Application of Cu$^{2+}$-attached Magnetite Nanoparticles Embedded Supermacroporous Monolithic Composite Cryogels for DNA Adsorption

Cu$^{2+}$-takılı Magnetit Nanopartikül Gömülü Süpermakrogözenekli Monolitik Kompozit Kriyojellerin DNA Adsorpsiyon Uygulaması

Research Article / Araştırma Makalesi

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ABSTRACT

Cryogels are known as a novel generation of stationary phases in the separation science, and they have been successfully used as efficient adsorbents for separation and purification of biomolecules, such as proteins, enzymes, DNA, cell organelles and viruses in downstream processes. In this study, DNA adsorption performance of Cu$^{2+}$-attached magnetite (Fe$_3$O$_4$) nanoparticles (Cu$^{2+}$-AMNPs) embedded supermacroporous monolithic composite cryogels were investigated. Firstly, Magnetite nanoparticles (NPs) coated with 3-aminopropyltriethoxysilane (APTES) were prepared by silanization reaction. Then, Cu$^{2+}$ ions were attached to silanized magnetite NPs through amine groups. After that, Cu$^{2+}$-AMNPs were embedded into the cryogels for DNA adsorption studies. The prepared cryogel was characterized by scanning electron microscopy (SEM), elemental analysis, FTIR and swelling. SEM analysis indicates that the cryogel have a heteroporous structure with interconnected pores of 10-50 μm size, which ascribed to the porogens effect of frozen water crystals. The maximum amount of DNA adsorption was 19.97 mg/g polymer, at pH 8.0.

Key Words
Affinity chromatography, DNA adsorption, metal chelate, cryogels.

ÖZET

Kriyojeller ayırma biliminde yeni nesil sabit faz olarak değerlendirilmektedirler. Kriyojeller, altakım işlemlerinde protein, enzim, DNA, hücre organelleri ve virüs gibi biyomoleküller ayırması ve saflaştırılması etkin adsorbent olarak kullanılmaktadır. Bu çalışmada, Cu$^{2+}$-takılı magnetit (Fe$_3$O$_4$) nanopartikül (Cu$^{2+}$-AMNP) gömülü süpermakrogözenekli monolitik kompozit kriyojellerin DNA adsorpsiyon performansı incelenmiştir. Öncelikle, 3-aminopropyltriethoxysilane (APTES) ile kaplanmış magnetit NPs'ler silanizasyon reaksiyonu ile hazırlanmıştır. Daha sonra, silanlanmış magnetit NPs'ler, amine grupları üzerinden Cu$^{2+}$ iyonları takılmıştır. Bundan sonra, Cu$^{2+}$-AMNPs'ler DNA adsorpsiyon çalışmaları için süpermakrogözenekli monolitik kompozit kriyojeller gömülmüştür. Hazırlanan kriyojel taramalı elektron mikroskobu (SEM), elementel analiz, FTIR ve şişme testi ile karakterize edilmiştir. SEM analizleri kriyojellerin, donmuş su kristallerinin porojen etkisi ile oluşan birbirine bağlı 10-50 μm boyutunda gözenekleri ile heterogözenekli bir yapıya sahip olduğunu göstermiştir. Maksimum DNA adsorpsiyon miktarı pH 8'de 19.97 mg/g polymer olarak bulunmuştur.

Anahtar Kelimeler
Afinite kromatografi, DNA adsorpsiyon, metal şelat, kriyojeller.

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INTRODUCTION

Immobilized metal affinity chromatography (IMAC) has been introduced by Porath et al. [1,2]. This technique has become a widespread analytical and preparative separation method for the purification of diverse biomolecules, such as enzymes, coenzymes, cofactors, antibodies, antigens, hormones, proteins and nucleic acids [3-8]. While the interaction of metal ions with nucleic acids is a long-standing and active field of research [9-10], there are limited studies including the adsorption of nucleic acids to the IMAC columns [11]. The low cost of metals and the ability to reuse adsorbents hundreds of times without any detectable loss of metal-chelating properties are the attractive features of immobilized metal affinity separation.

