

Preparation of Bentonite-Cysteine (Bent-Cys) micro-composite affinity sorbents for Ferritin adsorption

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Abstract

This study focused on developing bentonite-cysteine micro-composite affinity sorbents (Bent-Cys) (38-105 μm) for Ferritin adsorption from aqueous solutions. The pseudo-specific affinity ligand L-cysteine was immobilized by covalent binding onto the bentonite structure. X-ray diffraction and FTIR analysis of Bent-Cys composite affinity sorbents were performed. The specific surface area of the bentonite and Bent-Cys micro-composite structures were found to be 33.0 and 22.8 m^2/g , respectively. Elemental analysis of immobilized L-cysteine for nitrogen was estimated to be 541.3 $\mu\text{mol/g}$ bentonite. The non-specific ferritin adsorption onto bentonite structure was negligible (ca. 0.9 mg/g). Higher adsorption values up to 40.0 mg/g were obtained in which Bent-Cys micro-composite affinity sorbents were used from aqueous solutions. Adsorption studies were performed to evaluate the effect of initial concentration of ferritin, pH, temperature. The desorption characteristics of Bent-Cys micro-composite affinity sorbents were also studied. The maximum amount of ferritin adsorption from aqueous solution was observed at pH 4.0. Ferritin molecules could be repeatedly adsorbed and desorbed with the micro-composite affinity sorbent system without noticeable loss in the ferritin adsorption capacity.

Key Words: Bentonite, Ferritin adsorption, Composite systems, Affinity chromatography, Cysteine.

Introduction

The separation and purity of products resulting from biotechnology processes are assuming greater commercial importance. For this reason, the topic of bioactive molecules immobilized on a great variety of supports with various polymeric, organic and inorganic chemical compositions and shapes is of increasing current interest [1-4]. Clay and clay minerals are frequently used in many branches of industry such as enzyme immobilization [5], clarification of edible and mineral oils, paints, cosmetics, and pharmaceuticals [6], removal of pathogenic viruses, pesticides, herbicides, and other toxins [7-9], because of their high surface area, specific active sites and economical adsorptive properties. In aluminosilicate structures clay minerals, isomorphous substitution of Al^{3+} cation by Si^{4+} cation in

the tetrahedral layer and those of Mg^{2+} , Fe^{2+} etc cations by Al^{3+} cation in the octahedral layer result in a negative charge which is balanced by the cations such as Na^+ , K^+ , Ca^{2+} etc. These isomorphously substituted and hydrated cations strongly give rise to hydrophilic nature on the clay surface. It is known that natural clays are not very effective by themselves in different applications and in using as catalyst for the specific reaction. Therefore, the modification may be required for making clay and clays minerals of specific adsorption and catalytic properties. One of these modification processes is to interact the clays or clay minerals with various organic cations and molecules under certain conditions. Considering of the mechanisms of clay-organic interactions, the organic compound can bind the surface and/or penetrate into the interlayer space of clay minerals as a ligand [10, 11].

Organoclays prepared by treatment with large alkyl cations and small alkyl or aromatic organic cations can be regarded as organophilic features [12].

Proteins are assumed to interact mainly through the imidazole group of histidine and, to a lesser extent, the indolyl group of tryptophan and the thiol group of cysteine. Co-operation between neighboring amino-acid side chains and local conformations play important roles in protein binding. Aromatic amino acids and the amino-terminal of the peptides may also play a role [13].

Ferritin is the principal protein involved in storing iron in a soluble, non-toxic, yet bioavailable form in bacteria, plants and animals where it maintains up to 4500 atoms of hydrolysed and polymerized Fe^{3+} atoms in a soluble form within a protein shell [14]. Mammalian ferritins consist of a protein shell with 24 subunits surrounding a core of ferrihydrate [15-17]. In normal human subjects 25% of the total body iron is found in the storage protein ferritin.

The L-cysteine based ligands are much more stable than protein ligands because they do not require a specific tertiary structure for maintaining biological activity [18,19]. In addition, protein ligands are expensive and difficult to handle, sterilize and preserve its biological activity [20]. They can lose their activity by harsh elution and cleaning conditions. The L-Cysteine also offers several advantages over protein ligands in terms of economy, ease of immobilization and high adsorption capacity. It interacts with several proteins through its carboxyl, amino and -SH groups at near their isoelectric points and has shown particular efficacy in separating biomolecules [21].

