Electronic Supplementary Information

Surface immobilization of protease through an inhibitor-derived affinity ligand: a bioactive surface with defensive properties against inhibitor

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Experimental section

Materials

Gold-coated[1] silicon wafers (80 nm Au on a 10 nm chromium adhesion layer) were diced into 0.5 × 0.5 cm^2 pieces. The PAI-1-derived peptide (sequence ARMAPE) was purchased from GL Biochem (Shanghai) Ltd and synthesized using standard Fmoc-mediated solid-phase peptide synthesis. Oligo(ethylene glycol) methacrylate (OEGMA) of molecular weight 360 Da and hydroxyethyl methacrylate (HEMA) (99%) were purchased from Sigma-Aldrich Chemical Co. and passed through a basic Al_2O_3 column to remove the inhibitor before use. Triethylamine (TEA), 4-nitrophenyl chloroformate (NPC) were purchased from Sigma-Aldrich Chemical Co. and used as received. All other chemicals were purchased from Shanghai Chemical Reagent Co. and used without further purification, unless otherwise specified. Deionized water (DIW) was purified using a Millipore water purification system to give a minimum resistivity of 18.2 MΩ·cm. Fibrinogen was from Calbiochem (La Jolla, CA). The plasmin specific chromogenic substrate S-2251 was obtained from GL Biochem (Shanghai) Ltd. Recombinant tissue plasminogen activator (tPA) was purchased from Genentech (San Francisco, CA). Human serum albumin (HSA) and Plasminogen activator inhibitor-1 (PAI-1) was purchased from Sigma–Aldrich Chemical Co. $Na^{125}I$ was from Chengdu Gaotong Isotope Co., Ltd. (China).

Preparation of ARMAPE peptide-modified surface

ARMAPE peptide was immobilized on gold surfaces through POEGMA as a spacer. POEGMA was grafted on gold surface by surface-initiated atom transfer radical polymerization (SI-ATRP). The thiolated ATRP initiator (mercaptoundecyl bromoisobutyrate) was synthesized as described previously.[2] Gold-coated wafers were washed with acetone, treated for 30 min in an ozone plasma, washed with deionized water and ethanol, subsequently cleaned in a mixture of ammonia, hydrogen peroxide and deionized water ($NH_3{\cdot}H_2O$:H_2O_2:H_2O = 1:1:5 v/v/v) for 10 min at 75 ^oC, and then rinsed with deionized water and dried under nitrogen. The clean gold surfaces were immersed in 1 mM initiator in ethanol at ambient

temperature for 24 h. The surfaces were then thoroughly rinsed with ethanol and dried under a nitrogen stream. For surface polymerization of OEGMA by SI-ATRP, a reaction solution, consisting of 2.64 g OEGMA, 5 mL methanol, 5 mL H_2O, 12.5 mg bipyridyl, and 1 mL $CuCl_2 \cdot 2H_2O$ (0.04 M) was deoxygenized by ventilating under a N_2 stream for 15 min. Then 1 mL ascorbic acid (0.04 M) was added, followed by ventilation with a N_2 stream for another 15 min. Initiator-immobilized surfaces were immersed into the prepared solution and the polymerization proceeded for 1.5 h at ambient temperature under N_2 atmosphere in a glovebox. Finally, the reaction was stopped by rinsing the samples with deionized water and methanol thoroughly. The resulting surface is abbreviated as Au-POEGMA.

For peptide immobilization, hydroxyl groups of surface-grafted POEGMA were first activated by NPC. The Au-POEGMA surfaces were immersed in a solution composed of 0.1 g NPC, 5 mL anhydrous acetonitrile, and 50 mg trimethylamine at ambient temperature for 6 h. Then the surfaces were thoroughly rinsed with anhydrous acetonitrile and dried under a flow of nitrogen. The NPC-activated surfaces (Au-POEGMA-NPC) were immersed into the solution of ARMAPE peptide (0.5 $mg \cdot mL^{-1}$) in phosphate-buffered saline (PBS, pH 8.5) at 25 ^oC for 24 h. Finally, the surfaces were rinsed thoroughly with PBS (pH 7.4) and dried under nitrogen. Residual activated NPC sites were quenched in 0.5 M ethanolamine in PBS (pH 7.4). The resulting surface is abbreviated as Au-POEGMA-Pep.

