Supplementary Information

Bioluminescent nanosensors for protease detection based upon gold nanoparticle-luciferase conjugates

Young-Pil Kim,[a] Weston L. Daniel,[b] Zuyong Xia,[a] Hexin Xie,[a] Chad A. Mirkin,[b] and Jianghong Rao*[a]

Experimental Section

Chemicals. Propargylamine, 3-azidopropylamine hydrochloride, 2-mercaptoethanesulfonic acid (MESA), copper (II) sulfate pentahydrate ($CuSO_4 \bullet 5H_2O$), and ascorbic acid were purchased from Sigma-Aldrich. 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) was purchased from AnaSpec. Coelenterazine was purchased from Prolume. Ni-NTA agarose was purchased from Invitrogen. Active matrix metalloproteinase-2 (MMP-2) enzyme was purchased from Calbiochem. 5 nm Au NPs were purchased from Ted Pella, Inc. $HS(CH_2)_{11}(OCH_2CH_2)_3OH$ and $HS(CH_2)_{11}(OCH_2CH_2)_6OCH_2CO_2H$ were purchased from Prochimia.

Modification of Au NPs. Au NPs were suspended in a 9:1 water/ethanol solution at a concentration of 110 nM. $HS(CH_2)_{11}(OCH_2CH_2)_3OH$ and $HS(CH_2)_{11}(OCH_2CH_2)_6OCH_2CO_2H$ were mixed in ethanol and added to the Au NP solution to a final concentration of 4.5 and 45.5 μM, respectively. This mixture was incubated for 2 hours. The carboxy-modified Au NPs were purified by repeated centrifugation (21000 × *g*),

supernatant removal and resuspension in water. To modify the Au NP with propargylamine, carboxyl Au NPs (12.5 μL at 4 μM), propargylamine (5 μL at 10 mM), and EDC (5 μL at 10 mM) were sequentially added to 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 5.3) to give a final volume of 300 μL. The resulting mixture was incubated for 60 min at room temperature and purified from excess EDC and propargylamine by using a Microcon YM-100 centrifugal filter unit (100 kDa MWCO) and centrifugation (5000 × *g* for 5 min). The alkynated Au NPs were washed and resuspended in 0.1 M phosphate buffered saline (PBS, pH 8.0), and stored at 4 ºC until further use. The final concentration of the Au NPs in solution was calculated using the molar extinction coefficient (1.2×10^7 M^{-1} cm^{-1}) at 516 nm.

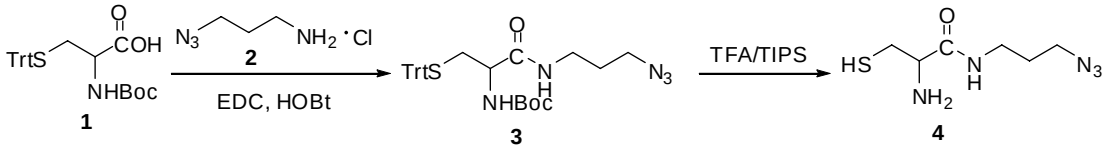
Plasmid constructions. For the insertion of *Mex* GyrA intein into the C-terminus of Luc8, two additional restriction sites EcoR I and Hind III were introduced into pBAD-Luc8 plasmid.[22] *Mex* GyrA intein (*Mycobacterium xenopi* gyrase A intein) is a 198 amino acid natural mini intein which lacks a central intein endonuclease domain. The intein GyrA mutant (N198A) gene was amplified from pTWIN-MBP1 (New England Biolabs) with two primers (forward 5′-A ATT GAA TTC TGC ATC ACG GGA GAT GCT and reverse 5′-A GCT AAG CTT GGT GAG GCC AGT AGC GTG-3′). The PCR product was digested by EcoR I and Hind III and ligated into the same enzyme digested pBAD-Luc8 plasmid to give the pBAD-Luc8-GyrA plasmid. To insert the MMP-2 substrate sequence between Luc8 and GyrA, the Luc8 gene was amplified from pBAD-Luc8 with two primers (forward 5′-A TGC CCA TGG CTT CCA AGG TGT AC-3′ and reverse 5′-ATGC GAA TTC ACC

ACC GCT ACG CAG ACT TAC AAT ACC ACC CTG CTC GTT CTT CAG-3′). The PCR products were digested with Nco I and EcoR I and ligated into the same enzyme-digested pBAD-Luc8-GyrA.

