Pluronic Additives: A Solution to Sticky Problems in Digital Microfluidics

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Digital microfluidics (DMF) is a promising technique for carrying out miniaturized, automated biochemical assays in which discrete droplets of reagents are actuated on the surface of an array of electrodes. A limitation for DMF is nonspecific protein adsorption to device surfaces, which interferes with assay fidelity and can cause droplets to become unmovable. Here, we report the results of a quantitative analysis of protein adsorption on DMF devices by means of confocal microscopy and secondary ion mass spectrometry. This study led us to a simple and effective method for limiting the extent of protein adsorption: the use of low concentrations of Pluronic F127 as a solution additive. This strategy has a transformative effect on digital microfluidics, facilitating the actuation of droplets containing greater than 1000-fold higher protein concentrations than is possible without the additive. To illustrate the benefits of this new method, we implemented a DMF-driven protein digest assay using large concentrations (1 mg/mL) of protein−substrate. The use of Pluronic additives solves a sticky problem in DMF, which greatly expands the range of applications that are compatible with this promising technology.

Introduction

A new paradigm for miniaturized bioassays has recently emerged and is called "digital" (or droplet-based) microfluidics. In digital microfluidics (DMF), droplets containing samples, reagents, and other liquids are manipulated on the surface of an array of electrodes by means of electrowetting[1,2] and/or dielectrophoresis.[3,4] By applying a sequence of 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 (as is the case for microchannels), DMF is a facile method for forming microreactors in which there is no possibility that reagents or samples will diffuse away. Perhaps most importantly, because DMF is implemented in an array geometry, it seems a natural fit for parallel-scale, multiplexed analyses.[5] As the popularity of this technique grows, it is being applied to an ever-expanding range of applications, including cell-based assays,[6] enzyme assays,[7–11] protein profiling,[12–14] and the polymerase chain reaction.[15]

Unfortunately, digital microfluidics is limited by a critical problem: nonspecific adsorption of biomolecules to device surfaces, or biofouling. DMF surfaces are typically formed from a hydrophobic material such as Teflon-AF, and although Teflon is marketed as a "non-stick" surface, it is actually quite prone to biofouling because of its hydrophobicity.[16,17] Teflon is not unique. In fact, most low-energy surfaces become fouled when exposed to solutes possessing nonpolar residues in aqueous solutions. This causes analytical problems for many applications (biosensors, medical implants, pipet tips, centrifuge tubes, etc.): molecules that adsorb to surfaces have reduced activities, become lost when samples are transferred to other vessels, and desorb unpredictably, leading to cross-contamination. Digital microfluidics also suffers from these analytical problems but in addition suffers from a mechanical problem: in DMF, droplet actuation is dependent upon smooth, homogeneous surfaces, and when appreciable biofouling occurs, droplets become stuck, rendering devices useless.[18–20] Clearly, if DMF is going to mature from being a technology for aficionados to being a technique that is

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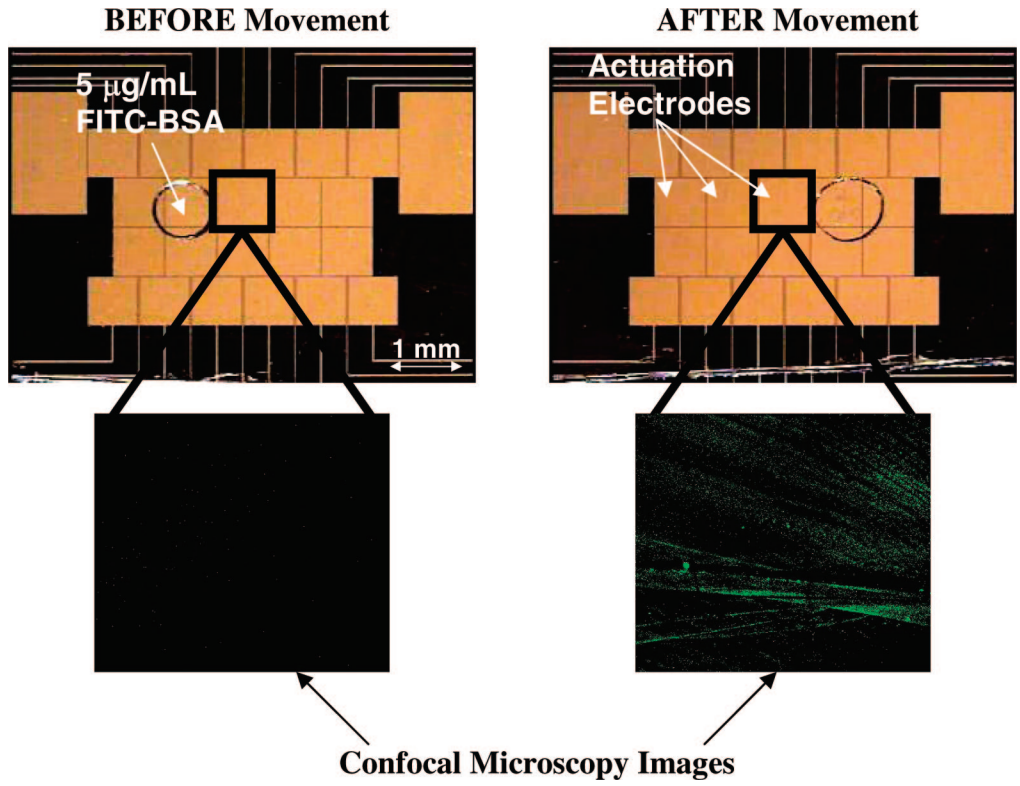

Figure 1. Protein adsorption from an aqueous droplet onto a DMF device. The left image shows a device prior to droplet actuation, paired with a corresponding confocal image of a central electrode. The right image shows the same device after a droplet containing FITC-BSA (5 μg/mL) has been cycled over the electrode four times, paired with a confocal image collected after droplet movement. The two images were processed identically to illustrate that confocal microscopy can be used to detect the protein that adsorbs to a device as a result of droplet actuation.

useful for biochemical applications at large, the problem of biofouling must be overcome.

