



Preparation and characterization of grafted cellulosic fibers and their applications in protein purification



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ABSTRACT

Fibrous adsorbents were subjected either to chemical treatment or gamma irradiation to introduce epoxide groups onto their cellulosic backbone using glycidyl methacrylate. These epoxide moieties were modified to have diethylaminoethanol (DEAE) as well as quaternary ammonium (Q) functionalities. The resulting anion-exchange adsorbents were characterized by their FTIR spectra and ionic capacities. The fiber-based adsorbent systems showed similar packing efficiency to the commercially available adsorbents, where the Péclet number values were ≥ 60 , suggesting near-plug-flow conditions. The total ionic capacities obtained for these chemically grafted adsorbents were *ca.* 400 mmol/L. These adsorbents showed dynamic binding capacities (DBC) of *ca.* 48 mg/mL for bovine serum albumin (BSA). Protein binding capacities obtained from chemical grafting initiation techniques were 18-fold-higher than radiation-induced techniques. The advantage of these adsorbents lies in their high operational flow rates while maintaining their high binding capacities.

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1. Introduction

Most downstream bioprocesses include several chromatographic steps amongst other traditional purification steps in order to produce highly purified bioproducts like plasmid DNA, virus-like particles, and monoclonal antibodies. With recent advancements in technology and increased demand for therapeutic biomolecules, the market value of the biopharmaceutical industry is expected to reach nearly \$166 billion USD by 2017 [1]. Consequently, there is an enormous demand for developing innovative and cost-effective bioprocessing techniques for reducing production costs, which mostly lie in downstream bioprocessing (up to 80% of the entire production cost) [2–4]. Ion-exchange chromatography is mostly preferred for downstream bioprocessing and porous resins with anion-exchange functionalities (DEAE and Q) have been extensively used for the separation and purification of biomolecules [5–9]. However, separation of biomolecules using these adsorbents is often hampered by restricted flow rates and low operating pressures [10]. Fiber-based materials show excellent physico-chemical

properties and offer several advantages, including large surface areas, high swelling capacities, mechanically robustness, and convenient usage [11]. These materials have been extensively used for the removal of metal cations [12], extraction and separation of analytes from aqueous media [13,14], and also for capturing the trace elements [15,16]. Recently, Gavara et al. [17] successfully demonstrated the potential use of cation-exchange fiber-based material for the separation and purification of biomolecules. In the current study, we examine the synthesis and performance of anion-exchange fiber-based variants. These adsorbents are easy to functionalize and offer very flexible operational conditions. Furthermore, their scale-up has previously been demonstrated up to the pilot scale without any pressure drop or bed compression issues [9,17].

Various kind of grafting techniques, like chemical, photo-initiation [18] and gamma irradiation grafting [19,20], have been used for introducing epoxy groups onto cellulosic substrates [21,22]. Graft polymerization of glycidyl methacrylate (GMA) through the chemical-initiation techniques onto substrates like nylon [23], acrylic [18], gelatin [24], polypropylene, poly (alginate acid), and cotton fabric has been reported for improving their properties for different applications [25]. In the present study, the cellulosic fiber backbone was graft-polymerized with GMA, both chemically as

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well as by gamma-irradiation. The free epoxy groups were further modified to have Q and DEAE functional moieties. The physico-chemical characteristics of these adsorbents were evaluated in terms of grafting percentages, swelling, porosity, as well as ionic capacity. The binding capacities of these adsorbents were assessed using BSA as a model protein.

2. Material and methods

2.1. Chemicals and reagents

Glycidyl methacrylate (GMA), diethylamine (DEA 99.5%), dimethylamine (40%), diethyl sulfate, ammonium cerium (IV) nitrate (CAN), *N,N*-dimethylacrylamide (DMA), and bovine serum albumin (Fraction V > 96%; Molecular Weight: 66.5 kDa; isoelectric point: 4.7) were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Acetone, acetonitrile, ammonium persulfate (APS), sodium dihydrogen phosphate, di-sodium hydrogen phosphate, sodium chloride (NaCl), sodium hydroxide (NaOH), and nitric acid (HNO₃, 65%) were from AppliChem GmbH (Darmstadt, Germany). Tris (99.3%, buffer grade) and nitromethane were purchased from Carl Roth (Karlsruhe, Germany). Alexa Fluor 488-labeled BSA was purchased from Molecular Probes, inc (Eugene, OR, USA). Ethanol absolute was purchased from Honeywell specialty chemicals Seelze GmbH (Seelze, Germany). Natural cotton was purchased from Gebrüder Otto GmbH & Co. Kg (Dietenheim).

