

Electrowetting-Based Microfluidics for Analysis of Peptides and Proteins by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

Aaron R. Wheeler,[†] Hyejin Moon,[‡] Chang-Jin "CJ" Kim,[‡] Joseph A. Loo,^{†,§} and Robin L. Garrell^{*,†}

Department of Chemistry and Biochemistry, Department of Biological Chemistry, David Geffen School of Medicine and Molecular Biology Institute, and Department of Mechanical and Aerospace Engineering, Henry Samueli School of Engineering and Applied Science, University of California, Los Angeles, California 90095

A new technique for preparing samples for matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is reported. The technique relies on electrowetting-on-dielectric (EWOD) to move droplets containing proteins or peptides and matrix to specific locations on an array of electrodes for analysis. Standard MALDI-MS reagents, analytes, concentrations, and recipes are demonstrated to be compatible with the technique. Mass spectra are comparable to those collected by conventional methods. Nonspecific adsorption of analytes to device surfaces is demonstrated to be negligible. The results suggest that EWOD may be a useful tool for automating sample preparation for high-throughput proteomics and other applications of MALDI-MS.

The push to sequence the human genome¹ brought an unprecedented level of attention to the field of genomics. In recent years, attention has been turned to the field of proteomics. First conceptualized in the mid-1990s,² proteomics has undergone a meteoric rise in popularity, with more than 2000 papers published in the field in 2003. Proteomics, like genomics, requires methods and instruments capable of collecting, storing, cataloguing, and analyzing vast amounts of information.³ The technological challenges for proteomics may be even greater than those for genomics, given that an organism has a single genome but may express hundreds of different proteomes,⁴ depending on environmental and developmental cues. The development of new methods and instrumentation with the capacity for rapid, high-throughput data collection is crucial for continued progress.

Current standard methods in proteomics rely on the pairing of two technologies: analytical separations (e.g., two-dimensional gel electrophoresis, 2DGE) and mass spectrometry (MS) detection.⁴ One mode of mass spectrometry, matrix-assisted laser desorption/ionization (MALDI) coupled with time-of-flight (TOF) analyzers, has become popular for high-throughput proteomics applications. In MALDI, which was introduced in the late 1980s,^{5,6} a protein sample is cocrystallized with an organic matrix. When the crystal is irradiated with energy of an appropriate wavelength, the sample is simultaneously desorbed and ionized.

The sample array geometry of most MALDI-MS systems makes it appealing for high-throughput proteomics applications. However, typical proteomics analyses require many steps; a crucial step is mixing the sample with matrix. Repetitive pipetting of reagents onto MALDI targets is time-consuming and can lead to sample loss, dilution, and contamination. High-end commercial instruments utilize robotically controlled deposition,⁷ but such instruments are expensive and require careful maintenance. Other methods for high-throughput deposition of sample and matrix include using lithographically patterned targets,^{8–10} microfabricated picoliter droplet delivery devices,^{11–15} or microfluidic channels.^{16–18} Of these methods, only patterned targets,^{8–10} which

* To whom correspondence should be addressed: (e-mail) garrell@chem.ucla.edu; (tel) (310) 825-2496; (fax) (310) 206–2061.

[†] Department of Chemistry and Biochemistry.

[‡] Department of Mechanical and Aerospace Engineering, Henry Samueli School of Engineering and Applied Science.

[§] Department of Biological Chemistry, David Geffen School of Medicine and Molecular Biology Institute.

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facilitate easier spot deposition but do not eliminate pipetting, have gained widespread use.¹⁹

We present here a new method for cocrystallizing sample and matrix for MALDI-MS. The method utilizes a solution handling technique based on a phenomenon that we have called electro-wetting-on-dielectric^{20–27} (EWOD). In EWOD, the local wettability of a surface is reversibly changed by applying potentials between electrodes buried beneath hydrophobic, dielectric layers. By applying a sequence of potentials to adjacent electrodes on an array, aqueous droplets can be made to travel across the surface. Several configurations of EWOD-based devices have been reported, including single-plate open air devices,^{28,29} parallel-plate devices filled with silicone oil,^{30–33} and parallel-plate open-air devices.^{20–27} EWOD-based devices are reconfigurable and can handle neutral and charged analytes, particulates,²³ and proteins.²⁴ We recently presented the design parameters required for open-air devices to dispense, merge, mix, and cut droplets in air.²⁵ We have further developed a method to realize EWOD actuation across a two-dimensional plane (rather than simply across one or two rows of electrodes)²⁶ and have used this technique to create a fully portable microfluidic device.²⁷

