The Role of Colloidal Stability and Charge in Functionalization of Aqueous Quantum Dots

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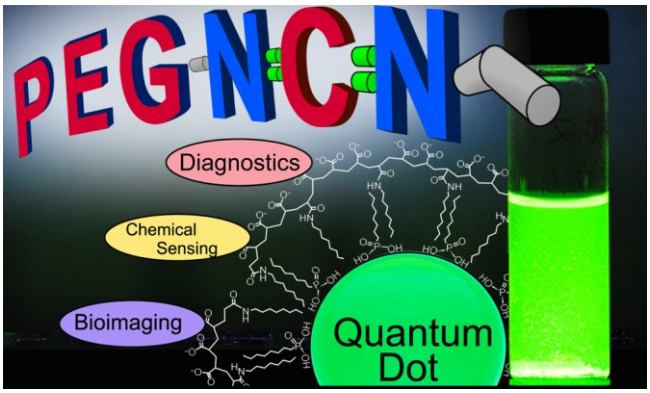
CONSPECTUS

Semiconductor quantum dots (QDs, also known as nanocrystals) have unique photophysical properties which allowed them to find utility in many applications, including television and display technologies. They also have significant potential as imaging agents in the biomedical field. To gain the most value from the use of QDs as health-related fluorescent probes, they must be biologically targetable, sensitive to metabolic analytes such as pH and O_2, and the resulting signal must be quantifiable. To achieve these goals, QDs need to be conjugated to vectors such as antibodies or environmentally-sensitive chromophores. Until recently, the functionalization of these nanomaterials required a fully "bottom-up," complex approach beginning with the synthesis of the QDs and subsequent manipulations. To simplify this process, our group set out to develop straightforward methods to prepare functionalized nanomaterials for biological imaging and sensing using low cost, commercially available aqueous QD dispersions.

In this Account we review the common problems and likely solutions when functionalizing QDs in water with chemical and biological vectors. Early in our investigations, we found that established protocols using a commercially available activating reagent resulted in either low reaction yields or QD precipitation. This was a consequence of the perturbation of the QDs' surface charges by the activating reagent and the conjugation substrate. These surface charges are derived from the anionic surfactants that are commonly employed for encapsulating water-soluble nanomaterials. Thus, cancellation of the surface charges by reagents or substrates results in colloidal instability. To address this problem, we devised conjugation methods that do not alter the overall charge balance of the system. Incorporating reactive moieties directly into the QD's water solubilizing polymer encapsulants negates the need for destabilizing activators, allowing for functionalization of aqueous samples without precipitation. The most successful approach was realized using neutral activating reagents, such as poly(ethylene glycol) carbodiimides (PEG-CD). PEG-CD binds to the carboxylic acid coating of water-soluble QDs, which primes them for amide bond formation with amine-functional substrates. Most importantly, this method can be applied to commercially available aqueous quantum dots. Using this method, we achieved reaction yields as high as 95%, allowing us to demonstrate a wide-range of QD functionalities and applications for chemical and biological sensing. Conjugation of environmentally-sensitive dyes to water soluble QDs results in reversible and ratiometrically reporting fluorescent probes for metabolic analytes such as pH, bisulfide, and O_2. QDs can also be functionalized with proteins for passive cell delivery or coated with poly(ethylene glycol) to enhance biocompatibility for *in vivo* studies. In the future, these capabilities



may be combined to realize the full potential for quantum dot nanotechnology for biological discovery.

INTRODUCTION

Semiconductor quantum dots (QDs) have interesting photophysical properties, such as size-dependent bandgaps and electronic fine structure due to quantum confinement, leading to significant research interest in the last 30 years.[1] Advances in QD syntheses in the early 1990's made them more accessible,[2] resulting in application-specific research reports and commercial products.[3] By the turn of the millennium there was significant enthusiasm for developing quantum dot technology for fluorescence-based biological imaging, mostly due to QDs' resistance to photobleaching and their potential for multi-functionalization. The continuously increasing density of states at energies greater than the bandgap[4] assures that almost any light source can be used for fluorescence excitation. The sharp crystallinity and surface passivation generally results in high fluorescence quantum yields.[5] These properties impart significant utility for long-term imaging / sensing studies, especially at the single chromophore level.

The development of biological imaging and sensing applications for QDs stalled due to significant technical challenges. For example, the highest quality QD materials are prepared with hydrophobic ligands (or 'caps') that render them insoluble in water. Replacing the caps with hydrophilic ones[6,7] resolves this problem, but these cap-exchanged QDs tend to precipitate quickly in water under ambient conditions.[8] Furthermore, cap-exchanged core nanocrystals are generally quenched when dispersed in water.

