Colloids and Surfaces B: Biointerfaces 88 (2011) 246–253

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

# Affinity adsorption and separation behaviors of avidin on biofunctional magnetic nanoparticles binding to iminobiotin

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ARTICLE INFO

*Article history:*
Received 10 May 2011
Received in revised form 26 June 2011
Accepted 28 June 2011
Available online 6 July 2011

*Keywords:*
Biofunctional magnetic nanoparticles
Avidin
Iminobiotin
Adsorption behaviors
Separation

ABSTRACT

Knowing the adsorption behavior of target proteins on biofunctional magnetic nanoparticles is of great importance for the separation and purification of proteins. Adsorption behaviors of avidin on biofunctional magnetic nanoparticles binding to iminobiotin were investigated under different conditions of temperature, pH, ionic strength, and feed avidin concentration. Biofunctionalization of the non-functional nanoparticles was performed, coupled with iminobiotin. Characterization of the particles was carried out using transmission electron microscopy (TEM) and Fourier transform infrared spectroscopy (FTIR). The results showed the avidin adsorption behaviors were mainly dependent on affinity interaction between avidin and iminobiotin coupled with the nanoparticles, which exhibited temperature, pH, ionic strength, and feed avidin concentration sensitivity. Maximum avidin adsorption capacity was achieved as 225 mg avidin/g biofunctional nanoparticles. Results were well fitted to the Langmuir isotherm model with the feed avidin concentration of less than 45 μg/ml. Based on the experiments above, the biofunctional magnetic nanoparticles were used to separate avidin from treated egg-white solution containing large amounts of other proteins. The avidin was isolated in 92% yield with an optical purity of more than 98.5% according to the HPSEC data. The regeneration of these nanoparticles was also studied and almost 87.3% of avidin could still be recovered by these regenerated nanoparticles.



## 1. Introduction

In recent years, much interest has been focused on biofunctional magnetic nanoparticles for the specific adsorption and separation of biomacromolecules, such as enzyme [1], proteins [2], and DNA. The adsorption and separation efficiency is mainly dependent on interface interactions between target biomacromolecules and the nanoparticles, which are commonly affected by nanoparticle surface properties, target biomolecules properties, and some external parameters, such as temperature [3,4], pH [5,6], ionic strength [7,8], and feed biomacromolecules concentration [5]. The driving forces for the adsorptions are electrostatic, hydrophobic, and ligand binding interactions. Suitable desorption conditions selected to release the absorbed biomolecules from the biofunctional magnetic nanoparticle were alkaline conditions [9], acidic conditions [10], ionic strength [11], and temperature [12].

Three types of model surfaces on biofunctional magnetic nanoparticles were used extensively for adsorption and separation of biomacromolecules. One type is organic/inorganic compound bound surfaces, which induces the nanoparticle surface with positive/negative charge at different pH conditions that adsorb tag protein with opposite charge, but repel other proteins with the same charge [13,14]. In fact, the separation efficiency of the proteins depends greatly on the difference of its isoelectric point. The second type is smart polymer coated surfaces which possess large surface area and surface properties, such as hydrophobicity or hydrophilicity which can be controlled under different conditions of temperature, pH, and ionic strength [15,16]. The third type is affinity ligands connected surfaces, such as $Cu^{2+}$ and other metal ions [17], which have advantage of low cost and high stability. The biofunctional magnetic nanoparticles can provide a mild condition for immobilized biomacromolecules, which is particularly suitable for biomolecule concentration and purification.

Avidin, a tetrameric protein, has a high degree of affinity and specificity with biotin ($K_D = 10^{-15}$ M) [18]. The protein has recently become an object of protein adsorption studies because the avidin–biotin complex exhibits the most stable non-covalent interaction found in nature. Recently, biofunctional magnetic nanoparticles coupling with biotin have been successfully employed for avidin adsorption [19,20]. However, the desorption and separation of avidin from the biofunctional magnetic nanoparticles has not been discussed in that case.

In this study, we prepared biofunctional magnetic nanoparticles binding to iminobiotin and these particles were used as a support

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doi:10.1016/j.colsurfb.2011.06.039

for adsorbing and recovering avidin under different conditions of temperature, pH, ionic strength, and feed avidin concentration. The biofunctional magnetic nanoparticles were also used to separate avidin from treated egg-white solution containing large amounts of other proteins. Furthermore, the regeneration and repeated use of the biofunctional magnetic nanoparticles were investigated in the experiments.

## 2. Materials and methods

### 2.1. Materials

The chemicals, $FeCl_3{\cdot}6H_2O$, sodium acetate, ethylene glycol, and ethylene imine polymer were analytical grade and used as received. EZ-Link®NHS-iminobiotin (>95%, cat no. 21117) was purchased from Thermo Scientific Pierce Protein Research Products. Biotin-4-fluorescein was purchased from Merck (Lot 698,049). Chicken egg white derived protein, avidin (>95%, cat no. A9275) and bovine serum albumin (≥98%, cat no. A7030, BSA): Sigma–Aldrich (USA). All other chemicals used were of analytical grade, and Milli-Q water was used throughout the experiments. Fresh chicken eggs were bought from local farmers' markets.

