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# A Digital Microfluidic Approach to Proteomic Sample Processing

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**A common characteristic for proteomic analyses is the need for extensive biochemical processing. Digital microfluidics (DMF), a technique characterized by the manipulation of discrete microdroplets (100 nL−10 μL) on an open array of electrodes, is a good match for carrying out rapid, automated solution-phase reactions. Here, we report a DMF-based method integrating several common processing steps in proteomics, including reduction, alkylation, and enzymatic digestion. Fluorogenic assays were used to quantitatively evaluate the kinetics and reproducibility of each reaction step, and MALDI-MS was used for qualitative confirmation. The method is fast, facile, and reproducible, and thus has the potential to be a useful new tool in proteomics.**

There is growing consensus that current proteomic analysis methods are limited by non-standardized sample handling and processing. For example, the Human Proteome Organization (HUPO) recently published a comprehensive study of "preanalytical variables that can potentially impact the outcome of results, but are not related to inherent sample differences."[1] According to the study, a key source of variance is the ad hoc nature of sample processing methods used by laboratories around the world. In fact, the error introduced to analyses by non-standard processing is so great that different laboratories analyzing the same sample can obtain orthogonal results. It seems likely that the promise of proteomics for clinical applications[2] will not be realized until these problems can be solved.

A technology that may provide some relief for ad hoc and irreproducible proteomic sample processing is miniaturization and integration in microfluidics.[3] For example, networks of enclosed microchannels have been used to implement the key proteomic processing step of enzymatic digestion in a variety of formats, including trypsin-coated beads[4,5] or membranes,[6] and immobilized enzymes in sol−gels[7,8] or polymer monoliths.[9] These formats, which have high surface-area-to-volume ratios, have favorable reaction kinetics and are a good match for applications requiring a single processing step. However, we posit that enclosed channels are not a good match for carrying out multistep processing regimens. This is illustrated in Figure 1a: diffusion and uncontrolled fluid flows[10,11] make it nearly impossible to control a complicated series of reactions between well-defined plugs of reagents in microchannels. Unfortunately, this challenge is typical in proteomics; for example, in shotgun proteomics, samples are subjected to a multiday, multistep procedure including acidification, denaturing, reduction, alkylation, enzymatic digestion (twice), purification, and dilution prior to analysis by separations and mass spectrometry.[12] We acknowledge that microvalves[13] may offer some relief from this problem, but we submit that in general, microchannels are ill-suited for implementing the complicated series of reactions necessary for many proteomic applications (with some exceptions noted[14]).

In contrast to microchannels, we propose that digital microfluidics (DMF) is a good match for implementing multistep series of reactions (Figure 1b). DMF[15,16] is a fairly new fluid handling technique characterized by the manipulation of discrete droplets on an open array of electrodes.[17,18] By applying a sequence of

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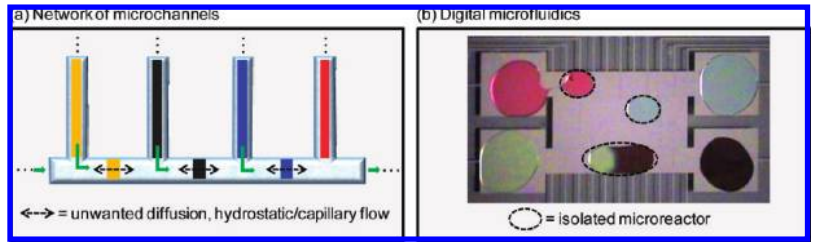


**Figure 1.** Cartoon (a) and picture (b) depicting multistep reactions in a network of microchannels and in a digital microfluidic device. The picture in (b) is adapted from reference 15 with permission from the American Association for the Advancement of Science (AAAS).

potentials to adjacent electrodes, a droplet of fluid can be dispensed from a reservoir, transported on the array, and merged with other droplets to implement nanoliter-scale reactions. Because each droplet is isolated from its surroundings rather than being embedded in a stream of fluid, DMF is a facile method for forming microreactors in which there is no possibility that reagents or samples will diffuse away. Most importantly, because each droplet is controlled individually, multistep methods involving many different reagents can be easily programmed.

