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Article

Catalytic Activities of Multimeric G-Quadruplex DNAzymes

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Abstract: G-quadruplex DNAzymes are short DNA aptamers with repeating G4 quartets bound in a non-covalent complex with hemin. These G4/Hemin structures exhibit versatile peroxidase-like catalytic activity with a wide range of potential applications in biosensing and biotechnology. Current efforts are aimed at gaining a better understanding of the molecular mechanism of DNAzyme catalysis as well as devising strategies for improving their catalytic efficiency. Multimerisation of discrete units of G-quadruplexes to form multivalent DNAzymes is an emerging design strategy aimed at enhancing the peroxidase activities of DNAzymes. While this approach holds promise of generating more active multivalent G-quadruplex DNAzymes, few examples have been studied and it is not clear what factors determine the enhancement of catalytic activities of multimeric DNAzymes. In this study, we report the design and characterisation of multimers of five G-quadruplex sequences (AS1411, Bcl-2, c-MYC, PS5.M and PS2.M). Our results show that multimerisation of G-quadruplexes that form parallel structure (AS1411, Bcl-2, c-MYC) leads to significant rate enhancements characteristic of cooperative and/or synergistic interactions between the monomeric units. In contrast, multimerisation of DNA sequences that form non-parallel structures (PS5.M and PS2.M) did not exhibit similar levels of synergistic increase in activities. These results show that design of multivalent G4/Hemin structures could lead to a new set of versatile and efficient DNAzymes with enhanced capacity to catalyse peroxidase-mimic reactions.

Keywords: G-quadruplex; DNAzymes; peroxidase; multimers; synergism

1. Introduction

Haem peroxidases use protein scaffolds that activate haem to react with H₂O₂ [1]. The reaction mechanism and properties of peroxidases have been extensively studied and they have several practical uses in research, bioanalysis and industrial applications. More recently, it was discovered that certain DNA aptamers have the ability to catalyse reactions similar to those carried out by haem peroxidases [2,3]. These DNA aptamers are Guanine-rich DNA oligonucleotides that form complexes with hemin to form G-quadruplexes (often referred to as G4/Hemin) with catalytic activities that mimic peroxidases [4–6]. The oligonucleotide sequences that form G-quartets are organised into G-quadruplexes in the presence of cations to form high-affinity binding sites for hemin turning these nanostructures into powerful catalysts that activate H₂O₂ for peroxidase-like oxidation reactions (Figure 1). These complexes (often referred to as DNAzymes) are more stable to extreme reaction conditions than protein enzymes, and are easier to synthesise and modify via chemical processes.

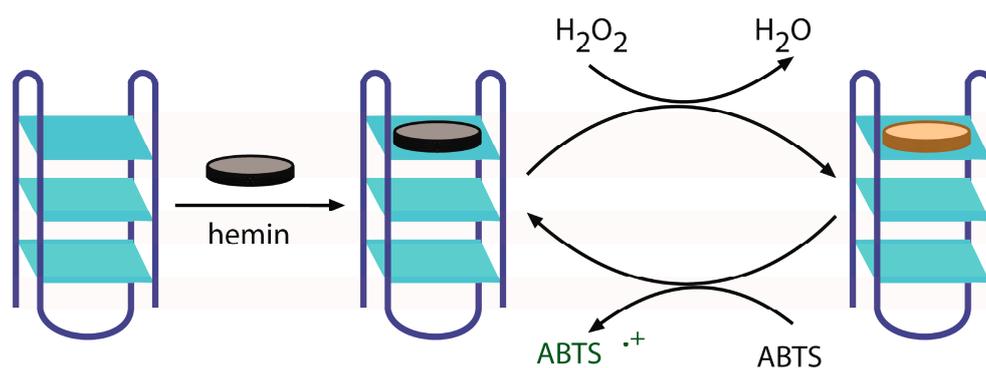


Figure 1. Assembly of G-quadruplex-hemin DNAzymes and H₂O₂-activated oxidation of ABTS. Guanine-rich DNA oligonucleotides fold to form G-quartet interactions (blue planes) which bind to hemin (grey discs). The resulting DNAzyme complex activates H₂O₂ to oxidise a donor substrate (in this instance ABTS) using a mechanism similar to haem peroxidase-catalysed reactions. The reduced haem (brown disc) can react with H₂O₂ and initiate another round of ABTS oxidation. The reaction product (ABTS^{•+}) has a bright green colour with absorption maximum around 412–422 nm.

G-quadruplex DNAzymes have the potential for development as nanodevices for applications in biosensors, diagnostics, and bioremediation and other aspects of biotechnology [7,8]. However, their relatively low catalytic activity in comparison to protein peroxidases currently restricts further development and applications of these systems. In order to develop DNAzymes into useful applications, it is necessary to characterize the structural and functional sequence parameters that influence the efficiency of DNAzyme reactions and their functionalities in applied devices.

Several strategies are being explored to improve the catalytic efficiency and reaction properties of peroxidase G-quadruplex DNAzymes [8]. One such approach is the finding that addition of polycationic amines such as spermine, spermidine, and putrescine significantly increases the activities of DNAzymes by favouring catalytically active conformations [9–11]. Similar rate-enhancements were observed by the addition of the nucleotide ATP to DNAzyme reactions [12,13]. It has also been reported that the conjugation of hemin with the G4-quadruplex moiety via covalent linkage [14] or with cationic peptides [15] can enhance the activities of DNAzymes. Other studies have focused on the rate-enhancing effects of flanking adenine or cytosine nucleotides on G-quadruplex activities [16–19].

