Bioconjugate Chemistry

Article

pubs.acs.org/bc

Isolation of Monovalent Quantum Dot–Nucleic Acid Conjugates Using Magnetic Beads

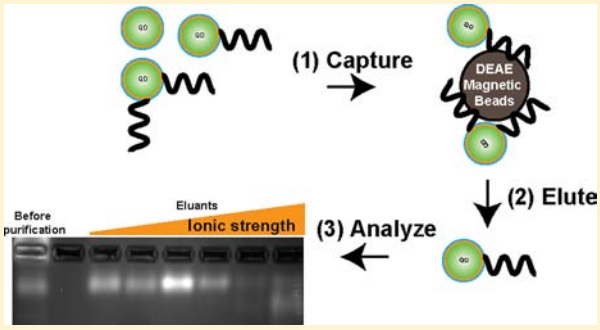
Uvaraj Uddayasankar,[†] Zhenfu Zhang,[†,‡] Ravi T. Shergill,[†] Claudiu C. Gradinaru,[†,‡] and Ulrich J. Krull*[,†]

[†]Department of Chemical and Physical Sciences, University of Toronto Mississauga, 3359 Mississauga Road, Mississauga, Ontario L5L1C6, Canada

[‡]Department of Physics, University of Toronto, 60 St. George Street, Toronto, Ontario M5S 1A7, Canada

Supporting Information

ABSTRACT: Control of the valency that is achieved in the decoration of quantum dots (QDs) remains a challenge due to the high surface area of nanoparticles. A population distribution of conjugates is formed even when reactions involve use of one-to-one molar equivalents of the ligand and QD. Monovalent conjugates are of particular interest to enable the preparation of multinanoparticle constructs that afford improved analytical functionality. Herein, a facile method for the formation and purification of QD-DNA monoconjugates (i.e., 1 DNA per QD) is described. Using diethylaminoethyl (DEAE) functionalized magnetic beads, a protocol was developed and optimized to selectively isolate QD-DNA monoconjugates from a mixture. Monoconjugates prepared with oligonucleotides as short as 19 bases and as long as 36 bases were successfully isolated. The monoconjugates were isolated in less than 5 min with isolation efficiencies between 68% and 93%, depending on the length of oligonucleotide that was used. The versatility of the method was demonstrated by purifying monoconjugates prepared from commercially available, water-soluble QDs. The isolation of monoconjugates was confirmed using agarose gel electrophoresis and single molecule fluorescence spectroscopy. Examples are provided comparing the analytical performance of monoconjugates to collections of nanoparticles of mixed valencies, indicating the significance of this separation method to prepare nanomaterials for bioassay design.



INTRODUCTION

Monovalent conjugates of nanoparticles with oligonucleotides have enabled the generation of unique complexes that have found use in fields such as biomedical imaging[1,2] and biosensing.[3,4] One nanomaterial of particular interest is quantum dots (QDs). QDs are fluorescent semiconductor nanoparticles with favorable properties such as high brightness, high photostability, and spectral properties that facilitate multiplexed detection.[5] Biosensing using QDs has focused on the use of multivalent conjugates, demonstrating unique transduction schemes[6] and improved analytical performance for some applications.[7] However, the ability to prepare monovalent conjugates can be essential, such as where cross-linking must be avoided. When multivalent conjugates are used, rather than the formation of a functional construct, a macromolecular aggregate can be formed. The accuracy and precision of quantitative binding assays can also benefit from the use of monoconjugates.[8] Multivalent conjugates may bind with an unknown number of target molecules. Facile strategies for preparation and purification are required to enable widespread accessibility to monovalent QD conjugates.

The challenge that is associated with the direct formation of monoconjugates stems from the high surface area of nanoparticles. Due to the presence of multiple active sites on the surface, incubation with even one equivalent of ligand results in a distribution of conjugates being formed.[9] Currently, there are two main approaches for the generation of QD monoconjugates. One exploits the use of either steric exclusion[2,10] or electrostatic repulsion[11] to minimize the number of molecules immobilized on the surface. The second, and most common approach, relies on purification of monoconjugates from a mixture of conjugates. An approach that relies on purification offers the flexibility to use a variety of surface coatings and conjugation methods. Common purification methods such as gel electrophoresis[1,8,12] and anion exchange chromatography[13] have demonstrated some success. Gel electrophoretic methods are the most accessible but require long run times to obtain sufficient resolution between the various conjugates, and the extraction of the monoconjugates from the gel is inefficient.[13] Methods based on chromatography overcome these limitations.[13] Anion exchange chromatography has been used to isolate QD-DNA monoconjugates, but the shortest length of single stranded nucleic acid that could be successfully isolated was 74 bases. Furthermore, current methods are not easily amenable to automation or high throughput sample processing. By implementation of alternative adjunct technologies there is

Received: May 7, 2014
Revised: June 9, 2014
Published: June 13, 2014

dx.doi.org/10.1021/bc5002032 | *Bioconjugate Chem.* 2014, 25, 1342–1350

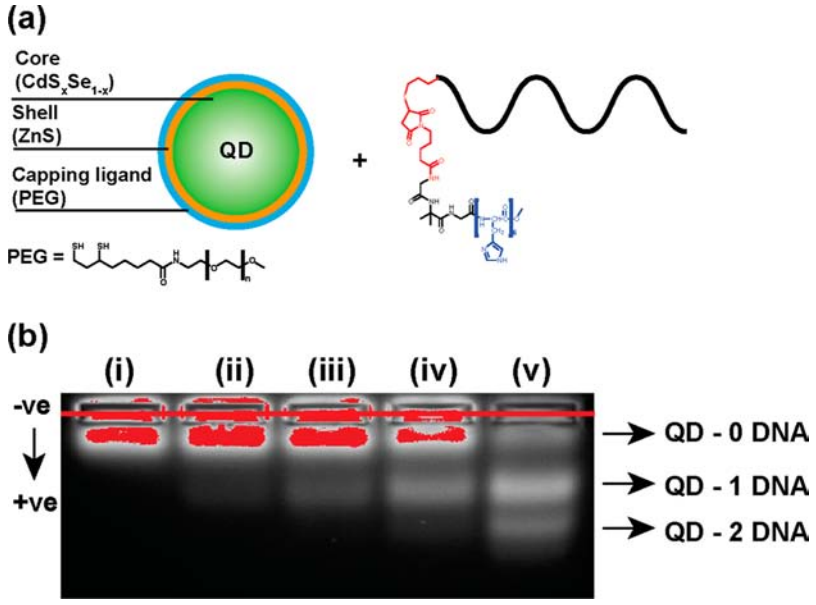


Figure 1. DNA conjugation onto PEG modified QDs. (a) Illustration of the surface coating of QDs with bidentate PEG ligands and the conjugation of DNA using hexahistidine modified DNA. (b) Agarose gel electrophoretic analysis of PEG-QDs that were incubated with increasing molar equivalent amounts of hexahistidine functionalized DNA (i) 0, (ii) 0.25, (iii) 0.50, (iv) 0.75, and (v) 1.0. The gels used were 2% agarose and electrophoresis was done in 0.5 × TBE at 3 V cm^{-1}.

significant opportunity for improvement of separation efficiency for conjugates prepared from short oligonucleotides. This would improve the versatility of the separation technique, and would also facilitate use of short probe strands that tend to offer much higher selectivity toward hybridization[14] as desired for determination of siRNA, some mRNA targets and polymorphism screening.[15] One such example of an adjunct technology is the use of magnetic beads as a basis for selective extraction.