Cryogels are known as a novel generation of stationary phases in the separation science [12], and they have been successfully used as efficient adsorbents for separation and purification of biomacromolecules, such as proteins, enzymes, DNA, cell organelles and viruses in downstream processes [13-17]. Cryogels are alternative column materials and have some advantages over other conventional ones, especially in purification of biomolecules, with their large pores, short diffusion paths, low pressure drop during processing and very short residence time during both adsorption and elution period [15]. However, the adsorption capacity of the cryogels for biomolecules is low due to low surface area of the interconnected supermacropores within the matrix [18]. Therefore, improving the binding capacity of supermacroporous cryogels has a great importance in bioseparation processes [19].

Different polymeric adsorbents have been suggested as supports for immobilization of DNA [20-25]. In order to treat some autoimmune diseases, such as systemic lupus erythematosus (SLE), DNA-affinity adsorbents have been used for removal of anti-double stranded DNA antibodies from the sera of patients suffering from SLE [26-29].

In this study, we prepared Cu²⁺-attached magnetite (Fe₃O₄) NPs embedded supermacroporous monolithic composite cryogel under the freezing-temperature. Due to the large surface area and high DNA adsorption capacity of these magnetite NPs, this adsorbent prepared by us may be considered as a good candidate for achieving higher removal rates for anti-DNA antibodies.

EXPERIMENTAL

Materials

DNA (from herring testes) was purchased from Sigma (St. Louis, MO, USA), (3-Aminopropyl)-triethoxysilane (APTES, 99%), Magnetite nanoparticle (< 50 nm) were purchased from Aldrich (Germany). N,N'-methylen-bis-acrylamide (MBA-Am) and ammonium persulfate (APS) were supplied by Sigma (St. Louis, MO, USA). Hydroxyethyl methacrylate (HEMA) was obtained from Fluka A.G. (Buchs, Switzerland), distilled under reduced pressure in the presence of hydroquinone inhibitor, and stored at 4°C until use. N,N',N',N'-Tetramethylethylendiamine (TEMED) was obtained from Fluka A.G. (Buchs, Switzerland). All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany). Deionized water was used in all experiment sets.

Silanization of Magnetite NPs with (3-aminopropyl)-triethoxysilane

Silane compounds as silane coupling agent easily react with the different supports having surface hydroxyl groups [30]. For the silanization of magnetite NPs with (3-aminopropyl)-triethoxysilane (APTES), 2.112 g Fe₃O₄ NPs were sonicated with 50 mL ethanol/water (volume ratio, 1:1) solution for 10 min to get uniform dispersion. Then 8.08 g of APTES was added, and solution stirred under N₂ atmosphere at 40°C for 2 h. The optimal surface modification molar ratio of APTES to Fe₃O₄ was found 4:1, previously [31]. After that the solution was cooled to room temperature. The silanized magnetite (Fe₃O₄) NPs (Figure 1) were collected with a magnet, and washed with ethanol and deionized water respectively. Finally, silanized magnetite (Fe₃O₄) NPs were dried in vacuum oven at 70°C.

Attachment of Cu²⁺ ions to silanized magnetite (Fe₃O₄) NPs

1.0 g of silanized magnetite NPs was treated with a Cu²⁺ solution [100 mg/L (pH 5.0), adjusted with
HCl and NaOH] at room temperature for 2 h. A 1000-ppm atomic absorption standard solution was the source of the Cu²⁺ ions. The concentration of the Cu²⁺ ions in the resulting solution was determined with a graphite furnace atomic absorption spectrometer (GFAAS, Analyst 800/PerkinElmer, USA). The instrument response was periodically checked with known metal solution standards. The experiments were performed in triplicate. The Cu²⁺ concentrations in the initial and final solutions were used to calculate the amount of Cu²⁺ ions adsorbed.

Cu²⁺ leakage from the Cu²⁺-AMNPs was investigated at pHs varied between 4.0 and 7.0 and also in a medium-containing 1.0 M NaCl. Cu²⁺-AMNPs were stirred for 24 h at room temperature. Then the concentration of Cu²⁺ ions in the supernatants was determined using an atomic absorption spectrophotometer.

