



Liquid chromatography using $\leq 5 \mu\text{m}$ open tubular columns

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ABSTRACT

Significant progresses have been made recently in liquid chromatography (LC) using $\leq 5\text{-}\mu\text{m}$ -i.d. open tubular (OT) columns. Peak capacities of 2000+ within 3 h, efficiencies of 10+ million plates/m and millisecond separations have all been obtained. Since all these magnificent progresses are achieved in the last 3–4 years and no one has reviewed these achievements, it is important to write a short review on this topic so that scientists can adopt it in their research and applications. In this article we first summarize the most recent progresses of LC separations using $\leq 5\text{-}\mu\text{m}$ -i.d. OT columns. We then review the procedures of how to prepare these columns and share our experiences on how to choose appropriate experimental components to construct apparatus and select appropriate experimental parameters to carry out these separations. We also discuss the pros and cons and provide a future perspective of this technique at the end of this article.

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1. Introduction

Chromatographic theory was developed in parallel with the advancement of gas chromatography (GC) [1–4]. Because early GC separations were carried out on packed columns, theoretical models had to consider the tortuous paths between packed particles. To simplify the mathematics of the flow of gas in a packed column with many tortuous paths, Golay used a model consisting of a single capillary tube and revealed theoretically that such a capillary tube coated with a layer of stationary phase could lead to high efficiency separations [5,6]. This finding led to the invention of open tubular (OT) GC, and packed columns were rapidly replaced by OT columns. It is estimated that more than 80% of all GC applications are presently run on OT columns, and the optimum inner diameters (i.d.) of these columns are around a few hundred μm [7]. Since the theory did not make any distinction between gases and liquids as the mobile phase, it was anticipated that OT columns would provide the best means to achieve high-efficiency liquid chromatography (LC) separations. Because the diffusivities in liquids is about 3 orders of magnitude lower than that in gases, the optimum inner diameter (i.d.) of the OT columns for LC was expected to be around a few μm [2].

However, performing open tubular liquid chromatography (OTLC) using columns in a few μm was challenging primarily because it was difficult to prepare such OT columns with sufficient sample loadability and retention power. To obtain an OT column with an enhanced sample loadability, Jorgenson et al. [8] etched a borosilicate-glass capillary having an i.d. of as small as 5 μm with hydrochloric acid and created a porous silica layer on the interior wall of the capillary by selectively removing the nonsilica components of the glass. This etching process increased the internal surface area by a factor of roughly 30. A layer of siloxane polymer stationary phase was then coated on the roughened surface to enhance the sample loadability. However, limited success was achieved toward ultrahigh efficiencies. Pesek and Matyska [9] described an alternative method to etch the surface of a 400 μm i.d. fused-silica capillary using ammonium hydrogen difluoride; the surface area was increased by a factor of around 1000. When a silane stationary phase was bonded to this surface, the sample loadability was increased. A more effective method to increase the loadability was to create a porous polymer layer on the interior wall of a capillary [2,3,10,11], and these columns were called porous layer open tubular (PLOT) columns. For example, a layer of poly(styrene-divinylbenzene) was prepared by Yue et al. [12] on the wall of a 4.2 m \times 10 μm i.d. capillary and ~ 100 fmol of angiotensin I and ~ 50 fmol of insulin could be loaded. A peak capacity of ~ 400 was obtained for the separation of a complex tryptic digest mixture. Unfortunately, it is challenging to prepare 5 μm i.d. or smaller PLOT columns reliably and reproducibly, although a 900-nm-i.d. PLOT column has been produced [13].

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The loadability issue has been mitigated with the work accomplished recently [13–17]. Using 2- μm -i.d. OT columns coated with octadecyltrimethoxysilane (OTMS), several million theoretical plates per meter were obtained in <10 min for separating amino acids and peptides [14], peak capacities of >2700 were achieved for separating pepsin/trypsin digested *E. coli* lysate [18], and milli-second separations were accomplished for separating amino acid mixtures [19]. Since most of these magnificent results were obtained using $\leq 5\text{-}\mu\text{m}$ -i.d. OT columns in the past few years, and no one has reviewed the achievements in this specific area so far, it is important to write a short review on this topic from a hands-on expert's point of view to alert and instruct the frontline analytical chemists about these progresses so that they can quickly adopt this technique in their research. In addition to reviewing the most recent progresses, we will also provide the basic knowledge and instructions of how to prepare $\leq 5\text{-}\mu\text{m}$ -i.d. OT columns and how to choose the appropriate experimental components to construct apparatus to carry out these separations. A future perspective, including the pros and cons of this technique, is also presented at the conclusion of this paper.

2. Most significant progresses of OTLC using $\leq 5\text{-}\mu\text{m}$ -i.d. columns

Ultrahigh efficiency OTLC separations were first experimentally demonstrated by Chen et al. [14] and Yang et al. [15] in 2018. The OT columns used in this work had a length of 48 cm (44 cm effective) and an i.d. of 2 μm and was coated with octadecyltrimethoxysilane (OTMS). The authors obtained efficiencies of several million theoretical plates per meter in <10 min [14] for separating amino acids and peptides and a peak capacity of 1640 within 172 min [15] for separating a peptide mixture from pepsin/trypsin-digested *E. coli* cell lysate. The efficiencies dropped by orders of magnitude if the column i.d. was increased to 5 μm [14]. An additional important benefit of this approach was that the separations were carried out under an elution pressure of around 50 bar [15] – a very moderate pressure compared with that used in high performance liquid chromatography (HPLC) or ultrahigh performance liquid chromatography (UPLC).

