# Fluorogenic assay and live cell imaging of HIV-1 protease activity using acid-stable quantum dot–peptide complex†

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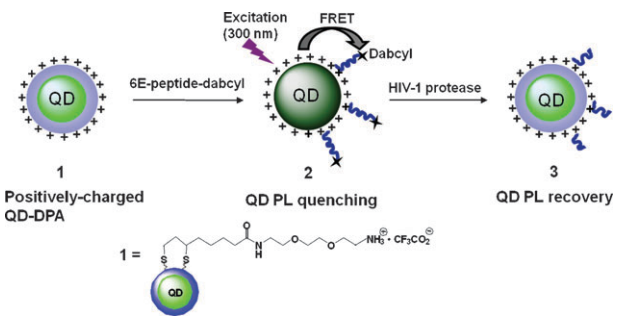
**A novel QD-peptide complex for detecting HIV-1 protease activity was prepared from simple one step electrostatic interaction. Fluorescence recovery of the pre-quenched QD through fluorescence resonance energy transfer allowed for *in vitro* assay and live cell imaging of the protease activity in HIV-1 transfected cells, proving the potential for cell-based protease inhibitor screening.**

The human immunodeficiency virus protease (HIV-1 PR) belongs to the family of aspartic proteases which are characterized by an optimum pH in the acidic range (pH 4–5) due to a conserved region of two aspartates in an active site.[1a] HIV-1 PR is essential for processing of newly synthesized viral polyproteins to create the mature protein components of an infectious HIV virion. Without effective HIV-1 PR, viral particles remain uninfectious, which makes this enzyme one of the key targets for anti-retroviral drug discovery.[1b] HIV-1 PR activity has been routinely assayed by the fluorescence resonance energy transfer (FRET)-based fluorogenic method using organic fluorophore-labelled peptide substrates.[1c] These assays, however, often suffer from several shortcomings including the instability of the dual dye-labelled peptides, high background, poor water solubility, and low quenching efficiency resulting in low sensitivity.[2]

Recently, CdSe/ZnS semiconductor nanocrystal, known as quantum dot (QD), has been widely used for *in vitro* diagnostics as well as biological imaging due to its unique fluorescent properties such as high photostability, broad excitation and tunable narrow emission spectra.[3] The QD, a novel type of fluorophore, has also been employed as an efficient FRET donor, which can be conjugated to acceptor dyes or gold nanoparticle-labelled peptidic substrates for sensing the activities of various enzymes such as caspases, thrombin, chymotrypsin,[4a,b] β-lactamase,[4c] matrix metalloproteinase,[4d] and collagenase.[5a–c] However, all of these enzymes require neutral or basic pH ranges for their optimum activity. Therefore, carboxylated QDs including QD-DHLA (dihydrolipoic acid), polymer-coated QD, and QD-streptavidin which are stable in neutral to basic pH ranges (pH 7–9) have been used in these enzymatic assays. The negatively charged QDs are thus not fully applicable to the family of acid proteases such as the HIV-1 PR.[1c] Furthermore, recombinant HIV-1 PR has $10^6$ times lower enzymatic activity (1 pmol/unit) than most of the aforementioned proteases (*e.g.* 1 μmol/unit for collagenase).[6] These challenges necessitate the development of more sensitive and robust probe systems for measuring HIV-1 PR activity *in vitro* as well as for live cell imaging applications.

Here, we demonstrate a novel QD–substrate probe for sensing HIV-1 PR activity through the fluorescence recovery of FRET-quenched QD prepared by electrostatic assembly with peptidic substrate (Scheme 1). The peptide sequence-specific fluorogenic property of the QD probe was validated in *in vitro* assays employing known protease inhibitors, which allows for determining the inhibition concentration at 50% ($IC_{50}$) values for pepstatin A and the FDA (Food and Drug Administration)-approved anti HIV-1 drug saquinavir (SQ). Importantly, we could also visualize the effect of SQ on protease activity in protease expressing live HeLa cells. These results eventually demonstrated the potential of this QD probe for the development and implementation of cell-based visual assays for protease-targeted anti-retroviral drug discovery.

The QDs that are rendered stable in acidic pH were prepared by exchanging trioctylphosphine oxide (TOPO) molecules on QD (QD495-TOPO, emission peak at 495 nm; Evidot®) with DPA (DHLA-PEG-Amine) ligand according to the method we previously reported.[7a,b] After the DPA ligand exchange of QD-TOPO, the QD becomes water-soluble. The excess DPA is removed by extensive membrane filtration with molecular weight cut-off (10 kDa) with deionized water. DPA-modified QDs (**1**) possessing both ethylene glycol and $NH_3^+ \cdot CF_3CO_2^-$ moiety on the surface ligand allowed for the stabilization in




**Scheme 1** Scheme for HIV-1 protease assay based on FRET-quenched QD–peptide complex. 6E-peptide-dabcyl indicates 6E-GLAib-SQNYPIVQ-K(dabcyl), or 6Glu-Gly-Leu-Aib-2Gly-Ser-Gln-Asp-Tyr-Pro-Ile-Val-Lys(dabcyl); Aib = amino-isobutyric acid.

