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SUPPORTING INFORMATION

Graphene oxide modified with aptamer-conjugated gold nanoparticles and heparin: a potent targeted anticoagulant

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Experimental

Materials.

Graphite powder (99%, 7–11 μm) was purchased from Alfa–Aesar (Heysham, Lancashire, UK). Potassium permanganate was purchased from SHOWA (Tokyo, Japan). 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 (≥1,000 NIH units/mg protein), fibrinogen, and BSA were obtained from Sigma–Aldrich (Milwaukee, WI, USA). Heparin (sodium salt) was purchased from Merck (Darmstadt, Germany), hirudin was purchased from AnaSpec (Fremont, CA, USA), and argatroban and warfarin were purchased from Sigma–Aldrich. The oligonucleotides and thiol–modified oligonucleotides listed in Table S1 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 (Australia). All other reagents were purchased from Sigma–Aldrich.

Synthesis of Graphene Oxide.

GO was synthesized using an improved Hummers' method.[1] Briefly, adding a mixture of graphite flakes (0.75 g) and $KMnO_4$ (4.5 g) to a 9:1 mixture of concentrated H_2SO_4 and H_3PO_4 (100 mL). The mixture was then heated to 50 °C and stirred for 12 h. The reaction was cooled to room temperature in an ice bath and then poured into 100 mL of deionized (DI) water containing 3 mL of 30% H_2O_2. The aqueous mixture was then centrifuged at a relative centrifugal force (RCF) of 35,000 *g* for 1 h, and the supernatant was decanted. The remaining pellet was repeatedly washed with 200 mL of DI water until the washings reached a pH of 6.0. The aqueous solution was then sonicated for 1 h and centrifuged at an RCF of 25,000 *g* for 0.5 h. The GO solution was collected and the

remaining pellet was discarded. The GO concentration in the supernatant was determined to be 1.20 g L^{-1}, using the freeze–drying method. A Zetasizer 3000HS analyzer (Malvern Instruments, Malvern, UK) was used for dynamic light scattering and zeta potential experiments. TEM was performed using an HT–7700 system (HITACHI, US), operated at 75 kV.

Preparation of TBA_{29}–Au NPs.

Spherical Au NPs (13.3–nm diameter) were prepared by reducing $AuCl_4^-$ using citrate ions.[2] The thiol–modified DNA oligonucleotides were attached to the Au NPs following a slightly modified procedure reported elsewhere.[3] The 5′–thiol–modified oligonucleotides were received in the disulfide form $HOCH_3(CH_2)_5S$–S–5′–oligo. The oligonucleotide–modified Au NP was prepared as follows. An aliquot of aqueous Au NP solution (980 μL), in a 1.5-mL tube, was mixed with the thio-oligonucleotides (100 μM, 20 μL) to give final concentrations of 15 nM Au NPs and 1000 nM oligonucleotides. The solution containing 1000 nM thiol-oligonucleotides was prepared and salt aged (with 200 mM NaCl). The oligonucleotide directly reacted with the Au NPs, attaching both $HO(CH_2)_6S$ and oligo–S units onto the Au NP surfaces. The mixture was centrifuged at an RCF of 30,000 *g* for 20 min, to remove excess thiol-oligonucleotides. The supernatant was removed, then the oily precipitate was washed with 5.0 mM Tris-HCl (pH 7.4). After three centrifuge/wash cycles, the colloid was re-suspended in 5.0-mM Tris-HCl (pH 7.4) and stored at 4 °C. The amount of TBA in the supernatant after centrifugation was measured using a single strain DNA labeling dye, OliGreen, to determine the number of TBA molecules on each Au NP.[4]

Preparation of TBA_{29}–Au NPs/GO and TBA_{29}–Au NPs/heparin/GO.

The TBA_{29}–Au NPs (0.5 nM) were mixed with GO (24 mg L^{-1}) in physiological buffer [25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM KCl,1 mM $MgCl_2$, 1 mM $CaCl_2$] and allowed to react for 1 h. The mixture was centrifuged at an RCF of 1000 *g* for 20 min to remove the free TBA_{29}–Au NPs. The supernatant was removed and the precipitate was washed

with physiological buffer. After three centrifuge/wash cycles, the colloid was resuspended in a sodium phosphate solution (5 mM, pH 7.4) and stored at 4 °C in the dark. To prepare TBA_{29}–Au NPs/heparin/GO, TBA_{29}–Au NPs/GO was mixed with heparin (Mw 13,500–15,000) with the concentration ratio of TBA_{29} to heparin of 2.0 in a sodium phosphate solution (5 mM, pH 7.4), which reacted for 2 h. Heparin is a highly sulfated glycosaminoglycan polymer.

Measurement of the Real–time Coagulation Kinetics.

The inhibitory potency of TBA_{29}–Au NPs/GO was assessed by conducting scattering light measurement. Briefly, an aliquot (500 μL) of physiological buffer (25 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5.0 mM KCl, 1.0 mM $MgCl_2$, and 1.0 mM $CaCl_{2)}$ was mixed with BSA (100 μM), and one of the oligonucleotides (free, Au NPs–bonded, or Au NPs–bonded with GO) and fibrinogen (1.14 μM) for 15 min. Thrombin (15 nM) was then added to, and a time–course of light scattering measurement was performed using an FP–6500 spectrophotometer (JASCO, Tokyo, Japan) at excitation and emission wavelengths both of 650 nm. The initial rate of increases in scattering light intensity, and hence the coagulation rate, was calculated from the linear portion of the early slope (0–800 s) of the scattering profiles. This initial rate represents the relative thrombin–inhibiting strength of the inhibitor.

Measurement of Thrombin Clotting Time.