In the past, digital microfluidics has primarily been used for relatively simple applications comprising one or two steps, including cell-based assays,[19] enzyme assays,[20,21] sample preparation for mass spectrometry,[22−24] immunoassays,[25,26] and the polymerase chain reaction.[27] Here, we report an integrated method for implementing three discrete processing steps, including proteomic sample reduction, alkylation, and digestion; this paper joins related work[28−30] in demonstrating the compatibility of DMF with multistep proteomic sample processing. The qualitative and quantitative data presented here suggest that DMF is capable of highly repeatable sample processing, and thus may be a useful new tool for standardized proteomics analyses.

## EXPERIMENTAL SECTION

**Reagents and Materials.** Bovine serum albumin (BSA), lysozyme from chicken egg white, insulin from bovine pancreas, TPCK-treated trypsin from bovine pancreas, pepsin from porcine gastric mucosa, endoproteinase Lys-C (Lys-C), iodoacetamide, Tris-HCl, Pluronic F127, Fluorinert FC-40, acetonitrile (ACN), and acetic acid were purchased from Sigma Chemical (Oakville, ON). Tris-(2-carboxyethyl)phosphine hydrochloride (TCEP) and E6638 EnzChek Protease Assay Kit were purchased from Invitrogen (Burlington, ON). Ziptip pipet tips ($C_4$ and $C_{18}$) were purchased from Millipore (Etobicoke, ON), and α-cyano-4-hydroxycinnamic acid (α-CHCA) and sinapinic acid (SA) were purchased from Waters Limited (Mississauga, ON). Unless otherwise indicated, all protein and processing reagent solutions were prepared in working buffer (100 mM Tris-HCl, pH 7.8, 0.08% w/v Pluronic F127) immediately prior to use.

Clean room reagents and supplies included Shipley S1811 photoresist and MF321 developer from Rohm and Haas (Marlborough, MA), AZ300T photoresist stripper from AZ Electronic Materials (Somerville, NJ), solid chromium from Kurt J. Lesker Canada (Toronto, ON), CR-4 chromium etchant from Cyantek (Fremont, CA), hexamethyldisilazane (HMDS) from Shin-Etsu MicroSi (Phoenix, AZ), and conc. sulfuric acid and hydrogen peroxide (30%) from Fisher Scientific Canada (Ottawa, ON). Piranha solution was prepared as a 3:1 (v/v) mixture of sulfuric acid and hydrogen peroxide. Parylene-C dimer was from Specialty Coating Systems (Indianapolis, IN), and Teflon-AF was from DuPont (Wilmington, DE).

**Device Fabrication.** Digital microfluidic devices were fabricated in the University of Toronto Emerging Communications Technology Institute (ECTI) facility using chrome-on-quartz photomasks printed at the University of Alberta NanoFab (Edmonton, AB). Briefly, glass wafers were cleaned in piranha solution (10 min), and then coated with chromium (200 nm) by electron beam deposition. After rinsing and baking on a hot plate (115 °C, 5 min), the substrates were primed by spin-coating with HMDS (3000 rpm, 30 s) and were then spin-coated with Shipley S1811 photoresist (3000 rpm, 30 s). Substrates were pre-baked on a hot plate (100 °C, 2 min) and exposed through a photomask using a Suss Mikrotek mask aligner. Substrates were developed in MF321 (3 min), and then post-baked on a hot plate (100 °C, 1 min). After photolithography, substrates were immersed in chromium etchant (30 s). Finally, the remaining photoresist was stripped in AZ300T (10 min).

After forming electrodes, devices were coated with parylene-C (2 μm) and Teflon-AF (50 nm). Parylene-C was applied using a vapor deposition instrument (Specialty Coating Systems), and Teflon-AF was spin-coated (1% w/w in Fluorinert FC-40, 2000 rpm, 60 s) and then post-baked on a hot-plate (160 °C, 10 min). To facilitate the application of driving potentials, the polymer coatings were locally removed from the contact pads by gentle scraping with a scalpel. In addition to patterned devices, unpatterned indium tin oxide (ITO) coated glass substrates (Delta Technologies Ltd., Stillwater, MN) were coated with Teflon-AF (50 nm, as above).

