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DNA Monofunctionalization of Quantum Dots

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Quantum dots (QDs) have been widely used to track cell components both in vitro and in vivo as they have a wide excitation spectrum, narrow emission, high efficiency and greater photostability than organic fluorophores. Single QDs can be identified by observing single-step blinking.[1] However, in spite of recent advances in surface chemistry, it is still nontrivial to control the stoichiometry of functionalized QDs such that a single QD labels a single molecule of interest.[2] Gold nanoparticles conjugated to DNA have been separated using gel electrophoresis,[3] anion exchange HPLC[4] and, when conjugated to His-tag proteins, by immobilized metal affinity chromatography.[5] Other work has concentrated on minimizing the number of multifunctional nanoparticles formed by linking through an initiator molecule that triggers a surface polymerization reaction[6] or by reaction on a sparsely functionalized solid support.[7] QDs monofunctionalized with streptavidin, an antibody or polyethylene glycol linker, have been purified using gel electrophoresis.[8] The limiting step of this method is the recovery of the QDs from the gel with typical extraction efficiencies of 30–50%. In this paper we describe a simple method by which commercially available QDs, monofunctionalized with a synthetic oligonucleotide, can be prepared quickly and with high yield by ion exchange using diethylaminoethyl (DEAE) Sepharose-packed spin columns.

Streptavidin-coated QDs (QD655, Invitrogen) were conjugated to biotinylated DNA duplexes as described in the Experimental Section. Streptavidin is a tetrameric protein with four biotin binding sites per molecule and there are, on average, ten protein molecules per QD (supplier's literature); although the average number of oligos attached to each QD can be controlled by varying the ratio of components, the population of QD–DNA conjugates is inhomogeneous. Figure 1A shows the resulting mixture analyzed by agarose gel electrophoresis. Bands corresponding to 0, 1 and 2 attached duplexes are clearly resolved. Figure 1B and C show that the band separation depends linearly on the length of the duplex.

Once the QD–DNA conjugate mixture was added to the ion exchange spin column as described in the Experimental Section, QDs were clearly visible as a red band in the DEAE resin. Unmodified QDs do not bind to the DEAE and were washed straight through by two column volumes of equilibration buffer. The contents of this, and subsequent, fractions are shown in Figure 2.

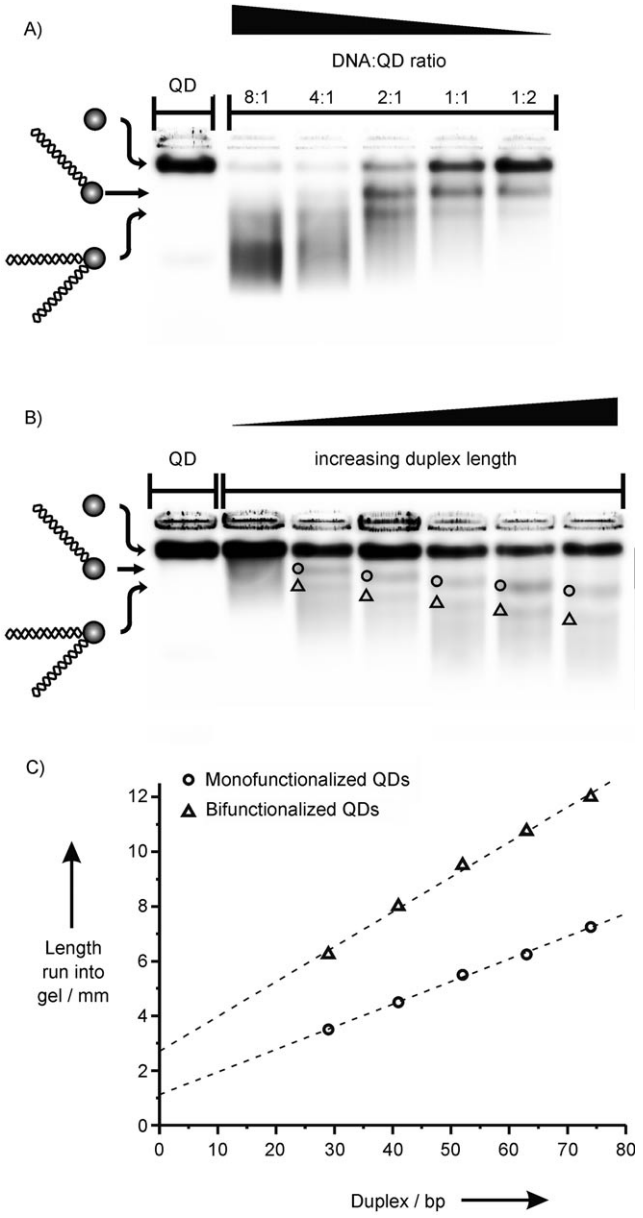

Figure 1. A) The distribution of streptavidin QDs and QD–DNA conjugates prepared using a range of concentrations of biotinylated DNA oligos. DNA:QD ratios are indicated above each lane of the 1% agarose, TAE gel. B) The mobility of the quantum dot is dependent on the length of the conjugated duplex. A 1% agarose gel was loaded with samples of streptavidin-coated QD655 after incubation with biotinylated duplexes of 19, 29, 41, 52, 63 and 74 base pairs. Sharp bands corresponding to monofunctionalized (○) and bifunctionalized (△) QDs are resolved in all but the lane containing a 19 bp duplex. C) The relative mobilities of both mono- and bifunctionalized quantum dots, where resolved, are linearly related to the length of the conjugated duplex.