All the buffer solutions were filtered with 0.45 μm filters (Sartorius, Goettingen, Germany).

2.2. Instrumentation

An ÄKTA explorer 100 system running on Unicorn 4.10 software and Tricorn chromatography column 5/50 (5 mm internal diameter (ID) × 55 mm length), were purchased from GE Amersham Bioscience (Uppsala, Sweden). Finite bath adsorption capacity was determined by measuring the absorbance at 280 nm using a Shimadzu UV-1700 PharmaSpec spectrophotometer. The fibrous adsorbents were scanned (averaged over 32 scans) from 4000 cm⁻¹ to 500 cm⁻¹ and analyzed with IRsolution Shimadzu 1.50 software (Shimadzu Corporation, Kyoto, Japan). Confocal microscopy images were taken using a Carl Zeiss LS510 laser scanning microscope (Jena, Germany; software version 3.0).

2.3. Adsorbent grafting and functionalization

2.3.1. Pre-treatment of the fibers (Mercerization)

The fibers were pre-treated with 30% (w/v) NaOH. The mercerization treatment was modified from the method as described in Rousselle et al. [26]. The fiber-containing solution (13 mL/g of fiber) was placed in a water bath at a constant temperature for 1.5 h. The treated fibers were washed with copious amounts of water followed by 0.1 M acetic acid, and once again with copious amount of water until neutral pH was achieved. The fibers were dried and used for further functionalization.

2.3.2. Chemical grafting (CG)

A 0.5 g of dried pretreated cotton was soaked in 33.75 mL of nitrogen-purged water containing 2.5 mL of GMA in 0.1 M nitric acid and 50 mg of CAN as an initiator for 3 h at 40 °C. The grafted material was washed with copious amount of water until neutral pH was achieved and then dried at 50 °C [27,28]. The concentration of the CAN (2–6 mM) was varied to achieve a different grafting percentage (12%, 45%, 100% and 112%) by this method.

2.3.3. Gamma irradiation (GIR) induced grafting

0.5 g of dried pretreated cotton was enclosed with 50 mL of nitrogen-purged grafting solution containing 3.2% (v/v) GMA and 7.6% (v/v) DMA in 1:1 v/v ethanol/water in a falcon tube. Gamma-irradiation was carried out at room temperature with a 10 kGy dose of gamma rays (Beta Gamma Service GmbH and Co. KG, Wiehl, Germany). After irradiation, the epoxy-grafted fibers were washed with 50% ethanol (v/v) and then with absolute ethanol and then dried at 50 °C [29].

2.3.4. Surface functionalization

Epoxide-functionalized fibrous adsorbents (0.5 g) were immersed in a 20 mL solution containing 5 mL each of diethylamine and dimethylamine and 10 mL of ethanol at 40 °C for 15 h under constant shaking to prepare weak anion-exchangers functionalized with DEAE. Subsequently, the adsorbent material was extensively washed with copious amount of water until neutral pH was achieved and then vacuum dried at 50 °C in an oven [30].

These DEAE-functionalized fibers were further alkylated into quaternary ammonium functionality using 4 mL of diethyl sulfate in 20 mL of nitromethane at 40 °C for 15 h under constant shaking [31]. Finally, the functionalized adsorbent was flushed with acetonitrile and water until a neutral pH was obtained and then dried at 50 °C in a vacuum oven.

2.3.5. Physico-chemical and functional characterization

The chemical changes involved in the backbone of dried fibrous adsorbents at each stage of functionalization were evaluated using IR Spectroscopy. The DEAE-fibers were investigated by confocal laser scanning microscopy (CLSM). Briefly, a known amount of the CG-DEAE fiber sample was incubated with Alexa Fluor 488-labeled BSA in 0.1 M phosphate buffer, pH 7.4 for 2 h at room temperature on a shaker. After incubation, the fiber samples were thoroughly washed with 0.1 M phosphate buffer, pH 7.4 and then observed directly by confocal microscopy. The obtained images were collected using the laser excitation sources at 488 nm. The percentage degree of grafting (%DG) was evaluated from the difference in weight before (W_0) and after (W_1) grafting [27] as shown in Eq. (1).

$$\%DG \text{ (g/g)} = \frac{(W_1 - W_0)}{W_0} \times 100 \quad (1)$$

Additionally, the degree of swelling (DS) for functionalized fibrous adsorbents was evaluated by immersing them in water for an hour and then weighing them in their wet (m_{wet}) and dried (m_{dry}) form till constant weight was achieved at 50 °C under vacuum. DS was measured using Eq. (2) [17].

