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DOWNSCALING QUANTITATIVE ISOTACHOPHORESIS: LIMITS AT THE SUB-PICOLITER SCALE

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ABSTRACT

Isotachophoresis (ITP), an important bioanalysis technique, was downscaled 600,000 times in volume in a 50 nm deep channel. The chip design enabled a quantitative injection volume of 200 femtoliters. To evaluate ITP as a tool in bioanalysis for sub-cellular volumes, an academic sample was injected. The sample, consisting of pretreated yeast lysate, spiked with two fluorescently labeled amino acids (10 attomoles per amino acid) was successfully focused. ITP in 0.33 µm heigh channels showed that the amino acids were also separated, contrary to ITP in 50 nm channels, indicating a limit in separation in ITP downscaling.

KEYWORDS: isotachophoresis, bioanalysis, single cell analysis, femtoliter, nanochannel, amino acid

INTRODUCTION

The developing field of single-cell analysis promises unprecedented insights in cell biology. However, to study a cell's time resolved metabolism in for instance, cellular division, differentiation, the effect of drugs etc., requires the analysis of metabolites in sub cellular samples. A single cell of 25 microns in diameter contains 8 pL. A sub-cellular aliquot extracted to study the cells workings over time, needs to small enough to not kill the cell, e.g. 5 % which would then be 400 fL. To analyze the multitude of compounds in such a sub-cellular sample quantitatively requires a method which is not available at the moment.

Isotachophoresis (ITP) is an electrophoretic technique known for its ability to focus and under favorable conditions separate analytes, including biological samples of high complexity [2]. ITP utilizes a discontinuous electric field along the separation axis, to focus analytes, and most preferably arrange them in order of their electrophoretic mobility, see Figure 1. The inherent properties of ITP to concentrate and separate are desirable in many analytical applications and have stimulated developments for its translation to chip format [3, 4].

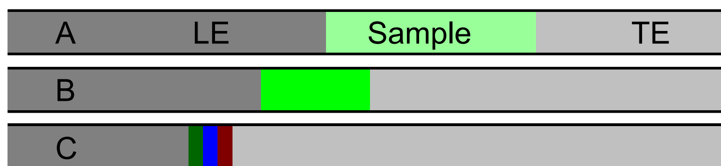

Figure 1: Conventional isotachophoresis. ***A*** *A sample is interposed between two chosen electrolytes, one with a higher, and the other with a lower electrophoretic mobility than the analytes, the leading electrolyte (LE) and the trailing electrolyte (TE) respectively.* ***B*** *When a voltage is applied, this imposes a discontinuous electric field. Consequentially, velocity normalization focuses the sample.* ***C*** *If conditions are favorable compounds are separated, arranging the analytes in order of their mobilities.*

For the downscaling of electrophoretic separations to the required sub-picoliter or even femtoliter volumes the emerging field of nanofluidics holds great promise [1]. Theoretically, downscaling ITP to channels with a nanodimension would allow low abundant compounds to be quantitatively analyzed in sub-picoliter injection volumes, as required for the analysis of an aliquot from a single cell. To validate this potential experimentally an ITP protocol was developed and validated in conventional micro capillary. The sample (0.12 µL) consisted of an academic biomodel, a pretreated yeast cell lysate spiked with amino acids, labeled with the fluorophore fluorescein isothiocyanate (FITC). A chip consisting of nanochannels was developed together with sophisticated interfacing, for the purpose of performing this ITP protocol quantitatively for a volume of 200 fL.

EXPERIMENTAL

Effectively translating quantitative ITP to a nanofluidic-chip format requires a sample of well-defined volume, to be interposed between a leading and a trailing electrolyte (See Figure 1). Therefore a channel structure was designed incorporating T-junctions, see Figure 2. The chip was fabricated in borofloat glass with chemical wet etching. Access holes were powderblasted in the top and bottom wafer. Two channel dimensions were used either 50 nm deep and 10 µm wide or 330 nm deep and 3 µm wide, corresponding to a sample volume of 200 fL and 400 fL.

The fluidic chips were placed in a custom-built interface, (see Figure 3) located on the xyz stage of a fluorescence microscope. It allows interfacing of one chip on a wafer. The interface ensures standardized placement of electrical and fluidic connections to the access holes, by providing electrodes from below and reservoirs from the top of the chip.

