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.

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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
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 (TiO2). 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 (Micro-mod 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 nSP1000, respectively). Silica particles were used following 5 min sonication and 1 min vortex.

2.2. Quantum 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 12:12 light/12-h dark cycle. Mice had free access to water and alfalfa-free forage (FR-2, Funa-bashi farm). The experimental protocols conformed to the ethical guidelines of the National Institute of Biomedical Engineering.

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% CO2).

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 nSP1000) or 0.5 μg/ml (QD) with PBS, respectively and the average particle size and zeta potential were measured using the Zetasizer Nano-ZS (Malvern Instruments). The mean size and the size distribution of silica particles were measured by dynamic light scattering method. The zeta potential was measured by laser diffraction (90° angle).

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 × 1011 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 (BIORÈVO, KEYENCE) with an 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% osmium tetroxide. 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 (3H-thymidine incorporation assay)

Proliferation of silica particle- or QD-treated HaCaT cells was measured by 3H-thymidine incorporation assay. Cells were cultured with varying concentrations of silica particles or QDs for 18 h at 37 °C and 1H-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-Aminofluorenone (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 particle was 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 × 104 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, nSP1000 or 0.2 mM H2O2 (positive control) or PBS (negative control) for 3 h. Cells from each group were resuspended at a density of 1 × 105 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, 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). Each slide was then analyzed using the Comet Analyzer (Yovenworks Corporation).

2.12. In vivo imaging

Bioluminescence 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 mm, 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.
filter set (ex/em 615-665/695-770). Tissues were then excised from the mice and fluorescent images of the tissues were obtained. Imaging parameters were selected and implemented using the instrument, Living Image 2.5 software. Bright field photographs were obtained for each imaging time. The merged bright field photogra-phs and fluorescent images were generated using Living Image 2.5 software.

2.13. Transmission electron microscopy (TEM) analysis of liver

BALB/c mice were treated with 0.6 mg/mouse (about 30 mg/kg, 70 nm) or 2 mg/mouse (about 100 mg/kg, 300 and 1000 nm) silica particles of each particle size and PBS (control) by intravenous injection. After 24 h, the tissues and organs such as brain, heart, lung, kidney, spleen and lymph node, were excised and fixed in 2.5% glutaraldehyde for 2 h. Small pieces of tissue sample were then washed with phosphate buffer three times and postfixed in sodium cacodylate-buffered 1.5% osmium tetroxide for 60 min at 4 °C, block stained in 0.5% uranyl acetate, dehydrated through a series of ethanol concentrations, and embedded in Epon resin (TAAB). Ultrathin sections were stained with uranyl acetate and lead citrate. The samples were examined under a Hitachi electron microscope (H-7650).

2.14. Living cell counting and DNA damage determination in isolated primary hepatocytes from silica particle-treated mice

Female BALB/c mice were treated with 2 mg/mouse (about 100 mg/kg) silica particles of each particle size (70, 100, 300 and 1000 nm) and PBS (control) by intravenous injection. After 5 h, parenchymal hepatocytes were isolated according to the in situ two-step collagenase perfusion technique. Briefly, the liver was perfused with 25 ml of 10 mM Hepes buffered calcium- and magnesium-free Hanks’ balanced salt solution (HBSS) containing 190 mg/ml EGTA (DOJINDO) for 5 min. The liver was then perfused with 40 ml of HBSS containing 250 mg/ml trypsin inhibitor, 500 mg/l collagenase and 550 mg/l CaCl2, for 10 min. The liver was then excised and the cells dispersed in HBSS. The cells were then centrifuged at 50 × g at 4 °C for 1 min. The resulting pellet was resuspended in 20 ml of 1.15 medium containing 5% FCS, 1 μM dexamethasone and 1 μM insulin and centrifuged at 50 × g at 4 °C for 1 min. This step was repeated 3 times. The resulting pellet was resuspended in medium and living cells were counted using trypan blue staining. Endogenous DNA damage in isolated primary parenchymal hepatocytes from mice treated with nSP70, nSP300, mSP1000 and PBS (control) was analyzed by alkaline comet assay as described above.