TCT tests were performed to assess the anticoagulation potency of TBA_{29}–Au NPs/GO for the common coagulation pathway. Thrombin (15 nM) was incubated at 37 °C in a biological buffer for 180 s, then incubated human plasma (37 °C) containing an inhibitor was added to initiate the clotting cascade. The human plasma collecting procedures were performed in compliance with relevant laws and institutional guidelines. Light scattering intensities of the mixtures at 650 nm were recorded. The TCT was taken as the point where the scattering signal was halfway between the lowest and highest points. The measurements were repeated three times, and each set of experiments was performed using a

single batch of plasma.

Determination of the Binding Constant for TBA_{29}–Au NPs/GO and Thrombin.

The dissociation constant K_d for TBA_{29}–Au NPs/GO and thrombin was determined by separately mixing an aliquot of each TBA_{29}–Au NPs/GO solution ([GO] = 96 mg L^{-1}) with different concentrations of thrombin (0–250 pM) for 15 min to form thrombin–TBA_{29}–Au NPs/GO complexes. Each solution was centrifuged (at an RCF of 30,000 *g*) for 20 min. Unbound thrombin in the supernatant was diluted with a biological buffer and quantified using fibrinogen-modified Au NPs (56 nm), as previously described.[5] Briefly, fibrinogen was added to a solution of Au NPs to prepare fibrinogen-conjugated Au NPs (Fib–Au NPs). Thrombin was then added to the as-prepared Fib–Au NPs solution (21.6 nM), causing the formation of insoluble fibrillar fibrin–Au NP agglutinates through the polymerization of unconjugated and conjugated fibrinogen. The mixture was then centrifuged, and the absorbance of each supernatant was measured at 534 nm. The saturation binding data were processed using the Scatchard equation (equation 1), to estimate the TBA_{29}–Au NPs/GO binding properties.

$$N_{Thr}/[\text{Free–Thr}] = N_{max}/K_d - N_{Thr}/K_d, \quad (1)$$

where N_{Thr} is the number of thrombin molecules bound to TBA_{29}–Au NPs/GO at equilibrium, N_{max} is the apparent maximum number of binding sites, [Free–Thr] is the free thrombin concentration at equilibrium, and K_d is the dissociation constant. The K_d and N_{max} values were calculated from the slope and intercept, respectively, of the linear plot of $N_{Thr}/[\text{Free–Thr}]$ versus N_{Thr}.

***In Vitro* Cytotoxicity.**

The transformed human embryonic kidneys cell line (293T) and human breast cancer cell lines (MDA-MB-231 and MCF-7) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in alpha-MEM supplemented with fetal bovine serum (10%), antibiotic–antimycotic (1%), L-glutamine (2.0 mM), and

nonessential amino acids (1%) in 5% CO_2 at 37 °C. Following the separate incubation of 293T, MDA-MB-231 and MCF-7 cell lines (approximately 2×10^5 cells/well) with culture medium in 24-well culture plates for 8 h at 37°C containing 5% CO_2, each of the culture medium was replaced with 400 μL of medium containing TBA_{29}–Au NPs/heparin/GO of different concentrations ([GO] = 0−24 mg L^{-1}) and then incubated for an additional 24 h. The total number of viable cells was determined by MTT cell proliferation assay. TBA_{29}–Au NPs/heparin/GO treated cell lines were collected at the bottom of the vial and the supernatant was discarded. Then MTT (1%) was added and the medium was removed after 1 h. The as obtained product was dissolved in 200 μL isopropanol. The absorbance was measured at 595 nm with a SynergyTM 4 Multi–Mode Microplate Reader (Biotek Instruments, USA).

Hemolysis Assays.

Hemolysis induced by TBA_{29}–Au NPs/heparin/GO was tested according to a previous report.[6] Fresh blood sample from a healthy volunteer (25 years old) was drawn from the vein into tubes containing ethylenediaminetetraacetic acid (EDTA) and immediately (within 30 min of collection) centrifuged (RCF 3,000 *g*, 10 min, 4 °C) to remove serum. Fresh red blood cells (RBCs) were then washed thrice with sterile isotonic PBS. Following the last wash, the RBCs were diluted with sterile isotonic PBS to obtain an RBC stock suspension (4 vol% blood cells). The RBC stock suspension (100 μL) was added to each TBA_{29}–Au NPs/heparin/GO solutions ([GO] = 0−24 mg L^{-1}) in 1.5–mL vials. After 4 h of incubation at 37 °C, each of the mixtures was centrifuged at RCF of 1,000 *g* for 10 min. Hemolysis activity was determined by measuring hemoglobin absorption at 576 nm (OD_{576}) in the supernatant (150 μL). Sterile isotonic PBS was used as a reference for 0% hemolysis. One hundred percent hemolysis was measured by adding ultrapure water to the RBC stock suspension. The hemolysis activity was calculated as follows:

Hemolysis (%) = $[(OD_{576\ TBA29–Au\ NPs/heparin/GO} - OD_{576\ blank})/(OD_{576\ ultrapure\ water} - OD_{576\ blank})] \times 100$

PT and aPTT Assays.

We used a prothrombin time (PT) reagent and an activated partial thromboplastin time (aPTT) reagent to test the potency of the anticoagulation of the inhibitors in both the contact activation pathway and extrinsic pathway of coagulation. For PT determination, human plasma (200 μL) was pre-incubated at 37 °C with inhibitor for 120 s and then PT reagent (200 μL) was added to initiate the extrinsic clotting cascade. The intensity of the light scattered from the mixtures was monitored until the signals reached saturation. For aPTT determination, human plasma (200 μL) was pre-incubated at 37 °C with inhibitor for 120 s and then aPPT reagent (200 μL) was added and incubated for another 200 s. Next, pre-warmed $CaCl_2$ (200 μL) was added to initiate the intrinsic clotting cascade. The intensity of the light scattered from the mixtures was monitored until the signals reached saturation. To calculate the PT and aPTT, the end time was chosen to be the point at which the scattering signal was halfway between the lowest and maximum points. The measurements were repeated three times; each set of experiments was performed using a single batch of plasma.

Table S1. DNA sequences of the thrombin–binding aptamers (TBAs) and rDNA used in this study.

