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A microfluidic affinity sensor for the detection of cocaine

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ABSTRACT

We present a microfluidic aptamer-based biosensor for specific cocaine detection. The device consists primarily of a microchamber packed with aptamer-functionalized microbeads that act as a sensing surface, integrated with an on-chip heater and temperature sensor. The sensor employs a Förster resonance energy transfer (FRET) system in which a fluorophore-quencher pair of carboxyfluorescein and Dabcyl generates a signal-on response to cocaine. We demonstrate device operation by successfully detecting cocaine with a four orders of magnitude linear response in micromolar to nanomolar concentrations. The detection limit of the device is further lowered to 10 pM by concentration of a highly diluted cocaine sample, which compares well with the most sensitive detection techniques currently available. The temperature-dependent binding of aptamer–analyte complexes is then used to effect thermal release of cocaine from the sensing surface. It is found that a temperature of 37 °C can fully regenerate the sensor in pure buffer. Furthermore, testing indicated that sensor response is consistent after repeated regeneration. These results demonstrate that aptamer-based sensing on a microfluidic platform has the potential to enable low-cost, rapid, and highly specific detection of cocaine in practical applications.

1. Introduction

The detection and quantification of trace amounts of illicit substances such as cocaine continues to be important for law enforcement and clinical medicine. Law enforcement officials must be able to detect trace amounts of cocaine in probationary offenders, and larger concentrations concealed by suspected users employing masking agents. In clinical medicine the detection of street drugs in patients of pain management practices requires frequent testing [1] that can be prohibitively expensive. Conventional methods available for cocaine detection include chromatography, presumptive testing, and immunoassays. Among these, gas chromatography–mass spectrometry (GC–MS) continues to be the gold standard in illicit material detection [2] given its sensitivity and reliability. Other chromatographic techniques, such as liquid chromatography (LC) and thin-layer chromatography (TLC) are also commonly used. In GC a sample of analyte is pushed through a column of adsorbent media, separating the constituents. LC systems are very similar, using a liquid as the carrier fluid instead of a gas, and TLC draws fluids across an adsorbent using capillary action. Unfortunately, GC–MS is inherently expensive, requiring a great deal of time, complicated equipment, and trained personnel. LC is typically even more expensive, and along with TLC gives poorer results due to its limited separation efficiency [3]. A more time-efficient alternative to chromatographic methods is presumptive testing using colorimetric reagents that rapidly react with a number of chemicals, but this approach is limited by a lack of specificity in that the reagents react non-specifically with target analytes [4,5]. To address the limitations of conventional techniques for detecting cocaine such as non-specificity and expense, the use of affinity sensing is highly attractive.

In affinity sensing, a target analyte is recognized by affinity binding with a receptor. That is, the analyte and receptor molecules join via specific hydrogen bonds, stacking of moieties, and analyte-induced receptor conformational changes which result in highly specific and reversible binding. Commonly used affinity receptors include antibodies, lectins, enzymes, and in particular, aptamers [6]. Aptamers are single stranded DNA and RNA oligonucleotides specifically selected for their binding affinity towards a specific target molecule [7]. Exposure to a target molecule induces conformational changes in aptamer structure facilitating binding that is both highly specific and reversible. Aptamers are easily chemically modified and are generated *in vitro*, resulting in no batch-to-batch variation in binding efficiency. As such, aptamers are attractive affinity receptors for cocaine detection. For instance, colorimetric sensing of cocaine was reported using aptamers conjugated to gold nanoparticles [8,9]. Additionally, UV absorption spectroscopy combined with aptamer-based solid-phase extraction was used to detect cocaine at micromolar concentrations in complex media on a large-scale liquid chromatography platform [10]. Cocaine sensing