2.15. Statistical analysis

Statistical comparisons between groups were performed by one-way ANOVA with Bonferroni test as a post hoc test. The level of significance was set at P < 0.05.

3. Results and discussion

3.1. Physicochemical properties of various sized silica particles and QD

We next used TEM to determine whether nSP with a particle size below 100 nm would penetrate the skin after topical application. As a result, the 28-day application of nSP70 to mice showed that nSP70 entered not only the skin (Fig. 2a), the regional lymph nodes (Fig. 2b) and the parenchymal hepatocytes present in liver (Fig. 2c, d) but also the cerebral cortex (Fig. 2e) and hippocampus (Fig. 2f). Surprisingly, penetration of nSP70 into the liver was also detected, and some of the nSP70 that entered the parenchymal hepatocytes were found to be distributed throughout the cytoplasm and inside the nucleus (Fig. 2c) and mitochondria (Fig. 2d). Localization of nSP70 in the nucleus was also detected in the skin and the lymph node (Fig. 2a, b). Next, the skin permeability of QD was evaluated. We found that QD penetrated the stratum corneum and entered the skin (Fig. 2g), lymph node (Fig. 2h), liver (Fig. 2i, j), cerebral cortex (Fig. 2k) and hippocampus (Fig. 2l). In addition, some of the QD that entered the skin (Fig. 2g), lymph node (Fig. 2h) and parenchymal hepatocytes (Fig. 2i) were detected inside the nucleus, similar to nSP70. We considered that the well-dispersed portion of QD showed skin permeability. It has been reported that QD can enter the skin by transdermal exposure under ultraviolet radiation [27]. However, for the first time we have revealed that nSP and QD penetrate the skin and enter tissues such as the lymph node, liver and brain under normal conditions.

3.3. Analysis of biological effects induced by nanosilica and QD

Thus, these particles are ideally suited as optimal sample to evaluate if and whether their biodistribution and biological effect depend on the particle size. As well as silica particles, the shape, size distribution and zeta potential of QD were evaluated. Surface of QD used in this study were coated with polyethylene glycol (PEG). QD were enhanced using silver for TEM analysis, because silver selectively deposits on the QD [27]. From the results of TEM analysis, QD were also spherical particles, and in terms of size category the primary particle sizes (about 35 nm) were approximately uniform. The size distribution spectrum of QD in a neutral solvent showed two peaks, and the average particle size of peaks 1 and 2 was about 35 and 300 nm, respectively.

3.2. Analysis of transdermal penetration and biodistribution of nanosilica and QD applied on the skin

The next step for the biosafety should include analyzing their biological effects against skin, brain, liver and lymph node. Consequently, first, in order to assess the biological response in the skin as a part of 28-day application of nSP70, we tried to detect the apoptotic cells by using Terminal Deoxynucleotidyl Transferase-Mediated X-dUTP Nick-End Labeling (TUNEL) staining. As a result, while a few TUNEL-positive cells were detected in water-applied mice skin (control) (Fig. 3a), a number of TUNEL-positive cells (expressed in red) were detected in nSP70-applied mice skin (Fig. 3b). The ratio of TUNEL-positive cells in the skin sections of mice transdermally-applied with nSP70 tended to increase compared to mice transdermally-applied with water (control). In one of two nSP70-applied mice, the ratio of TUNEL-positive cells in the skin section was dominantly increased (Fig. 3c). This result suggested that the transdermal application of nSP70 induced the cellular damage in the skin. On the basis of this result and transdermal absorption test results, we are now evaluating higher cerebral function, hepatic drug metabolism, and the immune system of mice after topical nSP exposure. Moreover, it is necessary to evaluate the influence of well-dispersed NMs on nuclear and mitochondrial functions, because we found that nSP70 and QD enter into these specific organelles. These results also suggest that systemic safety analysis (hazard analysis) of an NM is highly important for ensuring its safety. Because nSP70 and QD can
penetrate the skin barrier, which is the most rigid biological barrier, we believe that analysis of oral and pulmonary exposure should also be included in ensuring the biosafety of NMs.

