NANO LETTERS

2007
Vol. 7, No. 9
2618−2626

Cholera Toxin B Conjugated Quantum Dots for Live Cell Labeling

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Received April 26, 2007; Revised Manuscript Received July 13, 2007

ABSTRACT

Cholera toxin subunit B (CTB)–quantum dot conjugates were developed for labeling mammalian cells. The conjugates were internalized by all tested cell lines into small vesicles dispersed throughout the cytoplasm, while commercially available polyarginine conjugates rapidly accumulated in large perinuclear endosomes. Although a large proportion of CTB conjugates eventually also accumulated in perinuclear endosomes, this accumulation required several days, and even then many CTB conjugated quantum dots remained in small vesicles dispersed throughout the cytoplasm. Thus CTB conjugates are a practical alternative to polyarginine conjugates for the general labeling of mammalian cells.

Fluorescent labeling of cells allows tracking movement, cell division, and cellular interactions in vitro and in vivo. Cell labeling using quantum dot conjugates was originally reported by two groups.[1,2] Both groups emphasized that the excellent fluorescence properties of quantum dots (brightness, choice of many emission maxima, chemical stability, and photostability) would be well suited to cell labeling, especially cell tracking over long periods of time. However, a convenient method for cell labeling that would work for most cell types was needed. Our laboratory developed the use of polyarginine-conjugated quantum dots as a general cellular label.[3] One of the most useful aspects of the polyarginine labeling is speed; cells rapidly take up the quantum dot conjugates, reaching half-saturation at 12 min after addition of the quantum dots. Cells are readily labeled at high levels (>100 quantum dots per cell); quantum dots are taken up into endosomes and appear localized as very bright spots.[3] This method allows considerable flexibility in concentration of quantum dot conjugates administered, level of polyarginine substitution, and easy coupling of quantum dots to both polyarginine and other biotinylated reagents that are to be internalized along with the quantum dots.

A difficulty in using the polyarginine technique is that the quantum dots are taken up as large aggregates. Fluorescence correlation spectroscopy (FCS) showed that aggregates are formed very rapidly on mixing quantum dots with polyarginine, with or without serum in the medium (data not shown); even larger aggregates appear to be formed on the cell surface. Internalization results in irregular labeling, and tracing cells through several generations is difficult due to unequal division of QD contents. We have therefore sought methods that might permit labeling with less aggregation, allow quantum dots to remain dispersed in the cytoplasm for longer periods of time, be applicable to many cell types, and allow easy cellular labeling.

Cholera toxin B is the nontoxic, cell binding moiety of cholera toxin. CTB binds to gangliosides on the surfaces of nearly all mammalian cells; its pathway of uptake has been extensively studied.[4−6] CTB has been used to promote internalization of many conjugated and associated materials, including antigens for immunization.[7−10]

Many methods have been used to label living cells using quantum dots (see reviews, refs 11−21). Microinjection,[22] electroporation,[23] internalization via cationic detergents,[24−29] nonspecific binding of quantum dots followed by internalization,[30−39] binding, and internalization via cell-penetrating peptides,[3,40−43] and binding to many specific cell surface molecules via attached ligands, often followed by internalization[21,32,44−53] have all been demonstrated. However, most of the published methods demand manipulations other than simple addition of quantum dots to the cells.

Given the growth in both the synthesis and use of new quantum dot conjugates, it has become essential to provide adequate characterization so that results from different laboratories can be compared effectively. Larson et al.[54] used fluorescence correlation spectroscopy (FCS) to characterize water soluble quantum dots for use as multicolor probes in the multiphoton microscopy of live animals. They reported

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10.1021/nl0709930 CCC: $37.00
Published on Web 07/31/2007

the effective hydrodynamic radii of the quantum dots and the problems due to excitation saturation effects that arise from large two-photon absorption cross-sections of quantum dots. Theoretical work on such saturation effects[55] and the relation between nanocrystal size and two-photon absorption cross-section[56] has since been reported. Effective sizes and degrees of aggregation have also been reported for quantum dots stabilized by different surface chemistries[57−65] and Pons et al.[66] used dynamic light scattering methods in conjunction with TEM and XRD to characterize the sizes of quantum dots modified with different surface ligands. The influence of blinking on FCS and imaging has been explored, and correctives were demonstrated.[67−70] Uses of FCS for QD quantitation,[71,72] determining mobility[73] and aggregation,[74] have been demonstrated.

In this report, we demonstrate that carboxyl quantum dots are readily conjugated to CTB, that these conjugates are less prone to aggregation than their polyarginine counterparts by using FCS and gel filtration chromatography, and that they remain less aggregated after cellular uptake.

Quantum Dots. QDs: 605, 655, and 705 nm emitting ITK-CARBOXYL quantum dots; (605 and 655 are CdSe core with ZnS shell; 705 nm emitters are mixed CdTe−CdSe core with ZnS shell; the carboxyl coating was originally described by Wu et al.[54]) were purchased from Invitrogen Corporation (Portland, OR). CTB and triethylammonium bicarbonate buffer (1.0 M, pH 8.5; TEAB) were purchased from Sigma (St. Louis, MO) and EDC [(1-ethyl-3(3-dimethylamino propyl) carbodiimide HCl] was purchased from Pierce Chemical Company (Rockford, IL); all were used as purchased.

