Electronic Supplementary Information for:

Microarray-Based Resonance Light Scattering Assay for Detecting Thrombin Generation in Human Plasma by Gold Nanoparticle Probes

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Experimental section

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Experimental section

Materials and reagents. Factor Xa (FXa) was purchased from New England Biolabs (Beverly, MA, USA). Thrombin and avidin were purchased from Sigma-Aldrich Co. (USA). Human Antithrombin-III (Human ATIII) was purchased from Haematologic Technologies Inc. (USA). Bovine serum albumin (BSA) was purchased from GEN-VIEW Scientific Inc. (USA). Recombinant tissue factor (rTF) was purchased from Sekisui Diagnostics Ltd. (USA). Phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine were purchased from Avanti Polar Lipids Inc. (USA). Peptides: CALNN, CALNNGK(biotin)G and peptide substrates (see Table S1) were purchased from ChinaPeptides Ltd. (Shanghai, China). To obtain biotinylated peptide, biotinylated lysine (biotin-K) was used in the peptide synthesis process. 96-well polystyrene flat-bottomed microtiter plates were purchased from Thermo Fisher Scientific Inc. (USA). Aldehyde 3-D glass slides and polytetrafluoroethylene (PTFE) masker were purchased from CapitalBio Ltd. (Beijing, China). Other chemicals were of analytical grade and used as received. Milli-Q water (18.2 MΩ.cm) was used in all experiments.

Blood sampling. Approval was obtained from the Local Reasearch Ethics Committee (Jilin Blood Center Changchun Central Blood Station) and written informed consent for blood sampling was obtained from all subjects. Venepuncture was carried out with a 21-G needle using minimal suction with a light tourniquet. Venous blood (2.7 mL) was taken directly into BD citrate tubes containing 0.3 mL 0.109 mol/L sodium citrate. Platelet-poor plasma (PPP) was produced by

centrifugation (2000*g* for 15 min) and stored at -80 °C until assay.

Preparation of gold nanoparticle probes. The citrate stabilized 30 nm GNPs were synthesized by the traditional Turkevich-Frens method.[S1] GNP probes were prepared by our previous peptide capping procedure.[S2] Generally, an aqueous solution of peptide mixture (CALNN/CALNNGK(biotin)G=9:1 molar ratio) was added to the solution of 4 nM 30 nm GNPs to give a final concentration of total peptide of 20 M. After overnight incubation, excess peptides were removed by repeated centrifugation at 4000 rpm (1000*g*, 3 times) using an Eppendorf centrifuge (Eppendorf, Germany). The as-prepared GNP probes were resuspended in Probe Buffer (pH 7.5, 50 mM PB and 0.15 M NaCl) and stored at 4 °C. The GNP probes were characterized by a Hitachi H-600 transmission electron microscopy (TEM, Hitachi Ltd., Japan) at an accelerating voltage of 100 kV. The UV-visible spectra of GNP probes were recorded by a TU-1901 UV-visible spectrophotometer (Purkinje General Instrument Ltd., Beijing, China).

Peptide microarray fabrication and enzymatic hydrolysis. Peptide microarrays were manufactured by the standard procedure using a SmartArrayer 136 system (CapitalBio Ltd., Beijing, China).[S3] Generally, desired concentrations of peptide substrates in spotting buffer (0.3 M PBS (pH 8.5, 0.2 M NaCl) with 20 μg/mL BSA) were spotted on aldehyde 3-D slides, following overnight incubation under vacuum at 30 °C. The slides were washed with 30 mL of phosphate buffer (pH 7.5, 50 mM) containing 1% (w/v) BSA for 10 min and then immersed in blocking buffer (pH 7.5, 50 mM PB, 0.15 M NaCl containing 3% w/v BSA) for 1 h to inactivate any free

