AN INTEGRATED MICROFLUIDIC SYSTEM FOR AFFINITY EXTRACTION AND CONCENTRATION OF BIOMOLECULES COUPLED TO MALDI-MS

T.H. Nguyen[1], *C. Qiu*[2], *R. Pei*[3], *M. Stojanovic*[3], *J. Ju*[2], *and Q. Lin*[1]
[1]Department of Mechanical Engineering, [2]Department of Chemical Engineering, [3]Department of Medicine, Division of Experimental Therapeutics, Columbia University, New York, NY, USA

ABSTRACT

We present an innovative microfluidic device that accomplishes integrated, all-aqueous realization of specific extraction, concentration, and coupling to mass spectrometric detection of biomolecular analytes. The device uses an aptamer (i.e., oligonucleotide that binds specifically to an analyte via affinity coupling) immobilized on microbeads to achieve highly selective analyte capture and concentration. Here, we demonstrate specific extraction and concentration of adenosine monophosphate (model analyte) by approximately 100×, while providing a reusable platform via sufficiently low temperature release and regeneration of the aptamer surface at 38 °C.

1. INTRODUCTION

Presently, there is a strong desire to develop highly integrated biological analysis systems that can be used to perform general biochemical analysis. One important component in these systems is sample preparation, which involves extraction, purification and concentration (PC) of applicable analytes [1].

Preexisting systems for sample PC have generally employed solid-phase (SP) gels for retention of target molecules. A common shortcoming of SP systems is the indiscrimination of their capturing mechanisms. For example, hydrophobic [2] and ion-exchange [3] SP systems have the major limitation of extracting impure compounds with similar physical or chemical properties as the target. With applications in drug delivery or chemical assays, where specific molecules need to be released, introducing impurities can be problematic. In addition, elution of molecules using harsh pH or solvent gradients is common in SP systems. For certain biomedical applications, these elution schemes can present potential health hazards. An alternative retention media for specific PC systems is affinity stationary phases.

Affinity binding involves the reaction between a ligand and a specific receptor such as an antigen and antibody or, enzyme and substrate. The strong specificity stems from the ligand and receptor being ideally suited to one another both electrostatically and spatially. Additionally, ligand and receptor binding can be reversed by such stimuli as heat and ionic strength [4]. While antibody/lectin systems are the most common affinity pair, novel high-affinity aptamers derived from nucleic acid are drawing increased attention, since theoretically, they can be synthesized selectively towards any target molecule. Aptamers possess attractive advantages over antibody/antigen systems. They offer long-term stability, relatively straight forward synthesis, and the capability of modifiable end-chains to facilitate labeling or immobilization. Also, aptamers can reversibly bind to their targets within an aqueous environment, eliminating exposure of sensitive systems to harsh reagents [5].

Moreover, PC devices must release analytes effectively since sample preparation is naturally an initial step of a continuing process. A release step could prepare the analytes for an analysis stage, such as mass spectrometry. In addition, release of analytes enables surface regeneration, which allows for extensive long-term use if applicable. Here, we demonstrate PC of adenosine monophosphate (AMP), used as a model analyte, by an adenosine triphosphate aptamer (ATP-aptamer) on an integrated microfluidic device. The device is coupled to a matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) machine where AMP is analyzed.

2. PRINCIPLE AND DESIGN

Microfluidic Design

The device consists of a microchamber packed with aptamer-immobilized microbeads for analyte PC, a microheater and temperature sensor for thermally induced analyte release, and microchannels equipped with a passive valve using surface tension for spotting the released analyte onto a MALDI analysis plate (Fig. 1). Analyte and wash solutions are introduced via the sample inlet, while the matrix solution by the matrix inlet. The bead inlet facilitates packing the chamber with microbeads. A resistive heater and sensor are placed below the aptamer chamber to promote efficient heating and accurate sensing. The valve and deposition well are placed near the aptamer chamber to reduce analyte dilution after release due to adsorption to the channel walls or diffusion to dead fluid volumes.

The microfluidic chip structure is realized with three sandwiched polymer layers. L1 incorporates the inlets, passive valve, and waste outlet. To reduce bubble entrapment or dead volumes during sample spotting, L2 provides an air vent connected to the spotting well. It also encapsulates the fluidic network present in L1. L3 defines the spotting well and houses the air vent channel.

Passive Valving and Sample Spotting

During normal operation, AMP is introduced into the microchamber and extracted by the aptamer. A rinse follows to flush impurities through the waste outlet. For concentration, this procedure can be repeated to saturate AMP on the beads. Next, the microchamber is heated using the microheater to reverse the AMP/ATP-aptamer bond. This releases the analyte from the beads. In order to direct flow of a released AMP sample through a secondary channel leading to the spotting well, a valve based on surface tension is used.

