Research Article

In Vitro Toxicity Evaluation of Engineered Cadmium-Coated Silica Nanoparticles on Human Pulmonary Cells

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Cytotoxicity of cadmium-containing silica nanoparticles Cd-SiO$_2$NPs (0.05–100 μg/mL) versus SiO$_2$NPs and CdCl$_2$ was evaluated by an *in vitro* test battery in A549 by assessing (i) mitochondrial function, (ii) membrane integrity/cell morphology, (iii) cell growth/proliferation, (iv) apoptotic pathway, (v) oxidative stress, after short- (24–48 h) and long-term (10 days) exposure. Both Cd-SiO$_2$NPs and CdCl$_2$ produced dose-dependent cytotoxic effects: (i) *MTT-assay*: similar cytotoxicity pattern was observed at both 24 and 48 h, with a more Cd-SiO$_2$NPs pronounced effect than CdCl$_2$. Cd-SiO$_2$NPs induced mortality (about 50%) at 1 μg/mL, CdCl$_2$ at 25 μg/mL; (ii) *calcein-AM/PI staining*: decrease in cell viability, noticeable at 25 μg/mL, enhanced markedly at 50 and 100 μg/mL, after 24 h. Cd-SiO$_2$NPs induced higher mortality than CdCl$_2$ (25% versus 4%, resp., at 25 μg/mL) with further exacerbation after 48h; (iii) *clonogenic assay*: exposure for longer period (10 days) compromised the A549 proliferative capacity at very low dose (0.05 μg/mL); (iv) a progressive activation of *caspase-3 immunolabelling* was detected already at 1 μg/mL; (v) GSH intracellular level was modified by all compounds. In summary, *in vitro* data demonstrated that both Cd-SiO$_2$NPs and CdCl$_2$ affected all investigated endpoints, more markedly after Cd-SiO$_2$NPs, while SiO$_2$NPs influenced GSH only.

1. Introduction

The rapid development of nanotechnology worldwide is accompanied by massive generation and usage of engineered nanoparticles (ENPs), even though essentially most of these NPs have not been sufficiently examined for potential toxicity at this time [1, 2]. Thus, with the exponential growing production of ENPs, the potential for the respiratory system to be exposed to a seemingly countless number of unique NPs is expected to increase, and many aspects related to the size of these nanomaterials, smaller than cells and cellular organelles, have raised concerns about safety [2–4].

Among ENPs, silica/cadmium containing nanomaterials have attracted much attention in the latest years for their applications in medicine and industrial manufacturing, synthesis, and engineering [5–10]. Though silica nanoparticles

(SiO$_2$NPs) are generally considered to be nontoxic, experiments using cell cultures or animal models have indicated dose-dependent cytotoxicity, increased reactive oxygen species, and reversible lung inflammation [11–19]. On the other hand, a large body of evidence supports lung toxicity effects after cadmium exposure when inhaled [20, 21], and although its toxicity mechanisms are not yet fully understood, several reports have described pulmonary inflammatory changes and induction of oxidative stress in response to cadmium inhalation exposure [22].

Some NPs, such as metal-based nanomaterials should represent risk factors for lung diseases, as many of these metals in their native form are known to have fibrogenic, inflammogenic or carcinogenic effects in humans. Evaluation of the NPs biosafety is essentially required by tests examining general toxicity, target organ toxicity, and biocompatibility in

line with regulatory requirements and to identify molecular endpoints and multiple toxicity pathways.

The present study intended to elucidate the toxicological profile of a model nanomaterial namely cadmium-containing silica nanoparticles (Cd-SiO$_2$NPs) by an *in vitro* testing approach.

For instance, a key concept, developed from the strategy proposed by major institutions [23, 24] and international consensus meetings [25], indicates the use of multi-tiered testing protocols to address toxicological research and health risk assessment for NPs, based on (i) physic-chemical characterization, (ii) *in vitro* models by a battery of cytotoxicity tests, and (iii) *in vivo* experiments driven on the basis of the *in vitro* results. Information generated using *in vivo* studies will also provide a database from which to compare *in vitro* studies for identifying additional evidence that assists in explaining findings from *in vivo* nanomaterial toxicity or health effects. Comparing the *in vitro* and *in vivo* results may also help to assess the concordance/discordance between the alternative methods and the *in vivo* methods, and to test the predictability of the alternative methods for the *in vivo* results [26].

The identification of predictive *in vitro* toxicity assays is in line with the recommended attention that should be given to regulatory acceptance as means of promoting the use of alternative methods to animal testing in human safety assessment [27, 28].

Our recent *in vivo* investigation on Cd-SiO$_2$NPs indicated long-lasting lung damage, after intratracheal instillation (i.t.) of these nanoparticles in rats, characterized by morphological alterations, the occurrence of inflammation (accompanied by granuloma formation), stromal fibrogenic reaction, and enhancement of apoptotic phenomena followed by a consequent increased cell proliferation [29]. This pulmonary insult was also associated with an oxidative stress response [30].

In this work, a battery of *in vitro* tests have been used to examine the responses of human lung epithelial cells to Cd-SiO$_2$NPs exposure in terms of metabolic activity (by MTT assay), membrane integrity (by calcein-AM/Propidium Iodide staining), oxidative stress (by GSH content evaluation), apoptosis (by activated caspase-3 evaluation), and growth and cell proliferation (by clonogenic assay) to determine whether the modification of morphological and biochemical parameters evaluated by *in vitro* investigations are in accordance with *in vivo* pulmonary insult caused by these nanoparticles. The effects of Cd-SiO$_2$NPs were assessed in A549 cell line, which represents a widely used cell model to investigate alveolar cell function [31], after short- (24–48 hours) and long-term (10 days) exposure and compared with those caused by treatments with cadmium chloride (CdCl$_2$) and SiO$_2$NPs.

2. Materials and Methods

2.1. Chemicals. All cell culture reagents, chemicals, and cadmium chloride hemi (pentahydrate; CdCl$_2$) were obtained from Sigma-Aldrich (Milan, Italy). Caspase 3 and Alexa 488-labeled antibodies from Molecular Probes (Life Technologies, Monza, Italy) and the GSH quantification Kit from Oxis

International Inc. (Foster City, Ca, USA). Silica nanosize (SiO$_2$NP) was purchased from Degussa Gmbh (Germany) as HiSilTM T700, average pore size 20 nm, surface area 240 m^2/g, and pore specific volume of 0.4 cm^3/g.

2.2. Synthesis and Physico-Chemical Characterization of Engineered Cadmium-Coating Nanoparticles (Cd-SiO$_2$NPs). Synthesis and physico-chemical characterization of Cd-SiNPs are previously described in Coccini et al. [29]. Briefly, Cd-SiO$_2$NPs were produced by the impregnation of SiO$_2$NPs with cadmium nitrate dehydrate (CdNO$_3$ 3.56×10^{-2} M) in an aqueous solution with silica dispersed in a concentration ratio leading to a sample containing 40% Cd by weight. Powder was later subjected to grinding mills with high energy (200 rpm for 1.5 h, 400 rpm for 1.5 h, 600 rpm for 2 h) to get the most equal distribution of particle size and/or aggregates of particles. The entire synthesis preparation was performed under sterile condition to avoid NPs contamination.

