An integrated digital microfluidic chip for multiplexed proteomic sample preparation and analysis by MALDI-MS†

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To realize multiplexed sample preparation on a digital microfluidic chip for high-throughput Matrix Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS), several fluidic functions need to be integrated. These include the generation of multiple droplets from a reservoir and parallel in-line sample purification. In this paper, we develop two critical new functions in handling protein solutions and standard proteomic reagents with electrowetting-on-dielectric (EWOD) actuation, leading to an integrated chip for multiplexed sample preparation for MALDI-MS. The first is a voltage sequence designed to generate a series of droplets from each of the three reservoirs—proteomic sample, rinsing fluid, and MALDI reagents. It is the first time that proteomic reagents have been dispensed using EWOD in an air (as opposed to oil) environment. The second is a box-in-box electrode pattern developed to allow droplet passing over dried sample spots, making the process of in-line sample purification robust for parallel processing. As a result, parallel processing of multiple sample droplets is demonstrated on the integrated EWOD-MALDI-MS chip, an important step towards high-throughput MALDI-MS. The MS results, collected directly from the integrated devices, are of good quality, suggesting that the tedious process of sample preparation can be automated on-chip for MALDI-MS applications as well as other high-throughput proteomics applications.

Introduction

Proteomics is the study of the expression, function and interaction of proteins in health and disease, and is a steadily growing field of interest for applications such as drug discovery. Matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) has become a standard method for proteomics.[1,2] A typical sample preparation process for MALDI-MS requires a number of enzymatic and chemical processing steps. A critical step is mixing of the peptides and proteins with the organic matrices required for MALDI. Removing unwanted impurities that may interfere with the MALDI-MS signal is also important. These and other processing steps often are time-consuming and require repetitive pipetting of reagents onto MALDI sample targets, leading to sample loss and dilution as well as sample contamination by unwanted impurities, such as salts or denaturants.

Digital microfluidics involves manipulating liquids as discrete droplets, typically by using an electrically controlled driving mechanism such as electrowetting-on-dielectric (EWOD). Because EWOD is executed on a surface with an array of electrodes, digital microfluidics has the potential to become a good platform for parallel processing of samples for MALDI-MS analysis.[3,4] Standard MALDI-MS reagents and analytes have been shown to be compatible with EWOD actuation,[3] and EWOD has been used for in-line sample purification for MALDI-MS.[4] Our previous work was limited in that samples were deposited by pipetting, and were processed and analyzed serially. To achieve higher throughput, it is essential to be able to generate many droplets from a large reservoir, and to integrate that function on-chip. The capability to create and move droplets with different volumes would be useful to improve the performance and efficiency of in-line sample purification.[4]

In the present work, we demonstrate generation of multiple droplets from enclosed, on-chip reservoirs of proteomic samples, rinsing liquid, and MALDI-MS reagents. The sample droplets are transported to target areas where they are dried. After impurities are removed by rinsing droplets, matrix-containing droplets are delivered and dried and the samples analyzed *in situ* by MALDI-MS. These functions are integrated into a single chip for parallel processing of multiple samples. The integration is made possible for the first time by developing two new techniques over those previously reported[3,4]—a "box-in-box" electrode and an electronically programmed droplet dispenser. The new electrode design allows droplet passing over and past dried sample spots by accommodating two different droplet sizes during the in-line sample purification process, greatly improving the efficiency and reliability as needed for multiple-sample processing. We also develop a driving voltage sequence to dispense all the

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droplets (including proteomic solution) reliably and repeatably from the on-chip reservoirs in the air-filled device. In EWOD, the droplets are often dispensed and actuated in an immiscible medium or filling liquid, such as silicone oil. This secondary liquid eases most microfluidic functions by minimizing contact angle hysteresis (which decreases resistance to droplet movement) and by helping limit substrate fouling.[5,6] However, oil media are not compatible with many potential applications of EWOD, including MALDI-MS. One problem is that precious sample can be lost through emulsification and loss of protein to the oil phase. A second problem is that the sample droplets need to be dried on the chip surface, which is problematic if the device is filled with oil. Even if the oil is later removed, or the droplets are transferred to a different substrate for MS analysis, any residual oil can interfere with the MS signal. For these reasons, the devices here and in most of our previous work use air rather than oil as the ambient medium despite the more demanding standards in design and fabrication.[3,4,7–10]

Materials and methods

Reagents and materials

All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. Stock solutions of human angiotensin II (100 μM) and urea (9 M) were prepared in deionized (DI) water. A stock solution of bovine insulin (100 μM) was prepared in 0.2% trifluoroacetic acid (TFA). Stock solutions were kept frozen until use.

