Spectral Characterization of Lysozyme Adsorption on Dye-Affinity Beads

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ABSTRACT: Cibacron Blue F3GA-attached magnetic poly(2-hydroxyethyl methacrylate) [mPHEMA] beads were prepared by suspension polymerization of HEMA in the presence of magnetite (Fe_3O_4) nanopowder. Average diameter size of the mPHEMA beads was 150–200 μm. The characteristic functional groups of Cibacron Blue F3GA-attached mPHEMA beads were analyzed by Fourier transform infrared spectrometer (FTIR) and Raman scattering spectrometer. The lysozyme adsorption and desorption characteristics of Cibacron Blue F3GA-attached mPHEMA beads were also investigated using FTIR and Raman spectroscopic techniques. When the Raman spectrum of lysozyme adsorbed mPHEMA is evaluated characteristic Amide-I band appears at 1657 cm^{-1}. The intensity of this band decreases in the spectrum of lysozyme desorbed mPHEMA sample. When the characteristic bands of lysozyme adsorbed and desorbed mPHEMA samples are compared, the band intensities of desorbed sample are lower than those of lysozyme adsorbed sample except for the band appearing at 656 cm^{-1} (Tyr vC—S). © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 108: 3454–3461, 2008

Key words: dye-affinity chromatography; lysozyme; spectral characterization; Raman spectroscopy

INTRODUCTION

Reactive dye-affinity chromatography has been used extensively in laboratory and large scale protein purification.[1–3] Dye-ligands are commercially available, inexpensive, and can easily be attached, especially on matrices having hydroxyl groups. Although dyes are all synthetic in nature, they are still classified as affinity ligands because they interact with the active sites of many proteins mimicking the structure of the substrates, cofactors, or binding agents for those proteins.[4] A number of textile dyes, known as reactive dyes, have been used for protein purification. Most of these reactive dyes consist of a chromophore (either azo dyes, anthraquinone, or phathalocyanine), linked to a reactive group (often a mono- or dichlorotriazine ring). The interaction mechanism between dye and protein molecule includes the complex combination of electrostatic, hydrophobic, hydrogen bonding. Cibacron Blue F3GA is an anthraquinone textile dye that interacts specifically and reversibly with albumin.[5]

Lysozyme is found in a variety of vertebrate cells and secretions, such as spleen, milk, tears, and egg white. Lysozyme lyses certain bacteria by hydrolyzing the β-linkages between the muramic acid and *N*-acetylglucosamine of the mucopolysaccharides, which are present in the bacterial cell wall. Its common applications are as a cell disrupting agent for extraction of bacterial intracellular products, as an antibacterial agent in ophthalmologic preparations, as a food additive in milk products, and as a drug for treatment of ulcers and infections.[6] The potential for its use as an anticancer drug has been demonstrated by animal and *in vitro* cell culture experiments.[7] Lysozyme has also been used in cancer chemotherapy.[8] The studies conducted so far show that it induces the activity of phagocytizing cells, influences immunological processes by stimulating immunoglobulin synthesis, promotes interferon synthesis, and modulates tumor necrosis factor generation.[9] High purity requirements for both natural and therapeutic proteins along with the commercial pressures to reduce processing costs have stimulated more efficient, simple, and relatively less expensive separation techniques for lysozyme production in the recent years.[10]

A variety of methods have been used for measuring the adsorption of proteins, but none of them has been perfect.[11] The aim of this study is to investigate the spectral characterization of polymer preparation, dye-binding, and lysozyme adsorption–desorption on the dye-affinity magnetic beads by Fourier transform infrared (FTIR) spectroscopy and Raman spectroscopy methods.

EXPERIMENTAL

Materials

Lysozyme (chicken egg white, EC 3.2.1.7) and Cibacron Blue F3GA were supplied by Sigma (St. Louis, MO) and used as received. 2-Hydroxyethyl methacry-

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late (HEMA) and ethylene glycol dimethacrylate (EGDMA) were purchased from Fluka A.G. (Buchs, Switzerland), distilled under reduced pressure in the presence of hydroquinone inhibitor, and stored at 4°C until use. Magnetite nanopowder (Fe_3O_4, average diameter: 20–50 nm) was obtained from Sigma. Benzoyl peroxide was obtained from Fluka (Switzerland). Poly(vinyl alcohol) (98% hydrolyzed) was obtained from Aldrich Chemical Co. (St. Louis, MO) and had a molecular weight of 100.000 by viscosity. All other chemicals were guaranteed or analytical grade reagents commercially available and used without further purification. Laboratory glassware was kept overnight in a 5% nitric acid solution. Before use the glassware was rinsed with deionized water and dried in a dust-free environment. All water used in the adsorption experiments was purified using a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit with a high-flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANOpure® organic/colloid removal and ion-exchange packed-bed system.

