Focusing Review

Development of Micro-Flow-Controlled Techniques and Novel Stationary Phases in Capillary Liquid Chromatography

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Abstract

This focusing review summarizes our recent works on the dissemination of capillary liquid chromatography through various approaches, i.e. simple stepwise-gradient elution system, on-line sample enrichment system, alternate-pumping recycle chromatography system, on-line immobilized-enzyme reactor, development of novel stationary phases, etc. Capillary liquid chromatography saves energy and waste, and it possesses several advantages over the conventional liquid chromatography; however, it has not been much popularized since its appearance and most users still prefer to use conventional liquid chromatography if there is a choice between them. Our research focuses on, but not limited to, developing versatile capillary liquid chromatography systems that are not only environmental-friendly but also user-friendly, and novel stationary phases that could be operated under mixed modes conditions by manipulating the eluent, which are applicable to real samples analyses and will therefore eventually switch the users from “conventional” to “capillary”.

Keywords: Stepwise-gradient elution; On-line sample enrichment; Alternate-pumping recycle chromatography; Immobilized-enzyme reactor; Monolithic stationary phases

1. Introduction

Capillary columns were originally invented for gas chromatography (GC), but since approx. 85% of known compounds are neither volatile nor stable to be separated by GC, a novel technique for the separation of nonvolatile substances which encompasses the speed, efficiency, sensitivity, and versatility of GC is very much desired and indispensable. This vision was first realized in 1967 by Horvath et al. when they managed to use capillary tubes as well as small bore columns packed with glass beads in their Fast-LC (liquid chromatography) system in order to separate nucleotides [1]. Since then, several types of columns have been developed and the era of miniaturization of the LC systems has begun.

In the early days of modern LC, or by the name of High-Performance Liquid Chromatography (HPLC), which was developed in the 1970’s, the separation efficiency improved significantly with the development of column packing materials and on-line detectors [2-3]. The development of HPLC was then spurred by the discovery of DNA, and by the 1980’s, HPLC was commonly used for the routine analysis of various chemicals [4].

Table 1 shows the development history of capillary-based separation methods and their related techniques [5-14]. Ishii was the first person to use the word “micro” with LC [5], i.e. microcolumn LC (μLC). The earlier stage of development of μLC was focused on achieving higher resolution. For the case of densely packed columns, higher resolution could be achieved by using smaller sizes of the packing particles, but the pressure drop across the columns was a restricting parameter in most of the cases. Thus, open-tubular columns, i.e. the analogue of the GC capillary columns, were developed and they performed much superior permeability. Most of the development of open-tubular columns had been carried out by Ishii’s group [6, 15-16].

The application of capillary columns had a rapid growth after 1980, and this expansion was attributed to the
invention of fused-silica capillary in 1979 [8]. Apart of the densely-packed and open-tubular columns, the invention of fused-silica capillaries also encouraged the development of the electrically-based separation methods such as capillary zone electrophoresis (CZE) [9], electrokinetic chromatography (EKC) [10], and capillary electrochromatography (CEC) [11-12]. These electrically-driven systems have the potential to produce higher resolution (in term of theoretical plates) than the pressure-driven systems, and thus were rapidly accepted and are now widely used. In order to combat with the electrically-based systems, Tanaka’s group has developed a new type of stationary phase, i.e., the monolithic silica capillary columns, which have intermediate permeability between densely-packed and open-tubular columns and could produce much higher resolution at relatively low pressure in the LC mode [13].

As shown in Table 2, generally, LC can be classified into preparative LC, conventional LC, semi-micro-LC, micro-LC and nano-LC [17-18]. Until 2004, capillary columns in LC had been called microcolumns, microbore columns, small-bore columns and etc., where there was no specific definition made on their definition. In 2004, Takeuchi defined capillary LC as those involving nano-LC and micro-LC [14,18].

Table 1. Development of capillary-based separation methods and their related techniques.

