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The uptake and intracellular fate of a series of different surface coated quantum dots *in vitro*

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ABSTRACT

Quantum dots (QDs) are potentially beneficial semi-conductor nanocrystals for use in diagnostics and therapeutics. The chemical composition of QDs however, has raised concerns as to their potential toxicity. Although a thorough examination using specific biochemical endpoints is necessary to assess QD toxicity, an understanding of the interaction of QDs, specifically their uptake and intracellular fate, with biological systems is also essential in determining their potential hazardous effects. The aim of this study was to investigate the uptake and intracellular fate of a series of different surface coated QDs (organic, carboxylated (COOH) and amino (NH_2) polyethylene glycol (PEG)) in J774.A1 'murine macrophage-like' cells. Model 20 nm and 200 nm COOH polystyrene beads (PBs) were also studied. Results showed that COOH and NH_2 (PEG) QDs, as well as 20 nm and 200 nm PBs were located within lysosomes and the mitochondria of macrophages after 2 h. Additionally, elemental transmission electron microscopy confirmed both COOH and NH_2 (PEG) QDs to be located within membrane-bound compartments at this time point. The data from this study combined with current knowledge, indicates that the intracellular localisation of QDs could be directly related to their toxicity.

1. Introduction

In order to realise the advantages proposed by nanotechnology, it is imperative that the potential adverse effects that nanoparticles (NPs) may pose to human health is understood (Maynard et al., 2006; Maynard, 2007). Commonly, a wide and diverse range of biochemical tests are performed to investigate NP toxicity (Stone et al., 2009). In addition to this however, understanding the NP–cell interaction (specifically NP uptake and intracellular localisation) is also essential to assess the potential for NPs to cause adverse effects upon normal cell homeostasis.

Quantum dots (QDs) are an example of the many different forms of NPs being manufactured for use in nanomedicine, specifically as tools for diagnostics and therapeutics due to their novel fluorescent characteristics (Azzazy et al., 2006, 2007; Pison et al., 2006). Although potentially advantageous for nanomedicine, the ability to manipulate the surface of QDs with a variety of different biologically based molecules, such as lipids, as well as with different chemical surface coatings and polymers, thus affecting the specific surface charge of QDs, has been shown to significantly alter, as well as determine the specific uptake mechanism and subsequent intracellular localisation of QDs in a variety of different cell types (Lovric et al., 2005a,b; Maysinger et al., 2007; Hild et al., 2008). Despite this, the uptake mechanisms and intracellular localisation of QDs are not fully understood. Although the numerous different surface coatings are evidence for this lack of understanding, additional particle characteristics, such as particle size (Oberdorster et al., 2007), have also been reported to significantly affect the QD–cell interaction. This was highlighted by Lovric et al. (2005a) who showed that smaller QDs were able to penetrate into the nucleus, whilst larger QDs located in the cytoplasm of neuronal PC12 cells. It was subsequently reported that the smaller QDs induced a heightened toxicity compared to the larger QDs due to their intracellular localisation (Lovric et al., 2005a). It is essential therefore, that, in addition to their potential toxicity, the QD–cell interaction of the many different forms of QDs is determined prior to their manufacture for use in nanomedicine (Bailey et al., 2004).

The aim of this study was to further investigate the uptake, as well as the sub-cellular distribution and localisation of a series of different surface coated QDs. Carboxylate (COOH) modified polystyrene beads (PBs) with a diameter of 20 nm and 200 nm were also used to investigate the effects of size on particle uptake and their subsequent intracellular fate in comparison to the COOH coated QDs. It is hypothesised that the surface coating of NPs will

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determine their uptake and subsequent intracellular location in cells.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents used were purchased from Sigma–Aldrich, UK, unless otherwise stated.

2.2. Cells and cell culture

In a 75 cm^2 flask, J774.A1 cells were maintained in Rosewell Park Memorial Institute (RPMI)-1640 cell culture medium containing phenol red, L-glutamine (L-G) (5 ml at 200 mM) (Gibco®, UK), penicillin (5 ml at 100 U ml^{-1})/streptomycin (P/S) (5 ml at 0.1 μg ml^{-1}) (Gibco®, UK) and 10% heat-inactivated foetal calf serum (FCS) (Gibco®, UK) (hereafter known as '*complete medium*') in an atmosphere of 37 °C, 5% CO_2. J774.A1 cells were removed from the culture flask via gentle scraping and then re-suspended in complete medium prior to centrifugation at 380 × *g* for 2 min. Cell viability was determined using Trypan blue (0.4% solution diluted in phosphate buffered saline (PBS)) exclusion. Cells were adjusted to a suspension of 2.5×10^5 cells ml^{-1} in complete medium (hereafter known as '*cell suspension*') and used for all subsequent investigation, unless otherwise stated.

