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Author:	**George Kraus, Marit Nilsen-Hamilton, Pranav Shrotriya, Kyungho Kang, Aaron Kempema, Ashish Sachan**
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MicroCantilever (MC) based Robust Sensing Approach for Controlled Substances

Award Number:

Authors:

Principal Investigators: George Kraus, Marit Nilsen-Hamilton, Pranav Shrotriya

Graduate Students: Kyungho Kang, Aaron Kempema, Ashish Sachan

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ABSTRACT:

MicroCantilever (MC) based sensors can provide revolutionary sensitivity for forensic detection and identification of controlled substances, toxic species, biological molecules and DNA matching. Current sensor systems require extensive sample preparation and/or specialized instrumentation to identify molecules of controlled substances such as cocaine with high specificity and sensitivity. We aim to overcome these limitations by developing a novel sensing approach based on MC sensors coupled with aptamer-based receptor layers. To demonstrate the feasibility and forensic suitability of our approach, we are developing sensors for sensitive and specific identification of cocaine, cocaine metabolites and methamphetamine and metabolites. These objectives are being accomplished through the completion of tasks arranged in two phases so that knowledge and experience gained in the first phase may be utilized for successful completion of the second phase. In the ***first phase*** of the project, we have: **1)** Optimized the available DNA aptamer to improve its sensitivity for binding with cocaine; **2)** Characterized the sensitivity and specificity of MC-based sensors functionalized with an available DNA aptamer that has specific affinity for cocaine; and **3)** Initialed the selection of additional variant DNA aptamers with selective sensitivities to cocaine and its metabolites.

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TABLE OF CONTENTS

Abstract: ii

TABLE OF CONTENTS iii

Executive Summary 1

1 Introduction 9

1.1 Statement of problem 9

1.2 Review of relevant literature 10

1.2.1 Current approaches for detection and identification of controlled substances 10

1.2.2 Micromechanical cantilever based sensors 14

1.2.3 Aptamers 16

1.2.4 Aptamers for controlled substances 18

1.2.5 Summary of relevant literature 20

1.3 Underlying hypothesis and rationale for research 20

2 Methods 22

2.1 Biochemical characterization of the available DNA aptamer and cocaine molecules 22

2.2 Characterization of MC sensors functionalized with available DNA aptamers 23

2.2.1 Surface stress sensor 24

2.2.2 Experimental procedure for conventional sensing mode 25

2.2.3 Synthesis of thiolated cocaine molecules for competition sensing mode 27

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2.2.4 Experimental procedure for competition sensing mode 30

2.3 Selection of variant DNA aptamers with specific affinity to cocaine and cocaine metabolites: 31

2.3.1 Synthesis of biotinylated and cocaine and cocaine metabolites for SELEX 31

2.3.2 Selection of variant DNA aptamers through SELEX 32

3 Results 33

3.1 Biochemical characterization of the available DNA aptamer and cocaine molecules. 33

3.2 Characterization of MC sensors functionalized with available DNA aptamers 35

3.2.1 Results for conventional sensing mode 35

3.2.2 Synthesis of thiolated cocaine molecules 38

3.2.3 Results for competition sensing mode 39

3.3 Selection of additional DNA aptamers for cocaine and its metabolites 41

3.3.1 Synthesis of biotinylated and cocaine and cocaine metabolites for SELEX 41

4 Conclusions 43

4.1 Discussion of Findings 43

4.2 Implications for policy and practice 45

4.3 Implications for further research 47

5 References 49

6 Dissemination of Research Findings 60

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EXECUTIVE SUMMARY

The U.S. Department of Health and Human Services (HHS) has established a standard of cocaine metabolite cutoff levels of 150 ng/mL and 100 ng/mL for initial screening and confirmatory cutoff levels respectively. Current methods of initial screening and identifying biological samples for drugs of abuse can match the new standard for detection and identification of cocaine metabolite. However, current sensor systems require extensive sample preparation and/or specialized instrumentation to identify molecules of controlled substances with high specificity and sensitivity. To validate drug presence, samples often must be sent to the lab, which results in a significant delay in identification. MicroCantilever (MC) based sensors are increasingly being used to detect chemical and biological species in both gas and liquid environments and these devices could be developed for use in the field. The sensing strategy involves coating one surface of a micromachined cantilever with a receptor species that has a high affinity for the analyte molecule. The presence of the analyte is detected by resolving the surface stress change associated with absorption/adsorption of analyte molecules on the sensitized surface. The introduction of cantilevers substantially enriches the portfolio of sensing scenarios that can be used in high performance miniaturized analytical systems.

Aptamers are single-stranded DNA (ssDNA) or RNA molecules that form tertiary structures that recognize and bind to their respective targets. These nucleic acids are much smaller than their protein (antibody) counterparts, and unlike antibodies, ligand binding is often accompanied by a large structural change that can be utilized for

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detection of the target. Aptamers are promising candidates for MC sensors because: (a) aptamers recognize their target molecule with high specificity and affinity; (b) binding with targets is often accompanied by a structural change that can be utilized for detection; (c) binding reactions are reversible so that the receptor layers can be recharged for detection; (d) aptamers are much more stable than antibodies and can be reused in the same sensor many times. A sensing approach based on microcantilevers functionalized with aptamers provides an invaluable tool for forensic science because of its portability, capability for detection and capability for identification of analytes with high sensitivity and specificity.

To demonstrate the feasibility and forensic suitability of aptamer-functionalized MC sensors, we are developing sensors for sensitive and specific identification of cocaine, cocaine metabolites and methamphetamine and its metabolites. The central hypothesis for this work is that formation of affinity complexes due to the specific absorption of controlled substance molecules on sensitized surfaces will give rise to charge redistribution, configurational change and steric hindrance between neighboring molecules leading to a measurable surface stress change. We have formulated our hypothesis following these reported findings: a) aptamers have highly specific affinity to single analyte molecules; and b) formation of affinity complexes between surface bound aptamers and analyte molecules leads to a change in the surface stress state. The rationale for the proposed research is that, once suitable aptamers are identified and mechanisms governing surface stress generation are understood, it will become possible to apply similar approaches for identifying aptamers for other drugs of abuse

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and finally, to develop miniature surface stress sensors for controlled substance detection.

Our long term goal is to develop miniature sensors that integrate the high specificity and high sensitivity of aptamers for detection and identification of controlled substances. As a step towards this goal, the focus of this project is to develop microcantilever technology for detection of cocaine and methamphetamine as a proof of principle for this novel sensing technology. Cocaine and methamphetamine were chosen to establish the proof of concept since both of these are common drugs of abuse. Once the proof-of-concept of our sensing approach is established, it will become possible to apply this technology to the detection and identification of a variety of drugs of abuse

In the ***first phase*** of the project, we have: **1)** Optimized the available DNA aptamer to improve its sensitivity for binding with cocaine; **2)** Characterized the sensitivity and specificity of MC-based sensors functionalized with an available DNA aptamer that has specific affinity for cocaine; and **3)** Initialed the selection additional variant DNA aptamers with selective sensitivities to cocaine and its metabolites.

A severe limitation of the ssDNA cocaine aptamer that is currently available is its low affinity for target. Thus, although this aptamer is useful for demonstrating proof-of-concept, aptamers with higher affinities will be needed to increase the sensitivity of the cantilever sensors. The existing cocaine aptamer was tested by isothermal titration calorimetry to determine its affinity for cocaine. From these studies we found that acetonitrile, the common solvent for cocaine standards, causes a significant decrease in affinity of the aptamer for cocaine. By maintaining the acetonitrile concentration below 2%

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we realize a 5-fold increase in sensitivity of the aptamer compared with published data and with results from our own studies.

Our second approach to increasing the affinity of the aptamer has been to modify the aptamer sequence in portions of the molecule surrounding the presumed cocaine-binding site. This approach was particularly effective and has resulted in aptamer variants containing some unnatural bases with increased sensitivity for cocaine of about 8-fold. In combination with controlling the acetonitrile concentration these aptamer variants are about 40-fold more sensitive than reported so far for a cocaine aptamer.

The MC sensor response for cocaine detection has been characterized under two different sensing strategies. The first mode is a conventional mode that relies on resolving surface stress change associated with formation of affinity complexes between aptamer molecules and cocaine molecules on the sensing surface. The second mode is a novel “competition” mode that relies on resolving the surface stress changes associated with dissociation of affinity complexes on the sensing surface. A miniature sensor consisting of two adjacent micromachined cantilevers (a sensing /reference pair) was utilized for detection of cocaine molecules. Presence of the analyte species is detected by measuring the differential surface stress associated with adsorption/absorption of chemical species on the sensing cantilever. The unique advantages of the differential surface stress sensor are: 1) Direct detection of differential surface stress eliminates the influence of environmental disturbances such as nonspecific adsorption, changes in pH, ionic strength, and especially the temperature; and 2) sensitivity of the sensor is independent of distance between the sensing surface

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and detector, which results in the sensor being amenable for miniaturization and enables an array of sensors to be easily fabricated on a single MEMS device.

For the conventional sensing mode, thiolated cocaine aptamer molecules were immobilized on the sensing cantilever surface, while the reference cantilever was coated with a scrambled sequence of the cocaine aptamer. The solutions for the sensing experiments were prepared to ensure that acetonitrile concentration is below 2% for all cases and concentrations of cocaine molecules were varied from 25 to 500 μM. Surface stress changes from 9 to 51 mN/m were measured for the range of cocaine concentrations. Given a threshold sensitivity of 5 mN/m of surface stress, this sensor is able to detect cocaine with the lowest detectable concentration down to 5 μM in room temperature under the conventional sensing mode.

In the competition sensing mode, surface stress generated due to dissociation of aptamer/cocaine complexes was measured. A thiol modified cocaine molecule was synthesized to attach the cocaine molecules on the microcantilevers. The synthesis began with the known enol ether ester and was generated in one step via a cycloaddition to N-BOC-pyrrole. The eight-step synthesis of the thiolated cocaine molecule shown below generated a single enantiomer compound.