Name	Sequence[a]	TBA/Au NP[b]
$TBA_{29}–P_8T_{15}$	5′–T_{15} ATC TAG TCC GTG GTA GGG CAG GTT GGG GTG ACT AGA T–3′	–
$TBA_{29}–P_8T_{15}–SH$	5′–SH–T_{15} ATC TAG TCC GTG GTA GGG CAG GTT GGG GTG ACT AGA T–3′	70
$TBA_{15}–P_8T_{15}$	5′–T_{15} ATC TAG TCC GTG GTT GGT GTG GTT GGG GTG ACT AGA T–3′	–
$TBA_{15}–P_8T_{15}–SH$	5′–SH–T_{15} ATC TAG TCC GTG GTT GGT GTG GTT GGG GTG ACT AGA T–3′	72
rDNA–T_{15}–SH	5′–SH–T_{15} GCT GAC TAC ACG CTG TCA TTA CTG G–3′	105

[a]underlining indicates stem pairs; [b]number of TBA or rDNA molecules per gold nanoparticle (Au NP)

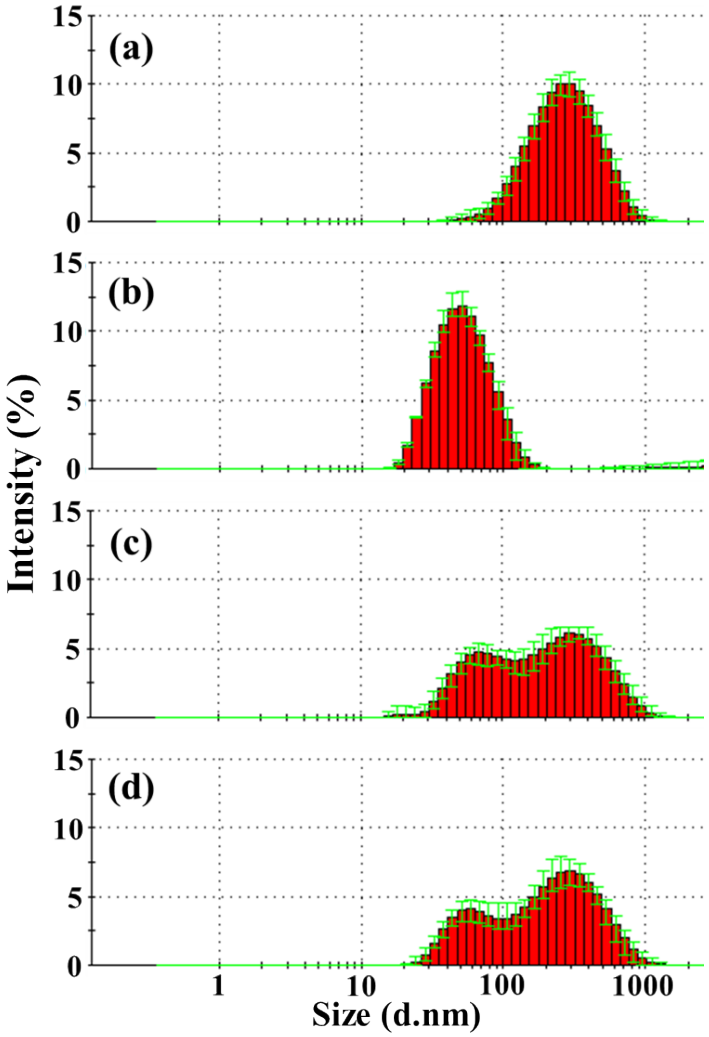

Fig. S1 DLS sizes of (a) as-prepared GO (6.0 mg L^{-1}), (b) as-prepared TBA_{29}–Au NPs (0.125 nM), (c) as-prepared TBA_{29}–Au NPs/GO (0.125 nM/6.0 mg L^{-1}), and (d) TBA_{29}–Au NPs/heparin/GO (0.125 nM/4.375 nM/6.0 mg L^{-1}) incubated in physiological buffer (25 mM Tris–HCl at pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$) for 2 months.

We noted the intensity of the DLS band of TBA_{29}–Au NPs at ~50 nm was decreased after adsorbed on the GO. Therefore, the first band in DLS spectra of TBA_{29}–Au NPs/heparin/GO was possibly attributed to unbound TBA_{29}–Au NPs.

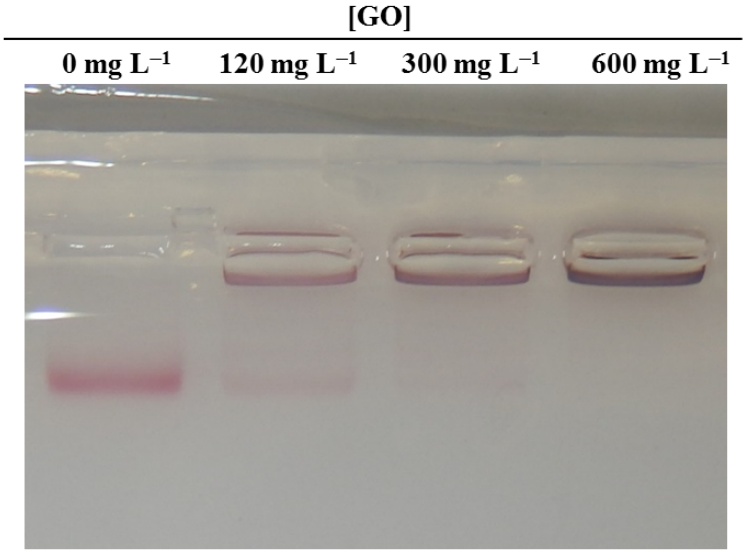

Fig. S2 Gel electrophoresis of TBA_{29}–Au NPs (10 nM) in the presence of GO (0–600 mg L^{-1}) in a 1.5% agarose gel containing 0.5X TBE buffer with electric field of 20 V cm^{-1}.

