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Chip-Based Protease Assay Using Fluorescence Resonance Energy Transfer Between Quantum Dots and Fluorophores

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Abstract

The analysis of proteolytic activity is essential for understanding the biological functions of living systems as well as for developing therapeutics and diagnosis. We developed a chip-based assay system for proteases that measures the fluorescence resonance energy transfer (FRET) between quantum dots (QD) and fluorophore-labeled peptides. In this system, while the photoluminescence (PL) of donor QD immobilized on a surface was quenched due to the presence of an energy acceptor (fluorophore) in close proximity, the protease activity caused modulation in the efficiency of the energy transfer between the acceptor and donor, thus enabling the highly sensitive assay. Unlike solution-based analyses, our chip-based format enables a more reliable analysis with no aggregation of QD, and it is able to function using a much smaller reaction volume. The system presented here will be useful for the detection of various proteases with high selectivity and sensitivity in a high-throughput manner.

Keywords: Chip, Protease, Enzyme assay, FRET, Peptide, Quantum dot

Introduction

The ability of enzymes to target the specific substrate in complex environments is essential for understanding the fidelity of most biological functions. Proteases are particularly important because a protease reaction is the final step in establishing the functional roles of many expressed proteins[1]. In addition, most proteases are involved in major human diseases such as cancer, AIDS, inflammation and neurodegenerative diseases[2], therefore, identification of these enzymes is crucial for revealing the mechanism by which their cellular processes occur, as well as for designing potential protease inhibitors for use in therapeutic drugs.

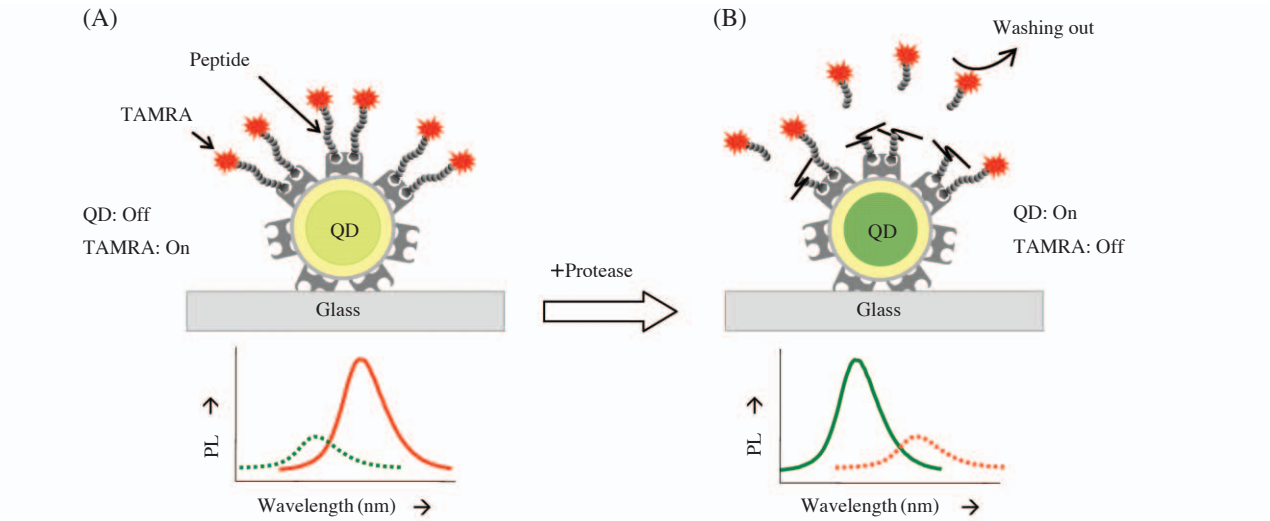
To date, many fluorescence-based assays that allow the identification of a specific protease through the labeling of its substrates have been developed. Typically, fluorescence (or Förster) resonance energy transfer (FRET) has been used to assay these enzymes using a method in which a specific peptide sequence is doubly labeled with a donor and an acceptor chromophore[3,4]. Because of its high specificity in relation to the distance-dependent energy transfer between the donor and acceptor, FRET has been useful for the detection of protease activity.

