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Advances in organic polymer-based monolithic column technology for high-resolution liquid chromatography-mass spectrometry profiling of antibodies, intact proteins, oligonucleotides, and peptides

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ABSTRACT

This review focuses on the preparation of organic polymer-based monolithic stationary phases and their application in the separation of biomolecules, including antibodies, intact proteins and protein isoforms, oligonucleotides, and protein digests. Column and material properties, and the optimization of the macropore structure towards kinetic performance are also discussed. State-of-the-art liquid chromatography-mass spectrometry biomolecule separations are reviewed and practical aspects such as ion-pairing agent selection and carryover are presented. Finally, advances in comprehensive two-dimensional LC separations using monolithic columns, in particular ion-exchange × reversed-phase and reversed-phase × reversed-phase LC separations conducted at high and low pH, are shown.

Contents

Abbreviations: DVB, divinylbenzene; ESI, electrospray ionization; FA, formic acid; HFBA, heptafluorobutyric acid; HIC, hydrophobic interaction chromatography; HILIC, hydrophilic interaction chromatography; IEX, ion-exchange chromatography; LC × LC, two-dimensional liquid chromatography; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; RP-LC, reversed-phase liquid chromatography; SEC, size-exclusion chromatography; S, styrene; TFA, trifluoroacetic acid; TIC, total ion current; TOF, time of flight; UHPLC, ultra-high-pressure liquid chromatography.

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1. Introduction

Rigid polymer-based monolithic columns have been introduced in the early 1990s as an alternative for packed-bed columns [1,2]. The first monolithic entities were based on methacrylate and styrenic precursors and were polymerized in large i.d. columns, *i.e.*, a mold, using conditions that were typically applied during a suspension polymerization for the preparation of porous polymer beads. The resulting column structure featured a macroporous interconnected structure of polymer globules. The potential of this novel column technology was readily demonstrated with high-speed separations of macromolecules, including intact proteins [3] and synthetic polymers [4]. The success of these columns, typically operated at high flow rates, in the separation of height molecular-weight analytes can be attributed to mass-transfer effects that are mainly based on convention (C_m-term). Since the polymer globules feature very few mesopores (pores in the size range of 2–50 nm) stationary-phase mass-transfer (C_s) greatly affecting dispersion characteristics when operating the column at high flow rate, are virtually absent. Although not within the scope of this review, it should be mentioned that the good kinetic performance for biomolecule separations is in sharp contrast with the efficiency typically reported for small-molecule separations [5–7]. However, considerable efforts and progress has been made to create monolithic columns suitable for small-molecule separations. The different strategies pursued were recently reviewed by Urban and Svec [8,9].

The ease of the preparation process of monolithic materials facilitated the development of miniaturized column formats, typically capillaries and microfluidic chips, which are used for peptide and protein profiling in clinical diagnostics and life-science LC–MS research. Since the monolithic interconnected structure is covalently linked to the inner capillary wall, frits used in particle-packed columns are no longer needed and very robust column formats are obtained. Geiser et al. demonstrated the possibility to conduct almost 2200 consecutive separations of a test mixture of three proteins without any significant shift in either retention time or column pressure [10]. More recently, Urban et al. demonstrated the durability of monolithic capillary columns with over 10,000 injections [11]. A landmark paper was published by the Huber research group in 2000, showing baseline resolved oligonucleotide LC–MS separations utilizing monolithic capillary columns based on ion-pair reversed-phase (RP) interactions [12]. The success of monolith chromatography is further amplified by the great variety of functional and crosslinking monomers available, allowing to create monoliths carrying the desired surface chemistry to achieve LC separations in different modes. In this way, a large variety of monolithic materials have been created that were then applied for biomolecule separations. For example, monolithic columns were developed enabling high-resolution biomolecule separations based on ion-exchange chromatography (IEX) [13–16]. Hilder's group reported macroporous polymer-monolithic stationary phases for hydrophobic interaction chromatography (HIC) of intact proteins [17].