The aim of this work was to investigate, experimentally, the potential of Bentonite-Cysteine (Bent-Cys) micro-composite affinity sorbent to adsorb ferritin using L-cysteine as a pseudo-specific affinity ligand. Bent-Cys micro-composite affinity sorbent surface examined by X-

Ray diffraction and FTIR analysis, elemental analysis and surface area measurement techniques. Laboratory batch kinetics and isotherm studies were conducted to evaluate the adsorption capacity of ferritin onto Bent-Cys micro-composite affinity sorbents. The effects of initial ferritin concentration, pH and reusability on adsorption capacity of ferritin were studied.

EXPERIMENTAL

Materials

The Wyoming bentonite (Cation Exchange Capacity:92 meq/100 g clay) was supplied by Sigma Chemical Co. (St. Louis, USA). Ferritin (from horse spleen, 440 kDa) was also supplied by Sigma (St Louis, MO, USA). L-cysteine methyl ester hydrochloride ($\text{C}_4\text{H}_9\text{NO}_2\text{S} \cdot \text{HCl}$, 98%) was supplied by Acros Organics (Fairlawn, NJ, USA). 2-Hydroxyethyl methacrylate (HEMA) and ethylene glycol dimethacrylate (EGDMA) were obtained from Fluka (Buchs, Switzerland), distilled under reduced pressure in the presence of hydroquinone inhibitor and stored at 4°C until use. Benzoyl peroxide (BPO) was also obtained from Fluka. Poly(vinyl alcohol) (PVAL; 100 kDa, 98% hydrolyzed) was supplied by Aldrich. All glassware was extensively washed with dilute nitric acid before use. All other chemicals were of analytical grade purity and were purchased from Merck (Darmstadt, Germany). All water used in the experiments was purified using a Barnstead (Dubuque, IA, USA) ROpure LP® reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANO pure® organic/colloid removal and ion-exchange packed-bed system. Buffer and sample solutions were prefiltered through a 0.2- μm membrane (Sartorius, Göttingen, Germany).

Preparation of Bentonite-Cysteine (Bent-Cys) Micro-composite Affinity Sorbents

CEC of the Wyoming bentonite sample [$\text{Al}_{1.50}$, $\text{Fe}_{0.17}$, $\text{Mg}_{0.33}$, Si_4 , $\text{O}_{10} \cdot (\text{OH})_2$] was estimated as 92 meq/100 g clay by using methylene blue method [22]. For the synthesis

of the Bent-Cys micro-composite affinity sorbents, bentonites interacted at 60°C with a 100 mL solution containing L-cysteine which was the same amount as the CEC value of the bentonites for 48 h. A washing procedure was applied after binding procedure to remove the any possible unreacted L-cysteine from the Bent-Cys micro-composite affinity sorbents. Bent-Cys micro-composite affinity sorbents were filtered and resuspended in deionized water. The suspension was stirred for about 1 h at room temperature and the sorbents were separated by filtration. The micro-composite affinity sorbents washed several times with deionized water using the same procedure. Then, Bent-Cys composite affinity sorbents were dried with vacuum oven at 60°C. Finally, Bent-Cys micro-composite affinity sorbents were screened by using Retsch Standard sieves (Model AS 200, Retsch GmbH, KG, Haan, Germany). In this study, Bent-Cys micro-composite affinity sorbents having the size range of 38-105 µm were used as a solid matrix for ferritin adsorption.

The leakage of the L-cysteine from the Bent-Cys micro-composite affinity sorbents was followed by the incubation of the fully wetted adsorbents with 10 mL of phosphate buffered (pH: 7.4) solution for 24 h at room temperature (25°C). The leakage experiments were carried out a stirring rate of 50 rpm. L-cysteine released after this incubation was measured in the liquid phase spectrophotometrically.

When not in use, the resulting sorbents were kept under refrigeration in 0.02% NaN₃ solution for preventing of microbial contamination.

