Nanocolumn Developments for LC-MS Proteomics

Nanocolumn technology has changed little over the years, but the applications in which they are used have changed significantly for the better

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During the last 30 years, low-flow liquid chromatography (LC), and especially nanoLC, has established itself as one of the main separation technologies for complex proteomics mixtures prior to mass spectrometry (MS). During these years, nanoLC has moved away from flow splitter supported LC pumps, manual split-injections, and low volume fused silica UV flow cells (1). Nowadays, nanoflow ultra-high-performance LC (UHPLC) has become available from a range of manufacturers, with optimised low volume flow paths and virtually zero-dead-volume connections.

Initially, the interest in nanoLC – despite all the adaptions that needed to be made to analytical LC systems – was due to the ability to analyse small sample amounts. Typically, a nanocolumn has an internal diameter (ID) of 75 µm and runs at 200-300 nL/min. This means the nanocolumn has a much-reduced column volume in comparison to a microflow 2.1 mm ID column. Taking the sensitivity factor calculation as a guideline, the nanocolumn would provide the same sensitivity as the 2.1 mm column with approximately 780 times less sample mass injected (2).

The introduction of nanospray ionisation in 1996 made the coupling of nanoLC to MS much more straightforward and helped push the number of labs taking up nanoLC-MS (3). The concentration-dependent nature of the nanospray ionisation comes together perfectly with the low-flow and high-sample concentration features of nanoLC. With the continuous improvements regarding MS capabilities and sensitivity, the development of nanoUHPLC with 2 µm and sub-2 µm particles in packed bed columns with a length of 50 cm and longer, and the already mentioned improved and virtually dead-volume-free connections, nanoUHPLC/MS has become easier to run and maintain.

However, despite all the developments to push performance and reliability of both the nanoUHPLC and MS systems, nanocolumn technology has changed little over the years. Initially, nanocolumns were packed with 5 µm particle C18 stationary phases, with 300Å pore size; however, the ID of the nanocolumn was 75 µm, and the typical length was 15 cm. A recurring comment, still made today, from new and experienced users is the 'perceived' lack of robustness and reproducibility of the nanocolumns they use. The low volume UHPLC connections have made quite an impact in making proper connections, no more self-assembly of sleeves, nuts, and ferrules, but exchanging the column is still one of the reasons to shut down the whole set-up, and restart after column installation. This is a time-consuming process, and especially when the column loses its performance faster than expected as more column exchanges are required.

With the introduction of nanoUHPLC in 2005, the increased pump pressure capabilities allowed for the use of smaller particles, moving from 3 µm particles to 2 µm and sub-2 µm particles. With the higher-pressure capabilities becoming available, up to 1,200 bars, longer columns could be employed as well, even with those smaller particles. Standard nanocolumn length has moved towards 50 cm, typically operated around 500-600 bars, and even 75 cm long nanocolumns are now available to investigate the most complex proteomic samples. However, they are still packed bed columns, with the particles of stationary phase tightly packed together within two frits to maintain the integrity of the packed bed. Packing the stationary phase is performed under high pressure and when in use the column switches from high pressure to low pressure and back rapidly. These pressure shocks could be quite large and will influence the stability of the packed bed, which might result in reduced column reproducibility and lifetime, and generate poor analytical results.

An extra point of attention in packing the perfect bed is the size distribution of the particles of the stationary phase. Ideally the particles are of identical size, allowing a homogeneous flow path through the nanocolumn. However, it is challenging to maintain a perfect size distribution, too many differences can have an influence on the quality of the packed bed, the reproducibility of the

