Cite this: *Nanoscale*, 2012, **4**, 6401

www.rsc.org/nanoscale

PAPER

In vivo assessment of CdSe–ZnS quantum dots: coating dependent bioaccumulation and genotoxicity†

A. Galeone,^{‡a} G. Vecchio,^{‡a} M. A. Malvindi,^a V. Brunetti,^a R. Cingolani^b and P. P. Pompa^{*a}

Received 12th July 2012, Accepted 4th August 2012

DOI: 10.1039/c2nr31826a

Semiconductor nanocrystals, or Quantum Dots (QDs), have gained considerable attention due to their unique size-dependent optical and electronic properties that make them attractive for a wide range of applications, including biology and nanomedicine. Their widespread use, however, poses urgent questions about their potential toxicity, especially because of their heavy metal composition that could cause harmful effects to human health and environment. In this work, we evaluated *in vivo* the long-term toxicity of CdSe–ZnS QDs with different surface coatings, probing oral administration in the model system *Drosophila melanogaster*. In particular, we found that all the differently coated QDs significantly affect the lifespan of treated *Drosophila* populations and induce a marked increase in reactive oxygen species (ROS) levels. Furthermore, we observed that these QDs induce severe genotoxic effects and increased rate of apoptosis in *Drosophila* haemocytes. These toxic effects were found to be mainly related to the *in vivo* degradation of QDs with consequent release of Cd²⁺ ions, while the coating of QDs can modulate their bioaccumulation in the organism, partly decreasing their overall toxicity.

Introduction

Quantum dots (QDs) are an important class of nanomaterials, holding great promise in many applications, from consumer products to several commercial systems of clinical significance.¹ Depending on their composition and size, they display unique optical and electronic properties of increasing potential,^{2–6} especially in biomedicine and biotechnology. In particular, QDs have interesting applications in optical imaging both *in vitro* and *in vivo*,^{7–10} and as carriers for drug and gene delivery, diagnostic imaging and cancer therapy.^{11–13} Due to such a wide range of use, human and environmental exposures are unavoidable, hence critical investigations to ascertain their toxicity are urgently required. The major concern for QDs is due to their possible toxicity caused by the presence of heavy metals in their core (such as cadmium, mercury, arsenic) with potential release of highly toxic ions in living biological systems.^{14–18} In this regard, several studies have recently demonstrated the toxicity of QDs *in vitro*,^{19,20} although many of these works have been performed in immortalized cells, with only a few studies in animal models to check accumulation and biocompatibility. Additionally, these

data are rather dissimilar because of the variety of QDs used in terms of size, dose, surface modification and administration route,^{21–27} so the extrapolation of definitive conclusions is challenging. QDs toxicity is mainly ascribed to metal ions release in biological systems, with consequent generation of reactive oxygen species.^{2,20,22,28} Based on this hypothesis, numerous strategies have been applied to minimize QDs toxicity. Among these, the most common in the literature is the modification of their surface through coating with different molecules (*e.g.*, BSA, PEG, GSH), which seem to improve the stability of the metal core, which is less susceptible to oxidative degradation.^{28–31} However, such strategies have been mainly validated by short-term treatments. In this work, we evaluated *in vivo* the long-term toxicity of CdSe–ZnS QDs with different surface coatings using *Drosophila melanogaster* as a model organism. Our results show that all the QDs strongly affect the life cycle of the organisms, causing generalized toxicity and DNA damage, primarily because of QDs degradation *in vivo* and release of cadmium ions. Furthermore, our data indicate that the QDs surface coatings can partially decrease their toxicity by reducing the bioaccumulation in treated animals.

Results and discussion

In this study, we have tested the *in vivo* toxicity of CdSe–ZnS QDs with different surface coatings (Fig. 1): mercaptoundecanoic acid (QD-MUA), polymer coating with poly-maleic anhydride octadecene (QD-PC) and polymer and polyethylene glycol (PEG) coating (QD-PC-PEG). These three differently coated QDs were characterized by TEM, DLS, Z-potential,

^aIstituto Italiano di Tecnologia (IIT), Center for Bio-Molecular Nanotechnologies@UniLe, Via Barsanti – 73010, Arnesano, Lecce, Italy. E-mail: pierpaolo.pompa@iit.it; Fax: +39-0832-295708; Tel: +39-0832-295714

^bIstituto Italiano di Tecnologia (IIT), Central Research Laboratories, Via Morego, 30 – 16136, Genova, Italy

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c2nr31826a

‡ Equally contributing authors.

UV-vis and fluorescence spectroscopy, gel-electrophoresis, and ICP-AES (ESI, Fig. S1†). We obtained stable and mono-disperse nanoparticles with a hydrodynamic radius of 15 ± 1.5 , 23 ± 1.6 , and 25 ± 1.5 nm for QD-MUA, QD-PC, and QD-PC-PEG, respectively. They displayed a similar surface charge (ca. $-25/-30$ mV).

