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Nano-LC: Big Data From Tiny Volumes

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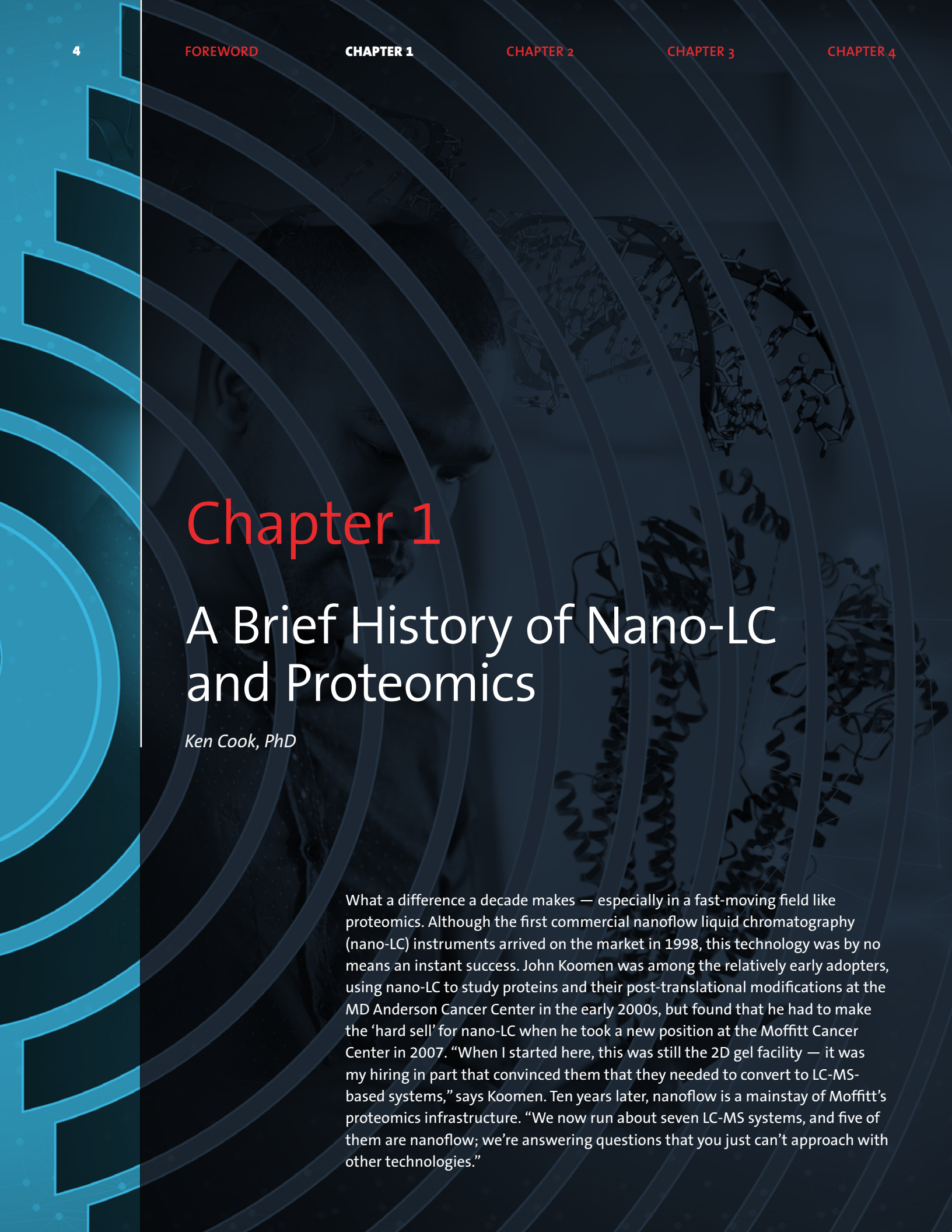
Foreword

The dominant technology of modern proteomics is certainly liquid chromatography-mass spectrometry (LC-MS). However, it is safe to say that the primary discussion point in terms of technology in proteomics has been the mass spectrometry part of this partnership. A great deal of emphasis is placed on the mass spectrometer and the data generated by it.

There has been much progress and innovation in mass spectrometry especially for proteomics and protein analysis. Researchers are pushing the boundaries of scientific research — multiplexed quantitation of low abundance peptides in complex matrices, characterization of positional isoforms of intact proteins, protein structure characterization and deep mining of post-translational modifications are few examples. Innovations in mass spectrometry have continued to deliver new levels of sensitivity, selectivity and versatility to enable life scientists to obtain the highest quality data to better enable our understanding of life and to better human health.

However, there has been less focus and emphasis placed on the separations of peptides and proteins prior to analysis by mass spectrometry in terms of the nano-LC chromatography systems and columns contributing to proteomics performance. One half of the LC-MS combination has been neglected.

Therefore it seemed to the authors that it was time this imbalance was redressed. The following handbook has been produced in an attempt to educate and inform on the relevance and significance of liquid chromatography to proteomics, in particular reversed-phase nano-LC directly coupled to MS.



Chapter 1

A Brief History of Nano-LC and Proteomics

Ken Cook, PhD

What a difference a decade makes — especially in a fast-moving field like proteomics. Although the first commercial nanoflow liquid chromatography (nano-LC) instruments arrived on the market in 1998, this technology was by no means an instant success. John Koomen was among the relatively early adopters, using nano-LC to study proteins and their post-translational modifications at the MD Anderson Cancer Center in the early 2000s, but found that he had to make the 'hard sell' for nano-LC when he took a new position at the Moffitt Cancer Center in 2007. "When I started here, this was still the 2D gel facility — it was my hiring in part that convinced them that they needed to convert to LC-MS-based systems," says Koomen. Ten years later, nanoflow is a mainstay of Moffitt's proteomics infrastructure. "We now run about seven LC-MS systems, and five of them are nanoflow; we're answering questions that you just can't approach with other technologies."

The delayed impact is understandable. Although the reduction of flow rates from tens or hundreds of microliters per minute to less than one microliter per minute yielded clear and significant gains in sensitivity, the earliest iterations of nano-LC required considerable effort and expertise. For example, researchers initially had to pack columns with particles themselves, a feat which was considerably more challenging at the nano-scale than with the larger capillary flow or analytical flow LC columns. "The variance in that was phenomenal," says Paul Taylor, who manages the SPARC BioCentre at Toronto's Hospital for Sick Children. "Now we're buying the columns and they're made with a degree of reproducibility." New users also discovered that even tiny flaws in the setup could have a disproportionate impact on separation success at the nanoscale. "And it's really hard to troubleshoot when you can't see the leaks," says Alex Hebert, a researcher in Joshua Coon's proteomics lab at the University of Wisconsin at Madison.

The technology soon evolved to become more robust and reliable. One of the first big leaps forward came in 2005, with the commercial launch of 'splitless' nano-LC systems

However, the technology soon evolved to become more robust and reliable. One of the first big leaps forward came in 2005, with the commercial launch of 'splitless' nano-LC systems. These replaced the early generation of split-flow instruments, in which only a small fraction of the solvents that were hooked up to the system was subjected to nanoflow separation while the rest went to waste. Further gains came with the development of ultra-high-performance LC (UHPLC) nanoflow systems, which have a considerably higher tolerance for pressure and therefore allow for the use of longer columns that deliver far superior separation for larger numbers of peptides. Indeed, Koomen estimated that nano-UHPLC essentially doubled the amount of data produced by a single proteomics experiment. Cumulatively, these developments made nano-LC far more accessible, eliminating much of the guesswork and unreliability that previously plagued the technology. "It was a great big jump," says Taylor. "The field went from making your own manual columns and guessing your flow rate — which could be anywhere from 50 to 750 nL/min depending on how you had the split flow set up — to actually having pumps that had built-in flow sensors and actually made a reproducible gradient."

The combination of nano-LC and MS has proven a major boon for efforts to thoroughly dissect proteomes in both basic and clinical research settings. One of the earliest milestone publications came in 2001, from John Yates' team at The Scripps Research Institute. The team developed a strategy called multidimensional protein identification technology (MudPIT)[1], which used two-dimensional nano-LC separation as a prelude to tandem MS to detect and identify nearly 1,500 proteins from the yeast *Saccharomyces cerevisiae*. At the time, this represented the most extensive characterization of a cellular proteome to date. In 2008, researchers led by Matthias Mann at the Max Planck Institute for Biochemistry achieved an even more comprehensive analysis of this organism[2], in which they were able to essentially characterize and compare the entire proteomic contents of both haploid and diploid *S. cerevisiae* cells. And in 2015, Coon, Hebert and colleagues published a study showing that similar analyses could be achieved with unprecedented speed[3]. "We figured out how to

do a yeast proteome in one hour, and a large part of that was due to using good chromatography," says Coon.

For biomedical research applications, such as the cancer proteomics studies conducted by Koomen's group, nano-LC is an ideal way to make the most of a small sample. "Whether it's animal models or even human tumor tissues, you really can't go back and ask people to bring you more sample — it really limits you in terms of the total amount of material that you can get," says Koomen. In one recent study, researchers led by Tamar Geiger at Tel Aviv University used a nano-LC-MS approach to quantitatively analyze 10,000 different proteins in tumor tissue samples collected from 88 different patients[4], as a step towards identifying diagnostically or prognostically relevant proteomic 'fingerprints'. Nano-LC-MS is also effective in surveying post-translational modifications, which are found on only a subset of proteins and may be present on those proteins only at low stoichiometry. For example, Mann teamed with Gianni Cesareni's group at University of Rome Tor Vergata to thoroughly assess how both protein expression levels and site-specific phosphorylation are altered in breast cancer cells treated with metformin, a drug for diabetes that also exerts antitumor effects[5].