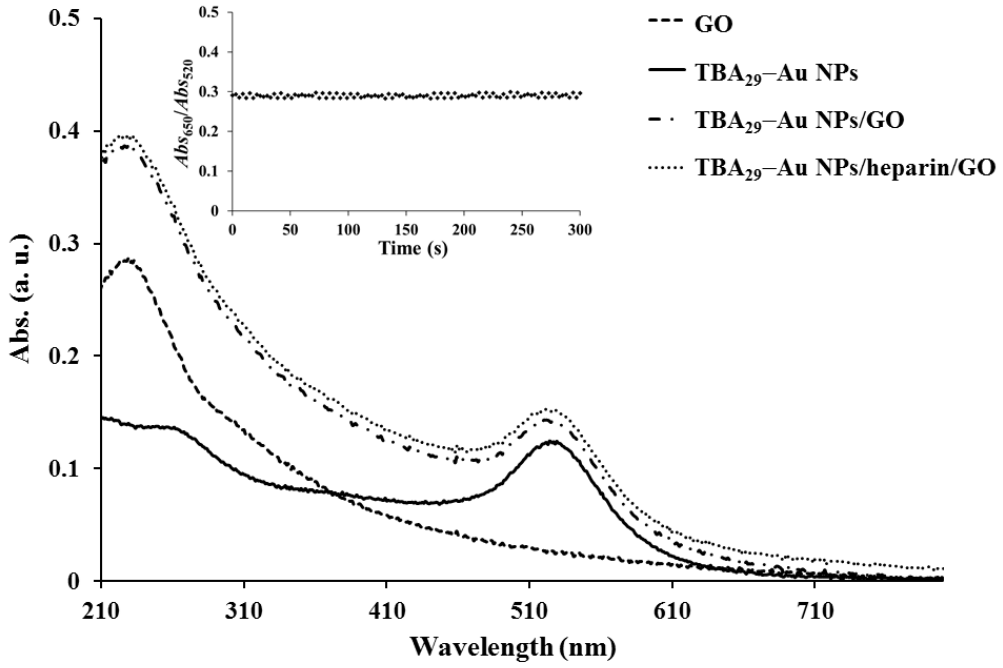

Fig. S3 The UV–vis absorption spectra of GO (at a concentration of 24.0 mg L^{-1}), TBA_{29}–Au NPs (0.5 nM), TBA_{29}–Au NPs/GO (prepared from 24.0 mg L^{-1} GO and 0.5 nM TBA_{29}–Au NPs), and TBA_{29}–Au NPs/heparin/GO (prepared from 24.0 mg L^{-1} GO, 0.5 nM TBA_{29}–Au NPs, and 17.5 nM heparin) in sodium phosphate solution (5 mM, pH 7). The inset: time course measurement of Abs_{650}/Abs_{520} value for TBA_{29}–Au NPs during their adsorption onto GO. The absorbance (Abs) is plotted in arbitrary units (a. u.).

Both the GO and TBA_{29}–Au NPs/GO spectra show an absorption band at 230 nm, suggesting that GO has a graphite structure.[7] This absorption band (230 nm) was attributed to the $\pi \rightarrow \pi^*$ transition of the C=C bond in the sp^2 hybrid region.[7] The shoulder at approximately 300 nm was caused by the $n \rightarrow \pi^*$ electronic transition of peroxide and/or epoxide functional groups in GO.[7] The peak at 520 nm in both the TBA_{29}–Au NPs and TBA_{29}–Au NPs/GO spectra was attributed to absorption by the dispersed Au NPs (13 nm).[8] The inset shows the time course absorption ratio of the TBA_{29}–Au NPs at 650 and 520 nm (Abs_{650}/Abs_{520}) after reacted with GO. The absorption coefficients at 650 and 520 nm are related to the quantities of dispersed and aggregated Au NPs, respectively.[8] Thus, we used Abs_{650}/Abs_{520}, the ratio of the absorption values at these two wavelengths, to express the molar ratio of the aggregated and dispersed Au NPs. Our results indicated the TBA_{29}–Au NPs were stable (no aggregation) after adsorption on GO.

