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# Digital Microfluidic Method for Protein Extraction by Precipitation

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**We present the first microfluidic method for extracting proteins from heterogeneous fluids by precipitation. The new method comprises an automated protocol for precipitation of proteins onto surfaces, rinsing the precipitates to remove impurities, and resolubilization in buffer for further analysis. The method is compatible with proteins representing a range of different physicochemical properties, as well as with complex mixtures such as fetal bovine serum and cell lysate. In all cases, the quantitative performance (measured using a fluorescent assay for % recovery) was comparable to that of conventional techniques, which are manual and require more time. Thus, this work represents an important first step in efforts to develop fully automated microfluidic methods for proteomic analyses.**

In the post-genome era, proteomics has emerged as the next great scientific challenge. While methodologies vary widely, a near-universal first step for proteomic analyses of physiological samples (e.g., blood, serum, tissue extract, etc.) is removal of the non-relevant solution constituents (e.g., nucleic acids and lipids).[1−4] A common method used to accomplish this task is protein precipitation. In this technique, one or more precipitants (organic solvents, salts, or pH modulators) is mixed with the protein-containing sample, which causes proteins to precipitate and settle to the bottom of the reaction vessel.[1−3,5−8] After centrifuging, removal of supernatant, and washing in appropriate rinse solvents, the precipitate can be redissolved, and the now-purified solution can be used for subsequent processing and analysis.

Here, we report the development of an automated microfluidic method for extracting proteins from heterogeneous fluids by precipitation. Although there have been myriad applications of microfluidic technologies to proteomics,[9] to our knowledge, there have been no papers describing protein extraction by precipitation in microchannels. (Note that precipitation has been used in channels to remove proteins for analysis of other analytes,[10] but not as a technique to extract and collect proteins for further analysis.) We speculate that this deficit is a function of complexity and heterogeneity—in protein extraction by precipitation, liquids (samples, precipitants, rinse solutions), solids (precipitates), and vapor phases (air for drying precipitates) all play prominent roles and must be precisely controlled. These requirements seem like a poor match for the conventional format of microfluidics, enclosed microchannels. Thus, in the current work, we chose to use the alternative format of digital microfluidics (DMF).

In DMF, discrete droplets of sample and reagents are controlled (i.e., moved, merged, mixed, and dispensed from reservoirs) by applying a series of electrical potentials to an array of electrodes coated with a hydrophobic insulator.[11,12] Although microchannels can also be used to manipulate droplets, DMF is a distinct paradigm; the principal difference is that in DMF, samples are addressed individually, while in channels, they are controlled in series.[13] DMF has recently become popular for a wide range biochemical applications including cell-based assays,[14] enzyme assays,[15−17] protein profiling,[18−20] and the polymerase chain reaction.[21] More importantly, the DMF format seems well suited for complex procedures such as protein extraction by precipitation, as DMF can be used to precisely control liquid-, solid-, and gas-phase reagents in heterogeneous systems.[22−25]

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In the work described here, we implemented an automated, DMF-based protocol for extracting and purifying proteins from heterogeneous mixtures, including the key steps of precipitation, rinsing, and resolubilization. The effectiveness of the new method was determined using electrospray ionization mass spectrometry (ESI-MS), and the % recovery was quantified using fluorescence. The method was demonstrated to be compatible with protein standards representing a range of different physicochemical properties, as well as with complex mixtures such as fetal bovine serum and cell lysate. In all cases, the performance of the new method was comparable to that of conventional techniques, with the advantages of automation and reduced analysis time. This work is an important first step in our efforts to develop fully automated microfluidic methods for proteomic analyses.

