Structural and functional study of a simple, rapid and label-free DNAzyme-based DNA biosensor for optimization activity

Narges Shahbazi[a], Saman Hosseinkhani[a,b], Khosro Khajeh[a,b], Bijan Ranjbar[a,c*]

[a] Department of Nanobiotechnology, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran

[b] Department of Biochemistry, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran

[c] Department of Biophysics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran

[*]Corresponding author at Departments of Biophysics and Nanobiotechnology, Faculty of Biological Sciences, Tarbiat Modares University, P.O. Box 14115-175, Tehran, Iran.
Tel/Fax.: +98 21 82883418
E-mail address: ranjbarb@modares.ac.ir (B. Ranjbar)

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Abstract

Peroxidase-mimicking DNAzyme has a potential to self-assemble into a G-quadruplex and shows peroxidase activity. In comparison to proteins, peroxidase-mimicking DNAzyme is less expensive and more stable. Herein, it is used in fabricating non-labeling biosensors. This paper investigates the structural and functional properties of a DNA biosensor based on split DNAzyme with a detection limit in nM range (9.48 nM). Two halves of DNAzyme were linked by a complementary sequence of DNA target. Hybridization of the DNA target pulled two DNAzyme halves apart and peroxidase activity decreased. This study can be divided into 3 stages. First, the characteristics of DNAzyme were studied by Circular Dichroism (CD) technique and UV-Vis spectroscopy to find out DNAzyme's optimum activity. It is worth to note that some divalent cations were used to form G-quadruplex, in addition to common monovalent cations. Furthermore, the hemin incubation was also optimized. Secondly, the structural and functional properties of two types of split DNAzyme were compared with DNAzyme. Thirdly, the hybridization of DNA target was monitored. The results revealed that peroxidase activities of split types decreased by half without any specific conformational changes. Interestingly, the catalytic activities of split DNAzymes could be promoted by adding Mg^{2+}. Besides, it was demonstrated that the structure, peroxidation reaction and DNA target hybridization of 2:2 and 3:1 split modes were almost alike. It was also illustrated that magnesium promoted the possibility of hybridization.

Keywords: DNAzyme, Biosensor, *Circular Dichroism*, G-quadruplex

1. Introduction

Guanine-rich sequences demonstrate the capability to fold a higher-order four-stranded structure termed as G-quadruplex, in which Hoogsteen hydrogen bonds form a square planar array called G-quartet [1,2]. G-quartets are predisposed to stack upon each other, leading to a considerable Van der Waals attraction. Some aromatic planar ligands including porphyrin and metalloporphyrin have a tendency to externally stack on the G-tetrad's terminal of G-quadruplexes. Hemin is one of the most important of these ligands that is able to bind to several quadruplexes with high affinity; thus, these types of G-quadruplex are also identified as aptamers for hemin [3]. The complex of G-quadruplex and hemin exhibits catalytic activity toward the H_2O_2-mediated oxidation reaction so it is known as a 'peroxidase-mimicking DNAzyme' (Fig. 1). It demonstrates that peroxidase-like activities of these types of DNAzyme are 250 times greater than hemin alone [4]. 2, 2-azino-bis (3-ethylbenzthiazoline)-6-sulphonic acid (ABTS) is commonly used as a reducing substrate which is oxidized by H_2O_2 into a colored product called '$ABTS^{+'}$ that is traced by an increase in absorption signal in 420 nm. ABTS is rapidly reacted, thus it is preferable for instrumental analysis. Peroxidase-mimicking DNAzymes are significantly steadier than proteins against hydrolysis and heat treatment. DNA is almost 1,000 times more stable than protein against hydrolysis and 100,000 times more stable than RNA, and unlike protein enzymes, DNAzyme can be denatured and renatured many times without losing its ability [5]. In addition, they can be synthesized, modified and labeled easily. Because of these valuable merits, DNAzymes are considered as cost-effective, flexible and promising tool for the design of biosensors so they are developed for detection of ions [6-9], nucleic acids [10-13], small molecules and proteins [14-17].

G-quadruplex demonstrates the capability of adopting several different topologies, depending on the nature of the G-quadruplex's sequence, the sequence and topology of the loop, and the ionic condition [18]. Due to strand orientation, G-quadruplexes have parallel, antiparallel and mixed parallel/antiparallel structures. Parallel intramolecular structure is the more favorable one for binding of porphyrins through end-stacking. In contrast, antiparallel quadruplex shows low activity owing to steric hindrance caused by the loops which makes hemin binding difficult. It is considerable that hemin attachment can also alter the conformation of G-quadruplex and promote the conversion of some G-quadruplex's structures from antiparallel to parallel form [19,20].

Metal ion is one of the most important factors which affect the structure and performance of peroxidase-mimicking DNAzymes. Cation is located in the center of quadruplex and stabilizes the hydrogen bonding of G-quartet. Ions can interact with two types of ligand to stabilize G-quadruplex's structure: the external phosphate and O6 guanines [21]. A variety of mono and divalent metal ions can stabilize or switch G-quadruplex's structure. Among monovalent cations, K^+ and Na^+ are the most studied types. Potassium cation fits into the cavity between two guanine tetrads and thus can coordinate with eight O6 atoms. This explains why potassium cations can better stabilize the G-quadruplexes than sodium ions, which are placed in the middle of tetrad planes [22]. Furthermore, the presence of K^+ is beneficial to formation of parallel structure,

compared to Na^+ which almost induces an antiparallel structure. Albeit it has been approved that K^+ significantly manifests a higher activity and there is no demand on cations in sensing systems, some investigations used other monovalent cations like NH_4^+. It was observed that the presence of NH_4^+ enhances the activity of G-quadruplex DNAzyme-base sensors [5, 23]. Indeed, higher initial rate of catalytic reaction and higher turnover number are obtained when ammonium cations are presented in the buffer, which can be due to the general acid-base catalysis. In contrast, low turnover number is observed in buffers including either excess potassium or sodium which causes less activity compared to NH_4^+[23, 24]. Despite this fact, some researches still use potassium and sodium in their DNAzyme-based biosensors [25, 26]. Hence, choosing the best type and concentration of cation is considered as the first step in optimization of DNAzyme-based biosensor's activity. Another crucial factor that influences the structure and function of peroxidase-mimicking DNAzyme is the loops that connect two adjacent G-tetrads. Generally, G-rich sequence with short loop has a tendency to form a parallel G-quadruplex, and the one with long loop usually folds into an antiparallel structure [27].

