Sample Prep Advances Extend Mass Spec

Improvements in Methodology Have Made MS an Analytics Option for Wider Variety of Studies

K. John Morrow Jr., Ph.D.

Sample preparation for mass spectrometry has been the topic of several recent symposia extensively covered in the pages of *GEN*. Mass spectrometry has grown over the years into an essential tool for macromolecular characterization due to the development of more economical and user-friendly instrumentation as well as more effective and accurate sample processing.

New wrinkles to the technology were considered at CASSS' recent "Practical Applications of Mass Spectrometry in the Biotechnology Industries." These include mass spec analysis of glycoforms in partial antibody fragments, more stringent characterization of disulfide bond formation, and innovative deglycosylation strategies.

Asish Chakraborty, Ph.D., a research scientist at **Waters** (www.waters.com), and his colleagues advanced the quantification of antibody glycoforms using liquid chromatographic (LC) separation combined with mass spec. "This area of investigation is critical for an understanding of the stability, biodistribution, and the target binding of antibodies," he stated.

mAbs are an excellent model system for mass spec investigations, as they are glycosylated at the asparagine residues in the Fc domain, and glycosylation heterogeneity at the Fc sites is well known, according to Dr. Chakraborty. Quantification of the glycan moieties frequently involves enzymatic release of the glycans from the proteins to which they are bound through N-linkages. Alternatively, fluorescent labeling and separation of labeled glycans using normal-phase chromatography with fluorescent detection are employed.

The presence of identical glycans in the protein, however, can make interpretation difficult. A superior interpretation of the structures may be obtained by both endopeptidase digestion of the antibody molecules, which results in the release of the Fc light chain fragment, and by reducing the sulphydryl linkages of the antibody to generate light- and heavy-chain fragments.

Dr. Chakraborty's group combined ultraperformance liquid chromatography (UPLC) and liquid chromatography/electrospray ionization with time-of-flight mass spectrometry (LC/ESI-TOF-MS) methods to optimize various sample-preparation schemes from several batches of the commercial recombinant IgG1 mAb trastuzumab. Using UPLC analysis of 2-AB labeled released glycans as a reference method, various LC/MS-based assays for glycan quantification were compared.

The MS-based assay of the Fc/2 fragment is accurate and provides results that are comparable to the N-glycan assay, Dr. Chakraborty explained. Each batch of antibody shares nearly the same relative abundance of each glycoform, but one specific glycoform was absent in some batches. Unlike N-glycan release assay, this LC/MS-based method cannot separate isobaric glycans.

"MS should be considered a complimentary technology to the N-glycan release assay, and though it may not give a scientist all the desired information, it can be a lot quicker and easier to use than before," Dr. Chakraborty remarked. "The beauty of the Xevo QT MS-based technology is that it is fast and easy to use, so results are yielded in an hour or two."

Direct Online Processing

Spark Holland (www.sparkholland.com), a provider of sample-prep hardware and software, specializes in online services for mass spec analysis. Dries Vrielink, SPE (solid-phase extraction) services and applications manager, and Marcel Jansen, global marketing director, discussed the company's extraction and separation technology for analytical systems, including HPLC, MS, GC, and NMR.

"There are several reasons why customers are interested in online sample preparation," commented Vrielink. "High recoveries and better sensitivity are the main assets of this technique. Offline sample prep using such procedures as protein precipitation are not sufficiently selective and liquid-liquid extraction is difficult to automate, slow, and labor intensive. Furthermore, because of the removal of aliquots of the samples and additional handling, there can be a substantial loss of material."