Monofunctionalized QDs were recovered at 300 mM NaCl and a mixture of monofunctionalized and bifunctionalized QDs were eluted at 320 mM NaCl. Multifunctionalized QDs were

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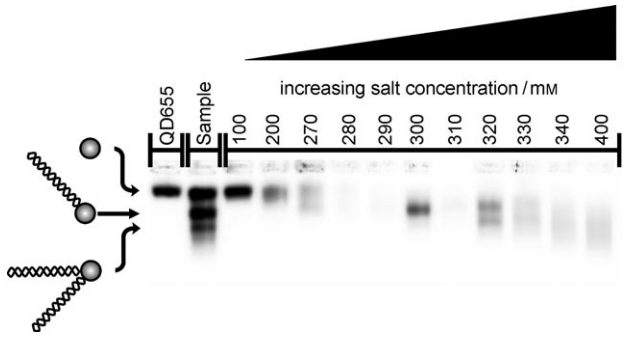

Figure 2. Purification of DNA-QD conjugates by ion exchange. The 1% agarose, TAE gel shows the heterogeneous sample of DNA-QD conjugates applied to a DEAE Sepharose spin column and the concentrated eluted fractions. The ionic concentration of the elution buffer is shown above each lane. The gel was run at room temperature at 3.5 $V cm^{-1}$ for 1.5 h.

eluted at $\geq$330 mM NaCl. Comparison of the fluorescence intensity of the monofunctionalized fraction with that of the monofunctionalized band in the sample before purification gave an estimate of 86% yield for the recovery of monofunctionalized QDs. See Figure S1 in the Supporting Information for details. The protocol also works with single-stranded DNA (ssDNA), see Figure S2.

In order to show that conjugation to the QD does not interfere with subsequent DNA hybridization, samples of differently coloured QDs QD565 and QD655 were functionalized with duplexes containing 20-nucleotide complementary sticky ends that had been purified and combined as described in the Experimental Section. Figure 3 shows analysis by gel electrophoresis of purified fractions and the resulting complexes. Mixing unfunctionalized QDs does not change the mobility of either the QD565 or QD655 band; this is consistent with the expected lack of interaction. The lane containing mixed multifunctionalized QDs contains no sharp band and little integrated fluorescence; this is consistent with the expected uncontrolled aggregation.[9] However, the product of combining the monofunctionalized fractions migrates as a single band that contains both quantum dots: The mobility of this complex is approximately equal to that of the larger monofunctionalized QD655 and clearly different from that of the smaller QD565. This shows that the monofunctionalized QDs have formed heterodimers linked by hybridization of the complementary sticky ends of the attached DNA duplexes.

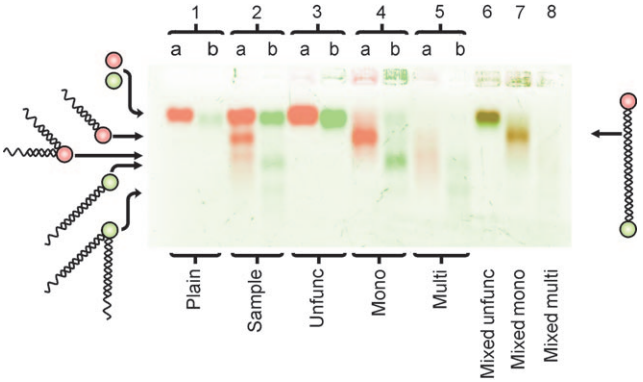

Figure 3. Interaction between QDs functionalized with complementary DNA. Lanes a) and b) of the 1% agarose TAE gel contain QD655 and QD565, respectively. Lane 1: as supplied. Lane 2: functionalized, unpurified. Lane 3: unfunctionalized dots purified from reaction mixture. Lane 4: purified monofunctionaized dots. Lane 5: purified multifunctionalized dots. Lane 6: mixed unfunctionalized dots. Lane 7: mixed monofunctionalized dots. Lane 8: mixed multifunctionalized dots.

We have shown that ion exchange can be used to purify QDs monofunctionalized with DNA oligonucleotides, and that the attached oligonucleotides can subsequently hybridize. Monofunctionalization will allow tight control of the self-assembly of more complex functional nanostructures based on DNA hybridization.[10] This is very practical preparation: it is quick, produces high yield and is relatively easy to scale up. Even a small spin column packed with 500 μL of resin can purify enough monofunctionalized QDs for several imaging experiments in one pass.

