ATTOLITER LIQUID CHROMATOGRAPHY USING EXTENDED-NANO CHANNEL FOR SEPARATION OF PROTEINS IN A SINGLE CELL

Hisashi Shimizu[1], Ryo Ishibashi[1], Kazuma Mawatari[1,2], Takehiko Kitamori[1,2]
[1]School of Engineering, The University of Tokyo, Japan, [2]CREST, Japan Science and Technology Agency, Japan

ABSTRACT

A liquid chromatography using 100-nm scale (extended-nano) channels was developed for single cell analysis. Our method, extended-nano chromatography makes possible an ultrasmall sample (100 aL-1 fL) injection and high efficient separation (~10^5-10^6 plates/m) using nanofluidic channel as separation column. In particular, hydrophilic interaction (HILIC) and reversed-phase chromatography was developed for separation of biomolecules including proteins in a single cell. In addition, an influence of a specific liquid property in extended-nano space was investigated for higher separation efficiency.

KEYWORDS

Nanofluidics, Chromatography

INTRODUCTION

Single cell analysis is a useful tool to reveal heterogeneity of cells such as stem cell differentiation and cancer metastasis [1]. Recently, some proteomics and metabolomics research are performed at single cell level using nanoliter liquid chromatography (nanoLC) or capillary electrophoresis (CE) combined with mass spectroscopy (MS) [2]. Nevertheless, quantitative analysis of biomolecules in a single cell is still difficult, mainly due to a large analysis volume and connection with MS. On the other hand, we have discovered specific properties of water confined in an extended-nano (10-1000 nm) fluidic channel [3] and proposed its analytical applications including chromatography. To date, the extended-nano chromatography realized separation of a smaller volume sample (180 aL) than a single cell (pL) [4], as well as a higher separation efficiency than that of conventional packed columns [5]. However, separation using aqueous mobile phase have not been implemented, which makes application to biological samples difficult. In addition, aqueous mobile phase indicates the specific liquid properties in extended-nano channel, which may have an influence on separation performance. In this paper, hydrophilic interaction (HILIC) and reversed-phase chromatography were firstly performed using extended-nano channel toward biomolecule separation. The separation performance was also investigated from a point of view of a specific liquid property in extended-nano space.

THEORY

Separation efficiency of chromatography is evaluated using plate height *H* which is expressed as,

$$H = \sigma^2 / L \tag{1}$$

where σ is the variance of the peak width and L is the length of the separation column. Here, a sharp peak gives a small σ and *H*, which means a smaller plate height gives higher separation efficiency. According to the theory, the plate height is expressed using flow velocity *u* as following.

$$H = A + B/u + C \cdot u \tag{2}$$

Here, the *A* term represents the eddy diffusion in a packed column which is caused by random pathways of sample molecules. The *B* term represents the dispersion in column length direction. The *C* term represents the dispersion in radial direction. In case of nanochannel, the *A* term is eliminated because the pathway is uniform by removing packing particles. Then, the equation can be written as,

$$H = \frac{2D_m}{u} + \frac{f_0}{105}\frac{d^2}{D_m}u \tag{3}$$

where D_m is the diffusion constant, f_0 is the width-to-depth ratio and *d* is the depth of the nanochannel. Therefore, the use of nanochannel also reduces the C term significantly, which results in high separation efficiency overcoming the limitation of conventional packed columns.

EXPERIMENT

Nanochannels were fabricated by electron beam lithography and reactive ion etching on a fused silica substrate. The substrate was bonded with a top substrate by fusion bonding at 1080° C, which was followed by surface treatments. In this paper, the silica surface was washed by 1% NaOH, 1% HCl and deionized water for HILIC mode or modified with trimethylsilylimidazole in the form of gas for reversed-phase mode as stationary phases. For mobile phase, water and acetonitrile (ACN) were used.

All chromatography experiments were performed using the silica chips, a pressure-driven nanofluidic control system [4] and fluorescent microscope. Figure 1 shows schematics and fluorescent images of an injection procedure. All the nanochannels were 910 nm wide, 220 nm deep, and 1.2 mm long. The injection was performed by switching pressures in 200 ms, which enabled highly reproducible injection of 100 aL-1 fL volume.

978-0-9798064-5-2/μTAS 2012/$20©12CBMS-0001

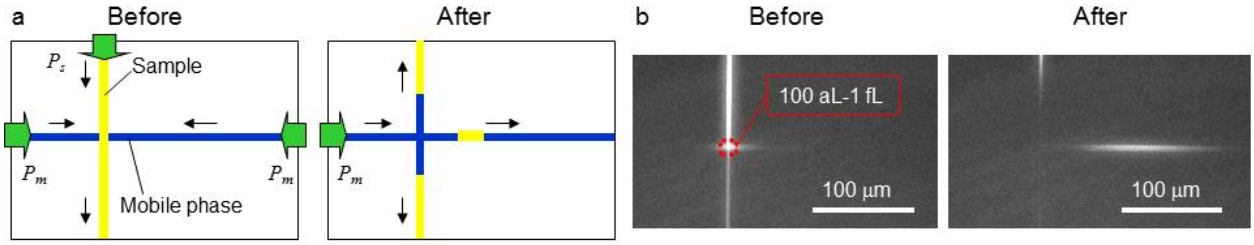

Figure 1. (a) Schematics and (b) fluorescent images of nanochannels before and after injection.

