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New Aspects in the Integration of Mass Spectrometry Technologies in the Biopharmaceutical Industry

Esme Candish, Andrew Dykstra, Alla Polozova, Da Ren, and Hao Zhang

In the past decade, advances in both separations and mass spectrometry (MS) technologies have enabled new, streamlined, and data-rich approaches to monitor product quality attributes and their relationship with process parameters throughout the lifecycle of therapeutic proteins. As we enter a new decade of technology and method development, MS-based approaches utilized in the biopharmaceutical industry are evolving further. In this mini-review, we explore key developments that could inspire and improve the future of therapeutic protein development.

Protein-based therapeutics are inherently heterogenous because of the presence of a wide range of post-translational modifications, resulting from either the manufacturing process or degradation during storage. These liabilities must be well characterized, monitored, and controlled to ensure product safety and efficacy. High-resolution mass spectrometry (HRMS) has become an increasingly critical analytical tool for identifying, characterizing, and monitoring these attributes (1). Advances in HRMS technology have led to both the development of capabilities to aid characterization and quantitation and to the simplification of instrumentation to permit routine monitoring. Advancements of HRMS capabilities include increased resolution and faster scan rates compatible with liquid chromatography (LC) and alternative fragmentation techniques. Electron-based dissociation (ExD) and ultraviolet photodissociation (UVPD) approaches have become increasingly robust in commercial instrumentation, and these approaches can be utilized for glycosylation and disulfide characterization, confirmation of aspartic acid isomerization, and top- or middle-down sequencing. With these developments, the implementation of HRMS within the biopharmaceutical industry continues to expand and enhance the understanding of process and product quality while streamlining development.

Multi-Attribute Methods

Considerable HRMS hardware innovation has seen the development of compact and robust instrumentation, with HRMS platforms now small enough to be part of the LC stack. Furthermore, advances in acquisition and data processing software have led to ease of use and inclusion of cGMP-compliant capabilities to ensure data integrity (21 CFR part 11). This growth is due, in part, to the industry-wide interest in multi-attribute methods (MAMs), which utilizes enzymatic digestion of the therapeutic protein followed by LC–HRMS analysis. MAMs affords residue-specific identification and quantitative monitoring of multiple product quality attributes, together with the capability of new peak detection during process development and release and stability testing (2–4). Direct attribute quantitation achieved with MAMs has been demonstrated to provide a high level of agreement with the output of several established purity assays, including released glycan analysis by hydrophilic-interaction liquid chromatography (HILIC), charged variants measured by cation exchange chromatography (CEX), and fragments determined by the capillary electrophoresis–sodium dodecyl sulfate (CE-SDS) assay (4). In addition, MAMs can also be leveraged for protein identity and the monitoring of process related impurities, including protein A and host cell proteins. As a result, several purity assays that have traditionally been leveraged to track all attributes can be potentially replaced with a single MAM assay. Despite the additional complexities of the assay (including multi-step sample preparation, sophisticated instrumentation and data analysis software requirements), the advantages of MAMs have been widely recognized by both the biopharmaceutical industry and regulatory agencies, with the MAM Consortium (www.mamconsortium.org) serving as a platform to drive collaboration and growth.

HRMS instrumentation is most often utilized for MAMs, although low-resolution quadrupole instrumentation has been explored because of low costs, the small footprint, and enhanced instrument robustness (5). However, a report evaluating HRMS and low-resolution quadrupole instrumentation for MAMs found HRMS to be the preferred platform because of the enhanced selectivity, the lower limit of quantitation, the ease of method development, and the new peak detection capabilities (6). Recognizing the value of LC–HRMS data has led to extended utilization of MAMs, with examples of upstream process cell culture monitoring becoming available (7). Broader implementation of MAMs is likely to continue, particularly with the development of automated sample preparation tech-
niques and artificial intelligence powered data analysis software to increase accuracy and consistency while reducing processing time (2).

**Coupling Established Purity Methods with MS**

HRMS-compatible purity assays for simultaneous attribute quantitation and identification is realistic with the latest technologies. The direct hyphenation of native size-exclusion chromatography (SEC) (8), CEX (9), and hydrophobic interaction chromatography (HIC) (10) with HRMS (in further discussion referred to as simply MS) has been achieved with volatile mobile phases, such as ammonium acetate. Additionally, capillary zone electrophoresis (CZE)–MS (11,12) and, more recently, imaged capillary isoelectric focusing (iCIEF)–MS (13) have matured to the point of commercial availability. These new approaches offer the rapid determination of attributes associated with each discrete chromatographic or electrophoretic peak. As with the established purity assays, the “single” attributes are typically quantitated using the peak area of each region (for example, the basic or acidic region in CEX). The deconvoluted spectra are evaluated for potential attribute identifications. These spectra are typically highly complex, especially in cases where multiple attributes make up each peak. For many glycoproteins, major glycoforms are often the most abundant species, and are easily identified by the characteristic series of +162 Da hexoses additions. The mass shifts relative to the main chromatographic or electrophoretic peak can assist with the identification of the separated variants. Highly encouraging proof-of-concept work has focused on the characterization of attributes with large mass shifts such as size variants, glycoforms, incomplete processing of C-terminal lysine, and drug-antibody ratios (12, 14–16). The assignment of attributes with small or nonexistant mass shifts, such as deamidation (Δ0.98 Da) and aspartic acid isomerization (Δ0 Da), remains challenging, but can be inferred based on a shift in retention or migration time.

Despite the improvements in both separation technologies and MS capabilities, in-depth characterization and monitoring of key product quality attributes at the intact level remains challenging, because of the inherent complexity and the size of the molecules (~150 kDa for an IgG). Further, the ability of intact analysis to localize the attribute to the amino acid residue is limited, and, hence, it is difficult to determine criticality. To improve MS-based identification and quantitation of intact proteins, it is essential to reduce this complexity by either increasing the peak capacity of the separation to resolve each variant, or reducing the complexity of the sample itself. Multidimensional analysis can be very effective to reduce complexity, and is increasingly being explored; a recent comprehensive review has discussed advances in this field (17). Nevertheless, the integration of these rapid separation techniques for at-line analysis during protein production for real-time process feedback has the potential to improve the future of therapeutic protein development.