Surface characterization

The chemical composition of the modified surfaces was determined by X-ray photoelectron spectroscopy (XPS, ThermoFisher K-Alpha instrument). Fourier transform infrared spectra (FTIR) of the modified surfaces were obtained by a Nicolet 6700 FTIR spectrometer (US Thermo Fisher Scientific Inc.). Static water contact angles of the surfaces were measured with a SL200C optical contact angle meter (USA Kino Industry Co., Ltd.) at ambient temperature. The density of peptide on the surface was determined by measuring the liberation of *p*-nitrophenol from the Au-POEGMA-NPC surface during peptide immobilization. The *p*-nitrophenol liberated (equivalent to the peptide on the surface) was determined from absorbance at $\lambda = 402$

nm measured using the Thermo Scientific™ Varioskan™ Flash Multimode Reader. A molar extinction coefficient of $\varepsilon = 17700$ L·mol^{-1}·cm^{-1} for *p*-nitrophenol was used.[3]

Protein adsorption

Protein adsorption on the surface was quantified by radiolabeling method. Fibrinogen, plasminogen and tPA were first labeled with ^{125}I. For studies of protein adsorption from buffer, labeled and unlabeled protein were mixed (1/49, labeled/unlabeled) at a total concentration of 1 mg·mL^{-1} for fibrinogen and HSA, and 0.1 mg·mL^{-1} for plasminogen and tPA. Prior to adsorption experiments, the wafers were immersed in PBS containing NaI (0.002 mg·mL^{-1}) for at least 30 min to inhibit the sorption of free ^{125}I to the gold surface.[1] For protein adsorption, surfaces were incubated in the radioactive protein solution (in PBS-NaI, pH 7.4) for 3 h at ambient temperature, rinsed three times (10 min for each) with PBS-NaI (pH 7.4), wicked onto filter paper and transferred to clean tubes for radioactivity determination. The radioactivity of the surfaces was measured by a Wallac 2480 Wizard 3" Automatic Gamma Counter (PerkinElmer Life Sciences, Shelton, CT). Protein adsorption was expressed as mass per unit surface area.

tPA adsorption on surfaces via different immobilization methods

Gold-coated wafers were used for physical adsorption of tPA. The wafers were washed with acetone, treated for 30 min in an ozone plasma, washed with deionized water and ethanol, cleaned in a mixture of ammonia, hydrogen peroxide and deionized water ($NH_3{\cdot}H_2O$:H_2O_2:H_2O = 1:1:5 v/v/v) for 10 min at 75 ^{o}C, and then rinsed with deionized water and dried under nitrogen. Au-POEGMA surfaces activated with *N,N*′-disuccinimidyl carbonate (Au-POEGMA-NSC) were used for covalent binding of tPA. The Au-POEGMA surfaces were added to an acetonitrile solution containing *N,N*′-disuccinimidyl carbonate (0.05 mmol mL^{-1}) and TEA (0.05 mmol mL^{-1}) and stirred at room temperature for 6 h. For tPA adsorption, surfaces were incubated in the radioactive tPA solution (0.1 mg·mL^{-1} in PBS-NaI, pH 7.4) for 3 h at room temperature, rinsed three times (10 min for each) with PBS-NaI (pH 7.4), wicked onto filter paper and transferred to clean tubes for radioactivity determination.