## EXPERIMENTAL SECTION

**Reagents and Materials.** Acetone, acetonitrile (ACN), chloroform, methanol, boric acid, 50% formic acid, fluorinert FC-40, sodium hydroxide, Pluronic F127, trichloroacetic acid (TCA), Triton X-100, phenylmethylsulfonyl fluoride (PMSF), sodium dodecyl sulfate (SDS), fluorescamine, ammonium sulfate, bovine serum albumin (BSA), fibrinogen (Fb), and myoglobin (Mb) were purchased from Sigma Chemical (Oakville, ON). Dulbecco's phosphate buffered saline (PBS) and fetal bovine serum (FBS) were purchased from Invitrogen Canada (Burlington, Ontario). 1,2-Dibutyroyl-*sn*-glycero-3-phosphocholine (PC) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). In all experiments, solvents of HPLC-grade and deionized (DI) water with a resistivity of 18 MΩ·cm at 25 °C were used.

Working solutions of all proteins were prepared in 10 mM borate buffer (pH 8.5) with 0.08% Pluronics F127 (w/v).[26] For qualitative analysis of protein extraction, a test solution containing 0.71 mM protein (Mb) and 62 mM lipid (PC) was prepared. For quantitative analysis of protein recovery, solutions of BSA (50 mg/mL), Mb (30 mg/mL), and Fb (20 mg/mL) were prepared. For experiments involving FBS and cell lysate, solutions were spiked with 0.08% Pluronics F127. Protein precipitation methods used trichloroacetic acid (TCA) (20% in DI water), acetonitrile (ACN), and ammonium sulfate (saturated solution in DI water) as the precipitants and acetone, chloroform/ACN (70/30 v/v), and chloroform/acetone (60/40 v/v) as rinse solutions.

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), parylene C dimer from Specialty Coating Systems (Indianapolis, IN), Teflon-AF from DuPont (Wilmington, DE), 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 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.

**Cell Culture and Analysis.** Jurkat T-cells were maintained in a humidified atmosphere (5% $CO_2$, 37 °C) in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 IU $mL^{-1}$), and streptomycin (100 $\mu g$ $mL^{-1}$). Cells were subcultured every 3−4 days at $5 \times 10^5$ cells $mL^{-1}$. Lysing medium was PBS with 0.08% (wt/v) F 127, 1% Triton X-100, and 1 mM PMSF. For precipitation experiments, cells were washed once in PBS, suspended in lysing medium at $6 \times 10^7$ cells $mL^{-1}$, incubated on ice (30 min), and centrifuged (13,000 rpm, 5 min). The supernatant was collected and stored at −80 °C until use.

**Device Fabrication and Operation.** Digital microfluidic devices were fabricated using conventional methods in the University of Toronto Emerging Communications Technology Institute (ECTI) cleanroom facility, using a transparent photomask printed at Norwood Graphics (Toronto, ON). Glass wafers (Howard Glass Co. Inc., Worcester, MA) were cleaned in piranha solution (10 min), and then coated with chromium (250 nm) by electron beam deposition. After rinsing and drying, the substrates were primed by spin-coating with HMDS (3000 rpm, 30 s) and then spin-coated again with Shipley S1811 photoresist (3000 rpm, 30 s). Substrates were prebaked on a hotplate (100°C, 2 min), and then exposed to UV radiation (35.5 mW $cm^{-2}$, 365 nm, 4 s) through a photomask using a Karl Suss MA6 mask aligner (Garching, Germany). After exposure, substrates were developed in MF-321 (3 min), and then postbaked on a hot plate (100 °C, 1 min). Following photolithography, substrates were immersed in chromium etchant (30 s). The remaining photoresist was stripped in AZ-300T (10 min). After forming electrodes and cleaning in piranha solution (30 s), substrates were coated with 2.5 $\mu$m of Parylene-C and 50 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, 2000 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 devices, unpatterned indium tin oxide (ITO) coated glass substrates (Delta Technologies Ltd., Stillwater, MN) were coated with Teflon-AF (50 nm, as above).

