Fibrous Adsorbents as Novel Chromatography Matrices for Enhancing Industrial Downstream Processing of Bioproducts

By

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Statutory Declaration

I, Poondi Rajesh Gavara, hereby declare that I have written this PhD thesis independently, unless where clearly stated otherwise. I have used only the sources, the data and the support that I have clearly mentioned. This PhD thesis has not been submitted for conferral of degree elsewhere.

Bremen, November 21, 2012

Signature _____

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Abstract

The high demand for the production of biopharmaceuticals is limited by the total available manufacturing capacity, process efficiency and cost in a biotechnology industry mainly due to bottlenecks in the downstream processing (DSP) of the products. Downstream purification by traditional packed bed chromatography adsorbents plays a central role which exhibit major limitations, as a consequence the biopharmaceutical industry requires alternative materials and systems to replace. This PhD thesis is aimed at developing adsorption systems based on novel ion-exchange fibrous adsorbents for efficient and cost effective solutions to industrial downstream processing of bioproducts. In this work, strong cation exchange composite fibrous adsorbents have been developed and characterized. The mentioned system showed high capacity and high throughput for chromatography performance that can be used in intermediate purification and also exhibited superior performance during early protein capture application utilizing a model protein from an artificial crude feed stock. Similarly, strong anion-exchange fibrous adsorbents have been developed and characterized as high capacity chromatography support and investigated for their mass transfer properties. The fibrous geometry system demonstrated the potential for use in column scalable operations with high binding capacity and high throughput. A scaling prototype chromatography cartridge system based on the functionalized fibrous adsorbents has been evaluated and demonstrated superior hydrodynamics and process performances compared to commercial columns for industrial downstream processing. Finally, in this research work, the real application of fibrous base adsorbents has been successfully demonstrated as chromatographic support as an alternative to commercial beads in a single step capture and large-scale purification of an algal product, beta-Phycoerythrin, from unicellular marine algae using the mentioned system.

Symbols

Symbol	Description
A_s	Asymmetry factor
B_i	Biot number
c	Solute concentration in the bulk liquid (kg/m^3)
C	Concentration of tracer (%)
c_i	Solute concentration in the fluid of micropore (kg/m^3)
c_0	Solute concentration at z=0 without dispersion and at z<0 with dispersion (kg/m^3)
C_0	Concentration of sample (mg/mL)
Cy	Cyanine
D_p	Effective pore –diffusion coefficient of the solute (m^2/s)
d_p	Average pore diameter (m)
IgG	Immunoglobulin
K	Relative elution volume
k	Permeability coefficient (cm^2)
k_f	Mass transfer coefficient (m/s)
kGy	Kilogray
L	Length of adsorber (m)
$NaCl$	Sodium Chloride
Pe	Peclet number (-), the rate of advection of a flow /rate of diffusion
pI	Isoelectric point
q	Total ionic capacity (mequiv./g)
Q_{dyn}	Dynamic binding capacity (mg/g)
q_0	Equilibrium capacity for feed concentration c_0 (kg/m^3)

r_p	Average radius of the particle (m)
t	Time (s)
T	Dimensionless throughput
t_R	Retention time (min)
V_c	Column volume (mL)
V_0	Dead volume (mL)
W_h	Peak width at half peak height (min)
W_0	Dry weight of backbone polymer (g)
W_1	Dry weight after grafting polymer (g)
z	Axial distance in the column (m)
u_0	Superficial velocity (m/s)
ε	Porosity of stationary phase or particle, dimensionless
ν	Interstitial velocity (m/s)
V	Volume at 10% breakthrough (mL)
$\Delta t(\mathrm{pH})_{50\%}$	Time between switching the mobile phase and 50% pH of the BTC (min)
μ_1	Viscosity of the mobile phase (poise)
ΔP	Pressure drop across the medium (psi)

Abbreviations

AEC	Anion exchange chromatography
APS	Ammonium persulphate
BSA	Bovine serum albumin
BTC	Breakthrough curve
CEC	Cation exchange chromatography
CLSM	Confocal laser scanning microscopy
CoPES	Component (mono) polyethersulfone
CV	Column volume (mL)
DBC	Dynamic binding capacity (mg/g)
DG	Degree of grafting (%)
DMAA	Dimethyl acrylamide
DMF	Dimethylformamide
DS	Degree of swelling (g/g)
DSP	Downstream processing
EBA	Expanded bed adsorption
EDS	Energy Dispersive Spectroscopy
FTIR-ATR	Fourier transforms infrared-attenuated total reflectance spectroscopy
FPLC	Fast protein liquid chromatography
GMA	Glycidyl methacrylate
GTMA	Glycidyl trimethyl ammonium chloride
HETP	Height equivalent to a theoretical plate (cm)
ID	Internal diameter (mm)

IEC	Ion-exchange chromatography
LFV	Linear flow velocity (cm/h)
NaOH	Sodium hydroxide
NTU	Number of transfer units
RTD	Residence time distribution
SEM	Scanning electronic microscopy
TEMED	N,N,N,N’-Tetramethylethylenediamine
UV	Ultraviolet

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Chapter 1

Introduction

1.1. Bioprocess technology and downstream processing

1.1.1 The scope of process biotechnology

Biopharmaceutical production either of recombinant antibody or non-antibody proteins, vaccines, enzymes, toxins, human or animal derived blood products like plasma, cultured cell or tissue products can perhaps be considered as one of the key goals in the biotechnology industry, with high expectations of fast and efficient production rates that lead to high purity quality products. Recent advances in the recombinant DNA and cell culture technology have permitted the large-scale production of recombinant monoclonal antibodies, which have increasing demand as therapeutics in treating human diseases [1]. Due to the emerging technology and increasing demand, the global biopharmaceutical market is expected to reach US$ 167 billion by the end of 2015 [2]. The modern life science industry is facing a real challenge to deliver new products to a highly demanding and regulated market. As a consequence, bioprocess technology options have become limited considering both total available manufacturing capacity and process efficiency and cost. The bioprocess technology can be divided into two main parts, namely upstream (fermentation) and downstream (separation and purification). The main obstacle to simplified and cost-effective bioproduct processing and inefficiencies associated with the recovery and purification of these macromolecules lies in the downstream side [3-5].

Downstream processing (DSP) may be defined as a series of steps that, when followed, result in a purified protein product after fermentation. DSP can be divided into several unit operations whose complexity depend on the final product purity required [6] and accounts for 50-80% of the total processing cost. The primary unit operation involves the isolation, concentration and dewatering of the target protein from cell harvest or cell debris by centrifugation, micro or ultrafiltration, and precipitation. The secondary unit operation involves protein purification, processing and stabilization by a broad range of column or membrane purification, sterilization and packing methods. The fermentation processes that are used by biopharmaceutical manufacturers have shown to lead to increasing quantities of therapeutic proteins. Producers are increasingly recognizing the present need for improvement and have shifted their focus from improving the production process (upstream) to improving the downstream process. Each additional unit operation will affect the overall process economy by increasing operational cost, process time and also produce a certain degree of

product loss [7]. Therefore the process economics, yield and time are interrelated so commercial manufacture requires a reliable process with high yield, high purity and most important cost-effectiveness in order to design a successful downstream process.

1.1.2. Current bottleneck in downstream processing

Experts state that a tenfold (from 0.1 to 1.0 g/L) increase in the fermentation titre causes the ratio of upstream to downstream costs in the process to drop from 55:45 to 30:70. This shows that the upstream costs are inversely proportional to the titre, but the same is not true for the downstream processing costs. However, this increase in turn leads to capacity bottlenecks in the subsequent purification processing and is associated with multiple numbers of conventional unit operations with limited efficiency. Downstream processing comprises up to 80% of the entire production costs. Product recovery takes place either from transgenics or yeast or *Escherichia coli* or mammalian cells, however, the industry needs improved downstream processes which are still an unresolved bottleneck" [8, 9]. Therefore, innovative and sustainable solutions and technologies in relation to the efficient use of production time, cost and resources are a key challenge for companies in the biotechnology sector to reduce costs, save jobs and maintain themselves in the marketplace [10].

The advances in technology development and rising global demand, particularly in the field of monoclonal antibodies, are positive indicators for continued growth of the industry in the future [11]. In industrial DSP, economic considerations play a central role in the production cost due to the demand from large cell culture densities and fermentations titers (antibody concentrations increase), which affects the capacities and quantities of used filters and chromatography media and the number of necessary chromatography cycles. The dominant factors here are high costs for chromatography and filtration materials, operating, cleaning and validation costs for chromatography systems as well as very large quantities of chemical reagents (especially buffer) and water [8]. As upstream productivity increased, the binding capacity of chromatographic materials that are used for the subsequent purification of proteins, increased by no more than a factor of three. Thus, the cost of specific chromatographic media (e.g. recombinant Protein A purifications of immunoglobulin"s in a fermentation volume of 10,000 liters) increases up to $4-5 million [12]. To save costs, this media is prepared with a large amount of buffers and water for reuse. As shown in recent studies, the required amount of buffers for the DSP of a 20,000 liter-bioreactor is of the order of 140,000 liters [13]. With a liter price of $2-12, depending on the chemicals used, this cost incurred up to $1.7 million [14]. However, real problems are expected to show up with the

isolation of proteins, since an increase in protein yields will require manufacturers to invest in larger equipment and more materials in operational costs, water consumption in order to handle the larger quantities of protein produced. Although large chromatographic columns are technically feasible, they require larger utilities and floor space to match the larger size of the columns, which is something that is not always possible for the existing facilities. The portion of floor space in a plant occupied by upstream and downstream installations has shifted due to the much higher liquid consumption for buffer preparation and the need for storage capacity that supports a modern, large-scale downstream process. The main reasons that bottlenecks are a topic for discussion are the time and costs involved to retrofit and revalidate existing plants.

It is, therefore, quite understandable that the biopharmaceutical industry is actively seeking less expensive alternatives, and not only in view of the growing cost pressure in the healthcare sector. One idea to overcome the problem at hand is to replace chromatography resins, for example Protein A (which can bind mammalian proteins), which is very costly, or to reduce the number of column chromatography steps from three to two, and eventually to one. Membrane absorbers might also be a potential alternative for counteracting the downstream bottleneck. In addition to these huge financial costs of DSP, which may make compliance with cGMP standards (current Good Manufacturing Practice) up to 50% of production [15] have environmental costs taken into account. However, the time required to establish a new purification process also plays a key role in process economics because there is no "one and only" purification process. Every substance requires a specific purification process, which is adjusted to the substance's specific biochemical and biophysical profile and its specific fermentation conditions.

Different bio-products from the biotechnological industry are mostly proteins, which are of concern because of their enzymatic activities, specific recognition interactions or other therapeutic actions. In most applications proteins require to have high degrees of purity. The products purity ranges from partially purified concentrates, e.g., food enzymes to highly purified preparations, e.g., purity demand $\geq$ 99.99% in the case of therapeutic proteins. proteins. Protein stability during downstream processing has become one of the major issues determining the success of a given process. The potential factors for product degradation can be divided into physical processes (shear, impingement, aggregation, precipitation), fluid phase system parameters (pH, ionic strength, buffer composition, temperature), biological (enzymatic degradation) or chemical processes (oxidation, deamidation, hydrolysis). Detailed knowledge on protein stability on a molecular basis is required as the purification process

includes high number of unit operations, holding time, and changes in pH, temperature, and solvent composition. This molecular understanding of structural changes of biomolecules as a result of environmental influences can help in process design [16].

1.1.3. The importance of chromatography as a purification unit operation

Chromatography, being a high-resolution and well-established method, is considered essential for the purification of bioproducts in the biotechnological industry in the past few decades. Chromatography encompasses methods, major aim of which is to isolate the greater part of a target compound by reversible adsorption onto a solid phase. Downstream chromatography purification has three phases: capture, intermediate purification and polishing. In the capture phase the objectives are to isolate, concentrate and stabilize the target product.

During the intermediate purification phase the objective is to remove most of the bulk impurities such as other proteins and nucleic acids, endotoxins and viruses. In the polishing phase the objective is to achieve high purity by removing any remaining trace impurities or closely related substances. The selection and optimum combination of purification techniques for capture, intermediate purification and polishing is crucial to ensure fast method development, a shorter time to a pure product, and process economy. The final purification process should ideally consist of sample preparation, including extraction and clarification when required, followed by two to three major purification steps. The number of steps used will always depend upon the purity requirements and intended use for the protein. In earlier applications of chromatography the protein purification involved relatively clean feedstock, generated by steps such as precipitation, centrifugation, dialysis and filtration. The role of chromatography was fine separation and polishing [15]. This purification scheme contains an extensive sequence of separation steps that adds greatly to the overall production costs (>50%) in downstream processing of biotechnological relevant proteins [16]and can result in significant loss of product. The economic feasibility of the production process is directly related to the number and efficiency of the processes required during purification in order to obtain the required product specifications [17].

1.1.4. Current platforms

With the growing importance of biopharmaceutical therapeutics in treating human disease such as cancer, purification in DSP is getting attractive in the manufacture of e.g. recombinant monoclonal antibodies, viral vaccines, and plasmid DNA [18, 19] made from the fermentation

approach, essentially by different adsorption chromatography. Here, the target molecule occurs because of its biophysical and chemical properties in specific interaction with a given stationary phase and is now disconnected from the remaining components of the fermentation broth. For the further isolation of the molecule, a significant additional number of steps are necessary. A typical purification process train for the manufacture of monoclonal antibodies [20] usually involves a native or recombinant Protein. A capture step combined with a cation exchange column for intermediate purification and an anion exchange column operating in flow through mode to remove negatively charged impurities such as DNA, host cell protein, endotoxins, and endogenous viruses is used.

The high share of biomass (high cell density) from the fermentation broth contains many relatively large undissolved particles. This leads to clogging of the system when a direct use on an adsorption material is accomplished and this would render it unusable. Therefore, different pre-filtration steps are necessary to prepare the clarified fermentation broth that consumes huge buffer preparation and operational costs. Another limitation arises from the physical properties of the current chromatography media. The treated fermentation broth or subsequent unit operation loads can be pumped only with a relatively slow flow rate through the system otherwise the material would compress as pressure is too high. In addition, all current adsorbent materials show a very low or no air tolerance. These circumstances limit the actual turnover of purification significantly in the biotechnology plant and that would lead to expensive operation responsibilities. Presently chromatographic adsorptive separations utilizing packed bed beaded supports, monoliths or membrane media are used for purification and recovery of protein and biological macromolecules. By use of beads, monoliths and membranes, separations are accomplished through similar physiochemical interactions but in hydro-dynamically different ways. A major difference between these three chromatographic media is the relationship between the adsorbing surface and the fluid flow through them.

1.1.5. Integration and intensification approaches

Fig.1.1 illustrates the overall downstream generic protein purification cascade which involves various unit operations for the primary recovery of the target product and is followed by secondary unit operations containing several chromatography purification steps. Since downstream processing is the dominant cost factor in most bioprocess scenarios, cost analysis has to be considered for a full evaluation of potential of any novel recovery and purification technology. Thus, there is need for development of new and innovative approaches/strategies for downstream processing of bioproducts. The challenge for biochemical engineers is to

design compact and clean integrated processes to efficiently separate unstable recombinant proteins, from dilute complex fermentation broths to the required pharmaceutical degree of purity. It provides knowledge of different techniques for solid-liquid separation, product release, refolding, concentration and purification of valuable bioproducts with a focus on the total integrated process.

Process integration is emerging as a leading theme in modern bioprocessing and one of the keys for the rational, cost-effective and productive design of (bio) separation processes by creation of a link between previously separate unit operations or combining individual steps in to one unit operation, by which product losses and process economics can be minimized. Integrated processes are required to reduce the number of processing steps, to simplify handling, to shorten the time a sensitive product has to be in contact with harsh conditions with an overall aim to reduce total processing cost. Strategies such as effective sequencing and dovetailing of operations to benefit overall processing could benefit the manufacture i.e. integration of purification and transformation steps can be applied for large-scale economic production of biotherapeutics. Final stages of downstream processing including crystallization/precipitation and drying (such as freeze drying, supercritical spray-drying and spray freeze drying) have been evaluated for preserving potency with increased stability and ease of formulation of biotherapeutics. The strategies developed have numerous benefits and can be effective over conventional ways of large-scale production of bioproducts [17].

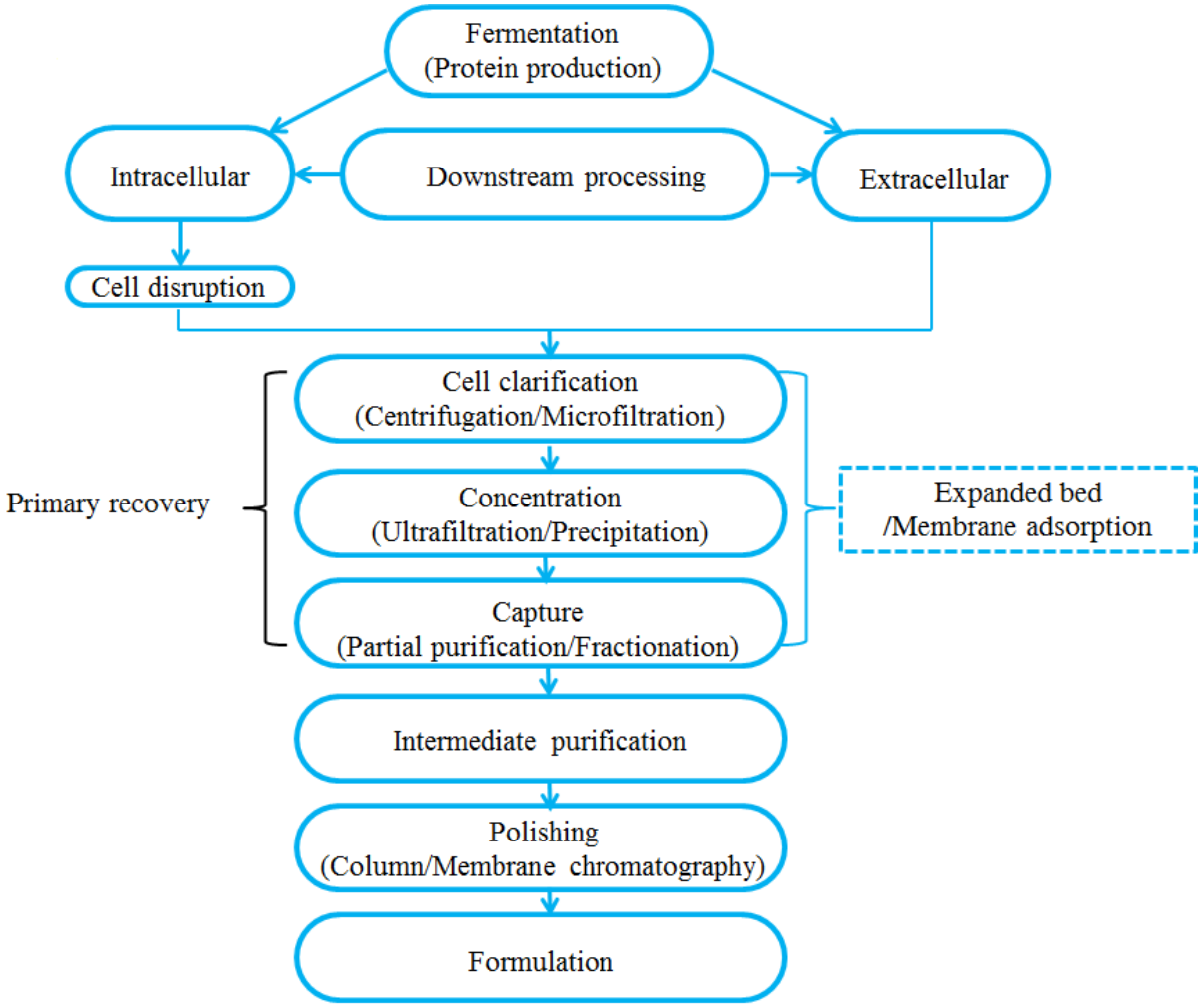

Fig. 1.1. Downstream generic protein purification cascade.

The operation of expanded bed adsorption (EBA) with unclarified feedstock's is described as achieving the objectives of solid elimination, dewatering and product fractionation which conventionally would be achieved by three discrete operations (e.g. centrifugation, ultrafiltration and fixed-bed chromatography [18].The operation of a fluidized-bed adsorber directly integrated with DSP of a cell disrupter to achieve online capture of intracellular products is an analogous application of direct product sequestration. Integration of anion-exchange membrane adsorption allows the large scale production of recombinant monoclonal antibodies in a flow-through mode and may provide a reasonable alternative to columns for the removal of low levels of impurities such as DNA, host cell proteins, and viruses [1]. However there will be some constraints and limitations for each integrate system in the current adsorptive recovery of bioproducts and details will be given in following section of types of adsorption.

1.2. Adsorption and chromatography

1.2.1. Adsorption operations

Currently, four different types of adsorption chromatography operations are being in use as follows: (i) packed bed adsorption; (ii) expanded bed adsorption (iii) membrane-based adsorption; and (iv) monoliths.

1.2.1.1. Packed bed of beads

Bead based media have convective flow occurring at the bead surface while most of the adsorbing surface is internal to the bead and can only be reached via diffusion, a slow process, especially in the case of macromolecules. As chromatography beads are porous and the selected entities to be captured must diffuse into the pores of the media to get captured, the speed and capacity of the whole system is diffusionally limited. At high flow rates, thus reducing the dynamic binding capacity and the apparent pressure, the efficiency of the system is affected. As a result, traditional chromatography has worked based on a compromise between resolution and capacity. This compromise is required mainly by differences between the velocity of diffusional mass transport inside in the particles and convective flow on the surface. Additionally, the permeability of the media is related to bead size as well as the media stability. Larger beads and beads with larger pores tend to have higher permeability. Beads that are not subject to or less subject to compression also tend to have greater permeability. However, at high flow rates, permeability does decrease and dynamic capacity also decreases. Dynamic capacity refers to the fraction of the total equilibrium capacity which is actually under process conditions i.e. when residence time is limited. That system also tolerates virtually no air or biomass and the recycling of the material, the validating and cleaning of the columns and the re-packing of the column requires a very high material and operational cost and is time consuming [21, 22].

Moreover, and since the mentioned soft beaded adsorbents are utilized in packed-beds, intensive clarification of the feedstock should be performed to avoid column blocking and backpressure development. Crude fermentation broth contains biological particles like cells or cell debris which can form very compressible filtration cakes and which can be adherent towards process surfaces. Therefore, extensive solids removal e.g. by filtration or centrifugation is typically performed in advance to any chromatography step. An important

challenge has been to find the best way to expose quickly and efficiently the mobile phase and the simple molecules to the entire surface of the chromatographic material, including the area inside the pores. For this reason, process integration has been proposed in the field of biochemical engineering and the reason could be one of the keys to the rational, cost-effective and productive design of (bio) separation processes.

1.2.1.2. Expanded-bed adsorption

Draeger and Chase [23] presented a novel integrated concept based on fluidized / expanded bed adsorption for the direct sequestration of bioproducts. Expanded bed adsorption (EBA) is an integrative primary recovery technology that combines solid-liquid separation with an adsorptive operation. During EBA operation, feedstock containing suspended biomass is introduced directly to the column and influences the expanded bed fluidization pattern and process performance. The accomplishment of the integrative process is related to detailed understanding of the biochemical principles involved and the constraints arising from feedstock complexity [17]. Even though the EBA has allowed early process integration by direct capture of targeted species from an unclarified biological feedstock, in many cases, fluidized bed performance could be impaired. This is due to unstable solid-liquid fluidization and / or diffusional restrictions [24, 25], blockage of the fluid distribution system, interactions between adsorbent beads and cells, cell debris and macromolecules (e.g. genomic DNA) [17] which, in return, cause a reduction of the overall system DBC for the target product.

The presence of suspended particles and the high viscosity of a feedstock may contribute to aggravating the mentioned limitations. In the case of application of biomass containing feed stock, distinct deviation is found from the ideal fluidization characteristics. This deviation eventually signifies that for the application of complex biological feedstock, the transport-limited sorption assumption is not valid and EBA system performance deviates significantly from packed bed results [26]. In the so-called EBA system, the throughput of the solution is from the bottom up and the beads float in the solution, making the system tolerate the biomass, while the pressure is reduced. This system allows an extensive waiver of pre-filtration; however, complications are the moving parts, the time-consuming and costly cleaning and validation. Besides, in order to remove and wash away sticky biological particles; more buffer is consumed during EBA process. These phenomena, i.e. decreased sorption performance due to biomass attachment to adsorbent and increased buffer consumption are the process challenges of EBA [27]. Thus, it is already known that interaction between biomass and the adsorbent phase may lead to the development of poor

system hydrodynamics and as a result of which a decrease in performance under real process conditions is found. EBA systems, through the construction of the pillars in itself, are currently not available in any sizes (up-scaling) and have in regard to the diffusion of the biomolecules the same restrictions as packed beads [28, 29].

1.2.1.3. Membrane-based adsorption

Membrane separation process is widely used in production, purification, and formulation of biotechnology products in the application of pressure-driven processes of ultrafiltration, microfiltration, and virus filtration [30]. Microfiltration membranes are applied in cell harvest for the removal of cell particles and cell debris larger than 0.1 µm and ultrafiltration membranes are used in ultrafiltration and diafiltration for concentration, buffer exchange and desalting applications where soluble macromolecules that have a size in solution of less than 0.1 µm. Membrane adsorbers are micro filtration membranes with functionalized surface, allowing for selective binding of biomolecules to be separated, where the binding capacity is based on the outer surface of the membrane pores. Membrane-based adsorbers are potentially applied for large-scale applications in capture and polishing steps for purification processing of large amounts of products in relatively short time. Currently, membrane adsorbers are focusing on the flowthrough application (negative chromatography) which is capturing the target product from the large volumes of the feed and polishing of target product form the trace impurities, such as virus, DNA and host cell proteins, viral vectors, plasmids as well as extremely large protein molecules [31]. The flow velocity independency of membrane adsorber makes it ideally suitable for the purification of very large molecules such as plasmids (MW >250 kDa) or rotavirus like particles for gene therapy applications [18, 31, 32].

Membrane-based adsorbers are available commercially but have the disadvantage that the capacity decreases with increasing pore size and can only operate for a given permeability due to the coupled flow and binding properties. Membrane adsorbers tolerate high process pressure and thus allow higher process speeds, but they do not tolerate any biomass, which means costly pre-filtration of the fermentation broth to prevent clogging of the adsorbent. A recycling is very expensive (buffer consumption, time), so the systems are mainly offered as disposable equipment and advertised. Commercially available membrane adsorbents have found limited applications that need to be overcome for routine application in bioprocess scale productions. The most important limitations are: (i) non-uniform membrane pore size distribution, (ii) irregular membrane thickness, (iii) distorted inlet flow distribution and poor

collection at the outlet, (iv) limited binding capacity [33, 34]. Polymer-based monoliths produced from different manufacturing technology but having similar pore morphology compete with macroporous membrane adsorbers, especially for ultrafast high-resolution separation [35, 36].

1.2.1.4. Monoliths

Porous polymer-based monoliths have been introduced in the early 1990s as a new class of chromatographic supports which offers some unique advantages, especially for separation of larger biomolecules. The main characteristic of these materials is the presence of large through-pores, which permits the use of high flow rates at low backpressures [37]. The synthesis of a suitable porous polymer monolithic stationary phase is an important step in the production using different polymerization methods, with thermal and photo-initiated radical polymerizations being the most commonly employed approaches. The use of a photo-initiated polymerization approach in the recent years using visible-region light emitting diodes (LEDs) [30, 31] overcomes the limitations related to conventional UV-initiation polymerization which require the use of UV-transparent molds (e.g. Teflon-coated capillaries) and monomers that do not absorb in the UV region (e.g. styrene-based monomers). The new approaches such as hyper crosslinking or the incorporation of nanoparticles developments in polymeric monolithic columns have shown significant performance benefits for the separation of small molecules. And also it can reasonably be expected that similar or other approaches can be used to improve the morphology of polymer monoliths designed for the separation of large molecules [38].

1.2.2. Chromatography modes

The bioseparation mechanism of chromatography depends on the mode of interactions involved and can be classified into ion-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), affinity chromatography (AFC), reverse-phase chromatography (RPC), immobilized metal affinity chromatography (IMAC) and other types of chromatography such as size exclusion chromatography (SEC) [39]. The binding of a desired protein to an immobilized ligand on the base matrix is due to one or several of the interactions [40] which include: (i) ion-ion or ion-dipole bonds (ii) hydrogen bonding (iii) hydrophobic interactions (iv) dispersion or van der Waals forces (v) aromatic or π-π interactions.

1.2.2.1. Ion exchange chromatography (IEC)

Ion exchange chromatography is a widely utilized technique for the recovery and purification of proteins, polypeptides, nucleic acids, polynucleotides and other charged biomolecules in life science industry. The advantages of this versatile method of separation is because of high resolution power, high-binding capacity, processing of large volumes in shorter period, simple buffer used in large scale protein purification process, [44] and can be adapted to capture, intermediate and polishing step at all three levels of purification. Proteins maintain their native configuration during IEC which is due to utilization of mild elution conditions, which guarantees bioactivity. The technique is based on the Coulomb-type interactions between charged (macro) molecules in the mobile phase and oppositely charged moieties that are immobilized onto a suitable stationary phase. The impact of separation performance is due to the variables of the nature of the stationary phase, differences in ligand density, and the molecular mass of the interacting species and the coupling chemistry to the support [45, 46]. IEC could be more complex during chromatographic separation due to non-electrostatic interactions and protein – protein association. Non-electrostatic interactions, such as hydrophobic interactions and hydrogen bonding, or other factors like the nature of the buffer ions also effect the protein separation with ion exchangers. Furthermore, protein – protein interaction may influence unexpected protein chromatographic behavior or lead to multilayer formation during process [47, 48]. The nature of the feedstock can also have an influence on protein separation due to its interaction with low molecular weight components and polymers in the mixture [49].

Porous ion-exchange resins have been the first to be commercialization [50]. The predominant electrostatic interactions of ion exchangers on the adsorbent with surface charges on the protein is governed under a given pH and salt concentration [2]. In IEC, elution of protein is performed either with decrease in pH or increase of salt concentration resulting in weakening of the ionic forces. Ion exchangers can be positively charged (anion exchanger) or negatively charged (cation exchanger) and be strong or weak depending on the permanence of charge over a broad pH range. Commonly, strong exchangers can maintain their charge over a broader pH range than weak ion exchangers [40]. A number of different functional groups are used [51].

1.2.2.1.1. Cation exchange chromatography (CEC)

Commonly used cation exchange resins are S-resin, (Sulfate derivatives); and CM resins, (carboxylate derived ions). The surface charge of the solutes (proteins, nucleic acids,

endotoxins), which bind, will be net positive. Thus, to get the binding of a specific protein, one should be below the pI of that protein. CEC is less commonly used compared to AEC, largely due to the fact that often proteins do not stick to this resin at physiological pH's and one is reluctant to titrate a protein through its isoelectric point to get it to adhere to the resin. Nonetheless, it is as powerful as AEC for initial separations with equivalently high capacity. Typically, CEC is performed using buffers at pH's between 4 and 7 and running a gradient from a solution containing just this buffer to a solution containing this buffer with 1 M sodium chloride (NaCl). Uses of CEC include initial clean-up of a crude slurry, separation of proteins from each other, concentrating a protein, and as a common first purification step for proteins expressed under acidic conditions such as in *Pichia pastoris*.

