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Rho, Hoon Suk; Hanke, Alexander Thomas; Ottens, Marcel; Gardeniers, Han J.G.E.

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Hoon Suk Rho[1]*
Alexander Thomas Hanke[2]
Marcel Ottens[2]
Han J.G.E. Gardeniers[1]

[1]Mesoscale Chemical Systems Group, MESA+ Institute for Nanotechnology, University of Twente, Enschede, The Netherlands
[2]BioProcess Engineering group, Department of Biotechnology, Faculty of Applied Sciences, Delft University of Technology, Delft, The Netherlands

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Research Article

A microfluidic chip with a staircase pH gradient generator, a packed column and a fraction collector for chromatofocusing of proteins

A microfluidic device for pH gradient chromatofocusing is presented, which performs creation of a micro-column, pH gradient generation, and fraction collection in a single device. Using a sieve micro-valve, anion exchange particles were packed into a microchannel in order to realize a solid-phase absorption column. To fractionate proteins according to their isoelectric points, elution buffer solutions with a stepwise pH gradient were prepared in 16 parallel mixing reactors and flowed through the micro-column, wherein a protein mixture was previously loaded. The volume of the column is only 20 nL, hence it allows extremely low sample consumption and fast analysis compared with a conventional system. We demonstrated separation of two proteins, albumin–fluorescein isothiocyanate conjugate (FITC-BSA) and R-Phycoerythrin (R-PE), by using a microcolumn of commercial charged polymeric particles (Source 15Q). The microfluidic device can be used as a rapid diagnostic tool to analyse crude mixtures of proteins or nucleic acids and determine adsorption/desorption characteristics of various biochemical products, which can be helpful for scientific fundamental understanding as well as instrumental in various industrial applications, especially in early stage screening and process development.

Keywords:
Microfluidics / pH gradient chromatofocusing / Protein separation
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1 Introduction

Over the last decades numerous microfluidic platforms have been developed for analytical chemical purpose, with advantages of extremely small sample consumption, high reproducibility, and a high level of automation and integration [1]. Especially in the field of separation and screening of bioproducts, microfluidic systems offer rapid evaluation of a target system with great selectivity and sensitivity, which has made miniaturized devices attractive for scientific and industrial applications including biochemical analysis [2, 3], biosynthesis [4, 5], proteomics [6–8], and bioprocess development [9–11].

One of the well-known microfluidic methods for the separation of proteins and nucleic acids is microfluidic electrophoresis [12–16]. Various combinations of microchannels and (micro-) electrodes were presented to identify and optimize the designs and operating conditions to achieve more efficient separation performance in the routine purification of biomolecules [12–16]. Compared with microfluidic electrophoresis, microfluidic solid-phase chromatography involving a column with packed resin particles has several beneficial aspects, such as a hydrodynamic flow-based driving principle, instead of electrokinetic flow, and a vast choice of commercial resins [4, 17–20], which facilitates the translation from small-scale microfluidic methods in the lab to large industrial systems for product purification. Especially the recent technical development in synthesis and functionalization of resin particles allows solid-phase chromatography to be used

Correspondence: Prof. Han J.G.E. Gardeniers, Mesoscale Chemical Systems, Faculty of Science and Technology, University of Twente, 7500 AE Enschede, The Netherlands
E-mail: j.g.e.gardeniers@utwente.nl

Abbreviations: **FITC**, fluorescein; **FITC-BSA**, albumin–fluorescein isothiocyanate conjugate; **R-PE**, R-Phycoerythrin

*Additional corresponding author: Dr. Hoon Suk Rho
E-mail: h.s.rho@utwente.nl

Color Online: See the article online to view Figs. 1–5 in color.

for many different applications, including ion exchange, size exclusion, and affinity binding [21].

Performing solid-phase chromatography with a microfluidic device requires the reliable formation of a microcolumn by packing resin particles, and accurate reagent manipulation, such as preparation of elution buffer solution, serial elution, and collection of fractions. The creation of a microcolumn on a chip has previously been achieved by adapting microstructures [22–24] and microvalves [17, 19, 20, 25, 26]. Although these micro-sized architectures successfully sieved particles from a flowing particle suspension, it is still challenging to handle the multiple reagents that are needed for completion of all experimental processes on a single chip [22–24]. Several devices with integrated microvalves, made by multilayer soft lithography, have been introduced, which provide a precise control of fluid flow, including loading, metering, and mixing of multiple reagents in microchannels [27–32] and the parallelization of reactors for solid-phase liquid chromatography [17, 19, 20]. An elegant example is a microfluidic device that successfully demonstrates automated on-chip ion exchange chromatography with linear gradient elution [19]. Flexible step gradient elution, based on the extensive parallelization of mixing units, could still bring additional benefits to such a device, for fast separation, low contamination, and low sample consumption.