Multimerisation of discrete units of G-quadruplexes to form multivalent DNAzymes is an emerging design strategy aimed at enhancing the peroxidase activities of DNAzymes [20,21]. In these multiple active site structures, individual DNAzyme molecular units are joined together by polynucleotide linkers that do not participate directly in the reactions. Some studies show that the linker length between individual DNAzyme molecules can be a determining factor in the activities of multimeric DNAzymes [17,19]. It is thought that this rate enhancement results from synergistic interactions between the individual subunits. In these arrangements, a multivalent DNAzyme sequence would be more active than the sum activities of an equal number of monomeric sequence units [20,21]. One hypothesis that explains the rate-enhancing effect of multimerisation is that multimeric DNAzyme structures potentially create additional high-affinity binding sites for haem incorporation, thereby resulting in more than one active site per DNAzyme monomer. However, in certain cases, there is no direct correlation between the number of units and the activity of the multimer [20,21]. This suggests that certain structural features do not necessarily support activity enhancement in multimers. Gaining a good understanding of the properties of such multivalent G-quadruplex DNAzymes would provide more insight into strategies for rational design of highly active DNAzymes.

While multimerisation has the potential to generate more active DNAzymes, only a handful of DNAzyme multimers have been reported [20–22]. It is not entirely clear what factors determine the catalytic properties of multimers when compared to their monomeric G-quadruplexes. For example, several key questions remained unanswered. Does multimerisation lead to cooperative and synergistic

rate enhancement of activity in all G-quadruplexes that exhibit peroxidase activities? Is there a direct correlation between the number of monomer subunits and catalytic efficiency? Do certain multimeric combinations result in limited rate enhancement or potential inhibition of catalytic activity? Does the number of G-quartets in a G-quadruplex aptamer determine catalytic efficiency? Can contiguous multimerisation result in a redistribution of catalytic units or generation of high-activity pockets?

In an effort to understand these questions, we started by designing dimers and trimers of selected DNazymes in order to study their catalytic properties in detail. In this report, we describe the activities of multimers of five different DNazymes with their corresponding monomers. Using the dinucleotide TT as the linker sequence, we made dimers and trimers of AS1411, also known as AGRO100 [23], Bcl-2 [11,24], c-MYC [11], PS5.M and PS2.M [23]. Using the standard H₂O₂/ABTS oxidation assay, we determined the effects of multimerisation on five G-quadruplex DNazymes. Our findings indicate that multivalency results in synergistic rate enhancements for some DNzyme sequences, while multimers of certain aptamers resulted in reduced activity per haem binding site.

2. Results and Discussion

2.1. Design of Multimeric DNazymes

To study the properties of multimeric G-quadruplex DNazymes, we selected five different DNzyme sequences (Table 1) that have been described in the literature. We selected AS1411, a highly-active DNzyme [11,23] that is also reported as an anti-cancer agent owing to its ability to target the protein nucleolin which is highly expressed on the surface of cancer cells [25]. PS5.M and its sequence-derivative PS2.M [3] were also chosen for their high activities. In addition, this group of DNazymes has been extensively studied and reported in the literature [26].

Table 1. Sequences of DNazymes used in this study. The DNA sequences are written in 5' to 3' direction.

DNzyme	Sequence
AS1411	GGTGGTGGTGGTTGTGGTGGTGGTGG
Bcl-2	GGGCGCGGGAGGAAGGGGGCGGG
c-MYC	GAGGGTGGGGAGGGTGGGGAAG
PS5.M	GTGGGTCATTGTGGGTGGGTGTGG
PS2.M	GTGGGTAGGGCGGGTTGG

c-MYC and Bcl-2 are less-characterised DNazymes in comparison to the three described above. The assumption is that these low-activity DNazymes would be an ideal model system to test any potential rate enhancement due to G-quadruplex multimerisation. c-MYC is derived from a G-rich region in the promoter sequence of the human *c-myc* gene [27], while Bcl-2 was identified as a G-rich segment of the Bcl-2 P1 promoter and shown to form mixed intramolecular G-quadruplex structures [28,29].

In each case, the multimers (dimers and trimers) were made by joining the nucleotide at the 3' end of the DNzyme sequence to the one on the 5' end of the same sequence using a TT dinucleotide linker (Figure 2F). It is known that the identity of the base composition and length of the linker sequence can influence the activities of monovalent and multivalent DNazymes [17]. To remove the potential for linker-dependent effects on our analyses, we chose to use the relatively simple TT linker sequence in order to obtain a relatively unconfounded effect of multimerisation on DNzyme activity [20,21].

