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NeutrAvidin Functionalization of CdSe/CdS Quantum Nanorods and Quantification of Biotin Binding Sites using Biotin-4-Fluorescein Fluorescence Quenching

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Abstract

We developed methods to solubilize, coat, and functionalize with NeutrAvidin elongated semiconductor nanocrystals (quantum nanorods, QRs) for use in single molecule polarized fluorescence microscopy. Three different ligands were compared with regard to efficacy for attaching NeutrAvidin using the “zero-length cross-linker” 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC). Biotin-4-fluorescein (B4F), a fluorophore that is quenched when bound to avidin proteins, was used to quantify biotin binding activity of the NeutrAvidin coated QRs and biotin binding activity of commercially available streptavidin coated quantum dots (QDs). All three coating methods produced QRs with NeutrAvidin coating density comparable to the streptavidin coating density of the commercially available quantum dots (QDs) in the B4F assay. One type of QD available from the supplier (ITK QDs) exhibited ~5-fold higher streptavidin surface density compared to our QRs, whereas the other type of QD (PEG QDs) had 5-fold lower density. The number of streptavidins per QD increased from ~7 streptavidin tetramers for the smallest QDs emitting fluorescence at 525 nm (QD525) to ~20 tetramers for larger, longer wavelength QDs (QD655, QD705, and QD800). QRs coated with NeutrAvidin using

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ASSOCIATED CONTENT

Supporting Information

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Figures S1–S7: B4F excitation and emission spectra, electron micrographs, B4F fluorescence vs B4F concentration, streptavidins per unit surface area, comparison of fluorescence anisotropy, TIRF microscopy. Tables S1–S3: extinction coefficients, and PEG and ITK quantum dot quenching intersections, biotin binding sites, and bound streptavidins (PDF)

The authors declare no competing financial interest.

mercaptoundecanoic acid (MUA) and QDs coated with streptavidin bound to biotinylated cytoplasmic dynein in single molecule TIRF microscopy assays, whereas Poly(maleic anhydride-*alt*-1-octadecene) (PMAOD) or glutathione (GSH) QRs did not bind cytoplasmic dynein. The coating methods require optimization of conditions and concentrations to balance between substantial NeutrAvidin binding vs tendency of QRs to aggregate and degrade over time.

Graphical abstract

INTRODUCTION

Semiconductor quantum dots (QDs) are fluorescent nanoparticles that are widely used in biochemical assays for labeling individual proteins both for in vivo imaging,¹ and high for in vitro precision tracking.^{2–4} QDs present some photophysical advantages over organic dyes because they are much brighter and do not photobleach over the time scale of typical fluorescence experiments.⁵ Applications for fluorescent nanoparticles are broad since their emission wavelengths can be tuned simply by changing the diameter and composition of the typically CdSe or CdTe core.^{6,7} This tunability of the emission wavelength, paired with their broad excitation spectrum, makes QDs ideal for multicolor imaging of biological molecules using a single excitation wavelength.⁸ Quantum nanorods (QRs) are elongated semiconductor nanoparticles that share many features with QDs such as material composition and bright, stable fluorescence, but unlike nearly spherical QDs, QRs exhibit polarized fluorescence emission which can be utilized to determine their three-dimensional orientation.^{9,10} A high degree of polarization, >20:1, is achieved when the aspect ratio of length to width is greater than 10:1.¹¹ Disadvantages of semiconducting nanoparticles are their larger size compared to visible organic fluorescent probes and fluctuations and blinking of their fluorescence. Adding a CdS¹² or ZnS¹³ shell reduces blinking and increases the brightness of nanoparticles.^{7,14} The size of the QD-coating hybrid can be minimized by choice of coating used to solubilize and conjugate the nanoparticles to the target biological system.

