

Boston University**OpenBU****<http://open.bu.edu>**

BU Open Access Articles

BU Open Access Articles

2017-11-09

Quantifying engineered nanomaterial toxicity: comparison of common cytotoxicity and g...

This work was made openly accessible by BU Faculty. Please [share](#) how this access benefits you. Your story matters.

Version	
Citation (published version):	Donald H Atha, Amber Nagy, Andrea Steinbrück, Allison M Dennis, Jennifer A Hollingsworth, Varsha Dua, Rashi Iyer, Bryant C Nelson. 2017. "Quantifying engineered nanomaterial toxicity: comparison of common cytotoxicity and gene expression measurements.." Journal of Nanobiotechnology, v. 15:79. https://doi.org/10.1186/s12951-017-0312-3

<https://hdl.handle.net/2144/29983>*Boston University*

RESEARCH

Open Access



Quantifying engineered nanomaterial toxicity: comparison of common cytotoxicity and gene expression measurements

Donald H. Atha^{1*}, Amber Nagy^{2,5}, Andrea Steinbrück³, Allison M. Dennis^{3,6}, Jennifer A. Hollingsworth³, Varsha Dua¹, Rashi Iyer⁴ and Bryant C. Nelson¹

Abstract

Background: When evaluating the toxicity of engineered nanomaterials (ENMs) it is important to use multiple bioassays based on different mechanisms of action. In this regard we evaluated the use of gene expression and common cytotoxicity measurements using as test materials, two selected nanoparticles with known differences in toxicity, 5 nm mercaptoundecanoic acid (MUA)-capped InP and CdSe quantum dots (QDs). We tested the effects of these QDs at concentrations ranging from 0.5 to 160 $\mu\text{g}/\text{mL}$ on cultured normal human bronchial epithelial (NHBE) cells using four common cytotoxicity assays: the dichlorofluorescein assay for reactive oxygen species (ROS), the lactate dehydrogenase assay for membrane viability (LDH), the mitochondrial dehydrogenase assay for mitochondrial function, and the Comet assay for DNA strand breaks.

Results: The cytotoxicity assays showed similar trends when exposed to nanoparticles for 24 h at 80 $\mu\text{g}/\text{mL}$ with a threefold increase in ROS with exposure to CdSe QDs compared to an insignificant change in ROS levels after exposure to InP QDs, a twofold increase in the LDH necrosis assay in NHBE cells with exposure to CdSe QDs compared to a 50% decrease for InP QDs, a 60% decrease in the mitochondrial function assay upon exposure to CdSe QDs compared to a minimal increase in the case of InP and significant DNA strand breaks after exposure to CdSe QDs compared to no significant DNA strand breaks with InP. High-throughput quantitative real-time polymerase chain reaction (qRT-PCR) data for cells exposed for 6 h at a concentration of 80 $\mu\text{g}/\text{mL}$ were consistent with the cytotoxicity assays showing major differences in DNA damage, DNA repair and mitochondrial function gene regulatory responses to the CdSe and InP QDs. The BRCA2, CYP1A1, CYP1B1, CDK1, SFN and VEGFA genes were observed to be upregulated specifically from increased CdSe exposure and suggests their possible utility as biomarkers for toxicity.

Conclusions: This study can serve as a model for comparing traditional cytotoxicity assays and gene expression measurements and to determine candidate biomarkers for assessing the biocompatibility of ENMs.

Keywords: Genotoxicity, Nanomaterials, Quantum dots, Cytotoxicity, Comet assay, Gene expression, Biomarkers

Background

Engineered nanomaterials (ENMs) are widely used in commercial and industrial products in agriculture, engineering and medicine. The small size of ENMs provides them with special properties such as enhanced surface

charge and a high surface area to volume ratio. Size and charge dependent interactions may increase the likelihood of biological effects on human cells [1]. Semiconductor nanocrystals, or quantum dots (QDs), are of particular interest in this regard due to their numerous applications in optics [2–4], biomedical diagnostics [5–7] and therapeutics [5, 8, 9]. This has created a critical need for the quantitative evaluation of ENM effects and determination of the sensitivity and reproducibility of the cytotoxicity assays used to measure these effects.

*Correspondence: donald.atha@nist.gov

¹ Biosystems and Biomaterials Division, National Institute of Standards and Technology, Bld. 227, Rm. A247, MS 8313, 100 Bureau Drive, Gaithersburg, MD 20899, USA

Full list of author information is available at the end of the article

Many studies have focused on the toxicity of specific ENMs, using common cytotoxicity assays yet few detail the specific cellular mechanisms that play a role in their toxicity [10–13]. Gene expression analysis affords the opportunity to evaluate these mechanisms of toxicity, through the monitoring of regulatory genes that are affected. Cellular processes such as the induction of inflammatory cytokines, autophagy, necrosis, and apoptosis have been shown to be affected by physical properties of ENMs, like size and charge, as well as chemical properties, including the core composition and surface functionalization [12–16]. In this respect, it is critical to know how gene expression data can be correlated with common cytotoxicity assays, to know what genes will be useful to monitor as potential indicators of toxicity and to characterize the sensitivity and reproducibility of the measurements.

In the current study we compare four common cytotoxicity assays: the dichlorofluorescein assay for reactive oxygen species (ROS), the lactate dehydrogenase assay for membrane viability (LDH), the mitochondrial dehydrogenase assay for mitochondrial function, and the Comet assay for DNA strand breaks. We compared the responses of cultured normal human bronchial epithelial (NHBE) cells to two types of semiconductor QDs that were chosen based on their known difference in cytotoxicity: cadmium selenide (CdSe) QDs, which are known to produce significant toxic effects in cultured mammalian cells and indium phosphide (InP) QDs, which are reported to induce minimal toxicity to mammalian cells [10, 11, 17–22]. Considering these previous studies, CdSe and InP QDs functionalized with negatively charged mercaptoundecanoic acid (MUA) were chosen as well-characterized test materials to compare the results of the cytotoxicity assays and to determine if certain transcriptional changes related to DNA damage and repair and mitochondrial function can be used as predictive toxicological indicators in conjunction with prototypical cytotoxicity assays.

