Mass spectrometry: gaining mass appeal in proteomics

The interest of the research community in analyzing large sets of proteins in biological samples is driving technological developments and a proliferation of commercially available tools for proteomics studies. Diane Gershon reports.

At its most basic level, proteomics involves the identification, quantification, structural characterization and localization of complete sets of proteins in a proteome, which can be studied at the level of the organism, organ, tissue, cell or organelle. The ultimate goal of proteomics is to provide a better understanding of protein function in healthy and disease states.

There is also intense interest in applying proteomics to the discovery of new biomarkers for the diagnosis and early detection of disease and to develop more effective therapeutic strategies. Undertaking a comprehensive analysis of, for example, the human serum or plasma proteome, a hot topic right now, presents a formidable challenge. What defeats everybody is the complexity of the sample (number of proteins present) and the dynamic range of protein concentration, which can vary by 10–12 orders of magnitude, putting it beyond the range of detection of most presently available technologies.

As an analytical approach, mass spectroscopy (MS) was initially used in the life sciences to measure the mass of small molecules in drug research. But the availability of genome sequence data and technical advances, most notably the development of soft desorption ionization methods, has allowed researchers to harness the power of mass spectrometry to analyze large molecules, such as proteins—a technical achievement that resulted in the 2002 Nobel Prize in Chemistry. MS may have become an indispensable analytical tool but, to date, MS-based proteomics has enjoyed most success when applied to small sets of proteins. The systematic and comprehensive analysis of larger protein sets will require improvements at every step along the way—from sample preparation to data analysis.

The new ProteomeLab IgY-12 kit of Beckman Coulter. (Courtesy of Beckman Coulter.)

Most MS-based proteomics strategies apply a two-pronged approach: separation of proteins or peptides followed by MS for identification and quantification. Two-dimensional (2D) gel electrophoresis/MS is relatively straightforward but is low-throughput and suffers from a lack of robustness and dynamic range when dealing with proteins in pH and molecular-weight extremes, as well as with membrane or low-abundance proteins. Gel-free methods have been developed to overcome these limitations but tend to require more up-front methods to reduce sample complexity prior to liquid chromatography (LC)-MS analysis.

Divide and conquer

Fractionation and depletion of proteins are two of the most widely used approaches for pre-analytical sample preparation, and several commercially available products are available. Researchers are also developing their own multidimensional separation strategies to address the issue of sample complexity and the dynamic range limitations of present analytical techniques.

A popular product for the selective immunoaffinity-based removal of high-abundance proteins is the Multiple Affinity Removal System (MARS) from Agilent Technologies (Palo Alto, California, USA), available in LC-column and spin-cartridge formats. Both use affinity-purified polyclonal antibodies to selectively deplete more than 98 percent of the six most abundant proteins (albumin, α-1-antitrypsin, haptoglobin, immunoglobulin A, immunoglobulin G (IgG) and transferrin) in human serum and the three

major proteins (albumin, IgG and transferrin) in mouse serum, with minimal nonspecific removal of nontarget proteins. These depleted proteins account for 85 and 80 percent of the total protein mass in human and mouse serum, respectively.

Beckman Coulter of Fullerton, California, USA offers a different approach. Its new immunoglobulin yolk (IgY) partitioning systems and chemistries are based on avian-generated antibodies bound to inert beads that selectively partition the 12 most abundant proteins in human serum and plasma, which constitute up to 95 percent of the total protein mass. The company exclusively licensed the IgY microbead technology from San Diego-based GenWay Biotech, which offers its own Seppro line of IgY microbead-based products and services for the immunoaffinity-based separation of proteins from serum, plasma, CSF and urine.

"The IgY partitioning [system] allows us to essentially put on the PF 2D 5 mg of enriched serum or plasma, which represents an original starting concentration of 100 mg of plasma or serum," says Jeff Chapman, director of the proteomics business center at Beckman Coulter. Compared to a 2D gel approach, where you would be loading micrograms of sample, "we're improving detectability 3–4 orders of magnitude just by combining these techniques", he says.

