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Titanium-scaffolded organic-monolithic stationary phases for ultra-high-pressure liquid chromatography☆

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ABSTRACT

Organic-polymer monoliths with overall dimensions larger than one millimetre are prone to rupture – either within the monolith itself or between the monoliths and the containing wall – due to the inevitable shrinkage accompanying the formation of a cross-linked polymeric network. This problem has been addressed by creating titanium-scaffolded poly(styrene-*co*-divinylbenzene) (S-*co*-DVB) monoliths. Titanium-scaffolded monoliths were successfully used in liquid chromatography at very high pressures (up to 80 MPa) and using gradients spanning the full range of water–acetonitrile compositions (0 to 100%). The kinetic-performance of (50-mm long) titanium-scaffolded monoliths was compared to that of similar monolith created in 1-mm i.d. glass-lined tubing at pressures up to 50 MPa. The peak capacities obtained with the titanium-scaffolded column was about 30% lower. An increased Eddy-diffusion, due to the pillar-structure, and a decreased permeability are thought to be the main reasons for this reduced kinetic-performance. No decrease in performance was observed when the titanium-scaffolded columns were operated at pressures of 80 MPa for up to 12 h. The column-to-column repeatability ($n = 5$) was acceptable in terms of observed peak widths at half heights (RSD *ca.* 10%) The run-to-run repeatability ($n = 135$) in terms of retention times and peak widths at half height were found to be good. Titanium-scaffolded columns coupled in series up to a combined length of (200 mm) were used for the analyses of a complex *Escherichia coli* protein sample. Our experiments demonstrate that columns based on titanium-scaffolded organic-polymer monolith can be operated under strenuous conditions without loss in performance. The titanium-scaffolded approach makes it feasible to create organic-polymer monoliths in wide-bore columns with accurate temperature control.

1. Introduction

Since their introduction in HPLC about 25 years ago organic-polymer monolithic stationary phases have gained a reputation for their ability to separate large biomolecules. In the late 1990s Svec et al. reported the separation of intact proteins within 20 s [1]. The suitability of organic-polymer monoliths for the fast separation of large molecules depends on the possibility to independently optimize the surface chemistry and the pore size. This optimization may result in a high efficiency (high plate counts), as well as a high permeability (low pressure drop) for the monoliths [2]. Organic-polymer monoliths allow post-polymerization changes in surface chemistry. Such surface modifications can be regionally controlled, which makes it possible to create monolith-based phase systems for multidimensional chromatography [3].

A known drawback of organic monolithic stationary phases is their shrinking during polymerization, which results in irregular monolithic structures at the column extremes. When polymerizing in fused-silica capillaries the ends of the column can be cut before use, so that the above-mentioned irregularities will not jeopardize the analytical performance. If an organic-monolithic structure is prepared in a confined (inflexible) structure the effects of shrinking cannot be circumvented. A few remedies to reduce these effects have been suggested, including the use of solvents that keep the stationary phase in the swollen-state [4] and polymerization under high pressures [5]. When scaling up columns from capillary-format (<1 mm) to larger internal diameters the heat generated during polymerization may lead to a temperature gradient and, therefore, highly non-homogenous structures inside the mould [6]. Peters et al. showed that slowly adding the polymerization mixture can help avoid effects of exothermal polymerization on the homogeneity of the monolithic structure [6]. The approach described by

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Podgornik et al. uses different annuluses, placed like Russian Matroshka dolls, circumvented this temperature gradient [7], but like all other proposed solutions [4–6] this is unattractive when the *in-situ* creation of a monolith in a (large) confined space is required for the separation of biomacromolecules.

In that case it is highly preferable to create monolithic stationary phases that do not break as a result of shrinkage during or after polymerization and that are created at a constant, well-controlled temperature. Shrinkage during polymerization is inevitable due to the cross-linking of the organic based monoliths. If the overall distance of the shrinkage is too large the polymer backbone will detached from the wall. The straightforward way to prevent breaking of the monoliths is to avoid attachment of stationary phase to the wall [4]. This results in large spaces between the monoliths and the walls (flow channels), unless conditions can be chosen such that the monolith swells significantly under the operating conditions, thus "refilling" the channels along the wall. This has been achieved by using poly(styrene-*co*-divinylbenzene) monoliths (poly(S-*co*-DVB)) with 100% THF as mobile phase [4]. For separations under typical RP-LC conditions attachment to the wall is crucial [8,9]. In that case, the distance across which the shrinkage is observed must be reduced to maintain covalent attachment of the monolith to the wall (*e.g.* by using capillary columns). In the present case overall shrinkage has been controlled by first creating a sparse, solid structure, spanning the full size of the confined space. Such a "scaffolding" provides a stable framework on which the functional monolithic stationary phase can be polymerized. Conceptually, shrinking or swelling of the polymer monolith will affect the pore size (permeability) of the stationary phase, but not its overall dimensions. The high forces associated with shrinking polymers, for example due to change in mobile-phase composition during gradient elution, which are responsible for breakage of monoliths or release from the column wall, the so-called depletion zones [8], are not expected to affect scaffolded monoliths. Additionally, the scaffold may reduce the temperature gradient if it is able to dissipate the heat generated during polymerization. Thus, scaffolding offers a path towards the *in-situ* creation of monoliths in wide columns and to the construction of large monolithic separation bodies in well-defined confined structures. The reinforcing properties of the scaffold allow their application under ultra-high-pressure (UHPLC) conditions with maximum column pressures exceeding 40 MPa.