**Subunit MS Analysis**

Reducing the size and complexity of protein-based therapeutics into smaller fragments improves the ability to confidently identify variant species without the need for multidimensional separation instrumentation. A simple reduction of the disulfide bonds of an IgG yields two heavy chain and two light chain species of ~50 kDa and ~25 kDa, respectively. The heavy chain and light chain species are routinely separated chromatographically or electrophoretically, and the simplification of the spectra assists with attribute identification and the localization of the attribute to the subunit. The enhanced resolution of modern MS instrumentation enables the acquisition of isotopically resolved spectra of both the light chain and heavy chain for improved confidence in primary sequence confirmation and attribute assignment. Two options for the relative quantitation of attributes at the subunit level have been described; the first more conventional approach relies on the chromatographic or electrophoretic separation of the modified species from the unmodified species. The second approach involves deconvolution of the raw spectra followed by the relative quantitation of the attribute based on the intensities of the deconvoluted spectral peaks separated by mass. The ability to quantitate using deconvoluted spectra reduces analysis time significantly because there is less dependency on the upfront separation. This approach was explored for the at-line bioreactor monitoring of glycosylation to support process improvements (18), and has recently been validated for the cGMP monitoring of mannose-5 in routine manufacturing (19).

Further reduction in the molecule size and complexity can be achieved with limited proteolysis and a suite of enzymes that facilitate a highly site-specific cleavage are gaining popularity. The most commonly employed is IdeS (immunoglobulin-degrading enzyme of S. pyogenes), which cleaves the IgG heavy chain below the hinge region, producing the F(ab’2) and Fc fragments with molecular weights of ~100 kDa and ~25 kDa, respectively (20). A successive chemical reduction of the disulfide bonds produces three ~25 kDa fragments—the light chain, the Fc, and the Fd. The simplified data permits a high confidence mass determination and the ability to localize key attributes to a fragment. Furthermore, the ~25 kDa fragments are highly amenable to middle down sequencing by ExD and UVPD fragmentation, which has the potential to localize the attribute at the amino acid residue. As above, attributes can be characterized and quantitated from the separation, or from the deconvoluted mass spectra. Chromatographically separated identifications of clips, select charge variants, and glycoforms have been successfully demonstrated (20–23). An interesting body of work, poised to be impactful, has further reduced the spectral complexity for attribute quantitation of glycoproteins using deconvoluted mass spectra. The endoglycosidases, EndoS or EndoS2, are employed to selectively cleave between the two N-acetyl glucosamine (GlcNAc) residues of the core glycan, leaving a single residue with or without fucose, thus reducing the complexity related to glycan heterogeneity.
This approach has been demonstrated for the quantitative monitoring of aglycosylation, afucosylation, glycation, and high mannose, and the correlation with the relevant bioassay output confirms the potential applicability of this assay (18, 24). The fast sample preparation and rapid analysis enables the same simple method to be extended for the quantitative monitoring of Fc methionine oxidation (MetOx) (25). The success of this work has led to the transfer, co-validation, implementation, and regulatory approval of the subunit Fc MetOx method in commercial QC laboratories for product release and stability testing, a key milestone for HRMS (26). Automated, compliant-ready software tools are becoming available to streamline the quantitation of deconvoluted subunit mass spectra and will likely increase the adoption of this workflow to support an wider number of attributes.

Future Perspective
The past decade has seen enormous advancements in MS instrumentation and software capabilities for improved characterization and quantitation of the attributes of protein-based therapeutics. MS-compatible separation approaches and new enzymes continue to be introduced to the market, breaking down the complexity of the protein-based therapeutics prior to introduction to the MS. As technology matures and software evolves to simplify data analysis, it will be exciting to see the increasing adoption of ExD and UVPD for primary sequence confirmation and attribute localization with top- and middle-down approaches. We expect to see increasing applications of HRMS, particularly MAMS, in the cGMP environment, and the global regulatory acceptance of HRMS-based methods to replace established assays. The sustained implementation of HRMS-based technologies will continue to advance process and product improvements.

References

Esme Candish is a Process Development Scientist at Amgen, in Cambridge, Massachusetts. Andrew Dykstra is a Process Development Principal Scientist at Amgen, in Thousand Oaks, California. Alla Polozova is a Director of Process Development at Amgen, in Cambridge, Massachusetts. Da Ren is a Process Development Scientific Director at Amgen, in Thousand Oaks, California. Hao Zhang is a Process Development Senior Scientist at Amgen, in Cambridge, Massachusetts. Direct correspondence to: ecandish@amgen.com
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The editors at Nature Methods named top-down mass spectrometry (MS) a “method to watch” in 2008 (1) because of the ability of this approach to fully characterize proteoforms, a term not coined until a few years later (2). It is remarkable how, in a fairly short period since then, top-down MS has matured to the point where top-down proteomics can be applied to complex samples on a fairly routine basis. This advancement can be largely attributed to the technological improvements made in all stages of analysis, including sample preparation, chromatography, high-performance MS, and data processing. Ultimately, however, it is important to keep in mind that differences between proteoforms are important primarily because they modulate intra- and inter-molecular interactions in vivo and potentially have different downstream biological effects.

Two types of experiments can be used when combining native ionization with top-down fragmentation (3,4). The first experiment involves the investigation of noncovalent complexes by native ionization followed by the gas-phase ejection of monomers. Subsequently, the fragmentation of these monomers is evaluated, which allows elucidating the proteoform-specific stoichiometry of noncovalent complexes. In a previous study, the triosephosphate isomerase complex was analyzed, and the presence of three proteoforms was demonstrated—an unmodified one, an N-terminally acetylated one, and one that was phosphorylated at residue Ser20. It was shown that the phosphorylated and acetylated proteoforms do not dimerize with themselves or each other, indicating that these modifications regulate (specifically, inhibit) the formation of the dimer (5). Because a complex is broken down into its constituent monomers prior to extensive backbone fragmentation, this approach has been referred to as “complex-down MS” (3,4) This name was introduced to distinguish this approach from the second type of experiment, which combines native ionization with top-down fragmentation. This experiment, referred to as “native top-down,” aims primarily at obtaining information on the folding (secondary and tertiary structure) of proteins existing in the solution either as monomers or as subunits within complexes. It accomplishes this process by inducing (top-down) backbone cleavage within a native-like protein in the gas phase. The difference between complex-down and native top-down is illustrated schematically in Figure 1. One very early example was provided through collision-induced dissociation of the peptide RES-701-1 (6). This peptide has a “lasso” structure, and it was shown that the C-terminal end, which passes through the “ring”, is protected from fragmentation. This embed-
ding of the C-terminus was not replicated in a synthetic version of the peptide, and abundant C-terminal fragmentation was observed in that analogue, showing that the higher-order structure modulated the fragmentation pattern. Nowadays, collision-based fragmentation is very rarely used in native top-down experiments, as phenomena such as (partial) unfolding and monomer ejection generally precede backbone fragmentation. Cases like the RES-701-1 peptide, where noncovalent interactions are so strong that they survive up to the internal energies required for backbone fragmentation, are the exception rather than the rule.