**Device Operation.** Devices were assembled with an unpatterned ITO/glass top plate and a patterned bottom plate separated

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by a spacer formed from one piece of double-sided tape (~70 $\mu$m thick). Droplets were sandwiched between the two plates and actuated by applying electric potentials between the top electrode and sequential electrodes on the bottom plate. The driving potentials of 60−80 $V_{rms}$ were generated by amplifying the output of a function generator operating at 18 kHz, and were applied manually to exposed contact pads on the bottom plate surface. Droplet actuation was monitored and recorded by a CCD camera mounted on a stereomicroscope with fluorescence imaging capability (Olympus Canada, Markham, ON). The devices used here had four 2.5 × 2.5 mm reservoir electrodes mated to a 4 × 6 array of 1 × 1 mm actuation electrodes, with interelectrode gaps of 5−40 $\mu$m. Each "unit" droplet covered a slightly larger area than a single actuation electrode such that the volume in each droplet was ~100 nL.

**Qualitative Analysis.** The proteomic processing work-flow was analyzed in four stages: (i) prior to reaction, (ii) after reduction, (iii) after alkylation, and (iv) after digestion. Insulin (40 $\mu$M in working buffer) was used as a model analyte for evaluating stages (i−iii). For stage (i), a droplet of insulin was dispensed by DMF and then analyzed. For stage (ii), droplets of insulin and TCEP (10 mM in working buffer) were dispensed and merged by DMF followed by incubation (room temp., 30 min). For stage (iii), droplets of insulin and TCEP were dispensed, merged and incubated (as in stage ii), and the product was then reacted with a third droplet of iodoacetamide (12 mM in working buffer) and incubated again (room temp., 2 min). Lysozyme (40 $\mu$M in working buffer) and BSA (10 $\mu$M in working buffer) were used as model analytes for stage (iv). In these experiments, droplets of lysozyme or BSA were dispensed and processed as in stage (iii) (above), followed by reaction with a droplet of trypsin (1:5 w/w of enzyme:protein in working buffer) and incubation (room temp., 60 min). In all cases, incubation steps were carried out in a humidified chamber (a Petri dish partially filled with deionized water, $dH_2O$) to limit evaporation. At least three replicate samples were evaluated for each condition.

After processing by DMF, each sample was purified using a ZipTip and then analyzed by matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). Briefly, ZipTips were wetted in 100% ACN containing 0.1% acetic acid (3×) and then equilibrated in 10% ACN containing 0.1% acetic acid (3×). Aqueous samples were drawn in and out of tips for 7−10 cycles, and then the solid medium was washed with 10% ACN containing 0.1% acetic acid (3×). Finally, samples were eluted in 100% ACN containing 0.1% acetic acid (2 × 1 $\mu$L) onto a stainless steel MALDI target plate. A 1 $\mu$L matrix solution was added to each sample: SA (10 mg/mL in 50:50 ACN:$dH_2O$ containing 0.1% acetic acid) for intact proteins (stage i), and α-CHCA (10 mg/mL in 50:50 ACN:$dH_2O$ containing 0.1% acetic acid) for reduced, alkylated, and digested products (stages ii−iv). After drying, spots were analyzed using a Micro-MX mass spectrometer (Waters, Milford, MA) operating in positive and reflectron modes over a mass to charge ratio ($m/z$) range from 500−6,500. At least one hundred shots were collected per spectrum, with laser power tuned to optimize the signal-to-noise ratio (S/N). Data were processed by normalization to the largest analyte peak, baseline subtraction, and smoothed with a 15-point running average. Spectra of enzyme digests were analyzed using the Mascot protein identification package searching the SwissProt database, with 3 allowed missed cleavages, a mass accuracy of ±1.2 Da, and with a fixed carbamidomethyl modification.

