Anal. Chem. **2005,** *77,* 534−540

Digital Microfluidics with In-Line Sample Purification for Proteomics Analyses with MALDI-MS

Aaron R. Wheeler,[†] Hyejin Moon,[‡] Christopher A. Bird,[†] Rachel R. Ogorzalek Loo,[§] Chang-Jin "C.J." Kim,[‡] Joseph A. Loo,[†,§] and Robin L. Garrell*[,†,∥]

Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90095-1569, Department of Biological Chemistry, David Geffen School of Medicine and Molecular Biology Institute, University of California, Los Angeles, California 90095-1569, Department of Mechanical and Aerospace Engineering, Henry Samueli School of Engineering and Applied Science, University of California, Los Angeles, California 90095-1597, and Biomedical Engineering Interdepartmental Program, University of California, Los Angeles, California 90095-1600

An in-line sample purification method for MALDI-MS, which relies on the electrowetting-on-dielectric (EWOD)-based technique for digital microfluidics, is reported. In this method, a droplet containing peptides and impurities is moved by EWOD and deposited onto a Teflon-AF surface. A droplet of water is subsequently moved over the spot, where it dissolves and removes the impurities. A droplet containing MALDI matrix is then moved to the spot, which is analyzed by MALDI-MS. This purification method reduces the number of salt adduct peaks caused by low concentrations of impurities (e.g., 20 mM sodium phosphate), and reduces or eliminates the catastrophic effects of high concentrations of impurities (e.g., 8 M urea). The method was used to purify spots made by depositing multiple droplets of contaminated peptides. Spectra from the purified spots showed an increase in the S/N ratio as a function of the number of droplets deposited; when not purified, the S/N ratio remained constant regardless of the number of droplets. Finally, the method was used to purify protein digests for peptide mass fragment (PMF) searches, and was shown to be more efficient than the conventional method of purification with reversed-phase-packed pipet tips. We anticipate this new, in-line sample purification technique for EWOD−MALDI-MS will enable development of integrated high-throughput proteomics analysis methodologies.

Mammalian cells typically express a proteome consisting of 5000−20000 proteins,[1] and may express hundreds of proteomes in response to environmental cues.[2] To meet the challenge of collecting vast amounts of proteomics data, several technologies have been developed to facilitate high-throughput analysis by mass spectrometry (MS). Lithographically patterned matrix-assisted laser desorption/ionization (MALDI) targets[3−5] and microfabricated picoliter sample delivery devices[6−10] have been used to prepare arrays with hundreds to thousands of spots for analysis by MALDI-MS. In another approach, microfluidic channels have been coupled with MALDI[11−14] or electrospray ionization[15−22] (ESI) MS for high-throughput proteomics analyses.

* To whom correspondence should be addressed at the Department of Chemistry and Biochemistry. Phone: (310) 825-2496. Fax: (310) 206-2061. E-mail: garrell@chem.ucla.edu.

[†] Department of Chemistry and Biochemistry.

[‡] Department of Mechanical and Aerospace Engineering.

[§] Department of Biological Chemistry.

[∥] Biomedical Engineering Interdepartmental Program.

(1) Apella, E.; Arnott, D.; Sakaguchi, K.; Wirth, P. J. In *Proteomics in Functional Genomics*; Jolles, P., Jornvall, H., Eds.; Birkhauser Verlag: Basel, Germany, 2000; pp 1−28.

(2) Wilkins, M. R.; Sanchez, J.-C.; Gooley, A. A.; Appel, R. D.; Humphrey-Smith, I.; Hochstrasser, D. F.; Williams, K. L. *Biotechnol. Genet. Eng. Rev.* **1995**, *13*, 19−50.

(3) Schuerenberg, M.; Luebbert, C.; Eickhoff, H.; Kalkum, M.; Lehrach, H.; Nordhoff, E. *Anal. Chem.* **2000**, *72*, 3436−3442.

(4) Gobom, J.; Schuerenberg, M.; Mueller, M.; Theiss, D.; Lehrach, H.; Nordhoff, E. *Anal. Chem.* **2001**, *73*, 434−438.

(5) Xu, Y.; Watson, J. T.; Bruening, M. L. *Anal. Chem.* **2003**, *75*, 185−190.

(6) Little, D. P.; Cornish, T. J.; O'Donnell, M. J.; Braun, A.; Cotter, R. J.; Koster, H. *Anal. Chem.* **1997**, *69*, 4540−4546.

(7) Onnerfjord, P.; Nilsson, J.; Wallman, L.; Laurell, T.; Marko-Varga, G. *Anal. Chem.* **1998**, *70*, 4755−4760.

(8) Ericsson, D.; Ekstrom, S.; Nilsson, J.; Bergquist, J.; Marko-Varga, G.; Laurell, T. *Proteomics* **2001**, *1*, 1072−1081.

(9) Ekstrom, S.; Ericsson, D.; Onnerfjord, P.; Bengtsson, M.; Nilsson, J.; Marko-Varga, G.; Laurell, T. *Anal. Chem.* **2001**, *73*, 214−219.

