Research Article

***In Vitro* Cytotoxicity of Nanoparticles: A Comparison between Particle Size and Cell Type**

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The reduction in size of Zinc oxide (ZnO) and Silicon dioxide (SiO₂) particles from micron to nano scale offers unique physical characteristics on one hand while making them cytotoxic on other hand. The present study was aimed at comparing cytotoxic effects of ZnO and SiO₂ nanoparticles with their micron size and secondary aim was to compare responses of these particles to two different cell types, namely, human lung epithelial cells (L-132) and human monocytes (THP-1). The L-132 and THP-1 cells were exposed to nano and micron size of ZnO and SiO₂ particles with different concentrations (5–500 µg/mL) for 24 h, and cytotoxicity was analyzed by MTT assay, live-dead staining, and TC-50 was calculated. ZnO and SiO₂ particles showed concentration-dependent cytotoxicity in both cell lines. In size-dependent study, ZnO particles exhibited nearly equal toxicity profile in L-132 cells while in THP-1 cells nano ZnO showed more toxicity than its micron size. The SiO₂ particles showed more toxicity in their nano size than micron size in both cell lines. Human monocytes, THP-1 cells, were more sensitive towards the toxicity of both particles than human lung cells, L-132. The results highlight the difference of cytotoxicity between particle sizes and differential sensitivity of cells towards the particles of same composition. In conclusion, ZnO and SiO₂ particles exhibited concentration-dependent toxicity, which was more in their nano size than micron counterpart. However, the toxic response varies depending on type of cell exposed due to differential sensitivity.

1. Introduction

Rapid advances in the field of nanotechnology led to creation of nano-sized particles with very different chemical and physical properties as compared to particles in their bulk form [1]. Nanoparticles (NPs) possess diverse applications in various aspects of human life, from cosmetics and medical products to water purification and solar energy capture [2]. Due to increasing widespread contact and interaction of nanomaterials (NMs) with human body, the potential hazard to human health and safety has become the matter of concern [3, 4]. Zinc oxide nanoparticles (ZnO-NPs) are commonly utilised for wide ranging applications from paint formulation and ceramic manufacture, to its use as a protective sunscreen in skin and hair care products [5]. Silica (SiO₂) is one of the most abundant oxides present in ambient air, typically in crystalline or amorphous form, and comprises up to 8%

of all total airborne NPs [6]. Silica nanoparticles (SiO₂-NPs) are produced on an industrial scale as additives to cosmetics, drugs, printer toners, varnishes, and food [7]. It is also widely used in biomedical applications as catalyst support, drug carrier [8], and gene delivery [9].

Despite the potential benefits of NPs, nanotoxicity has raised concern about its impact on human health. In NP research, particle size plays an important role in determining the particular biological behaviour of NMs. The large surface area of NPs makes the number of surface atoms or molecules increase exponentially to offer high reactivity. Numerous studies on the size and shape-dependent toxicity of NPs demonstrated biokinetic and biological impacts of NPs and exhibited altered cellular uptake, protein adsorption, accumulation in organelles, and distribution throughout the body [10–15]. One reason for these effects is the correlation between particle size and surface area. Recent studies showed

varied *in vitro* cytotoxicity of NPs with respect to size and cell type. NMs like quantum dots to silica nanotubes demonstrated size-dependent toxicity [10, 13, 16], while Zhang et al. [17] demonstrated differential toxicity of ZnO, TiO₂, SiO₂, and Al₂O₃ NPs of the same size (~20 nm) on human fetal lung fibroblast. Similarly, Yang et al. [18] also reported differential toxicity (ZnO > SWCNT > CB > SiO₂) in primary mouse embryo fibroblast. In addition to the NP form and size, type of cell exposed to the NPs also plays an important role in cytotoxicity. This is due to the variation in cell physiology (e.g., epithelial or lymphoid), proliferation state (tumoral or resting cells), membrane characteristics, and phagocyte characteristics among different cell types [19]. Lanone et al. [20] showed that two human cell lines, namely, A549 and THP-1, exhibited difference in sensitivity towards the toxic effects of 24 NPs. Kroll et al. [21], during analysis of 23 engineered NMs on 10 different cell lines, illustrated that individual cell lines differed significantly in their response depending on particle type and toxicity end point measured. Sayes and collaborators showed diverse sensitivity in terms of viability and inflammation of different nanosized particles (carbonyl iron, silica, and Zinc oxide, 90–500 nm) towards rat lung epithelial cells (L2 cell line) and primary alveolar macrophage [22].

