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REVIEW ARTICLE

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Protein analysis by desorption electrospray ionization mass spectrometry

Andre R. Venter

Department of Chemistry, Western Michigan University, Kalamazoo, Michigan, USA

Correspondence
Andre R. Venter, Department of Chemistry, Western Michigan University, 1906 W. Michigan Ave, Kalamazoo, MI, USA.
Email: andre.venter@wmich.edu

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Abstract

This review presents progress made in the ambient analysis of proteins, in particular by desorption electrospray ionization-mass spectrometry (DESI-MS). Related ambient ionization techniques are discussed in comparison to DESI-MS only to illustrate the larger context of protein analysis by ambient ionization mass spectrometry. The review describes early and current approaches for the analysis of undigested proteins, native proteins, tryptic digests, and indirect protein determination through reporter molecules. Applications to mass spectrometry imaging for protein spatial distributions, the identification of posttranslational modifications, determination of binding stoichiometries, and enzymatic transformations are discussed. The analytical capabilities of other ambient ionization techniques such as LESA and nano-DESI currently exceed those of DESI-MS for in situ surface sampling of intact proteins from tissues. This review shows, however, that despite its many limitations, DESI-MS is making valuable contributions to protein analysis. The challenges in sensitivity, spatial resolution, and mass range are surmountable obstacles and further development and improvements to DESI-MS is justified.

KEYWORDS
AIMS, ambient ionization, DESI, desorption electrospray ionization, protein analysis

1 | INTRODUCTION

Mass spectrometry (MS) generates information-rich spectra of proteins that can be used to identify, quantify, and perform fundamental characterization at the structural and functional levels (Benesch et al., 2007; Domon & Aebersold, 2006; Rogawski & Sharon, 2021). In most cases, protein characterization relies on purification before analysis, which can be laborious and time-consuming, depending on how rigorous the purification needs to be. Most instances of sample preparation also remove the protein from its native environment and in the process valuable information, such as spatial distribution and co-localization, is lost. With significant recent developments in instrumentation (Liu et al., 2023), the number of direct, chromatography-free, rapid MS-based assays for analysis of intact proteins from complex matrices, with limited or online purification steps, has been growing (Takano et al., 2020; Tousi et al., 2020; Vimer et al., 2020a, 2020b).

Abbreviations: AIMS, ambient ionization mass spectrometry; BMTs, boronic acid mass tags; DART, direct analysis in real time; DEFFI, desorption electro-flow focusing ionization; DESI, desorption electrospray ionization; EESI, extractive electrospray ionization; ELDI, electrospray laser desorption ionization; ESI, electrospray ionization; FAIMS, asymmetric waveform ion mobility spectrometer; FTICR, Fourier transform ion cyclotron resonance; LESA, liquid extraction surface analysis; LMJ-SSP, liquid microjunction-solid surface probe; MALDESI, matrix assisted laser desorption electrospray ionization; MALDI, matrix assisted laser desorption ionization; MS, mass spectrometry; MSI, mass spectrometry imaging; Nano-DESI, nanospray desorption electrospray ionization; NTA, nitriloacetic acid; PESI, probe electrospray ionization; PMMA, polymethyl methacrylate; PTMs, posttranslational modifications; TWIMS, traveling wave ion mobility mass spectrometry.

A special case of direct analysis MS is the so-called ambient ionization mass spectrometry (AIMS) methods. Over the past 20 years AIMS has made significant contributions to mass spectrometric analysis in many fields by reducing or removing the need for sample preparation before analysis. While AIMS was often defined as methods that do not require sample preparation (Harris et al., 2011; Huang et al., 2011; Takats et al., 2004; Venter et al., 2008) a more reasonable view is that the dependence on sample preparation *before* analysis is mitigated by the incorporation of in-situ sample processing during the analysis step. In AIMS, typically ions are formed outside of the mass spectrometer, or during the transport between the atmosphere and first vacuum stage, usually with some kind of selective extraction and/or desorption step that occurs proximal to and simultaneously with the ionization process (Javanshad & Venter, 2017). The inventions of desorption electrospray ionization (DESI) and (direct analysis in real time) DART around the year 2004, spurred the innovation of many derivative methods, and currently, more than 80 ambient ionization methods have been described. Many of these methods are close permutations of each other, yet the field has resisted structured naming schemes or formal organization. Not all ambient methods allow for the detection of protein molecules, and the limited mass range of most mass spectrometers favor those that produce multiple charged protein species. This is why protein analyses by AIMS have mostly been limited to those that rely on ESI such as DESI, EESI, nano-DESI, LESA, LAESI, and MALDESI. Techniques that rely on the application of matrixes together with short light pulses such as matrix-assisted laser desorption ionization (MALDI) are typically considered direct methods of analysis because of the significant amount of sample preparation that precedes analysis, even when those samples are analyzed under atmospheric conditions.

The distinction between direct methods and ambient methods is somewhat vague. Many methods that are frequently described as ambient are most often used in a manner more consistent with direct analysis. This is the case when extensive sample preparation precedes the analysis by AIMS, or when a purified solution is spotted on a sample substrate before analysis using an ambient method. Atmospheric pressure MALDI is not considered ambient, yet matrix-assisted laser desorption electrospray ionization (MALDESI) is usually included as an AIMS method. The classification between direct and ambient methods is however mostly an academic concern. AIMS, in general, has been reviewed frequently, a nonexhaustive list includes the following references (Feider et al., 2019; Huang et al., 2010; Javanshad & Venter, 2017; Monge et al., 2013; Morato & Cooks, 2023; Rankin-Turner et al., 2023; Shi et al., 2022; Venter et al., 2008; Venter et al., 2013). Its application is widespread, and examples can be found in the analysis of small molecules such as lipids, explosives, metabolites, pharmaceuticals, polymers, proteins, and peptides from both synthetic surfaces and biological tissues.

