This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the **Information for Authors**.

1　*In vitro* **evaluation of the cellular influence of indium tin oxide**

2　**nanoparticles using the human lung adenocarcinoma A549 cells**

3

4　Yosuke Tabei[*], Akinari Sonoda, Yoshihiro Nakajima, Vasudevanpillai Biju, Yoji Makita,

5　Yasukazu Yoshida, Masanori Horie

6

7　Health Research Institute, National Institute of Advanced Industrial Science and Technology

8　(AIST), 2217-14 Hayashi-cho, Takamatsu, Kagawa 761-0395, Japan

9

10　[*]Corresponding author

11　Address: Health Research Institute, National Institute of Advanced Industrial Science and

12　Technology (AIST), 2217-14 Hayashi-cho, Takamatsu, Kagawa 761-0395, Japan.

13　E-mail: y-tabei@aist.go.jp.

14　Tel: +81-87-869-3598

15　Fax: +81-87-869-3553

16

1 **Abstract**

2   Indium tin oxide (ITO) is widely used in liquid crystal displays (LCDs) or plasma

3 and mobile phone displays. Elevated production and usage of ITO in such displays have

4 increased concerns over the safety of industrial workers exposed to particulate aerosols

5 produced during cutting, grinding and polishing of these materials. However, the cellular

6 influences of ITO nanoparticles (NPs) are still unclear, although it has been reported that

7 micro-scale ITO particles induce cytotoxicity. This study aimed to examine the potential of

8 ITO NPs to induce cytotoxicity, oxidative stress, and DNA damage using human lung

9 adenocarcinoma A549 cells. Here, stable dispersions of a medium containing ITO NPs were

10 obtained using pre-adsorption and centrifugal fractionation methods, and the A549 cells were

11 incubated in this medium. The ITO NPs had low cytotoxic effects as shown by the WST-1 and

12 LDH assay. Transmission electron microscope observation showed cellular uptake of ITO

13 NPs. The ITO NPs increased the intracellular level of reactive oxygen species and the

14 expression of heme oxygenase 1 gene. Further, the results of alkaline comet assay showed that

15 ITO NPs induced DNA damage. Thus, these results suggest that ITO NPs possess a genotoxic

16 potential on human lung adenocarcinoma A549 cells.

17

18 **Key words:** Indium tin oxide, nanoparticle, lung epithelial cell, oxidative stress, genotoxicity,

19

1   **Introduction**

2   Indium tin oxide (ITO) is typically a sintered mixture of 90% indium oxide (In_2O_3)

3   and 10% tin oxide (SnO_2). Because of the unique physical properties of ITO, which include

4   high electrical conductivity and transparency, this material is widely used in liquid crystal

5   displays (LCDs) or plasma and mobile phone displays.[1,2] An increase in the demand for

6   computers and mobile phones had led to a rapid increase in the production of LCDs.

7   Increased production and use of ITO have increased concerns about the safety of industrial

8   workers exposed to particulate aerosols produced during cutting, grinding, and polishing of

9   these materials.

10   Epidemiological studies and studies of fatal cases of workers exposed to ITO show

11   that ITO induces occupational lung disease and is associated with the risk of interstitial lung

12   damage.[3,4,5,6,7,8] Lison *et al.*[9] showed that ITO particles were more toxic than its individual

13   components (In_2O_3 or SnO_2) or the unsintered mixture (In_2O_3 and SnO_2). Their results showed

14   that ITO particles are a novel toxicological materials, which have the potential to generate a

15   high amount of reactive oxygen species (ROS) and induce lung toxicity in experimental

16   animals. ITO particles induce cytotoxicity by cellular uptake of these particles by

17   macrophages and solubilization of indium ion via phagolysosomal acidification.[10] Thus, the

18   Ministry of Health, Labour and Welfare (MHLW) in Japan required a material safety data

19   sheet for indium, set a permissible concentration of 3×10^{-4} mg m^{-3} for indium compounds,

1   and stated that monitoring of the workplace environment must be conducted every 6

2   months.[11]

3   A nanoparticle (NP) is defined as a particle that has a diameter in the ranges of 1-100

4   nm (ISO/TS 27678:2008) and has unique physical and chemical properties such as high

5   catalytic activity and distinct absorbance spectrum compared to micro-scale particles.[12] These

6   properties render NPs beneficial for industrial use, and many NPs are used in products

7   familiar to consumers, such as sunscreen and cosmetics. Previous studies have shown

8   size-dependent adverse effects of particles. For example, micro- and nanoscale metal oxide

9   particles such as titanium dioxide (TiO_2), copper oxide (CuO), and nickel oxide (NiO)

10   particles shown different toxicity profiles.[13,14,15] Therefore, particle size must be important

11   parameter to be considered when evaluating the hazards of ITO particles. Many previous

12   studies have examined the toxicity of micro-scale ITO particles. However, the toxicity caused

13   by ITO NPs has not been examined thus far. We hypothesize that the unique properties of ITO

14   NPs, such as small size and large surface area, may exert biological effects that are quite

15   different from those of micro-scale ITO particles.

16   *In vitr*o studies provide important information, especially about the mechanisms

17   underlying NP toxicity.[16] Previous studies have shown that some NPs induce toxic effects

18   such as oxidative stress, apoptosis, cytokine production, and cell death.[17] This study aimed to

19   investigate the cellular influence of ITO NPs using human lung adenocarcinoma A549 cells as

4

1   an *in vitro* model of exposure to ITO NPs via inhalation. In particular, we examined the

2   potential of ITO NPs to cause oxidative stress and DNA damage. Subsequently, we examined

3   the mechanisms underlying these effects. We propose that exposure of A549 cells to ITO NPs

4   induce ROS production, which may further result in DNA damage.

5

1 **Experimental**

2 **Cell culture**

3   Human lung adenocarcinoma A549 cells (RCB0098) were purchased from RIKEN

4 Bioresource Center (Tsukuba, Ibaraki, Japan). The cells were cultured in Dulbecco's modified

5 Eagle's medium (DMEM; Gibco Life Technologies, Gaithersburg, MD) supplemented with

6 10% heat-inactivated fetal bovine serum (FBS; USDA Tested FBS; ThermoFischer Scientific

7 Inc., Waltham, MA), 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 250 ng mL^{-1}

8 amphotericin B (Nacalai Tesque Inc., Kyoto, Japan). The DMEM used in this study is referred

9 to as "DMEM-FBS". Cells in DMEM-FBS were placed in 80-cm^2 flasks (ThermoFischer

10 Scientific Inc.) and cultured at 37°C in an atmosphere of 5% CO_2.

11

12 **Preparation of ITO-medium dispersions**

13   ITO NPs were purchased from C. I. Kasei Co., Ltd. (Tokyo, Japan) and Xuan Cheng

14 Jing Rui New Material Co., Ltd. (Xuancheng, China). The surface area of the ITO NPs, which

15 were degassed at 120°C for 30 min, was determined with the Quantachrom Autosorb-1

16 instrument by using nitrogen adsorption and the five-point Brunauer-Emmett-Teller (BET)

17 method.