Quantum dots are available with a range of surface coatings, facilitating specific labeling of proteins both in vivo and in vitro. Although water-soluble, functionalized QDs are readily available, commercial availability of coated quantum rods is limited. Nanoparticles labeled with a biotin binding protein, such as streptavidin or NeutrAvidin, can be used to attach them to biotinylated proteins or nucleic acids.¹⁵ Here, we present several methods to

functionalize CdSe/CdS QRs with NeutrAvidin that can be readily applied for use in single molecule polarized fluorescence assays. Nanoparticles that have been synthesized in organic solvent and coated with a hydrophobic ligand¹⁶ are transferred to aqueous solution by exchanging the hydrophobic layer with a bifunctional ligand which contains a thiolate that binds to the particle surface and a polar carboxyl group that stabilizes the particles in aqueous media.¹⁷ The carboxyl group can be covalently cross-linked to an amine-containing compound or protein, in this case the biotin binding protein NeutrAvidin. Fluorescence polarization is retained after functionalization.

Knowing the number of binding sites available on avidin-coated QDs and QRs can be important in designing experiments requiring attachment to multiple or known numbers of proteins. Here we describe an improved method to quantify the number of avidin proteins attached to individual nanorods or quantum dots and compare the number of binding sites obtained using different methods for ligand exchange. We also compare the degree of NeutrAvidin functionalization achieved on QRs to that of commercially available functionalized QDs of different sizes and surface treatments. The same materials (CdSe, CdS, and ZnS) are used to manufacture the QDs and commercial QRs, but their shapes and sizes are different, which might affect the liganding chemistry. The comparable avidin protein density achieved indicates that the shape and size are not major determinants.

RESULTS

Determining Number of Biotin Binding Sites for Streptavidin and NeutrAvidin

Biotin-4-fluorescein (B4F) binds tightly to streptavidin and NeutrAvidin. Its fluorescence is strongly quenched (>90%, Figure S1) when bound. B4F quenching was used to determine the concentration of NeutrAvidin or streptavidin free in solution^{18–20} or conjugated to nanoparticles²¹ at 5 to 60 nM concentrations of protein tetramer. To verify and calibrate the technique and as a basis for reliably determining the amount of avidin in solutions of functionalized nanoparticles, we first measured B4F quenching over a range of known NeutrAvidin and streptavidin concentrations (Materials and Methods). While each tetramer contains four biotin binding sites, all four sites are not necessarily active and/or occupied simultaneously with B4F. Known concentrations of streptavidin and NeutrAvidin (based on absorbance at 280 nm) from 0 to 60 nM tetramers were combined with B4F at concentrations spanning 0 to 200 nM and the B4F fluorescence intensity was measured (Figure 1a). In the absence of protein the fluorescence increased linearly with increasing B4F concentration, but in the presence of streptavidin or NeutrAvidin the fluorescence was quenched until B4F binding became saturated, at which point the fluorescence increased linearly with a slope similar to that of B4F alone.

The effective concentration of biotin binding sites was determined from the point where the curve fitted the data at low B4F concentration and the line fitted the data at high B4F concentration intersect (Materials and Methods). The slopes of the curves in Figure 1b give the number of biotin binding sites per streptavidin or NeutrAvidin tetramer. Streptavidin binds an average of 3.46 B4F molecules per tetramer, close to the maximum occupancy of four, while NeutrAvidin binds 2.23 B4Fs per tetramer, close to the lower end of the range, 2.7–4.2 implied by the manufacturer's instructions (<https://tools.lifetechnologies.com/>

[content/sfs/manuals/MAN0011245_NeutrAvidin_Biotin_BindProtein_UG.pdf](#)). Incomplete biotin binding site occupancy could be due to steric hindrance of B4F binding or reduced activity of the lyophilized protein after resuspension in aqueous solution.

