Silica Monolithic Columns: Synthesis, Characterisation and Applications to Proteomics

3.1 Introduction

Proteomics deals with the analysis of large sets of proteins. Like genomics, proteomics research has fostered the development of novel technologies in the areas of separation science, mass spectrometry and bioinformatics. Analysis for proteomics has relied on two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), which provides unprecedented separation power for proteins. 2D-PAGE has long been the method of choice for the analysis of complex protein mixtures, as it enables the separation of thousands of proteins in a single run according to their isoelectric point (pI) in the first dimension and to their molecular weight in the second [1]. More recently, two- and multi-dimensional chromatographic approaches have proven complementary to 2D-PAGE. The chromatographic stationary phases employed need to exhibit very high resolving power and provide fast turnaround times per analysis.

Since 2D-PAGE has difficulties resolving either very small or very large proteins as well as polypeptides with an extreme pI and/or hydrophobicity [2], chromatographic alternatives have emerged. Making use of the high resolving power of high-pressure liquid chromatography (HPLC) for peptides, the so-called “shot-
gun” proteomics approach has been developed, where all proteins are digested with trypsin prior to separation [3]. While rendering the sample more amenable to HPLC separation, tryptic digestion increases the number of sample components by a factor of approximately 25 to 50. This overwhelming separation problem has initiated interest in coupled-column liquid chromatography (2D-LC) in order to increase peak capacity to a level that allows the mass spectrometer to detect, quantify and identify individual peptides. However, comprehensive 2D-LC of complex samples often implies spending 12 to 24 hours on the analysis of a single sample [4], due mainly to the rather long LC run times. As a result, great efforts have been made to optimise the separation efficiency of columns and reduce analysis times. Several approaches to achieve this goal are being explored, such as Ultra-High Pressure Liquid Chromatography (UHPLC), electrically driven separations, and columns with low flow resistance. While particle-based stationary phases continue to play a dominant role, it is the recent development of monolithic materials that promises to advance the possibilities of HPLC in proteomics. Silica-based monoliths have already gained an important place in separation science, but mainly for low molecular weight analytes.

In order to better understand the advantages of monolithic columns, the limitations of conventional chromatographic stationary phases should be considered. The following equations for plate height [5, 6] shed light on these limitations.

\[
H = Au^{1/3} + B/u + Cu 
\]

\[
H = \frac{1}{[(1/C_e d_p) + (D_m/C_m d_p u)]} + C_d D_m/u + C_{sm} d_p^2 u / D_m
\]

Equation 3.1 summarises the different terms contributing to band broadening, the "Eddy diffusion term" (A), the "axial diffusion term" (B) and the "resistance to mass transfer term" (C). The individual terms are given in more detail by Equation 3.2. Apart from some column specific constants \((C_e, C_m, C_d \text{ and } C_{sm})\) and the linear flow velocity \((u)\), particle diameter \((d_p)\) and solute diffusion in the mobile phase (characterised by the diffusion coefficient, \(D_m\)) determine separation efficiency to a large extent. The last term in Equation 3.2 describes diffusion-limited transport (i.e. relating to \(D_m\)) in the mobile phase over a distance related to the particle diameter (i.e. \(\approx d_p^2\)), required to reach the interface with the stationary phase over which the partitioning equilibrium takes place. Especially at high mobile-phase flow velocities (fast separations), this term determines separation efficiency to a large extent. In contrast, the A-term is responsible for most of the band broadening at low linear flow velocities [7].

Until recently, chromatographers have achieved more efficient, faster separations
by using smaller particles and thereby reducing the contribution of both the $A$- and the $C$-terms to band broadening. This was first demonstrated using 1 $\mu$m particles by Halasz et al. in 1975 [8]. However, improved efficiency also implied, in this case, much greater backpressure. Instrumentation to produce such high pressures was at that time not commercially available, which prevented the more widespread use of small particles. With the advent of novel, high-pressure-stable silica particles and special equipment, UHPLC was introduced by Jorgenson [9, 10]. This approach allows the use of 30-cm-long capillaries packed with 1 $\mu$m particles to yield efficiencies as high as 670 000 plates/m. Alternatively, electrically driven separations like capillary electrochromatography (CEC) can be used to obtain very high plate numbers, but it remains difficult to couple CEC with MS [11], which is the detector of choice in proteomic studies. Non-porous particles were used by Unger [12] to avoid peak broadening due to stagnant mobile phase transfer. However, such particles also show very limited binding capacity.

To avoid the problems related to the need for very high pressures and the low capacity of non-porous media, a new technique named perfusion chromatography was introduced in 1990 by Afeyan et al. [13]. This technique makes use of particles with very large pores (6000 to 8000 Å) that proteins can enter through a combination of convective and diffusional transport, resulting in relatively low pressures. However, up to a given pressure, the mobile phase and analytes tend to go around the particles without penetrating them. Shortly thereafter Tennikova, Svec and co-workers introduced a new support for chromatography in which separation takes place on very short, wide macroporous polymeric monoliths [14]. High porosity and low pressure are features characteristic of such monolithic materials. Instead of particle diameter (Equation 3.2), separation efficiency for these materials is related to structural parameters as discussed later. Monoliths can be prepared by one of two routes.