Quantitative analyses by scanning transmission electron microscopy (STEM) showed the aggregation of Cd-SiO$_2$NPs and the analysis of the elements in High Angle Annular Dark Field (HAADF) mode (energy-dispersive (EDS) spectra) confirmed the presence of Cd, Si, and O. X-ray diffraction demonstrated amorphous and crystalline phases of the sample. Dynamic light scattering (DLS) of the Cd-SiO$_2$NPs showed tendency to form aggregates and agglomerates of about 350 nm (zeta potential about −23 mV in DMEM). Particles presented spherical form, primary particle size range of 20–80 nm and specific surface area of about 200 m^2/g. Metal impurities (Ca (0.3%), Na (0.2%), K (0.2%), Fe (0.04%), and Mn (0.001%)) and the release of cadmium from nanoparticles dispersed in culture medium were determined by flame-atomic absorption analysis. Maximum cadmium release in culture medium (DMEM) was 28% after 16 h, and it was negligible in the subsequent 10-day period.

2.3. Cell Line and Cell Culture. Human lung epithelial cells (A549 cell line purchased from ECACC, Sigma-Aldrich, Milan, Italy) were used for *in vitro* study of the nanoparticle toxicity. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin. Cells were maintained at 37°C in a humidified atmosphere (95% air/5% CO$_2$).

Stock solutions were prepared by dissolving test materials (Cd-SiO$_2$NPs, CdCl$_2$, and SiO$_2$NPs) in culture medium (DMEM), then cells were exposed to concentrations ranging from 0.05 to 100 μg/mL. Fresh solutions of test materials were prepared shortly before each experiment.

Doses were chosen based on previous experiments in A549 cells showing toxic effects (e.g., apoptosis, necrosis) after cell exposure to concentrations ranging from 5 to 60 μM (corresponding: 0.916 to 10.99 μg/mL) of CdCl$_2$ [32–35].

2.4. Cytotoxicity Study: Short-Term Exposure (24–48 h)

2.4.1. Mitochondrial Function (MTT Assay) and Membrane Integrity (Calcein-AM/Propidium Iodide Staining). The viability was assessed by two dye-based methods: MTT assay (mitochondrial function) and calcein-AM/Propidium Iodide

(PI) staining (membrane integrity). Cells were seeded in 96-well plates at density of 1×10^4 cells/well in complete medium. After 24 h of cell attachment, the cells were exposed to Cd-SiO$_2$NPs at final concentration of Cd between 1 to 100 μg/mL for 24 or 48 h at 37°C and compared to equivalent amount of CdCl$_2$ or SiO$_2$NPs.

At the end of the incubation period, the mitochondrial function was assessed by 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 3 h at 37°C and was quantified spectrophotometrically at 550 nm in Biorad microplate reader. Data were expressed as a percentage of control (untreated cells).

The membrane integrity was evaluated by the co-incubation of the double staining: 2 μM calcein-AM and 2.5 μg/mL PI for 5 min at 37°C. Cells were examined under a Zeiss Axiovert 25 fluorescence microscope combined with a digital camera (Canon powershot G8). The fluorescence images were taken using 32x objective lens with an excitation wavelength of 400, 495, 570 nm; beamsplitter wavelength of 410, 505, 585 nm; and an emission wavelength of 460, 530, 610 nm. Viability was expressed as percent cells retained calcein compared to the total cells counted (calcein-positive plus PI-positive).

2.4.2. Oxidative Stress Evaluation: Glutathione (GSH) Measurement. The concentration of intracellular GSH was determined by colorimetric assay. Briefly, cells were seeded in six-well plates at density of 5×10^5 cells/well. After the treatments with 2 mL of Cd-SiO$_2$NPs, CdCl$_2$ and SiO$_2$NPs (final concentration ranging from 1 to 100 μg/mL in cell culture medium) for 24 and 48 h exposure, the medium was aspired and the cells was washed once with phosphate buffer saline (PBS). Then, the cells were scraped and centrifuged at 1100 rpm for 3 min at 25°C, the superrnatant was removed by aspiration. The cell pellets were resuspended in ice-cold metaphosphoric acid (MPA) and immediately homogenized (Ultra Turrax, Janke & Kunkel) then centrifuged at 3000 g, 4°C for 10 min. Subsequently, the samples were mixed with 4-Chloro-1-Methyl-7-Trifluromethyl-Quinolinium Methylsulfate and 30% sodium hydroxide reagents, and then were incubated for 10 min at room temperature (r.t.) in dark. The absorbance was measured spectrophotometrically (Spectrometer Lambda Bio 20, Perkin Elmer) at 400 nm, total glutathione content was determined with a standard curve.

2.4.3. Apoptotic Pathway: Immunofluorescence Detection of Activated Caspase 3. Cells were seeded in coverslips at density of 2×10^5 cells. After 24-h cell attachment, the cells were exposed to increasing concentrations of Cd-SiO$_2$NPs, CdCl$_2$, and SiO$_2$NPs (1–50 μg/mL) for 24 h at 37°C. At the end of incubation period, the cells were fixed with 4% paraformaldehyde for 20 min at r.t. and then in 70% ethanol over night at −20°C. After rehydration with PBS, the samples were incubated with blocking solution for 30 min at r.t., and then with polyclonal antibodies recognizing caspase 3 (dilution 1 : 200 in PBS). After washing, the bound antibodies were revealed with Alexa 488-labeled (dilution 1 : 100 in PBS) antibody recognizing rabbit-Ig. The slides were counterstained for DNA with 1 μg/mL Propidium Iodide, washed with PBS and finally mounted with Fluoroshield. Cells were examined under a CX41 Olympus fluorescence microscope, excitation light being provided by EPI LED Cassette (FRAEN, Settimo Milanese (MI), Italy) and combined with digital camera (Infinity2). Digital images were captured using 100x objective lens, measurement conditions were: 470 nm excitation ($T\% = 40$), 505 nm dichroic beamsplitter, 510 nm long pass filter.

2.5. Cytotoxicity Study: Long-Term Exposure (10 Days)

2.5.1. Clonogenic Assay. The procedure for clonogenic assay was adopted from Herzog et al. [36]. A549 cells were seeded in six-well at density of 400 cells/well, each well containing 2 mL of cell culture medium. After attachment (about 14 h, time was shorter than the population doubling time), the cells were washed with 2 mL PBS, and treated with 2 mL of Cd-SiO$_2$NPs, CdCl$_2$, and SiO$_2$NPs (final concentration ranging from 0.05 to 100 μg/mL in cell culture medium) over a time period required to form colonies (about 10 days). A colony being defined as at least 50 clones of one cell. At the end of the treatment, the medium was removed and the colonies were fixed, stained with Hematoxylin and then counted for the evaluation of cell survival after Cd-SiO$_2$NP, CdCl$_2$ and SiO$_2$NP treatments. The colonies were examined under Zeiss Axiovert 25 microscope combined with a digital camera (Canon powershot G8).

Digital photographs were taken from each well using 2.5x objective lens. The number of colonies that arose after treatment (surviving fraction) was expressed in terms of plating efficiency (PE). PE was calculated by dividing the number of colonies formed by the number of cells plated per 100.

2.6. Statistics. Data from acute exposure were obtained from three independent experiments each carried out in six replicates. Data from chronic exposure were obtained from two independent experiments and each experiment was carried out in three replicates. Results are expressed as mean ± SD. Statistical significance was assessed by one-way ANOVA. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cytotoxic Activity of Cd-SiO$_2$NPs Compared to CdCl$_2$ and SiO$_2$NPs in A549 Cell Line. *In vitro* cytotoxicity results after short- (24–48 h) and long-term (10 days) exposure of A549 cells to increasing concentrations of Cd-SiO$_2$NPs, CdCl$_2$, and SiO$_2$NPs (from 0.05 to 100 μg/mL) are reported and compared. Mitochondrial function, membrane integrity, oxidative stress, apoptosis were considered as endpoints of acute exposure, while the capacity to form colonies was considered as endpoint of chronic exposure.

