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Rosenzweig, Zeev, Shi, Lifang, Rosenzweig, Nitsa

Zeev Rosenzweig, Lifang Shi, Nitsa Rosenzweig, "Luminescent quantum dots for cellular analysis," Proc. SPIE 6370, Nanomaterial Synthesis and Integration for Sensors, Electronics, Photonics, and Electro-Optics, 637003 (19 October 2006); doi: 10.1117/12.690646

SPIE.

Event: Optics East 2006, 2006, Boston, Massachusetts, United States

Luminescent quantum dots for cellular analysis

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ABSTRACT

The paper describes the fabrication, characterization and applications of novel luminescent quantum dots fluorescence resonance energy transfer (FRET) based enzymatic activity probes. The luminescent probes are based on FRET between luminescent quantum dots that serve as donors and rhodamine acceptors that are immobilized to the surface of the quantum dots through peptide linkers that contain selective enzymatic cleavage sites. Upon enzymatic cleavage of the peptide linkers the rhodamine molecules no longer provide an efficient energy transfer channel to the quantum dots, which lightes up the initially quenched the quantum dots. The quantum dots based probes were applied for detecting enzyme activity and screening enzyme inhibitors. They were also used for the measurement of extracellular matrix metalloproteinases (MMPs) activity in normal and cancerous breast cells tissues.

Keywords-Quantum dots, FRET, enzyme inhibitor, enzyme sensor, cell analysis

1. INTRODUCTION

Fluorescence resonance energy transfer (FRET), a non-radiative energy transfer from the excited state of a donor (D) to an acceptor (A), is the result of long-range dipole-dipole interactions between the donor and acceptor (1, 2). FRET occurs when there is an overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor. FRET based sensing assemblies have been used successfully in biological applications including studying protein-protein interactions (binding affinity), protein conformational changes, and detecting nucleic acid and peptides (3-7). Organic fluorophores have been used as FRET donor/acceptor pairs. However, there are some problems of using organic fluorophores for FRET in biological systems including fast photobleaching and poor chemical stability. Additionally, the broad absorption and emission of organic fluorophores limits the choice of donor/acceptor pairs.

Luminescent quantum dots provide a potential solution to problems associated with use of organic fluorophores due to their high photostability, high emission quantum yield, narrow and symmetric emission peaks and size-dependent wavelength tunability (8). Quantum dots FRET based biosensors were recently used for detecting maltose (9), TNT (10), toxins (11) and β -lactamase (12). Recently, West and coworkers developed quantum dots based FRET protease probes by conjugating gold nanoparticles to CdSe/ZnS quantum dots through peptide linkers (13). The response time of these probes was long (48 hours) possibly due to aggregation of the quantum dots-gold nanocrystals clusters or strong interactions between the quantum dots and gold nanoparticles that did not involve their binding through the peptide linkers. This paper describes quantum dots FRET based probes with the capability to monitor enzymatic activity in real time. In these probes rhodamine molecules are attached to the surface of the quantum dots and used as molecular acceptors. Quantum dots-molecular acceptor systems are advantageous because of the simultaneous emission increase and decrease of the emission of quantum dots and molecular acceptors, which enables a ratiometric analysis method and results in high quantitative power.

To fabricate the quantum dots FRET based probes CdSe/ZnS quantum dots capped with tri-octyl phosphine oxide (TOPO) molecule were modified by exchanging the TOPO ligands with tetra peptide RGDC (Arginine-Glycine-Aspartic acid-Cysteine) molecules to form water-soluble quantum dots. The peptide molecules were bound to the ZnS shell of the CdSe/ZnS quantum dots through the thiol group of cysteine (C). The peptide-coated quantum dots were labeled with Rhodamine RedTM-X succinimidyl ester through the formation of amide bonds with the amino terminals of the coating peptides to form the quantum dots based FRET probes. In these FRET probes the quantum dots served as donors and the attached rhodamine molecules as acceptors. The emission of the quantum dots was quenched and the emission of the attached rhodamine molecules increased due to FRET between the quantum dots and the rhodamine molecules. Upon cleavage by enzymes (trypsin, collagenase, extra-cellular metallo-proteinases (MMPs)), the rhodamine molecules were removed from the surface of the quantum dots, which resulted in an increase in the emission peak of the quantum dots and a corresponding decrease in the emission of the displaced rhodamine molecules due to a decrease in FRET efficiency between the quantum dots and rhodamine molecules.