In this contribution we describe a microfluidic device that employs pH gradient chromatofocusing by the integration of a packed particle microcolumn, a fluidic multiplexer for the handling of protein sample and pH buffer solutions, parallel peristaltic mixing reactors, and fraction collectors. Chromatofocusing is a separation technique that allows resolution of single proteins from a complex mixture according to their difference in isoelectric point. The method is typically performed on a column filled with an ion exchange resin that has a certain buffering capacity and which is first adjusted to a specific pH. Next, a buffer of another pH is run through the column, and by equilibration of the buffer on the resin, a pH gradient along the column is generated, focusing the proteins along the pH gradient in accordance with their isoelectric point. This concept has been theoretically elaborated and experimentally tested in a series of papers by Sluyterman and co-workers [33, 34]. Sluyterman et al. developed a theoretical model [33] in which the running pH buffer is considered to be composed of N aliquots of a starting pH, that successively pass through the ion exchange column, and of which the pH becomes adjusted at each of the N sections of the column. The protein focusing action occurs because a protein with a specific isoelectric point, p*I*, will run through the column along with the buffer, up to the column section where the column material and the protein become oppositely charged, which is where the protein will be retained. Effectively this means that the protein moves through the column at the same velocity as the buffer section that has a pH slightly above the protein *pI*.

When the ion exchange column has little buffering capacity, such as is the case for the Source 15Q resin (a strong anion exchanger) used in our work, the pH gradient can be brought upon externally (*gradient chromatofocusing* [35]). The pH gradient can be changed monotonously, but also stepwise changing gradients have been applied [36]. In the field of isoelectric focusing, which is related to but not the same as chromatofocusing, alternative and very elegant configurations have been developed in which the pH gradient is build in into the ion exchanger by aligning polymers with different acidity/basicity values along the column length, either linearly or stepwise [37, 38].

Here, we achieved a solid-phase column for chromatofocusing by packing commercial monodisperse resin particles in a microchannel, using a microfluidic procedure. Elution buffer solutions with a stepwise pH gradient were prepared in 16 mixing reactors and flowed through the column to separate the proteins according to their isoelectric points, and fractionate them into separate integrated containers. We have demonstrated and quantified the successful separation of a protein mixture composed of two proteins with close *pI* values, albumin–fluorescein isothiocyanate conjugate (FITC-BSA) and R-Phycoerythrin (R-PE), on a replaceable packed column of anion exchange polymeric particles (Source 15Q).

2 Materials and methods

2.1 pH gradient chromatofocusing

Figure 1A shows the principle of pH gradient chromatofocusing of proteins. When protein mixture flows through a column where positively charged particles are packed, proteins bind onto the particles because they are negatively charged under the chosen pH conditions (Fig. 1A (a)) [21, 39, 40]. The bound proteins are released from the particles when there is no net electrical force between proteins and particles, which depends on their isoelectric point in combination with the pH at the location of the particles. Since the isoelectric points of different proteins are different, proteins bound to the particles can be separately eluted by controlling the pH of elution buffer (Fig. 1A(b)). The selective elution of pH gradient chromatofocusing starts from the high pH, and the pH value of elution buffer decreases linearly (linear gradient elution) or gradually (step gradient elution). Therefore the protein which has the highest isoelectric point will be eluted at first and selective elution of proteins based on their p*I* will continue. Figure 1B shows a conventional experimental setup for pH gradient chromatofocusing. The setup includes an anion exchange column, pumping systems for a crude sample and two pH buffer solutions, an a fractionation unit. The process flows through sample loading, pH gradient generation by mixing high pH buffer and low pH buffer, and elution. The microfluidic pH gradient chromatofocusing device adapts and scales down the complete process, and consists of a pH gradient generator, a micro-sized anion exchange column, and fraction collectors. The microcolumn is created by sieving anion exchange particles from a particle suspension. Since the volume of the column is nanoliter-scale (20 nL), the

