Published in final edited form as:

Nanomedicine. 2011 February ; 7(1): 88–96. doi:10.1016/j.nano.2010.09.001.

Doxorubicin conjugated quantum dots to target alveolar macrophages/inflammation

Krishnan V. Chakravarthy, PhD^{†,*}, Bruce A. Davidson, PhD^{†,‡}, Jadwiga D. Helinski, MS^{†,‡}, Hong Ding, PhD[§], Wing-Cheung Law, BS[§], Ken-Tye Yong, PhD[§], Paras N. Prasad, PhD[§], and Paul R Knight, MD, PhD^{†,§,‡}

Department of Microbiology and Immunology, State University of New York at Buffalo, Buffalo New York

Abstract

The ability to provide targeted therapeutic delivery in the lung would be a major advancement in pharmacological treatments for many pulmonary diseases. Critical issues for such successful delivery would require the ability to target specific cell types, minimize toxicity (i.e. inflammatory response) and to deliver therapeutic levels of drugs. We report here on the ability of nanoconjugates of CdSe/CdS/ZnS Quantum Dots (QDs) and doxorubicin (Dox) to target alveolar macrophages cells (aMØ), which play a critical role in the pathogenesis of inflammatory lung injuries. Confocal imaging showed the release of Dox from the QD-Dox nanoconjugate, as was evident by its accumulation in the cell nucleus and induction of apoptosis, suggesting that the drug retains its bioactivity after coupling to the nanoparticle. Inflammatory injury parameters (albumin leakage, proinflammatory cytokines and neutrophil infiltration) were recorded after *in vivo* administration of QD-Dox and Dox observing no significant effect after QD-Dox treatment compared with Dox. These results demonstrate that nanoparticle platforms can provide targeted macrophage-selective therapy for the treatment of pulmonary disease.

Keywords

Quantum dots; alveolar macrophages; drug delivery; cytokines; inflammation

Introduction

The immune/inflammatory modulation is important in various pulmonary disease processes including asthma, acute respiratory distress syndrome, and chronic obstructive pulmonary disease. The creation of stable, site-specific, therapeutic delivery platforms to direct drugs to a specific cellular target can tightly control therapy and reduce “collateral damage”. This is important for a immune/inflammatory therapy, where there is a constant balance between

© 2010 Elsevier Inc. All rights reserved.

*Corresponding authors: Department of Microbiology/Immunology State University of New York at Buffalo, 3435 Main Street, Buffalo, NY 14214, USA. kvc2@buffalo.edu, pknight@buffalo.edu.

[†]Department of Anesthesiology, State University of New York at Buffalo, Buffalo, NY

[§]Institute of Lasers, Photonics, and Biophotonics, State University of New York at Buffalo, Buffalo New York

[‡]Veterans Administration Western New York Healthcare System, Buffalo, NY

The authors declare no conflict of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

the beneficial effects of down-modulating an over-active immune response, while increasing the risk posed by suppressed immunity.

The aMØ is the sentinel cell involved in directing the host innate and adaptive immune responses involved in infectious and non-infectious lung diseases. The aMØ's central role in response to environmental influences makes these cells a candidate for targeted drug delivery in the modulation of the immune/inflammatory response. Due to the inhalational administration, the lungs offer a unique target for drug-nanoconjugate therapy.

We hypothesize that aMØ can be selectively targeted by Dox coupled to QDs. QDs are composed of atoms from groups II-VI or III-V or IV of the periodic table, and have a size comparable to or smaller than the Bohr radius [1, 2]. This size leads to a quantum confinement effect which results in size dependent optical properties. Furthermore, the QDs surface is easy to functionalize in order to provide support for covalent coupling of therapeutic agents [1, 3, 4]. We report here the successful use of colloiddally synthesized CdSe/CdS/ZnS QDs [5, 6] for the delivery of Doxorubicin to lung aMØ.

Methods

QD-Dox synthesis

CdSe/CdS/ZnS QDs were prepared by growing a CdS/ZnS-graded shell on a CdSe core. The detailed protocol for QD preparation used in these studies is described previously by our laboratory [9].

