A MICROFLUDIC APTASENSOR WITH INTEGRATED SAMPLE PRECONCENTRATION, ISOCRATIC ELUTION AND MASS SPECTROMETRIC DETECTION

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ABSTRACT

We present a microfluidic biosensor suitable for selective detection of analytes with integrated analyte preconcentration, isocratic elution and mass spectrometric detection. 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 arginine vasopressin (a peptide hormone) by a vasopressin specific atpamer. In addition, the aptasensor is capable of isocratic elution and microbead regeneration via thermally-induced reversibility of the aptamer-analyte binding mechanism, which renders the aptamer functionalized microbeads functional for repeated use. Furthermore, a microvalve directs the analyte onto an analysis plate for label-free detection by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).

KEYWORDS

Aptamer, aptasensor, preconcentration, MALDI, isocratic elution, temperature-dependent binding

INTRODUCTION

Micro- and nanofabrication has allowed the production of ultra-sensitive, portable, and inexpensive biosensors. The potential of this technology been increasingly realized in medical and biochemical fields, where such devices have been utilized for diagnostics, therapy, and sample preparation [1]. For example, diagnostics devices are generally employed to recognize the presence of a condition specific biomarker to enable accurate prognoses. Identification as well as thorough investigation of this biomarker may prove vital, at an early stage, in treating it successfully. Unfortunately for medical practitioners, disease related biomarkers are either physiologically present in minute quantity or severely contaminated by non-specific compounds in a patient's bloodstream or body fluid. Hence, highly sensitive as well as specific biosensors are required for effective detection of such biomarkers.

Biosensors have traditionally used affinity receptors to overcome non-selectivity and poor affinity in analytical applications. Antibody- and enzyme-based sensors are most common, but have limitations. Enzyme sensors require auxiliary reagents and/or separation steps [2], while antibody sensors exhibit limited shelf life and labeling degradation. Aptameric biosensors (aptasensors) based on RNA and DNA aptamers (synthetic oligonucleotides) can alleviate these problems. Using an in-vitro generation protocol, they can be engineered for bio-specificity toward possibly any target molecule that binds nucleic acid. In addition, aptamers offer room temperature stability, easy immobilization to surfaces and controlled functionality [3].

Here, we exploit aptamers for the selective concentration and detection of arginine vasopressin (AVP). This particular peptide hormone has been indicated for the diagnosis and treatment of immunological shock (hypotension and tissue death from inadequate perfusion) caused by hemorrhagic or infectious (septic) shock [4]. Standard methods of detecting AVP include fluorometry or ELISA which require time-consuming or complicated labeling of molecules. To address these limitations, our approach is based on a microfluidic platform that integrates specific preconcentration, isocratic elution, and label-free mass spectrometric detection for selective analyte biosensing and diagnostics [5]. The device uses aptamer functionalized microbeads to achieve highly selective analyte capture and enrichment. Additionally, the device exploits the thermally-induced aptamer-analyte reversibility to release the analyte, achieving isocratic elution and microbead regeneration. Furthermore, a microvalve directs the analyte onto an analysis plate for label-free detection by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Label-free detection reduces experimental time, which is significant for clinical applications.

PRINCIPLE AND DESIGN
Microfluidic Design

The aptasensor exploits a microfluidic platform that has been described elsewhere (**Fig. 1**) [5]. Briefly, the platform consists of a microchamber with aptamer-functionalized microbeads for analyte extraction and detection, a microheater and temperature sensor for thermally induced analyte release, and microchannels equipped with a surface-tension based valve for analyte transfer to MALDI-MS analysis. Three sandwiched polymer layers generate the device structure: L1 incorporates the inlets, passive valve, and waste outlet. To reduce bubble entrapment or dead volumes, L2 provides an air vent connected to the spotting outlet. L3 defines the spotting well and houses the air vent channel.

