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Comparison of DNAzyme activity for the development of an immobilized heme sensor

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Abstract

Point-of-care systems require highly sensitive, quantitative and selective detection platforms for the real-time multiplexed monitoring of target analytes. To ensure facile development of a sensor, it is preferable for the detection assay to have minimal chemical complexity, contain no wash steps and provide a wide and easily adaptable detection range for multiple targets. Current studies involve label-free detection strategy for relevant clinical molecules such as heme using G-quadruplex based self-assembly. We have explored the measurement of binding and kinetic parameters of various G-quadruplex/heme complexes which are able to self-associate to form a DNAzyme with peroxidase mimicking capabilities and are critical to nucleic acid research. The detection strategy includes immobilizing the G-quadruplex sequences within a polymer matrix to provide a self-assembly based detection approach for heme that could be translated towards other clinically relevant targets.

Keywords

devices; optical; sensor

INTRODUCTION

G-quadruplexes (G-quad) are stable structures formed via Hoogsteen hydrogen bonding between guanine bases in guanine rich DNA sequences.[1, 2] These interactions form tetrads that stack in a planar arrangement forming the G-quad. The amount of research that has explored G-quads has increased due to studies showing the importance of guanine-rich oligonucleotides, that can form a G-quad structure, modulating cellular processes such as replication, gene regulation, recombination and epigenetics.[3] DNA G-quad have also been widely involved in many functional DNAs and have been critical for their catalytic and target recognition activities. One such feature of G-quads is their ability to mimic peroxidase-like activity and form a DNAzyme. The underlying principal is dependent on the formation of a complex between a G-quad and hemin which can catalyze a H₂O₂ mediated oxidation of a peroxidase substrate. These sequences (with peroxidase-like ability) have been identified either via in vitro selection or traditional aptamer selection methods. The present study explores the different enzymatic activities of different DNAzymes present in the literature. Previous work has demonstrated that the strongest hemin-binding affinity was related to the best DNAzyme activity.[4] In addition, the binding of heme with the G-quad showing the highest activities are further probed using isothermal calorimetry. These studies

culminate towards developing a sensor for free heme which is pro-inflammatory and can cause oxidative damage to cells and tissues. Continuing studies show the efficacy of using an immobilized G-quad sequence within polymer scaffolds to serve as a sensor for heme.

EXPERIMENTAL DETAILS

The oligonucleotide sequences (including amine-terminated G-quad 1 sequence) and their complements were purchased from Integrated DNA Technologies, Inc (Coralville, IA). The lyophilized DNA samples were reconstituted with DI H₂O. Hemin was purchased from Frontier Scientific (Logan, UT). Aldehyde-terminated agarose microbeads were purchased from Thermo Scientific (Waltham, MA). Binding buffer contained 10mM Hepes (Sigma Chemical Co.), 100mM NaCl (Mallinckrodt AR) and 50mM KCl, pH 7.0, unless otherwise mentioned. Four different G-quadruplexes were utilized in this experiment as shown in Table 1[5–8].

DNAzyme activity

Each of these G-quads were diluted with DNase, RNase, and Protease-free water to a 100 μ M stock. The G-quadruplexes were further diluted to 5 μ M. Each of the four G-quadruplexes were placed into 1.5 μ L microcentrifuge tubes and diluted to 5 different concentrations: 0.01 μ M, 0.1 μ M, 0.5 μ M, 1.0 μ M, and 0 μ M. The G-quadruplexes were then heated at 95 degrees C for two minutes, followed by an ice bath for 1 minute. 10 μ L of Hemin (50 μ M stock in DMSO) was added into each sample respectively. The samples were covered in foil and incubated for 2–3 hours or used immediately. The peroxidase substrate was prepared using 0.0303 g 3,5-dichloro-2-hydroxy-benzenesulfonate (Sigma Chemical Co.), and 0.0218 g 4-aminoantipyrine (Sigma Chemical Co.) with 10 μ L of autoclaved water in a centrifuge tube respectively and mixed. Then, 3.5 mL of both the 4-aminoantipyrine and 3,5-dichloro-2-hydroxy-benzenesulfonate, and 3 mL of sodium phosphate buffer (0.1 M, pH 6.21) were mixed in a separate centrifuge tube to make up the peroxidase substrate used for subsequent experiments. 10 μ L of hydrogen peroxide was added into the cuvette, and the measurement was immediately started using the kinetics feature on a Beckman DU 800 Spectrophotometer. The enzymatic activity was determined using the change in absorbance versus time data.

Immobilization of G-quad 1 to microbeads

Amine-terminated G-quad 1 was coupled to the aldehyde-terminated agarose microspheres via reductive amination. The oligonucleotide-immobilized beads were rinsed 3 times with binding buffer. The efficiency of immobilization was determined by measuring the absorbance (at 260nm) of the G-quad solution before and after immobilization on the beads.