Formation of the QD-Dox nanoconjugate was accomplished by combining 30 µl of QDs ($\sim 1.41 \times 10^{-12}$ mol) and 20 µl of triethylamine ($\sim 1 \times 10^{-7}$ mol) with 203 µl HPLC grade water, followed by 67 µl of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide ($\sim 1.68 \times 10^{-7}$ mol) and mixing for several minutes. 7 µl of Doxorubicin ($\sim 3.02 \times 10^{-8}$ mol) was added, incubated for 2-4 hr, and the reddish solution was centrifuged at $9,900 \times g$ for 30 minutes. The pellets were washed once with water and the QD-Dox nanoconjugates were re-dispersed in water to a final volume of 330 µl (4.3×10^{-5} mol/µl). The conjugation efficiency of 47% was determined by spectrometry (absorption at 482 nm)[7-9].

Studies of QD-Dox nanoconjugate parameters: surface charge, size and photoluminescence were measured as described by KT Yong [9].

Animal Model

Male, Long-Evans rats (250-300 g) and C57Bl6 mice (25-30 g) were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and Charles River Laboratories (Wilmington, MA) respectively. The Institutional Animal Care and Use Committee of the Veterans Administration Western New York Healthcare System in Buffalo approved all experiments, and all animal work conformed to their guidelines for the humane care of animals.

Oro-pharyngeal aspiration delivery of nanoparticle solutions to the lungs

Rats/mice were anesthetized with 2% halothane in oxygen and, when unresponsive to a toe pinch, suspended by their incisors in a 60° supine position. The tongue was pulled out forward to raise the epiglottis, thereby opening the laryngeal inlet. Prior to solution instillation, the chest was squeezed and, following deposition of 300 µl (95 µl for mice) of the solution into the oropharynx with a pipette, released to facilitate aspiration of the instillate into the alveoli. A second 300 µl aliquot was instilled into the lungs 1 min later. The following instillate solutions were used: (i) H₂O (vehicle control), (ii) 10 nM Doxorubicin in H₂O, (iii) 10 nM equivalent of QD-Dox based on equivalent fluorescence

emission at 570 nm. Animals were sacrificed 4 or 24 hr post-instillation, with a minimum of 2 rats/time point/instillate group being performed during each experiment that was repeated 3 times (n=6 for each treatment and time point).

Arterial blood gas measurements

Arterial oxygenation (Pao₂) was collected from abdominal aorta following 2% halothane in 98% O₂ for 5 min and analyzed with an ABL₅ blood gas analyzer (Radiometer America, Westlake, OH).

Bronchoalveolar lavage

The bronchoalveolar lavage (BAL) was collected according with the protocol optimized by Knight, PR et al. [12].

BAL fluid albumin and cytokine ELISA

The degree of pulmonary injury was assessed by measuring the leakage of albumin into the airspaces following the procedure of Nemzek et al. [10]. BAL fluid MCP-1, MIP-2, and CINC-1 concentrations were determined with commercially available ELISA kits from R&D Systems (Minneapolis, MN) or PharMingen (San Diego, CA) following the manufacturers' instructions.

BAL fluid TNF α WEHI bioassay

TNF α bioactivity was measured using the WEHI 164 subclone 13 fibrosarcoma cell line bioassay, as previously described[11].

BAL fluid differential cell count

Following centrifugation of the collected BAL, the cell pellet was processed according with the protocol optimized by Knight PR et al. [12].

Histopathological Analysis

Following *in situ* flushing of the pulmonary vasculature, the trachea was cannulated, the lungs inflated at 20 cmH₂O with 10% neutral buffered formalin (NBF) while suspended in 10% NBF and incubated for ~24 hr at room temperature. Transverse sections, 2-3 mm thick, were prepared from the left lung, and from each of the 4 right lung lobes, embedded in paraffin, and 4 μ m sections were stained with hematoxylin and eosin. Slides were coded to "blind" the pathologist.

Alveolar macrophage cell isolation and in vitro nanoparticle exposure

BAL was performed on naïve rats, centrifuged and the cell pellet (>95% aM ϕ) resuspended in RPMI-1640 containing 1 mM glutamine, penicillin and streptomycin, amphotericin B and 10% fetal bovine serum to 1×10^6 aM ϕ /ml. Two hundred μ l/well (2×10^5 cells) was dispensed into wells of a low-binding, 96-well, flat-bottomed culture plate (Nunc, Thermo Fisher Scientific, Rochester, NY) and incubated at 37°C, 90% RH, 5% CO₂ for 20 hr. The cells were resuspended in 100 μ l fresh media and exposure to 20 μ l of Dox or QD-Dox was performed at 37°C, 90% RH, 5% CO₂ for 4 and 24 hr on a rocking-rotating platform (Nutator™, TCS Scientific, New Hope, PA). The supernatant was collected for cytokine analyses. The cells were washed and resuspended in 300 μ l of 2% BSA in PBS + 0.1% sodium azide for cytometric analysis.