### 2.2. Methods

#### 2.2.1. Preparation of magnetic nanoparticles

$Fe_3O_4$ nanoparticles were obtained via a facile solvothermal synthetic method, in which ethylene imine polymer was used as the protective agents to prevent the particles from aggregation. One typical synthetic procedure is as follows: $FeCl_3{\cdot}6H_2O$ (0.135 g), sodium acetate (1.8 g) and ethylene imine polymer (0.5 g) were added to ethylene glycol (20.0 ml) to form colloid mixture under vigorous stirring at room temperature for 45 min, then 18.0 ml of the mixture was sealed in teflon-lined stainless-steel autoclave of 20 ml capacity. Finally, the autoclave was heated and maintained at 200 °C for 12 h, and allowed to cool down to room temperature naturally. The back products were washed twice with absolute ethanol and twice with Milli-Q water, then the magnetic nanoparticles were dispersed in Milli-Q water and stored in sealed cell (50 ml) at room temperature.

#### 2.2.2. Biofunctionalization of magnetic nanoparticles with 2-iminobiotin

The procedure was anchoring 2-iminobiotin into magnetic nanoparticles. A general way to connect 2-iminobiotin from NHS-iminobiotin to the surface of magnetic nanoparticle is as follows: 40 ml of 0.1 mol/l borate buffer (pH 8.75) containing 25 mg magnetic nanoparticles was produced at 4 °C. One milligrams of EZ-Link®NHS-iminobiotin was dissolved in 0.2 ml of dry DMF and added incrementally at 4 °C to the gently stirred solution. After the addition, the solution was stirred for 2 h at 4 °C and allowed to warm to room temperature overnight. The biofunctional magnetic nanoparticles were separated from unconjugated 2-iminobiotin and other water-soluble chemicals by magnetically driven separation at room temperature for 5 min. The recovered biofunctional magnetic nanoparticles were redispersed in 40 ml of Milli-Q water to wash impurities of the nanoparticles surface. The washings were discarded and the recovered biofunctional magnetic nanoparticles were subsequently redispersed in the next 40 ml of Milli-Q water. At the end of the washing procedure, the nanoparticles were washed three times with Milli-Q water and then 40 ml solution containing the biofunctional magnetic nanoparticles was produced. 12.6 ml of solution was poured from the solution containing the biofunctional magnetic nanoparticles (25 mg), the solution obtained (containing 8.0 mg biofunctional magnetic nanoparticles) was used for avidin adsorption experiments. All of the solutions containing the biofunctional magnetic nanoparticles were stored at 4 °C until further use.

#### 2.2.3. Characterization of non-functional magnetic nanoparticles and biofunctional magnetic nanoparticles

The size and morphology of non-functional magnetic nanoparticles and biofunctional magnetic nanoparticles were measured by a bright-field TEM (Model JEM 2010, Japan). The TEM samples were prepared by coating a copper grid (200 mesh and cover with Formvar/carbon) with a thin layer of diluted particle suspension, the copper grid was then dried at room temperature for 24 h before the measurement. 2-Iminobiotin anchoring onto the surface of magnetic nanoparticles was monitored by Fourier transform infrared spectroscopy (FTIR). Data was collected on a Nicolet Avatar-330 spectrometer (Thermo Nicolet, US) with 4 $cm^{-1}$ resolution using the KBr pellet technique.

#### 2.2.4. Avidin affinity adsorption with biofunctional magnetic nanoparticles

A diagrammatic representation of the general procedure for the separation of avidin using biofunctional magnetic nanoparticles is present in Fig. 1. Unless mentioned otherwise, the binding buffer was a 0.1 mol/l sodium carbonate buffer (0.5 mol/l NaCl, pH 10.8) containing the indicated amount of avidin (supernatant: $S_0$). The washing buffer was a 0.05 mol/l Sodium phosphate buffer (0.1 mol/l NaCl and 1 mmol/l EDTA, pH 6.0). In the first step, the biofunctional magnetic nanoparticles (8.0 mg) were dispersed in 40 ml of the binding buffer, then the sample was incubated for 45 min at 18 °C (gentle stirring). The biofunctional magnetic nanoparticles were separated from the solution (supernatant: $S_1$) with the aid of a magnet. In the second step, the recovered biofunctional magnetic nanoparticles were redispersed in the washing buffer (pH 6.0 without avidin) and again separated ("washing step," $S_2$). In the third step, the biofunctional magnetic nanoparticles bound to avidin was redispersed in 0.1 mol/l ammonium acetate buffer (pH 4) containing 0.5 mol/l NaCl (the dissociation buffer) and they were then removed with the aid of a magnet. The dissociating step was usually repeated once (supernatant: $S_3$). After each separation, the respective supernatants were adjusted to pH 7.5 (1.0 mol/l HCl in the case of binding buffer, 1.0 mol/l NaOH in the case of dissociation buffer and washing buffer).

The effect of ionic strength on avidin adsorption was investigated at five different feed NaCl concentrations (0.1, 0.2, 0.3, 0.5, and 0.7 mol/l at pH 10.8) and the five experiments were carried out at 18 °C. The effect of temperature on avidin adsorption was investigated at five different reaction temperatures (4, 18, 25, 37 and 50 °C at pH 10.8 and NaCl 0.5 mol/l) and the feed avidin concentration was at 45 μg/ml in experiments. The effect of pH of binding buffer was studied at pH values of 7.0, 8.0, 9.0, 10.8 and at 18 °C. The effect of the feed avidin concentration on avidin adsorption was investigated at nine different feed concentrations (5, 10, 15, 25, 35, 45, 55, 65, and 75 μg/ml at pH 10.8) and the experiments were carried out at 18 °C.

