Portable FRET Sensing of Proteins, Hormones, and Toxins Using DNA Aptamers and Quantum Dots

G.W. Jackson[*], U. Strych[*,**], E. Frank[*], R.C. Willson[**], R. Ballerstadt[*] and R.J. McNichols[*]
[*]BioTex, Inc., Houston, TX, USA, bill@biotexmedical.com
[**]University of Houston, Dept. of Chemical and Biomolecular Engineering, Houston, TX, USA

ABSTRACT

There is an urgent need to develop methods and devices that make the monitoring of any compound of interest in the public water system more reliable, more sensitive and more economical. We are developing a platform sensing chemistry based on rapidly responsive fluorescent resonance energy transfer, or *FRET* with DNA aptamers as molecular recognition elements. In particular, we are using highly stable, highly fluorescent quantum dots and have implemented the sensing chemistry in a format compatible with readout by an inexpensive, portable, and yet highly sensitive fluorometer. Using different aptamers in the sensor formulation, we demonstrate the sensing and quantitation of the protein insulin and the small molecule hormone, thyroxine (T4). We are presently formulating similar sensors for the potent cyanobacterial toxin, anatoxin-a. Building on extensive previous glucose sensing experience, we also look towards the necessary modifications to develop a fully reversible, on-line sensor for similar analytes.

Keywords: FRET, aptamers, quantum dots, portable sensing

1 INTRODUCTION

There is mounting evidence that suggests an increasing presence of harmful or even toxic chemicals, including pharmaceuticals in public drinking water. Most recently an investigation in India detected enough ciprofloxacin in a village's water well to treat 90,000 people every day [1]. This issue is by no means confined to the developing world. A study published by the Associated Press in 2008 revealed the presence of contraceptives, analgesics and anti-cholesterol drugs in the drinking water of several U.S. cities [2]. Even though this controversial study is still being evaluated, it is unequivocal that the public water supply is threatened by environmental pollutants from industrial sources and, more recently, by the possibility of bioterrorism attacks. Thus there is an urgent need to develop methods and devices that make the monitoring of *any* compound of interest in the public water system more reliable, more sensitive and more economical.

We are developing a sensing chemistry based on rapidly responsive fluorescent resonance energy transfer (*FRET)* [3] using quantum dots as fluorescent reporters and DNA aptamers conjugated to quencher dyes as molecular recognition elements.

1.1 Aptamers

Aptamers are single-stranded DNA or RNA (ssDNA or ssRNA) molecules that can bind to various targets including proteins with high affinity and specificity. These molecules can assume a variety of shapes due to their propensity to form helices and single-stranded loops, explaining their versatility in binding to diverse targets. They are used as sensors [4], therapeutic tools [5], to regulate cellular processes [6], as well as to guide drugs to their specific cellular target [7]. Contrary to the actual genetic material, their specificity and characteristics are not directly determined by their primary sequence, but instead by their tertiary structure [8].

Aptamers are generated from large random libraries by an iterative process called *Systematic Evolution of Ligands by Exponential Enrichment* (SELEX) [9, 10]. The SELEX technique (see Figure 1 below) starts with a large library of random single stranded nucleotides or aptamers (ca. 10^{15} unique sequences). A typical library will contain a randomized region of ca. 40 nucleotides flanked by two constant regions for PCR priming. The library is exposed to a target and the bound aptamers are partitioned and amplified for the next round. With each round the stringency of the binding conditions is increased until the only remaining aptamers in the pool are highly specific for, and bind with high affinity to, the target.

