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Affinity monolith preconcentrators for polymer microchip capillary electrophoresis

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Abstract

Developments in biology are increasing demands for rapid, inexpensive, and sensitive biomolecular analysis. In this study, polymer microdevices with monolithic columns and electrophoretic channels were used for biological separations. Glycidyl methacrylate-co-ethylene dimethacrylate monolithic columns were formed within poly(methyl methacrylate) microchannels by *in situ* photopolymerization. Flow experiments in these columns demonstrated retention and then elution of amino acids under conditions optimized for sample preconcentration. To enhance analyte selectivity, antibodies were immobilized on monoliths, and subsequent lysozyme treatment blocked nonspecific adsorption. The enrichment capability and selectivity of these affinity monoliths were evaluated by purifying fluorescently tagged amino acids from a mixture containing green fluorescent protein (GFP). Twenty-fold enrichment and 91% recovery were achieved for the labeled amino acids, with a <25,000-fold reduction in GFP concentration, as indicated by microchip electrophoresis analysis. These devices should provide a simple, inexpensive, and effective platform for trace analysis in complex biological samples.

Keywords

Microchip; Monolith; Affinity column; Sample pretreatment

1 Introduction

Clinical assays, biological analysis, and pharmaceutical effectiveness studies increasingly require the monitoring of multiple analytes in complex mixtures. For instance, to detect cancer and other diseases at early stages, biomarker detection in bodily fluids is used widely [1]. However, these species often have low abundance and are in complex matrixes [2]. Consequently, it is an ongoing challenge to detect trace analytes in real samples.

Since the early 1990s, there has been strong interest in the miniaturization of chemical analysis systems [3]. Such instrumentation offers small volume analysis, fast separation, and the potential to combine multiple processes in a single device. Despite successful applications in areas such as biomarker assays [4], a major challenge with microfluidic devices is the detection limit, because small sample volumes (in the microliter range) can be loaded on chip [5], and the optical path for detection is short (typically <100 μm) [6]. In addition, the separation length in microdevices limits the resolving power, which is critical for analyzing complex mixtures [7]. As a consequence, sample preconcentration and pretreatment will play an important role in the determination of trace analytes in biological specimens using miniaturized devices.

Traditional sample concentration techniques in CE [8] such as sweeping and stacking have been shown for molecules like pharmaceutical species [9] and peptides [10]. Moreover, the stacking technique has been integrated into microdevices [11]. However, in stacking the conductivity of the sample matrix must be lower than the running buffer [12,13], constraining experimental conditions. Other online concentration methods have also been reported that utilize the size difference between analytes and buffer ions. These techniques take advantage of the inability of larger molecules to pass through a porous layer in a semipermeable hollow fiber [14], membrane [15] or joint [16], while smaller ions are allowed to transit. However, complex device fabrication and detection instrumentation are needed for these systems.

SPE is becoming a widely used method for sample preparation, in which a targeted analyte is retained on a column to separate it from the matrix and is then eluted for analysis [17]. The promise of enriching samples by SPE has led researchers to apply this approach in microdevices. In one study, microchip walls were coated with silanes to form a SPE column, and 80-fold preconcentration was observed [18]; however, due to the limited surface area, the loading capacity of this approach was relatively low. To address loading, silica bead [19] and polymer monolith [20,21] SPE columns have also been integrated into microdevices. Silica bead columns have disadvantages in terms of packing and frit fabrication, which complicate microdevice preparation. On the other hand, monoliths are an attractive alternative to packed particles because of low back pressure and relative ease of column formation [22].

However, SPE in as-formed monoliths typically has low selectivity. In addition, nonspecific binding sites hinder elution of desired analytes and decrease sample loading capacity due to competitive adsorption. One way to overcome these shortcomings is to introduce a precolumn to remove most interferences from the matrix. Landers' group [23] recently demonstrated a packed octadecyl bead precolumn coupled with monolith extraction on chip, which increased the loading capacity around 100-fold for DNA analysis. An alternative approach to improve selectivity is to immobilize enzymes or antibodies on a monolith. In fact, solid-phase supports have been used for the attachment of enzymes since the 1970s [24]. A recent review summarizes the application of monoliths as supports for attaching protease enzymes in protein mapping [25]. These studies indicate a promising future for monolithic materials as pretreatment columns for biological samples.

