



Flexible Electrospun Cellulose Fibers as an Affinity Packing Material for the Separation of Bovine Serum Albumin

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Abstract

Flexible, well-dispersed and continuous 100-nm diameter cellulose fibers were prepared from an ionic liquid solvent by a novel dry-jet wet-electro spinning process. The ribbon fibers formed were chemically activated and an affinity dye, cibacron blue (CB) was immobilized at 0.22 g CB/g dry fibers loading to the surface of these fibers. The resulting affinity matrix was packed into a chromatography column and the adsorption, desorption and specificity of this matrix for bovine serum albumin (BSA) was studied. These electrospun fibers had a BSA binding capacity of 230 mg/g, nearly twice that of CB-immobilized 100- μ m beads and over ten-fold higher capacity than CB-immobilized cellulose fibers prepared by a conventional electrospinning process. The results of this work suggest that chromatography supports of flexible, well-dispersed, continuous nanofibers may offer advantages over conventional supports in affinity separations.

Keywords: Cellulose; Electrospinning; Fibers; Affinity separation; Chromatography; Ionic liquid

Introduction

The rapid development of biotechnology and biomedicine requires more reliable and efficient separation technologies for the isolation and purification of biopolymers such as therapeutic proteins, antibodies, enzymes and nucleic acids. In particular, monoclonal antibodies are centrally important as therapeutics for the treatment of cancer and other diseases, leading to recombinant monoclonal antibodies that dominate today's biopharmaceutical pipeline [1,2]. The large-scale production of therapeutic biopolymers requires a manufacturing process that delivers reliability and in high-yield, as well as an effective purification process affording extremely pure products. Because of its high selectivity, affinity chromatography has been used extensively to isolate a variety of biopolymers. The retention of solutes is based on specific, reversible interactions found in biological systems, such as the binding of an enzyme with an inhibitor or an antibody with an antigen. These interactions are exploited in affinity chromatography by immobilizing an affinity ligand onto a support, and using this as a stationary phase. Traditional affinity chromatography relies on a column packed with gel beads. This approach has certain limitations including a high-pressure drop and low flow rates that lead to low productivities and difficulties in efficient scale-up.

Affinity separation membranes, consisting of electrospun nanofibers, have been developed recently. Affinity ligands are attached to the surface of the constituent fibers, offering a potential solution to some of the problems of traditional, column-based, affinity chromatography [3]. Electrospun fibers are good candidates for use in affinity separation because of their unique characteristics of high surface area to volume ratio, resulting in high ligand loading, and their large porosity, resulting in high throughput operation. A number of polymers have been used for electrospun fiber mesh-based affinity membrane separations including poly (ether-urethane-urea) [4], cellulose [5-7], poly(ethylene terephthalate)[8], polysulphone [9,10]

and polyacrylonitrile [11]. Typically, very thin electrospun fiber meshes are produced by electrostatically collecting negatively charged fibers on a collector electrode. These very thin 2D electrospun fiber mesh mats provide excellent solution permeability as compared to 3D column packed with affinity beads. However, many layers of individual thin membranes must be stacked to increase the effective area necessary for affinity separation. Such stacking also necessitates the use of O-rings to avoid leakage during the purification process.

Our laboratory has developed a new electrospinning method, which can be defined as dry-jet wet-electrospinning [12]. In this work a non-volatile imidazolium-based ionic liquid (IL) that acts as a versatile solvent for a variety of biomaterials, including unmodified cellulose, is used as an electrospinning solvent. Electrospun polymer fibers are collected in a coagulation bath containing water and/or alcohol that acts as a co-solvent for the IL and an anti-solvent for the biomaterial solutes, removing the IL, and coagulating the electrospun fiber. In this process, continuous and flexible fibers are obtained. The resulting flexible fibers formed in the coagulation bath can be subsequently packed homogeneously into any kind of spin column, chromatography column, or high-performance liquid chromatography (HPLC) column without special equipment. Since these are continuous fibers, they can

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easily be removed from these columns for cleaning, regeneration, or storage after use in a column-based separation.

