Programmed Affinity Extraction of Molecules on a Microfluidic Platform

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***Abstract*—Here, we present a "smart" microfluidic system for highly selective extraction of trace amounts of analytes using ribonucleic acid (RNA) aptamers. A micro-chamber consisting of streptavidin coated polystyrene beads was fabricated on a glass substrate using standard MEMS fabrication techniques. The beads were mobilized into the reaction chamber by an auxiliary channel and trapped using two weirs each connecting a sample carrier channel. After a thorough rinsing of the beads and chamber with buffer solution, a 20 μM solution of biotin labeled adenosine triphosphate aptamer (ATP-aptamer) was loaded into the chamber and allowed to bind with the streptavidin modified beads. An affinity matrix was realized. The extraction of an affinity analyte, adenosine monophosphate coupled with thiazole orange (TO-AMP), with acute discrimination, was demonstrated. Detection of the analyte was performed directly on the microfluidic device using an inverted fluorescence microscope. Additionally, we demonstrated "programmed" release and regeneration of the device using both competitive displacement and thermal energy. The effectiveness of both mechanisms is presented.**

Keywords-aptamer; controlled release; affinity sensor; microchip; purification; solid-phase extraction (SPE);

I. INTRODUCTION

Presently, there is an ever increasing demand for a selective and effective means to extract or purify specific analytes from biological sample solutions. Whether to simply detect specific molecules in environmental monitoring systems [1] or to concentrate compounds for further analysis on integrated separation systems [2], the need to extract only specific molecules while excluding non-specific molecules in the sample solution is significant. This is especially important in systems of multiple analytes producing similar detection signals that may confuse resulting analyses.

One of the shortcomings of many existing solid-phase extraction (SPE) systems is the indiscrimination of their capturing mechanisms. In particular, systems incorporating stationary matrices that capture analytes using hydrophobic [3] and ion-exchange [4] interactions have the negative side-effect of extracting all other compounds with similar physical or chemical interaction properties. In biomedical and biochemical related applications where specific molecules need to be released, such as in a drug delivery system, or during a specific chemical reaction, it is obvious that introducing impurities or non-specific compounds during processing can be problematic. However, affinity stationary phases show promise in resolving this issue with target molecule specificity in extraction systems.

Affinity interaction/binding is biochemical in nature. It involves the reaction between ligands and specific target molecules such as an antigen and antibody, enzyme and inhibitor, or aptamer and protein. The highly specific nature of these interactions results from the two participating compounds being ideally suited to one other both electrostatically and spatially. Affinity bonds are also reversible by such mechanisms as heat, altered solution pH or solution ionic strength [5]. In terms of ligand/target popularity, antibody/lectin systems are the most common, but novel high-affinity molecules such as oligopeptides, ribonucleic (RNA) and deoxyribonucleic (DNA) acid aptamers are receiving increasing attention [6, 7]. DNA and RNA aptamers are particularly attractive since they can be synthesized to produce high affinity towards possibly any target protein or molecule that binds nucleic acid as its function [8]. Aptamers present several advantages over antibody/lectin systems. DNA and RNA aptamers are more stable at room temperature, more easily synthesized and chemically modified at terminal sites as to facilitate attachment to stationary surfaces [9, 10]. This class of aptamers is also resilient under severe elution mechanisms such as extreme changes in pH, organic solvents, and detergents, all of which can denature ligand molecules [11].

Biochemical systems have increasingly incorporated the advantages and utility of miniaturization technology. Micro- and nanofabrication technology has facilitated this trend. Scaling of biochemical and biomedical applications is attributed to expensive reagents, fluorescent labels, and other chemicals needed for processing. Often the costs are hundreds of dollars (U.S.) per milligram for these substances and thus reducing the consumption by shrinking the dimensions of the system can greatly decrease expenses. Surface to volume ratios (SV) in microdevices can be much higher than those at conventional scales making diffusion distances much shorter. This effectively allows faster interaction between the surface and solution phases, which produces more efficient material transfer, quickening assay time. Several methods to provide high SV support matrices in microdevices include monolithic silicon arrays [12], porous silicon structures [13], and micro-bead gels [2, 14].

