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In vitro and *in vivo* assessment of nanotoxicity of CdS quantum dot/aminopolysaccharide bionanoconjugates

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ABSTRACT

The nanotoxicity of Cd-containing quantum dots (QDs) for biomedical applications is very controversial and not completely understood. In this study, we evaluated the cytotoxicity of surface-biofunctionalized CdS QDs with chitosan directly synthesized *via* aqueous route at room temperature. These core-shell CdS-chitosan nanoconjugates showed different degrees of cytotoxic responses using MTT cell proliferation assay toward three human cell cultures, human osteosarcoma cell line (SAOS), non-Hodgkin's B cell lymphoma (Toledo), and human embryonic kidney cell line (HEK293T), under three exposure times (1, 3, and 5 days) and three colloidal concentrations (10 nM, 50 nM, and 100 nM). The results clearly demonstrated that the CdS QDs, regardless to the fact that they were coated with a biocompatible aminopolysaccharide shell, induced a severe dose- and time-dependent inhibition of cell viability. In addition, the HEK293T and SAOS cell lines showed much more sensitive response compared to Toledo, which indicated that the cytotoxicity was also cell-type dependent. The exceptional resistance of Toledo cells to toxic effects of CdS nanoconjugates even at severe test conditions was assigned to specific role of B-lineage cells of the immune defense system. Remarkably, no conclusive evidence of toxicity of CdS nanoconjugates was observed *in vivo* using intravenous injections of CdS nanoconjugates in BALB/c mouse animal models for 30 days, but localized fluorescence was detected in *ex-vivo* liver tissue samples. Therefore, these results prove that there is no guarantee of "risk-free" use of CdS nanoconjugates for *in vivo* applications, even when functionalized with biopolymer ligands, as they can pose an excessive threat due to unpredicted and uncorrelated responses under *in vitro* and *in vivo* biological assays with highly toxic cadmium ions.

1. Introduction

Nanomaterials have been subject of intense research in the recent decades due to their huge potential of impact in all areas of human knowledge. This innovative class of materials has at least one of its dimension ranging from 1 to 100 nm, which offers a myriad of new opportunities to be explored in the field of nanoscience and nanotechnology. As the sizes of materials are reduced toward the nano-scale dimensions, after reaching a particular threshold state, their optical, electronic, magnetic and structural properties are drastically affected from those in the pristine bulk material. Thus, these size-dependent properties of nanomaterials make them very attractive to a broad range of applications, varying from biomedical imaging and nanomedicine to environmental science and nanotechnology [1–3]. Among several alternatives of the so-called low dimension materials (*i.e.*, 0D, 1D and 2D), colloidal semiconductor nanocrystals or quantum dots (QDs) have been intensively investigated for a wide range of biomedical and pharmaceutical applications based on their unique set of optical, electronic, and physicochemical properties [4]. However, despite after approximately three decades since its first report, the large majority of QDs are still produced based on cadmium chalcogenides (*e.g.*, CdX, X = S, Se, Te) using organometallic routes at high temperatures, which has raised increasing environmental and biological concerns regarding their toxicity [4]. Thus, the aqueous colloidal synthesis of semiconductor nanocrystals is an advantageous alternative to the widely organometallic process, because is environmentally friendly, easily scalable and simpler. In addition, the biofunctionalization of the surface has been commonly used as a strategy to minimize or even eliminate the potential toxicity of Cd-containing QDs for *in vivo* applications, which can be performed using distinct techniques, such as biocompatible capping ligands, ligand exchange, and surface passivation with non-toxic semiconductor layer. The use of biocompatible hydrophilic capping ligands *via* direct aqueous processing routes has called the attention of researchers due to their simplicity and compatibility with biological microenvironment. Some biomolecules such as carbohydrates [5,6], peptides [7], amino acids [8],