Characterization Studies

FTIR Spectrum

FTIR spectrum of the sample was obtained by using FTIR spectrophotometer (Shimadzu, EX 2000, Japan). The dry sample (about 0.1 g) was mixed with KBr and pressed into a tablet form. The FTIR spectrum was then recorded.

X-Ray Diffraction Analysis

X-Ray Diffraction (XRD) patterns were recorded by Rigaku 2000, USA automated diffractometer using Ni-filtered CuK_α radiation. Then basal spacing of each sample was calculated using Bragg's law:

$$2d \sin \theta = n \lambda \quad (1)$$

Where d is the basal spacing (Å), θ is the angle of diffraction (°), λ is the wavelength (nm), and n is the path differences between the reflected waves which equals an integral number of wavelengths (λ).

Surface Area Measurement

Surface area of the Bent-Cys micro-composite affinity sorbent was measured by nitrogen adsorption at 77 K using Quantachromosorb. Moisture and gases on the solid surface or penetrated in the open pores were removed by heating at 120°C for 2 h prior to the surface area measurement.

Elemental Analysis

The degree of L-cysteine immobilization in the synthesized Bent-Cys micro-composite affinity sorbent was determined by measuring the C, H, N, O contents with a Leco Elemental Analyzer (Model CHNSO-932, USA) Monosorb analyzer.

Effect of Initial Concentration and pH on Ferritin Adsorption

Adsorption of ferritin on the Bent-Cys micro-composite affinity sorbent was studied batchwise. Bent-Cys micro-composite affinity sorbents were incubated with 10 mL of ferritin solution at 25°C for 2 h. (i.e., equilibrium time), in flasks agitated magnetically at 100 rpm.

To observe the effects of the initial concentration of ferritin on adsorption, the initial concentration of ferritin was changed between 0.1-2.0 mg/mL. At the end of the pre-determined equilibrium period, the Bent-Cys micro-composite affinity sorbents were separated from the solution by centrifugation. Ferritin concentration

determined by measuring the absorbance at 280 nm. The amount of adsorbed ferritin was calculated as:

$$Q = \frac{(C_0 - C) \times V}{m} \quad (2)$$

Here, Q is the amount of ferritin adsorbed onto unit mass of Bent-Cys micro-composite affinity sorbents (mg/g); C_0 and C are the concentrations of ferritin in the initial solution and in the aqueous phase after treatment for certain period of time, respectively (mg/ml); V is the volume of the aqueous phase (ml); and m is the mass of the Bent-Cys micro-composite affinity sorbents used (g).

In order to study pH on coupling of ferritin to Bent-Cys micro-composite affinity sorbents, the pH of the solution was varied between 3.0-8.0 by using different buffer systems (0.1 M $\text{CH}_3\text{COONa}-\text{CH}_3\text{COOH}$ for pH: 4.0-6.0, 0.1 M $\text{K}_2\text{HPO}_4-\text{KH}_2\text{PO}_4$ for pH: 7.0-8.0). All the adsorption curves are averages of at least duplicated experiments.

Desorption and Repeated Use Studies

To determine the reusability of the Bent-Cys micro-composite affinity sorbents adsorption and desorption cycle was repeated five times by using the same micro-composite affinity sorbents. Desorption of ferritin was studied 1.0 M NaCl in aqueous solution. The ferritin adsorbed Bent-Cys micro-composite affinity sorbents were placed in the desorption medium and stirred continuously (at a magnetic stirring rate of 100 rpm) for 1 h at room temperature. Ferritin concentration within the desorption medium was determined by the above described method. The desorption ratio of ferritin was calculated by using the following expression:

$$\text{Desorption ratio (\%)} = \frac{[(\text{Amount of Ferritin released}) \times 100]}{[(\text{Amount of Ferritin adsorbed})]} \quad (3)$$

When desorption was achieved, the Bent-Cys micro-composite affinity sorbents were finally cleaned up with 50.0 mM NaOH solution in order to remove the remaining Ferritin and to regenerate them, and then re-equilibrated with the starting buffer.

