Anticoagulant Nanoparticles

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Gold Nanoparticles Presenting Hybridized Self-Assembled Aptamers That Exhibit Enhanced Inhibition of Thrombin**

Yen-Chun Shiang, Chia-Lun Hsu, Chih-Ching Huang, and Huan-Tsung Chang**

Aptamers are short single-stranded oligonucleotides that fold into specific three-dimensional structures that allow them to specifically bind targets, ranging from ions and small organic and inorganic molecules to biomacromolecules and living cells.[1] Relative to antibodies, aptamers have several advantageous properties, including stability, low cost, and ease of synthesis.[2] The potential use of aptamers for diagnostic and therapeutic applications is further enhanced upon their conjugation to nanoparticles (NPs).[3] Such aptamer-conjugated NPs exhibit unique optical, electrochemical, and magnetic properties, multivalency, and resistance against nuclease digestion, allowing them to be used as sensitive and selective probes for the detection of analytes or as effective drugs for the treatment of diseases.[4]

Aptamer-conjugated gold NPs (Apt-Au NPs) have been prepared previously and employed to detect and control the activity of thrombin,[5] a key serine protease in the coagulation cascade that activates upstream procoagulant factors to amplify coagulation and to convert soluble fibrinogen into insoluble strands of fibrin.[6a] The binding of thrombin to ligands is promoted by its exosites 1 and 2,[6b,c] which are positively charged domains. Fibrinogen, factors V and VIII, and protease-activated receptors (PARs) on platelets all bind to thrombin through exosite 1.[7] Relatively few ligands, such as heparin, bind to thrombin through exosite 2.[8]

The single-stranded 15-base thrombin-binding aptamer (TBA_{15}) binds thrombin specifically and inhibits its activity for blood coagulation through competition with fibrinogen for interaction with exosite 1 of thrombin (Figure 1 A).[9] In addition to TBA_{15}, the 29-base TBA (TBA_{29}) interacts with exosite 2 of thrombin, but has no enzymatic inhibitory function (Figure 1 B).[10] The dissociation constants (K_d) for the complexes of thrombin with TBA_{15} and TBA_{29} are ca. 100 and 0.5 nmol L^{-1}, respectively.[9,10] To further improve their binding affinities toward thrombin, TBA_{15} and TBA_{29} have been conjugated to Au NPs.[5a] The TBA_{29}-Au NPs exhibited improved anticoagulant potency (ca. 82-fold) through thrombin-mediated coagulation as a result of steric blocking effects and high binding affinity toward thrombin. Owing to a lack of flexibility and difficulty in controlling the aptamer density, however, the improved efficiency of that system was limited.

