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RESEARCH ARTICLE

Digital microfluidic hydrogel microreactors for proteomics

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Proteolytic digestion is an essential step in proteomic sample processing. While this step has traditionally been implemented in homogeneous (solution) format, there is a growing trend to use heterogeneous systems in which the enzyme is immobilized on hydrogels or other solid supports. Here, we introduce the use of immobilized enzymes in hydrogels for proteomic sample processing in digital microfluidic (DMF) systems. In this technique, preformed cylindrical agarose discs bearing immobilized trypsin or pepsin were integrated into DMF devices. A fluorogenic assay was used to optimize the covalent modification procedure for enzymatic digestion efficiency, with maximum efficiency observed at 31 μg trypsin in 2-mm diameter agarose gel discs. Gel discs prepared in this manner were used in an integrated method in which proteomic samples were sequentially reduced, alkylated, and digested, with all sample and reagent handling controlled by DMF droplet operation. Mass spectrometry analysis of the products revealed that digestion using the trypsin gel discs resulted in higher sequence coverage in model analytes relative to conventional homogenous processing. Proof-of-principle was demonstrated for a parallel digestion system in which a single sample was simultaneously digested on multiple gel discs bearing different enzymes. We propose that these methods represent a useful new tool for the growing trend toward miniaturization and automation in proteomic sample processing.

Keywords:
Agarose / Digital microfluidics / Electrowetting / Enzymatic digestion / Hydrogels / Technology

1 Introduction

This paper describes a new miniaturized method for eventual application to proteome profiling, the identification of all of the proteins that are present in a biological sample. Proteome profiling is an important research goal, and is being used to identify biomarkers for diagnosis and prognosis of disease [1–3], to compare protein expression levels for clinical monitoring of drug efficacy [4, 5] or toxicity [4, 6, 7], and may someday be useful for personalized medicine [8]. Unfortunately, large-scale protein identification is hampered by the complex, multistep sample processing regimens that are required prior to analysis. For example, proteomic samples are typically digested into smaller peptides by exposure to proteolytic enzymes prior to analysis; this step is widely recognized as being a significant bottleneck for proteome profiling [9]. In conventional in-solution (homogenous) enzymatic digestion, the enzyme concentration is kept low (1:50

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Abbreviations: Agr, Agarose; **DI**, deionized; **DMF**, digital microfluidics; **IAM**, iodoacetamide; **RT**, room temperature; **TCEP**, tris-(2-carboxyethyl)phosphine

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Colour Online: See the article online to view Figs. 1–5 in colour.

enzyme-to-proteomic sample ratio) to avoid autolysis (i.e. self-digestion of enzyme). These low ratios result in lengthy incubation times (often overnight) and require the use of elevated temperatures (37°C). Furthermore, these conditions lead to an increase in digestion artifacts such as transpeptidation and nonspecific cleavage [10] and deamidation/oxidation [11, 12], which complicate analysis and reduce confidence in the identification of proteomic analytes.

Many of the disadvantages of homogeneous (in-solution) enzyme digestion can be avoided by using systems in which liquid proteomic samples are exposed to proteolytic enzymes immobilized on solid supports [13]. In such heterogeneous systems, autolysis is virtually eliminated, such that much higher enzyme-to-substrate ratios can be used, which results in significant improvements in reaction rates [14, 15]. In addition, heterogeneous enzyme digestion systems have advantages of long-term storage stability and resiliency toward heat-induced denaturation and harsh solvents. Many formats exist for heterogeneous digestion systems, including microspin columns (http://www.sigmaaldrich.com/life-science/proteomics/mass-spectrometry/trypsin-spin-column.html), microspheres/beads [16,17] (https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=catProductDetail&productID=2-3128-00&catID=601950&backButton=true), polymer monoliths [18, 19], sol-gel polymers [20,21], the inner surfaces of capillary walls [22,23], and various types of membranes [24, 25]. But the most promising systems for heterogeneous enzyme digestion are likely those relying on three-dimensional hydrogels as scaffolds for housing immobilized enzymes [26–29]. Such systems are advantageous for several reasons. First, enzymes are intrinsically fragile and the hydrogel matrix serves as a near-native environment in which enzyme functions are preserved. Second, immobilization strategies for enzymes in hydrogels are typically simple and allow for optimal geometric congruence between the enzyme and solid support. Third, the high porosity of hydrogels allows for immobilization of very high densities of enzyme. And fourth, hydrogel supports can be easily molded to various shapes and sizes tailored to a specific application.