For phagocytic assays, 10.5 μ l of 100 μ g/ml cytochalasin D (Sigma Chemical, St. Louis, MO) was added to each well (5 μ g/ml final concentration) following the 20 hr incubation.

The cell suspensions were washed in either culture medium or serum-free medium (Macrophage Serum-Free Medium, GIBCO, Invitrogen, Carlsbad, CA) containing 5.6 $\mu\text{g/ml}$ cytochalasin D. Following centrifugation at $1,500 \times g$ for 3 min at 4°C , the cell pellets were resuspended in 180 μl fresh medium containing 5.6 $\mu\text{g/ml}$ cytochalasin D and transferred to wells of a low-binding, 96-well, flat-bottomed culture plate. Dox, QD-Dox, or H_2O (20 μl /well) was added, the cells incubated and analyzed by flow cytometry.

Confocal microscopic analysis

The aM ϕ and BAL-recovered cells were dispensed onto a 35 mm glass bottom microwell dish (MatTek, Ashland, MA) and treated with 200 μl of media, Dox, or QD-Dox solutions. Confocal images were obtained at 4, and 24 hr post exposure using a Leica confocal microscopy system (TCS SP2, Leica Microsystems, Bannockburn, IL) with an oil immersion objective (HXC PL APO CS 63×1.40). Hoechst 33342 was added to stain the nuclei and was imaged using a 405 nm laser while the fluorescent emission was collected at 430-480. A 488nm laser was applied to excite both Dox and the QDs. The fluorescence signal from Dox was collected at 540-580 nm, while the photoluminescence signal from the QDs was collected at 620-660 nm. For emission spectrum analysis, a 10 nm band pass filter was used and the scan was acquired from 500 nm to 700 nm in 10 nm steps.

Flow cytometry

Alveolar M ϕ staining was assessed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) with the acquisition threshold set using the forward scatter detector to exclude non-cellular events (i.e., cell debris). The fluorescence emissions of 10,000 cell events were collected with the FL2 (excitation = 488 nm, emission = 585 ± 21 nm optimized for Dox) and FL3 (excitation = 488 nm, emission = 661 ± 8 nm optimized for QD) detectors. QD-labeled aM ϕ exhibited no spectral overlap into the FL2 emission window. The label negative/positive boundary was set using the H_2O -treated aM ϕ samples such that $\approx 99\%$ was label negative.

WST-1 reduction assay

Following the overnight incubation, the aM ϕ culture was washed with warm PBS and 200 μl /well of warm culture medium containing the appropriate test solution was added. The culture was incubated at 37°C , 90% RH, 5% CO_2 on a rocking platform for 4 or 24 hr. Three hours prior to harvest, 10 μl of WST-1 Cell Proliferation Reagent (Roche Diagnostics, Indianapolis, ID) was added to appropriate wells. Following transferred to a clean 96-well plate, the absorbance was measured at 440 and 700 nm in a SpectraMax 190 microplate spectrophotometer (Molecular Devices, San Diego, CA). The difference between 700 nm and 440 nm is proportional to the culture viability.

Statistical analysis

All data were expressed as mean $\pm$ SEM. Comparisons of the assayed parameters at each harvest time point (4 and 24 hr) between the experimental and control groups ($n=6$ per group) were made using one-way ANOVA and Tukey's post-hoc tests.

Results

QD-Dox nanoconjugate

Dynamic light scattering indicated that the hydrodynamic diameter of the QDs was 11.1 ± 0.2 nm and 30.7 ± 2.9 nm for QD-Dox. The change in the hydrodynamic diameter after coupling is indicative of the surface presence of Doxorubicin. Covalent coupling was determined by a shift in zeta potential from -38.38 ± 2.3 mV (QDs) to -1542 ± 0.3 mV (QD-Dox), indicating

the formation of an amide bond between the amine group of the Dox and the carboxyl groups on the QD surface.

aMØ uptake of the QD-Dox

The uptake of Dox vs. QD-Dox was determined by flow cytometric analysis of aMØ after 1, 6, and 24 hr *in vitro* post-exposure. In Figure 1A, the percentage of cells positive for free Dox was $4.5 \pm 0.1\%$, $15.1 \pm 0.6\%$, $26.5 \pm 0.8\%$ at 1, 6, 24 hr, respectively. Following QD-Dox exposure, $15.7 \pm 0.5\%$, $31.5 \pm 1.4\%$, and $59.7 \pm 0.8\%$ of aMØ were positive, demonstrating significant increases ($p < 0.0004$) at each of the respective time points over the Dox-only groups. Thus, coupling of Dox to the QDs enhances uptake of the drug by the MØs.

