Functionalized Surface-Charged SiO2 Nanoparticles Induce Pro-Inflammatory Responses, but Are Not Lethal to Caco-2 Cells

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Revised Manuscript

Functionalized surface-charge SiO_2 nanoparticles induce pro-inflammatory responses, but are not lethal to Caco-2 cells

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Key words

Silica dioxide nanoparticles, Surface functionalization, Food additive; Caco-2 cells; Cytotoxicity, Inflammation.

ABSTRACT

Nanoparticles (NPs) are widely used in food and analysis of their potential gastrointestinal toxicity is necessary. The present study was designed to determine the effects of silica dioxide (SiO_2), titanium dioxide (TiO_2), and zinc oxide (ZnO) NPs on cultured THP-1 monocyte-derived macrophages and human epithelial colorectal adenocarcinoma (Caco-2) cells. Exposure to ZnO NPs for 24 hours increased the production of redox response species (ROS) and reduced cell viability in a dose-dependent manner in THP-1 macrophages and Caco-2 cells. Although TiO_2 and SiO_2 NPs induced oxidative stress, they showed no apparent cytotoxicity against both cell types. The effects of functionalized SiO_2 NPs on undifferentiated and differentiated Caco-2 cells were investigated using fluorescently-labeled SiO_2 NPs with neutral, positive or negative surface-charge. Exposure of both types of cells to the three kinds of SiO_2 NPs significantly increased their interaction in a dose-dependent manner. The largest interaction with both types of cells was noted with exposure to more negatively surface-charged SiO_2 NPs. Exposure to either positively or negatively, but not neutrally, surface-charged SiO_2 NPs increased NO levels in differentiated Caco-2 cells. Exposure of differentiated Caco-2 cells to positively or negatively surface-charged SiO_2 NPs also upregulated interleukin-8 expression. We conclude that functionalized surface-charge SiO_2 NPs can induce pro-inflammatory responses but are non-cytotoxic.

INTRODUCTION

Engineered nanoparticles (NPs) exhibit peculiar physicochemical features and biomedical properties at the nanoscaled level.[1,2] Nanomaterials have been used in many applications in the fields of medicine, microelectronics, catalysis, cosmetics, drug delivery, and imaging.[3-5] The use of engineered NPs in the food industry has been growing rapidly in recent years, based on their added benefits, such as improvement of taste and texture, prolongation of shelf life, and enhancement of nutritional values.[6,7] Among the engineered NPs, silicon dioxide (SiO_2), titanium dioxide (TiO_2), and zinc oxide (ZnO) NPs are manufactured in large quantities and used commercially as food additives.[8-10] Since NPs are currently widely used in food, analysis of any toxicity especially on the digestive system is necessary at this stage in order to understand their *in vivo* behavior as well as to detect any toxic effects.

Various genetic and environmental factors, such as food and stress, can cause intestinal barrier dysfunction. While the prevalence of inflammatory bowel diseases (IBD), such as Crohn's diseases and ulcerative colitis, is known to be high in Western countries, it has increased exponentially and rapidly in recent years in countries that had undergone shifts from local food style to westernized food, such as Asian countries.[11] Environmental risk factors for IBD include lifestyle (smoking and sleep), ecological factors (air pollution and water pollution), and pharmacologic agents.[12,13] IBD is thought to be associated with failure of immune response against intestinal flora by flares of inflammation.[14] Although intestinal macrophages play an important pathogenic roles in IBD, the mechanisms of inflammatory responses to environmental factors in the digestive system have remained unclear.

Recent studies have shown efficient uptake of NPs by intestinal epithelial cells and microfold (M) cells in an *in vitro* model.[15,16] Exposure of intestinal epithelial cells to NPs has been shown to be associated with accumulation of reactive oxygen species (ROS) and induction of DNA oxidative damage.[17,18] While other studies reported a similar increase in

intracellular ROS levels in the same cell model, no harmful effects were noted.[19] Thus, more investigation is needed on the intestinal toxicity of NPs.

NPs can enter the cells and/or translocate from the site of exposure to the circulation and secondary organs based on their minute size.[20,21] Since some NPs can impair cellular functions and be cytotoxic, it is crucial to understand the key aspects of interactions of NPs with the organism. Surface functionalization is a routine process to improve the behavior of NPs, but the induced surface properties, such as surface charge, may be associated with different toxicity profiles. For example, the surface charge of functionalized polymeric NPs was reported to determine their lung inflammogenicity.[22] We have also reported different responses based on the surface charge of NPs using the zebrafish embryo toxicity test method.[23] However, the effects of NP surface charge on the digestive organs remain elusive.

SiO_2, TiO_2, and ZnO NPs are manufactured in large quantities and SiO_2 NPs are abundantly used by the manufacturers of nanomaterials worldwide.[24] Therefore, it is important to investigate the effects of these NPs on the gastrointestinal tract and the cellular responses of intestinal cells for safety assessment of those NPs. The purpose of the present study was to determine the effects of exposure of THP-1 monocyte-derived macrophages and human epithelial colorectal adenocarcinoma (Caco-2) cells to SiO_2, TiO_2, and ZnO NPs. We also investigated the effects of fluorescently-labeled SiO_2 NPs with a neutrally charged pristine surface or with different surface functionalization that rendered them positively or negatively charged, on undifferentiated and differentiated Caco-2 cells.

EXPERIMENTAL METHODS

Nanoparticle Preparation and Characterization

SiO_2 NPs with a primary diameter of 30 nm, TiO_2 NPs; (AEROXIDE TiO_2 P25) with a primary diameter of 21 nm, and ZnO NPs with a primary diameter of 20 nm were purchased

from Micromod (Rostock, Germany), Degussa (Dusseldorf, Germany), and mkNANO (Mississauga, ONT, Canada), respectively. The NPs were suspended in culture media and dispersed using sonicator (80% pulsed mode, 100 W, 15 min, BRANSON Sonifier model 450; Danbury, CT), as described previously.[25] Rhodamine-labeled SiO_2 NPs, 30 nm in diameter, with hydroxyl groups on the surface (neutral surface charge; N) or functionalized with amino modified (positive surface charge; +q) or carboxyl modified (negative surface charge; –q) were purchased from Micromod. All NPs were suspended in water at 25 mg/L and then dispersed with the culture medium. The hydrodynamic sizes of the particles in media were measured four times after 1 hour on standing using the dynamic light scattering (DLS) technology with a Zetasizer Nano S (Malvern Instruments, Worcestershire, UK). The dispersion status was described by the intensity-weighted hydrodynamic average diameter (z-average) and polydispersity index (PdI), which reflect the broadness of the size distribution (scale range from 0 to 1, with 0 being monodispersion and 1 being polydispersion).[26] The zeta potentials of the particles in distilled water and culture medium were measured three times after 1 hour on standing with a Zetasizer Nano ZSP (Malvern Instruments). The pH of distilled water and culture medium containing the particles was measured three times using a pH meter (LAQUA F-52; HORIBA, Kyoto, Japan).