For MALDI experiments, insulin and angiotensin II were diluted into aqueous working solutions (1 μM solutions with 0.025% TFA) containing urea (5 M) and used within 1 day. Working solutions of the MALDI matrix 2,5-dihydroxybenzoic acid (DHB) (7.5 mg mL^{-1}) were prepared in 0.05% TFA with 5% acetonitrile and used within 1 day.

Teflon-AF 1600 resin was purchased from DuPont (Wilmington, DE). Stock solutions of 6.0% (wt/vol) were formed in Fluorinert FC-40 solvent and then diluted to 0.5% (v/v with FC-40). Clean room reagents and supplies were used as provided by the University of California at Los Angeles (UCLA) Nanoelectronics Research Facility (Nanolab).

Device fabrication

EWOD chips were fabricated at the UCLA Nanolab. To form the bottom plate of each device, 100 Å chromium and 1000 Å gold layers were deposited by electron beam evaporation on a glass substrate. Electrodes were patterned by photolithography and wet etching of gold and chromium. A silicon dioxide layer (8000 Å) was deposited using plasma enhanced chemical vapor deposition (PECVD) to form a dielectric layer, and contact pads were exposed by etching the oxide with buffered hydrofluoric acid. The devices were spin-coated (2000 rpm, 60 s) with 0.5% Teflon-AF and baked on a hot plate (160 °C, 10 min) followed by baking in an oven (330 °C for 30 min) resulting in a ~150 Å layer of Teflon-AF. A cover plate was formed from indium–tin oxide (ITO)-coated glass pieces (Delta Technologies, Ltd., Stillwater, MN) coated with a ~150 Å layer of Teflon-AF. The two plates were joined in a parallel-plate configuration with the gap between the plates determined by the spacers defined from SU-8 (MicroChem Corp., Newton, MA) Three different devices were used for this work: Device *X* for testing droplet generation (Fig. 1a), Device *Y* for demonstrating sample purification (Fig. 3a), and Device *Z*, the integrated EWOD-MALDI-MS chip (Fig. 4).

MALDI sample preparation

Aqueous droplets (0.02–4 μL) were sandwiched between the two plates, and moved by applying ac potentials (1 kHz, 50 V_{RMS}) between the blanket electrode on the top plate and successive electrodes on the bottom plate, in a manner similar to what has been described elsewhere.[3–9] After droplets were moved to the designated locations, the EWOD chip was placed in a chamber under house vacuum for dehydration. A typical droplet evaporated in 1–5 min, leaving the dried analyte(s) and matrices on the surface of the chip. Each experimental condition was repeated at least three times. When deposition was complete, the bottom plate of the EWOD chip was affixed with double-sided adhesive tape into a 1 mm deep recess that had been milled into a standard stainless-steel MALDI target. (As an aside, we note that although steps such as dehydration were done manually, automation of the entire sample preparation procedure should be relatively straightforward once all the microfluidic operations have been integrated on-chip.)