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enzymes, and proteins [9] have been investigated for the synthesis of fluorescent nanomaterials because they can concurrently join the chemical functional groups with the biological affinity for targeting specific cells and tissues. Aminopolysaccharides such as chitosan and its derivatives have been considered as a very interesting choice for the biological functionalization of QDs, due to their intrinsic biocompatibility, moderate water-solubility, good chemical stability against degradation in physiological medium, environmental compatibility, and abundance as a semi-processed byproduct extracted from natural sources (*e.g.*, chitin from crustacean exoskeletons) [10,11]. Nonetheless, some researchers claim that this approach of surface biofunctionalization of the Cd-based nanomaterials is not sufficient to guarantee the non-toxic behavior in the long term and at high dose of exposure, because they can undergo intracellular degradation processes releasing highly cytotoxic cadmium ions leading to cell death [12]. In contrast, other studies *in vitro* and *in vivo* reported that no toxicity was detected in Cd-based QDs when they were properly covered by a semiconductor outlayer (*i.e.*, ZnS or ZnSe) and additionally functionalized with a hydrophilic ligand. Recently, non-human primates were tested using intravenous injection of Cd-containing QDs and no signal of toxicity was observed in the tissues and organs after 90 days of examination [13]. Thus, despite all the apparently contradictory and inconsistent reports, some aspects have been consensually considered essential on investigating the toxicity of QDs, which are predominantly related to the nanoparticle size and distribution, surface charges, surface chemistry and chemical composition [14]. An interesting and the most comprehensive compiled study so far of Cd-containing QDs was published in *Nature Nanotechnology* by I. L. Medintz's research group [15], wherein the authors conducted a meta-analysis of cellular toxicity of over 300 papers. They examined over 20 attributes (*i.e.*, physicochemical properties and experimental conditions) associated with the toxicity and cell viability, such as QD size and concentration, surface ligand, exposure time, surface chemical modification, cell-line and biological assay type, among others, where they confirmed sub-groups with clearly correlated attributes, but some others with no observable correlation. For that reason, it is incontestably a rather complex coupling of attributes regarding the toxicity response of cells toward Cd-based QDs that poses great challenges for developing a comprehensive cause-effect relationship. Nonetheless, it is important to highlight that the eventual toxicity of Cd-based QDs may restrict some of their potential *in vivo* biomedical applications, but does not impair several *in vitro* applications. In fact, Cd-containing QDs have been intensively researched in the biomedical and pharmaceutical fields, to be used as fluorescent nanoprobes for *in vitro* detection and immunodiagnostic, bioanalytical assay, biosensors, cell labeling, bioimaging of cells and tissues, where potential toxicity of the nanomaterial is not considered a concern [16].

Thus, this study demonstrated for the first time that nanoconjugates made of CdS QDs biofunctionalized with chitosan, as a biocompatible aminopolysaccharide organic shell, showed no toxicity at low concentrations and short incubation times to three cell lines *in vitro*, but evidenced high toxicity at more severe conditions of high concentrations and long incubation period. Moreover, the toxicity responses were very dependent on the cell lineage, where embryonic HEK293T cells and SAOS cells showed very sensitive to physicochemical parameters and lymphoma cells (*i.e.*, Toledo) with an exceptional resistance toward the CdS nanoconjugates. Moreover, the *in vivo* assays performed in BALB/c mouse used as model animal did not indicated evidence of toxicity of the CdS nanoconjugates, but with localized fluorescence in *ex-vivo* liver samples.

2. Experimental

2.1. Materials

All of the reagents and precursors, including cadmium perchlorate hydrate (Aldrich, USA, $Cd(ClO_4)_2 \cdot 6H_2O$), sodium sulfide (Synth, Brazil, >98%, $Na_2S \cdot 9H_2O$), sodium hydroxide (NaOH, ≥99%; Merck, USA), and acetic acid (Synth, Brazil, ≥99.7%, CH_3COOH were used as received. Low molecular weight (LM_W) chitosan powder (Aldrich Chemical, USA, catalogue #448869, M_W = 60–70 kDa; degree of deacetylation = 96.1%; viscosity = 35 cPoise, 1 wt% in 1% acetic acid) was used simultaneously as capping ligand and surface biofunctionalization of QDs. Unless specified otherwise, deionized water (DI water, Millipore Simplicity™) with a resistivity of 18 MΩ·cm was used to prepare the solutions and the procedures were performed at room temperature (RT, 23 ± 2 °C).