Here, we present a quantitative evaluation of biofouling on digital microfluidic devices. Our primary tool was confocal microscopy. In assays similar to those reported recently by Tserepi et al.,[18] droplets containing labeled proteins were manipulated by DMF, and the extent of protein adsorption was characterized by fluorescence (Figure 1). This technique was versatile and robust and allowed us to evaluate several experimental conditions to develop a new strategy for preventing protein adsorption. To validate these results, we used an orthogonal technique, secondary ion mass spectrometry (SIMS).

We note that this is not the first attempt to characterize and/or prevent biofouling in digital microfluidics. Previous efforts have included the use of water-immiscible oils,[8,10] the application of specific pH and voltage biases, [19] and the formation of textured device surfaces.[20–22] In the first method,[8,10] aqueous droplets are manipulated through a matrix of oil (rather than air, as they are in our work), which functions to encapsulate the droplets, preventing contact with device surfaces. Although this technique is effective at reducing surface adsorption, it has several disadvantages, including incompatibility with miscible solvents such as ethanol or methanol, and partitioning of nonpolar analytes from aqueous droplets into the oil matrix. In the second method,[19] actuation parameters including voltage bias and pH are carefully tuned to limit the electrostatic attraction of proteins to device surfaces; however, this method does not prevent hydrophobic interactions, and it is unlikely to be useful for assays requiring reagents and solutions with different pI and pH values. The third method[20–22] may be the most promising because micro- or nanostructured device surfaces have reduced contact areas with aqueous droplets, which limits the amount of molecular adsorption. Thus far, however, such surfaces have proven difficult to integrate with DMF actuation because droplets tend to wet the interfeature regions, causing them to become stuck.[22] We assert that whereas these previous techniques to reduce biofouling in DMF may be useful in some circumstances, none of them represents a universal solution for the wide range of applications envisioned for this promising technology.

Here, we introduce a new method for minimizing the extent of biofouling in digital microfluidics. In this work, we have built on the extensive literature[23–25] regarding the use of poly(ethylene oxide) (PEO), also known as poly(ethylene glycol) (PEG), to reduce the surface adsorption of proteins and other solutes. Specifically, we evaluated the efficacy of PEO-containing triblock copolymers distributed under the trade name Pluronic (BASF, Florham Park, NJ). Whereas various kinds of Pluronics have been used for applications in microchannels,[26,27] we believe this to be the first report of the pairing of Pluronics and digital microfluidics. Because DMF is mechanically dependent upon device surface homogeneity (as described above), the Pluronic-based method reported here has a transformative effect on the technique, facilitating the actuation of droplets containing greater than 1000-fold higher protein concentrations than is possible for

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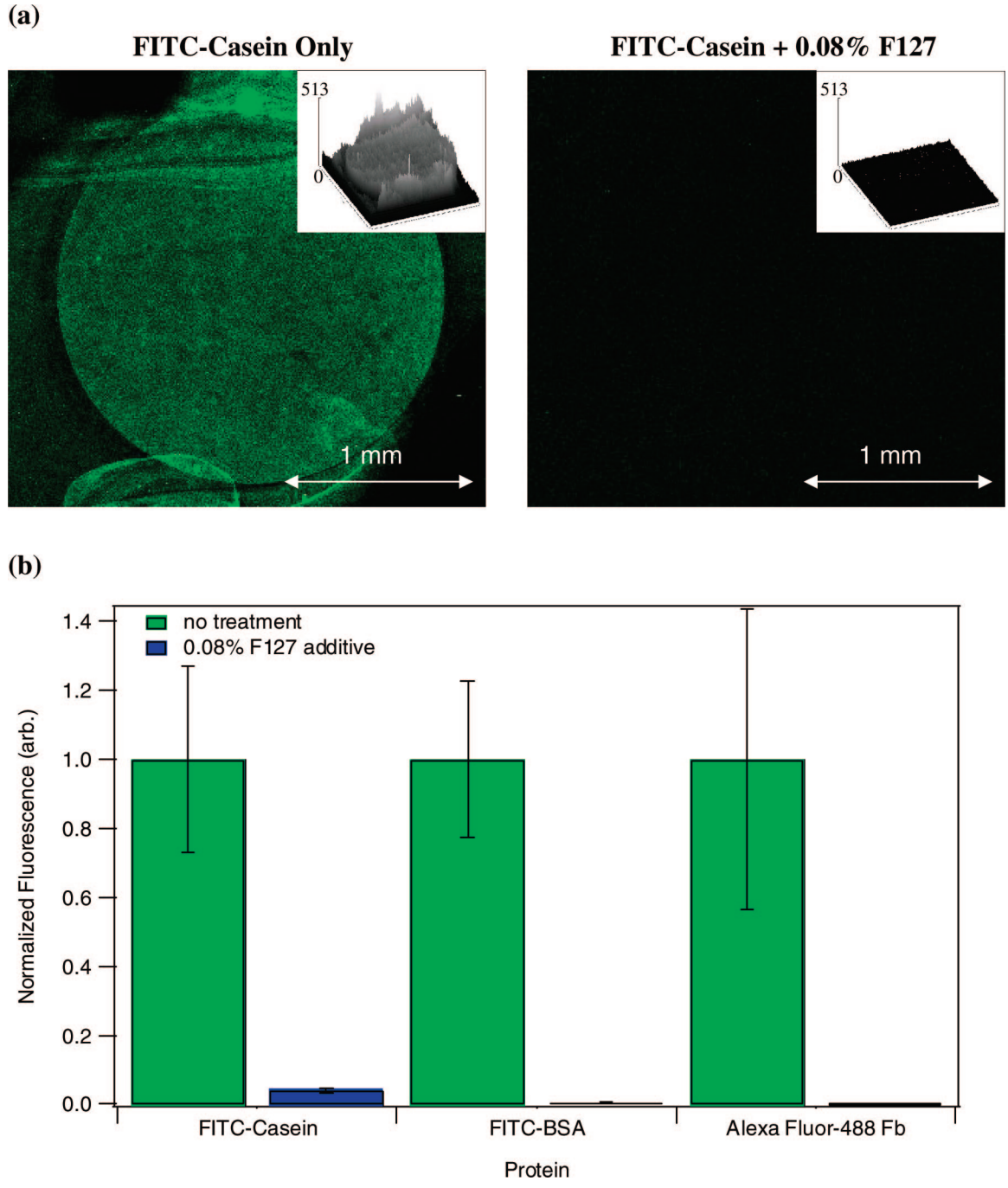