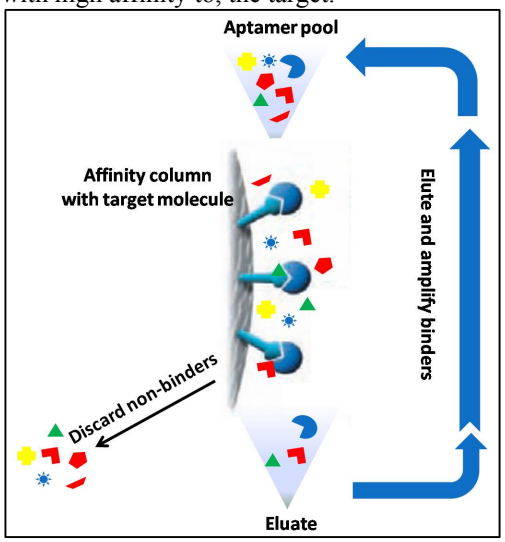

Figure 1: Simplified schematic of DNA aptamer selection (SELEX)

So far, aptamers are best known as ligands, binding proteins, in some cases rivaling antibodies in both affinity and specificity [11-14], and the first aptamer-based therapeutics are now emerging [13, 15]. More recently, however, aptamers have also been developed to bind small organic molecules and cellular toxins [16-20], viruses [21, 22], and even targets as small as heavy metal ions [23-27].

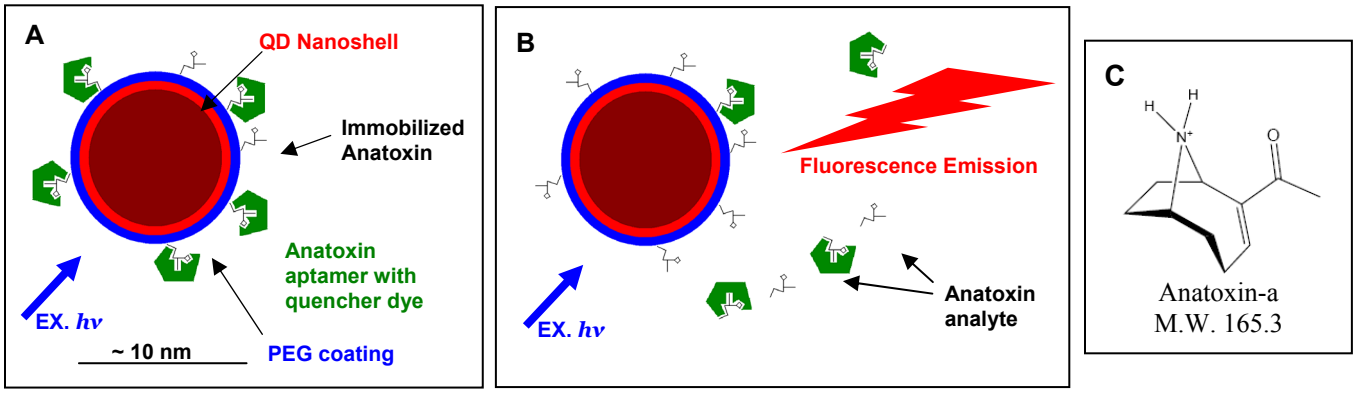

Figure 2. Schematic of anatoxin-a (ATX) sensing chemistry (approximate scale). *In a competitive-binding fluorescence resonance energy transfer (FRET) assay, quantum dots (QDs) are conjugated to ATX or ATX analogs. Aptamers which bind ATX with high specificity are identified by in vitro selection or "SELEX". Panel (A): Aptamers synthesized with a terminal fluorophore for quenching of the QD are bound to immobilized ATX. Panel (B) When free environmental ATX is exposed to this reagent mixture, QD-quenching aptamers are released from the QDs resulting in a fluorescence signal proportional to the ATX concentration. (C) Chemical structure of anatoxin-a (protonated form).*

1.2 FRET-Aptamer Sensing using Quantum Dots

Figure 2 above depicts our modular FRET-aptamer sensing scheme. In a competitive FRET format, an analog to the free analyte is covalently conjugated to highly fluorescent quantum dot (QD) nanoparticles. QDs are semiconductor nanocrystals (~2–100 nm) with unique optical and electrical properties currently applied in biomedical imaging and electronics industries [28-30]. Fluorescent organic dyes are often used for FRET sensors, but their functional limitations (e.g., spectral cross-talk and non-uniform fluorophore photobleaching rates) complicate subsequent analyses [31]. QDs provide significant advantages over organic fluorophores because of their unique photophysical characteristics, such as size-tunable photoluminescence spectra, higher quantum yields, broad absorption, and narrow emission wavelengths [29, 30]. Perhaps the most important advantage is a much increased resistance of QDs to photobleaching enabling development of more reproducible, stable assays.