- polymerisation of organic monomers
- polymerisation of alkoxysilane monomers

The first type of monolith has been the subject of intense research over the past years [15, 16]. The synthesis of organic monoliths involves mixing monomers, initiators, crosslinkers and porogenic solvents in a mould and subjecting them to UV light or heat to initiate polymerisation. In this approach, the porogenic solvent acts as an emulsifier and creates the porous structure. However, organic polymers are often subject to swelling or shrinking in organic solvents and show pores [17], whose size ($<2$ nm) hinders the motion of analytes in and out of the pore.
Figure 3.1: Scanning Electron Microscopy (SEM) of (a) the skeleton-throughpore network of a silica-based monolithic column, (b) the mesoporous structure on the surface of the skeleton and (c) a throughpore. (reprinted with permission from Cabrera, K., Journal of Separation Science 2004, 27, 843; copyright 2004 Wiley-VCH).
It was not until 1996 that a silica-based monolith was synthesised for use in HPLC [18], where the skeleton defines the throughpores and contains the mesopores (Figure 3.1). Whereas the latter provide the specific surface area and thus binding capacity, the flow-through pores are responsible for the hydraulic permeability of the monolith. Silica-based monoliths differ from their organic counterparts in that they only exhibit μm-size flow-through pores constituting a macroporous network and nm-size mesopores in the skeleton, but no pores smaller than 2 nm. Moreover, silica monolithic columns do not show the tendency to swell or shrink when they are used with organic liquid phases [17]. Desmet and coworkers [19] devised a model to investigate the effect of interstitial porosity (which can be assimilated to the porosity associated with throughpores) on column performance. It showed that increasing interstitial porosity from 0.4 to 0.9 yielded an up to a 2-fold improvement in separation efficiency. Therefore, the skeleton size, which refers to the average thickness of the silica network (Figure 3.1), is an important parameter when designing new and efficient separation media based on monoliths. Another structural parameter often referred to in publications related to HPLC on silica-based monoliths is the domain size, which is equal to the sum of the skeleton and the throughpore size.

This review will focus on silica monoliths first outlining the principle chemical reactions and their effect on the chemical and structural properties, which influence the chromatographic performance of the resulting columns. Subsequently, applications of silica monoliths to proteomics research will be outlined.

3.2 Synthesis

More than thirty years ago, Hansen and Sievers [20] prepared liquid chromatography columns from polyurethane. The very low backpressure allowed a very high speed of analysis. Similarly, Hjerten [21] successfully separated proteins using a compressed polyacrylamide gel. However, both stationary phases lacked mechanical strength and it was not until the beginning of the 90’s that monolithic stationary phases with satisfactory mechanical properties could be synthesised. The successful combination of sol-gel reaction and phase separation for the preparation of silica-based monoliths was first demonstrated by Kaji et al. [22]. Following acid and/or base catalysis, reactive alkoxy silanes polymerise to form a gel. Inorganic polymerising systems undergo a phase separation (or spinodal decomposition) driven by the increase in free energy for solvation due to the reaction of the different species present in the starting sol.
Figure 3.2: Schematic representation of the synthetic steps for the preparation of silica-based HPLC monoliths. (Heat treatment is optional).
### Table 3.1: Effects of the variation of different synthetic parameters on the structure of silica-based monolithic columns using acetic acid as catalyst of the sol-gel process. The parameters that were varied in the corresponding article are shown in italics. (NA) stands for not available.
The resulting gel separates into solid- and liquid-rich domains that further react with each other to give the gel its final structure. This process, called syneresis [23], results in shrinkage of the network and in an expulsion of liquid from the pores. The inorganic sol-gel transition to form an alkoxysilane network freezes the domains as permanent morphology [24]. After aging and drying of the gel, a highly porous monolith is obtained. A typical route for the preparation of silica-based HPLC monoliths is summarised in Figure 3.2. The pore structure and the mechanical strength of the monolith depend on the competition between the kinetics and thermodynamics of the sol-gel transition and of phase separation [23, 24]. Therefore, the nature and concentrations of the starting materials will be of great importance to control both the time and the speed at which both gelation and phase separation take place.

Brinker et al. investigated the dependence of hydrolysis and condensation reactions on pH [25]. The rate of hydrolysis at low pH is relatively high and decreases linearly with increasing pH up to 7. At higher pH, hydrolysis becomes faster again. At the same time, condensation is minimal around pH 3 and greatly increases over the pH range 7-9. At higher pH, condensation rate falls due to increased depolymerisation. Therefore, a low pH, resulting in long gelation and phase separation times, is favoured to obtain small throughpores [26]. Tanaka et al. [18, 24, 27, 28] advocated the use of tetramethoxysilane (TMOS) as silica monomer in the sol. This is now the most commonly used alkoxysilane to prepare HPLC grade monoliths. The mechanical strength of the monolith increases with increasing TMOS concentration whereas too low a concentration in alkoxysilane results in poor interconnectivity of the skeleton and, consequently, in poor mechanical strength [29].

