HYDROGEL DISCS ON DIGITAL MICROFLUIDIC DEVICES FOR PROTEOMIC APPLICATIONS

Vivienne N. Luk[1], Lindsey K. Fiddes[1], Alphonsus H.C. Ng[2], Eugenia Kumacheva[1] and Aaron R. Wheeler[1,2]

[1]Department of Chemistry, [2]Institute for Biomaterials and Biomedical Engineering, University of Toronto, CANADA

ABSTRACT

We introduce the use of immobilized enzymes in hydrogels for proteomic sample processing in digital microfluidic systems. In this technique, pre-formed cylindrical agarose discs bearing immobilized enzymes were integrated into digital microfluidic devices bearing arrays of electrodes. In some experiments, fluorogenic enzyme substrates were delivered to gel discs which served as integrated microreactors, in other experiments, droplets bearing proteomic samples were sequentially reduced, alkylated, and digested on gel discs via digital microfluidic droplet operation. We propose that these methods represent a useful new tool for the growing trend towards miniaturization and automation in proteomics and sample processing.

KEYWORDS: digital microfluidics, proteomics, enzymatic digestion, hydrogels, agarose, electrowetting

INTRODUCTION

Hydrogels are hydrophilic polymers that form porous networks structures swollen with water. They are uniquely useful for a wide range of applications because the porous networks serve as stable niches for immobilizing entities including proteins [1] and cells [2]. There has been great enthusiasm for combining hydrogels with microchannel-based lab-on-a-chip systems [3,4] for applications such as enzyme microreactors [5] and enzymatic digestion [6]. To our knowledge, however, there has only been a single report [7] of the combination of hydrogels with digital microfluidics (DMF), a technique in which discrete droplets are manipulated electrostatically on an open array of electrodes. [8] The first DMF/gel report (from a 2007 review article [7]) describes an experiment in which reagents were combined to form gels, but it was not clear from the brief discussion whether such gels could be subsequently addressed with droplets to enable applications. Here, we introduce a robust system combining hydrogels and digital microfluidics, in which cylindrical gel discs can be repeatedly and reproducibly addressed by reagent droplets on DMF devices, and we demonstrate that this system is useful for several types of enzyme applications. We propose that similar systems will be useful for a variety of applications.

EXPERIMENTAL

Hydrogel Enzyme Microreactor Disc Fabrication and Operation

2 μL aliquots of heated 4% wt. agarose solution (> 70^{o}C) were pipetted and sandwiched between two unpatterned Teflon AF-coated glass slides held together with 2 pieces of double-sided tape (140 μm spacer). Once cooled the cylindrical gelled discs were retrieved using tweezers. Enzymes were covalently attached onto agarose gel discs using methods similar to those developed by Guisan and coworkers [9] and discs were then positioned between top and bottom plates on DMF devices for analysis.

Enzyme Microreactor Assay

Gel discs conjugated with alkaline phosphatase were positioned onto DMF devices. A 2.5 μL droplet of fluorescein diphosphate (0 to 40 μg/mL) in reaction buffer (10 mM diethanolamine and 1 mM $MgCl_2$ in DI water) was dispensed and merged onto each disc and incubated for 3 minutes. Droplets were then dispensed from the gel and 2 μL of each dispensed droplet was collected and mixed with 18 μL DI water in a well in a 384-well plate. Fluorescence was measured using a multiwell plate reader. Each concentration of fluorescein diphosphate was evaluated three times on three different gels.

Proteomic Assay – Workup and Mass Spectrometry

Proteomic sample workup proceeded in three steps: (1) reduction - one 600 nL droplet of BSA (1 mg/mL) in working buffer (50 mM tris-HCl, pH 7.8, 0.08% w/v Pluronic F127 in DI water) 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 iodoacetamide (12 mM in DI water) was dispensed and merged with the combined droplet of analyte/TCEP and incubated again (room temp., 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/iodoacetamide. In condition (ii) (named "IN-GEL"), the combined droplet analyte/TCEP/iodoacetamide was actuated onto 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/iodoacetamide. 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 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 iodoacetamide) was split into two separate droplets (each ~900 nL) which 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 matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS).

