Thrombin Binding Aptamer, more than a simple aptamer: Chemically modified derivatives and biomedical applications

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ABSTRACT

The thrombin binding aptamer (TBA) is a well characterized chair-like, antiparallel quadruplex structure that binds specifically to thrombin at nanomolar concentrations and therefore it has interesting anticoagulant properties. In this article we review the research involved in the development of new TBA derivatives with improved anticoagulant properties as well as the use of the TBA as a model compound for the study of quadruplex structures. Specifically, we describe the impact of modified nucleosides and non-natural backbones in the guanine tetrads or in the loops and the introduction of pendant groups at the 3' or 5'-ends. The modified oligonucleotides are shown to be excellent tools for the understanding of the molecular structure of the TBA and its folding properties. Finally, we review the use of the TBA-Thrombin recognition system for the development of analytical tools based on the TBA folding.

Introduction

Aptamers are oligonucleotides that were originally derived from an *in vitro* selection and polymerase chain reaction process known as SELEX (systematic evolution of ligands by exponential enrichment) [1-4] which selects them on the basis of their specific and tight binding affinity to a target of choice from a library of sequences including proteins. Through this approach, a large number of aptamers with very high affinity have been developed for diagnostics, therapeutics and other technical applications [5], but there is still room for improvement in terms of increasing their binding properties and their pharmacokinetic properties [6].

The thrombin binding aptamer (TBA) is the first example of a potential nucleic acid therapeutic agent, targeted to a protein that does not physiologically bind nucleic acids with the following consensus sequence: 5'-$G^1G^2T^3T^4G^5G^6T^7G^8T^9G^{10}G^{11}T^{12}T^{13}G^{14}G^{15}$-3' [7]. This 15-base-long oligonucleotide binds specifically to thrombin at 10 nM concentrations and therefore, it has interesting anticoagulant properties. It inhibits specifically clot-bound thrombin and reduces arterial thrombus formation. In addition, it does not compete with other known active site inhibitors of thrombin [7-10]. Nevertheless, TBA binding to other serum proteins or proteolytic enzymes is essentially undetectable.

In an effort to identify the region of thrombin with which the TBA aptamer interacts, the inhibition of fibrinogen-clotting activity was studied using recombinant mutagenesis of anion-binding exosite of thrombin (exosite I) [10]. The results suggested that the single-stranded DNA binding site is located in the thrombin exosite I and overlaps the thrombin platelet receptor and thrombomodulin binding sites. The TBA binding site on thrombin was also examined by solid-phase plate binding assays [11] and by chemical modifications studies [12]. These studies showed that the TBA aptamer binds specifically to α-thrombin but not to γ-thrombin, which is a proteolytic cleavage product of α-thrombin in the fibrinogen-binding exosite. Both results suggest again that the thrombin exosite I is important for the aptamer-thrombin interaction.

The awareness of the folded structure of this aptamer, both free in solution or bound to thrombin, is essential to understand its biological activity and useful in the future development of oligonucleotide-based therapeutics or drug design. The TBA has been characterized by NMR spectroscopy [12-15] and X-ray crystallography [16-18]. These studies have led to the description of its compact and symmetrical chair-like, unimolecular antiparallel quadruplex structure. This structure consists of two G-tetrads connected by three edge-wise loops: two TT loops (T^3T^4 and $T^{12}T^{13}$) at one end and a single $T^7G^8T^9$ loop

in the other end (Figure 1A). The conformational distribution of the four co-planar 2'-deoxyguanosines in the G-quartets of the TBA aptamer is well defined and they are stabilized by cyclic Hoogsteen hydrogen bounding (Figure 1B). All sugar puckers are predominantly *South (S)* while the guanines on the same G-quartet plane display alternating 5'-*syn-anti-syn-anti-3'* conformations with respect to the glycosyl torsion angle (*syn*-G at positions G^1, G^5, G^{10} and G^{14}; *anti*-G at positions G^2, G^6, G^{11} and G^{15}, Figure 1), except for the G^8 and the thymines in the loops which are all *anti*. The two TT loops, both at one end of the quadruplex, span a narrow groove, while the TGT loop, placed at the other end, spans a wide groove.

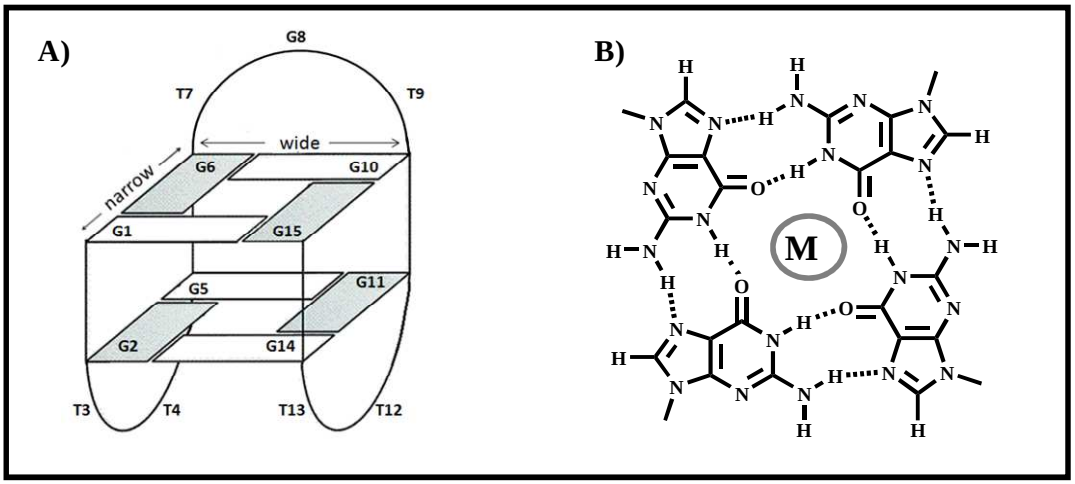

Figure 1: A) Folding topology of the intramolecular quadruplex adopted by the d($G^1G^2T^3T^4G^5G^6T^7G^8T^9G^{10}G^{11}T^{12}T^{13}G^{14}G^{15}$) thrombin-binding DNA aptamer containing three edge-wise loops. B) Structure of the G-quartet with cyclic array of four guanines formed by Hoogsteen-type H-bonds, M indicates a metal ion.

It has been known for several years that not only the primary nucleotide sequence, but also environmental conditions and in particular cations, play an important role in the formation, topology and stability of G-quadruplexes [19-25]. In the case of the thrombin binding aptamer, it was believed that the presence of K^+ in the medium was necessary to shift the equilibrium toward the quadruplex conformation, subsequently favouring thrombin binding, its ionic size fitting into the free space existing in the center of each quartet. Preliminary studies with Mn^{2+} suggested that it can bind strongly in two sites with one in the each narrow groove [26]. Both Mn^{2+} ions are released when the aptamer is complexed with thrombin, indicating that both narrow grooves are involved in the TBA-thrombin interactions. Some authors have used a combination of temperature-dependent UV spectroscopy, calorimetry, NMR and electrospray ionization mass spectrometry techniques to evaluate the effect in the stability, hydration and

thermodynamics of the monovalent and divalent metal ions in the formation of 3D structures of the TBA complexes [26-34]. Divalent ions (Pb^{2+}, Ba^{2+} and Sr^{2+}) and NH_4^+ bind and stabilize the quadruplex structure with even higher efficiency than K^+ while Li^+, Na^+, Cs^+, Mg^{2+} and Ca^{2+} form weaker complexes only at very low temperatures. These results have been rationalized in terms of their radii; cations with an ionic radius in the range 1.3-1.5 Å fit well within the two G-quartets of the complex while the other cations do not. The divalent cations like Pb^{2+}, Ba^{2+} and Sr^{2+} efficiently occupy the region between the two quartets in the TBA-ion complex in a 1:1 stoichiometry [27, 29, 33]. The aptamer complex with monovalent and divalent ions unfolds in a monophasic transition [30].