$$DS \text{ (g/g)} = \frac{(m_{wet} - m_{dry})}{m_{dry}} \quad (2)$$

Finally, the porosity of fibrous adsorbents was estimated using Eq. (3) [17], where 1 g of adsorbent was immersed in deionized water for an hour. The swollen weight of fibers was denoted as $m_{swollen}$. The excess water present in the pores of the fibers was removed by squeezing the fibers and denoted as $m_{squeezed}$.

$$\text{Porosity (\%)} = \frac{(m_{swollen} - m_{squeezed})}{m_{swollen}} \times 100 \quad (3)$$

Finite bath adsorption measurements were performed using 10 mg/mL of BSA as a model protein. The fibrous adsorbents were first equilibrated with 20 mM phosphate buffer (pH 7.4) and then further incubated for 3 h with BSA under mild shaking. The exact amount of BSA bound to the adsorbents was determined by measuring the differences in the absorbance (A_{280}) of BSA before and after incubation.

2.4. Column efficiency, ionic capacity and protein dynamic binding capacities

The dried fibrous adsorbent (0.2 g) was packed into a Tricorn 5/55 column (1 mL – column bed volume) and equilibrated with 20 mM Tris–HCl buffer (pH 7.4) at a flow rate of ca. 75 cm/h as reported previously by Gavara et al. [17]. The height of the bed was re-adjusted to avoid any free headspace in the column. The column efficiency and plate height for a packed column was evaluated by injecting a 10 μ L pulse of 5% acetone (v/v) at various linear flow velocities (75–600 cm/h).

The ionic capacities (or phosphate capacities) of the adsorbents were calculated using the transient pH phenomenon [28,32,33]. Two buffer solutions, having the same pH but differing in their ionic strengths, were used for evaluating the phosphate-ion capacities of the adsorbents. The packed fibrous column was first equilibrated with 500 mM phosphate buffer at pH 7.4 and subsequently switched to 20 mM phosphate at pH 7.4. The buffer switch induces a change in pH as a result of the release of ions that were bound to the adsorbent under high salt conditions. The time interval, Δt ($|\Delta\text{pH}| > 0$) was determined by measuring the actual time required to achieve 50% of the maximum pH value after switching the mobile phase. The volumetric flow rate (ϕ_v), column volume (CV), the concentration of elution buffer (C_2) as well as time interval (Δt) are used for calculating phosphate capacity (K) as shown in Eq. (4) [28,32,33].

$$K = \frac{[\Delta t(\text{pH})_{50\%} \times \phi_v]}{\text{CV}} \times C_2 \quad (4)$$

The dynamic binding capacities for Q and DEAE adsorbents were determined by frontal analysis [34]. The adsorbents were packed in Tricorn 5/55 columns, mounted onto an ÄKTA explorer 100 system, and then further equilibrated by passing 20 column volumes of 20 mM Tris–HCl buffer at pH 7.4. The DBC measurements were evaluated by saturating the column with 2 mg/mL solution of BSA at linear flow velocities of 75–600 cm/h while mon-

itoring the UV absorbance at 280 nm [35]. BSA was eluted from the column using 20 mM Tris–HCl buffer containing 1 M NaCl. The dynamic binding capacity (DBC) of BSA was determined using the 10% breakthrough volume (V), column void volume (V_0), initial BSA concentration (C_0), and finally using either the dry weight of adsorbent (W_g) or its column volume (CV), as shown in Eqs. (5) and (6) [27].

$$\text{DBC (mg/g)} = \frac{C_0 \times (V - V_0)}{W_g} \quad (5)$$

$$\text{DBC (mg/mL)} = \frac{C_0 \times (V - V_0)}{\text{CV}} \quad (6)$$

3. Results and discussion

The cellulosic fiber backbone consists of repeating units of glucose, where the OH groups present on the C-2, C-3 and C-6 positions play an important role in the formation of inter- and intramolecular hydrogen bonds, making cellulose nearly 70% crystalline in nature [36]. In this paper, the cellulosic adsorbents were pretreated with concentrated sodium hydroxide to disrupt these hydrogen bonds. Mercerization of fibers with NaOH helps in the swelling of the cell wall, which in turn increases the total available surface area [37] for polymer grafting.