EWOD should be well-suited to MALDI, as both techniques rely on array geometries. This stands in contrast to channel-based microfluidic devices for MALDI-MS,^{16–18} which require rastering or complex networks of holes to mate with MALDI-MS targets. We describe here the fabrication and use of EWOD devices for MALDI-MS analysis of peptides and proteins. This is the first report of using droplet-based digital microfluidics for proteomics or mass spectrometry applications.

EXPERIMENTAL SECTION

Reagents and Materials. All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. Stock solutions of analytes, including bovine insulin (100 μM), bovine insulin chain B (40 μM), horse heart cytochrome *c* (14.5 μM), and horse skeletal myoglobin (59 μM), were prepared in deionized (DI) water or with 0.2% trifluoroacetic acid (TFA). Stock solutions were kept frozen; working solutions were diluted and used within 1 day. Working solutions of matrixes, including 2,5-dihydroxybenzoic acid (DHB), ferulic acid (FA), and sinapinic acid (SA), were prepared in DI water containing TFA and acetonitrile and

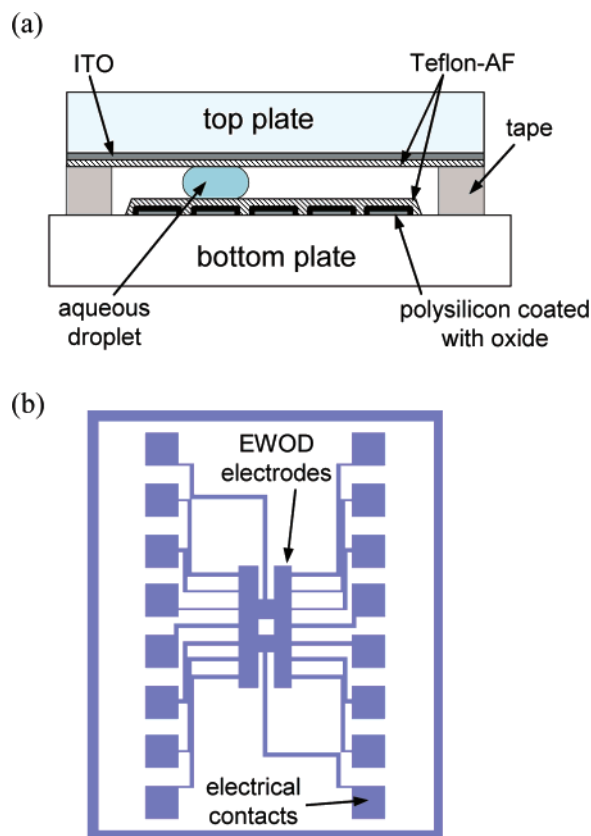


Figure 1. Side view (a) and top view (b) schematics of EWOD device (not to scale). The bottom plate served as the base for a pattern of polysilicon EWOD electrodes (1 mm², 4- μm gap) buried under thermal oxide. A top plate was formed from ITO on glass; both plates were coated with Teflon-AF. The plates were joined with double-sided tape as a spacer.

were used within 1 day. Solutions of acetonitrile and water (5, 10, 15, 25, and 50% acetonitrile, v/v) for contact angle tests were prepared and used within 1 day.

Various working concentrations of analytes and matrixes were evaluated for the feasibility of moving droplets by EWOD. The following concentrations were used to obtain the results presented here: insulin (1.75 μM , 0.025% TFA), insulin chain B (2 μM , 0.025% TFA), cytochrome *c* (1.85 μM , 0.025% TFA), myoglobin (1.45 μM , 0.0125% TFA), DHB (10 mg/mL, 0.05% TFA, with 5% acetonitrile), FA (3 mg/mL, 0.0375% TFA, with 15% acetonitrile), and SA (10 mg/mL, 0.1% TFA, with 33% acetonitrile).