3.2. Cytotoxicity Results after Short-Term Exposure (24–48 h)

3.2.1. Mitochondrial Function: MTT Assay. Data of mitochondrial function, evaluated by MTT after 24 and 48 h of exposure to increasing concentrations of Cd-SiO$_2$NPs,
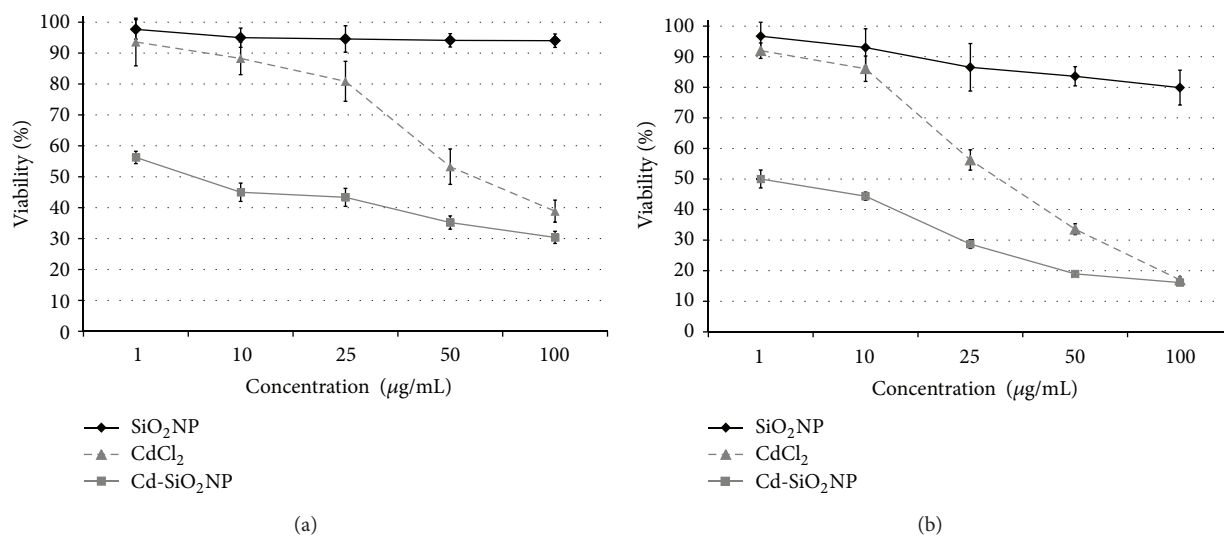
(a)

(b)

Figure 1: Cell viability measured by MTT assay in A549 cells exposed to increasing concentration (1–100 μg/mL) of Cd-SiO$_2$NPs (-□-), CdCl$_2$ (-△-), and SiO$_2$NPs (-◆-) after 24 (a) and 48 h (b). Data are mean ± DS of three separate experiments each carried out in six replicates. Error bars: ±SD.

CdCl$_2$, or SiO$_2$NPs (1–100 μg/mL) and expressed as percentage of the viability of control, are presented in Figures 1(a) and 1(b). Both Cd-SiO$_2$NPs and CdCl$_2$ produced a dose-dependent cytotoxic effect on A549 cells. The pattern of cytotoxicity was similar at both time points (i.e., 24 and 48 h) for either compound, but Cd-SiO$_2$NP cytotoxicity was more pronounced compared to CdCl$_2$. Cd-SiO$_2$NPs induced mortality (about 40% and 50% after 24 and 48 h exposure resp.) already at the lowest dose (1 μg/mL) (Figures 1(a) and 1(b)). The cytotoxic effect of CdCl$_2$ treatment was detected at 25 μg/mL with about 20% mortality after 24 h (Figure 1(a)) and 45% after 48 h exposure (Figure 1(b)). The maximum effect (about 80% mortality) of the two tested materials reached at the highest dose (100 μg/mL) after 48 h exposure (Figure 1(b)).

As graphically represented, in Figures 1(a) and 1(b) (see black line), SiO$_2$NPs did not show any significant cytotoxic effect after both time points considered (24 and 48 h).

3.2.2. Membrane Integrity: Calcein-AM/PI Staining.
Membrane integrity was evaluated by calcein-AM/PI staining after 24 and 48 h exposure to the compounds. Figures 2(a), 2(b), and 2(c) describe a panel of representative and randomly selected microscopic fields of A549 cells treated with increasing concentrations of Cd-SiO$_2$NPs, CdCl$_2$, and SiO$_2$NPs (1–100 μg/mL) after 24 h.

Calcein-AM/PI staining indicated cytotoxic effects. A similar dose-dependent cytotoxic effect was observed after both Cd-SiO$_2$NPs and CdCl$_2$ treatments (Figures 2(a) and 2(b)). A decrease in cell viability was observed as evidenced by the presence of numerous red coloured cells (indicating damage to the cell membrane), starting at 25 μg/mL dose and becoming markedly evident at the highest concentrations of 50 and 100 μg/mL (Figures 2(a) and 2(b)). Semi-quantitative analysis of selected microscopic fields, after 24 h exposure, in terms of cell counts and expressed as percentage of live cells

(green fluorescence), showed difference in cell death between Cd-SiO$_2$NPs and CdCl$_2$ treatments at dose of 25 μg/mL. Cd-SiO$_2$NPs mortality was higher than that caused by CdCl$_2$ treatment (about 25% versus 4%). In both Cd-SiO$_2$NP and CdCl$_2$ groups treated with the lowest dose (1 μg/mL) the green fluorescence was uniformly diffused in cell cytoplasm (indicating the maintained membrane integrity), and cell morphology was not altered (Figures 2(a) and 2(b)).

The effects of Cd-SiO$_2$NPs and CdCl$_2$ were exacerbated after 48 h exposure. Cell viability was decreased of about 60–100% at doses ranging 25–100 μg/mL (data not shown).

Images obtained from SiO$_2$NP treatment showed uniformly diffused green fluorescence and normal cell morphology for all treatment concentrations (1–100 μg/mL) when compared to control (Figure 2(c)). Semi-quantitative analysis with increasing concentrations of SiO$_2$NP treatment (1–100 μg/mL) showed no effects on A549 cells even at the highest dose investigated of 100 μg/mL and after both 24 (Figure 2(c)) and 48 h (data not shown).

3.2.3. Oxidative Stress: Evaluation of GSH Intracellular.
GSH levels were affected by all test materials at both time points considered (24–48 h). The reduction of intracellular GSH levels caused by Cd-SiO$_2$NP or CdCl$_2$ treatments was not dose-dependent: the GSH depletion was ranging from 35 to 40% and from 35 to 45% for Cd-SiO$_2$NPs and CdCl$_2$, respectively, after 48 h exposure (Figure 3). SiO$_2$NPs showed dose-depended depletion of GSH in cells when compared to control with about 55% decrease of GSH intracellular after 48 h exposure at the highest concentration investigated (100 μg/mL; Figure 3).