Oleg Krokhin's group at the University of Manitoba is using nano-LC to gain deeper insights into retention time, as a means for better understanding and predicting the migration of peptide fragments through the chromatography column. "Nanoflow has allowed us to get access to very large datasets of peptides," he says, noting that his team's current collection now includes data on more than one million peptide sequences. These retention time measurements can be critical for the accurate analysis of mixtures separated by chromatography. "You can use retention time prediction to filter your false-positive identifications," says Krokhin. "If you're not super confident about a result, you can look at your peptide's chromatographic retention time and ask if that peptide could come out of the column there."

Nano-LC does not yet represent a universal solution for all separations, and there are still applications for which higher flow rate capillary or analytical LC are better choices. "If I had the choice of working in proteomics with normal flow rate LC systems, I would — it's much more reproducible," says Krokhin. Accordingly, if maximal sensitivity and low sample quantities are not the most pressing issues, nano-LC may be less of an ideal fit. And although nano-LC is a great way to discover useful biomarkers, it is probably not the best way to routinely detect these markers in a clinical diagnostics setting. "The time commitment for a nanoflow experiment — even at the low end — is 30 to 45 minutes," says Koomen. "Whereas analytical scale experiments, typically the maximum time would be 10 to 15 minutes, and many can be done in two or three." And even with a cutting-edge system, the fundamental challenges of working at nanoscale can make it challenging to consistently perform successful nano-LC-MS analyses. "It would be a big deal to make these things super-bulletproof so that they just work," says Coon. "A lot of PhDs still struggle to run it."

“You just plug a cartridge in and it does all the chromatography you need.”

But the technology continues to evolve. For example, user-friendly chip-based systems are emerging that can minimize the amount of tinkering required to get an experiment underway. “You just plug a cartridge in and it does all the chromatography you need,” says Koomen. He notes that these are not a universal solution, and many applications still benefit from hand picked column configurations, but has found the systems to be valuable for training purposes. “It’s much easier to troubleshoot,” says Koomen. Alternatives to the standard reversed-phase chemistries could be a major asset for peptide identification — and Krokhin notes that up to one-fifth of all peptides may be lost in a conventional nano-LC column. His group is also working on extending separation performance by taking LC into the third dimension — using three different configurations of reversed-phase with distinct ion-pairing reagents and pH conditions to achieve three orthogonal stages of separation. “It provided very uniform delivery of peptides to the mass spectrometer, with 126 fractions that all had very even numbers of peptides and mass spectra,” says Krokhin.

For now, nano-LC is a field where extra effort can bring considerable rewards — and those benefits are what keep the proteomics researchers coming back. “When you’re going after low-abundance peptides, people quote 50 times more sensitivity for nano versus capillary, and I think that’s about right,” says Taylor. “Most of our projects involve very complex mixtures and we want to make sure we dig down as deep as we can.”

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3. The one hour yeast proteome. https://www.ncbi.nlm.nih.gov/pubmed/24143002
4. System-wide Clinical Proteomics of Breast Cancer Reveals Global Remodeling of Tissue Homeostasis https://www.ncbi.nlm.nih.gov/pubmed/27135363
5. Deep Proteomics of Breast Cancer Cells Reveals that Metformin Rewires Signaling Networks Away from a Pro-growth State. https://www.ncbi.nlm.nih.gov/pubmed/27135362

Chapter 2

Nano-LC-MS/MS in Proteomics: Putting it all in Context

Ken Cook, PhD

WHY DO LC BEFORE MS?

Liquid chromatography (LC) separation is a critical component of any experiment exploring the incredibly rich and complex protein mixtures obtained from biological samples such as cultured cell, tissue or blood. The separated samples are then subjected to tandem mass spectrometry (MS/MS), in which molecules such as peptides are fragmented for analysis, and the resulting spectra can subsequently be matched against a sequence database to produce identifications. The intensity of the signal in the mass spectrometer can also be converted into a quantitative measurement of the amount of peptide or protein. Thus, both identification and quantitation can be achieved in an LC-MS proteomics experiment.

This initial LC separation procedure is essential, as the MS instrument measures mass spectra serially rather than in parallel, and can only measure ten to twenty high quality mass spectra per second at most. More than that will overload the instrument, resulting in the identification of just a few hundred proteins rather than several thousand. Thus, MS analysis of a complex sample that may realistically contain hundreds of thousands of peptides could require tens of minutes or even hours. LC separation enables the different peptides to reach the mass spectrometer at an optimal rate of MS/MS acquisition. This simple proteomics workflow is illustrated below (Fig. 1).

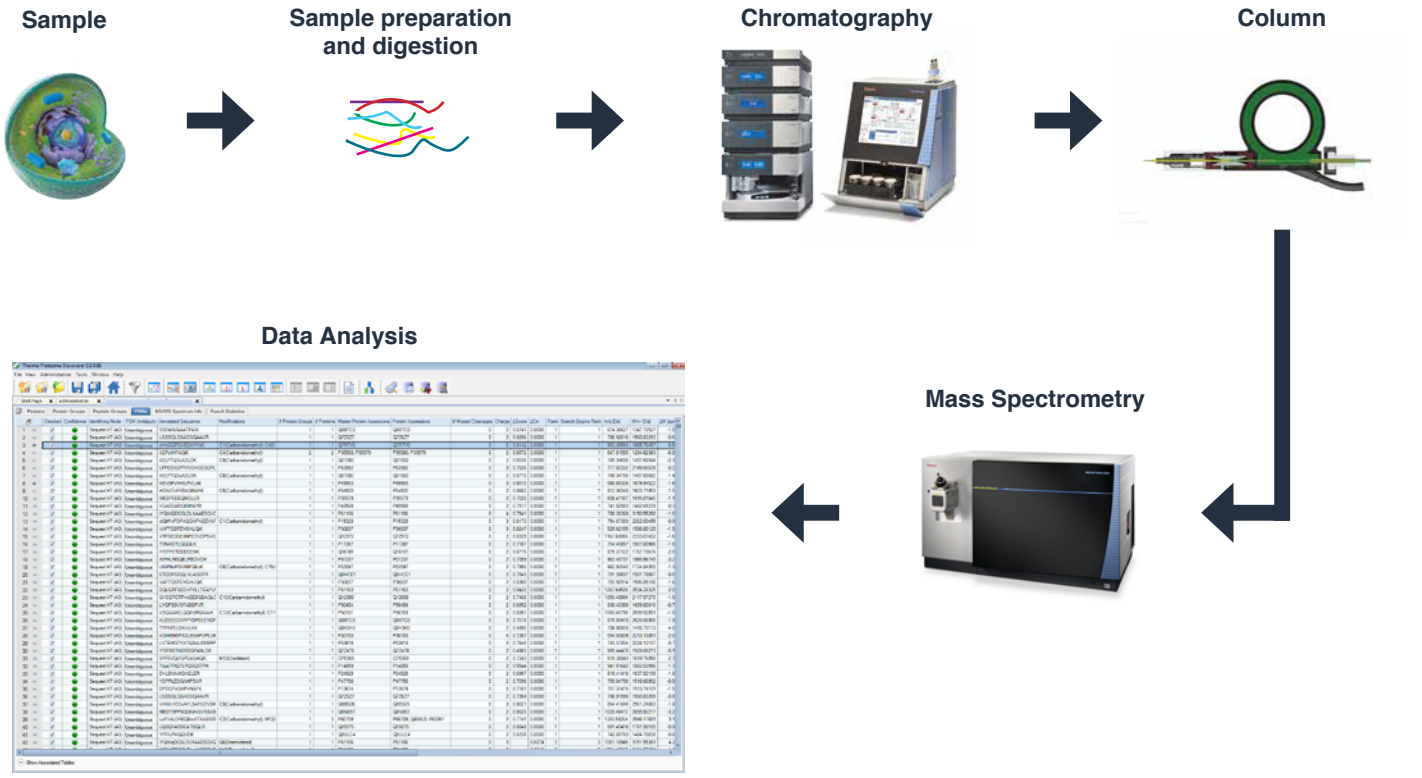


Figure 1. A typical LC-MS based proteomics workflow.

Here and in the chapters that follow, the focus will specifically be on the subset of LC applications that employ nanoflow-LC (nano-LC). Nano-LC analysis begins with the disruption of the sample, the solubilization and denaturation of the various cellular proteins, followed by the proteolytic digestion with an enzyme such as trypsin. The sample is then cleaned to remove buffer, salts, denaturants and detergents, all of which could interfere with LC-MS analysis. The peptide-enriched samples are then separated by nano-LC in the reversed-phase mode using an ion-pair reagent, followed immediately by on-line MS analysis for the identification and quantitation of the tryptic peptides. A range of software platforms can then be used to perform identification and quantitation, and to analyze and interpret the results. Optionally, an additional fractionation step termed two-dimensional (2D) chromatography may be performed, with the first separation offline prior to nano-LC-MS or both dimensions of separations online with nano-LC. This will separate the complex peptide mixture into several simpler fractions, which can then be introduced individually into the nano-LC-MS/MS workflow, producing better separation and further reducing the sample complexity and the rate at which peptides enter the MS instrument. This method gives the highest number of identified peptides, but also extends the analysis time.

There has been a great deal of technological development in the last twenty years or so in both nano-LC and MS, which has enabled today's capability to characterize the human proteome at a practical depth in less than a day. This progress will be discussed at greater length in chapter 3. For now, it is sufficient to say that the development of nano-LC has been profoundly important in terms of improving the performance of LC-MS in proteomics, resulting in considerably enhanced sensitivity at lower flow rates. The 'nanoflow' aspect reflects the extremely low flow rate and small column dimensions used in this form of LC. Proteomics samples are generally very small in terms of both volume and amount, and initially, most samples were obtained from small sodium dodecyl sulfate (SDS) polyacrylamide gel extracts with very small quantities of total protein. This made nano-LC essential, as the higher volume columns used in standard LC would overly dilute the sample.

There are critical differences between conventional analytical flow LC and nano flow-LC. A standard LC system will deliver flow rates ranging from 0.1 to 1 mL/min into the MS instrument. In contrast, the flow rates employed in nano-LC are far lower — on the order of 0.2 µL/min — and require highly specialized instrumentation. Whereas the flow of liquid from a standard chromatography system is clearly visible as it elutes from the system, it takes five minutes for 1 µL of liquid to pass through a nano-HPLC system (Table 1).

