MICROFLUIDIC WESTERN BLOTTING: CATIONIC SURFACTANT BASED PROTEIN SIZING INTEGRATED WITH ELECTROSTATIC IMMOBILIZATION

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ABSTRACT

We report on fully-functional Western blotting demonstrated in an automated microfluidic format. The novel microfluidic device incorporates contiguous polyacrylamide (PA) gel regions tailored for different biochemical/physical functionality in a microchamber. Our photopatterned polymer design strategy enables assay performance not possible with traditional bench-top Western blotting: rapid completion of multiple Western-blotting steps (~2 hours), minute sample consumption (< 10 ng), and no manual intervention. Using this format, three key Western blotting steps are seamlessly integrated in a single microfluidic device: (1) accurate protein sizing and separation via cationic detergent based polyacrylamide gel electrophoresis (Cat-PAGE), (2) immobilization of proteins after separation using electrostatic interaction with PA gel matrix, and (3) antibody-based detection and quantitation of immobilized protein targets. As a means to characterize system performance, a protein ladder consisting of protein G, ovalbumin (OVA), bovine serum albumin (BSA), and α-actinin is separated and then immobilized with mobility information preserved. Subsequent immunoblotting is also demonstrated. We see this format as forming the basis for unmatched protein blotting as is relevant to basic life sciences research.

INTRODUCTION

Western blotting is an indispensable bioanalytical tool – found in nearly every bioscience or biomedical research laboratory. Separations based on electrophoretic mobility in a polymeric sieving matrix (i.e., PAGE) provide molecular weight (MW) information for proteins. Subsequent transfer of proteins to a membrane and incubation with immunoaffinity probes allows highly specific detection of a target protein in complex biological fluids [1]. With this combination of assay steps, Western blotting is not only a powerful bioanalytical tool but also a diagnostic tool for diseases [2].

However, bench-top Western blotting (Figure 1) has critical performance drawbacks including large sample consumption (1-40 µg), long assay times (hours-to-days), and numerous manual intervention steps. As a solution to these problems, our research group laid out ground work for microfluidic Western blotting: 1D [2] and 2D [3] microfluidic PAGE and immunoblotting using antibody copolymerized in PA gels. Building on these initial studies, the present work introduces both device and assay innovation to integrate the three steps central to Western blotting in a microfluidic device without compromise. The new approach offers significant advances: (a) surfactant-based protein sizing to determine accurate MW, (b) electrostatic immobilization of all resolved proteins, and (c) subsequent use of an antibody probe for a protein target.

A distinct difference of the new approach is the sizing assay: a cationic surfactant that maintains protein activity is used in lieu of harsher anionic detergents [4]. The cationic surfactant (CTAB) binds proteins at an equal molar ratio and thus imposes the identical charge-to-mass ratio. As a result linear log-MW to mobility relation is observed for separation of CTAB-treated proteins. CTAB retains protein binding activity. Therefore, direct antibody-based blotting is possible – unlike *de-facto* standard anionic SDS (sodium dodecyl sulfate) system.

Most notably, a novel mechanism for post-sizing protein immobilization is devised for CTAB-PAGE. In sharp contrast to our previous antibody-based immobilization approaches [2, 3], we have developed charged PA gels. The net surface charges of proteins and gel matrix in opposite polarity enable electrostatic interaction. All separated proteins are strongly immobilized, conserving full separation

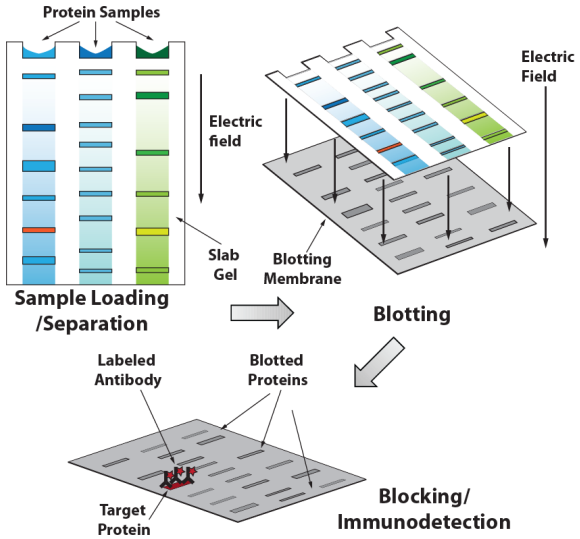

Figure 1: Conventional Western blotting steps. Samples are loaded into wells of a slab gel (Sample loading). The charged proteins are separated due to different mobility under electric field (Separation). The separated protein bands are transferred to a membrane support by electric field (Electrotransfer). After blocking of open charge sites (Blocking), an immunoaffinity probe is introduced for detection of a protein target (Immunodetection).

