Abstract: The purpose of the present work was to investigate the adsorption of BSA onto the clinoptilolite in aqueous media. In this study, batch adsorption experiments were carried out and the effects of pH, protein concentration, ionic strength and Si/Al ratio of zeolite on the adsorption process were examined. The optimum pH for adsorption was found to be 4.0. It was found that treatments applied to modify clinoptilolite had substantial effects on the adsorption. During adsorption, residual BSA concentration reached equilibrium in 60 min. Maximum adsorption capacity value for the raw clinoptilolite was determined as 388.3 mg BSA / g zeolite (23.2 mg BSA/m2) and for the treated clinoptilolite, was ranged between 500-660 mg BSA / g zeolite (12.8-31.8 mgBSA / m2).
Dear editor,

I would like to submit the following manuscript entitled "Adsorption of Bovine Serum Albumin (BSA) on Clinoptilolite.". I hope you will find it of interest and accept it for publication in "Biochemical Engineering Journal".

I am looking forward to hearing from you soon.

Yours sincerely

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ADSORPTION OF BOVINE SERUM ALBUMIN (BSA) ON CLINOPTIOLITE

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Abstract

The purpose of the present work was to investigate the adsorption of BSA onto the clinoptilolite in aqueous media. In this study, batch adsorption experiments were carried out and the effects of pH, protein concentration, ionic strength and Si/Al ratio of zeolite on the adsorption process were examined. The optimum pH for adsorption was found to be 4.0. It was found that treatments applied to modify clinoptilolite had substantial effects on the adsorption. During adsorption, residual BSA concentration reached equilibrium in 60 min. Maximum adsorption capacity value for the raw clinoptilolite was determined as 388.3 mg BSA / g zeolite (23.2 mg BSA/m²) and for the treated clinoptilolite, was ranged between 500-660 mg BSA/g zeolite (12.8-31.8 mgBSA/ m²).

Keywords: Zeolite; Bovine serum albumin; Protein; Adsorption; Ionic strength; Immobilisation

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INTRODUCTION

Latest applications in biotechnology such as artificial implants, protein-purification strategies, biosensors, and drug delivery systems includes the interactions between the proteins and non-biological surfaces. One of these applications, the investigation of interactions between the protein and inorganic support is an important concern widely handled[1,2]. Various chromatographic methods such as ion exchange, affinity, hydrophobic interaction, and so on have been used for the separation of various kinds of proteins, but the resins used in these methods have many problems. Some of these resins are very weak chemically and physically under the strong acid and alkali; high and low temperature and high pressure conditions[3,4]. Thus, generation of chemically and physically strong adsorbents is the main topic of the new studies. Zeolites are known to be chemically and physically strong and also have high surface area, thus, they can be considered as attractive candidates for applications in separation technologies[5]. Zeolites are crystalline porous solids consisting of corner-sharing AlO$_4$ and SiO$_4$ tetrahedra with pores and channel system. These physicochemical characteristics are the basis for their functions in the catalysis reactions, separation, and ion-exchange[6,7]. Macromolecules like proteins can be adsorbed on the surface of the zeolite particles. These properties of the zeolites make them potential carriers of various bio-molecules [8]. Zeolites have well defined pore and cage system which can be changed by thermal and chemical treatments. Also, they have the advantage that the basic/acidic nature of the material can be modified by varying the Si/Al ratio. Si/Al ratio of zeolites can be tuned during synthesis or can be varied after synthesis (e.g dealumination). The dealumination
process reduces the electrostatic charge in the structure of the zeolite and their adsorption properties become hydrophobic. Hydrophobic low molecular weight molecules show a high adsorption toward dealuminated zeolites. Previous studies indicated that proteins tended to bind well at or around their isoelectric point (pI) to zeolites with a higher Si/Al ratio, suggesting that the hydrophobicity has a strong influence on adsorption. In one of these previous studies, it was found that zeolites adsorbed the proteins and there were three physicochemical principles underlying the adsorption: (i) below the pI of each protein, it was mainly Coulomb’s attraction, similar to ion-exchange chromatography, (ii) at the pI, it involved hydrophobic interactions (a kind of van der Waals attraction) and (iii) above the pI, it was the sum of Coulomb’s repulsion and attraction [9,10].

