TOWARDS SINGLE-CELL PROTEOFORM PROFILING: ON-CHIP ISOELECTRIC FOCUSING IN IMMOBILIZED pH GRADIENT GELS

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ABSTRACT

Microfluidic tools are an essential capability in understanding cell-to-cell heterogeneity. Similarly, precision microanalytical tools can play an outsize role in measuring molecule-to-molecule heterogeneity, especially as protein post-translational modifications create proteoforms that execute regular and aberrant cellular functions. Although highly related, slight variations in protein charge contribute to proteoform heterogeneity. Electrophoretic methods, including immobilized pH gradient isoelectric focusing (IPG-IEF), provide the resolution to measure subtle protein-to-protein differences. However, conventional IPG-IEF can be low-throughput and requires large starting sample amounts, precluding single-cell analysis. Here, we present the first polydimethylsiloxane (PDMS)-based, immunoprobed, microfluidic IPG-IEF device to our knowledge. We demonstrate separation performance on par with our single-cell IEF device, including resolving analytes differing by only 0.1 pH.

KEYWORDS

Microfluidics, Electrophoresis, Proteoforms, Isoelectric Focusing, Immobilized pH Gradient.

INTRODUCTION

IEF can separate proteoforms arising from post-translational modifications of proteins (Figure 1a) [1,2]. Miniaturization of IEF is necessary for high-throughput analysis of proteoforms with reduced starting sample amounts, as is the case in single-cell studies. Microfluidic IEF has typically been carried out using carrier ampholytes (CAs) [3,4]. In CA-IEF, a mixture of CAs placed between an anolyte and catholyte region arrange themselves into a pH gradient under an applied electric field and then the sample proteins migrate to their isoelectric point, pI, in the gradient. CA-IEF suffers from gradient instability and the pH range cannot be easily engineered [2]. In the current single-cell isoelectric focusing assay, CAs are delivered using a hydrogel lid containing the CA, anolyte, and catholyte regions [3]. Since the CAs are free to diffuse throughout the hydrogel, the delivery lid must be prepared right before the assay is run, which adds a highly time-sensitive step to the assay and limits the throughput of the technique.

Another class of IEF employs IPG gels in which a pH gradient is already established before sample application by incorporating Immobilines into the polyacrylamide (PA) gel. Immobilines are small molecules containing either a carboxyl or tertiary amino group that buffer the gel to a different pH. By creating a gradient in Immobiline concentrations across the gel, a highly tunable pH gradient is created. Tunability is key, since a broad pH range might be desired in a multiplexing study with various proteins, while a narrow pH range increases the minimum resolvable pI difference and can be used to separate proteoforms close in pI. Since the pH gradient is immobilized, drift of protein bands towards the cathode is abolished and the protein band position is reproducible [2,5]. The microfluidic IPG-IEF device presented here is fabricated with a similar robust Immobiline chemistry employed by centimeter-scale IPG strips.

An IPG-IEF microfluidic device was previously reported, but it was fabricated in glass microchannels [6]. Glass microfluidic devices are difficult and expensive to fabricate [4]. Moreover, the sample proteins had to be pre-labeled with dye for detection. To overcome the limitations posed by glass fabrication, the IPG-IEF device presented here is made with PDMS. Additionally, we introduce a photo-active hydrogel overlayed on the IPG hydrogel to covalently immobilize proteins after irradiation with a UV light so that captured proteins can be read out via immunofluorescence.

