Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

Activation of the Catalytic Activity of Thrombin for Fibrin Formation by Ultrasound

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Experimental Procedures

Materials and Methods

All chemical reagents were of analytical grade and used without further purification. All oligonucleotide sequences were synthesized and HPLC-purified by Biomers Co. Ltd. (Germany). All sequences used in this work are listed in Table S1. T4 DNA ligase (5 Weiss U/μL), phi29 DNA polymerase (10 U/μL), dNTP Set (100 mM each) were purchased from Thermo Fisher Scientific. Human α-Thrombin and Human Research Grade Fibrinogen were obtained from Cellsystems. Chloroauric acid ($HAuCl_4$) and sodium citrate were ordered from Sigma. SensoLyte®520 Thrombin Activity Assay Kit was purchased from AnaSpec, Inc, USA. Roti®GelStain was received from Carl Roth (Germany). Other chemicals were purchased from Sigma-Aldrich unless otherwise noted. Ultrapure water with a resistivity > 18.2 MΩ·cm was used for all experiments. Ultrasonication experiments at f = 20 kHz were performed with a Qsonica Q125 sonicator (USA) equipped with a 3 mm diameter microtip probe (A12628PRB20). Sonication was performed using pulsed ultrasound (2.0 s on, 1.0 s off at 50% Amplitude). Ultrasonication experiments at f = 5 MHz were performed using a focused ultrasound system, which comprised a waveform generator with an integrated oscilloscope function (Model SDS1202X-E, Siglent.eu, Helmond, Netherlands), a second waveform generator (Model 33622A, Keysight Technologies, Böblingen, Germany), a radiofrequency (RF) broadband power amplifier (Model AG1021, T&C Power Conversion, Rochester, NY, USA) and a waterproof 5 MHz focused immersion transducer (Model V308-SU, Olympus Europa SE & Co. KG, Hamburg, Germany) in a water tank.

Gel Electrophoresis to Test Binding and Conjugation

The binding between $mTBA_{15}$ and thrombin was demonstrated by agarose gel electrophoresis (4%). The same amount of $mTBA_{15}$ (50 μM) in Tris-HCl binding buffer (25 mM, pH 7.4, 150 mM NaCl, 5.0 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$) was heated to 95°C for 5 min, then cooled down to room temperature over 30 min to form the secondary structure, which is favorable for binding of thrombin. The different amounts of thrombin (concentrations 0, 10, 25, 50, and 75 μM) were then introduced to above solutions respectively and the mixtures were kept at 37 °C for 30 min. Electrophoresis was carried out in 1× TAE buffer at 130 V for 30 min. After that, the gel was imaged with a gel imager. Binding of $pTBA_{15}$ with Cy3-thrombin was determined by gel electrophoresis as above, with 1% instead of 4% agarose gel. 3% agarose gel was used to test conjugation of TBA_{15}-L or TBA_{15}-R with AuNPs (100 nM, 5 μL).

Preparation of the Circular DNA Template for RCA

The circular template was formed using a linear thrombin aptamer template, a primer, and T4 DNA ligase. Briefly, 30 μL of 5'-phosphorylated template (100 μM) were hybridized with 45 μL of primer (100 μM) in 1× T4 DNA ligase reaction buffer (40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP, pH 7.8), followed by heating at 95 °C for 5 min then slowly cooling down to 25 °C for 35 min. Subsequently, 15 μL of T4 DNA ligase were added to above solution and the mixture was incubated at 22 °C overnight. The ligase was denatured by heating to 70 °C for 10 min. To remove excess primers, the resulting mixture was treated with 2 μL of exonuclease

l (20 U/μL) in reaction buffer (67 mM Glycine-KOH, 6.7 mM $MgCl_2$, 1 mM DTT) at 37 °C for 2 h and followed by inactivation at 80 °C for 15 min.

Synthesis of $pTBA_{15}$ by RCA

The $pTBA_{15}$ was synthesized through rolling circle amplification (RCA). Ligated thrombin template (0.4 μM) was incubated with phi29 DNA polymerase (0.2 U/μL) and dNTPs (0.5 mM each) at 30°C for 20 h in the reaction buffer (33 mM Tris-acetate, 10 mM Mg-acetate, 66 mM K-acetate, 0.1% (v/v) Tween20, 1 mM DTT, pH 7.9). The reaction was terminated by heating to 65 °C for 10 min.

Modification of Thrombin with Cyanine 3

Thrombin (50 μM) was dispersed in HEPES buffer (0.1 M, pH7.4) and a solution of Cyanine3-NHS (100 μM) in DMSO was added. The reaction mixture was reacted for 5 h with slight shaking. Subsequently, the Cy3-labelled thrombin was washed and purified by centrifugation with an ultrafiltration tube (MWCO 10 kDa) three times.

Synthesis and Characterization of Au Nanoparticles

Using the standard citrate reduction method, 13 nm Au@citrate NPs were prepared as reported previously.[1] All glassware used in the following procedures was cleaned in a bath of freshly prepared aqua regia, rinsed thoroughly in water and dried with N_2 prior to use. Briefly, 0.5 mL of $HAuCl_4 \cdot 3H_2O$ solution (1%, w/v) in 50 mL of Milli-Q water was heated to boiling and then 1.5 mL of sodium citrate solution (1%, w/v) was added to the boiling solution quickly with vigorous stirring. After the color change was completed in 5 min, the solution was kept boiling for another 15 to 30 min and was then allowed to cool down to room temperature while stirring. The 13 nm Au@citrate NPs were kept at 4 °C. The morphology of the AuNPs was examined using a Libra 120 Transmission Electron Microscope with 120 kV accelerating voltage. Particle hydrodynamic diameter was measured on a Malvern Zetasizer Ultra with a He-Ne laser (633 nm) and a backscattering angle of 173 °.

Preparation of AuNP-DNA Conjugates

AuNPs were functionalized with split aptamer fragments *via* gold-sulfur bonds. AuNP-DNA complexes were synthesized using a pH-assisted and surfactant-free method according to literature with minor modifications.[2] In brief, 2.5 μL of thiolated TBA_{15}-L or TBA_{15}-R split aptamer (100 μM) were added into 747.5 μL of AuNPs (5 nM), followed by adding 250 μL of citrate-HCl (500 mM, pH = 7.4) buffer into the solution. After incubation at room temperature for 1 h, the prepared AuNP-DNA conjugates were harvested through centrifugation (15000 rpm, 30 min, 4 °C) and the precipitate was rinsed three times with PBS (10 mM, pH = 7.4), and stored at 4 °C for further use. The molar feed ratio of each split aptamer to AuNPs was 66:1 during synthesis. The supernatant containing excess oligonucleotides was collected and the amount of DNA was determined according to UV-vis absorption spectroscopy. From this, the amount of DNA conjugated to the AuNPs was estimated as shown in Figure S16.

Ultrasound-Controlled Protein Release and Quantification of Activated Protein

To investigate the behavior of release of thrombin from aptamer and protein complex, the thrombin@$pTBA_{15}$ was treated by sonication with the probe immersed in the fluid for 30, 60, and 180 s. At defined times, 20 μL of solution were pipetted and taken out for further gel electrophoresis. For thrombin@Au@TBA_{15}, the sonication time was 15, 30, 60, 180, and 300 s. To measure the activated thrombin, after US treatment the solution was centrifuged at 15000 rpm at 4 °C for 30 min. The supernatants were collected, and the active amount of thrombin was calculated according to the standard curve (Figure S15). The sonication treatments were performed using a 3 mm diameter probe with an ice-water bath. The input power level was adjusted around 10 W/cm^2 with a constant frequency (20 kHz) and amplitude (50%). Sonication experiments were conducted with pure thrombin (1 nM) as a control.
Experiments with the focused 5 MHz US setup were done as follows: The center of the transducer was set 2.5 cm away from the ultrasonic-transparent 24 well plate (SARSTEDT AG & Co. KG, Nümbrecht, Germany), which allowed the focus point to align with the level of samples. The following acoustic parameters were chosen: a frequency of 5 MHz, a duty cycle of 20%, a sonication time of 6 min, and peak rarefaction pressure of 855 kPa at the focus (corresponding to a mechanical index of MI = 0.38).

