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Aptamer Conjugated RNA/DNA Hybrid Nanostructures Designed for Efficient Regulation of Blood Coagulation

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Abstract

Disruptions to the hemostatic pathway can cause a variety of serious or even life-threatening complications. Situations in which the coagulation of blood has become disturbed necessitate immediate care. Thrombin-binding aptamers are single-stranded nucleic acids that bind to thrombin with high specificity and affinity. While they can effectively inhibit thrombin, they suffer from rapid degradation and clearance in vivo. These issues are resolved, however, by attaching the therapeutic aptamer to a nucleic acid nanostructure. The increased size of the nanostructure-aptamer complex elongates the post-infusion half-life of the aptamer. These complexes are also immunoquiescent. A significant benefit of using nucleic acids as anticoagulants is their rapid deactivation by the introduction of a nanostructure made fully from the reverse complement of the therapeutically active nanostructure. These advantages make nanoparticle conjugated antithrombin aptamers a promising candidate for a rapidly reversible anticoagulant therapy.

Keywords

Thrombin; Anticoagulant; Aptamer; RNA/DNA hybrid

1 Introduction

Aptamers are emerging as a clinically relevant category of therapeutic nucleic acids. Owing to their low cost, high affinity, high specificity, and biodegradability, aptamers have become increasingly researched for their therapeutic potential [1, 2]. By binding a protein with a specifically targeted aptamer, the function of that protein can be disrupted [1, 3, 4]. A major benefit of using aptamers for anticoagulation is that the restoration of coagulation can be readily accomplished by introducing the reverse complement oligo, which inactivates the aptamer by forming a duplex with it [4–7]. A key issue with the use of aptamers as anticoagulants has been their rapid clearance, which has hindered their broader clinical use [8–10]. By attaching aptamers to RNA/DNA hybrid nanostructures, fibers in this example (aptamer fibers), the post-infusion half-life, and blood stability of the aptamer can be improved by increasing its size/molecular weight [1]. The nanostructure-aptamer complexes also retain the ability to be deactivated by the introduction of the reverse complement nanostructure to form short duplexes which are rapidly excreted [1]. The deactivation of the aptamers by the appropriate antidote oligonucleotide, typically the reverse-complement

strand of the aptamer, takes place, primarily, through Watson-Crick base pairing [1, 11, 12]. Through the disruption of the aptamer structure, the bound protein is released and able to return to its standard function [1]. In order to most effectively incorporate the aptamer to the nanostructure, the aptamer sequence can be added to the 5′ or 3′ end of one of the constituent oligos of the nanostructure or by using toe-hold regions [1, 13]. To ensure that no unintended structures will be formed, it is recommended that the sequences to be used are checked against each other and optimized, if necessary, with a secondary structure prediction tool, such as NUPACK [14].

The aptamer fiber used in this example is an adaptation of the NU172 formulation of ARC183, which was the first clinically tested thrombin-binding aptamer [1, 15–17]. This was done by extending the 3′ end of the DNA section of an RNA/DNA hybrid fiber with the NU172 sequence. The RNA fills gaps between the DNA toe-hold ends to maintain the double-helical structure of the construct. With one aptamer on each DNA strand in the fiber, the fiber is potentially able to bind multiple thrombin molecules. Due to the differences in thermodynamic stability of the fibrous structures as opposed to RNA/RNA and DNA/DNA duplexes that are fully complementary, the aptamer fiber is able to be deactivated by the administration of an antidote fiber, which is the reverse complement of the therapeutic fiber. This deactivation occurs through the isothermal association of fully complementary RNA and DNA duplexes as the aptamer and antidote fibers combine in solution [1].

Blood coagulation is a biochemical process that prevents blood loss due to blood vessel damage. It is complex as it involves not only the plasma proteins, thrombocytes, or platelets but other cells, such as leukocytes and endothelial cells [18]. The interaction among these components is critical in maintaining hemostasis and preventing life-threatening bleeding. When a nanoparticle formation is administrated systemically in blood, its effect on blood coagulation must be assessed. There are three main pathways involved in the coagulation cascade: intrinsic (refer to the contact activation pathway which is activated by damaged internal surfaces); extrinsic (refer to the tissue factor pathway which is activated by damaged external surfaces) [19]; and intrinsic and extrinsic pathways converge into the common pathway, specifically at thrombin. As thrombin is the enzyme where the intrinsic and extrinsic pathways meet, it is a promising target for anticoagulant medications and one of the most common targets for aptamer-based anticoagulants [1, 11].

