Electronic Supplementary Material (ESI) for ChemComm.

Electronic Supplementary Information

A Novel Method to Engineer Protease for Selective Enzyme Inhibition

Lingling Li[a], Hai Shi[b], Anzhi Sheng[a], Yi Yang[b], Liu Shi[b], Chao Li[b,c*], and Genxi Li[a,b*]

[a]Center for Molecular Recognition and Biosensing, School of Life Sciences, Shanghai University, Shanghai 200444, P. R. China

[b]State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing 210023, P. R. China

[c]School of Food and Biological Engineering, Hefei University of Technology, Hefei, Anhui 230009, P. R. China

* Emails: lchao@hfut.edu.cn (C. Li); genxili@nju.edu.cn (G. Li)

EXPERIMENTAL SECTION

Materials and Instruments. HAuCl4·3H2O were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Citrate and Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), proteinase K (No. V900887), bromelain (Product #No. B4882), trypsin (Product #No. T1426), chymotrypsin (Product #C4129), bovine serum albumin (BSA, Product #No. V900933) and matrix metalloproteinase 9 (Product #No. M8945) were purchased from Sigma-Aldrich (USA) and were used as received. Recombinant thrombin (Product #No. PRO-14220) was purchased from ProSpec-Tany TechnoGene Ltd (Israel). Tissue factor (Product #No. ab119148) was purchased from Abcam (USA). Activated coagulation factor X (Product #No. ACFP1063) was obtained from R & D systems (USA). Aegis Lysozyme was obtained from Hangzhou Kangyuan Tech Inc. Dulbecco's Modification of Eagle Medium (DMEM) medium, penicillin, streptomycin, and fetal bovine serum (FBS) were purchased from HyClone/Thermo fisher (Beijing, China). Thiolated PEG (Mw =5000) was purchased from Xi'an ruixi Biological Technology Co., Ltd (Xi'an, China). Other chemicals used in this work were of analytical grade and directly used without additional purification. The oligonucleotides and peptides were synthesized and purified by Sangon Inc. (Shanghai, China) that were listed in Supplementary Table S1 and Table S2. Deionized water (Milli-Q grade) with a resistivity of 18.2 MΩ-cm was used throughout this study.

The UV-vis spectra of nanoparticle solutions were recorded with a UV-1800 spectrophotometer (Shimadzu, Japan). The absorption intensities in the 96-well plates were collected by a Safire2 microplate reader (Tecan Group Ltd., Switzerland). Transmission electron microscopy (TEM) studies were performed with a JEM-2100 transmission electron microscope operating at 80 kV (JEOL, Japan). Specimens for TEM were prepared by drop casting 10 μL of the as-prepared samples onto a standard carbon-coated 230 mesh copper grid and drying at room temperature. Dynamic lighting scattering (DLS) measurements were performed through a Zetasizer Nano ZS (Malvern Instruments). To determine nanoparticle size distribution, a 1 mL sample was placed in a glass cuvette and DLS was performed at ambient temperature with a backscatter angle of 173° and an equilibration time of 100 seconds. Fluorescence spectra were measured on an F-7000 Fluorescence Spectrophotometer (Hitachi, Japan). The excitation and emission slit widths were each set to 5 nm.

Synthesis of Apt-ProK-GNPs. GNPs with an average of 13 nm were synthesized by the modified citrate reduction method. The procedure of preparing Apt-ProK-GNPs was amenable to all used nanoconjugates depicted in the manuscript. We here only take Apt29-ProK-GNPs as an example. First, the original citrate-covered GNPs solution (970 μL, 5 nM) was centrifuged and re-dispersed in PBS buffer (10 mM, pH 6.5). Then, a stock solution of proteinase K (1 mg/mL, 10 μL) was rapidly added into the citrate-GNPs solution with vigorous shaking to allow adsorption of protein molecules onto the GNP surfaces. Next, 10 μL of thrombin aptamer (Apt29, 100 μM) was added into the as-prepared ProK-GNPs solution and salt concentration of the solution was adjusted to