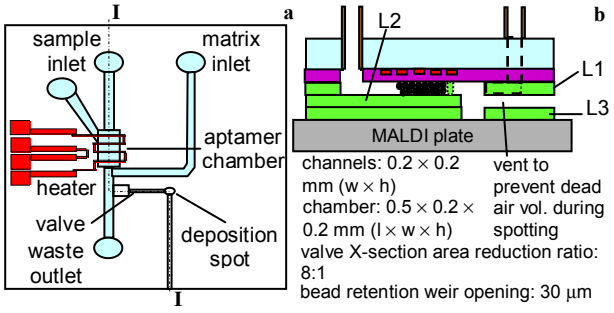

Figure 1. Device schematics. (**a**): *top view;* (**b**): **I-I** *cross-sectional view.* (*dashed line refers to hidden waste outlet.*) *Chip dimensions*: *2 × 2 × 0.16 cm* (*l* × *w* × *h*).

Passive microfluidic valves can employ surface tension for fluid regulation in low pressure systems. In principle, a hydrophobic channel generates a pressure difference across the air-liquid interface occurring within it, governed by a modified *Young-Laplace* equation (which includes the pressure drop induced by channel geometry) [6]:

$$\Delta p = 2\gamma\cos\left(\theta_c\right)\left[\left(\frac{1}{w_1}+\frac{1}{h_1}\right)-\left(\frac{1}{w_2}+\frac{1}{h_2}\right)\right] \qquad (1)$$

In Eq. 1, γ, θ_c, w, and h, are the surface energy, contact angle, width, and height of the channel, respectively at the air-liquid interface. This pressure drop allows the hydrophobic channel to act as a passive valve, and in our device, is used to regulate flow between two zones. Since the packed chamber is the primary flow resister in zone 1 (gray channel network in Fig. 1), a modified *Poiseuille* equation is used to determine its pressure drop [7]:

$$\Delta p = \frac{150\eta u\left(1-\varepsilon\right)^2 L}{d_0^2\varepsilon^2} \qquad (2)$$

Here, η, u, L, ε, and d_0 represent the dynamic viscosity, average fluid velocity, channel length, void fraction, and bead diameter, respectively. At a steady flow rate, the pressure difference imparted by the passive valve separate flow within zones 1 and 2 (white channel network in Fig. 1). During normal operation, where flow (10 µl/min) is directed through the waste outlet, the pressure drop in zone 1 is ~220 Pa (Eq. 2). To direct flow to the MALDI plate, the pressure drop in zone 1 must be greater than the valve's (1.7 kPa, Eq. 1). This is accomplished by plugging the waste outlet and maintaining a constant flow rate during deposition following thermally induced release of AMP from the aptamer. After sample spotting, the chip is removed from the MALDI plate for analysis.

3. EXPERIMENTAL

Materials and Equipment

Biotinylated ATP-aptamer is purified while AMP, cytidine, uridine, and guanosine triphosphate (C/U/G-TP) are synthesized. The matrix solution is prepared from 2,4,6-trihydroxy-acetophenone (2,4,6-THAP), 2,3,4-THAP, and diammonium citrate at 0.1, 0.05, and 0.075 M concentrations, respectively in a 3:5 (v/v) mixture of acetonitrile/water. Streptavidin coated agarose beads (~50 µm O.D.) provide support surfaces while a Voyager-DE time of flight mass spectrometer (Applied Biosystems) is used for mass analysis. DNA grade water is used in all experiments.

Device Fabrication

The device fabrication process is shown in Fig. 2. SU-8 molds for each microfluidic layer are first created, with which PDMS prepolymer is cast into an in-house built through-hole PDMS sandwiching jig and cured (60 °C, 8 h). Meanwhile, Cr/Au (5/100 nm) films are deposited, patterned, and passivated with SiO_2 on glass substrates, realizing the microheater and temperature sensor. Following plasma (O_2) treatment of each bonding interface, all three PDMS layers and the glass substrate are then aligned using optical microscopy and an x-y-z stage before permanently bonding them to each other consecutively. Finally, microbeads are packed into the aptamer chamber and the entire assembly is subsequently attached to a MALDI plate via spontaneous adhesion.

Experimental Procedure

The device is first rinsed with water (10 µl/min, 10 min). All following experimental washing and loading schemes are identical. ATP-aptamer is loaded (10 µM, 10 µl, 20 min) into the chamber to functionalize the bead bed. After a subsequent wash, a pure matrix mass spectrum (MS) is acquired for a negative control.