RESULTS AND DISCUSSION

Properties of Bent-Cys Micro-composite Affinity Sorbents

Bent-Cys micro-composite affinity sorbents were synthesized as a pseudo-biospecific affinity sorbent for the separation of Ferritin from the aqueous solution. The main criteria for selection of bentonite are its lack of toxicity, economical adsorptive properties, high surface area, specific active sites and hydrophilicity allowing easy fixation of biomolecules. L-cysteine molecules were immobilized covalently to the bentonite sorbents. L-Cysteine was coupled to the bentonite via its amino group; the thiol group remain free.

It is accepted that covalent bonds are formed between the amino groups of the L-cysteine and the Al^{3+} cations in the center of octahedral layer of the bentonite structures. L-Cysteine leakage also was investigated in PBS solution. Leakage was not observed from the Bent-Cys composite affinity sorbents.

Fourier Transform Infrared Spectroscopy (FTIR)

Wavenumber and vibration type of bentonite is presented in Table 1. The FTIR spectra of bentonite and Bent-Cys composite affinity sorbents in Figure 1.

In the FTIR spectrum, in relation to the covalent binding between bentonite and cysteine, the peak at 3489 cm^{-1} is probably N-H stretchings caused by NH_2 . The wide O-H band (approximately 3500 cm^{-1}) belonging to the carboxyl in the structure is invisible because it fits into the O-H stretchings of cysteine. The peaks at 1478 cm^{-1} is N-H bending and the band at 1416 cm^{-1} is a C=N stretching band. The CO carbonyl band was expected at 1630 cm^{-1} ; however, this band fitted into the O-H bending of water in bentonite. As a result, the O-H bending of bentonite appears different, which can be seen from the spectra. It can also be seen from the spectrum that the C-O stretching band is hidden by the peak. The peak at 2570 cm^{-1} is S-H bending. The peaks at 1380 and 779 cm^{-1} are carboxyl stretching and C-H bendings. From these

Table 1. FTIR vibrations of bentonite.

Wavenumber (cm ⁻¹)	Vibration Type
3640	O-H stretching (Mg, Al)-OH
3460	H-O-H hydrogen binding water
1650	H-O-H deformation
1115	Si-O stretching
1060	Si-O-Si stretching
936	2 Al ³⁺ binding OH deformation
808	Si-O stretching of quartz and silica
630	Si-O
528	Al-O-Si deformation
477	Si-O-Si deformation