Most native top-down studies today rely on interaction of protein ions with electrons (electron capture or transfer dissociation) or ultraviolet photons (ultraviolet photodissociation) (4). Unlike collision-induced dissociation, these methods allow backbone fragmentation without significant disruption of noncovalent interactions. As such, the fragmentation pattern is modulated by the higher-order structure. Of course, the implication is that—in contrast to complex-down experiments—full sequence coverage is not the goal of the native top-down approach because the absence of fragments from particular sequence regions can be informative. Approximately 20 years ago, the electron capture dissociation spectra of different charge states of ubiquitin were compared, showing that low charge states primarily release fragments from the terminal regions, with the release of fragments resulting from backbone cleavage in the central region of the sequence being observed mainly with high charge state precursors (7). This behavior was attributed to the progressive unfolding of the structure by salt bridge disruption with increasing charge state. A similar pattern was observed with temperature- or laser-induced disruption of the protein structure. Moving away from peptides and small proteins, an interesting model system is the alcohol dehydrogenase tetramer, which has been investigated with all three native top-down methods mentioned above (electron capture dissociation (8) electron transfer dissociation (9,10), and ultraviolet (UV) photodissociation (11)). For all three methods, studies are available where partial collision-induced unfolding was followed by backbone fragmentation, and this structural disruption consistently led to a change in the fragmentation pattern, which demonstrates the potential of native top-down for the rapid probing of protein folding and noncovalent interactions.

An interesting intermediate between the complex-down and native top-down approaches is the ejection of monomers or subcomplexes without (extensive) backbone fragmentation. Ejection of intact monomers is possible through carefully controlled collisional activation and can be helpful for determining the stoichiometry of a complex, because the mass of the complex and constituent subunits can be determined. This approach provides less detail than the complex-down workflow because detailed proteoform characterization is not performed. The ejection of subcomplexes is particularly interesting because it provides information on subunit connectivity within the original complex. Information on subunit connectivity in the original complex is usually not achieved through collisions with an inert gas because it requires deposition of sufficient energy to induce ejection of subcomplexes on a timescale that is too short to allow significant salt bridge rearrangement or monomer unfolding. Using a timescale that is too long typically results in the ejection of a highly-charged monomer as exploited in complex-down experiments.

![Native MS](native.png) + Complex-down

**FIGURE 1:** Illustration of native top-down and complex-down mass spectrometry (MS).
The best-characterized method for inducing the ejection of subcomplexes is surface-induced dissociation. In a recent example, the structure of the heterocomplex, Mnx, was investigated, and it was shown that it consists of a large (138 kDa) subunit connected to a heterohexamer made up of three 11.2 kDa and three 12.2 kDa subunits in an alternating sequence (12). As these approaches without extensive backbone fragmentation allow the virtual reconstruction (or “building up”) of the structure of a complex from the observation of subunits or subcomplexes, the name “complex-up MS” has been proposed for this type of experiment (3,4).

The coming years will see further adoption of the complex-up, complex-down, and native top-down approaches, as well as the expansion of the application field. The Consortium for Top-Down Proteomics has completed a number of initiatives to this end in recent years, including one on top- and middle-down characterization of monoclonal antibodies (mAbs) (13), one on the best practices for top-down MS (14), and one to define a standardized notation for proteoforms (15). The importance of the third aspect, and of broadly accepted standards for data handling in general should not be underestimated, because data processing and, in particular, confidence metrics for identification—including full or partial proteoform characterization—are important in allowing broad adoption of top-down proteomics (for example, in a clinical context). Consortium-led initiatives on combining native ionization with top-down analysis and using capillary electrophoresis for separating proteins and complexes prior to ionization are currently ongoing.

In addition to the deliberate push toward increasingly adopting these initiatives, there have recently been a number of exciting technological developments, particularly in regards to implementing advanced fragmentation methods on a range of mass analyzers. These technological developments allow greater experimental flexibility, particularly in combination with native ionization. For example, efficient electron capture dissociation through a commercially available instrument modification has been demonstrated on orbital trap MS (16,17) and quadrupole time-of-flight (QTOF) (18) instruments, with the latter also allowing the combination with ion mobility (IM) separation in a single measurement. UV photodissociation is also commercially available on certain orbital ion trap platforms, and recently surface-induced dissociation was launched commercially on an IM–mass spectrometer (MS) instrument. These are all high-end platforms, requiring significant capital investment and—for the time being—also significant user expertise. However, the fact that no in-house modification of instruments is required will significantly lower the barrier to entry into this field. These improvements will in turn pave the way for more widespread application of these methods, and will allow us to routinely start thinking about proteoforms in three dimensions.

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Frederik Lermyte is with the Conformation-Sensitive Mass Spectrometry Laboratory in the Department of Chemistry at Technische Universität Darmstadt, in Darmstadt, Germany. Direct correspondence to: frederik.lermyte@tu-darmstadt.de.
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To paraphrase the poet Robert Browning, a mass spectrometer (MS)’s reach should exceed its grasp. The reverse is currently true—although MS instrumentation has become more adept at grasping ions at the atmosphere-to-vacuum transition using wide inlet orifices, ion lenses and guides, and ion funnels, less research has been devoted to reaching out to analytes on a surface at a distance from the MS instrument. Although ambient sampling and ionization methods continue to diversify, most systems position the ion source, analyte, and MS inlet within centimeters or less of one another. Many objects and surfaces are simply too large (or have other complicating circumstances) to be positioned immediately adjacent to the instrument, ions pulled through atmospheric pressure transfer tubing become subject to egregious losses as the distance increases from centimeters to meters. Although a remote ion source and simple tube can be used, a truly capable analytical system requires a robust signal. Alternative models for direct, non-proximate sample analysis are neutral desorption and transfer of gas-phase analyte to an ion source/MS, condensed-phase extraction and transfer, and the extension of instrument vacuum to the sample.