Frequently used grafting procedures involve the use of single vinyl-containing monomers [28,30,38,39]. In the present investigation, the cellulose backbones were activated to introduce epoxide groups using GMA (see Fig. 1). CAN and gamma rays have been employed as radical initiators for the chemical and gamma irradiation grafting techniques, respectively. The free epoxide groups could be further chemically modified to have a range of different functionalities onto the backbone of the adsorbents, for example, anion-exchange [10,21,28,40,41] or cation-exchange [5,17,42]. The degree of grafting can be varied depending upon several parameters, like monomer concentration, initiator concentration,

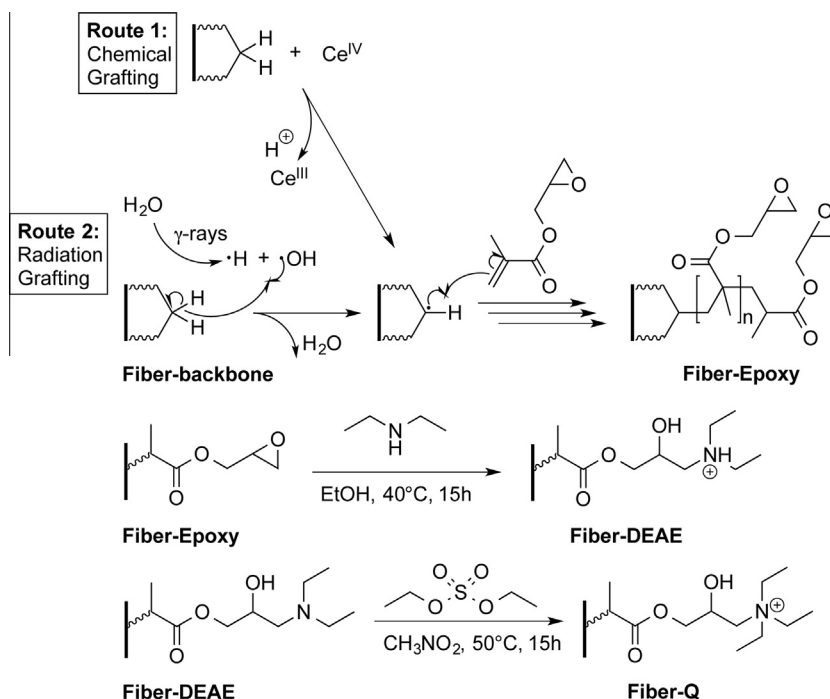


Fig. 1. Reaction scheme for the cerium ion-initiated and radiation-induced graft polymerization and derivatization of the epoxy groups to a DEAE functionality and quaternary ammonium functionality.

Table 1

Comparison of dynamic binding capacities of CG and GIR. 2 mg/mL solution of BSA was used for DBC measurements.

Adsorbents	DG (%)	10% DBC (mg/mL)
CG-DEAE fibers	12	26
	45	48
	100	44
CG-Q fibers	45	44
GIR-DEAE fibers	17	2.6

CG stands for chemical grafting; GIR is gamma irradiation; flow rate – 300 cm/h.

reaction time, and temperature of the reaction. In this study, various grafting percentages were achieved by varying the initiator concentration and determining its effect on the dynamic binding capacity [27]. The activated epoxy fiber-based adsorbents generated by two different grafting techniques were further modified to Q and DEAE functionality (see Fig. 1). A proprietary gamma irradiation protocol reported previously [17,28,29,38] was utilized for introducing the epoxy groups onto the fibers. The resulting amount of bound epoxy groups was relatively low, and hence, the gamma irradiation procedure for the fiber-based adsorbents needs further optimization for improving binding capacities. Due to the extremely poor protein binding capacities obtained for gamma-irradiated fibers (see Table 1), CG-DEAE (chemically grafted DEAE-fibers) and CG-Q fibers (chemically grafted Q-fibers) were further evaluated for chromatographic applications.

The chemical changes involved in the backbone of the anion exchange adsorbents were studied by FTIR. The IR spectra of pGMA, pure cotton (PC) base material (untreated), epoxy grafted (CG-Epoxy), and functionalized (CG-DEAE fibers and CG-Q fibers) adsorbents are shown in Fig. 2. The strong absorption band observed at 1730 cm^{-1} , represents the carbonyl groups of GMA. The untreated fiber backbone showed no absorption, while epoxy grafted adsorbents showed an absorption band at 1730 cm^{-1} . The striking differences in peaks at $2900\text{--}2800\text{ cm}^{-1}$ and $1100\text{--}1000\text{ cm}^{-1}$ indicates the presence of DEAE functionality. Absorption bands between $1060\text{--}1030$ and $672\text{--}666\text{ cm}^{-1}$ are due to the stretching modes of tetramethylammonium. Symmetrical and asymmetrical bands seen at $1460\text{--}1400\text{ cm}^{-1}$ and $1480\text{--}1440\text{ cm}^{-1}$ are due to the methyl groups from the quaternary ammonium [17,43]. The IR spectra clearly show the stepwise introduction of free epoxide groups, DEAE, and quaternary (Q) ammonium functionalities onto the backbone of the fibrous adsorbent. Material porosity for either the chemical grafting or gamma ray-induced grafting methods were $ca. 80 \pm 5\%$, which indicates that the physical integrity within the pores is not affected with grafting. Furthermore, all adsorbents showed a similar degree of swelling of $ca. 3.4 \pm 0.2\text{ g/g}$ in distilled water at $24\text{ }^\circ\text{C}$, which was suitable for having good convective flow properties [17].