2. SYNTHESIS AND CHARACTERIZATION OF QUANTUM DOTS BASED FRET ENZYMATIC ACTIVITY PROBES

2.1 Synthesis of quantum dots based FRET enzymatic activity probes

TOPO capped CdSe/ZnS quantum dots were prepared following a method developed by Peng with slight modifications (14, 15). The ligand exchange reaction used to replace the TOPO ligands with RGDC was carried out in a mixture of pyridine and dimethyl sulfoxide following a method first reported by Pinaud and coworkers (16). 1mL of 1 μ M TOPO coated CdSe/ZnS quantum dots were precipitated with methanol and re-dissolved in 2ml 9:1(V/V) pyridine:DMSO cosolvent. The use of this co-solvent effectively prevented aggregation of quantum dots during the reaction. Then, 200 μ l 5mg/mL peptide in DMSO was added to the reaction mixture. The pH was adjusted to 10 by adding tetra-methyl ammonium hydroxide (TAMOH) 20% (w/v) in methanol) to the reaction mixture. The TAMOH molecules were used to form anionic cysteine thiolates to facilitate binding of the peptide to the CdSe/ZnS quantum dots through the cysteine residues. The peptide coated quantum dots were vortexed for 30 minutes, precipitated by slow speed centrifugation (2000 rpm for 10 minutes) and re-suspended in DMSO. Then the quantum dots were precipitated by slow speed centrifugation and resuspended in Dulbecco's PBS buffer solution at pH 7.4. Unbound peptide molecules were removed by two repeated cycles of spin dialysis using an amicon centricon spin dialysis tube with a cutoff molecular weight of 30kDa (Microcon YM30, Millipore Corp.). In each spin dialysis cycle the sample was centrifuged at 2000 rpm for 20 minutes and washed with the Dulbecco's PBS buffer solution at pH 7.4. Peptide coated quantum dots were labeled with rhodamine to form the quantum dots based FRET enzyme activity probes. 150 μ L of 0.1 μ M peptide coated quantum dots were mixed with varying volumes (0 μ L to 150 μ L) of 4.8 μ M Rhodamine RedTM-X, succinimidyl ester in a PBS buffer solution of pH 7.4 to a final volume of 1.5 mL. The reaction mixture was incubated for 1 hour at room temperature.

2.2 Characterization of quantum dots based FRET enzymatic activity probes

Rhodamine molecules were conjugated to the peptide coated quantum dots. The effect of increasing concentration of rhodamine in the reaction mixture on the fluorescence resonance energy transfer (FRET) efficiency of the rhodamine labeled quantum dots is shown in figure 1a. When excited at 445 nm the emission spectra of the rhodamine labeled quantum dots show two clearly separated emission peaks of the quantum dots and the rhodamine molecules at 545 nm and 590 nm respectively. The emission peak of the quantum dots decreased with increasing rhodamine concentration indicating the occurrence of FRET between the quantum dots and the rhodamine molecules. The trends are consistent as a progressive increase in rhodamine emission follows the expected energy gain. Figure 1b depicts the emission intensity of quantum dots decay percentage as a function of the rhodamine/quantum dots ratio. Here, F_d is the emission intensity of unlabeled quantum dots and F_d is the emission intensity of rhodamine-labeled-quantum dots. The FRET efficiency increased with

increasing rhodamine concentration. Based on these results, we utilized rhodamine-labeled peptide-coated quantum dots that were prepared in solution containing a rhodamine:quantum dots at a ratio of 48:1 as our FRET-based enzymatic activity probes. Digital fluorescence microscopy images were used to provide another visual evidence of FRET between quantum dots and rhodamine (Figure 2). The emission color of peptide coated quantum dots is green (figure 2a). The emission color turns yellow-orange when rhodamine is bound to the peptide coated quantum dots (figure 2b). This emission color indicates the occurrence of FRET between quantum dots and the bound rhodamine molecule.

