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Characterization of the Functional Binding Properties of Antibody Conjugated Quantum Dots

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ABSTRACT

Antibody conjugated quantum dots are an emerging technology for high-resolution labeling of biological systems. In this work we determined the number of functional antibodies (i.e., antibodies that are sterically available for functional binding to target proteins) conjugated to semiconductor quantum dots. This is critical for the interpretation of biological data labeled with these methods. We found that the number of available functional antibodies varied significantly for different conjugation methods and are lower than previously estimated. These results may suggest potential strategies for improving quantum dot labeling of biological preparations.

Semiconductor quantum dots have physical and optical properties that make them useful tools for high-resolution labeling and imaging of proteins in cells. They are brighter than organic fluorescent dyes, exhibit minimal photobleaching, and have narrow emission spectra which support multiplexing of signals (i.e., the use of multiple quantum dots of different colors) in the same preparation.[1−5] By chemically conjugating antibodies and other peptides to their surface, quantum dots can specifically target cellular ligands of interest.[6−15]

One critical issue that has not been addressed is experimentally determining the number of antibodies bound to quantum dots which are functionally available for target protein binding. This is critical for the analysis and proper interpretation of biological data labeled using this method. For example, we have previously shown that immunoglobulin G (IgG) antibody functionalized quantum dots can be used for high-resolution imaging of fixed neurons and glial cells if conjugation and blocking conditions are optimized.[7] However, we also demonstrated reproducible nonspecific artifact labeling, which could be mistaken for specific labeling, if antibody−quantum dot conditions are less than optimal (Figure 1). While other groups have qualitatively characterized antibody-functionalized quantum dots using transmission electron microscopy, atomic force microscopy, UV spectroscopy, and gel electrophoresis[16−19] and in some cases have suggested estimates of the putative

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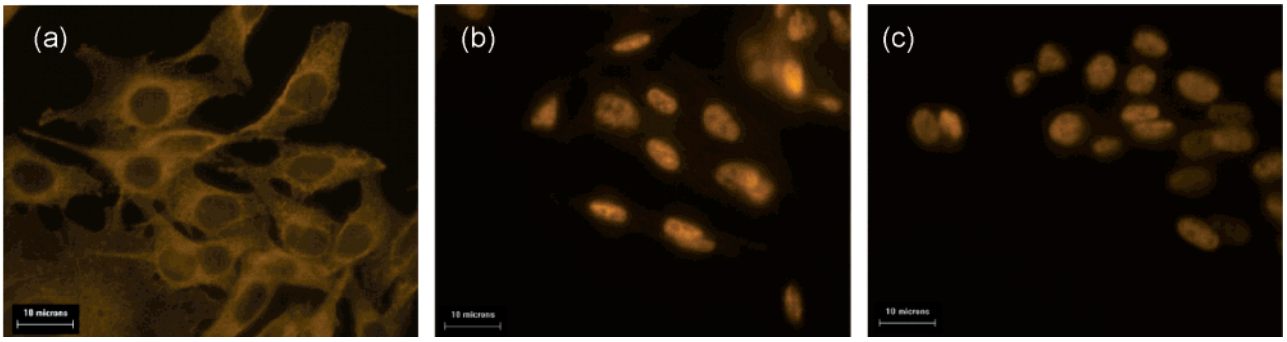

Figure 1. Examples of physiologically specific and nonspecific artifact labeling of cells (neural retinal Muller glial cells) using streptavidin−biotin IgG conjugation and covalent IgG conjugation to 605 nm quantum dots. (a) Specific labeling for the cytoskeletal intermediate filament glial fibrillary acidic protein (GFAP) using biotinylated anti-GFAP IgG conjugated to streptavidin-coated quantum dot complexes. This represents physiologically specific labeling. (b) Nonspecific nuclear staining using anti-GFAP IgG covalently conjugated to quantum dots in non-permeabilized cells. (c) Nonspecific nuclear CD90 labeling using anti-CD90 IgG covalently conjugated to quantum dots. This example is particularly striking because CD90 is a surface membrane protein expressed by populations of mesenchymal derived cells and is not expressed by Muller cells. Note that the same incubation times were used for all three samples; only the conjugation method varied.

number of total antibodies bound to quantum dots,[3] no calculations of the number of functional antibodies bound to quantum dots based on quantitative experimental results have been reported. In the present work we derived the number of functional IgG antibodies conjugated to quantum dots based on calculations of quantitative electrophoresis experiments using two different conjugation schemes: a common direct covalent conjugation using a reduced disulfide maleimide reaction, and biotinylated antibodies bound to streptavidin-functionalized quantum dots (see Methods in the Supporting Information). Antibody−quantum dot complexes were run in a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to separate the functional component of conjugated antibodies from the quantum dots. We then blotted the antibody fragments onto a membrane to determine the identity and amount of the antibodies and quantitatively compared the amount of transferred antibody to known antibody concentrations to derive the number of bound antibody.