Magnetic beads have become ubiquitous in the field of separation science.[16] These micrometer-sized particles possess a high surface area, which translates into a high binding capacity. The surface modification strategies have been well established and many commercial vendors provide magnetic beads with virtually any desired functional groups for common conjugation chemistries. This makes the technology widely accessible. Kinetics of capture and release are improved as the large surface area associated with the beads can be easily dispersed in a given volume and subsequently condensed at a single collection point using a magnet. The widespread adoption of this technique has already led to the development of many automated systems and is readily scalable.

In this work, diethylaminoethyl (DEAE) functionalized magnetic beads were used for the selective isolation of QD-DNA monoconjugates with the primary interest being isolation of monoconjugates that were prepared using oligonucleotides of less than 40 bases in length. QD-DNA conjugates were constructed using the well-established ability of hexahistidine functionalized nucleic acids to coordinate onto the inorganic shell of poly(ethylene glycol) coated QDs.[17] The DNA functionalized QDs were captured onto the DEAE magnetic beads using electrostatic interactions. Selective elution of the monoconjugated species was achieved by tuning the ionic strength of the wash solutions using sodium chloride. Analysis of the quality of the purified conjugates was done using agarose gel electrophoresis. Confirmation that monoconjugates were obtained was achieved using single molecule fluorescence techniques.[11,12,18] Commercial, water-soluble QDs were used to prepare conjugates in order to demonstrate the broad applicability of the purification method using materials that are widely available. The significance of the purification procedure in the preparation of QD-DNA monoconjugates for quantitative assays was investigated using fluorescence resonance energy transfer (FRET) to detect hybridization.[19]

RESULTS AND DISCUSSION

Quantum Dot Surface Chemistry and Bioconjugation.

Quantum dots, originally dissolved in organic solvents, need to be transferred to aqueous solution prior to functionalization with biomolecules. Quantum dots (QDs) were made water-soluble using a bidentate thiol poly(ethylene glycol) (DHLA PEG) coating (Figure 1a) that was previously introduced by Uyeda et al.[20] Successful ligand exchange was confirmed by the colloidal stability of the DHLA PEG-QDs in aqueous solutions and a hydrodynamic diameter (D_h) of 12.3 ± 0.2 nm as determined using fluorescence correlation spectroscopy (FCS); see Supporting Information, Figure S1. According to the manufacturer, the inorganic core of the original QDs measures 5.5−7 nm in diameter,[21] and the increase in hydrodynamic radius of the water-soluble conjugates is consistent with previous reports.[22] A PEG surface coating was chosen to provide excellent colloidal stability across a wide range of ionic strengths and also to minimize nonspecific adsorption onto the solid interface.[23] Both of these attributes facilitated the magnetic bead protocol that was used to isolate QD-DNA monoconjugates.

Nucleic acid conjugation onto the nanoparticles was achieved by implementation of the well-known interaction between a hexahistidine moiety and the inorganic shell of the QDs ($CdS_{(x)}Se_{(1-x)}$/ZnS; core/shell) (Figure 1a). This bioconjugation strategy has been demonstrated to be highly efficient, fast, and robust.[24] Hexahistidine functionalized nucleic acids were obtained by conjugating a thiol terminated nucleic acid with a maleimide-functionalized peptide that consisted of a hexahis-

dx.doi.org/10.1021/bc5002032 | Bioconjugate Chem. 2014, 25, 1342−1350

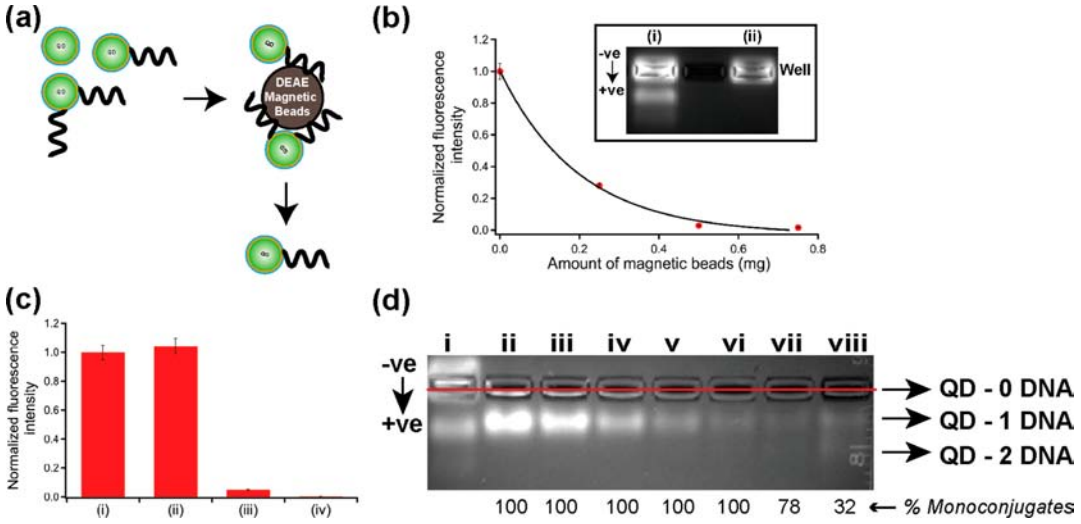


Figure 2. Magnetic bead based purification of QD-DNA (36-mer) monoconjugates. (a) Illustration of the purification procedure using magnetic beads to capture and selectively elute QD-DNA monoconjugates. (b) Determination of the binding capacity of QD-DNA conjugates onto the magnetic beads: (inset) agarose gel electrophoresis image of (i) crude mixture, and (ii) supernatant after extraction. (c) Optimization of the washing protocol: (i) QD solution prior to incubation with magnetic beads, (ii) QD solution after incubation with magnetic beads, (iii) first wash solution, (iv) second wash solution. (d) Analysis of eluent solutions using agarose gel electrophoresis: (i) crude mix of QD-DNA conjugates, (ii) 0.250 M NaCl, (iii) 0.275 M NaCl, (iv) 0.300 M NaCl, (v) 0.325 M NaCl, (vi) 0.350 M NaCl, (vii) 0.375 M NaCl, (viii) 0.400 M NaCl. The gels used were 2% agarose and electrophoresis was done in 0.5 × TBE at 3 V cm^{-1}.

tidine moiety. Successful immobilization of the hexahistidine-modified nucleic acids to onto the PEG-QDs was experimentally validated by agarose gel electrophoresis (Figure 1b). Unmodified nucleic acids did not bind onto the QDs (Supporting Information, Figure S2). In the gel electrophoretic analysis, bands of higher electrophoretic mobility were observed when the QDs were incubated with increasing molar equivalents of DNA. The observation of discrete bands rather than a smeared band demonstrates the ability of agarose gel electrophoresis to separate QD-DNA conjugates of varying stoichiometric valencies. The ability of agarose gel electrophoretic analysis to distinguish between conjugates of varying valences makes it a suitable technique to evaluate the performance of the proposed monoconjugate purification technique using magnetic beads.

