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Electronic Supplementary Information

Conjugation of polymers to proteins through an inhibitor-derived peptide: taking up the inhibitor "berth"

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Experimental section

Materials

The PAI-1-derived peptide (sequence ARMAPE) was from GL Biochem (shanghai) Ltd. and synthesized using standard Fmoc-mediated solid-phase peptide synthesis. Peptides were cleaved using trifluoroacetic acid (TFA) and purified using reverse-phase high-performance liquid chromatography (Kromasil 100-5 C18 column) on a water/acetonitrile gradient. Purified peptides were lyophilized and confirmed using mass spectrometry (Waters ZQ2000).

Recombinant tissue plasminogen activator (tPA) was from Genentech (San Francisco, CA). Plasminogen activator inhibitor-1 (PAI-1) was

purchased from Sigma. Dialysis membranes (molecular weight cut-off (MWCO): 8000-14,000 Da) were obtained from Solarbio Science & Technology Co., Ltd The centrifuge filters (Amicon Ultra-15, MWCO 30,000 Da) were purchased from Millipore Corporation. Deionized water (DIW) was purified using a Millipore water purification system to give a minimum resistivity of 18.2 MΩ•cm. All the other chemicals were purchased from Shanghai Chemical Reagent Co. and used without further purification, unless otherwise specified. Female BALB/c mice were purchased from Nanjing Peng Sheng Biological Technology Co. Ltd. and used under protocols approved by the Soochow University Laboratory Animal Center. All experiments were performed in compliance with the relevant laws and the guidelines of Soochow University Laboratory Animal Center. The Center has approved the experiments.

Matrix-assisted laser desorption ionization spectroscopy (MALDI-TOF).

MALDI-TOF spectra were collected using a Bruker Daltonics Ultraflextreme mass spectrometer equipped with a Nd:YAG laser (355 nm) in positive linear mode operated with Flex-Control 3.4 software. Protein samples were prepared by using sinapic acid (SA) as matrix.

Molecular docking

Docking calculations were also performed by means of Autodock Vina to investigate the binding mode between the peptide composed of 6 amino

acids (ARMAPE) and tPA. In order to match our experiment, we added a handle of 1 unit and 2 units of OEGMA monomer to the head of the peptide, respectively. The structure of tPA is the 2.9 Å resolution in complex with a substrate (2,7-BIS-(4-amidinobenzylidene)-cycloheptan-1-one) with accession code 1A5H in the Protein Data Bank (PDB) (http://www.rcsb.org). Default parameters were used as described in the AutoDock manual unless otherwise specified.

Preparation of peptide-modified surface and protein adsorption

The poly(HEMA) grafted PU surface was prepared by the method previously reported.[1] 4-Nitrophenyl chloroformate (NPC) was chosen to activate the hydroxyl groups of poly(HEMA) for peptide attachment. The activated surfaces were incubated with peptide in PBS (pH 8.2) for 12 h to allow ligand tethering, and residual activated NPC sites were quenched in 20 mM ethanolamine in PBS.

tPA, plasminogen, fibrinogen and HSA were labeled with ^{125}I using previous method.[2] Surfaces were incubated with the protein solution for 3 h at room temperature, rinsed three times with PBS, wicked onto filter paper and transferred to clean tubes for radioactivity determination. Protein adsorption was expressed as mass per unit surface area (ng/cm^2).

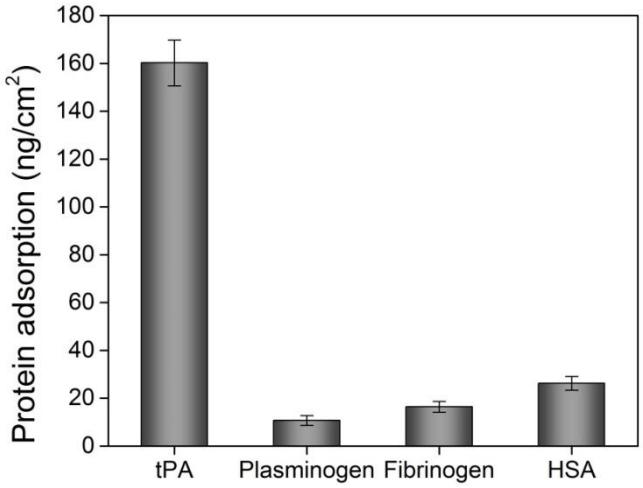

Fig. S1 Amounts of adsorbed protein on peptide modified surface.

Synthesis of peptide-terminated POEGMA.

The Peptide-functionalized ATRP initiator was synthesized using standard Fmoc-mediated solid-phase peptide synthesis by GL Biochem Ltd. In a typical polymerization, OEGMA (0.70 g, 1.47 mmol) monomer, Cu(I)Br (2.1 mg, 0.0147 mmol), and bpy (4.6 mg, 0.0294 mmol) were dissolved in methanol/H_2O (1:1, v/v, 8 mL). The solution was purged for 30 min with nitrogen to remove the oxygen. Deoxygenated peptide-initiator (12 mg, 0.0147mmol) was added to initiate the polymerization. The reaction was stirred at room temperature for 12 h. After polymerization, the polymer was purified by dialysis against Milli-Q water (MWCO 8000-14000) and lyophilized to remove the solvent.

