

<p>Yamashita K., Yoshioka Y., Higashisaka K., Mimura K., Morishita Y., Nozaki M., Yoshida T., Ogura T., Nabeshi H., Nagano K., Abe Y., Kamada H., Monobe Y., Imazawa T., Aoshima H., Shishido K., Kawai Y., Mayumi T., Tsunoda S., Itoh N., Yoshikawa T., Yanagihara I., Saito S., Tsutsumi Y.</p>	<p>Silica and titanium dioxide nanoparticles cause pregnancy complications in mice.</p>	<p>Nat Nanotechnology</p>	<p>6</p>	<p>321-328</p>	<p>2011</p>
<p>Nabeshi H., Yoshikawa T., Arimori A., Yoshida T., Tochigi S., Kondoh S., Hirai T., Akase T., Nagano K., Abe Y., Kamada H., Tsunoda S., Itoh N., Yoshioka Y., Tsutsumi Y.</p>	<p>Effect of surface properties of silica nanoparticles on their cytotoxicity and cellular distribution in murine macrophages.</p>	<p>Nanoscale Res Lett</p>	<p>6</p>	<p>93-98</p>	<p>2011</p>
<p>Nabeshi H., Yoshikawa T., Matsuyama K., Nakazato Y., Tochigi S., Kondoh S., Hirai T., Akase T., Yamashita T., Yamashita K., Yoshida T., Nagano K., Abe Y., Yoshioka Y., Kamada H., Imazawa T., Itoh N., Tsunoda S., Tsutsumi Y.</p>	<p>Amorphous nanosilica induce endocytosis-depende nt ROS generation and DNA damage in human keratinocytes.</p>	<p>Part Fibre Toxicol</p>	<p>8</p>	<p>1-10</p>	<p>2011</p>

Li X., Kondoh M., Watari A., Hasezaki T., Isoda K., Tsutsumi Y., Yagi K.	Effect of 70-nm silica particles on the toxicity of acetaminophen, tetracycline, trazodone, and 5-aminosalicylic acid in mice.	Pharmazie	66	282-286	2011
Isoda K., Hasezaki T., Kondoh M., Tsutsumi Y., Yagi K.	Effect of surface charge on nano-sized silica particles-induced liver injury.	Pharmazie.	66	278-281	2011
Yoshida T., Yoshioka Y., Fujimura M., Yamashita K., Higashisaka K., Morishita Y., Kayamuro H., Nabeshi H., Nagano K., Abe Y., Kamada H., Tsunoda S., Itoh N., Yoshikawa T., Tsutsumi Y.	Promotion of allergic immune responses by intranasally-administered nanosilica particles in mice.	Nanoscale Res Lett	6	195-200	2011
Hirai T., Yoshikawa T., Nabeshi H., Yoshida T., Tochigi S., Uji M., Ichihashi K., Akase T., Yamashita T., Yamashita K., Nagano K., Abe Y., Kamada H., Tsunoda S., Yoshioka Y., Itoh N., Tsutsumi Y.	Size-dependent immune-modulating effect of amorphous nanosilica particles.	Pharmazie	66	727-728	2011

Nabeshi H., Yoshikawa T., Akase T., Yoshida T., Tochigi S., Hirai T., Uji M., Ichihashi K., Yamashita T., Higashisaka K., Morishita Y., Nagano K., Abe Y., Kamada H., Tsunoda S., Itoh N., Yoshioka Y., Tsutsumi Y.	Effect of amorphous silica nanoparticles on in vitro RANKL-induced osteoclast differentiation in murine macrophages.	Nanoscale Lett Res	6	464-468	2011
Morishita Y., Yamashita K., Yoshikawa T., Terada Y., Nabeshi H., Yoshioka Y., Itoh N., Tsutsumi Y.	Detection of titanium dioxide particles on frozen tissue sections using synchrotron radiation X-ray fluorescence analysis.	Pharmazie	65	808-809	2011
Hasezaki T., Isoda K., Kondoh M., Tsutsumi Y., Yagi K.	Hepatotoxicity of silica nanoparticles with a diameter of 100 nm.	Pharmazie	66	689-703	2011

<p>Nabeshi H., Yoshikawa T., Matsuyama K., Nakazato Y., Arimori A., Isobe M., Tochigi S., Kondoh S., Hirai T., Akase T., Yamashita T., Yamashita K., Yoshida T., Nagano K., Abe Y., Yoshioka Y., Kamada H., Imazawa T., Kondoh M., Yagi K., Mayumi T., Itoh N., Tsunoda S., Tsutsumi Y.</p>	<p>Well-dispersed amorphous nanosilicas induce fatal toxicity and consumptive coagulopathy after systemic exposure.</p>	<p>Nanotechnol</p>	<p>23</p>	<p>1-8</p>	<p>2012</p>
<p>Hirai T., Yoshikawa T., Nabeshi H., Yoshida T., Tochigi S., Ichihashi K., Uji M., Akase T., Nagano K., Abe Y., Kamada H., Itoh N., Tsunoda S., Yoshioka Y., Tsutsumi Y.</p>	<p>Amorphous silica nanoparticles size-dependently aggravate atopic dermatitis-like skin lesions following an intra-dermal injection.</p>	<p>Part Fibre Toxicol</p>	<p>9</p>	<p>3</p>	<p>2012</p>
<p>Morishige T., Yoshioka Y., Inakura H., Tanabe A., Narimatsu S., Yao X., Monobe Y., Imazawa T., Tsunoda S., Tsutsumi Y., Mukai Y., Okada N., Nakagawa S.</p>	<p>Suppression of nanosilica particle- induced inflammation by surface modification of the particles.</p>	<p>Arch Toxicol</p>	<p>Epub print</p>	<p>ahead of</p>	<p>2012</p>

Yoshioka Y., Tsutsumi Y.	Recent topics of NanoTox studies for their safety - Foreword-.	Yakugaku Zasshi	131	193-194	2011
Nabeshi H., Yoshikawa T., Yoshioka Y., Tsutsumi Y.	Safety evaluation study of nanomaterials aimed at the promotion of the social reception.	Gene. Environ. (Genes and Environment)	33	21-26	2011
鍋師裕美, 吉岡靖雄, 吉川友章, 堤康央	食品分野におけるナノテクノロジーの安全性.	食品衛生研究	61	27-34	2011
東阪和馬, 堤康央	ナノ安全科学研究の最前線.	月刊『化学』（株式会社化学同人）	66	72-73	2011
森下裕貴, 鍋師裕美, 吉川友章, 吉岡靖雄, 堤康央	化粧品分野におけるナノマテリアルの安全性に関する国内外の報告.	コスメティックスページ	5	45-49	2011
Tsutsumi Y., Yoshioka Y.	Quantifying the biodistribution of nanoparticles.	Nat. Nanotechnol	6	755	2011
Yoshida T., Yoshikawa T., Tsutsumi Y.	Relation analysis between intracellular distribution of nanomaterials, ROS generation and DNA damage.	Yakugaku Zasshi	132	295-300	2011
Yamashita T., Yamashita K., Nabeshi H., Yoshikawa T., Yoshioka Y., Tsunoda S., Tsutsumi Y.	Carbon nanomaterials: efficacy and safety for nanomedicine.	Materials.	5	350-363	2012



Leading Opinion

Acute phase proteins as biomarkers for predicting the exposure and toxicity of nanomaterials[☆]

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ABSTRACT

Recently, nanomaterials have become an integral part of our daily lives. However, there is increasing concern about the potential risk to human health. Here, we attempted to identify biomarkers for predicting the exposure and toxicity of nanomaterials by using a proteomics based approach. We evaluated the changes of protein expression in plasma after treatment with silica nanoparticles. Our analyses identified haptoglobin, one of the acute phase proteins, as a candidate biomarker. The results of ELISA showed that the level of haptoglobin was significantly elevated in plasma of mice exposed to silica nanoparticles with a diameter of 70 nm (nSP70) compared to normal mice and those exposed to silica particles with a diameter of 1000 nm. Furthermore, the other acute phase proteins, C-reactive protein (CRP) and serum amyloid A (SAA) were also elevated in plasma of nSP70 treated mice. In addition, the level of these acute phase proteins was elevated in the plasma of mice after intranasal treatment with nSP30. Our results suggest that haptoglobin, CRP and SAA are highly sensitive biomarkers for assessing the risk of exposure to silica nanoparticles. We believe this study will contribute to the development of global risk assessment techniques for nanomaterials.