1.2.2.1.2. Anion exchange chromatography (AEC)

In anion exchange chromatography, negatively charged molecules are attracted to a positively charged solid support and the binding of a specific protein should be above the pI of that protein. AEC is often used as a primary chromatography step due to its high capacity, (matrices can bind from 10 to 100 mg of protein per mL) and ability to bind up and separate fragmented nucleic acids and lipopolysaccharides from the initial slurry. Commonly used anion exchange resins are Q-resin, (a quaternary amine); and DEAE resin, (Diethylaminoethane). Typically, AEC is performed using buffers at pH's between 7 and 10 and running a gradient from a solution containing just this buffer to a solution containing this buffer with 1 M NaCl. The salt in the solution competes for binding to the immobilized matrix and releases the protein from its bound state at a given concentration. Proteins separate because the amount of salt needed to compete varies with the external charge of the protein. Uses of AEC include initial clean-up of a crude slurry, separation of proteins from each other, concentrating a protein, and the removal of negatively charged endotoxins from protein preparations.

1.2.2.2. Hydrophobic interaction chromatography (HIC)

HIC separations are based on the interactions between hydrophobic groups on the protein and a hydrophobic matrix. In HIC, the sample is loaded at moderately high concentration of antichaotropic salt (cosmotropic/ lyotropic salt), which is having higher polarity and enhances hydrophobic interactions and protein precipitation (salting-out effect) [41]. Elution is achieved by a linear or stepwise decrease in the concentration of salt in the adsorption buffer, which provides a satisfactory recovery and elution under conditions of low salt concentration.

As the concentration of salts increases, the protein aggregates on the surface of the adsorbents. HIC is sensitive to used salt, pH, buffer type, and temperature is useful for separating the pure native protein from other forms [42]. The retention of protein on a reversed phase chromatography matrix is dependent on the relative hydrophobicity of the protein compared to the polarity of the fluid and solid phase. The sample is loaded in an aqueous buffer and eluted in an organic buffer when the relative hydrophobicity of the protein is decreased by either decreasing the polarity of the fluid phase or increasing the polarity of the protein through the addition of an ion-pairing agent.

1.2.2.3. Affinity chromatography (AFC)

AFC separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatographic matrix. Biological interactions between ligand and target molecule can be a result of electrostatic or hydrophobic interactions, van der Waals' forces and/or hydrogen bonding. To elute the target molecule from the affinity medium the interaction can be reversed, either specifically using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. Diversities of affinity chromatography have been used in bioprocessing. For instance, the most widely utilized Protein A affinity chromatography for large-scale antibody separation, or the dye ligand chromatography such as Cibacron blue, which mimics the nucleotide binding sites of enzymes. In IMAC, the high affinities of protein for specific metals are used. Biomimetic ligands mimic the interactions of biomolecules with their natural ligands. For the IMAC, the binding is to residues that are distributed over the protein surface [40, 43].

1.2.3. Chromatography performance

1.2.3.1. Column packing

The qualifications of packed chromatography columns are critical steps to ensure robustness and safety for both the purification process and the final product. Efficiency testing is done with a typical pulse response and its evaluation are summarized in Fig. 1.2 where an inert tracer was added to the liquid flow near to the column inlet (injection point) and the broadening of the pulse is analyzed as a chromatographic peak at the outlet. t_R is the retention time of the probe molecule, W_h is the peak width at half peak height and v_E the elution volume, is the volume from the point of injection to peak maximum. Column efficiency testing plays a key role in the qualification and monitoring of packed bed performance and

can be defined in terms of two parameters. The first one is peak broadening, which is typically described as height equivalent to a theoretical plate (HETP). This concept is equivalent to a tanks-in-series model reflecting the number of equilibrium stages represented by the column. The slower the velocity, the more uniformly analyte molecules may penetrate inside the particle, and the less different penetration affects efficiency. On the other hand, at the faster flow rates, the elution distance between molecules with different penetration depths will be high.

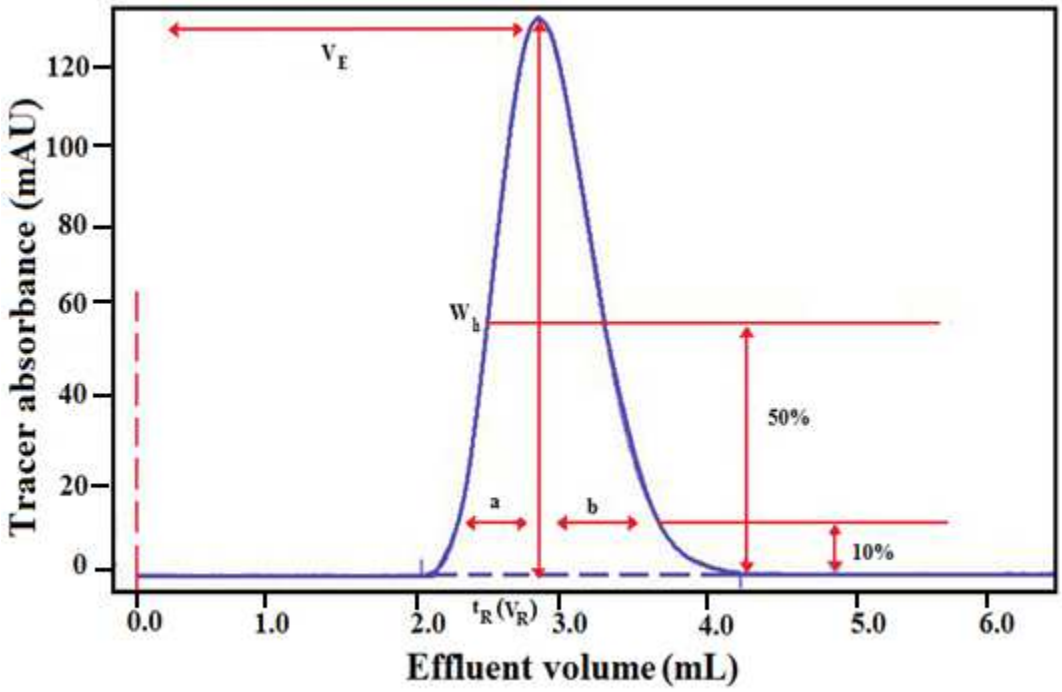

Fig. 1.2. Schematic overview of the pulse test.

HETP can be measured over a range of flow rates and the data fitted to the Van Deemter equation;

$$\mathrm{H} = \mathrm{A} + \frac{\mathrm{B}}{u} + C\,u \qquad (1)$$

where u (cm/h) is the average velocity of the mobile phase and A is Eddy's dispersion, B is molecular diffusion, C is mass transfer resistance and are factors which contribute to band broadening. HETP should be used as a reference value only since the calculated plate number will vary according to the test conditions like changes in solute, solvent, eluent, sample volume, flow rate, liquid pathway, temperature, etc. The second parameter is described by a peak asymmetry factor (A_s=*b*/*a*), where *a* is the peak width (left half) at 10% of peak height, *b* is the peak width (right half) at 10% of peak height a standard chosen to allow for simple

evaluation. Deviations from an ideal peak symmetry (A_s = 1) may be caused by irregularities in the packed bed itself, but also by unfavorable fluid flow within tubing and components external to the packed bed. In addition, peak asymmetry may arise from air trapped within the column or from clogged frits or screens in the fluid distribution system, resulting in flow disturbances In regard to the peak asymmetry, an asymmetry factor close to A_s = 1 is ideal. A typical acceptable range could be $0.8 < A_s < 1.8$ [20].

1.2.3.2. Capacity of functional groups

The total ionic capacity describes the number of charged groups on the ion exchanger. It is usually expressed as micromoles per gram dry ion exchanger or micromoles per mL swollen gel. However, total ionic capacity generally is not used when determining capacity of proteins and other multiply charged molecules. These must be determined empirically for each type of sample. For these types of adsorbents, available capacity (the actual amount of a sample which will bind to an ion exchanger under defined conditions) and dynamic capacity would determine the mass transfer rates to the binding sites and their availability for target substances. This includes the total capacity of functional groups of the adsorbent (total ion-exchange capacity) and the available binding capacity for target solutes under static and dynamic conditions (static and dynamic binding capacity).

The total ion-exchange capacity of weak cation exchange membranes can be determined via a static indirect titration [21] or by frontal analysis and elemental analysis [21, 22]. The accessible functional binding capacity can be determined in situ under flow-through conditions via the inadvertent pH transient method [22, 23]. The static binding capacity of fibrous adsorber is determined through mass balance after batch incubation or converted from the elution values of protein by UV spectro-photometry at 280 nm. The protein-binding isotherm can be measured in the same way and subsequent curve fitting for evaluation of parameters.

1.2.3.3. Dynamic binding capacity

The dynamic binding capacity of a chromatography media is the amount of target protein the media will bind under actual flow conditions before significant breakthrough of unbound protein occurs. This parameter reflects the impact of mass transfer limitations which may occur as flow rate is increased, so it is much more useful in predicting real process performance than a simple determination of saturated or static capacity. Breakthrough

analysis is evaluated by frontal chromatography separation in which the sample is fed continuously into the column. For a short time, the solute in the feed is taken up almost completely; however after a while, solute breakthrough occurs and in the effluent concentration increases with time as show in the Fig. 1. 3.

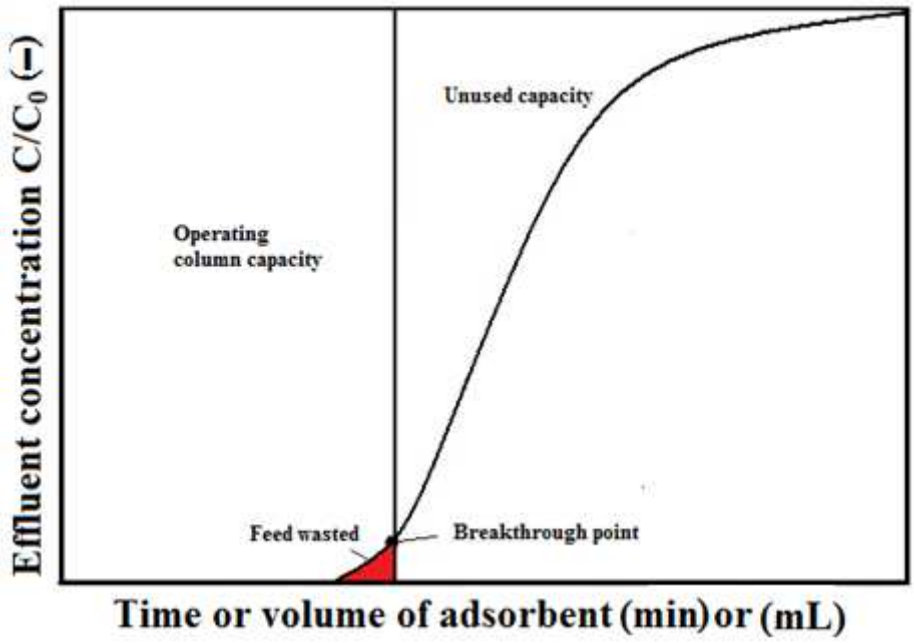

Fig. 1.3. Column loading process and breakthrough curve for frontal chromatography system.

For an adsorption step terminated at effluent concentration of breakthrough, a small amount of feed has been wasted and a portion of the column capacity remains unused. The required information to evaluate column performance is represented in these typical plots of effluent concentration vs. time or breakthrough curves (BTC). These curves can be used to determine (i) how much of the column capacity has been utilized, (ii) how much solute is lost in the effluent, and (iii) the processing time. This is precisely the performance information needed to optimize separation processing [1]. From the complete BTC, additional information about the maximum DBC ("max. DBC") and DBC value at 10% breakthrough (10% DBC) can be regained. For industry application, 10% DBC is more favorable because the feed is usually applied only until the concentration of the target solute in the flow-through reached up to 10% of its initial concentration. While the static binding capacity is dependent on the adsorbent structure, target molecule and buffer conditions, the dynamic value could be additional affected by the flow rates induced pressure drop and the design of packed bed configuration. With the introduction of new base materials, improvements in the structural characteristics of particles and an increase in the variety of organic polymers, rational procedures have been

developed in order to design composite media with improved physico-chemical stability, selectivity and efficiency. In polymer-coated media, chromatographic performances are strongly related to the chemical nature of the adsorbed polymer, as well as to its conformation in the deposited layer. Thus, a thin and densely packed layer can efficiently prevent non-specific protein adsorption, whereas flexible polymer chains can enhance site accessibility and decrease mass transfer resistance, provided that, in porous media, modification does not restrict pore access.

1.3. Novel adsorbent materials

1.3.1. Advantages of adsorbent fibers

Polymer-based porous fibrous materials are prominent among novel chromatographic supports with great potential for use in the separation and purification of biomolecules after proper surface activation and modification [24, 25]. Fibers show high performance due to having different characteristics: high tensile strength and ductility, chemically modifiable, low-density and biocompatibility. In general, the high surface area, higher porosities and interconnectivity of pores within the fibrous matrices ensure good mass transfer and support high cell densities and are easier for cell seeding [26, 29]. In particular, nonwoven fibrous matrices of polyethylene terephthalate (PET), and polyethersulfone (PES) have found widespread applications in tissue engineering [11, 13] as well as for production of recombinant proteins [29, 30] because of their low material cost, good biocompatibility, and excellent mechanical strength and stability.

The first organic polymers to attract interest were natural polysaccharides, including agarose, cellulose, cross-linked dextran and, to a lesser extent, cross-linked amylose and starch. These materials are produced with a suitable porosity, are stable over a wide pH range (pH 3-13) and are able to withstand alkaline washing. In addition, they possess a high content of hydroxyl groups available for activation and derivatization, they are hydrophilic and generally do not interact with proteins. However, under extreme conditions, they may exhibit weak ionic or hydrophobic properties. Specific interactions with proteins have also been reported, e.g., haemoglobin is able to recognize tx-l,4 glycosidic links in amylose [31] and the OH groups of polysaccharides are targets for the C3 protein of blood plasma. The main drawback of polysaccharides is their poor mechanical strength, related to their swelling ability. The extent

of polymer swelling depends on the chemical nature and on the concentration of polymer in particles and it decreases as the degree of cross-linking increases. Gel rigidity is also closely associated to the chain length of the cross-linking agent. Cellulose is the most abundant hydrophilic polysaccharide. It has long been available in fibrous or microgranular forms. Cellulose, a linear homopolymer of β-D- glucopyranose units joined with glycosidic bonds, is the most abundant natural polymer (normal softwood contains 37–43% of cellulose). Cellulose structure has three reactive hydroxyl groups in every constitutional hydroglucose unit and can be used as an alternative raw material for the preparation of various functional polymers [32].

1.3.2. Synthetic approaches to ion-exchange fibers

It is essential to modify the properties of polymer-based fibrous materials according to tailor-made specifications designed for target applications. There are several means to modify polymer properties, viz. blending, grafting, and curing. 'Blending' is the physical the physical mixture of two (or more) polymers to obtain the requisite properties. 'Grafting' is a method wherein monomers are covalently bonded (modified) onto the polymer chain, whereas in curing, the polymerization of an oligomer mixture forms a coating which adheres to the substrate by physical forces. Curing gives a smooth finish by filling in the valleys in the surface. Several grafting approaches to the modification of cellulose fibers are described, namely: (i) physical treatments such as corona or plasma treatments under different atmospheres; (ii) radiation grafting utilizing UV, gamma; (iii) chemical or photochemical grafting; (iv) enzymatic grafting.

1.3.2.1. Grafting initiated by chemical means

Grafting can proceed along two major paths in chemical means, viz. free radical and ionic. In the chemical process, the role of initiator is a very critical parameter as it determines the path of the grafting process. Apart from the general free-radical mechanism, grafting in the melt and atom transfer radical polymerization (ATRP) are also interesting techniques to carry out grafting. In the chemical process, free radicals are produced from the initiators and transferred to the substrate to react with monomers to form the graft co-polymers. In general, one can consider the generation of free radicals by indirect or direct methods. Surface grafting by controlled radical polymerization has been reported from polymeric substrates, colloidal particles and inorganic materials [35-44].

1.3.2.2. Grafting initiated by radiation means

In the radiation technique, the presence of an initiator is not essential but the medium is important in this case. The lifetime of the free radical depends upon the nature of the backbone polymer. Grafting proceeds in three different ways: (i) pre-irradiation (ii) peroxidation and (iii) mutual irradiation technique. In the pre-irradiation technique the polymer backbone is first irradiated in vacuum or in the presence of an inert gas to form free radicals [45-48] whereas in the peroxidation grafting method, the trunk polymer is treated to high-energy radiation in the presence of air or oxygen to form hydroperoxides or diperoxides, depending on the nature of the polymeric backbone and the irradiation conditions. The stable peroxy products are then treated with the monomer at higher temperature, whence the peroxides undergo decomposition to radicals, which then initiate grafting. The advantage of this technique is that the intermediate peroxy products can be stored for long periods before performing the grafting step. In case of mutual irradiation technique, the polymer and the monomers are irradiated simultaneously, to form free radicals and subsequent addition [49-51]. Since the monomers are not exposed to radiation in the pre-irradiation technique, the obvious advantage is that the method is relatively free from homopolymer formation, which occurs with the simultaneous technique. However, the decided disadvantage of the pre-irradiation technique is scission of the base polymer due to its direct irradiation, which can result in the formation of block co-polymers.

Plasma polymerization technique offers about the same possibilities as with ionizing radiation [52] and the main processes in plasmas are electron-induced excitation, ionization and dissociation. Thus, the accelerated electrons from the plasma have sufficient energy to induce cleavage of the chemical bonds in the polymeric structure, to form macromolecule radicals, which subsequently initiate graft co-polymerization. The principle involved in an enzymatic grafting method is that an enzyme initiates the chemical/electrochemical grafting reaction [53]. Several methods concerning graft copolymerization onto cotton and cellulose materials using chemical initiation, Ultraviolet (UV), cold plasma, electron-beam and gamma rays have been described in the last years. All of them made use of the pre-irradiation and peroxidation techniques. In general chemical methods utilize cerium salts, which low polymerization efficiency. UV and cold plasma methods yield mainly surface modified materials due to their low penetration efficiency. Electron beam is a high energetic source delivering energy in a very short time and thus increasing the unwanted degradation process of trunk polymers.

UV-initiate graft polymerizations of methacrylates onto cotton to reinforce these materials and the mechanical strength and wetting behavior of cellulose-based textiles have been improved [54]. The use of electron beam as a activation method where powdered samples of flax, cotton and viscose from textiles fibers were subjected to an electron beam (20-400 KGy) and further treated with glycidyl methacrylate (GMA) solution grafts the samples [55] A cotton fabric was grafted by gamma rays from a ^{60}Co source and epoxy groups present in the GMA grafted fabric was reacted with ethylene diamine and subsequently modified with phosphate [56]. Takacs *et al.* described a pre-irradiation to graft cotton cellulose by gamma rays ^{60}Co source where the degree of polymerization values for cellulose drops sharply from 1700 to 500 after 10 kGy irradiation [57]. Also grafting where GMA was graft polymerized to plasma treated fibers and where β-cyclodextrin was chemically bound to the grafted surface has been described [58]. However, reactive sites are only present on the polymer surface i.e. a fiber modification limited to its external surface is achieved. A traditional method to obtain grafting is based on purely chemical strategies utilizing various monomers, crosslinking agent and different concentration of ceric ammonium nitrate as initiator in aqueous nitric acid solutions at room temperature. This method is laborious, time consuming and requires successive chemical modification to produce adequate modification of the substrate. None of above mentioned methods are appropriate to produce potential chromatographic supports since grafting is inhomogeneous, limited to substrate external surface, swelling properties are difficult to control, ligand immobilization is difficult too due to steric hindrance and the mechanical and chemical properties of the resultant chromatographic support are poor and moreover there is lack of internal hydrogel which in turn reduces the swelling ability of the resulting composite and concomitantly the specific adsorption properties.

1.3.2.3. Grafting initiated by gamma radiation means

The fibrous material utilized in this research thesis was grafted to a hydrophilic polymer fiber in a gamma radiation induced simultaneous polymerization [59]. The gamma radiation induced polymerization performed with a radiation dose of 5-15 kGy and a dose velocity of 0.1-1 kGy/h in the absence of a polymerization of initiator substance, peroxides or azo compounds. The resultant composite fiber exhibits an internally grafted homogeneous hydrogel where reactive groups are introducible to perform ligand immobilization. Such homogenous grafting could not be achieved by pre-radiation grafting polymerization. It has been found that polymerization in liquid medium provides advantages over the use of gaseous monomers regarding homogeneity of grafting, swelling rate, pH stability, temperature

stability and general resistance against wear and tear. The composite exhibits a high swelling ratio, i.e. swelling factor of at least 10%, improving the mechanical stability of the chromatographic medium. Besides, the mentioned composite fiber is chemically very stable and advantageously allows to continually using a chromatography medium for many cycles. The composite after proper ligand immobilization can act as a chromatography medium, allows diffusion of target molecules into its composite core of the microfiber, and increases the dynamic binding capacity independent of the convective flow properties due to the large number of binding sites. The presence of reactive groups onto the composite material allows for the addition of various ligand types, which are useful or specific adsorptive process. The composite has a length of 0.5-10 cm with a thickness of 5-80 µm and fiber diameter increases 2-3 times upon swelling, further aiding in providing a good dynamic binding capacity with good convective flow properties. Another advantage of chromatography media of the mentioned composite media is the simplicity of handling. In or as chromatography media, the composite fibers are preferably arranged in the form of stacked disks, rolls or in parallel or twirled arrangement. Composite fibrous material can be easily arranged in a parallel configuration that can in preferred embodiments alleviate in combination with an appropriate surface shielding, deleterious biomass effects on system hydrodynamics and sorption performance and opportunity to offer as disposable cartridges.

1.3.3. Characterization of ion-exchange fibers

Fibers or membranes are typically described by their pore size, however the key characteristics in the design of a fibrous adsorber are really the selectivity, permeability, and the system capacity [60, 61]. The intrinsic selectivity of the fibrous adsorber is determined by the chemistry of fiber surface, i.e. the thermodynamic binding isotherm. The permeability is related to terms of pressure drop, especially at high flow rates. The system capacity is defined as the volume of feed that can be processed per unit of fiber area (volume). Using combination of biophysical and biochemical methods, the fiber morphology and structural changes after functionalization can be characterized. Scanning electronic microscopy (SEM) was used to visualize membrane morphology under dry state. Light microscopy and fluorescence microscopy have been employed for investigation of protein adsorption [58, 59]. Environmental scanning electron microscope (ESEM) has been used to study the swelling behavior of cellulose fibers in situ [64] and the wetting properties of macroporous polymer membrane [92]. Confocal laser scanning microscopy (CLSM) has been used long in medical research and microbiology for the identification of structures within cells and other tissues

[65]. This technique is now a powerful analytical tool [66] and has also been proposed for the characterization of adsorbent particles [67, 68] or porous membrane adsorbers in static state [69, 70]. The use of two different fluorescence dyes for labeled membrane and protein which can be detected independently enables simultaneous visualization of membrane pore structure and protein binding to the membrane functional layer by CLSM. Charcosset et al [69] investigated the membrane morphology by using CLSM in reflect mode. The Energy Dispersive Spectroscopy (EDS) is an analytical technique used for the chemical characterization of a specimen used in concert with SEM for compositional microanalysis. It is useful for material characterization since it can perform qualitative and semi-quantitative microanalysis on a specimen from a relatively low (~25X) to high magnification (~20, 000X) [71, 72]. FT-IR attenuated total reflectance (ATR) spectroscopy can be used for the fast characterization of cotton fabric scouring process based on these changes of spectra [73].

1.3.4. Limitations of the method

Effects of heterogeneity are also found in the modification with chemicals in the wet state, where it is necessary to work in conditions with vigorous agitation to improve the homogeneity of the system. The induced grafting technology of high-energy radiation can generate highly reactive radicals and initiate polymerization and extension graft chains on common polymeric materials of low reactivity chemistry. This consists of polymer radiation contact with a solution of one or more monomers. The use of chromatographic supports with a fibrous geometry is one novel approach to developing chromatographic systems for scaled-up separations. Systems based on fibrous supports, specifically, the randomly packed short-fiber (RPSF) columns were studied earlier [35, 36] but not in process scale level. RPSF columns are similar to conventional chromatographic columns, except that they use short, fibrous supports in place of spherical materials. The fibrous geometry gives a packing configuration that enables higher liquid flow rates, and the potential for significantly higher mass transfer rates. The major obstacle to the implementation of RPSF columns is the lack of fibrous substrates suitable for process bioseparations and also limits the absorption capacity of the column and consequently the throughput due to its poor mass transfer behavior [36]. So a number of modifications in the synthesis polymer based fibers will be investigated to achieve above said objectives.

1.4. Problem statement and outline of my research objectives

The high demand, as well as the innovative power and the therapeutic potential of the coming biopharmaceutical molecules with ever higher quality requirements is associated with increasing demands on the biotechnology industry. The mass flow rate in a biotechnology manufacturing facility is limited mainly by the need for recovery and purification of the downstream processing of products. Downstream processing (DSP) is characterized by complex conventional multi-step filtration and chromatography which have become limited in total available manufacturing capacity, process efficiency and cost. Desirable here is in environmentally and economically sound one-step purification (chromatography without water, energy, time consuming and costly pre-filtration). In industrial DSP, chromatography purification plays a central role in economic considerations due to advanced development in upstream large scale production of cell densities which effect on the capacities and quantities in the downstream purification by chromatography media and systems. The physical properties of the current chromatography media rise limitations in the direct use of adsorbent media for the capture of target product coming from the fermentation and have low or no air tolerance. Therefore different pre-filtration unit operations are required for the clarified broth thus not to clog the system which leads to high buffer and cost. The diffusional mass transfer properties associated within the porous spherical beads has influence in the reduction of the dynamic binding capacity (DBC) of the resins to capture desire target product. An increase in protein yields and number of purification steps will require manufacturers to invest in larger equipment and utilization of large volumes of buffer and water in the processes. The column design reduces throughput as a result of the increased pressure drop at higher flow rates and high material and operational (cleaning and validation) costs. These circumstances limit the actual turnover of a purification biotechnology plant significantly and are relevant to the operation expensiveness.

Therefore, efficient technologies and innovative and sustainable solutions required in relation to the use of production time, cost and resources is a key challenge for companies in the biotechnology sector [10]. As a consequence, the separation and purification schemes applied in the biopharmaceutical industry [74-78] requires alternative materials for adsorption chromatography to replace traditional packed-bed column resins and reduce the number of column chromatography steps from three to two, and eventually to one with efficient systems.

Although, alternative procedural approaches have been available for adsorption chromatography in the current market, but many of them giving inefficient and cost effective solutions to the industrial DSP. However, the introduction of expanded bed adsorption (EBA) has allowed early process integration by coupling solids removal, dewatering, and fractionation in a single step: this is usually referred as direct “capture” or sequestration from a crude feedstock [79]. A key drawback in EBA technology is the intrinsic difficulty of maintaining an appropriate (close to plug-flow) hydrodynamic condition of the fluidized/classified bed, which in turn causes a reduction of the overall system dynamic binding capacity for the target product [80]. Many commercial membrane adsorbers have been applied for removing of trace impurities and potential contaminants in flow-through mode and for separation of large solutes such as DNA, RNA and viruses in bind-and-elute mode and endotoxin in capture and polish steps [81-83]. Membrane absorbers might also be a potential alternative for counteracting the downstream but has the disadvantage of limited capacity and not tolerate any biomass which requires costly pre-filtration steps to prevent clogging of the adsorbent. However, lower surface-to-bed volume ratios generated lower binding capacity of membrane absorber and inefficient flow distributions of membrane modules remain a challenge for wide routine application in process-scale productions [84, 85].A recycling is very expensive (buffer consumption, time) and also not scalable to large scale process operations, so the systems are mainly offered as disposable devices.

A variety of chromatographic systems for bioseparations have been reported and the use of chromatographic supports with a polymer based fibrous geometry is a prominent among the novel systems. The adsorptive fibrous substrates which act as chromatography support for the functional ion exchange and affinity ligands used during a selective adsorption process [74, 86]. These fibers are coupled with synthetic polypropylene, poly (butylene terephthalate), PES (polyethersulfone) and nylon, which exhibit high surface area, variable and controllable porosity and interconnectivity of pores within the fabric matrices, have applications in the production of recombinant proteins [29, 87] because of their low cost, biocompatibility and excellent mechanical strength and stability. With these fabrics, ion exchange or affinity ligands could be directly attached to the fiber surface, thus eliminating slow intra-particle diffusion limitations as the process fluid is transported across the membrane. Besides, the controllable pore size and overall porosity of the membrane can reduce concerns of high pressure drops with elevated flow rates, resulting in increased throughput compared to column resin operations. These factors allow for much faster processing times and potentially binding for purifying valuable target biological molecules in terms of bound product per volume of

resin [76, 85]. Systems based on fibrous supports, specifically, the randomly packed short-fiber (RPSF) columns were studied earlier [34, 88] but not in process scale level. The major obstacle to the implementation of RPSF columns is the lack of fibrous substrates suitable for process bioseparations and also limits the absorption capacity of the column and consequently the throughput due to its poor mass transfer behavior [34]. The fibrous geometry could give a packing configuration that enables higher liquid flow rates and the potential for significantly higher mass transfer rates translate into product throughput in developing for scaled-up separations. However, none of the mentioned systems for fibrous substrates have been reported to act as potential chromatography support for high capacity and high throughput in bioseparation due to low porosity of the material, irregular column packing in process scale operation, development of back pressure with increasing operational flow rates resulting in lower productivity and poor resolution of target product form the crude mixture. And more significantly they are not suitable for process bioseparation due to lack of biomass compatibility [34]. For developing high performance fibrous adsorption operation systems with potential for using in industrial downstream processing one needs to explorer the following questions:

- Will there be any fibrous base adsorption systems available to produce high capacity and high throughput bioseparations to replace traditional beads and membrane adsorption?
- Could it be possible to design an intensified and integrated bioprocess adsorption system for direct capture of target product from fermentation and high resolution intermediate purification applications?
- Is there any available efficient and cost effective fibrous adsorption based cartridge system to replace traditional columns and membranes in bioprocess scale?
- Could it be possible to tackle real applications in industrial downstream process scale with the fibrous base adsorbent systems?