Figure 2. Confocal fluorescence analysis of the effect of Pluronic additive on protein adsorption to Teflon-coated surfaces. (a) Fluorescent images of test substrates exposed to FITC-casein without (left) and with (right) 0.08% w/v F127. The insets are 3D graphs of pixel intensity. (b) Graph depicting the normalized, integrated fluorescence of substrates exposed to FTIC-casein, FITC-BSA, and Alexa Fluor-488 Fb without (green bars) and with (blue bars) 0.08% Pluronic F127. The data represent six replicates per condition, with error bars of ±1 SD.

conventional DMF. This technique has the potential to be a near-universal solution for preventing biomolecular adsorption in digital microfluidics, thus greatly expanding the range of applications compatible with this promising technique.

Experimental Section

Reagents and Materials. Pluronic F68, Pluronic F127, Fluorinert FC-40, Tris-HCl, sodium azide, bovine serum albumin (BSA), fibrinogen (Fb), casein, and FITC-labeled casein (from bovine milk) were purchased from Sigma Chemical (Oakville, ON). Fetal bovine serum (FBS), FITC-BSA, Alexa Fluor-labeled fibrinogen from human plasma, and an E6638 EnzChek protease assay kit were purchased from Invitrogen (Burlington, ON). Parylene-C dimer was from Specialty Coating Systems (Indianapolis, IN), and Teflon-AF was from DuPont (Wilmington, DE).

Stock solutions (5.0−60 mg/mL) of all lyophilized proteins were prepared in 10 mM Tris-HCl (pH 7.8) buffer containing 0.1 mM sodium azide. Stock solutions of Pluronic F68 and F127 were formed in Tris-HCl/sodium azide buffer (1.0% w/v), and in dH_2O with no buffer (2.0% w/v). For quantitative protein adsorption measurements, working protein solutions were formed by diluting stock solutions to 1.0 mg/mL protein with x % w/v Pluronic F68 or F127 ($x = 0$, 0.08, 0.4, 0.8). For indirect-depletion measurements, working solutions were formed by diluting labeled BSA to concentrations ranging from 0.92 to 1.0 mg/mL in 10 mM Tris-HCl buffer. For DMF movability assays, unlabeled proteins were diluted to various concentrations in Tris-HCl buffer.

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 and gold from Kurt J. Lesker Canada (Toronto, ON), CR-4 chromium etchant from Cyantek (Fremont, CA), hexamethyldisilazane (HMDS) from Shin-Etsu MicroSi (Phoenix, AZ), and concentrated 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/hydrogen peroxide.

Device and Test Substrate Fabrication. Digital microfluidic devices were formed using conventional methods in the University of Toronto Emerging Communications Technology Institute (ECTI) fabrication facility. Glass wafers were cleaned in piranha solution (10 min) and then coated with chromium (60 nm) and gold (120 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 prebaked on a hotplate (100 °C, 2 min) and exposed through a photomask using a Suss Mikrotek mask aligner. Substrates were developed in MF321 (3 min) and then postbaked on a hot plate (100 °C, 1 min). After photolithography, substrates were immersed in gold etchant (50 s), followed by 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 postbaked on a hotplate (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 or the tip of a voltage probe. In addition to patterned devices, unpatterned indium tin oxide (ITO)-coated glass substrates (Delta Technologies Ltd., Stillwater, MN) were spin coated with Teflon-AF (50 nm, as above).

Unpatterned test substrates used to mimic DMF devices were prepared by cleaning glass slides in piranha solution (10 min), followed by coating with parylene-C (2 μm) and Teflon-AF (50 nm), as above.

Device Operation. Devices were assembled with an unpatterned ITO/glass top plate and a patterned bottom plate separated by a spacer formed from one piece of double-sided tape ($\sim$70 μm thick). As described previously,[2] 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 a 4 × 6 array of 1 mm × 1 mm actuation electrodes, with interelectrode gaps of 5−40 μm.