**Reaction Rate Analysis.** Fluorogenic assays were used to evaluate reaction rates of reduction and digestion on DMF devices. For the former, the analytes were BSA (10 $\mu$M in working buffer), insulin (40 $\mu$M in working buffer), and lysozyme (40 $\mu$M in working buffer), the reductant was TCEP (10 mM in working buffer), and the reporter was ABD-F (12 mM in working buffer). In each reaction, three droplets containing analyte, TCEP, and ABD-F were dispensed, merged, and rapidly mixed. Reaction progress was followed by fluorescence detection with $\lambda_{ex}$ = 390 nm and $\lambda_{em}$ = 510 nm. For the latter (digestion assays), the proteases were Lys-C (0.07 mg/mL in working buffer), pepsin (0.07 mg/mL in 100 mM HCl, pH 2.0 containing 0.08% w/v Pluronic F127), and trypsin (0.07 mg/mL in working buffer), and the reporter/analyte was the EnzChek reagent, intramolecularly quenched, fluorescently labeled BODIPY-Casein (0.33 mg/mL in working buffer). In each case, droplets of BODIPY-Casein and enzyme were dispensed, merged, and rapidly mixed, and then evaluated by fluorescence detection with $\lambda_{ex}$ = 485 nm and $\lambda_{em}$ = 520 nm.

In both types of assays, the fluorescence was quantified using a PheraStar multiwell plate reader (BMG Labtech, Durham, NC) as described previously.[19,20,31,32] In each assay, fluorescence from processed samples was measured in intervals of 10 min for 1 h. Between intervals, devices containing the reacting droplets were stored in a humidified chamber to prevent evaporation. Three replicate trials were conducted for each condition, and data were normalized and fitted with exponential functions.

**Reproducibility Analysis.** The reproducibility of the reduction and digestion assays was evaluated using methods similar to those described above. Briefly, for reduction, droplets containing 6 picomole aliquots of BSA, insulin, or lysozyme (~100 nL of 60 $\mu$M protein in working buffer) were dispensed and mixed with ~100 nL droplets of TCEP (10 mM in working buffer) and ABD-F (12 mM in working buffer). For digestion, droplets containing 0.17 $\mu$g aliquots of BODIPY-Casein (~100 nL of 1.7 mg/mL protein in working buffer) were dispensed and mixed with ~100 nL droplets of one of either Lys-C (0.3 mg/mL in working buffer), pepsin (0.3 mg/mL in 100 mM HCl, pH 2.0 containing 0.08% w/v Pluronic F127), or trypsin (0.3 mg/mL in working buffer). For both assays, the final combined droplets were allowed to incubate (45 min) in a humidified chamber and were then evaluated using a PheraStar multiwell plate reader. Three replicate trials were conducted per reaction.

## RESULTS AND DISCUSSION

**Multistep Proteomic Processing on DMF Devices.** The DMF array geometry is a natural fit for carrying out sequential biochemical assays, such as proteomic sample processing. The device shown in Figure 2 was designed for handling proteins and proteomic reagents to facilitate three sequential reactions: reduction of disulfides, alkylation of free thiols, and proteolytic digestion. As shown, in a typical analysis, a droplet containing the sample to be processed (in this case, insulin), was dispensed (Figure 2b) and merged with a droplet containing reducing agent (TCEP).

