Human Serum Albumin (HSA) Adsorption with Chitosan Microspheres

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ABSTRACT: In this study, chitosan microspheres were prepared and characterized for adsorption of human serum albumin (HSA) as affinity sorbent. The chitosan microspheres were obtained with a "suspension crosslinking technique" in the size range of 30–700 μm by using a crosslinker, i.e., glutaraldehyde. The chitosan microspheres used in HSA adsorption studies were having the average size of 170 $\pm$ 81 μm. Adsorption medium pH and the initial HSA concentration in the adsorption medium were changed as 4.0–7.0 and 0.5–2.0 mg HSA/mL, respectively, to investigate the HSA adsorption capacity of chitosan microspheres. Maximum HSA adsorption (i.e., 11.35 mg HSA/g chitosan microspheres) was obtained at pH 5.0 and 1.5 mg HSA/mL of the initial HSA concentration in the adsorption medium was obtained as the saturation value for HSA adsorption. A very common dye ligand, i.e., Cibacron Blue F3GA was attached to the chitosan microspheres to increase the HSA adsorption capacity. Actually, the HSA adsorption capacity was increased up to 15.35 mg HSA/g chitosan microspheres in the case of Cibacron Blue F3GA attached to chitosan microspheres used. © 2002 Wiley Periodicals, Inc. J Appl Polym Sci 86: 3035–3039, 2002

Key words: chitosan microsphere; HSA adsorption; affinity sorbent; Cibacron Blue F3GA

INTRODUCTION

The chromatographic separation of proteins is very important for both their analysis and purification in large-scale industries such as food and drug industries.[1] Up to now, many different types of chromatographic support have been developed for protein separation and purification. Unfortunately, no chromatographic support material has been developed as a unique one that can be used for the separation and purification of any desired protein yet. Furthermore, almost all of the developed chromatographic supports are very expensive, especially to use in large-scale industries. Therefore, investigators are still trying to develop novel chromatographic support materials, and chitosan is one of them.

Chitosan is a polyaminosaccharide, normally obtained by alkaline deacetylation of chitin. Chitin is a polysaccharide that is widely spread among marine, terestrial invertebrates, and lower forms of a plant kingdom, and it is the second most abundant natural polymer after cellulose.[2,3] Chitosan's availability and ability to be made into a variety of useful forms (i.e., films, fibers, and beads as well as powders and solutions) and its unique chemical and biologic properties make it a very attractive biomaterial. Therefore, it is extensively used in many different types of applications, i.e., treatment of wastewater,[4,5] chromatographic support,[6,7] enzyme immobilization,[8,9] and biomedical applications.[10–15] The main goal of this study is to develop an alternative adsorbent for HSA separation and purification by using very cheap biopolymer, i.e., chitosan. For this purpose crosslinked chitosan microspheres were prepared and used for HSA adsorption. In addition, a dye-ligand, i.e., Cibacron Blue F3GA was attached to the chitosan microspheres to increase HSA adsorption capacity.

EXPERIMENTAL

Preparation of chitosan microspheres

Chitosan was supplied commercially with the molecular weight of 650,000 Da (Fluka, Germany). The aqueous acetic acid solution was used as the solvent in microsphere preparation. Glutaraldehyde, 50% (Fluka, Germany) was used as the crosslinker. The following chemicals were all obtained and used as reagent grade from Fluka, Germany: petroleum ether, mineral oil, ethyl alcohol, acetic acid, sodium bisulphide, acetone. Suspension crosslinking technique was used for the preparation of chitosan microspheres.[16] In a typical procedure; 4% of chitosan solution was prepared by using the 5% of aqueous acetic acid solution; then it was poured into the suspension medium, as dropwise, which was composed of mineral oil and petroleum ether (25/35, v/v), and emul-

