Reversed-Phase Chromatography In Extended-Nano Space For The Separation Of Amino Acids

Adelina Smirnova, Hishashi Shimizu, Kazuma Mawatari, and Takehiko Kitamori*

The University of Tokyo, Department of Applied Chemistry,
7-3-1, Hongo, Bunkyo, Tokyo, JAPAN, 113-8656

*Corresponding author email: kitamori@icl.t.u-tokyo.ac.jp;

Phone: +81-3-5841-7231, Fax: +81-3-5841-6039

In this work we used reversed-phase chromatography in extended-nano channels to separate amino acids. A hydrophobic surface modification of extended-nano channels was established. A sample mixture of fluorescein and sulforhodamine B (0.5 and 0.05 mM respectively) was used for the demonstration of a reversed-phase separation mode. A small amount of sample band (30 fL) was injected into the separation channel, and two compounds were successfully separated. The maximum theoretical plate number of sulforhodamine B was 300,000 plates/m. Two sets of 3 amino acids (3.75 mM each) were separated using 0.01 M citrate buffer (pH 5.5) with 0.01 M sodium perchlorate and 12 and 25% of acetonitrile as a mobile phase. A successful separation (320,000 plates/m with plate height of 3.2 μ m for serine) was accomplished

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1. Introduction

Single-cell analysis has recently attracted much attention. An understanding of the mechanisms of cell-to-cell communication and cell signaling has helped in the creation of new medicines to fight cancer, autoimmune diseases and diabetes. The work at this level requires a novel analytical platform which has a much smaller volume (fL to aL) than that of a cell (pL) [1]. In line with emerging requirements for separation technique, high-performance liquid chromatography (HPLC) offers a definite advantage for the miniaturization of micro-columns to nano-columns with inner diameters ranging from 0.01-0.1 mm [2]. Various stationary phases (monolithic materials) exist in the small

spaces of prepared materials [3, 4]. To create a powerful analytical tool with high efficiency and fast separation following fast mass transfer, different small spaces have been studied, from packed fused silica capillaries [5, 6] and open tubular columns [7-9], as well as the analytical channels of microchips [10-13]. **Miniaturization of LC columns using capillaries packed with either monolithic materials or sub-2 μ m particles and application of OTCs with a sub-5 μ m diameter** led to a better performance in terms of the kinetic characteristics (reduced plate height, h , separation impedance, E , flow resistance factor, η , and permeability, K) along with stable thermodynamic properties (retention factor, selectivity and peak asymmetry) [8, 14, 15].

So-called extended-nano spaces, which range on a scale from 10^1 - 10^3 nm, are space bridges between a single molecule and normal liquid. The specificities of an extended-nano space for chromatography include an ultra-low sample injection volume and the elimination of the properties of packed column chromatography such as eddy diffusion and diffusion in the radial direction, all of which creates a unique mode of separation. Furthermore, due to the pressure-driven flow in the microchip the choice of solvents (aqueous or organic) is not limited, which is an advantage over an electrokinetic-driven flow. Our group has already developed a nanofluidic control system [16] and realized normal phase and HILIC modes of liquid-phase separation in an extended-nano space [17, 18], but reversed-phase HPLC in a nanochannel remains challenging. In this work we present the modification of a nanochannel with ODS and an evaluation of its performance under pressure-driven conditions. Reversed-phase HPLC in an extended-nano space for the separation of amino acids is described.

2. Materials and methods

Extended-nano channels were fabricated on a quartz glass plate by electron beam lithography (ELS-7500, Elionix, Tokyo, Japan) and plasma etching [19]. To protect free silanol groups on the surface from forming hydrophobic strained siloxane Si-O-Si bonds under high temperature, a substrate with channels was bonded to a cover plate using a low-temperature bonding technique after plasma surface activation [20]. The design of the nanochannels accommodated a loading, separation, and waste (Fig. 1) [21]. Each of the ends of the cross-shaped nanochannels (800 nm wide, 230 nm deep, 2000 μ m long from the cross-point) were linked to microchannels (250 μ m wide, 3 μ m deep). Each end of the microchannels was connected to the custom-made pressure driven system. Pressure-driven pinched injection was performed, as previously described [22]. The timing control was performed with a time resolution of 10 ms [16, 22].