BD Diagnostics of Franklin Lakes, New Jersey has also stepped into the pre-analytical protein separation arena with the acquisition last month of the technology and other assets of FFE Weber, a company based in Munich, Germany, that specializes in the separation and fractionation of complex proteins. The BD Free Flow Electrophoresis system, developed by FFE Weber, can perform fast matrix-free electrophoretic separation under native or denaturing conditions. Three main modes of operation offer separation according to isoelectric points (isoelectric focusing), net charge density (zone electrophoresis) and electrophoretic mobility (isotachophoresis).

Invitrogen of Carlsbad, California, USA, also has an answer to reducing sample complexity and enriching low-abundance proteins. Solution-phase isoelectric focusing with the company's ZOOM IEF fractionator, developed in part from technology that spun out of the Wistar Institute in Philadelphia, Pennsylvania, USA, is said to provide reproducible separations in three hours. The fractionated samples are then ready for analysis by one-dimensional and 2D gel electrophoresis or 2D LC-MS. The company also offers a Basic Protein Kit for use with the fractionator, which fractionates proteins in the 9–12 pH range.

The latest LC/MSDTrap XCT Ultra mass spectrometer. (Courtesy of Agilent, Inc.)

QIAGEN of Venlo, The Netherlands, is also getting in on the act. The company launched its major new Qproteome line in January 2005, which consists of eight kits that fractionate proteins on the basis of glycosylation patterns, subcellular localization, ionic charge, phosphorylation, affinity to nucleic acids or overall solubility. A monoclonal antibody–based kit is offered for the selective depletion of albumin and IgG from serum or plasma samples. Rather than launch the kits piecemeal, Joachim Schorr, QIAGEN's senior vice president of R&D says, "we waited till we had the complete package and solution for all these different questions".

Going with the nanoflow

Nanoflow-LC–MS (nano-LC–MS) has become a useful tool in proteomics applications in which there is demand for increased sensitivity or the sample is limited. But to obtain reproducible results at lower flow rates (nanoliters per minute), maintaining separation efficiency, flow accuracy and flow precision is critical and traditionally has required skilled operation of intricate nano-LC–MS equipment.

Dionex of Sunnyvale, California, USA, launched a new UltiMate 3000 nano-LC system for front-end MS protein and peptide separations in March 2005. With the UltiMate 3000, reliable generation of nano and capillary gradients is performed using an active flow splitting system (UltiFlow), said to significantly improve the reproducibility of gradients and flow accuracy down to flow rates of 50 nl/min, when compared to passive flow splitting approaches. The system is optimized for use with separation columns of inner diameters of 50 μm or more, and its modular design means it can be configured

for a range of separations, such as single or multidimensional LC, sample cleanup or sample preconcentration.

No relation to UltiMate, but the NanoMate HD system from Advion BioSciences of Ithaca, New York, USA, allows users to take full advantage of the capabilities of nanoelectrospray for protein analysis through this automated chip-based nanoelectrospray system. The lower flow rates (nl/min) that are characteristic of nanoelectrospray MS improve ionization efficiency and allow for longer analysis times, permitting users to extract the maximum information before the sample runs out. The centerpiece of the NanoMate HD system, which can be mounted on several ESI mass spectrometers, is the ESI chip, an array of 400 nanoelectrospray nozzles etched in silicon. Each nozzle is used only once to eliminate sample carryover.

Agilent Technologies also has a chip-based product that integrates multiple functionalities on a single microfluidics-based chip (see **Box 1**).

Mass spec marketplace

From an analytical standpoint, "there is no magic box out there that will answer all the questions," says Mark Flocco, business development manager for clinical proteomics and biomarker discovery at Bruker Daltonics, an operating company of Bruker BioSciences of Billerica, Massachusetts, USA. Bruker recently introduced a new benchtop MALDI–time-of-flight (MALDI-TOF) mass spectrometer, designed as a robust and affordable solution for clinical proteomics, as well as the routine expression analysis of peptides and proteins.

Detlev Suckau, head of MALDI application development (proteomics) at Bruker Daltonics in Bremen, Germany, says the Microflex LT "is optimized in price and volume". But it is not available as, nor can it be upgraded to, a MALDI-TOF(/TOF) instrument like other instruments in the Flex series—the mid-range, vertical Autoflex II and top-of-the-line Ultraflex II.

