Journal of Controlled Release 221 (2016) 9–17

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Gold nanoparticles modified with self-assembled hybrid monolayer of triblock aptamers as a photoreversible anticoagulant

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ARTICLE INFO

Article history:
Received 10 July 2015
Received in revised form 4 November 2015
Accepted 27 November 2015
Available online 28 November 2015

Keywords:
Aptamers
Self-assemble
Gold nanoparticles
Thrombin
Anticoagulants

ABSTRACT

We demonstrated that thrombin-binding aptamer-conjugated gold nanoparticles (TBA–Au NPs), prepared from a self-assembled hybrid monolayer (SAHM) of triblock aptamers on Au NPs (13 nm), can effectively inhibit thrombin activity toward fibrinogen. The first block poly(adenine) at the end of the triblock TBA was used for the self-assembly on Au NP surface. The second block, in the middle of TBA, was composed of oligonucleotides that could hybridize with each other. The third block, containing TBA_{15} (15-base, binding to the exosite I of thrombin) and TBA_{29} (29-base, binding to the exosite II of thrombin) provided bivalent interaction with thrombin. The SAHM triblock aptamers have optimal distances between TBA_{15} and TBA_{29}, aptamer density, and orientation on the Au NP surfaces. These properties strengthen the interactions with thrombin (K_d = 1.5×10^{-11} M), resulting in an extremely high anticoagulant potency. The thrombin clotting time mediated by SAHM TBA_{15}/TBA_{29}–Au NPs was >10 times longer than that of four commercially available drugs (heparin, argatroban, hirudin, or warfarin). In addition, the rat-tail bleeding assay time further demonstrated that the SAHM TBA_{15}/TBA_{29}–Au NPs were superior to heparin. The SAHM TBA_{15}/TBA_{29}–Au NPs exhibited excellent stability in the human plasma (half-life >14 days) and good biocompatibility (low cytotoxicity and hemolysis). Most interestingly, the inhibition by SAHM TBA_{15}/TBA_{29}–Au NPs was controllable by the irradiation of green laser, *via* heat transfer-induced TBA release from Au NPs. Therefore, these easily prepared (self-assembled), low cost (non-thiolated aptamer), photo-controllable, multivalent TBA_{15}/TBA_{29}–Au NPs (high density of TBA_{15}/TBA_{29} on Au NPs) show good potential for the treatment of various diseases related to blood-clotting disorders. Our study opens up the possibility of regulation of molecule binding, protein recognition, and enzyme activity using SAHM aptamer-functionalized nanomaterials.

1. Introduction

Hemostasis is a series of normal physiological processes; its main steps are vascular spasms, the formation of platelet plugs for temporary blockage of vessel breaks, and blood coagulation (formation of fibrin clots) [1]. Thromboembolic diseases induced by overactive blood clotting (thrombosis), such as arteriosclerosis, deep vein thrombosis, pulmonary embolism, myocardial infarction, and stroke, are a major cause of human mortality [2–4]. Anticoagulant therapy is used to prevent and control thrombosis-related diseases caused by hypercoagulability [5]. Thrombin (activated factor II) plays many vital roles in the blood coagulation system [6]. The most important function of thrombin is hydrolysis of soluble fibrinogen into insoluble strands of fibrin, which form a mesh for the thrombus scaffold [7]. Thrombin is also involved in many other coagulation-related reactions, such as feedback amplification of factors V, VIII, XI, and XII, and it initiates anticoagulation by activating protein C and platelets [6]. Therefore, thrombin is a very attractive target for the synthesis of inhibitors for the development of anticoagulants and treatment of thrombotic diseases.

