




This item was submitted to Loughborough's Institutional Repository (<https://dspace.lboro.ac.uk/>) by the author and is made available under the following Creative Commons Licence conditions.


 **creative commons**  
C O M M O N S D E E D


**Attribution-NonCommercial-NoDerivs 2.5**


**You are free:**

- to copy, distribute, display, and perform the work

**Under the following conditions:**

 **Attribution.** You must attribute the work in the manner specified by the author or licensor.


 **Noncommercial.** You may not use this work for commercial purposes.

 **No Derivative Works.** You may not alter, transform, or build upon this work.

- For any reuse or distribution, you must make clear to others the license terms of this work.
- Any of these conditions can be waived if you get permission from the copyright holder.

**Your fair use and other rights are in no way affected by the above.**

This is a human-readable summary of the [Legal Code \(the full license\)](#).

[Disclaimer](#) 

For the full text of this licence, please go to:  
<http://creativecommons.org/licenses/by-nc-nd/2.5/>

# Fast Cation-Exchange Separation of Proteins in a Plastic Microcapillary Disc

Darton N.J.<sup>\*</sup>, Reis, N.M.<sup>\*</sup>, Mackley M.R., Slater, N.K.H.

<sup>\*</sup>Authors contributed equally to this work, Department of Chemical Engineering and Biotechnology, University of Cambridge, Pembroke Street, CB2 3RA

## 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 microns, 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 Akta chromatography instrument for a range of superficial flow velocities,  $u_{LS}=5.5-27.7 \text{ cm s}^{-1}$ . The mean dynamic binding capacity for hen-egg lysozyme was found to be approximately 100  $\mu\text{g}$  for a 5 m length film, giving a ligand binding density of 413  $\text{ng cm}^{-2}$ . The dynamic binding capacity did not vary significantly over the range of  $u_{LS}$  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.

## 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 volume of adsorbent, at  $127 \text{ m}^2 \text{ ml}^{-1}$  [6] is ~4500 times that of an MCF with  $142 \text{ }\mu\text{m}$  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  $<300 \text{ cm hr}^{-1}$ , 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  $240 \times \text{EBC}$ . For MCF's, with no intraparticle diffusion resistance and high film mass transfer rates, the overall mass transfer resistance is only of order  $0.023 \times \text{EBC}$ . 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  $160 \text{ m h}^{-1}$  this MCF displays a similar number of

theoretical plates to a 20 cm Sepharose packed column, operated at  $100 \text{ cm h}^{-1}$ . 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  $\sim 50$  pence/m are estimated, such that the cost for a 5 m cation-exchange MCF would be  $\sim \text{£}2.50$ . 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. Material 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,  $\text{Na}_2\text{HPO}_4$ , MES, TRIS, HCl 3-amino-1-propanesulphonic, chick-egg lysozyme (pI 11,  $M_r$  14.3K), Bovine Serum Albumin (BSA)(pI 4.9,  $M_r$  67.0K [10]), bovine cytochrome-c (pI 9.3,  $M_r$  12.2K [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 (2005) [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 (Figure 1) and the two ends connected to an Upchurch 1/4 inch HPLC connector using slow-setting epoxy.

### *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 (1M) 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<sub>2</sub>HPO<sub>4</sub> (1M) containing 1g 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 hrs, 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 (0.4M) 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 ¼ 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 chromatography machine as illustrated in Figure 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.

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, loaded from the Akta syringe pump

A (Figure 2) for at least 10 disc volumes. Protein was then loaded by injecting a solution of 0.2 mg ml<sup>-1</sup> lysozyme in 20 mM Tris-HCl at pH 7.2 with a sample Smartline HPLC pump (Knaeur, Berlin, Germany) at flow rates of 1.0, 2.0, 3.0, 4.0 and 5.0 ml min<sup>-1</sup> corresponding to mean superficial flow velocities of 5.5, 11.1, 16.6, 22.2 and 27.7 cm s<sup>-1</sup>, respectively. Protein loading at each flow rate was continued until a plateau was reached in UV absorbance as measured by the Akta 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 Akta 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  $\theta$  is the fractional coverage of the surface,  $C$  is the concentration and  $\alpha$  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:

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

Combining equations (1) and (2):

$$q = q_{max} \frac{\alpha C}{1 + \alpha C}$$

(3)

Equation (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 Akta pump A (Figure 2) for at least 6 disc volumes. Then different concentrations of lysozyme were injected 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  $\text{ml min}^{-1}$  corresponding to a superficial mean flow velocity of 11.1  $\text{cm s}^{-1}$ . 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 Akta 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  $\text{mg ml}^{-1}$  BSA and 0.5  $\text{mg ml}^{-1}$  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  $2.0 \text{ ml min}^{-1}$ . A single step gradient of  $0.5 \text{ M NaCl}$  in  $20 \text{ mM Tris-HCl}$  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  $0.1 \text{ mg ml}^{-1}$  lysozyme and  $0.1 \text{ mg ml}^{-1}$  cytochrome-c in  $20 \text{ mM MES}$  at pH 6.0 using a  $100 \text{ }\mu\text{l}$  sample loop at  $2.0 \text{ ml min}^{-1}$ . A linear gradient of between 0 and  $1.0 \text{ M NaCl}$  in  $20 \text{ mM MES}$  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.

