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Short communication

Genotoxic effects of CdS quantum dots and Ag_2S nanoparticles in fish cell lines (RTG-2)

Q1 Marco Munari[a,1], Joachim Sturve[b,1], Giada Frenzilli[c,*], Matthew B. Sanders[d], Andrea Brunelli[e], Antonio Marcomini[e], Marco Nigro[c], Brett P. Lyons[d]

[a] Department of Biology, University of Padua, Padua, Italy
[b] Department of Biological and Environmental Sciences, University of Gothenburg, Göteborg, Sweden
[c] Department of Clinical and Experimental Medicine, University of Pisa, Italy
[d] Cefas Weymouth Laboratory, Barrack Road, The Nothe, Weymouth, Dorset DT4 8UB, UK
[e] Department of Environmental Science, Ca' Foscari University, Dorsoduro 2137, I-30123 Venice, Italy

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ABSTRACT

The increasing use of nanotechnologies will lead to significant releases of engineered nanoparticles into the aquatic environment, where their impact is still poorly characterized. In the present paper, the genotoxic and cytotoxic properties of CdS quantum dots (QDs) and silver sulphide (Ag_2S) coated with methyl polyethylene glycol (M-PEG) were investigated in a rainbow trout cell line (RTG-2). The results showed that CdS QDs were highly cytotoxic at high concentrations (10 and 50 μg/ml), and exhibited a concentration-dependent genotoxicity in the sub-toxic range (0.01–1 μg/ml) after 24 h exposure. Ag_2S showed neither genotoxic nor cytotoxic effects.

1. Introduction

Quantum dots (QDs) are semiconductor nanocrystals whose optical, electronic, and catalytic properties make them promising nanomaterials for applications in biology and medicine, as well as in solar cells and photovoltaics, security inks, photonics and telecommunications [1–3]. The potential toxicity of quantum dots has become a health concern due to their chemical composition and nanoscale features [4]. Literature data reports that QDs may be cytotoxic and/or responsible for changes in gene expression [5]. The existence of many different QDs, in terms of cores and coatings, responsible for differing chemical and physical proprieties, require extensive research on their toxicity.

The production and use of QDs are ever increasing and their release into the aquatic environment is to be expected. Therefore, robust approaches for assessing the impact of QDs on biota are needed. At present, data on the aquatic ecotoxicology of QDs are limited to a few species, including microalgae [6,7], freshwater mussels [8], and fish [9,10]. In most of the above-mentioned papers, engineered nanoparticle (ENP) toxicity was ascribed to redox imbalance. DNA strand breaks (single and double) represent one of the major oxidative damages to DNA *via* oxidative stress, often assessed by the comet assay [11]. This method has been used to evaluate the genotoxic effects of contaminants (as metals) in aquatic organisms [12,13] that are a potential target for nanoparticle genotoxicity [8,14–16].

In the present study, we have investigated the genotoxic potential of cadmium sulfide (CdS) QDs in an RTG-2 cell line, derived from gonads of the teleost fish, rainbow trout (*Oncorhynchus mykiss*) and representing a validated model for genotoxicity studies [17,18]. The study was extended to another nanosized metal sulphide, Ag_2S, the common reduction product of Ag which occurs in both wastewater treatment plants and the environment [19,20]. Although the very low water solubility of Ag_2S ENP may limit toxicity, its biological

Abbreviations: Ag_2S, silver sulphide; DLS, dynamic light scattering; ENP, engineered nanoparticle; FBS, fetal bovine serum; ICP, inductively coupled plasma (ICP) analysis; LDH, lactate dehydrogenase; MALDI, matrix assisted laser desorption ionization; MMS, methyl methanesulphonate; M-PEG, methyl polyethylene glycol; MPEG-SH, thiol-terminated methyl polyethylene glycol; QDs, quantum dots; RTG, rainbow trout gonadic (RTG)-2 cell line; TEM, electron transmission microscopy; XRD, X-ray diffraction (XRD) analysis.

* Corresponding author. Tel.: +39 050 2219111; fax: +39 050 2219101.
E-mail address: giada@biomed.unipi.it (G. Frenzilli).

[1] These authors equally contributed to the present work.

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impact remains an open question because of its stability and potential accumulation [21]. Quantum dots and Ag_2S ENP were modified by capping with methyl polyethylene glycol (M-PEG), which is often used to improve the biocompatibility of ENP in biological and medical applications. The comet assay was used to detect DNA damage (single- and double-strand breaks and alkali-labile sites) in RTG-2 cells. Cell viability was assessed with the LDH cytotoxicity assay.

