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Systemic distribution, nuclear entry and cytotoxicity of amorphous nanosilica following topical application

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ABSTRACT

Currently, nanomaterials (NMs) with particle sizes below 100 nm have been successfully employed in various industrial applications in medicine, cosmetics and foods. On the other hand, NMs can also be problematic in terms of eliciting a toxicological effect by their small size. However, biological and/or cellular responses to NMs are often inconsistent and even contradictory. In addition, relationships among NMs physicochemical properties, absorbency, localization and biological responses are not yet well understood. In order to open new frontiers in medical, cosmetics and foods fields by the safer NMs, it is necessary to collect the information of the detailed properties of NMs and then, build the prediction system of NMs safety. The present study was designed to examine the skin penetration, cellular localization, and cytotoxic effects of the well-dispersed amorphous silica particles of diameters ranging from 70 nm to 1000 nm. Our results suggested that the well-dispersed amorphous nanosilica of particle size 70 nm (nSP70) penetrated the skin barrier and caused systemic exposure in mouse, and induced mutagenic activity *in vitro*. Our information indicated that further studies of relation between physicochemical properties and biological responses are needed for the development and the safer form of NMs.

1. Introduction

A nanomaterial (NM) is defined as a substance that has at least one dimension of less than 100 nm in size. NMs can assume many different forms, such as tubes, rods, wires, spheres or particles. NMs have been widely used in consumer and industrial applications, such as medicine, cosmetics and foods, because they exhibit unique physicochemical properties and innovative functions [1]. However, NMs can also be problematic in terms of eliciting a toxicological effect by their small size. For example, exposure of cells or animals to carbon nanotubes, titanium dioxide nanoparticles or silver nanoparticles can induce cytotoxicity and inflammation [2–14]. We have previously shown that nSPs display a different intracellular localization compared with submicron- and micro-sized silica particles, and induce a greater cytotoxic response [15]. Whereas other studies reported that carbon nanotubes and titanium dioxide nanoparticles do not induce harmful effects [16–18]. Thus, despite intensive research efforts, reports of biological and/or cellular responses to NMs are often inconsistent and even contradictory. In addition, relationships among NMs physicochemical properties, absorbency, localization and biological responses are not yet well understood. In order to ensure the safety of NMs and open new frontiers in biological fields by the use of NMs, it is necessary to

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collect the information of the detailed properties of NMs from the point of view of biosafety and then, build comprehensive prediction system of NMs safety.

Accordingly, in this study, we evaluated the absorption properties and intracellular distribution of NMs, using typical NMs, amorphous nanosilica particles (nSP) and quantum dots (QD). nSP are one of the most widely applied NMs, and are used in cosmetics and food additives. nSPs and QD also have great potential for use as diagnostic imaging agents, gene delivery carriers and cancer therapies [19–23]; in addition, these NMs show overwhelmingly superior dispersibility as compared with carbon nanotubes, fullerene and nano-sized titanium dioxide (TiO_2). Thus, these NMs are ideally suited for determining how particle size influences the biodistribution and biological effects of NMs.

2. Materials and methods

2.1. Silica particles

Suspensions of fluorescent (red-F)-labeled amorphous silica particles (Micromod Partikeltechnologie GmbH) (25 mg/ml and 50 mg/ml) were used in this study; particle size diameters were 70, 300 and 1000 nm (designated as nSP70, nSP300 and mSP1000, respectively). Silica particles were used following 5 min sonication and 1 min vortex.

2.2. Quaautum dots

Quantum dots (QD) with emission maxima at 565 nm were obtained from Invitrogen (Hayward, CA). They were sold as Qtracker® Non-targeted Quantum Dots (PEG). QD were used after 5 min sonication and 1 min vortex.

2.3. Animals

BALB/c mice (female, 6–8 weeks) were purchased from Japan SLC, Inc. Mice were housed in a ventilated animal room maintained at 20 ± 2 °C with a 12-h light/12-h dark cycle. Mice had free access to water and alfalfa-free forage (FR-2, Funabashi farm). The experimental protocols conformed to the ethical guidelines of the National Institute of Biomedical Innovation.

2.4. Cell culture

HaCaT human keratinocyte cell line was kindly provided by Dr. Inui, Osaka University. HaCaT cells were cultured in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% heat-inactivated fetal bovine serum and 0.2 mM L-glutamine. The cells were grown in a humidified incubator at 37 °C (95% room air, 5% CO_2).

2.5. Physicochemical examinations of silica particles and QD

Silica particles and QD were diluted to 0.25 mg/ml (nSP70), 0.5 mg/ml (nSP300 and mSP1000) or 0.5 μM (QD) with PBS, respectively and the average particle size and zeta potential were measured using the Zetasizer Nano-ZS (Malvern Instruments Ltd). The mean size and the size distribution of silica particles were measured by dynamic light scattering method. The zeta potential was measured by laser Doppler electrophoresis. pH of each particles suspension were measured by ISFET-pH meter (SHINDENGEN, Japan). The size and shape of silica particles and QD were observed using transmission electron microscopy (TEM). Prior to TEM analysis, nSP70 were stained with 2% uranium acetate and QD were enhanced by silver using a standard AURION R-GENT SE-EM regent and protocol.