18   For cytotoxicity assays described in Fig. 1, ITO powder was dispersed in 1% BSA at

19 a concentration of 10 mg mL^{-1} by sonication. Subsequently, the dispersion was diluted with

1   DMEM-FBS at concentrations ranging from 1 to 1000 μg mL^{-1}. For other experiments, stable

2   dispersions were prepared using pre-adsorption and centrifugation method[18] with some

3   modification. Briefly, ITO particles were dispersed in 1% BSA at a concentration of 80 mg

4   mL^{-1} by sonication. Subsequently, the dispersion was centrifuged at 4000 × g for 20 min. The

5   precipitated ITO particles were re-dispersed in an equivalent volume of fresh DMEM-FBS.

6   The dispersion was centrifuged at 1000 × g for 20 min. The resulting fraction was applied to

7   A549 cells for evaluating the cellular responses.

8

9   **Characterization of ITO-medium dispersions**

10   The microstructure of ITO NPs in the ITO-DMEM-FBS dispersion was performed

11   using field-emission scanning electron microscopy (FE-SEM, JSM-6700F; JOEL Ltd., Tokyo,

12   Japan). The secondary particle size in the ITO-DMEM-FBS dispersion was determined using

13   the dynamic light scattering (DLS) particle size analyzer (LB-550; HORIBA Ltd., Kyoto,

14   Japan) at 25.0°C ± 0.1°C. The light source was a 650-nm laser diode of 5 mW. We determined

15   the particle size using 20-mL samples of ITO-DMEM-FBS dispersion without any

16   pretreatment.

17   The concentrations of components in the medium were determined using inductively

18   coupled plasma atomic emission spectrometry (ICP-AES, SPS7800; Seiko Instruments, Inc.,

19   Tokyo, Japan) and ICP-mass spectrometry (ICP-MS, X-Series II; ThermoFischer Scientific

1 Inc.). The ITO-DMEM-FBS dispersion was applied to the ultrafiltration membrane (Amicon

2 Ultra Centrifugal Filters Ultracel 30K; Merck Millipore, Billerica, MA) and centrifuged at

3 $7000 \times g$ for 20 min, and then the concentrations of Na, Ca, P, In, and Sn were determined.

4 The concentrations of In_2O_3 and SnO_2 included in the stable ITO-DMEM-FBS dispersions

5 were measured using ICP-AES (Thermo Jarrell Ash Corp., Franklin, MA).

6   The ITO particles included in the culture medium were removed by centrifugation at

7 $16,000 \times g$ for 20 min. The supernatant was collected for determination of the protein

8 concentration. The concentration of protein in the culture medium was determined using a

9 protein assay kit from Bio-Rad (Hercules, CA), where BSA was the standard.

10

11 **Determination of the viability of cells**

12   Cell viability was examined using the WST-1 solution (Premix WST-1 Cell

13 Proliferation Assay System; Takara Bio, Shiga, Japan). For the WST-1 assay, the cells were

14 seeded in 96-well plates (ThermoFischer Scientific Inc.) at a density of 2×10^5 cells mL^{-1}.

15 The cells were allowed to attach to the plate for approximately 20 h and treated with the

16 ITO-DMEM-FBS dispersion. For the determination of cell viability, the cells were incubated

17 with 10-fold diluted WST-1 solution at 37°C for 1 h. The optical density of formazan was

18 measured at 450 nm using a Tecan Infinite M200 (Tecan Group Ltd., Männedorf,

19 Switzerland).

1   To determine whether ITO NPs interfere with the WST-1 reaction product, WST-1

2   interference test was performed. The cells were seeded in 96-well plates and cultured for 24 h

3   at 37°C in 5% CO_2. The cell culture medium was removed and replaced by 100 μL 10-fold

4   diluted WST-1 solution. Following an incubation of 1 h at 37°C in 5% CO_2, the optical

5   density of formazan was measured as described above. Next, 10 μL DMEM-FBS and

6   ITO-DMEM-FBS dispersions were added to the well and the optical density was determined

7   once more. The result of interference test indicated that the interference of the ITO NPs with

8   WST-1 reaction product was negligible (data not shown).

9   For the lactate dehydrogenase (LDH) assay, the cells were seeded in 12-well plates

10   (ThermoFischer Scientific Inc.) at a density of 2×10^5 cells mL^{-1}. The cells were allowed to

11   attach for approximately 20 h and treated with the ITO-DMEM-FBS dispersion. LDH release

12   was measured with a tetrazolium salt by using Cytotoxicity Detection Kit[PLUS] (LDH) (Roche

13   Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. The

14   cytotoxicity was calculated as follows: cytotoxicity (%) = (experimental value – low

15   control)/(high control – low control) × 100. The low control, which refers to spontaneous

16   LDH release, was determined as the LDH released from untreated normal cells. The high

17   control, which refers to the maximum LDH release, was determined as the LDH released

18   from cells that were lysed using the lysis solution provided in the kit.

19

1   **Transmission electron microscopy**

2   A549 cells incubated in the ITO-DMEM-FBS dispersion were observed using a

3   transmission electron microscope. The cells were incubated in the ITO-DMEM-FBS

4   dispersion for 24 h and then fixed using 1.2% glutaraldehyde for 1 h. The fixed samples were

5   treated with osmium tetroxide (OsO_4) solution for 1 h, dehydrated in ethanol, and embedded

6   in epoxy resin. The resulting samples were then cut into ultrathin sections using a

7   diamond-knife ultramicrotome for examination under a transmission electron microscope.

8   Transmission electron microscopy (TEM) observations were performed using H-7600

9   (Hitachi Corp., Tokyo, Japan). The acceleration voltage was 80.0 kV.

