Improved RE31 Analogues Containing Modified Nucleic Acid Monomers: Thermodynamic, Structural and Biological Effects

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ABSTRACT

RE31 is a 31-nt DNA aptamer, consisting of the G-quadruplex and a duplex domain, which is able to effectively prolong thrombin time. This article reports on the influence of certain modified nucleotide residues on thermodynamic and biological properties as well as the folding topology of RE31. Particularly, the effect of the presence of canonical nucleosides in unlocked nucleic acid (UNA), locked nucleic acid (LNA) or β-L-RNA series was evaluated. The studies presented herein show that all modified residues can influence G-quadruplex thermal and biological stability in a position dependent manner. The aptamers modified simultaneously with UNA at the T\textsubscript{15} position and LNAs in the duplex part, possess the highest value of melting temperature and a two-fold higher anticoagulant effect. Importantly, RE31 variants modified with canonical nucleosides in UNA, LNA or β-L-RNA series exhibit unchanged G-quadruplex folding topology. Crucially, introduction of any of the modified residues into RE31 causes prolongation of aptamer stability in human serum.

Keywords: G-quadruplex, thrombin, anticoagulants, DNA, aptamer, unlocked nucleic acids
INTRODUCTION

The process of blood coagulation is a chain of reactions, during which serum soluble fibrinogen is converted into insoluble fibrin and forms clots. The aim of this phenomenon is to create a stable haemostatic plug, which will prevent blood from leaking out from blood vessels.\(^1\) A crucial coagulant factor, which plays the main role in converting fibrinogen into fibrin is thrombin, a serum multifunctional serine protease.\(^2\) In some cases, including coronary surgery, cancer therapy, and treatment of cardiovascular diseases to name a few, thrombin action is detrimental and needs to be inhibited. Currently, a wide variety of anticoagulant compounds have been identified, such as warfarin, low molecular weight heparin and dabigatran, and DNA aptamers constitute a unique class among them.

One the most widely known DNA aptamer is thrombin binding aptamer (TBA), which was discovered in 1992 by Louis Bock by \textit{in vitro} selection.\(^3\) The 15-nt DNA oligonucleotide folds into an antiparallel, intramolecular G-quadruplex structure with a chair-like conformation. The TBA core consists of the G-tetrads linked by one TGT and two TT edge-wise loops (Figure 1A).\(^4\) From the beginning of its discovery, TBA was thought to have interesting anticoagulant properties, although it failed in the clinical trials due to substantial therapeutic dose needed to achieve therapeutic effect. This, however, did not discourage scientists from seeking to take advantage of TBA favorable properties such as cost-effective and efficient chemical synthesis, small molecule size and reversibility of action.\(^5\) Significant improvements in TBA anticoagulant activity have been achieved by introduction of chemical modifications\(^6\)-\(^{11}\) and additional nucleotide residues into its sequence.\(^{12}\)
Figure 1. Structure of (A) thrombin binding aptamer (TBA), (B) RE31, (C) UNA nucleotide monomer, (D) LNA nucleotide monomer and (E) β-l-RNA nucleotide monomer.

One of the first attempts to create longer oligonucleotide constructs based on the TBA sequence were made by Ikebukuro and co-workers. They designed a pool of 31-nt oligonucleotides consisting of the prototype TBA G-quadruplex structure and five-base-pair long duplex regions at the 5′ and 3′ ends connected by three-nucleotide long loops, which exhibited higher than TBA inhibitory activity. In 2009, following these previous studies, Mazurov and co-workers designed RE31, a 31-nt DNA oligomer, which consists of the G-quadruplex and a duplex domain linked together by 4 nucleotides (Figure 1B). The G-quadruplex part is 15-nt fragment, which possesses identical to the TBA sequence and structure, while the duplex domain is composed of 6 pairs of complementary nucleotides. RE31 is able to prolong thrombin time more effectively than TBA does. According to crystallographic data, it was shown that the duplex part of RE31 gradually transforms into the G-quadruplex structure with similarly oriented helical axes of the two fragments. Therefore, the aptamer is believed to adopt a rod-like shape, which prevents simultaneous binding of both RE31 regions to a thrombin molecule. The binding to target protein involves only the G-quadruplex region and is assigned to take place in exosite I. Nevertheless, the duplex part, to
a certain extent, can provide additional contacts with symmetry-related thrombin molecules, which result in RE31 increased affinity for its target protein compared with TBA.\textsuperscript{15, 17}

Unlocked nucleic acid (UNA) is an RNA analog, which contains acyclic sugar moiety.\textsuperscript{18} The lack of a bond between the C2’ and C3’ atoms of the ribose ring results in increased flexibility of this monomer (Figure 1C) compared with the regular nucleoside, due to the enhanced rotational freedom of C-C bonds. It was previously reported that UNAs are able to improve the aptamer thermodynamic stability and anticoagulant activity in a position-dependent manner.\textsuperscript{6, 19} On the other hand, introduction of UNAs into RNA or DNA helix causes an unfavorable changes of thermodynamic parameters.\textsuperscript{20, 21}

Locked nucleic acid (LNA), in contrast, is a type of modification of nucleotide residues that demonstrate a favorable influence on duplex thermodynamic stability. LNA is an RNA analog, which possesses an additional methylene bridge connecting the C2’ and C4’ atoms of the ribose ring (Figure 1D).\textsuperscript{22, 23} Oligonucleotides containing LNA modifications form duplexes with increased thermal stabilities and improved selectivity according to the Watson-Crick base pairing rules.\textsuperscript{22} This findings refers to DNA, RNA as well as to DNA-RNA mixed helical fragments.\textsuperscript{23, 24} On the other hand, introduction of LNA into the G-quadruplex can destabilize its structure and decrease biological activity.\textsuperscript{8, 25}

The β-L-RNA derivative is characterized by inversion of the ribose ring stereogenic centers configuration (Figure 1E).\textsuperscript{26} Therefore, introduction of β-L-RNAs into duplex fragments results in the occurrence of a left-handed double helical structure. Homochiral L-RNA duplexes display unchanged thermodynamic stability in comparison to their naturally occurring β-D-RNA counterparts.\textsuperscript{27} What is more, introduction of β-L-RNAs into double stranded oligonucleotides improves their biological stability in human serum and resistance towards nucleolytic degradation.\textsuperscript{26}
Herein, the influence of modified nucleotide residues introduced into RE31 molecule was presented for the first time. In more details, the consequences of single and multiple substitutions of UNA-A, UNA-C, UNA-G, UNA-U, LNA-A, LNA-G, LNA-T, β-l.-RNA-A, β-l.-RNA-C, β-l.-RNA-G and β-l.-RNA-U on RE31 folding topology were examined. Moreover, the thermal and biological stability of the analyzed aptamers substituted with the modified nucleoside residues was assessed, as well as the anticoagulant properties of the novel RE31 variants were determined.