The devices had a double-cross geometry as shown in Figure 1a, with 1 × 1 and 1.5 × 1.5 mm actuation electrodes, 2.5 × 2.5 mm and 3.0 × 3.0 mm reservoir electrodes, and interelectrode gaps of 40 $\mu$m. Devices were assembled with an unpatterned ITO-glass top plate and a patterned bottom plate separated by a spacer formed from one or two pieces of double-sided tape (70 or 140 $\mu$m thick). Driving potentials (70−100 $V_{rms}$) were generated by amplifying the output of a function generator (Agilent Technologies, Santa Clara, CA) operating at 18 kHz. As described elsewhere,[14] droplets were sandwiched between the two plates and actuated by applying driving potentials between the top electrode (ground) and the 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.

**DMF-Driven Protein Extraction.** Protocols were developed using digital microfluidics to implement protein extraction by


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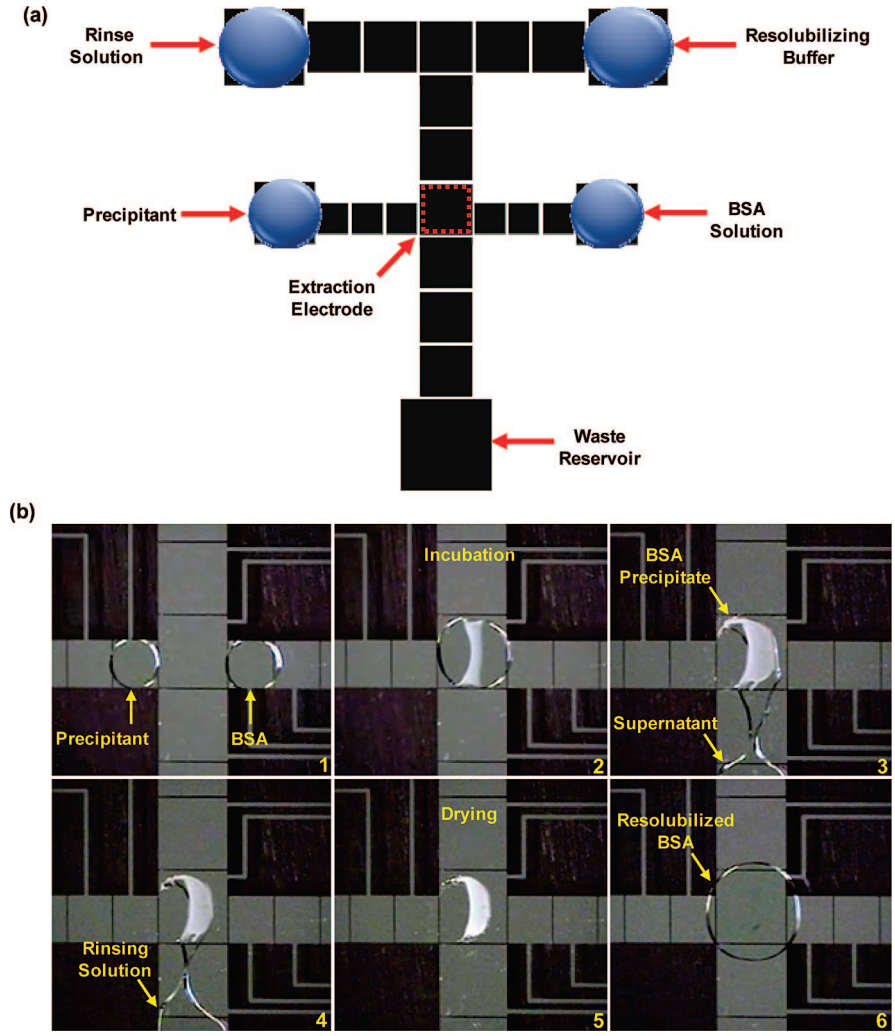


**Figure 1.** Digital microfluidic device and method for protein precipitation. (a) Schematic of device depicting the four reagent reservoirs, the waste reservoir, and extraction electrode. (b) Frames from a movie depicting the extraction and purification of BSA (50 mg/mL) in 20% TCA (precipitant) and washing with 70/30 v/v chloroform/acetonitrile (rinse solution). In the final frame, the precipitated protein is redissolved in a droplet of 100 mM borate buffer containing 1% SDS.