The number of functional antibodies covalently bound to commercially available quantum dots was on average much less than one functional IgG molecule per quantum dot (0.076 ± 0.014) and therefore of potential limited utility for biological experiments. In contrast, antibodies bound to quantum dots via the streptavidin−biotin system resulted in higher numbers of functional antibodies, with 0.60 ± 0.14 IgG molecules per quantum dot for a 1:1 IgG : quantum dot molar ratio and 1.3 ± 0.35 IgG molecules per quantum dot for a 2:1 ratio, thereby supporting biological labeling. In addition to these specific results, our methods may be of broader interest because our approach is easily extendable for experimentally deriving the number of functional antibodies or peptides bound to other classes of nanoparticles (e.g., magnetic nanoparticles).

We begin by considering the covalent conjugation of antibodies to quantum dots. Using a commercially available direct conjugation kit (Invitrogen) and following published protocols, antibodies were reduced using dithiothreitol (DTT), which generates three distinct fragments identifiable by their molecular weights: A 25 kD light chain, which importantly includes half of the specific antigen binding site for a particular IgG molecule, a 50 kD heavy chain, which includes the other half of the binding site, and a 75 kD partially cleaved chain consisting of a heavy chain and a light chain held together by an unreduced disulfide bond (Figure 2a). Following this, individual fragments were covalently bound to quantum dots via an SMCC linkage bond which cannot be broken by DTT treatment,[20] an important consideration for the interpretation of the experimental results that follow. This gives rise to three possible antibody fragment binding scenarios to quantum dots (Figure 2b): covalently bound light chains, covalently bound heavy chains, and covalently bound heavy−light chain partial fragments, of which only the latter can undergo further DTT reduction to remove the light chain fragment from heavy chains that remain bound to quantum dots, or heavy chains removed from light chains bound to quantum dots.

We first confirmed that antibodies were indeed covalently bound to the quantum dots by running IgG−quantum dot complexes though SDS-PAGE with and without DTT. For DTT reduced conditions we observed light chains cleaved from covalently bound partial fragments (Figure 3a, lanes 4−6). As expected, this separation occurred minimally in lanes without DTT (Figure 3a, lanes 2−3). The presence of a weak band at the 25 kD position in nonreduced lanes (Figure 3a, lanes 2 and 3) was due to low concentrations of reducing agents in the gel and running buffers. Interestingly, we saw no heavy chains being dissociated from light chain bound partial fragments. It is unclear why this was the case, although we hypothesize that the probability of the heavy chain portion of a partial fragment binding to a quantum dot is considerably higher than the light chain portion because there is twice the surface area for heavy chain binding and it is a condition that may be stereically favored (since the bend in the partial chain may tend to hide the light chain from the quantum dot). Another potential explanation for the lack of heavy chain band is methodological. Given the intensity of other bands in the membranes, small amounts of free heavy chain may have gone undetected given the exposure time we used to develop the membrane, which if it had been longer may have shown the presence of heavy chains but would have overexposed the other darker bands,

