CdSe nanocrystal based chem-/bio- sensors

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Semiconductor nanocrystals (NCs) have found application in biology mostly as optical imaging agents where the photophysical properties of the NCs are insensitive to species in their environment. This *tutorial review* examines the application of CdSe NCs as optical sensing agents where the NC's photophysical properties are sensitive to species in their environment. For this case, the NC is modified at the surface with a conjugate, which interacts with an external agent by physical (*i.e.* recognition) or chemical means. Signal transduction in these chem-bio (CB) sensitive NCs is derived primarily from energy transfer between the NC and the external agent, which functions as the energy transfer acceptor or donor. Signaling may be obtained by directly detecting luminescence from the NC and/or the conjugate. New developments for the use of NCs as gain materials in micro-lasing cavities (distributed feedback gratings and spherical resonators) opens the way to designing CB-sensitive NCs for high-gain sensing applications.

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Semiconductor nanocrystals as fluorophores and imaging agents

Inorganic semiconductor nanocrystals (NCs, also known as quantum dots) are a class of fluorophores that have attracted considerable interest owing to their unique photophysical characteristics. The electronic properties of NCs are determined by the physical confinement of excitons, which are excited electrons bound through Coulombic interactions to the holes left behind in the valence band. Quantum confinement in semiconductors occurs when the dimensions of the NC approaches that of the exciton. The effective bandgap of the NC widens with decreasing size, giving rise to unique, size-dependent optical and spectroscopic properties.[1] Broad absorption profiles and high extinction coefficients are complemented by a narrow (full-width-half-maximum ~30 nm) and spectrally tunable emission profile. Fig. 1 shows the size-dependent optical properties of CdSe nanocrystals. For

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Fig. 1 Size-dependent emission properties of highly luminescent CdSe/ZnS NCs. From left to right, solution emission maximums are at 470, 480, 520, 560, 594, and 620 nm. Photograph by Felice Frankel. Quartz cuvettes courtesy of Spectrocell Inc. (Reproduced with permission from *J. Phys. Chem. B*, 1997, **101**, 9463.[3] Copyright 1997 American Chemical Society.)

example, small (2.3 nm diameter) CdSe NCs emit blue light under optical excitation whereas their larger counterparts (5.5 nm diameter) emit red light. The NCs exhibit appreciable quantum efficiencies for emission, though coating the outer surface of NCs with higher band gap inorganic materials further improves the photoluminescence quantum yield. The coating presumably passivates sites associated with surface states that promote nonradiative recombination.[2,3] Along with higher quantum yields, ''overcoated'' core/shell CdSe/ZnS NCs are more robust and possess high photobleaching thresholds.[1] These properties of NCs contrast with organic dyes, which tend to possess low resistance to photobleaching, narrow absorption profiles, and emission spectra that tail to the red; the spectral congestion that results from the tailing emission profiles can complicate multi-color imaging applications.[4]

Despite the apparent advantages of NCs as compared to organic dyes, the implementation of NCs for fluorescence imaging was initially hampered by their insolubility in aqueous media as a result of the long-chained organic solvents needed for their high-temperature synthesis. This obstacle was overcome by further modifying the surface of the CdSe NCs.[3] Alivisatos and co-workers prepared bio-compatible CdSe/CdS or CdSe/ZnS NCs by adding a third layer of silica to the core/shell.[5] Nie and co-workers reported the use of mercaptoacetic acid to cap the surface of CdSe/ZnS NCs to impart water-solubility.[6] The synthesis of water-soluble NCs now includes modifications with phospholipids,[7] amphiphilic polymers,[8] dendrimers,[9] oligomeric phosphines[10] and cap-exchanging the hydrophobic surface of NCs with multidentate hydrophilic ligands.[11] These ligands have greatly facilitated the use of NCs as bio-imaging agents and probes because they can be conjugated to proteins and peptides, such as streptavidin for cell-labeling studies.[4] The stability and quantum yields of such water-soluble NCs greatly vary among the different solubilization methods. For example, mono-thiol caps are photochemically unstable,[12] whereas multidentate ligands are reported to yield an aggregate-free construct and to be stable in solutions of pH 5–12 over a course of one year with quantum yields around 25–30%.[11] Toxicity of NCs is a concern for biological *in vivo* experiments; however, most studies report no toxicity in live animals, even in embryos under standard conditions.[4] Oxidation of CdSe by air or UV light can cause Cd^{2+} to be released, which is toxic to cells.[13] Protection of NCs from oxidation reduces toxicity, but long term cadmium leaching from NCs has not been studied.

More recently, the utility of NCs has been expanded by their use as optical sensors.[14] Initial applications centered on physical sensing. Walker *et al.* used the temperature-dependent photoluminescence properties of CdSe/ZnS nanocrystals embedded in poly(lauryl methacrylate) to develop a temperature probe.[15] These initial studies have subsequently been elaborated for the measurement of temperature[16] and fluid flow near walls and in confined channels[17] opening the way for tandem flow measurements of temperature and velocimetry.

The water-solubility of overcoated NCs has rapidly led to their use as fluorescence chemical sensors. For this application, NC luminescence is perturbed by the presence of a target analyte in the NC's environment. In some cases, the fluorescence is suppressed by morphological changes to the lattice. For example, Ag^{+}, Pb^{2+}, and Cu^{2+} ions quench NC luminescence by replacing the Cd^{2+} ions in the nanocrystal lattice.[18] Only by overwhelming the NC with excess Cd^{2+} can luminescence be partially recovered. However, permanent quenching of NC luminescence is not practical as a sensing strategy, as it is usually not analyte specific, nor is it easily reversible. Recently, detection of specific chem-/bio- (CB) target analytes has been achieved reversibly using overcoated CdSe NCs that have been modified with a conjugate attached to the overcoated layer. We note that work has also been performed with NCs composed of materials other than CdSe, most notably CdTe.[19] However, the concepts of sensing and amplification strategies are most comprehensively embodied by CdSe. For this reason, CdSe NC constructs, together with the excited state mechanisms that underpin their sensing function, constitute the scope of this review.

Fluorescence energy transfer

Fluorescence resonance energy transfer (FRET) has been the primary photophysical mechanism by which NCs report the presence of a target analyte. FRET is a phenomenon in which photo-excitation energy is transferred from a donor fluorophore to an acceptor molecule. Förster theory correlates the rate for this energy transfer to the spectral overlap of donor emission and acceptor absorption and the donor–acceptor spatial arrangement.[20] The rate of energy transfer, $k_{D\rightarrow A}$, is given by eqn (1) and (2),

$$k_{\mathrm{D}\rightarrow\mathrm{A}} = \frac{1}{\tau_{\mathrm{D}}}\left(\frac{R_0}{r}\right)^6 \tag{1}$$

$$R_0 = \frac{9000\ \ln(10)\phi_{\mathrm{D}}\kappa^2}{128\pi^5 n^4 N}\int_0^\infty \mathrm{d}\nu \frac{f_{\mathrm{D}}(\nu)\varepsilon_{\mathrm{A}}(\nu)}{\nu^4} \tag{2}$$

where τ_D and ϕ_D are the lifetime and quantum yield of the donor, respectively, r is the distance between donor and acceptor, R_0 is the critical transfer distance, n is the refractive index of the medium, N is Avogadro's number, and κ^2 is a constant reflecting the relative orientation of donor and acceptor dipoles. Although the NC is a fairly large object in proximity to the dye, NC-FRET studies make an approximation to treat the NC excited state as an oscillating point dipole and use $\kappa^2 = 2/3$ for a random orientation. Even with the point dipole approximation, many studies described below report that treating the NC based energy transfer through Förster theory described experimental data consistently. The overlap integral is comprised of $f_D(\nu)$, the normalized fluorescence intensity of the donor in wavenumbers (cm^{-1}), and $\varepsilon_A(\nu)$, the extinction coefficient of the acceptor. The R_0 is the distance where 50% of the excited donor will decay back to its ground state and the other 50% will transfer its energy to the acceptor molecule. As shown in eqn (2), the transfer distance is dependent mainly on the spectral overlap between the emission of the donor and the absorbance of the acceptor.

