Characterization of the Functional Binding Properties of Antibody Conjugated Quantum Dots: Supplemental Materials

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Supplemental Materials

Materials and Methods

Quantum Dot Conjugation

605 nm quantum dots were conjugated to anti-GFAP and anti-CD90 IgG (BD PharMingen) using Quantum Dot Corporation's 605 antibody conjugation kit (catalog #2200-1). Briefly, quantum dots were activated using the SMCC crosslinker, which resulted in a maleimide functional group on the surface of the particles. Antibodies were simultaneously reduced with DTT to cleave the disulfide bonds and make –SH groups available for conjugation. Quantum dots were then added to the reduced antibody solution where covalent coupling occurred. The reaction was then quenched with β-mercaptoethanol. Excess antibody was removed with size exclusion chromatography. After conjugation, quantum dots were stored at 4°C.

Biotin-streptavidin-quantum dot complexes were synthesized using modifications of published protocols [1]. Briefly, biotin-streptavidin conjugates were formed by gentle vortexing followed by incubation of the biotinylated IgG with streptavidin coated 605 quantum dots (Invitrogen Corporation) for 30 minutes. Two reactions were performed: 1:1 and 2:1 molar ratios of biotinylated IgG to streptavidin coated quantum dots.

SDS-PAGE.

NuPAGE gels, 4-12% Bis-Tris (Invitrogen Catalogue # NP0321BOX), and 1M DTT in 1.0 mm X 10 mm wells were used for reduction of quantum dot conjugates. Briefly, 4X NuPAGE LDS sample buffer (Invitrogen Catalogue # NP0007), 1X NuPAGE sample buffer, DTT (in reduced samples only), and each sample were combined in a centrifuge tube according to standard

Western protocols. They were heated to 89°C for 15 minutes, centrifuged briefly, mixed gently, and loaded into the gels. Gels were run for 1.5 hours at 60 mA and 200 V in running buffer (760 mL of DI water and 40 mL NuPage MOPS SDS running buffer). Quantum dots were visualized in gels using a standard UV gel transilluminator. We ran 6 gels with covalently conjugated IgG containing a total of 32 unconjugated IgG controls and 13 IgG-quantum dot complexes. We ran 7 gels of streptavidin-biotin IgG-quantum dot complexes containing a total of 35 unconjugated IgG controls and 28 IgG-quantum dot complexes.

Membrane Transfer

Sponges, filter paper, and nitrocellulose membranes were soaked in transfer buffer (for one gel: 120 mL 100% methanol, 1020 mL DI water, and 60 mL NuPage transfer buffer concentrate (20X); for two gels: 240 mL 100% methanol, 900 mL DI water, and 60mL NuPage transfer buffer concentrate). After removing gels from their casings they were placed on soaked filter papers which were placed on top of two soaked sponges. Nitrocellulose membranes were cut, placed on the gels and covered with another piece of soaked filter paper. Two more sponges were placed on top of the second piece of filter paper and the entire sandwich was enclosed in the transfer apparatus. Transfer buffer was poured into the casings and run for 2 hours at 30 V.

Visualization

Membranes were blocked with 1.25 g of evaporated milk in 25 µl TBS (1 packet of Trizma Set Crystals (Sigma) in 2 L DI water, 17.6 g NaCl (200 mM), and 2 mL Tween-20) for 1 hour at room temperature. Secondary anti-mouse HRP conjugate was added and incubated for1 hour at room temperature. Membranes were rinsed 3 X with TBS for 5 minutes each. SuperSignal West

Pico Chemiluminescent Substrate (Pierce Product #34080) was added for detection of HRP and incubated for 1 minute with the membranes. Visualization took place 10 minutes later with films pressed against the membrane blots for 1 sec, 30 sec, or 1 min development points. The films were processed in a standard film developer.