Complementing all the above issues, systems based on novel fibrous ion-exchange adsorbents depicting high capacity and high throughput have been developed for the industrial downstream processing of bioproducts in this research work. Following outlines of research objectives have been presented in this dissertation:

In the first part of this thesis, chapter one, a general introduction to downstream chromatography purification and limitations in bioprocess scenario is be given and then followed by present practical methods alternative to packed bed adsorption operations. At the

end of the chapter the utilized fibrous adsorbents properties and comparative potential advantages with other methods will be presented. The second chapter of thesis describes preparation, characterization and process performance of innovative fibrous adsorbents (gPore) as cation exchangers for high capacity and high throughput bioseparations. Here the innovative gamma irradiated composite fiber harboring cation exchangers has been characterized by a plethora of physiochemical methods. A unique packing design and column efficiency with fibrous adsorbent and their functional characterization studies will be demonstrated. And importantly, superior performance of fibrous adsorption was demonstrated by an intensified and integrated chromatography process during early protein capture and intermediate-resolution applications. The third chapter will be devoted to synthesis of strong anion-exchange fibrous adsorbents using chemical grafting method and subsequently investigated the physical and functional characterization of the fibers. The hydrodynamic and sorption properties of these fibrous adsorbents will be presented for process scalable studies. Chapter four will be within the frame of confidential work with ChiPro GmbH and allocated to design and development of process technology scaling of a suitable prototype fibrous cartridge system for industrial downstream processing. The real application of fibrous adsorbent system as an efficient and cost effective solution to industrial downstream processing will be demonstrated in the case study of large scale purification of bioproduct. This part is done within the frame of confidential work in cooperation with Phytolutions GmbH. Chapter six, presents mass transfer properties of fibrous adsorbents in relation to process performance and compared with available conventional technologies. In the last chapter of this thesis, finally wrap up with the main important conclusions and future remarks.

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Chapter 2

Preparation, characterization, and process performance of composite fibrous adsorbents as cation exchangers for high throughput and high capacity bioseparations

2.1 Introduction

In the literature, numerous methods concerning graft polymerization onto cotton and cellulosic materials using chemical initiation, ultraviolet light (UV), cold plasma, electron-beam and gamma rays have been described in which all made use of pre-irradiation and peroxidation techniques. None of these methods, however, are appropriate to the production of potential chromatographic supports, since grafting is in-homogeneous and limited to substrate external surface, swelling properties are difficult to control, ligand immobilization is complicated due to steric hindrance and the mechanical and chemical stability are poor for the resultant composite. Moreover, there is a lack in internal hydrogel which reduces the swelling ability and concomitantly the specific adsorption properties [1-8]. In the recent years, only a few limited studies can be found on the activation of nonwoven membrane fabrics by surface grafting as an alternative to cotton fabric for bioseparations with low throughput and low dynamic binding capacities which are equal to or lower than commercial bead packed columns [9-11].

This chapter reports the advantages of the characteristics of a composite fiber which acts as a potential chromatography media which was synthesized by the mentioned procedure [12]. Using this method, it is possible to produce composite fibrous structures of high degree of grafting with homogeneous internal hydrogel which provides mechanical and chemical stability to the medium and also exhibits high swelling ratio, particularly good flow and adsorption properties can be ascertained. This composite fibrous chromatography media is having increased system productivity, which binds reversibly the target species and which is tolerant to biomass in order to allow direct processing of a crude feed stock. The composite fibers are having irreversible open compact structure with internally grafted homogeneous hydrogel, where reactive groups could be directly introduced to perform ion exchange or affinity ligand immobilization to the fiber surface, and thus reducing diffusion limitations as the process fluid is transported across the fiber. In addition, the pore size and overall porosity of the fiber can be reduce concerns of high pressure drops with increased operational flow rates, resulting in increased throughput compared to other column operations. Composite fibers also offer promising prospects in developing single use disposable bioseparation devices in process scale operations due to low production cost and relatively inexpensiveness of cotton fabric materials. In earlier works, the issue of column packing, high throughput and

high capacity for purification factor and process bioseparation, has not been addressed properly, so this is the first research to deal with these issues directly.

In this research chapter, the synthesized gamma irradiated simultaneously grafted composite fiber was further immobilized with functional ligands with proper surface activation thus being a strong cation exchange adsorbent and characterized for physical and functional properties. The fibrous columns, packed double roll form either in randomly for purification or non-randomly for process bioseparation, have been evaluated. Besides, permeability coefficient of the composite support was measured. Fibers were functionally characterized by a pH transition method to determine the total ionic capacity. For the column characteristics of the packed fibers, breakthrough analysis have been performed for operation in the frontal mode and compared with commercial resin values in the literature. The composite fiber as a chromatographic support was applied in lysozyme isolation from complex protein mixture and purification behavior results were compared with commercial strong cation-exchange resin. The performance of the mentioned composite material for capturing target product from biomass was explored in process bioseparation applications.

2.2 Experimental materials and methods

2.2.1 Materials

Glycidyl methacrylate (GMA), dimethyl acrylamide (DMAA), 2-(N-Morpholino) ethanesulfonic acid (MES), from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Acetone, ammonium persulphate (APS), sodium sulfite anhydrous, isopropanol, ethanol, sodium hydroxide, sulfuric acid and lysozyme were purchased from AppliChem GmbH (Darmstadt, Germany). N,N,N,N'-Tetramethylethylenediamine (TEMED) and low molecular weight protein marker were purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany). UV-visible spectrophotometer Specord 200 was purchased from Analytik Jena (Jena, Germany). Immunoglobulin (IgG) was obtained from Octapharma GmbH (Munich, Germany). Fluorescence dyes Cy3, chromatography columns, Tricorn [0.5 mm internal diameter (ID) × 5.5 cm length (L)], XK16 (16 mm ID × 20 cm L), SP Sepharose FF resin and the ÄKTA explorer system equipped with Unicorn 4.10 software for data collection and analysis were obtained from GE Amersham Bioscience (Uppsala, Sweden).

2.2.2 Sample preparation and characterization

2.2.2.1 Preparation of composite fiber by gamma radiation induced grafting

gPore - an innovative composite fibrous material was developed according to the mentioned procedure [32]. Briefly, a heterogeneous solution of 1.0 g of the dry mercerized hydrophilic polymeric material (TN & Platex, S.A., Buenos Aires, Argentina) was soaked in 50 mL of a monomer solution (GMA 1.6 % (v/v) and DMAA, 5% (v/v) in methanol/water, 1/1, (v/v)), previously degassed by nitrogen gas bubbling. The mixture was enclosed in a sealed tube to avoid oxygen diffusion during irradiation. Samples were irradiated with a 10 kGy dose at room temperature employing a ^{60}Co PISI semi-industrial irradiation source (CNEA, Ezeiza, Argentina). After irradiation, the resulting material was washed with an ethanol/water mixture (1/1) and subsequently with 96% ethanol. Washed composite fibrous material was dried for 24 h in a vacuum oven at 40 °C until constant weight was reached.

2.2.2.2 Ligand immobilization

Pendant epoxy groups from grafted polyGMA were reacted with a mixture of sodium sulfite / isopropanol / water (10:15:75 by weight) at 37 °C overnight and further treated with 0.5 M sulfuric acid solution at 80 °C for three hours, to hydrolyze the residual epoxy groups. After extensive washing with water, the functionalized fiber composite material was dried. Then the epoxy groups were quantified according to the mentioned procedure [13]. The extent of grafting / modification was determined gravimetrically and expressed as degree of grafting (DG) which was calculated as the percentage of increase in weight, as follows:

$$\mathrm{DG}\ (\%) = \frac{(W_1 - W_0)}{W_0} \times 100 \qquad (2.1)$$

Where W_0 and W_1 are the initial and grafted polymer weights, respectively

2.2.3. Scanning electron microscopy

Gold sputtered samples were examined at different magnification using a Joel JSM 5900 (Peabody Inc., USA) scanning electron microscope (SEM). Energy-dispersive X-ray spectroscopy (EDS) coupling to SEM for elementary analysis was performed in collaboration with our scientific partner at the Centro de Microscopías Avanzadas (FCEN-UBA) in Argentina and spectra were obtained with a Zeiss Supra 40 field-emission SEM and EDS Spectroscopy (Carl Zeiss NTS, LLC, USA). All samples were equilibrated in 3 M KCl in

phosphate buffer and rinsed with distilled water. Then the samples were dried in an oven at 45 ^{o}C up to constant weight.

2.2.4. Confocal laser scanning microscopy

The visualization of internal localization of grafted, functionalized sulfonate group composite fiber (SP gPore) morphology with protein adsorption was investigated by confocal laser scanning microscopy (CLSM). Briefly, a known amount of the SP gPore sample was incubated with an IgG labeled amino-reactive fluorescence dye Cy3 in adsorption buffer (0.1 M potassium phosphate pH 7.4) for 2 h at room temperature on a shaker. Then the samples were thoroughly washed with adsorption buffer and observed under confocal microscopy. SP Sepharose FF was utilized as a control cation exchange material. Confocal images were taken using a Carl ZeissLS510 (Jena, Germany; software version 3.0) with an oil immersion (63x /NA1.2 C-Apochromat objective lens). The obtained images were collected using the laser excitation sources ~550 nm, ~570 nm and recorded using a filter BP530-550.

2.2.5. Fourier transform infrared spectroscopy

To confirm the introduction of functional groups onto the composite fiber, the dried samples were measured directly using Fourier transform infrared spectroscopy affinity instrument equipped with attenuated total reflectance (FTIR-ATR) accessory, PIKE GladiATR diamond single reflection (Shimatzu Corporation). The FTIR-ATR spectra were acquired by scanning the specimens for 32 times in the wave number range from 550 to 4000 cm^{-1} with a resolution of 0.4 cm^{-1} and analyzed with IRsolution Shimatzu 1.50 software.

2.2.6. Measurement of degree of swelling

The degree of swelling (DS) was measured by weighing the composite fiber material in the wet state (m_{wet}) and after drying (m_{dry}) to constant weight at 60 ^{o}C in a vacuum oven as in the expression given below,

$$\text{DS (g } per \text{ g)} = \frac{(m_{\text{wet}} - m_{\text{dry}})}{m_{\text{dry}}} \qquad (2.2)$$

2.2.7. Determination of material porosity by water uptake

The total volume of composite fiber (Porosity, expressed as %) within the material body

was estimated by water uptake of the "squeezed" swollen sample, according to:

$$\text{Porosity}\ (\%) = \left[\frac{m_{\text{swollen}} - m_{\text{water-bound}}}{m_{\text{swollen}}}\right] \times 100 \qquad (2.3)$$

Where m_{swollen} is the mass of the material once water uptake has occurred, and $m_{\text{water-bound}}$ is the mass of the dry material once is has gained bound water by absorption of water vapor.

2.2.8. Column packing

In or as chromatography media, the composite fibers are preferably physically arranged in the form of stacked discs, simple rolls, or in parallel or twirled arrangement either in packed or open form by randomly packed bed (RPB) and non-randomly packed bed (NRPB). In this work, a unique packing technique was utilized where a defined amount of fibrous material was designed in a double roll on a thin flexible plastic net support structure considering proper aspect ratio (AR) and expansion factor (EF). This double roll composite fibrous material was randomly packed for the resolution applications and non-randomly for the process bioseparation applications in lab scale columns. The packed fabric bed was then swollen in place with 20 mM phosphate buffer (pH 7.4) at very low linear flow velocity (~75 cm/h) and the bed height of the column was fixed so that there was no visible head space in the column after swelling of the medium contained in the functionalized composite fiber. The column packing efficiency was evaluated in terms of height equivalent to a theoretical plate (HETP) by acetone pulse experiments and peak asymmetry factor (A_s) by acetone pulse experiments.

2.2.9. Flow permeability experiments

A fixed adaptor column (5 mm ID × 5.5 cm L), with 0.2 g of functionalized fibrous material, was prepared for flow permeability experiments. The experiments were conducted at room temperature on an ÄKTA explorer FPLC system with an internal pressure monitor. The height of the packed bed was 5.5 cm and pressure drops along the length of fibrous packed column were measured at different linear flow velocities between 150 and 600 cm/h of phosphate buffer. The extra-column pressure drop was accounted for by conducting the same measurements by passing the fibrous column in the system. The measurements of pressure drops were determined by the difference in the pressure at the inlet and the outlet of the

column observed in the pressure monitor.

2.2.10. Determination of the ionic capacity

Transient pH phenomenon can be employed to gather the ion exchange capacity of porous media [14]. System ionic capacity was measured by using two buffer solutions with different ionic strength yet the same pH value. For the measurement of the cation exchanger capacity the following solutions were utilized: 20 mM Tris–HCl pH 7.4 (buffer A) and the same buffer containing 1.0 M sodium chloride (buffer B). The functionalized fibrous composite material in a glass column (5 mm ID × 5.5 cm L) was mounted on ÄKTA explorer FPLC system, and a control bed of packed commercial adsorbent beads was equilibrated with buffer A. The mobile phase composition was then switched to buffer B. The time interval Δt (pH) recorded between the switching point of the mobile phase and the point at which 50% of the maximum reachable pH value (K) was actually obtained was recorded. Calculation of the ionic capacity q (mequiv./g) was done from the relative elution volumes, normalized by column volume, as described elsewhere [14, 15].

The following equation was utilized:

$$K = \left[\frac{\Delta t(pH)\phi_v}{V_c}\right] = a \times q + b \qquad (2.4)$$

where ϕ_v is the volumetric flow rate (mL/min), V_c is the column volume (mL), and $\Delta t(pH)$ was calculated from the pH transition profile experiments. For cationic systems, a = 1.57 and b = 0.5. These constants were previously calculated by Nika Lendero et al [15] for similar systems. SP Sepharose FF was utilized as a control ion-exchange material.

2.2.11. Protein adsorption equilibrium under static conditions

Lysozyme equilibrium adsorption isotherms under the static conditions were measured by batch experiments. A known of amount (0.1 g) of the SP gPore and SP Sepharose FF samples were taken and equilibrated with phosphate buffer (pH 7.4) in 15 mL falcon tubes. An initial protein concentration between 0.0 mg/mL and 10.0 mg/mL was prepared from stock solution of lysozyme at 10.0 mg/mL by mixing a known amount of protein with phosphate buffer. Appropriate combinations of stock solution and phosphate buffer were added to each test tube containing the sample to provide a final volume of 14 mL in each tube and an initial protein concentration between 0.0 mg/mL and 10.0 mg/mL protein. A 1.0 mL liquid sample from

each of the different initial protein concentrations was immediately taken and UV absorbance measured at 280 nm. Then the samples were then placed on a rotofor mixer rotating at ~40 rpm. After mixing for a minimum of 24 h, liquid from each sample was removed and the protein concentration was determined by UV-280-nm absorbance with a UV spectrophotometer. By difference, protein adsorbed to the fiber could be calculated. Likewise; controls were monitored to evaluate the non-specific binding of lysozyme to the fiber which was negligibly accounted. Langmuir adsorption isotherms were then prepared and modeling constants determined by least-squares regression fit to the equation:

$$Q = \frac{Q_{\max} C}{K_{\mathrm{d}} + C} \tag{2.5}$$

where Q is the adsorbed equilibrium concentration; $Q_{\max}$ is the equilibrium adsorption saturation capacity; C is the final lysozyme concentration, and K_{d} is the desorption constant.

2.2.12. Dynamic breakthrough by frontal analysis

The dynamic binding capacity (DBC) at 10 % breakthrough for the lysozyme with SP gPore material was determined by frontal studies. The columns (Tricorn 5/50, XK16) of different dimensions were packed with fibers in stacked discs, simple roll and double roll structures and mounted to an ÄKTA explorer FPLC system and equilibrated by passing the phosphate buffer pH 7.5. Lysozyme solutions of concentration (2 mg/mL) were loaded through a super loop at linear flow velocities of 75-600 cm/h. When the initial protein absorption was reached, then the column was washed with equilibration buffer until the effluent concentration fell back to a baseline value. The effluent concentration of lysozyme was measured with UV absorbance at 280 nm by internal UV detector. Bound lysozyme was eluted with 1 M NaCl in phosphate buffer and the column can be reused for next cycle. The dead volume of the system was determined by acetone injection. Complete breakthrough curves were obtained essentially according to Plieve et al. [14]. Calculations were performed as follows:

$$\mathrm{DBC} = C_0 \frac{V - V_0}{CV} \tag{2.6}$$

where C_0 represents the feed protein concentration (mg/mL); V is the volume of protein solution required to reach the 10% breakthrough (mL); V_0 is the chromatographic system dead volume (mL); and CV is the total column volume (mL).

2.2.13. Purification of lysozyme from a mixture of proteins

The SP gPore elution behavior of lysozyme from a mixture of proteins was compared with conventional commercial beads under 3 different superficial velocities. These experiments were performed with the columns (Tricorn 5/50) mounted to an ÄKTA explorer FPLC system. A 0.4 g of dry functionalized gPore material and 1 mL of Sepharose FF, were packed inside the columns and loaded with injection of 200 µL loop of mixture of proteins having concentrations of lysozyme (0.5 mg/mL, pI 11.0), concanavalin A (~0.2 mg/mL, pI 5.5) and α-chimitripsinogen (~0.2 mg/mL, pI 9.0). The unbound protein was washed out with equilibration buffer A (MES 50 mM, pH 6) and the target protein was separated by passing Elution buffer B (MES 50 mM + 1 M NaCl, pH 6) in a linear gradient of 0-90% B in 20 column volumes (CVs). The eluted purification profiles were analyzed and compared with an UV detector.

2.2.14. Lysozyme separation from crude feedstock

The biomass tolerance experiments were performed with an artificial mixture as a model process. The open structure fibrous material, double roll packed column (XK 16) mounted to a system (Pharmacia LKB Pump 500 Control LCC-501 plus with UV external AD 900 detector). The bed height was 6 cm and the linear flow rate was 150 cm/h (5 mL/min). The column was loaded with biomass containing disrupted 1.5% yeast and 5 g/L lysozyme. This artificial biomass mixture, correspondes to a stationary growth phase cell density of 1.2 $x10^{-9}$ cells/mL (a final optical density of ~1.0 at 600 nm). Then washing was done with the equilibration buffer A (20 mM PO_4+ 0.1M NaCl, pH 7.5) and elution of the lysozyme with elution buffer B (20 mM PO_4 + 1M NaCl, pH 7.5) by applying a step gradient of 10%, 60% and 100% B. The eluted fractions were collected and analyzed on SDS-PAGE.

2.3. Results and discussion

2.3.1. Fiber characterization

The SEM images of untreated and treated gamma irradiated grafted functionalized cation-exchange composite gPore fibers are shown in the Fig. 2.1, respectively. Though not a high resolution is shown, it can be noticed that the images obtained by SEM of a cross section of the material at high magnification, evidence the presence of a polymethylmethacrylate

(PGMA) grafted layer, that nearly homogenously covers the surface of the fibers and also the irradiated sample indicates not considerable deterioration as compared to the untreated sample. This, inside formed layer of polymer is, providing good dynamic binding capacity and good convective flow and presents mechanical support and chemical stability to the fiber to act as a cation-exchange chromatographic adsorbent.

The extent of epoxy activation was found to be s >290 (µmole epoxy groups /g material). The degree of grafting yield, as measured by gravimetry, for the gPore-fiber was obtained with 30%. The substantial differences between the modifications made in this work and other grafting techniques are that the same radiation dose of 10 kGy applied is 10-20 times lower because the polymerization is carried out simultaneously [16]. However, yields obtained in the simultaneous grafting technique are higher due to the avoidance of side reactions and the decomposition generated free radicals [17, 18]. The degree of grafting, depending on the concentration of monomer used, radiation dose, solvent type and the porosity of the base polymer [19], otherwise resulted in polymeric bodies with undesirable characteristics such as low porosity, poor swelling, or lack of physical robustness. Additionally, a study by micro-spectroscopy Raman of styrene grafts on various substrates by pre-irradiation confirms that the modification occurs not only on the surface but also inside the substrate, and the increase in total degree of grafting is accompanied by a proportional increase in the number of grafts within the mass of the substrate [20]. The structural changes in the support material produced by high grafting degree can affect the mechanical characteristics so the tested polymers are attractive for use as supports for immobilization. Therefore, it is desirable that the modification in base polymer is only skin deep and this is achieved through technique utilized in this work. The swelling of cellulose in water is limited as a consequence of the presence of crystalline regions which restrict the mobility of the polymer chains [4].

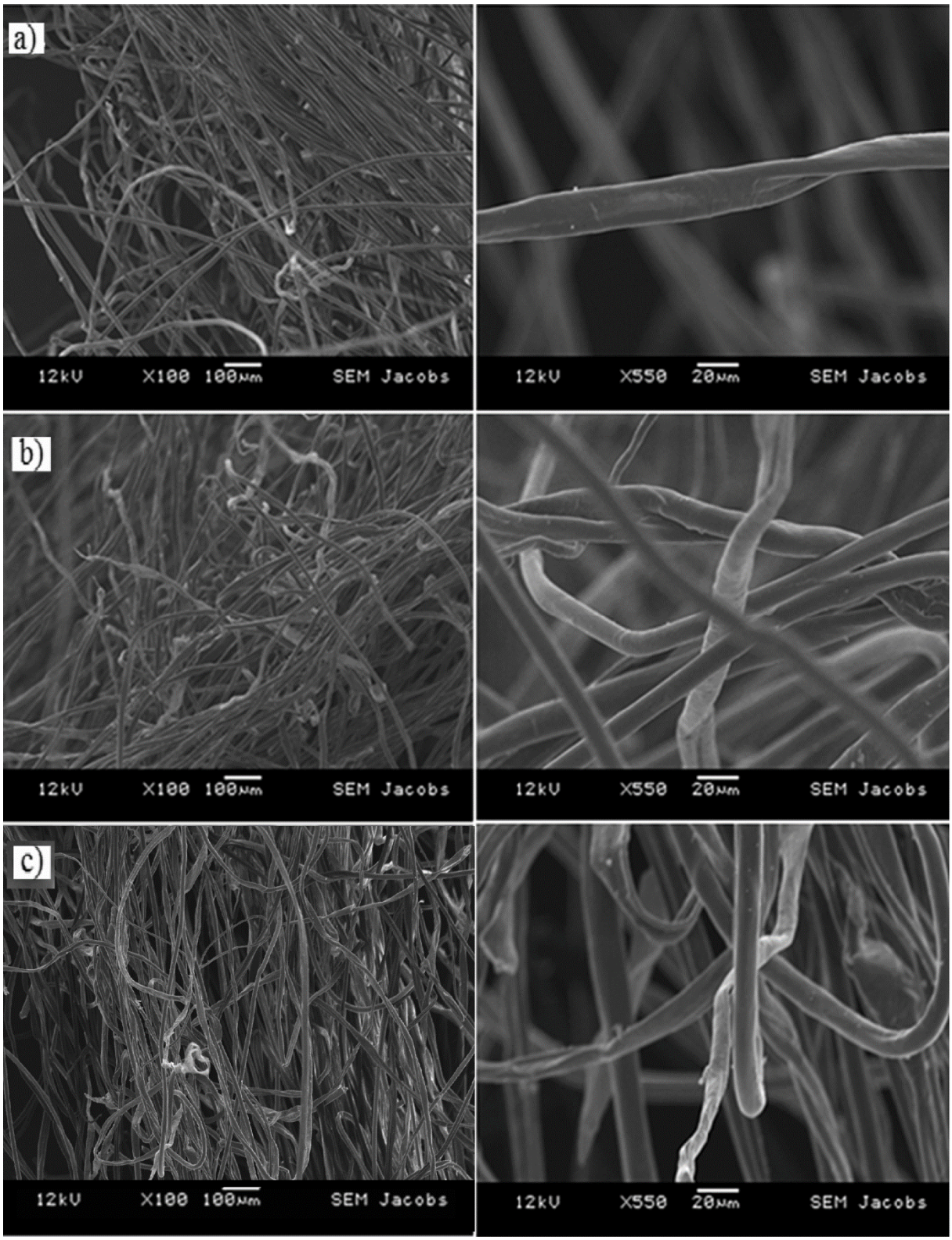

Fig. 2.1. Representative SEM images of (a) untreated, (b) gPore and (c) SP gPore in different scales.

The degree of swelling was observed as 2.95 g/g (~300%) in distilled water at 24 °C and having a substantially cross section; the fiber diameter increases upon swelling compared to the fiber in its completely dry state. The resulted composite fibers were having a thickness of 5-80 µm in their swollen state and a length of 0.5-10 cm [12]. With this swelling ratio, particularly good convective flow properties can be ascertained for the chromatography adsorbent. Water uptake experiments revealed a 75% porosity, which indicates that, the grafting procedure is not affecting the physical integrity of the pores present within the material. This is due to highly penetrative nature of gamma rays permitting their use in the homogenous and efficient modifications of the material.

To better ascertain the nature of the grafted and cation-exchange composite fiber (SP gPore), the EDS spectroscopy coupling to SEM and the obtained EDS spectra were collected inside the square drawn in the SEM pictures and are shown in Fig. 2.2. Visual inspections of the image do not reveal different morphologies onto the grafted surface or in the corresponding cross section of the material walls. A further structural insight was obtained by utilizing a grafted backbone containing pendant epoxy groups, which were further reacted with sulfonic acid. Table 2.1 is created from the peak integration of EDS spectra with elementary composition analysis. Some important parameters should be taken into an account during the analysis of EDS data, such as, (i) carbon percentage data could be higher than expected because the type used to steak samples to SEM holder is carbon based; (ii) potassium, chlorine and phosphate signals result from the salts used in sample equilibration; (iii) nitrogen signal is weak and close to carbon one, thus it is usually masked. Although the resolutions of the images were not properly selective because of the non-conducting properties of the material, however some predictable information can be achieved.

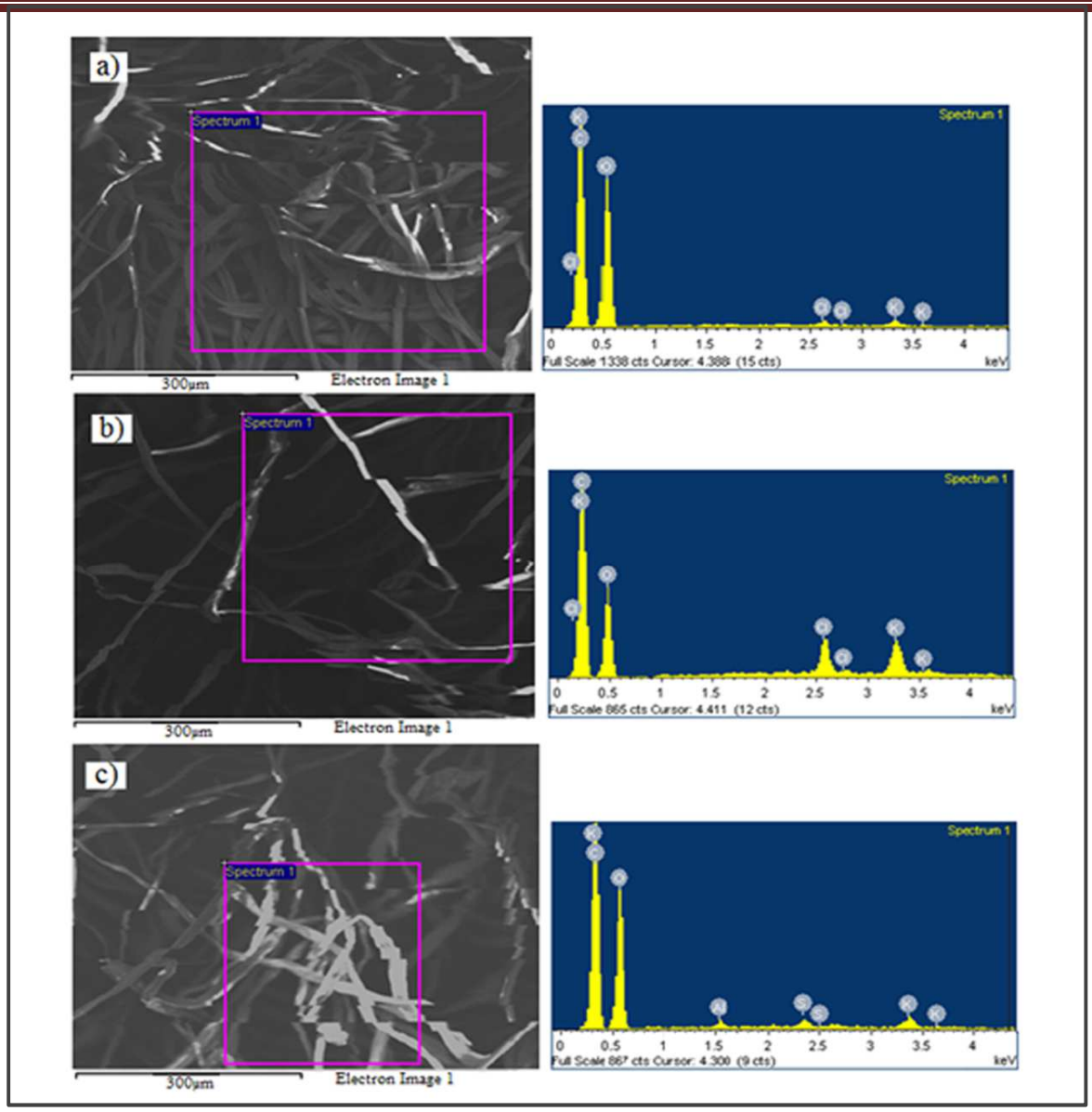

Fig. 2.2. Representative SEM images coupled EDS spectrum of a) untreated, (b) gPore and (c) SP gPore in full scales.

Thus, the EDS spectrum of the untreated and the grafted gPore fiber shows mainly carbon and oxygen. Chlorine and potassium signals are in low or high proportion (variable according to the efficiency of washing step) but in the same atom percentage. Sulfonate activated gPore material shows a sulfur signal and in addition the counter ion potassium signal can be detected. Thus this data reveals the direct information of the existence of negative charges onto the fiber.

Table 2.1: Elementary analysis of the samples with EDS.

Element	Original fiber[a]		Grafted fiber[a]		Composite with SP[a]	
	Weight (%)	Atomic (%)	Weight (%)	Atomic (%)	Weight (%)	Atomic (%)
C K	50.44	57.74	56.81	65.33	51.17	58.68
O K	48.90	42.02	37.87	32.69	47.23	40.66
Cl K	0.28	0.11	2.61	1.02	-	-
K K	0.38	0.13	2.72	0.96	0.75	0.26
[b]Al K	-	-	-	-	0.37	0.19
S K	-	-	-	-	0.49	0.21
Totals	100		100		100	

C-Carbon, K-Potassium, O-Oxygen, Cl-Chlorine, Al-Aluminium, S-Sulphur, [a] Number of iteration is 4; [b] Al Signal from background.

This implies that γ radiation-induced grafting polymerization has occurred with a nearly homogeneous distribution. These results are also in agreement with the observed swelling properties, mechanical and chemical compatibility between the grafting monomers and the backbone polymer.

2.3.1.1. Confocal laser scanning microscopy

The confocal images in Fig. 2.3b illustrates the typical morphology of hydrogel inside the composite fibrous structure, consisting of relatively thick cellulose-based fibers connected to a network of interconnected fibers and forms the macropores on the outer surface. The CLSM image (Fig. 2.3.b) of an IgG labeled with *Cy*3 fluorescence-stained fiber, reveals the very coarse structure containing the macroporous cellulose fiber network approximately, ~20 µm from the outer surface. The diameter of the larger pores is larger than ~15 µm. It had been found that fiber morphology in buffer can be visualized using CLSM in higher magnification, however in such conditions the complete profile cannot be obtained. Only a little part of matrix structure was visible as scale bar indicated in Fig. 2.3a and b. The control CLSM image of an IgG labeled with *Cy*3 fluorescence-stained commercial SP Sepharose FF is shown in 2.3a. The adsorbent gradually fills with the protein as expressed by penetration of fluorescing protein molecules into the internal regions of the beads. Protein binding as a

function of concentration and incubation times was also accounted by CLSM and related to the binding isotherms for these samples but needs further investigation. A study of the flow-through property and protein binding of the fiber can be monitored with CLSM, which might give important information on the dynamic behavior of porous membrane absorbers during separation.

