Affinity Separations Using Microfabricated Microfluidic Devices: *In Situ* Photopolymerization and Use in Protein Separations

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Abstract The use of microfabricated microfluidic devices offers significant advantages over current technologies including fast analysis time and small reagent requirements. In the context of proteomic research, the possibility of using affinity-based separations for prefractionation of samples using microfluidic devices has significant potential. We demonstrate the use of microscale devices to achieve affinity separations of proteins using a device fabricated from borosilicate glass wafers. Photolithography and wet etching are used to pattern individual glass wafers and the wafers are fusion bonded at 650°C to obtain enclosed channels. A polymer has been successfully polymerized *in situ* and used either as a frit for packing beads or, when derivatized with Cibacron Blue 3GA, as a separation matrix. Both of these technologies are based on *in situ* UV photopolymerization of glycidyl methacrylate (GMA) and trimethylolpropane trimethacrylate (TRIM) in channels.

Keywords: affinity separation, microscale devices, nanobiotechnology

INTRODUCTION

The field of proteomics, which describes the genome-scale study of proteins [1], is becoming increasingly important in modern life science research as more genome sequences are being completed and annotated. Proteomic studies typically involve the parallel analysis of many thousands of proteins in a single experiment. Such complexity of samples cannot be readily handled using standard one-dimensional separation strategies such as isoelectric focusing, reverse phase chromatography, or SDS-PAGE. Thus, proteomic analysis has relied on analytical methods that resolve complex mixtures using several orthogonal separation strategies such as two-dimensional protein electrophoresis in polyacrylamide gels or multi-dimensional liquid chromatography separations [2]. One of the challenges that exists is the need to prefractionate complex samples before performing such analyses. A common method for such prefractionation is to rely on affinity methods. For example, Gavin *et al.* [3] and Ho *et al.* [4] used an affinity column to purify affinity tagged proteins, and then used SDS-PAGE for further separation of affinity purified proteins before relying on mass spectrometry for further analysis. Affinity interactions are becoming increasingly important in proteomic studies [5]. Studies on the analysis of biomolecules by affinity are also driven by identifying diagnostic targets of disease, for example, diagnosis of certain central nervous system diseases [6].

Recently, microfabrication techniques developed for the semiconductor industry have been used to miniaturize analytical systems. These developments are of growing interest to the proteomics community for several reasons. The microfabrication techniques allow precise, complex, and reproducible construction of device structures, and microscale devices generally consume nanoliter quantities of sample and reduce analysis time. An end-goal of the use of microfabricated devices for biomolecule analysis is to develop 'micro total analytical system' (μTAS), which can be applied for proteomic study or other chemical analysis and detection. To achieve these goals, one must effectively scale down the traditional protein separation methods to the micron scale. A few devices have been developed which demonstrate this ability when studying simple mixtures of proteins. These devices separate proteins using ion-exchange chromatography [7], micellar electrokinetic chromatography [8], capillary electrophoresis [9,10], gel electrophoresis [11,12], and isoelectric focusing [13]. All of these devices are fabricated using various materials including glass, silicon, or plastic (PDMS, PMMA, *etc.*). Because of the increasing importance of affinity separations, many groups have been working to miniaturize affinity separation systems using various strategies. One approach is the immobilization of probe proteins on a functionalized surface [14]. This technology has particular application in biosensors.

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Bernard *et al.* [15] developed a contact printing technique to capture target proteins in solution and to print them onto various surfaces. Another scheme is to pack synthetic microbeads where the beads have been covalently immobilized with specific affinity ligands. With this strategy, a key challenge is the method used to pack and hold the microbeads in place. Fan *et al.* [16] used a magnetic stone to localize Dynal™ paramagnetic beads (~2.8 μm) in microchannels by a pneumatic pumping apparatus. Oleschuk *et al.* [17] created a double weir design on glass to construct a cavity in which silica beads (1.5-4 μm) could be trapped. In this device, electroosmostic flow was used to introduce beads into the cavity. Buranda *et al.* [18] used a microfabrication technique to pattern small obstructive features on PDMS. Wang *et al.* [19] constructed a big channel and a small channel connected to each other to hold beads on glass. No matter which methods are used to pack beads, some beads can be mislocalized; for example, they can be trapped in the corners. This limitation can cause substantial problems because of the difficulty of packing beads. Therefore, an alternate approach based on the use of monolithic polymer beds has been applied in microfluidic devices, which can permit reverse phase liquid chromatography [20] and ion exchange chromatography [7] in devices fabricated from glass or quartz. However, such an approach has not yet been reported for affinity-based separations.

