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Isolation/separation of plasmid DNA using hemoglobin modified magnetic nanocomposites as solid-phase adsorbent

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ABSTRACT

Hemoglobin (Hb) modified magnetic nanocomposites are prepared by immobilization of Hb onto the surface of amino-functionalized Fe_3O_4@SiO_2 magnetic nanoparticles via covalent bonding with glutaraldehyde as cross-linker. The obtained nanocomposites are characterized with FT-IR, SEM, XRD and surface charge analysis. A direct solid-phase extraction procedure for the isolation/separation of plasmid DNA using this nanocomposite as a novel adsorbent is thus developed. Some important experimental parameters governing the sorption efficiency, i.e., the pH of sample solution and the ionic strength, are investigated. The Hb modified magnetic nanocomposites provide a sorption capacity of 27.86 mg g^{-1} for DNA. By using 2.0 mg of the nanocomposites as sorption medium and a suitable acidity of pH 6.1, a sorption efficiency of 93% is achieved for 25 μg mL^{-1} of DNA in 1.0 mL of sample solution. Afterwards, the absorbed DNA could be readily recovered by using 1.0 mL of Tris–HCl buffer (pH 8.9, 0.01 mol L^{-1}), giving rise to a recovery of ca. 68.3%. The present solid-phased extraction protocol is applied for the isolation of plasmid DNA from *Escherichia coli* culture, resulting in comparable yield and purity of plasmid DNA with respect to those obtained by using commercial kits.

1. Introduction

The isolation/purification of DNA from complex biological samples is a critical procedure in forensic, clinic analysis and molecular biochemistry applications in order to get a relative purified template for subsequent procedures, such as polymerase chain reaction, cloning, sequencing and hybridization etc. There are usually two protocols, i.e., solvent extraction and solid-phase extraction, are adopted for the DNA isolation/purification. The solvent extraction approach generally involves the use of various organic solvent such as phenol and chloroform and repeated centrifugations, while the solid-phase extraction scheme employs a binding adsorbent in conjunction with a suitable binding buffer to extract the target DNA from a wide range of specimens [1]. Compared to the labor- and time-intensive solvent extraction process [2], the solid-phase extraction protocols is gaining more popularity in recent applications, due to the ability for achieving preconcentration along with fast and efficient purification, and avoid the use of volatile and harmful organic solvents. Up to now, various materials such as silica gel [3–5], calcium silicate [6], chelex (iminodiacetid acid) ionic resin [7], hydroxyapatite [8], aluminum oxide membrane [9], chitosan [10], cellulose [11] etc. have been used as effective adsorbents to extract genomic and plasmid DNA(pDNA) from biological samples via electrostatic interaction or (and) hydrogen bonding force under a suitable condition.

Recently magnetic techniques employing magnetic particles coated with different polymers (e.g., agarose, silica) have been used increasingly for molecular biological applications [12,13]. The isolation/extraction of target macrobiomolecules (e.g., protein, peptide, DNA, and RNA) from biological samples using magnetic micro- or nanoparticles as solid phase adsorbents offer many benefits over conventional SPE procedures, i.e., faster processing, low consumption of chemical reagents, ease of operation and potential for automation. The purification of genomic and plasmid DNA using magnetizable solid support (beads or matrix) has already been attempted from different biological sources, and an array of surface-modified magnetic nanoparticles, both chemically and biologically synthesized, have been successfully used for DNA purification [14,15]. Though pristine magnetic materials, i.e., Fe_3O_4, have the capacity of adsorbing DNA [16,17], surface modifications, including physical coating and covalent binding, have often been exploited to further facilitate the adsorption affinity [18–21]. Silica-coated core-shell magnetite nanoparticles, i.e., Fe_3O_4@SiO_2, which is based on the formation of magnetic iron oxide nanoparticles embedded in silica matrix, has been widely reported in literatures due to the ability to offer ease of succedent function of magnetite nanoparticles and subsequent improvement on the DNA purification efficiency [22–26].

