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Quantum Dot FRET-Based Probes in Thin Films Grown in Microfluidic Channels

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The objective of this study was to fabricate new sensing thin films, which make use of luminescent quantum dots and take advantage of their unique photophysical properties while minimizing their potential toxicity. To realize this goal, CdSe/ZnS luminescent quantum dots were incorporated into the inner layers of polyelectrolyte thin films, and molecular fluorophores were attached to the film surface. This resulted in fluorescence resonance energy transfer (FRET) between the quantum dots, which served as fluorescent donors, and molecular fluorophores, which served as fluorescent acceptors. Attenuation of the FRET interactions in the presence of target analytes was used as the sensing principle of these unique sensing films.

The layer-by-layer (LbL) deposition technique, which is based on alternate deposition of oppositely charged polyelectrolytes, to fabricate a polyelectrolyte multilayer (PEM) film has been widely used due to the simplicity and effectiveness of this method in creating high-quality polymeric thin films.[1] PEMs have found use in many applications and devices, including light-emitting devices,[2,3] bioactive material coatings,[4] electrically conductive polymers,[2,5] and hollow polyelectrolyte capsules for drug delivery.[6] Fluorescent dyes were previously immobilized to PEMs in order to study their physical properties, morphology, organization, and molecular orientation. For example, Richter et al. studied the properties of poly(styrene sulfonate) (PSS) and poly(allylamine hydrochloride) (PAH) films doped with fluorescent dyes.[7] In their study they used FRET between the conjugated polymer poly(*p*-phenylene vinylene) (PPV), which served as a fluorescent donor, and acceptor molecules, which were embedded in the PEMs. They were able to find the distance dependence between the donor and acceptor molecules by varying the number of polyelectrolyte layers and found that the FRET efficiency depended on $1/[1 + (d_0/d)]^4$, where d was the distance and d_0 the critical energy transfer distance. A deviation from this distance dependence at short distance between the donors and acceptors was attributed to low lateral density of the acceptor molecules and their distribution. Caruso et al. carried out a similar study in which they assessed the FRET between 6-carboxyfluorescein (6-CF) molecules as donors and rhodamine B-labeled melamine formaldehyde (RhB-MF) particles as acceptors.[8] PEMs of varying thickness were adsorbed on the RhB-MF particles. The energy transfer took place when 6-CF was adsorbed onto the RhB-MF particles. More recently, Schneider et al. used gold nanoparticles as acceptors in FRET studies of PEMs. In their study, PEM films containing fluorescent donor molecules were formed on the surface of gold nanoparticles. FRET measurements were used to characterize the properties of these composite particles.[9]

Luminescent quantum dots have been largely used as donors in FRET solution assays.[10,11] These unique luminescent nanoparticles are characterized by a broad excitation wavelength range, high photostability, narrow emission peaks, and size-dependent tunability. These properties enable fine-tuning of FRET interactions between the quantum dot donors and the molecular acceptors linked to their surface to maximize FRET efficiency. We have previously shown that it is possible to covalently link molecular fluorescent acceptors to the surface of single quantum dots and utilize these FRET probes for real-time monitoring of protease activity in solution.[12] Recent data describing FRET interactions between luminescent quantum dots and molecular acceptors support a model for these interactions based on the Forster theory as long as the quantum dot donors can be approximated to behave like point dipoles.[11]

In the current study, PEM films were grown in microfluidic channels to facilitate FRET-based assays of volume-limited samples. The microfluidic channels were fabricated using standard microlithography on poly(dimethylsiloxane) (PDMS).[13] The quantum dots-embedded PEM film was grown through alternate deposition of positively charged PAH and negatively charged PSS layers on the PDMS surface. Mercaptoacetic acid-modified CdSe/ZnS quantum dots, which were prepared following previously established ligand exchange protocols,[14] were deposited on a positively charged PAH layer of the PEM film. This was followed by alternate deposition of oppositely charged polyelectrolyte layers. Rhodamine molecules were then covalently immobilized to a PAH layer on the film surface. Figure 1 describes the fluorescence properties of PEMs that contain quantum dots and molecular acceptors and the FRET interactions between the quantum dots and molecular acceptors when separated by varying numbers of polyelectrolyte layers.