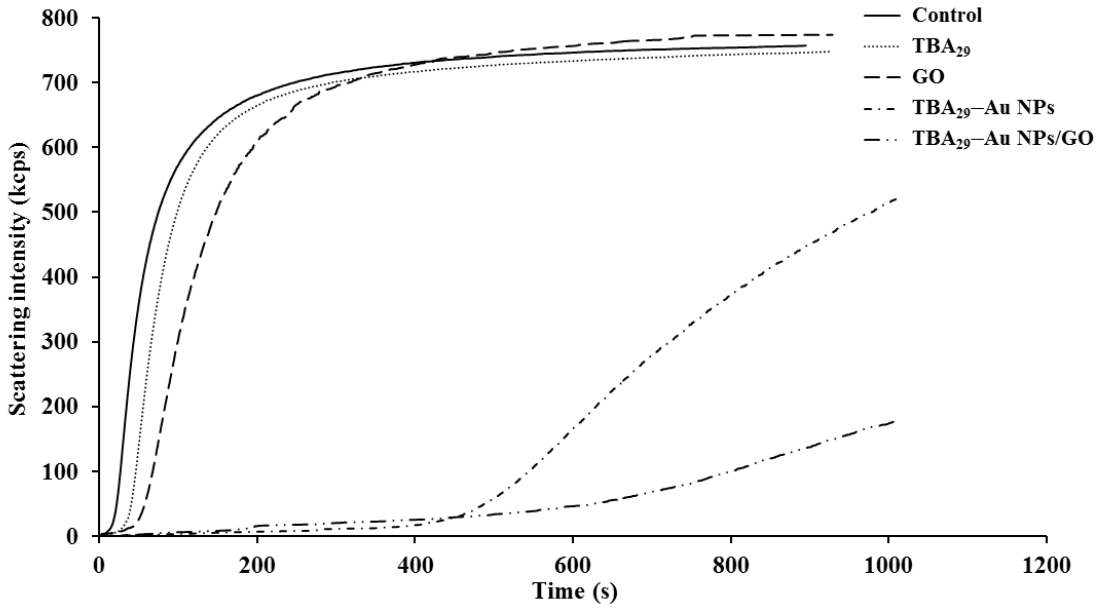

Fig. S4 Scattering intensity over time for coagulating mixtures of thrombin (15 nM), fibrinogen (1.14 μM), bovine serum albumin (BSA) (100 μM), and one of the following: TBA_{29} (35 nM), GO (24 mg L^{-1}), TBA_{29}–Au NPs (0.5 nM), TBA_{29}–Au NPs/GO (0.5 nM/24 mg L^{-1}). Coagulation was initiated by adding thrombin to each fibrinogen sample, and then light scattering was monitored at 650 nm. The control sample contained only thrombin, fibrinogen, and BSA in the physiological solution. The scattering intensity is plotted in units of kilocounts per second (kcps).

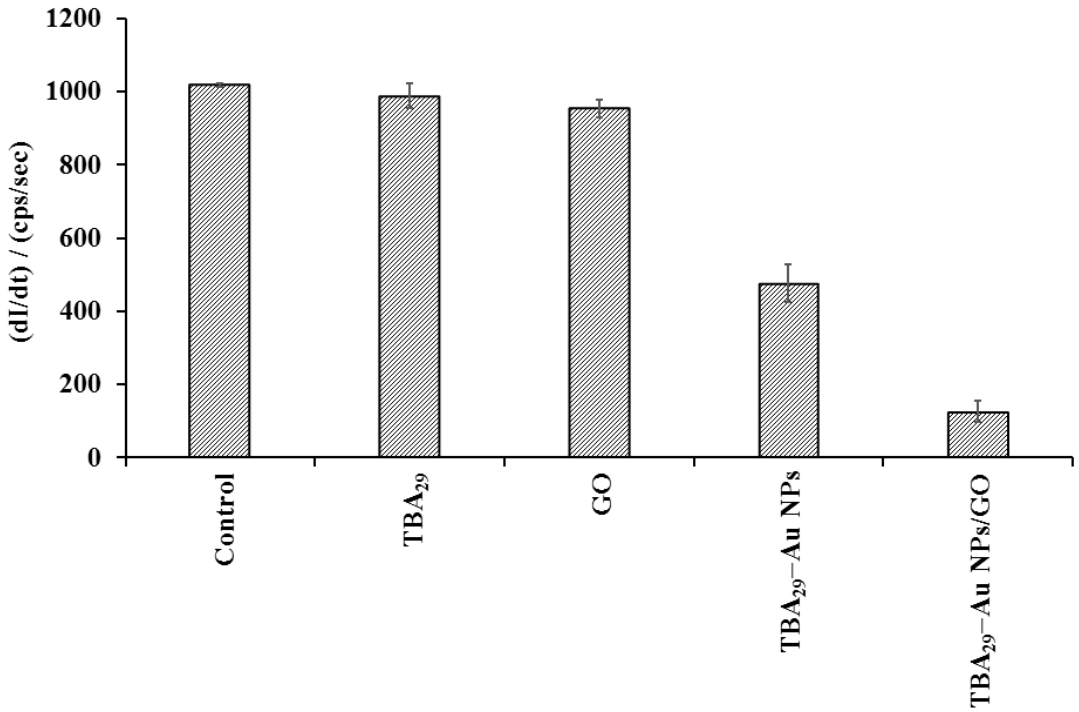

Fig. S5 Initial coagulation reaction rates (dI/dt) for mixtures of thrombin and fibrinogen containing different inhibitors in the presence of bovine serum albumin (100 μM) in physiological buffer. Light scattering in each sample was monitored at 650 nm. The thrombin (15 nM) samples, containing fibrinogen (1.14 μM) and TBA [TBA_{29} (35 nM), GO (24 mg L^{-1}), TBA_{29}–Au NPs (0.5 nM), or TBA_{29}–Au NPs/GO (0.5 nM/24 mg L^{-1})], were prepared in physiological buffer (25 mM Tris–HCl at pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$). The control sample contained only thrombin (15 nM) and fibrinogen (1.14 μM). The error bars represent the standard deviations of triplicate measurements. The other conditions were the same as those described in Figure S4.