Teflon-AF 1600 resin was purchased from DuPont (Wilmington, DE). Working solutions of 6% (w/v) were formed in Fluorinert FC-40 solvent; solutions were used as made or diluted (v/v with FC-40). Cleanroom reagents were used as provided by the UCLA Nanofabrication Facility.

Fabrication and Use of EWOD Devices. EWOD devices were fabricated at the UCLA Nanofabrication Facility and were similar to devices we have used in the past.^{20–23,25} As depicted in Figure 1, each device was formed from a bottom plate with individually addressable electrodes and a top plate with one contiguous electrode.

The bottom plate was formed from quartz wafers coated with $\sim 3500\text{-}\text{\AA}$ phosphorus-doped polysilicon (purchased from the Stanford University Nanofabrication facility). Polysilicon electrodes were patterned by photolithography and reactive ion etching,

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Thermal oxide (1500 Å) was grown on the polysilicon in an oxidation furnace. Holes through the oxide to the electrical contacts were formed with photolithography and wet etching with buffered hydrofluoric acid. The devices were then primed with hexamethyldisilazane vapor and spin-coated (2000 rpm, 60 s) with 5% Teflon-AF. The devices were postbaked on a hot plate (160 °C, 10 min) and in a furnace (330 °C, 30 min) to form a uniform $\sim 7500\text{-}\text{\AA}$ layer of Teflon-AF.

The top plate was formed from indium–tin oxide (ITO)-coated glass pieces (Delta Technologies, Ltd., Stillwater, MN). A $\sim 150\text{-}\text{\AA}$ layer of Teflon-AF was spin-coated (0.5%, processed as above) onto the ITO-coated glass. The two plates were joined with spacers ($\sim 300\text{ }\mu\text{m}$) formed from three pieces of double-sided tape. A typical EWOD pattern (Figure 1b) consisted of 16 1-mm^2 electrodes ($4\text{-}\mu\text{m}$ gap between electrodes) connected to electrical contact pads.

Aqueous droplets ($0.5\text{ }\mu\text{L}$) were sandwiched between the two plates and were moved by applying ac potentials (1 kHz, 75 V_{rms}) between the electrode in the top plate and successive electrodes in the bottom plate, as described elsewhere.^{20–23,25} Once the solution composition had been optimized (described below), droplet movement was facile and fast. Droplet movement was monitored and recorded by a CCD camera (Panasonic, Secaucus, NJ) mated to an imaging lens (Edmund Industrial Optics, Barrington, NJ) positioned over the top of the device.

Fabrication and Use of Contact Angle Test Substrates. To determine the solvent compositions that could be used for droplet translation on EWOD devices, unpatterned test substrates were used in conjunction with a contact angle goniometer (First Ten Ångströms, Portsmouth, VA) as described elsewhere.^{24,34,35} The test substrates, which were much simpler to fabricate than the patterned EWOD devices, were formed from pieces of phosphorus-doped silicon wafer coated with thermal oxide and Teflon-AF (as described above). For electrowetting experiments, a test substrate served as the bottom electrode, and a $100\text{-}\mu\text{m}$ -diameter platinum wire (Aldrich, Milwaukee, WI) served as a top electrode. As a first step, contact angles of $5\text{-}\mu\text{L}$ droplets of DI water were measured with (active) and without (resting) ac potentials applied. The contact angle change between active and resting substrates was noted; the lowest voltage at which the contact angle change was maximized (contact angle saturation)^{24,34–36} was used for subsequent experiments. Contact angle changes for $5\text{-}\mu\text{L}$ droplets of each solvent mixture (5, 10, 15, 25, and 50% acetonitrile in water) were then measured.