Protein expression and purification. The expression plasmid (pBAD-Luc8-pep-GyrA) was transformed into *E. coli* TOP10 competent cells (Invitrogen). The transformed cells were grown at 37 °C in 1 L Luria-Bertani broth containing 100 μg mL^{-1} ampicillin with reciprocal shaking (250 rpm/min) until the optical density of the solution reached 0.6. To induce fusion protein expression, 0.2% arabinose was added to the cultures, which were further incubated at 37 °C for 2 h. The cells were harvested by centrifugation and frozen at −80 °C. After thawing the next day, the cells were suspended in 25 mL of lysis buffer (20 mM Tris-HCl, 20 mM imidazole, 300 mM NaCl containing 1 mg mL^{-1} lysozyme, pH 7.5), followed by sonication for 5 min with a 30 s on and 30 s off cycle. After centrifugation (12000×g) for 30 min at 4 °C, the supernatants were collected and mixed with 1 mL of a 50% Ni-NTA agarose slurry (Invitrogen) equilibrated in lysis buffer. The mixture was incubated for 1h at 4 °C with reciprocal shaking to promote efficient binding. For purification, the lysate-Ni-NTA mixture was loaded into a column (1.5×5 cm) with the bottom outlet capped, and the flow-through sample was collected just after removing the bottom outlet. The column was rinsed twice with the washing buffer (20 mM Tris, pH 7.5, 300 mM NaCl, 20 mM imidazole) and the protein was eluted from the column using elution buffer (20 mM Tris, pH 7.5, 300 mM NaCl, 250 mM imidazole). The eluted fractions were further purified using a PD-10 desalting column, and the purified protein

was concentrated and exchanged into 0.1 M PBS (pH 7.4) by using a Microcon ultrafiltration device (YM-50, Millipore). The protein concentration was determined by measuring absorbance at 280 nm and using an extinction coefficient of 91010 M^{-1} cm^{-1}.

Synthesis of cysteine-azide.



The preparation of compound **4** has been described elsewhere.[28] Briefly, a mixture of Boc-(Trt)Cys-OH (525 mg, 1.1 mmole), 3-azidopropylamine hydrochloride **2** (129 mg, 0.94 mmole), triethylamine (0.32 mL, 2.3 mmole), HOBt (153 mg, 1.1 mmole) and EDC (216 mg, 1.1 mmole) in CH_2Cl_2 (10 mL) was stirred at room temperature overnight. CH_2Cl_2 (20 mL) and saturated sodium bicarbonate aqueous solution (20 mL) were added and the aqueous layer was extracted with CH_2Cl_2 (30 mL x 2). The combined organic layers were dried over $MgSO_4$ and purified by column chromatography to afford desired product **3** (419 mg, 81%). The ^{1}H-NMR spectrum of the compound was consistent with reported data. Compound **3** (100 mg, 0.18 mmole) was treated with a mixture of trifluoroacetic acid (3 mL), H_2O (0.1 mL) and triisopropylsilane (0.1 mL) at room temperature for 0.5 h. After the reaction was completed, the volatile solvent was then removed under vacuum and CH_2Cl_2 (10 mL) was added. H_2O (10 mL) was used to extract the organic solution. The aqueous layer was lyophilized to give compound **4** (35 mg, 96%). The ^{1}H-NMR spectrum of the compound was consistent with reported data.

Preparation for Luc8-pep-N_3 via intein-mediated splicing. MESA (40 μL at 100 mM) and Cys-N_3 (20 μL at 100 mM) were mixed with PBS (pH 8.0, 77.5 μL at 0.1 mM). Purified Luc8-pep-GyrA fusion protein (62.5 μL at 200 μM) was added to the mixture to give a final reaction volume of 200 μL. The reaction mixture was incubated overnight at 4 °C. To purify the cleaved product, the solution was mixed with a 50% Ni-NTA agarose slurry (100 μL) for 1 h at 4 °C with reciprocal shaking to promote efficient binding. The agarose mixture was then loaded into a column (1.5×5 cm) with the bottom outlet capped, and the flow-through sample was collected just after removing the bottom outlet. The collected protein was further purified by using a Microcon (YM-30, Millipore) to remove the excess MESA and Cys-N_3. The identity of the desired product (Luc8-pep-N_3) was confirmed using NuPAGE (Invitrogen) and quantified by measuring its absorbance at 280 nm using an extinction coefficient of 63495 M^{-1} cm^{-1}.