precipitation and resolubilization. In each experiment, droplets containing the sample (i.e., protein standards, mixtures, FBS, or cell lysate) and a precipitant were dispensed from their respective reservoirs and merged on the extraction electrode. In some cases, a third droplet containing a secondary precipitant was also dispensed and merged. The combined droplet was allowed to incubate until the protein was observed to precipitate from solution (~5 min, room temperature), after which the supernatant was actuated away from the extraction electrode (to the waste reservoir). The precipitate was then washed by dispensing and driving three droplets of rinse solution across the extraction electrode to waste. The precipitate was dried and a droplet of resolubilization solution was dispensed and driven to the extraction electrode to dissolve the protein. In some cases the precipitate was baked on a hot plate (95 °C, 5 min) prior to resolubilization to ensure complete removal of any solvent residue.

The composition of precipitant(s), rinse solutions, and resolubilization solutions were optimized for each analyte by trial-and-error and are recorded in Table 1. For protocols applied to standards, mixtures, and serum, sample and precipitant droplet volumes were ~140 nL, and rinse and resolubilization solution droplet volumes were ~315 nL. For the protocol applied to cell lysate, the corresponding volumes were ~70 and ~158 nL, respectively.

**Conventional Protein Extraction.** Protein samples were extracted on the macroscale by combining 10 $\mu$L of sample with 10 $\mu$L chilled precipitant in a microcentrifuge tube. The solution was incubated (5 min, 4 °C) and then centrifuged (13,000 rpm, 5 min), and the supernatant was discarded. The pellet was washed three times by iteratively suspending in rinse solution (22.5 $\mu$L) and centrifuging (13,000 rpm, 5 min) and discarding the supernatant. The final pellet was dissolved in 100 $\mu$L. The precipitant, rinse solution, and resolubilization solution for each analyte were identical to those used for DMF.

**Mass Spectrometry.** Extracts of PC/Mb mixtures were evaluated qualitatively by mass spectrometry. Briefly, 315 nL samples (prepared by DMF as described above) were diluted into 50 $\mu$L of 50/50 water/ACN containing 0.1% formic acid and injected into an LTQ linear ion trap mass spectrometer (Thermo Fischer Scientific, Waltham, MA) operating in positive ion mode. Samples were delivered via a fused silica capillary transfer line (100 $\mu$m i.d.) mated to a New Objective Inc. (Woburn, MA)

**Table 1. Solutions Used for Extracting, Purifying, And Resolubilizing Proteins**

| sample | primary precipitant | secondary precipitant | rinse solution | resolubilizing solution |
|---|---|---|---|---|
| Mb | ACN | N/A | chloroform/ACN (70/30 v/v) | 10 mM borate with 1% SDS |
| Fb | ACN | N/A | chloroform/ACN (70/30 v/v) | 10 mM borate with 1% SDS |
| Mb/PC | ACN | ammonium sulfate (saturated in DI water) | chloroform/ACN (70/30 v/v) | 10 mM borate with 1% SDS |
| BSA | 20% TCA | N/A | chloroform/ACN (70/30 v/v) | 100 mM borate with 1% SDS |
| FBS | 20% TCA | N/A | acetone | 100 mM borate with 10% SDS |
| cell lysate | 20% TCA | N/A | chloroform/acetone (60/40 v/v) | 100 mM borate with 10% SDS |

nanoelectrospray emitter (100 $\mu$m i.d. tapering to 30 $\mu$m i.d.). The samples were delivered at a flow rate of 0.5 $\mu$L min$^{-1}$, with an applied voltage of 1.7−1.9 kV and capillary temperature of 170 °C. Spectra were collected as an average of 50 acquisitions, and data shown here are representative of analysis of samples in triplicate.