SUPPORTING INFORMATION

Inhibition and Activation of Thrombin Monitored by Light Scattering

To test the inhibition and activation of thrombin by $mTBA_{15}$, $pTBA_{15}$, or $Au@TBA_{15}$, the conversion of fibrinogen (substrate of thrombin) into fibrin fibers was chosen as a reaction. More specifically, when fibrinogen is catalytically transformed by thrombin, the insoluble fibrin fibers form which results in an increase of the light scattering intensity. This was measured by a plate reader with a 100 µL quartz fluorescence cuvette. For monitoring scattering, the excitation and emission wavelengths were both 650 nm, and the emission was detected at a right angle relative to the light excitation so that the excitation light did not interfere with the light-scattering signal.[3] In general, 6 µL of fibrinogen (7.8 mg/mL) were added into 100 µL of $thrombin@mTBA_{15}$, $thrombin@pTBA_{15}$, or $thrombin@Au@TBA_{15}$ solution (with or without sonication) or pure thrombin (with or without sonication) as a control. The initial rate of scattering increase represented the relative thrombin-inhibition strength of the tested sample. Initial rates were calculated from the linear range of the early slope of the scattering profile.

Inhibition and Activation of Thrombin Characterized by Light Microscopy

The inhibition and activation of thrombin was also characterized by light microscopy. 10 µL of $thrombin@pTBA_{15}$ or $thrombin@Au@TBA_{15}$ solution (concentration of thrombin:10 nM) with or without sonication treatment were mixed with 3 µL of fibrinogen (23 µM). Subsequently, 10 µL of the mixture were transferred to a clean silicon wafer (1 × 1 cm), which was covered carefully with a coverslip and allowed to dry for 3 h. After that, the different samples were analyzed by light microscopy. $thrombin@mTBA_{15}$ and pure thrombin were treated with or without sonication as control.

Colorimetric Assay for Testing the Binding and Release of Thrombin

A color change assay based on AuNPs was used to monitor the binding and release process of thrombin from $thrombin@pTBA_{15}$. Briefly, different concentrations (0 - 2.5 nM) of AuNP solution were incubated with 4-nitrophenol (375 µM) and $NaBH_4$ (10 mM) to determine the concentration for inducing the color change from yellow to transparent solution. Then, different concentrations (0.094 µM - 1.5 µM) of fibrinogen were mixed with 13 nm-sized AuNPs (2 nM) in Milli-Q water at room temperature for 5 min separately. The prepared fibrinogen-wrapped AuNPs (F-AuNPs) were reacted with naked thrombin (0.1 µM, with or without US treatment) or $thrombin@mTBA_{15}$ and $thrombin@pTBA_{15}$ (with or without ultrasound treatment) at room temperature for 20 min. A mixture containing 4-nitrophenol (650 µL, 375 µM) and freshly prepared $NaBH_4$ (10 mM) was then transferred to the above solution (250 µL) and mixed thoroughly. After 30 min reaction, the mixture was centrifuged (12000 rpm) to remove the AuNPs and fibrin gels. The supernatant was collected carefully, transferred into a 1 mL cuvette, and absorption spectra were recorded using a plate reader (SpectraMax M5 microplate reader, Molecular Devices). For the colorimetric assay test with the 5 MHz ultrasound probe, the $thrombin@pTBA_{15}$ or $thrombin@Au@TBA_{15}$ were pre-mixed with F-AuNPs before ultrasound treatment.

Results and Discussion

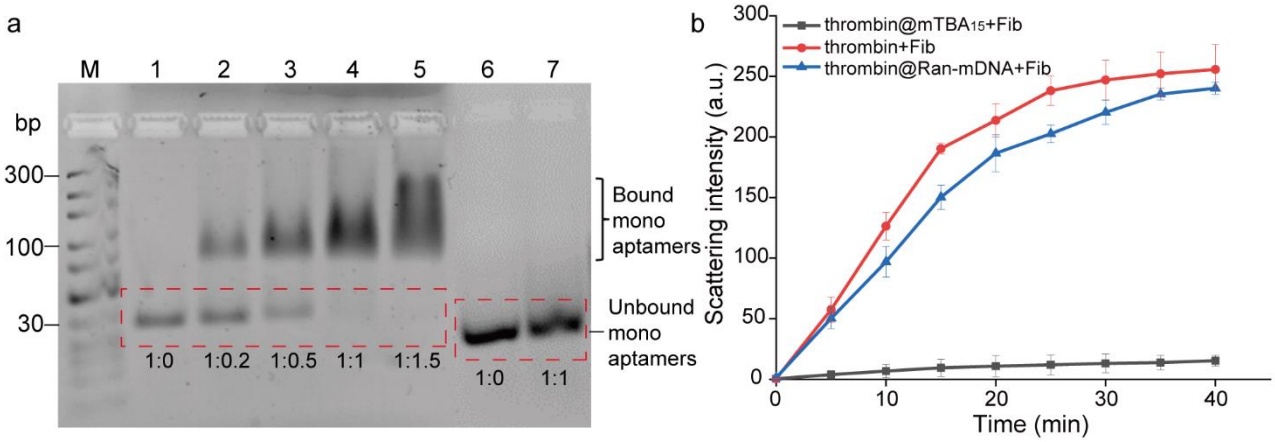


Figure S1. Binding and inhibition test of $mTBA_{15}$ aptamer with thrombin. a) Agarose gel (4%) characterization of binding between $mTBA_{15}$/Ran-mDNA and thrombin. Lane M: ultralow DNA marker; Lane 1-5: ratios of $mTBA_{15}$ to thrombin from 1:0, 1:0.2; 1:0.5; 1:1 to 1:1.5; Lane 6-7: ratios of Ran-mDNA to thrombin from 1:0 to 1:1. b) Real-time light scattering signal of fibrinogen solution with added thrombin (control), thrombin inhibited by $mTBA_{15}$, and Ran-mDNA.

SUPPORTING INFORMATION

Table S1. Sequences of DNA oligonucleotides used in this work.

Strand	Sequence (5'-3')
$mTBA_{15}$	GGTTGGTGTGGTTGGTTTTTTTTTTTTTTTTTTTTTTTT
$pTBA_{15}$ RCA template	Phosphate-GATCCTAAAAAAAAAAAAAAAAAAAAAAAAAAAACCAACCACACCAACCAAAAAAAAAACCACAC
Ran-mDNA	ATCAGGGCTAAAGAGTGCAGAGTTACTTAGTTTTTTTTTT
Ran-mDNA RCA template	Phosphate-GATCCTAAAAAAAAAAAAAAAAAAAAAAAAAAAACTAAGTAACTCTGCACTCTTTAGCCCTGATAAAAAAAAACCACAC
Primer	TTAGGATCGTGTGGTT
TBA_{15}-L	SH-TTTTTTTTTTTTTTTTTTTTTTTTTTGGTTGGTG
TBA_{15}-R	TGGTTGGTTTTTTTTTTTTTTTTTTTTTTTTT-SH

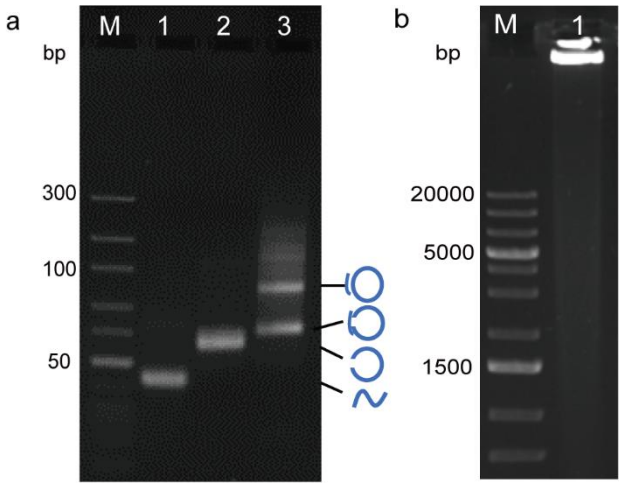


Figure S2. Ligation and $pTBA_{15}$ chain formation. a) Agarose gel (4%) characterization of ligation process. Lane M: ultra-low range DNA marker; lane 1: primer; lane 2: RCA template; lane 3: ligated template DNA with T4 DNA ligase. b) Agarose gel (1%) characterization of $pTBA_{15}$ chain. Lane M: 1 kb plus DNA marker; Lane 1: $pTBA_{15}$ product.