	Column Internal Diameter	Typical Flow Rate	Predominant Application	UHPLC Capabilities
Nano LC	20 - 100 µm	20 - 500 nL/min	Proteomics	High efficiency and sensitivity
Capillary LC	100 - 500 µm	0.5 - 10 µL/min	Proteomics, bioanalysis	Good compromise of efficiency and throughput with attention to sensitivity
Micro LC	0.5 - 2.1 mm	10 - 500 µL/min	DMPK, metabolomics, general LC-MS	Good compromise of efficiency and throughput with attention to loadability
Standard/Analytical LC	2.1 - 4.6 mm	05 - 2.5 µL/min	Protein fractionation, pharma, small molecule	High throughput analysis
Semi Prep LC	>4.6 mm	>2.5 mL/min	Protein purification	Only performed at pressures up to 200-400 bar

Table 1. LC Separations for Various Applications

Although 1 µL of extra 'dead' volume may go unnoticed with standard flow LC, that same dead volume will generate five minutes of delay and produce a disastrous mixing of peaks in a nano-flow system. Therefore, nano-LC can be a very unforgiving technique, and the equipment and connections at these low flow rates have to be carefully controlled. That being said, the current generation of nano-LC equipment has been specifically developed for these applications, and is very robust and relatively easy to use, including specially-designed ultra high-performance LC (UHPLC) systems. For example, the invention of nanoViper connections has revolutionized nano-LC by providing easy-to-use, low dead volume, finger-tight connections.

The low flow rate and the narrow column dimensions (75 µm internal diameter) of nano-LC offer benefits both in terms of LC separation and MS data quality. The samples from a typical proteomics experiment are often small in volume while exhibiting a high dynamic range of peptides. A large-volume column would swallow and dilute such samples to an extent that it would become impossible to detect low-abundance peptide species. In contrast, nano-LC columns keep these peptides concentrated, so that they can be efficiently detected by the MS instrument. Indeed, moving from a 4.6 mm diameter column to a column with 75 µm internal diameter gives a potential increase in sensitivity of up to 4,000-fold for the same amount of sample loaded onto the columns (Fig. 2).

From a mass spectrometry perspective, the advantage of nano-LC comes in the form of sensitivity and resolution, with the ability to detect very small amounts of peptide whilst separating a complex mixture. Electrospray ionization (ESI) is the standard for modern LC-MS, particularly for proteomics applications, as it enables biomolecules such as peptides to move

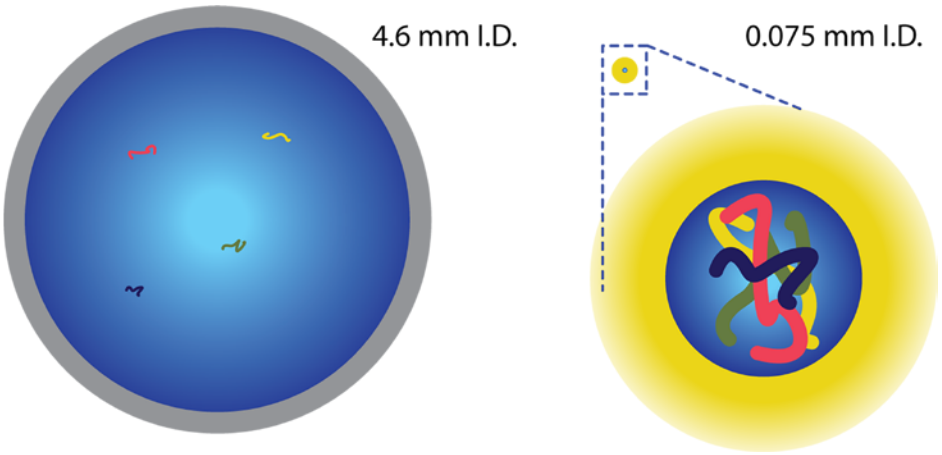
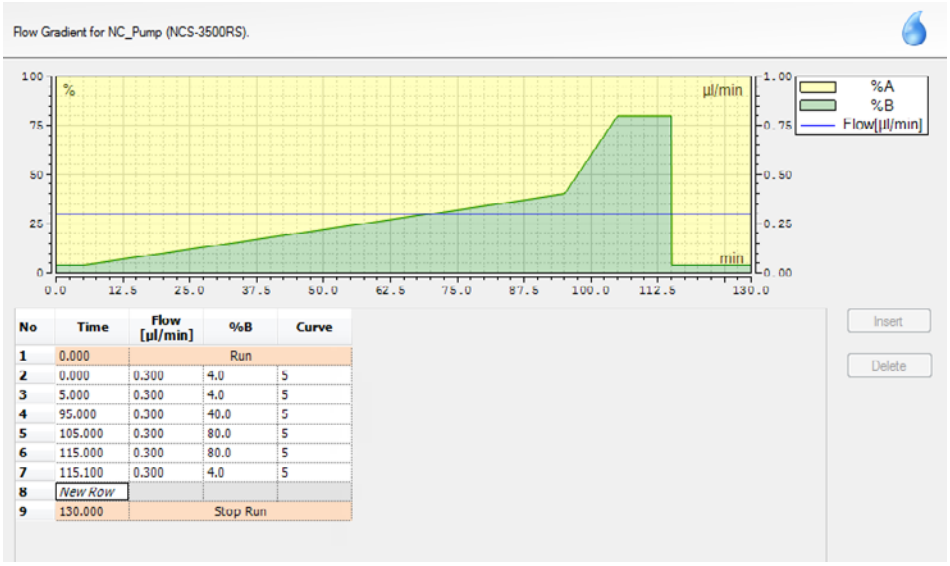


Figure 2. Mass concentration in a small-diameter LC column.

The efficiency of ESI increases as the flow rate of sample arriving at the electrospray emitter of the MS instrument decreases.

from solution- to gas-phase prior to mass spectrometric (mass to charge ratio) measurement. The efficiency of ESI increases as the flow rate of sample arriving at the electrospray emitter of the MS instrument decreases. In addition to increased ionization efficiency, the interfering background signal from the eluent is greatly reduced due to far smaller amount of eluent flowing into the system. As a result, even if nano-LC is more demanding of users, the benefits are also great.

Nano-LC is often performed using reversed-phase chromatography with a C18 bonded silica column, because the solvents used are volatile and give good solvation, which makes them highly compatible with MS. In reversed-phase, compounds are separated based on their hydrophobicity (Fig. 3); hydrophobic



No	Time	Flow [µl/min]	%B	Curve
1	0.000	Run		
2	0.000	0.300	4.0	5
3	5.000	0.300	4.0	5
4	95.000	0.300	40.0	5
5	105.000	0.300	80.0	5
6	115.000	0.300	80.0	5
7	115.100	0.300	4.0	5
8	New Row			
9	130.000	Stop Run		

Figure 3. Example of a reversed phase gradient for a nano-LC-MS proteomeics application suitable for intermediary complex samples. Peptide separation gradient from 4% B to 40% B lasts 90 minutes. Subsequently, the organic composition is ramped up to 80% B to elute all peptides and contaminations from the column. Finally, the column is re-equilibrated for 15 minutes at 4% B.

By gradually increasing the solvent strength during chromatographic separation, one can separate peptides in order of their inherent hydrophobicity.

molecules will bind more strongly to the stationary phase, and will elute from the column later. However, peptides are generally hydrophilic and would not normally bind strongly to a reversed-phase column. This can be remedied with the addition of an ion-pair reagent, usually formic acid. The acid lowers the pH and protonates carboxylic acid groups on the peptide, rendering them neutral. Formic acid also ion pairs with charged amine groups on the peptide to neutralize those charges. This transforms a charged, hydrophilic peptide into a more hydrophobic form for separation with reversed-phase chromatography. By gradually increasing the solvent strength during chromatographic separation, one can separate peptides in order of their inherent hydrophobicity.

Innovations in column technology have also raised the bar for nano-LC performance. Longer columns with greater peak capacity and resolution have resulted in an increase in the number of peptides that can be separated and detected, leading to higher protein identification rates and improved identification of low-abundance peptides (Fig. 4). Chromatography columns for

