Published in final edited form as:

Electrophoresis. 2017 July ; 38(13-14): 1743–1754. doi:10.1002/elps.201700054.

Integrated Electrokinetically Driven Microfluidic Devices with pH-Mediated Solid-Phase Extraction Coupled to Microchip Electrophoresis for Preterm Birth Biomarkers

Mukul Sonker, Radim Knob, Vishal Sahore, and Adam T. Woolley

Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT 84602, USA

Abstract

Integration in microfluidics is important for achieving automation. Sample preconcentration integrated with separation in a microfluidic setup can have a substantial impact on rapid analysis of low-abundance disease biomarkers. Here, we have developed a microfluidic device that uses pH-mediated solid phase extraction (SPE) for the enrichment and elution of preterm birth (PTB) biomarkers. Furthermore, this SPE module was integrated with microchip electrophoresis for combined enrichment and separation of multiple analytes, including a PTB peptide biomarker (P1). A reversed-phase octyl methacrylate monolith was polymerized as the SPE medium in polyethylene glycol diacrylate modified cyclic olefin copolymer microfluidic channels. Eluent for pH-mediated SPE of PTB biomarkers on the monolith was optimized using different pH values and ionic concentrations. Nearly 50-fold enrichment was observed in single channel SPE devices for a low nanomolar solution of P1, with great elution time reproducibility (<7% RSD). The monolith binding capacity was determined to be 400 pg (0.2 pmol). A mixture of a model peptide (FA) and a PTB biomarker (P1) was extracted, eluted, injected, and then separated by microchip electrophoresis in our integrated device with ~15-fold enrichment. This device shows important progress towards an integrated electrokinetically operated platform for preconcentration and separation of biomarkers.

1. Introduction

Biomarker analysis in bodily fluids is important for the diagnosis of diseases like cancer [1–3], and cardiovascular [4, 5], degenerative [6–8], genetic [9–11], and infectious [12, 13] disorders. However, these biomarkers are typically found in low concentrations such that a sample pre-concentration step is often required. Microfluidic devices offer advantages like integration, low cost, rapid analysis, portability, and low limits of detection for biomarker analysis [14, 15]. Integration in microfluidics is a key advantage which can allow automated analysis of multiple analytes without sample loss and contamination [14]. Considering this potential, integrated microfluidic platforms have gained significant attention recently in diagnosing and monitoring the progression of diseases through biomarker analysis [16–19]. Devices have been developed recently that can integrate preconcentration, solid phase extraction (SPE) and electrophoretic separation [20–22]. However, these devices report data for model analytes, high (micromolar) concentrations or both. Here, we report an integrated microfluidic device for SPE and microchip electrophoresis (μ CE) of multiple analytes

including a preterm birth (PTB) biomarker, demonstrating enrichment for low nanomolar concentration samples that are typical of PTB biomarkers [23, 24].

PTB is birth before 37 weeks of pregnancy. It is the most common complication of pregnancy, affecting over 500,000 births in the United States every year [25, 26]. Recently, Esplin et al. [23, 24] characterized and validated a biomarker panel in maternal serum, the analysis of which can provide a selective (81%) and sensitive (87%) diagnosis of PTB occurring four weeks in the future. One biomarker in this panel is a 19 amino acid, proline rich peptide (P1), which is a part of inter-alpha-trypsin inhibitor heavy chain 4, a glycoprotein previously indicated in inflammation. Although the serum concentration of P1 is not given in the literature, the concentrations of other PTB biomarkers in serum range from low nanomolar to micromolar levels [23]. Thus, a microfluidic analysis platform that detects nanomolar-level biomarker concentrations would allow the diagnosis of PTB risk and facilitate medical interventions to delay birth or increase fetus viability to improve outcomes [27, 28]. Our integrated microfluidic device that combines pH-mediated SPE of P1 with μ CE is a step toward addressing this need.

SPE is a common method for sample enrichment in which the analyte is concentrated on a solid support and thereafter selectively eluted for further analysis [29–31]. Solid supports used for SPE in microfluidics include packed materials [17, 32] and porous polymer monoliths [33–36]. Monoliths are desirable SPE materials in microfluidics due to their ease of fabrication without the need for retaining frits [37], high surface area, and low backpressures during fluid flow [38, 39]. Reversed-phase monoliths are often used for sample enrichment by hydrophobic interaction with analytes [40, 41]. Pruim et al. [42] used different methacrylate monoliths for preconcentration and gradient elution of peptides in a microfluidic setup. Ladner et al. [43] demonstrated electrochromatographic separations in lauryl methacrylate monoliths polymerized in cyclic olefin copolymer (COC) microchips. Although photopolymerization of monoliths in microfluidic systems requires additional device processing steps, masked UV exposure for controlled placement [39] makes monoliths amenable for scalable microfluidic production.

Here, we have modified a previously reported [44] pH-mediated SPE approach to utilize hydrophobic interaction with the stationary phase and electrokinetic migration of analytes at different pH values to enrich or elute them from a reversed-phase monolithic column in a microfluidic device. Related work in conventional capillaries includes preconcentration at a pH junction made using background electrolytes with different pH values [45, 46] and pH modulation for preconcentration via stacking and dynamic pH junctions [47, 48]. The pH-mediated preconcentration method described here is advantageous over previous microfluidic SPE work [35, 49–51], as it does not require the use of organic solvents like acetonitrile for extraction that may interfere with analyte separation. We achieved nearly 50-fold pH-mediated SPE enrichment for a PTB biomarker peptide (P1) at low nM concentrations, and found the monolith binding capacity for this peptide to be 400 pg (0.2 pmol). Importantly, the pH-mediated SPE approach was integrated with μ CE to simultaneously enrich and separate multiple analytes, resulting in a ~15-fold increase in PTB peptide peak area in the separation. Moreover, our application of SPE- μ CE to a peptide PTB biomarker (P1) expands over our published work demonstrating this approach only for

a protein PTB biomarker [50]. Overall, this study reports several innovations and lays the foundation for a superior integrated platform for the analysis of PTB biomarkers.

