Multiplexed Proteolytic Enzyme Assay Based on Energy Transfer between Quantum Dots and Gold Nanoparticles

Young-Pil Kim,[1,†] Young-Hee Oh,[1,†] Eunkeu Oh,[2] and Hak-Sung Kim[1,]*

[1]Department of Biological Sciences, Korea Advanced Institute of Science and Technology (KAIST), Daejeon 305-701, Republic of Korea
[2]Naval Research Laboratory (NRL) and Johns Hopkins University
†These authors contributed equally to this work.
*Corresponding author: E-mail address: hskim76@kaist.ac.kr

ABSTRACT

Nanoparticles are emerging materials in contemporary biotechnology. We have developed high sensitive biosensing systems by using nanoparticles for clinical diagnostic. In this study we offer that enzyme protease can be effectively detected by using an energy transfer between quantum dot (QDs) and gold nanoparticles (AuNPs) on a chip surface. By applying the peptide-conjugated AuNPs to multiple QD donors immobilized on the glass surface, the assay of various proteases (matrix proteinase, caspase, and thrombin) was distinctly achieved with low sample volume (~nL) (Figure. 1)

Detection of proteolytic enzymes will be also focused with chip-based approaches. Chip-based assay by the energy transfer between two nanoparticles will provide a great potential for analysis of enzyme proteases and their inhibitors with high specificity and sensitivity in a high-throughput manner. With different functionalities on multiple QD donors, energy transfer-based assay on a solid surface will provide the great potential for multiplexing as well as high-throughput assay with high specificity and sensitivity. This chip-based assay of proteases and their inhibitors will be expected to be a powerful diagnostic tool to address many other interactions such as DNA-protein, protein-protein, and protein-carbohydrate.

Keywords: protease, gold nanoparticle, quantum dot, microarray, multiplexed assay, enzyme activity

1. INTRODUCTION

Proteases have been of particular interest because they are involved in major human diseases such as cancer, AIDS, inflammation and neurodegenerative disease [1-3], and typical examples include matrix metalloproteinases (MMPs), caspases, and thrombin.

Thus, a method to assay proteases and their inhibition with high sensitivity in a multiplexed manner is of great significance in diagnosis of protease-relevant diseases and development of potential drugs [1-4]. In the FRET-based system, quantum dots (QDs) have been widely used due to their intrinsic optical properties including high quantum yield, less photobleaching, and size-tunable photo-luminescence with broad excitation and narrow emission bandwidths [5] In particular, multiple binding of an energy acceptor per quantum dot is expected to increase the overall energy transfer efficiency[6,7]. AuNP is known to have a superior quenching efficiency in a broad range of wavelengths compared to other organic quenchers.

We previously reported an inhibition assay of a target analyte in solution by using the energy transfer between QDs and AuNPs[8,9].

Here we demonstrate a multiplexed assay of proteases and their inhibition by using AuNP and QD conjugates as nanoprobes on a glass slide. The chemical feature of the nanoprobes was also investigated in comparison with QD-dye conjugates in terms of the energy transfer efficiency, quenching constant, and maximum effective distance.

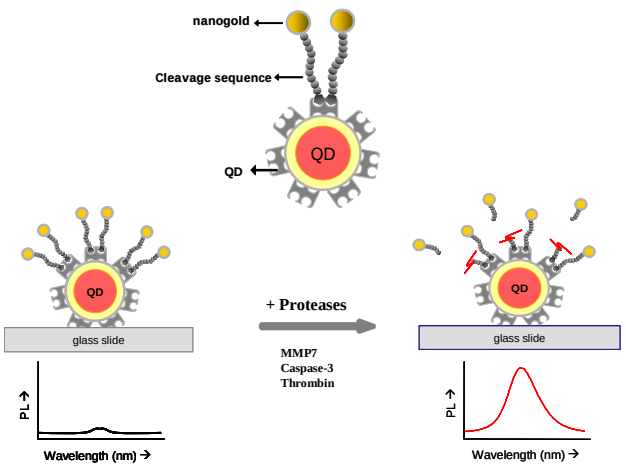

Figure 1. Principle of a chip-based protease assay by using energy transfer between QDs and AuNPs on a glass slide.

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2. EXPERIMENTAL

2.1 Synthesis of peptide-conjugated AuNPs.

Monomaleimide-functionalized AuNPs (Nanogold®, 1.4 nm in diameter) were dissolved. In the typical coupling reaction of AuNPs with respective biotinylated, cysteine-terminated peptide, the solution of AuNPs was added to equivalent volume of cysteine-terminated peptide dissolved in distilled water. Ten-fold molar excess of peptide to AuNP was used to obtain sufficient binding of peptides to AuNPs.

