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Research paper

Complex formation with protamine prolongs the thrombin-inhibiting effect of DNA aptamer in vivo

V.A. Spiridonova [a, *], T.M. Novikova [a], D.M. Nikulina [b], T.A. Shishkina [b], E.V. Golubkina [b], O.S. Dyukareva [b], N.N. Trizno [b]

[a] A.N. Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow, Russia
[b] Astrakhan State Medical University Ministry of Public Health of the Russian Federation, Astrakhan, Russia

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ABSTRACT

Antithrombin DNA aptamersRE31 are single-chain oligonucleotides that fold into three-dimensional forms allowing them to bind the enzyme with high affinity and inhibit its activity in vivo. They are rapidly degraded by a nonspecific nuclease, and, to prolong the lifetime of the aptamer DNA in the bloodstream, it is necessary to coat it with a polymer envelope. A new approach to solving this problem based on preparation of DNA–polyelectrolyte complexes with a minimal particle size that can circulate with blood flow. In our experiments, the negatively charged aptamer DNA RE31 was coated step-by-step with positively charged protamine. They had protamine/aptamer ratios of 0.2/1 and 0.4/1 by charge, with particle size being determined by dynamic light scattering. The aptamer DNA–protamine complexes were administered to rats, followed by ex vivo analysis of blood samples. The results showed that prothrombin time (PT) increased by a factor of 5.6–6.7 within 2 h after injection and remained at approximately the same level for 6 h, while injections of pure protamine did not lead to any noticeable change in clotting time. Thus, complexation with protamine proved to prolong the inhibitory activity of the RE31 DNA aptamer.

1. Introduction

Thrombin is a protease that plays a key role in the coagulation cascade. This enzyme initiates clotting by hydrolyzing fibrinogen, activating blood platelets and a set of plasma procoagulant factors. Direct thrombin inhibitors are a promising class of antithrombotic agents, and their development and introduction into clinical practice can significantly contribute to treatment and prevention of various thrombotic disorders [1,2].

Recent advancements in research methods have made it possible to produce a new class of direct thrombin inhibitors based on single-stranded DNA or RNA fragments (aptamers) that selectively bind with high affinity to the target molecule [3,4]. Aptamers are small DNA/RNA fragments isolated by selection from nucleic acid combinatory library [5]. In essence, aptamers are functional equivalents of monoclonal antibodies [6], but they show several advantages over the latter: they have low immunogenicity, are technologically feasible to produce, and can be rapidly inactivated by antidotes consisting of complementary nucleotide sequences [7–9]. Research on the production and properties of DNA aptamers to various protein targets as prospective drugs is underway in different countries [10,11].

Among the methods for producing nucleic acid aptamers, the best known is SELEX, a technology based on the ability of single-stranded nucleic acids to be copied and to fold into complex tertiary structures. This method has been discussed in several reviews [12–15].

Selection of a family of thrombin-inhibiting DNA aptamers with affinity constants from 25 to 200 nM (15TBA 5′-GGTTGGTGTGGTTGG) was first reported in 1992 [16]. Since then, structurally different families of aptamers against thrombin have been engineered in many laboratories around the world, nominally of lengths of 27–31 nucleotides (HD22 5′- AGTCCGTGGTAGGGCAGGTTGGGGTGAC and 31TGT 5′-CACTG GTA GG TT GG TGT GG TT GG GGC CAGTG) [17–19] which have prolonged clotting times several-fold.

We have previously created a new antithrombin RE31aptamer

* Corresponding author. A.N. Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Leninskie Gory, 1, build. 40, Moscow, Russia.
E-mail address: spiridon@belozersky.msu.ru (V.A. Spiridonova).

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based on the known 15-TBA quadruplex DNA, with additional sequences [20,21]. It consists of 31 nucleotides and carries 30 negative charges per molecule, or 1.8×10^{25} negative charges per mole (Fig. 1). Experiments have shown that this aptamer prolongs clotting time in vitro, but its lifetime in the bloodstream in vivo does not exceed 30 min, probably because of its hydrolysis by nonspecific nucleases and active renal removal.

To increase the stability of aptamers in the bloodstream, it has been proposed to modify the structure of the aptamers themselves, i.e. to modify the phosphodiester bond connecting nucleotides, or to introduce protective groups into the structure of the ribose or nitrogenous bases, which can dramatically change their affinity for protein targeting [22–26].