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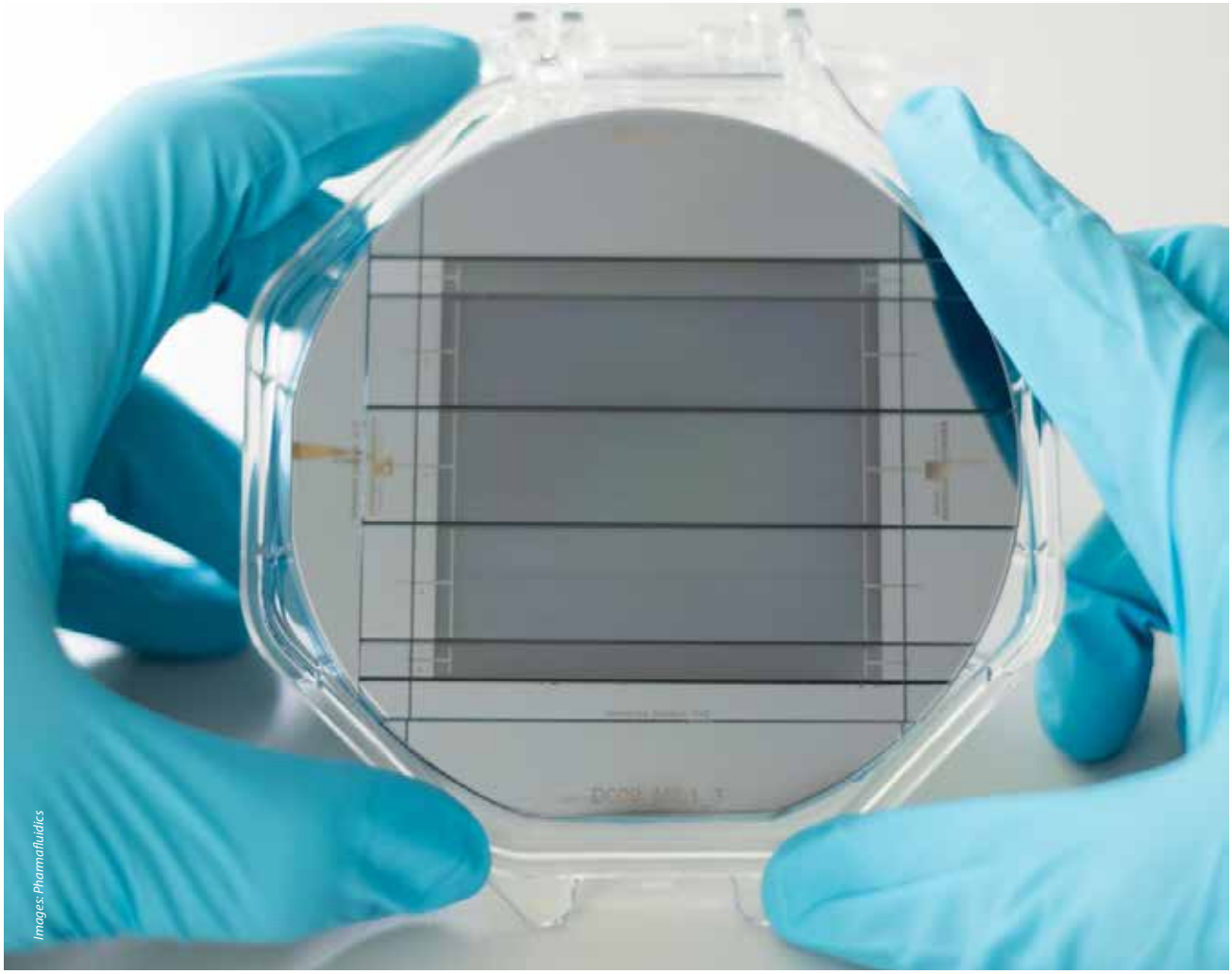
analysis, and even the reproducibility of the column quality in manufacturing.

Chip-cartridge designs have been developed to minimise the number of connections that need to be made from the nanoUHPLC to the MS. One advantage of such cartridges is that the nanocolumn and emitter are easily integrated; this is probably the most critical connection that has to be made. There are even cartridges where a trapping column – to help in quick sample loading and sample clean-up – is integrated. However, the nanocolumns employed in these designs are still based on packed-bed stationary phases, and still pose the possible issues aforementioned.

The same is valid for nanocolumns with integrated emitters. The emitters can be pulled at the tip of the fused silica nanocolumn, or attached during manufacturing. But, again, they are packed bed columns, and can demonstrate the same issues as single columns.

Another drawback of these is that they require specifically designed sources for the MS. This limits the use of the nanoUHPLC/MS to the column design that fits that particular source for that particular MS.