RESULTS AND DISCUSSION

Figure 2a shows a chromatogram of two fluorescent dyes separated by HILIC mode. As the samples had different emission wavelengths, the fluorescence was detected separately in four serial runs. The results were reproducible and well accorded with conventional HPLC's results, which verified the separation in HILIC mode. Next, separation efficiency was evaluated using the plate height as shown in figure 2b. The minimum plate height reached 2.9 µm (corresponding to 440,000 plates/m) which was one order of magnitude lower than that of conventional packed columns. Furthermore, in case of low water composition, the experimental the plate heights were lower than a theoretical curve which was derived from equation 3. This result suggests the unusual properties of liquid confined in extended-nano space had an influence on the separation efficiency.

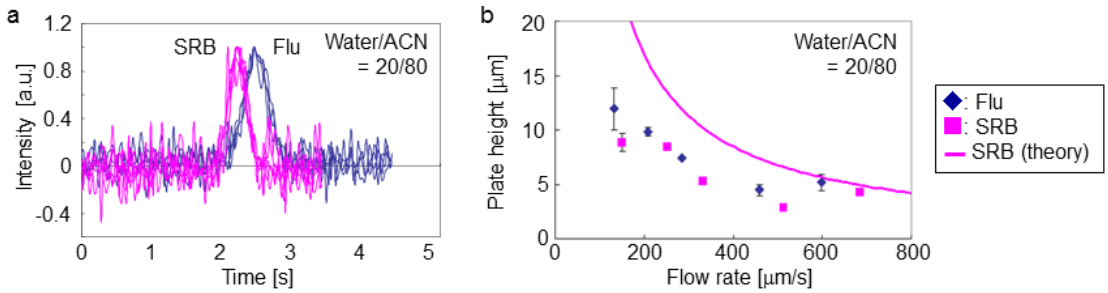

Figure 2. (a) Chromatogram of fluorescein (Flu) and sulforhodamine B (SRB) for HILIC mode (b) fluorescent images of nanochannels before and after injection.

In order to investigate this influence, diffusion coefficient under flow was measured for different compositions of mobile phase. From the theory of diffusion, the peak variance σ is associated with effective diffusion coefficient D_{eff} and elapsed time t as following.

$$\sigma^2 = 2D_{eff}t \tag{4}$$

At the same time, the peak variance σ is associated with the flow rate u using equation 1 and 3 as following.

$$\sigma^2 = \left(\frac{2D_m}{u} + \frac{f_0}{105}\frac{d^2}{D_m}u\right)L \tag{5}$$

Finally, equation 4 and 5 are correlated as following.

$$\sigma^2 = 2\left(D_m + \frac{f_0}{210}\frac{d^2}{D_m}u^2\right)t \tag{6}$$

$$D_{eff} = D_m + \frac{f_0}{210}\frac{d^2}{D_m}u^2 \tag{7}$$

Figure 3a shows the ratio of D_{eff} and D_m under flow plotted against mobile phase composition. From equation 7, the ratio D_{eff}/D_m should have a value larger 1 theoretically. However, the ratio was less than 1 only in low water composition, which means the D_{eff} is significantly decreased.

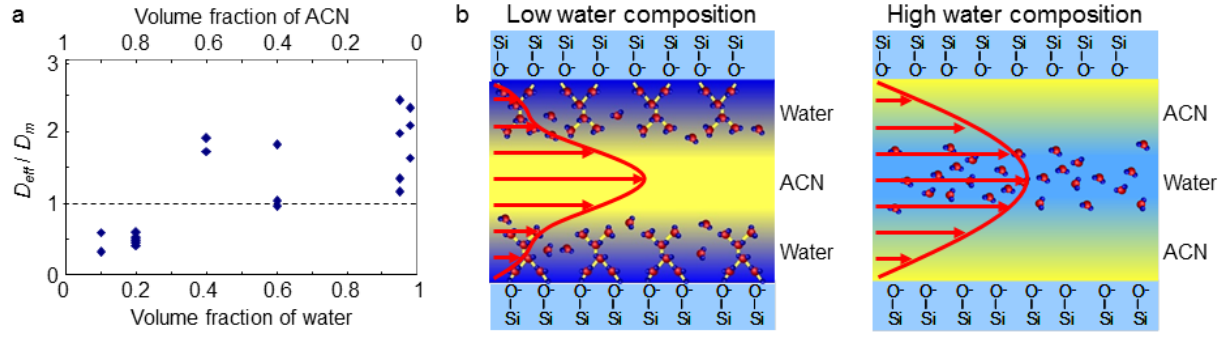

Figure 3. (a) Ratio of diffusion constant in flow (D_{eff}) and without flow (D_m) plotted against mobile phase composition. (b) Mobile phase partition and suggested flow rate distribution in nanochannel.

