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Size and surface modification of amorphous silica particles determine their effects on the activity of human CYP3A4 *in vitro*

Shunji Imai[1], Yasuo Yoshioka[1*], Yuki Morishita[1], Tokuyuki Yoshida[1], Miyuki Uji[1], Kazuya Nagano[2], Yohei Mukai[3], Haruhiko Kamada[2,4], Shin-ichi Tsunoda[2,4], Kazuma Higashisaka[1] and Yasuo Tsutsumi[1,4*]

Abstract

Because of their useful chemical and physical properties, nanomaterials are widely used around the world - for example, as additives in food and medicines - and such uses are expected to become more prevalent in the future. Therefore, collecting information about the effects of nanomaterials on metabolic enzymes is important. Here, we examined the effects of amorphous silica particles with various sizes and surface modifications on cytochrome P450 3A4 (CYP3A4) activity by means of two different *in vitro* assays. Silica nanoparticles with diameters of 30 and 70 nm (nSP30 and nSP70, respectively) tended to inhibit CYP3A4 activity in human liver microsomes (HLMs), but the inhibitory activity of both types of nanoparticles was decreased by carboxyl modification. In contrast, amine-modified nSP70 activated CYP3A4 activity. In HepG2 cells, nSP30 inhibited CYP3A4 activity more strongly than the larger silica particles did. Taken together, these results suggest that the size and surface characteristics of the silica particles determined their effects on CYP3A4 activity and that it may be possible to develop silica particles that do not have undesirable effects on metabolic enzymes by altering their size and surface characteristics.

Keywords: Nanomaterials; Silica nanoparticles; Size; Surface modification; CYP3A4; Human liver microsomes

Background

The small size and high surface area of nanomaterials (which are defined as materials with at least one external dimension in the size range of 1 to 100 nm) give them useful properties such as unique chemical reactivity, heat conductivity, and ability to permeate tissues. Therefore, nanomaterials are expected to be used for applications in many fields [1,2]. In particular, amorphous silica nanoparticles are among the most widely used nanomaterials because of their comparatively low cost, their straightforward synthesis, and the ease with which their surfaces can be modified [3]. Silica nanoparticles are already widely used in cosmetics, food, and medicines [4-6]. Therefore, collecting information about the safety of silica nanoparticles is important [7,8]. In previous work, we found that they can penetrate the skin and enter various tissues [9] and that at high doses, they are more likely to induce consumptive coagulopathy and liver damage than do silica microparticles [10].

Because silica nanoparticles are used in food and drugs, their effects on metabolic enzymes such as cytochrome P450s (CYPs) are of particular interest. Xenobiotics such as drugs are metabolized by CYPs which are expressed at the highest levels in the liver. Cytochrome P450 3A4 (CYP3A4) is the most abundant CYP isozyme expressed in human liver tissue and is involved in the metabolism of approximately half of the drugs in use [11,12]. Drugs, some foods and beverages, and various chemicals such as those in cigarette affect the activity of CYPs. For example, ketoconazole, cyclosporine A, ritonavir, and grapefruit juice inhibit CYP3A4 activity and thus can lead to side effects when taken with drugs metabolized by CYP3A4 [13,14]. In contrast, rifampicin and St. John's wort induce CYP3A4 and thus reduce the efficacy of some drugs that undergo CYP3A4-dependent metabolism [13,15].

* Correspondence: yasuo@phs.osaka-u.ac.jp; ytsutsumi@phs.osaka-u.ac.jp
[1]Laboratory of Toxicology and Safety Science, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan
[4]The Center for Advanced Medical Engineering and Informatics, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan
Full list of author information is available at the end of the article

Nanomaterials have also been reported to affect CYP3A4 activity. For example, nonmetallic carboxyl polystyrene nanoparticles (20 nm) inhibit the activity of CYP3A4 in microsomes isolated from baculovirus-infected cells expressing wild-type CYP3A4 [16], and silver nanoparticles dose-dependently decrease the amount of 6β-hydroxytestosterone, which is generated mainly by CYP3A4, in human liver microsomes (HLMs) [17].

Considering that silica nanoparticles are already used in foods and medicines, their effects on CYPs must be thoroughly explored. Silica nanoparticles are reported to be distributed to the liver after dermal, oral, intranasal, and intravenous administration [9,18,19]. In addition, we previously demonstrated that 70-nm silica nanoparticles are localized in the cytoplasm, which contains many enzymes related to metabolism such as CYPs [9]; therefore, silica nanoparticles have the opportunity to react with CYPs. Furthermore, Nishimori et al. and Li et al. showed that when administered to mice together, 70-nm silica nanoparticles and some drugs increased the toxicity on the liver relative to that observed when either is administered alone in mice [20,21]. These results suggest that silica nanoparticles may affect the activity of CYPs, but these potential effects have not been evaluated. In addition, little information is available about the effects of the size and surface characteristics of nanomaterials on CYP3A4 activity. In this study, we examined CYP3A4 activity in human hepatocellular carcinoma cells (HepG2) and in HLMs exposed to silica particles with various sizes and surface modifications.

Methods

Silica particles

Silica nanoparticles with diameters of 30 and 70 nm (nSP30 and nSP70, respectively), conventional silica microparticles with diameters of 300 and 1,000 nm (mSP300 and mSP1000, respectively), and nSP30 and nSP70 modified with carboxyl groups (nSP30-C and nSP70-C, respectively) or amine groups (nSP30-N and nSP70-N, respectively) were purchased from Micromod Partikeltechnologie GmbH (Friedrich-Barnewitz, Rostock, Germany). The silica particles were suspended in water, and the suspensions were stored at room temperature. Immediately prior to use, they were sonicated for 5 min and then vortexed for 1 min.

Physicochemical examination of the silica preparations

Silica particles were diluted to 0.1 or 0.2 mg/mL with ultrapure water or Dulbecco's modified Eagle's medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal calf serum (FCS) and 1% antibiotic-antimycotic mix stock solution (Ab) (Gibco, Carlsbad, CA, USA), and the average particle size and surface charge (zeta potential) were determined using a Zetasizer Nano-ZS (Malvern Instruments Ltd., Malvern, UK). The size of silica particles were measured by dynamic light scattering. The surface charge was measured by laser Doppler electrophoresis.

Reagents

Pooled HLMs (Xtreme 200) were obtained from XenoTech (Lenexa, KS, USA). Ketoconazole, a representative CYP3A4 inhibitor, was obtained from Wako Pure Chemical Industries (Osaka, Japan). Luciferin-isopropyl acetal (LIPA; Promega, Madison, WI, USA), which is metabolized specifically by CYP3A4 and releases luciferin, was used as a probe substrate to quantify CYP3A4 activity by luminescence after the reaction of LIPA with an ATP-luciferase reaction mixture [22].

Evaluation of CYP3A4 activity in HLMs

The inhibitory effects of silica particles at various concentrations (2, 10, 50, 200, and 800 μg/mL) and ketoconazole (200 nmol/L) were determined with HLMs (20 μg/mL) in the presence of NADPH Regenerating System (Promega). The incubation mixtures, which consisted of silica particles, ketoconazole, 10 μmol/L LIPA, and HLMs in potassium phosphate buffer (15 μL, respectively), were pre-incubated for 10 min at 37°C, and then the enzymatic reactions were initiated by the addition of 15 μL of NADPH. In addition, to determine whether LIPA was physically bound to the silica particles, we also started the reaction by adding 15 μL of LIPA after preparing a mixture of the silica particles, HLMs, buffer, and NADPH. Next, to determine whether the silica particles were physically bound to microsome proteins, we centrifuged a mixture of silica particles, LIPA, HLMs, and buffer at 1,000 × *g* or 5,000 × *g* for 20 min and then added NADPH (15 μL) to the supernatant (45 μL). For each of these procedures, the reactions were terminated after 10 min of incubation by the addition of reconstitution buffer (60 μL). Each plate was incubated at room temperature for 20 min, and then the luminescence was read with a luminometer for 1 s per well.

Cell culture

HepG2 cells were maintained in Dulbecco's modified Eagle's medium including 10% FCS and 1% Ab.

Lactate dehydrogenase release assay in HepG2 cells

The lactate dehydrogenase (LDH) activity in HepG2 cells exposed to silica particles was determined with a commercial LDH cytotoxicity test (Wako Pure Chemical Industries) conducted according to the manufacturer's instructions. In brief, HepG2 cells (1×10^5 cells/well) were pre-cultured in 24-well plates for 24 h, the pre-culture medium was removed, and the cells were incubated with silica particles (25 to 200 μg/mL) for 48 h. Then, 50 μL of the supernatant was used for LDH analysis. Absorbance at

570 nm was measured with a spectrophotometer. The percentage of cellular survival was calculated by means of the following equation:

$$\text{Cellular survival (\%)} = 100 - [100(A-B)/(C-B)]$$

where A is the absorbance measured for a well treated with silica particles, B is the absorbance measured for an untreated well, and C is the absorbance measured for a well treated with 0.1% Triton X.

