Full Paper

Detection of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) with a microbial sensor

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Jed O. Eberly,^{1,2,*} Michael L. Mayo,¹ Matthew R. Carr,¹ Fiona H. Crocker,¹ and Karl J. Indest^{1,*}

¹ U.S. Army Engineer Research and Development Center, Environmental Laboratory, Vicksburg, MS 39180, U.S.A.

² Montana State University, Central Ag Research Center, Moccasin, MT 59457, U.S.A.

Explosives such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) are common contaminants found in soil and groundwater at military facilities worldwide, but large-scale monitoring of these contaminants at low concentrations is difficult. Biosensors that incorporate aptamers with high affinity and specificity for a target are a novel way of detecting these compounds. This work describes novel riboswitch-based biosensors for detecting RDX. The performance of the RDX riboswitch was characterized in *Escherichia coli* using a range of RDX concentrations from 0–44 $\mu\text{mol l}^{-1}$. Fluorescence was induced at RDX concentrations as low as 0.44 $\mu\text{mol l}^{-1}$. The presence of 4.4 $\mu\text{mol l}^{-1}$ RDX induced an 8-fold increase in fluorescence and higher concentrations did not induce a statistically significant increase in response.

Key Words: aptamer; biosensor; riboswitch

Introduction

Training activities and explosives testing and production on U.S. Department of Defense (DoD) lands have led to explosives and propellant contamination of soils and groundwater at military facilities around the world. The extent of contamination on training ranges has been difficult to determine due to the heterogeneous dispersion of residues in soil and the lack of historical data regarding training site locations (Jenkins et al., 1998, 2001; Pennington et al., 2001, 2005; Premkumar et al., 2002). Energetic residues of these nitroaromatic and nitroamine compounds in soils have the potential to migrate into surface water and groundwater (Clausen et al., 2004;

Sheremata et al., 2001) and are significant hazards to human health and the environment (Klapötke, 2015). Currently, newer insensitive munition (IM) formulations, that include hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) among other components, are being widely adopted. However, very little is known about the associated environmental risks of their unintended release, because the presence of these potentially toxic compounds are difficult to detect and monitor effectively in the field.

Previous methods for detecting explosive contaminant residues have relied on immunoassays, electrochemical, and piezoelectric devices (Sapsford et al., 2002; Singh, 2007). However these methods are often cumbersome and tend to have poor sensitivity and dynamic range (Ehrentreich-Förster et al., 2008; Sapsford et al., 2002). The detection of explosives in the environment is necessary for assessing environmental and human health risk as well as remediation success. Current technologies that utilize high performance liquid chromatography (HPLC), gas chromatography (GC), or immunoassays are selective for specific explosive compounds and can approach sensitivity levels required to meet EPA guidelines for a screening level of 0.61 $\mu\text{g l}^{-1}$ in tap water (U.S. EPA, 2014). However, these methods are not suitable for continuous real-time monitoring under field conditions. There is a need for novel sensor technologies for these contaminants that provide real-time detection and monitoring at low concentrations, with low power requirements.

One novel approach for detecting these energetic compounds in the environment is with aptamer-based biosensors. Aptamers are short nucleic acid sequences that typically consist of a random sequence of 15–75 bases. They form 3D structures based on the primary sequence and bind to their targets through a combination of their 3D structure, stacking interactions between aromatic moieties, and electrostatic interactions (Stoltenburg et al.,

*Corresponding authors: Jed Eberly and Karl Indest, Montana State University, Central Ag Research Center, Moccasin, MT 59457, U.S.A.; U.S. Army Engineer Research and Development Center, Environmental Laboratory, Vicksburg, MS 39180, U.S.A.

Tel.: +1-406-423-5421 E-mail: jed.eberly@montana.edu

Tel.: +1-601-634-2366 E-mail: Karl.J.Indest@usace.army.mil

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Fig. 1. Overview of riboswitch selection process.

A. Schematic illustration of aptamer selection steps, B. Schematic illustration of selection of RDX responsive riboswitch clones.

