



Fast cation-exchange separation of proteins in a plastic microcapillary disc

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ABSTRACT

A novel disposable adsorbent material for fast cation-exchange separation of proteins was developed based on plastic microcapillary films (MCFs). A MCF containing 19 parallel microcapillaries, each with a mean internal diameter of 142 μm , was prepared using a melt extrusion process from an ethylene-vinyl alcohol copolymer (EVOH). The MCF was surface functionalised to produce a cation-exchange adsorbent (herein referred as MCF-EVOH-SP). The dynamic binding capacity of the new MCF-EVOH-SP material was experimentally determined by frontal analysis using pure protein solutions in a standard liquid chromatography instrument for a range of superficial flow velocities, $u_{1S} = 5.5\text{--}27.7\text{ cm s}^{-1}$. The mean dynamic binding capacity for hen-egg lysozyme was found to be approximately 100 μg for a 5 m length film, giving a ligand binding density of 413 ng cm^{-2} . The dynamic binding capacity did not vary significantly over the range of u_{1S} tested. The application of this novel material to subtractive chromatography was demonstrated for anionic BSA and cationic lysozyme at pH 7.2. The chromatographic separation of two cationic proteins, lysozyme and cytochrome-c, was also performed with a view to applying this technology to the analysis or purification of proteins. Future applications might include separation based on anion exchange and other modes of adsorption.

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1. Introduction

Downstream bioprocessing represents a substantial cost in the production of biopharmaceuticals. Chromatography processes allow separation of proteins based on their size, charge and/or net charge. The most commonly used chromatography separation operations are based on microporous matrices and packed bed columns, which have high protein capacities and present good separation characteristics [1]. However, due to their packed bed format there are several limitations to this approach including cost, bed compression leading to high pressure drop and low mass transfer rates due to the dependence on intra-particle diffusion for the transport of solute molecules to their binding sites within the pores of the media. In practice, these limitations necessitate slow column flow rates [2]. Radial and axial dispersion limitations and channelling arising from formation of flow passages due to cracking of the packed bed further impede operability [1].

To overcome some of the limitations of packed bed chromatography, synthetic microporous or macroporous membranes have been developed as chromatographic media [1–3]. In membrane chromatography processes the transport of solutes to their binding sites takes place predominantly by convection, thereby reducing

both process time and eluate volume. As the binding efficiency is largely independent of flow rate, and there is minimal pressure drop across the membranes, separations can be run at high flow rates [3]. Scale up of membrane chromatography is possible due to its modular format.

Here, the use of plastic microcapillary films (MCFs) as a basis for rapid bioseparations is presented. MCFs are a novel microstructured material containing continuous microcapillaries that can be extruded from a range of thermoplastic polymers with different material characteristics in a low cost scalable procedure [4,5]. In this work MCF was extruded from ethylene-vinyl alcohol copolymer and surface activated for cation exchange chromatography separation of proteins. As with membrane chromatography, transport of solutes to binding sites occurs mainly by convection. This feature, in combination with the low pressure drop for flows through the MCF, enables protein separations to be performed at high flow rates. For MCF's, with no intraparticle diffusion resistance and high film mass transfer rates, the mass transfer resistance is low compared to conventional porous adsorbents. However, the available surface area for protein binding in MCF is substantially lower compared to porous adsorbents.

Scale-up of MCF separations is similar to the linear scale-up of membrane processes. Throughput is particularly high relative to conventional adsorbents, due to a fundamental difference in the relationship between equilibrium and dynamic binding capacities. With a conventional cation-exchange adsorbent (e.g. SP Sepharose) the total surface area for protein adsorption per unit

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volume of adsorbent, at $127\text{ m}^2\text{ ml}^{-1}$ [6] is ~ 4500 times that of an MCF with $142\ \mu\text{m}$ capillaries and surface area $0.028\text{ m}^2\text{ ml}^{-1}$. For equal ligand densities, conventional adsorbents thus have ~ 4500 -fold higher equilibrium binding capacity (EBC). However, in bio-manufacturing a significant proportion of this surface is not accessed and the performance determining parameter is not the EBC but the dynamic binding capacity (DBC); the adsorbent capacity during flow.

Whereas conventional columns are operated with low superficial liquid velocities of $<300\text{ cm h}^{-1}$, MCF's can be run with superficial velocities up to 330-times higher since no matrix compression occurs and high pressures can be used (up to 50 bar pressure [7]). For a conventional porous adsorbent under typical operating conditions the overall mass transfer resistance to adsorption [8] is of relative magnitude $240\times$ EBC. For MCF's, with no intraparticle diffusion resistance and high film mass transfer rates, the overall mass transfer resistance is only of order $0.023\times$ EBC. Consequently, the rate of protein adsorption can be approximately 5-orders of magnitude higher in MCFs. All ligands are accessible and much higher dynamic binding capacities are obtainable when operated at high throughput.