Evaluation of CYP3A4 activity in HepG2 cells

HepG2 cells were treated with silica particles by means of the protocol described for the LDH release assay. After a 48-h incubation period, the incubation medium was aspirated, the cells were washed twice with phosphate-buffered saline, and 200 μL of LIPA (3 μmol/L) was added to each well. After 1 h, a 100-μL aliquot of culture medium including LIPA was transferred from each well to a 96-well opaque white luminometer plate at room temperature, and then 100 μL of Luciferin Detection Reagent was added to initiate the luminescence reaction. The plate was incubated at room temperature for 20 min, and then the luminescence was read for 1 s with a luminometer.

Statistical analysis

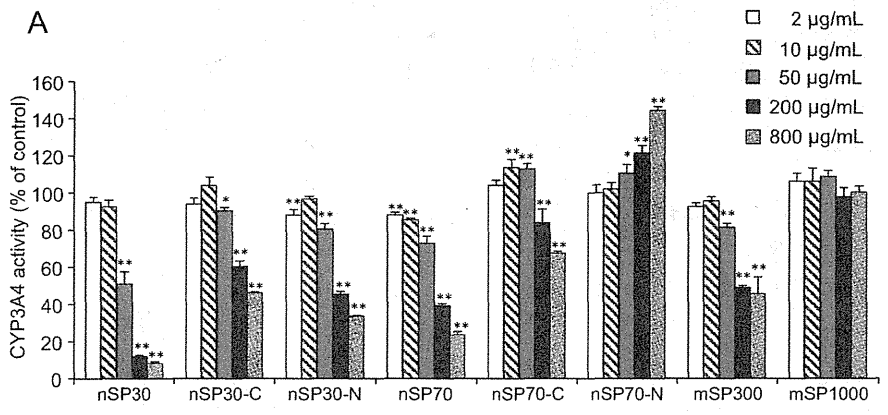
All data are presented as mean ± SD. Differences were compared by means of Dunnett's test. Differences between experimental groups and the control group were considered significant at $P < 0.05$.

Results and discussion

To evaluate the effect of silica particles on CYP3A4 activity, we measured CYP3A4 activity of HLMs and HepG2 cells after treating with silica particles. To elucidate the influence of size and surface modification of silica nanoparticles on their effect for CYP3A4 activity, we used silica particles with various sizes and surface modifications.

Physicochemical properties of silica particles

First, the particle size and surface charge of silica particles in water and medium were measured. The particle size and surface charge of several particles used in this study were reported in previous studies [9,23-26]. However, we measured these parameters again. Mean particle sizes of nSP30, nSP30-C, nSP30-N, nSP70, nSP70-C, nSP70-N, mSP300, and mSP1000 measured by dynamic light scattering method were 36.8 ± 0.3, 49.0 ± 1.7, 40.4 ± 0.9, 86.2 ± 2.7, 78.7 ± 0.3, 103 ± 0, 293.0 ± 2.7, and 1,253.3 ± 32.1 nm (in water), respectively, and 84.9 ± 1.9, 294.0 ± 45.0, 410.3 ± 48.2, 128.3 ± 2.3, 267.0 ± 28.6, 267.3 ± 2.1, 249.3 ± 24.0, and 1,083.3 ± 35.1 nm (in medium), respectively. The surface charge of nSP30, nSP30-C, nSP30-N, nSP70, nSP70-C, nSP70-N, mSP300, and mSP1000 measured by laser Doppler electrophoresis was −32.5 ± 1.4, −46.9 ± 1.8, −18.3 ± 1.9, −58.4 ± 0.2, −64.3 ± 1.9, −35.6 ±



B

Silica particle	nSP30	nSP30-C	nSP30-N	nSP70	nSP70-C	nSP70-N	mSP300	mSP1000
IC_{50} (μg/mL)	52	543	166	128	>800	>800	190	>800

Figure 1 Effect of silica particles on CYP3A4 activity in HLMs. (A) Silica particles were incubated with HLMs for 10 min. Then, CYP3A4 activity was measured, and the percentage of CYP3A4 activity was calculated relative to the activity of sterile water as a control. Data are presented as mean ± SD for three independent determinations. $^{**}P < 0.01$ and $^{*}P < 0.05$ versus the control group (Dunnett's test). **(B)** IC_{50} values for inhibition of CYP3A4 activity in HLMs by silica particles.

1.1, −56.4 ± 0.7, and −72.4 ± 0.6 mV (in water), respectively, and −9.0 ± 1.0, −11.2 ± 0.2, −11.1 ± 1.1, −10.0 ± 0.1, −10.2 ± 0.5, −10.1 ± 1.0, −9.3 ± 0.5, and −10.0 ± 1.6 mV (in medium), respectively.

Effects of silica particles on CYP3A4 activity in HLMs

Silica particles at concentrations of 2, 10, 50, 200, and 800 μg/mL were incubated with HLMs, and inhibition constants (IC_{50}) were determined (Figure 1). The activity of CYP3A4 decreased dose-dependently upon co-incubation with nSP30, nSP30-C, nSP30-N, nSP70, nSP70-C, or mSP300. In contrast, we observed no significant difference in the CYP3A4 activity of the mSP1000-treated group compared to that of the control group. For the unmodified silica particles, IC_{50} increased with increasing particle size. This result suggests that smaller silica particles have a greater potential to suppress CYP3A4 activity. In contrast, the IC_{50} values for nSP30 and nSP70 were lower than the values for nSP30-C, nSP30-N, and nSP70-C, indicating that surface modification changed the inhibitory potential of the particles. Surprisingly, we found that modification of nSP70 with amine groups resulted in increased CYP3A4 activity. Note, however, that differences in the size of nanomaterials reportedly affect their protein-binding mode [27]. Therefore, nSP30-N, which also has amino groups but did not activate CYP3A4, may interact with CYP3A4 differently than nSP70-N does.

Next, we investigated the mechanism of the effects of silica nanoparticles on CYP3A4 activity. First, we considered the possibility that the silica particles bound to the substrate and prevented interaction of the substrate and the enzyme (Figure 2A). We compared CYP3A4 activity under the following two conditions: (1) the enzymatic reaction was started by the addition of NADPH, which causes microsomal oxidation, after pre-incubation of the silica particles with a probe substrate, and (2) the enzymatic reaction was started by the addition of the probe substrate after pre-incubation of the silica particles with NADPH. Instead of silica particles, we also used ketoconazole which suppresses CYP3A4 activity without affecting the substrate. For all the silica particles and ketoconazole, the CYP3A4 activity was almost exactly the same under the two conditions, indicating that the silica particles did not affect the substrate-enzyme interaction.

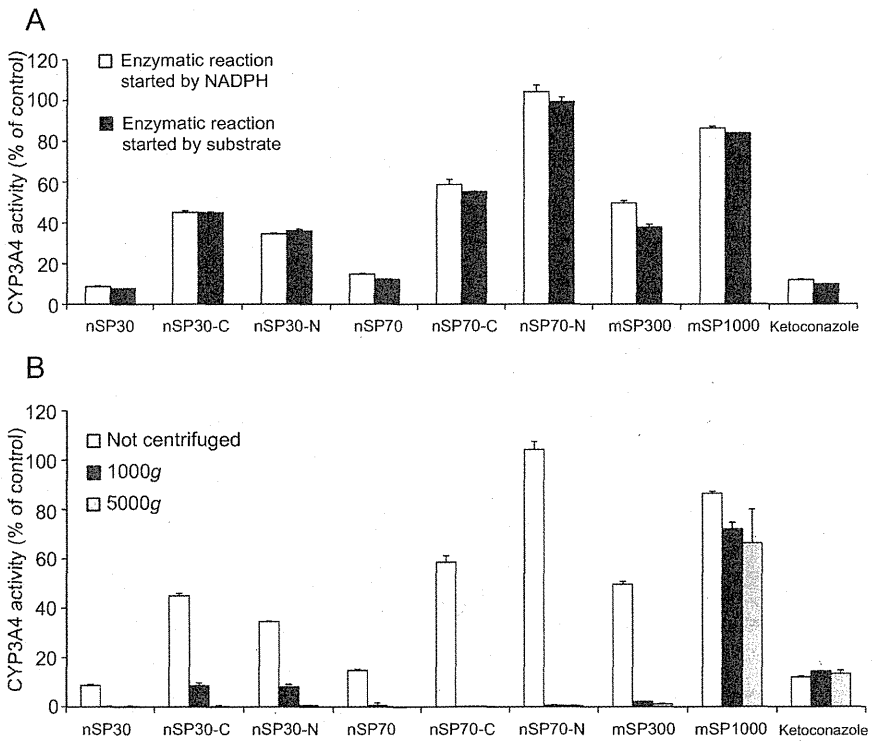


Figure 2 Evaluation of the interaction between silica particles and a probe substrate or CYP3A4. (A) Silica particles (200 μg/mL) or ketoconazole (200 nmol/L) was incubated with HLMs, and CYP3A4 activity was then measured in two ways: (1) the enzymatic reaction was started by the addition of NADPH after pre-incubation of the silica particles with the probe substrate, or (2) the enzymatic reaction was started by the addition of the probe substrate after pre-incubation of the silica particles with NADPH. **(B)** Mixtures of microsomes, the probe substrate, and silica particles were centrifuged at 1,000 × *g* or 5,000 × *g* for 20 min, or the mixtures were not centrifuged. The CYP3A4 activity of each supernatant was measured, and the percentage of CYP3A4 activity was calculated relative to the activity of sterile water as a control. Data are presented as mean ± SD for three independent determinations.