<table>
<thead>
<tr>
<th>Year</th>
<th>Separation methods and their related technique</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1978</td>
<td>Packed microcapillary LC</td>
<td>[7]</td>
</tr>
<tr>
<td>1979</td>
<td>Fused-silica capillary</td>
<td>[8]</td>
</tr>
<tr>
<td>1981</td>
<td>Capillary zone electrophoresis</td>
<td>[9]</td>
</tr>
<tr>
<td>1985</td>
<td>Electrokinetic chromatography</td>
<td>[10]</td>
</tr>
<tr>
<td>1987</td>
<td>Capillary electrophromatography</td>
<td>[11-12]</td>
</tr>
<tr>
<td>1998</td>
<td>Monolithic silica capillary column</td>
<td>[13]</td>
</tr>
<tr>
<td>2004</td>
<td>Capillary LC</td>
<td>[14]</td>
</tr>
</tbody>
</table>

Table 2. Classification of LC.

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Classification</th>
<th>I.D. / mm</th>
<th>Flow rate / mL min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytical</td>
<td>Nano-LC</td>
<td>&lt;0.075</td>
<td>&lt;0.00027</td>
</tr>
<tr>
<td></td>
<td>Micro-LC</td>
<td>0.2-0.8</td>
<td>0.002-0.030</td>
</tr>
<tr>
<td></td>
<td>Semi-micro-LC</td>
<td>1.0-2.1</td>
<td>0.047-0.21</td>
</tr>
<tr>
<td></td>
<td>Conventional LC</td>
<td>4.0-6.00</td>
<td>0.76-1.7</td>
</tr>
<tr>
<td>Preparative</td>
<td>Preparative LC</td>
<td>&gt;10</td>
<td>&gt;4.7</td>
</tr>
</tbody>
</table>

Saito et al., on the other hand, published a tremendously comprehensive review specifically on capillary columns in liquid chromatography (excluding electrodriven LC, i.e. CEC) in 2004 [19]. Besides elucidating the features as well as the advantages of small-bore columns, Saito also mentioned that this “intermediate technique” between conventional LC and microchip separations has its great benefit when combining with concentration sensitive detectors such as mass spectrometry (MS), and also its simple coupling to sample preparation as well as analytical columns makes it a facile technique as a comprehensive 2D LC system.

As mentioned before, capillary LC saves energy and waste, and it possesses several advantages over the conventional LC; nevertheless most users still prefer to use conventional LC if there is a choice between them. The main reason for this slow popularization is due to the fact that the column efficiency of capillary at this moment is not dramatically higher than that of the conventional LC. One of the main disadvantages is its poor concentration sensitivity caused by restricted sample injection volume and flow cell geometry. Besides, the column temperature is not stable due to its low heat capacity. In addition, even the smallest dead volume could be dreadful and cause band-broadening, that results in deterioration of its resolution. On top of these, commercially available micro pumping systems that allow gradient elution at low flow rates are limited and very highly priced, and it is still a challenging task to achieve reproducible micro/nanoflow-compatible gradient elution.

The main objective of the present research is to develop versatile capillary LC systems that solve all the above-mentioned drawbacks. Recently, micro unions and valves with low dead volume are commercially available in the market. By taking the advantages of the capillary system, these accessories can be easily applied into the capillary LC systems without affecting the column separation efficiency, and allowing us to conduct simple stepwise gradient elution [20], precolumn enrichment [21-25] and recycle separation [26], etc., with relatively good efficiency and tolerable band broadening.

2. Stepwise gradient elution

The ultimate goal of a separation is to achieve an adequate separation of all analyte compounds within an acceptable analysis time. In most cases, especially when the number of analyte components (n) is small, this objective is often achieved easily by changing and optimizing the separation conditions. However, as the number n increases, especially for complex samples containing compounds with great differences in the affinities to the stationary phase, the separation with desired resolution becomes increasingly difficult to separate all compounds in a single chromatographic run at a constant mobile phase composition (isocratic elution) or at a constant temperature (isothermal elution). This difficulty can be overcome, to some extent, by carrying out programmed elution...
techniques in order to improve the resolution for early eluted compounds while reduces the elution times as well as minimizes band broadening of the late eluted compounds [27].

Many parameters can be considered in a programmed elution, among these are solvent gradient (the most frequent in LC), thermal gradient (more efficient in GC), and dynamic gradients in CZE and CEC that include the factors such as pH, concentration of salt, and aqueous-organic medium [27-28]. In solvent gradient in LC, the elution strength of the solvent increases and the retention of samples solutes decreases as the concentration of a stronger solvent is increased during a single chromatographic run according to a pre-set program.