Results

Cytotoxicity measurements

All of the cytotoxicity and DNA damage data described in the following sections was collected using 5 nm diameter CdSe or InP cores rendered water soluble with MUA. MUA is a common thiol-based ligand used to stabilize colloidal QDs in aqueous media through the electrostatic repulsion of negative surface charge [23]. MUA itself was shown previously not to affect LDH release or DNA fragmentation [10]. The QD preparations tested here were characterized for UV absorbance, size and charge in aqueous media (see “Methods” section). A more detailed

description of other experimental methods used here also can be found in “Methods” section.

NHBE cells were exposed to increasing concentrations (0.5–160 $\mu\text{g}/\text{mL}$) of either 5 nm CdSe or 5 nm InP QDs and quantitatively evaluated for reactive oxygen species (ROS) generation over 120 min. The ROS levels were determined using the fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) measured at 10 min intervals. There was a significant increase in the ROS levels for the CdSe QDs for both the 80 and 160 $\mu\text{g}/\text{mL}$ exposures (relative to media only controls), but minimal increases in the measured ROS levels for the InP QDs at these same exposure concentrations (Fig. 1).

NHBE cells were exposed to increasing concentrations (0.5–160 $\mu\text{g}/\text{mL}$) of either CdSe or InP QDs for 24 h prior to assessing cell necrosis using lactate dehydrogenase (LDH) activity as an indicator of cell membrane viability. A significant increase in LDH release with CdSe QD exposure (relative to media controls) was observed at QD concentrations greater than or equal to 80 $\mu\text{g}/\text{mL}$, without a corresponding increase with InP exposure, as indicated by asterisks (Fig. 2).

NHBE cells were exposed to increasing concentrations (0.5–160 $\mu\text{g}/\text{mL}$) of either CdSe or InP QDs for 24 h and then evaluated for cellular viability. Cellular metabolism was determined by measuring the conversion of the water-soluble tetrazolium dye (WST-1) to formazan by mitochondrial dehydrogenase enzymes. A 75% loss of function with CdSe exposure and a 25% increase in

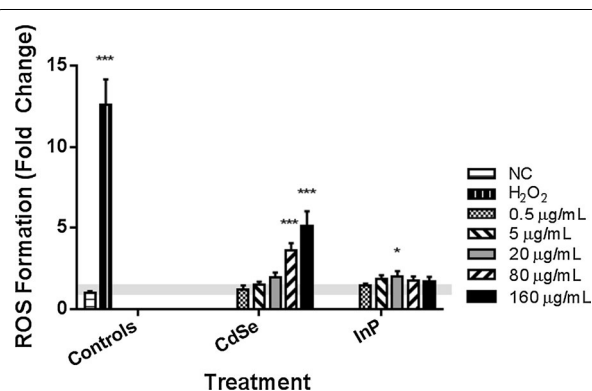
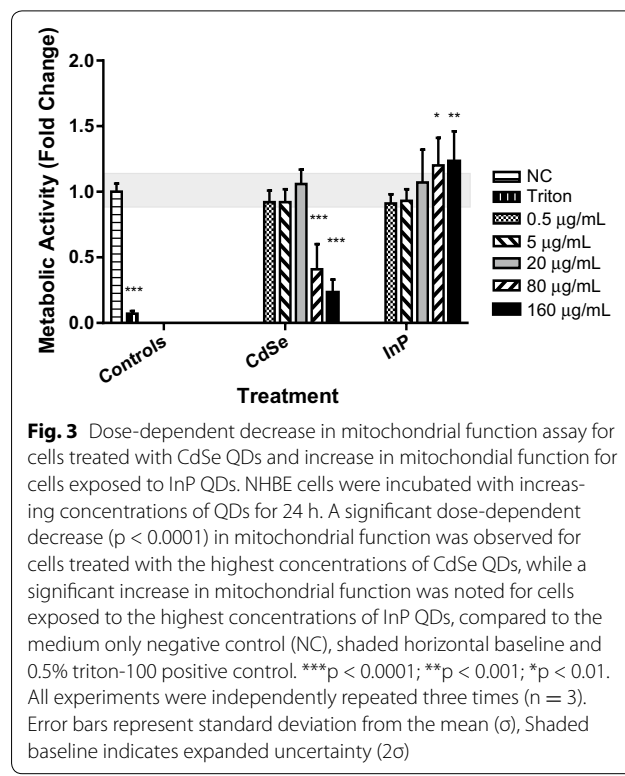
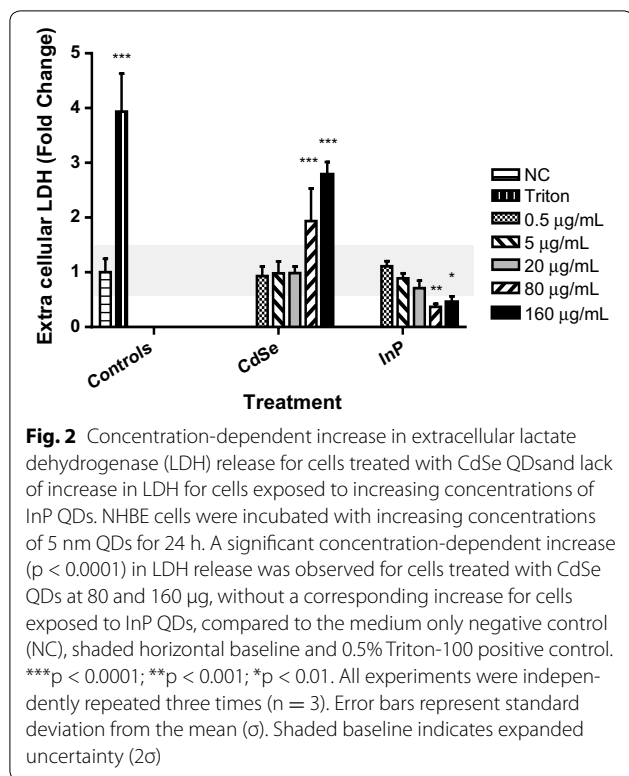


Fig. 1 MUA-coated CdSe QDs cause increased ROS formation in NHBE cells. NHBE cells were incubated with increasing concentrations of 5 nm CdSe and InP QDs for 24 h. A significant concentration-dependent increase ($p < 0.0001$) in ROS formation was observed for cells treated with CdSe QDs compared to the medium only negative control (NC), shaded horizontal baseline and 100 $\mu\text{mol}/\text{L}$ H₂O₂ positive control. InP QDs caused significant ROS after exposure to 20 $\mu\text{g}/\text{mL}$, but this response was not dose dependent. * $p < 0.01$, *** $p < 0.0001$. All experiments were independently repeated three times ($n = 3$). Error bars indicate one standard deviation from the mean (σ). Shaded baseline indicates expanded uncertainty (2σ)



function with InP exposure (relative to media controls—shaded baseline) was observed at QD concentrations greater than or equal to 80 $\mu\text{g}/\text{mL}$, as indicated by asterisks (Fig. 3). The drop in metabolic function with CdSe at 80 μg or greater is consistent with the loss of cell viability, as evidenced by the increase in LDH release at these high concentrations of CdSe (Fig. 2). This effect has also been observed previously with CdSe-CYST [11].