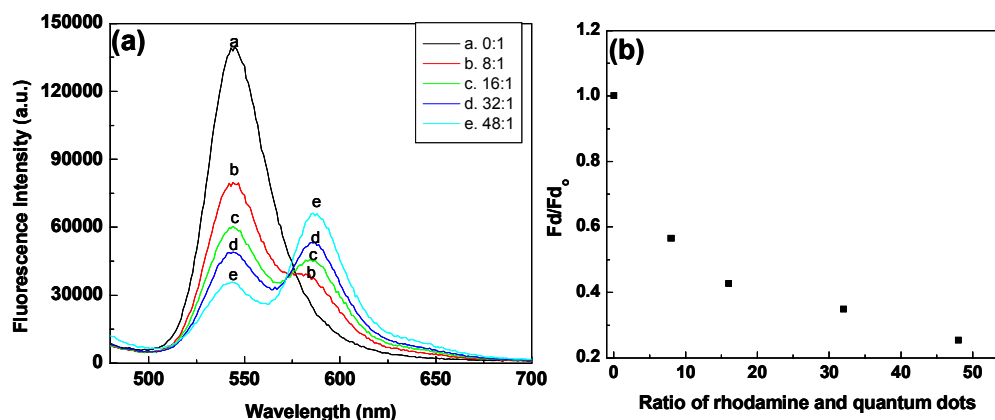


Figure 1 - a) Emission spectra of rhodamine-labeled peptide-coated quantum dots at increasing the rhodamine to peptide coated quantum dots ratio: (a)0:1 (black), (b) 8:1 (red), (c)16:1 (green), (d)32:1 (dark blue), e)50:1 (light blue); b) The emission intensity of peptide coated quantum dots decay percentage versus the ratio of rhodamine and quantum dots.

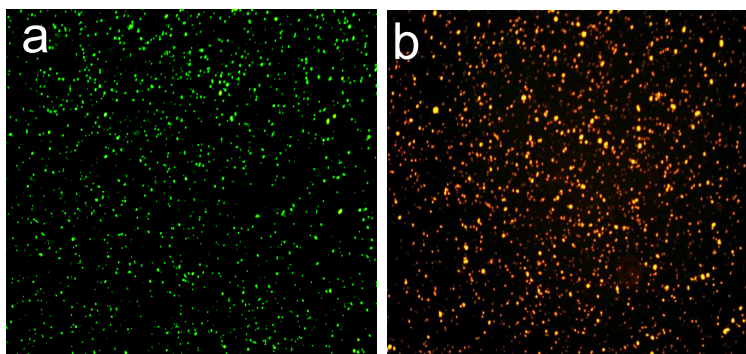


Figure 2 - Digital fluorescence images of (a) peptide coated quantum dots showing green emission, (b) rhodamine-labeled peptide coated quantum dots showing yellow-orange emission due to FRET between the quantum dots and rhodamine.