To assess the possible toxic effects of these QDs, we monitored populations of *Drosophila melanogaster* subjected to long-term chronic treatment for their entire lifespan (dose: $0.02 \mu\text{g g}^{-1}$ per day). A further aim of our work was to test different surface coatings of the QDs in order to understand how the surface may influence the biological response and overall toxicity. *Drosophila melanogaster* has a high homology with human DNA³² and represents a powerful and versatile tool to study human genetic disorders.^{33,34} In addition, having a short lifespan, well characterized development and anatomy, and simple transgenic analysis, *Drosophila* is a well-established model for mutagenesis and toxicity studies,^{35–37} especially in long-term exposure.^{38,39} Furthermore, very recently, it has been proved to be an excellent tool for nanotoxicology investigations, becoming a valuable prescreening model for nanomaterial risk assessment.^{40–42}

As a first step, we monitored the lifespan of *Drosophila* populations nurtured with food containing the three differently coated QDs ($0.02 \mu\text{g g}^{-1}$ Cd per day), compared with flies fed with normal food. As a further control, we also tested the effect of food containing only the solvent in which the QDs were resuspended (SN) in order to exclude any possible toxic effect of the synthesis mixture (such as precursors, surfactants, or contaminants). Fig. 2A shows that the survival curves of the three populations treated with the different QDs are significantly perturbed as compared to the controls (CTRL and SN), which present the typical trend of the *Drosophila* survival curve.⁴³ In particular, the QD-MUA treated flies show a strong lifespan decrease, with a half-life (τ_{50}) of ca. 58% with respect to the control. The decrease of the survival curves is coating-dependent and in the case of QD-PC and QD-PC-PEG is more attenuated (ca. 73% and 82% of the control value, respectively). These results indicate that all the tested QDs have a significant impact on the organism lifespan, although the different surface coatings may partly reduce their toxicity.

To investigate the possible mechanisms, we monitored the formation of reactive oxygen species (ROS) in the QDs treated populations. We observed a significant increase in the ROS levels (Fig. 2B), following the treatment with the CdSe-ZnS QDs,

Fig. 1 Schematic representation of QDs with the same core-shell (CdSe-ZnS) structure but different surface coatings: (MUA) mercaptooundecanoic acid; (PC) poly(maleic anhydride octadecene); (PC) + polyethylene glycol (PEG).

Fig. 2 (A) Lifespan curves of *Drosophila* flies nurtured with QDs treated food (QD-MUA, QD-PC, QD-PC-PEG) compared to two populations bred with normal food (CTRL) or supernatant treated food (SN); the lifespan curves were validated by the non-parametric log-rank (Mantel-Cox) test. (B) ROS measurements of QD treatments by DCF assay. Data are reported as mean $\pm$ SD from three independent experiments and as relative fluorescence intensity normalized to the control. *** $P \leq 0.01$ (one-way ANOVA statistical analysis).

confirming a correlation of toxicity with oxidative stress, in line with previous *in vitro* and *in vivo* reports.^{22,26} Also in this case we found a coating-dependent ROS generation, with a ROS level of ca. 210% for QD-MUA treated organisms and lower values for the other treatments (ca. 150% for QD-PC and 130% in QD-PC-PEG). Taken together, these results suggest that the lifespan reduction of QD-treated flies may be related to the overproduction of intracellular ROS which, eliciting the accumulation of oxidative damage, may be a major cause for general deterioration of cell and tissue functions.⁴⁴ The high level of ROS is likely ascribed to the intracellular release of Cd^{2+} ions (see below).

We also investigated the potential genotoxicity of CdSe-ZnS QDs. To this purpose, we monitored *Drosophila* haemocytes of third instar larvae, fed for 7 days with food containing the QDs. Haemocytes represent a well established cellular model for *in vivo* evaluation of DNA damage^{45,46} and response of the innate immune system.⁴⁷ These cells have similar function in mammalian cells⁴⁸ and are directly exposed to toxic substances absorbed by gastro-intestinal tissue. For these reasons, in recent years, *Drosophila* haemocytes have become a powerful tool to assess the genotoxicity of chemical compounds, as well as of engineered nanoparticles.^{37,49,50} We carried out two types of experiments,

Fig. 3 Genotoxicity effects of QDs on *Drosophila*. (A) TUNEL assay and (B) apoptosis/necrosis assay performed on circulating haemocytes. Data are reported as mean $\pm$ SD from three independent experiments. *** $P \leq 0.01$; ** $P \geq 0.05$ (one-way ANOVA statistical analysis).

namely TUNEL assay (to highlight DNA damage) and Annexin V/PI assay (to analyze the presence of apoptotic and/or necrotic cells). A quantitative analysis of TUNEL-positive cells in treated haemocytes is shown in Fig. 3A, revealing that all QDs elicit a high level of DNA damage (*ca.* 23, 14, 9% for QD-MUA, QD-PC, QD-PC-PEG, respectively) as compared to the physiological level of damage in the control sample (*ca.* 2%).⁵¹ Some representative images of TUNEL-positive QDs treated haemocytes are reported in Fig. S2.† Also in this experiment a consistent coating-dependence was observed. In the apoptosis/necrosis assay (Fig. 3B), the haemocytes of QD-MUA treated larvae revealed a considerable level of apoptotic cells (~20%), while QD-PC and QD-PC-PEG treatment showed lower values (12% and 6%, respectively). On the other hand, in the case of necrotic cells, only a weak increase was observed when compared to the control. Some representative images of QDs treated haemocytes are shown in Fig. S3.† Overall, these results highlight the ability of CdSe-ZnS QDs to induce apoptosis and DNA damage in *Drosophila* haemocytes. The genotoxicity trend is related to the surface coating of the nanoparticles.