Preparation of mPHEMA beads

mPHEMA beads were selected as the carrier for the synthesis of affinity adsorbent for lysozyme purification. mPHEMA beads were produced by suspension polymerization in an aqueous medium as described in our previous articles.[12–14] Polymerization procedure of mPHEMA beads was given as follows: The dispersion medium was prepared by dissolving 200 mg of poly(vinyl alcohol) (molecular weight: 100.000) within 50 mL of distilled water. The desired amount of benzoyl peroxide (0.06 g) was dissolved within the monomer phase 12/4/8 mL (EGDMA/HEMA/toluene) with 1.0 g of magnetite nanopowder. This solution was then transferred into the dispersion medium placed in a mechanically stirred (at a constant stirring rate of 600 rpm) glass polymerization reactor (100 mL) which was in a thermostatic water bath. The reactor was flushed by bubbling nitrogen and then sealed. The reactor temperature was kept at 65°C for 4 h. Then the polymerization was completed at 90°C in 2 h. After polymerization, mPHEMA beads were separated from the polymerization medium. The residuals (e.g., unconverted monomer, initiator, and other ingredients) were removed by an extensive cleaning procedure.

Cibacron Blue F3GA-attached mPHEMA beads

Ten gram of mPHEMA beads was mechanically stirred (at 400 rpm) in a sealed reactor at a constant temperature of 80°C for 4 h with 100 mL of the Cibacron Blue F3GA aqueous solution containing 4.0 g NaOH. The initial concentration of the Cibacron Blue F3GA in the medium was 1.0 mg/mL. After incubation, the Cibacron Blue F3GA-attached mPHEMA beads were filtered, and washed with distilled water and methanol several times until all the physically adsorbed Cibacron Blue F3GA molecules were removed. The dye-affinity beads were stored at 4°C with 0.02% sodium azide to prevent microbial contamination. The leakage of the Cibacron Blue F3GA from the beads was followed by treating the magnetic beads with buffer solutions for 24 weeks at room temperature. Cibacron Blue F3GA released after this treatment was measured in the liquid phase by spectrophotometry at 630 nm.

Characterization of mPHEMA beads

The surface morphology and internal structure of the mPHEMA beads were observed in a scanning electron microscope (JEOL, JEM 1200EX, Tokyo, Japan). mPHEMA beads were dried at room temperature and coated with a thin layer of gold (about 100 Å) in vacuum and photographed in the electron microscope with 1000× magnification. The particle size was determined by measuring at least 100 beads on photographs taken on a SEM.

The presence of magnetite nanopowder in the structure was determined by an electron spin resonance (ESR) spectrophotometer (EL 9, Varian, USA). Magnetism degree of mPHEMA was measured in a magnetic field by vibrating-sample magnetometer (Princeton Applied Research, Model 150A, USA).

The characteristic functional groups of the mPHEMA beads were analyzed by using a Fourier transform infrared spectrophotometer (FTIR, 8000 Series, Shimadzu, Japan). The samples were prepared by mixing with ~ 100 mg of dry, powdered KBr (0.1 g, IR Grade, Merck, Germany), and pressed into a pellet form. The FTIR spectrum was then recorded.

Polymer samples were investigated by Raman scattering spectroscopy, which was utilized in the range of 50–3000 cm^{-1} on a Labram 800 HR Raman Spectrometer (Jobin Yvon, France) equipped with a He-Ne laser source (emitting at 633 nm), a holographic grating with 600–1200 grooves/mm, and a charge coupled device detector.

Lysozyme adsorption–desorption experiments

Adsorption experiments were carried out on a magnetically stabilized fluidized bed system by using BioRad economic column (diameter: 1 cm, length: 5 cm). Magnetic beads suspended in pure water were put into a column equipped with a water jacket for temperature control. Expansion of the magnetic beads was done conventionally. During the experiment, the magnetic beads in the column were exposed to magnetic field which surrounded the column (Brms ≈ 24 Gauss, Bp-p ≈ 33 Gauss, $\phi = 50$ Hz).

Lysozyme solution (100 mL) was passed through the column equipped with a water jacket for temperature control containing magnetic beads, by a peristaltic pump for 2 h. Equilibration of Cibacron Blue F3GA-attached mPHEMA column was performed by passing four column volumes of sodium acetate buffer (pH: 5.2) before injection of the lysozyme solution. Lysozyme desorption was studied with 0.1*M* Tris/HCl buffer containing 0.5*M* NaCl. Desorption agent (50 mL) was pumped through the magnetically stabilized fluidized bed column at a flow rate of 1.0 mL/min for 1 h.

RESULTS AND DISCUSSION

Characterization studies

The surface morphology and bulk structure of nonmagnetic and magnetic PHEMA beads are shown by the scanning electron photographs in Figure 1. As seen in Figure 1(A), mPHEMA beads have a perfect spherical form and a rough surface containing macropores due to the abrasion of magnetite nanocrystals (diameter $<$ 20–50 nm) during the polymerization stage. However, the surface of the nonmagnetic PHEMA beads contained no macropores [Fig. 1(C)]. The photographs in Figure 1(B,D) were taken with broken beads to observe the internal parts of both nonmagnetic and magnetic PHEMA beads. The presence of pores within the bead interior was clearly seen in the photograph. It can be concluded that the mPHEMA beads have a macroporous interior surrounded by a reasonably rough surface, in the dry state. On the other hand, nonmagnetic PHEMA beads were in the uniform and spherical shape with smooth surface characteristics. Average diameter size of the mPHEMA beads was 150–200 μm.