(10) Miliotis, T.; Kjellstrom, S.; Nilsson, J.; Laurell, T.; Edholm, L.-E.; Marko-Varga, G. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 117−126.

(11) Liu, J.; Tseng, K.; Garcia, B.; Lebrilla, C. B.; Mukerjee, E.; Collins, S. D.; Smith, R. L. *Anal. Chem.* **2001**, *73*, 2147−2151.

(12) Brivio, M.; Fokkens, R. H.; Verboom, W.; Reinhoudt, D. N.; Tas, N. R.; Goedbloed, M.; van den Berg, A. *Anal. Chem.* **2002**, *74*, 3972−3976.

(13) Ekstrom, S.; Malmstrom, J.; Wallman, L.; Lofgren, M.; Nilsson, J.; Laurell, T.; Marko-Varga, G. *Proteomics* **2002**, *2*, 413−421.

(14) Gustafsson, M.; Hirschberg, D.; Palmberg, C.; Jornvall, H.; Bergman, T. *Anal. Chem.* **2004**, *76*, 345−350.

(15) Lion, N.; Gobry, V.; Jensen, H.; Rossier, J. S.; Girault, H. H. *Electrophoresis* **2002**, *23*, 3583−3588.

(16) Lion, N.; Gellon, J.-O.; Jensen, H.; Girault, H. H. *J. Chromatogr., A* **2003**, *1003*, 11−19.

(17) Gao, J.; Xu, J.; Locascio, L. E.; Lee, C. S. *Anal. Chem.* **2001**, *73*, 2648−2655.

(18) Pinto, D. M.; Ning, Y.; Figeys, D. *Electrophoresis* **2000**, *21*, 181−190.

(19) Kameoka, J.; Orth, R.; Ilic, B.; Czaplewski, D.; Wachs, T.; Craighead, H. C. *Anal. Chem.* **2002**, *74*, 5897−5901.

(20) Chen, S.-H.; Sung, W.-C.; Lee, G.-B.; Lin, Z.-Y.; Chen, P.-W.; Liao, P.-C. *Electrophoresis* **2001**, *22*, 3972−3977.

(21) Lazar, I. M.; Ramsey, R. S.; Sundberg, S.; Ramsey, J. M. *Anal. Chem.* **1999**, *71*, 3627−3631.

(22) Xie, J.; Miao, Y. N.; Shih, J.; He, Q.; Liu, J.; Tai, Y. C.; Lee, T. D. *Anal. Chem.* **2004**, *76*, 3756−3763.

10.1021/ac048754+ CCC: $30.25
Published on Web 12/17/2004

We have recently demonstrated on-chip sample preparation for MALDI-MS, enabled by the electrowetting-on-dielectric (EWOD)-based digital microfluidic technique in which droplets containing the sample and MALDI matrix are moved, mixed, deposited, and finally analyzed by MALDI-MS.[23] In EWOD, electrical potentials are applied sequentially between electrodes buried beneath a hydrophobic, dielectric layer; as the local wettability is changed, droplets can be made to travel across the surface.[24−27] Because EWOD and MALDI are both inherently array-based techniques, we believe that EWOD-based liquid actuation has the potential to be very useful for high-throughput proteomics.

One problem in developing proteomics methods is the number of chemical processing steps required. For example, for an analysis by peptide mass fingerprinting (PMF) with MALDI-MS, a sample is typically denatured, reduced, alkylated, proteolytically digested, and finally mixed with matrix and dried. This process takes many hours, requires several pipetting steps, and introduces unwanted impurities, such as salts or denaturants. These impurities affect the overall MALDI-MS sensitivity by reducing desorption/ionization efficiencies, and also by spreading out the analyte signal over many channels because of salt adduct formation. The resulting mass spectrum has diminished intensity, mass range, and resolution; furthermore, salt adduct peaks can obscure relevant analyte peaks.

To overcome the problems caused by salts and other unwanted impurities, samples to be analyzed by MS are often purified by solid-phase extraction (SPE). In SPE, a sample is adsorbed onto a solid hydrophobic medium, and hydrophilic contaminants are rinsed away. The purified sample is then desorbed in a nonpolar elution buffer. For MS, SPE is often accomplished using a popular commercial product, the ZipTip,[28] which incorporates chromatographic packing material in pipet tips.[29,30] Other methods for sample purification make use of hydrophobic media in microfluidic channels,[13−16] electrocapture in capillaries,[31,32] and hydrophobic MALDI targets[33−43] in place of conventional stainless steel targets. In the latter technique, a proteomic sample is allowed to dry on the hydrophobic surface; subsequently, the target is rinsed with a polar solvent to dissolve and remove the hydrophilic impurities. Much of the analyte remains on the surface, where it can be analyzed by MALDI-MS. The use of a hydrophobic surface has the added benefit of yielding more intense MALDI signals. This is because the spots that form are smaller than those that form on hydrophilic surfaces, resulting in increased analyte concentrations.[33−43] This technique provided the basis for developing the sample purification method described here.

