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

Sara Gosso, Daniela Gavello, Carlo N.G. Giachello, Claudio Franchino, Emilio Carbone, Valentina Carabelli

Department of Neuroscience, NIS Center, National Institute of Neuroscience, University of Torino, Corso Raffaello 30, 10125 Torino, Italy

ARTICLE INFO

Article history:
Received 4 July 2011
Accepted 10 August 2011
Available online 27 August 2011

Keywords:
Semiconductor nanocrystals
Adrenal chromaffin cells
Voltage-gated Ca2+ channels
Membrane capacitance changes
Exocytosis
Amperometry

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 Ca2+-channels functionality and Ca2+-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 Ca2+ currents by ~28% in a voltage-independent manner without affecting channel gating. Correspondingly, depolarization-evoked exocytosis, measured at ±10 mV, where Ca2+ 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 Ca2+-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 Ca2+ influx and severely interferes with the functionality of the exocytotic machinery, compromising the overall catecholamine supply from chromaffin cells.

© 2011 Elsevier Ltd. All rights reserved.

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 [12]. 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 Ca2+ 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 (Cd2+ and Zn2+), 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
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 endoplasmic reticulum, and impair voltage-gated Na+ channels [22,23]. JNFG 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 bio-imaging 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) function, focusing on the effects on Ca2+ influx through voltage-gated Ca2+ channels and related exocytosis. To the purpose we used conventional whole-cell patch clamp techniques to measure voltage-gated Ca2+ 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 Ca2+-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, 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 Ca2+- and Mg2+-free Locke’s buffer, which contained (mM): 154 NaCl, 3.6 KCl, 5.6 NaHCO3, 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 CaCl2, 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 CaCl2, 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-S-ornithine (0.5 mg/ml) and laminin (10 mg/ml in 1–5 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-arabinofuranoside-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% CO2, 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 mM Choline, 8 NaCl, 2 MgCl2, 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 TEA, 126 NaCl, 4 KCl, 2 MgCl2, 10 Glucose, 10 HEPES, pH 7.4, with NaOH. All the experiments were performed at room temperature. Ca2+ currents were sampled at 10 kHz and filtered at 2 kHz.

Depolarization-evoked exocytosis was measured as membrane capacitance increases (∆ΔCm) after depolarizing pulses. As described elsewhere [26], a sinusoidal voltage function was superimposed on the holding potential (+25 mV, 1 kHz), using the Lock-in extension of the Patch Master software. The amount of Ca2+ entering in the cells during a depolarization (quantity of charge) was calculated as the time integral of the Ca2+ current and normalized to cell capacitance (PC/Pf). The RBP size was estimated using the double-pulse protocol [27].

For the membrane resistance measurements, 30–50 μm current-clamp recordings the patch pipette was filled with an internal solution containing (mM): 135 KAsp, 8 NaCl, 20 HEPES, 2 MgCl2, 5 EGTA plus amphotericin B, (final concentration of 500 μg/ml), pH 7.3 using CsOH. The external solution contained (mM): 130 NaCl, 2 CaCl2, 4 KCl, 2 MgCl2, 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 AJA 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 in an extracellular solution containing (mM): 128 NaCl, 2 MgCl2, 10 glucose, 10 HEPES, 20 HEPES, 2 MgCl2, 10 CaCl2, 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 produced from Invivogen (Qdot® 585 ITK™, Q2111MP). These QDs have mean size of 7–8 nm, further coated with –COOH 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 MIT 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 μm² 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 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).
The same microscope settings (laser power, filters, detector gain, amplification gain) were used for both treated and control samples. Stacks of images from consecutive 1 μm-thick slices were processed with Adobe Photoshop (Adobe Systems, Mountain View, CA).

