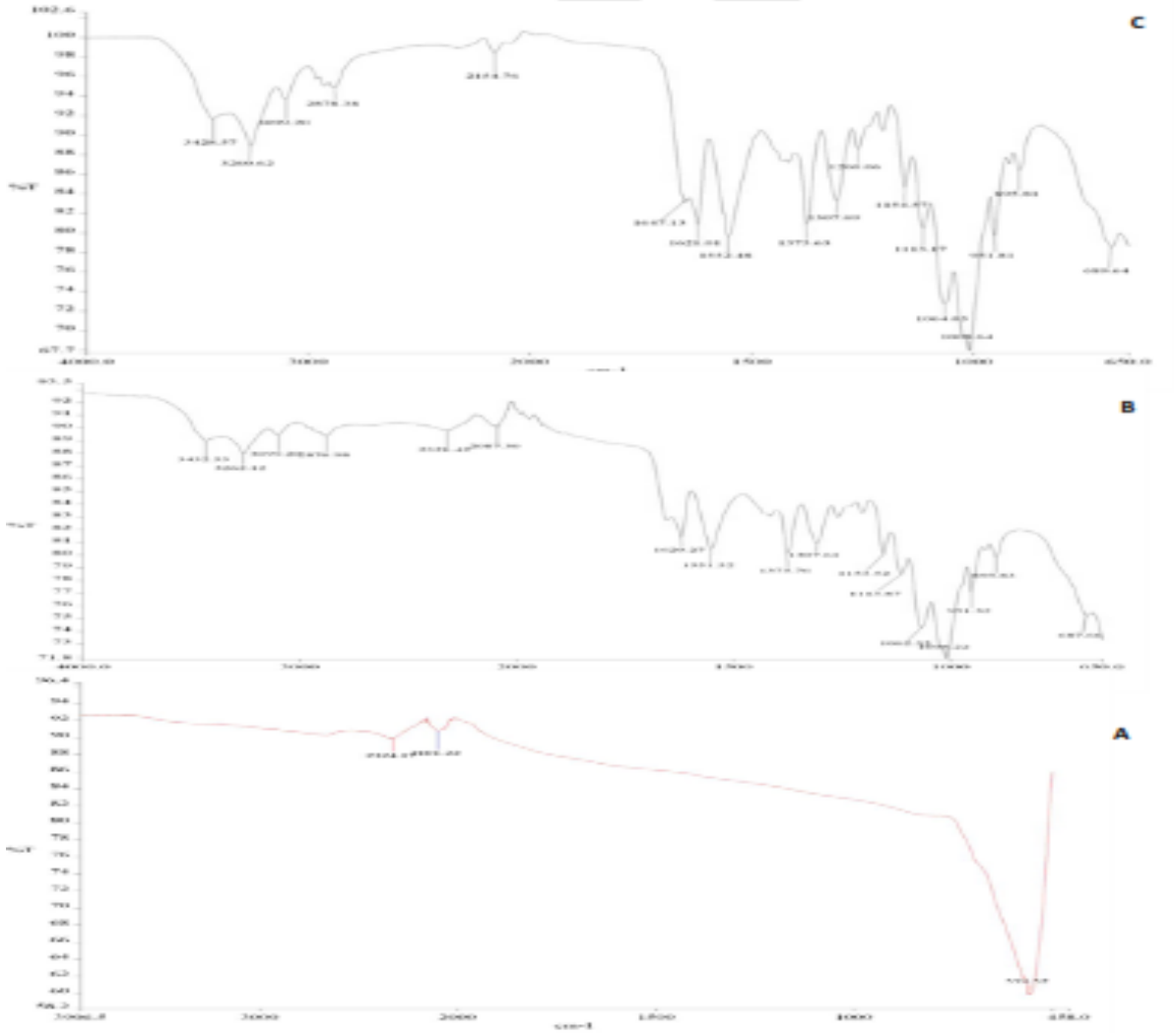

Fig. 5 FT-IR spectra of (A) naked MNPs, (B) CS-MNPs, and (C) pure chitosan.