Colloidal Blue Labeling

Colloidal Blue (Invitrogen Catalogue # LC6025) labeling was performed in some gels instead of the transfer step. Briefly, gels were fixed for 10 minutes in fixing solution (40 ml DI water, 50 ml methanol, 10 ml acetic acid), incubated for 3 hours with Colloidal Blue dye, and rinsed for 7 hours with DI water. Images were taken with a digital camera. Note that smaller proteins (light chains especially) diffused out of the gels at longer incubation times.

ImageQuant Analysis

Membranes were scanned with an HP PSC 2175 scanner and loaded into ImageQuant software (Amersham BioSciences), which calculated the size and density of each band, and plotted them against the known concentration of the controls. The data in Fig. 4 were fit to linear log curves given by $\ln y = ax - b$, where the parameters *a* and *b* were determined by ImageQuant. For quantification, gels were run with standards of 5 controls and 4 samples along with 1 lane of MagicMark Protein standard (Invitrogen LC5602). The 5 control lanes consisted of 1 μl, 0.75 μl, 0.5 μl, 0.25ul μl, and 0.1 μl of antibody from stock (BD PharMingin, 0.5mg/ml) to form the standard curves and 2 samples (4 μl and 2 μl) each of 1:1 and 2:1 IgG : quantum dot molar ratios for the biotin-streptavidin system.

Supporting Information

Additional sources of functional antibody loss

As described in the main text, any light chain that is covalently bound to the quantum dot surfaces will be unavailable for binding with target proteins due to steric considerations. Another potential source of antibody loss is free antibodies cross linking to other antibodies during the conjugation process (see supplementary figure panel a). The dark bands produced at the top of the membrane in Fig. 3a lanes 4-6 represent a significant fraction of antibodies that are cross-linked with each other. There is no fluorescence in the corresponding region of the gel, indicating that quantum dots were not present. Furthermore, non-reduced lanes (Fig 3a, lanes 2-3) do not have strong bands since quantum dots do not transfer to the membrane. It is only unbound networks of antibodies that are too large to run through the gel that remain in lanes 4-6. These cross-linked antibodies are also visible in the colloidal blue stain in the loading wells of Fig. 3b. This provides strong evidence that only cross-linked antibodies remained in the loading wells, and not antibodies conjugated to quantum dots. Cross linking does not occur in the biotin-streptavidin conditions (Figure 3c, lanes 2,3,5) creating another source of antibody loss in direct but not in biotin-strep- further increasing the difference in functionally available antibodies in the two methods.

Additional controls

Additional controls included non-functionalized quantum dots and partially conjugated quantum dots in order to ensure bands did not appear on membranes due to non-specific binding or other experimental artifacts (Fig. supplementary figure panel b). In lanes 2 and 4 of the supplementary figure, non-functionalized amino coated quantum dots were added; no bands were present on the corresponding membrane. Bare streptavidin quantum dots were also run with the same results:

no bands present on the corresponding membrane. Also, even though the concentration of antibody conjugated quantum dots was too low to show up in gels, protein bands were transferred to membranes (lanes 3 and 5). Other controls included using partially conjugated quantum dots. In particular, "excess IgG" quantum dots and "excess SMCC" (4-(N-maleimidomethyl)-cyclohexanecarboxylic acid N-hydroxysuccinimide ester) quantum dots (SMCC is used to cross-link amino and sulfhydryl groups [2]) were collected just after the filtration cut off step for the collection of the functionalized quantum dots. No antibody was present in either of these lanes, demonstrating that only conjugated IgG quantum dot complexes result in enough light chain dissociations to be detected.

NuPage versus NativeGel characterization of the number of bound antibodies

Since different numbers of antibodies bind to different quantum dots, in theory, quantum dot bands should separate according to the number of antibodies bound due to differences in molecular weight. We tried to detect the differences in molecular weight for different antibody-quantum dot complexes using NativeGel. The NativeGel prevents antibody reduction, leaving all antibodies attached to the quantum dot. Unfortunately, due to excessive smearing, we were unable to discern any differences in molecular weight. With the NuPage, antibody dissociation from quantum dots prevented any differences in molecular weight to appear for different numbers of conjugated antibodies. Our method therefore presents an average number of functional antibodies per quantum dot.