3. Results

3.1. CdSe–ZnS QDs internalization in MCCs

QD nanoparticles internalization may occur through different pathways [3], involving either clathrin-mediated endocytosis, macropinocytosis [32–34], or directed to the receptor system, in the case of ligand-conjugated QDs [35,36]. Concerning CdSe–ZnS QDs, internalization occurs passively in human mammary cells along three major pathways: endocytosis, sequestration in early endosomes, translocation to later endosomes or lysosomes [37]. Here we used confocal imaging to monitor CdSe–ZnS QDs (16 nM) internalization in living chromaffin cells after 24 h incubation. As illustrated in Fig. 1-left, consecutive confocal 2 μm z-stack images showed the presence of granule-like red fluorescence spots inside the QDs-treated cells. As observed in other cell preparations [38–40], there was no detectable QD fluorescence in the nucleus. In parallel, no signal was detected in control cells incubated with vehicle (control) solution (right panels). Co-immunolabeling with an antibody specific for β-actin (green image) confirmed the internalization of QDs and helped delineating cell borders and shape.

3.2. MCCs viability after QDs exposure

Although studying cytotoxicity and genotoxicity of CdSe–ZnS was beyond the purpose of the present study, we thought nevertheless important to check the effect of 24 h exposure to 16 nM QDs on MCC survival [41]. Chromaffin cell viability was monitored by the Trypan Blue exclusion assay (see Materials and methods). This organic dye selectively stains dead/dying cells. 24 h after incubation, the density of unstained living cells were approximately 14.6 ± 0.8 cells/mm² under control conditions (Fig. 2A) and decreased to 6.6 ± 0.5 cells/mm² with CdSe–ZnS QDs (p < 0.001). Since CdSe–ZnS QDs were suspended in the culture medium, we cannot exclude that some aggregation of nanoparticles may have occurred, down scoring cell toxicity [42]. Interestingly, also in hippocampal neurons treated with CdSe QDs, viability was

![Fig. 1. Confocal fluorescence images. (A) Representative images of MCCs captured 12 h after 16 nM CdSe–ZnS QDs solution incubation (left panels) or in the absence (control, right panels). Quantum dots are visible in red; cell cytoskeleton counterstained with an anti-β-actin antibody is shown in green. (B) Photomicrographs of 1-μm-thick optical confocal sections showing the same cells depicted in (A) through the z-axis at 2, 4, 6, and 8 μm above the bottom of the dish. Scale bars: A and B, 40 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image)
3.4. Saturation of Ca$^{2+}$ resistance (leakage currents) of MCCs (Fig. 2C). 

That QDs do not affect the membrane surface area and resting potential (Fig. 2B), in good agreement with previously reported values [43]. Comparable histograms representing mean membrane resistance in the absence (black) or presence of CdSe–ZnS QDs 16 nM (gray), measured injecting −10 pA for 1 s (inset). Data averaged from 18 and 19 cells, for controls and after QDs exposure, respectively.

3.3. Effects of CdSe–ZnS QDs on cell capacitance and input resistance

In order to investigate whether CdSe–ZnS QDs exposure (16 nM, 24 h) could alter cell membrane integrity, we measured the cell input resistance ($R_{m}$) by injecting −10 pA current pulse (1 s) from −80 mV holding potential ($V_{h}$) and monitoring the corresponding $\Delta V$ change. On average, controls and QDs-treated cells had the same $R_{m}$ of 41 ± 0.4 GΩ in control and after QDs exposure (Fig. 2B), in good agreement with previously reported values [43]. Membrane capacitance ($C_{m}$), estimated through the slow transient cancellation ($C_{slow}$), was 8.0 ± 0.4 pF in control conditions and 7.2 ± 0.3 pF after CdSe–ZnS QDs exposure ($p = 0.14$), suggesting that QDs do not affect the membrane surface area and resting resistance (leakage currents) of MCCs (Fig. 2C).

3.4. Saturation of Ca$^{2+}$ currents reduction at high CdSe–ZnS QDs concentrations

Chromaffin cells of the adrenal gland express L, N, P/Q and R-type Ca$^{2+}$ channels, which contribute to secretory responses according to their density of expression [44]. Thus, our aim was to understand whether CdSe–ZnS QDs exposure could affect Ca$^{2+}$ channels functionality and Ca$^{2+}$-dependent exocytosis. For this purpose, we first tested the effects of increasing concentrations of CdSe–ZnS QDs (5 nM–36 nM).