Hong *et al.* have determined the alkali metal binding site and constant by electrospray ionization (ESI) and infrared multiphoton dissociation (IRMPD) respectively [35]. The binding constant of potassium is 5-8 times greater than those for other alkali metal ions and the K^+ binding site is different from other metal binding sites. In a 1:1 TBA-metal complex, potassium coordinated between the bottom G-quartet and the two adjacent TT loops of the TBA. In a 1:2 ratio TBA-metal complex, the second potassium ion binds at the distant TGT loop. In the other hand, Na^+, Rb^+ and Cs^+ bind at the lateral TGT loop in both 1:1 and 1:2 complexes, presumably due to the formation of ion-pair adducts.

By contrast, some published works provide evidence that the TBA is able to bind thrombin in the absence of divalent and monovalent ions [24, 25, 36-37], which suggests that the binding to thrombin promotes the TBA folding to its 3D structure, even in the absence of salts.

Several groups have studied and suggested that molecular crowding causes a structural transition from an antiparallel to a parallel DNA G-quadruplex and they are an important factor to control the formation of G-quadruplex [38-39]. Miyoshi *et al*. have shown that different molecular crowding promotes and stabilizes the G-quadruplex structure of the TBA by a favourable enthalpic contribution that exceeds an unfavourable entropic contribution. Moreover, the thermodynamic effect correlates with the number of hydroxyl groups of the molecular crowding cosolute [39].

It is worth noting that despite the robust stability of the intramolecular quadruplex structure, alternative intermolecular quadruplexes are possible at high aptamer concentration, as detected by CD and electrophoresis migration experiments [40]. The crystal structure of the TBA-thrombin complex solved by Padmanabhan *et al*. [16] at 2.9 Å resolution differs in the aptamer quadruplex topology with the NMR structure. Indeed, the core of the two G-tetrads is the same in the two models, although structural differences exist in the way the central bases are connected. A difference concerning the disposition of the

two TT and the TGT loops with respect to the grooves. In an effort to resolve this ambiguity, the structure of the TBA-thrombin complex has been determined at 2.8 Á, built on the basis of the NMR structure of the aptamer [17]. The results confirmed that both structures fit the crystallographic data equally well, thus leaving the doubt on which binding model is the correct one. In both models, the TBA is sandwiched between two symmetry-related thrombin molecules and interacts with the exosite I of a thrombin molecule and exosite II of the second one. In particular, the two TT loops in the NMR structure interact with the fibrinogen-recognition site (exosite I) of the thrombin molecule and the TGT loop interacts with the heparin-binding site (exosite II) of the neighbouring thrombin, whereas in the X-ray structure the opposite occurs [16-17]. The structure of the complex between thrombin and TBA is shown in Figure 2.

The uncertainty between the two models was caused by the absence or poor electron density in the region of the TT loops and in the G^{10} for the X-ray structure and in the G^{14} and in the TGT loop for the NMR structure. In a more systematic analysis [18], eight orientations of the NMR aptamer were evaluated in an effort to reconcile the NMR and X-ray data [16-17]. The resulting crystallographic R-factors and the analysis of the aptamer-protein complexes clearly distinguished between the two possible oligonucleotides backbone directionalities. However, due to the missing density in the connecting loops of the aptamer, the details of the ligand protein interactions could not be properly addressed. Moreover, even recent papers still discuss modified aptamer-thrombin interactions on the bases of both models [39].

The recent solved high resolution structure of the complex of thrombin with a modified TBA (mTBA), which contains a 5'-5' inversion between T^3 and T^4, clarifies several questions regarding thrombin-aptamer interaction [41]. The aptamer tightly binds to thrombin exosite I by its TT loops, through a mix of hydrophobic and polar interactions in agreement with the results obtained in the systematic analysis [18]. However, the interaction details are different for the two aptamers due to the chain inversion of the mTBA. This chain inversion allows the formation of a great number of contacts with the enzyme and lead to an increase in shape complementarity. In addition, the quadruplex structure is efficiently stabilized by a potassium ion, which is sandwiched between the two quartets.

The antiparallel quadruplex structure of the TBA has a distinctive denaturation-renaturation profile that is reversible and observable by different techniques, particularly by NMR experiments, which suggest that the denaturation of the quadruplex occurs by the opening of the G-G base pairs that are not protected by a loop, followed by the opening of the TGT loop [42].

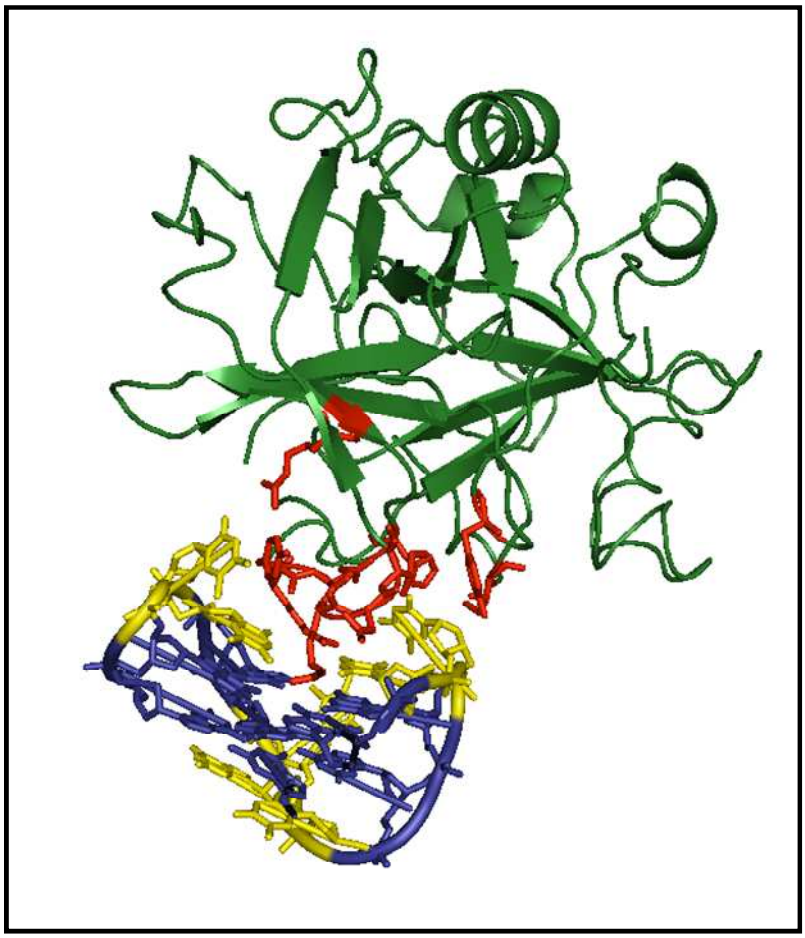

Figure 2. Structure of the complex formed by thrombin and the thrombin binding aptamer (TBA). Thrombin is coloured in green with the amino acid residues that contact the TBA in red (cartoon representation). The TBA is coloured in blue except the residues involved in the TT and TGT loops that are highlighted in yellow (stick representation).