2. Experimental Section

2.1 Chemicals and materials

Four-inch diameter single side polished silicon wafers were obtained from Desert Silicon (Tempe, AZ). A silicon dioxide layer of ~500 nm was oxidized on these wafers using a Bruce Tube Furnace (Bruce Industrial, New Castle, DE). S1805 positive photoresist and MF 26A photoresist developer were purchased from Dow Chemical (Marlborough, MA). Zeonor 1060R (6"×6"×1 mm thick and 6"×4"×2 mm thick) COC plates were purchased from Zeon Chemicals (Louisville, KY). Polyethylene glycol diacrylate (PEGDA, M_w 575), benzoin methyl ether (BME), 2,2-dimethoxy-2-phenylacetophenone (DMPA), 1-dodecanol, sodium tetraborate decahydrate, ethylene dimethacrylate (EDMA), dimethyl sulfoxide, Phe-Ala (FA, a model peptide), hydroxypropyl cellulose (HPC, M_w 100 kDa), and inhibitor remover packing beads were obtained from Sigma-Aldrich (St Louis, MO). Octyl methacrylate (C8) was from Scientific Polymer Products (Ontario, NY). Tween 20 and sodium hydroxide were obtained from Mallinckrodt Baker (Paris, KY). Cyclohexanol was from J. T. Baker (Phillipsburg, NJ). Isopropyl alcohol (IPA) and acetonitrile were from Fisher Scientific (Fair Lawn, NJ). Cyclohexane and methanol were purchased from Macron (Center Valley, PA). Anhydrous sodium carbonate, sodium bicarbonate, citric acid, sodium citrate, sodium phosphate monohydrate, anhydrous sodium phosphate, and boric acid were obtained from Merck (Darmstadt, Germany). Sodium chloride was from Columbus Chemicals (Columbus, WI).

All buffers were prepared using deionized water (18.3 MΩ) purified by a Barnstead EASYpure UV/UF system (Dubuque, IA) and filtered through a 0.45 μm Thermo Scientific Nalgene syringe filter (Waltham, MA). A fluorescein labeled PTB peptide (P1, QLGLPGPPDVPDHAAYHPF) [23, 52] was synthesized by GenScript (Piscataway, NJ). Ferritin was purchased from EMD Millipore (Billerica, MA). Fluorescein isothiocyanate (FITC) and Alexa Fluor 488 TFP Ester (AF 488) were obtained from Life Technologies (Carlsbad, CA). FA and ferritin were fluorescently labeled by adding 10 μL of 10 mM FITC in dimethyl sulfoxide to 100 μL of 10 mM FA or 50 μM ferritin and incubating overnight at room temperature. Excess FITC was filtered from ferritin using Amicon ultra 0.5 mL centrifugal filters (EMD Millipore, Billerica, MA). The concentration of filtered FITC-ferritin was determined with a Nanodrop ND-1000 spectrophotometer (Wilmington, DE). Residual, de-identified human serum was obtained from Prof. William Pitt at Brigham Young University.

2.2 Device design and fabrication

Device designs were made in CleWin software (Phoenix Software, Enschede, Netherlands). Silicon templates were fabricated in the BYU Integrated Microfabrication Lab using photolithographic patterning and wet etching techniques as described previously [53]. Devices were fabricated using COC plates, which were cut into the desired size using an industrial bandsaw. Patterns from silicon wafers were transferred to 1-mm-thick COC plates

by hot embossing for 26 min at 138°C. COC plates (2-mm-thick) had holes for reservoirs drilled with a 2-mm-diameter bit in a drill press (Cameron, Sonora, CA). Drilled COC plates were bonded to the imprinted COC plates at 110°C for 24 min. Enclosed devices were then further sealed by applying cyclohexane around the edges. Two different devices made from previously characterized masks were used for our experiments. One design was a straight 2-cm-long channel as shown in Figure 1A–B, for the optimization of pH-mediated SPE. The channel dimensions were ~80 µm wide and ~20 µm deep. A T-shaped four reservoir design (Fig. 1F–H) was used for integrated SPE-µCE experiments, and these channels were designed to be ~50 µm wide and ~20 µm deep.

2.3 Surface modification with PEGDA

A surface modification step was incorporated to ensure consistent electrokinetic sample flow. Channel surfaces of COC devices were photografted with PEGDA using an approach modified from Ladner et al [54]. First, polymerization inhibitor (methyl ether hydroquinone) was removed from PEGDA by slowly flowing it through a column filled with inhibitor remover packing beads and collecting the purified PEGDA. Methanol (94% w/w) was used to dissolve PEGDA to 3%, 5% or 7% w/w and BME (1% w/w) was used as the photoinitiator. This mixture was sonicated for 10 min and then used to fill the channels. Black tape was used to seal the reservoirs to prevent evaporation of the mixture, and then the whole channel was exposed to UV radiation ($>100 \text{ mW cm}^{-2}$) for 8 min using a SunRay 600 UV lamp (Uvitron international, West Springfield, MA). After exposure, the unpolymerized mixture was removed from the channel by applying vacuum, and the channels were cleaned with IPA.

Scanning electron microscopy (SEM) images of the PEGDA coating on channels were taken using a Philips XL30 ESEM FEG instrument in high vacuum mode at 5–20 kV electron beam potential. Devices were cut into small pieces around the channels using a laser cutter (VLS 2.30 Versa Laser, Universal Laser Systems, Scottsdale, AZ) and were glued to glass stubs using epoxy. Cross-sections of the channels were then microtomed using a glass knife. These samples were placed on an aluminum stub and coated with Au-Pd (60:40 ratio, 15–20 nm thickness) using a Q150T ES Sputterer (Quorum Technologies, Lewes, East Sussex, UK) to reduce charging.