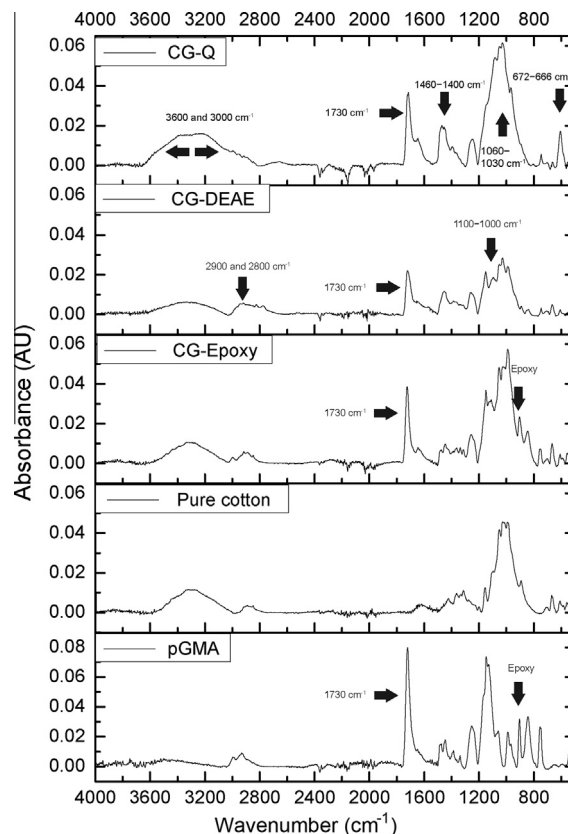


Fig. 2. IR-spectra of pGMA, pure cotton (untreated fibers), CG-Epoxy, CG-DEAE and CG-Q.

The confocal microscopy image (Fig. 3) indicates that the integrity of the material remains intact. Closer inspection shows a hydrogel-type behavior within the fibrous structure. The CLSM image of the Alexa Fluor-labeled BSA with stained-fiber also showed macro-pores within the adsorbents. The outer diameter of the adsorbent fiber was $\sim 20\text{ }\mu\text{m}$ whereas the inner diameter of the larger pores was found to be $\sim 15\text{ }\mu\text{m}$. The confocal microscopy also revealed homogenous grafting of the adsorbents.

Column efficiency of anion-exchange adsorbents is crucial for their application in chromatographic systems. The highly porous nature and structural integrity of fibrous adsorbents yields a relatively low packing density (0.2 g/mL). Consequent heterogeneity in packing may severely hamper the separation efficiencies of the columns [44–46], which is eliminated by uniformly swelling the packed adsorbent at low flow rates (75 cm/h) [17]. Fig. 4a shows the relation between theoretical plate height (H) and linear flow

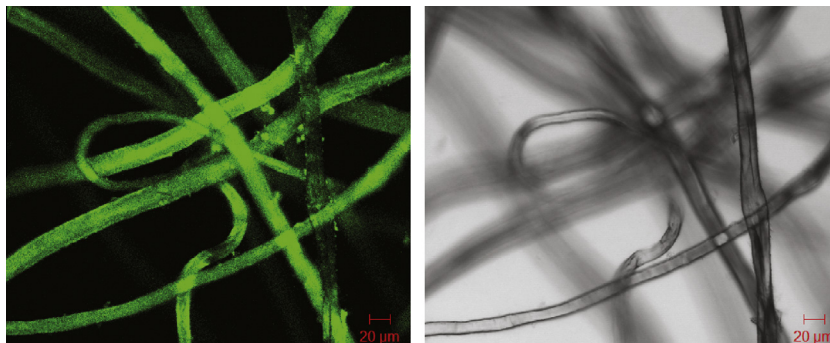


Fig. 3. Confocal microscopy of BSA adsorption onto CG-DEAE fibers.