The induced toxic effects of QDs were also investigated in *Drosophila* haemolymph in terms of cell morphology. A marked difference in cell structure and distribution was found in QD-MUA treated organisms (Fig. 4B) as compared to the control (Fig. 4A). In particular, in the untreated organisms, the major part of haemolymph cell population is represented by

Fig. 4 Representative images of *Drosophila* haemocytes. Bright field microscopy image of (A) control sample; (B) QD-MUA treated larvae showing an increase of granulocytes and abnormal cells. (C) Overlay of bright field and fluorescence images showing endocytosed QD-MUA (red). (The selected image is particularly rich of QDs in haemocytes and is not representative of the average situation.) (D) Higher magnification image showing the subcellular localization of QD-MUA in plasmocytes (scale bar: 25 μ m).

prohaemocytes. These are precursors of immune cells and have a small structure (about 1 μ m) with a compact nucleus and a very thin cytoplasmic layer. On the other hand, in the treated flies we observed a large number of plasmocytes and different forms of granulocytes; all these cells are larger phagocytes (about 10 μ m) with a huge nucleus (*ca.* 50% of the total cell volume) and several cytoplasmic granules. Such cell morphology is typically found in the case of bacterial infection or when it is necessary to engulf apoptotic circulating cells and debris resulting from damaged cells in other districts of the organism.^{52,53} These experimental data highlight a significant immune-stress condition in the organism and an increased phagocytic activity as a consequence of QDs-induced apoptosis. Additionally, in treated organisms we observed both a general increase of haemocytes and the presence of abnormal cells. These events indicate a significant stimulation of the *Drosophila* immune system that may induce the degeneration of haemolymphopoietic organs.⁵⁴ Interestingly, we observed that QDs are also bioaccumulated in *Drosophila* haemocytes (Fig. 4C and D). QDs were localized mostly in the cytoplasm of plasmocytes, where the strong fluorescence can be attributed to the presence of nanoparticle aggregates in endosomal/lysosomal vesicles after internalization. These experimental observations confirm that QDs are absorbed from the gastro-intestinal tract in the circulating *Drosophila* system and interact with haemolymph cells. The mechanism of entrance deserves further investigation but it is likely that the QDs uptake is due to the synergy of an active internalization of QDs combined to phagocytosis of cellular debris produced in other districts.

Fig. 5 (A) Time-dependent Cd^{2+} ions release of the differently coated QDs (QD-MUA, QD-PC, QD-PC-PEG) in *Drosophila* lysosomal environment (22 °C, pH 4.5). (B) ROS measurements by DCF assay of QD-MUA treatment compared to Cd^{2+} treatment (using the same cadmium concentration present in the QDs) and CTRL. Data are reported as mean $\pm$ SD from three independent experiments (normalized to the control). *** $P \leq 0.01$ (one-way ANOVA statistical analysis).

Fig. 6 QDs bioaccumulation in a single adult fly. Flies were treated with QDs ($0.02 \mu\text{g g}^{-1}$ Cd per day) for 20 days. Data are reported as mean $\pm$ SD from three independent experiments.

To better clarify the cause of these severe biological effects, we investigated the QDs stability and their possible ion release under *in vivo* conditions, since Cd^{2+} ions are the most plausible candidates in causing toxic effects.⁵⁵ To this aim, we incubated the differently coated nanoparticles under controlled conditions mimicking the lysosomal environment (where QDs are internalized after cellular uptake) in *Drosophila* (pH 4.5, 22 °C).^{56,57}

As shown in Fig. 5A, all the QDs exhibit a clear time-dependent degradation, with significant release of Cd^{2+} ions. Such release is considerable for all the QDs, although the surface coatings partly decrease the NPs degradation. Notably, for QD-MUA quite fast core dissolution was observed in the lysosomal environment, with a plateau after 2–3 weeks, possibly suggesting almost complete particle degradation. Such experimental evidence strongly corroborates the high toxicity observed for QDs when used under long-term exposure conditions, owing to a significant intracellular release of toxic ions, with a weak dependence on the surface coating (unless the coating is capable of guaranteeing complete long-term stability of a particle core under lysosomal conditions). To further support the hypothesis of ions-associated toxicity, we compared *in vivo* the effects of QDs treatment (QD-MUA) with those observed with Cd^{2+} ions (used at the same concentration present in QDs, namely mimicking the conditions of complete QDs core degradation). Interestingly, we found that the toxicity of the QDs treatment was very similar to that of Cd^{2+} (the ROS increase was slightly lower than that observed for Cd^{2+} treatment) (Fig. 5B). Therefore, the *in vivo* toxicity of QDs is mainly due to their *in situ* degradation, with intracellular release of metal ions.^{20,22,55} This, in turn, results in a high increase of oxidative stress and induction of cellular homeostasis response.^{20,58} The molecular mechanism underlying the overloading of ROS species by cadmium ions is not yet completely clear. However, it seems that intracellular cadmium ions, exceeding the capacity of the cellular antioxidant molecules (e.g., glutathione and metallothioneins), interact both with the cation-binding sites of enzymes and with reactive cysteine thiolates of proteins. These events, following chronic exposure, increase the oxidation state of cells and promote over-production of ROS, which can be further amplified by Fenton reactions with other physiological metal ions.⁵⁹

