Biofunctional Quantum Dots: Controlled Conjugation for Multiplexed Biosensors

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ABSTRACT Semiconductor quantum dots possess unique photophysical properties such as bright emission with narrow wavelength bandwidth and extremely broad and strong absorption. In combination with their size-dependent color tunability, quantum dots have been proposed as ideal candidates for multiplexed optical bioanalysis for more than a decade. However, the unavailability of stable, reproducible, biocompatible quantum dots with controlled and functional multiple biolabeling has restricted these nanocrystals to research applications. In this issue of *ACS Nano*, Jennings *et al.* demonstrate the versatile use of quantum dot antibody conjugates produced by commercially available kits that allow an easy and fast labeling. This Perspective highlights the potential of novel quantum dot bioconjugation approaches in combination with state-of-the-art detection methods and technologies for successful and widely applicable multiplexed biosensing.

Quantum dots (QDs) are inorganic nanocrystals composed of III–V (GaN, GaP, GaAs, InP, or InAs) or II–VI (ZnO, ZnS, CdS, CdSe, or CdTe) semiconductors with a size of approximately 1–10 nm. These small dimensions result in quantum mechanical behavior of the nanoparticles, which results in size-tunable absorption and emission wavelengths (the smaller the QD of the same material, the larger the band gap energy, and thus the smaller the emission and absorption wavelengths compared to the bulk material). Quantum confinement effects were first investigated around 30 years ago,[1−3] and since then, colloidal QDs have been developed with very highly photostability and brightness as well as large extinction coefficients over a wide wavelength range, which allows excitation of different QDs by a single excitation source (single wavelength).[4,5] Due to the many advantages over common fluorophores, use of QDs in biological applications has been quickly evolving over the last 10 years (Figure 1).[6,7]

Color Tunability. The color tunability in combination with the narrow (*e.g.*, ∼20–40 nm for CdSe) and very symmetric emission bands makes QDs attractive for multiplexed optical sensing applications. Multiplexing is an important technique for biotechnology applications, as several different parameters become accessible with a single measurement. For color coding (by using different wavelengths and intensities; *e.g.*, for microarray applications), more than 10 000 different codes can be achieved.[8] When the luminescence intensity is necessary for measuring the difference in concentrations and very low limits of detection are required (*e.g.*, for *in vitro* diagnostics), the number of parameters (*e.g.*, different biomarkers) is limited to approximately 10. In those diagnostic applications, Förster resonance energy transfer (FRET) is often used, and 4- and 5-fold multiplexing has already been realized for QDs as FRET donors and acceptors, respectively,[9,10] whereas 8-fold multiplexing has been achieved using charge transfer from QDs to ruthenium complexes.[11] Despite their outstanding photophysical properties and many demonstrations of their advantages for bioanalysis, QDs have never been integrated into commercial products for the diagnostics market. In this issue of *ACS Nano*, Jennings *et al.*[12] present a performance study of different QDs using sulfhydryl and amine reactive chemistry for antibody conjugation. These commercially available, easy-to-use labeling kits (eBioscience, Inc. San Diego, CA, USA) will

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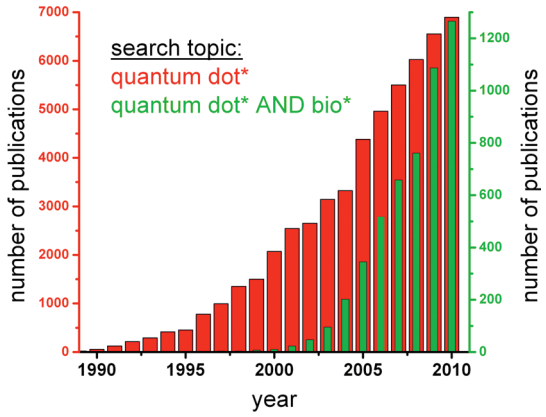

Figure 1. Publications from 1990–2010 concerning quantum dots in general (red) and quantum dots for bioapplications (green). Search performed on June 18, 2011 with ISI Web of Knowledge.

possibly be a helpful tool for many researchers and bring QDs a significant step closer to real-life multiplexed diagnostics.