2. Materials and methods

2.1. Nanoparticle synthesis and characterization

The CdS nanoparticles were prepared as previously reported by Sanders et al. [10] but at twice the dilution. ICP analysis showed the solution to contain 32% C, 5.7% H trace N, <0.1% chloride 9.7% S, 29.4% Cd, 0.4% Na. Cadmium measurements for the solution showed the concentration to be 1019 ppm for cadmium ions, which equates to 1.31 g/l for the CdS nanoparticles and gives an estimated capping agent concentration of 2.1 g/l. TEM analysis and UV absorption spectroscopy were performed in order to assess the frequency distribution of particle sizes.

The silver nanoparticles were prepared by reducing 2.3 g of silver nitrate in 2.3 l of methanol in the presence of 11 g of MPEG-SH (thiol-terminated methyl polyethylene glycol) as capping agent. The silver was reduced using a solution of 4.4 g of sodium borohydride in 200 ml methanol. The solution was stirred for 1 h and then reduced to oil by evaporation under vacuum at 50 °C. The particles were re-dissolved in a small amount of methanol (50 ml) and precipitated by addition of diethyl ether (~250 ml). The resulting precipitate was carefully isolated by decantation or centrifugation. The solid powder was dried under vacuum overnight, re-dissolved in distilled water, and scrubbed with a mixed-bed ion-exchange resin (Dow Marathon 3R) (300 g). The final solution was analyzed by ICP, showing the solution to contain 31.2% C 5.36% H 25.38% Ag, 3.53% Na and 1.54% B. This indicates possible contamination of $Na_2BO_2(OH)$ at about 12%. It also contains ~2 g/l of capping agent. ICP of the solution gives the nanoparticle concentration at 0.9 g/l.

2.1.1. Capping agent

Thiol-terminated methyl polyethylene glycol (M-PEG) capping agent was prepared as reported by Farkas et al. [22]. NMR analysis showed the material to be pure. Matrix Assisted Laser Desorption Ionization (MALDI) showed that the polymer had the nominal molecular weight of 796 Da and that the terminal groups were exclusively thiol (SH). Microanalysis showed that the material contained <0.1% Na^+ ions, no detectable bromine, and <0.1% phosphorus.

2.2. DLS analysis

The size distributions of CdS QD and Ag_2S ENP, dispersed in both water and L15, were determined as hydrodynamic diameters by Dynamic Light Scattering (DLS) with a Nicomp Submicron Particle Sizer Autodilute® Model 370 (Particle Sizing Systems, Port Richey, FL, USA), with a 632.8 nm laser and the detector set at 90°. This instrument can automatically recognize, through a patented software algorithm, a tri-modal size distribution of particles in the 0.5–6000 nm range.

The tested concentrations were 0.13 g/l and 0.09 g/l ENP in water samples and 10 mg/l ENP in L15 medium.

2.3. Exposure

Cells were maintained in 75-cm^2 flasks under 5% CO_2 at 22 °C, in Leibovitz medium (L15) plus 10% FBS, without antibiotics. Cells were passaged when the monolayer reached approximately 80–90% confluence. Cells were washed twice with Dulbecco's PBS, trypsinized (500–700 μl trypsin solution, 0.25%for each flask), stained with Trypan blue, and counted. Cells were transferred into 6-well plates (2 ml per well) at 10^5 cells per ml, in the same medium. After 24 h, cells were washed twice with PBS and exposed to five different concentrations (0.01; 0.1; 1; 10; 50 μg/ml) for each substance (CdS QD, Ag_2S ENP and M-Peg) for 24 and 48 h in 200 μl L.15 (1% FBS) at 15 °C. Dose selection and exposure time were based on previous studies [23–25]. After exposure, the cells were washed twice with PBS and trypsinized (about 500 μl of 0.25% trypsin in PBS). After washing, 1 ml supernatant was collected in Eppendorf tubes. Two independent experiments with two replicates each were carried out. Triton X, 1%, and the alkylating agent MMS (methyl methanesulphonate, 0.5 mM) were used as positive control for cytotoxicity tests and the comet assay, respectively.

2.4. Cytotoxicity

Two different methods for detection of cytotoxicity were used, the LDH and XTT assays.