The efficiency (E) of FRET, or the fraction of photons absorbed by the donor that are transferred to the acceptor, is the ratio of the transfer rate to the total decay rate of the donor and is described by eqn (3):

$$E = 1 - \frac{\tau_{D-A}}{\tau_D} = \frac{k_{D-A}}{k_{D-A} + \tau_D^{-1}} = \frac{R_0^6}{R_0^6 + r^6} \qquad (3)$$

Eqn (3) can be modified when more than one acceptor can interact equally with the donor as shown in eqn (4):

$$E = \frac{mR_0^6}{mR_0^6 + r^6} \qquad (4)$$

Therefore, eqn (4) shows that the efficiency of energy transfer is generally enhanced by increasing the number of acceptor molecules, m. It is important to note in a study that utilizes one donor and multiple acceptors, any distance measurements derived will represent a statistical average distance in a given solution of donor–acceptor pairs unless every pair has an identical fixed distance between the donor and acceptor and an identical number of acceptors per donor.[21] As the energy transfer rate constant and corresponding efficiency depend on the inverse sixth power of intermolecular separation of donor and acceptor molecules, usually on lengths scales between 20 to 90 Å, the study of FRET is useful for distances comparable to biological macromolecules and is often applied for sensing changes in protein conformation and analyte binding events.[20] Investigations of FRET with NCs that have formed the underpinning for the use of NCs as chemical sensors are presented in the section below.

NC energy transfer

Energy transfer among CdSe NCs was observed by Kagan *et al.* when they prepared thin films comprised of a mixture of closely-packed smaller 555 nm emitting and larger 620 nm emitting NCs.[22] Steady-state emission from the smaller NCs decreased concomitantly with an increase in emission from the larger NCs. Correspondingly, the lifetime of the smaller NCs shortened while that of the larger NC lengthened. Analysis of the lifetime data by Förster theory supported the occurrence of energy transfer. This study established that CdSe NCs can act as both FRET donors and acceptors.

Energy transfer involving CdSe NCs in aqueous media has been observed to occur between oppositely charged CdSe/ZnS NCs. Wargnier *et al.* prepared negatively charged CdSe/ZnS by treating the NCs with a mixture of mercaptosuccinic and mercaptosulfonic acids.[23] Positively charged ZnCdSe–ZnS NCs were synthesized by treating the NC surface with cystinamine. Upon mixing with larger NCs (which naturally carry a negative charge), emission from the positively-charged and smaller NCs was found to be quenched. On the basis of the diameters of the NC donor and acceptors, a Förster critical distance and efficiency were calculated to be 7.3 nm and 91%, respectively; these values were in reasonable agreement to the Förster critical distance and efficiency determined from the quenching of the PL decay times.

NCs as FRET donors

Energy transfer from a NC donor to an organic acceptor in aqueous solution was demonstrated by Willard *et al.*[24] Water-soluble CdSe/ZnS NCs were conjugated to a thiolated biotinilated bovine serum albumin (bBSA, 9 : 1 biotin : BSA ratio). Calculations revealed that 11 bBSA molecules were bound to the surface of the NC. The acceptor complex was prepared separately by conjugating streptavidin to tetramethylrhodamine. Upon titrating the dye-modified streptavidin to the NC–bBSA complex in PBS buffer, NC luminescence was quenched and TMR fluorescence was enhanced.

A penetrating study of energy transfer between donor NCs and acceptor dye molecules has been performed by Mattoussi and co-workers.[21,25] Water-soluble CdSe/ZnS NCs were prepared by exchanging ligands on the NC surface with dihydrolipoic acid, which possesses bidentate thiol groups. A maltose binding protein (MBP) from an engineered *Escherichia coli* containing site-specific labeled dye acceptors was assembled onto the surface of the NC by either: (1) electrostatic self-assembly of the negatively charged dihydrolipoic acid to a basic leucine zipper on the MBP or (2) metal-affinity coordination between the NC surface and a C-terminal oligohistidine chain on the MBP (see Fig. 2).[21,25] In synthesizing these constructs, the emission wavelength of the NC and the number of dye-labeled MBPs bound to the NC were systematically varied. The total number of MBPs per NC was maintained for the different-sized NCs in order to maintain the average distance between the NC and the acceptor dyes and hence the same NC quantum yield. As the fraction of dye-labeled MBPs was increased, emission from the dye increased while that from the NCs decreased. Time-resolved fluorescence experiments confirmed that the NC lifetime shortened as more dye-labeled MBPs surrounded the NCs.[21,25] Additionally, experiments varying the spectral overlap (by changing the NC donor emission wavelengths) revealed that the efficiency of energy transfer varied as expected from a Förster model. As expected from eqn (4), efficiency improved with increasing numbers of dye-labeled MBPs around the NC.[21] All of these studies support an energy transfer mechanism between a CdSe NC donor and an organic acceptor dye.

Energy transfer between a NC donor and a dye acceptor has also been observed at the single molecule level.[26] Hohng *et al.* used a commercially available streptavidin-coated NC to immobilize the NC on a quartz surface coated with bBSA. A cyanine dye, Cy5, was positioned near the NC surface by placing it with biotin on the same end of a duplex DNA. Energy transfer was monitored with the steady-state emission of the NC and Cy5. Observation of a single-molecule conformational change of the NC–Cy5 conjugate was also sought using a Holliday junction, which is composed of four DNA helices. The DNA junction changes its fold in the presence of divalent ions. Conformational change induced by the divalent ion would, in principle, lead to a change in FRET efficiency. However, very low signal intensity complicated such an interpretation. The low signal was ascribed, in part, to the very large energy transfer distance between the streptavidin-coated NC and biotinylated dye, emphasizing the need for simple conjugates in which the energy transfer distance can be short.

Zhou *et al.*[27] directly coupled an Alexa 594-labeled DNA acceptor to a NC donor through a thiol linker. Commercially obtained CdSe/ZnS NCs were treated with 3-mercaptopropionic acid (MPA) to produce a water-soluble NC. Coupling to Alexa 594-labeled DNA was achieved using a C_6 thiol linker. The ability to couple without protein linkers minimized the distance between the NC and the dye. Owing to the short donor–acceptor distance, the FRET efficiency in these constructs was found to be as high as 88% in ensemble and single-molecule constructs. A Förster critical transfer distance of 4.2 nm was calculated from the quenched luminescence.