Clinoptilolite is one of the zeolite species which is found in abundance in many deposits around the world. It belongs to the heulandite group, with a three-dimensional framework of silicon and aluminum tetrahedra, having the typical chemical formula Na₆[(AlO₂)₆(SiO₂)₃₀]·24H₂O [6].

Albumin is the most abundant protein in the circulatory system and it is responsible for the blood pressure and pH. BSA is a large protein containing 14% basic groups and 18% acidic groups, with a pI of 4.8. It is therefore negatively charged at pH 7.2 and positively charged at pH 4.7[11,12].

The aim of the present work is to explore the possibility of utilizing the raw and treated clinoptilolite for the adsorption of BSA in aqueous media. The effect of such factors
such as the pH value, protein concentration, adsorption time and Si/Al ratio value of the clinoptilolite was investigated.
EXPERIMENTAL

Zeolite sample

The zeolite sample used in this study was obtained from Bigadic region of Turkey. It was grinded and sieved. To determine the porosity and surface area characteristics of the zeolite samples nitrogen adsorption and desorption measurements were performed at -196 °C on Quantachrome NOVA 2200 surface area and pore size analyzer. Electron micrographs were obtained by using a Gemini scanning electron microscope equipped with Leo 32 Supra 35VP field emission scanning system and electron dispersive spectrometer was used for images and analysis. The infrared (IR) measurements were conducted with a Perkin Emler Spectrum One FT-IR Spectrometer. The spectra were recorded from 400 cm⁻¹ -4000 cm⁻¹. The sample was scanned 20 times at 2 cm⁻¹ resolution.

Adsorption studies

BSA was obtained from Sigma Chemical Co. (USA, catalog number A-7906, molecular weight = 69 KDa, isoelectric point(pI) = 4.7). BSA solutions were prepared with deionised water with a conductivity value of 18.2 MΩ supplied from Barnstead Nano pure Diamond. The effect of pH on the BSA adsorption was investigated using BSA solutions ranging between 50 and 450 ppm over the pH range 3.0-7.0. The pH values of
solutions were adjusted by appropriate use of buffer solutions (phosphate buffer
(Na$_2$HPO$_4$/NaH$_2$PO$_4$) and acetate buffer (CH$_3$COOH/ CH$_3$COONa).

BSA adsorption was measured by the classical batch equilibration method. Adsorption
isotherms were constructed by measuring the differences in the protein concentrations
free in solution that resulted from the addition of a specific amount of zeolite and are
averages of at least three experiments. In each adsorption study, 30 mg zeolite (dry
weight) was added to 50 ml of the BSA solution at 25°C and magnetically stirred
continuously. After 1.5h, the aqueous phase was separated from the zeolite by
centrifugation and the concentration of BSA in the solution was determined by diluting
the solution with suitable proportions and then by adding Bradford solution[13]. The
protein concentration in the supernatant were analyzed with a UV-Vis spectrophotometer
(UNICAM UV-Vis spectrometer) at 595.0 nm.

The effect of the initial BSA concentration on the adsorption capacity of the zeolite at
the optimum pH was determined using solutions with concentrations ranging from 50 to
450 ppm. Again, 30 mg zeolite (dry weight) was added to 50 ml of the BSA solution at
25°C and magnetically stirred continuously. After 1.5h, the aqueous phase was separated
from the zeolite by centrifugation and the concentration of BSA in that phase was
determined by using UV-vis spectrometer.
The amount of adsorbed BSA (mg BSA/g zeolite) was calculated from the decrease in the concentration of BSA in the medium by considering the adsorption volume and amount of the zeolite in the adsorption study:

$$q_e = \left[ (C_i - C_e) \cdot V \right] / m$$  \hspace{1cm} (1)

Here, $q_e$ is the amount of BSA adsorbed to the unit mass of the zeolite (mg BSA/g zeolite) at equilibrium; $C_i$ and $C_e$ are the concentrations of the protein in the initial solution and in the aqueous phase after treatment for certain adsorption time, respectively (ppm BSA); $m$ is the amount of zeolite used (g (gram)) and $V$ is the volume of BSA solution (l (liter)).

To determine the adsorption rate of BSA from aqueous solution, same batch adsorption and analysis procedure given above was used and optimum adsorption time was determined.