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sifier (i.e., Tween 80). During these processes the suspension medium was stirred with a mechanical stirrer in the range of 1000–2000 rpm at room temperature. Ten minutes later, 1 mL of glutaraldehyde was added into the suspension medium. Similarly, 1 h later 1.5 mL more of glutaraldehyde was added into the medium, and the stirring was allowed for another 2 h. At the end of this period, the chitosan microspheres were collected with centifugation and they were washed with petroleum ether, sodium bisulphide, and acetone consecutively. Then the microspheres were dried in an oven at 40°C for 2 days, and they were kept in a vacuum dessicator for further analysis and uses. The stirring rate of suspension medium, chitosan/acetic acid solution ratio, emulsifier concentration, and the amount of glutaraldehyde were changed for investigation of the effects of these parameters on the size and size distribution of chitosan microspheres and the obtained results were published in elsewhere with details.[16]

Characterization of chitosan microspheres

The size and size distributions of the chitosan microspheres were determined from the micrographs taken with an optical microscope (Nikon, Alphaphot, Japan). Average size and standard deviations of the microspheres on the micrographs (four micrographs and each containing approximately 25–50 microspheres) were evaluated.

The morphologic characterization of the chitosan microspheres was made with a scanning electron microscope (SEM, JEOL, Japan). A 100-μL aqueous suspension of chitosan microspheres was dropped on a sample holder (a stap) and placed in a vacuum oven at room temperature for 24 h to dry. The samples were coated with gold, and then SEM micrographs were obtained.

Swelling behavior of chitosan microspheres

Dynamic swelling properties of chitosan microspheres were determined by volumetric method. In this method; the chitosan microspheres of a known amount (50 mg) were placed in a tube (internal diameter 5 mm and height 100 mm) and the top point of the microspheres was marked. Afterwards the tube was filled with buffer solution and the height of the microspheres was marked periodically (i.e., for each 30 min). The percentage of swelling of microspheres in the tube was calculated from the following equation,

$$S_{CM} = \frac{h_t - h_o}{h_o} \times 100$$

where; S_{CM} is the percentage of swelling of microspheres, h_t denotes the height of the microspheres at time t, and h_o is the initial height of the microspheres in the tube. The crosslinker concentration in suspension medium was changed (i.e., 0.0083–0.066 mL glutaraldehyde/mL suspension medium) to obtain different swelling ratios. In these experiments the stirring rate, chitosan/acetic acid solution ratio, and emulsifier concentration were kept at 1500 rpm, 20 mg/mL suspension medium and 0.020 mL/mL suspension medium, respectively.

HSA adsorption with chitosan microspheres

In this part of the study, chitosan microspheres were used as affinity sorbent for HSA (Sigma, MO). The average size of chitosan microspheres was 170 $\pm$ 81 μm at the preparation conditions specified as stirring rate: 1500 rpm, chitosan/acetic acid solution: 20 mg/mL, emulsifier concentration: 0.020 mL/mL suspension medium and glutaraldehyde concentration: 0.0333 mL/mL suspension medium. In the HSA adsorption studies; around 100 mg of chitosan microspheres (swollen within the adsorption medium) were placed into a vial containing 5 mL of buffer solution (as HSA adsorption medium) and 7.5 mg HSA. The supernatant of the adsorption medium was pipetted out (1 mL) and tested with Bradford Technique for the determination of remained HSA in the medium.[17] All the measurements are the average values of three parallel studies. The pH of the adsorption medium was changed between 4.0 and 7.0 by using different buffer systems (0.1 *M* CH_3COONa—CH_3COOH for pH 4.0–6.0 and 0.1 *M* KH_2PO_4—K_2HPO_4 for pH 7.0) and the initial concentration of HSA in the adsorption medium was varied between 0.5–2.0 mg/mL to investigate the effects of pH and initial concentration of HSA on the HSA adsorption capacity of chitosan microspheres. On the other hand, a very common dye ligand, i.e., Cibacron Blue F3GA was attached to the chitosan microspheres to increase the HSA adsorption capacity. To incorporate the dye-ligand onto the chitosan microspheres, the following procedure was applied; 10 mL of the aqueous solution containing 300 mg Cibacron Blue F3GA (BDH, UK) was poured into 90 mL of the aqueous solution containing 1 g of chitosan microspheres and then 4.0 g NaOH was added. The medium was heated in a sealed reactor for 4 h at a stirring rate of 300 rpm, and at a constant temperature of 60°C. The amount of Cibacron Blue F3GA attached on the chitosan microspheres was determined by using an elemental analysis instrument (Leco, CHNS-932, USA) by considering the sulfur stoichiometry. In the HSA adsorption studies different types of chitosan microspheres prepared with different amounts of crosslinker were evaluated for the