Ca$^{2+}$ currents were measured from −40 to +40 mV, holding the cells to $V_{h} = −80$ mV. As shown in Fig. 3, exposure to 5 and 8 nM CdSe–ZnS QDs caused little changes to Ca$^{2+}$ current amplitudes with respect to control cells ($p > 0.05$). Ca$^{2+}$ currents were significantly reduced when applying 16 nM and showed no further reduction at 36 nM CdSe–ZnS QDs. Mean Ca$^{2+}$ current amplitudes measured at +10 mV showed maximal depression of 26 ± 6% (Fig. 3A), suggesting saturating conditions between 16 and 36 nM with an IC$_{50}$ of about 9 nM (Fig. 3B), in good agreement with previously reported IC$_{50}$ of QD cytotoxic effects [39]. Comparable IC$_{50}$ values were obtained at 0 and +20 mV. Representative Ca$^{2+}$ current traces at 5 and 36 nM CdSe–ZnS QDs are shown in Fig. 3A, inset.

3.5. Voltage-independent inhibition of Ca$^{2+}$ currents by CdSe–ZnS QDs

Once set the saturating concentrations of QDs we next studied the voltage-dependence of QDs effects by applying ramp commands from −80 mV to +60 mV for 150 ms. We found that CdSe–ZnS QDs reduced the maximum current amplitude from $-49 ± 4$ pA/pF ($n = 28$) to $-35 ± 3$ pA/pF ($n = 26$, $p < 0.01$), with no effects on the voltage of maximal Ca$^{2+}$ currents: 14.3 ± 1.5 mV and 13.5 ± 1.7 mV for controls and QDs-treated cells, respectively (Fig. 4A). Reduction of Ca$^{2+}$ currents was further investigated by applying step commands, consisting of voltage steps of increasing amplitude from −40 to +50 mV lasting 50 ms (Fig. 4B). Peak Ca$^{2+}$ currents ($I_{p}$) were similarly inhibited in the whole range of potentials examined (28 ± 4%), suggesting that the depressive action exerted by QDs was mostly voltage-independent and caused no changes to the Ca$^{2+}$ reversal potential (−58 mV). Reduction of Ca$^{2+}$ currents by CdSe–ZnS QDs was the same if either measured at the peak of the current or at the steady-state ($I_{ss}$) (27 ± 6%), indicating that QDs have little or no effects on fast Ca$^{2+}$ channel inactivation (Fig. 4D). Interestingly, a similar depression was observed on the tail current amplitude ($I_{t}$) measured on return to −80 mV. Comparing $I_{ss}$ at +20 mV and $I_{t}$ at −80 mV on the same cell, we found that the mean ratio $I_{ss}/I_{t}$ was nearly unchanged in control and QDs-treated cells (0.45 ± 0.03 vs. 0.43 ± 0.03 for $n = 14$ to 19 cells; $p > 0.5$), suggesting that Ca$^{2+}$ current depression is insensitive to voltage also at very negative potentials. This would exclude a possible block of Ca$^{2+}$ channels by free intracellular Cd$^{2+}$ or Zn$^{2+}$ ions released from the inner and outer shell of QDs. A hypothetical block of the Ca$^{2+}$ channel pore by internal Cd$^{2+}$ or Zn$^{2+}$ ions released from the inner and outer shell of QDs. A hypothetical block of the Ca$^{2+}$ channel pore by internal Cd$^{2+}$ or Zn$^{2+}$ ions released from the inner and outer shell of QDs. A hypothetical block of the Ca$^{2+}$ channel pore by internal Cd$^{2+}$ or Zn$^{2+}$ ions released from the inner and outer shell of QDs. A hypothetical block of the Ca$^{2+}$ channel pore by internal Cd$^{2+}$ or Zn$^{2+}$ ions released from the inner and outer shell of QDs. A hypothetical block of the Ca$^{2+}$ channel pore by internal Cd$^{2+}$ or Zn$^{2+}$ ions released from the inner and outer shell of QDs. A hypothetical block of the Ca$^{2+}$ channel pore by internal Cd$^{2+}$ or Zn$^{2+}$ ions released from the inner and outer shell of QDs. A hypothetical block of the Ca$^{2+}$ channel pore by internal Cd$^{2+}$ or Zn$^{2+}$ ions released from the inner and outer shell of QDs. A hypothetical block of the Ca$^{2+}$ channel pore by internal Cd$^{2+}$ or Zn$^{2+}$ ions released from the inner and outer shell of QDs. A hypothetical block of the Ca$^{2+}$ channel pore by internal Cd$^{2+}$ or Zn$^{2+}$ ions released from the inner and outer shell of QDs. A hypothetical block of the Ca$^{2+}$ channel pore by internal Cd$^{2+}$ or Zn$^{2+}$ ions released from the inner and outer shell of QDs.
and QDs-treated cells (2.09 ± 0.12 ms vs 2.4 ± 0.2 ms at +10 mV, Fig. 4C). Taken together these findings suggest that QDs do not alter Ca²⁺ channel gating, but rather reduce the overall Ca²⁺ channel conductance by either reducing Ca²⁺ channel availability (% of functioning channels), probability of channel opening or single channel conductance. Although a reduced Ca²⁺ channel availability appears the most reasonable cause, a final statement should wait for measurements of unitary Ca²⁺ currents in membrane micro areas [45]. In addition, a selective inhibitory effect on either one of the Ca²⁺ channel isoforms expressed by MCCs (L, N, P/Q, R) [43,46] cannot be excluded from the present data.