3.2.4. Apoptotic Pathway: Immunofluorescence Analysis of Activated Caspase 3.
Figure 4 displays a panel of representative randomly selected microscopic fields of A549
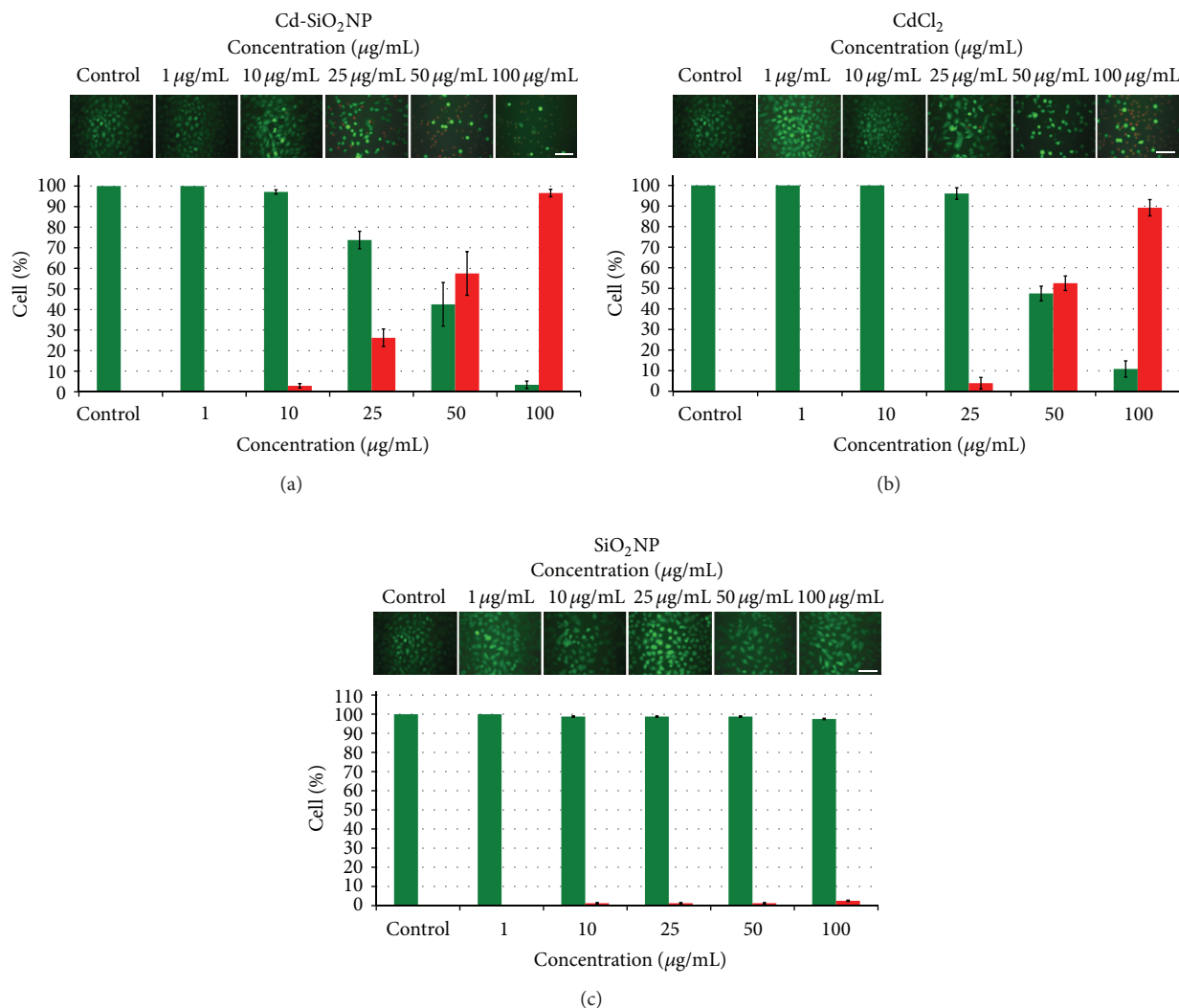
(a)

(b)

(c)

FIGURE 2: Representative images of randomly selected microscopic fields of A549 cells stained with calcein-AM/PI after 24 h exposure to increasing concentration (1–100 μg/mL) of Cd-SiO$_2$NPs (a), CdCl$_2$ (b), and SiO$_2$NPs (c). Dose-dependent cytotoxic effect in both Cd-SiO$_2$NP and CdCl$_2$ treatment groups: there was a strong decrease of viability at higher concentrations ranging from 50 to 100 μg/mL (low or no green fluorescence and red fluorescence indicating cell death). SiO$_2$NPs treated cells showed uniformly diffused green fluorescence at all tested doses similarly to cell controls. Quantitative analysis of the cell loss is shown for each treatment (Green Square Cell Live; Red Square Cell Death). Data are mean ± DS of three separate experiments, error bars: ±SD. (Scale bar: 100 μm).

cells treated with increasing concentrations of Cd-SiO$_2$NPs, CdCl$_2$, and SiO$_2$NPs (1–50 μg/mL). A549 cells exposed to Cd-SiO$_2$NPs or CdCl$_2$ showed a progressive activation of caspase 3 as brilliant green intracellular spots (Figures 4(a)–4(d) and Figures 4(e)–4(h), resp.) directly observed with the lowest dose tested of 1 μg/mL. To note, the advanced stage of apoptosis were observed at the highest dose of 50 μg/mL Cd for both Cd-SiO$_2$NP or CdCl$_2$ treatments (Figures 4(a)–4(d) and Figures 4(e)–4(h), resp.). In addition, a decrease in cell number along with a marked alteration of the morphology can be easily appreciated. On the contrary, the immunocytochemistry analysis to detect activated caspase 3 after 24 h exposure demonstrated no positivity for cells treated with SiO$_2$NPs after any tested doses (1–50 μg/mL;

Figures 4(i)–4(l)) and no appreciable morphology alterations compared to control.

3.3. Cytotoxicity Results after Long-Term Exposure (10 Days)

3.3.1. Clonogenic Assay. To determine whether the prolonged exposure (up to 10 days) to increasing concentrations (0.05–100 μg/mL) of Cd-SiO$_2$NPs, CdCl$_2$, and SiO$_2$NPs might have adverse effects, the proliferation ability and colony forming capacity of A549 cells were evaluated. Figure 5 shows representative images of randomly selected microscopic fields of the different treatment groups (Cd-SiO$_2$NPs, CdCl$_2$, and SiO$_2$NPs). The colonies of SiO$_2$NP groups (Figures 5(a)–5(e)) had roundish colony morphology and similar patterns
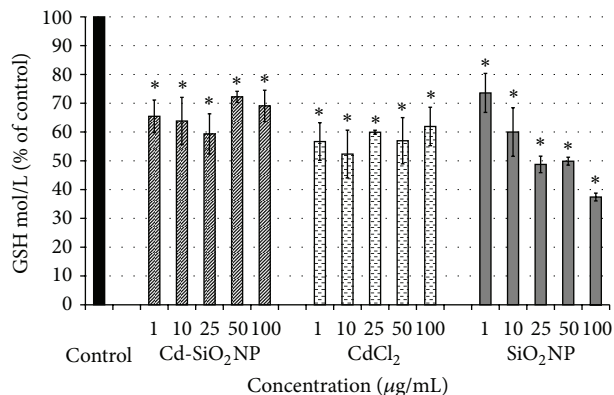
FIGURE 3: Glutathione (GSH) measurement in A549 cells unexposed (control) and exposed to increasing concentrations (1–100 μg/mL) of Cd-SiO$_2$NPs, CdCl$_2$, SiO$_2$NPs after 48 h. GSH levels were affected by all tested materials: the GSH depletion in A549 cells was not dose-dependent after Cd-SiO$_2$NP or CdCl$_2$ treatments, while was dose-depended after SiO$_2$NP treatments. Data are mean ± DS of three separate experiments each carried out in six replicates. $^*P < 0.05$ significant difference from control.

to control (data not shown), while, A549 cells treated with doses ranging from 0.05 to 10 μg/mL of Cd-SiO$_2$NPs and CdCl$_2$ presented few colonies and a drastic reduced size compared to control (data not shown, Figures 5(f)–5(j), and Figures 5(k)–5(o), resp.). At the higher concentrations of either Cd-SiO$_2$NPs or CdCl$_2$ (25 to 100 μg/mL) there was a complete inhibition of the colony formation.