Kinetics of protein adsorption measured by QCM-D

QCM-D measurements were carried out on a Q-Sense-E4 instrument (Q-Sense, Sweden) with control software (Resonant Probes GmbH, Goslar, Germany) to analyze the data. QCM-D chips (AT cut, 5 MHz, 14 mm diameter) were placed in the fluid chambers with the functionalized sides exposed to the solution. The normal sensitivity in liquid for protein adsorption in QCM-D experiments is typically ~1.8 ng·cm^{-2} for mass (multiple frequency modes) (Q-Sense, Sweden). All QCM-D experiments were conducted in flow-through mode at a rate of 50 μL·min^{-1} at 25 °C. A baseline was quickly established by passing PBS for 15 min. Then freshly prepared tPA solution with different concentration (5 μg·mL^{-1}, 10 μg·mL^{-1}, 20 μg·mL^{-1}, 40 μg·mL^{-1}, 80 μg·mL^{-1}, 160 μg·mL^{-1} and 320 μg·mL^{-1}) was allowed to flow for 10 min to the adsorption plateau. The affinity constant of the interaction was calculated according to Langmuir Model.

Enzymatic activity assay

Plasminogen activation by free or surface-immobilized tPA was determined according to a previously published method by monitoring the rate of plasmin production using a specific chromogenic substrate S-2251.[4] Briefly, 1.3 μM of substrate S-2251 and 0.24 μM plasminogen were added into the wells of a 96-well plate. The reaction medium was 50 mM Tris·HCl (pH 7.5) containing 50 mM NaCl and 0.01% Tween 80. Subsequently the surfaces with immobilized tPA were placed in the wells to initiate plasminogen activation. Free tPA of equal mass to that on the surface was used as control. The generation of plasmin was monitored by measuring the absorbance of the reaction mixture at 405 nm at 3 min intervals over a 60 min period using the Thermo Scientific™ Varioskan™ Flash Multimode Reader. Results are expressed as $\Delta A_{405}/min^2$.

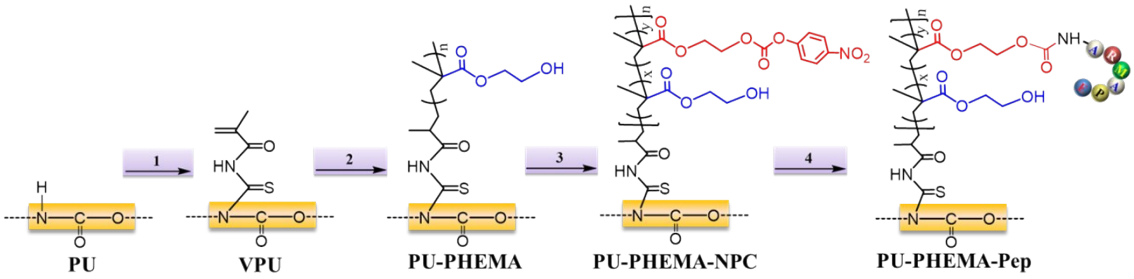
Enzymatic activity of both free tPA and surface-immobilized tPA were also measured in the presence of inhibitor PAI-1. Free tPA or immobilized tPA were mixed with PAI-1 at molar ratio of 1:1 for 5, 10, 20 and 30 min, respectively. Residual activities of tPA were then measured by the assay described above.

Preparation of peptide-modified PU surface (PU-PHEMA-Pep)

The ARMAPE peptide was immobilized on the PU surface through poly(2-

hydroxyethyl methacrylate) (PHEMA) as a spacer (Scheme S1). PU films were casted as reported previously[5] and punched into discs approximately 6 mm in diameter and 0.5 mm in thickness. The PU discs were immersed in 25 mL of an acetonitrile solution containing 8 mmol methacryloyl isothiocyanate. After stirring at 65 °C for 12 h, the vinyl group functionalized PU surface (VPU) obtained was washed with acetonitrile and dried in a vacuum oven at 40 °C for 24 h. PHEMA-grafted surfaces were prepared by free radical polymerization of HEMA on VPU surfaces. Briefly, HEMA (2.6 g, 20 mmol) and AIBN (0.033 g, 0.2 mmol) were dissolved in 10 mL purified methanol. VPU films were immersed in the mixed solution, which was then degassed by bubbling with nitrogen for 20 min. The polymerization was carried out at 60 °C for 5 h under a nitrogen atmosphere. The PU-PHEMA surfaces were rinsed successively with methanol, distilled water and methanol, and dried in a vacuum oven at 40 °C for 24 h. All the procedures were carried out as published previously.