While condensation of the alkoxysilanes proceeds, the resulting network becomes less soluble and hastens phase separation. Consequently, the use of other alkoxysilanes will lead to morphological and chromatographic differences. In addition, alkoxysilanes can be used to introduce functionalities for later derivatisation of the stationary phase or for direct tailoring of chromatographic properties. The following alkoxysilanes have been used separately or in combinations: tetraethoxysilane (TEOS) [30], methyltrimethoxysilane (MTMS) [31], ethoxytrimethoxysilane (ETMS) [32], 2-cyanoethyltriethoxysilane (CEOS) [33], (3-aminopropyl)triethoxysilane (APTES) [34] and diglycerylsilane (DGS) [30, 34]. Table 3.1 summarises the effects different synthetic parameters have on the monolith structure and Table 3.2 gives an overview over other reagents that have been used in the preparation of monoliths in order to vary their bimodal structure and/or their selectivity.
<table>
<thead>
<tr>
<th>Silanes</th>
<th>Catalysts</th>
<th>Porogens</th>
<th>Additives</th>
<th>Aging reagent</th>
<th>Throughpore size (m)</th>
<th>Mesopore size (nm)</th>
<th>Ref.</th>
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<td>Formamide</td>
<td>BPA antibodies in PBS</td>
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<td>TMOS</td>
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<td>Enzyme immobilised on beads</td>
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<td>NA</td>
<td>NA</td>
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<tr>
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<td>Tetraisopropyltitanate Urea</td>
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<td>Zirconium oxychloride NH4OH</td>
<td>NA</td>
<td>NA</td>
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<td>Formamide</td>
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<td>NA</td>
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<td>(HCl and Tris pH 8.25) or Tris</td>
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<td>Enzyme immobilised on beads</td>
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<tr>
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<td>HCl</td>
<td>Formamide</td>
<td>PEO + APTES + protease in HEPES buffer pH 7.5</td>
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<td>0.5-3.0</td>
<td>[54]</td>
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<td>Formamide</td>
<td>CTAB, F127</td>
<td>NA</td>
<td>0.5-3.0</td>
<td>[55]</td>
<td></td>
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<tr>
<td>TEOS + CH₃(CH₂)ₙSi(OR)₃</td>
<td>n-hexadecylamine</td>
<td>Formamide</td>
<td>n-hexadecylamine</td>
<td>NA</td>
<td>0.5-3.0</td>
<td>[56]</td>
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</table>

**Table 3.2:** Effects of the variation of different synthetic parameters on the structure of silica-based monolithic columns using also other catalysts for the sol-gel process. Different alkoxy silanes and porogens, a different pH as well as additives allowed to tailor the bimodal. The parameters that were varied in the corresponding article are shown in italics. (NA) stands for not available.
As alkoxysilanes are poorly water soluble, a chemical additive is often required to increase solubility. Additives also have two other functions. They can act as templates for the monolith’s pores (in which case they are also referred to as porogens or templates) and retard the phase separation process. For the preparation of HPLC monoliths, the most common porogen is poly(ethylene)glycol (PEG) [18,24,27]. An increase in PEG concentration leads to a decrease in both throughpore- and domain-size due to a retarded phase separation relative to the sol-gel transition [24,27,29,31]. The abundant cross-linking sites in monolithic structures having a small domain size give mechanically stronger monoliths [29]. The molecular weight of the porogen also has an impact on the morphology of the monolith; the larger the porogen, the larger the throughpores and mesopores [45]. Aging is the first step involved in tailoring the surface for use in chromatography after a monolith has been synthesised. Aging deals with the enlargement of the mesopores using a process called Ostwald Ripening that leads to round surfaces and a reduced contact area between liquid and solid due to local solubilisation. The most common method to tailor mesopores employs ammonium hydroxide [18,24,27,28], where an increase in concentration results in larger mesopores up to 400 nm [37]. Temperature can be used additionally to tailor mesopore size [37] as an increase in temperature results in a greater syneresis and thus in smaller pores [50]. Urea has been used as a precursor of ammonia in monolith preparation [31]. It hydrolyses to ammonia at high temperature and partially dissolves the silica backbone [41]. The most difficult step in preparing monoliths is the removal of liquid, as gels contract while the liquid evaporates from the pores [23,51]. The huge stress generated in the mesopores tends to exceed the strength of the network and can cause formation of cracks. This can be avoided by supercritical drying of the gel [52,53]. Shrinkage can also be eliminated by carefully choosing the solvent present in the monolith during the drying step. To this end, isobutanol, 2-pentanol and iso-octane led to large, crack-free monoliths [54]. Both drying temperature and pressure can be lowered below the critical value for these solvents while still obtaining crack-free monoliths if the pores are large enough [55]. Ionic solvents (e.g. 1-ethyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]-amide) have virtually no vapour pressure, thereby greatly simplifying the drying step. They were used as solvents for all steps of the synthesis of the monolith. Their use alleviated the need for solvent exchange [56]. The last step in preparing a silica-based monolithic HPLC column is derivatisation. It is generally performed using octadecyldimethyl-(N,N-diethylamino)silane [18,24,27] to obtain a stationary phase for reversed phase separations. Biomolecules have also been chemically attached to monoliths in the preparation
of affinity-based LC columns [57–59]. Affinity ligands can be attached to silica particles [40] before being added to the sol or directly put in the sol [39] prior to the sol-gel process. Alternatively, bio-macromolecules can be immobilised at the surface of monoliths by adsorption [33, 60]. Adsorption was also used to prepare Ti- [41, 61] and Zr-coated [42] monoliths. Tables 3.1 and 3.2 provide an overview of the materials used by various researchers and the parameters that have been varied in the synthesis of silica monoliths by sol-gel reactions.

### 3.3 Characterisation

#### 3.3.1 Physical characterisation of monoliths

Silica monoliths have to be physically characterised to relate these characteristics to their chromatographic behaviour. The link between morphology and chromatographic performance is of utmost importance in order to design better separation media. Several techniques are used for the physico-chemical characterisation of HPLC monoliths. Optical methods such as Scanning Electron Microscopy (SEM) [18, 24, 27] and to a lesser extent Transmission Electron Microscopy (TEM) [62, 63] are commonly used for the assessment of the structural parameters of monolithic columns. SEM is used to study the morphology of monoliths after the drying step. It gives an estimate of the size of the throughpores and of the skeleton [18, 24, 27] that will in turn determine the hydrodynamic properties and mechanical strength of the column. Whereas the methods outlined below are better suited to determine the exact size and the size distribution of the throughpores, SEM is the only method that allows gaining data about the skeleton size. However, SEM is mainly used to study transversal slices of a monolith, which does not allow to draw conclusions about the homogeneity of the pore size distribution and homogeneity over the entire column. A longitudinal cut of the monolith gives a better idea of pore size and especially of the homogeneity of its distribution [31].

