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Native and Synthetic G-quartet-based DNAzyme Systems – Artificial Enzymes for Biotechnological Applications

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Additional information is available at the end of the chapter

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Abstract

Catalysis of chemical reactions is crucial for both chemical industry and research. However, scientists are not the first ones to use catalysts in their laboratory. In fact, they are also essential for nature which designs plenty of biocatalysts, playing a pivotal role in living systems. For a long time, it was thought that only enzymes had this property. However, since the beginning of the 1980s, it is known that ribonucleic acids (also termed RNA) can acquire this ability, making them compulsory for key reactions (*e.g.*, for the translation of messenger RNA in the ribosome). Based on that, chemists designed several synthetic DNA catalysts (termed DNAzymes) for a large variety of reactions and applications. Among the DNA structures used, G-quadruplexes are guanine-rich noncanonical DNA structures (*i.e.*, differing from duplex DNA) composed of native G-quartets and particularly interesting for their ability to catalyze reactions of peroxidation. This peroxidase-mimicking system found plenty of applications detailed in this chapter. Moreover, optimizations of experimental conditions are also discussed and highlight the versatility and easy-to-use characteristics of G-quadruplexes DNA. Also, synthetic G-quartets, mainly TASQ (for *template-assembled synthetic G-quartets*), developed by chemists showed their ability to mimic G-quadruplexes, thanks to the presence of a G-quartet. Thus, synthetic G-quartets proved their capability to catalyze peroxidase-mimicking reactions, and these new exciting nature-mimicking catalytic systems are presented in detail in this chapter.

Keywords: DNAzyme, G-quadruplex, hemin, G-quartet, TASQ

1. Introduction

Catalysts are essential in the fields of both synthetic applications and biological functions. Indeed, while they make thousands of chemical reactions possible for industry and research,

they play a pivotal role in living systems. All along the years, nature develops strategies to catalyze biochemical reactions. The most representative catalysts are undoubtedly the enzymes, but they are not the only ones. Interestingly, ribonucleic acids (also known as RNA) are also able to play this role and to catalyze key reactions, like in the active site of the ribosome during the translation of messenger RNA (mRNA). Besides, it is strongly supposed that the origins of the prebiotic life were based on the use of RNA as both the carrier of the genetic information, and a catalyst: RNA was a self-sufficing molecule. This theory was termed "RNA world." [1–6]

Inspired by the role of RNA as a catalyst, chemists developed new catalytic systems based on deoxyribonucleic acids, also termed DNA. [7, 8] DNA offers more advantages, like its better stability compared to the RNA equivalent. [9] These aspects are developed in section 2 of this chapter.

Among all the DNA structures used as catalysts (*e.g.*, canonical duplex structures or noncanonical triplexes, etc.), author would like to highlight the reader on the G-quadruplex structures. [10] These noncanonical edifices, composed of a stacking of native G-quartets, are introduced in section 3. This presentation is followed by the story of the discovery of G-quadruplexes as catalysts, and then, by a rationalization of how this chemical mechanism works, and how chemists can modulate experimental conditions to obtain the efficiency desired. To complete the presentation, the large range of applications of these noncanonical structures is commented on and shows how versatile and effective quadruplex DNA are.

Based on the observation that the catalytic activity of G-quadruplex is mainly due to the presence of native G-quartets, [11] several groups designed new molecules able to form a synthetic G-quartet. The most representative examples are TASQ (for *template-assembled synthetic G-quartet*), [12–16] composed of four guanines grafted on a template, and able to self-assemble into an intramolecular G-quartet. In section 4 of this chapter, the concept of TASQ is first clarified and then their catalytic activity is specified. Finally, the very first applications proposed in the literature are described, and pave the way to the use of synthetic molecules to mimic natural enzymes, like peroxidases.

2. From enzyme to DNAzyme

2.1. From enzyme to ribozyme

Long regarded as the only biomolecules able to catalyze chemical reactions, proteins are not the exclusive edifices playing this pivotal role in biological systems. Indeed, at the beginning of the 1980s, it was discovered that RNA (for *ribonucleic acids*) share also this property and are involved in numerous biological process. [1, 2] Some of the most representative examples are certainly the RNase P catalyzing transfer RNA (or tRNA) maturation [17], the riboregulators (also termed riboswitches) incorporated in certain messenger RNA (or mRNA), and controlling transcription or translation *in cellulo*. [18, 19] Active site of the ribosomes, composed of ribosomal RNA (or rRNA) and catalyzing the protein synthesis in the cytoplasm, also has to be highlighted. [20] This ability of RNA to catalyze an enzymatic reaction was termed "ribo-

zyme,” obtained by contracting the words “ribonucleic acids” and “enzyme.” This fantastic biological and chemical breakthrough was honored in 1989 by the Nobel Prize in Chemistry, attributed to the two pioneers of this field: Sidney Altman and Thomas R. Cech.[3, 4] The potential of these ribozymes quickly drew the scientific community’s attention which developed the very first artificial ones in 1990.[21] The RNA sequences were first designed to catalyze single-strand RNA cleavage and then for RNA ligation, porphyrin metalation, or more classic organic chemical reactions like Diels–Alder and Michael reactions.[22]

2.2. From ribozyme to DNAzyme

Interestingly, not any natural catalytic deoxyribonucleic acids (or DNA) has been found as yet in nature. However, their higher chemical stability compared to RNA and proteins makes DNA catalysts of choice for chemists who want to develop innovative applications in a larger range of experimental conditions. Indeed, the DNA stability against heat treatment and hydrolysis is evaluated at 1.000 and 100.000 times higher than for RNA and proteins, respectively. [9] Furthermore, obtaining specific designed DNA sequences is increasingly easy, thanks to the automated DNA synthesizers for both academic laboratories and the industry. In parallel, several companies are specialized in the custom DNA synthesis, and offer the opportunity for everybody to work now with DNA catalysts. Other advantages of using DNA as a catalyst can be highlighted here, like the possibility to functionalize it (*e.g.*, fluorescent probes or specific other moieties), to graft it on a solid support (*e.g.*, polymers, gold surface), and on top of that, the virtually unlimited number of catalysts that may be obtained by modulating both the number and the nature of the nucleotides.[7, 23, 24] Thus, chemists can design the specific sequence meeting their requests in term of application and efficiency.

It was not until 1994 that the first instance of an artificial catalytic DNA, termed “deoxyribozyme” (by analogy with the ribozymes) or most widely named “DNAzyme,” was published. [25] Since then, plenty of applications has been developed, from the most original to the most complex ones, using different kinds of DNA structures. As a matter of fact, DNA is a highly versatile molecule that can self-assemble into several tridimensional organizations, depending of the sequences and conditions.[7, 23, 24] The most familiar form is undoubtedly the double-helix (also termed duplex) DNA form, used as a DNAzyme for enantioselective Diels–Alder and Friedel–Craft reactions.[7, 22] Nevertheless, other noncanonical DNA structures were also studied for their ability to catalyze chemical reactions. The two most representative structures are the triplex and the G-quadruplex DNA forms, with a clear predominance for the latter ones, which constitute the next section of this book chapter.[23, 24]

3. Native G-quartet-based DNAzymes: G-quadruplexes

3.1. Structure of G-quadruplexes

G-quadruplexes (Figure 1), formed from G-rich DNA strands, are composed of a stacking of several native G-quartets. Each quartet results from the self-assembly of four guanines in a same plan, self-stabilized by eight hydrogen bonds, from the Hoogsteen and Watson–Crick

faces of the guanine moieties. The additional π -stacking interactions between G-quartets, and the bonding of cationic ions (*e.g.*, K^+ , Na^+), increase the global stability of the tridimensional edifice.[10, 26, 27] In a structural point of view, the G-quadruplexes can mainly differ from the total numbers of constitutive G-quartets (from two to several thousands)[28–30] or strands (from one to four),[31–35] from the orientation of the strands (leading to several conformations named antiparallel, parallel, and hybrid), and finally from the length, DNA bases composition, and position of the loop(s) (which can be edgewise, diagonal, or chain-reverse).[36–41] All of these parameters are linked to the global stability of the edifice, like the number of G-quartets (as it is discussed later in this chapter), and are interdependent.

