

Principles of Nanoflow Liquid Chromatography and Applications to Proteomics

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Abstract: The low levels of endogenous proteins in biological samples and the large dynamic range of the proteome complicate global analysis of gene expression at the protein level. The use of liquid chromatography (LC) in analytical chemistry is well established. However, the relatively low sensitivity associated with conventional LC makes it unsuitable for the analysis of certain biological samples. Furthermore, the flow rates at which it is operated are not compatible with the use of specific detectors, such as electrospray ionization mass spectrometers. Therefore, due to the analytical demands of biological samples, miniaturized LC techniques were developed to allow for the analysis of samples with greater sensitivity than that afforded by conventional LC. In nanoflow LC (nanoLC) chromatographic separations are performed using flow rates in the range of low nanoliter per minute, which result in high analytical sensitivity due to the large concentration efficiency afforded by this type of chromatography. NanoLC, in combination to tandem mass spectrometry, was first used to analyze peptides and as an alternative to other mass spectrometric methods to identify gel-separated proteins. More recently, gel-free analytical approaches based on LC and nanoLC separations have been developed, which are allowing proteomics to be performed in faster and more comprehensive manner than by using strategies based on the classical 2D gel electrophoresis approach.

INTRODUCTION

The field of proteomics has experienced a fast evolution in recent years. This is due to completion of genome sequences and technological developments that allowed analysis of proteins in a high throughput manner. Genomics and proteomics are related disciplines and the latter is often defined as a branch of functional genomics. However, analysis of peptides and proteins derived from biological samples is limited by sensitivity issues due to the fact that, unlike the analysis of nucleic acids, there is not a method equivalent to the polymerase chain reaction for the amplification of proteins. Thus, methodologies for proteomics have to be sufficiently sensitive to be able to detect endogenous levels of proteins. Several reviews discussing approaches used in proteomics have been published recently [1-3]. This article will focus on one of these methods, namely nano-flow liquid chromatography (nanoLC), with emphasis on studies that used it in combination with mass spectrometry (MS) and tandem mass spectrometry (MS/MS). A brief discussion on the rationale behind miniaturizing LC will be followed by a description of the technical aspects of nanoLC, and an account on how this technique is being used as an indispensable tool in numerous proteomics studies. Other liquid phase separations carried out in capillaries can in principle also be used for analyzing proteins and peptides. These techniques, including capillary electrophoresis [4,5], capillary zone electrophoresis [6], and capillary electrochromatography [7,8], offer great sensitivity but are difficult to couple to MS and have limited sample

loading volumes. The present review will focus on nanoLC and the interested reader is directed to the cited reviews for more information on these other methods.

Traditionally, analysis by packed high pressure LC is carried out in columns with an internal diameter (ID) of 4.6 mm [Ref. 9]. These so called analytical columns have typical flow rates of 1 ml.min⁻¹. Columns with IDs of 1 and 2 mm are termed narrow bore columns and those packed in capillaries with IDs of 800 µm, 500 µm, 300 µm and 150 µm are known as microcapillary columns. Columns with narrower IDs are available; these are termed 'nanoflow' columns to denote that separations are performed using low nanoliter per minute flow rates and have IDs of 100 µm, 75 µm, and 50 µm.

None of the parameters used to measure the efficiency of a LC separation is influenced by the ID of the column. For example, the efficiency of a LC column is often measured in terms of plate number (N), which is related to peak width; therefore, this parameter is a measure of chromatographic resolution [9]. It has been found that N does not change as a function of the ID of the column [reviewed in Ref. 10]. If an analyte eluted from a chromatographic column producing a peak with a width at half height of 0.5 minutes, in conventional high performance LC, in which the flow rate is 1 ml.min⁻¹, the volume of the peak would be ~ 500 µl, whereas if the separation was carried out in a 75 µm ID column, in which the flow rate is 200 nl.min⁻¹, the volume would be ~ 100 nl. It follows that 75 µm ID columns can concentrate the analyte by a factor of 5000 when compared to 4.6 mm ID columns. Analyte concentration is important for its detection by UV absorption, which is the most commonly used detection method in LC. This is because, as indicated by the Lambert-Beer Law, absorbance is proportional to analyte concentration, the length of the path through the solution and the extinction coefficient of the

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analyte [11]. Thus, by increasing the concentration of analyte by a factor of 5000 the sensitivity of a chromatographic system with UV detection should in principle increase by the same factor. The sensitivity by other detection methods, such as electrospray ionization (ESI) MS, whose response is also concentration dependent, is also improved by column miniaturization. In the case of ESI, the observed increase of sensitivity is also attributable to a more efficient ionization at low flow rates (this will be discussed in more detail below).