To investigate the effects of the acid and base treatments on adsorption capacity, zeolite samples were treated with HNO$_3$, H$_2$SO$_4$, HCl, H$_3$PO$_4$ and NaOH solutions with concentrations ranging from 0.01-10 mol/l. Experiments were carried out in polypropylene bottles by adding zeolite sample to acid or base solution of known concentration. The slurry was then magnetically stirred at room temperature for 24h. Then, all samples were washed with water and dried at 120 °C. In the adsorption studies of the treated zeolite samples; 30 mg treated zeolite samples (dry weight) was added to
50 ml of the BSA solution at 25°C and magnetically stirred continuously. After 1.5h, the aqueous phase was separated from the zeolite by centrifugation and the concentration of BSA in that phase was determined by using Bradford method [13].

In the desorption studies, BSA loaded zeolite samples were treated with desorption agents; PEG (0.01 M), NaCl (1.0 M and 2.0M), NaSCN (1.0 M and 2.5M) aqueous solutions and NaCl (0.1 M) in ethanol/H₂O mixture (50/50, by volume). 30 mg (dry weight) portion of zeolite samples carrying 388 mg BSA /g zeolite were placed in desorption medium (50 ml) and stirred magnetically for 24h at 25°C. After 24h, the aqueous phase was separated from the zeolite and the concentration of BSA in that phase was determined by using Bradford method [13].
RESULTS AND DISCUSSION

Characterization of the zeolite samples

The physicochemical properties of the zeolite samples are listed in Table 1. Pore size value of all the materials studied is approximately 39.0 angström for acid treated zeolite samples. Base treated zeolite samples have higher pore size values than the acid treated ones.
Table 1. Properties of the zeolite samples with different Si/Al ratio.

<table>
<thead>
<tr>
<th>Modification agent</th>
<th>Concentration (mol/L)</th>
<th>Si/Al ratio</th>
<th>S(_{\text{BET}}) (m(^2)/g)</th>
<th>Pore size (angström)</th>
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<tr>
<td>HNO(_3)</td>
<td></td>
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<tr>
<td>10.0</td>
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<td>39.7</td>
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<tr>
<td>H(_2)SO(_4)</td>
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<td>5.80</td>
<td>49.0</td>
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</tbody>
</table>

Note: For the original clinoptilolite sample; Si/Al ratio = 4.46, \(S_{\text{BET}}\) (m\(^2\)/g) = 16.8 and Pore size (angström) = 39.7.
As it is evident in Table 1, all the adsorbents prepared by treating the clinoptilolite with mineral acids have a mean pore diameter value around 39 angström which is smaller than all the principal dimensions of BSA 40x40x140 (in angström) [14]. But, base treated zeolite samples especially those treated with 1.0, 0.1 and 0.01 M NaOH have higher pore size values.

**Figure 1a.** FT-IR spectra of the zeolite samples; raw clinoptilolite (—), BSA adsorbed clinoptilolite (…..) and pure BSA (.-.-.-.).

**Figure 1b.** FT-IR spectra of the zeolite samples, physical mixture of the raw clinoptilolite and BSA (—) and BSA adsorbed clinoptilolite (…..)

FTIR spectroscopic investigation of the zeolite samples showed that raw clinoptilolite sample contains various kind of silanol groups; interparticle hydrogen-bonded OH groups at about 3434 cm\(^{-1}\) and surface free OH groups at 3620 cm\(^{-1}\) as is shown in Fig.1a. Due to the majority of the surface hydroxyl groups of the raw zeolite were substituted to the adsorbed BSA, intensity of the band belongs to these hydroxyl groups has decreased. This decrease of OH band may suggest that adsorbed BSA molecules might assume a widely expanded form on zeolite surface. In addition, the interaction of BSA with zeolite is evidenced by analyzing the structure of Amide bands. The most dominant band component in Amide I region is the band at 1654 cm\(^{-1}\), which is usually attributed to \(\alpha\)-helical structure of BSA. The deformation motion of the NH groups have dominant contribution in the next band near 1544 cm\(^{-1}\) assigned as Amide II. In addition,
absorption peaks ascribed to the C-H stretching vibrations of the methyl and the methylene groups of BSA molecule, appeared between 2868 and 3065 cm\(^{-1}\). In summary, observed decrease at the intensity of OH band together with the appearance of signals in the aliphatic region after BSA adsorption argue for the covalent attachment of the BSA molecule to the zeolite edges (Fig.1.a). Also, IR spectra changes observed between 1400-1600 cm\(^{-1}\) confirm the covalent attachment (Fig.1.b).

