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Heme oxygenase expression as a biomarker of exposure to amphiphilic polymer-coated CdSe/ZnS quantum dots

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Abstract

Because of their unique optical properties quantum dots (QDs) have become a preferred system for ultrasensitive detection and imaging. However, since QDs commonly contain Cd and other heavy metals, concerns have been raised regarding their toxicity. QDs are thus commonly synthesized with a ZnS cap structure, and/or coated with polymeric stabilizers. We recently synthesized amphiphilic polymer coated TOPO-PMAT QDs which are highly stable in aqueous environments. The effects of these QDs on viability and stress response in five cell lines of mouse and human origins are reported here. Human and mouse macrophages, and human kidney cells readily internalized these QDs, resulting in modest toxicity. TOPO-PMAT QD exposure was highly correlated with the induction of the stress response protein heme oxygenase-1 (HMOX1). Other stress biomarkers (glutamate cysteine ligase modifier subunit, NAD(P)H, necrosis) were only moderately affected. HMOX1 may thus be a useful biomarker of TOPO-QDOT QD exposure across cell types and species.

Keywords

quantum dots; oxidative stress; biomarkers; heme oxygenase; in vitro toxicity

1. Background

Recent developments in the field of nanotechnology have produced a vast array of engineered nanoparticles (ENP) with a multitude of proposed applications. These include the use of ENPs in many consumer products such as electronics, paints, cosmetics, and sporting goods (NRC 2008). These particles possess many intriguing structural, physical, and chemical properties, yet also raise concerns about their safety and potential deleterious effects on biological systems. In this context, it is critically important that studies are

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Declaration of interest statement

The amphiphilic polymer coated quantum dots used in this study are the subject of pending US Patent Applications (#20090322327 & #20050136258). Otherwise, the authors declare no competing interests.

conducted to understand and characterize the potential toxicity of these newly created nanoparticles.

Semiconductor quantum dots (QDs) are fluorescent nanocrystals composed of a core that typically contains heavy metals such as CdSe or CdTe encased in another semiconductor shell of higher band gap (e.g., CdS and ZnS). QDs range in size from 2–10 nm and exhibit many unique physico-chemical properties such as size-tunable emission, high photostability, and large Stokes shifts leading to broad absorption profiles (Medintz et al. 2005; Michalet et al. 2005; Zrazhevskiy and Gao 2009). Because of these characteristics, QDs are superior to conventional organic fluorophores in terms of their imaging capabilities. Furthermore, the surface of QDs can readily be modified with various ligands rendering them ideal for *in vivo* targeting applications (Akerman et al. 2002; Gao et al. 2004).

There have been several recent publications investigating the potential *in vivo* toxicity of various QDs, primarily in rodent systems (Ballou et al. 2004; Yang et al. 2007; Geys et al. 2008; Hauck et al. 2010). These studies have demonstrated that while toxicity is limited in scope, it is also heavily dependent on the biochemical and biophysical characteristics of the specific QD being tested. Moreover, many studies have shown that surface chemistry and surface modifications play a critical role in QD uptake, cellular fate, and toxicity (Hardman 2006; Gopee et al. 2007; Robe et al. 2008; Clift et al. 2010b; Fischer et al. 2010).

In vitro systems can play a pivotal role in screening newly developed QDs and other nanomaterials for uptake and potential toxicity in a cost-effective manner prior to the initiation of the more costly *in vivo* studies. *In vivo* models are still necessary for testing but are limited by their cost and labor-intensive nature. The limitations of *in vitro* studies such as the lack of complexity relative to *in vivo* systems do not diminish their value as a rapid cost effective screening system to determine which QDs may pose unacceptable risk for potential *in vivo* uses (Clift et al. 2010c). Recently, Shaw and colleagues (2008) assessed the *in vitro* toxicity of fifty nanoparticle types (including 3 types of QDs) toward four cell lines using four different measures, in order to determine which physical and chemical aspects of these nanoparticles were most predictive of their ability to perturb biological systems. For the three types of QDs they investigated, it was found that perturbation of reducing equivalents (as measured by C₁₂-resazurin reduction) was a sensitive measure of toxicity for at least the NH₂-PEG modified QDs. Others have shown that QDs with various surface coatings can induce oxidative stress, usually at doses that cause toxicity (Green and Howman 2005; Ipe et al. 2005; Nel et al. 2006; Cho et al. 2007; Madl and Pinkerton 2009; Xia et al. 2009).