During operation, an analyte sample is extracted inside the aptamer microchamber via the sample inlet, while impurities are flushed away. Repeating this process concentrates the analyte. A resistive heater and sensor are placed below the aptamer chamber to promote efficient heating and accurate sensing during isocratic analyte elution. Thermally-induced isocratic elution also regenerates the device for subsequent samples. Fluorescence microscopy is used to detect a fluorescently tagged analyte, while an unlabeled analyte is detected using MALDI-MS via microfluidic routing to the deposition outlet provided by the surface-tension based valve [5].
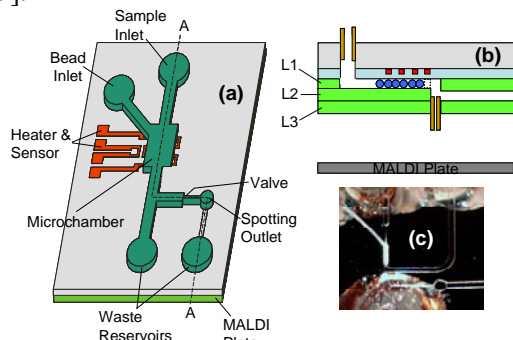

Fig. 1. *Schematic of device (a) isometric view (b) cross-section along the dashed line A-A in (a). (c) A fabricated aptasensor.*

EXPERIMENTAL
Materials and Equipment

Biotinylated AVP-aptamer is acquired through Integrated DNA Technologies. Tamra-AVP (TMR-AVP) and free AVP are purchased from American Peptide, whereas thiazole-orange adenosine monophosphate (TO-AMP) is synthesized in-house. Analyte samples are prepared in sterile water (Fisher). Streptavidin-immobilized polystyrene beads (50-80 μm in diameter) are acquired from Pierce. SU-8 2025 and 2100 (MicroChem), polydimethylsiloxane (PDMS), Torr Seal epoxy, and microscope grade glass slides (25 mm×75 mm) are purchased from MicroChem, Dow Corning, Varian, and Fisher, respectively. A Nikon Eclipse TE300 is utilized for fluorescence detection, while a Voyager-DE time of flight mass spectrometer (Applied Biosystems) is used for mass analysis.

Fabrication

Fabrication involves previously described negative soft lithography methods [5] (**Fig. 2**). Briefly, PDMS sheets for each microfluidic layer are first created. Meanwhile, Cr/Au (5/100 nm) films are deposited on glass substrates, realizing the microheater and temperature sensor. All three PDMS layers and the glass substrate are then aligned and permanently bonded. Finally, microbeads are packed into the aptamer chamber and the entire assembly is positioned on a manual x-y-z stage.
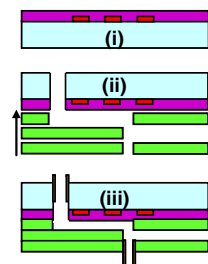

Fig. 2. *Device fabrication: i: patterning and passivation of resistive heater & sensor; ii: aligning and bonding PDMS microfluidic layers; and iii: packaging.*

Experimental Procedure

Microfluidic devices are initially rinsed thoroughly (10 μl/min) with water for 30 minutes (similar for subsequent rinses in all experiments). Sample solutions in varying concentrations of TMR-AVP, TO-AMP, and AVP-aptamer are prepared using the appropriate mass weights of the respective compound and water solution. Manual pressure is utilized to pack microbeads from the bead introduction channel of each device into the extraction chamber. Subsequently, this channel is sealed permanently. After another rinse step, an AVP-aptamer solution (20 μM) is injected (3 μl, 10 μl/min) and allowed to incubate (40 min) in the chamber. (This procedure is used for all sample injections.) Following a final rinse of each device, a baseline fluorescence signal is acquired by focusing a 10× objective at a specific location of the extraction chamber and averaging an 8-bit RGB signal over the entire recorded fluorescence image. MALDI-MS experiments utilized a device bonded (spontaneous adhesion of PDMS) to a MALDI plate.