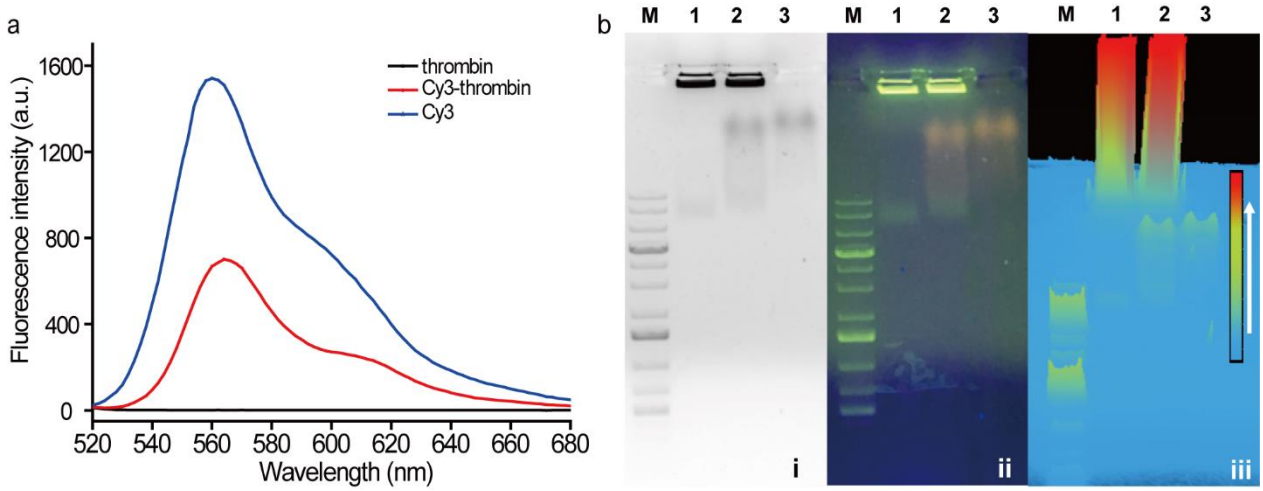


Figure S3. Characterization of binding between Cy3-Thrombin and Ran-pDNA. a) Fluorescence spectrum of Cy3-labelled thrombin. Excitation wavelength: 550 nm. Conditions: Tris-HCl binding buffer (25 mM, pH 7.4, 150 mM NaCl, 5.0 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$). Concentration of thrombin: 2.5 μM. b) Agarose gel (1%) for binding test of Ran-pDNA with Cy3-thrombin. Lane M: 1 Kb plus DNA marker; Lane 1: Ran-pDNA; Lane 2: Cy3-thrombin@Ran-pDNA; Lane 3: Cy3-thrombin. (i) Fluorescence image in grayscale; (ii) Corresponding fluorescence image in color scale; (iii) Corresponding 3D gel image. Compared with the $pTBA_{15}$ the fluorescence did not show any overlap and fluorescence intensity did not increase either, meaning that Cy3-thrombin binds $pTBA_{15}$ but not Ran-pDNA.

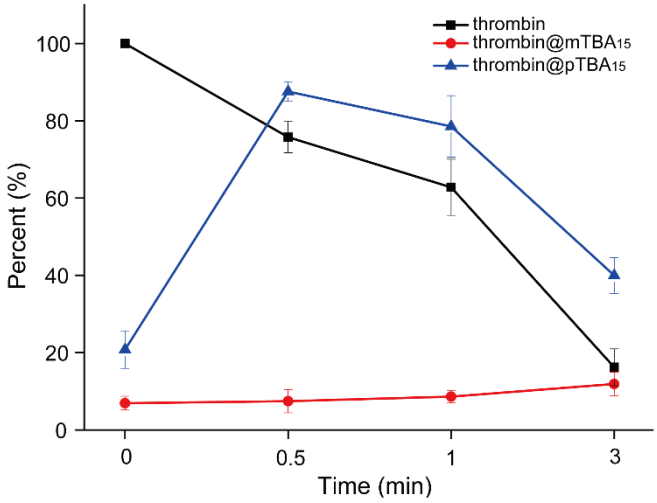


Figure S4. Normalized thrombin activity of thrombin (control), thrombin@mTBA$_{15}$, and thrombin@pTBA$_{15}$ treated with ultrasound for 0, 30, 60, and 180 s, respectively.

Figure S5. 1% agarose gel to trace ultrasound treatment of pTBA$_{15}$ for different time periods. From lane 1-5: 0, 30, 60, 180, and 300 s.

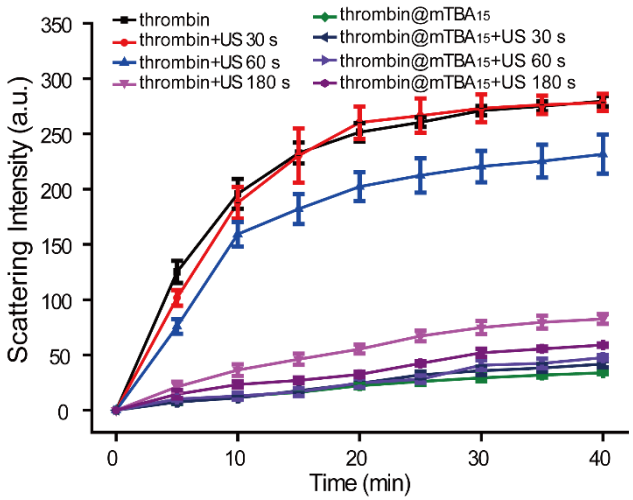


Figure S6. Real-time light scattering spectra of fibrinogen solution with added thrombin or thrombin@mTBA$_{15}$ with or without treatment of ultrasound. Ultrasound had little influence on uninhibited thrombin at short exposure times of 30 s and 60 s. Thrombin@mTBA$_{15}$ showed almost no response to ultrasound. Mean values ± SD from the mean, *N* = 3 independent experiments.