#### 2.2.5. Procedure for affinity adsorption and separation of avidin from treated egg-white solution

One egg-white (320 ml) separated from yolks was diluted with 0.5 vol. of water $(NH_4)_2SO_4$ was added to 65% saturation, and the mixture was stirred for 1 h and centrifuged at 10,000 × *g* for 20 min. The supernatant was adjusted to 100% saturation with $(NH_4)_2SO_4$ and stirred for 2 h, then the mixture was centrifuged at 10,000 × *g* for 20 min. The precipitate was dissolved in 200 ml of water and mixed with an equal volume of cooled ethanol and the mixture was constantly stirred for 15 min. During this procedure the temperature should not exceed 4 °C. After centrifugation (at 10,000 × *g* for 15 min), the precipitate was extracted with 50 mmol/l sodium

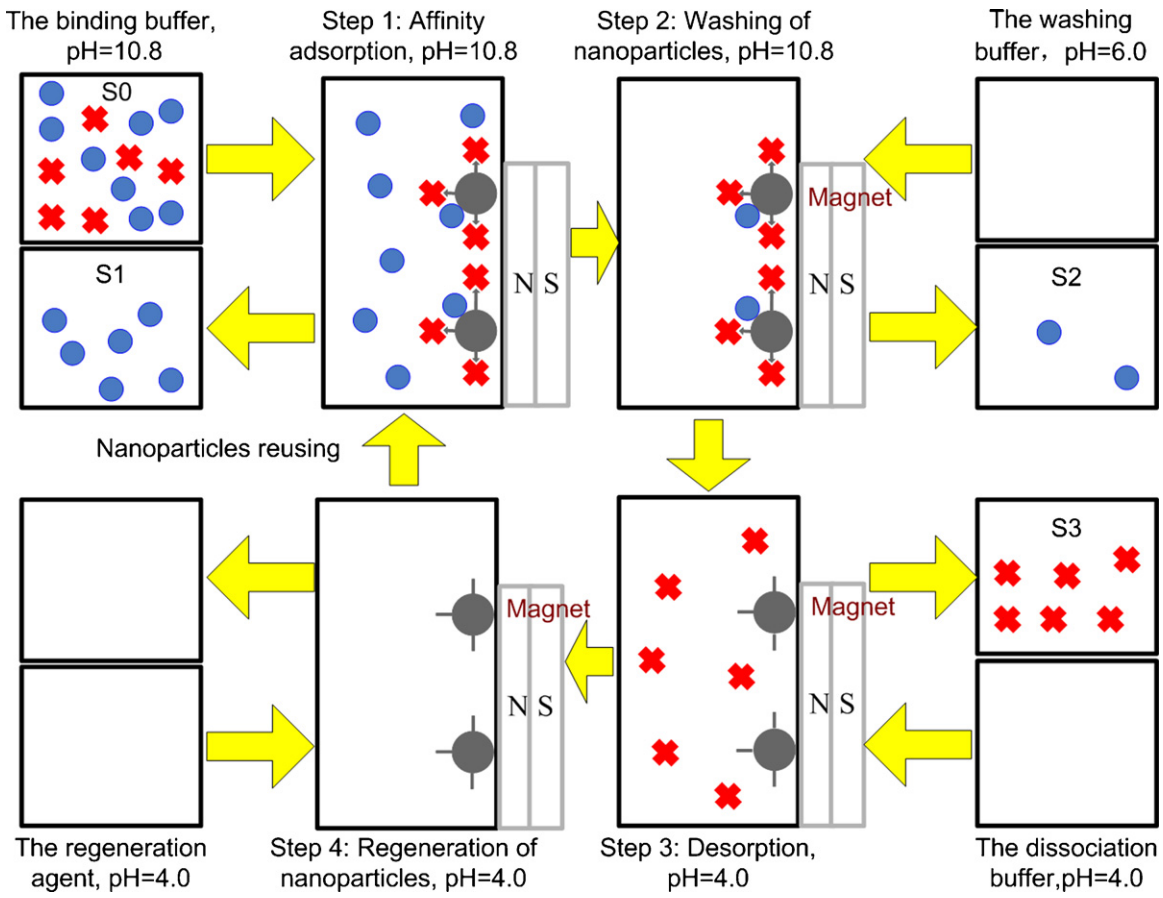


**Fig. 1.** Schematic presentation of an avidin purification by biofunctional magnetic nanoparticles: ( ) biofunctional magnetic nanoparticles, ( ) the avidin and ( ) impurities.

acetate buffer (pH 4.5). The extract was centrifuged at 10,000 × *g* for 10 min and the precipitate obtained was subjected to another extraction and centrifugation. The supernatants were combined, dialysed against water and freeze-dried. The sample ($S_0$) was kept at 4 °C until use.