In the experiments herein, the ratio of oligonucleotide to QD was kept small (DNA to QD ratio of less than 1) to maximize the proportion of monoconjugates that were prepared. The highest valency of conjugates of significant population was the diconjugate.

Selective Isolation of Monoconjugates. A protocol using magnetic beads was developed to isolate the QD-DNA monoconjugates. The main steps of the protocol are illustrated in Figure 2a. For all initial experiments, the DNA was 36 bases in length. Diethylaminoethyl (DEAE) functionalized magnetic beads were used for an ion exchange based purification. Upon incubation of the magnetic beads with the QD-DNA mixture, the QD-DNA conjugates were captured onto the magnetic bead, with any unmodified QDs remaining in solution. The absence of nonspecific binding of the QDs onto the beads was confirmed by measuring the fluorescence intensity of a solution of PEG QDs (without any DNA) before and after incubation with the magnetic beads (Figure 2c (i) and (ii)). Similar fluorescence intensities were obtained for both solutions, indicating the absence of any significant nonspecific adsorption.

The binding capacity of the magnetic beads for QD-DNA conjugates was determined using fluorophore (Cy5) labeled oligonucleotide that was conjugated to the QDs. QDs were functionalized with 1 equiv. of labeled oligonucleotide and then incubated with the magnetic beads for 1 min. The decrease in solution fluorescence intensity of Cy5 as the QD-DNA conjugates were captured onto the magnetic beads was used to monitor the binding capacity (Figure 2b). For QDs incubated with 1 equiv. of DNA, the binding capacity was determined to be 0.273 nmol of DNA mg^{-1} of magnetic beads. According to the manufacturer, the total surface area available for one milligram of magnetic beads is 1×10^3 cm^2.[25] Thus, the density of QD-DNA conjugates on the nanoparticle surface is 2×10^{11} DNA strands cm^{-2}. This immobilization density is consistent with those previously observed for nanoparticles immobilized on a solid substrate.[26,27] To further confirm the complete capture of QD-DNA conjugates, the supernatant was analyzed using gel electrophoresis (Figure 2b, inset). The absence of bands corresponding to the QD-DNA conjugates in the supernatant lane indicates complete capture.

Following capture, a washing step was required to remove fluid that was retained by the magnetic beads. The retained solution consisted mainly of unconjugated QDs, and the QD fluorescence intensity of the wash solutions was used to optimize the number of wash steps. Two washes were determined to be sufficient to remove >95% of the fluid retained by the magnetic beads (Figure 2c).

Selective recovery of the QD-DNA conjugates of varying valences was achieved by tuning the ionic strength of the elution solution. Ionic strength was selected for control of elution due to the ease of manipulation, especially for the small increments that were desired for adjustment of stringency conditions. Elution of the QD-DNA conjugates was achieved by incubating the magnetic beads for 30 s with solutions of varying ionic strength. Samples of the eluted solutions were run on the agarose gels for identification of the various QD-DNA valencies (Figure 2d). It is important to note that separation on the gels was only used to identify composition by comparing the position of bands and was not a part of the purification

dx.doi.org/10.1021/bc5002032 | Bioconjugate Chem. 2014, 25, 1342−1350

protocol. The lanes labeled "crude mixture" in Figures 2 and 3 represent a small portion of the initial solution (before purification). These were sometimes diluted to have sufficient volume to load onto the gel. The eluted fractions were oftentimes eluted into a smaller volume, which would appear as a brighter band due to its higher concentration. These changes in volume are responsible for the variations in band intensities between the crude mixture and the purified fractions.

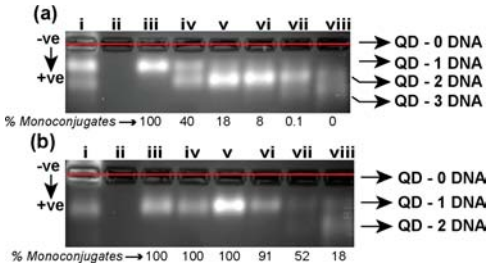


Figure 3. Monoconjugate purification of short nucleic acids (<30-mer). (a) 19-mer: (i) Crude mix of QD-DNA conjugates, (ii) blank, (iii) 0.10 M NaCl, (iv) 0.20 M NaCl, (v) 0.30 M NaCl, (vi) 0.40 M NaCl, (vii) 0.50 M NaCl, and (viii) 1.0 M NaCl. (b) 29-mer: (i) Crude mix of QD-DNA conjugates, (ii) blank, (iii) 0.10 M NaCl, (iv) 0.20 M NaCl, (v) 0.30 M NaCl, (vi) 0.40 M NaCl, (vii) 0.50 M NaCl, and (viii) 1.0 M NaCl. The gels used were 2% agarose and electrophoresis was done in 0.5 × TBE at 3 V cm^{-1}.

The QD-DNA monoconjugate of the 36 base oligonucleotide began elution at an ionic strength of 0.250 M NaCl with approximately 50% of the monoconjugates being eluted within an ionic strength range of 0.250−0.275 M NaCl. While the monoconjugates continued to elute at higher ionic strengths, the gel electrophoresis image indicates the coelution of QD-DNA diconjugates at an ionic strength of 0.375 M of NaCl. The purification method may be understood on the basis of probability of interaction of the QD-DNA conjugate with the magnetic bead. A larger number of oligonucleotides on a QD increases the probability of interaction with the magnetic bead. In the presence of sodium chloride, there is competition for binding onto the magnetic bead. Due to these competing interactions, as salt concentration is increased the QD-DNA conjugate with the lowest probability of binding (QD-DNA monoconjugate) is the first to elute. For short oligonucleotides in the diconjugate form, the DNA strands on the QD are likely not long enough to wrap around the nanoparticle for each to simultaneously interact with the magnetic bead.

The coelution of diconjugates is the main factor that limits the yield of the isolation procedure. The agarose gels were quantitatively analyzed using ImageJ, and the isolation efficiency calculated using eq. 1

$$\text{Isolation efficiency} = \frac{I_{\text{Pure Monoconjugate}}}{I_{\text{Total Monoconjugates}}} \quad (1)$$

where $I_{\text{Pure Monoconjugate}}$ represents the sum of band intensities of the monoconjugates that were present in fractions with 100% monoconjugates (indicated on the gel images) and $I_{\text{Total Monoconjugates}}$ represents the sum of band intensities of the monoconjugates in all fractions that were collected.

Based on this analysis, the isolation efficiency of the monoconjugates of 36-base-long DNA was calculated to be 93%. Isolation efficiency, rather then absolute yield of material, was used as a comparative variable, as it is independent of the composition of the crude material.