Comparing Coating Methods for Laboratory-Made Quantum Nanorods

The NeutrAvidin functionalization of laboratory-made QRs surface coated using different methods was measured using the B4F quenching assay on samples of QRs at known concentrations. Free NeutrAvidin was carefully removed from QR samples using sequential pelleting by centrifugation and resuspension in fresh buffer until the amount of free NeutrAvidin present in the QR solution was less than 1 tetramer per 30 QRs, based on the number of times the buffer was exchanged. B4F quenching was measured in either 0.25 or 0.5 nM solutions of QRs and compared to the NeutrAvidin calibration curve to determine the concentration of biotin binding sites present in each QR sample (Figure 2a). Poly(maleic anhydride-*alt*-1-octadecene) (PMAOD)-coated QRs bound an average of 63.1 NeutrAvidin tetramers per QR, glutathione (GSH)-coated QRs had an average of 30.8 tetramers per QR, and mercaptoundecanoic acid (MUA)-coated QRs had an average of 42.2 tetramers per QR (Figure 2b, summarized in Table 1). Nanorods had average dimensions of 56.3 nm long and 5.6 nm in diameter as determined by TEM (Figure S2), giving an average surface area, calculated assuming a cylindrical shape, of 1040 nm² per QR. On the basis of this surface area, the PMAOD-, GSH-, and MUA-QRs had NeutrAvidin surface densities of 0.061, 0.030, and 0.041 NeutrAvidins per nm², respectively (Figure 2c, Table 1). To confirm that the quenching observed with the nanorod samples was due to the bound NeutrAvidin and not the result of an interaction with the polymer coating, we compared B4F quenching of PMAOD, GSH, and MUA QRs before and after the NeutrAvidin conjugation reaction. The results showed that QRs do not quench B4F prior to NeutrAvidin conjugation, so the quenching observed with the NeutrAvidin functionalized QRs is due to the NeutrAvidin.

Throughout the optimization of the coating and functionalization methods, a number of conditions were observed that decreased stability or increased the rate of aggregation of the nanoparticles. The ligand exchange reaction was sensitive to the starting organic solvent. Beginning with the QRs in THF improved the yield compared to performing the reaction in chloroform. However, storage of QRs in THF for more than a day resulted in a transition from a brilliant pink color to brown, indicating loss of fluorescence. In contrast, QRs are stable for months to years when stored in hexane. Adding potassium *tert*-butoxide (K⁺BuO⁻) as a base for the reaction also improved the yield relative to adding KOH (Materials and Methods). Once in aqueous solution, QRs are prone to aggregation. Using avidin instead of NeutrAvidin (deglycosylated avidin) caused the nanoparticles to precipitate even in the absence of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) cross-linker. Zwitterions, which have a net neutral charge, have been shown to increase the stability of nanoparticles.^{22,23} NeutrAvidin, which has lower isoelectric point than glycosylated avidin (isoelectric point 10.5), may have a stabilizing effect similar to a zwitterion and so is more effective than avidin at stabilizing the nanoparticles. Nanoparticle stability was also sensitive to the ratio of EDC to NeutrAvidin; increasing EDC concentration without increasing the NeutrAvidin concentration accelerated precipitation. Exchanging aqueous QRs into

phosphate buffered saline (PBS) instead of borate buffer (pH 7.4 or pH 9) also precipitated them.

Quantifying Streptavidin Coating of Commercial Quantum Dots

To compare the functionalized QRs with commercial streptavidin-coated QDs we applied the B4F quenching method to quantify the number of streptavidins coating various samples of quantum dots obtained from Life Technologies, Inc. Mittal et al.²¹ also measured the streptavidin complement of QDs, but we consider our assay, the methods for estimating QD concentrations, and our estimate of B4F streptavidin binding stoichiometry more reliable than theirs. In contrast to the earlier study, we measured concentrations of QD stock solutions rather than assuming the listed concentration, and we experimentally determined the average number of functional biotin binding sites per streptavidin tetramer instead of assuming the maximum of four. We used a series of QDs coated via polyethylene glycol (PEG QD evaluation kit part #Q10151MP) and a series of QDs coated using ITK, an amphiphilic polymer As specified in the product literature, ITK quantum dots contained more biotin binding sites than the PEG-coated quantum dots (Figure 3a,b), so we used a lower range of B4F concentrations for the PEG QD measurements (Figure S3a,b).