The latter problem was resolved in 1996 by the development of core/shell materials,[9,10] such as CdSe/ZnS nanocrystals, that are resistant to surface damage and changes in the local solvent

environment. The stability of aqueous QDs dispersions was further improved through the use of multidentate ligands for cap exchange[11] and the development of the polymer encapsulation method for water solubilization.[12,13] Encapsulation coats the QDs with amphiphilic polymers or phospholipids to allow their native ligands to remain intact. In the case of polymer-based encapsulation, the use of hydrophobically-modified poly(acrylic acid) (PAA) was found to be very robust and became widespread.[14] A representation of an amphiphilic PAA-coated QD is shown in Figure 1A.

Most methods for water solublization produce QDs coated with carboxylic acids, which should be easy to functionalize using a carbodiimide activator such as N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC). However, Mattoussi et al. noted that EDC causes significant aggregation of cap-exchanged QD,[11] a phenomenon subsequently confirmed by others.[15-17] The development of EDC-based protocols for functionalizing polymer-encapsulated QDs is problematic as our group and others have shown that EDC also induces precipitation of these materials.[18] Other conjugation methods were available but required use of very expensive commercial substrates such as streptavidin- and aminoPEG-functionalized QDs. To address this challenge, we developed high-yielding functionalization protocols for aqueous nanomaterials, which was realized by acquiring a greater understanding of the electrostatics of colloids as discussed below.

DLVO THEORY

The central role of surface charges in stability of aqueous QDs is revealed in the works of Derjaguin, Landau, Verwey, and Overbeek (DLVO).[19] The paradigm of DVLO theory is that colloidal stability is defined by the resistance to coagulation. This is dictated by a particle's self-

interactions: a destabilizing attractive Van der Waals-type and, most importantly, a repulsive electrostatic component. The attractive force scales as $\sim {}^{A}/_{d^2}$ (d = interparticle surface separation distance), the magnitude of which is defined by the Hamaker constant (A). The repulsive $\sim\sigma^2 e^{-d}$ force is mostly determined by the surface charge per unit area (σ) of the colloidal particle and the concentration of electrolytes in solution. [19]

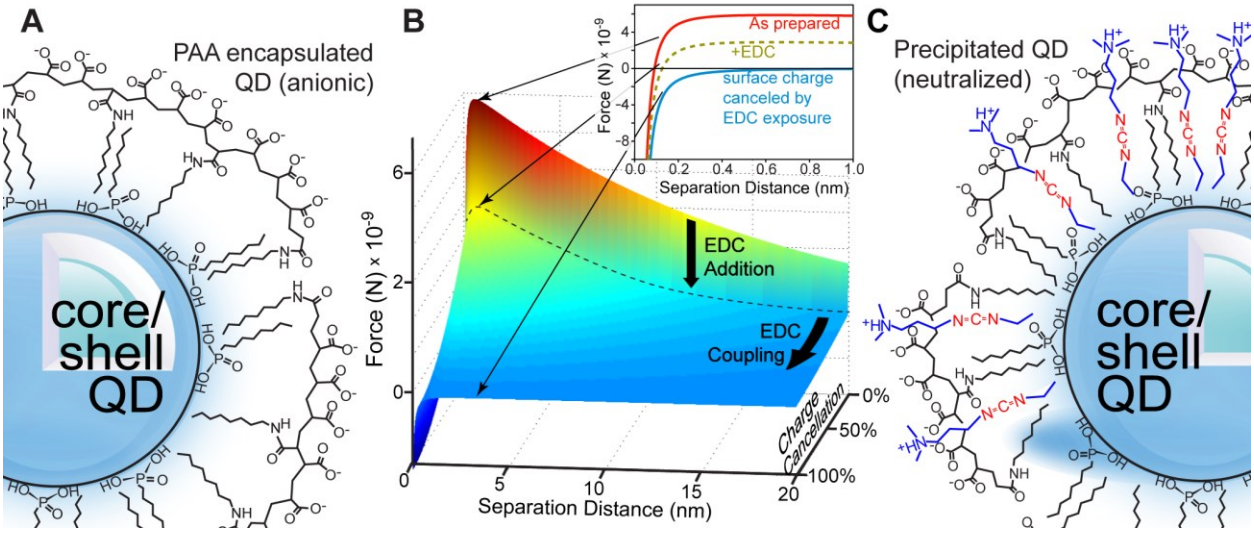


Figure 1. A. An idealized model of an octylamine-modified poly(acrylic acid) surfactant encapsulating a hydrophobic core/shell quantum dot. **B.** DLVO theory calculations describe interparticle forces between QDs as a function of surface separation distance. The repulsive forces become negligible if the surface charges are cancelled, which instigates QD precipitation. Inset are forces at small separation distances. **C.** A model for anionic QD surface charge cancellation due to cationic EDC interdigitation into the polymer coating.