2.3. Particles

2.3.1. QDs

Organic, COOH and amino NH_2 polyethylene glycol (PEG) semi-conductor, QD nanocrystals with a core material of cadmium telluride/selluride (CdTe/CdSe) mixture covered by a zinc sulphide (ZnS) shell were originally purchased from Qdots® (CA, USA) and provided by Invitrogen (Paisley, UK). Organic QDs were supplied at a 1 μM concentration in 4 ml of a lipophilic, organic solvent vehicle mixture, comprising of (wt.%) 95% decane, 2% trioctyl phosphine (TOP) and 2% trioctyl phosphine oxide (TOPO). COOH QDs, which had a carboxyl-derivative amphiphilic coating attached to the ZnS shell, were purchased at a concentration of 8 μM in 250 μl of 50 mM sodium borate ($Na_2B_4O_7{\cdot}10H_2O$) solution at pH 9.0. NH_2 (PEG) QDs were also provided at an 8 μM concentration in 250 μl of 50 mM $Na_2B_4O_7{\cdot}10H_2O$ solution (pH 8.3). The structure of the NH_2 (PEG) QDs consisted of an amide amphiphilic coating attached to the ZnS shell, with an amino-derivative PEG outer coating covalently attached to the positively charged, amide layer. The specific chemical composition of the amino-derivative PEG outer coating is the intellectual property of Qdots®. According to the manufacturer the average diameter (Ø) of each QD (including the core, shell and surface coating) was 15–21 nm. The amino-derivative PEG outer surface coating however, was between 40 nm and 50 nm in Ø, therefore resulting in a final Ø of 50–70 nm for each NH_2 (PEG) QD. The excitation and emission λ of each QD used was 488 nm and 545 nm, respectively. All QDs were investigated at 40 nM. QDs were suspended in complete medium only prior to experimental use.

2.3.2. PBs

Fluorescent, COOH-modified microsphere PBs at a size (Ø) of 20 nm and 200 nm (Molecular Probes, USA) were used (excitation λ 581 nm/emission λ 605). Both sized particles were used at a concentration of 50 μg ml^{-1}. Prior to use, PB stock solutions were vortexed for a total of 1 min in order to aid dispersal and then suspended in complete medium. The resultant particle suspensions were then vortexed for 1 min immediately prior to cellular exposure or experimental analysis.

2.4. Cellular uptake and intracellular localisation of particles

The location of NPs inside intracellular compartments was primarily determined by the staining of J774.A1 cells with a fluorescent antibody to detect the hydrophilic peripheral membrane protein early endocytic antigen-1 (EEA-1). EEA-1 is present in the cytosol of cells, attached to the surface of early endosomes and is essential to vesicle fusion processes during endocytic uptake by cells (Mu et al., 1995; Selak et al., 2004). In addition, this marker has recently been highlighted as an ideal marker for use in assessing the early uptake pattern and processes of NPs, particularly QDs (Zhang and Monteiro-Riviere, 2009).

2.4.1. Investigation to determine NP location inside endosomes

2.4.1.1. Fixed cell preparation. In a 24 well-plate containing 10 mm diameter (Ø) coverslips, 1 ml of complete medium was added. Following incubation for 24 h, cells were treated with 250 μl of QDs (40 nM) or PBs (50 μg ml^{-1}) for 10, 30, 60 and 120 min at 37°C, 5% CO_2. Control cells were treated with complete medium only. Samples were then washed twice with complete medium and fixed with 500 μl of 3% paraformaldehyde in phosphate buffered saline (PBS). Samples were then incubated at 4 °C for 24 h prior to immunofluorescent staining and laser scanning confocal microscopy, as described below.

2.4.1.2. Immunofluorescent staining and imaging. Cells were washed three times with 1% bovine serum albumin (BSA) (diluted in PBS) prior to treatment with 250 μl of 0.1% Triton X100 (diluted in PBS) for 20 min, at room temperature, to permeabilise the cell membrane. Cells were then washed three times with 1% BSA/PBS and treated with 250 μl of 1% BSA/PBS for 1 h, at room temperature, to block any non-specific antibody binding to the cell. Following specific investigation, no non-specific binding of the EEA-1 antibodies was observed (data not shown). After the 1 h treatment period, cells were washed three times with 1% BSA/PBS and then treated with 250 μl of a 1:250 dilution of rabbit polyclonal IgG antibody (Santa Cruz Biotechnology, CA, USA) (EEA-1) in 1% BSA/PBS for 1 h, at room temperature. Cells were subsequently washed three times with 1% BSA/PBS prior to treatment with 250 μl of a 1:200 dilution of goat anti-rabbit antibody (Molecular Probes, OR, USA) (fluorochrome) in 1% BSA/PBS for 45 min, at room temperature. Following the treatment period, cells were washed three times with 1% BSA/PBS. Cover-slips were then inverted and mounted onto microscope slides using Mowoil (Calbiochem, USA) and incubated for 24 h at 4 °C, in the dark. Samples were imaged via a confocal laser scanning microscope Carl Zeiss AG, LSM 510 META, Germany; containing (i) a helium–neon (He–Ne) laser, 5 mW maximum output, wavelength 543 nm, class 3a laser, (ii) a He–Ne laser, 15 mW maximum output, wavelength 633 nm, Class 3b laser and (iii) an argon laser, 200 mW maximum output, wavelengths 418–514 nm, Class 3B laser) using a 63× magnification (numerical aperture (N.A.) 1.3) with digital zoom. All images were captured using consistent optical parameters to enable a direct comparison between particles and over time. Investigation of possible particle uptake via endocytosis was repeated a total of three times ($n = 3$).