The functionality of each pathway can be evaluated separately using specialized tests. The activated partial thromboplastin time (APTT) assay is used to assess the intrinsic pathway, the prothrombin time (PT) assay measures the extrinsic pathway, and the thrombin time (TT) indicates the functionality of the common pathway. RNA/DNA nanostructures are incubated with fresh collected and pooled human plasma. If the nanoparticle interacts with any plasma proteins responsible for the blood clot formation pathway, this interaction may alter the protein function, which will be reflected by an increase in coagulation time when comparing the coagulation time with standard controls for each assay, as is the case for aptamer fibers [1].

2 Materials

1. Computer with appropriate oligonucleotide secondary structure prediction software (e.g., NUPACK) [14].
2. Human blood from at least three donors.
3. Neoplastine Cl. This reagent is supplied as lyophilized powder and the reconstitution buffer. Reconstitute condition according to the manufacturer's instructions, and use within the time specified by the manufacturer [20] (see Note 1).
4. Thrombin.
5. 0.025 M $CaCl_2$.
6. Owren-Koller buffer.
7. PTT-A reagent.
8. Normal and abnormal control plasma (CoagControl N + ABN).
9. RPMI-1640 Cell Culture Media.
10. Sterile and endotoxin-free phosphate buffered saline solution.
11. Pipettes covering the range of 0.05–10 mL.
12. 1.25 mL Finntip pipette tips.
13. 4-well cuvettes.
14. Metal balls for coagulometer.
15. Coagulometer (Diagnostica Stago Art4) (see Note 1).
16. Refrigerator.
17. Centrifuge capable of operating at 2500× *g*.
18. Purified oligonucleotides for the intended nanostructure.
19. Hybridization buffer (89 mM Tris, 80 mM boric acid (pH 8.2), 2 mM magnesium chloride, 2 mM potassium chloride) (see Note 2).
20. Dry heat bath.
21. Gel imager or UV-transilluminator.
22. 8% non-denaturing polyacrylamide (19:1) gel.
23. Power source for gel electrophoresis.
24. Gel electrophoresis chamber.
25. Ethidium bromide (0.5 μg/mL).
26. LAL assay kit (if using samples for further immunological or in vivo testing) (see Note 3).

3 Methods

3.1 Preparation of Study Samples

1. Assemble the aptamer fibers by combining individual monomers at their appropriate concentrations in hybridization buffer, made with LAL grade water, and dilute to the appropriate volume with LAL grade water.
2. Heat the solution to 95 °C for 5 min.
3. Incubate the solution at room temperature for 20 min.
4. Analyze the assembly of the nanostructures at 4 °C on 8 % non-denaturing native polyacrylamide (19:1) gel electrophoresis (native-PAGE) run for 30 min at 300 V in hybridization buffer.
5. Stain the gels with ethidium bromide (0.5 μg/mL) or by using the fluorescence of labeled oligonucleotides. Visualize the assemblies with a gel imager or UV-transilluminator.
6. All assemblies for immunological and in vivo studies should be further tested for bacterial endotoxins by a kinetic turbidity limulus amoebocyte lysate (LAL) assay.

3.2 Preparation of Test and Normal and Abnormal Control Plasmas

3.2.1 Blood Sample Preparation and General Testing Guidelines

1. Freshly collected whole blood is used within 1 h after collection.
2. Spin the blood for 10 min, 2500× *g* at 20–22 °C; collect plasma and pool from at least two donors.
3. Pooled plasma is stable for 8 h at room temperature. Do not refrigerate or freeze.
4. Analyze two duplicates (four total samples) of test plasma in each coagulation assay; to verify that the plasma functionality is not affected throughout the experiment, run one duplicate before the nanoparticle samples and the second duplicate at the end of each run.