The goal of the current study was to determine whether a stable, relatively low-toxicity and therefore popular type of QD (in this case tri-n-octylphosphine oxide - poly(maleic anhydride-alt-1-tetradecene (TOPO-PMAT)-modified QDs) could nonetheless cause a common biological effect across cell lines at doses that are not acutely lethal. Because of their high water solubility and long-term colloidal stability, amphiphilic polymer coated QDs (such as TOPO-PMAT QDs) are currently a preferred formulation for biological applications of QD technology (Rosenthal et al. 2011). A biomarker associated with TOPO-PMAT-QD exposure *in vitro* could therefore prove useful as an indicator of potential effects

of such exposures *in vivo*. Accordingly, we utilized both murine and human cell types (macrophages, endothelial cells, and epithelial cells) and employed various end point assays reflective of cellular stress or toxicity. The most robust and consistent indicator of TOPO-PMAT QD exposure across the cell lines tested was the induction of heme-oxygenase-1 (HMOX1) protein. This anti-oxidant enzyme is known to be responsive to a number of stressors, such as heavy metals (including cadmium), oxidants, and inflammation and can provide protection from such agents and conditions (Gozzelino et al. 2010). Although these amphiphilic polymer-modified QDs are stable and have relatively low toxicity, induction of HMOX1, coupled with the changes observed in the content of the intracellular NADPH and the expression of the modifier subunit of glutamate cysteine ligase (the rate-limiting enzyme in glutathione synthesis) suggests that they may nonetheless induce oxidative stress *in vivo*.

2. Methods

2.1 Quantum dot synthesis and characterization

For these studies, we synthesized amphiphilic polymer -coated TOPO-PMAT CdSe/ZnS QDs according to previously described methods (Pellegrino et al. 2004; Bagalkot and Gao 2011). Ocean Nanotech (Springdale, AR) kindly provided TOPO coated CdSe/ZnS core/shell QDs as a gift to Dr. Gao. Poly(maleic anhydride-alt-tetradecene) was from Sigma-Aldrich (St. Louis, MO). In brief, synthesis of the TOPO-PMAT QDs was as follows: 40 mg of PMAT was mixed with 17.7 nmol of TOPO QDs suspended in chloroform. The solvent was then allowed to evaporate, leaving a thin film of TOPO-PMAT QD complexes. The complexes were dissolved in 50 mM borate buffer (pH 8.5) using agitation or sonication. Any unbound PMAT polymer was removed by ultracentrifugation. A schematic for our QD composition and characterization is depicted in Figure 1A. Absorption and fluorescence emission spectra of the modified QDs were measured on a UV-2450 spectrophotometer (Shimadzu, Columbia, MD) and a Fluoromax4 fluorometer (Horiba Jobin Yvon, Edison, NJ), respectively. A CM100 transmission electron microscope (Philips EO, Netherlands) was used to measure the dry radius of these QDs, while their hydrodynamic diameter was measured using a Zetasizer NanoZS size analyzer (Malvern, Worcestershire, UK).

2.2 Cell culture

The RAW264.7 mouse macrophage cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and was cultured in RPMI medium with 10% fetal bovine serum (FBS; Hyclone, Ogden, UT), sodium pyruvate and 1 mM HEPES (complete medium). Every 2–3 days, the cells were passed by gently lifting them from tissue culture plates with sterile scrapers. This cell suspension was diluted with complete medium, triturated and re-plated. The THP1 human monocyte-like cell line (also obtained from ATCC) was maintained in RPMI medium supplemented with 10% FBS and 5 μ M β -mercaptoethanol. In order to differentiate these cells to macrophages, cells were treated with 80 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO) for 3 days. Twenty-four hours prior to QD treatment, the PMA was removed and replaced with fresh medium. The human kidney proximal tubule cell line, HK-2, was kindly provided by Dr. Richard Zanger (Fred Hutchinson Cancer Research Center, Seattle, WA) and was cultured in keratinocyte serum-free medium (Invitrogen, Carlsbad, CA). The A549 and SVEC cell

lines were both obtained from ATCC and cultured in F-12 K and DMEM media, respectively. Both media were supplemented with 10% FBS. All cell lines were maintained at 37 °C in a 95% air/5% CO₂ humidified atmosphere.

2.3 Quantum dot treatments

To prepare the QDs for cell culture experiments, QDs were vortexed and then filtered through a 0.22 µm syringe filter in the appropriate cell line-specific complete cell culture medium. This sterile QD containing medium was then again vortexed and used to replace the medium in which the cells had been growing. Treatments were for 24 hours and ranged from 0–40 nM. All experiments were performed in triplicate (n=3).

2.4 Assessment of cellular uptake of quantum dots

To quantitatively assess QD uptake by flow cytometry, we utilized a Beckman-Coulter Altra fluorescence activated cell sorter (Beckman-Coulter, Miami, FL). QD fluorescence excitation was at 488 nm, and fluorescence emission was collected with a 620 ± 30 nm band-pass filter.