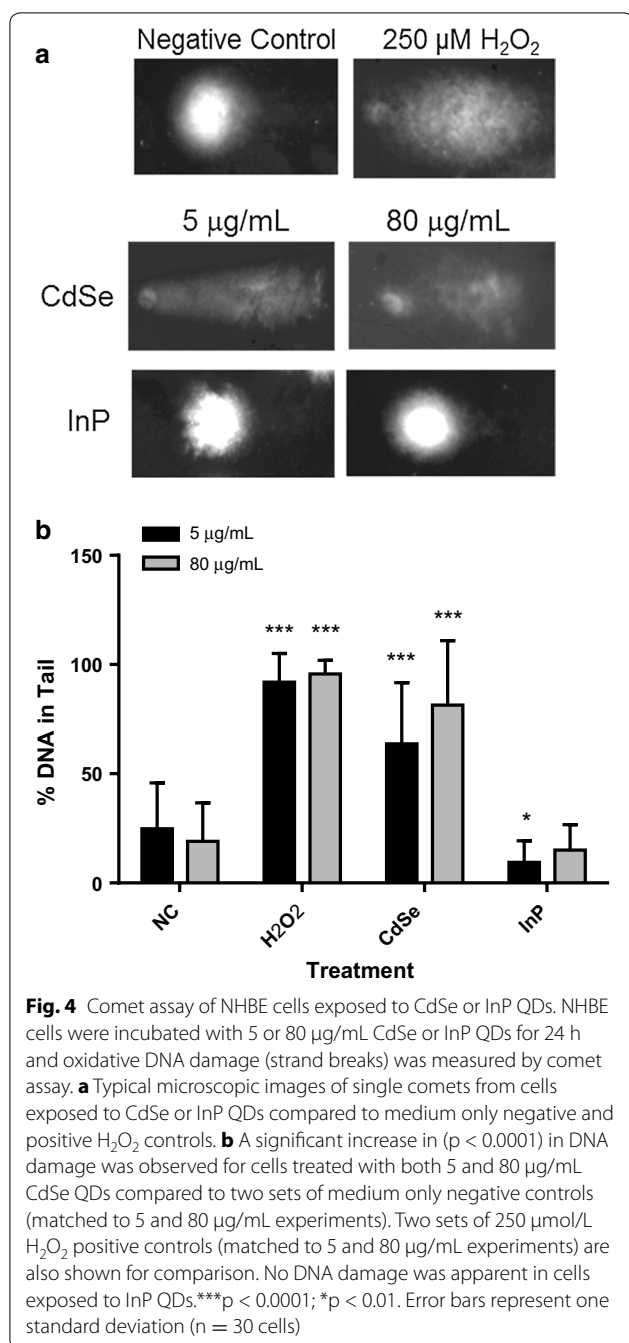
Measurements of DNA damage

DNA damage (strand breaks) was measured by the alkaline Comet assay in NHBE cells exposed to CdSe and InP QDs. Cells were imaged by fluorescence microscopy after staining with SYBR Green I. Characteristic comet shapes after exposure resulting from increased mobility of the fragmented nuclear DNA was evident after exposure to the CdSe QDs (Fig. 4a). InP QDs showed minimal effect. Images are representative single cells of control groups (media only and H_2O_2 -exposed cells) and QD-exposed cells. Data are expressed as percent of DNA in tail for QD-exposed NHBE populations compared to media only and H_2O_2 (250 $\mu\text{mol}/\text{L}$) controls. Significant differences between exposed cells and the media control are indicated by asterisks (Fig. 4b). DNA damage analyses revealed that CdSe QDs caused significant DNA strand breaks compared to InP QDs, which were equivalent to media-only treated cells. This response was observed in

NHBE cells exposed to even the lowest concentration of CdSe QDs (5 $\mu\text{g}/\text{mL}$), which indicates the high sensitivity of the Comet assay.

Effects on gene regulation

Cellular responses to a specific QD exposure can be considered both a function of QD concentration and the duration of the exposure. For our common direct cytotoxicity measurements, we chose a 24 h incubation at concentrations ranging from 0.5 to 160 $\mu\text{g}/\text{mL}$. In general, the QDs did not induce cytotoxic responses at (0.5, 5 or 20) $\mu\text{g}/\text{mL}$, therefore 5 $\mu\text{g}/\text{mL}$ was selected as the low exposure test point in the gene expression study. For the high exposure test point, the cells showed increasingly reduced viability over 24 h when exposed to either 80 or 160 $\mu\text{g}/\text{mL}$ CdSe QDs and the gene expression data could not be normalized using the actin gene. Hence, it was necessary to utilize a much lower exposure duration of 6 h and a high exposure concentration of 80 $\mu\text{g}/\text{mL}$ in order to ensure suitable normalization of the gene expression data. The gene expression results should be considered an average of a mixed population of cells at various stages of response. Since the data are normalized using the actin gene, the cells contributing to the gene expression must have at least minimal machinery and ability to express the actin gene. However, many of the cells may have lost the ability (i.e., resulting from DNA



damage) to express other genes. As in the direct cytotoxicity measurements, the gene expression analysis is used here as an indication of the average response of a mixed population of cells after specific QD exposure conditions.