2.2. Synthesis of nanoconjugates

The synthesis of CdS nanoconjugates followed a similar procedure reported by our group [17]. Essentially, a chitosan acetate solution (1%, w/v) was prepared by adding chitosan powder (0.5 g) to a 50 mL aqueous solution (2%, v/v) of acetic acid and stirring at room temperature until complete solubilization occurred (pH ~3.6). CdS colloidal nanoparticles stabilized by chitosan (CHI) were synthesized *via* a "green" aqueous processing route in a reaction flask at room temperature (RT) as follows: 2 mL of chitosan solution and 45 mL of DI-water were added to the flask reacting vessel and the pH was adjusted to 6.0 ± 0.1 (NaOH, 0.1 $mol \cdot L^{-1}$). Under moderate magnetic stirring, 4.0 mL of the Cd^{2+} precursor solution ($Cd(ClO_4)_2 \cdot 6H_2O$, 1.0×10^{-2} $mol \cdot L^{-1}$) and 2.5 mL of the S^{2-} precursor solution ($Na_2S \cdot 9H_2O$, 8.0×10^{-3} $mol \cdot L^{-1}$) were added to the flask (the S:Cd molar ratio was kept at 1:2) and stirred for 10 min. The QD colloidal dispersion produced was clear, stable and homogeneous. The QD colloidal solution was dialyzed for 24 h (with water changes after 2 h and 4 h) against 3 L of DI water using a Pur-A-Lyzer™ Mega Dialysis Kit (Sigma, cellulose membrane with molecular weight cut-off filter, MWCO of 12,000 Da) under moderate stirring at RT. After purification, the QD dispersion was stored at RT until further use.

2.3. Physicochemical characterization of quantum dot nanoconjugates

Ultraviolet-visible (UV–vis) spectroscopy measurements were performed using a Perkin-Elmer, Inc. (USA) equipment (Lambda EZ-210) in transmission mode with samples in a quartz cuvette over a wavelength range between 600 and 190 nm. All of the experiments were conducted in triplicate (n = 3) unless specifically noted, and the data were presented as the mean ± standard deviation.

The photoluminescence spectroscopy (PL) of the CdS nanoconjugates was performed based on spectra acquired at RT using a violet diode laser module at λ_{exc} = 405 nm (150-mW, Roithner LaserTechnik, Germany) coupled to a USB4000 VIS-NIR spectrophotometer (Ocean Optics, Inc., USA). All of the tests were performed using a minimum of four repetitions (n ≥ 4). Quantum yield (QY) was measured according to the procedure using Rhodamine 6G (Sigma, USA) in ethanol as the standard at $\lambda_{excitation}$ = 405 nm [18]. Digital color images of the fluorescence of the QDs in the visible range of the spectrum were collected using a "darkroom-chamber" excited with UV irradiation ($\lambda_{excitation}$ = 254 nm, 6 W, Boitton Instruments, Brazil).

Nanostructural characterization of the QDs was based on the images, electron diffraction patterns (ED) and *in situ* elemental chemical analysis using a Tecnai G2-20-FEI (FEI Company, USA) transmission electron microscope (TEM) coupled to an energy dispersive X-ray (EDX) microprobe at an accelerating voltage of 200 kV. In all of the TEM analyses, the samples were prepared by dropping the colloidal dispersion on a porous carbon grid. The QD size and size-distribution data were obtained based on the TEM images by measuring at least 100 randomly selected nanoparticles using image processing program (ImageJ, version 1.50, public domain, National Institutes of Health) [19].

Dynamic light scattering (DLS) and zeta potential (ζ-potential, ZP) analyses were performed using a Brookhaven Instruments Corporation (USA) ZetaPlus instrument with a laser light wavelength of 660 nm (35-