Journal of Synthetic Computability and Biology (JSCB)

https://utilitasmathematicapublishing.com

Vol: 1, ISSUE: 1, 2025

Pg No: 1-8

Environmental Fluoride Detection at the Point of Use Using a Cell-Free Riboswitch-Based Biosensor

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ABSTRACT

The development of programmable molecular systems to identify a variety of chemicals, diseases, and nucleic acids has been made possible by advancements in biosensor engineering. In this study, we design and evaluate a biosensor for fluoride, a significant groundwater contaminant of international concern. A DNA template that encodes a fluoride-responsive riboswitch that controls genes that provide a fluorescent or colorimetric output makes up the sensor's cell-free system. In both laboratory and field settings, individual reactions may detect fluoride at levels exceeding 2 ppm, the Environmental Protection Agency's strictest regulation standard, and can be lyophilized for long-term preservation. This work offers a crucial proof-of-principle for the future engineering of riboswitches and other biosensors to solve issues for the environment and global health by detecting fluoride on-site in a real-world water supply.

KEYWORDS: Riboswitches, Biosensor, Cell-free systems, Diagnostics, Water quality, Field use.

INTRODUCTION

The availability of safe drinking water plays a significant role in promoting public welfare.Nonetheless, a sizable section of the world lacks access to safe water sources; an estimated 3 billion people use water from sources that are either dangerous or pose serious hygienic problems.2. Fluoride is one extremely harmful pollutant that naturally seeps into groundwater. In areas with low resources, dental and skeletal fluorosis can be caused by prolonged exposure to fluoride concentrations above 2 parts per million.3. Large-scale remediation techniques do exist, but they need a lot of resources and are challenging to implement.3, 4 The dependence of gold-standard sensing techniques on pricey analytical tools exacerbates this issue by making detection challenging in places where the greatest need exists.4. Although there are numerous new fluorescent and colorimetric chemical fluoride sensors, their practical application is limited by the need for additional imaging equipment or the use of hazardous organic solvents.5. A more realistic, quick, and field-deployable method to check for fluoride in water is desperately needed in order to enable focused rehabilitation and empower impacted populations.

Recently, cell-free expression (CFE) systems have emerged as a viable platform for field-deployable molecular diagnostics due to their ability to be lyophilized and rehydrated on-demand.6–9 These systems usually have the requisite buffers, energy sources, and cofactors to support gene expression from other DNA templates, in addition to the cellular gene expression machinery.10. Cell-free platforms, as opposed to complete cells, provide an open, readily adjustable response environment, which speeds up the creation of genetically encoded programming.10. They also get beyond issues with host mutation, analyte toxicity, and biocontainment that restrict cellular sensors.11.
Using a fluoride-responsive riboswitch that controls the expression of the CrcB fluoride efflux pump in Bacillus cereus, we aimed to take advantage of the benefits of cell-free biosensing platforms to develop a novel method for checking for fluoride in water.Twelve We demonstrate that a cell-free gene expression system can activate both protein and RNA reporter expression in the presence of fluoride by setting up the B. cereus crcB fluoride

riboswitch to regulate the transcription of downstream reporter genes. We show that fluoride concentrations at the Environmental Protection Agency's (EPA) Secondary Maximum Contaminant Level of 2 ppm may be detected using an enzyme colorimetric reporter.14 Interestingly, compared to a number of evaluated commercial fluoride testing kits, these cell-free biosensors demonstrated more precise sensing with a lower limit of detection. Additionally, we show that our fluoride biosensor can be lyophilized for long-term distribution and storage, which enables us to identify fluoride in raw groundwater that was collected and tested on-site in Costa Rica. In addition to laying the groundwork for the use of cell-free biosensing systems in quick and field-deployable water quality diagnostics to address urgent global health issues, this work demonstrates the potential of riboswitches as useful biosensing instruments.

