Protease-activated quantum dot probes

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Abstract

We have developed a novel nanoparticulate luminescent probe with inherent signal amplification upon interaction with a targeted proteolytic enzyme. This construct may be useful for imaging in cancer detection and diagnosis. In this system, quantum dots (QDs) are bound to gold nanoparticles (AuNPs) via a proteolytically degradable peptide sequence to non-radiatively suppress luminescence. A 71% reduction in luminescence was achieved with conjugation of AuNPs to QDs. Release of AuNPs by peptide cleavage restores radiative QD photoluminescence. Initial studies observed a 52% rise in luminescence over 47 h of exposure to 0.2 mg/mL collagenase. These probes can be customized for targeted degradation simply by changing the sequence of the peptide linker.
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The design of protease-activatable probes with inherent signal amplification provides a unique tool to monitor specific molecular targets and pathways in vivo through optical imaging. Recently, progress has been made studying the energy transfer between two inorganic particles: a semiconductor nanocrystal (quantum dot, QD) and a nanometer-sized gold particle (AuNP), the donor and acceptor respectively [1,2]. Here, we utilize this non-radiative energy transfer to make a functional QD probe (see Fig. 1). This novel probe is optically suppressed in its native (quenched) state and becomes highly luminescent after proteolysis of the peptide linker, providing a new method for monitoring the expression and bioactivity of various proteases in tissues [3].

Organic fluorophores have been used to monitor protease activity in a variety of configurations. Most organic probes rely on fluorophores in close proximity to auto-quench fluorescence. Upon separation of the fluorophores by enzymatic cleavage of the labeled substrate, fluorescence is unquenched [4–6]. Other groups have utilized the loss of fluorescence resonance energy transfer (FRET) between donor and acceptor paired fluorophores linked by an enzymatically degraded peptide sequence to monitor protease activity [7,8]. More recently, probes consisting of a fluorescence emitter linked to a non-fluorescent absorber have been developed and used in imaging [9,10]. Despite promising results, these probes lack general tunability of wavelength, requiring specific pairing between the donor and acceptor. In addition, these probes utilize organic fluorophores whose optical properties are sensitive to their physical environment and are quickly photobleached under normal imaging conditions.

Optically quenched QDs have recently gained considerable interest. QDs possess strong luminescence, photostability against bleaching and physical environments

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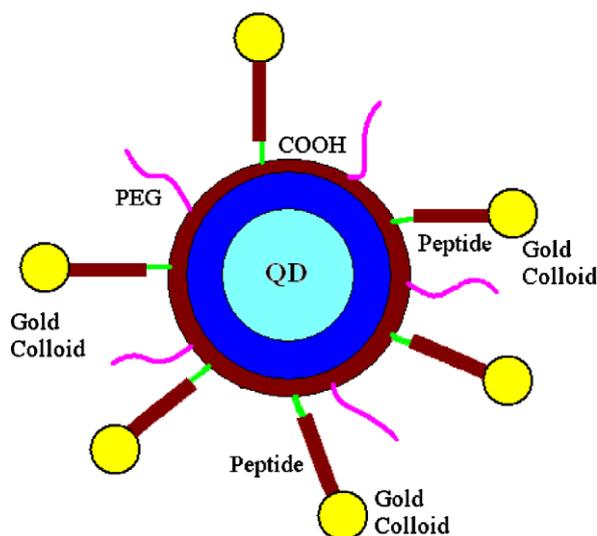 [3] Equal contribution.

Fig. 1. Schematic depicting activatable QD probe structure.

Fig. 2. Illustration of QD probe activation. Protease cleavage of the peptide linker releases the AuNPs that were suppressing QD photoluminescence.

such as pH and temperature, and optical tunability, overcoming many of the shortcomings apparent with organic fluorophores. These properties make them ideal for optical imaging and have proven to be useful as in vitro and in vivo biological labels [11,12]. Meanwhile, AuNPs have been shown as efficient fluorescence quenchers for organic dyes [1,13,14]. Combining the properties of QDs with AuNPs as a functionally activatable reporter for protease imaging would yield a highly advantageous and customizable agent for optical imaging. Several groups have demonstrated optical quenching of QDs. Gueroui et al. [2] demonstrated that AuNPs can quench QD luminescence at the single-molecule level. Clapp et al. [15] explored FRET phenomena in QD–protein–dye conjugates by accurately controlling the donor–acceptor separation distance to a range smaller than 100 Å. Medintz et al. [16] conjugated a β-cyclodextrin-QSY9 dark quencher in a MBP saccharide binding site and resulted in FRET quenching of QD photoluminescence. A QD–biomolecule assembly constructed using these methods may facilitate development of a novel class of hybrid sensing materials [16,17].

