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Surface modification of amorphous nanosilica particles suppresses nanosilica-induced cytotoxicity, ROS generation, and DNA damage in various mammalian cells

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ABSTRACT

Recently, nanomaterials have been utilized in various fields. In particular, amorphous nanosilica particles are increasingly being used in a range of applications, including cosmetics, food technology, and medical diagnostics. However, there is concern that the unique characteristics of nanomaterials might induce undesirable effects. The roles played by the physical characteristics of nanomaterials in cellular responses have not yet been elucidated precisely. Here, by using nanosilica particles (nSPs) with a diameter of 70 nm whose surface was either unmodified (nSP70) or modified with amine (nSP70-N) or carboxyl groups (nSP70-C), we examined the relationship between the surface properties of nSPs and cellular responses such as cytotoxicity, reactive oxygen species (ROS) generation, and DNA damage. To compare the cytotoxicity of nSP70, nSP70-N, or nSP70-C, we examined *in vitro* cell viability after nSP treatment. Although the susceptibility of each cell line to the nSPs was different, nSP70-C and nSP70-N showed lower cytotoxicity than nSP70 in all cell lines. Furthermore, the generation of ROS and induction of DNA damage in nSP70-C- and nSP70-N-treated cells were lower than those in nSP70-treated cells. These results suggest that the surface properties of nSP70 play an important role in determining its safety, and surface modification of nSP70 with amine or carboxyl groups may be useful for the development of safer nSPs. We hope that our results will contribute to the development of safer nanomaterials.

1. Introduction

The innovative development of nanotechnology has resulted in the creation of many nanomaterials (NMs), which are defined as materials with a diameter $\leqslant$100 nm. For example, amorphous nanosilica particles (nSPs) are NMs that have received wide attention in a variety of industries, such as the medical, cosmetic, and food industries [1,2]. Because nSPs are relatively inexpensive and their surface is easily modified, their use is expected to become more widespread. Since, human exposure to NMs, including nSPs, will increase, the development of more effective and safer forms of NMs is urgently needed. Recent studies have reported that NMs may have unforeseen biologic effects that conventional-sized materials do not. For instance, Poland et al., have suggested that carbon nanotubes induce mesothelioma-like lesions in mice in a manner similar to crocidolite asbestos [3]. Other groups have shown that titanium dioxide nanoparticles enter brain tissue and induce the production of reactive oxygen species (ROS) and inflammation [4]. Furthermore, nSPs have been reported to induce oxidative stress, genotoxicity, and inflammation both *in vitro* and *in vivo* [5–7].

ROS generation induces undesirable biologic effects such as inflammation or genotoxicity [8], and recently, many reports have

Abbreviations: DCF, 2′7′-dichlorofluorescein; DCFH-DA, 2′7′-dichlorodihydorofluorescein diacetate; D-MEM, Dulbecco's modified Eagle's medium; LDH, lactate dehydrogenase; NMs, nanomaterials; nSPs, nanosilica particles; ROS, reactive oxygen species; SD, standard deviation.

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suggested that various NM-mediated cellular responses are involved in ROS generation [9–11]. We have shown that nSPs induce inflammation or DNA damage via ROS generation *in vitro* [12,13]. Therefore, a method to avoid nSP-mediated ROS generation is needed for the development of safe nSPs.

It is well known that NM-mediated biologic effects are related to the physical characteristics of NMs, such as particle size and surface properties. Previously, we showed that nSP-mediated pregnancy complications or inflammation could be avoided by surface modification of the nSP with amino or carboxyl groups [13,14]. Another group has also reported that these surface modifications reduce nSP-induced hepatic toxicity [15]. In contrast, there are few studies that assess the relationship between the surface properties of nSPs and cellular responses such as ROS generation. In this study, we examined the relationship between the surface properties of nSPs and cellular responses such as cytotoxicity, ROS generation, and DNA damage.

2. Materials and methods

2.1. Silica particles

Amorphous nSPs (external diameter, 70 nm) with surfaces that were either unmodified or modified with amine or carboxyl groups were purchased from Micromod Partikeltechnologie GmbH, Rostock, Germany (designated nSP70, nSP70-N, and nSP70-C, respectively). The nSPs were sonicated for 5 min and vortexed for 1 min immediately prior to use.