aldehyde groups on slide surface. After blocking reaction, the slides were subjected to a series of washing steps: (1) 30 mL Milli-Q water for 3 min (3 times), (2) 30 mL of washing buffer (pH 7.5, 20 mM Tris, 0.15 M NaCl, 2 mM EDTA with 0.1% Triton X-100) for 10 min (2 times), and (3) 30 mL of thrombin buffer (pH 7.35, 20 mM HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid), 0.14 mM NaCl and 2 mM $CaCl_2$) for 10 min (2 times), respectively. Subsequently, the slides were dried by centrifugation (200*g* for 30 s) and divided into 10 independent subarrays by a PTFE masker. The slides were then incubated with thrombin, FXa or protease mixture (thrombin/FXa =10:1 molar ratio) which was diluted to the desired concentration with 30 μL thrombin buffer for 1 h at 37 °C. Corresponding control experiment was carried out by incubating one subarray with blank thrombin buffer on the same slide. After hydrolysis reaction, PTFE maskers were removed and slides were rinsed with 30 mL of washing buffer for 5 min (3 times), 30 mL of washing buffer without Triton X-100 for 5 min (3 times), and 30 mL Milli-Q water for 3 min (3 times), respectively. The slides were then dried by centrifugation (200*g* for 30 s) and subjected to the labeling procedure.

Attachment of GNP probes and detection. After enzymatic hydrolysis, the microarrays were incubated with avidin (3 μM) in 300 μL of probe buffer (pH 7.5, 50 mM PB, 0.15 M NaCl, supplemented with 0.1% Tween-20 (v/v) and 1% BSA (w/v)) at 37 °C for 1 h. Then, a series of washing steps were applied to the microarrays: (1) 30 mL of PBS buffer with 1% Tween-20 for 5 min (3 times), (2) 30 mL of PBS buffer for 5 min (3 times), and (3) 30 mL of Milli-Q water for 3 min (3 times), respectively.

Subsequently, the microarrays were treated with 300 μL of GNP probes (3 nM) in probe buffer for 1 h at 37 °C, washed and dried as described previously. Finally, the microarrays were read by a colorimetric microarray scanner (TeleChem International Inc., USA). According to the manufacturer's preset parameters, all images were collected with a broad spectrum white light source.

The background originating from the slides was recorded and subtracted from each image prior to evaluation. The mean value and standard deviation of the signal were determined from the 6 spot replicates per sample, respectively. The RLS signal changes (ΔR) were calculated using the following equation:

$$\Delta R=(R_0-R)/R_0\times 100\% \quad (1)$$

where R_0 and R are the mean RLS intensity of 6 spots on the same microarray without and with treatment of proteases, respectively.

Detection of thrombin generation in human plasma. Human plasma which was diluted to the desired concentration with prewarmed (37 °C) dilution buffer (pH 7.35, 20 mM HEPES, 21.7 mM citrate and 60 mg/mL BSA) mixed with 20 μL prewarmed (37 °C) trigger solution in a well of 96-well polystyrene flat-bottomed microtiter plate. The trigger solution was composed of 30 pM rTF and 24 μM procoagulant phospholipids (phosphatidylserine: phosphatidylethanolamine: phosphatidylcholine=1:1:3 molar ratio). After incubated at 37 °C for 5 min with gently shaken, 20 μL of prewarmed (37 °C) HEPES buffer (pH 7.35, 20 mM HEPES, 60 mg/mL BSA and 0.1 M $CaCl_2$) was added and mixed well. Then, 30 μL of the mixture was transferred to one subarray and incubated at 37 °C for 40 min. A control

experiment was carried out by incubating one subarray with non-activated human plasma on the same slide. After incubation, the microarrays were treated and read as described previously. The RLS signal changes were calculated using equation 1, where R_0 and R are the mean RLS intensity of 6 spots on the same microarray with incubation of non-activated and activated human plasma, respectively.

Table S1 Peptide substrate sequences used in the experiments

Code	Sequences	Reference
S01	CAEGGGVR-GPRVVK(biotin)[a]	S4
S02	CEGFFSAR-GHRPLK(biotin)	S4
S03	CAEGGVPR-SFRVVK(biotin)	S5
S04	CAEGGFPR-SFRVVK(biotin)	S5
S05	CAEGGVPR-SFKVVK(biotin)	S5
S06	CGGGARPR-SLLVGK(biotin)	S6
S07	CAEGGfPR-SFRVVK(biotin)[b]	S5
S08	CGGGVRPG-RVGGGEALFDK(biotin)	S4
S09	CGGGARSR-TVGGK(biotin)	S7

[a] '-'indicates the protease cleavage site. [b] Lowercase letter means the D-amino acid residue.