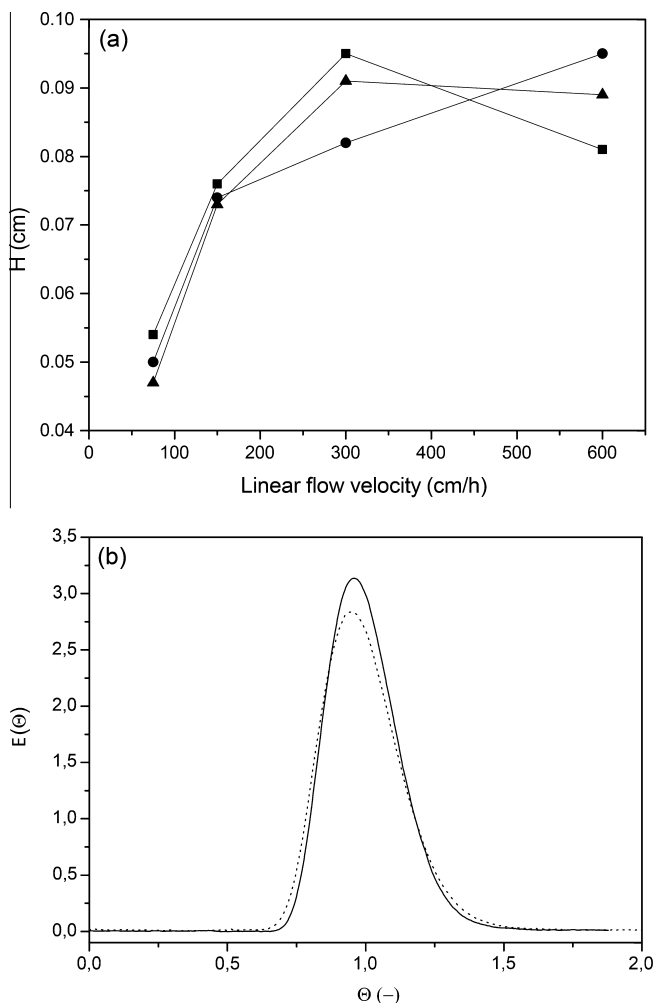


Fig. 4. (a) Theoretical plate height (H) versus linear flow velocity. Mobile phase: 20 mM Tris–HCl buffer (pH 7.4). Conditions:– (■) CG-DEAE fibers column: 5 mm ID \times 5.5 cm L; (●) Q Sepharose FF column: 5 mm ID \times 5.5 cm L; (▲) CG-Q fibers column: 5 mm ID \times 5.5 cm L; Sample: Acetone (5% v/v) of 1% column volume. (b) Column efficiency profiles of CG-DEAE (—) and CG-Q (---) fibers at 300 cm/h. Conditions:– Stationary phase: 0.2 g of CG-DEAE and CG-Q fibers; mobile phase: 20 mM Tris–HCl, pH 7.4; flow rate: 1 mL/min; UV detection at 280 nm.

velocity for CG-DEAE fibers and CG-Q fibers compared to Q Sepharose FF. In both, the CG-DEAE and CG-Q columns, a similar kind of trend was seen – when the linear flow velocity was higher than 75 cm/h, there was a slight increase in plate height, which then reached a plateau with linear flow velocities up to 600 cm/h indicating that the plate height in this regime was independent of the linear flow velocity. In contrast, in conventional porous media, like Q Sepharose FF, the plate height has previously been shown to be dependent upon flow velocity [47]. In addition, the peak asymmetries of a 5% v/v acetone pulse remained between 0.8 and 1.6 indicating favorable plug flow at variable flow velocities.

Total ionic capacities for CG-DEAE and CG-Q fibers measured by the pH transient method were 403 mmol/L and 386 mmol/L respectively (see Fig. 5). The ionic capacities obtained for these adsorbents are similar to that of ion-exchanger membranes [48]. Concomitant mass transfer studies of fiber-based adsorbents were conducted by determining the finite bath adsorption and DBC using BSA as a model protein. The effect of ligand density on protein binding capacities was determined by breakthrough analysis (see Table 1 and Fig. 6), where the highest DBC was obtained at