Low loadability was recognized as a major issue for OTLC, but this issue was greatly alleviated as the column i.d. decreased to low micrometers. In a recent paper Yang and Liu [16] demonstrated that a thin dense non-porous coating was well-suited for achieving ultrahigh efficiency or ultrahigh peak capacity results using 2- μm -i.d. OT columns. Based on the experimental results, these authors suggested that a key to achieve ultrahigh efficiency results was to match between stationary phase loadability with the mobile phase loadability. For OTMS coating in a 2- μm -i.d. OT column, a thickness of 10–20 nm seemed to have achieved such a match; peak capacities of >2000 in <3 h were commonly obtained using a 155-cm-long column prepared following the optimized protocol [16]. Fig. 1 presents a typical chromatogram of such separations.

The efficiency, peak capacity, and separation speed could be further improved through elevating the operation (or column) temperature; all these numbers could be improved without mutual compromises. In a recent report [18], Yang et al. showed that, by raising the operation temperature from 30°C to 70°C, the efficiencies improved by 34%–260% and the separation speeds could be improved by 7%–10%. Using a 155-cm-long column and a column temperature of 70°C, these authors obtained a peak capacity of 2720 within 143 min for separating a peptide mixture. Importantly, heating the column is easy to implement, and many commercial LC systems already have compartments to control column temperatures. Therefore, it is highly recommended to run OTLC separations at elevated temperatures whenever possible.

A narrow OT column was also proven to be excellent for performing ultrafast separations. Xiang et al. [19] utilized a 2- μm -i.d. and 6-cm-long (2.7 cm effective) OT column and resolved 6 amino acids (His, Gly, Ala, Arg, Trp and Phe) in <700 ms. This speed could be further increased by raising the gradient flow rate or shortening the column length at the expense of resolution. The 2.7 cm effective length was, however, constrained by the detector these authors were using in order to tune the optical alignment of the system conveniently. To demonstrate the feasibility of using this approach for practical applications, Xiang et al. separated complex protein digests; peptides were nicely resolved in ~ 1 min. In a separate paper, Xiang et al. [20] incorporated a 2- μm -i.d. OT column with flow injection chromatography (FIC) and separated an amino acid mixture at a throughput of 1800 samples per hour. For this work the column had a length of 3.5 cm (2.7 cm effective), and the sample contained 2 mM Gly, 6 mM Arg and 6 mM Phe.

3. Preparation of $\leq 5\text{-}\mu\text{m}$ -i.d. OT columns

Based on the types of stationary phases, OT columns can be roughly categorized into three groups: Bare Open Tubular (BOT), Wall Coated Open Tubular (WCOT) and Porous Layer Open Tubular (PLOT) columns.

3.1. BOT columns

A BOT column is basically an uncoated capillary. When the wall of a fused-silica capillary is in contact with an alkaline aqueous solution, the surface silanol groups will be dissociated forming a negatively charged surface. The negative surface will repulse anions and attract cations, and consequently anions will be accumulated in the center region and cations enriched near the wall of the capillary. For differently charged anions/cations, the highly charged anions/cations will be concentrated more in the center/wall region of the capillary than the lowly charged ones. As a pressure-driven (Poiseuille) flow is induced, the compounds residing in the center/wall region will move faster/slower than those in the wall/center region. Consequently, differently charged species will move forward according to the following order: the highly charged anions > the lowly charged anions > the neutral species > the lowly charged cations > the highly charged cations. Fig. 2A presents three example chromatograms using a 1.6- μm -i.d. BOT column for separating three fluorescence dyes: 2',7'-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF, -4 charged), fluorescein (-2 charged), and rhodamine B (neutral). Efficiencies of more than 100 000 plates per meter were obtained [21] in ~ 20 min, comparable to that in capillary zone electrophoresis. On the basis electrostatic interactions, a 2.3- μm -i.d. BOT column was also tested feasible for alkali metal cation separations [22]. Although no chemical coating must be synthesized, one needs to maintain a constant and stable surface charge (or zeta potential) to obtain reproducible separations. This requires an eluent with a stable pH and compositions and a stable temperature, and wall adsorption should be eliminated as well.

A BOT column can be used for high-resolution large molecule separations [23–27] via a hydrodynamic chromatography (HDC) mechanism. The concept of HDC was introduced in the mid-1960s when Small was asked to develop a method to determine the size of a plastisol resin with a diameter of $\sim 1\text{ }\mu\text{m}$ [28] and Small's first HDC paper was published in 1974 [29]. Packed-bed columns were employed for these separations. To make the separations effective, the pore sizes of a packed-bed column diameter should be larger than but comparable to (e.g., a few times with respect to) the sample particles.

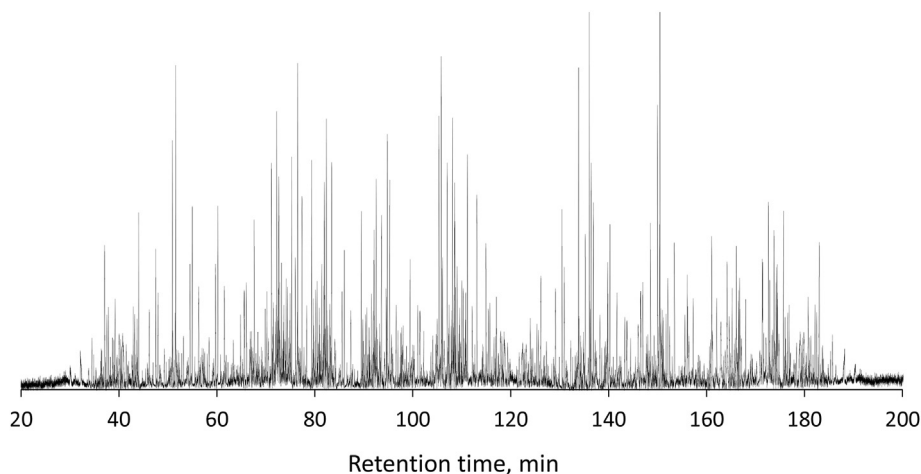


Fig. 1. Chromatogram for separating pepsin/trypsin digested *E. coli* lysate using a 160-cm-long OT column. The OT column had an i.d. of 2 μm , a length of 160 cm (155 cm effective), and a majority (~140 cm) of the column was maintained at 50°C. The column was prepared under the optimized conditions; 70% OTMS was flushed through the column at 1000 psi at 60°C for 18 h. Mobile phase A was 10 mM NH_4HCO_3 in DDI water, and mobile phase B was 80% acetonitrile in 10 mM NH_4HCO_3 . The volume of sample injected was ~120 μL . The elution pressure was 500 psi. The gradient was as set as mobile phase B increased from 5 to 100% within 3 h. A peak capacity of ~2200 within 160 min was computed using a method described in Ref. [15].