**Influence of variables on BSA adsorption**

1) **Effect of pH**

The pH dependence of BSA adsorption onto zeolite is shown in Fig. 2. Experiments were carried out using BSA solutions at different pH values which adjusted by using appropriate buffer solutions. The stability of the zeolite samples were checked at the pH used and the structures were found to be stable. As it is seen in Fig. 2, the highest value of \(q_e\) is obtained at pH 4.0 value near the isoelectric point of BSA. This result is in agreement with the results obtained in previous studies which reported the protein adsorption isotherms with different adsorbents for different pH values [1,15].

**Figure 2.** Variation of the adsorbed amount of BSA as a function of pH (BSA concentration = 50 to 450 ppm, temp. = 25°C); a) pH=3.0, b) pH=4.0, c) pH=4.5, d) pH=6.0 and e) pH=7.0
As it is known, pH has a significant effect on protein structure and due to this, pH is also effective at protein ↔ solid surface interaction [1,16,17]. A possible explanation for this behaviour has to be investigated by considering the electrostatic interaction between the protein and the surface of the adsorbent. The isoelectric point of BSA is 4.7, so when the pH = 4.0 protein is positively charged. In this case there is an electrostatic attraction between BSA and surface of the clinoptilolite. Usually, the maximum adsorption onto the zeolites tended to occur when the pH was at or just below the pI of the proteins. With increasing pH (higher than pI) the electrostatic repulsion between the protein and surface increases as both are negatively charged. Additionally, repulsion between adsorbed BSA molecules also increases at higher surface coverage. These two effects combine to reduce the adsorption capacity when the pH is increased above pI [18,19]. Following factors are effective during the adsorption of proteins onto the zeolite; 1) below pI, mainly the Coulombic attraction similar to ion-exchange chromatography; 2) at pI, probably hydrophobic interactions and the mesopore structure; and 3) above pI, hydrophobic interactions and substitution of water at the Lewis acid sites of Al. [9].

2) Effect of BSA concentration

Experiments showed that the amount of BSA adsorbed per unit mass of zeolite (i.e. the adsorption capacity) increases with the initial concentration of BSA (Fig.2). This increase continues up to the initial BSA concentration of 400 ppm and beyond this, there is not a significant at the adsorption capacity value. The plateau reached after 400 ppm represents the saturation of the active sites available on the zeolite to interact with
BSA and the maximum adsorption capacity. It can be concluded that the percent adsorption of BSA decreases with its increasing concentration in aqueous solutions. The maximum adsorption capacity was calculated as 388 mg BSA / g zeolite which corresponds to 23.2 mg BSA/m² when the surface area of the zeolite sample was considered. Different adsorbents have been reported for the adsorption of BSA. Silica was used and the adsorption capacity values of 2.2 mg/ m² and 3.5 mg/ m² were found for pH 4.7 and pH 5.0, respectively [1,15]. In another study, achieved adsorption capacities ranged between 3-32 mg BSA/ m² for ZnSe and 4-55 mg BSA/ m² for polyurethane-coated ZnSe [20]. In a recent work, MCM-41 was used and the adsorption capacity range was found to be 124-255 mg / g MCM-41 [8]. Thus, it can be concluded that the maximum adsorption capacity values obtained in this study for the raw and treated zeolite samples are comparable to those obtained with other adsorbents.

3) Effect of adsorption time

Fig.3 illustrates the adsorption of BSA onto the zeolite as a function of time. The slopes of the lines joining the data points in the figure reflect the adsorption rates. As it is seen, high adsorption rates were observed at the begining and then plateau values were reached within 90 min. In the previous studies in which the adsorbents Al-MCM-41, SiO₂ and siliceous MCM-41 were used, optimum adsorption times were given as 24h, 3h and 144h respectively [8,18,21]. Thus, the adsorption rate obtained with clinoptilolite seemed to be very satisfactory.
Figure 3. Variation of the adsorbed amount of BSA as a function of adsorption time (BSA concentration = 400 ppm, pH = 4.0).