Conjugation. The lyophilized CTB product was reconstituted in water to a final concentration of 1 mg CTB/mL. The manufacturer's buffer was 0.05 M Tris buffer, with 0.2 M NaCl, 3 mM NaN_3, and 1 mM Na_2EDTA at pH 7.5. The buffer was exchanged to TEAB buffer (0.01 M, pH 8.5) by using CENTRICON-10 centrifugal filters (Millipore, Billerica, MA); the final concentration was estimated by absorbance at OD 280 nm (we used the calculated value $E_{280} = 9.77 \times 10^3$ for the 11 600 molecular weight subunit B monomer; this differs by 9% from the value of 11.02×10^3 measured by Lai[75]). Note that CTB is normally a pentamer, hence the calculated pentamer molar absorption would be 4.8×10^4.

A solution of carboxyl quantum dots (50 μL, 8 μM, 605, 655, or 705 nm emitting quantum dots) was diluted by adding TEAB buffer (400 μL of 0.01 M, pH 8.5) containing 20 μL of EDC (1 mg/mL) and mixed at room temperature for 10 min. CTB (70 μL of 17.6 μM as pentamer) in 0.01M TEAB buffer was then added to the reaction mixture, thus giving an input ratio of 3CTB/QD. After 2 h constant mixing at room temperature, the CTB conjugate (CTB-QD) was either resuspended in 0.01 M sodium borate buffer by three cycles of centrifugation and resuspension by using CENTRICON-100 centrifugal ultrafilters (Millipore, Billerica, MA) or purified by gel filtration chromatography by using a Pharmacia FPLC system coupled with a UV-M II detector set at 280 nm (see details below).

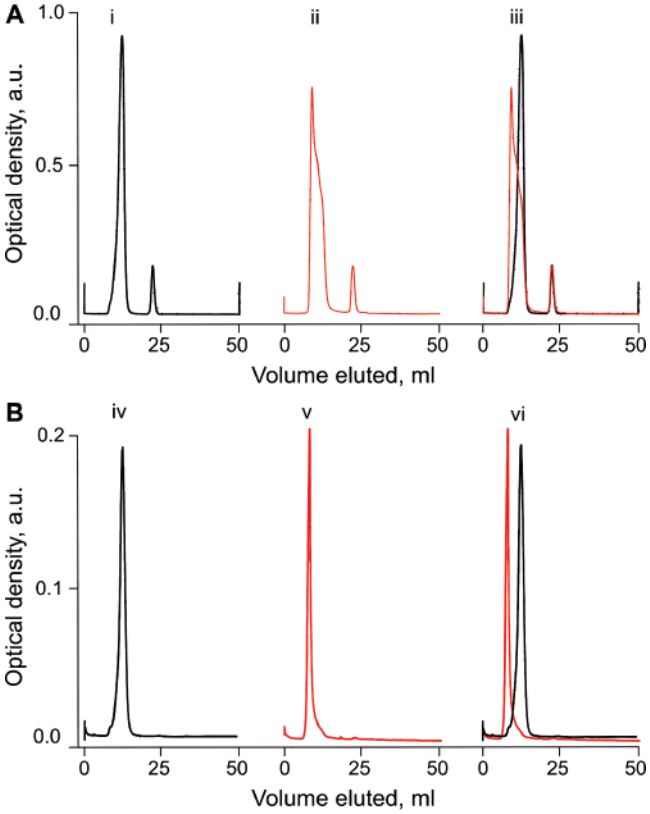

Figure 1. (A) Column chromatography of unreacted carboxyl quantum dots (i); CTB conjugates (ii); and overlay of i and ii (iii). (B) Rechromatography of main fractions from column in (A). Unreacted carboxyl quantum dots (iv); CTB conjugates (v); overlay of iv and v (vi).

The purified CTB-QD conjugates showed few or no visible aggregates or precipitated substances and retained the spectroscopic properties of the original purchased quantum dots. To characterize the effectiveness of the conjugation reaction and monodispersity of both the original carboxyl and CTB-QD preparations, we used column chromatography, agarose gel electrophoresis, and two-photon fluorescence correlation spectroscopy.

Gel Filtration Chromatography. Carboxyl quantum dots or CTB quantum dots (0.4 nmoles, typically 500 μL) were loaded onto a prepacked Superose 6 column (GE Healthcare, Piscataway, NJ), then chromatographed using an FPLC system (GE Healthcare). Buffer was 0.1 M NaCl, 0.05 M sodium borate, pH 8.5; flow rate 0.5 mL/min; fraction volume 2 mL; optical density monitored at 280 nm. A sample run is shown in Figure 1A, (i) unreacted carboxyl quantum dots; (ii) CTB conjugates; (iii) overlay. Note the absence of material eluting in the void volume in (i) and the decreased retention volume of the conjugate peak in (ii). Parts iv−vi of Figure 1B show a rerun of the peak fractions from the columns in Figure 1A. The trailing OD280 peaks in all columns in Figure 1A are unidentified but did not appear to have any toxic effects on cells.