pattern during subsequent assay steps. After blocking step that prevents non-specific antibody binding, immunoaffinity probes are electrophoretically injected, which eliminates the need for complex copolymerization of antibodies in the gel matrix to "blot" the target. The assay allows detection of target proteins without a complex renaturing process that SDS system requires.

DESIGN

A glass microfluidic chip (Figure 2a, b) was designed using Autocad software (Autodesk, San Rafael, CA). A rectangular microchamber (1.0×1.5 mm^2) forms the core of the device that accommodates three functional gel regions for different Western blotting steps (Figure 2c, the loading gel for sample loading, the separation gel for protein sizing, and the blotting gel for transfer and target probing). The chamber is connected with several microfluidic channels, namely the injection channel and control channels. The nominal width of the injection channel is 25 µm, and that of a control channel is 10 µm. The injection channel branches into thee channels (1, 2, 3) that constitute a double-T junction, where a narrow sample plug is formed by applying a pinch current. Control channels (4, 5, 6, 7, 8) are also linked to the microchamber. Constant current is injected via these highly-resistive narrow channels (i.e., current source). The electric fields are parallel to the currents. Current injection allows the electric field lines to be oriented parallel to the direction of material transport [5]. Thus, sample dispersion in the chamber can be minimized. Using the current injection method, the separated proteins migrate horizontally and vertically while preserving the resolution of the separation.

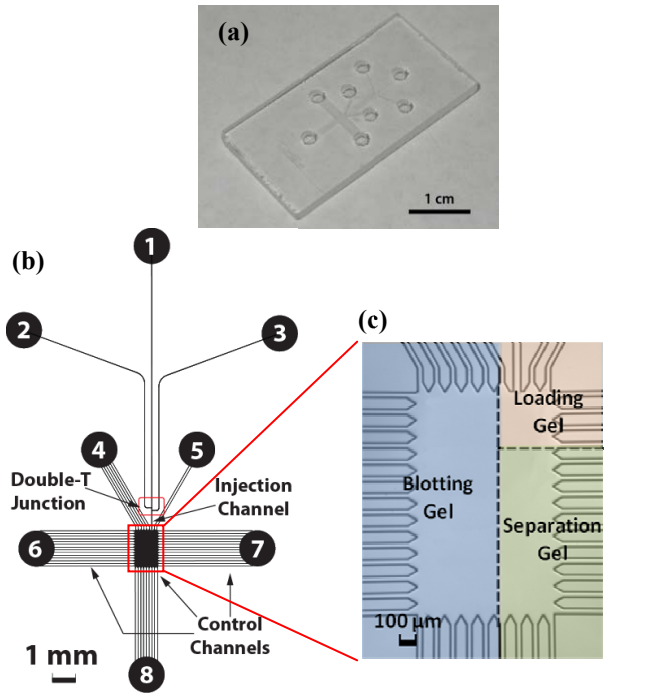

Figure 2: Microdevice design supports fully integrated Western blotting. (a) Photograph of the glass chip. (b) Microfluidic channels and a 2D microchamber. Electrophoretic sample manipulation is performed using a voltage control via access holes 1 to 8. (c) Gel regions of different biochemical and physical functionalities are photopatterned in the 2D chamber to integrate the different Western-blotting steps.

FABRICATION

Glass microfluidic chips were lithographed, isotropically etched, and diced in a foundry (Caliper Life Sciences, Hopkinton, MA). The etch depth was 20 µm. In house, eight access holes were drilled and the chip was cleaned thoroughly (e.g., piranha bath) then thermally bonded to a blank chip for 6 hours at 592 ºC in a programmable oven.