All these highly toxic/genotoxic effects were observed to be significantly reduced by decreasing the QDs dosage. In particular, ingestion of daily dosages $<0.001 \mu\text{g g}^{-1}$ Cd did not produce detectable toxicity.

To understand the different levels of toxicity observed for the differently coated QDs, we investigated the influence of the surface coating on the bioaccumulation of QDs in *Drosophila*. Interestingly, we found that after 20 days of treatment, the bioaccumulation of QDs is strongly dependent on the coating (Fig. 6). We observed, in fact, a significantly higher amount of cadmium in QD-MUA-treated *Drosophila* tissues with respect to the coated nanoparticles (especially in the case of PEGylation). It seems that polymer coating and pegylation strongly decrease the retention/bioaccumulation of QDs in the organism, considerably affecting the overall toxicity. These results are consistent with previous *in vitro* observations reporting that surface functionalization with PEG or other polymers may strongly reduce the cellular uptake of nanoparticles.^{60,61} Therefore, the difference in the toxicity observed in *Drosophila* for the three differently coated QDs can be mainly ascribed to their different cellular internalization and bioaccumulation.

Conclusions

In this study we investigated the *in vivo* toxicity of CdSe–ZnS QDs with different surface coatings. Our results indicate that

cadmium based QDs elicit a significant lifespan decrease, high levels of oxidative stress and genotoxicity, mainly due to the *in vivo* release of Cd²⁺ ions. The surface engineering of QDs may modulate the uptake/bioaccumulation in the organisms, decreasing the overall toxicity, but not eliminating it. In our opinion, these findings provide reliable data to classify CdSe–ZnS QDs as significantly toxic in long-term *in vivo* treatments. Actually, unless particularly stable coatings can guarantee long-term stability, preventing nanoparticles degradation and ions leakage, it seems reasonable to suggest careful limitation of human and environmental exposure to Cd-based QDs, akin to the World Health Organization (WHO) recommendations and legislation already developed for Cd exposure.⁵⁵ Alternatively, for *in vivo* applications, the development of alternative cadmium-free nanoparticles (such as InP QDs) could be envisaged.

Methods

Chemicals

All chemicals were used as received. Bis-(hexamethylene)tri-amine (#14506), poly(maleic anhydride-*alt*-1-octadecene) (#419117), sodium tetraborate decahydrate (#S9640), boric acid (#B1934), boric acid (#B1934), Tris–borate–EDTA buffer (#T3913), sucrose (#16104), and 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC, #E7750) were purchased from Sigma-Aldrich, and diamine-PEG 897 (#06703) was purchased from Fluka. Ultrapure grade water with a resistivity of 18.2 MΩ cm was used in all experiments.

Synthesis, water transfer and functionalization of quantum dots

TOP/TOPO capped CdSe–ZnS core–shell QDs emitting in the orange spectrum (λ_{max} 590 nm) were prepared by following standard colloidal synthesis procedures.^{62,63} The as-synthesized nanocrystals were transferred from the organic phase to the aqueous phase by exchanging the surface ligands to 11-mercaptoundecanoic acid (QD-MUA)⁶⁴ and by adopting a polymer coating procedure (QD-PC).^{65,66} The surface carboxylic groups of the polymer-coated QDs were used to bind one of the amino moieties of the diamino-PEG molecules through the formation of an amide bond.^{67,68} PEG molecules were introduced to increase the stability of the nanoparticles and to improve biocompatibility.

TEM analyses

The samples were prepared by dropping a dilute solution of nanocrystals in water on carbon-coated copper grids (Formvar/Carbon 300 Mesh Cu). TEM images were recorded on a JEOL Jem1011 microscope operating at an accelerating voltage of 100 kV.

Dynamic light scattering (DLS) and Z-potential measurements

A Zetasizer Nano ZS90 (Malvern, USA) equipped with a 4.0 mW He–Ne laser operating at 633 nm and an avalanche photodiode detector was used. Measurements were made at 25 °C in water, pH 7. Each sample was measured 5 times and the results were analyzed by Malvern Instruments Ltd software.

UV-vis absorption and photoluminescence spectra

UV-vis absorption spectra were recorded using a Varian Cary 300 spectrophotometer, while photoluminescence (PL) measurements were recorded by using a Jobin Yvon FluoroLog-3 spectrofluorometer. Experiments were performed at room temperature (20 °C).

