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The effect of CdSe–ZnS quantum dots on calcium currents and catecholamine secretion in mouse chromaffin cells

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ABSTRACT

Semiconductor nanocrystal quantum dots (QDs) possess an enormous potential of applications in nanomedicine, drug delivery and bioimaging which derives from their unique photoemission and photostability characteristics. In spite of this, however, their interactions with biological systems and impact on human health are still largely unknown. Here we used neurosecretory mouse chromaffin cells of the adrenal gland for testing the effects of CdSe–ZnS core–shell quantum dots (5–36 nM) on Ca^{2+} channels functionality and Ca^{2+}-dependent neurosecretion. Prolonged exposure (24 h) to commonly used concentrations of CdSe–ZnS QDs (≥16 nM) showed that the semiconductor nanocrystal is effectively internalized into the cells without affecting cell integrity (no changes of membrane resistance and cell capacitance). QDs reduced the size of Ca^{2+} currents by ~28% in a voltage-independent manner without affecting channel gating. Correspondingly, depolarization-evoked exocytosis, measured at +10 mV, where Ca^{2+} currents are maximal, was reduced by 29%. CdSe–ZnS QDs reduced the size of the readily releasable pool (RRP) of secretory vesicles by 32%, the frequency of release by 33% and the overall quantity of released catecholamines by 61%, as measured by carbon fibers amperometry. In addition, the Ca^{2+}-dependence of exocytosis was reduced, whereas the catecholamine content of single granules, as well as the kinetics of release, remained unaltered. These data suggest that exposure to CdSe–ZnS QDs impairs Ca^{2+} influx and severely interferes with the functionality of the exocytotic machinery, compromising the overall catecholamine supply from chromaffin cells.

1. Introduction

Quantum dots (QDs) are fluorescent semiconductor nanocrystals, whose basic structure is composed of a core semiconductor, typically CdSe or CdTe, enclosed in a shell of another semiconductor, such as zinc sulfide (ZnS). An additional coating can be added to the fluorescent nanocrystal and QDs functionalization may improve their solubility and preserve their non-aggregated state [1,2]. Shell coatings may also be useful for attaching conjugates to trace therapeutic and diagnostic macromolecules, receptor ligands, or antibodies [3].

QDs are among the most promising nanostructures for *in vitro* diagnostic applications such as cancer diagnosis and therapy [4,5]. Due to their robust and bright light emission, QDs are widely employed for *in vitro* and *in vivo* imaging and recent developments, related to their surface coating and bio-conjugation schemes, have made them most suitable as single particle tracking probes in living cell applications [6–8]. QDs have been used for distinguishing full collapse fusion from kiss and run events at small central nervous system (CNS) nerve terminals [9], or as indicators for movements of Ca^{2+} activated BK channels [10]. However, while the potential of these products holds great promise [11,12], QDs toxicity has been investigated in a variety of tissues and cell lines and it is not clear what may be their adverse effects on human health [13,14]. QDs toxicity varies among the tissues and depends on QDs core structure, coating and functionalization [15]. Most studies reveal that toxicity is mainly associated to the core metal constituents (Cd^{2+} and Zn^{2+}), which can be dispersed in the cytosol. Thus, while coating appears as a solution for limiting the release of free metals from the core, their release over prolonged periods has not yet been comprehensively understood [16].

Here we focused on the commercially available and mostly used carboxyl CdSe/ZnS QDs which are highly soluble in aqueous solutions and can be coupled to a variety of macromolecules. QDs functionalization with carboxyl, as well as with amino, hydroxyl and thiol groups, is rather critical. It varies the hydrodynamic radius of the nanoparticle and surface modifications may drastically alter the spectral properties, nanoparticle stability and the interaction with biological samples [14,17]. A detailed overview concerning the

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physical–chemical properties of QDs, their toxicity and biological fate has recently appeared [13,18].

Focusing on CdSe/ZnS QDs, the cytotoxic effects depend clearly on the biological sample, the QDs functionalization and coating. Internalized carboxyl CdSe/ZnS nanoparticles impair chondrogenesis in mesenchymal stem cells [19] and are retained in the cornea up to 26 days [20]. They also increase intracellular Ca^{2+} in macrophages [21] and rat hippocampal neurons, whereas unmodified CdSe QDs potentiate both Ca^{2+} influx and Ca^{2+} release from the endoplasmic reticulum, and impair voltage-gated Na^{+} channels [22,23]. βNGF peptide-conjugated QDs activate TrkA receptors and initiate neuronal differentiation in PC12 cells [24] while acute applications of steptavidin-conjugated QDs impair the synaptic transmission and plasticity in "*in vivo*" rat hippocampal neurons [25]. To our knowledge little is known about the cytotoxic effects of QDs on neuronal excitability, voltage-gated ion channels and neurotransmitter release. This is a key missing issue that would help assessing the potential risks of using QDs in bioimaging of neuronal and neuroendocrine tissues. For this reason we used the chromaffin cells of the adrenal gland as an experimental model of neuronal-like cell secreting neurotransmitters.

