MATRIX-FREE METHODS FOR LASER DESORPTION/IONIZATION MASS SPECTROMETRY*

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Matrix assisted laser desorption/ionization (MALDI) is a soft ionization mass spectrometric method that has become a preeminent technique in the analysis of a wide variety of compounds including polymers and proteins. The main drawback of MALDI is that it is difficult to analyze low molecular weight compounds (<1,000 m/z) because the matrix that allows MALDI to work interferes in this mass range. In recent years there has been considerable interest in developing laser desorption/ionization (LDI) techniques for the analysis of small molecules. This review examines the approaches to matrix-free LDI mass spectrometry including desorption/ionization on silicon (DIOS), sol-gels, and carbon-based microstructures. For the purposes of this review matrix-free methods are defined as those that do not require matrix to be mixed with the analyte and therefore does not require co-crystallization. The review will also examine mechanisms of ionization and applications of matrix-free LDI-MS. © 2006 Wiley Periodicals, Inc., Mass Spec Rev 26:19–34, 2007

Keywords: *MALDI; DIOS; laser desorption/ionization; mass spectrometry; matrix free*

I. INTRODUCTION

Soft ionization of analyte molecules has become very important in mass spectrometric analysis of chemical analytes. It is particularly important in the analysis of biomolecules and for analysis of synthetic macromolecules. One of the most important methods for the soft ionization of analytes is matrix assisted laser desorption/ionization mass spectrometry (MALDI). MALDI was introduced in the late 1980s (Karas & Hillenkamp, 1988). In general, the MALDI method involves co-crystallizing the analyte with a matrix, which is generally a low molecular weight compound that can absorb the energy of a laser light pulse. Absorption of the laser energy causes the matrix to undergo a phase transition (along with the analyte). The analyte is then ionized within the plume by intermolecular interactions, usually proton transfer. The ionized analyte can then be analyzed with the mass spectrometer.

However, the use of a matrix introduces a number of problems into the analytical method. First of all, the analyte must be miscible with the matrix and they must co-crystallize. This requirement has made MALDI somewhat difficult to implement because the selection of the proper matrix is often a matter of trial and error. Also, the co-crystallization process does not produce a uniform mixture, which causes "hot spots" to occur within the matrix film. MALDI is also rarely used for analysis of analytes of low molecular weight (<1,500 *m/z*). The use of MALDI for low molecular weight analytes is avoided because the matrix typically has a low molecular weight, which interferes with the analysis of the analyte. A recent article reviewed MALDI techniques which have been applied to the analysis of low molecular weight compounds (Cohen & Gusev, 2002), which identified several approaches for the analysis of molecules with a low molecular weight. The first approach is to pick a matrix which does not directly interfere with the analyte ions and pick out the analyte spectrum from between the matrix ion peaks. Another approach that has been used to analyze low molecular weight analytes has been to use a high molecular weight matrix. The difficulty in implementing this approach is that polymer matrices often do not co-crystallize with the analyte. In addition, it is difficult to analyze samples with a *m/z* similar to the polymer matrix, which means that the use of a higher molecular weight matrix still does not provide for a system that the entire range of analyte molecular weights can be analyzed. Another approach that has been used to analyze low molecular weight compounds is the use of inorganic matrices.

Because of the limitations of MALDI there has been a significant amount of work in the last several years to develop laser desorption/ionization techniques that can be performed without a matrix. The initial work on this was to use an inorganic matrix. This was demonstrated by Tanaka et al. (1988) in using cobalt powder mixed with glycerol as the matrix. One approach for the analysis of small molecules is the direct desorption/ionization of analytes. For instance, one study directly deposited tryptophan on a sample plate and then irradiated it with a laser to desorb and ionize the analyte (Zhan, Wright, & Zenobi, 1997). The method was limited to analytes with a *m/z* below 2,000, and is very dependent on the properties of the analyte. Furthermore, the direct desorption/ionization often promotes ion fragmentation because higher laser powers are required to desorb the analyte.

Another approach is to tailor the surface of the sample pate so that it is capable of transferring enough energy to the analyte to desorb/ionize the sample. In one study, a matrix is incorporated

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into agarose beads (Hutchens & Yip, 1993). These activated beads are then placed on the sample surface followed by the analyte. The analyte can then be analyzed by laser desorption/ionization mass spectrometry with the advantage that a low molecular weight background from the matrix molecules is not observed because the matrix molecules are immobilized within the beads. The study also introduced a method in which biological analytes can be captured by affinity interactions. This is accomplished by incorporating antigen or DNA functionalities onto the agarose beads. Surface enhanced laser desorption mass spectrometry (SELDI) has since evolved into a very important method for the analysis of biological analytes, particularly in extracting them from much more complex solutions (Issaq et al., 2003).

The first truly matrix-free laser/desorption ionization mass spectrometric method was introduced by the Siuzdak group in 1999 and involves placing the analyte on a sample plate of porous silicon (Wei, Buriak, & Siuzdak, 1999). The porous silicon targets are prepared by etching silicon wafers to produce a porous substrate with a nanostructured surface. The porous silicon surface can then be used directly for laser desorption ionization mass spectrometry with little or no small molecule interference. This demonstrated the analysis of small antiviral drugs, des-arg-bradykin, and other small peptide molecules. The technique of using porous silicon as a sample target for matrix-free analysis was named desorption ionization on porous silicon (DIOS). The performance of the porous silicon is determined by the silicon type and the etching conditions that are used to produce the porous silicon (Lewis et al., 2003). DIOS techniques have been most widely applied to analysis of biological analytes, especially proteins and peptides. Matrix-free methods have also been demonstrated for a wide range of analytes including drugs, explosives, polymers, and forensic analysis. There has also been a wide range of approaches to develop matrix-free LDI-MS methods. For this review matrix-free methods are defined as those that allow the sample to be place directly onto a surface and analyzed. This simplifies sample preparation because the analyte does not need to be mixed with the matrix and the analyte does not have to co-crystallize to produce an adequate signal.

II. APPROACHES TO MATRIX-FREE LDI-MS

A. Desorption Ionization on Silicon (DIOS)

The first method to utilize a true matrix-free desorption/ionization approach is called desorption ionization on porous silicon (DIOS) (Wei, Buriak, & Siuzdak, 1999). The properties of porous silicon are very useful for applications in chemical and biological analysis, as is discussed a review written a few years ago (Stewart & Buriak, 2000). Porous silicon is produced through an etching procedure in which a nanocrystyline surface is produced. The surface itself is then easily oxidized, which allows the surface of porous silicon to be chemically modified. Furthermore, porous silicon has been found to absorb UV light and can then also emit light through photoluminescence (Canham, 1990), electroluminescence (Halimaoui et al., 1991), and chemiluminesence (Mccort, Yau, & Bard, 1992). The use of Porous silicon has been widely used and studied in the literature. Another study focused on the effect that the preparation conditions have on the response of analytes in the MALDI instrument (Shen et al., 2001a). The preparation conditions of porous silicon are very important because a variety of conditions (i.e., crystal orientation, dopant type and level, etching solution, etching time, etc.) all affect silicon morphology including pore diameter and shape. Most post-etch solvents have no effect on the DIOS performance (the exception is ethanol, which reduces the intensity of background ions). Double etching (where the silicon plate is oxidized after etching and then treated with hydrogen fluoride) improves performance, although a higher laser power is required to desorb the analytes from the DIOS plate. The porosity of the DIOS plates was found to be 30%–40%, with pore sizes approximately 70–120 nm in diameter and spaced approximately 100 nm apart as shown in Figure 1. The article also demonstrated that the porous silicon surface can be chemically modified to prevent oxidation of the surface, which degrades the analytical utility of the DIOS plate. Another approach to produce porous silicon for DIOS is to use electrochemical etching (Gorecka-Drzazga et al., 2004). The article describes a procedure that produced a silicon surface that is hydrophilic, consisting of porous silicon dioxide. The hydrophilic section is surrounded by a hydrophobic region, which aids in placing the sample in the correct spot. The surface was then used to analyze a tripepdide down to 40 fmol/spot, and was also used to analyze catecholamines. Yet another article describes the preparation of porous n-type silicon plates using electrochemical etching and included both front side and back side illumination (Tuomikoski et al., 2002). The study found an optimal pore size in the range of 50–200 nm, and was used to analyze a number of biological compounds.

Another approach to forming surfaces applicable for DIOS is to form a silicon thin film. The thin film is fabricated using plasma-enhanced chemical vapor deposition (Cuiffi et al., 2001). The thin film forms a surface that does not require etching prior to its use for DIOS-MS. The surface produced by this method consists of vertical rod like columns that are approximately 10 nm in diameter and cluster into larger 100 nm size surface features. These thin films can easily be deposited onto a variety of surfaces including glass and plastic. It was found that the depth of the thin film needs to be at least 50 nm to obtain efficient desorption/ionization of the analyte, however no further advantage was found when the thickness of the film was greater than 100 nm. The thin film in the study was found to be effective in DIOS for the detection of low molecular weight compounds such as a tryptic digest of ubiquitin as well as for moderately large molecular weight compounds such as thyrocalcitonin (m/z 3605) and insulin (m/z 5735).