To determine whether the silica particles physically bound to microsome proteins, we centrifuged mixtures of microsomes, substrate, and silica particles and then measured the CYP3A4 activity in the supernatant (Figure 2B). We found that the CYP3A4 activity in groups treated with silica particles was dramatically lower in the centrifugation group compared to the uncentrifuged control, except in the case of the group treated with mSP1000 and ketoconazole. Microsome proteins and ketoconazole are not usually precipitated by centrifugation at either $1,000 \times g$ or $5,000 \times g$. Therefore, these results suggest that microsome proteins bound to the silica nanoparticles and to mSP300 to form complexes that were heavy enough to be precipitated by centrifugation at $1,000 \times g$ or $5,000 \times g$. Thus, we suggest that silica nanoparticles and mSP300 physically bound to microsome proteins and affected the CYP3A4 activity in HLMs. Bertoli et al. suggested the possibility that silica-coated magnetic 50-nm nanoparticles bind to CYPs in cells [28]. Therefore, mSP300 and the silica nanoparticles (except nSP70-N) may have bound to CYP3A4 and blocked its substrate-binding site or may have converted CYP3A4 to an inactive conformation, whereas nSP70-N bound to CYP3A4 and changed it to an active conformation.

Cytotoxicity of silica particles to HepG2 cells and CYP3A4 activity in HepG2 cells

To examine the influence of silica particles on CYP3A4 activity in hepatocytes, we evaluated cellular survival (Figure 3A) and CYP3A4 activity (Figure 3B) in HepG2

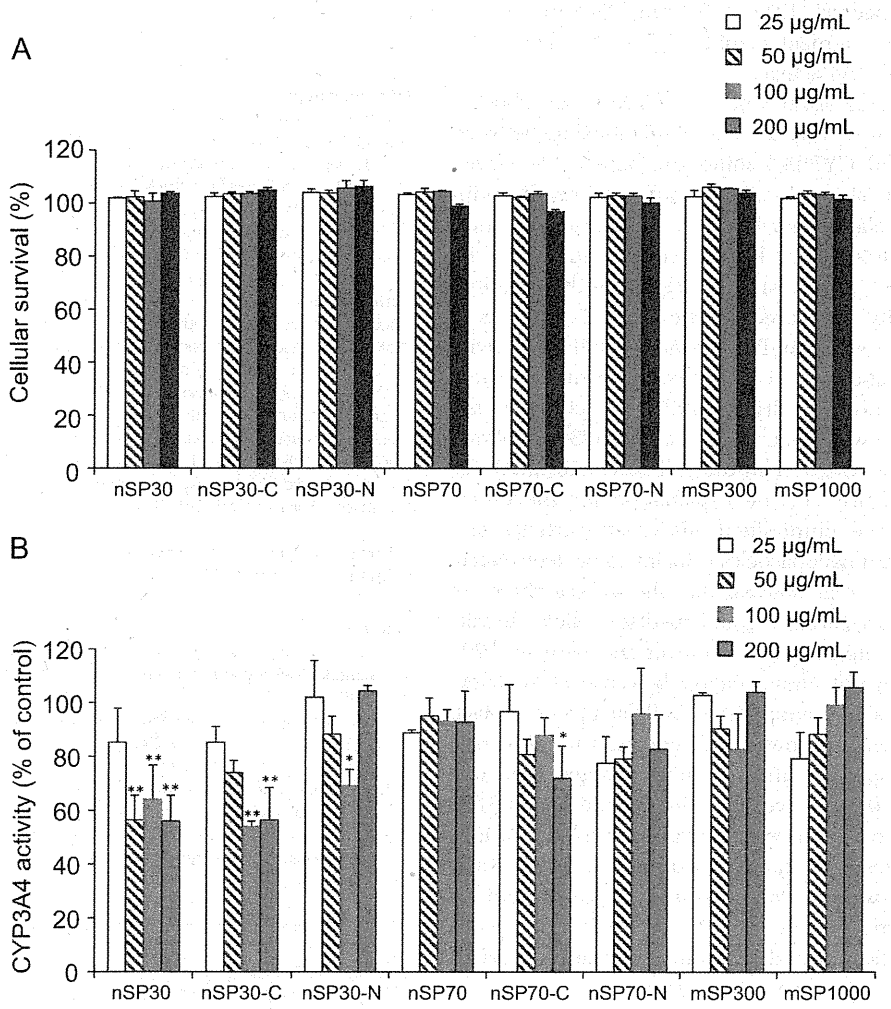


Figure 3 Effect of silica particles on membrane damage and CYP3A4 activity in HepG2 cells. HepG2 cells were incubated with silica particles for 48 h. **(A)** An LDH release assay was conducted with the supernatant, and the percentage of surviving cells was calculated. **(B)** CYP3A4 activity in HepG2 cells was measured, and CYP3A4 activity was calculated relative to that of the culture medium as a control. Data are presented as mean ± SD for three independent cultures. $^{**}P < 0.01$ and $^{*}P < 0.05$ versus control group (Dunnett's test).

cells after incubation with silica particles for 48 h. The survival rates in the groups treated with silica particles at concentrations of 25, 50, 100, and 200 μg/mL were almost 100%, indicating that none of the particles induced membrane damage in HepG2 cells under our experimental conditions (Figure 3A). In contrast, treatment with nSP30 at 50, 100, or 200 μg/mL, treatment with nSP30-C at 100 or 200 μg/mL, treatment with nSP30-N at 100 μg/mL, or treatment with nSP70-C at 200 μg/mL resulted in significantly lower CYP3A4 activity compared with the activity in the control group, whereas treatment with nSP70, nSP70-N, mSP300, or mSP1000 had no effect on CYP3A4 activity. These results suggest that smaller silica particles had greater potential to suppress CYP3A4 activity in cells. We confirmed that nSP30-N were aggregated at 200 μg/mL (mean particle size in medium, 410.3 ± 48.2 nm) compared to those at 100 μg/mL (mean particle size in medium, 121.0 ± 15.4 nm). That may be the reason why the treatment of nSP30-N at 200 ug/mL did not decrease CYP3A4 activity.

Both the HepG2 assay and the HLM assay showed similar results with regard to the relationship between particle size and CYP3A4 inhibition activity. However, the two assays showed slightly different results with regard to the relationship between surface modification and CYP3A4 activity. In HepG2 cells (Figure 3B), the nSP70-C-treated group showed significantly inhibited CYP3A4 activity compared to the control group at a concentration at which nSP70 did not inhibit the activity (200 μg/mL), and nSP30-C showed almost the same CYP3A4 inhibition activity as nSP30. In contrast, the HLM assay showed that nSP70 and nSP30 inhibited CYP3A4 more strongly than did nSP70-C and nSP30-C, respectively (Figure 1). The explanation for the difference between the unmodified silica nanoparticles and carboxyl-modified nanoparticles remains to be determined. However, Chung et al. showed that the surface charge of mesoporous silica particles could mediate cellular uptake rate or even cellular uptake route of the particles [29]. Therefore, change of surface charge by carboxyl modification may affect silica nanoparticles' cellular uptake. In fact, Ekkapongpisit et al. showed that carboxyl modification of 50-nm mesoporous silica particles changes their cellular uptake [30], and we have also found that nSP70 and nSP70-C have different intracellular localizations [25]. Thus, differences in cellular uptake or intracellular localization between unmodified and carboxyl-modified silica nanoparticles may explain the different results observed in cells and under conditions where the particles have direct access to microsomes.

Conclusions

We report for the first time that silica particles can either inhibit or activate CYP3A4 activity *in vitro*. We showed that smaller particles have a greater potential to inhibit CYP3A4 activity than larger particles and that surface modification of silica particles could change their effects on CYP3A4 activity. Our results suggest that optimization of the size and surface modification of silica particles will contribute to the development of safer applications of silica nanoparticles.

Abbreviations

CYP3A4: cytochrome P450 3A4; HLMs: human liver microsomes; LDH: lactate dehydrogenase; LIPA: luciferin-isopropyl acetal.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SI, YY, and TY designed the study. SI, TY, and MU performed the experiments. SI, YY, YuM, and TY collected and analyzed the data. SI, YY, and YuM wrote the manuscript. KN, YoM, HK, ST, and KH, provided technical support and conceptual advice. YT supervised the project. All authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

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Author details

[1]Laboratory of Toxicology and Safety Science, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan. [2]Laboratory of Biopharmaceutical Research, National Institute of Biomedical Innovation, 7-6-8 Saitoasagi, Ibaraki, Osaka 567-0085, Japan. [3]Laboratory of Innovative Antibody Engineering and Design, Center for Drug Innovation and Screening, National Institute of Biomedical Innovation, 7-6-8 Saitoasagi, Ibaraki, Osaka 567-0085, Japan. [4]The Center for Advanced Medical Engineering and Informatics, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan.