RESULTS

Control of Reporter Expression in Cell-Free Reactions by Fluoride Riboswitch. Our point-of-use diagnosis is a cell-free system with a lyophilizable and storable fluoride biosensor DNA template. The biosensor, which encodes the fluoride riboswitch and a reporter gene that generates a detectable output if fluoride is present, is activated by rehydration (Figure 1a,b). Our initial goal was to describe the B. cereus crcB riboswitch's regulatory activity in the cell-free reaction environment. We may employ the riboswitch in E. coli cell-free extract since its cotranscriptional folding process was previously characterized (Figure 1b), confirming that it works with E. coli RNA polymerase. Thus, we created a reporter plasmid that was positioned downstream of a constitutive E. coli σ70 promoter and contained the coding sequence of the reporter protein superfolder green fluorescent protein (sfGFP), a strong ribosome binding site (RBS), and the riboswitch sequence (all plasmid details are in Supplemental Table S1).

Following the optimization of the Mg^{2+} level within the reaction conditions for riboswitch function (Supplemental Figure S1), we titrated across a variety of NaF concentrations to ascertain the dose-response to fluoride of the fluoride sensor. Activation was observed at NaF concentrations as low as 0.1 mM, and all tested circumstances resulted in a discernible increase in expression over the OFF state (Figure 1c, green line and inset). This threshold is significant since the EPA's most stringent danger threshold for fluoride in drinking water, 2 ppm secondary maximum contamination limit, is equal to 0.1 mM NaF.14 However, we calculated the reliable lower limit of detection to be 0.2 mM NaF because the signal-to-noise ratio at 0.1 mM NaF is less than 3. Crucially, the system exhibits negligible leak as well; without NaF, we saw very little activation of gene expression. The riboswitch is very selective for fluoride, as evidenced by the fact that titration of similar doses of NaCl did not increase expression under any circumstances (Figure 1c, gray line). This outcome supports an earlier, more thorough description of the switch's fluoride-specificity in E. coli.Twelve Thus, the sensor can distinguish between health-relevant fluoride concentrations dosed into laboratory water samples without any riboswitch shape or function adjustment.

Modifying Reporters to Adjust Detection Threshold and Sensor Speed. An output that can be read rapidly with little additional equipment is necessary for biosensor field deployment.16 In 30 minutes at 30 °C, processes using the most activating fluoride concentration (3.5 mM) produced a detectable signal above the no-fluoride OFF state, and by the conclusion of the 8-hour experiment, the activation was 20 times more than that of the no-fluoride condition (Figure 2a). Nevertheless, even after being excited with a blue LED, the sensor's ON state was not visually discernible for several hours, indicating the need for a quicker reporter.

With a 3-way junction dimeric Broccoli (3WJdB)17 reporter, an RNA aptamer that activates fluorescence of its DFHBI-1T ligand upon transcription, we postulated that we could eliminate translational delays and speed up the sensor's response. Within 12 minutes at 30 °C, 3WJdB generated a signal discernible over background at all tested NaF doses (Figure 2b), which is more than twice as quick as sfGFP (Figure 2a). Interestingly, this outcome also demonstrates that, even though the fluoride riboswitch may misfold with the upstream riboswitch sequence, it is compatible with RNA reporters. The sensor's fluorescent output at the maximally activating testing condition was reduced by 50 times when sfGFP was substituted for 3WJdB, notwithstanding the speed improvement. Therefore, if a

plate reader is available, the RNA-level output is faster than the sfGFP output, but it is not bright enough to be used in the field.

