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Activatable FRET Nanosensor for Visualizing MT1-MMP Activity in Single Cancer Cell

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Activatable FRET Nanosensor for Visualizing MT1-MMP Activity in Single Cancer Cell

A Thesis submitted in partial satisfaction of the requirements of the degree Master of Science

in

Bioengineering

by

Eddie Yocon Chung

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Professor Michael J Heller
Professor Liangfang Zhang

2014
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The Thesis of Eddie Yocon Chung is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2014
DEDICATION

This thesis is dedicated to my parents and grandparents, whose unconditional support and love made me get to the place where I am today.
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ABSTRACT OF THE THESIS

Activatable FRET Nanosensor for Visualizing MT1-MMP Activity in Single Cancer Cell

by

Eddie Yocon Chung

Master of Science in Bioengineering

University of California, San Diego, 2014

Professor Yingxiao Wang, Chair

Membrane type 1 metalloproteinase (MT1-MMP) is an important marker for tumor malignancy since it can directly degrade extracellular matrix and promote cancer cell metastasis. Detecting MT1-MMP activity of cancer cells is an important index to monitor tumor malignancy in cancer diagnosis. However, there is lack of a simple method which can visualize MT1-MMP activity of a cancer cell with high resolution. Here, a MT1-MMP activatable quantum-dot (QD) nanosensor was
engineered to visualize the MT1-MMP activity at the single cell level. The QD-based Förster resonance energy transfer (FRET) nanosensor was cleaved by pericellular MT1-MMP of cancer cells, which resulted in a marked change of donor (QD)/acceptor (Cy3) emission ratio and activated cell penetration sequences allowing uptake by cancer cells. By quantifying cellular FRET images and intracellular accumulation of nanosensor, newly developed nanosensor can differentiate various levels of MT1-MMP activity in cancer cells. Thus our enzyme activatable peptide/QD-based FRET nanosensor can visualize the enzyme activity of a cancer cell, providing a simple method for monitoring tumor malignancy.
INTRODUCTION

Matrix Metalloproteinases (MMPs) are zinc dependent proteases that play important roles in regulating several physiological processes\(^1\). In cancer cells, MMPs are also involved in facilitating cancer metastasis such as angiogenesis\(^2\), extracellular matrix (ECM) degradation, and cancer cell invasion\(^3,4\). With MMPs, cancer cells can invade surrounding tissues, migrate into blood stream and disseminate to secondary organs\(^5,6\). Several MMP inhibitors has been utilized as anticancer agents for therapeutic intervention in cancer\(^7,8,9\). However, most of MMP inhibitors failed to achieve progressive-free and overall survival in phase III clinical trials. Therefore, it is essential to investigate each individual MMP with specific tumor type and stage, as well as the effectiveness of different combination of MMP inhibitors with other anti-cancer drugs\(^8\).

Human MMP family consists of 23 members, with 16 secreted and 7 membrane-anchored proteinases\(^1\). Among all human MMPs, membrane type-1 MMP (MT1-MMP, MMP-14) is a crucial metalloproteinase that has been reported as an important marker for tumor malignancy in multiple tumor types, and high levels of MT1-MMP directly correlate with enhanced cell migration. With its C-terminal transmembrane domain and a cytoplasmic tail, MT1-MMP has the ability to concentrate at the leading edge of several cancer cell types and facilitate remodeling of ECM \(^10-13\). Overexpression of MT1-MMP conferred tumorigenicity to nonmalignant epithelial cells and also activate pro-MMP-2 to further promote cancer cell invasion\(^12,14,15\). Therefore it is compulsory to develop a sensitive and selective MT1-MMP biosensor for cancer research and therapeutic diagnosis\(^16\).
In our previous publications, we have developed several FRET biosensors that can specifically monitor MT1-MMP activities on cell membrane\textsuperscript{17-19}. However, these biosensors are genetically encoded, which usually takes several days of effort prior taking images for quantification.