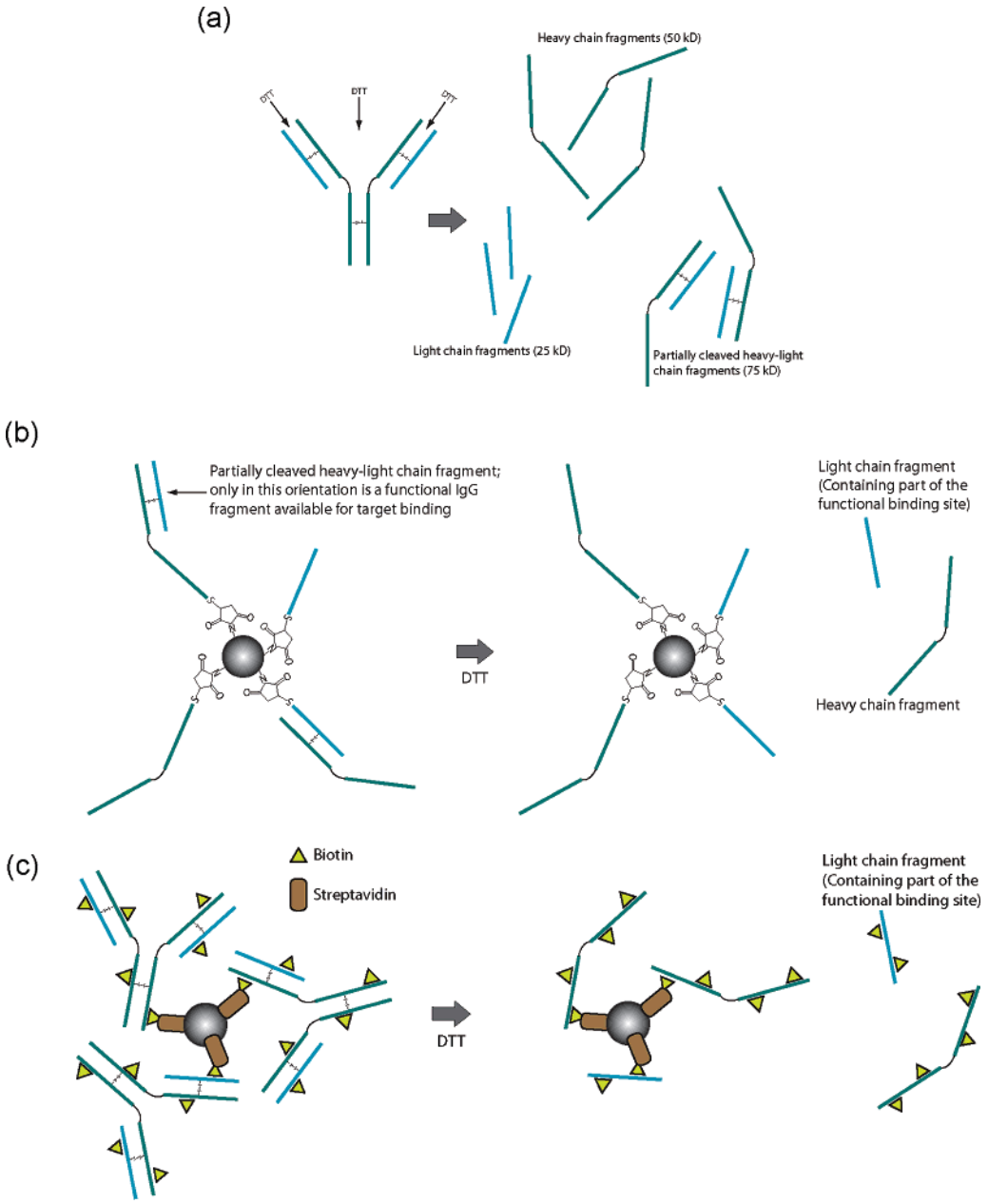

Figure 2. Antibody reduction and conjugation to quantum dots. (a) Schematic of antibody cleavage sites by DTT at disulfide linkages. The fragments that can result from DTT reduction include the light chain, heavy chain, and partially cleaved fragments due to incomplete reduction. (b) Schematic of direct SMCC covalent conjugation of antibodies to quantum dots. Further reduction with DTT following the primary reduction associated with the conjugation reaction yields the light chains which are counted in the derivation of the average number of functional IgG molecules originally on quantum dots. (c) Similar schematic for biotinylated antibodies conjugated to streptavidin-coated quantum dots.

resulting in uninterpretable smearing. Additional evidence that heavy chains covalently bound to quantum dots remained bound to the quantum dots is inferred by a nonspecific colloidal blue protein stain which labels any protein in the gel that did not transfer to the membrane (Figure 3b). Since blue bands appeared at the position in the gels that corresponded to the quantum dots, some amount of residual protein did remain on their surface. Given that most of the light chains were cleaved, since they transferred strongly to the membrane, this residual protein is mostly heavy chain. Regardless, for the purpose of calculating the amount of functional antibody on quantum dot surfaces this is of minimal importance, since it is the amount of available light chain that we are interested in. For an antibody to be functional, both the light chain and the heavy chain must be present. More specifically, it is the light chain in combination with the heavy chain that allows target binding. Molecularly, roughly the first 110 amino acids at the amino terminal end of both heavy and light chains form the variable, or V, regions which contain highly variable segments called complementary determining regions.[21,22] The pairwise association of V regions from both heavy and light chains is what actually forms the antigen binding site.[21,22] Anther important consideration to note is that the amount of partial fragments initially available for binding to quantum dots following the initial DTT reduction was very low, as evident