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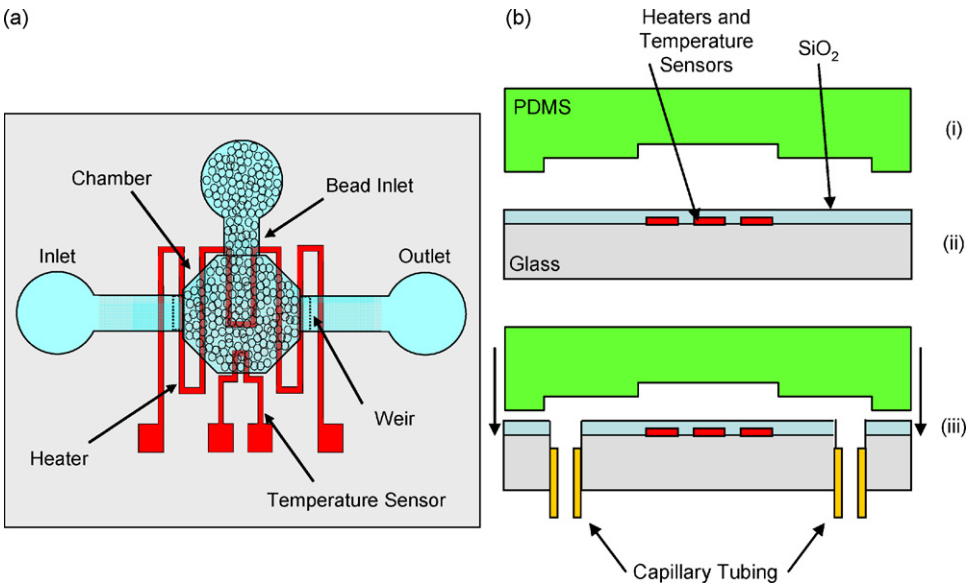

Fig. 1. (a) Schematic of the microfluidic cocaine sensor, and (b) fabrication process: (i) microfluidic features are defined in PDMS, (ii) heaters and temperature sensors are fabricated on a glass slide, (iii) the PDMS and glass slide are bonded together.

using aptamers has also been demonstrated using Raman scattering methods, in which aptamers bound to a silver colloid surface and labeled with a Raman reporter allowed detection of micromolar quantities of cocaine [11]. Furthermore, cocaine binding with a surface-immobilized aptamer can be measured via reduction of an electroactive species tethered to the distal end of the aptamer. This has allowed cocaine detection at the micromolar level using electrochemical methods [12], or with 1 nM detection limits using electrochemiluminescent methods [13]. Implemented on conventional platforms, these aptamer-based cocaine sensing approaches generally involve large reagent consumption, require bulky equipment, and are labor-intensive. These limitations hinder the realization of cocaine detection in portable, user-friendly systems.

Miniaturization technology holds potential for rapid, sensitive detection of cocaine with minute sample quantities. A microfluidic adaptation of a standard presumptive test for cocaine was fabricated, consisting of a microchannel into which was deposited a small amount of cobalt thiocyanate [14]. While still limited by conventional presumptive testing, the microfluidic device led to reduced sample consumption and allowed for device regeneration. In a different approach, a microfluidic sensor based on antibodies attached to a quartz crystal microbalance demonstrated rapid detection (<1 min) of nanograms of cocaine [15]. However, the use of antibodies makes the sensor regeneration difficult, and may also lead to batch-to-batch variations in sensor performance. More recently, a microfluidic continuous-flow aptasensor was reported that uses an electrochemical method for the detection of cocaine among other compounds in blood serum-based samples [16]. Micromolar detection limits were demonstrated.

This paper presents a microfluidic aptamer-based biosensor for cocaine that uses a cocaine-specific DNA aptamer modified with a fluorophore. Based on a device architecture designed for highly specific solid-phase extraction [17,18], this sensor is capable of low-cost quantitative cocaine detection in a highly specific, signal-on, and label-free manner. We demonstrate that the device is capable of specifically detecting cocaine at concentrations as low as 10 pM, with a linear response over four orders of magnitude. Additionally, the device can be conveniently regenerated for reuse by a modest temperature change via on-chip temperature control. As such, the device can potentially be used for sensitive and rapid detection of cocaine in practical clinical and law enforcement applications.