Micro-bead beds are known to produce the highest SV in microfluidic devices but are susceptible to high back-pressure. Hence, using beads in a design requires careful consideration of this problem. In this study, an RNA aptamer

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against adenosine triphosphate (ATP) was used as a high affinity surface [15] to extract and release target molecules of adenosine monophosphate coupled with thiazole orange (TO-AMP) on a microfluidic chip. Detection is carried out on a standard inverted fluorescence microscope allowing the easy integration of this system to many analytical settings. We present the design and fabrication of the chip and results from affinity micro-extraction experiments.

II. Experimental Methods

A. Materials

Biotinylated adenosine triphosphate aptamer (bio-ATP-40-1, or ATP-aptamer) was made and "DNA/RNAse free HPLC" purified by Integrated DNA Technologies, Inc. (Coralville, IA) and subsequently used as received. TO-AMP was synthesized by Jeffrey Rothsman (Medicine-Experimental Therapeutics, Columbia University, Fig. 1). Adenosine triphosphate (ATP) was purchased from Sigma-Aldrich Co. (Milwaukee, WI). Diethyl pyrocarbonate treated sterile water (SW), from Fisher (Pittsburgh, PA), was used for all biochemistry work. Buffer solution (pH 7.4) was prepared by mixing Tris-HCl (20 mM), NaCl (140 mM), KCl (5 mM), and $MgCl_2$ (5 mM) in SW. Chemicals for the buffer solution were purchased though Fisher. ATP aptamer, TO-AMP, and ATP working solutions were all prepared using Tris-HCl buffer. UltraLink immobilized streptavidin polystyrene beads (50-80 µm in diameter) were acquired from Pierce (Rockford, IL). All solvents, isopropyl alcohol (IPA), methyl alcohol, and acetone were of purified grade (Mallinckrodt Baker, Phillipsburg, NJ). SU-8 2025 and 2100 was bought from MicroChem (Newton, MA). Poly-dimethylsiloxane (PDMS) was acquired from Robert McKeown Company (Somerville, NJ). *Torr Seal* epoxy and silicone glue came from Varian (Palo Alto, CA) and Action Electronics (Santa Ana, CA), respectively. Glass slides (25 mm × 75 mm) were purchased from Fisher. Silica capillary tubing and Tygon poly-vinyl chloride (PVC) tubing were purchased from Polymicro Technologies (Phoenix, AZ) and McMaster Carr (Dayton, NJ), respectively. Thermal paste was provided by the author and Kapton Tape was purchased from Techni-Tool (Worcester, PA).

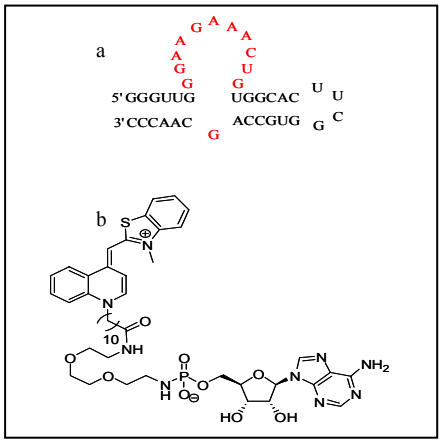

Figure 1. (a) Chemical structure of bio-ATP-40-1 aptamer. (b) Molecular structure of TO-AMP.