$S_0$ was dissolved in 50 ml binding solution containing biofunctional magnetic nanoparticles (200 μg/ml), then the mixture was incubated for 45 min at 18 °C (gentle stirring). The following procedures were carried out in accordance with the general procedure for the separation of avidin described above. For the investigation of the pH of washing buffer on avidin purity (the second step in the general procedure for the separation of avidin), the procedure was identical, but different washing buffers were used, namely 50 mmol/l sodium phosphate buffers (0.1 mol/l NaCl and 1 mmol/l EDTA, pH 4.2, 4.5, 5.0, 5.6, 6.0, 7.0, 8.0, 9.0 and 10.8). For the investigation of the number of washing cycles on avidin purity, one, two, and three washing cycles were performed in the experiments.

### 2.2.6. Regeneration studies of biofunctional magnetic nanoparticles

After an entire separation process of avidin, the biofunctional magnetic nanoparticles recovered were redispersed in a regeneration agent (0.1 mol/l ammonium acetate buffer at pH 4.0, 0.5 mol/l NaCl and 1 mmol/l EDTA), the sample was incubated for 10 min at 18 °C (gentle stirring). The biofunctional magnetic nanoparticles were subsequently removed with the aid of a permanent magnet and the washing step was usually repeated once. The recovered biofunctional magnetic nanoparticles were redispersed in Milli-Q water and the sample was incubated for 10 min at 18 °C (gentle stirring). The biofunctional magnetic nanoparticles were subsequently recovered with the help of a permanent magnet, then the regenerated biofunctional magnetic nanoparticles were achieved and redispersed in Milli-Q water and stored at 4 °C until further use.

To investigate the reusability of the regenerated biofunctional magnetic nanoparticles, the recovered biofunctional magnetic nanoparticles were applied in affinity separation of avidin from treated egg-white solution. The separation procedures were carried out in accordance with the general procedure for the separation of avidin described above.

### 2.2.7. Analytical methods

The amount of avidin present in the different supernatant solution was measured by fluorescence quenching or fluorescence polarization assay [21–23] on a spectrofluorophotometer (Shimadzu RF-5301). The total protein content of the various supernatants was measured by the Bio-rad protein assay according to the manufacturer's instructions. Calibration curves were constructed with BSA. For avidin, a calibration curve was established based on the results of fluorescence quenching or fluorescence polarization assay. Thereby the avidin contribution to the total protein concentration could be subtracted from the overall values in order to evaluate the avidin content adsorbed by the biofunctional magnetic nanoparticles and remained in the supernatant solution. High-performance size-exclusion chromatography (HPSEC) was also carried out for the chromatographic studies of avidin with a Waters model HPLC apparatus (Waters, USA) using a Waters 2998 photodiode detector (Waters, USA), processing the samples

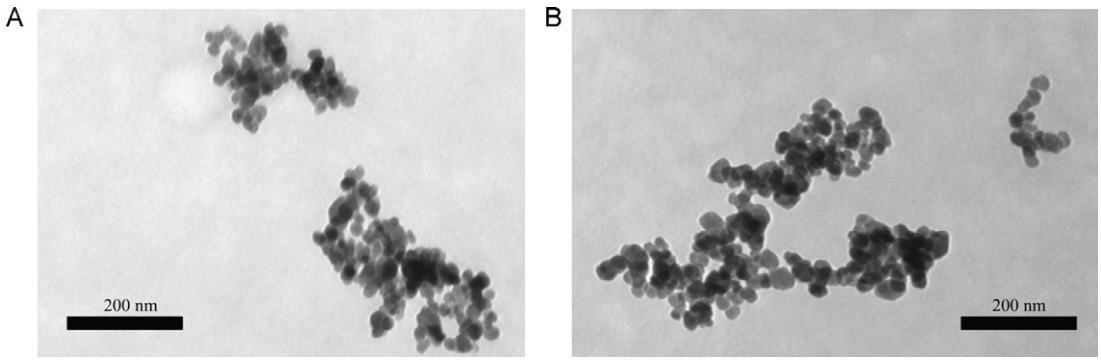


**Fig. 2.** TEM images of magnetic nanoparticles: (A) non-functional magnetic nanoparticles and (B) biofunctional magnetic nanoparticles.

on a SRT SEC-300 column (5 μm, 4.6 mm × 300 mm) (SEPAX Technologies, USA). The detection was performed by measuring the UV absorbance at 214 nm and evaluation of purity was performed by analyzing the peak area of avidin. The mobile phase was 0.15 mol/l NaCl, 0.02 mol/l sodium phosphate, pH 7.0, with a flow rate of 0.35 ml/min.

## 3. Results and discussion

### 3.1. Characterization of non-biofunctional magnetic nanoparticles and biofunctional magnetic nanoparticles

Typical TEM micrographs for non-biofunctional magnetic nanoparticles and biofunctional magnetic nanoparticles were shown in Fig. 2 which indicated that the diameter of these particles was approximately 30 nm. The size of the biofunctional magnetic nanoparticles was similar to non-biofunctional nanoparticles, assuming the avidin tetramer to be represented by a sphere whose calculated diameter, $D_0$, is 5.4 nm as obtained from the relationship using a molecular weight of 68,300 and a partial specific volume of 0.73 [24]. In the separation of proteins, the size of biofunctional magnetic nanoparticles should be slightly larger than or similar to the size of target proteins [16].

