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TRENDS

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Luminescent quantum dots in immunoassays

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Introduction

Luminescent semiconductor nanocrystals or quantum dots (QDs) are a relatively new class of fluorophores with unique photophysical properties that make them attractive to biologists for use in fluorescence assays. These properties include high quantum yields, large extinction coefficients, pronounced photostability, and more importantly, broad absorption spectra coupled to narrow size-tunable photoluminescent emission spectra [1–6]. Unlike conventional dyes, distinct populations of QDs can be simultaneously excited by a single excitation wavelength far removed from their respective emissions, which suggests they could be especially suited for multiplexing assays via simultaneous detection of multiple signals (Fig. 1).

Since biocompatible QDs were first described [2, 3], they have been utilized in many biological assays. These include DNA sorting, immunoassays, fluorescence resonance energy transfer (FRET)-based sensing, bio-optical coding, cellular and "animal" imaging. Three recent reviews detailed some of the progress made and potential applications of QD fluorophores [4–6]. The cumulative results of these reports indicate that the QD photophysical properties can circumvent some of the limitations encountered by conventional organic dye and protein fluorophores. These limitations include narrow excitation windows, small Stokes shifts and broad red-tailed emission spectra, and an inherent susceptibility to both chemical and photodegradation, which combined can seriously complicate any potential multiplex scenario. QD photo and chemical stability has allowed lower limits of detection and extended monitoring in assays, such as the tracking of receptor movement in live cells [7].

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Bioconjugation of quantum dots

Perhaps the single largest obstacle to a wider implementation of QD fluorophores for biological applications has been the lack of "one bioconjugation reaction fits all," whereby one can attach a variety of biomolecules, such as DNA or proteins, to QDs using a simple reproducible scheme. There is no technique that consistently allows preparation of QD bioconjugates with control over the ratio of biomolecules per QD and the subsequent orientation of the bioconjugate. This is due to two reasons: QD capping ligands and control over the attachment of biomolecules to the QD. QDs are synthesized from organometallic precursors and are inherently hydrophobic. Two approaches have been used to make QDs hydrophilic to render them compatible with biological media. The first requires "cap" exchanging the original hydrophobic capping on the QD surface with bifunctional ligands that can both mediate solubility and serve as a site for bioconjugation (reviewed in [4, 5]). The second approach involves encapsulating the native hydrophobic QDs with a "bifunctional hydrophobic–hydrophilic" shell (often made of a block copolymer). The hydrophilic functions of the shell mediate water-compatibility and can be used for further bioconjugation. Capping-ligand composition directly affects the final QD bioconjugate structure/function as the ligands serve as the subsequent site for bioconjugation. Currently no cap provides a suitable surface for every biological assay.

Strategies for creating QD bioconjugates can be divided into two general classes: (1) direct chemical coupling and