2012). Aptamers are used for a variety of applications including biosensors, electrochemical sensors, sensor arrays, molecular beacons, *in vivo* imaging, and targeted therapeutics (Bini et al., 2011; Famulok and Mayer, 2011; Mayer, 2009; Oh et al., 2010) and have been used to bind proteins, metals, antibiotics, dyes, fluorophores, cells, and many other small organic molecules (Famulok, 1999; Mosing and Bowser, 2007; Wang et al., 2011). Aptamers have a number of advantages over protein and antibody-based sensor elements. They are typically less complex and thus easier and cheaper to synthesize, they have good thermal and pH stability, more versatile chemistry, lower immunogenicity, and often have better affinity and specificity (Ahmad et al., 2011; Wang et al., 2011). They are also able to refold after denaturing to regain functionality (Mosing and Bowser, 2007).

One of the challenges of using aptamers is translating a binding event into a detectable signal. A variety of fluorescence, electrochemical, colorimetric, and biological methods have been developed to report aptamer-target interactions (Bin Seo and Gu, 2017; Carrasquilla et al., 2012; Cash et al., 2009; Mayer, 2009; Wang et al., 2011). Biological reporting mechanisms use an aptamer configuration known as a riboswitch. Riboswitches are cis-acting RNA genetic control elements that regulate gene expression by binding to target ligands (Lin and Thirumalai, 2012; Nudler, 2004). Riboswitches typically consist of an aptamer domain, which recognizes and binds the target ligand and a linker sequence (Beisel and Smolke, 2009; Lin and Thirumalai, 2012; Lynch et al., 2007). Substrate binding to the aptamer domain causes an allosteric change to the structure of the RNA which, in turn, controls the termination of transcription, the initiation of translation, or alternative splicing regulation (Barsacchi et al., 2016; Lin and Thirumalai, 2012). Development of riboswitches with the desired functionality generally consists of screen-

ing large clone libraries containing random linker sequences. The length and sequence of the linker determines the structure of the riboswitch which, in turn, determines its ability to regulate the target gene (Davidson et al., 2013; Lynch et al., 2007).

Naturally occurring riboswitches have been identified in numerous organisms. In *Bacillus subtilis* the biosynthesis of riboflavin, the precursor to flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), is regulated by an untranslated regulatory sequence located upstream of the riboflavin operon (Nudler, 2004). Extensive experiments have demonstrated that this region binds FMN and undergoes a conformational change that causes transcription termination (Nudler, 2004). Similar riboswitches have been reported in regulating thiamin, cyanocobalamin, and the amino acids methionine, cysteine, and lysine (Nudler, 2004). In addition to vitamin precursors and amino acids, riboswitches have also been implicated in the regulation of purine biosynthesis (Tucker and Breaker, 2005).

The purpose of this work was to determine if a reliable biological reporter system incorporating a RDX aptamer could be used for detecting low concentrations of RDX *in vivo*. Here, we report the construction and characterization of several RNA riboswitches that function as reporters of RDX exposure in *Escherichia coli*.

Materials and Methods

Aptamer selection and sequencing. Selection of aptamers for RDX was performed following the methods of Park et al. (2012) and Gu et al. (2016). An aptamer library consisting of a 30 bp random region flanked by 23 bp primer regions for a total construct size of 76 bp was obtained from TriLink Biotechnologies. An RNA library was prepared from the DNA aptamer template using Ambion T7

Fig. 2. Structure of RDX and counter selection targets. A. RDX, B. NTO, C. DNAN, and D. TNT.

RNA polymerase (Life Technologies, Grand Island, NY). After transcription, the DNA template was digested with RNase-free DNase I and the RNA library was subsequently cleaned and concentrated by ethanol precipitation. Graphene oxide SELEX (GO-SELEX) was performed following established methods (Gu et al., 2016; Park et al., 2012). Briefly, binding assays were performed in a reaction buffer consisting of 100 mM NaCl, 5 mM MgCl₂, and 25 mM Tris-HCl, at pH 6.5. Following each round of SELEX, the aptamer pool was reverse transcribed and PCR amplified. Following cleanup with a Zymo Oligo Clean and Concentrator kit (Zymo Research, Irving, CA, USA), the DNA library was used as a template for generating a new RNA library for the subsequent round of selection. Figure 1 shows a schematic of aptamer selection and riboswitch function. After four rounds of SELEX, >50% of the sequences were bound and a counter selection round was performed using trinitrotoluene (TNT) and 2,4-Dinitroanisole (DNAN), which are structurally similar to RDX (Fig. 2), to ensure that the aptamer pool was specific for the target compound. After counter selection, an additional round of SELEX was performed to ensure that the affinity of the aptamer pool was maximized.