Gel Electrophoresis. The conjugation of CTB to QDs was easily followed by gel electrophoresis (Figure 2). Electrophoresis was in 1% agarose (Fisher, molecular biology grade)

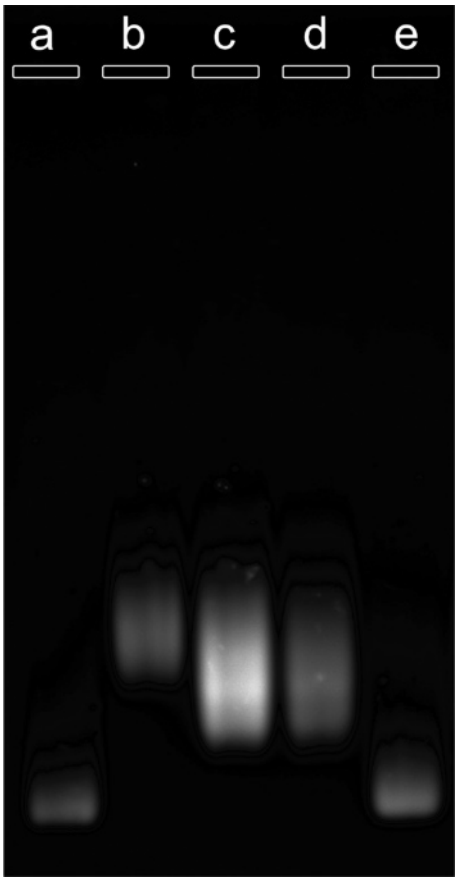

Figure 2. Gel electrophoresis of 655 nm quantum dots (a−e). Unreacted carboxyl quantum dots (a,e); first FPLC fraction (b); main FPLC fraction (c), FPLC tail peak (d). Only conjugates eluted in the main FPLC fraction (c) were used as labeling reagents.

in TAE buffer, pH 8; 12.5 V/cm, typical run time 1 h. Only the main fraction of the CTB-QD conjugates from the FPLC was used as a labeling reagent (Figure 2, column c), and given the small increase in hydrodynamic radius in the conjugate (see discussion below), it is reasonable to assume that there are two to three CTB protein molecules coupled to each quantum dot. Little or no material was retained at the origin.

Fluorescence Correlation Spectroscopy. The two-photon FCS instrument used in these experiments is based on the system described by Berland et al.[76] and will not be described in detail here. Briefly, a femtosecond titanium sapphire laser (Mira 900D, Coherent, Santa Clara, CA) operating at 76 MHz with a pulse width of ~120 fs and $\lambda = 800$ nm was used as an excitation source. The laser output was directed into a 1.4NA 63× Plan-Apochromat oil immersion objective mounted in an inverted microscope (Axiovert 200M, Zeiss, Jena, Germany). Liquid samples were prepared within COVERWELL perfusion chambers (PC8R-1.0, Grace Bio-Labs, Bend, OR) mounted on type 1 coverslips. Fluorescence emission was collected through the objective and passed through an appropriate two-photon bandpass filter (Chroma, Rockingham, VT) to block any stray excitation light. Detection was provided by two avalanche photodiodes (SPCM AQR-14, Perkin-Elmer, Quebec, Canada) whose TTL outputs were cross-correlated by an external correlator module (ALV5000/EPP, ALV Laser, Langen, Germany) connected to a data acquisition computer. Excitation volume calibration was performed by using a 50 nM solution of fluorescein dye in PBS, resulting in values for r_0 and z_0 of $0.2781 \pm 0.0046\ \mu$m and $0.6275 \pm 0.0065\ \mu$m, respectively (numbers denote one standard deviation). These spatial dimensions are in general agreement with those reported by Gratton and co-workers using similar laser intensities.[76]

Samples were prepared by passivating both the perfusion chamber and the coverslip with a 1% solution of bovine serum albumin for 15 min. This solution was carefully removed and a 1nM solution of the quantum dot preparation was added. To circumvent the artificial expansion of the excitation volume due to excitation saturation effects reported by Webb and co-workers,[37] very low excitation powers (~150 μW) were used in conjunction with small quantum dot concentrations (~1 nM).