We recently reported a new tool for lab-on-a-chip systems: the combination of hydrogels and digital microfluidics (DMF) [30]. Here, we report the application of this tool to proteolytic digestion for proteome profiling, and demonstrate its superiority to conventional techniques. DMF is a fluid-handling technique in which liquid samples are manipulated on the surface of an array of electrodes by electrostatic forces [31], and has been applied to a variety of applications related to proteomics including sample purification by precipitation [32], reduction of disulfides [33, 34], alkylation of thiols [33–35], proteolytic digestion [33–36], and combinations thereof [37]. These previous reports represent important steps toward the goal of integrated proteome profiling, but they are limited by the challenges inherent to homogeneous digestions described above. We speculate that the new techniques presented here, which combine the fluid manipulation and system integration capabilities of DMF with the rapid and efficient reaction rates afforded by heterogeneous hydrogel microreactors will be a useful addition to the growing trend in miniaturization of proteomic analysis tools [38], and may eventually contribute to a new generation of methods for automated proteome profiling.

2 Methods and materials

2.1 Reagents and materials

BSA, lysozyme from chicken egg white, insulin from bovine pancreas, trypsin from bovine pancreas, pepsin from porcine gastric mucosa, iodoacetamide (IAM), tris-HCl, sodium acetate, acetic acid, pluronic F127, fluorinert FC-40, ACN, TFA, FITC, fluorescamine, and low melting point agarose were purchased from Sigma Aldrich Canada (Oakville, ON, USA). Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) and E6638 EnzChek® Protease Assay Kit were purchased from Life Technologies/Invitrogen (Burlington, ON, USA). $Ziptip_{C18}$® pipette tips were purchased from Millipore (Etobicoke, ON, USA), and α-CHCA was purchased from Waters Limited (Mississauga, ON, USA). Deionized (DI) water had a resistivity of 18 MΩ · cm at 25°C. Unless otherwise indicated, all protein and processing reagent solutions were prepared in working buffer (50 mM tris-HCl, pH 7.8, 0.08% w/v Pluronic F127 in DI water) immediately prior to use.

2.2 DMF device fabrication and operation

DMF devices were fabricated in the University of Toronto Emerging Communications Technology Institute (ECTI) cleanroom facility, using a transparent photomask printed at Pacific Arts and Design (Markham, ON, USA). DMF device bottom plates bearing patterned chromium electrodes were formed by photolithography and etching as described previously [39], and were coated with 15 μm of Parylene-C and 200 nm of Teflon-AF. Parylene-C was applied using a vapor deposition instrument (Specialty Coating Systems), and Teflon-AF was spin-coated (1% wt/wt in Fluorinert FC-40, 1600 rpm, 60 s) followed by postbaking on a hot plate (160°C, 10 min). The polymer coatings were removed from contact pads by gentle scraping with a scalpel to facilitate electrical contact for droplet actuation. In addition to patterned DMF device bottom plates, unpatterned glass microscope slides and DMF device top plates formed from indium tin oxide (ITO) coated glass substrates (Delta Technologies Ltd, Stillwater, MN, USA) were coated with Teflon-AF (200 nm, as above).

The device used here featured an array of 68 square actuation electrodes (2 × 2 mm ea.) connected to ten reservoir electrodes (5 × 5 mm ea.), with interelectrode gaps of 50–100 μm. Devices were assembled with an unpatterned ITO–glass top plate and a patterned bottom plate separated by a spacer formed from two pieces of double-sided tape (total

spacer thickness 140 μm). To actuate droplets, driving potentials (200–250 V_{pp}) were generated by amplifying the output of a function generator (Agilent Technologies, Santa Clara, CA, USA) operating at 18 kHz. Droplets were sandwiched between two plates and were actuated by applying driving potentials between the top electrode (ground) and sequential electrodes on the bottom plate via the exposed contact pads. Droplet actuation was monitored and recorded by a CCD camera mounted on a lens.

2.4 Hydrorogel disc fabrication

Low melting point agarose was dissolved in DI water to a concentration of 4.0 wt% by incubating the mixture in an oven at 70°C for 1 h. Some mixtures included 10-μm-diameter polystyrene beads (Sigma Aldrich Canada) to make the gels easier to visualize. After removing the mixture from the oven, 2 μL aliquots were pipetted and sandwiched between two unpatterned Teflon AF-coated glass slides held together with two pieces of double-sided tape as a 140 μm spacer. These assemblies were placed on a cold pack to allow the droplets to gel (~2 min). The resulting gel discs were approximately 1 mm in diameter and 140 μm height (~440 nL) and could be retrieved from the glass–slide assembly using tweezers. For use with DMF, gel discs were sandwiched between a bottom plate and a top plate, with each disc positioned such that it straddled the interface between two electrodes on the bottom plate.