### **3. Results and discussion**

Figure 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 (Figure 1a). Dark field optical microscopy measurements of a cross section of the resulting extruded MCF (Figure 1b) indicates that the microcapillaries in the film have internal diameters of  $142 \text{ }\mu\text{m} \pm 9.8 \text{ }\mu\text{m}$ . The estimated total internal surface area available in the disc was  $423.8 \text{ cm}^2$ .

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 Figure 3 which shows the binding and elution of lysozyme in  $20 \text{ mM Tris-HCl}$  buffer at pH 7.2 in a “load” step then a “wash” step in  $20 \text{ mM Tris-HCl}$  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  $11.1 \text{ cm s}^{-1}$ . The binding of lysozyme with pI 11 to the SP treated MCF surface seen in Figure 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  $133 \text{ }\mu\text{g}$  as determined from the integration of the elution peak in the "Elute" phase in Figure 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 Figure 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  $300 \text{ cm h}^{-1}$  (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  $150 \pm 30.0 \text{ }\mu\text{g}$  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 throughout the column height.

Figure 4b shows the pressure drop through the disc increased linearly with the superficial flow velocity and follows Hagen-Poiseuille's equation for pressure drop in laminar flow in tubes. A mean internal hydraulic diameter of  $142 \text{ }\mu\text{m}$  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  $2.0 \text{ ml min}^{-1}$ , corresponding to a superficial flow velocity of  $11.1 \text{ cm s}^{-1}$  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. Figure 6 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 equation (3) of  $180 \mu\text{g}$  and a disassociation constant  $K_d$  (concentration at  $q_{max}/2$ ) is  $5.2 \mu\text{g ml}^{-1}$ . This equilibrium saturation binding density of lysozyme is equivalent to  $424.7 \text{ ng cm}^{-2}$  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 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  $26 \text{ pmol cm}^{-2}$ , 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 roughness 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 to subtractive chromatography a model impurity, lysozyme, was removed from a hypothetical bioproduct, BSA. Figure 6A demonstrates the efficient capture and subsequent elution of a 100  $\mu\text{l}$  pulse of lysozyme at a concentration of  $0.5 \text{ mg ml}^{-1}$  in the disc. About 97.4% w/w of protein was readily captured in the derivatised disc whilst flowing at high superficial flow velocity. Figure 6B shows a 100  $\mu\text{l}$  pulse of  $0.5 \text{ mg ml}^{-1}$  of anionic BSA passing straight through the derivatised disc without binding the activated surface. Figure 6C demonstrates the removal of the model impurity lysozyme from a mixture of BSA allowing the lysozyme 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  $0.5 \text{ mg ml}^{-1}$  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 at the outlet of the disc is shown in Figure 7, and confirms that BSA ( $M_r = 67\text{kDa}$ ) 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 ( $M_r = 14.4 \text{ kDa}$ ) can be seen in the flow through fraction 3, corresponding to unbound lysozyme breaking through the disc. The chromatogram shown above in Figure 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 Figure 8A, 100  $\mu\text{l}$  of cytochrome-*c* at a concentration of  $0.1 \text{ mg ml}^{-1}$  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 Figure 8B, about 95.4% w/w of lysozyme injected in a 100  $\mu\text{l}$  pulse at a concentration of  $0.1 \text{ mg ml}^{-1}$  was seen to bind the column and elute from the disc with the same NaCl gradient in the elution step. Figure 8C demonstrates that following injection of 100  $\mu\text{l}$  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 (Figure 9) confirmed that both cytochrome-*c* and lysozyme bound the MCF-EVOH-SP disc. At the start of NaCl gradient elution, cytochrome-*c* ( $M_r = 12.2 \text{ kDa}$ ) 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 ( $M_r = 14.4 \text{ kDa}$ ) elutes subsequently with its higher  $pI$  of 11 in fractions 14 to 18. The SDS-PAGE analysis shows a good level of separation of the two proteins, with the two proteins eluting in completely distinct fractions. However, the chromatogram in Figure 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.

#### **Acknowledgements**

The authors would like to acknowledge the financial support of BBSRC-BRIC scheme and European Commission (FP7 Marie Curie fellowship scheme), and assistance by Dr. Bart Hallmark, Dr. Christian Hornung and Murphy Oduwole. Professor Howard Chase is also acknowledged for the words of motivation to carry out this work and precious comments made during the preparation of the manuscript.