Collectively, these observations clearly show that nSPs and QD of less than 100 nm in diameter invade the body through the skin, suggesting that human beings are at high risk of exposure to NMs through the blood stream. Consequently, we analyzed the distribution and biological effects of NMs with a focus on the region level and the systemic level. Because nSPs have already been put into practical use in cosmetics, firstly, we evaluated the intracellular

Table 1
Summary of the physicochemical properties of silica particles.

<table>
<thead>
<tr>
<th>Primary particle size (nm)</th>
<th>Hydrodynamic diameter (nm)</th>
<th>Mean zeta potential (mV)</th>
<th>pH</th>
<th>Surface texture</th>
<th>Porosity</th>
<th>Surface area (m²/g)</th>
<th>Functional group</th>
</tr>
</thead>
<tbody>
<tr>
<td>nSP70</td>
<td>70</td>
<td>77.0 ± 0.4</td>
<td>-21.6 ± 4.5</td>
<td>7.4</td>
<td>Plain</td>
<td>Nonporous</td>
<td>43</td>
</tr>
<tr>
<td>nSP300</td>
<td>300</td>
<td>269.3 ± 2.1</td>
<td>-31.3 ± 6.5</td>
<td>7.5</td>
<td>Plain</td>
<td>Nonporous</td>
<td>10</td>
</tr>
<tr>
<td>mSP1000</td>
<td>1000</td>
<td>1187 ± 25.2</td>
<td>-37.7 ± 4.6</td>
<td>7.9</td>
<td>Plain</td>
<td>Nonporous</td>
<td>3</td>
</tr>
</tbody>
</table>

Mean particle size and zeta potential in solution of silica particles are expressed as mean ± S.D. (n = 3).

a Information from technical datasheet of products.

b The specific surface area was calculated by means of the following equation: \( s = \frac{6}{\rho d} \) (where \( s \), specific surface area (m²/g); \( \rho \), density (g/cc); \( d \), diameter (µm)).

Fig. 1. Transmission electron microscopy (TEM) analysis of silica particles. a–f, TEM photomicrographs of silica particles used in this study: nSP70 (a and b), nSP300 (c and d) and mSP1000 (e and f). Each type of sized silica particles existed as scattered and spheroidal. Scale bars: 1 µm (a and f), 100 nm (b), 500 nm (d) and 2 µm (c and e).
Fig. 2. TEM analysis of skin, lymph node, liver and brain samples from mice after 28-days of dermal exposure to silica nanoparticles and quantum dots. a–c, nSP70 (arrows) were present in the nucleus of skin (a), cervical lymph node (b), and parenchymal hepatocytes (c). d, nSP70 were also detected in the mitochondria of parenchymal hepatocytes. e–f, nSP70 were found in neuron of the cerebral cortex (e) and the hippocampus (f). g–h, QD (arrows) were present in the nucleus of skin (g), cervical lymph node (h) and parenchymal hepatocytes (i), similar to nSP70. j, In parenchymal hepatocytes, QD were detected in the mitochondria (j). k–l, QD were found in neuron of the cerebral cortex (k) and the hippocampus (l). N: nucleus; M: mitochondria. Scale bars: 200 nm (a, e–g, and j–l), 500 nm (b–d, h, and i).
distributions and biological responses of NM size in skin with a focus on nSP.