The choice for the scaffold-material is limited, because the scaffold must be rigid, so to provide a confined space for the monolith and so that the monolithic material will be anchored to the wall of the column (or the confined space). The latter is crucial for the stability of the monolithic stationary phase [9]. The scaffold should be inert under chromatographic conditions and, furthermore, should be producible in a suitable design. In chromatography stainless steel, aluminium, PEEK and fused silica have been used as column hardware-material. Not all of these materials may feasibly be used to produce a scaffold suitable for strengthening a monolithic stationary phases. Titanium is mechanically strong, relatively inert, and it can be produced in a confined and desired design [10]. It has previously been used as column-hardware [11]. Furthermore, the titanium surface can be oxidized [12] to titanium oxide, which is compatible with sample-preparation [13] and LC-separation processes, [14] and amenable to covalent anchoring of an organic monolith on the scaffold surface [11]. By using techniques akin to contemporary 3D printing, the titanium can be produced in very well-defined small-size structure [10], which makes it possible to use titanium for producing a scaffolded monolithic stationary phase for application in chromatography. The shrinking that accompanies the formation of a polymeric network still will occur, despite anchoring of the monolith to the scaffold. Also, shrinking and swelling due to changes in mobile-phase composition are still likely to occur. However, the total forces generated would be much smaller due to shorter distances between elements of the scaffolding structure. Even when the overall size of the scaffolded monolith is large, the requirements on the actual polymer are similar to those encountered when polymerizing the monolith in a smaller capillary format.

The ultimate objective is to create organic monoliths in large structures that do not suffer from overall shrinkage during or after polymerization. Furthermore, the scaffolded monolithic stationary phase should be reinforced, so that the monolith can be used even with low concentrations of organic solvents and UHPLC conditions. In the present study we prepared titanium-scaffolded organic-monolithic stationary phases in narrow-bore-column format, which allowed tested these under chromatographic conditions for the analyses of intact proteins. We present a thorough characterization of titanium-scaffolded monolithic stationary phases and we demonstrate their potential in liquid chromatography.

2. Experimental

2.1. Chemicals and materials

Styrene (S, >99.5%), divinylbenzene (DVB, 80%), 3-(trimethoxysilyl)propyl methacrylate (γ-MPS, 98%), 2,2′-azobisisobutyronitrile (AIBN, 98%), 1-decanol (99%), toluene (99.9%), aluminium oxide, ribonuclease A from bovine pancreas, myoglobin from equine heart (>90%), lysozyme from chicken egg white (>90%), carbonic anhydrase from bovine erythrocytes, cytochrome *c* from equine heart (>95%), β-lactoglobulin B from bovine milk (>90%), catalase from bovine liver, α-lactalbumin bovine milk (>85%), and conalbumin from chicken egg were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). *Escherichia coli* lyophilized protein was purchased from Bio-Rad (Veenendaal, The Netherlands). Methanol (MeOH), acetonitril (ACN), acetone, and tetrahydrofuran (THF, >99.8%) were purchased from Biosolve (Valkenswaard, The Netherlands). Thiourea (>99%) was purchased from B.D.H. Chemicals (Poole, England). Sodium hydroxide (NaOH) was purchased from Merck (Darmstadt, Germany). Hydrochloric acid (HCl) (37%) was obtained from Acros (Geel, Belgium). Milli-Q water (18.2 MΩ cm) was produced by a Sartorius Arium 611UV Ultrapure Water System (Goettingen, Germany). S and DVB were purified by passing the liquid precursors through a bed of activated basic alumina.

Glass-lined tubing (0.8 mm internal diameter × 50 mm length) was purchased from Da Vinci Europe (Rotterdam, The Netherlands). The titanium-scaffold tubes (1.3 mm internal diameter × 50 mm length) were developed for this project by FT Innovations (Boxmeer, The Netherlands). The titanium surface was chemically treated to create a titanium-oxide surface layer.

2.2. In-situ synthesis of monolithic stationary phases

Prior to the *in-situ* polymerization of the monolithic stationary phase, the internal surface of the glass-lined tubing and titanium-structured columns was modified to enable a covalent bonding between the wall and the monolithic stationary phase [9]. For this purpose the glass-lined tubing and the titanium-structured columns were subsequently flushed for 30 min at a flow rate of 10 μL/min with 1 M NaOH solution, water, 0.2 M HCl solution, and again water. Thereafter, the columns were dried with N_2. The glass-lined tubing was surface functionalized with a 10% (v/v) γ-MPS solution prepared in toluene through flushing for 1 h at 10 μL/min. The titanium structured columns were submerged in a 50% (v/v) γ-MPS solution [11] in toluene for 1.5 h. Thereafter, the surface-modified column housings were flushed with acetone and dried with N_2.