In the preceding discussion it is assumed that both columns have the same efficiency. There are technical problems associated with the reduced flow rates used in microcapillary LC and nanoLC. One of them is that relatively small dead volumes (i.e. the volume between the end of the column and the detector) can have detrimental effects in the resolution because of diffusion of the analyte after chromatographic separation and concentration. Reduction of the ID of the connecting tubes minimizes this dead volume but some extracolumn effects are commonly observed.

Solvent Delivery Systems

Flow rates in the nanoliter per minute range have been achieved using HPLC pumps operating at a relatively large flow rates (low microliter per minute) and then splitting the mobile phase to obtain the desired flow [12,13]. The flow splitter can be placed before or after the column. Although the latter configuration has been used for the analysis of small molecules [14], proteomics applications with limited sample amounts normally use systems in which a flow splitter is placed before the sample loop. The first commercial nanoLC system was marketed in the mid 1990's (by LC Packings), and recently, other companies (e.g., Dionex, Waters and Amersham) also have commercialized solvent delivery systems capable of low nanoliter per minute flow rates. In almost all cases, pumps deliver the mobile phase at a flow rate which is then split to nanoliter per minute ranges. Recent publications report systems that deliver the mobile phase at a flow rate controlled by switching valves [15,16] and systems capable of nanoLC without solvent splitting have also been developed lately [17] (commercially available from Eksigent).

Sample Injection

Overall sensitivity for real case applications is dependant on the injection of relatively large volumes of sample. In the hypothetical case that five microliters of sample were to be injected in a nanoLC column operating at 50 nL.min⁻¹, the loading time would be 100 minutes. Clearly, this loading time is impractical for routine applications, such that methods for fast sample injection were developed. The simplest of them is to inject the sample at a relatively large flow rate; after sample loading, the flow rate can then be reduced for gradient separation. This method is limited by the high back pressures that occur as a result of operating large flow rates through narrow packed capillaries. Thus, a more practical method is to load the sample using column switching configurations [18]. In this, a short, larger bore trapping column is placed in the sample loop. Analyte molecules are injected onto this column. After loading and

washing, a mobile phase of increasing organic content elutes the analytes from the trap column to the analytical column. This method is practical because samples can be loaded in a short time and then washed extensively with low organic mobile phase to remove salts and other weakly retained compounds, which could block the narrow capillaries and columns used for nanoLC. Consequently, column switching methods are ideal for the analysis of 'dirty' samples and for 2D-LC applications in which fractions from an ion exchange separation (with high salt concentration) are analysed by reversed phase (RP) nanoLC-MS. However, the use of this loading method may result in some loss of chromatographic resolution, such that other groups developed injection configurations based on a 'vented column' principle [19-21]. In this, a short concentrating column and the analytical column are connected by a T-junction. During loading to the short column, the T-junction is vented so that the flow downstream of the concentrating column is diverted to waste. After loading and washing, the T-junction is plugged so that the mobile phase used to elute peptides from the concentrating column is diverted to the analytical column. This configuration reduces the dead volume between the concentrating column and the analytical column, and as a result, the resolution and sensitivity of the chromatographic is not affected.

Capillary Columns for nanoLC and nanoLC-MS

Packing of chromatographic beads into capillaries of narrow diameter requires techniques of a different nature than those used for packing of conventional HPLC columns. One of the problems is the choice of frit to retain functionalized silica beads in capillaries. Several methods to construct porous frits have been developed, including sintering methods [22], restrictors and tapers [23,24], porous filters [12,25,26], porous ceramic plugs [27], and unions containing stainless steel screens [28].