This practical limitation has caused method development to look toward onsite residual collection for later analysis (bringing vestiges of sample closer to the MS) or instrument miniaturization for portability (bringing the mass analyzer closer to sample). However, the former is discontinuous analysis of material related to a target, and the latter is rarely available and does not currently offer the capabilities of high-resolution laboratory instrumentation. For continuous, direct MS analysis, there are four models for non-proximate MS.

**Atmospheric Pressure Ion Transfer**
Regardless of the predictable signal loss at atmospheric pressure, directing analyte ions into a long, atmospheric pressure transfer tube (Figure 2a) is otherwise convenient, cheap, and “good enough” for some applications. Early successes in ambient ionization source design led the Cooks laboratory to test the limits of pulling ions through a stainless steel tube. At the cost of orders of magnitude in signal reduction, the tubing conducted desorption electrospray ionization (DESI) analytes from sample surfaces 3 m distant (2). The Fournier laboratory SpiderMass system later equaled that distance with flexible polytetrafluoroethylene (PTFE) tubing and a supplemental vacuum to sample from laser ablation/ionization of biological tissues (3). The Smithsonian Museum Conservation Institute interrogated a wooden object 30 cm from the MS instrument with direct analysis in real time (DART) (4). The Müller laboratory has more recently integrated spray-based and plasma-based ionization techniques into a handheld probe, using flexible 60 cm polyetheretherketone (PEEK) or fluorinated ethylene propylene (FEP) tubing (5).

**Gas-Phase Neutral Transfer**
Notwithstanding the analytical success and cost savings that can be had transferring ions through air, even the high sensitivity of contemporary commercial MS instruments will inevitably be challenged by greater distances. A logical way to subvert this limitation is to desorb or ablate the analyte material from a distant surface without ionization, then transfer the neutral, gaseous material to an ion source proximate to the MS (Figure 2b). Perhaps the
most visible example of this systemic revision is rapid evaporative ionization MS (REIMS). The first non-proximate analysis method to examine organic molecules in complex samples, REIMS was originally conceived as generating ions from tissue vaporization in electrosurgery before transporting the ions through heated, flexible PTFE tubing to an MS instrument (6). However, after the technology was commercialized by Waters, a solvent addition/heated collision source was incorporated into the instrument vacuum interface to drive voltage-free inlet ionization of the remotely generated analyte aerosol (7). Recent publications with the commercial REIMS system have used laser ablation for non-proximate sampling of analyte from many different biomaterials (8). The Zhou laboratory built a non-proximate system not particular to an MS instrument manufacturer which uses solvent and an ultrasonic probe to desorb tissue aerosols, aspirates 70 cm through PTFE, and ionizes with heat and vacuum immediately external to the MS inlet (9).

Other surface-sampling systems desorb and transfer smaller amounts of material. The Vertes laboratory designed a laser ablation chamber to more efficiently transfer aerosolized analyte through 60 cm Tygon tubing to an MS-proximate electrospray emitter (10). The soft ionization by chemical reaction in transfer (SICRIT) ion source from Plasmion ionizes gas-phase analytes with an in-tube dielectric barrier discharge as they are pulled through by the instrument vacuum. Having sampled from laser-ablated surfaces 11 cm away (11), the instrument shows potential for sampling from greater distances, such as the Reynolds laboratory system for atmospheric pressure chemical ionization (APCI) of analytes desorbed 1 m away (12). Using longer rigid tubing, the Smithsonian is constructing a system to desorb analytes with a heated gas jet, transfer 2 m through an in-tube dopant permeator (13), and deliver the desorbed analytes to an atmospheric pressure photoionization (APPI) lamp (14). The Larriba-Andaluz laboratory is using computational fluid dynamics to design a more efficient inlet to collect such gaseous desorption products (15).

Condensed-Phase Transfer
Condensed-phase extraction is another model for neutral sampling. The most common form is liquid extraction surface analysis, in which microliters of the solvent are placed in direct contact with a sample surface to collect the analyte. Non-proximate sampling adaptations move the extract a significant distance—a grander scale than DESI or the Prosolia Flowprobe—before direct ionization and mass analysis (Figure 2c). The Eberlin laboratory developed the MasSpec Pen, a handheld interface used by a surgeon that creates a liquid micro-junction with biological tissue and then aspirates the droplet through 1.5 m PTFE tubing for inlet ionization (16). Similar systems have recently been demonstrated with analyses of more common objects. Oak Ridge National Laboratory created a handheld version of its open port sampling interface with liquid sample aspirated through 1 m of FEP tubing to an APCI source, successfully sampling from botanicals without peak broadening (17). Rather than transporting liquid droplets with the vacuum, a system constructed by the Spengler laboratory circulates extraction solvent through supply and return tubing between a handheld probe and a 50 cm-distant electrospray emitter (18). In addition to coupling to high-resolution instrumentation, the latter assembly was used with a miniature rectilinear ion trap to analyze vari-
ous irregularly shaped biological and synthetic objects. A probe designed by the Chen laboratory circulates silk suture string instead of liquid over a 2 m path, recovering sample residue from biological tissue for ionization with MS-proximate electrospray droplets (19). Each of the various neutral-transport systems may be constructed in an academic setting with some effort and tolerance for complexity.

**MS Vacuum Extension for Ion Transfer**

With atmospheric pressure transfer so inefficient or complex, one can “stretch out” the MS with front-end mobility devices to transport ions from a distant sample. Arguably, the most technologically sophisticated solution to ion loss over transfer distance has been realized with SPion, developed by Trace Matters. The SPion components together are operated fundamentally as an extension of a commercial mass spectrometer vacuum interface (Figure 2d), using a flexible, 1.2 m-long stacked-ring ion guide installed in place of standard fore-vacuum optics to transport analyte ions with performance similar to a factory inlet (20). Unlike other non-proximate sampling systems, a particular ion source is not intrinsically necessary to the function of an ultralong, lossless inlet. The SPion could, therefore, be used for large molecule analysis or with DESI, DART, and other third-party ambient ion sources if it can be fitted with supplemental evacuation as necessary. A similar system cannot be as easily constructed by laboratory tinkerers and will not be so affordable, but it opens exciting possibilities to those who can get it.

