Dimeric/Multimeric Anticoagulant DNA Aptamers

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Multivalent interactions frequently occur in biological systems and typically provide higher binding affinity and selectivity in target recognition than when only monovalent interactions are operative. Thus, taking inspiration by nature, bivalent or multivalent nucleic acid aptamers recognizing a specific biological target have been extensively studied in the last decades. Indeed, oligonucleotide-based aptamers are suitable building blocks for the development of highly efficient multivalent systems since they can be easily modified and assembled exploiting proper connecting linkers of different nature. Thus, substantial research efforts have been put in the construction of dimeric/multimeric versions of effective aptamers with various degrees of success in target binding affinity or therapeutic activity enhancement. Several dimeric and multimeric DNA-based aptamers, including those forming G-quadruplex (G4) structures, were designed as anti-inflammatory, antiviral, anticoagulant, and anticancer agents and their number is certainly bound to grow in the near future. In this content, we here focus on dimeric/multimeric constructs designed as anticoagulant agents.

Keywords: aptamer ; G-quadruplex ; design ; dimerization ; multivalency ; molecular recognition ; protein target ; therapy

1. Introduction

Nucleic acid-based aptamers are short single-stranded DNA or RNA molecules which, upon folding in their peculiar three-dimensional structure, can bind with high affinity and specificity a selected target of biological interest. They are also called “chemical antibodies”, but compared to protein-based molecules, oligonucleotide aptamers generally show lower immunogenicity, higher stability in a wide range of pH and temperature and the possibility to be differently modified or conjugated. Indeed, site-specific chemical modifications can be easily inserted in oligonucleotide aptamers to improve their stability to nuclease digestion or modulate binding affinity to their target [1][2][3][4][5][6]. These intriguing properties make oligonucleotide aptamers very attractive tools in both therapeutic [2][7][8][9][10][11][12][13][14][15] and diagnostic [16][17][18][19][20][21][22] applications [23].

Starting from a large pool of random oligonucleotide sequences, high affinity aptamers for a given target are generally identified through an in vitro selection process named Systematic Evolution of Ligands by Exponential Enrichment (SELEX) [24][25]. The outstanding progress achieved in this field resulted in a variety of selection methods and a large number of aptamers specific for very different kinds of targets—from small molecules, ions, proteins, cells, to even whole organisms, such as viruses or bacteria—have been thus far fished out [26][27][28][29][30][31][32].

Moreover, several aptamers are specifically internalized upon binding to cell membrane receptors and thus can serve as ideal selective delivery systems for different therapeutic targets, from small, conventional drugs to microRNAs or small interfering RNAs (siRNAs) [10][33][34].

Notably, among combinatorially selected aptamers, most of the oligonucleotides endowed with valuable biological activity are able to adopt stem-loop or G-quadruplex (G4) structures. The simplest architecture is represented by the stem-loop or hairpin rearrangement, i.e., an intramolecular conformation based on the coupling of complementary nucleobases in a single-stranded DNA sequence [35].

In contrast, oligonucleotides featured by guanine-rich sequences generally share the ability to fold into peculiar G4 structures [36][37][38][39][40]. The central core of a G4 architecture is the G-tetrad, a structural motif also named G-quartet, which consists of a cyclic planar arrangement of four guanine bases associated through Hoogsteen-type hydrogen bonds [38][40][41][42][43]. Stacking of two or more G-tetrads provides central cavities with a strong negative electrostatic potential, in which cations can be well accommodated, strongly influencing the formation, stability, and topology of the resulting G4 structure [44][45][46][47].

Considering that protein targets involved in specific diseases can (i) have more than one potential binding site recognized by different aptamers, (ii) be dimeric, tetrameric, or in general multimeric, (iii) dimerize or multimerize as a consequence of physiological or pathological events, multivalent aptamer constructs, especially in the simple dimeric forms, are of

particular interest [48][49][50][51]. Specifically, a multivalent aptamer is a construct composed of two or more units of the same or different aptamer motifs, containing or not additional structural elements or functional linkers, able to interact simultaneously with more protein binding sites, generally improving its overall efficacy.

Remarkably, SELEX often identifies oligonucleotide aptamers with a repeated sequence, suggesting high affinity recognition ability by dimeric aptamers for a given protein. As an alternative, since aptamers are largely amenable to chemical modifications [1][2][3][4][5][6], the oligonucleotide sequences initially discovered by SELEX can be easily modified to give dimeric or multimeric aptamers without linkers or using proper spacers of different nature (nucleotidic or not), length and flexibility, and exploiting different kinds of connecting interactions (base-pairs recognitions, covalent chemical linkages). Therefore, oligonucleotide aptamers represent a rich arsenal of finely tunable building blocks, which can be profitably joined to generate suitable constructs with improved functions and properties.