In some G-quadruplex DNAzyme-based sensors, G-rich sequence is divided into two or four parts which are called split DNAzymes. This property offers greater flexibility for design of a sensor. These parts can be located either in separated DNA strands or in only one strand. The two parts split DNAzyme, which is mostly used, can be either symmetric known as 2:2 (each part containing two G-tracts), or asymmetric known as 3:1 (one part including three G-tracts and the other containing one G-tract). Though the former is commonly used, some reports revealed 2:2 splitting mode can be easily assembled to form G-quadruplex, even a rigid duplex of oligonucleotides separate it and then a background signal is produced [5]. Both 2:2 [28, 29] and 3:1 [30, 31]modes have been employed in designing biosensors.

The current study presents a procedure to promote a split DNAzyme-based DNA biosensor's activity. It is noteworthy that PW17 was used as DNAzyme in this study (table 1). PW17 is one of the PS2.M variants designed by Willner *et al*. [32]. It was applied in peroxidase-mimicking DNAzyme-based sensors for detection of targets[33], such as metal ions [34, 35], nucleic acids [36] and small molecules [37, 38].The primary phase was the enhancement of DNAzyme peroxidase activity. First, the best ionic condition for DNAzyme formation was determined, which is routine in DNAzyme researches. Then the effect of hemin on structure was investigated, and the time of hemin incubation was optimized. The next phase was a comparative study between structure and function of DNAzyme and its split types to find out the effect of loop. Then some auxiliary ions were applied to promote split DNAzyme activity and diminish the loop effect. The final phase involved monitoring DNA target hybridization and improving it. The functions and hybridization capabilities of 2:2 and 3:1 split DNAzyme modes were compared, and then the more applicable type was selected. After that, the effects ions on hybridization were investigated to optimize DNA target attachment.

2. Materials and methods

2.1. Materials & reagents

All oligonucleotides were purchased from AnaSpec, USA (table 1). 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonicacid) diammonium salt (ABTS), 4-(2-hydroxyethyl) piperazine-1-ethane-sulfonic acid hemisodium salt (HEPES), hemin and salts (KCl, NaCl, $MgCl_2$, $CaCl_2$, $NH_4C_2H_3O_2$) were purchased from Sigma–Aldrich. Stock solution of hemin was prepared in dimethyl sulfoxide (DMSO) and stored in a dark place at −20°C.

2.2. Preparation of DNAzyme

1 μM of DNAzyme was diluted in HEPES buffer (25 mM, pH 7) and 100 mM of different types of cations were added to the solution. To ensure the formation of G-quadruplex structure, the mixtures were heated up to 88 °C for 15 min and then gradually cooled down to room temperature. Afterwards, an equal concentration of hemin (1 μM) was added to G-quadruplex solution and incubated for 1 hour at room temperature.

2.3. Colorimetric measurement assay

Cary 100 UV-VIS double beam spectrophotometer was used to measure ABTS anion radical produced by enzyme activity of DNAzyme (400–500 nm). The peroxidation reaction was evaluated by addition of H_2O_2 (0.3 mM) to DNAzyme and ABTS (1 mM) solution. Final concentration of DNAzyme was 100 nM in reaction solution. Absorbance of 420 nm was recorded till 3 min after initiation of reaction.

2.4. CD spectropolarimetry

Circular dichroism spectra were obtained using a J-715 spectropolarimeter at room temperature. The final concentration of the prepared DNAzymes and its split types were adjusted to 30 μM. Spectra were recorded between 200 and 320 nm in 1 mm path length cuvette with a bandwidth of 1nm. The final results were the mean of three measurements. The scan of buffer was used as a baseline and was subtracted from each spectrum. Spectra were smoothed by J-715 software and then normalized to have zero ellipticity at 320 nm [39, 40].

2.5. Fluorescence spectroscopy

PicoGreen is an ultrasensitive fluorochrome that selectively binds to dsDNA, and has characteristics similar to those of SYBR-Green. The PicoGreen is excited at 480 nm and has an

emission peak at 520 nm. In this study, PicoGreen is used to identify the double strand which separates two halves of DNAzyme. Fluorescence enhancement of PicoGreen is exceptionally high when bound to dsDNA, in comparison with G-quadruplex structure. The dilution procedure of PicoGreen contains dilution by HEPES buffer 25 mM (1:100) and then it was added to the G-quadruplex mixture to the final ratio of 1:2000. Fluorescence spectra were recorded on a Perkin Elmer fluorimeter [39].

3. Results & discussion

3.1 *Optimization of DNAzyme formation*

3.1.1 *Structural study*

G-quadruplexes are polymorphous structures due to the possibilities of assembling various numbers of oligonucleotides strands, the *anti* or *syn* glycosidic angel orientation of guanosines, and different topologies of loops. The structures of G-quadruplexes can be monitored through the specific signatures in their circular dichroism (CD) profiles. A huge positive band around 260 nm and a shallow negative band at 240 nm represent parallel structure, whereas a positive CD signal at 295 nm and a negative signal around 265 nm reveal antiparallel structure. Furthermore, the positive peaks at both 265 nm and 295 nm demonstrate the mixed hybrid-type structure or a mixture containing both parallel and antiparallel structures. Therefore, CD spectropolarimetry is a good indicator of change in G-quadruplex configuration [22, 23]. It is worth to note, parallel structure, in comparison with antiparallel type, shows better peroxidase activity. Hence, DNAzyme structure and peroxidase reaction were analyzed to answer the following questions: (a) Which cation forms better parallel structure? (b) Do divalent cations such as Mg^{2+} and Ca^{2+} form G-quadruplex like monovalent cations? (c) Is there a correlation between the topology and peroxidation rate of PW17? (d) Do concentrations of cations affect the performance and conformation of PW17?