B. Instrumentation

Mercury vapor lamp induced fluorescence using a Nikon Eclipse TE300 inverted epi-fluorescence microscope (Nikon, USA) was employed for detection. Fluorescence micrographs were recorded using a Q-Imaging model Retiga 2000R Mono-12-bit CCD and analyzed with Q-Capture Pro software (Austin, TX). Device temperature control was performed using a thermoelectric device from Melcor (model: CP1.4-71-06L, Trenton, NJ). DC potential was supplied to the thermoelectric device with an Agilent E3631 DC power supply (Santa Clara, CA). A type-K surface thermocouple model CO3-K and a model HHM-290 multimeter (Omega, Stamford, CT) were used to measure device temperature. Microfluidic flow was provided from a New Era model NE-1000 syringe pump (Farmingdale, NY), 5 cm^3 syringes, and 21 gauge (38.1 mm long) needles (Becton Dickinson, Franklin Lakes, NJ). Diamond-tipped drill bits (0.7 mm diameter) and a Model 7000 standard drill press were from Servo Products (Eastlake, OH).

C. Device Design and Fabrication

A typical device is shown in Fig. 2. The channels are numbered for reference. Channels c1 and c2 (5.1 mm × 400 µm × 40 µm) were used to deliver sample and buffer solution to the chamber (8.7 mm × 3 mm × 140 µm). Channel c3 was used to pack the polystyrene beads. The ports have radii of 1 mm each and are 140 µm thick. Hence, the chamber has an effective volume of 3.09 micro-liters with the tapers taken into consideration, whereas the microfluidic system volume (on-chip) is 3.60 micro-liters. Using Poiseuille-flow, the maximum pressure drop across this device (port to port), excluding beads, can be calculated from

$$Q = \frac{\pi (D_h)^4 \Delta p}{128 \mu l}. \qquad (1)$$

Here, Q is the flow rate, Δp is the pressure drop, µ is the dynamic viscosity of the fluid, l is the channel length, and D_h is the hydraulic diameter given by the expression

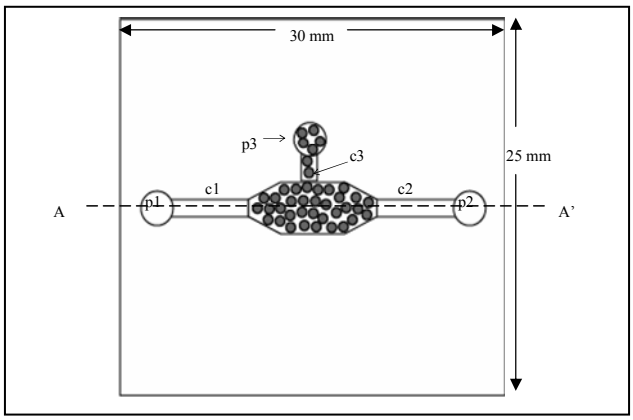

Figure 2. A cartoon presenting the layout of the microfluidic SPE device used for this study.

$$D_h = \frac{4A}{P}. \quad (2)$$

In (2), A is the cross-sectional area of the channel and P is the wetted perimeter. For water, the calculated pressure drop for Q = 50 µl/min used in experiments was 6.83 kilo-Pascal. When considering a packed chamber of micro-beads, the pressure increase was estimated to be 10-20 times greater.

Microchip SPE devices were fabricated on glass slides using standard PDMS processing. A simplified device process flow (Fig. 3) shows primary fabrication steps. An SU-8 mold for PDMS curing and channel fabrication was created on silicon wafers (101 mm) from Silicon Quest International (Santa Clara, CA). Fabrication began with deposition and patterning of 15 nm Cr alignment marks via thermal evaporation on an Edwards/BOC Auto306 thermal evaporator (Wilmington, MA), followed by lift-off in acetone overnight. Secondly, patterning of SU-8 2025 realized channels c1 and c2 (40 µm thick), whereas SU-8 2100 resist completed the mold, producing the reaction chamber and channel c3 (140 µm).