All Bruker machines in the Flex series offer a gridless ion source, as grids have been known to cause loss of signal, as well as AnchorChip technology, which provides exact positioning of the samples on the MALDI target for robust and fast automation. Next generation prespotted AnchorChip MALDI targets are also now available for MALDI-TOF(/TOF) instruments. These disposable, polymer-based

Acquity HPLC system from Waters. (Courtesy of Waters, Inc.)

MALDI targets, developed with Eppendorf of Hamburg, Germany, come in a 384-sample microtiter plate format conveniently prespotted with matrix and calibrant spots. This setup eliminates any problem of cross-contamination from previous samples inherent to reusable steel targets and makes MALDI sample preparation more convenient and less error-prone.

Waters Micromass of Milford, Massachusetts, USA, is pinning its hopes for future sales growth on its new end-to-end LC-MS solution for label-free quantitative proteomics and biomarker discovery. The Protein Expression System allows researchers to quantitatively assess changes in protein expression while performing qualitative protein identification—all within the same LC-MS run. Unlike other quantitative approaches (for example, using isotope-coded affinity tags), which necessitate the chemical modification, metabolic labeling or enzymatic derivatization and analysis of only a small proportion of the peptides associated with each protein within a sample, the Waters Protein Expression System does not require peptide derivatization and quantitatively profiles the majority of the tryptic peptides in a sample.

The system consists of the Waters nanoACQUITY Ultraperformance LC (UPLC) System interfaced to the company's Q-Tof Premier LC–tandem mass spectrometric (LC-MS/MS) instrument, which provides routine exact mass measurement in both MS and MS/MS modes. The nanoACQUITY UPLC system is based on 1.7-micron particles and allows uses to achieve high-resolution separations at nano-flow rates on columns with internal diameters ranging from 75 μm to 320 μm and without flow-splitting. Traditional MS/MS technologies operate in a data-directed analysis mode in which precursor ions are selected for MS/MS analysis in series where co-eluting low-abundance precursors can sometime be missed. The Protein Expression System uses a parallel fragmentation mode that allows precursor and fragment ions to be analyzed simultaneously, resulting in an improvement in the duty cycle and a significant increase in sequence coverage for each identified protein.

The quantitative aspects of the analysis were described in a recent paper in *Analytical Chemistry*[1]; a second paper, soon to be published in the same journal, will focus on protein identification aspects. The analysis combines accurate mass and chromatographic retention time measurements, with other measurements like fold change and ion intensities, to provide a unique signature for each peptide contained in a complex protein digest mixture. The report covered the analysis of human serum, but the method is applicable to other biological samples.

Building better traps

The workhorse in many proteomics research facilities is the ion trap. They are robust and reliable and relatively inexpensive. They offer good sensitivity but generally their mass accuracy and mass resolution is not that great. They do, however, allow multiple stages of MS to be performed. Ion traps can reproducibly generate high-quality MS/MS

data, greatly increasing the amount of structural information that can be obtained.

Improved scan cycle times that enable collection of mass spectra at rates up to three times faster than its predecessors, the XCT and XCT Plus, are the hallmark of Agilent's new LC-MSD Trap XCT Ultra, which was introduced in April 2005, and will be shipping in July 2005. According to Agilent, this increase in scan cycles can result in the identification of 60–80 percent more peptides from proteolytically digested proteins and can also significantly improve quantitation. The XCT Ultra, which is based on advanced multipole, nonlinear ion trap technology from Bruker Daltonics, has a full-scan MS/MS sensitivity specification of 250 femtograms for reserpine. The instrument also has two new automated data-dependent neutral-loss analysis modes that provide enhanced data quality for better analysis of post-translational modifications (PTMs), such as phosphopeptides and glycopeptides. The new XCT Ultra will also be compatible with Agilent's new HPLC-Chip technology (see **Box 1**).