2.5 Laser Scanning Confocal Microscopy

Laser scanning confocal microscopy was used to examine the internalization and subcellular localization of TOPO-PMAT QDs in all cell lines. Cells were exposed to TOPO-PMAT QDs, and after a 1 hr (RAW264.7, THP-1, HK-2, SVEC) or 2 hr (A549) incubation period, either examined live (mitochondria and golgi staining) or fixed in 4% paraformaldehyde/PBS (lysosomal and early endosomal staining). Mitochondria were stained with Mitotracker Green and the Golgi apparatus was stained with NBD Ceramide (Invitrogen). For mouse cell lines (RAW264.7, SVEC) early endosomes were stained with a rabbit polyclonal anti-mouse EEA1 antibody (Abcam, San Francisco, CA) followed by an Alexa 488-conjugated goat anti-rabbit IgG antibody (Invitrogen). For human cell lines (THP-1, HK-2, A549), early endosomes were stained with a FITC-conjugated mouse anti-human EEA1 antibody (BD Bioscience, San Diego, CA). Lysosomes were stained with Alexa 488 conjugated anti-mouse LAMP-1 antibody, or Alexa 488 conjugated anti-human LAMP-1 antibody, for mouse and human cell lines, respectively (eBioscience, San Diego, CA). Following staining, cells were examined on a laser scanning confocal microscope (Model LSM 510 NLO, Zeiss USA, Inc., Thornwood, NY) using 488 nm excitation. Emission of QD specific fluorescence was detected with a 620 ± 5 nm band pass filter. Fluorescence emission of organelle specific stains was detected using a 525 ± 5 nm band pass filter. Differential interference contrast (DIC) images were collected simultaneously with fluorescent digital images. Digital images were subsequently analyzed using the co-localization feature in Metamorph Software (Molecular Devices, Sunnyvale, CA).

2.6 Cell viability assays

Cells were cultured in 24-well tissue culture plates. After treatment with QDs for 24 hr, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 250 µg/ml) was added to each well and incubated for either 20 min (RAW264.7 cells) or 1 hour (all other cell lines) at 37 °C. Because RAW264.7 cells are much more active in their reduction of MTT

than the other cell lines, this shorter time period of incubation was necessary to maintain absorption readings within the linear range for these cells. Cells were washed with PBS, the formazan solubilized in DMSO, which was then transferred to 96-well microtiter plate and the absorbance read at 570 nm on a spectrophotometric plate reader (Molecular Devices, Sunnyvale, CA). In separate experiments we examined whether QDs interfered with formazan detection by UV spectrophotometry. HK-2 cells were treated with QDs, medium was removed, cells were washed with PBS, and the cells were then treated with pure DMSO. Subsequently, we observed by fluorescence microscopy that the QDs remained in the cells, that there was no QD specific fluorescence in the DMSO formazan solution, and that all formazan was solubilized and removed from the cells. Thus, in the way that this MTT assessment is done in the current study, there are no QDs present to interfere with the detection of formazan. Moreover, when TOPO-PMAT QDs were added to formazan/DMSO solutions obtained from cells not treated with QDs, there was no difference noted in formazan absorbance (data not shown).

As an alternative to the MTT cytotoxicity assay, we also utilized flow cytometric analysis for QD-induced cellular necrosis. Cells were removed from plates with NO-ZYME™ (Sigma) (THP-1 and RAW264.7 cells) or trypsin (HK-2, A549, and SVEC cells), and uptake of either 7-aminoactinomycin D (7AAD; 2 µg/ml final concentration) for THP-1 cells; or Hoechst 33258 for RAW264.7, A549, SVEC, and HK-2 cells was assessed. 7AAD fluorescence excitation was at 488 nm and fluorescence emission was collected with a 675 nm long-pass filter; Hoechst fluorescence excitation was at 351–362 nm, and emission was collected with a 460/20 nm band-pass filter. We used 7-AAD uptake as marker for loss of cell membrane integrity for the THP-1 cells because this allowed us to simultaneously measure NAD(P)H content or total thiols using monobromobimane (MBB) staining in this difficult to culture cell line. For the other cell types we used Hoechst 33258 uptake as a marker of cell membrane integrity, but measured NADPH and MBB fluorescence in separate aliquots to avoid spectral overlap with the Hoechst staining.

2.7 Glutathione, total cellular thiols and NAD(P)H determinations

Cellular lysates were prepared for fluorometric microtiter plate assay of GSH as previously described by measuring 1,2-naphthalene dicarboxaldehyde (NDA)-glutathione conjugate fluorescence (McConnachie et al. 2007).

We analyzed total cellular thiols by flow cytometry. Cells were removed from tissue culture plates with NO-ZYME™ (THP-1 and RAW264.7 cells) or trypsin, (HK-2, A549, and SVEC cells), and were stained for total thiols at approx 10⁶ cells/ml. One µl of 10 mM MBB in EtOH was added to the cells and incubated for 10 minutes at 37 °C followed by placement on ice. Probenecid (2 mM final) was also added to inhibit organic anion transporters from pumping bimane-GS conjugate out of the cell (Poot et al. 1995). Bimane fluorescence excitation was at 351–362 nm, and emission was collected with a 460/20 nm band-pass filter.