Here we use DLVO theory to understand why exposure to EDC results in the precipitation of colloidal QDs; the parameters are provided in the supporting information (S.I). In our approach, a solution of amphiphilic PAA polymer-encapsulated QDs was modeled before and after exposure

to EDC. As shown in Figure 1B ("as prepared"), anionic colloidal QDs are stable due to strong self-repulsive electrostatic forces. Upon addition of EDC, there is an initial drop in the force resulting from an increase in the solution's ionic strength ("+EDC"). However, QD colloids are stable in buffers of higher concentrations than modeled here, which suggests that this reduction of repulsive interaction is not enough to result in precipitation. Colloidal instability become apparent once we model EDC's cationic amine moiety coordinating to the anionic carboxylate ligands of the QDs as shown in Figure 1C, which phenomenologically results in negation of the QD's surface charges. Total surface charge cancellation removes the barrier to aggregation as seen in Figure 1B. To summarize, DVLO theory reveals that the perturbation of the QDs' surface charges by EDC enhances the potential for aggregation, which is the reason that the reagent may fail to functionalize colloidal QDs.

REAGENTLESS METHODS

The results above demonstrate that to develop an effective functionalization protocol, we need to preserve the surface charge density of colloidal QDs. To achieve this goal, our group used 40% octylamine-modified poly(acrylic acid) encapsulated CdSe/ZnS QDs as substrates due to their stability and similarity to commercially available QDs.[20] We also sought to avoid expensive reagents, which is why biotin-streptavidin coupling or NHS-based systems reacting with water-soluble amine-functional QDs were not examined.

We first explored the development of a QD coating that incorporated a functionalizable "chemical handle" that is the conjugation target of commercially available biologicals and other chemical vectors. Such a system has no need for external activators such as EDC. Thiol-maleimide coupling fit into this paradigm; however, we had to incorporate the thiol into polymer surface coating of the

QD. Fortunately, at this time, the reversible addition−fragmentation chain-transfer (RAFT) method of polymer synthesis was becoming well-established.[21] RAFT produces homogeneous polymers via radical polymerization in the presence of a dithiocarbamate reagent, which results in low polydispersity products. The RAFT agent is retained as the polymer head group, which is transformed into a thiol upon hydrolysis. The resulting -SH functionality has been used to coordinate RAFT-generated polymers to nanomaterials, as well as for further chemical modification.[22,23]

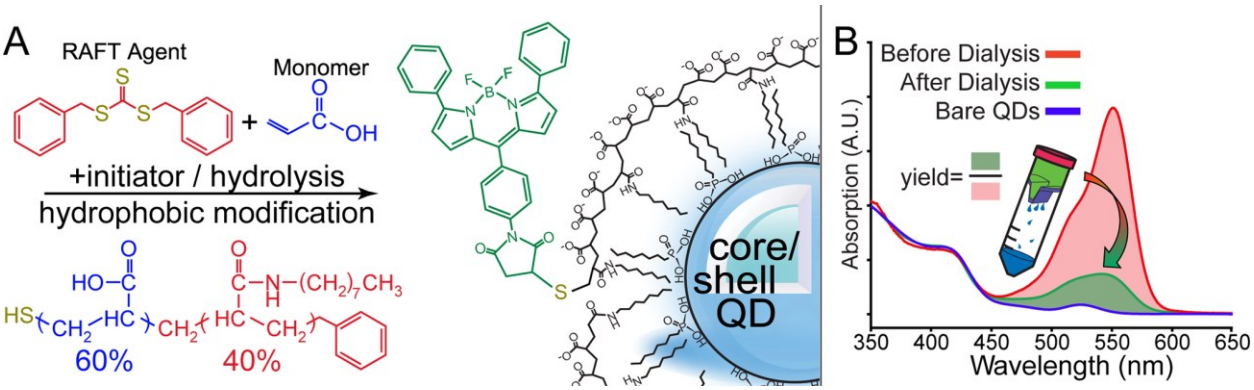


Figure 2. A. RAFT-mediated synthesis of poly(acrylic acid) and subsequent hydrophobic modification results in a QD water-solubilizing polymer with a thiol "chemical handle". The thiol was used to conjugate a maleimide-functional BODIPY dye to water-soluble CdSe/ZnS quantum dots in water with ~30% efficiency. **B.** Reaction yields are calculated by the ratio of the integrated absorption of a dye substrate before and after dialysis. Adapted with permission from ref. 24. Copyright 2008 American Chemical Society.