**Future Directions**

The analyst who wishes to perform non-proximate sampling will have to choose instrumentation based on the limitations of budget and the requirements of the analyte—to invest in a vendor-exclusive system, a commercial but vendor-neutral system, or the time cost to build or modify a custom system. The decision may also affect whether the MS instrument can be devoted entirely to non-proximate sampling or must be modular. The analyte object will impose requirements based on its size and tolerance for destructive sampling or solvent exposure, in addition to typical ambient analysis considerations like ionization type and potential for carry-over. Whether the required scale is 20 cm, 2 m, or more, non-proximate sampling technology will be increasingly valuable. Even when portable, high-resolution instrumentation becomes available, it will require an effective transfer pathway between the atmospheric pressure sampling point and the mass analyzer. Most of the systems discussed here use a handheld probe, which can be moved to access irregular shapes and offers intuitive user appeal to make it effective in exotic settings like the operating room and the industrial processing line. With efficient analyte transfer methodology, the ambient sampling mechanism need only be reproducible to make non-proximate analysis useful for stationary or mobile instruments in the laboratory and field.

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G. Asher Newsome is an analytical chemist with the Smithsonian Museum Conservation Institute in Suitland, Maryland. Direct correspondence to: newsomeg@si.edu.
Mass Spectrometry–Based Process Analytical Technologies for Cell Therapies

Ho-Tak Lau and Richard Rogers

Chimeric antigen receptor (CAR) T-cell therapies have demonstrated considerable clinical benefit in treating hematological diseases. Production of consistent CAR T-cell drug products from heterogeneous-starting materials is a key objective of CAR T-process development (PD). To achieve this, PD scientists employ process analytical technologies (PAT) to monitor upstream, downstream, and final product attributes. This article focuses on how the cell therapy field is leveraging mass spectrometry (MS)–based PAT.

Chimeric antigen receptor (CAR) T-cell (CAR-T) therapy has emerged as a promising treatment option for cancer patients. CAR-T therapy has demonstrated favorable clinical responses even for relapsed patients with poor prognoses (1,2). The manufacturing of the CAR-T drug product is a highly complex process involving the enrichment of T cells from leukapheresis material, the activation of the enriched T cells, the genetic engineering of T cells, the expansion of the engineered T cells, and finally the formulation of the drug product (Figure 1).

One of the goals of CAR-T drug product manufacturing is to optimize the development process to achieve a consistent product quality with each manufacturing batch. However, unlike small molecules and conventional biologic drug products, CAR-Ts are complex living drugs with dynamic cell states and multiple mechanisms of action. The cell therapy field strives to advance our understanding of T-cell biology and develop better analytical methods for characterization, in-process monitoring, and final drug product release. This article discusses the utilities of liquid chromatography–mass spectrometry (LC–MS) for immediate process support and a long-term vision in using LC–MS to better understand the cell therapy drug products.

In-Process Monitoring

During the manufacturing process for a cell therapy drug product, patient or donor cells are genetically engineered and expanded to a predetermined cell density. Various materials could be added during the manufacturing process (such as bovine serum albumin from culture media, antibodies for T-cell activation, and viral vectors for gene editing). These process reagents need to be removed or cleared from the cells before formulation. During the expansion stage, cells will also consume the culture media. It is essential to understand the clearance of process reagents and the consumption of media components. MS is a powerful tool for monitoring these process components.

For media analysis, the Rebel analyzer (908 devices), a specialized mass spectrometer, can monitor 34 analytes including amino acids, biogenic amines, vitamins, and dipeptides. The simple workflow involves diluting the samples, placing the samples in the auto sampler, and then initiating the analysis. Process development teams can operate the system to monitor daily culture conditions with little to no prior MS experience.

For characterizing and monitoring the clearance of protein-based process residuals, the development of MS-based assays like selection reaction monitoring (SRM), multiple reaction monitoring (MRM), and parallel reaction monitoring (PRM), have the potential to be faster than conventional methods such as enzyme-linked immunosorbent assay (ELISA) (Figure 2). ELISA-like methods require screening of antibodies for compatibility and strategic sourcing of critical reagents to support process development and clinical and commercial programs. Significant effort is consumed for qualifying these reagents and bridging to new reagent lots. Compared to these immunoassays, targeted proteomics allow antibody-free multiplex quantification of target proteins (Figure 2a). Furthermore, additional increases of sensitivity of targeted proteomics can be achieved through optional incorporation of antibody
enrichment. The most significant advantage of targeted MS-based assays is that they provide crucial support to process development teams in a timely manner (Figure 2b).

**Viral Vector**

Delivering gene editing cassettes to CAR-Ts frequently leverages viral vectors, such as gamma-retrovirus (RVV), lentivirus (LVV), and adeno-associated virus (AAV). Immune-based assays are available for rapid titer and purity determinations for these viral vectors. However, LC–MS analytical methods can provide detailed characterization of the protein profile of the viral vectors.

LVV is a subtype of RVV. They are RNA viruses that allow the long-term presence of CAR-T by integrating the genetic material into the host cell genome. However, it is also a concern that integration will cause insertional mutagenesis and changes of gene expression surrounding the insertion site (3–5). These viruses package proteins, such as reverse transcriptase and integrase, allow gene integration. They are also enveloped by the membrane of the production cells. Bottom-up proteomics approaches have demonstrated differences in the ratios between viral proteins (unpublished result) depending on the process. Whether such changes will affect viral titer is yet to be determined.