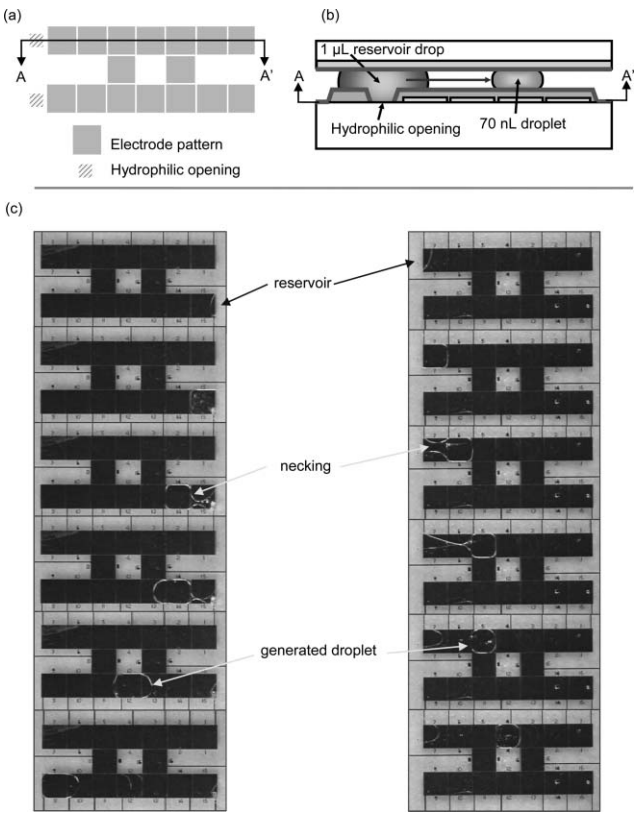

Fig. 1 Design of Device *X* and demonstration of droplet generation from the on-chip reservoir: (a) top view of electrode pattern; (b) side view of droplet generation (the much larger liquid reservoir is not drawn to scale); (c) video sequences (top-to-bottom) showing generation of 70 nL droplets of DHB solution (left) and aqueous insulin/urea (right).

Mass spectrometry

A Voyager DE-STR time-of-flight (TOF) mass spectrometer (Applied Biosystems, Foster City, CA) was used to collect MALDI-MS data. Mass spectra were collected in linear and reflector mode, typically with 200 shots per spectrum. Spectra were processed using Voyager Data Explorer (Applied Biosystems) and Igor Pro (Wavemetrics, Lake Oswego, OR). Spectra from rinsed (*i.e.*, purified) spots of peptide standards were baseline subtracted and normalized to the highest peak. Data from non-rinsed (*i.e.*, unpurified) spots were normalized to the highest peak from a corresponding rinsed spot.

Digital microfluidic functions for integrated chip

Generating reagent droplets from a reservoir

Generating droplets in air from an on-chip reservoir using only electrowetting forces (*i.e.*, without assistance, such as by applying external pressure) can be challenging. Although we have previously demonstrated generation of water droplets from a reservoir in an air environment,[7] it can be more difficult to generate droplets of reagents useful for proteomics, such as aqueous solutions of proteins, buffer salts, and MALDI matrices. This is because higher electrical potentials are required to generate droplets than are needed to simply move them. These elevated potentials may cause current leakage through the dielectric layer, resulting in electrolysis at the EWOD device surface. The electrolysis problem, which impedes droplet movement, is magnified for MALDI reagents, because (1) the reagents have high conductivity, accelerating the process of current leakage and dielectric breakdown, and (2) the affected surfaces have smaller contact angle changes when activated, necessitating the use of higher potentials for EWOD actuation.

Dielectric thickness is an important factor in determining the voltage required for droplet actuation by EWOD.[9] To evaluate this relationship, it is useful to rearrange the Lippmann–Young equation (eqn 1) in terms of electric field (eqn 2):

$$\cos\theta = \cos\theta_0 + \frac{1}{\gamma_{LV}}\frac{\varepsilon\varepsilon_0}{2t}V^2 \quad (1)$$

$$(V/t) = (\cos\theta - \cos\theta_0)^{\frac{1}{2}}\left(\frac{2\gamma_{LV}}{\varepsilon\varepsilon_0}\right)^{\frac{1}{2}}t^{-\frac{1}{2}} \quad (2)$$

where θ is the contact angle with applied potential, θ_0 is the initial contact angle, γ_{LV} is the surface tension of the liquid, ε is the dielectric constant of the dielectric layer, ε_0 is the permittivity of vacuum, V is the applied voltage, and t is the thickness of the dielectric layer. The electric field required to achieve a given contact angle change decreases as dielectric layer thickness is increased. By contrast, the electric field that causes dielectric breakdown is independent of thickness. Using a thicker dielectric layer in the EWOD device should therefore enable droplet actuation over a wider applied voltage range without electrolysis. In our reports describing progress toward the development of handheld EWOD-based devices, we used relatively thin dielectric layers (1000–2000 Å) so that a small voltage could be used for actuation.[7–9] In other applications, we have used thicker dielectric layers, ~8000 Å, and found that doing so effectively eliminates the electrolysis problem, even at 100 V.[10] Here, using an 8000 Å dielectric layer enabled the use of electric fields high enough to provide the larger actuation force needed to generate droplets of MALDI reagents, with no electrolysis.