10

11   **Cellular uptake of ITO NPs**

12   We measured the delivery of ITO NPs to the cell surface and the uptake of NPs by

13   the cells using ICP-MS (ThermoFischer Scientific Inc.). A549 cells in 6-well plates

14   (ThermoFischer Scientific Inc.) were incubated in the ITO-DMEM-FBS dispersion for 24 h,

15   washed with phosphate buffered saline (PBS) three times to remove loosely bound ITO NPs

16   on the cell surface, and detached using 0.25% trypsin. The cell pellet was resuspended in 1

17   mL PBS, and the number of cells was counted using a hemocytometer. The cells were mixed

18   with acid solution (water:nitric acid:hydrochloric acid = 4:1:3) and then heated at 80°C for 2 h

19   to dissolve the cellular contents. The solutions were diluted with water and used for ICP-MS

1 analysis.

2

Measurement of the level of intracellular ROS

4   The level of intracellular ROS was detected using 2′,7′-dichlorodihydrofluorescin

5 diacetate (DCFH-DA) (Sigma-Aldrich, St. Louis, MO). DCFH-DA was dissolved in DMSO

6 at 5 mM as a stock solution and stored at -20°C. After incubation of cells with the

7 ITO-DMEM-FBS dispersion, the medium was changed to a serum-free DMEM containing 10

8 μM of DCFH-DA, and the cells were incubated for 30 min at 37°C. Then, cells were washed

9 once with PBS, collected by 0.25% trypsinization, washed again with PBS, and resuspended

10 in 500 μL of PBS. Cell samples in PBS were excited with a 488-nm argon laser in the

11 FACSCalibur flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ). The

12 emission of DCF was recorded at 525 nm. Data were collected from at least 5000 gated

13 events.

14

Isolation of total RNA and quantitative real-time polymerase chain reaction analysis

16   For isolation of total RNA, the cells were cultured in 6-well plates (ThermoFischer

17 Scientific Inc.) and incubated in the ITO-DMEM-FBS dispersion for 24 and 72 h.

18 Subsequently, total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA)

19 according to the manufacturer's instructions. The concentration of the RNA was determined

11

1   using a Nanodrop ND-1000 spectrophotometer (ThermoFischer Scientific Inc.). The first

2   strand cDNA was synthesized from 0.5 μg of total RNA by using the SuperScript VILO

3   cDNA synethesis kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

4   The mRNA levels of heme oxygenase 1 (*HMOX-1*) were analyzed using the TaqMan gene

5   expression assay (IDs: HMOX-1, Hs01110250_m1, Applied Biosystems). The housekeeping

6   gene β-actin (Assay ID: Hs99999903_m1) was used as an internal control. The levels of each

7   target mRNA were measured using the Applied Biosystems 7300 Real Time PCR System.

8   The cycler conditions were as follows: 2 min at 50°C, 10 min at 95°C, and 60 cycles each of

9   15 s at 95°C and 1 min at 60°C. The mRNA expression levels of *HMOX-1* in each sample

10 were normalized to the β-actin mRNA levels and were then compared with those of the

11 untreated controls. The results are reported as fold change over control.

12

13 **Alkaline comet assay**

14   An alkaline comet assay was performed according to the instructions provided by the

15 manufacturer (Comet Assay; Trevigen, Gaithersburg, MD). For the comet assay, the cells (2 ×

16 10^5 cells mL^{-1}) were seeded into 6-well plates. After overnight incubation, the cells were

17 exposed to the ITO-DMEM-FBS dispersion for 24 and 72 h. Then, the cells were washed and

18 resuspended in Ca^{2+}- and Mg^{2+}-free PBS solution. The cell suspension (1×10^5 cells mL^{-1})

19 was mixed with LMagarose at a ratio of 1:10 (v/v). This mixture was immediately transferred

1   onto the CometSlide (Trevigen). The cells were lysed overnight at 4°C; then the CometSlides

2   were transferred to fresh dishes containing cold alkaline unwinding solution (0.2 M NaOH

3   and 1 mM EDTA, pH > 13) and incubated for 60 min at 4°C for the DNA unwinding step.

4   The CometSlides were placed on a horizontal electrophoresis unit and the unit was filled with

5   fresh buffer (0.2 M NaOH, 1 mM EDTA, pH > 13) to cover the CometSlides. Electrophoresis

6   was performed at 20 V for 35 min at 4°C in the dark and then staining was performed with a

7   silver staining kit (Trevigen). The comet tail length was defined as the distance between the

8   leading edge of the nucleus and the end of the tail. At least 50 comet tail lengths/slide were

9   quantified from three independent experiments performed in duplicate.

10

11   **Clonogenic assay**

12   The colony-forming ability (i.e., cell proliferation) was detected using a clonogenic

13   assay based on the methods described by Herzog *et al.*[19] and Franken *et al.*[20] Briefly, the cells

14   were seeded in 6-well plates (ThermoFischer Scientific Inc.) at a density of 300 cells/well.

15   Each well contained 2 mL of the cell culture medium. The cells were allowed to attach for 20

16   h and treated with 2 mL of the ITO-DMEM-FBS dispersion. Then, the cells were cultured for

17   the time period required for the control cells to form colonies (one colony was defined as ≤50

18   cells), i.e., 7 days. Subsequently, the dispersions were removed and cells washed with 2 mL

19   PBS. After fixation with 100% methanol for 20 min, the cells were stained with Giemsa's

1   staining solution (Kanto Chemical Co., Inc., Tokyo, Japan) for 20 min and rinsed with

2   distilled water. The number of colonies was counted.

3

4   **Statistical analysis**

5   Data are mean ± standard deviation values from at least three independent

6   experiments. Statistical analyses were performed with the analysis of variance (ANOVA) by

7   using Dunnett test for multiple comparisons.

8

14

1   **Results**

2   **Cellular influences of un-fractionated ITO-medium dispersion**

3   We used three kinds of ITO NPs in this study (Table 1). The ITO NPs were dispersed

4   in 1% BSA at a concentration of 10 mg mL^{-1} by sonication. Subsequently, the dispersion was

5   diluted with DMEM-FBS at concentrations ranging from 1 to 1000 μg mL^{-1}, and then, the

6   A549 cells were incubated in the dispersions. After incubation for 6 and 24 h, we examined

7   the cell viability and cell membrane damage by using WST-1 assay and LDH assay,

8   respectively. The cell viability was not influenced by ITO exposure even at ITO

9   concentrations of 1.0 mg mL^{-1} (Fig. 1A). The cell viability did not decrease despite prolonged

10   exposure (7 days) to ITO (data not shown). In addition, no cell membrane damage was

11   observed in the A549 cells exposed to ITO (Fig. 1B).