More recently, some of these FRET assays have been successfully used in conjunction with quantum dots (QDs)[5,6]. The QDs employ semi-conducting nanocrystals, which allow less photobleaching[7] as well as multiple binding of acceptors, thereby increasing the overall FRET efficiency[8]. However, these methods still face problems related to high-throughput assays due to difficulty with construction in a chip-based format. In addition, some issues related to the consumption of a large amount of reagents and unstable photoluminescence (PL) as a result of aggregation need be overcome before this method can be used for high-throughput screening. However, given the minimal volume required and stable response of the QD-based FRET system, it may offer a new assay format for the rapid and sensitive diagnosis of protease-relevant diseases in a high-throughput and multiplexing manner.

We developed a chip-based protease assay that utilizes a QD-based FRET system. By immobilizing QDs on a surface, this chip-based approach enables more effective energy transfer with no aggregation of the nanoparticles. In addition, only a very small volume of reagents, including the donor and acceptor, is necessary for a sensitive assay. Here the use of the developed assay system to detect a matrix metalloproteinase (MMP) using the FRET between QDs and organic fluorophores is described.

Results and Discussion

As illustrated in Scheme 1, our assay system relies



Scheme 1. Assay principle of protease based on the FRET between the donor (SA-QD in green) and the acceptor (TAMRA-labeled peptide in red) on a glass slide. The PL intensities of the donor and the acceptor are modulated (A) before and (B) after the protease reaction.

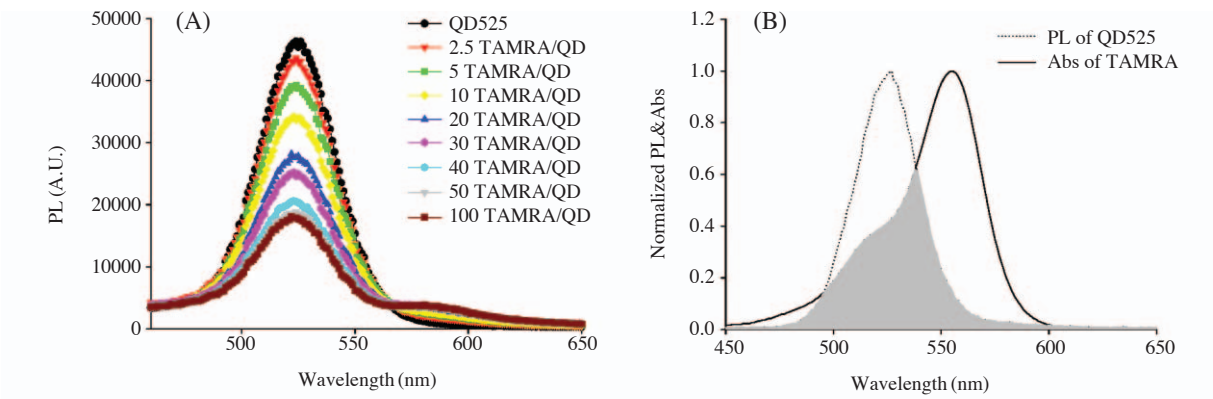


Figure 1. (A) Changes in the PL intensity as a function of the molar ratio between SA-QD525 and TAMRA-pep-biotin in solution. The ratios of acceptor to donor were varied from 2.5 : 1 to 100 : 1. (B) Emission and absorbance spectra of QD525 (dotted line) and TAMRA (solid line), respectively. The fluorescence intensity of QD525 was normalized to the maximum extinction coefficient of TAMRA ($6.5 \times 10^4\,cm^{-1}\,M^{-1}$ at 555 nm).

on modulations in the PL intensities between donor QDs and acceptor fluorophores before and after the protease reaction. The fluorophore-labeled peptide substrate (TAMRA-pep-biotin) was associated with streptavidin-coated QD525 (SA-QD525) that had been directly immobilized onto the amine-reactive glass surface (Slide H, Schott Nexterion). While the PL intensity of the donor QD is quenched by TAMRA dyes (Scheme 1A), the addition of proteases causes cleavage of the peptide substrate, which results in recovery of the PL of the QDs with a decrease in the TAMRA emission (Scheme 1B).