Once fritted, columns are slurry packed to a length of five to fifteen centimeters, although the use of long capillary columns packed to a length of 80 to 100 centimeters was reported by the group of Smith who use these unusually long columns routinely [28]. NanoLC separations carried out with long columns, in combination with extended gradients, afford greater resolution and peak capacities than those achieved with shorter columns. Another trend to increase chromatographic resolution is to construct columns with stationary phases consisting of particle sizes of less than 2 μm [Ref. 29]. A potential problem is that separations using long columns or those constructed with small particles require back pressures between 5,000 and 15,000 p.s.i. in order to deliver the mobile phase through the packed capillaries. Although until recently custom LC equipment was needed to achieve these pressures, a commercial LC system is now on the market that can withstand up to 15,000 p.s.i. (commercially available from Waters).

Columns for nanoLC-MS can also be constructed using fused silica capillaries tapered to a narrow end, such that the column has an integrated ESI emitter [19-21;24]. This type of columns is conceptually attractive because they reduce or completely eliminate dead volumes between the column and

the ESI source, thus aiding in increasing chromatographic resolution and consequently sensitivity.

Detectors

Another technical problem associated with nanoLC separations is that at low flow rates diffusion would occur in the flow cell of standard UV detectors, if these were used for nanoLC. As discussed above, in addition to concentration, absorbance is proportional to the optical path length in the UV detector. However, if this path length was too large diffusion of the analyte in the flow cell would occur such that this would lead to dispersal and peak broadening. To solve this problem, flow cells for UV detectors have been developed with U- and Z-shaped configurations that minimize dead volumes while maintaining a relatively long optical path [30]. However, due to different geometries and design, the sensitivity of UV detectors for nanoLC is less than that predicted by theoretical considerations [12].

Mass spectrometers are the most powerful detectors for nanoLC, and coupling of nanoLC to MS (nanoLC-MS) and MS/MS (nanoLC-MS/MS) allowed the development of several large scale and relatively comprehensive proteomic strategies. Although there is a recent interest in the coupling of LC to MALDI mass spectrometers, LC has been traditionally hyphenated to ESI-MS. This is because ESI is an ionization process that requires the analyte to be in the liquid phase, so that as they elute from LC runs, analytes can be ionized on-line by ESI and thus directly detected by MS. Wilm and Mann described an increased sensitivity of ESI when low flow rates in the inlet capillary are used and proposed the term nanoelectrospray or nanospray (nanoESI) for this variation of ESI [31-32]. Subfemtomole sample consumption was reported when using nanoESI emitters in combination with quadrupole MS [32]. Consequently, the increased sensitivity of nanoLC-MS is due to both an increased sample concentration capability of nanoLC and increased ionization efficiency during nanoESI. Low zeptomole (10^{-21} moles) detection limits have been reported for nanoLC-MS when high resolution chromatographic and mass spectrometric systems were combined [33].

BIOLOGICAL APPLICATIONS OF NANOLC-MS

Because of the technological developments on nanoLC briefly outlined above, the hyphenation of LC and MS has provided analytical biochemists with the opportunity to analyze peptides and proteins in biological fluids with unprecedented sensitivity. Microscale LC-MS/MS was first used to analyze peptides [34] and as an alternative to other MS methods, namely MALDI-TOF MS, to identify gel separated proteins [35]. In the last few years, other applications of nanoLC have been reported. A comprehensive review of all the different approaches that use nanoLC is clearly outside the scope of this article. Instead, examples will be given to illustrate how this technique has been used, and can be used in the future, to analyze peptides and proteins.

Analysis of Peptides in Biological Samples

Unlike the analysis of proteins above 10 kDa, peptides are too small for their efficient separation by gel

electrophoresis, a commonly used method for protein analysis. On the other hand, as discussed above, LC-MS methods can readily detect and sequence peptides with sensitivity and specificity. Therefore, nanoLC-MS has been used extensively to analyze this type of molecule. For example, nanoLC-MS was used to sequence major histocompatibility complex peptides from cells infected by the measles virus and from uninfected cells. Subtractive comparison of the data revealed peptides that were present in immunoprecipitates from the infected cells only, thus indicating that they were virus-specific peptides [36]. Similarly, Brockman *et al* also used a nanoLC-MS approach to identify peptides from *Trypanosoma cruzi* processed and presented by the MHC class I pathway [37].