3. APPLICATIONS OF QUANTUM DOTS BASED FRET ENZYMATICAL ACTIVITY PROBES

3.1 Enzyme activity measurements

The quantum dots FRET-based enzymatic activity probes were first used to determine the activity of the enzyme trypsin. Trypsin is a proteolytic enzyme with a molecular weight of 23,800 Daltons that cleaves proteins and peptides at the carboxyl end of lysine (K) and arginine (R). We anticipated that trypsin would cleave RGDC peptide to release rhodamine molecules to the solution, which would in turn affect the FRET signal of the quantum dots. In the RGDC particular peptide used in this study the cleavage site was between Glycine (G) and Arginine (R). The emission spectra, showing the effect of trypsin on the FRET signal of the rhodamine-labeled peptide-coated quantum dots, are shown in figure 3a ($\lambda_{\text{exc}} = 445\text{nm}$). The spectra were recorded 15 minutes after adding trypsin to the quantum dots solution. An increase in the quantum dots emission peak at 545 nm and a

decrease in the rhodamine emission peak at 590 nm are clearly seen indicating a significant decrease in the FRET efficiency. This is attributed to the enzymatic cleavage of the peptide molecules which leads to the expected release of rhodamine molecules from the surface of quantum dots. Figure 3b describes the trypsin concentration dependence of the ratio F_d/F_a . Here, F_d and F_a were the peak emission intensities of the quantum dot FRET-based probes when excited at 445 nm at 545 nm (quantum dot donors) and 590 nm (rhodamine acceptors) respectively. Figure 3c illustrates the quantum dots quenching by rhodamine and restore with peptide cleavage by 500 $\mu\text{g/mL}$ trypsin in 15 minutes.