Cell Culture

Human monocytic leukemia cells (THP-1 cell line, American Type Culture Collection; ATCC, TIB202, Manassas, VA) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, Carlsbad, CA) supplemented with 10% (*v*/*v*) fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. THP-1 cells were differentiated to macrophages with 0.1 μg/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St Louis, MO) for 72 hours, before experimentation. The human colon

colorectal adenocarcinoma cell line Caco-2, which are of intestinal epithelial origin, obtained from ATCC (HTB-37), were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium (Life Technologies) supplemented with 10% (*v*/*v*) FBS, 0.1 mM MEM Non-Essential Amino Acids Solution, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37 °C in 5% CO_2.

Differentiation of Caco-2 Cells

For differentiation of Caco-2 cell monolayer, the cells were seeded onto tissue culture polyethylene terephthalate membrane inserts (Falcon® Cell Culture Inserts, growth area 0.9 cm^2; Corning, Corning, NY) in 12-well Falcon® Cell Culture Insert Companion Plates (Corning) at a concentration of 4.2×10^4 cells/cm^2. The cells were cultured at 37°C, 5% CO_2 and 95% humidity for 21 days. The medium was replaced twice weekly with fresh medium during the differentiation process until day 21. To verify the monolayer integrity, the transepithelial electrical resistance (TEER) was measured using a Millicell® ERS (Merk, Darmstadt, Germany), as described previously.[27] Caco-2 cell monolayers with TEER values exceeding 600 Ω cm^2 were used in the experiments. Furthermore, alkaline phosphatase was used as a marker of differentiation of Caco-2 cells.[28] We confirmed that alkaline phosphatase activity increased about 3.5-fold in Caco-2 cells cultured for 21 days.

Cell Viability Assay

THP-1 monocytes were seeded at 1.5×10^4 cells/well onto 96-well plates and differentiated to macrophages with PMA before the experiment as described above. To use as undifferentiated Caco-2 cells, the cells were seeded overnight at 4.7×10^4 cells/cm^2 onto 96-well plates before the experiment. For differentiated Caco-2 cells, the cells were seeded at 4.7×10^4 cells/cm^2 onto 96-well plates and grown for 21 days. Particles were dispersed in each

serum-containing cell culture medium at a final concentration ranging from 1 to 100 μg/mL. The concentration range corresponded to the dose used in a round robin study to create the protocol for toxicological tests on NPs by the International Alliance for NanoEHS Harmonization (IANH).[29] We also used this concentration range in our previous studies.[30,31] Cell viability was determined after incubation with the dispersed SiO_2, TiO_2, and ZnO NPs for 24 hours in THP-1 macrophages and Caco-2 cells, by the MTS assay based on the CellTiter 96 AQueous One Solution (Promega, Madison, WI), which measures mitochondrial function; the latter correlates with cell viability. Cell viability assay was repeated one to four times in each set. Cell viability was also determined after 24-hrs incubation of undifferentiated and differentiated Caco-2 cells with the functionalized SiO_2 NPs. Cell viability assay was conducted three times in each set. After the incubation, the cells were incubated with fresh medium (phenol red-free) containing MTS reagent for 1 hour before measurements at 490 nm absorbance. The effect of the test particles on cell proliferation was expressed as percentage of inhibition of cell growth relative to the control.

Measurement of ROS Production

Cellular ROS production triggered by SiO_2, TiO_2, and ZnO NPs was assayed by staining with 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro fluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Life Technologies), followed by flow cytometry (FACS CantoII; BD Bioscience, Franklin Lakes, NJ). Before the experiment, THP-1 monocytes were seeded at 3 × 10^5 cells/well onto 24-well plates and allowed to differentiate into macrophages using PMA, as described above. After exposure to NPs for 3 hours, THP-1 macrophages were loaded with 5 μM CM-H_2DCFDA for 30 min at 37°C and analyzed by flow cytometry. Ten thousand cells per sample were acquired in histograms using FlowJo software (Flowjo, Ashland, OR). Dead cells and debris were subtracted by electronic gating using forward and

side scatter measurements. Measurement of ROS production was conducted three times in each set.

Interaction of SiO_2 Nanoparticles with Caco-2 cells

To measure the interaction of fluorescently-labeled SiO_2 NPs with the cells, the fluorescence intensity of rhodamine in Caco-2 cells was assayed with fluorescence microplate reader (SpectraMax M5e Multi-Mode Microplate Reader; Molecular Devices, San Jose, CA). To use as undifferentiated Caco-2 cells, the cells were seeded overnight at 4.7×10^4 cells/cm^2 onto 96-well plates before the experiment. For differentiated Caco-2 cells, the cells were seeded at 4.7×10^4 cells/cm^2 onto 96-well plates and grown for 21 days. Both undifferentiated and differentiated Caco-2 cells were exposed to SiO_2 NPs of three kinds of surface-charge for 3 hours. After the exposure, the fluorescence intensity of rhodamine was measured with the fluorescence microplate reader. Measurement of interaction of NPs with Caco-2 cells was conducted one to three times in each set.

NO Production of SiO_2 Nanoparticles in Caco-2 cells

Cellular NO production triggered by SiO_2 NPs was assayed by staining with diaminofluorescein-2 diacetate (DAF-2 DA) (GORYO Chemical, Sapporo, Japan) followed by measurement using the fluorescence microplate reader (SpectraMax M5e Multi-Mode Microplate Reader). To use as undifferentiated Caco-2 cells, the cells were seeded overnight at 4.7×10^4 cells/cm^2 onto 96-well plates before the experiment. For differentiated Caco-2 cells, the cells were seeded at 4.7×10^4 cells/cm^2 onto 96-well plates and grown for 21 days. Both the undifferentiated and differentiated Caco-2 cells were preloaded with 10 μM DAF-2DA for 30 min at 37°C and then exposed to three kinds of SiO_2 NPs for 24 hours. After the

exposure, the fluorescence intensity of DAF in the cells was measured with the fluorescence microplate reader. Measurement of NO production was conducted twice in each set.