Comparing to the isocratic mode, the main advantages of gradient elution are, (1) reduction of total analysis time, (2) improvement in overall resolution of a mixture, (3) possibility in obtaining better peak shapes, (4) increase in sensitivity, etc. However, not all LC methods can make use of gradient elution; gradient elution is impossible for size exclusion LC and is difficult for liquid-liquid LC. The most commonly used gradient technique is the linear solvent gradient in reversed-phase LC [27] in which solvents are often pumped together with turbulent mixing; each solvent is controlled by a programmer and the total flow rate is kept constant with the concentration of solvent increased or decreased linearly.

Besides the common linear gradient elution, stepwise gradient elution can also be employed for some purposes. Stepwise gradient elution is a sequence of more than two isocratic steps with successively higher concentration solvents. The sequence of the steps can be optimized by calculating the isocratic composition of the solvent necessary in each step, taking into account the corresponding part of the column migrated by the compounds in the preceding steps, i.e., the step changes must be programmed at the part of chromatograms where no peaks occur in order to avoid distortion of chromatographic peaks.

In the early stage of gradient elution in capillary LC, low μL min⁻¹ flow rate was achieved by splitting the eluent supplied from conventional HPLC pumping systems [29-30]. However, these systems face several drawbacks due to the fact that the split ratio is difficult to control, and most of the sample and eluent are not directly toward the column, causing a great waste of sample as well as the solvent.

Takeuchi and co-workers have proposed a simple stepwise gradient elution system using two switching valves in microcolumn LC, where solutions with different compositions filled in loops were supplied by using a single pump [31]. However, this method has a disadvantage that the flow rate decreased when the switching valves were turned on, causing poor repeatability. Although operating in the constant-pressure mode can solve this drawback, this in turn leads to another disadvantage that the flow rate is likely to vary depending on the column permeability.

For that reason, we assembled a system that allows postcolumn-controlled stepwise-gradient elution using a 6-way micro selection valve in capillary LC [20]. The developed system consisted of a gas cylinder, a 6-way selection valve, an injection valve (or a sample enrichment unit), a separation column, a UV detector and a syringe pump, as shown in Fig. 1. Eluents with different compositions were supplied by pressure applied from the argon gas cylinder and they were changed accordingly by switching the 6-way selection valve. The syringe located at the downstream of the detector regulated the flow rate of the system. The system gave very good repeatability results as the relative standard deviation for the retention time was between 0.4 and 0.6%. The system was then applied to the separation of phthalates and alkylbenzenes.

Fig. 1. Diagram of the gas-pressure-driven postcolumn-controlled flow system.
decreasing the size of the separation column also decreases
injection system for micro HPLC has been developed and
separation column in a LC system. On-line precolumn
method where a short column is used and placed before the
mode, it is more common and widely used in LC.

is more convenient, time- and cost-saving than the off-line
injector in a LC apparatus. Since on-line sample enrichment
the enrichment unit works as the alternative of the sample
using a short column or cartridge, or on-line mode where
which the enrichment of the analytes is done manually
C1 to C18 together with benzene were separated
within 20 min.

3. On-line sample enrichment system

By using capillary columns in LC, advantages such as
increased mass sensitivity due to a decrease in column’s
cross-sectional area, low consumption of solvent, reagent
and packing material, use of exotic mobile phase and
mobile phase additives, etc., can be expected. However,
decreasing the size of the separation column also decreases
its concentration sensitivity due to the limit in the sample
injection. In order to overcome this drawback, sample
enrichment technique has been proven to be useful and is
becoming crucial especially in trace analysis of
environmental pollutants [21-25].

Basically enrichment could be done in the off-line, in
which the enrichment of the analytes is done manually
using a short column or cartridge, or on-line mode where
the enrichment unit works as the alternative of the sample
injector in a LC apparatus. Since on-line sample enrichment
is more convenient, time- and cost-saving than the off-line
mode, it is more common and widely used in LC.

Precolumn enrichment is a kind of sample enrichment
method where a short column is used and placed before the
separation column in a LC system. On-line precolumn
injection system for micro HPLC has been developed and
applied to the rapid analysis of dibutyl phthalate in water
[32].

The dimensions (I.D. and length) and type of precolumn
can be varied in order to obtain maximum enrichment
 efficiency. Takeuchi and Ishii developed a pre-column
concentration method in ultra-micro HPLC and examined
its application to the trace analysis [33]. It was proven that
the precolumns, which were about 1/10 to 3/10 of the
length of the separation column, had shown good
enrichment property in the separations of phthalates in
water and corticosteroids in serum.