Additionally, breakthrough curves for MCFs are sharp even at high throughputs [9]. Indeed, when operated with a flow velocity of 160 m h^{-1} this MCF displays a similar number of theoretical plates to a 20 cm Sepharose packed column, operated at 100 cm h^{-1} . MCFs thus provide the ideal plug-flow behaviour that is needed for sharp chromatography breakthrough at high liquid flow rates. MCFs are fabricated from cheap polymer resins using a low cost extrusion process similar to that used for other disposable bioprocessing items (e.g. bags, filter housings and tubing). Materials and processing costs of ~ 50 pence/m are estimated, such that the cost for a 5 m cation-exchange MCF would be $\sim \pounds 2.50$. Module assembly and qualification, sales, distribution and manufacturer's margin would add to this cost to a similar extent as with current membrane and adsorbent technologies. Overall though, whereas MCF units might be physically larger than conventional columns, the manufacturing cost per unit will be significantly lower. Furthermore as the MCF is transparent, the separation can be tracked anywhere along its length.

2. Materials and methods

2.1. Chemicals

Ethylene-vinyl alcohol copolymer (EVOH) containing 32 mol% of ethylene was supplied by Eval Europe (Antwerp, Belgium). NaOH, cyanuric chloride, acetone, Na_2HPO_4 , 2-(N-morpholino)ethanesulfonic acid (MES), tris(hydroxymethyl)aminomethane (TRIS), HCl 3-amino-1-propanesulphonic acid, chick-egg lysozyme (pI 11, M_r 14.3 kDa), bovine serum albumin (BSA) (pI 4.9, M_r 67.0 kDa [10]), bovine cytochrome-c (pI 9.3, M_r 12.2 kDa [10]) were supplied by Sigma-Aldrich (St. Louis, Missouri).

2.2. Production of MCF-EVOH disc

An MCF was in-house extruded from ethylene-vinyl alcohol copolymer (EVOH) containing 32 mol% of ethylene following a patented extrusion method described in Mackley and Hallmark [11] using the set of temperature recommended by the manufacturer. After that, a 5 m piece of MCF-EVOH was wrapped into a disc to give a compact module (Fig. 1) and the two ends connected to an Upchurch 1/4 inch HPLC connector using slow-setting epoxy.

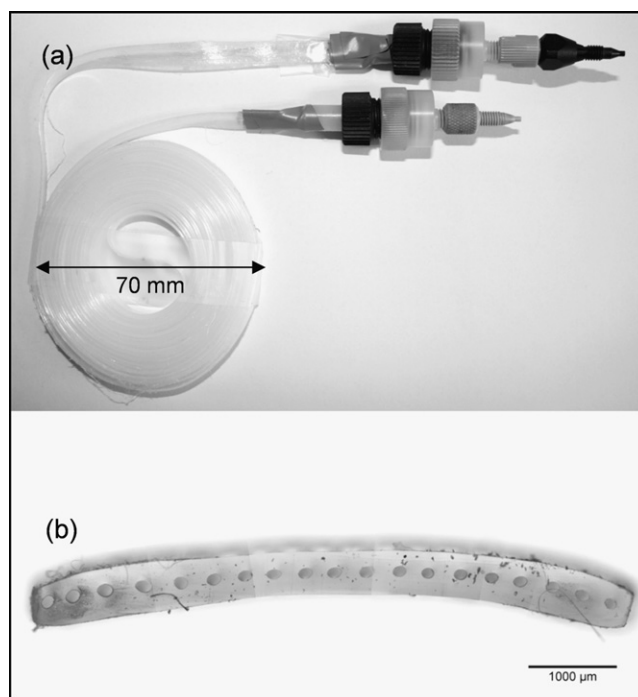


Fig. 1. Photograph and cross section of a plastic microcapillary film (MCF) disc extruded from ethylene vinyl alcohol copolymer (EVOH), containing 32 mol% of ethylene. The mean internal diameter of microcapillaries in the cross section of the film in (b) is $142 \pm 9.8\ \mu\text{m}$.

2.3. Surface modification of MCF-EVOH disc with SP groups

The internal surface of the microcapillaries in the MCF-EVOH disc was chemically treated to produce a cation exchange chromatography adsorbent using the method of McCreath et al. [12]. Briefly, the 5 m length of MCF-EVOH was first activated by flowing 30 ml ice cold NaOH (1 M) through the microcapillaries of the MCF in an ice bath for 30 min using an HPLC pump. This increased nucleophilicity by introducing alkoxide groups on the vinyl alcohol on the plastic surface. Then 20 ml ice cold cyanuric chloride (50 mM) in acetone was passed through the MCF in an ice bath for 20 min to activate the plastic with a linker. The MCF was then washed with 10 ml ice cold MilliQ water in an ice bath for 10 min.

For the covalent attachment of sulfonic acid (SP) groups to the activated surface of MCF-EVOH, 20 ml Na_2HPO_4 (1 M) containing 1 g 3-amino-1-propanesulphonic acid (pH 9.1) in a 40°C water bath was left recirculating through the MCF overnight using an HPLC pump. Following this step the water bath temperature was increased to 60°C for 5 h, after which 20 ml MilliQ water was flowed through the SP modified MCF for 20 min. Finally, the MCF-EVOH-SP was washed with 20 ml of NaOH (0.4 M) for 20 min followed by 20 ml MilliQ water for another 20 min. The MCF-EVOH-SP was stored at 4°C in 20 mM Tris-HCl, pH 7.2.