A solid-phase extraction system that allows one unattended run with online elution into HPLC and LC/MS systems was developed by the company in the 1980s and entered the market in the early 1990s. "We see big improvements in sensitivity with a notable economic advantage," Jansen commented. "Since the runs can be left unattended, the investigator can simply load the samples, start the method, and run them overnight. So while the first sample is being analyzed, the next sample is

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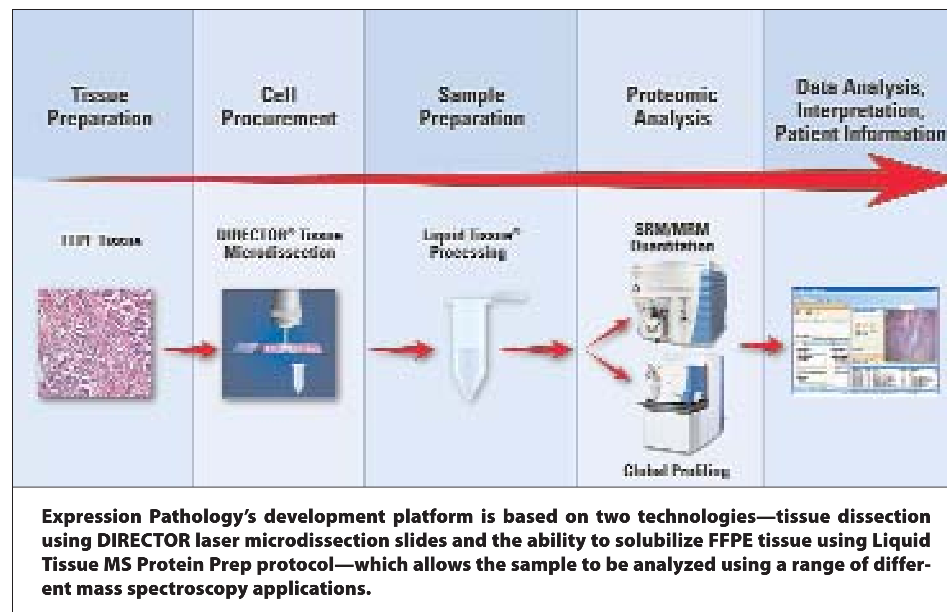

Expression Pathology's development platform is based on two technologies—tissue dissection using DIRECTOR laser microdissection slides and the ability to solubilize FFPE tissue using Liquid Tissue MS Protein Prep protocol—which allows the sample to be analyzed using a range of different mass spectroscopy applications.

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News Genomics & Proteomics

IBM Sets Out To Build Nanoscale DNA Sequencer

IBM Research (www.research.ibm.com) aims to build a nanoscale DNA sequencer which, if successful, could improve throughput and reduce cost to $100–$1,000 per genome sequenced, the company claims. A team of IBM scientists are working to master a technique that threads a long DNA molecule through a 3 nm wide hole, known as a nanopore, in a silicon chip. As the molecule is passed through the nanopore, it is ratcheted one unit of DNA at a time, as an electrical sensor reads the DNA.

To control the rate at which a DNA strand moves through the nanoscale aperture, IBM scientists have developed a device consisting of a multilayer metal/dielectric nanostructure that contains the nanopore. Voltage biases between the electrically addressable metal layers will modulate the electric field inside the nanopore. This device utilizes the interaction of discrete charges along the backbone of a DNA molecule with the modulated electric field to trap DNA in the nanopore. By cyclically turning on and off these gate voltages, scientists showed the plausibility of moving DNA through the nanopore at a rate of one nucleotide per cycle. IBM researchers believe that this rate would make DNA readable.

Integral Molecular Maps Cancer Biomarker for NCI

Integral Molecular (www.integralmolecular.com) was selected by the National Cancer Institute (NCI) to map epitopes for monoclonal antibodies directed against cancer biomarkers. Protein targets of interest to the clinical cancer proteomics community will be analyzed using shotgun mutagenesis mapping technology to identify amino acids that are essential for antibody binding, allowing the discovery and characterization of cancer-specific biomarkers. The $150,000 contract will be fully funded by the NCI.

Shotgun mutagenesis maps antibody epitopes by rapidly evaluating the effects of point mutations across an entire target protein. Using a high-throughput expression method, thousands of point mutations are concurrently evaluated for functional protein activity. Shotgun mutagenesis mapping identifies both linear and conformational epitopes on target proteins expressed in eukaryotic cells and in their native conformation. This approach is suited to cancer biomarkers because many are complex membrane proteins that are resistant to direct structural analysis.