Calculations of equivalent quantum dot conjugated antibody concentrations

The total number of quantum dot nanoparticles added to each well was calculated by multiplying the concentration from stock by the volume added. For direct conjugations, we used 2 μM and

for streptavidin quantum dots we used 1 μM. The resulting number (in moles) was then multiplied by Avogadro's number to obtain the total number of particles in the solution. Next, to find the total number of antibodies in solution, the equivalent antibody concentration in μl (obtained from ImageQuant) was converted to the equivalent antibody concentration in milligrams by multiplying by the antibody stock solution concentration (0.5 mg/ml). Taking the molecular weight of a single antibody to be 150kD (BD PharMigin) and converting it to grams (i.e. multiplying by $1.650e^{-24}$) yielded $2.475e^{-19}$ grams per antibody. Using Avogadro's number gives a similar value with no change in the final result. The antibody concentration in milligrams divided by the molecular weight of a single antibody in grams gives the total number of antibodies in solution. Finally, dividing the number of antibodies by the number of quantum dots in solution gives the number of antibodies per quantum dot.

For the direct conjugation method, 10μl of quantum dots were used at a 2μM concentration. To obtain the total number of quantum dots in 10μl:

$$\frac{2\mu mol}{L}\cdot\frac{L}{10^6\,\mu L}=2e^{-6}\,\frac{\mu mol}{uL}\cdot 10\mu L=2e^{-5}\,\mu mol\cdot\frac{mol}{10^6\,\mu mol}\cdot 6.022e^{23}\ \text{particles/mole}=1.2e^{13}\ \text{quantum dots/sample}$$

The average antibody equivalent concentration is 0.455 μl for a 0.5mg/ml stock solution.

$$\frac{0.5mg}{ml}\cdot 0.455\mu l\cdot\frac{ml}{1000\mu l}\cdot\frac{0.001g}{mg}\cdot\frac{1}{2.475e^{-19}\text{g/}antibody}=9.19e^{11}\ \text{antibodies}$$

$$\frac{9.19e^{11}\ \text{antibodies}}{1.2e^{13}\ \text{quantum dots}}=0.076\ \text{antibodies per quantum dot}$$

For biotin-streptavidin conjugations:

$$\frac{1\mu mol}{L}\cdot\frac{L}{10e^6\,\mu l}\cdot 4\mu l\cdot\frac{5}{8}\cdot\frac{mol}{10e^6\,\mu mol}\cdot 6.022e^{23}=1.505e^{12}\ \text{quantum dots per sample}$$

5/8 is used becaause 30 µl of antibody was reacted with 50 µl of quantum dots, so that

$\left(\frac{50}{80}\cdot \text{total volume}\right)$ yields the fraction in 1 µmol of quantum dots.