PDMS pre-polymer solution was mixed with a mass ratio of 10:1 and distributed on the mold. The pre-polymer was degassed by vacuum (30 min) and followed by semi-curing (70 °C, 50 min). In parallel, glass substrates were diced (25 mm × 30 mm) and drilled to create the access ports (p1-p3). The glass substrates were then cleaned using a solution of H_2SO_4 and H_2O_2 (7:4 vol/vol at 130 °C). Ports could also be fabricated in the PDMS blank layer. The semi-cured PDMS

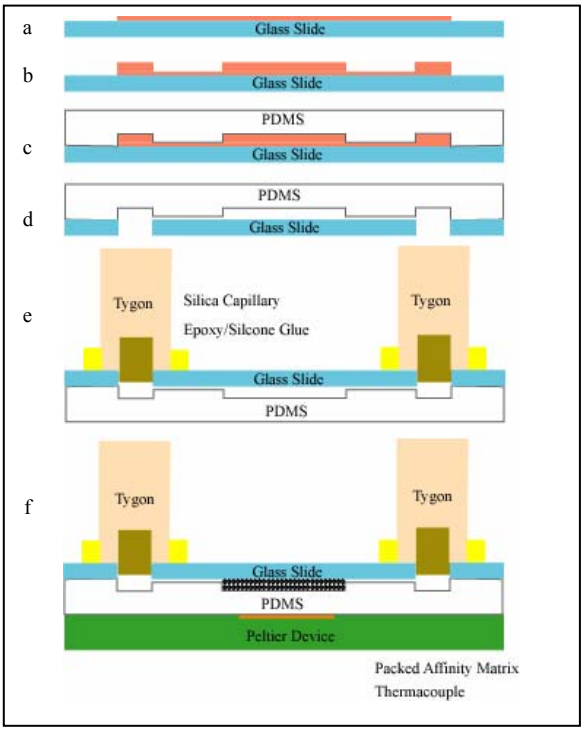

Figure 3. A simplified device process flow: (a -d) A microchannel realized with standard PDMS processing; (e & f) packaging. Use line (A-A') in the schematic of Fig. 2 as a cross-section reference.

sheet was removed from the SU-8 mold, aligned and bonded to the glass slides following O_2 plasma treatment of the bonding interface in a Technics Series 800 Micro RIE system (100 mtorr and 85 W) for 15 seconds. Permanent bonding and curing of PDMS to the substrate was performed by heating the chip (25 min at 85 °C).

Packaging was accomplished by inserting a combination of silica capillary tubing (0.6 mm ID, 0.7 mm OD) segments along with Tygon PVC tubing (0.6 mm ID, 3.18 OD) through the drilled access ports. The connection interfaces were sealed using silicone glue and Torr seal epoxy. For thermal related experiments, a thermocouple was subsequently sandwiched between a peltier device and the bottom of the microfluidic chip. The components were held together by thermal interfacing paste or Kapton Tape.

D. Experimental Procedure

All fluorescence detection was done using a Nikon TE300 and Q-Imaging Retiga 2000R system. During analyte binding, TO emission occurs at 530 nm when excited at 480 nm so a blue light filter and green-pass dichroic mirror were used accordingly. The device was mounted in the same position using double-sided scotch tape marks on the microscope stage. For each image, a 10× objective was used to collect emitted fluorophores from the same area of the chamber (Fig. 4). These operating conditions were identical for all images taken for fluorescence detection of the analyte.

The entire microfluidic system was completely flushed (50 µl/min) with the buffer solution for 30 minutes by using any port as an inlet and collecting waste from both remaining ports. Streptavidin coated beads were suspended in buffer (4 ml) and loaded into a 5 ml syringe. Manual pressure was used to pack the beads from channel c3 via port p3 into the chamber. Subsequently, channel c3 was sealed permanently near the port interface using silicone glue. The chamber and channels were washed (50 µl/min) with buffer (30 min) through c1. ATP-aptamer solution (20 µl, 20 µM) was injected (10 µl/min) and allowed to incubate (20 min) in the chamber. The channel was washed again (50 µl/min for 20 min) and a baseline fluorescence signal was taken.