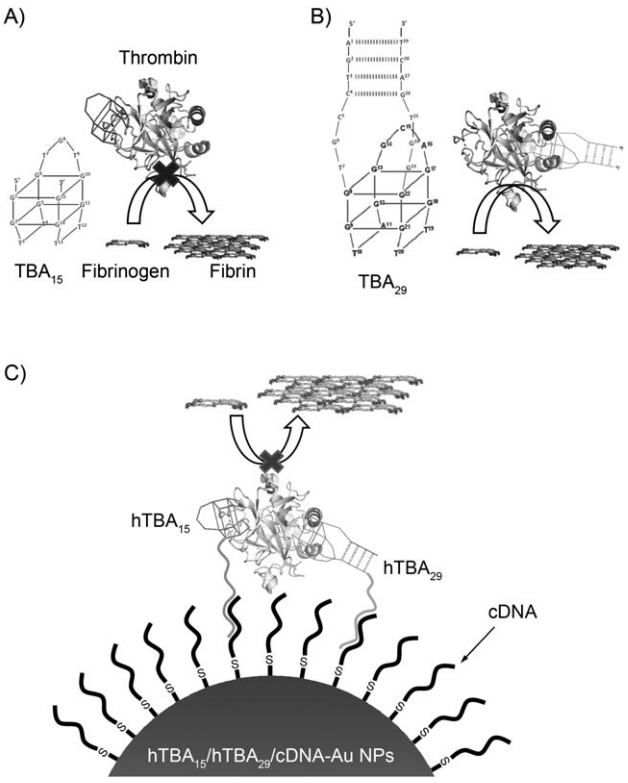

Figure 1. Representation of the binding and enzymatic inhibition of A) TBA_{15} that binds to exosite 1 of thrombin, leading to inhibition of the thrombin-mediated cleavage of fibrinogen to form fibrin, B) TBA_{29} that binds to exosite 2 of thrombin, and C) the $hTBA_{15}$/$hTBA_{29}$/cDNA-Au NPs that have bivalent interaction with thrombin for highly enzymatic inhibition.

A number of bivalent and multivalent TBAs have been developed to improve anticoagulant potency.[11] Preparation of these bivalent or multivalent TBAs is often difficult and results are limited for these systems. Herein, we used a self-assembled arranged monolayer (SAAM), based on DNA hybridization on the surfaces of Au NPs, to enhance the binding affinity of thrombin binding aptamers (TBAs) toward thrombin and, thereby improving its anticoagulant activity (Figure 1 C). We designed two TBAs, namely $hTBA_{29}$ (a 29-base sequence providing TBA_{29} functionality, a T_3 linker, and

[*] Y.-C. Shiang, Prof. H.-T. Chang
Department of Chemistry, National Taiwan University
1, Section 4, Roosevelt Road, Taipei 10617 (Taiwan)
Fax: (+886) 2-3366-1171
E-mail: changht@ntu.edu.tw
C.-L. Hsu, Prof. C.-C. Huang
Institute of Bioscience and Biotechnology, Center of Excellence for Marine Bioenvironment and Biotechnology (CMBB)
National Taiwan Ocean University 2
Pei-Ning Rd, Keelung 20224 (Taiwan)
Fax: (+886) 2-2462-2034
E-mail: huanging@ntou.edu.tw

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a 15-base sequence for hybridization) and $hTBA_{15}$ (a 15-base sequence providing TBA_{15} functionality, a T_3 linker, and a 15-base sequence for hybridization), and used them (see the Supporting Information, Table S1 for their sequences), through hybridization with Au NPs modified with captured DNA (cDNA-Au NPs), to prepare functional Au NPs ($hTBA_{15}$/$hTBA_{29}$/cDNA-Au NPs). We investigated the effect that the surface density of the hTBAs on the cDNA-Au NPs had on the enzymatic inhibition of thrombin and on the elongation of the thrombin clotting time (TCT), prothrombin time (PT), and activated partial thromboplastin time (aPTT) in plasma samples. We also employed DNA having an hTBA-complementary sequence to prepare antidote Au NPs (AD-hTBA-Au NPs), which reversed the activity of the $hTBA_{15}$/$hTBA_{29}$/cDNA-Au NPs toward thrombin through hTBA/AD-hTBA hybridization. Our simple SAAM aptamers/cDNA-Au NPs strategy can also be applied to prepare different SAAM aptamers/cDNA-Au NPs for targeting other coagulant related proteins, such as active protein C, factor VIIa, and factor IXa.[6a] Moreover, it can be applied more generally to create multivalent ligands, including metal complexing ligands and oligosaccharides on the surfaces of nanomaterials for sensitive and selective detection of metal ions and glycoproteins, respectively.