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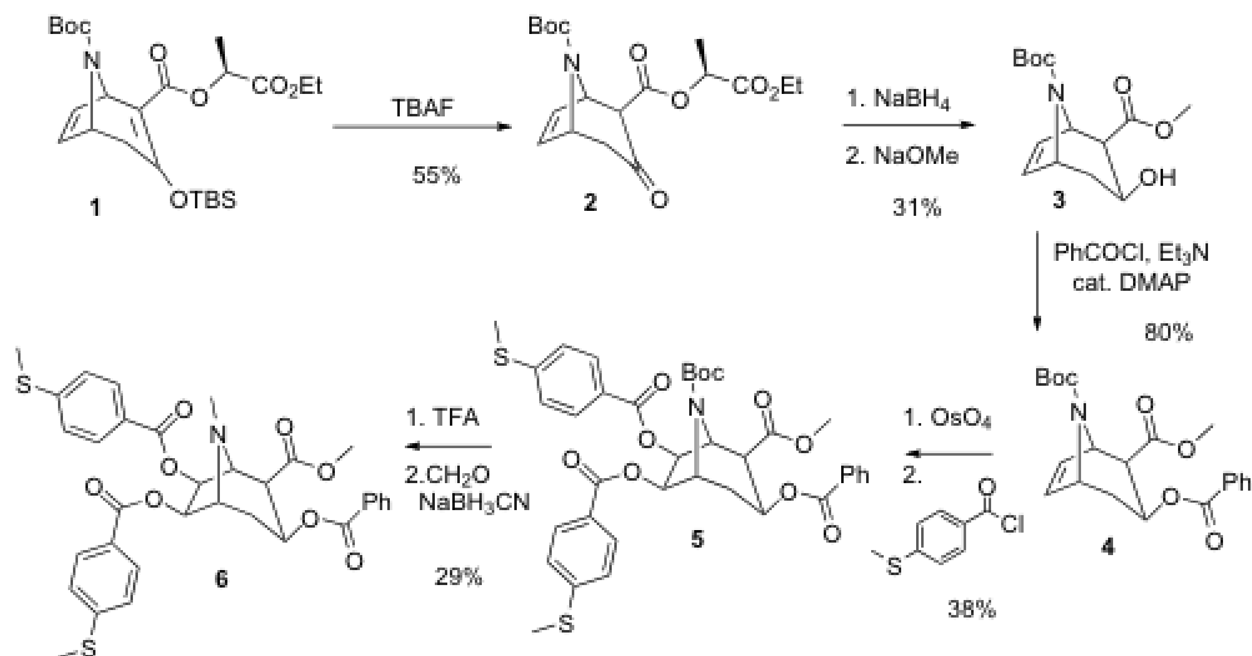

Our thiolated cocaine molecule has two points of attachment (via the two sulfur atoms) to the cantilever surface. The site of connection of the sulfur containing benzoate ester to the cocaine skeleton was designed to best present the key functional groups in the cocaine molecule (methyl ester, benzoate and amine) to the aptamer.

Thiol modified cocaine molecules were tethered to the gold surface using the sulphur link and immersed in a solution of the cocaine aptamers such that cantilever surface was covered with cocaine/aptamer affinity complexes. The functionalized cantilevers were then exposed to different concentrations of cocaine molecules and cantilever bending was monitored. The experimental results show that magnitude of bending in the functionalized cantilevers is not correlated with the cocaine concentration however the rate of bending demonstrates a good correlation with cocaine concentration. More importantly, the rate of bending correlation indicates that the lowest detectable cocaine concentration in competition sensing mode is approximately

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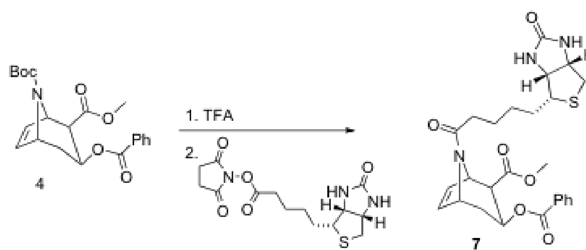
200 nM. To the best of our knowledge, this is the lowest detectable threshold reported for currently known cocaine aptamer based sensing.

We have synthesized biotin linked cocaine molecules for the selection of aptamers. The attachment of biotin to the cocaine molecule is shown below. This compound was prepared in one step from an intermediate in the synthesis of the thiolated cocaine molecule described above:



At present, the Kraus group is preparing cocaine metabolite-biotin adducts to be used in aptamer selection. The Nilsen-Hamilton group is currently using the biotin attached cocaine molecule for selection of aptamers that have specific binding to cocaine. Aptamer selection is being performed through the process of SELEX (**S**ystematic **E**volution of **L**igands by **Ex**ponential enrichment); a reiterative process of DNA amplification and selection.

Our experimental results from **the first phase** of this project have presented a novel modification of the aptamer structure that results in an order of magnitude improvement in affinity for cocaine. Increased affinity for an analyte is often associated with better discrimination of molecular structure and therefore these modified aptamers are expected to be more specific for cocaine. The modified aptamers need to be tested for their relative affinities for cocaine compared with various cocaine metabolites and other derivatives.

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Aptamer-functionalized microcantilevers used in the novel competition sensing mode have demonstrated an order of magnitude improvement in threshold sensitivity over the conventional sensing mode. However, the current sensing experiments have utilized only the currently available DNA aptamer. Further work is required to test the sensitivity of cocaine detection with the modified aptamer that demonstrates greater affinity. In addition, the specificity of cocaine detection needs to be tested in biological matrices as well as in real world samples.

We expect to select DNA aptamers with higher affinities to cocaine and its metabolites through the ongoing SELEX procedures. Increased accuracy of cocaine identification can also be achieved with a panel of aptamers of different affinities and specificities for target molecules. Compared with DNA, RNA possesses an additional hydroxyl group on the ribose associated with each base that provides more opportunity for interaction with target molecules and potentially higher affinity binding than does DNA. In order to increase the sensitivity of cocaine detection, it will likely be advantageous to select for DNA as well as RNA aptamers for cocaine and its derivatives.

In the ***second phase*** of the project, we propose to: 1) characterize the threshold sensitivity for cocaine detection using microcantilevers functionalized with the selected aptamers with higher affinity; 2) calibrate the sensor performance for detection of cocaine and its metabolites in biological matrices and simulated “real world” samples, 3) Select RNA aptamers with specific affinities to cocaine, cocaine metabolites and methamphetamine.

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1 Introduction

1.1 Statement of problem

Current sensor systems require extensive sample preparation and/or specialized instrumentation to identify molecules of controlled substances with high specificity and sensitivity. To validate drug presence, the sample often must be sent to the lab, which results in a significant delay in identification. Microcantilever (MC) based sensors are an intriguing new alternative because of their extremely high sensitivity and miniature sensing elements. The sensing strategy involves coating one surface of a micromachined cantilever with a receptor species that has a high affinity for the analyte molecule. The presence of the analyte is detected by resolving the surface stress change associated with absorption/adsorption of analyte molecules on the sensitized surface. The efficacy of microcantilever sensors has been successfully demonstrated for explosive detection, DNA sequence recognition and small molecule detection in chemical mixtures.

The goal of this project is to develop robust strategies for MC based sensors that will enable sensitive and specific detection of controlled substances such as cocaine, cocaine metabolites and methamphetamine. We plan to accomplish this goal through completion of tasks arranged in two phases so that knowledge and experience gained in the first phase may be utilized for successful completion of the second phase.

After the completion of the ***first phase*** of the project, we have: **1)** Characterized the sensitivity and specificity of MC-based sensors functionalized with an available DNA

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aptamer that has specific affinity for cocaine; **2)** Optimized the available DNA aptamer to improve its sensitivity for binding with cocaine; and **3)** Initiated the selection of additional variant DNA aptamers with selective sensitivities to cocaine and its metabolites.

In the ***second phase*** of the project, we will: 1) characterize the threshold sensitivity for cocaine detection using microcantilevers functionalized with the selected aptamers with higher affinity; 2) calibrate the sensor performance for detection of cocaine and its metabolites in biological matrices and simulated "real world" samples, 3) Select for RNA aptamers with specific affinities to cocaine, cocaine metabolites and methamphetamine.

1.2 Review of relevant literature

1.2.1 Current approaches for detection and identification of controlled substances

The U.S. Department of Health and Human Services (HHS) (Services 2008) has established a new standard of cocaine metabolite cutoff levels of 150 ng/mL and 100 ng/mL for initial screening and confirmatory cutoff levels respectively. Current methods of initial screening and identifying biological samples for drugs of abuse can match the new standard for detection and identification of cocaine metabolite. For instance, enzyme multiplied immunoassay technique (EMIT) (Mead, Niekro et al. 2003; Contreras, Hernandez et al. 2006; Baker and Jenkins 2008) and enzyme-linked immunosorbent assay (ELISA) (Lopez, Martello et al.; Kerrigan and Phillips 2001; Spiehler, Isenschmid et al. 2003) are the two predominant enzyme base immunoassays utilized for screening

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tests. . In both techniques, detection of controlled substance is based on optical absorbance resulting from enzymatic activity. Gas chromatography coupled with mass spectrometry (GC-MS) (Valente-Campos, Yonamine et al. 2006; Cristoni, Basso et al. 2007; Barroso, Dias et al. 2008) and high performance liquid chromatography (HPLC) (Nesmerak, Sticha et al.; Johansen and Bhatia 2007; Jagerdeo, Montgornery et al. 2008) can achieve detection levels required for confirmatory identification of controlled substances. These techniques require extensive sample preparation, long time and/or specialized instrumentation to validate drug presence. The sample often must be sent to the lab, which results in a significant delay in identification (Stojanovic, de Prada et al. 2001; Maurer 2005; Strano-Rossi, Molaioni et al. 2005; Cognard, Bouchonnet et al. 2006; Dixon, Brereton et al. 2006; Kaeferstein, Falk et al. 2006; Contreras, Gonzalez et al. 2007; Johansen and Bhatia 2007; Schaffer, Hill et al. 2007; Walsh, Crouch et al. 2007). Aptamer based biosensors (often called aptasensors) have been investigated as an alternative method to overcome these drawback. Sensitivity and detection times of conventional and aptamer based techniques are compared in Fig. 1.

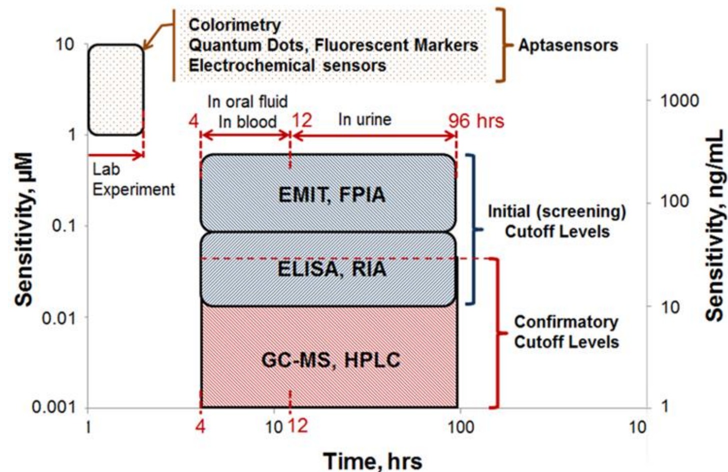

Fig. 1. Comparisons of sensitivity and time for conventional and aptamer based sensing techniques

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Current sensor systems require extensive sample preparation and/or specialized instrumentation to identify molecules of controlled substances with high specificity and sensitivity (Stojanovic, de Prada et al. 2001; Maurer 2005; Strano-Rossi 2005; Cognard 2006; Dixon 2006; Kaeferstein 2006; Contreras 2007; Johansen 2007; Schaffer 2007; Walsh 2007). To valiate drug presence the sample often must be sent to the lab, which results in a significant delay in identification. Toxicological analysis of the samples is performed through either mass spectrometry or chromatography. (Maurer 2005; Strano-Rossi 2005; Cognard 2006; Dixon 2006; Kaeferstein 2006; Contreras 2007; Johansen 2007; Schaffer 2007; Walsh 2007).

Current methods of screening biological samples for drugs of abuse employ immunoassays that require antibodies (that are often linked to enzymes) rather than aptamers. Of the various enzymatic immunoassay techniques available, enzyme multiplied immunoassay technique (EMIT) and enzyme-linked immunosorbent assay (ELISA) are the two predominant techniques currently found in forensic applications. Both differ from the proposed analytical technique in that detection is based on optical absorbance resulting from enzymatic activity rather than optical measurements of a metal surface deformation. However, like the proposed technique, EMIT utilizes competitive binding between drug molecules in the sample and drug labeled enzymes for a limited number of binding sites.