12   The ITO dispersion included large secondary particles that were extremely unstable.

13   Since the large secondary particles accumulated on the cells by gravity sedimentation, the

14   concentration of the dispersion did not reflect the concentration to which the cells were

15   exposed. Previous studies showed that stability of the dispersion is important for evaluating

16   the cellular responses of NPs.[21,22] Therefore, further cellular responses were examined by

17   using a stable ITO-DMEM-FBS dispersion.

18

19   **Characterization of fractionated ITO-DMEM-FBS dispersion**

15

1   The stable dispersion was prepared by pre-adsorption and centrifugal fractionation

2   methods as reported previously.[18] Examination of primary ITO particles by using FE-SEM

3   showed that most ITO NPs were globular particles (Fig.2A). The average sizes of ITO NPs in

4   sample A, sample B, and sample C were 20.8, 28.6, and 31.0 nm, respectively; the sizes were

5   calculated from the diameters measured for 100 particles (Fig. 2B).

6   The size of secondary ITO NPs in the dispersion was measured using DLS (Fig. 3A).

7   The sizes of the secondary particle in sample A, sample B, and sample C based on particle

8   numbers were 106.3, 63.4, and 57.3 nm, respectively, and those based on light scattering

9   intensities were 206.2, 128.1, and 130.7 nm, respectively. Although the sizes of secondary

10   particles in sample A gradually increased, those of particles in sample B and sample C did not

11   change during the experimental period. Further, we examined the stability of the dispersion

12   using DLS (Fig. 3B). The DLS results suggested that slight sedimentation occurred in sample

13   A and sample B, but we did not observe a marked decrease in the light scattering intensity.

14   Sample C did not show a significant change in the light scattering intensity for 4 days after

15   preparation. These results indicated that the ITO-DMEM-FBS dispersions prepared in this

16   study were suitable for *in vitro* experiments.

17   Results of the characterization of the ITO-DMEM-FBS dispersion are shown in

18   Table 1. The concentration of In_2O_3 in sample A, sample B, and sample C were 720, 490, and

19   480 µg mL^{-1}, respectively. The concentrations of SnO_2 in the sample A, sample B, and sample

16

1   C were 70, 50, and 50 μg mL^{-1}, respectively. The release of soluble indium and tin from ITO

2   NPs into the medium was very small. The slight decrease in the concentrations of sodium and

3   calcium and an increase in the concentration of phosphorus and protein in the ITO medium

4   dispersion did not have marked effects. These results indicated that the ITO NPs did not affect

5   the composition of the medium.

6

7   **Influence of the stable ITO-DMEM-FBS dispersion on cell viability and cell membrane**

8   The influence of ITO NPs on cell viability was examined using stable

9   ITO-DMEM-FBS dispersions prepared in the above step (Fig. 4). We found that the inhibitory

10   effect of ITO NPs on the cell viability were not remarkable, although the viability was slightly

11   inhibited at 72 h. Further, we measured the cell membrane integrity after incubation with the

12   stable ITO-DMEM-FBS dispersion for 6, 24, and 72 h (Fig. 5). We did not observe a

13   significant cytotoxic effect at all the tested concentrations.

14

15   **Uptake of ITO NPs by A549 cells.**

16   A549 cells were incubated with the ITO-DMEM-FBS dispersion for 24 h, and then,

17   the uptake of ITO NPs by the A549 cells was determined using TEM and by ICP-MS (Fig. 6).

18   TEM images showed that ITO NPs were incorporated into the A549 cells after 24 h

19   incubation (Fig.6A). Internalized ITO NPs existed in a lysosome-like structure in the

1   cytoplasm. We did not observe translocation of ITO NPs into the nucleus. We measured the

2   amounts of internalized ITO NPs in the A549 cells by using ICP-MS (Fig. 6B). After

3   incubating the cells with the ITO-DMEM-FBS dispersion for 24 h, the entire cell population

4   was detached and the cells were lysed and analyzed to determine the concentration of

5   internalized indium. ICP-MS measurements showed that the amount of indium uptake

6   correlated with the concentration of ITO NPs in the extracellular solution.

7

8   **Effects of stable ITO-DMEM-FBS dispersion on oxidative stress**

9   Previous studies indicated that NP-mediated cytotoxicity is associated with ROS

10   production.[23,24,25,26] Thus, we examined the effect of ITO NPs on oxidative stress. The stable

11   ITO-DMEM-FBS dispersions were exposed to A549 cells for 6, 24, and 72 h; subsequently,

12   we measured the intracellular ROS levels using the fluorescent dye DCFH-DA, which shows

13   enhanced fluorescence in the presence of oxidative stress (Fig. 7A). After 24 h exposure to

14   ITO NPs, a significant increase in ROS production was observed for sample B and sample C.

15   The A549 cells exposed to sample A showed a significant increase of ROS production at 72 h

16   in a concentration-dependent manner. Our results showed that exposure of A549 cells to

17   ITO-DMEM-FBS dispersion causes an increase in ROS generation reflected by an increase in

18   DCF fluorescence.

19   We measured the mRNA levels of *HMOX-1*, a marker of oxidative stress, to

1   determine the oxidative stress response of A549 cells exposed to the ITO-DMEM-FBS

2   dispersion (Fig. 8). The mRNA levels of *HMOX-1* gradually induced after incubation with the

3   ITO-DMEM-FBS dispersion. This up-regulation of *HMOX-1* mRNA was prominent at 72 h.

4   These results suggested that ITO NPs induced oxidative stress.

5

6   **DNA damage and colony-forming ability of A549 cells incubated with the stable**

7   **ITO−DMEM−FBS dispersion**

8   We examined the DNA single-strand breaks by using the alkaline comet assay. Here,

9   A549 cells were incubated with the stable ITO-DMEM-FBS dispersion as described in the

10   Experimental section. The representative images of DNA damage obtained using the alkaline

11   comet assay of A549 cells exposed to ITO NPs are shown in Fig. 9. The ITO NPs induced

12   DNA strand breakages at 24 h. The lesions were permanently induced in cells exposed sample

13   B and sample C. The tail length of cells exposed to sample B and sample C increased 2.9- and

14   2.6-fold, respectively, compared to that of the untreated cells at 72 h. Conversely, when cells

15   were exposed to sample A, the length of comet tail decreased at 72 h.

16   The influence of ITO NPs on cell proliferation was examined using a clonogenic

17   assay (Fig. 10). The results of the clonogenic assay showed a concentration-dependent

18   decrease in colony-forming ability for two out of the three samples, i.e., sample B and sample

19   C. Incubation   with   sample   A   induced   a   statistically   significant   but   not   a

19

1   concentration-dependent inhibition of colony formation.