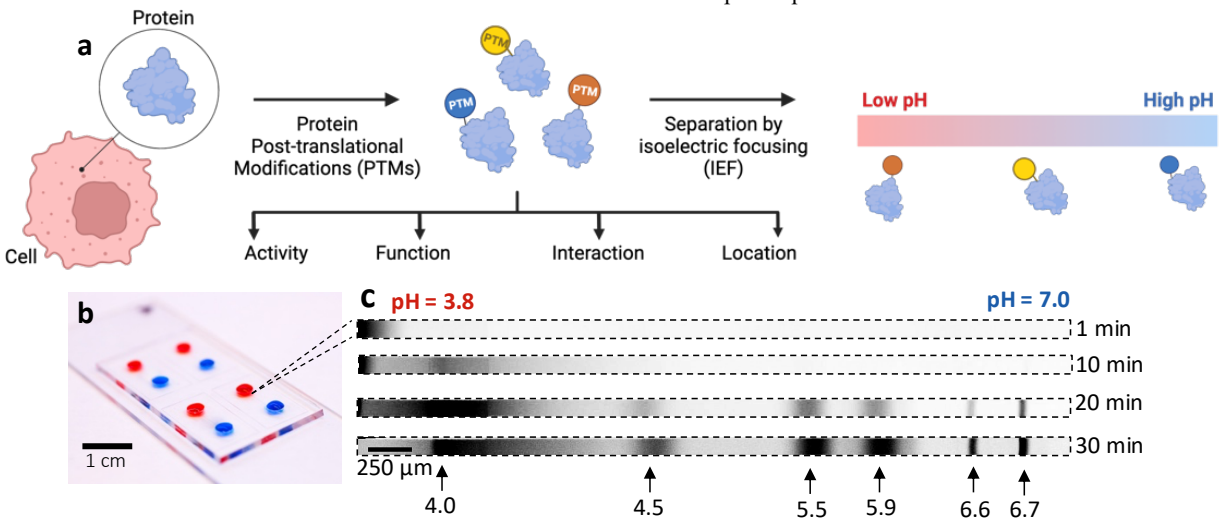

Figure 1: On-chip IPG-IEF enables separation by isoelectric point. (a) IEF can be applied to the analysis of proteoforms. Created with BioRender.com. (b) IPG-IEF device containing food dye-filled single channels. (c) Inverted fluorescent micrographs of IEF-separated pI markers at several time points demonstrates rapid focusing and gradient stability. Representative of n = 3 separations.

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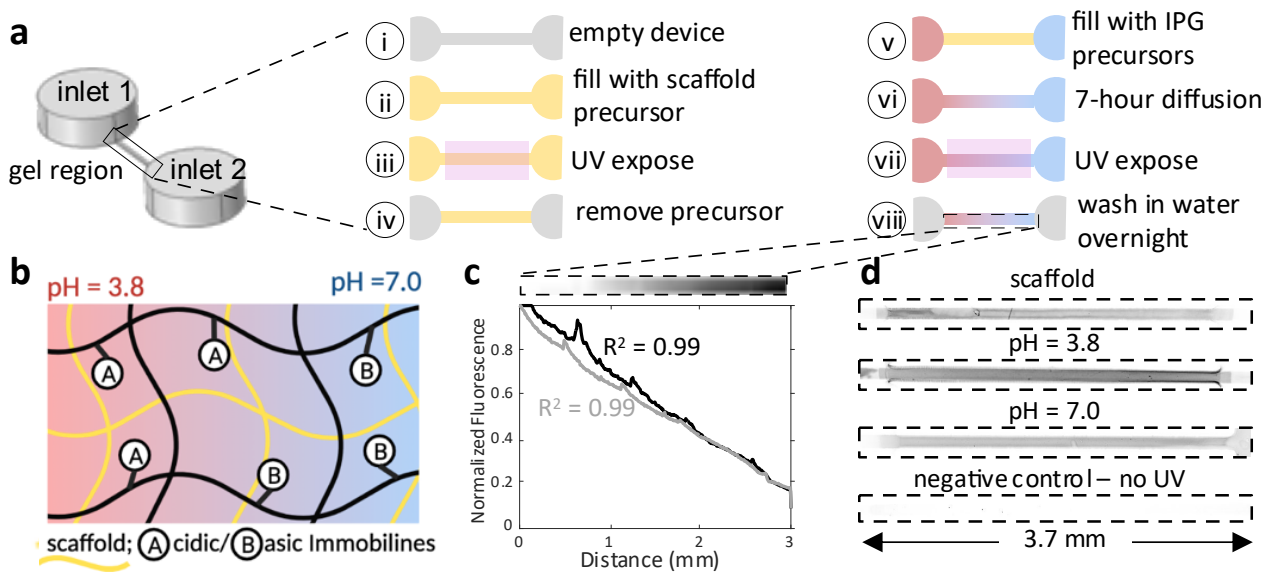

Figure 2: Development of IPG-IEF device. (a) IPG-IEF device fabrication using photopolymerization. b) IPG PA gel is overlayed on scaffold PA gel. Created with BioRender.com. (c) Linearity of gradient monitored with Nile Blue acrylamide dye. Plot shows the intensity profile after 7.5 hours for n = 2 IPG devices. (d) Confirmation of UV polymerization of 3 PA gel precursors individually in microchannels.

