Supporting Information

Real-time fluorescence turn-on assay for trypsin based on a conjugated polyelectrolyte

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**Experimental Section**

**Materials** The synthesis of PPE-CO2H referred to the method previously described.\(^1\) Four p-nitroaniline (pNA) modified peptides R\(_8\)G-pNA, R\(_6\)G-pNA, R\(_3\)G-pNA, and R\(_1\)G-pNA were purchased from GL Biochem Ltd. (Shanghai, China) and were used as received. Their stock solution was 4 mM in H\(_2\)O. Trypsin and p-toluenesulfonic acid monohydrate (p-TSA) were obtained from Aladdin Reagent Inc. (Shanghai, China). Acid phosphatase (ACP) from potato was obtained as lyophilized powder (3.0-10.0 units/mg solid) from Sigma (USA). 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) was purchased from Sigma-Aldrich Inc (Shanghai, China). Phenylmethylsulfonyl fluoride (PMSF) was purchased from Sangon Biotech Co. Ltd. (Shanghai, China). Sodium orthovanadate hydrate (Na\(_3\)VO\(_4\)) was purchased from Bio Basic Inc. (Toronto, Canada). Pyridine hydrochloride (PyH) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Protease inhibitor Cocktail Tablet (PIC) was purchased from Roche Applied Science (Mannheim, Germany).

**Instrumentation** Steady state fluorescence quenching experiments were carried out on a SPEX FL3 spectrometer with excitation at 400 nm wavelength and an emission range from 420 to 650 nm. Fluorescence turn-on assays and trypsin inhibition experiments were carried out on the same instrument with same spectroscopic parameters. The mass spectra were recorded on a Thermo LTQ XL™ ion trap mass spectrometer (Thermo Fisher Scientific, USA) operating with electron spray ionization (ESI) in positive ion mode. The HPLC characterization was performed on an Agilent 1260 series LC system (Agilent Technologies, USA) with a DAD detector and a reversed-phase kromasil 100-5C\(_{18}\) (4.6 mm \(\times\) 250 mm, E62001). 0.5‰ trifluoroacetic acid (TFA) in water (A) and 0.5‰ acetonitrile (B) were used as the mobile phase. The flow rate was kept at 1.0 mL/min during all experiments. The gradient proportion of B linearly increased from 2% to 35% over 30 min and then was rapidly returned to 2% to re-equilibrate 10 min prior to the next injection. The high-throughput screening (HTS) assays were carried out on a fluorescence-3000 spectrometer (Hitachi, Ltd. Japan) with excitation at 400 nm wavelength and an emission range from 420 to 650 nm. The photograph was taken with lighting of a portable UV lamp at 365 nm. The mean particle size was measured by Dynamic Light Scattering (Zetasizer Nano ZS90, Malvern Instruments LTD., Malvern, UK). Zeta potential of the particles was measured by Laser Doppler Anemometry (LDA; Zetasizer Nano ZS90, Malvern Instruments LTD., Malvern, UK).

**Steady-state fluorescence quenching:** Fluorescence quenching experiments of PPE-CO2H by R\(_8\)G-pNA, R\(_6\)G-pNA, R\(_3\)G-pNA, and R\(_1\)G-pNA were carried out in a quartz cuvette using micro-titration. In a typical titration quenching experiment, the fluorescence intensity of 2.0 ml PPE-CO2H (10 \(\mu\)M) were recorded before and after addition of a concentrated peptide stock solution and 1 min mixing.

**Fluorescence turn-on assay for trypsin and other enzymes:** The fluorescence turn-on assays for trypsin and other enzymes including thrombin, acid phosphatase, and lysozyme were carried out at room temperature. Each assay was composed of 2 ml of 10 \(\mu\)M PPE-CO2H and 2 \(\mu\)M R\(_8\)G-pNA in hepes buffer (10 mM, pH = 9.0). In a typical assay measurement, varying amounts of enzymes were added to the assay solution in a quartz cuvette. The fluorescence intensities at 460 nm were recorded at 30 seconds intervals with the excitation at 400 nm.
**Trypsin inhibition assay:** In inhibition experiments, trypsin (40 μM) was pre-incubated with varying amounts of AEBSF (0-200 μM) at room temperature for 30 min. Then 4 μL the enzyme-inhibitor mixtures were added to the assay solution. The final concentration of the trypsin was 40 nM and AEBSF was from 0 to 200 nM. The fluorescence intensities at 460 nm were recorded at 30 seconds intervals with the excitation at 400 nm.

**High throughput screening (HTS) assays:** The high-throughput screening (HTS) assays were carried on a 96-well plate. The inhibitor AEBSF and other molecules including PMSF, p-TSA, PyH, Na₃VO₄ and PIC were pre-incubated with 14 μM trypsin at room temperature for 30 min. The enzyme-inhibitor mixtures were added to the solution containing 20 μM PPE-CO₂H and 4 μM R₈G-pNA in hepes buffer (10 mM, pH = 9.0). the final concentration of the trypsin was 40 nM. The fluorescence spectrum was recorded by a fluorescence-3000 spectrometer with excitation at 400 nm and an emission range from 420-650 nm and 15 min mixing. The 96-well plate was photographed under ultraviolet rays at 365 nm from a portable UV lamp.

**Particle size and zeta potential determination:** The 20 μM PPE-CO₂H and 20 μM R₈G-pNA were mixed in deionized water by sonication before measurement. All measurements were done in triplicate.

**Results**

![Graph showing quenching of 10 μM PPE-CO₂H by 2 μM R₈G-pNA (green), R₃G-pNA (yellow) and R₁G-pNA (red) in aqueous solution with different sodium chloride concentrations at 0, 5, 10, and 20 mM.]

**Fig. S1.** Quenching of 10 μM PPE-CO₂H by 2 μM R₈G-pNA (green), R₃G-pNA (yellow) and R₁G-pNA (red) in aqueous solution with different sodium chloride concentrations at 0, 5, 10, and 20 mM.
**Fig. S2.** Relative fluorescence intensity of the solutions in the high throughput screen experiment. PICT is the solution of protease inhibitor cocktail and its concentration is 0.2×. The concentration of all other molecules was 400 nM.

**Fig. S3.** Structures of the chemical molecules used in HTS experiments.
**E**

![Mass Spectrum](chart1.png)

**F**

![Mass Spectrum](chart2.png)

**[CH₃CO-Arg-Arg-Arg+H]⁺**

**[Arg-Arg-Gly-pNA+H]⁺**
**E**

![Mass Spectrum](image)

- Mass peak at 529.55
- Peaks at 265.13, 256.61, 244.27, 149.02, 643.27, and 314.33

**F**

![Mass Spectrum](image)

- Mass peak at 508.38
- Peaks at 254.61, 225.24, 128.93, 352.39, 491.48, 622.29, and 644.37

**Molecular Formulas**

- [CH$_3$CO-Arg-Arg-Arg+H]$^+$
- [Arg-Arg-Gly-pNA+H]$^+$
Reference