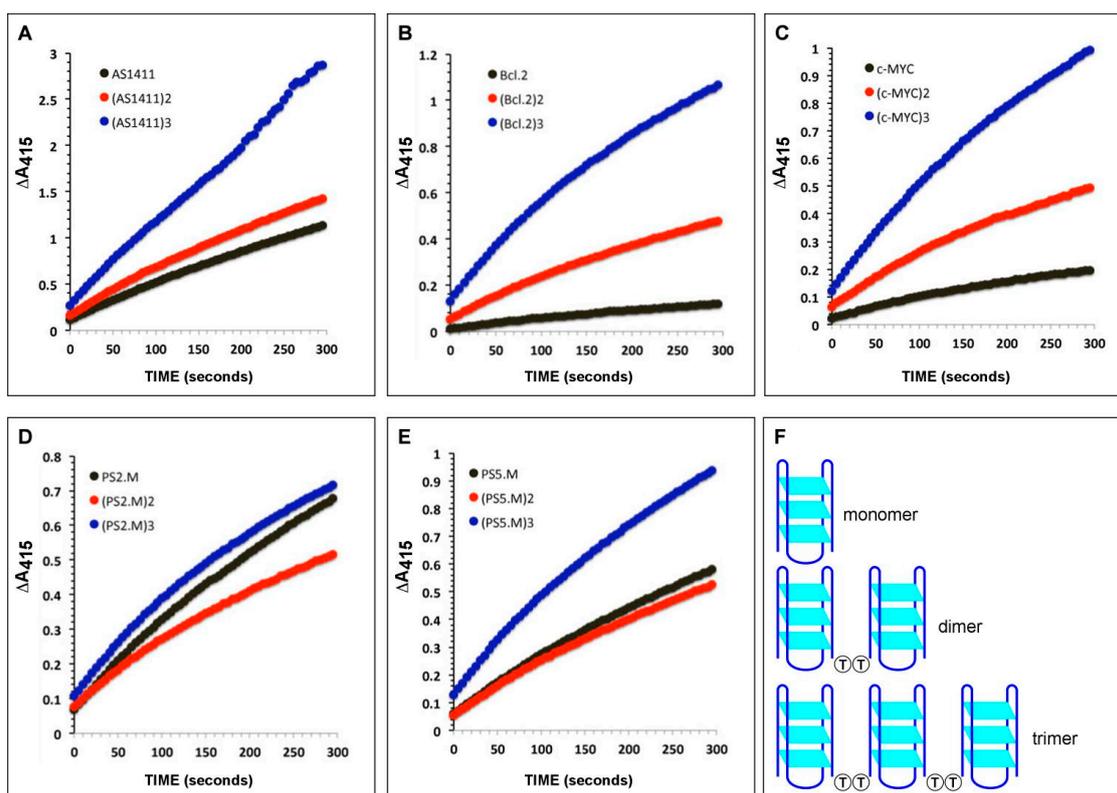


Figure 2. Time course of DNAzyme-catalysed reaction between H_2O_2 and ABTS (A) AS1411 and multimers; (B) Bcl-2 and multimers; (C) c-MYC and multimers; (D) PS5.M and multimers, (E) PS2.M and multimers. The numerals 2 and 3 behind each DNAzyme name refer to dimer and trimer respectively e.g., (AS1411)₂ and (AS1411)₃. Reactions were carried out in 25 mM HEPES-NaOH pH 7.4, 20 mM KCl, 200 mM NaCl, 0.05% Triton X-100, 1% DMSO. The activities of 0.25 μ M DNAzyme were assayed using ABTS (2.5 mM) and H_2O_2 (0.425 mM). Each reaction was started by the addition of H_2O_2 , and change in A_{415} was monitored immediately after starting the reaction for 5 min. The absorbance values shown were zeroed with those obtained in reactions that did not include the DNAzyme. Each trace is representative of three different reactions. (F) Schematic illustration of the design of G-quadruplex DNAzyme dimers and trimers. In the multimers, each monomeric unit is joined to the next by a TT dinucleotide.

2.2. Catalytic Activities of DNAzymes

Several buffer systems have been used to assay the activities of G4/Hemin DNAzymes, and it is clear that the reaction buffer pH as well as its composition play important role in the reaction rate and the stability of the product radical [3]. A common issue in peroxidation reactions is disproportionation of the reaction product [13], with certain buffers supporting the stability of the reaction product more than others. We used 25 mM HEPES-NaOH, pH 7.4, as the reaction buffer in these experiments since this buffer system supports both the actual peroxidase reaction as well as stability of the product [3,13,23,30].

All the 15 G-quadruplexes tested in this study displayed ABTS oxidation activities (Figure 2) and preliminary assays showed that a linear relationship exists between the concentration of G-quadruplex DNAzymes and peroxidase activities. In all cases, initial rates of ABTS oxidation increased linearly with DNAzyme concentration providing the concentrations of the substrates ABTS and H_2O_2 are not limiting (Figure 2).

AS1411: Consistently, AS1411 performed better in the reaction conditions used here than the other 4 DNAzymes tested. Similar high activities for AS1411 have been reported [23]. Their initial rate measurements showed that AS1411 is about 1.5-fold more active than PS5.M, and 2-fold more active than PS2.M. Our results generally agree with these measurements, both in initial rate and turnover

numbers (Figure 3). Initial rate measurements showed that the multimeric forms of AS1411 were more active than the monomer. While there was a slight increase in the activity of the dimer (819 nM/s) over the monomer (639 nM/s), a more remarkable effect was seen in the activity of the trimer (1472 nM/s). Similar trends were seen in the amount of ABTS• radical formed after 5 min of reaction (Figure 3B).