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1. Introduction

With the recent development of nanotechnology, nanomaterials such as silica nanoparticles are beginning to be used on a global scale. In comparison to conventional materials with submicron size, nanomaterials display unique properties such as high levels of

electrical conductivity, tensile strength and chemical reactivity [1]. Nanomaterials have already been used in various fields such as electronic engineering, cosmetics and medicine [2,3]. Because nanotechnology is emerging as a leading industrial sector, humans will be increasingly exposed to a wide range of synthetic nanomaterials with diverse properties.

The increasing use of nanomaterials has raised public concerns about the potential risks to human health [4–6]. For example, it is reported that carbon nanotubes induce mesothelioma-like lesions in mice in a similar way to crocidolite asbestos [7]. Other reports showed that exposure to titanium dioxide particles induce inflammatory responses and lung injury in mice [8,9]. In addition, our group showed that silica nanoparticles with a diameter of 70 nm can penetrate mouse skin and enter the circulatory system (unpublished data). Furthermore our group demonstrated that silica nanoparticles induce severe liver damage after systemic administration [10–12]. However, current knowledge of the potential risk of nanomaterials is considered insufficient. Indeed, concerns about the potential dangers of nanomaterials have led the World Health Organization and the Organization for Economic

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Co-operation and Development to call for an urgent and detailed evaluation of their safety. Therefore, it is extremely important to progress these safety evaluations in order to facilitate the development of nanomaterials that are harmless to humans, because nanomaterials have the potential to improve the quality of human life. In particular, it is hoped that a risk assessment system can be developed to estimate or predict the safety and toxicity of nanomaterials.

Molecular biomarkers, obtained from biological samples such as blood, urine and tissue, constitute an objective indicator for correlating against various physiological conditions or variation of disease state [13,14]. By using biomarkers, we are able to predict not only the present disease and clinical condition but the risk of acquiring disease in the future. Nowadays, biomarkers that act as predictors of cancer have already been developed and are commonly used in clinical practice [14]. Furthermore, such an approach is capable of predicting adverse effects of drugs and medicines [15,16]. By contrast, studies of biomarkers for nanomaterials have barely advanced. These biomarkers would represent the unity of local and systemic physiological responses induced as a result of the exposure. Therefore, biomarkers for nanomaterials will be invaluable for predicting their potential toxicity and establishing strategies for the safe development of nanomaterials production and use.

Here we attempted to develop potential biomarkers of nanomaterials using a proteomics analysis with the aim of developing safe forms of nanomaterials.

2. Materials and methods

2.1. Materials

Silica particles were purchased from Micromod Partikeltechnologie (Rostock/Warnemünde, Germany). The silica particles with diameters of 30, 70, 300 and 1000 nm (nSP30, nSP70, nSP300 and mSP1000, respectively), and nSP70 with surface functional groups such as carboxyl group and amino group (nSP70-C and nSP70-N, respectively) were used in this study. The silica particles were sonicated for 5 min and vortexed for 1 min prior to use.

2.2. Animals

Female BALB/c mice were purchased from Nippon SLC, Inc (Shizuoka, Japan) and used at 6–8 weeks of age. All of the animal experimental procedures in this study were performed in accordance with the National Institute of Biomedical Innovation guidelines for the welfare of animals.

2.3. Blood sample collection

For administration of silica particles through an intravenous route, BALB/c mice were treated with nSP70, nSP300, mSP1000, nSP70-C, nSP70-N or saline at 0.8 mg/mouse. At various times (6 h, 24 h, 3 day and 7 day) after treatment of these silica particles, blood samples were collected. For administration of silica particles through an intranasal route, BALB/c mice were treated with nSP30, nSP70 or saline intranasally at 0.5 mg/mouse. Blood samples were collected 24 h after the treatment of these silica nanoparticles.

2.4. Analysis of biomarkers for nanomaterials using a proteomics approach

BALB/c mice were treated with 0.8 mg/mouse nSP70 or saline intravenously. After 24 h, blood samples were collected and plasma was harvested by centrifuging blood at 12000 rpm for 15 min. Proteo prep (Sigma–Aldrich; Saint Louis, MO) was used to remove albumin and immunoglobulins from the plasma according to the manufacturer's instructions. Plasma samples were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Brilliant Blue staining. Plasma diluted into aliquots corresponding to 10 µg protein were mixed with an equal volume of Laemmli sample buffer (BIO-RAD, Tokyo, Japan) containing 5% 2-mercaptoethanol and boiled for 5 min prior to electrophoresis. Electrophoresis was performed at 15 mA for 10 min (stacking) followed by separation (600 V, 40 mA, 100 W) for approximately 45 min, using Precision Plus Protein Kaleidoscope molecular weight markers (BIO-RAD) as standards.

2.5. Identification of candidate proteins as biomarkers

Bands of interest were excised from the gel and then destained with 50% acetonitrile (ACN)/25 mM NH_4HCO_3 for 10 min, dehydrated with 100% ACN for 10 min, and then dried using a centrifugal concentrator. Next, 8 µl of 20 µl/ml trypsin solution (Promega, Madison, WI) diluted 5-fold in 50 mM NH_4HCO_3 was added to each gel piece and then incubated overnight at 37 °C. We used three solutions to extract the resulting peptide mixtures from the gel pieces. First, 50 µl of 50% (v/v) ACN in 0.1% aqueous trifluoroacetic acid (TFA) was added to the gel pieces, which were then sonicated for 30 min. Next, we collected the solution and added 80% (v/v) ACN in 0.1% TFA. Finally, 100% ACN was added for the last extraction. The peptide solution were dried and resuspended in 10 µl of 0.1% formic acid. The resulting peptide mixture was then analyzed by nano-flow liquid chromatography/tandem mass spectrometry (LC/MS; maXis, Bruker Daltonik GmbH, Bremen, Germany).

2.6. Measurement of acute phase proteins

Plasma levels of haptoglobin, C-reactive protein (CRP) and serum amyloid A (SAA) were measured by commercial enzyme-linked immunosorbent assay (ELISA) kits (Life Diagnostics, Inc.; West Chester, PA), according to the manufacturer's instructions.

2.7. Statistical analyses

All results are expressed as means \pm SD. Differences were compared by using the Bonferroni's method after analysis of variance (ANOVA).

3. Results

3.1. Identification of biomarkers of nanomaterials

We used silica particles as a model nanomaterial because it is one of the most common nanomaterials to have been developed. Silica particles are increasingly being used as additives in cosmetics and foods [17,18]. It is predicted that the global market for silica particles will soon grow to \$2 billion and a ton of silica particles is currently produced worldwide every year. Here, we used silica particles with a diameter of 30, 70, 300 and 1000 nm (nSP30, nSP70, nSP300 and mSP1000, respectively). The mean secondary particle diameters of the silica particles measured by Zetasizer were 33, 79, 326 and 945 nm, respectively (data not shown). The silica particles were confirmed to be well dispersed smooth-surfaced spheres by transmission electron microscopy (data not shown).

Initially, we attempted to identify protein biomarkers in mice by analyzing changes in the level of each plasma protein following treatment with silica nanoparticles using a proteomics approach. BALB/c mice were intravenously treated with nSP70 (0.8 mg/mouse) or saline and then plasma samples were collected 24 h later. Because albumin and immunoglobulins are known to account for the majority of plasma proteins, they were removed from the samples prior to analysis so that variation in the level of other proteins could be more closely monitored. The change of protein levels in plasma after treatment with nSP70 was assessed by SDS-PAGE analysis (Fig. 1). The intensity of a band of molecular mass 37 kDa was more intense in the plasma of nSP70 treated mice than that of saline treated control mice (Fig. 1). The band was excised and analyzed by LC/MS in order to identify the corresponding protein. This analysis identified the induced band after treatment with nSP70 as haptoglobin, one of the acute phase proteins.

3.2. The level of haptoglobin after treatment with silica particles

To assess the change of haptoglobin level in plasma after administration of silica particles, BALB/c mice were intravenously treated with nSP70, nSP300 or mSP1000 at 0.8 mg/mouse. We did not use nSP30 in the experiment, because nSP30 induced the toxic side effects after intravenous treatment at this dose. We confirmed that nSP70, nSP300 or mSP1000 at this dose did not induce any

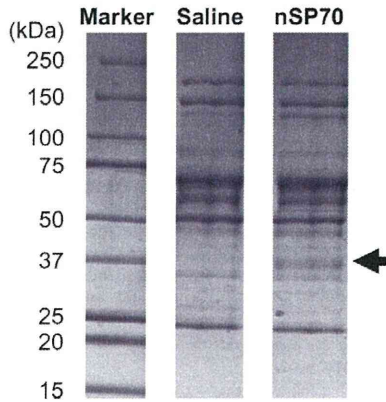


Fig. 1. SDS-PAGE analysis of plasma proteins. BALB/c mice were intravenously treated with nSP70 or saline at 0.8 mg/mouse. After 24 h, blood samples were collected. The change of protein levels in plasma after treatment of nSP70 was assessed by SDS-PAGE.

significant elevation of tissue injury and dysfunction markers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and blood urea nitrogen (BUN) (data not shown). After 24 h, the level of haptoglobin in the plasma was analyzed by ELISA (Fig. 2A). The levels of haptoglobin in the plasma of nSP70 treated mice were significantly higher than those of saline treated control mice. In contrast, the levels of haptoglobin in the plasma of mSP1000 treated mice were almost the same as those of the saline treated control group. The haptoglobin levels of nSP300 treated mice were slightly higher than those of saline treated control mice. These results indicate that the levels of haptoglobin in the plasma of mice increase as the silica particle size decreases. Thus, haptoglobin appears to be a valuable biomarker for exposure to silica particles of nanometer size.