We prepared thiol-functional amphiphilic poly(acrylic acid) polymers using the RAFT method outlined in Figure 2A. After water solubilization of CdSe/ZnS dots using these polymer encapsulants, the materials were reacted with maleimide- or iodoacetamide-functional dyes.[24,25] The conjugation yield was characterized by absorption of the dye before and after dialysis as

shown in Figure 2B, which demonstrated ~30% reaction efficiency. At the time, this was the best reported yield for covalent coupling to aqueous QDs, allowing us to demonstrate several applications for the QD-dye systems. Aqueous dispersions of non-toxic ZnSe:Mn doped QDs were also derivatized using this protocol.[26]

After our publication, several groups reported the synthesis of reactive, "spring-loaded" polymers for QD solubilization.[27-30] Most interesting was the addition of chemical handles as monomers in the polymer backbone,[28] including amine-functional PEGs and "click" type moieties such as norbornene.[27] These developments provided researchers with a toolkit of robust methods for QD functionalization.[31] Despite this momentum in the field, our group did not pursue this motif past our first report because it cannot be applied to commercially available water-soluble QDs. We sought to provide a protocol for functionalization that could be performed directly by end-users.

CARBODIIMIDE CHEMISTRY

Although the use of EDC is problematic, we conjectured that carbodiimide chemistry should and could be "rescued". Carbodiimides activate carboxylic acids into a reactive ester form. These in turn react with amines (i.e. a protein N-terminus or lysine) to create amide bonded conjugates via the mechanism shown in Figure 3A; further details using Density Functional Theory are provided in the supporting information. EDC has been used to functionalize QDs encapsulated with mixed PEG / carboxylic acid functionalities,[32] which requires a multistep preparation of such polymer encapsulants. However, we examined QDs encapsulated with pure carboxylate functionality as are generally present in the least expensive commercially available water-soluble samples. As such, we sought to synthesize carbodiimide activators that are electrostatically neutral to maintain the

integrity of the activated QDs' surface charge density. The reagent must also be water-soluble yet not self-reactive, a difficult task due to carbodiimides' rich chemistry.[33]

POLY(ETHYLENE GLYCOL) CARBODIIMIDES, PREPARATION AND USE

A reagent composed of a carbodiimde linked to a methyl-terminated poly(ethylene glycol) (MPEG) water-solubilizing moiety was found to meet the requirements stated above.[17] The synthesis proceeds by first reacting ethyl isothiocyanate with MPEG amine and desulfurizing the product with mercury oxide (Figure S1). The most effective reagent is methyl poly(ethylene glycol) carbodiimide with a 350 Da MPEG molecular weight ("MPEG-CD 350").[17] It was found that the aqueous colloidal QDs solubilized with either cap-exchange or polymer encapsulation are stable against exposure to significant quantities of MPEG-CD 350. In terms of the coupling conditions, there are several nuances to carbodiimide chemistry. The activation step is best performed under slightly acidic conditions while the subsequent amide bond formation requires a basic solution. The reaction must be performed quickly due to hydrolysis.[34] Given the above conditions, we perform coupling reactions on QDs dispersed in unbuffered DI water, which is slightly acidic due to atmospheric CO_2. After ~10 min incubation, the amine-functional substrate in buffer (typically 0.05M phosphate, pH 8→9) is added. The mixture is gently stirred overnight, after which the product was purified with dialysis. The reaction yields are determined by integrating the optical absorption of the substrate before and after dialysis (Figure 2B).

Our first publication on coupling polymer-encapsulated CdSe/ZnS QDs to amine-functional dyes using MPEG-CD 350 reported yields as high as 95%.[17] We also described the preparation of protein conjugates, as well as the functionalization of magnetic nanomaterials.[17] Over the ensuing years, we have used MPEG-CD 350 for a variety of purposes and have found that the reagent

empowers the preparation of almost any functional nanomaterial desired. We have modified cadmium-free $AgInS_2$/ZnS dots in water for biological imaging studies,[35] and we have successfully used MPEG-CD to functionalize commercially available aqueous CdSe/ZnS samples; examples are provided in the S.I. Data on functionalizing cap-exchanged QDs, which are notoriously difficult to derivatize with chemical or biological vectors, are also provided in the S.I.

Our group has also investigated the mechanistic details on the functionalization of aqueous QD colloids with MPEG-CD by modifying the structure of the activation reagents and varying the substrates. Discussed below are our mechanistic insights as well as strengths and limitations of the method, followed by a synopsis of the applications that were realized. A summary of the reagents and the best reaction yields is shown in Figure 3B.