PEG QDs had an average number of streptavidins per quantum dot ranging from 0.30 to 1.4 (Figure 4a, results summarized in Table S2), whereas the ITK quantum dots had between 7.4 and 18 streptavidins per quantum dot (Figure 4b, summarized in Table S3). Carboxylated ITK 655 quantum dots (without streptavidin) did not quench B4F, demonstrating that quenching for the main series was due to the streptavidin. Tables S2 and S3 give raw data as biotin binding sites (intersection between curves in the B4F assay) per OD of absorption at 350 nm to enable calculation of streptavidin content with alternate assumptions about QD extinction coefficients (e.g., values given by the manufacturer, which are generally higher than those estimated from the lowest energy absorption peak, resulting in lower concentration estimates).

QD shapes and sizes were estimated using TEM (Figure S4) to determine average surface areas, and streptavidin surface densities. Surface densities on PEG-QDs ranged from 0.0011 to 0.0083 streptavidins per nm² (Figure S5a, Table S2), while the densities on ITK quantum dots were ~10-fold higher, 0.094 and 0.17 streptavidins per nm² (Figure S5b, Table S3). Quantum dots increase in size with increasing emission wavelength and there was a trend for the number of streptavidins per QD to increase with QD size within a given type of coating, as expected (Figure 4a,b). The streptavidin surface density was more constant (Figure S5a,b).

DISCUSSION

We compared three different ligands in order to coat and water solubilize CdSe/CdS QRs synthesized in organic solvent. The QRs were then functionalized with NeutrAvidin using EDC and NHS. All three methods produced QRs with NeutrAvidin coating density comparable to the streptavidin coating of commercial ITK QDs. Nanorods maintained their polarization properties even after coating with NeutrAvidin (Figure S6). While QRs coated using PMAOD had the most NeutrAvidin, as measured using B4F quenching, they did not

bind biotinylated yeast cytoplasmic dynein in a single molecule binding assay (Figure S7c). GSH-QRs coated with NeutrAvidin also failed to bind to biotinylated dynein in the single molecule assay (Figure S7b). MUA-coated QDs bound to biotinylated GFP-tagged dynein at approximately 0.22 QDs per GFP (Figure S7a). However, binding of MUA QRs was lower than that of commercial ITK QDs which bound at ~0.8 QDs per GFP (Figure S7d). QRs prepared using the other two coating methods were never observed bound to dynein on axonemes.

B4F fluorescence quenching can be used to determine the concentration of biotin binding proteins in solution and attached to nanoparticles with high sensitivity and precision. At similar concentrations of NeutrAvidin and streptavidin, we found that NeutrAvidin binds fewer B4F molecules per tetramer than streptavidin.

The PMAOD-, GSH-, and MUA-coated QRs made in-house had more avidin tetramers per QR (30–60) than the commercial ITK QDs (7–22). The QRs are larger and when normalized to surface area, QRs exhibited an avidin surface density of roughly one-third that of the ITK QDs and 5-fold higher than the PEG QDs.

ITK quantum dots coated with amphiphilic polymer have more streptavidins per quantum dot than the PEG alternatives. As expected from the increase in size with wavelength, the number of streptavidins per QD tended to increase with emission wavelength and size. For a similar set of QDs obtained from Invitrogen, Inc. (now Life Technologies, Inc.) as used here, Mittal and Bruchez²¹ reported 40–80 B4F binding sites per ITK QD and 2–4 B4F sites per PEG QD (except 12 sites on 800 nm PEG QDs). They concluded that the binding capacity did not change systematically with QD size. Several earlier studies of streptavidin content of QDs are also listed by Mittal and Bruchez.²¹ Our values of 30–70 sites per ITK QD and 2–6 per PEG QD are similar overall, but we observed a substantial increase of content with size (Tables S2 and S3) leading to approximately constant surface density on both types (Figure S5). This is logical, as we would expect that a surface modification reaction would depend on the amount of surface present rather than the number of individual particles, assuming that surface curvature does not significantly impact reaction rates. Different bases for quantifying B4F, streptavidin, and QD concentrations and the number of B4F sites per streptavidin tetramer may be the cause of this apparent discrepancy. The largest difference is their use of the manufacturer's nominal stock QD concentrations, whereas we based the QD concentrations on measurements of the extinction coefficients where possible (Table S1). Except for the trend with QD size, though, the two studies are comparable.