These approaches can also profile the host cell proteins that are carried through the downstream process and identify post-translational modifications.
(PTM) associated with the viral vector proteins. AAV has been gaining more interest in recent years. AAV exists in the host nucleus as an episome, and on the rare occasion, integrates into a known genomic location at chromosome 19 (6,7). However, because the CAR construct is not usually integrated, expression of the CAR receptor eventually stops because of replication dilution. Recent efforts have combined CRISPR and AAV to integrate the CAR cassette to a specific genomic location (8). In AAV, the genetic material is encapsulated by a capsid consisting of three proteins, VP1, VP2, and VP3. Ratios between the three capsid proteins are either 1:1:8 or 1:1:10, depending on the serotype. For characterizing the AAV vector, the ratio between empty (no genetic material) and full vectors can be analyzed using an anion exchange chromatograph (9), and the ratio between the three capsid proteins can be acquired using intact protein mass spectrometry coupled to either reversed-phase LC (RPLC) (9) or capillary electrophoresis (CE) (10). Another advantage of the intact protein MS approach is that the MS data contains the molecular weight for each capsid protein. Finally, bottom-up proteomics have been used to profile the protein post-translational modifications (PTMs) associated with the capsid proteins (11).

**Donor Cells, Patient-Derived T-cells, and the Cell Drug Product**

So far, we have focused on how MS-based methods can provide support to process development by monitoring impurities, residual clearance, culture condition, and characterizing and quantifying gene editing materials. These methods can readily be integrated in the current process. However, the patient-specific cells are the most heterogeneous raw material in the CAR-T manufacturing process, and our understanding of it, is still very limited.

For the donor cell material, phenotypic information like cell density, cell size, and cell viability can be collected through using automatic cell counters. CD4 and CD8 cells can be further divided into various memory and effector subtypes through cell surface (CD45RO, CD45RA, CD27, etc.) and internal (FOXP3) markers (12) measured using flow cytometric methods. It is known that T cells with more naive phenotype generate CAR-Ts with better efficacy than more terminally differentiated T cells (13).

The current understanding of naive T-cell populations, categorized by flow cytometry, may not fully represent the optimal cell surface profile for successful CAR-T manufacturing. In fact, the Cell Surface Protein Atlas (14) has mapped more than 1000 proteins on the surface of T cells. With technological advancements of mass spectrometers, data acquisition algorithms, and data process tools, it is not uncommon to quantify >5000 proteins from a single LC–MS run today. We envision that by studying the donor cell materials, in-process cell materials, and the T-cell drug products, the data may provide us with more insights that could potentially benefit the CAR-T’s manufacturing process.

**Conclusion**

The autologous CAR-T manufacturing process is complex. This process includes collecting cells from patients, activating the T cells, transducing the T cells, expanding the T cells, and cryopreserving the final drug product. There are many opportunities for in-process monitoring to optimize and control the manufacturing process and ensure a quality final drug product. MS offers a powerful tool to identify and monitor key attributes throughout the CAR-T manufacturing process.

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Ho-Tak Lau and Richard Rogers are with Bristol Myers Squibb. Direct correspondence to: HoTak.Lau@bms.com.
In recent years, the pharmaceutical industry has been impacted by many significant product-related recalls, such as the 2,4,6-tribromoanisole (TBA)-related recall approximately 10 years ago, and, more recently, the nitrosamine for original and generic drug products, as well as benzene contamination issues in over-the-counter (OTC) consumer products. Those issues impacted the industry financially as well as tainted their reputation. The pharmaceutical industry initiated extensive investigations and implemented solutions to control and avoid those quality- and safety-related issues. In this article, case studies are presented to highlight the importance of mass spectrometry (MS)-based analytical solutions when extremely low detection limits are required, and when development resources are limited.

Case I

Roughly a decade ago, a case of trace level migrant chemicals resulting from wood pallets caused a multibillion dollar impact on the pharmaceutical and food industries. Companies, such as General Mills from the food sector, as well as Pfizer and Johnson & Johnson from the pharmaceutical industry, recalled multiple products because of contamination by a degradation product of a brominated fire retardant used to treat
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wood pallets (1–3). Since the trace level migrant of 2,4,6-tri bromoanisole has an extremely low sensory threshold, it can be easily detected by smell, and therefore it was relatively easy to identify its presence in the finished products. However, it was extremely difficult to detect and quantify this impurity under current good manufacturing practice (cGMP) conditions using a validated analytical method that had sensitivity close to the human olfactory threshold (4,5). The evaluation of these compounds in pharmaceutical formulations puts additional demands on method requirements. One of the most challenging aspects encountered in analyzing halophenol and halobromoanisole compounds in pharmaceutical formulations is the low detection limit required (about 10 pg/tablet or less for anisoles, and about 1 ng/tablet level for the phenols preferably) to match the detection limit of the human nose. The low level of detection requires eliminating any possible spectral interferences associated with finished pharmaceutical drug products and the siloxane-based gas chromatography (GC) column (see Figure 1).

It is clear that if the target compound peak is at a lower concentration level than the interference peak, then the majority of the signal is associated with the background, resulting in a false positive test result. Eliminating the spectral interference leads to the development of a selected reaction monitoring (SRM). Since the peak with a nominal mass of $m/z = 344$ is associated with two different structures and elemental compositions (see Figure 1), the CID fragmentation is different. Therefore, a unique transition can be selected for specific and

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**FIGURE 1:** (a) Isotope pattern for tribromo anisole molecular ion cluster, and (b) the isotope pattern of a common cyclic siloxane, present in a GC–MS background.

**FIGURE 2:** SRM traces for 2,4,6 tribromo-anisole at a 20 fg/mL level using stir bar sorptive extraction. 15 mL sample was extracted, resulting in 300 fg of target analyte on-column; (a) quantifier transition, and (b) qualifier transition.
sensitive detection of the target analyte (see Figure 2).

Such a low level detection requires a sample pretreatment to transfer a sufficient amount of the target compound to the GC−MS system. The most “popular” sample preparation option is based on solid phase microextraction (SPME), which was developed for trichloro-anisol (cork taint) in wine samples (4); however, the SPME option was successful only for the formulations with relatively “simple” excipient matrices. For more complex pharmaceutical formulations, a stir bar sorptive extraction based method was developed and validated to satisfy the request from the U.S. regulatory agency (5). The method was capable of detecting multiple haloanisols at 1–2 pg/tablet and halophenols at 70–95 pg/tablet levels, respectively, which has met the expectations of the regulatory agency set for 100 pg/tablet (5).

In summary, a GC tandem mass spectrometry (GC−MS/MS)-based technique was developed and implemented in a short period of time for the temporary release of finished pharmaceutical products.