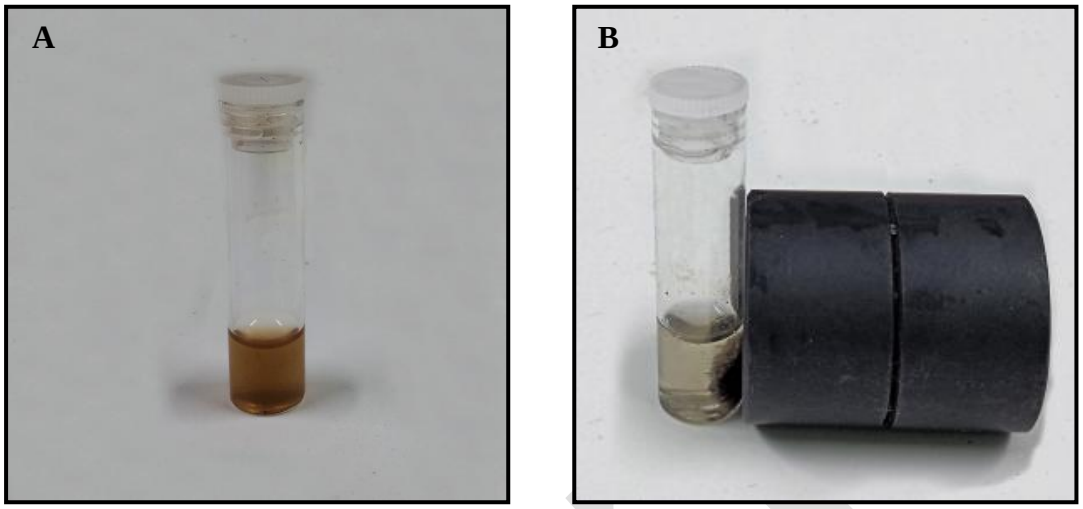

Fig. 6Photographs of adsorption system before (A) and after magnetic separation (B). The initial concentration of myoglobin= 2 mg mL^{-1}, the amount of CS-MNPs=20 mg, the pH=7, temperature=25 ºC

The effect of pH on adsorption of myoglobin on CS-MNPs was studied since proteins are amphoteric and the number of charges on the surface of protein is affected by the pH of the medium [29]. Figure 7 shows the effect of pH on the amount of adsorbed myoglobin on MNPs coated with chitosan. It was found that the maximum myoglobin adsorption was obtained and CS-MNPs had high maximum adsorption capacity when the pH value was 7. Figure 8 shows the difference in adsorption capacity between MNPs and CS-MNPs. The CS-MNPs have a larger adsorption capacity compared with MNPs. The adsorption equilibrium of myoglobin on to the fabricated materials was reached within 40 minutes due to the small diameter of fabricated nanoparticles. The adsorbed myoglobin was eluted from magnetic nanoparticles using 20 mM phosphate buffer (pH 5) containing 0.5 M NaCl and the extraction recovery was found to be 95%.

Stability of CS-MNPs was studied by five repeated adsorption-elution cycles using the same procedure. No remarkable reduction in the adsorption capacity indicated the stability of magnetic nanoparticles during five repeated adsorption-desorption operations.