2.4. Dynamic binding capacity analysis of a MCF-EVOH-SP disc

The Upchurch 1/4 inch HPLC connector glued to each end of the MCF-EVOH-SP disc facilitated its connection to a sample loop valve in a standard Akta liquid chromatography instrument as illustrated in Fig. 2. Using this configuration it was possible to control the injection of sample as well as buffer gradients of different salt concentrations through the disc in the same way as standard packed bed columns. All chromatographic protein separations were performed with the plastic disc embedded in an ice bath.

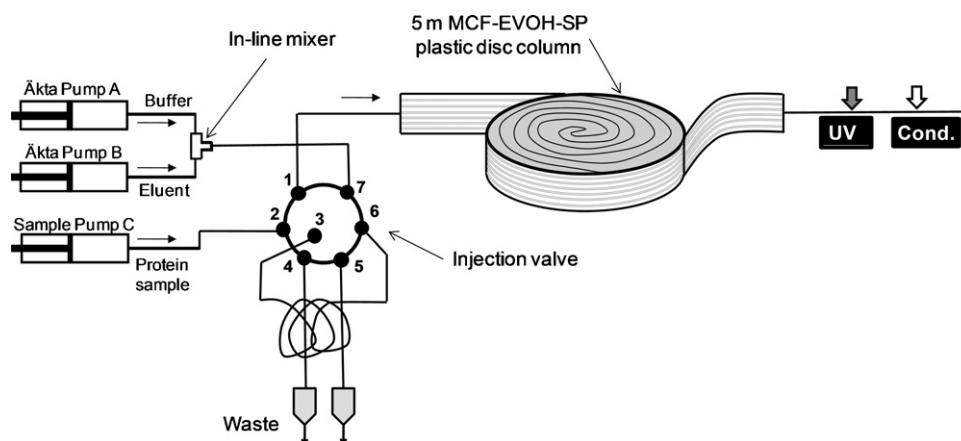


Fig. 2. Experimental setup used for frontal analysis experiments in MCF-EVOH-SP. A Knauer HPLC sample pump C was used for loading of protein samples.

The dynamic binding capacity was analysed to establish the effect of the superficial flow velocity on the chromatographic separation experiments using the newly fabricated 5 m long MCF-EVOH-SP disc with an internal disc volume of ~ 1.5 ml. The MCF-EVOH-SP disc was first equilibrated with the running buffer, 20 mM Tris-HCl, pH 7.2, loaded from the liquid chromatography instrument syringe pump A (Fig. 2) for at least 10 disc volumes. Protein was then loaded by injecting a solution of 0.2 mg ml^{-1} lysozyme in 20 mM Tris-HCl at pH 7.2 with a sample Smartline HPLC pump (Knauer, Berlin, Germany) at flow rates of 1.0, 2.0, 3.0, 4.0 and 5.0 ml min^{-1} corresponding to mean superficial flow velocities of 5.5, 11.1, 16.6, 22.2 and 27.7 cm s^{-1} or 40, 80, 120, 160 and 200 column volumes per hour. Protein loading at each flow rate was continued until a plateau was reached in UV absorbance as measured by the liquid chromatography instrument detector at the outlet of the disc. The disc was subsequently washed with the running buffer to remove the unbound protein. The bound protein was then eluted with a step gradient of 0.5 M of NaCl in 20 mM Tris-HCl pH 7.2 from liquid chromatography instrument pump B. The peak integration of protein absorbance measured at 280 nm allowed the calculation of the amount of lysozyme eluted at each flow rate which was assumed to be linearly proportional to the mass of protein initially bound. All experiments were done in duplicate.

2.5. MCF-EVOH-SP surface ligand binding density analysis

The ligand binding density on the internal surfaces of the newly fabricated MCF-EVOH-SP disc was measured using the Langmuir isotherm analysis [13]. The Langmuir equation states:

$$\theta = \frac{\alpha C}{1 + \alpha C} \quad (1)$$

where θ is the fractional coverage of the surface, C is the concentration and α is the diffusion constant. At equilibrium the saturation loading of bound protein is q_{max} , which is related to the amount of bound protein, q , at different starting protein loading concentrations by:

$$q = q_{\text{max}}^{\theta} \quad (2)$$

Combining Eqs. (1) and (2):

$$q = q_{\text{max}} \frac{\alpha C}{1 + \alpha C} \quad (3)$$

Eq. (3) allows q_{max} to be calculated by analysing the mass of protein bound, q , for different protein loading concentrations, C . This maximum, equilibrium saturation loading q_{max} represents the binding capacity of the MCF-EVOH-SP fabricated. Before this analysis the MCF-EVOH-SP disc was first equilibrated with the 20 mM Tris-HCl

running buffer using liquid chromatography instrument pump A (Fig. 2) for at least 6 disc volumes. Then different concentrations of lysozyme were inject in separate experiments into the MCF disc using an HPLC pump (Model 422, Kontron Instruments, UK) for at least 15 min (i.e. 10 disc volumes). This allowed protein concentration to homogenise along the length of the disc and provided the necessary time scale for protein diffusion to occur from the bulk solution to the capillary walls attaining equilibrium between bound and unbound protein molecules. The lysozyme concentrations tested were 0.1, 1.0, 5.0, 10.0, 20.0, 50.0, 80.0, $150.0 \mu\text{g ml}^{-1}$ in 20 mM Tris-HCl at pH 7.2 at a flow rate of 2.0 ml min^{-1} corresponding to a superficial mean flow velocity of 11.1 cm s^{-1} . The disc was subsequently washed with the running buffer for at least 5 column volumes to remove the unbound protein. The bound protein was then eluted at the same flow rate with a step gradient of 0.5 M of NaCl in 20 mM Tris-HCl pH 7.2 from liquid chromatography instrument pump B. The peak integration of protein absorbance measured at 280 nm allowed the calculation of the amount of lysozyme eluted at following each different lysozyme loading concentration. All experiments were done in duplicate.