Before photopatterning of PA gels inside the 2D chamber, the glass chip was thoroughly cleaned by flushing channels with 0.1 M NaOH for 10 min, and rinsing with DI water for 10 min and methanol for 5 min. For covalent linkage of PA gel to the inner glass surface, the surface was silanized using a solution consisting of a 2:2:3:3 ratio of 3-(trimethoxysilyl) propyl methacrylate, glacial acetic acid, DI water, and methanol (all from Sigma, St. Luis, MO). The solution was introduced into the chip and incubated for 30 min, and rinsed with methanol for 30 min. For photopatterning of three functional gels (Figure 2c), each gel precursor solution was introduced inside the chamber by capillary action, polymerized using UV light, and then evacuated and exchanged with subsequent precursor solution using vacuum suction. The 9%T (w/v) precursor solution for the blotting gel was prepared using 30 µL of 30%T acrylamide/bisacrylamide (Sigma), 10 µL of 1 mg/ml streptavidin acrylamide (Invitrogen, Carlsbad, CA), 40 µL of 2 mg/ml of capture protein, 20 µL of 5×TA buffer (1×TA buffer: 25 mM tricine-14 mM arginine, all from Sigma), and 5 µL of 1% (w/v) photoinitiator VA-086 solution (Wako Chemical, Richmond, VA). The 6%T separation gel and 3%T loading gel were photopatterned similarly except that streptavidin-acrylamide and capture protein were not included in gel precursors. 3%T loading gel was also polymerized in the microchannels 1, 2, 3 for sample loading.

A custom UV exposure system was used for photo-patterning. An inverted microscope (IX-50, Olympus, Melville, NY) equipped with mercury-lamp and UV objective lens (UPLANS-APO 4×, Olympus) allows manual alignment between a glass chip and a transparency mask (Fineline-Imaging, Colorado Springs, CO), and subsequent UV exposure for photopolymerization. The blotting and separation gels were exposed for 8 min at 13 mW/cm^2. The loading gel was blanket-exposed for 6 min at 9 mW/cm^2) under a UV lamp (Blak-Ray, Upland, CA).

Four standard proteins were conjugated with fluorophore (Alexa Fluor 488, Invitrogen): protein G (20 kDa, Abcam, Cambridge, MA), OVA (45 kDa, Sigma), BSA (68 kDa, Sigma), and α-actinin (95 kDa, Sigma). The proteins were solubilized in 1×TA buffer. 0.1% (w/v) CTAB (Sigma) was added to the proteins 5 min before loading to the chip.

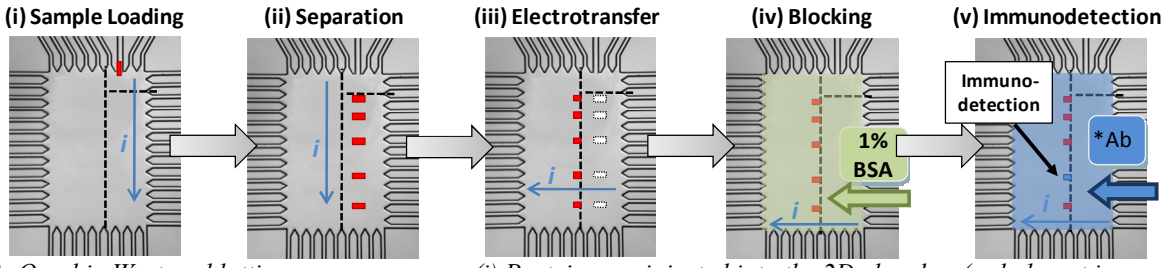

*Figure 3: On-chip Western blotting assay sequence: (i) Proteins are injected into the 2D chamber (red plug at image top). (ii) Proteins separate according to MW. (iii) Resolved proteins are transferred to the blotting gel by horizontal electric field, and immobilized by electrostatic interaction. (iv) Blocking reagent (1% BSA) prevents non-specific binding of antibody. (v) Finally, antibody probe (*Ab) is introduced to the immobilized sample.*

MICROFLUIDIC PAGE ASSAY

All assay steps (Figure 3) were performed by controlling voltage and current via 8 access holes with a custom high-voltage sequencer. Just before the sample loading step, 0.1% CTAB was electrophoretically introduced to the PA gels as insufficient CTAB concentration results in: (1) dilution mediated CTAB-protein dissociation, and (2) significant unwanted binding of proteins to the PA gels before the PAGE separation.