Neuropeptides and other peptide hormones have also been analysed by LC-MS [21,38-43]. The group of Kennedy used short 25 μm ID tapered columns containing integrated ESI emitters to quantitate neuropeptides collected from the *in-vivo* microdialysis of rat brain [40]. A sensitivity of low attomole (10^{-18} moles) of analyte on column was reported. Recently, the same group has used this system to discover peptides in brain that may prove to be novel neuropeptides [44].

Peptides in serum have also been detected and quantified by nanoLC-MS. Oosterkamp and colleagues used a nanoLC-MS method with a column switching set-up to analyse peptide hormones in plasma [45,46]. In this, an anion exchange column retains peptides while allowing non-polar compounds to waste. Peptides are then eluted directly from the anion exchange column to the analytical RP column, so that they can be analysed by nanoLC-MS. A column switching configuration was also reported for the analysis of neuropeptide Y in serum [47]. Peptides were retained in a restricted access cation exchange column, while proteins, organic anions and neutral matrix compounds were washed to waste. As with the previous example, elution of peptides from the ion exchange column directly to the RP column allowed for subsequent LC-MS analysis. Although this study used conventional high performance LC columns, rather than nanoLC ones, it is mentioned here to illustrate the principle of column switching for rapid and efficient enrichment of peptides by combining two modes of chromatography on-line.

A similar column switching method was applied for the analysis of small peptides in the urine of renal Fanconi patients with the difference that columns of narrower diameter were used for enhanced sensitivity [41]. This column switching system consisted of three columns connected on-line via two injectors. Two capillary LC pumps delivered the flow rate and one of them was modified for nanoLC operation. Eluting peptides were detected and sequenced online by a hybrid ESI-MS instrument capable of obtaining tandem mass spectra (MS/MS), which is used for primary structure determination of peptides (i.e., sequencing). A sensitivity of one nanomolar was observed for peptide standards spiked in urine when one microliter of sample was used for analysis (1 fmol of standard on column). About fifty different small peptides were identified in renal Fanconi patients, many of which were not found in healthy individuals.

The studies briefly discussed above demonstrated that column switching nanoLC-MS methods can be used for the analysis of peptides present in small volumes of biological samples. Column switching methods have the advantage that separation of peptides from matrix compounds, which could interfere with subsequent LC-MS analysis, is carried out on-line. This results in quantitative transfer of analytes from the concentrating column to the analytical column and sample losses are in this way minimized. However, due to their limited peak capacity, these methods are not suitable for comprehensive analysis of all the components of a biological fluid. Peak capacity can be increased by adding an extra dimension of the separation before RP nanoLC-MS. Thus almost two hundred peptides could be detected in the urine of renal Fanconi patients after extensive separation prior to nanoLC-MS [42]. In this study, strong cation exchange (SCX) was used to obtain fifteen fractions which were then analysed by nanoLC-MS. Of the peptides detected, many were only found in renal Fanconi patients and had not been reported in normal urine before, thus indicating that the appearance of these peptides in urine may be specific for this condition.

Proteomics Studies Based on nanoLC-MS and nanoLC-MS/MS

The invention and optimization of 2D gel electrophoresis in the mid 1970's allowed for the visualization of several hundred to thousand proteins in a single experiment [48,49]. This method has been extensively used to compare the protein patterns of biological samples after exposure to determined agents and to compare cancer cells with cells derived from normal tissue [50-54]. The identity of proteins present in gel spots showing an altered level of expression can then be determined by MALDI-TOF MS or nanoLC-MS/MS [35]. Although useful for the visualization of proteins and for obtaining proteome patterns, 2D gel electrophoresis has intrinsic limitations, which make this technique unsuitable for all proteomic applications. Of importance, due to its intrinsically limited dynamic range, 2D gel maps may only display the most abundant proteins [55] and important proteins such as those embedded in membranes may not enter the gels used for the first dimension of the 2D separation [56]. Furthermore, spots in 2D gels often contain more than one protein [66], thus making it impossible to obtain reliable quantitative information in these instances.