3.6. Decreased depolarization-evoked secretion after CdSe–ZnS QDs exposure

In order to test how QDs alter catecholamine secretion we measured the depolarization-evoked capacitance increases (∆Cm), the size of the readily releasable pool (RRP) and the Ca²⁺-dependence of exocytosis. In control conditions, cell depolarizations of 100 ms to +10 mV produced a mean quantity of charge equal to 2.8 ± 0.2 pC/pF and a corresponding mean ∆Cm of 41 ± 5 fF (n = 14). After 24 h incubation with CdSe–ZnS QDs (16 nM), the amount of mean charges decreased to 2.1 ± 0.3 pC/pF (p < 0.05), and correspondingly ∆Cm was reduced to 29 ± 3 fF (n = 17), showing a significant decrease with respect to control MCCs (p < 0.01, Fig. 5A and B). Thus, CdSe–ZnS QDs exposure significantly reduced both the quantity of Ca²⁺ entry and the related depolarization-evoked capacitance increases in MCCs. Furthermore, by measuring the size of the readily releasable pool (RRP), we estimated a mean RRP of 117 ± 16 fF in the absence of QDs (18 cells) that decreased to 80 ± 6 fF in QDs-treated cells (n = 17) (p < 0.05, Fig. 5C and D). Fig. 5D shows also that 16 nM QDs incubation causes a net decrease of the probability of vesicle fusion and secretion defined as p = 1 − ∆C2/∆C1 [27]: 0.57 ± 0.04 and 0.48 ± 0.02 (p < 0.05) in control and after CdSe–ZnS QDs treatment. In conclusion, our data suggest that CdSe–ZnS QDs impair secretion from MCCs mainly by reducing Ca²⁺ entry through voltage-gated Ca²⁺ channels, decreasing the size of RRP and lowering the probability of release. By plotting ∆C values versus the density of Ca²⁺ charges entering the cell during step depolarizations (+10 mV) of fixed length (100 s) we could also estimate the Ca²⁺-dependence of secretory responses (Fig. 5E). We found that CdSe–ZnS QDs (16 nM) reduced the slope of the linear Ca²⁺-dependence of secretion: 13.2 ± 1.4 fF/(pC/pF) (controls) versus 10.5 ± 1.2 fF/(pC/pF) for QDs, respectively.