RESULTS AND DISCUSSION

Thermal denaturation measurements

Thermal denaturation measurements were employed to verify the influence of single or multiple substitution of the nucleoside residues in UNA, LNA and β-l.-RNA series on RE31 thermal stabilities. This technique allows for recording changes in absorbance of the analyzed probe due to temperature increase. A valuable parameter, which allows for designation of oligonucleotide thermal stability is melting temperature (T_M) i.e. the temperature at which unfolded and structuralized forms of nucleic acids are in equal proportions. G-quadruplex thermodynamic studies were performed at 295 nm wavelength and characterized by the presence of hypochromic effect. This phenomenon relies on a decrease in absorbance connected with temperature increase and is a result of disappearance of interactions between G-tetrads-forming guanosine residues.

Herein, the attempts to increase the thermal stability of RE31 aptamer via modified residues introduction into its G-quadruplex core are presented for the first time. It was previously proven that introduction of UNA-U residues into the T_3, T_7 and T_12 positions of TBA increases its thermal stability. Based on this and taking into consideration the structural similarities of the G-quadruplex region of RE31 to the TBA molecule, the positions T_11, T_15
and T_{20} (equivalent to T_{3}, T_{7} and T_{12} in TBA) were chosen as positions of substitution of the nucleoside residues in the UNA series. Melting analysis revealed that replacement of thymine at position T_{11} and T_{20} with UNA-U causes a minor increase in thermal stability of RE31 variants in respect to unmodified aptamer (ΔT_{M} for aptamers O1 and O6 was 2.3 and 1.0 °C, respectively, Table 1). A similar stabilization was observed for oligomers O2 – O5 possessing UNA-A, UNA-C, UNA-G, UNA-U substitution at T_{15} position. In this case the ΔT_{M} values were in the range of 1.8 – 2.8 °C. Considerable improvement in thermal stability was achieved via simultaneous substitution of thymidine at T_{11}, T_{15} and T_{20} positions by UNA-U residues (ΔT_{M} for O7 was equal to 5.4 °C). Currently, there are no literature reports concerning the influence of modified residues on RE31 chemical and biological properties, however the structural similarities of this region to the TBA molecule allow for drawing certain comparisons. The above data are consistent with our previous reports indicating beneficial influence of introduction of UNA-U at T_{3}, T_{7} and T_{12} or other UNAs at T_{7} on TBA thermal stability (these positions are equivalent to T_{11}, T_{15} and T_{20} positions in RE31).6,19 Likewise in the TBA case, the favorable effect of UNAs at position T_{15} is most probably a consequence of increased flexibility or the presence of additional 2′ hydroxyl group. Thus, the type of heterocyclic base at position T_{15} seems to be negligible for stabilization of G-quadruplex structure. What is more, the lack of involvement in the intramolecular contacts of positions T_{11}, T_{15}, and T_{20} makes them tolerant to chemical modification.16

The characteristic structure of RE31 gives additional opportunities to increase its thermal stability via introduction of modified residues into the duplex fragment. It was previously proven that introduction of LNA residues into DNA or RNA duplexes increases their thermal stability and improves selectivity in comparison to the corresponding unmodified reference.23,29 What is more, it has been suggested that the most potent utilization of LNAs is through alternate spacing with unmodified nucleotides with the first LNA located at the
penultimate position from the 5’ duplex end.\textsuperscript{30} In the light of the above data, the T\textsuperscript{2}, A\textsuperscript{4}, G\textsuperscript{6} and A\textsuperscript{8} residues in RE31 compound were replaced by their LNA counterparts (O8), what results in a melting temperature increase by 10.8 °C (Table 1). Simultaneous presence of LNAs in duplex fragments and one of UNAs at T\textsuperscript{15} position also caused a significant rise in thermal stability in comparison to RE31 variants possessing only UNAs at T\textsuperscript{15} (the \(\Delta T_M\) values for O9 – O12 were in the range 9.2 – 12.6 °C). In contrast, the β-L-RNAs are said to have no influence on duplex stability, but exert notably protective effect for helical fragments against nucleolytic degradation.\textsuperscript{27} Therefore, the impact on the aptamer thermal parameters was studied following introduction of modified nucleotide residues in β-L-RNA series into RE31 helix and linker fragment (T\textsuperscript{2}-A\textsuperscript{8} and G\textsuperscript{24}-A\textsuperscript{30}). The achieved results stay in accordance with previously published data. The increase in G-quadruplex melting temperature was modest and \(\Delta T_M\) for O13 was equal to 1.6 °C (Table 1).

**CD spectroscopy**

Circular dichroism (CD) spectroscopy is a useful technique, which provides information about nucleic acids conformation and folding topology, based on the different absorbance of circularly polarized light.\textsuperscript{31} The characteristic CD spectrum is a result of different spatial orientation of optically active nucleotide residues in certain nucleic acid structures and gives information about folding topology of G-quadruplex. Based on experimental data analysis, there are distinguished two folding topologies for G-quadruplex: parallel and antiparallel.\textsuperscript{32, 33} The G-quadruplex with parallel folding topology has all strands oriented in the same direction and is characterized by CD pattern with one positive signal near 260 nm and one negative around 240 nm. In contrast, the CD spectra for the antiparallel G-quadruplex folding include one maximum near 290 nm and one minimum around 260 nm. It was previously reported that the CD spectrum for RE31 displays a shape, which is characteristic for antiparallel folding
topology with two positive CD bands near 245 and 295 nm and one negative signal around 265 nm.17, 34