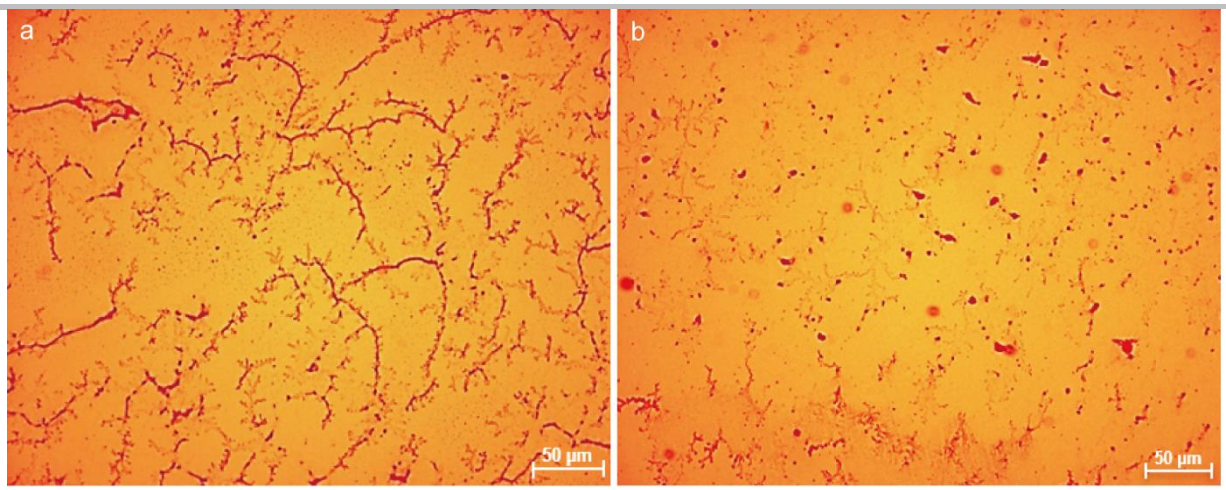


Figure S7. Optical microscopy images of fibrinogen treated with a) thrombin@pTBA$_{15}$ with ultrasound for 60 s; b) thrombin@pTBA$_{15}$ with ultrasound for 180 s.

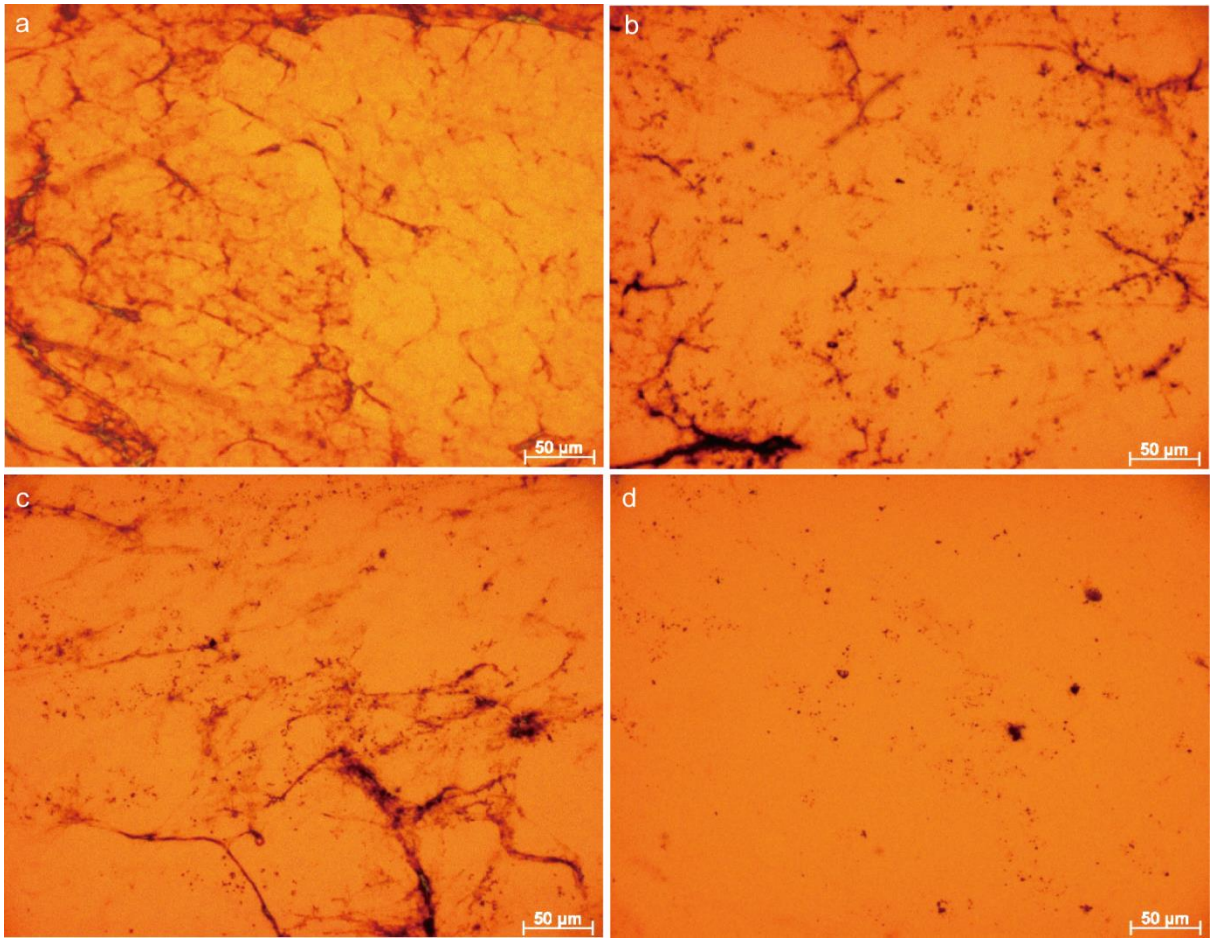


Figure S8. Optical microscopy images of fibrinogen treated with a) thrombin, b) thrombin exposed to ultrasound for 30 s, c) thrombin exposed to ultrasound for 60 s, and d) thrombin exposed to ultrasound for 180 s.