We have developed a new imaging agent that utilizes quenched QDs for imaging of proteolytic activity. Our reporter consists of a QD tethered to gold nanoparticles by user-defined peptide sequences specific for a protease of interest. To ensure energy transfer between the QD and AuNPs, peptide sequence length is the primary constraint controlling interparticle distance. The distance at which energy transfer between two molecules is 50% efficient is known as the Förster radius (typically less than 10 nm). The Förster radius is determined by a host of factors such as molecular dipole, quantum yield, refractive index, and spectral overlap [18]. Peptide sequences must not exceed the energy transfer distance necessary for AuNPs to suppress QD photoluminescence. Fig. 2
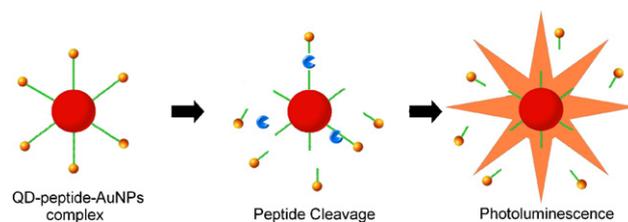
demonstrates the schematic activation of these probes. These QD–peptide–AuNPs imaging probes are activated upon peptide cleavage by a specific protease resulting in the release of AuNPs. Energy transfer no longer occurs between AuNPs and QD, allowing strong radiative emission by the QD. This novel functional agent offers tremendous flexibility and potential due to its optical tunability and adjustable peptide sequence for applications ranging from imaging proteolytic activity of cells to assessing the metastatic potential of cancerous lesions [4,19,20].

Materials and methods

Quantum dot synthesis. The QDs were core/shell structured CdSe/CdS synthesized as previously described [21]. Poly(ethylene glycol) (PEG, 750 Da) was used to increase the water-solubility and stability of the QDs. The resulting QDs are carboxylate-terminated with a peak emission at 620 nm [22]. QD photostability was tested under 4 h of continuous 100 W long-wave UV illumination with no measurable photobleaching or shift in emission wavelength. In addition, changes in buffer composition to HEPES-buffered saline or addition of collagenase (0.2 mg/mL concentration) to QDs resulted in either no change or a minimal decrease in QD photoluminescence. No increase in photoluminescence was observed.

Peptide synthesis. A collagenase-degradable specific peptide sequence (GGLGPAGGCG) was used in our study [23,24]. The GGLGPAGGCG degradable peptide sequence was synthesized using Fmoc solid phase peptide synthesis (Applied Biosystems, Foster City, CA). Cleavage from the polystyrene resin was effected with 95% trifluoroacetic acid, 2.5% water, and 2.5% triisopropylsilane. The cleaved peptide was precipitated in ether followed by dialysis against MilliQ water (Milli-Q Gradient, Millipore, Billerica, MA). The peptide was lyophilized and stored at −20 °C until use.

Conjugation reaction. QDs (2 nmol) in deionized water were activated with EDC and sulfo-NHS (Pierce, Rockford, IL) to form an active ester leaving group. The N-terminus of the synthesized peptide was then covalently linked to the QDs at the active ester site to form an amide. Activation of the C-terminus of the peptide was prevented by reacting residual EDC with β-mercaptoethanol prior to peptide addition. During the coupling reaction, peptide was added in a 30-fold molar excess to ensure sufficient coupling onto the QD. The reaction was allowed to proceed overnight in the dark at room temperature. The solution was then dialyzed with 5000 MWCO cellulose ester membrane (Spectrum Laboratories, Houston, TX) to remove any unreacted peptide or byproducts. Following dialysis, the solution was split into two aliquots. One aliquot was reacted with gold nanoparticles while the other aliquot served as control. The control underwent identical steps as the conjugate except it was reacted with equal volumetric amounts of deionized water rather than gold nanoparticles.

The QD–peptide conjugate was concentrated using a 50,000 MWCO Vivaspin Ultrafiltration concentrator (Vivascience AG, Hannover, Germany) and centrifuged at 2000g for 20 min. The purified QD–peptide conjugate was resuspended to 400 μL of deionized water.