The CD spectra for oligomers O1-O13 containing one or more nucleoside residues in UNA, LNA and β-l.-RNA series were recorded to determine the influence of certain modifications on aptamer conformation. All RE31 variants displayed the typical pattern for an antiparallel G-quadruplex structure with two maxima near 245 and 295 nm and one minimum around 265 nm (Figure 2A and 2B). However, a lack of positive band around 245 nm was observed for O13, which could be a consequence of a different chirality or stacking type. The CD spectra indicate that modified RE31 variants fold into antiparallel G-quadruplex topology, which is comparable to unmodified aptamer at physiological temperature,
Figure 2. Circular dichroism spectra (A, B) and thermal difference spectra (C, D) of unmodified RE31 (black, bold line) and RE31 variants modified with UNA-U at T\textsuperscript{11} (O1, red line), UNA-A at T\textsuperscript{15} (O2, blue line), UNA-C at T\textsuperscript{15} (O3, dark cyan line), UNA-G at T\textsuperscript{15} (O4, magenta line), UNA-U at T\textsuperscript{15} (O5, dark yellow line), UNA-U at T\textsuperscript{20} (O6, navy blue line), UNA-U at T\textsuperscript{11}, T\textsuperscript{15}, T\textsuperscript{20} (O7, dark red line), LNAs at T\textsuperscript{2}, A\textsuperscript{4}, G\textsuperscript{6}, A\textsuperscript{8} (O8, pink line), LNAs at T\textsuperscript{2}, A\textsuperscript{4}, G\textsuperscript{6}, A\textsuperscript{8} and UNA-A at T\textsuperscript{15} (O9, olive line), LNAs at T\textsuperscript{2}, A\textsuperscript{4}, G\textsuperscript{6}, A\textsuperscript{8} and UNA-C at T\textsuperscript{15} (O10, dark blue line), LNAs at T\textsuperscript{2}, A\textsuperscript{4}, G\textsuperscript{6}, A\textsuperscript{8} and UNA-G at T\textsuperscript{15} (O11, orange line), LNAs at T\textsuperscript{2}, A\textsuperscript{4}, G\textsuperscript{6}, A\textsuperscript{8} and UNA-U at T\textsuperscript{15} (O12, purple line), β-L-RNA at T\textsuperscript{2}-A\textsuperscript{8} and G\textsuperscript{24}-A\textsuperscript{30} (O13, violet line).

Thermal difference spectra

Thermal difference spectra (TDS) are considered to be complementary to CD spectroscopy, which allow to evaluate nucleic acid structures in solution.\textsuperscript{35} Various structures of nucleic acid possess subtle alterations in stacking interactions, which are reflected by characteristic TDS patterns.

The TDS profiles recorded for oligomers O1-O13 containing single or multiple substitution of the nucleoside residues in UNA, LNA and β-l-RNA series were characterized by TDS pattern with two positive peaks near 240 and 270 nm and a negative peak around 295 nm (Figure 2C and 2D). Herein, for the first time the TDS profiles for aptamer RE31 and its modified variants are published. Nevertheless, based on similarities to previously published data concerning the TDS patterns of unmodified TBA (characterized by presence of two maxima around 240 and 270 nm and two minima near 260 and 295 nm), it can be assumed that these spectral profiles are characteristic of an antiparallel G-quadruplex structure.\textsuperscript{6} Thus, according to TDS patterns it can be concluded that the presence of modified nucleotides in UNA, LNA or β-l-RNA series within RE31 has no influence on the overall folding topology of G-quadruplex structure. The results remain in compliance with the circular dichroism and thermodynamic studies.
Thrombin time assay

Thrombin time assay was employed to verify the influence of nucleotide residues in UNA, LNA or β-L-RNA series into RE31 on its inhibitory activity towards human thrombin. The main principle of the test is based on thrombin ability to convert plasma-soluble fibrinogen into insoluble fibrin, what results in occurrence of blood clots. The parameter that correlates to the process is thrombin time defined as the time, after which fibrin clot occurs following the addition of exogenous thrombin to blood plasma. Herein, the anticoagulant properties of RE31 variants were compared by analysis of the anticoagulant effect (AE). The AE parameter is defined as differences in the clotting times for (i) plasma with exogenous thrombin and oligonucleotides and (ii) plasma with exogenous thrombin only.19

It was previously reported that introduction of UNA residues into TBA molecule has particularly beneficial influence on its biological activity level.6, 19 Taking into account the structural similarities between G-quadruplex region of RE31 and TBA,16 we decided to verify whether it is possible to improve the aptamer anticoagulant properties through UNA-substitution. The measurements were performed at 0.165 μM concentration of each RE31 variant. The most favorable effect was achieved via introduction of UNA residues into T15 position of RE31. The UNA-C substitution at T15 position of RE31 turned out to be particularly beneficial (the AE for O3 was 17.08 s, Table 2). The prolongation of thrombin time, in comparison to unmodified RE31, was also possible due to introduction of UNA-G at the T15 position (AE for RE31 and O4 was 11.09 and 12.18 s, respectively). The high tolerance of T15 position towards substitution with UNA residues can be caused by the lack of involvement of the residue at this position in the direct interactions with thrombin or in an energetically favorable intramolecular interactions.16 Therefore, the increased flexibility in this loop fragment might result in more favorable arrangement of thrombin-RE31 complex. Surprisingly, the introduction of UNA-U at T15 position causes some decrease of RE31 anticoagulant activity
Moreover, the presence of UNA-U within the aptamer loops resulted in notable reduction of RE31 anticoagulant potential. The AE parameter measured for aptamers modified with UNA-U residues at positions T\textsuperscript{11} (O1) and T\textsuperscript{20} (O6) was 6.87 and 4.27 s, respectively. The above effect can be explained by involvement of these two residues in formation of hydrogen bonds between RE31 molecule and thrombin.\textsuperscript{16} Therefore, the increased flexibility of the RE31 loops via insertion of UNA residues perturbs favorable intermolecular interactions. The simultaneous introduction of UNA-U at positions T\textsuperscript{11}, T\textsuperscript{15} and T\textsuperscript{20} provoked the most significant reduction of RE31 anticoagulant properties (the AE for O7 was 0.65 s).

The introduction of LNA and β-L-RNA residues into aptamer duplex fragment results in decreased AE values in comparison to unmodified aptamer (AE for O8 and O13 was 10.09 and 8.13 s, respectively, Table 2). Surprisingly, this trend could be reversed through additional incorporation of UNA residues into T\textsuperscript{15} position. The most favorable effect was measured for aptamer O9 containing both, LNA and UNA-A residues (AE was 23.27 s). An almost equally beneficial outcome was found in case of coexistence of canonical LNAs at T\textsuperscript{2}, A\textsuperscript{4}, G\textsuperscript{6}, A\textsuperscript{8} positions and UNA-C at T\textsuperscript{15} position, the AE value for O10 was 21.58 s. Moderate improvement in anticoagulant properties was observed for oligomers O11 and O12 containing at the same time canonical LNAs at T\textsuperscript{2}, A\textsuperscript{4}, G\textsuperscript{6}, A\textsuperscript{8} positions and UNA-G or UNA-U at T\textsuperscript{15} position (the AE were 14.10 s and 10.68 s, respectively).