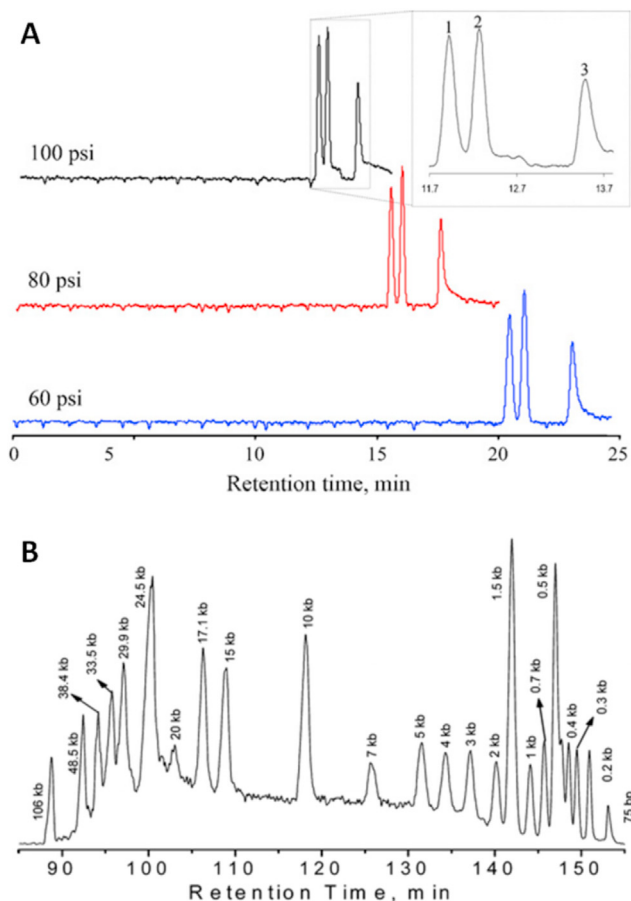


Fig. 2. Typical chromatograms using BOT columns. (A) Chromatograms yielded from a 44-cm-long (39-cm effective) and 1.6- μm -i.d. BOT column under different elution pressures. Sample injection condition: 80 psi for 10 s; eluent composition: 100 μM borax. Peak identification: 1—BCECF (−4 charged), 2—fluorescein (−2 charged) and 3—rhodamine B (neutral) (Figure was obtained from Ref. [21] with permission). (B) A typical chromatogram for DNA fragment separation using a 2.5- μm -i.d. and 445 cm long (440 cm effective) at an elution pressure of 360 psi. Eluent: 10 mM Tris-1 mM EDTA (pH = 8.0) (Figure was obtained from Ref. [17] with permission).

High resolution HDC separations were achieved for DNA fragments having a wide size range in a single run [23,30] using ≤ 5 - μm -i.d. OT columns, and a typical chromatogram is presented in Fig. 2B. A theoretical model [31] was developed to characterize these separations. Based on the separation mechanism, the surface charge on the capillary wall had limited effects on HDC separations.

3.2. WCOT columns

A WCOT column is defined as a column whose stationary phase consisted of a thin non-porous coating layer. A major concern of using a WCOT column for LC is the low loadability or loading capacity [32]. Efforts have been invested to alleviate this problem by roughing the wall surfaces [8] or attaching a porous layer of stationary phase to the wall [33,34]. In a recent study, Liu's group have demonstrated that this low loadability issue could be overcome when a narrow (e.g., 2- μm -i.d.) OT column with a thin non-porous dense coating was used [16]. This is because a reduced column i.d. increases the surface-to-volume ratio, leading to a good match between a stationary-phase loadability and mobile-phase loadability. To prepare the dense coating one may use a relatively high concentration of the coating reagent to derivatize the column wall. The following presents a protocol for synthesizing such an OTMS coating [14,16,35]. After the capillary was flushed with 1 M NaOH solution at 100°C for 2 h and rinsed with DDI water for 1 h, it was flushed with acetonitrile for about 30 min at an ambient temperature and dried with nitrogen overnight. The dried capillary was then flushed with 70% OTMS in toluene at 50°C for 16 h [Note: OTMS is extremely reactive with water, so care must be taken to avoid the coating reagent and capillary wall being exposed to moisture.] The column was ready for use after the OTMS-coated capillary was rinsed with toluene for 1 h and dried with nitrogen; the resulted coating had a thickness of 10–20 nm [16]. Columns prepared using the above method produced not only good retentions and reproducibility in the separation of amino acids, but also high peak capacities (>2000 within 3 h), at relatively low elution pressure (generally <1000 psi), as illustrated in Fig. 1.

Protocols to coat 4.6- μm -i.d. OT columns with poly (butadienesulfonic acid) or poly (butadiene-maleic acid) were

developed by Muller et al. [22] for amino acid and alkali cation analysis. A method to derivatize similar size OT columns with 3-sulfopropyltriethoxysilane or 3-(2-aminoethylamino)-propyltrimethoxysilane was also established for fast cation or anion separations. Göhlin and Larsson [36] prepared four different polyorganosiloxane coatings for 5- μm -i.d. OT columns, and Rodriguez et al. [37] attached C18 coating on the walls of a multi-lumen capillary (each with 4.2 μm i.d.). Because relatively low concentrations of coating reagents were used, very thin (single-molecule layer) coatings were generally obtained. Technically, a majority of the methods used to create coatings for larger i.d. OT columns can be applied to prepare ≤ 5 - μm -i.d. columns, but care must be taken not to clog these capillaries. Lam et al. provided a comprehensive review recently for preparing OT columns through both adsorption and chemical derivatization [38].