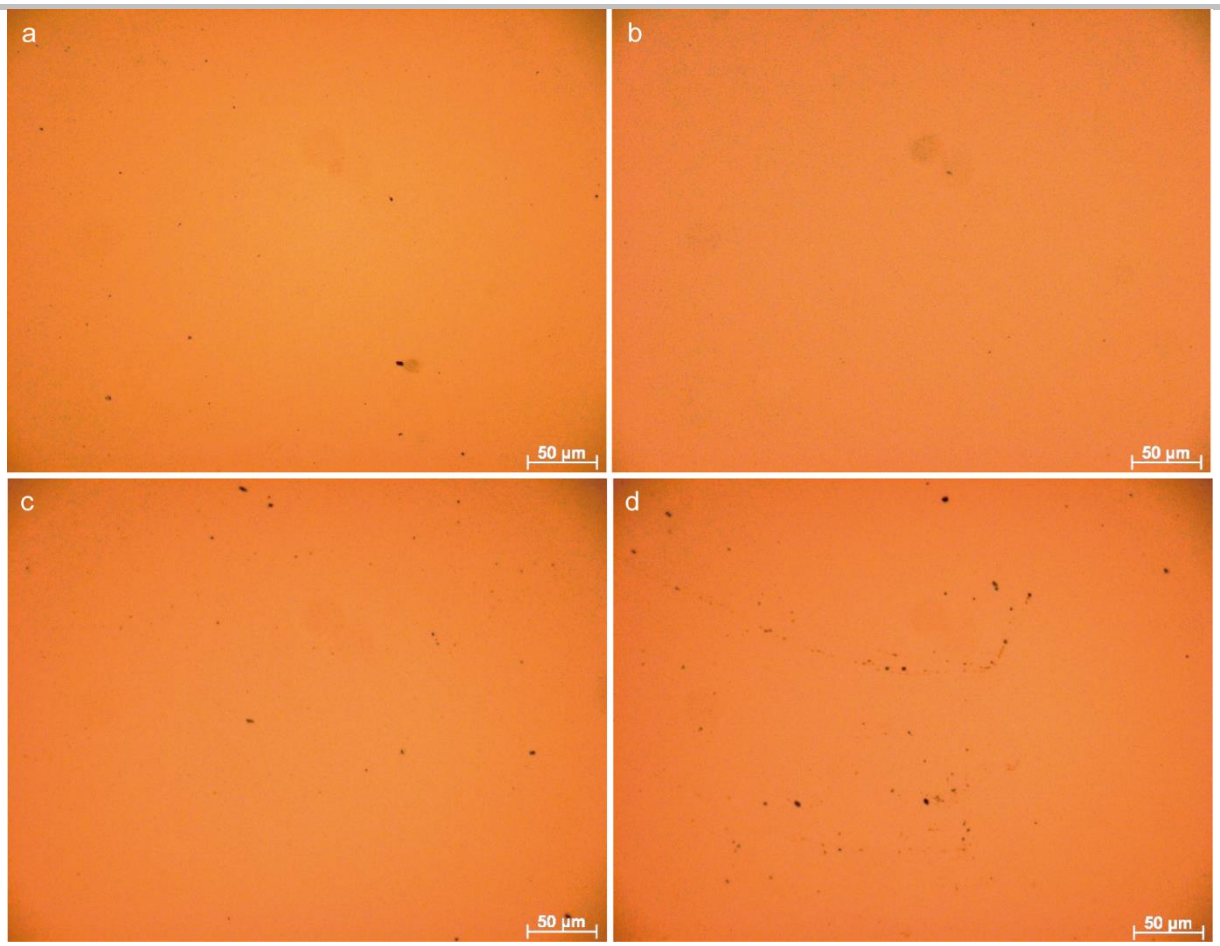
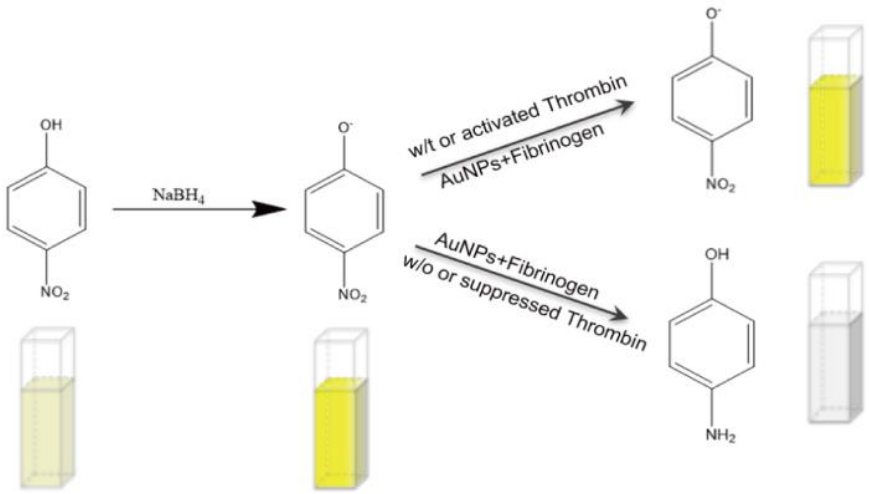


Figure S9. Optical microscopy images of fibrinogen treated with a) $mTBA_{15}$, b) thrombin@$mTBA_{15}$ exposed to ultrasound for 30 s, c) thrombin@$mTBA_{15}$ exposed to ultrasound for 60 s, and d) thrombin@$mTBA_{15}$ exposed to ultrasound for 180 s.



Scheme S1. Schematic representation of colorimetric assay to visualize the process of deactivation and activation of thrombin.

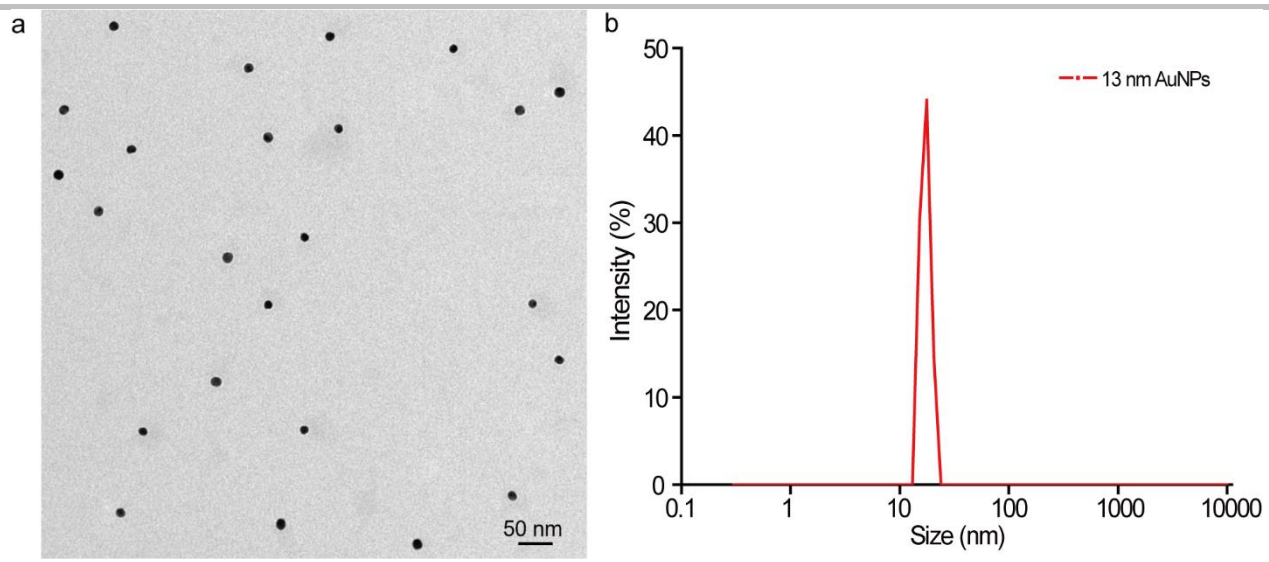


Figure S10. Fabrication and characterization of AuNPs. a) TEM image of AuNPs. Scale bar: 50 nm. b) DLS characterization for AuNPs.