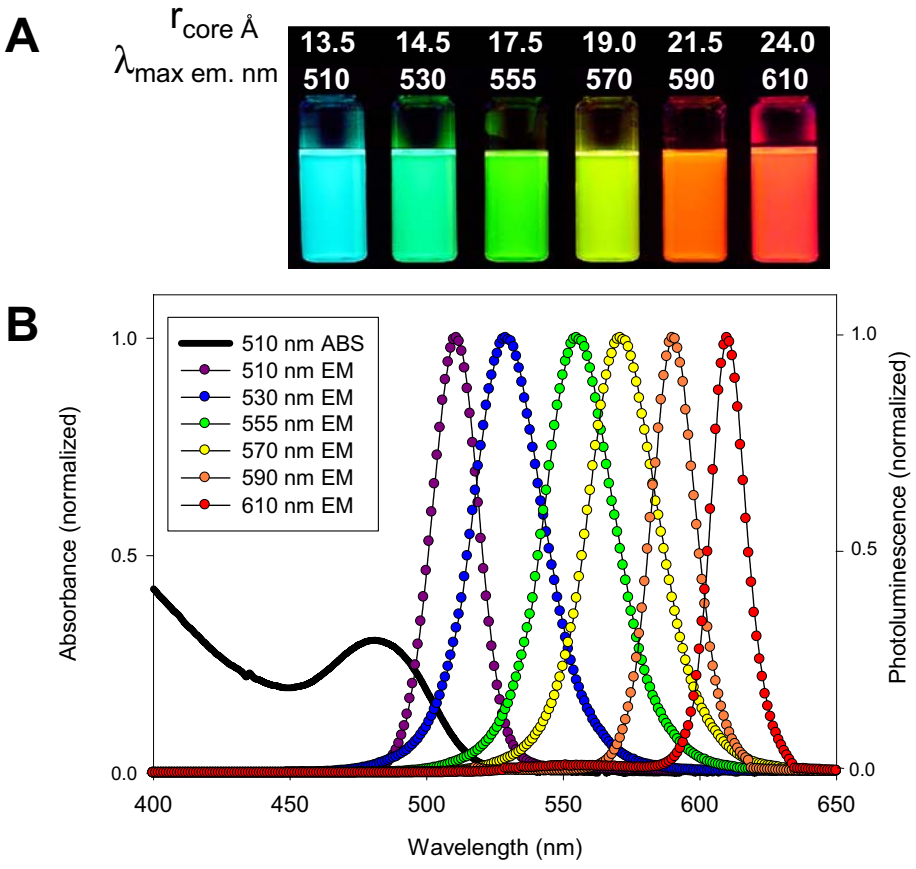

Fig. 1 **a** Six CdSe-ZnS QD dispersions with different core sizes all excited at 365 nm. **b** Normalized absorption and emission properties of the QDs. The *black line*shows the absorption of the 510 nm QDs. Molar extinction coefficients approach values of several million $cm^{-1}M^{-1}$ towards the UV. Figure reprinted by permission of the Nature Publishing Group [4]

(2) electrostatic and other self-assembly approaches [4, 5]. Each has its own benefits and liabilities depending upon the intended application(s). Chemical coupling, primarily based on modifying the COOH groups on QD surface caps with EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide] chemistry for subsequent attachment of amine groups is limited by problems of reproducibility and aggregation [4]. This arises from the inherent instability of the QD when chemically modifying its solubilizing cap and the cross-reaction of the numerous QD-COOH groups with multiple amines on biomolecules, i.e., lysines on proteins [4]. This strategy has been used to create QD-streptavidin conjugates which can then bind biotinylated antibodies [8]. Although avidin-biotin chemistry is robust, the biotin sites on large molecules such as antibodies cannot be controlled, and so the QD-antibody conjugate may not orient correctly, resulting in heterogeneous avidity.

We have developed an alternative approach to form more versatile QD-protein bioconjugates based on non-covalent self-assembly. Proteins engineered to express positively charged domains interact (via electrostatic attractions) with the negative surface of the QDs capped with dihydrolipoic acid (DHLA) ligands. This has allowed us to create QD bioconjugates with mixed-protein surfaces, where each protein imparts a different functionality [9–11]. Engineered maltose-binding protein dimer appended with a leucine zipper domain (MBP-zb) allows purification of the QD bioconjugate over amylose resin while a second protein (e.g., protein G or avidin, see Fig. 2) acts as a bridge for attaching antibodies to the QD [9–11]. In this configuration, avidin serves as a bridge for attaching almost any biotinylated protein to a QD, while engineered protein G specifically interacts with the Fc domain of antibodies. The engineered protein G allows a structured orientation of antibody on the QD with the binding sites facing outward; this can potentially decrease heterogeneity and improve conjugate avidity. We have also utilized metal-affinity-driven self-assembly between proteins appended with a poly-histidine attachment and DHLA-capped CdSe-ZnS QDs to form functional QD bioconjugates [5, 12]. Since many recombinant proteins already express these sequences for purification over metal-affinity media, this approach may allow the creation of a myriad of QD-protein bioconjugates.