2.6. Dermal administration of silica particles and transmission electron microscopy (TEM) analysis of skin, lymph node and liver

nSP70 (250 μg/ear/day) and QD (1.2 pmol/ear/day) suspension supplemented with 10% isopropyl myristate were applied to the inner side of both ears of BALB/c mice for 28 days. In both samples, the total number of particles applied over 28 days was 2.8×10^{13} particles. After 24 h of last administration, skin, lymph node and brain from each mouse were excised and fixed in 2.5% glutaraldehyde for 2 h. Then, small pieces of tissue sample were washed with phosphate buffer three times and post-fixed in sodium cacodylate-buffered 1.5% osmium tetroxide for 60 min at 4 °C, block stained in 0.5% uranyl acetate, dehydrated by dipping each of them through a series of ethanol solutions containing increasing concentration of ethanol, and embedded in Epon resin (TAAB). Ultrathin sections were stained with uranyl acetate and lead citrate (silica particles-treated samples) or AURION R-GENT SE-EM regent (QD-treated samples). The stained samples were subsequently observed under a Hitachi electron microscope (H-7650).

2.7. Detection of apoptotic cells in the nSP70-applied mice skin (terminal deoxynucleotidyl transferase-mediated X-dUTP nick-end labeling (TUNEL) staining)

The TUNEL staining was performed on paraffin-embedded skin sections of 28-day application of nSP70. The skin was fixed in 10% neutral buffered formalin and then embedded in paraffin. Paraffin-embedded skin was sliced and placed on glass slides. DNA strand breaks, which are associated with the apoptotic response, were detected with an *in situ* Cell Death Detection Kit, TMR red (Roche) according to protocol of this kit. Deparaffinization and rehydration of the skin sections were carried out according to standard protocols. Then, the skin sections were incubated with proteinase K for 30 min. After rinse of the skin sections twice with PBS, 50 μl of TUNEL reaction mixture were added on the skin sections and incubated for 60 min at 37 °C in the dark. The skin sections were rinsed 3 times with PBS and mounted with the mounting agent, ProLong Gold Antifade Reagent with DAPI (Invitrogen). The skin sections were analyzed under a fluorescence microscope (BIOREVO, KEYENCE) with excitation wavelength in the range of 520–560 nm and detection in the range of 570–620 nm. For counting the numbers of TUNEL-positive cells, approximately 1000 cells were randomly selected from 3 different areas in each section and examined under a fluorescence microscope at magnification of ×200.

2.8. Transmission electron microscopy (TEM) analysis of human keratinocyte cells

HaCaT cells were cultured in the presence of various sized silica particles (100 μg/ml) for 24 h on chamber slides, and then fixed in 2.5% glutaraldehyde followed by 1.5% osumium tetraoxide. The fixed cells were dehydrated and embedded in EPON resin. Ultrathin sections were stained with lead citrate and observed under an electron microscope.

2.9. Evaluation of the proliferation of silica particle- or QD-treated cells (^{3}H-thymidine incorporation assay)

Proliferation of silica particle- or QD-treated HaCaT cells was measured by ^{3}H-thymidine incorporation assay. 1×10^4 cells were cultured with varying concentrations of silica particles or QDs for 18 h at 37 °C and ^{3}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). The filter plates were then dried and counted by standard liquid scintillation counting techniques in a Top-Counter (PerkinElmer).

2.10. Mutagenicity assay (Ames test)

The mutagenicity assay was performed to evaluate the intrinsic mutagenic potency of the silica particles. For this purpose, the *Salmonella typhimurium* (*S. typhimurium*) mutagenicity test was performed according to the method of Ames [24–26]. Two strains of *S. typhimurium* bacteria were used, namely, TA98 and TA100. Experiments were conducted according to guideline of Health, Labour and Welfare Ministry. The test was carried out using 100 μl of well-dispersed solutions (10, 90, and 810 μg/ml) of silica particles. 2-Aminofluorene (2-AF) dissolved in DMSO was used as a positive control for the mutagenicity assay.

2.11. Determination of DNA damage (comet assay)

Damage of endogenous DNA in HaCaT cells after treatment with a given silica particles were analyzed by alkaline comet assay according to the Comet Assay Kit (Trevigen). All steps were conducted under dim yellow light to prevent additional DNA damage. Briefly, 3×10^4 HaCaT cells were seeded into each well of a 6-well plate and incubated for 24 h. After 24 h, cells were treated with 30 or 90 μg/ml nSP70, nSP300, mSP1000 or 0.2 mM H_2O_2 (positive control) or PBS (negative control) for 3 h. Cells from each group were resuspended at a density of 1×10^5 cells/ml in ice cold CMF-PBS and combined with molten LM Agarose (Trevigen) at a ratio of 1:10 (v/v). The cell-agarose mixture was immediately pipetted onto a frosted microscope slide (CometSlide; Trevigen). Each slide was then placed flat at 4 °C in the dark for 60 min, immersed in prechilled lysis solution (Trevigen), and left at 4 °C for 40 min to remove cellular proteins, leaving the DNA molecules exposed. The slides were then immersed in an alkaline solution (pH > 13, 0.3 M NaOH and 1 mM EDTA) for 40 min to denature the DNA and hydrolyze the sites that were damaged. The samples were electrophoresed for 10 min and stained with SYBR green I (Trevigen) according to the manufacturers instructions. Twenty-five cells on each slide, randomly selected by fluorescence microscopy, were then analyzed using the Comet Analyzer (Youworks Corporation).

2.12. In vivo imaging

Biodistribution of fluorescent-labeled silica particles was analyzed in live mice and excised tissues using the IVIS 200 imaging system (Xenogen corp.). Three female Hos: HR-1 mice were treated with 100 mg/kg DY-676 (excitation (ex) and emission (em) wavelengths 674 and 699 nm, respectively)-labeled silica particles of each particle size (70, 300 and 1000 nm) by intravenous injection. After anesthesia with isoflurane, live mouse fluorescence optical imaging was performed using the cy5.5