

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing the burden, to the Department of Defense, Executive Service and Communications Directorate (0704-0188). Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.					
PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ORGANIZATION.					
1. REPORT DATE (DD-MM-YYYY) 9/29/2011		2. REPORT TYPE Final		3. DATES COVERED (From - To) 2/1/2008-1/31/2011	
4. TITLE AND SUBTITLE Ready-to-use Aptamer Biosensors (RAB) for DNT and RDX				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER N00014-08-1-0469	
				5c. PROGRAM ELEMENT NUMBER	
				5d. PROJECT NUMBER	
6. AUTHOR(S) Soh, Hyongsok Tom				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California, Santa Barbara 3227 Cheadle Hall Santa Barbara, CA 93106-2050				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Office of Naval Research 875 North Randolph Street Arlington, VA 22203-1995				10. SPONSOR/MONITOR'S ACRONYM(S) ONR	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution is unlimited.					
13. SUPPLEMENTARY NOTES					
20111012013					
14. ABSTRACT See Attached					
15. SUBJECT TERMS Aptamers, Microfluidics, Directed Evolution, Smart Molecules					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 14	19a. NAME OF RESPONSIBLE PERSON Elizabeth Jensen
a. REPORT UU	b. ABSTRACT UU	c. THIS PAGE UU			19b. TELEPHONE NUMBER (Include area code) 805-893-4856

Abstract:

Abstract: Aptamers are nucleic acid-based reagents that bind to target molecules with high affinity and specificity. However, methods for generating aptamers from random combinatorial libraries (e.g., SELEX) are often labor-intensive and time-consuming. To address this problem, we have recently demonstrated the microfluidic SELEX (M-SELEX) technology that can accelerate aptamer isolation by enabling highly stringent selection conditions through the use of very small amounts of target molecules (X. Lou *et al*, PNAS 2009). In this work, we utilize the M-SELEX technology to generate a novel class of Self-Reporting Aptamers (SRAs) which are capable of a) specifically bind to their target, b) undergo conformational change triggered by this binding event, and c) activate a DNA enzyme moiety within the aptamer structure to fluorescently report the binding event. This technique is especially useful for the detection of small molecules because it does not require immobilization of the target molecules on solid support. As a proof of concept, in the last reporting period, we have demonstrated the isolation of SRAs that specifically bind to a target protein with low nanomolar affinity (S. Oh *et al.*, in review), and we report our progress in generating SRAs against small molecule targets.

Key words: Aptamers, Microfluidics, Directed Evolution, Smart Molecules

Ready-to-use Aptamer Biosensors for DNT and RDX

PI : H.T. Soh & Y. Xiao, University of California – Santa Barbara

Award Number: N000140810469

Scientific and Technical Objectives:

Aptamers are a class of synthetic, nucleic acid-based affinity reagents that are capable of specifically binding to a range of targets including small molecules, proteins, viruses, and whole cells. Aptamers offer many useful characteristics; these thermo-stable molecules are chemically synthesized, readily modified, and possess high affinity and specificity toward the target molecule, which makes them excellent candidates for biosensors. Previously, our group has demonstrated highly efficient aptamer selection methods using microfluidics technology (Lou et al, PNAS, 2009). In addition, we have shown the capability to perform real time detection of small molecules directly in complex sample matrices, using the aptamers that undergo “binding-induced structure-switching” (J. Swensen *et al.* JACS, 2009). However, in order to create such sensors, the challenge lies in the fact that previous methods of aptamer selection do not yield molecules with the desired switching function.

The main goal of this project is the development of a general strategy to generate such structure-switching biosensors that can detect small molecule targets. Towards that end, in this reporting period, we have made two major advancements, based on our work on the self-reporting aptamers (SRAs) (Oh et al. PNAS 2010). First, we have successfully selected SRAs that target small molecules and report the binding event through a colorimetric readout (module 1). More specifically, these new Self-Reporting Aptamers (SRAs) are capable of 1) specifically binding to their small molecule targets (e.g. ATP), 2) undergoing conformational changes during this binding event, and 3) activating a DNA enzyme moiety to catalyze peroxidation reaction, resulting in the color change as a read-out mechanism.