After the final round of screening, cDNA was prepared from the bound RNA aptamer pool and cloned using the pCR4-TOPO vector (Life Technologies, Grand Island, NY, USA) and *E. coli* TOP10. Positive clones were selected by blue/white screening on LB plates with 50 µg/ml kanamycin. Plasmids were extracted with a MiniPrep kit (Qiagen, Germantown, MD) and prepared for sequencing using the Big Dye terminator kit (Life Technologies, Grand Island, NY, USA) and sequenced on an ABI 3100 genetic analyzer (Life Technologies, Grand Island, NY, USA). Analysis of the sequence data was performed using Geneious R6 software (created by Biomatters, available from <http://www.geneious.com/>).

Riboswitch cloning. A riboswitch library was generated

consisting of the RDX aptamers described above, a 6N random linker, and the gene encoding the DsRed Express 2 red fluorescent protein. The 6N random linker library containing the RBS and Gibson assembly primer sequences was synthesized by TriLink Biotechnologies. The primer sequences for the linker region were 5'-TTGACTAGTACATGACCACTTGA-3' and 5'-CTTGCGTAATCATGGTCATAGC-3'. The *DsRed Express2* gene was amplified from the pDsRed-Express2 vector (Clontech Laboratories, Mountain View, CA) with the following forward and reverse primers: 5'-AGGAAACAGCTATGACCATGAT-3' and 5'-ATTCTAGAGTCGCGGCCG-3'. Assembly of the aptamer, linker, and *DsRed Express2* was performed using the NEB Gibson Assembly Master Mix (New England Biolabs, Ipswich, MA) and the resulting construct was ligated to a pMiniT plasmid (New England Biolabs, Ipswich, MA) and cloned into *E. coli* alpha-5 chemically competent cells.

The entire transformation mix was plated on LB plates with 100 µg ml⁻¹ ampicillin to select for clones with inserts. The resultant *E. coli* clone library was replica plated on LB plates with 100 µg ml⁻¹ ampicillin with 0 and 44 µmol l⁻¹ RDX and screened for clones that only expressed DsRed in the presence of RDX. The theoretical library size for 12 aptamer sequences with a 6N random library was 49,152 clones. Approximately 5,000 clones were screened for response to RDX.

Fluorescence assay. *E. coli* clones that expressed DsRed in the presence of RDX were assayed over a range of RDX concentrations to determine their sensitivity. Assays were performed in 48 well plates with 1 ml of LB media, 50 µg/ml ampicillin, and 0, 0.44, 4.4, and 44 µmol l⁻¹ RDX. RDX is a white solid that is colorless in aqueous or alcohol solutions, thus the presence of RDX was not expected to influence the fluorescence signal of DsRed express. Cultures were started at an initial OD₆₀₀ of 0.05 and the plates were incubated at 37°C with constant shaking at 150 rpm for 24 h. Fluorescence was measured using a BioTek Synergy HT plate reader (BioTek Instruments, Inc., Winooski, VT) with an excitation wavelength of 530 nm and an emission wavelength of 590 nm. To ensure specificity of the response, cells were also assayed with 44 µmol l⁻¹ TNT, NTO, and DNAN.

In order to verify that the response to RDX was not due to a transcriptional effect, the levels of DsRed-Express2 expression were measured in both RDX free samples and in the presence of 44 µmol l⁻¹ RDX by qPCR. Cultures were induced with RDX then incubated at 37°C for 24 h. Cells were harvested and the RNA was extracted with a Qiagen RNeasy kit (Qiagen, Germantown, MD, USA). Following extraction, RNA was treated with DNase to remove genomic DNA and the quality was checked on an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA was converted to cDNA and qPCR was performed using a PowerSYBR kit (Life Technologies, Grand Island, NY, USA) on an Applied Biosystems 7900HT system (ThermoFisher Scientific, Waltham, MA, USA).