Ion or nonelectrolyte solute concentrations affect both covalent and noncovalent processes. Change in ion's concentration influences the thermodynamic activities of both water (the osmotic effect) and biopolymers (preferential interactions). Influences of salt on processes including polyanionic nucleic acids are especially large. For example K^+, Mg^{2+}, or polyamine salts could affect midpoint temperatures (Tm) of conformational transitions even when these are at very low concentrations (≤0.01 M). Smaller but still very significant influences of changes in salt concentration in this low salt range are observed on processes involving short oligonucleotides [41]. According to this fact, the effects of salts on DNAzyme and split DNAzyme structures and activities, and DNA target hybridization were investigated in this study.

Fig. 2 represents the effects of different types of ions on PW17 structure. The weak positive peaks at 295 and 265 nm indicate that DNAzyme could slightly form both parallel and antiparallel structures without ions. In the presence of 100 mM of each K^+, NH_4^+ and Mg^{2+} G-quadruplex adopted parallel conformation featuring a sharp positive peak around 260 nm and a negative peak near 240 nm. Presence of Na^+ demonstrated mixed parallel and antiparallel conformations. In contrast to other cations, Ca^{2+} showed almost no effect on DNAzyme's structure. As mentioned before, PW17 was used as a DNAzyme in this study which is one of the PS2.M variants. Despite PS2M which forms antiparallel structure in the presence of Na^+[19], PW17 could shape both parallel and antiparallel types in the same situation. As it was expected, K^+ induced parallel conformation in PW17 like PS2M and other DNAzymes. NH_4^+ displayed parallel signatures like K^+. In contrast, PS2M is formed mixed parallel and anti-parallel conformations in the presence of NH_4^+[36]. In this study, CD results demonstrated that 100 mM of Mg^{2+} could stabilize the PW17 parallel structure against Ca^{2+}, although the effect of $Mg^{2+}$was not as strong as K^+ and NH_4^+. Consequently, CD spectra revealed that NH_4^+ and K^+ could well induce parallel structures and proved to be superior to the other cations for follow-up studies. Fig. 3 shows the effect of different concentrations of NH_4^+ and K^+ on PW17 conformation. It was illustrated that an increase in cation concentrations from 50 mM to 250 mM does not affects the conformation.

3.1.2 *Functional study*

The accumulation of the oxidized product of ABTS ($ABTS^+$) is recorded in order to follow up the peroxidase activity of DNAzyme in different situations. DNAzymes are capable of catalyzing the H_2O_2-mediated oxidation of ABTS and producing a green and soluble product with maximum absorbance at 420 nm. Fig. 4 displays the DNAzyme peroxidation rate in the presence of different types of cation. The catalytic data was recorded for 3 minutes when the kinetic spectrum reached the highest level. As expected, G-quadruplex did not show any peroxidation reaction without hemin. In addition, hemin catalytic reaction was negligible compared to its activity in the presence of cations. Some research studies have stated that excess of hemin cause a high background signal which is an intruding element in DNAzymes biosensing [42]. In this study, the hemin background signal was insignificant. Absorbance after 3 minutes in the presence of NH_4^+, K^+, Na^+, $Ca^{2+}$and Mg^{2+}were 1.5, 1, 0.6, 0.1 and 0.8, respectively. As demonstrated, the best catalytic activity was observed in the presence of NH_4^+ despite the fact that K^+ showed significant parallel signatures in CD spectra. This higher activity in the presence of NH_4^+ was perhaps caused by an increase in the turnover number of DNAzyme and an improvement in initial rate of catalytic reaction [5]. UV-VIS and CD spectra illustrated that Na^+ was the weakest cation in forming parallel structures and peroxidation reaction among other cations. Despite Mg^{2+}, DNAzyme showed no catalytic activity in the presence of Ca^{2+}.

Fig. 5 depicts the effect of different concentrations of NH_4^+ and K^+ on the peroxidase activity. In the presence of K^+, the peroxidase activity increased up to 100 mM of potassium ions and then

remained stable. In contrast, an increment in NH_4^+ concentrations caused an increasing peroxidation reaction. Consequently, the best concentrations of NH_4^+ and K^+ for peroxidase reaction of 100 nM DNAzyme were 250 mM and 100 mM, respectively. No structural change was observed in different concentrations (50 to 250 mM) of NH_4^+ and K^+ (Fig. 3). Taking into account, the enhancement in peroxidase reaction had no structural reasons. Overall, the structure and function of DNAzyme in the presence of different cations were surveyed to figure out the one which can form parallel structures and exhibits the best peroxidase activity. The results showed 250 mM NH_4^+ was the best candidate for follow-up studies.

3.1.3 *Optimization of hemin incubation*

As mentioned before, DNAzyme-hemin complex is an artificial system that increases the catalytic activity of hemin. Herein, the structural and functional study of hemin attachment could help to promote enzymatic activity. In addition, in the most DNAzyme-based biosensors, DNAzyme is incubated with hemin for at least one or two hours which is a time-consuming step [25, 26]. Hence, the optimization of hemin incubation time could speed up the detection. Fig. 6.a shows addition of hemin alters CD spectrums to some extent. In the presence of Mg^{2+} and NH_4^+, the magnitude of peaks increased slightly; in contrast, hemin caused a slight decrease in K^+ spectrum. In the presence of Na^+, parallel structures did not form completely but hemin induced a parallel structure, and a conformational change was observed. The CD spectra revealed that hemin did not significantly change the parallel structure. Therefore, UV-VIS was used only for monitoring DNAzyme-hemin attachment. Fig 6.b shows the peroxidase activity of DNAzyme during one hour after hemin addition. The catalytic activity increased until 30 min after hemin addition. Therefore, 30 min was determined as the minimum and optimum time for hemin incubation.