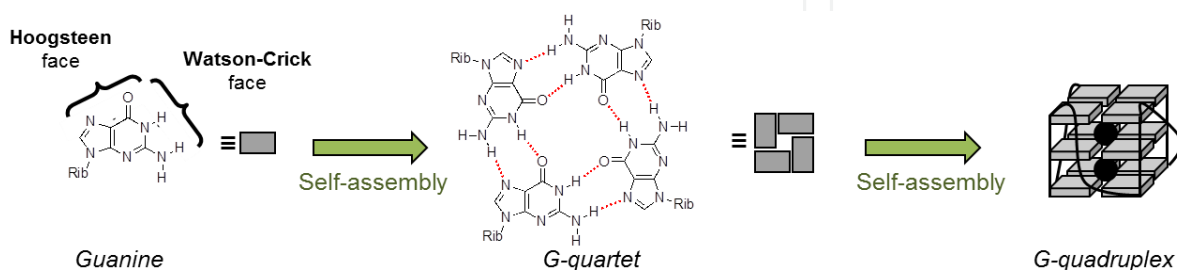


Figure 1. Schematic representation of the self-assembly of guanines *via* the Watson–Crick and Hoogsteen faces to form G-quartets, and G-quadruplexes.

These noncanonical structures are well known in a biological context, because they are strongly suspected to play important roles in key cellular events, like chromosomal instability, or regulation of gene expression. These aspects are far from the scope of this chapter, and author incites curious readers to have a look to some reviews cited hereafter.[26, 27, 42–44]

3.2. The seeds of the G-quartet ability to catalyze peroxidase-like reactions

In 1996, Y. Li and D. Sen developed and published the fourth known DNAzyme system,[45] able to catalyze the incorporation of metals (*i.e.*, $Cu(II)$ and $Zn(II)$) into a specific porphyrin, named mesoporphyrin IX, or MPIX. To select the best DNA catalyst for their system, they used the *in vitro* SELEX (for *systematic evolution of ligands by exponential enrichment*) method that highlighted one sequence, termed PS5.ST1, from an initial pool of DNA sequences.[45, 46] Interestingly, three main observations were crucial:

- a. the sequence of the strand was guanine-rich,
- b. the presence of alkaline cations was required (with a catalytic activity 300 times higher with K^+ than with Na^+), and
- c. the addition of another porphyrin derivative, the *N*-methyl mesoporphyrin IX (or NMM), well known for its interaction with G-quadruplexes and unable to be metallated due to the steric hindrance of the methyl group,[47–49] inhibited the incorporation of the metals into the MPIX. Altogether, these data suggested for the very first time that G-quadruplexes could adopt catalytic properties.

The subsequent step has been taken in 1998 when the same group showed that hemin, a Fe(III) cofactor playing a pivotal role in many enzymes (*e.g.*, in catalases, monooxygenases, and peroxidases), is activated by the presence of a G-quadruplex. More precisely, authors proved that the activity of the *horseradish peroxidase* (also termed HRP), composed of an hemin surrounded by a protein environment, can be mimicked using the same hemin but in the presence of G-quadruplexes.[50] Since then, many experiments using different sequences and morphologies of G-quadruplexes unambiguously confirmed the mandatory role of the G-quadruplex structure, for which the accessible G-quartet (*i.e.*, the external one), able to interact with hemin, constitutes the key step of the reaction.[24, 51–55]

3.3. Mechanism and factors influencing the catalytic activity

After almost 20 years of research on the G-quadruplex peroxidase-mimicking systems (Figure 2), the precise mechanism of the catalytic cycle is not fully understood. Nevertheless, in 2012, L. Stefan *et al.* proposed a first mechanism in nine main steps focused on the iron–porphyrin complex (*i.e.*, the hemin).[56] It was built on the basis of plenty of work published for the hemoprotein systems (primarily peroxidases and catalases).[57, 58] The mechanism is not scrutinized here, and readers are urged to see the references for additional information. However, the main factors influencing the catalysis are described hereafter and might be used as a roadmap.

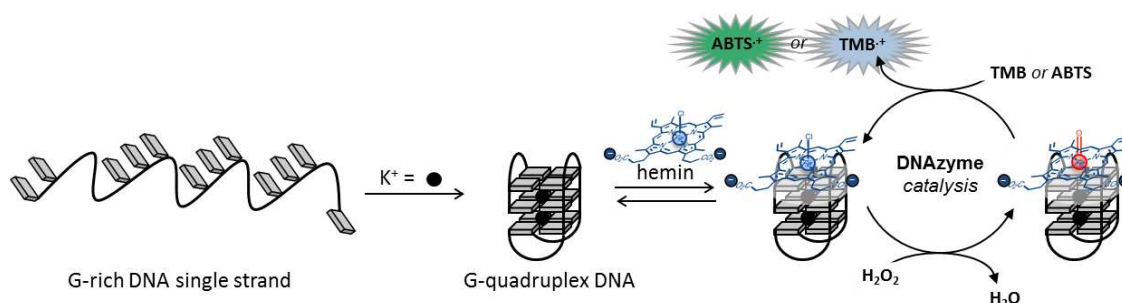


Figure 2. Schematic representation of the DNAzyme activity promoted by a G-quadruplex.

As explained before, the morphology of the G-quadruplexes used (*i.e.*, number of G-quartets, strands, type and length of loops, etc.) influences the catalytic efficiency of the reaction. The activation of the hemin rests on the presence of a hydrophobic binding site, playing the role of the “binding pocket” in enzymes. In this DNAzyme context, accessible G-quartets favor the interaction with hemin and, besides, protect the porphyrin from the oxidative degradation due to the oxygen peroxide. The existence of an axial ligand giving electronic density to the iron atom is another major key point.[11] Like in the native HRP in which an histidine has this function, it is assumed that in the G-quadruplex, one of the guanines of the external G-quartet is devoted to this, by flipping out of the plan (exactly like what was observed in another study with platinum complex binding to a G-quadruplex).[59, 60] Moreover, based on molecular modeling, it was also supposed that this effect could be due, in special cases, to a cytosine from a nearby loop, intercalated between the accessible G-quartet and the hemin, and creating π -

stacking interactions.[55] This insertion of the loop is clearly not a *sine qua non* condition, because several G-quadruplexes without loops and/or cytosine are effective as biotechnological catalysts (*e.g.*, $d((G_3T_n)_3G_3)$ ($n = 1-4$),[54] $d((TG_4)_4)$,[56] $d((T_4G_4)_4)$,[23] or $d((T_4G_6T_4)_4)$).[61] Notwithstanding, their activities are less high than G-quadruplexes with loops, for which the catalytic efficiency can be sorted as follows: antiparallel ones > hybrid forms > parallel ones. [62] The presence of a polar environment and several H-bond donors and acceptors on the distal face of the hemin also constitute a positive point.[11] All these aforementioned criteria are directly linked to the inherent design of the DNA sequence,[63] but other experimental parameters can be used to modulate the pseudo-enzymatic activity of the G-quadruplex. Among all the conditions (non-exhaustive list), chemists can easily inflect the pH, the nature of the buffer, the nature and concentration of salts (*e.g.*, K^+ and Na^+ are important for the G-quadruplex structuration), the presence and nature of a surfactant (*e.g.*, Triton X-100, Tween 20, Brij 56), the temperature, and, last but not least, the adjunction of an “additive.”[9, 50, 64–66] Indeed, some additional compounds can be used to amplify the response of the catalysis. In particular, the use of adenosine triphosphate (or ATP), and its derivatives, was studied by the groups of D.-M. Kong and D. Monchaud.[56, 67] Interestingly, the role of this small molecule is tricky, and Monchaud’s team tried to decipher its actual role. In fact, ATP was proposed to favor several equilibria in the hemin oxidation/reduction process, as it was described in detail in 2012.[56] The four main effects of ATP are:

- a. to facilitate the fixation of H_2O_2 on the iron atom of hemin,
- b. to fuel the two one-electron transfer steps of the catalytic cycle,
- c. to protect the reaction partners against oxidation, and
- d. to modulate the pH of the reaction mixture, like what is observed in cells.[68]

3.4. Applications of G-quadruplex-based DNAzymes

The *horseradish peroxidase*, mimicked by G-quadruplex-based DNAzyme systems, is a very well-known protein in a biochemical context in which, grafted to a biomolecule (mainly an antibody), it is used to oxidize a colorimetric or fluorogenic substrate in ELISA (for *enzyme-linked immunosorbent assay*). This high-throughput assay is, for example, used for the clinical detection of anti-HIV antibodies from biological samples.[69, 70] Moreover, the *horseradish peroxidase* is also used for the purification of waste waters,[71] to develop biosensors, or as a reagent for organic synthesis. This popular enzyme, extracted from the root of the horseradish, is able to oxidize many substrates (*e.g.*, aromatics, phenols, indoles, amines) in the presence of hydrogen peroxide. Its properties were discovered by L. A. Planche in 1810, who noticed that a tincture of guaiac resin became blue when a piece of fresh horseradish root was placed in it. The chemical reaction occurred here is now expected to be due to the peroxidase-catalyzed oxidation of 2,5-di-(4-hydroxy-3-methoxyphenyl)-3,4-diphenylfuran to the corresponding bis-methylenequinone (also known as “guaiacum blue”).[72]

Due to all the advantages described before concerning the use of DNA instead of protein to catalyze reactions, chemists have used for more than 15 years DNAzyme to develop and create plenty of new applications. Indeed, the far better modularity of the G-quadruplex-based

peroxidase-mimicking systems constitutes a new powerful and invaluable tool for scientists, mainly for *in vitro* detection of a large range of molecules.

Trying to be as exhaustive as possible, a list of the principal applications found in the literature is detailed hereinafter.

- a. Detection of monovalent cations like K^+ [73–76] or Ag^+ .[77–79] These corresponding methods are mainly based on the properties of monovalent cations to fold G-rich DNA strands to G-quadruplex. In other words, if the ion is present in the solution, the G-quadruplex is formed, and then it can interact with hemin, and subsequently, with the addition of H_2O_2 , it is able to catalytically oxidize a colorimetric, such as ABTS (for 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)[80, 81] or TMB (3,3',5,5'-tetramethylbenzidine)[82–84] or a fluorimetric probe.[85, 86]
- b. Detection of bivalent cations like Cu^{2+} ,[87–91] with a very low detection limit of 1 nM using the DNAzyme system published by F. Wang *et al.* [92] Also detection of Hg^{2+} [92–98] and Pb^{2+} [96, 99–108] are well represented, in particular because they cause huge damage to human body through food, water, and air. Recently, a dual label-free sensor was developed by H. Li *et al.*, and it can be used to measure the concentration of these ions to the limit of 3.9 nM and 4.8 nM, for lead(II) and mercury(II), respectively.[89]
- c. Detection of organic molecules. Among them, C. Yang *et al.* focused their research on the ochratoxin A, a toxin that can contaminate a large number of food commodities (*e.g.*, cereals, spices, coffee, milk).[109] The International Agency for Research on Cancer has classified this compound as a possible human carcinogen, and the European Commission has set the maximum level at 5 nM (equal to $2 \mu g \cdot kg^{-1}$) for grape-based beverages, like wine. In their publication, the authors proposed a powerful DNAzyme system with a detection limit of 2.5 nM, making it suitable for this analysis, respectful of the European standards. Moreover, the detection of glucose in urine by naked eyes can be performed using a sensor composed of both 23-mer G-quadruplex and glucose oxidase.[110] Thus, the DNAzyme system is coupled to a classic enzymatic reaction, and offers the opportunity to detect glucose from a concentration of 1 μM . Cocaine is also the focus of several studies, and DNAzymes have a high sensitivity up to 5 μM ,[111] or 2.5 μM ,[92] depending of the methodology used.
- d. Detection of proteins, like nucleolines,[112] thrombin,[113–120] lysozymes,[113, 116, 121], and the vascular endothelial growth factor (VEGF), which plays a major role in the cancer development.[122] Also, Z. Ye *et al.* designed a DNAzyme system able to evaluate the concentration of the estrogen receptor alpha ($ER\alpha$), a transcription growth factor involved in the hormone-dependent breast cancer development.[123] In the same way, a DNAzyme-based immunosensor for the highly sensitive detection of the spore wall proteins of *Nosema bombycis*, from biological samples (*i.e.*, silkworm blood), is a promising biotechnological tool for the diagnosis of pébrine disease, a silkworm infection with significant economic impacts.[124] Furthermore, a 96-well plate assay is reported by Y. He *et al.* for the detection of leptin, a protein playing a key role as a “satiety hormone” and influencing basal metabolism, hematopoiesis, reproduction, and angiogenesis.[125] This

method has a sensitivity up to 2 pg.mL^{-1} , 50 times better than the results obtained with ELISA (with a limit of 100 pg.mL^{-1}). Likewise, L. Stefan *et al.* reported the very first steps of a general strategy to make G-quadruplex DNA catalysts easily immobilizable, for the development of high-throughput ELISA-type assay.[126] In this study, a 96-well plate coated with streptavidin was functionalized using a cyclododecapeptide termed RAFT (for *regioselectivity addressable functionalized template*),[127] equipped on one side with a G-quadruplex (intra- or intermolecular), and with a biotin on the other side. The first step was the optimization of experimental conditions to access the best detection limit. In this case, it was found that for a catalysis limited to 2 hours (a fixed time decided by the authors to have a catalytic response in an acceptable amount of time), reactions have to be carried out in a cacodylic buffer (10 mM, with 10 mM KCl and 90 mM NaCl) at pH 4.4, with $1 \text{ }\mu\text{M}$ hemin, $400 \text{ }\mu\text{M}$ TMB, and finally 2 mM H_2O_2 to trigger the process. Using this protocol compatible with biochemical applications, the authors used only 2 pmol of the catalysis per well to detect streptavidin from a commercially available pre-coated microplate (300 pmol/well). This default of RAFT-quadruplex was chosen both to avoid unspecific associations and to limit the consumption of the catalyst. To propose scientists different ways to work with these kinds of DNAzyme systems, two protocols were developed. The more user-friendly one is a three-step procedure: first, a $200 \text{ }\mu\text{L}$ solution of RAFT-quadruplex + hemin solubilized in the *ad hoc* buffer described before was poured in the wells; after an incubation time of half an hour, a washing step (using a cacodylic buffer at pH 7.2) was performed to remove all unbound materials; and finally, a solution, including the luminescent probe TMB, was put inside all the wells. Reactions started when hydrogen peroxide solutions were put inside. The variation of absorbance at 370 nm was monitored using a 96-plate UV-Vis reader with one measure every 2 minutes. This work highlighted the fact that surface-immobilized DNAzymes are interesting alternatives to develop practically convenient, simple, and rapid biophysical assays. Nevertheless, this work constitutes another brick in the wall of the development of effective DNAzyme-based assays.[126]