2

1   **Discussion**

2   Owing to the wide-spread use of ITO, it has become important to investigate the

3   possible adverse effects of ITO on human health and the environment. Since inhalation is a

4   significant route of exposure to ITO NPs, we examined the effect of ITO NPs on human lung

5   adenocarcinoma A549 cells, a model cell line for lung exposure. In this study, we investigated

6   the possible hazards of exposure to a range of ITO NPs with respect to cytotoxicity, induction

7   of oxidative stress, and DNA damage. The results of the cytotoxicity assays (WST-1 and LDH

8   assays) showed that the ITO NPs had low acute toxic effects on the A549 cells. The results of

9   the genotoxicity assay (comet assay) showed severe DNA strand breaks in the A549 cells

10   exposed to ITO NPs.

11   Cellular uptake of NPs is an important process that governs the cellular effects of

12   NPs. Cellular uptake of NPs mediated by different processes, including phagocytosis[27] and

13   endocytosis.[28,29] Several studies have indicated that, since the uptake of ITO particles via

14   macrophages is more efficient than that via epithelial cells, ITO particles are cytotoxic to

15   macrophages but not to epithelial cells.[9,10] However, our results show that the ITO NPs are

16   internalized within the intracellular space in the A549 cells (Fig. 6A) and induce intracellular

17   oxidative stress (Fig. 7). The differences in the results of previous studies and those of our

18   study may be due to the size of ITO particles. Previous toxicological studies were performed

19   using micro-scale ITO particles. In this study, we examined the cellular effects of ITO NPs,

21

1   which consist of nanoscale primary and secondary particles. Recent studies showed that the

2   cellular uptake of particles is size-dependent.[30,31] The intracellular concentrations of indium

3   were correlated with extracellular ITO concentrations (Fig. 6B). Moreover, the sizes of

4   secondary particles in sample B and sample C were smaller than those of the particles in

5   sample A, and thus sample B and sample C showed higher genotoxicity (Fig. 9). Therefore,

6   although the mechanism underlying the cellular uptake of ITO NPs is unclear, our results

7   showed that ITO NPs were taken up by the A549 cells, and they induced oxidative stress and

8   DNA damage.

9   The stability of the NP dispersion medium is important for *in vitro* evaluation of

10 NPs.[21,22] While smaller particles reach the cells by diffusion, larger particles reach by gravity

11 sedimentation. Thus, the concentration of the dispersion is not the concentration to which the

12 cell is exposed. In this study, the secondary ITO particles of in DMEM-FBS were very stable

13 (Fig. 3). Neither the particle size nor the light scattering intensity changed during the

14 experiment period. Therefore, the cellular responses evaluated in this study were not

15 influenced by large sedimentation, and the concentrations of ITO in the dispersion accurately

16 reflected the concentration of cellular exposure.

17   The release of metal ions is an important factor associated with the cytotoxicity of

18 metal oxide NPs.[15,32] For example, high cytotoxicity of CuO NPs is associated with the

19 release of copper ions from the internalized CuO NPs.[33] Moreover, while NiO and chromium

1 oxide (Cr_2O_3) NPs easily dissolve in the culture medium and induce marked oxidative stress,

2 their micro-scale particles do not dissolve in the medium.[15,34] ITO is insoluble at

3 physiological pH.[6] The ITO NPs used in this study did not release large amounts of indium

4 ion into the medium (Table 1). Thus, the cellular effects of ITO NPs are different from those

5 of degradable NPs such as CuO, NiO and Cr_2O_3. Previous studies have shown that the

6 adsorption ability influences the cellular effects of NPs, especially of the insoluble NPs.[35,36]

7 TiO_2, cerium oxide (CeO_2) and zinc oxide (ZnO) NPs have strong adsorption ability; thus,

8 these NPs adsorbed the proteins and calcium ions in the medium thereby depleting the

9 medium of these components. In this study, we prepared a stable ITO-DMEM-FBS dispersion

10 by using pre-adsorption and centrifugation method to prevent indirect toxicity by medium

11 depletion. The concentrations of the medium components such as sodium, calcium, and

12 phosphorus were not affected by the ITO NPs (Table 1). Therefore, the cellular effects

13 observed in our study were not influenced by medium depletion and were induced by the ITO

14 NPs alone.

15 Several studies have indicated that oxidative stress is frequently associated with NP

16 toxicity.[17,37] Oxidative stress induces a wide variety of physiological and cellular effects

17 including inflammation, DNA damage, and apoptosis.[23,24,25,26] Moreover, studies on

18 indium-containing compounds suggest that the production of ROS is the mechanism

19 underlying NP-induced toxicity.[9,38] We hypothesized that the cellular effects induced by ITO

1   NPs in our studies were also mediated by the generation of oxidative stress. However, the

2   results of the intracellular ROS level (Fig. 7) were not correlated with the generation of DNA

3   damage (Fig. 9). Although the reasons of the difference remain unclear, the expression of

4   *HMOX-1* may be attributed to this differences. The induction of *HMOX-1* provides protection

5   against oxidative stress, chemical toxicity and certain chronic disorders.[39] In sample B and

6   sample C, the generation of ROS was observed at an early stages (< 24 h) (Fig. 7B and C), but

7   the expression of *HMOX-1* was not induced at 24 h (Fig. 8B and C). In sample A, the

8   intracellular ROS level increased after long-term exposure (72 h) (Fig. 7A). The expression of

9   *HMOX-1* was sufficiently induced at this time point (Fig. 8A). Therefore, our results suggest

10   that ITO NPs induced genotoxicity by generation of ROS, which were not neutralized by

11   antioxidant-related enzymes. Besides the generation of ROS, the impairment of the DNA

12   repair enzyme may be involved in the accumulation of DNA damage. Previously, it was

13   demonstrated that the activity of DNA repair enzyme was inhibited by the exposure of

14   indium.[40] Martin *et al*. demonstrated that the addition of hydroxyurea (HU) and cytosine

15   arabinoside (Ara-C) enhanced the sensitivity of the comet assay in response to certain

16   genotoxins.[41] This analysis is based on the accumulation of single-strand breaks during the

17   DNA repair process. Thus, the inhibitory effect of ITO NPs on the excision repair process will

18   be elucidated by performing the comet assay in the presence of HU and Ara-C.