The selective isolation of QD-DNA monoconjugates using shorter oligonucleotides was investigated. Oligonucleotides that were 19 bases and 29 bases in length were conjugated to QDs and purified using the magnetic bead method. Analysis by agarose gel electrophoresis identified the eluents that contained monoconjugates (Figure 3). The results shown in Figure 3 indicate that monoconjugates using a variety of oligonucleotide lengths could also be successfully isolated. Note that the monoconjugates would be expected to elute at a lower ionic strength, and 0.100 M NaCl was chosen as a value that offered good performance without any indication of contamination by conjugates of higher order. The only criterion for the lower limit of ionic strength is that there be sufficient selectivity to elute only the monoconjugate.

Slight variations in the composition of the crude mixture can be observed for the different oligonucleotides. The difference in composition of the raw materials can be attributed to two factors. First, slight variations in the incubation ratio of DNA to QD results in significant changes in the composition of the QD-DNA conjugates, as seen in Figure 1. Second, longer nucleic acids would have a higher steric hindrance toward immobilization on a PEG coated QD, reducing immobilization efficiency. This phenomenon has been previously observed for hexahistidine-functionalized proteins immobilized on PEG QDs.[28] While electrostatic repulsion between oligonucleotide strands decreases the immobilization efficiency onto nanoparticles, this becomes a significant concern only at large loading densities. At the ratios used in the experiments, electrostatic repulsive effects are expected to be minimal.

The length of the oligonucleotide correlated with the ionic strength required for the elution of the monoconjugate. The monoconjugates of the 19-mer eluted at a much lower ionic strength (0.100 M NaCl) as compared to the 29-mer and 36-mer oligonucleotides (>0.200 M NaCl). Longer oligonucleotides have stronger electrostatic interaction with the magnetic beads, requiring a higher ionic strength to displace the QD-DNA conjugate from the surface of the magnetic bead.

The yield of the isolation process was noted to decrease as the oligonucleotide length was decreased. The isolation efficiencies obtained for the three different lengths of oligonucleotides are listed in Table 1. The isolation efficiency that can be obtained is limited by the coelution of the diconjugate species. Above a certain ionic strength, a significant proportion of diconjugates is coeluted with the monoconjugates. Reduction of yield of pure monoconjugates occurs to a greater extent with the smaller oligonucleotides, as expected based on the electrostatic energetics responsible for adsorption on the magnetic beads. Increasing the ionic strength of the solutions using smaller increments provides for better yields (Supporting Information Figure S3). By using small increments of ionic strength, more fractions with pure monoconjugates

Table 1. Isolation Efficiency of the Purification Procedure Used to Isolate QD-DNA Monoconjugates

length of oligonucleotide (number of bases)	monoconjugate isolation efficiency (%)
19	68 ± 3
29	88 ± 6
36	93 ± 1

dx.doi.org/10.1021/bc5002032 | Bioconjugate Chem. 2014, 25, 1342−1350

could be identified, thus improving isolation efficiency. For example, the isolation efficiency of monoconjugate using the 19-mer oligonucleotide was improved from 57% to 68% by using smaller increments of ionic strength.

Characterization Using Single Molecule Fluorescence Spectroscopy. Single molecule fluorescence spectroscopy of conjugates that were spread on glass coverslips was used to confirm the extent of oligonucleotide conjugation on QDs. The QDs were functionalized with Cy5 labeled oligonucleotides and the monoconjugates were purified. Monitoring of the fluorescence intensities of both of the available fluorophores (QD and Cy5) provides insight into the valency of the conjugate. The dual color excitation allowed for determination of colocalized individual QDs and DNA-Cy5 molecules, as shown in Figure 4a and b. The two images shown are the

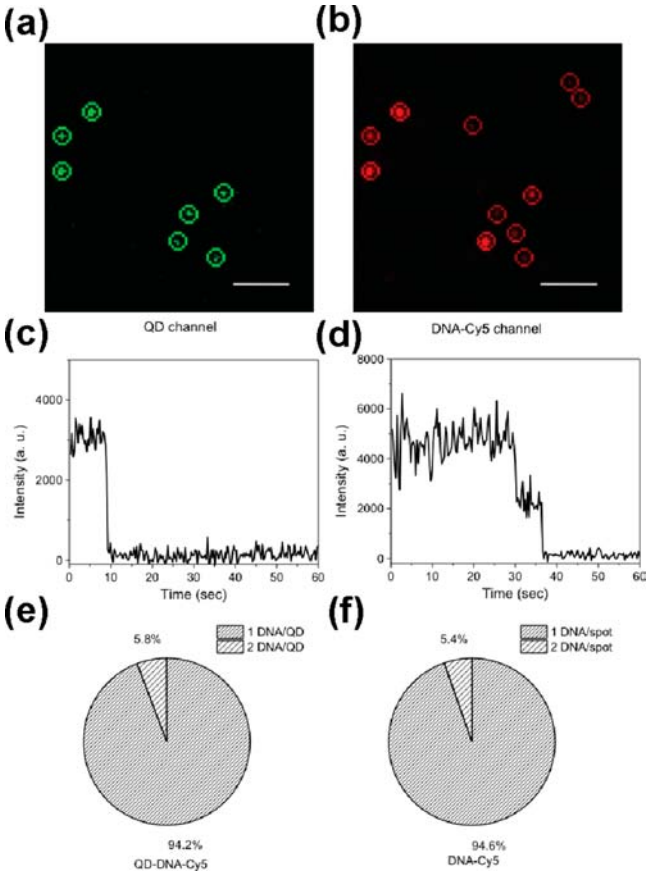


Figure 4. Total internal reflection fluorescence (TIRF) microscopy images of single QD-DNA-Cy5 particles spin-coated on glass surface: (a) image of the QD emission channel (green color), and (b) image of Cy5 emission channel (red color). Circles were added to emphasize the individual molecules. Scale bar length represents 5 μm. Intensity–time trajectories of Cy5 dyes showing (c) one photobleaching step, and (d) two photobleaching steps. Population distribution of (e) the QD-DNA-Cy5 particles, and (f) the DNA-Cy5 molecules, derived from the analysis of the number of photobleaching steps of the Cy5 fluorophore.

maximum intensity projection of image stacks using the ImageJ Z-project tool. A custom-written MATLAB program was used to first identify the position of individual conjugates based on an intensity threshold and a 2-D Gaussian MLE (Maximum Likelihood Estimation) fitting model.[29] Once conjugates had been spatially identified, the program then produced intensity–time trajectories of all the identified conjugates in the image field on the coverslip surface. From the analysis of a total of 1007 fluorescent sites, 53.5% of the DNA-Cy5 molecules were colocalized with a QD spot, while 46.5% did not overlap with a corresponding QD spot in the green channel. The low colocalization is most likely due of the frequent excursions of the QDs to a nonradiant dark state. This behavior is typically observed for QDs with water-soluble protective coatings, as reported previously by Webb and co-workers.[30]

In the red channel, stepwise photobleaching of intensity–time trajectories from individual DNA-Cy5 (Figure 4c and d) were observed. The results obtained from counting the photobleaching steps of the Cy5 fluorophore in 733 QD-DNA complexes are summarized in Figure 4e. The distribution of the QD-DNA conjugation was found to be 94.2% monoconjugate and 5.8% diconjugate. The small biconjugate fraction could be due to the fact that two QD-DNA-Cy5 molecules were localized within the same diffraction limited spot. The photobleaching behavior of 204 DNA-Cy5 molecules, spin-coated on a plasma-cleaned coverslip, was investigated using the same TIRF setup. As shown in Figure 4f, a similarly small population fraction (5.4%) also exhibited 2 photobleaching steps. These results confirm that the sample preparation method provides product that is highly homogeneous, consisting of monoconjugated QD-DNA constructs.