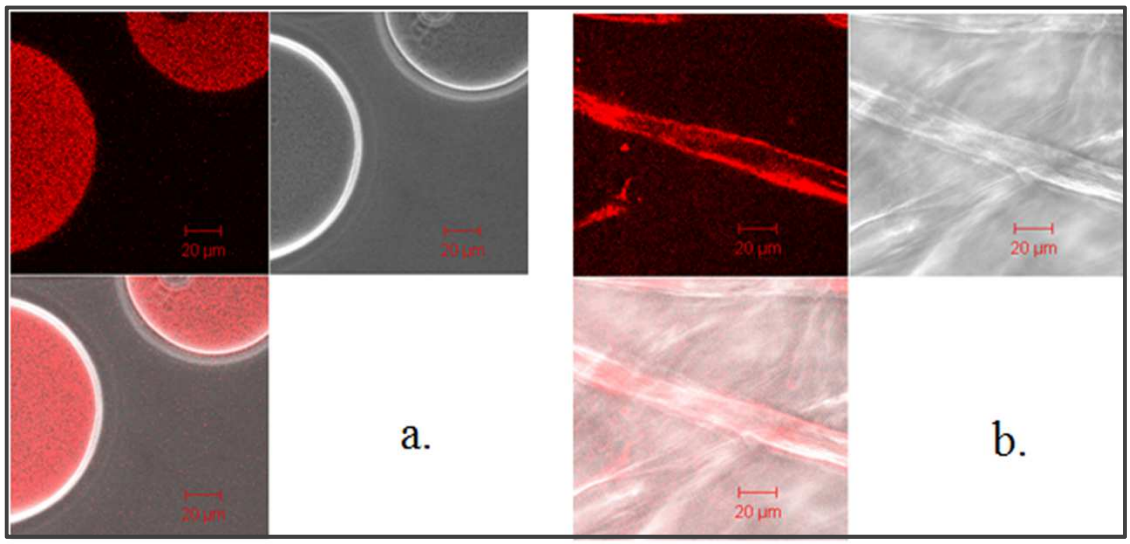

Fig. 2.3. Representative confocal microscopy studies on IgG adsorption: (a) SP Sepharose Fast Flow and (b) SP gPore.

2.3.1.2. Fourier transform infrared spectroscopy

To better understand the chemical changes involved in the sample preparation, the IR spectra of untreated, grafted (gPore) and cation-exchange composite fiber (SP gPore) are shown in Fig. 2.4. The appearance of characteristic peaks at 1720 cm^{-1} corresponding to the carbonyl of the GMA is not observed due to the overlap of this with characteristic peak of the cellulose base material and also epoxy signal (900 cm^{-1}) is masked due to many signals in that zone in the gPore and SP gPore. However, there are some indications that peak broadening (2900 cm) and a new signal at 2850 cm^{-1} both are attributed to the stretching of C–H bond in pGMA-DMAA. In gPore and gPore SP, the peak at 1640 cm^{-1} is corresponding to carbonyl of the DMA. There is an increase in absorption signal at 3400 cm^{-1} due to the stretching of C–N bond in DMAA. The characteristic absorption signals at 1000-1100 cm^{-1} and the observed area which show changes in the peaks shape and stretching of some characteristic bonds in sulfonic groups, are clear indications about the modifications in the SP gPore with respect to the untreated samples.

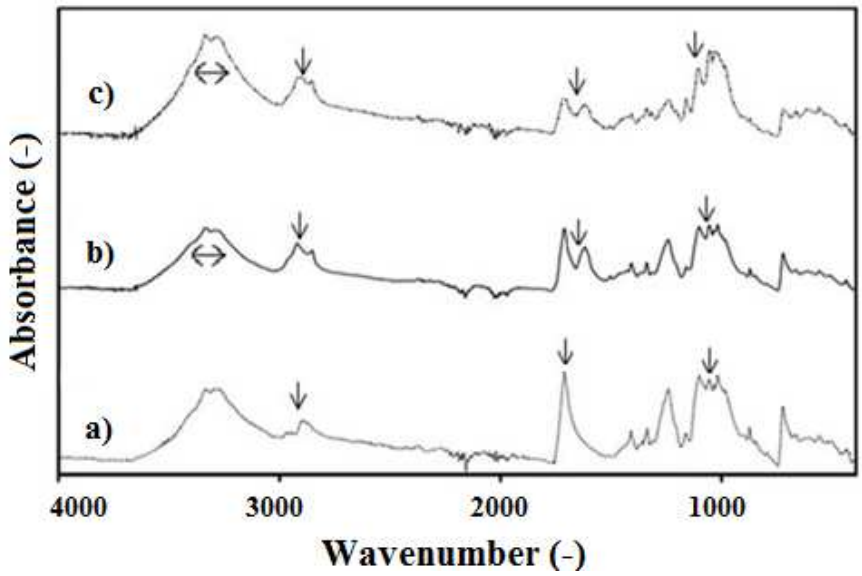

Fig. 2.4. FTIR-ATR spectra of (a) untreated, (b) gPore and (c) SP gPore.

2.3.2. Functional characterization

2.3.2.1. Column packing

Fig.5 illustrates a unique packing technique where a defined amount of fibrous material was designed in a double roll on a thin flexible plastic net support structure considering proper aspect ratio and expansion factor. The modified composite fibrous material as a chromatographic support in a packed column illustrates a unique situation where dry materials are swollen in a fixed column volume in which the chromatographic adsorbent is swelling. It has been known that while conditioning with equilibration buffer, the chromatographic adsorbent can expand 3–4 times its dry packing density (1.0 mL/0.2 g) due to its high swelling ratio. In order to ensure uniform column packing, swelling of the bed is done at very low linear flow velocity (75 cm/h), and therefore, hydrodynamic forces are not a factor in packing efficiency. It has been shown that the effect of packing heterogeneity in chromatographic columns might seriously affect separation efficiencies and lead to poor performance of chromatographic materials [21-24]. Fig. 2.1c evidences that the gPore fiber generated by gamma irradiated polymerization, has nearly homogenous distribution with internal hydrogel for mechanical support and good swelling properties. The best method of expressing the efficiency of a packed column is in terms of the height equivalent to a theoretical plate (HETP) and the peak asymmetry factor (A_s). It is of utmost importance to realize that the calculated plate number will vary according to the test conditions like changes

in solute, solvent, eluent, sample volume, flow rate, liquid pathway, temperature, etc. and it should therefore be used as a reference value only. In this paper, acetone was used as a probe solute under the non-retained condition in a fibrous packed 1 mL column volume, HETP and A_s were estimated at a linear flow velocity of 300cm/h and the resulting chromatogram is shown in Fig. 2.6. The resulted acetone peak shows a slight asymmetry (A_s ratio 1.5) indicative of some slight tailing with an HETP of 0.0647 cm/plate and a plate count of 1574 N/m. The plate count for this column is slightly less than the typical range and may reflect a slight loss in efficiency by operation of the column at a high flow rate (300 cm/h). Initial studies are usually carried out at 75 cm/h where the HETP and plate count will be optimal. The measured A_s values (0.8-1.8) are in a quite comparable range to commercial beads packed-bed columns operated at four different velocities as shown in the Table 2.2. This indicated that the uniform packing density of the resulting fiber-packed column is known to have good packed performance. Nevertheless, the resulted values for this column would be acceptable for use in an ion exchange purification process.

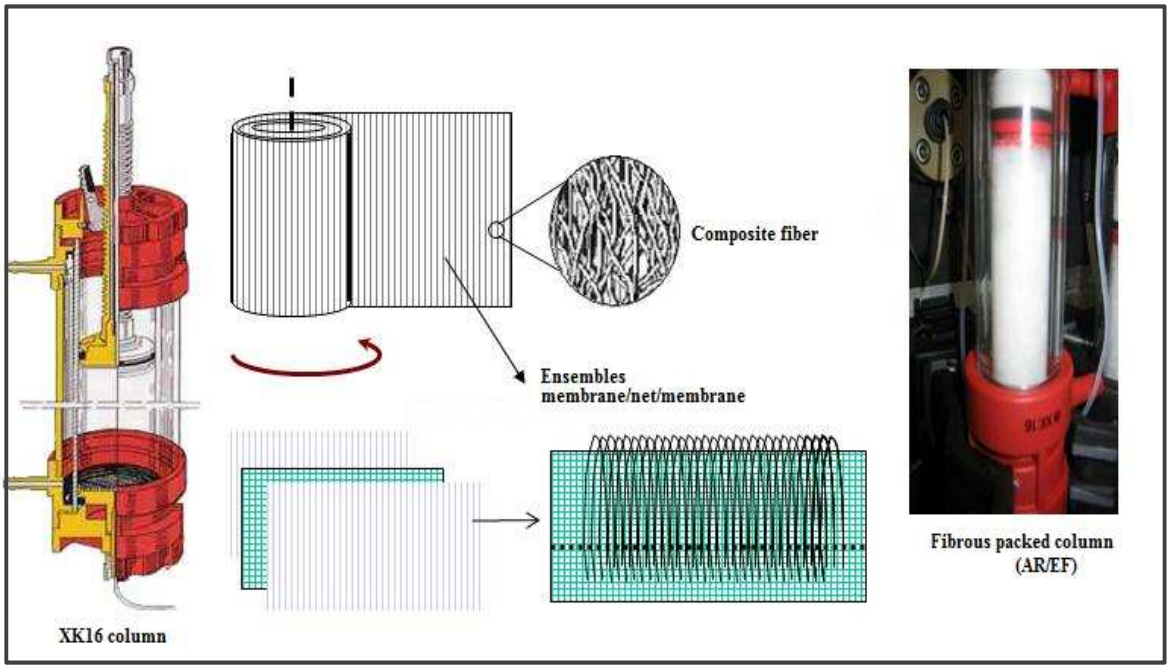

Fig. 2.5. Demonstration of a unique designed double roll column packing technique.

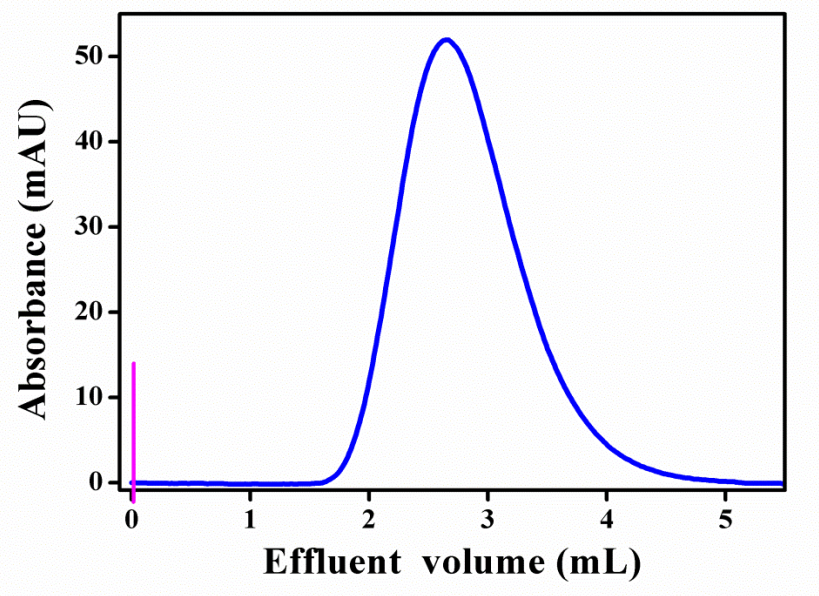

Fig. 2.6. Acetone peak profile measured on a column (5.5 mm ID × 0.5 cm L) packed with SP gPore, equilibrated in 20 mM phosphate pH 7.5 and run at a linear velocity of 300 cm/h.

2.3.2.2. Pressure drop measurements

The pressure drop in a porous medium which follows Darcy's Law at low Reynolds number is given by the expression [25, 26]

$$u_0 = \frac{k}{\mu}\frac{\Delta P}{L} \qquad (2.7)$$

where, u_0 is the superficial velocity; k is the permeability coefficient; μ is the viscosity of the mobile phase, which is 0.01 P for the phosphate buffer; ΔP is pressure drop across the medium and L is the height of medium. Fig. 2.7 illustrates the plot of superficial velocity versus the measured ΔP/Lμ value for a column packed with 0.2 g composite fibrous material. The slopes of the curves are the permeability coefficients, which are equal to 0.92×10^{-7} cm^2. This obtained value is 1-3 times higher than the literature values of membranes developed for the similar purposes [27] and 1-2 times higher than the values of columns packed with ion exchange beads [28,29]. However, many factors such as pore structure, shape, size, swelling degree and compatibility with mobile phase can change the back pressure of column.

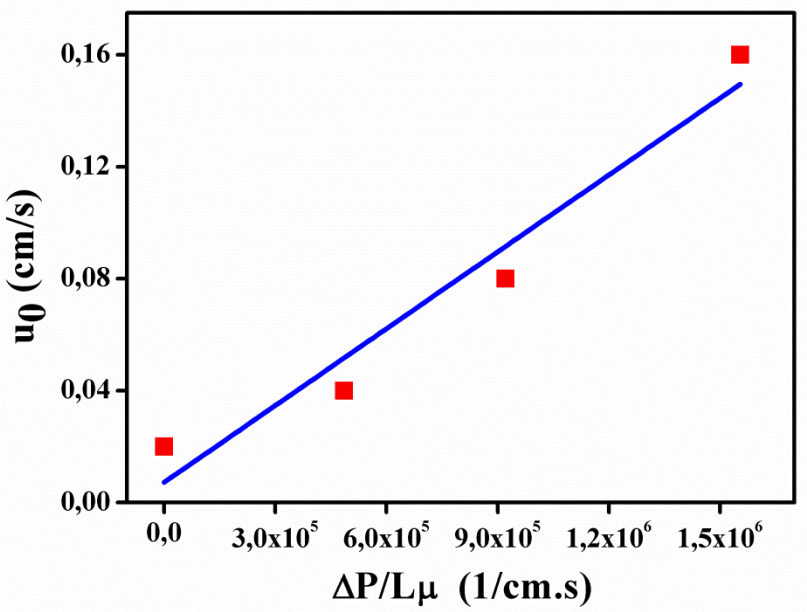

Fig. 2.7. Superficial flow velocity versus pressure drop for a column packed with SP gPore.

2.3.3. Total ionic capacity

The fibrous composite material may have build-in functionality as a strong cation-exchanger when the monomer utilized is selected accordingly i.e. the utilization of MAA during polymerization resulted in pendant carboxylic groups within the structure of the porous material. In this regard, total ionic capacity as measured by the pH transient method was reached with 6.45 mEq./g and the pH profile of the fibrous packed column is shown in Fig. 2.8. The obtained parameter is comparable to that of commercially available cation-exchanger prepared by gamma radiation grafted membranes [30]. For comparison, similar frontal analysis performed for the commercial bead cation exchanger SP Sepharose FF showed an ionic capacity of 1.8 mEq./g which is similar to the value mentioned in the literature. However, the strategy of build-in function is known to produce surface modification of composite fibrous structures with high number of charged groups on the ion exchangers. This is apparently due to the existence of internally grafted homogeneous hydrogel where reactive groups are easily introducible to perform effective ligand immobilization.

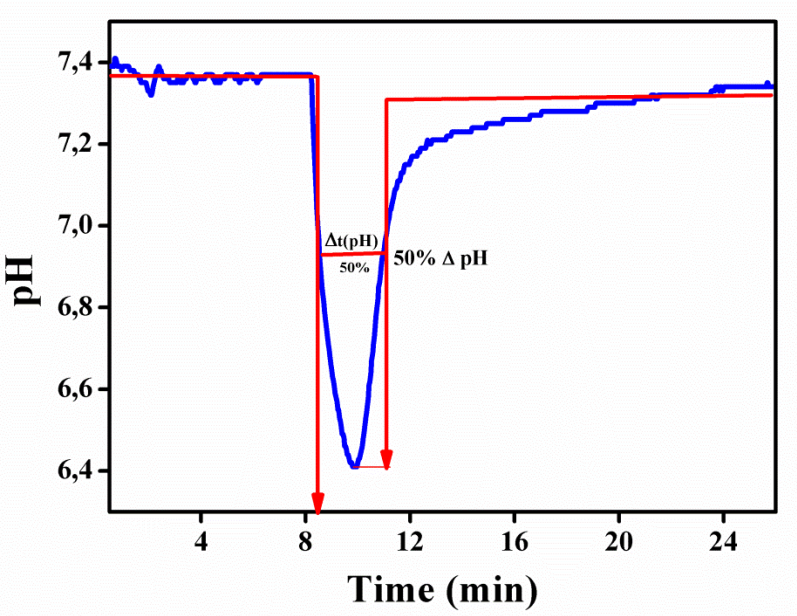

Fig. 2.8. Determination of $\Delta t(pH)_{50\%}$ (shown by arrows) from pH profile for SP gPore packed column. Conditions: buffer A: 20 mM Tris–HCl, pH 7.4; buffer B: 20 mM Tris–HCl + 1 M NaCl, pH 7.4; column (5 mm ID × 5.5 cm L); Method: 100 % buffer A (8 min) step to 100% buffer B (24 min). Flow rate: 150 cm/h. Detection: pH.

2.3.4. Adsorption equilibrium

Lysozyme was used as a model protein in the batch adsorption experiments to illustrate the differences in the adsorption properties of the functionalized gPore fiber and commercial beads. In Fig. 2.9, the adsorbed lysozyme is plotted as a function of lysozyme equilibrium concentration for static binding measurements and the corresponding Langmuir isotherm constants, $Q_{\max}$ (525 mg/g) and K_d (0.90 mL/mg) were determined for the SP gPore. The results indicated that the surface with functionalized composite fibrous adsorbent had significantly higher saturation capacity at all equilibrium liquid phase concentration than the literature values form the commercial ion-exchange beads. The high specific surface area with homogeneous grafted selective ligand immobilized composite morphology is allowed for almost two folds higher adsorption capacity, indicating a higher efficiency for bioseparation

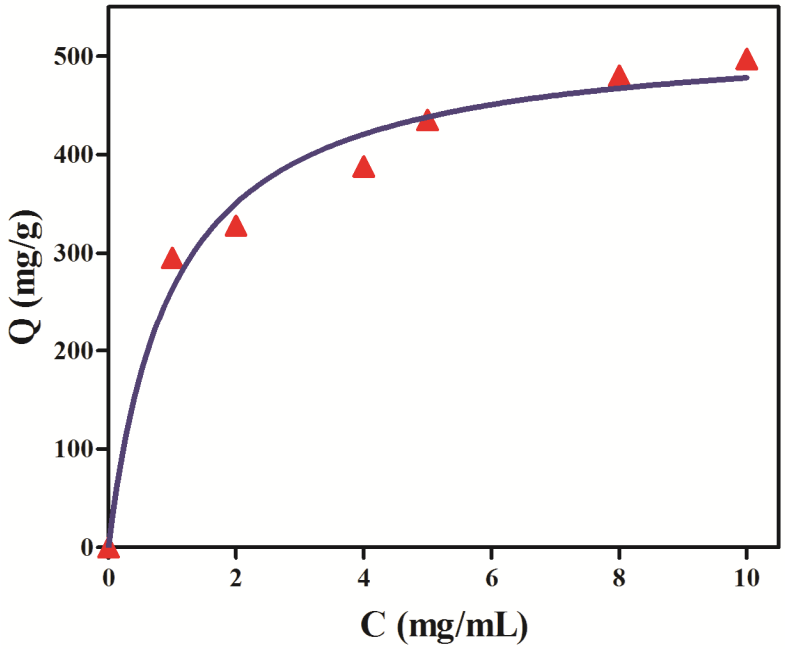

Fig. 2.9. Adsorption isotherm of lysozyme to the SP gPore where Q is adsorbed equilibrium concentration of lysozyme and C is the liquid phase equilibrium concentration of lysozyme.

2.3.5. Chromatography performance

2.3.5.1. Dynamic binding capacity

For any adsorption system is dynamic breakthrough analysis, a combination of equilibrium binding capacity, adsorption kinetics, and system dispersion. It is also a direct application of capacity for a flow through mode of operation where the bound molecule does not need to be selectively eluted for other molecules [10]. The DBC (dynamic binding capacity) parameter can change, for the same adsorbent, as a function of the molecular weight and the system superficial velocity. The most challenging applications are situations in which macromolecules (e.g. proteins and others) have to be sequestrated rapidly i.e. at high flow rates (or limited contact times). This is due to the existence of combined mass transfer resistance and dispersion effects. To determine such effects and their potential influence on performance, dynamic binding capacity was evaluated via breakthrough curve (BTC) analysis as a function of superficial velocity. Moreover, residence time distribution (RTD) experiments utilizing acetone as a conservative tracer were performed also as a function of flow velocity [31]; normalized profiles were interpreted by the dispersion model. The results of the breakthrough experiments carried out in a 1 mL column (5 mm ID × 5.5 cm L) as expressed by the quantities DBC and Peclet number (Pe) are shown in Fig. 2.10. It can be observed that DBC is decreasing with increasing the flow velocity and the maximum

capacity 283 mg/g was obtained at 75 cm/h. At higher flow rates, there is noticeable, however not dramatic reduction of the DBC (ca. 20%) up to a linear flow rate of 300 cm/h. The other parameter, Pe remains almost constant at values (≥70) except reaching a maximum value at a linear flow rate of 600 cm/h, Pe suggesting a close-to-plug- flow condition (i.e. negligible axial mixing). The transport of molecules by convective flow is assisted by the existence of the through-pores but limited by diffusion at the (diffusive) pore side. This pore architecture allows maintaining a loading level when flow rate increases [32]. On the other hand, a reduction of DBC at very high flow velocity can be explained by a decreased Pe, which translates into an increased axial dispersion within the system [33].

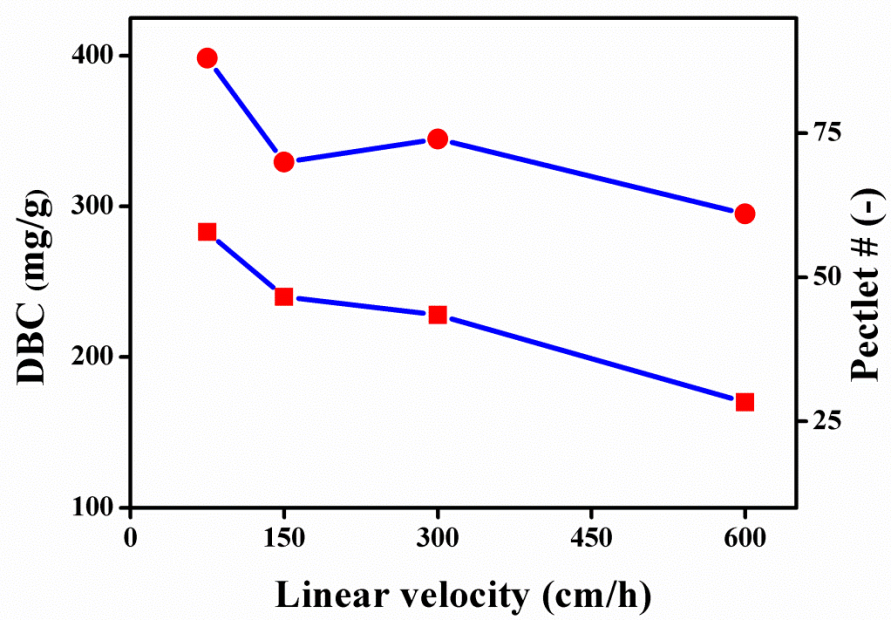

Fig. 2.10. Dynamic binding capacity (■) and Peclet number (●) of SP gPore, at four different flow rates. Conditions: 20 mM phosphate buffer pH 7.4 was used as equilibration buffer and sample (lysozyme 2 mg/mL) was dissolved in running buffer, column: 5 mm ID × 5.5cm L. Acetone was used as tracer for Peclet number determination. Different flow rates were applied: 75 cm/h, 150 cm/h, 300 cm/h, and 600 cm/h.

In another experiment, different packed forms of fibrous supports were tested in the columns by considering proper aspect ratio and expansion factor at two different flow velocities. Then the binding capacities were evaluated from the results of breakthrough experiments with lysozyme and compared with commercially available cation exchangers. The uniquely packed double roll fibrous support showed higher capacity (227 mg/g, 207 mg/g) compared to packed with simple roll (90 mg/g, 60 mg/g) and stacked disks (164 mg/g, 107 mg/g) at 150 cm/h and 300 cm/h, respectively. The decrease in binding capacity with packed simple roll is

due to lower interstitial volume available for holding fluid per unit dry mass of fiber. It can be observed that the unique packed double roll fibrous adsorbent shows higher binding capacity than the commercial cation exchange resins (92 mg/g – 178 mg/g) at 150 cm/h. It clearly shows that gPore has in comparison to commercial materials significantly superior dynamic binding capacity and can be used in a wide range of flow rates for purification processes.

2.3.5.2. Resolution behavior of the gPore adsorbent

The elution of a protein mixture in the use of SP gPore and a conventional cation-exchanger SP Sepharose FF, under different flow rates is shown in Fig. 2.11 and Fig. 2.12 respectively. SP gPore shows a higher resolution than the commercial SP Sepharose FF, since the three proteins of the protein mixture at the elution show clear dividing peaks, while the SP Sepharose FF displays four peaks. It can be clearly observed that the resolution was maintained with fibrous adsorbent even at high flow rates up to 900 cm/h. So increasing the flow rate by a factor of 10 is possible when using the gPore, as a high resolution is still valid. In contrast, SP Sepharose FF shows already a loss of resolution with an increase of factor 3. The increased resolution also affects the trend of gradients for the SP gPore; the elution of the proteins already allows a use of 50% of Elution concentration. The resolution performance is due to its selective adsorption of positively charged target molecules to the high ligand density on the homogeneous internal functional hydrogel grafted composite fiber which increases the specific adsorption properties. This clearly shows that the use of gPore allows a reduction of the buffer volume and the short processing time which enhances economics by intensifying the process.

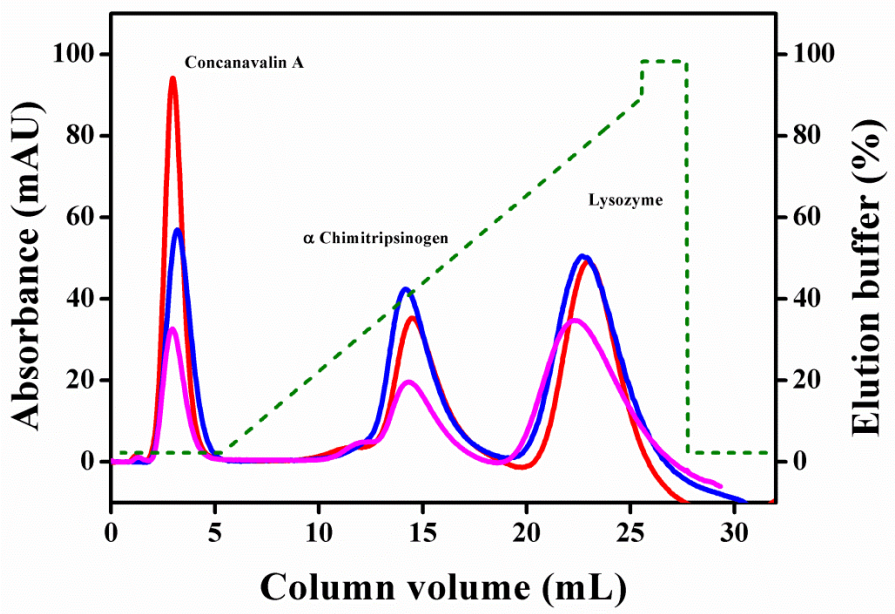

Fig. 2.11. Resolution behavior of SP gPore at an absorbance of 280 nm run at three different flow rates (—) 150 cm/h, (—) 300 cm/h and (—) 900 cm/h.

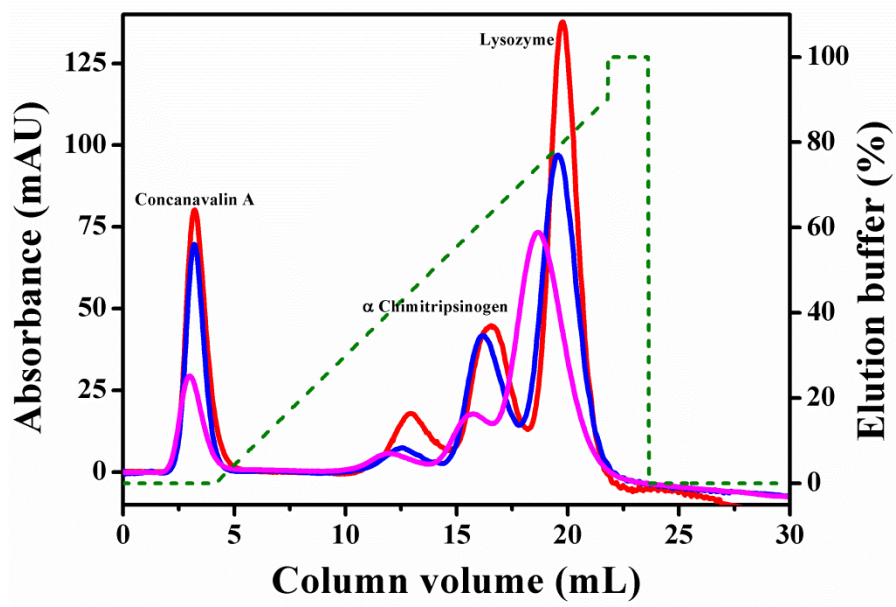

Fig. 2.12. Resolution behavior of SP Sepharose FF at an absorbance of 280 nm run at three different flow rates (—) 150 cm/h, (—) 300 cm/h and (—) 900 cm/h.

2.3.5.3. Bioprocess applications

The open structure non-randomly packed double roll material, was tested in the commercial column as shown in the fig. 2.13. The ability of these fibrous composite to pass whole cell feed streams was demonstrated in this section and also the binding specificity, and observation of any blockages, back-pressure-related development was observed.