Cell Growth. NIH 3T3 cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (CS) and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). Mouse muscle derived stem cells (MDSC) were kindly provided by Dr. Johnny Huard, isolated as previously described,[77] and cultured in DMEM (high glucose), 10% FBS, 10% horse serum, 1% chick embryo extract, 200 mM glutamine and 1% penicillin/streptomycin including prophylactic for mycoplasma (Invitrogen, San Diego, CA). Human mesenchymal stem cells (hMSC) were obtained from Lonza, Inc. (Walkersville, MD). Cells were certified positive for adipogenic, chondrogenic, and osteogenic potential assays by the manufacturer. Cells were maintained in mesenchymal stem cell basal medium (MSCBM), mesenchymal cell growth supplement, L-glutamine, penicillin, and streptomycin obtained from Lonza, Inc. (Walkersville, MD). M21 human melanoma cells were a kind gift from R. A. Reisfeld (Scripps, La Jolla, CA) and MH15 mouse teratocarcinoma cells were a kind gift from D. Solter and B. Knowles (Jackson Laboratories, Bar Harbor, ME); both were maintained in RPMI 1640 medium, supplemented with 10% FCS and antibiotics as described above for the NIH 3T3 cells. For labeling, CTB-QD conjugates were used at a concentrations between 250 pM and 4 nM in the cell growth medium, and labeling was normally for 12−18 h at 37 °C. Both the labeling duration and QD conjugate concentration can be adjusted to change the extent of labeling; however, aggregation at the cell surface becomes evident at higher concentrations (>4 nM) in some of the cell lines tested.

Cell Labeling. Figure 3 illustrates labeling of five different cell types with the CTB-QD conjugates. It is important to note that quantum dots are completely dispersed throughout the cytoplasm in each cell type, presumably in vesicles, which is in stark contrast to what is seen when cells are labeled by polyarginine conjugates. Figure 4a−c illustrates an example of an NIH 3T3 fibroblast cell labeled with 605 nm QTRACKER polyarginine conjugated quantum dots (1nM; Invitrogen, Portland, OR). Note the aggregation of the quantum dots and their preferential localization in

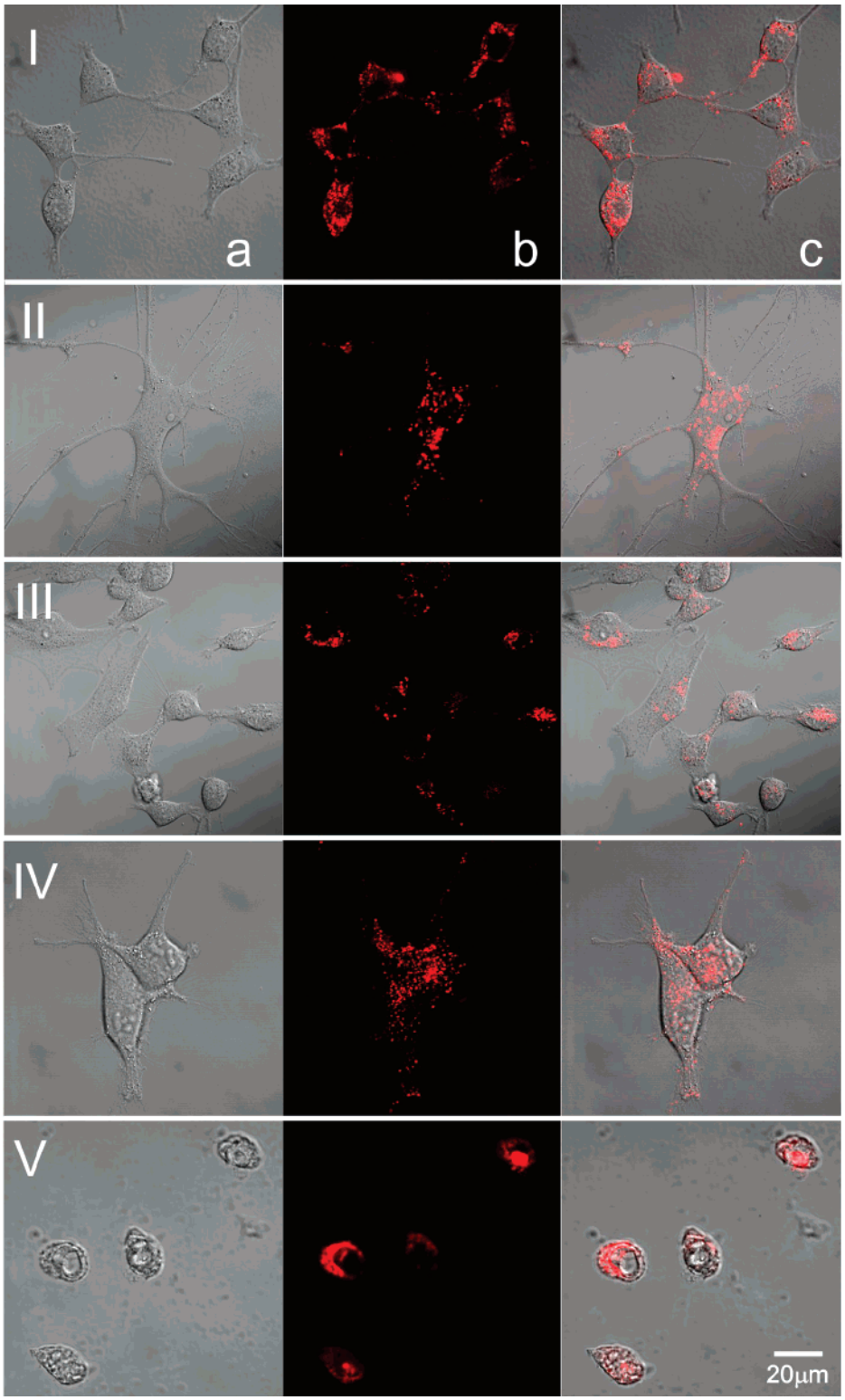

Figure 3. Labeling of live cells with 655 nm CTB-QD conjugates ((a) DIC, (b) confocal fluorescence, and (c) overlay). (I) NIH 3T3 fibroblasts, (II) human mesenchymal stem cells (hMSC), (III) mouse muscle derived stem cells (MDSC), (IV) M21 human melanoma, and (V) MH15 teratocarcinoma mouse tumor cells. All cells were imaged 18 h post labeling.