- e. Detection of nucleic acids is also the target of several G-quadruplex DNAzyme process. Indeed, they are applied for the detection of single-strand DNA,[128] or other DNA analytes,[62] including the smart “DNA machine” developed by I. Willner’s team in which its sensitivity is equal to 10^{-14} M of the target sequence (a 29-mer corresponding to a domain of single-strand DNA hepatitis B viral gene).[129] The detection of genetically modified organisms can also be done, as proved by B. Qiu *et al.* with both cauliflower mosaic virus 35S promoter, and lectin gene.[130] This approach was improved in 2014 to reach a detection limit of 5 nM .[131] Interestingly, a nonclassic fluorescence probe, the 2',7'-dichlorodihydrofluorescein diacetate (or H_2DCFDA), was used instead of the usual ABTS, TMB, or luminol.
- f. Moreover, DNAzyme is a powerful biotechnological tool to detect micro RNA (also termed mRNA),[132–134] particularly when it is coupled to a rolling circle amplification which allows a sensitivity of 0.3 fM ,[135] or as “DNAzyme Ferris wheel” like nanostructures, as proposed by W. Zhou *et al.* in 2015,[136] with a detection limit of $5 \times 10^{-13} \text{ M}$ for

miR-141 (a biological marker of the human prostate cancer) by naked eyes. Furthermore, scientists created DNAzyme sensors to detect small nucleotides like adenosine triphosphate (or ATP), [137–140] cyclic diguanilate c-di-GMP, [141] and also 8-OHdG (for 8-hydroxy-2'-deoxyguanosine) from the urine, that is associated with various cancers, diabetes, and neurological diseases. [142]

- g. The modification of one nucleotide inside a DNA sequence can also be verified using G-quadruplex-based DNAzyme. Actually, the method published by M. Deng *et al.* is able to detect the substitution of one DNA base in a 39-mer strand. [143] In parallel, I. Willner's team proposed an alternative method using functionalized magnetic particles, [144] whereas in 2015, D. Verga *et al.* described a smart methodology to discriminate by naked eyes a single nucleotide variation. [145] Enzymatic activities were also investigated in the presence of DNAzyme-based sensors. It was the case for the methyltransferase, [146] cholesterol oxidase, [147] glucose oxidase, [148] or telomerase. [149–151] This last example is of great interest because telomerase, overexpressed in a tumoral context, constitutes a high-potential biological marker for the diagnosis of cancers. [152, 153] The first DNAzyme method to detect it from cellular lysates was proposed by R. Freeman *et al.*, and is based on the concept that only cellular extracts containing telomerase (*i.e.*, the cancer cells) can elongate a primer. [154] Thus, the longer G-rich DNA strand, composed of several specific (TTAGGG)_n repeats, is able to form a G-quadruplex that can act as the catalyst of a DNAzyme process. Consequently, an "ON" signal, due to the catalytic oxidation of the colorless ABTS to the corresponding green oxidized product, is the proof of the telomerase activity, that is, that the lysate was from a cancer cell. Conversely, healthy cells contain nonactive telomerase, and the corresponding lysate has consequently no effect on the primer. Too short to form a G-quadruplex, the DNAzyme catalysis cannot occur, leading to no change of the media color/absorbance: it is the "OFF" signal. Inspired by this work, the D. Monchaud's team decided to use additives (*i.e.*, template-assembled synthetic G-quartet) to improve the detection limit of G-quadruplex composed of a repeat of the (TTAGGG)_n motif. [155] This research will be discussed later in this chapter. Another work from L.-J. Wang is based on the same concept as the Freeman's one, but using a 93-mer nucleotide telomerase substrate primer which leads to a detection limit as low as 0.1 aM (= 10⁻¹⁸) able to detect telomerase activity from HeLa cells. [156]
- h. Detection of antibodies to develop ELISA-type immunoassays [157, 158] or for immunohistochemistry assays are also extremely promising. G-quadruplex DNAzyme enables the visualization of the prostate-specific antigen (PSA), a high-potential tumor marker, directly in solid tissue sections. [159] In parallel, an assembly of antibody/gold nanoparticle/DNAzyme system was developed by M. Shi *et al.* in 2014 [160] and is used to quantify one of the most important carbohydrate tumor marker, the antigen CA19-9, from human serum samples. The detection limit obtained was 0.016 U.mL⁻¹, which is the lowest one reported in the literature. Using a close approach with a G-rich DNA sequence grafted on the gold nanoparticles surface, a new DNAzyme biosensor was proposed as a direct antigen-antibody detection assay. [161]

- i. Interestingly, the DNAzyme methodology was also used to detect bigger living systems, like the bacteria *Escherichia coli* O157:H7[162] or *Alicyclobacillus acidoterrestris*. [163] In the first example, authors used graphene oxide/thionine/gold nanoparticles coated SiO₂ nanocomposites to immobilize DNA, while for the second one, a more classic approach was used, in which G-quadruplex-hemin complexes oxidize the colorless guaiacol, produced by the bacteria, to tetraguaiacol, which is amber.
- j. Developed to target G-quadruplexes *in vivo*, *in cellulo*, or *in vitro* for biological applications, G-quadruplex ligands are molecules able to interact with G-quadruplexes and to stabilize them. [164–166] This ability can be evaluated, thanks to the DNAzyme process, because of a competition between the ligands researchers want to try, and the hemin. In other words, a good ligand takes the place of the hemin; hemin is subsequently not activated and, consequently, leads to a decrease of the signal intensity (measured by UV–Vis or by fluorescence). [61, 167–169]
- k. To finish this laundry list, it is essential to mention other ingenious applications for the G-quadruplex DNAzymes, like the development of logic gates [170–173] as the INHIBIT one published by T. Li *et al.*, [174] or as the AND one proposed by J. Chen *et al.*, described as a keypad lock security system. [175]

To summarize, all these cases, which represent the range from the more applied to the more conceptual scientific applications of the same DNAzyme catalysis, illustrate how using DNA instead of enzyme to catalyze a reaction puts out a new avenue in terms of polyvalence. It is believed that this list will increase more and more in the next years. But the precise understanding of the mechanism constitutes also an exciting challenge. On the one hand, this progress should offer scientists the possibility to fine-tune the experimental conditions (*e.g.*, sequence of the G-quadruplex DNA strand(s), length of the loop(s), addition of a boosting agent, etc.). On the other hand, a better comprehension should help to enlighten chemists about the mechanistic aspect of the oxidation states of the hemin, in both biological and DNAzyme systems.

4. Synthetic G-quartet-based DNAzymes: template-assembled synthetic G-quartets (or TASQ)

4.1. Concept and structure of TASQ

Based on the idea that G-quadruplexes, mimicking the natural *horseradish peroxidase*, lead to an increase of the range of applications, mainly due to the higher stability of the DNA compared to the protein and permitting a use in a bigger range of experimental conditions (temperature, buffer, ion strength, etc.), [7, 23, 176] few research teams decided to develop G-quadruplex-mimicking systems.

Because the pivotal step of the catalytic cycle is based on the activation of hemin by interaction with one of the external G-quartet, [11] the group of Dr. D. Monchaud decided to synthesize

the very first example of a water-soluble molecule, composed of four guanine residues, and able to form, intramolecularly, a synthetic G-quartet.[14]

Historically, the very first observation of synthetic G-quartets was made by I. Bang in 1910, who was able to form gels from a concentrate solution of guanosine monophosphate. However, the hypothesis of the self-assembly of guanine derivatives to G-quartet arrangements was only published more than half a century later by M. Gellert, M. Lipsett, and D. Davies in 1962. The dried fibers obtained could be analyzed by X-ray diffraction and helped the authors to propose the initial supramolecular structure shown in Figure 1.[177] Interestingly, a recent study proved that the length of the fibers can reach from 8 nm to 30 nm, corresponding to from 24 to 87 stacked G-quartets, respectively.[178]

During the last decades, plenty of examples using synthetic G-quartets as a supramolecular motif were developed and well summarized in several reviews.[10, 179, 180] The applications of these systems concern pH-sensitive hydrogel probes,[181] synthetic transmembrane Na⁺ transporter,[182] and other ionic channels,[183–185] enantioselective systems controlled by the cation used,[186] combinatorial chemistry,[187] and also molecular electronics and liquid crystals.[179] Among all these examples, in which the elementary brick is made of one or two guanines, only few stem from a four-guanine-based compound. This assessment is inquisitive and nonintuitive because G-quartets are composed of four guanines. It was why the team of J. Davis developed in 2000 and 2003 1,3-alternate calix[4]arene derivatives functionalized by four guanines.[188, 189] This smart system in which two guanines are on one side, while the two others are on the other side of the calixarene template, was used as both cation (inside the G-quartet) and anion (thanks to the H-bonds between protons of the amide groups and the anion) receptors.[188] The formation of the G-quartet is intermolecular between two guanines from one molecule, and two guanines from another one. It took the scientific community until 2008 to propose the first intramolecular synthetic G-quartet molecules, termed TASQ.