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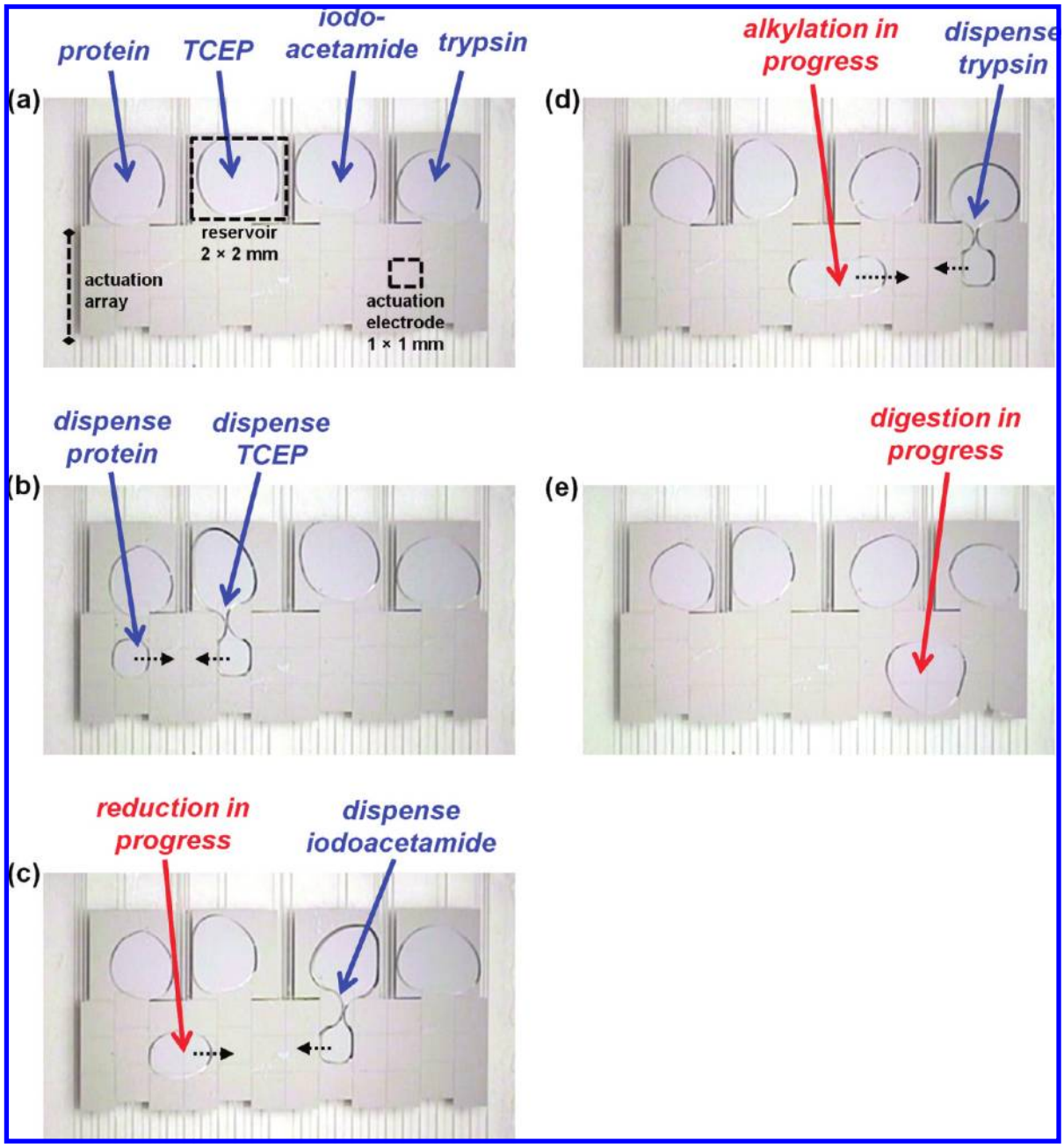


**Figure 2.** Sequence of images depicting a typical proteomic sample processing workup. (a, b) Droplets containing insulin (40 $\mu$M) and TCEP (10 mM) are dispensed from reservoirs, merged and actively mixed. After incubating, (c) a droplet of iodoacetamide (12 mM) is dispensed and merged with the sample droplet and actively mixed. After incubating again (d), the sample droplet is merged with a droplet of trypsin (1:5 enzyme to protein ratio w/w) and then (e) incubated a final time.

The stability, selectivity, and rapid reaction rate at room temperature makes TCEP a good replacement for the more commonly used dithiothreitol (DTT), which must be removed prior to further processing.[33] After reduction (Figure 2c), the sample droplet was merged with a droplet containing iodoacetamide (Figure 2d), which covalently binds to cysteine groups preventing reformation of disulfide bonds. Finally, the sample droplet was merged with a droplet containing protease (in this case, trypsin) to digest the protein into constitutive peptides (Figure 2e).