perinuclear endosomes. Figure 4d−f illustrates the same cell line but labeled instead using 655 nm carboxyl quantum dots (Invitrogen).

Note the lack of fluorescence in panel e, which demonstrates that the unconjugated carboxyl quantum dots are not able to pass through the cell membrane and label the cytoplasm under our labeling conditions.

Fluorescence Correlation Spectroscopy Analysis. The theory behind FCS is well established[78−81] and need not be discussed in any great detail. Briefly, each of the experi-

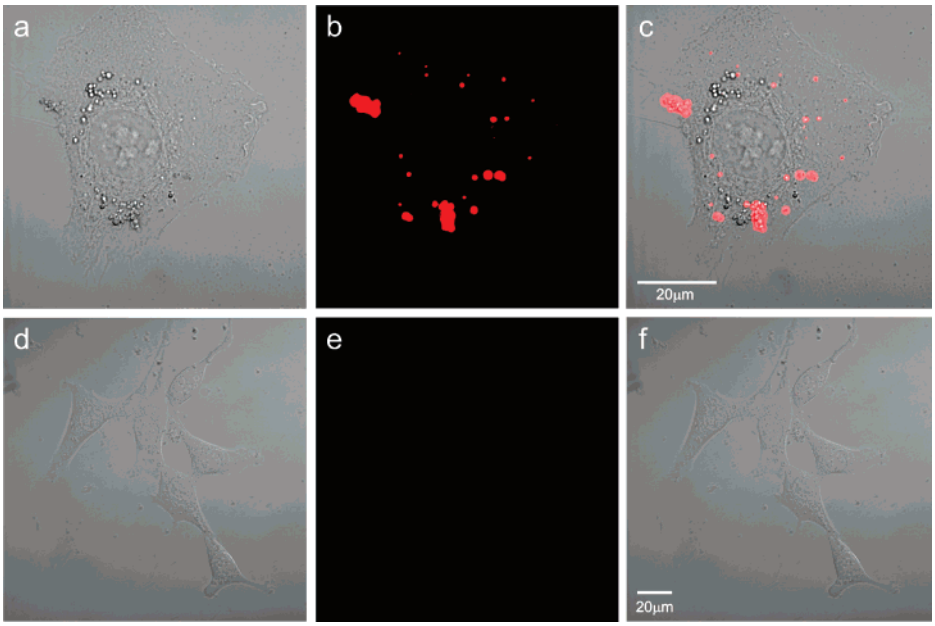

Figure 4. NIH 3T3 fibroblasts labeled with 605 nm QTracker polyarginine conjugated quantum dots (a−c) and 605 nm carboxyl quantum dots (d−f) ((a,d) DIC, (b,e) confocal fluorescence, (c,f) overlay). Cells were imaged 18 h post labeling.

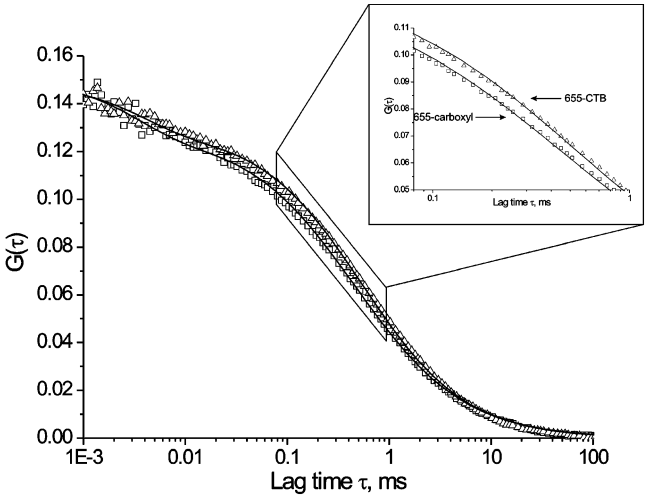

Figure 5. Correlation functions for the 655 nm carboxyl (□) and CTB (Δ) conjugated quantum dots. Inset highlights the differences in the two correlation functions.

mentally obtained correlation functions was fitted to the standard model of three-dimensional diffusion with a dark state contribution (as detailed in eq 1)

$$G(\tau) = G(0)\left\{\left[1+\left(\frac{\tau}{\tau_D}\right)\right]^{-1}\left[1+S^2\left(\frac{\tau}{\tau_D}\right)\right]^{-1/2} + Te^{-\tau/\tau_T}\right\} \quad (1)$$

In each of the fits to the experimental data, the values for G0 and τ_D were initially estimated by eye and the value of S^2 was kept fixed at the value determined in the calibration fits to the fluorescein data (0.1964).