Similarly, on-column concentration is also performed so
as to increase the sensitivity of the detection, but in this
case, the whole volume of sample is injected and is moved
by the mobile phase into the separation column, where the
analytes are being concentrated and separated. However,
the on-column concentration is normally performed when
using gradient elution or a trap column, or when diluting
the sample with weak solvent. In these cases, it is common
to inject large sample volumes in order to enhance detection
sensitivity. On the other hand, in the case of simple
isocratic mode, large volumes of sample would produce a
wide initial sample zone, and thus create a large pre-column
dispersion that severely degrades the resolution [34].

In our research, two types of sample enrichment methods,
i.e., on-column and on-line precolumn enrichments were
used for enhancing the analytes’ signal in capillary LC. The
on-column method gave a 50-fold improvement in sample
injection while the precolumn allowed injection of up to
5000-fold, which was the highest factor of enrichment that
has been reported [23]. Besides C18, C30, IC-Anion-SW
particles, monolithic C18 stationary as well as quartz fiber
coated with C30 were also used as the precolumn
enrichment materials. These systems were successfully
applied to the determination of inorganic anions in river
water sample, phthalates contained in tap water sample, and
polycyclic aromatic hydrocarbons in soil samples [25]. By
using a boronate precolumn, bisphenol A contained in PET
bottled drinks was also selectively determined.

Fig. 3 shows a particle-packed precolumn, which was
fabricated by packing the enrichment media into a Teflon
tube (0.25 × 10 mm I.D.; GL Sciences). As for the
monolithic C18 precolumn, since no frit is needed, it was
connected directly to the switching valve (i.e. M-435, as
shown in Fig. 4) via fused-silica tubings.

The designed particle-packed precolumn.
Fig. 4. A particle-packed precolumn connected to the M-435 micro injection valve.

The sample was loaded via a hand-made rubber band-driven pumping device during all concentrations. The connection capillary tubes attached to the M-435 micro injection valve were prepared from fused-silica tubing with 50 or 100 μm I.D. × 375 μm O.D. (GL Sciences). Careful precaution should be taken when connecting the fused-silica capillaries to the switching valve in order to minimize dead volume within the valve.

A separation of an authentic mixture of diethyl phthalate (DEP), benzyl n-butyl phthalate (BBP) and di-n-butyl phthalate (DBP) is shown in Fig. 5.

Fig. 5A was obtained when a 0.2 μL volume of 20 μg mL⁻¹ each of DEP, BBP and DBP were injected into the column, while Fig. 5B was obtained when 0.4 mL of 20 ng mL⁻¹ each of the same mixture was concentrated on-line into a monolithic precolumn before it was injected. It is interesting to note that though the injection volume was increased by 2000-fold in Fig. 5B, there was not any band broadening noticed; in fact the peak shape was improved as can be seen.

In addition, comparing to Fig. 5A, the theoretical plate number of the same separation column was improved by 4-fold in Fig. 5B. Even though the sample concentration was reduced by a factor of 1000 and the injection volume was increased by a factor of 2000, the peak heights of DEP, BBP and DBP were increased by 7.6, 5.5 and 7.5 times, respectively. It should be noted that these values are larger than two. The detection limits at a signal-to-noise ratio (S/N) equal to 3 were improved from 0.17, 0.26 and 0.36 μg mL⁻¹ (Fig. 5A) to 0.06, 0.13 and 0.13 ng mL⁻¹ (Fig. 5B) for DEP, BBP and DBP, respectively. Lower detection limits could be obtained by simply increasing the enrichment volume.

4. Alternate-pumping recycle chromatography system

In general, two alternate methods are available when a difficult separation cannot be accomplished by changing the column and mobile phase conditions. The most common approach is to increase the column length with identical packing material, but this might lead to a limitation to the separation efficiency due to an increased inlet pressure. The second approach is to recycle the sample through the column, in which no increase in the column inlet pressure is required. However, the dead volume in the recycle lines causes band broadening and peaks can only be recycled until they begin to overlap. Thus, the recycling technique has been limited to preparative chromatography where larger diameter columns are employed. In preparative chromatography, the recycling technique is well known as a useful tool to improve enantioseparation, production rate and recovery yield.