Here, we report the use of *in situ* UV photopolymerization of glycidyl methacrylate and trimethylolpropane trimethacrylate to create a frit in a microfluidic channel of glass. The frit can be used as a basis to pack poly (styrene-methyl methacrylate-glycidyl methacrylate) (PS-MMA-GMA) beads (average diameter ~2.1 μm). We also use the same copolymer to construct a monolithic bed in a microfluidic channel, and use Cibacron Blue 3GA as a model ligand to modify the polymer *in situ* to test the ability to separate simple mixtures of intact proteins by affinity.

MATERIALS AND METHODS

Materials

Lysozyme and Cibacron Blue 3GA were obtained from Sigma (St. Louis, MO, USA). Glycidyl methacrylate (GMA), trimethylolpropane trimethacrylate (TRIM), toluene, 2,2,4-trimethylpentane (isooctane), benzoin methyl ether, and ethylenediamine (99%) were purchased from Aldrich (Milwaukee, WI, USA). GMA and TRIM were freed from the polymerization inhibitor by an inhibitor remover, a disposable column from Aldrich. Fluorescein-5-isothiocyanate was purchased from Molecular Probes (OR, USA). PD-10 columns containing Sephadex G-25 were obtained from Amersham Pharmacia (Piscataway, NJ, USA). The 4" borofloat borosilicate glass wafers were purchased from Precision Glass & Optics (Santa Ana, CA, USA). Nanoport assemblies were supplied from Upchurch Scientific (Oak Harbor, WA, USA).

Device Fabrication

The microfabricated devices consist of a glass wafer with etched channels bonded to a blank glass substrate. Glass wafers were annealed at 565°C followed by RCA cleaning (soaking in H_2O_2+NH_4OH+H_2O (1:1:5) at 70-80°C for 10 min). A 500 Å layer of chromium followed by a 3,000 Å layer of gold was deposited on the surface of a 500 μm thick glass wafer (100 mm diameter, Precision Glass & Optics, Santa Ana, CA, USA). The glass substrate was then coated with a positive photoresist (Shipley 1827) and soft baked on a hot plate at 90°C for 5 min. The photoresist was patterned using a channel mask by exposing it to UV light. The exposed areas were developed by immersion in AZ MIF 300 and hard baked at 115°C for 30 min. Thus, the UV-exposed parts of the underlying materials were opened for subsequent etching. The metal layers were etched in a commercial gold etchant (Gold etchant TFA) and chromium etchant (CR-14). The accessible glass was then etched in a solution of hydrofluoric and nitric acid (7:3, v/v). The rate of etching was ~6 μm/min. After etching to the desired depth, the unexposed photoresist was stripped. The metal layers were removed using the respective etchants, and the wafer was rinsed in DI water and nitrogen dried. The final channel dimensions were 100 μm wide and 24 μm deep. The reservoirs that connected to the channels were drilled by an ultrasonic drilling machine. After rigorous cleaning, one glass wafer with channels and a blank glass wafer were bonded by using EV520 wafer bonder (provided by the Cornell Nanofabrication Center) followed by annealing at 650°C for 5 h. The individual devices on the bonded glass wafers were then diced.

Preparation of Polymer in Microfluidic Channels

After microfabrication, bonding, and dicing, nanoport assemblies (Upchurch Scientific, Oak Harbor, WA, USA) were attached to the holes. A solid adhesive ring prevented epoxy from leaking into the channels. Channels were extensively rinsed with DI water and methanol. The polymerization procedure described in detail elsewhere [20] is as follows. A reservoir was filled with degassed monomer mixture in toluene/isooctane (w/w, 30/70) solvent (30:70, monomer/solvent) which contains GMA/TRIM (w/w, 30/70) and 5 wt% initiator (benzoin methyl ether) with respect to the monomer. Capillary action allowed the mixture to fill into the whole channel. Portions of the channel were selectively exposed to a portable 365 nm UV light (Model UVGL-55, Upland, CA, USA) using different masks. To make a frit in a microfluidic channel, a high-resolution homemade (using traditional microfabrication technique) mask made from a 4" glass wafer was used. This mask was used because the feature size of the frit is relatively small (100 μm). To make a monolithic bed, a low-resolution mask made from aluminum foil attached to a 4" glass wafer offered sufficient resolution given the larger feature sizes (several millimeters). The mask was then put above the UV lamp. The