The hydrodynamic diameter (D_h) of the QD-DNA monoconjugate was measured using FCS (Supporting Information Figure S1). The D_h increased from 12.3 ± 0.2 nm (for QDs with no DNA) to 14.5 ± 0.3 nm (for QDs conjugated to one DNA strand). This increase in D_h may be attributed to the addition of DNA onto the QD. The colloidal stability of the QDs is preserved through the purification procedure, as demonstrated by the D_h of the QD-DNA monoconjugates.

Monovalent Conjugates of Commercially Available QDs. The ability to purify QD-DNA monoconjugates using an alternative bioconjugation chemistry was also demonstrated. QDs that are coated with amphiphilic polymers represent the predominant form of water-soluble QDs that are commercially available. These coatings encapsulate the QD in amphiphilic polymer, rather than displace the initial hydrophobic ligands that were present on the QD. This causes minimal disturbance to the nanocrystal surface, preserving the optical properties of the QD while making it water-soluble. The amphiphilic polymer prohibits the use of conjugation chemistries that rely on direct interaction with the inorganic surface of the QD, and this invokes need for implementation of other strategies to achieve covalent conjugation.[22] Commercially available QDs functionalized with an amphiphilic PEG amine coating were used to examine whether monoconjugates of QD-DNA could be isolated. Since these QDs were also coated with PEG, the advantages of using PEG coatings still apply. The amine QDs were activated with the heterobifunctional cross-linker SMCC to provide maleimide functional groups that were used to anchor thiol functionalized DNA (29 mer) (Figure 5a). Agarose gel electrophoresis was used to confirm the conjugation of DNA onto the maleimide-functionalized nanoparticles (Figure 5b). As previously observed, the electrophoretic mobility of the QD-DNA conjugates was higher than the unconjugated QDs. Based on the electrophoretic mobility, the unmodified QDs were also determined to have a small net negative charge. This negative charge could be due to the underlying polymer layer or the partial hydrolysis of maleimide groups to maleic acid. Incubation of the reaction

dx.doi.org/10.1021/bc5002032 | Bioconjugate Chem. 2014, 25, 1342–1350

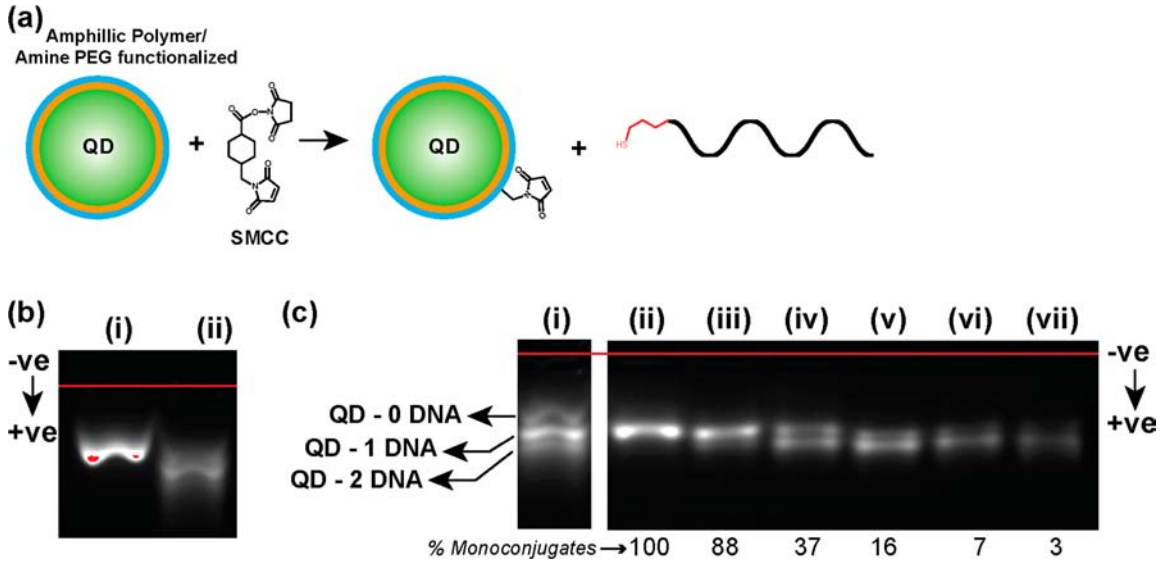


Figure 5. Isolation of monoconjugates using commercially available QDs. (a) Conjugation chemistry for the attachment of thiol functionalized DNA to amine functionalized QDs. (b) Agarose gel electrophoresis used for the confirmation of DNA conjugation onto the QDs: (i) SMCC modified QDs, and (ii) SMCC-QDs after incubation with DNA. (c) Agarose gel electrophoretic analysis of species that are eluted from the magnetic beads at varying ionic strengths: (i) crude mixture, (ii) 0.275 M NaCl, (iii) 0.300 M NaCl, (iv) 0.325 M NaCl, (v) 0.350 M NaCl, (vi) 0.375 M NaCl, and (vii) 0.400 M NaCl. The gels used were 2% agarose and electrophoresis was done in 0.5 × TBE at 3 V cm^{-1}.

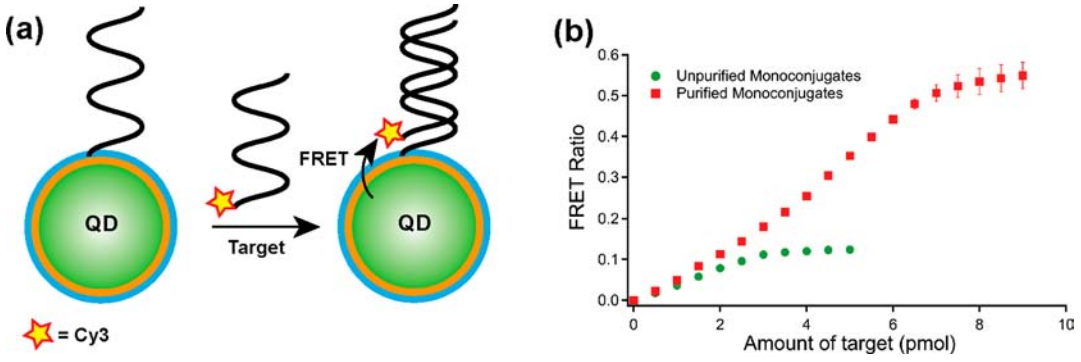


Figure 6. FRET based analysis for the investigation of the hybridization properties of QD-DNA monoconjugates. (a) Schematic illustration of the hybridization assay. (b) Dose response curves comparing the performance of QD-DNA monoconjugates (red squares) with QDs incubated with 1 equiv of DNA (unpurified) (green circles).

mixture containing QD and oligonucleotides with magnetic beads therefore resulted in all components with negative charge binding onto the beads. The unconjugated QDs bound to the magnetic beads due to their slight negative charge, and the wash buffer required an ionic strength of 0.100 M NaCl to remove the unconjugated QDs (see Supporting Information Figure S4). Further increase of the ionic strength resulted in the elution of the QD-DNA monoconjugates, as identified by gel electrophoretic analysis. The QD-DNA monoconjugates eluted at an ionic strength of 0.275 M NaCl. Additional monoconjugates were eluted with 0.300 M NaCl, but these only had a purity of 83% monoconjugates (with 17% of diconjugates being concurrently eluted). Smaller increments in the ionic strength could potentially provide for a higher yield of the monoconjugates, but under the conditions that have been noted, a 65% isolation efficiency of the monoconjugates was achieved.