By determining the fluorescence of the supernatant, we estimated the number of cDNA molecules on each NP to be 110.[12] Through hybridization, we prepared the hTBAs/cDNA-Au NPs merely by mixing the hTBAs and the cDNA-Au NPs. We then used the as-prepared hTBA/cDNA-Au NPs, namely $hTBA_{15}$/cDNA-Au NPs, $hTBA_{29}$/cDNA-Au NPs, and $hTBA_{15}$/$hTBA_{29}$/cDNA-Au NPs, to inhibit the clotting activity of thrombin (5.0 nM) in the presence of fibrinogen (1.14 μM) and BSA (100 μM). The total concentrations of the hTBAs were held constant (10 nM) in these experiments. Figure 2 A displays the scattering light intensities of the five mixtures. When the activity of thrombin was high (thereby inducing the formation of a fibrin gel through cleavage of fibrinogen), the scattering of light was strong; indeed, the scattering light intensity was proportional to the degree of coagulation. The efficiencies of inhibiting the activity of thrombin followed the order $hTBA_{15}$/$hTBA_{29}$/cDNA-Au NPs $\gg$ $hTBA_{29}$/cDNA-Au NPs $>$ $hTBA_{15}$/cDNA-Au NPs. For comparison, we conducted control experiments (in the absence of cDNA-Au NPs), which revealed (Figure 2 B) that only $hTBA_{15}$ exhibited significant inhibition of thrombin-induced coagulation, mainly because $hTBA_{15}$ targets the critical exosite 1, whereas $hTBA_{29}$ does not. The inhibition induced by the mixture of $hTBA_{15}$ and $hTBA_{29}$ was slightly better than that of $hTBA_{15}$ alone, presumably because of a synergistic effect. That is, simultaneous binding and blocking by the two aptamers of both of the exosites of thrombin led to strong and synergistic inhibition of the thrombin-dependent coagulant activity.[13]

To obtain more detailed information regarding the inhibition mechanism, we measured the initial reactions rates of the thrombin activity in the presence of the hTBAs and the hTBAs/cDNA-Au NP conjugates. Here, a high initial rate represents a low inhibition efficiency for thrombin coagulation. Table 1 summarizes the data.

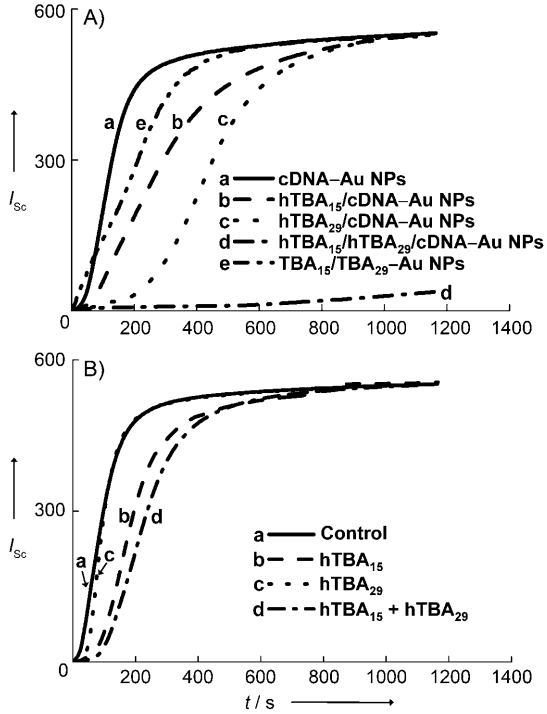


Figure 2. Real-time monitoring of light scattering during the coagulation of mixtures of thrombin, fibrinogen, and a) no inhibitor, b) $hTBA_{15}$, c) $hTBA_{29}$, and d) a 1:1 mixture of $hTBA_{15}$ and $hTBA_{29}$ (10.0 nM) in the A) presence and B) absence of cDNA-Au NPs (2.5 nM). Curve (e) in (A) represents the TBA_{15}/TBA_{29}-Au NPs inhibitory system developed in a previous study.[5a] After coagulation was initiated by adding fibrinogen to each thrombin sample, the light scattering from each sample was monitored at 650 nm. The control sample contained only thrombin (5.0 nM) and fibrinogen (1.14 μM) in physiological buffer. I_{sc} = scattering intensity in kilocounts per second.

Table 1: Initial reaction rates of thrombin-mediated fibrin formation.