Most exploited strategies involve the simple combination of two of more aptamer units concurrently binding two different domains of a target protein with key biological functions in physiological and pathological conditions.

Dimeric and multimeric DNA-based aptamers were developed as therapeutic tools targeting key proteins in different relevant diseases, such as inflammation, viral infection, thrombosis and cancer.https://www.mdpi.com/1420-3049/25/22/5227

We here describe the selection, design and properties of anti-thrombin multivalent DNA-based aptamers in terms of binding affinity and/or therapeutic efficacy.

2. Anticoagulant Aptamers

The most popular protein target for anticoagulant therapies is thrombin, a multifunctional “trypsin-like” serine protease able to bind fibrinogen and thus catalyze its conversion to fibrin clots in the last step of blood coagulation [52][53][54][55][56][57]. Considering its pivotal role in the coagulation cascade, the inhibition of thrombin activity is one of the most efficient antithrombotic strategies [58][59][60][61]. In this context, the development of effective antithrombin aptamers has been the focus of several investigations [61][62][63][64][65].

The most popular antithrombin aptamers able to inhibit thrombin activity are TBA15, TBA29, and TBA27 [61][65]. Besides differing for their overall length, these aptamers show distinct three-dimensional structures and recognize different thrombin binding sites [65].

Indeed, TBA29 and TBA27—also known as HD22-29 and HD22-27—adopt a mixed duplex/G4 architecture able to bind the exosite II of thrombin (heparin-binding site or ABE II) with high affinity (K_d values of 0.5 and 0.7 nM, respectively for TBA29 and TBA27) [66][67]. In particular, TBA27 is a truncated form of TBA29 (5'-AGTCCGTGGTAGGGCAGGTTGGGGTGACT-3') lacking the first and last residue of the parent aptamer. In contrast, TBA15 or simply TBA, of sequence 5'-GGTTGGTGTGGTTGG-3', folds into a stable chair-like, antiparallel G4 structure able to inhibit the conversion of soluble fibrinogen into insoluble fibrin strands by binding to thrombin exosite I (fibrinogen-binding site or ABE I) with a K_d of 26 nM [65][66][67][68][69][70][71][72][73][74][75].

Starting from these G-rich oligomers, several homo and heterodimeric constructs were developed as effective antithrombin agents [48][66].

RA-36 is the simplest TBA-based homodimeric aptamer. This 31-mer oligonucleotide comprises two TBA15 units, both in 5'-3' direction, covalently linked through one thymidine residue at position 16. As its monomeric precursor, RA-36 recognizes thrombin exosite I, inhibiting the binding between the protein and fibrinogen [76]. Notably, this dimer was able to exert its activity only in a K^+-rich solution, suggesting that the formation of a stable G4 structure is strictly required for effective inhibition of thrombin catalytic action [76][77].

The intriguing properties of RA-36 stimulated the design of other dimeric TBA15 variants, obtained by joining the 3'-ends of each G4 module and introducing inversion of polarity sites in the overall sequence. The connection between TBA15 motifs was realized using various symmetric linkers—i.e., deoxyadenosine or thymidine residues and/or a glycerol moiety—in place of the thymidine at position 16. Unfortunately, the direct comparison of the anticoagulant properties of the newly developed derivatives with the parent RA-36 was not performed, but the evaluation of prothrombin times revealed improved anticoagulant activity and higher T_m values for most of the designed dimers, compared to unmodified TBA15 [78].

Alternative dimeric constructs were based on the covalent connection between TBA15 and other antithrombin aptamers providing heterodimers able to recognize different thrombin exosites.

In particular, TBA15 and TBA29 were linked exploiting different spacers, such as a 15-nucleotide long poly(dA) linker providing HD1-22 [79][80] or poly(T) spacers of various length [81].

In all cases, TBA15/TBA29 dimeric derivatives showed improved thrombin affinity and/or inhibition activity with respect to each monovalent parent aptamer, especially when a poly(T) spacer of 5 residues was explored [79][80][81].