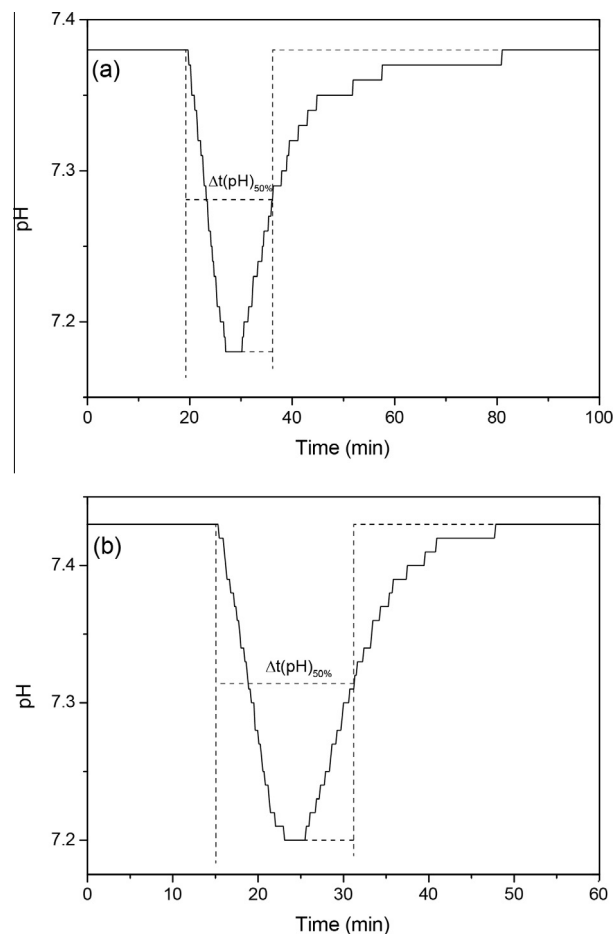


Fig. 5. Determination of Δt ($|\Delta pH| > 0$) from pH profile for (a) CG DEAE fibers and (b) CG Q fibers. Conditions:– Buffer A: 500 mM phosphate buffer, pH 7.4; Buffer B: 20 mM phosphate buffer, pH 7.4; Column (5 mm ID \times 5.5 cm L). Flow rate: 300 cm/h. Detection: pH.

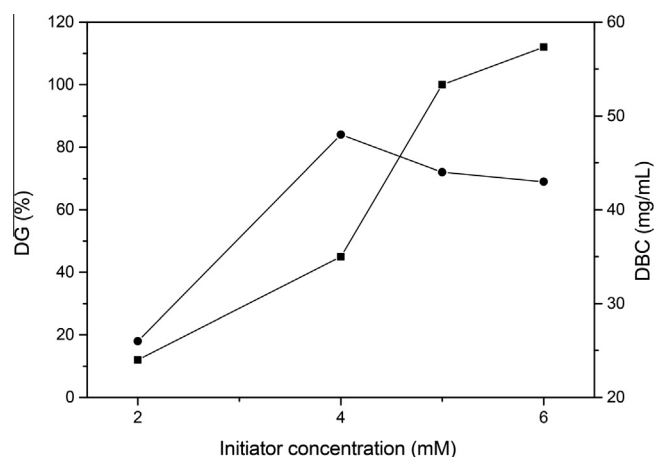


Fig. 6. Degree of grafting of GMA on fiber-based adsorbents as function of the initiator concentration versus DBC. Mobile phase: 20 mM Tris–HCl buffer (pH 7.4). Columns: (■) DG (%); (●) DBC (mg/mL); Sample: BSA (2 mg/mL).

45% DG with a value of 48 ± 4 mg/mL, which has a corresponding finite bath adsorption of 78 ± 4 mg/mL (Table 2).

It is clear that the DBC is not linearly dependent on the degree of grafting [30]. It showed a sharp increase at 45% degree of

Table 2
Comparison of DBCs of various anion-exchangers. 2 mg/mL solution of BSA was used for DBC measurements while 10 mg/mL solution of BSA was used for finite bath adsorption experiments (incubation time of 3 h).

Adsorbents	DBC at 10% 300 cm/h	Finite bath adsorption (mg/mL)	Ionic capacity (mmol/L)
CG-DEAE fibers	48 ± 4 mg/mL	78 ± 4	403
CG-Q fibers	47 ± 4 mg/mL	66 ± 4	386
DEAE Sepharose FF ^a	≤50 mg/mL	77	113
DEAE Sephacel	160 mg/mL	–	–

^a Taken from reference (200–400 cm/h) [49]. Capacity obtained by the vendors for DEAE Sepharose FF and DEAE Sephacel are 110 (HSA) mg/mL and 160 (HSA at 50 cm/h) mg/mL, respectively.

grafting, but no increase was seen by increasing the degree of grafting until 112% (see Fig. 6 and Table 1). Therefore, adsorbents with 45% grafting were selected for further studies. The effects of mass transfer resistance and dispersion on the DBC and column performance were studied at increasing flow rates. The DBC values