2.5 Covalent attachment of enzymes to gels

As depicted in Fig. 1, enzymes were covalently attached to agarose gel discs using methods similar to those developed by Guisan and co-workers [40–42] in four steps. In step (i), each agarose gel disc was suspended in 2.4 μL DI water. Six hundred eighty nanoliter of aqueous sodium borohydride (28 mg/mL in 1.7 N NaOH) was then added to the suspension. The reaction vessel was placed on an ice bath followed by an addition of 134 nL glycidol. The reaction was incubated overnight (18 h, room temperature (RT)). Afterwards, the gels were washed with DI water and filtered using a Buchner funnel. In step (ii), each gel was suspended in 18 μL of an aqueous solution of sodium periodate (typically 3.5 mM; see next section for details) for 75 min followed by the same wash and filtration step as above. In step (iii), each gel was then suspended in 18 μL of an enzyme solution (4.4 mg trypsin/mL in 50 mM sodium bicarbonate, pH 10, or 4.4 mg pepsin/mL in 50 mM sodium acetate, pH 4.5) where it was incubated (72 h, RT). Finally in step (iv), 2 μL of aqueous sodium borohydride (10 mg/mL) was added to the suspension and incubated for 30 min (RT). The gels were isolated using a wash and filtration step as described above and stored at 4°C until use.

To evaluate the uniformity of covalent attachment of enzymes to gel discs, FITC-labeled trypsin was prepared by combining 1 mL of 2 mg/mL trypsin solution (in 0.1 M sodium

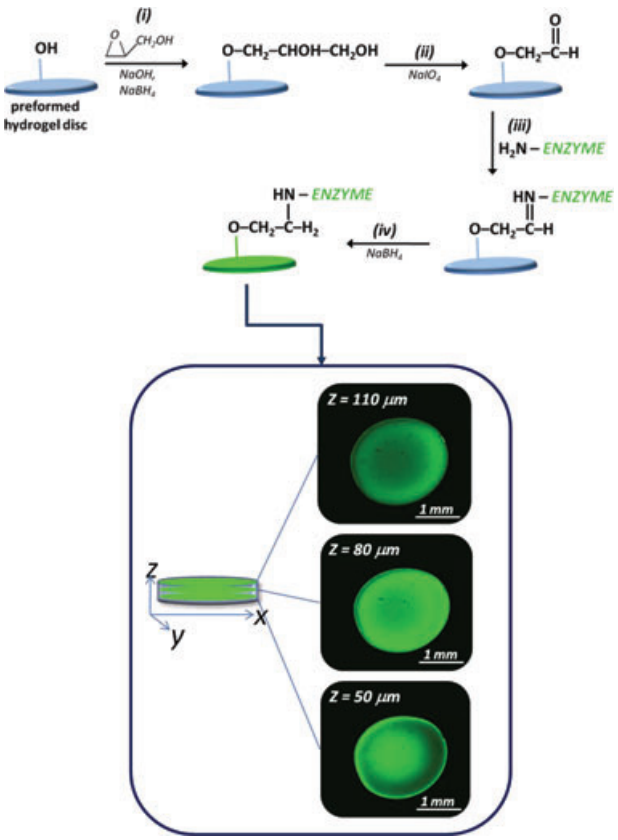

Figure 1. Immobilization of enzymes onto hydrogel discs. Reaction scheme (top): (i) preformed agarose discs (Agr–OH) are activated with glycidol (2,3-epoxypropanol) to form an intermediate glyceryl-agarose (Agr–O–CH_2–CHOH– $CH_2$0H); (ii) glyceryl-agarose is oxidized with periodate to generate glyoxyl-agarose (Agr–CH_2CHO); (iii) enzyme molecules are coupled to the aldehyde groups via the Schiff base reaction; (iv) reduction with sodium borohydride yields an enzyme-agarose derivative. *Z*-stack of cross-sectional fluorescent confocal images (bottom) of FITC-labeled trypsin attached to hydrogel discs. The scale bar is 1 mm.

carbonate buffer, pH 9) with 50 μL of 1 mg/mL FITC solution (in anhydrous DMSO). The reaction mixture was incubated for at least 8 h in the dark (4°C). Gels modified with this dye-labeled trypsin were formed as above, and were evaluated using a Leica TCS SP2 scanning confocal microscope (Leica Microsystems Canada, Richmond Hill, ON, USA) equipped with an Ar^+ laser (488 nm) with a 4× objective lens. Fluorescence was passed through a 510–525 nm band-pass filter, and digital images were acquired using Lecia Confocal acquisition software. Images were collected at 10 μm increments to from bottom (0 μm) to the top (140 μm) of the gel discs.