For SP purification/extraction, TO-AMP at different concentrations (400 nM, 500 nM and 10 µM) was loaded (10 µl at 10 µl/min) into the reaction chamber from channel c1. The solution was kept stagnant in the chamber for 10-15 minutes to allow complete interaction between the analyte and aptamer surface of the beads. Following the purification of analytes, the chamber was washed (50 µl/min for 15 min) with buffer to rid all non-specific compounds, un-reacted molecules, and impurities. A subsequent fluorescence image was taken. TO-AMP was released and collected in two ways: the first method used competitive displacement of TO-AMP by incubating different concentrations of ATP (800 µM and 3200 µM); the second method used elevated chip temperature (80 °C) while buffer was flowed (10 µl at 5 µl/min) through to collect analyte.

During purification, time resolved analyte adsorption experiments were conducted. For a 400 nano-molar

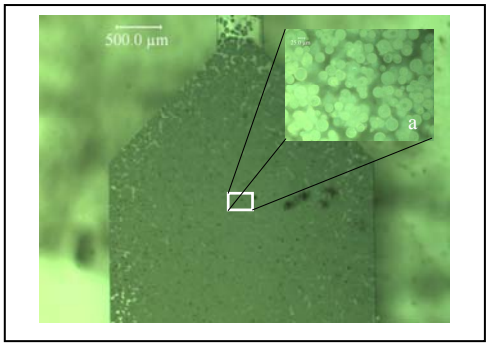

Figure 4. Bright-field micrograph of chamber. Inset (a) shows magnified area for fluorescence imaging and processing.

concentration of TO-AMP solution, fluorescence micrographs were recorded at time intervals of 1 minute. Images ceased to be taken after the observed fluorescence level showed no appreciable change.

III. Results and Discusion

A. Packing Stationary Material

An integrated SPE bed was prepared using a double weir design forming a cavity. Using PDMS for the channel material proved troublesome concerning this matter. Since PDMS is pliable, beads could be pushed under the weir structures under positive pressure resulting in immense backpressure. During bead introduction, port p3 was prone to clogging. Designs using drilled access ports in the PDMS blank layer proved a significant source of this problem. The holes in PDMS were plagued with burrs containing loose PDMS particles not cleared while drilling, which easily became obstacles and instigated clogging. This was mitigated by generating reversed flow and allowing beads to dislodge and flow back toward the source. On occasion, several forward/reverse pumping cycles were required to fully clear obstructions and continue filling the chamber. Drilling holes in the glass slides provided smoother, burr-free edges. Using this method, fully packed chambers were realized in over 90 percent of devices.

Another source of backpressure comes from the beads themselves, especially when using narrow uniform-width channels. To minimize backpressure of this sort, a widened chamber design was employed to contain the affinity matrix. Although the expansion ratio utilized (400 µm/3 mm) was not optimized, the back pressure was minimized, and the device functioned in a fashion similar to open micro-channels.

B. Time Resolved Binding of TO-AMP

To determine the approximate binding time of TO-AMP molecules to fully saturate the affinity matrix, fluorescence micrographs in discrete time intervals (1 min) immediately following a sample injection of TO-AMP (400 nM) were recorded. Fluorescence intensity measurements were obtained in a straight line direction (A-A') across each micrograph, averaged and then plotted as a function of time (Fig. 5). Similar analysis methods were used to quantitate all