Proof-of-principle droplet generation experiments were performed using Device *X* (Fig. 1). Fig. 1(a) and (b) illustrate small regions (~500 μm × 500 μm) where the Teflon-AF coating adjacent to the electrodes had been removed by oxygen plasma etching to reveal the hydrophilic oxide. Each hydrophilic patch could be used to anchor a ~1 μL liquid reservoir while pulling droplets from it. By applying 50 V_{RMS} (1 kHz) to the electrode adjacent to the anchored reservoir droplet and successively to the adjacent electrodes, liquid was pulled from the reservoir, formed a neck, and was cut into ~70 nL droplets. The droplet volume was defined by the size of the electrodes and the gap between plates. Droplets formed in this manner included aqueous insulin (1 μM in 5 M urea), the MALDI matrix, DHB (7.5 mg mL^{-1}), and DI water (for rinsing). Images from the video showing reagent and matrix droplets being generated and transported with this device are shown in Fig. 1(c).

Sample purification

During sample preparation for proteomics, unwanted impurities, such as salts, non-volatile solvents, and denaturants may be introduced.[11–13] High concentrations of these impurities may completely obscure the MS signal from the analyte(s) of interest. Low concentrations of impurities reduce the overall sensitivity of MALDI mass spectrometry, and relevant peaks can be obscured by salt adduct peaks. To overcome these problems, samples are often purified by solid phase extraction (SPE) prior to MALDI-MS analysis.[14] When the sample is applied to the SPE medium, hydrophobic analytes adsorb onto the hydrophobic surface. Hydrophilic impurities can be dissolved by a polar solvent and rinsed away. The hydrophobic analytes are then extracted into a less polar solvent. A common SPE tool used to purify samples for MS is the ZipTip®, which consists of reversed phase chromatographic media packed into pipette tips.[15,16]

We previously reported using EWOD device surfaces for in-line sample purification.[4] In this method, a proteomic sample is allowed to dry on a device surface; subsequently, the dried spot is rinsed with water to dissolve and remove the hydrophilic impurities. Much of the analyte remains on the surface, where it can be analyzed by MALDI-MS. This process is illustrated in Fig. 2. Because the purified analyte is already positioned on the target surface for MALDI analysis, this method circumvents the additional analyte desorption step that is required in SPE.

We introduce here several improvements upon our previous method for in-line sample purification, making use of a new EWOD electrode design. The first improvement is that the new design enables *multiple* samples to be processed from a single loading of sample, matrix, and rinse water. The second improvement is by introduction of a box-in-box electrode, incorporating multiple electrode sizes (Device *Y*, Fig. 3(a)).