Experimental Section

QD–DNA conjugation: DNA was synthesized by Integrated DNA Technologies, Inc. (Coralville, USA). Biotinylated oligonucleotides were purified by high performance liquid chromatography and unmodified strands by polyacrylamide gel electrophoresis. Sequences used can be found in the Supporting Information. The lyophilized products were resuspended in deionized water (MilliQ) and the concentration measured by absorption at 260 nm. Complementary oligonucleotides (final concentration 1 μM) were mixed stoichimetrically in hybridization buffer TE100 (10 mM Tris-HCl pH 8, 1 mM EDTA, 100 mM NaCl) and annealed from 95 °C to 20 °C over 20 min.

Conjugation of biotinylated DNA duplexes, or single strands, to streptavidin-coated QDs (Qdot® 655, Invitrogen) was performed at room temperature in low-protein-binding polypropylene 0.5 mL tubes (Sorenson BioScience, Inc., Salt Lake City, USA). QDs were added to conjugation buffer TE100BSA (TE100 supplemented with bovine serum albumin (BSA) final concentration 0.5 $mg mL^{-1}$) and the solution was gently mixed by pipette. After 5 min, a stoichiometric amount of biotinylated DNA was added. The final concentration of both QDs and DNA was 0.02 μM and typical reaction volumes were 20–40 μL. The solution was covered with foil and left at room temperature for 30 min then put on ice.

Purification of monofunctionalized QDs: QDs interact with the polypropylene frit of prepacked DEAE anion exchange columns (GE Healthcare), so a spin column with a single polyethylene frit (Bio-Rad) was packed with DEAE Sepharose resin (500 μL; GE Healthcare). This resulted in a column 6 mm in diameter and 8 mm in length with a void of 22 mm between the resin and the top of the column. The column was equilibrated with TE100 (2×1 mL). This buffer has a sufficiently low ionic strength to allow a 52 bp duplex to bind to the resin, but high enough to ensure that the duplex remains hybridized at room temperature.[11]

A sample of streptavidin-coated QD–DNA conjugate was carefully added to the top of the resin and the cap was pushed back on to the top of the column to apply pressure and removed to start the flow. After 5 min the column was washed with TE100 (2×1 mL). Care was taken not to disturb the resin as the buffer was added. The flow-through was collected and stored covered in foil on ice.

The ionic strength of the elution buffer was raised in steps (10 mM each) and for each salt concentration elution buffer (1 mL) was added and the flow-through collected. Enough time was allowed for the elution buffer to pass completely through the column at each step. Pressure was applied to the top of the column to increase the flow rate to approximately 1 mL min^{-1}. The sample was visible as a band of colour which remained in the resin until an elution buffer of sufficient ionic strength to elute the majority population of functionalized QDs was added.

Concentration of samples: The collected fractions were concentrated using Millipore Ultracel YM-10 microcon filters at 4 °C according to the manufacturer's instructions. A sample (500 μL) can be concentrated (to ~100 μL) by centrifuging at 14000 *g* for 30 min. If the sample was to be analyzed by gel electrophoresis the fraction was concentrated until the final volume was < 50 μL.

Agarose gel electrophoresis: Gels containing agarose (1 or 2%, as indicated in the caption) were run in 1×TAE (40 mM Tris Acetate pH8.3, 1 mM EDTA) for ≤2 h at 3.5 V cm^{-1} in the dark at room temperature. Samples were mixed with a loading buffer of glycerol (50%).

A Pharos FX Plus Molecular Imager and Quantity One analysis software (Bio-Rad) were used to measure band intensities, from which yields were estimated, using 488 nm laser excitation and 530 nm, 640 nm band pass filters for QD565, QD655 respectively. For presentational purposes the levels of gel images were rescaled linearly after analysis.

QD heterodimerization: Two populations of QDs with different sizes and peak emission wavelengths (QD565 and QD655) were functionalized with duplexes containing 20-nucleotide complementary sticky ends (see the Supporting Information for sequences) and purified as described above. The concentration of each fraction was determined by counting single-molecule photon burst frequencies in a small volume using confocal detection. A confocal volume ~1 fL was excited by a continuous-wave laser source (532 nm, Samba, Cobolt, Sweden). Fluorescence was spatially filtered and spectrally separated using a dichroic mirror (DRLP630, Omega Optical, Brattleboro, USA); fluorescence from QD565 and QD655 was spectrally filtered using 580 nm band-pass and 650 nm long-pass filters, respectively. Photon arrival times were recorded using avalanche photodiode detectors (SPQR-14, Perkin–Elmer) and processed with custom-written LabVIEW (National Instruments, Austin, USA) software. The relative concentrations of quantum dots in eluted fractions were estimated by comparing the frequencies of single-molecule events.

Pairs of samples of each QD (unfunctionalized, monofunctionalized and multifunctionalized) were mixed stoichiometrically and incubated for 1 h at room temperature.

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