Figure 1B is the confocal image of primary rat aMØ acquired 3 hr post-exposure to the QD-Dox, *in vitro*. Using separate acquisition channels for Dox (pseudo-green) and QDs (pseudo-red), we observed an accumulation of Dox in the nucleus, whereas the signal from the QDs remained in the cytoplasm, indicating separation. A mechanism of Dox action is its intercalation in DNA inducing apoptosis [13, 14]. Macrophages treated with QD-Dox show clumping and blebbing, which are signs of apoptosis.

Flow cytometry (Figure 1C) revealed that cytochalasin D treatment had no effect on Dox uptake, irrespective of the presence of serum in the medium, indicating that the uptake involves an actin polymerization-independent pinocytosis pathway. However, in the presence of serum, QD-Dox uptake was ablated by cytochalasin D, $2.0\% \pm 0.2\%$ compared to $25.0\% \pm 2.6\%$ ($p < 0.001$). In the absence of serum, cytochalasin D, the uptake was reduced from $74.9\% \pm 1.2\%$ to $45.7\% \pm 1.1\%$ ($p < 0.001$), indicating both actin polymerization-dependent (i.e., phagocytosis, macropinocytosis) and -independent (i.e., clathrin-mediated endocytosis, caveolin-mediated endocytosis) pathways are involved. We also compared QD uptake in the presence and absence of cytochalasin D. In serum, the QD uptake dropped from $93.4\% \pm 1.3\%$ to $58.1\% \pm 1.2\%$ upon treatment with cytochalasin D, but not in the absence of serum (data not shown). These findings suggest that serum-induced aggregation of QD enhances uptake through phagocytosis, while single suspension conjugated QDs are taken-up using an actin polymerization-independent pinocytosis pathway [15].

To determine whether coupling of Dox to QDs retains drug functionality, we performed a WST-1 reduction assay, to assess aMØ viability (Figure 1B). At 24 hr post exposure, there was ~40% reduction in viability of the aMØ, upon exposure to QD-Dox [$p < 0.001$ (Figure 2)]. Thus, our drug delivery system using QDs retains drug functionality comparable to treatment with free Dox.

We also assayed the *in vitro* medium following the exposure of a MØ to QD-Dox for TNF- α , the neutrophil chemoattractants, CINC-1 and MIP-2 (important in the acute neutrophilic inflammatory response), and the monotactic chemokine, MCP-1, a biomarker of later developing monocytic inflammation. Lipopolysaccharide (LPS- $1.5 \mu\text{g/ml}$) was employed as a positive control. Exposure to Dox or QD-Dox did not increase TNF- α , CINC-1, MIP-2, or MCP-1 levels compared to unexposed aMØ at both 4 and 24 hr post-exposure (Figure 3). Therefore, free Dox or QD-Dox induces alveolar macrophage apoptosis without causing a concurrent pro-inflammatory cytokine response.

In vivo delivery of QD-Dox by oro-pharyngeal aspiration

To translate our *in vitro* findings to *in vivo*, we delivered the QD-Dox nanoconjugates into the pulmonary airspaces of rats by oro-pharyngeal aspiration. At 24 hr post-instillation, the cells were recovered by BAL and imaged by confocal microscopy (Figure 4A-D). The presence of QD-Dox in the aMØ was demonstrated with z-stack analysis confirming intracellular translocation (data not shown). Identification of the emitting molecular species

was determined by spectral emission analysis (Figure 4E). Thus, oro-pharyngeal aspiration successfully delivered the QD-Dox to aMØ's, *in vivo*. Figure 5 demonstrates successful deposition of QDs, with mercaptosuccinic acid (MSA) surface modification, into the lung parenchyma of C57BL/6 mice following oropharyngeal aspiration delivery.