Inhibitor	Reaction rate [cps s^{-1}]
control	3170
$hTBA_{15}$	2280
$hTBA_{29}$	2980
$hTBA_{15}$ + $hTBA_{29}$	2150
cDNA-Au NPs	2985
$hTBA_{15}$/cDNA-Au NPs	1432
$hTBA_{29}$/cDNA-Au NPs	1290
$hTBA_{15}$/$hTBA_{29}$/cDNA-Au NPs	45
TBA_{15}/TBA_{29}-Au NPs	2119
rDNA/cDNA-Au NPs	3060

The initial reaction rate (3170 cps s^{-1}) of the control was the highest. Among the tested aptamers and conjugates, the $hTBA_{15}$/$hTBA_{29}$/cDNA-Au NPs possessed the highest inhibitory function (45 cps s^{-1}). The hTBAs/cDNA-Au NPs exhibited anticoagulation efficiencies higher than those of the free hTBAs, most likely through efficient blocking (through electrostatic interactions) of thrombin exosites or its active site for fibrinogen. The $hTBA_{29}$/cDNA-Au NPs led to coagulation of thrombin at an initial rate of 1290 cps s^{-1}, which is 2.3 times lower than that of the $hTBA_{29}$ (2980 cps s^{-1}). In the

presence of a random DNA strand (rDNA, with a 22-base random sequence, a T_3 linker, and a 15-base sequence for hybridization), we observed no enzymatic inhibition of thrombin in either the absence or presence of the cDNA-Au NPs. In control experiments, solution containing 15-mer TBA_{15} and 29-mer TBA_{29} in the absence or presence of cDNA-Au NPs had similar anticoagulant activity. Notably, the anticoagulant activity of the $hTBA_{15}$/$hTBA_{29}$/cDNA-Au NPs was 47-fold higher than that of our previously developed TBA_{15}/TBA_{29}-Au NPs (2119 cps s^{-1}),[5a] which we prepared by mixing TBA_{15}, TBA_{29}, and Au NPs, stabilized through Au–S bonding. This lower inhibition of TBA_{15}/TBA_{29}-Au NPs was mainly due to the lower flexibility and difficulty in controlling the aptamer density. For example, when the surface density was low (< 30TBA molecules per 13 nm Au NP), the TBA ligands most likely existed as flattened structures, leading to weak affinity toward thrombin and, thus, low anticoagulation capability. On the other hand, when the TBA-Au NPs presented more than 30TBA molecules per Au NP, stretched, linear TBA structures and/or intermolecular G-quadruplexes predominated on the Au NP surfaces, mainly because of steric effects and strong electrostatic repulsion among DNA molecules.[5a] As a result, the anticoagulation efficiencies of TBA-Au NPs decreased. Moreover, the less flexible nature of TBA_{15}/TBA_{29}-Au NPs made it difficult for enhancing inhibition to thrombin by multivalent interactions. The SAAM of $hTBA_{15}$ and $hTBA_{29}$ on the surfaces of the cDNA-Au NPs provided high flexibility and an appropriate orientation and distance between the $hTBA_{15}$ and $hTBA_{29}$ units for bivalent binding, allowing stronger interaction with thrombin ($K_d = 2.2 \times 10^{-12}$ mol L^{-1}; Supporting Information, Figure S1), leading to extremely high anticoagulant potency. Furthermore, electrostatic interactions and steric blocking effects between thrombin and $hTBA_{15}$/$hTBA_{29}$/cDNA-Au NPs might also have led to stronger inhibitions.[5a,14]