DNA aptamers to the analyte of interest (in this case anatoxin-a) are conjugated to a strongly absorbing chromophore dye. Upon exposure of the sensing cocktail to free analyte, many of the quenching aptamers are released from the QDs resulting in restoration of fluorescence. In order to demonstrate the broad utility of this sensing platform we developed sensing cocktails for both the small molecule hormone, thyroxine (also known as T4) and the protein insulin. Additionally, we have selected putative aptamers to anatoxin-a for testing in this platform.

1.2.1 Analyte Demonstrations

Anatoxin-a is a naturally occurring toxin produced by some strains of *Anabaena* (particularly, *A. flos-aquae*) and at least four other genera of freshwater cyanobacteria (commonly referred to as blue-green algae). Algal blooms are increasingly common due to eutrophication of surface waters with the resulting potential to produce significant levels of the toxin in the environment. Anatoxin-a is a strong nicotinic acetylcholine receptor antagonist. Because it is not degraded by cholinesterase or any other known cellular enzymes, muscle cells continue to be stimulated, causing muscular twitching, fatigue and eventually paralysis. Severe overstimulation of respiratory muscles may result in respiratory arrest and rapid death [32].

As a surrogate for working with cyanobacterial anatoxin (sometimes referred to as the "*Very Fast Death Factor*") while optimizing the chemistries for sensing, we have chosen a molecule of similar size, the hormone thyroxine (see Figure 3). Free thyroxine (fT4) is an analyte of clinical interest in patients with potential thyroid problems. Finally, we have chosen insulin as a model protein for our initial experiments in protein sensing.

2 MATERIALS AND METHODS

2.1 SELEX

Numerous protocols for the *in vitro* selection of aptamers have been described since the introduction of the method in 1990 [9, 10]. The fundamental requirement for all is the facile partitioning of the non-binding nucleic acid population from the binding one. In the case of insulin, the binding target adsorbed to 0.3 µm polystyrene microbeads (Bangs Laboratories, Fishers, IN). A starting nucleic acid library, 5'-ATACCAGCTTATTCAATT-N_{40}-AGATAGTA AGTGCAATCT-3' was synthesized (IDT, Coralville, IN) and applied to naked polystyrene beads. 10 rounds of SELEX were performed against protein-coated beads in a buffer of 20 mM Tris, 100 mM NaCl, pH 7.4. Following each round, increasingly stringent washes were performed by increasing the time and duration of the wash. Elution of the nucleic acids for the next round of amplification was achieved by exposing the beads to 7 M urea at 80ºC. "Asymmetric" PCR with a 100:1 ratio of forward to reverse primer was employed to create the necessary single-stranded DNA population for the next round of binding.

For SELEX against the small molecules thyroxine and anatoxin-a, the compounds were covalently immobilized onto "Amine - Link" agarose beads (Pierce) according to manufacturer's protocol.

For all 3 targets, 10 rounds of SELEX were employed. Following 10 rounds, the enriched ssDNA were amplified one more time and the PCR products were cloned in *E. coli* using the TOPO cloning system (Invitrogen, Carlsbad, CA). The cloned inserts from individual plasmids were sequenced using a commercial service. The aptamer sequences then were aligned using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and grouped by sequence motifs. Consensus sequences were chosen from each motif for future binding studies.