The nanoparticles prepared by this method had abundant amino groups on the surface which make it to be hydrophilic and bind to 2-iminobiotin in an optimized aqueous buffer system (see Section 2) based on the suggestion made by Brinkley [25] (Fig. 3). 2-Iminobiotin anchoring onto the surface of magnetic nanoparticles was verified by FTIR. Fig. 4 shows the FTIR spectra of non-functional magnetic nanoparticles and biofunctional magnetic nanoparticles in the wavenumber range of 500–4000 $cm^{-1}$. It showed that the characteristic adsorption bands of Fe–O bonds in the tetrahedral sites are 571 $cm^{-1}$ and 586 $cm^{-1}$. The spectrum of $Fe_3O_4$ nanoparticles showed peaks at 1620.28 and 3439.50 $cm^{-1}$, which was attributed to primary amides bond (R-$NH_2$). In FTIR spectra of biofunctional magnetic nanoparticles, the peak at 701.07 $cm^{-1}$ is characteristic peak of thiophene due to 2-iminobiotin connected to the biofunctional magnetic nanoparticles surface [26]. The iminobiotin coupled with biofunctional magnetic nanoparticles surface has been shown a high affinity to avidin (and avidin-tagged biomolecules) at elevated pH (9.5–10.8), but released the avidin easily at lower pH (≈4) [27–29]. In addition, the iminobiotin had a subtle advantage, which covered the surface of the nanoparticles and bound the target proteins quickly, thus reducing the overall unoccupied surface area that may be available for nonspecific adsorption of other proteins.

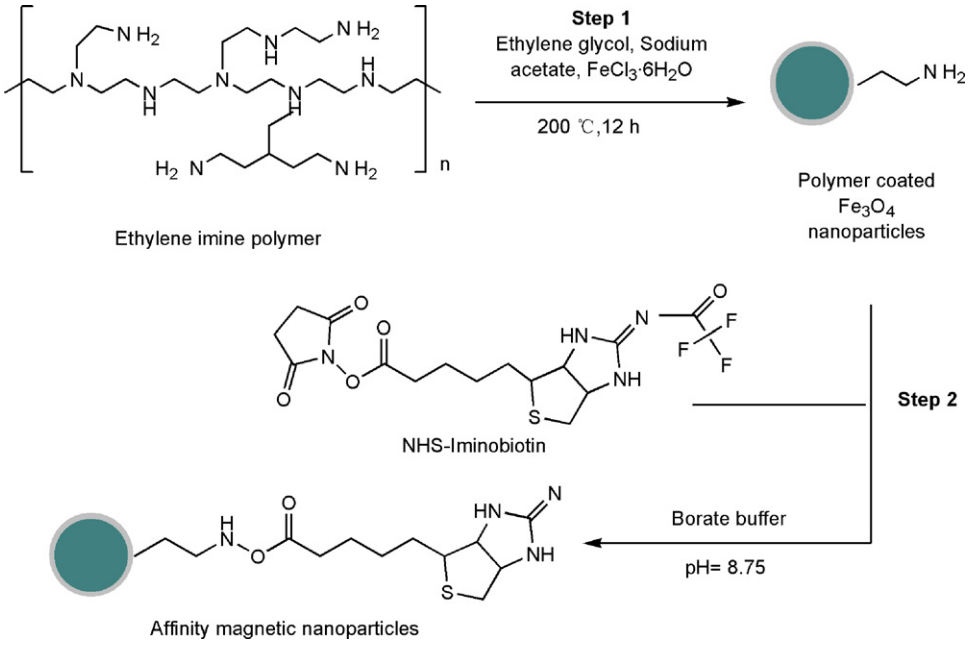


**Fig. 3.** Synthesis of the biofunctional magnetic nanoparticles using 2-iminobiotin as affinity mediator.

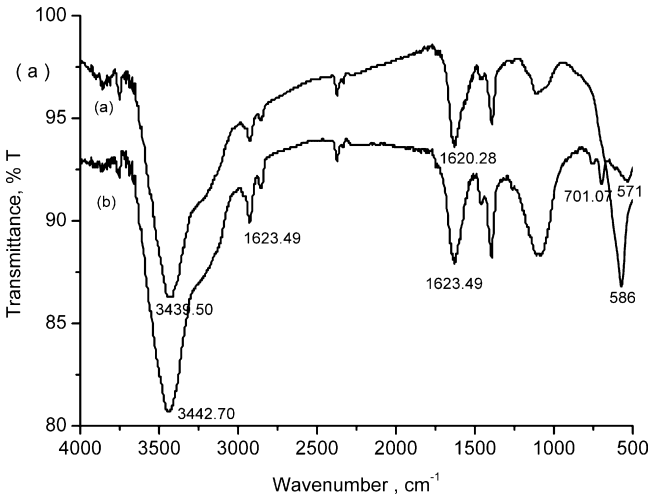


**Fig. 4.** FTIR spectra of (a) non-functional magnetic nanoparticles and (b) biofunctional magnetic nanoparticles.

### 3.2. Influence of ionic strength, temperature and pH on avidin adsorption and affinity adsorption isotherms of avidin

#### 3.2.1. Effects of ionic strength on avidin adsorption

The effects of ionic strength on avidin adsorption and recovery were shown in Fig. 5A. It was observed that avidin adsorption and recovery increased under low ionic strength, and the maximum of adsorption were observed in the presence of 0.5 mol/l NaCl. However, the solutions containing more than 0.5 mol/l NaCl resulted in a decrease in adsorption effectiveness. This observation was attributed to that affinity interaction between avidin and iminobiotin. The interaction would increase at low ionic strength [29], but it would be lowered because of the screening of the electrostatic interactions between avidin and charged layers on the nanoparticles at high ionic strength. Chiang et al. also found that the decreasing of plasmid DNA adsorption to magnetic particles was attributed to the screening of the electrostatic interactions between DNA and the charged ethylene imine polymer layers on the magnetic nanoparticles under conditions of high ionic strength [30]. Therefore, these results imply that ionic strength is an important factor for avidin adsorption and recovery.

