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GOx signaling triggered by aptamer-based ATP detection

Sarita Sitaula^a, Shirmir D. Branch^a, and Mehnaaz F. Ali^a

^a Department of Chemistry, Xavier University of Louisiana, 1 Drexel Drive, New Orleans, LA 70125, USA.

Abstract

Aptamer based ATP binding leads to the release of the cofactor FAD, which acts as a trigger to 'turn-on' the activity of apo-GOx and thus generate a measureable response.

Aptamers are single stranded nucleic acid molecules, which are identified via high throughput *in vitro* selection.¹ Aptamers can fold into unique three-dimensional shapes giving rise to clefts and pockets that bind specifically to a selected target.² Thus aptamers have been selected for a wide variety of targets³ with strong binding affinities and have the potential to be as widely applicable as antibodies within biosensing applications.⁴

However, in spite of their numerous attributes, aptamer use within label-free sensing applications has been limited.⁵ Label-free biosensors are advantageous since the modifications and optimizations required of labelling tags are laborious and in combination with required sensor functionalizations could hinder the conformational fit of aptamers to their targets. Towards this end we present a label free detection strategy for ATP using aptamer-based target recognition and enzyme reactivated signaling.

Glucose oxidase (GOx) is an ubiquitous enzyme that is commonly used within the home glucose meter.⁶ It is an example of an ideal recognition molecule that upon target (i.e. the substrate) recognition produces an easily measureable change in signal. Specifically GOx oxidizes β -D-glucose to gluconic acid and hydrogen peroxide, which is readily measured. GOx signaling has been adapted to a number of elegant detection strategies as either a signal amplification tool⁷ or as a trace tag⁸. These applications include examples where holo-GOx (with its co-factor intact) in conjunction with nucleic acids was used as an effective catalytic label for the detection of glucose and thrombin.⁹ Detection of glucose was carried out by using GOx-DNAzyme hybrids as a catalyst to enhance the oxidation of an HRP substrate in the presence of H₂O₂ (which is only produced in the presence of glucose). Other studies include ferrocenyl labeled anti-thrombin aptamers that utilized the glucose/GOx system to amplify the redox activity of ferrocene to improve sensitivity of thrombin detection. However, it is the extensive body of work carried out by Willner and co-workers that utilize flavin adenine dinucleotide (FAD) and pyrroloquinoline quinone for enzyme reconstitution within numerous elegant strategies to improve the electrical contact of redox enzymes with electrodes.¹⁰

In this present study we developed a label-free detection strategy for ATP by linking target recognition of a DNA aptamer to the reconstitution of apo-GOx with its cofactor FAD.

Specifically, FAD which is the redox center of GOx, is tightly but non-covalently bound to the protein with a $K_d < 10^{-10}$ M.¹¹ The removal of FAD produces apo-GOx (inactive enzyme) and reversibly curtails the activity of the enzyme. The modulation of the enzyme reactivation was linked to aptamer binding events via the suboptimal binding of FAD to the DNA aptamer for ATP. Studies from the literature elucidated that anti-ATP aptamer (ATP-A) binding was only to functional groups on the sugar and nucleobase of ATP.¹² Since FAD also consists of the identical sugar and nucleobase region as in ATP, its binding to ATP-A (Fig 1,i) will serve as a suboptimal target and the initial step to the detection scheme described herein. The presence of ATP (Fig 1,ii) will disrupt the weaker FAD/ATP-A complex since ATP-A was specifically selected for ATP. The released FAD is then available to reactivate apo-GOx (Fig 1,iii) in the presence of glucose and produce measureable amounts of H₂O₂. In order to quantitate the produced H₂O₂ a coupled peroxidase catalyzed secondary reaction is used to reflect changes in absorbance. Since only (ATP triggered) displaced FAD is able to reactivate apo-GOx, the final changes in absorbance are correlated to the concentration of ATP present.

Isothermal titration calorimetry (ITC) was used to thermodynamically characterize the binding of FAD to ATP-A (see Figure S3 in SI). Calorimetric data were fit to a single set of identical sites resulting in an enthalpy change of -8.4kcal/mol, and binding affinity of 12.9×10^{-6} M. The measured affinity for FAD to ATP-A corresponds well as a suboptimal target in comparison to the literature stated binding affinity of 6×10^{-6} M¹³ for ATP to ATP-A.