19   In this study, we investigated that cellular influences of ITO NPs and demonstrated

24

that ITO NPs have genotoxic effect on A549 cells. However, it is difficult to predict biological

responses to low-dose environmental exposures based on adverse effects induced by

high-dose concentrations. Recently, it was reported that Fourier-transform infrared

spectroscopy is useful to detect alterations induced by carbon NPs at environmentally relevant

concentrations.[42,43] Such studies will provide some insights into ITO-induced toxic

mechanisms at environmentally relevant exposure levels. Further, the precise cellular

signaling mechanisms underlying NP-mediated toxicity remains unclear. Therefore, further

studies are required to clarify the mechanisms underlying the cellular uptake and genotoxicity

of ITO NPs.

1 **Conclusion**

2   Our results showed that, similar to other NPs, ITO NPs were taken up into the

3   cultured human lung adenocarcinoma A549 cells. Furthermore, the ITO NPs possess potential

4   for inducing DNA damage in lung epithelial cells, which may be mediated through oxidative

5   stress. To our knowledge, this is the first study to report the toxic effects of ITO NPs on

6   human lung epithelial cells. The cellular effects of ITO NPs evaluated by *in vitro* experiments

7   may provide some insights into indium-induced lung disease.

8

1   **Acknowledgements**

2   The authors are thankful to Ms. Emiko Kobayashi, Bioproduction Research Institute,

3   National Institute of Advanced Industrial Science and Technology (AIST) for carrying out the

4   transmission electron microscopy.

5

6   **Conflict of interest**

7   The authors declare that there are no conflicts of interest.

8

1 **References**

2 1. U. Betz, M. Kharrazi Olsson, J. Marthy, M. F. Escolá and F. Atamny, *Surf. Coat. Technol.*

3 2006, **200**, 5751.

4 2. V. Fthenakis, *Renew. Sustain. Energy Rev.*, 2009, **13**, 2746.

5 3. T. Homma, T. Ueno, K. Sekizawa, A. Tanaka and M. Hirata, *J. Occup. Health*, 2003, **45**,

6 137-139.

7 4. S. Homma, A. Miyamoto, S. Sakamoto, K. Kishi, N. Motoi and K. Yoshimura, *Eur.*

8 *Respir. J.*, 2005, **25**, 200.

9 5. T. Chonan, O. Taguchi and K. Omae, *Eur. Respir. J.*, 2007, **29**, 317.

10 6. T. Hamaguchi, K. Omae, T. Takebayashi, Y. Kikuchi, N. Yoshioka, Y. Nishiwaki, A.

11 Tanaka, M. Hirata, O. Taguchi and T. Chonan, *Occup. Environ. Med.*, 2008, **65**, 51.

12 7. M. Nakano, K. Omae, A. Tanaka, M. Hirata, T. Michikawa, Y. Kikuchi, N. Yoshioka, Y.

13 Nishiwaki and T. Chonan, *J. Occup. Health*, 2009, **51**, 513.

14 8. K. J. Cummings, W. E. Donat, D. B. Ettensohn, V. L. Roggli, P. Ingram and K. Kreiss,

15 *Am. J. Respir. Crit. Care Med.*, 2010, **181**, 458.

16 9. D. Lison, J. Laloy, I. Corazzari, J. Muller, V. Rabolli, N. Panin, F. Huaux, I. Fenoglio and

17 B. Fubini, *Toxicol. Sci.*, 2009, **108**, 472.

18 10. W. M. Gwinn, W. Qu, C. J. Shines, R. W. Bousquet, G. J. Taylor, M. P. Waalkes and D. L.

19 Morgan, *Toxicol. Sci.*, 2013, **135**, 414.

1   11. Ministry of Health, Labour and Welfare, 2010, Japan, No. 1222-3.

2   12. G. Oberdörster, E. Oberdörster and J. Oberdörster, *Environ. Health Perspect.*, 2005, **113**,

3   823.

4   13. S. Q. Li, R. R. Zhu, H. Zhu, X. Y. Xue, X. Y. Sun, S. D. Yao and S. L. Wang, *Food Chem.*

5   *Toxicol.*, 2008, **46**, 3626.

6   14. H. L. Karlsson, J. Gustafsson, P. Cronholm and L. Möller, *Toxicol. Lett.*, 2009, **188**, 112.

7   15. M. Horie, K. Nishio, K. Fujita, H. Kato, A. Nakamura, S. Kinugasa, S. Endoh, A.

8   Miyauchi, K. Yamamoto, H. Murayama, E. Niki, H. Iwahashi, Y. Yoshida and J.

9   Nakanishi, *Chem. Res. Toxicol.*, 2009, **22**, 1415.

10   16. M. Horie, H. Kato, K. Fujita, S. Endoh and H. Iwahashi, *Chem. Res. Toxicol.*, 2012, **25**,

11   605.

12   17. A. Nel, T. Xia, L. Mädler and N. Li, *Science*, 2006, **311**, 622.

13   18. M. Horie, K. Nishio, K. Fujita, H. Kato, S. Endoh, M. Suzuki, A. Nakamura, A. Miyauchi,

14   S. Kinugasa, K. Yamamoto, H. Iwahashi, H. Murayama, E. Niki and Y. Yoshida, *Toxicol.*

15   *In Vitro*, 2010, **24**, 1629.

16   19. E. Herzog, A. Casey, F. M. Lyng, G. Chambers, H. J. Byrne and M. Davoren, *Toxicol.*

17   *Lett.*, 2007, **174**, 49.

18   20. N. A. Franken, H. M. Rodermond, J. Stap, J. Haveman and C. van Bree, *Nat. Protoc.*,

19   2006, **1**, 2315.

21. J. G. Teeguarden, P. M. Hinderliter, G. Orr, B. D. Thrall and J. G. Pounds, *Toxicol. Sci.*, 2007, **95**, 300.

22. H. Kato, M. Suzuki, K. Fujita, M. Horie, S. Endoh, Y. Yoshida, H. Iwahashi, K. Takahashi, A. Nakamura and S. Kinugasa, *Toxicol. In Vitro.*, 2009, **23**, 927.

23. P. V. AshaRani, G. Low Kah Mun, M. P. Hande and S. Valiyaveettil, *ASC Nano*, 2009, **3**, 279.

24. Y. H. Hsin, C. F. Chen, S. Huang, T. S. Shin, P. S. Lai and P. J. Chueh, *Toxicol. Lett.*, 2008, **179**, 130.

25. J. Ahmad, M. Ahamed, M. J. Akhtar, S. A. Alrokayan, M. A. Siddiqui, J. Musarrat and A. A. Al-Khedhairy, *Toxicol. Appl. Pharmacol.*, 2012, **259**, 160.