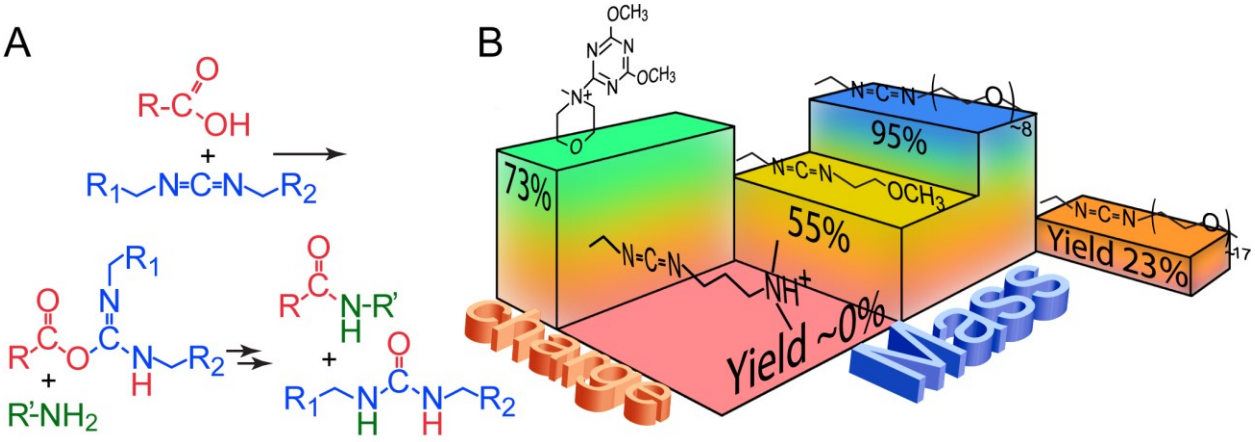


Figure 3. A. Mechanism of amide bond formation by carbodiimides. **B.** Compounds and their best reaction efficiencies (from left to right): DMTMM, EDC, EMC, PEG-CD 350 and PEG-CD 750. Note that DMTMM has a neutral activated state.

FACTORS THAT AFFECT CONJUGATION YIELDS: PEG MOLECULAR WEIGHT AND SOLUBILITY

There is perhaps some synergy in functionalizing polymer-coated quantum dots with polymer activators. We initially selected a high molecular weight MPEG (750 Da) to prepare the 1st MPEG-CD due to the fact that short-chain PEGs are not good QD ligands.[36] We found that MPEG-CD 750 can functionalize colloidal dots without inducing instability, however, the reaction efficiency was less than MPEG-CD 350. The conjugation efficiency was enhanced to the best result of 23% over very long reaction times (several days), which suggests that the reagent is resistant to hydrolysis. However, the reactivity and susceptibility to hydrolysis are two sides of the same coin, which led us to conclude that MPEG-CD 750 is overly sterically hindered. Therefore, we have not used this reagent since the initial report.

A low molecular weight reagent, 1-ethyl-3-(2-methoxyethyl)carbodiimide ($CH_3O(CH_2)_2N{=}C{=}NCH_2CH_3$, "EMC"), was synthesized to study how the reactivity of a molecular-scale activator contrasts with the polymer reagents.[37] EMC is not water soluble, but is none-the-less able to activate polymer-encapsulated QDs by a two-phase approach when added to a vigorously stirring sample. EMC is capable of functionalizing polymer-coated QDs (55% best result), but with a lower yield than realized with MPEG-CD 350. We attribute this to insolubility of EMC which, following excessive exposure, causes precipitation of QDs. This leads us to conclude that the activated complex is colloidally unstable resulting in lower reaction yields. These observations suggest that colloidal destabilization via activation of a QD's water-solubilizing moiety must be mitigated by an activating agent that can maintain the aqueous stability of the same colloidal particle.

FACTORS THAT AFFECT CONJUGATION YIELDS: ACTIVATED STATE

We further interrogated the nature of the activated ester state of the polymer that coats the QDs by examining functionalization via 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM), a carboxylic acid activator that, like EDC, is inherently cationic.[37] However, unlike EDC, this reagent sheds its positively charged 4-methylmorpholinium moiety upon reaction with the carboxylate to form a neutral, activated complex. The best reaction yield is high (73%) although still not as efficient as MPEG-CD 350. Exposure to excess reagent resulted in QD precipitation, but not due to free 4-methylmorpholine and at loading levels much higher than observed with EDC. We concluded that, much like EMC, the activated neutral QD requires a better water-solubilizing moiety than dimethoxy-1,3,5-triazine. For readers interested in developing functionalized QDs, the use of DMTMM is an attractive option due to its commercial availability.

FACTORS THAT AFFECT CONJUGATION YIELDS: SUBSTRATES

We used tetra-methylrhodamine-5-carboxamide (TAMRA) cadaverine in the initial report on MPEG-CD. This dye was employed for three reasons; it has a free primary amine functionality, the conjugation efficiencies were easy to characterize due to the dye's optical absorption, and the syntheses of QD-dye conjugates was topical due to their use in fluorescent, ratiometric sensing as discussed below. Conjugation of TAMRA to water soluble QDs allowed for the demonstration of appreciable reaction yields (~23%→95%) and multivalency via high dye:QD ratios (~150×). We have often used rhodamine B piperazine[38] for the same purpose, which has similar characteristics in terms of high conjugation efficiencies even with a secondary amine as the reactive functionality.