In recent years, studies on size-dependent toxicity between micro- and nano-scale particles [23–28] showed that toxicity of NP is more compared to larger particles and affirmed the hypothesis that NPs in general are more potent in causing damage. All these evidences suggested that NPs of the same or different size exhibited varied toxic response but the studies with reference to their micron size are lacking. Though there are some similar reports with regard to toxicity of various sizes of ZnO and SiO₂ NPs [27, 29–31], the comparative toxic effect of nano and micron size of ZnO and SiO₂ particles is not yet studied properly and requires further complimentary investigations. Thus, the present study investigated comparative toxic potential of nano and micron size of particles in two different cell lines. In present study, ZnO and SiO₂ particles of nano and micron size (ZnO: 100 nm and 5 μ m, SiO₂: 10–20 nm and 10 μ m) were selected. Among cell types, human lung epithelial cells (L-132) were selected because inhalation is one of the major routes of exposure for NPs and micro particles. Secondly, human monocytes (THP-1) were chosen due to their role in clearing foreign particles [32].

2. Materials and Methods

2.1. Reagents. Dulbecco's modified eagle's medium (DMEM), Roswell park memorial institute (RPMI) medium, penicillin-streptomycin-neomycin solution, fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and Zinc oxide nanopowder < 100 nm, Zinc oxide < 5 μ m, Silicon dioxide nanopowder < 10–20 nm, and Silicon dioxide ~0.5–10 μ m were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell Culture. Human lung epithelial cells, L-132, and human monocytes, THP-1, were obtained from the National

Centre for Cell Sciences (NCCS) Pune and maintained as a monolayer and suspension culture in plastic tissue culture flasks in DMEM and RPMI medium, respectively, supplemented with 10% FBS and 100 U/mL penicillin-streptomycin-neomycin mixture. The cells were incubated at 37°C, in a 100% humidified atmosphere containing 5% CO₂.

2.3. Characterization of Particles. Physicochemical properties of particles were analyzed using transmission electron microscopy (TEM), dynamic light scattering (DLS), and zeta potential analyzer. The morphology and size of NPs in the stock dispersion were determined by TEM. Dry powder of particles was suspended in distilled water at a concentration of 1 mg/mL and then sonicated at room temperature for 10 min to form a homogeneous suspension. After sonication, the TEM samples were prepared by drop coating of the stock suspension on carbon-coated copper grids. The films on the grids were allowed to dry prior to measurement. TEM measurements were performed at an accelerating voltage of 120 kV (Model 1200EX, JEOL Ltd., Tokyo, Japan). ZetaPALS (Brookhaven Instruments Corporation, Holtsville, NY) was used to determine the hydrodynamic size by DLS technique [27, 28] and zeta potential of particle suspension in cell culture medium.

2.4. Particle Preparation and Exposure. Before particle exposure to the cells, the particles were dispersed in cell culture medium to a concentration of 2 mg/mL. The dispersion was sonicated for 10 min using an ultrasonicator (Ultrasonic Homogenizer, Biologics Inc., USA.). Cells were plated into a 24-well plate at a density of 1.0×10^6 cells/well. The particle concentration range was selected based on the minimum concentration showing low toxicity to concentration showing maximum toxicity. Between the two cell types chosen for study, THP-1 cells were more sensitive compared to L-132. Based on the results of screening study done by taking different concentration range of particles (data not shown), sensitivity of cells, and previous reports [29, 33–36], a concentration range was selected. The L-132 cells were exposed to both sizes of ZnO particles at concentration of 5, 25, 50, and 100 μ g/mL and THP-1 cells were exposed to both sizes of ZnO particles at a concentration of 10, 20, 40, 80, and 100 μ g/mL for 24 h. Similarly L-132 and THP-1 cells were exposed to both sizes of SiO₂ particles at a concentration range of 100, 200, 300, 400, and 500 μ g/mL and 10, 50, 100, 150, 200, and 250 μ g/mL, respectively, for 24 h. Cells free of particles were used as control cells throughout each assay.