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Evaluation of silica nanoparticle binding to major human blood proteins

Katsutomo Hata[1†], Kazuma Higashisaka[1,2†], Kazuya Nagano[2], Yohei Mukai[3], Haruhiko Kamada[2,4], Shin-ichi Tsunoda[2,4], Yasuo Yoshioka[1,2*] and Yasuo Tsutsumi[1,4*]

Abstract

Nanomaterials are used for various biomedical applications because they are often more effective than conventional materials. Recently, however, it has become clear that the protein corona that forms on the surface of nanomaterials when they make contact with biological fluids, such as blood, influences the pharmacokinetics and biological responses induced by the nanomaterials. Therefore, when evaluating nanomaterial safety and efficacy, it is important to analyze the interaction between nanomaterials and proteins in biological fluids and to evaluate the effects of the protein corona. Here, we evaluated the interaction of silica nanoparticles, a commonly used nanomaterial, with the human blood proteins albumin, transferrin, fibrinogen, and IgG. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis showed that the amount of albumin, transferrin, and IgG binding to the silica particles increased as the particle size decreased under conditions where the silica particle mass remained the same. However, under conditions in which the specific surface area remained constant, there were no differences in the binding of human plasma proteins to the silica particles tested, suggesting that the binding of silica particles with human plasma proteins is dependent on the specific surface area of the silica particles. Furthermore, the amount of albumin, transferrin, and IgG binding to silica nanoparticles with a diameter of 70 nm (nSP70) and a functional amino group was lower than that with unmodified nSP70, although there was no difference in the binding between nSP70 with the surface modification of a carboxyl functional group and nSP70. These results suggest that the characteristics of nanomaterials are important for binding with human blood proteins; this information may contribute to the development of safe and effective nanomaterials.

Keywords: Nanomaterials; Protein corona; Biological interaction

Background

The development and use of materials at the nanometer scale already have multiple applications in various fields, including the food and cosmetics industries; such nanomaterials have thus become indispensable in our lives [1,2]. Silica nanoparticles have been widely used in many consumer products because of their useful properties, including relatively low production costs, easy separation, and ease of modification of their surface properties. Accordingly, they have attracted the attention of the pharmaceutical industry as materials for new drug delivery and diagnostic systems [3]. However, because nanomaterials possess novel properties that are different from conventional materials, concerns about their potential unanticipated effects have been raised. We previously found that silica nanoparticles with a diameter of ≤100 nm could cause pregnancy complications [4] or consumptive coagulopathy in mice after systemic exposure [5] compared with silica particles with a diameter of >100 nm. We also demonstrated that the surface-modified silica nanoparticles were unlikely to induce undesired inflammatory responses *in vitro* and *in vivo*, suggesting that it might be possible to decrease the adverse biological effects of nanomaterials and enhance nanomaterial safety by modifying their surface properties [6,7].

Nanomaterial-mediated biological effects are related to physical characteristics such as particle size and the

* Correspondence: yasuo@phs.osaka-u.ac.jp; ytsutsumi@phs.osaka-u.ac.jp
†Equal contributors
[1]Laboratory of Toxicology and Safety Science, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan
[4]The Center for Advanced Medical Engineering and Informatics, Osaka University, 1-6, Yamadaoka, Suita, Osaka 565-0871, Japan
Full list of author information is available at the end of the article

surface properties of the nanomaterials. When nanomaterials interact with proteins, a protein layer forms around the nanomaterials, referred to as the protein corona, which is dependent on the physical characteristics of the nanomaterial [8,9]. Many studies have shown that this protein corona plays a role in the biological effects induced by the nanomaterial and in the *in vivo* and *in vitro* kinetics of the nanomaterial. For example, Jiang et al. demonstrated that the uptake of FePt nanoparticles by HeLa cells is suppressed by the adsorption of human transferrin compared with that of the bare nanoparticles [10]. Similarly, the work of Ge et al. suggests that the binding of blood proteins to carbon nanotubes reduces those nanotubes' cytotoxicity [11]. Therefore, it is important to understand how nanomaterials interact with proteins to clarify both the biodistribution and safety of such nanomaterials. Although proteomics studies have comprehensively identified the blood proteins that make direct contact with nanomaterials, detailed analyses of the binding to individual blood proteins are incomplete lacking. To fully realize the potential of the protein corona, it is essential to understand and quantify the effects of individual proteins on the characteristics of nanomaterials. Furthermore, analyses of the amount of binding of each individual human plasma protein to nanomaterials with various properties would be invaluable in developing the safety profile of such nanomaterials.

In this study, we assess the differences in the interactions between major blood proteins and silica nanoparticles by changing nanomaterial characteristics such as size, specific surface area, and surface modification.

Methods

Materials

Human albumin (molecular weight 66 kDa), human fibrinogen (molecular weight 340 kDa), and human transferrin (molecular weight 78 kDa) were purchased from Wako (Osaka, Japan). Human immunoglobulin G (IgG) (molecular weight 146 kDa) was purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan) and human α1-acid glycoprotein (AGP) (molecular weight 44.1 kDa) was purchased from Sigma-Aldrich (Saint Louis, MO, USA).

Silica particles

Silica particles were purchased from Micromod Partikeltechnologie (Rostock/Warnemünde, Germany). Silica particles with diameters of 70, 100, 300, and 1,000 nm (nSP70, nSP100, nSP300, and mSP1000, respectively) and nSP70 with the surface modification of an added amino group (nSP70-N) or an added carboxyl group (nSP70-C) were used in this study. These particles were sonicated for 5 min and vortexed for 1 min prior to use.

Recovery of silica particles

Suspensions of silica particles (200 μL) were centrifuged at 21,500 × *g* for 20 min at 4°C. The pellets were washed three times with 500 μL of phosphate buffered saline (PBS) and then dissolved in 200 μL of 5% HNO_3 (Nacalai Tesque, Kyoto, Japan). The solutions (100 μL) were then transferred to centrifugation tubes and 5 mL of Milli-Q water (Millipore, Billerica, MA, USA) was added to each tube. The silicon content of the samples was analyzed by using inductively coupled plasma mass spectrometry (ICP-MS) (Agilent Technologies, Santa Clara, CA, USA). The recovery of the silica particles was calculated as the ratio of the silicon signal (cps) of the centrifuged samples to that of the uncentrifuged samples.

Gel electrophoresis

Each protein solution (200 μL), adjusted with PBS to obtain concentrations commonly found in human blood (albumin, 40 mg/mL; fibrinogen, 2 mg/mL; transferrin, 2 mg/mL; IgG, 10 mg/mL; and AGP, 0.5 mg/mL), was mixed with nSP70, nSP100, nSP300, mSP1000, nSP70-C, or nSP70-N (25 mg/mL) at 1:1 (*v*/*v*). To assess the effect of the specific surface area, each protein solution was mixed with nSP70 (1.08×10^6 mm^2/mL), nSP100 (7.54×10^5 particles/mL), nSP300 (2.54×10^5 mm^2/mL), or mSP1000 (7.54×10^4 particles/mL) at 1.75, 2.5, 7.41, and 25 mg/mL, respectively, which is about 1.5×10^4 mm^2 of specific surface area for each particle. The mixtures were then vortexed and incubated at 37°C for 1 h with constant rotating. The proteins bound to silica particles were separated by centrifugation at 21,500 × *g* for 20 min at 4°C. The pellets were washed three times with 500 μL of PBS to remove the unbound proteins. Proteins bound to the silica particles were suspended in 10% sodium dodecyl sulfate (SDS) buffer (sample solution). Then, the sample solution and Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) were mixed in equal amounts (mixture solution) and boiled for 5 min at 95°C. The IgG samples were diluted tenfold before being mixed with the sample buffer because their concentrations were too high to analyze the bands clearly. Each sample was separated by SDS polyacrylamide gel electrophoresis (PAGE) (ATTO, Tokyo, Japan). For the analysis of albumin, transferrin, and IgG, 10% to 20% e-PAGEL was used; for fibrinogen, 5% e-PAGEL was used. Electrophoresis was performed at 15 mA/gel for 10 min (stacking), followed by separation (600 V, 40 mA/gel) for approximately 45 min. After the electrophoresis, the gel was stained by Coomassie brilliant blue (CBB) staining, and the protein bands were quantified with the ImageJ software (http://rsb.info.nih.gov/ij/). The protein content of each band was estimated from its optical density compared with that of each standard solution. As standard solutions, the following protein concentrations were prepared: albumin at 0.08, 0.16, 0.4,

and 0.8 mg/mL; transferrin at 0.2, 0.4, 1.0, and 2.0 mg/mL; AGP at 0.05, 0.1, 0.25, and 0.5 mg/mL; IgG at 0.1, 0.2, 0.5, and 1.0 mg/mL; and fibrinogen at 0.2, 0.4, 1.0, and 2.0 mg/mL. The degree of binding was calculated by taking the theoretical concentrations calculated from the proteins added to the silica particles originally as 100%. Values less than the detection limit of the standard curve for each protein were recorded as 0%, even if bands were slightly detectable.

Statistical analyses

All results are expressed as means ± SD. Differences were compared by means of Tukey's multiple comparison test.