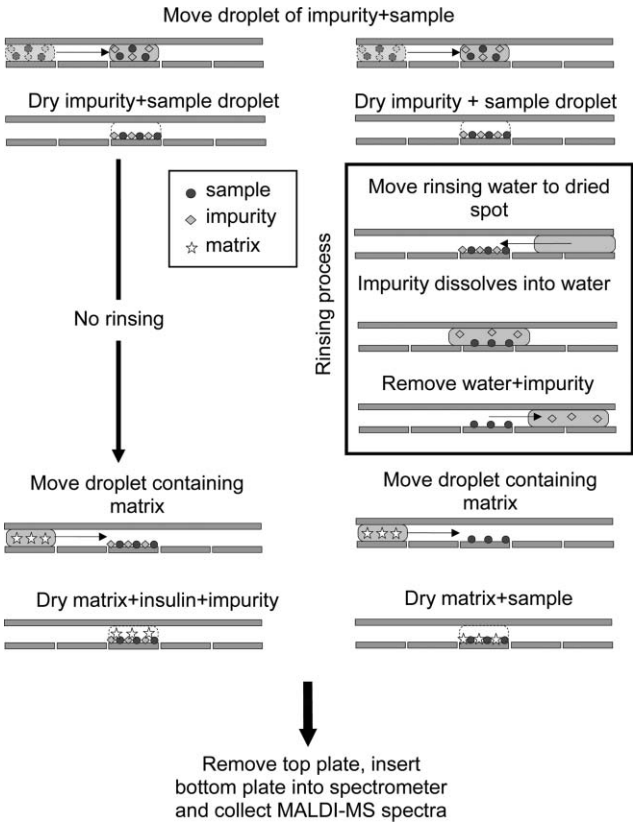

Fig. 2 Schematic of the on-chip sample purification process for EWOD-MALDI-MS. Left side: sample and matrix deposition with no rinsing step (control). Right side: after the sample is deposited and dried, but before the matrix is delivered, the impurities are dissolved and removed by a rinsing droplet.

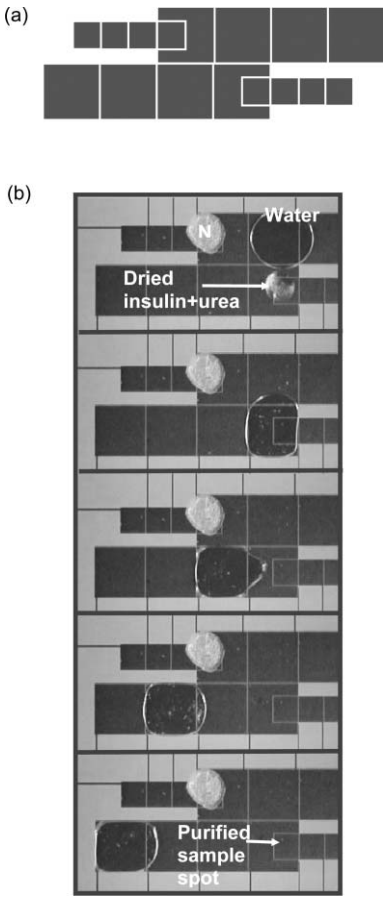

Fig. 3 Design of Device *Y* featuring box-in-box electrodes and demonstration of sample purification: (a) top view of electrode pattern; (b) video sequence (top-to-bottom) depicting EWOD-driven purification of a dried spot containing insulin and urea. The large electrodes are used to move a water droplet to the dried spot, where it selectively dissolves the urea. Because the rinsing droplet primarily touches clean surfaces on the surrounding electrodes, it is easily moved away, leaving behind an (invisible) insulin film. (In this demonstration, the dried insulin/urea spot marked "N" was not rinsed.)

When a protein droplet is allowed to dry on the device surface, the dried protein may coat most of the electrode. In previous work, we found that it is sometimes difficult to use EWOD to move droplets (*e.g.*, of a rinsing liquid) away from this relatively hydrophilic and possibly rougher surface, as the droplet pins.[4] To overcome this problem, small electrodes (0.7 mm × 0.7 mm) on Device *Y* are used to transport and deposit droplets of protein, along with buffers, denaturing agents and/or other "impurities", and larger electrodes (1.4 mm × 1.4 mm) are used to transport rinsing droplets. Certain small electrodes are designated as sample deposition sites; each site is surrounded by a larger, irregularly shaped electrode, forming a box-in-box electrode shape. This geometry makes it possible to move a larger rinsing droplet on and off the deposition site, because its contact line touches the surrounding clean hydrophobic electrodes.

Fig. 3(b) shows a video sequence demonstrating purification of a dried spot formed from a droplet containing insulin and urea that has been delivered from the series of small electrodes. The rinsing droplet was moved from a larger electrode over the deposition site, where it maintained contact with the adjacent/surrounding clean electrode. The rinsing droplet dissolved the urea (this process took less than 1 s), and was then moved away without difficulty. Matrix could then be delivered to the spot for subsequent MALDI-MS analysis. The incorporation of larger electrodes may be a generally useful technique to enable transport of liquids over areas where droplet pinning has proven problematic, including hydrophilic, rough or chemically heterogeneous patches.