**Fluorescence.** Extraction efficiency was evaluated quantitatively using a fluorescence-based assay. For samples prepared by DMF, 315 nL or 158 nL droplets (as above) of sample were diluted into 13 $\mu$L aliquots of pH 8.5 working buffer in wells in a 384-well low-volume microplate. The working buffers were identical to the resolubilizing buffers (Table 1). Upon addition of 2 $\mu$L of fluorescamine (5 mg/mL in acetone) the microplate was inserted into a fluorescence microplate reader (Pherastar, BMG Labtech, Durham, NC) equipped with a module for 390 nm excitation and 510 nm emission. The plate was shaken (1 min), allowed to sit (2 min), and then the fluorescence was measured.

As a control, for each analyte, identical samples that had not been extracted were evaluated using the same fluorescent assay. To ensure that controls were processed in identical volumes relative to extracted samples, each control was prepared by dispensing a droplet on a device, delivering it to the extraction electrode, and allowing it to dry. A droplet of resolubilization solution was then dispensed, driven to the dried spot, incubated to allow for full dissolution, and the droplet was collected and analyzed. Four replicate measurements were made for each sample and control.

For samples extracted using conventional methods, the quantitative analysis protocol was similar to that used for samples prepared by DMF, with identical working buffers and reagents. The difference was volume—in each case, precipitate from 10 $\mu$L samples was resolubilized in 100 $\mu$L of working buffer. Each sample was mixed with 13 $\mu$L fluorescamine (5 mg/mL in acetone) in a well in a 96-well plate. As with samples prepared by DMF, controls were evaluated, and four replicate trials were conducted for each sample and control.

## RESULTS AND DISCUSSION

**Device and Method Optimization.** In an automated method for protein extraction by precipitation, at least four different solutions must be managed: sample, precipitant, rinse solution, and resolubilization solution. To facilitate this goal, as shown in Figure 1a, we designed and built a digital microfluidic device with a "double T" pattern of electrodes, with four reservoirs (with dedicated droplet movement paths) for the various reagents, and a fifth reservoir for waste. The dimensions of the electrodes were chosen such that (in normal circumstances) the droplet volumes were 140 nL (sample and precipitant) and 315 nL (rinse and resolubilization solutions), respectively. As shown, a key position in the design is the extraction electrode—the surface above this electrode is where the precipitation and purification of proteins takes place.

In practice, samples were sequentially precipitated onto the extraction electrode, washed in rinse solution, and then resolubilized in solvent. Figure 1b shows a series of frames from a movie depicting this process for a sample containing BSA. As shown, a droplet containing BSA was merged with a droplet containing 20% TCA to precipitate the protein. After precipitation, the supernatant was driven away to the waste reservoir leaving the precipitate on the extraction electrode. The precipitate was then washed in three droplets of 70/30 chloroform/ACN and allowed to dry. Finally, the purified protein was resolubilized in a droplet of 100 mM borate (pH 8.5) buffer containing 1% SDS.

As is the case for conventional methods,[7] we found that the optimal DMF-driven recipe for protein precipitation varied from sample to sample (Table 1). For example, ACN worked well as a precipitant for highly concentrated solutions of Mb and Fb. Low concentrations (<1 mM) were more challenging; for example, reproducible precipitation and extraction of 0.71 mM myoglobin required two different precipitants, including concentrated ammonium sulfate. For samples containing proteins and phospholipids, chloroform was found to be a useful rinsing agent to remove residues of the phospholipid from the precipitate. However, because neat chloroform is not amenable to actuation at low driving potentials (as reported previously[27]), a mixture of chloroform and ACN (70/30 v/v) (which was readily movable) was used. As has been reported,[1,2,6] TCA was the best precipitant for large proteins and complex mixtures (BSA, FBS, and cell lysate). For BSA and FBS, acetone was found to be a useful rinsing agent to remove traces of TCA from the precipitate. For cell lysate, however, neat acetone was found to dissolve some of the precipitated protein; thus, a mixture of chloroform and acetone (60/40 v/v) was used. In all experiments, borate buffer containing SDS worked well for resolubilization, although in future work, we may experiment with alternatives (e.g., acid labile surfactants, urea, etc.).