## References

- [1] R. Ghosh, *Journal of Chromatography A* 952 (2002) 13.
- [2] D.Q. Yu, M.D. McLean, J.C. Hall, R. Ghosh, *Journal of Chromatography A* 1187 (2008) 128.
- [3] B.V. Bhut, K.A. Christensen, S.M. Husson, *Journal of Chromatography A* 1217 (2010) 4946.
- [4] B. Hallmark, F. Gadala-Maria, M.R. Mackley, *Journal of Non-newtonian Fluid Mechanics* 128 (2005) 83.
- [5] B. Hallmark, M.R. Mackley, F. Gadala-Maria, *Advanced Engineering Materials* 7 (2005) 545.
- [6] M. Barrande, I. Beurroies, R. Denoyel, I. Tatarova, M. Gramblicka, M. Polakovic, M. Joehnck, M. Schulte, *Journal of Chromatography A* 1216 (2009) 6906.
- [7] C.H. Hornung, M.R. Mackley, I.R. Baxendale, S.V. Ley, *Organic Process Research & Development* 11 (2007) 399.
- [8] W.C. Lee, *Journal of Chromatography B* 699 (1997) 29.
- [9] C.H. Hornung, M.R. Mackley, *Chemical Engineering Science* 64 (2009) 3889.
- [10] R.L. Moritz, R.J. Simpson, *Nat Methods* 2 (2005) 863.
- [11] M.R. Mackley, B. Hallmark, in U.o.C. (GB) (Editor), WO2005056272, GB, 2005.
- [12] G.E. McCreath, R.O. Owen, D.C. Nash, H.A. Chase, *J Chromatogr A* 773 (1997) 73.
- [13] I. Langmuir, *Journal of the American Chemical Society* 38 (1916) 2221.

## List of tables

**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. Scale up potential	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



## List of Figures

**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  $142 \pm 9.8 \mu\text{m}$ .

**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.

**Fig. 3.** Frontal analysis experiment of hen egg lysozyme in MCF-EVOH-SP at a superficial flow velocity of  $11.1 \text{ cm s}^{-1}$ . After proper initial equilibration, lysozyme was loaded to the column at  $0.2 \text{ mg ml}^{-1}$  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.

**Fig. 4.** Effect of superficial flow velocity,  $u_{LS}$ , on the (a) dynamic binding capacity of and (b) pressure drop,  $\Delta P$ , across the MCF-EVOH-SP column. The pressure drop in laminar flow through a tube is given by Hagen- Poiseuille equation,  $\Delta P = 32\mu.L.u_{LS}/(N_c d_m^2)$ , where  $\mu$  is the liquid viscosity,  $L$  the column length,  $N_c$  the number of microcapillaries and  $d_m$  the mean equivalent diameter of the microcapillaries.

**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 equation (3) of  $180 \mu\text{g}$ .

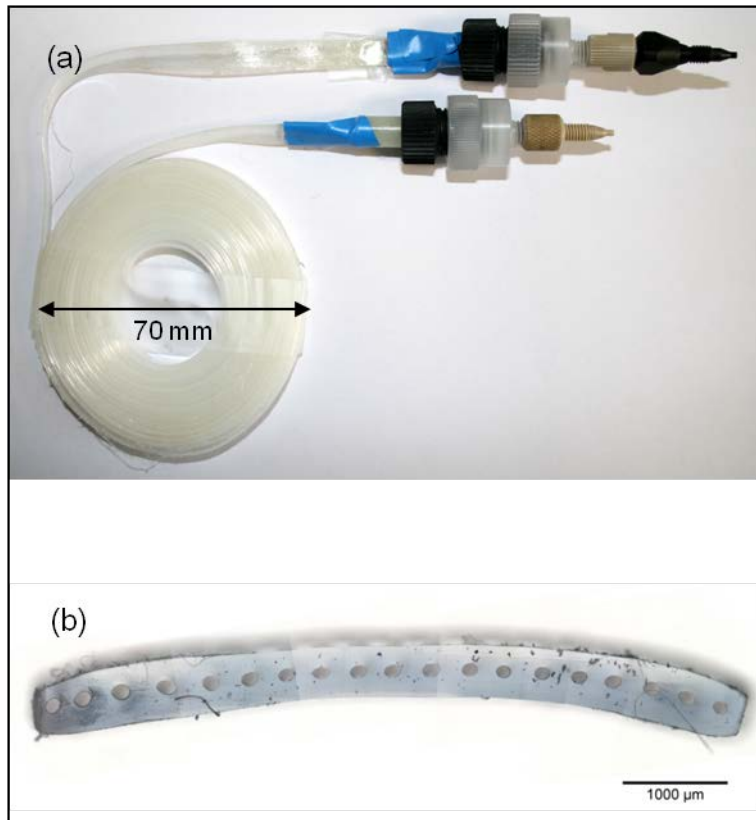
**Fig. 6.** Application of MCF-EVOH-SP as a subtractive adsorbent matrix. The adsorption of lysozyme ( $pI$  11) and BSA ( $pI$  4.9) was tested at a superficial flow velocity of  $11.1 \text{ cm s}^{-1}$  using 20 mM Tris-HCl (pH 6.0) as running buffer. Protein samples were injected into the plastic 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<sup>-1</sup>, (b) Breakthrough of 0.5 mg ml<sup>-1</sup> of BSA though the column. (c) Adsorption and elution of lysozyme from a sample containing 0.5 mg ml<sup>-1</sup> of lysozyme and 0.5 mg ml<sup>-1</sup> of BSA.