An arbitrary concentration of fluorescein solution is used to characterize the valving operation and sample spot characteristics. For valving experiments, solution is first flowed through zone 1 (10 µl/min) below the critical pressure of the valve. To operate the passive valve, the waste outlet is plugged while maintaining a constant flow rate. This increases the pressure in the flow stream adjacent to the valve to eventually overcome its critical pressure and actuate it. A 20× microscope objective is focused on the valve area during experimentation. To test sample spot characteristics, the waste stream is plugged while fluorescent solution is deposited from the chip using different flow rates (10-50 µl/min). Each spot is recorded and analyzed using a 20× objective.

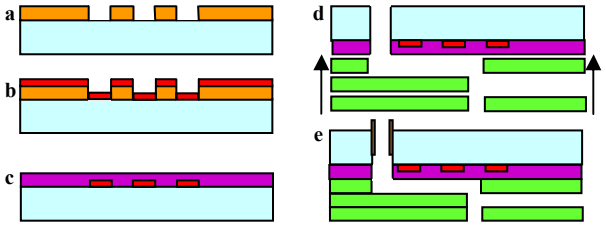

Figure 2. Fabrication flow as seen from cross-section **I-I** *in Fig. 1*: (**a**) *PR patterning for Cr/Au deposition*; (**b**) *thermal evaporation of Cr/Au bi-layer*; (**c**) *lift-off patterning of Cr/Au and PECVD deposition of* SiO_2; (**d**) *substrate drilled for fluidic ports and 3 through-hole PDMS layers aligned and permanently bonded*; (**e**) *packaged chip with tubing.*

For extraction/purification, 0.1 and 1.0 µM AMP samples are loaded into the aptamer chamber separately. A rinse follows to rid non-specific compounds. AMP is then released from the aptamer by raising the chamber temperature to 38 °C while introducing a matrix sample plug. The sample/matrix plug is then transferred to the spotting well and deposited onto the MALDI plate to be subsequently analyzed. Similarly, for specific extraction of AMP, a solution of AMP, CTP, UTP and GTP (1 µM) is loaded into the aptamer chamber. After an incubation (5 min) and wash step (to flush non-target molecules), matrix is loaded into the chamber and the heater is activated to release the molecules currently on the aptamer and deposit them onto the MALDI plate for analysis.

For preconcentration of AMP, a multiple injection scheme is used. The aptamer chamber is consecutively loaded with 10 nM injections of AMP sample. Each injection is incubated (5 min) and followed by a rinse. Upon suspected saturation of the aptamer with AMP, the chamber is heated to release the analytes into a matrix plug, which is deposited for analysis.

4. RESULTS AND DISCUSSION

In order to assure proper functionality of the valve, we regulate a fluorescence solution between zone 1 and 2 (Fig. 3). During normal operation, when the waste outlet is open, fluorescence solution bypasses the passive valve (Fig. 3a). Upon blocking the main outlet, we notice a gradual increase in fluorescence through the valve to the spotting outlet (Fig. 3b). This is due to increased pressure in zone 1, which overcomes the critical pressure of the valve to thus actuate it, and provide on-chip sample regulation.

Sample spot size is an important characteristic during MALDI analysis. Large volume spots can promote dissociation of matrix from sample upon spot crystallization, resulting in poor ionization. Additionally, non-uniformity in sample concentration throughout the spot can occur, degrading analysis. We measure spot size produced by our device as a function of driving flow rate (Fig. 4). For low flow rates (10-30 µl/min), spot sizes approximately equal to the well size is obtained (~500 µm). Higher flow rates (>40 µl/min) generate a larger spot diameter (~700-800 µm) since the seal between the PDMS and MALDI plate at the location of the spotting well tends to falter at the resulting higher pressures. Consequently, the sample spot broadens once the chip is removed from the plate to obtain a spot size.

