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Homogeneous immunoassays based on fluorescence emission intensity variations of zinc selenide quantum dot sensors

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ABSTRACT

The fluorescence emission intensity of ZnSe quantum dots (QDs) conjugated to proteins to form QD-based biomolecular sensors increases significantly upon binding of the sensors to target proteins in solution. This phenomenon enables the development of homogeneous, separation-free immunoassays for rapid quantitative detection of proteins in solution. Proof-of-principle assays were developed by dosing a solution containing a biomolecular target with a solution containing the corresponding QD-based sensor and monitoring the changes in the peak fluorescence emission intensity of the QDs. Direct immunoassays for detecting basic fibroblast growth factor (bFGF) and prostate-specific antigen (PSA) in solution were demonstrated using QD-anti-bFGF and QD-anti-PSA sensors. A competitive immunoassay for detecting human serum albumin (HSA) was also demonstrated by dosing samples containing HSA with QD-HSA sensors and free anti-HSA antibodies. The QD-HSA sensors were tested in 1000 × diluted human serum and found to be unaffected by interference from other proteins. The lower limit of detection of the assays was equal to the lowest sensor concentration in the solution that can be unambiguously detected, typically less than 1 nM. The dynamic range of the assays was determined by identifying the sensor concentration above which optical interference between QDs affected adversely the observed fluorescence emission intensity. The upper limit of this concentration was 2.5 μM for 4 nm QDs. The ZnSe QD-based sensors were stable and preserved ~80% of their initial peak emission intensity after two months in refrigerated storage. These biosensors have potential applications in rapid sensing of target proteins for emergency and point-of-care diagnostic applications.

1. Introduction

Semiconductor nanocrystals or quantum dots (QDs) are nanometer-sized inorganic crystals that have unique optical properties due to confinement of electron-hole pairs (excitons) by the grain boundary of the nanocrystals (Alivisatos, 1996; Brus, 1991). These include size-tunable photoluminescence, high quantum yields and molar extinction coefficients, and high photostability (Murray et al., 2000). QDs have attracted significant attention as fluorescent labels of proteins and cells for in vitro and in vivo imaging and for biological sensing applications (Michalet et al., 2005; Smith et al., 2006). The excellent photochemical stability and high brightness of QDs can increase detection sensitivity in immunoassays and their narrow and tunable emission spectra enable multiplexing (Goldman et al., 2005a, 2006; Sapsford et al., 2006).

A variety of targets have been detected using QDs as fluorescent labels, including small molecules, protein disease markers, bacteria, and viruses (Gill et al., 2008; Sapsford et al., 2006). For example, QDs have been employed in ELISA-type immunoassays (Wang et al., 2002; Woodbury et al., 2002), in Western blot analysis of proteins (Bakalova et al., 2005; Chen et al., 2009), in sandwich immunoassays that can detect prostate-specific antigen (PSA) (Kerman et al., 2007), in multiplexed sandwich immunoassays that can simultaneously detect four toxins (Goldman et al., 2004), and as donors for detection of small molecules and biological targets based on Förster Resonance Energy Transfer (FRET) between the QD and another fluorophore (Algar and Krull, 2008; Clapp et al., 2005; Goldman et al., 2005b; Medintz et al., 2003). However, the substitution of fluorescent proteins by QDs in biological sensing applications is not always practical due to the high cost of QDs and the high toxicity of typical CdSe-based QDs.

In this paper we demonstrate the development of novel homogeneous (separation-free) assays that enable direct detection of target analytes by monitoring the variations of the peak emission intensity of QD-labeled biomolecular sensors upon binding to specific target analytes. The variations in the peak emission intensity of the QDs are caused by surface-induced electronic perturbations (Cadars et al., 2009). The detection of a target analyte by these assays is accomplished without employing a second fluorophore and without immobilizing the probe, target, or probe-target complex

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onto the surface of a micro-well or micro-bead. This minimizes mass transport limitations that can dramatically decrease the response time of a sensor (Sikavitsas et al., 2002). Homogeneous assays that are robust and reliable can increase the speed and simplicity of detection (Kricka, 1994). These assays are typically designed to provide immediate response in the form of an optical or electrical signal when the specific target is detected, which is an important requirement for assays suitable for point-of-care or emergency care applications (Ligler, 2009). A potential limitation of homogeneous, separation-free assays is their reduced sensitivity due to interference of other molecules in the sample with the measured signal.

Homogeneous assays were developed using ZnSe QDs as responsive fluorescent beacons that are conjugated with a probe biomolecule. ZnSe QDs are less toxic when compared to Cd-based ones and allow more environmentally friendly processing and disposal. We demonstrate direct homogeneous assays for rapid quantitative detection of basic fibroblast growth factor (bFGF) and prostate specific antigen (PSA) targets in phosphate buffer saline (PBS) solution using ZnSe QD-anti-bFGF and ZnSe QD-anti-PSA sensors, respectively. A competitive homogeneous assay for detecting human serum albumin (HSA) in PBS and diluted human serum was also developed by dosing the HSA-containing solution with ZnSe QD-labeled HSA and anti-HSA antibody. In contrast with typical heterogeneous assays that require multiple steps and immobilization of the target and sensor on a substrate surface before measuring the signal, these assays were much simpler and faster to execute, making them attractive for applications requiring rapid quantitative detection of biological targets.