Water contact angles were measured for unmodified COC, photografted COC and a standard thermoplastic material, polymethyl methacrylate (PMMA, Evonik, Parsippany, NJ). For water contact angle measurements, ~2 cm × ~0.5 cm wide channels were imprinted on a COC plate using a silicon wafer, and photografting was performed using 3% and 5% PEGDA. COC layers were pulled apart and contact angle measurements were done on the channel after cleaning the surface with IPA and drying with nitrogen gas. Water contact angles were measured with a Ramé-Hart Contact Angle Goniometer (Model 100-00, Netcong, NJ) fitted with a manual syringe filled with high purity (18 MΩ) water. The droplets for measuring static water contact angles were ~10 µL. The success of photografting was confirmed by an observed increase in hydrophilicity of the surface.

CCD images of the injection region of unphotografted and photografted T-shaped devices were recorded during electrokinetic sample flow using a Photometrics coolSNAP HQ2

(Tucson, AZ) CCD camera. A 488 nm laser (JDSU, Shenzhen, China) was used to excite the desired section of the device using a 4× objective on an inverted Nikon Eclipse TE300 microscope. The CCD images (500 ms exposure time) were processed using NIH ImageJ software (<http://imagej.net/ImageJ>). These images confirmed the electrokinetic sample flow direction in photografted and unphotografted devices.

2.4 Monolith fabrication

Reversed-phase C8 monoliths were fabricated in the device channels by preparing a mixture of monomer, porogen, and surfactant first (see Table I), and then adding photoinitiator (1% by mass) to the mixture. This polymer mixture was sonicated for 15 min to ensure all components were completely dissolved, and channels were filled with the sonicated solutions. A Cr mask was used to cover the channel and expose only the desired area for UV polymerization. The length of the exposed region was 0.6 mm for all the monoliths used in this study. The observed monolith lengths were slightly longer (0.73 ± 0.10 mm, $n = 10$), either due to scatter of the UV source or minor solution movement at the ends during polymerization. UV exposure was carried out for 11 min and after exposure, the unpolymerized mixture was flushed and the channels were rinsed several times using IPA. SEM was performed on cross-sections of channels with or without polymerized monoliths, using the same conditions as described in section 2.3, except the SEM of the channel with a monolith was obtained in low vacuum mode without a sputtered metal coating. Bulk C8 monolith for SEM was prepared by adding 250 μ L of pre-polymer solution to a 1 mL Eppendorf tube and polymerizing it as described above. Then, the polymerized monolith was broken into pieces and stored in IPA to dissolve any unpolymerized mixture. These pieces were kept in a vacuum chamber overnight before being placed on a carbon taped aluminum stub for sputtering and SEM imaging.

2.5 Instrumentation

The laser induced fluorescence setup used for these experiments has been described previously [35, 53, 55, 56]. Briefly, a 488 nm laser (JDSU, Shenzhen, China) was directed through a 20× objective in an inverted Nikon Eclipse TE300 microscope to excite the fluorophores in the channels at the desired point. Collected fluorescence passed through a 505LD dichroic filter (Chroma, Rockingham, VT) and a D535/40 band-pass filter (Chroma, Rockingham, VT), and was detected by a photomultiplier tube (Hamamatsu, Bridgewater, NJ). A preamplifier (SR-560, Stanford Research Systems, Sunnyvale, CA) was used to process the detector output voltage. The fluorescence data was digitized by a NI USB-6212 analog-to-digital converter (National Instruments, Austin, TX) and recorded at 20 Hz using LabVIEW software (National Instruments, Austin, TX). Voltages were applied to the desired reservoirs using platinum electrodes by a custom designed voltage box connected to power supplies (Stanford Research Systems, Sunnyvale, CA). Eluted peak heights and areas were determined by OriginPro software (OriginLab Corporation, Northampton, MA).

2.6 Device operation

2.6.1 pH mediated SPE—Before experiments, the monoliths were washed several times using IPA and then preconditioned with (1:1 v/v) IPA and deionized water. The loading/

rinsing buffer (20 mM citrate, pH 5) was filled in the reservoirs and channels, which were optically inspected for any trapped bubbles that could interfere with fluid flow. The buffer was flowed electrokinetically through the monolith by applying +400 V (200 V/cm) to the sample waste reservoir and grounding the sample reservoir (Fig. 1A) for 2–3 min to fill in any air pockets left in the monolith. After electrokinetically rinsing the monolith and channel with citrate buffer, a blank elution was done to ensure no carryover contamination. Citrate buffer in the sample reservoir was replaced with 50 mM bicarbonate buffer (BCB, pH 10), and the voltage applied on the sample waste reservoir for elution was increased to +800 V (400 V/cm).

SPE samples were diluted in 20 mM citrate buffer (pH 5) and filled in the sample reservoir. The sample was injected electrokinetically on the monolith for the desired interval by applying +400 V (200 V/cm) on the sample waste reservoir and grounding the sample reservoir. After injecting the sample, the reservoirs were emptied, rinsed and refilled with fresh citrate buffer. A monolith rinsing step was carried out for 1 min at +400 V (200 V/cm). After rinsing, buffer in the reservoirs was replaced with 50 mM BCB, and the elution step was carried out as described above for the blank elution. During the elution step laser induced fluorescence was recorded in the channel after the end of the monolith as indicated in Fig. 1A. For monolith capacity experiments the detector gain was reduced to keep the signal from going off scale.

2.6.2 Integrated SPE- μ CE—In the integrated “T” shaped device, a monolith was fabricated in one of the arms of the T (Fig. 1 F–H), and was washed and filled as described in section 2.6.1. Reservoirs 2 and 3 were filled with citrate buffer (pH 5) while reservoirs 1 and 4 were filled with BCB having 0.05% HPC. Sample injection was carried out for 5 min, and monolith rinsing and sample elution steps were carried out as described in section 2.6.1, treating reservoir 2 as the sample reservoir and reservoir 3 as the sample waste reservoir, using +300 V (300 V/cm) for sample injection and rinsing, and +600 V (600 V/cm) for elution. Initially, the elution step was monitored at the intersection of the T (Fig. 1F) to determine the time required for the eluted sample plug to reach the intersection. For integrated SPE- μ CE, sample was loaded and rinsed as before, but during elution the voltage configuration was switched at the previously determined time to carry out μ CE of the eluted and injected plug in the intersection. During μ CE, +600 V was applied to reservoirs 2 and 3, +1400 V was applied to reservoir 4, and reservoir 1 was grounded, corresponding to ~400 V/cm on the separation channel. The detection point was positioned 5 mm beyond the intersection in the separation channel as indicated in Fig. 1G.