3.2.2 Test Plasma (With Aptamer-NANPs)—Combine 50 μL of aptamer-NANPs and 450 μL of test plasma in a microcentrifuge tube; mix well and incubate for 30 min at 37 °C.

3.2.3 Normal and Abnormal Control Plasmas

1. Use 1 mL of distilled water to reconstitute each of the lyophilized control plasmas.
2. Leave the solutions at room temperature for 30 min prior and mix thoroughly before use.
3. Keep the unused portion refrigerated, and use it within 48 h after reconstitution. These plasma samples are used as instrument controls.

3.2.4 Neoplastin, PTT-A Reagent, and Thrombin Preparation (Used to Initiate Plasma Coagulation)—

Lyophilized assay-specific reagents used in this assay are for initiating plasma coagulation, including Neoplastine for PT assay, PTT-A reagent for APTT assay, and thrombin for TT assay. Reconstitute the reagents according to the manufacturer's instructions and use fresh or refrigerated, use them within the time specified by the manufacturer.

3.3 Plasma Coagulation Assay Procedure

1. Allow the instrument to warm up for 5–10 min prior to use. Set up instrument test parameters for each assay (Table 1).
2. Prepare all reagents and warm them to 37 °C prior to use. All lyophilized reagents should be reconstituted at least 30 min prior to use.
3. Place cuvettes on the coagulometer into A, B, C, and D test rows (see Note 4.1).
4. Add one metal ball into each cuvette, and allow the cuvette and ball to warm for at least 3 min prior to use.
5. For PT or TT test, add 100 μL of control or test plasma to a cuvette. For APTT test, add 50 μL (Table 1). Prepare two wells for each test tube prepared in **step 3.2.2**.
6. This step is only for APTT: Add 50 μL of PTT-A reagent to plasma samples in cuvettes.
7. Pressing the A, B, C, or D timer buttons to start the timer for each test row. The timer will beep 10 s before the time is up. When this happens, immediately transfer cuvettes to the PIP row, and press the PIP button to activate the pipettor (see Note 4.1).
8. Depending on which assay is performed, add the corresponding coagulation activation reagent and volume to each cuvette, and record the coagulation time when the time is up (Table 1).

3.4 Calculations and Data Interpretation

1. For all three assays (PT, APTT, and TT), a percent coefficient of variation should be calculated for each control or test sample according to the following formula: $\%\mathrm{CV} = \frac{\mathrm{SD}}{\mathrm{Mean}} \times 100\%$. %CV between replicates of test plasma samples should be within 25%. If the test samples have a %CV greater than 25, the test samples should be reanalyzed. This assay performance requirement is based on the requirements for bioanalytical method validation guidance for the industry [21].
2. Normal and abnormal control plasma should have coagulation time within the time established by the certifying laboratory (e.g., for the most batches of control plasmas, normal coagulation time in the PT assay is ≤13.4 s, APTT is ≤34.1 s, and TT is ≤21 s; abnormal control plasma coagulation time should be above these limits) (Table 2). When the untreated plasma sample coagulates within

normal time limits, and normal and abnormal control perform as described above, both the instrument and the test plasma are qualified for use in this test.

3. When the coagulation time of the test plasma samples after exposure to nanoparticles is within normal limits, nanoparticles do not affect the assay coagulation cascade.

4. When the plasma coagulation time in plasma samples exposed to nanoparticles is prolongated, it suggests that the test particle either depletes or inhibits coagulation factors. There is no guidance on the degree of prolongation, but in general, a prolongation of twofold or more than that in the untreated control is considered physiologically significant.

4 Notes

1. This protocol is based on the semi-automatic STArt4 coagulometer from Diagnostica Stago [20]. If using a different instrument, please follow the operational guidelines recommended by the instrument manufacturer.

2. It is critical to ensure that the buffer contains a sufficient amount of K^+ as this ion is critical for the formation of G-tetraplexes, which are commonly found in thrombin binding aptamers [22].

3. While they are a necessary step in the development of any therapeutic agent, immunological and in vivo testing are outside of the scope of this chapter and, as such, will not be discussed here.