EMIT assays are homogeneous assays, meaning they do not require separation of bound fractions from unbound fractions. Systems commonly found in forensic laboratories typically screen for drug classes but do not differentiate between different

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substances with similar structures (i.e., amphetamine and methamphetamine or heroin and codeine). The systems are highly automated and can run numerous samples within a single sequence; however, screening for different drug classes, such as phenethylamines and opiates, requires different reagents and thus separate samplings. If a single sample is to be screened for an array of drug classes, the analysis time increases with the number of drug classes screened. An additional disadvantage of EMIT as commonly used for forensic samples is the physical size of the instrumentation. Instruments on the order of cabinet size render field analyses of samples impractical.

ELISA assays differ from EMIT in that they are heterogeneous – they require separating the unbound fraction from the bound fraction prior to analysis. The technique is similar to the proposed technique in that the walls of the sample wells are coated with a drug derivative analogous to the drug of interest. Competition occurs with the immobilized drug derivative and the free drug in samples to bind with an enzyme labeled anti-drug antibody. Following an incubation period where this competitive binding reaches equilibrium, the sample well is washed, leaving only the drug derivative-antibody complex and the drug concentration in the questioned sample can be calculated. ELISA has disadvantages similar to EMIT in addition to requiring a separation step. Each additional drug class requires both a different sample well and a different reagent. Likewise, the size of the instruments used to measure the ELISA outputs prohibits the use of this technique when the end use requires portability.

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1.2.2 Micromechanical cantilever based sensors

Micromechanical cantilever (MC) based sensors are increasingly being used to detect the presence of chemical and biological species in both gas and liquid environments. An increasing number of recent reports confirm the potential of MC sensors for environmental and biomedical applications. The multifaceted functionality of MCs indicates their uniqueness compared with more traditional sensor designs (Thundat, Oden et al. 1997; Sepaniak, Datskos et al. 2002).In the absence of external driving forces, the degree of deflection of a MC is directly related to the surface stress generated in the cantilever. A MC intended for chemical or biological sensing is normally modified by coating one of its sides with a responsive phase that exhibits high affinity to the targeted analyte. Consequently, the deflection of the MCs is governed primarily by the Gibbs free energy associated with the adsorption (surface interaction) or absorption (bulk phase interaction with thin films) processes on the modified surface. MC transducers are compatible with many responsive phases and can function in both gas and liquid environments.

Cantilevers with selective coatings have shown specificity to volatile organic compounds (VOCs), ionic species, proteins, and oligonucleotides (Betts, Tipple et al. 2000; Fagan, Tipple et al. 2000; Fritz, Baller et al. 2000; Hansen, Ji et al. 2001; Ji, Thundat et al. 2001; Lavrik, Tipple et al. 2001; Savran, Knudsen et al. 2004). Even cantilevers coated with generic polymers, such as polymethylmethacrylate, polystyrene, polyurethane and their blends, showed promise as detectors of VOCs. By applying the principal component and artificial neural network analyses to response patterns from

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arrays of such polymer-modified cantilevers, the concept of an artificial nose was successfully implemented (Lang, Berger et al. 1998; Lang, Baller et al. 1999; Baller, Lang et al. 2000; Archibald, Datskos et al. 2007). Notable discriminating power was observed for alcohol mixtures and certain natural flavors.

Thin films (~100 nm) of commercially available chromatographic stationary phases and chemically modified sol–gel coatings were used to create cantilever sensors with distinctive selectivity patterns for different classes of VOCs (Betts, Tipple et al. 2000; Fagan, Tipple et al. 2000). Using covalent attachment of mercapto-derivatized macrocyclic cavitands, such as crown ethers, calixarenes, and cyclodextrins, advanced molecular architectures of bio-mimetic receptors can be formed on gold-coated cantilevers. This approach results in cantilever sensors with remarkably low detection limits. In particular, sub-parts-per billion detection limits for vapors of aromatic compounds and 10^{-9} M concentrations of ionic species in solution were determined (Ji, Hansen et al. 2001; Ji, Thundat et al. 2001; Lavrik, Tipple et al. 2001). Self-assembled films of amino- and carboxy-terminated straight-chain thiols provide a convenient means to create cantilever sensors that can be used for pH measurements (Fritz, Baller et al. 2000).

The potential uses of cantilever transducers in biosensors, biomicroelectro-mechanical systems, proteomics, and genomics are intriguing trends in advanced biomedical analyses (Fritz, Baller et al. 2000; Hansen, Ji et al. 2001; Wu, Ji et al. 2001; Savran, Knudsen et al. 2004). When antibodies or small DNA fragments were immobilized on one side of a cantilever, the presence of complementary biological

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species produced cantilever deflections (Hansen, Ji et al. 2001; Wu, Ji et al. 2001). On the basis of the deflection behavior of MCs, even very small mismatches in receptor–analyte complementarity could be detected. A single base pair mismatch was resolved in oligonucleotide hybridization experiments performed on a cantilever surface (Fritz, Baller et al. 2000; Hansen, Ji et al. 2001; Wu, Ji et al. 2001).

As discussed above, the unusual multifaceted functionality of MC transducers inspires new sensing paradigms that can be used in high performance miniaturized analytical systems.(Sepaniak, Datskos et al. 2002). Successful development of MC based transducers is contingent on robust sensing strategies and identification of coating of responsive phases that have highly specific affinity to the target analyte

1.2.3 Aptamers

Aptamers are small RNA or DNA molecules (mostly15 to 60 nt) that bind other molecules with high specificity and high affinity (Hermann and Patel 2000). They are selected *in vitro* by a procedure called SELEX (**S**ystematic **E**volution of **L**igands by **Ex**ponential enrichment, Fig. 2), which is a reiterative process that results in the selection of oligonucleotides with high affinity for the chosen target (Ellington and Szostak 1990; Tuerk and Gold 1990). The procedure generally involves an immobilized target that can be covalently linked to a column support such as agarose to provide an affinity matrix or the target can be modified with the addition of biotin, which allows it to be captured by streptavidin on the filter or a column.

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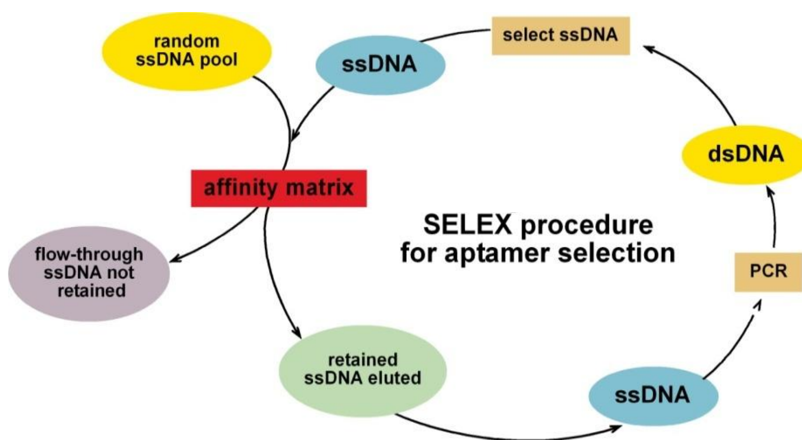

Fig. 2: SELEX (Systematic Evolution of Ligands by Exponential enrichment) procedure for aptamer selection

The investigator starts with a combinatorial mixture of oligonucleotides in which the specified base in each position of a central region of random sequence is varied independently of the remaining sequence. Thus, depending on the defined length (n) of the random sequence in the oligonucleotides of the pool, the mixture contains 4^n different sequences. This mixture of oligonucleotides is exposed to the target molecule and oligonucleotides that do not bind the target are removed. The remaining oligonucleotides are collected and amplified, using DNA polymerase under conditions that increase the probability of enzyme error, thereby increasing the variation in the selected pool. The resulting mixture is again selected on the immobilized target and the successful oligonucleotides are amplified by PCR. Negative selections are also performed in which the oligonucleotides are removed that bind related molecules for which the selected aptamer should not have affinity. Thus, with successive rounds of selection of increasing stringency, oligonucleotides are produced with high affinity and specificity for the target molecule.

Nilsen-Hamilton and mathematician Howard Levine developed a mathematical model that describes the SELEX process, which was used to design the experimental protocols for successful selection of two aptamers by the Nilsen-Hamilton lab (Levine

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and Nilsen-Hamilton 2007). One of these aptamers recognizes a protein and the other recognizes a small molecule. The latter will be discussed here as it is relevant to the aptamer selection for controlled substances. This aptamer recognizes a derivative (PDC) of an anticancer drug, PD173955, which is an Abelson tyrosine kinase inhibitor. Selection of the PDC aptamer was achieved using biotinylated PDC and streptavidin agarose to capture the aptamers on a filter. A pool of 5 $x10^{14}$ DNA molecules, each containing 53 bases of randomized sequence, was used to synthesize the RNA pool for selection of the aptamer. In each round, the RNA oligonucleotides that bound PDC were captured on a filter, eluted, reverse transcribed to cDNA, PCR amplified and transcribed back to RNA. Intermediate rounds of negative selection were performed in which the RNA was passed through the filter in the absence of biotinylated PDC and the oligonucleotides that bound the filter were discarded. Two SELEX experiments successfully yielded PDC binding populations. When cloned, these populations were found to contain the same two families of aptamers representing two different putative aptamers. The affinities of the putative aptamers for PDC were determined by isothermal titration calorimetry. One oligonucleotide was found to be an aptamer with an association constant (Ka) of 10^6 M^{-1}, which is a higher affinity than reported for other aptamers that recognize compounds related to PDC (Mehanovic, Kraus and Nilsen-Hamilton, manuscript in preparation).

1.2.4 Aptamers for controlled substances

Aptamers have also been selected that recognize other drugs including two drugs of abuse, which are cocaine (Stojanovic, de Prada et al. 2001) and codeine (Win, Klein et

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al. 2006), and many medicinal drugs and antibiotics including theophyline (Jenison, Gill et al. 1994), tobramycin (Wang and Rando 1995), neomycin (Wallis, von Ahsen et al. 1995), kanamycin (Lato, Boles et al. 1995), dopamine (Mannironi, Di Nardo et al. 1997), chloramphenicol (Burke, Hoffman et al. 1997), streptomycin (Wallace and Schroeder 1998) and tetracycline (Berens, Thain et al. 2001).The affinities (Ka) of these aptamers are in the range of 10^5 to 10^7 M^{-1}. Although most aptamers are currently produced by standard laboratory methods, recent developments in automation of aptamer selection suggests that the number of aptamers currently available that recognize a certain set of molecules could be rapidly expanded in the future.

Aptamers are much smaller than their protein (antibody) counterparts, and unlike antibodies, ligand binding is often accompanied by large structural changes in the aptamers that can be utilized for detection of the target (Stojanovic, de Prada et al. 2001). Because their specificities can be tuned by the selection conditions, aptamers can be selected that have exquisite discrimination between molecules. For example, aptamers have been isolated that distinguish between caffeine and theophylline, which differ by only a single methyl group (Jenison, Gill et al. 1994) and that distinguish tyramine and dopamine, which differ by a single hydroxyl group (Mannironi, Scerch et al. 2000). Very little change in the oligonucleotide sequence may be necessary to change the specificity of a nucleic acid. For example, two RNA aptamers were isolated that differ by only 3 nucleotides in 44 and that respectively specifically recognize only one of the two closely related amino acids, arginine and citrulline (Mannironi, Scerch et al. 2000).