To assess the potential of haptoglobin as biomarker more precisely, we examined the sensitivity and time dependency of changes in haptoglobin level after treatment with silica particles. BALB/c mice were treated with nSP70, nSP300 or mSP1000 intravenously at 0.8 mg/mouse. After 6 h, 24 h, 3 day and 7 day, we examined the level of haptoglobin in the plasma by ELISA (Fig. 2B). No elevation of haptoglobin in the plasma of mSP1000 treated mice was observed. However, nSP70 and nSP300 treated mice showed a maximum level of haptoglobin 24 h after treatment. Furthermore, at 3 days after treatment, the level of haptoglobin in nSP70 treated mice was significantly higher than saline treated control mice. Next, BALB/c mice were treated with 0.2 and 0.05 mg/mouse nSP70 intravenously. After 24 h, we examined the level of haptoglobin in the plasma by ELISA (Fig. 2C). Mice treated with 0.2 and 0.05 mg/mouse nSP70 did not show any elevated level of haptoglobin. These results indicate that the level of haptoglobin is elevated as the particle size of silica particles decreases and that an increase of haptoglobin is dependent on the concentration of silica particles.

3.3. Response of other acute phase proteins

Haptoglobin, CRP and SAA are typical acute phase proteins that are induced during infection and inflammation [19]. To assess the levels of CRP and SAA in plasma after administration of silica particles, BALB/c mice were intravenously treated with nSP70, nSP300 or mSP1000 at 0.8 mg/mouse. After 6 h, 24 h, 3 day and 7 day, we examined the level of CRP (Fig. 3A) and SAA (Fig. 3B) in the plasma of the mice by ELISA. At 6 h and 24 h, both the level of CRP and SAA in the plasma of mice treated with nSP70 was significantly higher than those of the saline treated control mice. Furthermore, the maximum level of CRP in nSP70 treated mice was observed at

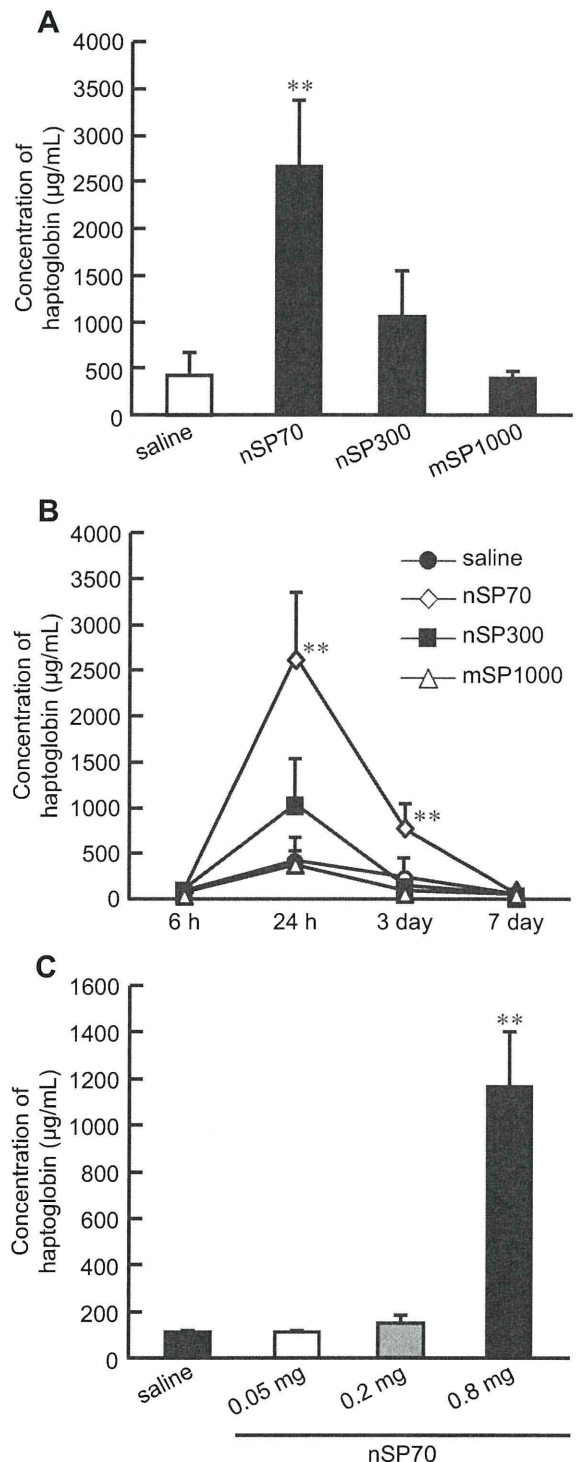


Fig. 2. The potential of haptoglobin as biomarker of nanomaterials. (A) The level of haptoglobin after treatment with silica particles. BALB/c mice were intravenously treated with nSP70, nSP300 or mSP1000 at 0.8 mg/mouse. After 24 h, the level of haptoglobin in the plasma of each mouse was examined by ELISA. (B) The time dependency of haptoglobin expression after treatment with silica particles. BALB/c mice were intravenously treated with nSP70, nSP300 or mSP1000 at 0.8 mg/mouse. After 6 h, 24 h, 3 day and 7 day, blood samples were collected. The level of haptoglobin in the plasma of the mice was determined by ELISA. (C) The sensitivity of haptoglobin after treatment of silica particles. BALB/c mice were intravenously treated with nSP70 at 0.8, 0.2 or 0.05 mg/mouse. After 24 h, blood samples were collected. The level of haptoglobin in the plasma of treated mice was determined by ELISA. Data are presented as mean \pm SD ($n = 5-6$; ** $P < 0.01$ versus value for saline treated group by ANOVA).

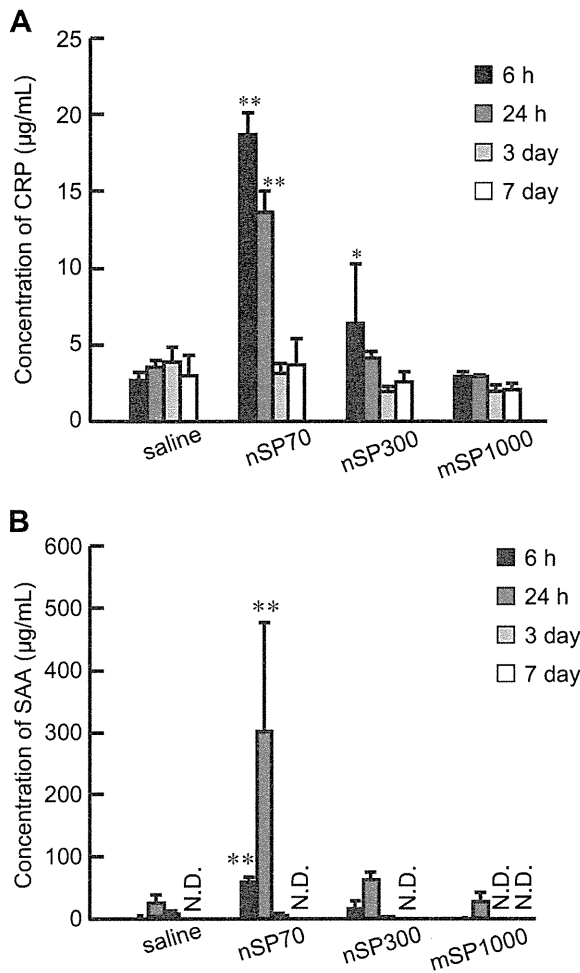


Fig. 3. Response of other acute phase proteins. BALB/c mice were intravenously treated with nSP70, nSP300 or mSP1000 at 0.8 mg/mouse. After 6 h, 24 h, 3 day and 7 day, blood samples were collected. The levels of (A) CRP and (B) SAA in the plasma of treated mice were examined by ELISA. Data are presented as mean \pm SD ($n = 5-6$; * $P < 0.05$, ** $P < 0.01$ versus value for saline treated group by ANOVA; N.D., not detected).