3.4. Analysis of intracellular distribution of silica particles in human keratinocyte

Presently, many modern cosmetic or sunscreen products contain nano-sized components, such as titanium dioxide (TiO2), zinc oxide (ZnO) and amorphous silica particles. Nano-sized TiO2 and ZnO are colorless substances and reflect/scatter ultraviolet (UV) more efficiently than their larger counterparts [28,29]. Amorphous nanosilica particles (nSPs) are used in large quantities and are one of the most important ingredients in the cosmetic industry, especially for their light-diffusing and absorption properties. Extensive consumption of these NM-supplemented cosmetic and food products has naturally raised the question as to whether these NMs could penetrate the skin, would eventually become absorbed systemically, and more importantly, whether they could be responsible for acute/chronic side effects. In the present study, we revealed that well-dispersed nSP could penetrate into and pass through the skin. Interestingly, we found that nSP migrated into the blood stream and passed into tissues such as liver. Moreover, nSP invaded specific organelles such as the nucleus and mitochondria. In view of these observations, we next examined the relation between the intracellular distribution and biological effects of nSP, which are the most important NMs in our daily life.

To determine the intracellular location of silica particles, we used TEM to examine the HaCaT cells that were treated with 100 µg/ml of nSP70, nSP300, or mSP1000. TEM examination revealed the presence of mSP1000 and nSP300 only in the endosome (Fig. 4a, b, arrows). mSP1000-treated cells were also found to contain a large number of lysosomes (Fig. 4a). In contrast, only in the nSP70-treated cells nSP70s were present in the cytoplasm as well as in the nucleus (Fig. 4c, d, arrow heads). Furthermore, nSP70s were accumulated in the nucleolus (Fig. 4e, f, arrows). Recently, it has been reported that the intercellular localization of NM is possibly linked to the induction of harmful effects. For example, the localization of silver nanoparticles in the nucleus and mitochondria may be related to mitochondrial dysfunction or oxidative stress [30]. Thus, analysis of intracellular localization enables us to provide important and useful information to predict the hazard to human health.

3.5. Analysis of cell-growth inhibition and genotoxicity induced by silica particles

Next, we investigated the biological effects of nSP. To this end, we assessed the effects of various particle size nSP on the proliferation of HaCaT cells. As shown in Fig. 5a, cell proliferation was inhibited following treatment with nSP70 and nSP300 in both dose and size dependent manner. The half maximal (50%) inhibitory concentration (IC50) of nSP70 and nSP300 for inhibiting cell proliferation was 323 and 3966 µg/ml, respectively. We were, however, unable to calculate the IC50 of nSP1000. Taken together, these results suggested that smaller sized silica particles inhibited the growth of HaCaT cells more strongly than the larger particles. In addition, we assessed the effects of QD on the proliferation of HaCaT cells. As the result, we indicated that the effect to cell proliferation of QD was predominantly lower than nSP70 at same
concentrations (particles/ml) (Fig. 5b). This result suggested that the biological effects of NMs were different by material.

On the basis of the nuclear entry of nSP70 in vivo and in vitro, we next evaluated the mutagenicity of silica particles using S. typhimurium strains TA98 and TA100 (Ames test). None of the nSP that we tested induced mutation in TA98 strain when used at the indicated concentrations (Fig. 6a). By contrast, nSP of all sizes induced mutagenicity in TA100 strain at the highest dose of treatment (30 and 90 mg/ml) (Fig. 6b). At lower doses (30 and 90 mg/ml) of treatment, only nSP70 induced mutation in TA100 strain (Fig. 6b). Thus, the results obtained from the Ames test suggest that the mutagenicity of the silica particles increased with the decreasing particle size. Next, we used the comet assay to analyze DNA single strand breaks in nSP-treated HaCaT cells. In cells treated with PBS (negative control) for 3 h, the average tail length was 23.3 mm (Fig. 6c). In cells treated with 90 mg/ml of nSP70, nSP100, nSP300, or nSP1000, the average tail lengths were 102.9 mm (Fig. 6d), 88.8 mm (data not shown), 30.5 mm (Fig. 6e), and 22.5 mm (Fig. 6f), respectively.