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In the present study, we describe an easy and inexpensive method for the preparation of highly functionalized and well-dispersed nanomagnetic supports and their use for DNA extraction. Magnetite nanoparticles are prepared by coprecipitation reaction of ferrous and ferric salts with ammonia, and then coated with silica, which is produced from sodium silicate solution directly. Herein, the surface of the magnetic silica is functionalized with amino-silane coupling agent, and then hemoglobin is covalently immobilized onto these supports by glutaraldehyde method. The magnetic nanocomposites are thus used as sorbent to harvest pDNA from cell culture after lyses. The quality and quantity of magnetic particle-purified pDNA are confirmed by comparison against pDNA obtained using a commercially available purification kit.

2. Experimental section

2.1. Materials

All the reagents used are at least of analytical reagent grade unless specified and 18 MΩ/cm deionized water is used throughout.

Hemoglobin (Hb, H2625) is purchased from Sigma (St. Louis, MO) and used as received. Super coiled DNA Marker is from Takara Biotechnology (Dalian, China). 3-aminopropyltriethoxysilane (APTES) is purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). Tris-(hydroxymethyl)aminomethane(Tris), $FeCl_3 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, $NaH_2PO_4 \cdot 2H_2O$, $Na_2HPO_4 \cdot 12H_2O$, HCl, methanol, Triton X-100, EDTA, Boric acid, glutaraldehyde and Salmon sperm DNA sodium salt are purchased from Sinopharm Chemical Reagent Co. (Shanghai, China).

2.2. Synthesis of magnetic silica nanoparticles

The magnetite nanoparticles are prepared by a conventional coprecipitation procedure. Briefly, 5 mmol of $FeCl_3 \cdot 6H_2O$ and 3 mmol of $FeSO_4 \cdot 7H_2O$ are dissolved in 150 mL de-gased water, and the mixture is heated to 40 °C under a nitrogen flow (40 mL min^{-1}). Then ammonia solution (25%, v/v) was added drop-wise into the reaction solution to adjust the acidity of the solution to pH 9.0 and the reaction mixture is further stirred at 40 °C for 30 min. The obtained Fe_3O_4 nanoparticles are washed repeatedly with deionized water until a neutral washout is obtained, and then are suspended in 150 mL deionized water.

To prepare the silica-coated Fe_3O_4 nanoparticles, the above suspension is heated to 80 °C under a nitrogen flow (40 mL min^{-1}). 10 mL of $NaSiO_3$ solution (0.64 mol L^{-1}) is then added drop-wise to the Fe_3O_4 suspension under vigorous stirring, and the mixture is adjusted to pH 6.0 with 2 mol L^{-1} HCl. The reaction mixture is further stirred at 80 °C for 3 h. The resulted silica-coated Fe_3O_4 nanoparticles are thoroughly washed with deionized water and collected by magnetic separation, followed by drying at 45 °C under vacuum for 12 h.

2.3. Surface treatment by amino-silane

Amino-functionalized Fe_3O_4@SiO_2 nanoparticles are preformed by using APTES as the silylation agent.

0.5 g of Fe_3O_4@SiO_2 nanoparticles, 63 mL of methanol, 1.0 mL of water and 3.0 mL of APTES are added to a 250-mL three-necked flask and ultrasonically dispersed for 20 min. Then 37 mL glycerol is added and the mixture is heated to 85 °C and kept stirring for 3 h. The resulted nanospheres were washed with deionized water and ethanol for several times, and then dispensed in 30 mL deionized water.

2.4. Hb immobilization

15 mL of amino-functionalized Fe_3O_4@SiO_2 suspension is added into a 50-mL beaker-flask, and then the supernatant is removed after separating by permanent magnet. 40 mL of Na_2HPO_4–NaH_2PO_4 buffer (0.1 mol L^{-1}, pH 7.4) and 10 mL of glutaraldehyde (25%, v/v) are added into the flask sequentially, the mixture was ultrasonically dispersed for 5 min, and then incubated at room temperature for 6 h. The obtained product is washed with deionized water thoroughly.