3. Results and Discussion

3.1 Surface modification with PEGDA

COC was chosen as the device material due to its compatibility with organic solvents that are required for monolith fabrication [35, 49]. However, one of the challenges with the use of COC is inconsistency in electrokinetic transport due to its hydrophobic surface. Reliable electrokinetic transport is an important characteristic to ensure constant sample injection for given parameters and a defined interval. Thus, a surface modification step was incorporated

before monolith fabrication. PEGDA was used for photografting of COC channels because it provides an optically transparent, inert, and hydrophilic coating which also decreases nonspecific adsorption [54, 57]. Initial photografting tests were performed using 3–7% PEGDA dissolved in methanol with BME (1%, photoinitiator) to modify the COC channels. We found that 3%, 5%, and 7% PEGDA formulations were able to make channels hydrophilic enough to facilitate sample injection. However, 3% PEGDA photografting was not reproducible, while 7% PEGDA photografting resulted in occasional blocking of channels. Thus, 5% PEGDA was used for subsequent channel photografting. SEM images were taken to confirm PEGDA photografting on COC channels (see supporting information, Fig. 1A). Water contact angle was measured for unmodified and photografted COC surfaces to confirm modification. The observed decrease in water contact angle on PEGDA photografted COC indicates an increase in the hydrophilicity of the COC surface (Table II). Additionally, visual inspection confirmed the flow of aqueous buffer by capillary action in photografted channels. Consistent forward sample flow was recorded in photografted devices by CCD imaging of fluorescence during electrokinetic injection of a fluorescent compound (see supporting information, Fig. 1B–D).

3.2 Monolith characterization

The monolith pre-polymer mixture was previously characterized and consisted of monomer (C8), cross-linker (EDMA), porogen (cyclohexanol and 1-dodecanol), surfactant (Tween 20) and a photoinitiator (DMPA) [35, 49]. C8 was chosen as the monomer because of its hydrophobic characteristics that can retain both proteins and peptides. For this study, a porogen-to-monomer ratio of 70:30 was used to ensure high surface area. Fig. 1C–D shows cross-sectional SEM images of a photografted channel without and with C8 monolith. The SEM shows that the monolith is well anchored to the PEGDA-photografted COC surface. Additionally, a magnified SEM image of bulk C8 monolith showed high porosity and round nodules of 200–500 nm diameter (Fig. 1E). In agreement with previous reports, these monoliths did not dislocate or move upon application of voltage during experiments [43].

3.3 Optimization of pH-mediated SPE

SPE was carried out on a monolithic support by altering the analyte charge, and hence hydrophilicity and electrophoretic mobility, using different pH solutions. Initially, pH 5 solution was used to enrich the sample on the reversed-phase monolith. The pH 5 buffer chosen for capturing P1 and ferritin is slightly above their native isoelectric points (~4.8; and fluorescence labeling moves the isoelectric point lower); at this pH these biomarkers have lower effective mobility and higher hydrophobicity, aiding in retention on the reversed-phase monoliths. Species with similar pI values and hydrophobicities would be similarly retained on our monoliths. Then, this sample was eluted from the monolith by switching to a higher pH buffer in which the analyte became more charged and hydrophilic. This pH-mediated SPE approach was fine-tuned using eluents with different pH and buffer concentrations.

3.3.1 Eluent pH—The effect of eluent pH from 7.0–11.5 was observed. The injection time (2 min) and buffer ionic concentration (50 mM) were kept constant. Experiments were carried out as described in section 2.6.1, and the elution profile of P1 (loaded at pH 5) was recorded for different pH eluents (Fig. 2A). Eluted peak heights and areas were determined

for each pH eluent (Fig. 2B). Elution buffer at pH 10 showed the highest peak height and peak area for eluting P1 from C8 monoliths, so this pH eluent was chosen for subsequent experiments.

3.3.2 Eluent buffer concentration—The effects of pH 10 BCB eluent concentration from 1 to 100 mM was also studied. Sample injection buffer and time were kept consistent from previous experiments. The elution profile for different buffers was recorded (Fig. 3A), and the peak height and area were plotted for different buffer concentrations (Fig. 3B). Although 1 mM BCB showed the largest peak area, it also had the lowest eluted peak height. The low ionic strength of 1 mM buffer resulted in a broader plug of P1 due to slow elution. A narrowing of the eluted peak was observed with increasing concentration of eluent. Considering both peak height and area, 50 mM BCB showed the best efficiency for eluting P1 from C8 monoliths, so we used 50 mM BCB in subsequent experiments.

3.4 pH-mediated SPE of PTB biomarkers

Fig. 4 shows the elution traces obtained after pH-mediated SPE of blank sample, two model dyes, and two PTB biomarkers (P1 and ferritin), on a C8 monolith under conditions optimized above. A blank run with no fluorescent sample showed only a small bump in the signal indicating a change in background fluorescence in changing from pH 5 to pH 10 buffer. 100 nM FITC showed a small peak and AF 488 showed a peak three times as tall as FITC at the same concentration after eluting from C8 monolith. An even larger elution peak was observed when FITC-labeled P1 at half the concentration of the dyes was loaded and eluted from the C8 monolith. Ferritin had a lower eluted peak height than P1, but the loaded ferritin concentration was 50-fold lower than P1. Additionally, ferritin showed a broader eluted band than the other analytes. Fluorescent dyes also showed lower peak heights than P1 on the monolith owing both to their higher mobility, which results in elution during electrokinetic loading and rinsing steps, and lower hydrophobicity [35, 50, 51], which makes them less retained on the monolith. Ferritin, being the most hydrophobic, showed the greatest retention. This experiment, done with 1 nM loaded ferritin, indicates potential for enrichment at pM concentrations. The hydrophobic characteristics of ferritin are also responsible for its broader eluted band. Our pH-mediated SPE extraction method allows low nM concentration analytes to be enriched and eluted.