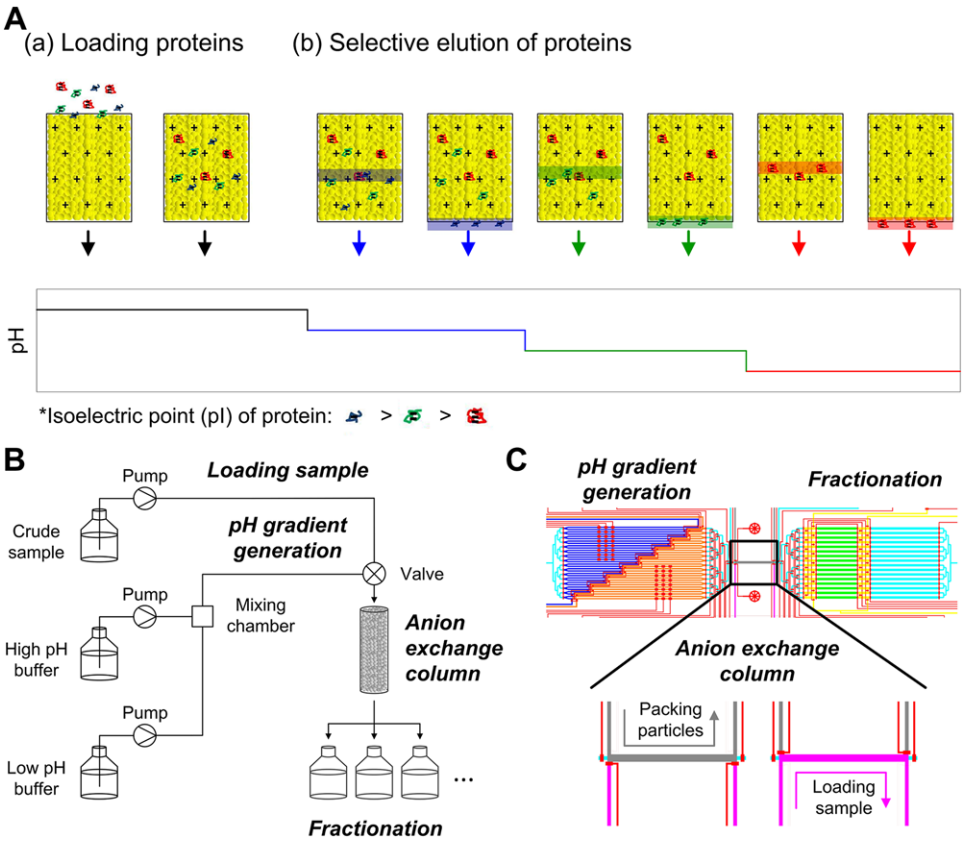

Figure 1. (A) Principle of pH gradient chromatofocusing. (B) Conventional experimental setup for pH gradient chromatofocusing. (C) A microfluidic method to separate proteins based on pH gradient chromatofocusing.

separation process is fast and sample consumption is extremely low compared with the conventional method.

2.2 Chip fabrication

The microfluidic chip has two polydimethylsiloxane (PDMS) layers, a fluidic layer and a control layer, and we fabricated the device using multilayer soft lithography [41, 42]. The protocols in our previous studies [26, 30, 43] were followed for fabrication of this device. Before use, the microfluidic channels were cleaned by flushing Milli-Q water (Millipore Co.) for 10 min, followed by air blowing.

2.3 Chip operation

We operated the microfluidic chip by an in-house built pneumatic control set up. The control system consists of pressure regulators, 3/2-way solenoid valves, and EasyPort USB digital I/O controller (all from Festo, Festo BV, The Netherlands) to apply compressed nitrogen gas into control channels for the actuation of microvalves. The operating set up was automated by a custom-built LabVIEW (National Instruments Co.) program. Reagents were loaded into fluidic channels by applying compressed nitrogen gas on the backside of solutions [44]. Our previous study shows that the calibration coefficient of loading solutions by pressure driven flow is 0.033 μL/min·mbar.

2.4 Data processing

We used a stereo microscope (Motic SMZ168, Lab Agency Benelux BV, The Netherlands) equipped with a CMOS camera (Moticam 3.0) for recording the optimization of device operation. We used an inverted fluorescent microscope (Leica DMI 5000M, 10X and 20X Objectives, Leica Microsystems BV, The Netherlands) equipped with an automatic XY-stage (Oasis PCI XY control unit), and a digital camera (Leica DFC300 FX, Leica Microsystems BV, The Netherlands) to acquire fluorescence images of the 16 fraction collectors. We processed and analysed all acquired images with Image J software (http://rsb.info.nih.gov/ij/).

2.5 Chip validation

A total of 100 μM of fluorescein (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) solution was prepared in Milli-Q water (Millipore Co.). The fluorescein solution and Milli-Q water were loaded into the buffer #2 loading site and buffer #1 loading site, respectively, in 16 mixing reactors. After mixing the solutions in the reactors for 1 min,

fluorescence images of the 16 reactors were acquired by a Leica I3 filter cube (excitation: BP 450–490 nm; emission: LP 515 nm).