Techniques such as mercury porosimetry, Size Exclusion Chromatography (SEC) and N₂ adsorption give additional data about the morphology of the investigated monolith. The specific surface area of a chromatographic column relates to the column selectivity and the amount of a given analyte that can be adsorbed. It is measured by N₂ adsorption via the Brunauer-Emmett-Teller (BET) method. This method involves measuring the volume of N₂ adsorbed on the surface of the column. In the case of liquid chromatography columns, monolayer adsorption is assumed. The specific surface area of a column can therefore be easily calculated
from the adsorbed volume of gas and the size of a N\textsubscript{2} molecule [64].

Mercury porosimetry allows determining both the volume of meso- and through-
pores in two experiments [65]. By wrapping the sample in a mercury-tight mem-
brane, the volume of throughpores is determined by linearly increasing the mer-
cury pressure. Under the action of pressure, the monolith will be crushed. The
volume of the throughpores is calculated as the reduction in volume of the mono-
lith. To assess the mesopore volume, the mercury pressure is linearly increased
without using a membrane so that the mercury can now invade the mesopores [65].

The shape of the adsorption curve gives an indication of the size and shape of
the pores, a bimodal pore distribution can be evidenced using this technique [66].
SEC makes use of a set of linear polystyrene standards with a molecular weight
between 600 and 3.7\times10\textsuperscript{6} Da (having molecular radii of 2.7 to 453 nm respec-
tively). The molecules that are too large to enter the porous structure elute in
the flow-through. By comparing the results obtained for a given monolith to those
obtained with a material of known porous structure allows determining the meso-
porosity. The results obtained by SEC and mercury porosimetry are comparable.
However, the pore size distribution obtained with SEC may appear somewhat
broader [67, 68]. SEC has the advantage of being a nondestructive method.

### 3.3.2 Chromatographic properties of silica monoliths

The macroporous structure of silica monoliths prepared by the sol-gel process is
controlled by the composition of the starting mixture. The size of silica skele-
tons and throughpores can be varied independently. The size of throughpores is
normally much larger than the thickness of the skeleton resulting in throughpore
size-to-skeleton size ratios up to 4.0 [31]. This porosity is much greater than in the
case of packed columns (0.25-0.4) resulting in considerably lower flow resistance.
The size of mesopores can be adjusted by varying pH, temperature and reaction
time [37]. The size of mesopores as well as their size distribution are important
when tailoring the monolith surface for HPLC separations. The smaller the meso-
pores, the higher the specific surface area and thus the higher the loadability of
the column. However, small mesopores will tend to hinder the movement of large
molecules such as peptides or proteins in and out of the pores thereby leading to
peak broadening.
The small diffusion pathlength provided by the large throughpores and small
skeletons results in efficient separations and low operating pressures. Because of
these properties, monoliths are able to efficiently separate analytes at flow rates
much higher than can be used with packed columns of similar dimensions. Because
monoliths are not prepared from particles, the impedance (number of theoretical plates per unit pressure drop [69]) is often used to compare monoliths to other HPLC columns as it emphasises the high efficiency obtained at low pressures. The size of throughpores for monoliths reported in the literature ranges from 1 µm [70] to 8 µm [28, 31]. In accordance with theory, the smallest throughpores (1.1-2.0 µm) give the lowest plate height (5-8 µm at 1.0-1.5 mm/s linear flow velocity for benzene derivatives with 80% methanol and ≈ 15 µm at 0.5 mm/s for insulin using 30% acetonitrile) [70, 71] but also a higher backpressure. Even though monoliths with large throughpores (8.0 µm) [28, 31] show a lower plate number per unit length, their backpressure is so low that very long monoliths can be used to compensate for this effect. Large throughpores also offer the advantage of being able to raise the flow rate and thus shorten analysis time [72]. Apart from the low operating pressures required by monolithic columns, the most prominent feature monoliths offer is a very shallow Van Deemter curve. The reduced contribution of mass transfer to plate height allows to accelerate the separation without sacrificing resolution and efficiency. To evaluate the efficacy of a commercial monolith (100*4.6mm, Merck, Japan) for high-speed RPLC of peptides [73], a digest of cytochrome C was injected at flow rates varying from 2.0 to 25 mm/s (10 mL/min). Little change in resolution and peak elution volume were observed. The morphology of silica monoliths is also described by the throughpore size-to-skeleton size ratio. When this ratio reaches very large values (≈ 4.0) [31], the pressure required to operate such a column is very low and thus the flow can be greatly increased. Such a large ratio is thus favoured when high-throughput analysis is required. However, if the sample is more complex and demands on separation efficiency increase, smaller ratios are preferable. Separation efficiency was also investigated in relation to domain size (throughpore + skeleton). The columns with the smallest domain size gave the lowest plate height. A column with a domain size of 5.8 µm showed a plate height of 15 µm while a column with a domain size of 2.3 µm gave a plate height of 5.0 µm for amylbenzene using 80% methanol [27]. Varying the size of the mesopores proved to be important in terms of both loading capacity and separation efficiency. Mesopores, if too small, can hinder the movement of analytes in and out of the pore. Insulin was more efficiently separated on a monolith with mesopores of 25 nm than 14 nm, where plate heights between 20 µm and 40 µm were obtained for the monoliths with large and small mesopores respectively [18]. When further increasing the size of mesopores (140 nm), it has been suggested that steric hindrance is no longer important and that a flow can be generated inside the mesopores [74]. This suggests that a perfusion mechanism
may take place in very large mesopores. However, using monoliths with mesopores of 12.5 and 20 nm, diffusion-limited mass transfer was still observed [75], while the possibility that convection takes place in larger mesopores was not ruled out though. The same study allowed to calculate equivalent sphere dimensions to compare monoliths and packed columns. An equivalent dispersion particle diameter ($d_{disp}$) was calculated in view of the contribution of the $C$-term to band broadening. An equivalent particle diameter based on permeability ($d_{perm}$) was also described based on the operating pressures obtained at different flow rates. $d_{perm}$ was equal to $\approx 15 \, \mu m$ while $d_{disp}$ was found to be $2.5 \, \mu m$ for angiotensin on both the 12.5- and 20-nm mesopore monoliths [75].