2.2 Conjugations and Sensor Cocktails

2.2.1 Thyroxine (T4) Sensor

Thyroxine, which contains a primary amine (Figure 3), was covalently immobilized on carboxyl terminated quantum dots fluorescing at 655 nm (QD655, Invitrogen) using EDC-NHS chemistry. QDs thus conjugated to T4 were separated from free T4 using a microfuge spin-filter (MWCO 100,000 Daltons, Millipore, Bedford, MA). QDs were then diluted as necessary to comprise various T4 sensor cocktails.

A carboxyl version of the quenching chromophore, QSY21 (Invitrogen) was conjugated to a DNA aptamer synthesized with a 5'-amine. Similar methods were employed to create the necessary components of an insulin sensor. In that formulation, however, insulin-specific aptamers were conjugated to the QD reporter while insulin was conjugated to the quenching dye in order to compete with the free analyte.

Once conjugations were complete, a typical sensing cocktail comprised approximately 4.0 µl QD/DNA, 1.0 µl QSY21/insulin, diluted in 400 µl PBS, pH 7.4. Dilutions of the sensing cocktails were adjusted to operate within the range of the portable Qubit fluorometer detector.

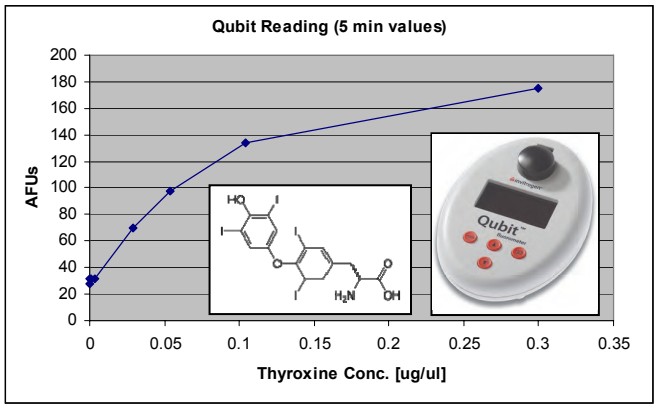

Figure 3. Quantitative detection of the small molecule thyroxine (T4) via FRET. *Insets show structure of T4 and portable (4.5 x 6.5 x 1.8 inch) Qubit™ fluorometer (Invitrogen) used to acquire data.*

3 RESULTS AND DISCUSSION

Figure 3 depicts the response of the sensing chemistry to free T4. While the sensor has not yet been optimized, we expect to improve sensitivity by at least 2 orders of magnitude by better tailoring of both the absolute and relative amounts of cocktail components. However, even without any further optimization in sensitivity, the limit of detection depicted is presently ~10 ppm. With pre-concentration steps often employed for environmental and clinical analytes such as methanol extraction, the current level of sensitivity may be acceptable for many applications (current ELISAs detect fT4 in the µg/dl range). Separately, we have confirmed that this sensor is insensitive to the structurally similar compound, phenol, as well as DMSO (data not shown).

Figure 4 shows the response of the sensor cocktail using insulin-specific aptamers. In this case, the limit of detection was approx. 20 nM. As a control experiment, we have confirmed that the sensor is not responsive to bovine serum albumin (BSA, data not shown).

At present we are testing anatoxin-specific aptamers in this formulation to enable portable sensing of the

