**Research Article**

**Synthesis, Characterization, and Properties of Sulfonated Chitosan for Protein Adsorption**

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Chitosan sulfate was prepared and characterized as a new chromatography media for protein separation. The degree of sulfonation of chitosan could be well controlled and impacted under conditions in the synthesis process. The prepared chitosan sulfate shows improved binding capacity with proteins. Sulfonated chitosan shows improved ion-exchange adsorption properties with proteins, which could have good potential in protein purification.

1. Introduction

With the rapid development of monoclonal antibody (mAb) therapeutics in the past decade, such as PD-1/PD-L1 immune checkpoint inhibitors, the worldwide demand of therapeutic proteins has been increasing dramatically, which raised the production capacity of therapeutic proteins accordingly [1]. Protein purification is one of the most important stages in GMP-compliant industrial scale mAb manufacturing process, and ion-exchange chromatography plays the crucial role of capturing targeted proteins during purification processes [2–6]. It is very important in the ion-exchange chromatographic systems to use porous adsorbent particles which can provide the highest possible breakthrough capacity for the desired bioactive molecules. To enhance breakthrough capacity, it is effective to covalently link positive or negative affinity ligands/groups to extenders, and one of the suitable extenders has been found to be polysaccharides [7–12].

Chitosan, as a natural polysaccharide, has a porous structure, suitable specific surface area, and good biological affinity [13–16]. Therefore, it has been widely studied as a good candidate of an ideal extender. However, it has rarely been studied as a bioseparation media for protein purification, partially due to its poor water solubility, limiting its applications in the field of protein purification [17–20]. Furthermore, its direct interactions with proteins are to form small emulsion droplets or the formation of soluble or insoluble complexes [21], and the electrostatic effect is not very good [22] too. It has been found that improving the surface hydrophilicity of chitosan can generate accommodated sites on the surface for protein adsorption [23, 24]. Therefore, chemical modification of chitosan becomes a feasible way to improve its surface properties for protein adsorption.

Chemical modification of chitosan can change its structures and physicochemical properties [25], e.g., biocompatibility, adsorption capacity, and electrostatic properties [14, 26, 27], thus extending its applications in the biopharmaceutical industry. Shi et al. [9] took polysaccharide to synthesize a cation exchanger, similar to chitosan. Saxena et al. [24] studied the chitosan/silica composite media as a membrane used in protein separation [28]. Due to its swollen property, chitosan could easily lose its physical structure, thus making it not a good candidate as the membrane separation media. As an amino polysaccharide, chitosan is a promising hydrophilic material. Therefore, considering its hydrophilic nature and high affinity for water, chitosan-based material could have great potentials in protein separation and purification [23, 24, 29, 30].

In this paper, sulfonated chitosan has been synthesized through a well-controlled process and then characterized by FT-IR, XRD, SEM, DSC, and TGA. Its adsorption properties to proteins have been studied by using bovine serum albumin (BSA).
2. Experimental

2.1. Materials. Chitosan with a degree of deacetylation $\geq 95\%$ was purchased from Shanghai Aladdin; bovine hemoglobin, bovine serum albumin (BSA), and the sulfonating agent-98% sulfuric acid, and chlorosulfonic acid were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai). All other chemicals and reagents are of analytical grade.

2.2. Method

2.2.1. Synthesis of Sulfonated Chitosan. Chitosan was dissolved in a diluted acetic acid solution and placed in a three-necked flask. Concentrated sulfuric acid (98%) was added into the chitosan solution dropwise with the water bath at $50^\circ$C, and the temperature was maintained for 3 hours. After the reaction was completed, the solution was poured into anhydrous ethanol at $5\%$ and placed overnight. Then, the solution was neutralized with ammonia, and white precipitate was obtained at pH = 7. The precipitate was washed with acetone and methanol sequentially and finally dried at $60^\circ$C afterwards.