6 h after treatment, whereas that of haptoglobin and SAA was observed at 24 h. In contrast, the level of CRP and SAA in plasma of mSP1000 treated mice were almost the same as that of the saline treated control mice at all time points. The level of CRP in the plasma of nSP300 treated mice was slightly higher than that of saline treated control mice at 6 h. Our results suggest that both SAA and CRP may be useful biomarkers for predicting the risk from exposure to silica nanoparticles as well as haptoglobin. Indeed, these biomarkers could give even better response and sensitivity when used in combination.

3.4. The level of acute phase proteins through various routes

Exposure to nanomaterials in our daily lives can occur through various different routes. For example, nanomaterials contained in foods and drug medicines are taken up orally, whereas nanomaterials spread in the environment generally enter the body intranasally. Therefore, there is a need to evaluate suitable biomarkers for the exposure of nanomaterials through various routes. To assess the response of acute phase proteins to

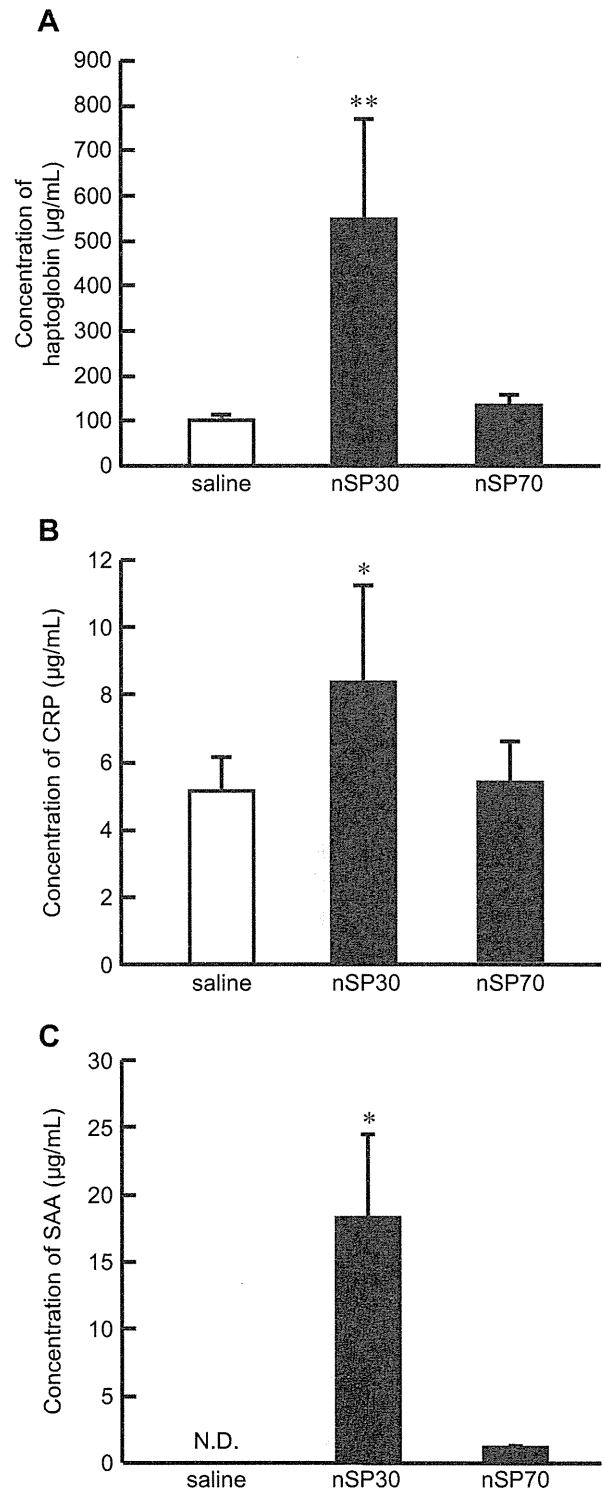


Fig. 4. Application of acute phase proteins to assess exposure of nanomaterials through various routes. To assess the administration of silica nanoparticles through an intranasal route, BALB/c mice were treated with nSP30, nSP70 or saline intranasally at 0.5 mg/mouse. Blood samples were collected 24 h after treatment. The level of (A) haptoglobin, (B) CRP and (C) SAA in the plasma were examined by ELISA. Data are presented as mean \pm SD ($n = 5-6$; * $P < 0.05$, ** $P < 0.01$ versus value for saline treated group by ANOVA; N.D., not detected).

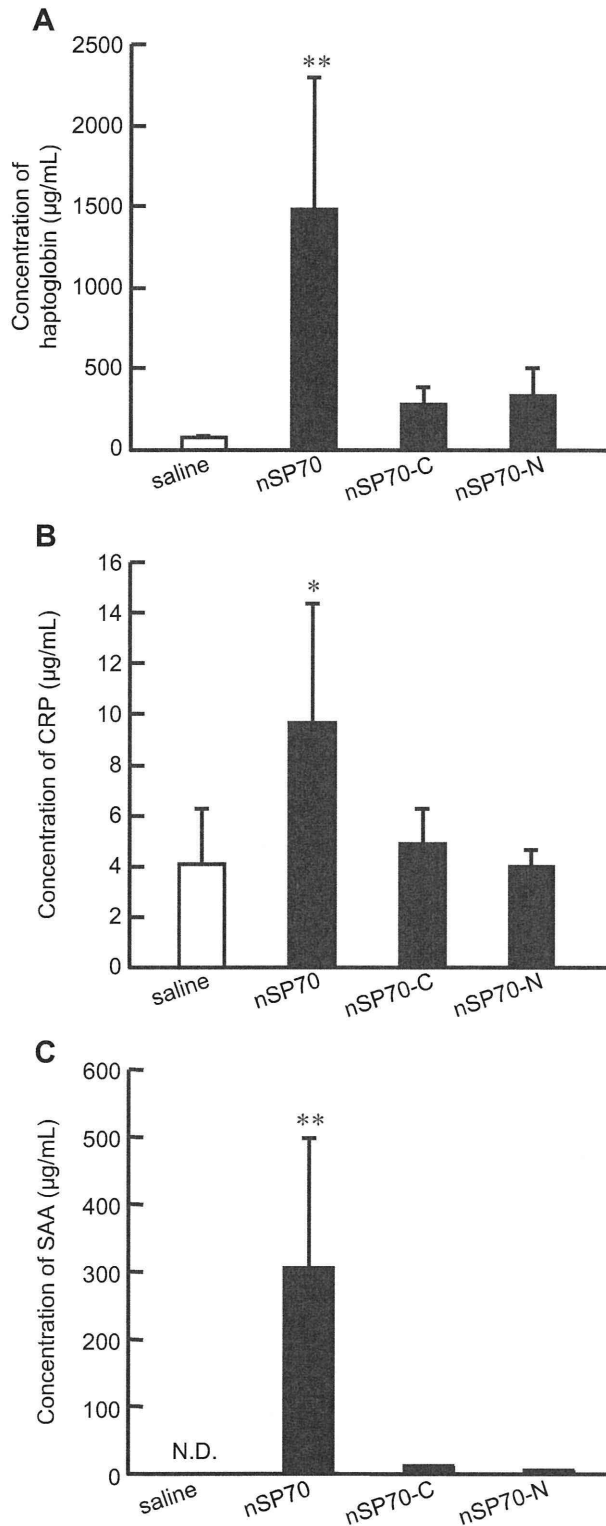


Fig. 5. Responses of acute phase proteins by the exposure to surface modified nSP70. BALB/c mice were intravenously treated with nSP70 modified with amino or carboxyl groups at 0.8 mg/mouse. After 24 h, the level of (A) haptoglobin, (B) CRP and (C) SAA in the plasma of treated mice were examined by ELISA. Data are presented as mean \pm SD ($n = 5-6$; * $P < 0.05$, ** $P < 0.01$ versus value for saline treated group by ANOVA; N.D., not detected).

silica particles introduced *via* different routes, we examined the level of haptoglobin, CRP and SAA in plasma after treatment of silica particles intranasally (Fig. 4). In this experiment, we used nSP30 and nSP70. For the administration of silica nanoparticles through an intranasal route, BALB/c mice were treated with nSP30, nSP70 or saline intranasally at 0.5 mg/mouse. After 24 h, we examined the level of haptoglobin (Fig. 4A), CRP (Fig. 4B) and SAA (Fig. 4C) in the plasma of the mice by ELISA. We showed that the level of haptoglobin, CRP and SAA in the plasma of mice treated with nSP30 intranasally was significantly higher than those of the saline treated control mice, although intranasal administration of nSP70 did not cause elevation in the plasma level of each acute phase protein in the treated mice. These results suggest that acute phase proteins could be useful biomarkers for predicting the risk arising from exposure to silica nanoparticles through various routes.