2.2 Protease assay in solution.

For quenching experiments, SA-QD605 was mixed with the varying amount of either peptide-conjugated AuNPs or Cy5 in a 96-well plate for 1 hr at room temperature to allow the specific association between SA and biotin. The final concentration of QDs in 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 by using a microplate reader (Infinite™ M200, TECAN, Austria). Protease assay was initiated by addition of a 10 μL protease solution to micro wells containing nanoprobes composed of QD-AuNP or QD-Cy5. Each enzyme (MMP-7, caspase, and thrombin) was dissolved in 10 mM HEPES buffer (pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$) at a final concentration of 100 ng mL^{-1}. Following a protease reaction, PL intensity in each well was measured.

2.3 Chip-based assay on a glass slide.

SA-QDs with different colors (QD525, QD605, and QD655) were arrayed directly onto NHS-derivatized hydrogel glass slide (Nexterion) in quadruple spot format by using a robotic arrayer (Microsys, Cartesian Technologies, Irvine, CA, USA) equipped with CMP 3 spotting pins (Telechem International, Sunnyvale, CA, USA). Following a spotting, the slide was incubated in a chamber at 70 % relative humidity and room temperature for 1 h, and then immersed in a solution of 2 % BSA (50 mM borate buffer pH 8.5) for 1 hr to block the remaining NHS groups. The slide was then rinsed with distilled water and subsequently incubated in a solution containing 500 nM peptide-conjugated AuNPs. For protease assay, a multiwell-type chambered silicon cover-slip (ϕ 3mm × H 1mm, Sigma) was overlaid onto a glass slide. A 10 μL of enzyme solution (10 mM HEPES buffer pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$) containing respective protease (100 ng mL^{-1} of MMP-7, caspase-3, or thrombin) was added to each well followed by incubation at 37 °C for 60 min. Inhibitor assay for respective protease was performed by incubation of protease solution in the presence of a specific inhibitor (NHDDPC, Z-VAD-FMK, or AEBSF). After fluorescence scanning, the spot fluorescence was analyzed by using an imaging software (GenePix Pro 4.0, Axon), and mean signal intensities of respective quadruple spots were corrected with a local background.

3. RESULTS AND DISCUSSION

Figure 1 illustrates our assay principle. Biotinylated peptide substrate for the protease is conjugated to monomaleimide-functionalized AuNPs, and the resulting AuNPs (Pep-AuNPs) are associated with streptavidin (SA)-conjugated QDs (SA-QDs) deposited on a glass slide to form nanoprobes of which photoluminescence (PL) is quenched. Addition of protease causes the cleavage of the peptide substrate on the nanoprobes, resulting in detachment of the AuNPs from QDs, and the PL of QDs is recovered. Protease inhibitor, however, prevents a recovery of PL of QDs by blocking the action of protease. Thus, our assay relies on modulations in PL intensities of nanoprobes on a glass surface by the protease activity. In contrast to a solution-based assay, a chip-based format allows a simple and sensitive assay of proteases and their inhibitors in a high throughput way. In addition, it enables more reliable analyses with no aggregation of nanoparticles and much smaller reaction volume (a few nanoliters). When considering our assay principle shown in Figure 1, it is highly desirable for a sensitive protease assay that nanoprobes have a background fluorescence level as low as possible, which is achieved by maximizing the PL quenching of SA-QDs by the Pep-AuNPs.

To check the usability of our system on proteolytic enzymes, we measured the relative PL intensity before and after enzyme reaction (Figure 2a). Matrix metallo-proteinases (MMPs) were employed in the present study, since they have been recognized as one of promising prognostic makers due to their ability to degrade extracellular matrix by up-regulation in malignant tissues. In our case, QD-SA can provide an accessible binding site of the peptides for the larger protease to recognize. When using QD605 with forty AuNPs-peptide acceptors, notable PL change was observed only for specific enzyme reaction (MMP-7), showing ~50% recovery of initial maximum QD PL. MMP-7 enzyme selectively cleaved Ala-Lys bond in the peptide sequence. As a control, non-specific enzyme (MMP-2) showed no significant change during the quenching state. The incomplete rise of the PL emission is mainly attributed to insufficient accessibility of the enzyme due to the steric hindrance in aqueous solution.