Based on data analysis of thrombin time assay conducted for the most favorable RE31 variants with its various solutions (0.0825, 0.165, 0.330, 0.495, 0.660, 0.825 and 1 µM) it could be concluded that the anticoagulant effect strictly depends on aptamers concentration. Noteworthy, it is possible to improve the effectiveness of action of particular RE31 variant via increase of oligonucleotide concentration. What is more, the results show that aptamers O3 and O4 containing single UNA residue at T\textsuperscript{15} position were characterized by greater AE value than their counterpart modified simultaneously with LNAs in duplex fragment and UNAs at T\textsuperscript{15}.
position (oligonucleotides O10 and O11, Figure 3). Aptamers O2 and O9 (modified with single UNA-A at T$^{15}$ or LNAs in duplex fragment and UNA-A at T$^{15}$, respectively) constituted the only exception of this rule (Figure 3). The sudden increase of anticoagulant properties of some RE31 variants, especially aptamer O3 (Figure 3), in respect to others could be also observed. This phenomenon may be attributable to differences in specificity and competition of interactions between the oligonucleotides and thrombin versus remaining plasma proteins. Different chemical modifications most probably modulate both kinds of interactions in a different way, which results in differences in the dose-response pattern of each oligonucleotide.

![Figure 3](image-url).

**Figure 3.** Concentration dependence of anticoagulant effect of unmodified RE31 (black symbol) and RE31 variants modified with UNA-A at T$^{15}$ (O2, blue symbol), UNA-C at T$^{15}$ (O3, dark cyan symbol), UNA-G at T$^{15}$ (O4, magenta symbol), LNAs at T$^2$, A$^4$, G$^6$, A$^8$ (O8, pink, open symbol), LNAs at T$^2$, A$^4$, G$^6$, A$^8$ and UNA-A at T$^{15}$ (O9, olive, open symbol), LNAs at T$^2$, A$^4$, G$^6$, A$^8$ and UNA-C at T$^{15}$ (O10, dark blue, open symbol), LNAs at T$^2$, A$^4$, G$^6$, A$^8$ and UNA-G at T$^{15}$ (O11, orange, open symbol).

In general, the data analysis shows a favorable influence of simultaneous substitution of LNA residues in duplex fragment and UNA-A, UNA-C or UNA-G at T$^{15}$ position on anticoagulant properties of RE31 variants. In addition, aptamers possessing UNA-A, UNA-C
or UNA-G instead of T$^{15}$ exhibited greater AE values than unmodified RE31. The observed effect is strictly concentration dependent.

**Amidolytic assay**

Thrombin molecule consists of four structural domains: the active site, two anion-binding exosites (I and II) and Na$^+$-binding site.$^{36}$ The active site is directly involved in hydrolysis of substrate peptide bond. The two anion-binding exosites are the most positively charged parts of thrombin, where binding of its substrates and activity modulator takes place. It was previously reported that both TBA and RE31 bind to exosite I.$^{14}$ Herein, the amidolytic assay was applied to assess the influence of modified nucleotides on RE31 aptamer binding to thrombin molecule. It is a useful method, which allows to determine the exact localization of interaction based on thrombin proteolytic activity. In situation of thrombin catalytic center accessibility, the peptide bond of chromogenic substrate H-D-Phe-Pip-Arg-pNA is hydrolyzed and p-nitroaniline is generated, what results in appearance of yellow color of probes. Therefore, the thrombin catalytic activity can be determined via investigation of the change in the sample absorbance. H-D-Phe-Pip-Arg-pNA binds to the thrombin active site even when other functional domains are involved in interactions with enzyme substrates or its inhibitors. The calculation of the percentage of thrombin activity maintenance (At) was used to determine thrombin ability to hydrolyze short peptide H-D-Phe-Pip-Arg-pNA in the presence of tested RE31 variants. The At parameter value of 100% corresponds to the absorbance for the thrombin solution measured at 405 nm.

The highest value of thrombin activity was measured for unmodified RE31 and its variants containing UNA-C (O3) and UNA-G (O4) at T$^{15}$ position or simultaneously UNA-G at position T$^{15}$ and canonical LNA residues in duplex part (O11) (Figure 4). The similar effect was noted for aptamers modified with UNA-U at position T$^{11}$ (O1) or simultaneously at T$^{11}$, T$^{15}$ and T$^{20}$ positions (O7) or β-i.-RNA residues in duplex part (O13, Figure 4). The At
parameter extended 100% for these RE31 variants. The remaining RE31 variants were characterized by a minor decrease in thrombin activity maintenance levels (90% < At <100%). The significance level of differences in thrombin activity in the presence of analyzed RE31 variants was determined via statistical analysis by Student t-test (data not shown). In all cases, the p-value was above 0.05, thus the observed differences in At values were not statistically significant. Taking together, the data demonstrate that thrombin maintains its ability to hydrolyze peptide substrate H-D-Phe-Pip-Arg-pNA in presence of the RE31 variants, and therefore it can be concluded that the aptamers inhibitory properties are a results of binding to exosite I of thrombin molecule.

**Figure 4.** The amidolytic activity of thrombin in the presence of unmodified RE31 (black bar) and RE31 variants modified with UNA-U at T\(^{11}\) (O1, red bar), UNA-A at T\(^{15}\) (O2, blue bar), UNA-C at T\(^{15}\) (O3, dark cyan bar), UNA-G at T\(^{15}\) (O4, magenta bar), UNA-U at T\(^{15}\) (O5, dark yellow bar), UNA-U at T\(^{20}\) (O6, navy blue bar), UNA-U at T\(^{11}\), T\(^{15}\), T\(^{20}\) (O7, dark red bar), LNAs at T\(^{2}\), A\(^{4}\), G\(^{6}\), A\(^{8}\) (O8, pink bar), LNAs at T\(^{2}\), A\(^{4}\), G\(^{6}\), A\(^{8}\) and UNA-A at T\(^{15}\) (O9, olive bar), LNAs at T\(^{2}\), A\(^{4}\), G\(^{6}\), A\(^{8}\) and UNA-C at T\(^{15}\) (O10, dark blue bar), LNAs at T\(^{2}\), A\(^{4}\), G\(^{6}\), A\(^{8}\) and UNA-G at T\(^{15}\) (O11, orange bar), LNAs at T\(^{2}\), A\(^{4}\), G\(^{6}\), A\(^{8}\) and UNA-U at T\(^{15}\) (O12, purple bar), β-L-RNA at T\(^{2}\)-A\(^{8}\) and G\(^{24}\)-A\(^{30}\) (O13, violet bar).
Surface plasmon resonance

The determination of the strength of interactions between thrombin and RE31 variants was conducted using real-time surface plasmon resonance (SPR) spectroscopy. This optical technique allows calculation of the dissociation constant (Kd value) according to the change in refractive index. The conventional SPR measurement requires immobilization of one of the binding components on the metallic surface of the sensor chip whilst the other is flowed across the sensor surface. Herein, the group of the most stable and potent RE31 variants were chosen for the binding affinity studies.