Results

Recovery of silica particles after centrifugation

In this study, we used silica particles with diameters of 70 nm (nSP70), 100 nm (nSP100), 300 nm (nSP300), and 1,000 nm (mSP1000), and nSP70 surface-modified with a carboxy group (nSP70-C) or an amino group (nSP70-N). The physical characteristics of these particles were assessed in previous studies [4,12]. Transmission electron microscopy analysis showed that they were smooth-surfaced spheres. Dynamic light scattering analysis revealed that the hydrodynamic diameters were 76, 106, 264, and 1136 nm for nSP70, nSP100, nSP300, and mSP1000 in PBS (pH7.4), respectively, and that the zeta potential of each particle was −19.5, −24.3, −25.8, and −33.2 mV, respectively. In addition, we previously reported that the mean secondary particle size of nSP70-C and nSP70-N was 69.6 and 71.8 nm, respectively, and that the zeta potentials were −76.3 and −29.0 mV, respectively. The size distribution spectrum of each set of silica particles showed a single peak, and the measured hydrodynamic diameter corresponded almost precisely to the primary particle size of each set of silica particles. These results indicate that the silica particles used in this study were well-dispersed in solution.

First, we wanted to evaluate the binding of silica particles with the major blood proteins after they were mixed and then centrifuged. Therefore, it was essential to assess how much of each silica particle was precipitated after centrifugation. We analyzed the recovery of each silica particle after centrifugation by using ICP-MS. ICP-MS analysis showed that the percentage recovery of nSP70, nSP70-N, nSP70-C, nSP100, nSP300, and mSP1000 was 71.0, 78.5, 71.5, 80.9, 82.6, and 77.2, respectively. These results show that the amounts of each silica particle recovered were similar, indicating that it would be appropriate to directly compare the degree of binding of each silica particle to the proteins.

Effects of particle size on binding to proteins

Next, we evaluated whether the differences in the size of the silica particles affected their binding to albumin, transferrin, fibrinogen, and IgG. We selected these proteins because these have high concentrations in human blood [13] and because the frequency of contact in the body is considered to be high. In addition, it was reported that these proteins were identified in protein corona on silica nanoparticles with human plasma [14,15]. SDS-PAGE analysis showed that there was no interaction between the silica particles and AGP (data not shown). For albumin (Figure 1A) and IgG (Figure 1C), the binding was dependent on the diameter of the silica particles. For transferrin, we found that nSP300 and mSP1000 did not bind (Figure 1B). In addition, the degree of binding of mSP1000 with fibrinogen was significantly lower than that of the other particles (Figure 1D). These results indicate that the binding of silica particles to albumin and IgG appears to increase as the particle size decreases. The degree of nSP70 binding was greatest with fibrinogen and smallest with albumin among these four proteins tested.

We also evaluated the effect of the specific surface area of the silica particles on binding to human plasma proteins. The specific surface areas of each particle were matched with that of 25 mg/mL of mSP1000 (about 1.5×10^4 mm^2), because mSP1000 was the largest particle in this study. The results showed that there was no significant difference in binding with each protein among each size of silica particle tested (Figure 2A,B,C,D), suggesting that the binding of silica particles to human plasma proteins is dependent on the specific surface area of the silica particles.

Effects of particle surface charge on binding to proteins

The physical properties of nanomaterials, including surface properties and morphology, are important factors in the biological responses to these materials. We previously demonstrated that nSP70 surface-modified with a carboxyl or amino group might induce abnormal activation of the coagulation cascade to a lesser extent than does unmodified nSP70 [12] and that differences in the properties of surface-modified silica nanoparticles directly affect the extent to which these particles interact with blood proteins, such as coagulation factor XII (unpublished data). Here, we assessed how surface modification affected the protein binding with silica nanoparticles. The surface modification of adding an amino group weakened the binding of nSP70 to albumin (Figure 3A), transferrin (Figure 3B), and IgG (Figure 3C), although the surface modification of adding the carboxyl-group made no noticeable difference to the binding of nSP70 to albumin and IgG. Similarly, there was no difference in fibrinogen binding among these three silica nanoparticles, suggesting that the physical properties associated with nanoparticle interaction with proteins are dependent on the kind of protein involved.

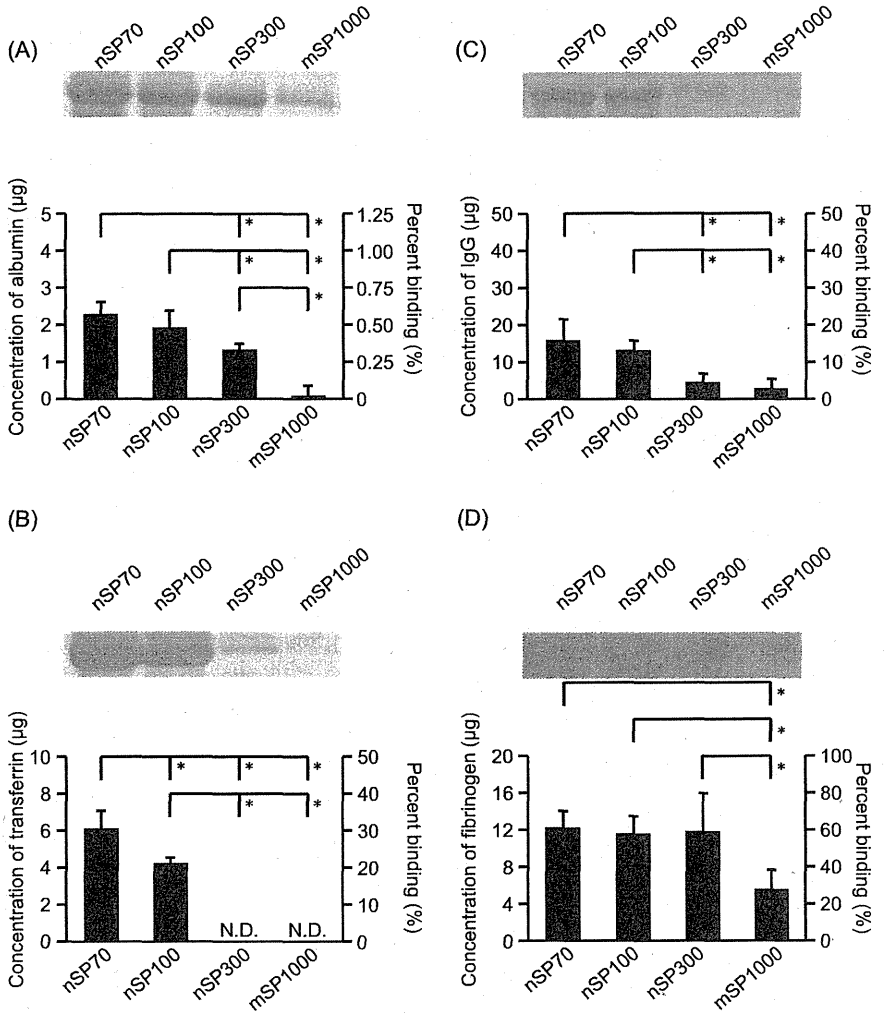

Figure 1 Effects of silica particle size on binding to human plasma proteins. Each protein solution was mixed with SP70, nSP100, nSP300, or mSP1000 (25 mg/mL). After centrifugation, each sample was separated by SDS-PAGE. The gel was stained by CBB staining, and the protein bands of albumin **(A)**, transferrin **(B)**, IgG **(C)**, and fibrinogen **(D)** were quantified with the ImageJ software. The protein content of each band was estimated from its optical density compared with the optical density of each standard solution. Data are presented as the mean ± SD; $n = 4$; $^{**}P < 0.01$ vs. nSP70-treated group; $^{*}P < 0.05$ vs. nSP70-treated group; N.D., not detected.

Discussion

Here, we assessed the effect of nanoparticle size on binding to proteins. We found that the binding of silica particles to albumin, transferrin, and IgG appears to increase as the particle size decreased (Figure 1A,B,C). However, we found no significant difference in binding to these proteins when the specific surface area of the different silica particles was the same (Figure 2A,B,C). The larger specific surface area of nSP70 relative to that of nSP300 and mSP1000 may have allowed the smaller particles to interact with biomolecules such as proteins. These results indicate that specific surface area plays a role in determining silica nanoparticle-protein interactions. How specific surface area influences nanoparticle binding to plasma proteins is poorly understood [16]. Several studies have shown that biological responses to nanomaterials could depend on specific surface area rather than on the mass of the particle [17]. Therefore, biological responses should be assessed in terms of the nanomaterial specific surface area-protein interaction.