The name “TASQ,” for *template-assembled synthetic G-quartet*, was introduced by the research group of J. C. Sherman to describe molecules built around a template and functionalized by four guanines, able to interact each other to self-assemble into an intramolecular G-quartet.[12] This concept probably derived from a modification of the TASP (for *template-assembled synthetic peptide*) development,[190] in which four peptide sequences were used instead of DNA bases. The aim of these models was the understanding of protein interactions, thanks to their spatial proximity when grafted to the same scaffold.

Thus, the first TASQ were synthesized from a highly lipophilic calixarene moiety substituted by four 2',3'-*O*-isopropylidene-guanosine.[12] The intramolecular formation of the G-quartet was demonstrated by ¹H NMR, NOESY, COSY, and HMQC and was definitively proved by X-ray diffraction in 2012.[191] Notwithstanding, no application of these systems was published, and their hydrophobic properties were probably the reason for that. Interestingly, they rectified this point using first phosphate groups to functionalize the calixarene on the opposite side of the guanosines[192] and then, subsequently, with the phosphate group intercalating between the guanine moieties and the scaffold (due to the use of 3'-monophosphate guanosines).[193]

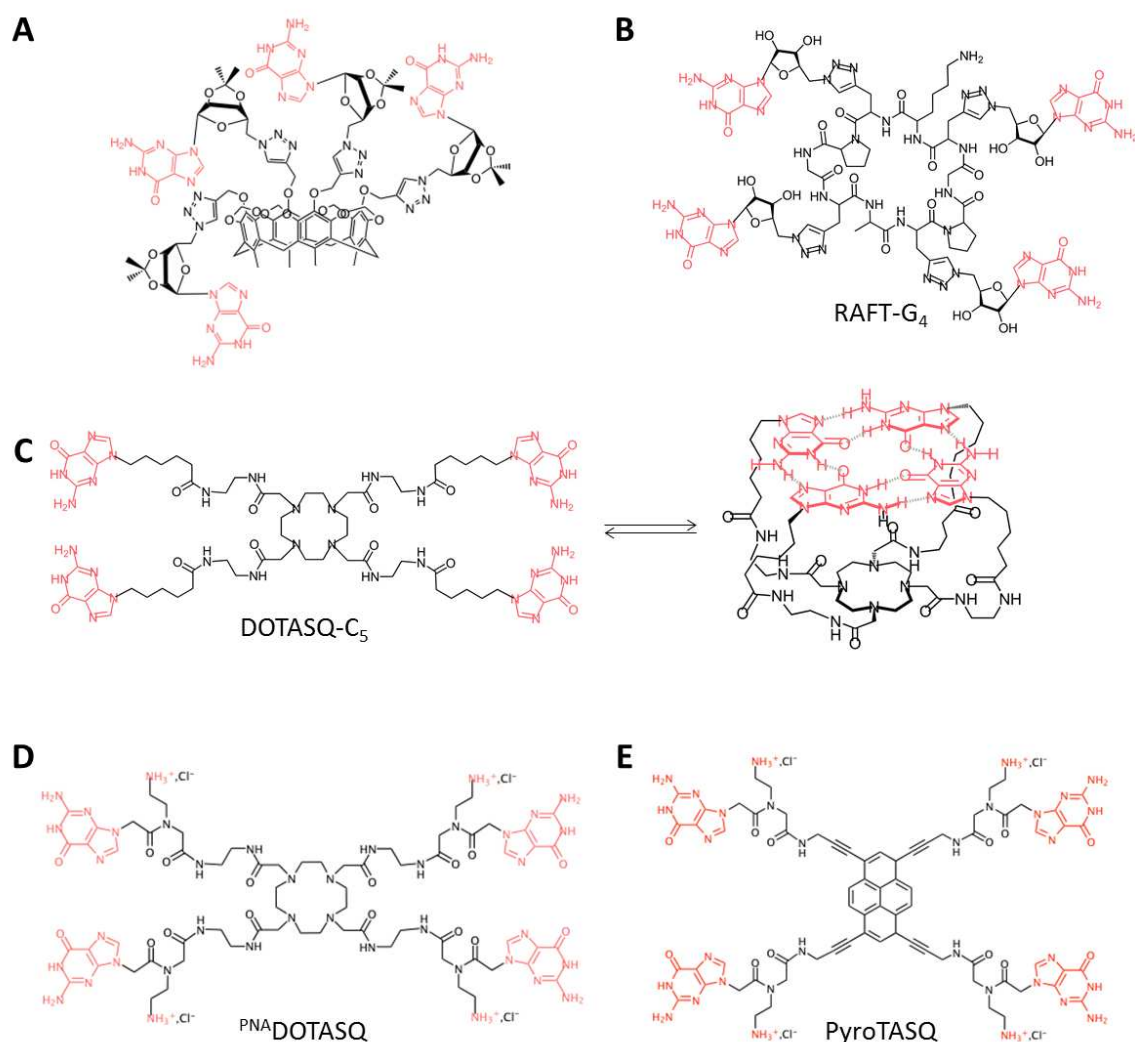


Figure 3. Chemical structures of the first TASQ developed by J. C. Sherman (A), [12] RAFT-G₄ (B), [13] DOTASQ-C₅ in equilibrium between its open (left) and closed conformations (right) (C), [14] PNA-DOTASQ (D), [15] and PyroTASQ (E). [16]

In parallel, the group of E. Defrancq was focused on the functionalization of a cyclodecapeptide termed RAFT (for *regioselectivity addressable functionalized template*) by DNA strands.[127, 194–196] This research led the team to synthesize a TASQ from a RAFT equipped with four guanosines.[13] Thanks to a collaboration with D. Monchaud's team, this molecule was studied as a DNAzyme (see section 4.2) and showed that the G-quartet intramolecular formation (confirmed by circular dichroism, and NMR studies) was able to catalyze the hemin oxidation/reduction cyclic reaction.[197]

However, the very first example of a water-soluble TASQ was proposed by L. Stefan *et al.* in 2011.[14] The key point was the choice of the *ad hoc* template, able to drive the G-quadruplex formation, and being fully soluble in water. It was why the cyclen macrocycle was chosen, for its high solubility, its ability of metal chelation, and its C₄-type symmetry, identical to the one of a G-quartet.[198, 199] Even if this last criteria was not a *sine qua non* condition, it seemed to

be favorable, like in the calixarene-templated TASQ developed by J. C. Sherman's group. Thus, DOTASQ were born from a DOTA (for 1,4,7,11-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid) moiety in which the four "arms" were functionalized by four alkylguanine groups. The term DOTASQ is a portmanteau word created from both acronyms DOTA (*i.e.*, the template) and TASQ (*i.e.*, the supramolecular property of the molecule). For the sake of comparison, four DOTASQ were synthesized: DOTASQ- C_1 and DOTASQ- C_5 (C_1 and C_5 suffixes indicating the length of the alkyl chain), and the terbium(III) equivalents Tb.DOTASQ- C_1 and Tb.DOTASQ- C_5 , respectively.[14]

Furthermore, another TASQ was developed with peptide nucleic acid guanine (also termed PNA guanines) arms and was called ^{PNA}DOTASQ.[15]

The peroxidase-mimicking catalytic activities of these compounds were evaluated and are described in the next sections. As a remark, other TASQ were developed by D. Monchaud's team like PyroTASQ (pyrene as a template),[16] NaphtoTASQ (naphthalene as a template) [200], and also PorphySQ and ^{PNA}PorphySQ (porphyrin moieties as templates).[201, 202]

4.2. Catalytic activities of synthetic G-quartets

The formation of a synthetic G-quartet was expected to have the same properties as a native one from a G-quadruplex. Indeed, this synthetic G-quartet is able to mimic the external (also termed accessible) G-quartet of the biological edifice.[12–16, 201] In this section, the main experimental data and results are highlighted to prove the feasibility of this new strategy of nature-mimicking catalysts. To begin with, the very first example of this approach, published by D. Monchaud's team, is presented and then the improvement with ^{PNA}DOTASQ is explained. A collaboration between this group and the E. Defrancq's one led to the study of the RAFT- G_4 , introduced in the previous section (see section 4.1), and detailed here.