20 mL of 2 mg mL^{-1} Hb solution is added and the mixture is incubated at 30 °C for 4 h, then the magnetic materials are separated by permanent magnet. The collected product is washed with 0.1 mol L^{-1} Na_2HPO_4–NaH_2PO_4 buffer (pH 8.0) until the washout has no absorbance at 406 nm. Afterwards, the product is dried at 40 °C under vacuum for 12 h.

2.5. Characterization of the Hb modified magnetic nanocomposites

The morphologies of the nanocomposites are observed on a Superscan SSX-550 Scanning Electron Microscope (Shimadzu, Japan) at 200 kV. FT-IR spectra are recorded using a Nicolet 6700 spectrometer (Thermo Electron, USA) from 400 to 4000 cm^{-1} with a resolution of 1.0 cm^{-1}. The X-ray diffraction (XRD) patterns are obtained by using a Rigaku D/max-a X-ray diffractometer (Rigaku, Japan) with CuKα radiation source. The surface charge property of the material is investigated by measuring Zeta potential with a ZEN3600 Nano Zetasizer (Malvern, England). Absorbance measurement is carried out on a T6 UV–vis Spectrophotometer (Beijing Purkinje General Instrument Co. Ltd., China).

2.6. DNA adsorption studies

2.0 mg of the Hb modified magnetic nanocomposites are added into 1.0 mL of DNA solution and the mixture is shaken vigorously for 15 min to facilitate the adsorption of DNA. After separation by permanent magnet, the supernatant is collected to quantify the DNA content remaining in the original solution by monitoring the Soret band absorbance of DNA at 260 nm. After DNA adsorption, the magnetic nanocomposites are mixed with 1.0 mL of Tris–HCl buffer solution (0.01 mol L^{-1}, pH 8) and the mixture is oscillated for 15 min to facilitate the desorption of the absorbed DNA from the nanocomposites. The supernatant is finally collected after magnetic separation for the ensuing investigations.

3. Results and discussion

3.1. Characterizations of the Hb modified magnetic nanocomposite

Evidence for the formation of silica-coated Fe_3O_4 nanoparticles was obtained from powder X-ray diffraction. Fig. 1 shows the XRD patterns of Fe_3O_4 nanoparticles (trace A) and its product after silica-coating (trace B). Diffraction peaks with 2θ at 30.29°, 35.67°, 43.43°, 53.65°, 57.20° and 62.81° were observed for Fe_3O_4, indicating the existence of (220), (331), (440), (422), (511), (440) crystal plane and a cubic spinel structure of the magnetite. The same sets of characteristic peaks were also observed for Fe_3O_4@SiO_2, indicating the stability of the crystalline phase of Fe_3O_4 nanoparticles during silica coating. The average size of the magnetite crystals was estimated by Scherrer equation to be 14.1 nm and 15.3 nm for Fe_3O_4 and Fe_3O_4@SiO_2, respectively.

It has been reported that there is a specific interaction between DNA and proteins, and biosensors based on DNA–Hb

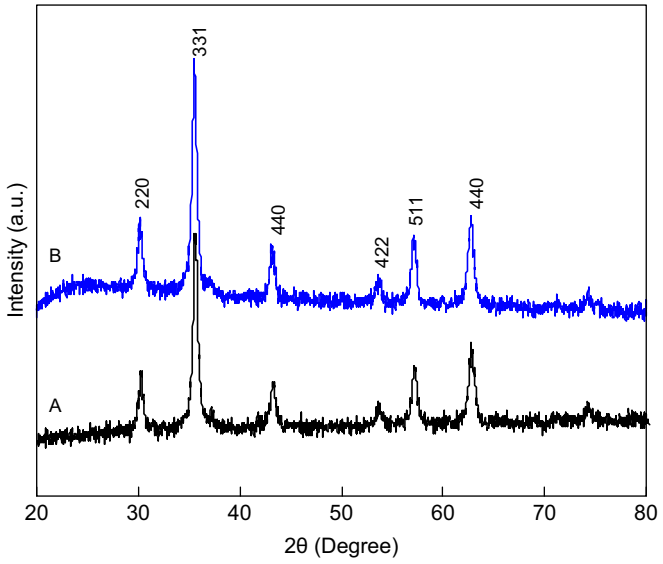

Fig. 1. X-ray diffraction patterns of Fe_3O_4 (A) and Fe_3O_4@SiO_2 nanoparticles (B).