TGen Named In Silico Center of Excellence

The Translational Genomics Research Institute (TGen; www.tgen.org) has been awarded a contract that could total about $2.07 million over three years to use computer simulations for brain cancer research. It joins four other national centers that have been selected by SAIC-Frederick under its prime contract with the NCI called the In Silico Research Centers of Excellence contract.

The award partners TGen with 5AM Solutions (www.5amsolutions.com), a Virginia-based life science software development firm. They will initially receive $691,930 for the first 12 months. The contract has two 12-month option periods that, if executed, would amount to an additional $1,373,582.

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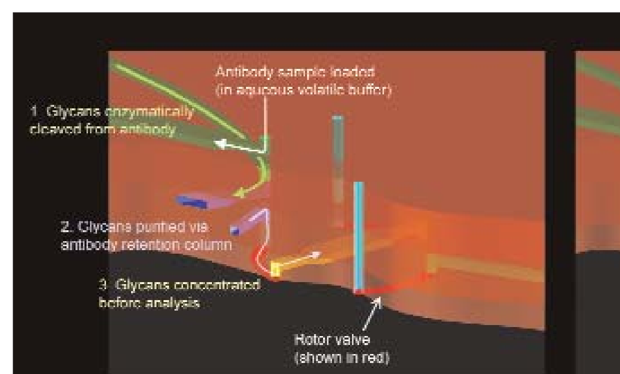
undergoing extraction."

Online SPE employs a technique similar to HPLC to isolate, enrich, and purify analytes from a sample matrix applied to a sorbent. All materials not adsorbed remain in the liquid phase and pass through the sorbent to waste. High pressures are applied to distribute the SPE solvents. The extracted sample is directly injected to the analytical column by a simple valve switch to avoid loss of the analytes of interest.

Online Deglycosylation Analysis

Improving and streamlining glycan analysis has also occupied a team of scientists at **Agilent Technologies** (www.agilent.com). "Our goal is to improve current workflows that use laborious enzyme-cleavage protocols of glycans followed by analytical characterization with mass spec and other time-consuming techniques," said Kevin Killeen, Ph.D., director of molecular separations.

To improve throughput and simplify analysis, Agilent has built an integrated microfluidic LC/MS device for enzymatic deglycosylation with immobilized PNGase F, constructed using laser ablation and lamination technologies. It performs complete sample preparation and analysis of n-linked glycans of IgG mAb samples. The microfluidic workflow system telescopes the required time for analysis from hours to a few minutes, according to Agilent.



Scientists at Agilent Technologies are working to improve and streamline glycan analysis. Steps 1 through 3 show antibody deglycosylation configuration, and steps 4 through 6 highlight glycan separation configuration (after switching rotor valve).

Glycans are released and purified from the antibody using an in situ porous graphitized carbon-trapping column. The deglycosylated recombinant antibodies are irreversibly trapped on the column, and the glycans are then eluted using a nanoseparation column with a formic acid gradient. Subsequently the sample undergoes electrospray ionization and identification by TOF-MS detection.

"We measure the amino form of the glycans instead of the hydroxyl form typically observed using in-solution cleavage," said Dr. Killeen, claiming several benefits to this strategy. "This an important advantage in characterizing minor components of the mAb glycan profiles. We also can better characterize the heterogeneity of deglycosylated antibodies with our on-chip workflow.

"Our approach reduces multiday solution-phase enzyme cleavage of glycans and sample clean-up workflows for MALDI, LC/MS, or fluorescent-labeling analysis to minutes for the HPLC chip approach," Dr. Killeen concluded.

Sensitive Detection of Antifoam

According to Mark Schenerman, Ph.D., of **MedImmune** (www.medimmune.com), "Antifoam C is widely used in the pharma and food industries, and while there are no known risks associated with its use, its presence is often monitored."

The compound, whose active ingredient is poly-dimethylsiloxane, is a silicon-containing material used to control foaming in industrial and manufacturing processes.

Although it is generally removed by subsequent processing, quantitative demonstration of this can be a challenge. Developing a sensitive detection method has proved challenging due to its lack of spectroscopic absorbability. Elemental silicon (Si), however, can be detected using inductively coupled mass spectrometry, a sensitive approach.

"This technology has come a long way in the last 10 years," Dr. Schenerman said.