We employed the colorimetric enzyme catechol (2,3)-dioxygenase (C23DO) as a reporter in place of a fluorescent output. Previously employed in genetically encoded biosensors for plant viruses18, C23DO oxidizes its colorless catechol substrate to the yellow-colored 2-hydroxymuconate semialdehyde, which generates a visible reporter output.19. This color shift makes it possible to read out gene expression either by the appearance of a yellow color that is visible to the unaided eye or by light absorbance at 385 nm on a plate reader. Based on our prior results, we empirically defined an absorbance of 0.8 as the visible output produced by all tested fluoride doses in 70 minutes at 30 °C (Figure 2c).18 Interestingly, the minimally and maximally activating conditions were separated by only 20 minutes, demonstrating the speed at which enzymatic reporters can amplify weak signals. In line with earlier applications of C23DO as a reporter in a cell-free reaction,19 we noticed a decrease in the absorbance signal following peak activation, which may have been brought on by the breakdown of 2-hydroxymuconate semialdehyde. Since variations in activation for an enzymatic reporter are based on variations in time to observable signal rather than final signal magnitude, which is based on the amount of substrate provided, this impact does not jeopardize sensor resilience. This strategy's drawback is that the sensor can only provide a binary presence/absence result within a given time window because activation time does not linearly correlate with fluoride concentration.16 We chose C23DO as our reporter of choice for a field-deployable diagnostic because of its sensitivity and low leak, which together with the benefits of an easily observable output and a respectable latency to detection, make this presence/absence result diagnostically relevant.

Lyophilization and Reaction Tuning for Biosensor Field Deployment. In order to detect fluoride close to the EPA's secondary maximum contamination limit of 2 ppm (100 µM), we then optimized our sensor. Our initial design produced a strong ON signal, but after 90 minutes the sensor started to leak fluoride-free (Figure 2c, gray line), making it more difficult to detect even minute levels of fluoride. By lowering the quantity of reporter DNA added to the process from 5 nM to 3 nM in an effort to reduce the sensor's output, we tried to address this issue. This resulted in a substantial delay in activation, but it also allowed us to detect 100 µM NaF over background while totally suppressing leak (Figure 3a). With our leakless sensor, we were able to detect as low as 50 µM NaF above background, but only after a lengthy incubation period that did not approach a visually visible threshold within six hours.

We looked for a method where tests could be read as "ON" only if the yellow color occurred inside a time window that was externally provided in order to resolve this problem and preserve a workable incubation period. As long as the time interval between the ON and OFF states is appropriately longer than the test duration, sensor leakage is not an issue under these restrictions. In order to carry out this plan, we raised the temperature of the CFE reaction to 37 °C and the concentration of biosensor DNA to 10 nM. With no discernible leak in the OFF condition, activation by 100 µM NaF produced a noticeable color shift in 60 minutes (Figure 3b, Supplemental Figure S2). Within 60 minutes, there was no color change under the same circumstances using a 3 nM DNA template. This finding emphasizes a significant benefit of the open reaction environment of cell-free systems: the limit of detection of the sensor may be easily adjusted by adjusting the biosensor's reaction time and DNA concentration.

According to recent research, CFE reactions can be lyophilized and rehydrated as needed for instructional purposes, nucleic acid detection, and on-demand biomanufacturing.6,7,20,21 We then sought to show that fluoride biosensor reactions continue to function after being lyophilized in order to extend these applications to point-of-use tiny molecule detection. By lyophilizing processes containing 10 nM C23DO reporter plasmid overnight, we were able to determine the effect of lyophilization on fluoride detection. After that, the reactions were rehydrated using either water containing 1 mM NaF (Figure 3c, bottom) or laboratory-grade Milli-Q water (Figure 3c, top), and they were incubated at 37 °C. In the 1 mM NaF condition, time-lapse photography demonstrates apparent activation within 60 minutes, while in the no-fluoride condition, no leak is shown within 100 minutes (Supplemental Video S1). This result

suggests that the lyophilization procedure does not interfere with fluoride riboswitch sensing in CFE reactions, which is in line with previous recent studies from lyophilized cell-free systems6,7,20,21.