As further support that our $hTBA_{15}$/$hTBA_{29}$/cDNA-Au NPs are a specific inhibitor for thrombin, we used a 5-FAM/QXL 520 (fluorophore/quencher pair) modified substrate (ca. 6 amino acids) that can be cleaved by thrombin at the site of Arg-Gly (R-G). Cleavage efficiencies of the substrate in the presence of various inhibitors were then monitored by changes in the fluorescence based on Forster resonance energy transfer (FRET). After adding various inhibitors separately to the mixtures of thrombin (5 nM) and the peptide substrate (50 nM), thrombin activity decreased as a result of a competitive reaction occurred between the inhibitors and the peptide substrate for the thrombin (Supporting Information, Figure S2). The order of the inhibition efficiency for thrombin toward the peptide substrate was $hTBA_{15}$/$hTBA_{29}$/cDNA-Au NPs $\gg$ $hTBA_{29}$/cDNA-Au NPs > $hTBA_{15}$/cDNA-Au NPs > $hTBA_{15}$/$hTBA_{29}$ > $hTBA_{15}$ > $hTBA_{29}$, which is in agreement with our scattering data (Figure 2 and Table 1).

In theory, the surface density of the hTBAs on the cDNA-Au NPs should play a role in determining the inhibition efficiency toward thrombin activity. Therefore, we investigated the effect of the concentration of hTBAs in the absence and presence of the cDNA-Au NPs (2.5 nM) on the enzymatic inhibition of thrombin (Supporting Information, Figure S3). In this study, we postulated that the initial rate would be proportional to the thrombin activity. The anticoagulation capabilities of the $hTBA_{15}$/$hTBA_{29}$/cDNA-Au NPs (2.5 nM) reached their maxima at the concentrations of $hTBA_{15}$ and $hTBA_{29}$ of 25 nM. From the bound TBAs (ca. 9 $hTBA_{29}$ and 9 $hTBA_{15}$) per cDNA-Au NPs (diameter $\approx$ 40 nm), we calculated the average distance between the $hTBA_{15}$ and $hTBA_{29}$ to be about 22 nm, which is much longer than the distance between the binding sites of exosites I and II of thrombin (ca. 5 nm).[6a,9b] This result supports our proposed mechanism that TBA ligands self-assembled and arranged to appropriate orientation and distance between the $hTBA_{15}$ and $hTBA_{29}$ units for bivalent binding. On the other hand, we note the inhibition of $hTBA_{15}$/cDNA-Au NPs (2.5 nM) or $hTBA_{29}$/cDNA-Au NPs (2.5 nM) increased upon increasing the concentration of $hTBA_{15}$ or $hTBA_{29}$ from 0 to 125 nM (Supporting Information, Figure S3). This result suggests a higher local concentration of TBA ligands could enhance the binding affinity and thus the inhibition toward thrombin.[15]

We defined the activity of thrombin in the absence of any inhibitor to be 100%. We then calculated the half-maximal inhibitory concentration (IC_{50}) of each inhibitor according to Equation (1):

$$Y = \frac{100}{1 + 10^{(\log IC_{50} - X)}} \quad (1)$$

where X is the logarithm of the inhibitor concentration and Y is the measured percentage activity at a given inhibitor concentration. We performed all of the enzyme activity inhibition assays for the determination of IC_{50} values in triplicate. The IC_{50} values for $hTBA_{15}$, $hTBA_{29}$, the mixture of $hTBA_{15}$ and $hTBA_{29}$, $hTBA_{15}$/cDNA-Au NPs, $hTBA_{29}$/cDNA-Au NPs, and $hTBA_{15}$/$hTBA_{29}$/cDNA-Au NPs were (41 ± 3), > 1000, (23 ± 3), (7.2 ± 0.3), (6.3 ± 0.2), and (1.6 ± 0.1) nM, respectively, further confirming that the $hTBA_{15}$/$hTBA_{29}$/cDNA-Au NPs provided high enzymatic inhibition toward thrombin (over 10-fold greater than that of $hTBA_{15}$).

