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Toxicity of silica nanoparticles depends on size, dose, and cell type

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Abstract

Monodisperse spherical silica nanoparticles (SNPs) of sizes of 20–200 nm in diameter were employed to study size, dose, and cell-type dependent cytotoxicity in A549 and HepG2 epithelial cells and NIH/3T3 fibroblasts. These uniform SNPs of precisely controlled sizes eliminated uncertainties arising from mixed sizes, and uniquely allowed the probing of effects entirely size-dependent. Cell viability, membrane disruption, oxidative stress, and cellular uptake were studied. The extent and mechanism of SNP cytotoxicity were found to be not only size and dose dependent, but also highly cell type dependent. Furthermore, the 60 nm SNPs exhibited highly unusual behavior in comparison to particles of other sizes tested, implying interesting possibilities for controlling cellular activities using nanoparticles. Specifically, the 60 nm SNPs were preferentially endocytosed by cells and, at high doses, caused a disproportionate decrease in cell viability. The present work may help elucidate certain contradictions among existing results on nanoparticle-induced cytotoxicity.

Key words: Silica nanoparticles; Cellular uptake; Cytotoxicity; Oxidative stress

Introduction

Silica is contacted extensively in our daily lives from both environmental sources, as well as industrial products, such as fumed silica and silica gel. Crystalline silica is known to induce chronic obstructive pulmonary disease, silicosis, and lung cancer,[1] whereas amorphous silica is considered relatively safe. Due to their large surface area-to-volume ratios, tunable pore size and connectivity, biodegradability, and ease of surface modification, amorphous silica nanoparticles (SNPs) have been investigated intensively for use in biomedical applications, including targeted drug delivery and multimodal imaging.[2] Such use of SNPs requires administration to target sites within the body via ingestion, inhalation, intravenous injection, or transdermal penetration. Therefore, investigation and understanding of SNP uptake, retention, cytotoxic potential, and cellular interactions in different tissues and organs are of critical importance as these technologies continue to develop.[3] Much work focusing on the toxic effects of SNPs both *in vitro* and *in vivo* has identified nanoparticle surface area, which is dependent on particle size, as one of the most critical factors contributing to toxicity,[4,5] and yet, previous studies have reported conflicting results regarding the relationship between SNP size and cytotoxicity. For example, investigation of SNPs with diameters of 30, 48, 118, and 535 nm on the viability of mouse keratinocytes revealed decreased viability with decreasing size at 10-200 μg/ml doses.[6] In contrast, another study using SNPs with diameters of 7, 20, and 50 nm at 20-640 μg/ml doses in human hepatocytes found viability to be reduced in the order of 20 > 7 > 50 nm.[7] As detailed in recent reviews,[8-11] uncertainty concerning the biological and toxic effects of SNPs, especially as they relate to particle size, is still widespread in the literature.

In order to clear this controversy, a number of factors relating to SNP effects on biological systems should be investigated. Specifically, it is critical to understand the toxicity of SNPs with diameters smaller than 100 nm, as this is the size that can avoid direct absorption by the reticuloendothelial system following

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intravenous administration.[12] However, few studies have been performed in this size range.[13,14] In addition, understanding the impact of size-dependent cellular uptake of nanoparticles on cytotoxicity is important, especially with regard to the following issues: (1) prevention of damage to cells and alteration of cellular function by nanoparticles, (2) controlling particle accumulation in cells, tumors, and organs for imaging, drug delivery, and therapeutic applications, and (3) ideal design of multi-functionalized nanoparticles for biomedical applications. For example, with regard to the impact of nanoparticles on cell function, *in vitro* studies have reported that SNPs induce endocytosis-dependent reactive oxygen species (ROS) generation leading to DNA damage,[15] the formation of nuclear inclusions and protein aggregates,[16] membrane disruption,[17] and promotion of both apoptotic signaling and cell cycle arrest.[18,19]

Herein, monodisperse spherical SNPs with diameters of 20-200 nm were employed to investigate how particle size and concentration affect the biological activities of human hepatoma (HepG2), mouse embryonic fibroblast (NIH/3T3), and human alveolar carcinoma (A549) cells. These three cell lines were chosen as they represent key tissues that would interact with SNPs during clinical administration or other *in vivo* contact, such as exposure to lung cells following inhalation or exposure to liver cells and fibroblasts via blood circulation.[7,12,21,22] For each cell type, the effect of size and concentration of SNPs on biological responses indicative of potential cytotoxicity, including mitochondrial dysfunction, membrane peroxidation, glutathione depletion, oxidative damage, and induction of morphological changes, were evaluated.

