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Ribozyme-Mediated Signal Augmentation on a Mass-Sensitive Biosensor

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The ability to detect biomolecules with high sensitivity is of paramount importance in biological research and in disease diagnosis. Today, most common biosensing platforms require the use of labels such as fluorophores and radiolabels. The labeling process introduces extra time, cost, and expertise required for the assays. Label-free detectors have been developed to alleviate this concern. These detectors include nanowires, cantilever-based sensors, surface plasmon resonance (SPR), and the quartz crystal microbalance (QCM).[1] The QCM has been commonly used to detect a variety of target analytes due to (1) being low cost (particularly when compared with SPR) and (2) having a relatively simple instrumentation. QCM operation is often approximated by a simple principle given by the Sauerbrey equation, which enables the user to quantify directly the mass adsorbed (Δm) onto the sensor surface for a given change in resonance frequency (Δf) of the crystal: $\Delta m = -(C_{QCM}/n)\Delta f$. C_{QCM} (17.7 ng·cm^{-2}·Hz^{-1} for the crystal used herein) is the crystal-specific mass sensitivity constant and n (=3 herein) is the overtone number. It should be mentioned that the Sauerbrey equation is only an approximation due to variation in viscosity of the adsorbed films; however a downward shift in resonance frequency is still accepted as a good indication of added mass.

The QCM's effectiveness in detecting the mass of model ligands including DNA and proteins[2] has been demonstrated. However, low-molecular-weight analytes can be difficult to detect based on mass addition alone. One successful solution has been the use of mass labels such as gold nanoparticles, which can be used in the context of an assay where free analyte is displaced with a mass-labeled or surface-bound version of the molecule.[3] In the latter case, additional amplification is achieved by appending the mass label to the receptor. In this communication, we present the use of effector-dependent ribozymes (aptazymes) as reagents for augmenting small ligand detection on a mass-sensitive device.

Aptazymes are allosteric ribozymes that have been selected (via *in vitro* selection) or engineered such that their activity is modulated by the presence of a particular analyte. The analyte-dependent activity of aptazymes has enabled their use as biosensors using a number of different detection schemes.[4] To date, label-free detection of aptazyme activity has not been presented.

Two distinct ribozyme platforms were chosen for this study: an L1-ligase-based aptazyme (L1-Rev), which is activated by a small peptide (MW $\approx$ 2.4 kDa) from the HIV-1 Rev protein, and a hammerhead cleavase-based aptazyme (HH-theo3) activated by theophylline (MW = 180 Da). Figure 1 shows the operation scheme of the two ribozymes: the ligase catalyzes the formation of a new phosphodiester bond between its own 5′ end and the 3′ end of the surface-bound substrate, while the cleavase ribozyme (which is hybridized to a surface-bound tether) catalyzes the hydrolysis of its own backbone, thus freeing the bulk of the RNA from the surface. In both cases, the catalytic activity in solution has been shown to be orders of magnitude greater in the presence of analyte than in its absence.[5] In this way, the presence of the analyte can be read via the change in the amount of surface-bound RNA. Because the ribozyme has a mass that is much greater than the analyte (for example, the liberated portion of HH-theo3 has a mass ~130 times greater than theophylline), their addition to or liberation from the surface is expected to be easily detectable.

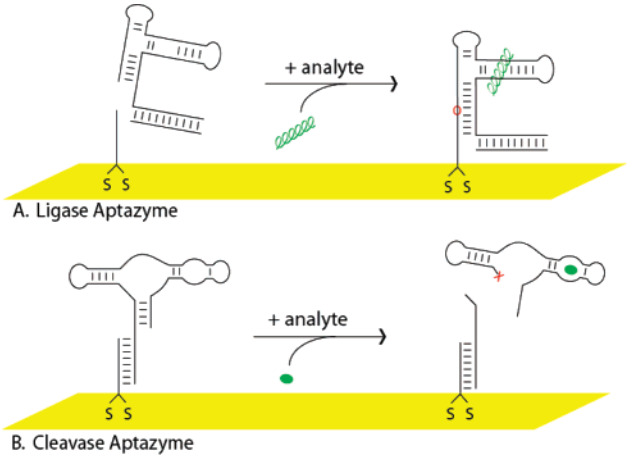

Figure 1. Aptazyme signaling on sensor surface. In the presence of its respective effector, a ligase aptazyme (A) attaches itself to a surface-bound substrate oligonucleotide, or a cleavase aptazyme (B) liberates itself from a surface-bound tether oligonucleotide. The corresponding sensor signal represents the presence of the small analyte. The site of ligation and cleavage are shown with a red O and X, respectively.

For ligase experiments, the surface was functionalized with substrate oligonucleotide SST20.sub, and the fluid in the reaction chamber was exchanged with ligation buffer. A typical experiment is shown in Figure 2. Control injections of tRNA and tRNA with Rev peptide show no significant drop in frequency, indicating that neither free RNA nor RNA with the Rev peptide has a significant nonspecific affinity for the functionalized surface. Between analyte injections, buffer injections are performed. The noise introduced by these injections is typically larger than that seen with analyte because a large volume buffer injection is done quickly in order to fully flush out the contents of the chamber. When the L1-Rev ligase ribozyme (50 nM final concentration) is injected after mixing with a blank sample (no peptide), a minimal signal is observed (which appears to be washed away upon buffer injection). When L1-Rev (50 nM) is mixed with a sample containing the Rev peptide (500 nM) prior to injection, significant ligation reaction is observed.