RESULTS AND DISCUSSION
Device Characterization

Using the aptasensor, we analyze AVP with an AVP-specific RNA aptamer. Fluorescently labeled TMR-AVP (peak absorption: 540 nm; peak emission: 580 nm) is used for systematic device characterization. First, we obtain a time-resolved fluorescence response after introducing a TMR-AVP sample into the aptamer microchamber. Hence, fluorescence micrographs are taken at discrete time intervals (5 s) following an injection of TMR-AVP in varying concentrations (0.01, 0.1, and 1 μM). To reduce the effect of fluorescent photobleaching, the shutter to the mercury lamp is closed for the time period between all signal measurements. Fluorescence signal measurements are obtained as described in the experimental procedure, averaged and then plotted as a function of time (**Fig. 3**). In all experiments, no appreciable increase in fluorescence intensity occurred after ~22 s of incubation time, delineating a ~10s binding time. This is approximated from the reaction time constants for each experimental concentration sample (0.01, 0.1, and 1 μM) being between 8 and 12 s. Subsequent experiments take this into

consideration while determining a suitable incubation time for a TMR-AVP sample within the microchamber before fluorescence is measured. This information also proves useful in establishing flow rates for experiments involving continuous preconcentration of TMR-AVP (below).
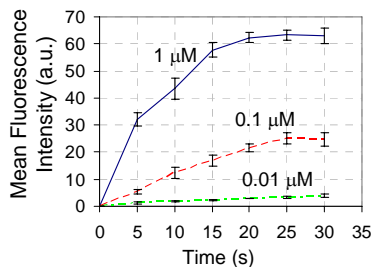

Fig. 3. *Binding time for TMR-AVP.*

The specificity of the aptamer to AVP is determined by observing the device responses (with/without aptamer functionalized beads) to TMR-AVP and fluorescently labeled TO-AMP (peak absorption: 480 nm; peak emission: 530 nm), a model impurity (**Fig. 4**). For example, samples of TMR-AVP and TO-AMP (1 µM) are injected into the microchamber under two conditions. Firstly, the chamber is packed beforehand with non-functionalized beads (only streptavidin-coated polystyrene: "bare beads"). The second condition utilizes beads functionalized with AVP-aptamer ("AVP-specific aptamer"). Non-functionalized surfaces produced negligible fluorescence after TO-AMP/TMR-AVP introduction. However, functionalized beads produced strong fluorescence only for TMR-AVP indicating good specificity between AVP-aptamer and AVP.

Extraction and Detection of TMR-AVP

To demonstrate aptamer-based capture and fluorescence detection of AVP, solutions of TMR-AVP at six different concentrations (0.001, 0.01, 0.1, 1, 10 and 1000 µM) were injected into the microchamber. For each sample introduction, fluorescence yield was quantified after an initial 30 s incubation time. Following the extraction of analytes, the chamber is washed with water to rid all non-specific compounds, un-reacted molecules, and impurities. Results are presented in **Fig. 5**. Below 1 nM, no significant signal above the background is detected. According to the data, TMR-AVP can be resolved at 1 nM (S/N ~ 3) with a dynamic range of 6 orders.
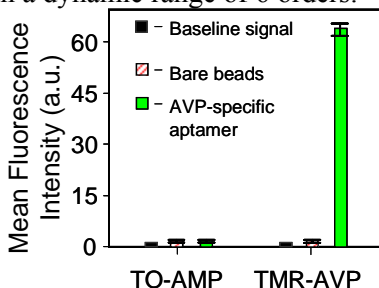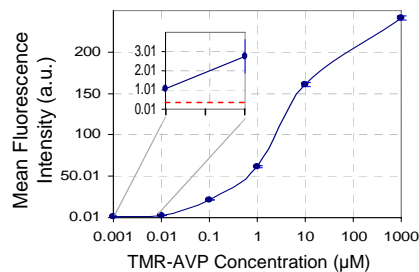

Fig. 4. *Selectivity of AVP-aptamer system.*

Fig. 5. *Single injection extraction and fluorescence detection of TMR-AVP. Graph inset shows a blow-up view of 1 nM & 10 nM data (dashed red line: baseline signal).*