For comparison to our direct cytotoxicity measurements, high-throughput quantitative reverse transcript polymerase chain reaction (qRT-PCR) was used to measure changes in expression of a selected panel of genes known to be involved in pathways of DNA damage and

repair, mitochondrial function and proliferation. The gene expression changes were assessed with beta-actin as the normalizer reference gene using a 96.96 dynamic chip array as described previously [13]. NHBE cells were exposed to MUA-functionalized CdSe and InP QDs. To study the changes in gene expression, NHBE cells were exposed to a low (5 μg/mL), but persistent level of QDs for 24 h or a high (80 μg/mL) level of QDs for 6 h. At the low QD exposure level, genes related to DNA damage, DNA repair, mitochondrial function and proliferation (CDK1, Gadd45A, BRCA1, BRCA2, XPC, AHR, CYP1A1, CYP1B1, DHFR and VEGFA) were generally unchanged or down regulated relative to untreated controls for both QDs (Table 1). This is consistent with the LDH, ROS and mitochondrial cytotoxicity measurements in which significant cytotoxicity effects were only observed at QD concentrations above 5 μg/mL. When cells were exposed to the high (80 μg/mL) QD concentration, with the exception of DHFR, most of the genes associated with these pathways were significantly upregulated relative to untreated controls. Most of these genes were more upregulated with exposure to CdSe QDs than with InP QDs. The exception of genes XPC and UPCI may be due to the loss of cell viability, metabolic function and in turn loss of gene expression in these pathways with exposure to high CdSe QDs. The cells exposed to the less toxic InP QDs appear to be capable of cellular responses in these genes, whereas the cells treated with the more toxic CdSe may not be capable of the same type of response. Other genes, such as SFN, BRCA2, CYP1B1, and VEGFA appear to be in stable cellular pathways that are activated upon CdSe treatment. GADD45A has been directly correlated to G2/M arrest under stress (excess Zn) in NHBE cells [24]. However, GADD45A gene expression was equivalent in both the CdSe and the InP QD exposed cells at 80 μg/mL, indicating that this pathway is unaffected.

Discussion

Compared to the CdSe QDs, InP QDs had minimal direct cytotoxic effects on the NHBE cells as measured by each of the common cytotoxicity assays. The increase in LDH release and ROS production that was observed with CdSe QD exposure was not observed upon exposure to InP QD. The correlation of LDH activity with the intracellular generation of ROS supports previous studies where QD cytotoxicity was found to be proportional to oxidative stress [25–27]. In addition, the minimal cytotoxic effects of the InP QDs also correlated with the DNA damage measurements showing minimal fragmentation/strand breaks with exposure to InP QDs. The common cytotoxicity measurements were also consistent in detecting the toxic effect of the CdSe QDs at the same

Table 1 High-throughput qRT-PCR of NHBE cells treated with CdSe or InP QDs

Cell response pathway	Gene name	Fold change (5 µg/mL, 24 h)		Fold change (80 µg/mL, 6 h)	
		CdSe-MUA	InP-MUA	CdSe-MUA	InP-MUA
DNA damage	CDK1	<i>0.50 ± 0.15</i>	<i>0.21 ± 0.09</i>	1.92 ± 0.10	1.15 ± 0.01
	GADD45A	1.18 ± 0.02	1.00 ± 0.09	4.17 ± 0.95	4.95 ± 0.66
	SFN	1.50 ± 0.04	1.75 ± 0.04	2.84 ± 0.50	0.86 ± 0.44
DNA repair	BRCA1	<i>0.55 ± 0.07</i>	<i>0.18 ± 0.03</i>	1.20 ± 0.05	0.96 ± 0.22
	BRCA2	<i>0.57 ± 0.07</i>	<i>0.20 ± 0.02</i>	3.06 ± 0.48	<i>0.43 ± 0.17</i>
	XPC	0.89 ± 0.02	0.79 ± 0.05	0.84 ± 0.15	2.29 ± 0.15
Mitochondrial function and repair	AHR	0.99 ± 0.04	1.11 ± 0.05	2.18 ± 0.29	2.35 ± 0.43
	CYP1A1	1.02 ± 0.11	1.17 ± 0.17	1.38 ± 0.35	<i>0.17 ± 0.04</i>
	CYP1B1	1.29 ± 0.02	1.16 ± 0.07	3.19 ± 0.46	0.70 ± 0.09
	DHFR	<i>0.57 ± 0.02</i>	<i>0.40 ± 0.02</i>	1.10 ± 0.10	1.15 ± 0.07
	UCP1	2.29 ± 0.50	2.20 ± 1.64	4.32 ± 0.87	7.20 ± 0.51
Proliferation	VEGFA	1.43 ± 0.05	1.44 ± 0.17	2.18 ± 0.48	1.21 ± 0.16

Cells were incubated with 5 µg/mL CdSe or InP QDs for 24 h or with 80 µg/mL CdSe or InP QDs for 6 h. Gene expression changes for different classes of cellular functions and processes were measured by qRT-PCR. Values are expressed as fold change relative to media controls (= 1.0). Italic values indicates a twofold or greater decrease in gene expression relative to untreated cells (≤ 0.5) while bold italics highlights twofold or greater increases in gene expression relative to untreated cells (≥ 2.0). All experiments were independently repeated three times ($n = 3$). Errors represent one standard deviation

range of concentration, and in a range consistent with earlier studies [10, 11].

Using CdSe QDs and InP QDs, we compared common cytotoxicity and gene expression measurements. The direct cytotoxic assays demonstrate that CdSe QDs induce severe DNA damage to NHBE cells when compared to InP QDs. The qRT-PCR gene expression data also revealed significant differences in certain DNA damage, DNA repair and mitochondrial function gene responses to CdSe and InP QDs, especially at higher concentration. The enhanced upregulation of markers BCR2, SFN, CYP1A1, CY1B1, CDK1 and VEGFA (Table 1) caused by CdSe QDs, indicate a consistent higher sensitivity and reaction to these QDs than for InP QDs. Based on our comparison to direct cytotoxicity measurements these genes, which are up-regulated specifically in response to increased CdSe QD exposure (i.e., BRCA2, SFN, CYP1A1, CYP1B1, CDK1 and VEGFA) may be possible biomarkers for cytotoxic damage from these types of ENMs.