In parallel, the work of H. O. Sintim's group, focused on intermolecular G-quartets (*i.e.*, not TASQ) using c-di-GMP (for cyclic diguanylic acid) as a catalyst, will be presented.[141, 203, 204]

DOTASQs as a pre-catalyst: As explained before, the main step of the catalytic reaction is the activation of the hemin by interaction with the G-quartet. To verify this *sine qua non* condition, titrations of 1 μ M hemin with DOTASQ were performed in cacodylic buffer.[205] The characteristic UV–Vis band of hemin from 350 nm to 400 nm was observed and, interestingly, the signal increased in all the cases with DOTASQ- C_1 , DOTASQ- C_5 , and 22AG, the telomeric G-quadruplex sequence d[AG₃(T₂AG₃)₃] used as a reference. Moreover, for the three systems, a stoichiometry of about 1:1 was found, and the dissociation constants were calculated using the following equation:

$$K_d = \frac{[\text{hemin}][\text{cat.}]}{[\text{hemin} + \text{cat}]} \quad (1)$$

Thus, the subsequent K_d were found: 170 nM for DOTASQ- C_1 , 135 nM for DOTASQ- C_5 , and 235 nM for the native G-quadruplex 22AG. These results were duplicated with other concen-

trations of hemin, and similar values were obtained. They show that both native and synthetic G-quartets are able to interact with hemin, forming the key step of the peroxidation reaction. Inspired by the optimal experimental conditions published by P. Travascio *et al.*, and developed for G-quadruplexes,[50] primitive experiments were carried out with 1 μM hemin, 2 mM ABTS, 600 μM H_2O_2 , and from 0 μM to 50 μM DOTASQ, in Caco.KTD buffer. This buffer designated a cacodylic acid buffer composed of 10 mM lithium cacodylate (Caco), 10 mM KCl (letter K), 90 mM LiCl, with addition of 0.1 % (v/v) DMSO (letter D) favoring the solubilization of hemin, and 0.05 % (w/v) Triton X-100 (letter T), a nonionic surfactant promoting disaggregation of hemin.

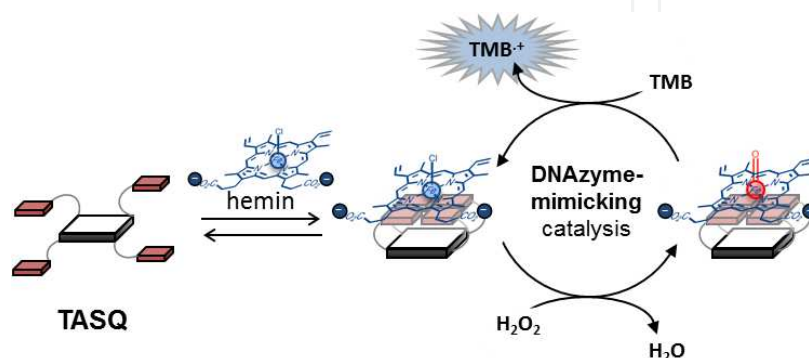


Figure 4. Schematic representation of the peroxidase-like activity promoted by the use of a TASQ. [205, 206]

The catalytic activity was therefore evaluated by UV–Vis absorbance, in 1-mL-quartz cuvettes, measuring the formation of ABTS^+ (also proposed as ABTS^- in the scientific literature), the oxidized product of ABTS which absorbs at 420 nm.[80, 81] All the results were compared to a control experiment, strictly composed of all the same reagents, except the lack of the pre-catalyst (*i.e.*, the hemin is alone with ABTS and hydrogen peroxide, and not catalyzed by G-quadruplexes or TASQs). After less than 1 hour, all the absorbance signals were constant and proved the feasibility of the TASQ-catalyzed peroxidation reaction concept.

However, to confirm that the catalysis of hemin was due to the formation of the intramolecular G-quartet, a DOTASQ- C_5 derivative, termed Prot.DOTASQ- C_5 , was synthesized. In fact, Prot.DOTASQ- C_5 has “protected guanines” with 6-O-benzyl groups in the four guanine moieties. Thus, the formation of G-quartet is impossible because of the rupture of the H-bonds involving the carboxylic group in position 6. The catalytic activity of this compound is null, confirming that the formation of an intramolecular G-quartet inside the TASQ is mandatory to activate hemin and to catalyze the reaction.[205]

For the sake of comparison of the efficiency of the DOTASQ, apparent rate constants k_{cat} were calculated, dividing the initial rate (V_0) by the concentration of the catalyst ($[\text{cat.}]$), using Eq (2).

$$k_{\text{cat}} = \frac{V_0}{[\text{cat.}]} = \frac{\Delta\text{Abs}_t}{\varepsilon_{\text{ABTS}^+}} \times \frac{1}{[\text{cat.}]} \quad (2)$$

Thus, k_{cat} of 0.36 h^{-1} and 0.29 h^{-1} were obtained for DOTASQ- C_5 and DOTASQ- C_1 , respectively. For structural reasons, DOTASQ- C_5 is able to form its intramolecular G-quartet easier than DOTASQ- C_1 . This better stability of the G-quartet favors the π -stacking of hemin and, consequently, the catalytic activity.

To compare TASQ with G-quadruplexes, a constant of $k_{\text{cat}} = 9.71 \text{ h}^{-1}$ was got for 22AG, which is 25 times more efficient than DOTASQ- C_5 . This difference seems to be curious because the K_d of DOTASQ and 22AG with hemin were close. A similar assessment was reported in the literature but with opposite results, with PS2.M and the RNA equivalent rPS2.M.[51] Indeed, their catalytic activities were similar, although the K_d values were 27 nM and 900 nM, respectively.

It can be hypothesized that, even if the key step was defined as the hemin/G-quartet interaction, the environment around the G-quartet plays also a pivotal role for the catalytic efficiency. The presence of loops, the accessibility of the G-quartet to hemin, and other factors described before (see section 3.3) are critical for all the steps of the catalysis.[11, 56]

Finally, to eliminate all the doubts, another substrate was used instead of ABTS, and TMB was chosen. Its oxidation is a two-step reaction producing first a charge transfer complex, with absorbance at 370 nm and 652 nm, and a final diimine product absorbing at 450 nm.[82–84] Using different experimental conditions than before, the catalytic activity of $50 \mu\text{M}$ DOTASQ- C_5 was evaluated in the presence of $1 \mu\text{M}$ hemin, $500 \mu\text{M}$ TMB, and 1.5 mM H_2O_2 in Caco.KTD buffer, and were compared with the same reaction with $2 \mu\text{M}$ 22AG. Like with ABTS, experiments with TMB approved the ability of TASQ to catalyze peroxidase-like catalysis. Obtained apparent catalytic constants were 0.02 h^{-1} for DOTASQ- C_5 against 0.27 h^{-1} for 22AG.[205] Altogether, these results constitute the proof of concept of the use of native and synthetic G-quartets as a universal platform for the peroxidation reactions. However, efficiency had to be improved, and better results were obtained with the second generation of TASQ: the $^{\text{PNA}}$ DOTASQ.