2. Principle and design

The device consists of a microfluidic chamber and channels fabricated from (poly)dimethylsiloxane (PDMS) and bonded to a glass substrate integrated with a resistive micro-heater and temperature sensor (Fig. 1a). The inlet and outlet microchannels are utilized for sample and buffer introduction and removal from the device, while an auxiliary microchannel is used for bead packing. Microweirs (15 μm high) separate the microchamber (130 μm high) from the inlet and outlet microchannels and serve to retain the beads in the microchamber. The on-chip microheater has a serpentine layout and directly covers the microchamber area to facilitate uniform heating of the microchamber area. A resistive temperature sensor is placed at the center of the microchamber to provide accurate temperature measurement for thermal control. The device was fabricated using soft lithography and lift-off techniques (Fig. 1b). A mold was fabricated from the negative photoresist SU-8, and a 10:1 mixture of PDMS pre-polymer and curing agent was cast over the mold to fabricate microchannels in a PDMS slab. This was bonded to a glass slide with integrated heaters and sensors. Details of the fabrication process are described elsewhere [18].

A Förster resonance energy transfer (FRET) system is utilized to signal cocaine binding to the aptamer. FRET systems involve the coupling of a fluorescent molecule that emits visible light (fluorophore) to another fluorescent molecule that absorbs visible light and emits at invisible wavelengths (quencher). We use carboxyfluorescein (FAM) as a visible-range fluorophore, which, attached to a cocaine-specific aptamer (below), is characterized by a peak absorption wavelength of 494 nm and a peak emission wavelength of 518 nm [19]. Dabcyl is employed as a quencher molecule, which absorbs light over a wide range of wavelengths and dissipates the light as infrared energy. When placed in close proximity to FAM, Dabcyl causes a drastic reduction in visible emission during excitation at 494 nm. To exploit the FRET system within the microfluidic device for aptamer-based cocaine detection, we employ a DNA aptamer that is adapted from the sequences originally obtained for free solution aptamer–cocaine binding [20]. A FAM molecule is attached to the 5′ end of the aptamer, and a biotin molecule at its 3′ end. This allows the aptamer to be immobilized onto a streptavidin functionalized surface via biotin–streptavidin interaction with the FAM molecule at the free end (Fig. 2a). In order to establish a baseline signal, a Dabcyl molecule, attached to the

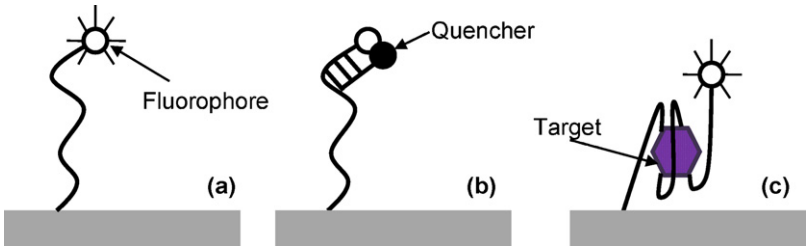

Fig. 2. Signal-on aptamer-based cocaine detection. (a) A fluorophore-labeled aptamer is immobilized onto a solid surface. (b) A quencher-labeled short complimentary strand is introduced, decreasing fluorescence. (c) Unlabeled cocaine binds competitively to the aptamer, displacing the quencher and reducing fluorescence.

3′ end of a short DNA strand complementary to the aptamer, is introduced. This strand hybridizes to the distal end of the aptamer (Fig. 2b), bringing the FAM fluorophore and Dabcyl quencher into close proximity. Thus, the baseline consists of a drastically reduced level of fluorescence. Upon introduction of unlabeled cocaine (i.e., cocaine molecules not altered by any labeling group for transduction), the aptamer selectively and conformably binds to the cocaine, which disrupts the interaction of the relatively weaker binding quencher strand, as shown in Fig. 2c. The displacement of the Dabcyl molecule results in a recovery of fluorescence (i.e., a signal-on response) in proportion to the concentration of cocaine. In this manner, the device accomplishes signal-on, label-free detection of cocaine.