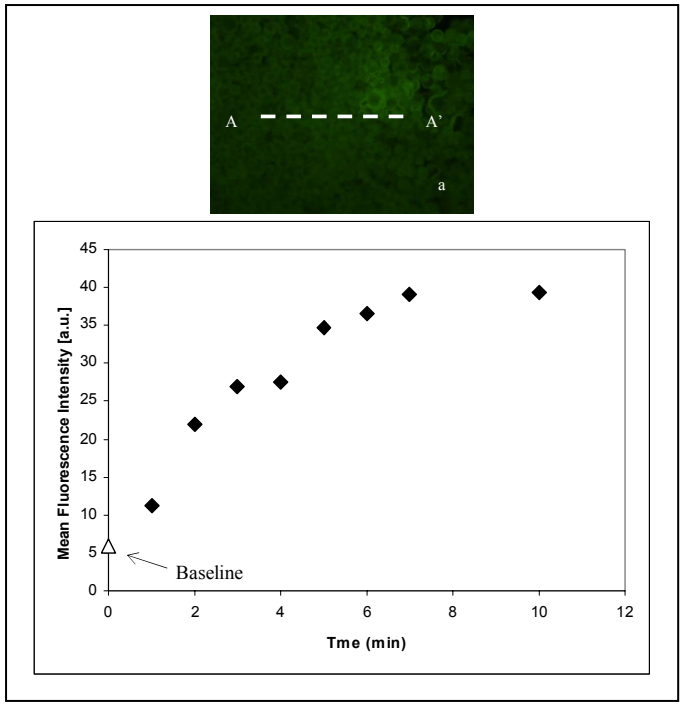

Figure 5. Time resolved extraction of TO-AMP (400 nM) by ATP-aptamer. Measurements made in the (A-A') direction of the linked micrograph inset (a). At each time step, fluorescence intensity was sampled, averaged, and normalized to produce a single value point for each interval.

ensuing data. No appreciable increase in fluorescence intensity occurred after 10 minutes of incubation time. Subsequent experiments took this into consideration when determining suitable interaction time between TO-AMP and ATP-aptamer. This information will also prove useful in future experiments using similar devices for the concentration of affinity analytes.

C. Solid-Phase Extraction of TO-AMP

Highly specific SPE is a valuable tool that can be used to selectively control biomolecules in many biomolecular applications. To determine the ability of this SPE matrix to retrieve specific analytes, three sample solutions of TO-AMP (400 nM, 500 nM and 10 µM) were injected as described in Section II. The solutions were allowed to interact with the matrix (10-15 min) and the chamber was then washed (50 µl/min for 15 min) with buffer before detection was performed. Imaging and fluorescence analysis conditions were similar to those used in the time resolved experiment, only without resolving the temporal dimension. Results are presented in Fig. 6. Notice that the signals are clearly distinct and non-overlapping for each concentration. The "noisy" nature of the signal comes from the dark areas in between individual beads as shown by the micrograph insets (Fig. 6a-d) rather than actual noise in the signal. The baseline fluorescence supported this explanation. No other radiation wavelength was detected from the micrographs other than that which is specific to TO emission (530 nm), further emphasizing the selective nature of the TO-AMP/ATP-aptamer interaction.