Table S2 Details of 13 normal, 7 hypocoagulability and 4 thrombophilia plasma samples

Gender (diagnosis)	Age, years	ΔR, %
Normal		
F	45	72.391±3.463[a]
F	48	66.282±2.878
F	54	57.627±2.740
F	59	75.029±1.180
M	38	73.262±2.379
M	42	68.736±3.925
M	42	62.285±2.027
M	47	68.384±2.577
M	48	72.476±3.880
M	51	73.673±1.945
M	57	61.510±3.880
M	60	65.502±5.260
M	65	62.540±6.133
Hypocoagulability		
F	48	48.868±5.164
F	54	50.427±2.260
F	59	54.676±3.542
M	47	46.857±4.214

M	48	59.799±4.454
M	57	42.012±2.457
M	60	48.710±1.568
Thrombophilia		
F (DVT)[b]	61	78.082±3.332
F (DVT)	71	74.398±1.877
M (DVT)	43	71.329±3.291
M (DVT)	60	68.803±3.079

[a] The standard deviations are derived from 6 reduplicate spots. [b] DVT, Deep vein thrombosis. The 4 patients with a history of idiopathic venous thrombosis had all had objectively documented evidence of thrombosis and had completed all anticoagulant therapy at least 3 months before blood sampling.

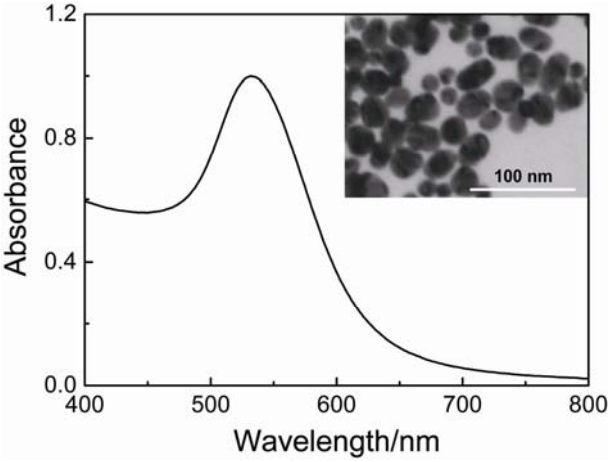

Fig. S1 TEM micrograph (inset) and corresponding UV-visible spectrum of GNP probes. The average size of GNP probes is 30±5 nm.