The most favorable Kd values, in comparison to unmodified RE31 (for which the Kd value was 1.34 nM, Table 2), were obtained for O3 modified with UNA-C residues at position T\textsubscript{15} (the Kd value was 0.43 nM). Kd values measured for RE31 variants modified with UNA-A, UNA-G and UNA-U at T\textsubscript{15} position were higher but still more favorable than for unmodified RE31 (the Kd values for O2, O4 and O5 were 0.76, 0.68 and 0.75 nM, respectively). The introduction of LNA residues into the duplex fragment of RE31 molecule caused a decrease in the strength of thrombin-RE31 interactions (the Kd value for O8 was 1.75 nM). Nevertheless, the unfavorable influence of LNAs could be alleviated by the presence of UNA-A or UNA-C at T\textsubscript{15} position (the Kd values for O9 and O10 were 1.15 and 0.90 nM, respectively).

In general, the Kd values did not directly correspond with the thrombin test results. This is most probably due to the complexity of blood coagulation processes and indicates that the dissociation constant parameter alone is not sufficient for the assessment of the anticoagulant potential.

Viability in human serum

Oligonucleotides are rapidly eliminated from the circulation and determination of their biostability in human serum is one the most crucial indicators, which allows for prediction of
their potential in vivo activity. The widely used measure of half-life ($T_{1/2}$), defined as time in which the amount of tested substance decays by half, was employed for determination of biostability of RE31 variants in human serum. Various approaches have been proposed to extend G-quadruplex serum viability and the chemical modification of the aptamer could help solve this issue.\textsuperscript{19,38}

Herein, the $T_{1/2}$ values for RE31 variants were designated via incubation in human serum at 37 °C. The RE31 variants (O8-O13) modified with LNA, β-L-RNA and simultaneously UNA and LNA residues were characterized by the most favorable biological stability. In all cases, $T_{1/2}$ was longer than 1440 min in comparison to 364 min for unmodified RE31 (Table 2). The $T^{15}$ substitution by UNA residues also improved the aptamer biostability. Although, in this instance the $T_{1/2}$ for UNA-A (O2) and UNA-C (O3) was equal to 1062 min and for UNA-G (O4) and UNA-U (O5) was 1047 and 1018 min, respectively. Only a slightly increased biological stability was observed for O6 containing UNA-U at $T^{20}$ position or O7 possessing simultaneously UNA-U at $T^{11}$, $T^{15}$ and $T^{20}$ positions ($T_{1/2}$ was 524 and 538 min, respectively). The achieved results correspond with previously published literature data stating that DNA G-quadruplexes modified with nucleotide residues in UNA series are characterized by extended in vitro stability in serum.\textsuperscript{38} The data presented here further support and extend the current state of knowledge in the field. The introduction of LNA residues may provide a convenient and effective tool to extend DNA G-quadruplex viability in human serum.

**CONCLUSIONS**

In this paper, examples of RE31 variants possessing artificial nucleotide residues have been presented for the first time, along with comprehensive thermodynamic and biological studies of RE31 variants modified with nucleotide residues in UNA, LNA and β-L-RNA series.

The most favourable influence on RE31 thermal stability had LNAs in the duplex fragment or a combination of LNAs in the helix and UNAs at $T^{15}$ (O8 – O12). Substantial
Improvement was also achieved via ternary substitution of T\textsuperscript{11}, T\textsuperscript{15} and T\textsuperscript{20} positions by UNA-U (O7). For the rest of the aptamers, only a minor stabilizing effect was observed. The results of structural studies confirmed that the overall folding topology of RE31 variants containing substitution with modified nucleotide residues at the studied positions was unaffected and that all analyzed aptamers form antiparallel G-quadruplex structure at 37 °C. What is more, it was possible to achieve RE31 derivatives characterized by significantly improved anticoagulant properties via simultaneous introduction of UNAs at T\textsuperscript{15} position and LNAs at helix fragment (O9 – O11). Slightly smaller but still significant anticoagulant effects were observed for aptamers possessing UNAs substitution at T\textsuperscript{15} position (O2 – O4). It is worth stressing that all analyzed RE31 variants possessed extended biological stability. Notably, introduction of LNAs and β-\textsuperscript{1}-RNAs into RE31 helical fragment caused meaningful prolongation of aptamers stability in human serum (O8 and O13). Additionally, it was proven that entire pool of tested RE31 variants exerted their inhibitory activity by binding to thrombin exosite I, but their affinity to target protein was strictly dependent on the type of modification.

The results described herein confirm that both LNA and UNA residues are suitable molecular tools to modulate RE31 thermal and biological stability with preservation or improvement of its anticoagulant properties. These findings provide new insight in the area of application of modified DNA aptamers as promising alternatives to classical antithrombin agents.

EXPERIMENTAL SECTION

Chemical synthesis of oligonucleotides

All oligonucleotides were synthesized on an automated RNA/DNA synthesizer using standard phosphoramidite chemistry and commercially available nucleoside phosphoramidites.\textsuperscript{39} The deprotection and purification of unmodified oligoribonucleotides or RE31 variants containing
nucleotide residues in the UNA, LNA and β-1-RNA series were performed as it was described previously.\textsuperscript{20, 40, 41} The purity of oligonucleotides was verified as being above 95\% by 20\% denaturing polyacrylamide gel, and the composition of all oligonucleotides was confirmed by MALDI-TOF mass spectrometry.