Semi-quantitative analysis showed a strong reduction of colony number (about 45%) already at the lowest tested dose (0.05 μg/mL) for both Cd-SiO$_2$NPs and CdCl$_2$ treatments, until reaching a total inhibition of cell proliferation at the doses from 25 to 100 μg/mL (Figure 6). SiO$_2$NP treatment did not produce adverse effects, indeed the proliferative activity of A549 cells was not inhibited after prolonged exposure to any SiO$_2$NP dose tested (0.05–100 μg/mL; Figure 6).

4. Discussion

The present *in vitro* investigations indicate that Cd-SiO$_2$NP treatment produced *in vitro* deleterious effects after short- (24–48 h) and long-term (up to 10 days) exposure with mitochondrial function severely impaired and activation of caspase-3, depletion of GSH, and inhibition of cell proliferation already observable at the lowest concentration doses (i.e., 1 μg/mL for the short-term exposure and 0.05 μg/mL for the prolonged exposure). Similar cytotoxic profile was observed after CdCl$_2$ treatment. However, the magnitude of effects caused by Cd-SiO$_2$NPs was more pronounced compared to that produced by CdCl$_2$.

A549 acutely exposed to Cd-SiO$_2$NPs showed dose-dependent alterations of mitochondrial function and membrane integrity, as well as activation of caspase-3 already after 24 h exposure. At 48-hr, a further exacerbation of these effects was assessed. CdCl$_2$ also influenced the same cellular

parameters although the effects were less pronounced than those caused by Cd-SiO$_2$NPs. On the contrary, SiO$_2$NPs did not induce cytotoxic effects in this cell model.

Intracellular GSH level changes (decreases) were also observed at both time-points (24 and 48 h) in A549 cells for all tested compounds (Cd-SiO$_2$NPs, CdCl$_2$, and SiO$_2$NPs) suggesting an induction of oxidative stress. Notably, GSH level was the only altered parameter following SiO$_2$NP exposure.

Clonogenic assay, used to evaluate the effects induced after prolonged exposure (10 days), showed the ability of both Cd-SiO$_2$NPs and CdCl$_2$ (at the lowest tested dose of 0.05 μg/mL) to drastically inhibit A549 cell proliferation, while, once again, SiO$_2$NPs were avoided of any effect.

In relation to cadmium toxicity, an extensive database is available on CdCl$_2$-induced pneumotoxicant effects by *in vivo* and *in vitro* models [22]. Mechanistically, Cd, at the cellular level, has been shown to cause oxidative stress by depletion of endogenous antioxidants such as glutathione that is associated with mitochondrial damage and induction of apoptosis [8, 34]. A recent *in vitro* study underlined that low-dose cadmium triggers apoptosis rather than outright necrosis [35]. Indeed CdCl$_2$ concentrations used in proximal tubule (PT) cell culture model to induce apoptosis ranged from 2 to 10 μg/mL (corresponding to 10–50 μM) [37]. For instance, these values are of similar magnitude as the threshold level of 50 μg/g kidney tissue for the development of signs of kidney dysfunction and PT damage indicated in *in vivo* experimental and human studies of chronic Cd^{2+} [38, 39]. Involvement of caspase-3 has been described in several animal models of chronic Cd^{2+} nephrotoxicity [40, 41].

Apoptosis (5-fold higher than control) was also observed in cultures, of rat lung epithelial cell line, exposed for 48 h to 20 μM CdCl$_2$ [42], and was preceded by the up-regulation of oxidant stress genes (glutathione S-transferase-alpha, gamma-glutamylcysteine synthetase, and metallothionein-1), activation of redox sensitive transcription factors (AP-1 and NF-κB), and changes in various forms of glutathione (reduced, oxidized, and protein-bound); thus, altogether, these features suggesting a key role played by the reactive oxygen species.

Our findings indeed evidenced, after both Cd-SiO$_2$NPs and CdCl$_2$, GSH depletion and activation of caspase-3 which is a critical executioner of apoptosis, as it is either partially or totally responsible for the proteolytic cleavage of many key proteins [43].

The observed cytotoxic effects induced by Cd-SiO$_2$NPs after short-and long-term exposure suggest a crucial role of the cadmium moiety in the biological response to Cd-SiO$_2$NPs although it seems unlikely that the changes produced by Cd-SiO$_2$NPs merely reflected the action of cadmium ions released from nanoparticles. Indeed, chemical experiments with Cd-SiO$_2$NPs have demonstrated limited release of cadmium ions from the nanoparticles dispersed in medium culture, the maximum metal release being ca. 28% over a 10-day period.