Direct Analysis of Protein Adsorption. Protein adsorption was assayed on two kinds of substrates: DMF devices and unpatterned test substrates. For the former, droplets containing labeled proteins were manipulated by DMF, after which the top plate and droplet were removed and the device was stored in the dark until analysis. For the latter, 3 μL droplets of protein working solutions were pipetted onto test substrates, forming circular footprints (radius $\sim$1 mm) on the surface. At least six replicates were evaluated for each experimental condition. Substrates held $\sim$28 droplets, including at least 1 background sample (containing no protein) for each unique condition. Adsorption was allowed to proceed for 45 min in a humidified chamber (a Petri dish partially filled with dH_2O), after which the substrates were immersed in dH_2O with gentle agitation for 10 min to remove nonadsorbed proteins (dilution factor $\sim$10 000). After rinsing, samples were air dried and stored in the dark until further analysis. In some experiments, substrates were prepassivated with Pluronic prior to exposure to proteins. For these experiments, substrates were immersed in 2% w/v F68 or F127 in dH_2O (10 min) and then air dried (with no rinse) immediately before protein adsorption. All assays involving Pluronics were carried out at room temperature (20−25 °C).

Confocal microscopy was the primary tool used to evaluate protein adsorption on surfaces. In these experiments, a Fluoview 300 scanning confocal microscope (Olympus, Markam, ON) equipped with an Ar^+ (488 nm) laser was used, in conjunction with a 100× objective (NA 0.95) for the analysis of proteins adsorbed to DMF device surfaces (Figure 1) or a 4× objective (NA 0.45) for the analysis of proteins adsorbed to test substrates (Figure 2). Fluorescence from adsorbed FITC and Alexa Fluor-488-labeled proteins was passed through a 510−525 nm band-pass filter, and each digital image was formed from the average of four frames using FluoView image acquisition software (Olympus). In all cases, experimental parameters were optimized such that the PMT was not saturated with signal from the most fluorescent samples. No attempt was made to quantify the density or numbers of molecules adsorbing to surfaces, thus nonlinear effects such as quenching and surface saturation were not considered.

For comparative analysis, images were processed using ImageJ (US National Institutes of Health, http://rsb.info.nih.gov/ij/). In each image, a circular (radius 1 mm) region of interest (ROI) was drawn around the fluorescent spot. For background samples and others for which no or low signal was observed, the ROI was arbitrarily defined in the center of the image. In each ROI, the fluorescence was quantified by integrating the brightness values over all pixels. All measurements were then background corrected by subtracting the integrated value collected from the corresponding background ROI. Replicate measurements were averaged and normalized relative to control measurements of labeled proteins containing no Pluronics. Two-tailed *t* tests assuming equal variances for each experimental condition were used to determine significance.

Secondary ion mass spectrometry (SIMS) was used as an orthogonal method to validate the results obtained by confocal microscopy. These experiments made use of a ToF-SIMS IV instrument (ION-TOF, Münster, Germany) at the Surface Interface Ontario facility located at the University of Toronto. Test substrates with adsorbed protein samples were probed by collecting negative ion spectra in static SIMS mode[28] (primary ion dose $<10^{13}$ cm^{-2}) using a 25 keV Ga^+ primary ion gun and a raster beam area of 500 μm × 500 μm (residence time 30 s). Charge neutralization was achieved using the electron flood gun associated with the instrument. SIMS images were processed using ION-TOF software and facilitated the comparison of multiple spectra, targeting selected *m*/*z* ratios.

Indirect-Depletion Analysis of Protein Adsorption. An indirect-depletion method was used to evaluate the amount of protein that adsorbs to DMF devices from aqueous droplets. In this analysis, aqueous droplets containing FITC-casein at various concentrations (1.00, 0.98, 0.96, 0.94, 0.92, and 0.90 mg/mL) were depleted by pipetting them onto test substrates (five replicates at each concentration). After incubation (45 min) in a humidified chamber, these depleted droplets were recollected into a pipet, deposited into a well in a multiwell plate, and diluted to 300 μL with buffer. A PHERAstar multiwell plate reader (BMG Labtech, Durham, NC) was used to measure the fluorescence (λ_{ex} = 485nm; λ_{em} = 520 nm) of droplets that were depleted on substrates (experimental) as well as droplets (five replicates at 1.00 mg/mL) that were directly dispensed into the multiwell plate, repipetted and dispensed again, and then diluted to 300 μL with buffer (control). This extra pipet step was included to ensure that all samples (experimental and control) were handled with the same number of pipet steps. Two-tailed *t* tests assuming equal variances for each experimental condition were used to determine significance.

Pluronc−Protein Interactions. The nature of pluronic−protein interactions in solution was probed by stimulated echo (STE) pulsed field gradient NMR (PFG-NMR). A description of this work can be found online in the Supporting Information.

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Table 1. Normalized Protein Adsorption (% Relative to Control) on DMF Substrates[a]

	% F127 in solution			% F68 in solution			
	0.08%	0.40%	0.80%	0.40%	0.80%	F127 on surface	F68 on surface
FITC-casein	4.0 (0.7)	2.6 (0.7)	3 (0.4)	16.0 (11.5)	5.6 (1.9)	68 (48)	81 (17)
FITC-BSA	0.7 (0.2)	0.5 (0.1)	0.5 (0.1)	0.6 (0.2)	0.3 (0.1)	49 (9)	67 (7)
Alexa Fluor-488 Fb	0.3 (0.1)	0.6 (0.3)	0.9 (1.0)	0.2 (0.1)	0.5 (0.1)	155 (21)	148 (25)

[a] SD in parentheses.