Excitation of the quantum dot donors at 445 nm resulted in two clearly separated emission peaks of the quantum dots at 540 nm and of the rhodamine at 590 nm. The ratio between the fluorescence

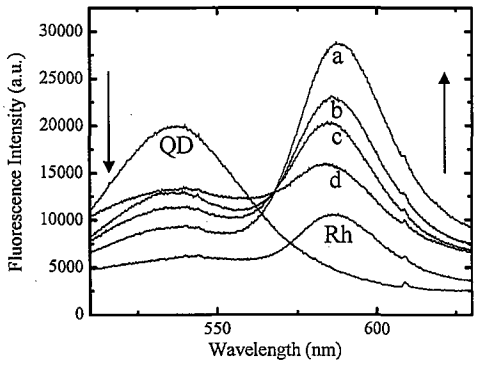

Figure 1. Fluorescence spectra of microfluidic channels containing control PEMs with quantum dots or rhodamine and microfluidic channels containing quantum dots and rhodamine, which are separated by 2 (a), 4 (b), 6 (c), and 8 (d) layers of polyelectrolytes.

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intensity of the quantum dots and the fluorescence intensity of the rhodamine acceptors (F_d/F_a) was found to be dependent on the number of polyelectrolyte layers separating the quantum dots from the rhodamine molecules. The maximum FRET efficiency was observed when the rhodamine molecules were separated from the quantum dots by two polyelectrolyte layers. Increasing the number of PEM layers resulted in lower FRET efficiency.

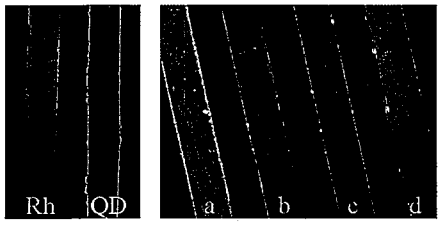

Figure 2. Fluoresence images of microfluidic channels (50 μm wide, 30 μm deep, and 3 cm long) containing control PEMs with rhodamine (Rh) or quantum dots (QD) and microfluidic channels containing quantum dots and rhodamine, which are separated by 2 (a), 4 (b), 6 (c), and 8 (d) layers of polyelectrolytes.

Fluorescence images of quantum dots (green emission) and rhodamine molecules (red emission) that were deposited in the PEMs (Figure 2) show that the emission color of quantum dot-containing PEM films was bright green in the absence of rhodamine. The emission color of the PEM films changed from green to orange in the presence of rhodamine due to FRET between the quantum dots and the rhodamine molecules. The FRET intensity reached a maximum when the quantum dots were separated from the rhodamine molecules by two layers of polyelectrolyes (channel a). The FRET intensity decreased with increasing number of polyelectrolyte layers separating the quantum dots from the rhodamine acceptors (channels b, c, and d).

The utility of the PEM-containing microfluidic channels as sensors was demonstrated through real-time monitoring of the enzymatic cleavage by trypsin of neurotensin, a peptide, which is localized in the gastrointestinal tract and the brain.[15,16] The FRET-sensing PEM film was fabricated as described above with one modification: rhodamine-labeled neurotensin molecules were conjugated to the PEM film surface. To carry out the enzymatic assay, the microfluidic channels were filled with a solution containing 0.25 mg/mL trypsin, and the flow was stopped to allow the cleavage of the rhodamine-labeled neurotensin molecules from the PEM surface. Figure 3 shows the effect of trypsin on the FRET signal between the quantum dots and the rhodamine-labeled neurotensin peptide molecules during trypsin proteolysis.

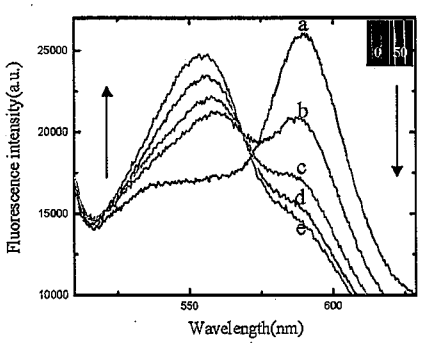

Figure 3. FRET spectra of the quantum dot FRET-based sensing PEM films prior to trypsin incubation (a) and following 10 (b), 20 (c), 30 (d), and 50 (e) min of incubation with 0.25 mg/mL trypsin at room temperature. The fluorescence intensity of rhodamine decreases while the emission intensity of the quantum dots increases. The inset box shows the FRET images of a PEM film prior to and following 50 min of incubation with trypsin.