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acidic aqueous media (pH 3–6) and overall positive charges (~66 amino groups per QD, zeta potential $\zeta = +30.6 \pm 5.8$ mV). Hydrodynamic size of the QD-DPA was $9.2 \pm 1.4$ nm in diameter based on dynamic light scattering (DLS) analysis; comparable with ~3.5 nm from transmission electron microscope (TEM) analysis (See the Supporting Information (S.I.) for the detailed characterization of the QD-DPA).

In order to create a FRET-quenched QD probe specific for HIV-1 PR, we designed a modular peptide (**Pep1**) consisting of six glutamic acid residues (6E) at the N-terminal site, a linker residue (Gly-Leu-Aib-2G), and HIV-1 PR recognition site (SQNYPIVQ),[8a] followed by a lysine residue for labeling with dabcyl quencher. Due to the presence of negatively charged 6E residues, the **Pep1** ($\zeta = -16$ mV) immediately forms an electrostatic complex with the positively charged QD **1** in deionized water, resulting in the DLS size (~12 nm). The binding of the dabcyl-labeled peptide with the QD-DPA surface resulted in FRET-induced quenching of the QD (Förster radius $R_o = 3.7$ nm; See the S.I. for the details) (Fig. 1a). The quenching efficiency of the QD probe depends on the molar ratio of **Pep1** to QD **1**, saturating FRET efficiency (over 0.95) at above the molar ratio of 3 (**Pep1**/QD). To prove that the quenching phenomenon is based on FRET rather than non-specific aggregation, we prepared the QD-DPA with longer emission peak (530 nm) for complexation with the **Pep1**. Negligible quenching was observed when compared with QD500-DPA, due to the lack of spectral overlapping of QD emission (530 nm) and dabcyl absorption ($\lambda_{max} = 454$ nm). With this QD530-DPA, a similar quenching phenomenon was observed when we replaced the dabcyl with 6-carboxy tetramethylrhodamine (TAMRA; $\lambda_{max} = 547$ nm). These results strongly suggest that electrostatic interaction between QD-DPA and 6E-modified peptides resulted in the FRET-induced quenching (See the S.I. for the details). This result also shows the simplicity of the preparation for QD–substrate complex **2** by mixing the two components in a similar manner to a kit.

To test the fluorogenic properties of the QD-based probe in the presence of HIV-1 PR, QD-**Pep1** **2** (0.18 μM) was incubated with HIV-1 PR (0.35 μg) for 1 h at 37 °C in the acidic buffer condition (10 mM acetate, 100 mM NaCl, 1 mM DTT, BSA 10 μg mL$^{-1}$, pH 4.5), resulting in ~8-fold increase of QD photoluminescence (PL) (Fig. 1b). The maximum fold-increase of PL in the QD-**Pep1** probe system was obtained after optimizing the incubation temperature (25 °C, 37 °C) and

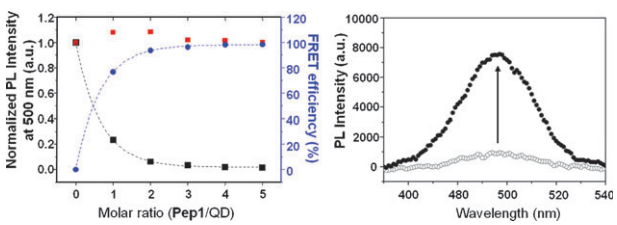


**Fig. 1** (Left) Normalized QD PL intensity at 500 nm in arbitrary units (a.u.) depending on the molar ratio of **Pep1** to QD **1** (black square) and the corresponding FRET efficiency (blue circle); the red square indicating QD PL in the presence of an equivalent amount of free dabcyl dye. (Right) Increase of QD PL after HIV-1 PR digestion of the QD-**Pep1** complex **2** in the acetate buffer (pH 4.5).

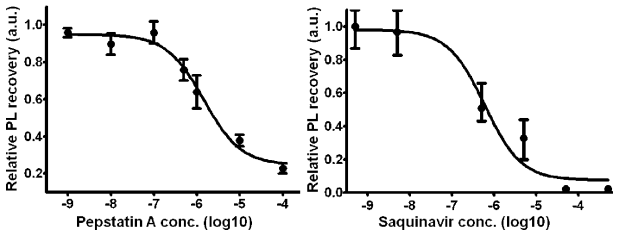


**Fig. 2** Determination of $IC_{50}$ values of the HIV-1 PR inhibitors (left) pepstatin A (2 μM) and (right) SQ (0.6 μM). 0% inhibition was defined as the normalized PL recovery ratio of QD-**Pep1** after HIV-1 PR treatment in the absence of inhibitor drug (pepstatin A or SQ), while 100% inhibition was shown in the presence of the highest concentration of inhibitor used (0.1 mM–1 mM). The data fitting was performed using a non-linear log(inhibitor) *vs.* response mode in GraphPad Prism software (ver 5.0).