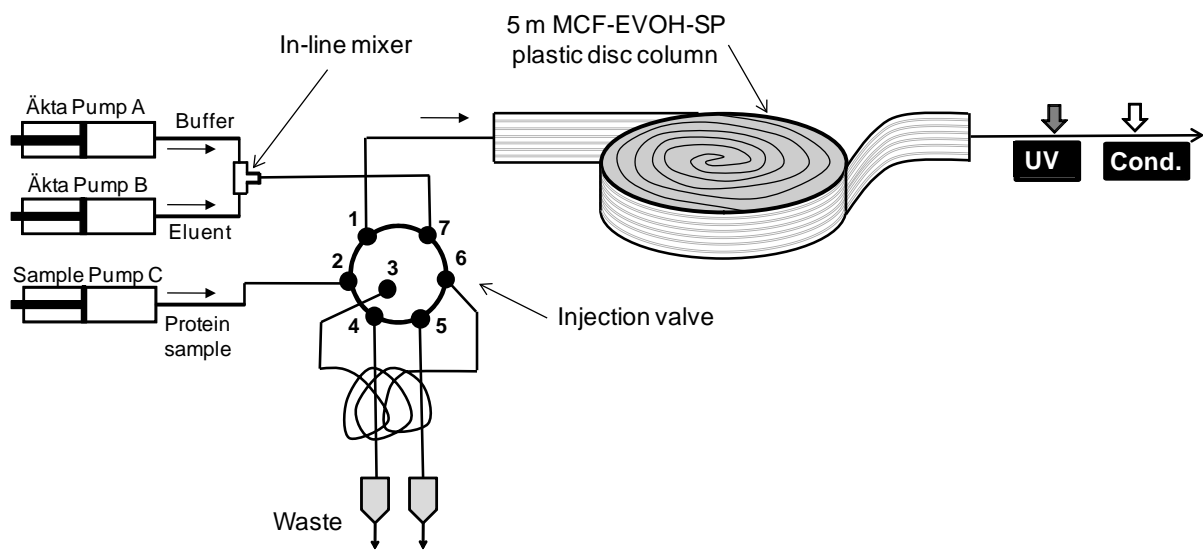
**Fig. 7.** SDS page of aliquots from subtractive ion-exchange separation of lysozyme from BSA as in Fig. 6(c). Samples were collected at the outlet of the disc in 1.0 ml fractions.

**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<sup>-1</sup>. Protein samples were injected into the plastic column using a 100 µl 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<sup>-1</sup> cytochrome-*c*. (b) Adsorption and elution of 0.1 mg ml<sup>-1</sup> lysozyme. (c) Separation of cytochrome-*c* and lysozyme from a protein sample containing 0.1 mg ml<sup>-1</sup> of cytochrome-*c* and 0.1 mg ml<sup>-1</sup> 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).