2.6. Proof-of-concept of bioseparations in the MCF-EVOH-SP disc

Potential application of the MCF-EVOH-SP as a new adsorbent for rapid capture of impurities (i.e. subtractive chromatography) was demonstrated by removal of lysozyme as a model impurity from a sample containing BSA. The MCF-EVOH-SP was first equilibrated with running buffer (20 mM Tris-HCl at pH 7.2) then a mixture of 0.5 mg ml^{-1} BSA and 0.5 mg ml^{-1} lysozyme in 20 mM Tris-HCl at pH 7.2 was injected into the MCF disc using a $100 \mu\text{l}$ sample loop at 2.0 ml min^{-1} . A single step gradient of 0.5 M NaCl in 20 mM Tris-HCl at pH 7.2 was used to elute bound protein in this experiment. The purity of the peaks recorded by UV absorbance was tested by analysing fractions collected in SDS-PAGE analysis. The experiments were then repeated with BSA and lysozyme separately.

Chromatographic separation of a solution of cationic proteins was demonstrated by injecting a mixture of 0.1 mg ml^{-1} lysozyme and 0.1 mg ml^{-1} cytochrome-c in 20 mM MES at pH 6.0 using a $100 \mu\text{l}$ sample loop at 2.0 ml min^{-1} . A linear gradient of between 0 and 1.0 M NaCl in 20 mM MES at pH 6.0 over 4 min was used to differentially elute bound protein. The purity of the peaks recorded by UV absorbance was tested by analysing fractions collected from the chromatographic separation in SDS-PAGE analysis. The experiments were then repeated for lysozyme and cytochrome-c separately.

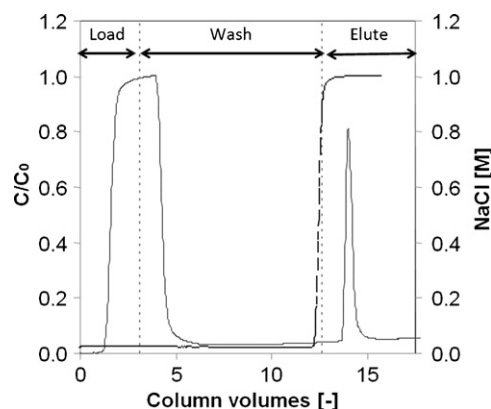


Fig. 3. Frontal analysis experiment of hen egg lysozyme in MCF-EVOH-SP at a superficial flow velocity of 11.1 cm s^{-1} . After proper initial equilibration, lysozyme was loaded to the column at 0.2 mg ml^{-1} in Tris-HCl buffer pH 7.2 with a HPLC sample pump and then washed with the running buffer and finally eluted with 1.0 M NaCl in a step gradient.

3. Results and discussion

Fig. 1 shows the MCF successfully produced from the EVOH copolymer. This thermoplastic material presented unique features that made it an ideal matrix for chromatographic applications, such as a high density of functional groups (up to 68 mol% of vinyl alcohol groups), a hydrophilic surface, good optical transparency, low water absorption and insolubility in aqueous solutions. A 5 m length section of MCF-EVOH could be wound into a disc module with 70 mm in diameter (Fig. 1a). Dark field optical microscopy measurements of a cross section of the resulting extruded MCF (Fig. 1b) indicates that the microcapillaries in the film have internal diameters of $142 \mu\text{m} \pm 9.8 \mu\text{m}$. The estimated total internal surface area available in the disc was 423.8 cm^2 .