NCs as FRET acceptors

Whereas there have been numerous studies on CdSe NCs as FRET donors, very few studies have reported CdSe NCs as FRET acceptors. Mattoussi and co-workers investigated the potential of CdSe NCs as energy acceptors from organic donor dyes by using a NC–dye MBP conjugate in which the dye emitted to the blue edge of the NCs.[28] AlexaFluor 488 and Cy3 dyes were employed to excite NCs of three different sizes. No evidence of energy transfer from the dye to the NC was found in steady-state and time-resolved emission experiments for dye : NC ratios up to 1 : 10. Tryptophan residues in the MBP were also excited to see whether FRET could occur between the amino acid and the NC. But again, there was no evidence of tryptophan quenching. Energy transfer from an organic dye to a CdSe NC is difficult to achieve for two reasons. First, the radiative decay rate of the dye donor excitation energy is fast compared to the decay rate for FRET from dye to the NC acceptor. In addition, the NC could be directly excited, which

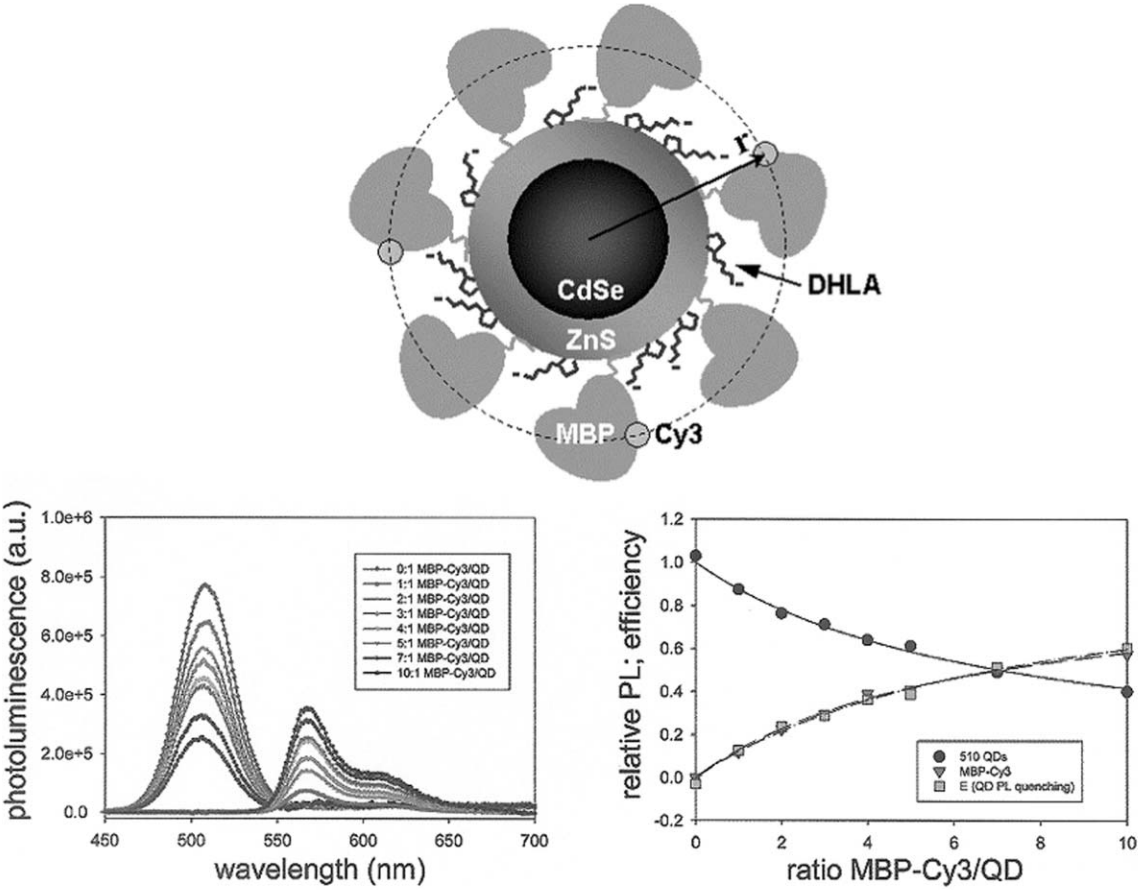


Fig. 2 (Top) A schematic representation of the NC-MBP assembly developed by Mattoussi and co-workers. The dihydrolipoic acid (DHLA) caps the NC and allows the MBP to self-assemble onto the NC. A Cy3 dye is attached to the MBP. (Bottom left) Photoluminescence spectra from 510 nm emitting NC and Cy3 dyes in the NC–MBP–Cy3 construct with increasing dye to NC ratio. (Bottom right) Experimental values for the NC emission decay percentage *versus* dye to NC ratio (circle), the rate of FRET extracted for the NC emission loss (triangle), and the rate of FRET deduced from acceptor gain (square). (Reproduced with permission from *J. Am. Chem. Soc.*, 2004, **126**, 301.[21] Copyright 2004 American Chemical Society.)

causes difficulties in ascertaining the FRET contribution to the observed NC emission. In order to test whether the lifetime of the donor was limiting FRET, a dye with a longer-lived excited state (a ruthenium–bpy–isothiocyanate complex with τ_o = 350 ns) was covalently appended to the MBP. Steady-state fluorescence experiments, however, were inconclusive owing to a high degree of emission spectral overlap between the ruthenium complex and the NC together with the low quantum yield of the complex. Time resolved measurements, however, did reveal quenching of the dye excitation lifetime, implying the occurrence of FRET.

Further support for the use of CdSe as energy acceptors has been provided by Acherman *et al.*, who have reported energy transfer between InGaN quantum wells and CdSe NCs.[29] A three-layer heterostructure was made by depositing a layer of CdSe NC through the Langmuir–Blodgett technique onto a GaN-capped InGaN quantum well. In this case, energy transfer rate of carrier varied as $1/d^4$, where d was the separation distance between the quantum well donor and the NC acceptor. The efficiency of energy transfer was found to be as high as 55%.

Blue-emitting polymers may act as FRET donors to NCs at 18 K.[30] Energy transfer from a conjugated organic polymer, {poly[(9,9-dihexylfluorenyl-2,7-diyl)-alt-co-(9,ethyl-3,6-carbazole)]} to CdSe/ZnS NCs was confirmed by the quenching of the polymer lifetime and by the appearance of NC emission upon excitation of the polymer. The calculated Förster radius of 8 nm (though this value is somewhat tenuous since it could not be corrected for direct excitation of the NCs) is comparable to that of organic FRET donor–acceptor pairs.

Commercially biotinylated NCs and streptavidin can be conjugated to a terbium ion ligated by two 6-carboxybipyridyl arms connected to a glutamate framework.[31] Carbodiimide coupling of the terbium complex yields an average of 3.5 terbium complexes per streptavidin protein. The NC was coated with 5 to 7 biotin molecules per NC. Upon titrating the biotinylated NC to the streptavidin–Tb complex, the lifetime of the NC is enhanced and the ratio of NC to Tb emission increased as well. These observations were attributed to the presence of FRET. An average donor–acceptor distance of 7.2–7.6 nm was determined for the FRET.

Anikeeva and co-workers have demonstrated energy transfer from a phosphorescent *fac* tris(2-phenylpyridine) iridium complex, $Ir(ppy)_3$, to a thin-film monolayer of CdSe/ZnS.[32] The $Ir(ppy)_3$ emission intensity decreased by 21% and the emission intensity of the CdSe/ZnS film increased by 55% when a monolayer of the NCs was printed on a film of 10% $Ir(ppy)_3$. A corresponding increase in the lifetime of the NC emission from 40 ns to 400 ns suggested the transfer of $Ir(ppy)_3$ excitons to the NC film. The distance between the iridium complex and the NC was estimated to be 4.0 nm, which is sufficiently short for Förster and Dexter (*i.e.*, correlated electron exchange) energy transfer to occur.

FRET schemes for sensing with NCs

The ability of NCs to participate in FRET provides a mechanism for signal transduction in optical sensing schemes. Fig. 3 summarizes the different sensing strategies that have been employed with NCs. Of the six signal transduction mechanisms, five rely on the NC as the FRET donor. The broad excitation spectrum of the NCs complicates their utilization as FRET acceptors since it is difficult to excite a dye selectively without exciting the NC acceptor. In Fig. 3A–3D, sensing is accomplished by modulating the FRET donor–acceptor distance. For Fig. 3E and 3F, the FRET distance is preserved but the analyte causes the spectral overlap function to change, thus modulating the efficiency of FRET upon analyte recognition. A discussion of systems that sense by each of the mechanisms presented in Fig. 3 follows.