Due to the limitations of 2D gel electrophoresis discussed above, some groups reported efforts directed at obtaining proteome maps using 2D-LC-MS. In each case, two orthogonal separation methods were used to increase the peak capacity of the system. Opitck and colleagues reported the application of size exclusion chromatography to separate cellular proteins into several fractions [57]. Proteins present in each fraction were then analyzed by nanoLC-MS. Similarly, Lubman and coworkers reported a method by which proteins are separated by isoelectric focusing in the first dimension followed by RP-LC-MS in the second [58]. This method was successfully implemented to analyze proteins derived from cancer cells [59,60]. Recently, other investigators have reported the use of free-flow electrophoresis followed by narrow bore RP HPLC for

proteome studies [61]. Separation of proteins by free-flow electrophoresis may provide advantages over gel electrophoresis in that some proteins, specially the large and hydrophobic ones, fail to enter isoelectric focusing and polyacrylamide gels. Moreover, recovery of these proteins for downstream analysis may be less problematic if proteins are kept in solution.

Although these publications demonstrated the feasibility of using separations in the liquid phase for obtaining proteome maps, it is not clear whether these methods provide greater proteome coverage than the 2D gel approach. Some proteins may precipitate during the RP separation due to the fact that these molecules tend to precipitate in the organic solvents used in this mode of chromatography. Furthermore, quantitation of proteins by nanoLC-MS remains problematic. Thus, 2D-LC at the protein level has not gained general acceptance as alternatives to 2D gel electrophoresis such that they may be regarded as complementary techniques, which will be useful for certain applications, but perhaps not as generic proteomic methods.

In contrast, 2D LC-MS at the peptide level is being increasingly used as an efficient alternative to 2D gels for proteomic studies [62]. In this approach, mixtures of proteins are digested with a protease; two orthogonal separation techniques are then used to separate the peptides generated as a result. In the first dimension, peptides are separated by SCX, which separates peptides according to their charge, and by RP nanoLC-MS in the second. The latter separates peptides based on hydrophobicity. Peptides eluting from the column are further separated according to their *m/z* by MS and sequenced by MS/MS. Due to the several levels of separation, 2D-LC-MS/MS is sometimes referred as multidimensional protein identification technology (MudPit) [63,64]. This method is also termed shotgun proteomics to indicate its large throughput sequencing capabilities. Yates and colleagues were one of the first groups to develop and implement 2D-LC-MS/MS for large scale proteomics [65]. They demonstrated that more than a thousand yeast proteins could be identified in a relatively short time (compared with the time needed to identify proteins from 2D gels). Several studies validated the usefulness of this and other related methods for cataloguing the protein composition of whole organisms [66-69], biological fluids [43,70-73], protein complexes [74-76] and subcellular compartments [77-79].

The major advantage of carrying out 2D-LC after proteolytic digestion is that peptides are more soluble than proteins, and because each protein generates several peptides upon proteolysis, it is more likely that at least some of the peptides derived from a particular protein have the right physicochemical properties to be amenable to LC separation and ESI-MS detection. Moreover, 2D-LC-MS at the peptide level is easily automated and faster than the 2D-gel approach. In addition, 2D-LC approaches offer the opportunity to increase sample loading capacity and dynamic range by increasing the number of fractions taken from the first dimension. The disadvantage is that some valuable information, such as molecular weight and the presence of isoforms, is lost when proteins in mixtures are digested prior to analysis.

Methods to obtain quantitative information from proteomic studies based on 2D-LC-MS have also been developed. Aebersold and co-workers invented a generation of reagents termed isotope coded affinity tags (ICAT), which are used to label cysteine containing peptides [80]. Since two different ICAT reagents are available with two different molecular weights (they differ in their content of heavy isotopes), they can be used to obtain relative quantitation of proteins by LC-MS after labeling, mixing, digestion and multidimensional separation of total cell lysates. Other strategies to obtain quantitative information from LC-MS have been developed. The groups of Yates [81] and Mann [82] reported approaches based on metabolic labeling of cells in culture using heavy nitrogen and heavy amino acids, respectively. Others employed isotopically labeled peptides that were used as internal standards to accurately quantify gel separated proteins [83]. Recently, isobaric reagents that label N-terminus of peptides have been commercialized (by Applied Biosystems). These are termed iTRAQ™ and have the advantage that since all peptides are labeled, this method can be used to follow changes in post-translational modifications. Other important advantages are that quantitation is performed from the MS/MS spectra, and because there are four different reagents (with four different Mr) they can be used to follow four different states simultaneously. iTRAQ™ reagents could have a great impact in quantitative mass spectrometry and this author looks forward for publications demonstrating their use.