Presence of the magnetite nanoparticles in the polymer structure was confirmed by ESR. The intensity of the magnetite peak against magnetic field (Gauss) is shown in Figure 2(A). mPHEMA has a relative intensity of 450. This value shows that polymer structure has a local magnetic field because of magnetite nanopowder in its structure. Magnetic

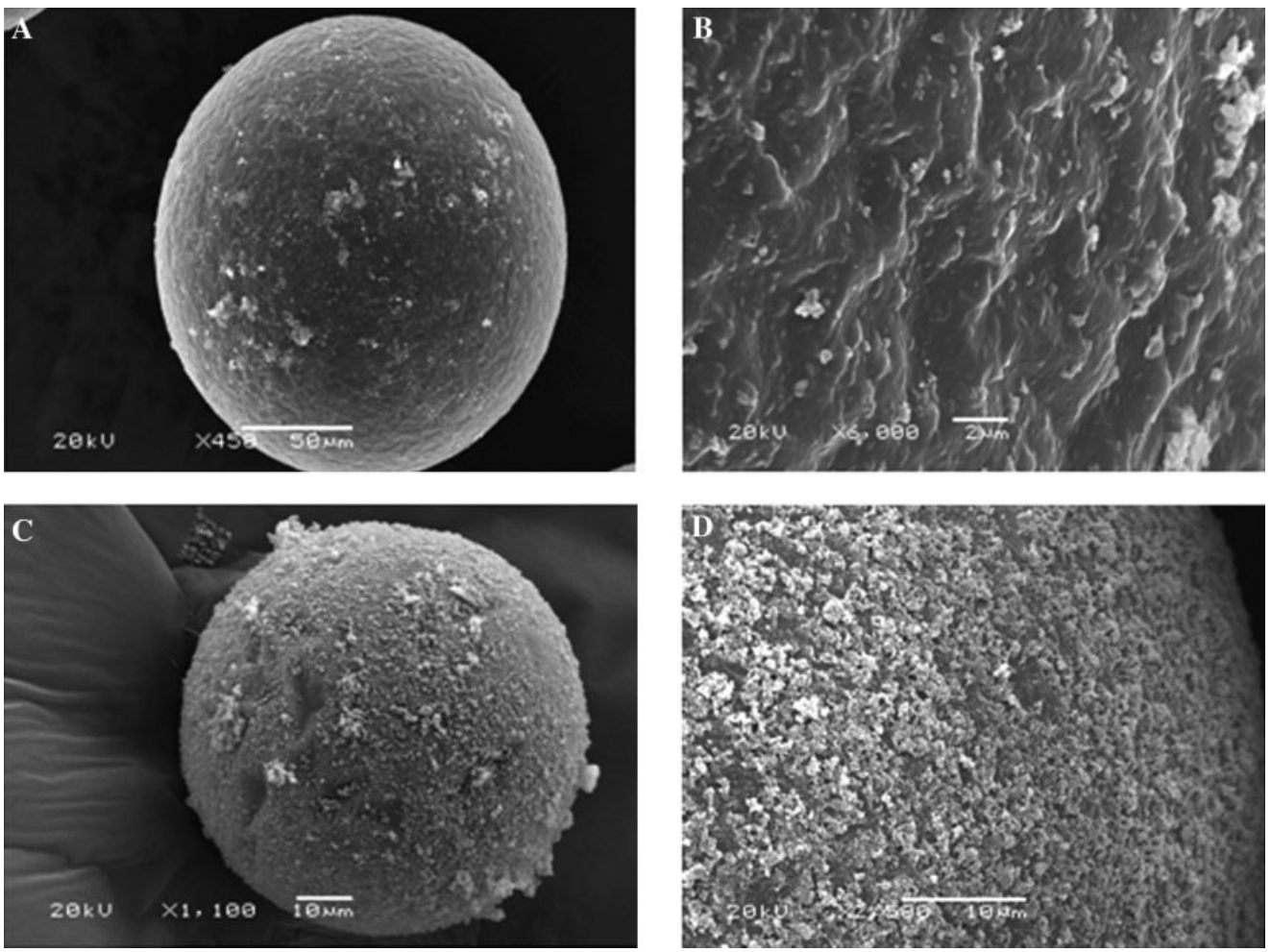

Figure 1 SEM pictures of polymeric beads: (A) mPHEMA beads; (B) cross section of mPHEMA beads; (C) Nonmagnetic PHEMA beads; (D) cross section of nonmagnetic PHEMA beads.