1. Clinical trials with the thrombin-binding aptamer

The anticoagulant properties of TBA were first evaluated on cynomolgus monkeys, and sheeps [8]. The rapid onset and the short half-life of TBA ($t_{1/2}$: 2 min) *in vivo* lead to an interest for the use of TBA for certain acute clinical settings such as surgical interventions where regional anticoagulation is required. TBA was able to inhibit clot-bound thrombin and platelet thrombus formation in an ex vivo whole-artery angioplasty model [9]. Moreover, when TBA was administered by infusion in a short-term canine cardiopulmonary bypass model it was shown that TBA could be used as anticoagulant safely and as efficiently as heparine [43]. Clinical trials evaluating TBA (ARC-183, HD1, Archemix Corporation) as anticoagulant during coronary artery bypass graft surgery were halted after phase I due to suboptimal dosing profiles, primarily caused by the restricted binding affinity of the aptamer [44]. A more potent

second-generation DNA aptamer (NU172, Archemix Coorp., Nuvelo Inc.) was developed showing clear inhibition of clot formation [45].

2. Modifications on the thrombin-binding aptamer

In recent years, several attempts to improve pharmacological properties of the TBA have been described, such as stability, higher thrombin affinity, longer life time in vivo etc. These modifications have included substitutions in the nucleosides [46], LNA [47-48], UNA [49], RNA [50-51] or 2'-*O*-methyl-RNA [50, 52], methylphosphonate or phosphorothioate internucleoside linkages [50, 52], partial inversion of the TBA polarity with and 5'-5' internucleoside linkage or change in the loop size and sequence [28]. In some cases, the modifications introduced are evaluated in different positions of the aptamer in order to increase the knowledge of the interactions between thrombin and the TBA which are critical for the biological activity. Besides these modifications, thrombin binding aptamer has been functionalized with different derivatives such as fluorescein, biotin or thiol groups to be incorporated in biosensors for the detection of thrombin. These derivatives will be described in section 7. Herein, we have classified the modifications of the TBA depending on the location: G-tetrads, loops or changes of the overall quadruplex structure.

2.1 Modifications of the guanine tetrad

Several modifications have been introduced in the G-tetrads, some of which are analogues of the guanine base. Other modifications are related with the sugar structure or with the internucleotide phosphate bonds in the guanine tetrad. The guanine analogues that have been introduced in the TBA are summarized in figure 3. Hypoxanthine, 7-deazaguanine and C^8-methylguanine were the first guanine analogues to be introduced into the TBA to understand its structure by NMR. These guanine derivatives are unable to form the hydrogen bond required for the formation of the G-tetrad and consequently cause significant disruption to the chair-like structure [12]. He and co-workers studied the N^2 and C^8-alkyl substituted of the G residues forming G-tetrads [53]. These positions are not forming the H-bonding of the tetrads and are available for attaching one or more groups pointing away from the chair-like structure. This is the main reason why these substitutions caused relatively small perturbation on the quadruplex structure. However, they can produce different effects on the thrombin activity. The increased activities for the substitutions on C^8 positions may be explained by the stabilization of *syn* conformation of the G

residues, while the increased activities for the substitutions on N^2 positions may be due to the interaction with thrombin.

6-Thioguanine reduced the quadruplex formation due to the increased radius and decreased electronegativity of the sulphur [54]. This modification caused a destabilization of the Hoogsteen hydrogen bonding of guanine tetrads. Moreover, the thiol group at position 6 disrupted the interactions with water molecules and with cations, becoming a weaker hydrogen bond acceptor than the oxo group. 8-Aminoguanine did not significantly alter the structure of the TBA quadruplex but it has a small destabilization effect on the TBA quadruplex. A detailed study of this modification in the G^2 position was carried out by molecular dynamics simulations, NMR, UV spectroscopy and circular dichroism. The presence of 8-aminoguanine did not affect hydrogen bonding or purine-ion interaction, but clearly reduced the strength of stacking interactions [55]. Nallagatla *et al.* prepared a library of all possible substitutions of guanine by isoguanine in the TBA by split and mix synthesis [56]. The library was screened for binding to human thrombin and selected sequences were individually resynthesized and their affinities were assayed by isothermal titration calorimetry. Three modified aptamers carrying one single isoguanine were found to have higher binding affinity for thrombin than the unmodified TBA. The thermal stability of these modified TBAs was not analysed although it is presumed that the effect of the modification will depend on the position of the aptamer.

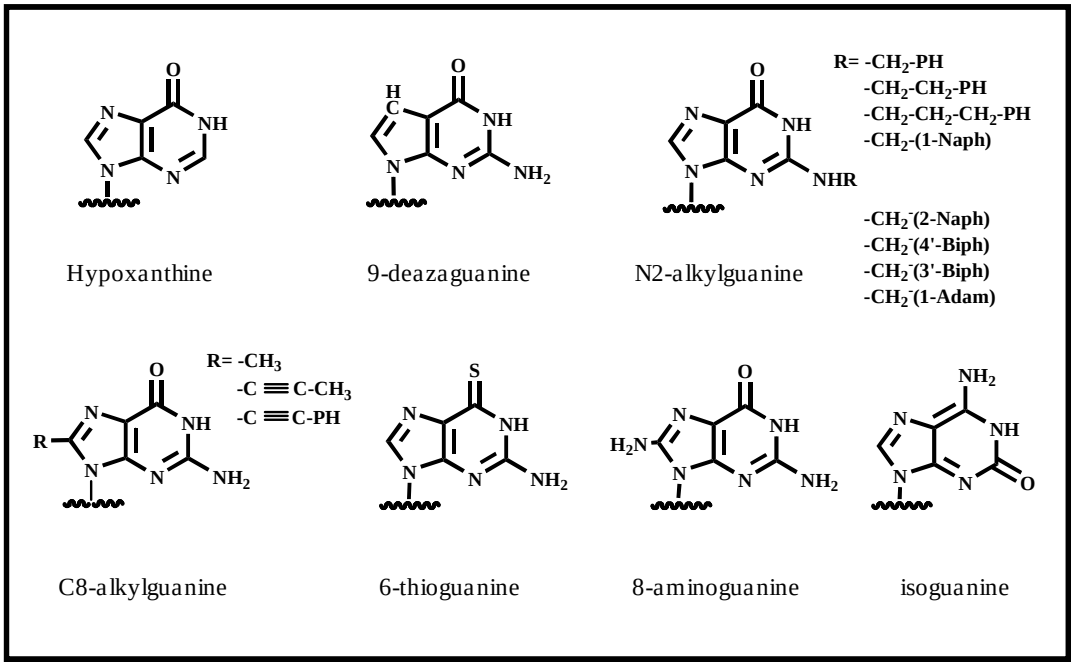

Figure 3: Chemical structure of the modified guanines in the TBA tetrads.

Finally, the effect of adding a third tetrad on the TBA aptamer has also been explored [28]. This modified thrombin binding aptamer is more stable than the native TBA due to the enthalpic contribution of the extra guanine tetrad.