3A: Sensing by nucleic acid recognition

Fig. 3A has been exploited to probe biological activity *via* FRET. Telomerization and DNA replication can be monitored with CdSe/ZnS NCs.[33] Patolsky *et al.* conjugated NCs to thiolated oligonucleotides with approximately 25 oligonucleotides per NC. Incubation with a dNTP (deoxy(nucleotide) triphosphate) mixture (dATP, dCTP, and dGTP) and Texas-Red labeled dUTP, in the presence of telomerase, initiated a change in the emission spectra of the NC and the dye over time. As telomerization proceeded, NC emission decreased and Texas-Red emission increased due to FRET. DNA replication was probed by labeling CdSe/ZnS NCs with a DNA primer, followed by incubation with the complementary DNA sequence to allow for hybridization. Replication was initiated by adding polymerase mixed with dNTPs and Texas-Red labeled dUTP. As replication progressed, the Texas-Red dUTP was brought in proximity to the NC, resulting in FRET from the NCs to the dye. These results suggested the application of NCs for the detection of cancer cells or for amplified detection of DNA on chip arrays.

Gill *et al.* have used FRET interactions of DNA to probe hybridization and cleavage.[34] In a similar manner to that discussed above, NCs were modified with a DNA strand and hybridized with a Texas-Red labeled nucleic acid. As hybridization proceeded, energy transfer occurred, and the NC emission progressively became weaker while the dye emission increased. The resulting CdSe NC–dye DNA duplex was cleaved with a hydrolytic enzyme DNase I. Cleavage of the DNA strand resulted in a partial restoration of the NC emission and a loss of dye emission. The incomplete recovery of the NC emission was attributed to the adsorption of dye molecules to the NC surface. Adsorption by the NCs presents a hurdle for their implementation as bioanalytical tools.

FRET from NCs can be used as a screen for small interfering RNAs (siRNA).[35] Bakalova *et al.* conjugated a single stranded siRNA to a NC to yield a hybridization probe while amplifying the target mRNA in the presence of Cy5 labeled nucleotides. The Cy5–mRNA was used as the hybridization template. A short hybridization time for siRNA–mRNA duplexes ensured for the selection of efficient, target-selective siRNA sequences. The affinity of the siRNA to the mRNA was detected by FRET from the NC to the Cy5. The Cy5 emission was only detected when there was good accessibility and high affinity between the two RNA strands.

The interaction of a HIV-1 regulatory protein, REV with its responsive element (designated RRE), has also been

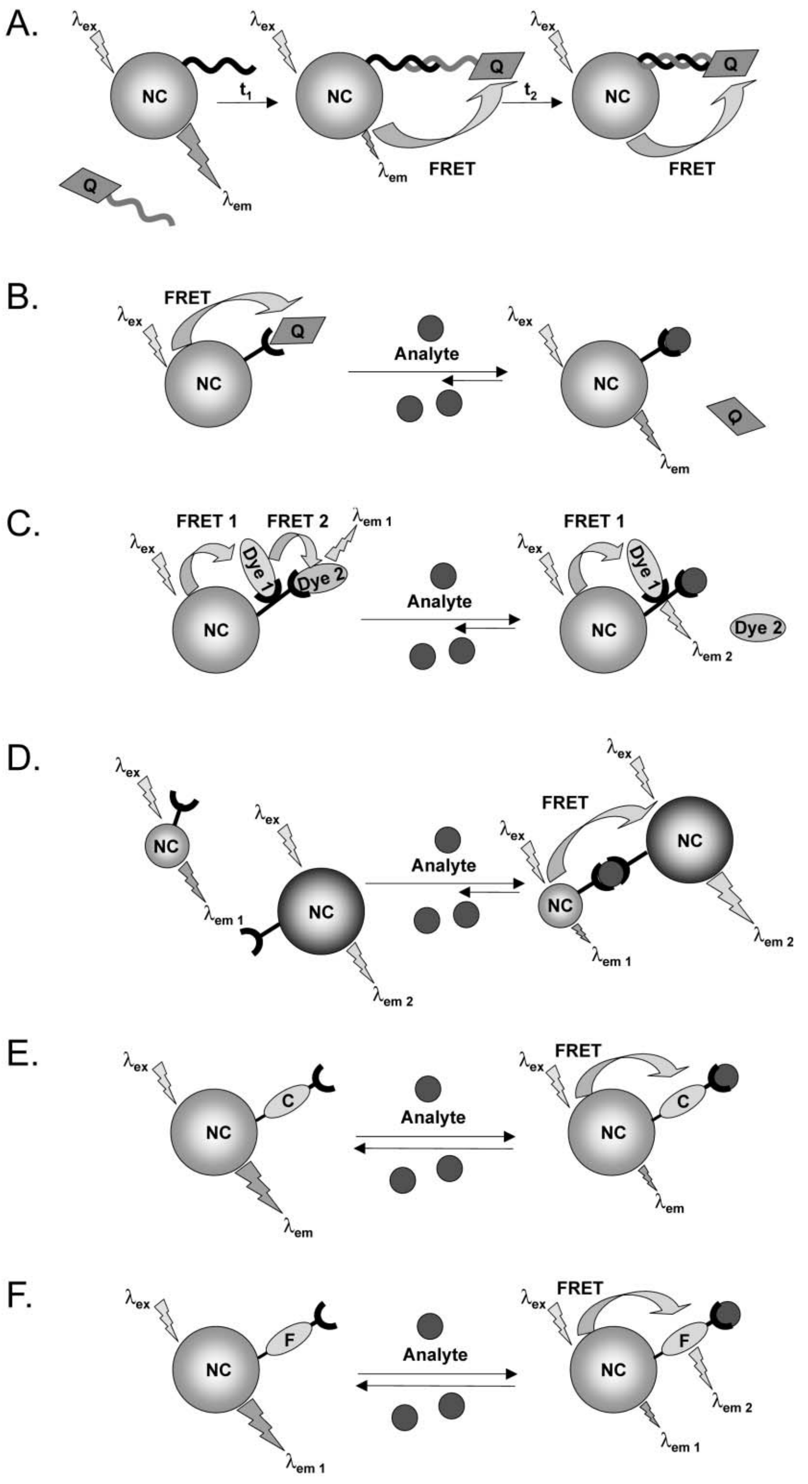


Fig. 3 Schematic diagram of the six different sensing strategies using FRET that are the focus of this tutorial review.

assayed by NC–dye FRET.[36] The 5′ end of the RRE RNA was biotinylated while a Cy5 dye was attached to the N-terminus of a model REV peptide sequence. A RRE–NC conjugate was synthesized by using a streptavidin-coated NC. The Cy5 modified REV was slowly titrated to the RRE–NC solution. Excitation at 488 nm, where the Cy5 does not absorb, yielded emission from both NC and Cy5 upon complexation of the REV to the RRE–NC. The ratio of the dye intensity to NC intensity increased with the addition of REV.

Per Fig. 3A, the foregoing bioassays rely on modulating the FRET distance between the NC and an acceptor dye upon biological recognition of different macromolecules. A change in emission lifetime and intensity may be detected. Applications based on this model assume that the NCs do not interfere with macromolecule binding as a result of either size or charge perturbations. This assumption must be assessed for the development of reliable bioassays.