3.2 Structural and functional study of split types of DNAzyme

Having proved split types of G-quadruplex DNAzymes are good candidates for non-labeling biosensors, Willner recomposed sequence of PS2M which consists of four GGG repeats [32]. Therefore, it is possible to split the full-length single stranded into two or four parts. Split DNAzymes are different types. One common type is named 2:2 in which the DNAzyme sequence is split into two equal parts. Some results showed that this type could assemble to G-quadruplex structure in the presence of DNA target and then produce a background signal in negative split DNAzyme biosensor [5]. Minggang Deng et al. reported another split type called 3:1(one part containing three GGG repeats and the other containing one GGG repeat) and found it useful in detection of nucleic acids [43]. In this study, both types were used and the following questions were answered: (a) Are enzymatic activities of split types of DNAzyme similar to DNAzyme? And how can we enhance peroxidation activities of split types? (b) In the presence

of DNA target, does split type assemble to G-quadruplex structure and does it produce a background? (c) Do two different split types of DNAzyme show the same enzymatic reaction?

Some reports were published on the effect of loops on the stability and topology of G-quadruplexes. They revealed that both length and sequence of loops determine the conformation and stability of G-quadruplex structures. It was also observed that loop-loop interaction, such as hydrogen binding, can affect the G-quadruplex's properties. It was observed that short loops generate parallel topologies, while long ones form antiparallel complexes. There are few reports about the effect of loop on enzymatic activity of G-quadruplexes [23]. Fig. 7.a elucidates a comparative study between peroxidation activities of DNAzyme and split types. As expected, a long loop caused a hindrance to the formation of G-quadruplexes. The enzymatic activities of split types were half of whole DNAzyme activity. It was hypothesized that applying an auxiliary cation helps to reduce the side effects of loop addition. Due to the fact nucleic acids are polyanions; they need positive ions to neutralize the charged phosphate groups. Moreover, ions can influence the structure of nucleotides indirectly through interaction with water molecules. Fig.7.b elucidates the effect of auxiliary cations on peroxidase reaction of split types in the presence of 250 mM NH_4^+.As mentioned before, the highest peroxidase activity of DNAzymes was observed in 250 mM NH_4^+, but in split types the enzymatic activity decreases due to insertion of loops. Hence, auxiliary cations were used to facilitate the formation of G-quadruplex. In the presence of Na^+, the enzymatic rate increased slightly up to a concentration of 5mM and became steady after that. In the presence of K^+, the peroxidase activity increased gradually but Mg^{2+} caused a dramatic increment up to 50 mM. Therefore, Mg^{2+} increased the catalytic activity of split types significantly. This can be justified by the fact that Mg^{2+} ion carries six-coordinated water molecules and then, when they attract a negatively-charged phosphate group, the hydrogen bonds can be formed. In addition, magnesium ions enhance the activity of O-H group of water and improve the strength of hydrogen bond. Therefore, hexahydrated magnesium cation binds with DNA electronegative elements (O, N) and stabilizes the nucleic acid structure like the effects of preferential hydration in osmolites on the structure of proteins [44]. In addition, other research studies proved that the presence of magnesium leads to the formation of more compact G4 structures that can better protect the bound hemin molecule from deactivation (oxidation by hydrogen peroxide) [45]. Accordingly, the presence of Mg^+ ions improves the split types' peroxidation activity as shown in Fig 7.c. In addition, both split types (2:2 and 3:1) were affected in the same pattern.

To investigate the effect of loop insertion on the DNAzyme structure, CD spectropolarimetry was used. Figure 7.d demonstrates the CD spectra of DNAzyme and its split types. A little red shift and an increase in signal intensity are observed in split type spectra, and both 2:2 and 3:1 types show the same structure. Split types formed parallel structures such as DNAzyme. Inserting loop in DNAzyme structure causes a weakness in hydrogen non-covalent bonds that causes the red shift in CD spectra of the split type. As mentioned before, Mg^{2+} enhances the peroxidation activity of split type, although Fig.7.d elucidates that addition of Mg^{2+} caused no structural changes in split types.

3.3 *Optimization of DNA analyte hybridization*

As mentioned before, two halves of DNAzyme were linked by a complementary sequence of DNA target. Hybridization of the target pulled two DNAzyme halves apart. The detection method scheme is illustrated in Fig 8. By hybridization of DNA target, the peroxidase activity decreases as illustrated in Fig 9.a. The hybridization potentials of two types of split DNAzymes were almost alike. It is worth to mention that the difference between DNAzyme's activity in the absence and presence of DNA target (ΔA 420 nm) is an indicator for sensing DNA target. In this study, we tried to optimize the DNA target hybridization and decrease the background noise to enhance biosensor efficiency and facilitate detection; in other word, we tried to increase ΔA 420 nm. It is noteworthy, producing background is one of the challenging sections in using split DNAzyme biosensors. Although a rigid duplex of DNA is designed to separate two halves of DNAzymes, G-quadruplexes can form. Hence, peroxidation reaction is observed even in the presence of DNA target.

The first step of optimization was monitoring DNA target hybridization. The fluorescence study was used to observe target detection. PicoGreen, a ds-DNA intercalating cyanine dye, was applied to monitor DNA target hybridization. PicoGreen shows significant different signals by intercalating with ds-DNA or Q-quadruplex structure. To shed more information, PicoGreen exhibits a significant difference in lifetime and quantum yield between double and single strands of DNA. Some researchers reported that, among deoxyoligonucleotides, PicoGreen shows maximum quantum yield in the presence of deoxy guanine. Hence, the dimeric association between PicoGreen and guanine rich single strand oligonucleotide is different from that of random single strand DNA. Accordingly, G-quadruplex shows a partial fluorescence signal in the presence of PicoGreen [39]. In this study, the complementary of probe A was used and named as probe B. Hence, probe A and B can form a duplex DNA. PicoGreen, as indicator of ds-DNA, is significantly attached to probe A and B duplex. Therefore, the highest intensity should be observed in probe A and B fluorescence assay. Subsequently, the fluorescence intensities of split types and probe A and B duplex were compared. If all probe A is attached to split type and blocks G-quadruplex formation, the fluorescence intensity of split type and probe A should be the same as probe A and B. Figure 9.b shows the fluorescence intensities of ds-DNA (probe A + probe B), split DNAzyme in the presence and absence of probe A, and DNAzyme alone. It is worth to note that oligonucleotides were formed in the presence of 250 mM NH_4^+ and 50 mM Mg^{2+}. As clearly observed, the fluorescence intensity of split type and probe A are less than probe A and B. Thus, it can be concluded that duplex forming in the middle of DNAzyme strand is incomplete and decreases the enzymatic activity. Therefore, even a duplex DNA destabilizes DNAzyme, G-quaduplex structure is formed to some extent. So it could be also concluded that there is a chance to strengthen the DNA target hybridization and weaken DNAzyme formation. Furthermore, the results show fluorescence intensities were the same in both split 2:2 and 3:1 in the presence and absence of probe A, so there was no significant difference between DNA target

