Mice model was prepared according to protocols approved by IACUC. The mesenteric model were conducted as previously described with some modifications.[42] Briefly, 6-8-wk-old male C57BL/6 mice from National Laboratory Animal Center (Taipei, Taiwan, ROC) were anesthetized with a mixture of Zoletil® (35 mg/kg) and Rompum (16 mg/kg) by intraperitoneal injection. Jugular vein was cannulated for continuous fluid supplement at 10 μl/min and aptamer administration; the mesentery was exteriorized via an abdominal

Coagulation Factor XIII-Binding Aptamers-Modified Gold Nanoparticles for Thrombus Targeting

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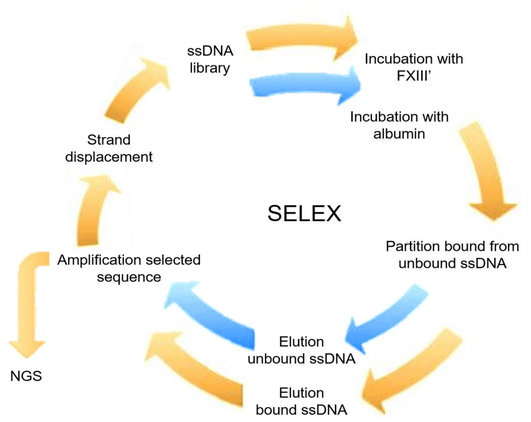
Abstract

Identification of the location of the thrombus, or thrombus-targeting, may facilitate diagnosis and target therapy of thromboembolic diseases. We developed novel single-strand DNA (ssDNA) aptamers with a high affinity to coagulation factor XIII (FXIII), which stabilizes the fibrin clot by cross-linking with fibrin in the thrombus. We hypothesize that the FXIII-binding aptamers may be immobilized on the surface of gold nanoparticles (Au NPs) and serve as targeting ligands of the nanocomposite. FXIII-binding aptamers were truncated with preservation of a conserved region based on entropy analysis. The truncated aptamers (FATs; 41-47 nt) exhibit dissociation constant (K_D) from 0.7 to 2.5 nM, as determined using bio-layer interferometry. Among them, FAT2 exhibits up to 4.2-fold signal of binding to platelet/fibrin clot or whole blood clot, compared to that from scrambled ssDNA. The 5′-thiol-modified FAT2 were conjugated on the Au NPs (13 nm; ca. 90 FAT2 molecules per Au NP) through Au–S bonding. The FAT2-conjugated Au NPs (FXIIIapt-Au NPs) and immobilizing it on graphene oxide (GO; lateral size~200 nm) to form FXIIIapt-Au NPs/GO, exhibit a much stronger binding ability to blood clots compared to free FAT2 due to the multivalent interactions. In addition, the FXIIIapt-Au NPs/GO possesses much superior anticoagulation abilities compared to free FAT2. We also prepared FXIIIapt-modified Fe_3O_4@Au NPs (FXIIIapt-Fe_3O_4@Au NPs) with magnetic resonance imaging (MRI) and X-ray computed tomography characteristics and demonstrated their specific targeting for fibrin clots. To evaluate the thrombus-targeting effect of FXIIIapt-Au NPs in anesthetized mice, FXIIIapt-Au NPs was administered *via* jugular vein, followed by induction of mesenteric thrombosis at the 2nd order branch by 10% $FeCl_3$, with thrombus formation visualized by $DiOC_6$ under fluorescence microscope. The microvessels including upstream and downstream of the section with thrombus were then tied, surgically removed and subjected to inductively coupled plasma mass spectrometry. In the FXIIIapt-Au NP group, the Au in the vessel with thrombus was 2.3- to 3.5-fold of that in adjacent vessel; whereas scrambled ssDNA-conjugated Au NP exhibited much less binding to the thrombus. In conclusion, immobilized FXIII-binding aptamers may be effective targeting ligands on nanocomposites for theranostic application of thromboembolic diseases.