This review will focus on the use of DESI-MS and closely related permutations thereof, such as liquid-DESI and DEFFI, applied to the analysis of proteins. A few other prominent ambient ionization methods capable of protein analysis are briefly discussed to place the capabilities of DESI-MS in context within the broader ambient ionization field. A selection of these methods is summarized in Figure 1. As mentioned, these are the AIMS methods that incorporate ESI as the major ionization process. Despite the tremendous ability that ESI has provided to the analysis of large biopolymers such as protein (Fenn, 2003), the MS analysis of proteins by ambient ionization has been challenging. Nevertheless, progress continues to be made in this regard, as exemplified by previous reviews on the application of AIMS to protein analysis. An early review (Yao, 2012) included DESI, nano-DESI, MALDESI, ELDI, and extractive electrospray or fused droplet electrospray ionization; while a 2018 review focused on liquid-extraction-based ambient methods of intact proteins especially LESA and the other liquid microjunction techniques (Kocurek et al., 2018). The capabilities of direct electrospray, MALDI, and ambient ionization methods to analyze intact proteins from crude samples were also compared (Vimer et al., 2020a). The current capabilities of surface-sampling MS to study intact proteins and protein complexes was again summarized in a review that compared matrix-assisted laser desorption/ionization (MALDI) with electrospray-based AIMS methods (Wong Kei et al., 2023).

Considering these previous reviews, we will focus in greater depth on one of these techniques, DESI-MS, and discuss the related techniques only to place it within the larger context of protein analysis by AIMS.

2 | PROTEIN LIBERATION FROM SURFACES DURING ESI-BASED AIMS METHODS

For solid samples, protein can be liberated from the matrix during analysis by liquid extraction, laser ablation, or physical removal, just-before, or during, electrospray ionization.

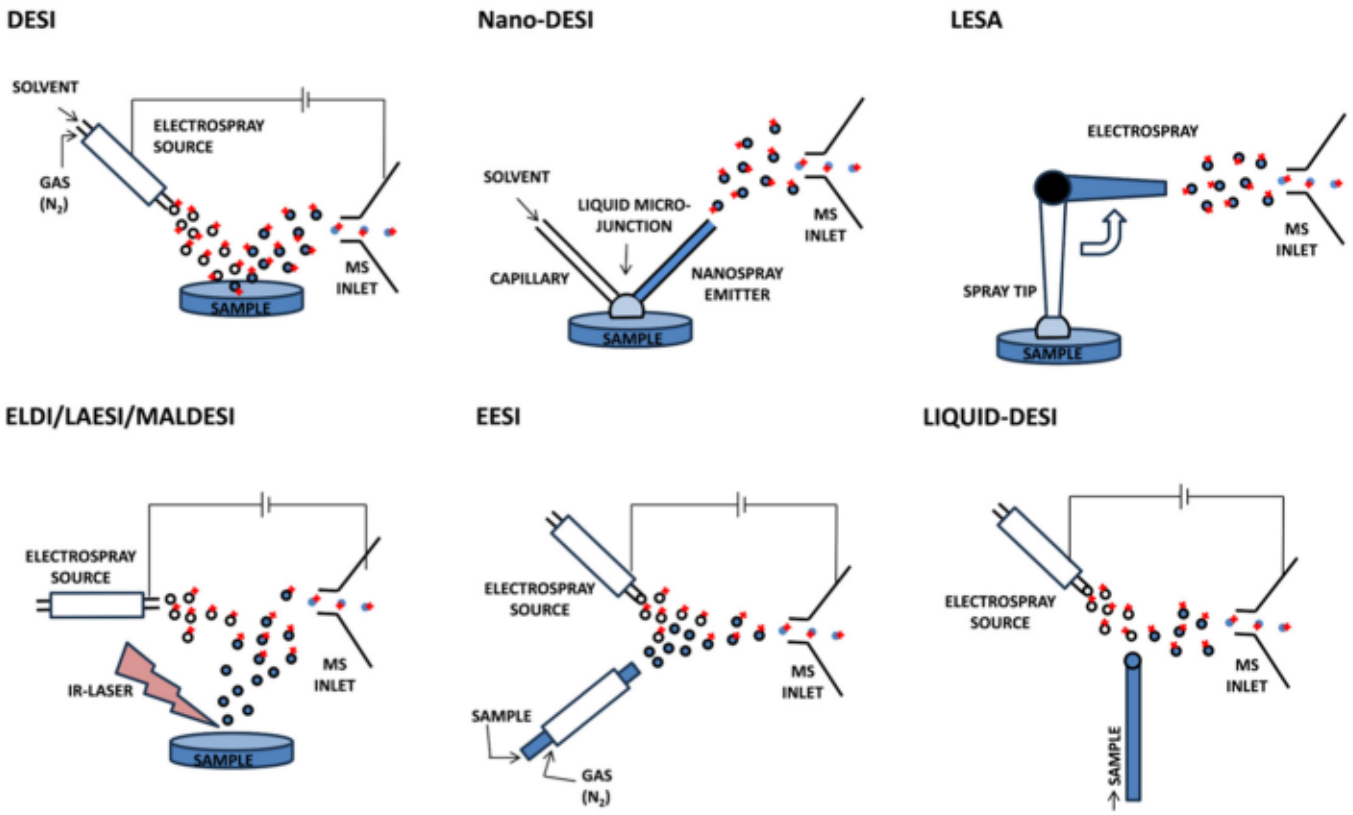

FIGURE 1 A selection of ambient ionization methods frequently used for protein analysis. Methods shown are desorption electrospray ionization, nanospray desorption electrospray ionization, liquid extraction surface analysis, electrospray laser desorption ionization/laser ablation electrospray ionization/matrix assisted laser desorption electrospray ionization, extractive electrospray ionization, and liquid sample desorption electrospray ionization. [Color figure can be viewed at wileyonlinelibrary.com]

Liquid extraction during AIMS can be initiated when the liquid phase is supplied as an energetic charged spray, forming a micro-localized liquid layer on the sample surface, followed by secondary liquid droplet desorption resulting from the energetic collisions of in-bound droplets (DESI). In other methods, an extraction solvent is supplied through a liquid microjunction by a set of capillaries, where a continuous flow of extract is aspirated through a capillary to a distant electrospray ionization source, exemplified by nanoDESI and the liquid microjunction-solid surface probe (LMJ-SSP). The extraction solvent can also be applied as a discreet droplet that is re-aspirated for analysis in a technique called liquid extraction surface analysis mass spectrometry (LESA-MS).

Hyphenated techniques based on laser desorption and subsequent electrospray ionization are also well-suited to protein analysis. Examples include ELDI (Huang et al., 2006), LAESI (Nemes & Vertes, 2007), and IR-MALDESI (Robichaud et al., 2014). Here, both IR and UV lasers have been used for sample ablation and extraneous matrix is not applied. More commonly IR lasers are used, where the water content of biological sample serves as the in-situ matrix. A plume of mostly un-ionized material is liberated, and this plume is intercepted by the ESI spray. Airborne neutral molecules are ingested into charged droplets leading to the creation of electrospray generated ions. Alternatively, droplets collide with airborne clusters and desorb sample material in a similar process as during a DESI experiment.