\textbf{UV melting analysis}

Oligonucleotides were dissolved in a buffer containing 100 mM potassium chloride, 20 mM sodium cacodylate and 0.5 mM Na\textsubscript{2}EDTA, pH 7.0. Single-stranded oligonucleotide concentrations were calculated based on the absorbance measured above 80 °C and the extinction coefficients were calculated, using Oligo Calculator (ribotask.com).\textsuperscript{42} The samples were denatured at 95 °C for 5 min and then cooled to room temperature overnight. The measurements were performed for nine different concentrations of G-quadruplex in the concentration range of 10\textsuperscript{−5} – 10\textsuperscript{−7} M. Absorbance versus temperature curves were obtained using the UV melting method at 295 nm in the temperature range of 4 – 90 °C with a heating rate of 0.2 °C/min on a JASCO V-650 spectrophotometer equipped with a thermoprogrammer. Melting curves were analyzed and the melting points determined by nonlinear curve fitting using the MeltWin 3.5 software. Melting temperatures calculated for a 10\textsuperscript{−4} M concentration of oligonucleotide are denoted by $T_M$, and melting points for any other concentration of oligonucleotide are denoted by $T_m$.

\textbf{Circular dichroism spectra}

CD spectra were recorded on a JASCO J-815 spectropolarimeter. The oligonucleotides were dissolved in a buffer containing 100 mM potassium chloride, 20 mM sodium cacodylate and 0.5 mM Na\textsubscript{2}EDTA, pH 7.0, to achieve a sample concentration of 3.0 µM. All samples were denatured at 95 °C for 5 min and then slowly cooled to room temperature overnight prior to data collection. The spectra were recorded in triplicate at 37 °C in the 205 – 320 nm wavelength
range. The buffer spectrum was subtracted from the sample spectra. The data analysis was performed using the Origin 8.0 software.

**Thermal difference spectra**

The TDS measurements were performed on a JASCO V-650 spectrophotometer equipped with a thermoprogrammer. The oligonucleotides were dissolved in a buffer containing 100 mM potassium chloride, 20 mM sodium cacodylate and 0.5 mM Na₂EDTA, pH 7.0, to achieve a sample concentration of 0.1 μM. Absorbance spectra were recorded in triplicate at 4 °C and 90 °C in the 220 – 335 nm wavelength range. The scan speed was 1000 nm/min with a 1 nm data interval. Thermal difference spectra were obtained by subtraction of the low temperature absorbance spectra from the high temperature absorbance spectra using the Origin 8.0 software. The differential spectra were normalized by dividing the data by its maximum value.³⁵

**Thrombin time assay**

The thrombin time assay was performed using commercially available Dia-TT kit (DIAGON®) and coagulometer K-3002 Optic. Each RE31 variant was dissolved in 100 µl Dia-TT reagent, to achieve a sample concentration of 0.33 μM. The reaction mixture was incubated at 37 °C for 5 min and put into sample well of coagulometer K-3002 Optic. Next, 100 µl of citrate plasma was added. Each RE31 variant was analyzed using plasma samples derived from five healthy volunteers. The sample from each volunteer was measured twice, and the errors of thrombin time values obtained from the double measurements did not exceed 6% (Table 2). The anticoagulant effect was obtained by subtraction of the time needed for plasma clotting in the presence of the aptamers from the time needed for clotting of plasma without aptamer. In addition, thrombin time for RE31 variants with the best anticoagulant properties in seven different concentrations: 0.0825, 0.165, 0.330, 0.495, 0.660, 0.825, and 1 μM was assigned
using plasma samples derived from three healthy volunteers. Each experiment was repeated twice and the result expressed as mean value.

**Surface plasmon resonance**

The value of dissociation constant ($K_d$) for chosen RE31 variants was measured using a Biacore™ X100 system (GE Healthcare Life Science), human alpha-thrombin solution (Haematologic Technologies, Inc.) and commercially available Biotin CAPture KIT (GE Healthcare Life Science). The 5′-biotinylated oligonucleotides were dissolved in a running buffer – 10 mM phosphate buffered saline (138 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.76 mM KH$_2$PO$_4$, 0.05% Tween-20, pH 7.4) to achieve a sample concentration of 1 µM. The samples were denatured at 95 °C for 6 min and then cooled to room temperature overnight. The oligonucleotides were immobilized on a chip surface via the streptavidin-biotin coupling method. The flow rate was set to 30 µl/min and the unbound oligonucleotide was removed by the treatment with 50 mM aqueous NaOH. Next, serially diluted thrombin solution in the concentration range of 12.5 - 200 nM along with a bovine serum albumin (BSA, Sigma-Aldrich®) and sample with only running buffer were injected. The measurements were performed at 25 °C. After each run, the system was regenerated by treatment with 50 mM aqueous NaOH. Data analysis was performed using the Biacore X100 Evaluation software and a 1:1 binding mode.

**Amidolytic assay**

Amidolytic assay was used to evaluate the ability of human alpha-thrombin (Haematologic Technologies, Inc.) to hydrolyze chromogenic substrate H-D-Phenylalanyl-L-arginine-p-nitroaniline (H-D-Phe-Pip-Arg-pNA, trade name: S-2238™, CHROMOGENIX) in the presence of chosen RE31 variants. The reaction mixture (150 µl), consisting of the chosen RE31 variants (1 µM), thrombin (0.4 U/ml) and reaction buffer (0.1 M Tris pH 8.2, 150 mM NaCl,
5 mM KCl, 0.1% BSA), was incubated at 37 °C for 5 min. Next, 50 µl of 2 mM chromogenic substrate H-D-Phe-Pip-Arg-pNA was added. The amount of released p-nitroaniline was measured at 405 nm using a microplate reader xMark™ (Bio-Rad). Data analysis was performed using Microsoft Excel 2013 software. Each experiment was repeated in triplicate and the result expressed as mean value.

**Viability of oligonucleotides in human serum**

The amount of 110 pmol of 5’-FAM labeled oligonucleotides was dissolved in 20 µl of 1 x PBS buffer containing 100 mM potassium chloride. The samples were denatured at 90 °C for 6 min and then cooled to room temperature overnight. Afterwards, 200 µl of human serum from human male AB plasma (Sigma-Aldrich) was added, and the samples were incubated at 37 °C. Aliquots of 5 µl were removed after 0, 10, 20, 40, 60, 120, 180 and 1440 min (for less stable aptamers) or after 0, 20, 40, 60, 120, 240, 480, 1440, 1680, 1920 and 2880 min (for more stable aptamers) of incubation and were then mixed with 5 µl of a 70% deionized formamide solution containing 50 mM EDTA and cooled on dry ice to quench the reaction. The samples were loaded on a 12% denaturing polyacrylamide gel prepared in 1 x TBE buffer. Polyacrylamide gel electrophoresis (PAGE) in denaturing condition was performed in 1 x TBE buffer at 20 W for 3 h at room temperature. The resultant gel was imaged and quantified using a Fuji Phosphorimager and MultiGauge Analysis Software.