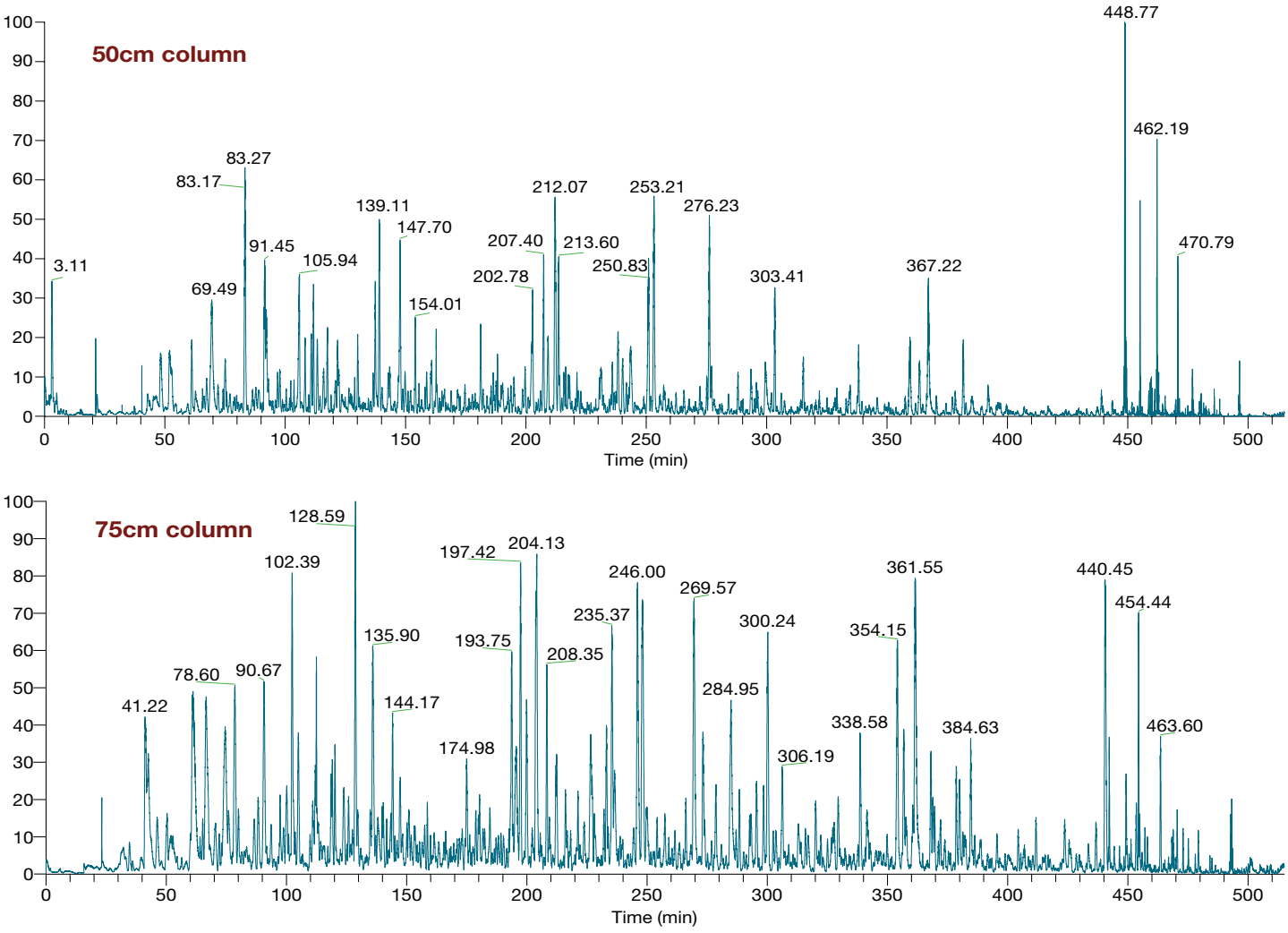


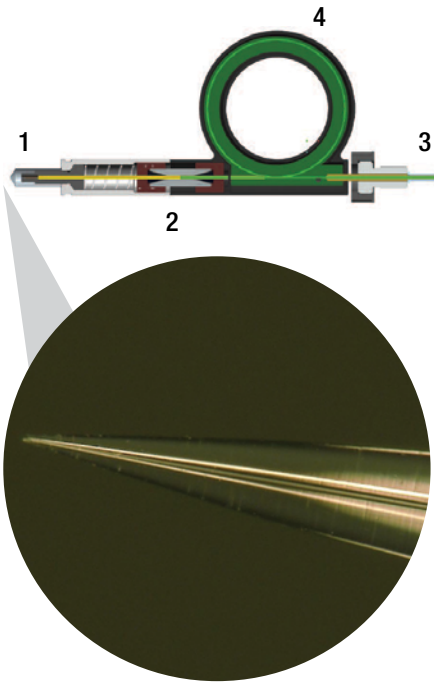
Figure 4. The effect of column length on peptide resolution.

proteomics have gone from 15 to 25 to 50 cm in length, and as of last year, 75 cm long columns are now commercially available. Nagaraj *et al.*[1] and Coon *et al.*[2] are among the research groups that have demonstrated the enhanced performance that becomes possible when longer reversed-phase columns are employed for nano-LC-MS/MS in proteomics.

Longer columns enable the separation of increasingly complex mixtures in a single analysis. This increase in resolution and capacity means that larger amounts of sample can be loaded onto the column and that run times can be extended. This produces a highly resolved stream of concentrated peptides for MS identification. Complex samples which would have previously required two-dimensional separation can now be analyzed in a single injection with a long gradient. This is a great improvement for quantitative experiments, where fractionation could lead to the same peptide being eluted in two or more fractions, which makes accurate quantitation difficult or impossible. However, these long columns can also be used in conjunction with 2D chromatography to enable deep proteomics experiments that are designed to identify as many proteins in the sample as possible. Ritorto *et al.*[3] identified 10,000 proteins from a HeLa cell lysate by using 2D fractionation followed by a four-hour nano-LC separation on 50 cm long columns.

It is also possible to fuse the nano-LC column directly to the electrospray emitter. This configuration has the advantage of enabling a simple 'plug and play' connection to the electrospray interface, and results in very low post-column dispersion of peaks. The voltage is applied through the integrated union, and produces a stable spray for the lifetime of the column. This allows long experimental sequences to be run without fear of spray failure during the sequence.

These innovations in nano-LC have arrived in parallel with major strides in high-performance instruments, enabling proteomics researchers to identify hundreds of thousands of peptides with much greater ease and speed, producing powerful data from 30 minutes to an hour. In 2016, the Journal Cell Systems published three papers that demonstrate the power of modern nano-LC-MS/MS, which were reviewed expertly in the same issue by Riley, Hebert and Coon[4]. In one of these studies, by Matthias Mann and colleagues, focused on clinical intent review in the context of plasma protein profiling[5], a new application for nano-LC that has been enabled by the more robust platforms. In typical clinical research assays, levels of relevant proteins are normally measured in individual immunoassays. In this paper, the authors employed a rapid, high-throughput and robust assay for plasma proteome profiling based on a simple MS approach without significant up-front sample prep. From as little as 1 µl of plasma obtained via finger prick, researchers were able to quantitate inflammatory proteins, apolipoproteins and nearly 50 biomarkers in just three hours. The extreme sensitivity afforded by nano-LC-MS/MS was essential to the identification of these plasma proteins. Pending further testing and validation, this approach could be employed to assess cardiovascular and metabolic health;



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   Easy to use, fingertight fittings eliminates column damage and prevents peak broadening
4. **Column with integrated temperature control**
   Column temperature control increases run to run reproducibility

with a more protracted separation time, it should be feasible to reproducibly quantify more than 1,000 proteins with relevance to different disease states.

The authors conclude that their plasma proteome profiling approach can deliver an informative portrait of a person's health state and can envision its large-scale use in biomedicine. Given the low resource requirements of this approach, it should be possible to conduct routine sampling of an individual's blood to produce regular plasma proteome profiles. In the future, such nano-LC-MS/MS-based profiling could be effective as a general approach for monitoring health and disease risk, providing early indications that could help clinicians to recommend appropriate lifestyle changes or pharmacological interventions. In this context, proteomics could become a powerful high-throughput tool for personalized medicine.

Ongoing advances in nano-LC technology are now enabling research that promises to be transformative.

SUMMARY

MS is not the only important element of proteomics — and the total MS-based workflow contains several other essential components. Before MS can enable peptide identification and quantification, the instrument must be 'fed' peptides and proteins that have been separated at high resolution at a rate that it can handle. Nano-LC is essential for achieving optimal sensitivity for the typically small sample quantities employed in proteomics research. Today, the combination of MS and nano-LC has moved from the cutting edge to become a routinely-used technology. Ongoing advances in nano-LC technology are now enabling research that promises to be transformative in terms of moving proteomics from basic research, to large scale translational research, and finally to the clinic, where it can greatly advance the quality and precision of medical care.

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Chapter 3

Nano-LC in Proteomics: The Technical Explanation

Stephan Meding PhD, Ken Cook PhD, Mike Baynham PhD

Nano-flow liquid chromatography (nano-LC) has become an indispensable tool in proteomics. In this chapter, we will look at the tremendous promise of proteomics mass spectrometry (MS) that has been made possible by its true partner, nano-LC.

THE NEED FOR SENSITIVITY

Nano-LC-MS provides greater sensitivity than conventional high-flow rate methods. This performance increase is caused by two major factors.

First, reducing the column diameter while keeping the sample amount constant results in a considerable signal increase (Fig. 1). This increase is proportional to the square of the ratio of the column diameters for concentration sensitive detectors, and electrospray ionization (ESI) MS detectors often behave as concentration sensitive detectors in LC-MS applications[1,2]. Based on this relationship, downsizing

from a 2.1 mm column to a 75 µm column would be predicted to increase sensitivity by nearly 800-fold[3]:

$$\left(\frac{\text{Diameter standard column}}{\text{Diameter nano column}}\right)^2 = \left(\frac{2.1\text{ mm}}{0.075\text{ mm}}\right)^2 = 784$$

It is critical to note that this boost in sensitivity is only obtained when the sample amount is kept constant. This increased sensitivity is especially valuable when sample material is limited or very expensive, which is very often the case in proteomics experiments. For example, samples such as tissue biopsies are by their nature limited, whereas isotopically-labeled samples become expensive due to the cost of chemical labeling.

Ionization efficiency increases markedly at lower flow rates, and this effect becomes especially prominent in the nano-flow range.

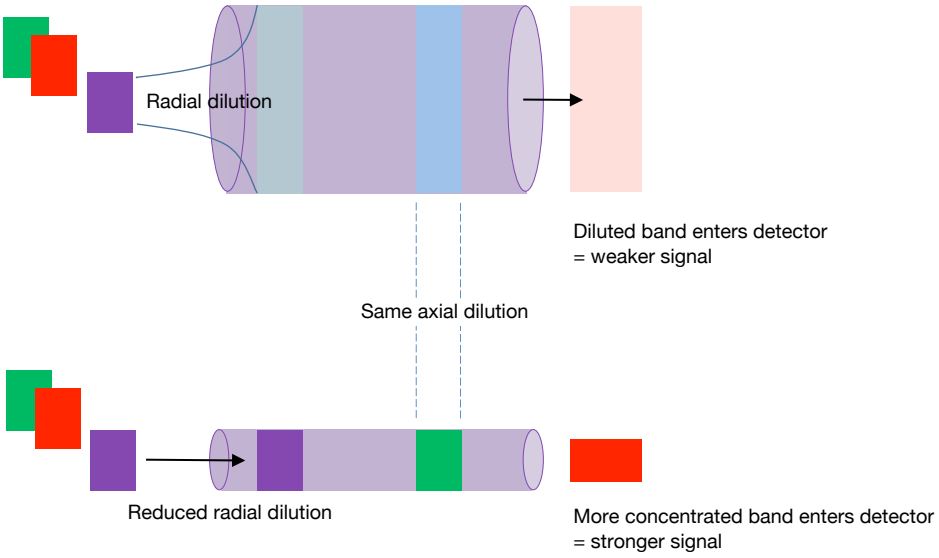

Figure 1. Conventional and narrow column showing chromatographic dilution process. A wider conventional column gives rise to increased radial dilution, resulting in a lower concentration and weaker signal. Radial dilution is a function of the square of the column radius. So, a narrow column results in a higher concentration of sample entering the mass spectrometer and hence a stronger signal.