Gel electrophoresis characterization

The samples were run on 2% agarose gel in TBE buffer for 1 h at 100 V. Prior to gel electrophoresis, a solution containing Orange G and 30% glycerol in gel-loading buffer corresponding to 20% of the sample volume was added to each sample. At the end of the run, the gel was observed under UV light, and a red filter was used to detect the fluorescence of the nanocrystals.

Elemental analysis

Elemental analysis was carried out by inductively coupled plasma atomic emission spectroscopy (ICP-AES) with a Varian Vista AX spectrometer. 150 animals were incubated overnight in 1.5 mL of concentrated HCl–HNO₃ 3 : 1 (v/v), diluted with ultrapure water, then were sonicated and filtered through membrane filtration; the resulting solutions were directly analyzed by ICP-AES.

Nanocrystals degradation

Nanocrystal degradation experiments were performed in sodium citrate buffer, pH 4.5, an acidic medium mimicking the lysosomal environment.^{56,57} The citrate buffer was prepared by mixing appropriate volumes of 20 mM aqueous solutions of citric acid and sodium citrate monobasic to achieve the final desired pH. Adequate volumes of stock QDs suspension were diluted in the buffer in order to achieve the final NP concentrations of 20 nM (corresponding to a Cd concentration of 13 μM). The nanoparticles were then allowed to degrade at the *Drosophila* organism temperature. The NPs degradation was analyzed at 1, 7, 14 and 21 days. At each time point, the NPs were separated from the rest of the solution through filtration using Amicon filters. Each solution was collected, digested by the addition of an HCl–HNO₃ 3 : 1 (v/v) solution and the intracellular Cd concentration was measured by means of ICP-AES.

Drosophila melanogaster strain and culture conditions

The flies and larvae of wild-type *Drosophila melanogaster* (Oregon R+) were cultured at 23 ± 1 °C on standard *Drosophila* food, containing agar, corn meal, sugar, yeast and nepagin (methyl-*p*-hydroxybenzoate).

Fly treatments

The differently coated QDs were formulated in the diet at the final concentration of 200 pM that corresponds to 130 nM concentration of Cd. Assuming that *Drosophila* typically eats 1.5 μL per day of food,⁶⁹ the dose of Cd administered to the flies correspond to 0.02 μg g^{−1} per day. For the experiments the flies were treated for an entire life-cycle. In particular, groups of 10 flies were transferred in vials containing normal, QD-MUA,

QD-PC and QD-PC-PEG treated food and after eggs deposition the flies were removed. The virgin male and female flies emerging from the control and differently coated-QDs treated food were transferred into new vials containing the respective food, and after eggs deposition (eggs-to-eggs) the adults were removed again. Finally, larvae and adult flies emerging from the sample treated food were used for all the experiments reported in this work.

Lifespan experiments

For longevity analyses, the newly eclosed flies were collected and housed at a density of 20 males and 20 females, separately, per each vial. At least 10 vials were used per treatment (a total of 100 male and 100 female flies per lifespan) for a total number of 1200 flies for each differently coated-QDs. Flies were transferred into fresh food every 3–4 days, and dead flies were counted every day until all died. We carried out this experiment using normal food, treated food containing QDs supernatant (SN) and treated food containing QDs with different coatings (QD-MUA, QD-PC and QD-PC-PEG).

Measurement of ROS

Twenty day-old flies were homogenized in tubes containing 1 mL PBST (PBS containing 0.1% Tween-20). The homogenate of each sample was divided into two different vials. The first vial was transferred into a 96-well plate. After adding 50 μ M DCF-DA to the samples, the plate was read every 5 min for 15 min with a fluorescent microplate reader (FLUOstar Optima, BMG Laboratory, Offenberg, Germany) for the quantification of fluorescence (485 nm excitation, 520 nm emission). The second vial was used for protein crude extract quantification. Following centrifugation at 2300g for 15 min at 4 °C in the presence of a protease inhibitor, the supernatant was quantified by the Bradford method.⁵⁶ The amount of proteins in the crude extraction was used to normalize the relative fluorescence measured by DCFH-DA in each samples. Three independent experiments with 20 flies in each were performed.

Haemocytes collection

Drosophila haemocytes were collected according to Irving *et al.*⁷⁰ with minor modifications. Chilled larvae 96 ± 2 h old were removed from food media, washed in water, sterilized in 5% bleach, and dried. The cuticle from 10 larvae was then disrupted with two fine forceps. The haemolymph and circulating haemocytes were collected in cold PBS solution. The number of haemocytes per larvae ranges from 1000 to 1500.

TUNEL assay

For TUNEL assay, third instar larvae haemocytes were fixed as previously described.³⁷ After washing in PBST (PBS, 0.1% Tween 20), the samples permeabilized in PBS 0.3% Triton X 100 for 15 min. Haemocytes were processed by Click-iT TUNEL Alexa Fluor647 Imaging Assay (Invitrogen) according to the manufacturer's instructions. The samples were then characterized by confocal microscopy (Leica TCS-SP5 AOBS). Quantitative analyses of TUNEL-positive nuclei were carried out by

examining different haemocytes samples (20 different microscopic fields each) from three independent experiments.