A similar method used a planar silica gel that was then drawn on with a graphite pencil to produce a carbon powder impregnated silica surface (Chen & Wu, 2001). The source of the silica gel was a TLC plate. In addition, a solution of sucrose/glycerol was added to the pencil line on the silica surface. The sample solution was then deposited on the pencil line and the sample was subjected to LDI-MS analysis. The silica surface was used to analyze small organic molecules such as methylpherine, cytosine, and small PEG polymers. The background signal was

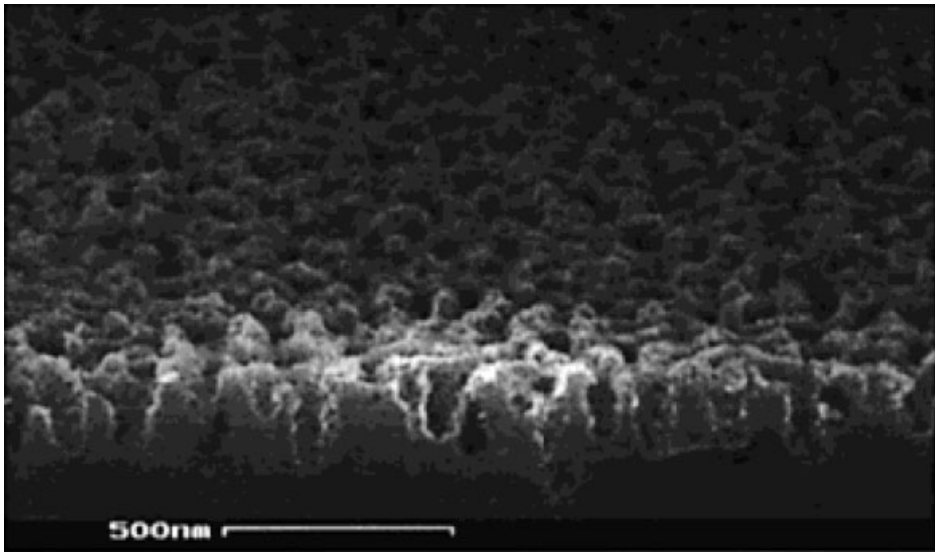

FIGURE 1. SEM analysis of the ''double-etched'' porous silicon surface prepared from low-resistivity n+-Si material (0.001–0.005 Ω · cm). Reprinted with permission from Shen et al. (2001a). Copyright 2001 American Chemical Society.

very low and it provided for a relatively simple approach to preparing a matrix-free method for LDI-MS.

MALDI in general uses lasers that operate in the UV part of the spectrum. Most MALDI instruments use a nitrogen laser with an operating wavelength of 337 nm. Two studies have demonstrated the use of lasers operating in the IR region of the spectrum for matrix-free LDI-MS. The first study introduced the use of a tunable IR laser operating with a wavelength between 1.45 and 4.0 μm (Bhattacharya, Raiford, & Murray, 2002). This system was used to obtain spectra of proteins such as bovine insulin, riboflavin, and bradykinin. The optimum wavelength for LDI-MS was found to be 2.94 μm, signal could also be obtained between 2.8 and 3.5 μm, although an abrupt loss of signal was observed when the laser was tuned to shorter wavelengths and a more gradual loss of signal when the laser was tuned to longer wavelengths. The loss of signal is thought to correspond to the OH stretch, which may be present on the surface in the form of residual water on the surface or from the sample. The use of IR DIOS was later further explored in another study using a 2.94 μm ER:YAG laser (Rousell et al., 2004). The IR desorption/ionization was compared using MALDI and matrix-free using a variety of surfaces including stainless steel, aluminum, copper, polyethylene, and porous silicon targets as seen in Figure 2. The best performance of these was found to be the porous silicon target. The detection limit for bradykinin was 300 fmol using the porous silicon surface. The IR technique was also used for the analysis of myoglobin, which indicates that the technique is useful even for analytes up to 17,000 *m/z*, although the laser energy that is required to desorb large biolmolecules is significantly higher than for small molecules.

Recently a technique has been introduced that allows for a MALDI plate to be placed outside the vacuum system of a MALDI instrument and still introduce analyte into the mass spectrometer (Laiko, Baldwin, & Burlingame, 2000). This technique is known as atmospheric pressure ionization MALDI (AP-MALDI). The AP-MALDI method has also been extended to produce an AP-DIOS method (Laiko et al., 2002). This study used a commercially available DIOS plate attached to the AP sample interface. The sample preparation for these plates used an acetonitrile/water mixture with 0.1% TFA. The solution was mixed 1:1 with the analyte solution and placed on the DIOS plate. The DIOS plate was then used to analyze small peptide analytes such as BSA digests. It was found that the AP-DIOS was superior to AP-MALDI at *m/z* below 1,100, however at higher *m/z* values AP-MALDI was shown to produce better data.

Clearly, the use of DIOS is strongly dependent on a wide range of properties of the silica surface. A recent study examined different silica surface structures and modifications to determine the requirements of the sample target to produce the optimal signal. The morphology of the silica substrate was examined by scratching with sandpaper, fabricating sub-micrometer grooves in the silicon, and coating porous alumina with silver. The study showed that surface roughness is important in allowing desorption/ionization for both stainless steel and silicon surfaces as seen in Figure 3. The study of silica surfaces using grooves showed that performance is enhanced using micrometer structures and appears to be the key factor in desorption/ionization on rough surfaces. When porous alumina or polyethylene were coated with metals, the analysis of small molecules was improved. This improvement is likely because of the improvement derived from the surface being electrically conductive. The alumina chip also had prolonged activity of up to a month even when the chip was stored in air.

B. Sol-gels

One approach that has been used to matrix-free LDI-MS has been to use sol-gels. Sol-gels are a polymeric structure formed by siloxane backbone (Si-O), and exhibit a range of useful electrical and optical properties (Schottner, 2001). Sol-gels have been used

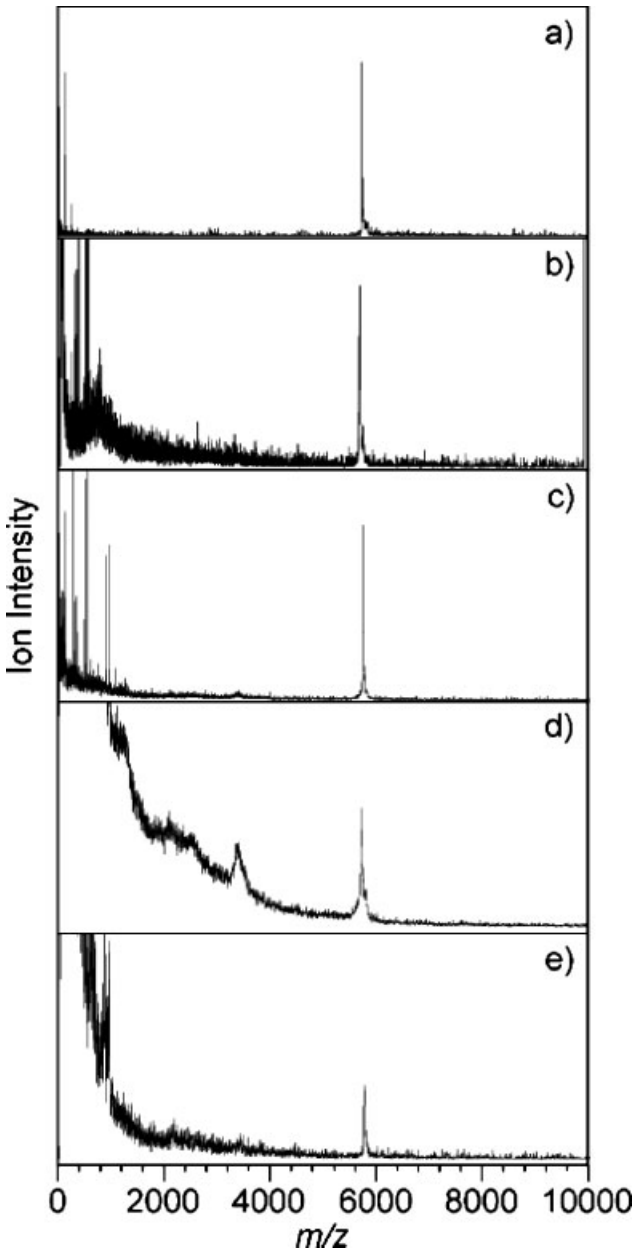

FIGURE 2. IR-LDI mass spectra of bovine insulin acquired from (**a**) silicon, (**b**) stainless steel, (**c**) aluminum, (**d**) copper, and (**e**) polyethylene targets (Rousell et al., 2004). Copyright 2004 John Wiley & Sons Limited. Reproduced with permission.