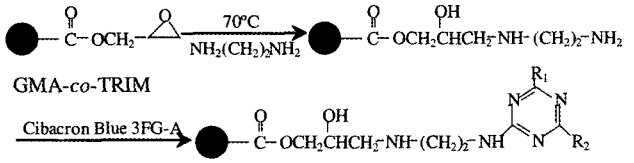

Fig. 1. Synthesis of Cibacron blue 3GA modified GMA-*co*-TRIM monolithic polymer.

distance between UV lamp and mask was 10 cm. The device was then affixed on the mask. The polymerization time was 20 min. After polymerization, any unreacted monomer was flushed out using methanol pulled into the channels by vacuum.

Modification of Monolithic Bed by Cibacron Blue 3GA

The chemical modification of the copolymer was carried out using the reaction of ethylenediamine with the epoxide groups of copolymer shown in Fig. 1 [22]. The resulting reactive amino groups can covalently attach to Cibacron Blue 3GA. The monolithic bed was first flushed by methanol pulled into the channels by vacuum. A mixture of methanol and ethylenediamine (v/v, 2:1) was injected into the channel with copolymer by syringe pump (Harvard Apparatus Inc., Holliston, MA, USA). Care was taken to make sure that the channel was filled with mixture and the whole system was sealed. The device was put on a hotplate at 70°C. The reaction was carried out for 6 h. During reaction, more mixture was injected every two hours. After the reaction ended, any unreacted reagent was washed out with methanol and then DI water. A solution was prepared by dissolving 0.015 g Cibacron Blue in a solution of 15 mL DI water and 25 mL methanol followed by the addition of 0.03 g NaCl, and titration to pH 12 by 2 M NaOH. The mixture was injected into the channel containing the ethylenediamine-modified monolithic bed. The operations were similar to the first reaction carried out in the channel. The reaction temperature was 60°C, and the reaction time was 3 h. The resultant monolithic bed was washed with DI water.

Fluorescent Labeling of Lysozyme and Cytochrome c

Lysozyme and cytochrome c were labeled prior to separation with fluorescein-5-isothiocyanate (FITC) following the manufacturer's protocol. 10 mg lysozyme or cytochrome c was dissolved in 1 mL of 0.1 M sodium bicarbonate buffer. Freshly prepared FITC solution (100 μL of 10 mg/mL in DMSO) was slowly added to the protein solution and vortexed. The mixture was incubated at 4°C in the dark overnight. The mixture (1 mL) was passed through PD-10 column (Amersham Pharmacia, Piscataway, NJ, USA) containing Sephadex G-25. The column was preequilibrated with 10 mM sodium phosphate buffer at pH=7.0. The FITC-labeled protein was eluted with 3.5 mL of 10 mM sodium phosphate buffer. The eluted protein concentration was approximately 2.5 mg/mL. The labeled protein was not further diluted prior to injection onto the device.

RESULTS AND DISCUSSION

Device Evaluation

The microscale device was used in chromatography mode thus requiring pressure-driven flow. Ideally, there should be no leakage during operation. To enclose the device channels, we used thermal annealing of the two pieces of borosilicate glass at high temperature. This approach gave high quality of bonding with no leakage. In the process of synthesizing the copolymer in the channel, some significant quantities of organic solvents, such as toluene and isooctane were used, which could dissolve certain epoxies used to attach peripheral components (*e.g.*, reservoirs) to the device. However, our nanoport assembly used in the device was specially designed to avoid leakage of any liquid during operation that might degrade the epoxy.