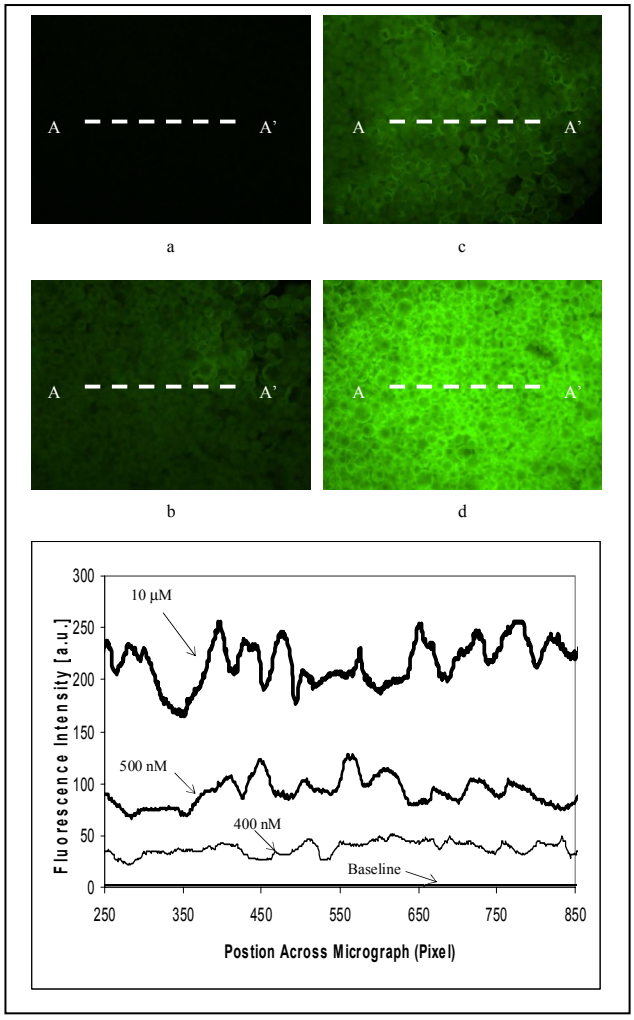

Figure 6. Micrographs displaying SPE extraction of 3 different concentrations of TO-AMP: (a) baseline fluorescence; (b) 400 nM; (c) 500 nM; and (d) 10 µM. Each signal appeared distinct with little noise.

A possible extension for many micro-extraction and purification devices is the concentration of analytes. As Fig. 6 acknowledges, our device presents strong potential for this subset of SPE. Three different concentrations of TO-AMP solution were injected into this device producing 3 separate normalized fluorescence profiles implying that gradual concentration can be achieved through time. This data alone suggested a potential concentration factor of 20 if identical injection and collection volumes are used. Although a 10 micro-molar solution of TO-AMP was the highest concentration used in our experiments, it by no means presented an upper limit. It is feasible that the saturation threshold of TO-AMP to ATP-aptamer had not been breached as of yet for the packed microfluidic matrix presented in this work.

D. Release of Analyte and Regeneration of the Device

A required attribute for "smart" microfluidic analysis devices is often the ability to intelligently capture and release target molecules. Attractive devices supplement this characteristic further with regeneration capabilities without loss of functionality. Our device was capable of capturing and releasing TO-AMP using two release methods: the first was competitive displacement using a concentration gradient of ATP analyte (Fig. 7a); and the second was thermal energy (Fig. 7b). After each competitive ATP solution (800 µM and 3.2 mM) injection (10 µl), fluorescence intensity was measured. Five extraction and release cycles were performed (2 shown here) using thermal energy. Solution conditions and sampling using TO-AMP (400 nM) mimicked those employed in SPE experiments. Fluorescence measurements were taken after each capture and release wash step.

Competitive displacement was used for only one cycle since it proved less efficient at releasing TO-ATP than thermal energy. Notice how competitive displacement after 2 gradient injections cannot bring the fluorescence signal to the base-line value whereas temperature release accomplished this each cycle. While thermal cycling, the extraction signal in cycle 2 deviated by 16.7 percent from that recorded during cycle 1. It was also found that the transition temperature required to release TO-AMP from ATP-aptamer existed between a narrow range (80-85 ^{o}C). Initial attempts used 60 and 95 degrees centigrade while flowing buffer solution (2 min at 5 µl/min) to collect the released analyte; the later caused denaturation of streptavidin and damage of the streptavidin-biotin (SB) bond whereas the former did not unfold the aptamer/target bond. It was reported [16] that denaturation of the SB bind occurs in water at 70 ^{o}C. Damage to the SB bind would rid our device of its functionality, so extremely high temperature elution was avoided. However, for this study TO-AMP could re-bind to the affinity matrix despite using relatively elevated temperatures (80-85 ^{o}C), which reportedly can denature the SB bind. This suggests that the SB bind was still intact even at these elevated temperatures and may be more resilient than originally estimated. Further investigation is required to isolate the exact temperature for controlled release of TO-AMP for this system as well as resolve the SB denaturation temperature discrepancy between our current results and previously published findings by [16]. Since thermally induced regeneration consumed far less buffer (fewer washing steps) and bio-compounds (no added ATP), it proved far more economical and required less processing time.