Quantum dots are inorganic fluorescent semiconductor nanocrystals. It has broad range of absorption and sharp, size-tunable symmetric emission spectra\textsuperscript{20, 21}. Compare to conventional organic dye, QD has an order of magnitude extinction coefficient and quantum yield, which make it brighter than most of dyes\textsuperscript{21, 22}. With its special core-shell inorganic composition, QD also has high photostability compare to conventional dyes\textsuperscript{23, 24}. It has been widely used in designs for life cell, \textit{in vivo} imaging\textsuperscript{25-27}, DNA hybridization\textsuperscript{28, 29}, and drug delivery\textsuperscript{30, 31}.

For live cell imaging and diagnosis, QD had shown its wide range of capacity with different functional modification which makes it a perfect tool for specific targeting without losing its brightness\textsuperscript{32, 33}. With peptide modified surface, QD can penetrate into live cell for imaging by either non-specific endocytosis or interaction with cell integrin\textsuperscript{34-36}.

Since quantum dot can serve as an ideal FRET donor\textsuperscript{37} for visualizing MT1-MMP activity, we conjugate QD with functionalized peptide contains MT1-MMP specific cleavage cite\textsuperscript{18} and activatable cell-penetrating peptide\textsuperscript{38, 39} to form a QD based FRET nanosensor to visualize MT1-MMP activity.
RESULTS AND DISCUSSION

Engineering design of the QD-based MT1-MMP nanosensor

The QD-Cy3-MT1-MMP nanosensor contains a QD as a FRET donor with high metal-affinity ZnS surface modification, allowing histidine-zinc sulfide self-assembly\textsuperscript{39} with multiple peptide-Cy3 modules (FRET acceptor) targeting MT1-MMP activity (Fig. 1). Each single peptide-Cy3 module consists of quantum dots binding site (6x histidine), cell-penetrating peptide\textsuperscript{38} (9x arginine) and its blocking peptide (8x glutamate), 3x RGD sequences for cell-targeting, the MT1-MMP cleavable sequence AHLR substrate\textsuperscript{18}, and a Cy3 dye (Fig. 1b). By this design, the electrostatic interaction between the positively charged arginine and negatively charged glutamate bends the peptide-Cy3 module in a hairpin-like shape, allowing FRET between QD and Cy3 when the peptide-Cy3 module is attached to the QD surface\textsuperscript{38, 40} (Fig. 1b). \textit{In vitro}, substrate sequence in the nanosensor can be cleaved by active MT1-MMP catalytic domain (MT1-CAT)\textsuperscript{12, 41}, which separates Cy3 from QD and disrupts FRET (Fig. 1b). The decrease of energy transfer between QD and Cy3 causes the QD intensity to increase and the FRET intensity (the Cy3 intensity with QD excitation) to decrease. As a result, the value of QD/FRET intensity ratio increases, which can be used to represent the level of MT1-MMP proteolytic activity (Fig. 1b).
Figure 1. Schematic illustration of the designs of the QD-peptide-Cy3 nanosensor and its activation mechanism. (a) The biosensor contains a QD coupled to multiple peptide-Cy3 to allow FRET between QD and Cy3. Panel (b) shows that the active catalytic domain of MT1-MMP (MT1-CAT) cleaves the biosensor in vitro and disrupts FRET. (c) The biosensors can be recruited to the surface of non-malignant cells, but they cannot be cleaved or efficiently penetrate cell surface. (d) When the sensor is recruited to cancer cells with active MT1-MMP, it is cleaved at the substrate (AHLR), energy transfer decreases and the emission of QD increases. Cell penetration is activated by the fully positive charged peptide sequence.
After incubation with cells expressing integrin surface receptors, the QD-Cy3 nanosensors can be recruited to the extracellular surface by the binding of RGD sequences with integrin\(^ {42}\) (Fig. 1c and 1d). At the surface if cancer cells with high MT1-MMP activity, the nanosensor will be cleaved at the specific substrate sequence, AHLR, by active MT1-MMP. The QD nanosensors are initially not charged. After cleavage, the negatively charged Cy3 component diffuses away from the extracellular membrane, and renders the QD component positively charged. Therefore, the positively charged cell-penetrating sequence of arginine is activated and allows the QD components to penetrate into the cell (Fig. 1c)\(^ {38}\). As a result, the cells with high MT1-MMP activity are expected to be observed with more intracellular QD components with high QD intensity and high QD/FRET ratio (Fig. 1d) while cells with low MT1-MMP activity will have mostly intact nanosensors at a much less amount with lower QD/FRET ratio within the cell body (Fig. 1c).