The surface charge of the nanomaterials affects the composition of the protein corona. We demonstrated that the surface modification of adding an amino group to nSP70 weakens the binding of nSP70 to albumin, transferrin, and IgG, although there were no significant differences between the binding of nSP70 and that of nSP70

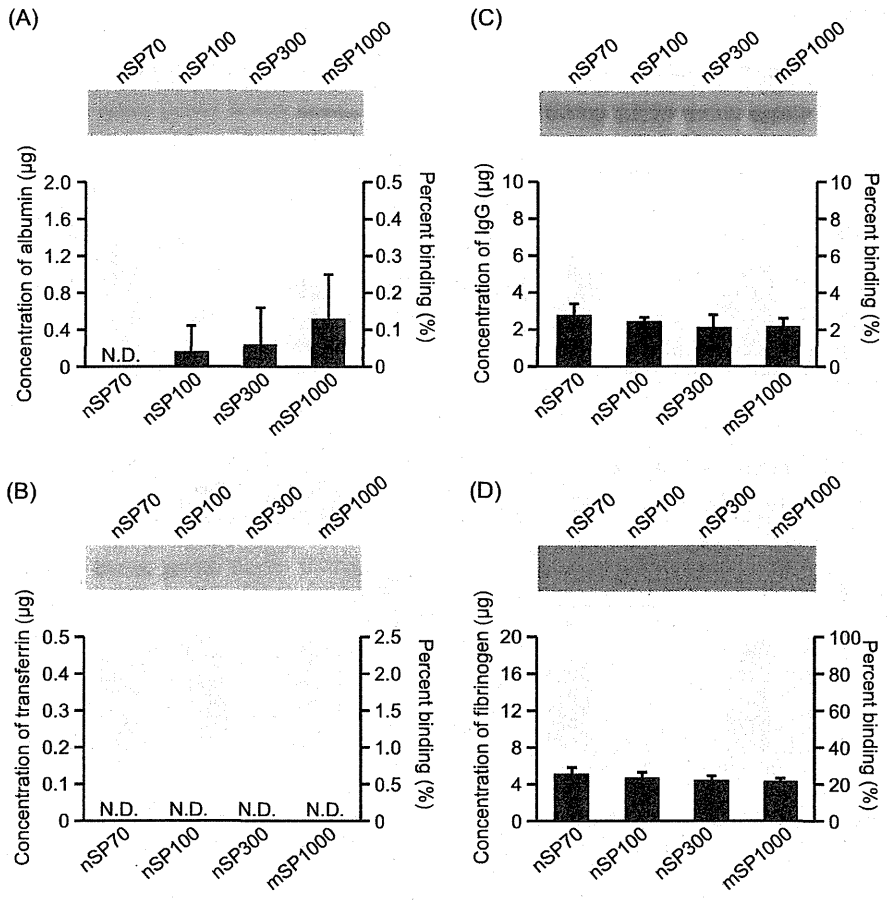

Figure 2 Effects of the specific surface area of silica particles on binding to human plasma proteins. Each protein solution was mixed with SP70, nSP100, nSP300, or mSP1000 at 1.75, 2.5, 7.41, and 25 mg/mL, respectively (i.e., approximately 1.5×10^4 mm^2 of specific surface area). After centrifugation, each sample was separated by SDS-PAGE. The gel was stained by CBB staining, and the protein bands of albumin **(A)**, transferrin **(B)**, IgG **(C)**, and fibrinogen **(D)** were quantified with the ImageJ software. The protein content of each band was estimated from its optical density compared with the optical density of each standard solution. Data are presented as the mean ± SD; $n = 3$; $^{**}P < 0.01$ vs. nSP70-treated group; $^{*}P < 0.05$ vs. nSP70-treated group; N.D., not detected.

with the surface modification of an added carboxyl group (Figure 3A,B,C). One study showed that polystyrene nanoparticles with a positively charged functional group were more likely to adsorb to proteins with an isoelectric point (pI) ≤5.5, whereas polystyrene nanoparticles with a negatively charged functional group were more likely to adsorb to proteins with a pI >5.5, and the distinct proteins appeared to show a preference for different functional groups [18]. Our results showed that the binding of nSP70-N with albumin (pI 5.92), transferrin (pI 6.81), and IgG (pI 6.4 to 6.9) was reduced relative to that of nSP70. It is conceivable that nSP70-N displayed a positive charge as $-NH_3^+$ in solution and that it did not show any preference for proteins with a pI >5.5. However, there was no significant difference in binding to fibrinogen (pI 5.7) between nSP70 and nSP70-N (Figure 3D). Fibrinogen binds to a wide variety of nanomaterials because it has several different binding domains that accommodate different nanomaterials [19]. Therefore, to understand the differences in the interactions between blood proteins and nanomaterials, it is essential to assess not only the characteristics of the nanomaterials but also the physical properties of the absorbed proteins such as their molecular weight, pI, and structure.

According to the technical data sheets posted in Micromod Partikeltechnologie, the nSP70 in this study became covered with silanol groups and was modified by the addition of an amino or a carboxyl group through its spacer structure. It is difficult to simply compare the difference between nSP70 and modified-nSP70 in terms of their interactions with proteins, but we believe that a difference in the binding domain affinities of the respective particles resulted in the difference in binding between nSP70 and nSP70-N.

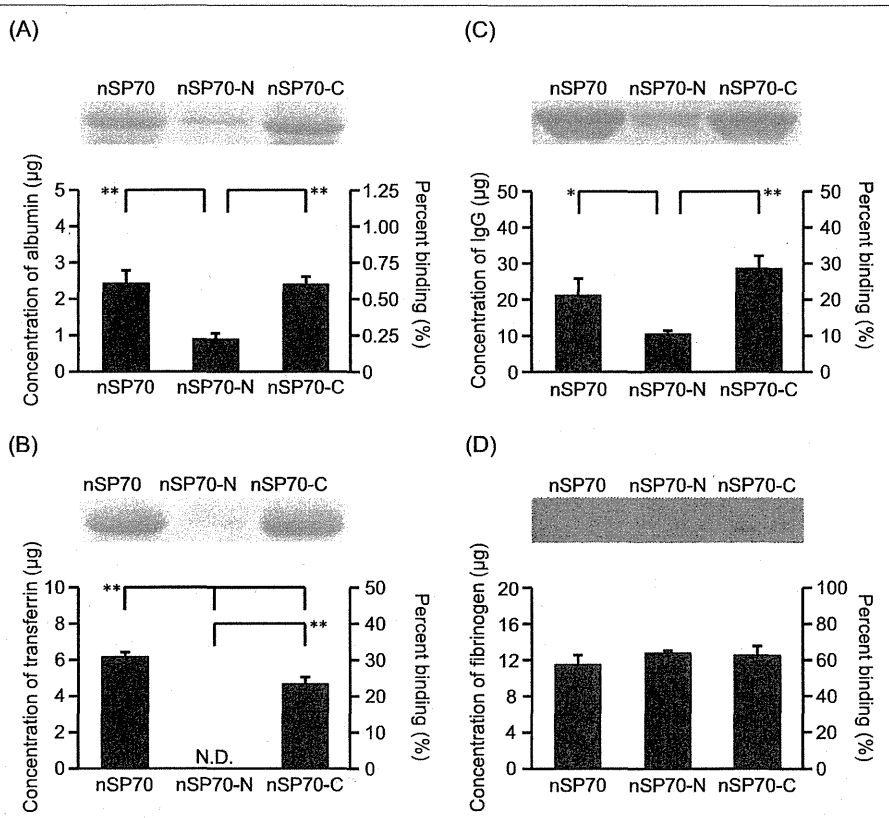

Figure 3 Effects of surface modification of silica particles on binding to human plasma proteins. Each protein solution was mixed with SP70, nSP70-N, or nSP70-C (25 mg/mL). After centrifugation, each sample was separated by SDS-PAGE. The gel was stained by CBB staining, and the protein bands of albumin **(A)**, transferrin **(B)**, IgG **(C)**, and fibrinogen **(D)** were quantified with the ImageJ software. The protein content of each band was estimated from its optical density compared with the optical density of each standard solution. Data are presented as the mean ± SD; $n = 4$; $^{**}P < 0.01$ vs. nSP70-treated group; $^{*}P < 0.05$ vs. nSP70-treated group; N.D., not detected.

The binding of protein with nanomaterials might play important roles in the ultimate fate of these nanomaterials in the body and in the subsequent nanomaterial-induced biological effects [20]. In this study, we showed that the degree of nSP70 binding to fibrinogen was greater than that to the other proteins tested (Figure 1A, B,C,D). The adsorption of opsonins such as fibrinogen and IgG creates a molecular signature that is recognized by immune cells and determines the route of particle internalization. This promotes phagocytosis and removal of the particles from the systemic circulation by cells of the mononuclear phagocytic systems [16,21]. Moreover, the nanoparticle-induced unfolding of fibrinogen has been shown to promote inflammation via Mac-1 receptor activation [17]. These data and our current results showing that the binding of nSP70 to fibrinogen was greater than that to other proteins are consistent with our previous finding that silica nanoparticles induce strong inflammatory responses [7]. We believe that this study on the interactions of nanomaterials with individual proteins provides essential information for evaluating the biological responses to nanomaterials and that it could lead to the development of safer and more efficacious nanomaterials.

Conclusions

Here, we showed that the binding of silica nanoparticles with albumin, transferrin, IgG, and fibrinogen is dependent on the specific surface area, not the size of the silica nanoparticles. We also showed that the binding rates of nSP70-N to albumin, transferrin, and IgG were weaker than that of nSP70 and nSP70-C; however, there was no difference in fibrinogen binding among nSP70, nSP70-N, and nSP70-C. We expect that it was connected for the development and use of safer and more effective nanomaterials by understanding the interaction between the nanomaterials and the proteins.

