SMALL SAMPLE PROTEIN ANALYSIS BY WESTERN BLOTTING UTILIZING A COUPON-BASED MICROFLUIDIC DEVICE

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ABSTRACT

We present a microfluidic strategy for performing complex microfluidic assays that require multiple operations. This approach utilizes low cost microfluidic paper coupons ("floupons") that can be sandwiched in microfluidic cassettes, allowing for a variety of different assays to be designed and configured by matching floupons with fluidic manifolds. This approach provides a convenient way to perform assays that have multiple steps, relieving the need to design highly sophisticated devices that incorporate all functions in a single unit, while still achieving the benefits of small sample size, automation, and high speed operation. We explore the use of this approach in Western blotting, which is an important example of an assay that requires multiple operations and can benefits from miniaturization.

KEYWORDS

Protein analysis, western blotting, immunoblotting, SDS-PAGE, electrophoresis

INTRODUCTION

Western blotting, electrophoretic separation followed by immunoblotting, is a well-known technique for quantifying protein composition in a complex biological sample [1]. Not only this procedure is an important confirmatory method for identifying diseases such as HIV [2], but it also is a powerful technique capable of identifying protein expression of cancer cells in respected tumor samples, thus providing significant information for applications in personalized medicine [3]. However, there is a major sensitivity limitation in the current technology in detection of low abundant proteins which are often the most important information. In cases of small biopsy samples, protein analysis of only a few hundred cells may be required.

Western blotting requires a number of steps including protein injection, separation, blotting, staining, rinsing, and imaging. The number of varied steps makes this a challenging assay to miniaturize into a single analysis unit. Excellent work has been done in this direction utilizing specially prepared gels [4, 5]. The most common way to ensure good separation in microfluidic electrophoresis is to utilize pinch injection to ensure a tight starting plug; however, this approach typically wastes over 80% of the sample which makes subsequent detection very challenging in small quantity analysis. For very small sample applications, an efficient injection schemes is needed.

In this project, we propose a multi-slab microfluidic device to perform SDS-PAGE followed by immunoblotting using a small portable gel-based platform (floupon). The floupon approach allows the system to utilize different manipulation strategies at different points in the assay by moving the floupon carrier into different manifolds during different stages of the assay. Specifically, we employ one manifold for injection and separation, then a second manifold for blotting, staining and rinsing. The use of different manifolds allows for flexibility in assay design, specialized operations, and greater use of three dimensional space in the assay. We describe the system here and focus on the injection manifold, which allows for efficient injection and concentration of proteins into the floupon gel.

EXPERIMENT

The design of the floupon system utilizes a paper-based carrier (2.5 cm x 5 cm x 300 µm) that has small grooves cut into it (2 mm x 3 cm) using a laser cutter. The paper coupon is impregnated with polyacrylamide gel, forming small running lanes (2 mm x 3 cm x 300 µm) in the grooves where protein separation will occur. The coupons can be handled without damaging the gel, and they may be stored over time in a humid package. The gel-filled coupons are sandwiched between two plastic fluidic slabs designed to fit together into a single unit. These components form a temporary 3D fluidic system that allows a portion of the assay to be performed. For injection, the top and bottom manifolds are mated to provide a tiny fluidic path from the top of a coupon to the underside. The system is illustrated in Figure 1.

Proteins are introduced into the top reservoir (which is filled with SDS buffer) then electromigrated through the gel towards the bottom outlet on the opposite side of the gel. Four electrodes are used, one at top, one at bottom (directly beneath), and two at the ends of the gel to produce a desired vertical electric field within the gel that will direct protein flow cleanly into the bottom outlet. To prevent proteins from leaving the gel, and to concentrate the proteins at a single point in the gel, a small membrane filter is incorporated into the bottom plastic manifold. This filter is composed of a track etched nano-pourus membrane (pore size 15 nm) which allows current to flow but provides a barrier to proteins which effectively build up on the surface. To focus the proteins into a small plug, the nanoporous membrane is coated with a polymer coating (photoresist, Shipley 1827) that is photolithographically patterned with a small opening (100 µm x 100 µm) at the center of the membrane. This forces all electric field lines to pass through a small region and effectively "pinches" the proteins into a small region. Proteins follow the field lines to build up at the surface—the entire process takes a few minutes.

Following the initial injection, electric fields are applied at ends of the protein slab to produce a lateral electric field that runs along the length of the gel slab. This field moves the protein plug along the length of the gel, performing electrophoresis. When gel separation is completed, the manifolds are removed and the coupon is moved to new

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manifolds which perform electroplotting onto a membrane, followed by immunostaining and wash. The use of different manifolds, each designed to mate with the coupon at different points in the assay, allows efficient design of fluidics for different tasks.