We further tested the potency of the anticoagulant $hTBA_{15}$/$hTBA_{29}$/cDNA-Au NPs in human plasma samples. We used citrated plasma to measure the TCT, PT, and activated partial aPTT. TCT is a common test performed in patients suspected of suffering from coagulopathy.[16] The measurement of TCT is a common screening test for factors I, IIa, and XIII of the common pathways. Measuring PT is a screening test for factors II, V, VII, and X of the extrinsic and common pathways, whilst aPTT is a screening test for factors II, V, VIII, IX, X, XI, and XII of the intrinsic and common pathways.[17] Figure 3 displays the dosage dependence of the delay of the TCT, PT, and aPTT. The results from these assays clearly reveal that the $hTBA_{15}$/$hTBA_{29}$/cDNA-Au NPs had highest inhibitory function when compare to the TBA_{15}/TBA_{29}-Au NPs and mixtures of $hTBA_{15}$ and $hTBA_{29}$ in plasma samples. The TCTs of the $hTBA_{15}$/$hTBA_{29}$ mixture and of the $hTBA_{15}$/$hTBA_{29}$/cDNA-Au NPs were approximately 2.1 and 11.5 times longer (t/t_0) in TCT, 2.9 and 3.7 times longer in PT, and 3.5 and 6.2 times longer in aPTT, respectively, than that obtained in the absence of any inhibitors (Figure 3). The vulues t_0 and t are the TCT, PT, or

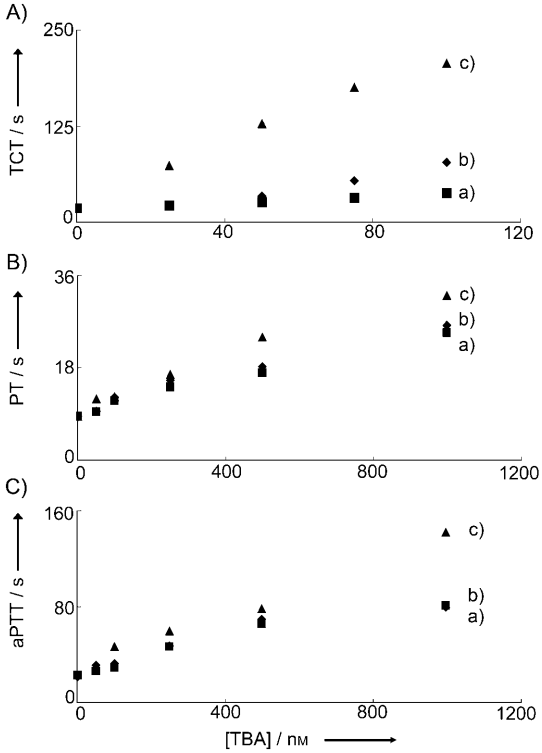
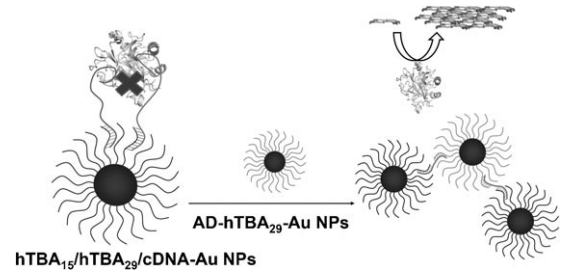


Figure 3. TCT, PT, and aPTT measurements of the anticoagulant potency of the a) mixtures of $hTBA_{15}$ and $hTBA_{29}$, b) TBA_{15}/TBA_{29}-Au NPs, or c) the $hTBA_{15}/hTBA_{29}$/cDNA-Au NPs in human plasma. To calculate the TCT, PT, and aPTT, the end time was chosen to be the point at which the scattering signal reached halfway between the lowest and maximum levels. Other conditions were the same as those described in Figure 2.