A microchip was placed in a custom-made chip holder, which was then fixed on the motorized stage (BIOS 105T, Sigma Kouki Co., Ltd., Tokyo Japan) of the fluorescence microscope (IX71, Olympus, Tokyo, Japan). We used an EMCCD camera (iXon, Andor Technology PLC) to detect the fluorescence signal, and special software (iXon) for the analysis.

Fluorescein (Flu) and sulforhodamine B (SRB) (0.5 and 0.05 mM respectively) were used as fluorescence dyes and a mixture of 0.1 mM Phosphate buffer (pH 8)/Acetonitrile = 75/25 (v/v) was used as a mobile phase. For separation of the amino acids, we used serine, alanine, proline, valine, leucine, and phenylalanine, labeled with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) [23]. The mobile phase was a mixture of acetonitrile and 0.01 M Citric buffer (pH 5.5) with 0.01 M NaClO₄. For channel surface modification we used octadecyldimethyl-N,N-diethylaminosilane (ODS-DEA) synthesized from diethylamine and octadecyldimethylchlorosilane (ODS-Cl), according to a previously established procedure [24]. Briefly, acetonitrile and dried toluene were continuously introduced into one of the microchannels for 30 min using a pressure controller (500 KPa) [16]. Then 20% (v/v) ODS-DEA in toluene was introduced and the microchip was placed on a Peltier heater (70 °C). The nanochannels were flashed with ODS-DEA for 30 min at 500 KPa, then for 2 hours at 80 KPa, in order to maintain a continuous flow and prevent solvent evaporation from the nanochannels. After the modification, the channels again were rinsed with toluene and acetonitrile through all microchannels for 30 min with pressure of 500 KPa. The following day, the procedure was repeated. The free silanol groups were protected by low-temperature bonding, and, therefore, we did not apply an alkali rinse before the ODS modification. The microchip was filled with mobile phase and stored under water. All chemicals were from Wako (Japan)

3. Results and discussion

3.1 ODS modification of the walls in an extended-nano channel

An extended-nano channel is similar to an open-tubular capillary and requires the on-column preparation of chemically bonded stationary phases. Direct modification with octadecyltrichlorosilane proved to be difficult due to polymerization and nanochannel clogging. Previously, hydrophobic nanochannel surface modification with trimethylsilylimidazole was accomplished by our group [18], but modification with C18 was still required.

108 We utilized an approach similar to monolith column modification using
 109 monofunctional silane with an amine in on-column bonding reactions. Reports using
 110 octadecyldimethyl-N,N-dialkylaminosilane showed maximum coverage with high
 111 reproducibility [24]. We applied this method for nanochannel modification and covered
 112 the nanochannel walls with C18 without clogging. Unfortunately, we could not evaluate
 113 the surface coverage achieved by the on-column reaction using existing techniques.
 114 Neither NMR nor CHN analysis is applicable to a single nanochannel, while
 115 chromatographic methods measuring the excess or adsorption isotherms via a UV
 116 detection system do not meet the requirements for such a small-detection volume (less
 117 than 10 fL). We could only use fluorescence detection and make comparisons with the
 118 retention of model fluorescence substances such as Flu (non-retained) and SRB
 119 (retained) under the same separation conditions. Therefore, we fabricated 2 microchips
 120 with different lengths of nanochannels, 2 and 5 mm, and modified them with ODS-DEA
 121 and checked the separation of Flu and SRB. The presence of the C18 phase on the
 122 surface was confirmed, as these dyes were well separated, while under the same
 123 conditions on a bare silica surface they would not have been [18]. The separation
 124 characteristics are presented in Table. 1. The retention coefficients, k' , of SRB for both
 125 lengths of nanochannel were similar, which proved that the surface coverage was the
 126 same. However, it was about 20 times less than that of a packed column ($k' = 30$), due
 127 to a much smaller phase ratio. We calculated the reduced plate height, $h=H/d_c$, and the
 128 reduced velocity, $v=ud_o/D_m$, using the plate height (H), the column diameter (d_c) for the
 129 plate nanochannel $d_c=2DW/(D+W)$, where D and W are the depth and width of the
 130 nanochannel respectively, the linear flow rate (u), the diffusion coefficient (D_m), and
 131 plotted the curve according to the Knox equation for open tubular columns, $h = \frac{B}{v} + Cv$,
 132 where B and C are system constants that are determined by longitudinal diffusion, and
 133 resistance to mass transfer, respectively. The obtained h and v are located on the left
 134 arm of the **theoretical** Knox curve shown in Fig. 2, and are compared with open tubular
 135 columns separation data [9]. In order to reach the optimal parameters, we can increase
 136 the reduced velocity values 2 ways: (1) increasing the linear velocity of the mobile
 137 phase, and (2) increasing the channel diameter. Due to the channel length difference at
 138 the same applied pressure, the h and v were varied by 2 and 5 mm, but the value for $B =$
 139 hv from the Knox equation was similar. Along with the open tubular columns, the
 140 values of B obtained were greater than the theoretical values of 2.