MALDI-MS was used to qualitatively evaluate DMF-driven processing reactions; Figure 3 shows representative spectra collected from each step in the process. As expected, the most significant feature in spectra of non-processed insulin (Figure 3a) is a peak at 5.7 kDa, corresponding to the singly charged intact analyte, while the smaller peak at 2.9 kDa is its doubly charged equivalent. Insulin is made up of two subunits, an α-chain (2.3 kDa) and $\beta$-chain (3.4 kDa), that are held together by disulfide bonds,[34] and peaks corresponding to each of them are apparent in spectra collected after reduction (Figure 3b). After reduction, reaction with iodoacetamide adds 57 Da to each thiol group; as

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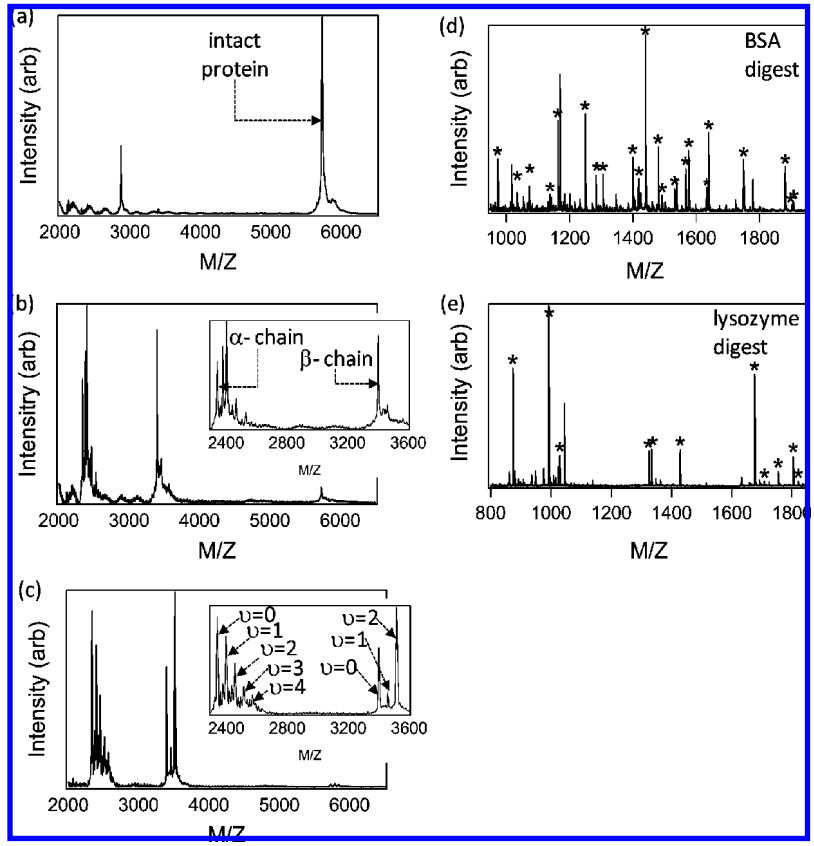


**Figure 3.** Representative MALDI-MS spectra of samples prepared by DMF-driven processing. (a) Non-processed insulin, (b) insulin after reduction, (c) insulin after reduction and alkylation, where $\upsilon$ denotes the number of alkylated thiols, and (d, e) BSA and lysozyme after reduction, alkylation, and tryptic digestion. In the latter, asterisks denote peptides identified by Mascot; Mowse scores were 167 for both spectra, yielding correct sample identifications ($p < 0.05$). Peptide coverages were 56 and 74%, respectively.

shown in Figure 3c, after intermediate reaction times, several peaks are observed corresponding to varying degrees of alkylation (where $\upsilon$ represents the number of reacted thiol groups). When reacted for longer times (~10 min), the analytes were completely alkylated (spectra not shown), but even after intermediate times, the peak representing the unreacted sample at 5.7 kDa has essentially disappeared. Spectra of insulin samples after digestion (not shown) confirm the efficacy of the complete regimen. We also applied on-chip reduction, alkylation, and digestion to larger analytes such as BSA (Figure 3d) and lysozyme (Figure 3e). Asterisks in these spectra denote peptides identified by the database search engine, Mascot. These data demonstrate that DMF is a robust tool capable of implementing all of the key steps in proteomic processing.