2.2 Modification of the 2'-deoxyribose of the guanine tetrad

Several modifications in the TBA have been applied to the sugar moiety of the guanine tetrad. The structures of the modified 2'-deoxyribose incorporated in the TBA are summarized in figure 4. In some of them, the 2'-deoxyribose moiety was replaced by a non carbohydrate structure. On the contrary, other modifications are based on the addition of different groups in the 2'-position. These groups may influence sugar puckering and glycosidic bond conformation of the G-tetrad.

In a very interesting work, Shafer's group [51] examined the influence of individual nucleoside conformation on the overall folding topology by selective replacement of deoxyG by riboG. The unimolecular antiparallel TBA is reversed to bimolecular parallel quadruplex by a specific ribonucleotides substitutions. The parallel quadruplex conformation implies that all nucleosides are in the *anti* conformation. The strong preference of guanine ribonucleosides for the *anti* conformation is the driving force for the change in topology and also impact in quadruplex molecularity.

The denaturation behaviour of the TBA derivatives carrying ribonucleotides was also described by Mergny's group [50]. The authors prepared a TBA analogue with all the G of the two tetrads replaced by riboG. In this case, the modified TBA presented a complex behaviour with a non-superimposable and multi-phasic response upon heating and cooling (hysteresis). The same authors also described the same TBA analogues with 2'-O-Me guanosine substitutions. This analogue, instead, showed reversible transitions with concentration independent of the melting temperature (Tm). In fact, substitution of the ribose 2'-H with a methoxy group destabilized the quadruplex structure.

LNA are 2'-O-4'-C-methylene-linked ribonucleotide nucleic acids analogues that bind with increased affinity to DNA and RNA. The bicyclic structure of DNA forces the sugar to be in the C3'-*endo* conformation, and nucleotides with a C3'-*endo* conformation prefer the glycosidic bond to be in the *anti* configuration. Three different works were addressed to study the effect of LNA in the TBA quadruplex [47-48, 57] . Mayol's group prepared four different TBA-based oligonucleotides containing LNA residues [47, 57]. The first analogue was fully substituted by LNA residues. This oligonucleotide was unstructured most probably due to the decreased flexibility of the oligomer. Oligonucleotides containing

G-LNA in the eight positions of the tetrads or in the first G^1 position (*syn* configuration) gave mixtures of several structures. On the other hand, the oligonucleotide containing G-LNA in the last guanine G^{15} (*anti* configuration) folded in the same TBA chair-like quadruplex. Bonifacio *et al.* also studied the effect of single LNA substitutions on different positions of the TBA [48]. The LNA substitutions had either a moderate stabilizing or destabilizing effect on the folded structure, depending on the position of the LNA in the TBA. The thermal stability of the substituted aptamers did not correlate to thrombin inhibition.

Damha and co-workers studied the impact of 2'-deoxy-2-fluoroarabinonucleoside residues (2'-F-araN) on the thrombin binding aptamer [58]. 2'-Deoxy-2'-fluoro-D-arabinonucleic acids (2'F-araN) confer DNA-like (*South*/*East*) conformation to oligonucleotides while rendering them more nuclease resistant. It was found that incorporation of 2'-F-araN G or T residues into the TBA stabilizes the complex (ΔTm + 3ºC /2'-F-araN modification). Oligonucleotides with all nucleotides replaced by 2'-F-araN in the G-*syn* positions or in the G-tetrads showed a moderated increase of the melting temperature compared to the unmodified TBA. The CD spectrum and the hysteresis observed in the heating and cooling processes of these analogues supported a parallel structure with all *anti*-dG and the existence of multimeric G-quadruplex structures. On the contrary, when the 2'-F-araN are replaced in the G-*anti* positions, in the loops or in both the resulted quadruplex structures correspond to antiparallel quadruplex with alternating *syn*-*anti* Gs. The lack of concentration dependence in the Tm data and the lack of hysteresis in the heating/cooling processes support a unimolecular G-quadruplex structure. Moreover, nuclease resistance of this modified TBA was increased up to 48-fold in 10 % fetal bovine serum (FBS).

Carbacyclic bicyclo [3.1.0] hexane locked nucleoside analogues are a different "locked" nucleoside from the previously presented LNA [52]. An advantage of this methanocarba nucleoside system over LNAs is that both *North (N)*- and *South (S)*-locked platforms can be prepared by shifting the position of the fused cyclopropane ring. It has been described the effects of replacing a single 2'-deoxyguanosine residue at the 3'-end of the TBA (positions dG^{14} and dG^{15}) with methanocarba nucleosides locked in either the *N*- or *S*-conformation [59]. These positions were selected to explore the combined effects of a constrained sugar pucker (*N* or *S*) and the corresponding biased glycosyl torsion angle (*anti* or *syn*) associated with a particular pseudosugar conformation. Experimental and theoretical results indicated that a *N*-pseudosugar conformation favours the *anti* glycosyl orientation, whereas the *S*-pseudosugar conformation favours the *syn* disposition of the base. The introduction of methanocarba nucleosides at positions G^{14} and G^{15} with locked-*N*(*anti*) and locked-*S* (*syn*) conformations fixed the

conformational state of these nucleosides and helped to understand the impact of conformational restrictions on the antiparallel, G-quartet DNA structure of the TBA. These results indicated that the glycosyl conformation is more restrictive for the TBA stability than the sugar puckering.

Wengel's group examined the influence of unlocked nucleic acid (UNA) on the thermodynamic stability, binding affinity and biological activity of the quadruplex TBA [49]. UNA is an acyclic RNA mimic, which misses the bound between the C2' and C3' atoms of the ribose ring. The modified variants are aptamers singly substituted with a UNA monomer in every possible position. UNA modified TBAs in positions U^3, U^7 and U^{12} showed an antiparallel folding topology. In contrast, modifications of any of the guanine monomers forming G-tetrads resulted in significant destabilization of the quadruplex structure. The modified TBA with UNA in position 7 resulted in the highest thrombin binding affinity. Recently, the same authors have also evaluated the effects of the modification of 2'-C-piperazino-UNA monomer [60]. This monomer is characterized by more efficient stabilization of quadruplexes structures in comparison to regular UNA and increases thermodynamic stability of TBA by 0.28-0.44 kcal/mol in a position depending manner with retained quadruplex topology and molecularity.