hybridizations in split 2:2 and 3:1. Since split 2:2 is more common in DNAzyme-based biosensors, split 2:2 was selected for further study. It is worth to note, figure 9.b elucidates that the fluorescence intensities of split types in the absence of probe A is higher than DNAzymes. This increment is possibly caused by the formation of hairpin in the loops.

In the next step, auxiliary cations were used to improve DNA target hybridization. Cations affect the structure and enzymatic proficiency of peroxidase DNAzymes; they also influence DNA-DNA hybridization. As illustrated in Fig. 10, cations play an important role in DNA target hybridization. High concentrations of sodium ions prohibit hybridization. By reducing the amount of sodium ions, hybridization increases slightly. In contrast, not only potassium ions have no effect on hybridization but also higher concentrations than 5 mM prevent hybridization. Unlike these two monovalent cations, magnesium ions significantly boost DNA target attachment. They quintuplicate the hybridization possibility. It could be suggested that cations can affect the nucleotides structure indirectly through interaction with water molecules, as mentioned before. Herein, magnesium ions are selected as an auxiliary ion in hybridization process.

Finally, enzymatic activity of DNAzyme-based biosensors was measured in the presence of different concentrations of DNA target (1-100 nM). As shown in Fig 11, the sensitivity of this biosensor was around nano molar range and the detection limit obtained was 9.48 nM. It is noteworthy, the detection limit was calculated according to equation: LOD=3.3*standard deviation of reference probe/slope of the calibration line.

4. Conclusion

The present study investigated the structural and functional properties of a DNA biosensor based on split DNAzyme for optimization activity. In the first step, DNAzyme formation was optimized. It was revealed that NH_4^+ was the most appropriate candidate for inducing parallel structure and good enzymatic activity. DNAzyme showed the highest peroxidation rate in the presence of 250 mM NH_4^+. Then, by monitoring the attachment of hemin, the minimum time for hemin incubation was determined as 30 min. In the next step, comparing conformations and functions of DNAzyme and its split types revealed that loops caused no significant structural change but decreased enzymatic activity by half. Hence, some auxiliary ions were used. Among them, Mg^{2+} stabilized nucleic acid structure and improved the peroxidase activity of split DNAzyme. In the final phase, hybridization of DNA target and production of background noise were monitored. Structure, function and hybridization of DNA target were almost the same in both symmetric and asymmetric split types. For optimization of hybridization, different types of cations were applied. Magnesium enhanced the hybridization propensity dramatically.

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Table 1. Sequence of oligonucleotides used in this study

	sequence
PW17	5' **GGGTAGGGCGGGTTGGG** 3'
Split 2:2	5' **GGGTAGGGTATGGTCGAATAAGTTAAGGGTTGGG** 3'
Split 3:1	5' **GGGTAGGGCGGGTATGGTCGAATAAGTTAAGGG** 3'
Probe A	5' **TTAACTTATTCGACCATA** 3'
Probe B	5' **TATGGTCGAATAAGTTAA** 3'

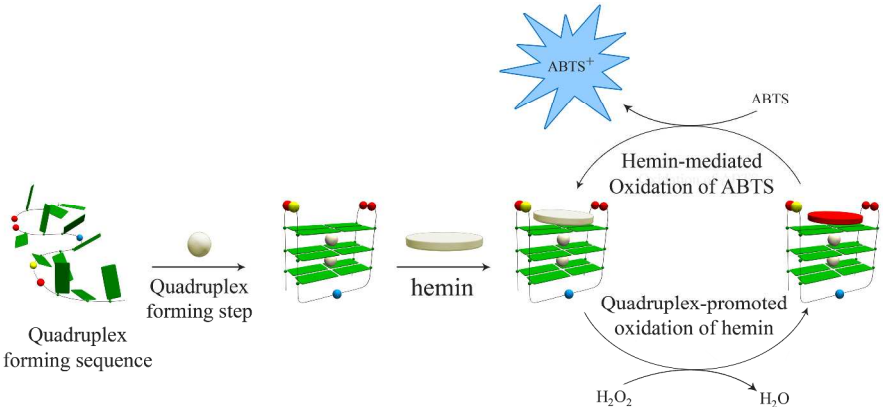


Fig. 1: Schematic representation of the quadruplex-mediated DNAzyme process

420x297mm (300 x 300 DPI)

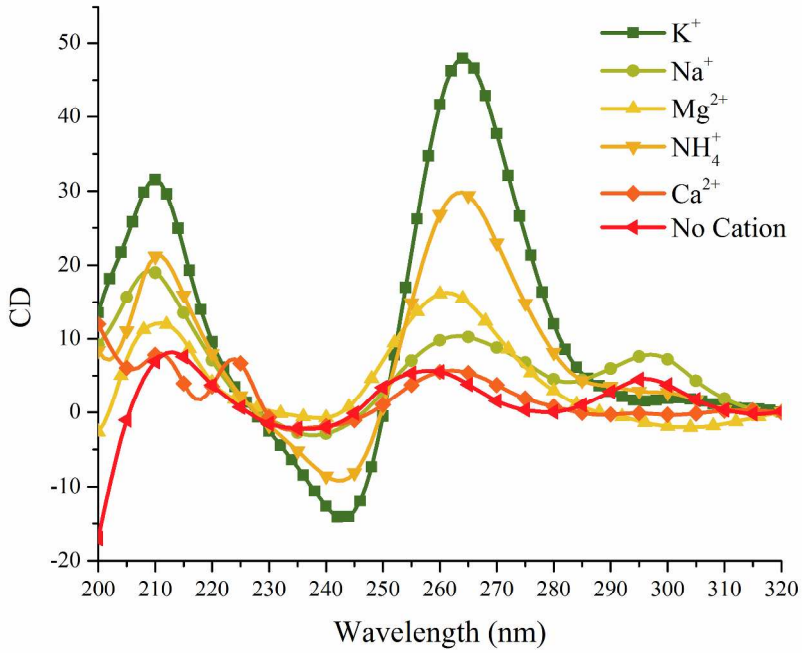


Fig. 2: CD spectra of DNAzyme in the presence of different cations

279x215mm (300 x 300 DPI)