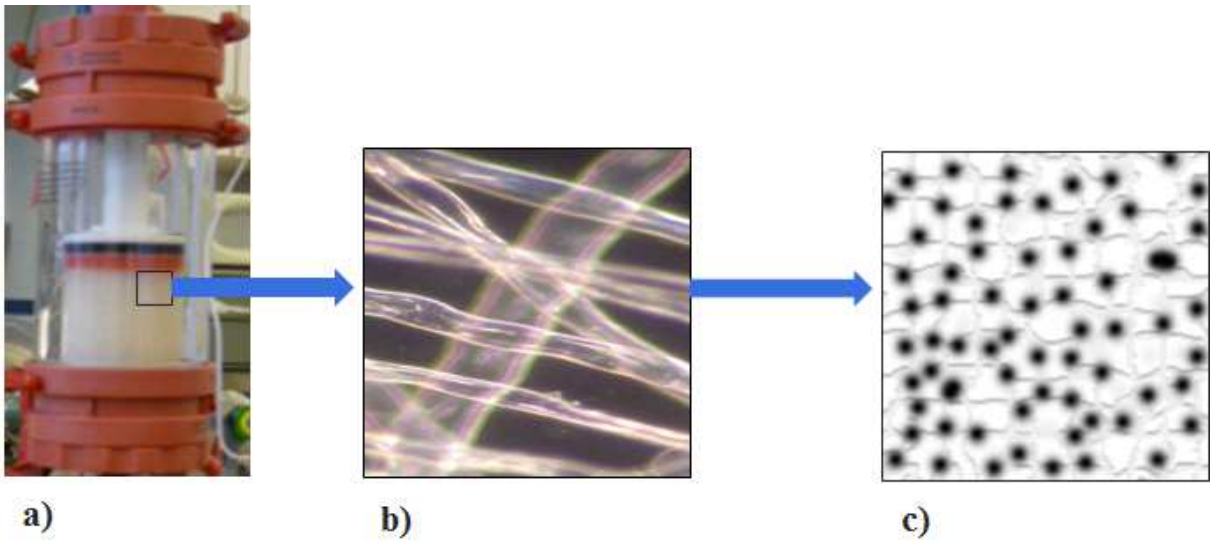

Fig. 2.13. Capture of lysozyme from artificial mixture on to the SP gPore; a) Composite fibrous material packed in a column b) Composite fibrous structures c) Specific adsorption of lysozyme (●) on to the high ligand density of SP gPore.

To simulate the prototype with the established expression systems of uncentrifuged and unfiltered yeast cell extracts, an artificial mixture of 5 g/L lysozyme (as a model protein to purify) and 1.5% yeast cells was produced, which realistically simulates the feedstock solution to a purification of macromolecules. The whole cell extract was given after the cell disruption on an FPLC system at a linear flow velocity of 150 cm/h. The material shows almost no pressure and no clumps, indicating high biomass compatibility. The elution of the model protein lysozyme was carried out and the resulted chromatogram elution profile is shown in Fig. 2.14. Lysozyme is selectively bound to gPore and then recovered as a very pure protein from unfiltered biomass as shown in the Fig. 2.15 b (gel photo: band E5). The biomass does not affect the adsorption of protein to gPore. The very high biomass compatibility and good binding properties allow high process speeds. These experiments demonstrate the strong tolerance of the biomass material, despite large cell density and without pre-filtration, expressed proteins can be purified with high flow rates and are eluted specifically. This in turn makes drastic reduction of process buffer volume and energy expenditure. The macro

porosity would allow for the capture of target soluble species while cells or cell-derived debris present in a raw fermentation / cell culture broth may be easily elutriated with the system outflow. A more detailed study will be carried out to investigate for its performance as a potential chromatography support, with real time applications which could be particularly useful in the capture of proteins from a non-clarified complex feed stream.

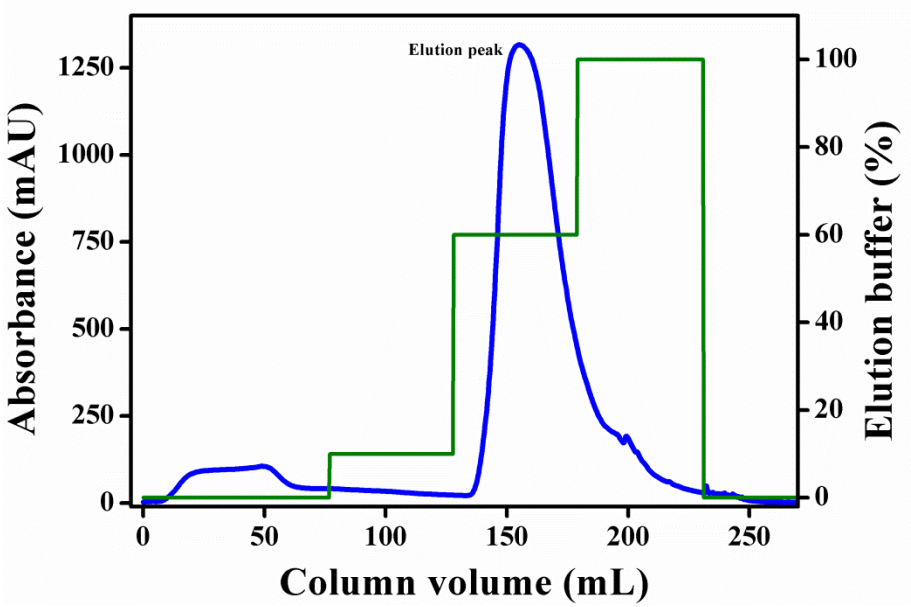

Fig. 2.14. Experiment for biomass tolerance of SP gPore under simulated process conditions. Lysozyme was mixed with yeast cells to the column packed with SP gPore. Subsequently, the column was washed and eluted the lysozyme (▬) peak at 150 mL with concentration of elution buffer (▬).

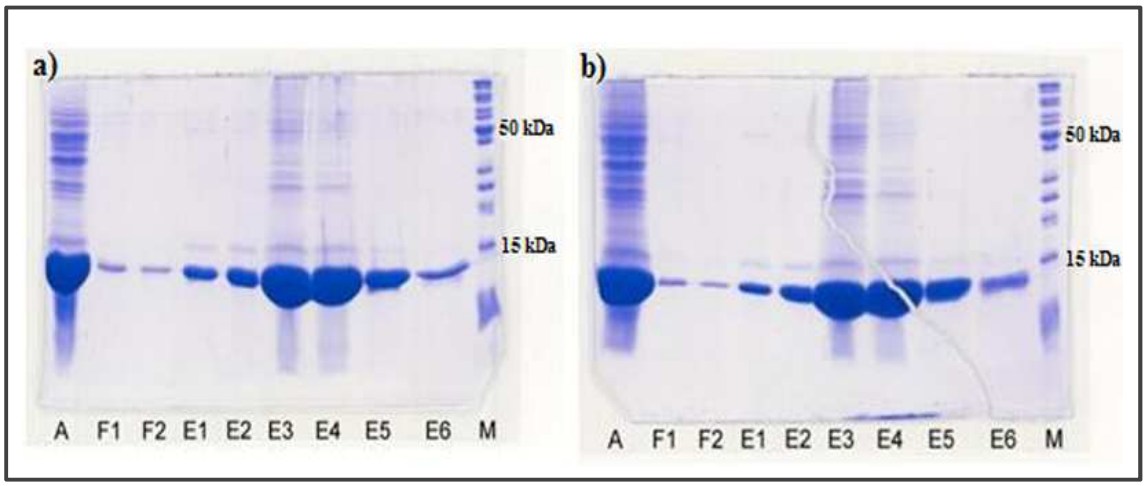

Fig. 2.15. Elution fractions from SP gPore column (a) filtered biomass and (b) unfiltered biomass (A- load, M- marker, F- flow-through, E- eluted fractions).

2.4. Conclusions

A novel type of composite fiber cation-exchange adsorbent has been prepared by gamma radiation graft simultaneous polymerization with subsequent ligand immobilization. This is the first study that demonstrates the utilization of composite fibers as a chromatography support for high throughput and high capacity bioseparations. The functionalized composite fiber was physically characterized by gravimetry, SEM, EDS, FTIR and the results indicated that a homogeneous internal hydrogel with high degree of grafting and swelling ratio was achieved which provides the mechanical and chemical stability to the chromatographic support. In this study, the potential of using column packing with fibrous support in chromatographic applications has been investigated and the results demonstrate its high column efficiency and high throughput. The results of the pressure drop experiments show that the modified fibrous adsorbent maintains very high flow permeability compared to other membrane types and to packed beds of chromatographic resins.

The total ion-exchange capacity of the synthesized fiber was determined by pH transition method and the obtained value is comparable to that of commercial available cationic-exchange based membranes. These modified composite fibers exhibited very high protein binding capacities for lysozyme under static and dynamic conditions. And also a high breakthrough performance is maintained at higher flow rates, indicating good mass transfer characteristics. The results obtained from the elution behavior of lysozyme indicate that high protein resolution at high flow velocities and reduction of buffer volume and processing time

is possible. However, the potential with different systems such as antibody proteins (monoclonal antibodies) could be explored when the composite support is immobilized with appropriate ligands. The experiments with feed stream demonstrated almost perfect compatibility with biomass and air, which is achieved by the structure of the material and eliminates the need for pre-filtration. In summary, the utilized cation exchange composite fibrous adsorbents have shown high binding capacity and high throughput with high resolution operated at higher flow rates. And the fibrous geometry is suitable for process bio separation applications. All these results from this chapter support the utilized innovative fibrous adsorbents can be used as potential chromatography matrices for the intensification and integrated bioprocess design for the recovery, separation and purification of biomolecules.

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Chapter 3

Synthesis, characterization and process performance of anion-exchange fibrous adsorbents for high throughput and high capacity bioseparations

3.1. Introduction

Industrial biopharmaceutical therapeutic product isolation and purification processes require alternative materials to replace traditional packed-bed column resins [1-8]. One promising alternative is to use adsorptive fibrous substrates which act as chromatography support for the functional ion exchange and affinity ligands used during selective adsorption process [9, 10]. These fibers are coupled with synthetic polypropylene, poly (butylene terephthalate), PES and nylon, which exhibit high surface area, variable and controllable porosity and interconnectivity of pores within the fabric matrices and ensure good mass transfer properties. With these fabrics, ion exchange or affinity ligands could be directly attached to the fiber surface, thus eliminating slow intra-particle diffusion limitations as the process fluid is transported across the membrane. Besides, the controllable pore size and overall porosity of the fibers can reduce concerns of high pressure drops with elevated flow rates, resulting in increased throughput compared to column resin operations. These factors allow for much faster processing times and potentially higher binding levels for purifying valuable target biological molecules in terms of bound product per volume of resin [11, 12]. It has been suggested from the literature that membrane properties and operating conditions have been significantly accounted before using fabrics as separation media in commercial operations. First, optimization of proper surface activation is crucial not only to minimize the non-specific protein adsorption, but also to allow for the covalent attachment of ion exchange or ligand functionalities to absorb the target product or contaminant. Second, it is essential to enhance high binding capacity by increasing available surface area for desire target product binding by reducing the fiber size to sub-micron diameters [10].

This chapter describes the synthesis, characterization and process performance of anion exchange fibrous adsorbents for high throughput and high capacity bioseparations. For the synthesis, the utilized fibrous material was surface functionalized by introducing amino groups by using glycidyl trimethyl ammonium chloride (GTMA) as etherifying agent under the catalytic action of sodium hydroxide. This optimized surface modification enables to introduce positive surface charges on the fiber surface and thus enhances the protein adsorption by electrostatic interactions [13-15]. The resulting modified anion exchange fibrous adsorbent was physically investigated by EDS-SEM and FTIR-ATR spectroscopy to study the nature of the functionalization. Fibers were functionally characterized by a pH transition method to determine the total ionic capacity and batch experiments for the static

binding capacity. The anion exchange fibers were oriented in randomly packed form in a linear scalable columns and the quality of packing efficiency was evaluated by measuring the HETP and Asymmetry factor (A_s). Besides, the interstitial and total porosities and permeability of the fibrous support were measured. For the column characteristics of the packed fibers, breakthrough analysis has been determined for operation in the frontal mode for different diameter columns. This research work describes the performance of the anion exchange fibers as a chromatography media that could be linearly scalable to a factor of column volume (110 mL).

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3.2. Experimental materials and methods

3.2.1. Materials

Fibrous material used in this study was made of 80% pure cotton and 20% CoPES (mono-component polyethersulfone) fusible adhesive fiber with a density of 1.3 g/cm^3 in a dry-laid process (EMS Chemie, Switzerland). Fiber diameters were in the range of 1–20 µm and this value was obtained by measuring fiber diameters from the SEM images. The surface textures of the fiber were also qualitatively analyzed from the SEM images. The fibrous material was washed in methanol and water, and dried in an oven prior to use. The dried samples were cut into small pieces before mercerization procedure. GTMA and BSA were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Dimethylformamide (DMF), acetone, and sodium hydroxide, were procured from AppliChem GmbH (Darmstadt, Germany). Chromatography columns, Tricorn [0.5 mm Internal Diameter (ID) × 5.5 cm length (L)], XK16 (16 mm ID × 20 cm L), XK 50 (50 mm ID × 20 cm L), Q Sepharose FF resin, and the ÄKTA explorer system equipped with Unicorn 4.10 software for data collection and analysis were obtained from GE Amersham Bioscience (Uppsala, Sweden).

3.2.2. Sample preparation and characterization

3*.2.2.1. Surface functionalization by cationization*

A known amount of washed fibrous material was first hydrolyzed by immersion in aqueous solution of sodium hydroxide (5 N to 7 N) for 10 to 12 h at 4 – 6 °C. Then the mercerized fiber was separated by decanting the alkaline solution and squeezed to remove excess liquid. Subsequently the hydrolyzed material was treated with a reaction mixture containing an

optimized concentration of GTMA in sodium hydroxide, with DMF, at room temperature for 24 h with stirring in a close vessel since higher concentrations of reactant are strongly exothermic. Finally the modified material was extensively washed with tap water until a neutral pH was achieved and then placed at 40 °C to completely dry.

The four experimental procedures of scanning electron microscopy, FTIR-ATR, measurement of degree of swelling, determination of material porosity by water uptake, were already described in the preceding chapter sections of **2.2.3, 2.2.5, 2.2.6, 2.2.7** respectively.

3.2.3. Column packing

The dried surface modified anion exchange fibrous material was homogenized into relatively short fibers (>1cm) by using an electric mixer (McMaster-Carr, Aurora, USA) and packed into three different diameter columns (5 mm, 16 mm and 50 mm, GE Amersham Bioscience, Sweden). A defined amount of dry functionalized fibrous material was packed in a fixed column by accounting its aspect ratio and expansion factor of the respective column volumes. The packed fibrous bed was then swollen in place with 20 mM phosphate buffer (pH 7.4) at very low linear flow velocity (~75 cm/h) and then the bed height of the column was fixed so that there was no visible head space in the column after swelling of the medium contained in the functionalized fiber. The quality of column packing efficiency was evaluated in terms of HETP and As by impulse injection experiments using acetone as tracer under a non-retained condition for mentioned columns at flow velocities ranging from 75 to 600 cm/h.

3.2.4. Pulse experiments

A 5 mm ID x 5.6 cm L with a fixed adaptor column (Tricorn 5/50), packed with 0.2 g of functionalized material was prepared for pulse experiments. The experiments were conducted at room temperature on an ÄKTA explorer FPLC system with a 10 µL sample loop and internal UV detector. The height of the packed bed was 5.5 cm. Aqueous acetone of 5.0 % (v/v) and lactoferrin solution of 10 mg/mL of 1% column volume were injected at different linear flow velocities between 150 and 600 cm/h of phosphate buffer. The extra-column volume was accounted for by conducting the same measurements bypassing the fibrous column in the system.

3.2.5. Flow permeability experiments

Pressure drops along the length of a column packed with modified fibrous material were measured at four different linear flow velocities between 150 and 600 cm/h of phosphate

buffer. The three different diameter utilized column dimensions were 5 mm ID × 5.5 cm L (Tricorn column); 6 mm ID × 10.5 cm L (XK16) and; 50 mm ID × 5.5 cm L (XK50). The mentioned fibrous packed columns were mounted on ÄKTA explorer FPLC system and the flow permeability experiments were conducted at room temperature with internal and external digital pressure monitors. The measurements of pressure drops were determined by the difference in the pressure at the inlet and the outlet of the column observed in the pressure monitor. The extra-column pressure drop was accounted for by conducting the same measurements without a fibrous column. A QF150 biotech pump (Quattroflow Fluid Systems, Hardegsen, Germany) was used to control linear flow velocity (600 cm/h) for the XK50 column since the ÄKTA explorer was not compatible to that flow velocity.

3.2.6. Determination of the total ionic capacity

Similarly, the anion exchanger capacity of fibrous adsorbent was determined by transient pH technique as described in the previous chapter [16] using the same equation where for anionic systems, a = 10.9 and b = 5.34 [17] and the following solutions were utilized: 500 mM phosphate pH 7.4 (Buffer A) and 20 mM phosphate pH 7.4 (Buffer B).

3.2.7. Protein adsorption equilibrium under static conditions

Bovine serum albumin (BSA) equilibrium adsorption isotherms under the static conditions were measured for a known of amount of the anion exchange fibrous support in phosphate buffer (pH 7.4) by batch experiments as mentioned in the chapter two.

3.2.8. Dynamic binding capacity by frontal analysis

The Dynamic Binding Capacity (DBC) at 10 % breakthrough for the BSA solutions of concentration (C_o 10 mg/mL) with fibrous material packed in three different columns (5 mm ID × 5.5 cm L; 16 mm ID × 5.5 cm L and 50 mm ID × 5.5 cm L) was determined by experimental procedure as described in the preceding chapter. A QF150 biotech pump (Quattroflow Fluid Systems, Hardegsen, Germany) was used to control linear flow velocity (600 cm/h) for determining the DBC of the XK50 fibrous column since the ÄKTA explorer was not compatible to that flow velocity. The breakthrough was calculated at 10% of the load concentration. The protein content was measured by UV absorption at 280 nm. There was no column compaction at high ionic strength and the column was reused in the next cycle after regeneration.

3.3. Results and discussion

3.3.1. Sample preparation and characterization

The surface modification of fiber by quaternization using GTMA as etherifying agent under the catalytic action of sodium hydroxide undergoes two step reaction. Firstly, the sodium hydroxide reacts with the hydroxyl groups of the fiber to yield sodium alkoxide groups. Secondly, these sodium alkoxide groups subsequently reacts with the epoxide group of GTMA and introduces amine groups which provide a positive charge to absorb anionic groups, which is used to evaluate the effectiveness of cationization as shown in the Fig. 3.1. Addition of DMF increases the susceptibility of the epoxide ring to attack the hydroxyl groups and permit subsequent attachment into cellulose. The optimization of cationization i.e. the addition of quaternary amino groups on the base material depends on the concentration of tryalkylammonium salt which reacts with hydroxyl groups. In this work, the influence of process conditions like concentration of GTMA, DMF and temperature and time of the cationization reaction were evaluated to find out the optimization conditions for surface functionalization of fibrous material to act as potential anion exchange chromatography support. From the experimental results it was shown that for one gram of source material in 40 mL reaction mixture of cationization, the required concentration of GTMA was found to be 35% for optimal surface modification with the addition of DMF (10%) at room temperature in order to achieve maximum binding capacity for modified anion exchange adsorbent to act as a potential chromatography support.

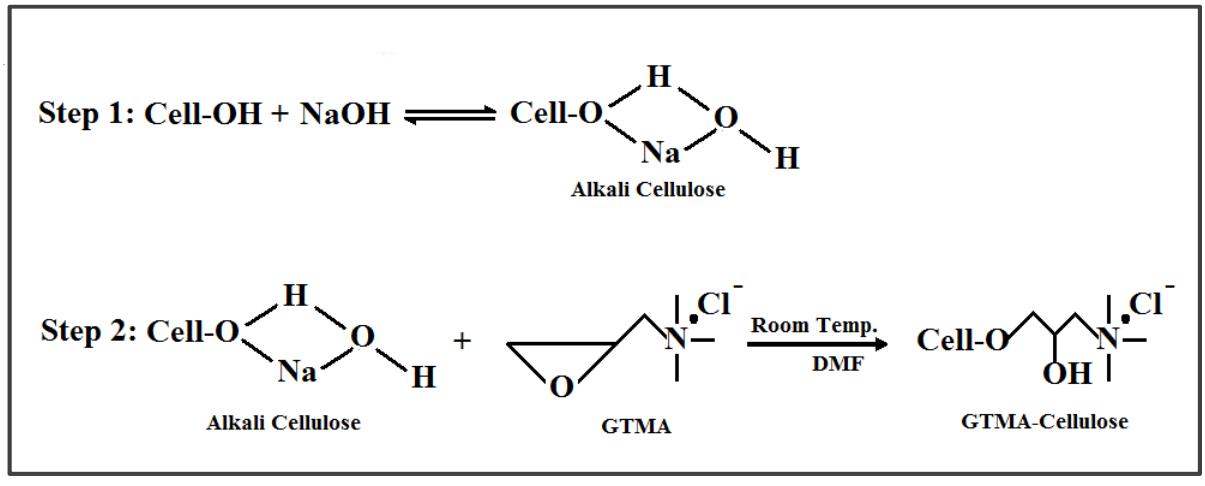

Fig. 3.1. Cationization reaction of the utilized fibrous material.

The SEM images in Fig. 3.2 show the representative morphologies of (a) untreated and (b) chemically modified anion-exchange fibrous material. The fibers however, were not uniform; having hydrogel inside the pores and the average diameter of the fiber was in the range of 8–20 µm. This value was obtained by measuring fiber diameters from SEM images. This may have been desirable, however, since the hydrogel with thicker fibers could provide greater mechanical and chemical stability while the thinner fibers could results in large specific surface area. The effect of cationization on fiber quality was reported in some of the previous researches; the strength of the fabric is slightly reduced due to the reduction in the number of intermolecular H-bonds by cross linking of molecules by the cationic reactants. Furthermore, the morphology of the fibers after surface functionalization (Fig. 3.2b), was not prominently deteriorated as compared to the untreated sample; the morphologies of the fiber before and after the surface functionalization were clearly distinguishable in terms of surface textures of the fibers which were qualitatively analyzed from the SEM images. This suggests that the fiber morphology could be more effective in bioseparations when compared to the foam-like morphology, which the commercial cellulose adsorptive membrane possesses [8]. With this hydrogel swelling ratio, particularly good convective flow properties can be ascertained for the chromatography adsorbent. Water uptake experiments revealed a ~92% porosity and the degree of swelling was observed as 3.95 g/g in distilled water at 24 °C. These results are also in agreement with the observed swelling properties, mechanical and chemical compatibility by introducing amino groups to the backbone polymer.

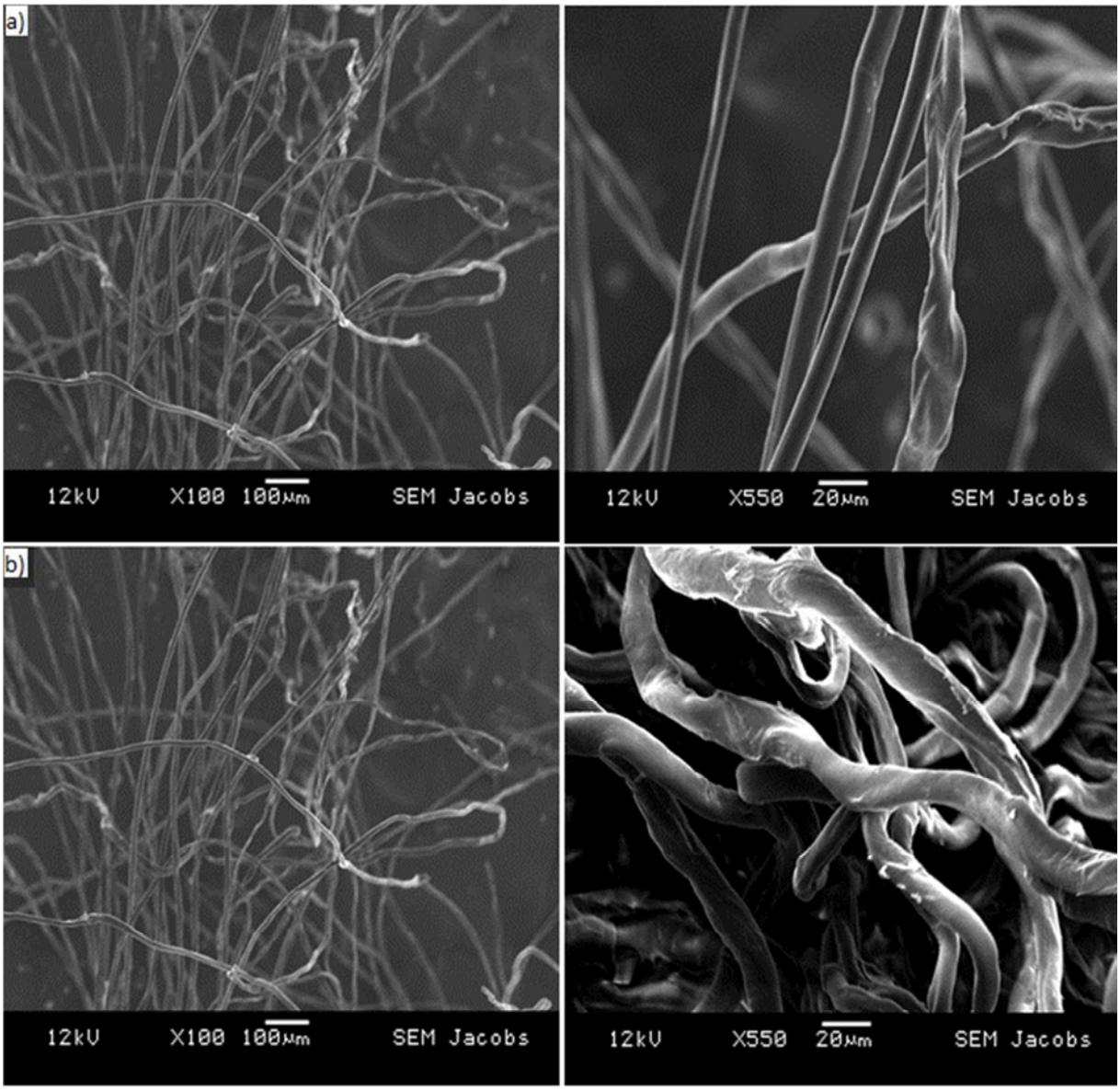

Fig. 3.2. Representative SEM images of (a) untreated, (b) chemically modified anion exchange fiber in different scales.

Fig. 3.3. illustrated the EDS-SEM spectra to better ascertain the nature of the modified fibrous material and a further structural insight was obtained by utilizing a cellulose backbone containing hydroxyl groups, which were further reacted with GTMA. Table 3.1 is created from the peak integration of EDS spectra with elementary composition data analysis and several parameters should be carefully considered when analyzing the EDS data as mentioned in the

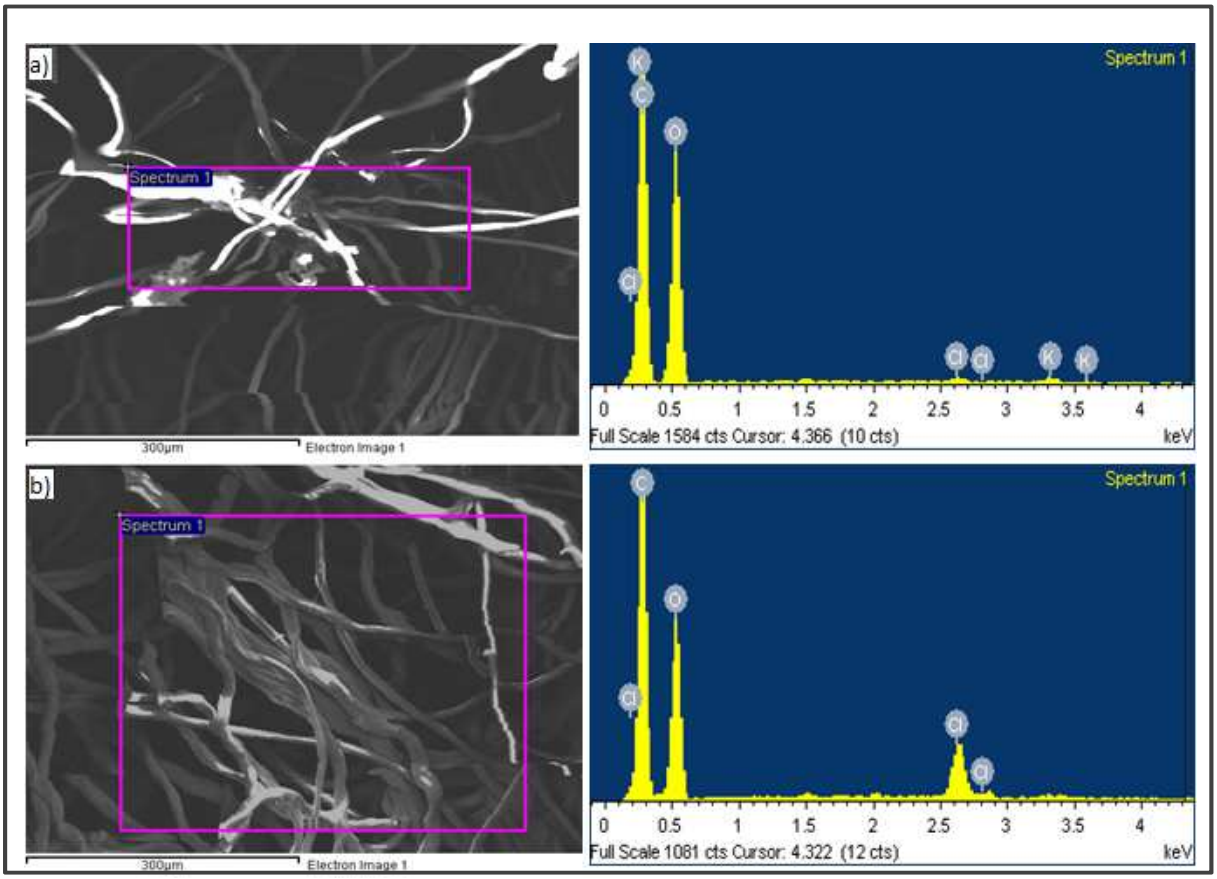

Fig. 3.3. Representative SEM coupled EDS images of (a) untreated, (b) chemical modified anion exchange fiber in full scales.

Table 3.1. Elementary analysis of the samples with EDS

Element	Untreated fiber *		Functionalized anion exchange fiber*	
	Weight (%)	Atomic (%)	Weight (%)	Atomic (%)
C K	50.19	57.45	54.11	61.83
O K	49.30	42.36	43.35	37.19
Cl K	0.25	0.10	2.54	0.98
K K	0.27	0.09	-	-
Totals		100		100

*Number of iteration is 4, C-Carbon, K-Potassium, O-Oxygen, Cl-Chlorine, S-Sulphur.

previous chapter. Thus, the EDS spectra of untreated material show mainly carbon and oxygen. Chlorine and potassium signals are in low or high proportion (variable according to the efficiency of washing step) but in the same atom percentage. In quaternary amine

activated material, the nitrogen signal is weak and close to carbon, thus it is quite difficult to find out at low nitrogen concentration as it is explained previously, however it shows chlorine signal but not potassium one. Thus this data reveals indirect information of the existence of positive charges onto the treated material.

3.3.1.2. Fourier transform infrared spectroscopy

To better understand the chemical changes involved in the sample preparation, the IR spectra of untreated and modified fibrous material are shown in Fig. 3.4. In the resulted spectra, the broad bands between 3000 and 3600 cm^{-1} are relevant to the O-H stretching from the intermolecular and intramolecular hydrogen bonds and the bands observed between 2840 and 3000 cm^{-1} refers to the stretching C-H from alkyl groups and the peaks between 1060-1030 and 672-666 cm^{-1} are due to the stretching modes of tetramethylammonium. The peak around 1750 cm^{-1} does not correspond to any signal that we expected, however this might be due to the additives that added during the making process of mixed fibrous material, which perhaps could interact with some reactive groups of cationization reaction. The most striking difference between untreated and modified anion exchange fiber spectra was the peaks positioned at 1460-1400 cm^{-1} and 1480-1440 cm^{-1} which corresponds to symmetrical and asymmetrical deformation band of methyl groups of quaternary ammonium respectively [18]. The IR spectrum shows evidence of the introduction of the quaternary ammonium salt group on PES fiber backbone.