3. Experimental

3.1. Materials and instrumentation

DNA was obtained from Integrated DNA Technologies (IDTDNA), Coralville, IA, in lyophilized form. The aptamer is of sequence 5′-FAM-A TCT CGG GAC GAC AGG ATT TTC CTC AAT GAA GTG GGT CGT CCC-Bio-3′, and the quencher-modified short complimentary strand is of sequence 5′-GTC GTC CCG AGA T-Dabcyl-3′. A stock concentration of each strand at 100 μM concentration was created using buffer solution consisting of pH 7.4 Tris–HCl, 150 mM NaCl, 5 mM KCl and 2 mM $MgCl_2$. Stock solutions were then diluted and aliquoted prior to testing. Ultralink Streptavidin-coated microbeads (80–120 μm in diameter) consisting of a copolymer of bis-acrylamide and azlactone were obtained from Thermo Scientific Pierce Protein Research Products. Microfabrication materials such as SU-8 2050, 2100 (MicroChem) and S1818 (Microposit), Sylgard 184 PDMS (Dow Corning), glass microscope slides (Fisher Science), and glass capillary tubing (Polymicro Technologies) were purchased from Microchem, Robert McKeown, Fisher Science, and Polymicro Technologies respectively. Fluorescence measurements were conducted on a Nikon Diaphot 300 Inverted Fluorescence Microscope coupled to a CCD digital camera (Micrometrics 190CU) and a Nikon HB-10103AF light source. Thermal measurement and feedback were conducted using a LabView program incorporating a proportional-integrative-derivative (PID) controller, 5½ digit NI PCI-4060 PCI digital multimeter card, and Agilent E3631A Power Supply. A New Era Pump Systems syringe pump model NE-1000 was used for pneumatic flow actuation.

3.2. Experimental protocol

The temperature dependence of resistivity of the temperature sensor was characterized using an environmental chamber, where the internal temperature was monitored with a platinum resistance temperature detector (RTD) probe (Hart Scientific 5628) and benchtop digital multimeter (Agilent 34420A). We observed that the temperature sensor resistance exhibited a linear response over the experimental temperature range. A temperature coefficient of resistance for the sensor can be calculated using equation (1).

$$R_{ref}\left[1+\alpha(T-T_{ref})\right]=R \quad (1)$$

Following thermal characterization, the microbeads were introduced into the biosensor prior to experimentation. A buffer diluted sample of microbeads was pumped through the bead inlet until complete packing was achieved. This inlet was then sealed and the sensor placed in an enclosure on the microscope stage to block ambient light. A 10 μL sample of 2 μM aptamer solution was then introduced into the microchamber at 2 μL/min via the solution inlet. Following incubation and a wash with 10 μL of pure buffer, this step was repeated. This procedure was then repeated with 2 samples of 5 μM quencher solution. In every such procedure, two samples of solution (aptamers or quenchers) were used to ensure adequate and uniform coating of the microbeads. Images were taken after each wash with buffer solution to provide baseline fluorescence readings. When testing the response of the sensor to a concentration of cocaine, similar procedures were used for sample introduction with the exception that one sample of cocaine was used, followed by a wash in pure buffer. When testing the ability of the sensor to concentrate cocaine, the subsequent buffer wash was omitted.

After each introduction, incubation and washing procedure involving aptamers, quenchers, or cocaine, the sensor was briefly (<5 s) excited with blue light using the fluorescent light source. Image acquisition was accomplished by opening the microscope shutter to the fluorescent light source and exciting a darkfield image of the transparent sensor. Resulting fluorescent emissions were captured by the inverted microscope and recorded by the attached Micrometrics CCD camera and associated software. Digital photographs were then analyzed using ImageJ software, brightness intensity was measured, and these values were used to calculate values of relative fluorescence. The sensor was otherwise kept in a dark environment to minimize the effect of photobleaching.

4. Results and discussion

4.1. Device calibration

Prior to testing with cocaine detection, the response of the on-chip temperature sensor and the effect of photobleaching on the system were characterized. Measurements show that the resistance of a typical temperature sensor varies linearly with temperature in the desired experimental range (Fig. 3) with a temperature coefficient of resistance of approximately $2.2\times10^{-3}\,^{\circ}C^{-1}$. The effect of photobleaching on the biosensor was then tested with an aptamer-functionalized bead packed microchamber (Fig. 4). Over a time period representative of our work, the signal changes by approximately 4%, a negligible rate of decay. These results indicate that photobleaching can be eliminated as a potential source of signal drift during our cocaine sensing experiments.