Secondly, we have developed a high throughput aptamer generation technology that integrates microfluidic aptamer selection (M-SELEX), next-generation sequencing and massively-parallel characterization through aptamer microarrays (module 2). We believe this combined capability to rapidly screen for high affinity aptamers provides promising applications in point-of-care diagnostics and rapid target detection in the field, which is of significant importance to U.S. Naval security.

Approach

Module 1. Development of Self-Reporting Aptamers (SRAs) targeting small molecules: In order to create a “smart” biosensor molecule that reports the presence of small molecule targets, the sensor must be capable of coupling the specific binding event with sensor signal transduction. Previously, post-selection modification of the aptamers sequences was performed to achieve this function. Unfortunately, this process is based on trial-and-error, and the resulting aptamers possesses significantly inferior affinity and specificity to the target molecule. Thus, a novel method of “screening” for this function is critically needed.

Toward this end, based on our previous work in targeting proteins, we developed an innovative microfluidic-screening method to directly generate SRA molecules to small molecules. As a model, we have chosen ATP as the target during the development of this technology. More specifically, the SRA molecules have the unique ability to adopt two different structures (Fig 1): a “duplex structure” which aptamer hybridizes with its antisense strand (Fig 1, left) and a “complex structure” which aptamer binds its specific target (middle). In the absence of target, aptamer stays its duplex structure. Upon target binding, the aptamer switches the structure from duplex to complex, which unmasks a hidden DNA enzyme that catalyzes the oxidation of ABTS upon intercalation with hemin. Our SRA molecules thus self-report the target-binding event via colorimetric signaling (right).

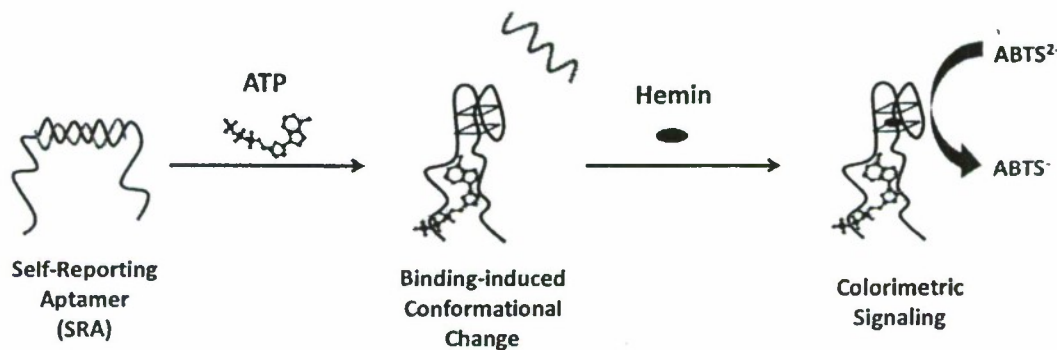


Fig 1. The signaling mechanism of SRA molecules. The specific binding between the SRA probe and the small molecule target (ATP) triggers a conformational change in the probe. This structural change “unmasks” the DNAzyme moiety hidden in the SRA sequence. Upon the addition of hemin, this catalyzes the process of ABTS oxidation for the colorimetric detection of target molecules.

Module 2. Integration of Microfluidic Selection, High Throughput Sequencing and Microarray Characterization. In vitro selection of aptamers can be divided into 3 main process: selection, sequencing and validation. With the use of Microfluidics technology (M-SELEX) and next-generation sequencing, we have addressed the first two bottlenecks during this project (Cho et al. PNAS 2010). However, the validation (i.e. measurement of affinity and specificity of the selected aptamers) remained as a slow, serial process. As a further step towards rapid and efficient identification of high-affinity aptamer sequences, we have successfully integrated the microfluidic selection, high throughput sequencing with microarray characterization (**MHM**) (Fig 2). More specifically, we developed the capability to efficiently generate high affinity aptamer pools within 3 rounds of selection using microfluidics, and to track the evolution of over $>10^6$ individual aptamer sequences via next generation DNA sequencing, and to characterize the binding affinity of $>10^5$ aptamer candidates ($> 10^5$) in a massively parallel manner. The details of experimental results are provided in subsequent sections.