Background

FXIII is a transglutaminase that stabilizes fibrin-fibrin interaction by cross-linking fibrin α and γ chains, which becomes part of the polymerized fibrin structure and plays a critical role in the final step of clot formation. As a tetramer, FXIII (FXIII-A_2B_2) is consisted of two catalytic A subunits (FXIII-A) and two carrier inhibitory B subunits (FXIII-B) in the plasma. FXIIIa also exists in platelets with approximately 60 fg per platelet, which is 150-fold greater than that in plasma. Platelet FXIII-A, also known as cellular FXIII, is largely stored in the cytoplasm of platelets, exposed on the surface upon platelets activation, and involved in cross-linking α2-antiplasmin to fibrin. Aptamers are small nucleic acid ligands that may be composed of RNA or single-stranded DNA (ssDNA), which can be used as targeting ligands for delivery of drugs, disease diagnosis, and therapeutic uses. Aptamers are screened by systematic evolution of ligands by exponential enrichment (SELEX), which is an experimental procedure that allows extraction, from a random pool of ssDNA, with a desired binding affinity for a target protein or cells. Compared to antibodies, aptamers may exert advantages including stability, low immunoreactivity and low batch-to-batch variability. These advantages exert great impact on potential application of aptamers in research, diagnostics and therapy. We hypothesizes that FXIII-binding aptamers (FXIIIapt) may serve as a targeting ligand that binds to the thrombus in theranostic application.



Materials and Methods

Platelet preparation Citrated human blood was centrifuged at 220 g for 20 min to obtain platelet rich plasma (PRP). After addition of PGI_2 (1 μM) to PRP followed by centrifugation at 850 g for 10 min, the platelet pellets were obtained, washed twice with PBS containing PGI_2, and re-suspended in binding buffer to a final concentration of 3 × 108 platelets/mL. In some experiments, the washed platelets were subjeced to a functional assay using aggregometer (AggRAMTM System; Helena Laboratories, Beaumont, TX, USA).

Mice thrombosis model Male C57BL/6 mice were anesthetized and prepared according to protocols approved by IACUC. Jugular vein was cannulated for continuous fluid supplemet and aptamer administration; the mesentery was exteriorized via an abdominal incision for visualization of the micro-vessels under fluorescence microscope. DiOC6 and FAM-labelled FAT2/Scr-DNA were injected from jugular vein for thrombus formation and aptamer targeting, respectively, followed by placement of a piece of cotton thread soaped with 10% $FeCl_3$ for 5 min to induce mesenteric thrombosis, which was obsered under a fluorescence microscope.

Preparation of FXIIIapt-Au NPs Spherical gold nanoparticles (Au NPs; 13.3-nm diameter) were prepared by reducing $AuCl_4^-$ using citrate ions. An aliquot of aqueous Au NP solution (980 μL), in a 1.5-mL tube, was mixed with the thio-FXIIIapt (100 μM, 20 μL) to give final concentrations of 15 nM Au NPs and 1000 nM thio-FXIIIapt and incubated for 12 h. The solution containing 1000 nM thiol-FXIIIapt was prepared and salt aged (with 200 mM NaCl). The mixture was centrifuged at an RCF of 30,000 g for 20 min, to remove excess thiol-FXIIIapt. 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 FXIIIapt 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.

Preparation of FXIIIapt-Au NPs/GO The FXIIIapt-Au NPs ([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 FXIIIapt-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.

Binding affinity and 3-D structure/docking prediction of FXIIIapt with FXIII

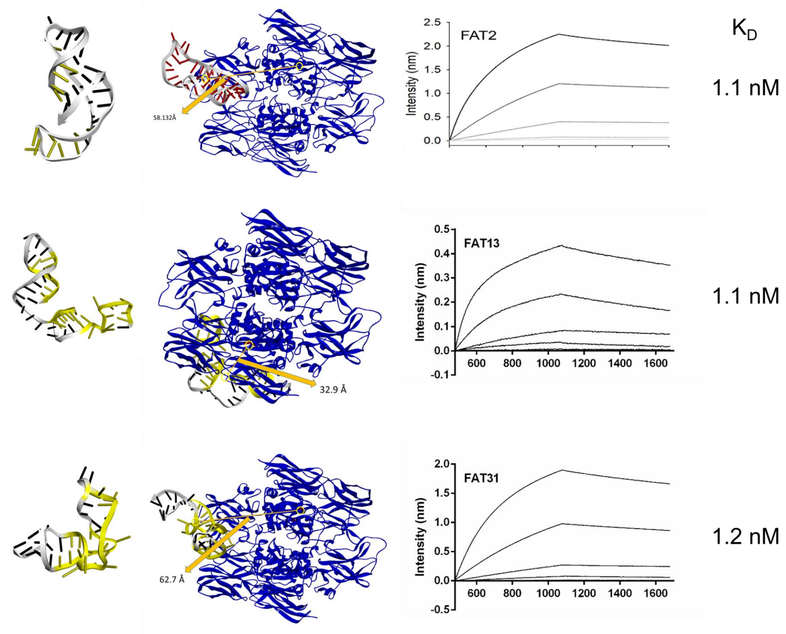


Figure 2. Putative binding fragment was noted as in red (B) A tertiary structure of FAT2 was derived using Discovery studio. (C) FAT2-FXIII complex was predicted using Discovery Studio; putative binding fragment was noted in yellow.