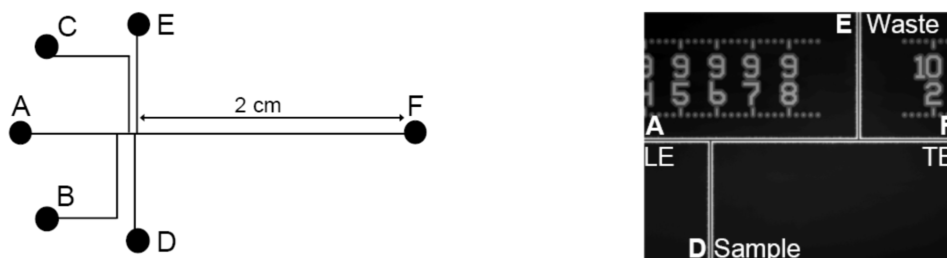

Figure 2: Left, schematic layout of the isotachophoresis chip. A to F are the access holes for liquid and electrodes. The side channels connect to the separation channel such that multiple, fixed-sample volumes may be chosen. This enables quantitative analysis and creation of the LE-sample-TE electrolyte configuration. Right, a brightfield image of the injection double-T cross used in this paper. The distance where channels D and E intersect with the separation channel is 400 µm. The ruler seen has a 20 µm spacing between tick marks.

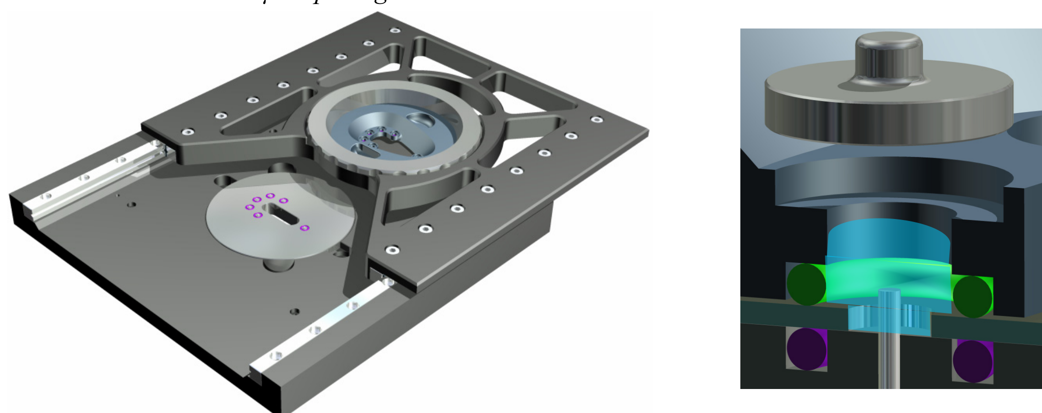

Figure 3: Nanofluidic interface module. The module is placed upon the xyz stage of a fluorescence microscope. The image depicts the interface (20x20 cm) in open position. When closed it allows a clear view of the injection area and the separation channel from the top by microscope. The disk seen on the bottom contains the electrode arrangement. It matches the positions of the access holes of the chip (Figure 2) and provides connectors for the power supply underneath. To assemble the interfacing, the wafer is placed upon this disc. The top of the interface is slid into place and pressed down placing reservoirs on top of the access holes. The image on the right shows such a reservoir sectioned along the electrode, in the assembled interface, with the two O-rings sealing the wafer from bottom and top. After placing the required electrolyte in the reservoir it can be sealed with the cap.

The developed protocol for capillary ITP was as follows. ITP electrolytes were introduced into the capillary (27 cm total length, 20 cm from inlet to detector, 50 µm ID) by pressure. It was first flushed with trailing electrolyte (TE), 5 mmol/L of HEPES. A plug of sample as then injected, typically 0.12 µL (22% of the capillary), consisting of 80:20:10 TE:pretreated yeast extract:labeled amino acid solutions (v:v:v, final concentration of each amino acid 50 µmol/L). Lastly that end of the capillary was placed in a vial with leading electrolyte (LE), 10 mmol/L of NaCl, the other in one with TE. ITP was then induced by applying 5 kV. The electrolytes were prepared anew in deionized water each day, adjusted to pH 9.50 with sodium hydroxide, and stored under argon until used.

RESULTS AND DISCUSSION

The protocol developed in capillary was transferred to the chip, for both channel depth varieties. To create the LE-sample-TE arrangement required for quantitative ITP, the chip (Figure 2) was initially filled, using capillary action [5], when TE was placed in well F. When the chip was completely filled, TE was also applied in well E. LE was placed in A and the yeast biomatrix containing the FITC-amino acids in D. To induce ITP, the appropriate voltage settings on the reservoirs were obtained empirically by observing the effects of manual voltage changes through the fluorescence microscope. Voltage settings were incorporated in an automated protocol using software controlling the power supply to perform the fast and/or stepwise voltage changes required. The voltages required to create an exclusive EOF flow from sample well D to waste E are given in Figure 4a and 4d. After an experiment, re-applying these plug formation settings for typically a few minutes would reform the stable plug, allowing repeated experiments. The plug created in Figure 4a corresponds to 600,000 times less sample volume compared to that used in capillary. Quantitativeness requires sample volume accuracy. Switching from plug creation settings to those for ITP, without losing or extracting sample into or from the sidechannels is therefore a crucial step. As a measure of quantitativeness, the surface area of the ITP graph in Figure 4f was compared to that of the plug and found to contain 8% more fluorescent compounds (30 fL injection error).