3.5. The level of acute phase proteins after treatment with surface modified silica nanoparticles

It has recently become evident that particle characteristics, including particle size and surface properties, are important factors in pathologic alterations and cellular responses [8,20–22]. Previously, our group also showed that surface modification of silica particles with functional groups such as amino or carboxyl group suppressed toxic biological effects of silica particles such as inflammatory responses [23]. To assess whether acute phase proteins could be useful biomarkers to predict risk factors associated with exposure to silica particles, we examined the level of haptoglobin (Fig. 5A), CRP (Fig. 5B) and SAA (Fig. 5C) in the plasma of mice after administration of nSP70 with amino or carboxyl group surface modifications. BALB/c mice were treated with 0.8 mg/mouse of these silica particles intravenously. After 24 h, we examined the level of haptoglobin, CRP and SAA in the plasma of the treated mice by ELISA. Our results showed that the level of these acute phase proteins in the plasma of nSP70 with amino or carboxyl group treated mice were significantly low compared with nSP70 treated mice.

4. Discussion

Our goal was to identify the biomarkers of nanomaterials for predicting their potential toxicity and to provide basic information for the creation of safe nanomaterials. To achieve these purposes, we tried to identify biomarkers in blood using a proteomics analysis. At first, we showed that the silica nanoparticles with small particle sizes (diameter < 100 nm) induced a higher level of acute phase proteins such as haptoglobin, CRP and SAA than larger silica particles (diameter > 100 nm) after intravenously treatment (Figs. 2 and 3). Previously, our group has shown that silica nanoparticles with relatively small particle size such as nSP70 induce a greater level of toxicity, including liver injury, compared to those of larger particle size [10,11]. Thus, there is a correlation between toxicity induced by the silica nanoparticles and the level of each potential plasma biomarker. Therefore, these acute phase proteins appear to be good biomarkers for predicting the strength of toxicity induced by silica nanoparticles.

The acute phase response is the nonspecific early response of an organism to infection and inflammation [24]. It comprises a whole array of systemic reactions and induction of a group of serum proteins called the acute phase proteins [25]. Monitoring the progression of infection and cancer by acute phase protein measurements in blood samples is used extensively in human patients. For example, haptoglobin is a biomarker of pancreatic cancer [26]. CRP is used as an index for the development of atrial fibrillation and maintenance [27], although mouse CRP is

synthesized only in trace amounts unlike its human counterpart [28]. In addition, both SAA and CRP are used as an index for adverse prognosis of breast cancer [29]. Therefore, we believe that these diagnostic systems using acute phase proteins for human health would be useful for predicting the risk of exposure to nanomaterials as well as their likely toxicities. In addition, we showed that the induction time for the maximum level of haptoglobin, SAA and CRP are different after treatment with the silica nanoparticles (Figs. 2 and 3). Therefore, the predictive quality of these biomarkers is improved when they are used in combination.

Epidemiological studies have suggested that increased levels of ambient particle including particle with nanometer size are associated with adverse effects in the respiratory and cardiovascular systems [30]. Some reports have shown that humans exposed to ambient particle have increased blood levels of CRP [31]. In addition, epidemiological studies have shown associations between increased concentrations of SAA and CRP in plasma, and increased risk of cardiovascular diseases [32] and cancer [33]. Therefore we consider that acute phase proteins would be biomarkers for predicting the risk of inflammatory disease, cardiovascular diseases and cancer after exposure by nanomaterials.

In recent years there has been increasing use of nanomaterials in cosmetics, due to their light-diffusing properties and absorbencies, as well as in foodstuffs, such as additives in powdered foods. In particular, silica particles have been extensively used in many consumer products. For example, in the US, the use of amorphous silica is limited to less than 2.0% by weight of common salt. Other limits are defined for finished foods (<1%) and dried egg products (<5%). We cannot avoid exposure to nanomaterials, either from the unintentional release of waste products into the environment or by exposure to medicines, cosmetics and foodstuffs. Thus, it is important to carry out a safety analysis of nanomaterials after exposure via various routes. In this study, we showed that the level of acute phase proteins in the plasma of mice treated with nSP30 intranasally was elevated, although nSP70 did not induce elevation of each acute phase protein (Fig. 4). Therefore we consider that nSP30 would induce any toxic biological effects after intranasally treatment. Now we are trying to examine the pharmacokinetics and biological effects of nSP30 after intranasally treatment.

We then examined the effects of surface modification of silica nanoparticles on the production of acute phase proteins, because it has become evident that surface properties are important factors in the biological effects of particles. We showed that nSP70 with amino or carboxyl group surface modifications did not induce the production of each acute phase proteins (Fig. 5). Previously, we showed that surface modification of silica particles with functional groups such as amino or carboxyl group suppressed toxic biological effects of silica particles such as inflammatory responses [23]. These results also suggest that acute phase proteins could be a promising candidate biomarker for predicting the strength of toxicity induced by silica nanoparticles, although it is need to examine the toxic biological effects of silica nanoparticles with functional groups. Over recent years, nanomaterials have been introduced into our everyday lives. For example, silica particles, titanium dioxide and fullerenes of various crystallographic structures and surface functional groups are used in a range of different consumer products. Therefore, we are now trying to evaluate the response of acute phase proteins to exposure from various nanomaterials.

In general, acute phase proteins are known to be released from the liver mainly as a result of inflammatory cytokines such as interleukin (IL)-6 [19]. However, we confirmed that the levels of IL-6 were not elevated in the plasma of mice treated with silica particles at 24 h after treatment (data not shown). Therefore it is unclear why nanomaterials induce the production of acute phase

proteins. We already showed that although silica particles with micrometer size tend to be taken up by Kupffer cells, silica nanoparticles with small particle sizes distribute around hepatic parenchymal cells (unpublished data). It is conceivable that instead of inflammatory cytokines, small silica particles act directly on the liver to induce the release of acute phase proteins. We are currently analyzing the detailed mechanism by which silica particles induce acute phase proteins in order to identify additional protein biomarkers.

5. Conclusions

We show here that acute phase proteins such as haptoglobin, CRP and SAA can act as useful biomarkers for analyzing the risk of exposure to nanomaterials and their associated toxicity. We believe that such information would be vital for the future development of predictive tests for estimation of the potential toxicity of new nanomaterials based on their physicochemical characteristics.

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References

- [1] Rutherglen C, Burke P. Nanoelectromagnetics: circuit and electromagnetic properties of carbon nanotubes. *Small* 2009;5:884–906.
- [2] Kaur IP, Agrawal R. Nanotechnology: a new paradigm in cosmeceuticals. *Recent Pat Drug Deliv Formul* 2007;1:171–82.
- [3] Cormode DP, Jarzyna PA, Mulder WJ, Fayad ZA. Modified natural nanoparticles as contrast agents for medical imaging. *Adv Drug Deliv Rev* 2010;62:329–38.
- [4] Nel A, Xia T, Madler L, Li N. Toxic potential of materials at the nanolevel. *Science* 2006;311:622–7.
- [5] Donaldson K, Murphy FA, Duffin R, Poland CA. Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma. *Part Fibre Toxicol* 2010;7:5.
- [6] Shvedova AA, Kagan VE, Fadeel B. Close encounters of the small kind: adverse effects of man-made materials interfacing with the nano-cosmos of biological systems. *Annu Rev Pharmacol Toxicol* 2010;50:63–88.
- [7] Poland CA, Duffin R, Kinloch I, Maynard A, Wallace WA, Seaton A, et al. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. *Nat Nanotechnol* 2008;3:423–8.
- [8] Morishige T, Yoshioka Y, Tanabe A, Yao X, Tsunoda S, Tsutsumi Y, et al. Titanium dioxide induces different levels of IL-1beta production dependent on its particle characteristics through caspase-1 activation mediated by reactive oxygen species and cathepsin B. *Biochem Biophys Res Commun* 2010;392:160–5.
- [9] Hougaard KS, Jackson P, Jensen KA, Sloth JJ, Loschner K, Larsen EH, et al. Effects of prenatal exposure to surface-coated nanosized titanium dioxide (UV-Titan). A study in mice. *Part Fibre Toxicol* 2010;7:16.
- [10] Nishimori H, Kondoh M, Isoda K, Tsunoda S, Tsutsumi Y, Yagi K. Silica nanoparticles as hepatotoxicants. *Eur J Pharm Biopharm* 2009;72:496–501.
- [11] Nishimori H, Kondoh M, Isoda K, Tsunoda S, Tsutsumi Y, Yagi K. Histological analysis of 70-nm silica particles-induced chronic toxicity in mice. *Eur J Pharm Biopharm* 2009;72:626–9.
- [12] Nishimori H, Kondoh M, Isoda K, Tsunoda S, Tsutsumi Y, Yagi K. Influence of 70 nm silica particles in mice with cisplatin or paraquat-induced toxicity. *Pharmazie* 2009;64:395–7.
- [13] Casado B, Iadarola P, Luisetti M, Kussmann M. Proteomics-based diagnosis of chronic obstructive pulmonary disease: the hunt for new markers. *Expert Rev Proteomics* 2008;5:693–704.