The work reported here is one of few examples of multistep reactions implemented by digital microfluidics. This work required close attention to two key phenomena: evaporation and non-specific adsorption. The former is particularly significant for procedures involving multiple incubation steps, as we observe droplets to evaporate at ~100−1000 nL/h, depending on the ambient humidity. To counteract this phenomenon, we positioned devices in a humidified chamber during all incubation steps, which eliminated any noticeable evaporation for the duration of experiments. While all the work reported here was performed at room temperature, in other contexts, we have observed this measure to be sufficient for keeping samples hydrated at 37 °C for >24 h.[19] The latter phenomenon, non-specific adsorption, is a particularly important concern for proteomics, as proteins are highly susceptible to sticking to hydrophobic surfaces (e.g., Teflon-AF-coated DMF devices). In the work reported here, protein sticking was controlled by the use of low concentrations of amphiphilic polymer additives.[31] In other contexts, we have observed that these additives have little or no effect on biochemical processes ranging from cell vitality[19] to enzyme kinetics,[20] and can prevent adsorption even in highly concentrated, heterogeneous solutions such as serum.[31] The combination of these strategies, that is, incubation in humidified chambers and the use of adhesion-reducing additives, facilitated the development of these digital microfluidic techniques.

**Processing Time.** Fluorogenic assays were used to determine the reaction rates of reduction and digestion by DMF. For reduction, droplets of sample, reductant (TCEP), and fluorogenic dye (ABD-F) were dispensed from reservoirs, merged and actively mixed, and allowed to react at room temperature. ABD-F is non-fluorescent until it reacts with a free sulfhydryl group, and thus can serve as a reporter for reaction progress in reduction of disulfide bonds.[35,36] Three analytes were evaluated, insulin (3 disulfides), lysozyme (4 disulfides), and BSA (17 disulfides + 1 free sulfhydryl group), and

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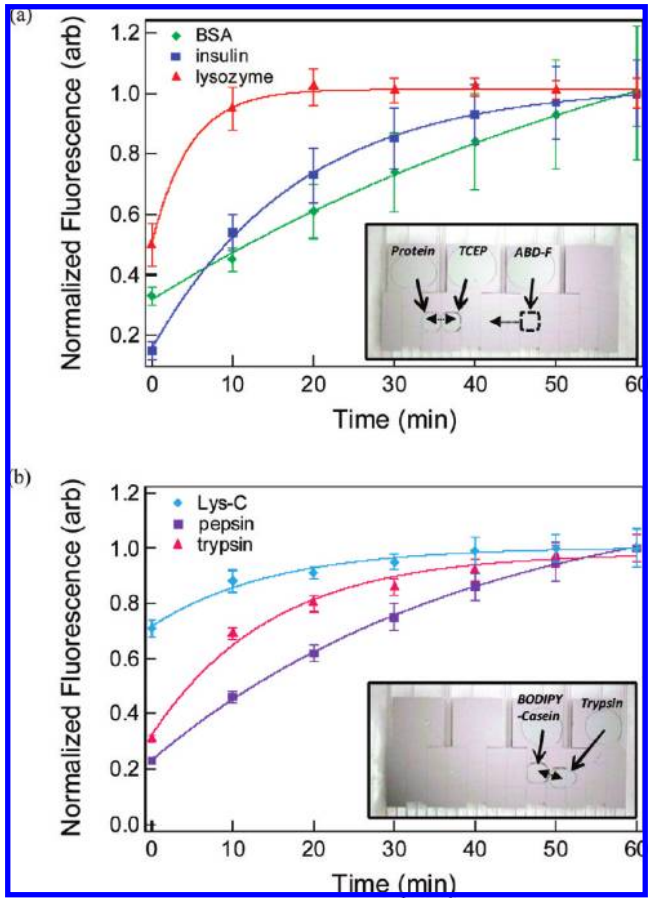


**Figure 4.** Rates of reactions. (a) DMF-driven reduction of BSA (10 $\mu$M), insulin (40 $\mu$M), and lysozyme (40 $\mu$M) with reductant, TCEP (10 mM), and ABD-F dye (12 mM) as a fluorogenic reporter. (b) DMF-driven digestion of BODIPY-Casein (0.33 mg/mL) with Lys-C (0.07 mg/mL), pepsin (0.07 mg/mL), and trypsin (0.07 mg/mL). Error bars are $\pm$ 1 SD.

representative data are shown in Figure 4a. Digestion was evaluated using an intramolecularly quenched, fluorescently labeled protein standard (BODIPY-Casein), which becomes progressively more fluorescent as it is digested.[37] Representative data for three proteases, trypsin, pepsin, and Lys-C, are shown in Figure 4b.