be 100 mM by NaCl. The mixture was shaken at 4 °C for 6 h. Apt29-ProK-GNPs conjugates were formed, and excess molecules could be removed by centrifugation (8000 g, 15 min) for two runs. After that, the remaining sites of GNPs were passivated by adding 10 μL of thiolated PEG (10 mM) in PBS solution into the as prepared Apt29-ProK-GNPs solution. The resulting mixture was shaken for 2 h and washed with PBS for three runs. The resulting pellet was dispersed in 1 mL of PBS (10 mM, pH 7.4) and the solution was store at 4 °C for further use.

To find the number of the bound aptamers and proteins, fluorescence and bicinchoninic acid (BCA) were used, respectively. Briefly, the concentration of GNPs was determined by using UV-vis spectroscopy measurements. These absorbance values were then related to the GNP concentration via Beer's law ($A = \varepsilon bc$). The extinction coeffient (ε) of GNP (d = 13 nm) is 2.7×10^8 L/(mol cm). To determine the concentration of oligonucleotides, the aptamer was chemically displaced from the GNPs surface by using 5 M DTT. The aptamers (both labeled with TAMRA) were completely released into solution during a 24 h incubation, and the precipitate was completely removed by centrifugation. To determine aptamer, fluorescence of the resulting supernatant was compared to a standard curve. The number of aptamers per GNP was calculated by dividing the concentration of fluorescent aptamers by the concentration of GNPs. As for ProK, 1 mL of the nanoconjugate was incubated with KCN to dissolve the GNPs and the resulting solution was ultrafiltrated by using a membrane with a cut-off of 10 kDa to remove interferent. Then, the ProK-containing solution was analyzed by BCA kit to determine the concentration of ProK in the

nanoconjugate. The number of ProK per GNP was calculated by dividing the concentration of ProK by the concentration of GNPs. All experiments were repeated three times using fresh samples to obtain reliable error bars.

Gel electrophoresis. BSA protein (1 mg/mL) was preincubated in 10 nM Tris buffer (pH 7.4) in the presence of proteases (in different concentrations) for 40 min at 37 °C. Then the solution was mixed with gel loading buffer (NuPAGE LDS sample buffer; Invitrogen), heated at 95 °C for 5 min, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 4-12%). Gel separation in running buffer proceeded at a constant voltage of 100 V for 1 h. After separation, the gels were stained with Coomassie Blue dye (GelCode Blue Stain Reagent, Thermo Scientific) for 1 h. Then the stained gels were washed and photographed using a Qinxiang Gel Imaging System (Shanghai, China).

Evaluation of inhibitory property. The thrombin inhibitory potency by GNPs, ProK-GNPs and Apt29-ProK-GNPs were measured using the fluorescent method. Briefly, thrombin (10 μg/mL) was preincubated with varying concentrations of inhibitors at 37 °C for 40 min in 10 mM PBS (pH 7.4) containing 140 mM NaCl, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$ and 5% (vol/vol) glycerol. After that, the prepared solution was centrifuged to remove nanoconjugates and the supernatant (90 μL) was incubated with peptide substrate (10 μL) to reach a final concentration of 100 nM. The enzymatic activity was followed by monitoring product formation for 400 seconds with a fluorescence spectrophotometer ($\lambda_{excitation}$ = 494 nm, $\lambda_{emission}$ = 518 nm). The normalized enzymatic activity was defined using the equation: As/Ac, where Ac is the thrombin

activity in the absence of inhibitors as control, and As is the thrombin activity after incubating with inhibitors. The enzymatic reaction was performed at least in triplicates.

Determination of enzymatic activity. A reaction mixture (1 mL) containing 2 mM peptide, 1 nM enzyme or nanoconjugate, and 10 mM PBS (pH 7.4) was incubated at 37 °C. After 30 min incubation, 0.05 mL of the mixture was extracted and cooled in an ice-bath to prevent further hydrolysis. The extent of cleavage was calculated by fluorescence change.