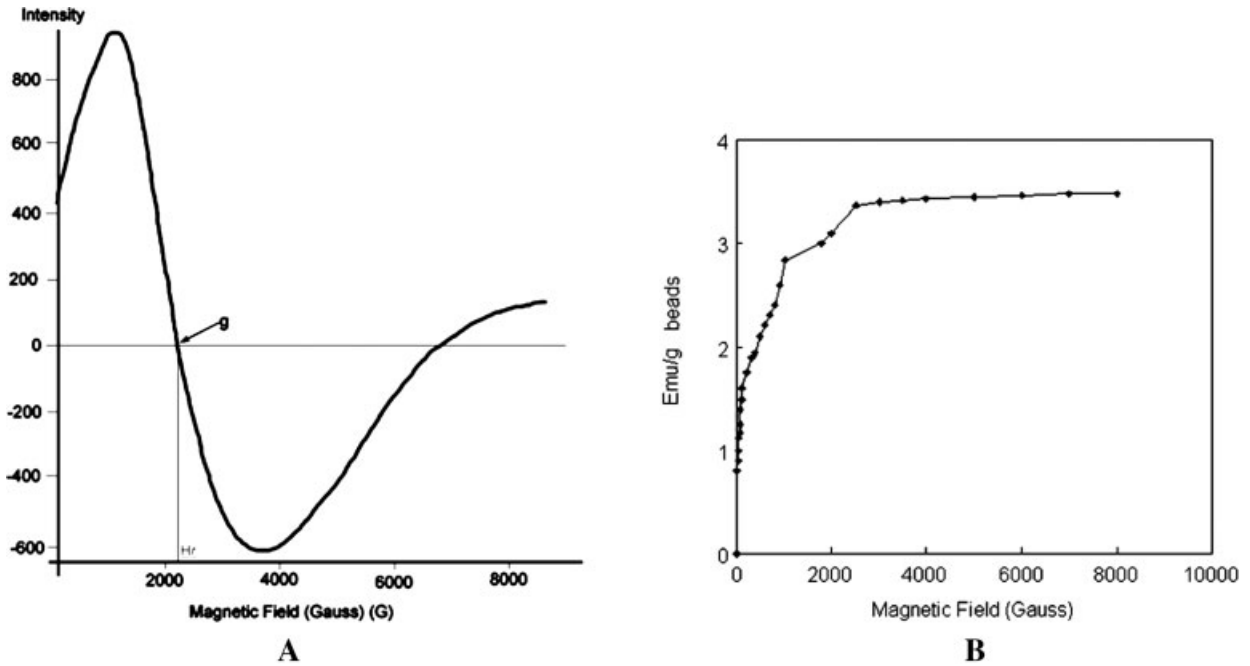

Figure 2 (A) ESR spectrum of mPHEMA; (B) Vibrating magnetometer curve of mPHEMA.

properties of the polymer structure were also expressed in electron mass unit (EMU), showing the behavior of magnetic beads in a magnetic field using a vibrating magnetometer, in Figure 2(B). In EMU spectrum, 2250 Gauss magnetic field was found sufficient to excite all of the dipole moments present in 1.0 g mPHEMA sample.

Cibacron Blue F3GA is covalently attached on the mPHEMA beads, via the reaction between the chloride groups of the reactive dyes and the hydroxyl groups of the mPHEMA beads (Fig. 3). The dye content was found to be 28.5 μmol/g by elemental analysis. The visual observations (the color of the beads) ensured attachment of dye molecules. Note that HEMA and other chemicals in the polymerization formula do not contain sulfur. This sulfur amount determined originated from only immobilized dye into the polymeric structure. The studies of Cibacron Blue F3GA leakage from the mPHEMA beads showed that there was no dye leakage in any medium used throughout this study, even in long period of time (more than 24 weeks).

FTIR Studies

Figure 4(A) shows the FTIR spectrum of HEMA monomer. When the evaluation of IR spectrum is performed; O—H stretching band is seen as a wide band at 3452 cm^{-1}. A strong C=O stretching vibration band of monomer appears at 1712 cm^{-1}. There is a characteristic band of C—C stretching of monomer at 1634 cm^{-1}. The C—O stretching vibration bands of monomer are displayed at 1079, 1169, and 1297 cm^{-1}.

When the evaluation of FTIR spectrum of EGDMA crosslinker that is given in Figure 4(B) is performed, a sharp peak can be observed clearly at 1721 cm^{-1} that shows the stretching vibration of C=O. The peak at 1635 cm^{-1} belongs to the C—C stretching vibration of the crosslinker. A sharp peak appears at 1318 cm^{-1}, 1293 cm^{-1}, and 1152 cm^{-1} showing the C—O stretching vibrations. A peak of the terminal —C=CH_2 group of EGDMA can be clearly seen at 941 cm^{-1}.

Evaluation of the FTIR spectrum [Fig. 4(C)] of crosslinked mPHEMA beads shows that the broad peak of O—H stretching vibration of HEMA monomer at 3452 cm^{-1} has disappeared. Multiple sharp peaks at 3500 ± 200 cm^{-1} frequency range characterized especially the O—H stretching vibration that is added and not added to the hydrogen bonding. However, the point which should be stressed is that, electrostatic interactions also occur between the Fe^{3+} ion and OH groups, besides the hydrogen bonds between the OH groups themselves. That is why the broad peak of OH in the spectrum of HEMA is not being manifested in the spectrum taken in the presence of Fe^{3+}. The sharp peak at 1727 cm^{-1} characterizes the C=O stretching vibration of the polymer. C—C stretching vibration bands at 1634 and 1635 cm^{-1} of the two monomers disappear in the spectrum of the polymer. Nevertheless it can be said that the weak band in the frequency range of 1637 ± 15 cm^{-1} comes from the terminal —C=CH_2 groups of EGDMA crosslinker. The broad peaks having a lot of shoulders at 1244 and 1164 cm^{-1} characterize the C—O stretching vibrations of the composite. The peak that is not determined in the IR spectrum of