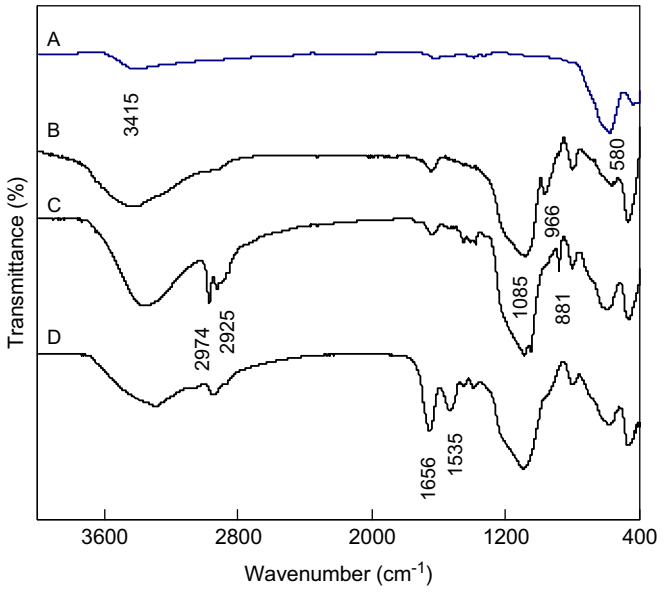

Fig. 2. Fourier transform infrared (FT-IR) spectra of Fe_3O_4 (A), Fe_3O_4@SiO_2 (B), Fe_3O_4@SiO_2-NH_2 nanoparticles (C) and Hb modified magnetic nanocomposites (D).

modified electrode have attracted much attention since the report of enhancement on electron transfer between electrode and heme proteins in myoglobin–DNA films [27]. In the present case, Hb is immobilized onto the surface of Fe_3O_4@SiO_2-NH_2 nanoparticles for the purpose of selective extraction of DNA. The FT-IR spectra of Fe_3O_4, Fe_3O_4@SiO_2, Fe_3O_4@SiO_2-NH_2 nanoparticles and the Hb modified magnetic nanocomposites are compared in Fig. 2. For the four nanoparticles, absorption peaks at 565 cm^{-1} were observed, corresponding to the Fe–O vibration from the magnetite phase [28]. The absorption bands at 1085 cm^{-1} and 966 cm^{-1} observed on Fe_3O_4@SiO_2 are ascribed to the stretching and deformation vibrations of SiO_2, reflecting the successful coating of silica on the magnetite surface. As for the Fe_3O_4@SiO_2-NH_2 nanoparticles, the absorptions at 2974 cm^{-1} and 2925 cm^{-1} correspond to the C–H bending vibrations from APTES. With the formation of Si–O–Si, the absorption of Si–OH at 966 cm^{-1} disappeared, while at the same time, an absorption peak at 881 cm^{-1} was observed, resulted from the N–H bending vibrations from amino group, indicating the successful aminopropyl functionalization of the silica layer on Fe_3O_4@SiO_2. As for the Hb modified magnetic nanocomposites, the appearance of absorption at 1656 cm^{-1} and 1535 cm^{-1}, which are the characteristic absorption peaks of amide in Hb, and the disappearance of N–H bending vibrations absorption at 881 cm^{-1} suggested well the successful immobilization of Hb on the surface of Fe_3O_4@SiO_2 after cross-linking.

Fig. 3 showed the SEM images of Fe_3O_4 (A), Fe_3O_4@SiO_2 (B) and the Hb modified magnetic nanocomposites (C). It could be seen that homogeneous spherical Fe_3O_4 nanoparticles were obtained after coprecipitation (Fig. 3A), while the size of the nanoparticles increased after silica-coating (Fig. 3B). After immobilization with Hb, the size of the nanoparticles is further increased and aggregation of the composites is also observed.