Mass Spectrometry. After droplet movement, EWOD devices were stored in a chamber under house vacuum; $0.5\text{-}\mu\text{L}$ droplets dried in $\sim 1\text{--}2$ min. Matrix and sample cocrystals were imaged by light microscopy. Typically, several spots were deposited on each EWOD device. When deposition was complete, the bottom plate of the EWOD device was affixed with double-sided tape into a 1-mm -deep milled-out groove on a standard stainless steel MALDI target.

A Voyager DE-STR-TOF mass spectrometer (Applied Biosystems, Foster City, CA) was used to collect MALDI-MS data.

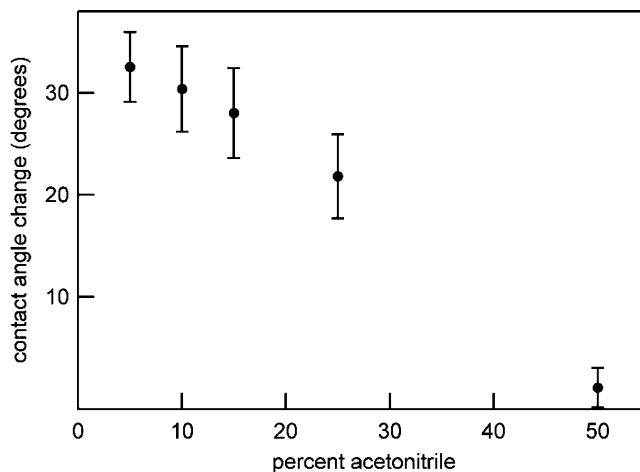


Figure 2. EWOD-driven contact angle changes of $5\text{-}\mu\text{L}$ aqueous droplets with varying amounts of acetonitrile. Error bars are ± 1 SD.

Typically, 500 shots were collected per spectrum, with the laser power adjusted for different matrixes. Data were normalized to the protein analyte peak; some data were baseline subtracted, smoothed with a running average of 15 points, or both. Data were analyzed with Voyager Data Explorer (Applied Biosystems and Igor Pro (Wavemetrics, Lake Oswego, OR). All MALDI data were replicated at least two times. To compare the new technique with conventional MALDI, five identical spots were prepared (insulin deposited first, followed by DHB) on an EWOD device and on a stainless steel MALDI target. The spectra were evaluated for rms noise, signal-to-noise (S/N), and resolution.

RESULTS AND DISCUSSION

Suitability of Solvent for EWOD-MALDI. We have previously demonstrated EWOD-driven translation, cutting, merging, and dispensing of droplets of DI water.^{20–23,25,26} Many potential applications of EWOD require the use of organic solvents. For example, acetonitrile is often used to increase the solubility of matrixes for MALDI-MS. Some matrixes, such as DHB, require little or no acetonitrile, while other matrixes, such as SA, require significant concentrations of acetonitrile. To determine which matrixes would be compatible with EWOD-MALDI-MS experiments, contact angles for droplets containing various concentrations of acetonitrile were evaluated using single electrode test substrates. Experience has taught us that if a change in contact angle of $\geq 25^\circ$ is observed between resting (no potential applied) and active (potential applied) electrodes on a test substrate, droplets of the same composition can be moved in open-air patterned EWOD devices.

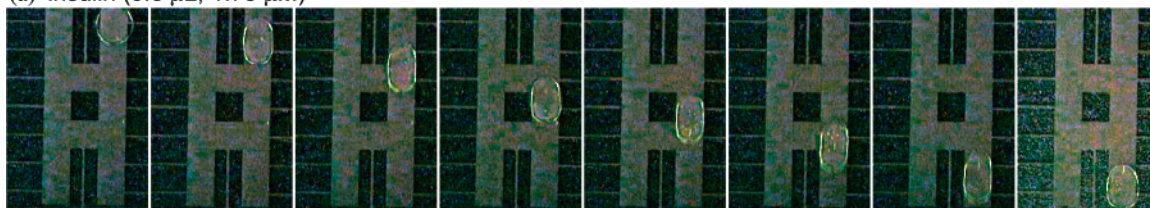
As a baseline, droplets of pure water were evaluated—contact angle changes of $\sim 35^\circ$ were observed upon application of voltage. As shown in Figure 2, droplets with acetonitrile concentrations of up to 15% exhibited a contact angle change of $\geq 25^\circ$. Higher concentrations of acetonitrile exhibited contact angle changes too low for droplet translation by EWOD. Thus, droplets composed of up to 15% acetonitrile were used for the EWOD experiments presented here. This enabled the use of the MALDI matrixes DHB and FA. SA, which requires at least 33% acetonitrile to dissolve, could not be moved by EWOD. It should be noted that the limit on acetonitrile content affects the crystallization process, which may in turn affect spectral quality.