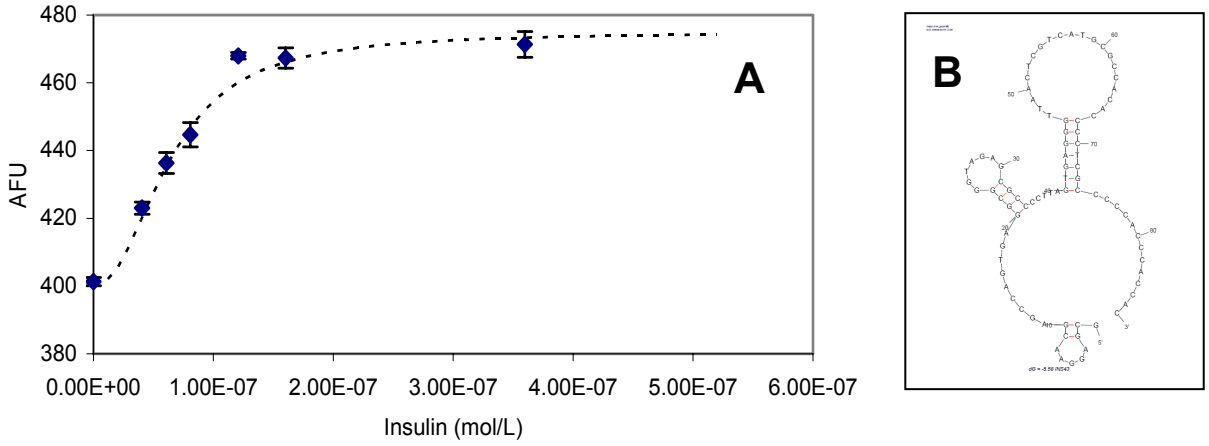

Figure 4. Response curve of displacement of insulin-specific aptamer#43-QD655 conjugate from QSY21-Insulin by free Insulin. *The increase in fluorescence with increasing insulin concentrations is due to FRET (A). The putative secondary structure of insulin-aptamer #43 (as predicted by Mfold (http://mfold.bioinfo.rpi.edu/)) is shown in B.*

compound in the field. Figure 5 (next page) shows two secondary structures for anatoxin-specific aptamers recently selected at BioTex. Predicted structures were generated at the RNAfold web server, Institute for Theoretical Chemistry, University of Vienna, http://rna.tbi.univie.ac.at/. Remarkably, aptamers "19b" and "42" having two completely different primary sequences yielded very similar secondary structures as shown in Figure 5.

Based on the preliminary results obtained so far, the specific and sensitive quantitation of environmentally relevant compounds is feasible. We are currently working on optimizing the sensing chemistries under laboratory conditions. In the future we plan to address the challenge of detecting individual contaminants in complex samples, i.e. detect insulin in blood serum, or detect anatoxin in a lake water sample. We are also currently investigating various rapid filtration and extraction approaches to pre-process those real-world samples.

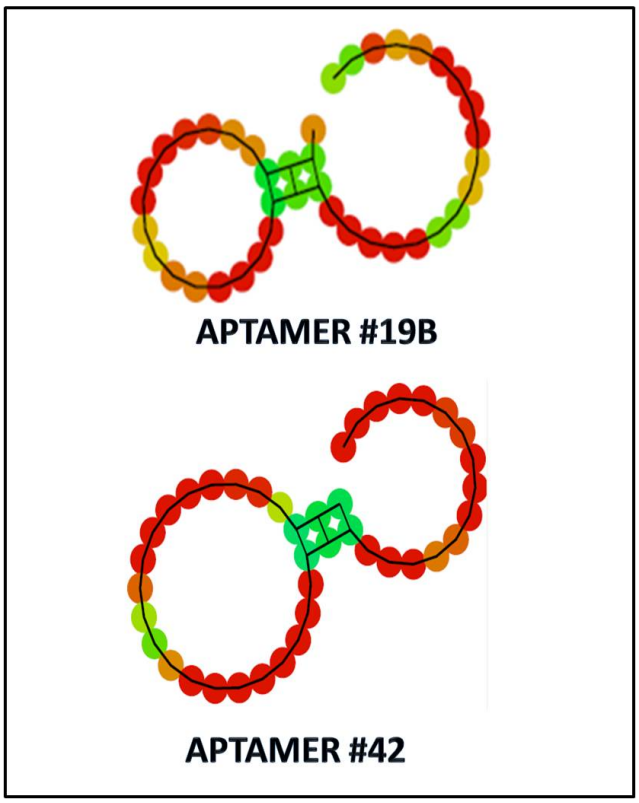

Figure 5. Secondary structure prediction for two conserved anti-anatoxin aptamer sequences, 19b and 42, selected in this study. *Colors represent the various probabilities for individual bases to pair as predicted. The structures were rendered using RNAfold (see text).*