for a wide range of analytical applications including enzyme encapsulation (Pierre, 2004), electrochromatography stationary phases (Allen & El Rassi, 2003), sensor applications, and ion exchange coatings (Collinson, 2002). The formation of a sol-gel is typically a three step process, first tetraethoxy silane (TEOS) is hydrolyzed to form a tetra hydroxyl silane, which is reacts by condensation, either with itself or with other hydroxyl containing compounds, the process then continues to polymerize and form the sol-gel. Sol-gels can be formed either in bulk or as a thin film. The first use of sol-gels for matrix-free LDI-MS involved incorporating a matrix molecule within the sol-gel structure (Lin & Chen, 2002). Incorporation of the matrix molecule was accomplished by condensation of the hydroxyl groups of 2,5-dihydroxybenzoic acid (DHB) with TEOS to form a sol-gel film with a matrix molecule immobilized. The surface that was produced will absorb the energy from the laser and allow the analyte to be desorbed, but which will produce a minor background signal. The sol-gel film was used to analyze for various biological proteins such as cytochrome c, insulin, and bradykinin. The background observed was very low and was only observed for films that were prepared with a great deal of DHB or when the laser power was very high as seen in Figure 4. The same group also examined the effect of introducing different isomers of DHB into the sol-gel (Ho, Lin, & Chen, 2003). The study showed that even though some isomers (2,6- and 2,3-DHB) only slightly absorb light at 337 nm, they experience a red shift in the sol-gel which makes them able to absorb the laser light. Furthermore, it was shown that the 2,5-DHB is the ideal isomer to incorporate into the sol-gel as it produces the signal with the highest S/N ratio. The approach of incorporating a matrix into a sol-gel was also performed by spin coating the sol-gel solution onto a glass chip (Lin, Yang, & Chen, 2004). Spin coating of the sol-gel was shown to produce a surface that produced very reproducible spectra for the analyte. The sol-gel surfaces were also found to be effective even a week after they were prepared. Furthermore, a solution containing a protein and trypsin was directly spotted on the plate to allow for a on-chip digestion of the protein and analysis of the peptide fragments. Another advantage of using a sol-gel material is that it can also be used to extract an analyte from solution. One study demonstrated this by coating an optical fiber with a DHB derived sol-gel (Teng & Chen, 2003). The coated optical fiber was then used to extract a benzo[a]pyrene from solution and then analyzed it by placing the fiber on a target in the mass spectrometer.

Sol-gels can also be formed from compounds other than silica containing compounds such as titanium and zirconium. Another approach demonstrated matrix-free LDI-MS from a sol-gel film formed with titania (Chen & Chen, 2004a). The advantage of using a titania sol-gel is that they are capable of directly absorbing energy in the UV region, and thus do not require a matrix molecule to be incorporated into their structure. The titania sol-gel was formed by mixing titanium (IV) *n*-butoxide/ethanol and a mixture of ethanol, water, and nitric acid. The solution was stirred in an ice bath for 10 min, after which polyethylene glycol (MW 600) was added and the mixture was stirred for 30 min. The titania sol solution was then spin coated onto an aluminum support. The titania chip was calcinated at 500° C for 1 hr. The titania surface that is produced is very hydrophilic (contact angle is 10.7°). However, to produce useful MS data a proton source needs to be added to the buffer of the analyte (such as a citric buffer). A UV spectra of the titiania surface shows that it absorbs very strongly in the region of 337 nm, which is the typical laser used for LDI-MS analysis. The chip was used for LDI-MS analysis of various proteins such as insulin, cytochrome c, and trypsinogen, which with a molecular weight of 23,982 *m/z* it is among the largest molecules analyzed using matrix-free LDI-MS methods. The titania chip was also shown to be functional as a surface for LDI-MS 1 month after it was prepared.

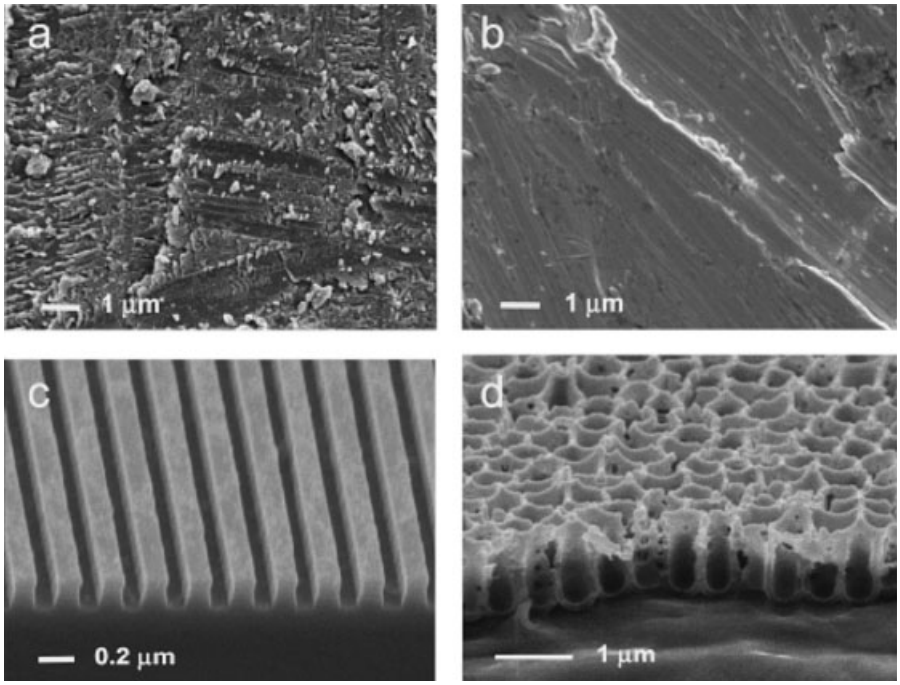

FIGURE 3. SEM images of manufactured surfaces: (**a**) silicon wafer scratched with No. 400 sand paper (surface), (**b**) stainless steel plate scratched with No. 400 sandpaper (surface), (**c**) submicrometer groove arrays on silicon (cross section), and (**d**) Au-coated porous alumina (cross section). Reprinted with permission from Okuno et al. (2005). Copyright 2005 American Chemical Society.

C. Carbon-Based Polymers & Microstructures

In addition to sol-gels, there has also been an interest in introducing matrix-free methods for LDI-MS that involve carbon containing structures. The use of carbon containing structures used graphite particles dispersed in glycerol (Sunner, Dratz, & Chen, 1995). This approach was further extended to the use of a TLC plate that was coated with 2 μm activated carbon particles to produce a system that incorporated both TLC functions and a matrix-free LDI technique (Chen, Shiea, & Sunner, 1998). These techniques were then further refined to contain only the surface that contained activated carbon (Han & Sunner, 2000). The surface was produced using mesoporous activated carbon (diameter 5–10 μm) which was placed on aluminum which had an adhesive applied. The excess activated carbon is then removed by blowing air or by shaking the paper. Obviously, this approach relies heavily on operator experience. However, the surface was used to analyze several compounds such as glucose, caffeine, and bradykinin. The activated carbon surface was also placed next to a TLC plate and used to extract samples from the plate and generate a spectra from the separated compounds.

One study immobilized metal or metal oxide particles in paraffin as an approach to allow matrix-free LDI-MS (Kinumi et al., 2000). The use of paraffin is significantly different than the glycerol dispersant that has traditionally been used to disperse metal particles. The use of the paraffin significantly reduced the matrix background analytes that were observed in the low mass region. This approach therefore produces a substrate that is useful for small molecule analysis and was used to analyze PEG200 and methyl state. The metal particles are typically in the micrometer to tens of micrometer size.

One approach that has been used in MALDI to allow for the analysis of small molecular weight compounds is to use a polymer as the matrix (Woldegiorgis et al., 2004). The polymers used in the study are based on oligothiophene or oligobenzodioxin and demonstrated an ionization process that involved electron transfer instead of the ordinary proton transfer. This study demonstrated that polymers can be used in the desorption/ionization, although it was not a matrix-free method. Porous polymer monolith structures have recently been applied as a matrix-free method for LDI-MS (Peterson et al., 2004). Porous polymer monoliths are rigid polymeric structures which have both micropores and mesopores, which transverse the length of the polymer. These structures have been used for a variety of applications in analytical applications including chromatographic stationary phases, enzymatic reactors, etc. Several monomer backbones were examined for their effectiveness as a matrix-free support. First a poly(butyl methacrylate-co-ethylene dimethacrylate) monolith was used, which could be photo patterned on the MALDI plate, however a relatively high laser power was required to desorb/ionize the analyte. Next, a poly(styrene-co-divinyl benzene) monolith was used, which required much less laser power to desorb and ionize the analyte, but locating the analyte on the monolith was more problematic. Finally, a poly(benzyl methacrylate-co-ethylene dimethacrylate) monolith, which incorporated phenolic groups to increase the UV absorption and energy transfer to the analyte, and also could be photopatterned on the sample plate. The solution that the sample