Our study is a comparison of methods commonly used to determine NP toxicity instead of the determination of the individual cellular mechanisms of this toxicity. Although the gene expression data presented in this report yields useful information on the cellular responses to CdSe and InP QD exposure, the mechanisms of these responses remains unclear. For example, despite its lower toxicity, InP QDs induced a transcriptional response in NHBE cells for markers GADD45A and AHR equivalent to CdSe. Markers XPC and UCP1 were even more

elevated in the case of InP QDs. One hypothesis is that the highly cytotoxic nature of CdSe QDs produces specific cellular damage that results in a reduced transcriptional response for certain markers such as XPC and UCP1. Cells exposed to the less toxic InP QDs, on the other hand, may be better able to respond in upregulating these markers. More extensive viability studies could be helpful to determine this. In addition, uptake studies would be helpful to determine the extent of internalization of the CdSe and InP NPs. Studies by Chau et al., using NHBE cells indicated it is the particle charge effects that affect the rate and route of transport [28]. However, the InP and CdSe nanoparticles used in the present study have the same MUA coating, which would be expected to have comparable properties of agglomeration and uptake. More extensive cellular response assays, such as time-dependent gene transcriptional profiles with additional markers, and alternative cell lines, could be performed on additional NPs at multiple concentrations to gain more insight into the mechanism of toxicity of ENMs.

Conclusions

This study can serve as a model for the comparison of toxicology methods. In combination with traditional cytotoxicity assays, gene expression profiles can be used to determine candidate biomarkers which would be helpful in assessing the biocompatibility of ENMs. However, the use of gene expression measurements can yield results for certain genes which apparently are inconsistent with common cytotoxicity assays. Quantifying the

cytotoxic interactions with cellular systems will require a thorough understanding of the biological responses produced by the ENMs.

Methods

QD preparation and characterization

QD synthesis

Cadmium oxide (CdO, 99.95%), and oleic acid (90%) were purchased from Alfa Aesar (Ward Hill, MA, USA), 1-octadecene (ODE, 90%), tetramethylammonium hydroxide (TMAH) and mercaptoundecanoic acid (MUA), from Acros Organics (Geel, Belgium), oleylamine (tech grade), selenium pellet ($\geq 99.999\%$), myristic acid ($\geq 98\%$), indium (III) acetate (99.99%), and dioctylamine (98%) from Aldrich (St. Louis, MO, USA), trioctylphosphine (TOP, 97%) trioctylphosphine oxide (TOPO, 90%), and tris(trimethylsilyl)phosphine ((TMS)₃P; 98%) from Strem (Newburyport, MA, USA). All chemicals were used without any further purification.

CdSe (5 nm) QDs were synthesized as previously described [10]. Briefly, cadmium oleate was prepared by heating 1.45 g CdO in 20 mL oleic acid at 170 °C until colorless and cooled to 100 °C prior to degassing under a vacuum. TOP-Se was prepared in 50 mL TOP. 3.95 g Se pellets were dissolved in an inert atmosphere glovebox to make a TOP-Se solution. In an air-free environment, 1 g TOPO, 8 mL ODE, and 0.75 mL cadmium oleate were combined. The reaction mixture was thoroughly degassed at room temperature, and again at 80 °C. The temperature was increased to 300 °C under an atmosphere of ultra high purity argon. A solution of 4 mL of TOP-Se, 3 mL oleylamine, and 1 mL of ODE were combined and quickly injected into the cadmium oleate solution. The temperature was subsequently lowered to 270 °C for 1 min to control CdSe QD growth [29]. The solution was cooled, yielding CdSe QDs with a diameter of 5 nm. InP QDs were synthesized using a modification of an existing protocol [20]. A 0.08 mol/L solution of indium myristate (1:4.1 In:MA) was prepared by heating 2 mmol indium (III) acetate (584 mg), 8.2 mmol myristic acid (1.87 g), and 25 mL of ODE to 120 °C under vacuum. After 20 min, the solution was backfilled with argon and heated for another 2 h at 120 °C. In a 100 mL round-bottom flask, 5 mL of indium myristate was heated to 188 °C. A syringe containing 0.2 mmol (60 μ L) of

(TMS)₃P and 1 mL di-*n*-octylamine was rapidly injected and the temperature stabilized at 178 °C. After one min, a second syringe containing 0.2 mmol (60 μ L) (TMS)₃P and 1 mL ODE were added dropwise at a rate of 1 mL/min. The reaction mixture was held at 178 °C for 15 min after the initial injection, when the heat was removed and the reaction was quenched with \sim 5 mL of degassed, room temperature ODE.

QDs were purified to remove excess ligands from the chemical synthesis as described [10]. QD concentrations were calculated according to Yu et al. [30] and Xie et al. [20] for CdSe and InP, respectively, on the basis of UV–VIS absorbance spectra. MUA was added to the toluene solution in amounts equivalent to 2 times the number of moles of QDs and incubated for 2 h. To facilitate the transfer of QDs from organic phase to water phase, a solution of TMAH in water (4 times the number of moles of QDs) was added dropwise. The water phase was precipitated with isopropanol, followed by centrifugation (5 min at 5000 rpm). The resulting pellet was redispersed in distilled water. Once the QDs transferred, the pH of the solution was brought back to \sim 6. Aggregates were carefully removed by centrifugation.

QD characterization

Absorption of aqueous suspensions of QDs were measured by UV–Vis spectroscopy and dynamic light scattering (DLS) using a Malvern Zetasizer [11]. Typical results in pure water are shown in Table 2 below.

DLS measurements (mean and standard deviation) indicated minimal aggregation in pure water. Zeta potentials indicated high stability. However, extensive aggregation of MUA capped CdSe (622 \pm 391 nm) was observed after 20 min in BEGM [11].