Finally, we examined inflammatory pulmonary injury (Figure 6), by assessing: 1) BAL cytokines (Figure 6A-D); 2) BAL albumin (Figure 6E); 3) arterial blood oxygenation (Figure 6F); 4) BAL leukocyte count (Figure 6G) and differential (Figures 6H-I); and 5) histopathology. *In vivo*, there were no increases in TNF α or MIP-2 at 4 and 24 hr in any of the experimental groups (Figure 6A-B). However, we observed that treatment with Dox produced significant increases in CINC-1 at 4 hr (Figure 6C), and the MCP-1 (Figure 6D) level at 24 hr ($p<0.05$). The increased CINC-1 level was consistent with increased neutrophil infiltration into the airways following Dox (Figure 6H). There was ~66% more neutrophil infiltration at 4 hr ($p<0.05$), and ~50% more neutrophil infiltration at 24 hr compared to QD-Dox treated rats. Increases in MCP-1 (Figure 6D) would indicate that Dox induces a sustained inflammatory response. At 4 hr post treatment (Figure 6E), the albumin concentration in the airways of animals treated with Dox alone was 88.1 ± 30.6 pg/ml of albumin, while the level following QD-Dox was 31 ± 4 pg/ml ($p<0.05$). The albumin concentration following the QD-Dox treatment at 4 and 24 hr was comparable to control. PaO₂/FiO₂ decreased to ~400 mmHg for both 4 and 24 hr post exposure to Dox compared to ~590 mmHg for controls ($p<0.01$) (Figure 6F). Collectively, these findings demonstrate less inflammatory response and functional lung impairment using QD-Dox compared Dox.

Post-instillation, histopathology of lungs at 4 and 24 hrs, did not demonstrate any differences between the groups that received control, Dox, or QD-Dox. The majority of the pulmonary parenchyma contained normal lung histology with intact alveolar architecture, thin septa, normal pleura, and the conducting and respiratory airways were normal. A few isolated, focal areas of parenchymal intra-alveolar cellular infiltrates, activated alveolar type II cells, thickened alveolar septa, and minor occurrences of perivascular edema were found. Samples could not be stratified, as all of the injury groups exhibited similar patterns of change.

Discussion

Nanoparticle delivery systems are currently being used for (1) new pharmaceutical moieties, (2) improved drug effectiveness with reduced side-effects, and (3) cell/tissue-specific targeting [16]. We have developed a nanotechnology-based approach for drug delivery to the aMØ. The rationale for using QD-Dox is based on the differential photoluminescence of these two agents. This allows for visual confirmation of the intracellular uptake, as well as dissociation of Dox in the nucleus, which is required for drug action. Our *in vitro* findings demonstrate that QD-Dox enhances intracellular uptake compared to free drug. Additionally, the uptake of QD-Dox by the aMØ did not elicit a significant pro-inflammatory cytokine response. We also demonstrate that Dox is released from the QD-Dox formulation and migrates to the nucleus (site of bioactivity), whereas the QDs remain in cytosol.

Administration of QD-Dox, *in vivo*, also resulted in uptake into aMØs without any evidence of acute lung injury or increase cytokine levels. A decrease in PaO₂/FiO₂ occurred both at 4 and 24 hrs following Dox administration, which was associated with increased levels of proinflammatory cytokines, and leakage of albumin and neutrophils into the airways. This decrease was not observed with QD-Dox, indicating that injury resulting from the administration of Dox was mitigated by complexing Dox to QDs. Finally, histopathology studies post oro-pharyngeal instillation found only minimal abnormalities. Collectively,

these findings demonstrate a novel drug delivery method targeting aMØ *in vivo*, with no discernible lung injury.

Previous reports have employed large porous particles[17], or large polymeric particles[18] for targeting cells within the lung alveolus. However, QDs as a carrier system presents distinct advantages of having both diagnostic and therapeutic benefits.[19] The ease of formulation and uniformity of QDs make a more efficient approach to lung cell targeting. Several studies have also assessed polysorbate-coated nanoparticles [20], and polyalkylcyanoacrylate (PACA)[21] nanoparticles loaded with Dox. These reports also demonstrated a therapeutic efficacy and decreased toxicity compared to the non-conjugated drug, consistent with our observations.