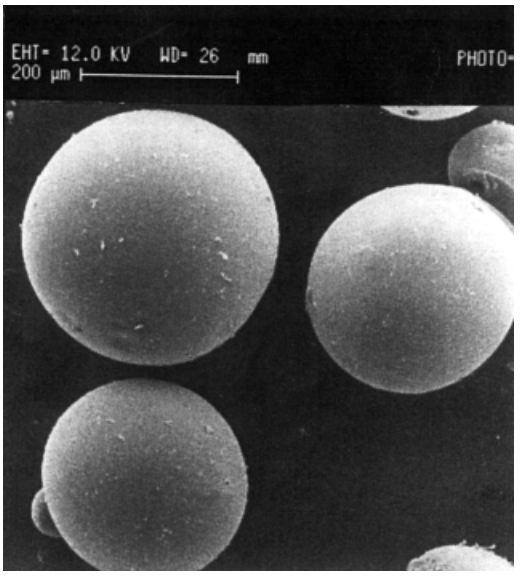

Figure 1 A representative SEM micrograph of chitosan microspheres.

investigation of the effects of swelling behavior of chitosan microspheres on HSA adsorption.

Effect of swelling behavior of chitosan microspheres on HSA adsorption

Chitosan microspheres are swollen with different swelling ratios depending on the crosslinking density of the microspheres because of the hydrogel structure of the chitosan. Hence, the volume and the surface area of the microspheres are increased by the increase in swelling ratio. Chitosan microspheres with different crosslinking densities (or swelling ratios) were evaluated for HSA adsorption to investigate the effect of swelling behavior of chitosan microspheres on HSA adsorption capacities. For this purpose, crosslinker (i.e., glutaraldehyde, GA) concentration was changed in the range of 0.0083–0.0666 mL GA/mL suspension medium during the chitosan microsphere preparation and those microspheres were used for HSA adsorption. The obtained HSA adsorption capacities were compared with the swelling ratios of chitosan microspheres.

Desorption studies of HSA

Desorption experiments were performed by using KSCN solution (0.5 *M* and pH: 8.0). HSA adsorbed chitosan microspheres (both plain and Cibacron Blue F3GA attached microspheres) were treated with KSCN solution for 1 h. The amount of HSA released was measured and the desorption ratio was calculated by using the following equation.

$$\text{Desorption Ratio (\%)} = \frac{\text{Amount of desorbed HSA}}{\text{Amount of adsorbed HSA}} \times 100$$

RESULTS AND DISCUSSION

Characterization of chitosan microspheres

Chitosan is a multifunctional polysaccharide, and has been the focus of much research in biomedical and pharmaceutical applications because of its biocompatibility, biodegradability, nontoxicity, and it is a very abundant naturally occurring raw material, i.e., chitin.[2] In this study, we prepared and characterized chitosan microspheres for the adsorption of HSA. Chitosan microspheres were prepared with the suspension crosslinking technique in the size range of 30–700 μm for different preparation conditions (i.e., stirring rate, chitosan/acetic acid solution ratio, emulsifier concentration, and glutaraldehyde concentration). The obtained chitosan microspheres were evaluated with the SEM micrographs to investigate the morphology of the microspheres. A sample of SEM micrograph is given in Figure 1. The produced chitosan microspheres are have a well-defined spherical shape and rather smooth surfaces as seen in this figure.