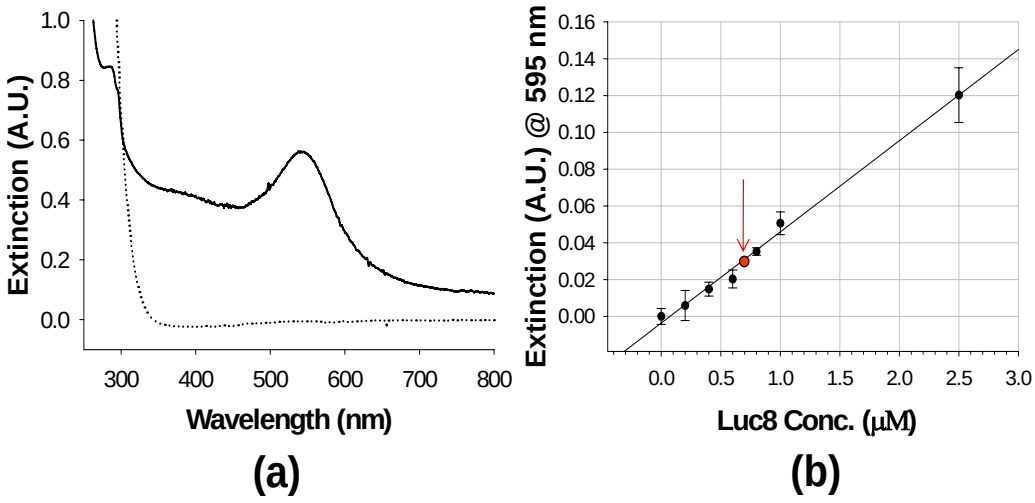
Conjugation of Au-≡ and Luc8-pep-N_3 via click chemistry. Alkyne-Au NP (80 μL at 0.5 μM) and Luc8-pep-N_3 protein (1 μL at 50 μM) were mixed in PBS (pH 8.0, 4 μL at 0.1 M). Ascorbic acid (5 μL at 100 mM) and $CuSO_4$ (10 μL at 10 mM) was added to the reaction mixture to give a final volume of 100 μL. The reaction mixture was incubated for 30 min at RT. After removing the excess small molecules using a mini-desalting column (Zeba Spin, Pierce Biotech), L-cysteine (10 μL, 100 mM) was added the solution and incubated for 10 min at RT to recover the activity of the luciferase. The solution was purified again using a mini-desalting column to remove L-cysteine followed by filtration using a Microcon YM-100 to remove unbound Luc8-pep-N_3. The final product was resuspended in 50 mM Tris

(pH 7.5) and stored at 4 °C until further use. To measure the ratio of Au NP to Luc8, the conjugate was first quantified by measuring the Au NP extinction at 516 nm and then treating the solution with 2.5% 2-mercaptoethanol overnight at RT. After confirming the disappearance of the extinction peak at 516 nm, the concentration of total protein was determined from a standard curve of free Luc8-pep-N_3 protein using a Bradford assay.

Control conjugate synthesis. As a negative control, a randomly-coupled conjugate was also synthesized. Carboxyl-Au NPs (12.5 μL at 4 μM), Luc8-pep-N_3 (10 μL at 50 μM) and EDC (5 μL at 10 mM) were sequentially added to PBS (pH 7.4) to give a final volume of 100 μL. The resulting mixture was incubated for 60 min at RT and purified from excess EDC and Luc8-pep-N_3 by using a Microcon YM-100 centrifugal filter unit (100 kDa MWCO) and centrifugation (5000×g for 5 min). The conjugate was washed and resuspended with 50 mM Tris (pH 7.5, containing 1.5 mM NaCl and 0.5 mM $CaCl_2$).