The aim of the current work is to prepare wet-electrospun cellulose fibers for use in an affinity separation. Cellulose is an excellent affinity matrix because it is inexpensive, biorenewable, physically strong, chemically resistant and highly biocompatible, allowing for separation of important biopolymers with minimal loss in biopolymer activity [13]. Hydrophilic cellulose, also shows low nonspecific protein adsorption, making it more suitable for protein purification than many traditional synthetic polymers. In the current study, electrospun cellulose fibers were modified with a model group-specific affinity ligand, Cibacron Blue F3GA (CB). CB immobilized cellulose fibers, packed into a column were used to investigate bovine serum albumin (BSA) adsorption/desorption properties.

Experimental

Electrospinning of cellulose

Cellulose (degree of polymerization 1100) 5% (w/w) in 1-ethyl-3-methylimidazolium acetate ([EMIM][OAc]) (Purchased as Cellionic™, Sigma-Aldrich, St. Louis, Missouri, USA) was diluted to 1.75% (w/w) cellulose with neat [EMIM][OAc] (Sigma-Aldrich). The mixture was then mechanically stirred using a magnetic stir bar (Fisher Scientific, Pittsburgh, Pennsylvania, USA) at 80°C in a temperature controlled oil bath until it formed a homogeneous solution.

This solution was loaded into a 5-mL syringe equipped with a 0.94 mm (inner diameter) stainless steel needle. The needle was connected to a high voltage power supply (CZE1000R, Spellman, Hauppauge, New York, USA), which is capable of generating a DC voltage up to 30 kV. A flat piece of aluminum foil (20 μm thick) was grounded and placed as the counter electrode on the bottom of a coagulation bath filled with water to remove IL and solidifying the fibers. The distance between needle tip and the aluminum collector was 9 cm. The electrospinning solution was fed at a constant rate using a mechanical syringe pump (NE-1000, New Era Pump System Inc., Wantagh, New York, USA) at 90 μL/min. The applied voltage was optimized to obtain good spinnability and continuous fibers, with a typical value of 18-19 kV. Fibers collected in the coagulation bath formed an entangled web of flexible fibers.

Cibacron Blue F3GA binding on fiber surface

Cibacron Blue F3GA (CB, Sigma-Aldrich) was covalently immobilized onto the surface of the electrospun cellulose fibers by a nucleophilic reaction between the chloride in the triazine rings of the CB and the hydroxyl groups of cellulose molecules under mild alkaline condition [14]. Conditions were optimized to enhance the CB loading as follows. CB was dissolved in 2 mL of distilled water (15 mg/mL), and then heated to 60°C in a water bath. Electrospun cellulose fibers (about 1 mg/mL) that had been collected in water were vacuum filtered to remove water, and then added into the CB solution. NaCl (220 mg/mL) was added into the reaction mixture and the temperature was maintained at 60°C for 1 h. The temperature of the reaction mixture was then increased to 80°C and 25 mg/mL Na₂CO₃ was added. The reaction then continued for 3 h at 80°C. The modified cellulose fiber was extensively washed with distilled water until the supernatant showed no optical adsorption at 600 nm, indicating that all un-reacted CB had been removed from solution. The modified cellulose fiber was freeze-dried and then precisely weighed to evaluate the CB capacity on cellulose fiber. The freeze-dried fiber was immersed in 2 mL distilled water with aqueous cellulase enzyme solution (700 U/g, Sigma-

Aldrich), which degrades cellulose to an oligomer. The solution was placed in a shaker at 37°C overnight. After degradation of the cellulose fibers in solution, the quantity of immobilized CB was measured by optical adsorption at 600 nm. The CB capacity (q) in mg/g of cellulose fiber was calculated by Eq. 1:

$$q = w / (W - w) \quad (1)$$

where w is the mass of CB immobilized on the cellulose fibers, and W is the total (dry) mass of CB immobilized cellulose fiber.

CB was immobilized onto cellulose beads with an average diameter of ~100μm under identical reaction conditions as a control. The CB capacity in mg/g of cellulose beads was also measured by the same procedure as described above.