RESULTS AND DISCUSSION

Fabrication of IPG-IEF Device

The IPG-IEF device presented here holds 4 separation lanes on a PDMS slab (Figure 1b) and is suitable for 42 of the 9x3 mm footprint lanes. Loaded analytes migrate along the pre-established pH gradient until reaching net zero charge (Figure 1c). Figure 2a details the 2-step photopolymerization of PA gel precursors, which is based on our previous work fabricating stable hydrogel gradients in a PDMS device [7]. Steps i-iv are modified from previous PDMS-based electrophoresis work [8], and steps v-viii were added in the present work to overlay the IPG onto the PA scaffold (Figure 2b). The presence of the scaffold prevents fluid flow and allows the establishment of a linear gradient (Figure 2c). The scaffold also contains a photo-active moiety for in-gel immunoprobing [3]. Next, we confirmed all precursors polymerized in the PDMS channel (Figure 2d). IPG-IEF device fabrication is easy and can be completed in 2 days with ~3 hours of hands-on time.

Performance of IPG-IEF Device

Our IPG-IEF device was designed to separate proteoforms having pI's in the pH range of 3.8 to 7.0. Figure 3a demonstrates separation of 6 pI markers (ranging from 4.0 to 6.7). The intensity profile of all 6 pI markers (Figure 3b) resolves pI markers 6.6 and 6.7, which differ by only 0.1 pH unit. The IPG-IEF device's pH gradient is linear from 4.5 to 6.7 (Figure 3c). A voltage ramp (Figure 4) was utilized to minimize protein precipitation [6]. Critically, the IPG-IEF device performance was comparable to our low-throughput single-cell IEF device capable of differentiating proteoforms differing by a single charge unit (Table 1) [3,9]. The minimum pI difference that can be resolved, Δ(pI)min = 0.20, is a conservative estimate as Δ(pI)min was calculated with pI markers that are 100X smaller than proteins by molecular weight, and therefore have broader peak widths. For context, a single phosphorylation event can cause a pI change of 0.3-0.4 [10].

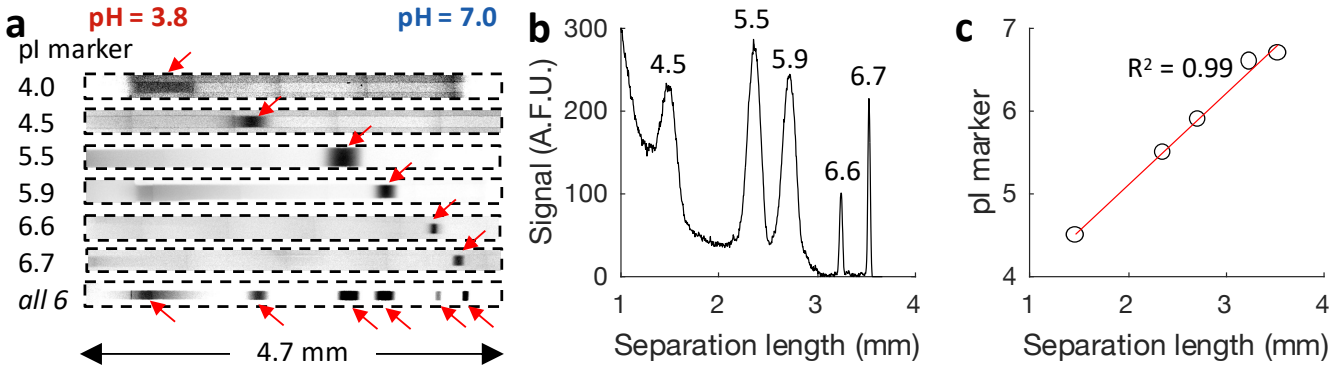

Figure 3: Characterization of pH gradient in IPG device. (a) pI markers were loaded at anode inlet and focused individually to verify identity of each band in the combined "all 6" condition. pI marker 4.0 overlaps with incomplete injection of other pI markers. Brightness and contrast adjusted for each micrograph for better visualization. (b) Intensity plot of "all 6" pI markers after 20 minutes of focusing. (c) Plot shows the position of 5 pI markers in (b) and linearity of pH gradient from pH 4.5 – 6.7. Representative of n = 3 separations.