#### 3.2.2. Effects of temperature on avidin adsorption

To determine optimum temperature for avidin adsorption, adsorption experiments were performed at various temperatures (4–50 °C) and the temperature-adsorption capacity profiles for the biofunctional magnetic nanoparticles were shown in Fig. 5B. It was observed that with the increase of temperature the avidin adsorption capacity increased firstly and then decreased gradually. The optimum temperature for avidin adsorption was 18 °C. The adsorption behavior was principally observed because the binding of molecules to specific receptor sites on proteins increased as the temperature was lowered [31–33]. However, the affinity for protein adsorption tended to decrease because the reaction rate on the affinity was low at 4 °C.

#### 3.2.3. Effects of pH on avidin adsorption

The effects of pH on avidin adsorption and recovery were reported in Fig. 5C which shows the avidin adsorption capacity enhanced with increasing pH values. It can be inferred that the high pH contributes in two ways to the observed improvement of the avidin adsorption capacity. It is known that at high pH the free base form of 2-iminobiotin retains the high-affinity ($K_a = 10^8\ M^{-1}$) spe-

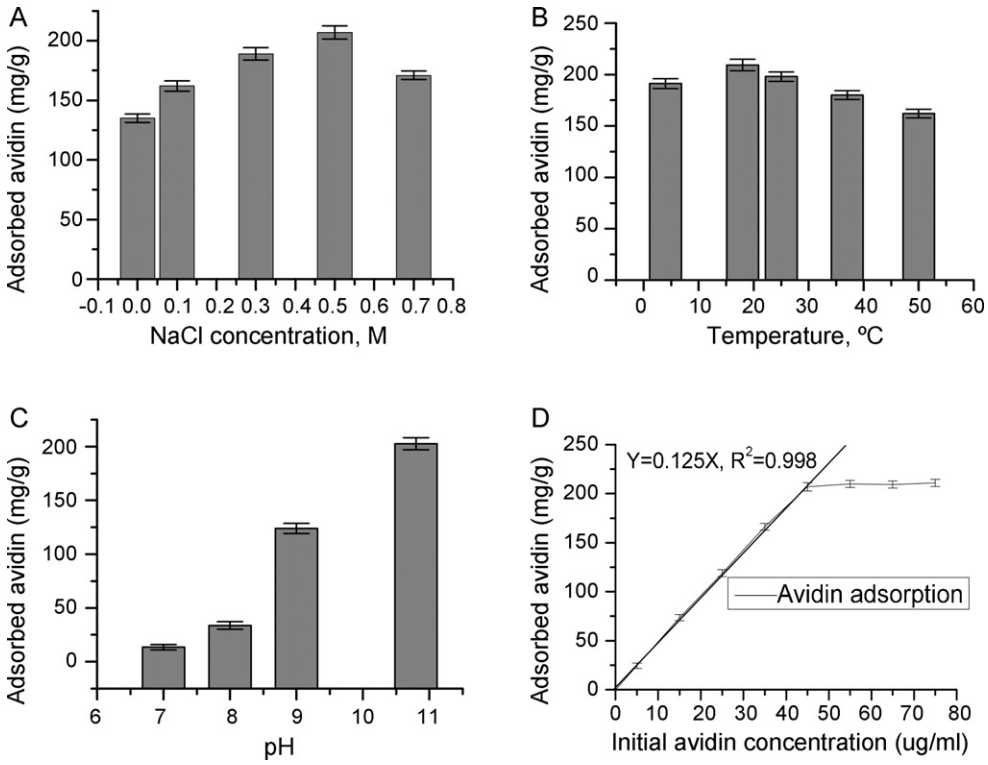


**Fig. 5.** Effect of ionic strength, temperature, pH and initial avidin concentration on avidin adsorption using biofunctional magnetic nanoparticles. (A) Effect of ionic strength of binding buffer with avidin feed concentration 45 μg/ml at pH 10.8 and temperature 18 °C, (B) effect of temperature of binding buffer with NaCl concentration 0.5 mol/l and avidin feed concentration 45 μg/ml at pH 10.8, (C) effect of pH of binding buffer with NaCl concentration 0.5 mol/l and avidin feed concentration 45 μg/ml at temperature 18 °C and (D) effect of initial concentration on avidin adsorption with NaCl concentration 0.5 mol/l. Adsorption isotherm of avidin on biofunctional magnetic nanoparticles when initial avidin concentration was less than 45 μg/ml. pH: 10.8; *T*: 18 °C. Each result is the average calculated in 95% confidence interval of five parallel studies.