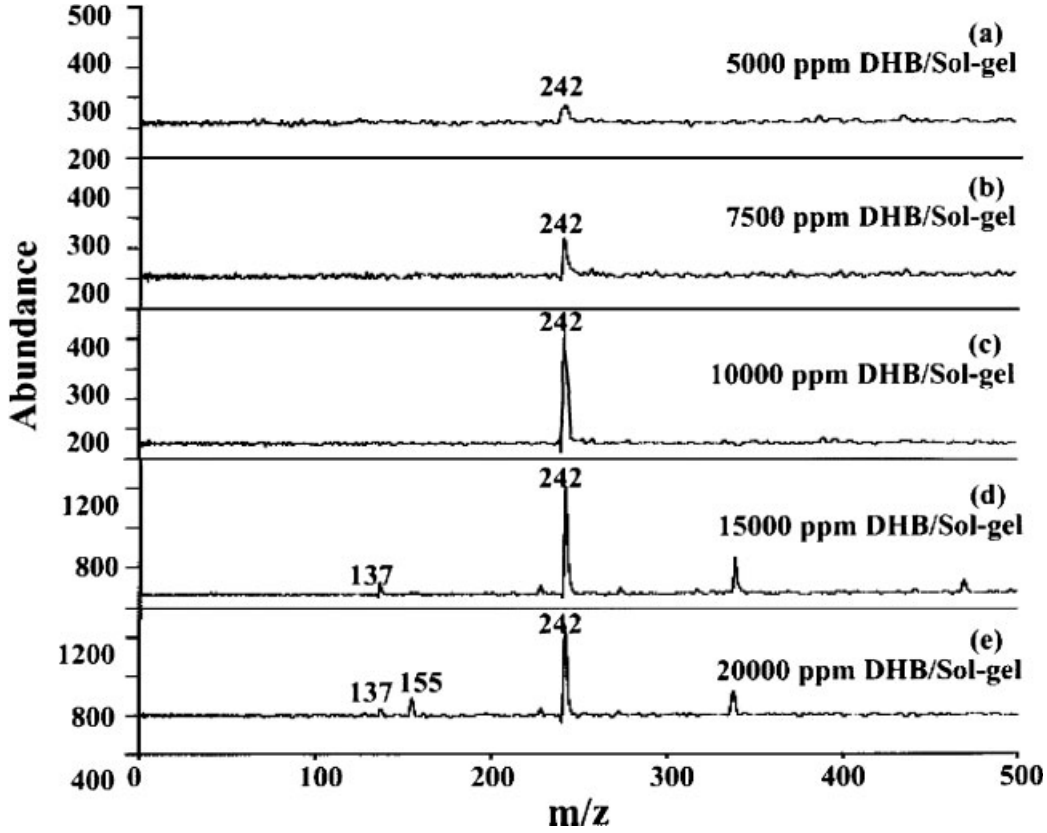

FIGURE 4. Laser desorption/ionization mass spectra of prometryn (MW 241.2, 100 ng) desorbed from the sol-gel-derived DHB films, which contained (**a**) 5,000, (**b**) 7,500, (**c**) 10,000, (**d**) 15,000, and (**e**) 20,000 ppm of DHB. Reprinted with permission from Lin & Chen (2002). Copyright 2002 American Chemical Society.

is dissolved in has a strong influence on the quality of the spectra obtained, the best results were obtained with 0.1% TFA. The pore size of the monolith was also examined, with the optimal pore size being between 200 and 900 nm. The monoliths were used to analyze caffeine, small peptides, acid labile compounds, and explosives.

D. Nanostructures

There have recently been several methods that use nanostructures to produce a matrix-free LDI-MS signal. The initial approach to using a nanostructure was to use carbon nanotubes as a matrix in MALDI (Xu et al., 2003). The nanotubes were found to be a useful matrix because they can trap analytes on their surface and they also efficiently transfer energy to the analyte. The nanotubes were shown to produce less background ions than other matrices and they also required less laser power to desorb/ionize analytes. However, preparation of a matrix containing carbon nanotubes is particularly difficult because nanotubes are insoluble in most solvents. Nanotubes were oxidized to improve their solubility (Ren & Guo, 2005). The oxidized nanotubes produced a phase that was more reproducible, and this technique was used to analyze a number of biological analytes. The advantages of using carbon nanotubes is quite clear, so there is interest in developing a method in which the nanotubes could be immobilized so that the sample preparation is simplified. One approach used a polyurethane adhesive to immobilize carbon nanotubes on a surface (Ren et al., 2005). The adhesive nanotube surface was shown to give a signal with a higher S/N ratio for a larger number of laser shots than for non-immobilized carbon nanotubes. The study used the method was used to analyze small sugar molecules including fructose at a concentration as low as 5 amol. The method was then used to distinguish a diabetic patient from a non-diabetic from an analysis of urine (glucose is present in the diabetic sample, but not in the healthy sample).

Another nanostructured material that has been used for matrix-free LDI-MS has been silicon nanowires (Go et al., 2005). The nanowires were synthesized using gold nanoclusters as a template on silicon wafers. Nanowires were then grown using silane vapor (SiH_4) as a reactant in a chemical vapor deposition furnace. The silicon wires could then be further modified using silating reagents. The best results for silicon nanowires was with nanowires 10–40 nm in diameter, with less than 10 nanowires/μm^2, and with lengths less than 5 μm. The silicon wire surface typically required much less laser power to desorb and ionize the analyte sample than other DIOS and MALDI methods (as much as 50 times less). The best detection sensitivity of the nanowire surface was found to be 500 amol of des-Arg^9-bradykinin. The silicon nanowires have high surface area, which allows them to easily wick fluids. This allowed the nanowire surface to be used as a TLC plate to perform a chromatographic separation of small

molecules, such as drugs. The Si-nanowire plate was then analyzed to identify the location of the different compounds on the plate as seen in Figure 5.

A second approach to fabricating a nanostructured surface for use in matrix-free LDI-MS is to make a surface from mesoporous tungsten and titanium oxides (Yuan et al., 2005). The synthesis involves using a polymer as a structure directing agent for the inorganic precursors, tungsten chloride, and titanium isopropoxide. The surface produced has a well-defined two-dimensional hexagonal mesostructure. The mesoporous surface also has a maximum UV absorption at 340 nm, well within the requirements for LDI-MS. The nanostructured surface was demonstrated to be applicable in the LDI-MS of Gramicidin (MW 1140). The mesoporous WO_3-TiO_2 surface was also compared to a non-porous surface. The porous surface was shown to produce a signal that is nearly three orders of magnitude more intense. This demonstrates the utility of using a nanostructured surface compared to one that is non-porous.

These studies demonstrate the utility of using a nanostructured material for performing matrix-free LDI-MS. However, these structures are still produced by a random process. There has also been some work fabricating a regular surface for matrix-free LDI-MS. One study generated a nanocavity array on a Si wafer and then used this surface for matrix-free LDI-MS (Finkel et al., 2005). The technique that was used to fabricate the nanocavity array is called nanosphere lithography, which involves SiO_2 nanoparticles (330 nm) that are deposited on an oxidized silicon wafer using convective assembly. The assembly is then subjected to reactive ion etching (RIE), which forms the nanocavities. The particles and the SiO_2 layer of the wafer are then removed and the chip is ready for analyte deposition and mass spectrometric analysis. The nanocavity array was used for the analysis of small peptides, and the detection sensitivity was comparable to other DIOS methods. The advantage of the nanocavity array is that the size (width and depth) and spacing of the nanocavities is controllable and should produces an optimized surface as shown in Figure 6. Another approach for fabricating a nanostructured surface for matrix-free LDI-MS involves the use of silicon microtips (Gorecka-Drzazga et al., 2005). The gated silicon microtip array was fabricated using semiconductor fabrication techniques. The size of each microtip was approximately 1 μm. The microtip array was used to analyze dopamine and a tripeptide. It was shown that few laser shots were required to obtain a signal of the analyte compared to MALDI and DIOS methods, suggesting that the energy transfer for the microtips is very efficient.

III. MECHANISMS OF IONIZATION

To understand the desorption/ionization process for DIOS and other matrix-free methods, it is necessary to understand the process with regular MALDI methods. A very extensive review on the MALDI desorption process was published in 2003 (Dreisewerd, 2003). A large number of parameters are important in the MALDI process including the irradiation parameters (laser