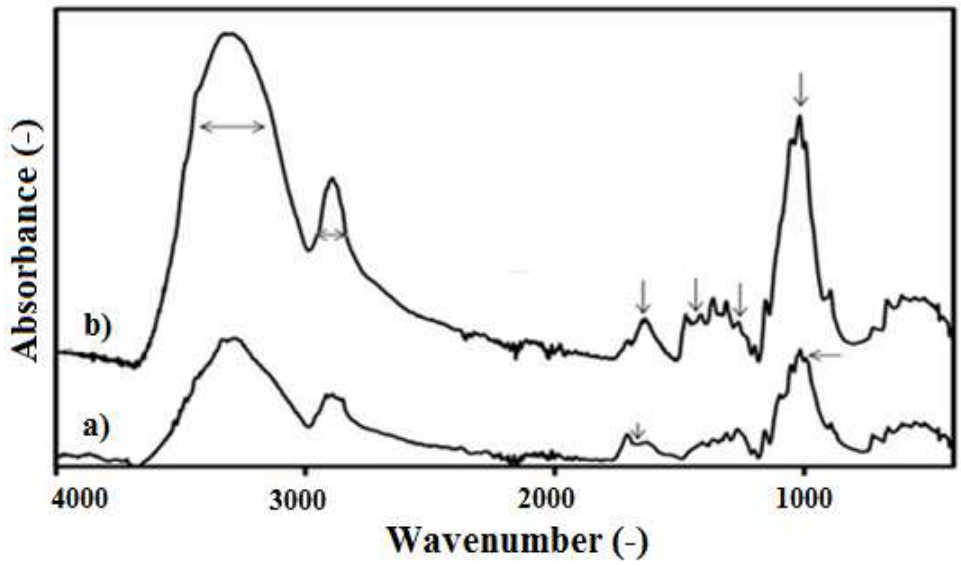

Fig. 3.4. IR-spectra of (a) untreated, and (b) chemically modified anion exchange fiber

3.3.2. Column packing

The surface modified anion exchange fibrous material was packed in a fixed column with conditioning buffer at very low linear flow velocity (75 cm/h). Homogeneous column packing could be achieved due to its high swelling ratio and could expand 3–4 times its dry packing density (1.0 mL/0.2 g). It has been shown that the effect of packing heterogeneity in current in widely used chromatographic columns might seriously impair the separation efficiencies of the columns that lead to deprived performance of chromatographic materials [19-22]. The homogeneity of the modified fibrous adsorbent is a significant factor that is closely observed and controlled during the surface modification of fibers that lead to act as a potential chromatographic support. Fig. 3.5 demonstrated three different diameter commercial columns randomly packed with anion exchange fibrous bed support. For the fibrous packed columns, when the flow velocity was higher than 75 cm/h, however the HETP only increased slightly and kept nearly constant (0.1321 cm/plate) with flow velocity up to 600 cm/h, which indicated that the uniform packing density of the resulting fibrous column, is known to have good packed performance. The measured peak asymmetric (A_s) values are found to be less than 1.6 for all three different diameter columns and showed quite comparable range to commercial beads packed-bed columns (A_s: 0.8-1.8).

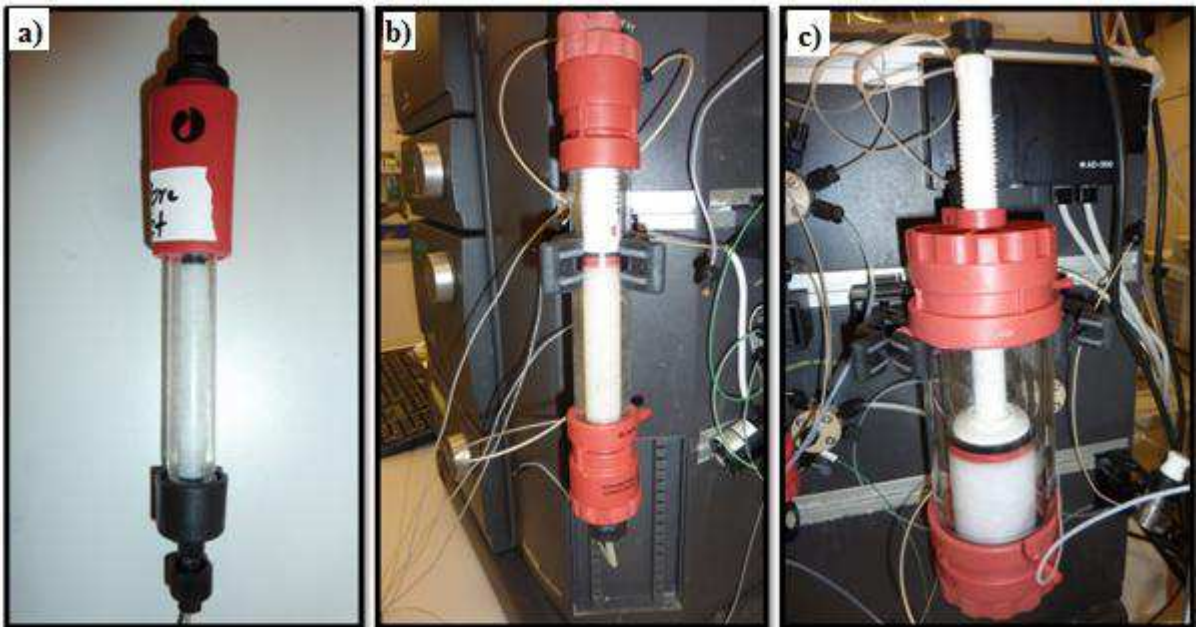

Fig. 3.5. Anion exchange fibrous adsorbents randomly packed in three different commercial columns: (a) column: 5 mm ID × 5.5 cm L; (b) column: 16 mm ID × 10.5 cm L; (c) column: 50 mm ID × 5.5 cm L.

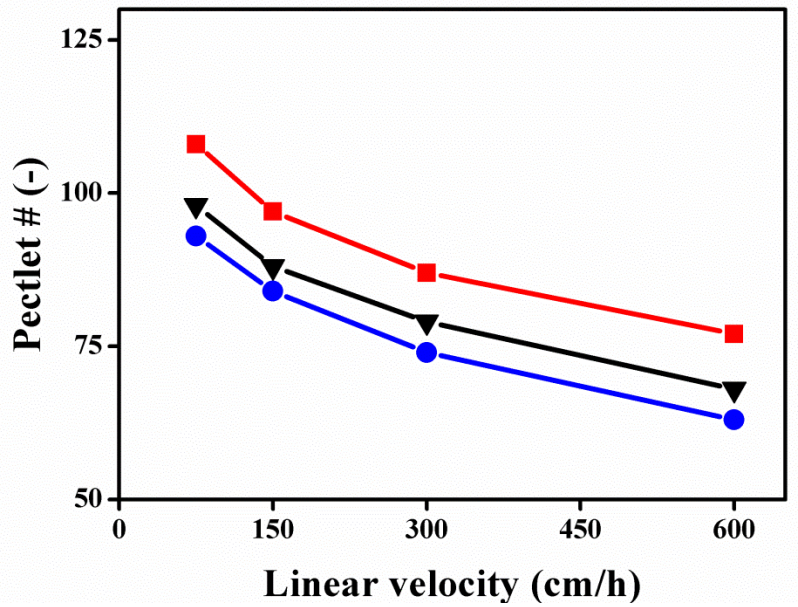

Fig. 3.6. Peclet number at different flow rates for three different diameter columns packed with anion exchange fibrous material. (▲) column: 5 mm ID × 5.5 cm L; (■) column: 16 mm ID × 10.5 cm L); (●) column: 50 mm ID × 5.5 cm L peak profiles of anion-exchange fibrous adsorbent at four different linear flow velocities (75 to 600 cm/h) for a column (5 mm ID × 5.5 cm L); mobile phase- 20 mM phosphate buffer (pH 7.4). As probe solute acetone was used (1% of column volume).

On the other hand, residence time distribution (RTD) analysis was performed to evaluate axial mixing. Response pulses obtained employing acetone as a tracer allowed for the determination of the Peclet number (Pe) using tank in series model and dispersion model [23] to fit the experimental data and interpreted normalized profiles. Fig. 3.6 describes the calculated values of Peclet at four different linear flow velocities for three different diameter columns packed with functionalized fibrous material. The obtained data demonstrates that the material has good liquid dispersion behavior and the Peclet number (>65) results in negligible axial mixing within the bed. The flow rates have no significant effect on the axial dispersion values of fibrous material. And also interestingly it can be noticed that with increasing the bed height along with column diameter, the quality of packing efficiency is not affected. The experimental results demonstrated that the chemically modified anion exchange fibrous materials have significant columns efficiency with good reproducibility comparable to commercial bead packed columns.

3.3.3. Porosity measurements

The total and interstitial porosities of a packed column were measured by pulse experiments using first absolute moment analysis which is expressed by the following equation

$$\mu_1 = \frac{\int_0^\infty C(t)tdt}{\int_0^\infty C(t)dt} \tag{3.1}$$

where μ_1 is the first absolute moment, *C* is the concentration of tracer (%) exhibiting the column and t is time. The relation between the first absolute moment and the total porosity ε is given by the expression

$$\mu_1 = \frac{L}{u_0}\varepsilon \tag{3.2}$$

where u_0 the superficial velocity (m/s) and *L* is the height of the column (m). If $\mu_1$is plotted versus L/u_0, the slope of the curve is porosity. This technique was previously utilized using small injections of different size tracers were applied to the packed column under nonbinding conditions [10, 24, 25].

In this work, acetone was commonly used to determine total porosity and a globular protein lactoferrin can be used to measure the interstitial porosity of a column packed with the modified fibrous adsorbent. Fig. 3.7 represents the plot of first absolute moments versus L/u_0 for the two tracers. This has been presumably shown that acetone is much smaller than lactoferrin and the obtained experimental results indicate that acetone has longer residence time and larger slope when compared to globular protein. This is due to fact that the pores easily available to acetone are inaccessible to lactoferrin. The porosity measured with acetone was 0.97 and with lactoferrin was 0.47. The voids, which are available to acetone may be associated with small gaps between fiber overlaps during fiber fusion process but too small for lactoferrin, account for ~50% of the total volume of the fibrous column.

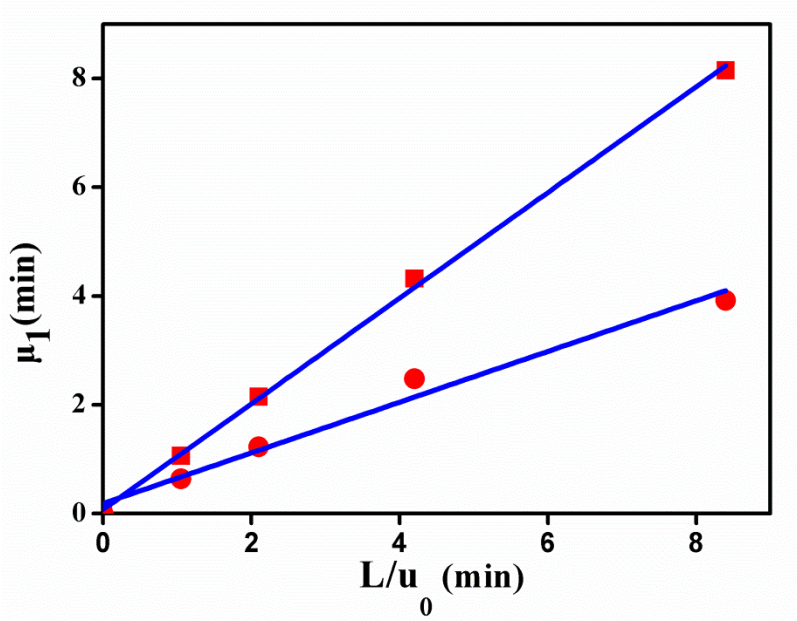

Fig. 3.7. First moment versus L/u_0 for pulse injects of acetone (■) and lactoferrin (●) to a column packed with anion exchange fibrous adsorbent.

3.3.4. Pressure drop measurements

The pressure drop in a porous medium follows Pre Darcy's Law at low Reynolds number and is given by the equation [26] and applied similarly as mentioned in the previous chapter. The superficial velocity is plotted as a function of the measured ΔP/Lμ for three different diameter columns packed with anion exchange fibrous material as shown in the Fig. 3.8. The slopes of the curves represent the permeability coefficients, which are found to be 0.95×10^{-7} cm^2, 1.81×10^{-7} cm^2 and 1.51×10^{-7} cm^2 for the 5 mm, 16 mm and 50 mm diameter columns respectively. These obtained values are between 1 and 3 times greater than the values of membranes [10, 26] and between 1 and 2 times greater than the values of columns packed with ion exchange beads [27, 28] utilized for the similar purposes reported in the literature. From the experimental results, it can be noticed that the permeability coefficient value is not affected even with increasing bed height of the functionalized fibrous adsorbent. Several significant parameters such as pore structure, shape, size, swelling degree and compatibility with mobile phase can influence the back pressure of column.

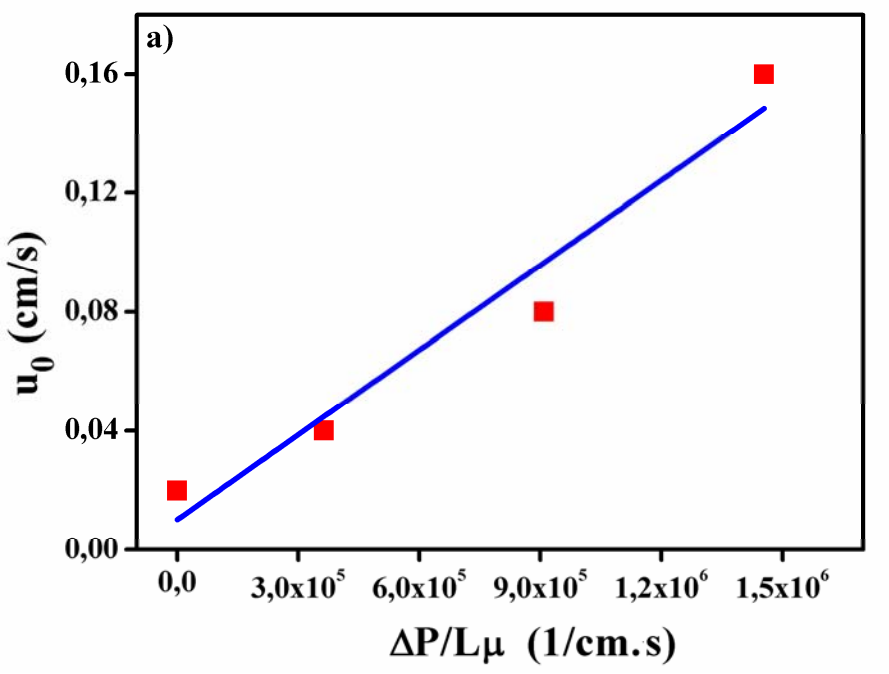

Fig. 3.8 a. Superficial flow velocity versus pressure drop for a column (5 mm ID × 5.5 cm L) packed with anion exchange fibrous adsorbent.

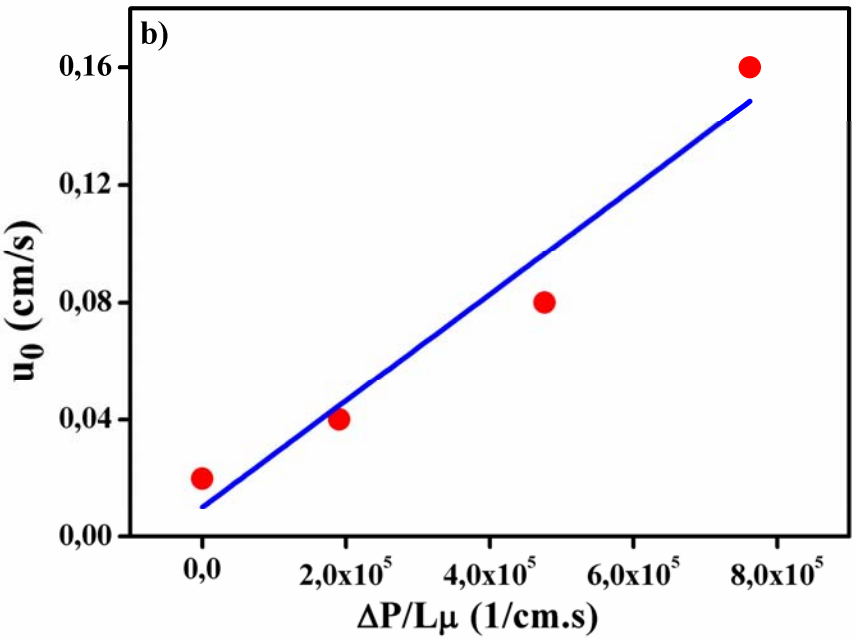

Fig. 3.8 b. Superficial flow velocity versus pressure drop for a column (16 mm ID × 10.5 cm L) packed with anion exchange fibrous adsorbent.

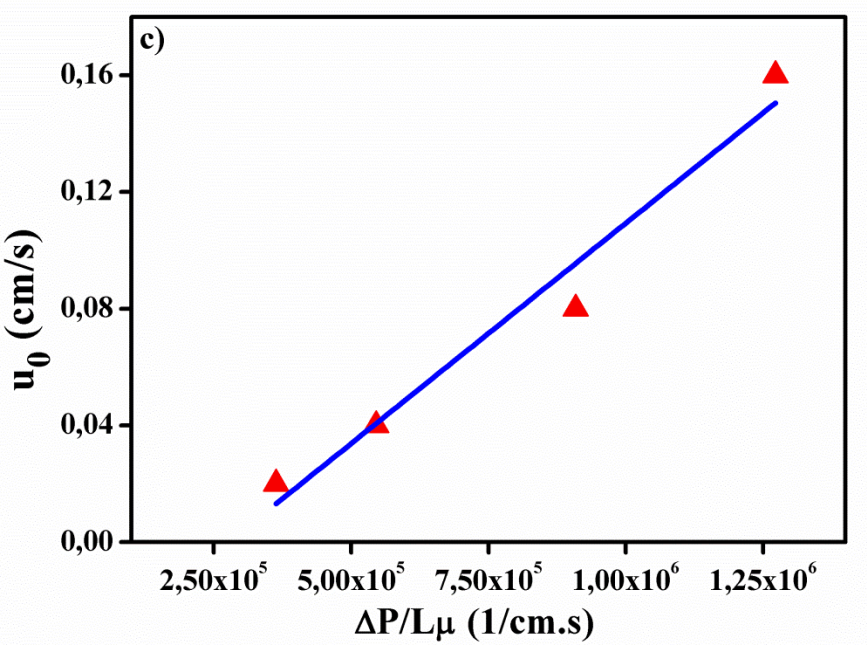

Fig. 3.8 c. Superficial flow velocity versus pressure drop for a column (50 mm ID × 5.5 cm L) packed with anion exchange fibrous adsorbent.

3.3.5. Total ionic capacity

The fibrous material may have build-in functionality as a strong anion-exchanger by optimizing the concentration of GTMA and the incubation time with alkali nature of the carboxylic groups within the structure of the porous material. In this work, the total ionic capacity as determined by the pH transient method and the value was found to be 2.8 mequiv./g and the pH profile of the packed fibrous column is shown in the Fig. 3.9. The resulted parameter is higher than commercially available ion-exchanger membranes [29]. Frontal analysis was evaluated in the same manner with commercial bead Q Sepharose FF that showed an ionic capacity of 1.5 mequiv./g which is nearer to the value mentioned in the literature. However, the strategy of build-in function is known to produce proper surface modification of fibrous structures due to the optimization of cationization reaction with high number of charged groups on the ion exchangers.

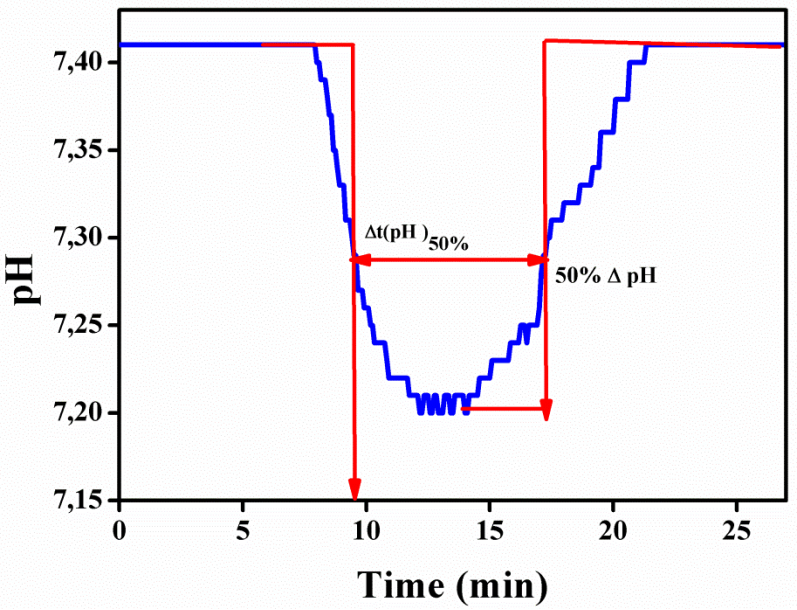

Fig. 3.9. Determination of $\Delta t_{(\mathrm{pH})50\%}$ (shown by arrows) from pH profile for anion exchange fibrous adsorbent packed column. Conditions: buffer A: 500 mM phosphate, pH 7.4; buffer B: 20 mM phosphate, pH 7.4.; Column (5 mm ID × 5.5 cm L); Method: 100% buffer A (> 10 min) step to 100% buffer B (30 min). Flow rate: 150 cm/h. Detection: pH.

3.3.6. Adsorption equilibrium

BSA was selected as a model protein to illustrate the differences in the adsorption properties of the anion exchange fibrous adsorbent and commercial beads. Adsorbed BSA is plotted as a function of BSA equilibrium concentration for static binding measurements and the corresponding Langmuir isotherm constants, $Q_{\max}$ (609 mg/g) and K_{d} (0.37 mg/g) were determined for the anion exchange fibrous adsorbent. As shown in Fig. 3.7, the surface with fibrous material exhibits a significant protein adsorption, almost three folds more than commercial beads as mentioned in the literature values. The high specific surface area combined with the unique fibrous structure with internal hydrogel after proper surface modification is attributed to the elevated binding capacity, indicating a higher efficiency for bioseparation.

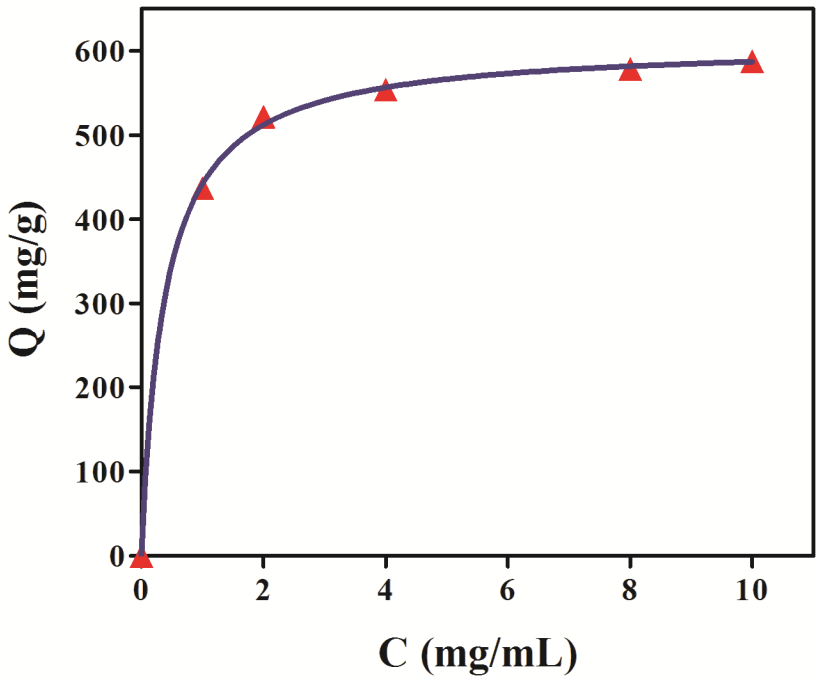

Fig. 3.10. Adsorption isotherm of BSA to the anion-exchange fibrous adsorbent (Q fiber) where Q is adsorbed equilibrium concentration of BSA and C is the liquid phase equilibrium concentration of BSA.

3.3.7. Dynamic breakthrough by frontal analysis

The dynamic breakthrough analysis is a combination of equilibrium binding capacity, adsorption kinetics, and system dispersion to evaluate any adsorption system [8]. The DBC parameter can change, for the same adsorbent for a flow through mode of operation, as a function of the molecular weight and the system superficial velocity. The most potential interesting applications are situations in which macromolecules (e.g. proteins and others) have to be sequestrated rapidly i.e. at high flow rates (or limited contact times). To determine the existence of combined mass transfer resistance and dispersion effects and their potential influence on performance, dynamic binding capacity was evaluated via breakthrough curve analysis (BTC) as a function of superficial velocity. The results of the breakthrough experiments carried out in an increasing diameter of three different columns 5 mm, 16 mm and 50 mm as expressed by the quantities DBC is shown in the Fig. 3.11. It was observed that DBC is decreasing with increasing the flow velocity with all the three mentioned diameter columns and the maximum binding capacities at 10% breakthrough obtained were 374 mg/g, 425 mg/g and 607 mg/g respectively at linear flow velocity of 75 cm/h. As the column volume increases with increasing diameter, the performance of the fibrous adsorbent in terms

of the DBC increases and the linear scalability on fibrous columns can be clearly noticed in the case of columns 5 mm, and 50 mm, and 50 mm. It's also interesting to observe that the performance of the fibrous adsorbent is not affected even though increasing the bed height of the medium as in the column with 16 mm. On the other hand, a reduction of DBC at very high flow velocity can be explained by relatively short residence time with binding sites on the adsorbent with mobile phase. The influence of mass transfer resistance on the breakthrough performance will be explained in the later chapter.

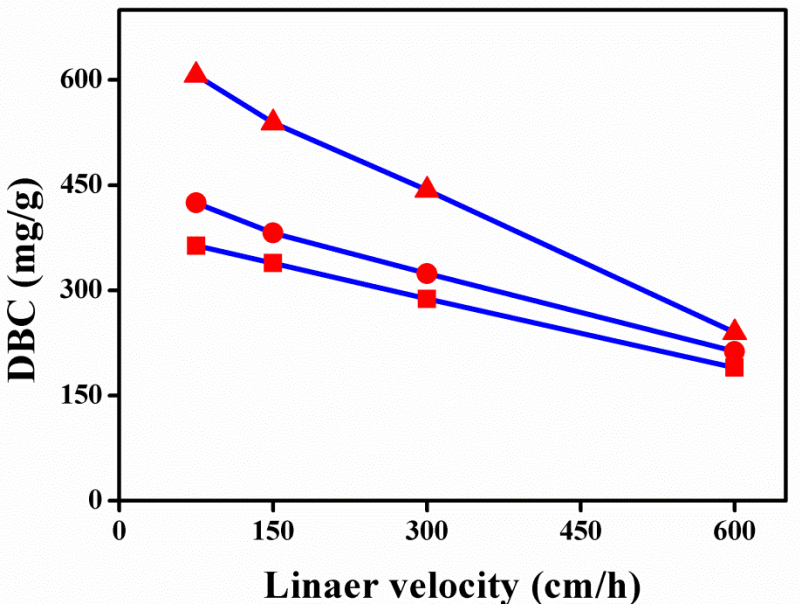

Fig. 3.11. Dynamic binding capacity versus flow velocity for three different diameter columns packed with anion exchange fibrous adsorbent at four different flow rates (75, 150, 300 and 600 cm/h). Conditions: 20 mM phosphate buffer pH 7.4 was used as equilibration buffer and sample (BSA 2 mg/mL) was dissolved in running buffer. (■) column: 5 mm ID × 5.5cm L; (●) column: 16 mm ID × 10.5cm L; (▲) column: 50 mm ID × 5.5 cm L.

3.4. Conclusions

The anion exchange fibrous adsorbent has been prepared by surface modification, using GTMA as etherifing agent under the catalytic action of sodium hydroxide. The introduction of amino groups was confirmed by physically characterization by SEM-EDS and FTIR-ATR and the results indicated that fiber morphology was well retained during hydrolysis and proper surface activation and modification was observed. In this study, the potential of fibrous column packing efficiency and flow permeability were evaluated for linear scalable columns and achieved reproducible column-to-column packing homogeneity and very high permeability coefficient compared to other membrane types and to packed beds of chromatographic resins. The results of the pulse experiments show that the modified fibrous

supports maintain very high porosity for both small and large solutes and have interstitial porosities greater than typical bead packed-beds. For the column characteristics of the packed fibers, breakthrough analysis have been determined for operation in the frontal mode and also compared effect of molecular weight of the sample on the DBC. The results from protein adsorption studies show that the fibrous materials have very high binding capacities under static and dynamic conditions with negligible non-specific protein binding and more significantly it has ability to bind high molecular weight proteins. This is the first paper that describes the performance of the fibrous adsorbent as a chromatography media in terms of high binding capacity at higher operational flow rates that could be linearly scalable to factor of column volume (110 mL). Dynamic breakthrough experiments of the fibrous adsorbents indicate that high binding capacities can be achieved with higher flow permeabilites thancommercial columns of ion exchange resin with similar or lower capacities.

In summary, all these results from this chapter support that the utilized anion-exchange fibrous materials have shown high binding capacity and high throughput at higher operational flow rates and the fibrous geometry is suitable for process scale operations. The mentioned high performance fibrous adsorbents have great potential in separation and purification of biomolecules in the capture and intermediate applications. And also may become attractive alternatives to other types of resins and membranes or monoliths currently being used in biopharmaceutical industry and offer the opportunity to develop disposable bioseparation devices due to low production cost and competitive process-parameters.

3.5. References

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Chapter 4

Process technology scaling a chromatography cartridge system based on functionalized fibers for industrial downstream processing

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The dissemination abstract for this chapter has been approved by ChiPro GmbH.

The abstract presented in this chapter is within the frame of confidential work in cooperation with ChiPro GmbH, Bremen.