A series of frontal analysis experiments using lysozyme allowed the dynamic binding capacity of the derivatised MCF-EVOH-SP to be determined for a range of high superficial flow velocities, i.e. $5.5\text{--}27.7 \text{ cm s}^{-1}$. A typical normalised breakthrough curve for lysozyme can be seen in Fig. 3 which shows the binding and elution of lysozyme in 20 mM Tris-HCl buffer at pH 7.2 in a “load” step then a “wash” step in 20 mM Tris-HCl at pH 7.2, followed by an elution step using 0.5 M NaCl in 20 mM Tris-HCl at pH 7.2 at a superficial flow velocity of 11.1 cm s^{-1} . The binding of lysozyme with pI 11 to the SP treated MCF surface seen in Fig. 3 is expected at this pH and demonstrates the cation exchange nature of this chemically modified thermoplastic substrate. The estimated mass of protein bound in this experiment was around $133 \mu\text{g}$ as determined from the integration of the elution peak in the “Elute” phase in Fig. 3, corresponding to a binding density of $\sim 313.8 \text{ ng cm}^{-2}$ for lysozyme. Results of the dynamic binding analysis with lysozyme for other superficial flow velocities can be found in Fig. 4a. The hashed area in the left hand side of the plot represents the typical superficial flow velocities used for packed bed chromatography columns, normally operated at very low superficial velocities such as 300 cm h^{-1} (i.e. $\sim 0.1 \text{ cm s}^{-1}$). The data shows no significant effect of the superficial flow velocity on the binding capacity of lysozyme in the derivatised disc, where a mean binding capacity of $150 \pm 30.0 \mu\text{g}$ was obtained. This quite remarkable feature of this new technology results from the non-porous nature and flow characteristics of small bore microcapillaries where mass transport of solutes occurs predominantly by convection. In microporous beads, the protein adsorption is controlled by diffusivity of proteins from the bulk solution to the core of the beads, therefore very low superficial flow velocities have to be used to provide the necessary time for the equilibrium to be attained and to minimise the axial dispersion of the solute through-

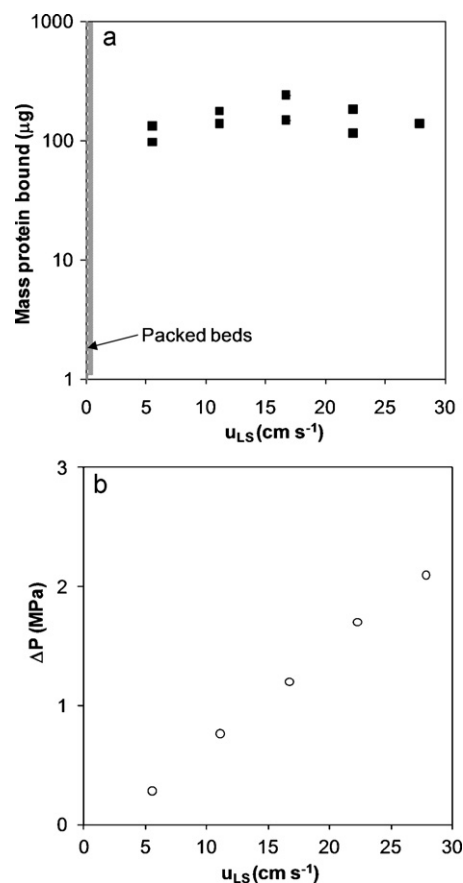


Fig. 4. Effect of superficial flow velocity, u_{LS} , on the (a) dynamic binding capacity of and (b) pressure drop, ΔP , across the MCF-EVOH-SP column.

out the column height. The process used to manufacture the MCF allows discs to be extruded to any length containing any number of embedded microcapillaries by adjusting the extruder die used.

Fig. 4b shows the pressure drop through the disc increased linearly with the superficial flow velocity. A mean internal hydraulic diameter of $142 \mu\text{m}$ and an even flow distribution through the 19 capillaries was assumed. The parallel array of microcapillaries provides an increased surface area whilst keeping modest pressure drops at high superficial flow velocities. A flow rate of 2.0 ml min^{-1} , corresponding to a superficial flow velocity of 11.1 cm s^{-1} was used for subsequent surface ligand binding density analysis and proof-of-concept separation experiments as both the binding capacity and pressure drop were favourable at that flow rate.

The density of ligands introduced to the inner surface of the capillaries in the MCF-EVOH-SP disc was found to be too low for analysis by FTIR or elemental analysis so the Langmuir isotherm analysis had to be used [13]. Egg lysozyme was used as model protein in this experimental set. Fig. 5 shows the mass of protein binding, q , achieved on each run at the different protein loading concentrations, C , tested. The line representing the Langmuir isotherm equation can be seen to fit the data very well and gives an equilibrium saturation binding density, q_{max} , from Eq. (3) of $180 \mu\text{g}$ and a disassociation constant K_d (concentration at $q_{\text{max}}/2$) is $5.2 \mu\text{g ml}^{-1}$. This equilibrium saturation binding density of lysozyme is equivalent to 424.7 ng cm^{-2} or $29.7 \text{ pmol cm}^{-2}$ in relation to the surface area of the disc, or $120 \mu\text{g ml}^{-1}$ in relation to the internal volume of the solution in the disc. The good fit of the Langmuir plot with the experimental data shows that lysozyme binds to the cationic support in a monolayer. With no porosity expected in the EVOH material, the total surface area estimated in a 5 m disc is equal to $\sim 423.8 \text{ cm}^2$. Assuming a spherical molecule

