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Journal of Chromatography A, 1044 (2004) 253–258
JOURNAL OF CHROMATOGRAPHY A
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Towards a miniaturised system for dynamic field gradient focused separation of proteins

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Available online 2 June 2004

Abstract

Separation and focusing of proteins is described in a miniaturised dynamic field gradient focusing device with a 2.5 cm × 0.1 cm channel filled with a porous polymer monolith. The separation channel is in contact with a parallel electric field channel with five individually addressable electrodes through a porous glass membrane so that a variable field can be generated that drives charged proteins electroosmotically against a constant hydrodynamic flow. Separated pre-stained proteins were detected by means of a digital camera and background subtraction.

Keywords: Dynamic field gradient focusing; Instrumentation; Miniaturisation; Monolithic channels; Proteins

1. Introduction

The increased interested in the areas of genomics and proteomics has driven the need for better analytical methods for the separation of proteins from complex samples. Capillary chromatography and multidimensional chromatographic techniques are being developed to complement, and now in some cases exceed, the existing established methods of both one-dimensional (1D) and two-dimensional (2D) gel techniques [1,2] which separate the analytes on the basis of physico chemical properties of the isoelectric point (p*I*) and the relative molecular mass. However, a serious limitation of the 2D gel methods is the problem of coupling the technique with mass spectrometry. Capillary HPLC techniques that are capable of being interfaced to mass spectrometry by electrospray are now driving the development of new multidimensional separation methods. These include combinations of capillary electrophoresis, capillary electro-chromatography, isoelectric focusing (IEF), and chromatofocusing (CF) [3–5].

Another alternative has been based on the pioneering work of O'Farrell [6] who showed how proteins could be focused at the interface of two different gel media packed into an electrochromatography column. Later, Ivory and Koegler [7] showed how charged proteins could be focused using an electric field gradient, namely electric field gradient focusing (EFGF). This approach led directly to Huang and Ivory [8] describing the technique of dynamic field gradient focusing (DFGF).

In this study a new DFGF chip has been developed together with a computer controlled optical real-time feedback that allows charged proteins to be focused and their axial position changed in a separating column.

2. Dynamic field gradient focusing

Dynamic field gradient focusing is an electrophoretic technique in which charged molecules are focused in a column at a point where their electrophoretic mobility is balanced against an opposing hydrodynamic flow [8]. Steady state bands of the charged species are formed in the separation channel when equilibrium focusing is reached, i.e. the electrophoretic migration is countered by an opposing balanced hydrodynamic flow (Fig. 1). The electric field is formed dynamically from a computer-controlled array of individual power supplies, so allowing the electronically generated field to take on various profiles. A single syringe pump generates the constant, opposing hydrodynamic field. DFGF offers some unique advantages over other electrically enhanced separation methods. Because of the dual channel system, problems found in both CE and capillary electrochromatographic (CEC) separations such as bubble

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doi:10.1016/j.chroma.2004.04.069

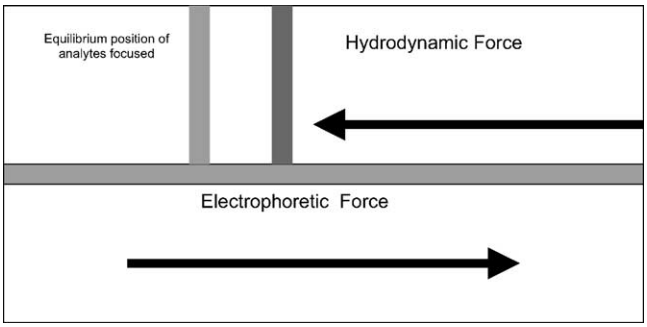

Fig. 1. Schematic of the dynamic field gradient focusing process.

formation and change of pH do not affect DFGF as these remain outside the separation channel. System and injection dead volume have a very limited effect on band broadening due to the focusing nature of the technique, the analytes can be both separated and concentrated, and finally, high concentrations of the analytes can be achieved as the focusing of the analytes does not occur at the isoelectric point.

3. Theory

The theory of DFGF has been published in other papers [9,10]. Basically the resolution R_s for positively changed species in DFGF is expressed as given by Lee [11]:

$$R_s = \frac{|u|}{4} \cdot \left(\frac{\overline{\mu}}{bD_T}\right)^{1/2} \cdot \frac{\Delta\mu}{\overline{\mu}^2} \quad (1)$$

where u is the hydrodynamic flow rate, D_T a coefficient that represents the sum of all contributions to the dispersion, b is the negative value of the electric field and μ is the mobility of the analyte.