The second, and arguably more important advantage of nano-LC arises from the relationship between the efficiency of ESI and sample flow rate. Ionization efficiency increases markedly at lower flow rates, and this effect becomes especially prominent in the nano-flow range. The exact reasons for this are still being debated, but the improved sensitivity obtained by MS following nano-LC seems to arise from a combination of better ionization, lower background noise and more efficient desolvation. Whatever the reason, the empirical results speak for themselves: changing from analytical- to nano-flow can increase sensitivity by two orders of magnitude[4]. As shown below, nano-LC-MS produces a considerable gain in sensitivity (Fig 2).

1969 First commercial High Pressure Liquid Chromatography system

1988 Concept of miniaturized LC Microcolumns

1989 Coupling of microbore LC (1mm ID) and ESI–MS for proteomics

1993 Coupling of narrower LC columns (100µm ID) to ESI–MS for proteomics

1994 Coupling nl/min flow rates with nanoelectrospray-MS

1996 First commercial Nano Flow LC system

2001 Nano 2D LC (MUDPIT) for proteomics

2003 First splitless microfluidic flow control in a commercial system

2003 Nano columns in chip format for LC-MS

2004 Introduction of UHPLC systems and columns

2005 Introduction of Nano-flow on UHPLC

2016 Introduction of the longest commercial nano columns (75cm)

Figure 3. A brief history of nano-LC.

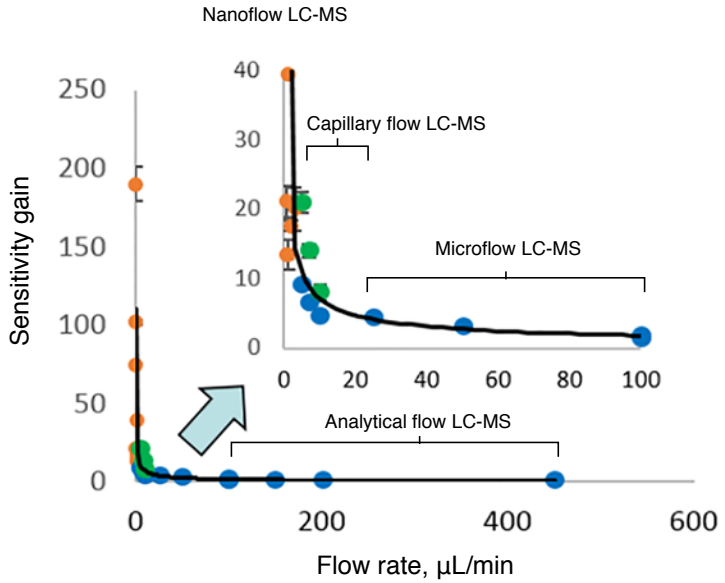

Figure 2. Nano-LC-MS offers greatly enhanced sensitivity. The relative sensitivity gain based on peak area for the EDLIAYLK peptide (m/z 482.77, charge +2) at flow rates ranging from 100 nL/min to 450 µL/min using different source types, emitter and column IDs. Inset shows close up of data for lower flow rates. Each dot represents a set of replicate measurements under the same LC-ESI-MS conditions. Blue dots, heated ESI (HESI) probe; Green dots, HESI probe with micro EASY-Spray transfer line; orange dots, HESI probe with nano EASY-Spray transfer line (adapted from Thermo Scientific Application Note PN64787-EN 0616S).

This increased sensitivity is of particular importance for proteomics, where scientists struggle with low abundance of analytes, high sample complexity and a wide dynamic range. So far, only nano-LC-MS has been able to deal with these challenges.

A BRIEF HISTORY OF NANO-LC

The development of dedicated instrumentation for nano-LC started in the late 1980s (Fig. 3). At first, flow splitters were used to deliver nanoliter flow rates to LC columns within systems operating at analytical flow rates of hundreds of microliters per minute. This flow-splitting design resulted in most of the consumed solvent being directly pumped to waste, with typically only one-thousandth of the solvent actually passing over the nano-LC column. Split-free, direct flow systems were eventually developed in 2003 and commercialized in 2005. From the very beginning, setting up a nano-LC system was considered an art. In particular, users typically struggled to make leak-tight, dead volume free connections.

The fluidic path of every LC system has a certain volume, and ideally this will exclusively comprise the volume within the capillaries, valves and column.

Dead volume is extra volume within the flow path, and is mostly caused by improper connections between the different components of the flow path (*e.g.* between the capillaries and the column) that result in small cavities. Within these cavities, the flow

of the liquid is disturbed, resulting in poor chromatographic performance, such as peak broadening. This is often caused by the use of tubing of incorrect length or internal diameter, or bad fittings. A dead volume free LC system eliminates these perturbations.

The latest generation nanoflow instrumentation provides repeatability similar to analytical flow ultrahigh-performance LC (UHPLC) systems.

Nano-LC separation can also be impeded by leaks and clogs. Leaks cause the eluent and analytes to take an alternative path rather than the intended route through the column. Leaks are very hard to detect visually at very low flow rates, and care must be taken to ensure a leak-tight system that maintains a fluidic path through the system that preserves robust chromatographic performance. Clogs can also be a common problem. In systems without a flow-splitter, they cause pump pressure to increase, whereas in flow-splitter systems clogs result in a reduced flow rate. The best practice for avoiding clogs is to ensure that samples are passed through a pre-column to remove particulate matter prior to the analytical column.

Because of these challenges, initial uptake of nano-LC was very limited. The few research groups that went through the painful exercise of setting up a nano-LC system did so because of the gains in sensitivity.

Köcher *et al.*[5] pushed sensitivity to a new maximum by successfully running chromatographic separations at 20 nL/min. This showed the performance capabilities of off-the-shelf instrumentation, but beyond proof-of-principle studies, such ultra-low flow rates have not been widely adopted by the proteomics community. Most scientists have opted for applications with flow rates between 200 and 400 nL/min. In this conservative range, one can achieve a good trade-off between sensitivity, repeatability and robustness. The latest generation nanoflow instrumentation provides repeatability similar to analytical flow ultrahigh-performance LC (UHPLC) systems, with relative standard deviations for retention time usually falling below 0.1% (Fig. 4)[6]. This strong reproducibility is also coupled with higher sensitivity measurements in the mass spectrometer.

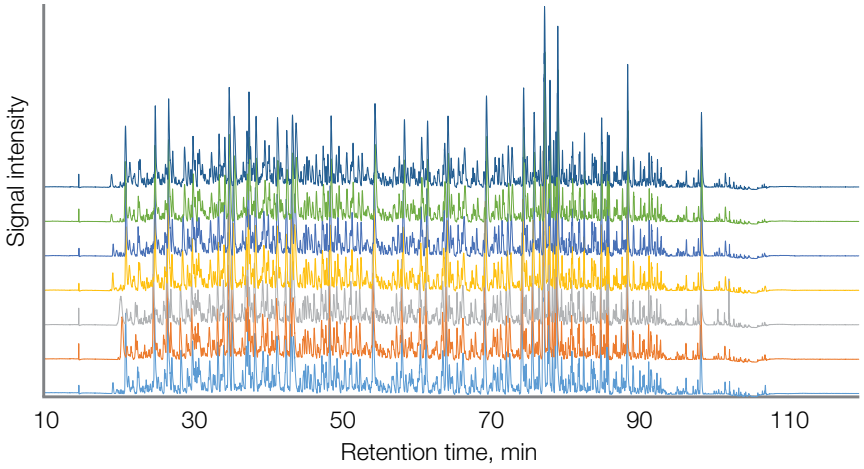

Figure 4. Retention times for multiple runs with a Thermo Scientific™ UltiMate™ 3000 RSLCnano system with Thermo Scientific™ ProFlow™ technology. Seven consecutive replicates of Thermo Scientific™ Pierce™ HeLa Digest Standard were run using a 75 cm Thermo Scientific™ EASY-Spray™ C18 LC column with a gradient duration of 90 min and at a flow rate of 300 nL/min

Such high levels of data reproducibility have become increasingly important. In the last decade, proteomics has moved from mostly qualitative to quantitative analysis. At first, identification lists were considered to be complete proteomics experiments: the larger the list, the better. Today, many proteomics experiments are comparative studies of large sample cohorts for large scale proteome profiling. In order to confidently align these large, complex data sets and thereby better enable relative quantitation, it is critical to minimize technical sources of variability in the system.

The use of nano-LC in proteomics has benefited considerably from parallel advances in MS technology coupled with nano-LC. The latest generation of Orbitrap™-based mass spectrometers achieve a mass resolution (not to be confused with chromatographic resolution) of up to half a million full width at half maximum (FWHM) and mass measurement accuracy levels of less than 1 part per million (ppm). These instruments currently offer the highest mass accuracy and resolution for routine use in proteomics. MS sensitivity, selectivity and versatility (MS^n and fragmentation modes) have also improved drastically in order to address the breadth and depth of contemporary proteomics analyses. State-of-the-art instruments can identify on the order of six thousand proteins in a single nano-LC-MS/MS experiment (Fig. 5) lasting approximately 2 to 4 hours using a long, narrow-bore (75-micron) nano-LC column. Mass spectrometers such as the Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer and Thermo Scientific™ Q Exactive™ HF hybrid quadrupole-Orbitrap mass spectrometer have an acquisition rate of approximately 20 Hz and an intrascan dynamic range of around 5,000 per scan. Thus, the integration of robust modern MS instrumentation with high resolution and sensitivity nano-LC can deliver a considerable boost in terms of identification rates.