2.5. Cell Viability Assay. To assess effect of particles on the viability of both cell lines, MTT assay [37] was performed. Following exposure, the cells were incubated with MTT (20 μ L/well of 5 mg/mL stock) for 4 h. Mitochondrial dehydrogenases of viable cells reduce the yellowish water-soluble MTT to water-insoluble formazan crystals, which were solubilized with DMSO. The medium was then removed and 200 μ L of DMSO was added into each well to dissolve formazan crystals. For suspension culture, medium was removed by centrifugation and then DMSO was added. After

TABLE 1: Particle characterization.

Particles	Description	Average size ^a	Size using TEM ^b (nm)	Size in media ^c (nm)	PDI ^d	Zeta potential ^e (mV)
Nano ZnO	Zinc oxide nanopowder	<100 nm	94.5 ± 7.7	253.6 ± 2.5	0.311 ± 0.003	17.50 ± 1.17
Micro ZnO	Zinc oxide	<5 μm	158.3 ± 9.8	391.2 ± 0.8	0.266 ± 0.006	−8.60 ± 3.29
Nano SiO ₂	Silicon dioxide nanopowder	<10–20 nm	19.1 ± 2.3	98.5 ± 11.1	0.241 ± 0.018	−36.74 ± 1.67
Micro SiO ₂	Silicon dioxide	~0.5–10 μm	530.9 ± 3.9	892.1 ± 16.7	0.172 ± 0.005	−33.21 ± 5.63

^aAccording to the manufacturer Sigma-Aldrich.

^bUsing transmission electron microscopy.

^{c,d}, and ^eUsing Zeta PALS.

PDI: polydispersity index.

thorough mixing, optical density at 570 nm was detected by microplate reader (Biotek, USA). Interferences of particles were corrected by taking their absorption effect. Control values (without stimuli) were set at 100% viable and all values were expressed as a percentage of the control and respective TC-50 (particle concentration inducing 50% cell mortality) concentrations were calculated by regression analysis using GraphPad Prism software.

2.6. Live-Dead Discrimination through Fluorescence Microscopy. After calculating TC-50 values of each sample, live-dead assay was performed to confirm 50% cell mortality. The cells were exposed to particles with their respective TC-50 value. L-132 cells were exposed to nano and micron ZnO particles at concentration of 37.67 μg/mL and 35.04 μg/mL, respectively, for 24 h; similarly THP-1 cells were exposed to nano and micron ZnO particles at concentration of 17.69 μg/mL and 38.35 μg/mL, respectively, for 24 h. Likewise, L-132 cells were exposed to nano SiO₂ at concentration of 312.5 μg/mL for 24 h while THP-1 cells were exposed to 17.69 μg/mL and 38.35 μg/mL of nano and micron SiO₂ for 24 h, respectively. Control and particle interacted cells were stained using Live/Dead double staining kit (Calbiochem, Germany) as per the manufacturer's instructions and then visualized under fluorescence microscope (Carl Zeiss, Germany).

3. Statistical Analysis

All experiments were done in triplicate and the results were presented as mean ± SEM. The experimental data were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni posttest using GraphPad Prism software version 4.00 (GraphPad Software, San Diego, California, USA). Differences were considered significant at $p < 0.05$. The TC-50 value was calculated using GraphPad Prism software (logarithmic transformation of X -values and nonlinear regression sigmoidal dose-response analysis with variable slope-with bottom and top constraints set at 0 and 100, resp.). Values were given ±95% confidence intervals.

4. Results

4.1. Characterization of Particles. TEM analysis showed that the particles were nearly spherical and cubical in shape (Figure 1). The primary sizes of the particles estimated from

TEM images are presented in Table 1. The hydrodynamic sizes of the dispersed particles were larger than the particle size measured by TEM (Table 1). The zeta potential measurement showed strong agglomeration of particles in aqueous solutions. The observed values were 17.50 mV for nano ZnO, −8.60 mV for micron ZnO, −36.74 mV for nano SiO₂, and −33.21 mV for micron SiO₂ and these values showed that the charge of the hydrated particles was not high enough to repel each other by electrostatic forces.