Solid probes such as needles (PESI) (Hiraoka et al., 2007) and other substrates including paper (Wang et al., 2010) have been used to sample substrates for analysis. This is followed by desorbing soluble sample material from the probe followed by ESI. Tissue samples or biological fluids have been placed on top of substrates, often paper or modified paper triangles (An et al., 2023, Jeng et al., 2005), with proteins and other components eluted directly from the tissue into the mass spectrometer for ambient analysis (Zhang et al., 2014). Here liquid extraction takes place, akin to what occurs in the microlocalized surface liquid layer during a DESI-MS experiment, but subsequent nebulization occurs due to electroosmotic flow to the substrate tip.

When the sample is a liquid, airborne neutral molecules can be created through nebulization of the sample, and ions are created after interception with a secondary electrospray

plume. The interception can create ions by either droplet fusion (Chang et al., 2002), or through glancing collisions with charged electrospray-created droplets, or extractive electrospray ionization (EESI) (Chen et al., 2006), respectively. The mechanism of EESI can be interpreted as DESI from the surface of liquid droplets. Another liquid sampling method known as liquid sample DESI achieves sampling from the meniscus of a liquid sample eluting capillary (Miao et al., 2010).

3 | DESI

Among the different ambient methods, DESI is still one of the most readily adopted and widely used techniques (Beneito-Cambra et al., 2020; Ferreira et al., 2019; Takats et al., 2004). The success of DESI can partly be attributed to the fact that building a DESI source is relatively easy and low-cost (Zemaitis & Wood, 2020), although recent developments in commercial designs offer many advantages such as smaller desorption footprints and increased sensitivity as shown in Figure 2. The use of desorption electro-flow focusing (DEFFI) also offers many advantages (Forbes et al., 2013; Wu et al., 2022) not yet fully explored for protein analysis. The typical DESI setup is made of two co-axial capillaries that form a pneumatically assisted electrospray. The outer capillary delivers N_2 as nebulizing gas, and the inner capillary delivers a continuous flow of solvent. Upon applying voltage to the sprayer, pneumatically accelerated solvent droplets, produced by electrospray process, are directed at a sample containing surface with velocities around 100 to 120 m/s (when using a standard in-house manufactured source) (Venter et al., 2006). The sample analysis in DESI occurs through five steps, known as the "droplet pickup" process (Badu-Tawiah et al., 2010; Venter et al., 2006); (1) formation of a spray plume (primary droplets) directed at the sample (Green et al., 2010; Olumee et al., 1998); (2) formation of a micro-localized liquid layer on the sample surface (Costa & Cooks, 2007, 2008; Zivolic et al., 2010); (3) dissolution/extraction of the analyte into the liquid layer (Badu-Tawiah et al., 2010; Green et al., 2010); (4) release of analyte-containing droplets (secondary droplets or also known as progeny droplets) from the liquid layer by pneumatically accelerated primary droplets (Costa & Cooks, 2007, 2008; Olumee et al., 1998); and finally (5) analyte ion generation from charged secondary droplets through ESI mechanisms.

In addition to parameters that can affect the electrospray process, many other parameters affect the signal obtained by DESI-MS (Campbell et al., 2012), including but not limited to solvent composition (Eberlin et al., 2011; Green et al., 2010), sprayer construction and geometry (Tillner et al., 2017), surface type (Kertesz & Van Berkel, 2008; Volný et al., 2008), relative humidity (Feider et al., 2018), and of course, the analyte's characteristics. DESI-MS is still mostly considered for analysis of smaller molecules such as metabolites and lipids, while reports of protein analysis by DESI-MS are few and far between.

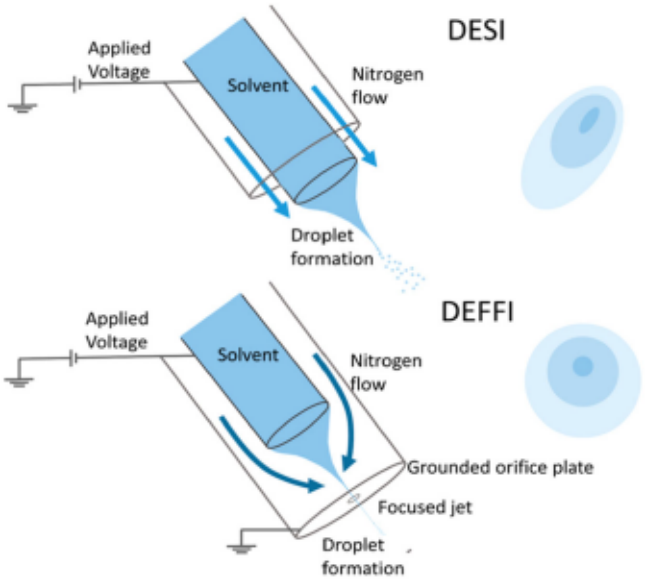

FIGURE 2 Desorption electro-flow focusing ionization (DEFFI) and desorption electrospray ionization (DESI) setups. (A) DEFFI and DESI sprayer setups (not to scale) and corresponding desorption footprints. The impact site of DESI spray is often elliptical due to asymmetry of the sprayer, while the DEFFI spray is rotationally symmetric and distorted only by the angle of incidence of the sprayer with the surface. Reprinted with permission from Wu et al. (2022) Copyright (2022) American Chemical Society. [Color figure can be viewed at wileyonlinelibrary.com]

4 | ADVANCES IN PROTEIN ANALYSIS BY DESI-MS

The analysis of lysozyme by DESI and even the ability for DESI to detect specific protein–ligand complexes was already shown in the first DESI publication (Takats et al., 2004). In a follow-up report (Takáts et al., 2005), several DESI-MS experimental parameters were optimized for the individual analysis of peptides and proteins varying in mass from 1 to 15 kDa, including the solvent system, source configuration, and sample surface. These parameters were then used to determine the detection limit for each peptide or protein. The samples consisted of 1–5 μL aliquots of air-dried protein standards on various surfaces. Although there were slight variations in

the optimal parameters for the individual molecules, the data showed that a smooth surface such as glass or polymethyl methacrylate (PMMA), as well as a high angle of incidence, was appropriate for DESI-MS analysis when MeOH/H_2O was used as the solvent system. The sensitivity of DESI-MS towards protein analysis was observed to be slightly less than that of AP-MALDI-MS, and the limits of detection for the proteins increased with increasing molecular mass.