Analysis of IL-8 Expression

To use as undifferentiated Caco-2 cells, the cells (2×10^5 cells) were seeded onto 12-well plates overnight before the experiment. For differentiated Caco-2 cells, the cells were seeded at 2×10^5 cells onto 12-well plates and grown for 21 days. Both undifferentiated and differentiated Caco-2 cells were exposed for 3 hours to 25 or 50 μg/mL of the three kinds of suspended SiO_2 NPs. Total RNA from the cells was isolated by using ReliaPrep RNA cell miniprep system (Promega) according to the protocol provided by the manufacturer. The concentration of total RNA was quantified by spectrophotometry (ND-1000; NanoDrop Technologies, Wilmington, DE). The RNA was reverse transcribed to single-strand cDNA using SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies). The complementary DNA (cDNA, $n = 4$ for each group) was subjected to quantitative PCR analysis with FastStart Universal Probe Master Mix (Roche, Basel, Switzerland) and primers for interleukin (IL)-8 using an ABI 7000 Real-Time PCR system (Life Technologies), as described previously.[31] Measurement of IL-8 expression was conducted twice in each set. The gene expression level was expressed relative to that of β-actin in the same cDNA.

Statistical Analysis

All values were expressed as mean±standard deviation (SD). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. A p value less than 0.05 was considered statistically significant.

RESULTS

Characterization of Nanoparticles in Suspension

In the present study, we analyzed the *in vitro* effects of metal oxide NPs and SiO_2 NPs with three different surface-charges. The intensity-weighted hydrodynamic average diameters and zeta potential of dispersed NPs were determined by the DLS technology. Table 1 shows the mean hydrodynamic diameters and PdI of three kinds of metal oxide NPs in two culture media. The hydrodynamic diameters of SiO_2, TiO_2, and ZnO NPs were 183±1.6, 183±0.6, and 175±3.5 nm, respectively, in RPMI and 197±5.8, 187±0.7, and 179±1.6 nm, respectively, in DMEM (Table 1), indicating aggregation or agglomeration of NPs in the culture media. Although DLS data provided the mean hydrodynamic diameters of >100 nm, the presence of nano-sized particles was confirmed in the media. The hydrodynamic diameters of neutrally, positively, and negatively surface-charged SiO_2 NPs were 36.3±0.33, 33.6±0.64, and 33.6±0.27 nm, respectively (Table 1). The zeta potentials were -33.2±1.85, -25.6±4.19, and -41.5±3.02 mV for neutrally, positively, and negatively surface-charged SiO_2 NPs in distilled water, respectively (Table 1). However, the zeta potentials of three kinds of SiO_2 NPs were almost the same in DMEM (23.3±1.87, 23.5±3.96, and 23.8±2.54 mV, respectively). The pH values were 7.00±0.05, 7.57±0.12, and 6.14±0.06 for neutrally, positively, and negatively surface-charged SiO_2 NPs in distilled water, respectively, whereas they were almost the same in DMEM (8.46±0.04, 8.50±0.02, and 8.46±0.02, respectively) (Table 1).

Effects of Exposure to NPs on Viability of THP-1 Macrophages

THP-1 macrophages were exposed to SiO_2, TiO_2, and ZnO NPs at concentrations ranging from 1 to 100 μg/ml for 24 hours. Exposure to SiO_2 and TiO_2 NPs for 24 hours had no significant effect on the viability of THP-1 macrophages (Figure 1A, B). In contrast, exposure to ZnO NPs dose-dependently reduced the cell viability (Figure 1C).

Effects of Exposure on Accumulation of ROS in THP-1 Macrophages

ROS production is considered one of the initial cellular responses to foreign material. Since a previous *in vitro* study found maximum ROS levels at 2–6-hour exposure to TiO_2 NPs[18], we examined the effects of 3-hour exposure to TiO_2, SiO_2, and ZnO NPs (1 and 10 μg/ml) on ROS production by THP-1 macrophages. Exposure to 10 μg/ml of TiO_2 and SiO_2 NPs significantly increased ROS levels in THP-1 macrophages (Figure 2). ROS levels were also increased in THP-1 macrophages exposed to ZnO NPs at concentrations of 1 and 10 μg/ml (Figure 2).

Effects of Exposure on Caco-2 Cell Viability

Caco-2 cells were exposed to SiO_2, TiO_2, and ZnO NPs at concentrations ranging from 1 to 100 μg/ml for 24 hours. There were no significant changes in Caco-2 cell viability after 24-hrs exposure to SiO_2 and TiO_2 NPs (Figure 3A, B). On the other hand, cell viability was significantly reduced after exposure to ZnO NPs at a concentration of 100 μg/ml (Figure 3C).

Effects of Functionalized SiO_2 NPs on Viability of Undifferentiated and Differentiated Caco-2 Cells

To determine the effects of SiO_2 surface-charge on cell viability, undifferentiated and differentiated Caco-2 cells were exposed for 24 hours to three kinds of SiO_2 NPs at concentrations ranging from 1 to 100 μg/ml. Cell viability, as estimated by the MTS assay, increased after exposure to neutrally surface-charged SiO_2 NPs at concentrations of 1 and 100 μg/ml in undifferentiated Caco-2 cells (Figure 4A) and positively surface-charged SiO_2 NPs at a concentration of 50 μg/ml in differentiated Caco-2 cells (Figure 4D). These results indicate that the surface-charge of SiO_2 NPs does not affect the viability of both undifferentiated and differentiated Caco-2 cells (Figure 4A-F).

Effects of Functionalized SiO_2 NPs on Interaction with Undifferentiated and Differentiated Caco-2 Cells

Next, we examined the effects of surface charge on SiO_2 NPs interaction with undifferentiated and differentiated Caco-2 cells. Exposure of both types of cells to the three kinds of SiO_2 NPs for 3 hours significantly increased their interaction in a dose-dependent manner (Figure 5A-F). The largest interaction with both undifferentiated and differentiated cells was noted with exposure to more negatively surface-charged SiO_2 NPs (Figure 5E,F).

Effects of Functionalized SiO_2 NPs on NO Production by Undifferentiated and Differentiated Caco-2 Cells

The production of cellular NO was examined in undifferentiated and differentiated Caco-2 cells exposed to SiO_2 NPs of different surface-charge (from 1 to 100 μg/ml). After exposure of the two types of Caco-2 cells to SiO_2 NPs for 24 hours, the positively and negatively surface-charged SiO_2 NPs, but not the neutrally charged NPs, increased NO levels significantly only in the differentiated Caco-2 cells, when applied at 100 μg/ml (Figure 6D, E).

Effects of Functionalized SiO_2 NPs on Expression of IL-8 in Undifferentiated and Differentiated Caco-2 Cells

Previous studies demonstrated that 1–6-hour exposure to NPs elicited the expression of various inflammatory cytokines.[19,20] Among them, IL-8 was used in Caco-2 cells as an inflammatory biomarker[32] since its expression was upregulated in the colonic mucosa of patients with IBD.[33] In the present study, the expression of IL-8 was measured in Caco-2 cells after 3-hour exposure to SiO_2 NPs with different surface-charge. The expression was

significantly increased in undifferentiated Caco-2 cells exposed to 50 μg/ml of negatively surface-charged SiO_2 NPs (Figure 7A). Furthermore, exposure of differentiated Caco-2 cell to 50 μg/ml positively and negatively surface-charged SiO_2 NPs significantly increased IL-8 mRNA expression (Figure 7B).