In a different approach, the same aptamers were joined by ethylene glycol spacers of different length. For instance, Tian and Heyduk prepared a covalent dimer of TBA15 and TBA29 featured by flexible connections based on 5'-$(OCH_2CH2)_6$-OPO_3-3' (spacer 18) repeated 5 or 10 times [82]. Five repeated units of the spacer provided an overall linker length of 12 nm, while 10 repetitions allowed reaching a 24 nm-long spacer. This longer version was used both to link the 3'-end of TBA29 with the 5'-end of TBA15 and vice versa. In all cases, the designed bivalent analogues proved to be more efficient in terms of thrombin binding affinity than the starting monomeric aptamers [82].

In turn, Hughes et al. inserted an inverted thymidine (^{i}T) at the 3'-end position of TBA15 and TBA29, thus providing RNV216A and RNV219, respectively [83]. These modified versions were then linked by using either a triethylene glycol (TEG) spacer (RNV220) or four thymidine residues (RNV220-T). Compared to both monovalent aptamers, RNV220 and RNV220-T showed significantly improved antithrombin activity in blood plasma [83].

As a valuable alternative to rational design, Ahmad and coworkers used an in vitro selection strategy to identify the optimal sequence joining TBA15 and TBA29 motifs. The randomized linker was 35-nucleotide long, covering the distance between the different thrombin binding exosites [84]. The resulting 119-mer bivalent aptamer (TBV-08) exhibited noteworthy thrombin binding affinity in the picomolar range (K_d of 8.1 pM) which well correlated with improved antithrombin activity. Similarly to previous approaches, the authors also prepared bivalent constructs presenting poly(T) or poly(dA) linkers. Interesting results in terms of K_d values were also found for the dimer containing a poly(T) spacer of 16 residues [84].

Only one study reported the covalent connection of TBA15 and the shorter HD22 aptamer, i.e., TBA27. In detail, from 2 to 10 units of the commercially available hexaethylene glycol (HEG)-based phosphoramidite were inserted to join the different monomeric aptamer units. The best results in terms of thrombin inhibitory activity were found for the analogue including 8 repeated units of the linker phosphoramidite 18, corresponding to a length of ca. 16 nm [85]. On the contrary, introduction of the shortest spacer of the series, i.e., the one constituted by 4 repeated units of phosporamidite 18, dramatically reduced the inhibitory activity of the obtained dimer analogue [85].

In order to simultaneously increase the resistance to nuclease degradation and the thrombin binding properties, Di Giusto and colleagues proposed circular multivalent constructs [86][87]. Indeed, circularization is a well-established strategy to improve the performance of aptamers [88][89] and has been efficiently applied to antithrombin aptamers targeting thrombin exosite I [66][90][91][92].

In this work, four different DNA aptamers were exploited as building blocks to obtain circular multivalent constructs: antithrombin aptamers TBA29 and GS-522 (i.e., a 15-mer with the sequence 5'-GGTTGGTGAGGTTGG-3' able to bind thrombin exosite I [68]), the L-selectin aptamer [93] and the aptamer against red blood cell marker. A DNA hairpin loop was included as an ancillary module between the aptamer motifs and all the resulting oligonucleotides were also elongated with flanking regions to allow extended stem-loop structure formation. Intra- or intermolecular DNA ligation approaches were used to provide each multivalent circular species, which finally involved two, three or four identical or different aptamer units [86]. The circular multivalent aptamers showed noteworthy stability in both serum and plasma along with improved anticoagulant activity compared to each parent antithrombin aptamer [86].

Another strategy to generate homo- or heteromultimers of a selected aptamer is based on the use of suitable nanoplatforms on which multiple copies of the monomeric aptamer can be linked [48]. This approach has been extensively investigated for TBA15, which thanks to its short sequence and well-known three-dimensional structure, well conserved also when bound to thrombin [94][95], is often exploited as a model system in proof-of-concept studies [48].

In this context, TBA15 was incorporated onto very different nanoplatforms, including magnetic [96][97][98], gold [99][100][101][102][103], silica-based nanoparticles [104][105][106][107], and graphene [108][109]. As a remarkable example, Hsu et al. proposed the multimerization of both TBA15 and TBA29 on the surface of gold nanoparticles (AuNPs). To reach this aim, both aptamers were equipped with thiol end groups allowing their attachment onto the gold surface through Au-thiol interactions [101]. The resulting multivalent nanoparticles, functionalized with about 15 molecules of each aptamer, exhibited an exceptionally high binding affinity for thrombin with a Kd value of 3.4 fM [101].

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