In a later report, the DESI-MS detection limits were determined for several proteins spanning a mass range of 12–66 kDa (Shin et al., 2007). The protein solutions were spray-deposited onto PMMA substrates at various surface concentrations. The spectra were deconvoluted and the detection limits were assigned to the smallest surface concentration for which a S/N > 5 could be obtained. Similar to the previous study by Takats et al., the limits of detection were observed to increase with increasing molecular mass, and a S/N > 5 was unable to be obtained from the largest proteins, ovalbumin, and bovine serum albumin. For these two proteins, the authors instead reported the lowest surface concentration from which ion peaks could be assigned to the respective proteins. A logarithmic plot of the detection limits (or lowest detectable surface concentrations) determined from both studies is presented in Figure 3. Although different methodologies and instruments were used in the two studies (e.g., the Thermo Scientific LTQ is about 10 times more sensitive than the Thermo LCQ, as seen by the lower limits of detection for identical proteins studied by both groups at around 12 kDa), there is a clear trend of increasing limit of detection with increasing molecular mass. This loss in sensitivity with protein size has often been attributed to the lack of efficient desorption of proteins from the sampled surface (Heaton et al., 2009; Myung et al., 2006; Shin et al., 2007). This hypothesis was disproven by separation of the desorption and ionization aspects of a DESI experiment using Spray Desorption Collection (SDC) (Douglass & Venter, 2013; Douglass et al., 2012). These experiments showed that proteins, large and small, desorb equally well; rather, undesirable protein-protein or protein-contaminant clustering and incomplete dissolution of the analytes was the major contributing factors to the poor performance of DESI for proteins (Douglass & Venter, 2013).

Progress has since been made to mitigate these effects by use of solvent additives. Optimizing the solvent composition (Towers et al., 2018), as well as using solution-phase additives such as ammonium bicarbonate (Honarvar & Venter, 2017) or ammonium acetate are

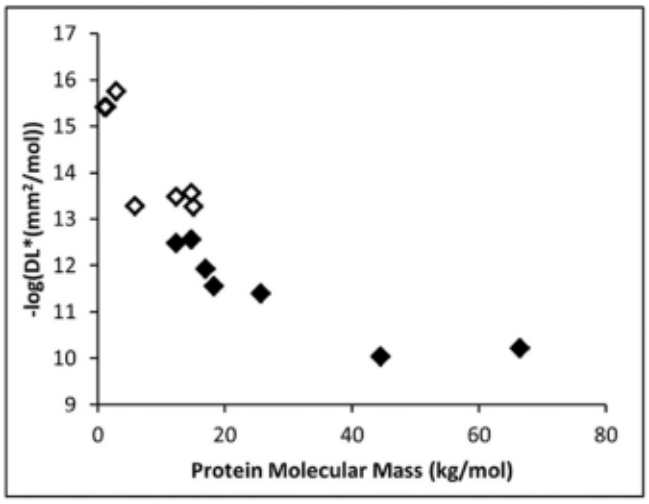

FIGURE 3 Logarithmic plot of protein desorption electrospray ionization-mass spectrometry detection limit versus molecular mass collected on Thermo Fischer Scientific LTQ◇ and LCQ (◆). Reprinted with permission from (Douglass & Venter, 2013), copyright 2013 (Wiley).

beneficial in proteins analysis by DESI-MS (Honarvar & Venter, 2018; Yan & Bunch, 2021). For instance, simply adding ammonium bicarbonate to the DESI solvent system increased signal to noise ratio (S/N) of proteins twofold to threefold compared to a formic acid solvent system, and up to sevenfold compared to aqueous methanol solvent systems. Ammonium bicarbonate, a better buffer at neutral pH compared to ammonium acetate (Konermann, 2017) can, however, unfold proteins when high source temperatures and spray voltages are used through a process known as electrothermal supercharging (Sterling et al., 2012).

L-serine was shown to increase signal intensity by up to five times when present during the analysis of native proteins with denaturing desorption solvents (Javanshad & Venter, 2021; Javanshad, Honarvar, et al., 2019). This likely works by preventing the formation of new protein–protein interactions during unfolding, thereby reducing non-specific aggregation.

Several approaches to improve protein detection by DESI have focused on instrumentation, such as integration of a high field asymmetric waveform ion mobility (FAIMS) ion mobility to DESI-MS (Garza et al., 2018), using a heated ion transfer inlet combined with traveling wave ion mobility separation (Towers et al., 2018), or shortening the ion transfer tube (Ambrose et al., 2017a, 2017b), using a two-step configuration for prewetting and delayed desorption of proteins (Maser et al., 2020), and employing DEFFI (Wu et al., 2022). Incorporation of an optimized geometry-independent DESI source can mitigate some of the intrinsic

irreproducibility of DESI due to the many variables in the source geometry (Abbassi-Ghadi et al., 2015), and maximize the efficiency of the analysis in general by tightly enclosing the process (Venter & Cooks, 2007).

Exposure of an enclosed DESI spray plume to polar organic vapors demonstrated the ability to change protein charge state distributions, and with some vapors to increase protein signal intensities. Ethyl acetate vapor addition was especially beneficial for improving the signal intensities of proteins, including proteins larger than 25 kDa, such as carbonic anhydrase II and bovine serum albumin (Javanshad, Maser, et al., 2019).

5 | ILLUSTRATIVE EXAMPLES OF DESI-MS PROTEIN ANALYSES APPLICATIONS

5.1 | Purified intact protein from prepared surfaces

Many early DESI analyses of proteins were performed by spotting dilute solutions of purified protein on specifically selected surfaces; this approach is still commonly employed today. These approaches are not ambient ionization, but rather direct analysis using ambient ionization methods. There is usually little advantage to spotting and drying purified protein standard solutions specifically for the analysis of proteins, per se. These solutions are analyzed with more ease and with better figures of merit directly by ESI. However, analyses of deposited solutions of purified standards are crucial in the optimization and development of the technique.