Elimination of thrombin in serum. Thrombin (1 μL, 1 mg/mL) was mixed with 7 μL of bovine serum (5%, diluted with 10 mM PBS (pH 7.4) containing 140 mM NaCl, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$) and then 2 μL of Apt29-ProK-GNPs (10 nM) was added to the solution to cleave the thrombin for 40 min at 37 °C. Then, the resulting solution was subjected to SDS-PAGE analysis.

Table S1. The used oligonucleotides in this study.

Oligonucleotide	**Sequences (5' to 3')**
Apt29	AGTCCGTGGTAGGGCAGGTTGGGGTGAC-$(T)_{10}$-$(CH_2)_6$-SH
Apt15	GGTTGGTGTGGTTGG
Apt-MMP9	TCGTATGGCACGGGGTTGGTGTTGGGTTGG-$(T)_{10}$-$(CH_2)_6$-SH
Apt-IXa	AUGGGGACUAUACCGCGAAUGCUGCCUCCCCAU-$(T)_{10}$-$(CH_2)_6$-SH
Apt-TF	UACAGAGGAGUACAAGUAGCAUGAUCCCUGGUGUAAA-$(T)_{10}$-$(CH_2)_6$-SH
Random DNA strand for thrombin assay	TACTCAGGCTGACTTCACGCTGTCATTAT-$(T)_{10}$-$(CH_2)_6$-SH
TAMRA- Apt29	TAMRA-AGTCCGTGGTAGGGCAGGTTGGGGTGAC-$(T)_{10}$-$(CH_2)_6$-SH

Table S2. The used peptides in this study.

Peptide	**Sequences (N to C terminus)**
Thrombin	FAM-LVPRGSGC-BHQ1
Common peptide substrate	FAM-LVPRGSPVGLIGCIEGRGCRRGC-BHQ1

Red sequence: substrate of thrombin; Green sequecne: substrate of matrix

metalloproteinase 9 (MMP9); Blue sequecne: substrate of activated coagulation factor IXa (FIXa); Brown sequecne: substrate of tissue factor (Tf).

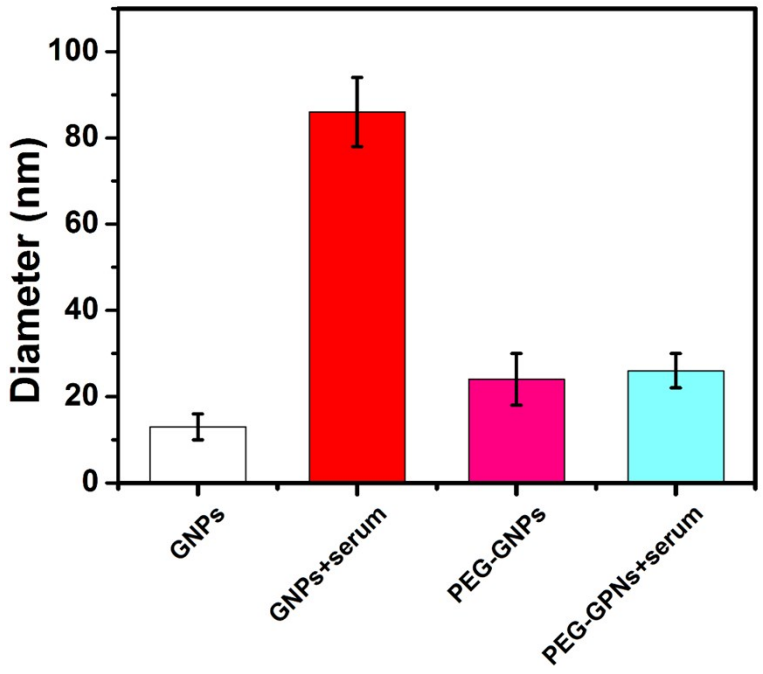

Figure S1. The antifouling ability of PEGylated-GNPs.