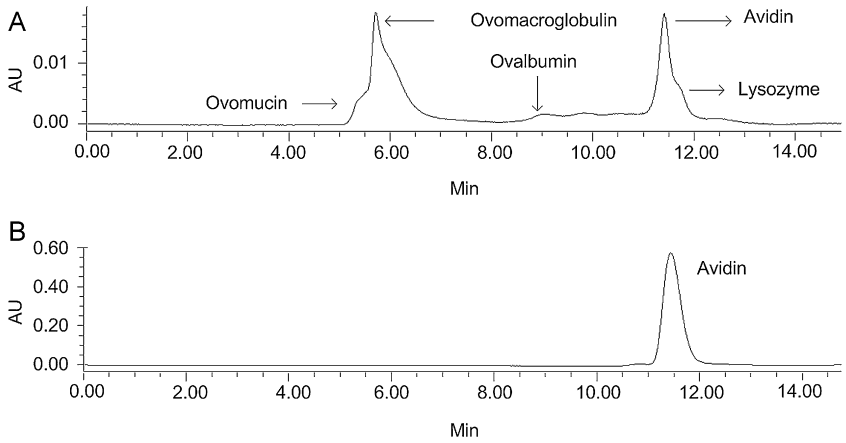


**Fig. 6.** HPLC analysis of the protein mixtures: (A) the proteins in $S_0$ and (B) the protein in $S_3$.

cific binding characteristics of biotin [34,35], therefore the binding interaction between iminobiotin and avidin increased at elevated pH. In addition, the isoelectric point of avidin is from 10.2 to 10.5. At pH 10.8 the avidin is negatively charged and biofunctional magnetic nanoparticles are positively charged. Therefore, there is an electrostatic attraction between avidin molecules and biofunctional magnetic nanoparticles. The attractive force can promote the adsorption of avidin onto the surface of the biofunctional magnetic nanoparticles.

### 3.2.4. Affinity adsorption isotherms of avidin

Adsorption isotherms have been used to characterize the interaction between the target molecules and the ligand [36]. Also, it is necessary to know the adsorption isotherm parameters, such as Langmuir and Freundlich adsorption isotherm parameters. This provides a relationship between the concentration of protein in the solution and the amount of protein adsorbed on the biofunctional magnetic particles when the two phases are at equilibrium. The Langmuir adsorption model has been widely used to design a affinity adsorption process for protein separation [37–41]. The model assumes that the molecules are adsorbed at a fixed number of well-defined sites, each of which can only hold one molecule [42]. The isotherms can be expressed as Langmuir equation:

$$q = \frac{q_m c}{k_d + c} \quad (1)$$

where $q$ and $c$ are the adsorbed protein density in equilibrium (mg/g) and aqueous phase protein concentration (mg/ml), respectively, $q_m$ (mg/g) is the maximum adsorption capacity, and $k_d$ (mg/ml) is the dissociation constant. It has been reported that the dissociation constant of avidin with iminobiotin is less than $10^{-9}$ M at pH 10.8 [29].

The adsorption isotherm was obtained from a series of experiments as represented in Fig. 5D. High $R^2$ values indicated that the Langmuir adsorption model predicted the adsorption behavior well when initial avidin concentration was less than 45 μg/ml, which means that avidin can only be bound with one iminobiotin molecule on the surface of biofunctional magnetic nanoparticles, although avidin can be theoretically linked with four iminobiotin molecules. The maximum avidin adsorption capacity calculated from experimental data using Langmuir model is 225 mg/g. The difference was attributed to steric and geosteric hindrance between the nanoparticles and avidin when it is close to the biofunctional nanoparticles. In addition, results from Fig. 5D show the avidin adsorption capacity was unchanged when avidin concentration was more than 45 μg/ml. The increase of feed avidin concentration needs more avidin binding sites of magnetic nanoparticles. When the magnetic nanoparticles binding sites concentration was not sufficiently high (saturation) with increasing avidin concentration, the amount of specific adsorption capability for avidin protein is unchanged. The molar ratio of the ligand to the tag proteins must be large enough in the biofunctional magnetic nanoparticles. The principle should also be taken into account for affinity chromatography and affinity precipitation.

### 3.3. Biofunctional magnetic nanoparticles for affinity separation of avidin from treated egg-white solution

To evaluate the biofunctional magnetic nanoparticles specificity for avidin, a series of experiments of the biofunctional magnetic nanoparticles for avidin separation were performed with the treated egg-white solution ($S_0$) containing ovomucin, ovomacroglobulin, ovalbumin, lysozyme and avidin. Fig. 6 shows the HPSEC Chromatograms of the protein mixtures. In Fig. 6A, the identification of the chemicals peak labeled with retention time indicated the presence of ovomucin, ovomacroglobulin, ovalbumin, lysozyme and avidin in supernatant $S_0$. Noticeably, the peak of the protein in the dissociation buffer (containing in solution the released avidin, $S_3$) only showed the existence of avidin in Fig. 6B. As expected, these results indicated that the avidin was released from the biofunctional magnetic nanoparticles in the dissociation buffer and mainly recovered in supernatant $S_3$. Less than 7.20% of the avidin was lost in the separation procedure and the total recovery yield of avidin was 92.80%. The residual protein contaminants of the purified avidin were below the detection limit and the purity of avidin was 98.52%. The biofunctional magnetic nanoparticles for affinity separation of avidin are a highly specific biofunctional nanomagnetic particles, which adsorb avidin nearly quantitatively, while concomitantly showing little or no adsorption of other proteins and contaminants.