Here we demonstrate a technique for *in situ* preparation of sample pretreatment monoliths in microfluidic devices. These monoliths are integrated readily into microdevices and used as SPE columns for sample preconcentration and pretreatment. We demonstrate the preconcentration of amino acids on monoliths to show the general nature of this approach. To enhance extraction selectivity, we immobilized antibodies on monoliths and blocked nonspecific adsorption sites. We have used these affinity monoliths to enrich fluorescently labeled amino acids 20-fold and purify them from a mixture containing a contaminant protein. Our results build a foundation for future fabrication of fully integrated sample preparation and separation microdevices for fast, sensitive, and inexpensive protein analysis.

2 Materials and methods

2.1 Reagents and materials

All amino acids except Trp were obtained from ICN Biomedicals (Aurora, OH). Lysozyme (95% protein), hydroxypropyl cellulose (HPC, average MW 100,000), Trp (99%), glycidyl methacrylate (GMA, 97%), ethylene dimethacrylate (EDMA, 98%), 2,2-dimethoxy-2-phenylacetophenone (DMPA, 99%), and 1-dodecanol (98%) were purchased from Sigma-Aldrich (Milwaukee, WI). PBS-EDTA coupling buffer (pH 7.2), sulfo-SMCC, and 2-mercaptoethylamine (MEA) were from Pierce (Rockford, IL). FITC was from Molecular Probes (Eugene, OR). Ethylenediamine (EDA) and Tris (electrophoresis grade) were from

Fisher (Fair Lawn, NJ). Cyclohexanol (100%) was from J.T. Baker (Phillipsburg, NJ). Anti-FITC was from Biomeda (Foster City, CA). Green fluorescent protein (GFP, 1.0 mg/mL) was from BD Biosciences (San Jose, CA). Sodium azide was from Merck (Darmstadt, Germany). All solutions were prepared with deionized water (18.3 M Ω -cm) purified by a Barnstead EASYpure UV/UF system (Dubuque, IA). Poly(methyl methacrylate) (PMMA, Acrylite FF, 3-mm thick) was from Cyro Industries (Rockaway, NJ) and was cut to 1.8×5.0 cm by a CO₂ laser cutter (C200, Universal Laser Systems, Scottsdale, AZ) before use.

2.2 Device fabrication

Two kinds of microdevices were utilized in this study (Figure 1): extractor and separation chips. The fabrication protocol was adapted from our previous work [26]. Briefly, the designed pattern was photolithographically transferred to silicon wafers, which were wet etched with 40% KOH and served as templates. PMMA substrates were imprinted by hot embossing using etched Si templates. The patterned PMMA was thermally bonded to an unimprinted PMMA substrate with drilled holes for reservoirs. Channel widths at half height were 50 μ m, and channel depths were 20 μ m.

Porous polymer monoliths (0.5-cm long) were prepared in the extraction microchip (Figure 1a) by photoinitiated *in situ* polymerization. Monoliths were made from GMA and EDMA monomers with DMPA as the photoinitiator. Cyclohexanol and 1-dodecanol were used as the porogen. The monolith preparation followed published procedures [27–29]. Briefly, 0.005 g DMPA, 0.4 g GMA and 0.6 g EDMA were mixed in a 4-mL glass vial. Porogen (0.3 g cyclohexanol and 0.7 g 1-dodecanol) was added slowly to the mixture. Before polymerization, the solution was sonicated in a water bath for 3 min followed by nitrogen purging for 3 min to remove dissolved oxygen. The degassed mixture was aspirated into the microchannels by vacuum, and excess monomer in the reservoir was removed by pipet to minimize siphoning during polymerization [30]. Next, the microchip was partially covered with electrical tape or aluminum foil to provide spatial control over polymerization. The microchip was then put on a cooled aluminum plate and exposed to UV light (200 mW/cm²) in the wavelength range of 320–390 nm for 10 min. Cooling the device helped eliminate undesired thermal polymerization [30]. Finally, unreacted monomer and porogen were removed by flushing isopropanol through the microchannels using a syringe pump.