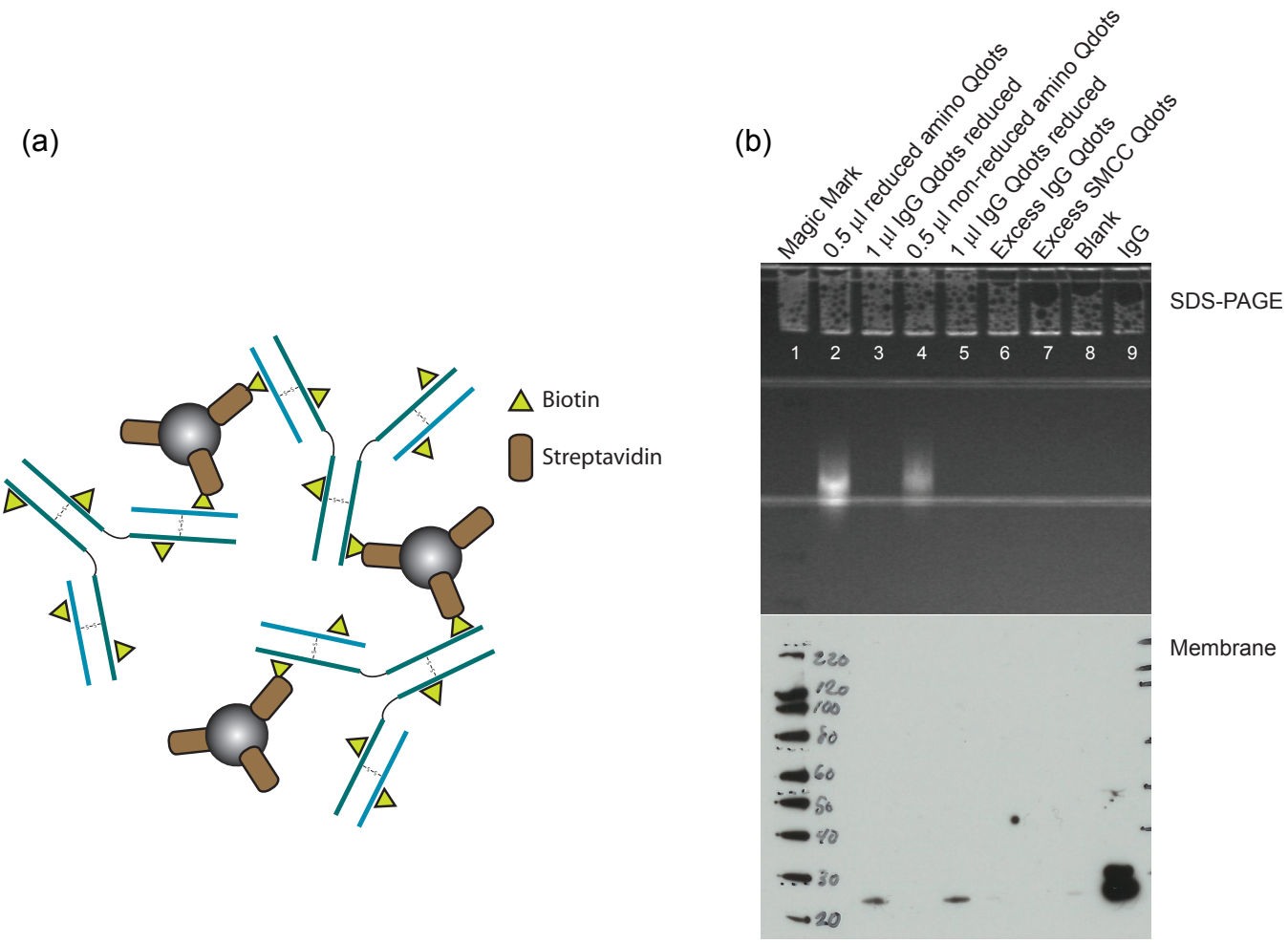
$$\frac{0.5mg}{ml}\cdot 0.943\mu l\cdot\frac{ml}{1000\mu l}\cdot\frac{0.0001g}{mg}\cdot\frac{1}{2.475e^{-19}\text{g/}antibody}=1.9e^{12}\text{ antibodies}$$

$$\frac{1.9e^{12}\text{ antibodies}}{1.5e^{12}\text{ quantum dots}}=1.3\text{ antibodies per quantum dot}$$

References

1. Vu, T.Q.; Maddipati R; Blute T.A; Nehilla B.J; Nusblat L.; Desai T.A. *Nano Lett.* **2005**, 5, 603.

2. Alivisatos, A.P.; Gu, W.; Larabell, C. *Annu Rev Biomed Eng* **2005**, 7:55.



Supplementary Figure. a. Schematic showing antibody-quantum dot cross-linking, which prevents antibody-quantum dot conjugates from running through the gel and is a source of antibody loss.
b. Additional control conditions (see text).