Our optimization strategy culminated in a field test of our sensor's accuracy in classifying fluoride-containing materials. In particular, we aimed to replicate an earlier environmental fluoride study that sampled and tested publicly accessible natural and municipal water sources close to the Irazu volcano in Cartago, Costa Rica, an area known to have elevated fluoride levels, using traditional methods (Supplemental Figure S4).23 In order to accomplish this, we manufactured lyophilized fluoride biosensor reactions and used our streamlined desiccant packaging (Supplemental Figure S5A) to transfer them to Costa Rica for field testing. Sampling areas noted in the earlier study23 We gathered samples in 50 mL conical tubes and performed a batch fluoride test by using single-use exact volume transfer pipettes to add unprocessed water to lyophilized reactions in PCR tubes (Supplemental Figure S5B).
All field testing was carried out on-site in Costa Rica without the use of lab apparatus or resources. Reactions were held in the armpit and incubated at about 37 °C. The reaction duration was extended to 5 hours in order to account for delayed activation brought on by low ambient fluoride concentrations and inaccurate body heat incubation.18 Within an hour, each positive control reaction turned a bright yellow, indicating that it was resistant to reaction poisoning caused by possible sample matrix effects (Supplemental Table S2). Cross-validation using a commercial fluoride-sensing electrode revealed no activation within 5 hours in any samples with fluoride concentrations below 50 µM (about 1 ppm). However, a water sample taken from a roadside ditch with a fluoride concentration of 60 µM showed a noticeable color change after 3.5 hours (Figure 4b). In detecting trace levels of fluoride below 100 µM, this delayed activation is consistent with our earlier characterization (Figure 3a). The results obtained from the cell-free sensors were validated by the commercial electrode measurement for all samples; no false positives or false negatives were detected in any of the situations (n = 9) (Supplemental Table S2). We have demonstrated that lyophilized fluoride biosensor CFE reactions can be used as low-cost, point-of-use diagnostics by accurately detecting fluoride levels relevant to public health concern thresholds in a real-world water source with minimal additional equipment. This shows the potential of engineered biosensor elements for small molecule detection in the field.

DISCUSSION

In this work, we have shown that a fluoride riboswitch can be used as a field-deployable diagnostic for environmental water samples by integrating it into a CFE system. As far as we are aware, this is the first instance of a cell-free riboswitch-based biosensor being used in the field to identify tiny chemicals that are important for health at regulatory levels. Crucially, this work offers significant simplification and cost savings over the gold standard electrochemical methods of fluoride detection, which cost hundreds to thousands of dollars and are difficult to use even for operators with scientific training. It also represents a significant improvement in efficacy over commercially available consumer kits (Supplemental Figure S6). Our biosensors, on the other hand, can be produced for $0.40 per reaction, just need a drop of water, and are resistant to temperature changes, allowing for incubation with body heat.
The ability to readily adjust biochemical parameters like cofactor and DNA concentration to minimize leak and enhance dynamic range—a historically challenging task for riboswitch fabrication in cells—is a fundamental advantage of cell-free biosensing. Moreover, riboswitches simplify the optimization space compared to trans-acting RNA or protein regulators because they are cis-acting, requiring only one DNA template concentration to be adjusted per sensor. We discovered that lyophilized reactions in PCR tubes were superior to paper-based reactions, which quickly dried up even when incubated in enclosed, humidified containers, as we were optimizing these reactions for the field. The longer incubation times needed for low analyte concentrations, variations in ambient temperature, and the practical challenge of incubating paper sensors without equipment using body heat all contributed to this effect, which made the tube format far more suitable for the difficulties of field deployment.

This study also shows that weak-binding RNA aptamers can be made into useful biosensors by utilizing transcriptional riboswitch-mediated gene expression. Given the complexity of its folding mechanism and transcriptional read-through seen both in vitro and in vivo13, we were taken aback by how successfully the B. cereus crcB riboswitch activated in an E. coli cell-free lysate environment.24 Because of the short time scales on which they make regulatory decisions, transcriptional riboswitches frequently exhibit weak activation, leading to kinetically rather than thermodynamically limited sensitivity.25 Because each reporter enzyme flips over many molecules of substrate, coupling transcriptional riboswitches to enzymatic outputs such as C23DO can enhance weak signals.26 Our sensor achieved a limit of detection of 50 μM, which is less than half of the lowest KD ever observed for any fluoride aptamer. This was made possible by the combined kinetic mechanism of switching and the signal amplification provided by a colorimetric reporter.27 This discovery is therefore a compelling illustration of how potential, diagnostically relevant sensors can be excluded when choosing aptamers based solely on thermodynamic binding affinities.