Morphological characterization

Field emission scanning electron microscopy (FE-SEM) was performed with a JEOL JSM-6332 FE-SEM (Tachikawa, Tokyo, Japan) equipped with a secondary electron detector at an accelerating voltage of 10 kV to study the surface morphology of the fibers. Prior to performing the FE-SEM analysis, fibers were sputter coated with gold to form a conductive film with a few nanometer thicknesses.

Equilibrium BSA adsorption test

Bovine serum albumin (BSA) was selected as a model protein to investigate the adsorption properties of the CB immobilized cellulose fibers. BSA adsorption capacities of the CB immobilized cellulose fibers were measured batch-wise at various equilibrium BSA concentrations. Two milligrams of CB immobilized cellulose fiber was immersed in 1 mL of BSA solution (0.2 mg/mL in phosphate buffered saline (PBS), pH 7.4). The solution was placed in a shaker at 37°C for 6 h. The adsorption capacity (q, mg/g fiber) was determined with Eq. 2:

$$q = [(C_i - C_f) * V] / m \quad (2)$$

where C_i and C_f are BSA concentration in the initial and final solution (mg/mL); V is the volume (mL); and m is the dry mass in grams of CB immobilized cellulose fiber. BSA concentration was determined by the bicinchoninic acid (BCA) method.

Dynamic BSA adsorption test

CB immobilized cellulose fiber (20 mg) and pristine cellulose fiber (20 mg) were introduced into a glass column (10 cm long, 1 cm inner diameter, Bio Rad, Hercules, California, USA), respectively. Packing height was fixed at 1 cm using a flow adaptor (Bio Rad). The resulting column was washed extensively with PBS (pH 7.4). BSA solution (10 mL of 0.2 mg/mL) was loaded into a 10-mL plastic syringe and fed into the column at a flow rate of 10 mL/h through the flow adaptor using an NE-1000 syringe pump. The BSA concentration at the column outlet was measured at different elution volumes. When feeding of 10 mL of BSA solution was finished, the column was washed with 10-mL of PBS to remove non-adsorbed BSA in the column. Then elution was performed using 10-mL of PBS containing 1.5 M NaCl (pH 10) as an elution buffer. After the elution, the CB immobilized fibers were completely regenerated within the column by washing with the elution buffer followed by PBS. Then reusability of these fibers was evaluated by following the procedure described above in an iterative process. CB immobilized cellulose beads and pristine cellulose beads were evaluated in a same procedure except the packing height was changed to 0.5 cm.

Results and Discussion

Electrospinning of cellulose fiber using RTIL

Some room temperature ionic liquids (RTILs) such as 1-butyl-3-methylimidazolium chloride ([BMIM][Cl]) [12], 1-allyl-3-methylimidazolium chloride ([AMIM][Cl]) [15] and 1-Ethyl-3-methylimidazolium acetate ([EMIM][OAc]) [16], have been previously reported as solvents for electrospinning unmodified cellulose. In this study [EMIM][OAc], having a lower viscosity and higher conductivity than either [BMIM][Cl] or [AMIM][Cl], was used as a solvent for unmodified cellulose in order to obtain small diameter fibers. Unmodified cellulose can be directly regenerated into nanoscale fibers through dry-jet wet electrospinning using ILs. This fiber fabrication process is much simpler than the electrospinning of cellulose acetate followed by alkaline hydrolysis [5-7,17]. In dry-jet wet electrospinning, a coagulation bath containing co-solvent for the IL such as water or alcohol must be used to remove the non-volatile IL. When water was used as a coagulation solvent, ribbon fiber morphology was observed, as shown in (Figure 1a, b). This is due to the relatively slow IL extraction speed of water. In the process of IL extraction, the fiber morphology slowly changes from cylindrical form into ribbon form. When a mixture of water and ethanol (50:50 w/w) was used cylindrical fiber morphology was observed (Figure 1d), since water/ethanol mixtures have higher IL extraction speeds than water alone. Ribbon fibers have higher specific surface areas as compared to that of cylindrical fibers, therefore the binding capacity of ribbon fibers for CB are expected to be higher than that cylindrical fibers. For this reason ribbon fibers collected from a water bath were used in subsequent experiments.

