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## Size-dependent cytotoxic effects of amorphous silica nanoparticles on Langerhans cells

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Amorphous silica nanoparticles (nSPs), are widely used in medicines, cosmetics and food. However, due to their reduced particle size they are suspected to pose new risks induced by changes in biological reactivity and kinetics, which differ from those of bulk materials. In a previous study, we showed that silica particles with a diameter of 70 nm penetrated the stratum corneum (SC) of mouse skin and were taken up by living cells such as keratinocytes and Langerhans cells. To clarify the relationship between particle size, distribution and cellular response, we have evaluated size-dependent intracellular localization and cytotoxicity of silica particles, using the mouse epidermal Langerhans cell line XS52. On treatment with silica particles of diameters 70, 300, and 1000 nm, cellular uptake and cytotoxicity increased with reduction in particle size. These results suggest that smaller sized silica particles induced greater cytotoxicity against Langerhans cells, which was correlated with the quantity of particle uptake into the cells.

### 1. Introduction

The recent development of nanoscale engineering represents a current dynamic area of interdisciplinary research, incorporating nanomaterials (NMs) into a diverse product matrix such as diagnostics, food additives and cosmetics. Because amorphous silica nanoparticles (nSPs) and titanium oxide nanoparticles, etc. are colorless and reflect ultraviolet more efficiently than micro-sized particles, nSPs and titanium oxide nanoparticles are already used as cosmetic vehicles or functional ingredients in many cosmetics such as foundation creams and sunscreens. However, because NMs may possess novel properties, kinetics, and biological effects different from those of micro size bulk materials, their potential harmful effects on humans are raising concerns about their safety. Thus, there is an urgent need for risk assessment of NMs. To achieve this, it is most important to analyze the relationship between particle-size parameters, cellular distribution and biological effects, allowing prediction and avoidance of risk in using NMs.

In a previous study, we showed that silica particles with a diameter of 70 nm penetrated the stratum corneum (SC) of mouse skin and were taken up by living cells such as keratinocytes and Langerhans cells. So, to reveal the relationship between particle size, distribution, and cellular response, we evaluated size-dependent intracellular localization and cytotoxicity of silica particles, using the mouse epidermal Langerhans cell line XS52.

### 2. Investigations, results and discussion

To assess cellular uptake of nSPs, we observed XS52 cells treated with 100 µg/ml nSP70, nSP300 and mSP1000 using transmission electron microscopy (TEM). We found that nSP300 and mSP1000 were located in cytoplasm only (Fig. 1c and d), while nSP70 was surprisingly located in nucleus as well as cytoplasm (Fig. 1a and b). Furthermore the quantity of silica particles taken up by the cells increased as particle size decreased. These results suggested that the uptake and localization of silica particles altered with particle size.

We next investigated biological effects of various-sized silica particles in XS52 cells. To assess the effect of treatments with nSPs on cellular proliferation, the [<sup>3</sup>H]-thymidine incorporation assay was performed. As shown in Fig. 2, XS52 cell proliferation was dose-dependently inhibited by treatment with silica particles of all sizes. IC<sub>50</sub> values for nSP70, nSP300 and mSP1000 were 4.2, 32.6, and 75.0 µg/ml, respectively. These results showed that the growth of XS52 cells was more strongly inhibited by smaller-sized nSP.

To study the mechanism responsible for the effects on XS52 cells treated with various-sized silica particles, we measured the quantity of lactate dehydrogenase (LDH) released. LDH is released into culture medium after the cellular membrane disruption that constitutes the last step of the in vitro cell death process. After 24 h of exposure (Fig. 3), no LDH release was observed in mSP1000-treated cells, while dose-dependent LDH