The methods for coating and functionalizing QRs described here and for quantifying avidin content should be applicable to other semiconductor nanocrystal reagents and shapes.

MATERIALS AND METHODS

Water Solubilization of CdSe/CdS Quantum Nanorods

QRs with CdSe nanorod cores and CdS/ZnS double shells were made in three steps according to the literature methods: first, CdSe nanorods (14.8×5.3 nm) were synthesized;²⁴ second, CdSe nanorods were coated with an elongated CdS nanorod shell;¹¹

third, CdSe/CdS nanorods were coated with a thin layer of ZnS in trioctylphosphine oxide for a total size of 56.3 nm × 5.6 nm.¹⁶ Excess ligand, solvent, and unreacted precursor were removed by three cycles of precipitating the QRs using ethanol (a nonsolvent) and centrifugation. The resulting particles formed stable dispersions in nonpolar organic solvents such as hexanes, toluene, chloroform, and tetrahydrofuran (THF). We tested different carboxylated ligands for reactivity and solubilization of the QRs in aqueous media. Glutathione (GSH) and mercaptoundecanoic acid (MUA), each containing both a carboxyl and a thiol, bind covalently to the QR shell via their sulfur atoms, replacing the TOPO. Amphiphilic polymer poly(maleic anhydride-*alt*-1-octadecene) (PMAOD) does not replace TOPO, but rather, its alkyl chains intercalate among the alkyl chains of the TOPO, and its carboxyl groups render the nanoparticles water-soluble.

Glutathione Coating of Nanorods—Hexane was evaporated under vacuum and nanoparticles were resuspended in THF to a concentration of approximately 4 μM. QRs were coated with GSH following a protocol adapted from Jin et al.²⁵ 500 μL of QRs in THF was combined with 200 μL of 10 mg/mL GSH and heated to 60 °C in a water bath. The mixture was centrifuged at 14 000g for 10 min to pellet the QRs. The supernatant was removed and discarded and QRs were resuspended in 1 mL of water. 5 mg of potassium *tert*-butoxide (K⁺BuO⁻) was added to the QR solution and sonicated for 15 min. The aqueous QRs were centrifuged at 3000g to remove aggregates and insoluble QRs and the supernatant was recovered.

Mercaptoundecanoic acid Coating of Nanorods—Hexane was evaporated and QRs were resuspended in THF to a concentration of approximately 4 μM. QRs were coated with MUA following a modified protocol from Jin et al.²⁵ 10 mg of MUA was added to 500 μL of nanoparticles in THF. The mixture was heated to 60 °C in a water bath. 5 mg of K⁺BuO⁻ was added to the THF solution and the sample was returned to the 60 °C water bath. The QRs precipitated and were pelleted by centrifuging at 14 000g for 10 min. The THF supernatant was removed and discarded and QRs were resuspended in 1 mL of water. Aggregates and insoluble particles were removed by centrifuging at 3000g for 10 min and recovering the supernatant.

Poly(maleic anhydride-*alt*-1-octadecene) Coating of Nanorods—QRs were solubilized by intercalating PMAOD into the hydrophobic TOPO coating.^{26,27} 1 mL of 10 mg/mL PMAOD in chloroform was combined with 1 mL of QRs at approximately 1–2 μM in chloroform. The mixture was stirred at room temperature for 2 h. The chloroform was evaporated under vacuum and QRs were resuspended in 1 mL of aqueous 50 mM sodium borate, pH 8.3. This solution was sonicated for 10 min and then centrifuged at 3000g for 10 min to remove aggregates. The supernatant was recovered and the sonication and centrifugation steps were repeated.

All three types of QRs were stored in the dark at room temperature.