A good match is required between sample solvent composition and surface. Porous polytetrafluoroethylene (PTFE) is a popular surface choice for many DESI-MS analyses (Takats et al., 2004), however low wettability of this surface by aqueous, nondenaturing solvent systems, presents problems for native-state protein analysis. Hydrophobic porous polyethylene is a good alternative, as proteins can be applied directly out of water in the native state even as signal intensities are lower (Honarvar & Venter, 2017). Other surfaces such as PMMA (Shin et al., 2007; Takáts et al., 2005), and glass have often been used. A problem with most surfaces is the creation of the coffee-ring effect when proteins are spotted mechanically by micropipette and allowed to dry. To avoid this problem, protein standards are sometimes spray-deposited on surfaces. This produces a more uniform and reproducible sample deposition (Ambrose et al., 2017a, 2017b, Honarvar & Venter, 2018).

The short interaction time during DESI-MS analysis between the desorption solvent and the natively deposited protein leads to the observation of mostly native state protein charge states, and the conservation of protein complexes, even when equimolar concentrations of methanol–water mixtures are used. This was demonstrated by the combination of DESI-MS with ion mobility measurements (Myung et al., 2006). Using optimized conditions for native DESI-MS several selected protein standards and protein complexes with molecular weights ranging from 8.6 to 66.4 kDa were recently shown to produce spectra with narrow charge state distributions (CSD) and peaks showing noncovalent bond-assembled intact protein complexes (Yan & Bunch, 2021). These observations speak to the low energy deposited during the desorption and ionization processes when proteins are analyzed by DESI-MS. While the energy deposition was not yet formally and quantitatively studied in the realm of native protein analysis by DESI-MS, early work compared the degree of fragmentation of p-methoxybenzylpyridinium thermometer ions between DESI and ESSI. The results show similar trends in the survival yields as a function of the nebulizing gas pressure, solvent flow rate, and distance from the sprayer tip to the MS inlet (Nefliu et al., 2008). Modern commercial DESI-MS instrumentation often have extended heated ion transfer tubes. While these dramatically improve the detection of less abundant proteins (Towers et al., 2018) additional energy deposition and protein unfolding may take place during the brief residence time in the ion transfer capillary, but this has not been investigated yet.

By modification of the standard DESI setup, excitingly large proteins and protein assemblies could be observed. Proteins with molecular weights ranging from 66 to 800 kDa were reported from a smooth glass surface by implementing the following changes to standard DESI conditions: Reverting to the standard vendor-supplied ion transfer tube from the extended one often used with DESI-MS experiments, wet spray conditions due to low nebulizing gas pressures, and short sample to inlet distances (Ambrose et al., 2017a, 2017b). The DESI conditions employed here are speculated to cause solvent to be aspirated into the ion transfer tube, leading to an interesting hyphenation between DESI and Solvent Assisted Inlet Ionization (Pagnotti et al., 2011) although this hypothesis needs verification. Upon addition of different lipids, detergents, and other small molecules to the DESI spray solvent, native protein complexes were detected in the mass spectra. For example, outer membrane protein F (OmpF) was detected in its trimeric state (110 kDa, average charge state detected = 24+) when octyl glucoside micelles were added to the DESI spray solvent. Upon substitution of the octyl glucoside with a different detergent, lauryldimethylamine *N*-oxide, a shift to higher charge

states was observed suggesting detergent exchange. These results suggest that DESI could be used as a tool for rapid screening of detergents in membrane protein studies (Ambrose et al., 2017a, 2017b).

5.2 | Proteins by DESI-MS directly from liquid samples

Spotting and drying of solubilized proteins onto surfaces causes proteins to aggregate and is a leading cause of the loss in proteins signal, especially for those with molecular weights above 25 kDa (Douglass & Venter, 2013). A neat solution to this problem is the analysis of solubilized proteins directly from the liquid phase (Miao & Chen, 2009). Measurement of liquid samples significantly extends the mass range of DESI-MS allowing for the analysis of such high-mass proteins as 150 kDa immunoglobulin G. Protein complex ions (e.g., superoxide dismutase, enolase, and hemoglobin) desorbed from solution by liquid sample DESI remain intact, indicating the capability of DESI for preserving weak noncovalent interactions (Ferguson et al., 2011).

The direct sampling feature of liquid sample DESI allows the ionization of protein solutions without adding acids/organic solvents. As a result, native protein ions are generated from proteins in water by DESI and protein conformational changes such as heat-induced unfolding can be studied in water without influences of organic solvents and acids (Miao et al., 2010). Further, selective noncovalent adduct protein probing (SNAPP) was used to evaluate protein structural evolution in liquid sample DESI. With SNAPP, protein structure is explored as a function of side-chain availability as determined by a specific interaction between lysine and 18-crown-6 ether. The results showed that with liquid sample DESI, an acidic desorption solvent could generally be used to increase ionization efficiency, without denaturation of protein structure, as would occur when using acidic spray solvent in ESI (Moore et al., 2012).

Another approach to sample proteins from liquids is by the techniques called extractive electrospray ionization (EESI) (Chen et al., 2006) or fused-droplet electrospray ionization mass spectrometry (Chang et al., 2002). In EESI, neutral molecules are nebulized into the charged plume generated by electrospraying the reagent solvent (e.g., methanol/water solution). One mechanistic interpretation of the process is that a DESI-like droplet-pickup-mechanism operates from the liquid surface of nebulized liquid samples. Sample nebulization increases the surface area of the liquid with subsequent increases in sensitivity (Chen et al., 2006). EESI tolerates highly complex matrices and biological samples, with a high tolerance to matrices containing a high concentration of salts. Trace amounts of proteins spiked into various raw samples such as urine, *Escherichia coli* mixtures and tear samples were directly detected in their native conformations by EESI-MS without sample pretreatment (Hu et al., 2011). Using EESI, lysozyme added into untreated human saliva samples was quantified, showing a linear dynamic range of more than 3 orders of magnitude (Chen et al., 2010).

5.3 | DESI-MS imaging and protein detection from biological samples

An early, proof-of-principle experiment involved the identification of intact hemoglobin α and β chains (~16 kDa) from lysed red blood cells, by coupling DESI to a high-resolution FTICR instrument (Takats et al., 2008). Here, high mass spectrometric resolution provided the specificity required to identify charge states from the complex biological matrix. High-resolution mass spectral analysis was later shown crucial for high fidelity determination of molecular distributions in biological tissues (Manicke et al., 2010).