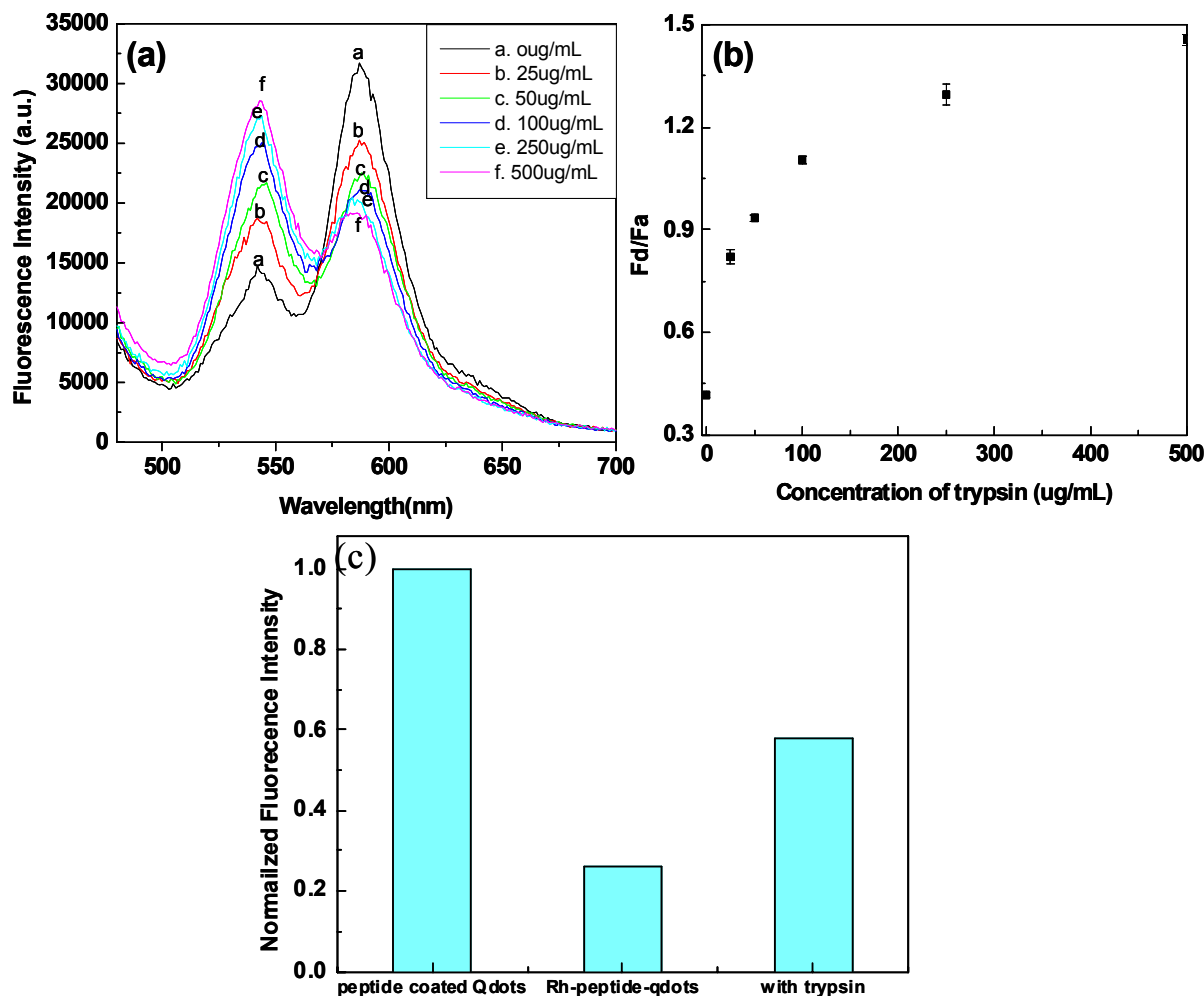


Figure 3 - a) Emission spectra of the quantum dot FRET-based probes at increasing trypsin concentration: (a) 0 $\mu\text{g/mL}$, (b) 25 $\mu\text{g/mL}$, (c) 50 $\mu\text{g/mL}$, (d) 100 $\mu\text{g/mL}$, (e) 250 $\mu\text{g/mL}$, (f) 500 $\mu\text{g/mL}$. ($\lambda_{\text{ex}} = 445 \text{ nm}$). b) Trypsin concentration dependence of F_d/F_a , 15 minutes following the addition of trypsin to solutions of quantum dot based probes c) This chart demonstrates the reduction in quantum dots fluorescence intensity after labeling with rhodamine and then restore after peptide cleavage by 500 $\mu\text{g/mL}$ trypsin in 15 minutes, $\lambda_{\text{ex}} = 445 \text{ nm}$.

3.2 Inhibition assays

The quantum dots FRET based probes were used to determine the inhibition effect of there organic trypsin inhibitors, 4-(2-Aminoethyl) benzene-sulfonyl fluoride hydrochloride, 4-Amidinophenylmethane-sulfonyl fluoride hydrochloride and 1,10-phenanthroline. Trypsin was incubated with different inhibitor concentration prior to adding quantum dots based probes and monitored with digital fluorescence imaging microscopy. Figure 4 show the temporal dependence of the ratio F_d/F_a at different concentration of 4-(2-Aminoethyl) benzene-sulfonyl fluoride hydrochloride. With concentration of 2500 $\mu\text{g/mL}$, 4-(2-Aminoethyl) benzene-sulfonyl fluoride

hydrochloride can 74% inhibit activity of trypsin after 15minutes. The inhibition assay is concentration and time dependent.

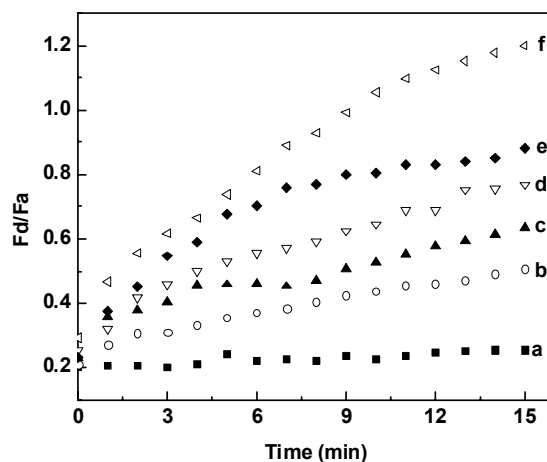


Figure 4 - Temporal dependence of the inhibition effect of -(2-Aminoethyl) benzene-sulfonyl fluoride hydrochloride to trypsin in the quantum dots based probes: a) without enzyme, b) 2500 μ g/mL, c) 1250 μ g/mL, d) 250 μ g/mL, e) 50 μ g/mL, f) 0 μ g/mL.