4.2. Effect of ZnO Particles. ZnO particles showed concentration-dependent toxicity profile in both cell lines (Figures 2(a) and 2(b)). On exposure to L-132 cells, ZnO particles of both sizes illustrated significant decrease in cell viability at concentration from 25 to 100 μg/mL. Nano ZnO and micron ZnO particles showed decrease in cell viability from 73% to 21% and 63% to 23%, respectively, in L-132 cells. Similar trend was observed in THP-1 cells, showing decrease in cell viability from 77% to 21% and 82% to 35% at concentration from 5 to 100 μg/mL.

4.3. Effect of SiO₂ Particles. SiO₂ particles on interaction to both cell lines demonstrated concentration-dependent toxicity (Figures 3(a) and 3(b)). On exposure to L-132 cells, nano SiO₂ particles showed significant reduction in cell viability from 44% to 33% at concentrations from 300 to 500 μg/mL but micron size of SiO₂ particles showed less reduction in cell viability that is only up to 75%. In case of THP-1 cells, nano and micron size SiO₂ particles decreased the cell viability from 71% to 12% and 66% to 48%, respectively, at concentration from 50 to 250 μg/mL.

4.4. Relative Cytotoxicity. TC-50 values for all the particles were calculated from concentration response curve (Table 2). The cytotoxicity data and TC-50 values (Figures 2 and 3 and Table 2) varied significantly between particle size and cell type. The TC-50 values of nano and micron size ZnO particles in L-132 cells were nearly equal (namely, 37.67 μg/mL and 35.04 μg/mL, resp.). However, in THP-1 cells, nano ZnO has lesser TC-50 value (17.69 μg/mL) than its micron size (38.35 μg/mL). In case of SiO₂ particles, only nano SiO₂ showed toxic effect on L-132 with TC-50 value of 312.5 μg/mL cells while its micron size exhibited relatively weak toxic effect only at higher concentrations and did not allow the calculation of TC-50 value. In contrast to these results, in

TABLE 2: Calculated TC-50 concentrations.

Cell type	ZnO particles		SiO ₂ particles	
	Nano ZnO	Micro ZnO	Nano SiO ₂	Micro SiO ₂
L-132	37.67 $\mu\text{g/mL}$ (30.62 to 46.35)	35.04 $\mu\text{g/mL}$ (29.76 to 41.25)	312.5 $\mu\text{g/mL}$ (280.1 to 348.7)	nd
THP-1	17.69 $\mu\text{g/mL}$ (15.75 to 19.87)	38.35 $\mu\text{g/mL}$ (24.52 to 58.13)	88.39 $\mu\text{g/mL}$ (75.74 to 103.2)	231.0 $\mu\text{g/mL}$ (144.4 to 369.6)

nd: not determined (TC-50 can not be calculated).

FIGURE 1: Transmission electron microscope (TEM) images of (a) nano ZnO, (b) micro ZnO, (c) nano SiO₂, and (d) micro SiO₂. Scale bar is 100 nm for all images.

THP-1 cells the TC-50 values of nano and micron size SiO₂ particles were 88.39 $\mu\text{g/mL}$ and 231.0 $\mu\text{g/mL}$, respectively.

4.5. Live-Dead Assay. The 50% cell mortality was further confirmed by discrimination of live/dead cells at respective TC-50 values using fluorescent microscopy. Live cells appeared green (Figures 4 and 5) and cells with compromised membrane were seen red in colour. The fluorescent microscopic

pictures depicted 50% cell mortality as indicated by red staining with propidium iodide at its TC-50 values.

5. Discussion

The small size and the relatively large surface area of NPs resulted in increased toxicity when compared to particles in micrometer size. Therefore, the present study was carried out with objectives to compare the toxic potentials of nano and

FIGURE 2: Cytotoxicity of ZnO particle in (a) L-132 cells and (b) THP-1 cells. Concentration-response curve to obtain TC-50 value. Results were mean $\pm$ SEM of three independent experiments each carried out in triplicate. * $p < 0.05$ in comparison to untreated controls. # $p < 0.05$ indicates significant difference between nano and micron size group.

FIGURE 3: Cytotoxicity of SiO₂ particle in (a) L-132 cells and (b) THP-1 cells. Concentration-response curve to obtain TC-50 value. Results were mean $\pm$ SEM of three independent experiments each carried out in triplicate. * $p < 0.05$ in comparison to untreated controls. # $p < 0.05$ and ## $p < 0.01$ indicate significant difference between nano and micron size group.

micron size of ZnO and SiO₂ particulates on exposure to human cell lines.