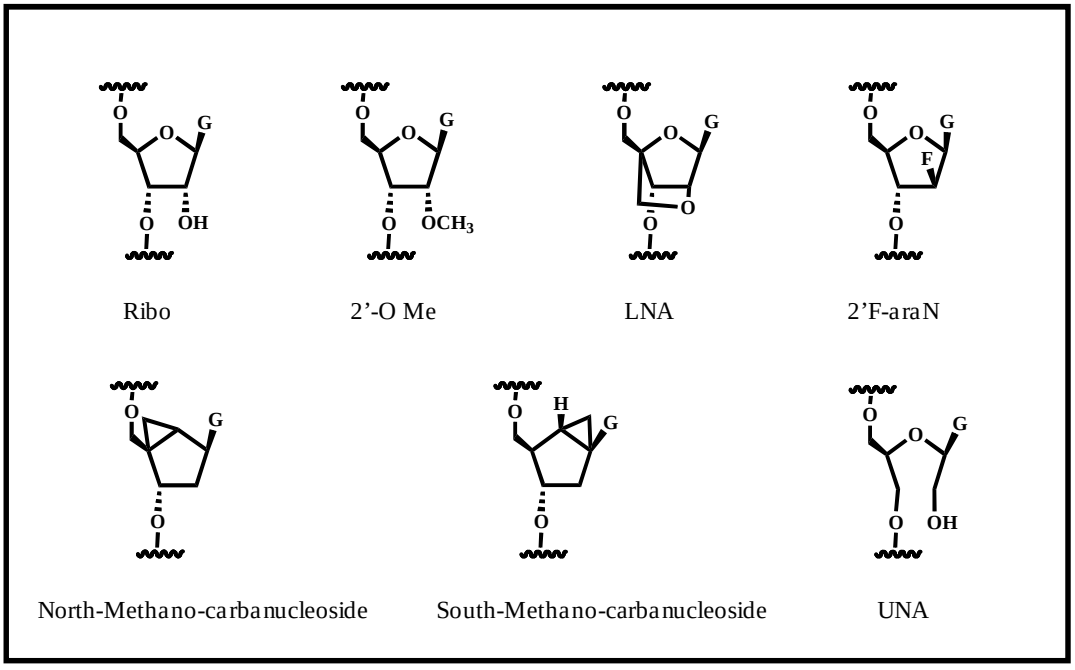

Figure 4: Chemical structure of the modified carbohydrate moiety in the guanine TBA tetrads. LNA: locked nucleic acid, UNA: unlocked nucleic acid.

2.3 Modifications in the internucleotide phosphates of the guanosine tetrad

The TBA has been modified with three different phosphate linkers, phosphorothioate, methylphosphonate and formacetal. The structures of these linkages are summarized in figure 5. The modified TBA oligonucleotides containing thiophosphoryl substitutions at different internucleotide sites were studied. It was found that these linkages do not disrupt the antiparallel ntramolecular quadruplex [52]. The substitutions placed between planes of G-quartets led to a drop in formation free energy, and the stability decreases linearly with the number of these modifications. The TBA containing phosphorothioate linkages have more resistance to various nucleases. In this way, the *in vivo* half-life of the modified TBAs are increased. Mergny's group also studied the introduction of phosphorothioate bonds in all of G-forming tetrads [50]. The resulting modified TBA was less stable than the unmodified TBA. It also had an intramolecular G-quadruplex structure with concentration independent melting temperatures showing a reversible quadruplexes to random coil transitions. The same group studied the modified TBA carrying backbone methylphosphonate in the two G- tetrads [50]. The methylphosphonate TBA variant suffered a loss of negative charge at the level of the phosphate backbone that led to a strong destabilization. Non observable melting transition was detected. The negative charge of the oxygen atoms in the phosphate groups was found to be involved in a complex pattern of water bridges with the sugar group and the edges of the guanine units. A series of TBA analogues were synthesized containing one or more phosphodiester linkages replaced by a natural formacetal group [61]. The formacetal group is achiral and the incorporation of these moieties into oligonucleotides decreased the tissue uptake and increased the *in vivo* half-life. Unfortunately, no structural studies were carried out with these TBA analogues.

In summary, several G-tetrad modifications of the TBA have been studied. Some of them have destabilized or disrupted the quadruplex structure because the introduced modification have changed directly the H-bonding tetrad arrangement, sugar puckering or glycosidic guanine orientation. In addition, some modifications have destabilized the antiparallel quadruplex to form a more undefined multimer quadruplex structures.

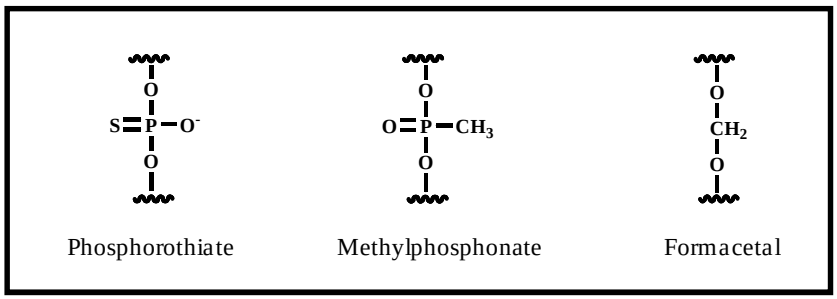

Figure 5: Chemical structure of the modified internucleotide phosphate bonds in the guanine TBA tetrads.

3. Modification of the loops

The TBA is formed by two guanine tetrads connected by three edge-wise-loops: a central TGT loop and two TT loops as it is shown in the Figure 1A. The loops are important in the folding of the aptamer and in the interactions with the thrombin. Most of the modifications are based in changes of the composition or in the length of the loops. Thymine bases in the loop region exclusively prefer the *anti* orientation. The literature reports that G^8 shows base stacking interactions with the first tetrad, but the conformation of the nucleoside is not mentioned.

3.1 Modifications of the loop composition by natural nucleosides

Shafer's group has undertaken a systematic examination of the thermodynamic stability of thrombin aptamer analogues containing sequence modifications in one or more loops [28]. The results indicated that changes in loop sequences had a significant impact on the aptamer stability. Most of the changes in the central loop led to a decrease in thermodynamic stability, indicating that, at least among the sequences explored, the TGT loop sequence is optimal for stability. These effects may involve changes in both stacking interactions and cation binding. The impact of replacing single Ts in the external loops with Cs provide evidence for hydrogen bond formation between these loops, as observed in the NMR structures. The stability of aptamers containing a C in position 3 or 12 was similar or slightly higher than the unmodified TBA. A recent paper has examined the stability of the G-quadruplex of TBA in which thymine residues were substituted by adenine. The G-quadruplexes formed by T^4A and $T^{13}A$ were more stable and T^3A, T^7A, T^9A and $T^{12}A$ were more unstable than that of the wild-type [62].

3.2 Modifications of the loop composition by non-natural nucleosides

Replacement of *anti* thymines in the loops with *anti* conformationally biased 2'-F-araT increased the thermal stability to different degrees depending on the number and position of the modification. In addition, the modification of G^8 with 2'-F-araG resulted in an increase of the stability. Overall, 2'-F-araN modifications in the loops stabilizes the formation of a unimolecular G-quadruplex [58]. LNA substitutions in the loops demonstrate a position dependent effect on the stability of the TBA. The substitution of G^8 for G-LNA increased the stability, but the substitution of T^7 decreased the stability. Nevertheless, substitution of T^4 disrupted the aptamer [48]. On the other hand, single UNA modification

of the TBA in U^3, U^7 and U^{12} positions did not affect the stability of the unimolecular antiparallel structure. However, these same modifications in positions U^4, G^8, U^9 and U^{13} resulted in significant destabilization of the quadruplex structure [49].

Borbone's group modified the different positions of the TBA with flexible acyclic thymidines [63]. They obtained the same pattern of thermodynamic stabilities than UNA modifications. These analogues were able to fold into a bimolecular or monomolecular quadruplex structure depending on the nature of the monovalent cations (sodium or potassium) coordinated in the quadruplex core. Thermal stability was in agreement with the structural model in which T^9, T^4 and T^{13} are stacked on the adjacent G-quartet. These interactions were totally or partially disrupted by the introduction of the acyclic nucleotide at these positions. The TBA analogues containing an acyclic residue at positions T^3, T^7 or T^{12} resulted in a similar stability than the observed for the unmodified TBA, thus suggesting a marginal role of these positions on the structural stability [63].