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Table 1

Types of plasma coagulation assay, its control, instrument setting, plasma and coagulation activation reagent volumes, and normal coagulation time

		Instrument settings				Volumes		
Assay	**Control**	**Max time (s)**	**Incubation time (s)**	**Single/ Duplicate**	**Precision**	**Plasma and reagent volumns**	**Coagulation activation reagent**	**Normal Coagulation time (s)**
PT-prothrombin time (Neoplastine)	Coag. control N + ABN	60	120	Duplicate	5%	100 µL plasma	100 µL Neoplastine (PIP position 4)	≤13.4
APTT-activated partial thromboplastin time	Coag. control N + ABN	120	180	Duplicate	5%	50 µL plasma + 50 µL PTTA reagent	50 µL $CaCl_2$ (PIP position 2)	≤34.1
TT-thrombin time	Coag. control N + ABN	60	60	Duplicate	5%	50 µL PTTA reagent	100 µL thrombin (PIP position 4)	≤21

Table 2

Plasma coagulation assessment: (Top) Results of Prothrombin Time (PT), Activated Partial Thromboplastin Time (APTT), and Thrombin Time (TT) of Anti-Thrombin Fibers for Their Abilities in Delaying the Coagulation in Donors from the United States and Brazil, Displaying Some Minor Regional Variations; (Bottom) Restoration of Normal Coagulation Time by Addition of Kill-Switch Fibers [1]

	PT		APTT		TT	
Sample	**U.S. data (13.4 s)**	**Brazil data (12.1 s)**	**U.S. data (37.0 s)**	**Brazil data (36.5 s)**	**U.S. data (21.0 s)**	**Brazil data (16.6 s)**
NU fiber	17.3 ± 0.2	16.6 ± 0.3	>120.00	89.4 ± 1.4	>60.00	26.8 ± 0.2
RA fiber	16.6 ± 0.4	19.7 ± 0.2	70.9 ± 5.0	104.7 ± 1.9	>60.00	31.6 ± 0.6
NU/RA fiber	16.8 ± 0.4	17.7 ± 0.0	81.3 ± 4.3	100.4 ± 1.5	>60.00	28.6 ± 0.0
DNA-RNA fiber	11.8 ± 0.3	13.5 ± 0.2	36.7 ± 0.8	40.2 ± 0.1	15.8 ± 0.7	19.3 ± 0.1
Plasma	10.8 ± 0.2	13.6 ± 0.2	33.4 ± 1.2	28.6 ± 0.3	16.6 ± 0.3	19.4 ± 0.1
NU172 aptamer	10.9 ± 0.2	14.1 ± 0.2	38.0 ± 1.5	39.5 ± 0.1	15.3 ± 0.8	19.0 ± 0.2
RA-36 aptamer	11.3 ± 0.4	15.3 ± 0.5	41.5 ± 3.2	45.5 ± 0.8	16.6 ± 0.8	26.5 ± 0.5

Sample	**PT (13.4 s)**	**APTT (37.0 s)**	**TT (21.0 s)**
Control (normal)	12.7 ± 0.2	36.8 ± 1.5	21.4 ± 0.6
Control (abnormal)	20.7 ± 0.4	71.8 ± 1.4	41.3 ± 1.4
Plasma	9.7 ± 0.0	29.4 ± 0.2	16.1 ± 0.3
NU172 aptamer	16.1 ± 0.5	37.9 ± 0.7	25.3 ± 1.8
RA-36 aptamer	17.3 ± 0.3	44.2 ± 0.6	45.0 ± 1.4
NU fiber	18.6 ± 1.7	101.7 ± 2.0	59.5 ± 0.5
NU fiber + kill-switch	10.9 ± 0.1	36.7 ± 0.2	17.5 ± 0.4
RA fiber	20.8 ± 0.1	89.9 ± 1.1	>60.00
RA fiber + kill-switch	13.7 ± 0.1	42.9 ± 0.3	44.7 ± 0.1
NU/RA fiber	16.8 ± 0.9	95.3 ± 3.0	>60.00
NU/RA fiber + kill-switch	10.6 ± 0.3	38.5 ± 0.4	18.4 ± 0.6