$^{\text{PNA}}$ DOTASQ as a pre-catalyst: The main structural modifications made from DOTASQ to $^{\text{PNA}}$ DOTASQ were the substitution of the original alkyl-arms by PNA guanine moieties. Thus, the new properties worn by this new TASQ are numerous and not detailed here (this molecule was also developed as a smart G-quadruplex ligand),[15] but the main point which must be highlighted here is the introduction of a total of four cationic charges, thanks to the presence of four pendant primary amine side chains. These positive charges (at physiological or acidic pH) are of the utmost importance to increase the interaction with hemin. Indeed, hemin is an Fe(III)-porphyrin holding two anionic charges due to two carboxylic groups. Electrostatic interactions were expected to facilitate the hemin approach and association with the G-quartet.

To verify this hypothesis, R. Haudecoeur *et al.* performed the catalytic reaction with TMB, using the same protocol described before, from $0 \mu\text{M}$ to $50 \mu\text{M}$ $^{\text{PNA}}$ DOTASQ. For the sake of comparison, experiments with the same range of concentration were carried out with DOTASQ- C_5 (*i.e.*, the most efficient DOTASQ) and also with 22AG at $2 \mu\text{M}$ as a reference.[206]

Interestingly, the results showed the far better catalytic ability of $^{\text{PNA}}$ DOTASQ compared to the DOTASQ. Indeed, the initial rates were evaluated at $1.25 \mu\text{M}\cdot\text{min}^{-1}$ and $0.05 \mu\text{M}\cdot\text{min}^{-1}$ at

50 μM , respectively, corresponding to an average increase of efficiency of 2600 %. At this stage, $\text{PNA}^{\text{DOTASQ}}$ is the more active TASQ catalyst for peroxidase-like reactions, and is closer to the activity of G-quadruplex, with a factor of 11 between it and 22AG (*i.e.*, in the experiments, 22 μM $\text{PNA}^{\text{DOTASQ}}$ offers the same catalytic response than 2 μM of the natural G-quadruplex). $\text{PNA}^{\text{DOTASQ}}$ confirms the role of intramolecular G-quartets to activate hemin and to perform DNAzyme-like catalysis, and also that improvement of the efficiency is just at the very beginning of the development. The modification of the arms is a positive point, but the role of the template, directly linked to the ability of synthetic G-quartet formation, is by consequence indirectly linked to the catalytic properties.

RAFT- G_4 as a pre-catalyst: To evaluate the role of the template on the DNAzyme-like activity, the TASQ developed by E. Defrancq's team was tested.[197] Termed RAFT- G_4 , the molecule is composed of a cyclodecapeptide and four guanosine arms in which one triazole per arm is intercalated between the ribose and the peptide scaffold (playing a role in the stabilization of the intramolecular G-quartet).[13] As a first step, UV-Vis titrations of hemin by RAFT- G_4 were performed to check the interaction, and compared with DOTASQ- C_5 . Interestingly, very similar signals shifted from the signal of hemin alone were obtained in the presence of both TASQ for the two peaks at 363 nm and 394 nm, corresponding to the aggregated and disaggregated forms of the hemin (*i.e.*, catalytically active), respectively.[50]

Afterwards, catalytic experiments were carried out with 1 μM hemin, 2 mM ABTS, and 600 μM H_2O_2 in Caco.KTD buffer, with concentrations of DOTASQ- C_5 and RAFT- G_4 from 10 mM to 100 mM. Positively, the results revealed the ability of RAFT to be a catalyst, even if the efficiency was lower than for the DOTASQ- C_5 . To optimize the catalysis, the use of 10 mM ATP in Caco.KTD buffer at pH 4.8 (protocol based on previous studies with G-quadruplexes) was performed and showed the positive effect of these modifications of the experimental conditions. More precisely, this optimization led to an improvement of DOTASQ and RAFT- G_4 efficiency by factors 1.8 and 5.1, respectively.[197]

These series of experiments triggered two main conclusions. First, the ability of a TASQ to be a good catalyst is dependent on the template, the nature of the guanine arms, and the stability of the G-quartet, and it seems to be for now difficult to rationalize their impacts. The multiplication of examples of TASQ in the literature will be an invaluable chance to understand the role of each structural part of the molecules. Author wagers that new molecules like Pyro-TASQ[16] or NaphtoTASQ[200] will help to decipher a little more the reasons of these differences.

Another nice synthetic G-quartet system used as DNAzyme-like catalyst was developed by H. O. Sintim's team.[141] In this case, the molecule is not a TASQ forming an intramolecular G-quartet, but the cyclic diguanylic acid (also termed c-di-GMP), composed of two guanine residues. The authors proposed that c-di-GMP are able to form a discrete G-quadruplex formed by two intermolecular G-quartets (*i.e.*, from four c-di-GMP molecules) at the micromolar level, but with the *sine qua non* presence of the intercalating proflavin molecule. In this case, it can be considered that proflavin assumes the role of a non-covalently linked template, unlike in TASQ. Experiments carried out with 0.5 μM hemin, 30 μM proflavin, 2 mM ABTS, and 2 mM H_2O_2 in Tris-HCl buffer (50 mM, pH 7.9) revealed that a catalytic activity can be distinguished

for concentrations of c-di-GMP higher than 2 μ M. Several control experiments were performed and clearly highlighted the fact that the formation of intermolecular G-quartets is required to observe catalytic activities.

All these data firmly confirm the role of synthetic G-quartets, both intramolecular and intermolecular, as efficient and promising nature-mimicking systems. In spite of the increasing excitement around this field, it is nevertheless honest to confess that the use of TASQ as catalysts for peroxidase-like experiments is, for the moment, far from the success of the DNAzyme, mainly in terms of applications.[7, 23, 24] However, the very first ones were developed and are presented hereinafter.

4.3. Applications of synthetic G-quartets

One of the main topics of natural G-quartets is the biological role of G-quadruplex structures, found in many sequences in the human genome (estimated at about 350,000 sequences).[207, 208] This alternative higher-order DNA structure (*i.e.*, a noncanonical double-helix one)[209] is strongly suspected to play important roles in key cellular events, like chromosomal instability or regulation of gene expression.[26, 27, 42–44] Notably, the extremities of chromosomes, termed telomeres, are composed of a G-overhang strand with several (TTAGGG)_n repeats from an average of 100–200 bases.[26, 27, 210] Interestingly, this single strand (stabilized by a protein complex named shelterin) is the substrate for a key enzyme, the telomerase.[211–213] Although this field is of particular interest and extremely exciting, it is far from the scope of this chapter, and the author recommends some excellent reviews to go further insight. In few words, telomerase is able to synthesize TTAGGG repeats, leading to the elongation of the telomeres. It is however only active in a vast majority of cancer cells (85 % of the tested cell lines) but inactive in somatic healthy cells.[214] Telomerase is thus considered as a cancer marker with a strong therapeutic potential.[152, 153] Consequently, direct or indirect evaluation of the activity of this enzyme from cell lysates could be of the utmost importance for the tumor diagnosis.

Based on the DNAzyme technology, R. Freeman *et al.* developed an assay to detect telomerase activity in an indirect way. If it is active, a primer is elongated to long (TTAGGG)_n strands able to form G-quadruplexes.[154] However, long single-strand sequences can form multimers, [215] composed of several G-quadruplex structures, with a “stacking”[216–218] or a “beads-on-a-string”[219, 220] global structure. In the first case, several G-quadruplexes from the same strand are stacked together, while in the second case, G-quadruplexes are independent, without any interactions between them.