This result is discussed further in figure 3b. In the conventional theory of HILIC chromatography, water is partitioned on the stationary phase surface in low water composition, while acetonitrile is on the surface in high water composition [6]. On the other hand, we have found an increase of water viscosity in extended-nano space, probably due to structuring from the nanochannel surface [3]. Therefore, the higher viscosity of loosely structured water might result in a deformation of flow rate distribution in nanochannel. This is the first example to suggest the liquid property in extended-nano space can improve analytical performance.

Figure 4a shows a chromatogram of the two fluorescent dyes separated by reversed-phase mode. The result was also reproducible and well accorded with conventional HPLC's result, which verified the separation in reversed-phase mode. The minimum plate height was 1.1 μm (corresponding to 910,000 plates/m) which means 20 times improvement of separation efficiency compared to packed columns.

Furthermore, separation of proteins was attempted using reversed-phase mode (figure 4bc). As a result, reproducible peaks of two fluorescently-labeled proteins were observed in different mobile phase compositions. Although gradient elution system is required further to separate these proteins, extended-nano chromatography was verified to be available for separation of proteins. The numbers of injected protein molecules were calculated from concentration (1×10^{-5} M) and injection volume (5 fL). The number was ~10^4 for extended nano chromatography and ~10^6 for commercial micro HPLC. Thus, extended-nano chromatography realized 6 orders miniaturization and could be a prospective method for separation analysis of proteins in a single cell.

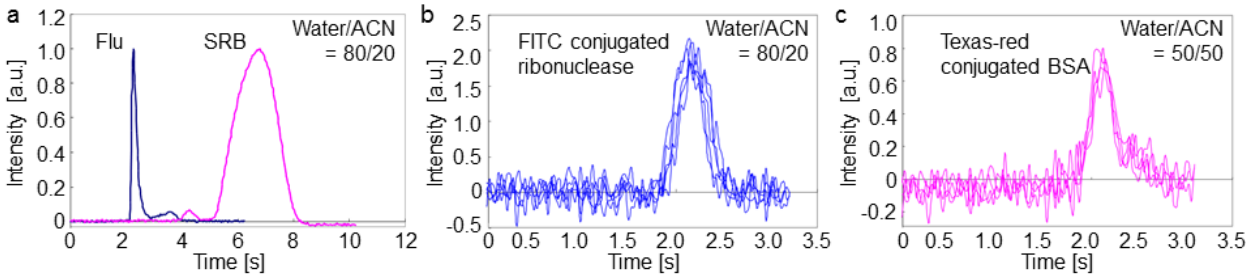

Figure 4. Chromatograms for reversed-phase mode. (a) fluorescein (Flu) and sulforhodamine B (SRB). (b) and (c) fluorescently-labeled proteins.

CONCLUSION

In this paper, HILIC and reversed-phase mode separation were firstly performed using extended-nano channel. In HILIC mode, a possibility of higher separation efficiency due to a specific property of liquid in extended-nano space was suggested. Using HILIC mode, separation of small hydrophilic molecules such as sugar and amino acids was made possible. In reversed-phase mode, a possibility of protein separation was verified. Because an injection volume of extended-nano chromatography (~100 aL-1 fL) is much lower than single cells (~pL), separation of proteins in a single cell (~10 molecules) is highly expected by an improvement of the detection system (single molecule level).

ACKNOWLEDGEMENT

This work was partially supported by JSPS Core-to-core program and Grant-in-Aid for Specially Promoted Research.

REFERENCES

[1] Daojing Wang and Steven Bodovitz, *Single cell analysis: the new frontier in 'omics'*, Trends in Biotechnology 28, pp. 281–290, (2010).

[2] Andrea Amantonico, Pawel L. Urban and Renato Zenobi, *Analytical techniques for single-cell metabolomics: state of the art and trends*, Analytical and Bioanalytical Chemistry 398, pp. 2493–2504, (2010).

[3] Takehiko Tsukahara, Akihide Hibara, Yasuhisa Ikeda, and Takehiko Kitamori, *NMR study of water molecules confined in extended nanospaces*, Angewandte Chemie International Edition 46, pp. 1180–1183, (2007).

[4] Ryo Ishibashi, Kazuma Mawatari, Katsuyoshi Takahashi and Takehiko Kitamori, *Development of a pressure-driven injection system for precisely time controlled attoliter sample injection into extended nanochannels*, Journal of Chromatography A 1228, pp. 51–56, (2012).

[5] Ryo Ishibashi, Kazuma Mawatari and Takehiko Kitamori, *Highly efficient and ultra-small volume separation by pressure-driven liquid chromatography in extended nanochannels*, Small 8, pp. 1–6, (2012).

[6] Fabrice Gritti, Alberto dos Santos Pereira, Pat Sandra and Georges Guiochon, *Comparison of the adsorption mechanisms of pyridine in hydrophilic interaction chromatography and in reversed-phase aqueous liquid chromatography*, Journal of Chromatography A 1216, pp. 8496–8504, (2009).

CONTACT

Hisashi Shimizu 81-3-5841-7232 or shimizu@icl.t.u-tokyo.ac.jp