Preconcentration of TMR-AVP by Continuous Infusion of a Dilute Solution

We next investigate preconcentration of TMR-AVP using continuous infusion of a dilute sample (**Fig. 6**). Here, sample enrichment of a trace TMR-AVP sample (100 pM) occurs by continuous infusion of the dilute solution into the microchamber until there is observed fluorescence saturation. Taking into consideration the required residence time determined above, we choose a conservative flow rate of 1 µl/min to insure complete analyte-aptamer interaction, although 25 µl/min is possible. Fluorescence signals are obtained periodically (every 70 min). After approximately 280 min, we notice no observable increase in fluorescence suggesting saturation. This corresponds to the apparent fluorescence intensity of TMR-AVP equal to that of a single injection of 0.1 µM TMR-AVP solution, suggesting an analyte concentration factor $\sim 10^3 \times$, which suggests a TMR-AVP resolution of ~1 pM (with S/N ~ 3) is possible with potentially improved hardware.
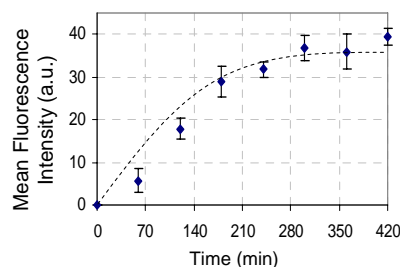

Fig. 6. *Concentration of a 100 pM dilute TMR-AVP sample by continuous injection.*

Thermally Activated Release of TMR-AVP

In following, the temperature-dependant reversibility of our aptasensor is studied. To demonstrate this, a 1 µM TMR-AVP solution is first extracted. The captured TMR-AVP is then released by heating the surface to an elevated device temperature (**Fig. 7**). After extraction of TMR-AVP on the aptamer surface, a high intensity fluorescence signal is initially obtained. The temperature on-chip is increased to a predetermined setpoint and held for 2 min. We repeat the experiment for several setpoint temperatures (32-60 °C). A sharp decrease (93%) in signal intensity

occurs at 50 °C until fully suppressed at 58 °C, indicating nearly complete release of the captured TMR-AVP. This establishes the capability of our aptasensor for thermally activated release and isocratic elution of a captured target analyte. Moreover, thermal regeneration using this technique is demonstrated with repeated extraction and release cycles (5) at 58 °C of 1 µM TMR-AVP samples (**Fig. 8**). The fluorescence signals resulting from TMR-AVP extraction and release in all subsequent cycles are comparable to those obtained in the first cycle. This indicates that the thermal stimulation did not affect the functionality of the aptamer molecules and successfully allowed aptasensor regeneration
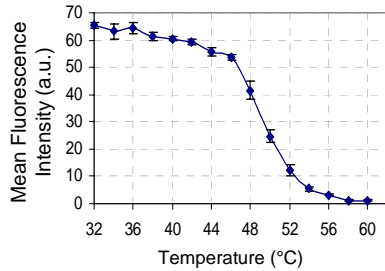

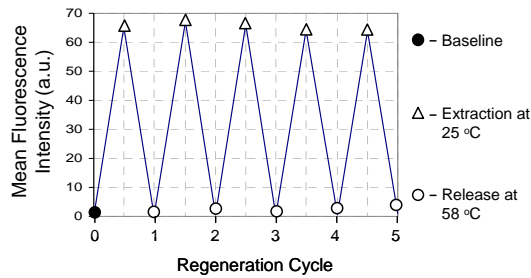*Fig. 7. Thermally induced release of TMR-AVP.*

Fig. 8. Thermal release allows the aptamer surface to be regenerated for multiple extraction-release cycles.