Case II

Recent voluntary recalls of finished pharmaceutical products and consumer products (6,7) associated with benzene levels above the USP safety limits (8,9) highlight the importance of the analytical testing for toxic substances in finished pharmaceutical and consumer products. In both cases, the recalls were preceded by citizen petitions made by Valisure. The impacted products included hand sanitizers, primarily in gel form, and sunscreens, primarily in pressurized container packaging, using isobutane as the propellant. Benzene is a known carcinogen substance (10), and it is defined as Class I solvent in USP General Chapter <467> (8), and in the Food and Drug Administration (FDA) residual solvent guidance (11,12).

Residual solvents are classified into three classes based on risk assessment. Class 1 (residual solvents) represents “solvents to be avoided.” These solvents are known to be human carcinogens, or are strongly suspected human carcinogens. The USP chapter indicates “if (the solvent’s) use in order to produce an official product with a significant therapeutic advance is unavoidable, their levels should be restricted.” As benzene is not necessary for manufacture of hand sanitizer or sunscreen, it should not be present in the finished products. The FDA published a method for determination of benzene in hand sanitizer, and utilized the 2 ppm limit. To assign the associated risk for sunscreen, we need to understand the daily exposure level for benzene when the finished product is being used as intended. Approximately 28 grams/applications, and up to four applications/day (9), which results in a maximum daily use of 112 grams of the sunscreen, could result in a 224 µg/day benzene exposure if the products reach the limit set in the USP <467> standard. However, the USP chapter limits are intended for a maximum daily use of 10 grams of a finished product, consistent with the US FDA published permissible limit of 20 µg (0.02 mg)/day (13). Application of the 20 µg per day limit and the Option 2 approach listed in USP <467> for a dose of 112 grams result in a 0.18 ppm limit in sunscreen products (9).

\[
\text{Concentration (ppm)} = \frac{1000 \times \text{PDE (mg/day)}}{\text{Maximum daily dose}} = \frac{1000 \times 0.02 \text{ mg/day}}{112 \text{ g}} = 0.18 \text{ ppm}
\]

As the FDA already published a method for determining benzene in hand sanitizers, the
The method in the USP <467> standard uses FID detection, appropriate for detecting and quantifying benzene, at or around the 2-ppm safety level. However, FID detection may not be sufficient for small sample sizes and lower levels of analysis (9). Another alternative is to use a single-stage MS-based detection that provides better selectivity and lower detection limit if an extracted ion trace specific for the benzene is being used. This approach requires a quantifier ion (for quantitation) and a qualifier ion (for confirming absence of spectral interferences).

To enhance the specificity of the detection method, and reduce the potential chemical and spectral interferences, an MS/MS method was developed for the detection. A transition of \( m/z = 78\rightarrow52 \) was selected based on the product ion spectra. The test results using the two different detection methods for benzene for a compressed propellant sample, which contains benzene below the USP limit of 0.18 µg/g, are presented in Figure 3.

Both methods provided distinct chromatographic peaks, making the integration and quantitation straightforward. However, the peak in the MS/MS trace shows much higher signal-to-noise (S/N) value, and a little higher peak intensity compared to the extracted ion data (Figure 3). It is important to note that the S/N achieved for such a level of benzene is superior compared to the GC-FID based residual solvent method for Class I solvents (14,15).

In summary, a GC–MS/MS-based method was rapidly developed as a reliable alternative method for detecting trace level of benzene in compressed gas excipients. The mass spectrometry based method is more reliable to test samples with relatively high daily doses, where the GC-FID-based USP <467> method has serious limitations.

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In-Depth Analysis of Host Cell Protein (HCP) Impurities by LC–MS/MS to Augment Routine HCP-ELISA Testing of Biotherapeutics

Ying Zhang and Jason C. Rouse

The enzyme-linked immunosorbent assay (ELISA) is the industry standard for quantitative host cell protein (HCP) analysis in biopharmaceutical product and process development. HCP-ELISA affords total HCP measurements (ng/mg) with broad HCP coverage in a straightforward and reliable analysis format. HCP-ELISA supports batch release testing of bulk drug substance, as well as step-by-step confirmation of downstream process performance. Failure to remove residual HCPs sufficiently during downstream processing can potentially affect the quality, safety, and efficacy of the biotherapeutic. In recent years, liquid chromatography–tandem mass spectrometry (LC–MS/MS) has gained momentum as an orthogonal approach to HCP-ELISA. LC–MS/MS provides identification and quantitation of individual HCPs, and helps ensure that no HCPs evade HCP-ELISA detection above a reportable limit (for example, 10 ng/mg). The individual HCP information from LC–MS/MS is used to facilitate a multi-departmental risk assessment if one or more residual HCPs are present in final drug substance above 10 ng/mg.
peptides from potential HCPs and the intended product are separated by reversed-phase LC (RPLC) and subsequently detected by an ultrahigh resolution, accurate mass research grade mass spectrometer. A majority of these peptides are fragmented by collisional activation in the gas phase to yield amino acid sequence information, which is used to identify both HCP- and product-related peptides through protein database searching. The protein databases are set up to contain the proteome of the host organism, sequence of the intended product, proteases, and common contaminants. Additionally, known problematic HCPs (28) should be included as a separate database to enhance confidence scores during database searching, especially since these HCPs are typically present at low ng/mg levels. Each HCP should be identified with at least two unique peptides. Acceptable peptide identifications include <1% false discovery rate (FDR) for a stringent search and <2–5% FDR for a less stringent search. Typically, individual HCPs above the quantitation limit (QL) of 10 ng/mg (if present) are reported for the final DS. For in-process samples, many HCPs will be observed prior to downstream processing with exponentially less and less HCPs after the affinity capture and polishing steps. Therefore, in addition to tabulating the extensive list of individual HCP identifications and relative abundances for each process step, it is equally powerful to report the number of HCPs before and after each purification step to demonstrate clearance, as well as use Venn diagrams to compare pre- and post-change samples to evaluate the effectiveness of process improvements in demonstrating product comparability.