An epifluorescence microscope (IX-70, Olympus) equipped with a Peltier-cooled CCD camera (CoolSNAP HQ2, Photometrics, Tucson, AZ) was used to capture fluorescence image of proteins during all assay stages. The images were later analyzed for protein quantitation using ImageJ software (NIH, Bethesda, Maryland).

RESULT

The CTAB-treated molecular-weight standard was pipetted into the hole 3. A narrow plug was formed at the double-T junction. Due to the large pore size (3%T) of the loading gel, sample loading was fast (< 1 min). The sample was injected into the 2D chamber (Figure 3i). Upon reaching the separation gel (6%T), the sudden decrease in pore size results in sample stacking. While migrating downstream, the protein mixture separates into multiple bands based on their molecular weight due to sieving action (Figure 3ii). As seen in the separation montage (Figure 4a), the protein plug stacked and then separated into compact bands (except smearing α-actinin of a large MW) within 30 s. The linear log-MW vs. mobility relation was observed (Figure 4b), promising accurate MW determination when using CTAB-PAGE.

After separation, horizontal electric field was applied to transfer the separated protein bands to the blotting gel (Figure 3iii). Upon reaching the blotting gel, the separated protein bands are compressed and immobilized due to electrostatic interaction with PA gel (Figure 5). In alkaline TA buffer (pH 8.2), PA is hydrolyzed and bears net negative charges [6]. When biotinylated "capture proteins" of large MW and low-pI value such as IgG (pI=5.5-8.0, MW=150 kDa [7]) and β-galactosidase (pI=4.61, MW=465 kDa) are copolymerized in PA gel using streptavidin-acrylamide linker, the charge density was dramatically enhanced, which was evidenced by much stronger immobilization. Completion of electrotranfer takes about 42 s. Based on fluorescence intensity, substantial amounts of separated proteins are retained. Retention of separated proteins after the immobilization is 75, 77, 65, 78% for protein G, OVA, BSA, and α-actinin respectively. The near 1:1 mapping between separation pattern and immobilization is noted (e.g., separation resolution between protein G and OVA is 1.4 before and after the blotting), indicating a superior control of protein transport. Assay from sample loading to electrotransfer is completed in merely 63 s, which is ~10^2 less than the conventional Western blotting.

After immobilization, a horizontal electric field is continuously applied to wash off residual CTAB from the blotting gel. This step is critical for subsequent probing antibody introduction as antibody tends to precipitate when expose to CTAB. In the following quenching step, open charge sites on the gel are "blocked" by electrophoretically introducing 1% BSA (w/v) solubilized in TA buffer to prevent non-specific antibody binding (Figure 3iv). Residual BSA was washed off by applying reverse electric field. After the blocking, an antibody probe conjugated with Alexa Fluor

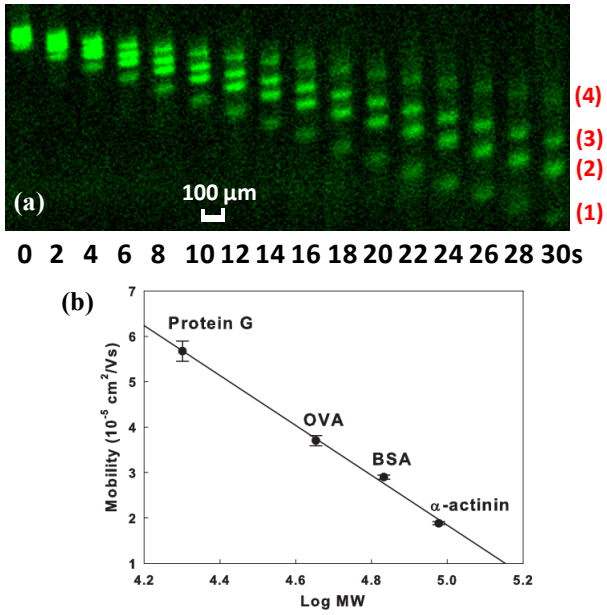

Figure 4: Separation montage of a wide MW protein ladder: (1) protein G, (2) OVA, (3) BSA, (4) α-actinin. (a) sizing and separation completes in <30 s. (b) Linear log MW vs. mobility graph indicates CTAB protein sizing.