In order to facilitate the separation steps between bound and unbound species involved within this detection approach, ATP-A was 5' biotinylated and linked to avidin coated magnetic beads (Fig 2A,i). This step allows for easy removal of excess unbound aptamer by washing with binding buffer (see SI for buffer specifics) followed by the addition of 1.2×10^{-6} M FAD to the individual ATP-A coated bead aliquots (Fig 2A,ii). In order to determine efficiency of FAD binding, the residual unbound FAD (supernatant) was removed and used to reactivate apo-GOx (Fig 2A,iii). Figure 2B shows the initial rates of the enzyme reactivated with the residual FAD from each bead aliquot indicating the repeatability (% CV of ~5) of forming the FAD/ATP-A complex. The data also includes controls showing the signal derived from the incubation of the beads and FAD in the absence of aptamer (Fig 2B data point: No ATP-A). It is seen that ~70% of the FAD added to the bead/aptamer aliquots forms the FAD/ATP-A complex. A negative control without (Fig 2B data point: No FAD) FAD shows low signal intensities and is indicative of the background signal arising from GOx containing undissociated FAD.

The sensitivity of this binding assay towards ATP was determined by adding varied concentrations of ATP to the preequilibrated FAD/ATP-A/bead complex (Fig 2A,ii). Upon the addition of ATP (Fig 2A,iv) the suboptimal ATP-A/FAD complex is dissociated to release FAD (v) and reactivate the apo-GOx. As seen in Figure 2C, when the ATP concentration is increased so does the enzyme activity (reactivation of apo-GOx) showing saturation in signals above ATP concentrations of 250 μ M. A linear relationship between enzyme activity and concentrations of ATP was obtained in the range of 10 μ M to 100 μ M (See Fig S4 in SI). The detection limit of ATP is 10 μ M and corresponds to 3 σ above the mean background level. Thus this displacement strategy is sensitive to the lower end of ATP concentrations that are typically found under physiological conditions (0.1mM-3.0mM).¹⁴ A detection limit of 10 μ M is comparable to or exceeds a few other DNA aptamer-based detection methods.¹⁵ However most electrochemical based techniques have detection thresholds in the sub-nanomolar range.¹⁶ Towards this end, on going efforts involve transferring the assay towards an electrochemical system.

The selectivity of the displacement strategy was probed with the use of two other nucleotide analogues, cytidine triphosphate (CTP) and guanosine triphosphate (GTP) all at concentrations of 250 μ M. As seen in Figure 3A, ATP has ~3.5 fold higher signal increase in comparison to GTP and CTP. The selectivity data compares well with existing DNA aptamer-based ATP detection literature.¹⁷

From literature studies, it is seen that in the ATP/ATP-A complex, two ATP moieties are bound predominantly via direct contact with positions G9 and G22 of ATP-A.¹² The previously mentioned ITC studies (See Fig S3 in SI) of FAD binding to ATP-A resulted in an apparent stoichiometry of 1:1. However, ATP binding to ATP-A resulted in an apparent stoichiometry of ~2:1 (see Table S1 in SI), which correlates well with the literature studies indicating two ATP moieties per ATP-A. In order to further probe the mechanism of FAD binding to the ATP-A aptamer, mutants of ATP-A were generated where G was replaced by A at the position of 9 and/or 22 that are the putative ATP contacting sites. Aliquots of 1.2×10^{-6} M FAD (Fig 3B,i) were added to the four aptamers respectively (ATP-A, G9M, G22M and G9/22M aptamers were initially immobilized onto avidin coated magnetic beads via a 5'biotin). In order to determine any change in affinity due to the G9 or G22 mutation specifically or both together, the bound FAD moieties were displaced via the complementary sequence (Fig 3B,ii) to the individual aptamers. As shown in Figure 3B (iii and iv), the displaced FAD was used to reactivate the apo-GOx and thus indicate the amount of FAD bound to each of the mutated aptamers in comparison to ATP-A (native ATP aptamer). The FAD dissociated from the double mutated aptamer (G9/22M) shows little or no change in enzyme activity in comparison to the control. However there is a ~51% decrease between the G22M and the native aptamer (ATP-A).