3B and 3C: Sensing by analyte-induced displacement

FRET coupled to quenching, as schematically represented in Fig. 3B and 3C, provides alternative strategies for sensing. Here, luminescence due to FRET is turned on by the appearance of analyte, which displaces a quencher (Fig. 3B) or a terminal energy acceptor (Fig. 3C).

Mattoussi and co-workers have developed a sensor for maltose by adapting their CdSe–MBP conjugates for both analyte-displacement strategies depicted by Fig. 3B and 3C.[37,38] In the first construct, a β-cyclodextrin (β-CD) conjugated to a non-fluorescent QSY9 quencher dye was docked to the MBP saccharide binding site of the CdSe/ZnS–MBP. Maltose displaces the β-CD–QSY9 conjugate to restore NC emission. In their second strategy, defined by Fig. 3C, the CdSe/ZnS–MBP construct is labeled with two different cyanine dyes: Cy3, which is bound directly to the MBP, and a β-CD conjugated to a Cy3.5, docked to the MBP binding site. Prior to maltose binding, Cy3.5 emission prevails by a two-step energy transfer from Cy3 which in turn accepts energy from CdSe. Upon binding to maltose, the β-CD–Cy3.5 conjugate is displaced and, in the absence of the terminal acceptor, Cy3 is the predominant emitting species.

A hybrid NC–antibody that senses TNT operates by the mechanism shown in Fig. 3B.[38] In place of MBP, an anti-TNT specific antibody fragment is appended with an oligohistidine sequence, which is bound to the surface of a CdSe/ZnS NC. A TNT analogue pre-labeled with a quencher dye (Black Hole Quencher-10, BHQ-10) was pre-docked in the recognition site of the antibody. The docked dye quenches NC emission. The NC emission is ''turned-on'' when TNT displaces the quencher from the sensor, as shown in Fig. 4. The specificity of the TNT sensor was tested by comparing the effectiveness of FRET from the NC to the quencher dye for three different TNT analogues. The analyte specificity of the original antibody fragment was conserved after being bound to the NC. This approach is general and constructs involving an antibody fragment bound to a NC surface through noncovalent self-assembly should find wider use as a target of other analytes of interest.

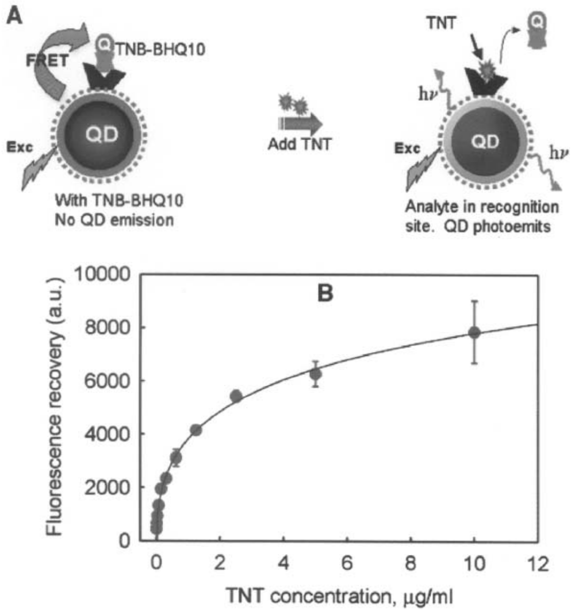


Fig. 4 (A) A schematic diagram of the quencher-displacement mechanism to sense TNT. (B) Increase in the NC photoluminescence *versus* concentration of TNT. (Reproduced with permission from *J. Am. Chem. Soc.*, 2005, **127**, 6744.[38] Copyright 2005 American Chemical Society.)

3D: Sensing by NC-to-NC FRET

The NC–dye constructs incorporating analyte-displacement strategies are excellent single-response sensors. To realize maximum sensitivity and utility, however, a more ideal sensor would be reversible and/or self-referencing (or ratiometric). One such scheme is shown by Fig. 3D. FRET between small (green) and large (red) CdSe/ZnS nanocrystals has been implemented to sense potassium ions.[39] 15-crown-5 ethers were adsorbed onto the surface of the NCs through the bidentate thiol of dihydrolipoic acid. Prior to adding K^+ ions, both green and red NC emissions are present; the NC solutions were dilute enough to prevent energy transfer between the two NCs. Upon adding $KClO_4$, the green emission of the smaller NCs gradually decreased, while the red emission of the larger NCs increased. This synchronous change in emission intensities was ascribed to an energy transfer mechanism caused by aggregation of the NCs. The aggregate formed by the recognition of K^+ ions by the 15-crown-5 ethers to furnish a 15-crown-5–K^+–15-crown-5 sandwich complex. It was unclear whether the sandwich complex was formed from the coordination of two ether molecules from the neighboring arms of the same NC (intraparticle association) or from the two ether molecules of different NCs (interparticle association). A response was observed over a 10^{-6}–10^{-4} M concentration of $KClO_4$; the NCs were observed to precipitate from solution at higher $KClO_4$ concentrations. The sensing construct showed excellent sensitivity; however, high association between the K^+ ion and the crown ether as well as aggregation between the NCs at high concentrations makes reversible sensing difficult.

3E: Sensing by NC conjugation to analyte-sensitive chromophores

A reversible CdSe/ZnS NC sensor of pH operates according to Fig. 3E.[40] A pH-sensitive chromophore was conjugated to NC with a lipoic acid derivitized with a [1,3]oxazine ring. The [1,3]-oxazine ring may be opened by hydroxide anion to generate a 4-nitrophenylazophenolate chromophore. The absorption spectrum of this chromophore overlaps with the emission spectrum of the NC. This spectral overlap activates FRET, leading to a diminution of NC luminescence. Specifically, the NC emission intensity decreases by 35% over the pH from 7.1 to 8.5. The sole emission from the NC results in a detection signal that is not referenced, making calibration difficult.

3F: Sensing by NC conjugation to analyte-sensitive lumophores

In general, pH, or for that matter, the concentration of any analyte is difficult to quantify with a simple change in emission intensity of donor and/or acceptor. An accurate measurement of analyte concentration is unattainable if the overall background intensity changes, emission from species in the environment is present, or if the dye emission is sensitive to an interferent. These problems can be circumvented by constructing a reversible and ratiometric sensor according to the mechanism shown in Fig. 3F.

The construction of a CdSe/ZnS NC sensor of pH per Fig. 3F[41] was begun by encapsulating a CdSe/ZnS NC in an amphiphilic polymer in a similar manner as reported by Wu *et al.* to impart water solubility.[8] The polymer scaffold provides a hydroxy functionality for coupling organic molecules to the NC. Using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) coupling, a pH-sensitive squaraine dye was covalently attached *via* ester linkages. The absorption and the emission spectra of the squaraine dye are pH sensitive. The dye is highly absorptive and emissive at low to neutral pH. With increasing pH, the overall emission intensity decreases monotonically as shown in Fig. 5. The ratiometric nature of the sensor is derived from the modulation of the FRET efficiency arising from the engineered overlap of the pH-dependent squaraine dye absorption spectrum with the (pH-insensitive) NC emission spectrum. Upon excitation of the CdSe/ZnS NC, the NC may either fluoresce or transfer energy to the squaraine dye. At low pH, the overlap of the dye absorption spectrum and NC emission spectrum is small owing to the low absorption cross-section of the former. Under these conditions, FRET is efficient and the dye is more emissive than the NC owing to energy transfer. As the pH is raised, energy transfer from the NC to the dye is less efficient owing to poorer spectral overlap. The inset of Fig. 5 depicts the variation of the critical transfer distance (R_0) with solution pH due to the modulation of the overlap integral of eqn (3). The efficiency of the sensor is approximately a linear function of the modulation of the overlap of the NC and dye emission. Time-resolved fluorescence spectroscopy and excitation spectra provided further support for the energy transfer mechanism.