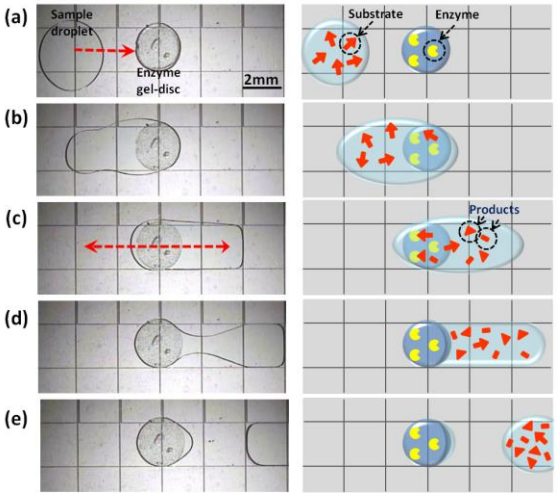

Figure 1: Hydrogels and digital microfluidics. A series of images from a movie (left) and a schematic (right) depicts an enzyme assay in a 2 mm dia. gel disc on a DMF device. A 2 μL droplet containing a sample is delivered to the gel (a, b), actively incubated (c), dispensed from the gel (d), and then isolated (e). The gel disc contains a suspension of 10 μm dia. beads to make it visible.

RESULTS AND DISCUSSION

A first question in our experiments was whether agarose gel discs would remain stationary or would become mobile when exposed to moving droplets on DMF devices. We hypothesized that adhesion and friction would keep the gel discs stationary, and Figure 1 demonstrates that this is the case. In fact, over the course of hundreds of experiments, gel discs were never observed to move. A second question was whether liquid droplets could be dispensed from the gels, or whether the liquid would remain permanently bound to the hydrophilic discs. In fact, as shown in Figure 1, droplet dispensing was not only possible but quite straightforward -- when the three electrodes adjacent to a gel disc are actuated in succession, a droplet (connected by a tail) is pulled away from the gel until it pinches off. After dispensing, a small amount of the original droplet volume remains on the gel. To characterize this quantitatively, ten 2.5 μL droplets of water were merged and dispensed from hydrogel discs, and on average, ~10% of the volume (178 ± 13 nL) remains bound to the gel, and ~90% is dispensed.

To demonstrate the capacity of digital microfluidics and hydrogels to form enzyme microreactors, gel discs were formed from agarose covalently functionalized with alkaline phosphatase. Droplets containing fluorescein diphosphate (which is non-fluorescent) were dispensed and merged onto the gels to allow for the enzyme to cleave off the phosphate groups, generating the fluorescent product, fluorescein. Figure 2 shows the fluorescence of the dispensed droplets as a function of initial fluorescein diphosphate concentration. As expected, the fluorescence increased linearly with substrate concentration.

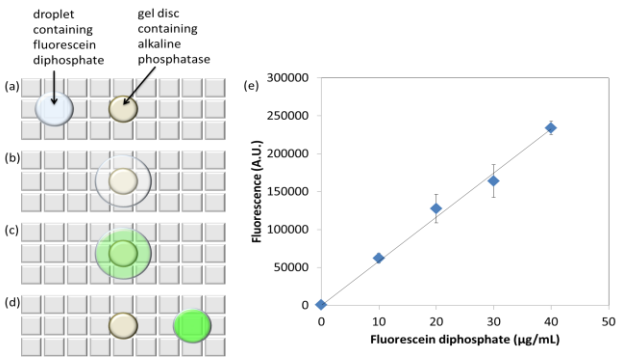

Figure 2: Enzyme Microreactor. Gel discs were formed with covalently linked alkaline phosphatase. Droplets containing fluorescein diphosphate (a) were merged onto the discs (b) and upon incubation, the fluorescein diphosphate was converted to fluorescein (c). Droplets containing the fluorescent products were dispensed from the gel (d). Fluorescence of dispensed droplets as a function of the concentration of fluorescein diphosphate (e). The experiments were replicated 3 times and error bars are ± 1 S.D. A least squares line of best fit through the data has an $R^2 = 0.9929$.