2.3. Characterization

2.3.1. Infrared Spectroscopy (FTIR). FTIR spectra were recorded on a Perkin Elmer model 1600 IR spectrometer (Nicololi, USA), operating from 4000 to 400 cm$^{-1}$, in a resolution of 4 cm$^{-1}$, obtained after cumulating of 64 scans.

2.3.2. Wide Angle X-Ray Scattering. X-ray diffraction was performed by a Rigaku 18 KW Rotating Anode X-ray Generator Xray. Diffractometer was used to investigate the solid-state morphology of sulfonated chitosan in film form (Cermet X-ray tubes, power 1200w (tube voltage 40 kV, tube current 30 mA)). X-rays with a wavelength of 1.5406Å were generated using a CuKa source. The angles were chosen as the starting angle 5° and the ending angle 50°.

2.3.3. Thermal Gravimetric Analysis. TGA was performed on the HCT-1 microcomputer differential thermobalance (Beijing) at a heating rate of $10^\circ$/min under a nitrogen flow rate of 100 mL/min.

2.3.4. Differential Scanning Calorimetry. Integrated Thermal Analyzer was utilized to test the effect of heating on chitosan and sulfonated chitosan. The instrument used for this study is DSC200-F3 (German NETZSCH).

2.3.5. Scanning Electronic Microscopy. SEM was used to examine surface morphologies of chitosan sulfate. JSM-IT100 scanning electron microscope (Japan Electronics Corporation) with the magnification 50x–300,000x and Tungsten filament zoom spotlight is used in this study.

2.3.6. Specific Surface Area and Pore Size Analysis. Surface and pore information were obtained by using Automated Adsorption Instrument of Autosor-IQ-C-TCD (Quantachrome Corporation). Specific surface area and pore size analysis (BET) was tested at room temperature, through the nitrogen adsorption and desorption.

2.3.7. Protein Adsorption. The binding properties (capacity) of bovine serum albumin (BSA) onto sulfonated chitosan and chitosan in citric acid buffer were investigated, respectively. Experiments were performed in citric acid buffer at pH = 3. BSA was added into accurately weighed 7 parts of 0.5 g of sulfonated chitosan and chitosan in an Erlenmeyer flask and put them into a shaker thermostat at a temperature of $25^\circ$C for 24 hours. The binding behavior of BSA and the adsorption amount was measured by an ultraviolet spectrophotometer.

3. Results and Discussion

3.1. Characterization of Sulfonated Chitosan

3.1.1. FTIR. Chitosan and sulfonated chitosan were characterized by Fourier Transform Infrared Spectrometer. Figure 1 shows FTIR spectra of both chitosan and sulfonated chitosan.

It can be seen from Figure 1 that there existed the characteristic bands of the chitosan in the spectrum, including characteristic broad peaks at 3300-3400 cm$^{-1}$ which corresponds to the hydrophilic groups in Chitosan, such as -OH and -NH2, demonstrating the strong hydrogen interactions, and at 2850 cm$^{-1}$ which corresponds to the stretching vibration of -CH2-, and also the absorption peaks at 1650 cm$^{-1}$ and 1600 cm$^{-1}$ which correspond to the stretching vibration of -NH2 group. The peak of 1470 cm$^{-1}$ is the deformation vibration of -CH2- and the peak of 1422 cm$^{-1}$ is the associative secondary alcohol -OH deformation vibration, and also, the peak of 1345 cm$^{-1}$ is the group -CH deformation vibration, and the peak at 1030 cm$^{-1}$ is the primary alcohol C-O stretching vibration. The above results are consistent with the structure of chitosan.