Both spectra and image, shown as the inset, show that the fluorescence intensity of the green-emitting quantum increases while the orange emission due to FRET between the quantum dots and rhodamine molecules decreases as the proteolytic cleavage of neurotensin molecules removes the rhodamine acceptors from the PEM film surface. Control experiments with trypsin free solutions show no FRET signal change and confirm our conclusion that the observed FRET changes were due to enzymatic cleavage of neurotensin and not due to nonenzymatic modification of the PEM film.

In conclusion, we have successfully fabricated PEM films in microfluidic channels with FRET sensing capabilities and employed them in enzymatic assays. The employment of FRET sensors enables real-time monitoring of the assays since it eliminates the need to remove cleaved rhodamine molecules from the channels during the assays. The presence of the cleaved fluorescent rhodamine molecules in the channels precludes the use of conventional fluorescence techniques to monitor the enzyme assays. On the other hand, it is possible to monitor the assays by FRET since the cleaved rhodamine molecules do not affect the FRET properties of the film. Incorporating the quantum dots into sensing films enables us to take advantage of their photophysical properties and use them as luminescent probes while minimizing their toxicity. Since it has already been shown that PEM films are biocompatible and exhibit excellent adhesion properties suitable for cell growth,[13,17] we are currently pursuing this direction by employing the FRET-based sensing films introduced in this paper to monitor the proteolysis activity of CAPAN-2 pancreatic cancer cells. The ability to grow the PEM sensing films in microfluidic channels will also enable us to perform the assays on tissue cultures, taking advantage of the *in vivo*-like environment created when PEM films are grown in microfluidic platforms. It will also be possible to carry out cellular assays and study cell-substrate interactions at the single cell level.

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Supporting Information Available: Experimental procedures. This information is available free of charge via the Internet at http://pubs.acs.org.

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Supportive Information

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Experimental Section

Materials and methods - Poly (sodium 4-styrenesulfonate) was purchased from Scientific Polymer Products. Sodium chloride was obtained from J. T. Baker. Poly (allylamine hydrochloride) (MW ~70 000), cadmium oxide, lauric acid, trioctylphosphine oxide (TOPO), hexadecylamine (HAD), lauric acid, chloroform, diethylzinc, trioctylphosphine (TOP), methanol and selenium powder, were purchased from Sigma Aldrich. Thioglycolic acid and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide; N-Hydroxysulfosuccinimide sodium salt were purchased from Fluka. Phosphate buffered saline was purchased from Gibco. Neurotensin was purchased from American Peptide Company. PDMS, silicone elastomer kit was purchased from Dow Corning Corporation. Dialysis cassettes with 2,000 and 3500 MWCO were purchased from Pierce. Rhodamine Red ^{TM} –X succinimidyl ester was purchased from Invitrogen. Water was purified using a UV ultra pure water purification system from Barnstead. Polydimethylsiloxane (PDMS) films were oxidized using a plasma etcher (Anatech LTD-Plasma Series). Photolithographic masks were fabricated at NIST using standard microlithography protocols. Atomic force microscopic measurements were performed using a Dimension 5000 AFM microscope from Digital Instruments.

Digital Fluorescence Microscopy – Luminescence images were obtained using a digital luminescence imaging microscopy system. The system consisted of an inverted fluorescence microscope (Olympus IX 70) equipped with a 100 W Hg lamp as a light source. The fluorescence images were collected via a 20X microscope objective with NA = 0.4. A filter cube containing a 450 ±10 nm band-pass excitation filter, a 470 nm

dichroic mirror, and a 500 nm long pass emission filter was used to ensure spectral imaging purity. A high performance 16 bit resolution, back illuminated CCD camera of Roper Scientific was used for digital imaging. Luminescence spectra of quantum dots were obtained using the same microscopy system by passing the collected luminescence signal through a 250 mm Acton spectrograph. The spectra were obtained with a high performance 16 bit resolution, back illuminated CCD camera (Roper Scientific). The Roper Scientific software WinSpec 32 was used for the acquisition of digital images and spectra. The exposure time was 0.1 seconds.