For further hexapeptide immobilization, PU-PHEMA surfaces were activated with 4-nirophenyl chloroformate (NPC) (a solution composed of 0.1 g NPC, 5 mL anhydrous acetonitrile, and 50 mg triethylamine) at ambient temperature for 6 h. The surfaces were thoroughly rinsed with anhydrous acetonitrile and then dried under a flow of nitrogen. The NPC-activated PU-PHEMA surfaces were immersed into a 0.5 mg·mL^{-1} hexapeptide phosphate-buffered saline (PBS, pH 8.5) solution at 25 °C for 20 h. Finally, the surfaces were removed from the solution, rinsed thoroughly with PBS and dried under nitrogen. Residual activated NPC sites were quenched in 0.5 M ethanolamine in PBS solution. The resulting films are referred to as PU-PHEMA-Pep.



Scheme S1. Fabrication of peptide-functionalized PU surface. (1) Immobilization of methacryloyl isothiocyanate on the PU surface (VPU); (2) graft copolymerization of HEMA on the VPU surface; (3) activation of hydroxyl groups with 4-nitrophenyl

chloroformate (NPC); (4) attachment of ARMAPE peptide via covalent bonding.

Clot lysis activity of tPA

Surfaces were incubated in tPA solution (0.1 $mg \cdot mL^{-1}$) for 3 h at ambient temperature. The surfaces were rinsed three times with PBS (pH 7.4) to remove any unbound proteins and then placed in clean wells of a 96-well plate. Clot lysis activity of surface-immobilized tPA was assessed using a modified plasma recalcification assay.[6] Platelet-poor-plasma (100 μL) was added to the wells containing the samples. Following 5 min equilibration at 37 °C, 100 μL $CaCl_2$ (0.025 M) was injected into the wells to initiate the clot formation. Absorbance at λ = 405 nm was measured at 30 s intervals over a 2 h period.

For the assessment of clot-lytic activity in the presence of PAI-1, tPA-immobilized surfaces were incubated with PAI-1 (equivalent mole with tPA) in PBS (pH 7.4) for 30 min. Then the surfaces were rinsed and placed in clean wells. Clot lytic activity of tPA was assessed using the method described above.

Results

Surface characterization

Table S1 XPS results and static water contact angles of unmodified and modified gold surfaces.

Surface	C (%)	O (%)	N (%)	Au (%)	Br (%)	WCA (°)[a]
Au	27.1	8.6	N.D.	64.3	N.D.	73
Au-Br	52.4	12.2	N.D.	34.5	0.9	65
Au-POEGMA	70.1	29.7	N.D.	0.2	N.D.	46
Au-POEGMA-NPC	67.3	31.4	1.3	N.D.	N.D.	82
Au-POEGMA-Pep	66.9	29.5	3.5	0.1	N.D.	72

[a]Standard deviations are within ±2°.

For surface-initiated ATRP of OEGMA, a monolayer of bromoisobutyryl-containing initiator was first assembled on the gold surface. This was evident by the

appearance of bromine element detected by XPS (**Table S1**). After polymerization of OEGMA, the disappearance of the bromine element and significant increase of oxygen content shown in XPS results indicated the presence of the polymer on the surface. In addition, the POEGMA-grafted surface showed a much smaller contact angle as compared with the Au-Br surface due to the hydrophilic nature of POEGMA (**Table S1**). For peptide immobilization, the introduction of the *p*-nitrophenyl groups resulted in the appearance of nitrogen element in XPS results.

Protein adsorption

The amounts of tPA adsorbed on the three surfaces are shown in Fig. S1.