Figure 5 show the inhibition ability of trypsin inhibitors. The inhibition ability was calculated using equation [1].

$$\text{Inhibition} = ((\text{Fd/Fa})_o - (\text{Fd/Fa})_i) / ((\text{Fd/Fa})_o - (\text{Fd/Fa})_b) \quad [1]$$

$(\text{Fd/Fa})_0$ is the ratio of emission intensity of quantum dots and rhodamine with trypsin (without inhibitor) after adding the quantum dots based probes for 15mins. $(\text{Fd/Fa})_i$ is the ratio of emission intensity of quantum dots and rhodamine without different concentrations of inhibitor after adding quantum dots based probes for 15mins and $(\text{Fd/Fa})_b$ is the ratio of emission intensity of quantum dots and rhodamine in Dulbecco's PBS buffer solution at pH 7.4 without trypsin and inhibitors. The inhibition efficiency of 4-(2-Aminoethyl) benzene-sulfonyl fluoride hydrochloride is higher than that of 4-Amidinophenylmethane-sulfonyl fluoride hydrochloride, which is reversible inhibitor of metallo-proteinases and of metal activated proteinases. It is clearly seen that 1, 10-phenanthroline is the best performing inhibitors. With a concentration of 250 μ g/mL, 1,10-phenanthroline, inhibits 100% of enzymatic activity of trypsin, while 4-(2-Aminoethyl) benzene-sulfonyl fluoride hydrochloride and 4-Amidinophenylmethane-sulfonyl fluoride hydrochloride inhibits trypsin by 45% and 20% respectively.

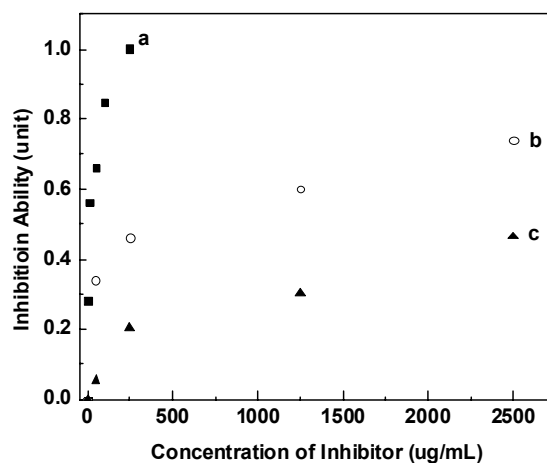


Figure 5 - Inhibition of trypsin by a) 1,10 phenanthroline, b) 4-(2-Aminoethyl) benzene-sulfonyl fluoride hydrochloride and c) 4-Amidinophenylmethane-sulfonyl fluoride hydrochloride.

3.2 Monitoring the proteolytic activity of MMPs in normal and cancerous breast cells

Extracellular metallopeptinases (MMPs) are natural proteinases that share a common modular domain structure and are capable of degrading the extracellular matrix components (ECM) (17). Numerous studies have shown a correlation between the expression of MMPs and the invasive behavior and metastasis potential of tumors (18, 19). For example, MCF-7 breast cancer cells in culture produce both soluble and membrane-bound factors that stimulate the production of pro-MMPs (20). A higher level of MMP1 was found in the breast cancer cell lines MDA-MB-231 and MCF7 compared to normal breast cell lines (21). Therefore, it is of high importance to develop tools for the measurement of MMPs as a mean for the detection of breast cancer cells in biopsies. Variety of methods and probes were previously developed to detect and enable better understanding of the functions of MMPs *in vivo*. These include the design of biocompatible near infrared fluorochromes, novel imaging probes specific to MMPs, fluorescent nanoparticles and fluorescence molecular tomography (FMT) (22, 23). Recently, Bremer et al described a probe which is based on FRET between organic fluorophore donor and acceptor molecules via a peptide linker which is cleaved by MMPs (24).