buffer formulation (BSA 0–0.2 mg mL$^{-1}$, NaCl 0–100 mM), which are important factors for the reproducibility of the assay (See the S.I. for the details). The sequence specificity of the QD PL recovery was also confirmed with the control QD **1** which was complexed with non-binding peptide **Npep1** (6E-linker-Val-Asn-Cys-Ala-Lys-Lys-Ile-Val-Lys(dabcyl)).[8b] The optimized assay condition and the sensitivity of the QD-**Pep1** probe system allowed us to reproducibly measure the $IC_{50}$ values of the HIV-1 PR inhibitors, pepstatin A (2 μM) and SQ (0.6 μM) (Fig. 2). $IC_{50}$ values are varied depending on the assay system used as reported in the literature, for example, 1 μM for pepstatin A and 0.024–1.4 μM for SQ.[1,9]

To further test the feasibility of the QD-**Pep1** probe for cell-based drug screening, we attempted to visualize the HIV-1 PR activity in living cells. To create a HIV-infected cellular model, HeLa cells were transfected with a HIV-1 plasmid, pCMV-dR8.74, which encodes for the *gag* and *pol* genes of HIV-1.[10a,b] The gag genes encode the structural components of the viral capsid and the *pol* genes the essential viral enzymes including HIV-1 PR, respectively. The Gag proteins of HIV-1, like those of other retroviruses, are necessary and sufficient for the assembly of virus-like particles, which however remain non-infectious due to the absence of viral envelope proteins and genomes. To confirm that the HeLa cells were transfected with HIV-1 plasmid, we performed the p24 ELISA assay which identifies the HIV-1 specific antigens.[10c] The live HeLa cells containing non-infectious HIV-1 were incubated with QD-**Pep1** probe (50 nM) for 3 h at 37 °C before confocal microscope imaging. Fig. 3 shows the punctate QD signals detected in the cytosol of the HIV-1 infected HeLa cells (See the S.I. for the z-section images in 7 different focal middle planes at 1 μm interval). These signals were not observed in the control naïve cells (not shown). Further, to confirm the sequence specificity of the QD probe in live cells, we also incubated the QD-**Npep1** with the HIV-1 infected cells at the same conditions, which didn't show any significant recovery of QD signals inside the cells, confirming the negligible false-positive signals.

With the successful demonstration of the QD-**Pep1** probe for HIV-1 PR activity in live cells, we performed cell-based visual drug screening ([SQ] = 0.025–10 μM) on the HIV-1 infected HeLa cells. QD PL (green channel) was recovered at 0.025 μM of SQ treatment, indicating the low inhibitory

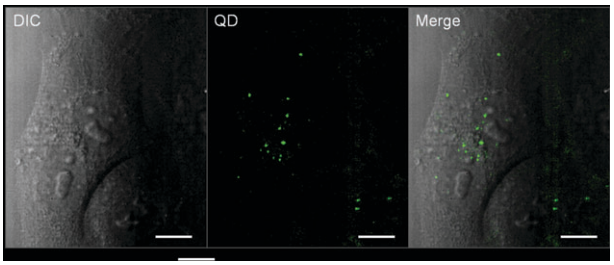


**Fig. 3** Representative confocal microscope images of HIV-1 plasmid transfected HeLa cells incubated with QD-**Pep1** probe (50 nM, 3 h), which shows the recovered QD signal indicating HIV-1 protease activity inside living cells. Differential interference contrast channel (DIC) image, green QD channel (bandpass emission filter 500–525 nm) with 488 nm laser excitation, and the merged image of the DIC and the QD channel are shown with scale bar of 10 μm.

activity of SQ allowing HIV-1 PR activity. However, the QD PL can not be recovered when HIV-1 PR activity is significantly inhibited by the high concentration of SQ (0.25–10 μM) (See the S.I. for the images). This result indicates the potential of this probe for cell-based protease inhibitor screening. The effective concentration of SQ was found to vary according to the assay system used, for example, 0.05–0.5 μM using HIV-1 infected macrophages.[10a]

In conclusion, these data showed the potential of the acid-stable QD probe for sensing aspartic proteases such as HIV-1 PR which requires an acidic pH for optimum activity. The simplicity in preparation (simple mixing), reproducibility due to the photostability of QDs, and enzymatic sensitivity can truly allow this QD-based probe to be applied to assays of other aspartic proteases such as β-secretase or cathepsin D. Moreover, the present approach provided the potential of QDs to be applied as a cell-based visual screening platform for the development of novel protease inhibitors as well as visual target identification in living cells. Finally, the studies to make this strategy universal for multiplexing and other enzymatic assays are underway.

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