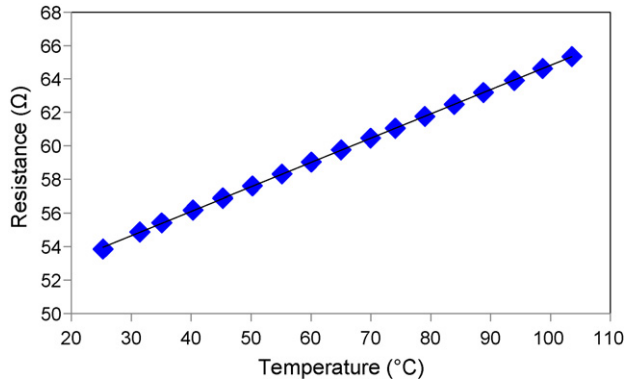

Fig. 3. Measured electrical resistance of the temperature sensor as a function of temperature.

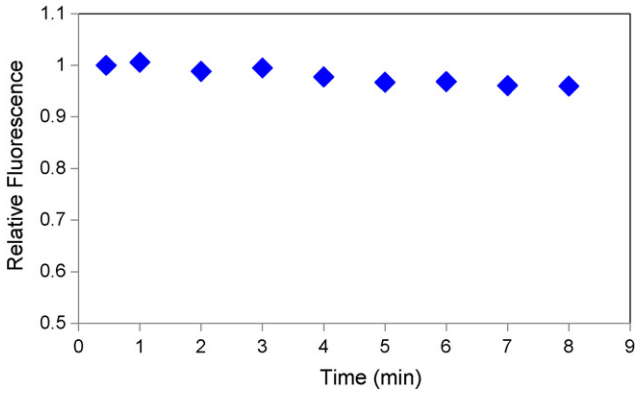

Fig. 4. Effect of photobleaching on the relative fluorescence over time.

4.2. Transient response

For accurate fluorescence measurements, the aptamer-binding system must have reached equilibrium prior to exciting the fluorophores and acquiring a signal. To ensure this was achieved during experimentation, the transient response of the biosensor following introduction of all sample types (aptamers, quenchers, and cocaine) was characterized. The transient fluorescent response to addition of aptamers was found to be near instantaneous (data not shown). To test the transient response of the quenchers, a standard 5 μM sample of quencher solution was introduced and fluorescence response was measured intermittently over a period of 8 min (Fig. 5). The transient response of the biosensor to quencher introduction was slower than that of aptamer introduction, with full quenching occurring within 8 min. This time response is most likely

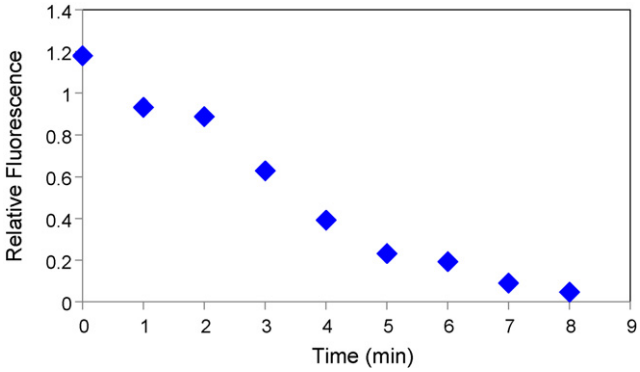

Fig. 5. Transient response of the sensor to introduction of quencher solution.