The best performing RDX riboswitch clones (Clones 4

and 11) were subsequently sequenced to determine the aptamer and linker sequence. Sequencing was performed by amplifying the riboswitch and flanking regions with primers 5'-ACCTGCCAACCAAAGCGAGAA-3' and 5'-TCAGGGTTATTGTCTCATGAGC-3'. Amplicons were subsequently labeled with a BigDye Terminator® v3.1 sequencing kit (Thermo Fisher Scientific, Waltham, MA) and sequenced on an ABI 3500xL Genetic Analyzer (Life Technologies, Grand Island, NY, USA).

Results

Aptamer selection and sequencing

After four rounds of SELEX, recovery of target bound RNA aptamers was saturated (Fig. 3). No binding was observed in the counter selection round against TNT and DNAN and a subsequent round of selection showed no additional increase in the ratio of the pool that was bound to the target. Sequencing of the clone library from the

Fig. 3. SELEX enrichment of RNA aptamers for RDX. Counter selection was performed with TNT and DNAN.

Fig. 4. Fold change in fluorescence response of RDX riboswitch regulated DsRed Express2 clones when induced with 44 μ M RDX. Experiments were performed in triplicate. Error bars represent standard deviation.

Fig. 5. *E. coli* fluorescence response to increasing RDX concentrations.

Fluorescence response of *E. coli* clone 4 (A) and clone 11 (B) expressing DsRed Express2 in the presence of increasing concentrations of RDX. The excitation wavelength was 530 nm and the emission wavelength was 590 nm. Experiments were performed in triplicate. Error bars represent standard deviation.

Table 1. Riboswitch sequences for the two best performing clones.

Clone	Sequence
4	UAGGGAAGAGAAGGACAU <u>AUGAUGCAGUCAACUGUACGGGGUUAGUCUUGCGG</u> UUGACUAGUACAUGACCACUUGAC <u>CUUUAGGAAA</u>
11	UAGGGAAGAGAAGGACAU <u>AUGAUGCAGUCAACUGUACGGGGUUAGUCUUGCGG</u> UUGACUAGUACAUGACCACUUGA <u>UGAGAAAGGAAA</u>

Linker sequence is underlined. DsRed Express 2 gene is not shown for clarity.

round 5 SELEX pool resulted in 12 unique sequences which were subsequently used for generating a riboswitch library (Table S1).

DsRed Riboswitch screening

A total of six clones with a 2-fold or greater increase in fluorescence in the presence of RDX were identified (Fig. 4). Clones 4 and 11 had the greatest activation ratio (fold change) with an 11 and 5-fold change in fluorescence, respectively. These clones were used for all subsequent analyses. The qPCR results showed no statistically significant difference in DsRed-Express2 mRNA levels between RDX free controls and $44 \mu\text{mol l}^{-1}$ RDX with average ΔC_q of 20.2 (Fig. S1). This suggests that the increased fluorescence in the presence of RDX was due to riboswitch activation at a translational level rather than transcriptional regulation.

The activation ratio was calculated as the fold change in fluorescence between cells induced with $44 \mu\text{M}$ RDX and uninduced cultures. The two clones with the lowest background fluorescence and greatest activation ratio (Clones 4 and 11) were selected for further analysis over a range of RDX concentrations. The results of this assay showed a rapid response between 0 and $4.4 \mu\text{mol l}^{-1}$ RDX. Above $4.4 \mu\text{mol l}^{-1}$ RDX the fluorescence response was saturated (Fig. 5). Clone 11 had significantly higher fluorescence reaching a maximum fluorescence of 5700 RFUs compared to Clone 4 which only achieved a maximum of 280 RFUs with $44 \mu\text{mol l}^{-1}$ RDX. The difference in DsRed Express2 expression was visually discernable between the RDX free control and cells that were exposed to RDX (Fig. S2). Neither clone showed a significant response relative to the control when exposed to 10 ppm NTO, DNAN, or TNT (Fig. S3). Sequencing results showed that each clone had a unique linker sequence (Table 1).