In addition, the tendency to form aggregates and agglomerates of these doped NPs [29] may also have contributed
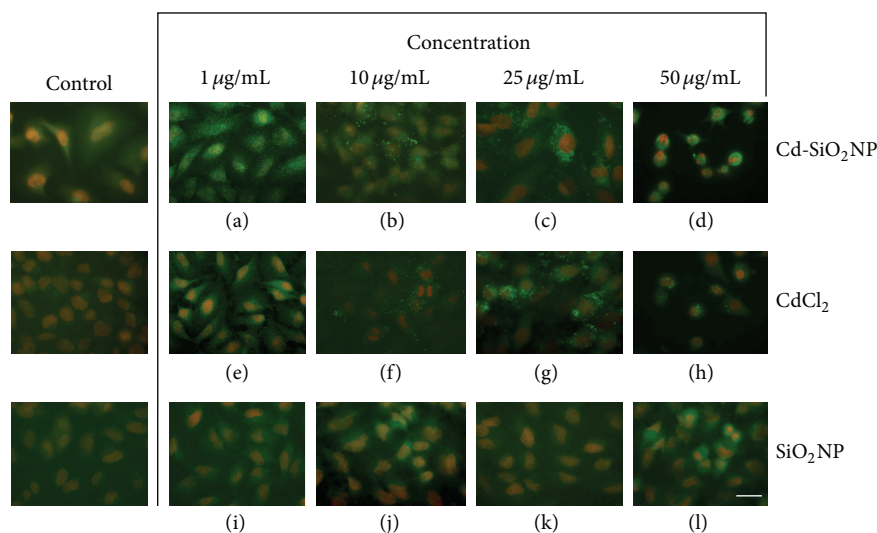
FIGURE 4: Representative images of randomly selected microscopic fields of A549 cells marked with caspase 3 antibody after 24 h incubation with: Cd-SiO$_2$NPs ((a)–(d)), CdCl$_2$ ((e)–(h)), SiO$_2$NPs ((i)–(l)) at the doses indicated in the figure. Activation of caspase 3 is indicated by green spots already at the lowest tested dose of 1 μg/mL in both Cd-SiO$_2$NP and CdCl$_2$ treatment groups, while there was no positivity in SiO$_2$NPs treated cells at all the tested doses similarly to the unexposed cells (control). (Scale bar: 100 μm).
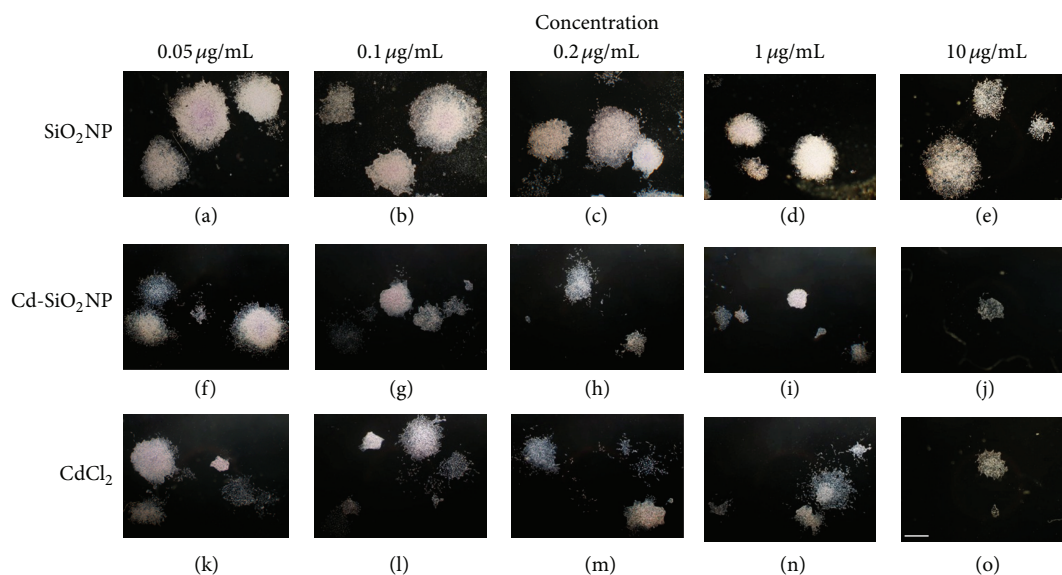

FIGURE 5: Representative images of randomly selected microscopic fields of the colonies formed after 10 consecutive days exposure to increasing concentrations (0.05–100 μg/mL) of Cd-SiO$_2$NPs, CdCl$_2$, and SiO$_2$NPs. SiO$_2$NP colonies showed similar patterns at the all tested doses to the control (control data not shown). Few colonies with reduced size compared to control were observed at the lower concentrations (0.05–10 μg/mL) of Cd-SiO$_2$NPs and CdCl$_2$, while there was a total inhibition of the colonies at the higher tested doses (25–100 μg/mL) for both Cd-SiO$_2$NPs and CdCl$_2$. (Scale bar: 600 μm).

in triggering the described Cd-SiO$_2$NP effects: the DLS data demonstrated an agglomeration and aggregation extent of Cd-SiO$_2$NPs (about 350 nm) greater than that measured for SiO$_2$NPs (about 120 nm). Whether those agglomerated particles retain toxic properties of the individual nanoparticles or are capable of subsequently is a critical question [44].

An additional hypothesis may be related to the nano dimension of the material investigated. In our *in vitro* experiments, the nano dimension may have facilitated the

cell concentration and thus toxicity of the administered cadmium. A "Trojan horse"-type mechanism involving silica nanoparticles as effective carriers for the cellular uptake of toxic metals has been described [45]. In the same type of lung cells used in this study, exposure to SiO$_2$NPs doped with metals such as iron, manganese, cobalt, or titanium was shown to generate higher concentrations of reactive oxygen species and induce more severe oxidative stress compared to equivalent amounts of the respective metal ions [46]. In our
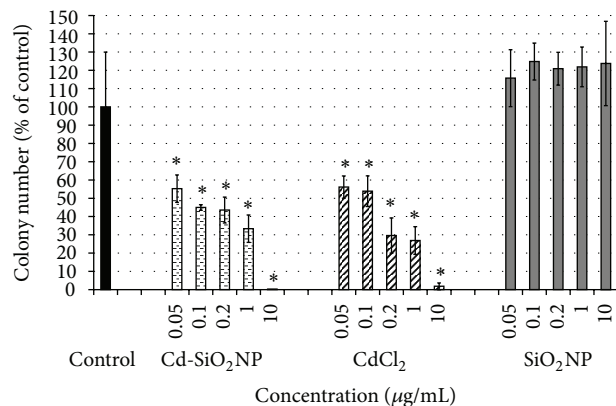
FIGURE 6: Histograms show the number of colonies formed after 10 consecutive days exposure to increasing concentrations of Cd-SiO$_2$NPs, CdCl$_2$, SiO$_2$NPs at the doses indicated in the figure. The formation of A549 colonies appeared to be serious compromised by both Cd-SiO$_2$NP and CdCl$_2$ treatments already at the lowest dose of 0.05 μg/mL, while SiO$_2$NPs had no effects on their formation. Data are mean ± DS of two separate experiments each carried out in three replicates, expressed as percentage of control colonies. $^*P < 0.05$ significant difference from control. Error bars: ±SD.

study, incorporation of Cd into SiO$_2$NPs may have increased the metal dose delivered to target cells although no specific data supporting this process are presently available.

With regard to SiO$_2$NPs, no cytotoxic effects were observed for all concentrations tested and for all exposure times (acute or chronic), with the only exception for the observed GSH depletion after 48 h treatment. Several recent literature data are consistent with the present findings: SiO$_2$NPs penetrated A549 cells and did not cause significant toxic effects at the molecular and cellular levels below 100 μg/mL [47]; it induced low cytotoxicity at concentrations up to 200 μg/mL [48], and generated oxidative stress reflected by reduction of GSH levels [16] or oxidant generation [49]. On the other hand, other investigations indicated A549 cell viability decreases after SiO$_2$NPs exposure down to 100 μg/mL [16], as well as a proinflammatory response triggered by SiO$_2$NPs without blocking cell proliferation or causing cell death in A549 cells [50].

For comparison, our previous *in vivo* results indicated early and persistent lung damage after i.t. instillation of Cd-SiO$_2$NPs in terms of enhanced apoptotic phenomena followed by a significant increase of proliferating cells [29], as well as pulmonary inflammation and fibrosis in rats evidenced by a wide-spread immunoreactivity of both cytokines/chemokines and collagen, respectively. The effects were detectable at the earliest time point, 24 h, and persisted until the 30th day post exposure. Similar pattern of toxic insult was also revealed after i.t. instillation of equivalent amount of CdCl$_2$, although it was less marked than Cd-SiO$_2$NP treatment. The dose of CdCl$_2$ per animal was 400 μg (2.1 μmol) $\equiv$ 247 μg Cd. Cd-SiO$_2$NPs and CdCl$_2$ also showed the capacity to cause long-lasting oxidative stress by increasing the tissue F$_2$-isoprostane levels and pulmonary SOD1, COX-2, and iNOS expressions [30].

On the contrary, no changes involving these markers were observed in animals treated with SiO$_2$NPs.