The authors use five different QDs with emission wavelengths from 525 to 650 nm, which are labeled with antibodies with only a few mixing steps, fast reaction times, and easy spin column purification. Although amine or sulfhydryl chemistries are not new for QD bioconjugation, the presented applications show impressive results concerning the optical and biological functionality of the antibody conjugates, which are the most important aspects for realizing successful multiplexed bioanalysis. The different fluorescent conjugates were successfully and efficiently applied to multiplexed immunoassays (three colors), flow cytometry (four colors), direct cell membrane staining (three colors), immunocytochemistry (two colors), immunohistochemistry (five colors, *cf.* Figure 2B), and endocytosis (two colors), demonstrating the versatile possibilities of using these nanocrystals for real-life multiplexed optical biosensing.

Commercial Availability. The company eBioscience (www.ebioscience.com) currently offers nine different biofunctional QD nanocrystals (based on the technology of Evident Technologies, NY, USA) with emission wavelengths from 490 to 700 nm. Antibody conjugation kits are available at emission wavelengths of 605 and 650 nm. Another color will be added this year, and three more
are expected for 2012. Currently, the choice of different companies providing colloidal QDs is already quite large (*cf.* Table 1). Although the most frequently used and also the best characterized QDs are CdSe/ZnS-based nanocrystals, a trend toward cadmium-free materials is apparent, and even IR-emitting PbS dots are commercially available. In general, it is good news that the former Evident QDs (which have been used quite successfully by many researchers) are available again, and that companies are still investing in optimizing QDs for straightforward and functional biolabeling. This aspect is of paramount importance for successful integration of QDs into multiplexed biosensing.

Approaches for Labeling. There are several reasons why controlled, selective, as well as stable bioconjugate chemistry reactions are needed for QDs: (i) neither the activity of the biomolecule nor the functionality of the QD should be compromised; (ii) the biomolecular orientation should be controlled such that the attachment points of the biomolecule to the QD surface, the distance between the QD and a given moiety of the biomolecule, as well as the amount of biomolecules per QD are predictable and reproducible; and (iii) the QD bioconjugates should be stable in bioassay media (*e.g.*, serum, plasma, blood, *etc.*) and under most assay and storage conditions (*e.g.*, time, temperature, pH, *etc.*). In a recent review, Algar *et al.* provided an interesting overview of the controlled display of biomolecules on nanoparticles (Figure 2C) toward the concept of "bioorthogonal" chemistry.[13] These conjugation concepts offer control over the orientations and positions of biomolecules on the QD surface and therefore go far beyond the random labeling approaches (such as standard EDC chemistry). Especially for nanoparticles, which are of similar or larger size than biomolecules, it is extremely important that binding (or interaction) sites point toward the target of interest and that the biomolecules do not hinder each other from efficiently binding to (or interacting with) this target. Thus, QD labeling concepts require different approaches than, for example, bioconjugation with relatively small fluorescence dyes.

Quantum Dots for Biosensors. Although controlled bioconjugation is necessary for successful life science measurements, biosensors (*in vitro* or *in vivo*) usually have to fulfill several other important requirements in order to be used for commercial applications. In addition to the desire for a multiple-parameter measurement, the detection of the target biomarkers must be extremely sensitive, very selective, and offer high spatial resolution, and the measurement should be reproducible, fast, inexpensive, and miniaturized. For all of these requirements, QDs possess specific advantages compared to other fluorescent markers (Table 2). Of course, QDs also exhibit specific disadvantages. Some disadvantages are unavoidable as they result from the QD specifications, such as the relatively large size, which might alter the natural behavior of small biomolecules. Other disadvantages, such as instability in