Characterization

^{1}H NMR spectra were recorded on an INOVA 400 MHz nuclear magnetic resonance instrument, with D_2O used as solvents. The number-average

molecular weight (M_n) and molecular weight distribution (M_w/M_n) of the resultant polymer was determined using a Waters 1515 gel permeation chromatograph (GPC) with PS as standard sample and DMF was used as the eluent at a flow rate of 0.8 mL/min operated at 30 ℃.

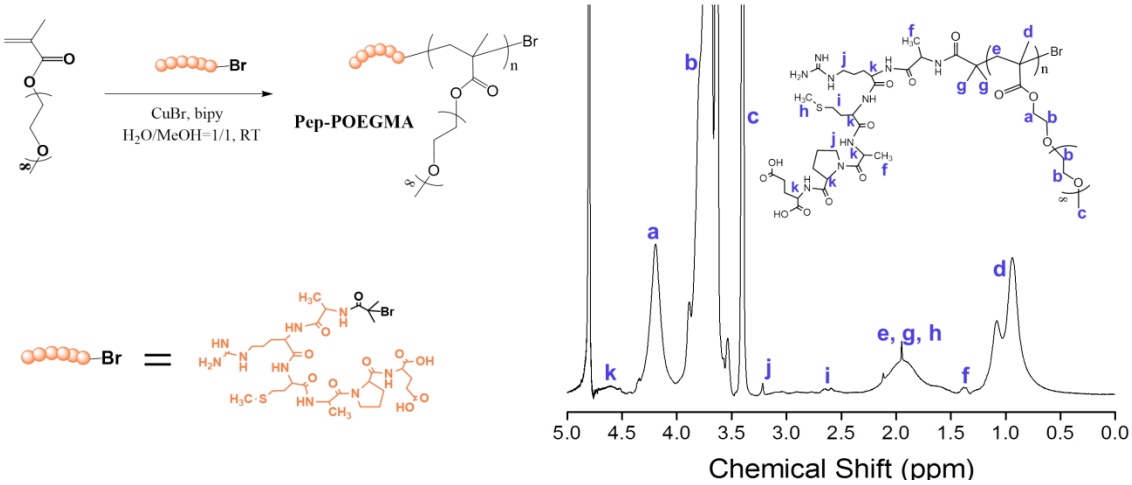

Fig. S2 Synthesis of peptide-terminated POEGMA (Pep-POEGMA) and the ^{1}H NMR spectrum of Pep-POEGMA.

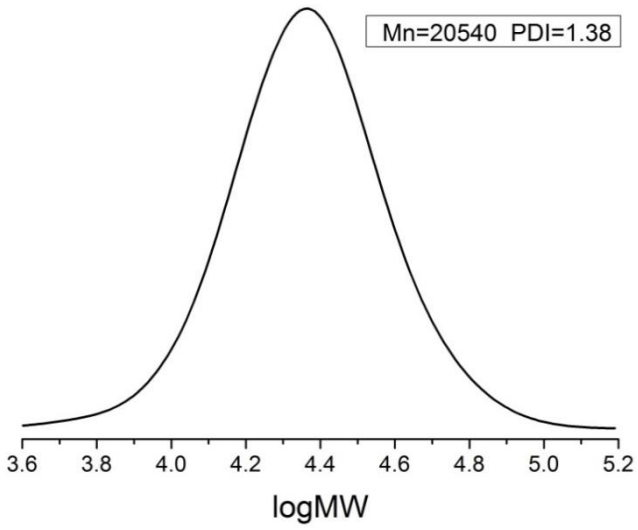

Fig. S3 Molecular weight of Pep-POEGMA determined by GPC.

Conjugation between tPA and Pep-POEGMA

0.5 mL fresh prepared tPA solution (1 mg/mL, PBS buffer, pH 7.4) was added to a small plastic vial, followed by the addition of 100 μL Pep-POEGMA solution (50 mg/mL). The vial was incubated at 37 ℃ with gentle shaking for 3 h. The conjugates were purified and concentrated by ultrafiltration using a 30 kDa molecular weight cut-off membrane (12,000 rpm, 3 times, 10 min per time) with PBS (pH 7.4). The collected conjugate was then characterized by aqueous GPC and HPLC.

Conjugate characterization

RP-HPLC was carried out using an Aligent 1200 Series LC system equipped with a C18 column (250 × 4.6mm; 5μm). Solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in acetonitrile. Samples were eluted using a linear gradient from 15 to 45% B in 30 min at a flow rate of 0.8 mL/min. Sample detection was carried out using a UV detectors connected in series at $\lambda = 280$ nm.