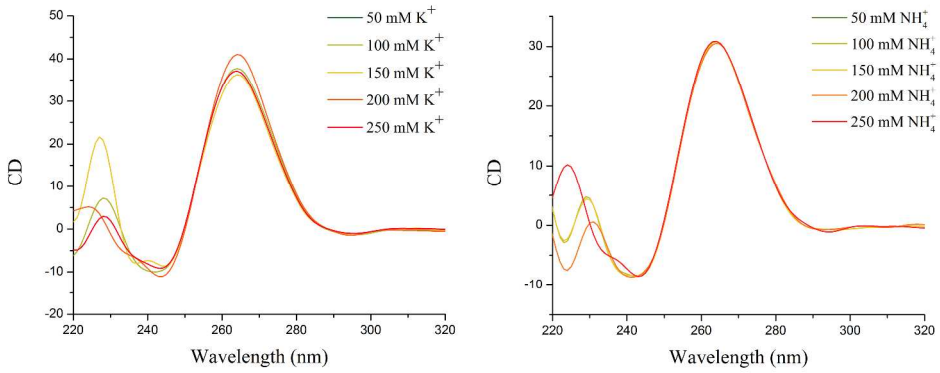


Fig. 3: CD spectra of DNAzyme in the presence of different concentrations of K+ and NH4+

500x199mm (300 x 300 DPI)

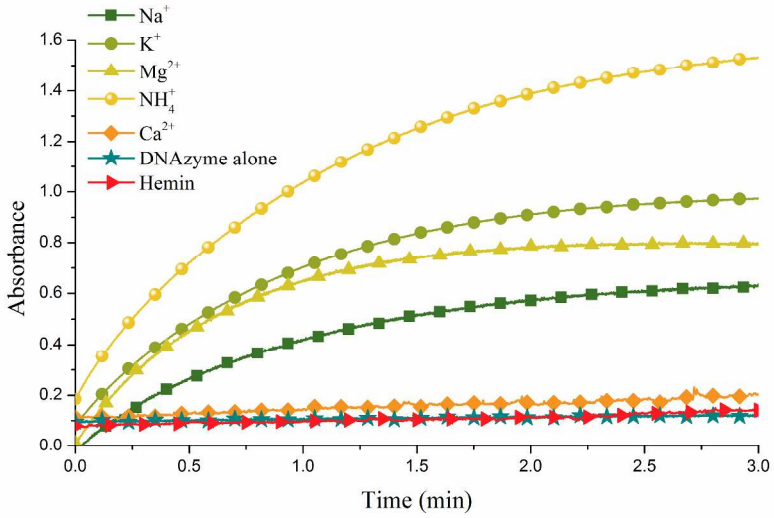


Fig. 4: Peroxidase activity of DNAzyme in the presence of different cations

381x215mm (300 x 300 DPI)

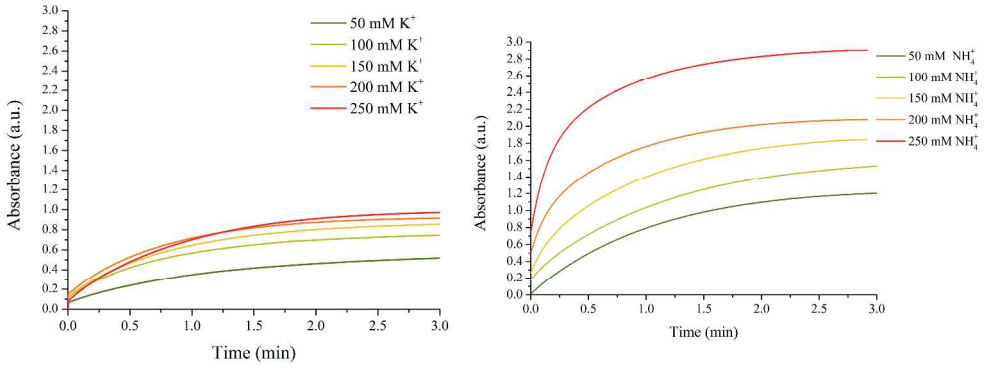


Fig. 5: Peroxidase activity of DNAzyme in the presence of different concentrations of K+ and NH4+

500x199mm (300 x 300 DPI)

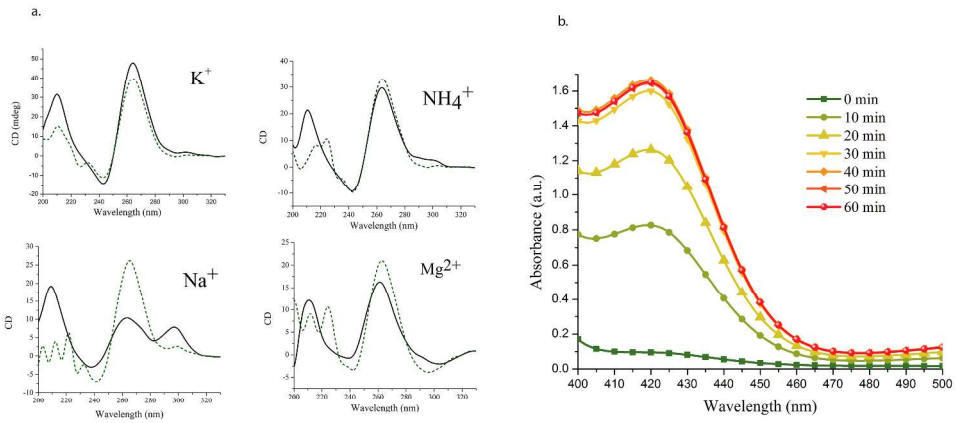


Fig.6: (a) CD spectra of DNAzyme in the presence (dash green line) and absence (solid line) of hemin. (The concentration ratio of hemin and DNAzyme is 1:1) (b) Peroxidase activity of DNAzyme during one hour after hemin addition