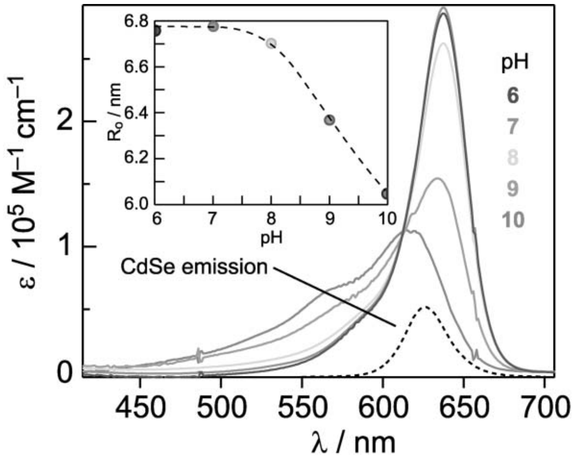


Fig. 5 The pH-dependent absorption profile of a squaraine dye overlaid with the emission of the CdSe/ZnS NC. The inset depicts the variation of the critical transfer distance due to the modulation of the spectral overlap integral by solution pH.

As a result of the FRET efficiency modulation, the emissions of the NC and dye were found to be naturally ratiometric. For a simple two-state system, with modulated energy transfer, an isosbestic point should be maintained in the photoluminescence spectrum. This is observed in Fig. 6. The relative emission intensities vary significantly with the largest changes occurring near the dye pK_a; an isosbestic point is maintained at 640 nm. The solution pH can be read out precisely by taking the ratio of emission peak intensities (NC and dye) to the intensity at the isosbestic point, which functions as an internal reference. This ratiometric approach is powerful when compared to typical CBsensors that display a single intensity-based response to analytes (*i.e.* either brightening or darkening) because the ratiometric construct is not sensitive to fluctuations of light excitation or collection efficiency as sensing is self-referencing. This is shown in Fig. 7. The pH may be determined accurately to 5% in highly scattering environments or when the excitation intensity is highly fluctuating. Such precision is impossible to achieve for the dye alone. In addition, the continuous absorption manifold of the CdSe NCs results in an excitation-wavelength independent bandshape, a feature that is not present in single molecular ratiometric sensors. Thus, the NC–dye conjugate does not need to use two independent excitation sources (or alternatively a single excitation specifically at an absorptive isosbestic point) for proper function. The approach is completely general for any analyte that modulates FRET involving the NC. The reversible and ratiometric nature of the approach presented by Fig. 3F makes NCs versatile agents for chemical and biological sensing.

CdSe as gain material in lasing cavities

The physical properties and dimensions of NCs naturally lend them to sensing on small length scales. On this count, NCs have a significant advantage over conventional molecular chemosensors. Owing to their photostability and spectrally narrow gain profiles, NCs can function as gain materials for

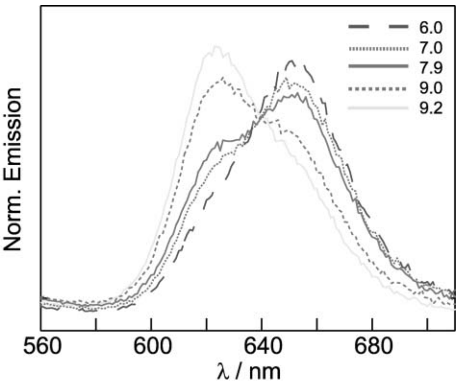


Fig. 6 Emission spectra of the NC–squaraine dye construct at different pH. The isosbestic point at 640 nm allows for ratiometric sensing.

laser cavities on micro-dimensions.[42] The ability to amplify NC output signals in such cavities offers unique opportunities for detecting the signal from NC sensor constructs on small length scales.

As CBsensing is scaled down, the number of receptor sites decreases on a sensing platform. Device sensitivity and performance is compromised because the optical sensing mechanism of typical chemosensors, single molecule detection–single photon generation, yields signals that are too weak to be detected. In principle, the linear, single photon response of a molecular chemosensor can be replaced with the non-linear one of a NC that is part of a laser cavity (see Fig. 8). An example of this sensing strategy with TNT-sensing semiconducting organic polymer as gain material has already been reported by Rose *et al.*; enhanced sensitivity to TNT was found to be most pronounced when the polymer-incorporated distributed feedback films were pumped at intensities near their lasing threshold, exhibiting non-linear sensing.[43] Because the NCs are robust, they can withstand the high fields generated in such cavities. This review concludes with a discussion of lasing cavities incorporating NCs and their potential for high gain CB sensing.

Theory and motivation on high gain sensing

The intensity of light can build exponentially as light propagates in a cavity with an inverted excited state population. This exponential increase in intensity is given by,[14]

$$I = I_0 e^{g(v)z} \tag{5}$$

where I_0 is the seed intensity, I is the intensity at some distance z into the cavity, and $g(v)$ is the gain coefficient given by eqn (6).

$$g(v) = [N_u B_u(v) - N_l B_l(v)]\frac{hv}{c} \tag{6}$$

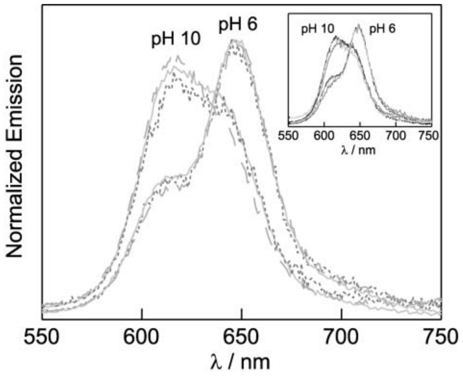


Fig. 7 Sensing of the local pH by the NC–squaraine dye construct with variations in the excitation intensity and local environment. The ratio of NC to dye emission varies such that pH is determined within 5% when altering the slit entrance of the Xe lamp excitation of the fluorimeter (--- 0.5 mm; —, 2.00 mm) and when examining the construct within a highly scattering media, which consisted of a solution of silica microspheres (— —). The inset shows that emission was independent of excitation wavelength (—, 380 nm; —, 450 nm; —, 520 nm).

where N_u and N_l are the upper and lower level populations, respectively, and $B_u(v)$ and $B_l(v)$ are the emission and absorption probability, respectively, at frequency v. Separation of Einstein B_u and B_l coefficients from the population inversion allows eqn (5) to be recast as,

$$I = I_0 e^{\sigma(v)\Delta N_{ulz}} \tag{7}$$

where $\sigma(v)$ is the stimulated emission cross-section. Eqn (7) provides an immediate inroad to sensing. If an analyte can affect the photophysics of the NC by removing or adding energy to the cavity (in a FRET process or by another signal transduction mechanism), the gain coefficient will be perturbed, leading to an exponential change in the laser output. A non-linear, highly sensitive "turn-off" or "turn-on" sensor can potentially be made.