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Author Contributions

W.K. performed all experiments and wrote the paper. J.W. supervised the SPR measurements. C.J.S. proofed the paper. A.P. performed oligonucleotide synthesis, supervised all the experiments and proofed the paper. All authors have given approval to the final version of the manuscript.

ACKNOWLEDGMENTS

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ABBREVIATIONS USED

AE, anticoagulant effect; At, percentage of thrombin activity maintenance; Kd, dissociation constant; LNA, locked nucleic acid; SPR, surface plasmon resonance; TBA, thrombin binding aptamer; TDS, thermal difference spectra; Tm, melting temperature; T1/2, half-life; UNA, unlocked nucleic acid

REFERENCES

Table 1. Melting temperatures of RE31 variants modified with UNA-A (A\textsuperscript{U}), UNA-C (C\textsuperscript{U}), UNA-G (G\textsuperscript{U}), UNA-U (U\textsuperscript{U}), LNA-A (A\textsubscript{L}), LNA-G (G\textsubscript{L}), LNA-T (T\textsubscript{L}), β-L-RNA-A (A\textsubscript{β}), β-L-RNA-C (C\textsubscript{β}), β-L-RNA-G (G\textsubscript{β}) and β-L-RNA-U (U\textsubscript{β}).

<table>
<thead>
<tr>
<th>Name</th>
<th>Position of modification</th>
<th>Sequence (5’-3’)</th>
<th>Melting curve fits</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>T\textsubscript{M} (˚C)</td>
<td>ΔT\textsubscript{M} (˚C)</td>
</tr>
<tr>
<td>RE31</td>
<td></td>
<td>GTGACGTAGGTTGGTTGGGGCGTCAC</td>
<td>51.6 ± 0.4</td>
<td>0</td>
</tr>
<tr>
<td>O1</td>
<td>T\textsuperscript{11}</td>
<td>GTGACGTAGG\textsuperscript{U}GTGGTTGGGGCGTCAC</td>
<td>53.9 ± 0.4</td>
<td>2.3</td>
</tr>
<tr>
<td>O2</td>
<td>T\textsuperscript{15}</td>
<td>GTGACGTAGGTTGG\textsuperscript{A}GTGGTTGGGGCGTCAC</td>
<td>53.4 ± 0.2</td>
<td>1.8</td>
</tr>
<tr>
<td>O3</td>
<td>T\textsuperscript{15}</td>
<td>GTGACGTAGGTTGG\textsuperscript{C}GTGGTTGGGGCGTCAC</td>
<td>54.0 ± 0.1</td>
<td>2.4</td>
</tr>
<tr>
<td>O4</td>
<td>T\textsuperscript{15}</td>
<td>GTGACGTAGGTTGG\textsuperscript{G}GTGGTTGGGGCGTCAC</td>
<td>54.2 ± 0.3</td>
<td>2.6</td>
</tr>
<tr>
<td>O5</td>
<td>T\textsuperscript{15}</td>
<td>GTGACGTAGGTTGG\textsuperscript{U}GTGGTTGGGGCGTCAC</td>
<td>54.4 ± 0.2</td>
<td>2.8</td>
</tr>
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<td>O6</td>
<td>T\textsuperscript{20}</td>
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<td>52.6 ± 0.5</td>
<td>1.0</td>
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<td>O7</td>
<td>T\textsuperscript{11}, T\textsuperscript{15}, T\textsuperscript{20}</td>
<td>GTGACGTAGG\textsuperscript{U}GTGGTTGGGGCGTCAC</td>
<td>57.0 ± 0.1</td>
<td>5.4</td>
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<td>O8</td>
<td>T\textsuperscript{2}, A\textsuperscript{4}, G\textsuperscript{6}, A\textsuperscript{8}</td>
<td>GT\textsuperscript{G}G\textsubscript{A}A\textsubscript{C}G\textsubscript{U}TA\textsubscript{A}GGTTGGTTGGGGCGTCAC</td>
<td>62.4 ± 0.4</td>
<td>10.8</td>
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<td>O9</td>
<td>T\textsuperscript{2}, A\textsuperscript{4}, G\textsuperscript{6}, A\textsuperscript{8}, T\textsuperscript{15}</td>
<td>GT\textsuperscript{G}G\textsubscript{A}A\textsubscript{C}G\textsubscript{U}TA\textsubscript{A}GGTTGGTTGGGGCGTCAC</td>
<td>61.9 ± 0.9</td>
<td>10.3</td>
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<td>O10</td>
<td>T\textsuperscript{2}, A\textsuperscript{4}, G\textsuperscript{6}, A\textsuperscript{8}, T\textsuperscript{15}</td>
<td>GT\textsuperscript{G}G\textsubscript{A}A\textsubscript{C}G\textsubscript{U}TA\textsubscript{A}GGTTGGTTGGGGCGTCAC</td>
<td>63.1 ± 0.6</td>
<td>11.5</td>
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<tr>
<td>O11</td>
<td>T\textsuperscript{2}, A\textsuperscript{4}, G\textsuperscript{6}, A\textsuperscript{8}, T\textsuperscript{15}</td>
<td>GT\textsuperscript{G}G\textsubscript{A}A\textsubscript{C}G\textsubscript{U}TA\textsubscript{A}GGTTGGTTGGGGCGTCAC</td>
<td>64.2 ± 0.4</td>
<td>12.6</td>
</tr>
<tr>
<td>O12</td>
<td>T\textsuperscript{2}, A\textsuperscript{4}, G\textsuperscript{6}, A\textsuperscript{8}, T\textsuperscript{15}</td>
<td>GT\textsuperscript{G}G\textsubscript{A}A\textsubscript{C}G\textsubscript{U}TA\textsubscript{A}GGTTGGTTGGGGCGTCAC</td>
<td>60.8 ± 0.5</td>
<td>9.2</td>
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<tr>
<td>O13</td>
<td>T\textsuperscript{2}, A\textsuperscript{4}, G\textsuperscript{6}, A\textsuperscript{8}</td>
<td>GT\textsuperscript{G}G\textsubscript{A}A\textsubscript{C}G\textsubscript{U}TA\textsubscript{A}GGTTGGTTGGGGCGTCAC</td>
<td>53.2 ± 0.2</td>
<td>1.6</td>
</tr>
</tbody>
</table>

\(a\) buffer: 100 mM KCl, 20 mM sodium cacodylate, 0.5 mM EDTA\(\textsubscript{(Na)}\), pH 7.0.