**Fig. 1.**



**Fig. 2.**

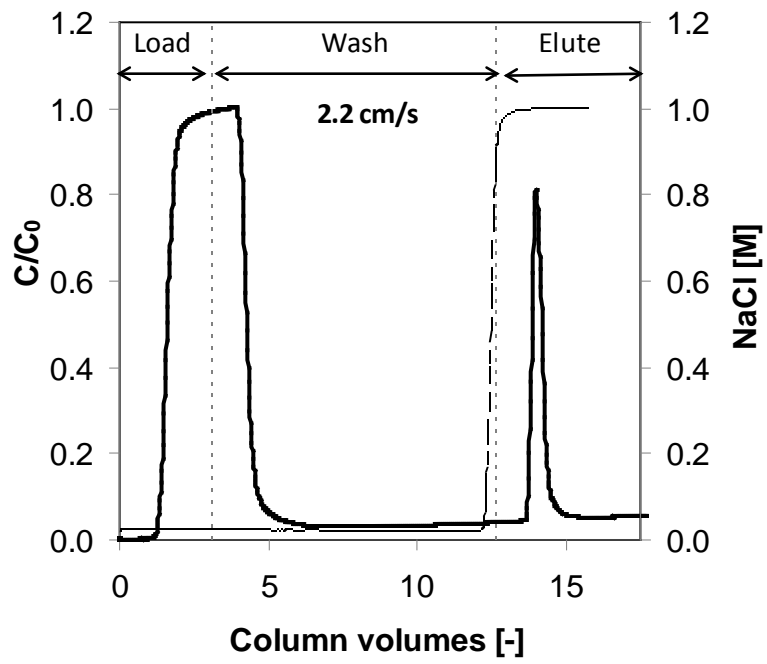


Fig. 3

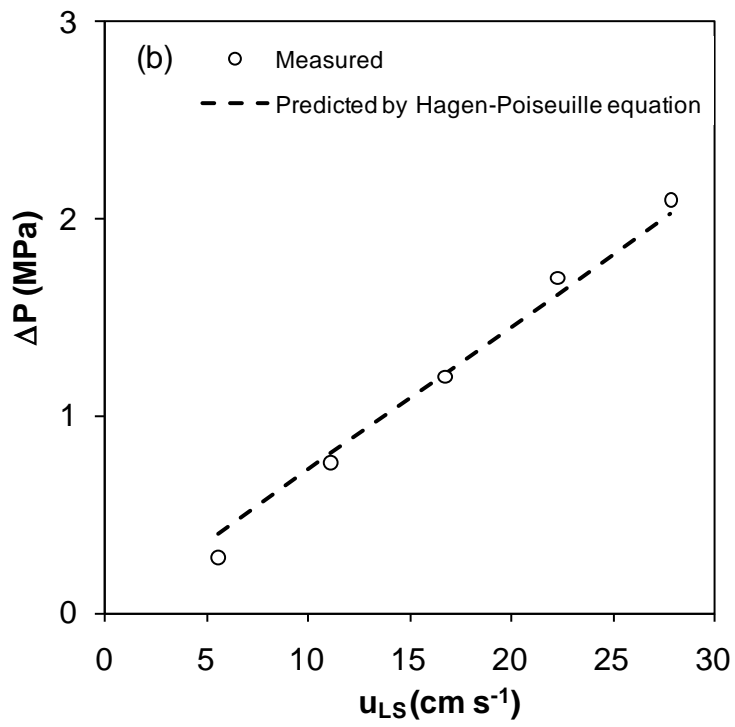