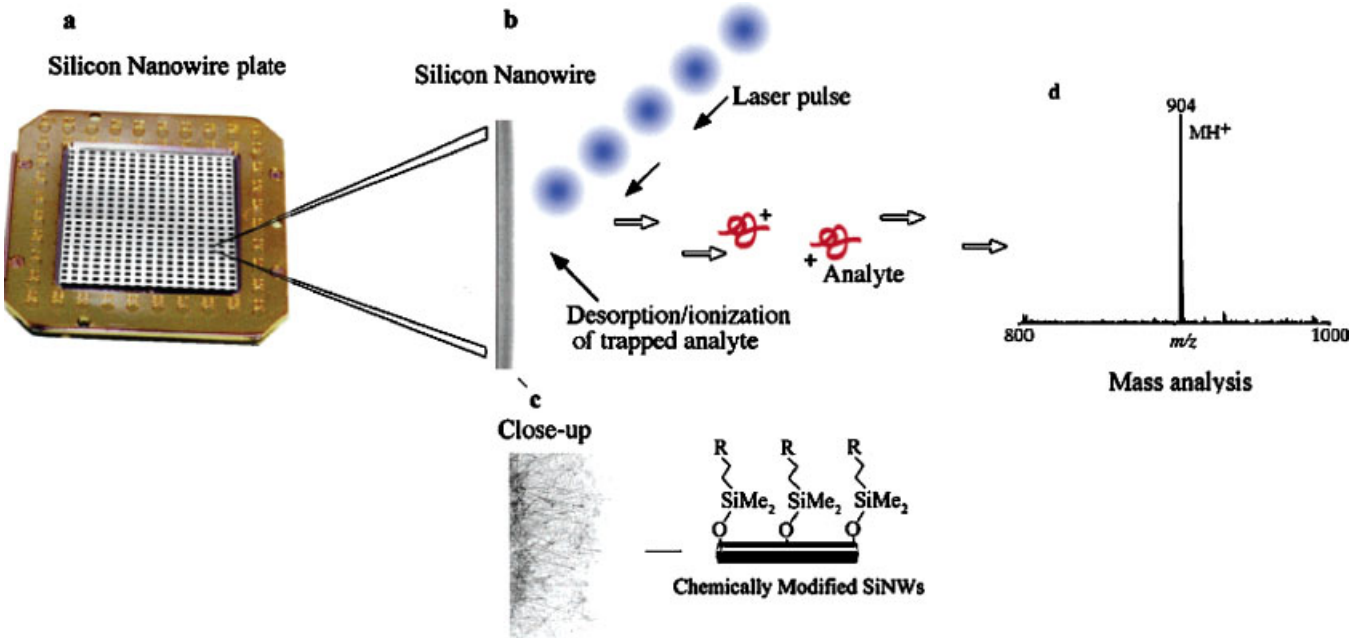

FIGURE 5. Configuration of laser desorption/ionization mass spectrometry experiment showing (**a**) patterned SiNWs grown on a Si substrate attached to a modified MALDI plate, (**b**) schematic of laser desorption/ionization of trapped analytes within the Si nanowire mesh, (**c**) close up SEM image of SiNWs and an illustration of the functionalities by silylation, and (**d**) mass spectra of 500 amol of des-Arg9-bradykinin using a 40-nm diameter SiNW. The measured signal-to-noise ratio was 600 to 1. Reprinted with permission from Go et al. (2005). Copyright 2005 American Chemical Society. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

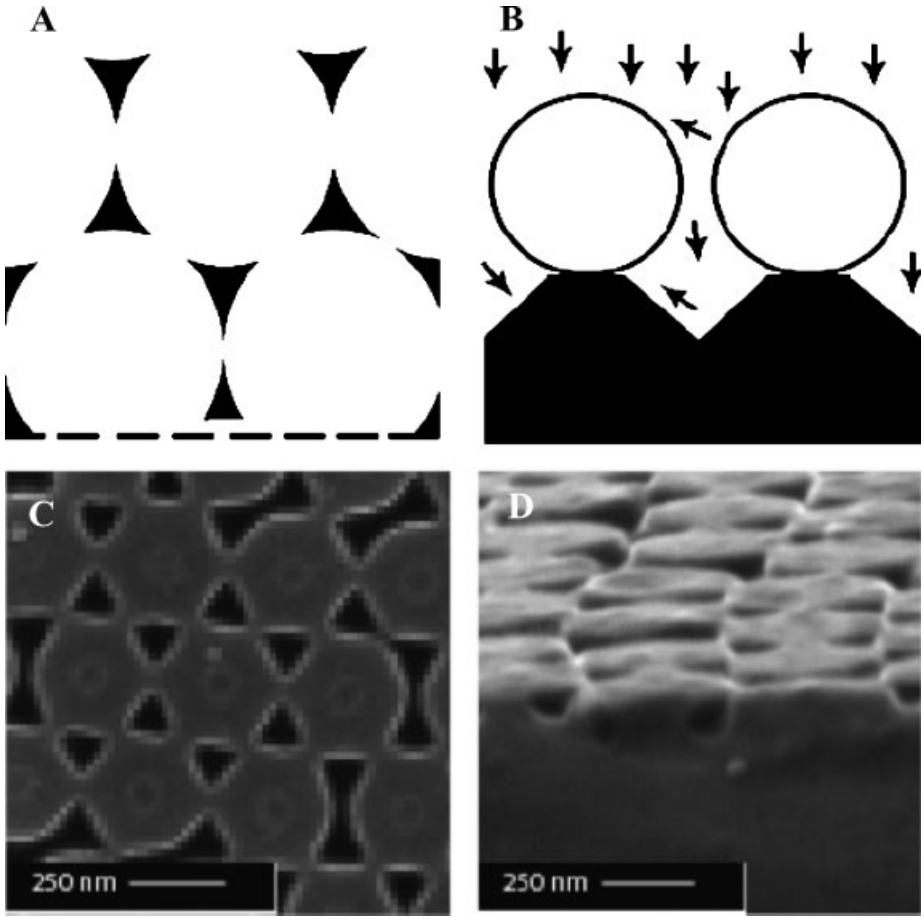

FIGURE 6. Schematic drawings of the top (**A**) and the side view (**B**) of a SiO_2 nanoparticle-masked surface during RIE. The dotted line in **panel A** indicates the cross-sectioning position for the side view scheme; the arrows in **panel B** represent etching molecules. Corresponding FE-SEM images are shown in **panels C** and respectively. The surface was prepared with 1-min etching. Reprinted with permission from Finkel et al. (2005). Copyright 2005 American Chemical Society.

wavelength, pulse duration, laser fluence, etc), material parameters (sample morphology, preparation, analyte-matrix interaction), and the plume dynamics (initial velocity, velocity distribution, gas phase dynamics, internal energies). Typically the best MALDI performance is obtained when the absorption maxima of the matrix is near the wavelength of the laser used. The increased efficiency is due to the most energy being absorbed by the matrix and transferred to the analyte in the desorption/ionization process. Typically there is a threshold laser fluence above which an analyte signal is obtained, above this level the ion signal is roughly linear with increasing laser fluence. The duration of the laser pulse is also important in generating the signal, it must be short to rapidly desorb the analyte, although longer laser pulses have the effect of degrading the analyte before it is desorbed. In addition to the laser conditions, the main factor in the MALDI process is the selection of the matrix. The selection of the correct matrix is heavily dependent on the analyte and the solution that it is prepared in. The analyte/matrix ratio is also very important in crystal formation and quality. The dependence of signal quality on crystal formation also makes quantitative analysis very difficult. The morphology is significantly different for each matrix molecule although the specific effects of the matrix morphology is unclear. Many different sample preparation methods produce good signal, which indicates signal generation in MALDI is relatively insensitive to the morphology. After the sample has been desorbed from the sample plate, the plume dynamics become very important in the ionization and analysis of the volatilized sample. In particular the initial velocity of the ions and molecules in the plume is very important. This initial velocity is produced because of the expansion of solid matrix mixture into a gas in a vacuum chamber.

On the surface, matrix-free methods of desorption/ionization have a similar mechanism to MALDI, although there are some significant differences between the two methods. Both use a laser pulse to excite and desorb the analyte, but with matrix-free methods the surface must be able to transfer the laser energy to the analyte. One study examined the effect that various parameters have on signal generation in DIOS (Kruse et al., 2001a). This study examined the effect of the morphology of the porous silicon (prepared using different approaches) as well as

the effects of laser wavelength, pH, and solvent have on ion signal generation. It was found in that the ability of the porous silicon to produce luminescence when illuminated with UV light is related to the porous silicon's ability to produce a signal for DIOS. However, these properties do not appear to be influenced by the same morphological features and the two events are both created by a similar optical absorption event. It was also found that the mass spectra generated with DIOS is less dependent on the wavelength of the laser. The lack of wavelength dependent signal generation is because instead of the matrix needing to absorb the energy of the laser (which is highly dependent on the molecular structure of the matrix) the porous silicon needs to absorb a photon that exceeds the band gap of crystalline silicon (1.1 eV). The DIOS process, like the photoluminescence of porous silicon, is affected by pore morphology. It was found that the pore size and the porosity of the silicon must be large enough to provide for an efficient surface for energy transfer to the analyte. In general the pore size must be larger than 10 nm to provide for an efficient DIOS surface as summarized in Figure 7. It was also found that the solvent is very important in the production of DIOS signal. The solvent dependence is likely due to the surface wetting properties of the solvent, and thus the ability of the solvent to deposit the analyte in a position that is favorable to the DIOS process. Another study also examined the mechanism of ionization for DIOS and also matrix-free desorption from etched carbon surfaces (Alimpiev et al., 2001). It was also found that the surface must be rough to obtain an ion signal; however, it is not required that the surface be porous. Although surfaces with a thick porous layer were found to produce a very good signal, which may be due to the surface structure and not the porosity of the surface. The study used both a 337 nm UV laser and a 3.28 μm IR laser to examine the difference in the ionization mechanisms. The IR fluence needed to desorb/ionize the analyte was approximately 20 times higher than for the UV laser. The IR desorption also required that a solvent layer be present on the surface to provide a mechanism for the light to be absorbed. It was hypothesized that the surface using either laser is heated to the same temperature (1,000 K) although the laser power required for the IR laser to heat the surface was much greater.