2.4.1. LDH assay

The cytotoxicity detection kit (LDH) Roche (Cat. Nr. 11 644 793 001) was used. L.15 medium (100 μl from each well) from the 96-well plates was taken after the 24 and 48 h exposures and split into 96-well plates (100 μl per well). To this volume, 100 μl reagent was added and the plate was incubated for 30 min at room temperature, protected from light. The reaction mixture was obtained by mixing 0.25 ml catalyst with 11.25 ml dye solution. The absorbance was measured at 490 or 492 nm by a Wallac 1420 VICTOR^{2TM} plate reader spectrophotometer.

2.4.2. Cell proliferation kit II (XTT) Roche (Cat. No. 11 465 015 001)

This assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye in metabolically active (viable) cells. After the exposure, 100 μl medium was taken from each well and 100 μl XTT labelling mixture was added. The plates were incubated for 4 h before measuring absorbance at 450–500 nm with a Wallac 1420 VICTOR^{2TM} plate-reader spectrophotometer. The XTT labelling mixture was obtained by mixing 5 ml XTT labelling reagent + 0.1 ml electron coupling reagent, immediately before use. The 0% cytotoxicity value was assigned to the well without ENP or MMS; cytotoxicity in the other wells is determined relative to the untreated cells. As results of LDH release measurements were consistent with those obtained with the XTT assay, only XTT assay data are presented.

2.5. Comet assay (Single Cell Gel Electrophoresis)

The comet assay was carried out following Guidi et al. [26] with slight modifications.

Eppendorf tubes containing cells were spin down at 3000 rpm for 2 min. Pellets were resuspended in LMP (low-melting-point agarose) 0.5%, 170 μl. Two drops (85 μl) were placed on each 1% NMP (normal-melting-point agarose) agarose-coated slide. A coverslip was placed on top of each gel and the slides chilled to allow coverslip removal. After a few minutes at 4 °C, the slides without coverslips were placed in chilled lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, 8 g NaOH and 1 l dH_2O; 1% Triton X-100 and 9% DMSO added prior to use) for 1 h and immersed in electrophoresis buffer (300 mM NaOH, 1 mM EDTA in 1 l dH_2O; pH 13) for 20 min at 4 °C. Electrophoresis lasted 20 min (25 V and 300 mA; 0.75 V/cm) before slides were washed in neutralization buffer (0.4 M Tris; pH 7.5).

Air-dried slides were stained with ethidium bromide (2 μg/ml in dH_2O) and scored using Comet Assay IV (Perceptive Instruments Ltd). % Tail DNA was chosen as a reliable comet assay parameter [27]. 50 cells for each half slide were scored. 100 cells were scored per replicate treatment ($n = 4$). Means were analyzed by Analysis of Variance (ANOVA). Before and at the end of the experiments, an aliquot of both exposed and control cells was used for the assessment of cell viability by the trypan blue dye exclusion technique, mixing 0.4% trypan blue solution with the cell pellet, smearing the mixture on a Bürker chamber, and scoring white (live) and blue (dead) cells.

3. Results

3.1. Nanoparticle characterization

TEM analysis and UV absorption spectroscopy showed that CdS quantum dots and Ag_2S ENP had average diameters 4 ± 1 nm and 13 ± 7 nm, respectively (Fig. 1a–d). Powder XRD showed that both particles were in the cubic phase.

3.2. Dynamic Light Scattering (DLS) analysis

Slight agglomeration was observed in the CdS QD samples, after dispersion in L15 medium. Gaussian size distribution of CdS QD increased from 9.5 ± 6 nm in water to 28 ± 17.4 nm in L15 (Fig. 2a and b), remaining stable 24 h and 48 h after analysis. Nicomp size distributions of Ag ENP samples were stable over the time range of the experiments: 56 ± 6.2 nm in water and 57 ± 7.6 nm in L15 (Fig. 2c and d), reaching the same values 24 h as well as 48 h after analysis.

3.3. Cytotoxicity

Results obtained from the cytotoxicity test showed that Cadmium QD was cytotoxic at the two higher concentrations (10 and 50 μg/ml) after both 24 and 48 h exposure (Table 1). In contrast, cytotoxicity of Ag_2S was very limited (Table 1). The capping agent M-PEG was also tested for cytotoxicity over the whole concentration range used for metal sulphide. Results showed the absence of cytotoxicity between 0.01 and 1 μg/ml but very high cell death at the higher concentrations (10 and 50 μg/ml) (Table 1).

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