Modifications of the TBA loops by thiophosphoryl internucleotide bonds were evaluated [52]. No destabilization was observed in each of the loop regions, although the stability against nuclease was increased in comparison to that of the native TBA. Finally, a new TBA aptamer modified with 4-thio-2'-deoxyuridines replacing some Ts in the loops was described [64]. This substitution was based on previous experiments showing that oligonucleotides with 4-thio-2'-deoxyuridines showed high-affinity binding to HIV-1 reverse transcriptase [65]. No thermodynamic data were performed but TBA modified with 4-thio-2'-deoxyuridines has an increased anticoagulant and antithrombotic properties [64].

3.3 Modification of the loop length

Loop length plays an important role in intramolecular quadruplex formation. When the central loop was replaced by four nucleotides, the resulting aptamer had a lower stability compared to unmodified TBA [28]. The thermodynamic analysis indicated that the central loop sequence in the parent aptamer is optimal for stability. Reduction of the two external TT loops to a single T led to a complete disruption of the quadruplex structure. This was expected due to the difficulty of forming a single base loop. On the contrary, extension to TTT loops had the same stability as the unmodified TBA. Addition of a single G at the 5'-end decreased the stability of the aptamer while addition of a G at the 3'-end increased the stability [28].

4. Synthesis of different constructs based on the TBA

In an effort to select more potent and selective DNA ligands to thrombin, several authors have synthesized different constructs. Most of them have modified the TBA structure itself or others have incorporated additional structures or molecules to the TBA. The first approach was comprised of an unimolecular quadruplex motif and complementary flanking sequences capable of forming an additional Watson-Crick duplex motif [66]. After that, following the same approach, a new 29 nucleotide single stranded oligonucleotide based on a quadruplex/duplex structure was described to bind the heparin-binding exosite of thrombin [67]. Seela's group proposed a new construct arising by replacement of the TGT loop of the TBA by a mini-hairpin 5'-GCGAAGC-3'. This fused oligonucleotide exhibited a two-phase thermal transition indicating the presence of the two unaltered moieties [68].

A new interesting architecture demonstrated that the combination of bivalent TBA aptamers, which simultaneously targeted and accordingly inhibited the regulatory exosites I and II of thrombin [69]. This approach turned out to be a combination of features of the individual aptamers in one molecule: high affinity binding and anticoagulant activity. A new quadruplex structure was studied in the d($G^2T^4G^2CAG^2GT^4G^2T$) sequence, which differs from the TBA in having longer first (T^4) and third (GT^4) loop and a shorter (CA) middle loop. This oligonucleotide has different strand directionalities, loop connectivities and *syn*/*anti* G-tetrad distribution [70]. Circularization is an attractive alternative to chemical modification for improving aptamer stability. This new approach was used in the design and construction of a TBA aptamer. The new construct has increased target binding affinity and much improved stability in biological fluids [71].

Mayol and co-workers described a new topology of the TBA that consists of a series of oligonucleotides containing 3'-3' or 5'-5' inversion of polarity sites [72]. The oligonucleotide d(3'-GGT-5'-5'-TGGTGTGGTTGG-3') was characterized by an unusual folding, three strands parallel to each other and only one strand oriented in an opposite manner. This led to an *anti-anti-anti-syn* and *syn-syn-syn-anti* arrangement of the Gs in the two tetrads. The thermal stability of the modified oligonucleotide was higher than the corresponding for the unmodified TBA.

Several intercalating agents have been conjugated to the 3'-end of the TBA and they have been found to stabilize the aptamer. Moreover, the hydrophobicity and fluorescent properties may be used to enhance the bioavailability of these conjugates [73]. Finally, the capping of the 3'-end of the TBA with bridged nucleosides was described. The bridged nucleosides increased the nuclease resistance 36-27 fold

and the stability in serum 1.5-4 fold without affecting the binding affinities of the aptamers to thrombin [74].

5. Binding activity of modified thrombin binding aptamers

The reported bibliography concerning the modified TBAs might give an insight into the variables involved in the mode of action of the TBA. Nevertheless, the mode of action of the TBAs actually requires a more wide recognition process that involves even locally a single residue. Several assays are described to study the binding or interaction of the modified TBAs to thrombin such as nitrocellulose filter binding assay [58], isothermal tritation calorimetry [53, 56, 62, 75], surface plasmon resonance [49] or by non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) [74]. Recently, the interaction of the TBA with thrombin was evaluated by differential pulse voltammetry at a glassy carbon electrode and atomic force microscopy at a highly oriented pyrolytic graphite electrode [76].

Thrombin binding affinity of the modified TBAs in the tetrads was studied by different researchers. The thrombin binding of a TBA containing 2'-deoxyinosine or 7-deaza-2'-deoxyguanosine was significantly decreased as the residues were unable to form the hydrogen bonds required for the formation of the G-tetrad [77]. On the contrary, the TBA containing isoguanine showed an enhanced binding activity to human α-thrombin compared to the unmodified TBA determined by isothermal titration calorimetry [56]. The effect of 2'-F-araN modifications was conducted using nitrocellulose filter binding assay. The binding of 2'-F-araN aptamers to thrombin was always adversely affected when the modification was on G tetrads themselves. Some loop modifications with 2'-F-araN also reduced thrombin binding. However, the two loop modified aptamers in positions 7, 9, 12, 13 or 3, 4 , 7, 9 showed a 4-5 fold enhancement in thrombin binding affinity [58].

On the other hand, real-time measurements of the interaction between thrombin and the TBA containing UNA modifications were performed by surface plasmon resonance. The modification of the G^1 position showed similar affinities to the unmodified TBA. The rest of the Gs involved in the tetrads showed a higher dissociation constant or were not measurable, presumably due to a lack of significant affinity towards thrombin after the incorporation of UNA in these positions. The U^7 UNA modification located in the central loop was the only UNA modified aptamer that showed small but significant

improvement in affinity [49]. Similar results were obtained with 2'-C-piperazino-UNA-U monomer, but in this case the presence of a positively charge decreased the thrombin affinity [60].

Another interesting work has focused on the replacement of thymine loop residues by adenine. Isothermal titration calorimetry (ITC) measurements indicated that the binding constant of the interactions between $T^{13}A$, $T^{7}A$, $T^{9}A$ and $T^{12}A$ aptamers and thrombin was close to that of the unmodified TBA, whereas $T^{13}A$ was significantly lower and $T^{4}A$ did not appear to bind thrombin [62]. The binding energy of the modified TBA containing a 5'-5'-site of polarity inversion to thrombin was characterized by means of ITC. The equilibrium constant for the interaction of the modified TBA was about one order of magnitude higher than that for the TBA. The binding process was enthalpically driven with a larger favorable enthalpy for the modified aptamer [75]. The construct formed by a quadruplex core of the TBA and a duplex interacted with a 20 to 50 –fold higher affinity to the heparin-binding exosite than the unmodified TBA by nitrocellulose filter [67]. Finally, thrombin binding affinities of capped TBAs with 2',4'-bridged nucleotides were measured using non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) [74]. The binding abilities were almost the same level than the native TBA.