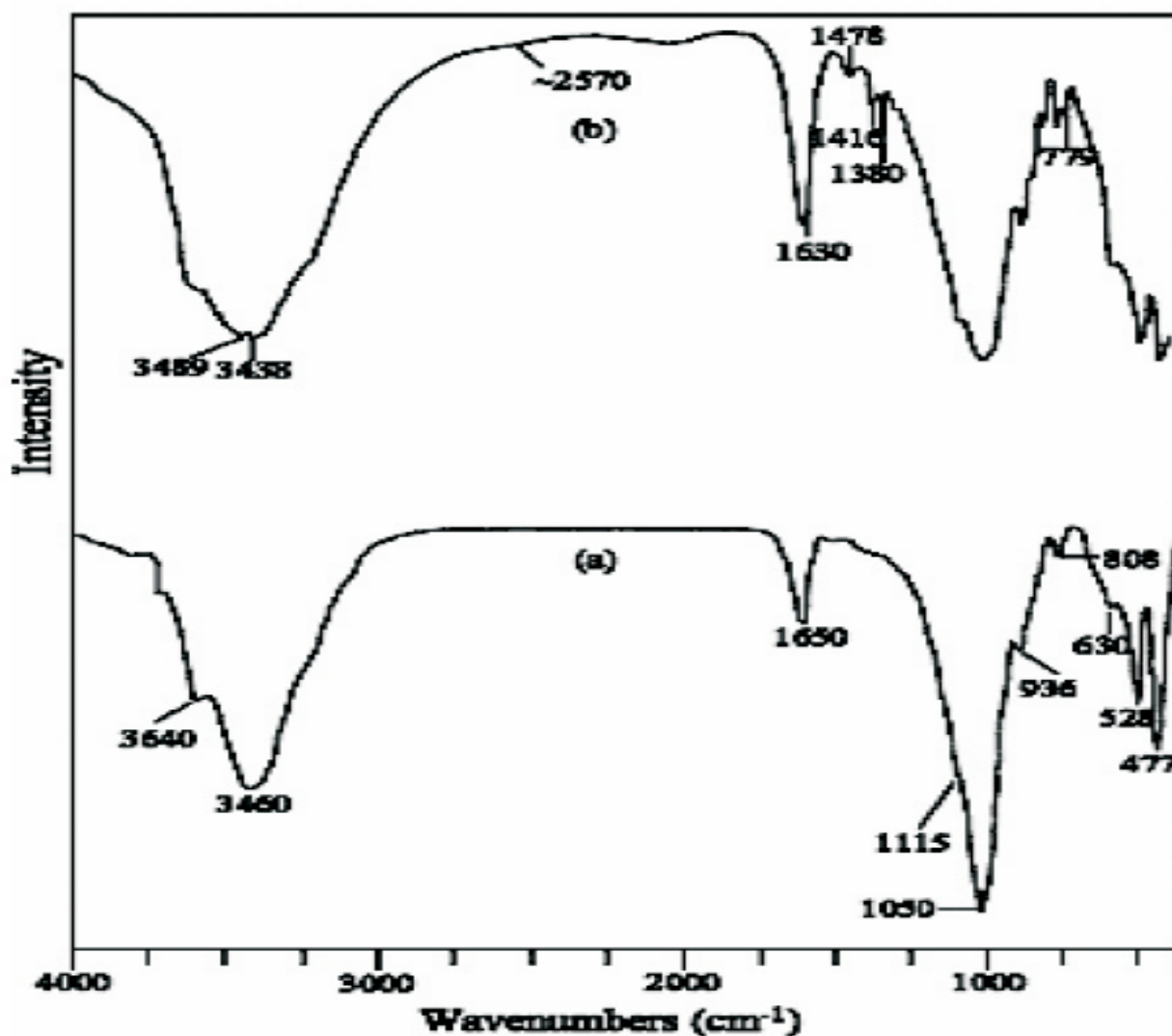


Figure 1. The FTIR spectra: (a) Bentonite (b) Bent-Cys micro-composite affinity sorbent.

results, it seems that bentonite interacts with the NH_2 groups of cysteine. Consequently, it can be concluded that the bentonite structure interacted with the NH_2 group of cysteine. This is also proved by the N-H stretching and bending and the C=N stretching bands in the FTIR spectrum. It is clearly seen that the Al-OH stretching bendings in the FTIR table of bentonite lost sharpness as a result of the interaction with cysteine, in particular, the Al-OH stretching bending at 3640 cm^{-1} . Since the Al^{3+} cation in the center of the octahedral layer of bentonite has the Lewis acid characteristic, it is coordinated by the amine group of cysteine, which is an expected results. All these results may be caused by the interaction between the Al^{3+} cation in the center of the octahedral layer of bentonite and the NH_2 group of cysteine.

X-Ray Diffraction (XRD) Studies

When the X-Ray spectrum of Bent-Cys micro-composite affinity sorbent was examined, firstly a sharp peak whose d_{001} distance (approximately $6.080 (2\theta)$) was 14.0 \AA was seen. The fact that the 12.70 \AA d_{001} distance ($6.960 (2\theta)$) for bentonite went up to 14.0 \AA proves that the cysteine molecule went into between the bentonite layers and was bound to them. The tetrahedral-octahedral-tetrahedral (TOT) silicate layer of bentonite has a width of nearly 9.6 \AA [23]. However, according to previous studies, the width is about 9.0 \AA [24]. It can be concluded that when cysteine molecule was bound between the bentonite layers, it created an inner layer space of 5.0 \AA . Organic cations such as short chain alkyl amines form single or double layers up to their cation exchange capacity (CEC). On the other hand, longer chain cations settle as pseudo-triple molecules or parafine complex [25-26]. In the light of this data, it can be inferred that the cysteine molecule is paralel to the TOT layer and is a monolayer in the inner layer space. In addition, in the x-ray spectrum of Bent-Cys, the peak with a 4.51 \AA d_{020} distance ($34.800 (2\theta)$) and the one with a 2.56 \AA d_{006} distance ($34.960 (2\theta)$) belong to the major

montmorillonite component. Also, a quartz non-clay component with a $26.480 (2\theta)$ d distance was seen in the same spectrum.