The application of DESI-MS to in situ analysis of intact proteins directly from tissue sections has been an ambitious goal. However, inefficient dissolution of large biomolecules and chemical noise arising from the complex tissue matrix have impeded detection of proteins directly from biological tissue sections by DESI-MS, until recently. One concern is fouling of the mass spectrometer interface with biological material. On the one hand, the inlet is somewhat protected as only compounds soluble in the aqueous spray solvent are removed, however, under aggressive conditions sample material can be ablated from the sample surface. High salt concentrations in most biological samples, and dissolved but un-ionized compounds can also coat ion optics requiring frequent source cleaning. While challenging, such applications are starting to emerge, but so far, only for small and abundant proteins. Successful demonstrations have depended on ion mobility to separate low-abundance protein signals from the chemical background of the biological milieu.

Traveling wave ion mobility mass spectrometry (TWIMS) coupled to DESI-MS allowed identification and spatial distribution of proteins in liver tissues. The intact mass and spatial distribution of the hemoglobin α and β chains (~16 kDa), a fatty acid binding protein (14.3 kDa), and a 10 kDa heat shock protein was determined (Towers et al., 2018).

In a similar effort to increase sensitivity to detect proteins that would have otherwise remained buried in

the noise, DESI-MS was coupled to a high-field asymmetric waveform ion mobility spectrometer (FAIMS) (Garza et al., 2018). Successful protein detection was achieved after washing tissue slices with ethanol and chloroform to remove lipids and other abundant small molecules and optimizing for spray conditions specific to tissue analysis. Key changes in spray conditions involved a higher solvent flow rate, a larger distance between the sprayer and the sample, a shorter sample-to-ion transfer tube distance, and a lower desorption angle. Many small proteins (6–16 kDa) were *detected*, and their spatial distributions were determined from mouse brain, human ovary, and human breast tissue samples. *Identification* of proteins was limited by low ion signals when using from-tissue -CID or -UVPD. Thus, only highly abundant protein species can be identified using top-down proteomics approaches when doing CID or UVPD in tandem with DESI-MS (Garza et al., 2018).

Circumventing the need to desorb reticent protein directly from biological tissues a recent method uses immunoassay-based desorption electrospray ionization mass spectrometry imaging (Immuno-DESI-MSI) (Song et al., 2023). Provided that antibodies are available to the targeted molecules of interest, Immuno-DESI-MS can provide the spatial distribution of protein, beyond what can be observed by conventional DESI-MSI. In this method, a set of boronic acid mass tags (BMTs) label antibodies are used as MSI probes. The BMT is released from its tethered antibody by the acidified desorption spray solvent. The fluorescent moiety enables the BMT to work in both optical and MS imaging modes, while the positively charged quaternary ammonium group enhances ionization efficiency. The introduction of the boron element also makes mass tags readily identified because of its unique isotope pattern. As a proof-of-concept implementation, a clinical therapeutic target, the epithelial growth factor receptor, as well as its cascade signaling factors and associated enzymes were shown to be mainly distributed in parenchyma and shared a similar distribution pattern across a tumor section (Song et al., 2023).

5.4 | Protein digests by DESI-MS

One way to overcome the lack of sensitivity to larger proteins is tryptic digestion before DESI-MS analysis. This approach also allows for bottom-up protein identification with reasonable demonstrated sequence coverage. Proteins can be digested off-line or in situ. Off-line digestion before DESI-MS analysis was demonstrated early on (Takats et al., 2004), and without the need for sample clean up, salt removal, or chromatography, especially when combined with ion mobility to separate peptide charge states and to remove chemical noise (Kaur-Atwal et al., 2007). This approach enabled the identification of skeletal muscle proteins for species differentiation between beef, pork, horse, chicken, and turkey meat samples (Montowska et al., 2014).

Offline tryptic digestion followed by liquid sample DESI-MS presents a more streamlined approach that proceeds without the need to apply and dry samples before analysis, providing reasonably good sequence coverage of 52% to 97% for a variety of proteoforms (Miao & Chen, 2009).

While DESI-MS can often be performed without sample clean up and chromatography, these operations can be implemented to further improve DESI-MS analysis. This is especially useful for applications with variable protein concentrations, and possible ion suppression effects from other analytes. To this point, high-performance thin-layer chromatography was used to pre-separate tryptic digests before analysis directly from the silica gel or cellulose plates leading to sequence coverages approaching 70% (Pasilis et al., 2008).

An innovative approach to analyze large proteins directly from surfaces, while retaining protein spatial distribution, involves depositing trypsin solution onto a tissue slice for in-situ digestion and subsequent mass spectrometric detection and imaging of tryptic peptides (Casadonte & Caprioli, 2011). Proof-of-principle experiments of this approach, when combined with DESI-MS analysis, was first demonstrated for spiked proteins from archeological artifacts (Heaton et al., 2009). A similar approach was used to detect tryptic digests from protein arrays printed by inkjet onto Permanox surfaces (Rao et al., 2013), and for mixtures of up to five model proteins (Montowska et al., 2014).

Immobilizing trypsin on polymer surfaces before sample deposition was found to significantly increase enzyme bioactivity (Peterson et al., 2002). Reactivity increases of up to 2000 times higher than in bulk solution at room temperature have been reported when the enzyme was covalently bound to an organosiloxane surface. The combination of trypsin immobilized on organosiloxane surfaces with DESI-MS provided excellent sequence coverages of 100% for melittin, 100% for cytochrome *c*, 90% for myoglobin, and 65% for bovine serum albumin (Dulay et al., 2015).

Another creative approach to protein digestion combined with DESI-MS involves reagentless atmospheric thermal degradation using a so-called "on-probe pyrolyzer DESI-MS" system (Zhang et al., 2007). Atmospheric pressure pyrolysis induces a variety of products that include site-specific cleavages at aspartic acid, as well as dehydration reactions in peptides and proteins.

The pyrolysis residues of peptides and the protein lysozyme retain sequence information useful for proteomic-based protein identification (Zhang & Basile, 2007).

5.5 | Protein binding assays by DESI-MS

As was the case for many of the other DESI-MS applications presented in this review, the possibility of protein functional analysis was first demonstrated in the seminal DESI-MS paper (Takats et al., 2004). Demonstrated with lysozyme, it was shown that substrates, such as *hexa-N-acetyl chitohexaose*, can easily be delivered to on-surface protein and that noncovalent protein-substrate complexes could subsequently be observed in the mass spectrum.

This application was further investigated using liquid sample DESI-MS where intact protein–ligand complex ions could be formed when the ligand was incorporated into the desorption solvent sprayed toward separate protein sample solutions. This type of "reactive-DESI" methodology, where the ligand is present in the desorption spray solution and the protein as part of the sample, can provide rapid information on binding stoichiometry, selectivity, and kinetics. This was demonstrated by the binding of ribonuclease A (RNaseA, 13.7 kDa) with cytidine nucleotide ligands and the binding of lysozyme (14.3 kDa) with acetyl chitose ligands. A higher throughput method for ligand screening by liquid sample DESI was demonstrated in which different ligands were sequentially injected as a segmented flow for liquid sample DESI ionization (Liu et al., 2013).