Mono-maleimide functionalized gold nanoparticles (1.4 nm; Nanoprobes, Yaphank, NY) were covalently linked to the sulfhydryl group on the cysteine residue of the QD–peptide conjugate at a ratio of 6:1 AuNP:QD. A centrifuge filter (Vivaspin 6 MWCO 50,000) was then used to remove unbound AuNPs, and the probe was resuspended in sterile HEPES-buffered saline (HBS: 135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1.8 mM $CaCl_2$, and 10 mM HEPES, pH 7.4). Luminescence measurements were made on the control and conjugate to compare quenching of the QDs by the gold nanoparticles.

Activation of probe. Following initial luminescence measurements, collagenase type XI (Sigma–Aldrich, St. Louis, MO) was added to the probes at a final concentration of 0.2 mg/mL. Control samples (QD probe without collagenase) were monitored simultaneously. Extinction measurements were made of varying concentrations of collagenase in HBS to examine effects on turbidity which may affect luminescence measurements. Studies demonstrated minimal effects on turbidity at wavelengths >450 nm for concentrations of 0.2 mg/mL and lower. Higher concentrations of collagenase resulted in decreased QD photoluminescence due to increased turbidity. Fluorescence measurements of collagenase (0.2 mg/mL) in HBS ($n = 3$) were used to subtract autofluorescence from collagenase in all probe samples containing collagenase. QD probes with collagenase were incubated at room temperature in the dark. Samples were removed briefly from the dark to measure photoluminescence of QDs over time. Studies were limited to less than 48 hours due to the progressive loss of collagenase bioactivity in solution [23].

Spectroscopy measurements. All measurements were performed at room temperature with a 500 μL stoppered quartz cuvette to prevent evaporation (Starna Cells, Atascadero, CA) on a Horiba Jobin Yvon SPEX FL3-22 Fluorimeter (Edison, NJ) with dual excitation and emission monochromators. Time-integrated photoluminescence was measured before and after conjugation to the AuNP to observe quenching of QD probes. Photoluminescence measurements were also taken over time to observe proteolytic activation of the probe. Baseline values were taken for all measurements of QD probes in deionized water and HBS. QDs were excited at 360 nm, and emission scans measured from 400 to 700 nm. Bandpass slits and integration time were set to 3 nm/3 nm and 100 ms, respectively, on the fluorimeter. All values were normalized over time to a rhodamine 6G standard to avoid any artifacts that could arise from possible lamp fluctuations. Extinction measurements from 200 to 800 nm were also acquired for each sample on a Varian Cary 50 Bio spectrophotometer (Walnut Creek, CA).

Results

Results of QD quenching by AuNPs are shown in Fig. 3. All measurements reported consist of a sample size $n = 3$. For a 6:1 ratio of AuNP:QD, QD–peptide–AuNP luminescence indicates a 71% quenching of signal compared to unreacted QD–peptide. In addition, initial measurements at $t = 0$ h indicate no statistical difference between probe without collagenase and probe with collagenase (0.2 mg/mL) after subtraction of collagenase autofluorescence.

Luminescence of each sample was measured at the following time points: 0, 18, 38, and 47 h. Fig. 4 illustrates the spectral profile of increased QD luminescence over time. At collagenase concentrations of 0.2 mg/mL,
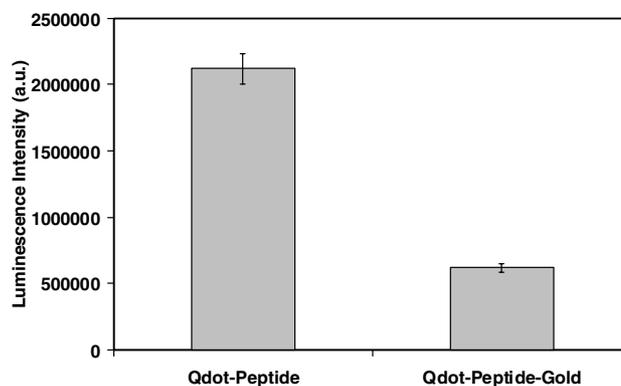

Fig. 3. This chart demonstrates the reduction in QD luminescence after covalent bonding of AuNPs. Fluorescence intensity at 620 nm was measured ($n = 3$).
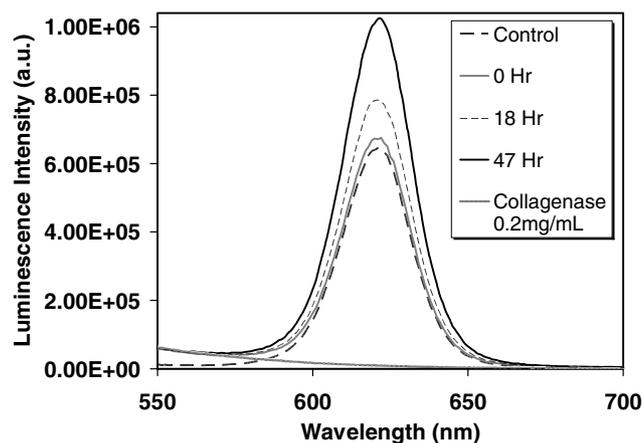