Characterization of Frit in Microfluidic Channel

Compared to frits made in capillaries which are typically several millimeters long, frits in microfluidic channels should be relatively short (hundreds of microns). If the polymerization proceeds too quickly, the heat generated cannot be liberated quickly and it may cause the monomer to polymerize in undesired regions. If the polymerization is too slow, there is a risk of significant changes in the small volume (nL) of the solution caused by evaporation. Therefore, it is critical to maintain control of the reaction rate of the polymerization. The GMA/TRIM system was used by Chen *et al.* [23] to make frits in capillaries less than 2 mm. They used a 1 h reaction time which is not appropriate for our system. Here more initiator benzoin methyl ether (5 wt% with respect to monomer) was added to the monomer solution to shorten the reaction time to 20 min. Viklund *et al.* [21] commented that if the concentration of initiator is higher than 4%, cracks in the continuous polymer structure may appear; however, we did not observe any cracks in our system. We observed that the length of the polymer is significantly greater than that of the mask, probably caused both by diffusion of the free radicals to masked regions and by diffraction of the light source. The window open to UV light in the mask was 100 μm long, and the frit made in the channel was around 250 μm as shown in Fig. 2(a) and Fig. 2(b).

Frits are typically used in the flow through mode. Thus, it is important to minimize the flow resistance. Back pressures at different flow rates were measured in our device. Fig. 3 shows the plot of back pressure versus flow rate in the range of 0.2~2 mL/min. The pressure drop is proportional to the flow rate, which obeys the Hagen-Poiseuille law. Even at very large flow rates (1 mL/min is very large in 100 μm by 24 μm channel), the pressure drop is very small compared to typical larger scale HPLC operating conditions. Although no specific functionaliza-

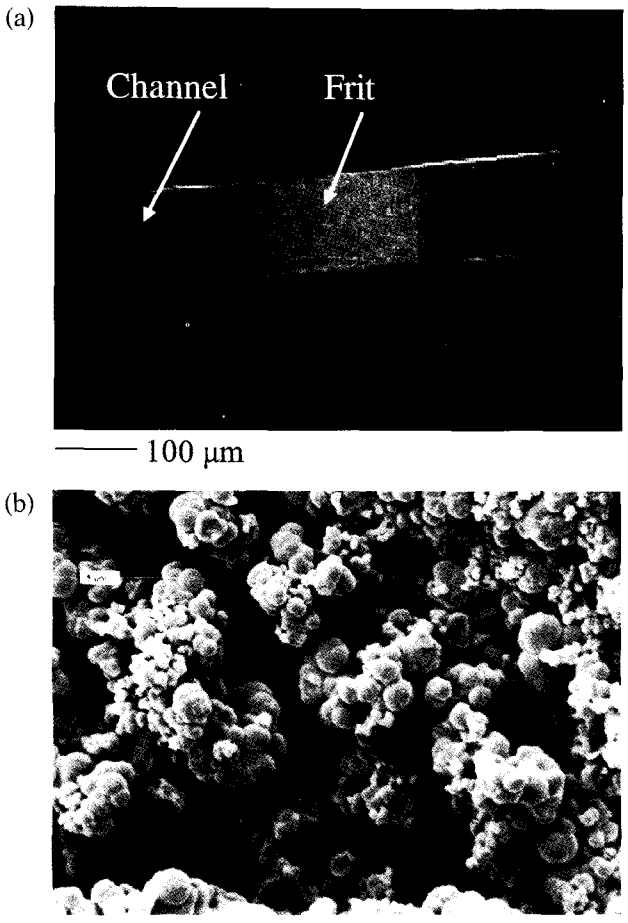

Fig. 2. (a) Optical microscope image of a microfluidic channel frit (the length of the frit is ~250 μm; the channel is 100 μm wide), (b) SEM image of GMA-*co*-TRIM polymer as a frit.

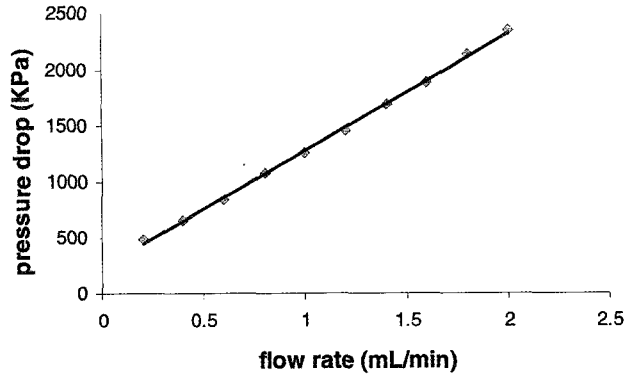

Fig. 3. Back pressure induced by the frit at different flow rates.

tion of the inner wall of channels was carried out, the attachment of the frit to the channel wall was found to be stable. Nonporous copolymerized particles of styrene, methyl methacrylate, and glycidyl methacrylate (~2.1 μm)