Although different from a morphological point of view, monolithic and packed columns are subjected to the same mechanisms involved in band broadening: molecular diffusion, liquid hold-up in the mesopores and stagnant mobile phase at the liquid-solid interface. Therefore, theoretical investigations performed for packed columns may also be applicable to monoliths. The contribution of Eddy diffusion to plate height is mainly affected by the homogeneity of the chromatographic bed, which is inversely related to particle diameter for spherical, particulate LC columns. It was modelled that an improvement in bed homogeneity could yield a reduction in plate height by as much as 50% [19]. Using computational fluid dynamics software, the theoretical band broadening in an ideal monolith was simulated [76]. Reduced plate heights as small as $h_{min}=0.8$ (equivalent to appr. $1.8 \, \mu m$) and separation impedances as small as $E_{min}=120$ for a retained component were predicted. Especially with respect to the Eddy diffusion term, significant improvements (about a factor of 10) as compared to current state-of-the-art monoliths were predicted. This indicates that, as with packed columns, more homogeneous monoliths would give more efficient separations. A mathematical model specifically designed to describe the dynamic behaviour of an analyte in a chromatographic column [77] could help in designing better monoliths with regard to separation efficiency. The model predicts that monoliths should preferably have relatively large throughpores with high interconnectivity and small-sized skeletons with mesopores large enough not to hinder the passage of molecules in and out of the pore [77]. For particulate columns, the effect of the layer of immobilised ligands (e.g. hydrophobic, ionic) on the intraparticle interstitial velocity is rather small, while the effect of the value of the pore connectivity, $n_T$, on the intraparticle interstitial velocity is very large [78]. It can be assumed that this is also valid for monoliths as they exhibit mesopores in the skeleton comparable to the pores in silica particles. The ideal stationary phase for liquid-based separations should provide a large
3.4. LC-MS of peptides and proteins using silica-based monoliths

Surface contact area between stationary and mobile phases, form a homogeneous channel network for the facile transport of mobile phase through the column and maximise channel interconnectivity to limit peak broadening [77, 79]. Silica monoliths possess these characteristics and show very efficient separations, but like porous particles, they still suffer, though to a much lesser extent, from band broadening originating in diffusion-limited mass transfer. Convective mass transfer is very advantageous as it helps to establish an efficient exchange of molecules between stationary and mobile phases, and thus permits raising the velocity of the mobile phase without significantly decreasing column efficiency to achieve fast analysis. The performance of silica-based monoliths under isocratic conditions are well documented [18, 24, 27]. However, they have not been as thoroughly investigated under gradient conditions using proteins and peptides.

3.4 LC-MS of peptides and proteins using silica-based monoliths

The use of three silica-based monoliths exhibiting different morphologies (100*4.6mm, 2.0 μm and 13 nm; 50*4.6 mm, 2.5 μm and 25 nm; 100*4.6 mm, 2.5 μm and 25 nm) was investigated for peptide mapping of a tryptic digest of cytochrome C homologues (from equine, bovine, canine and avian origin) by gradient elution RPLC-MS [72]. Each monolith showed efficient and reproducible separation allowing small differences in amino acid sequence to result in minute and reproducible differences in chromatographic profiles. The main difference between the 2.0-μm and the 2.5-μm throughpore monolith is the flow rate at which they can be operated. The monolith with wider throughpores could be used with flow rates twice as high as the monolith with the smaller throughpores, though at the cost of decreased capacity and selectivity due to the loss of surface area. Monolithic columns (100*4.6mm [37]; 150*0.1mm and 500*0.1mm [80], and 560*0.05 mm, Chapter 4) were used to evaluate their efficacy for high-speed gradient elution RPLC of peptides using tryptic digests of cytochrome c from bovine heart [73] and horse heart [80]. Resolution and retention volume were found to vary very little when the linear flow was increased from 2.0 to 25 mm/s (10 mL/min) for use with the 4.6-mm id monolith. Similar results were obtained with both the 50- and the 100-μm monolithic columns (Figure 3.3). The 50-μm capillary column was operated at flow rates up to 2.0 μL/min (≈20 mm/s), which is more than 10 times the flow rate applicable with a packed column of similar dimension (Chapter 4). This confirms the applicability of silica monoliths to very
fast separations.