- [14] Ferte C, Andre F, Soria JC. Molecular circuits of solid tumors: prognostic and predictive tools for bedside use. *Nat Rev Clin Oncol*; 2010 Jun 15 [Epub ahead of print].
- [15] Vaidya VS, Ozer JS, Dieterle F, Collings FB, Ramirez V, Troth S, et al. Kidney injury molecule-1 outperforms traditional biomarkers of kidney injury in preclinical biomarker qualification studies. *Nat Biotechnol* 2010;28:478–85.
- [16] Ozer JS, Dieterle F, Troth S, Perentes E, Cordier A, Verdes P, et al. A panel of urinary biomarkers to monitor reversibility of renal injury and a serum marker with improved potential to assess renal function. *Nat Biotechnol* 2010;28:486–94.
- [17] Villota R, Hawkes JG. Food applications and the toxicological and nutritional implications of amorphous silicon dioxide. *Crit Rev Food Sci Nutr* 1986;23:289–321.
- [18] Merget R, Bauer T, Kupper HU, Philippou S, Bauer HD, Breitstadt R, et al. Health hazards due to the inhalation of amorphous silica. *Arch Toxicol* 2002;75:625–34.
- [19] Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med* 1999;340:448–54.
- [20] He X, Nie H, Wang K, Tan W, Wu X, Zhang P. In vivo study of biodistribution and urinary excretion of surface-modified silica nanoparticles. *Anal Chem* 2008;80:9597–603.
- [21] Nabeshi H, Yoshikawa T, Matsuyama K, Nakazato Y, Arimori A, Isoe M, et al. Size-dependent cytotoxic effects of amorphous silica nanoparticles on Langherhans cells. *Pharmazie* 2010;65:199–201.
- [22] Yamashita K, Yoshioka Y, Higashisaka K, Morishita Y, Yoshida T, Fujimura M, et al. Carbon nanotubes elicit DNA damage and inflammatory response relative to their size and shape. *Inflammation* 2010;33:276–80.
- [23] Morishige T, Yoshioka Y, Inakura H, Tanabe A, Yao X, Narimatsu S, et al. The effect of surface modification of amorphous silica particles on NLRP3 inflammasome mediated IL-1beta production, ROS production and endosomal rupture. *Biomaterials*; 2010 Jun 17 [Epub ahead of print].
- [24] Baumann H, Gaudie J. The acute phase response. *Immunol Today* 1994;15:74–80.
- [25] Kushner I. The phenomenon of the acute phase response. *Ann N Y Acad Sci* 1982;389:39–48.
- [26] Firpo MA, Gay DZ, Granger SR, Scaife CL, DiSario JA, Boucher KM, et al. Improved diagnosis of pancreatic adenocarcinoma using haptoglobin and serum amyloid A in a panel screen. *World J Surg* 2009;33:716–22.
- [27] Korantzopoulos P, Kalantzi K, Siogas K, Goudevenos JA. Long-term prognostic value of baseline C-reactive protein in predicting recurrence of atrial fibrillation after electrical cardioversion. *Pacing Clin Electrophysiol* 2008;31:1272–6.
- [28] Szalai AJ, McCrory MA. Varied biologic functions of C-reactive protein: lessons learned from transgenic mice. *Immunol Res* 2002;26:279–87.
- [29] Pierce BL, Neuhaus ML, Wener MH, Bernstein L, Baumgartner RN, Ballard-Barbash R, et al. Correlates of circulating C-reactive protein and serum amyloid A concentrations in breast cancer survivors. *Breast Cancer Res Treat* 2009;114:155–67.
- [30] Mauderly JL, Chow JC. Health effects of organic aerosols. *Inhal Toxicol* 2008;20:257–88.
- [31] Ruckerl R, Ibald-Mulli A, Koenig W, Schneider A, Woelke G, Cyrus J, et al. Air pollution and markers of inflammation and coagulation in patients with coronary heart disease. *Am J Respir Crit Care Med* 2006;173:432–41.
- [32] Albert MA, Ridker PM. The role of C-reactive protein in cardiovascular disease risk. *Curr Cardiol Rep* 1999;1:99–104.
- [33] Siemes C, Visser LE, Coebergh JW, Splinter TA, Wittteman JC, Uitterlinden AG, et al. C-reactive protein levels, variation in the C-reactive protein gene, and cancer risk: the Rotterdam Study. *J Clin Oncol* 2006;24:5216–22.



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

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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 (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 mSP1000, 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-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% 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 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 reagent 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 × 10¹³ 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 reagent (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% 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. 1 × 10⁴ cells were cultured with varying concentrations of silica particles or QDs for 18 h at 37 °C and ³H-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 × 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, mSP1000 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, 0.3 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

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 photographs 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, liver, 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/l EGTA (DOJINDO) for 5 min. The liver was then perfused with 40 ml of HBSS containing 250 mg/l 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 L15 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

The first step for ensuring the biosafety of NMs are to evaluate whether the NMs could penetrate the epithelial barriers, could eventually become absorbed systemically, and more importantly, whether they could be responsible for acute/chronic side effects. In this context, here, we evaluated whether the nSP and QD could penetrate into the skin of BALB/c mice following dermal exposure. Prior to undertaking the skin penetration study, we first analyzed the physicochemical properties of the commercially available silica particles of 70, 300 and 1000 nm in diameter (nSP70, nSP300 and mSP1000, respectively). Close examination of the silica particles of different particle sizes by TEM revealed that all silica particles used in this study were smooth-surfaced spherical particles and in size category the primary particle sizes were approximately uniform (Fig. 1a–f). According to technical datasheet, surface textures of all silica particles were plain and nonporous. The specific surface area was calculated by means of the following equation; $s = 6/d\rho$ (where s , specific surface area (m²/g); ρ , density (g/cc); d , diameter (μm)). The specific surface area of nSP70, nSP300 and mSP1000 calculated using this equation was 43, 10 and 3 m²/g, respectively. In addition, all silica particles used in this study was not modified with any functional groups. These physicochemical properties were summarized in Table 1. From the results of mean particle size in solution, it was suggested that the silica particles used in this study remained as stable well-dispersed particles in solution, and not as aggregates.

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

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 the 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