The surface charge measured as zeta potential is presented in Fig. 4 for Fe_3O_4@SiO_2, Fe_3O_4@SiO_2-NH_2 and the Hb modified magnetic nanocomposites. It could be seen that the surface of the three nanoparticles all turned from positively charged to negatively charged with the increase of pH. Due to the existence of abundance of Si–OH group, the isoelectric point (pI) of Fe_3O_4@SiO_2 is 2.5, which is nearly identical to that of the amorphous silica [29], indicating successful wrapping of the Fe_3O_4 core by the formation of a fully covered silica layer. Considering that the intrinsic p*Ka* of aminopropyl is 9.8, the presence of amine group would shift the pI of the amino-functionalized materials towards higher pH values, i.e., 8.1 for the Fe_3O_4@SiO_2-NH_2 nanoparticles. After the immobilization of Hb onto the surface of Fe_3O_4@SiO_2-NH_2 nanoparticles, the charge characteristics of the composites is governed by Hb, thus a decrease of the pI for the Hb modified magnetic nanocomposites, i.e., pI 5.5, is observed.

The magnetization curves measured for Fe_3O_4, Fe_3O_4@SiO_2, and the Hb modified magnetic nanocomposites are compared in Fig. 5. There was no hysteresis in the magnetization for the three tested nanoparticles. Neither coercivity nor remanence was observed, suggesting that the three nanoparticles are superparamagnetic. The saturation magnetization value was measured to be 66.63 emu g^{-1} for Fe_3O_4, and it is decreased to 41.56 emu g^{-1} for Fe_3O_4@SiO_2 as the surface of the Fe_3O_4 core was coated by a layer of silica. The saturation magnetization value further decreased to 33.21 emu g^{-1} for the Hb modified magnetic nanocomposites as Hb was immobilized on the surface. Although the saturation magnetization decreased after Hb immobilization, complete magnetic separation of Hb modified magnetic nanocomposites could be achieved within 2 min by placing a magnet near the vessels containing the aqueous dispersion of the nanocomposites.

3.2. Adsorption studies

The effects of sample pH on the adsorption efficiency of DNA were investigated and the results were showed in Fig. 6. It can be seen that favorable adsorption is achieved within pH 4.1–6.1. At pH >6.1, a significant drop of the adsorption efficiency is observed, and virtually no adsorption of DNA is recorded at pH 9.9. These observations could be well explained by the electrostatic interaction between DNA and the Hb immobilized on the surface of the magnetic composites. DNA is a polyanion and which is negatively charged within the pH range tested, while Hb is a kind of neutral protein and its pI is about 6.8–6.9. In the present case, at pH <6.1, the surface of the composites is positively charged, thus the electrostatic attraction between DNA and the nanocomposites facilitates the adsorption of DNA. As the pH value exceeds the pI of Hb, the surface of the

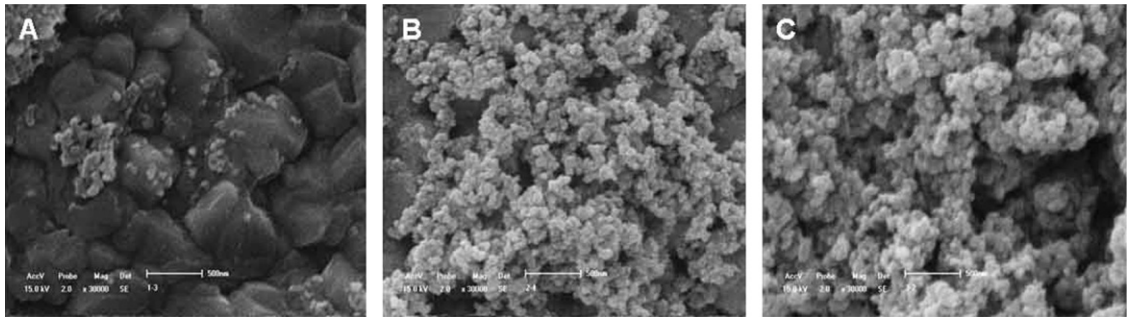

Fig. 3. SEM images of Fe_3O_4 (A), Fe_3O_4@SiO_2(B) and Hb modified magnetic nanocomposites (C).