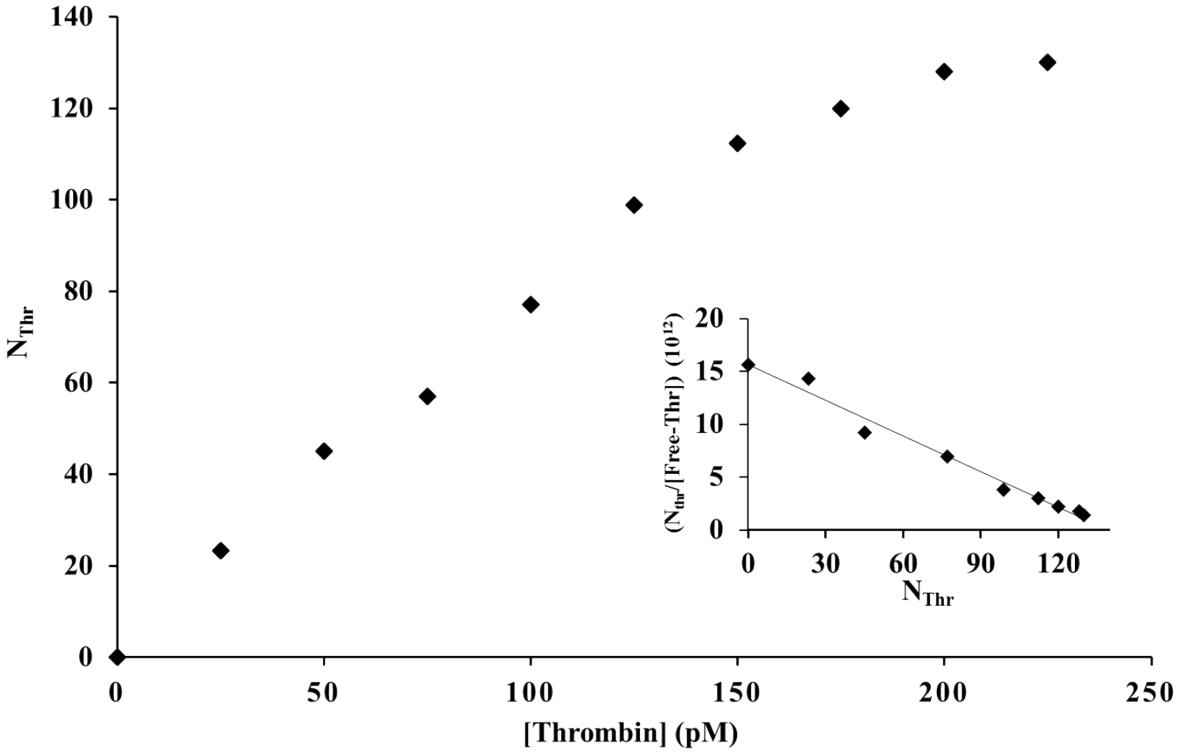

Fig. S6 Plots for calculating the dissociation constant K_d for thrombin and TBA_{29}–Au NPs/GO). N_{Thr} is the number of thrombin molecules bound to TBA_{29}–Au NPs/GO at equilibrium and [Free–Thr] is the free thrombin concentration at equilibrium.

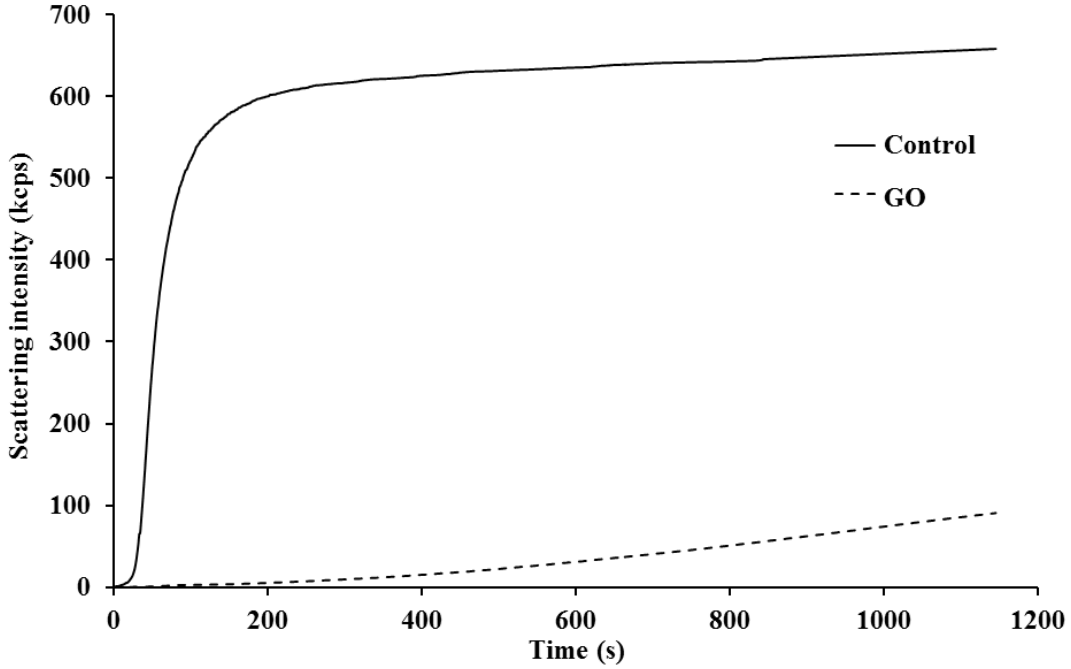

Fig. S7 Scattering intensity over time for coagulating mixtures of thrombin (15 nM) and fibrinogen (1.14 μM) in the absence and presence of GO (at a concentration of 60 mg L^{-1}). Coagulation was initiated by adding thrombin to each fibrinogen sample, and then light scattering was monitored at 650 nm. The control sample contained only thrombin and fibrinogen in physiological solution.

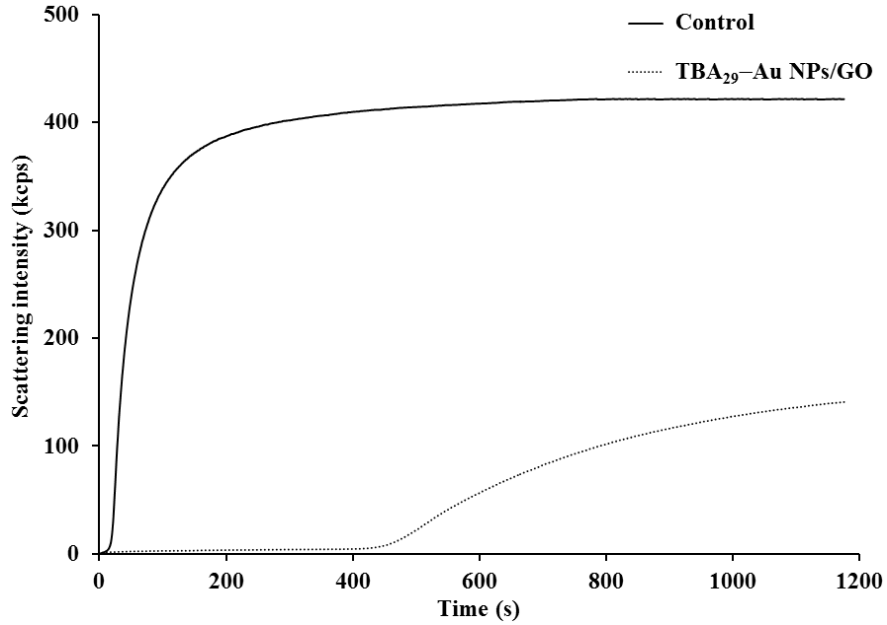

Fig. S8 Representative scattering intensity as a function of time for TBA_{29}–Au NPs/GO in human plasma samples, which allows the thrombin clotting time (TCT) to be calculated. The TCT was used to assess the anticoagulant potency.