Methods

Synthesis and characterization of SNPs

SNPs and FAM-conjugated SNPs were synthesized using a modified Stöber method.[23] The size and external surface area of the SNPs were determined. Further details are available in the Supplementary Materials.

Cell culture and SNP exposure

All cell lines were purchased from American Type Culture Collection (ATCC) and maintained as a monolayer in tissue culture dishes. The cells were used between passages 10 and 20 and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. SNP suspensions of 10-500 μg/ml were prepared in cell culture medium and applied to cells seeded in a 24-well plate at a density of 5×10^4 cells/well for 24 or 72 h at 37 °C in a 5% CO_2 humidified environment. Cells not exposed to particles (0 μg/ml) served as a control (Con) in each experiment.

Cytotoxicity assays

Cell viability was assessed using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction on cells exposed to SNPs of each size at concentrations of 10-500 μg/ml for 24 and 72 h. Further details are supplied in the Supplementary Materials. The lactate dehydrogenase (LDH) leakage was determined from cells exposed to 20-200 nm SNPs at concentrations of 10-500 μg/ml for 24 or 72 h (Supplementary Materials). Intracellular ROS and cellular glutathione (GSH) levels were determined from cells exposed to 20, 60, 100, and 200 nm SNPs at concentrations of 50, 100, 200, and 500 μg/ml for 24 h using fluorogenic probes (Supplementary Materials).

Cellular morphology and SNP uptake

The morphology of A549 and NIH/3T3 cells in the presence of SNPs and the time-dependent uptake of FAM-conjugated SNPs on NIH/3T3 cells were visualized by phase-contrast microcopy and confocal fluorescence microscopy (Supplementary Materials). For transmission electron microscopy (TEM) observation, suspensions containing 10 and 200 μg/ml of 20, 60, and 200 nm SNPs in cell culture medium were applied to A549 cells at 37 °C in 5% CO_2 for 24 h. TEM sample preparation is described in the Supplementary Materials.

Results

Characterization of SNPs

Figure 1 shows TEM and scanning electron microscopy (SEM) images of SNPs with diameters of 21.06 ± 3.38 (20), 40.26 ± 3.14 (40), 59.72 ± 4.58 (60), 81.05 ± 4.99 (80), 100.33 ± 4.78 (100), 149.87 ± 5.70 (150) and 199.89 ± 6.57 (200) nm, indicating that the particles are spherical and well-defined in size. The 20, 60, 100, and 200 nm SNPs were subjected to dynamic light scattering (DLS) to measure hydrodynamic sizes, which were 37.82 ± 6.17, 70.12 ± 17.5, 105.5 ± 38.58 and 203.3 ± 50.75 nm in DI water, respectively. The sizes measured by the two different methods indicate that the smaller SNPs, 20 and 60 nm, aggregate significantly in DI water. Corresponding zeta potentials were measured to be −24.5 ± 3.74, −38.1 ± 9.85, −31.7 ± 8.73 and −36.3 ± 8.08, respectively, indicating good overall particle stability. DLS measurements were also attempted in culture media, but found to be unreliable due to protein aggregates.[24] Finally, the external surface areas of the 20, 60, 100, and 200 nm SNPs were calculated to be 88.905, 49.724, 29.289, and 13.762 m^2/g, respectively, by the Brunauer–Emmett–Teller (BET) method.

Cell viability

Cytotoxicity of SNPs was evaluated using MTT assay after 24 and 72 h of nanoparticle exposure at doses of 10-500 μg/ml (Figure 2) since the internalization of SNPs at doses over 10 μg/ml is known to typically affect the viability of mammalian cells.[25-27] Although SNP doses over 100 μg/ml are unrealistic for inhaled nanoparticles, SNPs can accumulate in specific cells/organs following administration either by normal actions of the body (clearance by the mononuclear phagocyte system), as a result of long-term exposure, or by specific targeting for drug delivery applications.[28,29] For the A549 cells, SNPs of all sizes caused a reduction in viability at doses ≥50 μg/ml ($p < 0.05$) with no significant differences between doses after 24 h of exposure (Figure 2, *A*). After 72 h of nanoparticle exposure (Figure 2, *B*),