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With recognition of the obvious benefits of aptamers in developing microsensors, many applications have been developed as discussed in a recent review (Mairal, Cengiz Ozalp et al. 2007). The efficacy of aptamers has been shown on a number of biosensing platforms. Examples include the use of surface plasmon resonance (Win, Klein et al. 2006), electrochemistry (Schlecht, Malave et al. 2006), fluorescence spectrometry (Ozaki, Nishihira et al. 2006), nanotube field-effect transistors (Maehashi, Katsura et al. 2007) and microcantilever technology (Savran, Knudsen et al. 2004).

1.2.5 Summary of relevant literature

Aptamers are promising candidates for MC sensors because: (a) they recognize their target molecules with high specificity and affinity; (b) aptamer specificity can be "tuned" according to the selection strategy, (c) binding with targets is often accompanied by a structural change that can be utilized for detection; (d) binding reactions are reversible so that the receptor layers can be recharged for detection; (e) aptamers are much more stable than antibodies at ambient temperatures and can be reused in the same sensor many times. A sensing approach based on microcantilevers functionalized with aptamers will provide an invaluable tool for forensic science because of its portability, capability for detection and capability for identifying analytes with high sensitivity and specificity.

1.3 Underlying hypothesis and rationale for research

The central hypothesis for this work is that formation of affinity complexes due to the specific absorption of controlled substance molecules on sensitized surfaces will give

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rise to charge redistribution, configurational change and steric hindrance between neighboring molecules leading to a measurable surface stress change. We have formulated our hypothesis following these reported findings: a) aptamers have highly specific affinity to single analyte molecules; and b) formation of affinity complexes between surface bound aptamers and analyte molecules leads to a change in the surface stress state. The rationale for the proposed research is that, once suitable aptamers are identified and mechanisms governing surface stress generation are understood, it will become possible to apply similar approaches for identifying aptamers for other drugs of abuse and finally, to develop miniature surface stress sensors for controlled substance detection.

Rationale for choosing cocaine and methamphetamine for this study: Our long term goal is to develop miniature sensors that integrate the high specificity and high sensitivity of aptamers for detection and identification of controlled substances. As a step towards this goal, the focus of this project is to develop microcantilever technology for detection of cocaine and methamphetamine as a proof of principle for this novel sensing technology. Cocaine and methamphetamine are chosen to establish the proof of concept since both of these are common drugs of abuse. Once the proof-of-concept of our sensing approach is established, it will become possible to apply this technology to the detection and identification of a variety of drugs of abuse

Our research team uniquely combines a chemist, biochemist and mechanical engineer, each of whom has a long standing research interest in sensing of controlled substances with suitable aptamers, and who have collaborated for the purpose of

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developing the technology for forensic applications. In addition, we have established collaboration with Jeremy Morris a Drug Chemist and Trace Analyst for improving our understanding of current practices on samples and matrices used for drug identification.

2 Methods

In the **first phase** of the project our work has focused on: characterizing and optimizing the available DNA aptamer through biochemical studies; characterizing the response MC based sensor functionalized with an available DNA aptamer for specific detection of cocaine; and selecting additional variant DNA aptamers with selective sensitivities to cocaine and its metabolites. Experimental design and methods for each of these tasks is explained below

2.1 Biochemical characterization of the available DNA aptamer and cocaine molecules

DNA cocaine aptamers were chemically synthesized and purchased from Intergrated DNA Technologies (IDT), (Coralville, Iowa). Aptamer binding affinity for the cocaine target was measured by isothermal titration calorimetry (ITC) under the same buffer conditions of monovalent and divalent cations and pH as used for the microcantilever studies. In studies in which the effect of acetonitrile on aptamer binding was evaluated, the acetonitrile was first removed from the cocaine stock and then replaced at various concentrations for measurement by ITC.

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2.2 Characterization of MC sensors functionalized with available DNA aptamers

The MC sensor response for cocaine detection has been characterized under two different sensing strategies as schematically represented in Fig 3. The first mode is a conventional mode that relies on resolving surface stress change associated with formation of affinity complexes between aptamer molecules and cocaine molecules on the sensing surface. The second mode is a novel “competition” mode that relies on resolving the surface stress changes associated with dissociation of affinity complexes on the sensing surface. Aptamer molecules are much larger than cocaine molecules therefore their removal from the sensing surface in the competition sensing mode is expected to give a larger surface stress change as compared to the conventional sensing mode and consequently to result in a lower threshold sensitivity of cocaine detection. In both modes of operation, a DNA aptamer will be used to detect cocaine molecules. The aptamer has been previously selected to recognize cocaine molecules (Stojanovic, de Prada et al. 2001). In the absence of cocaine the termini of the aptamer are believed to be separated, but in its presence they form a stem and a three-way junction. The extent to which the aptamer changes in structure is a function of the number of bases in its terminal stem (Neves, Reinstein et al.) Surface stress associated with this major structural change will be investigated to characterize sensor response to the aptamer-ligand binding and as a function of aptamer stem length.

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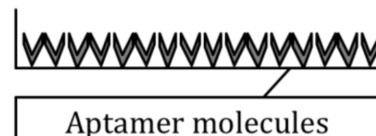
2.2.1 Surface stress sensor

A miniature sensor consisting of two adjacent micromachined cantilevers (a sensing /reference pair) was utilized for detection of cocaine molecules (Kang, Nilsen-Hamilton et al. 2008). Presence of the analyte species is detected by measuring the differential surface stress associated with adsorption/absorption of chemical species on the sensing cantilever. The unique advantages of the differential surface stress sensor are: 1) Direct detection of differential surface stress eliminates the influence of environmental disturbances such as nonspecific adsorption, changes in pH, ionic strength, and especially the temperature; and 2) Sensitivity of the sensor is independent of distance between the sensing surface and detector, which results in the sensor being amenable for miniaturization and enables an array of sensors to be easily fabricated on a single MEMS device.

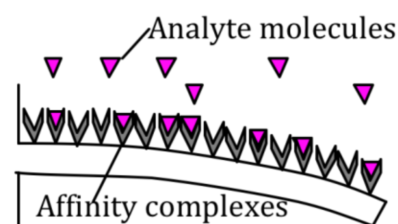
A novel interferometric technique was utilized to measure the differential surface stress induced bending of the sensing cantilever with respect to the

Conventional sensing mode

Sensing surface coated with aptamer molecules

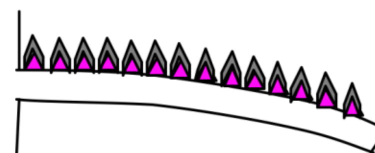


Exposure to analyte leads to affinity complex formation on sensing surface and surface stress change



Novel "competition" sensing mode

Sensing surface coated with affinity complexes of analyte and aptamer



Exposure to analyte leads to affinity complex dissociation on sensing surface and surface stress change

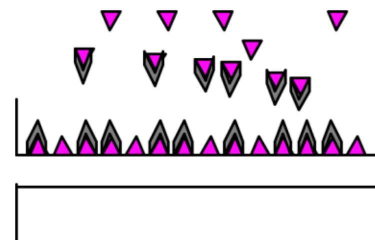


Fig 3: Schematic representation of the two sensing strategies for cocaine detection.

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reference cantilever. The principle of differential surface stress measurement is schematically presented in Fig 4. The sensor consists of two microlens arrays (MLA1 and MLA2) and microcantilevers (sensing/reference pair) arranged such that a pair of light beams can reflect from the microcantilevers. As a result, the two beams collect a phase difference proportional to the differential displacement of the two beams. Monitoring of the phase difference as a function of time is utilized to determine the development of differential surface stress between the two cantilevers.

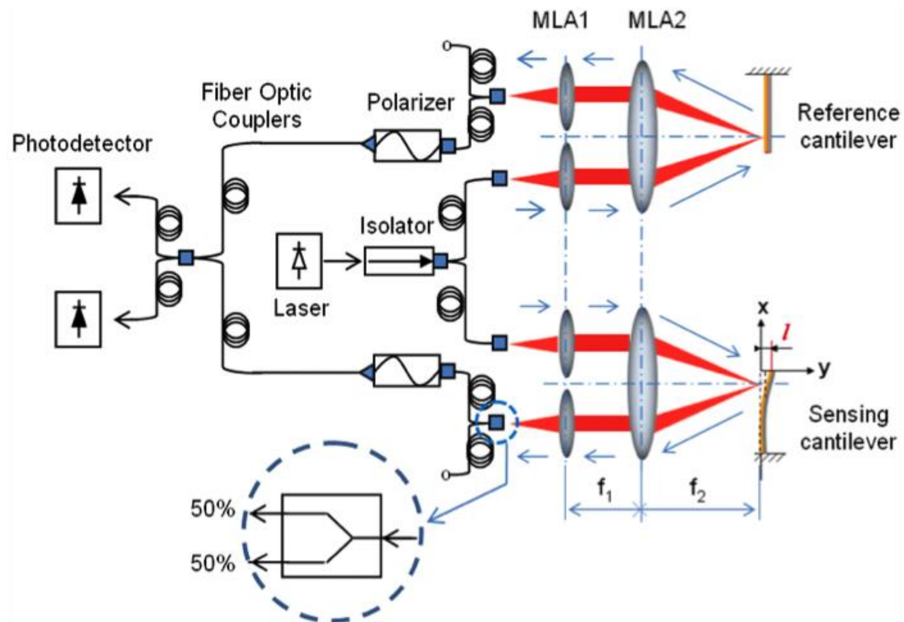

Fig 4: Novel Differential surface stress sensor

2.2.2 Experimental procedure for conventional sensing mode

Cantilever functionalization: For the conventional sensing mode, sensing and reference microcantilevers were coated with DNA cocaine aptamer and scrambled DNA sequence with same bases as aptamers, respectively in order to functionalize them for cocaine detection. Thiol-modified cocaine aptamers with the sequence of 5'- HS-$(CH_2)_6$ - GAC AAG GAA AAT CCT TCA ATG AAG TGG GTC -3' and a thiol modified DNA with scrambled sequence were purchased from IDT (Coralville, Iowa). Gold-coated

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microcantilevers with nominal dimensions of 500 μm length x 100 μm width and 1 μm thickness were purchased from Nanoandmore.com (Lady's Island, South Carolina). Microcantilevers were cleaned by the Piranha solution (70% H_2SO_4 and 30% H_2O_2) for 30 minutes, rinsed in deionized water and dried in the gentle N_2 flow. Thiol-modified cocaine aptamer were heated to 60 °C to cleave any disulfide bonds and mixed with the saline sodium citrate buffer (20×SSC), pH 7.4) to obtain a 0.5 μM aptamer solution. Cleaned microcantilevers were immersed in the aptamer solution for three hours in order to immobilize the thiol-modified DNA aptamer on the gold-coated surface. A similar procedure was used to immobilize DNA strands with scrambled sequence on the reference cantilever. Subsequently, functionalized microcantilevers were immersed in 6-mercapto-1-hexanol solution (3 mM concentration) for one hour to displace any adsorbed DNA strands.

Sensing Experiments: Standard cocaine samples dissolved in acetonitrile were purchased from Sigma Aldrich (St. Louis, Missouri). As received, the cocaine samples were diluted in deionized water and heated in order to eliminate acetonitirile from the solutions used to measure sensor response. The functionalized sensing and reference cantilevers were mounted in the differential surface stress sensor and exposed to different concentrations of cocaine from 0 to 500 μM in 140 mM phosphate-buffered saline solution (PBS, pH 7.4) to determine the sensor response as a function of the cocaine concentration.