According to the different strategies used to measure the thrombin binding, we can conclude that no important binding changes are observed when the modification does not disrupt the quadruplex structure. In addition, some modifications in the loops or in the overall construct structure do not affect or increase the binding affinity.

6. Thrombin inhibition by modified thrombin binding aptamers

Prothrombin Time (PT) is the more used assay to study the inhibition of thrombin. PT assay is a routine diagnostic assay that evaluates *in vitro* the activation of extrinsic pathway of the coagulation cascade. This ultimately measures the conversion of fibrinogen in fibrin by thrombin, with the consequent formation of a solid gel clot. When this assay is performed in presence of the aptamer TBA, the binding of fibrinogen to the thrombin is inhibited and a longer time is required to form a clot. Moreover, other important assays are fibrinopeptide A release assay, platelet aggregation and thrombus growth.

The effects of the substitutions at N^{2} and C^{8} of the G residues which form the G-tetrad on the thrombin inhibitory activity measured by PT were relatively small. The introduction of a benzyl group into N^{2} of G^{6} and G^{11} and naphthylmethyl groups into N^{2} of G^{6} increased the thrombin inhibitory activity, whereas other substituents in these positions had almost no effect or decreased the activity. Particularly,

the oligonucleotides carrying a 1–naphthylmethyl group in the N2 position of G^6 showed an increase in activity by about 60% *in vitro* and *in vivo.* The introduction of a relatively small group such as methyl and propynyl, into the C8 positions of G^1, G^5, G^{10} and G^{14} increased the activity, presumably due to the stabilization of the quadruplex, whereas the introduction of a large substituent group, decreased the activity, probably due to steric hindrance [53]. The 2'-C-piperazino-UNA-U monomer modification showed an unfavorable impact of the piperazino moiety on the inhibition with thrombin [60]. The biological effect of the UNA-modified TBAs was tested in a prothrombin time assay. The TBA modified with UNA-U^7 showed an increased inhibitory effect relative to the unmodified TBA, while inhibition of coagulation by G^1, U^3, G^8, U^9 and U^{12} was near two fold decreased, and U^4, U^{13} and G^{15} showed no influence on fibrin-clot formation [49].

The TBA modified with LNA showed a different thrombin inhibition according the position of the modification. Stable aptamers with LNA in positions G^5, T^7 or G^8 showed a decreased thrombin inhibition measured by fibrin clotting assay. Nevertheless, a less stable aptamer with LNA at G^2 was as active as the unmodified aptamer [48]. In addition, Mayol's group described that the TBA modified by LNA in the G^{15} position displayed a prolonged PT [47]. The modification of the phosphate linkages by formacetal [61] or thiophosphoryl [52] groups exhibited a similar prothrombin time to the one found for the unmodified TBA. The effect of the modified loops on the thrombin inhibitory activity was also studied using acyclic nucleosides. In this case, the analysis of PT assays confirmed that the highest PT value was obtained for a modified TBA containing an acyclic thymidine in position 7 [63]. On the other hand, the TBA modified with four 4-thiodeoxyuridine showed a 2-fold increased inhibition of thrombin catalyzed fibrin clot formation, fibrinopeptide A release and thrombus formation [46].

The structural changes in the overall structure that have been described do not seem to affect too much the thrombin inhibition [75]. The TBA containing a 5'-5' inversion of polarity site affected sensibly the biological inhibition. Cook and co-workers presented a series of constrained unimolecular quadruplex/duplex molecules with increased thrombin inhibition using clot formation assay and release of fibrinopeptide A [66]. Moreover, Steiner also described a quadruplex/duplex molecule construct that binds the heparin-binding exosite with 20-50 fold higher affinity measured by clotting time [67].

Finally, the new construct assembled by two distinct aptamers that targets thrombin combines features of the individual aptamer subdomains with enhanced activities regarding both functionalities; these are probably due to an enhanced affinity of the bivalent fusion aptamer. This structure displayed

enhanced anticoagulant activity when compared to the TBA, however, affinities were improved only two to three fold compared to those of the individual precursors [69].

Similar conclusions could be obtained for the thrombin inhibition of the modified TBA. It is important to mention that T^7 position seems very sensitive to different modifications in terms of increasing the thrombin inhibition. Moreover, the addition of different constructs with improved pharmacokinetic properties to the TBA could be a reasonable idea that probably would not compromise the inhibitory activity.

7. Novel applications using thrombin binding aptamer.

In addition to the anticoagulant properties, a large number of analytical tools based on the folding and refolding of the TBA have been developed. In the following section some of these new developments are reviewed.

7.1. The TBA as model for the analysis of binding mode of drugs with affinity to G-quadruplex.

G-quadruplexes have become structures of special interest for drug development due to their possible implications in anticancer research. The potential role of G-quadruplexes has been highlighted with the development of strategies designed to stabilize telomere ends as G-quadruplex structures using specific small molecules, which can destabilize telomere maintenance in tumour cells [78]. G-quadruplexes are also found in transcriptional regulatory sequences of critical oncogenes such as *c-myc* and *c-kit* [79, 80]. Ligands that selectively bind and stabilize these structures were studied as potential anticancer drugs of interest [81]. The TBA was used as a model for the analysis of the interaction of several drugs with G-quadruplex structures. In one of the first studies, Joachimi *et al*. described the potential role of porphyrins in the modulation of the anticoagulant properties of the TBA [82]. Later, Del Toro *et al*. confirmed the formation of an interaction complex with a stoichiometry 1:1 between the porphyrin (TmPyP4) and the TBA [83]. Ultraviolet melting and circular dichroism data reflected that the initial G-quadruplex structure of the TBA was stabilized in the interaction complex: being slightly disordered by the presence of the ligand. The interaction between the porphyrin (TmPyP4) and the TBA was also studied by time-resolved fluorescence anisotropy. Based on the anisotropic decay curves, a sandwich-type binding mode was proposed in which both terminal G-quartet and T-T base pairs stack on

the porphyrin ring [84]. The interaction between the TBA and the bipyridinium salts was studied by cyclic voltammetry. A strong interaction between G-quadruplex forming DNA sequences and viologens was observed [85].

7.2. TBA as sensing element for thrombin and metal ions

The conformational change of the TBA during the folding /unfolding process was exploited for building sensors for metal ions and for detection of thrombin. This work together with the use of other aptamers as sensors has been summarized in several recent reviews [86-90]. One of the most relevant studies is the development of probes for the detection of intracellular potassium concentration [91-92]. Oligonucleotides containing the TBA sequence functionalized with a fluoresceine derivative as fluorophore and a rhodamine dye as quencher at the 3' and 5'-ends were prepared. Upon binding of potassium, the TBA probes folded in the intramolecular quadruplex. The quadruplex folding induced by potassium was observed by a decrease of fluorescence due to fluorophore-quencher interaction [91-92]. The development of quadruplex DNA-based FRET probes with special emphasis on the TBA quadruplexes were reviewed [93].

A similar FRET experiment was adapted recently for the detection of the activity of human O^6-alkylguanine-DNA alkyltransferase (hAGT) [94]. The modified TBA probe contained one O^6-methylguanine residue that prevented quadruplex formation. Upon removal of the O^6-methyl group in the guanine by hAGT, the natural TBA sequence is formed and it folds into the quadruplex, inducing a decrease of fluorescence due to fluorophore-quencher interaction [94]. A colorimetric assay for the determination of mercury (II) using the TBA was also reported [95]. The binding of mercury to the TBA induced the folding of the molecule that triggered salt-induced gold nanoparticle aggregation [95].