Synthesis of Luminescent Quantum Dots - CdSe/ZnS quantum dots were prepared following a method previously reported by Peng and coworkers[1] with slight modifications[2]. It involves dissolving 12.7 mg CdO in 160 mg lauric acid under nitrogen at T >200° in a three neck bottle flask. After cadmium oxide is fully dissolved, 1.94 g trioctyl phosphine oxide (TOPO) and 1.94 g hexa decyl amine (HAD) are added to the mixture and the temperature is raised to 250-300K. The heat mantles are removed and 80 mg of selenium powder in 2mL of trioctyl phosphione (TOP) is injected into the reaction. After the reaction temperature is decreased to ~200 °C, the ZnS coating is formed by injecting into the reaction mixture 2 ml TOP solution containing 250 µl hexamethyldisilathiane ($(TMS)_2S$) and 1 ml diethylzinc ($Zn(Et)_2$) .The reaction mixture is kept at 180°C for one hour and then cooled to room temperature. The resulting CdSe/ZnS quantum dots are washed three times with methanol and chloroform. The hydrophobic quantum dots turn hydrophilic and water miscible through a ligand exchange reaction of TOPO with mercapto acetic acid[3]. 2ml of TOPO coated quantum

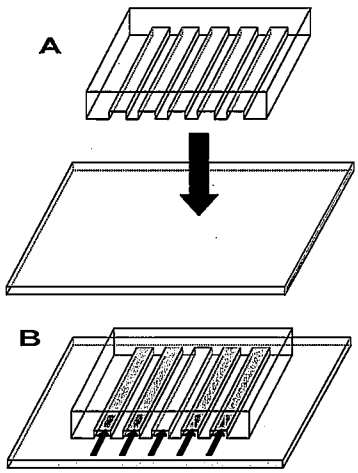
dots are suspended in 3mL of chloroform and incubated with 2ml of thioglycolic acid overnight under continuous stirring. The resulting water soluble quantum dots are extracted in 10 ml water and washed three times with water and chloroform.

Synthesis of PAH-Neurotensin-Rhodamine conjugate – 2 μM rhodamine Red™ –X succinimidyl ester were reacted with 1mg/ml neurotensin at pH 7.4 in a 2 ml total reaction volume. The reaction volume was incubated for 2 hours at room temperature under continuous stirring. The unreacted rhodamine was separated out of the solution using a dialysis cassette with a 2000 MWCO cutoff filter. The remaining rhodamine labeled neurotensin solution was added to a 1 ml solution containing 1mg/ml PAH, 20 μM EDC and 50 μM sulfo NHS. The reaction mixture was incubated for 4 hours at room temperature under continuous stirring. The labeled PAH was separated from the reaction by-products by using a dialysis cassette with a 3500 MWCO cutoff filter. It should be noted that the separation of unbound rhodamine and rhodamine-labeled neurotensin as well as the separation of PAH-neurotensinn-rhodamine from smaller molecular by products could be realized as effectively by using other commercially available means like ultra centifugal filtering devices and size exclusion chromatography columns.

Fabrication of microfluidic channels on PDMS surface - Glass masters containing the channels geometry (parallel lines, 50 μm wide, 30 μm deep, 3 cm long) were fabricated following a procedure described by Martynova et al[4]. A PDMS mask was fabricated by first using a Sylgard 184 silicon elastomer kit to prepare a PDMS solution. The kit contains a silicon elastomer base (liquid resin) precursor and a silicon curing agent (cross

linking reagent). The two components were mixed in a 10:1 ratio and degassed for 45 minutes under vacuum. The PDMS solution was then poured to fill the channels of the glass master and then was cured at 100 °C for 1 h to form the PDMS mask. The PDMS mask was peeled off the glass master and was placed on an oxidized PDMS-treated glass slide (25 x 75 mm, thickness 0.93-1.05 mm pre-cleaned with sulfuric acid) to form microfluidic channels of 50 μm wide, 30 μm deep and 3 cm long (scheme S1 step A). The glass slides were coated with PDMS since PDMS was previously shown to promote polyelectrolyte deposition through surface oxidation[5]. The flat PDMS substrate was first oxidized in an O_2 (approximately 2.6 Pa) plasma for 120 seconds and then covered with the molded PDMS. This was followed by alternate deposition of polyelectrolytes to form the PEM film in the microfluidic channels following a procedure previously published by Reyes et al.[5] (scheme S1 step B).