The aim of the present study was to investigate whether carboxyl CdSe–ZnS core–shell quantum dots impair mouse chromaffin cell (MCC) functioning, focusing on the effects on Ca^{2+} influx through voltage-gated Ca^{2+} channels and related exocytosis. To the purpose we used conventional whole-cell patch clamp techniques to measure voltage-gated Ca^{2+} currents and the associated secretory responses viewed through membrane capacitance increases, whereas single exocytic events were detected by amperometric recordings. CdSe–ZnS QDs internalization was confirmed by confocal laser-scanning microscopy and reduced MCCs survival after CdSe–ZnS QDs exposure, evaluated by means of the Trypan Blue exclusion assay. Our results mainly concern QDs toxicity on cell viability and Ca^{2+}-dependent events controlling neurotransmitter release in adrenal chromaffin cells.

2. Materials and methods

2.1. Isolation and culture of mouse adrenal medulla chromaffin cells

Mouse chromaffin cells (MCCs) were obtained from young C57BL/6J male mice (Harlan, Milano, Italy), which were killed by cervical dislocation and cultured as previously discussed [26]. All experiments were conducted in accordance with the guidelines on Animal Care established by the Italian Minister of Health and were approved by the local Animal Care Committee of Turin University After removal, the adrenal glands were placed in Ca^{2+} and Mg^{2+} free Locke's buffer, which contained (mM): 154 NaCl, 3.6 KCl, 5.6 $NaHCO_3$, 5.6 glucose, and 10 HEPES (pH 7.3, at room temperature). The glands were decapsulated, and the medullas were precisely separated from the cortical tissue. Medulla digestion was achieved after 20 min at 37 °C in the enzyme digestion solution, containing DMEM enriched with: 0.16 mM L-cysteine, 1 mM $CaCl_2$, 0.5 mM EDTA, 20 U/ml of papain (Worthington Biochemical, Lakewood, NJ, USA) plus 0.1 mg/ml of DNAse (Sigma, Milan, Italy). The digested glands were then washed twice, with a solution containing DMEM, 1 mM $CaCl_2$, 10 mg/ml BSA, resuspended in 2 ml DMEM supplemented with 15% fetal bovine serum (FBS) (Invitrogen, Grand Island, NY, USA) and pipetted up and down gently to mechanical disaggregating of the glands.

Cells were plated in four-well plastic dishes pretreated with poly-L-ornithine (0.5 mg/ml) and laminin (10 mg/ml in L-15 carbonate). After 1 h, 1.8 ml of DMEM supplemented with 15% FBS, 50 IU/ml penicillin, and 10,000 IU/ml streptomycin (Lonza Group Ltd., Basel, Switzerland), 10 μM Cytosine b-D-arabino-furanoside-hydrochloride (Sigma), 10 μM 5-Fluoro-2′-deoxyuridine (Sigma) was added to the culture medium. Dishes were then incubated at 37 °C in a water-saturated atmosphere with 5% CO_2, and used within 2–4 days after plating.

2.2. Electrophysiological recordings

Voltage-clamp recordings were performed in the whole-cell perforated configuration by using an EPC-10 amplifier and Patch Master software (HEKA Elektronik, Lambrecht, Germany). Patch pipettes were made in borosilicate glass (Kimax 51; Witz Scientific, OH, USA) and filled with an internal solution containing (mM): 135 $CsMeSO_3$, 8 NaCl, 2 $MgCl_2$, 20 HEPES plus amphotericin B, used at a final concentration of 500 μg/ml; pH 7.3, using CsOH. Pipettes' series resistance was 1–2 MΩ. The external bath used as "control solution" contained (mM): 4 TEACl, 126 NaCl, 10 $CaCl_2$, 4 KCl, 2 $MgCl_2$, 10 Glucose, 10 HEPES, pH 7.4, with NaOH. All the experiments were performed at room temperature. Ca^{2+} currents were sampled at 10 kHz and filtered at 2 kHz.

Depolarization-evoked exocytosis was measured as membrane capacitance increases (ΔC_m) after depolarizing pulses. As described elsewhere [26], a sinusoidal wave function was superimposed on the holding potential (±25 mV, 1 kHz), using the Lockin extension of the Patch Master software. The amount of Ca^{2+} entering in the cells during a depolarization (quantity of charge) was calculated as the time integral of the Ca^{2+} current and normalized to cell capacitance (pC/pF). The RRP size was estimated using the double-pulse protocol [27].