338x180mm (300 x 300 DPI)

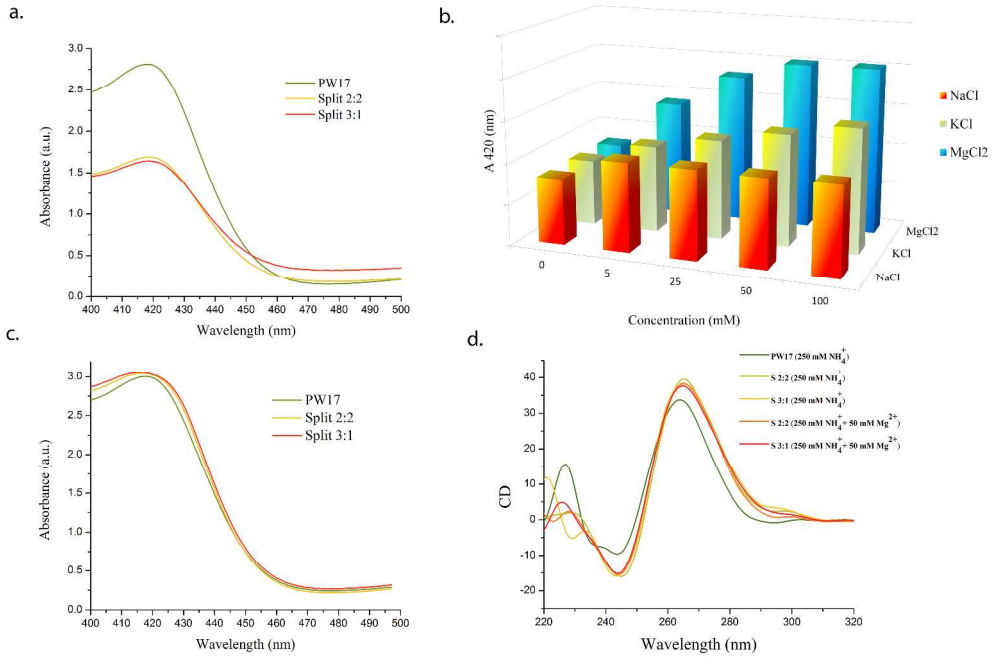


Fig. 7: Peroxidation activity of DNAzyme and its split types (a) in the presence of 250 mM NH4+ (b) in the presence of different type and concentration of cations (the standard deviations are below than 10%) (c) in the presence of 250 mM NH4+ + 50 mM Mg2+ (d) CD spectra of PW17 and its split types in the presence of 250 mM NH4+ and 50 mM Mg2+

600x399mm (300 x 300 DPI)

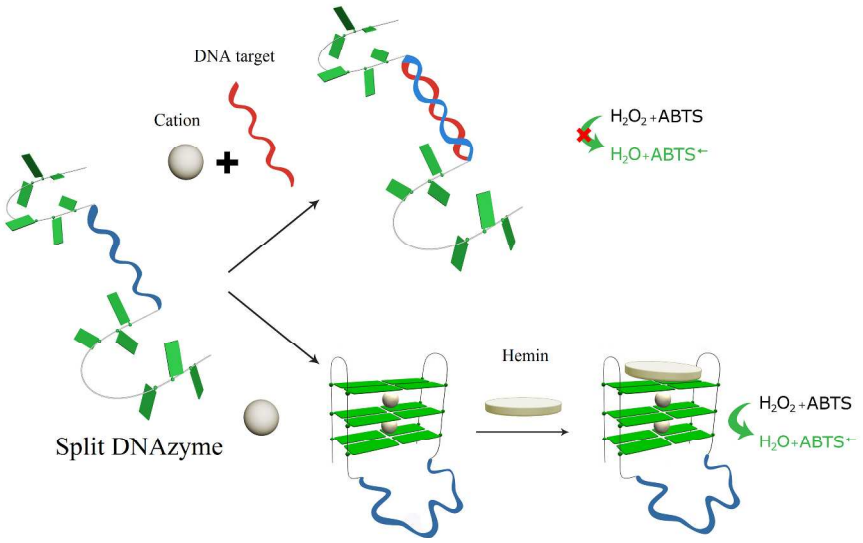


Fig. 8: The scheme of detection method

312x213mm (300 x 300 DPI)

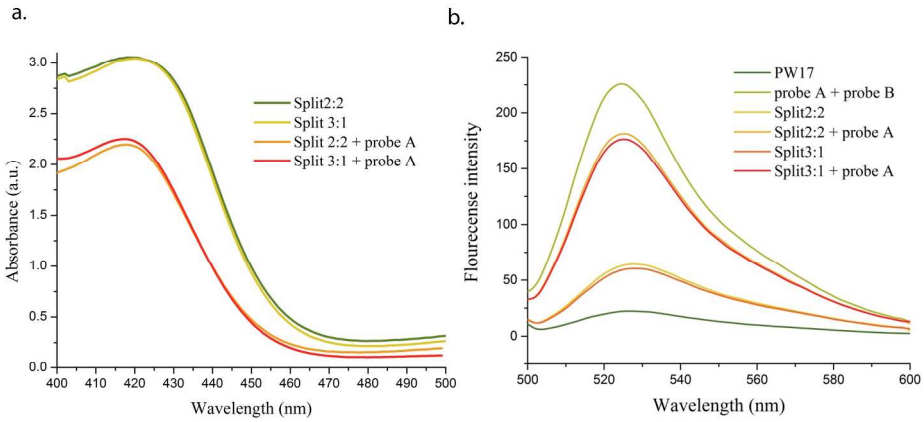


Fig. 9: (a) The catalysis spectra curve of split types in the presence and absence of DNA target (probe A) (b) Emission (521 nm) spectra of PicoGreen in the presence of DNAzyme, probe A + probe B and split types in the presence and absence of probe A