Preparation of Cu²⁺-AMNPs embedded cryogel column
Preparation of supermacroporous monolithic composite cryogel embedded with Cu²⁺-AMNPs is described as follows: Monomers (50 mg MBAAm and 0.3 mL HEMA) were dissolved in deionized water (5 mL) and the mixture was degassed under vacuum for about 5 min to eliminate soluble oxygen. The cryogel was produced by free radical polymerization initiated by TEMED (20 µL) and APS (100 µL) [10% (w/v)]. After adding APS, the solution was cooled in an ice bath for 2-3 min. In this step, 15 mg of Cu²⁺-AMNPs were added to the polymerization mixture. TEMED was added and the reaction mixture was stirred for 1 min. Then, the reaction mixture was poured into a plastic syringe (5 mL, id. 0.8 cm). The polymerization solution in the syringe was frozen at -12°C for 24 h and then thawed at room temperature. For removal of unconverted monomers and initiator, washing solutions (i.e., a dilute HCl solution and a water-ethanol mixture) were recirculated through the monolithic composite cryogel column, until cryogel column is clean. Purity of the monolithic composite cryogel was followed by observing the change of optical densities of the samples taken from the liquid phase in the recirculation system. After washing, the cryogel was stored in buffer containing 0.02% sodium azide at 4°C until use.

The porosity of Cu²⁺-AMNPs embedded cryogel column
For the measurement of volume and free water content of the cryogel sample, ϕ value was estimated. A piece of cryogel sample was saturated with deionized water, then it was immersed in water having volume \( V_1 \) after that, the total volume of cylinder was measured as volume \( V_2 \). Water-saturated cryogel volume \( V_0 \) was calculated by the volume difference, i.e., 
\[
V_0 = V_2 - V_1.
\]

The mass of water-saturated cryogel, \( m_w \), was weighted. After squeezing the cryogel sample to remove the free water within the large pores, the mass of the cryogel sample without free water, \( m_s \), was weighted as described previously [32]. The porosity was calculated by the following formula:
\[
\phi = \frac{(m_w - m_s)}{\rho_w V_0} \times 100
\]

where \( \rho_w \) is the density of deionized water. Then the cryogel sample was dried in the oven at 60°C for 12-24 h to a constant and the dried cryogel mass \( m_d \) was determined, and the total water fraction.
(TWF) was calculated by the following formula:

\[
TWF = \frac{(m_w - m_d)}{w \times V_0} \times 100
\]  

(2)

**Surface morphology**

The morphology of a cross section of the dried cryogel was coated with gold-palladium (40:60) and investigated by SEM (FEI Quanta 250 FEG, Holland).

**FTIR**

FTIR spectra of the unmodified and APTES-incorporated magnetite NPs were obtained by using a FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). The dry cryogel (about 0.1 g) was thoroughly mixed with KBr (0.1 g, IR Grade, Merck, Germany), and pressed into a tablet, and the spectrum was then recorded.

**Elemental analysis**

Elemental analysis (Leco elemental analyzer, Model CHNS-932, USA) was performed to determine Nitrogen atom in APTES incorporated into the magnetite NPs.

**Chromatographic procedures**

**DNA adsorption from aqueous solutions**

Investigation of DNA adsorption was carried out in a recirculating system equipped with a water jacket for temperature control. After washing of cryogel with water, it was equilibrated with 0.02 M Tris buffer containing 0.02 M NaCl (pH 8.0). Then the prepared DNA solution was pumped through the column by recirculating for 2 h. The adsorption was followed by monitoring of the decrease in UV absorbance at 260 nm. The effects of flow rate, DNA concentration and pH of the medium on adsorption capacity were studied. The flow rate of the solution (i.e., 20 mL of the aqueous DNA solution) was varied in the range of 0.5-2.0 mL/min. To observe the effects of the initial DNA concentration on adsorption, it was varied between 0.1 and 4.0 mg/mL. To determine the effects of pH on adsorption, pH of the medium was varied between 6.0 and 9.0.

**Desorption and repeated use**

In all cases, adsorbed DNA molecules were desorbed using 0.1 M phosphate buffer containing 1 M NaCl (pH 9.0). In a typical desorption experiment, 20 mL of desorption agent was pumped through the cryogel at a flow rate of 1.0 mL/min for 30 min. The final DNA concentration in the desorption medium was spectrophotometrically determined. When desorption was achieved, the cryogel was cleaned with IM NaOH and then re-equilibrated with 0.02 M Tris buffer containing 0.02 M NaCl (pH 8.0). The desorption ratio was calculated from the amount of DNA adsorbed on the cryogel and the final DNA concentration in the desorption medium. To test the repeated use of this composite cryogel, DNA adsorption-desorption cycle was repeated for 30 times using the same cryogel column. To regenerate and sterilize, after desorption, the cryogel was washed with IM NaOH solution.