We report the integration of sample purification with the EWOD−MALDI-MS[23] technique. EWOD was used to (1) move droplets containing peptides and impurities to desired locations and deposit them, (2) pass droplets of water over the dried spots to dissolve the impurities and rinse them away, and (3) bring droplets containing matrix to the spots for analysis by MALDI-MS. This is the first report of using digital (i.e., droplet-based) microfluidics for in-line sample purification and cocrystallization for proteomics.

EXPERIMENTAL SECTION

Reagents and Materials. All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. Stock solutions of human angiotensin II (100 μM), urea (9 M), and sodium phosphate buffer (100 mM, pH 7.0) were prepared in deionized (DI) water. A stock solution of bovine insulin (100 μM) was prepared in 0.2% trifluoroacetic acid (TFA). Stock solutions of equine myoglobin (150 μM) and bovine ubiquitin (150 μM) were prepared in 9 M urea. A stock solution (1 mg/mL) of endoproteinase Lys-C (Wako BioProducts, Richmond, VA) was prepared in DI water. 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 (0.1, 1, or 8 M) or sodium phosphate buffer (20 or 95 mM) and used within 1 day. Working solutions of the MALDI matrix 2,5-dihydroxybenzoic acid (DHB) (7.5 mg/mL) were prepared in 0.05% TFA with 5% acetonitrile and used within 1 day. For PMF digests, myoglobin or ubiquitin solutions were diluted with DI water and Lys-C to contain 100 μM protein, 4 μM Lys-C, and 6 M urea, and incubated at 37 °C for 2 h. After digestion, the solution was diluted 1:50 into 6 M urea and frozen (−80 °C) until use.

Teflon-AF 1600 resin was purchased from DuPont (Wilmington, DE). Stock solutions of 6% (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) 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.[23] Briefly, the bottom

(23) Wheeler, A. R.; Moon, H.; Kim, C.-J.; Loo, J. A.; Garrell, R. L. *Anal. Chem.* **2004**, *76*, 4833−4838.
(24) Lee, J.; Moon, H.; Fowler, J.; Schoellhammer, T.; Kim, C.-J. *Sens. Actuators, A* **2002**, *95*, 259−268.
(25) Moon, H.; Cho, S. K.; Garrell, R. L.; Kim, C.-J. *J. Appl. Phys.* **2002**, *92*, 4080−4087.
(26) Cho, S. K.; Moon, H.; Kim, C.-J. *J. Microelectromech. Syst.* **2003**, *12*, 70−80.
(27) Pollack, M. G.; Fair, R. B.; Shenderov, A. D. *Appl. Phys. Lett.* **2000**, *77*, 1725−1726.
(28) Millipore ZipTip pipet tips, http://www.millipore.com/catalogue.nsf/docs/C5737 (accessed on July 12, 2004).
(29) Kussmann, M.; Nordhoff, E.; Rahbek-Nielsen, H.; Haebel, S.; Rossel-Larsen, M.; Jakobsen, L.; Gobom, J.; Mirgorodskaya, E.; Kroll-Kristensen, A.; Palm, L.; Roepstorff, P. *J. Mass Spectrom.* **1997**, *32*, 593−601.
(30) Gobom, J.; Nordhoff, E.; Mirgorodskaya, E.; Ekman, R.; Roepstorff, P. *J. Mass Spectrom.* **1999**, *34*, 105−116.
(31) Astorga-Wells, J.; Jornvall, H.; Bergman, T. *Anal. Chem.* **2003**, *75*, 5213−5219.
(32) Astorga-Wells, J.; Bergman, T.; Jornvall, H. *Anal. Chem.* **2004**, *76*, 2425−2429.
(33) Zaluzec, E. J.; Gage, D. A.; Allison, J.; Watson, J. T. *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 230−237.
(34) Liu, Y.-H.; Bai, J.; Liang, X.; Lubman, D. M.; Venta, P. J. *Anal. Chem.* **1995**, *67*, 3482−3490.
(35) Blackledge, J. A.; Alexander, A. J. *Anal. Chem.* **1995**, *67*, 843−848.
(36) Vestling, M. M.; Fenselau, C. *Mass Spectrom. Rev.* **1995**, *14*, 169−178.
(37) McComb, M. E.; Oleschuk, R.; Manley, D. M.; Donald, L.; Chow, A.; O'Neil, J. D. J.; Ens, W.; Standing, K. G.; Perreault, H. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 1716−1722.
(38) Ogorzalek Loo, R. R.; Mitchell, C.; Stevenson, T. I.; Loo, J. A.; Andrews, P. C. *Int. J. Mass Spectrom. Ion Processes* **1997**, *169/170*, 273−290.
(39) Brockman, A. H.; Dodd, B. S.; Orlando, R. *Anal. Chem.* **1997**, *69*, 4716−4720.
(40) Worrall, T. A.; Cotter, R. J.; Woods, A. S. *Anal. Chem.* **1998**, *70*, 750−756.
(41) Hung, K. C.; Ding, H.; Guo, B. *Anal. Chem.* **1999**, *71*, 518−521.
(42) Yuan, K.; Desiderio, D. M. *J. Mass Spectrom.* **2002**, *37*, 512−524.
(43) Botting, C. H. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 598−602.

plates of the devices were formed by depositing 3500 Å thick phosphorus-doped polysilicon on a quartz wafer; EWOD electrodes were formed by patterning with photolithography and reactive ion etching. The design (reported previously[23]) consisted of 16 1-mm^2 electrodes with a 4 μm gap between them. A layer of thermal oxide (3000 Å) was grown on the polysilicon, 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 postbaked on a hot plate (160 °C, 10 min) and in a furnace (330°C, 10 min) to form 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) covered with a ~150 Å layer of Teflon-AF (processed as above). The two plates were joined with spacers (~300 μm thick) formed from three pieces of double-sided tape.