3.5 Effect of analyte concentration on pH-mediated elution

Different concentrations of FITC-labeled P1 were loaded on a C8 monolith to determine the effect of analyte concentration on pH-mediated electroelution. Fig. 5A shows the elution profile from the monolith for different loaded concentrations (50–500 nM) of P1. A linear plot (Fig. 5B) was obtained for the peak heights as a function of loaded P1 concentration with an R^2 value of 0.997. The eluted peak migration time RSD for Fig. 5A data was determined to 3.9% ($n=7$). These results demonstrate the ability of pH-mediated SPE with electroelution to provide reproducible and quantitative results, similar to earlier μ CE systems that reported quantification either by standard addition or calibration curve [58].

3.6 Analyte enrichment

Reversed-phase monoliths can be used for preconcentration of dilute analytes, especially with the use of increased loading time. To determine the analyte enrichment with pH-mediated SPE, 50 nM FITC-labeled P1 was loaded on a C8 monolith for 1–5 min, and elution profiles were recorded as shown in Fig. 6A. Peak heights in the elution traces increased with loading time. The peak heights were used to calculate enrichment based on the average signal obtained from 50 nM P1 injected directly into the channel (see supporting information Fig. 2). Fig 6B shows the average peak height and calculated enrichment factor for injection times from 1–5 min. An enrichment of nearly 50-fold was observed for 50 nM P1 with a 5 min injection time. The eluted peak migration time RSD for Fig. 6A results was calculated to 6.4% (n=6), indicative of good reproducibility of this approach. Increasing the sample injection time further could lead to even higher enrichment factors.

3.7 Monolith capacity

Monolith capacity is an important parameter to determine because it gives an estimate of the enrichment capabilities of the system. The monolith capacity was determined by injecting 500 nM FITC-labeled P1 on the C8 monolith for different times and monitoring elution as seen in Fig. 7A. A tenfold higher concentration of P1 was used than in Fig. 6 so saturation could be achieved more readily. Traces during elution for 1–6 min loading times (Fig. 7A) showed narrow peaks, with heights that increased with injection time through a certain range. Fig. 7B shows the eluted peak heights as a function of injection time. A plateau in the eluted peak height is seen starting at 4 min injection time, indicating monolith saturation. We adapted a photobleaching flow measurement method previously described by He et al. [59] to determine the migration velocity of P1 under our injection conditions (see supporting information, Fig. 3). A dip in fluorescent signal due to photobleaching was detected at 5.5 s, indicating the time taken for the photobleached plug to migrate 5 mm. The migration velocity of P1 was thus $900 \mu\text{m s}^{-1}$, which for 500 nM P1 loaded for 4 min corresponds to a monolith binding capacity of 400 pg or 0.2 pmol. The binding capacity RSD is <15%, limited by the RSD of the monolith lengths, and consistent with the peak height variations in Fig. 7B. Although the binding capacity for P1 is less than 1 ng, for the nL volumes used in microfluidics this capacity is sufficient for typical SPE experiments.

3.8 Effects of blood serum on P1 binding capacity

Sample matrix affects the retention and elution capabilities of our system. Fig. 8A shows the elution profile from these monoliths of 50 nM P1 loaded in buffer and diluted human serum (buffered at pH 5). Fig. 8B shows the eluted peak heights of P1 from the monoliths for each sample matrix. For P1 loaded from 0.05%, 0.5% and 5% serum, the eluted peak height was reduced by 20%, 45%, and 75%, respectively, compared to P1 loaded in buffer. This indicates a decrease in binding of P1 to the monolith due to serum components competing for retention. Additionally, more peak tailing was observed in Fig. 8A for samples loaded in a serum matrix because interfering components slowed the electroelution of P1 from the monolith. A similar effect was observed for the higher P1 concentrations in Fig. 7. Importantly, our results show that pH-mediated extraction and elution could be used to enrich target peptides from diluted serum samples.

3.9 Integrated SPE and μ CE of a PTB biomarker

Integration of SPE sample enrichment and μ CE can enable automated analysis of low-concentration analytes. To demonstrate this potential, pH-mediated SPE was combined with μ CE using the device design detailed in Fig 1F–H. Fig. 9A shows an electropherogram after extraction, pH-mediated electroelution, injection, and μ CE of 50 nM P1 in buffer. As a comparison μ CE of 50 nM P1 (unenriched and buffered at pH 9.5; injection time 1 min) run in a standard PMMA device [36, 60] with the same injection volume as our SPE- μ CE device is also shown. A 10-fold increase in the peak area of enriched P1 was observed, with a small decrease in the number of theoretical plates per meter (N/m) from 210,000 to $\sim$ 140,000. Furthermore, a mixture of FITC-labeled P1 and FA in buffer was used for integrated SPE and μ CE to demonstrate the separation ability of this device. Fig. 9B shows the electropherogram of a mixture of P1 and FA extracted, eluted, injected, and separated on our device, along with the electropherogram of the same unenriched mixture. An 8.5- and 15-fold increase in peak areas was observed for FA and P1, respectively. Decreases in N/m from $\sim$ 560,000 to $\sim$ 140,000 and $\sim$ 380,000 to $\sim$ 25,000 were also observed for FA and P1, respectively. The decrease in N/m for P1 is due to a closely migrating impurity peak that is no longer resolved in the enriched mixture. The enrichment for FA was lower than for P1 due to its higher electrophoretic mobility, which results in its partial elution during the rinsing step. Additionally, the enrichment factor for P1 was less than what was observed in electroelution, because during μ CE only a part of the eluted plug is injected into the separation channel. From the 50 μ m channel width and $\sim$ 700 μ m eluted plug length, we estimate that $\sim$ 7% of the eluted plug was injected for μ CE. The peptide enrichment reported in this study is comparable to what we recently reported in an integrated pressure-driven microfluidic platform [50].