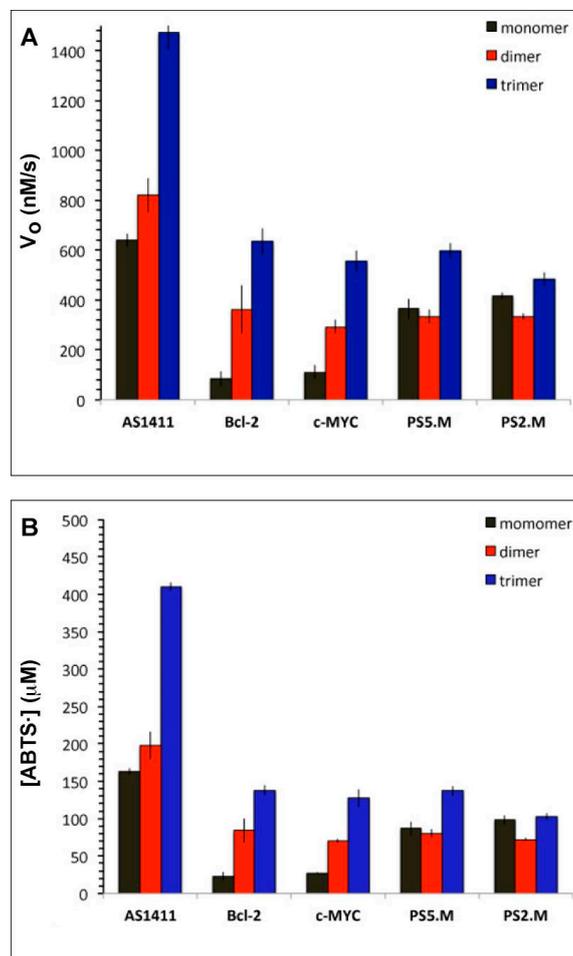


Figure 3. DNAzyme activities of DNAzymes. (A) Initial Rates (expressed as V_0 , nM/s) were calculated from the slope of the initial linear portion (typically, the first 30 s) of the reaction curves shown in Figure 2. (B) The amount of ABTS• radical produced after 300 s from the activity of 1 μ M DNAzyme were determined from the endpoint of the reaction time course shown in Figure 2. See *Materials and Methods*.

Bcl-2: In comparison to AS1411, Bcl-2 is a relatively low activity DNAzyme [11,24]. As shown in Figure 2B, multimerisation had a remarkable rate-enhancing effect on Bcl-2 activity. The initial rate measurements (monomer, 83 nM/s; dimer, 360 nM/s; trimer, 634 nM/s) showed a 4-fold enhancement effect from dimerisation and nearly 8-fold from trimerisation (Figure 3A). Similar trends were observed in the total amount of ABTS• product formed (Figure 3B).

c-MYC: In a pattern similar to that seen with Bcl-2, multimerisation significantly enhanced the ABTS oxidation activity of c-MYC. (Figure 2C). The initial rate measurements obtained were monomer (111 nM/s), dimer (291 nM/s), and trimer (556 nM/s). The amount of product formed showed a similar trend (Figure 3). These values represent approximately 3- and 5-fold increases as a result of dimerisation and trimerisation, respectively.

PS5.M and PS2.M: PS5.M and PS2.M are two of the best-studied peroxidase DNAzymes and have been used as model systems for characterising aspects of G-quadruplex DNAzymes catalytic

mechanism. Indeed, PS5.M is one of the first DNA oligonucleotide aptamers shown to have the ability to bind hemin and acquire peroxidatic catalytic activities [2,3]. The 18-nucleotide PS2.M was derived from rational redesign of the 24-nucleotide PS5.M to achieve better catalytic activity [3]. In our experiments, the activity of PS2.M (416 nM/s) (Figure 2D,E) was higher than that of PS5.M (364 nM/s) (Figure 3A).

In contrast to the DNAzymes described above (AS1411, Bcl-2, and c-MYC), PS5.M and PS2.M dimers were slightly less active than their corresponding monomers (Figure 2D,E and Figure 3A). The initial rate values determined for PS5.M monomer (365 nM/s) and dimer (333 nM/s) were similar, an indication that dimerisation of this aptamer did not have a synergistic effect on activity. Furthermore, the initial rate value determined for the trimer (597 nM/s) is about 1.5-fold higher than that of the monomer. Rather than having a rate-enhancing effect, these results suggest that multimerisation resulted in reduced activity per haem binding site in PS5.M. Similar effects were seen in the activities of PS2.M constructs. The dimer was 20% less active than the monomer, while the activity of the trimer (485 nM/s) was about 16% higher than that of the monomer (416 nM/s) (Figure 3A).

2.3. AS1411 Multimers Display High Activities at Low DNAzyme Concentrations

The results presented above (Figures 2 and 3) show that AS1411 DNAzymes were generally more active than the other aptamers tested in this study. The high activity seen with AS1411 trimer indicates that this construct could be the starting point for further optimisation experiments aimed at designing more active aptamers. From a catalytic perspective, it is desirable to have DNAzymes that display high activities at low concentrations, i.e., those with high turnover per unit catalyst concentration.