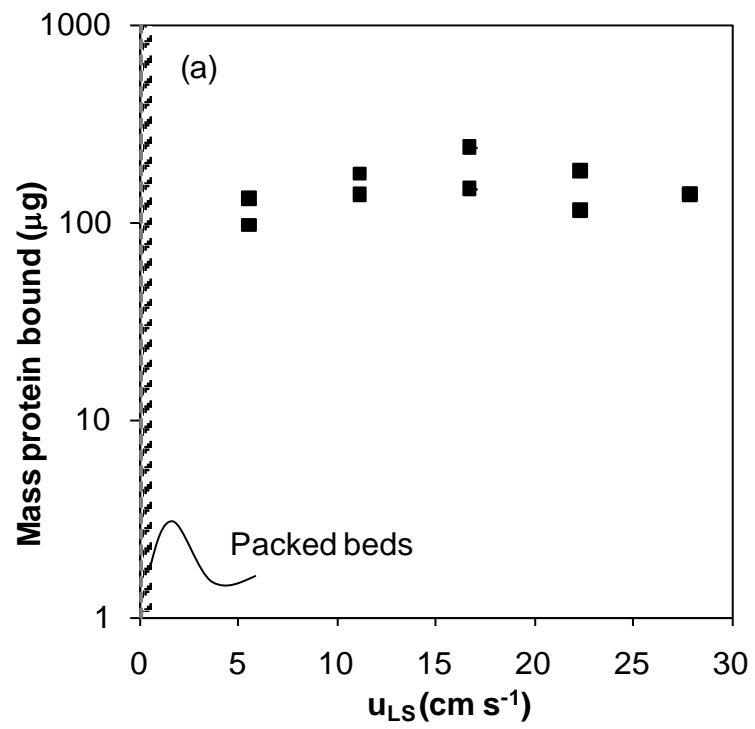
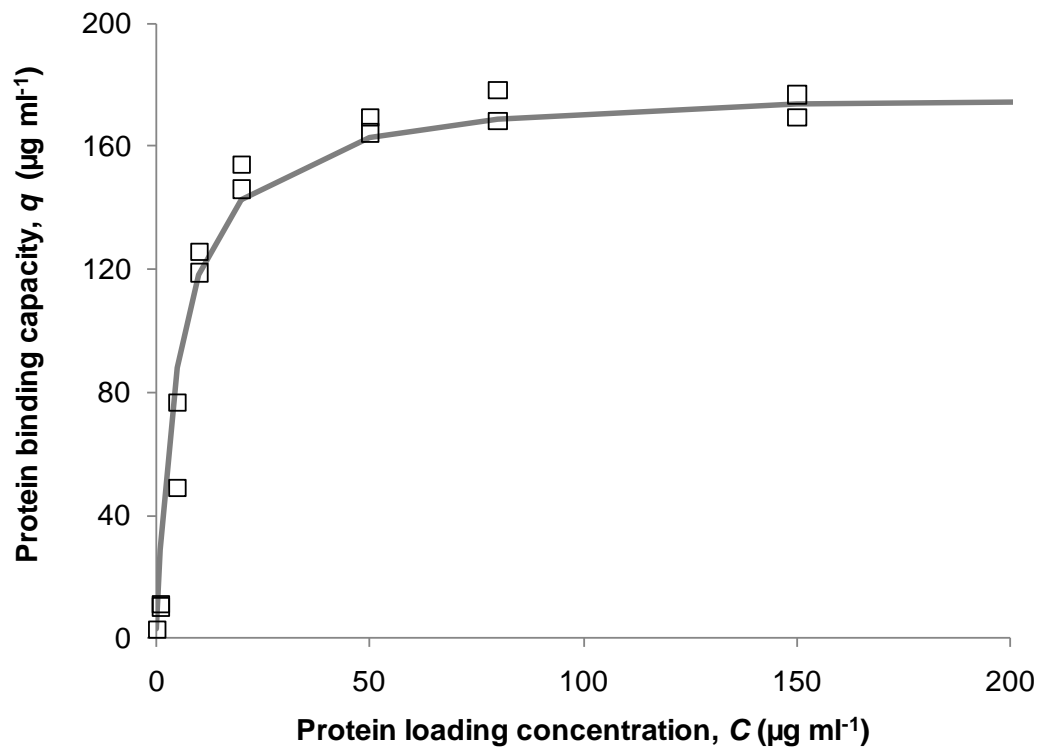


Fig. 4.