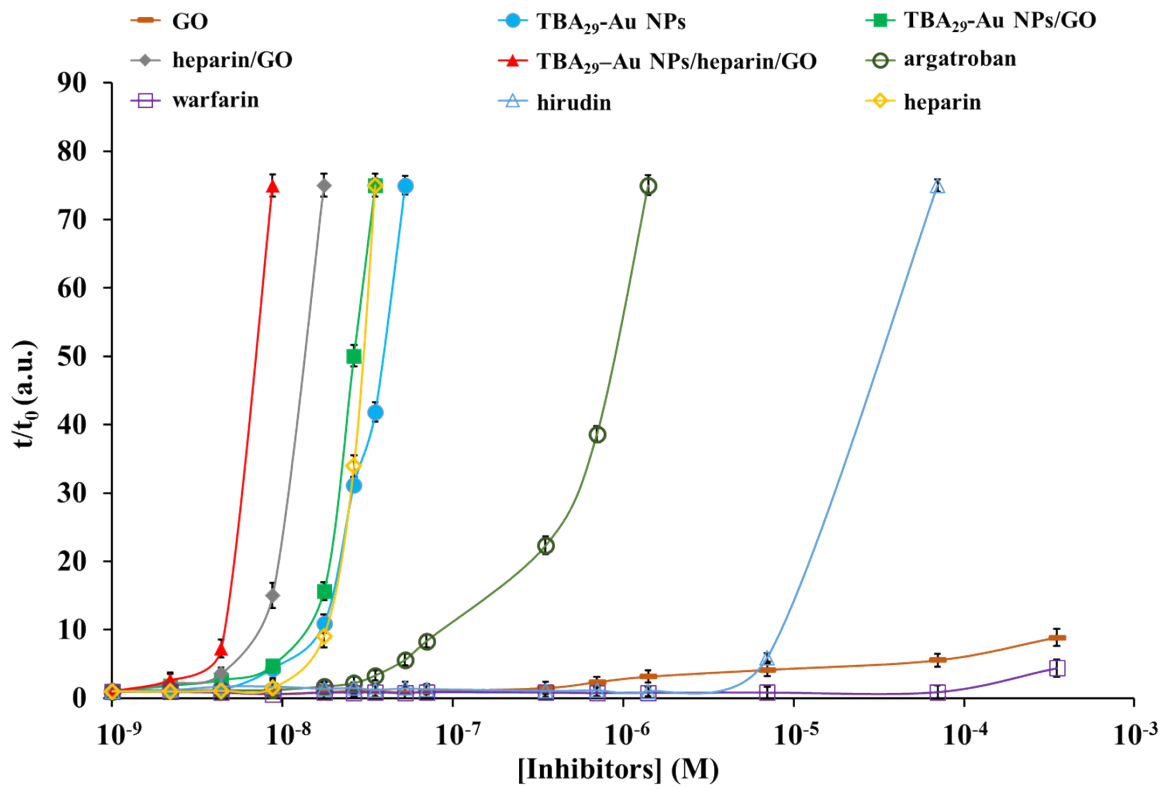

Fig. S9 Dependence of the delay in the TCT (t/t_0) on the dose of commercial drugs (heparin, argatroban, hirudin, and warfarin) and the nanomaterials tested GO, TBA_{29}–Au NPs, TBA_{29}–Au NPs/GO, heparin/GO and TBA_{29}–Au NPs/heparin/GO in the human plasma samples. The error bars represent the standard deviations of triplicate measurements. Other conditions were the same as shown in Figure 2.