A similar liquid sample DESI-MS approach was followed to quantify protein–carbohydrate interactions in vitro. Here, the protein and various carbohydrates were mixed in the liquid sample, while the desorption spray contained only the solvents components. Association constants for the interactions between carbohydrates and lysozyme or a single chain variable fragment of a monoclonal antibody were in good agreement with values measured by isothermal titration calorimetry and the direct ESI-MS assay (Yao, Shams-ud-doha, et al., 2015). A comparison of the results between *reactive* liquid DESI and premixing the ligands and proteins (Liu et al., 2013) suggests that the equilibration time in reactive-DESI-MS experiments might be too short for the protein–ligand binding equilibrium to be established. This presents the concern that reactive-DESI-MS may potentially underreport equilibrium affinity values.

While the previous methods analyzed the intact ligand–protein complexes, another tactic involves analyzing only the small molecules binding to proteins. This approach is similar to protein microarrays, often using optical readout, but here the detection is by MS (Pacholarz et al., 2012). This has the advantages of label-free detection and additional assurance against false positives based on the mass spectra. To achieve a high-throughput ligand screening experiment, proteins were immobilized on agarose gels in micro-array format, and ligands were applied to surface-bound protein sports in buffered solutions. After washing the array, the bound ligands were desorbed and detected by DESI-MS (Yao, Wang, et al., 2015). The method was used to determine specific binding of 34 known ligands for seven proteins, requiring only 4 s to identify ligands in each sample spot. Subsequently, it was used to screen 88 small molecular compound ligands for binding to matrix metalloproteinase-9 (MMP-9) and good ligand binding correlation was found between DESI-MS and molecular docking experiments.

5.6 | Functional analysis of proteins by DESI-MS

Moving beyond ligand–protein binding, a few studies have shown that enzymatic reactions, and even their kinetics, can be studied by DESI-MS. These types of experiments do not analyze the protein per se, but rather its function.

When proteins are embedded in agarose gels, as shown above, specifically bound ligands can be detected, but the protein is held too tightly to be released for analysis. Making use of the reversible and specific binding of poly-histidine tags to metal affinity surfaces, both the enzymatic reaction products, and subsequently the protein, can be analyzed. It was shown that recombinant proteins can be captured and purified out of biological milieu by 6-His binding to divalent copper and nickel ions immobilized on surfaces through nitrilotriacetic acid complexation (Cu-NTA and Ni-NTA). Proteins remain biologically active and enzymatic reactions can be followed by DESI-MS. This approach can potentially be used to identify substrates for so called "orphan" proteins—those without known homology or substrates. With subsequent acidification of the desorption solvent, proteins can be released from the metal affinity surface by the desorption spray of DESI-MS for analysis to confirm identity, and potentially to determine post-translational modifications (PTMs). Two recombinant proteins, S-adenosylhomocysteine nucleosidase (His-SHAN) and caffeine synthase (His-CS), expressed in *E. coli* were immobilized on Cu-NTA or Ni-NTA surfaces.

The proteins were purified on surface, and the nucleosidase reaction products for His-SAHN and the methylation product of His-CS (theobromine to caffeine) were detected. A desorption solvent containing 50% methanol with 0.2% formic acid was sufficient to release proteins and to allow the detection of the denatured proteins (Javanshad et al., 2023).

To fully characterize proteins and their biochemistry, not only do substrates need to be identified, but chemical kinetics must also be understood, as they play an important role in understanding biochemical reaction mechanisms. While optical methods dominate this application, MS offers many advantages by being label-free, and more specific. On the other hand, ideal mass spectrometric conditions are very different from typical biological conditions for enzymatic reactions. Enzymatic reactions require buffered aqueous environments with constrained pH and ionic strength, usually maintained though inorganic salts or nonvolatile buffers, while MS performs better under acidified conditions with volatile buffers. Reaction rates are also significantly accelerated in microdroplets (Lee et al., 2015) leading to substantial overestimation of bulk reaction rate constants. Liquid sample DESI was able to overcome these barriers by incorporating a quenching step (Cheng et al., 2017). Enzymatic reactions including the hydrolysis of 2-nitrophenyl-β-D-galactopyranoside by β-galactosidase, and the hydrolysis of acetylcholine by acetylcholinesterase were studied. This approach resulted in the fast and accurate measurement of kinetic constants in good agreement with literature values. Time-resolved liquid sample DESI-MS also allowed the determination of K_m and turnover number k_{cat} for trypsin digestion of angiotensin II (Cheng et al., 2017).

5.7 | Reactive DESI-MS and determination of protein PTMs

During DESI, in situ chemical reaction or derivatization of analytes on a sample surface can be performed by delivering chemical reagents that can readily react with the target analyte through the charged solvent spray (Chen et al., 2006). This modified DESI-MS method, called reactive DESI-MS, is used for enhancing ionization efficiency of target analytes or for selectively modifying target analytes to discriminate it from other interfering species.

The ability to detect PTMs in situ by reactive DESI was demonstrated by the selective derivatization of citrullinated peptides. A citrulline derivatization agent, phenylglyoxal, was added to the DESI solvent system, allowing detection of citrullinated peptides. This reaction is selective to citrulline residues, but can potentially be further refined to characterize other PTMs (Feider et al., 2019), and extended for MS imaging experiments as was shown for MALDI tissue imaging (Harkin et al., 2022).

6 | DESI-MS IN RELATION TO OTHER AIMS METHODS FOR PROTEIN ANALYSIS

6.1 | Mass range

The analysis of very large protein assemblies has been demonstrated by some AIMS methods when analyzing proteins from prepared surfaces. For example, the tetradecameric GroEL (~800 kDa) were detected by native LESA-MS (Mikhailov et al., 2017). MALDESI has also been shown to analyze 150 kDa protein routinely and at high throughput up to 1.5 Hz/sample (Pu et al., 2022). In contrast, DESI-MS, under most circumstances, is limited to protein below 66 kDa, although liquid sample DESI-MS extended this range to around 150 kDa (Ferguson et al., 2011).