Another way the lifetime of a DNA aptamer in circulating blood can be prolonged is by complexing it with a polymer carrier (e.g., a protein) [27]. A promising candidate for such a carrier is protamine, a polycationic peptide that has various applications in clinical practice. In particular, it is used for drug delivery and is known to prolong the absorption of injected insulin suspension [28]. Protamine has a molecular weight of 5.8 kDa, is rich in basic amino acids, especially arginine (70–80%) [29], and therefore has 1.26×10^{25} positive charges per mole. Thus inclusion in a complex with DNA, protamine neutralizes the negative charge of the DNA phosphate groups. Commonly called a Poly-Electrolyte Complex (PEC), this is comprised of polymers of biological macro-molecules, containing groups capable of dissociation in water, which may interact to create an ensemble of charged components.

To address the important problem of creating antithrombin drugs with optimal properties within animal models, we wanted to test the suggestion that in a complex with protamine, the DNA aptamer would be retained longer within in the blood stream before disintegration by non specific nuclease activity.

In this study, we prepared RE31-protamine complexes containing different proportions of polycations, and tested them for the thrombin specific anticoagulant activity.

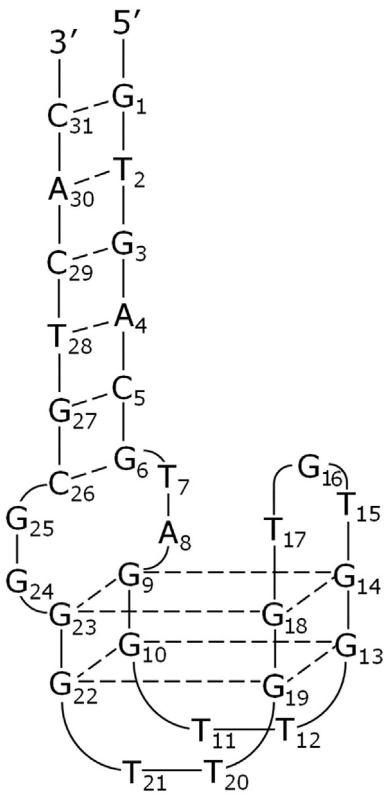

Fig. 1. The structure of RE-31DNA-aptamer.

2. Materials and methods

2.1. Reagents and chemicals

Buffer solution salts, protamine sulphate (from Salmon sperm, Mw 5.8 kDa) and other reagents were purchased from Sigma-Aldrich (US). Aptamers were synthesized by Syntol (Moscow, Russia).

2.2. Preparation of polyelectrolyte complexes (PEC)

Protamine (3.1 mg) was dissolved in Milli-Q water to a concentration of 620 μg/mL (0.107 mM), while aptamer RE31 [30] was at a concentration of 400 μM. Complex formation aptamer RE31 with protamine were prepared at different rations.

2.3. Characterization of the size of the aptamer/protamine complexes

We produced RE31/protamine complexes with different charge ratios and have been observing a change in the size of the resulting particles. The formation of PEC was monitored by dynamic laser light scattering, using a Zetasizer Nano-ZS instrument (Malvern Instruments, UK). Laser wavelength was set at 633 nm Measurements were conducted at 20 °C. Analysis utilized three parallel samples, each of 5 measurements taken for 10 runs. The confidence interval was determined by using the software package “Statistical processing of AtteStat”. These resulted in a narrow specific fractionation range for the ratios of aptamer/protamine. Complex size for these ratios was comparable, and an increase in the protamine component had little effect on overall particle diameter (Table 1).

For our analysis, we chose preparations of particles containing aptamer/protamine charge ratios of 1/0.2 and 1/0.4.

2.4. Animals

Experiments were done in 8–10 week white outbred rats. There were seven animals in each four experimental groups and five animals in the control group. All the animals were maintained under standard environmental conditions and were provided with feed and water ad libitum.

All experiments on animals followed the protocol was approved by local ethical committee of Astrakhan State Medical University according International recommendations (ethical code) to the conduct of biomedical research using animals (CIOMS, Geneva, 1985). Drug delivery was administered with sodium ethaminal (40 mg/kg) intraperitoneally.

2.5. Experiments in vivo

The formation of PEC (RE31 with protamine) was prepared in the PBS buffer. Rats of experimental groups received intraperitoneal injections of (1) aptamer RE31 alone (0.5 ml of 10 μM solution); (2) 0.5 ml PEC solution with an aptamer/protamine charge ratio of 1/0.2, (3) 0.5 ml PEC solution with an aptamer/protamine charge ratio of 1/0.4; and (4) protamine alone (0.5 ml of 1.46 μM and 2.9 μM solutions). (5) Control animals were injected with 0.5 ml of saline.

2.6. Clotting assays

Blood samples were taken 2, 6, and 12 h after injection (in the

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