2.6 pH gradient chromatofocusing test

Albumin–fluorescein isothiocyanate conjugate (FITC-BSA), R-Phycoerythrin (R-PE), ethylenediamine (≥99.5 %), and 1-Methylpiperazine (≥99.5 %) were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). 0.5 g/L of FITC-BSA solution, 0.5 g/L of R-PE solution, 1:100 diluted Source 15Q (∅ 15 μm particles based on rigid polystyrene/divinyl benzene polymer matrix, GE Healthcare Life Sciences, GE Healthcare Europe GmbH, Eindhoven, The Netherlands) suspension were prepare in 50 mM ethylenediamine buffer (pH 7.0). The FITC-BSA solution and R-PE solution were mixed in a 1:1 volume ratio before the experiment. 1-Methylpiperazine buffer solutions (pH 5.0 and pH 4.0) were prepared for the generation of pH gradient of elution buffer solutions in the microfluidic device. After fractionation of proteins, fluorescence images of the 16 reactors were acquired by a Leica I3 filter cube (excitation: BP 450–490 nm; emission: LP 515 nm) to obtain FITC-BSA fluorescence intensity and a Leica N 2.1 filter cube (excitation: BP 515–560 nm; emission: LP 590 nm) to monitor R-PE fluorescence intensity.

3 Results and discussion

3.1 Design of the microfluidic device

The microfluidic device consists of two layers, a layer for the liquid flow channels and another layer for the pneumatic control channel channels. Figure 2 shows the overlay design of the device with the connections of channels, reagent inlets and outlets, and control ports. The fluidic channels for loading reagents, pH buffer #1 (blue colour), pH buffer #2 (orange colour), spacer (yellow colour), protein mixture (light red colour), particle suspension (gray colour), pushing reagents (light blue colour), and collecting fractions (green colour) are connected to the inlets and outlets. The control channels for valve operation (red colours) are connected to control ports. The design comprises three parts, a pH gradient generator, a microcolumn, and a fraction collector. The pH gradient generator consists of 16 parallel mixing reactors and it can produce non-linear pH gradient by mixing two pH solutions with different volume ratios. The microcolumn is connecting 16 parallel mixing reactors to 16 fraction collectors one by one. Stepwise elution is performed by operating eight valve control channels (a multiplexer) implemented between the gradient generator and the fraction collector.

3.2 Creation of a solid-phase microcolumn in a microchannel

Figure 3 shows the generation of a microcolumn on a chip. The column unit consists of five flow channels, one central channel for elution, two upper side channels for loading particle suspension, and two bottom side channels for loading protein solution. The column was realized by closing six valves which are located at the junctions of the flow channels. Particle suspension was introduced by controlling two valves on two upper side flow channels. The valve connected to the inlet of the particle suspension was opened and the other valve connected to the outlet was partially opened to sieve the particles in the suspension. Valve openings according to various applied pressures were tested and optimized to sieve particles based on their size. Figure 3A shows the column generation by packing polystyrene particles (∅ 3 μm, 100 particles/nL, Polysciences, Inc., Polysciences Europe GmbH, Eppelheim, Germany). We applied 1.3 bar for control ports to close valves

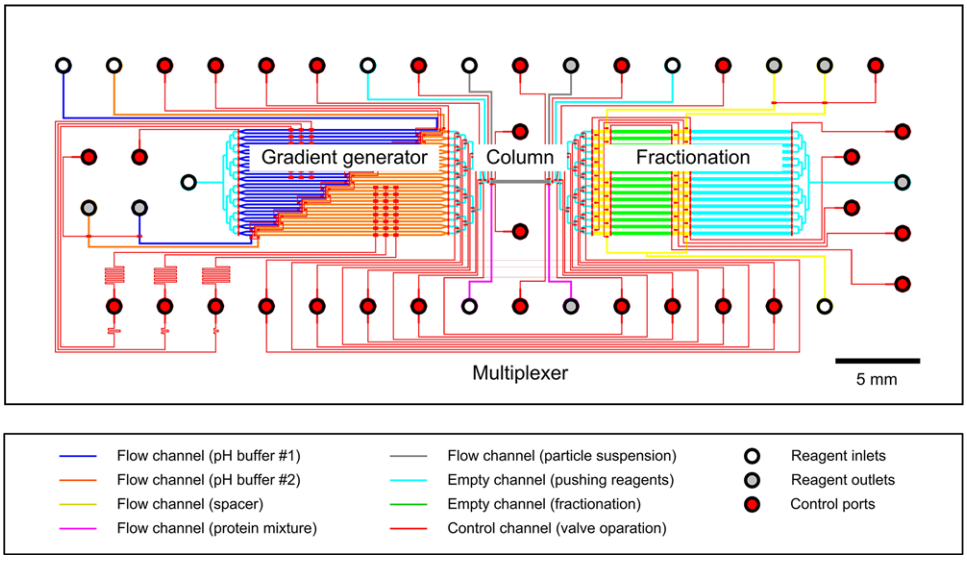

Figure 2. Design of the microfluidic device. The device comprised two layers, one layer for flow channels and another layer for control channels. The overlay CleWin (software used to design photolithographic masks; CleWin 5, Phoenix Software, The Netherlands) design shows the connection of the channels, inlets/outlets, and control ports.