Discussion

To our knowledge, this work is the first report of a RNA riboswitch that responds to RDX resulting in an *in vivo* fluorescence signal. While aptamer response was sensitive to low ($<1 \mu\text{mol l}^{-1}$) concentrations of RDX (Fig. 5), it was rapidly saturated which limited the dynamic range of the response. While this detection range is within the recommended EPA tap water screening level of $135 \mu\text{mol l}^{-1}$ (U.S. EPA, 2014), further optimization of the aptamer specificity may be needed to improve the range of this response.

The observable color change that occurred when cells were exposed to RDX (Fig. S2) suggests that an *E. coli* strain harboring the riboswitch could be used as a biosensor for qualitative detection of RDX. In addition, the results of the fluorescence measurements (Fig. 5) indicate that at least semi-quantitative detection at sub $\mu\text{mol l}^{-1}$ concentrations are possible. The fold change and limits of detection are similar to reports for a 2,4-Dinitrotoluene (DNT) responsive riboswitch which showed an activation ratio up to 9.8 (Davidson et al., 2013). This is similar to the 11-fold change in fluorescence observed with Clone 4 when exposed to $44 \mu\text{mol l}^{-1}$ RDX (Fig. 4). However, the response curves also indicate that the RDX riboswitches

were more sensitive to low concentrations of RDX. The RDX riboswitch was sensitive to as low as $0.4 \mu\text{mol l}^{-1}$ and the response was saturated by $4.4 \mu\text{mol l}^{-1}$. In contrast, the DNT riboswitch had a detectable increase in fluorescence at around $50 \mu\text{mol l}^{-1}$ (Davidson et al., 2013).

A significant level of background fluorescence was observed with both clones indicating that the riboswitch functions as a rather leaky repressor of DsRed-Express2 expression in the absence of RDX. However, the difference was visually discernable between cultures exposed to RDX and control cultures (Fig. S2). While the observed fold change is similar to the 8-fold change in expression reported for a theophylline riboswitch (Desai and Gallivan, 2004; Lynch et al., 2007), a higher signal to background ratio may be desirable. The length of the linker sequence between the aptamer domain and RBS is known to effect the function and dynamic range of a riboswitch (Desai and Gallivan, 2004). The length and sequence of the linker likely determines the interaction of the aptamer with the RBS and start codon. Previous work has indicated that base-pairing between the aptamer domain and the region surrounding the RBS is important for riboswitch function (Lynch et al., 2007). Further optimization of this linker sequence is one possible approach to reducing background expression.

Realistically, *E. coli* remain in the exponential growth phase for less than one day, depending on the growth conditions. Therefore, rapid growth rates (or lack thereof) could be problematic for measuring and inferring reliable DsRed reporter response. This fact remains a major challenge for environmental detection via microorganisms. One approach to overcome this challenge would be through encapsulation of the cells in a support matrix such as sol-gel which would limit growth while stabilizing the cells to maintain activity (Premkumar et al., 2002). An additional challenge is that the complex soil and water matrices encountered in the environment may pose additional difficulties to reliable detection of RDX potentially limiting the applicability of the reported biosensor to surface and ground water samples.

In summary, several RNA aptamers were characterized that were capable of binding RDX. This work demonstrated that these aptamers could function as riboswitches to regulate detectable expression of the fluorescence gene DsRed-Express2. The riboswitches provided a semi-qualitative method for detecting RDX with a limit of detection around $0.44 \mu\text{mol l}^{-1}$ and demonstrated the utility of a riboswitch-based biosensor for the detection of explosives.

An observable color change could be seen in cultures even at low ($<1 \mu\text{mol l}^{-1}$) RDX concentrations (Fig. S2) indicating that these riboswitches may have utility at environmentally relevant concentrations of RDX which are in the $\mu\text{mol l}^{-1}$ range for many State and EPA regulations (U.S. EPA, 2014). An *E. coli* strain containing a riboswitch represents a novel means of detecting RDX and could be incorporated into a bio-based sensor device used for remote, low power, continuous monitoring. Further work is needed to determine the dynamic range of the signal response and to explore the response of the biosensor under environmental conditions.

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Supplementary Materials

Supplementary figures and table are available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

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