Swelling behavior of chitosan microspheres

The swelling ratio (or crosslinking density) is dependent on the amount of crosslinker that was used for crosslinking, significantly.[18,19] Therefore, in this study the amount of crosslinker was evaluated as the most effective parameter on the swelling behavior of the chitosan microspheres. The swelling ratio variations with time are given in Figure 2, depending on the amount of crosslinker. As seen in this figure in all

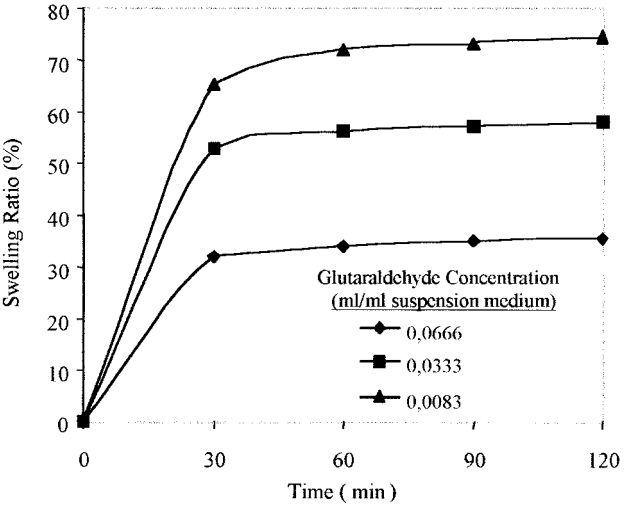

Figure 2 Swelling behavior of chitosan microspheres.

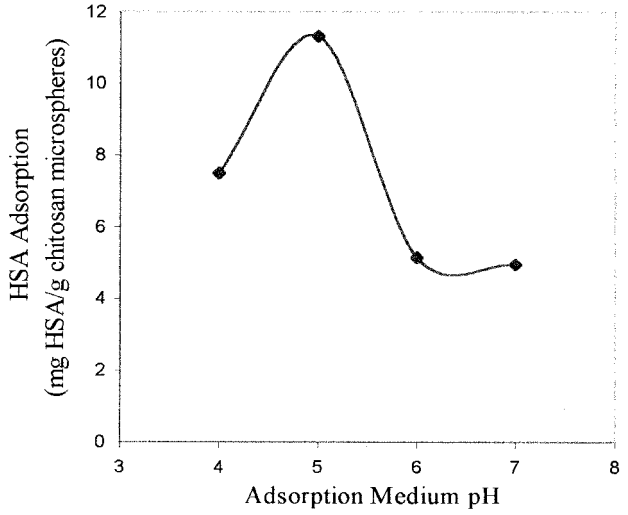

Figure 3 Effect of pH on the HSA adsorption with chitosan microspheres (initial HSA concentration is 1.5 mg/mL).

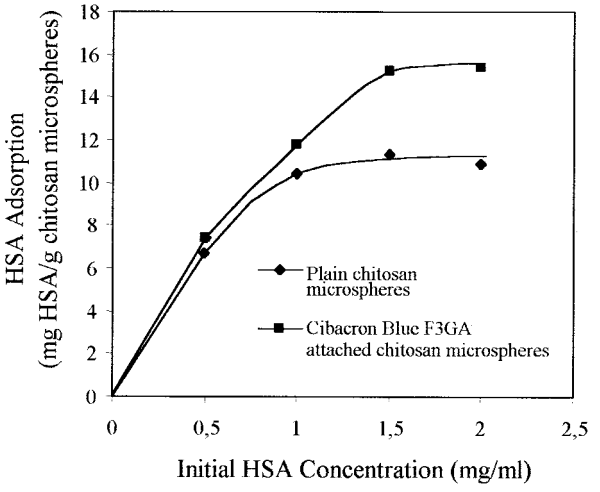

Figure 4 Effect of initial HSA concentration on the HSA adsorption with chitosan microspheres.

cases the microspheres reached the equilibrium value of swelling within 30–60 min. The maximum swelling ratio, i.e., 75%, was achieved by using the lowest crosslinker concentration, and the swelling ratio was decreased by increasing the crosslinker concentration.