Another group also studied matrix-free desorption/ionization mass spectrometry using an IR laser (2.94 μm ER:YAG) and compared these results to those obtained with a UV laser (Rousell et al., 2004). This study examined the effects that different target materials had including aluminum, copper, stainless steel, poly(ethylene), and silicon. The spectra for these surfaces were quite similar despite very different thermal conductivities and IR absorption properties. This suggests that the important factor in matrix-free IR LDI-MS is any solvent molecules that are present in the vicinity of the analyte. The silicon and aluminum targets produced spectra with little low mass interference and good signal intensities. However, the other surfaces produced have a relatively large low mass background, which was suggested to be due in part to the fragmentation of the analyte (insulin). Analyte fragmentation was widely observed for analytes using the IR

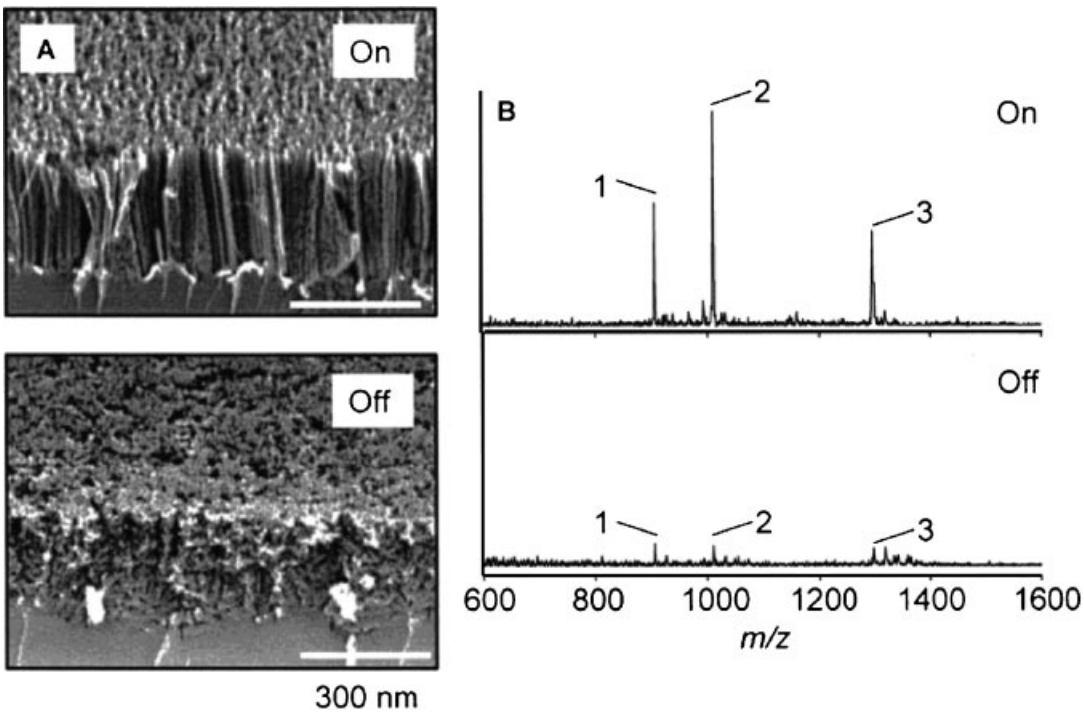

FIGURE 7. **A**: Cross-sectional SEM images on and off Au-patterned spots of p+-PSi showing differences in pore size and overall porosity. The wafer is tilted 30° from normal to reveal both top surface and the cross section. **B**: Mass spectra generated from a 1.5-pmol mix of des-Arg1 bradykinin (1), R-Bag Cell Peptide1-8 (2), and angiotensin I (3) on and off the Au-patterned area show the extent to which pore morphology dictates DIOS performance. Reprinted with permission from Kruse et al. (2001a). Copyright 2001 American Chemical Society.

laser for irradiation. The IR laser fluence that was required to analyze larger molecules (i.e., cytochrome c) was found to be an order of magnitude higher than that required for analysis of smaller analytes, and the upper mass limit for analysis of biomolecules was approximately 17 kDa. The detection limit with the matrix-free IR method was found to be 10 times higher than with a similar UV-MALDI method.

Fully understanding the analysis process for MALDI and DIOS involves not only the desorption and ionization process, but also any chemistry that the analyte undergoes within the ionization plume. One group recently examined the reduction of organic dyes in the analyte plume for MALDI and DIOS (Okuno et al., 2004). Reduction of the analyte in the plume is important because it makes structural determination very difficult. These processes also give insight into the processes that the analyte undergoes when it is desorbed and ionized. Dyes that were easily reduced were analyzed whereas different amounts of Cu(II) were added as an electron scavenger. The addition of the copper suppressed the reduction of the dye in MALDI, which suggests that the reduction is due to electron transfer from the target surface to the analyte. The reductions of the dye were more pronounced in DIOS as compared to MALDI. In addition, using copper did not reduce the reduction reactions in DIOS, which the authors attribute to an ample supply of electrons when the porous silicon DIOS plate is used.

IV. APPLICATIONS OF MATRIX-FREE LDI-MS

A. Forensics

One area that DIOS has been applied to is forensics. In particular, it has been used to analyze ethoxylate polymers present in a contraceptive lubricant (Thomas et al., 2001a). The rational of using DIOS to analyze contraceptive polymers in a forensic environment is that DIOS has a high tolerance for contaminants and also has a low background for the low molecular weight *m/z* region. The polymers that were present in the contraceptive included nonoxynol-9, octoxynol-9 (these two compounds are both spermicides), and PEG polymers of various molecular weights. The DIOS technique was also applied to the analysis of cotton swabs collected from both male and female users of the product. The technique was then applied to an actual sexual assault case (Shen et al., 2004b). This case had no sources of DNA, however DIOS analysis of swabs from the suspect and from the victim (extracted with methanol) both contained traces of nonoxynol-9, PEG 1000, and PEG 1450. These studies demonstrate that DIOS is applicable to very complex samples and will produce data that is legally defensible.

B. Drug Analysis

One area of research that has attracted a significant amount of interest for matrix-free LDI-MS is in the area of drug analysis. The analysis of small molecules like drugs and other biologically important small molecules is one of the strengths of matrix-free LDI-MS methods. The applications are for the basic research into biological processes and for drug development and also for forensic identification of illicit drugs. One application of DIOS for drug development is in determining enantiomeric excess of a synthetic process (Shen et al., 2001b). While the initial report of determining enantiomeric excess was used for only secondary alcohols, it has the promise of being useful for use in high throughput applications in which the optimal reaction conditions can be quickly and easily determined (Finn, 2002).

One study demonstrated the use of atmospheric pressure DIOS as a technique to analyze for small molecules, particularly biological and drug molecules such as testosterone and midazolam (Huikko et al., 2003). It was shown that compounds with high proton affinity, especially amino containing groups, are efficiently ionized in a DIOS system. The linear dynamic range was three orders of magnitude and the analysis was reproducible, which demonstrates that the AP-DIOS system is applicable for quantitative analysis of small biological molecules and drugs. Another study then extended the use of AP-DIOS to the forensic analysis of drug seizures, particularly for the analysis of amphetamines and fentanyls (Pihlainen et al., 2005). The AP-DIOS method was selected to provide for a rapid analysis of illicit drug seizures. The analytes were also subjected to MS/MS analysis to identify the analyte. The detection limits for the amphetamines and fentanyls by AP-DIOS-MS/MS was found to be in the range of 1–3 pmol, which was 5–10 times lower than a comparable AP-MALDI-MS/MS method. The study also describes the analysis of an ''authentic'' sample seized by the Finnish Police. The sample was an Ecstasy tablet, the active component was determined to be 3,4-MDMA. No other major interfering or active components were identified. A seized powder containing 3-MF was also analyzed by the AP-DIOS-MS/MS method. The powder was found to contain 3-MF at a concentration of 0.3% or 0.6 μg/ml.

Another application of matrix-free methods has been to directly record tetracycline antibiotics separated on a TLC plate onto a particle suspension and read them in a LDI-MS instrument (Crecelius et al., 2002). The separation of the tetracycline antibiotics was performed on silica gel that was prepared on aluminum plates, the mobile phase was a mixture of dichloromethane, methanol, and water (59:35:9, v/v). The analytes were eluted for a distance of 7 cm and were visualized using a UV lamp. The developed TLC plate was attached to a MALDI plate and particle suspensions were applied to the TLC strip. A number of different particle suspensions were compared including cobalt, TiN, TiO_2, graphite, and silicon particles. The diameters of the particles ranged in the 20–40 nm range for cobalt and TiN up to 40 μm for silicon. The technique was also compared to typical organic matrices for comparison. The best data was collected from the suspension of graphite particles in ethylene glycol.

One application of DIOS was to analyze catecholamines, which are important neurotransmitters and perform essential functions in the central nervous system (Kraj et al., 2003). DIOS was applied to analysis of these compounds to demonstrate that the technique could be used in the analysis of important biological analytes. The technique was used to analyze dopamine and norepinephrine in the pmol/spot levels. It was also suggested that this technique would be useful in metabolomics, which is the study of metabolites and metabolic pathways in a biological organism.