3.3. PLOT columns

A PLOT column is defined as an OT column with a relatively thick and porous coating. PLOT columns are so far the most popularly exploited amongst the three types of OT columns due to their high sample load abilities. In the application of PLOT columns, a milestone achievement was made by Karger's group who incorporated 10- μm -i.d. PLOT columns with mass spectrometry (MS) for proteomic analysis [12,39,40]. A porous poly(styrene-divinylbenzene) coating was synthesized for these columns as a stationary phase. Preparing a PLOT column with an i.d. of ≤ 5 - μm is challenging because it can get clogged easily, especially during the polymerization step. Crego et al. [41] converted a protocol used to produce 50- μm -i.d. PLOT columns to a method for preparing 5- μm -i.d. columns. The coating was created via a hydrolysis and polycondensation of tetraethyl orthosilicate (TEOS) in a water-ethanol solution, followed by a functional group (C18) attachment. Clogging can occur but this problem can be mitigated by reducing the pre-gelling time.

An organic polymer PLOT column can be produced using a thermally initiated radical polymerization process. The process includes three main steps: activation, modification, and polymerization. An alkali solution, such as a NaOH solution, can be used to activate wall, yielding an increased number of silanol groups on the surface. To accomplish this, for example, Yue et al. [12] used 1.0 M NaOH to flush their capillary at a room temperature overnight. Rogeberg et al. [42] filled their capillary with a 1.0 M NaOH solution, sealed both ends, and heated the assembly at 100°C for 2 h. In the modification step, the capillary wall is reacted with a silanization reagent, creating reactive sites for the polymerization. The goal of the modification is to generate anchors for the attachment of a polymer coating. Typically, the wall of the activated capillary is reacted with a silane reagent containing a moiety such as 3-(trimethoxysilyl)propyl methacrylate (γ -MAPS) for participation in the next-step polymerization reaction. In the polymerization step, monomer, cross-linker, porogen and thermal initiator are mixed and introduced into the capillary, both ends of the capillary are blocked, and polymerization is initiated by heating the capillary. Common monomers include styrene [12,39], divinylbenzene [12,39], [2-(methacryloyloxy)ethyl] dimethyl-(3-sulfopropyl) ammonium hydroxide [43], and benzyl methacrylate [44]. Either a single porogenic solvent [40,45] or two porogenic solvents [13] can be used as the porogen; potential porogenic solvents include methanol, ethanol, cyclohexanol, and 1-dodecanol. In 2017, Li et al. [13] developed a protocol for preparing 900 nm-i.d. PLOT columns. Briefly, the capillary was first activated by flushing 1 M NaOH through the capillary for 5 h and subsequently rinsed with water for 3 h. The capillary was then flushed with acetone and dried with nitrogen. The activated capillary was then filled with a mixture of

γ -MAPS and acetone (v/v = 1) and stored in the dark for 24 h, followed by washing with acetone and drying with nitrogen. A solution containing 3.98% o-9-[2-(methacryloyloxy)-ethyl-carbamoyl]-10,11-dihydroquinidine (MQD), 7.92% 2-hydroxyethyl methacrylate (HEMA), 7.98% ethylene glycol dimethacrylate (EDMA), 39.77% cyclohexanol, 40.00% 1-dodecanol and 0.35% 2,2'-azobisisobutyronitrile (AIBN) was vibrated and ultrasonically mixed and then purged with nitrogen. After this solution was introduced into the modified capillary and both ends of the capillary were blocked, the capillary was put inside an oven at 60°C for polymerization for 2 h. The OT column was ready for experiment after the capillary was rinsed with methanol.

The porous polymer coating sometimes needs to be further functionalized. For example, a poly[vinylbenzyl chloride (VBC)-divinylbenzene (DVB)] PLOT column was first prepared, and ethylenediamine was then introduced to react with VBC to yield an amine-bonded poly(VBC-DVB) HILIC-PLOT column for separations of glycans [40].

The preparation of an inorganic silica PLOT column also consists of three major steps [46]: capillary pretreatment, hydrolysis and polycondensation, and silanization. In the capillary pretreatment step, the wall of the capillary is etched by an alkaline solution. In the second step, a silicon alkoxide precursor such as tetraethoxysilane or tetramethoxysilane is hydrolyzed and condensed into a gel with an acid or base as a catalyst. After aging and drying, a xerogel (or a porous silica layer) is formed on the capillary wall. In the last step, the silica gel is functionalized with specific groups via silanization. As an example, Hara et al. [47] synthesized a mesoporous silica layer with a thickness of 300–550 nm on the wall of a 5- μm -i.d. capillary. A solution containing tetramethoxysilane (TMOS), urea, polyethylene glycol, and 0.01 M acetic acid was pushed into the capillary for the gelation at 25°C for 20 h. Mesopores in the gel were created by treating the gel with ammonia at 95°C. After the column was washed with methanol, it was dried at 120°C in an oven for 24 h. Functionalization of the mesopore surfaces was carried out by flushing the porous gel with a 20:80% (v/v) octadecyldimethyl-N,N-dimethylaminosilane (ODS-DMA)/toluene mixture solution at 60°C for 24 h. Preparations and applications of PLOT column in LC are comprehensively reviewed lately by both Li et al. [48] and Lam et al. [38].