Recycle chromatography (RC) is an approach where the sample is being recycled through the column(s), thus producing the same separation effect as when a relatively long column is being used. The advantages of RC are that it allows the separation of compounds with very near retention time, no increase in inlet pressure, and it also solves the problem of having difficulties in making long columns. On the other hand, once the analytes are being separated, it would be mixed again during recycling process, and thus peaks can only be recycled until they begin to overlap.

Basically, there are two operating modes in RC, i.e., direct-pumping (DP) and alternate-pumping (AP). In
DP-RC, a pump, a sample injector, a column and a detector are connected in series in a closed loop. The effluent from the detector is fed back to the pump, and the unresolved sample is recycled within the loop until sufficient resolution is attained. Whereas in AP-RC, the pump is removed from the recycle loop, and the sample is recycled exclusively through the column(s).

The development of an alternate-pumping recycle chromatography system for capillary LC with an aid of a commercially available low dead volume 6-way switching valve was investigated in our research. Our system was assembled by using two monolithic silica capillary columns and the dead volume of the recycling lines was kept to a minimum by avoiding passing the sample though the pump chamber and sample injector, as shown in Fig. 6.

![Fig. 6. Schematic diagram of the AP recycle system for monolithic capillary columns. Reproduced from ref. [26] with permission.](image)

Fig. 7 shows the separation of benzene-d₆ and benzene via the recycle system using two monolithic capillary columns. It was observed that the theoretical plate number (N) of the whole system increased linearly with increasing number of cycle, and the N per unit time increased with increasing inlet pressure. When supplying the eluent under 1.0 MPa, a baseline-separation of benzene and deuterated benzene was achieved after 5 cycles, and the N were 160,000 and 150,000 for benzene and deuterated benzene, respectively. The system realizes high resolution (with high N) and high speed separation (at low pressure) in capillary LC.

For difficult isotopic separation that requires long analysis time, the recycle system utilizing capillary columns reduces the consumption of mobile phase. The RC also provides a relatively simple and inexpensive mean of attaining higher separation efficiency. In other words, any difficult separation could possibly be achieved by recycling the sample through the columns until sufficient resolution is attained and this actually reduces the turnaround time as optimization of the separation method on a conventional chromatographic system could be very time consuming.

5. Immobilized-enzyme reactor for on-line protein digestion

Since protein comprises the most abundant class of all biological molecules and is found in all living cells, proteomics has become of vital need in the ever growing world of discovery. Proteomics refers to the large scale study of proteins, particularly their structures and functions. Although there is a huge number of commercially available sophisticated high-throughput screening methods to help scientists to analyze protein in an accurate and efficient manner, the sample preparation step, i.e. the digestion of protein, is still very time consuming and it varies from 12 h [35] to 20 h [36] and sometimes up to 24 h [37]. This drawback is mainly due to the fact that the sample is still needed to be prepared off-line via enzymatic digestion in solution (conventional batch-wise method), which in most cases, is unavoidable.

Therefore, in order to reduce the time taken during the sample preparation step, immobilized-enzyme reactors were developed and have been investigated since then. The immobilized-enzyme reactors are used to digest proteins through a heterogeneous liquid-solid interface, and this concept of enzyme immobilization on a solid support for protein digestion has gained much attention because it offers advantages such as good reproducibility, attributed to the reduced enzyme denaturation rate, and reusability, attributed to the reduced or almost avoidable enzyme autoproteolysis during or after the digestion [38-51].

Immobilization of enzyme normally takes place in a 2-step or 3-step process, in which silanised reactors are then
treated with glutaraldehyde in a buffer solution. The buffer solution is important because it reduces self-digestion or autoproteolysis of the enzyme. The first solid support used for enzyme immobilization was a fused-silica capillary [38], in which the inner surface of the 50-µm-I.D. capillary was immobilized with trypsin for on-line digestion of protein. Years later, supports based on microchips [39-42], porous beads [43-46], micro membranes [47-48], micro channels [49], silica-based monoliths [50-51] have been developed. These supports have a similarity, i.e. immobilization increases the stability of the enzyme. And in most cases, trypsin was of the popular choice.