DISCUSSION

In the present study, we examined the effects of SiO_2, TiO_2, and ZnO NPs in THP-1 monocyte-derived macrophages and Caco-2 cells. Inflammatory cells, such as macrophages, play important pathogenic roles in the inflammatory responses in the intestine, as well as intestinal epithelial cells.[34] Therefore, THP-1 cells, a monocyte-derived macrophage-like cell type, commonly used for cellular studies on the pro-inflammatory effects of nanomaterials.[35] While TiO_2 and SiO_2 NPs increased the levels of ROS in THP-1 macrophages in the present study, they had no cytotoxic effects on these macrophages. On the other hand, exposure to ZnO NPs significantly reduced cell viability of THP-1 macrophages. The results also showed increases in the levels of NO and IL-8 in differentiated Caco-2 cells exposed to positively or negatively surface-charged SiO_2 NPs. Our results indicate that functionalized surface-charge SiO_2 NPs induce pro-inflammatory responses, but are not lethal to Caco-2 cells.

Exposure to TiO_2 NPs increased the levels of ROS in THP-1 macrophages, but the results of the MTS assay indicated that these NPs are not cytotoxic to either THP-1 macrophages or Caco-2 cells. We examined previously the different interactions and cellular responses related to the type and size of TiO_2 NPs[31] while another group examined the different responses to morphologically different TiO_2 NPs in an i*n vitro* model of intestinal barrier.[36] In other studies, we found that ZnO NPs are cytotoxic to human umbilical vein endothelial cells (HUVECs) and human endothelial colony-forming cells.[30,37] Similarly, ZnO NPs significantly reduced cell viability of the two cell types employed in the present study.

Consistent with these findings, other investigators showed that ZnO NPs were cytotoxic through overproduction of ROS, as well as genotoxic through micronuclei and DNA damage in Caco-2 cells.[38,39] Gerloff et al.[40] also showed that ZnO NPs were cytotoxic in both undifferentiated and differentiated Caco-2 cells.

Previous *in vitro* studies showed that amorphous SiO_2 NPs reduced cell viability in time- and dose-dependent manners of cultured human alveolar epithelial cells,[41] 3T3 fibroblasts, and RAW 264.7 macrophages.[42] Although SiO_2 NPs were not cytotoxic to Caco-2 cells in the culture media, significant cytotoxicity was detected in another study in which cells exposed to SiO_2 NPs dispersed in the fasting-state simulated intestinal fluids.[43] On the other hand, another study showed significant cytotoxicity in cells exposed to both native and digestion-stimulated SiO_2 NPs.[40] However, our results indicated that SiO_2 NPs are not cytotoxic to both THP-1 macrophages and Caco-2 cells. Since the different surface areas of materials appear to drive the different adverse biological effects,[44] the status of aggregated and agglomerated SiO_2 NPs in the media may affect the extent of cytotoxicity.

SiO_2 NPs are widely used as food additives because of their relatively low toxicity among the currently available NPs. The physico-chemical properties (e.g., size, surface area) and surface properties (e.g., surface charge) have been demonstrated to play key roles in their toxicity or biological effects.[45] Accordingly, we also investigated in the present study the effects of SiO_2 NPs of different surface-charge on both undifferentiated and differentiated Caco-2 cells. The results confirmed that positively and negatively surface-charged SiO_2 NPs induced increased NO production by differentiated Caco-2 cells. The present study also showed IL-8 over-expression in undifferentiated Caco-2 cells exposed to negatively surface-charged SiO_2 NPs and differentiated Caco-2 cells exposed to positively and negatively surface-charged SiO_2 NPs. These results suggest that SiO_2 NPs with functionalized surface-charge induces pro-inflammatory responses in Caco-2 cells.

Our results showed that the zeta potentials of all three kinds of SiO_2 NPs was negative and the zeta potential of negatively surface-charged SiO_2 was the lowest among them in distilled water. For pH values above the isoelectric point (IEP), the zeta potentials of silica become more negative with the increase in pH.[46] Since the present results showed that the pH values were above 6 (IEP of silica is 2-3), negative zeta potentials were observed in all cases. The pH values change in accordance with the surface charge of particles when suspended in distilled water.[47] In the present study, the pH values were 7.00±0.05, 7.57±0.12, and 6.14±0.06 for neutrally, positively, and negatively surface-charged SiO_2 NPs in distilled water, respectively, Moreover, the zeta potential changes with changes in pH values.[48] In our study, the zeta potential values were -33.2±1.85, -25.6±4.19, and -41.5±3.02 mV for neutrally, positively, and negatively surface-charged SiO_2 NPs, respectively, indicating that the differences in zeta potentials corresponding to pH values reflect the differences in the surface status of NPs among the three kinds of SiO_2 NPs. The zeta potentials of SiO_2 NPs of the three different surface-charges were almost the same in the medium. The pH values do not change because the cell culture medium is a buffer solution, resulting in no differences in zeta potentials of three kinds of SiO_2 NPs in DMEM. The earliest cellular responses to NPs are mediated through the interactions between NPs and cells, and the receptors involved in innate immunity, such as toll-like receptors (TLRs) are critical to these interactions.[49] Therefore, these receptors-mediated interactions with the cells might vary among the three different surface-charges SiO_2 NPs despite of almost the same zeta potential of NPs in DMEM. These differences are considered to explain the differences in toxicity in Caco-2 cells.

Evidence suggests that the differentiated Caco-2 cells have much lower uptake ability of NPs compared with the undifferentiated Caco-2 cells.[50] On the other hand, Ye et al.[51] demonstrated that although SiO_2 NPs were taken up better on the undifferentiated cells than

on the differentiated cells in serum-free media, there were no significant differences in uptake of NPs between the undifferentiated and differentiated Caco-2 cells in serum-containing media. Our study showed that the significant increase in the interaction of the three SiO_2 NPs with the cells was almost similar in both undifferentiated and differentiated Caco-2 cells. The results could be due to exposure to SiO_2 NPs in both cells in the serum-containing media. It has been reported that the transport efficiency (rate of transport to cell uptake) across Caco-2 cells is significantly higher for negatively charged NPs compared to positively charged NPs.[52] Consistent with the above findings, our study demonstrated that the interaction of negatively surface-charged SiO_2 NPs with both undifferentiated and differentiated Caco-2 cells was significantly higher than that of neutrally or positively surface-charged SiO_2 NPs.