Integrated EWOD-MALDI-MS chip

This and prior work have shown that droplets of proteomics reagents and samples can be generated and transported by digital microfluidics and that on-chip purification is possible. Device *Z* in Fig. 4 represents the first fully integrated device for processing multiple droplets in parallel. An SU-8 layer (~40 μm) was spin-coated (3000 rpm, 30 s) and patterned by photolithography to form reservoir walls, which also serve as the spacer between the top and bottom plates. This process was followed by Teflon-AF spin coating. This particular device includes four reservoirs: two smaller reservoirs (~1 μL) are used for sample and matrix solutions, while the two larger ones (~4 μL) are used for rinsing water and waste collection.

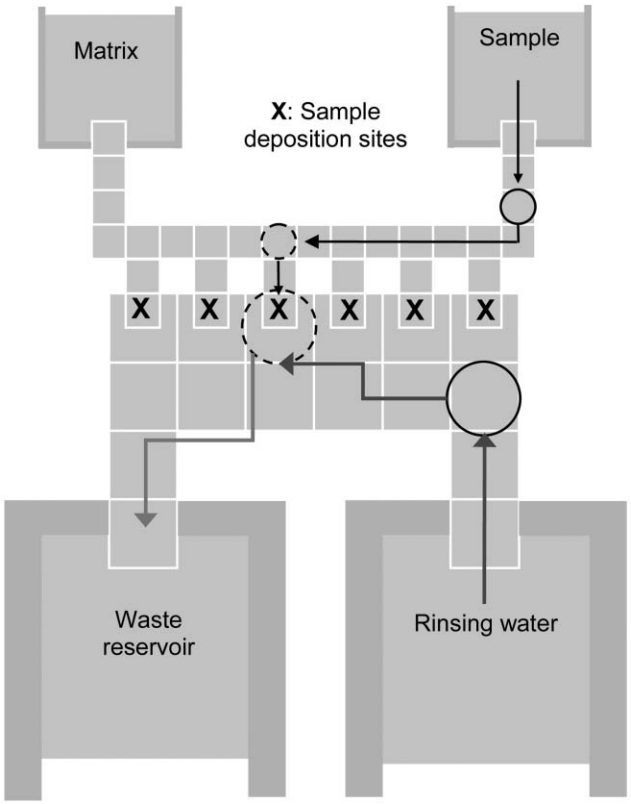

Fig. 4 Schematic of Device *Z*, the integrated EWOD-MALDI-MS device.

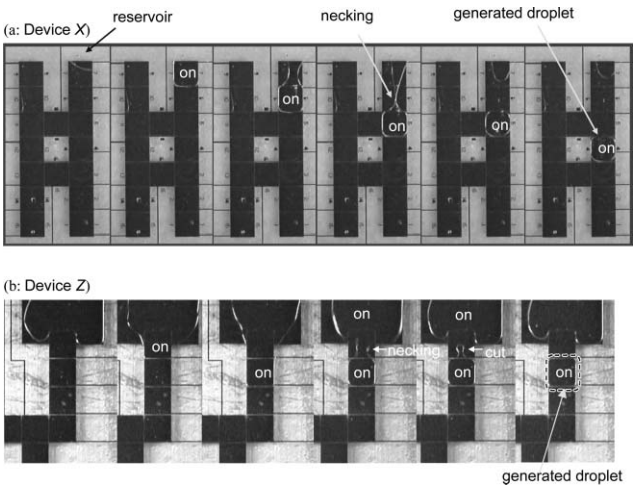

Fig. 5 Droplet generation with Devices *X* and *Z*. (a) On Device *X*, which was designed for passive dispensing, a long neck formed and it was necessary to activate at least three electrodes to dispense a droplet. (b) On Device *Z*, which was designed for active dispensing, only a short neck formed, and only two electrodes were needed to dispense the ~20 nL droplet. In the images, "on" indicates the electrode is activated with a voltage; all other electrodes are floated.

