Electronic Supplementary Information

An Ultrasensitive Chemiluminescence Turn-on Assay for Protease and Inhibitor Screening with a Natural Substrate

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**EXPERIMENTAL SECTION**

**Chemicals**

All chemicals were of analytical grade and used without further purification. Deionized water (18.2 MΩ) was obtained from a Milli-Q Academic water purification system (Millipore, MA, USA) and used throughout. Trypsin from porcine pancreas was purchased from Amresco LLC (Solon, OH, USA). Cyt c and bovine serum albumin (BSA) were purchased from Bio Basic Inc. (Markham, Ontario, Canada). The trypsin stock solution (30 mg/mL) was prepared freshly in 50 mM Tris-HCl (1 mM CaCl$_2$, pH 8.2) before the experiments. Ca$^{2+}$ was added to the stock solution to activate trypsin and increase its stability against autolysis according to a previous report.$^{S1}$ 200 μM cyt c stock solution was prepared in 50 mM Tris-HCl (1 mM CaCl$_2$, pH 8.2).

Luminol was purchased from Acros Organics (Geel, Belgium). H$_2$O$_2$ (30%, v/v) was purchased from Beijing Chemical Works (Beijing, China). Hemoglobin (from bovine red cell) was purchased from Worthington Biochemical Corp. (Freehold, NJ, USA). Papain, proteinase K, and chymotrypsin were obtained from Sangon Biotech Co. Ltd. (Shanghai, China). Lysozyme was from Genview (Houston, TX, USA). S1 nuclease was from Fermentas Inc. (MBI Canada). Alkaline phosphatase (ALP, from calf intestine) was from Takara Biotechnology Co. Ltd. (Dalian, China). The luminol stock solution (0.01 M) was prepared in 0.1 M NaOH and stored at 4 ºC before use. Working solutions of luminol were prepared by proper dilution of the stock solution.
with a borate buffer (100 mM, pH 9.0) to the desired concentrations.

**Apparatus**

CL reactions were carried out using an IFFM-E flow injection chemiluminescence (FI-CL) analysis system (Remex Analytical Instrument Co. Ltd., Xian, China). A peristalsis pump was set at 40 rpm to deliver luminol and \( \text{H}_2\text{O}_2 \) separately in inner diameter 0.5 mm PTFE tubing. They were mixed in a mixer. Sample was injected through an injector valve, and the CL signal was detected with an IFFS-A CL detector (Scheme S1). 1 mM \( \text{H}_2\text{O}_2 \) and 0.1 mM luminol in 100 mM borate buffer at pH 9.0 were used for the FI-CL reactions throughout. The error bars in the figures represent the standard deviation from three independent measurements.

UV-vis absorption spectra were obtained using a Cary 50 Bio Spectrophotometer (Varian Inc., CA, USA) equipped with a xenon flash lamp. Quartz cuvettes with 10 mm path length and 2 mm window width were used.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) measurements were performed using a Bruker autoflex III smartbeam MALDI-TOF/TOF instrument. 400 \( \mu \text{M} \) cyt c and 100 \( \mu \text{g/mL} \) trypsin were mixed in 50 mM Tris-HCl (1 mM CaCl\(_2\), pH 8.2), and incubated at 37 °C for 4 hours before the analysis. The spectrum was scanned from 500 to 2100 (m/z).
Trypsin activity assay

Trypsin was investigated as a model protease. It is a serine protease, and can specifically catalyze the hydrolysis of peptide and ester bonds containing lysine and arginine residues at the C-terminus.\(^{52}\)

The assay procedures are as follows: a high concentration trypsin stock solution was prepared before the experiments. 50 nM cyt c and the buffer solution were first mixed, various concentrations of trypsin (0, 2, 5, 10, 20, 50, 100, 150 and 200 ng/mL) were added to the sample tubes (final sample volume: 3 mL) and incubated at 37 °C. 200 µL of the sample solutions were taken at 10 min intervals and injected into the FI-CL analysis system, and the CL signals were detected.

Trypsin inhibition

Trypsin (200 ng/mL) and the soybean inhibitor of different concentrations (0, 2, 5, 10, 20, 50, or 100 ng/mL) were mixed first and then added to the sample solution containing 50 nM cyt c in 50 mM Tris-HCl (1 mM CaCl\(_2\), pH 8.2), and incubated at 37 °C (total sample volume: 3 mL). 200 µL of the sample solution was taken at 10 min intervals, injected into the FI-CL analysis system, and the CL signals were recorded.

Selectivity study
Lysozyme, BSA, ALP, and S1 nuclease were chosen to test the selectivity of our assay. A sample mixture containing 50 nM cyt c, 200 ng/mL trypsin or a potential interference protein (BSA, lysozyme: 1 μg/mL each; ALP, S1 nuclease: 10 U/mL each) was incubated at 37 ºC for an hour. The CL signal of these samples were then recorded.

**Activity assay of other protease**

Proteinase K, papain, and chymotrypsin are all commonly used protease. They catalyze the hydrolysis of different peptide bonds. The assay procedure is as follows: a protease was mixed with 50 nM cyt c in 50 mM Tris-HCl (1 mM CaCl₂, pH 8.2) and incubated at 37 ºC for 1 hour. Samples were taken every 10 min, injected into the FI-CL analysis system, and the CL signals were recorded.

**References**


**Scheme S1.** Schematic illustration of the FI-CL system.
Fig. S1. (a) CL intensity changes with or without the addition of trypsin. Substrate: 1, cyt c; 2, hemoglobin; 3, lysozyme; 4, BSA; 5, collagenase (5 nM each). Trypsin: 10 μg/mL. (b) CL spectra of 5 nM cyt c without and with 10 μg/mL trypsin (three repeated injections). Samples were incubated at 37 °C in 50 mM Tris-HCl (1 mM CaCl₂, pH 8.2) for 1 hour.
**Fig. S2.** UV-vis absorption spectra of a) 1 µM hemoglobin only. b) 1 µM hemoglobin + 20 µg/mL trypsin. Conditions: incubation in 50 mM Tris-HCl (1 mM CaCl₂, pH 8.2) at 37 °C for 1 hour.
**Fig. S3.** MALDI-TOF mass spectrum of the trypsin digested cyt c (from 500 to 2100, m/z). Inset: structure of the heme-peptide conjugate.
**Fig. S4.** UV-vis absorption spectra: (a) 4 μM cyt c only. (b) 4 μM cyt c + 20 μg/mL trypsin. Conditions: incubation at 37 ºC for 1 hour.
**Fig. S5.** Assay for different proteases. CL intensity changes were plotted against the enzymatic reaction time. Proteinase K and chymotrypsin: 200 ng/mL each; papain: 1 μg/mL.
Fig. S6. Time-dependent CL intensity changes with inhibitor concentration (0, 2, 5, 10, 20, 50, 100 ng/mL). Enzymatic reaction conditions: 50 nM cyt c, 200 ng/mL trypsin with different concentrations of the inhibitor in 50 mM Tris-HCl (1 mM CaCl₂, pH 8.2).