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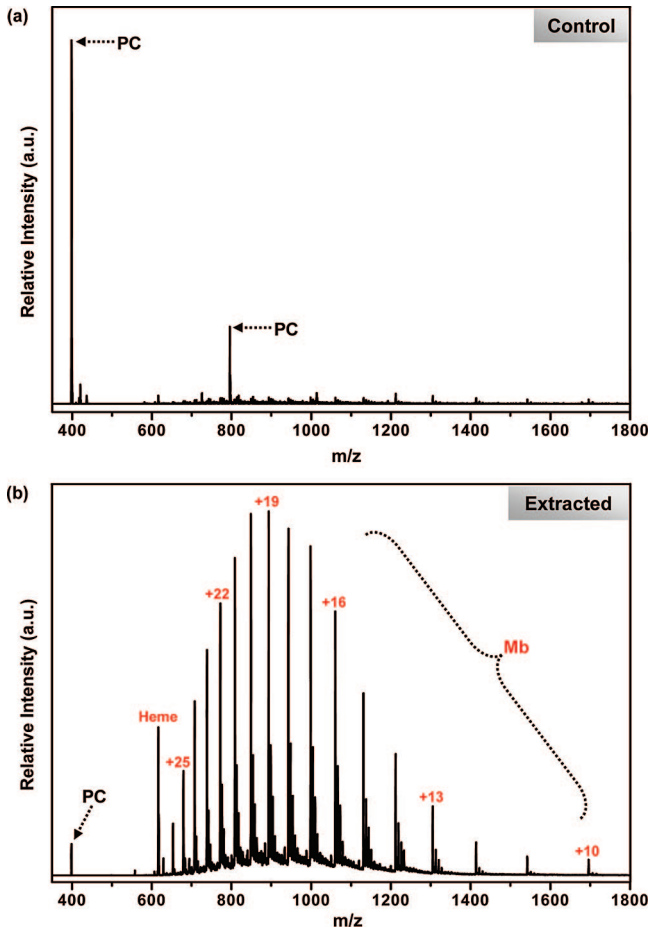


**Figure 2.** ESI-MS spectra of (a) control and (b) extracted samples containing model analyte, Mb (0.71 mM) and model contaminant, PC (62 mM).

The protein extraction method reported here differs from conventional techniques in many respects, but one difference stands out: no centrifugation is required. In initial experiments, we observed that precipitates in droplets settle much more quickly than they do in centrifuge tubes. This is largely a function of distance—the maximum path in DMF samples is ~0.1 mm, while the comparable parameter in microcentrifuge tubes or well plates is several millimeters. Moreover, once settled, precipitates in DMF devices adhere strongly to the Teflon-AF device surfaces. This is fortuitous, as it facilitates the process of separating the liquid phase from solid (Figure 1b frames 3−4). These characteristics make the DMF-based extraction procedure faster by a factor of 2 relative to the macroscale equivalent (from ~30 to ~15 min).

**Evaluation of Method Efficiency.** To qualitatively evaluate the effectiveness of on-chip protein extraction, we used a model system comprising a protein analyte (Mb) and a phospholipid contaminant (PC). To approximate a demanding scenario, the concentrations were chosen to have a large excess (80:1) of contaminant. Figure 2 shows representative mass spectra generated from control and extracted solutions. As shown, in the spectrum of the control sample (Figure 2a), there are two prominent peaks at $m/z$ 398 and 795 representing the $[M + H]^{+1}$ and $[2M + H]^{+1}$ ions of PC. A close look at the spectrum reveals a noisy baseline in the 600−1,800 $m/z$ region corresponding to a low signal from Mb, which is suppressed by the high concentration of contaminant. In the spectrum of the extracted sample (Figure 2b), the peaks in the 600−1,800 $m/z$ region correspond to the multiply charged ions of Mb (+27 to +10). An additional peak at $m/z$ 616 represents the $[M + H]^{+1}$ ion of the dissociated Heme cofactor. A very small peak at $m/z$ 398 corresponds to the trace PC that remains in the solution. As shown, this method is a qualitative success, transforming a contaminated sample with very little analyte signal into a much purer solution that contains primarily analyte.