Abbreviations

AGP: α1-acid glycoprotein; CBB: Coomassie brilliant blue; ICP-MS: inductively coupled plasma mass spectrometry; IgG: immunoglobulin; nSP70: 70-nm-diameter silica nanoparticles; nSP70-C: nSP70 surface-modified with carboxyl groups; nSP70-N: nSP70 surface-modified with amino groups; nSP300: 300-nm-diameter

silica particles; mSP1000: 1000-nm-diameter silica particles; PBS: phosphate-buffered saline; pI: isoelectric point; SDS-PAGE: sodium dodecyl sulfate- polyacrylamide gel electrophoresis.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
KH and YY designed the study. KH and KH performed experiments and collected and analyzed data. KH, KH, and YY wrote the manuscript. KN, YM, HK, and ST gave technical support and conceptual advice. YT supervised all of the projects. All authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

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Author details
[1]Laboratory of Toxicology and Safety Science, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan. [2]Laboratory of Biopharmaceutical Research, National Institute of Biomedical Innovation, 7-6-8, Saito-Asagi, Ibaraki, Osaka 567-0085, Japan. [3]Laboratory of Innovative Antibody Engineering and Design, Center for Drug Innovation and Screening, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan. [4]The Center for Advanced Medical Engineering and Informatics, Osaka University, 1-6, Yamadaoka, Suita, Osaka 565-0871, Japan.

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Letter

Silica nanoparticle-induced toxicity in mouse lung and liver imaged by electron microscopy

Katsuhiro Isoda[1,2], Masuo Kondoh[2], Yasuo Yoshioka[3], Yasuo Tsutsumi[3], Takayoshi Imazawa[4], Tetsuji Nishimura[1], Isao Ishida[1] and Kiyohito Yagi[2]

[1]Graduate School of Pharmaceutical Sciences, Teikyo Heisei University, 4-21-2 Nakano, Nakano-ku, Tokyo 164-8530, Japan
[2]Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka 565-0871 Japan
[3]Laboratory of Toxicology and Safety Science, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka 565-0871, Japan
[4]Bioresources Research, Laboratory of Common Apparatus, National Institute of Biomedical Innovation, 7-6-8 Asagi Saito, Ibaraki, Osaka 567-0085, Japan

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ABSTRACT — Nanomaterials have been proposed as novel substrates for medical and commercial applications. However, such materials also may have novel toxicities, thus posing environmental and health concerns. We previously reported hepatic injury in mice following the intravenous administration of unmodified silica particles with diameters of 70 nm (SP70); this toxicity was not observed following administration by the same route of micro-size particles with diameters of 300 nm (SP300) or 1,000 nm (SP1000). In the present study, we used electron microscopy to investigate the dynamics of silica nanoparticles administered in mice. SP70 was observed in hepatocytes and in lung epithelial cells. Inclusion within hepatocytes was associated with accumulation of SP70 in the liver sinusoidal endothelial cells and passage through the space of Disse. In contrast, SP300 and SP1000 were not observed within the hepatocytes. To our knowledge, our report represents the first demonstration that silica nanoparticles accumulate in hepatocytes, liver sinusoidal endothelial cells, Kupffer cells, and lung tissue; accumulation of SP70 in liver sinusoidal endothelial cells correlated with the induction of liver injury.

Key words: Silica nanoparticles, Electron microscope, Hepatocytes, Liver sinusoidal endothelial cells, Kupffer cells, Lungs

INTRODUCTION

Recently, nanomaterials have been proposed for use in a widening range of industrial, pharmaceutical, and technical applications. Nanomaterials are frequently used in microelectronics, cosmetics, and sunscreens, and their potential use in drug-delivery systems is being investigated (Dobson, 2006; Caputo *et al.*, 2008; Nohynek *et al.*, 2008). Nanomaterials have unique physicochemical qualities compared to micromaterials with regard to size, surface structure, solubility, and aggregation. Thus, the reduction in particle size from the micro- to nanoscale is expected to facilitate many industrial and scientific applications. However, nanomaterials have potential toxicities that are not found in micromaterials, and it is, therefore, essential to understand the biological activity and potential toxicity of nanomaterials (Warheit *et al.*, 2008; Bystrzejewska-Piotrowska *et al.*, 2009). Silica nanoparticles are intended for use in cosmetics and for systemic and local delivery of drugs (Vallet-Regi *et al.*, 2007). Previously, we found that intravenous administration of 70-nm, but not 300- and 1000-nm, silica particles caused liver injury (Nishimori *et al.*, 2009c; Nishimori *et al.*, 2009a; Nishimori *et al.*, 2009b). However, the mechanism of hepatotoxicity of the silica nanoparticles remains poorly understood.

In the present study, we investigated the dynamics of the silica nanoparticles using electron microscopy (EM). As we report here, silica nanoparticles invaded hepatocytes following parenteral administration in whole animals.

Correspondence: Katsuhiro Isoda (E-mail: k.isoda@thu.ac.jp)

MATERIALS AND METHODS

Materials

Silica particles with diameters of 70, 300, or 1,000 nm (SP70, SP300, or SP1000, respectively) were obtained from Micromod Partikeltechnologie GmnH (Rostock, Germany). The size distribution of the particles was analyzed using a Zetasizer (Sysmex Co., Kobe, Japan), and the mean ± S.D. diameters were 57.5 ± 20.3, 326 ± 32.1, and 966 ± 36.3 nm, respectively. The silica particles were amorphous substance with chemically unmodified surfaces. The particles were spherical and nonporous and were stored as aqueous suspensions at 25 or 50 mg/mL. The suspensions were thoroughly dispersed by sonication before use and then diluted in ultrapure water. All reagents used were of research grade.

Animals

Eight-week-old BALB/c male mice were purchased from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan) and were maintained in a controlled environment (23 ± 1.5°C; 12-hr/12-hr light/dark cycle) with *ad libitum* access to standard rodent chow and water. The mice were permitted to adapt to the new environment for 1 week before commencing with the experiment. Mice were intravenously (i.v.) injected with silica nanoparticles (10, 30, or 100 mg/kg body weight) or vehicle. Mice were then anesthetized, bled for serum, and sacrificed at 24 hr after i.v. injection. The experimental protocols conformed to the ethical guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University.

Biochemical analysis

Serum alanine aminotransferase (ALT) was measured with a commercially available kit according to the manufacturer's protocols (Wako Pure Chemical Industries, Osaka, Japan).

Ultrastructure of liver tissue

The mice were sacrificed on 24 hr after injection of nanoparticles. The liver and lung tissues were removed, immersed in 2.5% glutaraldehyde fixative, dissected into small blocks, and then returned to the same fixative at 4°C for 2 hr. Liver and lung fragments then were washed several times in 0.1 M phosphate buffer (pH 7.4), and post-fixed in phosphate-buffered 1% osmium tetroxide for 60 min at 4°C. After dehydration by passage through a series of ethanol concentrations and QY-1, liver and lung fragments were embedded in EPON resin (TAAB). Semi-thin (1-μm) sections were stained with toluidine blue and examined by light microscopy. Ultra-thin (10-nm) sections were stained with uranyl acetate and lead citrate, and examined under a Hitachi EM (H-7650, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed by two-way ANOVA, followed by *post-hoc* Student's t-test where overall significance was indicated.*$P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

We initially observed the silica nanoparticles by EM. The shapes of SP70 (Fig. 1A), SP300 (Fig. 1B), and SP1000 (Fig. 1C) were globular, and none of the silica nanoparticles exhibited flocculation. Intravenous injection of SP70 at 100 mg/kg resulted in liver damage in mice, as demonstrated by a significant rise in serum ALT levels at 24 hr post-injection (Fig. 1D). Next, to examine the mech-

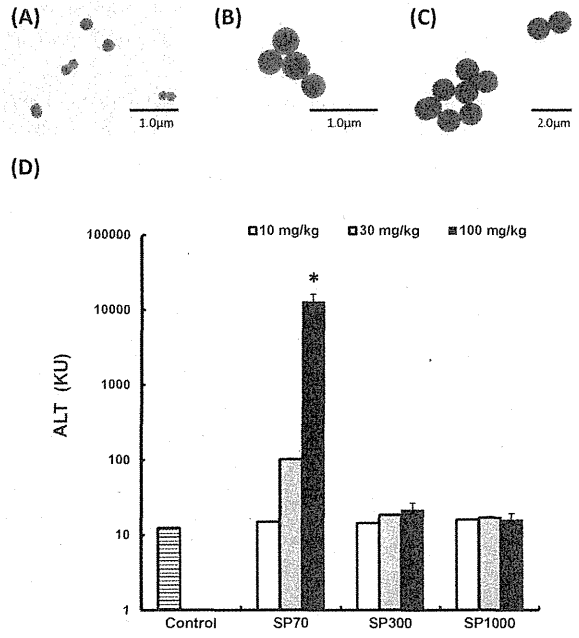

Fig. 1. Ultrastructure and acute liver toxicity of silica nanoparticles. Ultrastructure of silica nanoparticles SP70 (A), SP300 (B), or SP1000 (C) examined under the electron microscope. Silica nanoparticles of the indicated sizes were administered i.v. at the indicated doses. At 24 hr after administration, blood was collected, and the resultant serum was used for the ALT assay (D). Data are mean ± S.E.M. (n = 4). Significant differences were observed between the vehicle groups (*$p < 0.05$).