2.6 Gel disc microreactor optimization

To determine the optimum density of enzyme molecules for proteolytic digestion, trypsin-labeled gels were formed

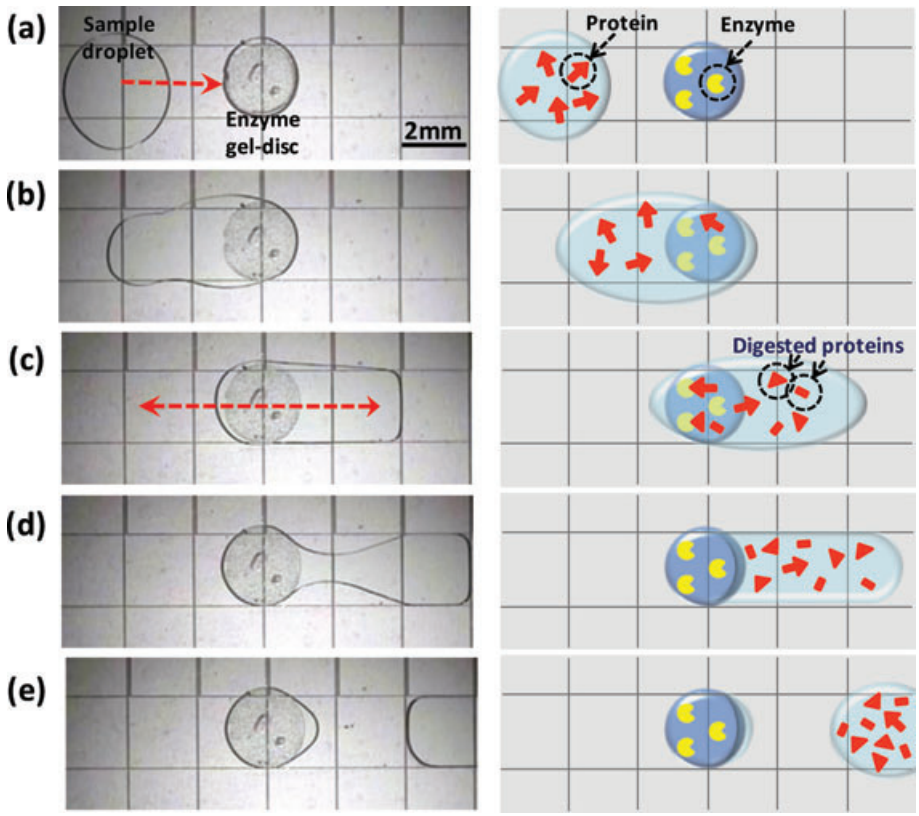

Figure 2. Hydrogel proteolytic enzyme microreactors. A series of images from a movie (left) and a schematic (right) depicts a typical digestion in a 2-mm-diameter gel disc on a DMF device. A 2 μL droplet containing a proteomic sample is delivered to the gel (a and b), actively incubated (c), dispensed from the gel (d), and then isolated (e). The gel disc contains a suspension of 10-μm-diameter beads to make it visible.

as above, with exposure to 0.5, 2, 3.5, 5.5, or 7.5 mM sodium periodate in step (iii). Each gel was placed onto a DMF device. A 2.5 μL droplet of quenched bodipy-casein (100 μg/mL in working buffer) from an EnzChek® Protease Assay Kit was dispensed and merged onto the gel and moved back and forth across the gel for a few seconds. The device was placed in a humidified chamber (a petri dish partially filled with DI water) and incubated for 1 h (RT). After incubation, the droplet was dispensed from the gel, 2 μL was collected by pipette and deposited in a multiwell plate, and was diluted to 50 μL with DI water. A PHERAstar multiwell plate reader (BMG Labtech, Durham, NC, USA) was used to measure the fluorescence ($\lambda_{ex} = 485$ nm; $\lambda_{em} = 520$ nm; focal height = 7.0 mm; gain = 300) of digested BODIPY-Casein. Three replicates were carried out per condition. Gels exposed to 3.5 mM sodium periodate were found to have the highest digestion efficiency, and thus this condition was used for all subsequent experiments.

The amount of trypsin attached to gel discs under the optimal conditions described above (with 3.5 mM sodium periodate) was determined using a fluorogenic depletion assay. Gel discs were treated as in steps ([i]–[iii]) above, and after incubation, 15 μL of the supernatant (containing unbound trypsin) was collected by pipette and deposited in a multiwell plate and combined with 5 μL of 3 mg/mL fluorescamine in acetone. This solution was diluted to 50 μL with DI water and evaluated using a PHERAstar multiwell plate reader ($\lambda_{ex} = 355$ nm; $\lambda_{em} = 460$ nm; focal height = 7.0 mm; gain = 50). The average value of fluorescence intensity of the supernatant from three gels was compared to a calibration curve of fluorescamine-derivatized trypsin (range: 0.275–4.40 mg/mL) to determine the amount of unbound trypsin after reaction with the gels. The amount of trypsin bound to the gels was assumed to be the difference between the initial concentration (4.4 mg/mL) and the concentration in the supernatant after incubation with gels.