NeutrAvidin Coating of PMAOD, MUA, and GSH Quantum Nanorods

The carboxyl groups on GSH, MUA, and PMAOD were covalently linked to amine groups in NeutrAvidin using the “zero-length crosslinker” 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) with *N*-hydroxysuccinimide (NHS) present to increase cross-linking efficiency. Aqueous GSH, MUA, or PMAOD coated QRs were pelleted at 62 000*g* at 4 °C for 30 min. The supernatant was removed, and QRs were resuspended in one-tenth to one-third volume of 50 mM sodium borate, pH 8.3. 30 µL of buffer-exchanged QRs were combined with 30 µL of a 1 mM EDC, 5 mM NHS solution made fresh from powder immediately prior to use. The mixture was incubated for 5 min at room temperature and combined with 30 µL of 10 mg/mL NeutrAvidin in 10 mM sodium borate, pH 7.4. This solution was incubated at room temperature for 5 min then stored at 4 °C for 4–16 h.

Free NeutrAvidin was removed from the QR solution through sequential centrifugation steps. 90 µL of NeutrAvidin-QRs was ultracentrifuged at 35 000*g* for 20 min at 4 °C until the nanorods pelleted. 80 µL of supernatant was removed and replaced with 80 µL of fresh 50 mM sodium borate, pH 8.3. This was repeated four times to achieve ~6500-fold dilution of unconjugated NeutrAvidin, to an estimated final concentration of ~8 nM NeutrAvidin tetramers and ~250 nM QRs, or about one unbound tetramer per 32 QRs.

Determining Concentrations of Nanoparticles

QDs conjugated to streptavidin using PEG and emitting fluorescence at 525, 565, 585, 605, 655, and 705 nm (termed PEG QDs) were purchased as an evaluation kit from Life Technologies, part no Q10151MP. QDs conjugated to streptavidin using ITK and emitting fluorescence at 525, 545, 565, 585, 605, 655, 705, and 800 nm (termed ITK QDs), part nos Q10041MP, Q10091MP, Q10031MP, Q10011MP, Q10001MP, Q10021MP, Q10061MP, and Q10071MP, respectively, were kindly donated to us by Life Technologies, Inc. Measurements of the number of Streptavidin or NeutrAvidin molecules conjugated to the QDs or QRs depended on their estimated concentrations. Molar extinction coefficients (ϵ) for nanoparticles depend on their size, shape, and composition.²⁸ Molar extinction coefficients of QDs as a function of their longest wavelength absorption peak have been well characterized,²⁸ and this method was used to determine the concentrations of the commercial 525, 565, 585, and 605 QDs, each of which had a distinct absorption peak 10–25 nm below their quoted emission peak. The 655, 705, and 800 QDs, however, did not exhibit a distinguishable lowest energy absorption peak. For these QDs, an extinction coefficient of 1 700 000 M⁻¹ cm⁻¹ at 550 nm, as provided by the manufacturer, was used to determine concentration. Additional extinction coefficients provided by Life Technologies at other wavelengths are listed in Table S1 for comparison. In most cases, the spectral method for determining molar extinction resulted in somewhat higher estimated concentrations than those provided with the commercial samples.

Although the molar extinction coefficients of QDs have been calculated experimentally, no such calibrations are available for the more complex CdSe/CdS/ZnS-type core/shell/shell QRs as used in this study. Therefore, the extinction coefficient for CdSe/CdS/ZnS core/shell/shell QRs was calculated by combining information on the sizes of the CdSe core and the