Ion traps with a linear configuration offer faster scanning speeds and higher trapping and detection efficiencies compared to conventional three-dimensional (3D) ion traps. Applied Biosystems of Foster City, California, USA, together with joint venture partner MDS Sciex of Toronto, Canada, offers a hybrid linear ion trap as part of its Q TRAP series of LC-MS/MS systems. In April 2005, they launched a new mid-level platform in that series, which, like its predecessors the Q TRAP and the 4000 Q TRAP LC-MS/MS systems, combines the specificity and quantitation capabilities of triple quadrupole technology with the full-scan MS/MS sensitivity of an ion trap.

The 3200 Q TRAP system is part of the new BIOiTRAQ systems used in proteomics for the identification and absolute and relative quantitation of biomarkers. It will also incorporate the NanoSpray source and nanoflow interface that can also be found on the 4000 Q TRAP system. For nanoflow applications, the heated nanoflow interface is said to provide increased stability, ruggedness, flexibility and ease of use, especially for negative ion and polarity switching analyses.

The LTQ linear ion trap from Thermo Electron (San Jose, California, USA) is about 20–50 times more sensitive than the older ion traps and the scan rate is about five times faster, says John Yates, head of the proteomic mass spectrometry lab at The Scripps Research Institute's Department of Cell Biology in La Jolla, California, USA. The improvement in performance is so marked that Yates, who has three LTQs thermo in his lab, quips: "I'm having trouble getting people to use the older [3D] ion traps." Thermo sells the LTQ as a standalone instrument or as part of a ProteomeX LTQ workstation complete with integrated HPLC and application-specific kits for high-throughput protein identification, maximum sequence coverage, phosphorylation site mapping and multidimensional LC-MS (MudPIT).

"The hot technology at the moment is the hybrid LTQ-FTMS," says Yates. "It has turned Fourier transform-MS (FTMS) into a very usable technology". Historically, FTMS had

a reputation for being operationally complex and not sufficiently robust. The Finnigan LTQ FT from Thermo is a hybrid mass spectrometer that combines linear ion trap and FT-ion cyclotron resonance (FT-ICR) technologies in a single instrument. According to Yates, who is about to take delivery of his first LTQ FT, the instrument provides very high mass measurement accuracy in the 1-3 ppm range and very high mass resolution of 70,000-150,000. "This is not easily achievable with other current technology, certainly not in an LC-MS environment," he says. But they don't come cheap at around $800,000, which is considerably more than a standard ion trap.

The strength of the LTQ-FT is in its extraordinary mass accuracy. This enables the detailed analysis of intact proteins, providing identification, primary structure determination and characterization of PTMs at the protein level, without the need for protein digestion and analysis of the peptides using 'bottom-up' approaches. "Some of the modifications can be very similar but the mass accuracy of the LTQ-FT can resolve those issues," says Tim Schlabach from Thermo. The company also introduced two new protein analysis software products earlier this year: ProSight, dedicated to top-down protein sequencing, and Sequest Sorcerer, designed to accelerate protein database searching up to 50-fold. "This single PC-sized solution will give you almost cluster-type performance without the need for an IT department," says Schlabach.

Bruker's hybrid FTMS instrument is the apex-Qe. The instrument is now fitted with the Apollo II ion funnel technology, which is said to significantly improve the analysis speed of the apex-Qe FTMS platform for proteomics research, and to provide an order-of-magnitude improvement in sensitivity for all modes of FTMS accurate mass measurement. The apex-Qe can selectively isolate and accumulate ions in the FTMS analyzer to enrich their abundance for subsequent structural experiments. This is useful for the analysis of low-abundance molecules, and particularly for PTMs, where accurate mass measurement on the MS/FTMS-fragments is necessary for structural characterization.

Mass spectrometry has come a long way since the early mass spectrometers and does a marvellous job of identifying materials. It does an even better job if the complexity of the material is simplified beforehand. And, while there is still no 'one-size-fits-all' solution for MS-based proteomics, from a hardware standpoint, "we're in pretty good shape", says Suckau. On the software side, however, "this is really an open field where there is no end in sight".