The final voltages on wells A and F, applied for ITP (Figure 4b and 4e), are the highest potential differences applicable over the separation channel, for that channel height. They represent an optimum between strong focusing, and dispersive effects. However, a higher optimal potential difference could be applied over the 0.33 µm channel for ITP (approx. 75% of that in capillary ITP) compared to the 50 nm channel. This marks an important difference for ITP in the two channel

heights, as although focusing was demonstrated in the 50 nm channels, the amino acids were not separated. The 0.33 µm channels allowed higher optimal field strengths for ITP, with the amino acids partially separated.

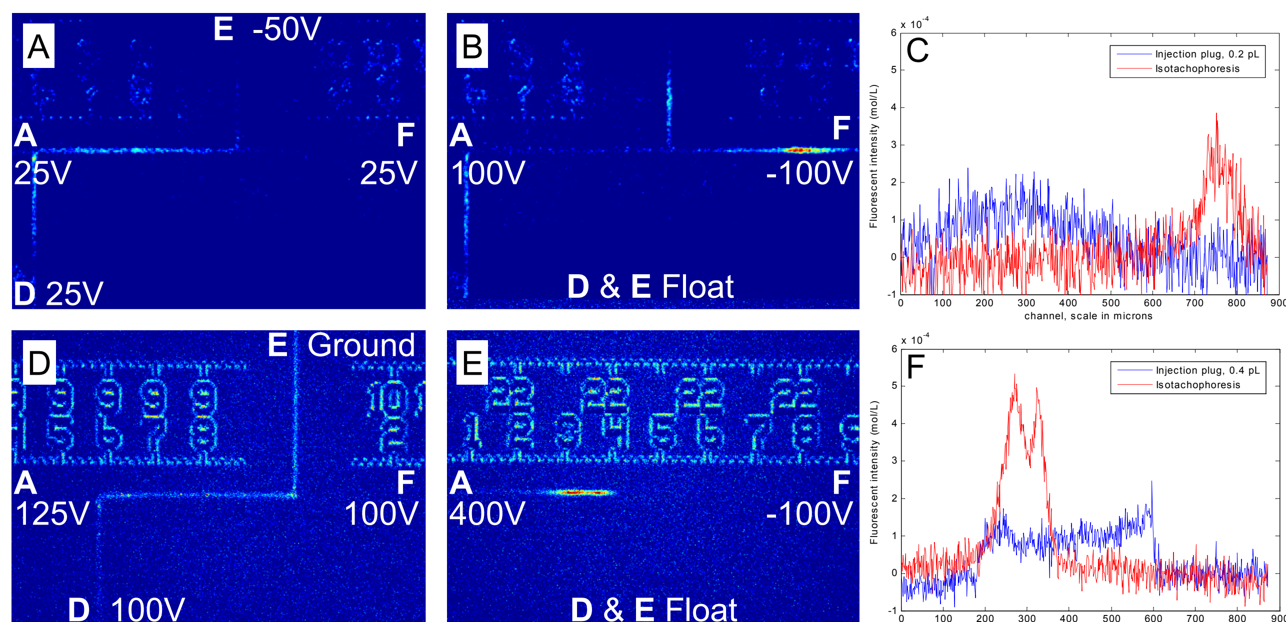

Figure 4: On-chip ITP results in 50 nm (top) and 0.33 µm channels (bottom). Figures a & d, are false colored fluoresence images showing the created stable injection plug in the chip (see Figure 2) of the yeast biomatrix spiked with Glu-FITC and Phe-FITC. The white letters A, D, E and F designate the access holes, with LE present to the left of the plug (well A) and TE to the right (well F). Figures b & e show images of the resulting ITP in the two channel height regimes, 50 nm and 0.33 µm respectively. Voltages indicated in Figures a & d are those applied to create the stable sample plugs shown, corresponding to 200 and 400 fL respectively. In Figures b & c the applied voltages to perform ITP are indicated. Figures c & f show fluorescent intensities, scaled to concentration, along the separation channel in the nano and sub micron channel respectively. Figures a & b are background corrected.

CONCLUSIONS

ITP of a quantitative sub-picoliter sample volume was performed. The sample consisted of an academic biomodel, to evaluate the technique for bioanalytical applications. It was found that the focusing effect of ITP can be applied in 50 nm deep channels. In addition, compounds were partially separated in sub-micron channels, due to higher optimal potential differences applicable. This optimal potential difference, depending on channel height, was unexpected and was found to impose a fundamental limitation on the effectiveness of further downscaling of ITP separations. Development of the fluidic chip and particularly the interfacing enabled analysis on a minute scale, so far beyond the platforms and techniques presently available. Capillary ITP bioanalysis protocols can now be translated to sub-picoliter volumes using this setup. It opens the way for further exploration of fundamental principles of minute channels and ITP in general, e.g. plateau concentrations, analysis time, resolving power etc., as well as ITP hyphenated methods. Specifically, it can be used to answer biological questions requiring sub-picoliter analysis, such as samples from single cells.

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