To check the applicability of the assay system to other proteases, we tested caspase-3 and thrombin. As shown in Figure 2b, addition of caspase-3 and thrombin to respective nanoprobes also gave rise to a significant recovery in the PL of QDs. TEM images of the nanoprobes before and after addition of MMP-7 also revealed a distinct difference in association of AuNPs with QDs; few AuNPs were observed around QDs due to cleavage of the peptide substrate by MMP-7 (Figure 2c).

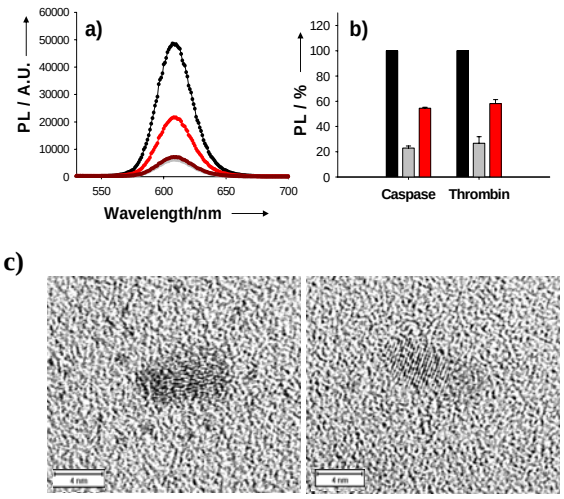

Figure 2. a) Changes in the PL intensity of nanoprobes by MMP-7: QD only (black), QD-Pep-AuNP (gray), additions of MMP-7 (red) and MMP-2 (brown). b) Changes in relative PL of respective nanoprobes by caspase and thrombin: SA-QDs only (black), QD-Pep-AuNP (gray), and additions of caspase and thrombin (red). C) High-resolution TEM images of QD-Pep$_{MMP7}$-AuNP assembly before (left) and after (right) incubation with MMP-7.

For the chip-based assay, an ellipsoidally shaped QDs were immobilized in a quadruple spot format on the amine-reactive surface, and then subsequent quenching and enzyme reaction were carried out with appropriate control set (Figure 3). Similar to the solution-based assay, PL intensity of QD-AuNP showed a significant change by the successive reaction. Significantly, enzyme-induced PL recovery as well as PL quenching by AuNP were successfully achieved on surfaces (Figure 3c). When the specific cleavage of peptide-conjugated AuNPs was additionally confirmed by silver staining (Figure 3), inversely proportional to the QD fluorescent image. This obviously indicates that AuNPs-peptide was cleaved by protease, and then the digested AuNPs-peptide was flushed out by the additional washing step. When compared to the control set (Figure 3b), it was shown that our energy transfer-based assay for protease activity has distinct usability on a glass surface.

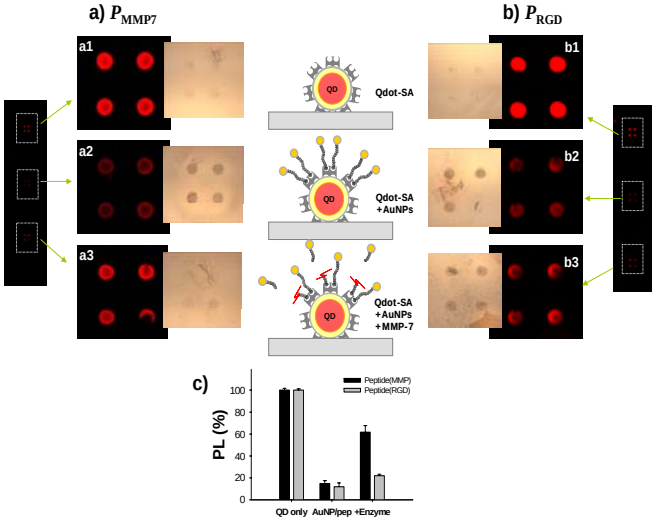

Figure 3. Chip-based energy transfer analysis between QDs and AuNPs for enzyme assay. Subsequent reactions were performed with a) specific (PMMP7) or b) non-specific (PRGD) peptide-conjugated AuNPs after immobilizing QD-SA through microarraying. Fluorescent images from those spots were obtained by using a chip-based scanner, and the corresponding silver-staining on surfaces (bright field images) was shown to confirm the amount of tethered AuNPs. Relative PL changes in graph c) were calculated from the intensities of the quadruple spots.