For the bead assay, 200 μ L of beads were separated into three vials. The first vial contained beads without the G-quad immobilized. The other two vials consisted of 200 μ L of G-quad 1 immobilized beads. 1 μ L and 10 μ L of heme (50 μ M stock) was added to the positive vials respectively. Hepes buffer was added to both vials to make up a total volume of 500 μ L. The control vial received 10 μ L of the heme along with the Hepes buffer. The vials were incubated for two hours after which 10 μ L of hydrogen peroxide and 490 μ L of the peroxidase

substrate were added and color changes were observed. Transmission images of the beads were taken using a Nikon Eclipse Ts2 Inverted routine microscope and accompanying software and a 20× objective.

Isothermal titration calorimetry

G-quad solutions (400 μ L, 50 μ M) and Heme 200 μ L (700 μ M) were prepared in binding buffer (10mM Hepes, 100mM NaCl, 50mM KCl, pH 7.0). All the samples and buffers for the washing syringe were sonicated for 15 mins at RTP. A solution of G-quad 1 or 2 (400 μ L) was loaded in a deep well plate assigned as the target while Heme solutions were loaded in a deep well plate assigned as the ligand, for syringe injection. Upon completion of the injections, the data was processed using MicroCal Auto_{itc} Origin Software.

DISCUSSION

The formation of DNAzyme complexes, were carried out using the four different G-quad sequences specified above. Due to the importance of the detection of heme, the activity of the four DNAzymes were studied either directly in solution or after immobilizing the G-quads on a solid scaffold.

DNAzyme activity

Figure 1 shows the activity for the four different sequences that are being studied for heme detection. The data clearly delineates G-quad 1 and 2 as having the highest enzyme activities. These two sequences with the highest enzymatic activity were further explored to probe time of incubation to enhance activity. Using the G-quad with highest enzymatic activity will ensure a low detection threshold for the detection of free heme.

Figures 2 (a) and (b) show the comparison between G-quad 1 and 2 with respect to incubation times of heme and the G-quad with increasing concentrations of G-quadruplex. The bar graph shows increased activity of both DNAzymes with a two hour incubation period. However, G-quad 2 shows the highest activity (>2.5fold) for the 0.5 μ M concentration of aptamer with a two hour incubation.

Isothermal titration calorimetry

In order to confirm the literature binding constants and isolate the strongest binders prior to transferring the detection system to a solid scaffold, ITC was used to thermodynamically characterize the binding of heme to G-quad 1 and 2 respectively (see Figure 3 a and b). Calorimetric data for G-quad 1 and 2 were fit to a single set of identical sites resulting in enthalpy change of -2.19×10^4 and -3.89×10^5 joules/mol, and binding affinity of 1.5×10^{-6} M and unresolved respectively. The measured affinity for heme to G-quad 1 corresponds well with the literature but G-quad 2 does not and needs further optimization.

Microporous bead

Figure 3 (c) shows a transmission image of the agarose beads with size ranges from 45–200 μ m. Although, most prior efforts for immobilized biological sensors have used the attributes of a planar surface, the trend towards satisfying the necessity for high binding

capacity sensors has moved towards the use of porous high surface area polymer ensembles. [9] After immobilization of the amine-terminated G-quad 1 sequence, the efficiency of immobilization was determined to be in the 96% range. These G-quad 1 immobilized porous matrices were used within an assay for heme. Figure 3 (d) shows the color changes associated with the negative control, 1 μ L and 10 μ L heme conditions as specified earlier. The addition of the hydrogen peroxide and peroxidase substrate shows a clear difference between the three vials with the more concentrated heme vial showing a larger contrast in color in comparison with the 1 μ L heme addition.

CONCLUSIONS

The preliminary study described above demonstrates the efficacy of using G-quadruplexes as signaling probes for the detection of heme. The comparison of enzymatic activity between the four G-quads demonstrates the importance of G-quadruplex formation with respect to heme detection. The combination of the selective formation of DNazymes and the porous agarose scaffold as a sensing platform will allow for fast, quantitative determination of free heme thresholds.

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Figure 1.
Enzymatic activity of four different G-quadruplexes.

Figure 2.

Enzymatic activity of (a) G-quad 1 and (b) G-quad 2 with varied sequence concentrations of 0.01, 0.1 and 0.5 μM . Each G-quad concentration was added to 10 μL of Heme (50 μM stock) and either immediately measured for enzyme activity after the addition of hydrogen peroxide and peroxidase substrate or incubated for 2hr prior to measuring the enzymatic activity.

Figure 3.

(a and b) Profiles for the ITC of heme into a 50µM of G-quad 1 and 2 respectively in binding buffer at 25°C. (c) Transmission images of agarose scaffolds taken with a 20× objective. (d) the agarose bead samples (i) without heme, (ii) with 1µL heme and (iii) with 10µL heme respectively.

Table 1

List of G-quadruplexes with references

Name	Sequence	Reference
G-quad 1	5'-TTT GGG TAG GGC GGG TTG GG-3'	Willner et al; Anal Chem. 2010
G-quad 2	5'-GTG GGT AGG GCG GGT TGG -3'	Shi et al; Chem Comm. 2011
G-quad 3	5'-TTA GGG TTA GGG TTA GGG TTA GGG TT-3'	Ambrus et al; Nucleic Acids Res. 2006
G-quad 4	5'-AAT GGG AGG GAT TGG GTG GG-3'	Yang et al; Anal Chem. 2012