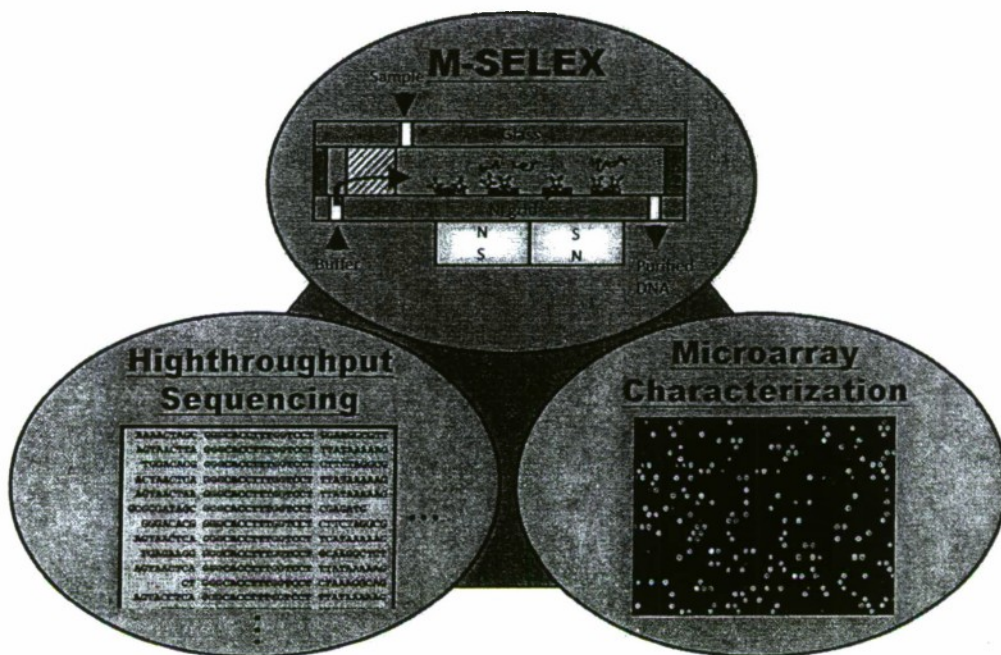


Fig 2. The integration of M-SELEX, high throughput sequencing and microarray characterization. This integration method to identify high affinity aptamers in high throughput begins with three rounds of M-SELEX. The enriched aptamer pool is sequenced in high throughput ($>10^6$ sequences), and the candidate aptamers are characterized in massively parallel manner ($>10^5$ sequences) using microarray technology.

Concise Accomplishments

As described in our proposal, the project is organized into three phases:

Phase 1 (year 1): Develop the Microfluidic-SELEX (M-SELEX) Platform

Phase 2 (year 2): Utilize M-SELEX to isolate SRAs targeting Human α -thrombin as a model target.

Phase 3 (year 3) : isolate SRAs for targeting a small molecule (ATP)

- Phase I has been successfully completed and the following journal articles have been published as a result.
 1. X. Lou, J. Qian, Y. Xiao, L. Viel, A. E. Gerdon, E. T. Lagally, P. Atzberger, T. M. Tarasow, A. J. Heeger, and H. T. Soh "Micromagnetic Selection of Aptamers in Microfluidic Channels," *Proceedings of the National Academy of Sciences, USA*, 106 (9) 2989-2994 (2009)
 2. Y. Xiao, K.J.I. Plakos, X. Lou, J. Qian, R.J. White, K.W. Plaxco, and H. T. Soh, "Fluorescence Detection of Single Nucleotide Polymorphism via a Single Self-Complementary, Triple-Stem DNA Probe" *Angewandte Chemie International Edition* (48) 4354-4358 (2009)
 3. J. Qian, X. Lou, Y. Zhang, Y. Xiao, and H.T. Soh, "Generation of Highly Specific Aptamers via Micromagnetic Selection" *Analytical Chemistry* Vol. (81) No. (13) 5490-5 (2009)
- Phase 2 had been successfully completed, and the following journal articles have been published as a result.
 1. Minseon Cho, Yi Xiao, Jeff Nie, Ron Stewart, Andrew Csordas, Seung Soo Oh, James A. Thomson, H. T. Soh, "Quantitative Selection of DNA Aptamers through Microfluidic Selection and High Throughput Sequencing," *Proceedings of the National Academy of Sciences, USA*, 107 (35) 15373-15378 (2010)
 2. Seung Soo Oh, Kory Plakos, Xinhui Lou, Yi Xiao, H. Tom Soh, "In Vitro Selection of Structure-Switching, Self-Reporting Aptamers," *Proceedings of the National Academy of Sciences, USA*, 107 (32) 14053-14058 (2010)
- Phase 3 has been successfully completed (see expanded accomplishment section below), and the following journal articles are in preparation
 1. Seung Soo Oh et al, "In vitro selection of multi-functional molecular machines," (in preparation)
 2. Minseon Cho et al., "Integration of microfluidic selection, high throughput sequencing with microarray characterization for massively parallel aptamer selection: (in preparation)"
- In addition, the PI (H.T. Soh) received two major honors:

2010 Guggenheim Fellowship (One of two recipients in Engineering)
2011 NIH Edward Nagy Award

Expanded Scientific Accomplishments

Module 1: Development of Self-Reporting Aptamers (SRAs) In order to generate the SRAs molecules, we first designed a random DNA library as shown in Figure 3. Each library molecule contains a central 18-base nucleotide sequence, which can fold into a G-quadruplex structure to form a DNA enzyme in the presence of hemin as shown in Fig 3 (red). This domain is flanked by two 17-base random sequence domains (blue), each further flanked by two specific 20-base primers (green) for polymerase chain reaction (PCR). During the development of this technology, we used ATP as the target as a proof of principle.

The selection scheme is shown in Fig 4, which began with an incubation step (Fig 4, step A & B) of the DNA library consisting of $\sim 10^{14}$ unique molecules with biotinylated antisense DNA strand. This antisense DNA is complementary with the DNAzyme sequence inserted in the library. The double-stranded (ds) DNA molecules were then attached to streptavidin-coated magnetic beads via biotin (step C). Next, the bead-library assemblies were challenged with the target. The single-stranded (ss) aptamer molecules capable of binding-induced conformation change were released and separated from the ds-DNA modified beads (step D). The separation step was performed on a Micro-Magnetic Separation chip (MMS) (step E). The selected aptamers were then PCR-amplified with biotinylated reverse primers (step F), and ss-DNA was generated as the library for next round (step G & H).

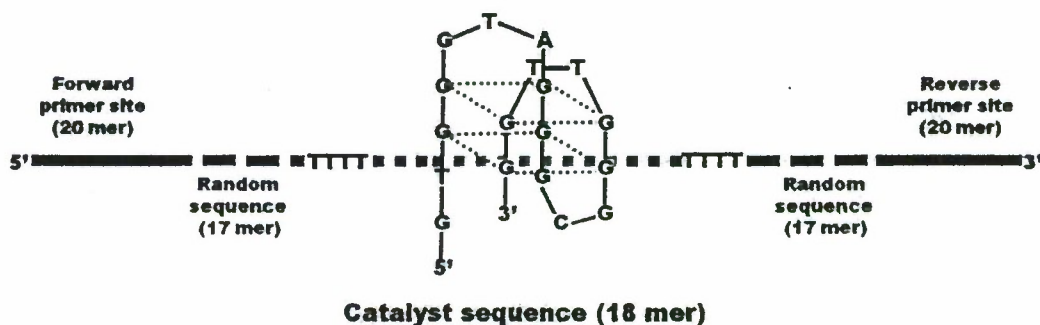


Fig 3. The design of SRA library. The structures includes the central quadruplex region that functions as the DNAzyme moiety.