Protein Movability Assay. The relationship between protein concentration and movability by DMF was probed using solutions of unlabeled proteins (Casein, BSA, and Fb) as well as fetal bovine serum. Droplets containing various concentrations of these test solutions with and without Pluronics (0.08% w/v F127) were assayed for movability, which is defined as the capacity to translate a droplet across a series of four electrodes, back and forth twice (i.e., a total of 16 electrode-to-electrode steps). Driving potentials were typically ~60 V (enough for contact angle change saturation[29]), but when a given droplet resisted movement, the driving potential was increased until either (a) the droplet moved or (b) the device experienced dielectric breakdown (i.e., became unusable). At least three replicates of each experimental condition were evaluated on three separate devices to account for interdevice variation, and the maximum movable concentration for each protein was recorded.

Enzyme Assays. Stock solutions of 100 μg/mL trypsin and 1 mg/mL labeled, quenched bodipy-casein (each with 0.08% w/v F127 additive) were prepared using the E6638 EnzChek Protease Assay Kit. For microscopy, 70 nL droplets of each stock solution were actively dispensed from separate reservoirs onto the electrode array and then merged and mixed by moving the coalesced droplet around a loop of six actuation electrodes. For quantitative analysis, the procedure was replicated at lower concentrations of protein (10 μg/mL trypsin, 2.5 μg/mL bodipy-casein, both with 0.08% F127), and after mixing, the device was positioned on the top of a microtiter plate and inserted into a PheraStar multiwell plate reader for fluorescence detection (λ_{ex} = 485 nm, λ_{em} = 520 nm, focal height = 15.0 mm, gain = 900). The fluorescence from the merged droplet was measured in intervals of 60 s for 28 min. Four replicate trials were conducted.

Results and Discussion

Protein Adsorption on DMF Devices. Digital microfluidic device surfaces are typically formed from Teflon-AF, which is prone to unwanted protein adsorption from aqueous solutions. As a consequence, DMF applications involving proteins suffer from analytical problems (i.e., loss of analyte, cross-contamination, etc.) and mechanical problems—when enough protein adsorbs to a DMF device surface, droplets become stuck, rendering the device useless. This phenomenon is illustrated in Figure 1—a droplet containing FITC-BSA (5 μg/mL) was manipulated between four electrodes until the droplet became stuck (which occurred after 16 electrode-to-electrode steps). As shown, the FITC-BSA that adsorbed to the surface of the device as a consequence of the droplet movement was detectable by confocal microscopy. This result (i.e., a detectable amount of protein) is fortunate because it allowed us to conduct comparative experiments evaluating conditions for reducing the level of biofouling (described in the following section).

In contrast to confocal microscopy, an indirect-depletion method assaying the concentration of protein in solution after exposure to a Teflon-coated substrate (described in the Experimental Section) was not sensitive enough to detect the depleted protein. For example, a t test could distinguish between the fluorescence from standard solutions of 0.92 and 1.00 mg/mL FITC-casein ($p < 0.05$) but could not distinguish between 1.00 mg/mL FITC-casein and an identical sample that had been predepleted on a Teflon surface ($p = 0.18$). This result is encouraging for those who wish to use DMF to develop analytical methodologies involving proteins because it suggests that the number of molecules in solution that adsorb to a DMF device surface is low (i.e., less than 8%). But clearly, the amount that does adsorb causes mechanical problems because droplets become stuck and can no longer be moved. In the following sections, we describe our efforts to characterize this phenomenon and develop strategies to minimize it.

Worst Case Scenario. One strategy that might be used to reduce the effects of biofouling would be to translate droplets very rapidly to limit the amount of adsorption to any given electrode. (DMF is capable of actuation at 25 cm/s.[30]) Unfortunately, this strategy is untenable for many applications that require that reagents be merged and incubated in a static (i.e., not moving) droplet for a period of time to allow a reaction to complete. With this in mind, we developed an assay to estimate the relative amount of protein that adsorbs to a Teflon-coated surface in a worst case scenario in which a droplet remains static on one spot for 45 min. Surfaces were exposed to droplets containing fluorescently labeled casein, BSA, or Fb and were then rinsed, dried, and evaluated by confocal fluorescence microscopy.

In this worst case scenario analysis, two strategies for reducing the extent of protein adsorption were investigated: (1) protein samples were spiked with Pluronics as additives in different concentrations (0.08, 0.4, and/or 0.8% w/v), and (2) devices were prepassivated with surface-adsorbed Pluronics (a strategy that has proven useful for reducing nonspecific adsorption for other applications[25,31]). For both strategies, two different kinds of Pluronics, F68 and F127 (with $PEO_x/PPO_y/PEO_z$ molecular ratios of 76:30:76 and 99:67:99, respectively), were evaluated. The critical micellar concentrations (CMCs) for F68 and F127 are approximately 10 g/dL (0.1% w/v at 20 °C)[32] and 2.5 g/dL (0.025% w/v at 25 °C), [33] respectively; thus, all solutions used in strategy 1 contained micelles. Additionally, F127 is known to form gels at high concentrations and temperatures[34–36] (i.e., 15−30% w/v, 37 °C); thus, the parameters in our experiments were well below those required for gelation.