The absorption of Cy3 significantly overlap with the emission, but not the absorption, of 525 nm emitting QD (Fig. 2a). Therefore, when QD is excited, the energy can be directly transferred to an adjacent Cy3, resulting in Cy3 emission, named FRET emission. In addition, the emission peaks of QD and Cy3 are separated by 45 nm. Therefore, QD (donor) and Cy3 (acceptor) are expected to be an ideal FRET pair (Fig. 2a). Indeed, our results show that after self-assembly of QD with Cy3-peptide, with the excitation of QD, the QD emission peak dropped significantly and Cy3 emission peak rose at 570 nm (Fig. 2b). This result indicated that the energy had been transferred from QD to proximal Cy3. We also noticed that QD emission peak had slightly red-shifted to 528 nm, therefore in this study we choose 528 nm as
QD emission. The binding was further confirmed by adding imidazole as binding competitor to pull out histidine containing Cy3-peptide construct from QD, which shows immediate recovery of QD emission peak (Fig. 3).
Figure 2. The spectra of QD, Cy3 and the biosensor. (a) Normalized absorption and emission spectrums of QD-donor and Cy3-acceptor. (b) The spectrums of QD and 2 hrs after Cy3-labeled peptide assembling (1:31). The sensor was excited at 410±5 nm.

Figure 3. Spectra of FRET nanosensor with (blue line) and without (red line) addition of binding competitor imidazole (100 mM). The spectra were obtained after incubation for 3 min.
**In vitro cleavage shows fast signal response and correlates with MT1-MMP activity**

To assess the sensitivity of the QD-Cy3 nanosensor to MT1-MMP cleavage, the nanosensors were cleaved in vitro using the active MT1-CAT. As shown in Figure 3, when purified FRET nanosensors (32 nM) were co-incubated 1:4 with MT1-CAT stock solution (0.7μM) at 37 °C, a dramatic increase of QD emission peak was observed with a significant drop of Cy3 emission peak around 570 nm within 3 minutes (Fig. 4a). This trend continued up to 120 min after incubation, causing a sustained increase of QD/FRET intensity ratio (Fig. 4b). This results indicates that the QD-Cy3 biosensor can be efficiently cleaved by MT1-CAT at the substrate, allowing peptide-Cy3 to diffuse away from QD, causing the decrease of energy transfer and an increase of QD/FRET ratio (Fig. 1). As shown in Figures 4a and 4b, the increase of QD/FRET ratio was mainly due to a strong recovery of QD emission after cleavage. The decrease of FRET intensity was less than expected, possibly due to the bleedthrough from the increased QD emission intensity. These results indicate that the QD-Cy3 FRET biosensor can be efficiently and robustly cleaved by catalytically active MT1-CAT in vitro.

In vitro FRET nanosensor response to different MT1-CAT concentration was also investigated. Figure 4c shows the spectrums of the QD/Cy3 nanosensors after they had been incubated with different concentrations of MT1-CAT for 15 min. The resulting QD/FRET ratio is proportional to the concentration of MT1-CAT, thus allowing a quantitative assay. The overall time courses of QD / FRET ratio with different MT1-CAT concentration confirmed the robustness of the assay (figure 4d). The results indicate that our FRET nanosensor has the capability to detect different
MT1-CAT concentration within 15 min. Therefore our FRET nanosensor can be applied to determine different levels of MT1-MMP with a large signal response in a short period of time.
Figure 4. QD-peptide-Cy3 cleavage assay with MT1-CAT. (a) QD signal recovered dramatically after treatment of 140 nM MT1-CAT. (b) The plot of donor (QD) / acceptor (FRET) intensity ratio (n=3, error bar: SEM). (c) Representative spectrum of QD-peptide-Cy3 cleavage (15 min, 37°C) with different concentration of MT1-CAT and ratio of QD/FRET intensity (inner plot). (d) The time courses of QD/FRET ratio with different concentration of MT1-CAT. The excitation of the spectrometer 410±5 nm and emission collection 528±5 nm (QD), 570±5 nm (FRET) were chosen for MT1-MMP cleavage essay.
The FRET nanosensor can be activated by cancer cells with high MT1-MMP activity