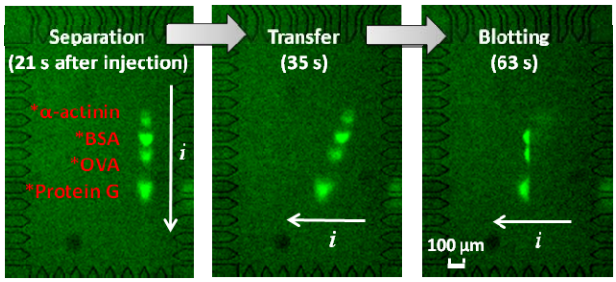

Figure 5: Electrostatic immobilization allows capture of all sized proteins

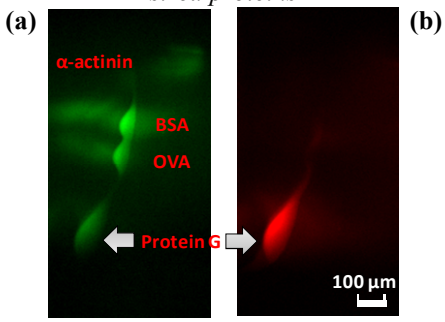

Figure 6: Two-color images for immunoblotting results. (a) Immobilized proteins (green) and (b) subsequent antibody binding of protein G (red). Protein G binds specifically with anti-protein G indicating successful immunodetection.

568 (Invitrogen) was introduced, incubated for 10 min and washed off by applying reverse field. The immunoaffinity probe binds specifically with immobilized target as seen in two color imaging (Figure 6). Protein G was successfully detected without complicated protein-renaturing required for conventional Western blotting.

CONCLUSION

Microfluidic CTAB-PAGE harnesses the true integration potential of microfluidics to yield 'hands-free' Western blotting; sizable tank blotting cell is miniaturized in a tiny glass chip and the total assay is automated via multi-channel voltage/current control. Importantly, the assay performance is also notably enhanced: 10^2-$10^3\times$ reduced sample consumption (~10 ng) and rapid completion times (~2.5 hours vs. 1-2 days). CTAB-PAGE is an attractive alternative to the canonical SDS-PAGE in microfluidic format. Electrostatic interaction enables electrotransfer of all resolved protein to the blotting region. The immunodetection of target becomes more versatile with the assistance of CTAB. There is no need for copolymerization of matched-pair antibodies to immobilize the separated protein targets. Instead, immunoaffinity probes are introduced after immobilization. This means that enzyme-linked secondary antibody can also be used to boost signal, which allows label-free detection and significant improvement of the detection limit. Finally, immunoblotting is performed without complex and time-consuming protein renaturation steps.

This novel suite of microfluidic bioanalytical assays is currently under optimization. We see the reported format as forming the basis for the full suite of immunoblotting assays, as well as for other multi-dimensional separations. Regarding Western blotting, we are currently developing a high sensitivity (pM) version of this assay with >90% capture efficiency of target protein after sizing separation. High sensitivity detection, combined with the quantitation potential of the reported approach, would enable analysis of low-abundance protein biomarkers of disease, even in limited volume samples (e.g., clinical samples housed in repositories, specific to longitudinal assessment of disease development). Further, the ability to blot proteins from a small group of cells – or even single cells – is a major thrust of our current studies.

ACKNOWLEDGEMENTS

The authors acknowledge generous financial support from the University of California (UC) Discovery Grant and the Industry-University Cooperative Research Program (IUCRP). Infrastructure support was provided by the California Institute of Quantitative Bioscience (QB3) Biomolecular Nanofabrication Center (BNC) on the UC Berkeley campus. A.E. Herr is an Alfred P. Sloan Research Fellow (chemistry).

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