CdS shell as determined by TEM imaging and the extinction coefficient of the individual components, and adding their contributions together (Figure S2). To determine the contribution of the CdSe nanorod core to the extinction coefficient, a literature calibration was used based upon TEM measurements of the nanorod size: at 350 nm, the absorption of the CdSe core scales with the volume,²⁴ which was measured to be $3.22 \times 10^{-21} \text{ cm}^3$ on average, giving an extinction coefficient at 350 nm of $1.09 \times 10^7 \text{ M}^{-1} \text{ cm}^{-1}$ for the CdSe core alone. No direct measurement for the extinction coefficient of CdS rods is currently available, but the wavelength-dependent *linear* extinction coefficient of CdS ($\alpha(\lambda)$, in units of cm^{-1}) can be estimated from the reported imaginary index of refraction k of 5.3 nm CdS QDs according to $\alpha(\lambda) = 4\pi k/\lambda$.²⁹ Using this literature report of the value of k (0.389) at 350 nm, we obtained $\alpha(\lambda) = 1.40 \times 10^5 \text{ cm}^{-1}$. The *linear* extinction coefficient may be converted into a *molar* extinction coefficient ($\text{M}^{-1} \text{ cm}^{-1}$) if the volume V of the material (e.g., CdS, in cm^3) is known according to $\epsilon(\lambda) = N_A V \alpha(\lambda)/1000 \ln(10)$, in which N_A is Avogadro's number (mol^{-1}), the factor $\ln(10)$ converts the extinction coefficient from a base e exponential (standard for linear absorption) to a base 10 exponential common for molar extinction coefficients; and the factor of $1/1000$ converts volume in cm^3 to L.³⁰ Using TEM to calculate the volume of the total structure and subtracting the volume of the core, we obtained a volume of $1.145 \times 10^{-20} \text{ cm}^3$ and a molar extinction coefficient at 350 nm attributable to the CdS shell of $4.17 \times 10^7 \text{ M}^{-1} \text{ cm}^{-1}$. The extinction coefficients of the CdSe core and CdS shell can be added together resulting in the extinction coefficient of the whole nanorod $\epsilon_{350}(\text{rod}) = 5.26 \times 10^7 \text{ M}^{-1} \text{ cm}^{-1}$. The concentration of QRs in solution was determined using this extinction coefficient based upon the absorption measured at 350 nm. The amount of ZnS in the QRs and its absorption at 350 nm are both negligible and therefore contribution was not included.

Biotin-4-Fluorescein Quenching Assay to Quantify NeutrAvidin and Streptavidin Coating

Powdered B4F (Invitrogen) was resuspended to an approximate concentration of 2.5 mg/mL, or ~3.9 mM in 30 mM sodium borate, pH 8.3, and filtered through a 0.2 μm syringe filter. Absorbance at 495 nm was used to determine the actual concentration of the stock solution using an extinction coefficient of $68\,000 \text{ M}^{-1} \text{ cm}^{-1}$.²¹

Streptavidin (Thermo Scientific) and NeutrAvidin (Thermo Scientific) were dissolved in 10 mM sodium borate, pH 7.4, and the concentrations were measured using the absorbance at 280 nm and extinction coefficients of $41\,940 \text{ M}^{-1} \text{ cm}^{-1}$ per monomer and $23\,615 \text{ M}^{-1} \text{ cm}^{-1}$ per monomer calculated from their amino acid sequences.³¹

Fluorescence of the B4F was determined using a Tecan GENios plate fluorescence reader with 485 nm excitation and 535 nm emission. Solutions and cartridges for the plate reader were prepared in a 4 °C cold room. 180 μL of each solution with known or unknown avidin protein concentration was added to wells in a 96-well plate. 20 μL of B4F at a range of concentrations was added to each well. Final dye concentrations after mixing ranged from either 0 nM to 40 nM or 0 nM to 200 nM depending on the approximate concentration of avidin protein in the sample. The plates were incubated overnight at 4 °C and measured the following morning in the plate reader.