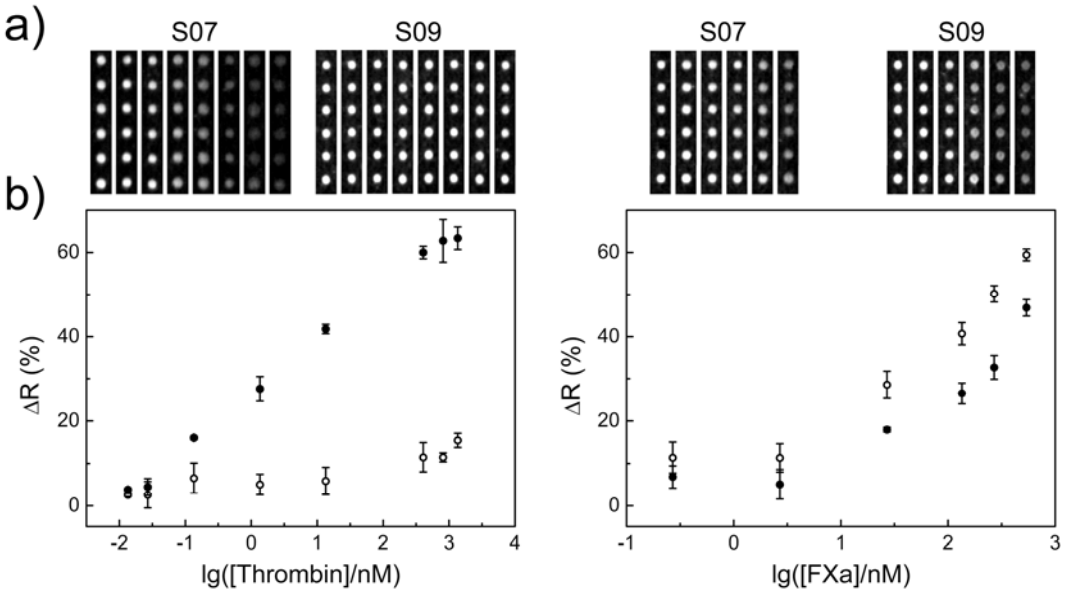

Fig. S2 (a) RLS images and (b) corresponding curves of integrated RLS intensity change as a function of the concentration of pure thrombin or FXa. The concentrations of pure thrombin are 0.0135, 0.027, 0.135, 1.35, 13.5, 405, 810 and 1350 nM, the concentrations of pure FXa are 0.27, 2.7, 27, 135, 270 and 540 nM, respectively. The concentrations of S07 (●) and S09 (○) in spotting solution are 0.5 mg/mL, respectively.

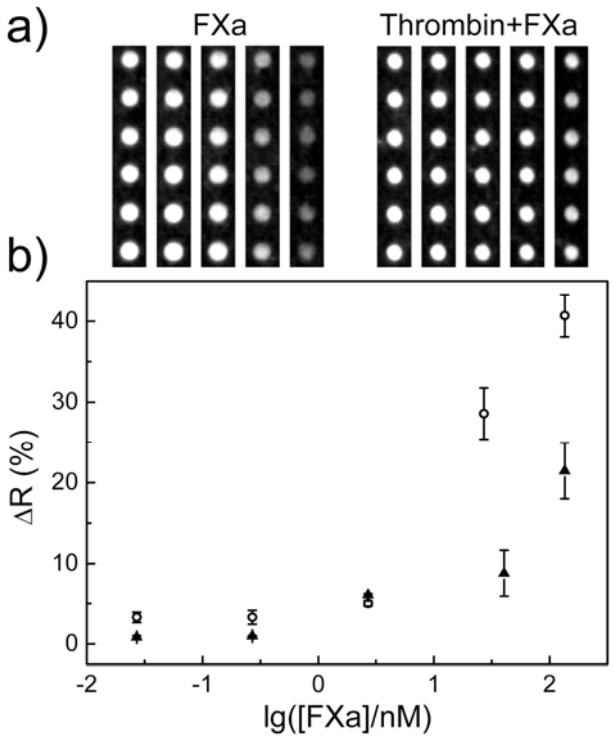

Fig. S3 (a) RLS images and (b) corresponding curves of integrated RLS intensity change as a function of the concentration of pure FXa (○), or the corresponding FXa concentration in protease mixture (▲). The concentrations of pure FXa are 0.027, 0.27, 2.7, 27 and 135 nM. The corresponding concentrations of FXa in protease mixture are 0.027, 0.27, 2.7, 40.5 and 135 nM. The molar ratio of thrombin and FXa in protease mixture is 10:1. The concentration of S09 in spotting solution is 0.5 mg/mL.

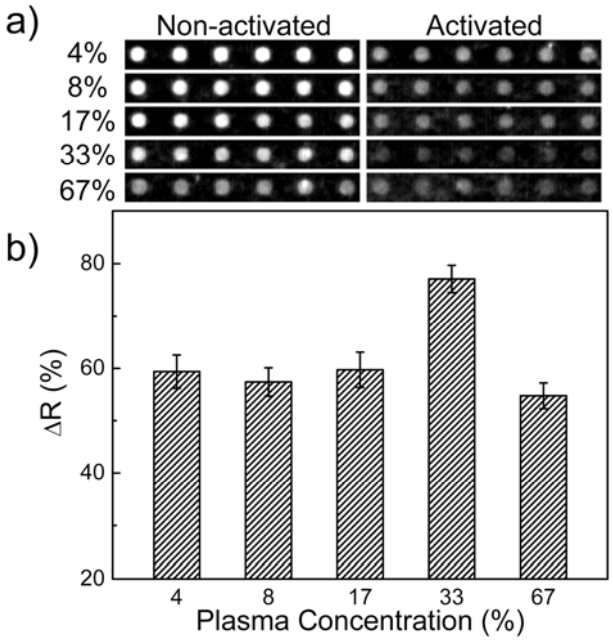

Fig. S4 (a) RLS images of peptide microarrays incubated with different concentrations of non-activated/activated human plasma. (b) The corresponding RLS intensity changes of different concentrations of human plasma. The concentration of S07 in spotting solution is 0.5 mg/mL.