Annexin/PI binding staining

The analyses of annexin V/PI binding were carried out with an Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Invitrogen) according to the manufacturer's instruction. Briefly, haemocytes from 10 larvae were resuspended in binding buffer in the range from 1000 to 1500 haemocytes per larva. Then, a solution containing PI/Alexa Fluor® 488 annexin V was added to haemocytes. Cells were gently vortexed and incubated for 15 min at room temperature in the dark. Finally, the samples were analyzed by confocal microscopy (Leica TCS-SP5 AOBS). Quantitative analyses of Annexin V/PI-positive cells were carried out by examining different haemocytes (20 different microscopic fields each) from three independent experiments.

Optical and fluorescence microscopy analyses of haemocytes

For optical images, chilled larvae 96 ± 2 h old were removed from food media, washed in water, sterilized in 5% bleach, and dried. The cuticle from 10 larvae was then disrupted with two fine forceps. The haemolymph and circulating haemocytes were collected in 40 μ L of Schneider's medium. This solution was mounted in the slides and observed in bright field microscopy (Leica TCS-SP5 AOBS). For fluorescence images of QDs internalization, we collected haemocytes from 10 larvae and placed them on a polylysine slide to allow electrostatic adhesion of cells. Subsequently the sample was fixed in 4% paraformaldehyde in PBS for 15 min. After washing in PBST (PBS, 0.1% Tween 20), the cells were scored for the presence of QDs by confocal microscopy.

Statistical analyses

GraphPad Prism 5 statistical analysis software was used in all statistical analyses performed in this work (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA). In particular, the survival distributions (lifespan curves) were assessed in terms of significance using the nonparametric log-rank (Mantel-Cox) test; the TUNEL and annexin V/PI assays were evaluated by the *t*-test; the Reactive Oxygen Species (ROS) tests were analyzed by one-way ANOVA and compared to the control by the Bonferroni posttest.

References

- 1 H. M. E. Azzazy, M. M. H. Mansour and S. C. Kazmierczak, *Clin. Biochem.*, 2007, **40**, 917–927.
- 2 A. M. Derfus, W. C. W. Chan and S. N. Bhatia, *Nano Lett.*, 2004, **4**, 11–18.
- 3 D. Gerion, F. Pinaud, S. C. Williams, W. J. Parak, D. Zanchet, S. Weiss and A. P. Alivisatos, *J. Phys. Chem. B*, 2001, **105**, 8861–8871.
- 4 M. Bruchez, M. Moronne, P. Gin, S. Weiss and A. P. Alivisatos, *Science*, 1998, **281**, 2013–2016.
- 5 W. C. W. Chan, D. J. Maxwell, X. Gao, R. E. Bailey, M. Han and S. Nie, *Curr. Opin. Biotechnol.*, 2002, **13**, 40–46.
- 6 M. Nirmal and L. Brus, *ChemInform*, 1999, **30**, DOI: 10.1002/chin.199929266.
- 7 A. May, S. Bhaumik, S. S. Gambhir, C. Zhan and S. Yazdanfar, *J. Biomed. Opt.*, 2009, **14**, 060504.