To determine cellular NAD(P)H levels, cells were analyzed with the same experimental set-up as used for total-thiol assessments. However, they were not treated with

monobromobimane, rather the amount of UV-induced blue autofluorescence was measured instead.

2.8 Lipid peroxidation determination by NAO

We assessed the peroxidation of cardiolipin in the inner mitochondrial membrane, using the fluorescent dye acridine orange 10-nonyl bromide (nonyl acridine orange, NAO) which binds cardiolipin with a high affinity, thus providing a quantitative measure of mitochondrial lipid peroxidation (Petit et al. 1992; McMillin and Dowhan 2002; Wright et al. 2004). NAO (Chemicon International, Temecula, CA) was dissolved in EtOH, and diluted in appropriate cell culture medium prior to incubating with cells for 10 min at 37 °C (final concentration 156 nM). NAO fluorescence was determined by flow cytometry using 488 nm excitation and a 525/40 nm band pass filter to measure fluorescence emission.

2.9 Western immunoblot analyses

Total cellular protein was extracted with Mammalian Protein Extraction Reagent (M-PER, Thermo, Rockford, IL) and subjected to SDS-PAGE and Western blot analysis with enhanced chemiluminescence detection. Evaluation of GCL catalytic (GCLC) and modifier (GCLM) protein expression was carried out as previously reported (Botta et al. 2004; Botta et al. 2006) using rabbit polyclonal anti-GCL peptide antisera. To determine heme oxygenase-1 protein expression, we utilized a mouse anti-HMOX1 monoclonal antibody (R&D Systems, Minneapolis, MN). The detection of appropriately directed and bound HRP-conjugated secondary antibodies was accomplished with enhanced chemiluminescence and X-ray films. Exposed films were imaged using a BioRad Gel-Doc system (BioRad, Hercules, CA) and band intensities determined with NIH Image software.

2.10 Data analyses

Individual assay results were subjected to one-way or two-way analysis of variance (ANOVA), and a post-hoc Dunnett's test when appropriate (Prism, Graph Pad Software, San Diego, CA). A Bonferroni post-hoc test was used for confocal microscopy co-localization analysis. Differences with $p < 0.05$ were considered statistically significant. The raw data presented in Figures 2, 5 and 6 (in addition to the NAD(P)H and NAO assay data) were converted to z-scores (within cell lines and assays). The raw data for a particular assay and cell line were converted to z-scores by subtracting the mean value and dividing by the standard deviation. This calculation was repeated for all the assays and cell lines. The z-scores were clustered using unsupervised hierarchical clustering analysis (R/Bioconductor software, <http://www.bioconductor.org>) similar to the approach taken by Shaw and colleagues (Shaw et al. 2008). The clustering results are shown in a two-dimensional heat map in Figure 7. Multilinear regression analysis comparing assay z scores with uptake z score was performed using the online statistical analysis tool Rweb v1.03 (<http://bayes.math.montana.edu/cgi-bin/Rweb/AnalysisModules/runRegression.cgi>). Results of this analysis are given in Table 1. Cross-correlation among assays was also performed and the results of this analysis are given as a two-dimensional heat map in Supplemental Figure S1. The strength of correlation of individual assays with TOPO-PMAT QD uptake was used as the basis for ordering the assays.

3. Results

3.1 Quantum dot characterization

A molecular self-assembly approach was used to prepare the TOPO-PMAT encapsulated QDs (a schematic of preparation for these QDs is presented in Figure 1). QDs with the hydrophobic ligand TOPO already attached were used, and these were subsequently mixed with PMAT. The TOPO coating and PMAT bind to each other via multivalent hydrophobic interactions forming complexes that are highly stable in aqueous solution. Transmission electron microscopy (TEM), dynamic light scattering (DLS), and spectroscopy were used to characterize the size and optical properties of purified QDs. TOPO-PMAT QDs have a narrow size distribution and quantum yield values similar to that of the starting TOPO-modified QDs suspended in chloroform. TOPO-PMAT QDs have a hydrodynamic diameter of 12.7 ± 0.5 nm as measured by dynamic light scattering and the core of these particles is 6.8 ± 0.5 nm in diameter.

3.2 Cellular uptake

The 5 cell lines we evaluated varied widely in their apparent uptake and retention of TOPO-PMAT QDs (Figure 2). The human and murine macrophage cell lines, THP1 and RAW264.7 cells respectively, each exhibited a significant and dose-dependant degree of TOPO-PMAT QD uptake ($p < 0.01$ for the 20 and 40 nM doses, relative to the background fluorescence of vehicle treated controls within each cell line). Avid uptake was similarly observed in the human kidney proximal tubule cells (Figure 2A). Conversely, mouse endothelial cells (SVEC) and human lung epithelial cells (A549) exhibited only minimal uptake (Figure 2B). This uptake, albeit low, achieved statistical significance ($p < 0.05$) at the highest TOPO-PMAT QD concentration, relative to the background fluorescence of vehicle treated controls.