Altogether, the *in vivo* results showed a higher Cd-SiO$_2$NPs reactivity (regardless of whether form type is present: original, agglomerate, or with sorbed material at NP surface) than SiO$_2$NPs and CdCl$_2$ in the lung tissue.

Both *in vitro* and *in vivo* findings pointed out that Cd-SiO$_2$NP exposure produces a complex and multicomponent insult leading to an exacerbated toxicity response compared to the toxic pattern caused by CdCl$_2$ treatment and essentially much more than SiO$_2$NPs.

5. Conclusions

In vitro experiments in pulmonary cells have provided effective means of screening and ranking the tested materials (Cd-SiO$_2$NPs > CdCl$_2$ > SiO$_2$NPs) using multiple toxicological endpoints (i.e., mitochondrial and membrane alterations, induction of apoptosis, inhibition of growth and proliferation, and intracellular GSH depletion). Coherently, the *in vivo* results have systematically characterized the tissue damage evidenced by lung parenchyma injury and fibrosis, apoptotic phenomena, the occurrence of inflammation, and pulmonary oxidative stress in rats. The *in vivo* targeted tests have complemented and addressed the *in vitro* findings to ensure the adequate evaluation of nanoparticle hazard potential, also in terms of time of appearance and persistence of the toxicological features on living organism.

Highlights

(i) Cd-SiO$_2$NPs produced in vitro toxic effects after short- and long-term exposure;

(ii) similar toxic profile was observed after CdCl$_2$, the effect rank: Cd-SiO$_2$NPs > CdCl$_2$;

(iii) SiO$_2$NPs influenced oxidative stress pathway only;

(iv) *In vitro* tests on lung cells provided effective means of ranking the tested materials.

Conflict of Interests

The authors declare that they have no conflict of interests and they alone are responsible for content and writing of the paper.

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References

[1] F. Marano, S. Hussain, F. Rodrigues-Lima, A. Baeza-Squiban, and S. Boland, "Nanoparticles: molecular targets and cell signalling," *Archives of Toxicology*, vol. 85, no. 7, pp. 733–741, 2011.

[2] A. D. Maynard, R. J. Aitken, T. Butz et al., "Safe handling of nanotechnology," *Nature*, vol. 444, no. 7117, pp. 267–269, 2006.

[3] G. Oberdörster, E. Oberdörster, and J. Oberdörster, "Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles," *Environmental Health Perspectives*, vol. 113, no. 7, pp. 823–839, 2005.

[4] G. Oberdörster, A. Maynard, K. Donaldson et al., "Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy," *Particle and Fibre Toxicology*, vol. 2, article 8, 2005.

[5] N. Ahmed, H. Fessi, and A. Elaissari, "Theranostic applications of nanoparticles in cancer," *Drug Discovery Today*, vol. 17, no. 19-20, pp. 1147–1154, 2012.

[6] M. Mahmoudi, V. Serpooshan, and S. Laurent, "Engineered nanoparticles for biomolecular imaging," *Nanoscale*, vol. 3, no. 8, pp. 3007–3026, 2011.

[7] V. Mamaeva, C. Sahlgren, and M. Lindén, "Mesoporous silica nanoparticles in medicine-recent advances," *Advanced Drug Delivery Reviews*, vol. 65, no. 5, pp. 689–702, 2013.

[8] B. A. Rzigalinski and J. S. Strobl, "Cadmium-containing nanoparticles: perspectives on pharmacology and toxicology of quantum dots," *Toxicology and Applied Pharmacology*, vol. 238, no. 3, pp. 280–288, 2009.

[9] S. Simovic, N. Ghouchi-Eskandar, A. M. Sinn, D. Losic, and C. A. Prestidge, "Silica materials in drug delivery applications," *Current Drug Discovery Technologies*, vol. 8, no. 3, pp. 269–276, 2011.

[10] J. L. Vivero-Escoto, I. I. Slowing, B. G. Trewyn, and V. S.-Y. Lin, "Mesoporous silica nanoparticles for intracellular controlled drug delivery," *Small*, vol. 6, no. 18, pp. 1952–1967, 2010.

[11] W.-S. Cho, M. Choi, B. S. Han et al., "Inflammatory mediators induced by intratracheal instillation of ultrafine amorphous silica particles," *Toxicology Letters*, vol. 175, no. 1–3, pp. 24–33, 2007.

[12] H.-J. Eom and J. Choi, "Oxidative stress of silica nanoparticles in human bronchial epithelial cell, Beas-2B," *Toxicology in Vitro*, vol. 23, no. 7, pp. 1326–1332, 2009.

[13] E. Gazzano, M. Chiazza, M. Polimeri et al., "Physicochemical determinants in the cellular responses to nanostructured amorphous silicas," *Toxicological Sciences*, vol. 128, no. 1, pp. 158–170, 2012.

[14] T. Kaewamatawong, A. Shimada, M. Okajima et al., "Acute and subacute pulmonary toxicity of low dose of ultrafine colloidal silica particles in mice after intratracheal instillation," *Toxicologic Pathology*, vol. 34, no. 7, pp. 958–965, 2006.

[15] W.-K. Lee, B. Torchalski, N. Kohistani, and F. Thévenod, "ABCB1 protects kidney proximal tubule cells against cadmium-induced apoptosis: roles of cadmium and ceramide transport," *Toxicological Sciences*, vol. 121, no. 2, pp. 343–356, 2011.

[16] W. Lin, Y.-W. Huang, X.-D. Zhou, and Y. Ma, "In vitro toxicity of silica nanoparticles in human lung cancer cells," *Toxicology and Applied Pharmacology*, vol. 217, no. 3, pp. 252–259, 2006.

[17] D. Napierska, L. C. J. Thomassen, D. Lison, J. A. Martens, and P. H. Hoet, "The nanosilica hazard: another variable entity," *Particle and Fibre Toxicology*, vol. 7, article 39, 2010.

[18] A. Panas, C. Marquardt, O. Nalcaci et al., "Screening of different metal oxide nanoparticles reveals selective toxicity and inflammatory potential of silica nanoparticles in lung epithelial cells and macrophages," *Nanotoxicology*, vol. 7, no. 3, pp. 259–273, 2013.

[19] E.-J. Park and K. Park, "Oxidative stress and pro-inflammatory responses induced by silica nanoparticles in vivo and in vitro," *Toxicology Letters*, vol. 184, no. 1, pp. 18–25, 2009.

[20] S. Nogué, P. Sanz-Gallén, A. Torras, and F. Boluda, "Chronic overexposure to cadmium fumes associated with IgA mesangial glomerulonephritis," *Occupational Medicine*, vol. 54, no. 4, pp. 265–267, 2004.

[21] G. F. Nordberg, K. Onawa, M. Nordberg, and L. T. Friberg, "Cadmium," in *Handbook of Toxicology of Metals*, G. F. Nordberg, B. A. Fowler, M. Nordberg, and L. Friberg, Eds., Elsevier, Amsterdam, The Netherlands, 2007.

[22] Agency for Toxic Substances and Disease Registry (ATSDR), "Toxicological profile for Cadmium," Atlanta, Ga, USA, Department of Health and Human Services, Public Health Service, 2008.

[23] US EPA, "Draft nanomaterial research strategy," (NRS) EPA/600/S-08/002, United States Environmental Protection Agency, 2008.

[24] US National Cancer Institute—Nanotechnology Characterization Laboratory, http://ncl.cancer.gov/working_assay-cascade.asp.