Biological experiments

Cell culture and QD exposure

Normal human primary bronchial epithelial cells (NHBEs) were purchased from Lonza (Walkersville, MD, USA) and propagated in bronchial epithelial cell growth media (BEGM, Clonetics Bullet Kit Lonza, Walkersville, MD, USA) on 100 mm petri dishes coated with Type I 50 μ g/mL rat tail collagen (BD Biosciences, Bedford, MA, USA) diluted in Dulbecco's phosphate buffered saline (DPBS). Cells were passaged weekly and fed

Table 2 Characterization of InP MUA and CdSe MUA QDs

Label name	Total mass (g)	Total moles (mol)	Absorption max. (nm)	Size from UV–Vis (nm)	Size from DLS (nm)	Zeta potential (mv)
5 nm InP MUA	0.00197	6.778×10^{-6}	613	4.61	3.99 ± 0.34	-45.8 ± 8.7
5 nm CdSe MUA	0.0094	4.899×10^{-5}	617	5.43	8.84 ± 2.45	-54.9 ± 11.6

by replacing spent media with fresh media every (2–3) days. For necrosis, apoptosis, reactive oxygen species (ROS) production and mitochondrial function assays, cells from passages 3 to 7 were seeded at 2.5×10^4 cells per well in 96-well flat bottom tissue culture plates and acclimated overnight. For comet assays and RNA isolation, cells were seeded at 1.5×10^5 cells per well in 6-well tissue culture dishes. Cells were allowed to acclimate prior to QD exposures. QD suspensions ranging from 0.5 to 160 $\mu\text{g}/\text{mL}$ and appropriate controls were prepared in DPBS or BEGM and immediately added to aspirated wells (150 $\mu\text{L}/\text{well}$ for 96 well plates and 2 mL/well for 6-well plates). While the data is reported as $\mu\text{g}/\text{mL}$ of QDs added to the cells, these concentrations equate to 0.3 to 97.0 $\mu\text{g}/\text{cm}^2$ (96-well plates) and 0.1 to 33.3 $\mu\text{g}/\text{cm}^2$ (6-well plates). Cells were incubated for 6 or 24 h in a humidified atmosphere at 37 °C and 5% CO_2 during QD exposures.

Oxidative stress (ROS levels)

Intracellular ROS formation in NHBE cells exposed to QDs was quantified using 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA, Molecular Probes, Eugene, OR, USA). NHBE cells exposed to Dulbecco's phosphate buffered saline (DPBS) only served as negative and 100 $\mu\text{mol}/\text{L}$ H₂O₂ served as positive controls. QD controls at the highest concentrations were included in wells without cells to determine if QDs induce spontaneous fluorescence of CM-H₂DCFDA. Fluorescence was measured using an excitation wavelength of 490 nm and an emission wavelength of 535 nm every 10 min post exposure for 120 min. Readings beyond 120 min resulted in errant readings due to cell starvation. Experiments were performed in triplicate on three independent occasions. Representative data from the 60 min reading are presented.

Cell viability assays

Cell membrane integrity was measured by assaying lactate dehydrogenase (LDH) activity in cellular supernatants. LDH kits were purchased from Roche (Indianapolis, IN, USA) [31, 32]. The 96-well plates were centrifuged at $200 \times g_n$ for 5 min to pellet uninternalized QDs. Supernatants (75 μL) were transferred to a clean plate, and LDH activity was assessed per the manufacturer's instructions. Cells exposed to 0.5% Triton-100 were utilized as the positive control. Experiments were performed in triplicate on three independent occasions. QDs incubated with LDH reaction mix in a cell free environment were used to determine if QDs caused assay interference. Reactions were read colorimetrically on a BioTek plate reader at an absorbance–wavelength of 490 nm and a reference wavelength of 600 nm after 15 min.

Mitochondrial activity, as measured using water-soluble tetrazolium dye (WST-1, Roche, Indianapolis, IN, USA), was assessed after incubation with QDs as described previously [10]. Cells exposed to 0.5% Triton-100 were utilized as the positive control. Experiments were performed in triplicate on three independent occasions. WST-1 reagent was added to each well (7.5 μL) of 96-well plates; plates were briefly vortexed and then incubated at 37 °C and 5% CO_2 for 2–3 h prior to reading at an absorbance–wavelength of 420 nm and a reference wavelength of 600 nm. QD suspensions were also incubated with the WST-1 reagent alone to determine potential assay interference.

DNA damage

NHBE cells were exposed to QDs for 24 h, washed three times with DPBS, harvested by trypsinization, counted and resuspended at 2.5×10^5 cells/mL in freezing media consisting of 70% BEGM, 20% fetal bovine serum and 10% dimethyl-sulfoxide (DMSO) prior to storage in liquid nitrogen until comet assay analyses. Cells treated with media only or exposed to 250 $\mu\text{mol}/\text{L}$ H₂O₂ for 1 h served as controls for DNA strand breaks. DNA strand breaks were measured by alkaline comet assay, otherwise known as single cell gel electrophoresis (SCGE) as described previously [11]. The percentage of DNA in the tail was calculated for each cell and averaged ($n = 30$ cells) for each treatment group. Percent DNA damage was determined as a function of treatment concentration and graphed as percent DNA in tail.

RNA isolation, high-throughput quantitative real-time polymerase chain reaction

NHBE cells were exposed to 5 or 80 $\mu\text{g}/\text{mL}$ MUA InP or CdSe-QDs for 24 or 6 h, respectively. The number of viable cells was too low after treatment beyond 6 h at high QD concentrations. Cells were washed 3 times with DPBS to remove residual QDs prior to lysis. RNA was harvested and purified using Qiagen RNeasy mini-prep kits (Valencia, CA, USA) per manufacturer's recommendations. For RNA samples used for transcriptomics, two DNA digestions were performed using Qiagen's RNase free DNase set (Valencia, CA, USA). Gene expression changes for 96 targets were assessed using the BioMark real-time PCR high throughput chip system and 96.96 dynamic arrays (Fluidigm, CA, USA) as described previously [11]. The 96 TaqMan assays tested in this report include regulatory genes for pathways including mitochondrial function, inflammation, DNA damage and repair, autophagy and matrix formation. Real-time PCR was performed on the BioMark instrument using BioMark HD Data Collection Software v3.0.2. Data analyses were performed using Fluidigm Real Time PCR Analysis

Software. Sample delta Ct values were calculated by using media only values as the negative control. Delta Ct values were calculated for the TaqMan assays using beta-actin as the normalizer reference gene.

RNA sequence experiments revealed many genes with altered expression. To select significantly regulated genes with confidence, we defined a gene as significantly regulated if it had an adjusted p value (p-adj) less than 0.05 ($n = 3$). This adjusted p value helps to lower the false positives, and is considered a more stringent test compared to the traditional p value [11]. Using p-adj with a threshold value of 0.05, we got a list of 118 genes of significant regulation. While not discounting the relevance of genes that do not show notable changes relative to media controls, we felt that focusing on the genes that were altered at least twofold would be more relevant. These 31 genes were found to be altered at least twofold at the high or low NP concentrations (see Additional file 1: Table S1). We then selected genes that we felt were the most relevant to compare with our cytotoxicity measurements. DNA damage and repair genes were selected for comparison to the comet assay. Mitochondrial function and metabolism genes were selected for comparison to our metabolic activity measurements and the proliferation gene was selected to compare with the extracellular LDH as an indicator of cell membrane viability.