A lower monoconjugate isolation efficiency for the same length of DNA (29-mer) was achieved for the amphiphilic PEG amine coating in comparison to the DHLA PEG coating. This reduction in yield is attributed to the negative charge of QDs with the amphiphilic coating. The additional negative charge serves as a constant background and results in a decrease of the difference in electrostatic stabilization energy between the monoconjugate and the diconjugate, thus decreasing the selectivity of differential retention.

Hybridization Analysis of Monoconjugates. Forster resonance energy transfer (FRET) based analysis[19] was performed to characterize the hybridization properties of the monoconjugates (Figure 6a). In FRET, energy is nonradiatively transferred from a donor (the QD) to an acceptor (Cy3 on the DNA). The energy transfer efficiency is dependent on a number of factors (information about the FRET pairs used in these experiments can be found in the Supporting Information), one of them being the distance between the QD and the dye. Hybridization of the complementary target with the probe results in bringing the dye into close proximity to the QD, facilitating efficient energy transfer. The FRET ratio, defined as the ratio of fluorescence intensity of the acceptor to the donor, can be used to quantify the amount of target hybridized.

It should be noted that the monoconjugate based on the 19-mer oligonucleotide, and DHLA PEG-QDs, was used for

dx.doi.org/10.1021/bc5002032 | Bioconjugate Chem. 2014, 25, 1342−1350

hybridization analysis. Short oligonucleotides are affected more by the nanoparticle due to the reduced extension away from the nanoparticle surface.

The importance of purifying the monoconjugates prior to use in analysis is illustrated in the response curve shown in Figure 6b. For the unpurified samples, the FRET response saturated at a lower limit when compared to the purified monoconjugates. This difference arises from the presence of a significant population of QDs that do not have a recognition element (oligonucleotide probe molecule). These QDs cannot take part in the FRET assay; yet, they contribute to the emission of the QDs, lowering the overall FRET response. In addition, the amount of probe used is also lower since the QD concentration is usually correlated to the probe concentration, which will lead to a lower amount of probe for the unpurified conjugates. Oligonucleotides that were not conjugated to the nanoparticles have not influenced the results as these were removed using spin ultrafiltration prior to assay of target oligonucleotides.

■ CONCLUSIONS

In this work, DEAE modified magnetic beads were used to selectively isolate QD-DNA monoconjugates. The benefits offered by magnetic beads in comparison to chromatographic methods lie in the speed of purification, the reduced number of interfaces that are encountered where sample loss can occur and the ability to readily produce a concentrated sample. The DNA strands were relatively short in comparison to the previously reported conjugates that can be separated by anion exchange chromatography. Three different lengths (19-mer, 29-mer, and 36-mer) were conjugated onto PEG modified QDs to demonstrate the capability of the method. Incubating the QD-DNA conjugates with magnetic beads resulted in their capture onto the bead surface. Ionic strength was used to tune the stringency of the elution solutions to selectively isolate the QD-DNA monoconjugates. The isolation efficiencies were found to depend on the length of the DNA, and ranged from 68% for the 19 mer to 93% for the 36 mer. In addition to achieving high isolation efficiencies, less than 5 min was required to isolate the monoconjugates. Monoconjugates prepared from commercially available, water-soluble QDs could be prepared, demonstrating the versatility of the method. The native negative charge of the commercially available QDs reduced the isolation efficiency. Without optimization, the isolation efficiency for monoconjugates prepared using the commercially available QDs was 67%. The significance of this work lies in the ability to easily purify monoconjugates of short oligonucleotides, which has been a challenge to date. The method also has potential applications for other nanomaterials. The separation mechanism is dependent on the oligonucleotides immobilized on the particle, making it independent of the type of nanoparticle used. The benefits offered by magnetic beads, such as potential for automation and availability, promises to make this technique a powerful addition to the toolbox available for the production of modified nanoparticles.

■ METHODS AND MATERIALS

All chemical reagents were obtained from Sigma-Aldrich (Oakville, ON, Canada), unless otherwise stated. Details of common instruments used are included in the Supporting Information.

Buffers. TB: 100 mM Tris-borate buffer; pH 7.4. PBS: 10 mM phosphate buffer, 2.7 mM potassium chloride, and 137 mM sodium chloride; pH 7.4. TBE: 50 mM Tris-borate buffer with 2 mM ethylenediaminetetraacetic acid; pH 6.9.

Quantum Dots. CdS_xSe_{1-x}/ZnS (core/shell) quantum dots (Cytodiagnostics Inc., Burlington, ON, Canada), with an emission peak of 525 nm, were made water-soluble using a bidentate poly(ethylene glycol) based coating (DHLA-PEG750). The ligand was synthesized as reported by Mei et al.,[1,8,12,31] and the reaction scheme is presented in the Supporting Information, Scheme S1.

Ligand Exchange. DHLA-PEG750 (~18.7 mg, 2×10^{-5} mol) was dissolved in 1 mL of ethanol, followed by the addition of QDs (1 nmol) that were dissolved in toluene. The resulting mixture was heated to 70 °C for at least 12 h. The QDs were precipitated from the ethanol solution using a mixture of hexanes and chloroform. The resulting solid was dispersed in 1 mL water and then purified using Amicon Ultra 0.5 mL centrifugal filters (100 kDa MWCO; Millipore, Billerica, MA, USA), as per the manufacturer's instructions.

Peptide Functionalized Nucleic Acid. Disulfide functionalized nucleic acids (sequences available in Table 2; obtained from IDT, Coraville, IA, USA) were reduced using dithiothreitol (DTT). An excess of DTT (~500 equiv) was incubated with the DNA in PBS buffer for 1 h. DTT was then removed by extracting the aqueous solution with ethyl acetate. The resulting nucleic acid solution was quantified using UV−vis spectrometry. The DNA was incubated with 5 equiv of the peptide, PEP1 (sequence available in Table 2; obtained from CanPeptide, Montreal, QC, Canada). Following overnight incubation at room temperature, the DNA−peptide chimera was purified using illustra NAP-5 desalting columns (GE Healthcare Life Sciences, Montreal, QC, Canada) as per the manufacturer's protocols.

