Supplementary Material (ESI) for Chemical Communications

A Quantum-Dot Based Protein Module for *in vivo* Monitoring of Protease Activity Through Fluorescence Resonance Energy Transfer†

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Experimental Procedures

Construction of plasmids

To construct an expression vector for His6-MA/CA-Cys-ELP105K-TAT (H-MA/CA- ET), plasmid p08-ELP105K-EC20 described by Lao et al (Lao 2007) was digested with *Sma*I and *Hin*dIII and a DNA fragment encoding TAT peptide (amino acid sequence: GGTKTGRRRQRRKKRGY) was inserted between the same restriction sites. The resulting plasmid p08-ELP105K-TAT was cut with *Bam*HI and *Nde*I and 1.7 kb fragment was ligated to plasmid pET14 (Novagen, Madison, WI) treated with same enzymes to generate pET-ELP105K-TAT. An artificial gene carrying hexa-histidines and the MA/CA peptide with cysteine was prepared by heating and annealing two oligonucleotides, CATGGGC CAT CAC CAT CAC CAT CAC TCC CAG GTC AGC CAA AAT TAC CCT ATA GTG CAG AAC CTG CAG TTC TGC CA and TATG GCA GAA CTG CAG GTT CTG CAC TAT AGG GTA ATT TTG GCT GAC CTG GGA GTG ATG GTG ATG GTG ATG GC C and ligated to the 6.3kb fragment obtained from pET-ELP105K-TAT by digesting with *Nco*I and *Nde*I to create pET-H-MA/CA-ET.

Expression and purification of peptides

The expression vector for H-MA-ET was constructed by inserting DNA fragments coding for the TAT peptide, the hexa-histidine, and the MA/CA cleavage sequence into plasmid p08-ELP105K-EC20.[30] For protein expression, *E. coli* BL-21(DE3) (Novagen, Madison, WI) carrying the expression plasmid

was grown in terrific broth media containing 100 μg/ml ampicillin at 30°C until OD_{600} = 5. Cells were harvested by centrifugation, resuspended in 50mM Tris/HCl (pH 8.0) with 0.1M NaCl and 1 mg/ml of lysozyme, and lysed by sonication. After removing the cell debris, proteins were purified by repeated temperature transition cycles. Briefly, for each cycle, NaCl was added to the sample at a final concentration of 2M and the sample was heated to 37°C and centrifuged at 16,000 g at 30°C for 15 min. The pellet containing the proteins were resuspended in ice-cold 50mM Tris/HCl (pH 8.0) with 0.1M NaCl and centrifuged at 16,000 g at 4°C for 15 min to remove undissolved proteins. This temperature transition cycle was repeated once more, and the pellet containing H-MA/CA-ET was finally resuspended in ice cold 50mM Tris/HCl (pH 8.0) with 0.1M NaCl. The purity of the protein was determined by SDS-PAGE electrophoresis.

Conjugation of peptides with fluorescent dye

The labeling of purified protein modules with Alexa 568 maleimide was follows. Purified proteins in 50mM potassium phosphate buffer (pH 7) at a final concentration of 40 μM were mixed with Tris-(2-carboxyethyl) phosphine (TCEP) and Alexa 568 maleimide (Invitrogen, Carlsbad, CA) (thiol reactive dye) at 10-fold molar excess for 2 h at room temperature in the dark. The reaction was stopped and the unreacted dyes were removed by two or three thermal precipitation cycles.

Assembly and characterization of QD-protein-dye conjugates

Conjugation of QDs to the Alexa-labeled protein module was adapted from Clapp et al.[31] TOPO-capped CdSe/ZnS QDs (Evident Tech. Inc) were made water soluble by performing a ligand exchange reaction with dihydrolipoic acid (DHLA). DHLA-capped QDs were added to different ratios of Alexa568-labeled proteins, resuspended in 10mM HEPES buffer (pH 8.2), and incubated at room temperature overnight. After conjugation, the FRET efficiency was measured using a fluorometer by exciting at 430 nm and the spectrum was recorded from 475 nm to 650 nm

***In vitro* protease assay**

For the *in vitro* assay, HIV-1 PR was diluted in 200 mM sodium acetate pH 5.5, 10 % glycerol, 5 % ethylene glycol and 1mM DTT at a concentration of 500 nM. The protease assay was initiated by addition of 45 µL protease solution to microwells containing the QD probes. The 96-well plate was incubated at 37°C with a tight seal to prevent water evaporation. The fluorescence intensity in each well was measured using a microplate reader (Synergy^{TM} 4, BIOTEK® Instruments, Inc., Winooski, VT).