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(a) insulin (0.5 μ L, 1.75 μ M)



(b) ferulic acid (0.5 μ L, 3 mg/mL)

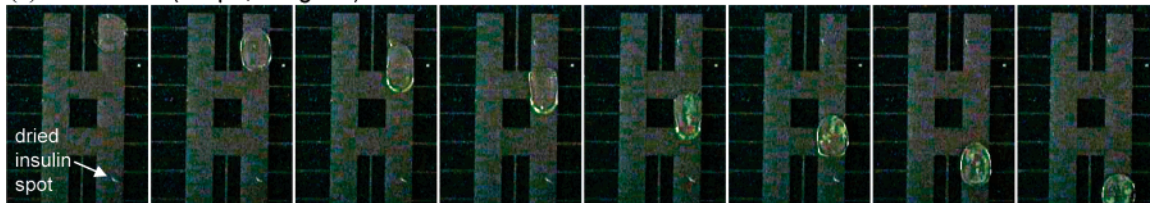


Figure 3. Video sequence (left to right) of EWOD-driven movement of 0.5- μ L droplets of insulin and FA down the right side of the electrode pattern. In (a), an insulin droplet (1.75 μ M, in 0.025% TFA) was moved and dried. In (b), a droplet of FA (3 mg/mL, in 0.0375% TFA, with 15% acetonitrile) was driven onto the dried insulin spot. Movies of these sequences can be found online in the Supporting Information.

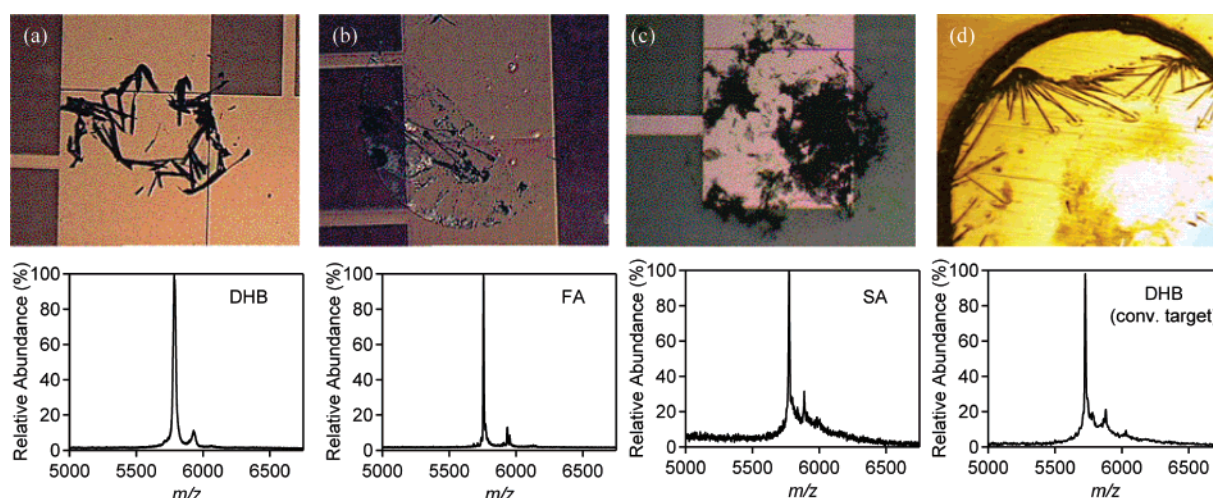


Figure 4. Images and MALDI mass spectra of insulin (5733 Da) cocrystallized with (a) DHB, (b) FA, and (c) SA on the surface of an EWOD device. For each trial, a 0.5- μ L droplet of insulin was moved by EWOD to a designated electrode and dried. In (a) and (b), insulin deposition was followed by EWOD-driven deposition and drying of a 0.5- μ L droplet of matrix. In (c), insulin deposition was preceded by manual deposition of a 0.5- μ L droplet of SA. The image and spectrum in (d) were obtained by manually pipetting and drying 0.5- μ L droplets of DHB followed by insulin on a conventional stainless steel target.