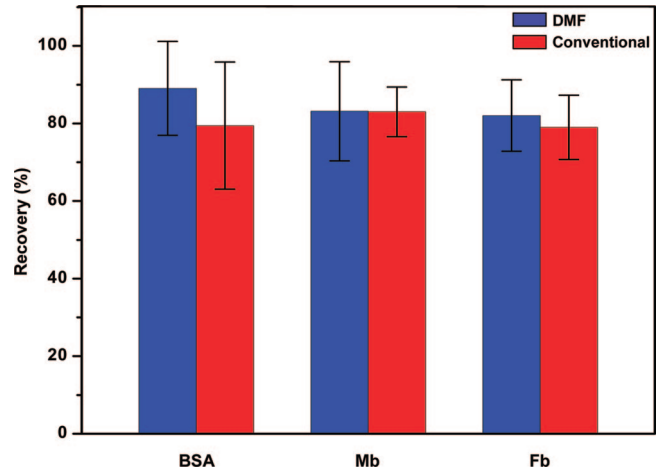


**Figure 3.** Bar graph comparing the recovery efficiency for protein standards using the DMF method (blue bars) and conventional macroscale techniques (pipet, centrifuge, etc.; red bars). In each experiment, BSA (50 mg/mL), Mb (30 mg/mL), or Fb (20 mg/mL) samples were precipitated, washed, resolubilized, and reacted with fluorescamine, and the fluorescence intensity was compared with that of a control. The data represent the mean ± SD of four extractions for each condition.

To quantitatively evaluate the extraction efficiency of the new DMF techniques, we used a fluorescence-based assay similar to the widely used absorbance-based Bradford test.[28] The fluorescent method relies on fluorescamine, a fluorogenic reagent that exhibits no fluorescence until it reacts with primary amines, such as those at the N-terminus of proteins.[29,30] The reaction is immediate and is used for non-specific labeling and quantification of proteins in solution.[31,32] Three proteins representing a range of physicochemical characteristics, BSA (MW 66 kDa, pI 4.7), Mb (17 kDa, pI 7.3), and Fb (MW 340 kDa, pI 5.5) were evaluated at different concentrations to determine the range of linear response. Concentrations found to be in this range (50, 30, and 20 mg/mL of BSA, Mb, and Fb respectively) were chosen for quantitative analysis, and % recovery was determined by comparing the fluorescence intensity of multiple samples before and after extraction. For comparison, the same samples and concentrations were also extracted from 10 $\mu$L aliquots in microcentrifuge tubes.

As shown in Figure 3, the new digital microfluidic method proved to be very efficient—over 80% was recovered for each protein standard evaluated. In initial experiments, we hypothesized that macroscale methods might have better recovery, as the

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centrifugation step might facilitate collection of very small, non-settling precipitate particles. However, we did not observe this to be the case—as shown, the efficiencies determined for the new method were comparable or better than those calculated for standard techniques. We note that protein recovery in conventional precipitation methods scales with concentration, and the use of lower concentrations results in reduced recovery rates (low concentrations were not evaluated here). Regardless, for the concentration ranges described here (which overlap with those commonly encountered in clinical samples) the new microfluidic method seems to be an analogous procedure to the macroscale technique, with the advantages of automation and shorter processing times.

**Application to Complex Solutions.** Protein precipitation is most useful as a sample cleanup step applied to complex mixtures; for example, precipitation is often used to purify proteins from plasma for proteome profiling.[33,34] To evaluate the potential for the new digital microfluidic method for such applications, we tested two model systems: fetal bovine serum (FBS) and cell lysate (CL). In both cases, the samples were found to be compatible with digital microfluidic manipulation and extraction. Figure 4 shows images of dried protein precipitates generated from droplets of FBS and CL.