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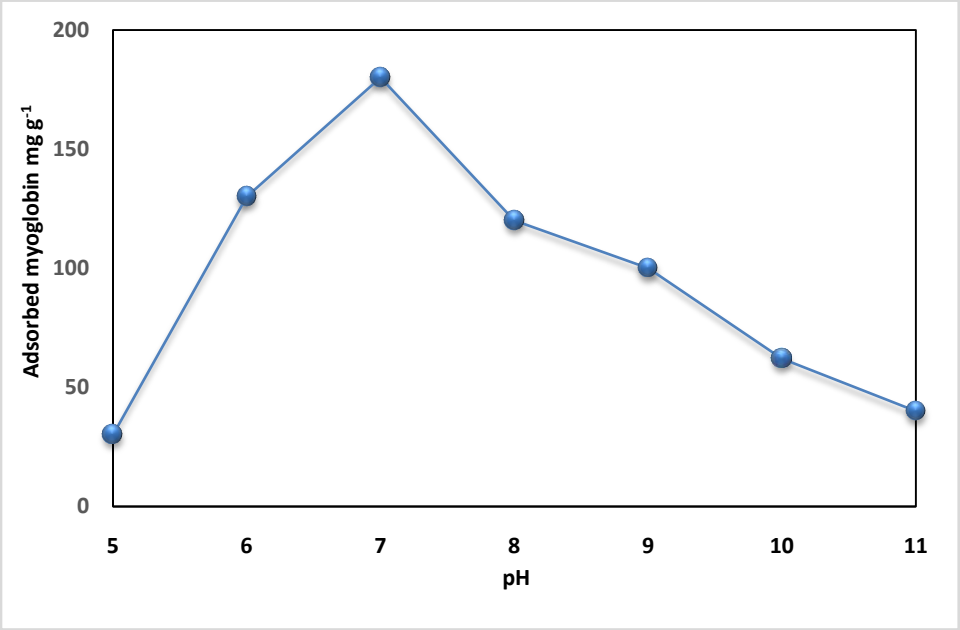

Fig. 7 Effect of pH on adsorption of myoglobin on CS-MNPs.

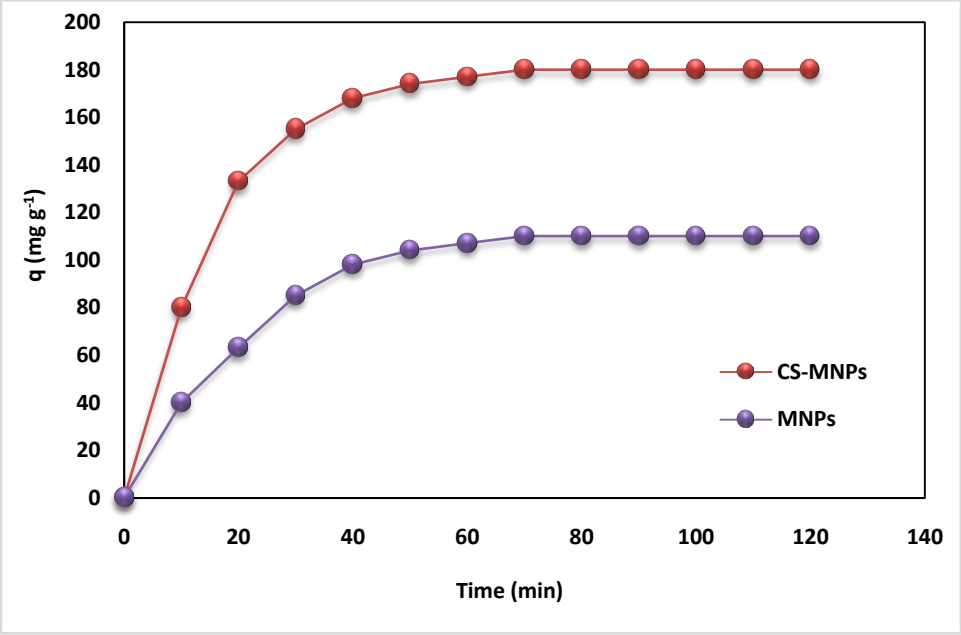

Fig. 8 Comparison the maximum adsorption of myoglobin using MNPs and CS-MNPs.

4. Conclusion

The extraction of biological samples is complicated, labour-intensive and time consuming. For this purpose, magnetic nanoparticles coated with chitosan were fabricated. The presented TEM, SEM, XRD,EDAX, and FT-IR spectroscopy confirmed formation of the magnetic material with a mean diameter of 23 nmand the modification of the surface of MNPs did not change the spinel structure of Fe_3O_4. Magnetic Fe_3O_4-chitosan nanoparticles possess the advantages of excellent adsorbency for myoglobin and easier separation from the reaction system. Therefore, this type of magnetic nanoparticle would bring advantages to the conventional extraction beds of proteins.

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