2.2. Cell culture

HaCaT cells (human keratinocyte cell line) were kindly provided by Dr. Inui, Osaka University, Japan [16]. C8-B4 cells (murine microglial cell line), PC12 cells (rat pheochromocytoma cell line), and IEC-6 cells (rat normal small intestinal cell line) were obtained from American Type Culture Center (ATCC, Manassas, VA). TLR-1 cells (murine hepatocyte cell line) were provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University, Japan. HaCaT cells were cultured in Dulbecco's modified Eagle's medium (D-MEM; Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum, 0.2 mM L-glutamine, and 1% Antibiotic–Antimycotic Mix stock solution. C8-B4 cells were cultured in D-MEM supplemented with 10% heat-inactivated fetal bovine serum, and 1% Antibiotic–Antimycotic Mix stock solution. PC12 cells were cultured in RPMI-1640 medium (Wako) supplemented with 10% heat-inactivated fetal bovine serum, 10% heat-inactivated horse serum, and 1% Antibiotic–Antimycotic Mix stock solution. IEC-6 cells were cultured in D-MEM supplemented with 10% heat-inactivated fetal bovine serum, 1 μg/mL insulin (Sigma–Aldrich, Saint Louis, MO), and 1% Antibiotic–Antimycotic Mix stock solution. These cell lines were grown in a humidified incubator at 37 °C (95% room air, 5% CO_2). TLR-1 cells were cultured in RITC80-7 medium supplemented with 10 μg/mL transferrin, 1 μg/mL insulin, 10 ng/mL human Epidermal Growth Factor (Sigma–Aldrich), and 1% Antibiotic–Antimycotic Mix stock solution. The cells were grown in a humidified incubator at 33 °C (95% room air, 5% CO_2).

2.3. ^{3}H-thymidine incorporation assay

The cytotoxicity of the nSP70s against C8-B4 cells, PC12 cells, IEC-6 cells, and TLR-1 cells was measured by using a ^{3}H-thymidine incorporation assay. Cells (2×10^4 cells/well seeded in 96-well plates) were cultured with various concentrations of nSP70, nSP70-C, or nSP70-N for 18 h at 37 °C; ^{3}H-thymidine (1 μCi/well) was then added into each well. After a further 6 h, cells were harvested and lysed on glass fiber filter plates using a cell harvester (PerkinElmer, Waltham, MA). The filter plates were then dried and counted by standard liquid scintillation counting techniques in a microplate counter (TopCount; PerkinElmer).

2.4. Lactate dehydrogenase release assay

The cytotoxicity of the nSP70s against HaCaT cells was evaluated by measurement of lactate dehydrogenase (LDH) release. Cells (5×10^3 cells/well seeded in a 96-well plate) were cultured with various concentrations of nSP70, nSP70-C, nSP70-N, or 0.2% Tween 20 (positive control) for 24 h at 37 °C. LDH activity of the supernatant of the culture medium was then determined using a commercial LDH cytotoxicity test (Wako) according to the manufacturer's instructions.

2.5. Detection of ROS

The generation of total intracellular ROS in HaCaT cells and TLR-1 cells was measured by monitoring the increasing fluorescence of 2′7′-dichlorofluorescein (DCF). The cell permeant 2′7′-dichlorodihydorofluorescein diacetate (DCFH-DA; Sigma–Aldrich) enters cells where intracellular esterases cleave off the diacetate group. The resulting DCFH is retained in the cytoplasm and oxidized to DCF by ROS. Cells (3×10^4 cells/well seeded in a 96-well plate) were cultured for 24 h and then treated with various concentrations of nSP70, nSP70-C, or nSP70-N for 3 h at 37 °C. Cells were then washed once with phenol red-free medium, and incubated in 100 μL of a working solution of DCFH-DA at 37 °C for 30 min. Using a fluorescence reader (ARVO MX; Perkin Elmer), the fluorescence of DCF was monitored at the excitation and emission wavelengths of 485 and 530 nm, respectively.