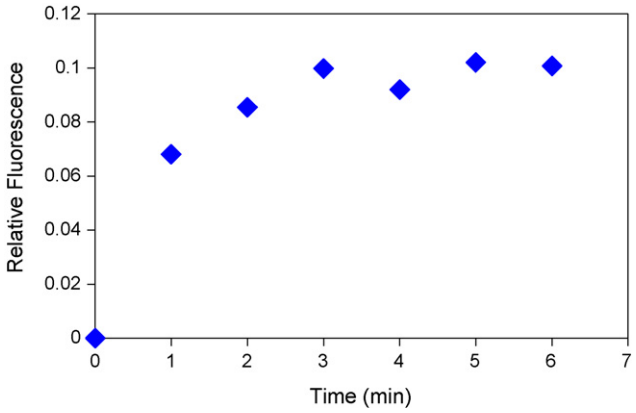

Fig. 6. Transient response of the sensor to introduction of cocaine (1 μM).

a result of the difference in kinetics between biotin–streptavidin binding and the hybridization of the complimentary strand to the aptamer. As a result, all future quenching solutions were allowed to incubate for at least 10 min before cocaine introduction.

Subsequently, the time-dependent fluorescence response of the sensor to introduction of cocaine was measured. Specifically, a 1 μM sample of cocaine was introduced to a prepared sensor and the fluorescence response was measured versus time for 6 min (Fig. 6). Fluorescence intensity appeared to achieve steady state after a period of approximately 3 min, indicating that the sensor has a time constant of approximately 1.2 min, and a settling time of almost 5 min with respect to cocaine. This result was expected, as the aptamers express a higher affinity for the cocaine molecules than the quencher-labeled short complimentary strands but lower than that of a biotin–streptavidin interaction. As a result, equilibrium with the target analyte was achieved at a faster rate than with the quencher molecules, but slower than with introduction of aptamers alone. This test was repeated at several other cocaine concentrations with similar results (data not shown), indicating that the transient response was independent of the concentration of cocaine being tested. During subsequent experiments, a cocaine sample would be incubated before washing for at least 5 min to ensure complete interaction of the cocaine and aptamer for maximum fluorescence response. This information was also used to estimate a flowrate necessary for experiments in which a device would be exposed to a continuous flow of cocaine.

4.3. Detection of cocaine

The response of the sensor to various concentrations of cocaine was then tested. The sensor was tested using practically relevant concentrations of cocaine, from 1 nM to 100 μM, in a buffer solution designed to mimic the environmental conditions (pH, salt content) of human bodily fluid (Fig. 7). Previously published data indicated that for a single dosage of cocaine, the maximum concentration of cocaine in the blood plasma of the user would be 1 μM [21]. As concentrations higher than 1 μM may be of interest in law enforcement forensics, a test at a concentration of 100 μM was included as well. The sensor exhibits a considerably linear response over the range of concentrations tested, indicating a four orders of magnitude linear range. As an experimental control, introduction of a control molecule, deoxycholic acid (DCA) in the identical concentration range, was also performed using the biosensor. DCA was chosen given its structural similarity to cocaine and common presence in the body. We observed no appreciable signal above a relative fluorescence of 0.2 for even the largest tested concentration (1 mM), which is equivalent to that of approximately 10 nM cocaine. Although this would indicate detection ambigu-

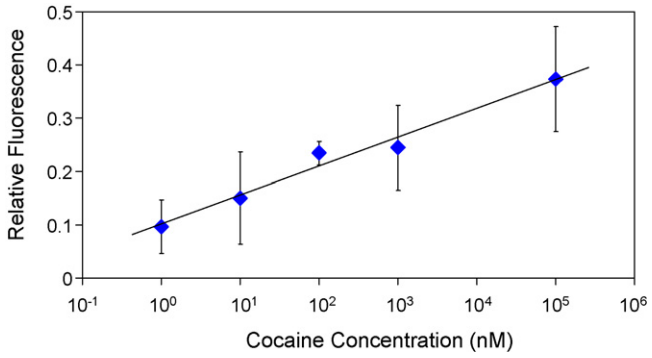

Fig. 7. Response of the sensor to cocaine at varying concentrations.