CB immobilization

Reaction parameters, CB concentration, NaCl concentration and Na_2CO_3 concentration in reaction solution were optimized to maximize the amount of CB immobilized. The content of immobilized CB on electrospun cellulose fiber was increased with increasing CB concentrations. If no NaCl was added in reaction solution no CB was immobilized. This is believed to be due to the repulsive interaction between cellulose fiber and CB since both surfaces are negatively charged. The effect of reaction solution pH was evaluated by changing the Na_2CO_3 concentration in the solution. The amount of CB immobilized increased with increasing the Na_2CO_3 concentration reaching a maximum at 0.02 mg/mL Na_2CO_3 . The final optimized reaction parameters were 0.015 mg/mL CB, 0.220 mg/mL NaCl and 0.020 mg/mL Na_2CO_3 .

An SEM image of CB immobilized fiber is shown in (Figure 1c). There was no obvious morphological change caused by CB immobilization. CB immobilized fibers were next degraded by reaction with cellulase, and then the content of CB on the electrospun cellulose fibers was evaluated. The maximum CB content on electrospun cellulose fiber prepared using optimized reaction parameters was 0.22 g CB/g dry fiber. This value was considerably higher by than 0.09 g/g on cellulose beads (~100- μm diameter) prepared using the same optimized reaction conditions. This higher CB content on electrospun fibers is certainly due to higher specific surface area than that of cellulose beads. It is well known that cellulose materials swell to a certain extent in aqueous solution and then form gel layer. While the specific surface area of cellulose under dry conditions can be measured by Brunauer-Emmett-Teller (BET) isotherm analysis, it is difficult to make the same measurement of specific surface area under wet conditions. Optical microscopic images of cellulose fiber and cellulose beads in

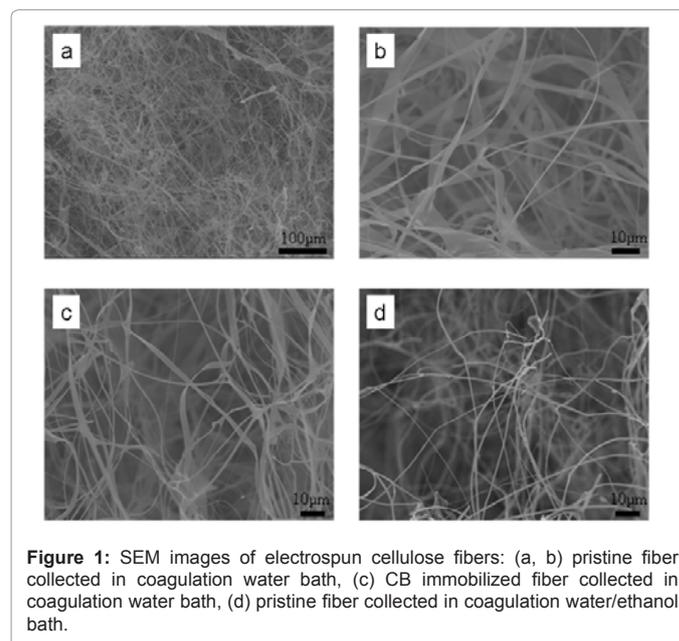


Figure 1: SEM images of electrospun cellulose fibers: (a, b) pristine fiber collected in coagulation water bath, (c) CB immobilized fiber collected in coagulation water bath, (d) pristine fiber collected in coagulation water/ethanol bath.