Statistical analyses

Biological data are presented as fold change above or below the media control and graphically represented as mean fold change. Statistical significance was calculated by One-way Analysis of Variance (ANOVA) using multiple comparisons versus control group (Bonferroni t-test). Analyses were performed using SigmaPlot version 11.0 (Systat Software, Inc., San Jose, CA, USA) using a minimum of three independent experiments for cell viability assays (LDH, mitochondrial function and apoptosis). Numerical transformations of the data were performed as necessary to satisfy equivalence of variance and normality parameters before statistical analyses were conducted. For the comet assays, statistical differences among treatment groups were evaluated by Student's t test. $p < 0.001$ are indicated.

Additional file

Additional file 1: Table S1. Quantitative RT-PCR of NHBE cells treated with CdSe or InP QDs cells.

Abbreviations

QDs: quantum dots; ROS: reactive oxygen species; NHBE: normal human bronchial epithelial cells; DNA: deoxyribonucleic acid; MPA: mercaptopropionic acid; MUA: mercaptoundecanoic acid; CYST: cysteamine; 53BP1: p53 binding

protein 1; BEGM: bronchial epithelial cell growth media; LDH: lactate dehydrogenase; PBS: phosphate buffered saline; CAM: camptothecin; CM-H₂DCFDA: 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; WST-1: water-soluble tetrazolium.

Authors' contributions

Wrote paper, conceived and designed biological experiments: DHA, AN. Synthesized and characterized nanomaterials: AS, AMD. Critically reviewed manuscript and designed synthetic methods: JAH. Conducted comet analysis: VD. Planned and critically reviewed project: RI, BCN. All authors read and approved the final manuscript.

Author details

¹ Biosystems and Biomaterials Division, National Institute of Standards and Technology, Bld. 227, Rm. A247, MS 8313, 100 Bureau Drive, Gaithersburg, MD 20899, USA. ² Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM, USA. ³ Center for Integrated Nanotechnologies, Materials Physics & Applications Division, Los Alamos National Laboratory, Los Alamos, NM, USA. ⁴ Defense Systems and Analysis Division, Los Alamos National Laboratory, Los Alamos, NM, USA. ⁵ Present Address: Navy Medical Research Unit—San Antonio, 3650 Chambers Pass, Bldg. 3610, Fort Sam Houston, TX 78234-6315, USA. ⁶ Present Address: Department of Biomedical Engineering and Division of Materials Science and Engineering, Boston University, Boston, MA, USA.

Acknowledgements

We thank Dr. Norman Doggett, Priya Dighe, and Melinda Wren of Los Alamos National Laboratory for the use of the BioMark high through-put real time PCR system.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Consent for publication

Not applicable.

Disclaimer

Certain commercial equipment, instruments and materials are identified in this paper to specify an experimental procedure as completely as possible. In no case does the identification of particular equipment or materials imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the materials, instruments, or equipment are necessarily the best available for the purpose.

Ethics approval and consent to participate

The manuscript was approved by the NIST internal review board. There are no human subjects or animals involved in the study.

Funding

This work was supported by the National Institutes of Standards and Technology and the Los Alamos National Laboratory LDRD-DR program. This work was performed, in part, at the Center for Integrated Nanotechnologies, a US Department of Energy, Office of Basic Energy Sciences facility in support User Projects RA2012B0008 and U2010A979. JAH also acknowledges partial support by NIH-NIGMS Grant 1R01GM084702-01. Los Alamos National Laboratory is operated by Los Alamos National Security, LLC, for the National Nuclear Security Administration of the US Department of Energy under Contract DE-AC52-06NA25396.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 10 August 2017 Accepted: 24 October 2017