Further research using other substrates revealed that the cationic nature of TAMRA cadaverine and rhodamine B piperazine is responsible in part for the high reaction yields. Specifically, we

suspect that the dyes coordinate in close proximity to the anionic QDs before conjugation. The basis for this observation is the fact that working with anionic species has been problematic. For example, we reported that conjugating anionic single-stranded amine-functional DNA to QDs using MPEG-CD is facile, so long as the length of the DNA substrate is less than 10 basepairs (bp).[39] However, above 10 bp, the additional negative charge resulted in reaction efficiencies that were unmeasurably low.

Conjugation with cationic substrates suffers from similar limitations as revealed in our unpublished investigations on the functionalization of QDs with cell delivery vehicles. We began by examining the TAT peptide, which is known to be ineffective for QD cell delivery.[40] We hypothesized that the problem could be solved by creating a QD probe with a high TAT valency, potentially synthesized with MPEG-CD. However, we found that exposure of the QDs to large quantities of TAT peptide in the preparation stage resulted in precipitation. Identical results were obtained using the cationic cell delivery vehicle polyethylenimine. This series of experiments taught us that certain cationic species can precipitate QD colloids just like EDC, likely by the same charge cancellation mechanism. We also explored the conjugation of neutral, semi-water-soluble vectors such as PEG-modified pyrene and perylene.[41,42] We found that there is a critical valency threshold above which the QDs lose colloidal stability by charge neutralization. While this finding seems disheartening, the critical valency threshold is quite high (~100:1 pyrene:QDs in ref. 42).

We have frequently used MPEG-CD chemistry to conjugate QDs to peptides and proteins,[17,43-45] although one study revealed an issue with MPEG-CD chemistry.[17] Our attempt to conjugate tetrameric streptavidin to CdSe/ZnS QDs resulted in the precipitation of the activated nanomaterials due to a polymerization-like reaction. Essentially, streptavidin functioned as mortar to bind QDs together. The problem was partially resolved by increasing the streptavidin to QD

ratio to 100:1 during conjugation; the final product was found to have ~2 proteins per QD resulting in a significant loss of biologicals.

To summarize, functionalizing aqueous polymer-encapsulated and cap-exchanged QD colloids can be realized using water-soluble, neutral activators. Substrates should be modestly charged, and cationic chemical and biological vectors have the highest conjugation yields. Note that there is a risk for disrupting the colloidal stability of the QDs with highly cationic species. Neutral systems work well up to a critical valency; however, anionic substrates become increasingly problematic if repulsive forces dominate the QD-substrate interactions. Biological functionalization is not without pitfalls, which reveals the need for functionalization strategies beyond those discussed in this Account.[31]

APPLICATIONS

The development of effective conjugation methods dramatically increased the number of substrates that could be attached to aqueous QDs to achieve additive and/or synergistic functionalities. Given this ability, our group developed QD-organic dye hybrids as ratiometric fluorescent sensors, performed surface modification for *in vivo* imaging, and prepared cell-penetrating protein conjugates. These developments follow the timeline of progression illustrated in Figure 4.