EWOD-Driven Droplet Movement. The primary goal for this work was to develop EWOD devices for deposition of matrix and protein/peptide samples for MALDI-MS. Figure 3 represents a four-step droplet movement experiment on an EWOD device: (1) a droplet of insulin was moved to a designated electrode (Figure 3a); (2) the droplet was allowed to dry; (3) a droplet of FA was moved to the dried spot (Figure 3b); and (4) the droplet was allowed to dry. Droplets were routinely driven on and between each line of electrodes on each device. Sometimes, matrixes were observed to precipitate during or prior to droplet movement. This phenomenon, caused by evaporation, created difficulties for EWOD-driven droplet movement. To determine acceptable droplet compositions, the reagents were diluted until there was consistently no interference to droplet movement from precipitation. We performed several variations of this procedure with different reagents, as described below.

Mass Spectrometry. MALDI-MS was used to analyze spots of protein and matrix prepared by EWOD. Images and mass spectra of insulin cocrystallized with DHB, FA, and SA are shown

in Figure 4. The appearance of crystals on EWOD devices (Figure 4a–c) was similar to those formed on a standard stainless steel target (Figure 4d). Likewise, the EWOD-MALDI spectra of insulin exhibit the expected strong signal at 5.7 kDa, with similar peak shape as for spectra collected from a standard target. The small peaks at higher mass were usually observed and were likely caused by the matrix forming a photochemically induced adduct with the peptide.³⁸

DHB (Figure 4a) and FA (Figure 4b) spots were prepared as shown in Figure 3—an insulin droplet was moved and dried, followed by a droplet of matrix. DHB, which is known as a “universal matrix” used for peptides, proteins, nucleotides, and synthetic polymers,³⁷ was found to work quite well with EWOD. Because SA requires more than 15% acetonitrile for solubility, SA-

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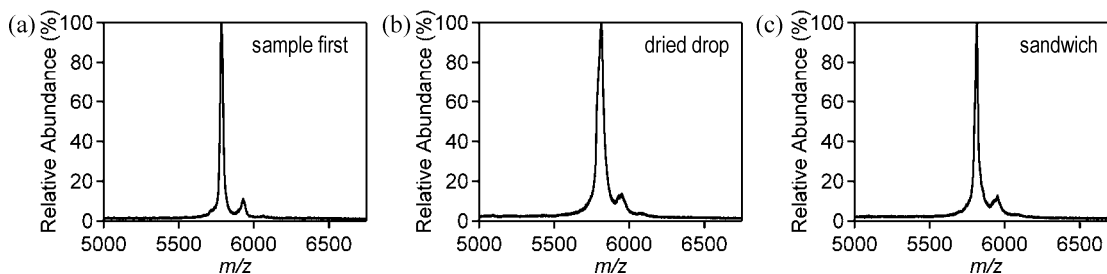


Figure 5. MALDI mass spectra of insulin cocrystallized with DHB. In (a), a droplet of insulin was deposited first, followed by a droplet of DHB. In (b), individual droplets of insulin and DHB were merged and the combined droplet was dried. In (c), a droplet of DHB was deposited, followed by a droplet of insulin, and then another droplet of DHB.