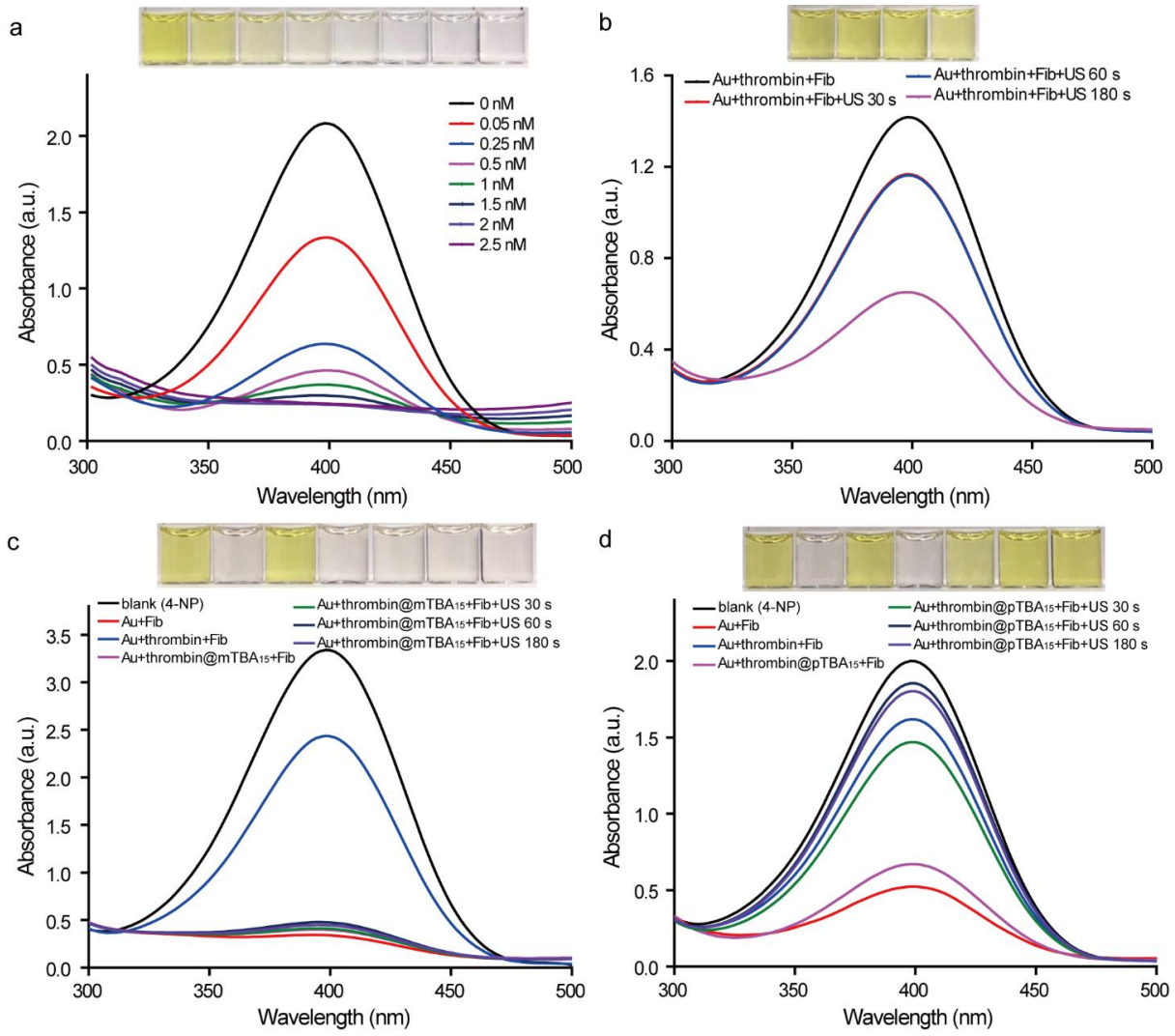


Figure S11. Absorption spectra and color change of colorimetric assay to follow the process of deactivation and activation of thrombin. a) Different concentrations of AuNPs induced color change; b) The influence of ultrasound on pristine thrombin (note: the red and the blue spectra lie over each other); c) The influence of ultrasound on thrombin@mTBA$_{15}$; d) The influence of ultrasound on thrombin@pTBA$_{15}$.

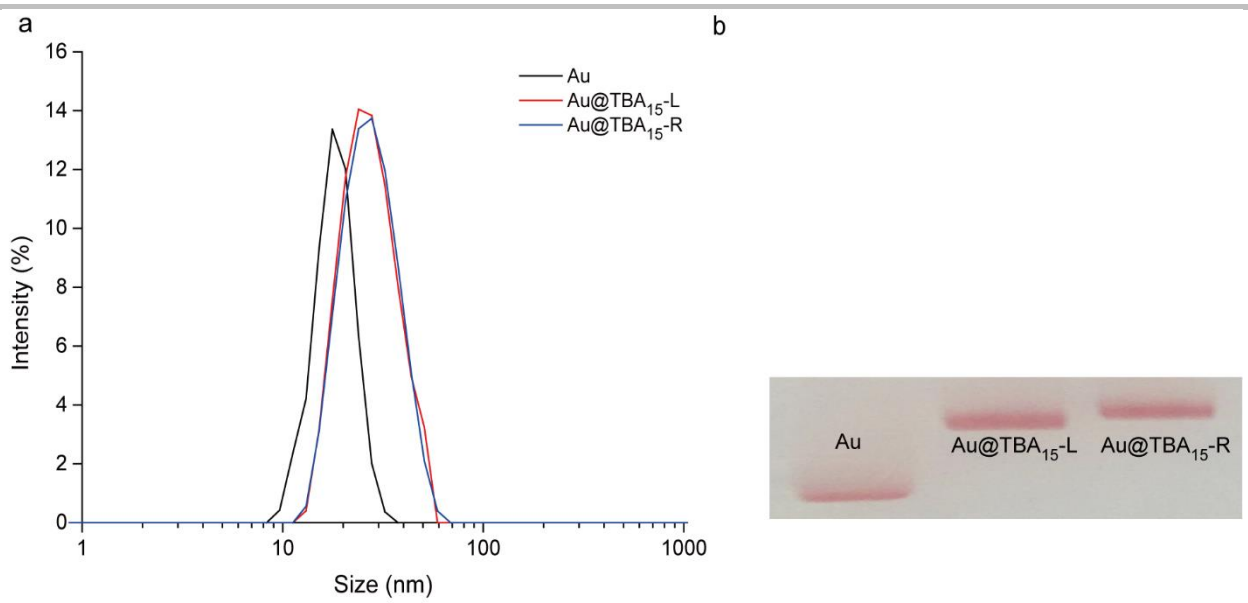


Figure S12. Modification of AuNPs with the split aptamer. a) Dynamic light scattering analysis of Au, Au@TBA_{15}-L and Au@TBA_{15}-R. b) Agarose gel (3%) electrophoresis of Au, Au@TBA_{15}-L and Au@TBA_{15}-R.

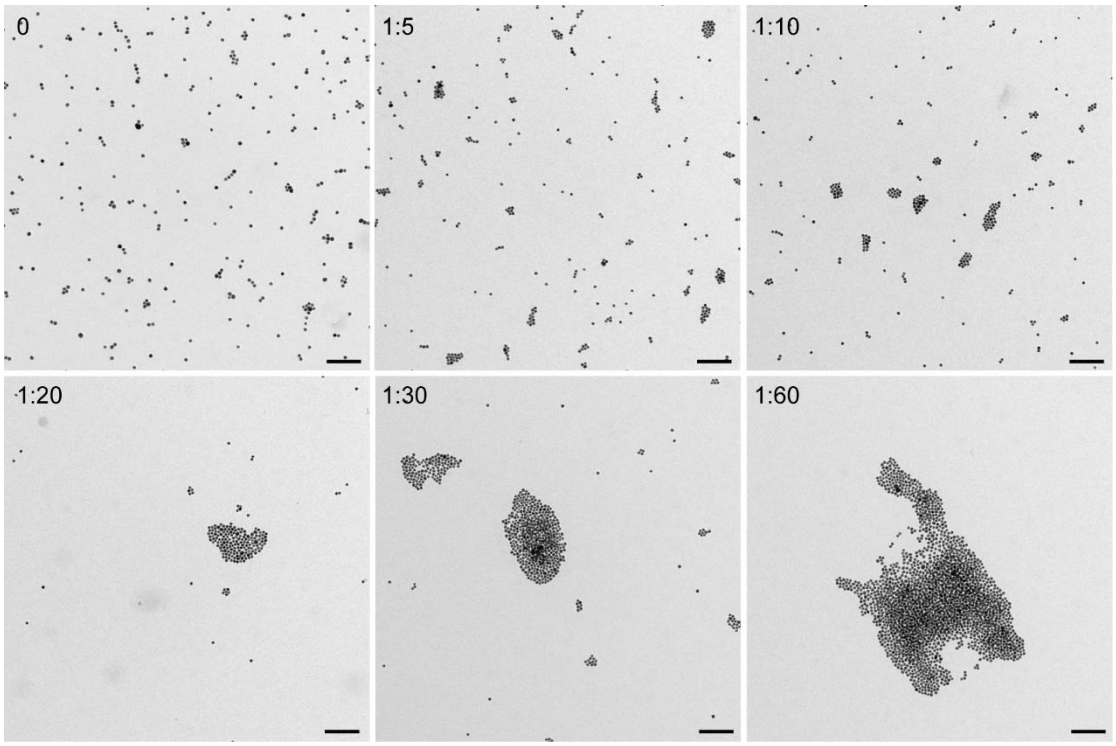


Figure S13. Transmission electron microscopy (TEM) images of the AuNP assembly in the presence of different molar ratios of NPs to thrombin (Scale bar: 100 nm).