Table 2. Sequences of Nucleic Acids and Peptides

DNA	
19-mer	5′-Thiol-ATT TTG TCT GAA ACC CTG T-3′ (Probe)
	3′-Cy3-TAA AAC AGA CTT TGG GAC A-5′ (Target)
29-mer	5′-Thiol-TTT TTT TTT TCC CTC CCC CAT GCC ATC CT-3′
36-mer	5′-Cy5-CCA CTG CAA ACA CTG GGC TGC AGC TTA TTT GGC CAG-Thiol-3′
Peptide	
PEP1	(6-Maleimidohexanoic acid)−G(Aib)GHHHHHH

DNA-QD Conjugation. The QDs were functionalized with the peptide modified DNA by incubation in the required ratio, in TB buffer, for at least 4 h.

SMCC Mediated DNA-QD Conjugation. Qdot 605 ITK Amino (PEG) Quantum Dots (Invitrogen, Oakville, ON, Canada) were buffer exchanged into 1 × PBS, as per the manufacturer's protocols. Following buffer exchange, the QDs were incubated with a 200 times molar excess of 4-(*N*-maleimidomethyl)cyclohexanecarboxylic acid *N*-hydroxysuccinimide ester (SMCC). After incubating for 1 h at RT, excess SMCC was removed using a NAP-5 column.

Disulfide modified oligonucleotides were reduced to thiols as described above. The SMCC activated QDs were then mixed with the thiol oligonucleotides in the required ratio, and incubated at RT overnight. The QD-DNA conjugates were then buffer exchanged into TB using spin ultrafiltration.

Monoconjugate Purification. The DEAE functionalized magnetic beads (Bioclone Inc., San Diego, CA, USA) were

washed twice in TB buffer supplemented with Tween-20 (0.05%, w/v). The QD-DNA conjugates were incubated with the magnetic beads for 1 min, after which the magnetic beads were collected to the side of the tube using an Ambion Single Tube Magnetic Stand (Invitrogen, Oakville, ON, Canada). The supernatant was removed and replaced with TB buffer as a wash. The magnetic beads were then sequentially incubated, for 30 s each, with solutions of increasing ionic strength. The ionic strength was controlled using sodium chloride. Once the fractions consisting of the monoconjugates were identified (using agarose gel electrophoresis), they were combined into a single aliquot and could be further concentrated using spin ultrafiltration. After use, the magnetic beads were rinsed with a 2 M sodium chloride solution and then stored in ethanol at 4 °C.

Agarose Gel Electrophoresis. Agarose gels were prepared in 0.5 × TBE buffer at a concentration of 2% (w/v). The gels were run for at least 2 h at 3 V cm^{-1}.

Single-Molecule Fluorescence Microscopy. A sample volume of 50 μL from a 100−200 pM solution of QD monoconjugates made with a Cy5 labeled oligonucleotide (36-mer; see Table 2 for sequence) in TB was spin-coated on plasma-cleaned glass coverslips (CA48366-089-1, VWR, USA). A custom-built multicolor total internal reflection fluorescence (TIRF) microscope[32,33] was used to measure the fluorescence intensity−time trajectories of immobilized QD-DNA complexes. The QD excitation was at 473 nm and the dye (Cy5) was excited at 633 nm, using CW lasers controlled by an acousto-optic tunable filter (Gooch & Housego, USA). The excitation light was reflected by a quad-edge dichroic mirror (Di01-R405/488/532/635, Semrock, USA) and illuminated the sample after passing through an oil-immersion microscope objective (1.45NA/60X Plan-Apochromat, Olympus, USA). The green (QD) and red (DNA-Cy5) fluorescence emission were collected using the same objective and transmitted through the quad dichroic mirror. In order to remove scattering and cross-talk signals, the QD fluorescence images were collected using a combination of a long-pass (BLP01−488, Semrock, USA) and a bandpass filter (HQ 520/66, Chroma, USA). The DNA-Cy5 images were collected with a long-pass (BLP01−647, Semrock, USA) and a bandpass filter (HQ 685/80 nm, Chroma, USA). A sequence of images was collected using a cooled, ultrasensitive electron-multiplied charge-coupled device (EMCCD, DU-897BV, Andor Technology, USA). The exposure time was 100 ms per frame and the laser excitation intensities used in these experiments was approximately 50 W cm^{-2}.

Hybridization Analysis. The probe was prepared at a concentration of 0.1 μM (defined by QD concentration; see Supporting Information for information on QD quantification) in 50 mM TB buffer along with 0.1 M NaCl. The probe was then incubated with varying amounts of target. Hybridization was allowed to proceed for 1 h at room temperature. The FRET ratio was then determined by measuring the fluorescence spectra (see Supporting Information for data analysis). Data was collected for purified monoconjugates and also unpurified conjugates to identify the impact of purification of some aspects of analytical performance.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Schematic of ligand synthesis, descriptions of the instruments used, fluorescence correlation spectroscopy analysis of QD-DNA conjugates, UV−vis analysis of QD-DNA conjugates, information on the FRET pair and others as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: ulrich.krull@utoronto.ca. Phone: 905 828 5437. Fax: 905 569 4388.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We would like to acknowledge the Natural Sciences and Engineering Research Council of Canada (NSERC) for financial support of this research. U. U. is thankful to NSERC for the provision of a graduate fellowship.

■ ABBREVIATIONS

QD, quantum dot; DNA, deoxyribonucleic acid; DEAE, diethylaminoethyl; RNA, ribonucleic acid; FRET, Förster resonance energy transfer; DHLA PEG, dihydroxylipoic acid poly(ethylene glycol); FCS, fluorescence correlation spectroscopy; Cy5, Cyanine 5; TIRF, total internal reflection fluorescence; D_h, hydrodynamic diameter; DTT, dithiothreitol; SMCC, succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate

■ REFERENCES

(1) Howarth, M., Liu, W., Puthenveetil, S., Zheng, Y., Marshall, L. F., Schmidt, M. M., Wittrup, K. D., Bawendi, M. G., and Ting, A. Y. (2008) Monovalent, reduced-size quantum dots for imaging receptors on living cells. *Nat. Methods 5*, 397−399.

(2) Farlow, J., Seo, D., Broaders, K. E., Taylor, M. J., Gartner, Z. J., and Jun, Y.-W. (2013) Formation of targeted monovalent quantum dots by steric exclusion. *Nat. Methods 10*, 1203−1205.

(3) Claridge, S. A., Mastroianni, A. J., Au, Y. B., Liang, H. W., Micheel, C. M., Fréchet, J. M. J., and Alivisatos, A. P. (2008) Enzymatic ligation creates discrete multinanoparticle building blocks for self-assembly. *J. Am. Chem. Soc. 130*, 9598−9605.

(4) Choi, J. Y., Kim, Y. T., and Seo, T. S. (2013) Polymerase chain reaction-free variable-number tandem repeat typing using gold nanoparticle−DNA monoconjugates. *ACS Nano 7*, 2627−2633.

(5) Petryayeva, E., Algar, W. R., and Medintz, I. L. (2013) Quantum dots in bioanalysis: a review of applications across various platforms for fluorescence spectroscopy and imaging. *Appl. Spectrosc. 67*, 215−252.