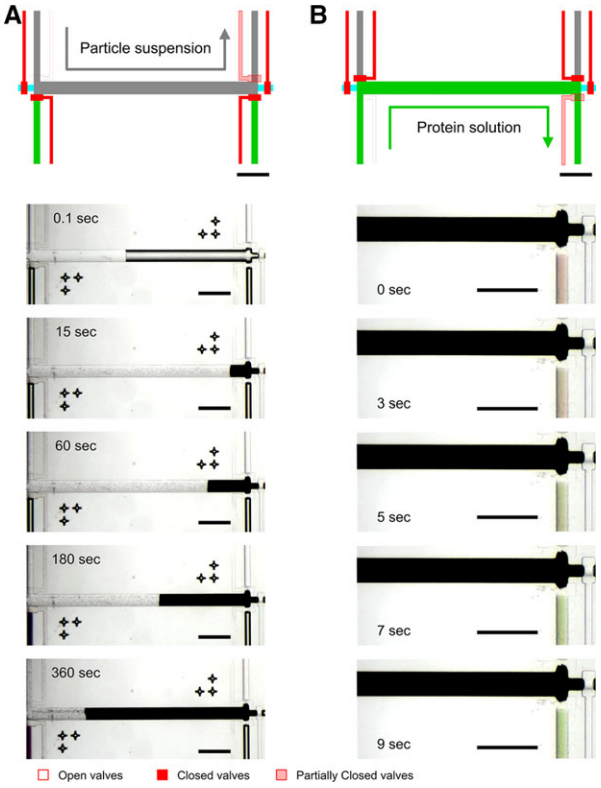

Figure 3. Creation of a microcolumn in a microchannel. (A) Particle packing by sieving particles from particle suspension, and (B) Elution through the microcolumn. 500 μm scale bars are shown.

completely and 1.0 bar for a control port to close a valve partially while the particle suspension was loaded into the channel by applying 0.5 bar from the backside of the solution. The time-lapse microscope images show the process of particle packing. The complete packing of particles was achieved by loading the suspension for 10 min. After packing the particles, the two valves on the upper side channels are closed and the two valves on the bottom side channels are opened to load protein mixture in a buffer with a suitable pH in the column (Fig. 3B). The valve connected to the inlet is opened and the other valve connected to the outlet is partially opened. To visualize the process of sample loading we transferred red colour dye solution though the suspension inlet and directed this solution into the outlet of protein loading channel. Then we introduced green colour dye solution into the protein solution channel through the microcolumn. The colour changes in the channel from red to green in time series microscope images present the fluid flow of solution through the column. Particles stayed in the column without displacement during loading of solution, which shows the capability of the device for reliable packing of the microparticles.

3.3 Generation of pH gradient on a chip

Figure 4 shows the pH gradient generator of the microfluidic pH gradient chromatofocusing device. The pH gradient generator was designed by adapting 16 parallel peristaltic mixers. The final pH in a mixing reactor is determined by the volume ratio of two solutions, pH buffer #1 and pH buffer #2, in the reactor. The volume ratios of the buffer #1 sites are varied from 0.941 to 0.059 with a decrement of 0.059 while the volume ratios of the buffer #2 sites are ranged from 0.059 to 0.941 with an increment of 0.059. Figure 4B shows the processes of loading buffer solutions and peristaltic mixing in a reactor among 16 reactors. Initially a mixing reactor is divided by two loading sites, buffer #1 loading site and buffer #2 loading site by closing metering valves (Fig. 4B (a)). After loading, the metering valves are open and side valves are closed to form a single loop-shaped reactor (Fig. 4B (b)). Then the mixing valves are actuated in a sequence, (001), (011), (010), (110), (100), (101), where 0 is open and 1 is closed, to create fluid flow for active mixing of the solutions. Due to the design of the connections of mixing valves an anticlockwise fluid flow is generated in mixing reactors from #1 to #8 while a clockwise fluid flow is created in the mixing reactors from #9 to #16. Figure 4C shows on-chip colour gradient generation. For visualization of mixing phenomena in mixing reactors, blue colour dye solution and Milli-Q water were introduced into buffer #1 site and buffer #2 site, respectively (Fig. 4C (a)). After operation of mixing valves with an operating frequency of 15 Hz for 30 s two solutions were completely mixed (Fig. 4C (b)). Peristaltic mixing of the solutions in 16 parallel reactors by operating three valves with the sequence is shown in Supporting Information Movie 1.