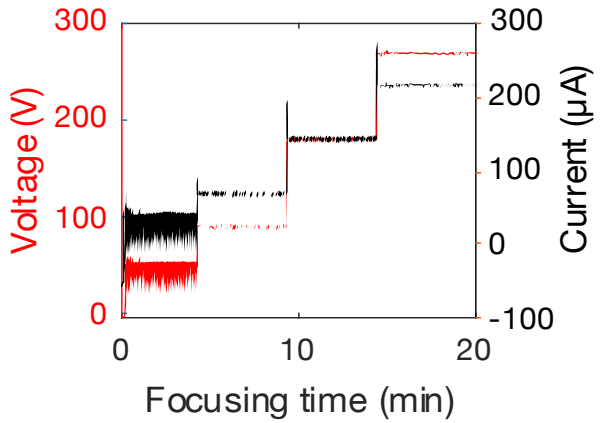

Figure 4: Voltage and current trace under constant voltage conditions. Representative of n = 3 separations.

Immunoprobed Readout of IPG-IEF Device

Finally, we performed IPG-IEF of TurboGFP in our device (Figure 5). To immunoprobe, the PDMS was delaminated from the glass slide and incubated with primary and secondary antibodies. TurboGFP typically has 3 proteoforms in IEF [3,6]. While the proteoforms are not fully resolved, the presence of peak "shoulders" suggests that detecting all 3 proteoforms should be possible with optimization.

CONCLUSION

Answering the National Academy of Engineering's call to "Engineer the Tools of Scientific Discovery," we have developed the first immunoprobed, microfluidic IPG-IEF device in PDMS. In next steps, we will advance to single-cell analysis in an all-in-one microdevice with on-chip lysis, separation, and readout for high-throughput proteoform profiling.

EXPERIMENTAL SECTION

Device Fabrication

The IPG-IEF device was fabricated by standard soft lithography methods. RTV615 PDMS (Momentive) was mixed at a 10:1 ratio, degassed, and poured over a silicon wafer mold of the microchannel features. Each microchannel is 3.5-mm long, 100-µm wide, and 50-µm tall. After the PDMS was cured at 80ºC for 2 hours, a biopsy punch was used to make the 3-mm diameter inlet and outlet holes. To reversibly attach the PDMS to the glass slide substrate, we did not perform plasma bonding.

The next step is to polymerize PA gel in the PDMS channels. Since PA polymerization is oxygen inhibited and PDMS is known to absorb oxygen, the channels are first incubated with 10% benzophenone in acetone for 3 minutes. Benzophenone serves as an oxygen scavenger when exposed to UV [8]. A 6 %T PA gel precursor containing acrylamide/bis-acrylamide (Sigma-Aldrich), the photo-active moiety N-[3-[(3-benzoylphenyl)formamido]propyl] methacrylamide (BPMAC, PharmAgra Laboratories), and VA-086 photoinitator (Wako Chemicals) is then flowed through the channels and photopolymerized with 2 minutes of UV light exposure at 17 mW/cm^2 (Figure 2ai-iv). The inlets were then filled with 6% T PA acidic (pH = 3.8) and basic (pH = 7.0) precursors containing acrylamide/bis-acrylamide, Immobilines (Sigma-Aldrich), and VA-086. The acidic and basic IPG precursor recipes were adapted from previous work [6]. The precursors were allowed to diffuse into the microchannels for 7 hours to establish a linear gradient and then were photopolymerized with 2 minutes of UV light exposure at 17 mW/cm^2 (Figure 2av-viii). The devices were then washed in deionized water overnight and could be stored at 4ºC in deionized water for at least 9 days.

Table 1. Comparison of IPG device with single-cell IEF device[3,7]. Only pI markers with Gaussian profile were used for analysis of IPG device (n = 3; mean ± standard deviation).