To further examine the sensitivity and specificity of the nanosensor in detecting MT1-MMP activity in live cells, the FRET nanosensor was incubated with invasive human breast cancer cell line, MDA MB – 231 (MDA). Broad spectrum MMP inhibitor GM6001 (GM), which can form bidentate complex with zinc at active site, has been applied to MDA cells as a negative control to inhibit MT1-MMP activity. The images of MDA cells cultured on cover glass were quantified after 3 hrs of co-incubation with FRET nanosensor. After removal of excess nanosensors, the MDA cells without GM showed high QD/FRET ratio and high intracellular QD intensity, while the cells with GM showed low QD/FRET ratio and low intracellular QD intensity (Fig. 5a). The cells were randomly picked and have similar average cell area. All cells were focused at the bottom of the cell to capture real nanosensors signals inside cell body. These results indicate that MDA cells can cleave the QD-Cy3 biosensor and facilitate the penetration QD clusters inside the cell. It is possible that the cleaved QD
Figure 5. Quantification of MDA cell MT1-MMP enzyme activity. (a) The representative images of cells with (left) and without GM (right) pre-treatment. The QD/FRET ratio images were overlaid with cell edge (white line, top panels), the QD intensity images were overlaid with detected QD clusters (red lines, middle panels), with the DIC images at the bottom row. (b) The pixelwise histogram of QD/FRET ratio (top) and QD intensity (bottom) within the detected QD clusters of the cell. (c) Comparison of the QD/FRET ratio, QD intensity, and cell sizes between the cells with (n=20) and without GM pre-treatment (n=22). * indicates statistically significant difference, p < 0.002. Error bar: SEM, scale bar: 10 um.
components of the nanosensor formed clusters at the cell surface as mediated by RGD-integrin interaction. Then the QDs entered the cell membrane by endocytosis and take over most of the cell body.

Next, we examined the cell penetrating behavior of the MT1-MMP activated nanosensor by analyzing the histogram of pixelwise QD intensity and QD / FRET ratio (Fig. 5b). The histogram revealed that in non-inhibited MDA cells, the main population of FRET nanosensor, both QD / FRET ratio and QD intensity, have shifted to higher value, while GM inhibited MDA cells have significantly lower values and total counts. A second population of pixels with higher QD/FRET ratio and QD intensity can be clearly identified (Fig. 5b). This result suggests that large amount of activated nanosensor can be uptake by non-inhibited MDA cell, and this phenomenon is universal, exists in 82 % of the cell samples. Although the histogram of non-inhibited MDA cells has overlapped with GM inhibited MDA cells, we believe that such phenomenon is the contribution of RGD sequences of the linker peptide, which allowed non-activated nanosensor to be uptake by the cell as well. These results suggest our FRET nanosensor can serve as a powerful tool for profiling cellular MT1-MMP.

Since most of the nanosensors formed clusters after cellular uptake through self-aggregation and endocytosis, we quantified QD / FRET ratio by accumulation of all ratio values within the selected region based on intensity threshold, then divided by the area of the region to get the average QD / FRET ratio of each single cell. Since largely spread cells usually have stronger cell activities and surface areas. The normalized intensity was obtained by averaging the QD emissions
intensity above the threshold and normalizing by the cell size (Fig. 5c). The bar graph in Figure 4c shows significant differences between MDA cells with and without GM in both QD / FRET ratio and normalized QD intensity. We also confirmed our hypothesis by monitoring the ratio of FRET nanosensor on the cell surface with real-time GM wash out. A massive QD / FRET ratio change of nanosensor was observed within 5 minutes after GM inhibitor had been washed away, indicating fast activation of the nanosensor exerted by MT1-MMP. This phenomenon couldn’t happen when nanosensor was uptake by the cell (Fig. 6).
Figure 6. Visualize MT1-MMP activity in breast cancer cell line with GM wash out assay. (A) QD / FRET ratio change from $t=0$ min (middle) to $t=5$ min (right) after GM wash out. (B) QD / FRET ratio change after GM wash out with MDA cells co-incubated with nanosensor for $>6$ hours. Scale bar: 10 um
The dual-index read-out of QD-Cy3 nanosensor can be applied to classify different cell lines according to MT1-MMP activity and nanosensor penetrability.