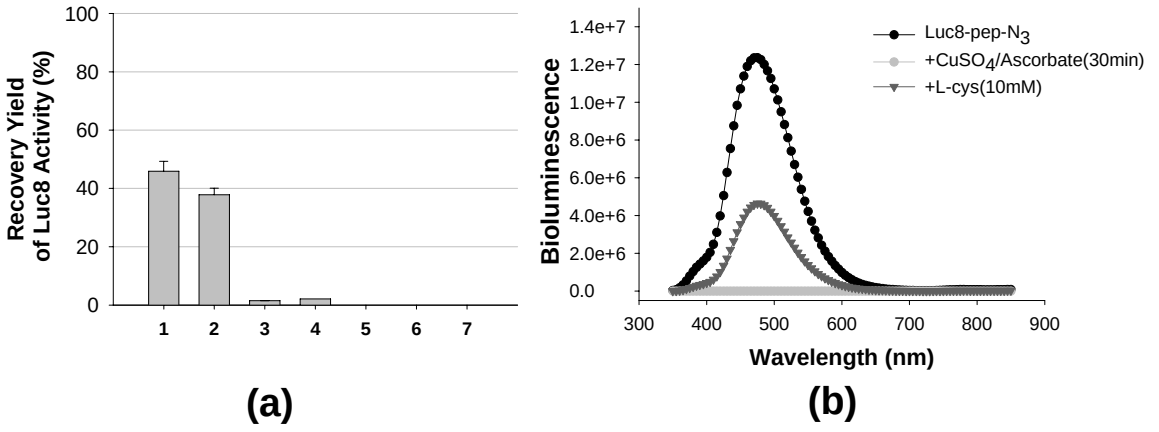
MMP-2 assay. The Luc8-pep-Au NP conjugate (10 μL at 0.36 μM) was mixed with 50 mM Tris buffer (80 μL, pH 7.5, containing 1.5 mM NaCl and 0.5 mM $CaCl_2$). The protease assay was initiated by the addition of the MMP-2 enzymes (Calbiochem, 10 μL of each stock solution), giving final concentrations ranging from 50 ng mL^{-1} to 1 μg mL^{-1}. The reaction mixture was incubated at 37 °C for 1h. Bioluminescence emission spectra were collected on a Fluoro Max-3 (Jobin Yvon Inc.) with the excitation light blocked. The mean and standard deviation of the intensity were calculated from two experiments. The control experiments with the randomly coupled probe followed the same procedure.

Fig. S1. Determining the ratio of Luc8 to Au NP in the conjugate



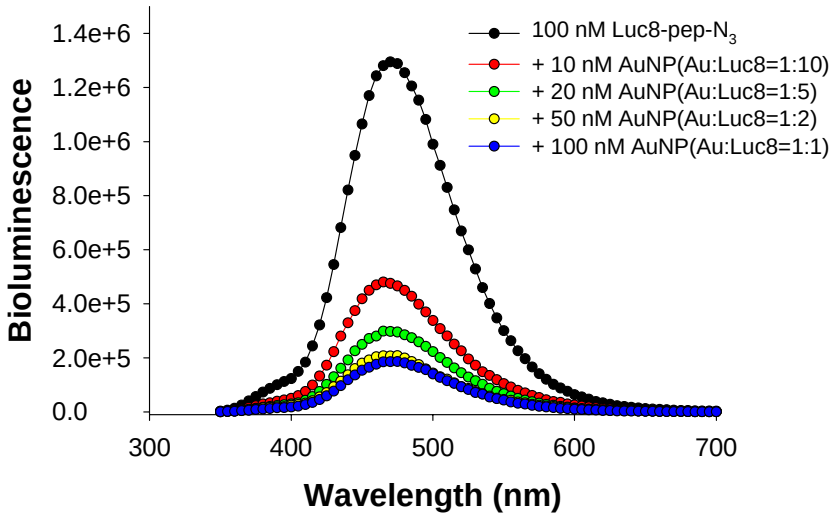
(a) UV-visible spectra of the Luc8-pep-Au NP conjugate in the absence (solid line) and presence (dotted line) of 2.5% 2-mercaptoethanol (2-ME). (b) Determination of the concentration of Luc8 protein using the Bradford assay. The mixture of Luc8-pep-Au NPs and 2-ME was used for extinction analysis and the Bradford assay after incubation for 24 hr at RT. The concentration of the Luc8-pep-Au NPs was calculated to be 0.18 μM. The absorbance at 595 nm from the Au NP-decomposed solution corresponded to a concentration of 0.7 μM (indicated with the red arrow). The ratio of Luc8 to the Au NPs was 3.9 (0.7/0.18). The mean value and standard errors were 4.7±1.2 from two independent experiments.