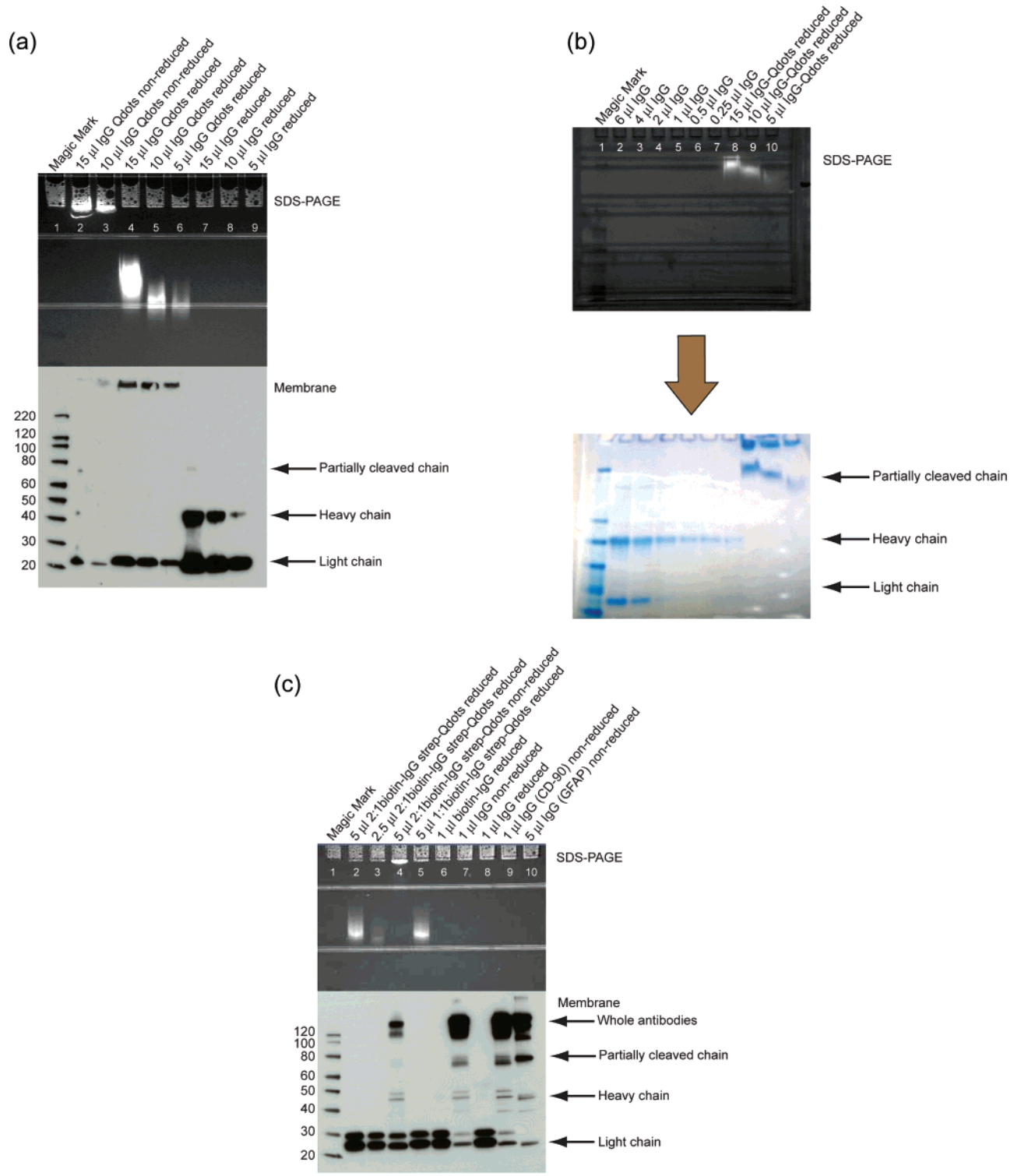

Figure 3. Separation of IgG antibodies into fragments using SDS-PAGE and membrane transfer under different experimental and control conditions. (a) Covalently conjugated IgG to quantum dots via an SMCC linker and controls. (b) Colloidal Blue nonspecific stain for proteins in gels for the direct conjugation method. (c) Biotinylated IgG bound to streptavidin-coated quantum dots and controls.