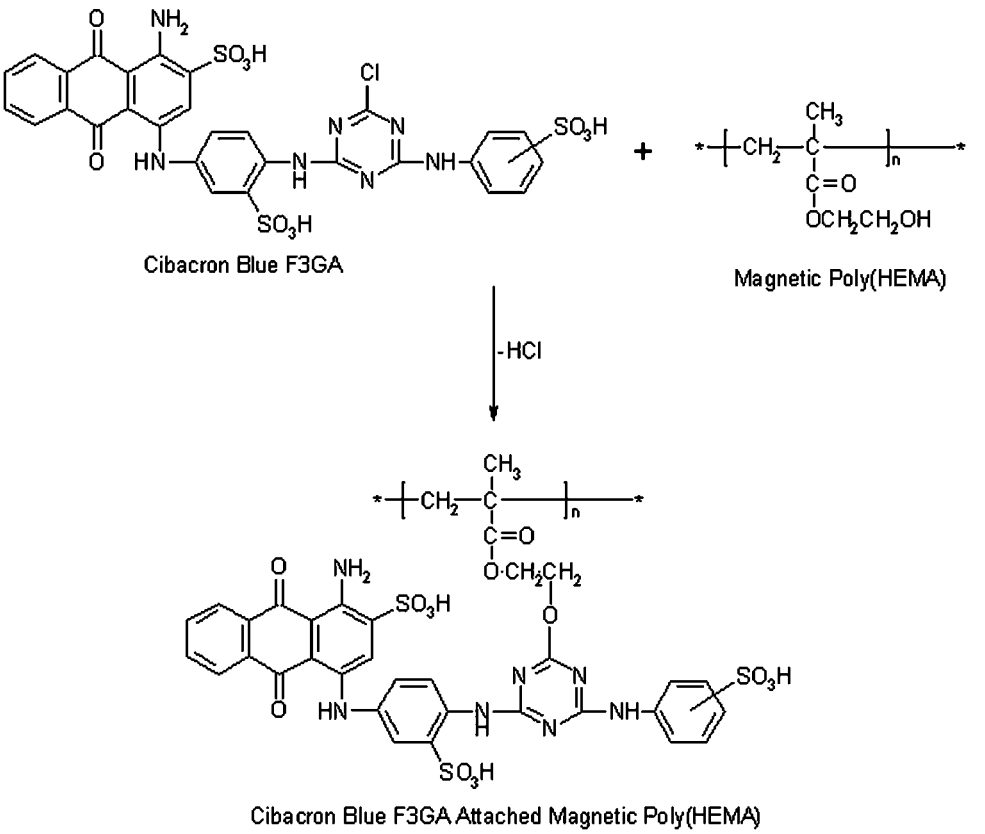

Figure 3 Chemical Reaction between mPHEMA and Cibacron Blue F3GA.

the two monomers is determined in the spectrum of the composite at 753 cm^{-1} and characterizes the Fe—O stretching vibration.

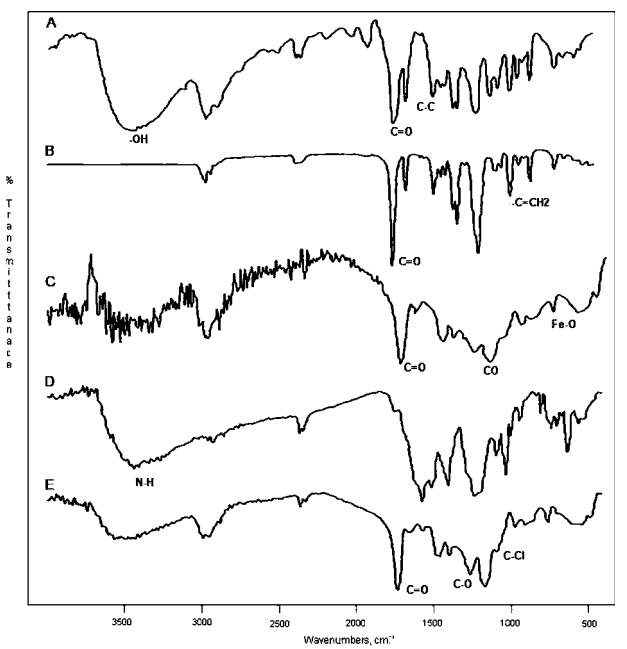

Figure 4 FTIR spectra: (A) HEMA monomer; (B) EGDMA crosslinker; (C) mPHEMA beads; (D) Cibacron Blue F3GA; (E) mPHEMA-Cibacron Blue F3GA.

FTIR spectrum of the Cibacron Blue F3GA is given in Figure 4(D). The N—H stretching vibration bands of this dye appear at 3433 ± 50 cm^{-1} frequency range

TABLE I
Some Characteristic Vibration Bands Collected from FTIR Spectra

	Wavenumber (cm^{-1})	Assignment
HEMA	1079, 1169, 1297	C—O stretching
	1634	C—C stretching
	1712	C=O stretching
	3452	O—H stretching
EGDMA	941	$-C=CH_2$
	1152, 1293, 1318	C—O stretching
	1635	C—C stretching
	1721	C=O stretching
mPHEMA	753	Fe—O stretching
	1164, 1244	C—O stretching
	1637 ± 15	$-C=CH_2$
	1727	C=O stretching
	3500 ± 200	O—H stretching
Cibacron Blue F3GA	500–700 and 1085	C—Cl stretching
	1022	S—O stretching
	1226 ± 25	S=O stretching
	1507, 1565	C=C stretching
	1650	C=N stretching
	1739	C=O stretching
	3433 ± 50	N—H stretching
mPHEMA-Cibacron Blue F3GA	1158, 1262	C—O stretching
	1727	C=O stretching