3.3 Confocal Microscopy

Using confocal microscopy, we determined that TOPO-PMAT QDs were indeed internalized (not associated with the cell membrane) and primarily co-localized with the lysosomal protein LAMP-1, regardless of cell type (Figure 3), suggesting that these QDs are internalized via the endosomal-lysosomal pathway. Some co-localization with other subcellular structures (early endosomes, mitochondria, golgi) was observed in all cell types (Figure 4 and Supplemental Figures 2–4). Nonetheless, there was a significant difference between the percent QDs localized to lysosomes vs. localization in all other organelles in all cell lines (Table S1). Two-way ANOVA testing with “cell line” and “organelle” as factors showed that there was a significant effect of organelle but not of cell line on the variance. These data indicate that irrespective of the cell type, the lysosome was the principal intracellular location of these QDs.

3.4 Cellular viability

Cell viability was evaluated initially with the MTT assay. This method gives a measure of mitochondrial dehydrogenase activity via the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a formazan precipitate by metabolically active cells. Even

though RAW264.7 cells and THP1 cells both avidly internalize TOPO-PMAT QDs, only THP1 cells exhibited a significant attenuation in mitochondrial dehydrogenase activity after exposure to these nanoparticles (Figure 5A). As the SVEC and A549 cells did not exhibit a high degree of uptake, relative to the other cell lines, they appeared to be spared from any effect, as evidenced by their unperturbed MTT activity. Interestingly, the HK-2 cell line actually displayed increased mitochondrial dehydrogenase activity with TOPO-PMAT QD treatment (Figure 5A).

Using flow cytometry, the effects of TOPO-PMAT QDs on cellular necrosis were evaluated as measured by cell membrane permeability toward 7-aminoactinomycin D (7-AAD) in THP1 cells, or Hoechst 33528 in the other cell types (Figure 5B). While only the RAW264.7 cells exhibited statistically significant increases in cellular necrosis at concentrations of 10 nM TOPO-PMAT QD and higher, there was a trend toward increasing necrosis for the THP1 cells. These data are consistent with the observed uptake data reported in Figure 2. This trend toward necrosis was not observed however with the HK2, A549 or SVEC cells as the percent of necrotic cells was unaffected by TOPO-PMAT QD exposure.

3.5 Cellular thiol levels and lipid peroxidation

Regardless of cell line or concentration of TOPO-PMAT QDs, there was no statistically significant effect of treatment on total cellular thiols within a cell line as measured by monobromobimane (MBB) fluorescence and flow cytometry, or on cellular glutathione (GSH) levels as measured by the NDA plate assay (data not shown).

Furthermore, NAD(P)H concentrations were likewise not significantly changed with treatment within any of the cell lines tested (data not shown), but there was a correlation between TOPO-PMAT QD uptake and NAD(P)H content overall (see below). Lastly, there was no effect of TOPO-PMAT QD treatment on peroxidation of the mitochondrial inner membrane lipid cardiolipin as measured by nonylacridine orange (NAO) staining (data not shown).

3.6 Western immunoblot analysis

To investigate whether QDs may elicit more subtle effects on cellular redox status, the effect of TOPO-PMAT QDs on expression of the redox sensing proteins glutamate cysteine ligase catalytic (GCLC) and modifier (GCLM) subunits, and heme oxygenase-1 (HMOX1) was determined (Figure 6). GCLC expression was not significantly altered following QD exposure in all cell types. There were trends (although not statistically significant) for decreasing expression of GCLC in RAW264.7 cells and increasing expression in SVEC cells. GCLM expression was unaffected by TOPO-PMAT QD exposure within each cell line, with the exception of the THP1 cell line. In this case, there was a modest yet significant induction of GCLM expression. In contrast to GCLC and GCLM, there was a robust and significant induction of HMOX1 expression in response to TOPO-PMAT QD treatment for both RAW264.7 and THP1 cells. Although the fold induction changes did not achieve statistical significance, this was also the case for HK-2 cells.