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3.2 Kinetic behavior in a reversed-phase nanochannel

To study the kinetic characteristics (separation impedance, E , and permeability, K) of extended-nano channels, we evaluated the wetting properties first. These are dependent on both fluidic and interfacial parameters. We studied the water properties of multiple nanochannels modified by C-18 (same sizes as a single chromatography channel 2 mm in length) using the capillary filling method previously developed by our group [25]. The meniscus motion of the Flu in the mobile phase through the nanochannels was recorded. The relationship between the ratio of the square of the meniscus displacement to the time x^2/t and the external pressure P_{ex} has a linear dependency and the fitting line intercepts the axes at 0, which allowed us to neglect the surface tension ($\cos \theta$) due to the hydrophobicity of the channel walls and omit the second portion of the equation derived from the Hagen-Poiseuille law and the Young-Laplace equation:

$$\frac{\Delta x^2}{\Delta t} = \frac{4.5d_c^2}{64\eta} P_{ex} + \frac{4.5\gamma d_c}{16\eta} \cos \theta$$

where $x = x(t) - x_0(t_0)$ is the meniscus displacement, $t = t - t_0$ is the time difference, η is viscosity, γ is the surface tension, and θ is the dynamic contact angle [25].

Because we were using an open system, the inner pressure in the nanochannels and the viscosity remained unknown, but we did establish the dependency of the external pressure and could apply it to Darcy's law.

The solvent velocity in a chromatographic column can be described as $u = \frac{F}{A}$ or

$u = K \frac{\Delta P}{\eta L}$, where u is the flow velocity, F is the volumetric flow rate through the column

A is the cross-sectional surface of the empty column, K is the specific permeability, ΔP is the pressure drop over the column, and η is the viscosity of the solvent (1 Cp for a water/acetonitrile 75/25 (v/v) mixture). This formulation is also known as Darcy's law. Unlike conventional HPLC, in an open-tube nanochannel the interstitial velocity as well as the influence of the interparticle porosity, bed morphology and particle size and shape is eliminated. So the calculation of the permeability coefficient can be simplified as:

$$K = F L / A P$$

To solve this equation we can borrow the ΔP from the capillary filling results, so the calculated value, K , of the nanochannel permeability is about $5-6 \times 10^{-14} \text{ m}^2$, which overcomes the restrictions of a conventional packed column ($0.4 - 2 \times 10^{-14} \text{ m}^2$)

174 [26] and becomes closer to a monolithic one ($8\text{--}130 \times 10^{-14} \text{ m}^2$) [5]. However, the inner
 175 diameter of a nanochannel is smaller than that of monolith pores, so resistance is higher.
 176 Total column performance was evaluated by estimating the separation impedance
 177 (E), that describes the compromise in speed-efficiency-pressure $E = \frac{t_0 \Delta P}{N^2 \eta}$, where t_0 is
 178 the elution time of an unretained solute, and N is the number of theoretical plates. A
 179 smaller separation impedance relates to a higher degree of total column performance.
 180 For conventional columns, impedance values of 3,000–5,000 are generally obtained
 181 [15], while for monolithic columns minimum values of approximately 100–300 are
 182 reached [5]. The separation impedance, obtained by the nanochannel was 530–580, and
 183 it decreased with the flow velocity; however, the minimum impedance value
 184 (corresponding to 1–2 mm/s flow) had not yet been reached. Both higher applied
 185 pressure and a wider and longer nanochannel improved the separation performance in
 186 the nanospace.