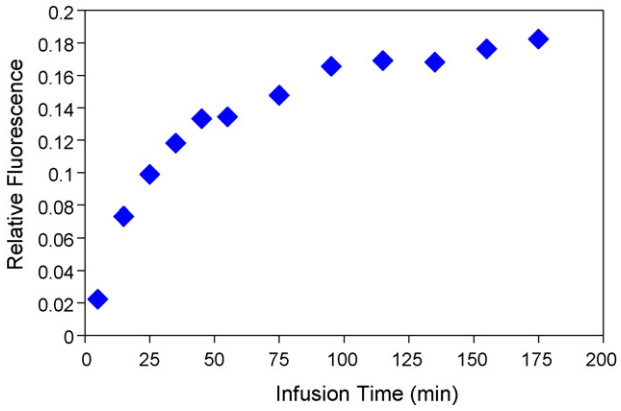

Fig. 8. Time-resolved relative fluorescence as cocaine is concentrated from a continuous flow (2 μL/min) of a highly dilute solution (10 pM).

ity during experiments involving low concentrations of cocaine, we do not anticipate this to occur practically since physiological DCA concentration is relatively low compared to tested levels [22]. From this data we conclude that the sensor is capable of detecting practically relevant concentrations of cocaine and compares well with previously reported aptamer-based cocaine sensors [9,12]. A relatively large degree of variability from test to test can be observed from Fig. 7, where error bars are computed based on three independent tests. Our analysis suggests that this can be largely attributed to the limited resolution of our rudimentary fluorescence microscopy system. Therefore, it is anticipated that improved fluorescence imaging would considerably reduce this variability. Additional sources of error may include variations in bead packing density, the presence of bubbles in the sensing chamber, and loss of sample due to dead volumes. These issues will be investigated in future work.

4.4. Improving detection limits by continuous-flow concentration

Previous work has demonstrated the ability of aptamers to concentrate and purify target analytes [18]. Due to the equilibrium-binding nature of the aptamer–analyte system, repeated exposure of the sensing surface to low concentrations of target analyte results in continued extraction of the analyte from the solution. Here, we exploit this phenomenon to concentrate dilute samples of cocaine for subsequently improved detection. The sensor was continuously infused with a sample of 10 pM cocaine solution at a rate of 2 μL/min. This flowrate was calculated based on the size of the sensing chamber (1 μL) and the transient response of the sensor to introduction of cocaine. As a result of the increasing concentration of cocaine at the sensing surface, the relative fluorescent signal increased to a value similar to a much higher concentration of cocaine (Fig. 8). At time intervals of 5–30 min, fluorescence intensity was measured, which increased until a saturation value of approximately 0.18 relative units was achieved after 175 min. This fluorescence value corresponds to a single sample of 5 nM cocaine, indicating that the sensor concentrated the cocaine by a factor of 500. This technique allows for the detection of trace amounts of cocaine in concentrations otherwise undetectable by other aptamer-based cocaine sensors [12,13].

4.5. Thermally based device regeneration

There are many device applications for which continued reuse of a single sensor would be advantageous. Our device makes use of the temperature-sensitive nature of aptamer–analyte binding to render it fully reusable. In order to investigate the ability of the sensor to be regenerated, the effect of temperature on the sensing surface was tested (Fig. 9). Following introduction of a 100 μM sample of cocaine, the on-chip heater and temperature sensor were used to raise the temperature of the chamber from room temperature to 37 °C while flowing pure buffer solution at 10 μL/min. Upon starting the test, a small increase in relative fluorescence at low temperatures was witnessed. This is likely attributed to hydrodynamic forces removing a small amount of quencher strands prior to the onset of temperature-induced conformational changes. As the chamber temperature was increased, a larger change in fluorescence intensity was seen at approximately 33 °C. This indicates that at this temperature, changes in the secondary structure of the aptamer result in the release of the cocaine molecule and leftover quencher strands. Signal increase continued until it saturated at approximately 37 °C at a value of 1, demonstrating successful regeneration of the aptamer surface. This indicates that the sensor can be heated to a relatively modest temperature to effectively release cocaine using a single homogeneous phase.