To demonstrate the capacity of digital microfluidics and hydrogels for proteome profiling applications (in which proteins are identified after digestion into smaller peptides), hydrogel discs were modified to bear covalently attached digestive proteases (trypsin or pepsin). MALDI-MS was used to evaluate the quality of the digestions generated by each of the conditions. Figure 3 shows representative spectra (normalized to the highest peak intensity) of processed BSA collected using three digestion conditions. In each of these experiments, the digesting samples were allowed to incubate for four hours at room temperature. The SOLUTION-LOW condition represents traditional enzyme-to-protein ratios used for protein identification; although this ratio results in slow digestions, it is conventionally 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 (likely caused by partially digested peptides). In contrast, in the spectra generatedby the IN-GEL condition, almost all of the major peaks (peaks with intensity ≥10% of the most intense peak) were identified as digest peptides, indicating that the digestions were substantially complete. This is notable, given that the digestion was carried out for 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 analyte. This suggests that the improved performance of the IN-GEL condition is a function both of the high concentration of the enzyme and the benefits provided from the immobilization of enzymes on gels.

To demonstrate the potential for multiplexing, devices were formed with gels bearing trypsin and pepsin. As illustrated in Figure 4, by splitting droplets and delivering them to the different gels, more peptide diversity was generated, resulting in greater sequence coverage (data not shown) was obtained.

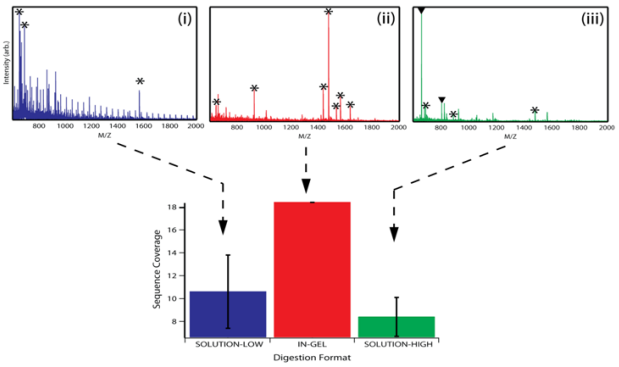

Figure 3: Comparison of digestion efficiency. Representative MALDI-MS spectra (top) and their corresponding sequence coverage data (bottom) for BSA digested (i) in-solution with low trypsin concentration (0.02 mg/mL), (ii) in-gel and (iii) in-solution with high trypsin concentration (40 mg/mL). Asterisks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

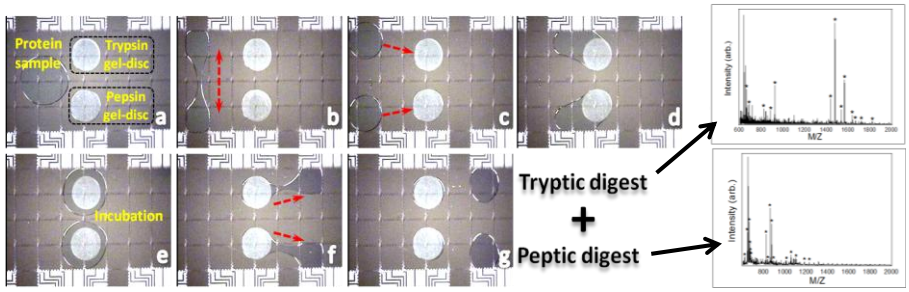

Figure 4: Frames from a movie (left) of a BSA droplet being split into two daughter droplets (a-c) which are then delivered to hydrogel discs bearing trypsin (top) and pepsin (bottom). Samples were incubated (d,e) and then isolated (f,g) for analysis by MALDI-MS Representative spectra (right) of tryptic and peptic digests. Asterisks (*) denote peptides originating from BSA.

CONCLUSIONS

In conclusion, we introduce the marriage of hydrogels and digital microfluidics, and propose that this combination may be useful for many applications in the future, particularly, in the field of proteomics.

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