2. Materials and methods

2.1. Materials

Trioctylphosphine (TOP, 90%), selenium powder (−100 mesh, 99.5%), 1-hexadecylamine (HDA, 98%), diethylzinc (Et_2Zn, 1.0 M solution in heptane), 11-mercaptoundecanoic acid (MUA, 95%), potassium tert-butoxide (95%), *N*-(3-Dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC), and *N*-hydroxylsulfosuccinimide (Sulfo-NHS) were purchased from Sigma-Aldrich. Methanol, ethylether, butanol, and hexane were purchased from Fisher. 10× Phosphate buffered saline (PBS) was purchased from Gibco. Human serum albumin (HSA, 99%, MW 67.6 kDa), monoclonal anti-human serum albumin antibody produced in rabbit (anti-HSA, MW 150 kDa), human serum from platelet poor human plasma (sterile-filtered, mycoplasma tested and virus tested), polyclonal anti-prostate specific antigen antibody (anti-PSA, MW 150 kDa) produced in rabbit, and prostate specific antigen (PSA, MW 28.4 kDa) from human semen were purchased from Sigma-Aldrich. Recombinant human basic fibroblast growth factor (bFGF, MW 16 kDa) and mouse anti-human basic fibroblast growth factor (anti-bFGF, MW 150 kDa) were purchased from Biolegend.

2.2. Synthesis and functionalization of ZnSe QDs

ZnSe QDs were synthesized by injecting a mixture of diethylzinc and Se powder dispersed in TOP into hot HDA at 310 °C (Hines and Guyot-Sionnest, 1998). Samples were drawn at specific time intervals and the ZnSe QDs contained in them were characterized to determine their size and optical properties. Samples containing ZnSe QDs with sizes between 3 and 5 nm were used for the biological sensing experiments. These QDs were modified with MUA according to published protocols (Chan and Nie, 1998) and were dispersed in aqueous solutions.

2.3. Preparation of ZnSe QD-antibody and QD-antigen sensors

A standard stock solution of HSA was prepared by directly dissolving 10 mg HSA into 1 mL of 1× PBS buffer. Anti-HSA, bFGF and anti-bFGF stock solutions were prepared by dissolving 100 μg of each one of the as-purchased proteins in 1 mL of sterile 18 MΩ-cm water. As-purchased solutions containing 1 mg anti-PSA/mL and 2.83 mg PSA/mL were diluted with PBS buffer to prepare stock solutions of the two proteins. All protein solutions were filtered through a 0.20 μm filter into sterile glass vials and stored at 4 °C. ZnSe QDs functionalized with MUA were conjugated with proteins by a two-step procedure using EDC and Sulfo-NHS as cross-linkers, following a standard protocol (Hermanson, 1996). For example, 8 mg of EDC and 40 μL of freshly-made NHS-water solution containing 10 mg NHS/mL were added to 2.0 mL of QD PBS buffer solution containing 1.06×10^{15} particles/mL which is equivalent to a QD concentration of 1.75 μM. The solution was stirred slowly for 30 min at room temperature before adding 3 to 4 nmol of HSA. The ratio of the number of ZnSe QDs to the number of biological molecules available for conjugation was controlled to be close to 1:1. The mixture was incubated at room temperature for 4 h to complete the covalent coupling reaction and allowed to stay overnight to enhance QD stabilization.

2.4. Immunoassay

Calibration lines for the immunoassays were established by measuring the peak emission intensity of the free sensor and the equimolar sensor+target complex as a function of sensor concentration. Two sets of 15 vials were prepared, each containing a progressively increasing concentration of sensor. One set consisted of fifteen 1 mL samples of sensor solution, each containing a progressively increasing amount of sensor. This reference set was used to generate the sensor-only calibration line. The second set was used to generate the equimolar sensor+target calibration line as follows: 15 vials were prepared, each containing less than 1 mL of sensor solution with a progressively increasing concentration. A small volume of a concentrated solution containing free antibody (or antigen) was added to each vial to deliver an amount of antibody (or antigen) that was equal to the amount of sensor contained in the vial. PBS buffer was subsequently added to make the final volume of the mixture in each vial to be equal to 1 mL. After a short mixing period on an orbital shaker, the fluorescence spectrum of each sample was measured and the peak emission intensity was plotted as function of the concentration of sensor in the final 1 mL mixture. The sensor-only calibration line provided the lower limit of fluorescence intensity during assay execution and the equimolar sensor+target line the upper limit.

Each immunoassay was executed manually by dosing a sample solution containing the target with a solution containing the corresponding sensor. For direct immunoassays, the sensor solution contained a QD-labeled antibody. For competitive immunoassays, two dosing solutions were used, one containing QD-labeled antigen and the other free antibody. For the assay examples discussed in this paper, a series of samples with volume less than 1 mL was prepared, each containing the same amount of target. An increasing amount of QD-labeled antibody (for direct immunoassays) or labeled antigen and free antibody solution (for competitive immunoassays) was added into each vial, always starting with an amount that is smaller than the amount of target to be detected. The final volume in each vial was adjusted to be equal to 1 mL by adding PBS buffer. After mixing, the fluorescence spectrum of each solution was recorded and the peak emission intensity was plotted as a function of the concentration of the QD-labeled sensor that was added to each sample. For commercial applications, such an immunoassay can be