After the sensing experiments, the sensing and reference cantilevers were heated in deionized water at 80 °C to regenerate the aptamer sequence and to remove any

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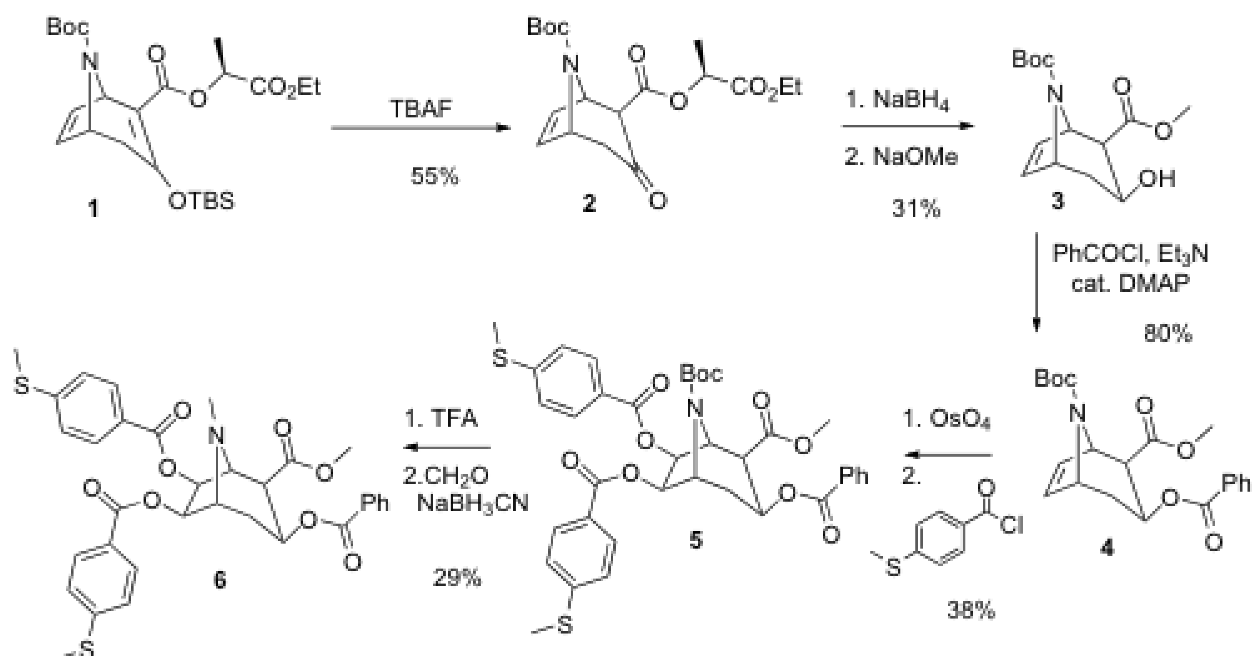
bound cocaine molecules. The regeneration allows the sensing cantilevers to be used a number of times and each cantilevers was used for three sensing experiments.

2.2.3 Synthesis of thiolated cocaine molecules for competition sensing mode

For the "competition" sensing mode, thiol-modified cocaine molecules were attached to the cantilever surface. The thiol-modified cocaine molecules were synthesized according to the following steps:



(1R,2R,3S,5R)-8-tert-butyl 2-methyl 3-(benzoyloxy)-8-azabicyclo[3.2.1]oct-6-ene-2,8-dicarboxylate(4)

To a stirred solution of enol silyl ether **1** in THF (0.10M), TBAF (1.0 M in THF) (1 eq) was added dropwise at 0 °C. The reaction mixture was stirred at this temperature for 5 min. After completion of the reaction, it was quenched with water and extracted with ethyl acetate (EtOAc), washed with water, brine and dried over $MgSO_4$, followed by

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filtration and concentration *in vacuo*. The residue was purified by flash chromatography (2:1 hexanes:EtOAc). The product was obtained as a light yellow oil in 55 % yield.

To a stirred mixture of ketone **2** in methanol was added to a solution of $NaBH_4$ (5 eq) in methanol (0.10 M) at -30°C. The reaction was stirred at this temperature for 7 h. After completion of the reaction, it was quenched with 4 M HCl until acidic. The reaction mixture was concentrated *in vacuo*. Ammonium hydroxide (NH_4OH) was added until the solution was basic, next the residue was extracted with EtOAc and washed with water, brine and dried over $MgSO_4$, followed by filtration and concentration *in vacuo*. The residue was obtained as a clear oil and taken on to the next step without further purification.

The crude alcoholic product dissolved in MeOH (0.10 M) was added to a NaOMe solution (3 eq) and the reaction was stirred for 1 h at 0°C. Upon completion of the reaction mixture, it was concentrated. The residue was poured into a sat. NH_4Cl solution and extracted with Et_2O, washed with water, brine and dried over $MgSO_4$, followed by filtration and concentration *in vacuo*. The residue was purified with flash chromatography (1:1 - 1:2 Hexanes:EtOAc). The product was obtained as a clear oil in 31% over two steps.

To a stirred mixture of alcohol **3**, Et_3N (6 eq), DMAP (0.1 eq) in CH_2Cl_2 (0.10 M) at 0°C had benzoyl chloride (1.4 eq) added to the reaction. The reaction was heated to reflux and stirred at this temperature for 8 hrs. Extracted with CH_2Cl_2, washed with water, brine and dried over $MgSO_4$, followed by filtration and concentration *in vacuo*.

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The residue was purified with flash chromatography (5:1-3:1 Hexanes :EtOAc). The product was obtained as a clear oil in 80 % yield.

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.51 (s, 9 H), 1.71 (br, 1 H), 2.24 (br, 1 H), 2.97 (m, 1 H), 3.65 (s, 3 H), 4.73 (m, 2 H), 5.49 (q, J = 9.3 Hz, 1 H), 6.20 (br, 2 H), 7.42 (t, J = 7.5 Hz, 2 H), 7.53 (t, J = 7.2 Hz, 1 H), 7.98 (d, J = 7.2 Hz, 2 H); m/z: 410 $[M+Na]^+$, 288, 166; HRMS: calcd. for $C_{21}H_{25}NO_6$: 410.1574 $[M+Na]^+$; found 410.1574.

(1S,2R,3S,5R,6R,7S)-3-(benzoyloxy)-2-(methoxycarbonyl)-8-methyl-8-azabicyclo[3.2.1]octane-6,7-diyl bis(4-(methylthio)benzoate) (6)

To a stirred solution of olefin **4** in acetone (0.03 M), H_2O (0.10 M), and TMNO dehydrate (2 eq) had OsO_4 (2.5 wt. % in t-BuOH) (0.05 eq) added dropwise, and the reaction was stirred for 18 hrs at rt. The reaction mixture was concentrated. Extracted with EtOAc, washed with water, brine and dried over $MgSO_4$, followed by filtration and concentration *in vacuo*. The residue was taken on to the next step without further purification.

To a stirred solution of the crude diol, and DMAP (2.2 eq) in CH_2Cl_2 (0.05 M) had 4-(Methylthio)benzoyl chloride (2.1 eq) added at 0°C and the reaction warmed up to rt. The reaction was stirred at this temperature for 8 h. After completion, the reaction was quenched with water, extracted with CH_2Cl_2, washed with water, brine, and dried over $MgSO_4$, followed by filtration and concentration *in vacuo*. The residue was purified with flash chromatography. (3:1 - 1:1 Hexanes:EtOAc) The product was obtained as a clear oil in 38% over two steps.

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To carbamate **5** in CH_2Cl_2(0.05 M) was added TFA (10 eq), and the reaction was stirred for 8 h at rt. The reaction mixture was concentrated *in vacuo*. NH_4OH was added until the solution was basic, then the residue was extracted with EtOAc, washed with water, brine, dried over $MgSO_4$, followed by filtration and concentration *in vacuo*. The residue was taken on to the next step without further purification.

To a stirred a solution of the crude secondary amine, formaldehyde (37 wt. % in H_2O solution) (1.5 eq) in acetonitrile (0.10 M) along with a flake of bromocresol green had $NaCNBH_3$ (1.5 eq) added. Acetic acid was added periodically over 3 h to maintain a pH of 4 until completion of the reaction. $NaHCO_3$(s) was added, followed by addition of 1 M NaOH until the solution was basic. The residue was extracted with EtOAc, washed with water and brine, dried over $MgSO_4$, followed by filtration and concentration *in vacuo*. The product was purified by preparative TLC. (1:1 Hexanes:EtOAc) The product was obtained as a white solid in 29% over two steps.

^1H NMR (300 MHz, $CDCl_3$) δ 2.20 (br, 1 H), 2.55 (br, 1 H) 2.50 (s, 6 H), 2.78 (s, 3 H), 3.40 (br, 1 H), 3.64 (br, 1 H), 3.74 (s, 3 H), 3.93 (br, 1 H), 5.15 (m, 1 H), 5.69 (s, 2 H), 7.10 (dd, J = 8.4 Hz, J= 3.6 Hz, 4 H), 7.45 (t, J = 7.2 Hz, 3 H), 7.55 (t, J = 7.2 Hz, 2 H), 7.76 (dd, J = 2.7 Hz, J = 8.3, 2 H), 8.06 (d, J = 7.7 Hz, 2 H). m/z: 636 $[M+H]^+$, 151.

2.2.4 Experimental procedure for competition sensing mode

Microcantilevers were cleaned using Piranha solution (70% H_2SO_4 and 30% H_2O_2), and immersed into 200 μM of thiolated cocaine molecules in ethyl acetate for 15 h in order to form monolayers of thiolated cocaine on gold surface of the sensing cantilever. Thiol-cocaine coated microcantilevers were incubated in 400 μM aptamer solution for 30

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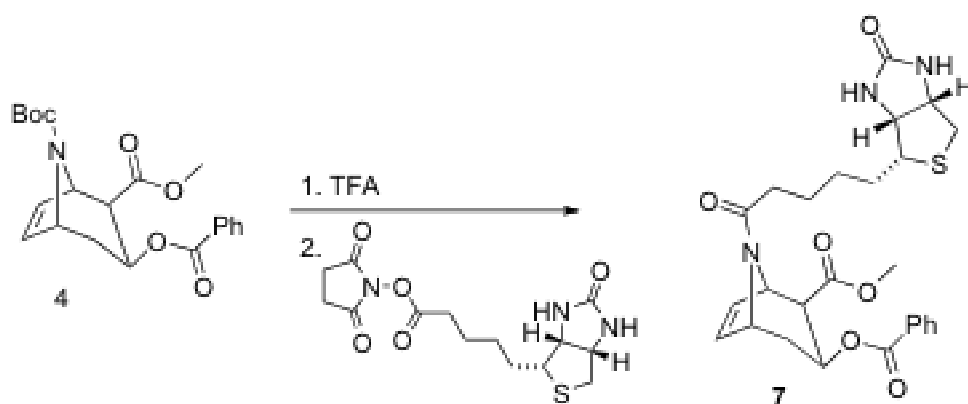
minutes such that aptamer-cocaine affinity complexes are formed on the cantilevers (as schematically depicted in Fig 4). The reference cantilevers were coated with the scrambled DNA sequence of same length and base composition as the cocaine aptamer so that exposure to cocaine molecules leads to affinity complex dissociation only on the sensing surface.