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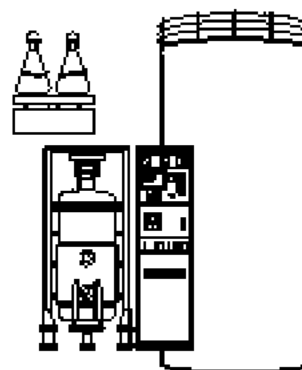

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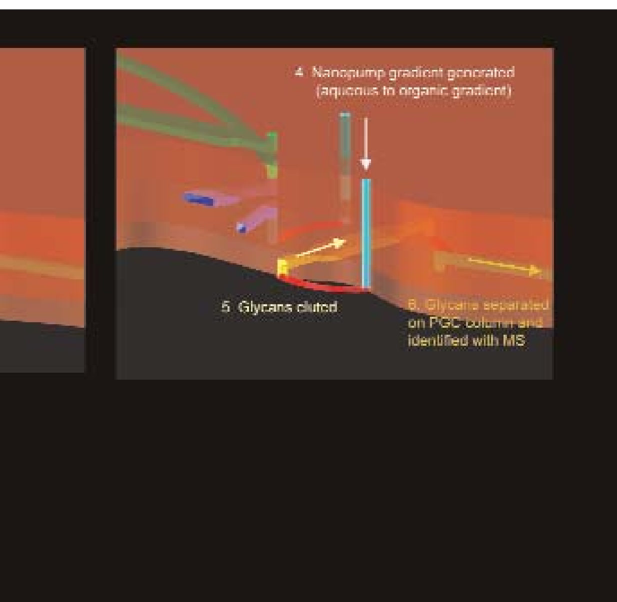

It uses an ionized gas, or plasma, and any material introduced into the plasma will itself be ionized and can be identified in the mass spectrometer. The procedure can measure Si at parts per trillion. It is widely used in trace-element identification throughout the industrial world.

MedImmune demonstrated that this approach to the quantification of Antifoam C is highly specific and reproducible. A number of different diluents and spiked solutions were monitored with a high degree of accuracy. Thus this method is suitable for determination of Antifoam C levels in production samples. It should be noted that this technique is applicable to the detection of any compound that has a unique and identifiable atom as part of its structure.

Formalin-Fixed Tissues

Ordinarily, materials analyzed by mass spectrometry are crude or partially purified samples. **Expression Pathology** (www.expressionpathology.com), however, has introduced technology for direct isolation and evaluation of formalin-fixed histological specimens, according to David Krizman, Ph.D., CSO.

The technology uses a laser-based microdissection technology termed Director® to collect specific cells from slides, which are then introduced into a protein buffer. Samples in the range of 30,000 cells are prepared for mass spectrometry using Liquid Tissue® MS reagents, which bring about complete solubilization and capture of the entire protein content in a mass spec friendly format, Dr. Krizman said.

When tissues at various stages of malignant transformation were analyzed for increasing levels of the Her2 peptide with the Director and Liquid Tissue technologies, there was a virtually perfect quantitative agreement with comparable data obtained using both immunohistochemistry and FISH measurements, he added.

The approach can also be used in biomarker discovery, according to the company, which has analyzed eight primary cutaneous melanomas and 16 metastatic brain lesions by LC/MS/MS global profiling of Liquid Tissue lysates. The team observed 120 significant protein changes in metastatic tumors as compared to the primary melanomas.

"We offer SRM assays on a collaborative/fee-for-service basis," Dr. Krizman noted. "We are willing to undertake collaborative protein biomarker discovery and validation collaborations either for clinical or basic research purposes." At present the company is gearing up to move assays based on its collection of putative biomarker proteins into clinical trials.

The disciplines of mass spectrometry and sample preparation are intimately bound together, since MS cannot be an effective analytical tool without the specialized methods for removing trace contaminants that might otherwise create unmanageable noise. Interfering materials including protein aggregates, protein fragments, and low molecular weight compounds can all render the technology ineffective. So sample preparation must be effectively married to mass spectrometry instrumentation to optimize performance. The procedures described in this article, while incremental in scope, make mass spectrometry an option for analytical tasks that would not otherwise be available. GEN

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