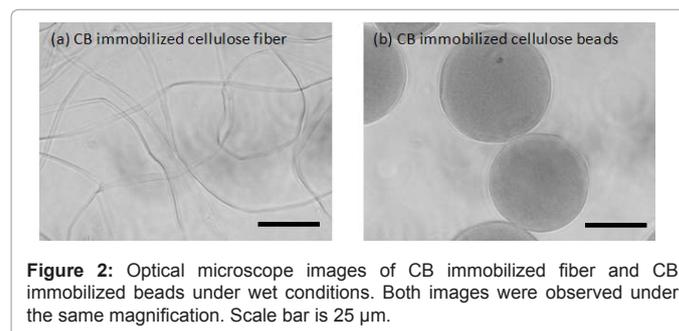


Figure 2: Optical microscope images of CB immobilized fiber and CB immobilized beads under wet conditions. Both images were observed under the same magnification. Scale bar is 25 μm .

wet conditions are shown in (Figure 2). The specific surface area of electrospun cellulose fibers having small diameters is considerably higher than that of cellulose beads.

Static adsorption of BSA

BSA was selected as a model protein to investigate the adsorption properties of CB immobilized cellulose fibers. Albumin, which has many important physiological functions, is the most abundant protein in blood plasma. Research on albumin separation has attracted significant attention because of its importance in blood protein manufacture. First, the adsorption rate of BSA on CB immobilized fibers and beads were studied at an initial BSA concentration of 1 mg/mL (Figure 3a). High adsorption rates were observed initially and saturation values were then gradually achieved in about 4h. Adsorption isotherms of BSA were then evaluated at fixed adsorption time, 6h (Figure 3b). Note that the major requirement in affinity chromatography is the specificity of the adsorbent [18]. Non-specific interactions between the carrier matrix and the molecules to be adsorbed must be kept at a minimum to have high specificity. As seen in (Figure 3b), non-specific BSA adsorption on pristine cellulose fiber was only 7.8 mg/g, while CB immobilization significantly increased BSA adsorption capacity of the fiber (233 mg BSA per g fiber). The non-specific BSA adsorption on pristine cellulose beads was 7.5 mg/g, which is close to the value observed for pristine cellulose fibers. BSA adsorption capacity was

saturated at higher equilibrium BSA concentration, which represents saturation of the active adsorption sites on CB immobilized fiber.

This adsorption behavior can be expressed using a conventional Langmuir isotherm (Eq. 3),

$$Q = (q_{\max} C)/(K_d + C) \quad (3)$$

This can be transformed into a linear form (Eq. 4),

$$C/q = C/q_{\max} + K_d/q_{\max} \quad (4)$$

Where, C (mg/mL) is the equilibrium concentration of BSA in solution, q (mg/g) is the adsorption capacity, q_{\max} (mg/g) is the maximum adsorption capacity and K_d is the effective dissociation constant. The solid lines shown in (Figure 3b) were calculated using the Langmuir equation. When the equilibrium BSA concentration was increased, BSA adsorption capacity gradually saturated. The equilibrium BSA concentration was calculated when BSA adsorption capacity was 95 % saturated. The maximum adsorption capacity (saturated) of CB immobilized fiber, 233 mg/g, which is much higher than that observed for CB immobilized beads, 111 mg/g. The equilibrium BSA concentration is 8 mg/mL for CB immobilized fibers at 95% saturation