This integrated pH-mediated SPE- μ CE method shows promising results for peptide enrichment and electrophoretic separation. We expect that N/m can be improved by further optimization of buffers and applied voltages. Higher enrichment factors can also be obtained by increasing the length of the monolith or injecting for longer times. Furthermore, this simple pH-mediated SPE approach can be extended for analysis of a wide range of analytes like PTB biomarkers.

4. Conclusions

A pH-mediated SPE method was developed using hydrophobicity and electrophoretic mobility modulation in different pH buffers, and applied in preconcentration of biomarkers related to preterm birth. Surface modification of COC microchannels was performed using PEGDA to increase the hydrophilicity to make electrokinetic flow more reproducible. Porous, reversed-phase C8 monoliths polymerized in COC channels were used for SPE. Eluents of different pH and ionic concentrations were used to determine the best elution conditions. The optimized eluent was then used to preconcentrate multiple analytes on C8 monoliths. An enrichment factor of nearly 50 was observed for a PTB peptide biomarker (P1) eluted from a monolith after 5 min of injection, with reproducible elution ($\sim$ 6% or better migration time RSD). A linear relation between peptide concentration and eluted peak height was also observed, indicating the potential for this approach to be used for

quantitative analysis. The monolith binding capacity for P1 in our devices was determined to be 400 pg (0.2 pmol). Importantly, this pH-mediated SPE approach was integrated with μ CE to enable combined enrichment (~15-fold) and μ CE separation of a peptide biomarker related to preterm birth (P1). Further optimization of monolith composition and separation buffer should result in increased enrichment and separation efficiency.

The device and methods we developed show potential for integrated on-chip sample preparation, including purification and preconcentration. In the future, this work can be further optimized to facilitate on-chip fluorescent labeling of analytes along with preconcentration. Additional reservoirs could be introduced to minimize sample contamination and increase automation, allowing fluids to be manipulated electrokinetically. Our pH-mediated SPE method could also work with open tubular columns [61] or micro pillars [62]. Since a complex matrix like serum contains additional components that compete for monolith binding sites, an affinity monolith could be introduced before the reversed-phase monolith for selective capture of multiple PTB biomarkers before enrichment [56, 58, 63]. This could enable the analysis of low concentrations of PTB biomarkers directly from serum samples using an affinity monolith for selective capture, and then enriching these biomarkers on a reversed-phase monolith (in a buffered matrix), followed by electrophoretic separation. Such a device could offer a prompt and cost-effective analysis platform for PTB biomarkers, with further potential to be adapted for any specific panel of biomarkers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support for this work was provided by the National Institutes of Health through grant R01 EB006124. We are thankful to Prof. Matthew Linford for letting us use a goniometer, and to Dr. Anubhav Diwan and Tuhin Roychowdhury for their help with contact angle measurements. We are also thankful to Prof. William Pitt for providing blood serum samples. We also thank the Integrated Microfabrication Laboratory at Brigham Young University for device fabrication facilities. The authors declare no conflict of interest.

References

1. Hayes J, Peruzzi PP, Lawler S. Trends Mol Med. 2014; 20:460–469. [PubMed: 25027972]
2. Zhou L, Wang R, Yao C, Li X, Wang C, Zhang X, Xu C, Zeng A, Zhao D, Zhang F. Nat Commun. 2015; 6:6938. [PubMed: 25907226]
3. Yotsukura S, Mamitsuka H. Crit Rev Oncol Hemat. 2015; 93:103–115.
4. Pavo N, Raderer M, Hülsmann M, Neuhold S, Adlbrecht C, Strunk G, Goliasch G, Gisslinger H, Steger GG, Hejna M, Köstler W, Zöchbauer-Müller S, Marosi C, Kornek G, Auerbach L, Schneider S, Parschalk B, Scheithauer W, Pirker R, Drach J, Zielinski C, Pacher R. Heart. 2015; 101:1874–1880. [PubMed: 26416836]
5. Manson JE, Bassuk SS. Metabolism. 2015; 64:S33–S39. [PubMed: 25487190]
6. Bateman RJ, Xiong C, Benzinger TLS, Fagan AM, Goate A, Fox NC, Marcus DS, Cairns NJ, Xie X, Blazey TM, Holtzman DM, Santacruz A, Buckles V, Oliver A, Moulder K, Aisen PS, Ghetti B, Klunk WE, McDade E, Martins RN, Masters CL, Mayeux R, Ringman JM, Rossor MN, Schofield PR, Sperling RA, Salloway S, Morris JC. N Engl J Med. 2012; 367:795–804. [PubMed: 22784036]
7. Jack CR Jr, Bennett DA, Blennow K, Carrillo MC, Feldman HH, Frisoni GB, Hampel H, Jagust WJ, Johnson KA, Knopman DS, Petersen RC, Scheltens P, Sperling RA, Dubois B. Neurology. 2016; 87:539–547. [PubMed: 27371494]