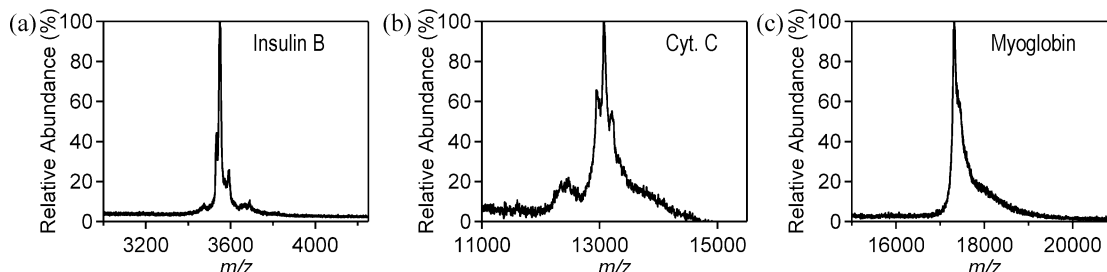


Figure 6. MALDI mass spectra of (a) insulin chain B (3495 Da), (b) cytochrome *c* (12.4 kDa), and (c) myoglobin (16.9 kDa). For each spectrum, a 0.5- μ L droplet of peptide or protein was moved with EWOD and dried, followed by DHB.

containing droplets were unmovable by EWOD. Thus, SA spots (Figure 4c) were deposited manually followed by EWOD-driven movement and drying of an insulin droplet. This result demonstrates that if matrixes that are not water soluble are desirable, the technique of precoating a high-throughput target with matrix¹⁵ could be used for EWOD-MALDI devices.

For a comparison of EWOD-MALDI to conventional MALDI, five spots were prepared by depositing insulin and then DHB on an EWOD device and on a stainless steel target. The two kinds of spectra had similar S/N (103 ± 43.1 and 136 ± 68.4 for conventional MALDI and EWOD-MALDI, respectively). Conventional MALDI had slightly better resolution (508 ± 62 for conventional MALDI and 275 ± 88 for EWOD-MALDI), but EWOD-MALDI had lower noise (60 ± 10 for conventional MALDI and 29 ± 6 for EWOD-MALDI). No attempt was made to optimize EWOD-MALDI for spectral properties; even so, the EWOD devices proved to be an effective alternative to conventional targets for MALDI-MS. It should be noted that another form of EWOD, in which droplets are suspended in silicone oil,^{30–33} would probably not be suitable for this technique because MALDI signals are severely degraded by the presence of nonvolatile liquids.³⁷

Cocrystallization of sample and matrix for MALDI-MS is process-driven, with many recipes and variants to choose from.^{37,39} We have replicated three common cocrystallization recipes using EWOD, including the following: (1) the “sample first” technique,⁴⁰ for which sample is deposited first, followed by matrix, (2) the “dried drop” technique,⁶ for which sample and matrix are mixed and dried together, and (3) the “sandwich” technique,⁴¹ for which a layer of sample is deposited between two layers of

matrix. Sample MALDI spectra of insulin–DHB created with each of these methods are shown in Figure 5.

Spots formed with the sample first technique (Figure 5a) were prepared as depicted in Figure 3. Spots formed with the dried drop technique (Figure 5b) were prepared by using EWOD to merge a droplet of insulin and DHB and allowing the combined droplet to dry. Prior to drying, the droplet was moved back and forth between electrodes several times, which has been shown to increase mixing efficiency.^{22,32,33} Spots formed with the sandwich technique (Figure 5c) were prepared by using EWOD to deposit a droplet of DHB, insulin, and then DHB again. The signal-to-noise ratios were similar for spectra formed by each technique, with the sample first and sandwich techniques giving a slightly narrower analyte peak. The compatibility of EWOD-MALDI-MS with common matrix/sample preparation recipes demonstrates that EWOD should be useful for many applications of MALDI-MS.

In addition to insulin (Figures 4 and 5), several other proteins and peptides were analyzed with EWOD-MALDI, as shown in Figure 6. Samples with a wide range of molecular weights were probed, including insulin chain B (3495), cytochrome *c* (12 400), and myoglobin (16 900). A close look at Figure 6 reveals that the measured molecular weights for cytochrome *c* and myoglobin were higher than the expected values. This may have been caused by the use of a custom sample target that changes the ion flight path length, by dielectric charging,^{40,42,43} or by a combination of the two factors. In future experiments, EWOD-MALDI mass spectra will be calibrated with known standards.