- 8 I. L. Medintz, H. T. Uyeda, E. R. Goldman and H. Mattoussi, *Nat. Mater.*, 2005, **4**, 435–446.
- 9 M. A. Walling, J. A. Novak and J. R. E. Shepard, *Int. J. Mol. Sci.*, 2009, **10**, 441–491.
- 10 M. Frasco and N. Chaniotakis, *Anal. Bioanal. Chem.*, 2010, **396**, 229–240.
- 11 V. Bagalkot, L. Zhang, E. Levy-Nissenbaum, S. Jon, P. W. Kantoff, R. Langer and O. C. Farokhzad, *Nano Lett.*, 2007, **7**, 3065–3070.
- 12 X. Michalet, F. F. Pinaud, L. A. Bentolila, J. M. Tsay, S. Doose, J. J. Li, G. Sundaresan, A. M. Wu, S. S. Gambhir and S. Weiss, *Science*, 2005, **307**, 538–544.
- 13 K. Jain, *BMC Med.*, 2010, **8**, 83.
- 14 J. A. Ryan and L. E. Hightower, *Environ. Toxicol. Chem.*, 1994, **13**, 1231–1240.
- 15 J. C. Wataha, P. E. Lockwood, A. Schedle, M. Noda and S. Bouillaguet, *J. Oral Rehabil.*, 2002, **29**, 133–139.
- 16 R. L. W. Messer, S. Bishop and L. C. Lucas, *Biomaterials*, 1999, **20**, 1647–1657.
- 17 R. N. Ratnaik, *Postgrad. Med. J.*, 2003, **79**, 391–396.
- 18 Y. Chen, F. Parvez, M. Gamble, T. Islam, A. Ahmed, M. Argos, J. H. Graziano and H. Ahsan, *Toxicol. Appl. Pharmacol.*, 2009, **239**, 184–192.
- 19 J. L. Pelley, A. S. Daar and M. A. Saner, *Toxicol. Sci.*, 2009, **112**, 276–296.
- 20 N. Chen, Y. He, Y. Su, X. Li, Q. Huang, H. Wang, X. Zhang, R. Tai and C. Fan, *Biomaterials*, 2012, **33**, 1238–1244.
- 21 T. S. Hauck, R. E. Anderson, H. C. Fischer, S. Newbigging and W. C. W. Chan, *Small*, 2010, **6**, 138–144.
- 22 W. Liu, S. Zhang, L. Wang, C. Qu, C. Zhang, L. Hong, L. Yuan, Z. Huang, Z. Wang, S. Liu and G. Jiang, *PLoS One*, 2011, **6**, e24406.
- 23 D. K. Tiwari, T. Jin and J. Behari, *Int. J. Nanomed.*, 2011, **6**, 463–475.
- 24 A. Tino, A. Ambrosone, L. Mattered, V. Marchesano, A. Susa, A. Rogach and C. Tortiglione, *Int. J. Biomater.*, 2011, **2011**, 792854.
- 25 R. S. H. Yang, L. W. Chang, J.-P. Wu, M.-H. Tsai, H.-J. Wang, Y.-C. Kuo, T.-K. Yeh, C. S. Yang and P. Lin, *Environ. Health Perspect.*, 2007, **115**, 1339–1343.
- 26 R. Hardman, *Environ. Health Perspect.*, 2006, **114**, 165–172.
- 27 K. Leigh, J. Bouldin and R. Buchalan, *J. Toxicol.*, 2012, **2012**, 397657.
- 28 C. Kirchner, T. Liedl, S. Kudara, T. Pellegrino, A. Muñoz Javier, H. E. Gaub, S. Stölzle, N. Fertig and W. J. Parak, *Nano Lett.*, 2004, **5**, 331–338.
- 29 D. Maysinger, M. Behrendt, M. Lalancette-Hébert and J. Kriz, *Nano Lett.*, 2007, **7**, 2513–2520.
- 30 T. Jin, F. Fujii, Y. Komai, J. Seki, A. Seiyama and Y. Yoshioka, *Int. J. Mol. Sci.*, 2008, **9**, 2044–2061.
- 31 A. Hoshino, K. Fujioka, T. Oku, M. Suga, Y. F. Sasaki, T. Ohta, M. Yasuhara, K. Suzuki and K. Yamamoto, *Nano Lett.*, 2004, **4**, 2163–2169.
- 32 G. M. Rubin, M. D. Yandell, J. R. Wortman, G. L. Gabor Miklos, C. R. Nelson, I. K. Hariharan, M. E. Fortini, P. W. Li, R. Apweiler, W. Fleischmann, J. M. Cherry, S. Henikoff, M. P. Skupski, S. Misra, M. Ashburner, E. Birney, M. S. Boguski, T. Brody, P. Brokstein, S. E. Celniker, S. A. Chervitz, D. Coates, A. Cravchik, A. Gabrielian, R. F. Galle, W. M. Gelbart, R. A. George, L. S. Goldstein, F. Gong, P. Guan, N. L. Harris, B. A. Hay, R. A. Hoskins, J. Li, Z. Li, R. O. Hynes, S. J. Jones, P. M. Kuehl, B. Lemaitre, J. T. Littleton, D. K. Morrison, C. Mungall, P. H. O'Farrell, O. K. Pickeral, C. Shue, L. B. Vosshall, J. Zhang, Q. Zhao, X. H. Zheng and S. Lewis, *Science*, 2000, **287**, 2204–2215.
- 33 B. H. Jennings, *Mater. Today*, 2011, **14**, 190–195.
- 34 L. T. Reiter, L. Potocki, S. Chien, M. Gribskov and E. Bier, *Genome Res.*, 2001, **11**, 1114–1125.
- 35 M. D. Adams and J. J. Sekelsky, *Nat. Rev. Genet.*, 2002, **3**, 189–198.
- 36 D. St Johnston, *Nat. Rev. Genet.*, 2002, **3**, 176–188.
- 37 G. Vecchio, A. Galeone, V. Brunetti, G. Maiorano, L. Rizzello, S. Sabella, R. Cingolani and P. P. Pompa, *Nanomed.: Nanotechnol. Biol. Med.*, 2012, **8**, 1–7.