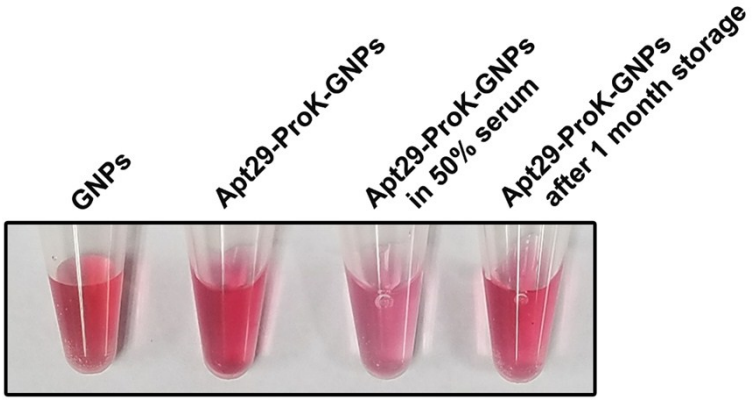

Figure S2. Photographs of the synthesized nanoconjugates.

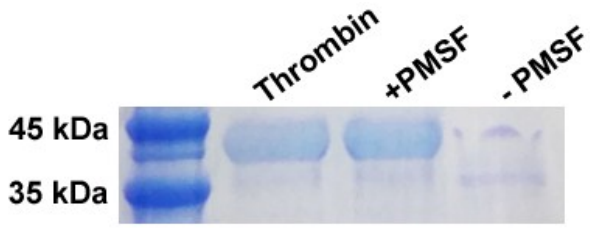

Figure S3. SDS images of thrombin, thrombin incubated with PMSF-pretreated Apt29-ProK-GNPs and thrombin incubated with Apt29-ProK-GNPs.

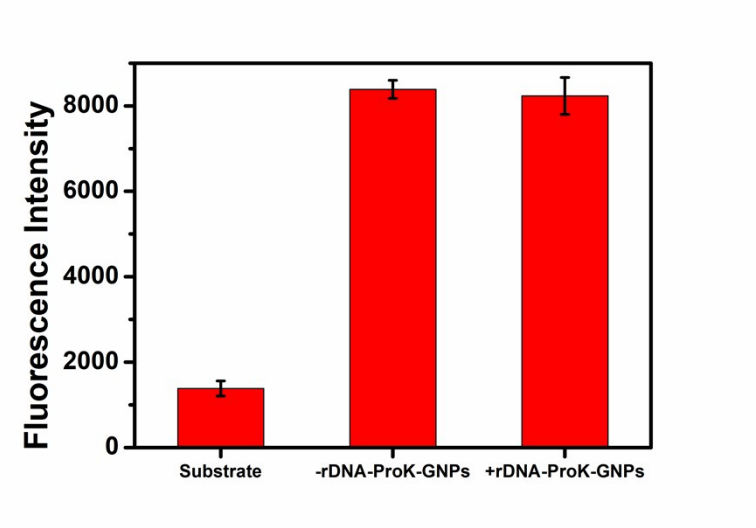

Figure S4. Fluorescence intensity of thrombin substrate and substrate with or without treatment of rDNA-ProK-GNPs and then thrombin for 40 min. [rDNA-ProK-GNPs] = 1 nM, [Thrombin] = 10 μg/mL, [Substrate] = 100 nM.

Table S3. Kinetic parameters for ProK and Apt29-Prok-GNPs-catalyzed hydrolysis of substrate peptide. (pH 7.4 and 37 °C)

Enzyme	[S] (μM)	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} M^{-1})
ProK	0-200	120	14	116000
ProK-GNPs	0-200	5.8	10.4	1790000
Apt29-Prok-GNPs	0-200	6	5.6	930000