2.4.2. Investigation of NP location inside lysosomes and the mitochondria

Additional investigation of the location of NPs inside cells was performed via the use of fluorescent cell permeable markers to assess the potential for NPs present within lysosomal compartments and the mitochondria of live cells (i.e. non-fixed cells). The specific fluorescent markers used to determine this consisted of (i) Lysotracker®, which has a high selectivity for acidic, spherical organelles within cells, and (ii) Mitotracker® which creates a fluorescent conjugate visible via fluorescent microscopy, following an oxidative reaction with the accessible thiol groups on peptides and proteins present within the mitochondria of the cell.

2.4.2.1. Preparation for live cell analysis. In a 45 mm Petri dish (Iwaki, Scientific Laboratory Supplies, Yorkshire, England), 8 ml of J774.A1 cell suspension was added to a sterile, glass microscope cover-slip (Ø – 42 mm) and then incubated for 24 h in an atmosphere of 37 °C, 5% CO_2.

2.4.2.2. Investigation of QD intracellular localisation using Lysotracker®. After the culture period, cells were washed twice with cell culture medium (RPMI 1640 containing L-G without phenol red, P/S and 10% FCS) and then treated with 2 ml of QDs (40 nM) or PBs (50 μg ml^{-1}) (suspended in cell culture medium) for 10, 30, 60 and 120 min at 37 °C, 5% CO_2. Control cells were treated with cell culture medium only. During the final 10 min of each treatment period, cells were treated with 2 μl of a fluorescent cell permeable lysosomal marker (Lysotracker®, Molecular Probes, OR, USA), at a final concentration of 50 nM, diluted in cell culture medium (1 mM stock solution in dimethyl sulfoxide (DMSO)), subsequently diluted to 50 μM working solution in cell culture medium), at 37 °C, 5% CO_2.

2.4.2.3. Investigation of QD intracellular localisation using Mitotracker®. After the culture period, cells were washed twice with cell culture medium (RPMI 1640 containing L-G, without phenol red, P/S and 10% FCS) and then treated with 2 μl of a fluorescent cell permeable mitochondrial marker (Mitotracker®, Molecular Probes, OR, USA) at a final concentration of 500 μM, diluted in cell culture medium (1 mM stock dilution in DMSO), for 30 min at 37 °C, 5% CO_2. Cells were then washed twice with cell culture medium and subsequently treated with 2 ml of QDs (40 nM) or PBs (50 μg ml^{-1}) (suspended in cell culture medium) for 10, 30, 60 and 120 min at 37 °C, 5% CO_2. Control cells were treated with cell culture medium only.

2.4.2.4. Lysotracker® and Mitotracker® image analysis. Following the incubation and treatment periods, samples were washed twice with cell culture medium. The cover-slip was then transferred into a 58 mm × 55 mm aluminium, black anodised perfusion, open and closed cultivation (POC-R) observation chamber (PeCon GmbH, Germany) and 2 ml of cell culture medium (RPMI 1640 containing L-G without phenol red, P/S and 10% FCS) was immediately added to maintain cell viability. Samples were imaged via confocal laser scanning microscopy using the parameters previously described for EEA-1 sample image analysis. Investigation of possible particle localisation in lysosomes and the mitochondria of J774.A1 cells was repeated a total of three times ($n = 3$).

2.5. Transmission electron microscopy (TEM)

The investigation of NP uptake and intracellular fate was also assessed via TEM, with the aim to visualise the precise location of NPs inside cellular compartments and organelles at a high resolution.

2.5.1. Sample preparation

In a 24 well-plate (Helena Biosciences, Gateshead, UK), 1 ml of a 10×10^5 cells ml^{-1} J774.A1 cell suspension, in complete medium, was added to each well and then incubated at 37 °C, 5% CO_2 for 24 h. After the incubation