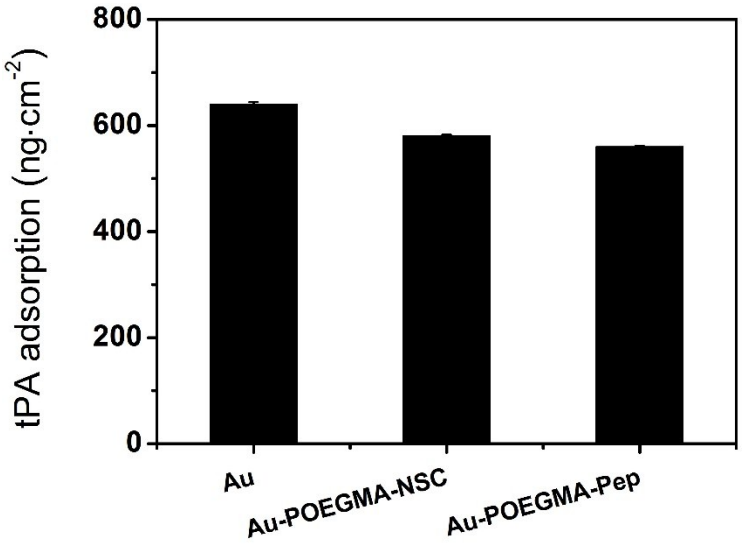


Fig. S1 Amounts of tPA adsorbed on Au, Au-POEGMA-NSC and Au-POEGMA-Pep surfaces measured by radiolabeling method. (mean ± SD, n=3)

The interactions between tPA and the Au-POEGMA-Pep surface was investigated by QCM. After equilibration with PBS, tPA solutions with concentration ranging from 5 to 320 $\mu g \cdot mL^{-1}$ were successively injected and the corresponding frequency shifts were shown in Fig. S2a. The extent of frequency shift decreased with the increase of tPA concentration. The adsorption isotherm seems to conform to the Langmuir adsorption curve, thus the binding of tPA to the Au-POEGMA-Pep surface was analyzed with the Langmuir adsorption model. The binding affinity can be obtained by plotting according to the following equation:

$$\frac{C}{\Delta F_{eq}} = \frac{C}{\Delta F_{max}} + \frac{1}{\Delta F_{max} K_a}$$

Where C is tPA concentration, ΔF_{eq} is the frequency shift at equilibrium under C, ΔF_{max} is the frequency shift at an infinite tPA concentration, and K_a is the association constant. By linear fitting the plot of C/K_a vs. C, K_a was calculated as 1.55 M^{-1} (Fig. S2b).

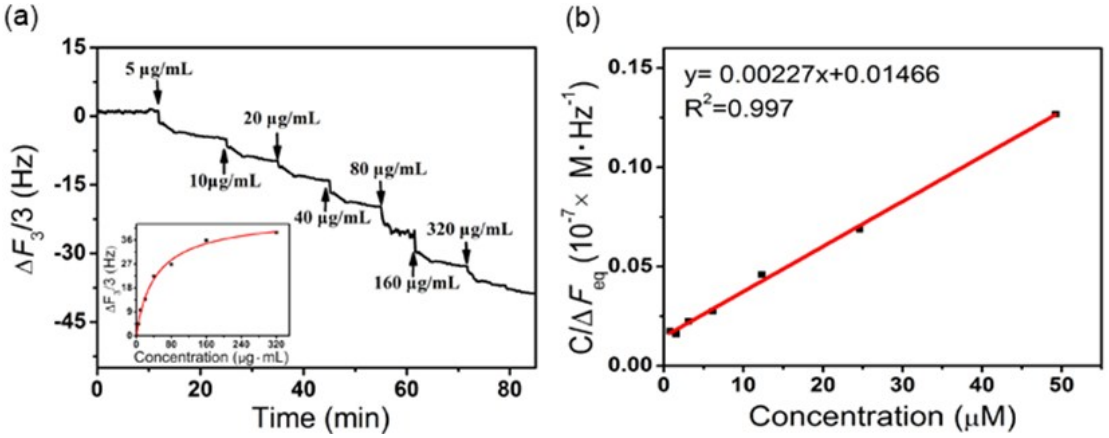


Fig. S2 (a) Frequency shift (ΔF) of tPA binding on Au-POEGMA-Pep surface with QCM analysis. Inset: the frequency shifts varying with the concentrations of tPA on Au-POEGMA-Pep surface; (b) The binding affinity corresponding plot analyzing by QCM.

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