As discussed above, our results showed similar cellular interaction of surface-charged SiO_2 NPs with undifferentiated and differentiated Caco-2 cells. However, exposure of differentiated Caco-2 cells to either positively or negatively surface-charged SiO_2 NPs was associated with high levels of NO and IL-8 compared with undifferentiated Caco-2 cells. In other words, it seems that the cellular interaction of NPs did not affect the pro-inflammatory response of these cells. The bio-reactivity to NPs is influenced by adsorption of biological molecules onto the surface of NPs, forming a protein corona surrounding the core of NPs.[53] Previous studies showed that the protein composition of the protein corona varied with the surface chemistry of NPs.[54] Since the unique protein corona formed on NPs plays an important role in determining the biological reactivity,[55] differences in the protein corona of SiO_2 NPs associated with surface charge may explain the different response in Caco-2 cells. Further studies are needed to determine the effects of surface properties of NPs on the protein corona composition and the effects of such composition on the biological activities of NPs.

The gastric pH is known to range from 1.5-2.0 in the fasting state and to rise up to pH 7.0 after ingestion of a meal.[56] Zeta potentials change with the pH value of the solution, and

in the case of silica, the potentials decrease with increases in pH values.[48] In the intestinal fluid where the pH is acidic, the zeta potentials of SiO_2 NPs might be higher than those in the present model. Previous studies demonstrated that the zeta potential of polystyrene NPs correlated negatively with cytotoxicity of nonphagocytic lung epithelial cells but positively with cytotoxicity of phagocytic macrophages,[57] suggesting that changes in zeta potentials that parallel the changes in pH values affect the responses of cells to NPs. As discussed above, our results showed more robust pro-inflammatory responses to positively or negatively compared to neutral SiO_2 NPs with the same zeta potentials. Thus, it is conceivable that functionalized surface-charge SiO_2 NPs seem to cause a more pronounced inflammatory responses than neutral NPs even under acidic conditions.

In the present study, SiO_2 NPs with functionalized surface charge induced inflammatory responses, but did not exhibit cytotoxicity towards both undifferentiated and differentiated Caco-2 cells. Previous studies demonstrated passage of orally ingested NPs through the mucosal lining of the intestinal tract and that they were taken up by Peyer's patch M cells, which play an important role in intestinal immunity, as well as epithelial cells.[58] For proper assessment of the effects of NPs contained in food on the function and viability of intestinal cells, it is important to clarify the interactions between NPs and various intestinal cells (e.g., mucosal, M and epithelial cells) using various advanced *in vitro* intestine models.[59] In addition, changes in cellular access of NPs by intestinal mucus through both ionic and hydrophobic interactions and changes in NPs properties by significant changes pH throughout the intestinal tract need to be considered.

Conclusion

The present *in vitro* study demonstrated that all tested NPs (TiO_2, SiO_2 and ZnO) increased the levels of ROS in THP-1 macrophages, whereas only ZnO, but not TiO_2 or SiO_2 NPs were

cytotoxic to these cells and Caco-2 cells. The present study also investigated the effects of the surface charge on SiO_2 NPs. While exposure to all types of SiO_2 NPs did not induce cytotoxicity towards Caco-2 cells, the positively and negatively surface charged SiO_2 NPs induced inflammatory responses in differentiated Caco-2 cells, as reflected by increased production of NO and IL-8, compared with neutral SiO_2 NPs. Our results indicate that the functionalized surface-charge SiO_2 NPs can induce pro-inflammatory responses but are non-cytotoxic.

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Author Contributions

ST-O performed the experiments, analyzed the data, and wrote the manuscript. ME and MY were involved in cell culture and sample preparation. KI and AI contributed to nanomaterial characterization. SV, SB, LT, and GI designed the study, reviewed the manuscript, and provided comments. SI designed the study, supervised the project, and contributed to data interpretation and manuscript revision. All authors read and approved the final manuscript.

Funding

This work was supported by grant (Z9013040) from the Japan Society for the Promotion of Science (JSPS) postdoctoral fellowships for foreign researchers, JSPS KAKENHI Grant Number JP26293149, and a grant from Casio Science Promotion Foundation.

Declaration

The authors declare no conflict of interest.

ACKNOWLEDGMENT

The authors thank Ms. Yumiko Tateno for the excellent help in data analysis and Dr. Kunichi Miyazawa for the valuable discussion and comments.

ABBREVIATIONS

Caco-2, human epithelial colorectal adenocarcinoma cells; DLS, dynamic light scattering; IBD, inflammatory bowel diseases; IL-8, interleukin-8; NO, nitric oxide; NPs, nanoparticles; PdI, polydispersity index; PMA, 12-myristate 13-acetate; ROS, redox response species; SiO_2, silica dioxide; THP-1, human monocytic leukemia cells; TiO_2, titanium dioxide; ZnO, zinc oxide

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Figure Legends

Figure 1. Effects of metal oxide NPs on cell viability. THP-1 macrophages were incubated for 24 hours in the presence of (**A**) SiO_2, (**B**) TiO_2, and (**C**) ZnO NPs at final concentrations of 0, 1, 10, 25, 50, and 100 μg/ml. Data are mean±SD (n = 6, *p<0.05 vs. 0 μg/ml; control of each NP).

Figure 2. Effects of metal oxide NPs on ROS production. THP-1 macrophages were incubated for 24 hours in the presence of SiO_2, TiO_2, and ZnO NPs at final concentrations of 0, 1, and 10 μg/ml. Data are mean±SD (n = 3, *p<0.05 vs. 0 μg/ml control (CTL)).

Figure 3. Effects of metal oxide NPs on cell viability. Caco-2 cells were incubated for 24 hours in the presence of (**A)** SiO_2, (**B**) TiO_2, and (**C**) ZnO NPs at final concentrations of 0, 1, 10, 25, 50, and 100 μg/ml. Data are mean±SD (n = 6, *p<0.05 vs. 0 μg/ml control; control of each NP).

Figure 4. Cell viability of undifferentiated (**A, C, E**) and differentiated (**B, D, F**) Caco-2 cells exposed for 24 hrs to SiO_2 NPs of different surface-charges (N: negative, +q: positive, and -q: negative). The three types of SiO_2 NPs were used at concentrations of 0, 1, 10, 25, 50, and 100 μg/ml. Data are mean±SD (n = 5, *p<0.05 vs. 0 μg/ml; control of each NP).

Figure 5. Uptake of SiO_2 NPs of different surface-charges (N: negative, +q: positive, and -q: negative) by undifferentiated (**A, C, E**) and differentiated (**B, D, F**) Caco-2 cells for 3 hours. The three kinds of SiO_2 NPs were used at concentrations of 0, 1, 10, 25, 50, and 100 μg/ml. Data are mean±SD (n =6, *p<0.05 vs. 0 μg/ml; control of each NP).