aPTT in the absence and presence of inhibitor, respectively. Prolonging the TCT, PT, and aPTT in plasma samples by TBA_{15}/TBA_{29}-Au NPs and $hTBA_{15}/hTBA_{29}$/cDNA-Au NPs led to values of 4.3/11.5, 3.1/3.7, and 3.7/6.2 times, respectively (Figure 3). The results clearly demonstrate the advantages of $hTBA_{15}/hTBA_{29}$/cDNA-Au NPs over TBA_{15}/TBA_{29}-Au NPs with respect to blood coagulation time. We further compared our $hTBA_{15}/hTBA_{29}$/cDNA-Au NPs with commercial drugs in whole blood clotting time (CT) assays (Supporting Information, Figure S4). The whole blood clotting time (CT) using $hTBA_{15}/hTBA_{29}$/cDNA-Au NPs (10 nM; [hTBA] = 100 nM) was (321 ± 18) s, which is longer than the CT of (162 ± 20) s in the absence of the inhibitor. The CT was also longer than using two commercial drugs; (280 ± 12) s and (265 ± 15) s when using hirudin (100 nM) and argatroban (100 nM), respectively. These results indicate the anticoagulant ability of our $hTBA_{15}/hTBA_{29}$/cDNA-Au NPs in whole blood was better than the two commercial drugs.

The reversibility of the binding between an inhibitor and thrombin directly affects the pharmacology of a potential drug. The hTBAs and their complementary sequences (AD-hTBAs) are effective drug/antidote pairs for thrombin (Scheme 1). Therefore, we investigated the effects of two kind of antidotes, the AD-hTBAs and the AD-hTBAs-conjugated Au NPs (AD-hTBAs-Au NPs), on the anticoagulant properties of the $hTBA_{15}/hTBA_{29}$/cDNA-Au NPs. We



Scheme 1. Representation of the antidote effect of the AD-$hTBA_{29}$-Au NPs to $hTBA_{15}/hTBA_{29}$/cDNA-Au NPs via hTBA/AD-hTBA hybridization for recovery of the activity of thrombin.

prepared two reacted clotting mixtures of thrombin, fibrinogen, and the $hTBA_{15}/hTBA_{29}$/cDNA-Au NPs separately with 100 nM AD-hTBAs and 2.5 nM AD-hTBAs-Au NPs (ca. 40 AD-hTBA molecules per Au NP). The total concentration of AD-hTBAs in these solutions was maintained at a constant level (100 nM). Figure 4A shows that the scattering of light from the mixture containing AD-$hTBA_{15}$ (100 nM), AD-$hTBA_{29}$ (100 nM), or 50 nM AD-$hTBA_{15}$/AD-$hTBA_{29}$ (1:1) increased slightly over time, but the activity of thrombin was