Elemental Analysis

Elemental analysis of the bentonite and Bent-Cys composite affinity sorbents were performed, and the attachment of the L-cysteine was found as $541.3\text{ }\mu\text{mol/g}$ bentonite from the nitrogen stoichiometry.

Surface Area Measurement

When the surface area of bentonite and Bent-Cys micro-composite affinity sorbent were examined, the surface area of the bentonite and Bent-Cys micro-composite structures were found as 33.0 ± 0.3 and $24.7\pm 0.3\text{ m}^2/\text{g}$, respectively. The surface area decrease of Bent-Cys micro-composite structures may be explained in terms of the micropore openings blocked by the cysteine molecule embedded in the interlayer space which forms a macroporous structure [27]. The surface area decrease of Bent-Cys micro-composite structures ($25.8\pm 0.3\text{ m}^2/\text{g}$) in comparison with that of the bentonite ($33.0\pm 0.3\text{ m}^2/\text{g}$) can be related to micropore (2 nm) openings covered by the cysteine molecule retained in the interlayer space which result in the form of the structure with mesopores ($2\text{-}20\text{ nm}$).

Ferritin Adsorption from Aqueous Solutions

Effect of pH

The amount of ferritin adsorbed onto the Bent-Cys micro-composite affinity sorbent exhibits two adsorption domains as a function of pH, as shown in Figure 3. (i) The amount of ferritin adsorbed onto the Bent-Cys micro-composite affinity sorbent shows a maximum at pH 4.0, with a decrease at lower and higher pH values (isoelectric point of ferritin: 4.2). Specific interactions (electrostatic and coordination) between ferritin and Bent-Cys micro-composite affinity sorbent at pH 4.0 may result both from the ionization states of several groups on amino-acid side chains in ferritin structure, and from the conformational state of ferritin molecules (more folded

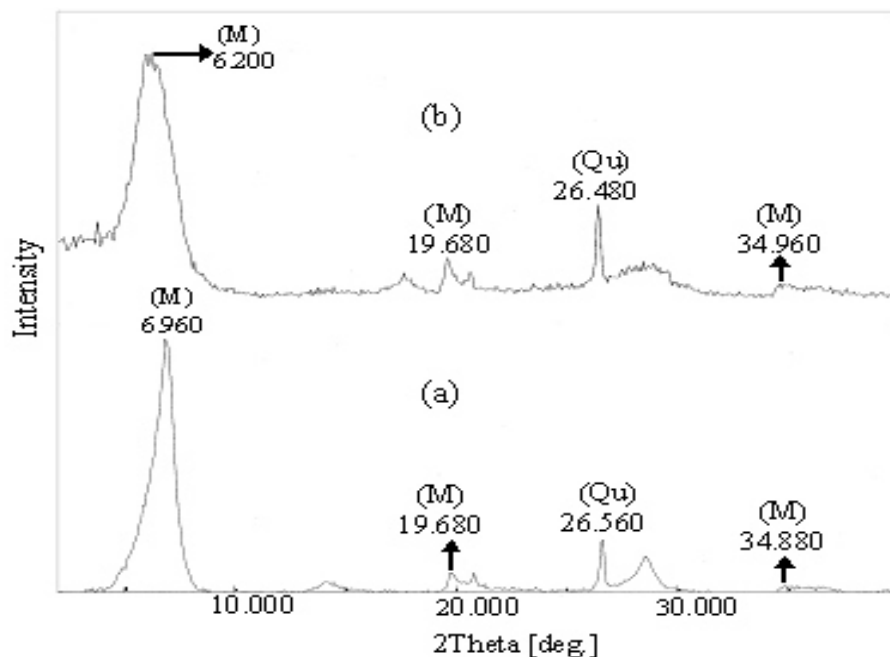


Figure 2. XRD spectrum of: (a) Bent-Cys (b) bentonite structures.

structure) at this pH. (ii) At pH values lower and higher than pH 4.0, the adsorbed amount of ferritin drastically decreases. This could be created from the ionization state of ferritin and could be caused repulsive electrostatic forces between adsorbed ferritin molecules and the Bent-Cys micro-composite affinity sorbent.