When attempting endogenous protein assemblies directly out of tissue, unfortunately, most AIMS techniques, including DESI-MS, have been limited to proteins of low molecular weight (<20 kDa), or those at very high abundance. A recent breakthrough shows that this limitation can be overcome. Intact endogenous protein assemblies up to 145 kDa were detected directly from tissue by nano-DESI (Hale et al., 2022).

6.2 | Spatial resolution

Improvements in spatial resolution has been a major driving force in AIMS innovation. Innovations includes oversampling, optimization of capillary dimensions in nano-DESI (Yang et al., 2023), improvements in sprayer designs (Forbes et al., 2013), and the use of exotic nebulizing gasses such as helium (Javanshad et al., 2022). These approaches are, in some cases, able to increase not only spatial resolution but also sensitivities. Resolutions rivalling or even improving upon MALDI analysis is now available in both DESI-MS, reported at sub 50 μm, and with nano-DESI below 10 μm. The challenge of reduced sensitivity concurrent with decreases in extraction zones has limited the implementation of very small footprints for protein analysis by AIMS, especially out of biological tissues. For protein analysis by DESI-MS, spatial resolution has been demonstrated

at 150–200 μm (Feider et al., 2016; Towers et al., 2018), while most nano-DESI MSI of proteins has been performed with a spatial resolution of 80–200 μm. Both DESI-MS and nano-DESI provide spatial resolutions significantly smaller than LESA, where spatial resolution of 1 mm was reported (Griffiths et al., 2019). Nano-DESI currently holds the spatial resolution record for imaging a set of small proteins out of biological tissues at 7 μm! (Yang et al., 2023).

6.3 | Quantitation

Quantitation is a weakness for direct and ambient analysis of complex mixtures such as biologically relevant samples. This is because of the simultaneous analysis of hundreds of molecules extracted from each location on the sample without sample clean-up or separation, leading to severe matrix effects during analysis. This is especially true for intact protein analysis due to low protein abundance, often with distinct proteoforms, and the distribution of protein signal over multiple charge states. Furthermore, protein and interferents are non-uniformly distributed through tissues (Unsihuay et al., 2021). To quantify proteins at tissue level homogenization followed by LC-MS is a better approach, although by no means straightforward (Donnelly et al., 2019).

However, it is often important to know the distribution of protein (or proteoforms) in a tissue, requiring MS imaging. This is the area where ambient ionization methods can make a substantial contribution, although most of this work is still done by MALDI-MS (Stoeckli et al., 2001; Unsihuay et al., 2021).

Absolute quantification of intact proteins observed in MSI experiments is challenging especially with ambient ionization methods, due to the uncertainty in extraction/desorption efficiencies, the ionization efficiency, and signal suppression in different parts of the tissue, leading to indeterminable response factors. Therefore, most of the studies in this field are focused on the spatial distribution of the proteins rather than quantification.

To determine spatial distributions of molecules relative quantitation is sufficient, rather than absolute quantitation. Signal is most typically normalized to the TIC, or to a selected endogenous molecule in the sample, although internal standards have been used to account for differences in matrix effects for the analysis of small molecules and lipids. Standards have been applied by spotting (Nilsson et al., 2010), spraying (Vandenbosch et al., 2023), or inkjet printing (Luo et al., 2018; Pirman et al., 2013). However, the addition of standards in MSI experiments is complicated, and labeled or equivalent standards are not always readily available, especially for proteins.

Standards can also be spiked into the extraction solvent. This strategy works well with probe-type liquid sampling techniques in which desorption and ionization stages are decoupled. For example, Lanekoff et al. used nano-DESI MSI with a phosphatidylcholine internal standard added to the extraction solvent to show the different types of matrix effects observed in MSI of mouse brain tissue (Lanekoff et al., 2014). In the authors laboratory, using DESI-MS, adding internal standards to the desorption spray solvent has not yet been successful because some of the nebulized extraction solvent is sampled directly into the ion transfer tube without surface interaction, and therefor differences in surface geometry, tissue inhomogeneity, or extraction efficiencies are not necessarily accounted for.

A different approach for spatially resolved absolute quantification involves the creation of an external calibration sample produced from tissue homogenates spiked with known amounts of isotopically labeled analyte of interest. The "mimetic tissue model" initially developed for MALDI imaging (Groseclose & Castellino, 2013), has since been adopted for the targeted quantification of proteins in tissue sections in LESA-MSI experiments (Havlikova et al., 2019). This approach can potentially readily be extended to other sampling/ionization techniques, such as DESI-MS, but this has not been demonstrated yet.

7 | CONCLUSIONS

This review shows that, despite its many limitations, DESI-MS is making valuable contributions to the analysis of proteins and that the challenges in sensitivity, spatial resolution and mass range are surmountable obstacles. Intact protein or digested protein have been analyzed from prepared surfaces, directly from solutions, and out of biological samples. Spatial distributions of protein can be obtained at 10's to 100's of micrometer resolution either directly or indirectly through reporter molecules. PTMs can be identified, and specific binding of co-factors and their binding stoichiometry can be determined. Substrates and their enzymatic transformations can be measured by DESI-MS and their kinetic values estimated, promising the potential for the comprehensive functional analysis of orphan proteins.

While DESI-MS does not currently offer the same spatial resolution, mass range, or sensitivity other solvent extraction-based ESI-driven techniques provide, the

noncontact nature of DESI signifies an important advantage in terms of in robustness, as is the relative ease with which a rudimentary DESI-MS set-up can be created.

Thus, despite superior performance in in situ surface sampling of intact proteins from tissues currently offered by other ambient techniques such as LESA (Griffiths & Cooper, 2016, Griffiths et al., 2019), and nano-DESI (Hale & Cooper, 2021, Hale & Hughes, & Cooper, 2021, Hsu et al., 2015), further development and improvements of DESI-MS for protein analysis is justified.

ORCID

Andre R. Venter http://orcid.org/0000-0001-6912-7446

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AUTHOR BIOGRAPHY

Andre R. Venter completed his formal postsecondary education at the University of Pretoria, South Africa, culminating in a PhD in 2003 with Prof. Egmont Rohwer. He worked as a postdoctoral researcher at Purdue University with Prof. Graham Cooks, West Lafayette, Indiana. At Purdue University he investigated fundamentals of desorption electrospray ionization mass spectrometry and other ionization methods. At Western Michigan University since 2008, and as full professor since 2019, his research group studies ionization mechanisms involving solvent-air and solvent-surface interfaces, towards further development of (ambient) ionization mass spectrometry.

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