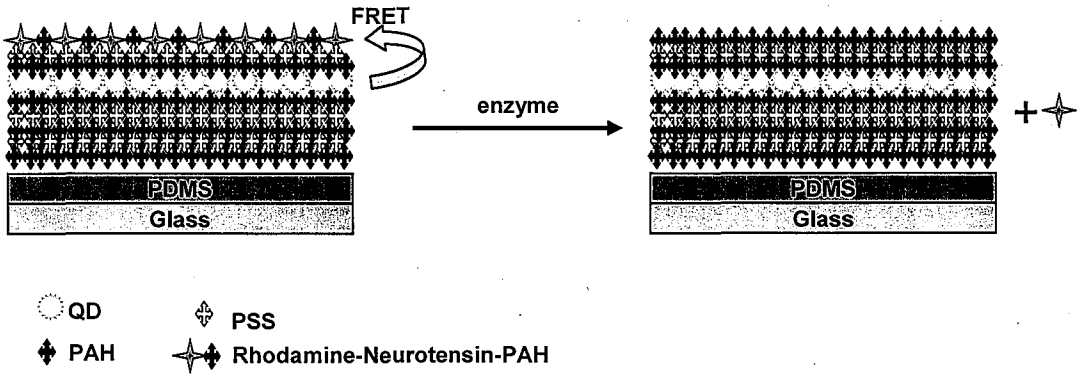
Scheme S1



Preparation of the FRET sensing film using the layer by layer (LbL) deposition method - . Scheme S2 illustrates the fabrication of PEMs in microfluidic channels by

alternate deposition of positively charged polyallylamine hydrochloride (PAH) and negatively charged polystyrene sulfonate (PSS). PAH and PSS solutions were prepared by dissolving 1 mg/ml PAH or 1mg/ml PSS in a solution of 0.1M NaCl in deionized water. The pH of the PAH and PSS solutions was adjusted to 5 and 6, respectively. The micro channels were first filled with PAH using a vacuum pump and allowed to incubate (no flow) for 30 min. The channels were then rinsed with water and dried. Five alternating layers of PSS and PAH were deposited to form the polyelectrolyte multilayer (PEM) film. Each layer was deposited by filling the channels with a PAH or PSS polyelectrolyte solution and incubating it for 10 minutes before flashing the solution out of the channels. Then, quantum dots were deposited on the PAH surface layer. The negatively charged quantum dots were then coated with another PAH layer, which was followed by the deposition of a PSS layer. Rhodamine-neurotensin-PAH was then deposited on the film surface.

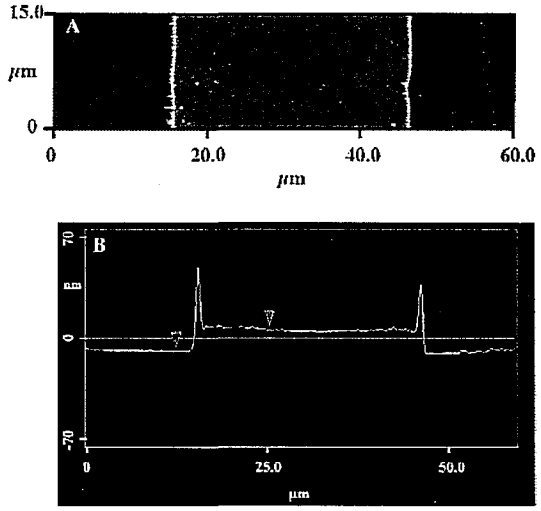
Scheme S2



Atomic Force Microscopy (AFM) - Atomic force microscopy measurements were carried out using a Dimension 5000 AFM microscope (Digital Instruments, Santa

Barbara, CA). AFM measurements were acquired in tapping mode, to obtain topographical (height) data of the deposited PEMs following Reyes *et al*[5]. Heights were measured in three different cross-sectional areas of the lines for a number of layers. All AFM measurements were made on dried PEMs channels. Figure S1A represents a top view image of a 9-layer PEM film (the yellow area) on a PDMS substrate. The left and right brown areas show the bare PDMS substrate. Figure S1B shows a cross-section (average) graph of the image shown in S1A. The average thickness of 9 layer PEM film was 14.2± 0.6 nm. Based on these AFM measurements it was concluded that the thickness of a single PEM layer is about 1.5nm.

Figure S1 –Atomic force microscopy (AFM) of 9 layers PEMs on PDMS substrate.



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