4. Experimental configurations to execute sample injection and OTLC separation

4.1. System with flow splitter

To use a ≤ 5 - μm -i.d. OT column, a flow splitter is usually employed for sample injection and analyte elution, although a micro-HPLC setup [49] had been utilized for large-i.d. OT columns [50]. A flow splitter is utilized to reduce the flow rate of a stream by splitting it into multiple (usually two in LC applications) streams. Fig. 3A presents a picoLC-MS system described recently [51], and we use it here as an example to describe how an OTLC system for ≤ 5 - μm -i.d. OT columns is constructed. Referring to Fig. 3A, a ≤ 5 - μm -i.d., 150- μm -o.d. and 80-cm-long OT column was coated with OTMS. An Upchurch micro-cross was used to construct the flow splitter. A 10-cm-long \times 150- μm -i.d. \times 360- μm -o.d. capillary was used to connect the injection valve (a six-port valve, VICI Valco, Houston, TX) and the flow splitter. A capillary with appropriate i.d. (e.g., 5- μm -i.d.) and length (e.g., 50 mm) was used as the sampling loop of the six-port injection valve. Inside the flow splitter, the OT column head was inserted ~ 1 mm deep into this connection capillary. [Note: an appropriate portion of the polyimide coating of the OT column may be removed to facilitate the insertion, if necessary.] A restriction capillary (RC) was used to control the

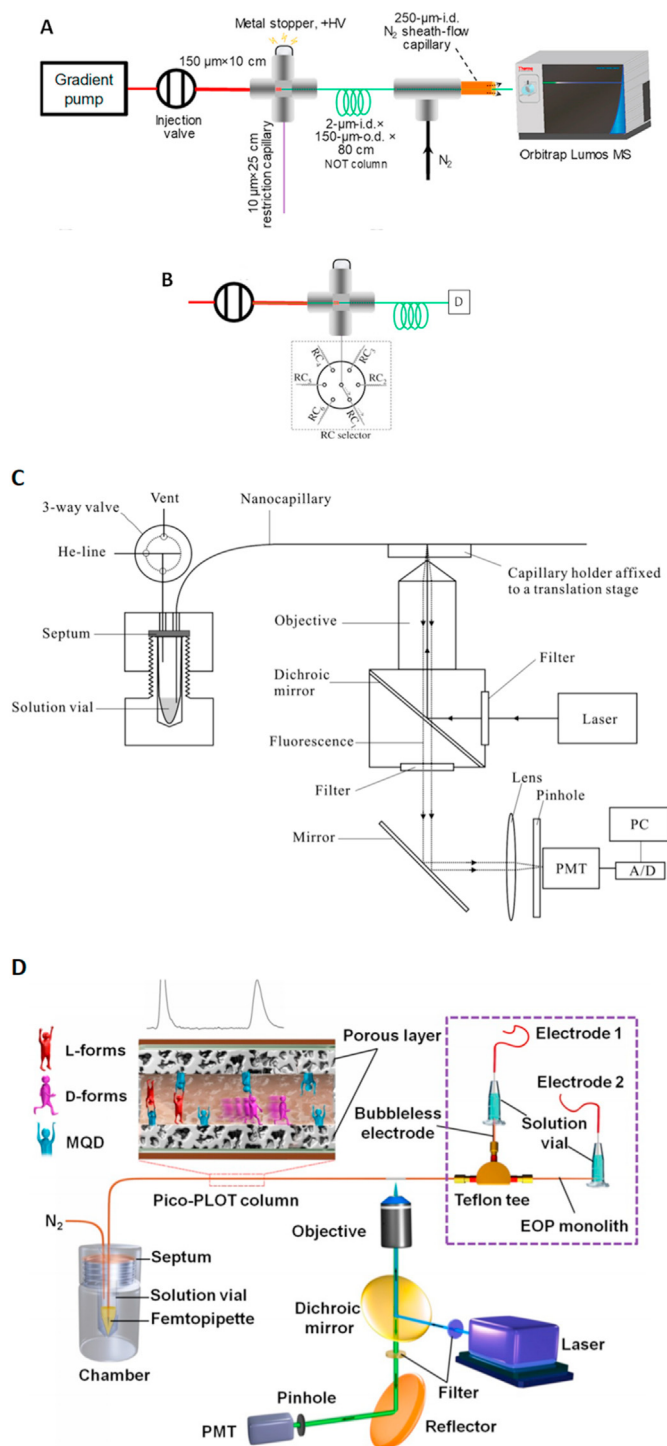


Fig. 3. Experimental configurations to execute sample injection and separation using $\leq 5\text{-}\mu\text{m-i.d.}$ OT columns. (A) A system with a flow splitter for sample injection and elution (Figure was obtained from Ref. [51] with permission). (B) A device that was used to change splitting ratio (Figure was obtained from Ref. [52] with permission). (C) A system without flow splitter (Figure was obtained from Ref. [24] with permission). (D) A pico-HPLC system using a 900-nm i.d. OT column (Figure was obtained from Ref. [13] with permission).

splitting ratio. In this particular work [51], a Dionex NCP3200RS UPLC gradient pump (ThermoFisher) operating at 700 nL/min was employed, the splitter had a splitting ratio of 1:886, and implementing the sample injection and OTLC separation were similar to that for a non-flow-splitter LC systems.

Fig. 3B presents a modification to the apparatus in Fig. 3A, in which the restriction capillary is replaced with a selection valve connected to a series of RCs. A desired RC can be selected by changing the positions of the selection valve; this allows one to conveniently change the splitting ratio. Construction and characterization of this device has been described in details previously [52].

4.2. System without flow splitter

Alternatively, sample introduction and elution can be executed using a pressure chamber [24], and this approach avoid using a flow splitter. Fig. 3C presents a schematic experimental setup to illustrate its working principle. The helium-pressurized chamber consisted of a cap and a base, machined from poly(methylmethacrylate). The chamber space had an i.d. of ~ 8 mm and a depth of ~ 30 mm, which was used to accommodate a 600 μL microcentrifuge tube to serve as a sample or an eluent vial. A septum was put between the base and the cap to ensure a seal. A high-pressure regulator was used to control the pressure inside the chamber. The line connecting the helium pressure line from the vent valve (Fig. 3C) to the chamber space contained a stainless-steel hypodermic needle tubing (25-gauge, 3.5 cm long), and this restriction protected against explosive helium release, even if the pressurized chamber failed. The sampling end of the OT column was inserted through the septum into the solution vial.