Following the successful demonstration of the ability of quantum dots FRET based probes to monitor the activity of trypsin and trypsin inhibitors and taking advantage of quantum dots compared to organic fluorophores, we utilized the same probes to measure in real time the activity of collagenase. We hypothesized that since collagenase has a wide range of proteolytic activity it would be at least as effective as trypsin in cleaving the RGDC peptide that was attached to our quantum dots. Figure 6a shows the emission spectra of rhodamine labeled peptide-coated quantum dots following 15 mins incubation at room temperature with collagenase levels ranging from 0 to 5 $\mu\text{g/mL}$. Similarly to trypsin, the FRET signal between the quantum dots and rhodamine decreased with increasing concentration of collagenase. It should be noted however that a 20-fold lower level of collagenase compared to trypsin was needed to similarly affect the FRET signal of the quantum dots. The higher cleavage rate by collagenase could be attributed to its non-selective peptide cleavage activity. The temporal dependence of the ratio F_d/F_a of the quantum dots at increasing collagenase concentration ranging from 0 to 5 $\mu\text{g/mL}$ provided information on the rate of the enzymatic reaction (figure 6b). The ratio of F_d/F_a increased with time and increased faster at higher concentration of collagenase. Therefore, the ratio F_d/F_a was concentration dependent and time dependent. We can see that with a concentration of 5 $\mu\text{g/mL}$ collagenase the enzymatic reaction was completed in 15 minutes.

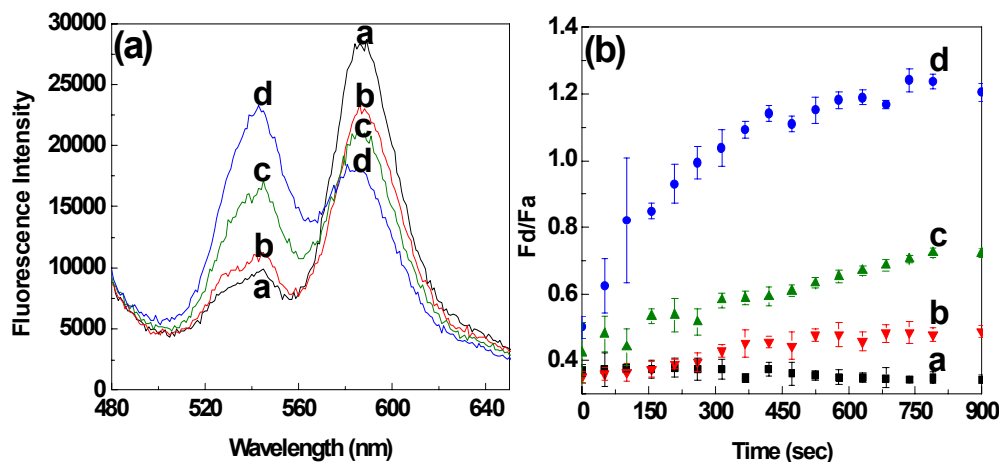


Figure 6 - (a) Emission spectra of rhodamine labeled peptide-coated quantum dots 15 minutes following the addition of collagenase of increasing concentration. (b) Time dependence of the ratio F_d/F_a (see text) of the rhodamine-labeled peptide-coated quantum dots at increasing collagenase concentration. a) 0 $\mu\text{g/mL}$ (black), b) 0.5 $\mu\text{g/mL}$ (red), c) 2.5 $\mu\text{g/mL}$ (green), d) 5.0 $\mu\text{g/mL}$ (blue)