One concern for methodologies using EWOD for biochemical applications is the potential for molecules such as peptides and proteins to nonspecifically adsorb on the hydrophobic Teflon-AF

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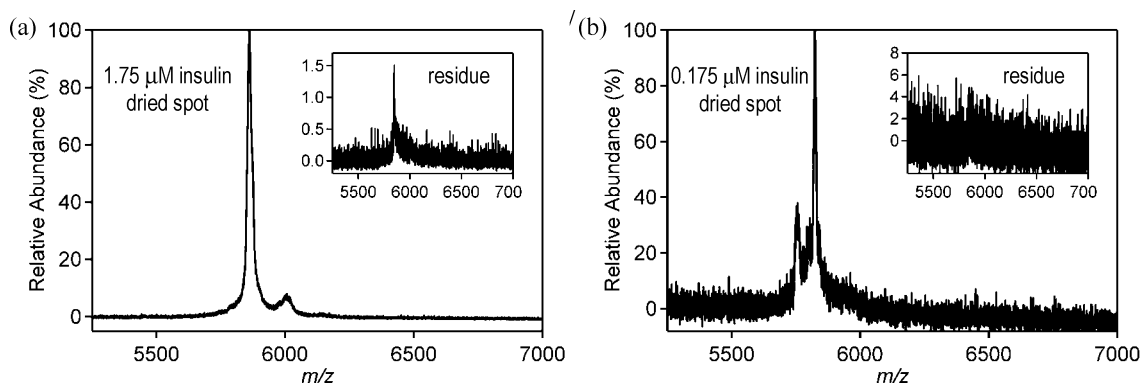


Figure 7. MALDI mass spectra indicating the extent to which insulin is left behind on the EWOD device surface after movement of a droplet of (a) 1.75 μM insulin, and (b) 0.175 μM insulin. The main panels depict spectra from insulin/DHB spots prepared in the same manner as for Figure 5a. The inset panels depict spectra from DHB spots that were deposited onto electrodes over which the insulin droplets were moved. In (a), a small signal from insulin residue with peak height of less than 2% of the main peak is observed; in (b), no residue is detected.

surface. As shown in Figure 7, MALDI-MS proved to be a convenient tool to probe this phenomenon. Two sets of spectra are shown, formed from droplets containing 1.75 or 0.175 μM insulin (Figure 7a and b, respectively). For each concentration, a droplet of insulin was moved across a series of electrodes to a designated point and dried. Two DHB droplets were then moved and deposited—one to the dried spot of insulin (main panels of Figure 7) and one to an electrode over which the insulin had traveled (inset panels). Each inset spectrum was scaled to the parent ion peak of the main panel spectrum. Although MALDI-MS is typically not a quantitative technique, the spectra qualitatively suggest that the level of surface fouling at high concentrations of insulin is low, and the amount of fouling at low concentrations is below the detection limit of the technique. In future work, the effects of nonspecific adsorption on EWOD-MALDI experiments will be evaluated quantitatively. If adsorption proves to be a nuisance, we will use techniques developed previously to reduce its effects.²⁴

CONCLUSION

This work demonstrates that EWOD is compatible with matrixes, samples, concentrations, and recipes typically used for MALDI-MS analyses. The results indicate that EWOD-MALDI-MS has the potential to be useful for high-throughput proteomics analyses. The proteomics strategy employing 2DGE and MS utilizes the measurement of proteolytic peptide fragments as the means for protein identification. Future work will be focused on

integrating EWOD-MALDI with proteolytic digestion. For these experiments, droplets containing trypsin and protein analyte will be merged, incubated at room temperature, and dried, followed by deposition of a third droplet containing matrix. We are also working to extend this technique to devices with dense arrays of electrodes; we anticipate that this technique will be a useful tool for automating sample preparation for high-throughput proteomics analyses.

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SUPPORTING INFORMATION AVAILABLE

Video clips of EWOD-driven movement of droplets of insulin and ferulic acid. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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