To perform sample introduction, one needed to open the pressure chamber, put the vial containing a sample solution in the chamber space, cap the chamber and seal it. Then the sampling end of the OT column was dipped into the sample vial, and helium was introduced into the chamber space to pressurize the sample into the OT column. The amount of sample injected depended on the pressure inside the pressure chamber and the injection time. After sample injection, one needed to open the pressure chamber again, replace the sample vial with an eluent vial, cap and seal the pressure chamber, and release helium to the pressure chamber to execute the separation. Because many manual operations were involved, operation automation could be difficult. Because only one solution valve was put inside the pressure chamber, this setup was limited to isocratic separations.

Fig. 3D presents a pico-HPLC system using a 900-nm i.d. OT column [13]. The system consisted of a pressure chamber, an electroosmotic pump (EOP) and a LIF detector. The OT column was connected to an EOP monolith and a bubbleless electrode through a PEEK tee. The free ends of the monolith capillary and the bubbleless electrode were immersed into two vials containing 3 M sodium acetate. For sampling, a high voltage was applied across the EOP monolith to aspirate the sample into the OT column. Subsequently, the EOP (shown inside the dotted rectangular) was removed, the sample vial in the pressure chamber was replaced with an elution vial, and nitrogen was introduced into the pressure chamber to drive the eluent through the OT column to execute the separation. The approach by sharpening the column tip and using the tip to withdraw sample can potentially provide a viable scheme for single cell sampling and the subsequent analysis [53].

Other systems have been developed, and often these systems were applied to specific applications such as microchip HPLC [54]. In this review, microchip HPLC is not covered, and related information may be obtained from recent review articles [38,55].

5. Detection

LC with $\leq 5\text{-}\mu\text{m-i.d.}$ OT columns requires sensitive detectors because the quantity of sample loaded for the separation is extremely low. Detection has hindered the exploitation of using

$\leq 5\text{-}\mu\text{m}$ -i.d. OT columns for high-resolution LC separations for many decades. The most popularly employed detection scheme in current LC, the ultraviolet and visible wavelength absorbance detection, can be virtually ruled out due to the short optical path length for on-column detection and low sample quantity for off-column flow cell detection.

5.1. LIF detection

LIF is one of the most sensitive techniques for molecular detection, and it is widely used in LC including OTLC. Current LIF systems are applicable only for large-i.d. capillaries. Because LIF detections for LC with sub-micrometer to low micrometer i.d. OT columns require much stricter alignment and focusing, such LIF detector are not commercially available yet. To address this issue, Weaver et al. [56] constructed a confocal LIF detector, and the detector has been successfully utilized for various ultra-narrow OTLC separations [13,15,17]. The optical paths are schematically presented in Fig. 3C. Briefly, the excitation light, a 488 nm laser beam from a solid-state laser module (Melles Griot, Rochester, NY), passed through a 488-nm laser line filter (Melles Griot), reflected by a dichroic mirror (491 nm cut-on wavelength, Semrock, Rochester, NY) and focused on to the optical window of OT column by an objective lens (0.32 \times NA, 16 \times magnification, Melles Griot). Fluorescence from the inside of the column was collected and collimated by the same objective lens, passed through the same dichroic mirror, reflected by a mirror (Melles Griot), filtered by a 510 nm long-pass filter (Semrock, Rochester, NY), and focused by a lens (Melles Griot) through an 800- μm pinhole onto a photo-multiplier tube (H5784-01, Hamamatsu, Shizuoka, Japan). The output signal was acquired by a USB data acquisition card (Measurement Computing, Norton, MA) and displayed on a computer via an in-lab developed LabView program.

Accurate alignment of the optical window with the objective lens is critical to achieve a sensitive detection. For the LIF detector presented in Fig. 2C, an optical window was created at an appropriate position on the column by removing the polyimide coating with flame, and the optical window was affixed on an x-y-z translation stage above the objective lens. After a fluorescent dye (e.g., 1 μM fluorescein in 10 mM TE buffer at pH \sim 8.0) was set to flow through the column at a constant flow rate, the LIF detector was turned on, and the fluorescence signal was continuously monitored. The position of the optical window was then adjusted via the translation stage until the maximum signal output was obtained. At this time, the positions of the translation stage were locked; the optical window was aligned with the LIF detector. After the dye solution inside the OT column was washed out, sample injection and OTLC separation could be performed. Fig. 4A presents a picture of the prototype of this LIF detector.

An advanced version of such a detector (see Fig. 4B) was recently described by Zhang et al. [57]. The detector had a concentration LOD of 10 pM and a mass LOD of <7 ymol of fluorescein, and it had been used for monitoring fluorescently-labeled amino acid enantiomers resolved using a 900-nm-i.d. PLOT column [13]. In particular, a visual and real-time imaging scheme was adopted for the optical alignment. The detector consisted of three sets of light paths: the excitation light path, the fluorescence collection light path, and an image calibration light path. The excitation and the fluorescence collection light paths were similar to that shown in Fig. 3C. The image calibration light path started with a light emitting diode (LED) (M625L3, Thorlabs, USA) to illuminate the detection window of the capillary, followed by a filter (ZET 635/20X, Chroma, USA) to narrow the bandwidth of the LED light and then an aperture (SM1D12, Thorlabs, USA) to tune the light intensity. After the LED light passed through the capillary, the light signal (or the

image of the OT column) was collected by the objective below, passed through the two dichroic mirrors, reflected by a reflector (PFE10-P01, Thorlabs, USA), focused by a lens (AC254-075-A, Thorlabs, USA) and received by the camera (DCC1545M, Thorlabs, USA). Real-time images recorded by the camera were processed and displayed with the ThorCam software (Thorlabs, USA). Because the capillary-bore image and fluorescence light went through the same optical path for collection and collimation, if a sharp image of the capillary bore could be recorded by the camera, the OT column was aligned with the LIF detector.