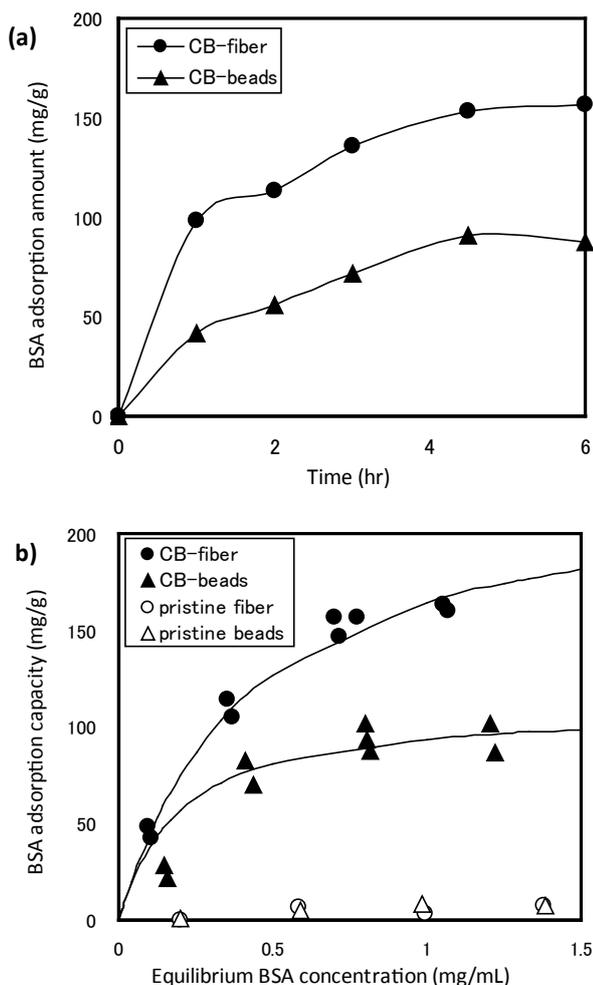


Figure 3: BSA adsorption properties on CB immobilized fiber, CB immobilized beads, pristine fiber and pristine beads: (a) at different adsorption times, (b) at various BSA concentrations.

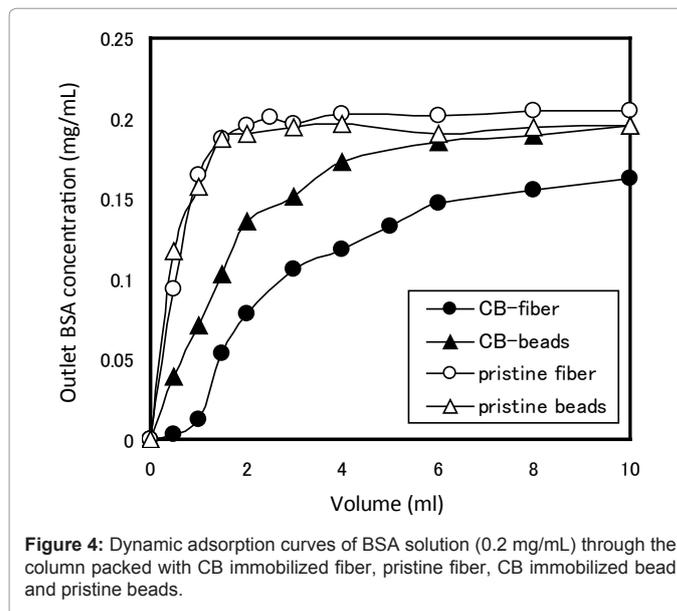


Figure 4: Dynamic adsorption curves of BSA solution (0.2 mg/mL) through the column packed with CB immobilized fiber, pristine fiber, CB immobilized bead and pristine beads.

(221 mg/g) and 3.6 mg/mL for CB immobilized beads at 95% saturation (106 mg/g). The higher BSA adsorption capacity of fiber is primarily due to the higher CB content. This value is also much higher than the 13 mg/g determined for an electrospun cellulose fiber mat reported by Ma et al. [5]. This cellulose fiber was derived from electrospun cellulose acetate, the resulting saponified cellulose fibers were densely collected and adhered to one another and the CB content on their cellulose fiber was considerably lower [15]. The improvement of BSA adsorption capacity in fibers prepared through our dry-jet wet-electrospinning method resulted primarily from the flexible and loose fiber, network providing efficient interaction between BSA and CB molecules, and to a lesser extent from their higher CB content. The dense and adherent fiber structure in the conventional electrospun fiber mat results in less surface area and makes it more difficult for BSA to reach CB molecules on the fiber. By contrast, our flexible and loose fiber would provide efficient interaction between BSA and CB molecules easily.

Dynamic adsorption of BSAs

The dynamic adsorption properties of CB immobilized fibers, pristine fibers, CB immobilized beads and pristine beads were investigated (Figure 4). A controlled weight of packing material was used in these experiments as an alternative to regulating the bed height. Non-specific adsorption on pristine fibers will also result in lower outlet BSA concentration than inlet BSA concentration. However, due to very low non-specific adsorption capacity of pristine fibers, BSA quickly reaches saturation on the pristine fiber surface and flows through without further adsorption. Thus, the outlet BSA concentration becomes same as feed concentration of 0.2 mg/mL. The non-specific adsorption capacity of pristine fibers and pristine beads can be calculated by same method as the maximum BSA adsorption capacity of CB immobilized fibers from Eq. 3, and we assume the non-specific adsorption follows a Langmuir isotherm. BSA non-specific adsorption is much lower on the pristine fiber than on pristine beads (Figure 4).