Figure 2a shows typical images collected in a confocal microscopy assay for a surface exposed to labeled protein under the worst case scenario conditions. As shown in the left panel, in an area exposed to a droplet containing only labeled protein, a circular fluorescent feature was observed that corresponded to the droplet footprint. In contrast, in an area exposed to a droplet containing labeled protein and 0.08% w/v F127, very little fluorescence was observed. As shown in Table 1, all of the

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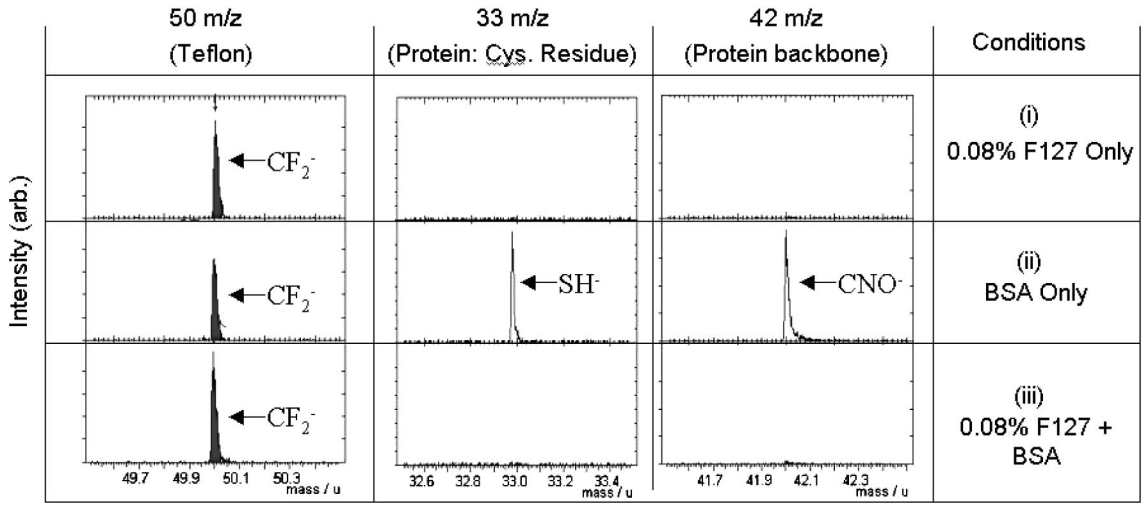

Figure 3. Negative ion SIMS spectra of Teflon-coated substrates exposed to (i) 0.08% F127 in Tris-HCl buffer, (ii) 1.0 mg/mL BSA in Tris-HCl buffer, and (iii) 1.0 mg/mL BSA in 0.08% F127 in Tris-HCl buffer. *m/z* 50, 33, and 42 correspond to CF_2^-, SH^-, and CNO^-, respectively. The data shown are representative of four samples evaluated with a scan area of 500 μm × 500 μm.

pluronic-additive treatments (strategy 1, above) reduced the level of protein adsorption to surfaces. Each condition resulted in a >99% reduction in signal from adsorbed BSA and Fb, but F127 was significantly more effective at reducing the adsorption of casein, which suggests that F127 may be a more widely applicable adsorption blocker. There are small but significant differences[37] in the effectiveness of the different concentrations of F127. The results for one case, 0.08% F127, are shown graphically in Figure 2b.

In comparison to strategy 1 for blocking protein adsorption (solution additives), strategy 2 (prepassivated surfaces) was not nearly as successful. In fact, as shown in Table 1, in the case of fibrinogen, prepassivation of surfaces appeared to *increase* the level of protein adsorption relative to the control. We are unsure of why this was the case because surface passivation has proven effective for other applications;[25,31] we speculate that higher concentrations of Pluronics and longer passivation times may be required to block protein adsorption more effectively. Alternatively, covalent attachment of Pluronics or other PEO-containing polymers to surfaces[38,39] might be a more effective method. Regardless, because strategy 1 was so successful, we chose to adopt it, and in all of the experiments described in the following sections, 0.08% w/v F127 was used to prevent protein adsorption.

Validation of Results. To verify the confocal microscopy results described above, SIMS was used to qualitatively assess the effects of Pluronic additives on protein adsorption. In these experiments, unlabeled BSA was used as a model protein, and three experimental conditions were evaluated in which Teflon-coated surfaces were exposed to (i) 0.08% F127 alone, (ii) 1 mg/mL protein alone, and (iii) 1 mg/mL protein + 0.08% F127. As shown in Figure 3, several *m/z* ratios were identified that correlated with particular chemical groups. For example, *m/z* 50 corresponds to Teflon (CF_2^-) and appears in all samples, whereas *m/z* 33, 42, and 26 (not shown) correspond to moieties found only in proteins (SH^-, CNO^-, and CN^-, respectively), and were detected only on surfaces exposed to protein alone (condition ii). The fact that these peaks were not detected in the sample containing protein and F127 (condition iii) supports the conclusion drawn from the confocal data that 0.08% F127 blocks the adsorption of proteins onto Teflon-coated surfaces.

In an attempt to interrogate the potential adhesion of Pluronic molecules to DMF substrates, we also evaluated several *m/z* ratios that correspond to chemical groups in Pluronics (*m/z* 59, 73, and 87) but were unable to detect any such peaks that were unique to conditions i and iii. Whereas F127 may adsorb to surfaces at levels below the detection limit of ToF-SIMS, it does not appear to do so to a significant extent.

Effects and Mechanism of Pluronic Additives. The additive strategy for preventing protein adsorption in digital microfluidics has a potential drawback—chemical additives can interfere with sensitive assay constituents such as enzymes or cells. Fortunately, this does not appear to be a problem for pluronics. For example, PEG-containing polymers have been shown not to cause problems for protein−protein interactions[40] and in fact can be used as biomimetic additives to enhance protein stability.[41,42] For this reason, Pluronics are widely used in tissue engineering studies[43,44] and is even included in some formulations of commercial cell culture media.[45] These data are supported by our own work, which has demonstrated little or no effect of Pluronic additives on cell viability[6] or enzyme kinetics.[7] Finally, we note that the concentration reported here, 0.08% w/v F127, is probably not the lower-limit on conditions that can reduce biofouling; it is simply the lowest concentration we evaluated. Thus, if 0.08% F127 ever proves problematic for a given assay, lower concentrations might be effective.