Aqueous GPC was performed on a Agilent 1200 HPLC system equipped with a UV detector and a Sepax Zenix SEC-300 column (7.8 × 300 mm column, pore size 300 Å, particle size 3μm) using a 150 mM phosphate buffer at pH = 7.0 as the mobile phase at 25 ℃ (flow rate: 1.0 mL/min).

Enzymatic activity assays.

Plasminogen activation by tPA was determined according to a previously published method by monitoring the rate of plasmin production using its specific chromogenic substrate S-2251.[3] Briefly, 1.3 μM of substrate S-2251, 0.24 μM plasminogen and 0.5 μg/mL tPA were taken in a 300 μL well of 96-well plate. The reaction medium was 50 mM Tris-HCl (TBS) containing 50 mM NaCl with 0.01% Tween 80 at pH 7.4. The initial rate of hydrolysis of S-2251 by plasmin produced from plasminogen as a result of tPA activity was determined by measuring the absorbance at 405 nm at different time intervals using a microplate reader. The slope of absorbance against square of time ($\Delta A/min^2$) was expressed as the initial rate of S-2251 hydrolysis.

The rate of tPA inhibition by recombinant PAI-1 was determined by mixing equal molar amounts of t-PA and activated PAI-1 for 30 min and subsequently measuring the activity of uninhibited tPA. Inhibition reaction samples containing 0.5 nM t-PA, 0.5 nM active PAI-1 in 200 μL of TBS (pH 7.4, containing 0.05% Tween 80) were incubated at 37 ℃ for various time. Residual tPA activities are then measured by the assay described above.

***In vivo* pharmacokinetic study**

Both free tPA and tPA/Pep-POEGMA conjugate were diluted with TBS (pH 7.4) and calculated amount of each formulation equivalent to 6500 IU/kg were slowly administered for about 10 s via tail vein of the mice.

After dosing, 0.5 mL blood was withdrawn at 1, 5, 10, 15, 30 min and 1 h, respectively. Blood samples were collected into micro-centrifuge tube containing 13 mM sodium citrate to prevent blood coagulation and centrifuged at 4000 rpm for 10 min to separate plasma from other blood elements. The remaining activity of tPA was determined in the same way of the previous method.

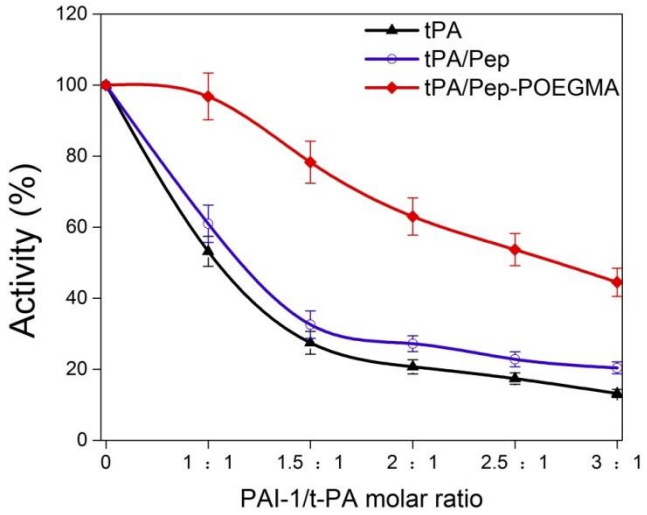

Fig. S4 Relative activity of tPA, tPA/Pep and tPA/Pep-POEGMA conjugate after 30 min incubation with different amounts of PAI-1.

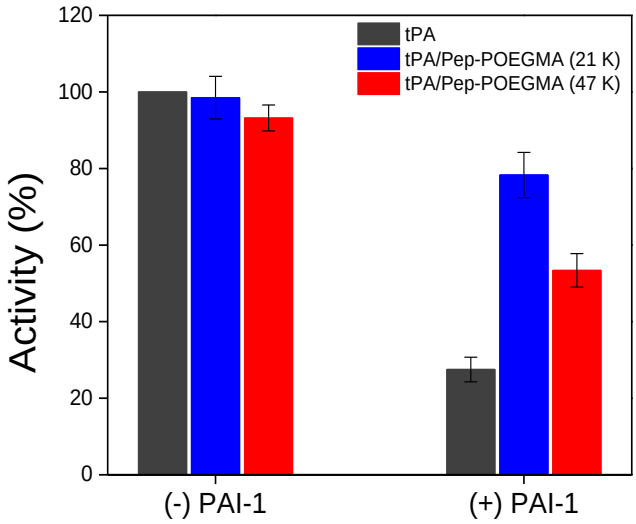

Fig. S5 Relative activity of tPA and tPA/Pep-POEGMA conjugates (with different molecular weight) in the absence/presence of PAI-1 (molar ratio of PAI-1 to tPA is 1.5:1).

References

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3 S. Absar, S. Choi, F. Ahsan, E. Cobos, V. C. Yang and Y. M. Kwon, *Thromb. Res.*, 2013, **131**, e91.