Figure 3.3: Total Ion Current (TIC) chromatograms corresponding to the injection of 2.0 µL of a cytochrome c tryptic digest (0.01 µg/µL) on a Chromolith CapRod RP18e silica-based monolithic capillary column (0.1 mm ID, 15 cm length) run at two different flow rates: A = 4.5 µL/min and B = 2.0 µL/min. Mobile phase A consisted of 0.1% formic acid in water. Mobile phase B consisted 0.1% formic acid in acetonitrile. The gradient slope was kept constant. (reprinted with permission from B. Barroso, D. Lubda, R. Bischoff, Journal of Proteome Research 2003, 2, 633; copyright 2003 American Chemical Society [80])

The very high linear flows (up to 25 mm/s) that can be used with monolithic columns are a great advantage in terms of analysis time. However, they raise several problems when coupling monolithic columns to MS. Large-internal diameter columns are coupled to MS using conventional pneumatically assisted electrospray ionisation sources (ESI). A nebuliser gas and a drying gas are used to help evaporation of the eluent and favour the transfer of the analyte to the gas phase. The low flow rates (<0.5 µL/min) used with narrower packed columns (<100-µm id)
normally enables the coupling of LC columns to MS by means of a nanospray interface, without using a nebuliser gas. Due to their very high porosity, capillary monoliths are often used with higher flow rates. Barroso [80] used flows up to 4.5 \( \mu \text{L/min} \) with a 100-\( \mu \text{m} \) id reversed-phase silica monolith, coupled to an ion trap mass spectrometer via a commercially available pneumatically-assisted microESI source. In an attempt to eliminate post-column dead volumes altogether, a 100-\( \mu \text{m} \) id silica monolith was also directly coupled to the mass spectrometer [81]. The electrospray voltage was connected to the stainless steel union positioned in front of the monolithic column, allowing the effluents to be sprayed directly from the monolith. First demonstrated by Koerner et al. [82] with an organic-based monolith, this set-up was also successful with a silica-based monolith [81]. The good-quality ionisation is claimed to result from the small-sized throughpores that act as a set of tapered-nanospray tips [82]. Such a set-up alleviated the need of a tapered tip, which is easily blocked and whose coating is often fragile and short-lived. A stable spray was obtained at flow rates up to 1.0 \( \mu \text{L/min} \) for a wide range of mobile phase compositions. In another application, a 50-\( \mu \text{m} \) ID reversed-phase monolithic column (560 mm) was coupled to an ion trap mass spectrometer via an in-house modified nanospray interface or a commercially available microESI source (Agilent, Germany) (Chapter 4). In the nanospray-configuration, the monolithic column was butt connected to a gold coated nanospray emitter using a 360-\( \mu \text{m} \)-ID Teflon sleeve and subsequently mounted in front of the nanoESI source entrance. Such a set-up introduced very little post-column dead volumes and allowed to obtain a stable spray at most flow rates and gradient steepnesses. However, spray stability was negatively influenced by higher flow rates and extreme gradient steepnesses (>9% ACN/min). Using the nanospray source, a wide range of flows (up to 1.95 \( \mu \text{L/min} \)) and gradient steepnesses (up to 9% ACN/min) could be used. However, efficient ionisation and long-lasting nanospray tips were only possible if the flow rate was kept below 1.0 \( \mu \text{L/min} \). When using a 50-\( \mu \text{m} \) ID monolith, a compromise between sensitivity and analysis time has to be reached. Monolithic columns with a smaller internal diameter could be more easily coupled to MS via a nanospray interface while retaining optimum sensitivity and analysis time, however, they are not yet available.

Silica monoliths were mostly used to shorten the analysis time of a single analyte in isocratic elution mode or a relatively simple mixture of peptides in gradient elution. With more complex samples, the excellent separation efficiency also enabled to combine shorter analysis time with good resolution as will be discussed below. Methanolic extracts of microcystins (hepatotoxic cyclic peptides) were prepared from samples of Microcystis PCC7820 and Anabaena sp. [48] and added...
to a commercial standard of microcystin-YR and methanolic extracts of Baltic Sea Nodularia. The pooled extracts were diluted with water and concentrated on an SPE cartridge. A reversed-phase monolithic column (100*4.6mm) was used for their separation. The analysis time was reduced from 45-60 to 4.3 min when compared with packed columns of similar dimensions [83] without any loss in resolution or sensitivity.

The analysis of the proteome often requires attaining high sensitivity next to separation efficiency. To analyze combinatorial, synthetic peptide libraries, a 100-µm monolithic column (500 mm long) was coupled to a Fourier Transform mass spectrometer [84]. The first synthetic peptide library was based on the sequence, VSXLY (X = one out of all 20 natural amino acids), whereas the second library had the following characteristics, CWXXG (X = amino acids E, N, R, F, P, S, W, Y, L, or H). The high resolving power and low operating pressure of the silica monolith added to the high selectivity of the Fourier Transform mass spectrometer allowed to separate all peptides from these libraries in 30 min, while sensitivity remained adequate due to the capillary format of the monolith. A similar monolith (150*0.1mm) was coupled to an ion-trap mass spectrometer for the analysis of different proteomics samples using a commercially available microESI source [80]. Samples of high complexity and of very different nature were used to demonstrate the capacity of reversed-phase silica monoliths for the analysis of these biomedical samples. In one example, elastin, a rather hydrophobic protein, was digested by various proteases and the digests analysed by LC-MS as a basis for discovering biomarkers of pulmonary disease. Peptide identity was ascertained by MS/MS. Broncho-Alveolar Lavage Fluid (BALF) was collected during fiberoptic bronchoscopy. It contains phospholipids, nucleic acids, proteins and peptides in very low concentrations and high amounts of salt. Good peak shape and chromatographic resolution as well as clear MS/MS spectra were obtained. As part of another biomarker discovery study, a sample of serum from a cervical cancer patient was spiked with 1.26 pmol/µL cytochrome c to act as internal standard and depleted of albumin and γ-globulins [85], digested with trypsin and the resulting tryptic peptides fractionated using a strong cation exchange (SCX) column. The sample was analysed by LC-MS on a 50-µm ID reversed-phase monolith. The very short and fast gradient enabled analysis of one sample every 30 min, which is 6 times faster compared to a packed column. This is a great asset in the comparative analysis of biomedical samples.
3.4. LC-MS of peptides and proteins using silica-based monoliths

Figure 3.4: Base Peak Chromatogram (BPC) corresponding to the injection of 2.0 µL of an in-gel digest of an excised spot on a nanoLC-MS system making use of a Chromolith CapRod RP18e silica-based monolithic capillary column (0.05 mm id 56 cm length) and a Zorbax 300 SB-C18 trap column (0.3 5 mm, 5 µm d<sub>p</sub>). After 5 min loading, elution took place at 1.0 µL/min. The gradient steepness was 1% acetonitrile/min. Mobile phase A consisted of 0.1% formic acid in water. Mobile phase B consisted 0.1% formic acid in acetonitrile.