in the reduced unconjugated IgG controls (Figure 3a, lanes 7−9). This point is an important consideration for why the number of available functional antibody in the covalently conjugated condition was calculated to be so low. (Note that no partial fragments were visible for the quantum dot lanes because the entire partial chain cannot be cleaved intact from the quantum dot since the SMCC linkage cannot be broken by DTT.) Furthermore, additional evidence that antibodies were covalently attached, not electrostatically attached, comes from the fact that several bands would have shown up in nonreduced lanes if they were electrostatically attached (Figure 3a, lanes 2 and 3) because the gel would have separated the antibodies from the quantum dots according to their molecular size and weight. Further indirect evidence that antibodies were covalently bound is implied by the fact that quantum dots in nonreduced lanes did not travel through

the gels but remained in the loading wells due to the large size of the unreduced complex (visible as intense signals in the loading wells for lanes 2 and 3 of the SDS PAGE in Figure 3a).

We ran the same experiments with biotinylated antibodies and streptavidin-coated quantum dots at 2:1 and 1:1 antibody to quantum dot molar ratios.[23] Biotinylated antibodies have four−eight biotin molecules attached at random locations throughout the entire antibody, which results in the IgG molecules being conjugated to quantum dots presumably in all possible spatial arrangements (Figure 2c). Importantly and very differently from the direct covalent conjugation reaction, using the biotin−streptavidin system, the entire antibody molecule is conjugated to the quantum dot; it is not reduced into light chain and heavy chain fragments prior to binding. Similar to the covalent antibody conjugation method, nonreduced conditions resulted in quantum dots remaining in the loading wells (Figure 3c, lane 4) while reduced conditions allowed quantum dots to run through the gels (Figure 3c, lanes 2, 3, and 5). Some amount of antibody did transfer in nonreduced conditions for biotin−streptavidin IgG−quantum dot complexes because of the reducing agents in the running buffers and the gel, causing the light chain to dissociate in the same manner as for the covalent conjugation. Since all bands were much stronger in the biotin−streptavidin method in general, bands for the nonreduced condition were correspondingly stronger. Bands in non-DTT treated antibody lanes (i.e., Figure 3c, lanes 7, 9, and 10) show the reduction process in greater detail since reduction agents in the running buffers reduced the antibodies less efficiently than DTT-treated conditions (Figure 4a, lanes 2, 3, 5, 6, and 8). Additional controls (supplementary figure) are discussed in the Supporting Information.

On the basis of these data and the qualitative models introduced above that describe the different putative binding scenarios for antibodies directly covalently conjugated to quantum dots and for antibodies bound to quantum dots via biotin and streptavidin (Figure 2), we derived the average number of functional IgG conjugated to quantum dots. We use the term "functional antibody" to describe the amount of F_{ab} light chain, which includes a part of the target protein binding epitope, that is physically oriented outward from a quantum dot and presumably able to interact with its ligand. As such, only a partial fragment bound to the quantum dot would be functional since it contains both the light and heavy chains required to bind to the target protein. Furthermore, because of the structure of the antigen binding site, a partial fragment covalently bound to the quantum dot oriented with the light chain facing the nanoparticle would almost surely prevent ligand binding. Since it is the F_{ab} light chain portion of the antibody that actively binds to proteins, quantifying the amount of light chain fragments not directly bound to quantum dots and oriented outward gives a good approximation of the functional activity of antibody−quantum dot complexes.

To determine the number of functional IgG bound to quantum dots, we measured the density of the 25 kD light chain bands and compared them to controls of known antibody concentrations. Using image analysis software that measures the band density of electrophoresis gels (ImageQuant TL, GE Healthcare; see Methods in the Supporting Information), we fitted curves to known concentrations of unconjugated IgG to obtain standard curves of IgG band densities (Figure 4a,b; all $r^2 \geq 0.89$). Using these curves, we then determined the concentration of IgG bands associated with covalently bound IgG and 2:1 and 1:1 IgG:quantum dot molar ratio streptavidin-biotin conjugation conditions (Figure 4c,d). Finally, we calculated the number of functional antibodies bound to the quantum dots for each condition (Figure 4e; see Supporting Information for detailed calculations).