The sensing and reference cantilevers were mounted in the surface stress sensor and sensor response was measured for exposure to cocaine solutions of concentration varying from 1 to 200 μM in PBS (pH 7.4). After the sensing experiment, the sensing and reference cantilevers were regenerated through heating in deionized water at 80 °C and each cantilever was reused three times for the sensing experiments.

2.3 Selection of variant DNA aptamers with specific affinity to cocaine and cocaine metabolites:

2.3.1 Synthesis of biotinylated and cocaine and cocaine metabolites for SELEX

Cocaine metabolites and degradation products will be attached to biotin for use in selecting aptamers.



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(1R,2R,3S,5R)-methyl 3-(benzoyloxy)-8-(5-((3aR,4R,6aS)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoyl)-8-azabicyclo[3.2.1]oct-6-ene-2-carboxylate (7)

To carbamate **4** in CH_2Cl_2(0.05 M) was added TFA (10 eq) and the reaction was stirred for 8 h at rt. The reaction mixture was concentrated *in vacuo*. NH_4OH was added until the solution was basic, then the residue was extracted with EtOAc. Washed with water, brine, and dried over $MgSO_4$, followed by filtration and concentration *in vacuo*. The residue was taken on to the next step without further purification.

To a stirred solution of the crude secondary amine, Et_3N in DMF was added NHS-biotin. The reaction was stirred at 45°C for 8 h. The reaction mixture was concentrated *in vacuo*. The product was purified by preparative TLC (98:2 EtOAc:MeOH). The product **7** was obtained as a white solid in 5% over two steps.

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.80-1.80 (m, 10 H), 2.26 (br, 1 H), 2.42 (br, 1 H), 2.81 (m, 3 H), 3.06 (m, 2 H), 3.51 (m, 1 H), 3.67 (d, J = 8.4 Hz 1 H), 4.90 (m, 1 H), 5.39 (m, 2 H), 5.45 (m, 1 H), 6.27 (br, 2H), 7.45 (t, J = 7.6 Hz, 2 H), 7.56 (d, J = 8.0 Hz, 1 H), 7.99 (d, J = 6.4 Hz, 2H). m/z: 391 $[M-PhCO_2H]^+$, 288, 149

2.3.2 Selection of variant DNA aptamers through SELEX

Aptamer selection is being performed through the process of SELEX (**S**ystematic **E**volution of **L**igands by **Ex**ponential enrichment); a reiterative process of DNA amplification and selection [Fig 2]. For PCR, biotinylated DNA primers are used, which provides a means to select the single stranded DNA aptamers (the biotinylated strand being removed leaving a single-stranded non-biotinylated strand). For RNA aptamers

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the DNA primer will contain the sequence of the T7 polymerase promoter to provide a means of producing RNA from the PCR-amplified DNA. Currently single-stranded DNA aptamers are being selected and streptavidin is used to capture the target molecules by way of their biotin tags. The streptavidin-bound target molecules are collected on filters along with the bound ssDNAs. Recent mathematical modeling studies by Levine and Nilsen-Hamilton have provided guidance for aspects of the selection process ((Levine and Nilsen-Hamilton 2007; Seo, Chen et al. 2010), Seo et al., in preparation). These parameters (number of positive and negative selections, ration of RNA/target, rate of increasing RNA/target ratio) are being used for selection. We are also interested in obtaining aptamers with different ranges of specificity to cocaine and its derivatives. Therefore, the selection will be split into several portions at about the 5th or 6th round when it is expected that a binding population is evolving and each of these portions treated with a different combination of cocaine and metabolites to select for aptamers with different specificities for cocaine and its metabolites.

3 Results

3.1 Biochemical characterization of the available DNA aptamer and cocaine molecules

A variety of assays were investigated to interrogate the binding of target to the aptamers. The only reliable method was shown to be ITC (Fig. 5). Using this method we found that the affinity of the currently available cocaine aptamers is between 11 and 22 μM. We also found that the aptamer's binding affinity is highly sensitive to the presence of acetonitrile, which is the solvent of available cocaine standard solutions

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(Fig. 5(A)). This observation probably explains why the aptamer has been reported to have a range of affinities (Stojanovic, de Prada et al. 2001; White, Phares et al. 2008; Neves, Reinstein et al. 2010).

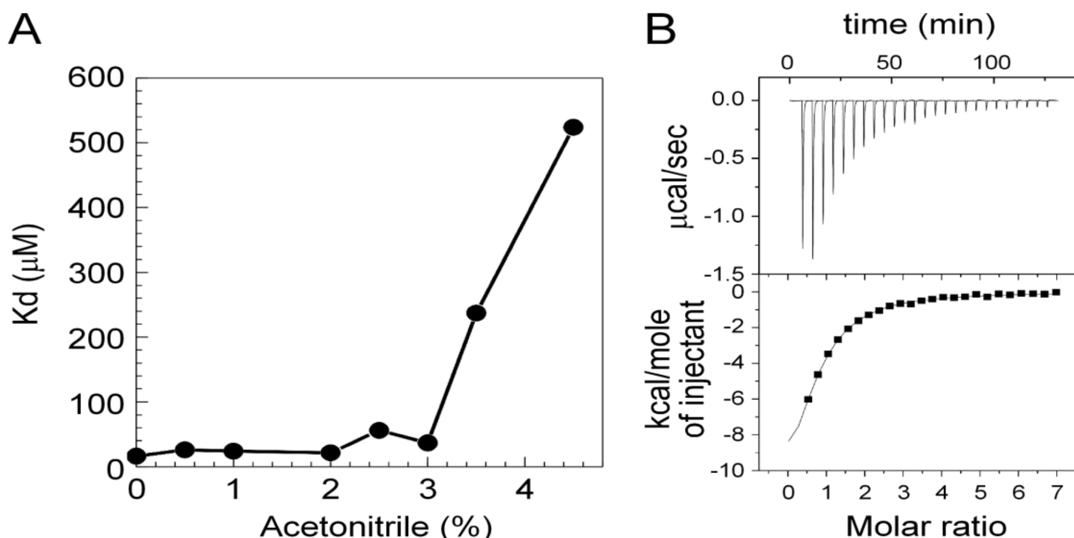

Fig. 5 Isothermal titration calorimetry (ITC) was performed to determine the equilibrium constants for the cocaine aptamer **(A)**: Effect of acetonitrile on the dissociation constant of the cocaine aptamer. **(B)** The affinity of the cocaine aptamer for cocaine was measured by

With the goal of increasing the aptamer affinity for cocaine, we explored the effect of modifications to the aptamer sequence and discovered that one modification resulted in an increase in aptamer affinity of about 8-fold to 6 $x10^6$ M^{-1}. The affinity of this aptamer for its ligand can be determined by ITC and by changes in fluorescence quenching of 2-AP in the aptamer. The disassociation constant for the

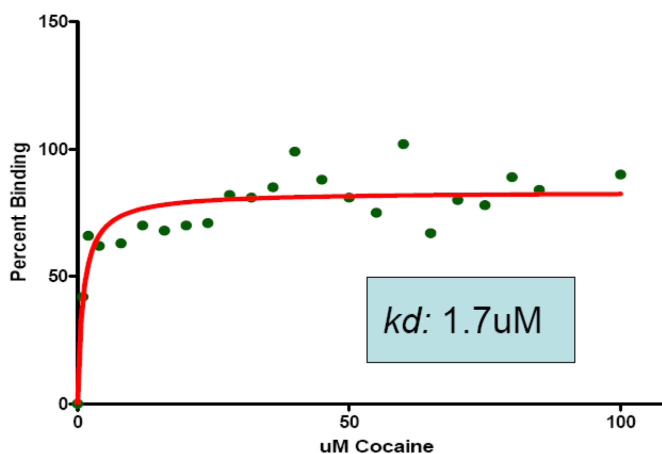

Fig. 6: Binding efficiency and dissociation constant determined using fluorescence quenching experiments for the modified aptamer

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modified aptamer determined from the fluorescence quenching is shown in Fig. 6.

3.2 Characterization of MC sensors functionalized with available DNA aptamers

3.2.1 Results for conventional sensing mode

Differential surface stress developed on the functionalized cantilevers was measured as a function of cocaine concentrations in the PBS buffer. Sensor response was measured for 10 different cocaine concentrations - 0 (pure buffer), 25, 50, 75, 100, 150, 200, 300, 400, 500 μM. At each concentration, the sensing experiments were repeated three times to assess the repeatability of the experimental measurement. Two typical experimental observations of surface stress development during direct sensing corresponding to a cocaine concentration of 50 μM and pure buffer are plotted in Fig 7. As shown in the Fig 7, the surface stress starts developing as soon as the cocaine solution is injected in the sensor and saturates to a constant value after a period of approximately 25 minutes. In the case of pure buffer, there is no surface stress buildup indicating the specificity of sensor response to cocaine solution.

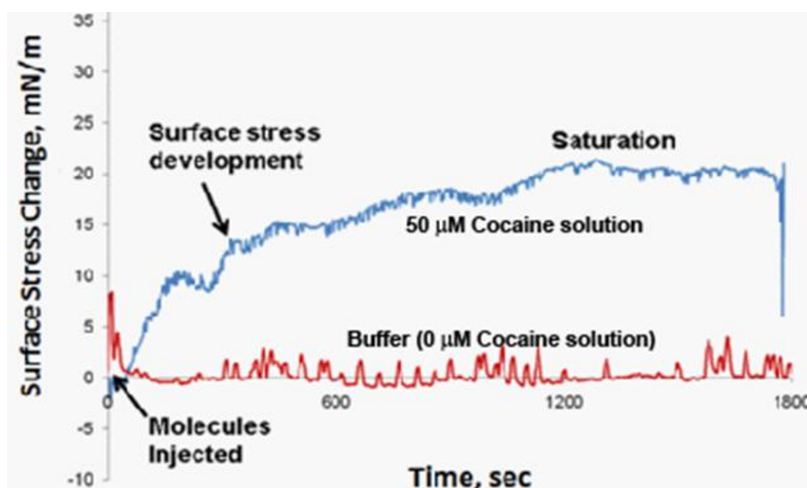

Fig 7: Surface stress build up during direct sensing for two different cocaine concentrations (50 μM and and pure buffer (0μM))

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The saturated surface stress values were recorded for each direct sensing experiment and are plotted as a function of cocaine concentration in Fig 8. Saturated surface stress increases as the cocaine concentration during the sensing experiment is increased. At the lower concentration range from 25 to 100 μM, surface stress values increase almost proportionally with concentration. However as the cocaine concentration is increased beyond 100 μM, the surface stress values demonstrate a weaker dependence on the cocaine concentration. Based on above results and a sensitivity of 2 mN/m for the surface stress measurement, the lowest detectable threshold for the cocaine concentration is estimated to be 5 μM under the conventional sensing mode.