Flow cytometry assessment of FAM-labeled aptamer binding to resting *vs* activated platelets

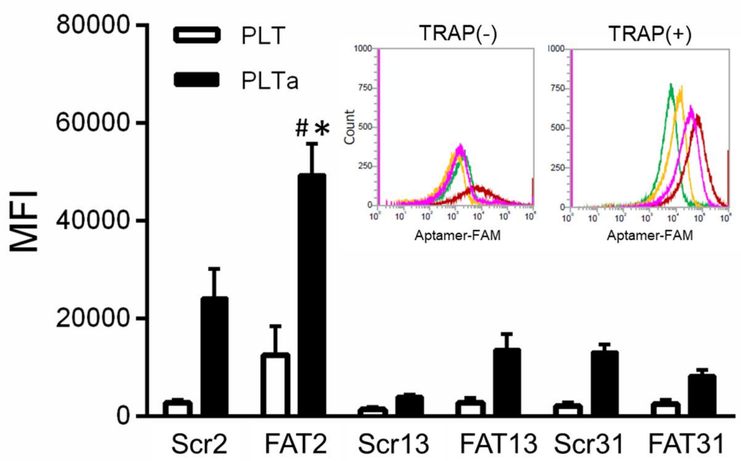


Figure 3. The FAM-labeled aptamers binding to resting platelets (PLT) vs. activated platelets (PLTa) using flow cytometry. Platelets were activated by thrombin receptor activating peptide (TRAP). Quantitative analysis of aptamers was presented as fluorescence intensity (n=9~13). *,#, p<0.05 compared with corresponding PLT and other aptamer groups, respectively.

Binding effect of FXIIIapt to mice mesentery thrombus *in vivo*

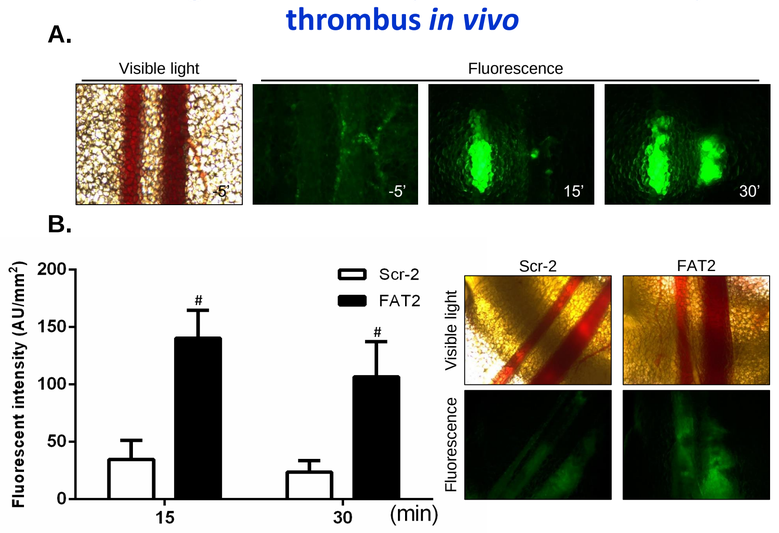


Figure 4. Binding effect of DiOC6/FAT2/Scr-2 to thrombus *in vivo*. (A) Fluorescence and visible light imaging of mice mesentery model treated with DiOC6. (B) The FAM-labeled FAT2 or Scr-2 binding to thrombus by $FeCl_3$-treated mesentery thrombosis in mice mesentery model. Quantitative analysis of aptamers was presented as fluorescence intensity (n=6). #, P<0.05 compared with corresponding Scr-2 groups, respectively.