From Eq. (1) it can be seen that the resolution is inversely proportional to the slope of the electric field, so that resolution is increased by reducing the field gradient, raising the conductivity and/or increasing the hydrodynamic flow rate. As shown in this paper, DFGF can separate charged analytes, such as proteins and both their resolution and position inside the separation channel can be controlled by changing the profile and magnitude of the electric field.

In the work of Ivory the electric field channel is also used as a cooling channel, and as in his device the separation channel was packed with a support material to reduce diffusion of analytes and hence band broadening in the separation channel. By miniaturising both channels the surface to volume ratio is increased in the electric field channel; Joule heat dissipation will hence be increased and the need to use cooling buffers is no longer needed. By reducing the dimensions of the separation channel the diffusion in this channel can be reduced and hence the need for a packing material. This paper takes the first steps to confirm the benefits of miniaturisation in the area of focused separations.

4. Experimental

4.1. Chemicals and materials

Corning Vycor 7930 porous glass was used in the membrane and was ground down to a thickness of 1 mm by Advanced Glass Ceramics (Holden, MA, USA); it was then cut into sheets 3.0 cm by 0.5 cm. Untreated fused silica tubing (535 μm i.d. × 693 μm o.d.) was purchased from PolyMicro Technologies (Phoenix, AZ, USA). Kaleidoscope pre-stained proteins with a molecular mass range of approximately 200 000–6500 daltons were purchased from Bio-Rad Labs. (Hertfordshire, UK). The buffer solutions were prepared with filtered (0.22 μm) deionised water and degassed before use.

4.2. Instrumentation

The separation device (schematic: Fig. 2) was made from two blocks of Plexiglas machined using a Roland MDX-20 desktop milling machine [12].

The base and separation chamber were cut from two blocks 5 cm × 2 cm × 5 mm for the field gradient chamber

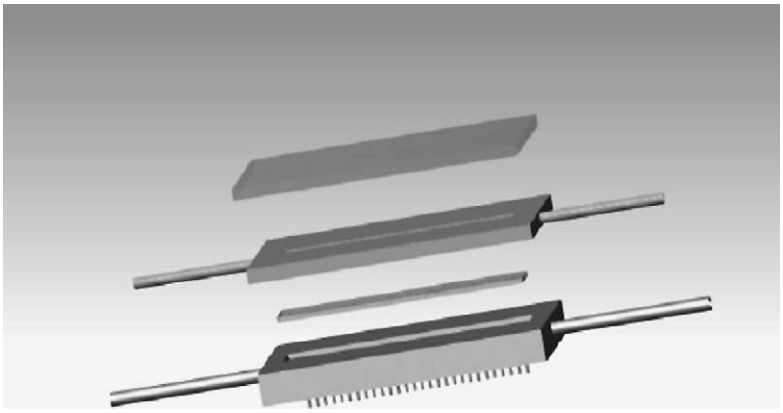

Fig. 2. Schematic showing the base, membrane, separation channel and top cover of the separation device.

and 5 cm × 2 cm × 1 mm for the separation chamber. The separation chamber has a trough machined 2.5 cm × 0.1 mm wide and the field chamber a trough 2.5 cm × 2 mm wide. This field chamber also holds the five individual control electrodes for controlling the electric field with a spacing of 0.5 cm. The separation channel is separated from the field chamber by a slotted membrane of Vycor glass [13], with an approximate specific gravity (dry) 1.5 and a void space 28% of the material volume, an internal surface area of 250 $m^2 g^{-1}$ average and pore diameter of 4 nm. The separation chamber is packed with a styrene divinylbenzene PolyHipe monolith [14] having the general structure as shown in Fig. 3.

The top cover of 100 μm polyether ether ketone (PEEK) film was bonded onto the top of the separation channel using a cyanoacrylate glue. This was found to be stable for 48 h before the top had to be resealed. The assembled device is shown in Fig. 4.