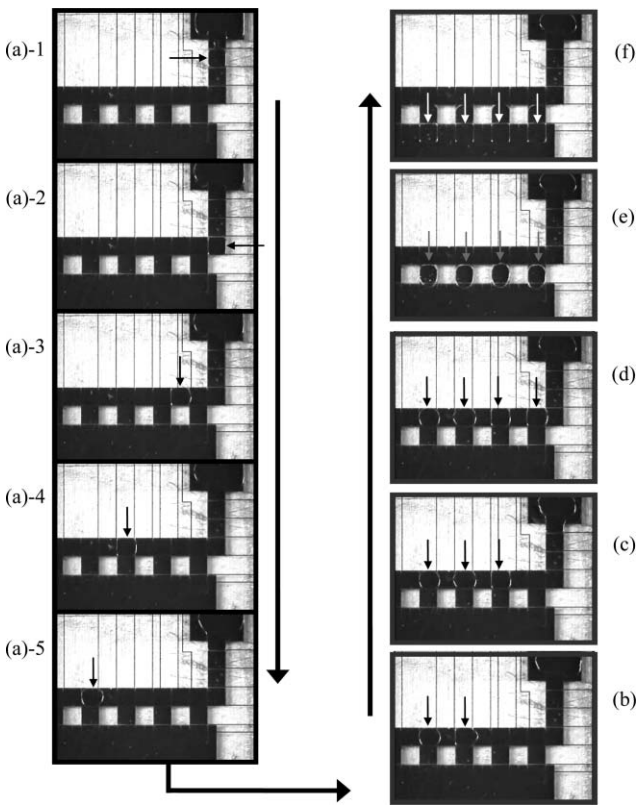

Fig. 6 Video sequence depicting the generation and positioning of multiple droplets of angiotensin II with Device *Z*. Arrows indicate the droplet positions. Frames (a)-1 through (a)-5 show the first ~20 nL droplet being generated from the reservoir. Frames (b) through (d) show three additional droplets being generated in the same manner as in (a). Frames (e) and (f) show the four droplets being moved simultaneously to the deposition sites.

Device *X* was formed with permanent, hydrophilic anchors for reservoirs. Dispensing depends solely on the passive narrowing of a liquid neck when the liquid is pulled away from a reservoir. Often in this process a long neck forms, and several electrodes are required for successful dispensing (Fig. 5(a)). Further, the number of electrodes required to dispense depends on the volume of the reservoir drop. Device *Z* was designed to make droplet generation more efficient and repeatable. In Device *Z*, EWOD "reservoir electrodes" were patterned at the center of each reservoir. A small portion of liquid is pulled away from the reservoir by sequential actuation of two adjacent electrodes. A voltage is then applied to the reservoir electrode, actively pulling the liquid back towards the reservoir. A narrow neck forms quickly and then breaks, releasing the dispensed droplet (Fig. 5(b)). In this active dispensing mode, the number of electrodes required is fixed at two, regardless of the reservoir or dispensed droplet volumes. Quantitatively, active dispensing was observed to yield more consistent droplet volumes than passive dispensing.

The operation protocol for Device *Z* consisted of seven steps: (1) generate up to six droplets of sample from the sample reservoir (upper-right corner in Fig. 4, ~60 s); (2) move droplets to the deposition sites (2 s) and dry them (~1–2 min in vacuum chamber); (3) generate three or more droplets of rinsing water from the water reservoir (lower-right corner in Fig. 4, ~15 s); (4) remove impurities by moving the water droplets over the deposition sites (~5 s for rinsing six deposition sites); (5) send rinsing water droplets to the waste

reservoir (lower-left corner in Fig. 4, ~15 s); (6) generate multiple droplets from the matrix reservoir (upper-left corner in Fig. 4, ~60 s); and (7) move matrix droplets to the deposition sites (~2 s) and dry them (~1–2 min in vacuum chamber).