Due to the low backpressure of monolithic columns, column length is not a limiting factor anymore when the efficiency of a separation needs to be improved (Figure 3.4). To this end, a long monolithic column (900*0.2 mm) was used for the analysis of the metabolome of Arabidopsis thaliana [86]. A cold methanol extract of 100 mg fresh weight of ground leaves was diluted with water and injected. A great variety of analytes was detected comprising glucosinates, flavonoids, phenolic compounds, anthocyanines, membrane lipids, porphyrins and chlorophylls. The length of the column proved to be an effective way to limit ionisation suppression by increasing resolution between analytes while analysis time was kept to about 2h.

An alternative to very long columns in order to separate very complex mixtures is the use of 2D-LC. A 2D-LC system was developed for the separation of complex peptide mixtures taking a tryptic digest of bovine serum albumin (BSA) as example [87]. Fractionation took place on a polymer-based cation exchanger (50*2.1 mm, 5µm d<sub>p</sub>) followed by analysis of the resulting peptidic fractions on a very short RP monolith (25*4.6 mm) coupled to a time-of-flight mass spectrometer via an ESI interface. Alternatively, a capillary-based monolith (100*0.1mm) was
employed as $2^{nd}$ dimension with split injection and flow. The monolith in the capillary format resulted in better MS spectra and sensitivity. Runs were 40 min in the $1^{st}$ and 2 min in the $2^{nd}$ dimension for the 4.6-mm ID monolith. Due to its greater length, runs making use of the capillary monolith were 4 min long. From the analysis of a BSA digest, a peak capacity of 700 was calculated for the 2D-LC set up (Figure 3.5).

![Figure 3.5: Representation of a two-dimensional separation of a bovine serum albumin tryptic digest. A silica-based monolithic capillary column (0.1 mm ID, 10 cm length) was used in the second dimension following prefractionation by strong cation-exchange chromatography. The MS spectra for the spots A, B & C are shown. (reprinted with permission from Kimura H.; Tanigawa T.; Morisaka H.; Ikegami T.; Hosoya K.; Minakuchi H.; Nakanishi K.; Ueda M.; Cabrera K.; Tanaka N.; Journal of Separation Science 2004, 27, 897; copyright 2004 Wiley-VCH).](image1)

A shotgun approach (digestion of the whole sample and separation by 2D-LC [3]) was used for the analysis of the proteome of Arabidopsis thaliana after depletion of Rubisco, a high-abundance protein found in plant leaves [88]. The first dimension was based on SCX whereas the second dimension was performed using RPLC. The silica monolith (500*0.1mm) was coupled to the SCX column by means of a 10-port valve and two C$_{18}$ trap columns to desalt the fractionated sample prior to injection on the monolith. Individual RPLC runs were in excess
of 2h. Using this set-up, a total of about 3500 MS/MS spectra were acquired during each run, enabling to identify about 300 unique proteins. Monoliths appear to be a viable alternative for fast, high-resolution separations of complex samples to the recently introduced UHPLC systems, which require special HPLC pumps and extremely pressure stable stationary phases. We expect that rapid separations by LC-MS using silica-based monoliths will extend the possibilities of LC-MS in proteomics, notably for the comparative analysis of larger sets of samples in biomarker discovery research.