**RESULTS AND DISCUSSION**

The amine groups of APTES compounds show high affinity site for heavy metal ions (e.g., Cu²⁺) [33]. Magnetic NPs with large surface area have high metal chelating capacity. The amount of chelated Cu²⁺ on silanized magnetite NPs and amine content in APTES were measured as 558 μmol/g magnetite NPs, and 1032 μmol/g magnetite NPs, respectively. These results may be interpreted that, about two APTES molecules may be incorporated one Cu²⁺ ion (Figure 1). Investigation of leakage of Cu²⁺ ions from the Cu²⁺-AMNPs embedded cryogel column show no detected leakage in any of the adsorption and desorption media, suggesting that the washing procedure was satisfactory for the removal of the nonspecifically adsorbed Cu²⁺ ions from the cryogel.

**SEM observation**

The SEM images of the internal structures of Cu²⁺-AMNPs embedded supermacroporous monolithic composite cryogel are shown in Figure 2. As seen in Figure that, Cu²⁺-AMNPs were uniformly distributed into the PHEMA cryogel network. Prepared composite cryogel has large continuous interconnected pores (10-50 μm in diameter) with thin polymer walls that provide channels for the mobile phase to flow through. Pore size of the matrix is much larger than the size of the DNA molecules, allowing them to pass easily. As a result of the convective flow of the solution
through the pores, the mass transfer resistance is practically negligible.

Swelling test
The porosity, $\phi$, and the total water content, TWC, of the cryogel column embedded with Cu$^{2+}$-AMNPs was measured with deionized water as 72.4% and 93.9% (v/v) respectively. These results indicate that, about 21.5% of the total water was bound by the small pores of polymer matrix where almost no flowing liquid passed through. However, the large pores constituting 72.4% of the total pores were filled with free water and most of the fluid flowing channels were taken place within these large pores.

FTIR
The FTIR spectra of magnetite NPs (a), silanized magnetite NPs (b) and Cu$^{2+}$-attached silanized magnetite NPs (c) are shown in Figure 3. Bands at 2921 and 1461 cm$^{-1}$ can be referred to the N-H stretching vibration and NH$_2$ bending of free NH$_2$ group, respectively. A medium intense band from S-CH$_2$ at 1350 cm$^{-1}$ is also confirm the immobilization of APTES to magnetite NPs.

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Figure 2. SEM micrographs of Cu$^{2+}$-AMNPs embedded PHEMA cryogel.

Figure 3. FTIR spectra of magnetite NPs (a), silanized NPs (b) and Cu$^{2+}$-attached silanized NPs (c).
DNA adsorption from aqueous solutions

Effects of pH

Figure 4 shows the effect of pH on the adsorption of DNA on Cu$^{2+}$-AMNPs embedded supermacroporous monolithic composite cryogel. The maximum adsorption of DNA was observed at pH 8.0 (0.02 M Tris buffer containing 0.02 M NaCl) above and below the pH 8.0, the DNA adsorption capacity decreased. The DNA adsorption on IMAC is mainly through chelation between metal ions and the phosphate backbone or the DNA bases [34]. DNA adsorption in IMAC is mainly at a weakly alkaline pH favors the reaction and therefore induces DNA adsorption on the metal-immobilized matrixes.

The optimal pH for DNA adsorption on Cu$^{2+}$-AMNPs embedded supermacroporous monolithic composite cryogel is around 8.0. This result has a correlation with nucleic acid separation by IMAC [11]. It should be also noted that nonspecific adsorption (i.e., adsorption on PHEMA based cryogel) was independent of pH and it was observed at the same at all the pH values studied.