8. Lewczuk P, Mroczko B, Fagan A, Kornhuber J. *Adv Med Sci.* 2015; 60:76–82. [PubMed: 25579841]
9. Mussap M, Noto A, Cibecchini F, Fanos V. *Semin Fetal Neonatal Med.* 2013; 18:56–64. [PubMed: 23164809]
10. Ruggeri B, Sarkans U, Schumann G, Persico AM. *Psychopharmacology.* 2014; 231:1201–1216. [PubMed: 24096533]
11. Ross CA, Aylward EH, Wild EJ, Langbehn DR, Long JD, Warner JH, Scahill RI, Leavitt BR, Stout JC, Paulsen JS, Reilmann R, Unschuld PG, Wexler A, Margolls RL, Tabrizi SJ. *Nat Rev Neurol.* 2014; 10:204. [PubMed: 24614516]
12. Carinelli S, Martí M, Alegret S, Pividori MI. *New Biotechnol.* 2015; 32:521–532.
13. Punyadee N, Mairiang D, Thiemme S, Komoltri C, Pan-ngum W, Chomanee N, Charnkaew K, Tangthawornchaikul N, Limpitikul W, Vasanawathana S, Malasit P, Avirutnan P. *J Virol.* 2015; 89:1587–1607. [PubMed: 25410854]
14. Nge PN, Rogers CI, Woolley AT. *Chem Rev.* 2013; 113:2550–2583. [PubMed: 23410114]
15. Araci IE, Brisk P. *Curr Opin Biotechnol.* 2014; 25:60–68. [PubMed: 24484882]
16. Kim HJ, Park JW, Park JW, Byun JH, Vahidi B, Rhee SW, Jeon NL. *Ann Biomed Eng.* 2012; 40:1268–1276. [PubMed: 22302320]
17. Zhang H, Liu L, Fu X, Zhu Z. *Biosens Bioelectron.* 2013; 42:23–30. [PubMed: 23202325]
18. Nahavandi S, Baratchi S, Soffe R, Tang SY, Nahavandi S, Mitchell A, Khoshmanesh K. *Lab Chip.* 2014; 14:1496–1514. [PubMed: 24663505]
19. Pagaduan JV, Sahore V, Woolley AT. *Anal Bioanal Chem.* 2015; 407:6911–6922. [PubMed: 25855148]
20. Long Z, Shen Z, Wu D, Qin J, Lin B. *Lab Chip.* 2007; 7:1819–1824. [PubMed: 18030406]
21. Kang QS, Li Y, Xu JQ, Su LJ, Li YT, Huang WH. *Electrophoresis.* 2010; 31:3028–3034. [PubMed: 20872608]
22. Ramsey JD, Collins GE. *Anal Chem.* 2005; 77:6664–6670. [PubMed: 16223254]
23. Esplin MS, Merrell K, Goldenberg R, Lai Y, Iams JD, Mercer B, Spong CY, Miodovnik M, Simhan HN, van Dorsten P, Dombrowski M. *Am J Obstet Gynecol.* 2011; 204:391.e391–391.e398. [PubMed: 21074133]
24. Graves SW, Esplin MS. *Am J Obstet Gynecol.* 2011; 204:S46. [PubMed: 21514920]
25. Goldenberg RL, Culhane JF, Iams JD, Romero R. *Lancet.* 2008; 371:75–84. [PubMed: 18177778]
26. Romero R, Dey SK, Fisher SJ. *Science.* 2014; 345:760–765. [PubMed: 25124429]
27. Goldenberg RL, Iams JD, Mercer BM, Meis PJ, Moawad A, Das A, Miodovnik M, VanDorsten PJ, Caritis SN, Thurnau G, Dombrowski MP. *Am J Obstet Gynecol.* 2001; 185:643–651. [PubMed: 11568793]
28. Goldenberg RL, Goepfert AR, Ramsey PS. *Am J Obstet Gynecol.* 2005; 192:S36–S46. [PubMed: 15891711]
29. Augusto F, Hantao LW, Mogollón NGS, Braga SCGN. *TrAC Trends Anal Chem.* 2013; 43:14–23.
30. Duan YP, Dai CM, Zhang YL, Ling C. *Anal Chim Acta.* 2013; 758:93–100. [PubMed: 23245900]
31. Majors, RE. *Handbook of Sample Preparation.* John Wiley & Sons, Inc; 2010. p. 53-79.
32. Malmstadt N, Yager P, Hoffman AS, Stayton PS. *Anal Chem.* 2003; 75:2943–2949. [PubMed: 12964737]
33. Yu C, Davey MH, Svec F, Fréchet MJ. *Anal Chem.* 2001; 73:5088–5096. [PubMed: 11721904]
34. Aggarwal P, Tolley HD, Lee ML. *J Chromatogr A.* 2012; 1219:1–14. [PubMed: 22169193]
35. Yang R, Pagaduan JV, Yu M, Woolley AT. *Anal Bioanal Chem.* 2015; 407:737–747. [PubMed: 25012353]
36. Yang W, Sun X, Pan T, Woolley AT. *Electrophoresis.* 2008; 29:3429–3435. [PubMed: 18702050]
37. Oleschuk RD, Shultz-Lockyear LL, Ning Y, Harrison DJ. *Anal Chem.* 2000; 72:585–590. [PubMed: 10695146]
38. Vázquez M, Paull B. *Anal Chim Acta.* 2010; 668:100–113. [PubMed: 20493286]
39. Knob R, Sahore V, Sonker M, Woolley AT. *Biomicrofluidics.* 2016; 10:032901. [PubMed: 27190564]