(37) Significance values reported here are for $p < 0.05$. For FITC-casein, spots formed from solutions containing 0.4% Pluronic F127 exhibited less fluorescence than did spots formed from solutions containing 0.08% Pluronic F127 and exhibited equivalent fluorescence to solutions containing 0.8% Pluronic F127. For FITC-BSA, all conditions were equivalent. For AlexaFluor 488-Fb, 0.08% exhibited less fluorescence than 0.8% and exhibited equivalent fluorescence to 0.4%. Because no trend was observed and because the differences were small, we chose to focus on the lowest concentration, 0.08% F127.

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Table 2. Maximum Movable[a] Protein Concentrations by DMF ($n = 3$)

	casein	BSA	Fb	100% FBS
protein only	0.005 mg/mL	0.005 mg/mL	0.0015 mg/mL	not movable
protein + 0.08% F127	at least 10 mg/mL (1:69)[b]	at least 50 mg/mL (1:120)[b]	at least 50 mg/mL (1:25)[b]	movable

[a] Definition of movability: droplet moves across four electrodes back and forth for two cycles on three different devices. [b] Molar ratios of Pluronic/protein.

The data presented in Figures 2 and 3 do not suggest an obvious mechanism: how does a small amount of additive so effectively reduce the level of protein adsorption onto Teflon-coated substrates? The molar ratios of 0.08% Pluronics/protein in these experiments were 1:6.9, 1:2.4, and 1:0.5 for casein, BSA, and Fb, respectively; thus, it is conceivable that the observed effect on adsorption is a function of interactions between additive and protein molecules (e.g., it is possible that Pluronic molecules coat the proteins, preventing them from adsorbing to surfaces). In experiments described in the Supporting Information, we probed this phenomenon using stimulated echo (STE) pulsed field gradient (PFG) NMR to calculate diffusion coefficients for Pluronic F127. As shown in Figure S1, the presence of BSA has little effect on the diffusion coefficient of the additive, suggesting that molecular interactions (if any) are limited. More study is warranted, but it seems unlikely that the effects reported above can be explained by additive-protein interactions.

We hypothesize that the observed effects on protein adsorption are a result of layers of Pluronics that form at the phase boundaries of aqueous droplets. We base this hypothesis on the results of Chen et al.,[46] who used vibrational spectroscopy to demonstrate that Pluronics dissolved in water at concentrations between 0.0001% and 0.5% w/v form an ordered layer at the air/liquid and hydrophobic solid/liquid interfaces (PPO units oriented toward air or solid, PEO oriented toward water). In similar studies,