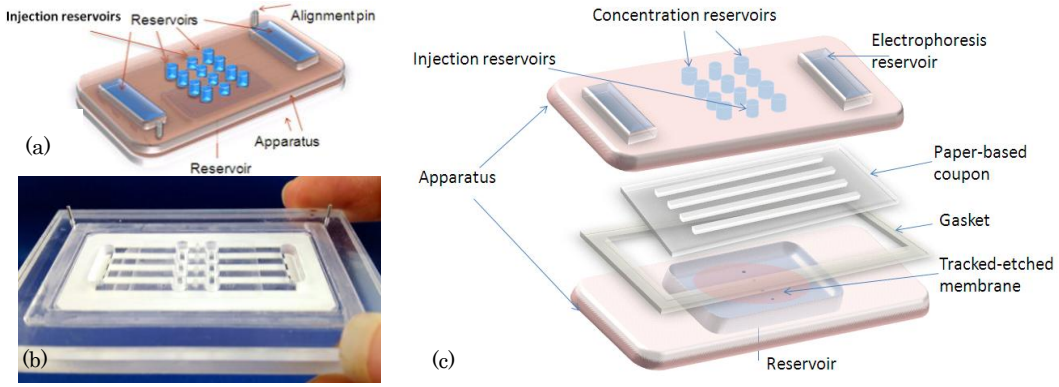

Figure 1: (a) Schematic of pre-concentration/electrophoresis floupon system (b) A photograph of the electrophoresis apparatus (c) Schematic of the layers of pre-concentration/electrophoresis system.

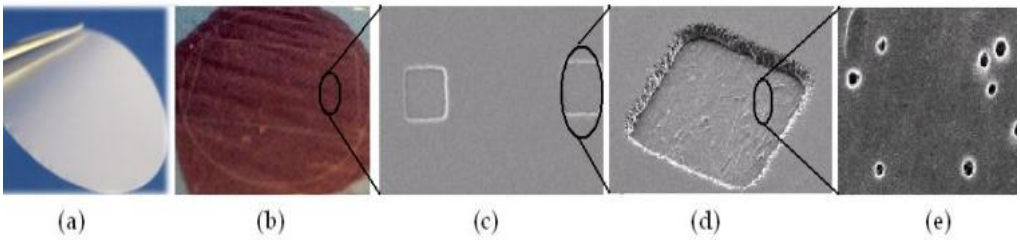

Figure 2: (a) Image of tracked-etched membrane prior to patterning (b) Patterned membrane with Shipley resist coating (c) SEM image of patterned membrane where the openings are patterned using photolithography. (d) SEM image of one of the openings in the membrane (e) SEM image of the 15 nm pores inside the opening in the membrane

To monitor the progression of protein at the injection reservoirs, several experiments were run at different times within 12 minutes after protein injection. Images of the protein in the gel were captured using Olympus IX71 inverted microscope which is equipped with TRITC filter. Alexa-fluor goat anti-rabbit 532 antibody (20 ng/µL) which was negatively charged and denatured using SDS solution was injected into the injection reservoir. Negative electric field was applied at injection and concentration sites while positive electrode was connected at the bottom reservoir (Figure 3a). After each experiment, the coupon was taken out of the apparatus and the gel was cut across the length to image the cross section view (Figure 3b), indicating the location of proteins during transit from top of the gel to the bottom. The efficiency of injection was calculated by collecting the sample left in the loading reservoir, blotting on a membrane and imaging the spot to measure the light intensity of the proteins left at the injection reservoir. Light intensities were compared with control protein light intensity. In all experiments efficiency was calculated to be over 80% after four minutes of injection. After injection of the proteins, the electrodes at the injection and concentration sites were removed and 335 V was then applied to the ends of the gel slab. After 3 minutes of running the gel, two alexa-fluor labeled proteins (immunoglobulin heavy and light chains, 22 and 55 kD molecular weight, respectively) were successfully separated (Figure 3c). The next step is the process of immunoblotting which takes place in a separate apparatus.

RESULTS AND DISCUSSION

We have found that we can efficiently inject and capture proteins in a sample by injecting vertically through a microslab of polyacrylamide gel, using microfluidic reservoirs, electric field shaping, and a micropatterned nanoporous membrane. Cross sectional images taken at different times during the injection indicate that proteins follow field lines and pinch the sample at the small openings of the membrane. This injection captures proteins at approximately 80% efficiency. The small injection plug allows for protein separation to be performed in a few minutes which was also demonstrated. This is enabled by the use of a microfluidic gel coupon which can be placed into a 3D system specifically designed for injection, and which can be moved to a second unit designed for blotting and immunostaining. The technique promises to allow Western blotting to be performed with small protein samples such as from cells taken from biopsies or tissue sections.