***In Vivo* monitoring of HIV-1 Pr activity**

For intracellular delivery, HeLa cells were seeded into 96-well clear bottom black wall plates (Nunc Scientific) at 37°C in 5% CO_2 in air and cultured to >90% confluence. After washing, cells were incubated at 37°C in the dark with 1× Leibovitz L-15 medium (Invitrogen) containing 150 nM QD probes for 2 h. After washing with PBS, cells were observed under a Zeiss Axiovert 40 CFL inverted fluorescence microscope. For *in vivo* monitoring of HIV-1 PR activity, HeLa cells containing QD probes were transfected with the plasmid pNL4-3.HSA.R^{-}E^{-}[19-20] using the Lipofectamine^{TM} 2000 transfection reagent (Invitrogen). After 6 h, the medium was changed to the normal growth medium and cells were propagated for another 18 h and were observed under a fluorescent microscope. For the inhibitor experiments, different concentrations of inhibitors were added to the medium prior to transfection. The protease inhibitors were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

Fluorescence Microscopy and image processing

Cell imaging was performed on a Zeiss Axiovert 40 CFL inverted microscope equipped with a 12-V, 35-W halogen lamp (for the phase-contrast images) and an HBO 50 W/AC mercury lamp (for the fluorescence images). The objectives used were a 5×/0.12 A-Plan, a 10×/0.25 A-Plan, a 20×/0.50 EC Plan-NEOFLUAR, and a 40×/0.50 LD A-Plan (Zeiss). Fluorescent probes were detected by using two

different filter sets; QD filter consisting of a D436-nm exciter, a D535/50-nm emitter, and a 475 nm-dichroic long pass beam splitter (Chroma Technology) and FRET filter consisting of a D436-nm exciter, a D610/50-nm emitter, and a 475 nm-dichroic long pass beam splitter. Images were acquired by using a ProgRes MFscan Monochrome CCD camera (Jenoptik). Both phase-contrast and fluorescence images were analyzed by using Image-Pro PLUS analysis software (Media Cybernetics). All settings for image processing were kept constant, and the exposure time for image capture was adjusted, if necessary, to maintain output levels similar to those observed under the fluorescence microscope. The intracellular distribution of the QD- based probes were analyzed by merging the bright field images with the fluorescence images. Composite merged images were produced by superimposing the fluorescence images from the QD filter and from the FRET filter**.** The QD and Alexa fluorescence intensity was quantified by randomly choosing five fields from each well using the Image-Pro PLUS analysis software.