In order to be fully regenerable, the device must retain functionality following thermal release of cocaine. We subjected the device to multiple sensing cycles using a changing concentration of cocaine for each cycle (Fig. 10). In each cycle, we used the standard procedure for testing a concentration of cocaine, followed by thermal regeneration that was implemented by raising the temperature of the sensing chamber to 37 °C while flowing pure buffer through the chamber at 10 μL/min. The hatched bars indicate relative fluorescence following regeneration (surfaces coated with aptamers only). Gray bars indicate quenched sensors, and the black bars indicate sensor response to incubation of a sample of cocaine. The data indicates that the device behaves predictably without significant drift, with the response to cocaine concentration changing

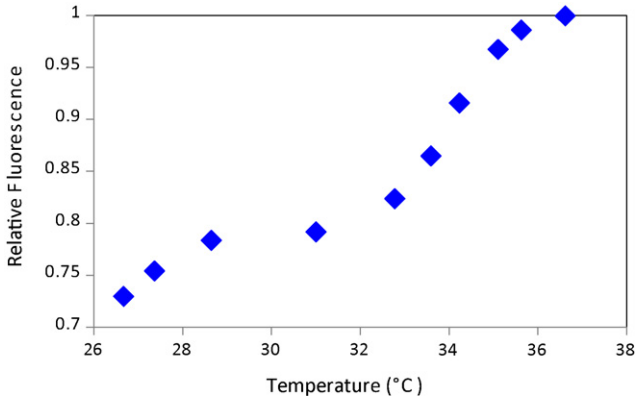

Fig. 9. Effect of temperature on relative fluorescence from a sensor incubated with 100 μM cocaine solution. Return of relative fluorescence to 1 indicates successful regeneration of the aptamer surface.

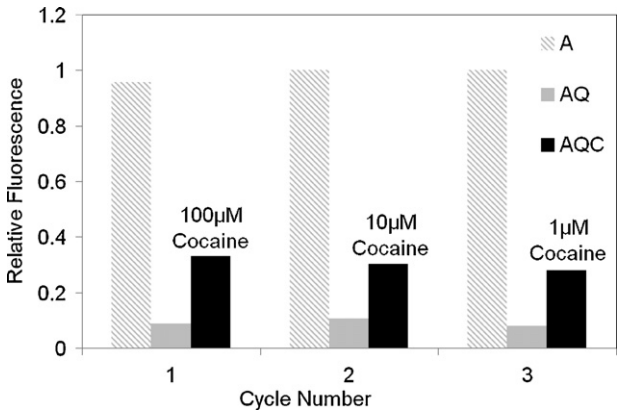

Fig. 10. Reuse of the sensor after regeneration. In each cycle, the sensor was first thermally regenerated at 37 °C (A), quenched (AQ), and finally exposed to cocaine (AQC).

predictably with each consecutive test. The device was capable of detection of 1 nM cocaine concentration after regeneration (data not shown). Thus, modest heating and washing grants our device repeated use without loss of functionality, an improvement over previously reported cocaine sensors [12,15].

5. Conclusions

Detection of trace amounts of illicit substances such as cocaine continues to be an important function in law enforcement and clinical medicine. Prevalent methods of detection in these fields such as GC–MS and presumptive testing allow for either accurate, specific detection involving expensive testing or rapid, economized detection that is unreliable due to its lack of specificity. We describe a novel microfluidic fluorescence aptamer-based biosensor which can rapidly detect low concentrations of cocaine. The device is characterized by high selectivity and sensitivity (10 pM), as well as rapid response and reduced sample consumption. The transient response of the sensor (equilibration in less than 5 min) is comparable to the detection time of cocaine in a GC-based system, but with the potential to bypass the need for cumbersome equipment and highly trained technicians. When testing samples of cocaine, the sensor responded linearly to cocaine concentration from 1 nM to 100 μM using only 10 μL of cocaine sample with a 5 min response time. Due to the nature of aptamer–target binding, which is sensitive to external stimuli, it was shown that an increase in temperature from ambient to 37 °C results in the release of the bound cocaine. Full reusability of the device was then demonstrated by multiple tests involving sensing and regeneration following a wash with pure buffer and a mild increase in temperature. As the sensor is designed along standard microfabrication principles, mass production to reduce manufacturing costs should be possible. This represents an improvement over previous aptamer-based cocaine sensors as well as a viable alternative to conventional methods of cocaine detection. Further work will include possible improvements to the cocaine sensing system, including alternate choices of chamber geometries and fluorophore-quencher pairs.

Acknowledgements

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