Effects of DNA concentration

The important role of some divalent cations play in binding of DNA to the mica has been studied [34]. Affinity of DNA bases to some divalent cations increases throughout this series: Mg$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, and Cu$^{2+}$ [35,36]. In this study Cu$^{2+}$ ion was chosen as DNA binding agent to the adsorbent contains APTES molecule as ligand. Figure 5 shows the DNA adsorption isotherm of the plain (as a reference surface) and Cu$^{2+}$-AMNPs embedded supermacroporous monolithic composite cryogel. DNA adsorption on plain PHEMA cryogel was low (about 1.5 mg/g), although adsorption of DNA molecules on Cu$^{2+}$-AMNPs embedded supermacroporous monolithic composite cryogel through Cu$^{2+}$ ions was significant (up to 13.7 mg/g). As expected, the amount of DNA coupled to cryogel almost reached a plateau of around 4.0 mg/mL because of saturation of the active binding sites.

Adsorption Isotherms

During the experiments, adsorption isotherms were used to evaluate adsorption properties. The Langmuir adsorption isotherm used to characterize the binding process of DNA to Cu$^{2+}$-AMNPs embedded supermacroporous monolithic composite cryogel is generally formulated by Eq. (3), which is utilized in a linear form as in Eq. (4).

\[
Q = Q_{\text{max}} b C_e / (1 + b C_e) \tag{3}
\]

\[
1/Q = (1/Q_{\text{max}} b)(1/C_e) + 1/Q_{\text{max}} \tag{4}
\]

where $Q$ is the concentration of DNA bound to the adsorbent (mg/g), $C_e$ the equilibrium DNA concentration in solution (mg/mL), $b$ the Langmuir constant (mL/mg) and $Q_{\text{max}}$ is the maximum adsorption capacity (mg/g). A linear plot of $1/Q$, the reciprocal of the bound DNA concentration versus $1/C_e$, the reciprocal of the unbound DNA concentration gave rise to an intercept of $1/Q_{\text{max}}$ and a slope expressed by $1/(Q_{\text{max}} b)$. The maximum adsorption capacity ($Q_{\text{max}}$) for the Cu$^{2+}$-AMNPs embedded supermacroporous monolithic composite cryogel was determined from the intercept $1/Q_{\text{max}}$ to be 54.35 mg/g, and the respective Langmuir constant
was extracted from the slope \((1/(Q_{\text{max.}}b))\) to be 0.093 mL/mg. The high correlation coefficient \((R^2)\) was 0.9976 for \(\text{Cu}^{2+}\)-AMNPs embedded supermacroporous monolithic composite cryogel, indicating that the Langmuir adsorption model can be applied in this affinity adsorbent system.

It has been reported that the effect of isotherm shape with a view to predicting if an adsorption system is “favorable” or “unfavorable”[37]. The essential features of a Langmuir isotherm can be expressed in terms of a dimensionless constant separation factor or equilibrium parameter, \(R_L\); which is defined by Eq 5.

\[
R_L = \frac{1}{1 + b C_0}
\]

(5)

where, \(C_0\) is the initial DNA concentration (mg/mL), and \(b\) is the Langmuir adsorption constant (mL/mg). The parameter indicating the isotherm shape is given in Table 1.

<table>
<thead>
<tr>
<th>Value Type of isotherm</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R_L &gt; 1) Unfavorable</td>
</tr>
<tr>
<td>(R_L = 1) Linear</td>
</tr>
<tr>
<td>(0 &lt; R_L &lt; 1) Favourable</td>
</tr>
<tr>
<td>(R_L &lt; 1)Irreversible</td>
</tr>
</tbody>
</table>

Table 2. \(R_L\) values based on the Langmuir equation.

<table>
<thead>
<tr>
<th>DNA Concentration (mg/mL)</th>
<th>(R_L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.991</td>
</tr>
<tr>
<td>0.25</td>
<td>0.997</td>
</tr>
<tr>
<td>0.5</td>
<td>0.955</td>
</tr>
<tr>
<td>1.0</td>
<td>0.915</td>
</tr>
<tr>
<td>2.0</td>
<td>0.843</td>
</tr>
<tr>
<td>3.0</td>
<td>0.882</td>
</tr>
<tr>
<td>4.0</td>
<td>0.729</td>
</tr>
</tbody>
</table>

Figure 6. Linear representation of Langmuir equation of DNA with \(\text{Cu}^{2+}\)-AMNPs embedded supermacroporous monolithic composite cryogel column.