Fig. 8 shows a schematic diagram of the apparatus we have designed for the on-line protein digestion by using an immobilized-enzyme reactor (which was a micro column), and then the peptide separation by reversed-phase HPLC [52]. The enzyme reactor was prepared by using aminopropyl controlled-pore glass (200/400 mesh, 505 Å average pore size; CPG, NJ, USA) that was reacted first with glutaraldehyde, and then with trypsin in the presence of phosphate buffer. This partially micro LC system consists of three main stages. The first stage, i.e. the first dimension, is the separation of a mixed protein solution by size-exclusion chromatography using aqueous NaCl containing phosphate buffer (pH 6.8) as the eluent. At the second stage, on-line digestion of the isolated protein was achieved by continuously directing the effluent from the UV detector used for size-exclusion chromatography instantly to the immobilized-enzyme column. In the final stage, i.e. the second dimension, tryptic fragments from each isolated protein were collected at a second injector down-stream of the enzyme reactor and injected for peptide separation by the reversed-phase HPLC on a conventional-sized column with UV detection under gradient elution mode. Fig. 9 shows the separation and on-line digestion of BSA (bovine serum albumin) and myoglobin, as well as their individual tryptic fragments separations.

Fig. 8. Schematic diagram of the fractional micro 2D liquid chromatography system used for rapid proteomics with on-line protein digestion. Reproduced from ref. [52] with permission.

Fig. 9. Size-exclusion separation of mixed protein (A) and the separation of tryptic fragments of on-line digestion of BSA (B) and myoglobin (C). Inner chromatograms show tryptic fragments underwent in-solution digestions, respectively. Reproduced from ref. [52] with permission.
6. Novel stationary phases

Typically, there are four types of capillary columns that are currently in use, i.e., open tubular, partially packed, densely packed, and the newly developed monolithic columns. Open tubular and monolithic capillary columns are expected to achieve higher column efficiency due to their higher permeability. On the other hand, if operating pressure is not a limiting factor, densely packed columns are of the popular choices due to their robustness and promising efficiency. The most common and widely used column support material is silica-based microspheres and the typical stationary phase is octadecyl-functionalized silica (ODS; C18). Alternatively, stationary phases such as C30, C8, C4, cyano, phenyl, and etc., are also used for certain purposes.

In recent years, core-shell particles (i.e. a solid core with a porous shell, which is also known as fused-core, solid core or superficially porous particles) have been used increasingly due to their highly efficient separation ability operating at fast flow rate while having a relatively low back pressure [53-54]. Core-shell particles have smaller pore volume that reduces the band broadening from the longitudinal diffusion (i.e. B term in the van Deemter equation); shorter diffusion path length also reduces the contribution of the C term due to fast mass transfer [55-56]. Since retention or separation of analytes occur on the stationary phase, developing and/or fabricating new functional materials in order to improve the separation efficiency is still an ongoing challenging task. Capillary LC is convenient for developing new stationary phases as only a very small amount of the developed stationary phases is sufficient for investigating the retention behavior as well as column efficiency. Our attempts of developing novel stationary phases for capillary ion chromatography (IC) will be discussed in this section, with focus on non-charged or neutral polyoxyethylene stationary phases and ionic or chargeable monolithic stationary phases.

6.1. Polyoxyethylene-based stationary phases for capillary IC

Polyethylene glycol (PEG) stationary phases have been widely used in capillary GC, LC, countercurrent chromatography (CCC) as well as IC. PEG moieties provide not only hydrophobic interaction but also hydrogen bonding as well as dipole-dipole interaction. Our earliest attempt of using PEG as stationary phase in IC was due to the observation that this water soluble compound became inhomogeneous when high concentration of sulfate salt (for example, 1 M sodium sulfate) was added to the PEG aqueous solution. It was found that the phase separation is related to both cation and anion of the salt as well as to the salt concentration; compared to less-hydrating ions, i.e. chaotropic ions, strongly-hydrating ions, i.e. kosmotropic ions, tend to separate the PEG phase from the salt-rich aqueous phase. For example, sulfate and magnesium ions are kosmotropic ions while ammonium and perchlorate are chaotropic ions. Since PEG could provide hydrophobic interaction, it can be easily fixed on hydrophobic supports such as the common C18 stationary phase [57].