A syringe pump NP70 Harvard Apparatus (Edenbridge, UK) was used to deliver low concentration buffer into the electric field (bottom) chamber and another NP70 pump was used to deliver higher concentration buffer and sample into the top separation channel. The dynamic electric field was generated using five individual 1–500 V Emco (Sutter Creek, CA, USA) d.c. to high voltage d.c. converters. These where mounted on a breadboard and driven by a simple digital I/O. The voltage from the Emco high voltage cube was connected to the DFGF chip using a series of five gold electrodes 0.5 cm spaced in the base of the electric field channel. The voltage at each individual electrode was controlled from an in-house computer program providing individual control over each electrode as shown in Fig. 5.

Detection was achieved in the visible spectrum using a high definition digital camera subtraction method as developed at the University of York [15]. The principle used was to capture an image of the separation channel wetted with the buffer solution and then capture an image of the separation channel with the loaded sample. The two images were then digitally subtracted using Abobe Photoshop (Adobe Systems UK, Middlesex, UK) and the subtracted image scanned using software provided by Scion (Fredick, MD, USA).

The principle is outlined in Fig. 6.

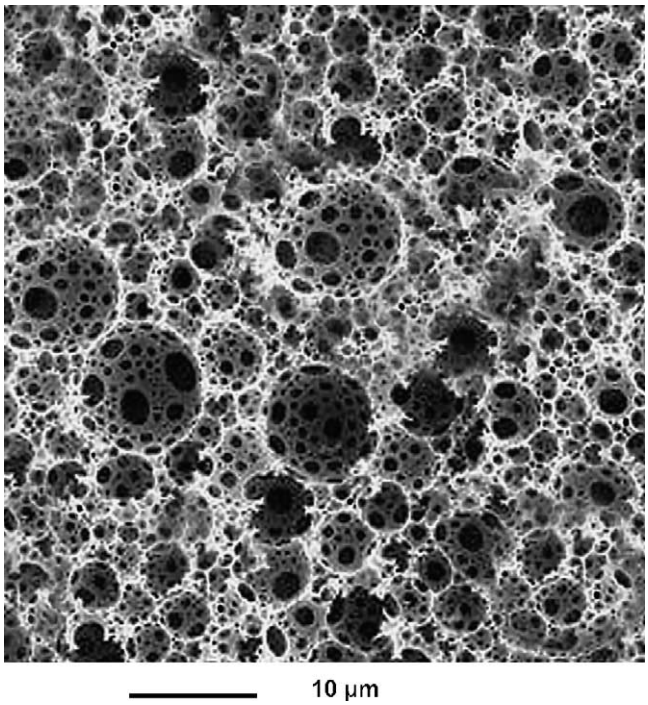

Fig. 3. Pore structure of the monolith use in the separation chamber.

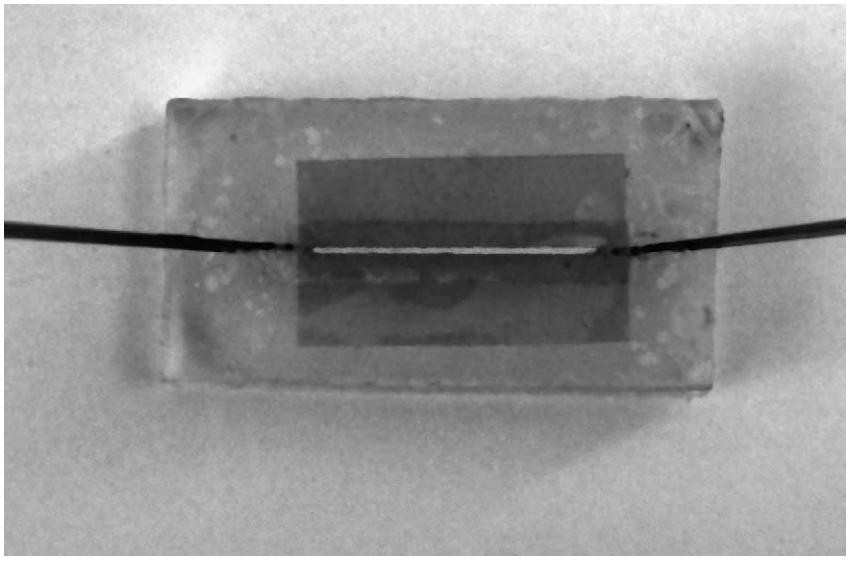

Fig. 4. The assembled DFGF chip showing the input/output tubing and the top PolyHipe separation channel. This assembly differs from that shown in Fig. 2 in that the inlets and outlets are inserted at 45° to the main channel.