increases. The adsorption capacity decreased significantly from 3.7 to 2.8 mg/g polymer with the increase of the flow-rate from 0.5 to 2.0 mL/min. An increase in the flow rate reduces the solution volume treated efficiently until breakthrough point and therefore decreases the service time of cryogel column. This is due to decrease in contact time between the DNA molecules and \(\text{Cu}^{2+}\)-AMNPs embedded supermacroporous monolithic composite cryogel at higher flow rates. These results are also in agreement with those referred to the literature [38]. When the flow-rate decreases the contact time in the column is longer. Thus, DNA molecules have more time to diffuse to the pore walls of cryogel and to bind to the ligand, hence a better adsorption capacity is achieved.

**Effect of flow-rate**

The adsorption amounts at different flow-rates are given in Figure 6. Results show that the DNA adsorption capacity on \(\text{Cu}^{2+}\)-AMNPs embedded supermacroporous monolithic composite cryogel decreases when the flow-rate through the column increases. The adsorption capacity decreased significantly from 3.7 to 2.8 mg/g polymer with the increase of the flow-rate from 0.5 to 2.0 mL/min. An increase in the flow rate reduces the solution volume treated efficiently until breakthrough point and therefore decreases the service time of cryogel column. This is due to decrease in contact time between the DNA molecules and \(\text{Cu}^{2+}\)-AMNPs embedded supermacroporous monolithic composite cryogel at higher flow rates. These results are also in agreement with those referred to the literature [38]. When the flow-rate decreases the contact time in the column is longer. Thus, DNA molecules have more time to diffuse to the pore walls of cryogel and to bind to the ligand, hence a better adsorption capacity is achieved.

**Desorption and reusability of adsorbents**

Desorption of DNA from \(\text{Cu}^{2+}\)-AMNPs embedded supermacroporous monolithic composite cryogel was also carried out in column system. The desorption of DNA is expressed in percentage of totally adsorbed DNA. Due to economic restraints, there is a growing interest in the preparation and use of effective low-cost and reusable adsorbents [29]. Up to 96.39% of the adsorbed DNA was desorbed by using 0.1 M phosphate buffer containing 1M NaCl (pH 9.0) as elution agent. The addition of elution agent reduced electrostatic
interactions, resulting in the release of the DNA molecules from the adsorbent. Note that there was no Cu$^{2+}$ release from the cryogel. With the desorption data given earlier, we concluded that 0.1 M phosphate buffer containing 1 M NaCl (pH 9.0) is a suitable desorption agent, and allows repeated use of the affinity cryogel used in this study. To show the reusability of Cu$^{2+}$-AMNPs embedded supermacroporous monolithic composite cryogel, the adsorption-desorption cycle was repeated 30 times using the same cryogel. There was no remarkable decrease in the adsorption capacity of the cryogel. The DNA adsorption capacity decreased only 4% after 30-cycle.

CONCLUSION

A wide variety of functional molecules, (i.e., enzymes, antibodies, proteins, and nucleic acids) may be used as ligands in the design of novel adsorbents [39]. These ligands are extremely specific in most cases. However, they are expensive, due to high cost of production and/or extensive purification steps. The main advantage of IMAC consists in its simplicity, universality, stability, and cheapness of the chelating supports [40]. Experiment results of this new metal-immobilized affinity adsorbent prepared as Cu$^{2+}$-AMNPs embedded supermacroporous monolithic composite cryogel indicated that this novel adsorbent can effectively adsorb DNA from aqueous solution, and the excellent adsorption-desorption (i.e., up to 19.97 mg/g-96.39%, respectively) of the adsorbent promise it to be useful in practical applications. The formation of coordinated compound between DNA and Cu$^{2+}$ should be considered to be the major binding mode. DNA immobilized affinity sorbents is commonly used in the therapy of some autoimmune diseases by removal of anti-DNA antibodies from patient sera [12,20,22]. The correlation of amount of DNA immobilized on affinity sorbents with anti-DNA antibody adsorption capacity was exhibited [16,17]. Therefore, our prepared adsorbents carrying higher amounts of DNA may be considered as a good candidate for achieving higher removal rates for anti-DNA antibodies.

REFERENCES