187

188 3.3 Separation of amino acids

189 We applied our system for the separation of 6 amino acids (serine, alanine, proline,
 190 valine, leucine and phenylalanine), representing the entire range of retention on a C18
 191 column. The driven pressure was 3 MPa ($\sim 6.5 \text{ pL/min}$) with an injection time of about
 192 0.1 – 0.7 s, which corresponded to an injection volume of 10–60 fL.

193 First, the 6 NBD-F labeled amino acids were separated by mixture of 25%
 194 acetonitrile and 0.01 M citrate buffer (pH = 5.5) with 0.01 M sodium perchlorate. The
 195 chromatogram showed several peaks that were not well resolved (data is not presented).
 196 To explore the possibility of separation under isocratic conditions we used 2 sets of 3
 197 amino acids each (A: serine, alanine and proline; and, B: valine, leucine and
 198 phenylalanine (3.75 mM each)) eluted by the same buffer containing 12 (for A) and
 199 25% (for B) of acetonitrile. Figure 3 shows the corresponding chromatograms of the
 200 successful separation. The estimated efficiency for each amino acid is listed in Table 2.
 201 The plate height was calculated using the half-height peak width. Plate counts for amino
 202 acids differs from that of Flu and SRB due to the different (more selective) mobile
 203 phases that are used for separation.

204 Using a gas cylinder instead of a compressor pump can further increase an applied
 205 pressure by more than 4 MPa and improve the separation conditions. However, the
 206 sensitivity of the detection was still very low due to the short detection distance (230
 207 nm). Detection in a nanochannel requires a high sensitivity detection system such as a

208 laser induced fluorescence microscopy or differential interference contrast thermal lens
209 microscopy (DIC-TLM)

210 We applied the LIF detection system for the separation of amino acids. Using
211 highly sensitive detection, the concentration of tested substances was reduced by 2
212 orders to approximately 20 M. The signal noise fluctuations were lowered as well.
213 However, peak capacity for the 2 mm channel under isocratic conditions was limited by
214 the maximum of possible separated peaks, which is approximately 7-12. To improve the
215 separation efficiency, therefore, we must optimize the channel sizes (depth, width and
216 length), increase the applied pressure, and use the gradient flow of a mobile phase.
217 These points are currently under study in our laboratory.

218

219 Conclusion

220 To summarize, reversed-phase chromatographic separation with a femtoliter
221 injection volume was performed in a nanospace. This is a prospective technique that
222 could be used for unique single-cell analyses such as determining the content of
223 intracellular amino acids following single-cell trapping and lysis in a nanochamber [27].

224

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311 Figure Caption

312 Fig. 1 Photograph of the microchip and a close-up of the design of the extended-nano
313 channels. Nanochannels were 2.2 mm in length (from cross-section), 800 nm wide and
314 230 nm deep. Microchannels were 250 mm wide and 3 mm deep.

315

316 Fig. 2 **Theoretical** Knox curve, plot of h against v , as calculated from the Knox equation.
317 Comparison of separation parameters used on extended-nano channels versus open
318 tubular columns.

319

320 Fig. 3 Chromatograms of the separation of NBD-F labeled amino acids, (3.75 mM). Mobile
321 phase: 10 mM Citric buffer (pH 5.5) with 10 mM NaClO₄ and acetonitrile (A – 12%, B – 25%
322 v/v); injection time: A – 0.2 s, B – 0.5 s.

323

Figure 1

Figure 2

Figure 3

Table 1. Comparison of experimental performance data for 2 and 5 mm length extended-nano channels

Parameters	2 mm length nanochannel	5 mm length nanochannel
k' (SRB)	1.44	1.45
B	2.76	2.86
h	15.2	37.7
v	0.181	0.076
d_c , μm	0.357	0.357

Table 2. Performance characteristics of a separation of NBD-F labeled amino acids on an extended-nano space.

	Serine	Alanine	Proline	Valine	Leucine	Phenylalanine
N, plates	640	390	580	140	380	820
N/m, plates/m	320.000	190.000	290.000	70.000	190.000	400.000
H, plate height, μm	3.2	5.2	3.5	14	5.3	2.5