Aqueous droplets were sandwiched between the two plates, and moved by applying ac potentials (1 kHz, 45 V_{rms}) between the electrode in the top plate and successive electrodes in the bottom plate, in a manner similar to what has been described elsewhere.[23−27] Figure 1 in our previous EWOD−MALDI paper[23] depicts a schematic of the operation of a similar EWOD device. For all experiments reported here, the EWOD filler (medium surrounding the droplets) was air[23−26] rather than oil.[27] 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.

The minimum droplet volume that can be moved on EWOD devices depends on the dimensions of the EWOD electrodes and the interplate spacing. For the devices described in this work, this minimum volume was determined empirically to be ~0.5 μL. Droplet movement was facile and fast for solutions with peptide concentrations of 2 μM or less; droplets with higher concentrations moved sluggishly or not at all.

Sample Preparation. After droplet movement, the bottom plates of the EWOD devices were dehydrated in a chamber under house vacuum; 0.5 μL droplets dried in ~1−5 min. Each experimental condition was repeated at least three times. To ensure high mass accuracy for proteomics database searches, a calibration spot containing insulin and angiotensin II (1 μM each in DHB solution) was manually deposited next to each digest spot and dried. Matrix crystals were imaged by light microscopy. When deposition was complete, the bottom plate of an EWOD device was affixed with double-sided tape into a 1 mm deep groove milled out from a standard stainless steel MALDI target.

For comparison to conventional methods, Lys-C digest in 6 M urea was purified using a C_{18} ZipTip (Millipore, Billerica, MA). Samples of 2 μM digest (10 μL) were adsorbed onto the ZipTips, rinsed in 0.1% TFA, and eluted in 0.1% TFA with 50% acetonitrile (2.5 or 10 μL), as per the manufacturer's instructions. Aliquots of the eluent (0.5 μL) were mixed with DHB solution (0.5 μL), deposited onto a stainless steel MALDI target, and dried.

MS and PMF Searches. 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 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 nonrinsed (i.e., unpurified) spots were normalized to the highest peak from a corresponding rinsed spot. Spectra of PMF digests were calibrated with spectra of standards and analyzed with the Mascot protein identification package[44] searching the SwissProt database. The database was searched with no missed cleavages, a mass accuracy of 0.05%, and variable modification of methionine oxidation.

RESULTS AND DISCUSSION

EWOD-Driven Sample Purification. We recently demonstrated that droplets containing peptides and MALDI matrix can be moved by EWOD and analyzed with MALDI-MS.[23] The present work builds upon that technique by integrating in-line sample purification. Sodium phosphate, a buffer, and urea, a denaturant, were selected as model impurities because of their ubiquitous use in biology.

Figure 1 schematically illustrates a typical purification experiment. A droplet containing peptides and impurities was moved by EWOD to a specific location on the array and then dried to form a spot. Subsequently, an EWOD-driven droplet of DI water was moved onto the spot; after visual confirmation that the impurity had dissolved, the droplet was moved away from the spot. For low concentrations of impurity (e.g., 20 mM sodium phosphate), dissolution was instantaneous; for high concentrations (e.g., 8 M urea), it required a few seconds. After this rinsing step, a droplet containing MALDI matrix was moved by EWOD to the same spot and dried; finally, the spot was analyzed by MALDI-MS. Control experiments were performed without the water rinse.

The purification method was originally evaluated using the same volume, 0.5 μL, for the sample and rinsing droplets. Moving the rinsing droplet away from the dried spot proved challenging and often impossible under these conditions because the droplet was pinned on the protein-fouled surface.[45] We solved this problem by using larger rinsing droplets that touched the adjacent clean electrodes. Rinsing droplets of 1 μL were easily moved over spots formed from 0.5 μL sample droplets. We are currently developing new device geometries for manipulating smaller volumes, such as might be required for high-throughput analyses.

For many applications, protein adsorption to EWOD devices is undesirable.[45] The structure of Teflon-AF, the material typically used for EWOD device surfaces, is shown in Figure 2. Proteins and peptides adhere to Teflon-AF, just as to other nonpolar materials,[33−43] through nonspecific hydrophobic interactions. Fair and co-workers have proposed that protein adsorption to Teflon-AF is such a problem that silicone oil, rather than air, should be used as an EWOD filler to minimize this effect.[46] For our sample purification technique, however, the use of silicone oil or any other method to inhibit protein adsorption would be counterproductive. Furthermore, silicone would make the process of drying droplets for analysis difficult, and would cause spectral interference. We are also concerned that moderately polar analytes, such as MALDI matrixes and many other biochemical reagents, may partition out

(44) Perkins, D. N.; Pappin, D. J. C.; Creasy, D. M.; Cottrell, J. S. *Electrophoresis* **1999**, *20*, 3551−3567.
(45) Yoon, J.-Y.; Garrell, R. L. *Anal. Chem.* **2003**, *75*, 5097−5102.
(46) Srinivasan, V.; Pamula, V. K.; Fair, R. B. *Lab Chip* **2004**, *4*, 310−315.