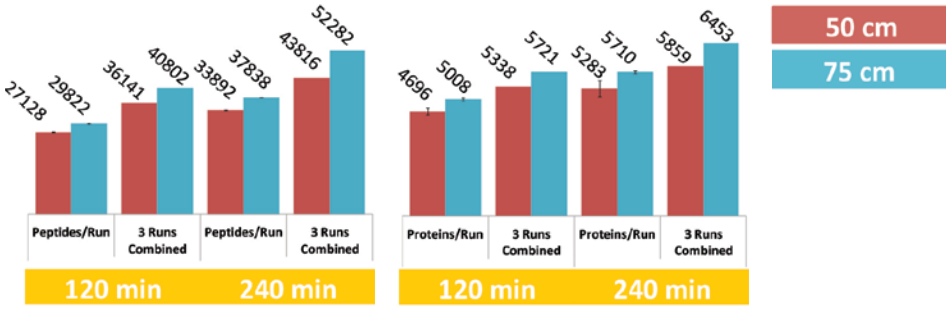

Figure 5. Number of identified peptide and protein groups. From a proteomics perspective, researchers in the field are commonly interested in the number of peptides identified, either in terms of peptide spectral matches, unique peptides, or protein groups. The 75 cm column consistently resulted in the highest total number of peptides and protein identifications by at least a 7% margin.

OPTIMIZING NANO-LC SEPARATIONS

Proteomics samples are typically extremely complex, containing both highly-abundant proteins as well as very low-abundance proteins, with dynamic ranges that may span many orders of magnitude (Fig. 6)[7]. This range can be as high as six orders of magnitude in human cell extracts or even 12 orders of magnitude in plasma specimens.

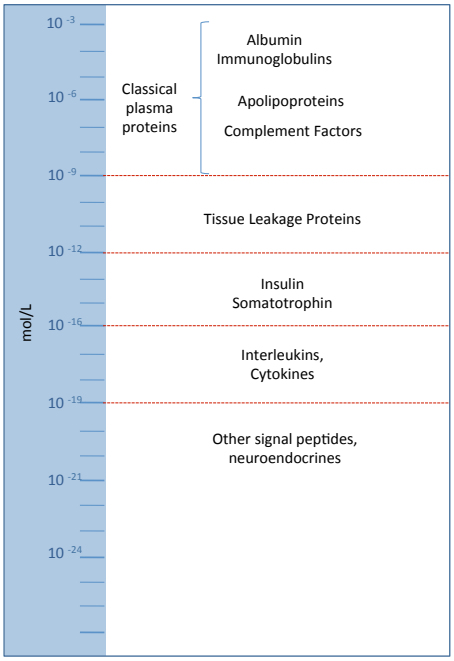

Figure 6. High dynamic range of proteins found in blood showing various classes of proteins. At the top, classical plasma proteins are detectable in 1 µL of sample or less. Typically after depletion of abundant proteins, tissue leakage proteins typically require, at least, 1 mL plasma volumes. Another 3 orders of magnitude lower are the concentration ranges of secreted signal peptides/proteins like insulin, and somatotropin. Interleukins and other cytokines really push current MS systems to their very limit, whereas other neurosecretory signal peptides require extensive concentration steps to reach levels detectable by MS.

In order to cope with such complexity and dynamic range, LC systems need to feed the mass spectrometer with a pre-separated sample at an adequate rate — ideally no faster than the speed of data acquisition in the mass spectrometer — and separate high-abundance from low-abundance peptides. There are four primary parameters that can be optimized to increase peptide resolution and thus provide an optimally separated sample for mass spectrometric analysis: gradient duration, column length, particle size in the stationary phase, and temperature control.

Thermo Scientific™ EASY-nLC™ 1200 system

Gradient duration and column length

In addition to extending the length of the gradient to improve separation performance, one can also optimize the chromatography liquid phase (solvents) and the slope of the gradient; once optimized, however, these will remain unchanged for the analysis of all samples in an experiment. It is important to note that gradient length increases only work when accompanied by a concurrent increase in column length, rather than by simply lengthening gradients on a short column.

Longer columns can also produce superior separation.

Longer columns can also produce superior separation (Fig. 7). However, column backpressure increases in proportion to column length increase, and is also inversely proportional to the square of particle size reduction. As a result, this approach to improving resolution is time-consuming and/or requires instrumentation that is able to handle higher system pressures. For the 10 – 15 cm columns used in the early days of nano-LC, standard HPLC systems (<400 bar system pressure) were sufficient. With the demand for higher chromatographic

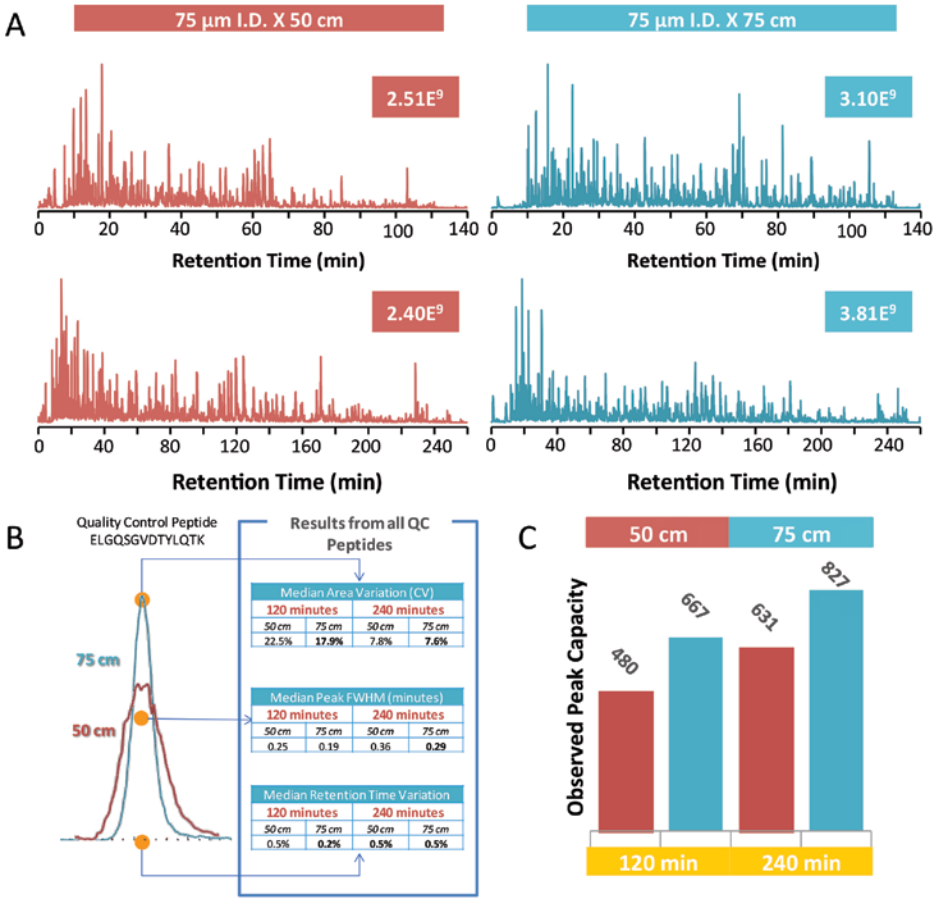

Figure 7. Reproducibility of the chromatographic separation is the most important requisite for a reliable comparison among different runs. A) Representative chromatograms obtained using different gradient and column lengths. B) Extracted ion chromatogram for one of 15 representative QC peptides, and average chromatographic metrics for all 15 QC peptides obtained from different experimental configurations. C) Histogram comparing the peak capacity obtained for each experiment.

resolution, UHPLC systems capable of handling higher pressures are now required in order to accommodate longer columns. Currently, 75 cm columns are the longest available on the market.

Chromatographic performance (CP) increases linearly with increasing gradient length and column length. CP is defined as follows, where n is the number of peaks used for the calculation, T_G is the gradient length and W_p is the width at half maximum of the peak height:

$$C_p = 1 + \frac{T_G}{\frac{1}{n}\sum_1^n W_p}$$

Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer

The 75 cm column achieves a chromatographic performance of over 800 employing a 240-minute gradient, almost doubling the previous peak performance achieved by MacCoss and coworkers[8].

As mentioned above, running such columns requires the use of nano-UHPLC systems. The Thermo Scientific™ EASY-nLC™ 1200 system currently offers the highest pressure rating (1200 bar), and this system enables the analysis of complex peptide and protein samples with 75 cm columns.

From a proteomics perspective, researchers in the field are commonly interested in the number of peptides identified, either in terms of peptide spectral matches, unique peptides, or protein groups. The 75 cm column consistently produces the highest total number of peptides and protein identifications, outperforming shorter columns by at least a 7% margin (Fig. 8). Whereas in the past, reproducibility among replicates was typically around 80%, this study obtained highly reproducible results, where less than 5% of the peptide/protein identifications for a given dataset were not shared with any of the others in a set of three replicates. Efficient chromatography allows not only for better separation of the analytes, but also improves ionization, and when combined with a high performance mass spectrometer such as the Orbitrap Fusion Lumos MS, most of the ions obtained are likely to yield positive peptide identifications.

Column Particle Loading Capacities

Not all C18 resin materials are created equal. Resolution and capacity are among the important factors that one must consider in selecting a resin for proteomics. The vast dynamic range of proteins in most biological samples means that to see enough of the very low abundance proteins in the mixture, one must also load a large amount of the high-abundance peptides present in the same sample. This requires a column of high carbon content to achieve sufficient loading ability while also maintaining peak shape and resolution. Most columns will produce a good peak shape at 100 fmol loading onto the column. However, beyond this point, the column will suffer from overloading effects, and the peak shape and resolution quality will be negatively affected, reducing the number of quantifiable peptides.