1. Silva, J.C. *et al. Anal. Chem.* **77**, 2187–2200 (2005).
2. Yin, H. *et al. Anal. Chem.* **77**, 527–533 (2005).
3. Fortier, M.H., Bonneil, E., Goodley, P. & Thibault, P. *Anal. Chem.* **77**, 1631–1640 (2005).

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BOX 1 CHIPS WITH EVERYTHING

Obtaining reproducible results from nanoflow high-performance liquid chromatography (HPLC) can be an art. Many researchers feel that the technique, which typically operates at flow rates between 150 and 300 nanoliters per minute (nl/min), is still not robust or reliable enough to use on a routine basis for MS-based proteomic analyses. Those who do take this approach often find they spend more time optimizing the chromatography than identifying proteins.

Conventional column-based nanoflow LC-MS can offer maximum sensitivity with minimal sample sizes but requires substantial user skill. The complex arrangement of equipment, with intricate fittings and connections, makes it difficult to maintain separation performance and obtain reproducible results at nanoliter flow rates.

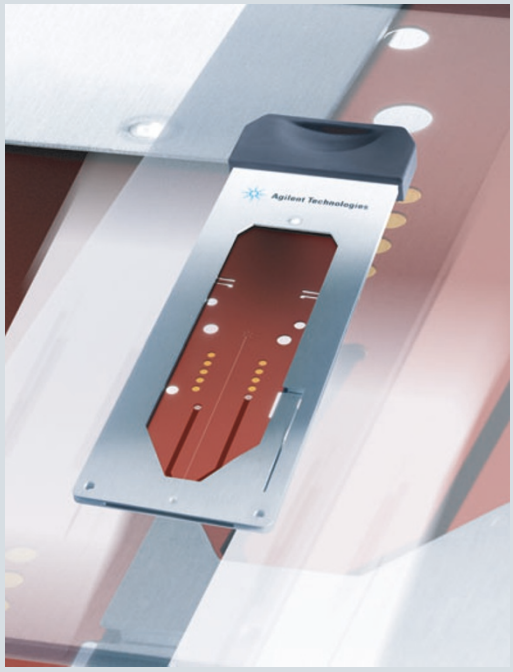
But help is at hand. In February, Agilent Technologies of Palo Alto, California, USA, introduced a new HPLC-Chip/MS system for protein identification that should simplify the life of researchers not versed in the operation of nanoflow LC equipment. The microfluidics-based device integrates the sample enrichment and separation capabilities of a nanoflow LC system with the intricate connections and spray tips used in nanoelectrospray MS—all on a reusable inert polymer chip the size of a credit card.

Microfluidic integration of the nanoflow-LC components onto a single chip not only simplifies the workflow and improves ease of use, but also eliminates 50% of the fittings and connections typically required in a nanoflow LC-MS system. This reduces the possibility of leaks and dead volumes that can lead to post-column peak broadening and a reduction in sensitivity.

For operation, the chip is inserted into a HPLC-Chip/MS Cube interface, which mounts onto the Agilent ion trap mass spectrometer. In addition to positioning the sprayer tip in the optimal position for mass analysis, the HPLC-Chip Cube MS interface makes all the electrical connections and establishes leak-free high-pressure fluid connections. Chips can be replaced in seconds, considerably quicker than changing out conventional nano-LC columns.

"Since you have virtually no dead volumes, we're finding out that the efficiency of the chromatography is much better," says John Michnowicz, LC-MS Marketing Manager at Agilent Technologies. An evaluation of the microfabricated approach to nanoflow-LC was recently published in *Analytical Chemistry*[2] in which reversed-phase gradient separations of tryptic protein digests at flow rates between 100 and 400 nl/min allowed separation with subfemtomole detection sensitivity.

Pierre Thibault, of the Institute for Research in Immunology and Cancer at the University of Montreal in Quebec, Canada, beta-tested the HPLC-Chip/MS system for Agilent. He says, it represents "a step towards more ease of operation for people that are not accustomed to high-performance separation



The new HPLC -Chip/MS system from Agilent (Courtesy of Agilent, Inc.)

devices operating at nl/min flow rates". In a paper, also published in *Analytical Chemistry*[3], his group was able to detect variations in peptide abundance as low as twofold for spiked tryptic digests present at 2–5 fmol in plasma samples.