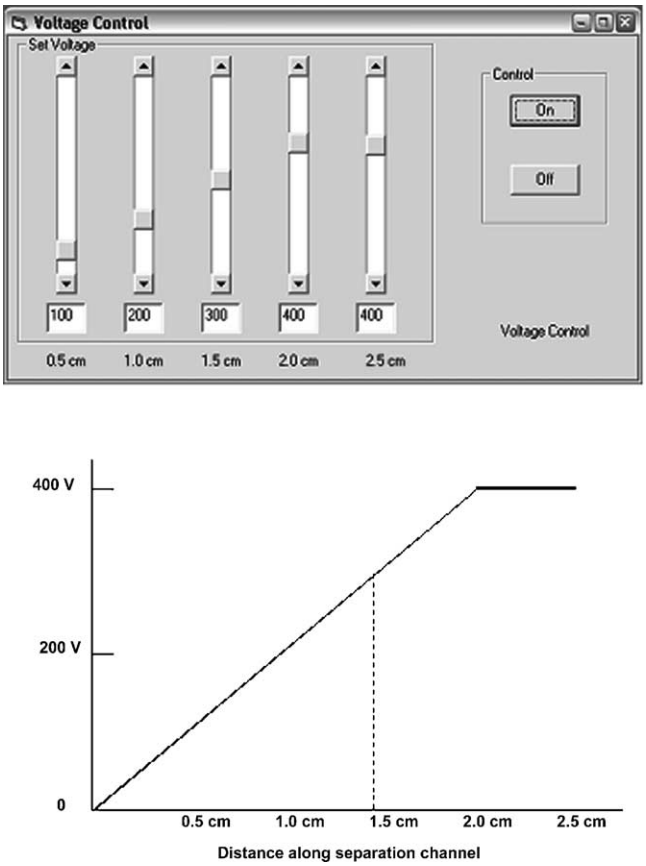

Fig. 5. Control program visual showing the generated electric field profile.

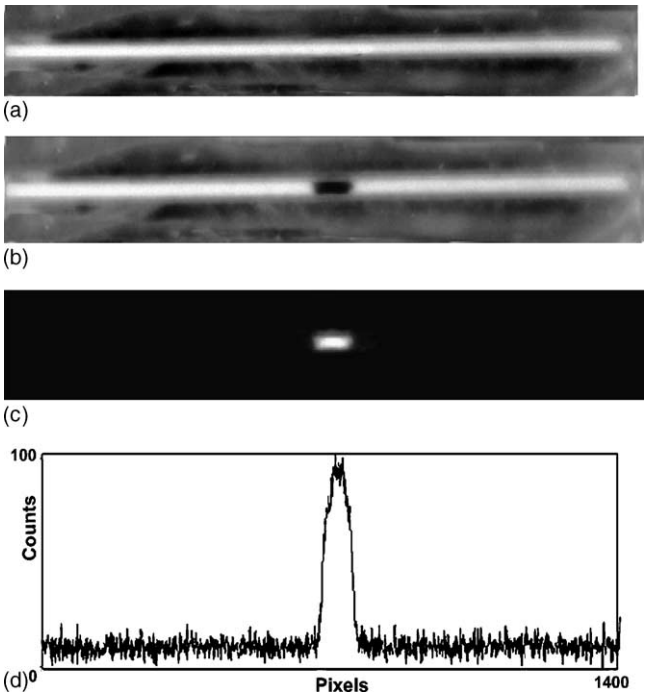

Fig. 6. (a) Digital image of the wet separation channel; (b) the channel with a loaded sample of Bio-Rad Kaleidoscope prestained proteins; (c) digitally subtracted image of (a) and (b); (d) scanned image of (c).

4.3. Procedure

For the experiments described in this paper, a buffer concentration of 1 mmol L^{-1} Tris–HCl at pH 8.7 was used in the electrode channel at a flow rate of 150 μL min^{-1}, linear velocity of 1 cm min^{-1} and a high concentration of 50 mmol L^{-1} Tris–HCl, pH 8.7 in the separation channel at a flow rate of 5 μL min^{-1} with a linear velocity of 2 cm min^{-1}. For the separation an injection of about 10 μL of the Kaleidoscope pre-stained proteins was made after the solution had been heated at 40 °C for 1 min.