Several direct thrombin inhibitors (DTIs), such as unfractionated heparin (UFH), hirudin, argatroban, dabigatran, and pradaxa have been clinically proven and are widely used as anticoagulant drugs [8,9]. Although these drugs are highly effective thrombin inhibitors, their clinical use in thromboembolic diseases is hampered by a range of adverse reactions [10]. For example, UFH and low-molecular-weight heparin (LMWH) increase the risk of thrombocytopenia and osteoporosis [11,12]. Other serious disadvantages of existing DTIs are the lack of efficiently reversible control of thrombin activity and low stability or a short lifetime in the blood. Thus, we need novel thrombin inhibitors with high efficacy, selectivity, and safety for treating coagulation-related cardiovascular diseases.

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http://dx.doi.org/10.1016/j.jconrel.2015.11.028
0168-3659/

The development of enzyme inhibitors based on nucleic acid aptamers using an *in vitro* selection process called SELEX (systemic evolution of ligands by exponential enrichment) has been a promising approach to this challenge [13,14]. Aptamers represent a very interesting class of molecules with high affinity [dissociation constant (K_d) ranging from pico- to nano-molar] for the target proteins (enzymes) [15]. The aptamers directed against the two thrombin exosites [exosite I (fibrinogen recognition exosite) and exosite II (heparin-binding exosite)] have been selected [16,17]. A 15-mer thrombin-binding-aptamer (TBA_{15}) is a potential anticoagulation drug because it can inhibit the activity of thrombin through its interaction with fibrinogen-binding exosite I [16,18]. However, high concentrations (>sub-micromolar) of TBA_{15} are required for an effective anticoagulant response because of its low binding affinity (K_d was approximately 10^{-7} M). Moreover, TBA_{15} has an extremely short *in-vivo* half-life because of degradation by blood nucleases. It has been demonstrated that modified nucleosides, bivalent TBAs, and dendritic and polymer TBAs can enhance inhibition of thrombin or improve anticoagulation [19–23]. However, the toxicity of artificial modified aptamers has to be considered. Moreover, the enhancing effect of bivalent-linking aptamers and dendritic and polymeric TBAs is limited [19,21]. These molecules have low flexibility and it is difficult to control the density and orientation of aptamers. Previously, we have demonstrated that 29-mer TBA-modified Au NP nanoparticles (TBA_{29}–Au NPs) are strong anticoagulants because of their steric blocking effects and high binding affinity for thrombin [24]. However, the expensive thiol end label is required to anchor the TBAs on Au NPs through the strong Au–thiol interactions (approximately 160 kJ/mol). Moreover, the precise control of the density and conformation of the tethered TBAs and reversible control of the binding between TBAs and thrombin are yet a challenge.

To control the orientation and distances between bivalent TBAs on Au NPs, we used a self-assembled hybrid monolayer (SAHM) conjugate process, based on tethering of the non-thiol, poly(adenine)-terminal, hybrid TBAs on the surfaces of Au NPs (Scheme 1A). The aim was to enhance the binding affinity of TBAs for thrombin, thereby increasing the anticoagulant activity. We investigated the effect of the bivalent structures of TBAs located on the Au NPs on the inhibition of thrombin. Irradiation with a green light laser (532 nm) was employed to reverse the effect of SAHM TBA–Au NPs on thrombin (Scheme 1B). Furthermore, the anticoagulation potency of SAHM TBA–Au NPs was evaluated by *in vivo* assay of rat-tail bleeding.

2. Materials and methods

2.1. Materials

Calcium chloride, hydrochloric acid, magnesium chloride, potassium chloride, sodium chloride, tris(hydroxymethyl)aminomethane (Tris), and trisodium citrate were purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA). Human α-thrombin (≥1000 NIH units/mg of protein), fibrinogen, and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Milwaukee, WI, USA). Commercial anticoagulants, argatroban, warfarin and heparin (sodium salt; Mw is approximately 5000 Da) were purchased from Sigma-Aldrich and hirudin and SensoLyte 520 kit for thrombin substrate were purchased from AnaSpec (Fremont, CA, USA). The oligonucleotides and thiol-modified oligonucleotides listed in Table S1 (ESI†) were purchased from Integrated DNA Technologies (Coralville, IA, USA). Reagents for the measurement of prothrombin time (PT) and activated partial thromboplastin time (aPTT) were purchased from Helena Laboratories (Beaumont, Texas, USA). All other reagents were purchased from Sigma-Aldrich.