The folding/unfolding of the TBA can also be regulated by light. The incorporation of *o*-nitrobenzyl thymidine derivatives (caged nucleosides) in the TBA sequence did not allow the folding of the TBA, preventing thrombin binding. Photoremoval of the nitrobenzyl groups on thymidines generated the native aptamer which now is capable of binding thrombin, which prevented blood clotting [96]. Also, the effect of a photoactive nitrobenzyl group on a guanine residue of TBA has been studied using classical molecular simulations [97]. Theoretical calculations are able to describe the change in the structure when the modified residue is incorporated in the TBA as well as the formation of the quadruplex after photolysis [97]. The photodeprotection of the nitrobenzyl groups is irreversible and for this reason,

Ogazawara *et al.* [98] developed a guanine derivative carrying a fluorenylvinyl group at position 8. The fluorenylvinyl guanine derivative may undergo to *cis-trans* photoisomerization that is reversible. The *cis-trans* isomerization affected the formation of the quadruplex structure and subsequently the binding of thrombin. In this way, the binding of thrombin to the TBA derivatives carrying guanines with the fluorenylvinyl group can be reversibly modulated by light [97].

The conjugation of several derivatives of the TBA to gold nanoparticles was studied [99]. Some of the TBA-gold nanoparticles are highly efficient as anticoagulants [99]. Moreover, the functionalization of iron oxide nanoparticles with TBA has described [100]. The TBA magnetic nanoparticles conjugates showed a clear magnetic resonance imaging (MRI) signal when binding to thrombin [100].

Several electrochemical sensing platforms based on the TBA quadruplex were developed for the detection of thrombin. A label-free electronic detection system for the direct detection of thrombin based on electrochemical impedance spectroscopy was developed [101]. The TBA carrying an amino group was covalently linked to multi walled carbon nanotube disposable screen-printed carbon electrodes by amide formation and the resulting electrodes were able to sense thrombin at a detection limit of 105 pM [101]. The incorporation of ferrocene to the TBA increased the sensitivity of the detection reaching a detection limit for thrombin to 0.5 pM [102]. Conjugation of the TBA to silver nanoparticles and to gold nanoshells allowed the detection of thrombin by surface-enhanced Raman spectroscopy [103-106]. The interaction of thrombin with the TBA was also studied on quantum dots and in surface plasmon resonance [107].

The absorption and redox behavior of the TBA and the complex thrombin-TBA was evaluated by differential pulse voltammetry at a glassy carbon electrodes [76, 108]. The TBA guanine oxidation peak was found to be sensitive to G-quadruplex formation and to thrombin binding, showing a higher oxidation potential [76, 108]. Recently, the excellent binding properties of a 29-base-long thrombin-binding aptamer linked to gold nanoparticles were used for the development of a sensitive detection of DNA that relied on the modulation of the thrombin activity on the surface of the nanoparticles [109].

7.3. Single-molecule experiments on the TBA

One of the first single-molecule experiments using the TBA-thrombin interaction was performed by atomic force microscopy (AFM) [110]. An AFM gold-coated tip was functionalized with the thiolated TBA. The thrombin was linked covalently to a gold-coated glass slide. The rupture force for a single

aptamer/thrombin complex was determined as 4.45 pN. The analysis of the system revealed that the rupture forces corresponded to the melting of the G-quadruplex of the aptamer bound to the thrombin and subsequent dissociation of the complex [110].

Recently, the TBA folding and unfolding induced by ions was studied using nanopores encapsulated with single molecules. The TBA quadruplex was formed rapidly in the presence of potassium ions and had a slow unfolding reaction. The sodium and lithium complex of the TBA were similar but the folding and unfolding of the sodium complex was faster than the folding and unfolding of the lithium complex [111].

The excellent molecular recognition properties of DNA were exploited to incorporate functionalities in molecular constructs and for the design of 2-dimensional arrays with well defined structures [112-113]. A remarkable development in this field was the use of stable DNA Holliday junctions with addressable sticky ends to form two-dimensional DNA crystals [113]. The so-called DNA tile system was used for the assembly of bidimensional DNA arrays, containing thrombin binding aptamer sequences [114-115]. The DNA arrays templated the formation of ordered thrombin arrays that were visualized by AFM [114-117]. Origami DNA is a new method for the rational organisation of structures that uses a circular viral single stranded DNA (M13 DNA) and about two hundred oligonucleotides (staple strands) that are designed to fold the viral DNA into a rationally designed shape [118]. The TBA sequences were also introduced in DNA origamis, showing a nanometric control of the deposition of thrombin molecules on the origami [119-120].

Conclusions

Aptamers are a novel class of nucleic acids with affinity to proteins that may be used for therapeutic or diagnostic purposes. The thrombin-binding aptamer was one of the first aptamers developed by SELEX and probably one of the most studied aptamer. The TBA is a relatively short sequence, easy to synthesize with a well-defined structure and has a good affinity for thrombin. For all these reasons, it can be considered a paradigm of the potential applications of the aptamers. During the last 20 years, several authors have defined the structural facets of the TBA molecule exploring several potential variables such as sugar puckering, glycosidic bond conformation, H-bonding groups, backbone modifications, etc… Some of the modified TBA derivatives have a good affinity for thrombin, as well as a large stability in physiological conditions, which has led to the setting of some clinical assays. The

lessons learned in this process are important not only for the anticoagulant properties of the TBA but also to improve the understanding of G-quadruplex structures present in telomeres and some promoter regions of oncogenes.

An important development in the last years has been the conjugation of the TBA to nanomaterials such as gold and iron oxide nanoparticles, which may increase the stability in plasma as well as it may open the opportunity of adding receptor-mediated systems for efficient *in vivo* targeting. Moreover, the TBA-thrombin recognition system is already being used for the development of sensors based on both electrical and optical methods, and more recently for the DNA-templated directed assembly of nanomaterials. As the time goes on, the potential applications of this relatively simple DNA molecule are increasing exponentially. This intense activity will help to further develop the aptamer field and it may also span the knowledge about other useful nucleic acids for therapeutic or diagnostic purposes.

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LEGENDS

Figure 1: A) Folding topology of the intramolecular quadruplex adopted by the d($G^1G^2T^3T^4G^5G^6T^7G^8T^9G^{10}G^{11}T^{12}T^{13}G^{14}G^{15}$) thrombin-binding DNA aptamer containing three edge-wise loops. B) Structure of the G-quartet with cyclic array of four guanines formed by Hoogsteen-type H-bonds, M indicates a metal ion.

Figure 2. Structure of the complex formed by thrombin and the thrombin binding aptamer (TBA). Thrombin is coloured in green with the amino acid residues that contact the TBA in red (cartoon representation). The TBA is coloured in blue except the residues involved in the TT and TGT loops that are highlighted in yellow (stick representation).

Figure 3: Chemical structure of the modified guanines in the TBA tetrads.

Figure 4: Chemical structure of the modified carbohydrate moiety in the guanine TBA tetrads. LNA: locked nucleic acid, UNA: unlocked nucleic acid.

Figure 5: Chemical structure of the modified internucleotide phosphate bonds in the guanine TBA tetrads.

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