To check the efficiency of the energy transfer, we measured the PL intensity that occurred when varying molar ratios of acceptor (TAMRA-pep-biotin) to donor (SA-QD525) in aqueous solution were used (Figure 1A). As the molar ratio of acceptor to donor increased, the PL intensity of the donor QDs decreased gradually with a marginal enhancement in the PL of the acceptor TAMRA dye. When the quenching efficiency (Q_E) was calculated according to Eq. 1, which is described in the Materials and Methods section, the maximum Q_E of the donor QD reached approximately 62% at a molar ratio of 50 : 1. Further increases in the molar ratio did not result in greater enhancement of Q_E, therefore the molar ratio of 50 : 1

Table 1. FRET-based quenching efficiency (Q_E) and separation distance (r) between QDs and TAMRA in solution.

Donor	Acceptor	Estimated distance (nm)[a]	R_0 (nm)[b]	Q_E (%)[c] (n=50)[d]	r (nm)[e]
SA-QD525	TAMRA-RPLALWRSK-biotin	10.9–13.4	7.0	62.0±5.9	12.3±0.5

[a]An estimated distance is a theoretical maximum distance from the core of the donor to the surface of the acceptor that takes into account the full extension of the peptide sequence and the radius of the donor SA-QD. [b]The Förster distance (R_0) value of QD-TAMRA was calculated from the overlap integral in Figure 1B. [c]The quenching efficiency (Q_E) was calculated using Eq. 1. [d]The binding ratio (n) was estimated from the saturated molar ratio of the donor to the acceptor where Q_E did not increase further. [e]The separation distance (r) was determined using Eq. 2.

represents the maximum binding number of TAMRA-labeled peptides per QD. Based on these results, as well as the overlapping integral spectra between the donor and acceptor (gray area in Figure 1B), we calculated the Förster distance (R_0=7.0 nm) and separation distance (r=12.3 nm) between the donor QD and the acceptor dye (Table 1). Assuming the full length of the peptide (end-to-end distance of *N*-residue peptide in a nearly extended conformation is about 3.8Å × *N*) and the radius of SA-QD (7.5-10.0 nm), this separation distance was well correlated with the estimated distance (from 10.9 to 13.4 nm) in the regime of FRET. Despite the longer separation distance of 10 nm, which typically corresponds to the limited distance of traditional FRET, the multiple binding of acceptors per single QD seems to allow the energy transfer to occur, even when there is an excessive FRET distance. It is important to note that the multiple binding (54 : 1) of Cy5-labeled DNA per QD allowed an efficient energy transfer, even at a distance of ≈$2R_0$, compared to the 1 : 1 binding ratio that occurs when an acceptor-to-donor method is used[9].

To examine the utility of the chip-based protease assay, we constructed the FRET system on a glass slide. To accomplish this, SA-QDs were immobilized on a glass slide, and then conjugated with a 50-fold excess of TAMRA-pep-biotin, which functions both as a substrate for MMP-7 and as an energy acceptor. Because MMP-7 selectively cleaves the Ala-Lys bond in the peptide sequence (-RPLALWRSK-), the energy transfer between the QD and TAMRA can discriminate the presence of MMP-7. The modulations in the PL of TAMRA and the QD on a glass slide were monitored using a fluorescence scanner at different emission wavelengths (525 nm for donor QD signal and 595 nm for acceptor signal) with a fixed excitation wavelength (460 nm). Compared to the control spots (a in Figure 2A and B), the conjugation of acceptors on SA-QDs caused a drastic reduction (b in Figure 2A) in the PL intensity of QD, which resulted in a strong acceptor signal being observed (b in Figure 2B). This indicates that the FRET between the acceptor and the donor effectively functions on a chip surface. Furthermore, addition of MMP-7 protease onto the assembly of the acceptor-to-donor resulted in an inverse change in the PLs of the acceptor and the donor (c in Figure 2A and B), however, no significant change was observed when MMP-2 was used as a negative control (d in Figure 2A and B). This result strongly indicates that a chip-based FRET system can effectively detect the activity of MMP-7 with specificity.