For the detection of metabolites and ions important to environmental and human health monitoring, the methods we describe here could be used to maximize the performance of numerous natural riboswitches.28 Furthermore, the straightforward structure of our DNA expression construct and the suitability of CFE reactions for high-throughput screening29 may be utilized to describe the hundreds of "orphan" riboswitches that have been bioinformatically discovered but bind to unidentified ligands.30 We even think that riboswitches could be re-engineered to have new functions using these techniques.31–33 With a fuller knowledge of the principles governing riboswitch mechanisms, we seek to develop their functional characteristics to satisfy the demand for field-deployable environmental and health diagnostics worldwide.

MATERIALS AND METHODS

Plasmid Building. Gibson assembly (New England Biolabs, Cat#E2611S) was used to assemble the plasmids, and a Qiagen QIAfilter Midiprep Kit (QIAGEN, Cat#12143) was used to purify them. pJBL3752 was used to build pJBL7025 and pJBL7026. Supplemental Table S1 has a list of every plasmid sequence.

Prepare the extract. Sonication and postlysis processing were used to obtain extracts from the Rosetta2 (DE3) pLysS strain in accordance with established methods.10. To put it briefly, cells are utilized to inoculate a 20 mL overnight starter culture for a 1 L final culture after being plated on a chloramphenicol-selective agar plate and cultured for the entire night. After being cultivated to an optical density (OD600) of 3.0 ± 0.2, this culture is sonicated to pellet and lyse it, and then it is centrifuged for 10 minutes at 4 °C and 12,000g. Following lysis, the extracts were shaken for 80 minutes at 37 °C and 200 rpm before being recentrifuged in the same manner. Following injection into a 10K MWCO dialysis cassette (Thermo Fisher, 66380), the supernatant was dialyzed for three hours at 4 °C. It was then centrifuged one more under the same conditions and snap-frozen in liquid nitrogen.

CFE Test. The standard protocols were followed in the preparation of CFE reactions.10. Reactions consist of a mixture of template DNA and inducers in a roughly 30/30/40 ratio, cell extract, a reaction buffer that contains NTPs, amino acids, buffering salts, crowding agents, and an energy source. Only the DNA template and concentration, the inducer concentration, and the buffering magnesium glutamate concentration—the latter of which is tuned by extract—changed between reactions. For field deployment and shelf stability tests, the ideal magnesium glutamate concentration was 20 mM, but for all other data, it was 12 mM. Using the proper ideal magnesium concentrations, there was minimal variation in extract efficacy between batches (Supplemental Figure S7).

See the Supplemental Experimental Design Spreadsheet for a sample reaction setup. At the 10 μL scale, triplicates of each kinetic CFE reaction were prepared on ice. To prevent bubbles, 10 μL of a mixture containing the appropriate reaction components was pipetted into three wells of a 384-well plate (Corning, 3712) after 33 μL of the mixture had been prepared. For sfGFP (20 nM reporter plasmid, emission/excitation: 485/520 nm every 5 min for 8 h at 30 °C), C23DO (variable reporter plasmid concentration, 385 nm absorbance every 30 s for 4−6 h