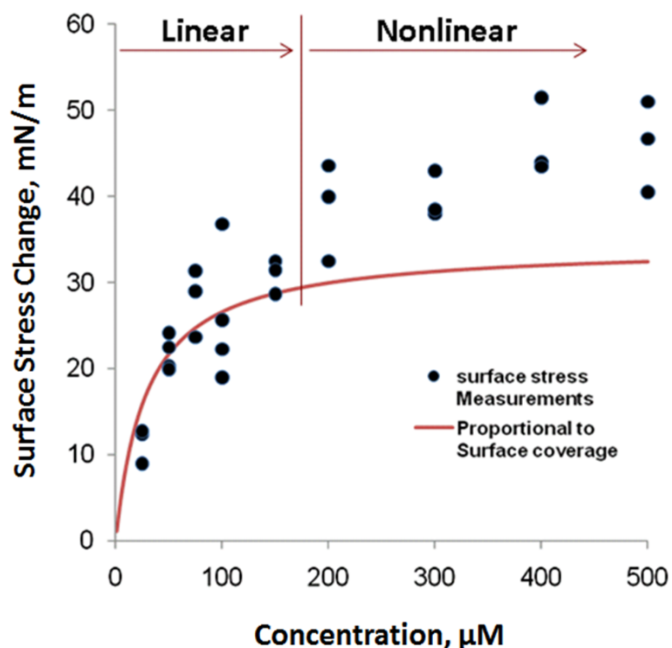

Fig. 8: Saturated surface stress values as a function of cocaine concentrations measured during direct sensing.

In order to understand the relationship between aptamer cocaine binding and surface stress development, the dissociation constant measured using ITC (see biochemical characterization section) was used to predict the surface coverage of cocaine-aptamer complexes for each cocaine concentration. White et. al. (2008) have shown the functionalization procedure followed here leads to initial cocaine aptamer coverage equivalent to 4.4×10^{12} molecules/cm^2 The dissociation constant is utilized

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to estimate the fraction of initial aptamer molecules that form the cocaine aptamer complexes as well as the relative distance between the cocaine-aptamer complexes. Measured surface stress values were assumed to be directly proportional to coverage of cocaine-aptamer complexes. The fit to measured surface stress under this assumption is also plotted in Fig 8. The separation distances are normalized by the separation between the immobilized aptamers. The proportionality assumption describes the measured surface stress values lower values of cocaine concentrations (till 200 μM, marked as linear regime in the curve) but at the higher concentrations, the measured values keep on increasing whereas the predicted values on the surface coverage remains nearly constant (indicated as the non-linear regime).

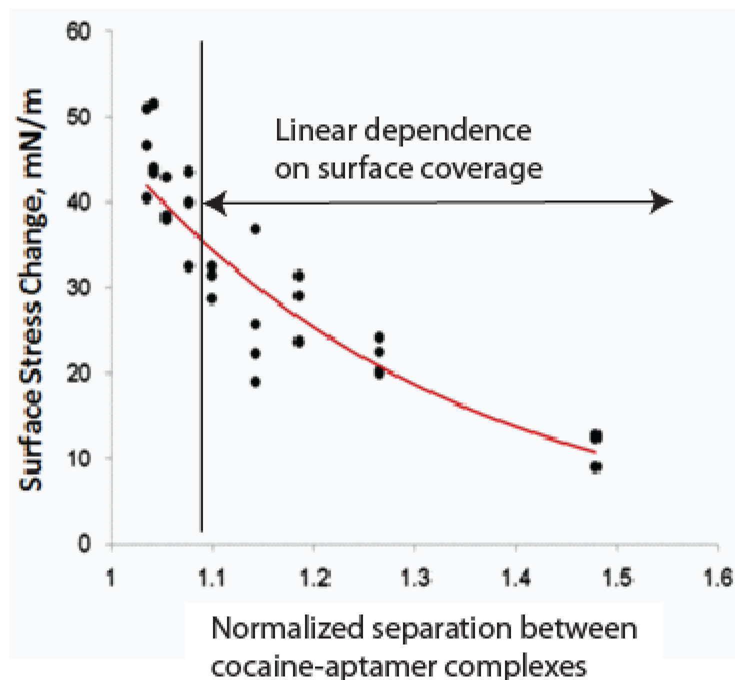

Fig. 9: Surface stress as a function of normalized separation between cocaine-aptamer complexes.

The measured surface stress is plotted as a function of the calculated separation between the aptamer-cocaine complexes in Fig 9. As indicated on the two plots, the surface stress developed is proportional to coverage of cocaine-aptamer complexes when the aptamer complexes are spaced at distance larger than 1.1 times the initial separation between

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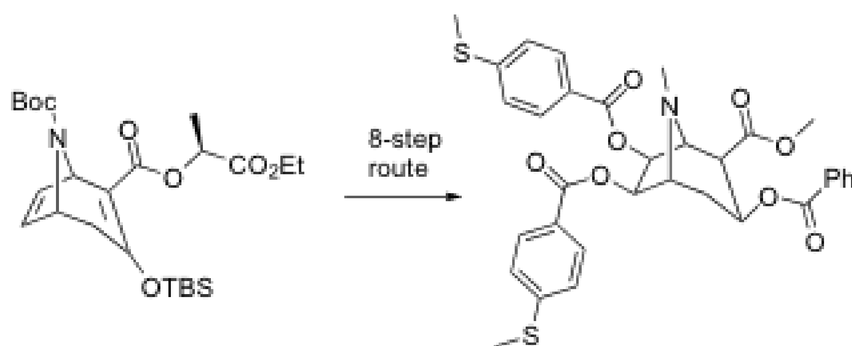
the immobilized aptamers. As the cocaine-aptamer complex separation becomes smaller than 1.1 times the immobilized aptamer separation, developed surface stress is no longer proportion to estimated surface coverage. This transition may be due to the intermolecular repulsion between the cocaine-aptamer complexes. The data presented here can be utilized to estimate the functional form of the interchain repulsion between the cocaine-aptamer complexes and also to determine the mechanism underlying the surface stress generation.

3.2.2 Synthesis of thiolated cocaine molecules



The eight-step synthesis of the thiolated cocaine molecule shown above generated a single enantiomer compound. Our thiolated cocaine molecule has two points of attachment (via the two sulfur atoms) to the cantilever surface. The site of the connection of the sulfur containing benzoate ester to the cocaine skeleton was designed to best present the key functional groups in the cocaine molecule (methyl ester, benzoate and amine) to the aptamer.

The synthesis began with the known enol ether ester shown above. The starting material was generated in one step via a cycloaddition to N-BOC-pyrrole. We have optimized the yields for each step in the synthesis. The synthesis is direct and

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reproducible. This synthetic route is now being adapted for the preparation of cocaine metabolites. That work will be completed by the end of the project period.

3.2.3 Results for competition sensing mode

In the competition sensing mode, the sensing cantilevers were coated with thiolated cocaine molecules and incubated in cocaine-aptamer solution such that exposure to cocaine solution leads to disassociation of cocaine-aptamer complexes on the cantilever surface. According to the initial hypothesis, the surface stress change was expected to be dependent on the concentration of cocaine solution. The measured surface stress change corresponding to cocaine concentration of 50 μM measured under the competition sensing mode is shown in Fig. 10. It is important to note that surface stress changes during competition sensing mode are of opposite sign as compared to direct sensing mode. Only the magnitude of the surface stress change is plotted in Fig 10 in order to facilitate the comparison with direct sensing mode. Under the competition sensing mode, the final magnitude of the saturated surface stress change is considerably larger than that measured for direct sensing

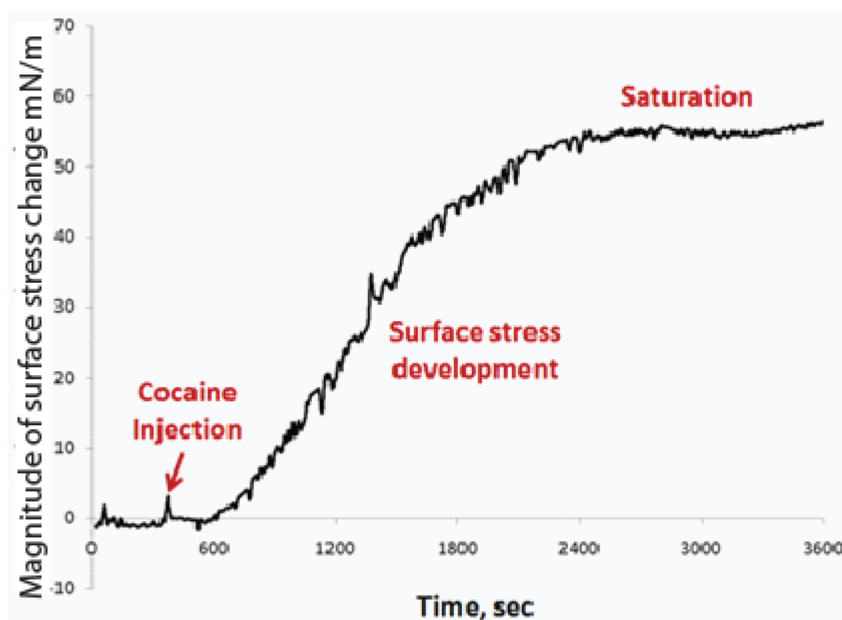

Fig. 10: Measured magnitude of surface stress change under competition sensing mode for 50 μM cocaine concentration.

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mode (see Fig. 7) for the same concentration. However, the initial experiments showed that the magnitude of saturated surface stress change was almost independent of the cocaine concentration introduced into the sensor. In addition, the surface stress change did not saturate till almost 2 h after injection of the cocaine solution in the sensor.

It was also interesting to observe that the initial slope of the surface stress change seemed to be dependent on the concentration of cocaine solution introduced into the sensor. Initial slope of the surface stress change during competition sensing mode was measured for seven different cocaine concentrations: 0 (pure buffer), 1, 2, 5, 10, 50 and 100 μM. The normalized slope of the surface stress buildup during the first 20 min after introduction of cocaine, is plotted as a function of cocaine concentration for indirect sensing in Fig 11. Observed values of the normalized slope demonstrate a strong dependence on the concentration in the range from 0 -10 μM cocaine. Given the sensitivity of surface stress measurements and the experimental results in Fig. 11, the lowest detectable cocaine concentration in

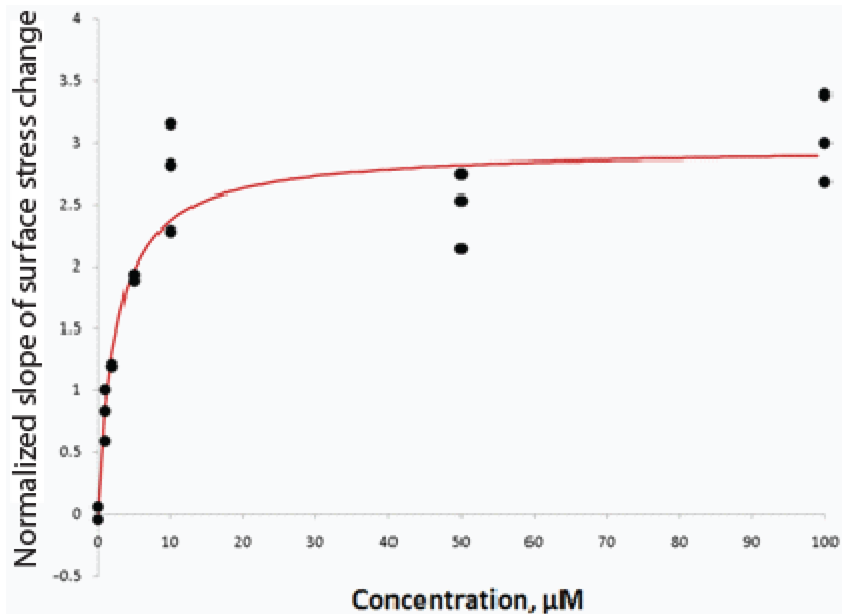

Fig. 11: Normalized slope of surface stress development during the first 20 minute after introduction of cocaine solution under competition sensing mode.