As for Ca²⁺ currents, secretory responses were studied at increasing doses of QDs, starting from 5 to 36 nM. Fig. 6 shows the decrease of quantity of charge (Ca²⁺ ions), and related ∆Cm versus increasing CdSe–ZnS QDs concentrations. Data were averaged over a variable number of cells (see figure legend) obtaining IC₅₀ equal to 9 ± 5 nM. Depolarization-evoked capacitance changes decreased from 41 ± 5 fF (controls) to 28 ± 4 fF, when the maximal concentration was applied (36 nM); in this case IC₅₀ was 6 ± 2 nM. Increasing concentrations of QDs reduced the size of the RRP, as well. Already at CdSe–ZnS QDs 5 nM, we estimated a significant decrease from 117 ± 16 fF to 74 ± 12 fF (p < 0.05).

3.7. Effects of CdSe–ZnS QDs on the frequency and shape of quantal secretory events

Single exocytotic events in MCCs were investigated using carbon fiber electrodes polarized to +800 mV [26]. Exocytosis was stimulated by a KCl-enriched external solution (30 mM, see Methods). Amperometric currents, from control and CdSe–ZnS QDs-treated cells, were monitored over 2 min (Fig. 7A) and analyzed as previously described [28]. Frequency of amperometric spikes was the only parameter significantly modified by QDs (Fig. 7B). Mean frequency was 0.52 ± 0.07 Hz (n = 20 control cells, 1253 spikes) and decreased to 0.35 ± 0.04 Hz (n = 22 QDs-treated cells, 919 spikes, p < 0.05), thus confirming that QDs reduce the probability of vesicle fusion and catecholamine release, as determined by the double-pulse protocol used for estimating the RRP (Fig. 5D). The decreased frequency of secretory events gave rise to a drastic reduction of the cumulative secreted charges (oxidized adrenaline and noradrenaline) over 2 min recordings. Fig. 7B shows the mean cumulative secreted charges averaged over n = 20 control and n = 22 QDs-treated cells. At the end of the stimulation, maximal cumulative charge decreased by 61 ± 12%, from 17 ± 4 pC (control) to 6.6 ± 1.3 pC (QDs), thus confirming that the reduced frequency of released secretory granules drastically affects exocytosis in MCCs.

On the contrary, the analysis of the amperometric spike parameters revealed that QDs did not significantly alter either the maximal oxidation current (Iₘₐₓ, from 39 ± 5 pA to 32 ± 6 pA), the cube root of the charge (Q₁/3, from 0.54 ± 0.02 pC₁/3 to 0.50 ± 0.02 pC₁/3) or the spike’s kinetic parameters (Table 1). By analyzing the distribution of Q₁/3 values, control data were fitted with a double Gaussian function with peaks at 0.47 ± 0.01 pC₁/3 (59%) and 0.69 ± 0.1 pC₁/3 (41%), in good agreement with previously reported data [26,47]. In the presence of QDs, the two peaks of the
bimodal distribution were not significantly changed, centered around $0.40 \pm 0.04$ pC$^{1/3}$ and $0.7 \pm 0.2$ pC$^{1/3}$, and respectively contributing by 80% and 20% to the total area (Fig. 7C); furthermore, it is worth noticing that the percentage of vesicles with higher charge was drastically reduced in presence of QDs. As a final remark, it should be noted that despite all the parameters of Table 1 ($I_{\text{max}}$, $Q$, $t_{1/2}$) are statistically unchanged, they are systematically smaller by 10%–20% with respect to control. Thus, it is likely that taken altogether and summed to the 33% decreased frequency of amperometric bursts they account for the 61% depression of the total quantity of catecholamine released, which is the most depressive effect of QDs on MCCs functioning.