The physicochemical characteristics of nano and micron particles of both ZnO and SiO₂ samples revealed that the primary size of micron ZnO and SiO₂ particles provided by the supplier was not compatible with the measurements made in laboratory. The supplier reported that the mean size of micron ZnO particle was $<5 \mu\text{m}$ and micron SiO₂ particle was in the range of 0.5 to 10 μm ; however, the measured particle sizes (Table 1) by TEM were not identical for micron sizes of both ZnO and SiO₂ particles. The average size of nano ZnO and nano SiO₂ was in agreement with the size provided by the

supplier. In comparison to measurements in the dry phase (by TEM) the mean particle sizes and size distributions of particles (measured by DLS) were enhanced when measured in aqueous media. Due to high specific surface area and high surface energy level, NPs have the propensity to aggregate together to form micro-size particles that are more stable in the environment [38, 39]. To minimize the effects of particles aggregation and sedimentation, colloidal suspension of particles was always freshly prepared and sonicated before each experiment.

Nanomaterials induce cell specific responses resulting in variable toxicity [40–42]. Our results indicated that the sizes

FIGURE 4: Fluorescent microscopic images of L-132 cells stained with Cyto-dye and propidium iodide. Cells were exposed at TC-50 concentrations of ZnO and SiO₂ particles for 24 h. Live cells appeared green and dead cells were seen red in colour. Cells were cultured with (a) DMEM only (control); (b) nano ZnO; (c) micro ZnO; (d) nano SiO₂; and (e) TC-20 (i.e., 485 µg/mL) of micro SiO₂. The magnification was 400x.

of particles as well as the target cell type are critical determinants of intracellular responses and degree of cytotoxicity. In the present study, exposure of ZnO and SiO₂ particles caused concentration-dependent cytotoxicity as revealed by cell viability assay. The comparative results indicated that nano ZnO exhibited more toxicity than its micron size in THP-1 cells. The results were consistent with Prach et al. [32] who demonstrated higher toxicity of ZnO-NPs on human T cells than ZnO microparticles and supported the findings. However, in L-132 cells, equal toxicity profile was observed

with nano and micron size of ZnO. These results were in agreement with the results of Lin et al. [30] and Deng et al. [43] who found similar toxicity ZnO particles of different sizes irrespective of size. The equal toxicity profile of ZnO particles observed in this study is possibly due to the lack of difference in nano and micron size of ZnO as revealed from its TEM and DLS data.

In case of SiO₂ particles, nano SiO₂ exhibited more toxicity than its micron size as reported in previous investigations [28, 31, 35] and corroborates the findings. SiO₂ particles

FIGURE 5: Fluorescent microscopic images of THP-1 cells stained with Cyto-dye and propidium iodide. Cells were exposed at TC-50 concentrations of ZnO and SiO₂ particles for 24 h. Live cells appeared green and dead cells were seen red in colour. Cells were cultured with (a) RPMI medium only (control); (b) nano ZnO; (c) micro ZnO; (d) nano SiO₂; (e) micro SiO₂. The magnification was 400x.

possessed more toxic effect on THP-1 cells than on L-132 cells. A comparable response between the cell lines suggested difference in sensitivity of cells towards the same particles. The TC-50 values obtained with L-132 cells were higher than those obtained with THP-1 cells and revealed more sensitivity of THP-1 cells than L-132 cells. These results were consistent with the findings of Lanone et al. [20] who also reported differential sensitivity of human alveolar and macrophage cell line towards various NPs.

To summarize, there is a strong size-selective difference in NP toxicity. As the cellular targets are not the same, one can

expect the cellular response not to be identical, depending on the cell death mechanism [44]. Possible reason to explain the cellular target specificities even for similarly sized particles may be attributed to the function of phagocytosis, which characterizes monocytes (THP-1 cells), but not lung epithelial cells [20]. It is the preliminary study to compare cytotoxicity between particle sizes, although, to find out possible mechanism in its differential toxicity, further studies need to be carried out. Hence the parameters like target cell type and size of the particle have to be carefully taken into account when assessing NP toxicity.

Competing Interests

The authors declare that they have no competing interests.

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