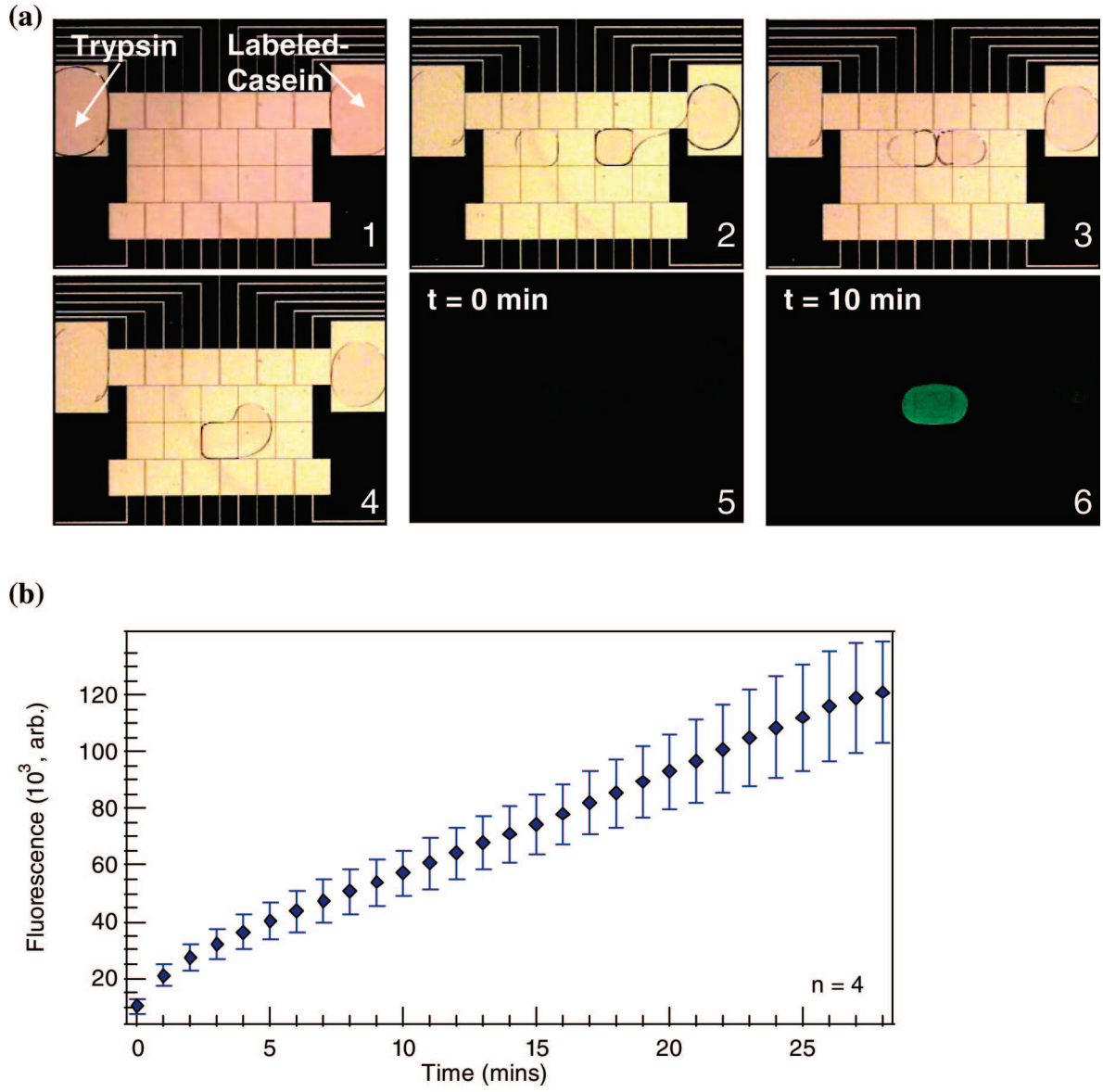

Figure 4. Digital microfluidic driven proteolysis assay. (a) Sequence of images depicting the fluorescent assay for tryptic digestion. (1, 2) Droplets containing bodipy-casein (1 mg/mL) and trypsin (100 μg/mL) are dispensed from reservoirs, (3) merged, and (4) actively mixed. (5, 6) Fluorescent images of a merged droplet before and after digestion. Fluorophores on undigested casein are quenched (i.e., nonfluorescent); as the protein is cleaved, the fluorescence signal increases. (b) Graph depicting the kinetics of tryptic digestion on a DMF device as measured in a fluorescence plate reader. Error bars are ± 1 SD, and the error in the data points ranged from 6−13% RSD.

Phipps et al.[47] used neutron reflection to probe the same phenomenon, revealing that ordered layers of Pluronics form at interfaces when dissolved at concentrations >0.001% w/v. If Pluronic layers form at the solid/liquid interface in droplets manipulated by digital microfluidics (even if the layers are not permanent, as indicated by the fact that we did not detect Pluronics-derived signals by SIMS), this could prevent protein adsorption, even if proteins are present at much higher concentrations than Pluronics in the bulk solution (as is the case in the following section).

Non-Stick Digital Microfluidics. The experiments described above demonstrate that Pluronic additives significantly reduce the extent of biofouling on Teflon surfaces. To test the efficacy of this strategy for digital microfluidics, we evaluated the maximum movable concentrations of three unlabeled proteins (casein, BSA, and Fb) with and without 0.08% F127. Movability was defined as the capacity to manipulate droplets back and forth over four electrodes on three different devices. As shown in Table 2, in control solutions without Pluronic additive, the maximum movable concentrations were ~5−15 μg/mL (similar to what has been reported previously[12–14]). In comparison, when droplets contained 0.08% F127, the maximum movable concentrations were more than 1000× higher (i.e., 10−50 mg/mL). In fact, the concentrations reported in Table 2 for Pluronic-containing samples are not limits; they are simply the highest concentrations we tested (and are likely much higher concentrations than are needed for most assays).

In an attempt to probe the limits of this phenomenon, we evaluated the movability of fetal bovine serum (FBS), a complex, concentrated mixture of proteins and other constituents. Somewhat surprisingly, this very sticky solution, when paired with 0.08% F127, was movable by DMF. The translation of FBS-containing droplets was sluggish relative to that of less concentrated solutions (probably caused by viscosity), but the results were reproducible and no signs of adsorption (i.e., irregularities in the circular droplet shape) were observed. These compelling results suggest that Pluronic additives transform DMF into a technique compatible with a much wider range of reagents, solutions, and assays than was previously thought to be possible.

To illustrate the new kinds of applications that are possible for digital microfluidics when paired with Pluronic additives, we implemented a DMF-driven protein digest assay. The reporter in this assay, bodipy-labeled/quenched casein, has low fluorescence when intact but becomes highly fluorescent when digested, making it useful for evaluating the kinetics of proteolysis. In this assay, a concentrated (1 mg/mL) solution of bodipy-casein was used to ensure that the fluorescence would be visible in a stereomicroscope. Note that this would be impossible without Pluronics because 1 mg/mL is more than 100× higher than the maximum movable concentration without the additive. Figure 4a shows a sequence of images from a movie depicting the assay: 70 nL droplets containing trypsin and bodipy-casein were dispensed from reservoirs and then merged, mixed, and allowed to react. As shown in frame 6 (Figure 4a), the droplet becomes fluorescent after ~10 min. In addition to visualization, the reaction was implemented (at lower concentrations) with quantitative detection using a fluorescence plate reader. As shown in Figure 4b, the reaction comes close to completion in ~30 min. Throughout the reaction, droplets were observed to be movable, suggesting that the combination of Pluronic additives with DMF will facilitate procedures composed of sequential reactions that require extended periods of time.

Conclusions

We have demonstrated that confocal microscopy is a useful tool for probing the extent of biofouling that occurs on digital microfluidic devices. Using this tool, we identified a simple strategy for limiting protein adsorption: the use of low concentrations of Pluronic additives. Secondary ion mass spectrometry was used to corroborate these results. On the basis of previous studies, we hypothesize that this effect is a result of the formation of ordered layers of Pluronics at the phase boundaries of aqueous droplets. Regardless of the mechanism, the additive strategy significantly limits the amount of biofouling such that 1000-fold higher concentrations are movable when compared to DMF without the additive. These compelling results suggest that Pluronic additives transform DMF into a technique compatible with a much wider range of reagents, solutions, and assays than was previously thought to be possible.

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Supporting Information Available: Investigation of Pluronic−protein interactions by NMR. This material is available free of charge via the Internet at http://pubs.acs.org.

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