2.3 Tris-reacted monoliths

For general analyte preconcentration, the reactive GMA epoxy groups were blocked using Tris buffer (100 mM, pH 8.4) pumped through the monolith and incubated for 24 h at room temperature [31]. This protocol is based on the chemical reaction between GMA epoxy groups and Tris amine groups. The monolith was then rinsed with water using a syringe pump. The reservoirs were filled with deionized water, and the device was stored in a humidified Petri dish until use.

2.4 Immobilization of antibodies on monoliths

To provide analyte specificity, monolithic columns were functionalized with immobilized antibodies, as illustrated in Scheme 1 [32]. Briefly, amine groups were first introduced on the monolith surface by EDA reaction with the GMA epoxy groups; neat EDA was flowed into the monolith with a syringe pump (Harvard Apparatus, Holliston, MA) and incubated at room temperature for 24 h. The monolith was next washed with 100 mM PBS-EDTA buffer at 10 μ L/min for 30 min to remove any remaining EDA. Pendant amines were reacted subsequently with a heterobifunctional crosslinker, sulfo-SMCC, which contains an amine-reactive N-hydroxysuccinimide ester and a maleimide group to react with thiols. Consequently, the sulfo-SMCC can link reduced antibodies to the amine-modified monolith. The crosslinker was prepared at 2 mg/mL in PBS-EDTA and was pumped through the monolith at 1 μ L/min for 2

h. Then, 1 mg of anti-FITC was mixed with 200 μ L of 6 mg/mL MEA and incubated for 2 h at 37 $^{\circ}$ C. MEA preferentially reduces disulfide bonds in the antibody hinge region, largely leaving the remainder of the antibody intact (see Scheme 1) [33,34]. The partially reduced antibody was purified using a desalting column (Pierce) equilibrated with PBS-EDTA. The fractions were monitored by measuring UV absorbance at 280 nm with a Nanodrop ND-1000 spectrophotometer (Nanodrop Inc., Wilmington, DE). Once the protein concentration was >10 μ g/mL, the solution was pumped into the monolith and incubated for 4 h at room temperature to attach the antibodies. Unbound antibodies were washed out using PBS-EDTA, and the devices were stored in PBS-EDTA buffer containing 0.02% sodium azide until use.

2.5 Electrophoresis experiments

FITC labeling of amino acids followed a literature procedure [35]. Operation of the separation chip (Figure 1b) has been described previously [26,36]. Briefly, microchannels were filled with 10 mM carbonate buffer (pH 9.1) containing 0.5% (w/v) HPC using a syringe pump. The solution in reservoir 3 was removed by pipet and replaced with 15 μ L of sample in running buffer. A platinum electrode was placed in each reservoir to provide voltage, and all electrodes were interfaced with a custom-built switch which connected to PS310 (providing 0.6 kV) and PS350 (providing 1.6 kV) high-voltage power supplies (Stanford Research Systems, Sunnyvale, CA). For injection, reservoirs 3, 5 and 6 were grounded while reservoir 4 was at 0.6 kV. For separation, reservoirs 3 and 4 were at 0.6 kV, reservoir 5 was grounded, and reservoir 6 was raised to 1.6 kV. Laser-induced fluorescence was used to detect FITC-tagged analytes and GFP. The detection system and data collection setup have been reported before [26], and the sampling rate for data acquisition was 10 Hz; higher sampling rates may be desirable for quantitative work. Peaks in the electropherograms were identified by spiking 3 μ L of 10-fold more concentrated analyte into reservoir 3 and repeating the separation under the same conditions.