FBS and CL solutions were more challenging to work with than protein standard solutions. First, dispensing droplets of cell lysate was difficult because the solution was so viscous. To solve this problem, the spacing between the top plate and the patterned bottom plate was decreased from 140 to 70 $\mu$m (resulting in a smaller droplet volume)—this strategy (reducing the spacing between plates) has been shown to enhance dispensing of viscous solutions in DMF.[35] Second, precipitates formed from FBS and CL were particularly sticky, and in rare cases, the supernatant droplet could not be driven away from the extraction electrode. In such cases, we found that if a supplemental droplet of rinse solution was dispensed and driven to the extraction electrode, the combined droplet (rinse solvent + supernatant) could be successfully driven to waste. Upon application of these strategies (reduced interplate spacing and supplemental rinse droplets), the extraction process for FBS and CL became reliable and reproducible.

Protein recovery efficiencies from FBS and CL were determined using the fluorescent assay described above. In each case, performance was excellent (mean ± S.D.): 84.0 ± 7.8% for FBS and 82.4 ± 11.5% for CL. These values are comparable to those reported for macro-scale techniques.[2] We note that higher protein recovery can be achieved by using less rigorous rinsing; however, multiple rinse steps are typically used to ensure high purity of the processed sample. In summary, the performance of the new DMF-driven method is comparable to conventional techniques even for complex mixtures, which bodes well for our plans to integrate this procedure with other processing steps (such as enzymatic digestion[26]) for automated, miniaturized proteomic analysis.


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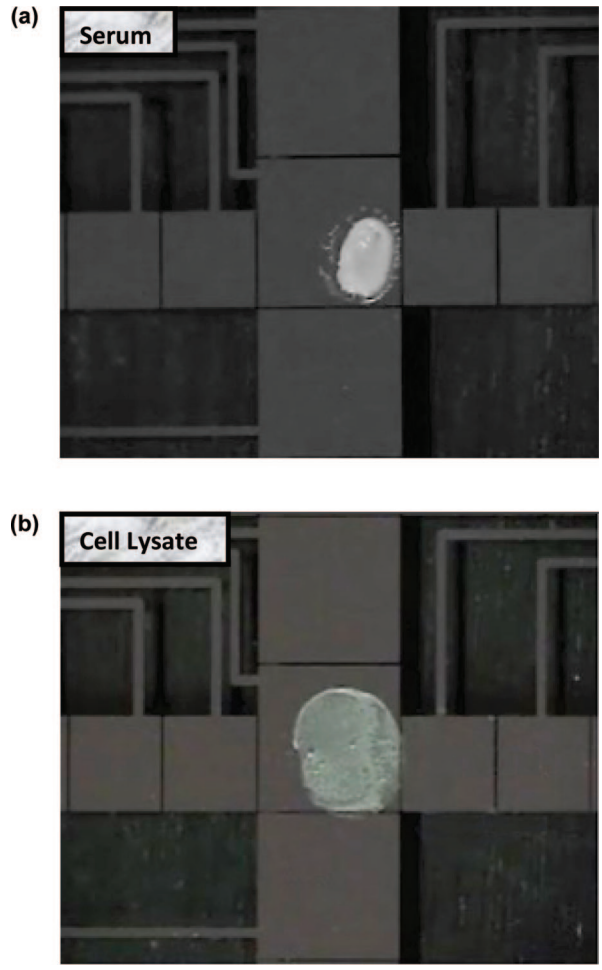


**Figure 4.** Images of protein precipitates generated from droplets of (a) fetal bovine serum (140 nL) and (b) cell lysate (70 nL) using the DMF method.

## CONCLUSION

We report here the first application of microfluidics to protein extraction by precipitation. In this work, digital microfluidics (DMF) was used to extract and purify proteins from protein standards and heterogeneous mixtures. The new method had comparable performance relative to conventional techniques, combined with the advantages of reduced reagent and sample consumption, no centrifugation, and automated liquid handling. These results suggest great potential for the development of integrated, multistep processes incorporating sample reduction, alkylation, and digestion. Thus, this work represents an important first step in our efforts to develop fully automated microfluidic methods for proteomic analyses.

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