Immunoassay development and multiplexing using QD-bioconjugates

The ability to multiplex QD-based immunoassays is where these fluorophores will potentially have the greatest impact in the near future. This includes in vitro assays as well as immuno-labeling in both live and fixed cell specimens. Several immunoassay studies using QDs have been carried out since Chan and Nie reported the initial work on antibody-induced agglutination of QDs [3, 13–15]. For example, QD-based immunological assays to detect the waterborne pathogen *Cryptosporidium parvum* have been

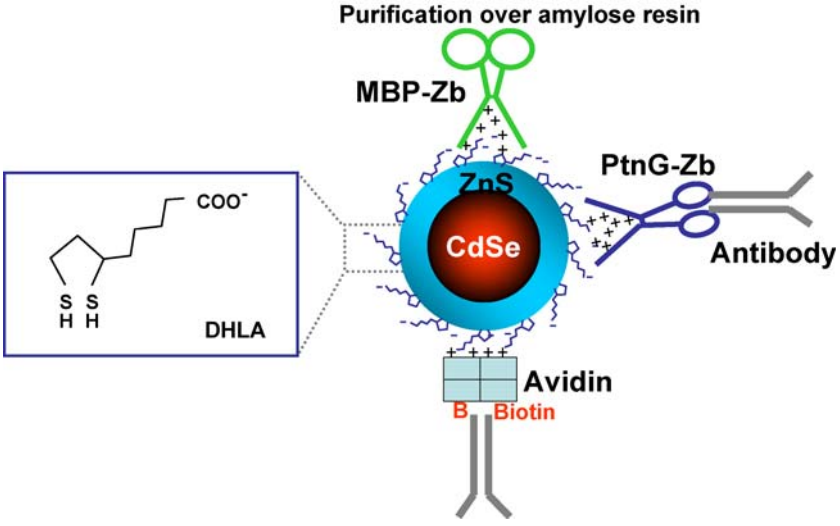

Fig. 2 Schematic of a mixed surface CdSe-ZnS core-shell QD surface-capped with DHLA. Two approaches for creating QD immunoreagents are highlighted. A protein G dimer expressing a positively charged leucine zipper domain (*PtnG-zb*) assembles on DHLA-capped QD surface. The protein G binds to the Fc domain of IgG. Positively charged avidin can also be used as a bridge to attach biotinylated antibodies to QDs. A maltose-binding protein leucine-zipper dimer (*MBP-zb*) allows purification of the QD-bioconjugate over amylose resin [8–10, 19]

demonstrated [14, 15]. By coupling biotinylated antibodies to streptavidin-coated QDs, they showed improvement over the two most commonly used commercial staining kits, due to QD enhanced photostability. They showed higher signal-to-noise ratios (1.5- to 9-fold) and sensitive detection in environmental water samples. There have been other examples including both direct and indirect immunoassays with QDs in microarrays, detection of tumor markers, Western blotting and pathogenic *E. coli* detection [16–19].

We have performed multiplexed sandwich immunoassays using QD-antibody conjugates for the simultaneous detection of cholera toxin, ricin, shiga-like toxin 1, and staphylococcal enterotoxin B (see Fig. 3) [20]. Antibodies specific to each toxin were conjugated to a different population of QDs (identified by their emission peaks): 510 nm QDs for anti-cholera toxin, 555 nm QDs for anti-ricin, 590 nm QDs for anti-shiga-like toxin, and 610 nm QDs for anti-staphylococcal enterotoxin B. A single wavelength was used to excite the nanocrystals, and photoemission spectra were collected using a commercially available microtiter plate reader after examining toxin concentrations of 1,000 and 30 ng/ml. A simple linear equation-based algorithm was used to deconvolute the signal, facilitating simultaneous quantification of all four toxins. Performing the same assay using four different organic fluorophores would require numerous excitation sources/emission windows and complex processing [21]. These same benefits can apply to QD-based immunofluorescent staining in cells; antibody-conjugated QDs have been used to demonstrate multicolor cellular staining [8]. Wu et al. [8] double-labeled both the nuclei and microtubules within the same cell, and showed that QDs were much more resistant to photobleaching than conventional organic dyes under