2.7 Proteomic work up and mass spectrometry

Proteomic sample work up proceeded in three steps: (1) reduction—a 600 nL droplet of analyte (BSA or lysozyme, 1 mg/mL in working buffer) and a second 600 nL droplet of TCEP (10 mM in working buffer) were dispensed and merged by DMF followed by incubation (60 min, RT); (2) alkylation—a third 600 nL droplet of IAM (12 mM in DI H_2O) was dispensed and merged with the combined droplet of analyte/TCEP and incubated again (room temperature, dark, 45 min); (3) tryptic digestion. The third step was implemented in one of three different conditions. In condition (i) (named "SOLUTION-LOW"), a 600 nL droplet of trypsin at 0.02 mg/mL in working buffer was dispensed and merged with the combined droplet of analyte/TCEP/IAM. In condition (ii) (named "IN-GEL"), the combined droplet analyte/TCEP/IAM was actuated onto a gel disc containing trypsin. In condition (iii) (named "SOLUTION-HIGH"), a

600 nL droplet of trypsin at 40 mg/mL in working buffer was dispensed and merged with the combined droplet of analyte/TCEP/IAM. In all cases, the digestion mixture was allowed to incubate (4 h, RT) in a humidified chamber (a petri dish partially filled with DI water) to limit evaporation. Samples on gels were then dispensed away from the gels, and all samples were then quenched by dispensing and actuating a 600 nL droplet of 2.5% TFA to merge with the droplet. At least three replicate samples were evaluated for each condition.

In some cases, a modified IN-GEL procedure was used, in which the droplet at the end of step 2 (a mixture of analyte, TCEP, and IAM) was split into two separate droplets (each ~900 nL) that were individually actuated onto two different gel discs. One gel disc contained trypsin and the other contained pepsin. The droplets were allowed to incubate in a humidified chamber and were then quenched as above.

After processing by DMF, each sample was purified using a Ziptip$_{C18}$® and then analyzed by MALDI-MS. Briefly, Ziptip$_{C18}$® were wetted in 100% ACN containing 0.1% TFA (3×) and then equilibrated in 5% ACN containing 0.1% TFA (3×). The quenched digest samples generated by DMF were drawn in and out of tips for 7–10 cycles, and then washed with 5% ACN containing 0.1% TFA (3×). Finally, samples were eluted in 100% ACN containing 0.1% TFA (2 μL) onto a stainless steel MALDI target plate. A 1 μL matrix solution (10 mg/mL α-CHCA in 50:50 ACN:DI H_2O containing 0.1% TFA) was added to each sample. After drying, spots were analyzed using a Micro-MX mass spectrometer (Waters, Milford, MA, USA) operating in positive and reflectron modes over a *m/z* range from 600 to 2000. At least 100 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 evaluated visually.

To characterize digested peptides in mass spectra, peaks with intensities below 10 and 2.5% signal relative to the largest peak intensity were rejected for single and double enzyme digestion experiments, respectively. The masses of expected peaks were determined using the PeptideMass prediction/characterization tool on the ExPASy proteomic server taking protein sequences from the UniProt Knowledgebase (Swiss-Prot and TrEMBL), with one allowed missed cleavages, cysteines treated with IAM and a mass accuracy of ± 1.2 Da.

3 Results and discussion

3.1 Gel disc microreactor formation and use

Fiddes et al. [30] recently introduced a new model for miniaturized systems: the marriage of hydrogel discs and DMF. Here, we applied this combination to implement a critical step in many proteomic applications, enzymatic digestion. Using an adaptation of methods reported previously [26, 40–42], agarose gel discs were modified to bear proteolytic enzymes (trypsin or pepsin), such that when droplets containing proteomic analytes were exposed to the modified discs, the proteins were enzymatically cleaved.