4 CONCLUSIONS

We have demonstrated successful FRET-aptamer mediated sensing of the protein insulin and the small molecule hormone, thyroxine (T4) using highly stable and fluorescent quantum dot nanocrystals. The selected nucleic acid aptamers have a number of advantages over other molecular recognition elements such as antibodies. These include low cost of synthesis, zero chemical variability, and the ability to be selected under non-physiological conditions. At present, we are implementing a sensor using recently selected aptamers against anatoxin-a, a particularly potent neurotoxin produced by harmful algal blooms of cyanobacteria and a concern for its potential use as a weapon of bioterrorism.

5 ACKNOWLEDGEMENTS

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REFERENCES

1. Mason, M. (2009) Associated Press,
2. Donn, J., et al. (2008) Associated Press,
3. Clapp, A.R., et al., J Am Chem Soc, 2004. **126**(1): p. 301-10.
4. Holthoff, E.L. and F.V. Bright, Anal Chim Acta, 2007. **594**(2): p. 147-61.
5. Kaur, G. and I. Roy, Expert Opin Investig Drugs, 2008. **17**(1): p. 43-60.
6. Toulme, J.J., et al., FEBS Lett, 2004. **567**(1): p. 55-62.
7. Chu, T., et al., Curr Opin Mol Ther, 2007. **9**(2): p. 137-44.
8. Breaker, R.R., Nature, 2004. **432**(7019): p. 838-45.
9. Ellington, A.D. and J.W. Szostak, Nature, 1990. **346**(6287): p. 818-22.
10. Tuerk, C. and L. Gold, Science, 1990. **249**(4968): p. 505-10.
11. Lee, J.F., et al., Nucleic Acids Res, 2004. **32**: p. D95-100.
12. Srisawat, C. and D.R. Engelke, RNA, 2001. **7**: p. 632-41.
13. Ruckman, J., et al., J Biol Chem, 1998. **273**(32): p. 20556-7.
14. Jayasena, S.D., Clinical Chemistry, 1999. **45**(9): p. 1628–1650.
15. Chan, M.Y., et al., J Thromb Haemost, 2008. **6**(5): p. 789-96.
16. Sazani, P.L., et al., J Am Chem Soc, 2004. **126**(27): p. 8371.
17. Babendure, J.R., et al., J Am Chem Soc, 2003. **125**: p. 14716-7.
18. Hirao, I., et al., Nucleic Acids Symp Ser, 1997. **37**: p. 283-4.
19. Liu, J. and Y. Lu, Angew. Chem., Int. Ed., 2006. **117**: p. 90-94.
20. Chu, T.C., et al., Cancer Res., 2006. **66**(Jun 15): p. 5989-92.
21. Gopinath, S.C.B., et al., J Gen Virol, 2006. **87**: p. 479-87.
22. Gopinath, S.C.B., et al., Journal of Biochemistry 2006 139(5):837-846, 2006. **139**(5): p. 837-46.
23. Wernette, D.P., et al.
24. Chang, I.-H., et al., Environ. Sci. Technol., 2005. **39**: p. 3756.
25. Swearingen, C.B., et al., Anal. Chem., 2005. **77**: p. 442-8.
26. Wrzesinski, J. and J. Ciesiolka, Biochemistry, 2005. **44**(Apr 26): p. 6257-68.
27. Stefan, L.R., et al., Nucleic Acids Res, 2006. **34**: p. D131-D134.
28. Dabbousie, B.O., et al., J Phys Chem B, 1997. **101**: p. 9463-75.
29. Bruchez, M., et al., Science, 1998. **281**: p. 2013-6.
30. Chan, W.C.W. and S. Nie, Science, 1998. **281**(5385): p. 2016-8.
31. Zhang, C.-y. and L.W. Johnson, Anal Chem, 2006. **78**: p. 5532-7.
32. EPA, U.S. Environmental Protection Agency, 2006. **EPA/600/R-06/137**.