An accurate and highly reproducible gradient profile is necessary for reliable chromatofocusing of biomolecules. To evaluate the capability of the device for the generation of a wide pH gradient range we created, as a proof of principle, concentration gradients of fluorescein (FITC) and obtained fluorescence intensities in 16 mixing reactors. We introduced 100 μM of FITC solution and Milli-Q water into two loading sites in the reactors. Figure 4D (a) shows the volume ratio of two solutions in each reactor. The calculated concentration of FITC in the reactors ranged from 29.4 to 470.6 μM with an increment of 29.4 μM. The fluorescence intensities of FITC in the reactors were obtained after mixing for 30 s, the relationship between the measured fluorescence intensities of FITC and the concentration of FITC were plotted as a standard curve (Fig. 4D (b)). The standard curve produced by a serial dilution of the FITC solution in 16 reactors shows an accurate on-chip gradient generation. Error bars in the plot represent the standard deviation of three measurements.

3.4 On-chip fractionation

We designed parallel reactors to construct a wide range of gradient for on-chip chromatographic separation, however massive numbers of valves and control ports are required to control the manipulation of reagents in the reactors. To minimize the number of control inputs we adapted a microfluidic multiplexer, a combinatory array of binary valve architecture [27]. The connections between 16 reactors in the

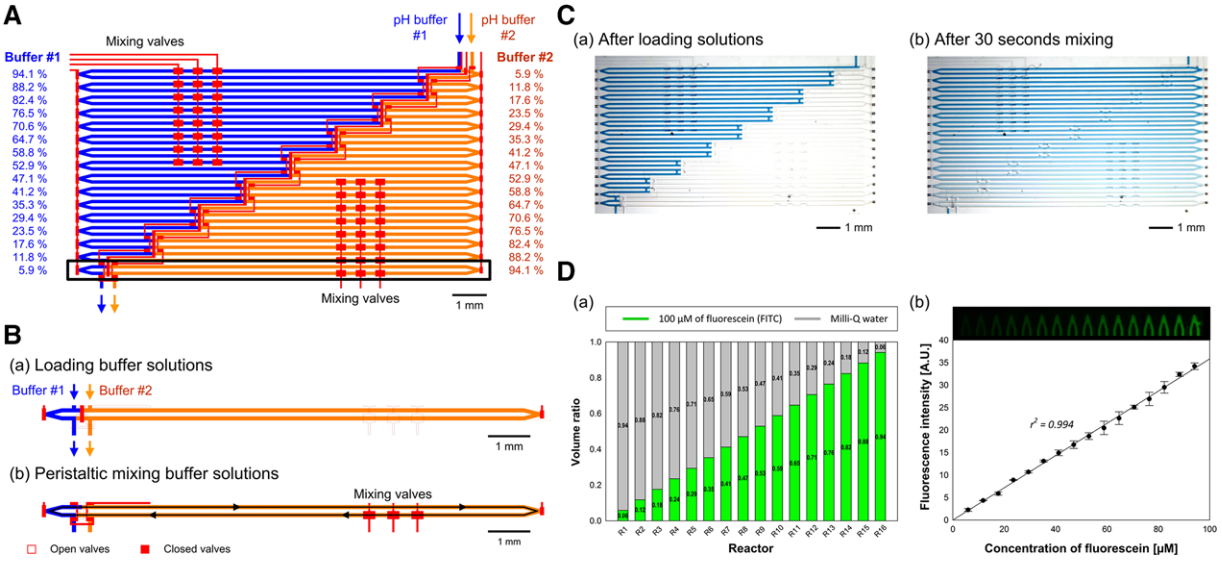

Figure 4. Parallel mixing reactors for pH gradient generation. (A) Design of the 16 mixing reactors. (B) Process flow in a reactor. (C) Microscope images of loading and mixing blue dye solution and Milli-Q water. (D) The gradient of concentration of fluorescein (FITC) obtained by Milli-Q water and loading 100 μM of FITC solution into buffer #1 sites and buffer #2 sites, respectively. The concentration of FITC in each reactor was calculated by volume ratios of the two loading sites (a) and the relationship between the measured fluorescence intensities of FITC and the concentration of FITC were plotted as a standard curve (b).

pH gradient generator (R_M) and 16 reactors in the fraction collector (R_F) were controlled by eight combinations of binary valves and the multiplexing control allowed the multiple step elution and collection of fractions through a single column (the detailed design and valve operations are shown in Supporting Information Fig. 1). Since dead volume still remained through the multiplexing unit during stepwise elution and all the procedures were based on fluid flow analysis, there were risks to have a cross contamination between neighbouring fractions by diffusion of biomolecules. To prevent this, we considered the first and last 19% of each fraction as waste, and pushed these parts out with an immiscible oil phase after completing the multiple step elution. Inserting spacers of an immiscible liquid between fractions also allowed collecting the fractions out of the microchannel separately for further analysis of the samples based on the concept of plug-based microfluidics [8]. In Supporting Information Fig. 2 we show a demonstration of the sampling process.