In contrast to the approaches to obtain quantitative information from nanoLC-MS briefly discussed above, which rely on some sort of chemical labeling, Chelius *et al.* reported that ion counts obtained during 2D-LC-MS can be used to compare protein abundances in related samples^{84;85}, even when these analysis were performed without adding internal standards. These results may seem surprising given that ionization by ESI could be suppressed when several molecules co-elute from LC runs. The lack of signal suppression observed by Chelius could be due to extensive separation by 2D-LC. Indeed, increased separation efficiencies by using longer gradients [86] or introducing a further dimension [87] have been shown to reduce signal suppression effects. Another reason why signal suppression may not be a problem in nanoLC-MS experiments is that, as discussed above, the ionization efficiency of ESI increases when operated at low flow rates. Thus, when analysis by LC-MS was replaced by nanoLC-MS signal suppression was significantly reduced [88].

In line with the idea that ion counts from nanoLC-MS may be used for the relative quantitation of proteins and peptides, it was observed that the quantitative information obtained by nanoLC-MS correlated with those derived from ICAT and 2D gel electrophoresis experiments [71]. Quantitative information can also be obtained from gel separated proteins by spiking gel pieces with a known amount of protein during the in-gel digestion procedure (manuscript in preparation). This protein serves as an internal standard to correct for differences in protease activities and for differences of sample losses that may occur during extraction of digested proteins from the gel pieces. The ion intensities of analyte peptides can then be corrected to that of a closely eluting internal standard peptide to obtain

relative quantitation. As discussed above, new generations of ultrahigh pressure nanoLC systems are available which offer increased chromatographic resolution. Better resolved peptides would result in less competition for ionization during ESI. Therefore, an important application of this mode of chromatography may be in quantitative mass spectrometry approaches that do not use chemical labeling.

CONCLUDING REMARKS AND FUTURE PROSPECTS

The methodology for nanoLC is now relatively mature and several instruments capable to perform this type of chromatography have been commercialized recently. The hyphenation of LC to MS and MS/MS is currently allowing the fast and comprehensive detection of proteins and peptides in a myriad of biological samples. A current trend in the development of miniaturized LC is the fabrication of microfluidic devices for fast and sensitive analyses [89,90]. These designs will extend the applications of nanoLC and will be useful for high throughput screens of clinical samples.

Further developmental efforts should be directed at developing quantitative techniques based on LC-MS, such that comprehensive quantitation of proteins in a simple manner becomes possible. The currently available quantitative methods rely on isotopic or chemical labeling and need custom reagents or culture media incorporating heavy isotopes of either amino acids or nitrogen. Due to their expense and because these reagents are difficult to obtain, not many groups are using isotope-based quantitative proteomics routinely. Moreover, these quantitative approaches have fundamental limitations. The ICAT approach can only be used to quantify cysteine containing peptides [80], and thus this method cannot be used as a generic tool to quantify posttranslational modifications and proteins that do not contain cysteines cannot be quantified with this method [1,71]. As for metabolic labeling (e.g. the SILAC approach [91]), media to perform these experiments have to be depleted of at least one common amino acid before adding an amino acid labeled with a stable isotope. During the depletion process other essential nutrients are also removed such that not all cell types can be grown in this type of media. In addition, metabolic labeling is restricted to the analysis of proteins derived from cultured cells and can not be used for tissues obtained from model animals or biopsies. The iTRAQ reagents can overcome some of these limitations. Nevertheless, these are expensive reagents used to derivatize peptides, a chemical reaction which could, in principle, be a source of variability. Therefore, simplicity and affordability are key factors if these types of analyses are to be performed routinely in biochemistry research laboratories. In this respect, non isotope-based quantitative approaches are attractive due to their inherent simplicity, affordability, and because they can be applied to any biological sample. The limiting factor will be the development of computer software capable of extracting and exploiting the quantitative information already present in nanoLC-MS runs.

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ABBREVIATIONS

LC	=	liquid chromatography
nanoLC	=	nanoflow LC
MS	=	mass spectrometry
MS/MS	=	tandem mass spectrometry
RP	=	reversed phase
SCX	=	strong cation exchange
2D	=	two-dimensional
ESI	=	electrospray ionization
MALDI-TOF	=	matrix assisted laser desorption/ionization-time-of-flight
ID	=	internal diameter

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