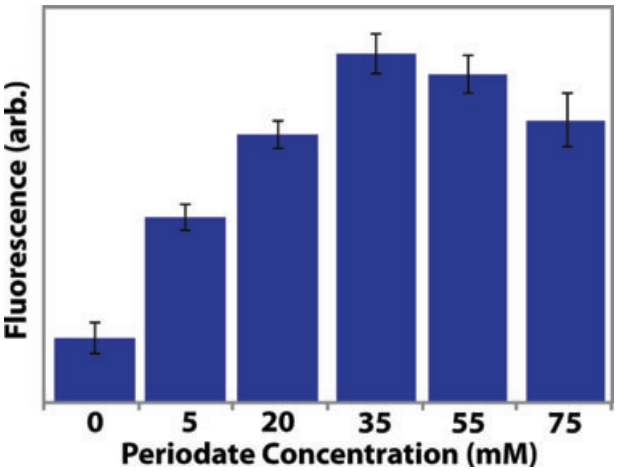

Figure 3. Gel disc microreactor optimization. Gel discs were exposed to various concentrations of $NaIO_4$, (0.5, 2, 3.5, 5.5, or 7.5 mM), which determines the density of enzyme immobilized. A droplet of a fluorogenic reporter, quenched bodipy-casein (which becomes progressively more fluorescent as it is digested) was dispensed, merged onto the gel, incubated, and then collected for determination of fluorescence intensity. The data represent three replicates per condition, with error bars of ±1 SD. The amount of trypsin immobilized in the optimal conditions (i.e. after exposure of gel discs to 3.5 mM sodium periodate) was found to be 31 ± 4 μg trypsin per 2-mm-diameter gel disc.

Figure 1 illustrates the mechanism by which enzymes are immobilized on gel discs. In step (i), preformed agarose discs (Agr–OH) are exposed to an activating agent, glycidol (2,3-epoxypropanol), to form an intermediate, glyceryl-agarose (Agr–O–CH_2–CHOH–CH_2OH), which is then oxidized in step (ii) with sodium periodate to form glyoxyl-agarose (Agr–CH_2CHO). Subsequent exposure of the oxidized gel in step (iii) to an enzyme solution results in the covalent attachment of the enzyme via a Schiff's base reaction. Finally in step (iv), a reduction in sodium borohydride transforms the weak Schiff's bases into stable secondary amide bonds and all remaining aldehyde on the solid support into inert hydroxy groups. The inset of Fig. 1 contains a *z*-stack series of cross-sectional confocal images of a gel disc that was modified with FITC-trypsin. The images reveal that the enzyme is distributed throughout the gel, which indicates that a large surface area is used for enzymes immobilization.

Gel disc microreactors are easily integrated onto DMF platforms by placing them directly onto the area/electrode of interest. Gel discs on DMF devices remain stationary, such that liquid droplets can be manipulated relative to them. Figure 2 demonstrates the process for a proteolytic microreactor. A droplet containing a protein sample is delivered to the gel and is then incubated such that the covalently bound proteolytic enzyme can digest the sample into peptides.