4. Discussion

Using chromaffin cells as an experimental model for monitoring Ca$^{2+}$-dependent secretory processes [26,28,44,48], we provided evidence that CdSe–ZnS quantum dots reduce Ca$^{2+}$ fluxes and Ca$^{2+}$-dependent catecholamine secretion. Given the wide usage of QDs to trace biomolecules and molecular processes in living tissues, these findings are of relevance for understanding QDs cytotoxicity on adrenal chromaffin cell functioning. Further experiments should clarify whether our conclusions can be extrapolated to other hormone-releasing cells, belonging to zona fasciculata, glomerulosa and reticularis of the same gland or to other neuroendocrine tissues, such as pancreatic and pituitary cells, and thus help understanding how QDs could interfere with the molecular mechanisms regulating Ca$^{2+}$-dependent vesicle release in central synapses.

Concerning the action of QDs on voltage-gated Ca$^{2+}$ channels (Figs. 3 and 4), the mean Ca$^{2+}$ current reduction of $28 \pm 4\%$ that we observed at saturating concentrations (16 nM) appears of functional relevance for two reasons. First, because QDs significantly reduces the quantity of released catecholamines (adrenaline and

![Fig. 4. Ca$^{2+}$ currents reduction after CdSe–ZnS QDs exposure.](image-url)
noradrenaline). Second, because QDs attenuates the high-threshold Ca\(^{2+}\) currents (L, N, P/Q and R-type) that regulate the action potential shape and the spontaneous firing frequency of MCCs at rest [43] through the activation of Ca\(^{2+}\)-dependent BK and SK potassium channels. BK and SK channels are highly expressed in MCCs and regulate the speed of AP repolarization, burst firing duration and interspikes interval [49]. Thus, a reduction of available Ca\(^{2+}\) channels by QDs may cause significant changes to action potential shape and cell firing that are not easily predictable. Apart from this issue, we can draw interesting conclusions concerning the voltage-independent action of QDs on Ca\(^{2+}\) currents reduction that we observed in a broad range of voltages (+50 to −80 mV). A voltage-independent reduction of Ca\(^{2+}\) currents would exclude a possible blocking effect of Ca\(^{2+}\) channels by free intracellular Cd\(^{2+}\) (or Zn\(^{2+}\)) ions released from the core or the outer shell of QDs. Direct block of Ca\(^{2+}\) channels by Cd\(^{2+}\) (or Zn\(^{2+}\)) would be strongly voltage-dependent, i.e., maximal around 0 mV and largely removed at very negative voltages (−80 mV) due to the massive inward passage of Ca\(^{2+}\) ions and/or lowering of the energy barrier that would facilitate the exit rate of Cd\(^{2+}\) or Zn\(^{2+}\) ions from their binding sites inside the channel pore [50] (see Results). It is likely therefore that the QDs-induced cytotoxicity observed here derives from the generation of free radicals or other toxic factors associated to the partial release of the core/shell metal constituent that act by...
decreasing the number of functioning Ca$^{2+}$ channels with little or no effects on their gating properties and membrane integrity (Figs. 2 and 4). Notice that this nanoparticle toxicity is markedly different from that induced by multi-walled carbon nanotubes (MWCNT), which lower membrane resistance without affecting Ca$^{2+}$ currents in the same cell preparation [51]. In addition, it is also worth noticing that QDs action on ion channels is rather heterogeneous, depending on nanoparticle functionalization and cell model. CdSe–ZnS QDs (nanomolar range) had no effect on hERG and inward rectifying K$^+$ channels in rat basophilic leukemia (RBL) and CHO Chinese hamster ovary (CHO) cell lines [52]. Acute exposure of unmodified CdSe QDs have been shown to potentiate Ca$^{2+}$ influx and enhance Na$^+$ channels inactivation in hippocampal neurons [23,25].

Our data are in-line with a number of reports in which the core metal constituents of QDs are shown to exert toxic effects on cell functioning, mostly through the oxidation of QDs, decomposition of CdSe/CdTe nanocrystals, and release of free Cd$^{2+}$ ions and/or CdSe complex from the core [15,53,54]. It is shown in fact that QDs-induced cellular damage can be limited by protecting the core from degradation using surface coating (a ZnS layer or a silica shell). This limits Cd$^{2+}$ leaks from the core and reduces free radical generation. In support of this, ZnS core/shell particles are shown to reduce apoptosis and JNK activation [55], although it should be noticed that the ZnS shell does not completely eliminate cytotoxicity and that degradation of the shell or capping material occurs over prolonged QDs exposures [56,57].