A study from L. Stefan *et al.* [155] showed that mainly for a 46-base long strand (d[AG₃(T₂AG₃)₇] composed of two G-quadruplexes separated only by three nucleobases), two different kinds of G-quartets able to activate hemin can be defined. Indeed, the first accessible G-quartets are the external ones, and are identical to the ones found in a single G-quadruplex. However, the second hemin interaction site is at the interface between two G-quadruplexes. The teams of L. Petraccone and H. Sugiyama demonstrated that this “pocket” (composed of two G-quartets) is more hydrophobic than the classic external G-quartet and favors therefore the interactions with hydrophobic organic molecules, like hemin.[221, 222] Interestingly, this internal binding site clearly has a positive effect on the hemin binding and on its catalytic

activity. The efficiency of the DNAzyme response is due to the hydrophobic properties of this internal site that favors hemin fixation and its protection against degradation. Moreover, once stacked to an internal G-quartet, the distal face of the Fe(III)–porphyrin is “through contact with” the second G-quartet that can play a key role during the oxidation/reduction process of the iron during the catalysis (*e.g.*, mimicking the action of histidine like in the natural proteins, favoring H₂O₂ deprotonation).[11] This hydrophobic site, composed of two native G-quartets able to “sandwich” the hemin, is thus more active than only one G-quartet. The increase of activity leads surely to a better detection limit.

With this in mind, it was decided by D. Monchaud’s group to create an artificial high-activity hemin binding site to improve the detection of telomeric G-quadruplex sequences, thanks to the use of TASQ. For the detection of 22AG, the addition of TASQ was expected to modify the characteristics of one or two of the external sites, to one or two pseudo-internal sites. Indeed, thanks to a like-likes-like process, TASQ interact with G-quadruplexes *via* a synthetic G-quartet/native G-quartet recognition, identical to a classic G-quartet/G-quartet interaction. Thus, the “binding pocket” created between the external native G-quartet of the G-quadruplex and the intramolecular synthetic G-quartet of a TASQ is an artificial high-activity hemin binding site.[155]

To verify it, a DNAzyme experiment was carried out in Caco.KTD in a 96-well plate with 1 μM hemin, 2 mM ABTS, and 600 μM H₂O₂, in the presence or absence of 50 μM DOTASQ-C₅, with different concentrations of 22AG (from 65 nM to 8 μM). Results highlighted the fact that the catalysis is more efficient in the presence of TASQ, and offer the possibility to decrease the detection limit from 4 μM to 500 nM. This improvement confirms the concept of the pseudo-internal high-activity hemin binding site that can be considered as an equivalent of the “binding pocket” of natural enzymes. In term of initial rates, whereas the optimal concentration of DOTASQ-C₅ can increase it by factor 2.7 at 40 equivalents, ^{PNA}DOTASQ was able to double it only at one equivalent.[155]

To conclude, TASQ can be used as “boosters” of the catalytic activity of DNAzyme, and permit to improve the detection limit by speeding up the rate of oxidation of the substrate (*e.g.*, ABTS or TMB). This effect could be very interesting to detect smaller concentrations of G-quadruplexes, in particular of telomeric G-quadruplexes, to determine with a better signal-to-noise ratio telomerase concentrations from cell lysates.

Another application detailed here is the development of a DNAzyme-mimicking system to detect the bacterial signaling molecule c-di-GMP, published by H. O. Sintim *et al.*[141] The detection of this molecule, able to form biofilms in several clinical relevant bacterial pathogens, is crucial to limit hospital infections. Interestingly, the target molecule is also, by itself, the catalyst of the peroxidation reaction (see section 4.2), because of its ability to self-assemble to form G-quartets. This method was validated with *E. coli* overexpressing a diguanylate cyclase WsprD70E from crude bacterial lysates.

The last but not the least application is the use of TASQ to create fully synthetic process able to mimic nature. Indeed, from a fully natural process with the *horseradish peroxidase*, DNA strands and hemin are still natural products in DNAzyme. However, in TASQ-based catalysis,

only hemin is natural, because organic synthetic molecules (*i.e.*, TASQ) replace DNA. To pursue the evolution, another extremely soluble Fe(III)–porphyrin, the 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrin (also termed FeTPPS), was used instead of hemin. This molecule was well known for its excellent water solubility, its stability, its resistance to highly oxidative conditions, and known to perform H₂O₂-mediated oxidations for more than two decades.[223–225] After a first titration of the FeTPPS with DOTASQ-C₅, the catalytic experiments were carried out in Caco.K (without Triton X-100 and DMSO, because of the excellent solubility of this porphyrin compared to hemin). The same protocol was used in the presence of ABTS, and results showed an initial rate of 0.36 h⁻¹ for DOTASQ-C₅, while no activity was detected with 22AG. This point must be due to electrostatic repulsions between the four negative charges of the sulfonate derivative and the negatively charged phosphate groups of the DNA strand. In sum, the use of FeTPPS with a TASQ was the last step to create a fully synthetic process mimicking a well-known natural process of peroxidation.[205]

5. Conclusion

As demonstrated all along this chapter, native and synthetic G-quartets are powerful catalysts for peroxidase-like process.

On the one hand, the DNAzyme field is fed by the numerous examples of G-quadruplexes used as a native catalytic platform, that found dozens of applications, from the detection of products in biological samples, to the evaluation of heavy metal concentrations, proteins activity, or to develop logic gates for new DNA-based nanotechnologies.[7, 23, 24] G-quadruplex DNA are extremely versatile structures that can be folded from plenty of sequences, in several media, and their functionalization to add probes, functional groups, or to graft them on a solid support are important and invaluable advantages. The design of the G-quadruplex structures, that is, of the catalyst, is far easier than for enzymes, because small modifications of a protein commonly lead to a modification of the active site and then, consequently, to a partial or total loss of activity. Indeed, all the G-quadruplexes are virtually able to catalyze peroxidase-mimicking reactions, because the key part of these noncanonical structures is one of the external G-quartet which is, by definition, the basic unit of G-quadruplexes.[10, 180] As a result, these easy-to-use DNAzyme systems are ready to be applied in chemistry and biology laboratories, mainly for their adaptability and stability, but also in the medical field in which DNAzyme can be considered as a cheaper alternative to the natural *horseradish peroxidase*, mainly to tag relevant biomolecules, like antibodies in ELISA protocols.

On the other hand, the only use of the minimal catalytically active part of the G-quadruplexes, the G-quartet, was presented in this chapter, mainly thanks to the use of TASQ. These template-assembled synthetic G-quartets, able to form an intramolecular G-quartet, proved that a small synthetic molecule can selectively interact with hemin to catalyze peroxidase-mimicking reactions. Interestingly, all the TASQ highlighted here (DOTASQ,[14, 205] ^{PNA}DOTASQ, [15, 206] or RAFT-G₄)[13, 197]) offer different activities, and the best edifice is definitely ^{PNA}DOTASQ, which is closer to the efficiency of G-quadruplex-based DNAzymes, even if a

decrease by a factor of 10, approximately, is observed. However, the use of synthetic molecules instead of natural structures (*i.e.*, enzymes or DNA) is an undeniable advantage, in particular because these TASQ are easily synthesized in four straightforward steps from commercially available products, with good yields. Their modifications and improvements are only limited by imagination, and the new molecules designed by D. Monchaud's team clearly demonstrate this point.[16, 200] Interestingly, a fully synthetic system based only on nonnatural components was described in this chapter for its ability to reproduce the natural enzymatic process. In other words, TASQ permit to mimic a natural catalysis originally made by the *horseradish peroxidase* with only synthetic molecules made by chemists.[55] It can be postulated that the mechanism behind this reaction is probably very close to the natural one, even if more data are needed to confirm this proposition.

To conclude, this chapter showed the role of nature-mimicking catalytic systems, using noncanonical DNA G-quadruplex structures as native G-quartets, or synthetic G-quartets with TASQ. Even if the efficiency of these systems is for now not as high as the natural *horseradish peroxidase*, it is weighted against plenty of advantages in terms of applications, experimental conditions, versatility, and chemical modifications. Step by step, the scientific community puts new bricks in the wall and paves the way to more efficient nature-mimicking catalytic systems, closer and closer to what nature is able to do.

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