Fig. 6 shows a video sequence depicting the sequential generation of four droplets of angiotensin II solution (steps 1 and 2, above) and their simultaneous positioning at four deposition sites. After the sample droplets had dried, rinsing water droplets were generated from the water reservoir and moved to the deposition sites where they selectively dissolved urea from the dried sample spot. The water droplets were then moved to the waste reservoir. Subsequently, four droplets of DHB solution were generated from the matrix reservoir and deposited at the four protein deposition sites. After all seven steps had been completed, MALDI mass spectra were collected. The duration of each step of electrode activation sequence is 1 s. The entire process (seven steps above) took approximately 10 min, from sample solution loading of the reservoirs through drying. Fig. 7 shows spectra obtained from not-rinsed and rinsed spots. No signal was observed in the spectrum from the non-rinsed spot (top), while a strong angiotensin signal (1046 Da) was observed for the spectrum from the rinsed spot (bottom).

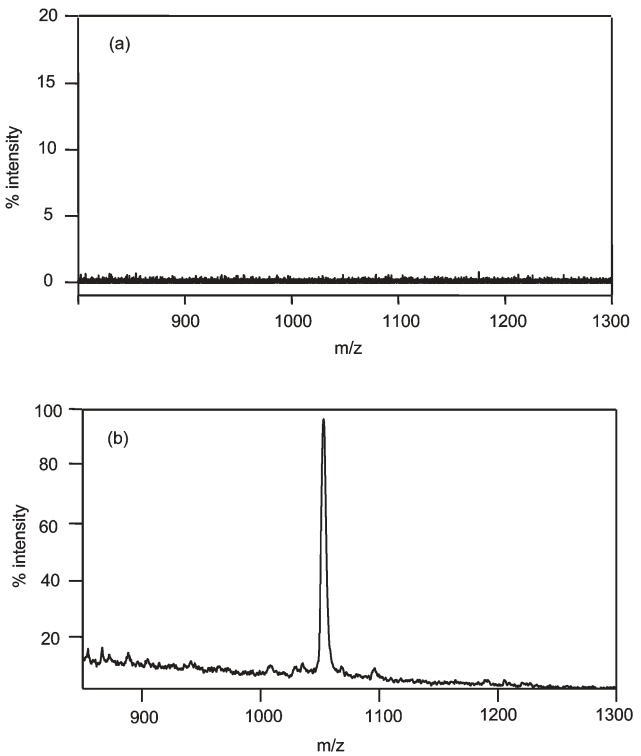

Fig. 7 MALDI mass spectra of non-rinsed and rinsed angiotensin II/urea spots that were prepared and processed with fully integrated Device *Z* as described in the text and depicted in Fig. 4. Multiple spots were processed in parallel on a single device; these mass spectra are representative. (a) Without the rinsing (purification) step, the analyte peak is completely suppressed because of interference from urea. (b) With the rinsing step, the angiotensin $(M + H)^+$ peak is strong and the signal-to-noise ratio is very good.

Conclusions

An integrated EWOD chip has been developed that enables multiple samples to be processed through several steps for MALDI-MS. Innovations include a new electrode design that enables efficient and reproducible generation of droplets from on-chip reservoirs, and the incorporation of several different size electrodes to facilitate liquid transport over areas where droplets may be prone to pin. The integrated device was demonstrated in the processing of four angiotensin peptide/urea samples in parallel through a sequence of seven actuation steps: (1) generation of sample droplets; (2) transport and drying of sample droplets; (3) generation of rinsing droplets; (4) their transport to the sample sites for selective dissolution of urea; (5) transport and disposal of the rinsing droplets; (6) generation of DHB (MALDI matrix) solution droplets, and (7) their delivery to the dried angiotensin spots. MALDI mass spectra of the rinsed spots showed strong analyte signals, while no analyte signal could be detected from the non-rinsed spots.

The success demonstrated here in multi-step parallel sample processing represents a significant advance in digital microfluidics for proteomics and other bioanalytical applications. Scale-up to larger devices with additional reservoirs and many sample paths for high-throughput proteomics appears feasible. We are now evaluating the technique for processing more complex biological samples of clinical relevance. Ongoing work includes the integration of additional protein chemistry-related processing steps, including disulfide bond reduction, cysteine alkylation and proteolytic digestion.

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