We compared the activities of AS1411 monomer, dimer and trimer in reactions that contained 0.1, 0.25, 0.5 and 1.0 μM DNAzyme concentrations. We included PS5.M for comparison with the AS1411 constructs since it is one of the most studied high activity DNAzymes reported to date. The results (Figure 4) show that all four DNAzymes are active at lower concentrations, and that there is a linear relationship between DNAzyme concentration and ABTS oxidation activity. At 0.1 μM , the lowest concentration studied, the initial rate for AS1411 trimer was 139 nM/s, which was approximately 4.5 times the value for PS5.M (31 nM/s) at the same DNAzyme concentration. The high activity of AS1411 trimer at nanomolar concentrations could have practical use in applications where low concentrations of the G4/Hemin DNAzyme are desirable.

2.4. Effects of Reactants' Concentrations on Activities of AS1411 Multimers

To further understand the properties of the highly active AS1411 multimers, we examined the effects of ABTS and H_2O_2 concentrations on the activities of the DNAzymes. We chose a range of ABTS and H_2O_2 concentrations that will allow us to determine the nature of the relationship between reactant concentration and DNAzyme activities. Assays were carried out in reactions that contained 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, and 2.5 mM ABTS, representing a 10-fold increase from the lowest to the highest concentration. The effect of H_2O_2 concentration was investigated in reactions that contain 0.106, 0.213, 0.319, 0.425, 1.063, 2.125, 3.188, and 4.250 mM, representing a 40-fold increase from the lowest to the highest concentration.

The results (Figure 5) show that the rates of DNAzyme reactions are effectively controlled by the concentration of H_2O_2 in the reaction mixture. The initial rates remained largely unchanged over a 10-fold increase in ABTS concentration (Figure 5G), an indication that the activities of the DNAzymes do not depend on the concentration of the donor substrate. However, as shown in Figure 5A–C, the amount of reaction product formed, as indicated by the endpoint after 5 min of reaction, is dependent on the concentration of ABTS. It is observed that the time course curves show complete depletion of the substrate at lower concentrations. The rate of this depletion is faster in the more active multimers.