4) Desorption study

Although the proteins can be adsorbed on zeolites, it is not easy to desorb them by conventional eluents. NaCl is a widely used desorption agent in the desorption studies of the proteins. Na\(^+\) and Cl\(^-\) ions could destabilize the hydrophobic interaction between the protein and the adsorbent [22]. Also, polyethylene glycol (PEG) could be used to desorb the proteins adsorbed onto the zeolites at their pIs without loss of activity. PEG stabilizes protein structure, reduces protein aggregation, and enhances protein refolding[23,24].

In the desorption study PEG (0.01M), NaCl (≥ 1.0 M) and NaCl (0.1 M in ethanol/water mixture; 50/50, by volume) were used as eluent. The zeolite samples loaded with the maximum amount of BSA were placed into the desorption medium and after 24h mixing, the amount of desorbed BSA was measured. It was observed that there is not any significant BSA release (only 0.25-2.50 mg BSA) from the zeolite to the desorption medium. This result is in agreement with those obtained in the previous studies in that silica based adsorbents have been used and in these studies it was reported that the adsorption of protein is said to be practically irreversible[21].
5) Effect of acidic and basic treatments

In this part of the study, dealumination technique was applied to the clinoptilolite, to produce zeolites with higher Si/Al ratios. In order to understand the effect of Si/Al ratio on the adsorption of BSA, raw clinoptilolite treated with HNO₃, H₂SO₄, HCl, H₃PO₄ and NaOH solutions at concentrations ranging between 0.01 to 10 mol/l. Then, obtained zeolite samples with different Si/Al ratios were used in BSA adsorption (Table1). Data for the amount of BSA adsorbed onto the treated clinoptilolites are presented in figures 4 and 5. Fig. 4 shows the adsorption isotherms for BSA adsorbed onto the zeolite samples at pH 4.0. H₃PO₄ - treated zeolite sample has a significantly higher adsorption capacity than the other adsorbents. The adsorption capacity for BSA decreased with increasing concentration of acid used in the treatment. During the acid treatment of the silica based materials -Si-O-Si + HOX → -Si-OH + -SiOX reaction occurs (HOX is the acid). In this reaction, siloxane bonds interacting with acid are broken to yield the silanol group. This interaction increases the relative amount of the Si-OH groups used in the adsorption of BSA. This explains the higher qₑ values obtained during the adsorption studies using the acid treated zeolite samples[25].

It was shown that the adsorption capacity of clinoptilolite increased with decreasing of the Si/Al ratio, probably due to the increased affinity of BSA for the external surface of the adsorbents for the acid treated zeolite samples. The external area plays a predominant role in BSA adsorption on zeolites. Since the zeolite structures have microporous pores which are too small with respect to the kinetic diameter of the BSA, the adsorption occurs
on the external surface [8]. In the case of acidic treatment there is not a significant change at BET surface area and pore volume values. But, due to the removal of aluminum from the zeolite structure during the acid treatment, Si-OH groups become less acidic (due to the absence of interaction between aluminum, lewis acid center, (from zeolite) and oxygen (from Si-OH), thus the interaction between the functional groups of BSA and hydrogen of the silanol group on the zeolite surface becomes weaker. In general, proteins with diameter larger than the pore diameter adsorb on the outer surface of the zeolite through hydrogen bonding interaction between the OH groups of zeolite and the carboxylic or amino groups in the protein structure [25]. As it is seen in Table 1, alkali-treated zeolite samples have the higher surface area and pore diameter values than the other zeolite samples listed. These data explains the higher BSA adsorption with these solids. After alkali treatment, pores become big enough to allow the movement of BSA molecule into the interior regions of the zeolite and to ensure the interaction with Si-OH groups placed in these regions (Fig.4 and 5). And also, it is known that alkali treatment increases the amount of Si-OH via the breakage of the Si-O-Si linkage [26].

**Figure 4.** Variation of the adsorbed amount of BSA as a function of acid concentration (BSA concentration = 400 ppm, pH = 4.0)

Fig.5 shows that the adsorption capacity of zeolite decreases with increasing alkali concentration used at the treatment. This is mainly due to the decreasing amount of hydroxyl groups on the surface of zeolites as a result of the Si removal from the surface
which means the decrease of the silanol groups with increasing base concentration[26]. Due to their high pore size and surface area values, base treated zeolite samples have higher $q_e$ values than both original and acid treated zeolite samples.