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competition sensing mode is approximated to be 200 nM. To the best of our knowledge, this is the lowest detectable threshold reported for cocaine aptamer based sensing.

The large magnitude of the saturated surface stress change observed during the competition sensing mode indicated that aptamer-cocaine complex dissociation was responsible for the surface stress change as removal of larger aptamer molecules leads to larger deformation of the surface. The long time period required to reach the saturation value as well as the constant magnitude of saturated surface stress change irrespective of different cocaine concentrations indicates that the equilibrium is only achieved when significant number of cocaine-aptamer complexes on the cantilever surfaces have undergone dissociation. Dependence of the initial slope of surface stress change on the cocaine concentration indicates that initial rate of disassociation for cocaine-aptamer complexes on the cantilever surface is proportional to the number of cocaine molecules in solution.

3.3 Selection of additional DNA aptamers for cocaine and its metabolites

3.3.1 Synthesis of biotinylated and cocaine and cocaine metabolites for SELEX

Cocaine metabolites and degradation products will be attached to biotin for use in selecting aptamers. The attachment of biotin to the cocaine molecule is shown below. This compound was prepared in one step from an intermediate in the synthesis of the thiolated cocaine molecule described above.

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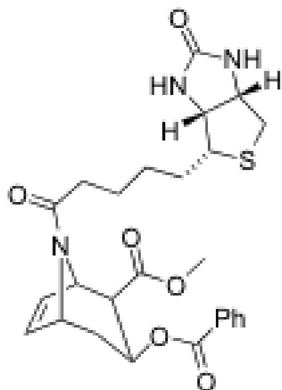
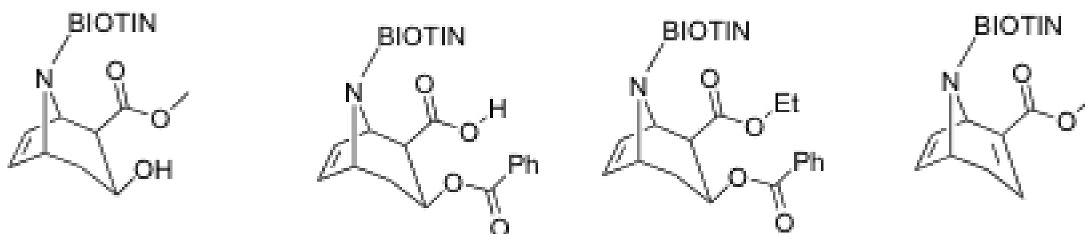


By the end of the project period, the Kraus group will have prepared the following cocaine metabolite-biotin adducts to be used in aptamer selection. With these compounds, SELEX experiments will be performed to obtain aptamers that bind to specific metabolites or to cocaine. Currently SELEX experiments are underway in the Nilsen-Hamilton group. Although it is too early to determine if an aptamer has been selected, this group has a record of successfully selecting aptamers with the last four attempts at obtaining aptamers to different molecules having been successful. The group attributes its success to experience and optimization of the procedure based on mathematical modeling.



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4 Conclusions

4.1 Discussion of Findings

In the ***first phase*** of the project, we have: **1)** Optimized the available DNA aptamer to improve its sensitivity for binding with cocaine; **2)** Characterized the sensitivity and specificity of MC-based sensors functionalized with an available DNA aptamer that has specific affinity for cocaine; and **3)** Initiated the selection additional variant DNA aptamers with selective sensitivities to cocaine and its metabolites.

The existing cocaine aptamer was tested by isothermal titration calorimetry to determine its affinity for cocaine. From these studies we found that acetonitrile, the common solvent for cocaine standards, causes a significant decrease in affinity of the aptamer for cocaine. By maintaining the acetonitrile concentration below 2% we realize a 5-fold increase in sensitivity of the aptamer compared with published data and with results from our own studies.

Our second approach to increasing the sensitivity of the aptamer has been to modify the aptamer sequence in portions of the molecule surrounding the presumed cocaine-binding site. This approach was particularly effective and has resulted in aptamer variants containing some unnatural bases with increased sensitivity for cocaine of about 8-fold. In combination with controlling the acetonitrile concentration these aptamer variants are about 40-fold more sensitive than reported so far for a cocaine aptamer.

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Differential surface stress sensor was utilized in both conventional as well as competition sensing mode. In the conventional mode, the surface stress generated due to binding of cocaine molecules to the existing cocaine aptamer was determined. As reported above, the cocaine aptamer/cocaine molecule binding was found to be dependent on the acetonitrile concentration. The solutions for the sensing experiments were prepared to ensure that acetonitrile concentration was below 2% for all cases and concentrations of cocaine molecules were varied from 25 μM to 500 μM. Surface stress changes from 9 to 51 mN/m were measured for the range of cocaine concentrations. Given a threshold sensitivity of 5 mN/m for surface stress measurements, this sensor is able to detect cocaine with the lowest detectable concentration down to 5 μM in room temperature under the conventional sensing mode.

In the competition sensing mode, surface stress generated due to dissociation of aptamer/cocaine complexes was measured. A thiol modified cocaine molecule was synthesized to attach the cocaine molecules on the microcantilevers. the modified cocaine molecules were tethered to the gold surface using the sulphur link and immersed in a solution of the cocaine aptamers such that cantilever surface is covered with cocaine/aptamer affinity complexes. The functionalized cantilevers were then exposed to different concentrations of cocaine molecules and cantilever bending was monitored. The experimental results show that magnitude of bending in the functionalized cantilevers is not correlated with the cocaine concentration however the rate of bending demonstrates a good correlation with cocaine concentration. More importantly, the rate of bending correlation indicates that the lowest detectable cocaine

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concentration in competition sensing mode is approximately 200 nM. To the best of our knowledge, this is the lowest detectable threshold reported for currently known cocaine aptamer based sensing.

We have synthesized biotin attached cocaine molecules for the selection of aptamers. At present, the Kraus group is preparing cocaine metabolite-biotin adducts to be used in aptamer selection. The Nilsen-Hamilton group is currently using the biotin attached cocaine molecule for selection of aptamers that have specific binding to cocaine. Aptamer selection is being performed through the process of SELEX (**S**ystematic **E**volution of **L**igands by **Ex**ponential enrichment); a reiterative process of DNA amplification and selection.

4.2 Implications for policy and practice

Current sensor systems require extensive sample preparation and/or specialized instrumentation to identify molecules of controlled substances with high specificity and sensitivity (Stojanovic, de Prada et al. 2001). Current methods of screening biological samples for drugs of abuse employ immunoassays, which require antibodies rather than aptamers. Of the various enzymatic immunoassay techniques available enzyme multiplied immunoassay technique (EMIT) and enzyme-linked immunosorbent assay (ELISA) are the two predominant techniques current found in forensic applications. These systems are highly automated and can run numerous samples within a single sequence; however, screening for different drug classes, such as phenethylamines and opiates, requires different reagents and thus separate samplings. If a single sample is to be screened for an array of drug classes, the analysis time increases with the number

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of drug classes screened. An additional disadvantage of the EMIT and ELISA assays that are commonly used for forensic samples is the physical size of the instrumentation. Instruments on the order of cabinet size render field analyses of samples impractical. Aptamer-based MC sensors will provide an invaluable tool for forensic science because of its portability, capability for detection and identification with high sensitivity and specificity. Forensic scientists such as Dr. James Siefert (Forensic Science Laboratory, Michigan State Police, 989-777-9300) believe that aptamer-based sensors have the potential of contributing valuable new technology for laboratory and field applications.

Experimental results presented here have demonstrated a proof-of-concept for cocaine detection with aptamer-functionalized microcantilevers at very low concentrations. Aptamers are promising candidates for MC sensors because: (a) they recognize their target molecules with high specificity and affinity; (b) aptamer specificity can be "tuned" according to the selection strategy, (c) binding with targets is often accompanied by a structural change that can be utilized for detection; (d) binding reactions are reversible so that the receptor layers can be recharged for detection; (e) aptamers are much more stable than antibodies at ambient temperatures and can be reused in the same sensor many times. A sensing approach based on microcantilevers functionalized with aptamers will provide an invaluable tool for forensic science because of its portability, capability for detection and capability for identification of analytes with high sensitivity and specificity.

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4.3 Implications for further research

A severe limitation of the ssDNA cocaine aptamer that is currently available is its low affinity for target. Thus, although this aptamer is useful for demonstrating proof-of-concept, aptamers with higher affinities will be needed to increase the sensitivity of the cantilever sensors. Our experimental results from **the first phase** of this project have presented a novel modification of the aptamer structure that results in an order of magnitude improvement in affinity for cocaine. Increased affinity for an analyte is often associated with better discrimination of molecular structure and therefore these modified aptamers are expected to be more specific for cocaine. The modified aptamers need to be tested for their relative affinities for cocaine compared with various cocaine metabolites and other derivatives.

Aptamer functionalized microcantilevers used in the novel competition sensing mode have demonstrated an order of magnitude improvement in threshold sensitivity over the conventional sensing mode. However, the current sensing experiments have utilized only the currently available DNA aptamer. Further work is required to test the sensitivity of cocaine detection with the modified aptamer that demonstrates greater affinity. In addition, the specificity of cocaine detection needs to be tested in biological matrices as well as in real world samples.

We expect to select DNA aptamers with higher affinities to cocaine and its metabolites through the ongoing SELEX procedures. Increased accuracy of cocaine identification can also be achieved with a panel of aptamers of different affinities and specificities for target molecules. Compared with DNA, RNA possesses an additional

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hydroxyl group on the ribose associated with each base that potentially provides more opportunity for interaction with target molecules and higher affinity binding than does DNA. Another approach to increase the sensitivity of cocaine detection, will be to select RNA as well as DNA aptamers that recognize cocaine and its derivatives.

In the ***second phase*** of the project, we propose to: 1) characterize the threshold sensitivity for cocaine detection using microcantilevers functionalized with the selected aptamers with higher affinity; 2) calibrate the sensor performance for detection of cocaine and its metabolites in biological matrices and simulated “real world” samples, 3) Select for RNA aptamers with specificity and high affinities to cocaine, cocaine metabolites and methamphetamine.

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6 Dissemination of Research Findings

Results of our current work have been reported through presentations at NIJ grantees meeting and 47th annual technical meeting of the Society of Engineering Science held at Iowa State University, Ames, Iowa. We have prepared two invention disclosures based on: 1) 2-Aminopurine modification of aptamer that results in higher affinity for cocaine; and 2) Competition sensing mode for MC sensor based detection of cocaine that results in higher threshold sensitivity. These invention disclosures have been filed through Ames Laboratory which houses the Midwest Forensic Resource Center (MFRC).

We plan to disseminate the research results through peer-reviewed journal publications including the Journal of Forensic Sciences and presentations at annual meetings of American Academy of Forensic Science (AAFS) and Mid-western Association of Forensic Scientists (MAFS). To facilitate wide dissemination of research finding in the forensic community, we will continue to work with MFRC to share the developed sensor technology with partnering crime laboratories. We will also communicate research results to the Journal of Clandestine Lab Investigating Chemists Association, a national publication only available to law enforcement scientists.