Not all C18 resin materials are created equal. Resolution and capacity are among the important factors that one must consider in selecting a resin for proteomics.

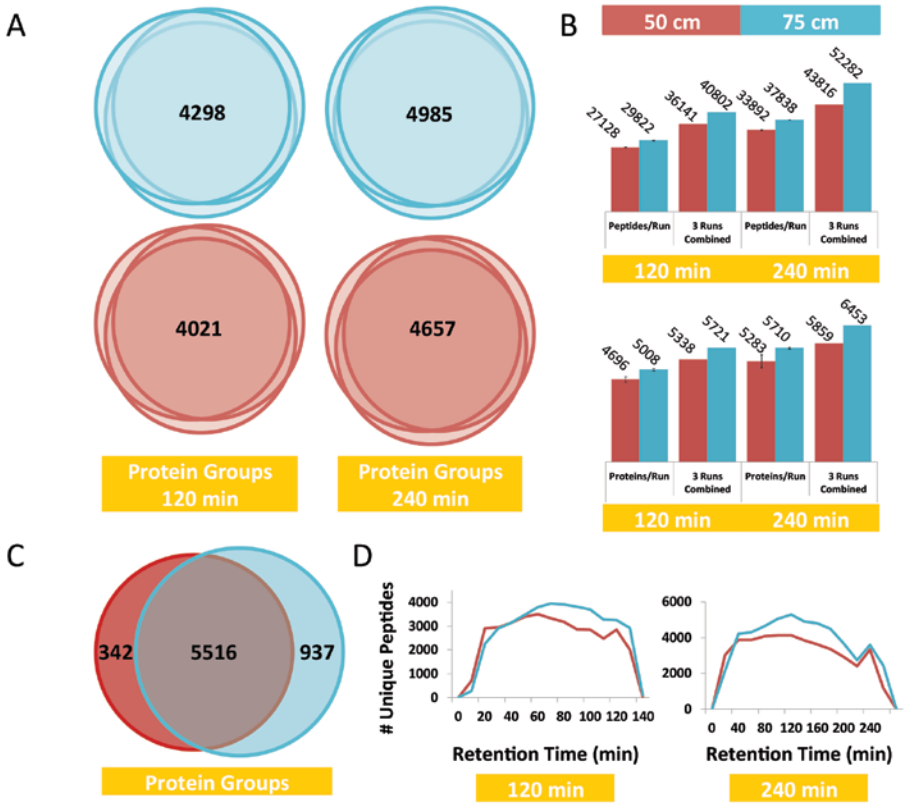

Figure 8. The 75cm column results in higher numbers of peptides and protein identifications. A) Venn diagrams showing the overlap among technical replicates for the identified proteins from varying column and gradient lengths. B) Number of identified peptide and protein groups. C) Venn diagram showing the total number of overlapped proteins for both column lengths. D) Line graph displaying the trend of identified peptides versus retention time during the LC-MS analysis.

An example of 2 different C18 columns is shown below (Fig. 9). In this example, the Thermo Scientific™ Acclaim™ PepMap™ LC column has a very high loading capacity for peptides, which has helped it to become the gold standard column in proteomics experiments. Here we see it compared to a more standard C18 resin in the Reprosil AQ. Both columns show good peak shape at 100 fmol loading, however this deteriorates with the Reprosil column at 250 fmols. In comparison

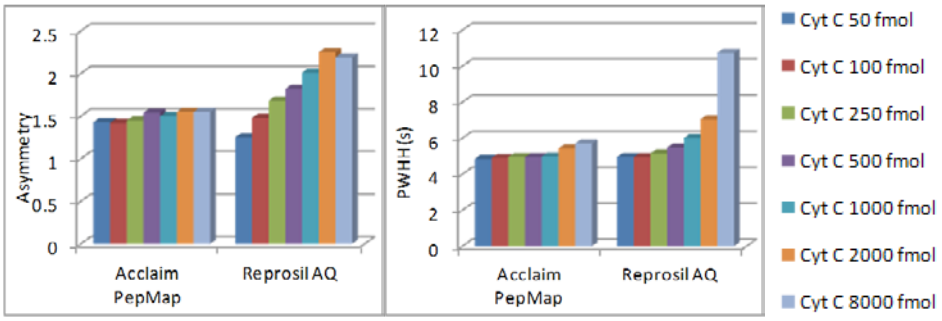

Figure 9. The PepMap resin with its high loading capacity maintains good peak shape at peptide injections on column of over 8,000 fmoles on a 15 cm long column.

the Acclaim PepMap column is still showing good peak shape even at 8000 fmols. Peak width analysis confirms this and shows a similar pattern.

Temperature Control

Temperature rules retention, selectivity and efficiency in your chromatography performance. With long run times in particular, the temperature of the column must be controlled to maintain retention time precision from run to run, as retention times will change with temperature, and it is common for the ambient temperature in a building to change significantly over the course of a day.

The temperature of the column must be controlled to maintain retention time precision from run to run, as retention times will change with temperature.

Many HPLC systems provide the option to control column temperature with the use of a thermostatted column compartment, which usually comes in one of two types: a peltier heater or a forced air circulation device. For most users, the most common running temperature is 40°C which places the column above ambient temperature fluctuations. Column heating is essential in nano-LC, and without temperature control, the retention time can vary considerably between injections. By stabilizing the column temperature, retention time precision of 0.2% RSD can be easily achieved (Fig. 10).

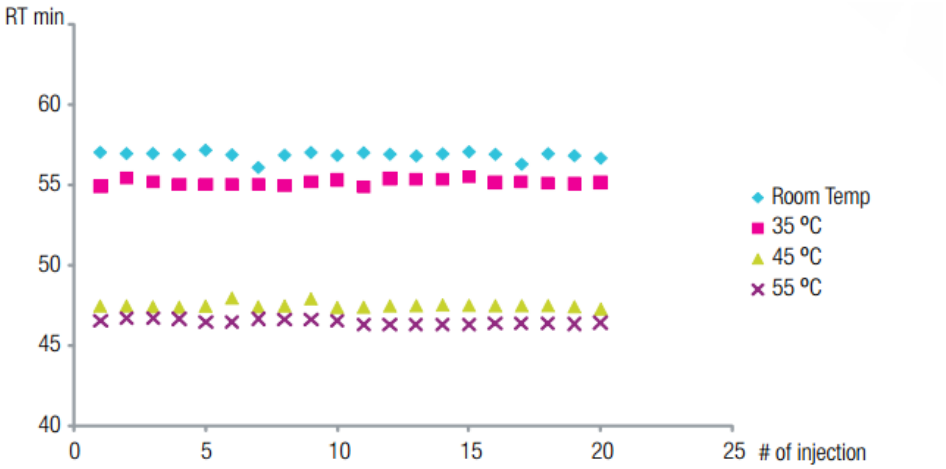

Figure 10. Temperature control results in high retention time precision.

Temperature can also be used to increase the performance of the chromatographic separation in a number of ways. As the temperature is increased, the viscosity of the mobile phase flowing through the column is reduced. Since the backpressure generated by the column is dependent upon the viscosity, this allows for the use of a longer column or smaller particles to improve column efficiency or of higher flow rates to reduce analysis time. For example, the Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system features a built-in column oven, which means that it can be used at slightly lower backpressure limits. Column temperatures between 45 to 50 °C are commonly used for this type of high-resolution chromatography.

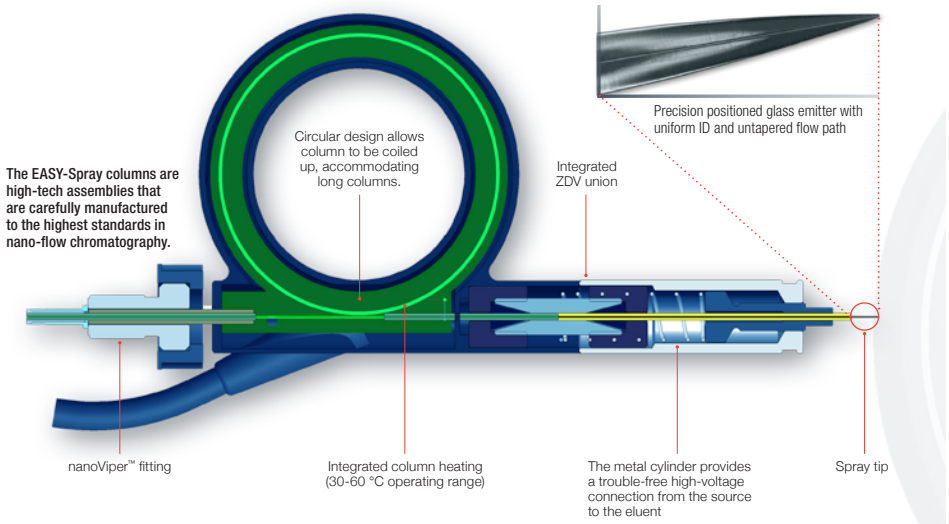

Figure 11. The EASY-Spray columns are high-tech assemblies that are carefully manufactured to the highest standards in nano-flow chromatography.

Even with the latest-generation columns and UHPLC systems, highly complex samples such as tissue or blood cannot always be sufficiently separated for mass spectrometers

Innovations in column technology, such as the EASY-Spray nano-LC column, allow the temperature control to be incorporated into the column assembly and powered from the ESI source. This assures high run-to-run reproducibility while removing any capillary connections between the column and ESI source (Fig. 11).

TWO-DIMENSIONAL LC

Even with the latest-generation columns and UHPLC systems, highly complex samples such as tissue or blood cannot always be sufficiently separated for mass spectrometers to comprehensively identify their proteomic contents. In these scenarios, multi-dimensional (mostly two-dimensional) separation methods are widely used to improve peptide resolution, thereby increasing protein identification rates. Two-dimensional (2D) chromatography describes orthogonal separations, in which each dimension is based on different chemical properties of the sample, ensuring maximal separation of the peptides in the sample. In 2D chromatography, the goal is to spread the peptides evenly across fractions in the first dimension, and then to do so again during the second-dimensional analysis.