The use of laser ablation to manufacture the chips provides tremendous flexibility for creating HPLC-Chip designs with additional functionality. The Protein ID HPLC-Chip will be only the first of many chips to roll off the production line. In the short term, Agilent plans to introduce a chip with a longer column length of 150 mm in place of the 43 mm × 75 μm column. The longer column, arranged in a serpentine configuration on the chip, will allow for greater peak capacity. And, over the next 12–18 months, chips designed for multidimensional chromatography (ion exchange in the first dimension followed by reverse phase in the second), as well as for affinity separations and on-chip tryptic digests, should hit the market.

The Protein ID HPLC-Chip now only interfaces to Agilent equipment, specifically the XCT series of ion traps, which includes the recently launched LC/MSD Trap XCT Ultra. Agilent is also developing the HPLC-Chip interface for its electrospray TOF mass spectrometer and expects to have ironed out the kinks in the software to make that available this fall. There are also plans to develop the HPLC-Chip interface for a new quadrupole TOF (Q-TOF) instrument Agilent is planning to introduce in 2006.

SUPPLIERS GUIDE: COMPANIES OFFERING MASS SPECTROMETRY PLATFORMS AND RELATED EQUIPMENT AND BIOINFORMATICS SOLUTIONS FOR PROTEOMICS RESEARCH

Company	Web Address
Advion BioSciences	http://www.advion.com
Agilent Technologies	http://www.chem.agilent.com/scripts/PHome.asp
Applied Biosystems	http://www.appliedbiosystems.com
Becton, Dickinson and Company	http://www.bd.com
Beckman Coulter	http://www.beckmancoulter.com
Biacore	http://www.biacore.com/lifesciences
Bio-Rad Laboratories	http://www.bio-rad.com
Bruker Biosciences	http://www.brukerbiosciences.com
Cerno Bioscience	http://www.cernobioscience.com
Ciphergen	http://www.ciphergen.com
Decodon	http://www.decodon.com
Dionex	http://www1.dionex.com/en-us/index.html
Eksigent Technologies	http://www.eksigent.com
Eldex	http://www.eldex.com
EMD Biosciences	http://www.emdbiosciences.com/home.asp
Eprogen	http://www.eprogen.com
ESA Biosciences	http://www.esainc.com
FFE Weber	http://www.f-f-e.com
GE Healthcare (formerly Amersham Biosciences)	http://www1.amershambiosciences.com/aptrix/upp01077.nsf/content/na_homepage
GeneBio	http://www.genebio.com
GenoLogics Life Sciences Software	http://www.genologics.com
Genomic Solutions (subsidiary of Harvard Biosciences)	http://65.219.84.5/index.html
GenWay Biotech	http://www.genwaybio.com
Gyros	http://www.gyros.com
Imaxia	http://www.imaxia.com
Invitrogen	http://www.invitrogen.com
IonSpec	http://www.ionspec.com
Jeol	http://www.jeol.com
Kratos Analytical/Shimadzu	http://www.kratos.com
Lumicyte	http://www.lumicyte.com
Matrix Science	http://www.matrixscience.com
Merck KgaA	http://www.merck.de/servlet/PB/menu/1001723/index.html
MDS Sciex	http://www.mdsciex.com/home/low%20res/default.asp?s=1
NextGen Sciences	http://www.nextgensciences.com
New Objective	http://www.newobjective.com
Nonlinear Dynamics	http://www.nonlinear.com
Oxford Genome Sciences	http://oxfordgenomesciences.com
Perkin Elmer	http://las.perkinelmer.com
Proteome Sciences	http://www.proteome.co.uk
Proteome Systems	http://www.proteomesystems.com
Qiagen	http://www.qiagen.com
Shimadzu	http://www.shimadzu.com
Sigma-Aldrich	http://www.sigma-aldrich.com
Syngene	http://www.syngene.com
Tecan	http://www.tecan.com
Thermo Electron	http://www.thermo.com
Waters	http://www.waters.com/watersdivision/Contentd.asp?ref=CEAN-5KUSS8