26. I. Passagne, M. Morille, M. Rousset, I. Pujalte and B. L'Azou, *Toxicology*, 2012, **299**, 112.

27. R. Weissleder, H. C. Cheng, A. Bogdanova and A. Bogdanov, *J. Magn. Reson. Imaging*, 1997, **7**, 258.

28. U. Schoepf, E. Marecos, R. Melder, R. Jain and R. Weissleder, *BioTechniques*, 1998, **24**, 642.

29. A. Moore, J. P. Basilion, E. A. Chiocca and R. Weissleder, *Biochim. Biophys. Acta*, 1998, **1402**, 239.

30. F. Lu, S. H. Wu, Y. Hung and C. Y. Mou, *Small*, 2009, **5**, 1408.

1   31. P. O. Andersson, C. Lejon, B. Ekstrand-Hammarström, C. Akfur, L. Ahlinder, A. Bucht

2   and L. Österlund, *Small*, 2011, **7**, 514.

3   32. H. L. Karlsson, P. Cronholm, J. Gustafsson and L. Möller, *Chem. Res. Toxicol.*, 2008, **21**,

4   1726.

5   33. A. M. Studer, L. K. Limbach, L. Van Duc, F. Krumeich, E. K. Athanassiou, L. C. Gerber,

6   H. Moch and W. J. Stark, *Toxicol. Lett.*, 2010, **197**, 169.

7   34. M. Horie, K. Nishio, S. Endoh, H. Kato, K. Fujita, A. Miyauchi, A. Nakamura, S.

8   Kinugasa, K. Yamamoto, E. Niki, Y. Yoshida and H. Iwahashi, *Environ. Toxicol.*, 2013,

9   **28**, 61.

10   35. A. Casey, E. Herzog, F. M. Lyng, H. J. Byrne, G. Chambers and M. Davoren, *Toxicol.

11   Lett.*, 2008, **179**, 78.

12   36. M. Horie, K. Nishio, K. Fujita, S. Endoh, A. Miyauchi, Y. Saito, H. Iwahashi, K.

13   Yamomoto, H. Murayama, H. Nakano, N. Nanashima, E. Niki and Y. Yoshida, *Chem. Res.

14   Toxicol.*, 2009, **22**, 543.

15   37. Y. Ju-Nam and J. R. Lead, *Sci. Total Environ.*, 2008, **400**, 396.

16   38. H. H. Liu, C. Y. Chen, G. I. Chen, L. H. Lee and H. L. Chen, *Int. Arch. Occup. Environ.

17   Health*, 2012, **85**, 447.

18   39. A. K. Jaiswal, *Free Radic. Biol. Me*d., 2004, **11**, 549.

19   40. J. Bustamante, L. Dock, M. Vahter, B. Fowler, and S. Orrenius, *Toxicology,* 1997, **118,**

1   129.

2   41. F. L. Martin, K. J. Cole, M. H. Orme, P. L. Grover, D. H. Phillips, and S. Venitt, *Mutat.*

3   *Res.,* 1999, **445,** 21.

4   42. J. Li, R. Strong, J. Trevisan, S. W. Fogarty, N. J. Fullwood, K. C. Jones and F. L. Martin,

5   *Environ. Sci. Technol.,* 2013, **47**, 10005.

6   43. M. J. Riding, J. Trevisan, C. J. Hirschmugl, K. C. Jones, K. T. Semple and F. L. Martin,

7   *Environ. Int.,* 2012, **50,** 56.

8

1 **Table**

2 **Table 1** Characterization of the ITO−DMEM−FBS dispersion used in this study.

Sample	Primary particle size[a] (nm)	Specific surface area ($m^2 g^{-1}$)	Secondary particle size[b] (nm)	Metal oxide conc. ($\mu g\ mL^{-1}$)	Soluble metal conc. (μM)	Protein conc. (mg mL^{-1})	Salt composition of the dispersion		
							Na (mM)	Ca (mM)	P (μM)
A	30	30.8	d_l: 206.2 d_n: 106.3	In_2O_3: 720 SnO_2: 70	In: 0.03 Sn: 1.80	3.4	144.7	0.8	86.6
B	30	16.0	d_l: 128.1 d_n: 63.4	In_2O_3: 490 SnO_2: 50	In: 0.08 Sn: 0.14	5.1	155.3	1.3	69.6
C	30	15.3	d_l: 130.7 d_n: 57.3	In_2O_3: 480 SnO_2: 50	In: 0.07 Sn: 0.18	4.1	146.3	1.3	87.8
DMEM-FBS					In: 0.02 Sn: 0.00	3.4	170.6	1.9	66.1

3 [a]According to manufacture's data sheet

4 [b]d_l and d_n mean secondary particle size based on the light scattering intensity and particle

5 number, respectively.

6

1   **Figure legends**

2   **Fig. 1** Effects of un-fractionated indium tin oxide (ITO) NPs on cell viability and the cell

3   membrane. (A) Effect of ITO-DMEM-FBS dispersion on cell viability. A549 cells were

4   incubated with ITO-DMEM-FBS dispersions for 6 and 24 h. The cell viability was measured

5   using the WST-1 assay, and results were given as percent related to untreated controls. (B)

6   Effect of the ITO-DMEM-FBS dispersion on cell membrane damage. A549 cells were

7   incubated with ITO-DMEM-FBS dispersions for 6 and 24 h, and then, cell membrane damage

8   was determined by measuring the intracellular release of LDH. The method of calculating

9   cytotoxicity is described in the Experimental section. No significant differences were

10   observed between the untreated cells and ITO-exposed cells (Dunnett, analysis of variance

11   [ANOVA]).

12

13   **Fig.2** Size distributions of indium tin oxide (ITO) NPs. (A) Field-emission scanning electron

14   microscopy (FE-SEM) observation of ITO NPs in stable ITO-DMEM-FBS dispersions. (B)

15   Histograms of the particle size distribution of ITO NPs.

16

17   **Fig. 3** Stabilities of ITO-DMEM-FBS dispersions. (A) ITO secondary particle sizes (*d*) based

18   on particle number (open circles) and based on light scattering intensities (closed circles) were

19   measured using dynamic light scattering (DLS). (B) Light scattering intensities of ITO

1 secondary particles were measured by DLS. A plot of relative light scattering intensities of

2 ITO secondary particles versus time (day). The ITO-DMEM-FBS dispersions were prepared

3 at day 0.

4

5 **Fig. 4** Effect of stable ITO-DMEM-FBS dispersions on cell viability. A549 cells were

6 exposed to ITO-DMEM-FBS dispersions for 6, 24, and 72 h. The cell viability was measured

7 using the WST-1 assay, and the results were given as percent related to untreated controls. **

8 *P* < 0.01 (versus control, Dunnett, analysis of variance [ANOVA]).