3.5 Separation of two proteins by pH gradient chromatofocusing

To demonstrate the feasibility of on-chip pH gradient chromatofocusing, we performed a chromatographic separation of the mixture of two standard proteins, albumin–fluorescein isothiocyanate conjugate (FITC-BSA) and R-Phycoerythrin (R-PE). The two proteins were chosen as a model system for the evaluation of the selective separation on the device because their isoelectric points (pI) are different but relatively close (4.7–4.8 for FITC-BSA and ~4.4 for R-PE) [45, 46].

Note that in principle it would be possible to work with larger numbers of p*I* markers in the same mixture, the reason for not doing so is that usually the p*I* values of commercial p*I* ladder standards extend over a large pH range, whereas we would like to investigate a more delicate p*I* difference. Furthermore, due to the limited number of fraction collection containers that we could embed in our microfluidic device without increasing the number of required valves to unpractical numbers, working with a larger number of proteins would not allow to see the distribution of proteins over the different collection "bins" and therewith would not allow to determine the "peak width" (i.e. focusing quality) for each of the proteins. Finally, a large range of proteins with different p*I* would also require that each protein preferably gives fluorescence at a different wavelength, which would imply the use of a spectrometric camera to analyse the distribution of the proteins over the different collection "bins". For these reasons we decided to perform a demonstration with only two fluorescent proteins.

Source 15Q particles were packed in the column microchannels of the device by sieving the particles during loading a 1:100 diluted Source 15Q suspension by applying 0.5 bar from the backside of the suspension. The mixture of 0.5 g/L of FITC-BSA and 0.5 g/L of R-PE was prepared and 2 μL of this protein cocktail was transferred into the Source 15Q column through the sample loading channel. After loading the mixture, the column was washed by flushing 10 μL of 50 mM ethylenediamine buffer (pH 7.0). The non-linear pH gradient ranged from 4.94 to 4.06 with a decrement of 0.059 was produced by mixing pH 5.0 buffer and pH 4.0 buffer in the 16 peristaltic mixing reactors (R_M) (Fig. 5A). We

A

Reactor number	Buffer #1 (pH 5.0)	Buffer #2 (pH 4.0)	Final pH
R_M #1	94.1 %	5.9 %	4.94
R_M #2	88.2 %	11.8 %	4.88
R_M #3	82.4 %	17.6 %	4.82
R_M #4	76.5 %	23.5 %	4.76
R_M #5	70.6 %	29.4 %	4.71
R_M #6	64.7 %	35.3 %	4.65
R_M #7	58.8 %	41.2 %	4.59
R_M #8	52.9 %	47.1 %	4.53
R_M #9	47.1 %	52.9 %	4.47
R_M #10	41.2 %	58.8 %	4.41
R_M #11	35.3 %	64.7 %	4.35
R_M #12	29.4 %	70.6 %	4.29
R_M #13	23.5 %	76.5 %	4.24
R_M #14	17.6 %	82.4 %	4.18
R_M #15	11.8 %	88.2 %	4.12
R_M #16	5.9 %	94.1 %	4.06

B

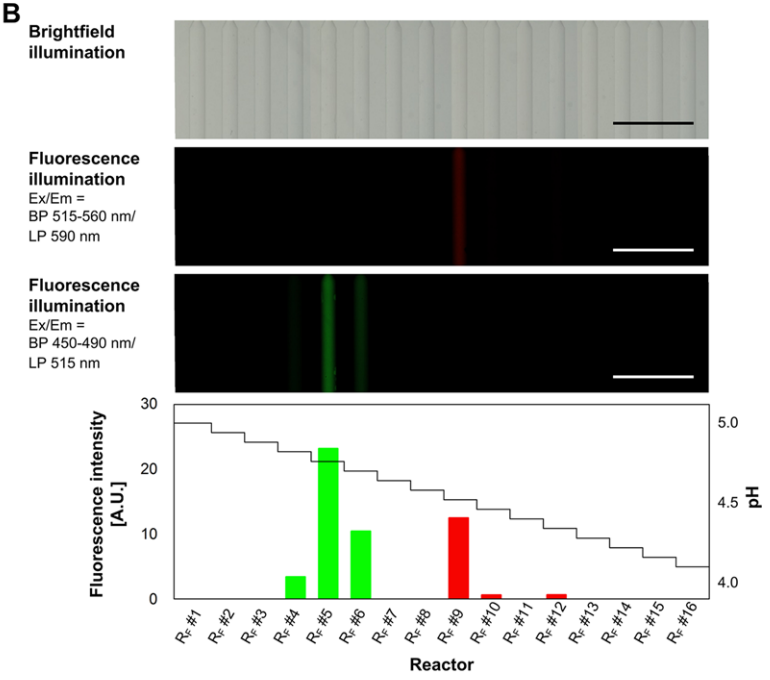

Figure 5. On-chip pH gradient chromatofocusing to separate two proteins from the mixture. (A) Preparation of elution buffers with pH gradient in 16 mixing reactors. (B) Microscope images of 16 parallel fractionation reactors acquired by brightfield illumination and fluorescence illumination. The change of pH value of elution buffer solution and obtained fluorescence intensities of the reactors are shown in the graph (1 mm scale bars are shown).