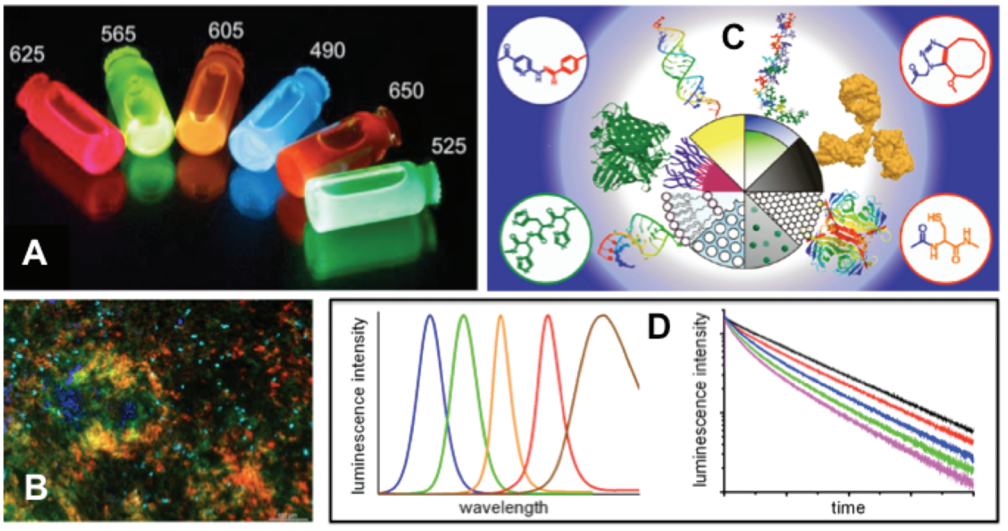

Figure 2. Quantum dots (QDs) offer bright multicolor emission for one single excitation wavelength (A). In this issue of *ACS Nano*, Jennings *et al.* present their successful application in 5-fold multiplexed immunohystochemistry (B). In a recent review, Algar *et al.* presented the importance of controlled bioconjugation of nanoparticles. Reproduced from ref 13. Copyright 2010 American Chemical Society (C). QDs exhibit narrow emission bands and usually multiexponential luminescence decay curves (D), which both can be efficiently exploited with steady-state and/or time-resolved optical technologies for multiplexed biosensing.

TABLE 1. Commercially Available Colloidal QDs (in Alphabetical Order of the Companies)

company	Web site	materials	wavelengths
CAN Hamburg	can-hamburg.de	CdSe, CdSe/CdS and CdSe/CdS/ZnS	*ca.* 480—620 nm
Crystalplex	crystalplex.com	CdSeS/CdS/ZnS and CdSeS/CdS/ZnCdS	*ca.* 450—680 nm
eBioscience	ebioscience.com	CdSe/ZnS and InGaP/ZnS	*ca.* 490—700 nm
Invitrogen/LifeTechnologies	invitrogen.com	CdSe/ZnS and CdSeTe/ZnS	*ca.* 525—800 nm
mkNANO	mknano.com	CdSe, CdS, CdSe/ZnS, CdTe, InP/ZnS, and PbS	*ca.* 380—1500 nm
Nanoco Group PLC/Sigma-Aldrich	nanocotechnologies.com	CdSe, CdS, and CdSe/ZnS	*ca.* 480—640 nm
NN-Labs	nn-labs.com	CdSe, CdS, CdSe/CdS, CdTe, CdSe/ZnS, and InP/ZnS	*ca.* 390—660 nm
Ocean NanoTech	oceannanotech.com	CdSe and CdSe/ZnS	*ca.* 520—630 nm
PlasmaChem	plasmachem.com	CdTe, CdSe/ZnS, and ZnCdSe/ZnS	*ca.* 440—780 nm

biological media or batch-to-batch variations of QD production, could possibly be overcome. Some can even be turned into advantages, such as blinking effects, which have been shown to be useful for super-resolution imaging.[14] A widely discussed issue concerning QDs for *in vivo* applications is their toxicity, which can possibly be overcome by nanoparticle coatings or by using cadmium-free QDs (*e.g.*, InP-based QDs). Although a general evaluation of QD toxicity does not exist and different QDs in different environments show different behaviors concerning toxicity issues, recent *in vivo* studies have revealed no evident toxic effects, even for cadmium-based QDs.[15,16]