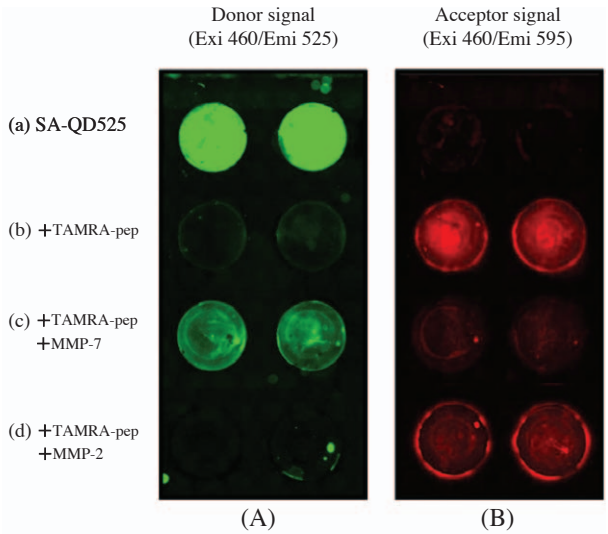


Figure 2. Chip-based assay of MMP-7 based on the FRET between SA-QD525 and TAMRA-pep-biotin. (A) Donor and (B) acceptor signals were displayed in the absence and presence of MMP-7. MMP-2 protease was used as a control. The duplicate spots were used for checking the reproducible reaction.

We also determined the standard curve and detection limit for MMP-7 activity on a glass surface. With the assistance of a multiwell-type silicon coverslip for high-throughput assay, and following the method described in a previous study[10], different concentrations of MMP-7 were loaded onto each well overlaid onto the surface comprising the assembly of the QDs and TAMRA-peptides. This allowed the fluorescence image (Figure 3) and the standard curve (Fig-

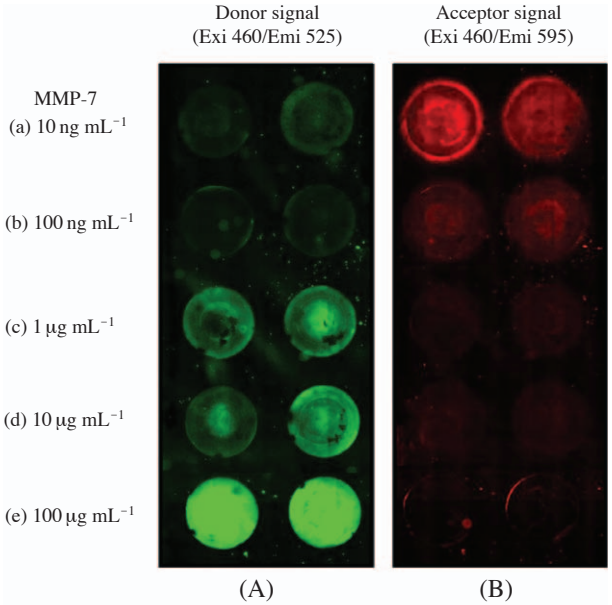


Figure 3. Chip-based assay of protease activity as a function of MMP-7 concentration. Fluorescence images were displayed with (A) donor and (B) acceptor signals from the chipbased FRET system.