2.6. Comet assay

Damage of endogenous DNA in HaCaT cells after treatment with nSP70, nSP70-C, or nSP70-N was analyzed by using a Comet Assay Kit (Trevigen, Gaitherburg, MD, USA) as described previously [12,17,18]. HaCaT cells (3×10^4 cells/well seeded in a 6-well plate) were cultured for 24 h; cells were then treated with 30 μg/mL nSP70, nSP70-C, nSP70-N, or PBS (negative control) for 3 h. Cells from each group were resuspended at a density of 1×10^5 cells/mL in ice-cold calcium- and magnesium-free-PBS and combined with molten Low Melting Agarose (Trevigen) at a ratio of 1:10 (v/v). The cell–agarose mixture was immediately pipetted onto a frosted microscope slide (Comet Slide; 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 and leave 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 manufacturer's instructions. Twenty-five cells on each slide, which were randomly selected by use of fluorescence microscopy, were then analyzed by using a Comet Analyzer (Youworks, Tokyo, Japan). All steps were conducted under dim yellow light to prevent additional DNA damage.

2.7. Statistical analysis

All results are presented as means ± standard deviation (SD). Differences were compared by using Tukey's method after analysis of variance (ANOVA).

3. Results

To assess the relationship between the surface properties of nSPs and cellular responses, we used nSPs with diameters of 70 nm whose surface was either left unmodified (nSP70) or was chemically modified with amine (nSP70-N) or carboxyl groups (nSP70-C). In a previous study, the mean secondary particle diameter of each type of nSP was shown to be 64.2, 72.7, and 76.2 nm (nSP70, nSP70-N, and nSP70-C, respectively) [19]. The surface charge (zeta potential) of each type of silica particle was -42.1 mV, -29.8 mV, and -72.0 mV (nSP70, nSP70-N, and nSP70-C, respectively) [19].

To compare the cytotoxicity of nSP70, nSP70-N, and nSP70-C, we used a $_3$H-thymidine incorporation assay to examine cell proliferation after treatment with the nSP70s in TLR-1 cells (murine hepatocyte cell line), C8-B4 cells (murine microglial cell line), PC12 cells (rat pheochromocytoma cell line), and IEC-6 cells (rat normal small intestinal cell line). The doubling time of HaCaT cells (human keratinocyte cell line) was long, so evaluation of cytotoxicity by means of $_3$H-thymidine incorporation was unsuitable. Therefore, we examined cytotoxicity in HaCaT cells by using an LDH release assay. Although the susceptibility of each cell to the nSPs was different, nSP70 showed higher cytotoxicity than nSP70-C and nSP70-N in all cell lines (Fig. 1A–E).

Previously, we showed that nSP70 enters keratinocyte cells, and translocates to tissues throughout the whole body, such as liver, after dermal exposure in mice [18]. Therefore, we focused on HaCaT cells (keratinocyte cells) and TLR-1 cells (hepatocyte cells) to evaluate the effect of surface modification of nSP70 on ROS generation and DNA damage. Total intracellular ROS generation was measured in nSP70-, nSP70-N-, or nSP70-C-treated HaCaT cells or TLR-1 cells by assessing 2′7′-dichlorofluorescein fluorescence. In both cell lines, nSP70 induced intracellular ROS generation in a dose-dependent fashion (Fig. 2A and B). In contrast, generation of ROS by nSP70-N or nSP70-C treatment was significantly lower in both cells compared with nSP70 treatment at the same concentration (Fig. 2A and B).

In a previous study, we showed that nSP70-induced ROS generation resulted in DNA damage in HaCaT cells [12]. We therefore analyzed DNA damage in nSP70-, nSP70-N-, or nSP70-C-treated HaCaT cells by using a comet assay. The DNA damage was evaluated by tail length (Fig. 3A) and tail moment (Fig. 3B), which were defined as the product of the comet length and the amount of DNA in the tail, and the ratio of the tail length to head diameter (Fig. 3C). In cells treated with nSP70, DNA damage was induced significantly compared to negative control cells (PBS treated cells) (Fig. 3A–C). In contrast, in the nSP70-N- or nSP70-C-treated cells, DNA damage was significantly lower than that in nSP70-treated cells, and the levels of DNA damage were equal to that of negative control cells (Fig. 3A–C).

Taken together, these results indicate that the undesirable cellular effects of nSP70, such as cytotoxicity, ROS generation, and DNA damage, are reduced by surface modification of the nSP.