Our approach for targeting of the aMØ is based on their role as a scavenger of small particles that enter the alveoli. Uptake of “dust” particles does not usually elicit an inflammatory response. Previous studies reported that aMØ possess receptors for polyanionic particles [22] that enable them to uptake negatively charged particles more efficiently than neutral and positive ones. Both the size of the QD and composition of the solution used appear to be a critical factor in aMØ uptake mechanism. Uptake in serum was clearly dependant on a phagocytic mechanism and in the absence of serum, non-actin polymerization requiring pinocytotic process(es).

The efficient scavenging of QD-Dox by the aMØ provides a compelling mechanism for the prevention of injury to other cells of the lung by Dox. The observed BAL cytokine levels following QD-Dox exposure are indistinguishable from those obtained from control animals. It is reasonable to speculate that decreases in inflammatory lung injury are directly related to a decrease of other cells of airways and alveoli to which there is free Dox uptake.

The potential of using a nanoparticle drug delivery approach provides a novel therapeutic strategy for treating lung diseases. The ability to target specific cells in the lung without exposing other pulmonary tissue or distant organs to detrimental actions of drugs, is an exciting avenue to explore. Many of the complications associated with immune/inflammatory therapy are caused by the inability to limit life threatening systemic responses. Potential applications could lead to therapeutic strategies to selectively up-regulate and down-regulate key aMØ regulatory and effector functions. Our study has shown that QD's can also serve as a diagnostic resource with minimal toxicity regionally and systemically[23]. However, we must acknowledge that ongoing studies need to address in greater depth what parameters (i.e. surface coating and functionalization, charge, size, duration of treatment) with QD formulations result in reduced regional and systemic toxicity. We point the reader to several papers that also address the issue of QD cytotoxicity [24, 25].

Based on our study, we propose that the formulation inclusive of surface coating, and side groups are critical in modulating lung injury following instillation. Therefore, MSA QDs may have tremendous potential for diagnosis and treatment of lung injury compared to other formulations primarily due to their selective uptake into alveolar macrophages, and should be further assessed for future lung pharmacotherapy applications.

Acknowledgments

This study was supported by National Institutes of Health Grant AI084410 (to P.R.K., P.N.P.); National Cancer Institute Grant CA119397 (to P.N.P.); and National Institutes of Health Grants HL48889 (to P.R.K.), and AG031035 (to K.V.C.). This work was also supported by the Chemistry and Life Sciences Division of the Air Force Office of Scientific Research (P.N.P.).

This work was supported by NIH grants HL48889 (PRK), AG031035 (KVC), CA119397 (PNP), and the John Oishei Foundation 9351-526578 (PNP).