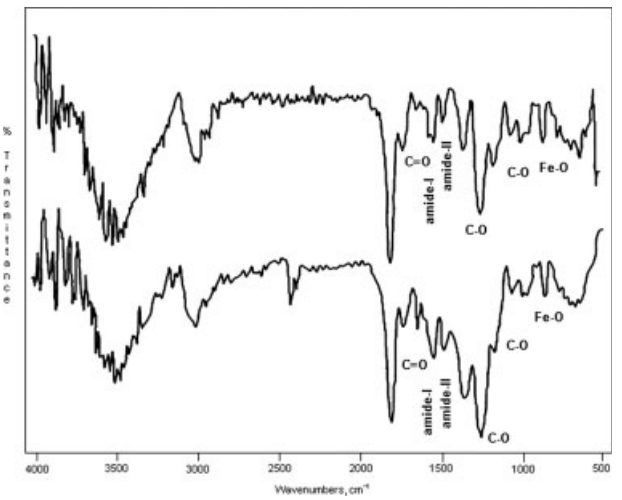

Figure 5 FTIR spectra: (A) lysozyme adsorbed mPHEMA beads; (B) mPHEMA beads after desorption.

as multiple bands. The different amine groups of the dye could have a tendency to be free and H-bonded. That is why this spectral situation is regular. The band having moderate intensity at 1739 cm^{-1}, a shoulder at 1650 cm^{-1}, and then sharp bands at 1565 and 1507 cm^{-1} are the spectral evidences that characterize the C=O, C=N, and C=C stretching vibrations of the structure. The band at 1226 cm^{-1} and the broad band at nearly 25 cm^{-1} below and above this frequency characterize the S=O stretching vibrations. The band that can be seen obviously at 1085 cm^{-1} belongs to the C—Cl stretching. At the lower wavenumber such as 1022 cm^{-1} S—O stretching vibration band is also seen in the spectrum. In addition, it is possible to see the C—Cl stretching vibrations of the dye at different wavenumbers in the range of 500–700 cm^{-1}.

Figure 4(E) shows the IR spectrum of mPHEMA-Cibacron Blue F3GA system. The C=O band is observed at 1727 cm^{-1} as a sharp peak. It implies that the strength of the band at 1650 cm^{-1} is very low. The peaks at 1262 and 1158 cm^{-1} characterize the C—O stretching vibrations. The points required to be emphasized are the following: the conversion of the C—Cl stretching vibration of the original Cibacron Blue F3GA at 1085 cm^{-1} to a weak shoulder at 1081 cm^{-1} of the IR bands; in addition, the decrease in the intensity of the same stretching vibrations of Cibacron Blue F3GA at 500–700 cm^{-1} frequency interval. The FTIR evaluations are given in Table I with the data obtained from this study.

Lysozyme adsorption–desorption dynamics of mPHEMA

FTIR spectroscopy

The lysozyme adsorption and desorption dynamics of Cibacron Blue F3GA-attached mPHEMA beads