Interestingly, the signal from the G9M mutated aptamer shows a comparable signal to the native aptamer (ATP-A). The reactivation data from the mutation analysis suggests that the G22 site has a dominant role in FAD binding. This data was corroborated via ITC studies (see Fig S5 in SI) of FAD binding to the mutated aptamers. The binding of the G9M aptamer to FAD resulted in a binding affinity of 19.5×10^{-6} M with an apparent stoichiometry of ~1:1 (see SI for raw ITC data). Since the binding affinity for the G9M aptamer to FAD is in the same order of magnitude as the native aptamer for FAD (12.9×10^{-6} M) this confirms the previous postulation that the native aptamer (ATP-A) has one predominant binding site for FAD as opposed to two for ATP. This difference in binding could be attributed to steric hindrance within the target binding loop of the aptamer due to the larger FAD moiety (with the additional riboflavin region).

In conclusion, we have shown a label-free detection scheme for ATP using a DNA aptamer and a reactivated enzyme system. By separating GOx from its prosthetic group FAD, we were able to modulate the activity of the enzyme. ATP binding to ATP-A was shown to release bound FAD and thus directly turn-on the activity of apo-GOx. The displacement strategy was used to detect ATP in a dose dependent manner down to 10 μ M, which is well below the physiologically relevant concentrations of ATP. However the sensitivity could be further improved with continuing work of adapting the detection strategy towards an electrochemical output. Additionally, this system was specific for ATP over other nucleotide analogs such as CTP and GTP. The stoichiometry of FAD binding to ATP-A was probed via ITC measurements as well as generating mutations of the putative ATP binding sites on the aptamer. These studies determined G22 as the dominant binding site for FAD to the ATP binding aptamer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Design of ATP triggered enzyme reactivation. (i) FAD as a suboptimal target is bound to the ATP aptamer (ATP-A). (ii) The presence of ATP, which is the native target for ATP-A, displaces the pre-bound FAD. (iii) The displaced FAD is able to re-associate with apo-Glucose oxidase and oxidize glucose to generate H_2O_2 , which is subsequently coupled with peroxidase (POD) to generate a measurable signal.

Fig. 2.

(A) 5'Biotinylated ATP-A (i) is immobilized onto avidin coated magnetic beads. (ii) FAD is added to the ATP-A linked beads. The presence of a magnetic field helps separate the beads from the unbound excess FAD. (iii) The excess FAD from each bead aliquot is used to reactivate apo-GOx and in the presence of glucose, generate a measurable response. This strategy can be used to determine the level of FAD loading on the beads. (iv) The addition of ATP to the FAD/ATP-A/bead complex disrupts FAD/ATP-A interactions and releases FAD. (v) The displaced FAD can reactivate apo-GOx and generate signal. (B) This data shows the initial velocity measured from the residual FAD signal from five different bead aliquots (F1-F5) respectively. The residual unbound FAD is used to reactivate apo-GOx and generate a response. The No-ATP-A control represents the maximum amount of reactivation in the absence of aptamer. The No FAD control helps determine the residual signal from undissociated holo-GOx present in the apo-GOx sample and sets the lower limit for sensitivity. (C) Dose dependent curve of initial velocity versus ATP concentration. Error bars were obtained from three or more trials.

Fig. 3.

(A) Bar graphs showing selectivity of detection strategy in the presence of 250 μM ATP, GTP and CTP. (B) Mutation analysis to determine stoichiometry of FAD binding to ATP-A was carried out by generating mutated ATP-A sequences. Mutations (G to A) were introduced at the individual putative ATP binding sites (G9M and G22M) as well as at both (G9/22M) respectively. The 5' biotinylated ATP-A and mutated aptamers were bound to separate bead aliquots. After binding with FAD, the complementary sequence to the individual aptamers were introduced to displace bound FAD. The released FAD was used to reactivate GOx and determine amount of FAD released. (C) FAD binding analysis of ATP-A (see SI table) and selected mutations: G9/22M, G22M and G9M. Bar graphs represent initial velocity measured of reactivated apo-GOx from FAD displaced from each of the aptamers respectively. The FAD was displaced using complementary sequences of the individual aptamers.