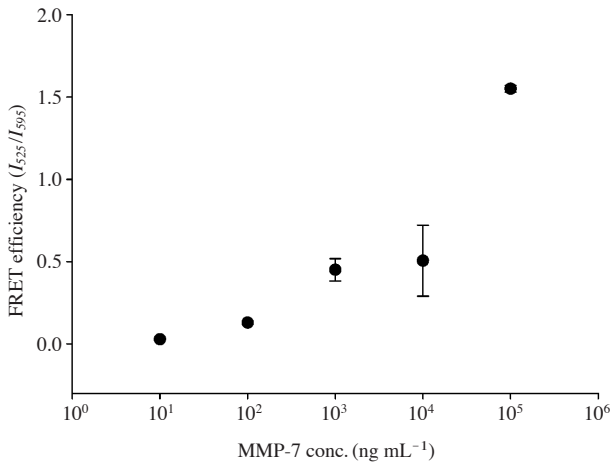


Figure 4. Standard curve of MMP-7 activity generated using the chip-based FRET system. FRET efficiency between the QD and TAMRA was plotted as a function of MMP-7 concentration, which ranged from 10 ng mL^{-1} to 100 μg mL^{-1}. The error bar indicates the standard deviation in duplicate spots (I_{525}: PL intensity of donor; I_{595}: PL intensity of acceptor).

ure 4) to be obtained. The FRET efficiency was then calculated based on the ratio (I_{525}/I_{595}) of the PL intensity between the acceptor and the donor, and then used to analyze the output signals. The FRET efficiency induced by MMP-7 was found to be linearly dependent on the logarithmic concentration of MMP-7, ranging from 100 ng mL^{-1} to 100 μg mL^{-1}, which is a favorable range when the secretion levels in malignant tissues (< 600 ng mL^{-1})[11] and in cancer patient sera (< 126 ng mL^{-1}) are considered[12]. No significant FRET efficiency was observed between systems treated with a low concentration of MMP-7 (10 ng mL^{-1}) (a in Figure 3A and B) and those that contained no MMP-7 (b in Figure 2A and B). The detection sensitivity of our assay system, therefore, appears to be approximately 100 ng mL^{-1}, which is comparable to that of other FRET-based systems[6]. Taken together, these results indicate that a chip-based FRET system could be very useful for sensitive and high-throughput assays of proteases.

Based on these results, our chip-based FRET system has several advantages over FRET assays that are conducted in aqueous solution. The system described here requires a relatively small sample volume, which facilitates high-throughput screening. In addition, the stable immobilization of QDs on a surface enables the reliable analysis of enzyme activity. Moreover, since size-tunable QDs with different colors can be employed as energy acceptors on a surface, we anticipate that a multiplexed assay of proteases will possible using our chip-based FRET system.

Conclusions

In conclusion, we described a chip-based assay of protease that uses the FRET between QDs and fluorophores. Immobilizing the SA-QDs on a glass surface allowed the multiple binding of acceptors (TAMRA-pep-biotin) onto the SA-QD to have an efficient energy transfer, even at a distance approaching $2R_0$. As a result, a high level of sensitivity was attained in a broad concentration range, which enabled the activity of MMP-7 to be assayed. In contrast to a solution-based analysis, the chip-based format allowed more reliable analysis, with no aggregation of QDs. In addition, this format required a much smaller reaction volume. Overall, these results indicate that the system described here has the potential to screen the activity of disease-associated proteases for the development of therapeutics and diagnostics in a high-throughput manner.

Materials and Methods

Materials

Matrix metalloproteinase-7 (MMP-7) and matrix metalloproteinase-2 (MMP-2) were purchased from Calbiochem. Streptavidin-conjugated quantum dots (SA-QD525) were obtained from Invitrogen. A

hydro-gel-type amine-reactive glass (Nexterion™ Slide H) was obtained from Schott Nexterion (Germany). TAMRA (5(6)-carboxytetramethylrhodamine) -labeled peptide (TAMRA-RPLALWRSK-biotin, TAMRA-pep-biotin) was synthesized from Peptron Inc. (Korea). A chambered silicon coverslip (50 wells, 3 mm × 1 mm, sterile) was purchased from Sigma-Aldrich.