Figure 1 also presented the FTIR spectra of sulfonated chitosan. It can be shown from Figure 1 that many characteristic peaks for chitosan sulfate existed, including the peak of 691 cm$^{-1}$ which corresponds to N-SO3, the strong absorption peak between 800 cm$^{-1}$ and 813 cm$^{-1}$ which correspond to the characteristic peaks of sulfate-based O-S and the symmetrical vibration of C-O-S, the weak peak of around 915 cm$^{-1}$ which corresponds to para-sulfamoyl, the peak at about 1034 cm$^{-1}$ which corresponds to O-S-O stretching vibration, the peak at 1180-1270 cm$^{-1}$ which corresponds to S=O asymmetrical stretching vibration in SO3$^-$ group, the peak at 1385 cm$^{-1}$ which corresponds to the asymmetric stretching vibration absorption and symmetrical stretching vibration absorption of sulfate, the peak at 1419 cm$^{-1}$ which corresponds to N-SO3, the peak at 1525 cm$^{-1}$ which corresponds to the bending vibration of C-N-C, and the peak at 1670 cm$^{-1}$ (a weak peak) which corresponds to the bending vibration absorption of the NH-SO3H group. All the above results reveal the presence of sulfonic groups in the modified chitosan.

Comparing FTIR spectra between chitosan and chitosan sulfate, it can be seen that the absorption peaks of chitosan sulfate at 3400 cm$^{-1}$ and 1422 cm$^{-1}$ are obviously weakened, and the disappearance of the absorption peak at 1030 cm$^{-1}$ indicated that the primary -OH group is involved in the sulfonation reaction. The weakening of the absorption peak at
Figure 1: Continued.
2850 cm\(^{-1}\) is due to the degradation of the chitosan backbone. In addition, the FTIR spectrum of sulfonated chitosan showed numerous characteristic peaks of sulfonic acid groups, as discussed in the above paragraph. Altogether, these results demonstrate that the sulfonation reaction of chitosan was well controlled.

### 3.1.2. XRD Results

XRD patterns of chitosan and sulfonated chitosan are shown in Figure 2. It can be seen from Figure 2 that chitosan shows two reflections at \(2\theta = 20^\circ\) and \(2\theta = 12.5^\circ\), which correspond to crystal form II and I (Samuels, 1981). The sulfonated chitosan has the peak intensity decreased at the position of \(2\theta = 20^\circ\), and the peak for chitosan at \(2\theta = 12.5^\circ\) has been shifted to \(2\theta = 15^\circ\) for sulfonated chitosan, with deceased peak intensity, too. These results show that the sulfonation has reduced the capability to form a hydrogen bond in chitosan sulfate than those in chitosan, thus causing the decrease of the crystallinity of both chitosan crystal form II and form I. In addition, it cannot be ignored that there exist many unassigned peaks on the XRD pattern of sulfonated chitosan, which may be caused by the perturbations during XRD experimental process.

### 3.1.3. Thermal Analysis

Figure 3(a) shows the thermogravimetric analysis (TGA) thermograms of chitosan and sulfonated chitosan. It can be seen from Figure 3(a) that the degradation of chitosan is divided into two stages, and the degradation of sulfonated chitosan is divided into three stages. The degradation process of chitosan is divided into the dehydration stage (25-100°C) and the main chain degradation stage (250-500°C), while the degradation process of sulfonated chitosan is divided into three stages: the dehydration stage (25-100°C), the sulfuric acid group degradation stage (100-250°C), and the main chain degradation stage (250-500°C). Therefore, these results clearly show that the sulfonic group has been successfully incorporated into chitosan sulfate.

Figure 3(b) shows the differential thermal analysis (DTA) thermograms of both chitosan and sulfonated chitosan. It can be seen from Figure 3(b) that the DTA thermograms of the two materials are quite different. The endotherm of chitosan at 100°C is due to the evaporation of water of crystallization. A strong exotherm peak at about 300°C is due to the decomposition of amine groups (GlcN, see Figure 3(c)) in chitosan [31]. The DTA results of chitosan sulfate show that
at a temperature of 100°C, the oscillation amplitude for sulfonated chitosan is less than that of chitosan, which indicates that the absorbed crystallization heat of sulfonated chitosan is significantly less than that of chitosan. Also, strong endothermic peaks appear at 325°C and 400°C, respectively, which correspond to the two major degradations of the materials.