High intensities and maximal response from the laser cavity can most easily be achieved by confining light within the cavity of the laser resonator. In a conventional Fabry-Perot resonator, confinement is achieved by reflection of light off a front and back mirror. With CdSe NCs as gain materials, Distributed Feedback (DFB) and spherical Whispering Gallery Mode (WGM) cavities (see Fig. 9) have been used to confine light to obtain lasing devices.[44–48]

CdSe incorporated DFBs

Optical gain and stimulated emission using CdSe NCs at room temperature was reported by Klimov *et al.*[42] In this work, gain-induced narrowing of the fluorescence emission spectrum (or amplified spontaneous emission, ASE) was achieved in close-packed drop-cast films of NCs. In order to achieve ASE, high volume fractions of NCs as well as ultrafast optical pumping (100 fs pulses) were shown to be essential in overcoming competitive nonradiative processes. With the parameters to achieve gain in NC films defined, the chemistry needed to incorporate them into feedback structures to attain lasing was developed.[44,45]

The architecture of a second-order DFB comprises a slab waveguide imprinted with a grating of period (Λ) that satisfies the Bragg condition,

$$\Lambda = \frac{\lambda_B m}{2n_{eff}} \tag{8}$$

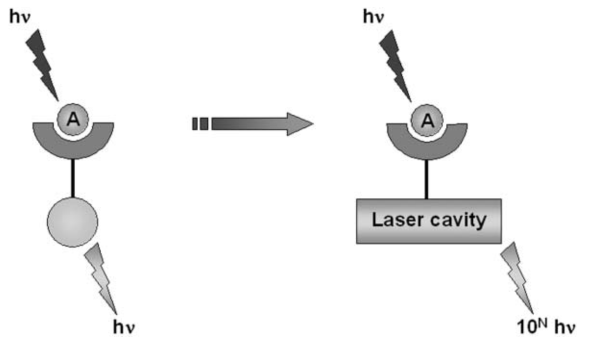


Fig. 8 Design concept to achieve CB sensing on small lengths scales. A single photon emitting center is replaced by a laser, which can be turned off or on by the recognition of a target. The strategy allows for high gain responses in the presence of analyte at low concentrations.

where λ_B is the Bragg wavelength that is supported by period Λ, m is the order of the grating reflection, and n_{eff} is the effective refractive index.[14] The lasing medium must have a gain profile that is coincident with λ_B. Stimulated emission of light is achieved as the propagating wave traverses the DFB grating and builds up gain from the constructive feedback caused by the grating.

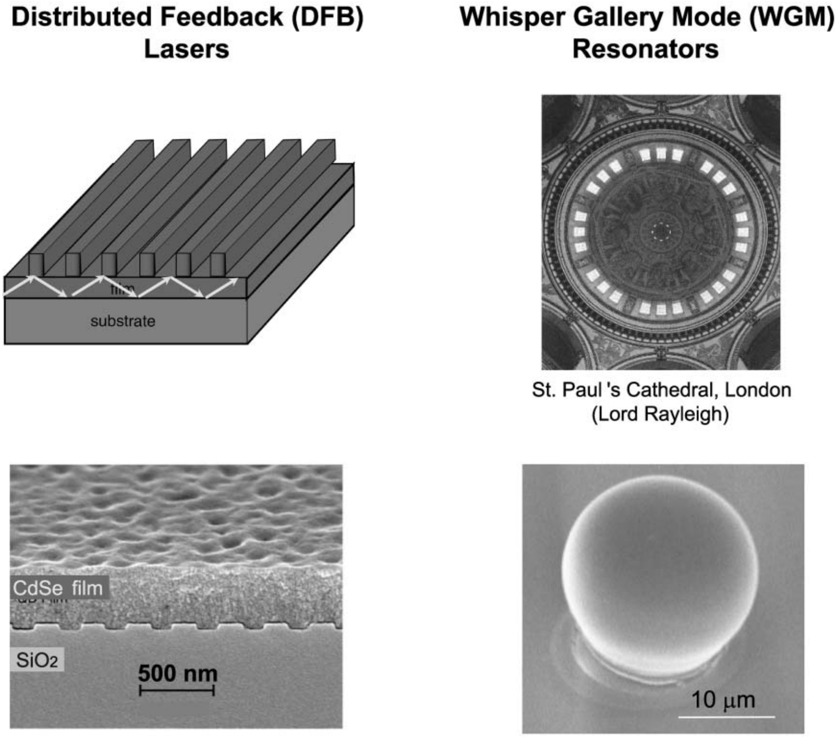
Eisler *et al.* encapsulated CdSe NCs within feedback structures by spin coating a NC–titania thin film on top of a DFB grating; the grating was generated using interference holography and patterned onto a silica wafer through reactive-ion-etching (RIE).[44] The thickness of the film was controlled by the speed of the spin coating, and the volume fraction of the NCs, which controls the refractive index. As defined by eqn (8), n_{eff} was adjusted to match the Bragg condition of the grating for the given ASE of the NC–titania film. Using different size NCs, different DFB lasers were produced over a wavelength range from 560 nm to 625 nm. The technology was further expanded by embossing a DFB pattern directly onto the NC–titania thin film.[45] In this work, a poly(dimethylsiloxane) (PDMS) elastomeric stamp was patterned with a DFB grating, brought into contact with a freshly spin-coated NC–titania pre-polymer, which was transferred to a hot plate to initiate polymerization. The pattern of the PDMS stamp is preserved upon polymerization and lasing, as shown in Fig. 10, is achieved. Soft lithography allows multilayer structures to be prepared by overlaying DFB gratings of different periodicity (and accordingly different sized NCs). With a multi-layered DFB laser, simultaneous lasing of multiple colors may be observed.

The sol–gel derived NC–titania films of Fig. 10 degrade rapidly after exposure to water and short chain alcohols. Recent work by Chan *et al.* describes the development of a solvent stable NC–silica composite laser.[48] CdSe/ZnS NCs were modified with 5-amino-1-pentanol to impart ethanol solubility. The silica precursors were 3-aminopropyltrimethoxysilane and (triethoxysilyl)propyl isocyanate were added to the NC–ethanol solution. The film fabrication and grating pattern transfer were performed in the same manner as described above to yield a device that was stable in several solvents. Stable lasing was observed from these DFBs in the presence of water as well as short-chain alcohols. These results enable NC-based DFBs to be used for high gain sensing in aqueous environments.

CdSe incorporated spherical resonators

Lasing from NCs can also be achieved in spherical cavities that support Whispering Gallery Modes (WGM). The WGM is formed from the constructive interference of successive total internal reflections of light off the concave inner surface of the spherical cavity.[14] Due to the strong confinement of photons within the modal volume, the laser Q-factor of these modes can be as high as 10^8.[14] CdSe/ZnS NCs are particularly suited to spherical resonators because they can withstand the high power densities that are developed within the small volume of the microsphere.

WGM lasing structures may be fabricated by assembling thin films of CdSe NCs onto 10 μm polystyrene spheres using a layer-by-layer wet chemical procedure; however, the highly


Distributed Feedback (DFB) Lasers

Whisper Gallery Mode (WGM) Resonators

St. Paul 's Cathedral, London (Lord Rayleigh)

Fig. 9 Two different types of laser cavities incorporating CdSe NCs as gain media. (A) Schematic representation of a distributed feedback (DFB) grating and a SEM image of the grating. (B) The reflection of sound waves that can be found at the Whispering Gallery of the St. Paul's Cathedral in London can be adapted on a smaller length scale to trap light waves in a spherical microcavity. An SEM of a NC-coated microsphere is shown.

loaded fraction of NCs that is necessary for light amplification is difficult to achieve by this method.[46]

Kazes *et al.* reported lasing from CdSe/ZnS NCs and quantum rods by using a cylindrical microcavity constructed from an optical fiber within a glass capillary tube.[49] A solution of the NCs or the quantum rods filled the space between the fiber and the capillary tube. The microcavity was pumped with a nanosecond Nd-YAG laser. The lasing threshold for the NC microcavity was 3 mJ whereas the threshold of the quantum rod microcavity was at 0.08 mJ. The linearly polarized emission of the CdSe quantum rods was observed from the lasing microcavity. Along similar lines, Petruska *et al.*[50] incorporated the matrix formed from NCs tethered to a titania sol–gel matrix by using alcohol-terminating amines into glass microcapillary tubes to promote WGM resonance. Lasing was obtained at room-temperature using a femtosecond excitation source.