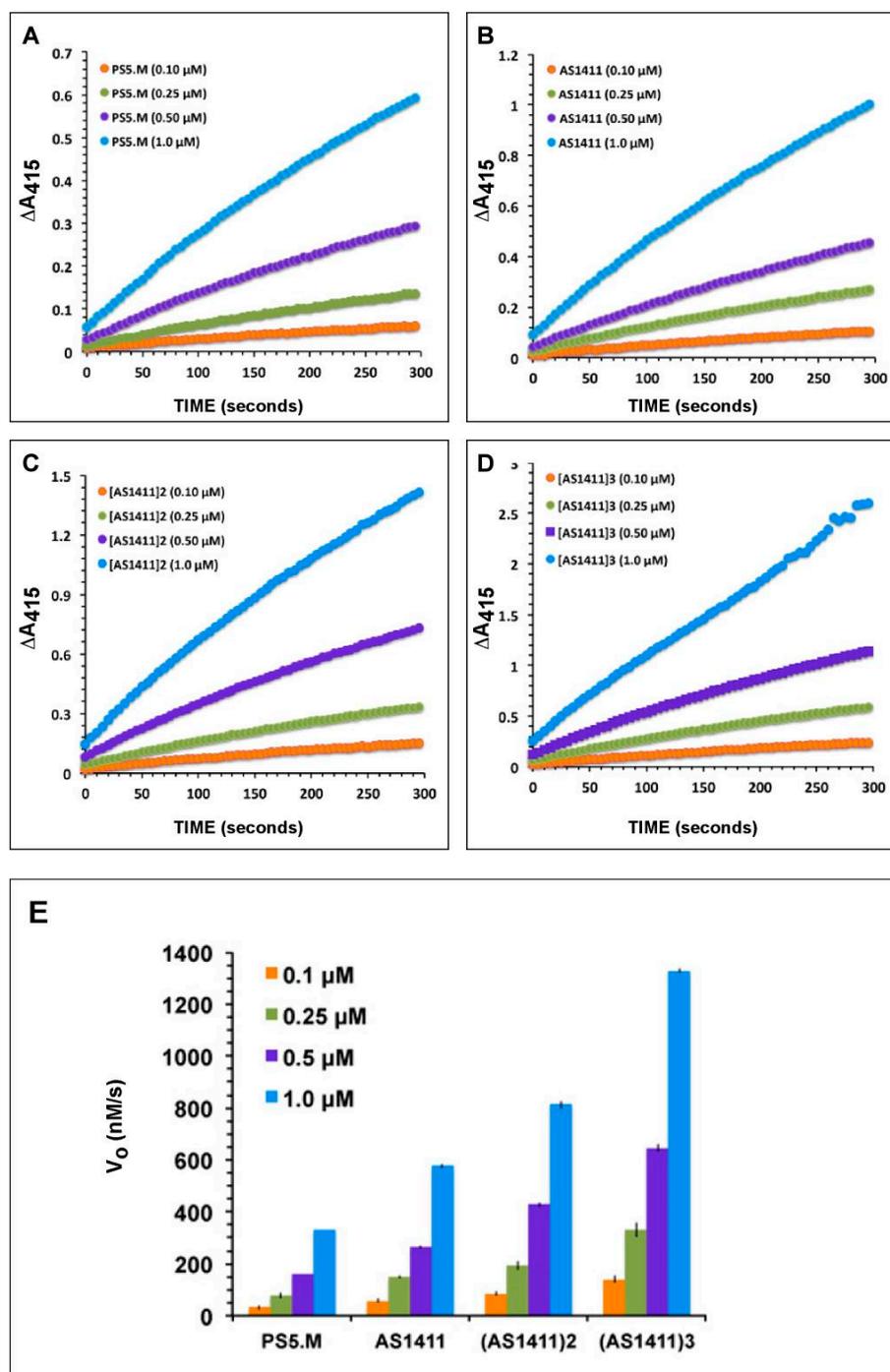


Figure 4. Effect of DNAzyme concentrations on activities of AS1411 multimers and PS5.M. (A) PS5.M; (B) AS1411 monomer; (C) AS1411 dimer; (D) AS1411 trimer. In (A–D), the curves show time courses of ABTS oxidation by 0.1, 0.25, 0.5, and 1.0 μM DNAzyme. (E) Initial Rates (expressed as V_0 , nM/s) were calculated from the slope of the initial linear portion of the curves shown in (A–D).