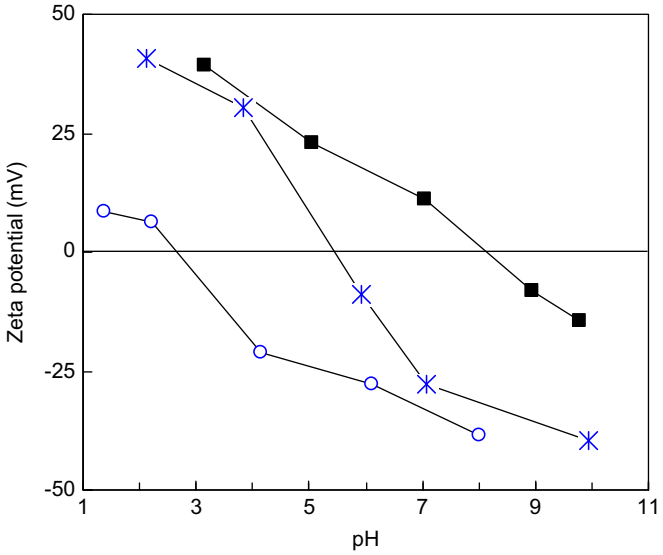

Fig. 4. Zeta potentials of Fe_3O_4@SiO_2(○), Fe_3O_4@SiO_2-NH_2(Delta;) and Hb modified magnetic nanocomposite(■) at different pH values.

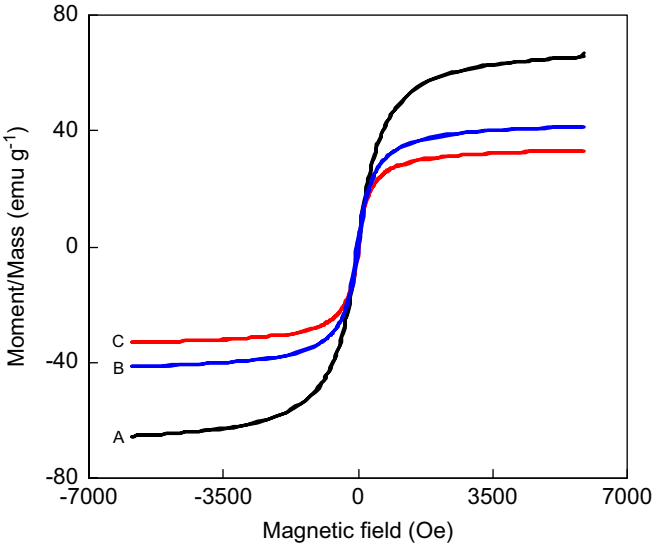

Fig. 5. Magnetization curves for Fe_3O_4 nanoparticles (A), Fe_3O_4@SiO_2 nanoparticles (B) and the Hb modified magnetic nanocomposites (C).

nanocomposites became negatively charged and thus the electrostatic repulsion deteriorated the adsorption of DNA. Consequently, a sample acidity of pH 6.1 is employed for the ensuing investigations.

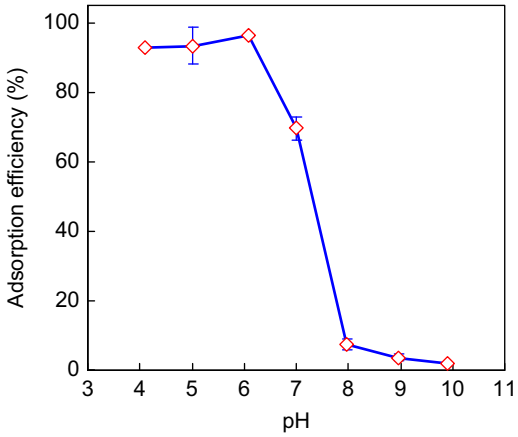

Fig. 6. Effect of sample pH value on the adsorption efficiency of DNA. Hb modified magnetic nanocomposite: 2.0 mg; DNA: 25 μg mL^{-1}, 1.0 mL; adsorption time: 15 min.