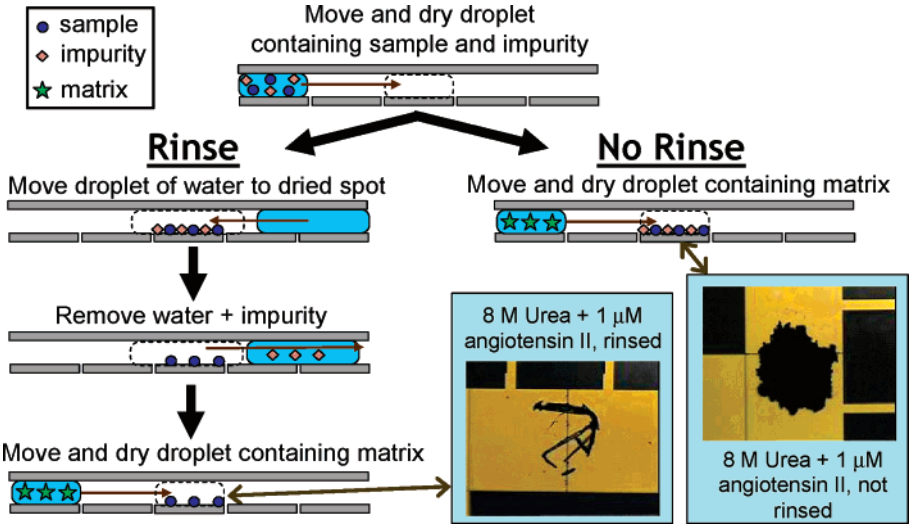

Figure 1. Schematic of EWOD-driven sample purification and micrographs of dried spots. Insets: The rinsed sample (left) appears to be primarily composed of DHB crystals, while the not-rinsed sample (right) is dominated by urea.

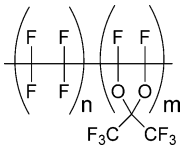

Figure 2. Chemical structure of Teflon-AF, a copolymer of tetrafluoroethylene and 2,2-bistrifluoromethyl-4,5-difluoro-1,3-dioxole (PDD).

of aqueous droplets into silicone.[47,48] For these reasons, air was used as a filler medium for the experiments reported here.

MS of Standards. In MALDI-MS, impurities at low-to-intermediate concentrations form adducts with analyte molecules that can obscure the relevant data and reduce the overall signal intensity.[49] Figure 3 contains representative EWOD−MALDI-MS spectra for rinsed and not-rinsed (control) samples of angiotensin II prepared with 20 mM sodium phosphate. In the spectrum of the control sample (Figure 3b), peaks at M + 23, M + 45, and M + 67 correspond to the mono-, di-, and trisodium adducts of the parent molecule. The adduct peaks are barely visible in the spectrum of the rinsed sample (Figure 3a). The intensities of the molecular ion peaks in the rinsed spectra were ~2× greater than those in the control spectra.

At high impurity concentrations, the MALDI-MS signal may be extinguished completely.[49] Figure 4 shows representative spectra for rinsed (main panels) and control samples (inset panels) prepared with high levels of impurities. In the control spots, impurities completely suppressed the MALDI-MS analyte signal ($n \geq 3$ for each condition), while the rinsed spots reproducibly yielded intense signals. This dramatic improvement was achieved by using a single rinsing droplet; additional rinsing droplets had little effect for any of the conditions tested (data not shown).

Many clinically relevant protein and peptide samples are too dilute for detection by MS. Such samples may be concentrated

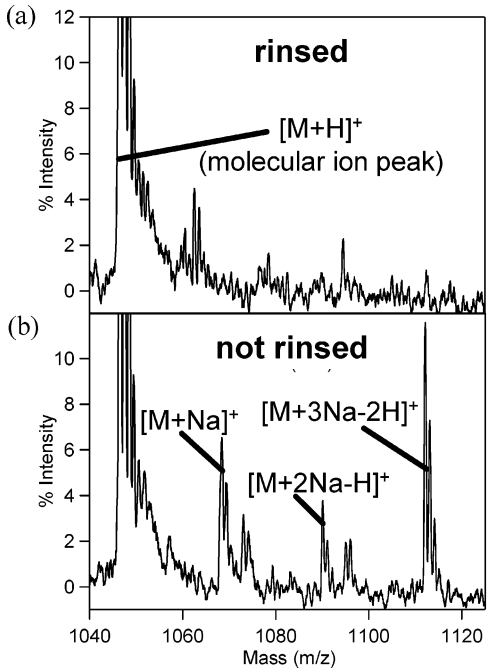

Figure 3. MALDI-MS spectra of (a) rinsed and (b) control spots containing angiotensin II, 1046 Da (0.5 pmol of total peptide), and 20 mM sodium phosphate. The clusters of peaks, apparent in all high-resolution MALDI-MS spectra, reflect $^{12}C/^{13}C$ isotope distributions.