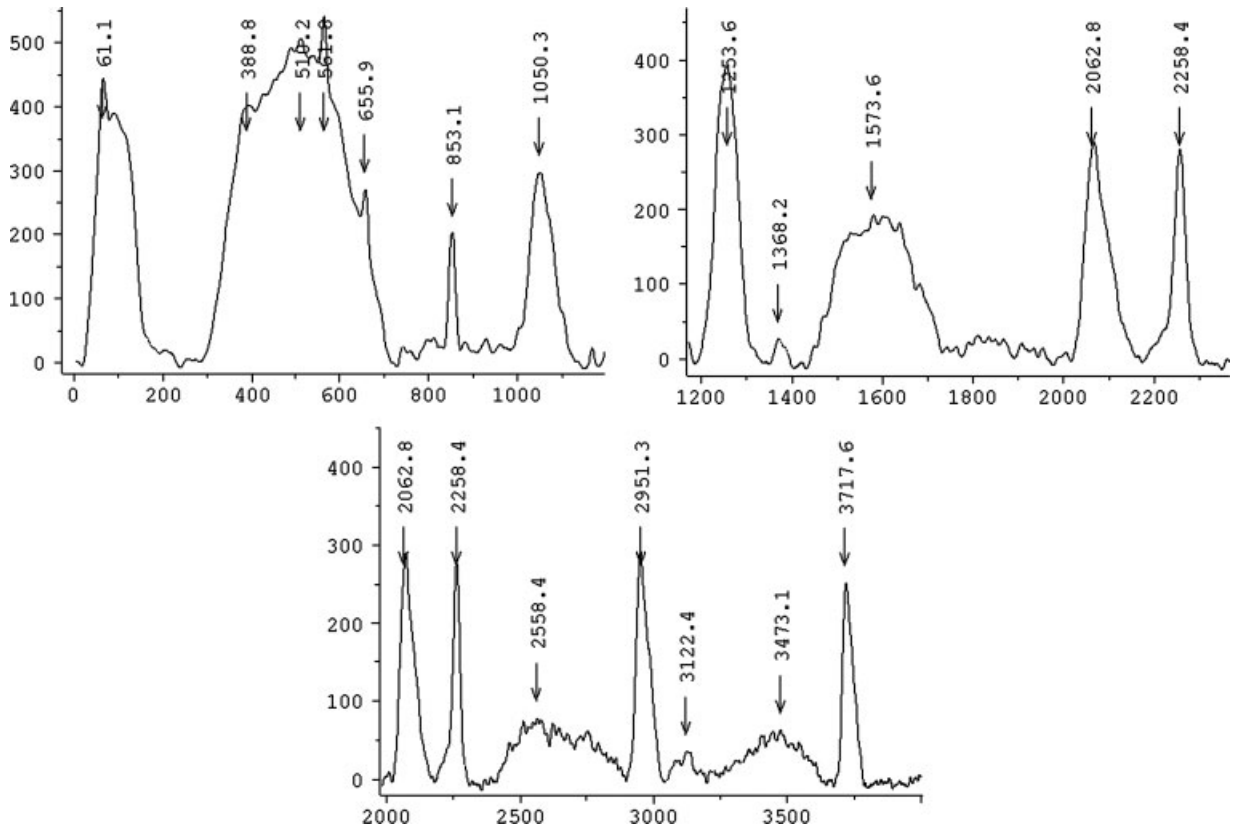

Figure 6 Raman spectrum of lysozyme adsorbed mPHEMA beads.

TABLE II
Some Characteristic Vibration Bands Collected from Raman Spectra

Raman wavenumbers (cm^{-1})			
Ref. 24	Ref. 25	In this study	Assignment
508	508	510	S—S (g-g-g)
525	524	–	S—S (g-g-t)
–	538	–	Trp
–	622	–	Phe
–	644	656	Tyr, v C—S
–	721	740.6	C—S
760	759	756.5	Trp
850, 830	853, 830	851.5	Tyr
936–946	926	927.6	v Cα—C—N (α-helix)
965–970	958	961	v Cα—C—N (random) Trp
980–990	974	–	v Cα—C—N (β-sheet) Tyr

were investigated by using FTIR and Raman spectroscopic methods. When hydration of lysozyme is investigated,[15] spectral changes of functional groups of lysozyme (especially amide bands) are evaluated and C=O vibrations coming from the peptide groups at the frequency interval of 1600–1750 cm^{-1} can be determined. In our spectral evaluation, we can see the amide-I band at 1646 cm^{-1} (Fig. 5). The decrease in the intensity of this peak is determined from the spectrum of mPHEMA sample after desorption. The amide-II band can be observed at 1500–1550 cm^{-1}. In this study, this band is displayed at 1556 cm^{-1} with a very low intensity.

Yasumoto and coworkers made a series of evaluations of the IR spectrum using the second derivatives and thus characterizing the interactions between lysozyme and water.[16]

In the spectral evaluation of FTIR spectrum of the lysozyme by Kumosinski et al., amide-I bands were characterized at lower wavenumbers with a spectral evaluation difficulty between determined wavenumbers and functional group characterization.[17]

Freeze-dried lysozyme stabilization,[18] thermal denaturation of hen egg-white lysozyme,[19] and the change in structure of lysozyme during the lyophilization[20] are the studies where structure characterizations/spectral changes are observed by using FTIR.

When the spectrum of this sample is evaluated we can see the vibrations of mPHEMA characterized previously. These vibrations are the C=O stretching vibrations (1726 cm^{-1}), the C—O stretching vibrations (1154–1157 cm^{-1}, 1263–1268 cm^{-1}), and the Fe—O stretching vibrations (750–965 cm^{-1}) which occur as the basic characteristic bands of the polymer.

Raman spectroscopy

In recent years, usage of the Raman techniques in spectroscopic characterization of the lysozyme has gained great importance and attracted large interest of the researchers.[21–23] In the Raman study of crystalline hen egg-white lysozyme,[24] Amide I (1657