obtained at a flow rate of 300 cm/h for CG-Q fibers and CG-DEAE fibers were 47 mg/mL and 48 mg/mL, respectively. Furthermore, our fibrous adsorbents showed similar binding capacities for BSA at high flow rates when compared to the commercial beaded adsorbents (Fig. 7) (see Table 2) [49]. The values reported are also higher when compared to monolithic adsorbents (≤20 mg/mL of CIM-DEAE disks [50], 30 mg/mL of CIM-QA (BIA Separations, Villach, Austria) [51] and 40 mg/mL of UNO-Q monoliths (Bio-Rad, Hercules, California, U.S.) [51]). Although our adsorbents showed a slight decrease in DBC at very high flow velocity, which can be explained by a small increase in axial dispersion within the system [17,52], the DBC values for CG-DEAE fibers and CG-Q fibers (see Fig. 8) were independent of flow rates until 300 cm/h, indicating convective flow, since there was a negligible reduction in binding capacity. Additionally, the Péclet number for CG-DEAE (≥120) and CG-Q (≥60) remained constant, further confirming plug flow characteristics with minimal axial mixing [53,54].

4. Conclusions

In this study, we have presented the synthesis and evaluated the performance of fiber-based anion-exchange adsorbents. The results of this study demonstrate that chemically grafted adsorbents (CG-DEAE fibers/CG-Q fibers) exhibit higher binding capacities for BSA as compared to radiation-induced functionalized adsorbents (GIR-DEAE fibers). The highest binding capacity of 48 ± 4 mg/mL and 47 ± 4 mg/mL, respectively, for CG-DEAE fibers and CG-Q fibers was seen at 45% degree of grafting, which could be carefully controlled. The mercerization step for opening of the fiber structure helped to increase the grafting efficiency. These adsorbents provide high capacities at high flow rates with low to negligible pressure drops. Furthermore, they preserve their hydrodynamic properties and hence may provide an additional alternative to the adsorbents available in the market for the purification of proteins.

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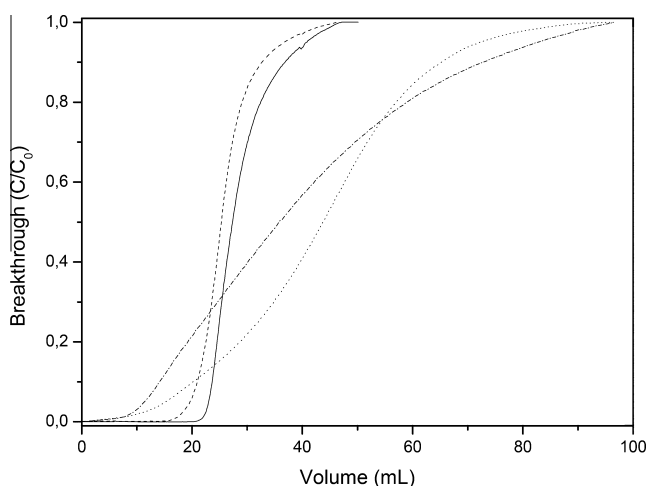


Fig. 7. Breakthrough comparison of different adsorbents at 300 cm/h. Conditions:– Stationary phase: 1 mL each of CG-DEAE, CG-Q, DEAE Sepharose FF, and Q Sepharose FF; mobile phase: loading buffer – 2 mg/mL of BSA in 20 mM Tris–HCl, pH 7.4; flow rate: 1 mL/min; UV detection at 280 nm. (–) CG-DEAE; (– –) CG-Q; (···) DEAE Sepharose FF and (– · –) Q Sepharose FF.

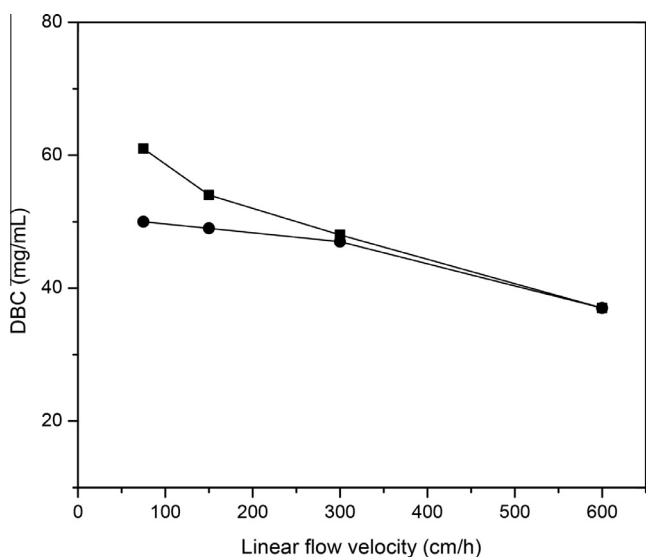


Fig. 8. DBC of CG-DEAE (■) and CG-Q (●) fiber-based adsorbents at different flow rates. Mobile phase: 20 mM Tris–HCl buffer (pH 7.4). Sample: BSA (2 mg/mL).

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