Collagenase belongs to the MMPs family. We employed the quantum dots probes to measure the activity of MMPs in cell cultures. Digital fluorescence microscopy images were used to measure the FRET between

quantum dots and rhodamine and the effect of MMPs in normal and cancerous breast cells on the FRET signal. Figure 7 shows images of the quantum dot FRET probes in normal (images a,b) and cancerous (images c,d) breast cells when taken at $t = 0$ and $t = 15$ minutes following the addition of the probes. It can be seen that the emission color of the quantum dots (orange) did not change when incubated with normal breast cells. Whereas there was a significant change of emission color from orange to green when the quantum dot probes were incubated with breast cancer cells, which is attributed to the over expression of MMPs in breast cancer cells. The quantum dots probes could be used to discriminate normal and cancerous breast cells based on the different level of MMPs expressions between normal and cancerous breast cells.

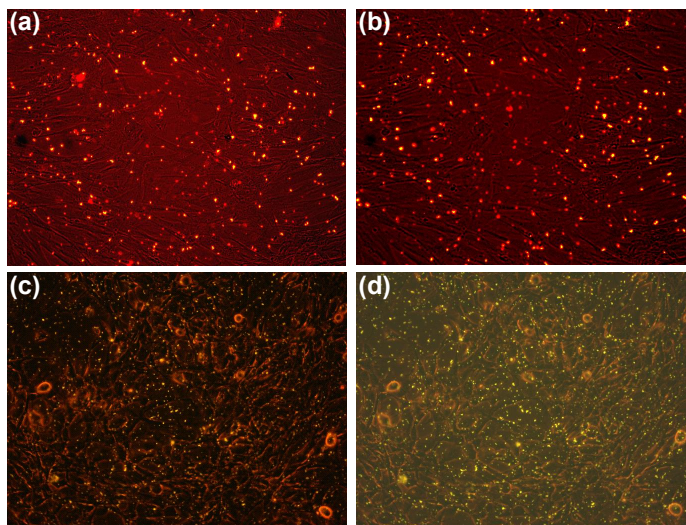


Figure 7 - Digital fluorescence microscopy images of rhodamine labeled peptide-coated quantum dots in cell culture. (a) incubated in HTB 126 cell line for 0 minute, (b) incubated in HTB 126 cell line for 15 minutes; (c) incubated in HTB 125 cell lines for 0 minutes, (d) incubated in HTB 125 cell lines for 15minutes

4. SUMMARY AND CONCLUSIONS

We have designed and developed novel quantum dots based FRET enzymatic activity probes. First the probes were used to test the enzymatic activity of trypsin in solution. The FRET signal changes were found to be trypsin concentration dependent. The probes were used successfully to determine the inhibition efficiency of three organic trypsin inhibitors, 4-(2-Aminoethyl) benzene-sulfonyl fluoride hydrochloride, 4-Amidinophenylmethane-sulfonyl fluoride hydrochloride and 1, 10 phenanthroline. We also found that the probes could not be used to determine the inhibition efficiency of larger trypsin inhibitors like protein molecules. It is possible that protein molecules could displace the peptide molecules from the quantum dots. This would release rhodamine molecules to the solution even in the absence of trypsin. The quantum dot based probes used effectively to discriminate between normal and cancerous breast cells based on differences in the enzymatic activity of metalloproteinases in the extracellular matrix (MMPs). Future studies in our laboratory will focus on improving the stability of the quantum dots in biological system. For example, metallothionin will be used as an improved capping ligand since it binds to the quantum dots through multiple cysteine residues. We will also change the peptide linkers to enable wider application of the quantum dot based probes.

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ACKNOWLEDGEMENTS

This work was supported by NSF grant CHE-0314027. The authors wish to acknowledge the support of Laurie Locascio and Michael Gaitan of NIST and the NIST administration for enabling the completion of this research project at NIST at the aftermath of Hurricane Katrina.