The signal obtained from ligation assays could potentially be increased by using more ribozyme RNA. However, at high RNA concentrations, nonspecific binding to the surface was observed. It is likely that MCH blocking of the gold surface was not ideal, as nucleic acid binding to gold has been observed in other studies.[6]

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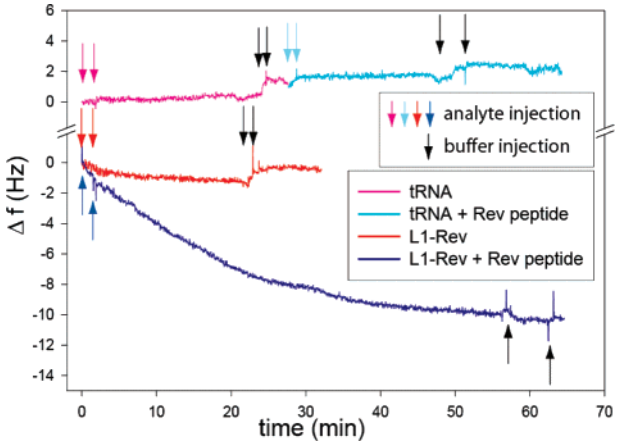

Figure 2. Ligase-based detection. The change in QCM frequency is plotted versus time for each analyte injection (performed successively on the same surface). The starting point of each data set was set to zero, and control injections of tRNA are shown above an axis break for clarity. Ligation is seen as a drop in frequency due to the addition of mass to the surface.

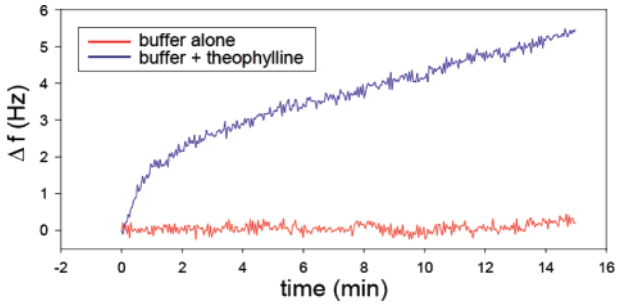

Figure 3. Cleavase-based detection. The change in QCM frequency is plotted versus time for successive injections to the same surface. The loss of ribozyme from the surface is seen as a rise in frequency.

Increasing the surface density of the ribozyme substrate, and hence the number of sites available for ribozyme ligation, should also increase the observed signal.

For cleavase experiments, the surface was functionalized with oligonucleotide SS20.hyb. The fluid in the reaction chamber was exchanged with hybridization buffer in which HH-theo3 RNA (50 nM) is injected and allowed to hybridize to the surface-bound tether. To initiate ribozyme cleavage, the fluid in the chamber is replaced with cleavage buffer. A typical experiment is shown in Figure 3. Buffer-only injections reveal little cleavage or dissociation from the surface (which are indistinguishable in this assay). However, when theophylline (1 mM) is included in the cleavage buffer, the resonance frequency increases as cleaved ribozymes liberate themselves from the surface. Nonetheless, it should be noted that the cleavase platform is inherently subject to greater background, due to nonspecific cleavage or degradation of ribozyme sensors.

The use of an oligonucleotide tether to capture the RNA creates a potentially reusable surface, as remnants of reacted ribozymes could be removed with a denaturant, and new ribozymes introduced in their places. This strategy could also be employed for ribozyme ligases by hybridizing ligase substrates to a tether. In the current configuration, a substrate-functionalized sensor surface can be used only once. A reusable surface should enable analyte detection to be quantitative, since a dose−response curve could be generated under uniform conditions.

The QCM detection of small molecule-mediated changes in aptazyme immobilization can be compared with direct detection of small molecule binding. The binding of glucose to the *E. coli* glucose/galactose receptor and of nogalamycin to duplex DNA have been observed.[7] Neither example is as generalizable as our aptazyme models, since in the former case a large structural change in the receptor enables detection, while in the latter the surface is densely packed with duplex DNAs that each intercalate numerous analytes. In order to directly compare the signal obtained via aptazyme ligation to that for binding alone, an experiment was carried out in which the surface-bound receptor was an anti-Rev aptamer, rather than the substrate for the L1-Rev aptazyme. Binding of the Rev peptide to this aptamer produced an almost undetectable decrease in frequency, while the aptazyme could readily detect the peptide (Figure 2). In contrast, when the Rev protein was added, a frequency change was readily observed, demonstrating that the aptamer receptors were functional and accessible (see Supporting Information, Figure S2).

The experiments performed herein demonstrate for the first time the use of aptazymes to enable mass-enhanced detection of small analytes. However, their use does not preclude the subsequent use of other methods of signal amplification, such as secondary binding elements. Ribozymes can be modified with biotin to append streptavidin-coated nanoparticles, or a secondary mass-label could be attached to a short oligonucleotide that would hybridize to ribozyme sequence. Our future efforts will be aimed at creating more uniform and, ultimately, reusable aptazyme-based detection methods as well as applying the method to various platforms including SPR and cantilever-based sensors.

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Supporting Information Available: QCM operating conditions, oligonucleotide and peptide sequences, buffer conditions, HH-theo3 design and activity in solution, comparison to direct analyte binding. This material is available free of charge via the Internet at http://pubs.acs.org.

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