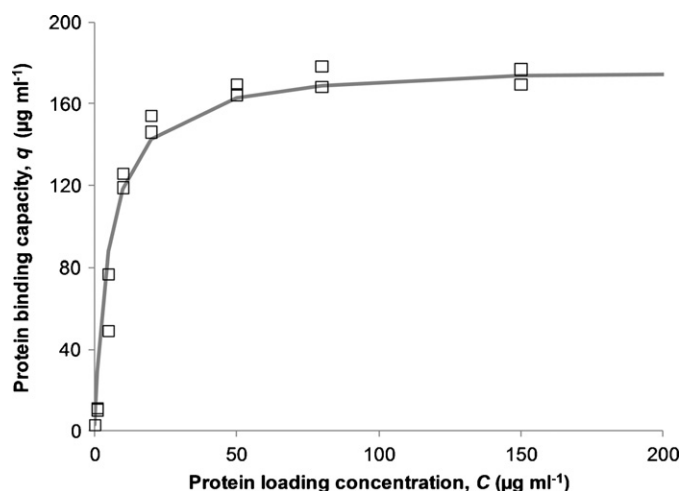


Fig. 5. Langmuir isotherm analysis of ligand binding density in the MCF-EVOH-SP disc using hen lysozyme. The different values of protein mass bound, q , achieved at the different protein loading concentrations, C , are plotted (squares). The line representing the Langmuir isotherm equation can be seen to fit the data very well and gives an equilibrium saturation binding density, q_{\max} , from Eq. (3) of $180 \mu\text{g}$.

diameter for lysozyme of 3 nm (measurement taken from crystal structure Protein Data Bank entry 3IJU), approximating hexagonal packing geometry the estimated binding density for lysozyme binding as a monolayer on the surface is 26 pmol cm^{-2} , which gives a total of $157 \mu\text{g}$ in the whole disk. This value is 13% lower than q_{\max} obtained, which can be explained by a level of rugosity observed in the inner surface of the microcapillaries in the MCF-EVOH-SP disc (results not shown).

The binding density value measured is several orders of magnitude lower than those typically obtained for packed bed column media such as $\sim 55 \text{ mg ml}^{-1}$ of SP Sepharose High performance beads (GE Healthcare). This apparent lower binding capacity of the MCF disc per unit volume is largely due to the protein binding the capillary walls as a monolayer in absence of porosity present in standard packed bed media. As a result this MCF chromatography approach is better favoured for high speed analytical applications rather than preparative mode applications.

Two sets of experiments were performed to demonstrate usability of MCF-EVOH-SP in the downstream processing of protein solutions: the first set aimed at “subtractive mode” operation; the second in “separation mode”.

In some downstream bioprocess steps it is desirable to remove trace impurities from a solubilised target protein product by adsorbing the impurity to an inert surface which is ideally low-cost and disposable. This is commonly referred to as “subtractive” chromatography. To demonstrate the utility of the novel MCF-EVOH-SP adsorbent produced in this work in subtractive chromatography

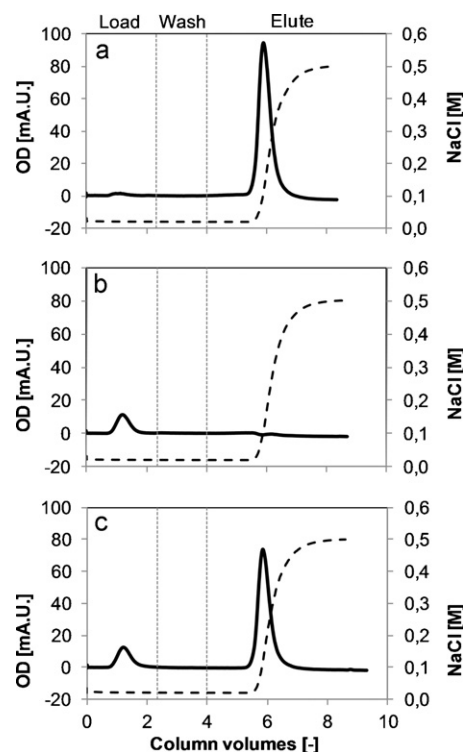


Fig. 6. Application of MCF-EVOH-SP as a subtractive adsorbent matrix. The adsorption of lysozyme (pI 11) and BSA (pI 4.9) was tested at a superficial flow velocity of 11.1 cm s^{-1} using 20 mM Tris-HCl (pH 6.0) as running buffer. Protein samples were injected into the plastic column using a $100 \mu\text{l}$ sample loop and washed with the running buffer before being eluted with a step gradient using 0.5 M NaCl . (a) Adsorption and elution of lysozyme at 0.5 mg ml^{-1} . (b) Breakthrough of 0.5 mg ml^{-1} of BSA through the column. (c) Adsorption and elution of lysozyme from a sample containing 0.5 mg ml^{-1} of lysozyme and 0.5 mg ml^{-1} of BSA.

a model impurity, lysozyme, was removed from a hypothetical bioproduct, BSA. Fig. 6A demonstrates the efficient capture and subsequent elution of a $100 \mu\text{l}$ pulse of lysozyme at a concentration of 0.5 mg ml^{-1} in the disc. About 97.4% w/w of protein was readily captured in the derivatised disc whilst flowing at high superficial flow velocity. Fig. 6B shows a $100 \mu\text{l}$ pulse of 0.5 mg ml^{-1} of anionic BSA passing straight through the derivatised disc without binding the activated surface. Fig. 6C demonstrates the removal of the model impurity lysozyme from a mixture of BSA allowing the BSA to pass directly through the MCF unbound. In this figure, the two proteins were loaded in a single $100 \mu\text{l}$ pulse each protein at the same 0.5 mg ml^{-1} concentration. The different peak heights and areas in the chromatograms are related to the higher extinction coefficient of lysozyme at the selected wavelength. The SDS-PAGE analysis of the 1.0 ml sample fractions collected in this experiment

Table 1

Benefit comparison analysis of plastic microcapillary disc chromatographic with current packed beds and membranes protein chromatography technologies.

