A nanoLC-MS-based platform for peptide analysis

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2006

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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NanoLC-MS-based platforms present interesting features for the study of the biochemical processes underlying biomedical events. This thesis reports on several studies in which various aspects and components of such a nanoLC-MS system have been optimised, with the aim to rapidly, precisely and accurately quantify peptides in complex matrices. Limitations such as low signal reproducibility, lengthy analysis times and poor system robustness were overcome. Though much progress has been made generally in the field, nanoLC-MS is not yet a user-friendly technique and still requires dexterity. Nevertheless, the latest technological developments have started to make the use of nanoLC-MS more straightforward for inexperienced researchers.

### 7.1 Nanospray interface

A nanoESI interface was built in-house and the different parameters likely to influence the quality of the spray and the spectra were investigated. The position and dimensions of the nanospray, as well as the composition and flow rate of the mobile phase, were shown to greatly influence the spray and the resulting spectra. Positioning the nanospray tip at a 20° angle with respect to the heated capillary provided the best S/N, whereas signal intensity was highest when the interface was positioned axially and close to the heated capillary. While a larger %ACN
in the mobile phase required a lower spray voltage to obtain a stable spray, the spray voltage had to be carefully adjusted at low % ACN (i.e. 10%). Varying the FA concentration had a clear impact on both signal intensity and the charge envelop. An increase in the %FA in the mobile phase resulted in a shift toward lower m/z. 1% FA, though, led to a strong reduction in signal intensity. Larger intensities were found for coated needles with a small-ID tip.

7.1.1 Perspectives

Ideally, sprays should be stable at every flow rate, under every mobile phase composition and at every analyte concentration. Such a situation has yet to be accomplished, however. For example, it has been demonstrated by Schmidt et al. [1] that there was no ionisation suppression when using very small ID (1 µm) tapered emitters at very low flow rates (<20 nL/min). Emitters allowing the unsuppressed ionisation of analytes at larger flow rates would thus be an attractive addition for these systems. To this end, multiple emitters should be built following the principle described by Tang et al. [2] As already stated, the flow rate through the individual emitters should not be larger than 20 nL/min and the emitter opening should be approximately 1 µm. Therefore, the flow rate to be used determines the number of emitters on the multiple-emitter interface. As an example, to optimally couple a 50-µm-ID packed column used at a flow of 100 nL/min to MS would thus require an emitter with 5 openings of approximately 1 µm ID. The distance between each emitter is likely to strongly influence the sprays formed at each emitter. If the emitters are too close, the sprays formed could (partially) overlap and result in the collision of the small droplets emitted, thereby producing larger droplets and reducing ionisation efficiency. Chip technology is the most amenable way to tackle this problem. The design of the masks used for photopatterning can easily be changed to vary the distance between pairs of emitters. Additionally, the chip could be (partially) made of a conductive polymer that would not require coating of the emitter with gold or other metal, thereby ensuring greater durability of the emitters.

7.2 Column technology

Silica-based monoliths allow high-efficiency separation of complex samples in a fraction of the analysis time required with particle-packed columns. The bimodal pore structure of silica monoliths exhibits large, interconnected throughpores and smaller mesopores inside a thin silica skeleton, resulting in high porosity and only
small distances over which analytes must diffuse. There are two main approaches to preparing monoliths. Monoliths either exhibit large throughpores, offering very low flow resistance at the cost of separation efficiency, or small throughpores and a relatively thick skeleton, allowing very efficient separations though at the cost of higher operating pressures. The monolith we characterised exhibited throughpores of 2-3 μm, a skeleton of 1.0-1.5 μm and mesopores of 18 nm, and appears to be a compromise between the two aforementioned synthesis approaches. Its high efficiency and low flow resistance allowed the separation of very complex mixtures in a much shorter time than what is usually achieved with packed columns. Peak widths at half height were on the order of 10 s and separations were very repeatable. A wide range of flow rates (up to ≈ 20 times the flow used with packed columns) and gradient slopes (up to 9% ACN /min) could be used, depending on the complexity of the separation. Moreover, the specific surface area was sufficient to load large sample volumes and achieve good detectability of low-level analytes.

7.2.1 Perspectives

Though silica-based monoliths allow high-efficiency separation at a fraction of the analysis time required with particle-packed columns, several improvements still remain to be achieved. Even more efficient and faster separations could be attained by optimising the synthesis of silica monoliths. Ideally, the skeletal porosity should be very high to achieve very fast separations. Our monolith had a 1.0-to-1.5-μm thick skeleton and was 90 to 95% porous. Monoliths with ”paper-thin” skeletons should thus be more porous and allow faster separations. Moreover, computer simulations predict that an increased porosity should result in more efficient separations [3]. Smaller throughpores were shown to result in more efficient separations, due to the limited distance over which analytes have to diffuse to interact with the stationary phase. Additionally, mesopores should be interconnected and large enough not to hinder the passage of molecules in and out of the pore and to allow convective transport inside the pores. Our monolith had mesopores of 18 nm, which is comparable to the pore size of porous silica beads, which do not exhibit convective transport inside their pores. Thus, we can assume that separations on our monoliths were still limited by the diffusion of analytes in and out of mesopores. Monoliths with large mesopores (>400 nm) have been reported [4], but did not fulfill the promise of more efficient separations. Since separation efficiency is strongly correlated with homogeneity of the chromatographic bed, it is safe to assume that the large mesopore size distribution observed was underlying the fact that separation effi-
ciency did not improve. Therefore, novel monoliths allowing more efficient and faster separations should have well-interconnected pores in the sub-micron range and skeletons as thin as possible. There might be only one type of pore and not two as is the case now. Moreover, both the pore and skeleton size distribution should be as narrow as possible. Such structural characteristics would allow the use of very long LC columns much like GC columns in conjunction with UHPLC equipment to achieve plate numbers of several hundreds of thousands. However, a very thin silica skeleton might not be strong enough to withstand the pressures generated in UHPLC, in which case the use of oxides other than silica might lead to the synthesis of stronger monoliths. The limited stability of silica at extreme pHs presents another reason to turn to alternative monolith materials. Moreover, such monoliths could exhibit a selectivity different from that of silica. For example, metal oxide-based (e.g. TiO$_2$) stationary phases show great affinity for phosphorylated analytes. Additionally, TiO$_2$ is much more resistant to extreme pHs than silica and would offer more flexibility in choosing the conditions under which a column can be operated.