For covalently conjugated IgG we calculated that on average there is much less than one antibody molecule (0.076 ± 0.014) per quantum dot. In other words, adding 10 μL of antibodies directly conjugated to quantum dots is equivalent to adding 0.455 μL from a 0.5 mg/mL stock. This suggests that covalently conjugated antibodies have low amounts of functionally available antibodies and are of potentially inadequate sensitivity for reliable specific labeling of target proteins (see Figure 1). In contrast, the number of antibodies bound to quantum dots via the strepavidin−biotin system resulted in a more biologically reasonable 0.60 ± 0.14 IgG molecules per quantum dot for a 1:1 IgG/quantum dot molar ratio and, as would be expected, 1.3 ± 0.35 IgG molecules per quantum dot for a 2:1 ratio. This is equivalent to a functional volume of 0.943 μL of antibody for a 2:1 molar ratio or 0.53 μL for 1:1 molar ratio starting from 4 μL from a 0.5 mg/mL stock concentration. We acknowledge that these numbers are an approximation, since light chains near the quantum dot surface attached to a heavy chain bound to the quantum dot as part of a partial fragment would be sterically unavailable for antigen binding but could still dissociate following DTT reduction. However, we suspect this represents a small source of error because it is likely sterically difficult for bound heavy−light chain domains to bind to the quantum dot; it thermodynamically favors the functional partial fragment orientation (see Figure 2b). In any case, this error would contribute to an overestimation of the number of functional antibodies conjugated to a quantum dot. Therefore it represents an estimation of an upper bound on the number of putative functional antibodies, further emphasizing the significance of these results.

These results are significantly less than suggested estimates of between 2−10 antibodies conjugated per quantum dot.[3,24,25] To the best of our knowledge no conjugation reaction can control the binding orientation of IgG molecules. Consequently, due to Brownian motion the number of bound functional antibodies is almost certainly less than the number of total bound IgG. An important question is: Why did covalent conjugations result in lower numbers of functional antibodies compared to streptavidin−biotin conjugations? One explanation is that DTT-reduced antibody fragments attaching to the surface of quantum dots leave few opportunities for light chain fragments to be properly oriented outward and available for protein binding. Only partial fragments result in functional antibodies, and even then the