The aim of this chapter is to design and develop a procedural scaling of a prototype chromatography cartridge system based on functionalized anion-exchange fibers and evaluate the platform technology that allows a resource-efficient downstream processing with high throughput and high capacity for various bio products. In this work, the prototype cartridge was validated by evaluating their hydrodynamic properties and chromatography process performance. The experimental results indicate that the prototype system which has a unique design with a total cartridge volume of 150 mL that allows excellent hydrodynamic properties and resistance to mechanical stress with a pressure drop less than 1.5 bar operated at higher flow rate (600 cm/h). And the breakthrough experiments for the mentioned system show optimum dynamic binding capacity for bovine serum albumin (250 mg/g) at same flow rate. The performance of the mentioned prototype cartridge packed with the fibrous material was quite comparable to systems with similar dimensions of commercial columns in having better hydrodynamic properties and chromatography process performance but further optimization and validation studies required before using as separation systems in commercial operations. In summary, all the experimental results support that the utilized fibrous material have great potential for high throughput and high binding capacity in separation and purification of bioproducts and also may become attractive alternatives to other types of resins, membranes and particles currently are being used in biopharmaceutical industry. And also offer the opportunity to develop disposable bioseparation devices due to low production cost, relatively inexpensiveness of fibers material and cartridges and competitive process-parameters. However, several important properties and operating conditions have emerged from the literature that suggests careful considerations should be made before using the packed cartridges as disposable separation devices in commercial operations.

Chapter 5

Industrial case study: Purification and scale-up of beta-Phycoerythrin using fibrous based adsorbents by prototype cartridge chromatography

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The dissemination abstract for this chapter has been approved by Phytolutions GmbH.

The abstract presented in this chapter is within the frame of confidential work in cooperation with Phytolutions GmbH, Bremen

In this chapter, the real application of fibers was demonstrated as a potential chromatography support packed in the prototype cartridge in the case study of purification and scale-up of beta-Phycoerythrin (BPE) from the unicellular red marine algae. This is addressed with one step anion-exchange chromatography capture method using anion-exchange fibrous adsorbents in the small scale, replacing two step conventional chromatography operations. The recovery in the capture step was ~50% and the purity was greater than 4 which is equal to commercial value (defined as the absorbance ration of A 545 nm to A 280 nm greater than 4.0). Then the captured process was successfully scaled to large scale purification utilizing an in-house prototype chromatography cartridge of volume ~250 mL with reproducible recovery and purity in the capture step by applying the same optimized process parameters. The purified eluted pink colored B-PE solution was concentrated by ultrafiltration and finally formulated the product. For further increase the purity of B-PE required for various applications, there is the possibility to utilize a polishing step by using gel filtration chromatography. The results of the application of fibers in the purification of B-PE with high purity and recovery have shown that the fibers can be utilized as chromatographic support as an alternative to the commercial beads. The downstream processing of B-PE developed here with novel fibrous adsorbents is efficient and cost effective when compared to conventional chromatography process existing in the industrial downstream processing.

Chapter 6

Mass transfer properties of fibrous adsorbents in relation to process performance

6.1. Introduction

The design and scale up of large-scale chromatographic operations for the recovery and purification of biomolecules is of major industrial importance. Adsorption chromatography is performed in frontal mode where the shape and rate of appearance of the breakthrough profile, represents the mass transfer limitations that control the performance and dynamic behavior of a fixed bed adsorber. The main available technologies for adsorption separations are: purely diffusive (convectional) and perfusion particles and membrane-based affinity chromatography [1]. This chapter describes the mass transfer properties of the innovative fibrous adsorbents in relation to process performance characteristics and compared with conventional available technologies. It includes the resistance encountered by the molecules and the effect of these resistances in the design of process for the recovery and purification. Mass transfer occurs in mixtures containing local concentration variation. The mechanism of mass transfer is considered for the transport of material from high concentration to the regions with low concentration. There are various situations in bioprocessing where the concentration of compounds are not uniform. Hence the mass transfer plays a vital role in the process design in many reaction systems like the transport of oxygen to the cells, extraction of expressed product from the fermentation broth and the transport of substrate to the adsorption site. The mass transfer occurs as result of diffusion or convection and both are important for the uniform availability of the mass to the reaction site. Frontal chromatography is an extensively operated process for the purification of biomolecules by utilizing adsorption supports, which differ with respect to the base material as well as to the surface chemistry. The functionality of an adsorbent is conferred to the surface chemistry with ionic, hydrophobic and affinity ligands and the performance are influenced by ligand density and spatial accessibility [2]. The influence of adsorption performance depends on numerous factors including; the physical and chemical properties of the adsorbate and the adsorbent, the particular rate limiting mechanism, the operating conditions like liquid flow rate, fluid viscosity and adsorbent bed height. The adsorption phenomena onto the adsorbents are not instantaneous but highly specific. Thus mass transfer limitations must also be considered during process characterization, which depends on the relative importance of the above factors. Therefore, the adsorption dynamics of such processes and the characteristics of a fixed-bed adsorber breakthrough profile can be explained and predicted through mathematical modeling.

6.1.1. Diffusion theory

Molecular diffusion is a process by which the component molecules move under the influence of concentration difference in the system. This process can be analyzed in two ways. First, it can be described by Fick's law and diffusion coefficients. Second, it can be explained in terms of mass transfer coefficient. Fick's law of diffusion states that the mass flux is proportional to concentration gradient as [3]

$$J_A = -D_{AB}\frac{dC_A}{dy} = \frac{N_A}{a} \qquad (6.1)$$

where J_A is the mass flux (gmol/s m^2) of component A, N_A is the rate of mass transfer (gmol/s), a is the area across which mass transfer occurs (m^2), D_{AB} is the binary diffusion coefficient or diffusivity (m^2/s) of component A in a mixture of A and B, C_A is the concentration (gmol/cm^3) of component A and y is distance (m).

6.1.2. Transport mechanism

The adsorption phenomena onto an adsorbent are not instantaneous. Because there are some resistances occurring at particular level, this must be considered during process characterization. These resistances can be described as depicted in Fig 6.1.

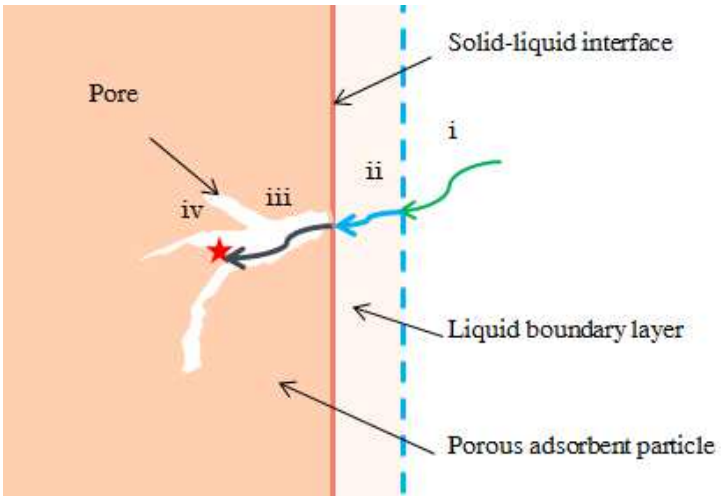

Fig. 6.1. Steps involved in adsorption of solute from liquid to porous adsorbent particle.

i. Transfer from the bulk liquid to the liquid boundary layer surrounding the particle (bulk diffusion)
ii. Diffusion through the relatively stagnant liquid film surrounding the particle (film diffusion)
iii. Transfer through the liquid in the pores of the particle to internal surfaces (porous adsorbents) or diffusion into the solid (gel type particles), that is intraparticle diffusion
iv. The actual adsorption process i.e. binding at the reaction site

In general, points (i) and (iv) are assumed to be negligible because most of the resistance to the mass transfer resides in the liquid film rather than in the bulk. Among these resistances the film and the pore resistance are the main resistances to be considered for efficient system engineering [4] which will be addressed in the following sections.

6.1.3. Film diffusion

The film theory states that mass transfer of solute from one phase to another involves transport of bulk of one phase to phase boundary interface and then from the interface to the bulk of the second phase. A relatively thin stagnant fluid film exists on either side of the interface and the mass transfer through this film is affected exclusively by molecular diffusion. The resistance of this film is called film resistance. The rate of mass transfer is proportional to the driving force to the transfer and the area available for the transfer process to takes place. Therefore the rate of mass transfer to a phase boundary is given by the equation [5]

$$N_A = k_f \, a \, \Delta \, C_A \qquad (6.2)$$

where N_A is the rate of mass transfer (gmol/cm^3 sec) for component A, k_f is the mass transfer coefficient (m/s), a is the area available for mass transfer (m^{-1}), C_A is the concentration gradient (gmol/cm^3).

The resistance to the mass transfer is defined as

$$R_m = \frac{1}{k_f a} \qquad (6.3)$$

In general, reduction in the thickness of the boundary layer or improving the diffusion coefficient in the film will result in enhancement of the k_f and improvement in the rate of mass transfer.

6.1.4. Pore diffusion

As mentioned in above, there is another important resistance due to the pore diffusion, which is called pore resistance where the target of the mass transfer is located inside of the solid particles. According to the equation 6.1, the rate of mass transfer is expressed as below

$$N_A = - D_{AB} \frac{dC_A}{dy} a \qquad (6.4)$$

The rate of mass transfer can be increased by increasing surface area, increasing of the concentration gradient or enhancing the diffusivity. The concentration gradient and diffusivity could be the option to increase the mass transfer but increasing the surface area is economically unfavorable. The effective diffusivity inside the pores i.e. pore diffusivity is less by certain magnitude from the diffusivity which can be expressed by the equation below [6]

$$D_p = \frac{D_{AB}\, \varepsilon\, t\, \delta}{\tau} \qquad (6.5)$$

where D_p is the effective pore diffusivity, $\varepsilon\, t$ is the porosity, δ is the constrictivity and τ is tortuosity.

6.1.5. Mathematical framework and dimensionless analysis

The parameters of mass transfer mechanisms are difficult to measure by direct methods but it is possible to estimate by fitting experimental data with appropriate mathematical models. Frontal experiments are useful to understand the mass transfer phenomenon by determining the breakthrough performance in packed beds [7-10]. Several models have been reported in the literature based on diffusional mass transport models, such as the external film diffusion model [11-13], the intraparticle diffusion model [14, 15], the film–surface diffusion model [16, 17] and the film–pore diffusion model [18-21]. The model selected and analyzed for predicting breakthrough performance of high capacity fibrous adsorbent in this work is based on a classical mathematical film and pore diffusion model. The purpose is to apply an easy-to-use analytical solution to the sorption of protein onto fibrous adsorbents. This model is

characterized by external mass transfer limitation and by intraparticle diffusion occurring through the liquid-filled voids (pores) of the adsorbent particles. A solid diffusion model was also applied to data obtained with the same fibrous material [22]. Development of analytical expressions to simulate different adsorption systems implies the following assumptions:

i. The adsorbent particles are spherical, with uniform size and density and the functional groups of the ion-exchangers are distributed evenly throughout the interior of the particle.
ii. The mass transfer from the bulk liquid to the liquid boundary layer surrounding the particle is negligible.
iii. The mass transfer to the surface of the adsorbent is governed by film diffusion, which is characterized by a mass transfer coefficient (k_f).
iv. The adsorbent is a porous material in to which the solute must diffuse. This is characterized by the pore diffusivity (D_p).
v. Surface reaction between the adsorbate and an adsorption site is both irreversible and infinitely fast. Adsorption is isothermal and its equilibrium behavior can be represented by a rectangular isotherm.
vi. Axial-dispersion is negligible in packed bed systems.

In order to achieve a mathematical description of the frontal chromatographic process, the transport mechanisms for the velocity of the adsorption at the local level can be studied. For one solute molecule to be adsorbed on the solid particle it should at first cross from the bulk of the liquid phase to the surface of the adsorbent. Mass transfer resistance is primarily found at the particle level; nevertheless, diffusion on the bulk is usually not considered a major limitation mechanism. Generally, external mass transfer and kinetic of adsorption are very rapid in the liquid phase so the rate limiting processes are influenced by the film diffusion and transport inside the pore system. For transport, resistance in the pore is the dominant contribution to the total mass transfer resistance for particle diameter is larger than 5 µm in most preparative chromatography [23]. The dimensionless treatment of the breakthrough behavior is based upon analytical solutions for the rate-limited breakthrough behavior in fixed-bed processes [24, 25]. When a step change in solute feed concentration is applied to an initially unsaturated column, the breakthrough behavior can be determined for the case of favorable equilibrium (i.e $R_{eq} < 1$). The effect of breakthrough is important at unfavorable

equilibrium, the transition zone moves through the adsorber as a concentration wave, resulting in a proportionate-pattern breakthrough behavior. In contrast, at nonlinear (irreversible) equilibrium, the transition zone is self-sharpening and leads to a constant-pattern curve on a real-time scale. The case of linear equilibrium is found between those two limits. A "constant pattern"of liquid phase concentration develops as the solute passes through the bed and the average solid and liquid phase concentration no longer needs to be considered separately. The shape of the constant pattern breakthrough curve depends only on the rate-determining mass transfer step in the absence of axial dispersion. The breakthrough can be represented in terms of a dimensionless effluent concentration, X= c/c_0 and a dimensionless mean solid phase concentration (adsorbed phase), Y= q/q_0, where c_0 is the feed solute concentration and q_0 is the adsorbent concentration in equilibrium with c_0. The assumption of a constant pattern in the bed leads to X=Y everywhere. For the dimensionless analysis, time or volumetric throughput may be scaled upon the dimensionless throughput T:

$$T = \frac{c_0}{q_0}\frac{u_s}{L}\left(t - \frac{L\varepsilon_b}{u_s}\right) \qquad (6.6)$$

where c_0 (constant) feed concentration (kg/m^3), q_0 is the equilibrium capacity for feed concentration c_0 (kg/m^3), u_s is the superficial velocity (m/s), L is the length of adsorber (m), t is time (s) and ε_b is the porosity of packing dimensionless.

For every single rate-limiting mechanism, a dimensionless number of mass-transfer units, N (abbreviated as NTU), can be defined characterizing the shape and sharpness of the transition zone as a ratio of mass-transfer efficiency and column residence time:

$$N = k\frac{L}{u_s} \equiv \frac{L}{HETP} \qquad (6.7)$$

where k is a mass-transfer coefficient, an adsorption rate constant or simply a lumped kinetic rate constant and the parameter HETP is the height equivalent to a theoretical plate.

6.1.6. Pore and film resistances under irreversible equilibrium

Hall et al. [24] have solved the nondispersive model under the assumption of irreversible equilibrium and constant-pattern approach. The model includes, both pore-diffusion and fluid-

film mass transfer and were transformed in dimensionless equations by the following equations [24, 26] :

$$T = 1 + \left(\frac{1}{N_{pore}} + \frac{1}{N_{film}}\right)\left[\frac{\emptyset(X) + \frac{N_{pore}}{N_{film}}(\ln X + 1)}{\frac{N_{pore}}{N_{film}} + 1}\right] \quad (6.8)$$

$$N_{pore} = \frac{15 \times D_e \times L \times (1-\varepsilon)}{r_p^2 \times u} \quad (6.9)$$

$$N_{film} = \frac{3\, k_f \times L}{r_p \times u} \quad (6.10)$$

where T is the dimensionless throughput. N_p and N_f are mass transfer units. D_e is effective diffusion coefficient, L is the length of the adsorber (m), r_p is radius of the particle (m) and u is the supervficial velocity (m/s) and k_f is the mass transfer coefficient.

If pore-diffusion alone is rate-determining (N_p -----> 0), then the above equation simplified to:

$$X = 1 - [\tfrac{2}{3} - \tfrac{N_p\,(T-1)}{3.66}]^2 \quad (6.11)$$

When film resistance alone is rate-determining (N_p -----> ∞), then Eq. 18 is simplified to:

$$X = \exp[\, N_f\, (T-1) - 1] \quad (6.12)$$

6.1.7. Mass transfer in packed beds based on bead adsorption

For frontal separations in conventional packed bed column or fixed bed chromatography bead systems, the solute should first diffuse into the porous bead to access available adsorption sites. Generally in bioprocesses, large contaminant molecules such as DNA or viruses with a hydrodynamic diameter (20~300 nm) are restricted. The adsorption process is controlled by the rate of pore diffusion due to limited mass transfer diffusion and higher resistant associated long solute-ligand diffusion distances and even that lead to blocking of pores. In order to achieve proper efficiency of a system, a good quality of column packing and low band broadening due to axial dispersion and mass transfer resistance are required. As a

consequence, a compromise between efficiency and column pressure drop involved in an optimization of chromatographic process is achieved.

6.1.8. Mass transfer in membrane adsorption

Membranes as chromatographic adsorbents in preparative scale have emerged from the mass transfer characteristics of conventional packed-bed adsorption processes. Theses membrane adsorbers are based on microporous supports and subsequent surface modification for the attachment of functional groups acting as binding sites throughout the surface of the pores. The pore structure of the membrane allows a convective flow through the majority of the pores with high throughput and the characteristic distances for pore diffusion will be drastically reduced [27]. The mass transport of solute to adsorption sites occurs thus mainly by convectional flow, film diffusion (film transport through the boundary layer to the porous pore wall, and for diffusion into the pores which are not accessible by convection) [28-30]. Surface modifications with graft-functionalized polymers of macroporous membranes have a significant advantage as it improves higher mass transfer kinetics due to parallel mechanism of surface and pore diffusion coupled with higher ligand density [31]. However, this assumption is not well defined with respect to the influence of the surface chemistry. For membrane adsorbers with photo-grafted polyacrylate layers to the internal pore structure, the overall mass transfer coefficient for the adsorbing solute is also influenced by diffusion to the binding sites in this layer. This effect may become significant when the adsorption is rapid and the thickness of this layer is relatively large, as it is reported in porous membrane adsorbers [32, 33]. Membrane adsorber media allows high efficiency and high flux separations due to greater mass transfer rates than that of standard bead based absorbent media [34]. With proper process technology and subsequent optimized surface modification of ligand functionalization, membrane adsorbers could yield 10 to 100 times higher chromatographic process efficiency than standard bead columns [35]. The ion-exchange membranes utilized at low loads are often comparable to those of commonly used beads in terms of breakthrough performances [27, 36, 37].

Membrane adsorbers exhibit convective flow and have a lower pressure drop at a higher operational flow rate and thus resulting in increased throughput with significant reduction in buffer consumption and process time. It can be integrated in process scale bioseparation operations due to low production cost and relatively inexpensiveness of material and is easy to scale-up/down. Moreover, they offer to utilize as disposable devices and save on cleaning

and validation costs [38, 39]. Despite broad range of applications in process scale operations, membrane adsorbers suffer from several major limitations due to non-identical membrane

pore size distribution (mono-modal or bi-modal), uneven membrane thickness, distorted inlet flow distribution and poor collection at the outlet [30, 40]. For the membranes with a wide pore size distribution and uneven thickness, the solute transfer favorably takes place through large pores, where the transport resistance is minimum and very little target solute can be passed through the smaller pores. Consequently, the available adsorption sites are low that leads to limited binding capacity and a large dispersion effect of membrane adsorber. These limitations can be reduced by utilizing membranes with narrow pore size distribution and smaller thickness due to the lower trans-membrane pressure drop.

6.1.9. Mass transfer in fibrous based adsorption

Although some applications of commercially available high-capacity membranes [41] and nonwoven fabric [42] have already been described but their mass transfer characteristics have not been studied in detail. Until now, theoretical analysis was based upon a more or less ideal and uniform interaction between protein and ligand only limited by slow adsorption kinetics and axial dispersion [8, 43, 44]. Thus, an 'extended' model is necessary, similar to general rate models for fixed-bed adsorption with porous particles [45]. In the present study, the mass transfer behavior of chemically grafted anion-exchange fibrous material in fixed-bed chromatography resulting mass transfer coefficients was evaluated by a dimensionless analysis of the breakthrough zone in frontal analysis. This enables a comparison of the high-capacity fibrous adsorbers with conventional bead matrices.

6.1.10. Mass transfer in monoliths

In contrast to the conventional chromatographic columns packed with porous particles, the monolithic column is a single piece of porous structure of uninterrupted and interconnected channels of specific controlled size with large inner channels diameter (1-1.5µm). In a monolith column, due to high porosity, the pressure drop is low and stationary phase structure is intact even at a high flow rate [46]. The sample is transported through the monolith via convection, leading to very fast mass transfer between the mobile and stationary phase. Moreover, the separation and purification process based convective flow can result in flow-independent resolution and dynamic binding capacity even for macromolecules [47]. However, monolith based metal affinity chromatography exhibits concentration independent

efficient removal of RNA and endotoxin and complete recovery of pDNA at high flowrates. Therefore monolith provides economically viable new platform for pDNA purification [48].

6.2. Experimental materials and methods

6.2.1. Materials

The anion-exchange fibrous absorbents used in this study were with a density of 11 g/cm^3 (EMS Chemie, Switzerland). The model protein used in this work was bovine serum albumin (BSA) from Sigma Aldrich Chemie (Germany). Chromatography resin Q Sepharose FF, columns, Tricorn [0.5 mm Internal Diameter (ID) × 5.5 cm length (L)], XK16 (16 mm ID × 20 cm L) and the ÄKTA explorer system equipped with Unicorn 4.10 software for data collection and analysis were obtained from G E Amersham Bioscience (Uppsala, Sweden).

6.2.2. Breakthrough experiments by frontal analysis

The breakthrough curves for the BSA were determined by frontal studies using the columns (Tricorn 5/50) packed with fibrous material and commercial Q Sepharose FF. The column was loaded with the protein concentration of 2 mg/mL at linear flow velocity of 150 cm/h. For the effect of molecular weight of the sample on DBC at 10% breakthrough was determined by loading separately, low (BSA) and high (thyroglobulin) molecular weight proteins of 2 mg/mL concentration in a column (5 mm ID × 5.5 cm L) and run at a linear flow velocity (300 cm/h). In another study, the fibrous adsorbent was packed at two different bed heights (5 cm and 10 cm) in a column (XK 16) and the protein concentration utilized was 2 mg/mL**.** The experimental procedure was as described in the preceding chapter 3. The dynamic binding capacity was calculated at 10% of the breakthrough. The protein content was measured by UV absorption at 280 nm.

6.3. Results and discussion

6.3.1. Mass transfer properties fiber vs. bead

In a packed–bed configuration, a combined liquid film and pore diffusion limitations can be anticipated when the porous nature of the fibrous adsorbent is utilized. To quantify the effect

of mass transfer on the breakthrough behavior, mass transfer units (N) can be calculated with using pore and film diffusion model after Hall 1966 using a analytical solution to determine for mass transfer coefficient and pore diffusivity from the packed bed experiments. The sharpness of the breakthrough curve increases with increasing number of transfer units and the film transfer can be influenced by the slope at the beginning of the breakthrough curve. The analytical solution was fitted to the experimental data with fibrous adsorbent by non-linear regression based on the sum of the least squares of the residuals (SRS) using Microsoft Excel Premium Solver V11.5 program as shown in Fig. 6.2 and the mass transfer parameters were found to be 2.7×10^{-6} m.s^{-1} for k_f and 1.6×10^{-11} m^2.s^{-1} for D_p, respectively. Similar experiments were performed with commercial beads as illustrated in Fig. 6.3 and the values of k_f as 3.2×10^{-6} and D_p as 8.1×10^{-12} were obtained. These values were compared with experimental data obtained with beads for published data in packed bed configuration which Show values in agreement with the ones reported for the adsorption of BSA [8] and for the adsorption on β-galactosidase on porous silica [48]. Process parameters and the effective mass transfer coefficients calculated for the systems are summarized in Table 6.1. From the obtained results, the mass transfer coefficient values for k_f with fibrous adsorbents from the model may illustrates that the influence of film resistance to the mass transfer from the bulk mobile phase does not play a significant role in the overall mass transfer as expected in the beads. On the other hand, the coefficient values obtained for D_p with fibrous adsorbents were higher than beads which influence the mass transfer and enhancing breakthrough performance of fibers higher than beads due to its convective flow properties.

Table 6.1. Process characteristics of the packed-bed systems for the experimental determinations of breakthrough curves employing anion-exchange fibrous support and bead.

Column parameters	**Q fibrous adsorbent**	**Q Sepharose FF bead**
Pore diameter (m)	4×10^{-5}	2×10^{-5}
Bed length (m)	0.055	0.055
Column internal diameter (m)	0.005	0.005
Initial feed concentration (kg/m^3)	2	2
Void fraction (-)	0.8	0.4
Matrix volume (m^3)	1.08×10^{-6}	1.08×10^{-6}
Volumetric flow rate (m^3/s)	1.67×10^{-8}	1.67×10^{-8}

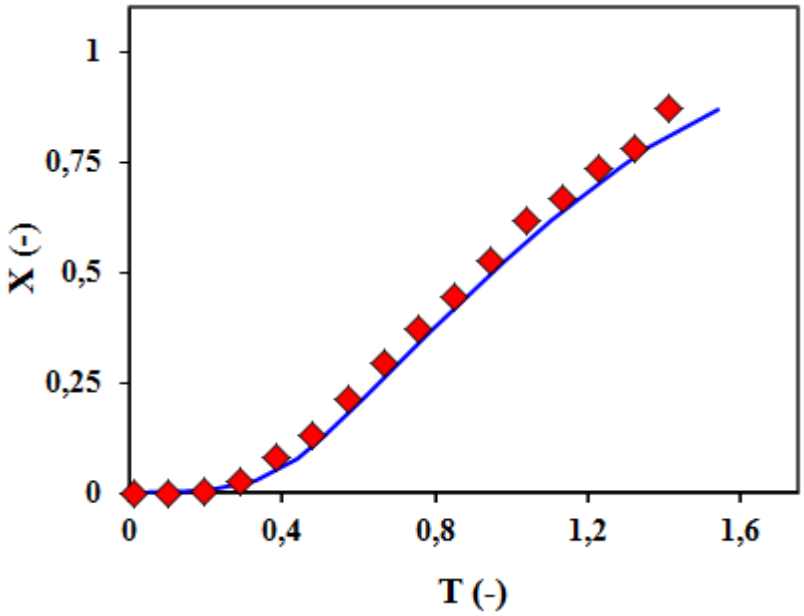

Fig. 6.2. Breakthrough curve analysis for the Q fibrous adsorbent in a packed-bed. Normalized experimental data were fitted using the pore and film diffusion model after Hall. (■) experimental values; (—) calculated from equation.

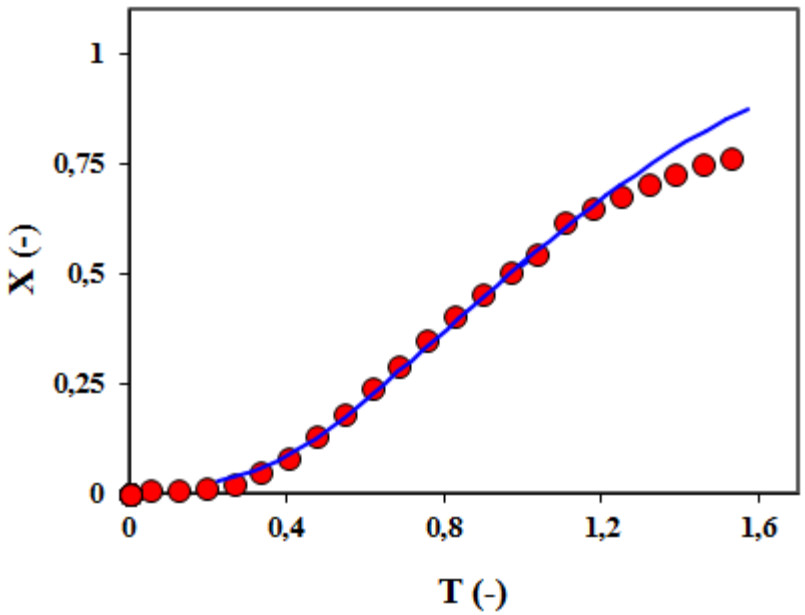

Fig. 6.3. Breakthrough curve analysis for the Q Sepharose bead in a packed-bed. Normalized experimental data were fitted using the pore and film diffusion model after Hall. (●) experimental values; (—) calculated from equation.

Besides, the breakthrough curve allowed the calculation of both dynamic and equilibrium capacities of the adsorbent for BSA from the frontal analysis. Q_{dyn} obtained from the curve was 260 g/kg of anion-exchange (Q) fiber at 10% of breakthrough. This represents 46% of the total available equilibrium capacity (564 g/kg) under the superficial velocity conditions fixed during this experiment.

For the porous adsorbent materials, a dimensionless number called Biot number (B_i) was used in the mass transfer phenomenon to study the relative importance of pore diffusion resistance to film diffusion resistance. The Biot number expresses the ratio of time for film mass transfer to the time it takes for a molecule to diffuse through the adsorbent particle and it can be calculated by the following equation;

$$B_i = \frac{k_f\, L}{D_p} \qquad (6.12)$$

where k_f the mass transfer coefficient (m/s) is, D_p is the pore diffusivity (m^2/s) and L is the length of the particle (m).

If B_i number is less than one, then the external mass transfer resistance is controlling the mass transfer where as if the value is more than 100 then it indicates pore diffusion is predominate mass transfer mechanism. The value of Biot number between 1 and 100 indicate that both pore and film mass transfer resistances are involved in the particular process [49]. For these fibrous adsorbents, the observed Bi value was 6.8 which indicate the combined mass transfer resistance from film and pore for the adsorption of BSA onto the fibrous support but the pore diffusion is the controlling parameter in this case.

6.3.2. Effect of protein size

The protein size on the breakthrough performance with chemically grafted fibrous adsorbent influences the mass transfer properties in a packed bed operation. Fig. 6.4 show the breakthrough curves for low and high molecular weight proteins (BSA 66.5 kDa and thyroglobulin 650 kDa) againt normalized sample concentration respectively. The 'S' shape profile in fixed-bed adsorption is generally associated with solutes of smaller molecular weight and simple structure, and is not observed in adsorbates with larger molecular weights [50]. From the obtained results, the breakthrough at 10% saturation for the thyroglobulin (30 mg/g) was early when compared to BSA (374 mg/g) that indicates that the fiber has less utilization of bed capacity for high molecular weight proteins. It is observed that the adsorbent

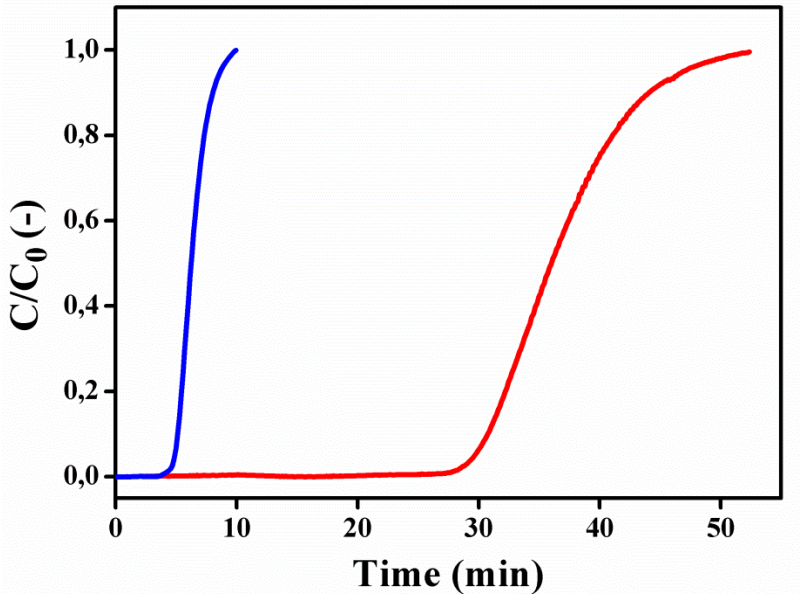

Fig. 6.4. Breakthrough curves for (—) BSA and (—) Thyroglobulin on Q fibrous adsorbent packed in a column (2 g/L Protein concentration in 20 mM Tris-HCl buffer pH 7.4 at a linear flow velocity 300 cm/h).

with a larger protein will have an earlier breakthrough and the slope of the breakthrough curve increases with a decrease in protein sizes. However, the resulted parameter for thyroglobulin is almost 4-5 folds higher than for the commercial resins. These experiments show that the advantages of greater dynamic binding capacity for higher molecular weight proteins (up to 650 kDa) with fibrous adsorbent were even more evident when compared to packed bed adsorption. An engineering estimation of protein diffusion coefficients is often required in biochemical process design for preliminary design work or order-of-magnitude estimation. Based on a global parameter, particle diffusion coefficient (D_p), a correlation is presented for predicting protein diffusion coefficient and assuming a spherical particle can be calculated with the following expression;

$$D_P = \frac{-k\,T}{3\,\pi\,\mu\,d_p} \tag{6.13}$$

where D_p is the stokes-Einstein particle diffusion coefficient (cm^2/sec), k is the Boltzmann constant (m^2 Kg/s^2 k^{-1}), μ is the viscosity of the protein solution (Kg/m.sec), T is absolute temperature (Kelvin) and d_p is the particle diameter (nanometer). The calculated diffusion coefficient (D_p) for BSA (5.98×10^{-7}) was higher than that of Thyroglobulin (2.0×10^{-7}) which is in agreement with the predicted values [51].