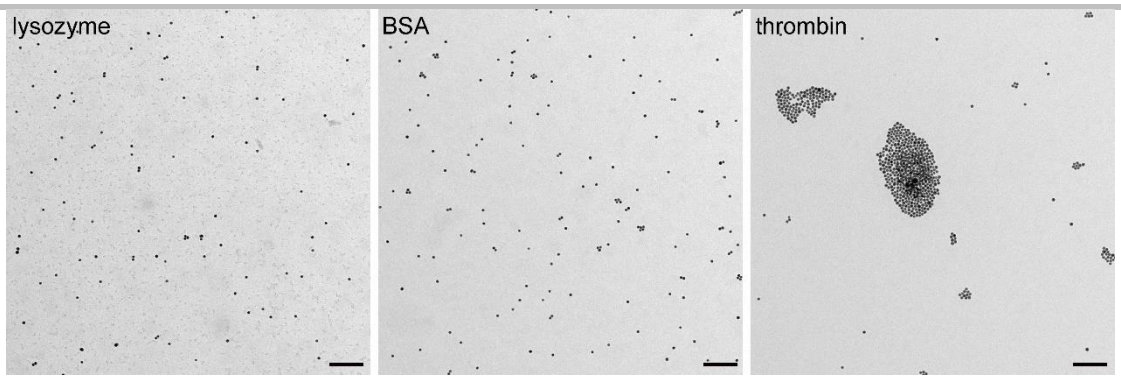


Figure S14. Transmission electron microscopy (TEM) images of binding specificity test of Au@TBA_{15}. (Scale bar: 100 nm).

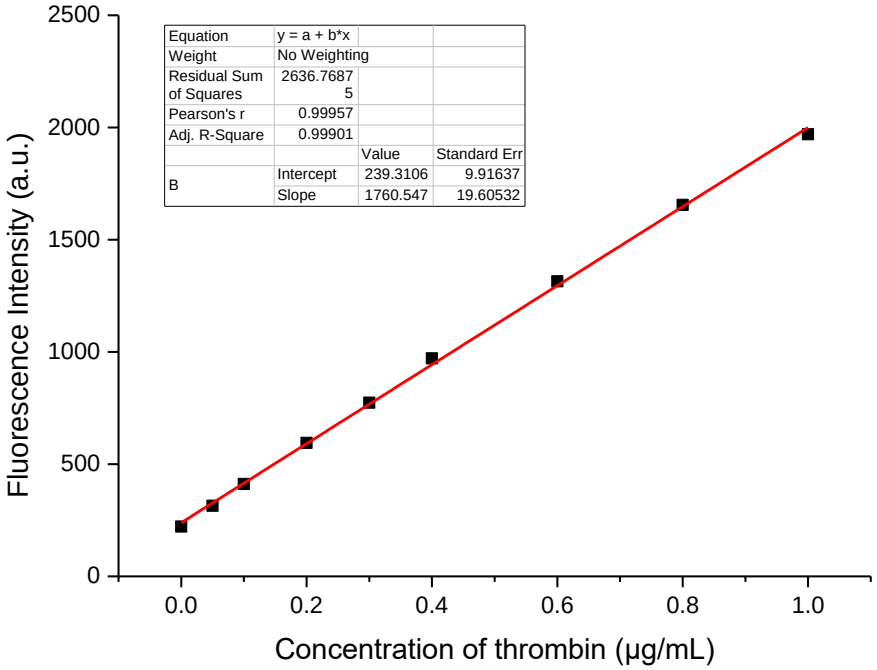


Figure S15. Linear relationship between fluorescence intensity and concentration of thrombin (5-FAM, λ_{exc} / λ_{em} = 490 nm / 520 nm).

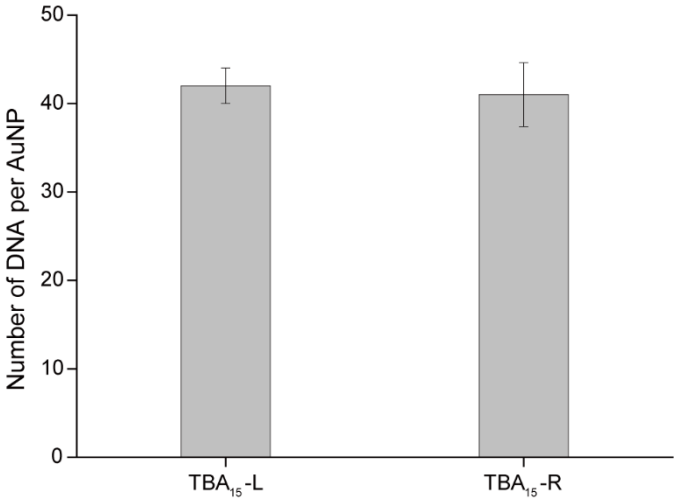


Figure S16. Quantification of TBA_{15}-L and TBA_{15}-R DNA loading on AuNPs.

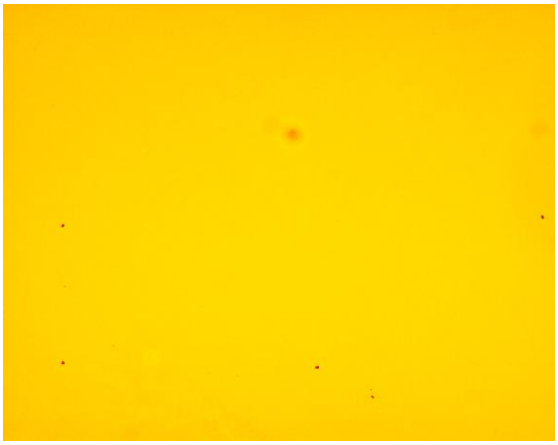


Figure S17. Optical microscopy images of fibrinogen treated with Au@TBA_{15}.

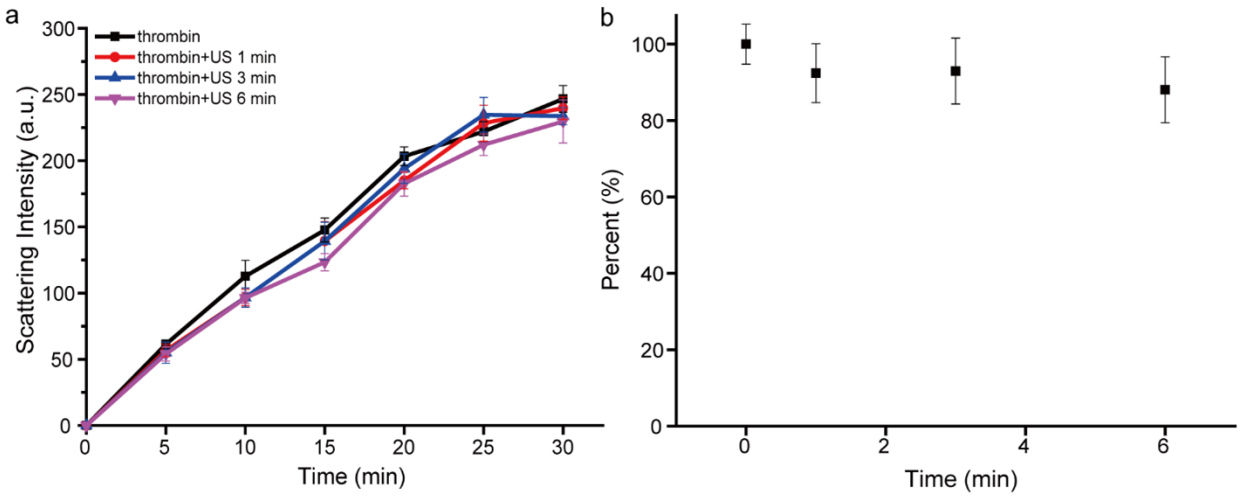


Figure S18. a) Real-time light scattering spectra of fibrinogen solution with thrombin with or without treatment of 5 MHz US. b) Normalized thrombin activity. Mean values ± SD from the mean, *N* = 3 independent experiments.