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

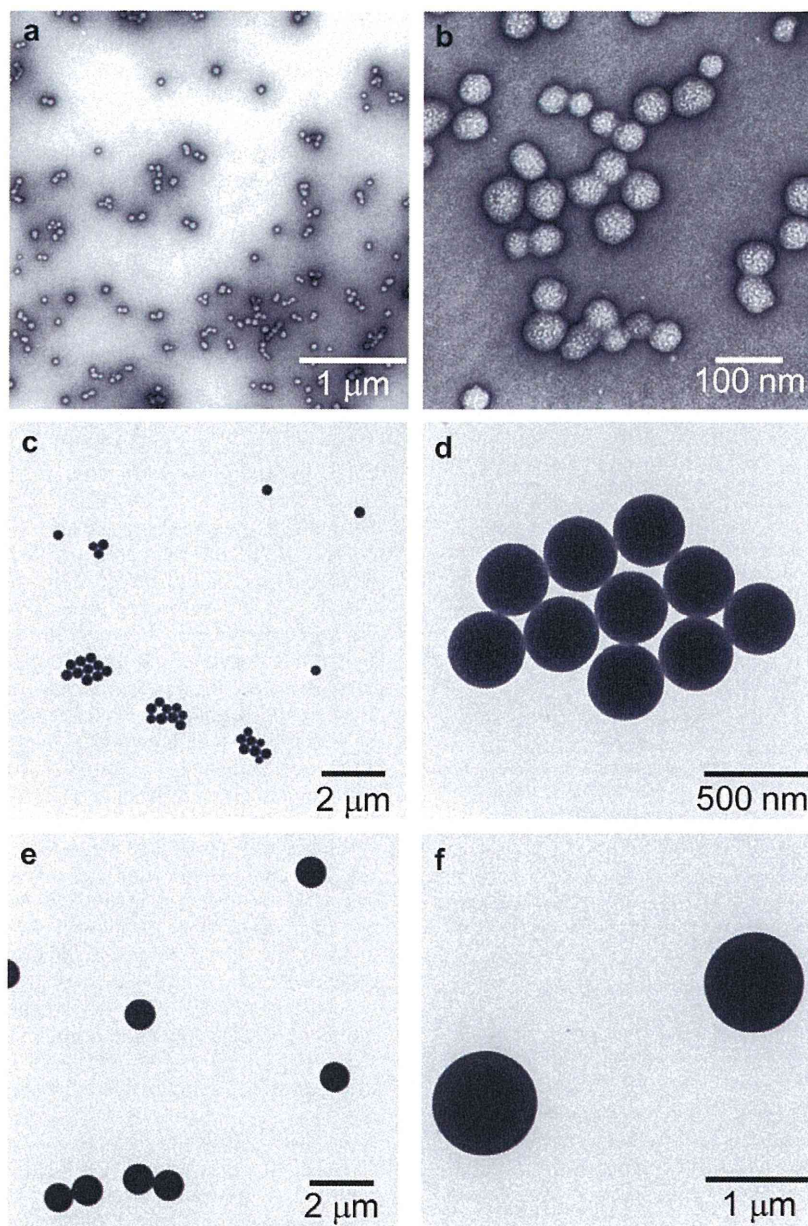


Fig. 1. Transmission electron microscopy (TEM) analysis of silica particles. a–f, TEM photomicrographs of silica particles used in this study: nSP70 (a and b), nSP300 (c and d) and mSP1000 (e and f). Each type of sized silica particles existed as scattered and spheroidal. Scale bars: 1 μm (a and f), 100 nm (b), 500 nm (d) and 2 μm (c and e).

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.

	Primary particle size (nm) ^a	Hydrodynamic diameter (nm)	Mean zeta potential (mV)	pH	Surface texture ^a	Porosity ^a	Surface area (m ² /g) ^b	Functional group ^a
nSP70	70	77.0 \pm 0.4	-21.6 \pm 4.5	7.4	Plain	Nonporous	43	None
nSP300	300	269.3 \pm 2.1	-31.3 \pm 6.5	7.5	Plain	Nonporous	10	None
mSP1000	1000	1187 \pm 25.2	-37.7 \pm 4.6	7.9	Plain	Nonporous	3	None

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

^a Information from technical datasheet of products.

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

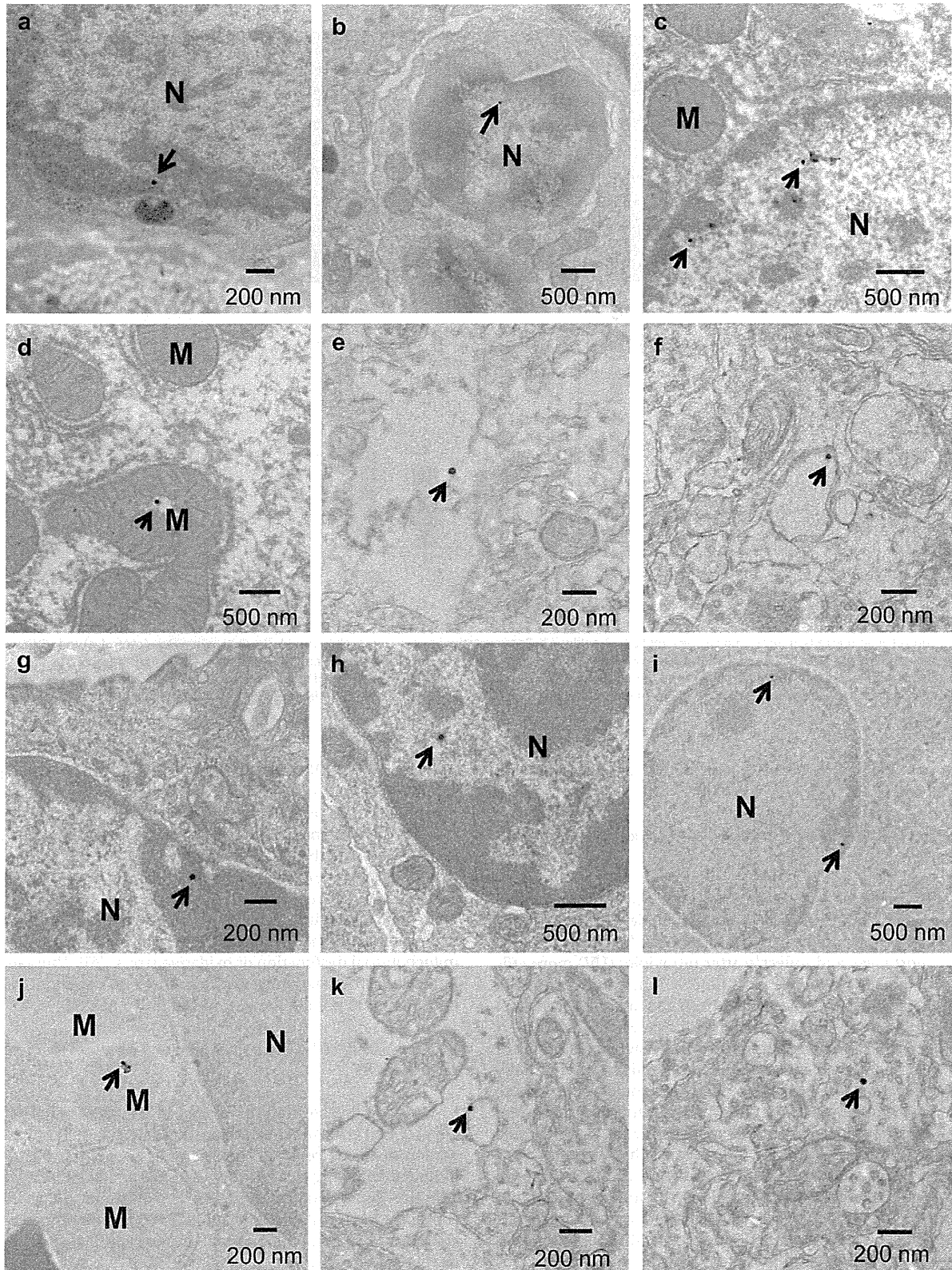


Fig. 2. TEM analysis of skin, lymph node, liver and brain samples from mice after 28-days of dermal exposure to silica nanoparticles and quantum dots. a–c, nSP70 (arrows) were present in the nucleus of skin (a), cervical lymph node (b), and parenchymal hepatocytes (c). d, nSP70 were also detected in 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).

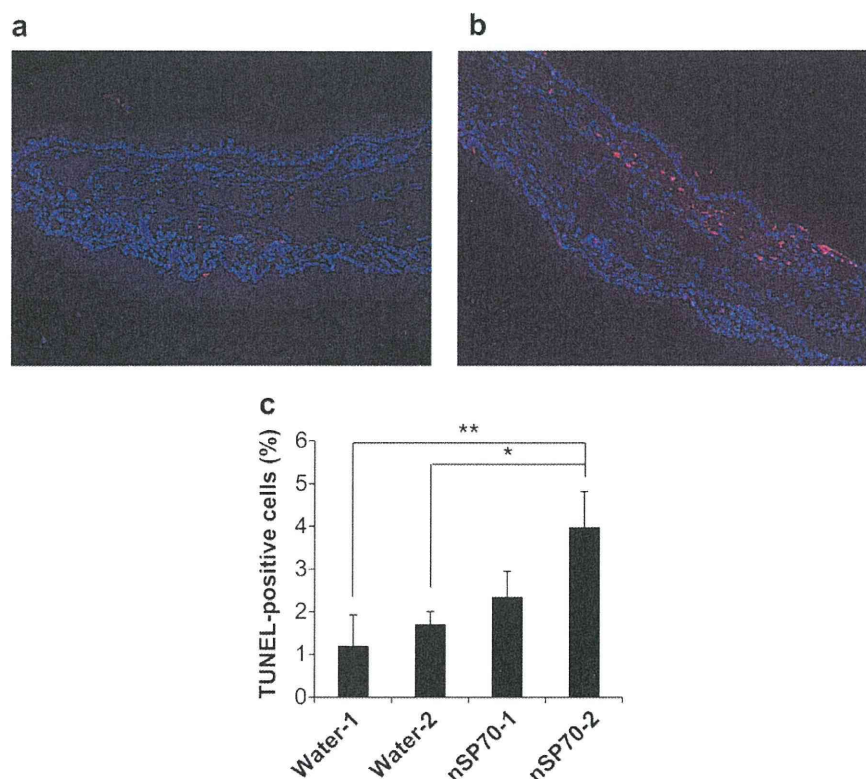


Fig. 3. Detection of apoptotic cells in silica nanoparticles-applied mice skin. a, b, Representative images from TUNEL stained skin section in water- (control, a) and nSP70- (b) applied mice were shown. Nuclei and TUNEL-positive cells were represented in blue and red, respectively. Original magnification, $\times 200$. c, For quantification of TUNEL-positive cells, approximately 1000 cells were randomly selected from 3 different areas in each section and two animals and counted the numbers of positive cells expressed as a percentage of the total (c). Data shown are average means (\pm SD) of each treated group. * Significant increase ($P < 0.005$) compared with Water-2. ** Significant increase ($P < 0.001$) compared with Water-1.