**Fig. 4.** TEM analysis of HaCaT cells treated with silica particles. a–e, Silica particles (arrows) were found in HaCaT cells treated for 24 h with 100 mg/ml of mSP1000 (a), nSP300 (b), and nSP70 (c, d, and e). In panels c and d, arrow heads show the presence of nSP70 in the nucleus, and in panels e and f, arrows show the presence of nSP70 in the nucleolus. Panels d and f are same as panels c and e at higher magnifications, respectively. N: nucleus; NU: nucleolus. Scale bars: 1 mm (a and b), 2 mm (c and e) and 500 nm (d and f).
The average tail lengths increased depending on the dose and size of the silica particles (Fig. 6g). The tail lengths found in the nSP70- and nSP100-treated cells were longer than those found in the positive control cells (0.2 mM H2O2 treated cells). These findings suggest the possibility that nSP with particle sizes below 100 nm could induce mutation.

3.6. Analysis of in vivo biodistribution of silica particles in mice

We next analyzed biodistribution and biological effects in systemic level using silica particles-injected mice, because it was suggested that nSP moved to the blood stream from skin as described above. To elucidate the in vivo distribution of silica particles, we determined the distribution of silica particles following intravenous injection, by optical imaging analysis (Fig. 7a–c). Intense fluorescence was observed near the liver in all silica particle-treated mice immediately after treatment and this signal migrated to the near the intestinal tract with time. Imaging of dissected liver from nSP300- or mSP1000-treated mice revealed that intense fluorescence was observed only around the gall bladder. In contrast, nSP70-derived fluorescence was observed throughout dissected liver. In addition, our preliminary results revealed that all silica particle-derived fluorescence was also observed in intestinal tract and feces (data not shown), suggesting that silica particles might be excreted in the bile after circulating systemically, in a manner independent of particle size.

To clarify detailed localization of silica particles in liver of nSP-injected mice, next we perform transmission electron microscopy (TEM) analysis (Fig. 7d–g). While silica particles of all sizes were found to be ingested into Kupffer cells, nSP70 and nSP300 were also observed in parenchymal hepatocytes. In the nSP70-treated group, particles were shown to be localized in the cytoplasm and nucleus of various tissues such as lung, kidney, spleen and lymph node (data not shown). Microscopic findings showed that with reduction in particle size, silica particle uptake into Kupffer cells tended to be decreased and in contrast, particle uptake into the cytoplasm of parenchymal hepatocytes tended to be increased (Fig. 7h).

Subsequently, we confirmed whether the biological effects induced by nSP in liver in which silica particles accumulated. The liver is one of the most important tissue in the body, because the liver takes an important role in metabolism, discharge, detoxification, maintenance of homeostasis of the body fluid. Especially, hepatocyte plays a vital role as functions of the liver. Using primary hepatocyte isolated from silica particles-injected mice intravenously, cytotoxicity and DNA damage of hepatocyte were analyzed. As the result, cytotoxicity of hepatocyte from nSP300 and mSP1000-injected mice little occurred. On the other hand, hepatocyte from nSP70-injected mice indicated higher cytotoxicity than nSP300 and mSP1000-injected mice (Fig. 8a). Furthermore, DNA damage of hepatocyte was detected only in nSP70-injected mice as well as in HaCaT cells (Fig. 8b). These results also indicate that differences in biological effects such as cytotoxicity and genotoxicity are caused by differences in biodistribution of silica particles. And it suggests that accumulation of nSP into the liver and/or nucleus may lead to genotoxicity.

Thus, we also highly recommend including carcinogenicity test and reproductive and developmental toxicity test for ensuring biosafety of NMs. Additionally, because nSP70 were accumulated in the nucleus, we suggest evaluating the effect of an NM on protein synthesis to further ensure its biosafety.