Analysis of FRET in Solution

For quenching experiments, SA-QD525 was mixed with varying amounts of TAMRA-pep-biotin (in 25 mM HEPES, pH 7.4) in a 96-well plate for 1 hr at room temperature to allow a specific association between SA and biotin to form. The final concentration of the QDs in the aqueous solution was typically 10 nM (corresponding to 1 pmol in a 100 μL reaction volume). After incubation, the fluorescence was measured at an excitation wavelength of 430 nm using a microplate reader (Infinite™ M200, TECAN, Austria). The PL intensities of the donor and acceptor were then monitored as a function of the molar ratio of the acceptor to the donor. The concentrations of the donor (SA-QD525) and the acceptor (TAMRA-pep-biotin) were then determined using the extinction coefficients of the QD ($1.3 \times 10^5\ M^{-1}\ cm^{-1}$ at 488 nm) and TAMRA ($6.5 \times 10^4\ cm^{-1}\ M^{-1}$ at 555 nm), respectively.

Chip-based Protease Assay

For chip-based analysis, a multiwell-type chambered silicon cover-slip (ϕ 3 mm × H 1 mm, Sigma) was overlaid onto an NHS-derivatized hydrogel glass slide (Schott Nexterion). A solution of 10 nM SA-QD525 (10 μL in 50 mM borate buffer pH 8.5) was then dropped on the wells formed by the chambered silicon cover-slip. Next, the slide was incubated for 1 hr at room temperature, and the QD-immobilized wells were then immersed in a solution of 2% BSA (in 50 mM borate buffer pH 8.5) for 1 hr to block the remaining NHS groups. After being rinsed with distilled water, the wells were incubated in a solution of TAMRA-pep-biotin (10 μL at 500 nM). The slides were then incubated for 1 hr at room temperature. Next, the wells on the slide were rinsed three times with distilled water and then dried with a stream of N_2. For the protease assay, 10 μL of a MMP-7 solution (50 mM HEPES buffer pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$) with varying concentrations of MMP-7 (10 ng mL^{-1} to 100 μg mL^{-1}) was added to each well. The plates were then tightly sealed to prevent evaporation and then incubated at 37°C for 60 min. MMP-2 protease was used as a negative control. After incubation, the surface was rinsed three times with distilled water, dried with a stream of N_2, and then subjected to fluorescence analysis.

Fluorescence Readout

Fluorescence scanning was carried out using an ArrayWoRx^e slide scanner (Applied Precision, USA) equipped with a white-light CCD camera. For measurements of the PL intensities on the surfaces, the glass slides were independently scanned at 460 nm/525 nm (for the QD signal) and at 460 nm/595 nm (for the FRET signal) using an excitation/emission filter set (Chroma Tech. Corp., USA). After scanning, the fluorescence images were analyzed using imaging software (Multi Gauge ver 3.0, Fujifilm), and the mean value and standard deviation were calculated using duplicate wells.

Calculations of Quenching Efficiency and Separation Distance

The Förster distance (R_0) value of QD-TAMRA was estimated using the Förster formula and the spectral overlapping between the emission spectrum of the donor, QD, and the absorbance spectrum of the acceptor, TAMRA, as described elsewhere[13]. We assumed that the quantum yield of SA-QD525 (Lot No. 47989A) and the refractive index of the biomolecules were 0.59 and 1.4 in aqueous solution, respectively. In addition, the orientation factor was assumed to be 2/3.

Energy transfer between QD and TAMRA can be determined based on the quenching efficiency (Q_E) of the experimentally obtained PL data. Considering multiple acceptors per quantum dot, the overall efficiency can be written as:

$$Q_E = 1 - \frac{PL_{DA}}{PL_D} = \frac{nR_0^6}{nR_0^6 + r^6} \quad \text{(Eq. 1)}$$

Where PL_D is the PL intensity of the donor alone, PL_{DA} is the PL intensity of the donor in the presence of the acceptor (s), r is a separation distance from the QD centre to the acceptors, and n is the number of surface-bound acceptors.

From the experimentally determined quenching efficiency (Q_E), the above equation can be used to determine the donor-to-acceptor distance (r) with calculated R_0:

$$r = \cdot \left(\frac{n(1-Q_E)}{Q_E}\right)^{1/6}, R_0 \quad \text{(Eq. 2)}$$

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