The CB immobilized fiber showed a gradual increase in the outlet BSA concentration that failed to reach the feed concentration over the 10 mL feed volume. The lower BSA outlet concentration observed for the CB immobilized fiber is due to the specific BSA adsorption by the

CB molecule which was evidenced by PBS wash after BSA adsorption to remove non-specific adsorption.

Dynamic desorption of BSA

After the BSA adsorption test, the column was washed with 10 mL PBS to remove non-adsorbed BSA. Then PBS containing 1.5 M NaCl (pH 10) was fed into column to elute captured BSA on CB immobilized fiber and beads. (Figure 5) shows desorption curves for the CB immobilized fiber and beads. Very sharp elution of highly concentrated BSA was obtained from CB immobilized fiber as compared with that of the CB immobilized beads. The maximum outlet BSA concentration from the column packed with CB immobilized fiber was 0.47 mg/mL at 1.5 mL feed volume of the eluent. This represents an approximately 2.5-fold concentration of BSA solution in the CB-beads column eluent. Therefore, the higher adsorption capacity results in higher desorption concentration as shown in (Figure 4 and Figure 5). This column should afforded excellent BSA purification from the mixture with other proteins and contaminant [19].

Reusability and stability

After the BSA desorption study, the column was washed with additional 20 mL elution buffer without removing fibers from column to completely elute captured BSA. Then PBS was fed into column to replace the elution buffer, resulting in a regenerated column, which was then reused for another dynamic BSA adsorption experiment. This cycle was repeated three times to evaluate the reusability and stability of the column. Each adsorption behavior was similar (Figure 6a). However, there were significant differences in desorption behavior, although the elution volume of maximum desorption concentration was same for all three cycles (Figure 6b). The maximum elution concentrations were 0.47 mg/mL, 0.34 mg/mL and 0.41 mg/mL in the first, second and third cycles, respectively. The recovery for 1st and 3rd cycle of adsorption and desorption was >99%, and recovery for 2nd cycle of adsorption and desorption was 81%, as calculated from (Figure 6). The variability in recovery is likely caused by loose packing of fibers in the column, which affords a low-pressure drop. The fiber packing conditions were then changed and the experiment was repeated. The solution permeation was homogeneous to obtain optimal adsorption. Further studies on packing methods are required to improve packing homogeneity, packing amount and packing density, and better control