Increase in conformational size and the lateral electrostatic repulsions between adjacent adsorbed ferritin molecules may also cause a decrease in adsorption efficiency. Also, the change of coordination interaction at high and low pH should have a great influence.

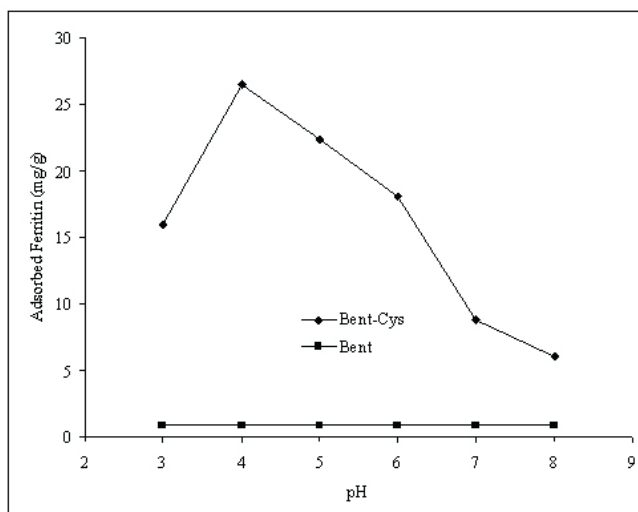


Figure 3. Effect of pH on Ferritin adsorption on Bent-Cys micro-composite affinity sorbents; L-Cysteine loading: 541.3 $\mu\text{mol/g}$ bentonite; Ferritin concentration: 0.5 mg/mL; T: 25°C.

Table 2. The surface area of Bentonite and Bent-Cys micro-composite affinity sorbent.

Structure	Surface Area (m^2g^{-1})
Bentonite	33.00 \pm 0.30
Bentonite-Cysteine	24.70 \pm 0.30

Effect of ferritin concentration

Figure 4 shows the effects of ferritin concentration on ferritin adsorption. As presented in Figure 4, pseudo-specific adsorption (i.e., adsorption of ferritin molecules onto the Bent-Cys micro-composite affinity sorbents through L-cysteine molecules) was significant (40.0 mg ferritin/g) and increased with increase in the initial concentration of ferritin in the incubation medium. As expected, the amount of ferritin coupled to Bent-Cys micro-composite affinity sorbents via L-cysteine

molecules reached almost a plateau value around 1.0 mg/mL, due to the saturation of active binding sites.

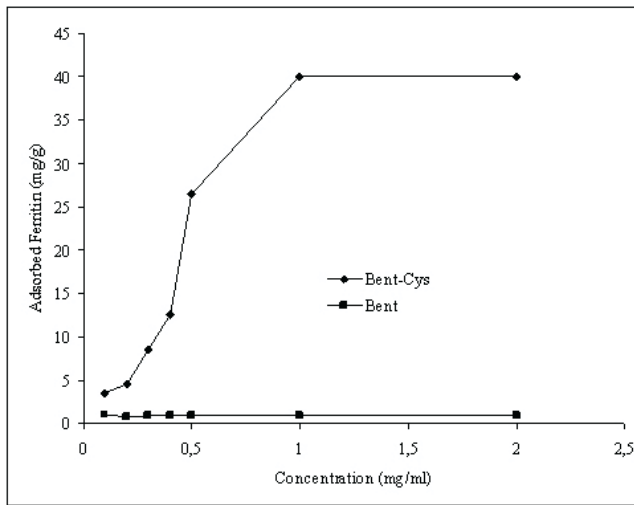


Figure 4. Effect of Ferritin initial concentration on Ferritin adsorption through Bent-Cys micro-composite affinity sorbents; L-Cysteine loading: 541.3 $\mu\text{mol/g}$ bentonite; pH: 7.0; T: 25°C.