40. Stachowiak TB, Rohr T, Hilder EF, Peterson DS, Yi M, Svec F, Fréchet MJ. Electrophoresis. 2003; 24:3689–3693. [PubMed: 14613194]
41. Liu K, Aggarwal P, Lawson JS, Tolley HD, Lee ML. J Sep Sci. 2013; 36:2767–2781. [PubMed: 23813977]
42. Pruim P, Öhman M, Schoenmakers PJ, Kok WT. J Chromatogr A. 2011; 1218:5292–5297. [PubMed: 21733523]
43. Ladner Y, Crétier G, Faure K. J Chromatogr A. 2010; 1217:8001–8008. [PubMed: 20800231]
44. Nordman N, Barrios-Lopez B, Laurén S, Suvanto P, Kotiaho T, Franssila S, Kostianen R, Sikanen T. Electrophoresis. 2015; 36:428–432. [PubMed: 25043750]
45. Britz-McKibbin P, Bebault GM, Chen DDY. Anal Chem. 2000; 72:1729–1735. [PubMed: 10784134]
46. Britz-McKibbin P, Chen DDY. Anal Chem. 2000; 72:1242–1252. [PubMed: 10740866]
47. Baidoo EEK, Benke PI, Neusüss C, Pelzing M, Kruppa G, Leary JA, Keasling JD. Anal Chem. 2008; 80:3112–3122. [PubMed: 18384203]
48. Imami K, Monton MRN, Ishihama Y, Terabe S. J Chromatogr A. 2007; 1148:250–255. [PubMed: 17382949]
49. Nge PN, Pagaduan JV, Yu M, Woolley AT. J Chromatogr A. 2012; 1261:129–135. [PubMed: 22995197]
50. Kumar S, Sahore V, Rogers CI, Woolley AT. Analyst. 2016; 141:1660–1668. [PubMed: 26820409]
51. Sonker M, Yang R, Sahore V, Kumar S, Woolley AT. Anal Methods. 2016; 8:7739–7746. [PubMed: 28496521]
52. Sahore V, Kumar S, Rogers CI, Jensen JK, Sonker M, Woolley AT. Anal Bioanal Chem. 2016; 408:599–607. [PubMed: 26537925]
53. Kelly RT, Woolley AT. Anal Chem. 2003; 75:1941–1945. [PubMed: 12713054]
54. Ladner Y, Bruchet A, Crétier G, Dugas V, Randon J, Faure K. Lab Chip. 2012; 12:1680–1685. [PubMed: 22430301]
55. Yu M, Wang HY, Woolley AT. Electrophoresis. 2009; 30:4230–4236. [PubMed: 19924700]
56. Sun X, Yang W, Pan T, Woolley AT. Anal Chem. 2008; 80:5126–5130. [PubMed: 18479142]
57. Rogers CI, Pagaduan JV, Nordin GP, Woolley AT. Anal Chem. 2011; 83:6418–6425. [PubMed: 21728310]
58. Yang W, Sun X, Wang HY, Woolley AT. Anal Chem. 2009; 81:8230–8235. [PubMed: 19728735]
59. He M, Bao JB, Zeng Y, Harrison DJ. Electrophoresis. 2010; 31:2422–2428. [PubMed: 20568261]
60. Pagaduan JV, Ramsden M, O'Neill K, Woolley AT. Electrophoresis. 2015; 36:813–817. [PubMed: 25486911]
61. Zeng H, Glawdel T, Ren CL. Electrophoresis. 2015; 36:2542–2545. [PubMed: 26101201]
62. Mery E, Ricoul F, Sarrut N, Constantin O, Delapierre G, Garin J, Vinet F. Sens Actuators, B. 2008; 134:438–446.
63. Yang W, Yu M, Sun X, Woolley AT. Lab Chip. 2010; 10:2527–2533. [PubMed: 20664867]

Figure 1.

Device layout, photograph, operation and SEM images. (A) Single channel SPE device layout showing reservoirs for sample and sample waste, monolith, and voltage configuration for sample injection and elution. (B) Photograph of a device showing reservoirs and monolith (white) in the channels. SEM cross-sectional image of (C) an empty microfluidic channel, (D) a channel containing a C8 monolith, and (E) a magnified view of a C8 monolith prepared in bulk. (F–G) Integrated SPE- μ CE device layout showing monolith position, sample flow, detection point and voltage configuration for (F) pinched sample injection/elution and (G) separation with pullback voltages. (H) Device photograph. Reservoir numbering is given in the text.

Figure 2.

Effect of eluent pH on electroelution. (A) Elution of P1 (50 nM loaded concentration) from a C8 monolith with increasing eluent pH. Traces are offset vertically and horizontally. (B)

Eluted peak height and area for different pH eluents.

Figure 3. Effect of eluent buffer concentration. (A) Elution of P1 (50 nM loaded concentration) from a C8 monolith using different BCB eluent concentrations. Traces are offset vertically and horizontally. (B) Peak height and area for different eluting BCB concentrations.

Figure 4. pH-mediated SPE of sample dyes and PTB biomarkers for 2 min loading times. Traces are offset vertically. Concentrations: ferritin (1 nM), P1 (50 nM), AF 488 (100 nM), and FITC (100 nM).

Figure 5. Effect of loaded P1 concentration on eluted peaks. (A) Elution from a C8 monolith of different concentrations of P1 loaded for 1 min. Traces are offset vertically and horizontally. (B) The peak height of eluted P1 plotted against concentration ($n=3$).

Figure 6. P1 (50 nM loaded) enrichment with injection time. (A) Elution profiles. (B) P1 enrichment and peak heights (n=3) with injection time.

Figure 7. Monolith saturation. (A) Elution profiles for P1 (500 nM loaded) for increasing injection times. Traces are offset vertically and horizontally. (B) Peak heights of eluted P1 for increasing injection times (n=3).

Figure 8. Effect of a serum matrix on P1 retention and elution. (A) Elution profiles of P1 (50 nM) loaded on a monolith in buffer and various human serum matrices. (B) Peak heights of eluted P1 for different matrices (n=3).

Figure 9. Integration of pH-mediated SPE with μ CE. (A) Electropherogram of 50 nM P1 loaded with and without on-chip enrichment using a C8 monolith. (B) Electropherogram showing separation of FA and P1 (50 nM each loaded) with and without enrichment. Traces are offset vertically.

Table IReversed phase octyl methacrylate prepolymer mixture^{*}

Name	Functional role	Mass (%)
Octyl methacrylate (C8)	Monomer	20%
EDMA	Cross-linker	10%
Cyclohexanol	Porogen	25%
1-dodecanol	Porogen	25%
Tween-20	Surfactant	20%
DMPA	Photoinitiator	1%

^{*}
sum >100% due to rounding

Table II

Contact angle measurements

Material	Contact angle (mean $\pm$ std. dev.) [*]
COC	90.7° $\pm$ 1.5°
PMMA	71.7° $\pm$ 2.1°
3% PEGDA photografted COC	67.7° $\pm$ 1.5°
5% PEGDA photografted COC	54.0° $\pm$ 2.6°

^{*}
n=3