Published online: 09 November 2017

References

- Borm PJ, Robbins D, Haubold S, Kuhlbusch T, Fissan H, Donaldson K, Schins R, Stone V, Kreyling W, Lademann J, Krutmann J, Warheit D, Oberdorster E. The potential risks of nanomaterials: a review carried out for ECETOC. Part Fibre Toxicol. 2006;3:1–35.
- Pal BN, Ghosh Y, Brovelli S, Laocharoensuk R, Klimov VI, Hollingsworth JA, Htoon H. Giant CdSe/CdS core/shell nanocrystal quantum dots as efficient electroluminescent materials: strong influence of shell thickness on light-emitting diode performance. *Nano Lett*. 2012;12:331–6.
- Milliron DJ, Hughes SM, Cui Y, Manna L, Li J, Wang L-W, Paul Alivisatos A. Colloidal nanocrystal heterostructures with linear and branched topology. *Nature*. 2004;430:190–5.
- Mocatta D, Cohen G, Schattner J, Millo O, Rabani E, Banin U. Heavily doped semiconductor nanocrystal quantum dots. *Science*. 2011;332:77–81.
- Hu M, Yan J, He Y, Lu H, Weng L, Song S, Fan C, Wang L. Ultrasensitive, multiplexed detection of cancer biomarkers directly in serum by using a quantum dot-based microfluidic protein chip. *ACS Nano*. 2010;4:488–94.
- Diagaradjane P, Orenstein-Cardona JM, Colón-Casasnovas N, Deorukhkar A, Shentu S, Kuno N, Schwartz DL, Gelovani JG, Krishnan S. Imaging epidermal growth factor receptor expression in vivo: pharmacokinetic and biodistribution characterization of a bioconjugated quantum dot nanoprobe. *Clin Cancer Res*. 2008;14:731–41.
- Walker K-AD, Morgan C, Doak SH, Dunstan PR. Quantum dots for multiplexed detection and characterisation of prostate cancer cells using a scanning near-field optical microscope. *PLoS ONE*. 2012;7:e31592.
- Bagalkot V, Zhang L, Levy-Nissenbaum E, Jon S, Kantoff PW, Langer R, Farokhzad OC. Quantum dot-aptamer conjugates for synchronous cancer imaging, therapy, and sensing of drug delivery based on bi-fluorescence resonance energy transfer. *Nano Letters*. 2007;7:3065–70.
- Muthu MS, Kulkarni SA, Raju A, Feng S-S. Theranostic liposomes of TPGS coating for targeted co-delivery of docetaxel and quantum dots. *Biomaterials*. 2012;33:3494–501.
- Nagy A, Steinbrück A, Gao J, Doggett N, Hollingsworth JA, Iyer R. Comprehensive analysis of the effects of CdSe Quantum dot size, surface charge, and functionalization on primary human lung cells. *ACS Nano*. 2012;6:4748–62.
- Nagy A, Hollingsworth JA, Hu B, Steinbrück A, Stark PC, Valdez CR, Vuyisich M, Stewart MH, Atha DH, Nelson BC, Iyer R. Functionalization-dependent cellular survival pathways by CdSe quantum dots in primary normal human bronchial epithelial cells. *ACS Nano*. 2013;7:8397–411.
- Peng L, He M, Chen B, Wu Q, Zhang Z, Pang D, Zhu Y, Hu B. Cellular uptake, elimination and toxicity of CdSe/ZnS quantum dots in HepG2 cells. *Biomaterials*. 2013;34:9545–58.
- Stern ST, Zolnik BS, McLeland CB, Clogston J, Zheng J, McNeil SE. Induction of autophagy in porcine kidney cells by quantum dots: a common cellular response to nanomaterials? *Toxicol Sci*. 2008;106:140–52.
- Soenen SJ, Montenegro J-M, Abdelmonem A, Manshian BB, Doak SH, Parak WJ, DeSmedt SC, Braeckmans K. The effect of nanoparticle degradation on poly(methacrylic acid)-coated quantum dot toxicity assessment in toxicology: the importance of particle functionality assessment in toxicology. *Acta Biomater*. 2014;10:732–41.
- Bradburne CE, Delehanty JB, Gemmill KB, Mei BC, Mattoussi H, Susumu K, Blanco-Canosa JB, Dawson PE, Medintz IL. Cytotoxicity of quantum dots used for in vitro cellular labeling: role of QD surface ligand, delivery modality, cell type, and direct comparison to organic fluorophores. *Bioconjug Chem*. 2013;24:1570–83.
- Pathakoti K, Hwang H-M, Xu H, Aguilar ZP, Wang A. In vitro cytotoxicity of CdSe/ZnS quantum dots with different surface coatings to human keratinocytes HaCaT cells. *J Environ Sci (China)*. 2013;25:163–71.
- Brunetti V, Chibli H, Fiammengo N, Galeone A, Malvindi MA, Vecchio G, Cingolani R, Nadeau JL, Pompa PP. InP/ZnS as a safer alternative to CdSe/ZnS core/shell quantum dots: in vitro and in vivo toxicity assessment. *Nanoscale*. 2013;5:307.
- Haubold S, Haase M, Kornowski A, Weller H. Strongly luminescent InP/ZnS core-shell nanoparticles. *ChemPhysChem*. 2001;2:331–4.
- Xu S, Kumar S, Nann T. Rapid synthesis of high quality InP nanocrystals. *J Am Chem Soc*. 2006;128:1054–5.
- Xie R, Battaglia D, Peng X. Colloidal InP nanocrystals as efficient emitters covering blue to near infrared. *J Am Chem Soc*. 2007;129:15432–3.
- Li L, Reiss P. One-pot synthesis of highly luminescent InP/ZnS nanocrystals without precursor injection. *J Am Chem Soc*. 2008;130:11588–9.
- Thomas A, Nair PV, Thomas KG. InP quantum dots: an environmentally friendly material with resonance energy transfer requisites. *J Phys Chem*. 2014;118:3838.
- Laaksonen T, Ahonen P, Johans C, Kontturi K. Stability and electrostatics of mercaptoundecanoic acid-capped gold nanoparticles with varying counterion size. *ChemPhysChem*. 2006;7:2143–9.
- Shih RS, Wong SH, Schoene NW, Lei KY. Suppression of Gadd45 alleviates the G2/M blockage and the enhanced phosphorylation of p53 and p38 in zinc supplemented normal human bronchial epithelial cells. *Exp Biol Med (Maywood)*. 2008;233:317–27.
- Li KG, Chen JT, Bai SS, Wen X, Song SY, Yu Q, Li J, Wang YQ. Intracellular oxidative stress and cadmium ions release induce cytotoxicity of unmodified cadmium sulfide quantum dots. *Toxicol In Vitro*. 2009;23:1007–13.
- Lovrić J, Cho SJ, Winnik FM, Maysinger D. Unmodified cadmium telluride quantum dots induce reactive oxygen species formation leading to multiple organelle damage and cell death. *Chem Biol*. 2005;12:1227.
- Yan M, Zhang Y, Xu K, Fu T, Qin H, Zheng X. An in vitro study of vascular endothelial toxicity of CdTe quantum dots. *Toxicology*. 2011;282:94–103.
- Chau E, Galloway JF, Nelson A, Breyse PN, Wirtz D, Searson PC, Sidhaye VK. Effect of modifying quantum dot surface charge on airway epithelial cell uptake in vitro. *Nanotoxicology*. 2012;7:1143–51.
- Mahler B, Lequeus N, Dubertret B. Ligand-controlled polytypism of thick-shell CdSe/CdS nanocrystals. *J Am Chem Soc*. 2010;132:953–9.
- Yu W, Qu L, Guo W, Peng X. Experimental determination of the extinction coefficient of CdTe, CdSe and CdS nanocrystals. *Chem Mater*. 2003;15:2854–60.
- Martin R, Wang HL, Gao J, Iyer S, Montano GA, Martinez J, Shreve AP, Bao Y, Wang C-C, Chang Z, Gao Y, Iyer R. Impact of physicochemical properties of engineered fullerenes on key biological responses. *Toxicol Appl Pharmacol*. 2009;234:58–67.
- Gao J, Wang HL, Shreve A, Iyer R. Fullerene derivatives induce premature senescence: a new toxicity paradigm or novel biomedical applications. *Toxicol Appl Pharmacol*. 2010;244:130–43.

Submit your next manuscript to BioMed Central and we will help you at every step:

- We accept pre-submission inquiries
- Our selector tool helps you to find the most relevant journal
- We provide round the clock customer support
- Convenient online submission
- Thorough peer review
- Inclusion in PubMed and all major indexing services
- Maximum visibility for your research

Submit your manuscript at
www.biomedcentral.com/submit