FXIIIapt-Au NPs and FXIIIapt-Au NPs/GO nanocomposites for thrombus targeting

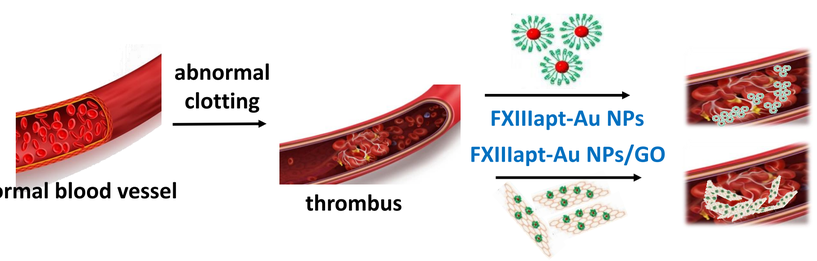


Figure 5. Preparation of FAT2 (FXIIIapt)-modified gold nanoparticles (FXIIIapt-Au NPs) and FXIIIapt-Au NPs-anchored graphene oxide (FXIIIapt-Au NPs/GO) for improving the targeting ability toward thrombus.

Conclusions

FXIIIapt-modified nanocomposites may be potentially used for targeting thrombus in theranostic applications.

Comparison of binding capability of free FXIIIapt and FXIIIapt-modified nanoparticles to fibrin clots

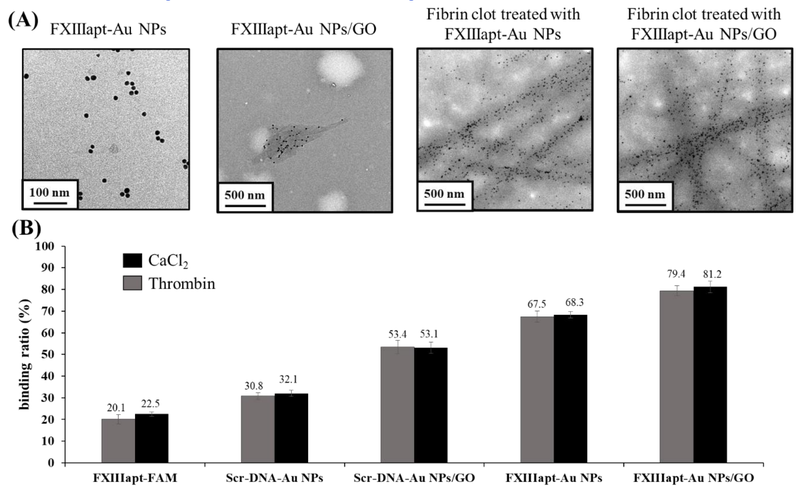
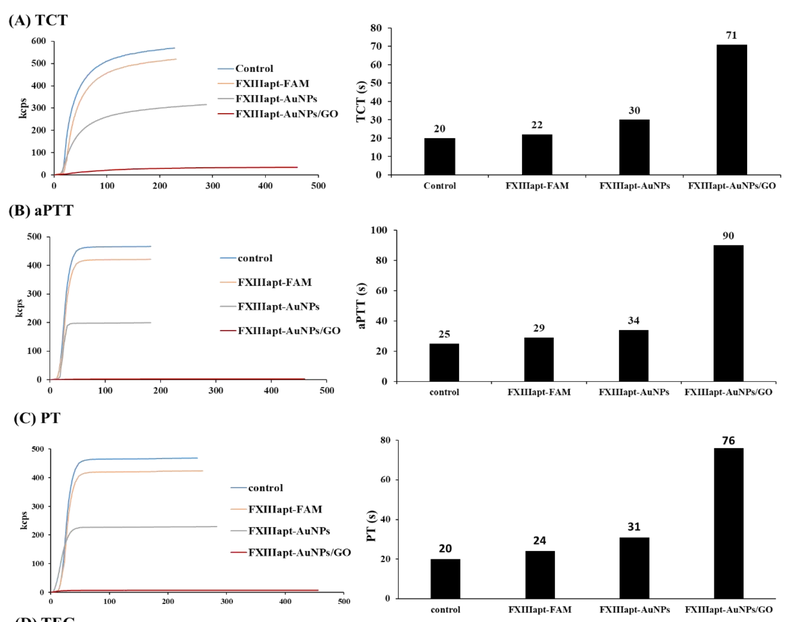