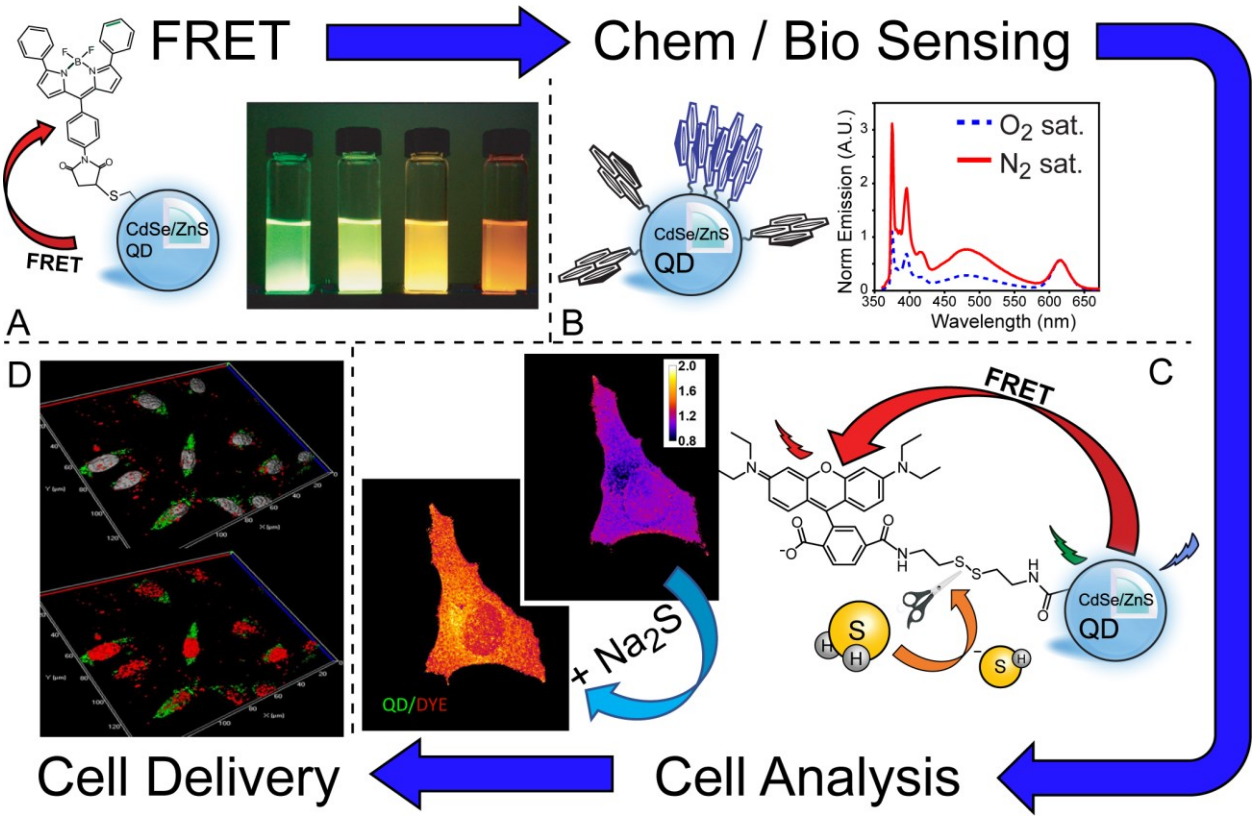


Figure 4. Timeline of the development of applications for biological imaging and sensing with functionalized QDs. **A.** FRET between aqueous polymer-encapsulated QD donors to organic dye acceptors is surprisingly efficient. When the QD is coupled to an analyte-sensitive chromophore, the result is a ratiometric, or "color-changing" sensor. Adapted with permission from refs. 24 and 17. Copyright 2008, 2009 American Chemical Society. **B.** Ratiometric sensing of oxygen required modification of pyrene to conjugate it to aqueous CdSe/ZnS dots. Adapted with permission from ref. 42. Copyright 2017 American Chemical Society. **C.** A QD-dye FRET displacement strategy for sensing hydrogen sulfide was found to be active after microinjection into live cells. Adapted with permission from ref. 46. Copyright 2016 American Chemical Society. **D.** Conjugating chimera proteins of Runx2-DSS allows QDs to localize in the nuclei of mesenchymal cells (top: grey from DAPI staining, bottom: red from CdSe/ZnS QD-Runx2-DSS conjugates). Adapted with

permission from ref. 44. Copyright 2013 American Society for Biochemistry and Molecular Biology.

Quantum Dot-Based Sensors. Due to their supermolecular size and surface passivation, core/shell QDs are inherently insensitive to their environment. Thus, an analytical strategy for detecting chemical or biological targets necessitates their functionalization with another species that has an analyte-dependent response. We accomplished this goal using reagentless thiol-maleimide conjugation to synthesize QD-organic dye coupled chromophores.[24] These systems were designed such that the nanomaterials are FRET energy donors to dye acceptors (Figure 4A). If the dye has an analyte-dependent absorption, the FRET efficiency is modulated, which results in a dual-emission ratiometric, or "self-calibrating," sensing response. Furthermore, several dyes can be conjugated to a single QD, enhancing the overall photostability. We used a QD-dye coupled chromophore to prepare a ratiometric fluorescent pH sensor, where an enhancement in the dye's absorption due to pH resulted in a shift of the emission owing to greater FRET efficiency.[17,24,47] Other groups have extended this concept to measuring pH in live cells.[48]

Next, we developed QD-dye motifs to study analytes relevant to cancer. We created ratiometric O_2 sensing and imaging agents by conjugating oxygen sensitive pyrene or perylene dyes to water soluble QDs[41,42] (Figure 4B). Both CdSe/ZnS and cadmium-free $AgInS_2$/ZnS QDs were coupled to these polyaromatic hydrocarbons using MPEG-CD 350 and were delivered to the cytoplasm of live HeLa cells using microinjection. The O_2 levels were modulated by incubating two cell populations under normal (20%) and hypoxic (7%) conditions overnight. We used optical filters to separately visualize the QD and dye emissions, the data from which were combined to create ratiometric maps of the cells' oxygen levels that delineated the normal vs. hypoxic cells. Other probes with possible use in cancer research were developed by conjugating fluorescent CdSe/ZnS

dots to an energy-accepting rhodamine dye via a disulfide-functional amine-terminated linker.[46] The disulfide bond is stable against the conjugation conditions, and the QD-dye conjugate displayed efficient FRET energy transfer and ratiometric emission. However, the disulfide linker is cleavable with good selectivity by bisulfide ion (SH^-, the aqueous form of H_2S), which is an endogenous gasotransmitter and a cancer biomarker.[49] Microinjection and imaging the QD and dye emissions demonstrated the potential for measuring bisulfide in biological environments (Figure 4C).