We further attempted a chip-based assay of various proteases in a multiplexed manner. For this, respective peptide substrates for three kinds of proteases (MMP-7, Caspase-3, and thrombin) were conjugated to AuNPs (*i.e.* Pep$_{MMP7}$-AuNPs, Pep$_{CAPS}$-AuNPs, and Pep$_{THR}$-AuNPs). When three kinds of SA-QDs (SA-QD525, SA-QD605, and SA-QD655) with different colors were separately spotted onto a glass slide, a strong PL intensity was observed from each spot at specified wavelength (Figure 4a). Addition of respective Pep-AuNPs to QDs (*i.e.* Pep$_{MMP7}$-AuNPs for QD-525, Pep$_{CAPS}$-AuNPs for QD605, and Pep$_{THR}$-AuNPs for QD655) resulted in a significant decrease in the PL intensity as shown in Figure 4b. However, when three proteases were subsequently added to the nanoprobes constructed with QDs showing different colors and respective Pep-AuNPs (MMP-7 in Figure 4c, caspase-3 in Figure 4d, and thrombin in Figure 4e), only the spots with corresponding peptide substrate yielded a distinct recovery of PL intensity. Cross-reaction images for proteases against other peptide substrates were found to be negligible, which confirms the specificity of the assay system. With protease-specific inhibitors, no recoveries in the PL of QDs were observed (Figure 4f).

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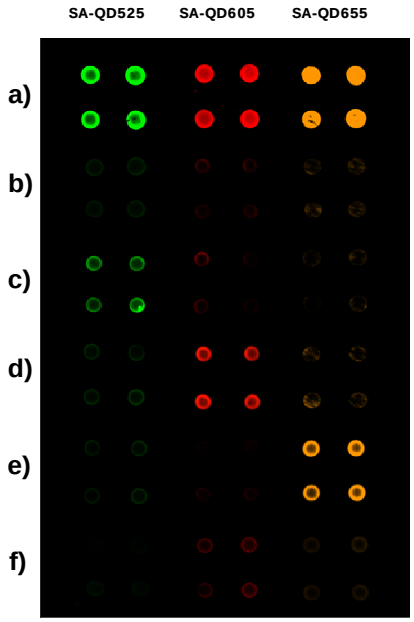

Figure 4. Chip-based multiplexed assay of proteases by using QDs with different colors. SA-QD525, SA-QD605, and SA-QD655 were used. Biotinylated peptide substrates for MMP-7, caspase-3, and thrombin were conjugated to AuNPs, respectively. a) SA-QDs only. b) SA-QDs + respective Pep-AuNPs. c) SA-QDs + Pep-AuNPs + MMP-7. d) SA-QDs + Pep-AuNPs + caspase-3, e) SA-QDs + Pep-AuNPs + thrombin. f) QDs + Pep-AuNPs + mixture of respective protease and its inhibitor.

This result clearly illustrates that the developed system is generally applicable to a chip-based assay of various proteases and their inhibitors in a multiplexed way.

4. CONCLUSIONS

In conclusion, we have demonstrated that the combination of QDs and AuNP with distinct photo-physical properties allows a high ratio of acceptor to donor to achieve an efficient energy transfer. In this regard, chip-based analysis using these nanoparticles can extend the versatility of energy transfer-based assay by overriding the limitations of solution-based assay. First, small sample volume (~nL) facilitates high throughput screening with the development of microarraying technologies, compared to the use of high sample volume in solution. Second, chip-based construction of the nanoparticles allows the use of stringent washing steps to perform subsequent reactions without the need for considering different environments. Once QDs are immobilized on a chip surface, there is no concern for aggregation that can be expected in solution due to the unstable conditions. In contrast, when the conjugates and enzymes (or inhibitors) are mixed in aqueous solution, it is not easy to discern the actual enzymatic signal from various conditions such as pH, ionic strength, and salt concentration etc. Furthermore, mixing with different buffers resulted in significant losses in the optimal photo-physical properties of QDs. Finally, the surface immobilization of protein-coated QDs can serve as capturing molecules, providing a unique configuration for the efficient energy-transfer, which can not be achieved with conventional dyes for chip-based FRET assay. With different functionalities on multiple QD donors, energy transfer-based assay on a solid surface will provide the great potential for multiplexing as well as high-throughput assay with high specificity and sensitivity, and will be expected to be a powerful analytical tool to address many other interactions such as DNA-protein, protein-protein, and protein-carbohydrate.

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