	IPG device	single-cell IEF[3,9]
Linearity of pH gradient (R^2)	~0.99	~0.90
$\Delta(pI)_{min}$	0.20 ± 0.0069	0.16 ± 0.02
Peak Capacity	16 ± 2.0	12 ± 5.9
Slope of pH gradient (dpH/dx)	1.18 ± 0.11 mm^{-1}	~0.35 mm^{-1}
Separation length	3.5 mm	9 mm
Focusing time	20 min	~7.5 min
Device can be stored for:	>= 9 days	must be prepared fresh

Gradient Formation and Polymerization Experiments

Nile Blue acrylamide (NB) was used as a proxy for Immobiline chemicals since it has a similar molecular weight and would therefore be expected to diffuse through the PA gels at a similar time scale. For the diffusion experiment in Figure 2c, NB was spiked into the acidic gel precursor at a concentration of 17.4 µM. For the polymerization test experiment in Figure 2d, AlexaFluor-647-labeled donkey anti-rabbit antibody (Invitrogen A-31573) was spiked into the scaffold, acidic, and basic gel precursors at a concentration of 0.01 mg/mL. For the polymerization experiment, the antibody simply served as a large molecular weight dye.

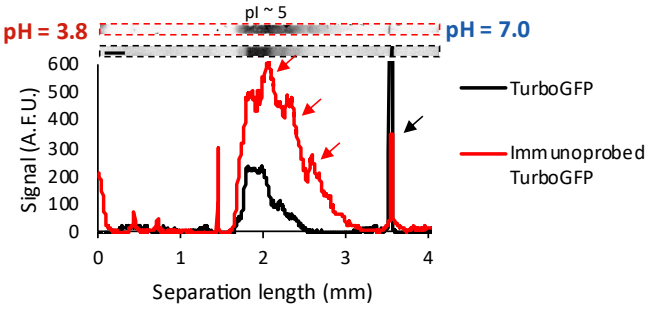

Figure 5: Immunoprobing IPG-IEF device. Inverted fluorescence images and intensity plots of IPG-IEF separated TurboGFP and immunoprobed TurboGFP. Red arrows point to possible TurboGFP proteoforms. Black arrow points to unknown species. Representative of n = 2 separations.

Isoelectric Focusing Experiments

Fluorescent pI markers (Sigma-Aldrich) were used at a concentration ranging from 3.33 µg/mL to 6.67 µg/mL to provide similar band intensities and were diluted in deionized water. The pI marker sample was applied at the anode inlet (near pH = 3.8) and the cathode inlet (near pH = 7.0) remained filled with deionized water. TurboGFP (Evrogen) protein was used at a concentration of 50 nM and applied to the cathode end. For protein experiments, the anode inlet was filled with 1X IEF Anode Buffer and the cathode inlet was filled with 1X IEF Cathode Buffer (Bio-Rad). The inlets were connected to a programmable high voltage power supply, LabSmith HVS448LC 3000V High Voltage Sequencer, with platinum electrodes. An electric field was applied using the following voltage ramp: 50 V/cm for 4 minutes, 100 V/cm for 5 minutes, 200 V/cm for 5 minutes, and 300 V/cm for 6 minutes. For the protein experiments, TurboGFP was immobilized in the IPG gel by irradiation of UV light at 100% intensity for 45 seconds with the Hamamatsu LC8 Spot Light Source.

Immunoprobing

To expose the IPG gels for immunoprobing, the PDMS layer of the IPG-IEF device was gently peeled from the glass slide. Since the PA is covalently bonded to the PDMS and not the glass slide, the PA remains on the PDMS layer. The IPG gels were rinsed in tris-buffered saline with Tween-20 (TBS-T, Cell Signaling Technologies) for 30 minutes and blocked in 2% bovine serum albumin (BSA, Sigma-Aldrich) in TBS-T for 30 minutes. The antibodies used for immunoprobing were primary rabbit anti-tGFP antibody (Pierce PA5-22688) at a concentration of 33 µg/mL and secondary polyclonal antibody AlexaFluor-647-labeled donkey anti-rabbit (Invitrogen A-31573) at a concentration of 67 µg/mL. After primary (2 hour) and secondary (1 hour) antibody incubation steps, the IPG gels were washed in TBS-T for 1 hour. The IPG gels were then rinsed with deionized water briefly and gently dried with a nitrogen stream before fluorescence imaging on an Olympus IX50 inverted epifluorescence microscope.

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