DMF-driven reduction and digestion reactions are 90% complete within 15−45 min and 10−50 min, respectively. This suggests that a full sample processing workup (including protein extraction by precipitation[32]) would require approximately 2 h to complete. This time frame is fast relative to conventional methods,[12] but we note that longer times will likely be required for more complex samples (e.g., serum or cell lysate). Regardless, the automation and potential for scaling to parallel sample preparation suggests that the new DMF method will be characterized by improved throughput relative to the manual techniques that are typically used.

**Reproducibility.** A critical issue in proteomics is a lack of reproducible, standardized sample handling and processing methods.[1] We used the fluorogenic assays described above to evaluate the experimental error in DMF-driven on-chip reduction and digestion. As shown in Figure 5, the digital microfluidic approach has relatively good precision, with %RSD ranging from 3−7% for the reduction assay and 2−5% for the digestion assay. The reduced precision for the reduction assay compared to the digestion is likely caused by the extra dispensing step (three droplets instead of two); we estimate that the %RSD for dispensing one droplet in these devices is ~1−3%. The dispensing methods used here (i.e., application of driving potentials to 3 electrodes adjacent to a reservoir, resulting in necking and formation of a unit droplet[24]) are relatively crude; methods relying on more sophisticated active-feedback control can facilitate higher precision metering.[38,39] Regardless, the advantage of processing on a single substrate is significant, as the multiple containers and transfers inherent in macroscale/manual sample handling can lead to loss of sample and introduction of contaminants such as human keratin.[40] In addition, if widely adopted, the automation of DMF would eliminate the human error introduced by technicians working in different laboratories.

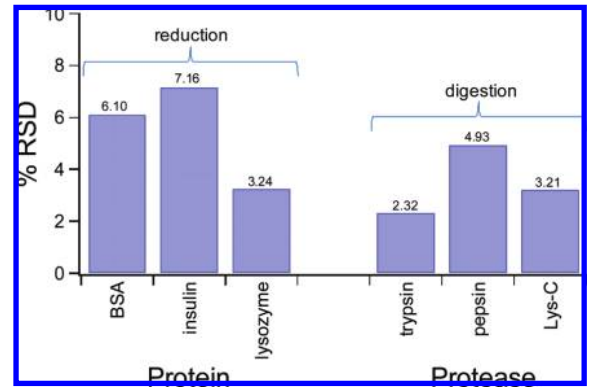


**Figure 5.** Experimental precision in DMF-driven assays. The precision in reduction was evaluated for BSA, insulin, and lysozyme (6 picomoles ea.) using TCEP (10 mM) as a reductant and ABD-F (12 mM) as the fluorogenic reporter. The precision in digestion was evaluated using BODIPY-Casein (0.17 $\mu$g) combined with trypsin (0.03 mg/mL), pepsin (0.03 mg/mL), and Lys-C (0.03 mg/mL) proteases. All samples were analyzed in triplicate.

The data in Figure 4 demonstrates that digital microfluidics can be used for multistep proteomic processing with high reproducibility. This is an important step forward in our plans to develop a fully integrated proteomic processing platform. Because DMF is compatible with real-world samples (e.g., undiluted serum[31,32]) and large volumes (up to milliliters[41]) and can be integrated with separations,[42] we speculate that this technique may someday contribute to a solution to the well-documented repeatability problems in sample preparation for proteome profiling.

## CONCLUSION

The lack of standardized sample handling and processing in proteomics is a major limitation for the field. Here, we have shown

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that digital microfluidics (DMF) is a powerful tool for rapid, reproducible, automated sample processing. We propose that DMF has the potential for being useful across a broad range of applications for standardized analysis, as it can be precisely duplicated in any laboratory across the world.

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