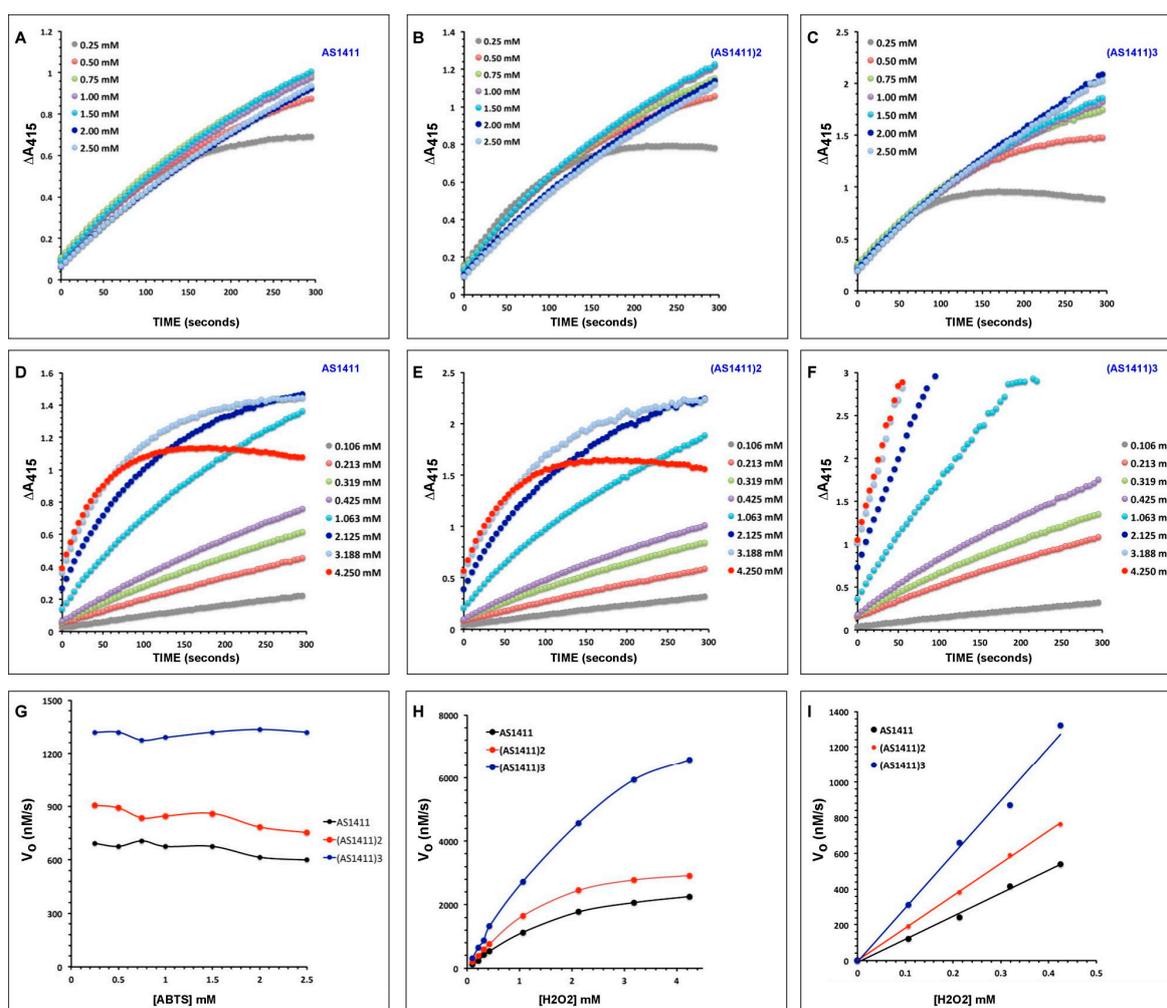


Figure 5. Effects of ABTS and H₂O₂ concentrations on activities of AS1411 multimers. The effects of ABTS concentrations on the time course of DNAzyme activities are shown in Panels (A–C). The final ABTS concentrations in the reaction mixtures are 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, and 2.5 mM. Reactions were carried out at a fixed H₂O₂ concentration (0.425 mM), and 1.0 μM DNAzyme. Similarly, Panels (D–F) show the effects of H₂O₂ concentrations on the time course of reactions at a fixed ABTS concentration (2.5 mM), and 1.0 μM DNAzyme. The final H₂O₂ concentrations are 0.106, 0.213, 0.319, 0.425, 1.063, 2.125, 3.188, and 4.250 mM. The relationship between substrate concentrations and Initial Rates are shown in Panel (G) (ABTS) and Panel (H) (H₂O₂). Panel (I) shows the linear relationship between Initial Rates and H₂O₂ concentration at lower concentrations of the reactant (0.106, 0.213, 0.319, and 0.425 mM). Initial Rates (expressed as V₀, nM/s) were calculated from the slope of the initial linear portion of the curves in (A–F).

In contrast to the effect of ABTS concentration, results shown in Figure 5D,E indicate that the initial rates of the three DNAzymes are dependent on the concentration of H₂O₂. Figure 5H shows the dependence of initial rates on H₂O₂ concentration. The pattern indicates a linear correlation between H₂O₂ concentration and initial rates at the lower H₂O₂ concentration (Figure 5I). The trend changed to what appears at first to be a saturation kinetics at higher H₂O₂ concentration, especially with AS1411 and (AS1411)2. However, the time course of the reactions at high H₂O₂ concentration (over 2 mM) shows an early plateau of activity, an indication of inactivation of the DNAzymes at high oxidant concentration. Suicide inactivation of natural protein peroxidases and G-quadruplex peroxidase DNAzymes at high H₂O₂ concentration is a well-known phenomenon that limits the use of peroxidases in certain applications [1,6,8]. In addition, high concentration of H₂O₂ is known to

accelerate the disproportionation of ABTS[•] radical cation [23]. The response of the activities of the DNAzymes to changes in ABTS and H₂O₂ concentrations observed here has also been reported in a previous study where it was shown that DNAzymes behave differently from HRP, a natural protein peroxidase [23].

Interestingly, the plot of Initial Rates versus H₂O₂ concentration (Figure 5H) shows that the trimer, (AS141)₃, appears to be more resistant to the reduction in activity at high H₂O₂ concentration. This could be due to presence of multiple active sites or as a consequence of a more stable structural arrangement that protects the hemin at the reaction centre. This property could make (AS141)₃ particularly useful in reaction systems that require the use of H₂O₂ at high concentrations. Further structural studies will provide more insights into the basis for this stability.

2.5. Determinants of Rate Enhancement by Multivalent DNAzymes

The goal in designing multivalent G4/Hemin DNAzymes is to achieve increased catalytic performance. While it is expected that molecular coupling of DNAzyme units as dimers, trimers or tetramers could have synergistic effects by resulting in a single molecule with activity higher than that from the sum of the parts, this may not necessarily be the case. One of the key objectives of this study is to gain an understanding of the factors that determine the outcome of multimerising G-quadruplexes in terms of rate-enhancement or lack of it. The activities of G4/Hemin structures are strongly related to the topological structures formed by the DNA sequence [31].

Generally, DNAzymes with parallel G-quadruplex structures tend to have higher activities than those with antiparallel arrangements. It is suggested that the lower activities of antiparallel structures are due to reduced hemin binding as a result of the steric hindrance caused by protruding loops at their outer G-quartets, while parallel G-quadruplexes have no such constraints [32]. Based on several studies, the activities of the different topological forms of G-quadruplexes follow the pattern: parallel > mixed parallel/antiparallel > antiparallel [8,17,26]. The topology of a G-quadruplex sequence depends on the length of the loops that connect two adjacent G-tracts. G-tracts with short loops tend to form parallel structures, while long loops give antiparallel topologies [33,34]. In addition, the nature of cations (Na⁺, K⁺, Mg²⁺, NH₄⁺) present in the G-quadruplex structure can determine its topological arrangement [8].

Our results show that the G-quadruplexes that are known to form predominantly parallel structures (AS1411, Bcl-2, and c-MYC) showed increased activity upon multimerisation. AS1411 forms a parallel bimolecular quadruplex structure that consists of four pairs of stacked G-tetrads [25]. Similarly, c-MYC forms a parallel structure [11,35]. These parallel structural arrangements favour external stacking of the hemin ligand [36]. Earlier studies using NMR showed that Bcl-2 forms mixed parallel/antiparallel structure [28,29]. More recently, it was shown that Bcl-2 formed intramolecular parallel G4 structures in experiments where CD spectra were measured in a buffer containing 10 mM NaH₂PO₄/Na₂HPO₄, 100 mM KCl, and 2 mM MgCl₂ [11]. It follows that certain buffer conditions might favour a predominance of parallel G4 arrangements, which could explain the high rate enhancement caused by multimerisation of Bcl-2 aptamers into multivalent unimolecular structures.