5.2. C^4D detection

Zhang et al. [58,59] developed an admittance detector that could be used for $\leq 5\text{-}\mu\text{m}$ -i.d. OT columns. The authors used two exterior electrodes, separated along the column axially, to measure the solution admittance inside the column; the method was commonly called a capacitively coupled contactless conductivity detection (C^4D). Fig. 4C presents the detection cell configuration, and Fig. 4D presents a schematic diagram of the admittance detector. Shielded cables and SMB or BNC connectors were used throughout. Resistors ≥ 1 G Ω were of the glass-sealed type. The excitation source was either (a) a benchtop function generator (4011, www.bkprecision.com), or (b) a 555 timer (NE555N, www.st.com) based oscillator, powered by two 9 V batteries. The electrodes were tubular stainless-steel electrodes (0.4/0.64/7 mm i.d./o.d./length). The ac signal was integrated by an RMS-DC converter (AD536A, www.analog.com), and the data were acquired after low pass filtration (time constant 0.1 s) by a nominally 22-bit low-cost data acquisition card (EMANT300, www.emant.com) at 10 Hz. The capillary detection cell and the transimpedance amplifier (TIA) were kept in one metal enclosure, and all other components in a second one. Advantages of C^4D include on-column detection, facile placement, noninvasive monitoring without galvanic contact (hence no possibility of electrode deterioration), system simplicity and manufacturing inexpensiveness.

The admittance detector was tested for monitoring KCl inside a 5- μm -i.d. capillary in a flow injection format; a limit of detection (LOD) of 0.32 μM KCl was obtained. When the capillary i.d. was reduced to 2 μm , the LOD increased to 2.1 μM . Ion exchange chromatographic separations were attempted using a 2- μm -i.d. BOT column for Li^+ , Cs^+ and Ca^{2+} , and the admittance detector was used to monitor the resolved ions. Possibly due to the high background signal of the eluent (50 μM tartaric acid), concentrations of 200 $\mu\text{eq/L}$ for all three analytes were injected for these separations. Improvements are still required before such admittance detectors can be broadly used for $\leq 5\text{-}\mu\text{m}$ -i.d. OTLC.

5.3. MS detection

The advancement of MS instrumentation has made it widely accepted and sometimes mandatory for molecular detection and identification. Advanced electrospray ionization sources, such as nanospray and sub-ambient-pressure ionization source [60] have allowed for $>50\%$ of analytes initially in solution phase to be transmitted to the MS detector. Shen et al. [61] have reported a detection of 10 zmol (~ 6000 molecules) from a protein digest using a 15- μm -i.d. packed column running at nLs-per-min flow rates.

Xiang et al. [51] have demonstrated the feasibility of using MS detection for $\leq 5\text{-}\mu\text{m}$ -i.d. OT columns. The overall system configuration is presented in Fig. 3A. The exit-end of the column was sharpened via HF etching to achieve efficient electrospray and ionization (ESI) at a flow rate of pL per min. The chemically sharpened tip also helped to prevent tip clogging during ESI [62], compared with pulled tips in which the column i.d. was reduced as