We found that permanently coated PEG on a hydrophobic C30 stationary phase retained anions in the partition mode and since the retention of anions increased with increasing eluent concentration for most of the eluents, this stationary phase allowed us to use high concentration eluents and direct determination of trace anions contained in seawater samples could be achieved without any interference caused by the matrices [58]. The determination sensitivity (calculated as S/N values) of the PEG-coated stationary phase was improved by 15-fold (for iodide) when a conventional-sized column (4.6 mm I.D.) was used [59]. In order to improve the durability of the stationary phase, PEG was chemically-bonded to diol-functionalized C30 via oxalyl chloride [60]. On the other hand, when poly(ethylene oxide) (PEO; PEGs with molecular weights >100,000) was chemically bonded on aminopropylsilica, the selectivity for anions improved, in which anions were retained in ion-exchange mode [61-62]. Similar retention mechanism was observed when polyoxyethylene (POE) was used, the resulting stationary phases retained not only anions but also cations; the retention behavior suggested that the eluent cations could be trapped among multiple POE chains via ion-dipole interaction, and the trapped cations worked as the anion-exchange sites [63]. Depending on the eluent employed, the POE-bonded stationary phases could also work in reserved-phase mode for nonpolar analytes as well as in hydrophilic interaction chromatography (HILIC) mode for polar organic analytes [63].

Fig. 10 shows the main structures of these non-charged stationary phases.

Fig. 10. Expected structures of the permanently coated PEG stationary phase (A), chemically-bonded diol-PEG via oxalyl chloride (B), as well as the POE-based stationary phases (C).
We proposed an imaginary stationary phase model to show that multiple POE chains trap eluent cations via ion-dipole interaction, on which anions are retained by electrostatic interaction [63]. This model was verified by using crown ethers as the eluent additive in the reversed-phase mode [64]. By using this cation-trapping ability, chemically-bonded crown ether stationary phases were developed and the retention of both anions and cations on the same stationary phase was investigated [65-66]. Besides the retention of anions in the ion-exchange mode, by using eluent with higher pH would favor the retention and separation of the metal cations. It was also found that without the obstruction of nitrogen atom within the cavity of crown ether, 2-aminomethyl-18-crown-6-ether (2AM18C6) could easily form complexes with metal cations, and the retention as well as elution order were dependant on the complex formation constant between crown ether and the metal cations [66]. The chemical structures of the crown ethers used as well as the expected structure of the resulted stationary phase are shown in Fig. 11.

![Chemical structures of the crown ethers used](image)

**Fig. 11.** Chemical structures of the crown ethers used (upper trace; 1A15C5: 1-aza-15-crown-5-ether, 1A18C6: 1-aza-18-crown-6-ether, 2AM18C6) and the resulted 1A15C5-bonded stationary phase (lower trace). Adapted from ref. [66] with permission.

6.2. Monolithic stationary phases for capillary IC

Unlike the particulate-packed columns, as mentioned earlier in the previous section, monolithic capillary columns are expected to achieve higher column efficiency due to their higher permeability. The high permeability of the monolithic column is mainly attributed to its double-pore structure, i.e. μm-sized through-pores and nm-sized meso-pores on the surface of the skeletons. In recent years, monolithic columns have been studied intensively due to their higher permeability with ultra low flow resistance, which make it suitable for high speed separation required in modern analysis nowadays [67-68]. Generally, monolithic columns can be divided into silica- and organic polymer-based materials; the silica monolith has some advantages over the polymer monolith, such as good mechanical strength, well-controlled pore structure and high column efficiency especially for small molecules. On the other hand, polymer monolith is easy to prepare (column reproducibility is more promising than the silica ones) and it is robust over a wide range of pH, which make it ideal for the use in ion chromatography.

At the present time, there are a limited number of commercially available monolithic columns for IC, and most of the applications of monolithic columns in IC at the conventional column size scale are mostly dynamically or chemically modification of commercially available silica-based monolithic columns [69]. In addition, the routine use of PEEK housings for IC columns causes the difficulty in fixing the monolith to the wall of the conventional-sized columns. Therefore, the development of monolithic IC columns has been focused on the use of capillary columns/systems, in which fixing the monolith inside the column is less troublesome and less possibility for shrinkage of the monolith to occur. It was also reported that dynamically modification of bare silica monolithic columns with latex nanoparticles achieved 50% higher efficiency than the similar type monolithic ODS column coated with cationic surfactant didodecylmethyl ammonium bromide (DDAB) [70].