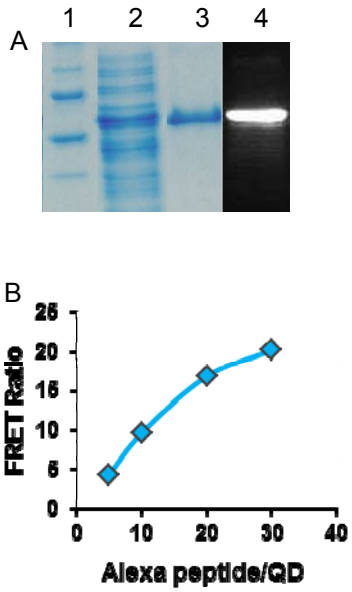
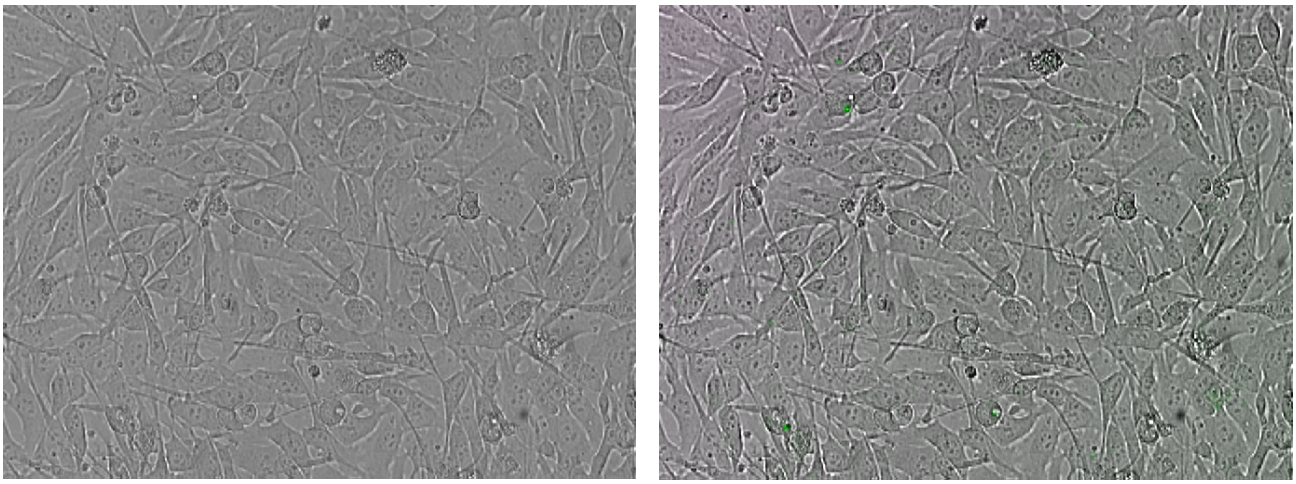


Figure S1. Protein purification and conjugation. A) Production and purification of protein module. Lane 1: protein marker, lane 2: total cell lysate, and lane 3: purified protein module. Conjugation of the protein module with Alexa 568 maleimide was confirmed under UV light (lane 4). B) FRET ratios of different QD:Alexa-module conjugates. The ratio is expressed as the Alexa (610 nm)/QD (540 nm) emission intensity when excited at 430.

Fig. S2. Intracellular delivery of QD to HeLa cells. In the absence of the TAT peptide, virtually no fluorescence was observed inside HeLa cells after 2 h of incubation.



DIC Merged

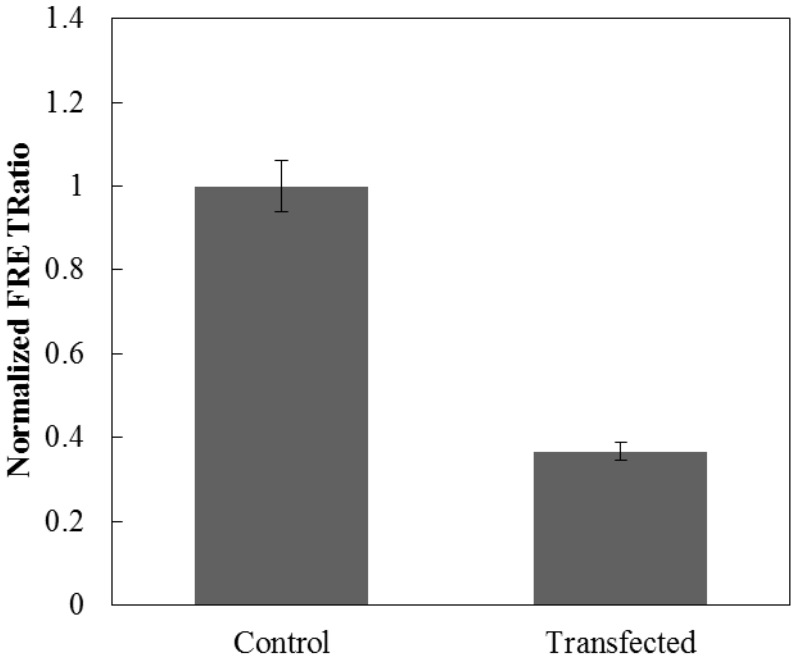


Figure S3. Normalized FRET ratio for untransfected control cells and transfected cells.

Fig. S4. Inhibition of HIV-1 Pr activity by Ritonavir. The merged fluorescence images of cells treated with different dosages of Ritonavir.