**Fig. 5.**

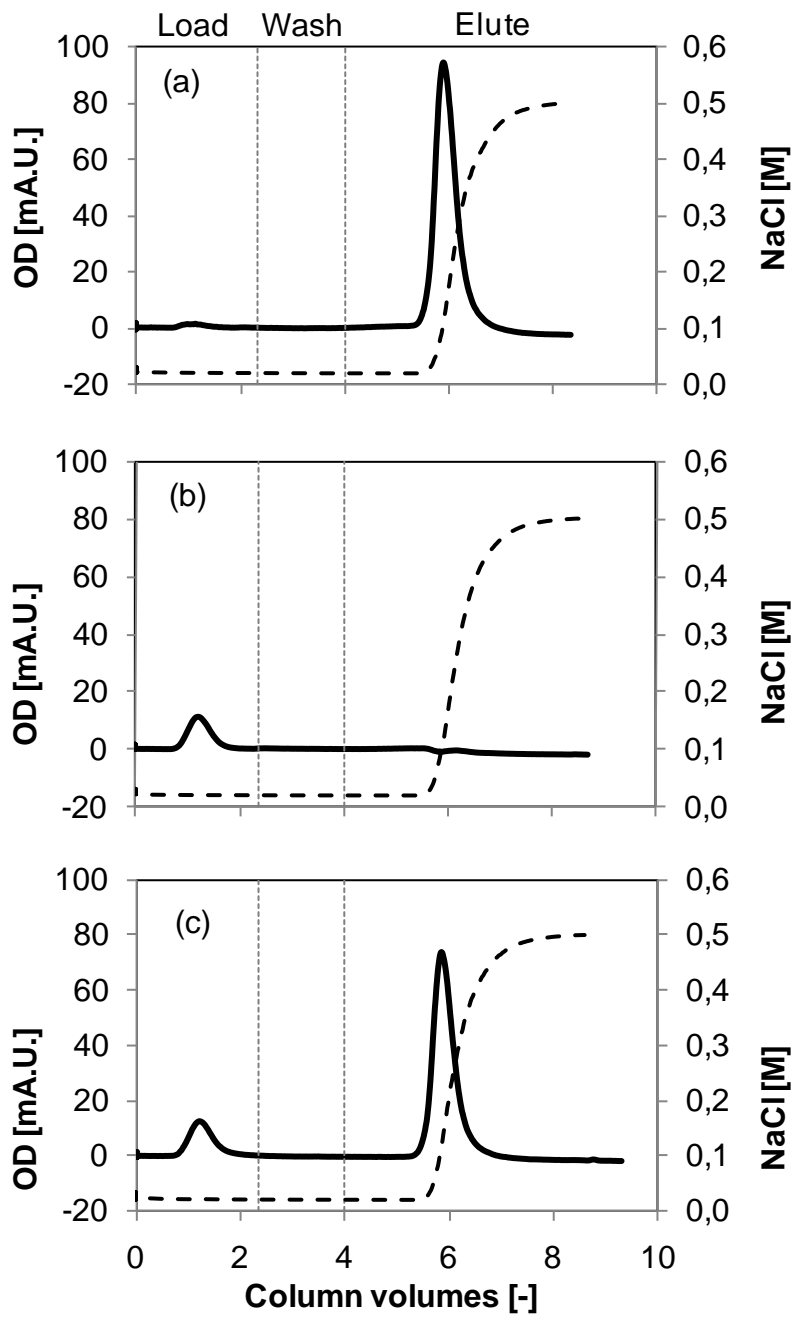
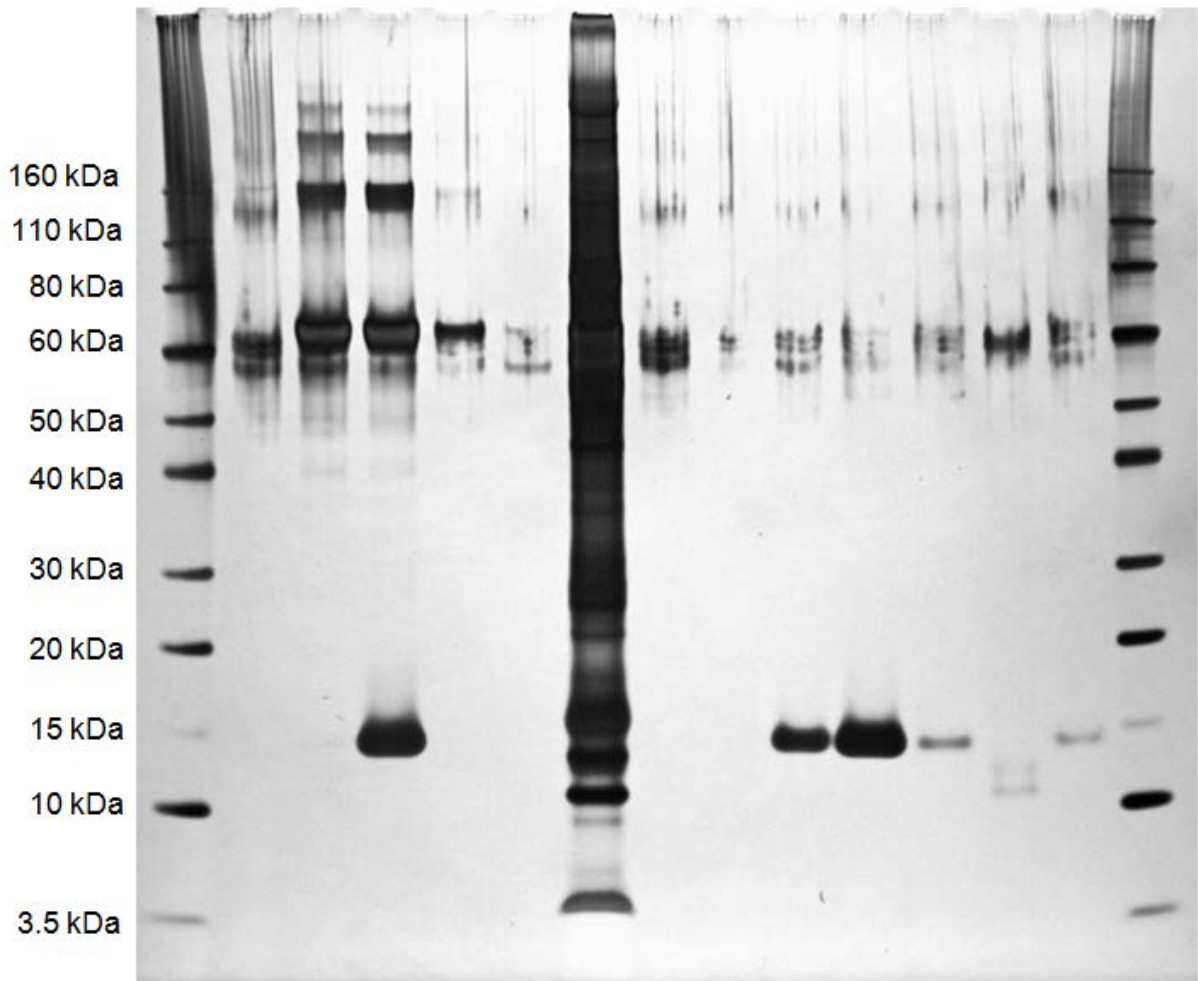