4. Discussion

Recently, the biodistribution of NMs through different exposure pathways has been revealed. For example, some reports have shown that NMs enter through neuronal pathways such as the olfactory bulb–brain translocation pathway [20–22]. In a previous study, we showed that after intravenous administration nSP70 tends to accumulate in the liver [18]. Thus, it is important to gather safety information on NMs using *in vitro* tests and cell lines from a range of tissues.

In the current study, we used five cell lines to show that surface modification of nSPs reduces cytotoxicity, ROS generation, and

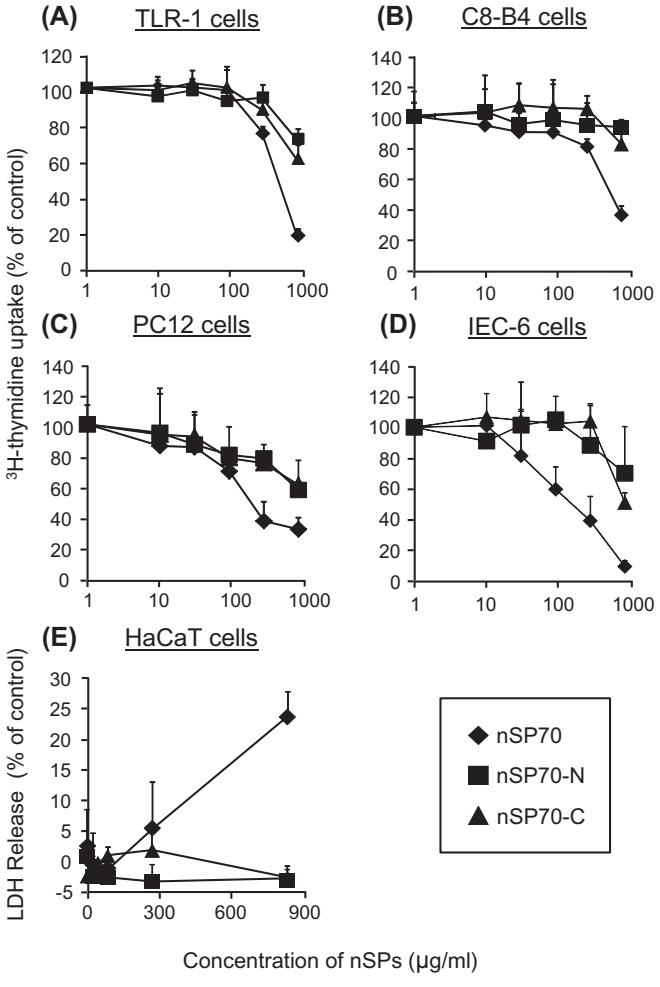

Fig. 1. Effect of surface modification of nSP70 on cytotoxicity. The cytotoxicity of (A) TLR-1 cells, (B) C8-B4 cells, (C) PC12 cells, (D) IEC-6 cells, and (E) HaCaT cells after incubation with nSP70, nSP70-N, or nSP70-C at various concentrations for 24 h was evaluated by use of a ^{3}H-thymidine incorporation assay an LDH release assay. The percentage increase in cell proliferation was calculated relative to the negative (medium) control. Data are presented as means ± SD.

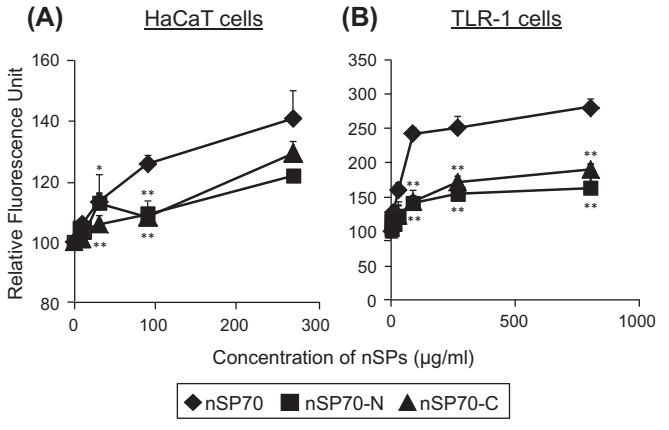

Fig. 2. Effect of surface modification of nSP70 on total ROS generation. Total ROS generation in (A) HaCaT cells and (B) TLR-1 cells incubated with nSP70, nSP70-N, or nSP70-C at various concentrations for 3 h was assessed by using a 2′7′-dichlorodihydorofluorescein assay. Total ROS induced by treatment with nSP70s is expressed as relative fluorescence units. Data are presented as means ± SD. $^{**}P < 0.01$, $^{*}P < 0.05$ vs value for same dose of nSP70.