Figure 5 shows the correlation data obtained from the 655 nm emitting carboxyl quantum dots and those that were conjugated with CTB. The fitted parameters and extracted diffusion constants and hydrodynamic radii are shown in Table 1. The decrease in the diffusion constant measured by FCS translates into an increase in hydrodynamic radius by 2.8 nm. As such, the overall diameter of the CTB-QD conjugate was increased by 5.6 nm. These data confirm that the both the initial and the modified quantum dot solutions were monodisperse and void of aggregates in solution. Both gel electrophoresis and column chromatography show one principal fraction in the conjugated product; the thickness of the CTB pentamer is ∼3.1 nm and the diameter is ∼6.2 nm as determined by X-ray diffraction;[82] these measured conjugate sizes would be compatible with as little as one CTB protein with its long axis oriented on edge, or two proteins flattened on the quantum dot surface in various orientations with respect to each other, or three pentamers in a pyramidal orientation flattened on the quantum dot surface.

Table 1. Fitted Diffusion Times and Extracted Diffusion Constants and Hydrodynamic Radii of 655 nm Carboxyl and CTB Conjugated Quantum Dots.

quantum dot	τ_D/ms	D/cm^2 s^{-1}	r_H/nm
655-carboxyl	0.4345	2.2245×10^{-7}	9.8
655-CTB	0.5618	1.7208×10^{-7}	12.6

Effect on Stem Cell Gene Expression. To specifically address the issue of toxicity, we have studied the effects of the CTB QDs on differentiation and marker expression in both human and mouse muscle derived and mesenchymal stem cell lines. In all cases described below, cells were labeled in growth medium (see above) overnight (12−18 h) by using indicated concentrations of quantum dots, then trypsinized and replated under the described conditions.

Stem cells labeled with CTB-QD conjugates appear to maintain their differentiation potential as well as stem cell properties. hMSCs were labeled successfully with a range of doses of CTB-QD −655, from 16 nM down to 250 pM (Figure 6A). After labeling, hMSC were placed under

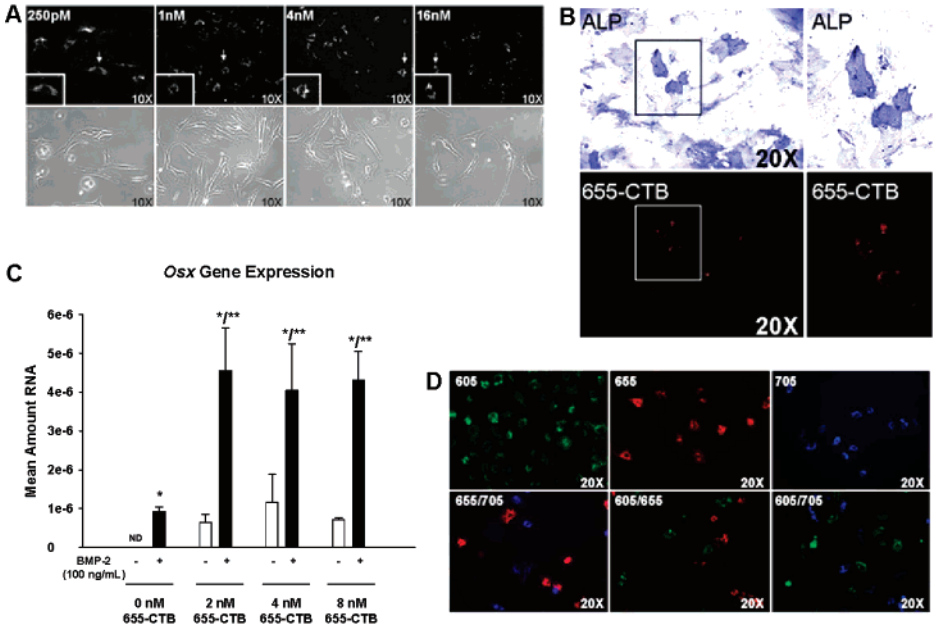

Figure 6. hMSC cell behavior after labeling with CTB-Qdots. (A) hMSC were labeled with 250 pM, 1, 4, or 16 nM 655-QD-CTB overnight, trypsinized, and replated onto glass-bottom dishes. (B) 655-QD-CTB labeled (250 pM) hMSC exhibited induction of ALP activity with 7 days of treatment with osteogenic medium. (C) qPCR analysis for *Osx* gene expression after 24 h treatment in the presence (solid bars) or absence (open bars) of 100 ng/mL BMP-2. Bars represent mean ± SEM, $n = 3$. * Differs significantly from control (−BMP-2/0 nM CTB-QD-655), $p < 0.05$. ** Differs significantly from −BMP-2 group for each CTB-QD-655 dose, $p < 0.05$. (D) hMSC co-cultures of 4 nM CTB-QD-605, −655, or −705 labeled hMSC.