We performed ten rounds of positive selection to obtain SRAs with affinity for ATP. To gradually increase the stringency, we systematically decreased ATP concentrations from 1 mM to 20 μ M during the selection process. In order to ensure that the SRAs are specific to ATP, we performed negative selection with 1 μ M streptavidin in rounds three through ten. We chose streptavidin as the target for negative selection because it was used for the immobilization of the aptamer library onto the beads, and also used to generate single-stranded products after PCR amplification. To monitor the convergence of the selection process, we used real-time PCR (RT-PCR) to measure the enrichment ratio of

DNA molecules eluted by ATP over those eluted by the selection buffer after each round. We amplified fractions of these DNA samples through an optimized PCR cycle, analysed them by gel electrophoresis and calculated the enrichment ratio from the intensity of bands. After the selection, we cloned individual SRA sequences from the pool via insertion into the pCR4-TOPO vector and transformation into competent bacterial cells. Sequencing of 45 randomly picked colonies revealed three dominant consensus sequences, which constituted 100 % of the population (Fig 5).

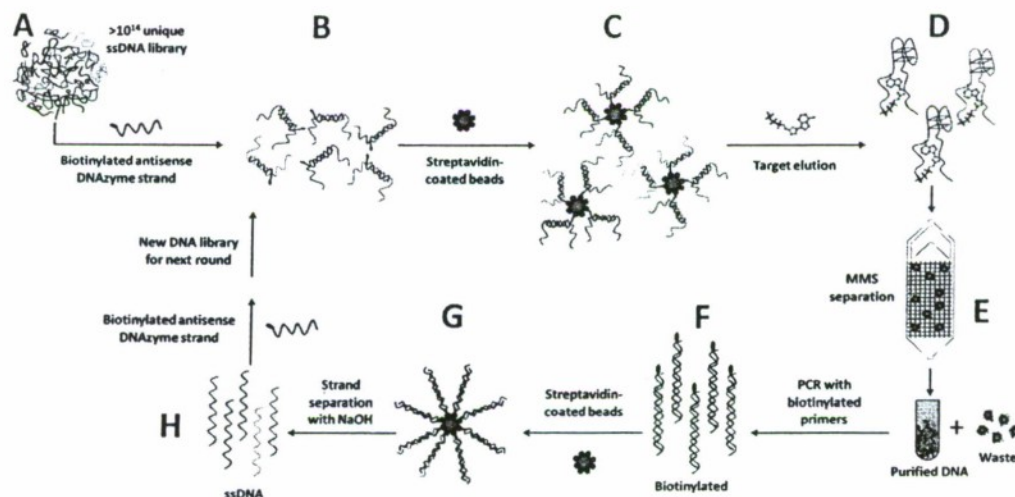


Fig 4. The overview of micro-fluidic selection process to select SRAs. A) Preparation of ssDNA library consisting of $\sim 10^{14}$ unique sequence. B) Incubation of the DNA library with the biotinylated antisense DNA strand. C) Immobilization of the ds-DNA to streptavidin-coated magnetic beads. D) Elution of SRAs molecules from beads upon challenging with thrombin. E) Magnetic separation of the selected SRAs molecules from beads with the micro-fluidic MMS chip. F) PCR amplification of the eluted SRAs molecules. G-H) Generation of single-stranded SRAs for the next round of selection.

Aptamer ID (Percentage)	Selected Sequence 5'-end	DNAzyme Sequence	Selected Sequence 3'-end
KP3 (58 %)	AGGCTCGTCAACTCGGCTTTT	GTGGGTAGGGCGGGTTGG	TTTCCGGCTTGAAGGAGACGA
KP4 (38%)	TTAATTCTGGGGGAGCCTTTT	GTGGGTAGGGCGGGTTGG	TTTTGCCCCGGAGGAGGAATT
KP5 (4%)	AAGCGAGATCAGACTACTTTT	GTAGGTAGGGCGGGTTGG	TTTACGGAGGTTTGAAGGAG

Fig 5. SRA sequences that recognize ATP.