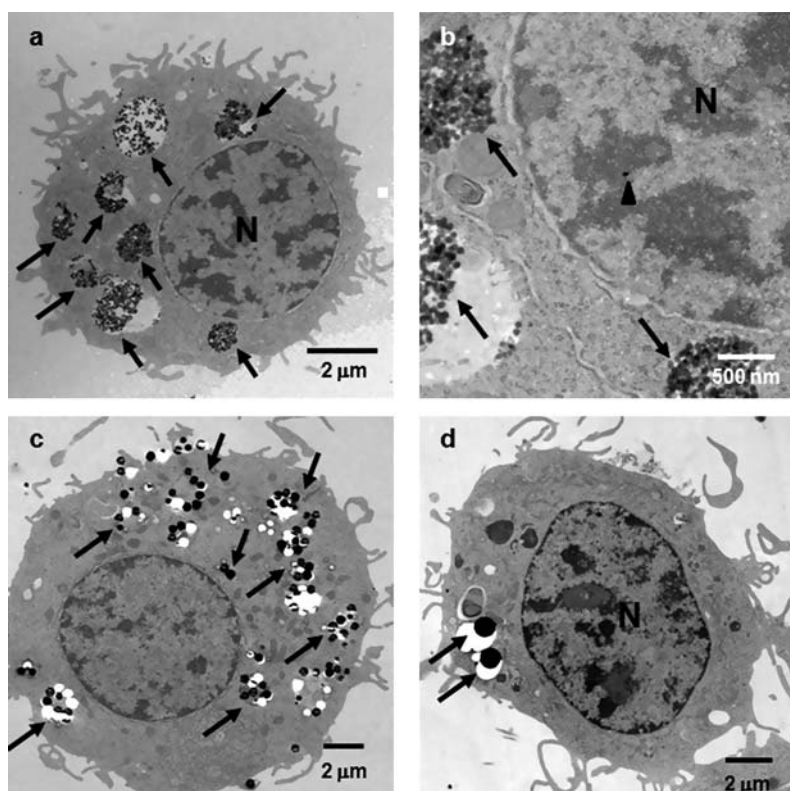


Fig. 1: Localization of silica particles in XS52 cells (arrows). Cells were treated for 24 h with nSP70 (a and b), nSP300 (c) and mSP1000 (d). nSP300 and mSP1000 were located in cytoplasm only. On the other hand, nSP70 was located in the nucleus as well as in cytoplasm (b, arrow head). Scale bars; 2  $\mu\text{m}$  (a, c and d), 500 nm (b)

release was observed in nSP70- and nSP300-treated cells. The highest LDH release was recorded on treatment with 30  $\mu\text{g/ml}$  nSP70 ( $193 \pm 6.8\%$  of control). This result therefore indicated that the cytotoxicity to XS52 cells may be due to cellular membrane damage. Consequently, it appears that the difference in the quantity of silica particles taken up by the cells may explain the size-dependent toxicity to XS52 cells.

As reported elsewhere, we had shown that nSP70 penetrated the stratum corneum (SC) of mouse skin and was taken up by living Langerhans cells (Nabeshi et al. 2010). Furthermore, in the present study we showed that the difference in the quantity of silica particles taken up by the cells was linked to size-

dependent toxicity and nSP70 taken up by Langerhans cells entered the nucleus. Thus, our previous and present results suggest that transdermal exposure to nSPs may (i) risk dysfunction of Langerhans cells, as shown by the cytotoxicity to XS52 cells, (ii) induce immune disruption by altering the immune response (Tinkle et al. 2003; Fifi et al. 2004) and (iii) induce dysfunction of the nucleus and genotoxicity via aggregation of intranuclear protein or inhibition of RNA transcription (Chen and von Mikecz 2005) following entrance of nSPs into the nucleus.

Collectively, the data obtained in this study offer highly useful information for prediction and avoidance of harmful effects mediated by nSPs used commercially in cosmetics. Thus, cor-

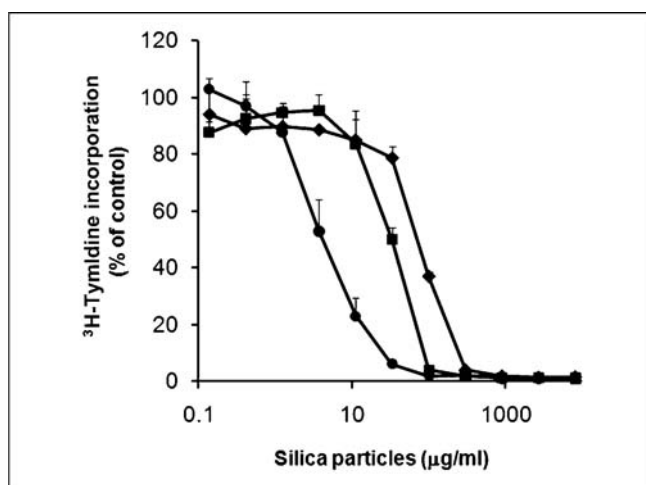


Fig. 2: Effect of various-sized silica particles on proliferation of XS52 cells. The proliferation of cells after incubation with nSP70 (circle), nSP300 (square) and mSP1000 (diamond) for 24 h was evaluated using the [ $^3\text{H}$ ]-thymidine incorporation assay. The percentage increase in cell proliferation was calculated relative to the negative control. Data are presented as means  $\pm$  SD