Chromatographic beds with very well defined structures, which can be compared to that of monoliths, were prepared by microfabrication [5]. However, though channels (that we can compare to the throughpores of a monolith) were only a few µm wide and deep, the pillars (the equivalent of the skeleton in monoliths) were still too thick to allow very fast separations. Following the idea developed by Regnier, monolith-like chips could be prepared with greater permeability in order to speed up analysis. However, the fabrication of a chip with structural features similar to that of the ideal monolith will require technologies developed for nanotechnologies, such as e.g. e-beam writers.

7.3 Multidimensional chromatography

Due to the great complexity of the proteome of an organism, multiple-step analyses are required in order not to lose a considerable amount of information. Very abundant proteins (e.g. albumin) often mask proteins and peptides of lower abundance and thus interfere with their detection and identification. Therefore, samples must be effectively depleted of these very abundant proteins. For this purpose, three columns were coupled on-line in a novel, though simple, and robust set-up for multidimensional nanoLC-MS (Chapter 5). No additional switching valve or pump was required to couple the three different columns. A RAM cartridge was coupled to nanoLC-MS for the analysis of a neuropeptide and, after optimisation, virtually all BSA was discarded (Chapter 5). Separations were very
efficient with peak widths at half height of 10s. Such narrow chromatographic peaks were rather unexpected due to the large size difference between the RAM cartridge and the nanoLC trap column, introducing significant pre-trap-column dead volume. The total cycle time including albumin depletion, separation of the analytes, washing and re-equilibration of the system, was about 40 min.

### 7.3.1 Perspectives

Further developments of this modular system would involve the use of stationary phases of different selectivity in the cartridge and the trap column. RAM particles derivatised with strong cation-exchange functionalities would reduce the risk for peptides breaking through on the RP trap column. Additionally, the sample could be more easily fractionated using salt concentration steps, thereby reducing the complexity of the sample and increasing the system peak capacity. Alternatively, an immobilised-enzyme reactor (IMER) could be coupled on-line with set-ups similar to those described in Chapter 5 and Chapter 6. The use of IMERs reduces the digestion time and allows automation of the digestion step. Improvements of digestion rates and sequence coverage can be obtained by derivatising the immobilised enzymes according to the concept presented by Freije [6], using a range of derivatives. Digestions are often performed in mixed aqueous-organic media in order to increase sequence coverage and speed up the digestion step. However, such samples cannot be directly injected onto an RPLC system because peptides are likely to break through the trap column. A multidimensional nanoLC system, based on the set-up described in Chapter 6, would benefit from a mixed-mode trap column containing both HILIC and RP stationary phases, to allow every peptide to be trapped.

The set-up described in Chapter 5 does not contain an additional switching valve or pump, and is therefore amenable to the implementation of multidimensional chromatography on a chip. On-chip sample manipulation would benefit from the implementation of novel microfluidic concepts like that of the use of recirculating flows in microchannels to trap large molecules or particles [7]. Recirculating flows could be used for sample preconcentration before and/or after albumin depletion as well as for digestion. Recirculating flows are likely to improve digestion rates and increase sequence coverage by dragging the proteins to be digested repeatedly along the protease-coated microchannel.
7.4 Non-specific interactions

It has been shown that adsorption of proteolytic peptides to the vial surface is the underlying cause of repeatability problems in the quantitative analysis of peptides. Improved peptide recovery and greater signal repeatability are obtained by improving peptide solubility, which directly influences the adsorption equilibrium for the peptides between the solution phase and the vial surface. Addition of varying amounts of DMSO to the sample vial reduced peptide adsorption by up to 80%, consequently leading to greater repeatability and lower LOD.

7.4.1 Perspectives

Adsorption of peptides to system surfaces is underlying repeatability problems in quantitative analysis. As in vials and in CE, peptides and proteins are also likely to adsorb non-specifically to the capillary or channel walls used in miniaturised analytical systems. Based on the work by Righetti, derivatisation of the capillary walls with non-ionic and zwitterionic [8] surfactants or large polymers [9, 10] led to a reduction in protein adsorption of more than 90%. The same principle is applicable to vials, capillary walls in LC analysis and microchannels in chip-based analysis. Alternatively, novel hydrophilic polymers could be developed to serve as substrate materials for microfluidic device fabrication.
References