Prior to any injection the flows were established for 10 min and a linear voltage profile applied with a voltage of 400 V on the electrode on the input port of the separation channel. All other voltages on the electrodes were scaled in linear steps to the voltage on the exit electrode being zero.

5. Results and discussion

A schematic of the miniaturised DFGF system is shown in Fig. 7. The first syringe pump provides a high concentration buffer to the separation channel and the other pump a low concentration buffer to the electrode channel. Sample is introduced via a simple low-pressure sample loop. No attempt was made to reduce or minimise any dead volumes in

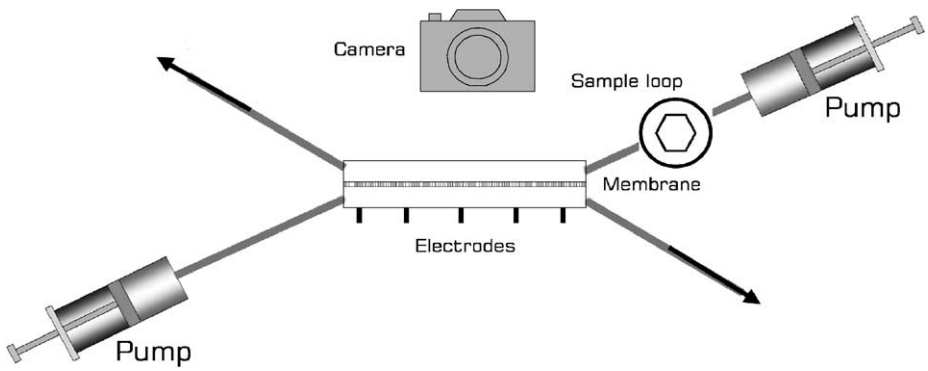

Fig. 7. Schematic of the dynamic field gradient focusing system.

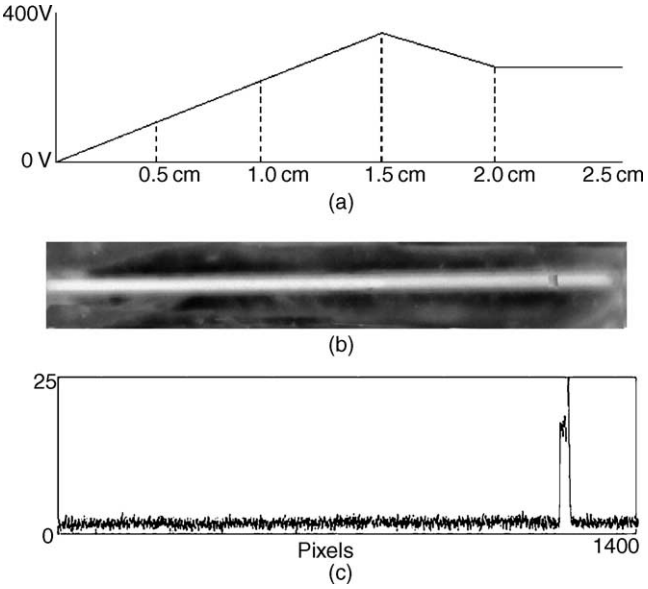

Fig. 8. (a) Voltage profile applied for sample injection; (b) digital image of injected prestained proteins held at the top of the column; (c) scanned subtracted image of (b).

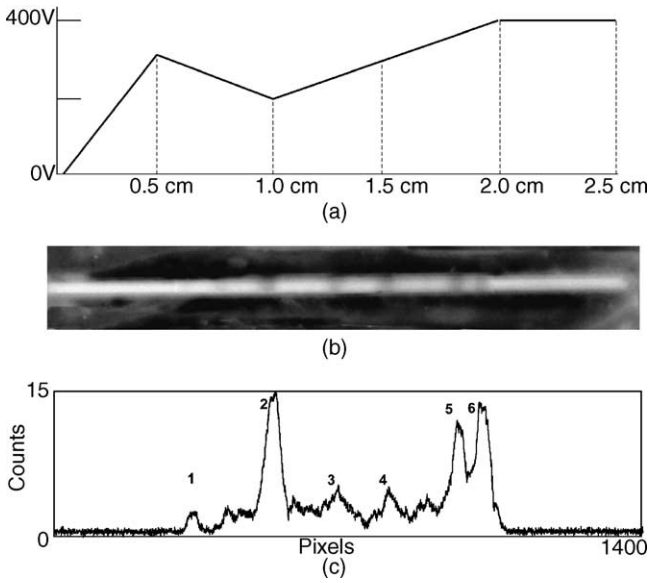

Fig. 9. (a) Voltage profile applied for the separation of the proteins; (b) digital image of focused prestained proteins; (c) scanned subtracted image of (b). Peak assignments: (1) aprotinin; (2) bovine serum albumin; (3) galactosidase; (4) soybean trypsin inhibitor; (5) carbonic anhydrase; (6) myosin.