3.7 Clustering analysis

While one goal of these studies was to investigate the toxic effects of TOPO-PMAT QDs within various cell lines utilizing a battery of assays, it was also useful to ascertain whether a particular cell type or particular assay was more informative in predicting toxicity relative to the others. To do so would enable us to streamline the screening of newly developed QD nanoparticles for uptake and potential toxicity. To address this question, the raw data presented in Figures 2, 5 and 6 (in addition to the NADPH and NAO data) were converted to z-scores and then subjected to unsupervised hierarchical clustering analysis similar to the approach taken by Shaw and colleagues (2008). The results from this analysis are depicted in Figure 7. Several striking features regarding the relationship between TOPO-PMAT QD uptake among the different cell types and the toxicological assays performed are evident. The first observation is that HMOX1 expression is the biomarker most highly correlated with TOPO-PMAT QD uptake, followed by cellular necrosis and then GCLM expression. Induction of both HMOX1 and GCLM are commonly used markers of oxidative stress. Other biomarkers analyzed showed weaker associations with TOPO-PMAT QD uptake (e.g. NAD(P)H content; MBB fluorescence), while others (NAO, GCLC, GSH, MTT) showed very little association with either uptake or with HMOX1, or GCLM expression, even though some of these were moderately correlated with each other in pair-wise linear regression analyses (e.g. GCLC with GCLM; MTT with GSH; Supplemental Figure S1).

In order to more fully understand the relative strength of association between the functional assays and TOPO-PMAT QD uptake, multiple linear regression analysis of assay z score vs. uptake was performed using an online web tool (<http://bayes.math.montana.edu/cgibin/Rweb/AnalysisModules/runRegression.cgi>). Table 1 shows the results of this analysis. Again, HMOX1 expression was most highly correlated with TOPO-PMAT QD uptake, followed by GCLM expression, and then NAD(P)H content.

4. Discussion

The TOPO-PMAT QDs particles used in these studies are in a class of QDs that are useful for biological imaging, primarily because of their high water solubility and exceptional stability. Furthermore, the carboxyl functional groups present in this coating makes it convenient for ligand attachment. Similarly modified QDs have shown promise for tumor targeting *in vivo* using antibodies directed against surface receptors on cancer cells (Gao et al. 2004; Yang et al. 2009); and for siRNA delivery (Qi and Gao 2008). The larger hydrodynamic radius of TOPO-PMAT QDs that was observed in aqueous buffers is likely due to the presence of the polymer, and its strong interaction with H₂O (Larson et al. 2003).

The cell lines selected for these studies are representative of the types of cells TOPO-PMAT QDs are likely to encounter following *in vivo* exposures. Specifically, following inhalation exposure, they will potentially come in contact with alveolar macrophages. For this reason the murine macrophage or human macrophage-like cells lines, RAW264.7 and THP1, respectively were selected for study. The avid uptake of TOPO-PMAT QDs by macrophage cells is consistent with the central role this cell type plays in particle scavenging. The HK-2 cell line was selected for these studies because the renal proximal tubule cells in the kidney are known targets of cadmium mediated toxicity, and QDs commonly distribute to this

organ (Lin et al. 2008; Lin et al. 2009; Yong et al. 2009; Ma et al. 2010; Vibin et al. 2011; Yeh et al. 2011). Furthermore, the observed high degree of TOPO-PMAT QD uptake in HK-2 cells was not surprising as these cells have well characterized solute transporters, including organic anion and cation transporters (Motojima et al. 2003; Gunness et al. 2010), and have recently been shown to avidly accumulate ZnO and CdS nanoparticles (Pujalte et al. 2011). The relatively low TOPO-PMAT QD uptake observed in the present study with the A549 and SVEC cell lines was not unexpected as these cells are not necessarily known to be particle scavengers. However, Praetner and colleagues (2010) have recently shown that QD nanoparticles can be taken up *in vivo* by capillary endothelial cells. Because these epithelial and endothelial type cells would potentially be exposed to QDs following intravenous administration for medical purposes, examination of the potential QD mediated toxicity in an additional battery of assays was warranted.

It must be acknowledged, however, that some differences in cellular uptake may be due, in part, to the different media in which the cells were cultured. The HK-2 cells, for example, were grown in keratinocyte serum-free medium supplemented with bovine pituitary extract and human recombinant epidermal growth factor. The other cell lines were all cultured in medium supplemented with 10% fetal bovine serum. It is conceivable that the differing medium components resulted in varying protein corona effects, which could potentially impact the availability of TOPO-PMAT QDs for cellular internalization, as has been described for other nanoparticles (Clift et al. 2010a). That these particles were internalized (as opposed to attaching the cell surface) was confirmed using confocal microscopy analysis.

The localization to early endosomes and especially lysosomes indicates that these particles likely follow an expected route of cellular uptake and trafficking for particles of this size. We did observe some localization with mitochondria and golgi as well, suggesting that some of the particles may either exit the lysosome or directly pass through the cell membrane, enter the cytosol and transit to these organelles. No appreciable uptake into the nucleus was noted, as indicated by overlays of fluorescence images with DIC images. The subcellular localization may be related to the toxicity, as we noted an inverse correlation between the percentage of QDs localized to lysosomes early on, and the eventual degree of necrosis among the cell lines ($r^2=0.33$), suggesting that retention in the lysosomal compartment may be protective.