Snee *et al.* developed a facile method of incorporating NCs onto the surface of micron-sized silica or polystyrene microspheres to produce a lasing cavity that is both reproducible and stable.[46] This synthetic methodology entails mixing a NC–titania sol precursor with silica microspheres before spin casting onto a glass substrate and annealing at high temperature (see Fig. 11). The disparity in the size of the titania film and the microsphere feedstock results in a conformal coating of the titania film onto the microspheres. Fig. 12 shows the lasing obtained from these WGM resonator composites. A clear threshold is observed for biexcitonic lasing on the low energy side of the quantum dot fluorescence. The facile and robust method of incorporating NCs as gain material for spherical resonators was found to be general; different NCs can be used to achieve lasing at different wavelengths,[47] and WGM resonant cavity structures can be introduced using a variety of commercially available microsphere templates.[46] The overcoating of NC–titania sols onto the spheres produces hundreds of uniform microresonators in a single spin-coating process, which opens up the potential for multiplexed sensing modalities.

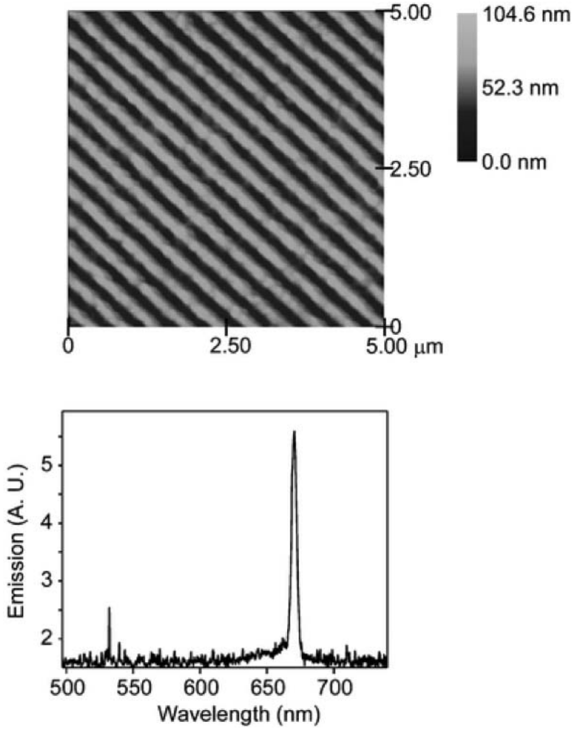


Fig. 10 (Top) AFM of NCs incorporated into a thin film. (Bottom) The emission spectrum of a NC-titania DFB laser device. (Reproduced from *J. Mater. Chem.*, 2005, **15**, 2697.[14])

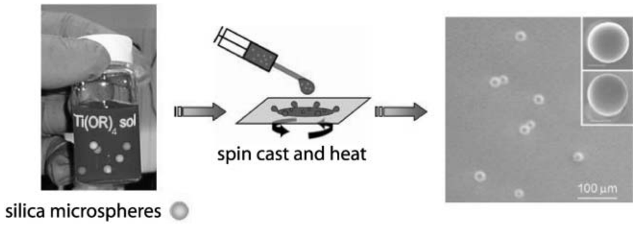


Fig. 11 A schematic representation for the synthesis of NC coated microspheres to produce a NC-microsphere that exhibits lasing from a whisper gallery mode. The silica microspheres are on the order of microns; their size is exaggerated in the container on the left in order to emphasize the nature of the synthetic procedure.

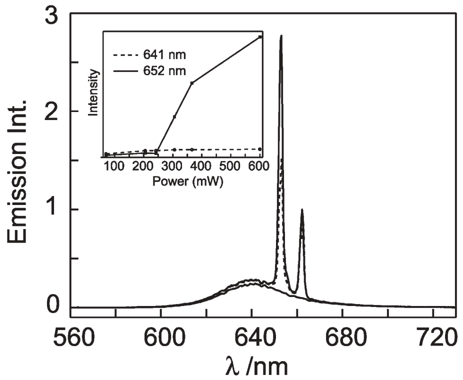


Fig. 12 Fluorescence from the CdSe NCs (bottom trace) transforms into discrete sharp lines once the lasing threshold is crossed. Inset shows the non-linear increase in intensity at threshold pump intensity at 652 nm while the fluorescence at 641 nm shows a linear response.

Prospects for non-linear sensing using CdSe as gain material

The development of environmentally responsive CdSe NC constructs, together with NC-based DFB thin film and WGM spherical lasing platforms, opens the way for the development of high gain CBsensors. The gain coefficient of NC-based laser cavities may be altered through the modulation of energy transfer by the various mechanisms shown in Fig. 3. The excited state population may be reduced through resonance energy transfer loss channels. Consequently, the loss of the excited state concentration (decrease in ΔN_{ul}) will represent a significant portion of the total population inversion resulting in a large change in the amplified laser intensity. When the loss of energy within the laser cavity is greater than the gain, the laser turns off, which can then be easily observed. Alternatively, the energy transfer mechanisms may be constructed to enhance the excited state population through excitation of the NC by the analyte. In either of these strategies, the response of the analyte by the NC is perturbed in a highly non-linear fashion, thereby facilitating signal detection. Alternatively, the surface of CdSe NCs, which are part of a lasing cavity, are amenable to modification with receptor sites. Recognition of target analytes that absorb or emit at the propagating frequency can in principle add to the gain or loss mechanism.

The ability to produce very high Q laser cavities using standard sol–gel chemistry offers other opportunities for highly non-linear sensing. The optical Q factor is proportional to the timescale that energy is stored in the cavity compared to the rate of energy loss. Consequently, higher Q cavities have longer photon storage times, increasing the effective length scale over which the laser light is amplified. The change in intensity and sensitivity as a result of the loss or gain of excited state population is exponential with respect to the length scale of laser light amplification. Additional sensitivity may be achieved by the direct modification of the Q factor itself, which will shift the wavelengths of lasing emission. The wavelengths of DFB and WGM, lasing emission lines are very sensitive to the refractive index of the gain medium. Adsorption of an analyte on the surface of the lasing device has the potential of altering the Q-factor and changing the optical properties of the structure, which would change the lasing wavelength. Because the laser lines are narrow, the shift in the lasing wavelength can be monitored easily.

Summary

The attractive properties of broad absorption profiles, narrow tunable emissions, high photostability, and high quantum yields for NCs have led to their development as CBsensors. The narrow emissions of the NCs as well as their resistance to photobleaching compared to those of organic dyes hold promise for multiplexing applications in a biological environment. In addition, the development towards self-calibrating NC sensors has expanded the utility of NC biosensors to be comparable to that of organic dyes. For sensing, as opposed to imaging, the NC photophysics must be sensitive to their environment. Most applications to date have focused on utilizing FRET as the mechanism to perturb the photophysics. However, other mechanisms exist. A recent report by Sykora *et al.* has demonstrated that CdSe emission is completely quenched by carboxylated Ru–polypyridyl complexes.[51] This result, accompanied by the fact that the spectral overlap is small, suggests charge transfer as the predominant mechanism for NC quenching as opposed to FRET. Considering the effectiveness of electron transfer as an excited state quenching mechanism, the development of NC CBsensors whose signal transduction is derived from charge transfer appears to be a particularly fruitful line of inquiry.[52] Finally, the use of NCs as gain materials in lasing cavities lays the foundation for non-linear sensing strategies that allow for the rapid and ultrasensitive detection of target analytes by NC-based CBsensors.

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