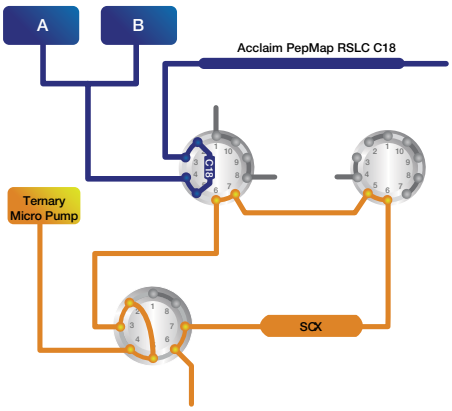

Figure 12. Flow schematic for offline 2D-LC.

The advantages of the online approach are that it is better for reducing carry-over contamination between samples and uses smaller sample amounts.

2D separation can be performed offline or online. Offline separation is when the two dimensions are performed on different systems (Fig. 12); this is often considered a more robust approach, and minimizes idle time for the mass spectrometer.

For online separation, both dimensions are performed on the same LC system, which is in turn coupled to the mass spectrometer. The advantages of the online approach are that it is better for reducing carry-over contamination between samples and uses smaller sample amounts. However, the options for second-dimensional separation in the online configuration are limited by the solvent phase compatibility with the ESI process and the mass spectrometer. Therefore, reversed-phase separation in acidic conditions is most commonly used. There are more possibilities for the first dimension.

Cation- or anion-exchange separation are often used, but basic pH, reversed-phase, hydrophilic interaction liquid chromatography (HILIC) and electrostatic repulsion interaction liquid chromatography (ERLIC) have also been used successfully (Fig. 13). If the first dimension is used online, the elution solvent has to be compatible with loading conditions for the second dimension — for example, high organic solvent elution is not suitable for reversed-phase loading, which must be low in organic solvent in order to ensure that peptides and proteins bind to the column. With offline 2D separation, buffer exchange is

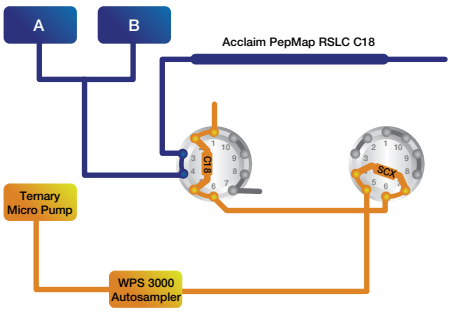

Figure 13. Flow schematic for online 2D-LC with cation exchange in the first dimension (salt plugs) followed by reversed phase in the second dimension.

possible and the sample fractions do not necessarily have to be eluted in a solvent that is compatible with loading onto the second dimension.

Multidimensional separations are also useful for performing sample enrichment or pre-concentration (Fig. 14). Most often, this is done for phosphopeptides or other modified peptides. For example, a column utilizing titanium dioxide — or alternately, immobilized metal ion affinity chromatography (IMAC) — is used to separate phosphorylated from unphosphorylated peptides.

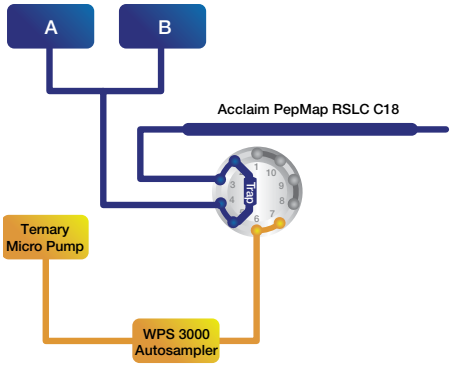

Figure 14. Flow schematic for online 2D-LC with a trapping column in the first dimension, such as IMAC in the first dimension followed by reversed phase in the second dimension.

EASE OF USE

There have been other innovations in nano-LC in areas besides performance and specifications. Advances in usability have been critical in enabling broad adoption of this technology in the research world. And although scientists in the first days of nano-LC sometimes struggled with making leak-tight connections at very high pressures and without dead volume, setting up a system today is far easier.

Specially designed chromatography systems are required to produce accurate flow at 50 – 300 nL/min, and the effects of dead volume in the connections can be profound. A 0.5 µL dead volume may not be that noticeable in an HPLC setup at 1 mL/min, but at a flow rate of 0.3 µl/min this same dead volume is large enough to ruin the chromatographic performance. This was without a doubt the greatest challenge in setting these systems up in the early days, and it still remains a problem with some commercially available systems. This is possibly why some manufacturers recommend higher flow rate columns.

The original method for establishing connections was to take the column tubing, which was typically a 20 µm internal diameter silica capillary, put a PEEK sleeve on the end, and then try to clamp the sleeve against the silica with a nut and ferrule to hold it in place in the connection port. This created many potential areas for dead volume to arise, especially for inexperienced users. It would also not hold against any

Figure 15. A nanoViper fitting.

backpressure over 400 bar, limiting use to relatively short columns. Such connections were the major cause of system failure, due both to dead volume and the appearance of leaks at very low flow rates that were difficult to find and troubleshoot.

To address this problem, Thermo Fisher Scientific has developed nanoViper fittings (Fig. 15), in which the capillary is already nipped in place on a finger-tight fitting, which seals against the port face perfectly every time with a backpressure rating of 1,200 bar. This allows tool-free, reproducible installation of capillaries within seconds, so that getting the fluidics correctly set up takes only a few minutes. This places modern nano-LC into the same category of ease of use as a standard HPLC system, using only finger-tight connections.

To further increase usability, as shown in figure 11, nano-LC columns can be directly connected to an ESI emitter, minimizing post-column volume and eliminating the additional step of connecting the emitter, saving time. This combines an ease of use to nano-LC chromatography with an assurance of good connections which ensures optimum performance every time.

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Chapter 4

Is Nano-LC Ready For Routine Applications?

Stephan Meding, PhD

Numerous peer reviewed articles in the field of proteomics have described the use of nano-LC-MS/MS for basic life science or translational clinical research. However, you will be hard pressed to find nano-LC-based systems in routine environments where standard methods are run day in, day out with high throughput. In the previous chapter, we have highlighted the advantages of nano-LC-MS versus analytical flow LC-MS in basic research. Enhanced sensitivity is clearly a good reason to consider nano-LC-MS when studying proteins in matrices such as blood and plasma — but is the technology ready for routine proteomics analyses?

In order for this to happen, nano-LC must be able to deliver clear benefits and meet certain essential requirements that allow it to reach similar levels of high throughput analysis, robustness, and ease of operation as higher flow LC systems.

High throughput analysis is one of the most important aspects when moving from basic discovery research with tens of samples to large scale translational research with several hundred samples where time is an important factor as well as being cost effective. Moving to higher flow LC such as UHPLC (1mm column ID) has reduced analysis time in many other research areas, but for proteomics applications, the loss of speed with nano-flow (conditioning, stabilizing flow rates before analysis) is considered a major disadvantage, whilst trying to maintain the sensitivity levels needed to analyze the low abundant protein biomarkers.

Despite these challenges, several groups have successfully used nano-LC-MS in clinical research and published the findings in peer reviewed journals. A highly relevant review introducing nano-LC into clinical research demonstrated highly reproducible results could be achieved[1]. This group demonstrated that 11 laboratories using 14 nano-LC-MS systems were able to develop, and apply highly multiplexed (multiple reaction monitoring) MRM-MS assays targeting 125 peptides derived from 27 cancer-relevant proteins and 7 control proteins to precisely and reproducibly measure the analytes in human plasma.

For large targeted screening and validation studies, where throughput is more important and only intermediate sensitivity is required, capillary flow is instead being adopted. Nano-LC-MS and capillary LC-MS together may offer a valuable combination, with the first helping to identify new putative clinical markers and the latter helping to verify/validate them in large patient cohorts. Thus, capillary flow LC-MS delivers good sensitivity coupled with high throughput. Capillary flow LC is usually defined by flow rates between 1-10 µL/min, and 150 or 300 µm columns are most commonly used. As mentioned in chapter two, MS sensitivity increases as the flow rate is reduced. This theoretically results in up to a 50 fold increase in sensitivity from a 2mm analytical column to a 0.3mm capillary column.

Capillary-flow methods also permit cycle times of 15 minutes or less, eliminating the major pain-point associated with nano-LC-MS. This results in increased separation performance compared to nano-LC, because small dead volumes or minute leaks will have less effect at this scale. The availability of commercially-available dedicated instrumentation and solutions for capillary LC also makes set-up simple, delivering performance that is comparable to that of analytical LC-MS.

Chromatographers using nano flow rates and who require higher throughput can move to capillary flow rates because it still provides good sensitivity in combination with modern MS detectors.

Ligand binding assays, such as ELISA, are the method of choice for protein quantification. However, antibody specificity has been questioned recently,

High throughput analysis is one of the most important aspects when moving from basic discovery research with tens of samples to large scale translational research with several hundred samples.

with several studies finding that antibodies may target a lot more than only the intended protein. This consideration has resulted in the search for alternative methods and hence, the adoption of LC-MS analyses and for better sensitivity capillary flow LC-MS analyses.

The latter provides sufficient sensitivity to detect most proteins at physiologically relevant levels; its selectivity is unambiguous if the right ion targets are selected, and LC and MS instrumentation has matured enough to provide robust methods.

In conclusion, nano-LC technology has made huge strides towards becoming a suitable tool for routine applications. It has established a firm position in basic proteomics discovery research, where the highest sensitivity is needed. For now, its greatest value lies in discovery phase research where analytical depth is of the utmost importance.

Although nano-LC has not yet been broadly embraced by the translational and clinical research community because of its relatively low throughput capabilities, and robustness, the emergence of capillary flow LC will have utility with this community where relatively high sensitivity, throughput and robustness is needed.

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