However, there are two completely different formats of nanocolumns that have been developed as well. In the early 2000s, monolithic columns, either silica or polymer



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based, were introduced commercially, including monoliths with an internal diameter of 100 µm and 200 µm.

Consisting of a single continuous rod of separation media, they did not need any frits, removing the potential clogging sites that packed bed column typically has. With a structure that consists of macropores, to accommodate the flow of the mobile phase, and the mesopores, which form the surface area structure where the interaction with sample compounds occurs, these columns were particularly suited for the separation of peptides.

Additionally, the macropores are typically larger than the spaces between particles in a well packed bed, reducing the risk of clogging due to particulates or precipitated sample compounds, helping to increase the robustness of the column. Despite multiple research groups demonstrating the performance of monolithic columns, they have largely disappeared from the market due to the lack of column to column reproducibility (4).

The second format utilises modern lithographic micro-machining techniques to create an array of perfectly ordered and precision positioned free-standing micro-pillars in a narrow channel on a silicon wafer. This is a procedure that can be repeated identically for every new wafer, overcoming possible column-to-column reproducibility issues. This allows for a most homogeneous flow path of the sample through the micro-pillar column, maintaining a higher concentration of the compounds in the column and reducing the band broadening effect. Additionally, the number, size, and positioning of the micro-pillar have a direct impact on the column backpressure. With the freedom of lithography to create such pillars, typical backpressures can be reduced to 100 bars or less, while at the same time extending the column length up to 200 cm or more. Like the monolithic columns aforementioned, these column contain no frits, and the pillar backbone has a high permeability, reducing the clogging risk.

Next to these nanocolumn developments, the applications in which they are used are also progressing. Proteomics is the main field of research for nanocolumns, and, as described above, has moved from digested single protein and SDS-gel band analysis to 2D LC-MS workflows, to comprehensive proteomic LC-MS analysis of thousands of digested proteins using 50 cm and 75 cm long packed bed columns, or 50 cm and 200 cm long micro-pillar array columns. With these changes in applications, the trend has been to use longer gradient times to obtain more details of the proteome analysed.

One of today's eye-catching applications is clinical proteomics, aiming at discovering protein differences related to diseases, to provide biomarkers for diagnostic and prognostic purposes. Clinical proteomics investigations also look at the possibilities of predicting therapeutic responses (5-6). However, there are larger patient sample cohorts that need to be analysed (7). Shorter analysis times, and more robustness of the methods are becoming more essential to generate the information at the required time.

This is where separation columns come in again, looking at microlitres per minute flow rates, rather than nanolitres. More sample injections per column, higher column to column reproducibility, without the loss of resolution and sensitivity to find these low abundant protein biomarkers. Keep an eye out for these developments, and see for yourself where and how you can utilise the power of such columns in your daily LC-MS work.

References

1. *Chervet M* et al, *Instrumental requirements for nanoscale liquid chromatography,* Anal Chem *68(9): pp 1507-12, 1996*
2. *Meiring E* et al, *Nanoscale LC–MS: Technical design and applications to peptide and protein analysis,* Sep Sci *25(9):2002*
3. *Wilm M* et al, *Analytic properties of the nanoelectrospray ion source,* Anal Chem *68(1): pp1-8, 1996*
4. *Hayes R* et al, *Core-shell particles: preparation, fundamentals and applications in high performance liquid chromatography,* J Chromatogr A, *29(1357): pp36-52, 2014*
5. *Verrills NM, Clinical proteomics: present and future prospects,* Clin Biochem Rev, *27(2): pp99-106, 2006*
6. *Frantzi M* et al, *Clinical proteomics: closing the gap from discovery to implementation,* Proteomics, *18(14): 2018*
7. *Visit: www.evosep.com/project/what-matters-in-clinical-proteomics-1-challenges-of-cohort-studies/?et_fb=1*

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Robert van Ling started 25 years ago with LC Packings, one of the front runners in the development of nanoLC and nanoUHPLC, and was responsible for the introduction of the UltiMate systems and PepMap nanocolumns. Since then, he has been responsible for the chromatography part of proteomics and protein analysis at Dionex Corp. and Thermo Fisher Scientific. Robert joined **PharmaFluidics** in 2018, and is an avid fan of their µPAC™ technology.