Fig. 4. Emission scan of control (probe without collagenase), 0 h activation of probe by collagenase, 18 h activation of probe by collagenase, 47 h activation of probe by collagenase, and collagenase. Probes were activated with collagenase (0.2 mg/mL) in HEPES-buffered saline. Settings: excitation at 360 nm with bandpass slits of 3 nm/3 nm on excitation and emission sides. Integration time of 100 ms.

there is minimal collagenase autofluorescence past 550 nm. A rise in luminescence over time of the probe with collagenase is observed in Fig. 5. At 47 h, an average luminescence rise of 52% is observed. The control group containing inactivated probe (QD probe without collagenase) in buffer solution indicated statistically insignificant changes in luminescence during the same time intervals.

Discussion

Preliminary results demonstrate proof-of-principle that activatable QD probes are highly promising agents not only for extending the limits of optical imaging by providing an inherent increase in the signal:background ratio but also for monitoring proteolytic activity which may provide information about the metastatic potential
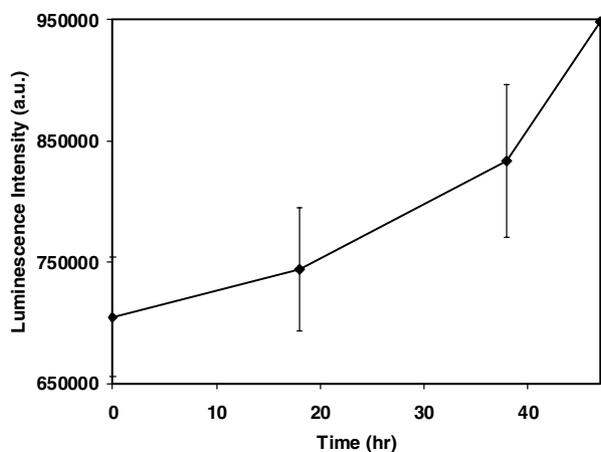
Fig. 5. Plot of probe activation with collagenase demonstrating increased luminescence over time. Data are reported as means ± SD ($n = 3$).

of a tumor. Furthermore, these probes have the added advantages of photostability and emission tunability not present with organic fluorophores.

Several parameters need further optimization for the development of clinically applicable QD protease probes. An important issue in proteolytic activity is peptide accessibility, which is affected by interparticle distance between the QD and AuNP, the density of AuNPs surrounding the QD, and the PEG surface coat on the QD. All of these parameters will be individually characterized to tailor reaction rates of proteolytic activation in future studies. To minimize steric effects and provide better accessibility for collagenase, a short 750 Da PEG polymer coating on the QD was used, and two spacer glycine amino acids were added to both ends of the LGPA sequence. In addition, 6:1 ratio of AuNP:QD was chosen because it provided a strong degree of optical quenching while preserving photoluminescence recovery. Higher ratios of AuNP:QD resulted in a greater degree of optical quenching but had minimal photoluminescence recovery (not reported) possibly due to increased steric hindrance from AuNPs, decreasing substrate accessibility by the enzyme.

In conclusion, we report engineering a functional QD probe for imaging proteolytic activity. Current fluorescent imaging contrast agents provide increased contrast by accumulation of fluorophores at specific targets of interest. These agents provide limited functionality in the ability to observe molecular specific processes. Developing 'smart' optical contrast agents that respond to molecular processes, such as protease activity, not only provides the advantages of observing time- or spatial-dependent processes, but also provides an inherent increase in signal:background ratio greater than conventional targeted fluorophores. Moreover, due to the narrow emission bandwidth of QDs, it is conceivable to construct a library of different wavelength emitting biocompatible quenched probes, each with its own enzyme

specificity to allow for simultaneous imaging from many different proteases.

This probe has tremendous biological applications due to its customizable peptide linker sequence for monitoring a protease of interest and optical tunability. Near infrared QDs could be easily fabricated, developing probes that would be ideal for in vivo imaging due to tissue's high optical transmissivity in the near infrared spectrum. Furthermore, the inherent increase in photoluminescence with these probes may be used to overcome the limited signal:background ratio problem often present in targeted imaging. Future studies will focus on optimizing and enhancing the quenching and unquenching efficiency of these 'smart' probes.

Acknowledgment

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