[25] D. B. Warheit, P. J. A. Borm, C. Hennes, and J. Lademann, "Testing strategies to establish the safety of nanomaterials: conclusions of an ECETOC workshop," *Inhalation Toxicology*, vol. 19, no. 8, pp. 631–643, 2007.

[26] M. A. Maurer-Jones and C. L. Haynes, "Toward correlation in in vivo and in vitro nanotoxicology studies," *The Journal of Law, Medicine & Ethics*, vol. 40, no. 4, pp. 795–801, 2013.

[27] S. Creton, I. C. Dewhurst, L. K. Earl et al., "Acute toxicity testing of chemicals—opportunities to avoid redundant testing and use alternative approaches," *Critical Reviews in Toxicology*, vol. 40, no. 1, pp. 50–83, 2010.

[28] REACH (Registration Evaluation AuthorizationandRestriction of Chemicals), Regulation (EC) No 1907/2006, 2006, http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=oj:l:2006:396:0001:0849:en:pdf.

[29] T. Coccini, S. Barni, R. Vaccarone, P. Mustarelli, L. Manzo, and E. Roda, "Pulmonary toxicity of instilled cadmium-doped silica nanoparticles during acute and subacute stages in rats," *Histology and Histopathology*, vol. 28, no. 2, pp. 195–209, 2013.

[30] T. Coccini, E. Roda, S. Barni, C. Signorini, and L. Manzo, "Long-lasting oxidative pulmonary insult in rat after intratracheal instillation of silica nanoparticles doped with cadmium," *Toxicology*, vol. 302, pp. 203–211, 2012.

[31] C. Jumarie, "Cadmium transport through type II alveolar cell monolayers: contribution of transcellular and paracellular pathways in the rat ATII and the human A549 cells," *Biochimica et Biophysica Acta*, vol. 1564, no. 2, pp. 487–499, 2002.

[32] K. Y. Kwon, J. H. Jang, W. I. Choi, S. Ramachandran, C. H. Cho, and P. T. Cagle, "Expression of apoptotic nuclei by ultrastructural terminal deoxyribonucleotidyl transferase mediated dUTP nick end labeling and detection of FasL, caspases and PARP protein molecules in cadmium induced acute alveolar cell injury," *Toxicology*, vol. 218, no. 2-3, pp. 197–204, 2006.

[33] W.-K. Lee and F. Thévenod, "Novel roles for ceramides, calpains and caspases in kidney proximal tubule cell apoptosis: lessons from in vitro cadmium toxicity studies," *Biochemical Pharmacology*, vol. 76, no. 11, pp. 1323–1332, 2008.

[34] F. Thévenod, "Cadmium and cellular signaling cascades: to be or not to be?" *Toxicology and Applied Pharmacology*, vol. 238, no. 3, pp. 221–239, 2009.

[35] H. Zhang, D. R. Newman, J. C. Bonner, and P. L. Sannes, "Over-expression of human endosulfatase-1 exacerbates cadmium-induced injury to transformed human lung cells in vitro," *Toxicology and Applied Pharmacology*, vol. 265, no. 1, pp. 27–42, 2012.

[36] E. Herzog, A. Casey, F. M. Lyng, G. Chambers, H. J. Byrne, and M. Davoren, "A new approach to the toxicity testing of carbon-based nanomaterials-The clonogenic assay," *Toxicology Letters*, vol. 174, no. 1–3, pp. 49–60, 2007.

[37] W.-K. Lee, M. Abouhamed, and F. Thévenod, "Caspase-dependent and -independent pathways for cadmium-induced apoptosis in cultured kidney proximal tubule cells," *American Journal of Physiology—Renal Physiology*, vol. 291, no. 4, pp. F823–F832, 2006.

[38] L. Järup, M. Berglund, C. G. Elinder, G. Nordberg, and M. Vahter, "Health effects of cadmium exposure—a review of the literature and a risk estimate (Scandinavian Journal of Work, Environment and Health (1998) 24, suppl 1 (52))," *Scandinavian Journal of Work, Environment and Health*, vol. 24, no. 3, p. 240, 1998.

[39] S. Thijssen, J. Maringwa, C. Faes, I. Lambrichts, and E. Van Kerkhove, "Chronic exposure of mice to environmentally relevant, low doses of cadmium leads to early renal damage, not predicted by blood or urine cadmium levels," *Toxicology*, vol. 229, no. 1-2, pp. 145–156, 2007.

[40] T. Aoyagi, K. Hayakawa, K. Miyaji, H. Ishikawa, and M. Hata, "Cadmium nephrotoxicity and evacuation from the body in a rat modeled subchronic intoxication," *International Journal of Urology*, vol. 10, no. 6, pp. 332–338, 2003.

[41] Y. Liu, J. Liu, S. M. Habeebu, M. P. Waalkes, and C. D. Klaassen, "Metallothionein-I/II null mice are sensitive to chronic oral cadmium-induced nephrotoxicity," *Toxicological Sciences*, vol. 57, no. 1, pp. 167–176, 2000.

[42] B. A. Hart, C. H. Lee, G. S. Shukla et al., "Characterization of cadmium-induced apoptosis in rat lung epithelial cells: evidence for the participation of oxidant stress," *Toxicology*, vol. 133, no. 1, pp. 43–58, 1999.

[43] X.-M. Sun, M. MacFarlane, J. Zhuang, B. B. Wolf, D. R. Green, and G. M. Cohen, "Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis," *The Journal of Biological Chemistry*, vol. 274, no. 8, pp. 5053–5060, 1999.

[44] J. M. Balbus, A. D. Maynard, V. L. Colvin et al., "Meeting report: hazard assessment for nanoparticles-report from an interdisciplinary workshop," *Environmental Health Perspectives*, vol. 115, no. 11, pp. 1654–1659, 2007.

[45] E.-J. Park, J. Yi, Y. Kim, K. Choi, and K. Park, "Silver nanoparticles induce cytotoxicity by a Trojan-horse type mechanism," *Toxicology in Vitro*, vol. 24, no. 3, pp. 872–878, 2010.

[46] L. K. Limbach, P. Wick, P. Manser, R. N. Grass, A. Bruinink, and W. J. Stark, "Exposure of engineered nanoparticles to human lung epithelial cells: influence of chemical composition and catalytic activity on oxidative stress," *Environmental Science and Technology*, vol. 41, no. 11, pp. 4158–4163, 2007.

[47] Y. Jin, S. Kannan, M. Wu, and J. X. Zhao, "Toxicity of luminescent silica nanoparticles to living cells," *Chemical Research in Toxicology*, vol. 20, no. 8, pp. 1126–1133, 2007.

[48] Y. Shi, S. Yadav, F. Wang, and H. Wang, "Endotoxin promotes adverse effects of amorphous silica nanoparticles on lung epithelial cells in vitro," *Journal of Toxicology and Environmental Health A*, vol. 73, no. 11, pp. 748–756, 2010.

[49] M. J. Akhtar, M. Ahamed, S. Kumar et al., "Nanotoxicity of pure silica mediated through oxidant generation rather than glutathione depletion in human lung epithelial cells," *Toxicology*, vol. 276, no. 2, pp. 95–102, 2010.

[50] S.-J. Choi, J.-M. Oh, and J.-H. Choy, "Toxicological effects of inorganic nanoparticles on human lung cancer A549 cells," *Journal of Inorganic Biochemistry*, vol. 103, no. 3, pp. 463–471, 2009.