In contrast, PS2.M forms mixed parallel/antiparallel structure and its structural state seems to be dependent on the nature of the cation used in inducing the folding of the sequence into the G-quadruplex structure [3,32,37]. Hence, it is likely that a tendency toward antiparallel topology may not support favourable binding of hemin thereby explaining the relatively modest rate enhancement in multimeric PS2.M and PS5.M. Further mechanistic studies are required to understand why the multimers of PS2.M and PS5.M behaved differently from those of the parallel topology-forming AS1411, Bcl-2 and c-MYC.

It is anticipated that highly active DNAzymes, such as the ones reported in this study, will have applications in designing highly sensitive sensors based on G-quadruplex peroxidase system. The notably high activities of AS1411 multimers would require detailed mechanistic investigation considering that AS1411 forms intermolecular G4 structures. Detailed spectroscopic and structural

insights into the basis for the high activities of the multimers could provide valuable information on how to design better DNAzymes. Such studies, which are beyond the scope of the work reported here, will be a focus of our future investigation into the properties and applications of multimeric G4/Hemin DNAzymes.

The effect of multimerisation on Bcl-2 and c-MYC shows that the strategy can be used to enhance the activities of G4/Hemin sequences with low activity but which possess other desirable properties. Certain DNAzymes have been designed for optimal activities in conditions of extreme pH, high temperature or other non-standard conditions [8,17,19].

It is worth noting that a different buffer condition, especially one with a different composition of cations, could yield a result pattern that is different from what we observed in this study. Careful variations in ABTS and H₂O₂ concentration as well as the concentration of the DNAzyme could reveal further aspects of the reaction properties of multivalent G4/Hemin structures. Further efforts will focus on how flanking residues, linker length and sequence, and rate-enhancing boosting agents (ATP and polyamines) affect the catalytic properties of multivalent G-quadruplex DNAzymes.

3. Materials and Methods

3.1. Reagents and DNA Oligonucleotides

HPLC-purified DNA oligonucleotides were purchased from Integrated DNA Technologies, IDT (Leuven, Belgium) without further purification. These were dissolved in TE (1×) buffer (10 mM Tris-HCl pH 7.5, 0.1 mM EDTA) as 100 μM solutions and stored at −20 °C when not in use. DNA concentrations were determined using A₂₆₀ and the molar extinction coefficient calculated for each oligonucleotide according to its base sequence. All reagents and substrates were purchased from Sigma-Aldrich, Dorset, UK, and were of analytical grade. The buffer reagent HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid) was prepared as a 200 mM stock solution and stored at 4 °C. Buffers were prepared freshly each week and stored at 22 °C. The standard peroxidase substrate, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) was freshly prepared in water as 50 mM solution, and H₂O₂ was prepared freshly daily from a 30% aqueous stock solution (Sigma-Aldrich, UK).

3.2. Preparation of G4/hemin DNAzyme

G4/hemin DNAzymes were prepared as 10 μM solutions and stored at −20 °C. Briefly, 50 μL of 100 μM oligonucleotide solution in TE (1×) was added to 450 μL of a buffer that contains 25 mM HEPES-NaOH pH 7.4, 20 mM KCl, 200 mM NaCl, 0.05% Triton X-100, 1% DMSO. The sample was heated at 95 °C for 10 min, and cooled rapidly by dipping in ice. The sample was left at 22 °C for 30 min after which equivalent concentration of hemin was added and mixed thoroughly. The sample was kept at 22 °C for a further one hour after which it was stored at −20 °C.

3.3. Determination of G4/hemin DNAzyme Activity

The activities of the G4/Hemin DNAzymes were determined in reactions that contained 25 mM HEPES-NaOH pH 7.4, 20 mM KCl, 200 mM NaCl, 0.05% Triton X-100, 1% DMSO, 0.25 μM DNAzyme, ABTS (2.5 mM) and H₂O₂ (0.425 mM). Each reaction was started by the addition of H₂O₂, and change in A₄₁₅ (characteristic for the reaction product ABTS[•]) was monitored immediately after starting the reaction over 5 min on a CLARIOstar Plate Reader (BMG LABTECH). Absorbance changes were converted to the amount of ABTS[•] formed using a molar extinction coefficient of 36000 M^{−1} cm^{−1} for ABTS[•] [38]. The initial reaction rate, V_o, expressed as nM/s, and the amount of ABTS[•] formed after 5 min reaction, expressed as μM, were determined from the time course of the reaction. The absorbance intensity at λ = 415 nm (corresponding to the amount of ABTS[•] formed) was measured as a function of time. The initial rates (V_o) were calculated from the slope of the initial (30 s) linear portion of the increase in absorbance [15,39].

4. Conclusions

In this study, we have shown that multimeric G4/Hemin peroxidase DNAzymes derived from parallel-forming G-quadruplexes show synergistic and cooperative catalytic activities when compared to their monomeric units. The striking activity of AS1411 trimer makes it a candidate for further designs with potential applications in sensor technologies and other aspects of biocatalysis. The simple multimeric constructs reported in this study provides the basis for further investigation into how this approach could be used to build more efficient catalytic DNA nanodevices. The evidence from this work suggests that parallel topology-forming G-quadruplexes are more likely to form highly active multimers. However, different linker lengths and additional rate-enhancing features could improve the activities of multimers derived from antiparallel G-quadruplexes. Future studies on kinetic characterisation and structural analyses will provide more insights into how multimeric G4/Hemin systems fold and activate peroxidase catalysis.

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