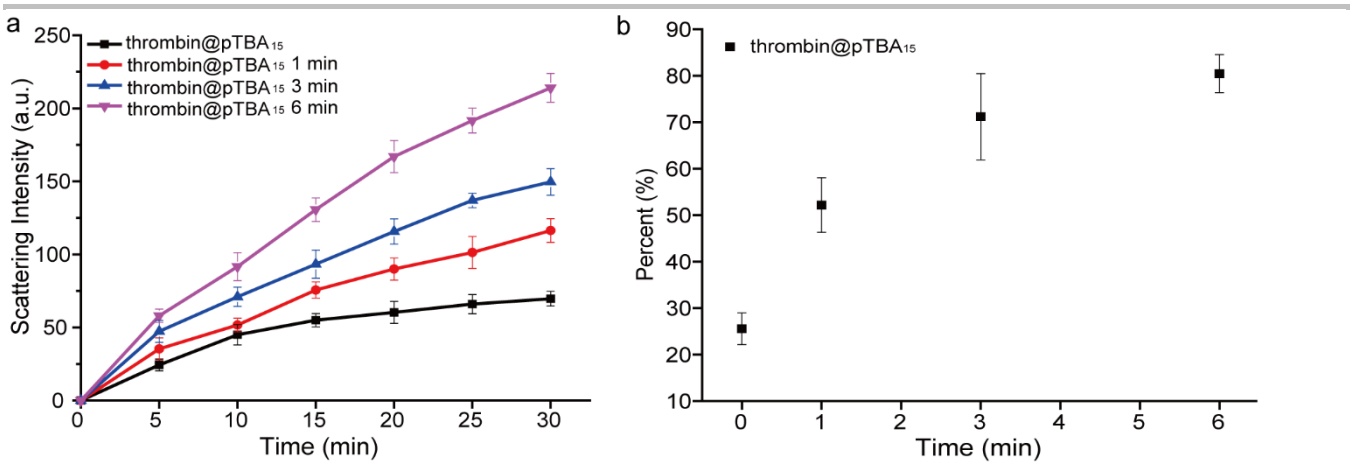


Figure S19. a) Real-time light scattering spectra of fibrinogen solution with thrombin@pTBA$_{15}$ with or without treatment of 5 MHz US. b) Normalized activity of thrombin. Mean values ± SD from the mean, *N* = 3 independent experiments.

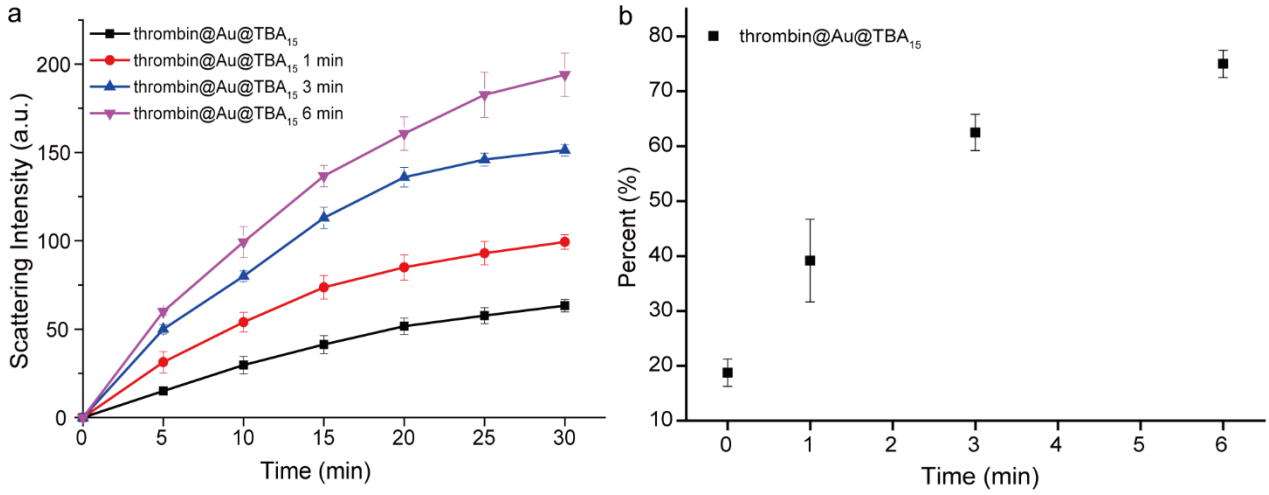


Figure S20. a) Real-time light scattering spectra of fibrinogen solution with thrombin@Au@TBA$_{15}$ with or without treatment of 5 MHz US. b) Normalized activity of thrombin. Mean values ± SD from the mean, *N* = 3 independent experiments.

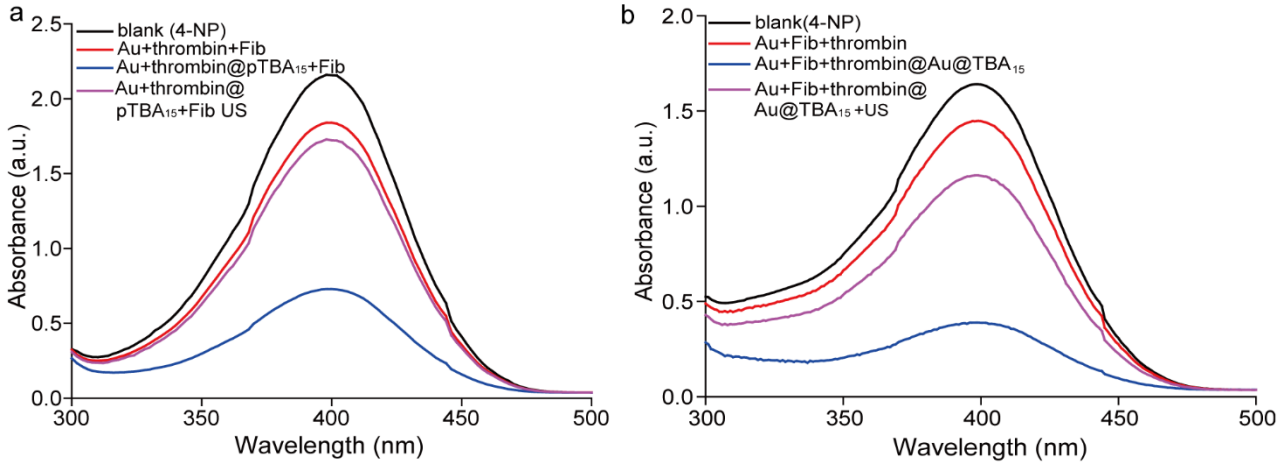


Figure S21. Absorption spectra to follow the process of deactivation and activation of thrombin during colorimetric assay from a) thrombin@pTBA$_{15}$ and b) thrombin@Au@TBA$_{15}$.

References

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[3] Y. Kim, Z. Cao, W. Tan, *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 5664–5669.

Author Contributions

P. Z. performed all synthetic and analytic experiments involving (poly)aptamers and evaluated the results thereof. S. H. performed AuNP synthesis and analysis. J. F. conducted TEM measurements. J. C. and F. K. supported experiments on the 5 MHz US setup. S. H., A. J. B., R. G., and A. H. conceived and supervised the project. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.