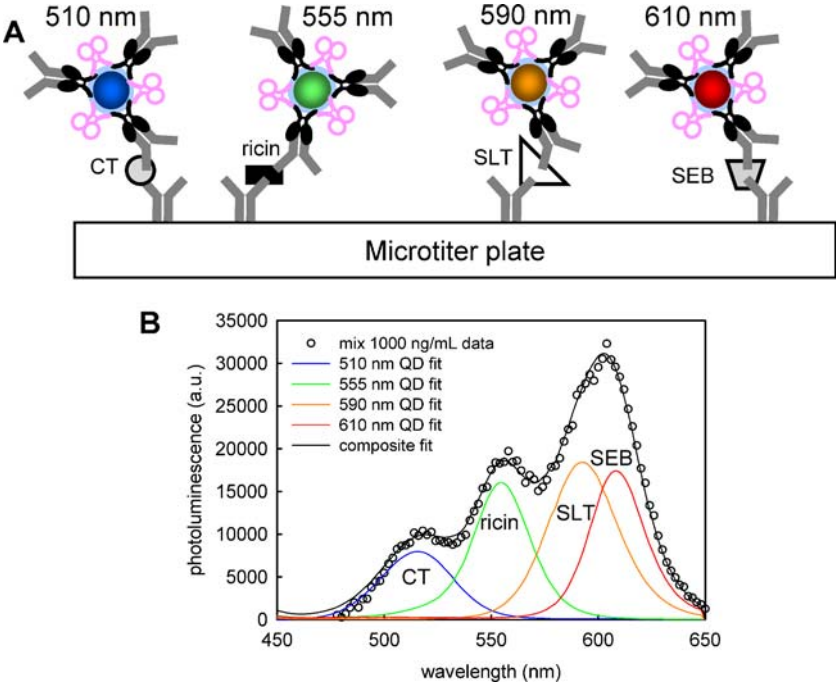

Fig. 3 **a** Schematic of multi-analyte toxin detection. Four toxins [cholera toxin (*CT*), *ricin*, shiga-like toxin (*SLT*), and staphylococcal enterotoxin B (*SEB*)] were detected simultaneously in a sandwich assay format from a single well of a microtiter plate. Antibodies against all four toxins were adsorbed on the wells of 96-well plates, exposed to a mix of all four toxins, and toxins were detected by anti-toxin antibodies conjugated to the various QDs. **b** Data from an experiment in which all four toxins were added at a concentration of 1,000 ng/ml and detected with the QD reagents. Both the composite fluorescence signal and the deconvoluted individual contributions of each color/toxin are shown. Reprinted from [19] with permission from the American Chemical Society

continuous illumination. Multiplexing with QDs may enable multi-target co-localization studies within the same cell. It can potentially allow simultaneous monitoring of multiple cellular events and thus help in the elucidation of complex cellular processes.

There are, however, limits on the number of independent signals achievable for multiplexed immunoassays. The first consideration is the ability to resolve independent QD spectra. Emission spectra separated by only 15 nm in their intensity maxima (where the distributions have similar full width at half max, FWHM ~25–35 nm) can be resolved without difficulty [20]. It may be possible to reduce this to 10 nm or less, but it will be difficult to reduce below some threshold value (~7 nm). Use of CdSe-ZnS core-shell QDs (the most refined for biological purposes) limits the emission window to the 490–630 nm range. Assuming one can resolve populations having emission maxima separated by 10 nm, this suggests an upper limit of ~15 signals and represents a significant improvement over today's technology.

Outlook

QD reagents are commercially available from Quantum Dot Corporation (http://www.qdots.com) and Evident Technologies (http://www.evidenttech.com), and detailed monographs are available describing both synthesis and capping [1, 9, 22]. Some recent reports have raised questions about QD toxicity when used for in vivo cellular labeling and two issues are of primary concern: (1) the presence of heavy metal ions (i.e. Cd, Te, Se) and (2) QD phototoxicity due to reactive oxygen generation [23]. Use of materials that combine a ZnS overcoating with a dense capping layer can prevent metal leeching and minimize phototoxicity. Phototoxicity may be harnessed and exploited for targeted cell killing; QDs have been used as sensitizers in photodynamic therapy [24].

QDs are attractive fluorophores that complement traditional organic dyes and fluorescent proteins and may be superior for certain applications such as multiplexing. Given the high priority currently placed on developing assays for detecting multiple analytes, it is probable that QD fluorophores can contribute to enhanced throughput in this area by facilitating multiplex assays. Researchers have made much progress with the QD materials and have proven their value.

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