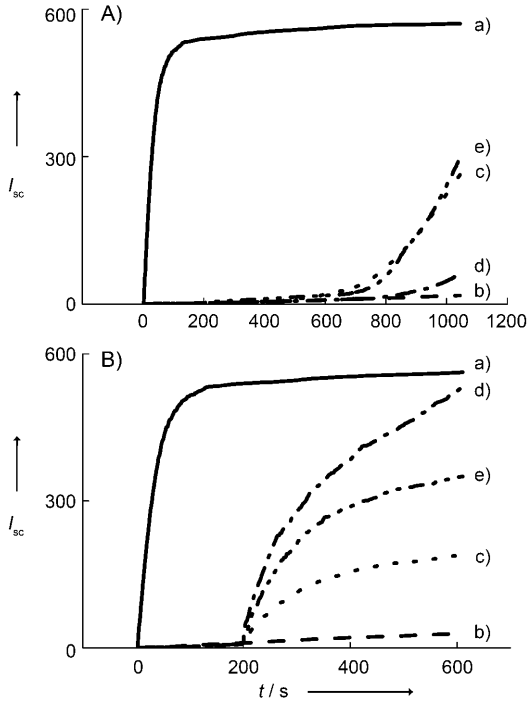


Figure 4. Reversible inhibition of the function of the $hTBA_{15}/hTBA_{29}$/cDNA-Au NPs by A) AD-hTBAs (100 nM) and B) AD-hTBAs-Au NPs (2.5 nM). a) Fibrinogen was added to thrombin immediately in the absence of any inhibitors. b) $hTBA_{15}/hTBA_{29}$/cDNA-Au NPs (2.5 nM) were incubated with thrombin and fibrinogen. Solutions of c) 100 nM AD-$hTBA_{15}$, d) 100 nM AD-$hTBA_{29}$, e) a 1:1 mixture of AD-$hTBA_{15}$ and AD-$hTBA_{29}$ (100 nM) in (A) and solutions of c) AD-$hTBA_{15}$-Au NPs (2.5 nM), d) AD-$hTBA_{29}$-Au NPs (2.5 nM), e) a 1:1 mixture of AD-$hTBA_{15}$-Au NPs and AD-$hTBA_{29}$-Au NPs (2.5 nM) in (B) were added separately to a mixture of $hTBA_{15}/hTBA_{29}$/cDNA-Au NPs, thrombin (5.0 nM), and fibrinogen (1.14 μM) that had been incubated for 200 s. Other conditions were the same as those described in Figure 2.

not completely reversed within 20 min. In the presence of the AD-hTBA-Au NPs, the scattering of light from the mixture increased immediately (Figure 4B). The initial reaction rates of fibrin formation as a result of thrombin activity in the presence of the AD-$hTBA_{15}$-Au NPs, AD-$hTBA_{29}$-Au NPs, and a mixture (1:1) of the AD-$hTBA_{15}$-Au NPs and AD-$hTBA_{29}$-Au NPs were 726, 1853, and 1375 cps s^{-1}, respectively. Thus, the AD-$hTBA_{29}$-Au NPs exhibited the highest antidote potency toward the mixture of hTBA, mainly because of the higher binding affinity of TBA_{29} toward thrombin. Although the $hTBA_{15}$ hybridized with its complementary sequence of the AD-$hTBA_{15}$, the remaining $hTBA_{29}$ on the Au NP surfaces still possessed anticoagulant activity. The scattering intensity of the clotting mixture in the presence of the AD-$hTBA_{29}$-Au NPs was close to that obtained in the absence of any thrombin inhibitors, revealing the ready recovery of the activity of thrombin. In contrast, we observed no change in the scattering signal of the clotting mixture in the presence of 29-mer oligonucleotides having random sequences, either alone or as Au NP conjugates (data not shown); that is, their effect at reversing the inhibition of thrombin was limited.

The $hTBA_{15}$/$hTBA_{29}$/cDNA-Au NPs exhibit high anticoagulant activity as a result of inhibiting the thrombin-mediated cleavage of fibrinogen. Instead of directly conjugating functional aptamers onto Au NP surfaces through Au–S bonding, in this study we hybridized $hTBA_{15}$ and $hTBA_{29}$ with a complementary sequences that were themselves covalently bound to the Au NPs. The $hTBA_{15}$/$hTBA_{29}$/cDNA-Au NPs exert their high inhibitory effect toward thrombin through a combination of multivalent interactions and steric blocking effects. Relative to the TBA_{15}/TBA_{29}-Au NPs inhibitory system that we developed in a previous study,[5a] the $hTBA_{15}$/$hTBA_{29}$/cDNA-Au NPs, which were stable in biological buffer and plasma samples, provided a greater (47-fold) anticoagulant activity toward thrombin. The system containing the $hTBA_{15}$/$hTBA_{29}$/cDNA-Au NPs had a 11.5-fold-longer TCT relative to that tested in the absence of any inhibitor. The addition of the antidote NPs restored the TCT to its original value in the absence of any inhibitors. Thus, our newly developed anticoagulant drug/antidote pair has high potency and potential biomedical applications. Our results suggest that SAAM techniques can be effective at improving the activity of aptamers toward proteins, opening a new avenue for developing efficient drugs, including those for anticoagulation.

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