2.6 Characterization and use of Tris-reacted monoliths

To quantitatively monitor sample loading and elution, we used 0.1 mM Trp in Tris buffer (pH 8.4). Because Trp absorbs at 280 nm, the UV absorbance (Nanodrop ND-1000) of the solution eluted from the monolith in reservoir 2 (Figure 1a) was measured. A UV absorbance calibration curve of Trp in Tris buffer ($R^2=0.991$) was generated to allow quantitation of the Trp eluted from the column. Tris buffer was first pumped through the monolith to reduce surface wetting losses. Trp solution was pumped through the monolith, and 50- μ L fractions in reservoir 2 were collected in a 0.5-mL microcentrifuge tube. A 2- μ L aliquot from each fraction was pipetted onto the Nanodrop system to probe UV absorbance at 280 nm. Once the UV absorbance reached a plateau (indicating column saturation), the monolith was rinsed with deionized water, and 50 μ L fractions were collected. To elute Trp from the column, 10 mM phosphate buffer (pH 2.1) was pumped through the monolith, and 20 μ L fractions were collected. The pump rate was 2 μ L/min for all steps. The elution efficiency was calculated by dividing the amount of Trp collected in the elution step by the total retained amount of Trp. The quantity of retained Trp was determined by subtracting the amount of Trp eluted in the loading step from the total amount of Trp pumped into the monolith.

To evaluate the enrichment achieved with Tris-reacted monoliths, electropherograms of 200 nM FITC-Asp were obtained before and after monolith extraction. Briefly, a 200- μ L sample was separated into two parts. A 100- μ L aliquot was pumped through the Tris-reacted monolith at 2 μ L/min and rinsed with 10 μ L deionized water. The retained analyte was then eluted with 20 μ L of 10 mM phosphate buffer (pH 2.1). The pH of the eluted sample was adjusted to ~ 9 by mixing with 0.4 μ L of 1 M NaOH solution. The monolith-enriched and control samples were subsequently analyzed by microchip CE as described in section 2.5.

2.7 Characterization and use of affinity monoliths

The amount of anti-FITC affixed on the monolith was determined by the 280 nm UV absorbance difference of the antibody solution before and after immobilization [37]. Briefly, 500 μ L of 10 μ g/mL partially reduced antibody solution was separated into two parts; 250 μ L of the solution was used for immobilization and the other 250 μ L were retained as a control. After derivatization, the affinity column was flushed with 10 μ L PBS buffer. All solution removed from the column was combined, and the volume was determined by micropipette. The control antibody solution was diluted to the same volume with PBS, and the UV absorbance of both solutions was analyzed by the Nanodrop system.

Because nonspecific adsorption can cause carryover contamination and decrease sample loading capacity due to competitive effects, column performance can be improved if nonspecific adsorption sites are removed. To do so, 40 mg/mL lysozyme in PBS buffer [38] was flushed through the affinity monolith at 2 μ L/min for 20 min after antibody immobilization. Then the affinity column was rinsed with deionized water at 5 μ L/min for 10 min to wash out any unbound lysozyme. To examine the effectiveness of lysozyme treatment for blocking nonspecific adsorption, 50 μ g/mL GFP solutions were used as a fluorescence probe and pumped through both control and lysozyme-blocked affinity columns. The monoliths were next rinsed with 10 mM Tris buffer (pH 8.4) at 10 μ L/min for 3 min. A $\sim$ 300- μ m-long segment of the affinity column was illuminated with a laser at 488 nm, and fluorescence images were taken with a Nikon digital camera (Coolpix 995, Tokyo, Japan) [39]. Quantitative fluorescence intensities were monitored by a cooled CCD camera (Coolsnap HQ, Roper Scientific, Tucson, AZ); the signal was determined from the average intensity on the monolith for each CCD image. Data processing and CCD parameter adjustments were carried out using V++ Precision Digital Imaging Software (Version 4.0, Auckland, New Zealand), and the CCD exposure time was 300 ms.

The extraction efficiency of lysozyme-treated affinity columns was measured somewhat differently from the Tris-reacted monoliths. FITC-Gly (1 mM) was used as the indicator, and the concentration of eluted analyte was monitored by CCD. To quantitatively determine the amount of FITC-Gly, a calibration curve was generated from the average fluorescence signal of standard FITC-Gly solutions in reservoir 2 (concentration range 0.01–10 mM, $R^2=0.996$).