onto MALDI targets by depositing multiple droplets at a single spot, a strategy that has been used successfully with microfabricated picoliter droplet delivery devices.[6−10] Unfortunately, any impurities present in the sample are concentrated along with the analytes.[8] We have examined this effect for EWOD−MALDI-MS, as shown in Figure 5. Spots were created by moving and drying one, two, or three droplets containing 0.33 μM angiotensin II and 100 mM urea. After the final sample droplets were dried, some spots were rinsed with an EWOD-driven droplet of water. MALDI matrix was deposited, and spectra were collected from the rinsed (Figure 5a, main panel) and control spots (inset). As shown in Figure 5b, the signal-to-noise (S/N) ratio for the rinsed spots increased as a function of the number of droplets deposited, but was constant for the control spots. The capacity to deposit multiple

(47) Mayer, P.; Vaes, W. H. J.; Hermens, J. L. M. *Anal. Chem.* **2000**, *72*, 459−464.
(48) Yoon, B. O.; Koyanagi, S.; Asano, T.; Hara, M.; Higuchi, A. *J. Membr. Sci.* **2003**, *213*, 137−144.
(49) Yao, J.; Scott, J. R.; Young, M. K.; Wilkins, C. L. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 805−813.

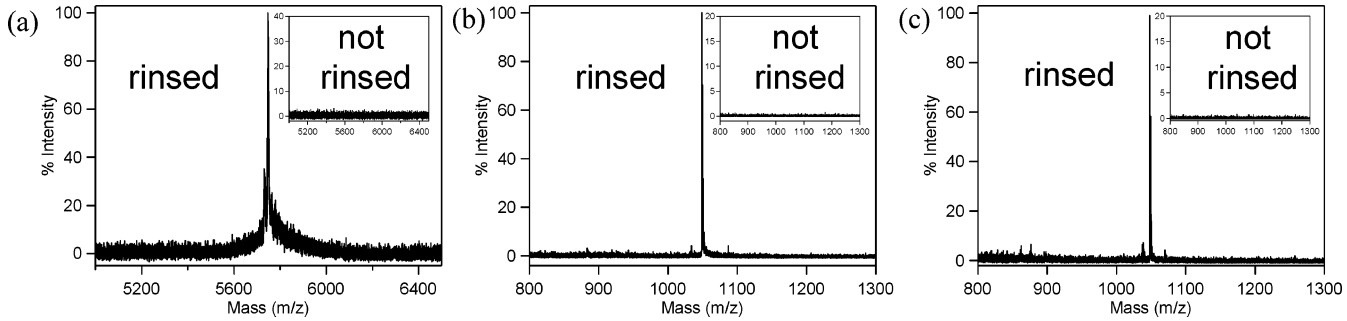

Figure 4. MALDI-MS spectra of (a) insulin, 5733 Da, with 8 M urea, (b) angiotensin II, 1046 Da, with 8 M urea, and (c) angiotensin II with 95 mM sodium phosphate. All spots contained 0.5 pmol of total peptide. The spectra in the main panels are from rinsed spots; the spectra in the inset panels are from spots that were not rinsed. Each inset panel spectrum was scaled to the maximum signal in the corresponding main panel spectrum.

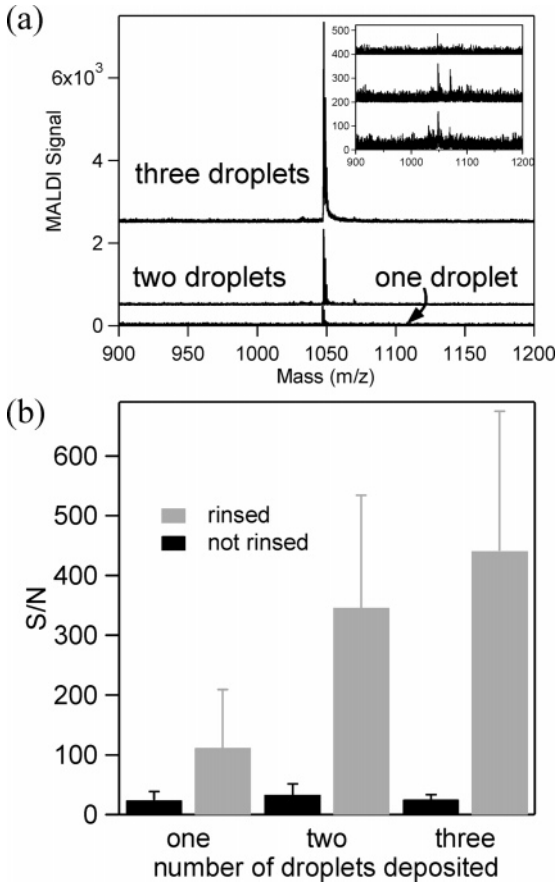

Figure 5. MALDI-MS spectra (a) and bar graph (b) comparing the spectral S/N ratios from spots of 0.33 μM angiotensin II + 100 mM urea. Spots were prepared by moving and drying one, two, or three droplets (0.17, 0.33, and 0.66 pmol of total peptide, respectively) at the same place on an EWOD device. $n = 3$ for each condition. Spectra in the main panel of (a) are from spots subsequently rinsed with an EWOD-driven water droplet, while spectra in the inset panel are from not-rinsed spots.