For the membrane resistance measurements in current-clamp recordings the patch pipette was filled with an internal solution containing (mM): 135 KAsp, 8 NaCl, 20 HEPES, 2 $MgCl_2$, 5 EGTA plus amphotericin B, (final concentration of 500 μg/ml), pH 7.3 using CsOH. The external solution contained (mM): 130 NaCl, 2 $CaCl_2$, 4 KCl, 2 $MgCl_2$, 10 Glucose, 10 HEPES, pH 7.4 with NaOH. The membrane resistance was evaluated by injecting −10 pA current pulse for 1 s, and then measuring the membrane potential difference at the end of the pulse.

2.3. Amperometric recordings

We performed the amperometric recordings by using carbon fibers purchased from ALA Scientific Instrument Inc. (Westbury, NY, USA) and a HEKA EPC-10 amplifier. Carbon fibers (5 μm diameter) were cut at an angle of 45°, polarized to +800 mV and positioned next to the cell membrane. MCCs were maintained into an extracellular solution containing (mM): 128 NaCl, 2 $MgCl_2$, 10 glucose, 10 HEPES, 10 $CaCl_2$, 4 KCl and then stimulated using a KCl-enriched solution, containing (mM): 100 NaCl, 2 $MgCl_2$, 10 glucose, 10 HEPES, 10 $CaCl_2$, 30 KCl.

Amperometric currents were sampled at 4 kHz, low-pass filtered at 1 kHz, monitored over 120 s. Finally, we analyzed the recordings by using IGOR macros (Wave-Metrics, Lake Oswego, OR, USA) as previously described [28].

2.4. Cell staining and cytotoxicity assays

Carboxyl QDs with CdSe core and ZnS shell, have been purchased from Invitrogen (Qdot® 585 ITK™, Q21311MP). These QDs have mean size of 7–8 nm, further coated with $-COO^{-}$ surface groups to achieve a polymer layer that allows facile dispersion of the QDs in aqueous solution with retention of the optical properties. TEM images of the core–shell are given in the Invitrogen data sheet as well as the absorption and emission spectra of CdSe–ZnS QDs nanocrystals. These latter are narrow and symmetrical with emission maxima near 585 nm. Hydrodynamic diameter of CdSe–ZnS QDs was estimated around 9.3 nm by means of dynamic light scattering (DLS) analysis [29]. The same authors characterized as well the synthesized CdSe/ZnS QD nanoparticles, furnishing TEM images and absorption/emission spectra.

Stock solution of the QDs (8 μM in 50 mM borate, pH 9), was diluted in culture medium to reach a final concentration of 5, 8, 16 or 36 nM. One day after plating, MCCs were incubated for 24 h with CdSe–ZnS QDs; then culture medium was replaced and cells were ready for experiments.

Given our main interest on the effects of QDs on chromaffin cell functionality, cell viability after QDs exposure was tested using the Trypan Blue exclusion assay, which is a simple and rapid technique that stains dead/dying cells with compromised membrane integrity. Although less precise than the MTT assay [30], the Trypan Blue exclusion assay gives good estimates of drug toxicity on living cells [31]. Cells were counted by comparing the number of living (unstained) cells before and after 24 h QDs incubation. Cell counting was performed over 4 dishes of the same cultures for both QD-treated and control cells. Each dish was divided in 49 square areas of 500 × 500 μm^2 and counting of the cells was in a total of 196 areas. Trypan Blue incubation (Sigma; 0.4% final dilution) was performed 10 min before cell counting.

2.5. Confocal microscopy

Mouse chromaffin cells were exposed for 24 h to 16 nM CdSe–ZnS QDs and then washed twice with media to remove any cell-associated dye. In each experiment, a parallel culture incubated with vehicle solution was used as control.

Conventional immunofluorescence procedures were performed to counterstain samples for actin filaments. Briefly, after fixation with 4% paraformaldehyde in phosphate-buffered saline (PBS), cells were permeabilized (PBS^{+}, PBS containing 0.2% Triton X-100) and blocked (1.5% normal donkey serum in PBS^{+}). Actin cytoskeleton was visualized by staining for 2 h with a monoclonal anti-β actin mouse antibody (A5441, Sigma) diluted 1:500 in PBS^{+}, followed by an Alexa Fluor 488-labeled donkey anti-mouse IgG (1:500 in PBS^{+} for 1 h; A21202, InVitrogen, Molecular Probes, Oregon, USA).

Fluorescence signals were detected with a Fluoview 300 confocal laser-scanning microscope (Olympus, Hamburg, Germany). Image acquisition has been performed over 4 control and 4 treated dishes. Stacks of images from consecutive 1 μm-thick slices were processed with Adobe Photoshop (Adobe Systems, Mountain View, CA).