Desorption of Ferritin

Desorption and regeneration are crucial steps in all affinity chromatography techniques. It was thus necessary to evaluate the regeneration efficiency of the affinity adsorbents after each cycle. The micro-composite affinity sorbents adsorbed different amounts of ferritin were contacted within the desorption medium, and the amount of ferritin desorbed in 1 h was determined. In this study, more than 95% of the adsorbed ferritin molecules were removed easily from the micro-composite affinity sorbents in all cases when 1 M NaCl was used as desorption agent. In order to show reusability of the Bent-Cys micro-composite affinity sorbents, the adsorption-desorption cycle was repeated 5 times using the same micro-composite affinity sorbents. For sterilization, after one adsorption-desorption cycle, micro-composite affinity sorbents were washed with 50 mM NaOH solution for 30 min. After this procedure, micro-composite affinity sorbents were washed with distilled water for 30 min, then equilibrated with phosphate buffer for the next adsorption-desorption cycle. It is observed that the adsorption behavior of

ferritin to the micro-Bent-Cys micro-composite affinity sorbents was little changed over 5 cycles. These results demonstrated that the stability of the present Bent-Cys micro-composite structures as an affinity sorbent.

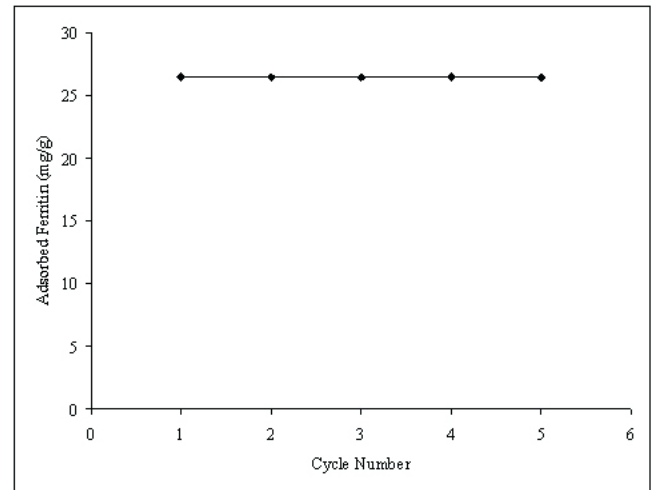


Figure 5. Repeated use of Bent-Cys micro-composite affinity sorbents; L-Cysteine loading: 541.3 $\mu\text{mol/g}$ bentonite; T: 25°C.

4. CONCLUSION

Bentonite has several advantages for use as a support, including its lack of toxicity, chemical reactivity and hydrophilicity allowing easy fixation of biomolecules. Surface properties of such hydrated minerals can be improved by different binding mechanism of inorganic cations with a number of organic molecules such as quaternary ammonium cations and cationic surfactants so that these organo-clays using in removal of hazardous materials from ground water and soil [28-31]. The adsorption characteristics of organic compounds on the organo-clays and clays depend on the type of the component, and intrinsic properties of solution phase [32-34]. It is known that natural clays are not very effective by themselves in different applications and in using as catalyst for the specific reaction. Therefore, the modification may be required for making clay and clays minerals of specific adsorption and catalytic properties. One of these modification processes is to interact the clays or clay minerals with various organic cations and molecules under certain conditions. In addition to these,

montmorillonite is acidic in nature; the acid sites can serve as centres of binding through the NH₂ group of proteins [1].

Bent-Cys composite affinity sorbents were synthesized as a micro-composite affinity sorbent for the separation of ferritin from the aqueous solution. The main criteria for selection of bentonite are its lack of toxicity, economical adsorptive properties, high surface area, specific active sites and hydrophilicity allowing easy fixation of biomolecules. The adsorption behaviours of ferritin onto Bent-Cys micro-composite affinity sorbents were investigated using various reaction conditions. This approach for the preparation of pseudo-specific affinity adsorbent has many advantages over conventional techniques. An expensive and critical step in the preparation process of affinity adsorbent is immobilization of an affinity ligand to the matrix. In this procedure, cysteine acted as the pseudo-specific ligand, and there is no need to activate the matrix for the ligand immobilization. Cysteine was covalently bonded to bentonite and there is no ligand leakage. The time consuming and high cost of ligand immobilization procedure has inspired a search for suitable low-cost adsorbents. Bent-Cys micro-composite affinity sorbents were also cheap. The properties of the micro-composite affinity sorbents seem to provide an adequate approach to ferritin isolation and separation. The cysteine containing bentonite micro-composite structures revealed good properties as an affinity sorbent and will be useful for adsorption of ferritin.

5. REFERENCES

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