Fig. 6.



	Load			Wash		Elution								
Marker	Fractions											BSA	Lysozyme	Marker
	1	2	3	4	5	6	7	8	9	10	11			



**Fig. 7.**

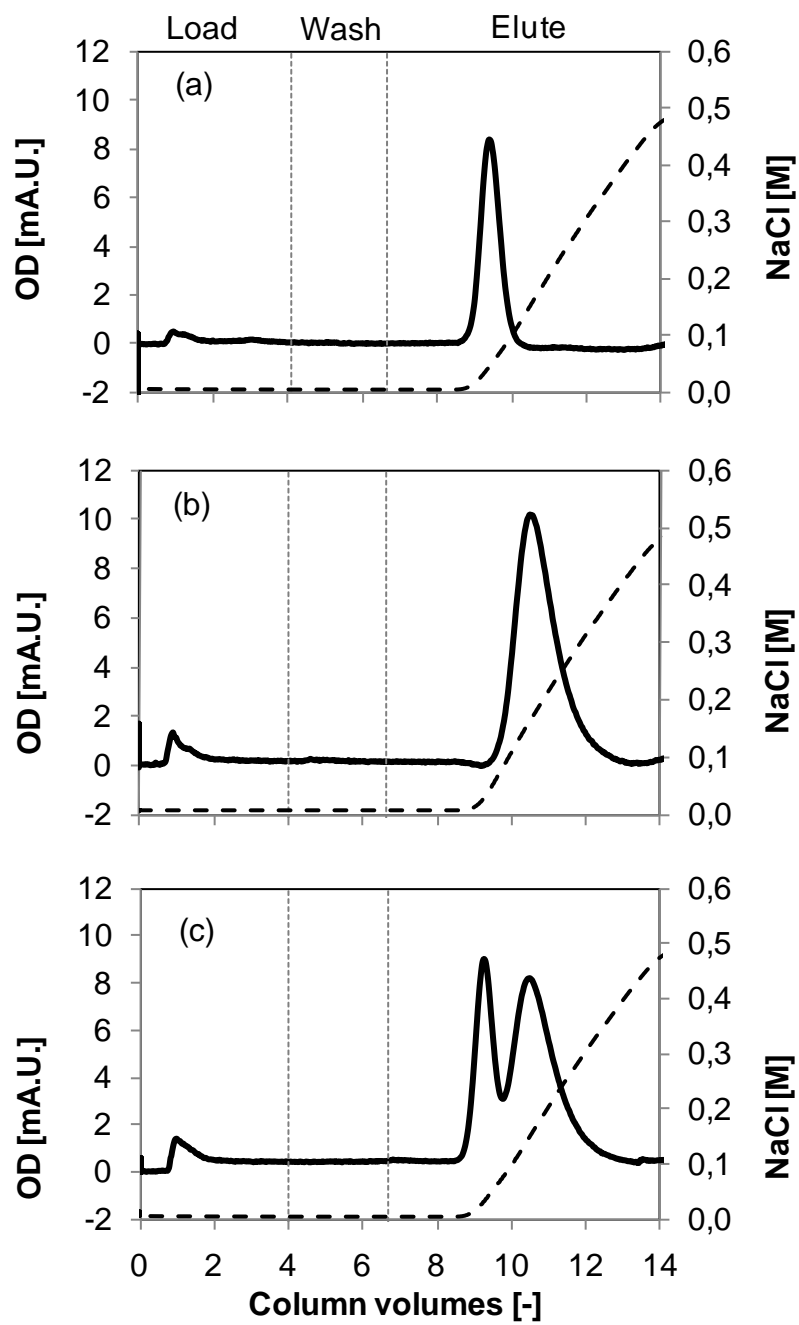


Fig. 8.

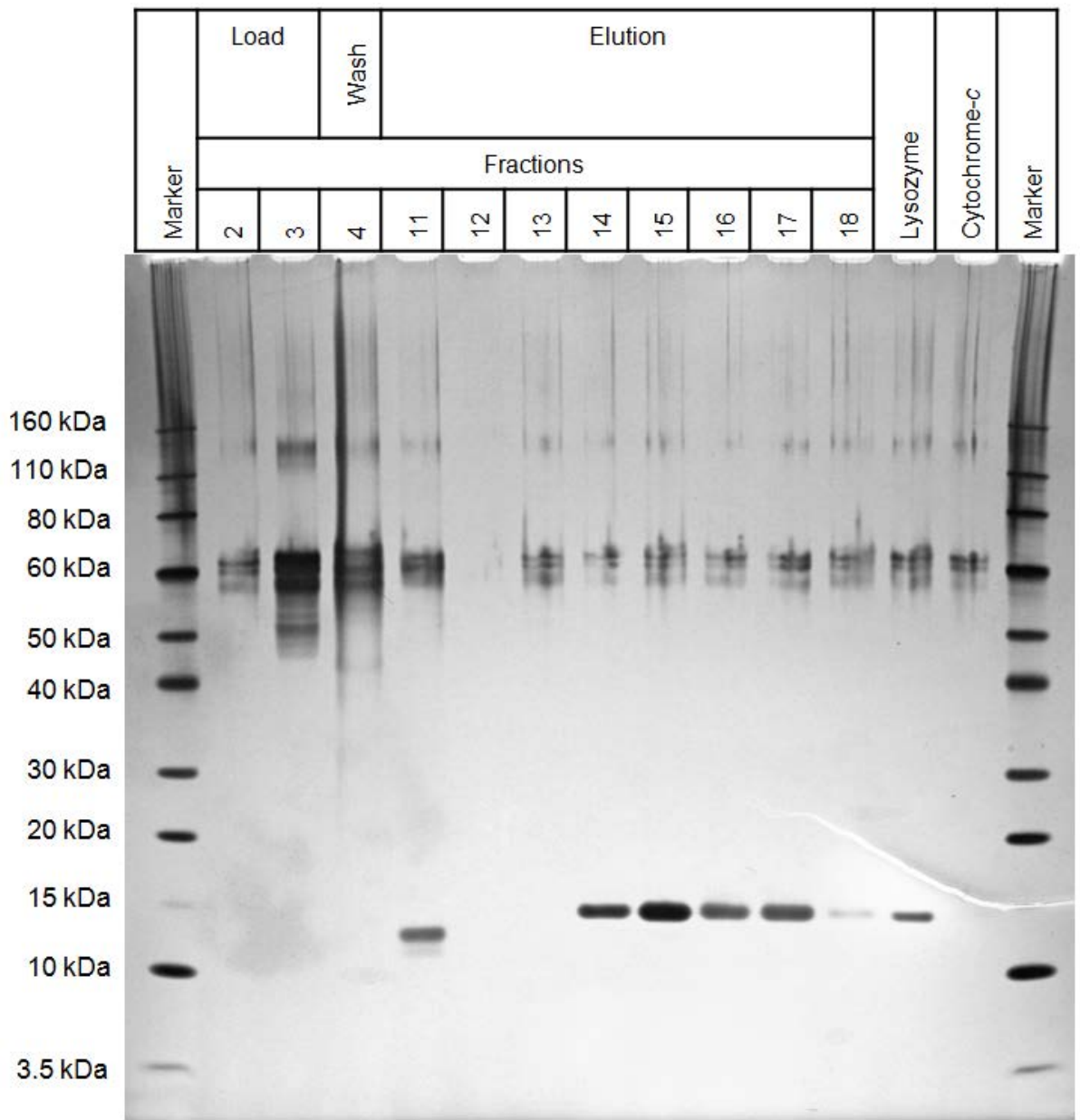


Fig. 9.