Figure 6. NO production by undifferentiated (**A, C, E**) and differentiated (**B, D, F**) Caco-2 cells exposed for 24 hours to SiO_2 of different surface-charges (N: negative, +q: positive, and -q: negative). The three kinds of SiO_2 NPs were used at concentrations of 0, 1, 10, 25, 50, and 100 μg/ml. Data are mean±SD (n = 6, *p<0.05 vs. 0 μg/ml; control of each NP).

Figure 7. Effect of 3-hour exposure of undifferentiated (**A**) and differentiated (**B**) Caco-2 cells to SiO_2 NPs of different surface-charges (N: negative, +q: positive, and -q: negative) on IL-8 expression. The three kinds of SiO_2 NPs were used at concentrations of 0, 25, and 50 μg/ml in the. Data are mean±SD (n = 4, *p<0.05 vs. 0 μg/ml control (CTL)).

Table 1. Characterization of nanoparticles

Nanoparticles	Primary diameter (nm)	Hydrodynamic size (nm)		PdI		Zeta potential (mV)		pH	
		RPMI	DMEM	RPMI	DMEM	Distilled water	DMEM	Distilled water	DMEM
SiO_2	30	182.8±1.6	197.1±5.8	0.266±0.006	0.877±0.142	—	—	—	—
TiO_2	21	183.4±0.6	186.6±0.7	0.273±0.008	0.147±0.014	—	—	—	—
ZnO	20	175.3±3.5	179.3±1.6	0.244±0.004	0.242±0.014	—	—	—	—
SiO_2 N	30	—	36.3±0.3	—	0.175±0.003	-33.2±1.8	-23.3±1.9	7.00±0.1	8.46±0.0
SiO_2 +q	30	—	33.6±0.6	—	0.150±0.019	-25.6±4.1	-23.5±4.0	7.57±0.1	8.50±0.0
SiO_2 -q	30	—	33.6±0.3	—	0.110±0.015	-41.5±3.0	-23.8±2.5	6.14±0.1	8.46±0.0

Data are mean±SD of three or four independent experiments.

PdI: polydispersity index, RPMI: Roswell Park Memorial Institute, DMEM: Dulbecco's Modified Eagle's Medium.

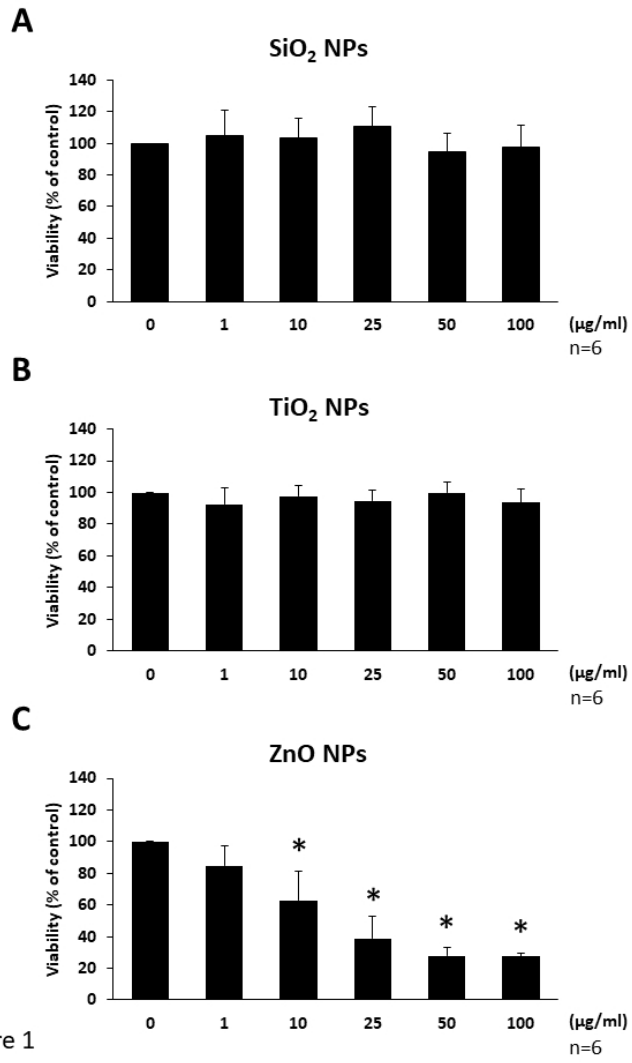

Figure 1

Figure 1. Effects of metal oxide NPs on cell viability. THP-1 macrophages were incubated for 24 hours in the presence of (A) SiO2, (B) TiO2, and (C) ZnO NPs at final concentrations of 0, 1, 10, 25, 50, and 100 μg/ml. Data are mean±SD (n = 6, *p<0.05 vs. 0 μg/ml; control of each NP).

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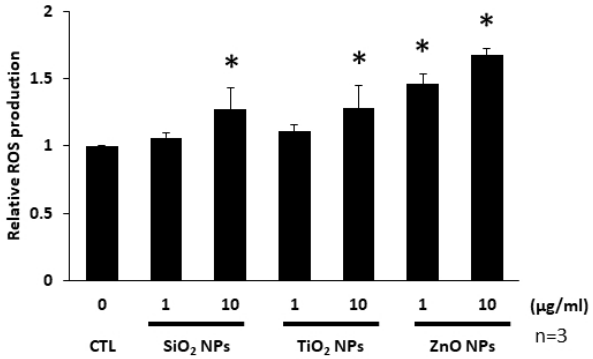

Figure 2

Figure 2. Effects of metal oxide NPs on ROS production. THP-1 macrophages were incubated for 24 hours in the presence of SiO2, TiO2, and ZnO NPs at final concentrations of 0, 1, and 10 µg/ml. Data are mean±SD (n = 3, *p<0.05 vs. 0 µg/ml control (CTL)).