\(b\) The measurements were performed at 295 nm.

\(c\) The measurements of selected RE31 variants were also performed in 1 x PBS buffer, pH 7.4. The data are in Table S1 (Supporting Information).
Table 2. The anticoagulant properties (TT, AE)\(^a\), dissociation constant (K\(_d\)) and biostability (T\(_{1/2}\)) of RE31 variants modified with UNA-A (A\(^\beta\)), UNA-C (C\(^\beta\)), UNA-G (G\(^\beta\)), UNA-U (U\(^\beta\)), LNA-A (A\(^\lambda\)), LNA-G (G\(^\lambda\)), LNA-T (T\(^\lambda\)), β-l-RNA-A (A\(^\beta\)), β-l-RNA-C (C\(^\beta\)), β-l-RNA-G (G\(^\beta\)) and β-l-RNA-U (U\(^\beta\)).

<table>
<thead>
<tr>
<th>Name</th>
<th>Position of modification</th>
<th>Sequence (5'-3')</th>
<th>TT(^{\pm\delta}) [s]</th>
<th>AE [s]</th>
<th>K(_d) [nM]</th>
<th>T(_{1/2}) [min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>RE31</td>
<td></td>
<td>GTGACGTAGTTGGTGTTGGGGGCGTCAC</td>
<td>30.07 ± 0.40</td>
<td>11.09 ± 0.48</td>
<td>1.34 ± 0.05</td>
<td>364</td>
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<tr>
<td>O1</td>
<td>T(^{11})</td>
<td>GTGACGTAGGUL(^{U})TGTTGGTTGGGGGCGTCAC</td>
<td>25.80 ± 0.67</td>
<td>6.87 ± 0.39</td>
<td>n.d.</td>
<td>378</td>
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<tr>
<td>O2</td>
<td>T(^{15})</td>
<td>GTGACGTAGTTGGGTAC(^{U})TGTTGGGGGCGTCAC</td>
<td>30.53 ± 1.23</td>
<td>11.55 ± 1.13</td>
<td>0.76 ± 0.02</td>
<td>1062</td>
</tr>
<tr>
<td>O3</td>
<td>T(^{15})</td>
<td>GTGACGTAGGTTGGC(^{U})GTGGTTGGGGGCGTCAC</td>
<td>35.88 ± 1.71</td>
<td>17.08 ± 2.31</td>
<td>0.43 ± 0.01</td>
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<tr>
<td>O4</td>
<td>T(^{15})</td>
<td>GTGACGTAGTTGGGTGG(^{U})GTGGTTGGGGGCGTCAC</td>
<td>30.98 ± 1.98</td>
<td>12.16 ± 1.64</td>
<td>0.68 ± 0.05</td>
<td>1018</td>
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<tr>
<td>O5</td>
<td>T(^{15})</td>
<td>GTGACGTAGTTGGGTGG(^{U})GTGGTTGGGGGCGTCAC</td>
<td>27.83 ± 0.28</td>
<td>9.66 ± 0.48</td>
<td>0.75 ± 0.05</td>
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<tr>
<td>O6</td>
<td>T(^{20})</td>
<td>GTGACGTAGTTGGTTGCTG(^{U})TGGGGCGTCAC</td>
<td>22.90 ± 0.73</td>
<td>4.27 ± 0.46</td>
<td>n.d.</td>
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<tr>
<td>O7</td>
<td>T(^{11}), T(^{15}), T(^{20})</td>
<td>GTGACGTAGGUL(^{U})TGTTGGTTGGGGGCGTCAC</td>
<td>20.80 ± 0.80</td>
<td>0.65 ± 0.30</td>
<td>n.d.</td>
<td>538</td>
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<tr>
<td>O8</td>
<td>T(^{2}), A(^{4}), G(^{6}), A(^{8})</td>
<td>GT(^{G})A(^{L})CG(^{G})T(^{A})GTGGTTGGTTGGGGGCGTCAC</td>
<td>29.10 ± 0.37</td>
<td>10.09 ± 0.75</td>
<td>1.75 ± 0.12</td>
<td>&gt; 1440</td>
</tr>
<tr>
<td>O9</td>
<td>T(^{2}), A(^{4}), G(^{6}), A(^{8}), T(^{15})</td>
<td>GT(^{G})A(^{L})CG(^{G})T(^{A})GTGGTTGGGGGCGTCAC</td>
<td>42.12 ± 2.51</td>
<td>23.27 ± 3.06</td>
<td>1.15 ± 0.02</td>
<td>&gt; 1440</td>
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<tr>
<td>O10</td>
<td>T(^{2}), A(^{4}), G(^{6}), A(^{8}), T(^{15})</td>
<td>GT(^{G})A(^{L})CG(^{G})T(^{A})GTGGTTGGGGGCGTCAC</td>
<td>41.17 ± 1.18</td>
<td>21.58 ± 0.33</td>
<td>0.90 ± 0.03</td>
<td>&gt; 1440</td>
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<tr>
<td>O11</td>
<td>T(^{2}), A(^{4}), G(^{6}), A(^{8}), T(^{15})</td>
<td>GT(^{G})A(^{L})CG(^{G})T(^{A})GTGGTTGGGGGCGTCAC</td>
<td>33.05 ± 1.18</td>
<td>14.10 ± 0.98</td>
<td>n.d.</td>
<td>&gt; 1440</td>
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<tr>
<td>O12</td>
<td>T(^{2}), A(^{4}), G(^{6}), A(^{8}), T(^{15})</td>
<td>GT(^{G})A(^{L})CG(^{G})T(^{A})GTGGTTGGGGGCGTCAC</td>
<td>29.58 ± 1.29</td>
<td>10.68 ± 0.18</td>
<td>n.d.</td>
<td>&gt; 1440</td>
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<tr>
<td>O13</td>
<td>T(^{2}), A(^{4}), G(^{6}), A(^{8}), U(^{15})</td>
<td>GT(^{G})A(^{L})CG(^{G})T(^{A})GTGGTTGGGGGCGTCAC</td>
<td>26.77 ± 0.42</td>
<td>8.13 ± 0.81</td>
<td>n.d.</td>
<td>&gt; 1440</td>
</tr>
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</table>

\(^a\) Thrombin time value for plasma without aptamers was in the range of 17.4 – 21.6 s.

\(^b\) The measurements were performed for 0.165 μM aptamer concentration.
TABLE OF CONTENTS GRAPHIC