Data Analysis

Because biotin–avidin affinity is very high, the concentration, C_1 , of added biotin at which quenching saturates and fluorescence begins increasing linearly gives a good estimate of the concentration of binding sites on the avidin protein in the sample. Below C_1 , fluorescence increased gradually as B4F increased according to $F = [B4F] F_{\text{Sat}} / ([B4F] + K_{\text{half}})$, the nonlinearity presumably due to mutual quenching of B4Fs in addition to quenching by the avidin,²⁰ where F_{Sat} is the maximum fluorescence at high [B4F] and K_{half} is the half-saturating B4F concentration (Figure 1). Above C_1 , fluorescence increased linearly according to $F = S[B4F] + \text{Int}$, where S is the slope, similar to that in the absence of any avidin protein, and Int is an intercept. The intersection between the quenched curve at low [B4F] and the unquenched line at high [B4F] was found by minimizing least-squares fits of the curve and the linear functions fit to the quenched and unquenched regions, respectively. A MatLab routine successively tested partitioning the data between quenched and unquenched regions, fitting the two relations for each partition to the data and tabulating the resulting correlation coefficient, R^2 . For the partitioning with the highest R^2 value, the [B4F] value at the intersection between the two curves was taken to be C_1 . The chosen partitioning was also required to contain C_1 between quenched and unquenched B4F concentration regions. Data sets with fewer than three points in the linear regime were excluded due to unreliability of the fit. Confidence intervals for C_1 were determined by bootstrapping using the same fitting algorithm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) B4F fluorescence vs B4F concentration with NeutrAvidin present at concentrations listed in the legend. Quenching data for each NeutrAvidin concentration are fit with a curve and a straight line (solid lines; see Methods) to determine the B4F concentration, C_I , at the intersection. The B4F series without NeutrAvidin ("Buffer") is fit to a straight line only. Error bars are standard deviations. (B) C_I vs streptavidin (blue) and NeutrAvidin (red) tetramer concentrations with fitted lines given in the boxes. The slopes give the apparent number of B4F binding sites per streptavidin (3.46) or Neutravidin (2.23). Error bars are 95% confidence interval as determined by bootstrapping.

Figure 2.

Measurement of NeutrAvidin content of laboratory-made QRs coated with different surface treatments. (A) B4F and QRs were combined at known concentrations but with unknown surface density of NeutrAvidin. Data are fit to a curve and a line as in Figure 1 to determine the intersection, C_I . B4F fluorescence was also measured in the presence of surface-treated QRs without NeutrAvidin to verify that quenching is due solely to the NeutrAvidin. Error bars are standard deviations. (B) Average numbers of NeutrAvidins per QR and (C) per unit surface area as determined from the C_I in (A) and the effective numbers of B4F binding sites

per tetramer from Figure 1. Error bars are 95% confidence interval as determined by bootstrapping.

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Figure 3.

Measurement of streptavidin coating of commercial (A) PEG and (B) ITK QDs. B4F and QDs were combined at known concentrations and the B4F fluorescence was measured. C_I values were determined as before. Error bars are standard deviations.

Figure 4.

Average number of streptavidin tetramers per (A) PEG or (B) ITK QD as determined by C_1 values from Figure 3 and the apparent number of B4F binding sites per streptavidin tetramer (3.46) from Figure 1. Error bars are 95% confidence interval as determined by bootstrapping.

Summary of NeutrAvidin Quantification Parameters for QRs with Different Surface Treatments^a

Table 1

sample	B4F quenching intersection, C_1 (nM)	biotin binding sites per 350 nm (nM/AU)	biotin binding sites per nanorod	NeutrAvidins per nanorod	NeutrAvidins per unit surface area (1/nm ²)
PMAOD QRs	50.7; +15.7; -4.24	3854; +1197; -322.6	203; +62.3; -17.0	63.1; +28.2; -7.61	0.0607; +0.0272; -0.00732
GSH QRs	49.8; +2.93; -3.68	1894; +111.4; -140.0	99.6; +5.86; -7.36	30.8; +2.63; -3.30	0.0297; +0.00253; -0.00318
MUA QRs	62.5; +4.58; -6.37	2378; +174.1; -242.4	125; +9.16; -12.7	42.2; +4.11; -5.72	0.0406; +0.00395; -0.00550

^a Values denoted by + and - indicated the upper and lower bounds, respectively, of the 95% confidence interval determined by bootstrapping. The concentrations of biotin binding sites per QR absorption unit or per QR are listed as C_1/OD and $C_1/[QR]$, respectively. The number of NeutrAvidin tetramers per QR (NAV/QR) were calculated as $NAV/QR = (C_1 - 15.49)/(2.23 \cdot [QR])$, coefficients determined from the linear fit of C_1 vs NeutrAvidin tetramer concentration shown in Figure 1, where C_1 and $[QR]$ are both given in nM. NeutrAvidins per unit surface area is listed as NAV/QR divided by the surface area of individual QRs.