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 (TiO_2), zinc oxide (ZnO) and amorphous silica particles. Nano-sized TiO_2 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 nSP70 migrated into the blood stream and passed into tissues such as liver. Moreover, nSP70 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 intercellular location of silica particles, we used TEM to examine the HaCaT cells that were treated with 100 $\mu\text{g}/\text{ml}$ 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 (IC_{50}) of nSP70 and nSP300 for inhibiting cell proliferation was 323 and 3966 $\mu\text{g}/\text{ml}$, respectively. We were, however, unable to calculate the IC_{50} 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

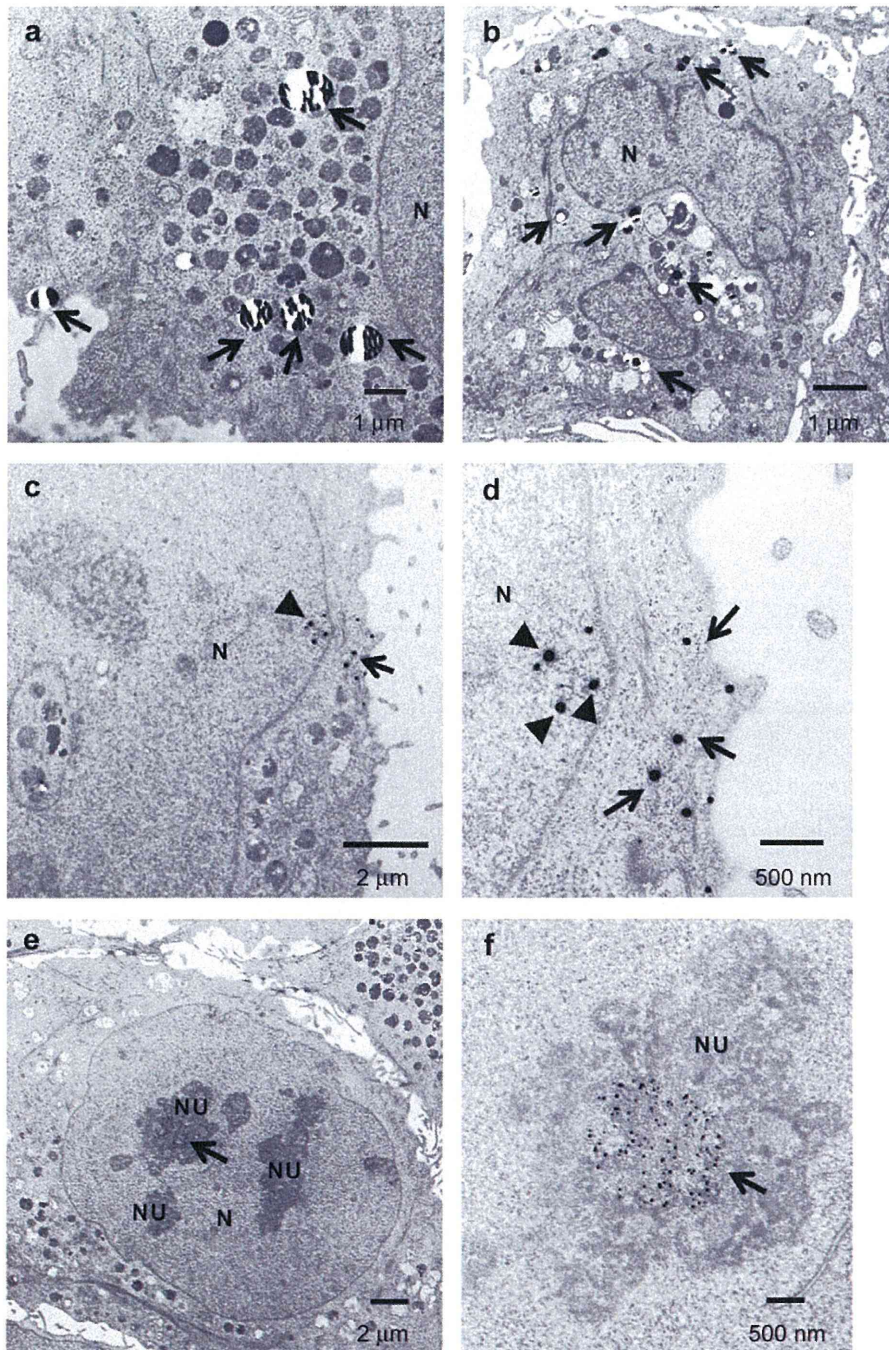


Fig. 4. TEM analysis of HaCaT cells treated with silica particles. a–e, Silica particles (arrows) were found in HaCaT cells treated for 24 h with 100 µg/ml of mSP1000 (a), nSP300 (b), and nSP70 (c, d, and e). In panels c and d, arrow heads show the presence of nSP70 in the nucleus, and in panels e and 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 µm (a and b), 2 µm (c and e) and 500 nm (d and f).

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 (810 µg/ml) (Fig. 6b). At lower doses (30 and 90 µg/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 µm (Fig. 6c). In cells treated with 90 µg/ml of nSP70, nSP100, nSP300, or mSP1000, the average tail lengths were 102.9 µm (Fig. 6d), 88.8 µm (data not shown), 30.5 µm (Fig. 6e), and 22.5 µm (Fig. 6f), respectively.

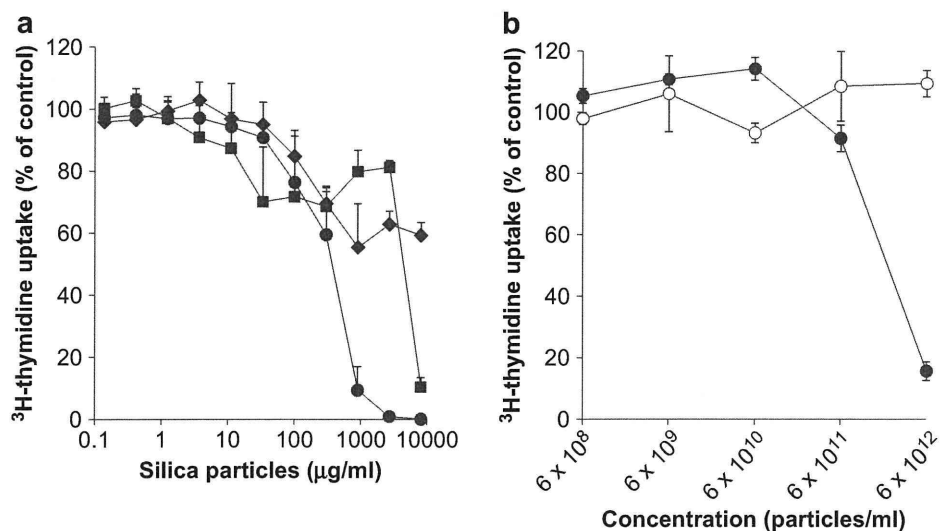


Fig. 5. Effect of various sized silica particles and QDs on cell proliferation. a) In order to assess the biological effects according to particle size, proliferation of HaCaT cells following 24 h of incubation with the indicated concentrations of nSP70 (closed circle), nSP300 (closed square) and mSP1000 (closed diamond) were measured using the tritium thymidine uptake assay. b) In order to assess the biological effects according to the material, proliferation of HaCaT cells following 24 h of incubation with the indicated concentrations of nSP70 (closed circle) and QD (opened circle) were measured. Results shown as relative rates (% of control). Each data point represents mean \pm SD ($n = 3$).

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 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). Surprisingly, sub-nuclear localization of particles was observed in nSP70-treated groups (Fig. 7e). These results confirmed that the

distribution of nSPs with particle size less than 100 nm differ from those of submicron-sized silica particles. These data strongly suggested that we must distinguish nSPs from existing submicron-sized silica particles, and address specialized risk assessment for nSPs.

3.7. Analysis of cytotoxicity and genotoxicity in primary hepatocyte induced by silica particles

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