Figure 6. FXIIIapt-FAM, FXIIIapt-Au NPs and FXIIIapt-Au NPs/GO targeting fibrin clots. (A) TEM images of FXIIIapt-Au NPs and FXIIIapt-Au NPs/GO binding to fibrin clot. (B) The binding ratio of to FXIIIapt-FAM, FXIIIapt-Au NPs and FXIIIapt-Au NPs/GO fibrin clot. The fibrin clot was formed from human plasma after triggered by thrombin (5 nM) or $CaCl_2$ (8 mM). The FXIIIapt-FAM and FXIIIapt-modified nanoparticles ([FXIIIapt]=100 nM) incubated with as-formed fibrin clot at room temperature for 30 min. The binding ratios ([apt]b/[apt]0) are determined after separation of fibrin clot and unbounded FXIIIapt-FAM, FXIIIapt-Au NPs or FXIIIapt-Au NPs/GO through fluorescence (FXIIIapt-FAM) or UV-Vis absorption (FXIIIapt-Au NPs or FXIIIapt-Au NPs/GO) spectroscopic measurements, which [apt]b and [apt]0 is the concentration of as-incubated FXIIIapt (100 nM) and fibrin clot-bound FXIIIapt, respectively. The scramble DNA (scr-DNA) modified-Au NPs (scr-DNA-Au NPs) and -Au NPs/GO (scr-DNA-Au NPs/GO) were conducted as controls. The mean value and standard deviations are determined from three independent experiments.

Prolonged clotting time of FXIIIapt-modified nanoparticles



Inhibitor	R (min)	K (min)	αAngle (deg)	MA (mm)	LY30(%)
Control	4.8	2.6	67.1	55.9	11.1
scrDNA-Au NPs	3.9	2.3	72.1	52.1	8.2
FXIIIapt-Au NPs	3.4	2.4	63.3	41.7	27.1
FXIIIapt-Au NPs/GO	2.3	3.7	59.2	**35.7**	37.6
FXIIIapt-FAM	3.6	3.2	68.5	45.8	13.0

Figure 7. Comparison of anticoagulation of free FXIIIapt (i.e., FXIIIapt-FAM), FXIIIapt-Au NPs and FXIIIapt-Au NPs/GO. (A) TCT, (B) aPTT, (C) PT, and (D) TEG assays. Error bars represent the standard deviation of four repeated measurements. Light scattering was monitored from samples containing plasma, inhibitor and clotting reagent. The clotting time obtained from the intensity of light scattering as a result of the coagulation process in the presence of inhibitor (control), FXIIIapt, FXIIIapt-Au NPs and FXIIIapt-Au NPs/GO ([FXIIIapt] = 100 nM). The mean value and standard deviations are determined from three independent experiments.

Targeting of FXIIIapt-Fe_3O_4@Au NPs to fibrin clots

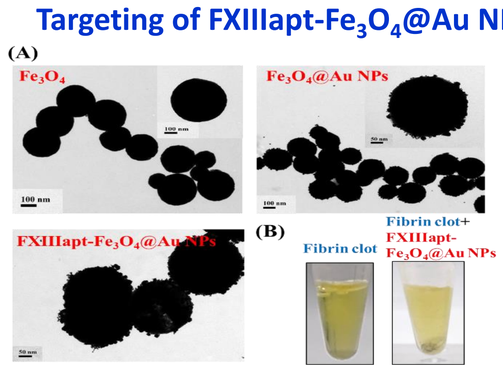


Figure 8. Characterization of FXIIIapt-Fe_3O_4@Au NPs and their targeting to fibrin clots. (A) TEM images of Fe_3O_4 NPs, Fe_3O_4@Au NPs and FXIIIapt-Fe_3O_4@Au NPs. (B) Photographs of fibrin clots in the absence and presence of FXIIIapt-Fe_3O_4@Au NPs.

Summary

- The functional structures of FXIII'-binding aptamers were determined and optimized based on binding affinity assay and software.
- The FATs bind to activated platelets but not resting platelets.
- FAT2 exerts much more binding signal than scr-DNA on whole blood and platelet/fibrin clot from both human and rats.
- FXIIIapt-Au NPs/GO has a ultrastrong biding affinity (K_d = 6.94 × 10^{-12} M) toward FXIII protein.
- FXIIIapt-Au NPs/GO exhibits extraordinary targeting of fibrin clots and anticoagulation activity .
- FXIIIapt-Fe_3O_4@Au NPs possesses specific binding to fibrin clots and will be used for bimodal imaging of thrombus *in vivo*