**Figure 5.** Effect of NaOH treatment on BSA adsorption (BSA concentration = 400 ppm, pH = 4.0).

6) **Effect of ionic strength**

By adding different amount of NaCl into the adsorption medium, effect of the ionic strength on the adsorption was quantitatively investigated. Figure 6 shows that increasing salt concentration in adsorption medium has a detrimental effect on BSA adsorption but $q_e$ values are greater then than those obtained in the absence of NaCl. Previous studies showed that interactions between proteins and surfaces are affected by the ionic strength. In the presence of salt at low concentration, charge-mediated repulsion is quenched by ions and excess adsorption ocurred [27-29]. In addition to this, $\text{Na}^+$ and $\text{Cl}^-$ ions could destabilize the hydrophobic interaction between the protein and the adsorbent when they present at high concentration[22]. With increasing salt concentration this destabilization effect becomes stronger and the adsorption capacity decreases (Fig.6).

**Figure 6.** Variation of the adsorbed amount of BSA as a function of NaCl concentration (BSA concentration = 400 ppm, pH = 4.0).
CONCLUSION

In the present study, adsorption of BSA onto the raw and treated clinoptilolite was investigated. The method used in this study allows different angles of the protein/zeolite surface/solution system to be probed. It has been observed that zeolite adsorbents show a very high adsorption capacity for BSA. Adsorption capacity values obtained with raw and treated clinoptilolite samples are comparable to those obtained with other adsorbents reported in the literature and, the treated clinoptilolite samples adsorbed greater amount of BSA than the raw clinoptilolite. The results indicated that the surface area, chemical composition and structure of the framework influence the BSA adsorption.

ACKNOWLEDGMENTS

The authors extend their gratitude to Arife Karakas from Gazi University for her support in the present work.
REFERENCES


FIGURES

**Figure 1a.** FT-IR spectra of the zeolite samples; raw clinoptilolite (−), BSA adsorbed clinoptilolite (…..) and pure BSA (--.--.--.).
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Figure 3. Variation of the adsorbed amount of BSA as a function of adsorption time (BSA concentration = 400 ppm, pH = 4.0).
Figure 4. Variation of the adsorbed amount of BSA as a function of acid concentration (BSA concentration = 400 ppm, pH = 4.0).

Figure 5. Effect of NaOH treatment on BSA adsorption (BSA concentration = 400 ppm, pH = 4.0).
Figure 6. Variation of the adsorbed amount of BSA as a function of NaCl concentration (BSA concentration = 400 ppm, pH = 4.0).
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Note: For the raw clinoptilolite sample; Si/Al ratio = 4.46, \( S_{BET} (m^2/g) = 16.8 \) and Pore size (angström) = 39.7.
FIGURE CAPTIONS

Figure 1a. FT-IR spectra of the zeolite samples; raw clinoptilolite (—), BSA adsorbed clinoptilolite (…..) and pure BSA (.-.-.-.-.).

Figure 1b. FT-IR spectra of the zeolite samples, physical mixture of the raw clinoptilolite and BSA (─) and BSA adsorbed clinoptilolite (…..)

Figure 2. Variation of the adsorbed amount of BSA as a function of pH (BSA concentration = 50 to 450 ppm, temp. = 25°C); a) pH=3.0, b) pH=4.0, c) pH=4.5,d) pH=6.0 and e) pH=7.0.

Figure 3. Variation of the adsorbed amount of BSA as a function of adsorption time (BSA concentration = 400 ppm, pH = 4.0).

Figure 4. Variation of the adsorbed amount of BSA as a function of acid concentration (BSA concentration = 400 ppm, pH = 4.0)

Figure 5. Effect of NaOH treatment on BSA adsorption (BSA concentration = 400 ppm, pH = 4.0).

Figure 6. Variation of the adsorbed amount of BSA as a function of NaCl concentration (BSA concentration = 400 ppm, pH = 4.0).
TABLE CAPTIONS

Table 1. Properties of the zeolite samples with different Si/Al ratio.