Nuclear pores are made of large protein complexes that cross the nuclear envelope, the membrane bilayer that surrounds the nucleus of the eukaryotic cell, and the pores are about 30 nm in diameter [31,32]. Thus, it is unlikely that the nSP70, which has a mean diameter of about 70 nm, entered the nucleus through the nuclear pore. We hypothesize that the nSP70 might interact with the nuclear transporting proteins via specific- or non-specific
interactions, and the nSP70/protein complexes are then transported into the nucleus. To test this hypothesis, we are currently pursuing a proteome-based approach to identify nSP70-interacting proteins.

Recently, commercially available amorphous silica-based products were subjected to various toxicological tests including acute and repeated dose toxicity, genotoxicity, carcinogenicity and reproductive toxicity [33]. According to this report, amorphous silica particles are non-toxic. Although the primary particle sizes of the amorphous silica used in these toxicological studies were between 1 and 100 nm, the ECETOC 2006 report stated that they did not exist as primary particles, but existed only as aggregates of particle sizes between 100 nm and 1 μm. The ECETOC 2006 report, however, did not exclude the possibility that materials having particle sizes below 100 nm might be developed and available for use in the future. In contrast to the results described in the ECETOC report, results of our present
Fig. 7. Biodistribution analysis of silica particles at macro- and micro levels. A–C, Macro level analysis: optical imaging of fluorescently labeled silica particles in live mice and excised liver. DY676-labeled silica particles (a, nSP70, b, nSP300 and c, nSP1000, 100 mg/kg) were intravenously injected into female hairless mice. Twenty min and 6 h after injection, optical images were acquired using a Xenogen IVIS 200 imaging system. The signal intensity in the region of interest is expressed as photons (p) per second (sec) per centimeter squared (cm²) per steradian (sr) (a steradian is a unit of solid angle). d–f, Micro level analysis: BALB/c mouse liver injected with 30 mg/kg (nSP70) or 100 mg/kg (nSP300 and nSP1000) nSPs was observed by TEM. d, arrow, nSP70, f arrow, nSP300, g, nSP1000 were phagocytosed in Kupffer cells. d, arrow head, nSP70 and f, arrow head, nSP300 were also detected in cytoplasm of parenchymal hepatocytes. Interestingly, e, nSP70 entered the nucleus of the parenchymal hepatocytes. Arrows and arrow heads indicate silica particles. Scale bar, d, 2 μm, e, 1 μm and f, g, 5 μm. H, Localization of each silica particle in liver is summarized. Amount of silica particles were shown as follows; −: Not detected, +: small, ++: middle, +++: large.
study showed that well-dispersed nSP70 could indeed penetrate the skin barrier and cause systemic exposure, thus suggesting that the well-dispersed NMs have to be viewed as new entities and tested accordingly for ensuring their biosafety. It is known that the asbestos-related health hazard, symptoms of mesothelioma, appears after prolonged exposure to asbestos particles (average of 40 years, shortest around 20 years) [34–37]. Because of this and also in view of the growing demand for the NMs in various fields, there is a clear and urgent need for in depth risk assessment of all NMs for safety use. Keeping in line with this idea and because it is not known whether exposure to NMs might cause initiation and/or progression of various diseases (e.g., atopic dermatitis, infectious disease, etc.), we have initiated more detailed and extensive safety analysis studies including relationships between the physicochemical properties (i.e., size, shape, and surface property) of an NM and its biodistribution, and analysis of the interaction of the NMs with allergens, gastrointestinal flora, and resident floras as contributing factors to human health in order to further ensure its biosafety.

4. Conclusions

This study revealed that, as compared with the bulk material of particle sizes above nanoscale (above 100 nm), well-dispersed amorphous nanosilica with a particle size of 70 nm shows different bio-properties with respect to skin penetration and nuclear entry. These bio-properties of nanosilica show the potential as a new functional material, but, reflecting these differences, nSP70 exert various adverse biological effects in regional and systemic level, such as DNA fragmentation. We consider that more information which provided by further studies of relation between physico-chemical properties and biological responses, would lead to realization of an affluent society by the use of safe and useful NMs.

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