at 30 °C), and 3WJdB (20 nM reporter plasmid, emission/excitation 472/507 nm every 30 s for 2 h at 30 °C), the plates were sealed (Thermo Scientific, 232701). Kinetic data was then tracked using a BioTek Synergy H1m plate reader. 1 mM catechol was added to C23DO reactions, while 20 µM DFHBI-1T was added to 3WJdB reactions. A no-DNA negative control was made in triplicate for each extract under test in all fluorescence tests. The average of three samples from the no-DNA condition has been removed from the baseline for all reported fluorescence levels. Since reaction progress is measured by time to activation rather than peak absorbance value, baseline subtraction was not carried out for catechol reactions. NaF and NaCl titrations were carried out in different experiments for the findings shown in Figure 1c.
Calibration of Mean Equivalent Fluorescence. Using a previously defined process, fluorescence measurements were calibrated to a standard curve of fluorescein isothiocyanate (FITC) fluorescence to provide standard fluorescence units of µM equivalent FITC.37 A 50 µM stock was produced in a pH 9.5, 100 mM sodium borate buffer, and serial dilutions were carried out. These samples' fluorescence levels were measured at 485 nm excitation and 520 nm emission wavelengths for sfGFP and 472 nm excitation and 507 nm emission wavelengths for 3WJdB. A linear conversion factor connecting the plate reader's output in arbitrary units to the FITC standard curve was then computed using these values.
Lyophilization. A Labconco FreeZone 2.5 Liter −84 °C Benchtop Freeze-Dryer (Cat# 710201000) was used for all lyophilization. In PCR strip tubes, a CFE reaction master mix was made and divided into 20 µL aliquots. Following a pin puncture of the tube caps, the strips were wrapped in aluminum foil, frozen in liquid nitrogen, and lyophilized for a whole night at 0.04 mbar. The punctured PCR strip tube caps were replaced during lyophilization. After being parafilm-sealed, the tubes were immediately put into Drierite (Cat#11001) to be stored at room temperature (Supplemental Figure S5A).
Sensors made of paper. With a Swingline Commercial Desktop Punch (A7074020), individual sensors were punched out of Whatman 1 CHR chromatography paper (3001−861). After that, the tickets were put in a Petri dish, submerged in 4% BSA for an hour, moved to a different dish, and allowed to air dry for the entire night. Tickets were dried, spotted with 20 µL of CFE reaction, and then put in plastic jars (QOSMEDIX 29258). These jars were then covered in aluminum foil, loosely closed, and frozen in liquid nitrogen before being lyophilized for a whole night at 0.04 mbar. Tickets were rehydrated using 20 µL of sample solution after being moved to fresh jars for testing. Following closure and parafilm sealing, the jars were incubated for one hour at 37 °C. deployment in the field. 10 nM pJBL7025 and 1 mM catechol were used to create 20 µL lyophilized reactions. Additional reactions were pre-enriched with 1 mM NaF and then lyophilized as a positive control. A comprehensive list of sample site locations and water sources evaluated is provided in Supplemental Table S2. Without being processed or filtered, 50 mL samples of water were gathered and kept in Falcon tubes (Fisher Scientific, Cat# 14-432-22). Using 20 µL precise volume transfer pipettes (Thomas Scientific, 1207F80) to extract from the collected samples, reactions were rehydrated. A positive control reaction was rehydrated with the sample, a blank reaction was rehydrated with the sample, and a negative control reaction was rehydrated with filtered water to check for reaction leaks. Three reactions were conducted at each sample site. Using standard procedures, reactions were put in a plastic bag and incubated for five hours at body temperature in the armpit. If a noticeable yellow tint appeared, the reaction was considered active.18 Using an Extech ExStik Waterproof Fluoride Meter (Cat# FL700), quantitative measurements of the fluoride concentration of the same sample were made. The Open Data Commons Open Database License (https://www.openstreetmap.org/copyright) governs the use of OpenStreetMap's geographic data.
Taking pictures. With the exception of clipping the image borders, no post-capture editing was done and all photos were taken with a cell phone camera. A desk lamp was used to illuminate the tubes from below in order to accentuate the color shift of the reaction. Without being taken out of the plastic jars used for incubation, paper sensors were photographed while being lighted from above by a desk lamp.

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