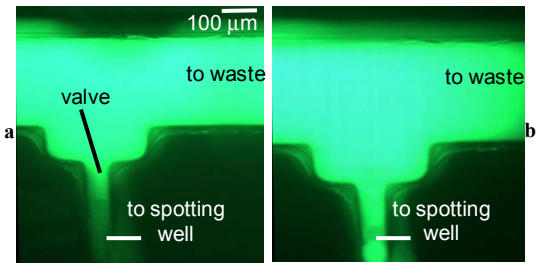

Figure 3. Operation of passive valve: (**a**) *fluorescein solution flow through the waste outlet bypassing valve*; (**b**) *valving fluorescein solution through valve.*

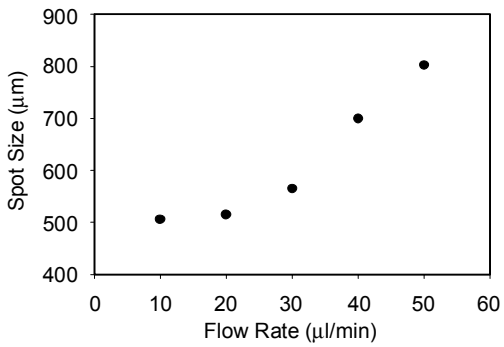

Figure 4. Spot size on MALDI plate as a function of flow rate used to transfer sample to deposition well.

However, this is of no detriment to the overall performance of the device compared to conventional spotting (with syringe or pipette), where crystallized spots are larger (>1 mm). Future work will address further decreasing the spot size to what is allowed by more established methods of MALDI sample spotting (e.g., electro-spray ionization).

To demonstrate AMP extraction by ATP-aptamer, we first inject two sample solutions of AMP (0.1 & 1.0 µM) into the chamber. AMP is released and deposited onto a MALDI-MS plate and analyzed (Fig. 5). The MS of a spot obtained from a 0.1 µM AMP solution (Fig. 5a) shows a distinctive mass peak of 348 Da, which corresponds to AMP (established value: 347.22 Da). Since AMP concentration is relatively low, the magnitude of this peak is comparable to several peaks from the MALDI matrix (338, 392, 468 & 502 Da). A mass spectrum obtained from a 1.0 µM AMP solution (Fig. 5b) improves the analyte-to-reference peak contrast. In this case, the AMP peak dominates reference peak amplitudes suggesting that concentrating dilute samples can improve analyte recognition.

Purification of analytes is a valuable tool for selectively controlling analytes in biochemical applications. We selectively extract AMP from a homogeneous solution of AMP, CTP, UTP, and GTP (1.0 µM each) by loading the sample into the aptamer chamber and subsequently washing the chamber to isolate AMP. A deposited sample spot is obtained similarly to previous protocol. Fig. 6 represents the MS of an analyte sample originating from the homogeneous solution. The ratio of AMP to noise is comparable to that seen in Fig. 5b, where only AMP is present in the solution. Additional non-target peaks are observed (483, 484, & 523 Da). However, their intensities are significantly lower than the AMP peak suggesting the amount of non-specific binding is negligible. This confirms the ability of our system to selectively extract and concentrate biomolecules for analytical applications.

As a sample preparatory technique, PC is useful for sample conditioning and analyte signal improvement. We test PC performance of our device by loading a dilute AMP sample into the aptamer chamber multiple times to saturate the analyte on the aptamer bed before release for MS analysis. Dilute sample concentration is chosen to be much lower (~0.01 µM) in order to highlight the detection enhancement due to PC. We inject 20 consecutive dilute

AMP samples into the aptamer chamber, release the captured AMP with heat, and transfer the concentrated plug to the spotting well. A MS is obtained from the resulting sample spot (Fig. 7a). We notice an AMP peak to noise ratio slightly higher than that seen in Fig. 5a, demonstrating the successful concentration of AMP by ~10×. This initial result does not reveal an intuitively anticipated 1:1 relationship between injection number and concentration factor (i.e., we do not see an AMP peak to noise ratio corresponding to 2× that of Fig. 5a). The relationship between injection number and concentration factor will be further investigated in future work.

More consecutive injections of dilute AMP solution are attempted to obtain the maximum PC factor of our device. A maximum of 200 injections are performed. Following the final injection, a sample spot is obtained and analyzed with MALDI-MS similar to the protocol with 20 injections (Fig. 7b). Notice that the AMP peak dominates those of reference peaks and the AMP peak to noise peak ratio is comparable to Fig. 5b. This suggests a PC factor of nearly 100×. This is a significant PC factor, comparable to that seen in reverse-phase systems [1], but with the advantage of higher specificity. An interesting point to highlight is that we stop AMP sample injections after 200 due to experimental practicality, not because of actual saturation of the analyte. This suggests the possibility for even larger PC factors with our system. The maximum PC factor will be investigated in future research where continuous injections will be used to facilitate experimental protocol.

5. CONCLUSIONS

Our device offers distinct advantages over current SP systems. We demonstrate specific PC, release and regeneration at 38 °C, and integration with MALDI-MS. With the possibility of over 100-fold preconcentration, this device makes an attractive sample preparation tool for biochemical applications.