C. Protein Analysis

One of the most important areas for matrix-free LDI analysis is in the analysis of biological compounds. Proteins are of particular interest because they are important in determining cellular processes and including disease processes. One article demonstrated the variety of analysis that can be performed on proteins using DIOS (Thomas et al., 2001b). First, several enzymes were analyzed, although proteins >20,000 *m/z* could not be desorbed, and the most useful mass range was <3,500 *m/z*. The enzymatic activity of enzymes was monitored by analyzing for the product and enzyme kinetics could be determined. Tryptic digests were analyzed and used to identify proteins. It was found that the addition of ammonium citrate to the biological materials helps to reduce contaminant interferences present in biological solutions. Another study used perflorinated surfactants to clean up tryptic digests prior to DIOS analysis and identification of the peptide fragments (Nordstrom et al., 2006). The use of DIOS to determine enzymatic activity was also further expanded to obtain high throughput screening information (Shen et al., 2004a), including DIOS plates with 96 and 384 spots to accomplish high throughput screening of enzyme activity and inhibition. There has also been some interest in using enzymes immobilized on a DIOS plate to facilitate on-probe tryptic digestion of proteins (Xu et al., 2004). This was accomplished by functionalizing the DIOS surface with 3-aminopropyl trimethoxy silane. The amino-functionality was then modified with 2,4,6-trichloro-1,3,5-triazine. Finally, trypsin is added to the wafer, where an amino group attaches to the surface. The V_{max} of the trypsin immobilized on the chip was 1.0 μM/s and the K_m was found to be 0.0053 mM. These values are very close to those determined for free trypsin. The chip was then used to digest proteins such as cytochrome c and identify protein from the digest fragments. However, in some cases matrix was added to improve the ionization efficiency of the digested peptides.

One article compared the quality of the data from DIOS TOF/TOF MS with that obtained from ESI-MS/MS (Go et al., 2003a), which focused on the analysis of proteins, in particular a BSA digest, (cimetidine and chlorpromazine). These biological analytes were analyzed with tandem MS to aid in the identification, and was compared to tandem ESI-MS data. The DIOS-MS and MS/MS data is shown in Figure 8. This data in the article show the quality of the DIOS data to be superior, particularly for the second mass spectrometric dimension.

One of the disadvantages of performing analysis of biological samples is that there is typically a great deal of sample preparation that must be done to analyze it. One approach to minimize the amount of sample preparation required is to directly analyze a whole cell. The analysis of a whole cell was demonstrated in the direct analysis of *Aplysia californica* atrial gland cells (Kruse et al., 2001b). The analysis of cells was accomplished by first slicing tissue sample and applying them onto the porous silicon surface by blotting, which was then followed by washing the sample with a methanol and ammonium citrate solution, which released the proteins from the cell onto the DIOS surface. The method was used to identify several proteins from the cells, and was useful despite the thickness of the analyzed cells (approximately 1 μm thick).

D. Analyte Capture

The ultimate application of matrix-free LDI-MS methods is to combine it with other powerful analytical techniques, such as analyte capture embodied by SELDI-TOF MS (Merchant & Weinberger, 2000). The SELDI approach is made possible by combining a matrix-free surface and then modifying the surface to allow analyte capture (Hutchens & Yip, 1993). The SELDI approach was introduced in two articles in 2004 by the Siuzdak group. The first of these articles silated the oxidized porous silicon using hydrophobic silanes to produce a surface that is resistant to oxidation and hydrolysis, and also provides a surface that allows analytes to absorb via hydrophobic interactions (Trauger et al., 2004). The SELDI approach described provides a convenient approach to sample clean up on the DIOS chip. The modification of the porous silicon was performed using derivitization procedures similar to those used for chromatographic stationary phases. This approach was used to produce a variety of modified surfaces including trimethylsilyl, amine, C_8, C_{18}, and perfluorinated surfaces. The attachment of these functional groups was accomplished simply by placing a small amount of the silating reagent on the porous silicon surface and incubating for 15 min. The surface of the modified surface was soaked in methanol prior to use to remove any hydrophobic species. The sample was then spotted onto the sample plate and then re-aspirated. The spotting/aspiration step was repeated to maximize the sample loading. The technique was demonstrated for the extraction of various proteins and peptides from solution, which included des-Arg^9-brdykinin, for which the detection was demonstrated for 0.8 zeptomoles. It was also demonstrated that the method is useful for monitoring of the inhibition of proteins, as the data shows in Figure 9. The second article which demonstrated analyte capture by tailoring the surface using azide and alkyne cycloaddition (Meng, Siuzdak, & Finn, 2004b). These surfaces were able to capture sample azides with a biotinylated alkyne, which could then capture avidin and then analyze the analyte using DIOS. The analyte capture method was used to probe the omega class glutathione transferase (GSTω). This method was used to covalently label and identify the active site of GSTω.

E. Polymer Analysis

One area in which MALDI has been widely applied is the analysis of polymers. The analysis of polymers by MALDI is very important because unlike ESI-MS methods the analysis of polymers by MALDI produces a single mass, giving a very good indication of the molecular weight and polydispersity of a polymer sample. The area of using MALDI for polymer analysis has been recently reviewed (Murgasova & Hercules, 2003). MALDI is however unsuitable to analysis of low molecular weight polymers and for analysis of low molecular weight components of polymer mixtures. DIOS in particular has been applied to the analysis of polymers. One study used DIOS to study low molecular mass polyesters (Arakawa et al., 2004). This study directly compared the analysis of low molecular weight polyester components analyzed by MALDI and by DIOS. This found that the DIOS method produced a

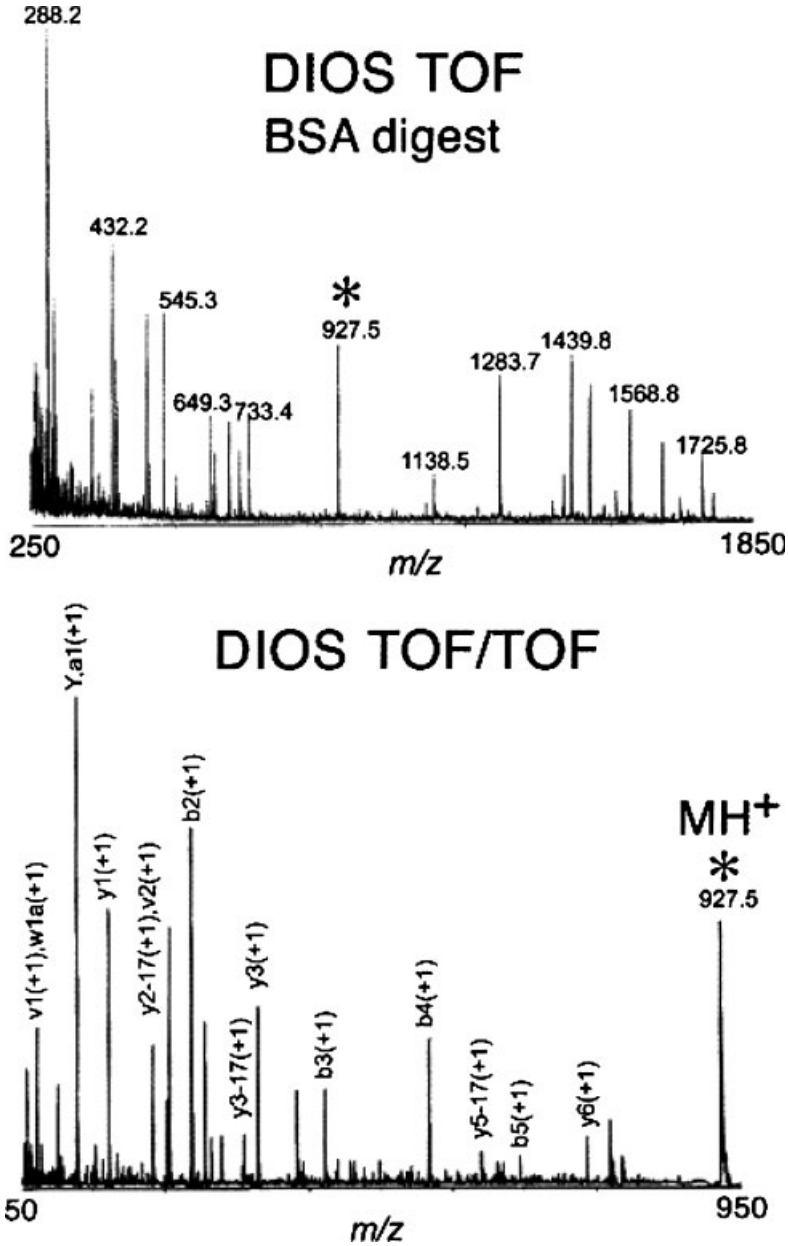

FIGURE 8. DIOS TOF/TOF spectrum of BSA digest and the TOF/TOF fragmentation mass spectrum of the singly charged ion, MH+ = 927.5. Reprinted with permission from Go et al. (2003a). Copyright 2003 American Chemical Society.

mass spectrum that was simpler and easier to analyze because of the absence of low molecular weight matrix ions. This absence of the low molecular weight matrix ions also allowed for the rapid optimization of the analysis procedures for the analyte. Another recent study used DIOS to quantitatively analyze poly(propylene glycol) mixtures (Okunoa, Wada, & Arakawa, 2005). The actual PPG polymers analyzed were mixtures of diol and triol structures. The careful selection of analyte concentration and solvent allowed for reliable quantitative analysis of the polymer mixtures. In fact the DIOS method was found to be more suitable to quantitative analysis of the polymer mixtures than MALDI because the standard deviation of the weight percent of the different polymers was much lower with DIOS.