3.5 Other applications of silica-based monoliths in proteomics

In proteomics analysis, the digestion step is of critical importance. In-solution digestion often takes 12h or more. By immobilising proteases on a solid support, digestion kinetics can be greatly enhanced bringing digestion time down to minutes or even seconds [89]. Moreover, immobilisation often results in diminished autolysis [90,91]. Trypsin was adsorbed on monoliths exhibiting different morphological characteristics [33]. Immobilised trypsin was 10-20 times more active than in its free form and was stable for 4-6 weeks when stored at 4 or 25°C. Trypsin activity was monitored by following the catalytic hydrolysis of N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) [92]. The immobilised trypsin could be reused for up to 6 cycles. At that point, 40% of the original amount of trypsin remained adsorbed. It is important to note that washing and reequilibration of the immobilised-trypsin monolith led to a loss of 10-15% trypsin every time. It was shown that catalytic efficiency (kcat/Km) increased with increasing mesopore diameter indicating that diffusional mass transfer played an important role. When trypsin was adsorbed onto the monolith with the largest pores (average pore size: 18 nm), the catalytic efficiency was almost 30 times higher than for trypsin in solution. Even when trypsin was immobilised on the monolith with the smallest pores (average pore size: 3.3 nm), the catalytic efficiency was twice as high as in solution [33], which shows that accessibility in large pores is crucial for enhanced digestion kinetics. The immobilisation of other enzymes on monoliths was also investigated. For example, the influence of the pre-immobilisation of glucose oxidase on silica beads prior to the sol-gel reaction on their activity was studied [40]. Glucose oxidase was first immobilised on a porous support prior to incorporation in a sol for the sol-gel reaction. Activity was found to decrease by only 10% when the enzyme was pre-immobilised whereas it lost as much as 60% when put directly in the
sol. Moreover, pre-immobilisation eliminated bleeding of the enzyme [40]. When protease P (from Aspergillus melleus) was immobilised, the internal diameter of the monolith was varied and found not to have any influence on the conversion rate of the substrate when the linear flow was kept constant [47]. Three monoliths of the same diameter were butt-connected and the conversion rate increased by as much as 50%. This protease P micro-bioreactor performed better at high flow rate than the control batch experiment whereas it did not match the conversion rates of the batch experiment at lower flow rate. It suggests that convective flow took place in the enzyme reactor at high flow rates and thereby enhanced the digestion rates. Frequently, proteomic samples are separated by 2D-PAGE and individual spots in-gel digested. These samples need to be desalted and enriched prior to off-line nanospray or MALDI analysis. To this end, a platform making use of pre-treatment tips based on monolithic stationary phases with various surface chemistries corresponding to biomolecules of various characteristics (hydrophilic, hydrophobic, phosphorylated) was developed [61]. C_{18} tips were employed for the desalting and concentration of peptide samples. Titania-coated tips were applied to the isolation of phosphopeptides (Figure 3.6), as titanium dioxide recognises phosphorylated substances. These tips exhibited satisfactory sample capacity and dead volumes for protein/peptide analysis and can be integrated in automated sample preparation systems. Further investigations towards the chemical derivatisation of silica monoliths will likely extend possibilities for sample pretreatment. MALDI is widely used for generating gas-phase ions from thermolabile bio-macromolecules notably in proteomics. To enhance its possibilities, a MALDI plate was modified with a silica monolith for the analysis of di- and tri-peptides [44]. The MALDI matrix, \(\alpha\)-cyano-4-hydroxycinnamic acid, and the analytes were added to the sol before the sol-gel process took place on the MALDI plate. Different monoliths with varying pore sizes were synthesised but they all gave similar spectra. Though this approach may be beneficial with respect to sample capacity, it still needs to prove its practical relevance for complex proteomics samples.
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Figure 3.6: Pretreatment of a tryptic digest of β-casein using a titania-coated silica-based monolithic extraction tip to enrich phosphorylated peptides. (A) Chromatogram of sample without pretreatment, (B) Chromatogram of sample with pretreatment. LC analysis took place on a MonoCap silica-based capillary column (0.1 mm ID, 15 cm length). Mobile phases A and B consisted of 0.05% trifluoroacetic acid in water and acetonitrile respectively. (reprinted with permission from Miyazaki S., Morisato K., Ishizuka N., Minakuchi H., Shintani Y., Furuno M., Nakanishi K.; Journal of Chromatography A; 1043, 19; copyright 2004 Elsevier)
3.6 Conclusion

One of the latest developments in column technology is the development of monolithic columns, which overcome some of the limitations associated with packed columns when it comes to throughput and diffusion-limited mass transfer. Monolithic columns were first developed using organic monomers. Several years later, progress in silicium chemistry allowed preparing silica-based monoliths. The bimodal pore structure of silica monoliths exhibits large, interconnected through-pores and smaller mesopores inside the thin skeleton defining the throughpores. This results in high porosity and small distances over which the analytes diffuse. These characteristics allow very efficient separations at low pressures thereby enabling to speed up the analysis of complex samples. Two approaches seem to prevail in HPLC-related research on monoliths. The first is to obtain very efficient separation media by synthesising monoliths with very small throughpores and fine skeletons at the cost of an increased operating pressure. The second is to prepare monoliths with large throughpores requiring very low pressures thereby allowing very long columns to be used. Even though the effects of most synthesis conditions on the morphology of monoliths are understood, more research is needed to design more homogeneous and efficient stationary phases.

3.7 List of abbreviations

- APTES: (3-aminopropyl) triethoxysilane
- BAPNA: N-α-benzoyl-DL-arginine-p-nitroanilide
- BET: Brunauer-Emmett-Teller
- BSA: Bovine serum albumin
- CEC: Capillary electrochromatography
- CEOS: 2-Cyanoethyltriethoxysilane
- DGS: Diglycerilsilane
- ESI: Electrospray ionisation
- ETMS: Ethyltrimethoxysilane
- HPLC: High-pressure liquid chromatography
- LC: Liquid chromatography
- MALDI: Matrix assisted laser desorption/ionisation
- MS: Mass spectrometry
- MS/MS: Tandem mass spectrometry
- MTMS: Methyltrimethoxysilane
- PEG: Poly-(ethylene)glycol
3.8 List of symbols

- pI: Isoelectric point
- RPLC: Reversed-phase liquid chromatography
- SCX: Strong cation exchange
- SEC: Size exclusion chromatography
- SEM: Scanning electron microscopy
- TEM: Transmission electron microscopy
- TEOS: Tetraethoxysilane
- TMOS: Tetramethoxysilane
- TOF: Time-of-flight
- TSC: Tetrahedral skeleton column
- UHPLC: Ultra high-pressure liquid chromatography
- 2D-LC: Two-dimensional liquid chromatography
- 2D-PAGE: Two-dimensional polyacrylamide gel electrophoresis

### List of symbols

- $C_d$: Axial diffusion in the mobile phase
- $C_e$: Eddy dispersion
- $C_m$: Mass transfer in the mobile phase
- $C_{sm}$: Mass transfer in the stationary phase
- $d_{disp}$: Equivalent dispersion parameter
- $D_m$: Solute diffusion coefficient in the mobile phase
- $d_p$: Particle diameter
- $d_{perm}$: Equivalent permeability parameter
- $E$: Separation impedance
- $H$: Plate height
- $k_{cat}/K_m$: Catalytic efficiency
- $\eta T$: Pore connectivity
- $P_t$: Pressure of transition
- $u$: Linear flow velocity


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