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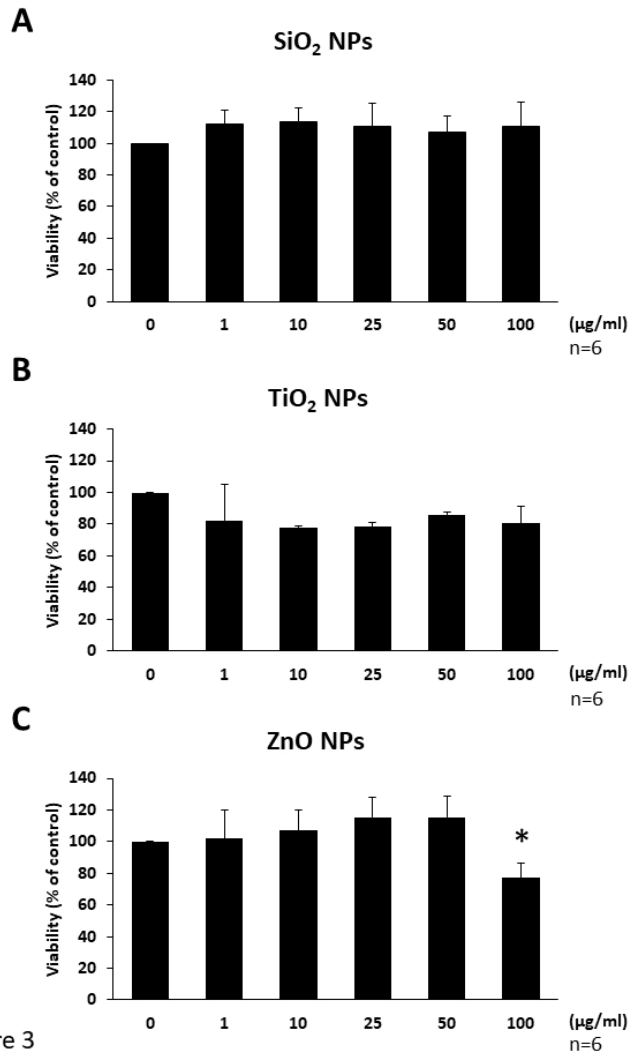

Figure 3

Figure 3. Effects of metal oxide NPs on cell viability. Caco-2 cells were incubated for 24 hours in the presence of (A) SiO2, (B) TiO2, and (C) ZnO NPs at final concentrations of 0, 1, 10, 25, 50, and 100 μg/ml. Data are mean±SD (n = 6, *p<0.05 vs. 0 μg/ml control; control of each NP).

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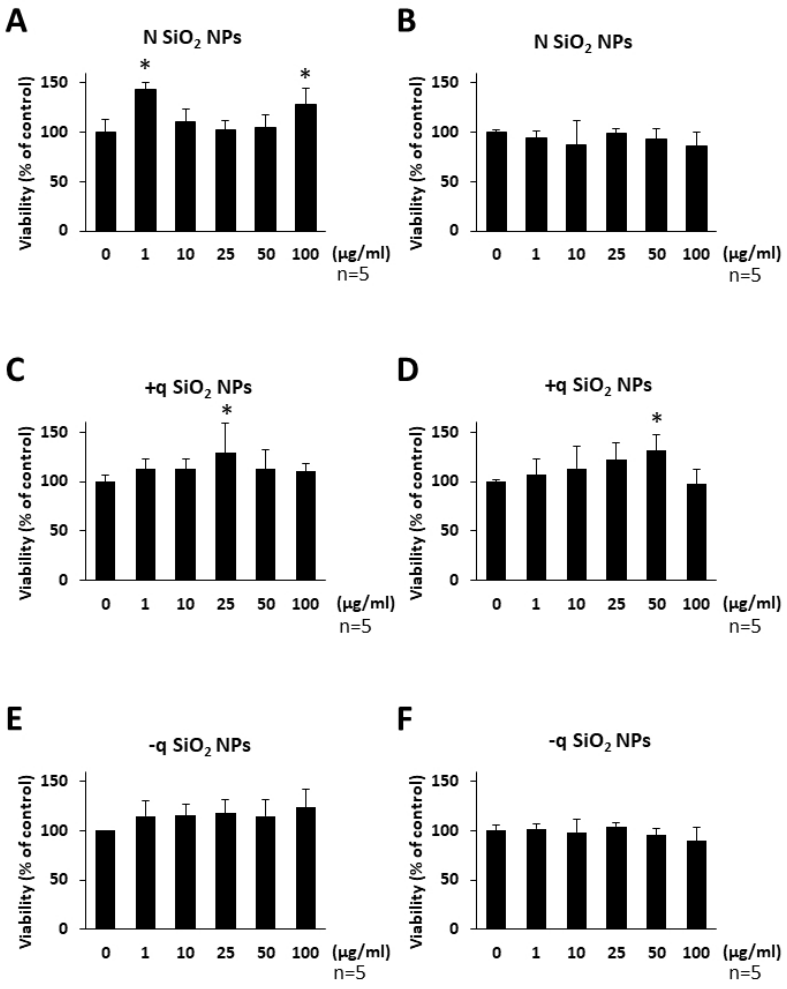

Figure 4

Figure 4. Cell viability of undifferentiated (A, C, E) and differentiated (B, D, F) Caco-2 cells exposed for 24 hrs to SiO2 NPs of different surface-charges (N: negative, +q: positive, and -q: negative). The three types of SiO2 NPs were used at concentrations of 0, 1, 10, 25, 50, and 100 µg/ml. Data are mean±SD (n = 5, *p<0.05 vs. 0 µg/ml; control of each NP).

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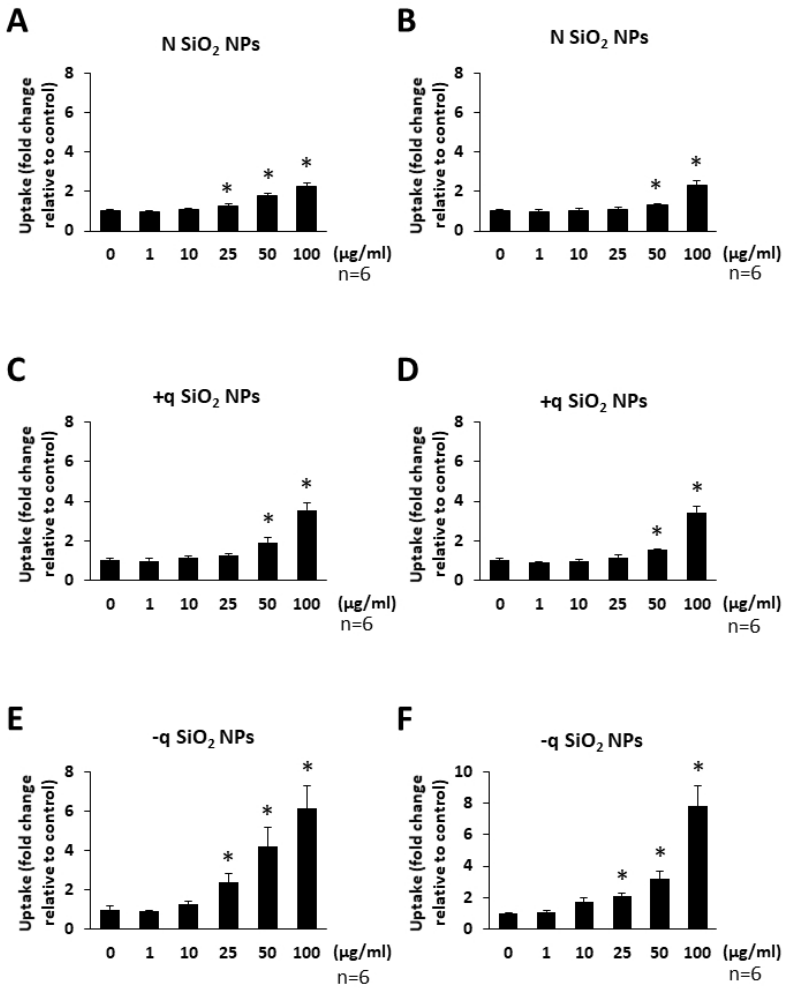

Figure 5

Figure 5. Uptake of SiO2 NPs of different surface-charges (N: negative, +q: positive, and -q: negative) by undifferentiated (A, C, E) and differentiated (B, D, F) Caco-2 cells for 3 hours. The three kinds of SiO2 NPs were used at concentrations of 0, 1, 10, 25, 50, and 100 μg/ml. Data are mean±SD (n =6, *p<0.05 vs. 0 μg/ml; control of each NP).