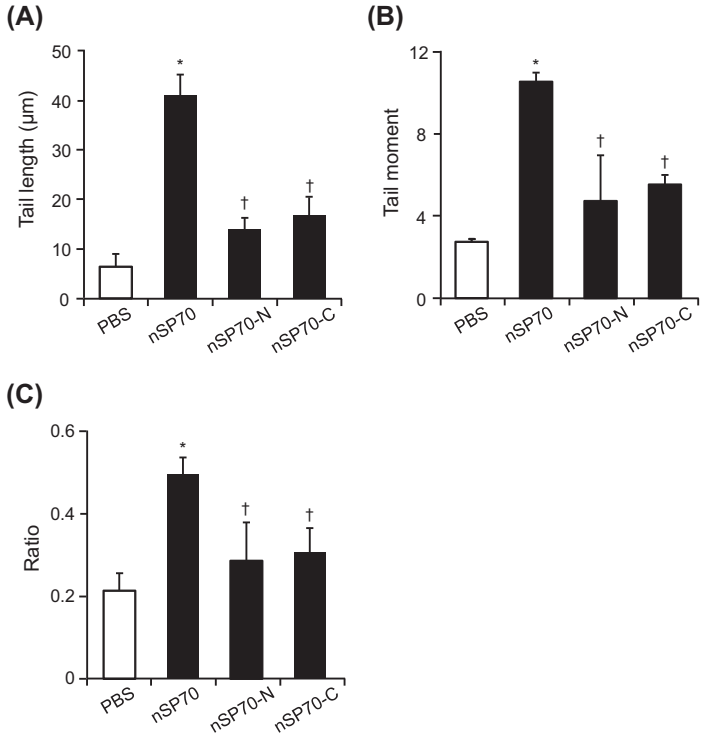

Fig. 3. Effect of surface modification of nSPs on DNA damage in HaCaT cells. HaCaT cells were incubated with nSP70, nSP70-N, or nSP70-C at 90 μg/mL for 3 h. The DNA damage was measured by (A) the tail length, (B) the tail moment, and (C) the ratio of tail length to head diameter by use of a comet assay. Data are presented as means ± SD. $^{*}P < 0.01$ vs value for negative (PBS) control, $^{\dagger}P < 0.05$ vs same dose of nSP70.

DNA damage. It has been suggested that one of the factors in NM-mediated cellular responses is the cellular uptake or distribution of NMs based on their interactions with biologic proteins [23–25]. Previously, we found that although unmodified nSP70 and nSP70-C were taken up equally into cells, only nSP70 induced inflammatory responses *in vitro* [13]. Therefore, we speculated that each nSP was taken up into various cells equally in this study. On the other hand, we showed that differences in the cytotoxicity of nSP70, nSP70-N, or nSP70-C in mouse macrophage cells might be attributed to the different cellular distributions of each nSP [19]. We next plan to precisely examine the relationship among the cellular uptake/distribution of nSP70s, ROS generation and DNA damage.

ROS are primary mediators of DNA damage [26,27]. Previously, we showed that activation of NADPH oxidase, which is an important enzymatic source of ROS [28], via phagocytosis of nSP70 is a factor in DNA damage [12,29]. Therefore, we speculated that the surface modification of nSPs alter the level of activation of NADPH oxidase, and this result in the reduction of nSP70-C and -N induced-ROS generation and DNA damage. We plan to examine the relationship between NADPH oxidase activation and surface modification of nSPs; this information will help in the creation of safer forms of nSPs.

In summary, our results indicate that modification of the surface of nSPs with amine or carboxyl groups may be effective for the creation of safer nSPs. We hope that our studies will contribute to improving the safety of NMs.

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