Ratiometric fluorescent sensing of proteins by the modulation of FRET between QD-dye coupled chromophores requires a more complex structure. We created such a system by conjoining a protein binding agent to a rhodamine dye, both of which were attached to the surface of water-soluble CdSe/ZnS QDs using lysine as a 3-spoke wheel.[50] We conjectured that the binding of the protein to the agonist would perturb the dye and result in a ratiometric response, a mechanism that was found to function surprisingly well. Specifically, a QD-biotin-rhodamine coupled chromophore exhibited an enhanced QD:dye emission ratio upon addition of streptavidin. Non-targeted proteins failed to elicit a response and the detection limits were low (1.16×10^{-5} g/L). The assay is homogeneous, which imparts significant ease for use compared to heterogeneous methods such as ELISA. A similar reporter was created for thrombin, although the specificity of that sensor was not as robust.[50]

Surface Modifications of QDs for Bioimaging. Considerable effort has gone into improving the performance of QDs in biomedical applications. In this regard, it is important to minimize non-specific binding of nanomaterials to cells and tissues.[51] Surfaces coated with PEG,[52] zwitterions,[53] and mixed-charged peptides[54] are known to resist biofouling, while nanoscale objects can use highly tailored ligand geometries and densities for the same effect.[55] PEGylation[56] of quantum

dots is a good strategy and is usually performed by cap-exchanging as-prepared QDs with PEG-functional ligands[36] or by pre-modifying encapsulating polymers with PEG.[27-30,57] For those researchers who cannot work from the bottom up, we examined the post-modification of water-soluble polymer-encapsulated QDs with PEGamine using MPEG-CD. The resulting PEGylated QDs were found to have no adhesion to live cells.[37] To study the efficacy for such PEGylated nanomaterials for *in vivo* imaging studies, cadmium-free NIR emissive $AgInS_2$/ZnS QDs were PEGylated and were successfully used to study the microvasculature of a mammary gland tumor in a BALB-neuT mouse.[35]

Live Cell Delivery. The passive delivery of sensing QDs into the cytoplasm of living cells will have a major impact on the ability to perform biochemical assays in complex environments. Unfortunately, QDs are non-cooperative in this regard as they generally become sequestered in endosomes, even if they are conjugated to well-established cytosolic delivery vehicles.[40] The George group of UIC discovered that a polypeptide composed of (Asp-Ser-Ser, "DSS"), an acidic domain of the calcium-binding agent dentin phosphophoryn,[58] is an effective cell penetrating agent.[44] As part of a collaborative project, we conjugated a chimera of DSS and Runx2, a nuclear localizing factor, to water-soluble CdSe/ZnS QDs using MPEG-CD 350.[44] After incubating the QDs in cells for 24 hours, their emission (Figure 4D, red) co-localized with the nuclear stain DAPI (grey). We are presently following up on this report to demonstrate cellular metabolic sensing using the same delivery vehicle.

CONCLUSION

Described herein is the development of methods for aqueous QD functionalization with chemical and biological vectors. DLVO theory demonstrates that QD colloids need significant surface

charge density to maintain stabilizing repulsive forces. Charged activating reagents for functionalization may be disruptive in this regard, and their use results in low conjugation yields or precipitates the nanomaterials. This prompted our development of reactive "spring-loaded" amphiphilic polymers for encapsulating QDs such that no destabilizing reagents are required. To assist end-uses of commercially available QDs, we synthesized neutral carbodiimide activating reagents that were found to be highly effective for preparing functional nanomaterials in water. Some limitations exist due to the electrostatic and structural nature of the conjugation substrates. These reagents were employed to create dye- and protein- coupled QD systems that can ratiometrically report on chemical and biological analytes and deliver nanomaterials into live cells. In the near future, we hope to see combinations of the two functionalities, so that the biological imaging community will reexamine the use of quantum dots in biological assays. Further advancements will be made using smaller nanomaterials with reduced toxicity and minimal "blinking" behavior.

ASSOCIATED CONTENT

Supporting Information Available. DVLO theory parameters, additional information on PEG-CD synthesis and usage with commercially available QDs, and DFT results on carbodiimide activation.

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ABBREVIATIONS

DMTMM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EMC, 1-ethyl-3-(2-methoxyethyl)carbodiimide; MPEG, methyl polyethylene glycol; MPEG-CD, methyl polyethylene glycol carbodiimide; PAA, poly(acrylic acid); PEG, poly(ethylene glycol); QD, quantum dot.

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