osteogenic conditions for 7 days; they then exhibited up-regulation of alkaline phosphatase (ALP) activity, which is an early marker of osteogenic lineage progression in vitro. hMSCs exhibiting positive staining for ALP activity also contained the quantum dot label, indicating that CTB-QD-labeled hMSCs maintained their differentiation potential (Figure 6B). Members of our group have previously reported that hMSC express osteogenic genes such as *Osterix* (*Osx*) in response to BMP-2 stimulation in vitro.[83−88] Here, we report that hMSC induces *Osx* gene expression within 24 h of treatment with BMP-2 (Figure 6C) after labeling with CTB-655. Interestingly, the quantum dot label appears to have a synergistic effect with BMP-2 for induction of *Osx* gene expression. We also labeled hMSC cells with three different color CTB-QD conjugates as individual cultures and created two-color co-cultures of hMSC (Figure 6D).

MDSCs also appear to maintain their stem cell properties under CTB-QD labeling. CTB-QD labeled MDSC maintain similar percentages of expression for surface markers indicative of the stem cell phenotype such as stem cell antigen 1 (Sca-1) and CD34, compared to nonlabeled cells (Figure 7A).

MDSCs are inherently myogenic and readily fuse to form myotubes under serum deprivation.[77] Here, we demonstrate that CTB-QD-655 labeled MDSC can also form myotubes under serum deprivation for 72 h, hence maintaining their myogenic potential following labeling with CTB-QD conjugates. Fusion of individual cells, each labeled with a different color CTB-QD conjugate, to form myotubes was observed after serum deprivation for 72 h (Figure 7B,E). Note the presence of both colors in the myotube connecting the fused cells in Figure 7B (cf. Murasawa et al.[30]). Finally, as MDSCs are also known to be osteogenic,[89] we have demonstrated that CTB-QD labeled MDSC retain their osteogenic potential and up-regulate *Osx* and *Alp* gene expression under BMP-2 stimulation (Figure 7C,D). Although BMP-2-induced *Osx* gene expression was slightly reduced in CTB-655 labeled cells compared to non-QD labeled cells, the fold induction is several orders of magnitude over cells not treated with BMP-2. The difference is mathematically significant, but we do not consider this slight decrease to be biologically significant. Moreover, the decrease was not correlated with dose. In the case of *Alp*, BMP-induced gene expression was equal to or higher than that in unlabeled cells and again was not correlated with QD dose. Taken together with the gene expression analysis of *Osx* in hMSC, the effect of CTB-QD conjugates on gene expression during differentiation is minimal; labeling of either hMSC or MDSC with CTB-QD conjugates does not appear to drastically influence their differentiation potential.

Our results may be contrasted with studies of the effect of EviTag quantum dots internalized using either Lipofectamine or TAT-protein mediated cell uptake.[27,28] In one paper, the osteogenic potential of immortalized human mesenchymal stem cells appeared unimpaired, but alkaline phosphatase activity and the expression of mRNA for osteopontin and osteocalcin were reduced relative to cells exposed to the internalizing agent only.[28] In the second paper, quantum dot labeled mesenchymal stem cells showed reduced chondriogenesis and reduced expression of chondriogenesis-associated proteins and their mRNAs (type II collagen and aggrecan).[27] In neither case was viability or growth affected. In our hands, there was no consistent impairment in cell differentiation or impairment of marker expression. Unfortunately, the differences in origin of