(6) Algar, W. R., Kim, H., Medintz, I. L., and Hildebrandt, N. (2014) Emerging non-traditional Förster resonance energy transfer configurations with semiconductor quantum dots: Investigations and applications. *Coord. Chem. Rev. 263−264*, 65−85.

(7) Hildebrandt, N., Wegner, K. D., and Algar, W. R. (2014) Luminescent terbium complexes: Superior Förster resonance energy transfer donors for flexible and sensitive multiplexed biosensing. *Coord. Chem. Rev.*, DOI: 10.1016/j.ccr.2014.01.020.

(8) Liu, H. Y., and Gao, X. (2013) Engineering monovalent quantum dot−antibody bioconjugates with a hybrid gel system. *Bioconjugate Chem. 22*, 510−517.

(9) Mullen, D. G., Desai, A. M., Waddell, J. N., Cheng, X.-M., Kelly, C. V., McNerny, D. Q., Majoros, I. J., Baker, J. R., Sander, L. M., Orr, B. G., and Banaszak Holl, M. M. (2008) The implications of stochastic synthesis for the conjugation of functional groups to nanoparticles. *Bioconjugate Chem. 19*, 1748−1752.

(10) Tikhomirov, G., Hoogland, S., Lee, P. E., Fischer, A., Sargent, E. H., and Kelley, S. O. (2011) DNA-based programming of quantum dot

valency, self-assembly and luminescence. *Nat. Nanotechnol. 6*, 485−490.
(11) You, C., Wilmes, S., Richter, C. P., Beutel, O., Liße, D., and Piehler, J. (2012) Electrostatically controlled quantum dot monofunctionalization for interrogating the dynamics of protein complexes in living cells. *ACS Chem. Biol. 8*, 320−326.
(12) Clarke, S., Pinaud, F., Beutel, O., You, C., Piehler, J., and Dahan, M. (2010) Covalent monofunctionalization of peptide-coated quantum dots for single-molecule assays. *Nano Lett. 10*, 2147−2154.
(13) Carstairs, H. M., Lymperopoulos, K., Kapanidis, A. N., Bath, J., and Turberfield, A. J. (2009) DNA Monofunctionalization of quantum dots. *ChemBioChem 10*, 1781−1783.
(14) Suzuki, S., Ono, N., Furusawa, C., Kashiwagi, A., and Yomo, T. (2007) Experimental optimization of probe length to increase the sequence specificity of high-density oligonucleotide microarrays. *BMC Genomics 8*, 373.
(15) Cissell, K. A., Shrestha, S., and Deo, S. K. (2007) MicroRNA detection: challenges for the analytical chemist. *Anal. Chem. 79*, 4754−4761.
(16) Ríos, A., Zougagh, M., and Bouri, M. (2013) Magnetic (nano)materials as an useful tool for sample preparation in analytical methods. A review. *Anal. Methods 5*, 4558.
(17) Medintz, I. L., Berti, L., Pons, T., Grimes, A. F., English, D. S., Alessandrini, A., Facci, P., and Mattoussi, H. (2007) A reactive peptidic linker for self-assembling hybrid quantum dot-DNA bioconjugates. *Nano Lett. 7*, 1741−1748.
(18) Casanova, D., Giaume, D., Moreau, M., Martin, J.-L., Gacoin, T., Boilot, J.-P., and Alexandrou, A. (2007) Counting the number of proteins coupled to single nanoparticles. *J. Am. Chem. Soc. 129*, 12592−12593.
(19) Algar, W. R., and Krull, U. J. (2007) Towards multi-colour strategies for the detection of oligonucleotide hybridization using quantum dots as energy donors in fluorescence resonance energy transfer (FRET). *Anal. Chim. Acta 581*, 193−201.
(20) Uyeda, H. T., Medintz, I. L., Jaiswal, J. K., Simon, S. M., and Mattoussi, H. (2005) Synthesis of compact multidentate ligands to prepare stable hydrophilic quantum dot fluorophores. *J. Am. Chem. Soc. 127*, 3870−3878.
(21) Cytodiagnostics Inc.
(22) Dennis, A. M., Sotto, D. C., Mei, B. C., Medintz, I. L., Mattoussi, H., and Bao, G. (2010) Surface ligand effects on metal-affinity coordination to quantum dots: implications for nanoprobe self-assembly. *Bioconjugate Chem. 21*, 1160−1170.
(23) Susumu, K., Uyeda, H. T., Medintz, I. L., Pons, T., Delehanty, J. B., and Mattoussi, H. (2007) Enhancing the stability and biological functionalities of quantum dots via compact multifunctional ligands. *J. Am. Chem. Soc. 129*, 13987−13996.
(24) Boeneman, K., Deschamps, J. R., Buckhout-White, S., Prasuhn, D. E., Blanco-Canosa, J. B., Dawson, P. E., Stewart, M. H., Susumu, K., Goldman, E. R., Ancona, M., and Medintz, I. L. (2010) Quantum dot DNA bioconjugates: attachment chemistry strongly influences the resulting composite architecture. *ACS Nano 4*, 7253−7266.
(25) Bioclone Inc.
(26) Noor, M. O., Tavares, A. J., and Krull, U. J. (2013) On-chip multiplexed solid-phase nucleic acid hybridization assay using spatial profiles of immobilized quantum dots and fluorescence resonance energy transfer. *Anal. Chim. Acta 788*, 148−157.
(27) Uddayasankar, U., and Krull, U. J. (2013) Analytical performance of molecular beacons on surface immobilized gold nanoparticles of varying size and density. *Anal. Chim. Acta 803*, 113−122.
(28) Boeneman Gemmill, K., Deschamps, J. R., Delehanty, J. B., Susumu, K., Stewart, M. H., Glaven, R. H., Anderson, G. P., Goldman, E. R., Huston, A. L., and Medintz, I. L. (2013) Optimizing protein coordination to quantum dots with designer peptidyl linkers. *Bioconjugate Chem. 24*, 269−281.
(29) Abraham, A. V., Ram, S., Chao, J., Ward, E. S., and Ober, R. J. (2009) Quantitative study of single molecule location estimation techniques. *Opt. Express 17*, 23352−23373.
(30) Yao, J., Larson, D. R., Vishwasrao, H. D., Zipfel, W. R., and Webb, W. W. (2005) Blinking and nonradiant dark fraction of water-soluble quantum dots in aqueous solution. *Proc. Natl. Acad. Sci. U.S.A. 102*, 14284−14289.
(31) Mei, B. C., Susumu, K., Medintz, I. L., and Mattoussi, H. (2009) Polyethylene glycol-based bidentate ligands to enhance quantum dot and gold nanoparticle stability in biological media. *Nat. Protoc. 4*, 412−423.
(32) Saleem, Q., Zhang, Z., Gradinaru, C. C., and Macdonald, P. M. (2013) Liposome-coated hydrogel spheres: delivery vehicles with tandem release from distinct compartments. *Langmuir 29*, 14603−14612.
(33) Liu, B., Mazouchi, A., and Gradinaru, C. C. (2010) Trapping single molecules in liposomes: surface interactions and freeze−thaw effects. *J. Phys. Chem. B 114*, 15191−15198.