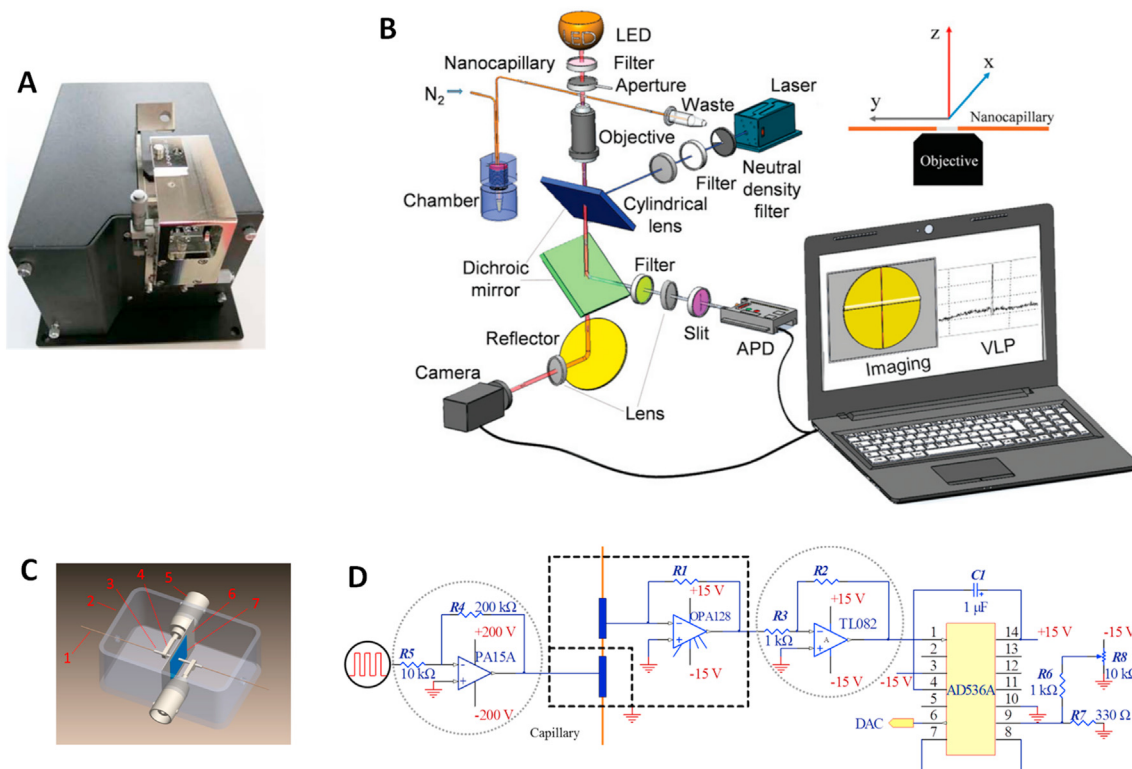


Fig. 4. Common detection schemes for $\leq 5\text{-}\mu\text{m}$ -i.d. OT columns. (A) Image of a prototype LIF detector (Figure was obtained from Ref. [56] with permission). (B) Schematic diagram of a confocal LIF detection system with visual and real-time imaging focusing (Figure was obtained from Ref. [57] with permission). (C) Cell configuration of an admittance detection. 1 - capillary; 2 - grounded metal box; 3 - electrode cast by Woods metal alloy; 4 - crimp-snap connectors; 5 - BNC connector; 6 - grounded Faraday shield; and 7 - adhesive paper tape for insulation (Figure was obtained from Ref. [58] with permission). (D) Schematic diagram of the admittance detector. Components enclosed by light dashes are optional; those enclosed by heavy dark dashes indicate a grounded enclosure. R1: 106–1011 Ω . R2: 104–106 Ω . (Figure was obtained from Ref. [59] with permission).

well. A high voltage was also applied through metal stopper on the cross to initiate and maintain ESI. A nitrogen sheath flow was applied at the emitter through a Tee junction to improve electrospray stability. An Orbitrap Fusion Lumos Tribrid Mass Spectrometer was used as the detector.

This system was experimented for proteomic analyses. Information-rich chromatograms were obtained when 7.5 pg of a tryptic peptide sample from *Shewanella oneidensis* was loaded into the OT column and eluted using a 30 min LC gradient. When 75 pg of this sample was loaded, ~ 1000 proteins could be profiled, representing over 10–100-fold improvement in sensitivity compared with previously developed 15 or 30- μm -i.d. packed column LC [61,63] and CE MS systems [64]. Because a flow splitter was used in this the system, only a small portion of the sample was loaded into the column; the rest of sample was wasted.

Other detection strategies such as ion-selective microelectrode [65] and potentiometric microelectrode [22] can be used for $\leq 5\text{-}\mu\text{m}$ -i.d. OT columns, but making such detectors to perform reliably and reproducibly is challenging. A recent review [66] has covered this kind of detectors.

6. Future perspective

A constant goal of analytical chemistry is to develop analytical instruments toward better, faster, and cheaper analysis, and OTLC provides a relatively easy route toward this goal. Significant progress has been made using ultra-narrow OT columns, and superior efficiency and outstanding peak capacity have been demonstrated [12,67]. An OT column with an i.d. of $\leq 5\text{-}\mu\text{m}$ takes only sub-pL sample for its separation. Since a single mammalian cell generally

has a volume of <500 fL (while slightly larger volumes are possible, for example, a Hela cell could reach a volume of 1.2–4.4 pL [68,69]), such column can be used for direct cytosol analysis for these cells. The extraordinarily high efficiencies, combined with the power of a modern MS, makes such columns an excellent means for single cell deep proteomic analysis [51]. By sacrificing a small portion of the efficiency, one can use a $\leq 5\text{-}\mu\text{m}$ -i.d. OT column to perform ultrafast LC separations [19], presenting a great alternative for the 2nd-dimension separation in 2-dimensional separations; for example, using a configuration similar to that described in literature [70]. Fast OTLC can be particularly useful in the area of process online analysis, where real-time feedback based on analytical data acquired directly from the manufacturing process stream is often needed during drug production [71]. All above accomplishments come from using narrow OT columns, and OT columns with large i.d. cannot achieve these magnificent performances.

We, the authors, have heard many times a concern of column clogging, but this was not really a problem in our experience. Here we would like to explain why. A fine particle or fiber can clog a channel when it travels from a large channel (that permits the particle/fiber to enter) to a small channel (that stops the particle/fiber to pass through). For any OT column, as long as it has a uniform diameter throughout, column clogging can hardly occur, because if a particle/fiber can get in the column, it can get (or be flushed) out. [Note: a PLOT column may not have a uniform diameter throughout, and it can be clogged.] A particle/fiber can block the entrance of an OT column though. If that happens, a backflush should recover the column. In a worst scenario, one can trim off a short segment of the column head to solve the problem. Nevertheless, care must be taken to avoid particles/fibers entering

solutions; all solutions must be filtered (e.g., using MF-Millipore™ Membrane Filter, 0.22 µm pore size) before use.

Although excellent performances have been obtained, there is a lot of rooms for improvements. The use of a flow splitter is often not favored because stable elution rate is difficult to maintain, leading to irreproducible separations. The splitter may be eliminated if an electroosmotic pump [72–74] is employed. Although a ≤5-µm-i.d. OT column takes sub-µL sample for each separation [45], one often needs to prepare nLs or even µLs of sample to carrying out such an injection; most of the sample went to the waste or was lost. This is generally not acceptable for practical trace analyses or proteomics analyses. This problem can be mitigated using a microfabricated injection scheme [48]. A variety of materials (polymers, silica, nanoparticles, graphene oxides and metal–organic frameworks) have been tested and utilized for OT column coatings, and these coatings, if applicable to ≤5-µm-i.d. columns, can increase the application possibilities via different separation mechanisms [38].

In conclusion, we expect LC using ≤5-µm-i.d. OT columns to play a pivotal role in future chemical separation and analysis, especially single cell analysis. We also anticipate MS to be a primary detector for these systems, because it is sensitive and can provide enormous amount of information of the molecules detected. LIF, admittance detectors and microelectrodes are used currently, but they will be used mainly for research purposes.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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