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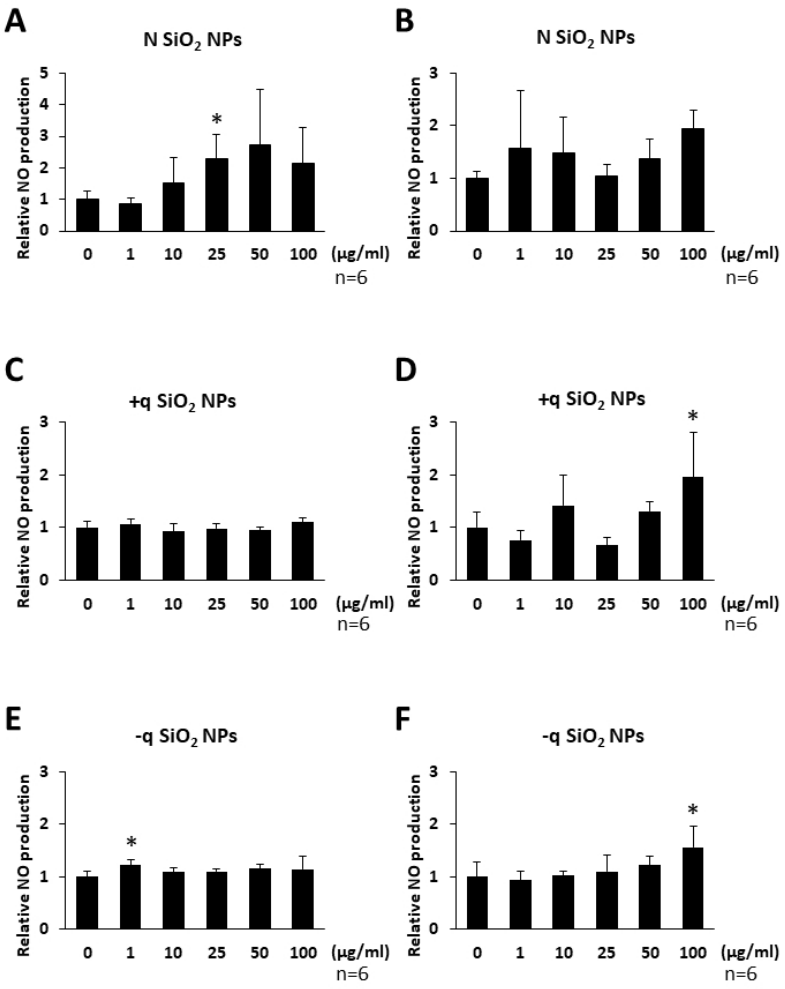

Figure 6

Figure 6. NO production by undifferentiated (A, C, E) and differentiated (B, D, F) Caco-2 cells exposed for 24 hours to SiO2 of different surface-charges (N: negative, +q: positive, and -q: negative). The three kinds of SiO2 NPs were used at concentrations of 0, 1, 10, 25, 50, and 100 μg/ml. Data are mean±SD (n = 6, *p<0.05 vs. 0 μg/ml; control of each NP).

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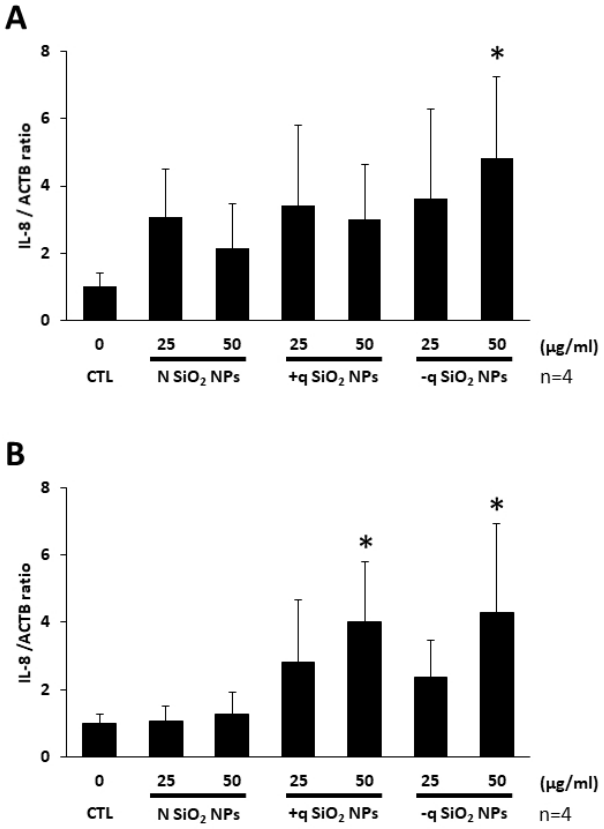


Figure 7

Figure 7. Effect of 3-hour exposure of undifferentiated (A) and differentiated (B) Caco-2 cells to SiO2 NPs of different surface-charges (N: negative, +q: positive, and -q: negative) on IL-8 expression. The three kinds of SiO2 NPs were used at concentrations of 0, 25, and 50 µg/ml in the. Data are mean±SD (n = 4, *p<0.05 vs. 0 µg/ml control (CTL)).

190x275mm (96 x 96 DPI)

Materials

Neutral SiO_2 NPs

Positively surface-charged SiO_2 NPs

Negatively surface-charged SiO_2 NPs

Characterization

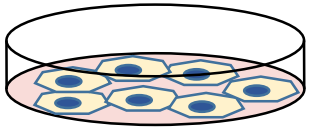
Hydrodynamic diameter

Polydispersity index

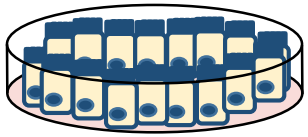
Zeta potential

pH

Exposure



undifferentiated Caco-2 cells



differentiated Caco-2 cells

Effect of SiO_2 NPs

Cell viability

Interaction with cells

NO production

Interleukin-8 expression