Substrate feature	Chromatography substrate		
	Microporous beads	Membrane	Plastic microcapillary disc
1. Binding capacities	High	High	Medium/low
2. Superficial flow velocities	Low	High	High
3. Separation resolution	High	Medium	Medium
4. Pressure drops	High	Low	Low
5. Ease of scale up	Poor	Good (modular)	Good (modular)
6. Mass transfer rates	Low	High	High
7. Cost	High	High	Low
8. Possibility of mid-separation tracking	No	No	Yes
9. Possibility of parallel analysis	No	No	Yes

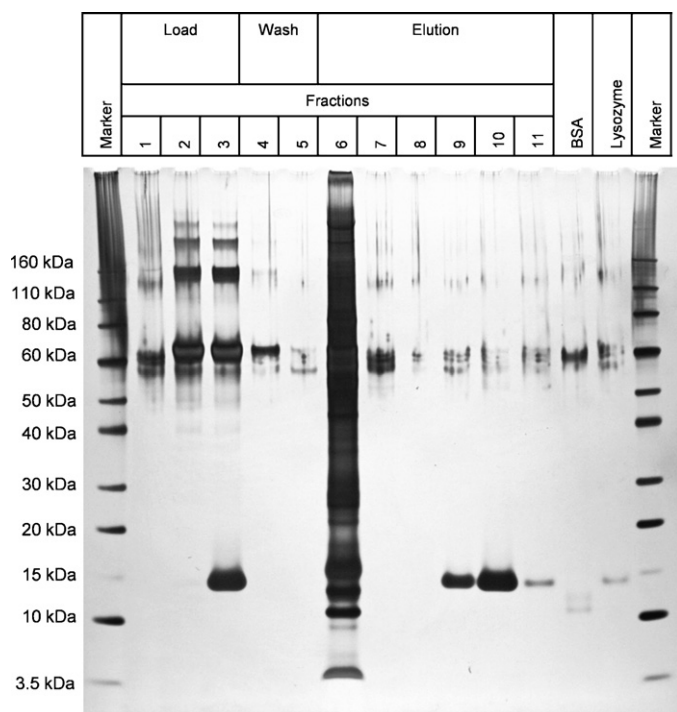


Fig. 7. SDS page of aliquots from subtractive ion-exchange separation of lysozyme from BSA as in Fig. 6(c). Samples were collected at the outlet of the disc in 1.0 ml fractions.

at the outlet of the disc is shown in Fig. 7, and confirms that BSA ($M_r = 67$ kDa) flows unhindered through the MCF-EVOH-SP during the protein loading step (fractions 1–3). Due to the sensitive silver staining protocol used for visualising the protein bands some lysozyme ($M_r = 14.4$ kDa) can be seen in the flow through fraction 3, corresponding to unbound lysozyme breaking through the disc. The chromatogram shown above in Fig. 6A has revealed that about 2.6% w/w of lysozyme does not bind, breaking through the disc by the end of the loading stage. Bound lysozyme was subsequently eluted from the MCF disc by introducing a step salt input into the disc, which corresponds to the eluted fractions 6–11 analysed. Some additional protein fragments were detected in fraction 6 possibly from some low level proteolytic activity producing lysozyme cleavage products. Two lanes in the SDS-PAGE show a BSA and lysozyme control sample run for comparison.

The second set of experiments aimed at testing the feasibility of separating two cationic proteins, lysozyme and cytochrome-c in the cation exchange disc fabricated. In Fig. 8A, 100 μ l of cytochrome-c at a concentration of 0.1 mg ml⁻¹ was injected into the chromatography disc. About 95.0% w/w of protein bound the disc and eluted upon a application of a linear NaCl gradient. Similarly in Fig. 8B, about 95.4% w/w of lysozyme injected in a 100 μ l pulse at a concentration of 0.1 mg ml⁻¹ was seen to bind the column and elute from the disc with the same NaCl gradient in the elution step. Fig. 8C demonstrates that following injection of 100 μ l of a mixture of these two proteins, about 95.8% w/w of the total protein has bound the disc. In the elution stage, two elution peaks could be detected during the NaCl gradient elution as expected. The SDS-PAGE analysis of the eluate collected at the outlet of the disc in 1.0 ml fractions (Fig. 9) confirmed that both cytochrome-c and lysozyme bound the MCF-EVOH-SP disc. At the start of NaCl gradient elution, cytochrome-c ($M_r = 12.2$ kDa) eluted from the disc first in fraction 11 (corresponding to 8–9 column volumes in the chromatogram) due to its low pI of 9.3 then lysozyme ($M_r = 14.4$ kDa) elutes subsequently with its higher pI of 11 in fractions 14–18. The SDS-PAGE analysis shows a good level of separation of the two pro-