Regardless of the degree of TOPO-PMAT QD uptake, relatively mild toxicity, as measured by cellular necrosis or decrease in mitochondrial dehydrogenase activity was observed in all cell types. Interestingly, an increase in mitochondrial dehydrogenase activity with increasing doses of these QDs was observed in the HK-2 cell line. This may be due to the activation of metabolic pathways that increase the availability of NAD(P)H, which has been shown to occur in bovine kidney epithelial cells exposed to mild oxidative stress (Naoi et al. 2010). Nevertheless, since these assays of necrosis are measures of frank toxicity (i.e. compromised membrane permeability), these cellular viability data collectively indicated only mild to moderate acute toxicity caused by TOPO-PMATQD uptake in the various cell lines. This finding is in agreement with several previous reports indicating minimal toxicity associated with negatively charged QDs (Clift et al. 2008; Clift et al. 2010b; Clift et al. 2010d).

Because these QDs are localized to a large extent in lysosomes, it is possible that the QDs may be degraded at the lower pH present in this acidic intracellular organelle (Stern et al. 2008). Indeed, in a recent publication, a greater than 50% loss in PMAT-QD fluorescence (a measure of stability) at pH 4 -5 has been observed (Hu and Gao 2010). Thus, it is possible that some of the QD constituents such as Cd ion could be released, resulting in the mild toxicity we observed.

To investigate a potential mechanistic explanation for the observed toxicity, cellular thiols were initially investigated as an indicator of cellular redox status. This end-point was chosen because many nanoparticles, including QDs, are known to induce oxidative stress, and because QDs can release Cd (Hardman 2006; Gopee et al. 2007; Robe et al. 2008; Clift et al. 2010d; Fischer et al. 2010), a potent oxidant and thiol-binding heavy metal. Furthermore, free Cd is known to deplete cellular thiols such as GSH via direct binding or through the generation of reactive oxygen species (Rikans and Yamano 2000). Nevertheless, the data for total cellular thiols, cellular GSH levels, and lipid peroxidation, collectively indicate that stable TOPO-PMAT QDs do not greatly perturb cellular redox status. The exact reasons for this are unclear, but may be related to the relative stability of these particles and hence low levels of Cd ion release, the balance between GSH consumption and de novo synthesis in the face of oxidative stress, or compensatory upregulation of other antioxidant factors (such as HMOX1) that are protective of redox status in the cells.

To investigate whether TOPO-PMAT QDs may elicit more subtle effects on cellular redox status, additional studies were designed to measure the expression of the redox sensing proteins glutamate cysteine ligase catalytic (GCLC) and modifier (GCLM) subunits, and heme oxygenase-1 (HMOX1). Glutamate cysteine ligase is the rate-limiting enzyme in GSH synthesis and the expression of both subunits is known to be increased by variety of oxidative stress inducing treatments (Franklin et al. 2009). Similarly, HMOX1 has been shown to be responsive to both oxidative insults and inflammation (Pae et al. 2010; Paine et al. 2010). Although GCLC and GCLM were relatively non-responsive in all the cell lines, HMOX1 protein was robustly and consistently induced by TOPO-PMAT QD treatment. HMOX1 converts heme to biliverdin, and in the process releases carbon monoxide (CO). CO in low concentrations has been proposed to have antioxidant properties by virtue of its ability to inhibit the production of reactive oxygen species by the mitochondrial respiratory chain and to inhibit nitric oxide production by nitric oxide synthase (Pae et al. 2010). In addition, biliverdin can be further metabolized by biliverdin reductase to bilirubin, and both biliverdin and bilirubin have antioxidant properties (Gozzelino et al. 2010). Thus, the induction of HMOX1, which is thought to occur secondary to activation of Nrf2, AP1 or Egr1 under conditions of oxidative stress and inflammation (Chen et al. 2010; Gozzelino et al. 2010), appears to be a highly sensitive endpoint for TOPO-PMAT QD exposure, especially in those cell types that display a high cellular loading of these QDs.

5. Conclusions

In conclusion, the data presented in this study indicate that TOPO-PMAT modified QDs are only slightly to moderately toxic at the doses and exposure duration tested in the selected cell lines. Because TOPO-PMAT QDs are relatively stable, significant toxicity toward these

cell types was not expected. Compared to previous reports on less stable QDs that readily degrade and release Cd (Hardman 2006; Gopee et al. 2007; Robe et al. 2008; Fischer et al. 2010), the relative stability of these QDs appears to be a distinct advantage. In support of this contention is the fact that only mild toxicity was seen, and there were no consistent changes seen in GSH content, metabolic activity (as measured by MTT assay) or lipid peroxidation. However, other sensitive measures of cellular stress (e.g. HMOX1 expression, GCLM expression, and NAD(P)H content), showed significant associations with TOPO-PMAT QD uptake as exhibited by hierarchical clustering (Figure 7), and multilinear regression analysis of assay z scores (Table 1).