Because our FRET nanosensor has two indexes, QD/FRET ratio and QD intensity, to determine MT1-MMP activity, we further applied our nanosensor to HeLa and HT1080 cell lines to evaluate its potential to distinguish different cell lines (Fig. 7).

The result shows that MDA-MB-231 and HT1080 cells have significantly higher QD/FRET ratio than the HeLa cells, suggesting the nanosensors can be cleaved by active MT1-MMP (Fig. 7c). This result is also consistent with previous reports that MDA and HT1080 have high MT1-MMP expression while HeLa cells have low MT1-MMP expression\(^46,47\). MDA and HT1080 cells are not distinguishable with only ratio index since both MDA and HT1080 have high activity of MT1-MMP. Therefore we also applied the second index, normalized intensity (Fig. 7b). The normalized intensity showed significant difference between MDA cells and other two cell lines. Here, MDA and HT1080 cells become distinguishable, we believe that this is due to different cell endocytosis behaviors among different cell lines. For example, after autocatalytic processing HT1080 had delayed endocytosis of active MT1-MMP and enhanced activation of pro-MMP-2 while MDA cells had no effect on activation of pro-MMP-2\(^48\). Therefore, by combining both QD / FRET ratio and normalized intensity, we have the capability to further distinguish different cell lines with similar MT1-MMP activity by cell behaviors on endocytosis (Fig. 7c).
Figure 7. Detection of MT1-MMP activity in different cancer cell lines. (a) Representative QD/FRET ratio images of MDA-MB231 (left), HeLa (middle), and HT1080 (right) cell lines. (b) Comparison of the QD/FRET ratio and QD intensity between MDA (n=22), HeLa (n=23), and HT1080 (n=23). ** indicates statistically significant difference from the other two groups, $p < 0.001$. Error bar: SEM. (c) 2D plots with MDA (blue), HeLa (red), and HT1080 (green) cell lines. The shaded regions indicate sufficient separation of the cell lines by classification with QD intensity and FRET ratio. Scale bar: 10 µm
CONCLUSION

In this study, we have demonstrated that our FRET nanosensor can serve as an ideal diagnosis tool to visualize MT1-MMP activity with high resolution and easy approach. With QD as the donor of FRET nanosensor, the resolution of MT1-MMP activity has been amplified. Furthermore, with MT1-MMP activatable construct, we can establish another intensity index to further confirm the activity of MT1-MMP by different level of cell penetration. These features confer our FRET nanosensor the capability to serve as a tool for quick cancer drug screening. The two indexes of nanosensor also give us another easy approach to distinguish different cell lines.

This is just the proof-of-concept. The future work of this FRET nanosensor will be focused on more stable QD conjugation and more sensitive linker peptide. The combination of FRET nanosensor and microchip\textsuperscript{49, 50} is also applicable to achieve lab on the chip with minimum patient sample and reagent. This will light up the new era of personalized cancer therapy.
EXPERIMENTAL DETAILS

Peptide construction and dye conjugation

The FRET nanosensor linker peptide was constructed by fusing several functional motifs in a designed order. The quantum dots binding site (6x histidine) was coded by pRsetB vector at the N-terminus of the linker peptide; The cell-penetrating peptide (9x arginine) and its blocking peptide (8x glutamate) were derived from reported cell-penetrating peptides\textsuperscript{38}; Cell-targeting peptide (triple RGD motifs) was derived from α5β1 integrin binding motif of fibronectin; The MT1-MMP cleavable peptide CRPAHLRDSG was reported in pervious publication\textsuperscript{17} with a C-terminal cysteine for Cy3 labeling. These motifs were flanked by GGSGGT linker peptides. The oligonucleotide coding were synthesized and inserted into pRsetB vector using BamH I/Bgl II restriction site with a stop codon. The construct was confirmed by sequencing.