Label-Free Detection of AVP with MALDI-MS

While using fluorescence for device characterization, we further investigate label-free detection of AVP using MALDI-MS. Here, temperature-dependent aptamer-analyte binding allows isocratic elution and MS detection of unlabeled AVP. Following extraction of a range of AVP samples (10 & 100 pM; 0.001 & 1 µM), the microchamber is heated (58 °C) to transfer the released AVP to the deposition outlet via the surface tension-based valve for MALDI-MS. A distinct spectral peak for AVP can be observed for concentrations approaching 10 pM with S/N > ~3 (**Fig. 9**). Thus, when samples are also enriched in the device (see **Fig. 6**), we anticipate AVP detection at three-orders-of-magnitude lower concentrations (~10 fM). Hence, the realization of ultrasensitive, label-free detection of AVP for clinical shock diagnosis and treatment is possible.
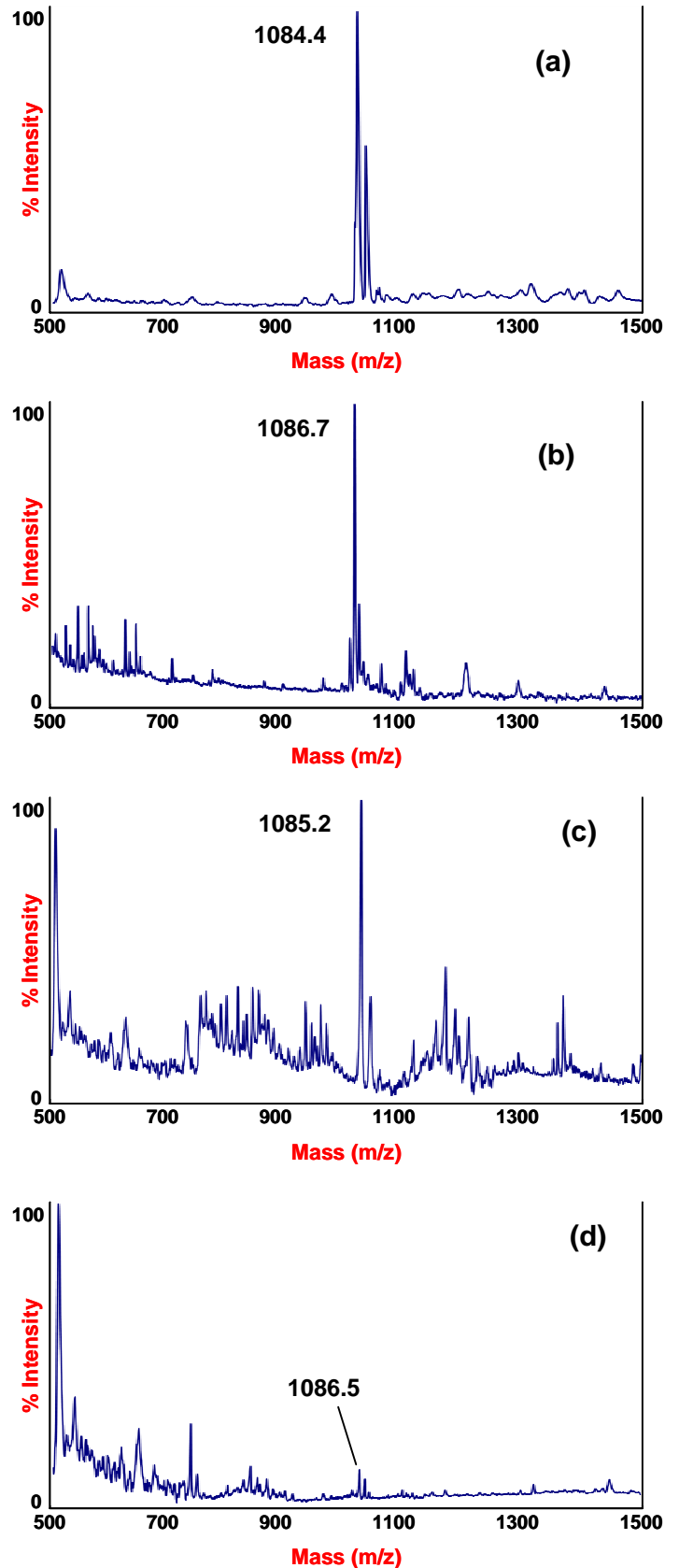

Fig. 9. MALDI-MS spectra: (a) 1 mM AVP dominant peak is 1084.4 Da; (b) 1.0 nM AVP; (c) 100 pM AVP; (d) 10 pM AVP

CONCLUSIONS

We present an aptasensor capable of selective preconcentration, isocratic elution and MALDI-MS detection of analytes. This system offers sensitive detection of AVP and demonstrates a promising practical shock indicating biosensor.

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