It can be explained that the performance of the breakthrough increases with low molecular weight proteins which influences low mass transfer resistance as diffuse easily and interact strongly with charged functional groups attached to the polymeric support.

6.3.3. Effect of bed height

The influence of bed height on the breakthrough performance at four different velocities was investigated for the Q fibrous adsorbent from the frontal analysis. From Equation (N_{pore}) it can derived that the number of transfer units is proportional to the adsorbent bed height. Furthermore, it can be found that Peclet number (>94) increased with increasing bed height, improving the adsorption performance from a dispersion point of view. Therefore, breakthrough steepness is supposed to increase with the bed height, resulting in higher adsorption capacity. This is due to an increase in the amount of adsorbent mass, which results in greater adsorption sites with increasing bed height. Breakthrough experiments with BSA on Q fibrous adsorbent this assumption, as evidenced by the ratio of Q_{dyn}/Q_{max} are shown in

Fig. 6.5. For small bed heights there is a noticeable decrease of this ratio with increasing flow rate, which is less obvious for taller beds

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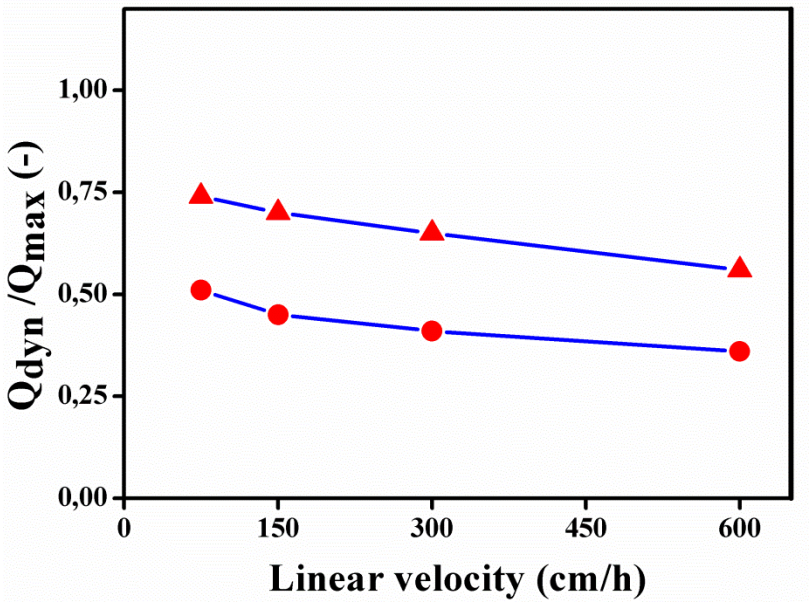

Fig. 6.5. Ratio of breakthrough capacity to equilibrium capacity for an anion-exchange fibrous adsorbent at different linear velocities and bed heights (2 g/L BSA in 20 mM Tris-HCl buffer pH 7.4; Bed heights; (●) 5 cm and (▲) 10 cm).

6.3.4. Process performance of fibrous based adsorption system

The optimal bioprocess designed for the recovery and purification of bioproducts by intensification and integration of two or more separation schemes increases the process efficiency and reduces the total production cost. This can be possible by synthesis of innovative composite fibrous adsorbents which were tested and analyzed at laboratory and pilot scale. It was the first attempt to investigate the mass transfer resistance limitation for the fibrous adsorbent material. The fibrous materials are supposed to have composite macro porous structure that work like interstitial place in beaded particles and perfusive pores which behave like porous bead adsorbents. The mass transfer studies revealed that film diffusion is not the primarily controlling mass transfer mechanism as anticipated with beads but due to pores as depicted from pore diffusivity and Biot number. This indicates that the real sorption takes place in the small perfusive pores like beaded particles but still the larger pores are bale

to allow the crude biomass from fermentation to pass through without any pressure drop and clogging the system while using in a packed bed system.

The innovative composite fibrous (gPore) adsorption system offers distinct advantages when compared to conventional technologies as shown in the table 6.2. The experiments with fibrous chromatography support for the separation of model proteins from artificial crude feed stock as described in the previous chapters revealed that gPore is compatible to biomass and air without any pressure drop and clogging during the capture process and can be operated even at higher flow rates. Despite large cell density and without pre-filtration expressed protein can be purified with high flow rates and are eluted specifically with maximum recoveries. It can be possible to apply gPore in primary recovery operation in bioprocess where several unit operations can be integrated into a single capture step without need for prefiltration and clarification steps. It can be competent to other conventional systems in reducing buffer preparation, volume storage, and short processing time that is enabled by convection flow and energy utilization.

The gPore fibrous system has minimal dependence for the back pressure as a function of flow rates. This was clearly depicted in the flow permeability experiments in the preceding chapters where the back pressure is low operated at flow rate of 600 cm/h when compared to commercial beads in a packed bed. Based on its physical properties of macro porous composite fibrous nature, it is distinguished from existing systems by a nearly optimal ratio of convective mass transport and diffusion and showed excellent hydrodynamics properties even at higher flow rates. In case of packed bed beads, limitations include a relatively time-consuming and high-pressure packing process, a high-pressure drop in the columns and the slow diffusion of solutes within the pores of the bead matrix and therefore high dependence of back pressure as a function of flow rate.

Table 6.2. Unique advantages of gPore fibrous base adsorption system

	Fibrous adsorbers (multimodal)*	Membrane adsorbers**	Adsorbents in packed bed**	Adsorbents in EBA**
Material Structure	composite fiber	micro-porous	meso-porous beads	mesoporous tungsten carbide
Mechanical resistance	yes	Yes	no	no
Chemical resistance	yes (up to 1 N)	yes	yes	yes
Ligand chemistry	any	any	any	any
Useful Lifecycles	50-100	50-100	50-100	50-100
Operational flow rates (cm/h)	up to 600	ca. 500 (Satobind MA15-DEAE)	ca: 600 (Nuvia Q)	ca. 400 (Streamline DEAE)
Backpressure as a function of flow rates	minimal dependence	moderate dependence	high dependence	no– elutriation of the particles
Need for clarification	no (≤ 10 % biomass)	yes (extensive clarification)	yes (extensive clarification	no (≤ 10% biomass)
Buffer consumption factor	0.3	1	1	1.6
DBC of BSA (mg/Ml) at flow rate 300 cm/h	60-70	ca. 38	ca. 170	ca. 100
Process flexibility	yes	no	no	no
Product flexibility	yes	no **	no	no
Resolution power	yes	no	yes	no
Disposability	yes	yes	no	no

* Experimental data

** Data from literature and or from experience

*** Column volume

The single component gPore adsorbent system is compared with existing fibrous based process systems available in the literature and market as shown in table 6.3 and is competent in terms of chromatography capture, purification and polishing applications. The presence of functional hydrogel with high surface area in the gPore facilitates the available adsorption sites to the protein molecule. The breakthrough experiments revealed that gPore has good sorption properties even at higher flow rates. The results show that gPore has in comparison to commercial materials significantly surviving dynamic binding capacity and can be used in a wide range of flow rates. The mass transfer due to the film is very less as compared to the beaded particles and the pore diffusivity increased with increase in the flow rate. It depicts that mass transfer resistance due to the pore is very less as compared to the beaded particles. The pore size of the fibrous material is huge as compared to the beaded material. The increase of the pore diffusion coefficient with respect to increase in the flow velocity indicate the presence of perfusive pores, where the increased flow rate results in the convective flow in such a type of pores and thus reducing the mass transfer limitation.

The rate of diffusion is reciprocal to the resistance and can be enhanced by reducing the resistance. The decrease of the film resistance can be helpful in this regard. The rate of diffusion depends on the distance to be travelled by solute, which can be decreased by decreasing the particle size. And the breakthrough experiments with fibrous material packed in different range of columns and cartridges prototype indicates that the fibrous adsorption system is feasible to operate in an industrial level and also handling of large amount of sample is easy and it takes less time to process the sample as compared to fixed bed adsorption. gPore offers product flexibility and can be utilized in different modes of chromatography by immobilization with proper functional ligand chemistry. In this thesis work, only strong ion exchangers were discussed however, the potential with different systems such as antibody proteins (monoclonal antibodies) could be explored when the composite support is immobilized with appropriate affinity ligands. This means you can integrate the new matrix not only at all levels of chromatography, but also in virtually every process (biotechnology, food) for the purification of any products to make the process more economical and ecological.

Table 6.3. Comparison of novel single component gPore system with existing fibrous systems

	[1]gPore fiber	[2] US Patent by Yavorsky et al. (Millipore Corp)	[3]Qyu Speed D (Asahi KASEI)	[4]Natrix
Adsroptive media	functional composite fiber with hydrogel	functionalized shaped (winged) synthetic fiber presents fibrillated or rigid structure	hollow fiber membrane adsorber	macroporous hydrogel polymer support
Ion-exchange functionality	cation exchange (SP) and anion exchange (Q) fibers	cation exchange (SP) and anion exchange (Q) fiber	anion exchange (DEA ligands)	cation exchange (SP) and anion exchange (Q/C) fiber
Ligand chemistry	any	any	Na	Any
DBC	60-70 mg/mL BSA	28-37 mg/mL BSA 20-60 mg/g IgG	>40 mg/mL BSA (MV)	85 mg/mL IgG
Flow rates	* 100-900 cm/h	200-1500 cm/h	5-13 MV/min	5-20 MV/min
Chemical compatibility (1N NaOH)	yes	na	Yes	Na
Scalability	easy and linear	na	easy and linear	Na
Virus removal	na	LRV ~3.18	LRV>5	Na
Buffer consuption	low	na	Low	Low
Reusability after regeneration	less	na	Yes	Yes
Impurity removal	efficient	efficient	Efficient	Efficient
Processing time	less	less	Less	Less
Purification application	yes	yes	Yes	Yes
Capture application	yes	yes	Yes	Yes
Flow through applications	yes	yes	Yes	Yes
Recovery	≥ 90%	≥ 90%	High	≥ 95%
Available in market	no	no	Yes	Yes
Disposable	yes	yes	Yes	Yes

*Recommended flow rate 600 cm/h

Na-not available

[1] Own experimental data and or experience

[2] Based on the information available in the US patent (# US 2012/0029176A1 Yavorsky et al.) "Chromatography media and method"

[3]http://www.planovafilters.com/index.php?eID=tx_nawsecuredl&u=0&file=fileadmin/documents/pdfs/Products/QyuSpeed/QSD_English.pdf&t=1346160626&hash=0171d10d6a4677a12ee337140d7b983c

[4] http://natrix.accelhost.com/accelsite/media/2985/Natrix%20HIC%20Poster%20-%20BPI%202011.pdf

The gPore adsorption system facilitates process flexibility by process integration which can be applied in a capture step or process intensification for applying in intermediate purification at reasonable resolution even at higher flow rates so that buffer volume and process time can be reduced. And also gPore fibrous adsorption system offers the opportunity to develop disposable bio separation devices with suitable prototype chromatography cartridges due to low production cost of fibers and systems and competitive process-parameters.

6.4. Conclusions

The impact of mass transfer properties in breakthrough performance using high capacity anion-exchange fibrous adsorbents for BSA were studied and compared with commercial beads. A combined pore and film diffusion model predicted the breakthrough binding behavior which attributed to mass transfer coefficient (k_f) and pore diffusion coefficient (D_p). From the results it can be observe that the k_f value of fibrous system is in good agreement to the beads as the mass transfer resistance from the bulk fluid does not influence the breakthrough performance. The value of D_p for fibers was higher which effect the mass transfer and enhancing the breakthrough performance of fibers greater than beads due to its convective flow properties. In addition, the breakthrough curve from the frontal analysis under the superficial velocity conditions represents 46% of the total available equilibrium capacity (564 g/kg). The obtained values explained the performance of the breakthrough increase with low molecular weight proteins and correlated with the calculated D_p for BSA which was higher than that of Thyroglobulin in enhancing the access of available binding sites inside the pore. The influence of bed height on the dynamic capacity at different flow rates were investigated for the Q fibrous adsorbent from the breakthrough analysis. From the obtained results, an increase in bed height increases the breakthrough time, resulting in increasing mass transfer properties which leads to higher adsorption capacity. The unique grafted surface-functionalized novel composite fibrous system offers a platform technology for recovery, separation and purification of bioproducts by a variety of adsorptive and chromatographic separations in downstream processing. The unique grafted surface-functionalized novel composite fibrous system offers a platform technology for recovery, separation and purification of bioproducts by a variety of adsorptive and chromatographic separations in downstream processing. And also the process performance of gPore fibrous

adsorption system with unique advantages have been presented and compared with conventional adsorption systems with the available literature data.

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Chapter 7

Summary and future remarks

7.1. Summary

In this final chapter, I will briefly summarize the main aspects of the thesis and its implications for the downstream processing of bio products in the biotechnology industry. The main aim of the thesis was to develop innovative fibrous adsorbent materials and systems for efficient and cost effective solutions to industrial downstream processing. To design an intensified process for the recovery and purification of different bio-molecules by the integration of numerous steps of downstream processing into less number of steps in order to increase process efficiency and reduce overall cost of the product. For this purpose, fibrous materials were proposed as novel chromatography support for efficient adsorption system to integrate the early recovery and intermediate purification steps.

Downstream processing of bio-molecules needs several unit operations involving cell disruption, cell clarification, volume reduction, fractionation, capture, intermediate purification, and polishing and buffer exchange in order to formulate the final purified product. This conventional multistep downstream processing is often time consuming, labor-intensive requiring large hold-up volumes of buffer, water and energy consumption and usually accounts for up to 80% of the total process costs. Hence the biopharmaceutical industry has begun to target on process intensification and integration to improve economics by merging two or more unit separation schemes into one, thus reducing process times, cost of goods, and capital investments. Nevertheless, industrial chromatographic processes mandatorily require an extensively clarified feed stream to prevent packed bed clogging and backpressure development. Alternatively, the introduction of expanded bed adsorption (EBA) has allowed early process integration as direct “capture” or sequestration from a crude feedstock. However, the poor hydrodynamic condition of the fluidized/classified bed causes a reduction of the overall system dynamic binding capacity for the target product. The chromatographic beads exhibit several major limitations due to slow intra-particle diffusion within the porous beads which tend to limit the dynamic binding capacity of the resins to capture the desired target product, the column design reduces throughput as a result of the increased pressure drop at higher flow rates and high material and operational cost. Although membrane adsorbers offers capture and polishing applications, they do not tolerate biomass and capacity decreases with increasing pore size, limited system design and can only operate for a given permeability due to the coupled flow and binding properties.

As a consequence, the separation and purification schemes applied in the biopharmaceutical industry for the purification of biotherapeutics requires alternative materials to replace traditional packed-bed column resins. For the process intensification via process integration of several steps, it is important to choose the efficient and cost effective unit operation that has ability to modify and integrate several unit operations. The process intensification is feasible if the adsorbent is modified in a way that it could have high adsorption capacity and high throughput with less expensive material and operational cost.

Fibrous materials are prominent among novel chromatographic supports depicting high throughput and high product capacity for the separation and purification of biomolecules after proper surface modification. However, fibrous adsorbents have not been reported for high throughput and capacity due to low porosity, irregular packing, low pressure drops and lack of biomass compatibility. Since the surface modified and activated fibers with numerous graft polymerization using preirradiation and peroxidation techniques reported in the literature are not appropriate for potential chromatography adsorbent because grafting is in-homegeneous, ligand immobilization is complicated, and a lower surface area with limited capacity, poor mechanical and chemical stability and lack of hydrogel is achieved.

The utilized composite fibrous material (gPore) in this research work was obtained from our scientific partner. The gPore was synthesized by gamma-irradiated simultaneously grafted polymerization where a monomer (glycidyl methacrylate) was polymerized on the pore surface in order to increase the surface area of the material. The chosen monomer has a dual functionality i.e. the ability of polymerization and the inherent epoxide. The conversion of epoxide to any functionality is possible under mild conditions. The gPore was chemically converted to cation exchanger (SP gPore) by immobilization with sulfonate groups under optimized conditions. The SP gPore has been characterized and showed physical properties of high porosity, high degree of swelling (300%), high degree of grafting (30%), mechanical stability and water permeability. The fiber morphology from microscopic studies revealed that gPore exhibited irreversible open compact structure with internally grafted homogenous hydrogel. The grafting of epoxide and its chemical modification to cation exchanger was confirmed by FTIT-ATR.

In this work, the fibrous packing configuration was done in the column utilizing a unique designed “double roll” support structure with appropriate aspect ratio and subsequently tested for packing efficiency and chromatography performance. The mentioned system showed

similar packing efficiency of height equivalent to a theoretical plate (HETP) value to the packed bed beads and reasonable asymmetry values. These indicate the uniform column packing which influence the chromatography performance during the process. The permeability coefficient of gPore is 1-2 times higher than membranes and to the packed beds of chromatographic resins. Experimentally determined Peclet number (Pe) values from residence time distribution experiments were suggesting a close-to-plug-flow condition. The composite fiber with functional hydrogel provides mechanical and chemical stability to the chromatographic support and good swelling properties. A uniform packing efficiency enables particularly good flow and adsorption properties otherwise the effect of packing heterogeneity might seriously affect separation efficiency and lead to poor process performance of the chromatography support. The total ionic capacity of the SP gPore fibrous adsorbent was more than that of commercial available cation-exchange based membranes. Moreover, high equilibrium protein binding capacities for lysozyme under static and dynamic conditions were found to be 525 mg/g and 283 mg/g respectively (Chapter 2). This might be due to composite fiber with functional hydrogel having higher surface area where reactive groups could be directly introduced to perform ion exchange immobilization to the fiber surface and thus reducing the diffusion limitations as the process fluid transported across the fiber.

The gPore fibrous adsorbent has the ability to separate a target model protein (lysozyme) from a mixture of proteins in a packed bed. These experiments demonstrated that gPore can be employed in intermediate purification application where resolution is maintained even at high flow velocities and utilizing shorter gradient development routines. The bioprocess can be intensified with gPore by operating at higher flow with high resolution minimizes the buffer volume, process time and operational cost. On the other hand, direct sequestration of a model protein (lysozyme) was also possible from an artificial mixture containing 1.5% yeast homogenate. The lysozyme selectively bound to the SP gPore and eluted with reproducible purity and recovery without prefiltration and clarification of the crude stock. This demonstrated almost perfect compatibility with biomass and air without pressure drop and clogging. The so-called gPore fibrous adsorption system was tested for its potential for intermediate purification for high resolution and capture application with superior performance. Here it can be concluded that the gPore fibrous adsorbents has the ability to intensify the purification process by integration of various primary unit operations into a single purification step. To summarize the work from the second chapter, this is the first study that demonstrates the utilization of cation exchange composite fibrous adsorbents as a

chromatography support for high throughput and high capacity and suitable for intensified and process integration applications in bioseparation. However this gPore fibrous adsorbent system could be tested in industrial scale for recovery and separation of bioproducts which will be explained in the later section.

The functionality of fibrous adsorbents can be flexible so here the work was extended for other strong anion exchange adsorbents. This was modified by using a chemical grafting method after amination with glycidyl trimethyl ammonium chloride (GTMA) under optimized reaction conditions. The chemical synthesis of the material was a critical step as the material's physical properties should be compatible with the chromatographic systems. The concentration of GTMA, dimethyl formide (DMF), reaction temperature and time are critical for the optimal functionalization yield. The optimization of cationization is important for the fibrous adsorbent to act as a potential chromatography support. This material was characterized by many ways to visualize the effect of chemical modification on the porosity. For pulse experiment with acetone and lactoferrin as tracers under nonbinding conditions, the total porosity (to acetone) and the interstitial porosity (to lactoferrin) were measured respectively and high porosity for both small and large solutes was maintained and interstitial porosities greater than typical bead packed-beds were achieved. The modified anion exchange fibrous adsorbents showed good packed performance in linear scalable columns which can be determined through residence time distribution. The fibrous adsorbents have higher permeability coefficients ($>0.9 \times 10^{-7}$ cm^2) than other commercial membranes and columns packed with resins. The protein adsorption studies indicated that the functionalized fiber had a high static binding capacity (609 mg/g) for bovine serum albumin (BSA). The breakthrough experiments from frontal analysis revealed that the sorption properties of anion-exchange fibrous adsorbents and the maximum dynamic binding capacity achieved was 607 mg/g and proved to be linearly scalable to a factor of a column volume (>110 mL). Additionally this work describes the performance of the anion-exchange fibrous adsorbents packed in commercial columns as a chromatography media in terms of high binding capacity operated at higher operational flow rates that could be linearly scalable. Here it can be concluded that the anion exchange fibrous adsorbents have high capacity and high productivity for bioseparations.

The mass transfer resistance of the fibrous adsorbents has been investigated in a packed bed. The relative impact of mass transfer parameters on the breakthrough performance for bovine

serum albumin (BSA) were predicted with a combined pore and film diffusion model by fitting breakthrough data to mathematical models. The results show that the film diffusion is the not primarily controlling mass transfer mechanism as anticipated with beads. The large impact of pore diffusion coefficient indicated that the pore diffusivity was controlling the mass transfer for breakthrough performance. The effect of protein size on the breakthrough performance of fibrous adsorbent illustrated that low molecular proteins have high mass transfer properties which leads to increase of the binding capacity compared to high molecular weight proteins. And interestingly, the fiber had the ability to bind a high molecular weight protein (thyroglobulin) significantly higher than the commercial beads. Furthermore, the effect of flow rate and bed height of absorbent on the dynamic binding capacity (Q_{dyn}) revealed that an increase in bed height increases the breakthrough time as a function of flow rate. Overall, the innovative fibrous adsorbents after proper surface modifications resulted in less mass transfer resistance thereby offering good hydrodynamic properties and process performance for the integration and intensification of the bioprocess.

However, these innovative fibrous adsorbents could be tested in an industrial scale for recovery and purification of bioproducts for efficient and cost effective solution to downstream processing. For this reason a procedural scaling of a suitable prototype chromatography cartridge system based on functionalized fibers has been developed and worked in cooperation with ChiPro GmbH. It was investigated by evaluating their hydrodynamic and process performance parameters and the experimental results indicate that the cartridge prototype system has a unique design that allows excellent hydrodynamic properties, resistance to mechanical stress and process performance with optimum dynamic binding capacity operating at higher flow rates. The results were not shown here due to copyright reasons. The performance of the mentioned prototype cartridge packed with the fibrous material is quite comparable to systems with similar dimensions of commercial columns but further optimization and validation studies required before using as separation device systems in commercial scale operations. The innovative gPore cartridge system presented here has been practically validated in a case study of algal product (beta-Phycoerythrin) recovery and purification from unicellular marine algae in a food industry. The aspect of application was done within the frame of confidential work with Phytolutions GmbH. The innovative fibrous adsorbents were successfully utilized in a single ion-exchange capture step for large scale chromatography purification by using the mentioned cartridge system with reproducible recovery and purity required for the commercial purpose. The

mentioned process is efficient and more economical compared to available processes in the literature. The bioproduct case study demonstrated successfully that the fibrous adsorbents can be utilized as chromatographic support as an alternative to the commercial beads in the industrial downstream processing.

The designed innovative fibrous adsorbents system is an intensified single component system which can be applied in the intensified process for the recovery and purification of biomolecules from the crude biomass in a single capture step without need for clarification, prefiltration steps and large requirements of buffer, water and energy. The fibrous adsorbents have the high resolution power to separate the target product from the mixture of contaminants even at higher flow rates. Thereby reducing the buffer volume, process time and cost in intermediate purification applications and allows designing an intensified process. The ability of product recovery from biomass in the capture step and high resolution in intermediate purification step offers process flexibility depending on the user specific requirement. The functional hydrogel with high surface area and increased porosity with less mass transfer resistances and proper surface activation favors the high dynamic capacity to capture the target product in a relatively short process time. In addition, the pore size and overall porosity of the fiber can be controlled to operate at very low pressure drops, resulting in increased throughput compared to column bed beaded operations. The design of cartridge format increases the throughput as a result of reduced pressure drops at higher flow rates and also occupies less floor space. The fibrous material has the ability of easy modification of any functional ligands and makes them suitable for the more specific purification in a bioprocess step and offers product flexibility. The fibrous adsorbent systems can be scalable in industrial scale since the physicochemical properties and process performance of the material does not change in higher scale and the production of the material is easy to handle. The production cost of a cartridge system is very low and the fibrous materials are relatively inexpensive as result the newly adsorption system offers opportunity to develop bioseparation devices. Consequently reducing the operational, cleaning and validation cost for the chromatography process and also may become attractive alternatives to other types of packed or expanded beads, membranes adsorption and particles currently being used in biopharmaceutical industry. Therefore the designed fibrous adsorbent system can be targeted on process intensification and integration to improve economics by merging two or more separation units into one thus reducing process time, cost of goods and capital investments. Hence the newly developed cartridge fibrous adsorption system presented here has the potential impact in

relation to the efficient use of production time, cost and resources for companies in the life science industry to reduce costs, save jobs and maintain itself in the marketplace.

7.2. Conclusions

- Will there be any fibrous base adsorption systems available to produce high capacity and high throughput bio separations fibrous to replace traditional beads and membrane adsorption?

  The gPore fibrous adsorbent system has been demonstrated for high capacity and high throughput with high resolution that could fulfill the criteria to replace the traditional adsorption systems with beads and membranes.

- Could it be possible to design an intensified and integrated bioprocess adsorption system for direct capture of target product from fermentation and high resolution intermediate purification applications?

  The capture and intermediate purification application of gPore fibrous system has been demonstrated and therefore could be possible to design an intensified and integrated system to improve process economics.

- Is there any available efficient and cost effective fibrous adsorption based cartridge system to replace traditional columns and membranes in bioprocess scale?

  The design and development of prototype cartridge chromatography system is available for the cost effective and efficient solutions to replace traditional columns and membranes in bioprocess scale.

- Could it be possible to tackle real applications in industrial downstream process scale with the fibrous base adsorbent systems?

  The case study with alga beta product recovery and purification in industrial downstream process scale with gPore fibrous base adsorbent system has been successfully tackled.

7.3. Future remarks

Europe indeed has the potential to be a world leader in the field of industrial biotechnology and offers excellent research base and skilled labor force across European member states. Europe has taken a decisive step toward integrating biotechnology into policy making process and that is a great benefit to the biotechnology sector which is now central to the main aims of Horizon 2020, to tackle societal challenges; promote industrial leadership; and boost European excellence in research, development and innovation (RDI). The biopharmaceutical market is one of the fastest-growing segments of the life sciences industry and expected to represent 30% of all marketed drugs, estimated to be about $40 billion annually within the next five years. The increased demand for the production of biopharmaceuticals drives the importance for the improvement in cost-effective and efficient purification technologies in the downstream processing which provides new opportunities and major challenges for the key market drivers and barriers for the biotech industry. The market drives for the biopharmaceuticals mainly due to increased competition, rising development and production costs, safety and immunogenicity concerns and rise of follow on biologics (biosimilars). The technical improvement of automation, streamlined expression (high product titers) and high quality platform technologies in the upstream processes is resulting in pressure on downstream processing which contributes 50-80% manufacturing costs for commercialization of bio production. The number of processing steps, cleaning and validation cost needs to reduce which in turn significantly demands the development of new process chromatography adsorbents and flexibility and speed in facility, process design and cost efficient solutions to downstream processing.

The potential solutions to technical needs to be considered in the 2020 downstream processing policy includes the development of enhanced capacity adsorption systems with high throughput and low pressure devices like fibers, membranes and monoliths with reduced elution volumes. Implementing efficient non-chromatographic approaches like precipitation, crystallization and improved cell removal approaches like flocculation steps and replacement of protein A product capture with robust, inexpensive ligands for the process integration and intensification. The adoption from stainless steel to single-use bioprocessing equipment and process products for high capacity and high throughput chromatography adsorbents in downstream processing particularly single-use disposable technology reduces capital

investment costs and time to start-up. Implementation of more extensive validation by rapid process analysis and development techniques with high throughput screening, expert systems, improved process models and monitoring process parameters and attributes using process analytical technology are necessary. Enhancing flexibility, speed in facility and process design by facility of the future (FOF) with disposables, purification platforms and smaller foot prints would be significantly required in the production. This improves to optimize the production efficiency and cost-effectiveness, which provides key data, information and analysis of the Contract Manufacturing Organization (CMO) industry, and the downstream processing and reagent market. In addition to manufacturing in biopharmaceutical production, they also offer clinical trials, logistics, packaging, and even marketing. According to industry experts, almost 60% of the CMO market is dominated by manufacturing functions, out of which more than 50% is dominated by the downstream process. Exploration of potential investment opportunities, understanding and analyzing upcoming trends in licensing will be poised to drive the future growth of the downstream processing.

The main aspects of the work done here is to develop process chromatography adsorbents for efficient and cost effective solutions to industrial downstream processing of bioproducts. The approach developed for the surface modification of fibers in this thesis have provided the basis for a promising strategy for product flexibility and it can be further extrapolated depending on the application requirement i.e. development of other ion-exchange (weak) fibrous adsorbents in order to have a complete ion exchange product portfolio. The potential with different systems such as antibody proteins (monoclonal antibodies) could be explored when the composite fibrous support is immobilized with appropriate functional affinity ligands or peptides for the affinity chromatography adsorbents. It could be further explored by studying the combination improved chemistry and surface energetics to assess biomass attachment onto anion-exchange fibrous adsorbents for direct capture applications from crude feed stock. Based on the hydrodynamics and process performance results obtained, a detailed study with help of design of experiment (DOE) could provide more insightful information for the fabrication of the prototype chromatography cartridge that can be further optimized for large scale industrial applications. The immediate requirement of the work to be done here is the practical application of the newly fibrous base adsorbent cartridge system in the real case scenario in the area of biopharmaceuticals, food, cosmetics, and dye industries for large scale downstream processing in the European sector.