We synthesized KP3 and KP4 sequences and measured their binding affinity to ATP. To do so, we prepared DNA/bead assemblies and challenged them with ATP at varying concentrations from 1 to 100 μM . The quantities of released SRAs were determined by RT-PCR with iQTM 5 multi-color detection system, and we determined the threshold cycle value (C_T) for each target concentration. Both KP3 and KP4 sequences exhibited high affinity for the ATP target (Fig 6). Assuming a 1:1 Langmuir binding model, we calculated the equilibrium binding

affinities (K_d) to be $14.2 \pm 2.2 \mu\text{M}$ and $12.2 \pm 3.3 \mu\text{M}$ for KP3 and KP4, respectively. The two SRA sequences were also specific to ATP. For example, KP 3 showed 5.5 and 7.4 fold higher affinity to ATP compared to GTP and CTP, respectively (Fig 6).

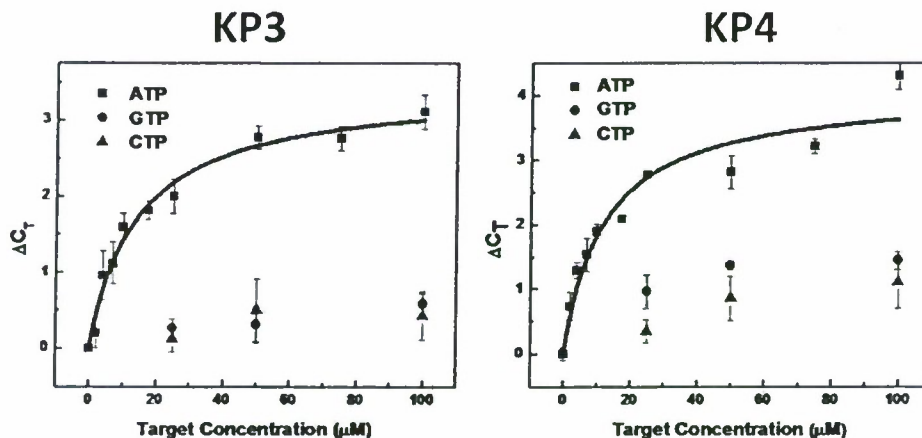


Fig 6. KP3 and KP4 sequences show specific affinity for ATP target with micromolar dissociation constants. RT-PCR determination of dissociation constants for KP3 and KP4 yields K_d values of $14.2 \pm 2.2 \mu\text{M}$ and $12.2 \pm 3.3 \mu\text{M}$, respectively. KP3 shows 5.5 and 7.4 fold higher affinity to ATP compared to GTP and CTP, respectively.

Finally, we show that the KP4 sequence exhibits SRA biosensor activity, where ATP indeed triggers the structure-switching action to activate the DNAzyme to catalyze the ABTS oxidation process. This oxidation results in a colorimetric readout (Fig 7).

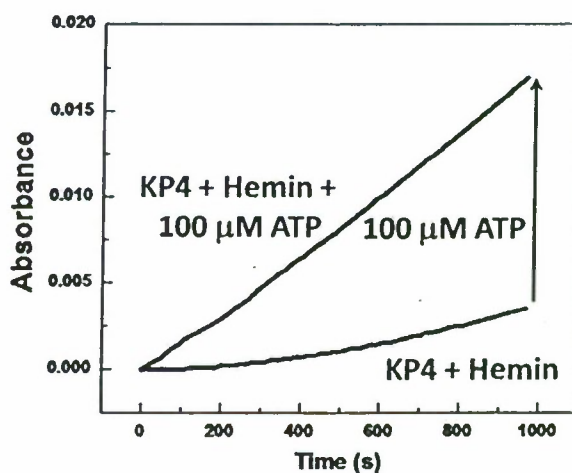


Fig 7. Measurement of absorbance change. In the absence of ATP, KP4 + hemin shows baseline catalytic activity (Red). When 100 μM ATP is added, DNAzyme within the KP4 sequence is activated, which significantly increase the catalytic reaction of ABTS, resulting in a color change.

Module 2: Integration of Microfluidic Selection, High Throughput Sequencing and Microarray Characterization

In this module, we sought to combine microfluidic selection (M-SELEX) and high throughput sequencing with microarray technology to generate DNA aptamers in a high throughput manner. More specifically, we first performed three rounds of microfluidic selection using human α -thrombin as a model target. Then we measured the bulk affinity of each enriched pool for thrombin with bead-based fluorescence assay. As expected, the initial random library displayed negligible binding affinity to the target. The first round (R1) and second round (R2) pool showed a slight increase in affinity to the target, but the third round pool (R3) showed a significant increase, with a bulk dissociation constant (K_d) of 11.2 ± 2.3 nM.