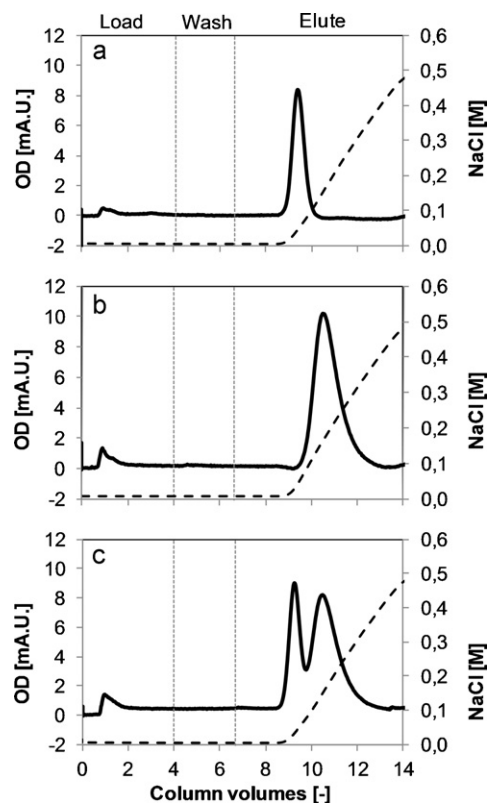


Fig. 8. Ion-exchange separation of cytochrome-c (pI 9.3) and hen egg lysozyme (pI 11) in the MCF-EVOH-SP in 20 mM MES buffer (pH 6.0) at a superficial flow velocity of 11.1 cm s⁻¹. Protein samples were injected into the plastic column using a 100 μ l sample loop and washed with the running buffer before being eluted with a linear gradient 0–0.5 M NaCl in 8 ml (i.e. about 5.3 column volumes). (a) Adsorption and elution of 0.1 mg ml⁻¹ cytochrome-c. (b) Adsorption and elution of 0.1 mg ml⁻¹ lysozyme. (c) Separation of cytochrome-c and lysozyme from a protein sample containing 0.1 mg ml⁻¹ of cytochrome-c and 0.1 mg ml⁻¹ of lysozyme.

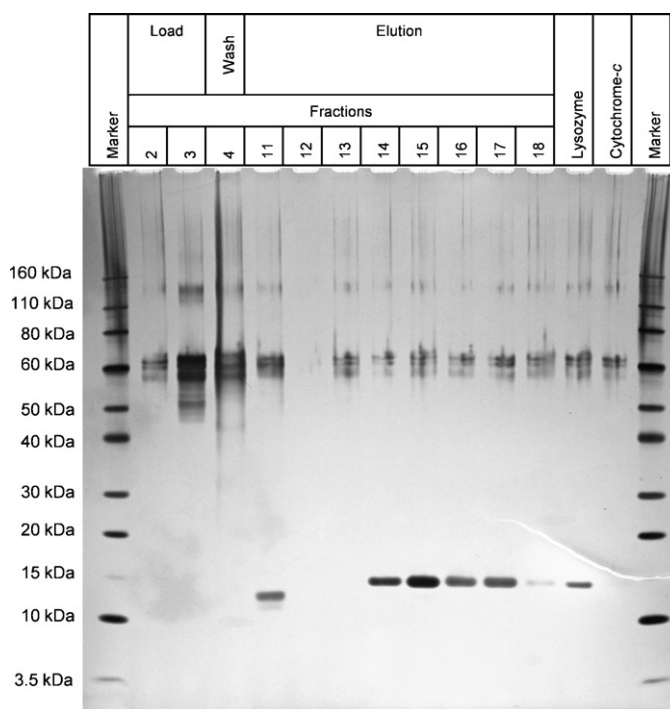


Fig. 9. SDS page of aliquots from ion-exchange separation of cytochrome-c and lysozyme in the MCF-EVOH-SP as seen in Fig. 8(c).

teins, with the two proteins eluting in completely distinct fractions. However, the chromatogram in Fig. 8C shows a level of overlapping of the two protein peaks, presumably as a result of axial dispersion in the UV cell detector (where the dead volume is not insignificant). This shows that small amounts of proteins with close isoelectric point can be quickly and effectively separated with this new plastic disc chromatography technology.

Table 1 summarises the current benefits of this new chromatographic technology in comparison with the two main chromatography substrates: microporous beads and chromatography membranes. Although the binding capacities presented by microporous beads and membranes are clearly higher than those herein reported for the EVOH-MCF-SP, this new plastic chromatography disc technology has the potential for offering a cheaper reasonable resolution method for rapid protein analysis. Additionally the MCF substrate allows mid-separation tracking and parallel analysis as separation in each individual capillary can be tracked separately with a simple UV area imaging system as a result of the flat external surfaces of the plastic film.

4. Conclusions

We have demonstrated a novel extruded plastic chromatography substrate can be used in the fast cation exchange separation of proteins. A 5 m plastic film melt-extruded from EVOH containing an array of 19 microcapillaries embedded in it was chemically derivatised with SP groups to produce a cation exchange chromatography disc. Frontal analysis and equilibrium Langmuir isotherm analyses using model protein solutions allowed full characterisation of this new chromatographic material. Although the obtained binding

capacities were comparatively low for applications in preparative chromatography this technology shows great promise as a low pressure alternative for low cost, high throughput qualitative chromatographic analysis of proteins.

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