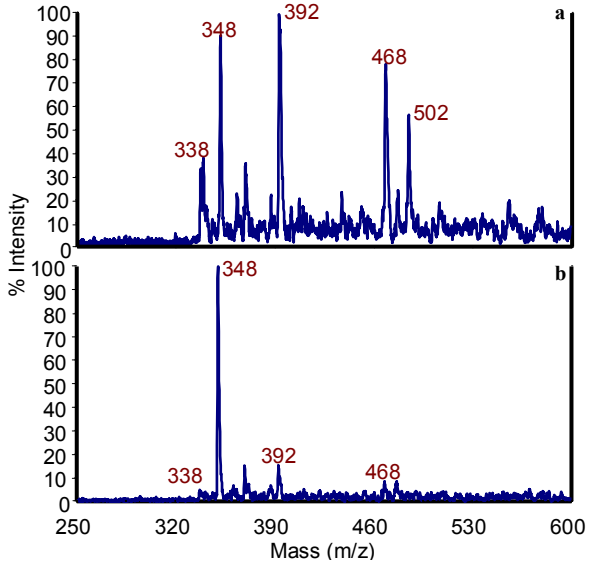

Figure 5. MS from (**a**) *0.1 μM AMP*; (**b**) *1.0 μM injected sample.*

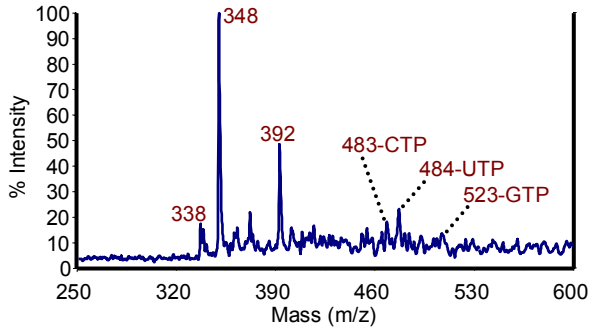

Figure 6. MS from an injected sample of AMP, CTP, UTP, & GTP (1 μM each) where AMP has been isolated.

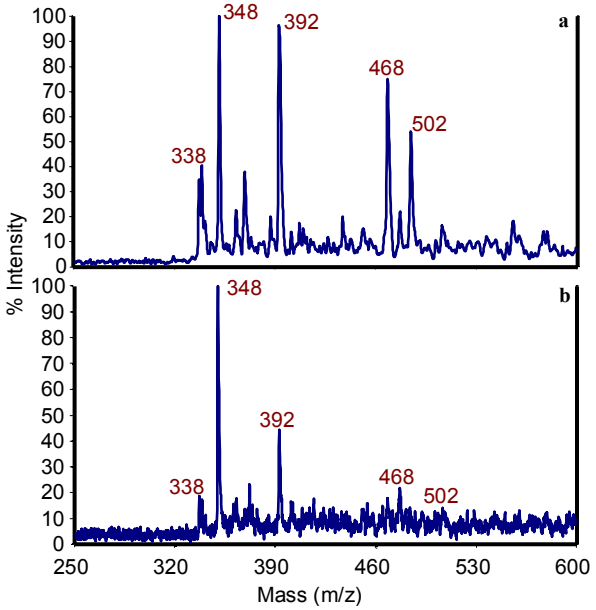

Figure 7. MS from a sample spot obtained from (**a**) *20 injections*; (**b**) *200 injections of 10 nM AMP solution.*

6. ACKNOWLEDGEMENTS

This research has been funded by the National Science Foundation (Grant #s CBET-0693274 & EIA-0324845).

7. REFERENCES

[1] N. Guzman and R. Majors, "New directions for concentration," *LC GC Europe*, pp. 1-9, 2001.

[2] R. Ramsey and D. Collins, "Integrated microfluidic device," *Anal. Chem.*, vol. 77, pp. 6664-6670, 2005.

[3] C. Yu et al., "Monolithic porous polymer," *Anal. Chem.*, vol. 73, pp. 5088-5096, 2001.

[4] V. Meyer, *Practical High-Performance*, John Wiley & Sons, Chichester, England, 1994.

[5] S. Jayasena, "Aptamers: an emerging class," *Clin. Chem.*, vol. 45, pp. 1628-1650, 1999.

[6] Y. Feng et al., "Passive valves based on," *Sens. Actuators, A*, vol. 108, pp. 138-143, 2003.

[7] R. Darby, *Chemical Engineering Fluid Mechanics*, Marcel Dekker, New York, 2001.