In agreement with most in vitro studies, we also found a decreased chromaffin cell viability after 24 h of CdSe–ZnS QDs exposure (≥16 nM) [23,27,58], confirming that cell apoptosis occurs to some degree unless a specific nanoparticle functionalization is performed, as in the case of SiO$_2$-doped QD internalized into cortical neurons that drastically increases cell viability [59]. In this respect, it is worth mentioning that while unmodified CdSe/CdTe QDs cause morphological changes such as loss of plasma membrane integrity, chromatin condensation and damage to mitochondria and nuclei [54], CdSe–ZnS QDs internalization in MCCs preserves membrane integrity, as monitored by the unaltered cell membrane resistance (Fig. 2) [42,60]. This action is again distinct from that of MWCNTs, which are shown to increase membrane leakage (lower membrane resistance) and penetrate the cell nuclei during prolonged exposures [51].

Concerning QDs internalization, we found that 24 h exposure to CdSe–ZnS QDs (16 nM) were sufficient for their complete uptake during resting cell conditions. Although other pathways cannot be ruled out, it is likely that in MCCs, QDs internalization occurs mainly through clathrin-mediated endocytosis following the basal secretory activity of chromaffin cells at rest [9,32,34,61]. This explains the bright fluorescent dots in correspondence of the secretory granules of 200–400 nm diameters and the absence of diffused fluorescence in the nucleaus [Fig. 1], as observed in other cell preparations [38–40]. However, since the mechanism of cellular uptake strongly depends on nanoparticles size and shape and is still largely unknown [3,62], it is also possible that a fraction of CdSe–ZnS QDs enters the chromaffin cells by diffusion across the plasma membrane. These nanocrystals may interfere with the cell membrane, the cytoskeleton and the microfilaments controlling the movements of secretory granules near the cell membrane, and alter the rate of membrane vesicle fusion and catecholamine release (Figs. 5 and 7).

An important issue of our findings is the reduced amount of catecholamine release following exposure to CdSe–ZnS QDs (Fig. 7). Part of the decrease is certainly due to the reduced amount of Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ channels during
depolarization-induced exocytosis. However, QDs decrease also the size of the RRP and the probability of vesicle release as shown by the increased ΔC2/ΔC1 capacitance ratio during double-pulse depolarizations and the lower rate of amperometric bursts during KCl-induced exocytosis. The depression of exocytosis is more dramatic when observing the quantity of released catecholamines during 2 min long KCl depolarization (61%; Fig. 7B) where besides the RRP also the "slowly releasable" pool (SRP) of vesicles contributes to the sustained secretion. This occurs with little changes of both the catecholamine content of single granules and their kinetics of release measured from amperometric recordings, suggesting that QDs internalization interferes with the secretory apparatus without much affecting the mechanism of single vesicle replenishment or catecholamine detachment from the intragranular matrix. Most likely the decreased size of the RRP and probability of vesicle release derive from either an altered coupling 

Table 1

<table>
<thead>
<tr>
<th></th>
<th>I_{max} (pA)</th>
<th>Q (pC)</th>
<th>Q^{1/3} (pC^{1/3})</th>
<th>t_{1/2} (ms)</th>
<th>m (nA/s)</th>
<th>t_p (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>39 ± 5</td>
<td>0.23 ± 0.02</td>
<td>0.54 ± 0.02</td>
<td>6.2 ± 0.3</td>
<td>18 ± 3</td>
<td>4.2 ± 0.2</td>
</tr>
<tr>
<td>QDs</td>
<td>32 ± 6</td>
<td>0.21 ± 0.04</td>
<td>0.50 ± 0.02</td>
<td>6.0 ± 0.5</td>
<td>14 ± 2</td>
<td>4.1 ± 0.2</td>
</tr>
</tbody>
</table>