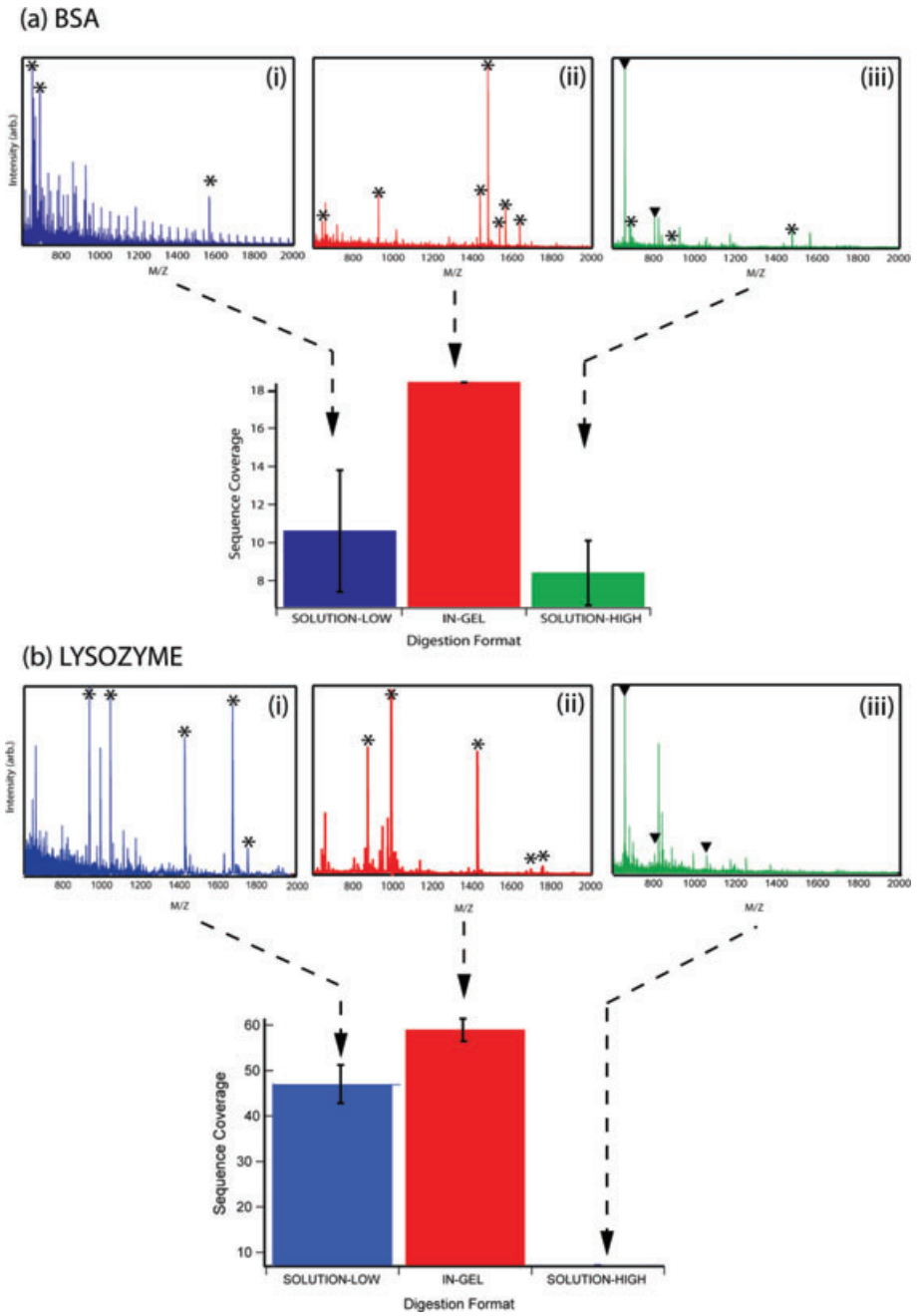

Figure 4. Comparison of digestion efficiency. Representative MALDI-MS spectra (top) and their corresponding sequence coverage data (bottom) for (a) BSA and (b) lysozyme-digested (i) in-solution with low trypsin concentration (0.02 mg/mL), (ii) in-gel, and (iii) in-solution with high trypsin concentration (40 mg/mL). Asterisks (*) in the spectra denote tryptic peptides of the analytes, and upside down triangles (▼) denote tryptic autolysis peaks. The sequence coverage data represent three replicates ($n = 3$) per condition, with error bars of $\pm$ 1 SD.

After incubation, the droplet (now containing digest peptides) can be dispensed away from the gel for further processing or analysis.

3.2 Gel disc microreactor optimization

For gel disc microreactors, the density of immobilized enzymes determines the overall reaction efficiency. If the density is too low, the small number of catalytic sites will contribute to a slow reaction rate, while if the density is too high, the enzyme molecules can sterically hinder each other, which can also contribute to suboptimal rates. To determine the optimum density of enzymes for the current application, we evaluated a series of different trypsin densities on gel discs.

The critical step that determines eventual enzyme density in the process reported here is the number of aldehyde groups generated on the gel (step [ii] in Fig. 1). A series of gels were prepared bearing different densities of aldehyde moieties by exposure to different concentrations of sodium periodate. These gels were then modified with trypsin, incorporated onto DMF devices, then exposed to droplets (as in

Fig. 2) containing quenched BODIPY-casein, a reporter that becomes progressively more fluorescent as it is digested. The data from the assay are shown in Fig. 3. As shown, exposure of gels to 3.5 mM sodium periodate resulted in the highest signal, which correlates to the highest rate of digestion. This condition was used for all subsequent experiments. The density of trypsin molecules attached under these conditions was determined using a fluorogenic depletion assay to be 31 ± 4 μg trypsin per 2-mm-diameter gel disc.

3.3 Comparison of in-gel and in-solution digestion quality

DMF has been touted as being a useful new tool for proteomic sample preparation, particularly for implementing proteolytic digestion [32–37]. But until now, all enzyme digestion methods implemented by DMF have been homogeneous (i.e. solution only). As noted in the introduction, there are significant benefits of using heterogeneous digestion systems (i.e. those with proteolytic enzymes immobilized on a solid substrate). We hypothesized that the new DMF methods with gel microreactors reported here would benefit from these same advantages. To test the hypothesis, we evaluated three conditions: proteins digested on a gel disc microreactor (named "IN-GEL"), proteins digested in solution at 1:50 enzyme:protein concentration (named "SOLUTION-LOW"), and proteins digested in solution at 40:1 enzyme:protein concentration (named "SOLUTION-HIGH"). In all cases, the samples were reduced, alkylated, and then digested, with all reagent manipulation steps implemented by DMF droplet actuation.

MALDI-MS was used to evaluate the quality of the digestions generated by each of the conditions. Figure 4 shows representative spectra (normalized to the highest peak intensity) of processed (a) BSA and (b) lysozyme collected using the three digestion protocols. In each of these experiments, the digesting samples were allowed to incubate for 4 h at room temperature. The SOLUTION-LOW condition is the enzyme-to-protein ratio that is conventionally used for proteomic applications; although this ratio results in slow digestions, it is used to circumvent the appearance of autolysis peaks. As shown, the SOLUTION-LOW condition resulted in reasonable spectral quality, but the spectra contained many peaks that could not be identified, which likely originated from partially digested peptides. In contrast, in the spectra generated by the IN-GEL condition, almost all of the major peaks were identified as digest peptides, indicating that the digestions were substantially complete. This is notable, given that the digestion was carried out for relatively short time and at room temperature.