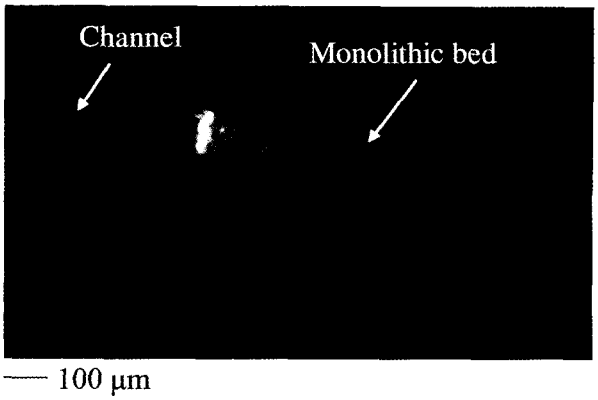

Fig. 4. Fluorescence image of a monolithic bed after capturing FITC-labeled lysozyme.

were used to test the packing with the frit. After packing, sodium phosphate buffer was flushed using a syringe pump. No particles of the packing were found to pass through the frit pores during the packing and flushing, but some particles were found in the corners of the channels even after flushing with buffer solution. A method for the reproducible elimination of these stray particles is necessary before this approach can be used for reliable bead packing.

Affinity Capture by Cibacron Blue 3GA Modified Monolithic Polymer

One of the key features of microfluidic devices is the ability to study very small amounts of sample in a short time. Cibacron Blue 3GA modified monolithic polymer (2 mm long in a microfluidic channel) was used as a model affinity system to test the affinity capture of lysozyme. 1 nL of 2.5 mg/mL FITC-labeled lysozyme in 0.01 M sodium phosphate buffer (pH 7.0) was injected into the device. After injection, the monolithic polymer that captured lysozyme was washed by 20 nL of buffer solution at 10 nL/min. With this flow rate only a few minutes were required to capture and wash the affinity bed. Fig. 4 shows the fluorescence of the monolithic bed with captured FITC-labeled lysozyme taken by PentaMax cooled CCD camera under Olympus BX-50 fluorescence microscope. The intense fluorescence observed at the leading edge of the polymer bed may be caused by precipitation of lysozyme.

We also tested the ability of the affinity bed to separate a mixture of lysozyme and cytochrome c. 1 nL of a mixture of 1 mg/mL unlabelled lysozyme and 1.5 mg/mL FITC-labeled cytochrome c in 0.01 M sodium phosphate buffer (pH 7.0) was applied into the device. After injection, the same wash procedure as the lysozyme adsorption experiment was processed. Fig. 5 shows the fluorescence of the monolithic bed taken by the same CCD camera and under the same exposure time (2,000 ms). No fluorescence is observed which suggests that there is no significant adsorption of cytochrome c.

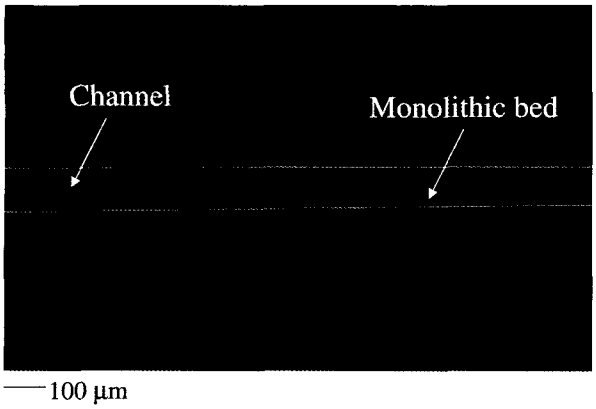

Fig. 5. Fluorescence image of a monolithic bed after capturing unlab lysozyme and FITC-labeled cytochrome c mixture (note: the frame of the channel was added manually after taking the picture).

CONCLUSION

Two technologies have been developed to achieve affinity chromatography on glass microscale devices. An *in situ* photopolymerized porous polymer was made as a frit in a microfluidic channel. The frit was short and porous which brought a very low flow resistance making it suitable for packing beads in microscale devices. However, the same copolymer was used as a matrix for affinity chromatography on microscale devices. Cibacron Blue 3GA was used as a model affinity ligand to test the affinity modification and demonstrated the ability to capture FITC-labeled lysozyme and separate a mixture of lysozyme and cytochrome c.

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