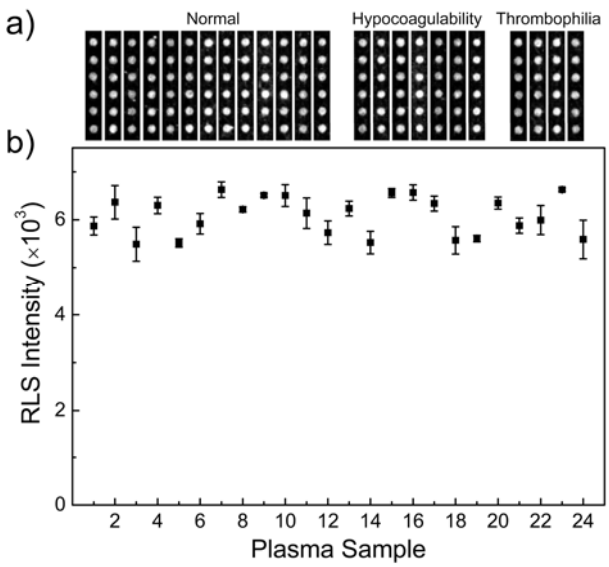

Fig. S5 (a) RLS images and (b) corresponding RLS intensities of non-activated human plasma samples (1-13, 14-20 and 21-24 are normal, hypocoagulability and thrombophilia plasma samples, respectively). The concentration of S07 in spotting solution is 0.5 mg/mL.

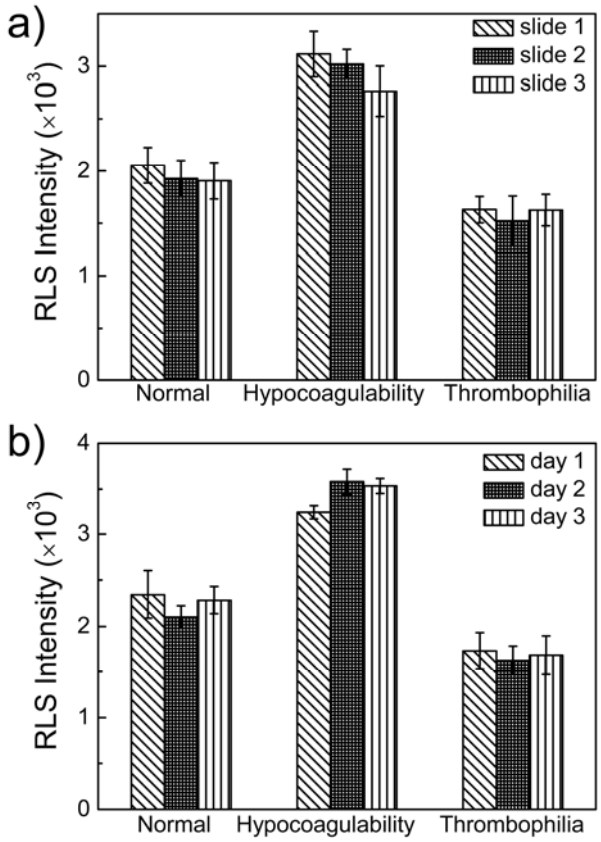

Fig. S6 RLS intensities of normal, hypocoagulability and thrombophilia plasma samples. Diluted plasma samples (33%) were incubated on (a) 3 peptide microarray slides which fabricated at different batches at same day, and (b) 3 peptide microarray slides which fabricated at different batches at different days, respectively. The concentration of S07 in spotting solution is 0.5 mg/mL.

The arrays in (a) and (b) were incubated with same plasma samples, respectively. The coefficient of variations (CVs) of the RLS signal intensities of these samples were between 5 to 10 %.

Additional references

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