Collectively, these data indicate that TOPO-PMAT QD uptake is highly variable among cell types, that they are slightly to moderately toxic, and that their effects *in vivo* will likely be highly dependent upon the cell type and organ being exposed. Moreover, HMOX1 protein expression may be a very promising general biomarker for TOPO-PMAT QD exposure *in vivo*. Indeed, Lin and co-workers (Lin et al. 2010) have recently shown that intravenous injection of CdTe QDs into mice caused an up-regulation of HMOX1, as well as other biomarkers of oxidative stress and inflammation, in the livers of these animals. While other organs were not evaluated, this report confirms that HMOX1 is a promising candidate biomarker of exposure to QDs in general.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A

B

C

D

Figure 1. Synthesis scheme and characterization of QDs encapsulated with amphiphilic polymer, PMAT

(A). TOPO-PMAT QDs schematic representation. Possible attachments for ligands are shown. The solid sphere represents the CdSe/ZnS core/cap structure, surrounded next by TOPO, and then the PMAT polymer coating. (B). Transmission electron microscope (TEM) image of TOPO-PMAT coated QDs. Scale bar = 30 nm. (C). Dynamic light scattering results. QDs have a hydrodynamic diameter of 12.7 ± 0.5 nm. (D). Absorbance (black line) and fluorescence (red line) of TOPO-PMAT QDs suspended in PBS.

Figure 2. Quantum dot uptake in five cell lines

Uptake of TOPO-PMAT QDs as measured by flow cytometry. In panel A, RAW264.7 cells, THP1 and HK-2 cells were exposed to the indicated concentrations of TOPO-PMAT QDs for 24hrs, removed from the plates and assessed for up-take. In panel B, A549 and SVEC4.10 cells were exposed and analyzed as in Panel A, but the y-axis scale has been expanded to emphasize the minimal uptake of TOPO-PMAT QDs for these two cell types. Data represent the mean $\pm$ S.E.M. of at least 3 replicates. (* $p < 0.05$ and ** $p < 0.01$ relative to untreated vehicle control).

Figure 3. Laser scanning confocal microscopy analysis of subcellular localization of TOPO-PMAT QDs with LAMP-1 in five cell lines

Cells were loaded with the QDs and stained for LAMP-1 as indicated in Materials and Methods. Separate images were taken for QDs (left column, red) and LAMP-1 (middle column, green). Merged images are shown as an overlay onto a differential interference contrast (DIC) image in the right hand column.

Figure 4. Percentage of TOPO-PMAT QDs co-localized with organelle stains in five cell lines
Confocal images of subcellular localization of QDs and organelle stains were analyzed using Metamorph Software as indicated in Materials and Methods. The percentage of colocalized signals was then plotted for each organelle within the 5 cell lines.

A.

B.

Figure 5. The effect of TOPO-PMAT QD treatment on cellular viability

(A). Effect of quantum dots (QD) on mitochondrial dehydrogenase activity as measured by the MTT assay. (B). Effect of QD on the percent of cellular necrosis as measured by Hoechst 33258 or 7-AAD uptake and analyzed by flow cytometry. Data represent the mean $\pm$ S.E.M. of 3 replicates. (* $p < 0.05$ and ** $p < 0.01$ relative to untreated vehicle control).

Figure 6. The effect of TOPO-PMAT QD treatment on stress-response protein expression
 (A). Effect of quantum dots (QD) on GCLC expression. (B). Effect of quantum dots (QD) on GCLM expression. (C). Effect of quantum dots (QD) on Heme-oxygenase-1 expression. Data represent the mean $\pm$ S.E.M. of 3 replicates. (* $p < 0.05$ and ** $p < 0.01$ relative to untreated vehicle control).

Figure 7. Unsupervised Hierarchical Clustering Analysis

Results from 10 assays (right side of figure) were converted to z scores for each assay within a cell line. Z-scores from cell lines and their treatments were clustered against assay type using the methods of Shaw and colleagues (Shaw et al. 2008).

Table 1

Multilinear regression analysis. HMOX1 expression, GCLM expression, and NAD(P)H content are strongly associated with PMAT QD uptake.

Assay	Estimated coefficient	Std. Error	t value	Pr(> t)
HMOX1 *	0.551	0.206	2.684	0.014
GCLM *	-0.348	0.140	-2.489	0.022
NADPH *	0.464	0.190	2.433	0.025
Necrosis	0.381	0.192	1.987	0.061
MBB	0.364	0.203	1.790	0.089
NAO	-0.275	0.203	-1.355	0.191
GCLC	0.172	0.174	0.991	0.334
MTT	-0.160	0.210	-0.763	0.455
GSH	0.104	0.141	0.739	0.468

*
p<0.05