droplets to a single spot and subsequently remove unwanted impurities represents an advantage for EWOD−MALDI-MS over techniques lacking the latter capacity.[6−10]

MS of Proteolytic Digests. Modern proteomics relies on identification of unknown proteins by enzymatic digestion and comparison of the resulting peptide mass fragment (PMF) spectra to those predicted from protein sequence databases. Many proteins can be digested under mild conditions, but some, such as globular and membrane proteins, require a denaturant for complete digestion. Myoglobin and ubiquitin require harsh conditions to digest completely;[50] we have used these proteins to investigate EWOD−MALDI with integrated sample purification for PMF identification.

The lysine-specific protease Lys-C, which is active at high concentrations of urea, was used to digest myoglobin and ubiquitin in 6 M urea solutions. Digest solutions were then moved, purified, and analyzed by EWOD−MALDI-MS. Digest spectra were compared to the SwissProt database using the Mascot protein identification search engine,[44] as shown in Figure 6. In spectra of EWOD-purified samples (Figure 6a,b, main panels), peptides corresponding to myoglobin fragments 1−16 (m/z 1815), 17−42 (m/z 2858), 64−77 (m/z 1379), 80−96 (m/z 1853), 103−118 (m/z 1884), 119−133 (m/z 1502), and 134−145 (m/z 1360) were identified, as well as peptides corresponding to ubiquitin fragments 12−27 (m/z 1787), 34−48 (m/z 1668), 49−63 (m/z 1779), and 64−76 (m/z 1450). In comparison, spectra of control samples (Figure 6a,b, inset panels) had a few of the predicted peaks, but with much weaker signals than those from rinsed samples.

It should be noted that the mass accuracy observed for peaks detected by EWOD−MALDI was ~0.01−0.04%, which is slightly less than the 0.01% accuracy that is typical for PMF searches. Mass shifts have been observed in spectra collected from other dielectric MALDI targets.[33,37,43] They are caused by charging and by slight flight path differences that result from using a surface with a nonstandard height. These effects were minimized by using the reflector mode and by calibrating with standards. For future experiments requiring higher mass accuracy, we will use an orthogonal time-of-flight mass spectrometer (e.g., quadrupole TOF) to negate charging effects.

EWOD−MALDI-generated spectra were compared to data generated using ZipTips.[28] Five spots of a myoglobin digest in 6 M urea were prepared by each method. (ZipTip eluent was deposited onto an unmodified stainless steel MALDI target.) Spectra were evaluated in terms of maximum signal strength (the height of the most intense peak), the number of peptides assigned by Mascot, the percent of the sequence coverage, and the Mowse score.[51] As shown in Table 1, EWOD−MALDI was superior in each criterion. While the higher signal is attributed to the reported

(50) Russell, W. K.; Park, Z. Y.; Russell, D. H. *Anal. Chem.* **2001**, *73*, 2682−2685.