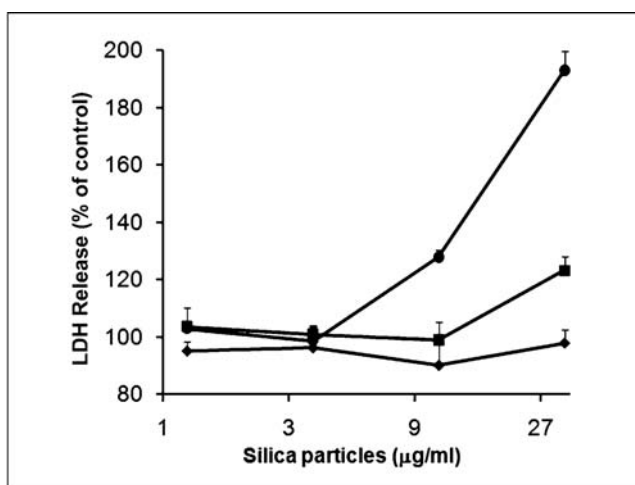


Fig. 3: Effect of silica particles on membrane damage. Cellular membrane damage in XS52 cells after incubation with nSP70 (circle), nSP300 (square) and mSP1000 (diamond) for 24 h was evaluated by the LDH release assay. The percentage cellular membrane damage was calculated relative to the negative (medium) controls. Data are presented as means  $\pm$  SD

related analysis of physicochemical properties, harmful effects and biodistribution as performed in our study may offer valuable readouts for toxicity of nanomaterials and help to develop non-toxic nanomaterials in the future.

### 3. Experimental

#### 3.1. Silica particles

Fluorescent (red-F)-labeled silica particle suspensions (25 mg/ml or 50 mg/ml) with a diameter of 70, 300 and 1000 nm (Micromod Partikeltechnologie GmbH, Rostock, Germany; designated nSP70, nSP300, mSP1000, respectively) were used in this study. In each case, silica particles were used after 5 min sonication and 1 min vortexing.

#### 3.2. Cell culture

Cells from the Langerhans cell-like line XS52 (a kind gift of Akira Takashima, University of Toledo, Health Science Campus, Toledo) were expanded in complete medium containing 2 ng/ml murine GM-CSF and 10% culture supernatants from skin-derived stromal NS47 cells (a kind gift of Akira Takashima). Complete medium was RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 1% non-essential amino acids, 1% L-glutamine, 1 mM sodium pyruvate, 1% 2-mercaptoethanol, 10 mM HEPES buffer, and 1% Antibiotic-Antimycotic Mix stock solution.

#### 3.3. Transmission electron microscopy (TEM) analysis of Langerhans cell line

XS52 cells were cultured with various-sized silica particles for 24 h on chamber slides, then fixed at 4 °C in 2.5% glutaraldehyde and washed three times in 0.1 M phosphate buffer (pH: 7.4); cells were then post-fixed in phosphate-buffered 1% osmium tetroxide for 60 min at 4 °C, dehydrated through a series of ethanol concentrations and embedded in EPON resin (TAAB, Watford, UK). Ultrathin sections were stained with lead citrate and examined under an electron microscope (Hitachi H-7650).

#### 3.4. [<sup>3</sup>H]-Thymidine incorporation assay

Proliferation of silica particle-treated XS52 cells was measured by [<sup>3</sup>H]-thymidine incorporation assay.  $1 \times 10^4$  cells were cultured with varying concentrations of nSPs for 18 h at 37 °C and [<sup>3</sup>H] thymidine (1 µCi/well) was then added into each well. After a further 6 h, cells were harvested and lysed on glass fiber filter plates using a Cell harvester (PerkinElmer, MA, USA). The filter plates were then dried and counted by standard liquid scintillation counting techniques in a TopCounter (PerkinElmer, MA, USA).

#### 3.5. LDH release assay

Lactate dehydrogenase (LDH) activity of XS52 cells exposed to nSP70, nSP300, mSP1000 was determined using a commercial LDH cytotoxicity test (WAKO, Osaka, Japan) according to the manufacturer's instructions. In brief,  $5 \times 10^3$  cells were seeded into each well of a 96 well plate. After 24 h incubation, cells were treated with nSP70, nSP300, mSP1000 or 0.2% Tween 20 (positive control). After a further 24 h incubation period, 50 µl of medium overlying cells was used for LDH analysis. Absorption of light at 560 nm was measured using a spectrophotometer.

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