In our earlier attempt, we dynamically modified monolithic silica capillary columns with quaternary ammonium ions such as cetyltrimethylammonium chloride (CTAC); the first layer of CTAC was introduced by electrostatic interaction while the second layer was introduced by hydrophobic interaction, as shown in Fig. 12. The second layer worked as the anion-exchange sites; this capillary column was applied to the rapid separation of five inorganic anions and it also allowed the determination of bromide in seawater (Fig. 13) without tedious pretreatments [71].
As shown in Fig. 13, an addition of 0.1 mM CTAC into the eluent was necessary to maintain a stable retention time. Even though this monolithic silica column achieved rapid separation at lower inlet pressure, the retention time of anions gradually decreased when no CTAC was added into the eluent. This drawback was improved by modification with a more hydrophobic modifier such as DDAB [72]. It should be noted that seawater samples were directly injected onto the modified monolithic silica column without any interference of matrix ions because high concentration sodium chloride (i.e. 500 mM) could be used as the eluent.

On the other hand, polymer-based monoliths, which are the preferred stationary phase for IC due to the routine use of eluents with high pH, are often prepared by polymerization of functional monomers with a cross-linking agent and some porogens in a capillary tube. The methacrylate-based polymers are one of the most widely researched monoliths for use as a separation media due to the fact that they are highly stable even under extreme pH conditions and the porous structure is easier to optimize. In addition, when glycidyl methacrylate (GMA) is used as the monomer, the epoxy group in GMA could be easily further modified with other functional groups such as primary, secondary or tertiary amines, etc., for the separation of various compounds. We have successfully fabricated methacrylate-based monolithic columns modified with trimethylamine [73], diethylamine [74], triethylamine [75] as well as PEG [76] for the separation of inorganic anions. Compared to silica-based monoliths, these polymeric monoliths generally exhibit poorer column efficiency especially for the separation of small molecules and ions; however, the chromatographic results showed that trimethylamine- or PEG-modified monoliths could be a good alternative for the rapid determination of anions without significant swelling or shrinkage occurred in the capillary.

Besides the dynamic coating of the silica-based monoliths as well as polymerization of methacrylate-based monoliths with postmodification of the functional groups, we also fabricated a hybrid organic-silica monolithic capillary column with sulfonate functionality for the rapid and simultaneous separation of cations via a simple and easy “one-pot” reaction [77]. Allylsulfonate that worked as an organic monomer in the single-step reaction provided strong cation-exchange moieties directly, and this stationary phase was successfully applied for the rapid and simultaneous separation and determination of inorganic cations in groundwater and the effluent of onsite domestic wastewater treatment system.

7. Conclusions

This focusing review summarizes our recent endeavors in developing versatile capillary LC systems that allow stepwise-gradient elution at low μL min⁻¹ flow rate, large-volume injections, improvement of separation efficiency by using the recycling technique, on-line protein digestion using a novel immobilized-enzyme micro column, and selected novel stationary phases developed for capillary IC. It has taken more than four decades to make capillary LC a well-known and entrusted separation, determination as well as identification technique in a wide range of analyses, and the development of capillary LC will continue to grow into a mature method and will shift users from conventional to capillary LC, as can be seen in the development of capillary GC.

The development of ancillary techniques and novel stationary phases described hereby has significantly contributed to solving problems related to the low popularization of capillary LC. Besides the innovations of new technologies for the development of the hardware aspect of capillary LC, on-line coupling to a spectroscopic detection method that provide structural information of the analytes are getting equally crucial. Highly priced MS, however, still remain as the only and ultimate concentration sensitive universal detector, which is commercially available, for capillary LC.

Acknowledgments

The author would like to express her earnest heart-felt gratitude to Professor Toyohide Takeuchi for his sincere and continuous guidance throughout the author’s research life. Sincere appreciation is also dedicated to all collaborators and coworkers as well as members of the author’s laboratory.

The author is especially grateful for the financial supports from the Japan Society for the Promotion of Science (JSPS; Grant-in-Aid for Young Scientists), Japan
Science Association (Sakawa Scientific Research Grant), Koshiyama Science Foundation, Sajiro Endo Science and Technology Foundation, as well as the Support Fund for Leading Researcher and Research Activation Grant offered by the Faculty of Engineering and Gifu University, respectively.

Last but certainly not least, the author’s sincere gratitude and appreciation to the award committee and all members of the Japan Society for Chromatographic Sciences.

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