The effects of ionic strength on the adsorption of DNA by the Hb modified magnetic nanocomposites are investigated by the addition of various amount of NaCl at pH 6.0 by fixing the DNA concentration at 25 μg mL^{-1}. The results indicated that the adsorption efficiency of DNA decreased substantially with the increase of the ionic strength, i.e., the NaCl concentration. This further indicated that the adsorption of DNA onto the surface of magnetic nanocomposites is mainly governed by electrostatic interactions [30].

The typical adsorption isotherm of DNA onto the surface of the magnetic nanocomposites is illustrated in Fig. 7. As mentioned previously that electrostatic interaction is the main driving force for the adsorption, thus the adsorption is ascribed to monolayer coverage and the experimental data are then fitted with the Langmuir model, as expressed in the following:

$$Q^* = \frac{Q_m \times C^*}{K_d + C^*}$$

C^* (μg mL^{-1}) is the DNA concentration in aqueous solution and Q^* (μg mg^{-1}) denotes the amount of DNA retained by the Hb modified composites. Q_m (μg mg^{-1}) represents the maximum adsorption capacity and K_d is the dissociation constant. By fitting the experimental data to this equation, Q_m is estimated to be 27.86 μg mg^{-1}.

3.3. Recovery of the retained DNA from the Hb modified composites

As discussed previously, electrostatic interaction is the driving force for the binding of DNA onto the Hb modified magnetic composites, and the adsorption of DNA is strongly dependent on the variation of pH value and ionic strength of the reaction system. Therefore, the recovery of the adsorbed DNA could be

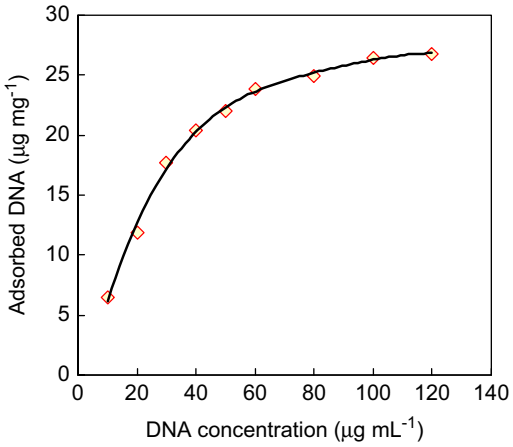

Fig. 7. The adsorption isotherm for DNA. Hb modified magnetic nanocomposite: 2.0 mg; DNA: 1.0 mL; adsorption time: 15 min; sample pH: 6.1.

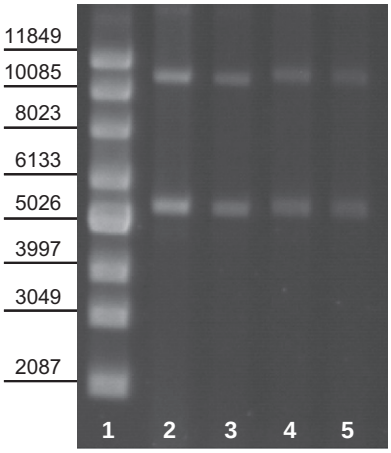

Fig. 8. The agarose gel electrophoresis of pDNA from colibacillus. Lane 1: Super coiled DNA Marker; Lanes 2 and 3: pDNA extracted with a commercial kit; Lanes 4 and 5: pDNA extracted with the present sold-phase extraction protocol.