The effect of cleaning cycles and the pH of washing buffer on the avidin purity and recovery yield were investigated. It was observed that at pH 6.0 or greater the number of washing cycles had no effect on avidin recovery yield, but two or three washing cycles were sufficient to rid non-specific adsorption of proteins and increase avidin purity. Fig. 7 shows the effects of pH on the avidin purity and recovery yield. Clearly, the purity of avidin increased rapidly as the pH of washing buffer decreased, and the recovery yield of avidin maintained a high level as the pH of washing buffer was more than 5.6. It is possible that there are strong electrostatic attraction between the impurities (such as ovomucin, pI 4.1, ovomacroglobulin, pI 4.5, ovalbumin, pI 4.5) and the biofunctional magnetic nanoparticles, because at elevated pH the impurities are negatively charged and biofunctional magnetic nanoparticles are positively charged while

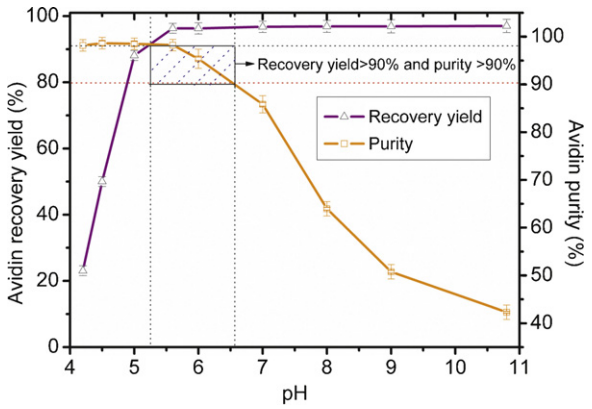


**Fig. 7.** The effect of pH of washing buffer on avidin purity and recovery yield. Each result is the average calculated in 95% confidence interval of five parallel studies.

at low pH, the electrostatic attraction is weakened considerably. In the case, the washing buffer at low pH succeeded in lowering the ovomucin, ovomacroglobulin and ovalbumin contamination of the avidin to below the detection limit. As expected, these washing cycles also cleaned the lysozyme contamination of the avidin. The results showed that at pH 5.6–7.0 the affinity interactions between iminobiotin and avidin are still present. Sugawara et al. reported that iminobiotin binds to avidin at pH 5.6–8.9 [43]. When these washing cycles were performed between pH 5.6 and pH 7, more than 92% of avidin could be recovered in the general procedure for the separation of avidin. The biofunctional magnetic particles for affinity separation of avidin, like affinity chromatography and affinity precipitation, need the washing step which is the redispersion and reseparation of biofunctional magnetic particles in the washing buffer without breaking the affinity interaction. Several cleaning cycles and the adjustment of the pH of washing buffer can be performed to increase the avidin purity to the extent possible.

### 3.4. Regeneration of biofunctional magnetic nanoparticles

The regeneration of biofunctional magnetic nanoparticles was investigated as a function of the regeneration agent (0.1 mol/l ammonium acetate buffer at pH 4.0, 0.5 mol/l NaCl and 1 mmol/l EDTA), and the regeneration procedures were carried out in accordance with Section 2.2.6. To verify the reusability of the regenerated nanoparticles, adsorption–desorption cycles were repeated twice using the same biofunctional magnetic nanoparticles. It was observed that almost 87.3% of avidin could still be recovered by the regenerated nanoparticles. It can be concluded that at pH 4.0 there should be a little electrostatic repulsion between the residual protein molecules (ovomucin, ovomacroglobulin, ovalbumin and lysozyme) and the biofunctional magnetic nanoparticles. In addition, the residual avidin can also be released from the biofunctional magnetic nanoparticles [35]. Thus, the recovered biofunctional magnetic nanoparticles could be regenerated by the suitable regeneration agent, which made possible repeated usage of the biofunctional nanoparticles and maximized its value.

## 4. Conclusion

This work aimed and described affinity adsorption and separation behaviors of avidin on biofunctional nanoparticles binding to iminobiotin. Affinity adsorption is a powerful technique that enables the use of biofunctional nanoparticles for rapid, high-efficient, and selective protein separation and purification. From the experimental data, it can be reported that affinity adsorption and separation of avidin was achieved effectively and the obtained results proved that biofunctional magnetic nanoparticles have good adsorptive properties for these purposes. Avidin adsorption capacity of biofunctional magnetic nanoparticles was strongly depended on interactions between avidin and iminobiotin coupled with biofunctional magnetic nanoparticles, which exhibited sensitivity to temperature, pH, ionic strength, and feed avidin concentration. The experimental data were well fitted to the Langmuir model when feed avidin concentration was less than 45 μg/ml, and the maximum avidin adsorption capacity obtained was 225 mg/g. Based on the experimental results above, the biofunctional magnetic nanoparticles were successfully used to adsorb and separate avidin from treated egg-white solution containing large amounts of other proteins, and avidin can be obtained in high recovery yield (>92%) and high optical purity (>98.5%). These studies on regeneration of biofunctional magnetic nanoparticles, reported here, prove reusability of the nanoparticles. Thus, it can be concluded that biofunctional nanoparticles binding to iminobiotin can be used as a specific affinity nanoparticles for affinity adsorption and separation of avidin in biotechnological and industrial applications.

## Acknowledgement

The work was supported by the earmarked fund for Modern Agro-industry Technology Research System (Project code no: nycytx-41-g22).

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