References

1. Hild WA, Breunig M, Goepferich A. Quantum dots - nano-sized probes for the exploration of cellular and intracellular targeting. *Eur J Pharm Biopharm.* 2008; 68:153–68. [PubMed: 17869074]
2. Prasad, PN. Introduction to Biophotonics. Wiley; New York: 2003.
3. Medintz IL, Uyeda HT, Goldman ER, Mattoussi H. Quantum dot bioconjugates for imaging, labelling and sensing. *Nat Mater.* 2005; 4:435–46. [PubMed: 15928695]
4. Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Doose S, Li JJ, et al. Quantum dots for live cells, in vivo imaging, and diagnostics. *Science.* 2005; 307:538–44. [PubMed: 15681376]
5. Peng KY, Chen SA, Fann WS. Efficient light harvesting by sequential energy transfer across aggregates in polymers of finite conjugational segments with short aliphatic linkages. *J Am Chem Soc.* 2001; 123:11388–97. [PubMed: 11707115]
6. Peng X, Manna L, Yang W, Wickham J, Scher E, Kadavanich A, et al. Shape control of CdSe nanocrystals. *Nature.* 2000; 404:59–61. [PubMed: 10716439]
7. Chan WC, Maxwell DJ, Gao X, Bailey RE, Han M, Nie S. Luminescent quantum dots for multiplexed biological detection and imaging. *Curr Opin Biotechnol.* 2002; 13:40–6. [PubMed: 11849956]
8. Xu G, Yong KT, Roy I, Mahajan SD, Ding H, Schwartz SA, et al. Bioconjugated quantum rods as targeted probes for efficient transmigration across an in vitro blood-brain barrier. *Bioconjug Chem.* 2008; 19:1179–85. [PubMed: 18473444]
9. Yong KT, Qian J, Roy I, Lee HH, Bergey EJ, Tramosch KM, et al. Quantum rod bioconjugates as targeted probes for confocal and two-photon fluorescence imaging of cancer cells. *Nano Lett.* 2007; 7:761–5. [PubMed: 17288490]
10. Nemzek JA, Call DR, Ebong SJ, Newcomb DE, Bolgos GL, Remick DG. Immunopathology of a two-hit murine model of acid aspiration lung injury. *Am J Physiol Lung Cell Mol Physiol.* 2000; 278:L512–20. [PubMed: 10710523]
11. Davidson BA, Knight PR, Helinski JD, Nader ND, Shanley TP, Johnson KJ. The role of tumor necrosis factor- α in the pathogenesis of aspiration pneumonitis in rats. *Anesthesiology.* 1999; 91:486–99. [PubMed: 10443613]
12. Knight PR, Davidson BA, Nader ND, Helinski JD, Marschke CJ, Russo TA, et al. Progressive, severe lung injury secondary to the interaction of insults in gastric aspiration. *Exp Lung Res.* 2004; 30:535–57. [PubMed: 15371091]
13. Kalyanaraman B, Joseph J, Kalivendi S, Wang S, Konorev E, Kotamraju S. Doxorubicin-induced apoptosis: implications in cardiotoxicity. *Mol Cell Biochem.* 2002; 234-235:119–24. [PubMed: 12162424]
14. Wang S, Konorev EA, Kotamraju S, Joseph J, Kalivendi S, Kalyanaraman B. Doxorubicin induces apoptosis in normal and tumor cells via distinctly different mechanisms. Intermediacy of H₂O₂- and p53-dependent pathways. *J Biol Chem.* 2004; 279:25535–43. [PubMed: 15054096]
15. Dobrovolskaia MA, McNeil SE. Immunological properties of engineered nanomaterials. *Nat Nanotechnol.* 2007; 2:469–78. [PubMed: 18654343]
16. PN, P. Introduction to Biophotonics. N.Y.: Wiley; 2004.
17. Edwards DA, Hanes J, Caponetti G, Hrkach J, Ben-Jebria A, Eskew ML, et al. Large porous particles for pulmonary drug delivery. *Science.* 1997; 276:1868–71. [PubMed: 9188534]
18. Lai SK, O'Hanlon DE, Harrold S, Man ST, Wang YY, Cone R, et al. Rapid transport of large polymeric nanoparticles in fresh undiluted human mucus. *Proc Natl Acad Sci U S A.* 2007; 104:1482–7. [PubMed: 17244708]
19. Jacobsen, Nicklas Raun; M, P.; Jensen, Keld Alstrup; Vogel, Ulla; Ladefoged, Ole; Loft, Steffen; Wallin, Håkan. Lung inflammation and genotoxicity following pulmonary exposure to nanoparticles in ApoE^{-/-} mice. *Particle and Fibre Toxicology.* 2009; 6:2. [PubMed: 19138394]

20. Steiniger SC, Kreuter J, Khalansky AS, Skidan IN, Bobruskin AI, Smirnova ZS, et al. Chemotherapy of glioblastoma in rats using doxorubicin-loaded nanoparticles. *Int J Cancer*. 2004; 109:759–67. [PubMed: 14999786]
21. Soma CE, Dubernet C, Barratt G, Nemati F, Appel M, Benita S, et al. Ability of doxorubicin-loaded nanoparticles to overcome multidrug resistance of tumor cells after their capture by macrophages. *Pharm Res*. 1999; 16:1710–6. [PubMed: 10571276]
22. Wilhelm C, Billotey C, Roger J, Pons JN, Bacri JC, Gazeau F. Intracellular uptake of anionic superparamagnetic nanoparticles as a function of their surface coating. *Biomaterials*. 2003; 24:1001–11. [PubMed: 12504522]
23. Yong KT, Roy I, Ding H, Bergey EJ, Prasad PN. Biocompatible near-infrared quantum dots as ultrasensitive probes for long-term in vivo imaging applications. *Small*. 2009; 5:1997–2004. [PubMed: 19466710]
24. Derfus AM, Chan W, Bhatia S. Probing the cytotoxicity of semiconductor quantum dots. *Nanoletters*. 2004; 4:11–18.
25. Qi L, Gao X. Emerging application of quantum dots for drug delivery and therapy. *Expert Opin Drug Deliv*. 2008; 5:263–267. [PubMed: 18318649]