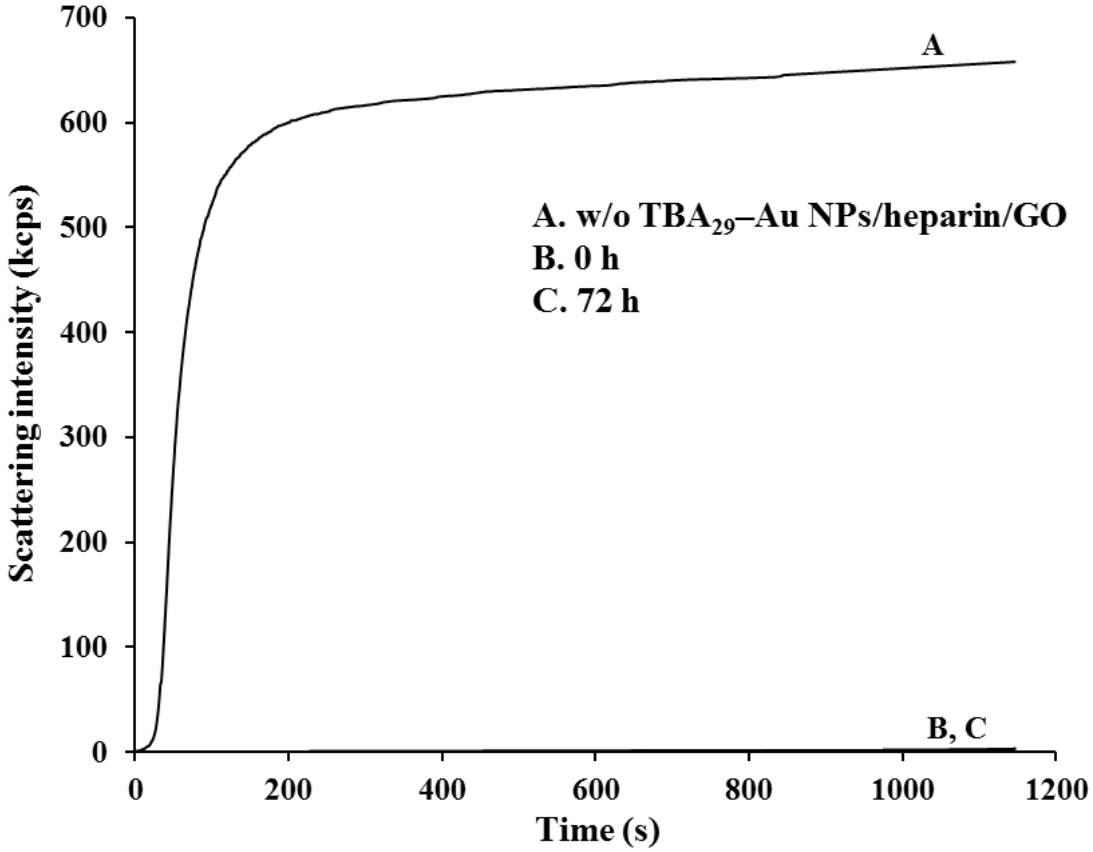

Fig. S10 Scattering intensity as a function of time, validating the use of TBA_{29}–Au NPs/heparin/GO as a stable anticoagulant agent in a representative human plasma sample. TBA_{29}–Au NPs/heparin/GO (prepared from 0.5 nM TBA_{29}–Au NPs, 24 mg L^{-1} GO, and 17.5 nM heparin) was incubated in a diluted (by a factor of two) human plasma sample for 0 h or 72 h, then thrombin (15 nM) was added. Other conditions were the same as those described in Figure 2.

Curve A is a control experiment of thrombin clotting time (TCT) measurement of human plasma in the absence of TBA_{29}–Au NPs/heparin/GO. The control experiment confirms the thrombin containing plasma is quickly coagulated (TCT~20 s) in the absence of TBA_{29}–Au NPs/heparin/GO.

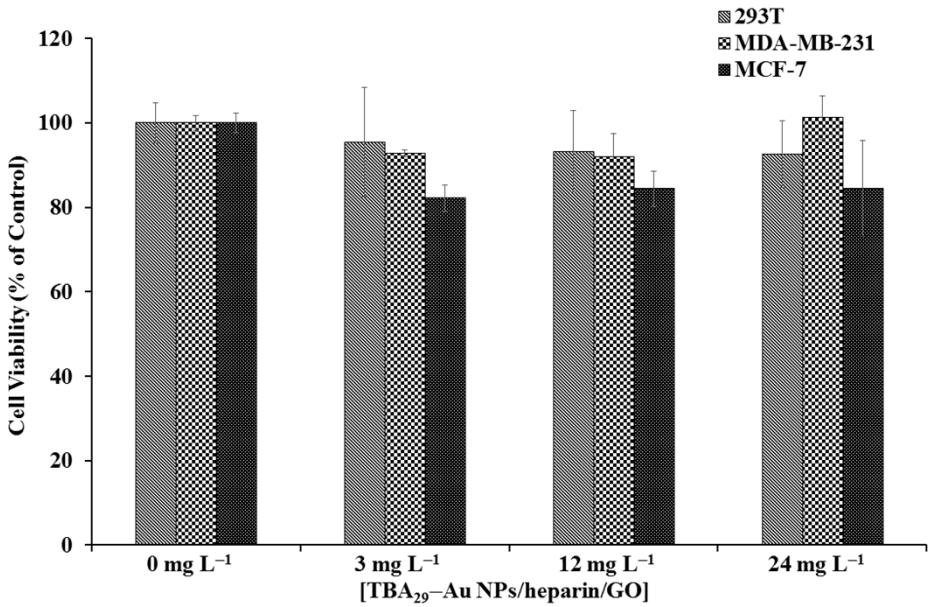

Fig. S11 Cell viability of 293T, MDA–MB–231 and MCF–7 cells incubated with TBA_{29}–Au NPs/heparin/GO ([GO] = 0, 3, 12, and 24 mg L^{-1}) in alpha–MEM at 37 °C with 5% CO_2 atmosphere for 24 h. Isotonic saline solution without TBA_{29}–Au NPs/heparin/GO was used as a control. Error bars represent the standard deviation of three repeated measurements.

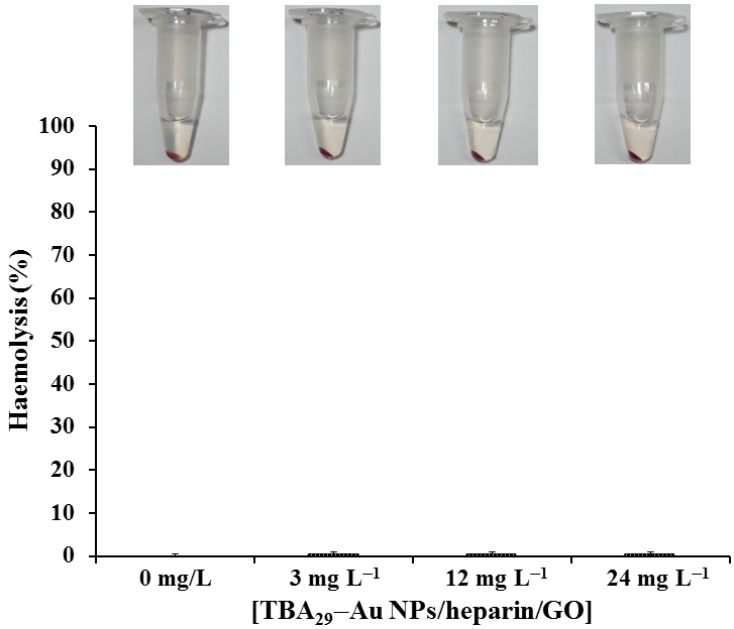

Fig. S12 Comparison of hemolytic activities of various concentrations of TBA_{29}–Au NPs/heparin/GO ([GO] = 0, 3, 12, and 24 mg L^{-1}). Error bars represent the standard deviation of four repeated measurements.

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