- 38 M. P. Singh, K. Ravi Ram, M. Mishra, M. Shrivastava, D. K. Saxena and D. K. Chowdhuri, *Chemosphere*, 2010, **79**, 577–587.
- 39 M. D. Rand, *Neurotoxicol. Teratol.*, 2010, **32**, 74–83.
- 40 G. Vecchio, A. Galeone, V. Brunetti, G. Maiorano, S. Sabella, R. Cingolani and P. P. Pompa, *PLoS One*, 2012, **7**, e29980.
- 41 A. Panacek, R. Prucek, D. Safarova, M. Dittrich, J. Richtrova, K. Benickova, R. Zboril and L. Kvitek, *Environ. Sci. Technol.*, 2011, **45**, 4974–4979.
- 42 X. Liu, D. Vinson, D. Abt, R. H. Hurt and D. M. Rand, *Environ. Sci. Technol.*, 2009, **43**, 6357–6363.
- 43 B. M. Zid, A. N. Rogers, S. D. Katewa, M. A. Vargas, M. C. Kolipinski, T. A. Lu, S. Benzer and P. Kapahi, *Cell*, 2009, **139**, 149–160.
- 44 M. C. Wang, D. Bohmann and H. Jasper, *Dev. Cell*, 2003, **5**, 811–816.
- 45 E. R. Carmona, A. Creus and R. Marcos, *Mutat. Res., Genet. Toxicol. Environ. Mutagen.*, 2011, **718**, 33–37.
- 46 P. Irving, J.-M. Ubeda, D. Doucet, L. Troxler, M. Lagueux, D. Zachary, J. A. Hoffmann, C. Hetru and M. Meister, *Cell. Microbiol.*, 2005, **7**, 335–350.
- 47 S. Cherry and N. Silverman, *Nat. Immunol.*, 2006, **7**, 911–917.
- 48 M. Meister, *Curr. Opin. Immunol.*, 2004, **16**, 10–15.
- 49 E. Demir, G. Vales, B. Kaya, A. Creus and R. Marcos, *Nanotoxicology*, 2010, **5**, 417–424.
- 50 S. Sabella, V. Brunetti, G. Vecchio, A. Galeone, G. Maiorano, R. Cingolani and P. Pompa, *J. Nanopart. Res.*, 2011, **13**, 6821–6835.
- 51 B. Charroux and J. Royet, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 9797–9802.
- 52 M. J. Williams, *J. Immunol.*, 2007, **178**, 4711–4716.
- 53 W. Wood and A. Jacinto, *Nat. Rev. Mol. Cell Biol.*, 2007, **8**, 542–551.
- 54 M. Meister and M. Lagueux, *Cell. Microbiol.*, 2003, **5**, 573–580.
- 55 M. P. Waalkes, *J. Inorg. Biochem.*, 2000, **79**, 241–244.
- 56 A. S. Arbab, L. B. Wilson, P. Ashari, E. K. Jordan, B. K. Lewis and J. A. Frank, *NMR Biomed.*, 2005, **18**, 383–389.
- 57 M. Levy, F. Lagarde, V. A. Maraloiu, M. G. Blanchin, F. Gendron, C. Wilhelm and F. Gazeau, *Nanotechnology*, 2010, **21**, 395103.
- 58 H. Yepiskoposyan, D. Egli, T. Fergestad, A. Selvaraj, C. Treiber, G. Multhaupt, O. Georgiev and W. Schaffner, *Nucleic Acids Res.*, 2006, **34**, 4866–4877.
- 59 J.-M. Moulis, *BioMetals*, 2010, **23**, 877–896.
- 60 Arnida, A. Malugin and H. Ghandehari, *J. Appl. Toxicol.*, 2010, **30**, 212–217.
- 61 C. Freese, M. I. Gibson, H.-A. Klok, R. E. Unger and C. J. Kirkpatrick, *Biomacromolecules*, 2012, **13**, 1533–1543.
- 62 B. O. Dabbousi, J. Rodriguez-Viejo, F. V. Mikulec, J. R. Heine, H. Mattoussi, R. Ober, K. F. Jensen and M. G. Bawendi, *J. Phys. Chem. B*, 1997, **101**, 9463–9475.
- 63 Z. A. Peng and X. G. Peng, *J. Am. Chem. Soc.*, 2001, **123**, 183–184.
- 64 R. Costi, A. E. Saunders, E. Elmaleh, A. Salant and U. Banin, *Nano Lett.*, 2008, **8**, 637–641.
- 65 T. Pellegrino, L. Manna, S. Kudara, T. Liedl, D. Koktysh, A. L. Rogach, S. Keller, J. Rädler, G. Natile and W. J. Parak, *Nano Lett.*, 2004, **4**, 703–707.
- 66 R. Di Corato, A. Quarta, P. Piacenza, A. Ragusa, A. Figuerola, R. Buonsanti, R. Cingolani, L. Manna and T. Pellegrino, *J. Mater. Chem.*, 2008, **18**, 1991–1996.
- 67 A. Williams and I. T. Ibrahim, *Chem. Rev.*, 1981, **81**, 589–636.
- 68 M. A. Malvindi, R. Di Corato, A. Curcio, D. Melisi, M. G. Rimoli, C. Tortiglione, A. Tino, C. George, V. Brunetti, R. Cingolani, T. Pellegrino and A. Ragusa, *Nanoscale*, 2011, **3**, 5110–5119.
- 69 W. W. Ja, G. B. Carvalho, E. M. Mak, N. N. de la Rosa, A. Y. Fang, J. C. Liong, T. Brummel and S. Benzer, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 8253–8256.
- 70 P. Irving, J. M. Ubeda, D. Doucet, L. Troxler, M. Lagueux, D. Zachary, J. A. Hoffmann, C. Hetru and M. Meister, *Cell. Microbiol.*, 2005, **7**, 335–350.