facilitated by either regulating the pH value or the concentration of electrolyte in the stripping reagent. For this purpose, a series of buffers, e.g., Tris–HCl buffer, Britton–Robinson buffer, phosphate buffer and some electrolyte solutions, e.g., NaCl and Na_3PO_4, were exploited to strip the adsorbed DNA with a same concentration of 0.01 mol L^{-1} and a fixed volume of 1.0 mL. The results indicated that all these stripping reagents were able to recover certain extent of the adsorbed DNA. However, the highest elution efficiency was obtained by using Tris–HCl buffer as eluent. The dependence of DNA recovery efficiency on the pH value of the Tris–HCl buffer was further investigated within a range of pH 7.1–8.9. The increase of the buffer pH value provides an obvious improvement on the recovery of the adsorbed DNA up to pH 8.9. For the ensuing experiments, a Tris–HCl buffer of pH 8.9 was adopted for the elution of the retained DNA, giving rise to a recovery of ca. 68.3%.

3.4. Isolation of plasmid DNA (pDNA) from Escherichia coli culture

The extraction protocol consists of cell lysis to release plasmid DNA and binding of DNA to the Hb modified magnetic nanocomposites, followed by elution of the purified DNA. Briefly, 2 mL of the *Escherichia coli* culture was taken into a centrifuge tube and the culture supernatant is discarded after centrifugation for 2 min at 10000 rpm. The captured cell are first mixed with 150 μL of solution I (25 mmol L^{-1} Tris–HCl, 10 mmol L^{-1} EDTA, pH 8.0), then 200 μL of solution II (0.2 mol L^{-1} NaOH and 1.0% SDS) is added into the mixture. The tube is placed on ice for 5 min after gentle mixing. Then 150 μL of solution III (3 mol L^{-1} KAc, 2 mol L^{-1} HAc, pH 6.0) is added and the resultant mixture is incubated on ice for additional 5 min to facilitate the precipitate of genomic DNA, proteins and other cell debris. After separation by centrifuging at 12,000g for 5 min, 350 μL of cleared alkaline lysate supernatant is transferred to a fresh centrifuge tube for pDNA extraction. 2.0 mg of Hb modified magnetic nanocomposite is added to the tube and the isolation procedure is followed as described in the experimental section. To compare the developed technique against the existing approaches for DNA purification, pDNA extraction is performed using a commercial kit (Axygen Biotechnology, China) according to the manufacturer's instructions.

The integrity, yield, and purity of the extracted pDNA are checked with agarose gel electrophoresis and UV-spectrophotometry. As shown in Fig. 8, the electrophoresis analysis revealed no notable differences between pDNA extracted with the Hb modified magnetic nanocomposites and the commercial kit. The extracted pDNAs are of large molecular sizes with molecular weights greater than 5 kb. Smeared bands at 10 kb observed in the electropherogram are most likely due to the breakdown of super coil pDNA during the extraction process. The quantity and quality of the extracted DNA are estimated on measurements of the absorbance at 260 nm (A_{260}) and the absorbance ratio at 260 nm/280 nm (A_{260}/A_{280}), respectively. About 10.5 μg of pDNA is extracted from 1 mL of cells cultured overnight. The yields of the DNA isolated using the Hb modified magnetic nanocomposites are comparable to those obtained by the reported extraction process [16,31]. The A_{260}/A_{280} ratio of the DNA samples obtained is about 1.84, suggesting that the extracted DNA templates are of relatively high purity with negligible protein contamination.

4. Conclusions

Hb modified magnetic nanocomposites is used as efficient sorbent for the isolation/purification of pDNA. pDNA can be isolated from the crude bacteria culture efficiently and recovered easily with a Tris–HCl buffer, and the adsorption/desorption operations can be easily manipulated under external magnetic field. This developed solid-phase extraction approach with Hb modified magnetic nanocomposites proved to be a time- and cost-effective pDNA preparation technique independent of centrifugation and hazardous organic solvents. It is well suited for routine laboratory use. At the same time, the simplicity of this approach indicates its potential for automated pDNA isolation/purification. Moreover, the mild adsorption and recovery circumstance is beneficial to retain the biological activity of the purified DNA.

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