the very simple connections. Using the conditions described in Section 4.3 and applying the voltage profile as shown in Fig. 8 the Kaleidoscope sample was injected and held on the separation channel. The voltages shown in Fig. 8 are the assumed potentials that are applied onto the electrodes based on the digital output from the I/O board on the computer, the principle of this starting voltage profile being to have a step field at the end of the column leading to an electric field well that will trap the sample. Since there is also a concentration difference between buffers in the two channels, a concentration, and hence a conductivity gradient will also form along the length of the membrane separating the two channels.

The profile shown in Fig. 8 was obtained five minutes after the introduction of the sample and was found to be stable. On changing the voltage profile to the one shown in Fig. 9a the sample plug separated into the individual bands (Fig. 9b) for which a densitometer scan is shown in Fig. 9c. Essentially the voltage profile is reduced from the top of the column to give an electric field well at the end of the column; this was designed to retain the bands in the column. This focused equilibrium profile was obtained after the voltage profile had been held for 15 min. Identification of the bands is based on computer colour matching of the colour chart provided with the Kaleidoscope mixture.

The proteins with high electrophoretic mobilities should focus in the high electric field and the proteins with the lowest electrophoretic mobilities should focus in the low electric field region.

When the voltage profile was reduced to that shown in Fig. 10 then the electrophoretic migration of all species was less that the hydrodynamic flow, the focusing was lost and the proteins were eluted from the channel.

The peak shapes obtained in these experiments are of interest. Previous workers have shown that the peaks should have a Gaussian distribution [7]. In Fig. 6 the peak is fairly symmetrical; the spikes most probably arise from the irregular nature of the surface of the PolyHipe and the top cover. However, the peak shown in Fig. 8 where the Kaleidoscope sample is held on the top of the column has a square profile with an indication of a separation occurring at the rear of the peak. Once the sample has been focused then the peaks still have a somewhat flat top. This effect could be due to either the colour resolution or saturation of the camera, which has a resolution of eight bits with six usable, or a result of the background correction to enhance the image quality.

The Kaleidoscope mixture contains seven proteins; in Fig. 9c we only attempted to identify them from colour matching the Bio-Rad colour chart to the image in Fig. 9b. However, the assignment is tentative, and only six of the proteins in the mixture were identified.

Fig. 9b is also interesting in that the colour density of each band is not uniform, as it should be across the column; this may be a result of the surface of the channel, as it was not a polished surface, or the non-uniformity of the PolyHipe monolith. There is clearly a requirement to develop a UV detection system to view unstained proteins.

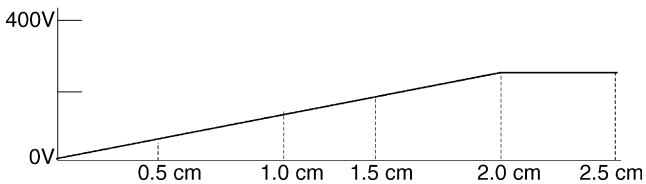

Fig. 10. Elution voltage profile.

The device made shows that DFGF can be miniaturised; by reducing the dimension further then benefits of not having to use a packing material in the separation could help to deliver better peak shapes.

6. Conclusions

A prototype miniaturised DFGF system has been constructed from laboratory machined Plexiglas. A porous glass membrane separates the 2.5 cm × 0.1 cm channel, which is packed with a PolyHipe monolith to reduce diffusional band broadening. The electric field channel has five electrodes, each with an individual computer controlled high-voltage supply. No cooling was necessary because of the small dimensions of the device. Separation and focusing of the constituents of a mixture of prestained proteins (molecular mass 6500–200 000) was achieved by opposing pumped hydrodynamic flow in the separation channel with a computer generated electric field profile, and the narrow focused protein bands were detected by processing data from digital camera images with background subtraction. By changing the voltage profile, proteins could also be moved up and down the channel and diverted from the channel.

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