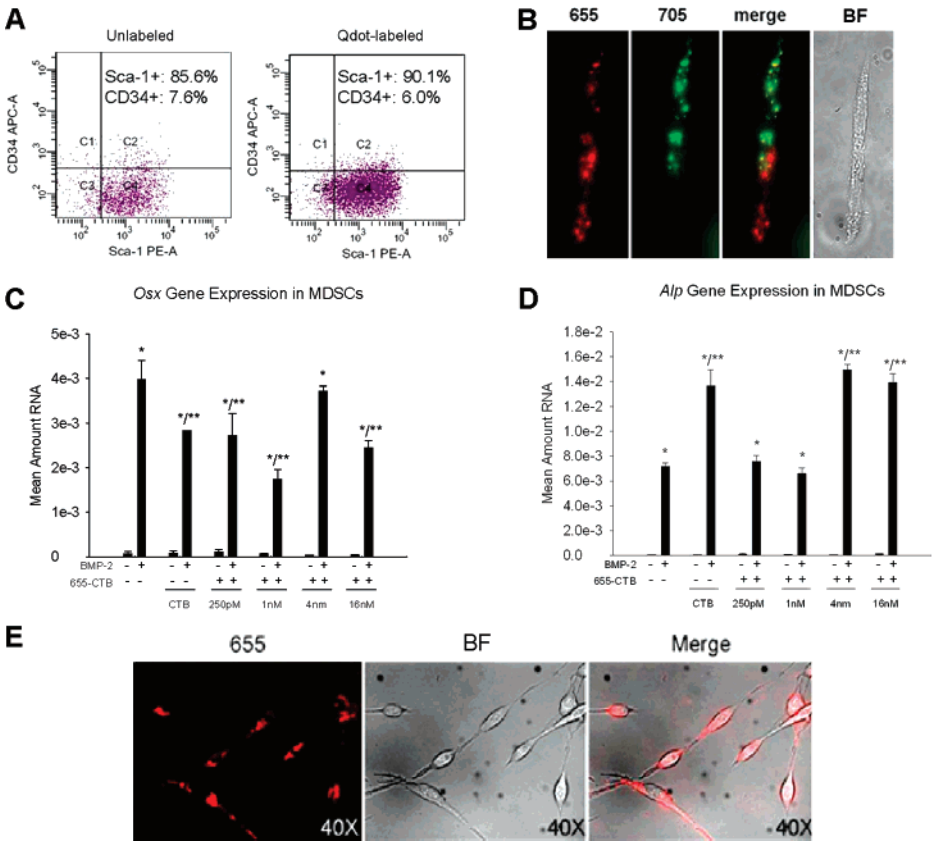

Figure 7. MDSC cell behavior after labeling with CTB-Qdots. **(A)** Labeled and unlabeled MDSC were analyzed by flow cytometry for Sca-1 and CD34 expression. Cells were labeled using 4 nM CTB-QD-655. (B) CTB-QD-655 labeled MDSC form myotubes under serum deprivation (2% serum) for 72 h. Co-cultures of MDSCs labeled with 4 nM of either CTB-QD-655 or −705 under serum deprivation for 72 h show fusion of two differently labeled cells to form a myotube that contains both labels. (C) qPCR analysis for *Osx* gene expression after 24 h treatment in the presence (solid bars) or absence (open bars) of 100 ng/mL BMP-2. Bars represent mean ± SEM, $n = 3$. * Differs significantly from control (−BMP-2/0 nM CTB-655), $p < 0.05$. ** Differs significantly from −BMP-2 group for each CTB-655 dose, $p < 0.05$. (D) qPCR analysis for expression of Alp gene after CTB-QD labeling. Significances as in (C). (E) Myotube formation in CTB-QD-655 labeled MDSC.

quantum dots (Quantum Dot Corporation (now part of Invitrogen) vs Evident), mode of internalization, cells used, and markers tested make comparison of our results to theirs difficult. The most extensive test of gene expression after quantum dot internalization was by Zhang et al.,[65] who found minimal effects from internalization of PEG-silica-clad quantum dots. We note, however, that neither we nor the groups cited above assessed gene expression or toxicity relative to the amount of internalized quantum dots (cf. Chang et al.[90])

We have shown that CTB-QDs are a practical alternative method for labeling several different cell types. In our hands, labeling was more uniform than that given by polyarginine conjugates. However, the label was incorporated more slowly and cell labeling never attained the very high levels seen when using polyarginine conjugates. This did not prove a significant limitation; cells labeled using either conjugate were readily imaged. We expect that the more uniform cytoplasmic distribution given by CTB-QD conjugates would allow better following of cells through several generations, as the distribution of the quantum dots between daughter cells should be more uniform. We have also observed that the CTB conjugated quantum dots remain in cells over several generations with no obvious toxicological effects. However, the more rapid and high-level labeling given by polyarginine conjugates may be suitable for many other purposes.

Presumably the uptake of CTB-QD conjugates is by a pathway similar to that of the uptake of CTB labeled using conventional fluorophores; that is, mainly mediated by uptake in caveolae and retrograde transport through the Golgi apparatus and thence into the endoplasmic reticulum.[4−6] This mechanism would explain the initial uniform labeling that we observe. We note that CTB internalization may occur by several alternate pathways;[4,6,91] given the high sensitivity and stability conferred by quantum dots, CTB-QD conjugates may aid in exploring the minor pathways.

Each of the many methods described for labeling live cells with QDs in the literature has its advantages; many are directed toward specific cell surface receptors. The advantages of the CTB-QD conjugates are that they should be usable with essentially all mammalian cell types; there are no reagents to mix, conjugates may be used in the presence or absence of serum, aggregation is minimal, and the resultant cytoplasmic labeling is far more uniform than that obtained using polyarginine conjugates. Thus the CTB-QD conjugates are likely to be more suitable for long-term, multigeneration cell tracking.

Acknowledgment. This work was supported by NIH under grants R01 EB 000364 and R01 EB 004343. We thank Dr. Johnny Huard (Growth and Development Laboratory, Children's Hospital of Pittsburgh, Department of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, PA 15260) for mouse muscle-derived stem cells and for the use of his facilities to prepare and maintain them. We thank R. A. Reisfeld, D. Solter and B. Knowles for kind gifts of M21 and MH15 cell lines. We also thank Dr. Justin Crowley for the initial concept of using CTB labeled quantum dots, Dr. Newell Washburn (Department of Chemistry at Carnegie Mellon) for the use of the two-photon FCS apparatus and Elvira Garcia-Osuna for invaluable assistance in cell growth and in image quantification and analysis.

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NL0709930