performed step pH gradient elution by pushing elution buffer solutions from R_M #1 (pH 4.94) to R_M #16 (pH 4.08). 16 fractions were collected in fractionate reactors (R_F) and illuminated by Leica N 2.1 filter cube (excitation: BP 515–560 nm; emission: LP 590 nm) and Leica I3 filter cube (excitation: BP 450–490 nm; emission: LP 515 nm) for monitoring the fluorescence intensities of FITC-BSA and R-PE, respectively. The fluorescence microscope images in Fig. 5B show the obtained fluorescence intensities of 16 fraction collectors (R_F). We measured the fluorescence intensities of FITC-BSA in R_F #4 (3.44 A.U.), R_F #5 (23.17 A.U.), and R_F #6 (10.48 A.U.) and fluorescence intensities of R-PE in R_F #9 (12.53 A.U.), R_F #10 (0.68 A.U.), R_F #11 (0.01 A.U.), and R_F #12 (0.70 A.U.). The isoelectric points obtained by on-chip pH chromatofocusing, 4.65–4.76 for FITC-BSA and 4.29–4.47 for R-PE, shows good agreement with the values from the references, 4.7–4.8 for FITC-BSA and $\sim$ 4.4 for R-PE [45,46]. Not shown here is that after a separation, the ion exchange resin can be flushed out of the channels, the channels can be cleaned, and new resin can be introduced and packed to form new columns, allowing repetitive use of the chip.

The above data allow a semi-quantitative evaluation of the chromatofocusing quality. FITC-BSA is distributed over three "bins" (fraction collectors), each with a "width" of 0.06 pH units, with concentrations proportional to the fluorescence intensities mentioned above. Assuming a Gaussian peak, we estimate a peak width at half maximum of ca. 0.08 pH units for this protein (the peak maximum being at a pH of 4.70). For R-PE, it is impossible to estimate a peak width with the given data, but presumably it is about the same as for FITC-BSA. From this, a very rough estimate of the separation resolution is obtained of $4.70–4.47/0.08 = 2.9$. Assuming that a resolution of 1.25 is necessary for baseline separation, our method in the current configuration would be able to separate proteins with an estimated difference in p*I* of 0.1 pH units, which to the best of our knowledge is a competitive value, considering that we did not make attempts to optimize it for our system. A possible way of optimization would be to apply a mixture of buffers with pH values closer together than the 1 pH unit we have used now, however in that case also two proteins with closer p*I* should be used to evaluate the separation. Our resolution is not as good as the best available commercial systems, for which a chromatofocusing resolution as low as 0.02 pH units has been claimed [47].

4 Concluding remarks

In conclusion, we have developed a new approach for performing pH gradient chromatofocusing on an integrated microfluidic chip. All the processes of chromatography separation including loading reagents, pH gradient generation, particle packing in a column bed, stepwise elution, collecting fractions, and on-chip detection were automated in a single device by the integration of microvalves. We demonstrated the device for chromatofocusing of two fluorescent proteins with a p*I* difference of ca. 0.3–0.4 pH units, and obtained a peak width at half maximum of 0.08 pH units, as well as an

estimated baseline resolution of 0.1 pH units. A drawback of working with the low amounts of protein as was done in our work is that concentration measurements of the separated proteins is a serious challenge. Therefore, we have used fluorescent proteins only in this study, however, a modification of our microfluidic device may be envisioned, in which each fraction collector is connected to an ESI nozzle and measurement is performed by mass spectrometry, which has the required detection sensitivity. Examples of chromatofocusing combined with MS have been reported in literature [48].

The demonstrated rapid on-chip analysis with the extremely small sample consumption is beneficial for the characterization of rare and expensive materials and identification of process conditions for bioprocessing. In addition, the large variety of commercial resins that is available nowadays for the creation of a microcolumn, and the flexible generation of pH or concentration gradients, will allow the device to be used as a rapid diagnostic tool to analyse crude mixtures of proteins and determine adsorption/desorption behaviour of various biochemical molecules. We believe that such fully automated and integrated microfluidic platforms for high-throughput protein screening offer a promising approach for fast biopharmaceutical process development.

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The authors have declared no conflict of interest.

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