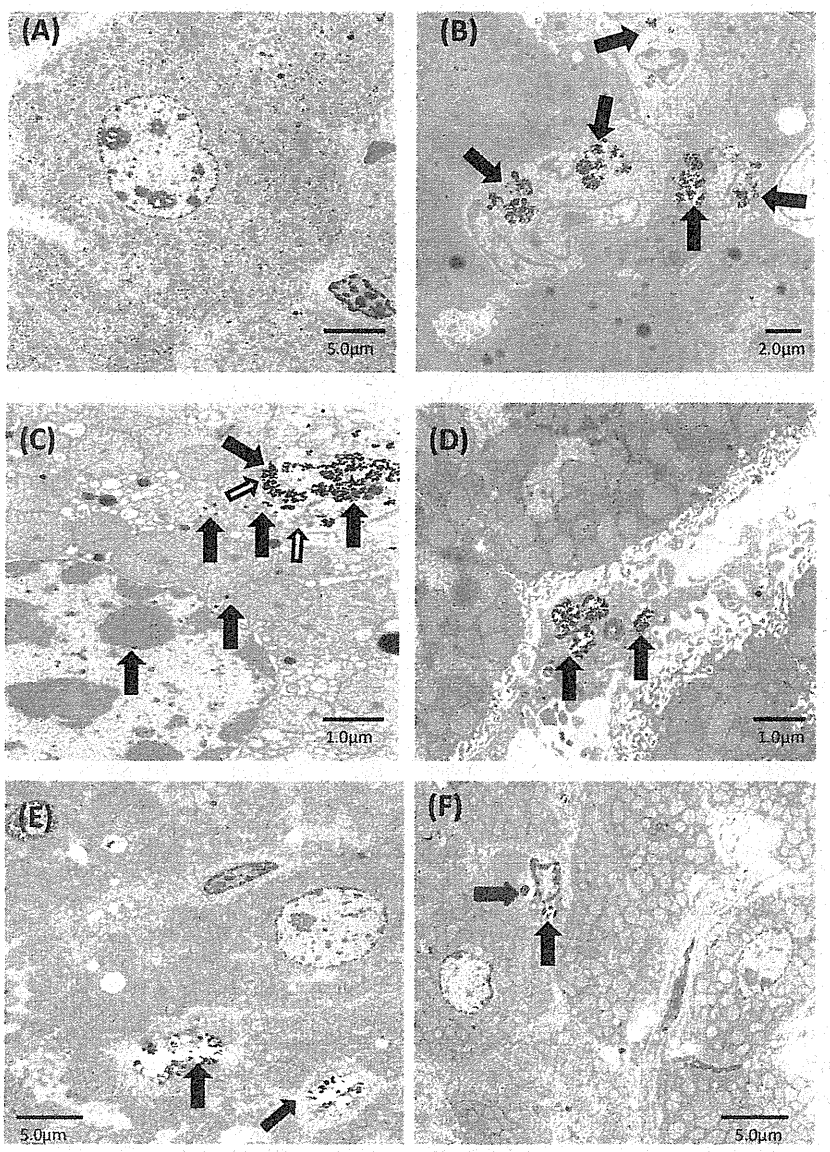

Fig. 2. Ultrastructure of liver tissue following injection of silica nanoparticles. Ultrastructure of liver tissue in silica nanoparticle-treated mice. Animals were administered i.v. with vehicle (A) or with silica nanoparticles (at 100 mg/kg) SP70 (B, C, D), SP300 (E), or SP1000 (F). At 24 hr after administration, livers were collected, fixed, and processed; ultrathin sections were stained with uranyl acetate and lead citrate and examined under the electron microscope. Black arrows indicate silica nanoparticles. White arrows indicate the space of Disse.

anism whereby silica nanoparticles induced liver toxicity, we used EM to observe liver tissue in silica nanoparticle-injected mice at 24 hr post-injection (Figs. 2, 3). SP70 was detected in the hepatocytes, with associated necrosis (Fig. 2B). In addition, SP70 accumulated in the liver sinusoidal endothelial cells; particles apparently accessed the hepatocytes by passing through the space of Disse (Fig. 2C). SP70 was in some instances observed within the nuclei of the hepatocytes. Particles were engulfed by, and accumulated within, Kupffer cells (Fig. 2D). In contrast, SP300 (Fig. 2E) and SP1000 (Fig. 2F) were not observed within the hepatocytes of 30 sections. The results of these EM observations confirmed our previous observation (Nishimori *et al.*, 2009c) that SP70 nanopar-

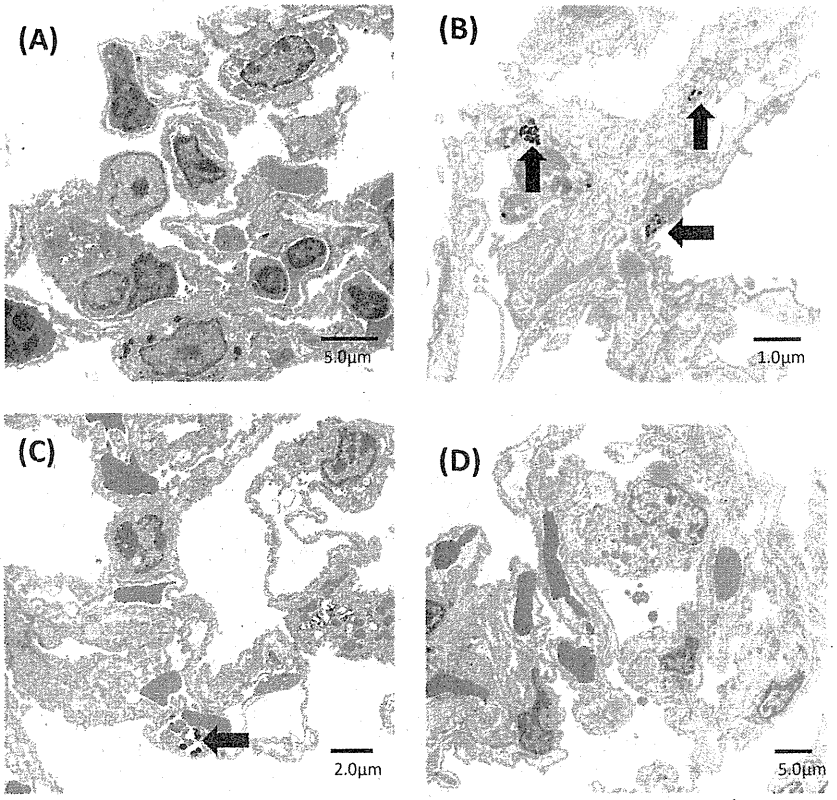

Fig. 3. Ultrastructure of lung tissue from mice treated with silica nanoparticles. Ultrastructure of lung tissue from mice injected i.v. with vehicle (A) or with silica nanoparticles (at 100 mg/kg) SP70 (B), SP300 (C), or SP1000 (D). At 24 hr after administration, lungs were collected and fixed with 2.5% glutaraldehyde; ultra-thin sections were stained with uranyl acetate and lead citrate and examined under the electron microscope. Black arrows indicate silica nanoparticles.

ticles, but not SP300 or SP1000, caused hepatocellular necrosis. The results of the present study further suggested that liver sinusoidal endothelial cells contributed to the internalization of silica nanoparticles by hepatocytes.

We extended our analysis by examining lung following i.v. administration of the silica nanoparticles (Fig. 3). We observed SP70 (Fig. 3B) and SP300 (Fig. 3C) within the lung epithelial cells. SP1000 was not observed in the lung epithelial cells of 28 sections (Fig. 3D). Similarly, silica nanoparticles were not observed in kidney tissue from any of the nanoparticle-dosed animals (SP70, SP300, or SP1000; data not shown).

Thus, we observed by EM that SP70 accumulated in the liver endothelial cells and hepatocytes. Liver sinusoidal endothelial cells were shown to participate in the accumulation of SP70 nanoparticles. In addition, we observed the phagocytosis (engulfment) of SP70 by Kupffer cells. These processes presumably contribute to liver injury. Our EM observations confirmed our previous results (Nishimori *et al.*, 2009c) indicating that liver sinusoidal endothelial cells are directly involved in silica nanoparticle-induced liver injury. The necrosis observed in the present work (e.g., Fig. 2B) was similar to the liver necrosis detected following exposure to aryl alcohol or to hepatitis B virus (Sell, 1997). Other sources have reported that silica nanoparticles induce necrosis in human epithelial cells (Napierska *et al.*, 2010).

The present work also demonstrated that SP70 and SP300 silica nanoparticles could be recovered from lung epithelial cells following i.v. injection. We are not aware of previous reports of nanoparticle accumulation in the lung following parenteral administration. The possible role of these nanoparticles in lung injury is unclear and will require further investigation.

To our knowledge, this report represents the first demonstration that silica nanoparticles are incorporated intra-

cellularly within hepatocytes, liver sinusoidal endothelial cells, Kupffer cells, and lung epithelial cells. Further studies based on these data are expected to provide useful information regarding the safety of these and other nanomaterials.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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