Next, we analyzed the DNA sequences in the library, R1, R2 and R3 pools by performing high-throughput DNA sequencing with the Genome Analyzer II (Illumina, San Diego, CA) and used Illumina Pipeline software to process the resulting data. We obtained $>10^7$ raw sequences from each selection round. We subsequently filtered out unnecessary sequences that have incorrect length or primer sequences, and trimmed out the two PCR primer sites from the remaining sequences. As a result, we obtained $>10^6$ unique aptamer sequences from each pool. We observed that the number of unique sequences decreased and the percentage of duplicate sequences increased in later selection rounds, indicating the convergence of aptamers (data not shown).

To identify sequences the highest affinity from the sequencing data, we ranked the sequences based on their copy number, and synthesized the top 100 sequences from the R1, R2 and R3 pools using the Agilent Custom Microarray Platform (Agilent Technologies, Mountain View, CA). In order to measure the affinity of aptamers, we first labeled the thrombin with Alexa647 fluorophore. Then we incubated the 20nM of the labeled proteins with the microarray for one hour. After washing the microarray, we mapped the fluorescence signals of each pixel, and measured the relative binding affinity to thrombin based on the signal amplitude (Fig 9).

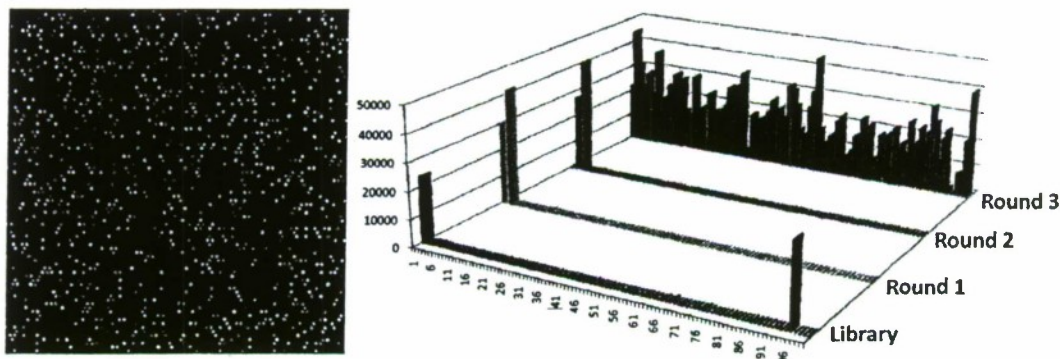


Fig 9. Massively parallel binding affinity determination using microarray. The 100 top-ranked sequences of the library, R1, R2 and R3 are in situ synthesized using the Agilent Microarray Platform (left). After incubating 20 nM of Alexa647-labeled thrombin with the array, the fluorescence intensities from every printed sequence are mapped. In contrast to the sequences from R1 and R2, the sequences from R3 pool show significant binding affinity for thrombin (right). In this way we are able to precisely measure the affinity of a large number of aptamer sequences in a massively parallel manner.

In summary, we show the first high throughput integration of aptamer selection, sequence analysis, and binding measurement. We believe that the integration of M-SELEX process with high throughput sequencing and microarray characterization is a significant step towards greatly accelerating the process to discover high affinity aptamers. Our methodology displays a number of advantages over conventional SELEX methods. First, the selection can be performed rapidly—we demonstrate the isolation of specific aptamers for protein targets with $K_d \sim 10$ nM within 3 rounds. Second, this integration method effectively identifies the highest affinity aptamers in a massively parallel manner with unprecedented throughput. Beyond this initial demonstration, we plan to continue the work in selecting aptamers for a range of different biomolecules.

Work Plan

This is the final year of support on this project.

F. Major Problems / Issues

We have successfully demonstrated SRAs which self-report the binding to target protein via significant fluorescence without additional post-modification. Throughout phases I, II and III, we have not encountered any major problems.

G. Technology Transfer

One provisional patent has been filed during the reporting period, and another patent disclosure is being prepared.