The synthesized plasmid was further expressed for linker peptide with BL21 Competent E. coli, and then purified by nickel chelation chromatography extraction described as our previous publication\textsuperscript{51}. For Cy3 conjugation, the purified protein was dissolved at 1 mg/ml in degassed PBS buffer and incubated at room temperature for 30 minutes. 100 molar excess of Tris-(2-Carboxethyl) Phosphine (TCEP) was added and incubated with nitrogen gas at room temperature for 10 minutes. Mixed 50 μl of anhydrous Dimethylformamide to one pack of Cy3 (GE Healthcare) and added to TCEP reduced protein. The final solution was further incubated with Nitrogen at room temperature for two hours with additional mixing every 30 minutes, then left the reaction overnight (~10 h)
at 2-8°C. The conjugated peptide was separated using Ni-NTA beads followed the common protein purification procedures. The final concentration of conjugated peptide-Cy3 construct was determined by an UV-visible spectrophotoreader (Fisher Scientific) with absorbance on 550 nm and Cy3 extinction coefficient 150000M⁻¹cm⁻¹.

**QD Nanosensor Assembling**

The Quantum Dot-peptide-Cy3 nanosensor was fabricated by incubating 32nM of 525 nm emitting ITK carboxyl quantum dots (Invitrogen) with 31-fold (1μM) excess of previously purified Cy3 labeled linker peptide (Concentration of Cy3-peptide is determined in Supplementary Fig. S3) for 2 hrs at 4 ℃. Dilute the mixture 10 times with proteolysis assay buffer (50 mM HEPES, 10 mM CaCl2, 0.5 mM MgCl2, 50 μM ZnCl2, and 0.01% Brij-35, pH 6.8), remove the excess dye-labeled peptide with a centrifugal 50 kDa MW cut-off filter (Millipore) at 4000 rpm for 5 min, then recover to the original concentration with proteolysis assay buffer.

**MT1-CAT enzyme cleavage essays**

The MT1-MMP catalytic domain (MT1-CAT) was expressed and purified as described¹¹,⁵² and stored at -80 ℃ prior to use. The cleavage essays were carried out in 96-well polystyrene half area transparent plate (Greiner). MT1 Cat. with different concentration was added 1:4 into the freshly made nanosensor in proteolysis assay buffer. The reactions were carried out for 2 h at 37 ℃ in a fluorescence plate reader (TECAN Infinite M1000 Pro) with intensity gain 90, bandwidth 10 nm.
Intracellular Imaging

All cells were cultured following ATCC instructions. The glass bottom dishes for cell imaging were coated with 20 μM fibronectin solution at 37 °C for 5 h before cells were transferred. Cells were transferred on the coated class bottom dish a night before experiment with culture media contains 1 % BSA. The freshly made FRET nanosensor solution was mixed 1:1 with DMEM culture medium (with 1 % BSA) and substitute the original medium in the glass bottom dish. The dishes were further incubated at 37 °C incubator for 3 hrs with additional mixing every 30 min to avoid sensor aggregation. After incubation, cells were fixed by covering 4% cold paraformaldehyde solution for 10 min. Dishes were flushed and covered with cold PBS to remove QD nanosensors outside cells before imaging.

Quantitative cell images were obtained by Nikon Eclipse Ti inverted microscope with a cooled charge-coupled device camera using MetaFluor 6.2 software (Universal imaging). The emission ratio and intensity values were quantified by Matlab (The Math Works) with our Fluocell package and Excel (Microsoft). The cell region was drawn manually and then set QD intensity threshold 500 to pick QD nanosensor inside cells. The statistical outlier was chosen based on average QD intensity ± 3 times STD to remove aberrant cells.
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