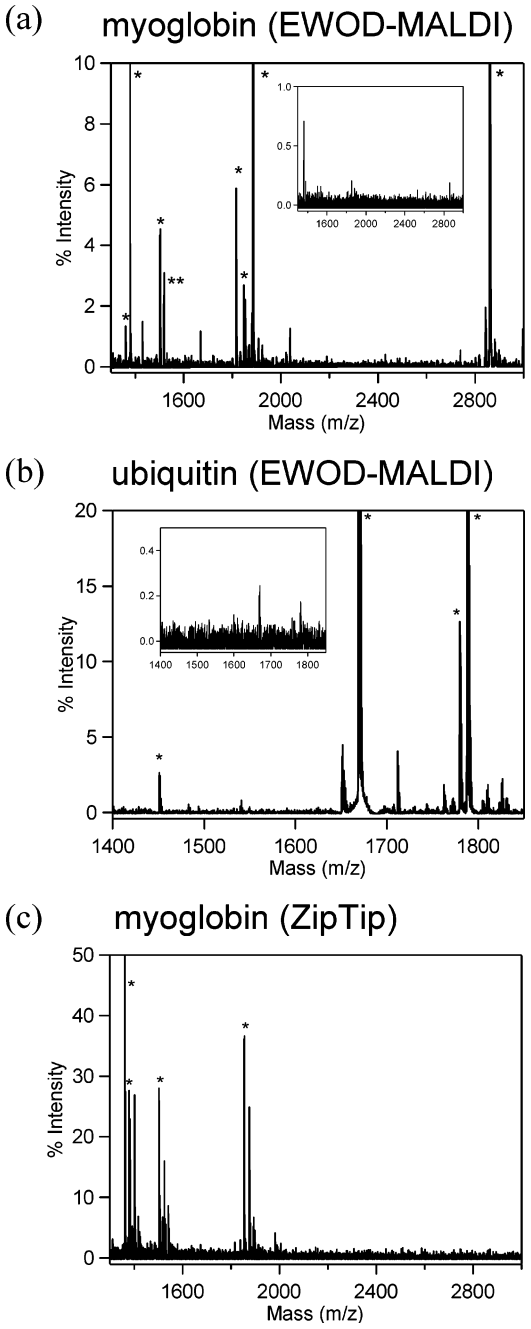

Figure 6. MALDI-MS spectra of Lys-C digests of (a) equine myoglobin, 16951 Da, and (b) bovine ubiquitin, 8565 Da, purified by an EWOD-driven water droplet, and (c) equine myoglobin purified with a ZipTip. All spots contained digest peptides of 1 pmol of total protein. For (a) and (b), the main panels show spectra of rinsed spots, and the insets spectra of control spots, all obtained directly from the EWOD device. For (c), an aliquot of myoglobin digest was purified on a C_{18} ZipTip column and eluted onto a stainless steel MALDI target. In the spectra of purified samples, peaks corresponding to peptides predicted by the Mascot protein identification search engine are marked with an asterisk. In (a), a double asterisk at $m/z = 1518$ marks an oxidized variant of fragment 119−133 (m/z = 1502). The main panel spectra are shown with the largest peaks off-scale to better reveal the smaller peaks. Each inset panel spectrum was scaled to the maximum signal in the corresponding main panel spectrum.

gain for spots prepared on hydrophobic surfaces,[33−43] we believe that the improvement in peptide coverage arises from mechanistic differences between the two methods, discussed below.

Table 1. Comparison of the EWOD−MALDI Method to the ZipTip Method[a]

	most intense peak height	no. of peptides identified	% coverage	Mowse score
EWOD-MALDI	2900 (1500)	6.2 (1.8)	64 (12)	120 (30)
ZipTip	960 (650)	2.8 (1.0)	25 (9)	40 (20)

[a] SD in parentheses.

The critical difference between ZipTip and EWOD−MALDI methods is that analytes purified by the former method must be desorbed and eluted, while EWOD-purified analytes are already in place for analysis. Analyte desorption is problematic for larger, more hydrophobic peptides that adsorb tightly to the C_{18} packing material of the ZipTip.[42] For example, the largest peptide in the myoglobin digest (m/z = 2858, corresponding to fragment 17−42) was not detected in the ZipTip spectra (Figure 6c). By contrast, it generated the most intense peak in the EWOD−MALDI spectra (Figure 6a).

In this comparison, the analyte concentrations were kept constant for the two purification techniques by desorbing the analytes from ZipTips in 10 μL of 50% acetonitrile with 0.1% TFA. When eluted with only 2.5 μL, the peak at m/z = 2858 was observed, although it was still weaker than in the EWOD−MALDI spectra (data not shown). An analogous increase in concentration and signal may be achieved for EWOD−MALDI-MS by depositing multiple sample droplets to a single spot (Figure 5). For 1 pmol of myoglobin digest peptides, multiple spotting was not necessary. In fact, the high S/N ratio in the Figure 6a spectrum indicates that much lower quantities should be detectable.

CONCLUSION

A new electrowetting-based method for sample purification has been demonstrated to reduce or eliminate the adverse effects of moderate and high concentrations of contaminants on MALDI-MS spectra. The method also out-performs the conventional technique of ZipTip purification for a PMF database identification of a myoglobin digest. The utilization of a Teflon-AF EWOD device surfaces is advantageous, in that a wide range of proteins and peptides adsorb to them. We anticipate that this novel coupling of EWOD−MALDI-MS with in-line sample purification will facilitate development of fully integrated proteomics methodologies, in which samples will be moved, reduced, digested, purified, and analyzed by MALDI-MS. If future applications require specificity, devices might be constructed to incorporate spots that are charged,[52] or spots to which antibodies or other molecular recognition elements have been covalently attached.[53] More generally, the digital microfluidics purification method described here may be useful in any application that would benefit from the removal of unwanted, hydrophilic impurities.

(51) Probability that a particular protein assignment is not random, given as −[log(P)]. For the conditions used here, a Mowse score of >49 is significant (p < 0.05).

(52) Xu, Y. D.; Bruening, M. L.; Watson, J. T. *Anal. Chem.* **2004**, *76*, 3106−3111.

(53) Nedelkov, D.; Nelson, R. W. *J. Mol. Recognit.* **2003**, *16*, 15−19.

ACKNOWLEDGMENT

We thank Debalina Chatterjee for help with device fabrication. The Center for Cell Mimetic Space Exploration (CMISE) [a NASA University Research, Engineering and Technology Institute (URETI), under Award No. NCC 2-1364], the NSF XYZ-On-Chip Program, and the DARPA BioFlips Program have supported this work. The W. M. Keck Foundation provided support to establish the UCLA Mass Spectrometry and Proteomics Technology Center. A.R.W. thanks the NIH for an NRSA postdoctoral fellowship.

Received for review August 20, 2004. Accepted October 29, 2004.

AC048754+