3.1.4. SEM. Figure 4 shows the SEM micrographs of both chitosan and sulfonated chitosan. It can be seen from Figure 4 that the surface of chitosan (Figure 4(a)) itself is porous and has a large specific surface area, and the surface of sulfonated chitosan (Figure 4(b)) is more rough and more porous than those of chitosan, which due to the sulfonic group to reduce the cross-linking density, thus increasing the capacity to holding water.

3.1.5. BET. The specific surface area of the sulfonated chitosan was obtained by adsorption and desorption measurements based on nitrogen adsorption by BET and Langmuir methods, as is shown in Figure 5. It can be found that the specific surface area obtained by the BET method is 17.20123 m²/g, whereas the specific surface area obtained by the Langmuir method is 25.26701 m²/g. Since the Langmuir method assumes a single layer adsorption, there is a certain gap between the actual adsorption conditions of the material and the hypothesis, so the BET method could be more accurate to reflect the actual specific surface area. If it is protein adsorption, considering the single layer adsorption, the Langmuir method can better represent the actual surface area. In addition, the Langmuir adsorption isotherm of the sulfonated chitosan is a type III isotherm. The amount of adsorption in the low-pressure zone is relatively small, and the forces between sulfonated chitosan and nitrogen are relatively weak.

From Figure 6, it has been shown that the majority of pores are within the ten nanometers, which is the suitable size for protein transportation and adsorption onto the internal surfaces of sulfonated chitosan. It can be also shown from Figure 6 that the distribution of the pore size is mainly limited in the range of micropores and mesopores, which is adaptable to general protein size to provide channels for protein intraparticle transportation and further adsorption onto intraparticle internal surfaces. Moreover, these intraparticle pores increase the pore volume for the ion exchange ligands to be located inside, thus further improving protein adsorption onto the ligands. Therefore, this new sulfonated chitosan material could have great potential for applications in protein adsorption.

3.1.6. Optimization of Reaction Conditions. It should be noted that there exist optimal reaction conditions to control the degree of sulfonation of chitosan. The conditions include sulfonating reagent, sulfonation auxiliary reagent, neutralizing reagent, reaction time, and reaction temperature. By changing these conditions, the degree of sulfonation of chitosan can be well controlled, and these conditions could be called optimal reaction conditions. This work has tremendous experiments, and it is suitable to be depicted in other place.

3.2. Binding Activity. Macromolecular interaction between polysaccharides and proteins could always be observed through the phenomena of flocculation formation in the
Figure 3: Continued.
solutions. In this case, the binding behaviors of BSA with both sulfonated chitosan and native chitosan were examined. It was clearly shown in Figure 7 that the binding amounts of BSA onto sulfonated chitosan and onto chitosan are quite different. The enhancement of protein binding is originated from the sulfonation of chitosan. The possible mechanism is that the introduction of sulfate groups on chitosan chains increases the negative charges, and BSA, as a polyelectrolyte, exhibits partial charges on multiple points on protein structures, which enhance the capability to interact with the sulfate group, thus improving its binding capacity onto sulfonated chitosan.

Figure 3: (a) TGA and (b) DTA thermograms of chitosan and sulfonated chitosan. (c) Structural formula for GlcN and GlcNAc.

Figure 4: Scanning electron micrographs of chitosan (left) and sulfonated chitosan (right).

Figure 5: Specific surface area measured by BET method and Langmuir method.
4. Conclusions

Sulfonated chitosan was prepared and well characterized by FTIR, TGA/DTA, XRD, SEM, and BET/Langmuir. The results show that sulfonic groups have been successfully incorporated into chitosan. The ionic exchange adsorption properties of the prepared chitosan sulfate to BSA show remarkable improvements than those of chitosan. Sulfonated chitosan has great potential to be used in the ion-exchange separation process of protein purification as a new chromatography media.

![Figure 6: Distribution of pore size and pore volume.](image1)

![Figure 7: The binding capacity of BSA on sulfonated chitosan and chitosan.](image2)
Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interests.

Acknowledgments

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