IV. CONCLUSION

The preparation of an affinity interaction SPE bed on-chip offers distinct advantages over current on-line extraction/purification methods. High selectivity, fast processing times, and simple fabrication makes the presented device an attractive analysis tool in the biomedical and biochemical industries. It is apparent that the overall microchip design presented here is not optimized as a SPE device. However, it did demonstrate the benefits of high specificity analyte/matrix interaction and the potential as an efficient solid-phase concentrator. Solid-phase regeneration by thermal energy and a fabrication process tailored to standard microfabrication techniques primed our device for future integration of MEMS components such as on-chip sensors and heaters.

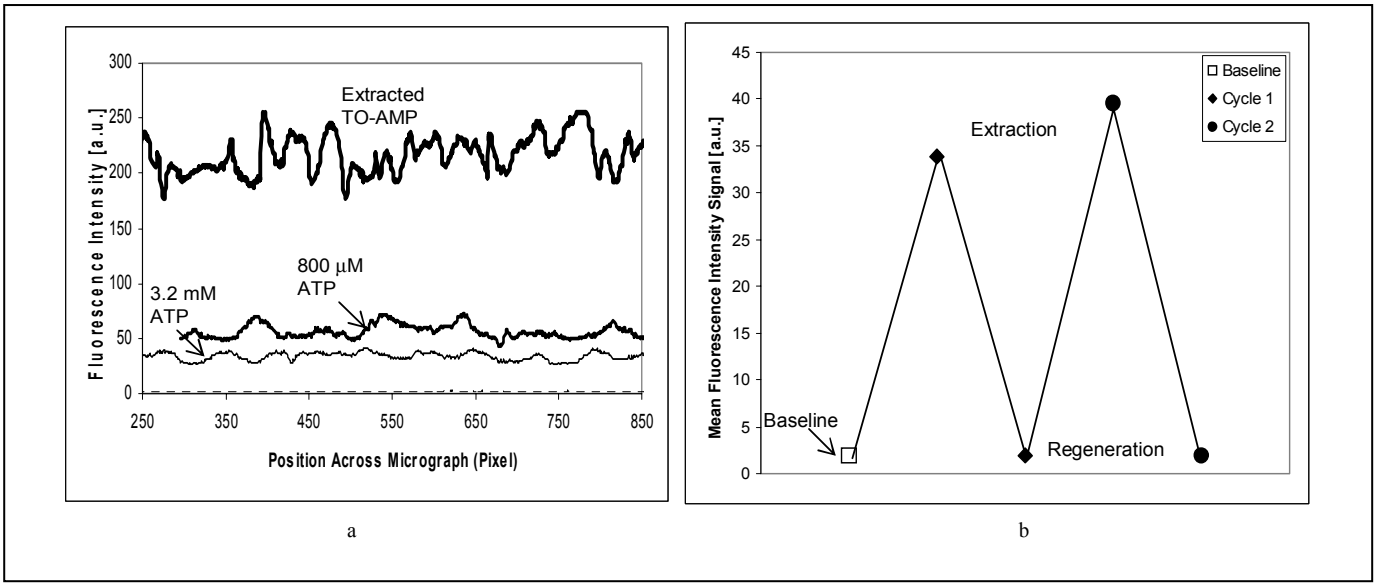

Figure 7. Controlled release of TO-ATP and regeneraion the SPE device (baseline colinear w/horizontal axis). Graph (a) shows competitive displacement with ATP (800 µM & 3.2 mM). Graph (b) presents thermally induced release and regeneration. Single-valued points were obtained similarly to time-resolved data.

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