To evaluate the selectivity of affinity monoliths, electropherograms of a FITC-amino acid/GFP mixture were obtained before and after monolith extraction. The procedures were similar to those described earlier for Tris-reacted monoliths. The mixture consisted of FITC-Gly, FITC-Phe, FITC-Arg and GFP. All amino acid concentrations were 10 nM while the concentration of GFP was 50 μ g/mL. A 500- μ L solution was separated into two parts: a 50- μ L control and a 450- μ L monolith-extracted sample. The 450- μ L aliquot was pumped through the affinity monolith at 2 μ L/min. The rinsing, elution, and pH adjustment were the same as described in section 2.6. The monolith-extracted and control samples were subsequently analyzed by microchip CE as outlined in section 2.5.

3 Results and discussion

3.1 Monolith characterization by SEM

SEM images of a typical monolith in a microdevice and more detailed monolith features are shown in Figure 2. Under our synthesis conditions the monolith (see Figure 2b) has good porosity, which provides low backpressure, and a large surface area to enhance loading capacity.

3.2 Preconcentration of amino acids on Tris-reacted monoliths

A typical concentration profile for Trp over the course of loading, rinsing and elution from a Tris-reacted monolith is presented in Figure 3. Mean recovery volumes were 46 μ L for the 50- μ L fractions, indicating collection losses of <10%. During the loading step (Figure 3a–b), the Trp concentration in the reservoir after the monolith was near zero until after 350 μ L of flow, and then increased with the flow volume until the maximum loading of 0.1 mM Trp was reached at around 500 μ L (Figure 3b). During washing (Figure 3c), a small amount of Trp was removed from the column (<5%), indicating strong Trp retention on the monolith. Over 70% of the retained Trp was collected in 60 μ L of elution buffer (Figure 3d). We define the elution efficiency as the moles of analyte eluted divided by the moles retained. The average elution efficiency was 82%, the run-to-run variability was 6.1% (n=3), and the chip-to-chip variability was 1.2% (n=3). These results demonstrate that monoliths can be integrated reproducibly in microdevices and have good SPE functionality.

To evaluate the feasibility of combining a monolith column with microchip CE separation, 200 nM FITC-Asp was loaded onto a Tris-reacted column, eluted and subsequently separated by microchip CE (Figure 4). In comparing the control (Figure 4a) and extracted sample (Figure 4b), the retention time of Asp was about the same, but the peak height of the extracted solution was threefold higher. Since a 100- μ L sample was loaded and elution occurred in a fivefold smaller volume (20 μ L), the threefold signal increase corresponds to ~60% recovery of FITC-Asp. This type of Tris-reacted column is useful for preconcentration and can be combined readily with microchip CE, but the extraction and preconcentration is not selective.

3.3 Characterization of affinity monoliths

Under our reaction conditions, the amount of anti-FITC immobilized on the 0.5-cm-long monolith column was 250 ± 70 mg/g (n=3). This result is similar to published data on immobilized trypsin on a GMA-co-EDMA monolith (~320 mg/g) [37], and is somewhat higher than what was reported using the 1,1'-carbonyldiimidazole (CDI) method for immobilizing anti-FITC on the same monolith (61 mg/g) [31]. Thus, our protocol yields comparable results to other methods and has several advantages. Unlike direct reaction with epoxy groups [40], our technique works with tenfold lower antibody concentrations (~10 μ g/mL) and sixfold shorter reaction times (~4 h). Furthermore, compared to our approach, the CDI method needs water-free conditions [40]; while the Schiff base and hydrazide techniques involve hydrolysis of the epoxy ring, which requires catalyst optimization [41].

Fluorescence images comparing lysozyme-treated and unblocked monoliths are shown in Figure 5. Considerable GFP was adsorbed on the surface of monoliths not treated with lysozyme, and bright fluorescence was observed throughout the column. On the other hand, after lysozyme blocking very low fluorescence (near background) was found on the monolith. The ratio of background-subtracted GFP signals in unblocked and blocked monoliths was 16, indicating that lysozyme passivation significantly reduces nonspecific protein adsorption on our affinity monoliths.