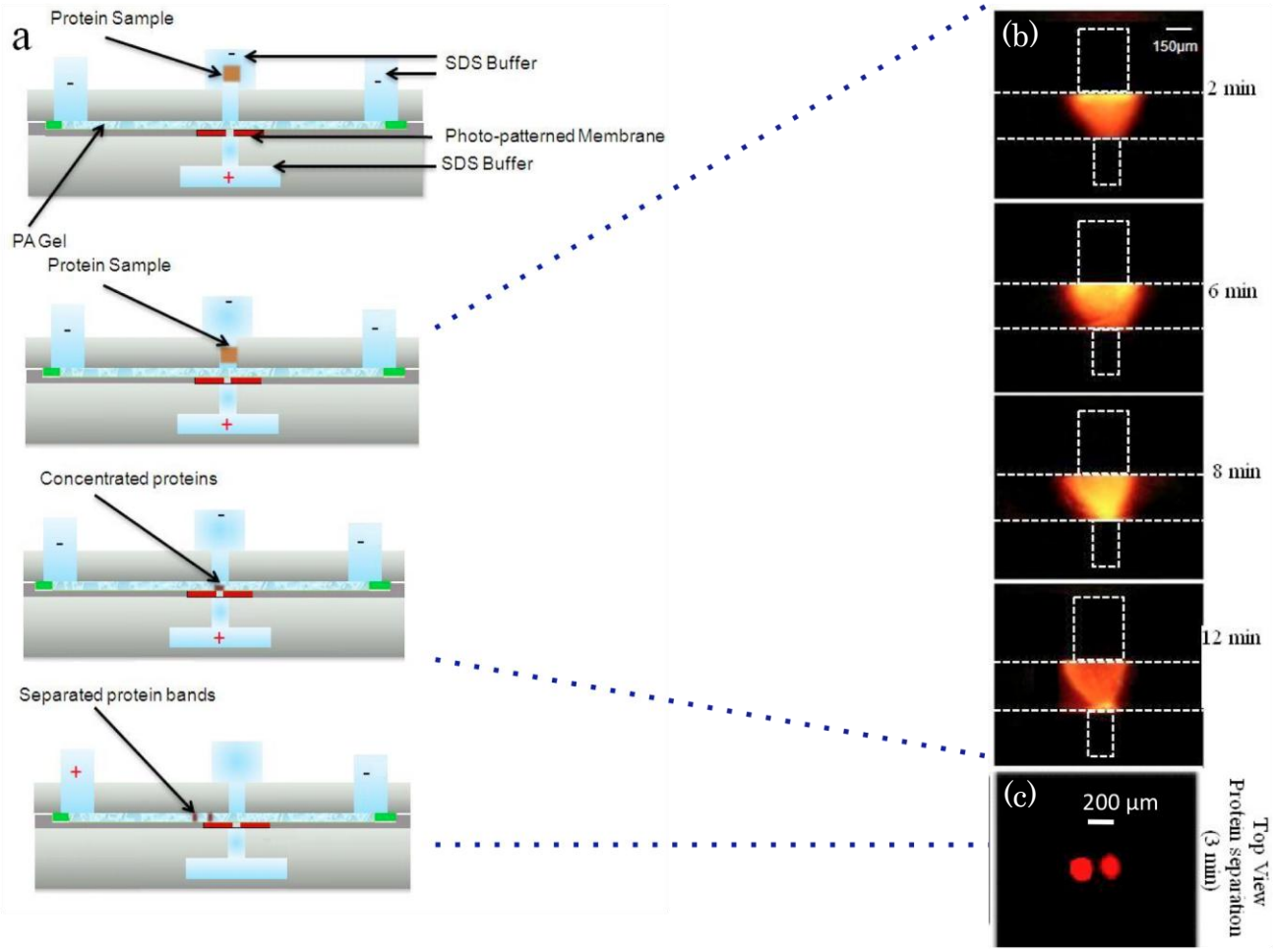

Figure 3: (a) Schematic Side view of pre-concentration apparatus. Photo- patterned membrane allows the concentration of proteins by trapping the proteins on the membrane while allowing electric field line to pass through goat anti-rabbit (c) Top view image of goat anti-rabbit Alexa flour 532 antibody after 3 minutes of separation into heavy and light chain.

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