This review discusses advances in the preparation, characterization, and application of polymer-monolithic column technology with a focus on fast and high-resolution peptide and protein LC–MS separations. First, material properties and the possibilities and limitations in controlling the macropore structure and surface chemistry are described. Next, selected examples of separations are provided demonstrating the potential to analyze a wide range of biomolecules, from peptides to monoclonal antibodies, using one-dimensional and comprehensive multi-dimensional separation strategies. Finally, challenges and perspectives for high-resolution monolith chromatography are shown.

2. Preparation and properties of polymer-monolithic capillary columns

2.1. Column and material properties

Fused-silica capillary column formats are typically applied in proteomics research because of their flow-rate compatibility with nanoelectrospray ionization in mass-spectrometric (ESI–MS) detectors. The polymerization mixture typically comprises the initiator dissolved in a homogenous solution of monovinyl and divinyl monomers and inert pore-forming diluents (porogens). Typically, the surface of the fused-silica inner capillary wall is first functionalized with a silane spacer, such as (trimethoxysilyl)propyl methacrylate, where the methoxy groups react with the silanol groups situated at the activated fused-silica capillary surface. Pendant vinyl groups subsequently react with the monomers present in the polymerization mixture. A detailed investigation of monolith anchoring approaches was conducted by Courtois et al. [18]. The best silanization approach for fused-silica used toluene as solvent for the silanization agent. Nesterenko et al. optimized the bonding procedure to establish a covalent bond between polymer monoliths and the wall of titanium housings [19]. Monolithic capillary columns prepared using this approach proved to be very robust and stable even at ultra-high-pressure operating conditions (ΔP = 80 MPa) [20].

Recently, procedures to chemically anchor polymer monoliths to the wall of polyetheretherketone tubing (PEEK) and to polyimide microfluidic chips have also been described [21–23].

Two most often applied polymeric monolithic stationary phases for biomolecule separations are based on (meth)acrylate and styrenic monomers. Both methacrylate-ester-based monoliths and styrene-based monoliths exhibit good stability in separations using acidic and basic mobile phases. Whereas methacrylate monoliths are stable between pH 2 and 12 [24], styrene-based monolithic columns were stable even at a pH up to 14 [25]. Both types of these materials typically feature a very small volume of mesopores, and as a result, those monoliths typically exhibit surface areas of less than 50 m^2/g [26]. Whereas this may enhance the separation efficiency by minimizing the C_s-term contribution to band broadening, the smaller surface area compared to columns packed with fully porous particles negatively affects mass loadability. The loading capacity of oligonucleotides on 200 μm i.d. capillary poly(S-*co*-DVB) monolithic columns was investigated by Oberacher et al. by measuring the peak width while applying a 10 min RP-LC gradient [27]. The maximum mass loadability was found to be 500 fmol (2.4 ng). Detobel et al. found that the mass loadability for a similar type of monolith for intact proteins strongly affected peak width, *i.e.*, injecting 10 pg carbonic anhydrase resulted in 2–3 s wide peaks (measured at half height), which increased linearly with injected protein mass [28]. Asymmetric peaks, clearly indicating overloading, were observed in a range of 50–100 pg. The limited mass loadablity of polymer monolithic stationary phases should be taken into account when profiling low-abundant biomarkers.

The DVB crosslinker comprises a 2:1 mixture of *meta*-DVB and *para*-DVB and is commercially available in two grades with 65% and 80% purity with the rest being mostly ethylvinylbenzene [29]. During the course of polymerization, DVB is depleted earlier than S, which results in the formation of a crosslink density gradient with the outer layer of the polymer globules being less crosslinked. Poly(S-*co*-DVB) monolithic material prepared in bulk quantity (~1 g) was investigated applying thermal analysis techniques [30]. Experiments confirmed the presence a broad distribution of crosslinking densities and at a temperature of 100 °C the least crosslinked outer shell of the globules in monolithic material devitrified. In practice however, the thermal stability of proteins depends on the specific protein tested and the exposure time. To