F. Solid Phase Synthesis Applications

It is important to note that DIOS methods are becoming mature enough to allow them to be applied to a variety of useful and interesting applications. One recent application attached a photocleavable Diels-Alder reagent on the surface of the DIOS chip (Meng et al., 2004a). This compound could then capture azides on the silicon surface. Analysis by DIOS causes the Diels-

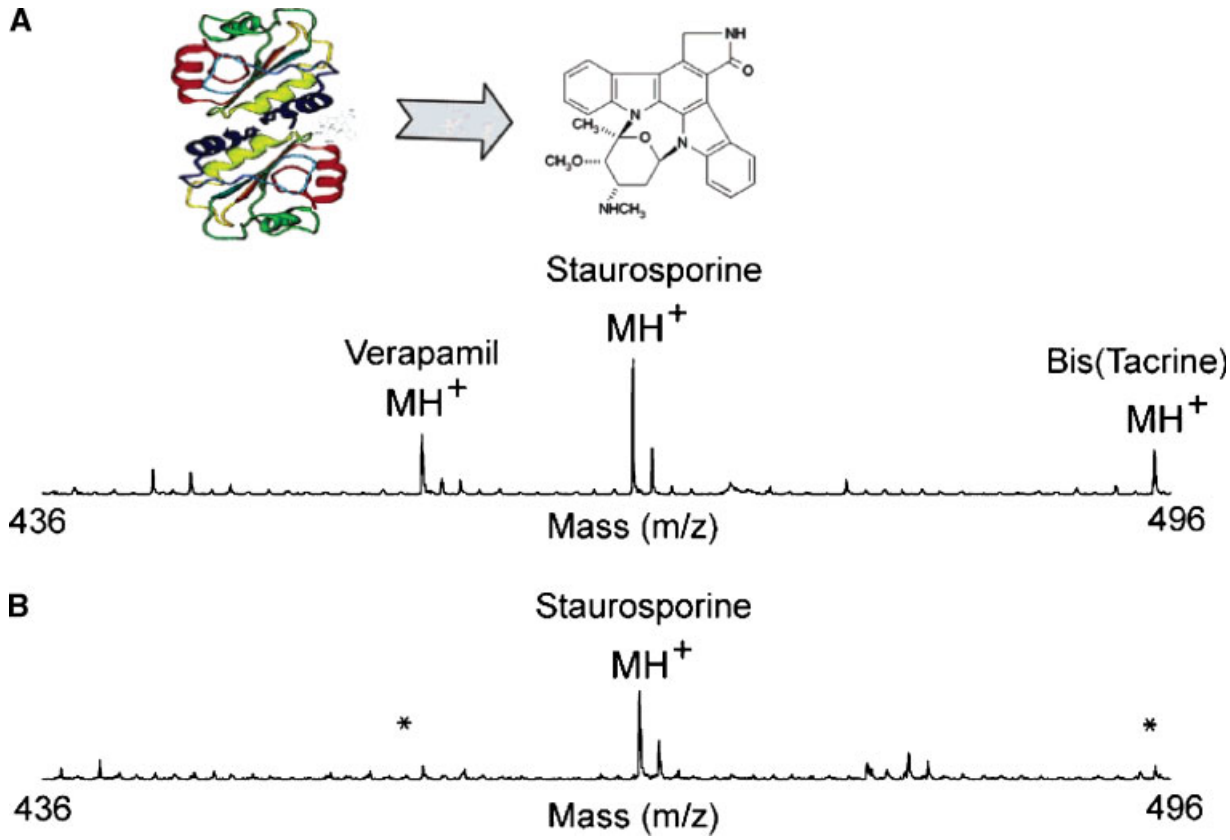

FIGURE 9. Monitoring of enzyme inhibitors with DIOS-MS following sample cleanup: Rho-kinase II, staurosporine (a potent kinase inhibitor), verapamil, and bis(tacrine) before (**A**) and after (**B**) passage through a 10,000 MW cutoff filter in the presence of a stabilizing buffer allowing for the identification of a known inhibitor. Reprinted with permission from Trauger et al. (2004). Copyright 2004 American Chemical Society.

Alder structure to undergo retro-fragmentation, which allows the probe to be analyzed directly by mass spectrometry, bypassing workup steps that are normally required when analyzing photo-clevable groups in sold-phase synthesis. This method was used to probe the sold-phase selectivity of enzyme-catalyzed reactions.

G. Sample Prep Methods

One of the strengths of matrix-free methods is that they can greatly simplify sample preparation that must be performed prior to analysis of a sample. One study demonstrated a sol-gel supported surface doped with diaminobenzoic acid as the surface (Chen & Chen, 2003). The sol-gel part of the surface was able to desalt oligionucleotide samples. This sample was useful for samples that contained SDS at a concentration up to 0.1%. Another approach that has been used for sample preparation used magnetic nanoparticles to concentrate peptides from a digest mixture (Chen & Chen, 2005). The nanoparticles were then used as effective SALDI matrix. The same group also used a molecularly imprinted sol-gel for sample preparation (Chen & Chen, 2004b). The selective selection of a cyclodextrin was accomplished by imprinting the sol-gel material and spin coating it onto the sample plate, which demonstrates a matrix-free method is useful for molecular recognition applications.

One of the difficulties that has been observed in sample preparation for MALDI as well as for matrix-free methods is that when a sample is spotted on a sample plate and analyzed there are often areas of the sample plat that are more productive than others. These 'hot spots' makes analyzing samples difficult, particularly if an automated instrumental method is used to analyze multiple spots. One approach that has been introduced to minimize the problem is electrospray deposition of the analyte on the sample plate, including for DIOS samples (Go et al., 2003b). Electrospray deposition of the analyte is accomplished by pumping the solution through a capillary and applying a voltage to the solution (1.5–1.7 kV), as illustrated in Figure 10. The technique produces a stable electrospray between the capillary and the DIOS plate. This sample preparation method produces a sample spot that is much more reproducible (<7% RSD) than the typical dried droplet method of preparing samples (<18% RSD). The improved sample reproducibility makes it more appropriate for quantitative analysis and allows for quantization down to a lower analyte concentration.

V. FUTURE DIRECTIONS

The application of matrix-free methods has been shown to be very useful for a wide range of applications. However there are

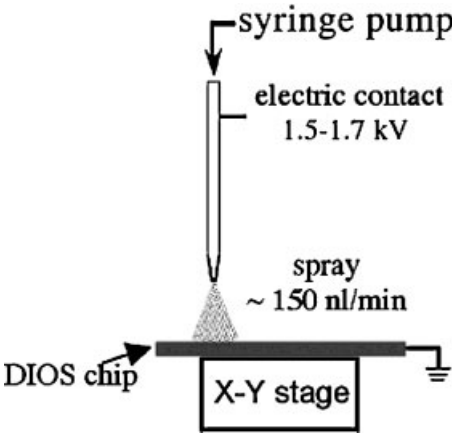

FIGURE 10. Schematic diagram of the electrospray sample deposition setup. Reprinted with permission from Go et al. (2003b). Copyright 2003 American Chemical Society.

still several areas in which they can be improved. Matrix-free methods are typically limited to relatively low molecular weight analytes (<3,000 *m/z*). There should be an effort to extend the range of molecular weight that these methods can analyze for. If the mass range could be extended to 50,000 or 100,000 Da, matrix-free methods would be suitable for most biological proteins of interest. Improving this range will likely require making target surfaces modified with light absorbing and acidic species so that the energy transfer and the ionization efficiency are improved. There may also be some advantage to moving to IR lasers, as they have been shown to analyze biomolecules up to approximately 17 kDa. There is likely to also be very keen interest in modifying targets so that matrix-free SALDI can be performed, which has been demonstrated and was discussed in this review. However more surface chemistries will need to be evaluated and studied to demonstrate the usefulness of analyte capture on matrix-free target plates and tailor the plates for specific applications. In recent years there has also been intense interest in interfacing liquid separations (i.e., LC, CE, etc) with MALDI (Foret & Preisler, 2002). These techniques would be even more useful if they would be performed using sample targets that do not require matrix to be mixed with the solvent used in the separation technique. Finally, in MALDI methods there is a shift to using atmospheric pressure (AP) ionization. The move to AP instrumentation is to make the sample preparation and analysis easier for the typical analyst. Matrix-free methods for laser desorption/ionization are an ideal to be combined with AP methods. This would provide a very easy method for sample preparation and analysis for the casual user of LDI-MS methods.

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