399x199mm (300 x 300 DPI)

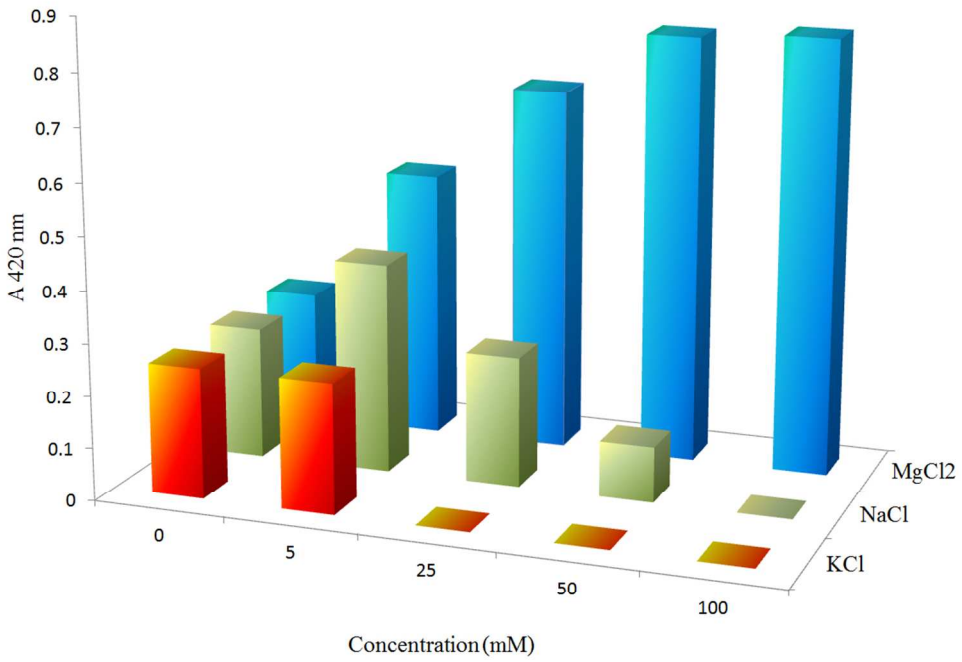


Fig. 10: Effect of different cations on DNA target hybridization (the standard deviations are below than 10%)

99x80mm (300 x 300 DPI)

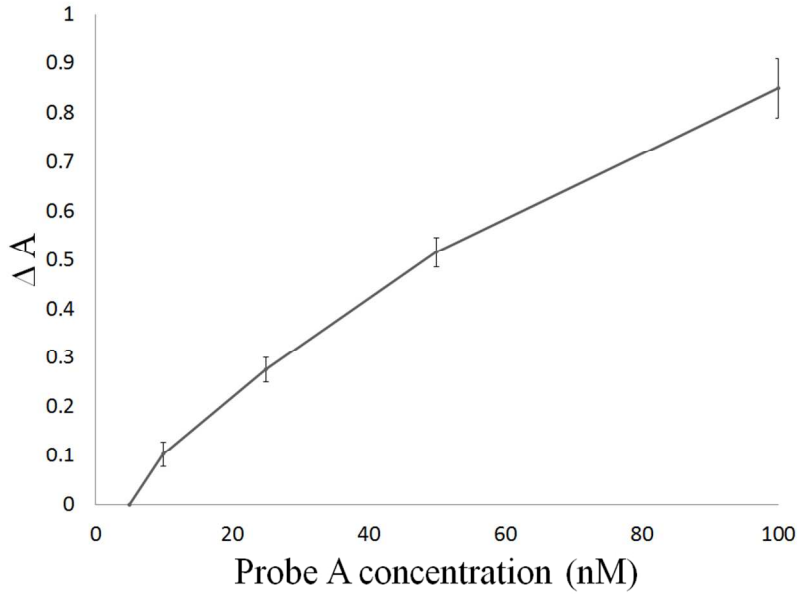
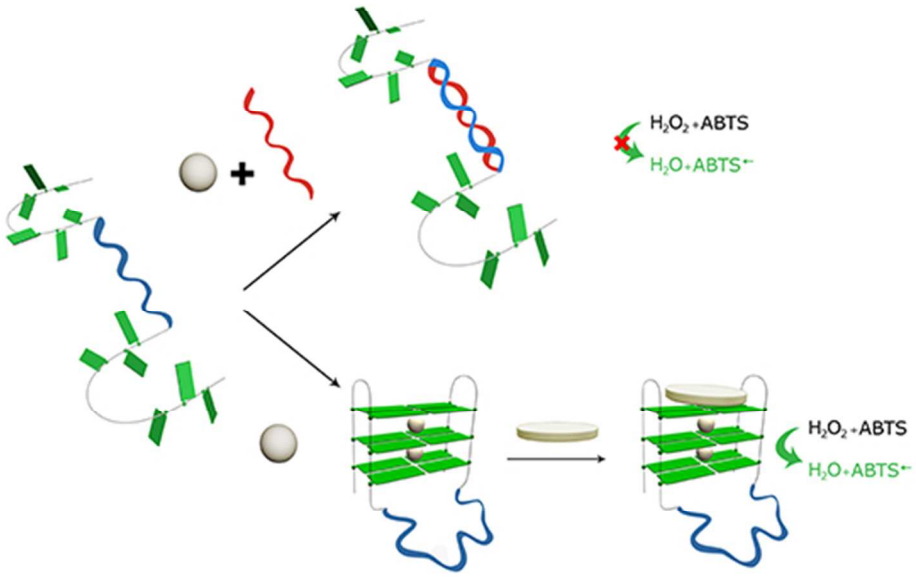


Fig. 11: Sensitivity assay of split 2:2 in the presence of different concentrations of oligonucleotide target (probe A). Δ A= (absorbance of peroxidation reaction of split 2:2 in the absence of probe - absorbance of peroxidation reaction of split 2:2 in the presence of probe)

99x70mm (300 x 300 DPI)



50x50mm (300 x 300 DPI)