In order to exploit the superior properties of QDs for biosensing more fully, the high-performance materials need to be accompanied by high-performance methodologies and technologies. Sensitive detection and discrimination of multiple emission spectra and/or excited state lifetimes (Figure 2D) without optical crosstalk between the different species is extremely important for successful integration of QDs in multiplexed sensing. Highly sophisticated methods such as super-resolution imaging,[17] multiphoton absorption,[18] and energy and charge transfer,[19] in combination with advanced excitation and detection technologies such as pulsed lasers, tunable light sources, confocal microscopes, and time-resolved spectrometers are used to push the QD performance for biosensors to the limits. For all of these specialized applications, quantum dots' photostability, strong and broad absorbance, high brightness and quantum yields, and size tunability are their main advantages over conventional fluorescent markers.

OUTLOOK AND FUTURE CHALLENGES

Despite the disadvantages that have thus far blocked the use of QDs in real-life clinical applications or commercial diagnostic kits, these nanoparticles have a bright future. Keeping in mind the youth of the research field of QDs (Figure 1) and

TABLE 2. Pros (Bold) and Cons (*Italics*) of Quantum Dots for Biosensors

keyword	biosensor requirement	quantum dot properties
sensitivity	very high signal-to-noise ratios	**strong absorbance, high quantum yield, high brightness** *large QD size might lower binding efficiency*
specificity	controlled bioconjugation with highly selective biomolecules	**large surface for many biomolecules; various conjugationmethods available** *large QD size might interfere with biomolecular recognition*
multiplexing	distinction between many single parameters	**color-tunable narrow emission bands; one excitation source for different QDs**
reproducibility	very low coefficients of variation; stable and reproducible materials	*lack of control of bioconjugation; batch-to-batch variations of QD production; stability problems in bioconjugated form*
spatial resolution	super-resolution and/or molecular ruler (*e.g.*, FRET)	**strong absorbance, high quantum yield, high brightness,large surface, and blinking** *large size and blinking*
economy	small (lab-on-a-chip), fast (high-throughput), and inexpensive	**integration into lab-on-a-chip is possible; multiplexed approach allows fast acquisition of multiple parameters at low costs** *biocompatible QDs are expensive*

the vast amount of different QD materials and applications for the life sciences that have appeared over the last 10 years (and still keep appearing), it is not surprising that the necessary but often tedious optimization and regulatory steps (which can be quite difficult, especially for nanomaterials for bioapplications) that are required in order to launch a successful product have not yet been accomplished. Researchers will continue to produce novel and interesting results regarding multiplexed biosensing with semiconductor quantum dots. The fact that more and more companies are developing and providing market-compatible QD products for the life science sector, including optimized stability and bioconjugation, is a good indication that highly sensitive, multiplexed biosensing with QDs may soon be available for detecting real-life biomarkers under real-life experimental conditions. Fruitful cooperation between public and industrial research (as successfully demonstrated by Jennings *et al.* in this issue of *ACS Nano*) will likely be a key issue in reaching this goal. One of the major future challenges remains the development and large-scale production of stable and reproducible QDs with thin, biocompatible coatings that completely preserve the inner functions of the semiconductor quantum dot and provide a biofunctional and biocompatible outside surface for successful implementations into *in vitro* as well as *in vivo* biosensing. Let us see who will arrive there first—publicly supported research, industry, or perhaps both together?

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