As a second test for the new system, a SOLUTION-HIGH condition was tested, which is homogeneous, but contains a similar amount of enzyme as the IN-GEL system. As shown, the spectra generated using this condition were dominated by autolysis peaks, with few peptide peaks originating from the analytes. This suggests that the improved performance of the IN-GEL condition is a function of both the high concentration of the enzyme and the benefits provided from the immobilization of enzymes on gels.

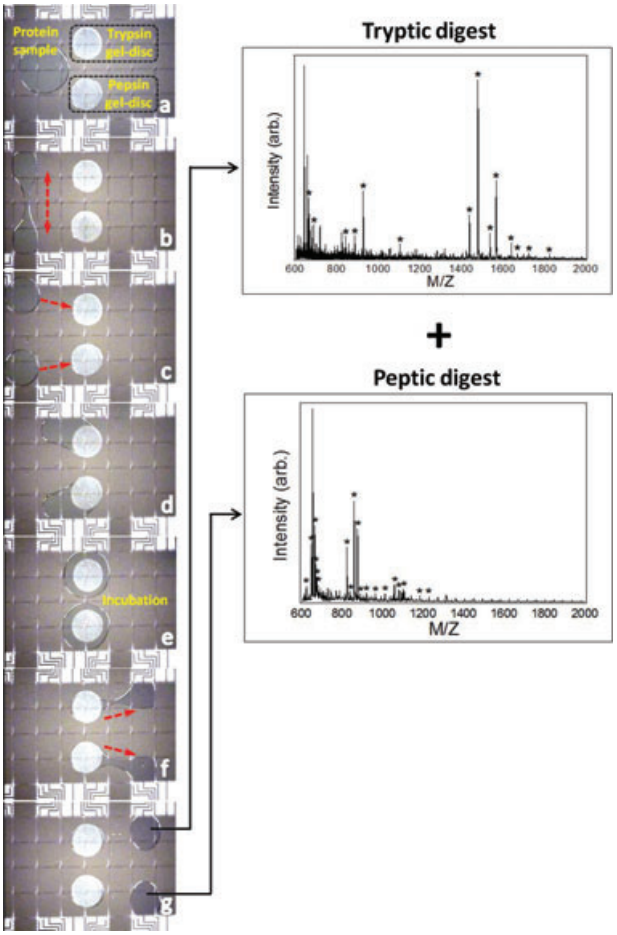

Figure 5. DMF-driven multienzyme digestion of a protein sample using hydrogel discs. Frames from a movie (left) depicting a droplet containing BSA being split into two daughter droplets (a–c) that are then delivered to hydrogel discs bearing trypsin (top) and pepsin (bottom). Samples were actively incubated (d and e) and then isolated (f and g) for analysis by MALDI-MS. The gel discs contain a suspension of 10 μm-diameter beads to make them visible. Representative MALDI-MS spectra from three replicate experiments (right) of tryptic and peptic digests. Asterisks (*) denote tryptic and peptic peptides originating from BSA.

3.4 Multiplexed digestion

For complex samples, the list of peptides formed after digestion with a single enzyme is often not sufficient to identify the proteins in question. For this reason, parallel digestions using different enzymes can improve sequence coverage by identifying more peptides resulting in increases confidence in protein identification in proteome profiling [43, 44]. DMF

seems well suited to such tasks, given that droplets containing samples can be manipulated, split, and delivered to multiple locations in a device in parallel. To test this idea, we developed a parallel digestion scheme in which an initial droplet was reduced and alkylated, and was then split and delivered to two different gel microreactors—one containing trypsin, and one containing pepsin. The process is shown in Fig. 5. MALDI-MS results generated from the two different gels reveals a large number of unique tryptic and peptic peptides. An analysis of the peptides identified confirms that the sequence coverage from both enzymes (34%) is indeed higher than that of either enzyme alone (19% and 19% for trypsin and pepsin, respectively). These results illustrate the potential for parallel proteomic sample processing for DMF paired with gel discs. We propose that this capacity may eventually prove to be a useful part of an automated platform for proteome profiling.

4 Conclusion

Here, we demonstrated the utility in combining DMF and hydrogels disc for proteomics applications. Gel discs bearing covalently attached proteolytic enzymes were found to be useful for proteomic sample preparation, with better performance than conventional homogeneous techniques. We propose that these methods represent a useful new tool for the growing trend toward miniaturization and automation in proteomic sample processing.

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