Figure 1. Uptake of free Doxorubicin and QD-Dox by aMØ *in vitro*

A) Flow cytometric analysis of Dox and QD-Dox uptake by rat aMØ isolated by BAL and exposed *in vitro* for 1, 6, and 24 hr. Data is expressed as %aMØ positive for Doxorubicin fluorescence (n=3 for all conditions). *p<0.0001 compared to H₂O-exposed control at the same time point. †p<0.0005 compared to free Doxorubicin group at the same time point. B) Confocal image of cells obtained 3 hr post-instillation of QD-Dox conjugate by BAL. Arrowheads indicate aMØ positive for Dox nuclear staining (green) and QD cytoplasmic fluorescence (red). C) Flow cytometric analysis of Dox and QD-Dox uptake by aMØ isolated by BAL and exposed for 6 hr in the presence or absence of 5 µM cytochalasin D and the presence or absence of serum in the medium. Data expressed as %aMØ positive for

Dox or QD emission (n=3 for all conditions). §p<0.001 compared to QD-Dox, non-cytochalasin D exposed control within the same type of medium.

Figure 2. Effect of free Doxorubicin and QD-Dox exposure on WST-1 reduction by aMØ *in vitro*
 Rat aMØ were isolated by BAL and exposed *in vitro* for 4 or 29 hr to QD-Dox, Dox, or LPS in a 96-well culture plate. WST-1 was added to each well 4 hr prior to measurement. At 4 or 29 hr post-exposure the absorbance of the culture medium was assessed at 440 and 700 nm. The decrease in WST-1 reduction in aMØ exposed to Dox or QD-Dox indicates Doxorubicin-induced cytotoxicity, potentially due to apoptosis. *p<0.001 compared to aMØ only group at 24 hr. †p<0.001 compared to Dox exposed cells.

Figure 3. Effect of free Doxorubicin and QD-Dox on *in vitro* cytokine production in primary rat aMØ

Rat aMØ were isolated by BAL and exposed *in vitro* for 4 or 24 hr to Dox, QD-Dox, or 1.5 µg/ml LPS in a 96-well culture plate. Culture medium was assayed for A) TNFα using the WEHI bioassay, and B) MIP-2, C) CINC-1, and D) MCP-1 by ELISA. Only exposure to LPS produced a significant response of each cytokine or chemokine at both time points compared to unexposed aMØ, * $p < 0.01$.

Figure 4. *In vivo* drug delivery of QD-Dox at 24 hr post oro-pharyngeal aspiration instillation in rats

Confocal microscopy images of aMØ isolated from rats exposed to QD-Dox, *in vivo*, for 24 hr using the A) Dox emission (pseudo-colored green); B) QD emission (pseudo-colored red); and C) merged image of both Doxorubicin and QD emission (co-localization of Dox and QD pseudo-colored yellow). D) Differential interference contrast transmission image superimposed on merged confocal image. Z-stack analysis indicates intracellular uptake of QD-Dox by aMØ (data not shown). White bar = 10µm. E. Spectral scans of aMØ from rats exposed to Dox, QD, or QD-Dox for 24 hr.

Figure 5. Frozen lung sections following QD instillation

Confocal fluorescence images of frozen lung sections (30 μm) from C57BL/6 mice receiving, A) no QD instillation (normal lung tissue), or B) 160 μg of QD in 95 μl instilled into the lungs by pharyngeal aspiration 45 min prior to harvest. Insets indicate normalized emission spectra of A) pulmonary tissue autofluorescence (green), or B) QDs in pulmonary tissue (red-orange). Data indicate deposition of QDs (red-orange) into the respiratory lung parenchyma that are easily differentiated from lung tissue autofluorescence (green). Objective = 20X, white bar = 50 μm .

Figure 6. Effect of free Dox and QD-Dox on *in vivo* pulmonary cytokine production, injury parameters, and cellular inflammatory response

Rats were exposed to water (control), Dox, or QD-Dox by oro-pharyngeal aspiration. At 4 or 24 hr post-exposure an arterial blood sample was taken and BAL performed. The recovered BAL fluid was assayed for: A) TNF α bioactivity by WEHI bioassay, and B) MIP-2, C) CINC-1, and D) MCP-1 by ELISA Lung injury was assessed by: E) BAL albumin by ELISA, and F) PaO₂/FiO₂. The pulmonary cellular inflammatory response was assessed by: G) total number of leukocytes, H) neutrophils, and I) aM ϕ recovered in the BAL fluid.

*p<0.05 compared to the water control group.