Fig. 7. Characterization of quantal secretory events from MCCs after CdSe–ZnS QDs exposure. (A) Amperometric spikes were monitored for 2 min and evoked by external perfusion with 30 mM KCl solution, starting from the arrow. Exposure to CdSe–ZnS QDs (16 nM) caused a significant reduction of frequency of the amperometric currents. Inset: representative amperometric spikes in the two conditions. (B) Left panel: mean cumulative distribution of the amperometric charge without and after CdSe–ZnS QDs (16 nM) exposure. Error bars were indicated every 10 000 points uniquely for simplifying the figure. Right panel: bar histograms of mean cumulative charge and mean spike frequency averaged over 22 and 20 control and treated cells, respectively. (C) Q^{1/3} distribution for controls (left) and with CdSe–ZnS QDs (right). Experimental data were fitted to a double Gaussian function (see text for details).
between Ca\textsuperscript{2+} channels and the secretory apparatus or from an altered functionality of microfilaments and membrane anchoring proteins, which regulate vesicle docking to the plasma membrane and vesicle movements from the SRP to the RRP pool. Concerning this issue, there are reports showing that QDs binds to F-actin and shrinks actin cytoskeleton rings of renal epithelial cells [38], moreover Cd\textsuperscript{2+} ions are shown to depolymerize F-actin and affect the cadherin–catenin complex in the same cell type [63]. As F-actin is postulated to play a role in controlling secretory granule access to the plasma membrane [64] it is thus possible that Cd\textsuperscript{2+} ions released from QDs reduce neurosecretion by disassembling the F-actin cytoskeleton rings near the plasma membrane and the formation of the SNARE complex which regulates the “kiss-and-run” and “full-collapse fusion” processes preceding catecholamine release. A consequence of this would be a marked reduction of the RRP and frequency of secretory events.

Despite a number of studies on QDs cytotoxicity, little is known about the effects of nanoparticles on neurosecretion and synaptic transmission. The only report to our knowledge deals with the acute action of CdSe and CdSe–ZnS QDs on field-evoked paired-pulse ratio and LTP synaptic plasticity in in-vivo pulse ratio and LTP synaptic plasticity in hippocampal dentate gyrus area of anesthetized rats [25]. Both parameters are altered functionality of microfilaments and membrane anchoring proteins, which regulate vesicle docking to the plasma membrane and vesicle movements from the SRP to the RRP pool. Concerning this issue, there are reports showing that QDs binds to F-actin and shrinks actin cytoskeleton rings of renal epithelial cells [38], moreover Cd\textsuperscript{2+} ions are shown to depolymerize F-actin and affect the cadherin–catenin complex in the same cell type [63]. As F-actin is postulated to play a role in controlling secretory granule access to the plasma membrane [64] it is thus possible that Cd\textsuperscript{2+} ions released from QDs reduce neurosecretion by disassembling the F-actin cytoskeleton rings near the plasma membrane and the formation of the SNARE complex which regulates the “kiss-and-run” and “full-collapse fusion” processes preceding catecholamine release. A consequence of this would be a marked reduction of the RRP and frequency of secretory events.

5. Conclusions

Given the rapidly growing use of QDs for medical diagnosis and therapy, in-vitro and in-vivo studies using QDs demand for a careful evaluation of their potential cytotoxic effects on cell viability and functionality, as well as on health hazard. Future experiments should be addressed to investigate more deeply the QDs-induced alterations of the molecular events regulating Ca\textsuperscript{2+}-dependent neurotransmitter release in chromaffin cells with the ultimate idea that these findings could help better understanding the cytotoxic effects of semiconductor nanocrystal QDs in other neuroendocrine cells and central neuron synapses.

Acknowledgments

This work was supported by the Foundation Compagnia di SanPaolo “Neuroscience program” to VC, Regione Piemonte “Nanofase program” to EC and by CRT Foundation “Alfieri project” to EC and VC.

References