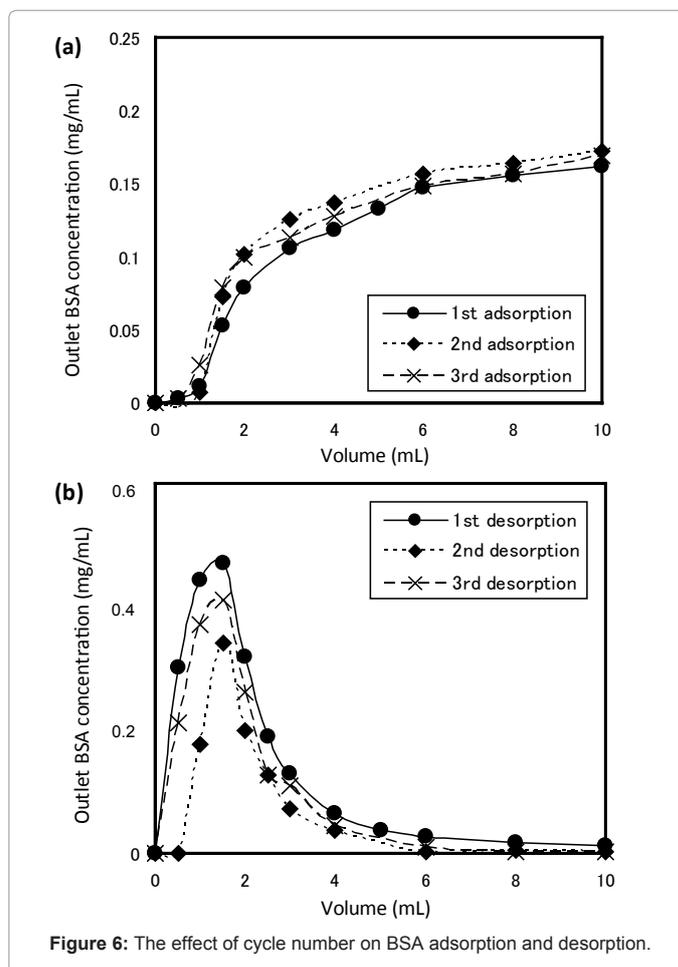


Figure 6: The effect of cycle number on BSA adsorption and desorption.

of solution feed rate and BSA concentration are required to optimize adsorption/desorption behavior.

Pressure drop at low flow rates

As a demonstration experiment, a height adjustable HPLC column (Diba, Danbury, CT, USA) was packed with 0.5 cm height and 0.66 cm inner diameter of CB immobilized cellulose fibers, then the column was mounted on AKTA HPLC to test pressure drop at several flow rates. At flow rate of 0, 1, 2, 3, 4 and 5 mL/min, the pressures were recorded as 0.12, 0.18, 0.22, 0.26, 0.29 and 0.33 MPa, respectively.

Conclusions

A flexible and continuous cellulose fiber was prepared by dry-jet wet electrospinning using room temperature ionic liquids. Cellulose fibers collected in a coagulation water bath showed ribbon morphology with a high specific surface area. The resulting fiber was then chemically activated and an affinity dye ligand, CB, was coupled to the fiber surface. CB immobilized fiber showed an affinity adsorption capacity for BSA as high as 233 mg/g with little non-specific adsorption. CB immobilized fibers, used as an affinity chromatography packing, showed good adsorption behavior for 0.2 mg/mL BSA solution as compared with a column packed with CB immobilized beads. BSA captured on CB immobilized fiber could be eluted with 1.5 M NaCl solution to give a five-fold higher eluant. These characteristics indicate that while flexible and continuous cellulose fibers prepared by dry-jet wet-electrospinning represents a promising packing material for

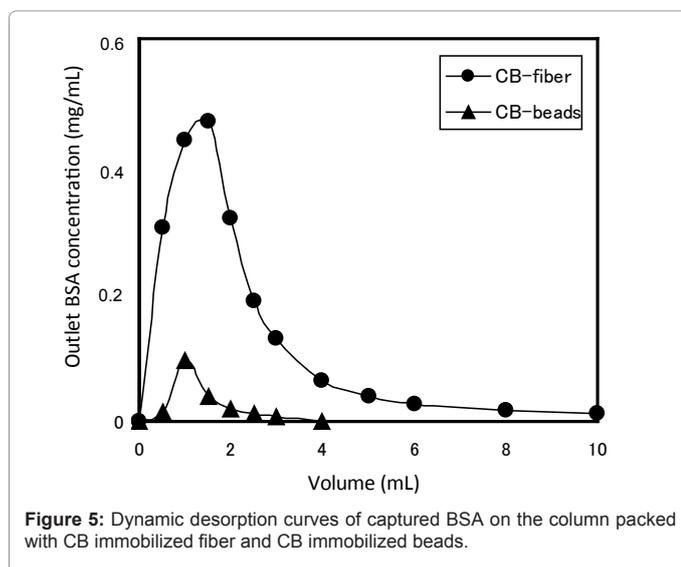


Figure 5: Dynamic desorption curves of captured BSA on the column packed with CB immobilized fiber and CB immobilized beads.

affinity chromatography, additional studies will be required to establish the manufacturability and scalability of these materials. Flexible fibers can also be fabricated from many kinds of polymers, since this method can be applied to conventional electrospinning using volatile solvents.

Acknowledgements

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