HCP quantitation via LC–MS/MS is achieved on either a relative or absolute basis. Relative quantitation is often performed by the “top three” label-free method, where the summed peak areas for the three most abundant peptides from each HCP are compared against the summed peak areas of the three most abundant peptides obtained from either the biotherapeutic product or spiked-in protein standards. Both data dependent acquisition (DDA) (21,24,27) and data independent acquisition (DIA) (23,26,29,30) modes can be used. DDA is most often applied, whereas DIA offers better sensitivity for low abundant HCPs (since these peptides may be missed by DDA). However, confidence scores could be negatively impacted for coeluted peptides in DIA. Building a spectral ion library using in-process samples can be implemented to solve the issue, but the process is typically laborious, and the library needs to

FIGURE 1: A typical LC–MS/MS-based proteomics workflow for host cell protein (HCP) analysis.

FIGURE 2: Characterization roadmap for HCP analysis by LC–MS/MS to facilitate early and late-stage bioprocess development (FIH: first in human).
be updated when manufacturing process improvements are made (26). Conversely, for absolute quantitation, heavy-isotope-labeled peptide analogues from select HCPs can be spiked into the samples at known levels. Targeted proteomics tools, such as multiple reaction monitoring (MRM), parallel reaction monitoring (PRM), and selected reaction monitoring (SRM), can be applied for quantitative measurements (22). Additionally, the accuracy of both relative and absolute quantitation can be improved by applying either external or internal calibration curves based on spiked-in standards (13,26). Therefore, it is possible to obtain a summed value of all HCPs present via LC–MS/MS and compare trends of various samples with HCP-ELISA results. However, analysts need to be cautious in drawing conclusions because detection principles for HCP-ELISA and LC–MS/MS are fundamentally different. On the other hand, correlation of LC–MS/MS and HCP-specific ELISA results for a particular HCP can usually be established (12,27). In summary, LC–MS/MS offers opportunities to obtain identification and quantitation of individual HCPs down to low ng/mg levels in a single experiment, which ensures development of a robust and well-controlled manufacturing process and enables enhanced process understanding.

Because the purified product is usually significantly more abundant than individual HCPs, as is the case for monoclonal antibodies (mAbs), HCP analysis challenges not only the sensitivity of the mass spectrometer but also the dynamic range. Multiple strategies, such as product depletion or HCP enrichment, can be applied to improve HCP detection (7,31). For example, some commonly used technologies, including molecular weight (MW) cut-off filters (32), LC fractionation (33,34), electrophoresis (35), and 2D sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (36), effectively separate HCPs from the intended product based on the physiochemical properties of proteins, such as size, hydrophobicity, and isoelectric point. In addition, mAbs can be depleted effectively via a digestion protocol under native condition (37). On the contrary, HCPs can be enriched by affinity capture (7) or substrate binding (6,8). However, all depletion or enrichment approaches could potentially miss HCPs that bind to the intended product or have similar physiochemical properties as the product, as well as introduce additional variables for accurate quantitation. Alternatively, proteolytic peptides from HCPs can be analyzed by coupling two-dimensional LC with tandem MS (2D-LC–MS/MS) to further improve sensitivity (22,23,38). Furthermore, analysts can also apply nano-LC to improve sensitivity, although it is possible to sacrifice robustness and throughput with this approach (23).

In practice, HCP-ELISA is used routinely for in-process sample analysis and DS batch release in quality control laboratories. HCP-ELISA provides evidence for HCP clearance along purification train and confirmation of a high-quality DS. However, one of the known limitations of HCP-ELISA is that the polyclonal antibodies (generated from immunization of animals by null cell line lysate) often have no or limited coverage to certain HCPs that are either non- or weakly immunogenic, leading to under-representation of these HCPs in the final readout. Therefore, LC–MS/MS can be applied to fill this gap during different stages of bioprocess development (Figure 2). At an early stage prior to start of clinical trials, LC–MS/MS analysis is used to verify that HCP-ELISA does not miss any HCPs in the pilot-scale DS at more than 10 ng/mg (a common reportable limit for HCPs across the industry). During subsequent commercial process development, several iterations of LC–MS/MS experiments are applied to ensure HCPs are effectively removed in the final pilot-scale DS before the process is locked (finalized), especially for those products without an affinity purification step. Upon manufacturing process validation, LC–MS/MS analysis of both the DS and in-process samples from the same batch is used to provide confirmation of HCP clearance. At any stage of the product development lifecycle, LC–MS/MS results enhance process understanding and help the bioprocess team develop and optimize the upstream and downstream manufacturing process conditions to minimize HCP production or optimize HCP removal. In addition, LC–MS/MS can be applied to enhance understanding of ELISA critical reagent coverage (39,40) to facilitate the periodic bridging activity between old and new critical reagents. More importantly, LC–MS/MS enables the required knowledge-based risk assessment to allow decision making during process development and discussions with regulatory agencies around control strategies (19,41). When higher level HCPs are observed during manufacturing process development, LC–MS/MS results can clarify if it is because of the emergence of new HCPs or increased level of existing HCPs. This practice is important because information on existing HCPs could be potentially correlated to historical toxicology or clinical experiences. On the contrary, lower level HCPs observed in a new batch by HCP-ELISA is not necessarily an indication of lower risk because of potential exis-
tence of problematic HCPs, which need to be considered during risk assessment.

To summarize, one of the advantages of LC–MS/MS is that it allows direct measurements of individual HCPs in the final DS and throughout the purification process. Knowledge of individual HCPs in DS is critical for biotherapeutic manufacturers to ensure quality, safety, and efficacy of the medicine. Therefore, LC–MS/MS has gradually become an important orthogonal approach to HCP-ELISA, in addition to supporting in-depth process characterization and control. More importantly, LC–MS/MS could potentially reveal HCPs that are under-represented in HCP-ELISA and facilitate potential risk assessments, ensuring a comprehensive analytical characterization and process development data package for regulatory submissions. It is recommended to qualify the LC–MS/MS protocol for HCP analysis to ensure suitable method performance, confidence, and applicability to future projects and process improvement situations. To date, reliably detecting individual HCPs at <1 ng/mg levels remains challenging by LC–MS/MS, but work is ongoing at different biopharmaceutical companies and vendor partners to develop methods and instrumentation with better sensitivity, selectivity, and robustness (28). Hopefully, future technological advances will provide real-time bioprocess development support for HCP analysis and more.

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Ying Zhang and Jason C. Rouse are with Pfizer Inc., in Andover, Massachusetts. Direct correspondence to: ying.zhang06@pfizer.com.