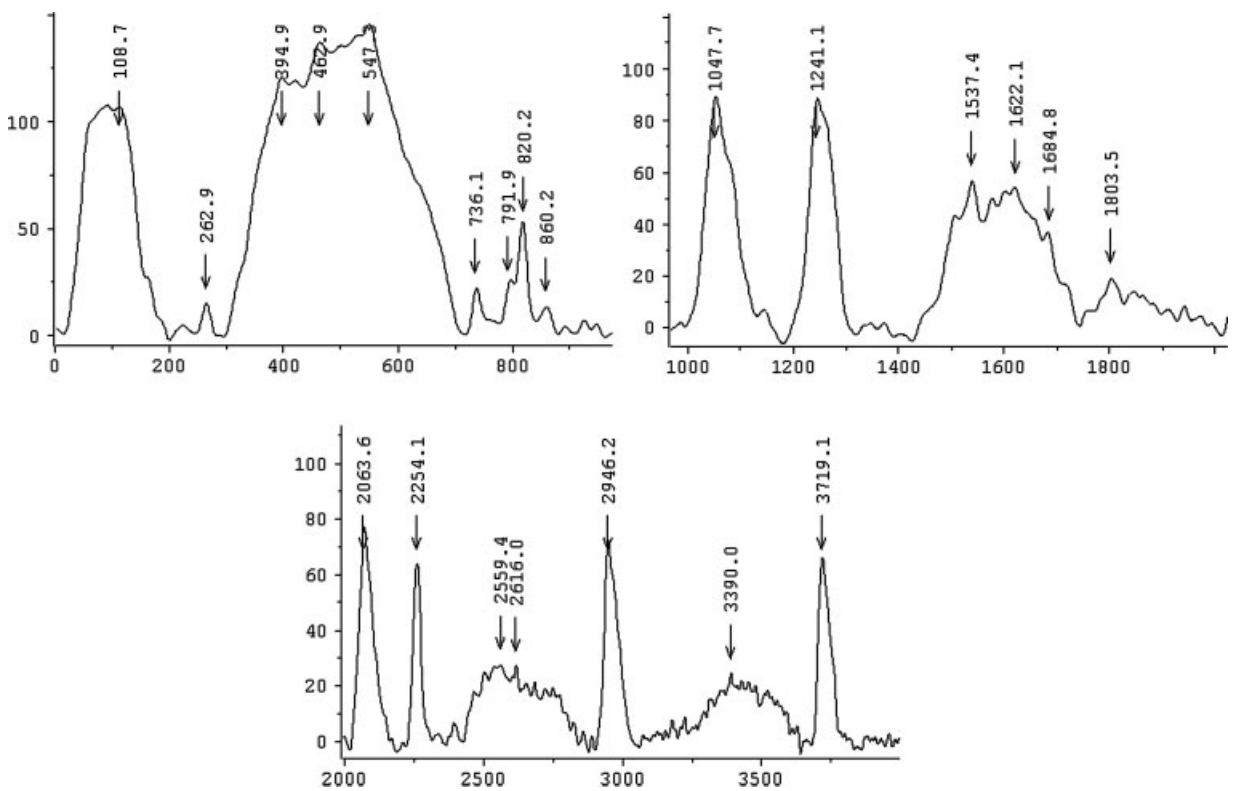

Figure 7 Raman spectrum of mPHEMA beads after desorption.

cm^{-1}) and Amide III (1259 cm^{-1}) bands can be observed, respectively. The band at 1554 cm^{-1} is characterized as Trp residue. The bands at 1032 and 1005 cm^{-1} are characterized as a phenyl ring of phenylalanyl residue. The peak at 1013 cm^{-1} belongs to the Trp residue.

When the Raman spectrum of lysozyme adsorbed mPHEMA is evaluated amide-I band appears at 1657 cm^{-1} (Fig. 6). The intensity of this band decreases in the spectrum of desorbed sample. Amide-III bands appear at 1254 and 1241 cm^{-1} in the spectrum of lysozyme adsorbed sample and desorbed sample, respectively, and the decrease in intensity is very significant when these two bands are compared. Similarly, the Raman band of lysozyme adsorbed sample appearing at around 1574 cm^{-1} was displayed at nearly 1537 cm^{-1} with a lower intensity in the desorbed sample spectrum. The spectral changes at 1000–1030 cm^{-1} are not very clear for the two samples. Howell[25] and Torreggiani[26] explain the Raman characterization of lysozyme in detail. These two Raman evaluations are given in Table II with the data obtained from this study.

When the characteristic bands of adsorbed and desorbed samples are compared the band intensities of desorbed sample is lower than those of lysozyme adsorbed sample except for the band appearing at 656 cm^{-1} (Tyr vC—S) (Fig. 7). This band can be clearly seen in lysozyme-adsorbed mPHEMA. However, it has been converted to a weak shoulder at the same wavenumber in the spectrum of desorbed mPHEMA sample.

CONCLUSIONS

Proteins will undergo structural changes during the process of adsorption on supports,[27] while elution agents will also affect the conformation of the protein.[28] Structural changes in proteins may result in changes in their bioactivity. However, only a limited amount of work has been performed on the effects of adsorption and elution conditions on the structural changes of proteins.[29] Here, lysozyme adsorption–desorption dynamics of Cibacron Blue F3GA-attached mPHEMA beads were investigated by using FTIR and Raman spectroscopic methods. Spectral changes of functional groups of lysozyme, especially amide-I band at 1646 cm^{-1} are evaluated and C=O vibrations coming from the peptide groups at the frequency interval of 1600–1750 cm^{-1} can be determined due to the hydration of lysozyme. The decrease in the intensity of amide-I peak at 1646 cm^{-1} is determined from the IR spectrum of mPHEMA sample after desorption. Raman spectrum of lysozyme adsorbed mPHEMA has also amide-I band at 1657 cm^{-1}. The intensity of this band similarly decreased in the spectrum of desorbed polymer sample. The bands in Raman spectra for lysozyme adsorbed mPHEMA at 1554 cm^{-1}, at 1032 and 1005 cm^{-1}, at 1013 cm^{-1} belong to the Trp residue, phenyl-ring or phenylalanyl residue, and Trp residue, respectively. Similarly the Raman band of lysozyme adsorbed sample appearing at around 1574 cm^{-1} was displayed at nearly 1537 cm^{-1} with a lower intensity in the desorbed sample spectrum.

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