Based on the FITC-Gly CCD signal in reservoir 2 generated as described in section 2.7, the average elution efficiency of the lysozyme-treated affinity columns was 86%, and the chip-to-chip variability was 3.1% (n=3). These results indicate that the elution efficiency of our affinity columns is comparable to that of Tris-reacted monoliths, and our column performance is reproducible. Importantly, affinity monoliths have analyte selectivity, as we show in the following section.

3.4 Selective extraction by affinity monoliths

To evaluate the selectivity of our affinity columns, a mixture of FITC-labeled amino acids and GFP was pumped through an affinity monolith and then analyzed by microchip CE. In Figure 6a, the amino acid peak heights were about tenfold smaller than GFP. Contrastingly, in Figure 6b the GFP peak is reduced significantly after affinity purification, while the FITC-amino acid peak heights increased around 20-fold. Based on the 22-fold reduction in volume during extraction, we calculate a 91% recovery of FITC-tagged amino acids. The higher recovery in this experiment compared to Tris-reacted monoliths may be attributed to reduced nonspecific adsorption with the affinity monolith. After extraction, a FITC peak appeared in the electropherogram (Figure 6b). The FITC peak was identified by spiking 3 μ L of 500 nM FITC into reservoir 3 (Figure 1b) and repeating the separation under the same conditions. Uncoupled FITC, which is retained by the affinity monolith, was not visible above noise in the raw sample (Figure 6a). Based on the signal ratio of Arg to GFP in Figure 6a–b, a 25,000-fold reduction in GFP concentration was achieved after immunoaffinity extraction. Our results clearly demonstrate that microchip affinity monoliths can selectively concentrate and purify target analytes through specific antibody-antigen interactions.

4. Concluding remarks

Pretreatment and selective analyte enrichment are essential in many applications where the samples are complex, including trace protein analysis. In this study, photo-defined monoliths were applied as sample preconcentrators and affinity purification columns in polymer microfluidic devices. Successful antibody immobilization and nonspecific adsorption blocking have also been shown. These results demonstrate that microchip immunoaffinity monoliths can selectively enrich desired species in complex biological mixtures for subsequent CE analysis. The good reproducibility in amino acid work indicates the excellent potential for use of these affinity monoliths in fully integrated on-chip sample preparation and separation. These systems offer the possibility of fast, simple and sensitive protein analysis.

Abbreviations

PMMA, poly(methyl methacrylate); GMA, glycidyl methacrylate; EDMA, ethylene dimethacrylate; DMPA, 2,2-dimethoxy-2-phenylacetophenone; GFP, green fluorescent protein; EDA, ethylenediamine; MEA, 2-mercaptoethylamine..

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Figure 1. Schematic diagram of microchips; (a) an extractor chip and (b) a separation chip. In (a) the 0.5-cm-long monolith is formed between reservoirs 1 and 2. In (b) reservoir 3 is for sample and reservoir 4 is for injection waste. The separation channel connects reservoirs 5 and 6. The distance from the intersection to reservoirs 3–5 is 0.5 cm, and the distance to reservoir 6 is 3.5 cm.

Figure 2.
SEM images of (a) a typical monolith in a microchannel and (b) a close-up view showing detailed monolith morphology.

Figure 3.

Concentration of Trp collected in reservoir 2 (Figure 1a) as a function of volume flowed through a Tris-reacted monolith at 2 $\mu\text{L}/\text{min}$. Intervals: (a) loading, (b) monolith saturation, (c) washing, and (d) elution. Fractions in reservoir 2 were collected at increments of 50 μL in (a–c), and 20 μL in (d). Fractions were quantified by UV-Vis measurement.

Figure 4. Microchip CE of FITC-Asp (a) before and (b) after Tris-reacted monolith extraction. CE conditions are described in section 2.5.

Figure 5. Fluorescence images of retained GFP on monoliths (a) without and (b) with lysozyme blocking.

Figure 6. Microchip CE of amino acids and GFP (a) before and (b) after affinity column extraction. Peaks 1–5 are Gly, Phe, Arg, FITC, and GFP, respectively. CE conditions are described in section 2.5.

Scheme 1.
Protocol for attaching antibodies onto a monolith.