2.2. Preparation of self-assembled TBA-modified Au NPs

Spherical Au NPs (13.3-nm diameter) were prepared by reducing $AuCl_4^-$ using citrate ions. The thiol-modified DNA oligonucleotides were attached to the Au NPs following the procedure reported elsewhere [24]. The preparation of nonthiolated DNA-modified Au NPs followed a slightly modified, already reported procedure [25,26]. An aliquot of aqueous citrate-stabilized Au NP solution, in a 1.5-mL tube, was mixed with the triblock DNA to give final concentrations of 8 nM Au NPs and 1.6 μM DNA and 5 mM sodium phosphate buffer pH 7.4 and 50 mM NaCl. After incubation for 2 h, the triblock DNA-adsorbed Au NP samples were salt-aged (with 500 mM NaCl) for another 12 h. The mixture was centrifuged at a relative centrifugal force (RCF) of 30,000 *g* for 20 min to remove excess triblock DNA. The supernatant was removed, and the oily precipitate was washed with physiological buffer solution [25 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5.0 mM KCl, 1.0 mM $MgCl_2$, and 1.0 mM $CaCl_2$]. After three centrifuge/wash cycles, the purified triblock DNA-modified Au NPs were stored at 4 °C. The amount of triblock DNA in the first supernatant after centrifugation was measured using a single-strain DNA labeling dye, OliGreen, to determine the number of triblock DNA molecules on each Au NP.

2.3. Measurement of real-time coagulation kinetics

The inhibitory potency of TBA-modified Au NPs (TBA–Au NPs) was assessed using scattered light measurements. Briefly, an aliquot (500 μL) of physiological buffer was mixed with BSA (100 μM), one of the TBAs (free or Au NP-bonded), and fibrinogen (1.14 μM) for 15 min. Thrombin (10 nM) was then added, and light scattering measurements (time-course) were performed using an FP–6500 spectrophotometer (JASCO, Tokyo, Japan) at excitation and emission wavelengths of 650 nm. The initial rate of increases in scattered light intensity, and hence the coagulation rate, was calculated from the linear portion of the early slope (0–300 s) of the profiles. This initial rate represents the relative thrombin-inhibiting strength of the inhibitor.

2.4. TCT assay

TCT tests were performed to assess the anticoagulation potency of free TBAs, TBA–Au NPs, and four commercial anticoagulants (heparin, argatroban, hirudin, and warfarin) for the common coagulation pathway. Thrombin (10 nM) was incubated in a physiological buffer (250 μL) at 37 °C for 180 s, then the incubated human plasma (37 °C; 250 μL) containing an inhibitor was added to initiate the clotting cascade. The human plasma collection procedures were performed in compliance with the relevant laws and institutional guidelines. Scattered light intensities of the mixtures were recorded at 650 nm. The TCT was obtained as the point where the intensity of the scattered light was halfway between its lowest and highest values. The measurements were repeated three times, and each set of experiments was performed using a single batch of plasma.

2.5. PT and aPTT assays

For PT determination, human plasma (200 μL) was pre-incubated with an inhibitor at 37 °C for 120 s. Then, PT reagent (150 μL) was added to initiate the extrinsic clotting cascade. The intensity of the scattered light was monitored until the signals reached saturation. For aPTT determination, human plasma (160 μL) was pre-incubated with an inhibitor at 37 °C for 900 s and then the aPTT reagent (100 μL) was added and incubated for another 200 s. Next, pre-warmed $CaCl_2$ (100 μL) was added to initiate the intrinsic clotting cascade. The intensity of the scattered light was monitored until the signals reached saturation. To calculate the PT and aPTT, the end time was chosen as the point at which the scattered light signal was halfway between its lowest and maximal values. The measurements were repeated three times; each set of experiments was performed using a single batch of plasma.