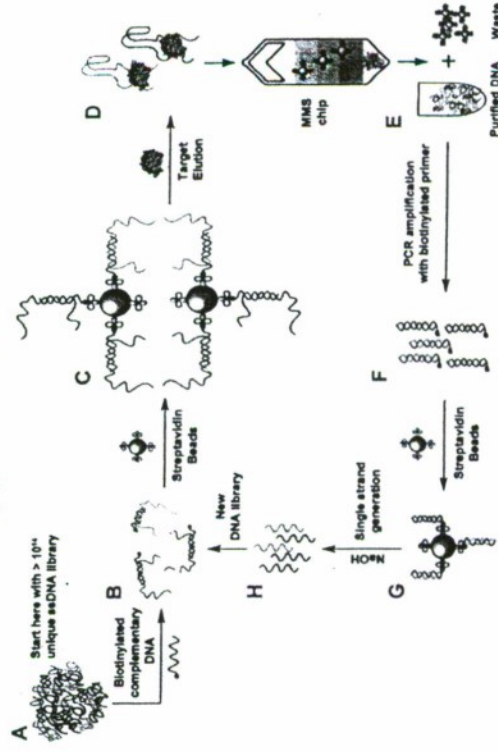
H. Foreign Collaborations and Supported Foreign Nationals

PI (H. Tom Soh) is a citizen of South Korea and Permanent Resident of the U.S.
Co-PI (Yi Xiao) is a citizen of China who is in the process for receiving her Permanent Residency in the U.S.

Project Objectives:

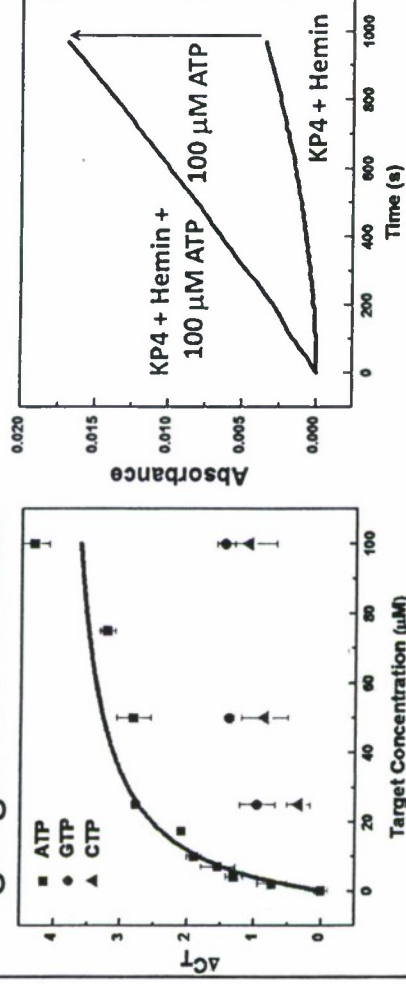
- Aptamers are highly stable nucleic acid molecules which are *in vitro* selected to specifically bind to their molecular targets with high affinity and specificity.
- The goal of this work is to develop a highly efficient approach to generate a new class of aptamers (Self-Reporting Aptamers, SRA) that can detect small molecules using the mechanism of binding-induced-folding.

Technical Approach:



The overview of microfluidic selection process to generate SRAs.

Highlight of Results:



- A) Affinity & Specificity measurements show low micromolar Kd.
- B) Colorimetric signaling upon target binding

Accomplishments:

- Developed the first "Self Reporting Aptamer" capable of a) specifically binding to their target, b) undergoing conformational change triggered by this binding event, and c) activating a DNA enzyme moiety within the aptamer structure to fluorescently report the binding event.
- Using a protein (Human α -thrombin) and a small molecule (ATP) as a model, we showed proof of principle with high affinity and specificity.
- Developed methods of combining microfluidic selection, high throughput sequencing with microarray technology to rapidly identify high affinity aptamers.

[illegible]

Participants	Male non-minority	Female non-minority	Male minority	Female minority
Pls	1			
UnderGrad Students				
Grad Students			1	
Post Docs		2		