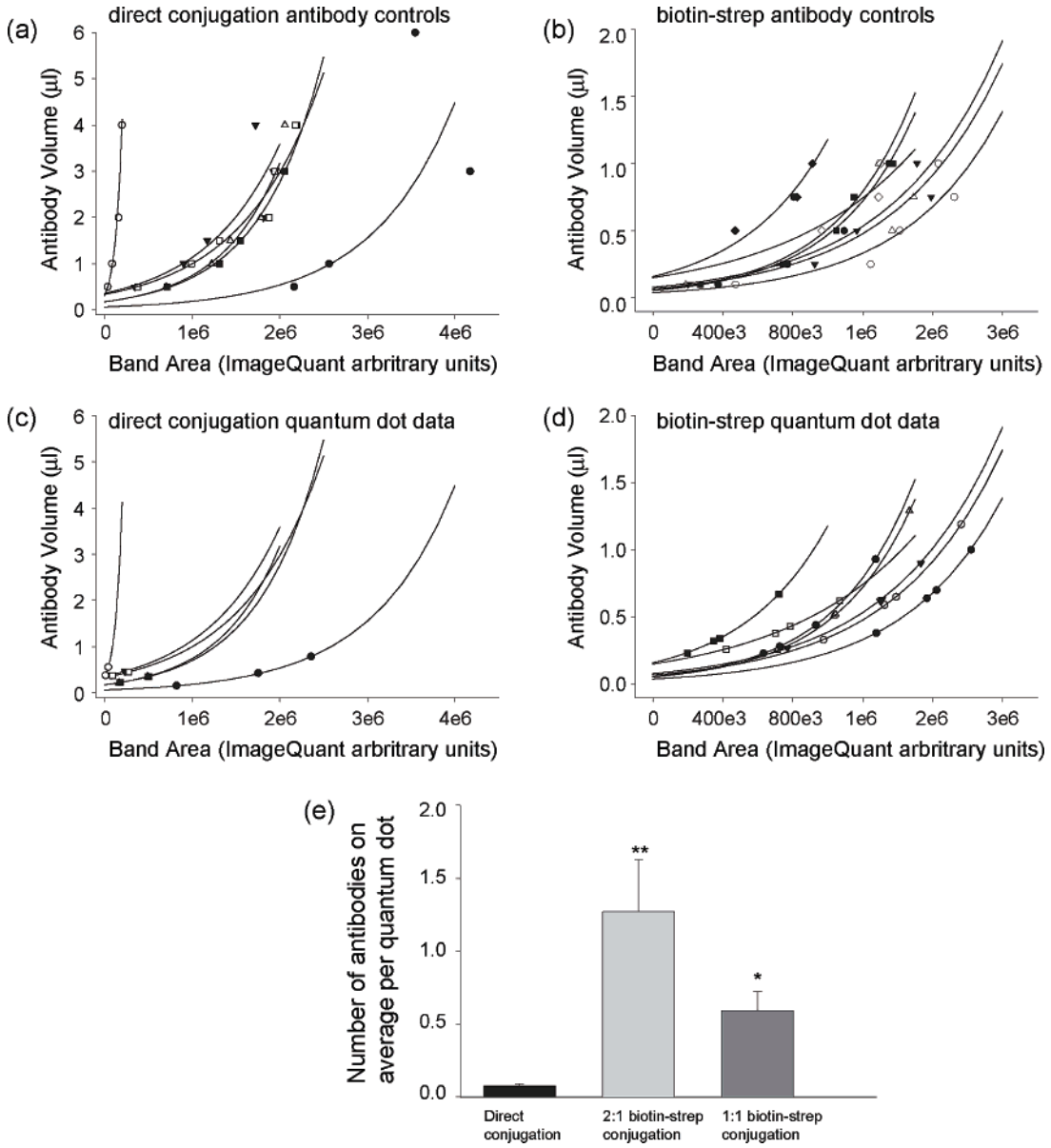

Figure 4. Derivation of the average number of functional antibodies on both covalently conjugated and streptavidin−biotin conjugated quantum dots based on measurements of the bound density for different concentrations. (a and b) Fitted linear log control curves ($\ln y = ax - b$) for known volumes of unconjugated IgG antibody band densities in SDS-PAGE gels. Note that the data for each gel were fitted with its own curve in order to control for inter-gel variability. Each symbol represents a different gel ($n = 6$ gels for covalently conjugated IgG conditions containing a total of 32 unconjugated IgG controls and 13 IgG−quantum dot complexes, and 7 gels for streptavidin−biotin IgG−quantum dot complexes containing a total of 35 unconjugated IgG controls and 28 IgG−quantum dot complexes). (c and d) Corresponding derived volumes from SDS-PAGE band densities for conjugated and streptavidin−biotin conjugated antibody−quantum dot complexes using the curves plotted in panels a and b, respectively. (e) Calculated values for the average number of antibodies conjugated to quantum dots for both conditions based on the derived measurements of functional antibody volumes (* and ** $p < 0.01$). See Supporting Information for detailed calculations.

orientation of the partial fragment binding to the quantum dot surface must be correct to allow the light chain fragment to point outward in order to interact with its ligand. In biotin−streptavidin conjugations the antibody is never cleaved, leaving the whole molecule bound to the quantum dot surface and structurally offering more opportunities for light chain fragments to bind their targets. It is plausible that other covalent conjugation chemistries result in higher yields of functional antibodies, comparable to those we report for streptavidin−biotin conjugates or even higher, but it cannot simply be assumed so since, as we show here, at least one well established and commonly used covalent conjugation reaction results in low numbers of functional antibody on quantum dots. We propose that functionalized quantum dot labeling of biological preparations need to be preceded by the experimental determination of the number of functionalized antibodies per quantum dot, especially given the variability in conjugation methods between different labs. These considerations have a direct impact on the quality, interpretation, and relevance of biological or physiological results obtained using quantum dot labeling nanotechnologies.

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Note Added after ASAP Publication. The figure showing a schematic of antibody−quantum dot cross-linking and additional control conditions was omitted from the Supporting Information file posted May 31, 2007; the corrected version was published ASAP June 27, 2007.

Supporting Information Available: Descriptions of materials and methods including quantum dot conjugation and membrane transfer, additional information regarding additional sources of functional antibody loss, additional controls, and calculations of equivalent quantum dot conjugated antibody concentrations, and a figure showing antibody−quantum dot cross-linking and additional control conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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