Fig. S2. Recovery of the luciferase activity using copper chelators



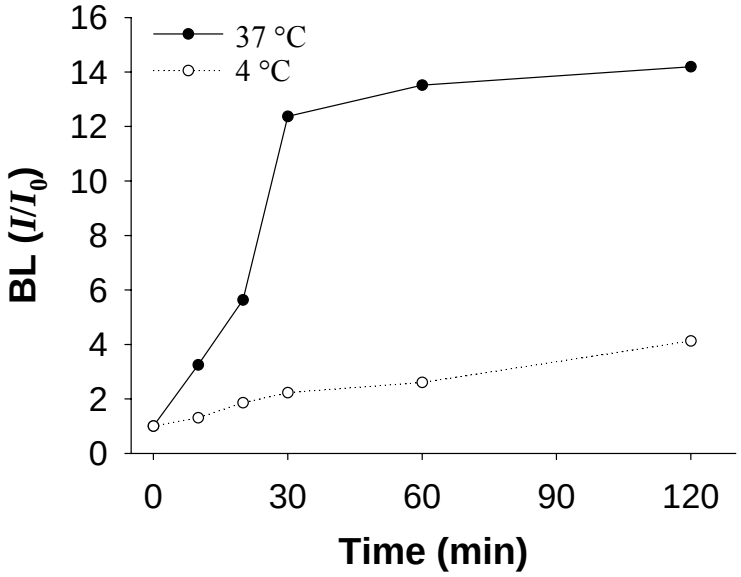
(a) Recovery yield (%) of Luc8-pep-N_3 activity after 30 mins of treatment with $CuSO_4$ (1 mM) and ascorbic acid (5 mM) using various copper chelators. 1: dithiothreitol (DTT), 2: L-cysteine, 3: imidazole, 4: ethylenediaminetetraacetic acid (EDTA), 5: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), 6: pyridine, 7: ammonium thetratholmolybdate (ATM). Luc8-pep-N_3 protein (1 μM) was first mixed with $CuSO_4$ (1 mM) and ascorbic acid (5 mM) in 0.1 M PBS (pH 8.0) for 30 min at RT. Each chelator was then added to the reaction solution at a final concentration of 10 mM, and incubated for a further 30 min, followed by a bioluminescence measurement. (b) Change in bioluminescence of the Luc8 protein: initial (black), after 30 min of click conditions (gray), and 30 min after adding L-cysteine to the reaction (dark gray). Approximately 40 % signal was recovered compared to the initial bioluminescence.

Fig. S3. Test of the quenching efficiency of Au NP on Luc8



Different concentrations of carboxyl-Au NPs (final concentration of 10–100 nM in HEPES pH 7.4) were mixed with Luc8-pep-N_3 protein (final concentration of 100 nM in HEPES pH 7.4), and EDC (final concentration of 100 μM) was then added to the reaction solution. After incubating for 30 min at RT, the BL of each reaction solution was measured using a fluorometer (Fluoro Max-3). The Au NP:Luc8 ratios (1:10, 1:5, 1:2, and 1:1) showed different quenching efficiency (64%, 77%, 84% and 85%, respectively).

Fig. S4. Time-dependent monitoring of MMP-2 activity



The nanosensor (360 nM) was treated with MMP-2 (500 ng mL^{-1}) in 50 mM Tris buffer (pH 7.5, containing 1.5 mM NaCl and 0.5 mM $CaCl_2$). The reactions were monitored over 120 min at 37 °C (closed circle) and 4 °C (open circle). The BL intensity (***I***) was normalized to the initial BL ($\boldsymbol{I}_0$ at 0 min).