HSA adsorption studies

In the adsorption studies, the effects of pH and initial HSA concentration (in adsorption medium) on the HSA adsorption capacity of chitosan microspheres were investigated. The amount of Cibacron Blue F3GA attached on the chitosan microspheres was calculated as 0.155 mg Cibacron Blue F3GA/mg chitosan microspheres by stoichiometric calculations. The amount of attached Cibacron Blue F3GA and HSA adsorption capacities for chitosan microspheres were very close to Cibacron Blue F3GA attached polyethersulfone-supported chitosan sorbents.[20] The obtained results were given with details in following subsections.

Effects of ph

Figure 3 shows the effects of adsorption medium pH on the HSA adsorption of chitosan microspheres. In all cases investigated, the maximum adsorption of HSA was observed at pH 5.0. Significantly lower adsorption capacities were obtained in more acidic and more alkaline pH regions. The decrease in adsorption capacity with increase/decrease in pH can be attributed to electrostatic repulsion effects. It has been shown that proteins have no electrical charge at their isoelectric points, and therefore, the maximum adsorption from aqueous solutions is usually observed at their isoelectric point.[21–23] Actually, the isoelectric point of the HSA was reported as 4.9 in the literature.[24]

Effects of initial HSA concentration

In this part of the study; the pH of the adsorption medium was fixed at pH 5.0 and initial HSA concentration was changed in the range of 0.5–2.0 mg HSA/mL. Figure 4 shows the obtained results as the effects of the initial HSA concentartion on the HSA adsorption of chitosan microspheres. As seen in this figure, HSA adsorption was increased by the increasing of initial HSA concentration up to the value of 1.5 mg HSA/mL. This means that this value is the maximum (or saturation) HSA adsorption value and even if the initial HSA concentration is increased the adsorption will be the same. On the other hand, in the case of Cibacron Blue F3GA attached chitosan microspheres, the HSA adsorption capacity was clearly increased, as can be seen in Figure 4.

Effects of swelling behavior of chitosan microspheres

The amount of crosslinker was evaluated as the most effective parameter on the swelling behavior of chitosan microspheres as discussed in previous subsections. The surface area of the microspheres are increased by increasing the swelling ratio (or by decreasing the crosslinker amount); therefore, it is expected that the HSA adsorption would be increased, depend-

TABLE I
Effect of Swelling Ratio of Chitosan Microspheres on HSA Adsorption*

Glutaraldehyde Conc. (ml/ml Sus. Medium)	Swelling Ratio (%)	HSA Adsorption (mg/g microsphere)
0.0083	71	12.04
0.0333	54	11.33
0.0666	36	3.50

* Adsorption medium pH: 5.0 and initial HSA concentration is 1.5 mg/ml.

TABLE II
Desorption of HSA from Chitosan Microspheres

Microsphere Type	Desorption Ratio (%)*
Plain Chitosan Microsphere	88
Cibacron Blue F3GA Attached	86

* Initial HSA concentration is 1.5 mg/ml and glutaraldehyde concentration is 0.0333 ml/ml suspension medium.

ing on the degree of swelling. Different amounts of crosslinker was used to obtain chitosan microspheres with different swelling ratios, and these microspheres were used for HSA adsorption studies. The obtained results were given in Table I.

HSA desorption studies

In the desorption studies, HSA adsorbed chitosan microspheres (both plain and Cibacron Blue F3GA attached chitosan microspheres) were treated with KSCN for 1 h and the desorption ratio of HSA was calculated by using the amount of desorbed and adsorbed HSA as explained before. The obtained results are given in Table II.

As shown in Table II, around 85–90% of the adsorbed HSA could be easily desorbed from both the plain and Cibacron Blue F3GA attached chitosan microspheres. This is because of the specific interaction forces (such as electrostatic and hydrophobic forces) between the albumin molecules and the solid matrix. In addition, SCN^- ions easily bind albumin molecules.[21]

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