9

10 **Fig. 5** Effect of stable ITO-DMEM-FBS dispersions on the cell membrane. A549 cells were

11 exposed to ITO-DMEM-FBS dispersions for 6, 24, and 72 h, and then, cell membrane

12 damage was determined by measuring the intracellular LDH release. The method of

13 calculating cytotoxicity is described in the Experimental section. No significant differences

14 were observed between untreated cells and ITO-exposed cells (Dunnett, analysis of variance

15 [ANOVA]).

16

17 **Fig. 6** Cellular uptake of indium tin oxide (ITO) NPs by A549 cells. (A) Examination of A549

18 cells incubated with the ITO-DMEM-FBS dispersions under the transmission electron

19 microscope. The A549 cells were incubated with the ITO-DMEM-FBS dispersions for 24 h.

35

1   Overview of the cell (left) and details of the part in white frames (right). The concentrations

2   of indium oxide (In$_2$O$_3$) in sample A, sample B, and sample C were 720, 490, and 480 μg

3   mL^{-1}, respectively. (B) Internal concentrations of indium obtained by inductively coupled

4   plasma mass spectrometry (ICP-MS). The A549 cells were incubated with the

5   ITO-DMEM-FBS dispersion for 24 h, and then, the intracellular concentrations of indium

6   were measured using ICP-MS.

7

8   **Fig. 7** The levels of intracellular reactive oxygen species (ROS) in A549 cells exposed

9   ITO-DMEM-FBS dispersions. Cells were exposed to stable ITO-DMEM-FBS dispersions for

10   6, 24, and 72 h; then, we measured the intracellular ROS levels with the DCFH method, using

11   a flow cytometer. The value of the DCF fluorescence standardized untreated control was 1. *

12   $P < 0.05$, ** $P < 0.01$ (versus control, Dunnett, analysis of variance [ANOVA]).

13

14   **Fig. 8** The mRNA transcript levels of *HMOX-1* in A549 cells exposed ITO-DMEM-FBS

15   dispersions. The cells were exposed to stable ITO-DMEM-FBS dispersions for 24 and 72 h,

16   and then, we measured the expression levels of *HMOX-1*using real-time PCR. Each transcript

17   level was normalized to corresponding *β-actin* value and presented as relative units compared

18   to untreated control. * $P < 0.05$, ** $P < 0.01$ (versus control, Dunnett, analysis of variance

19   [ANOVA]).

1

2   **Fig. 9** Effect of indium tin oxide (ITO) NPs on DNA integrity. A549 cells were exposed to

3   stable ITO-DMEM-FBS dispersions for 24 and 72 h. The tail length values of DNA were

4   obtained by analyzing at least 50 random comet images from each treatment. The left panels

5   are comet images of untreated and ITO-exposed cells at 24 and 72 h. ** $P < 0.01$ (versus

6   control, Dunnett, analysis of variance [ANOVA]).

7

8   **Fig. 10** Clonogenic assays of cells exposed to stable ITO-DMEM-FBS medium dispersions.

9   A549 cells were treated with stable ITO-DMEM-FBS dispersions. Cell proliferation was

10   measured using the clonogenic assay. After the cells were cultured with the ITO-DMEM-FBS

11   dispersion for 7 days, the number of colonies were counted. Cell proliferation was

12   standardized to the untreated cells (100%). ** $P < 0.01$ (versus control, Dunnett, analysis of

13   variance [ANOVA]).

14

(A) WST-1 assay

(B) LDH assay

Figure 1

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

(A) FE-SEM

(B) Size distribution
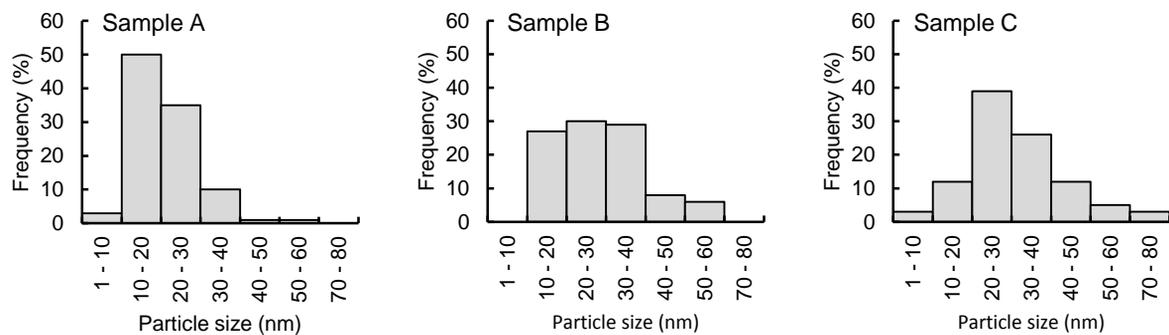
Figure 2

(A) Secondary particle size
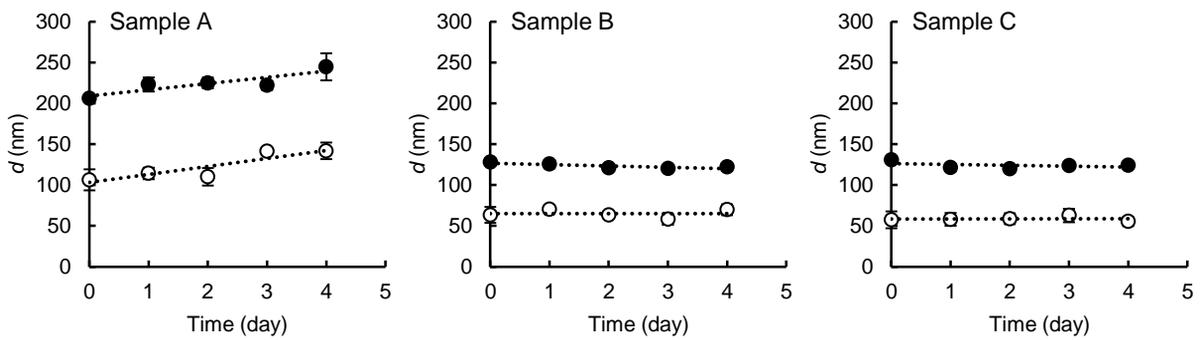
(B) Light scattering intensity

Figure 3

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

(A) Sample A

(B) Sample B

(C) Sample C

Metallomics Accepted Manuscript
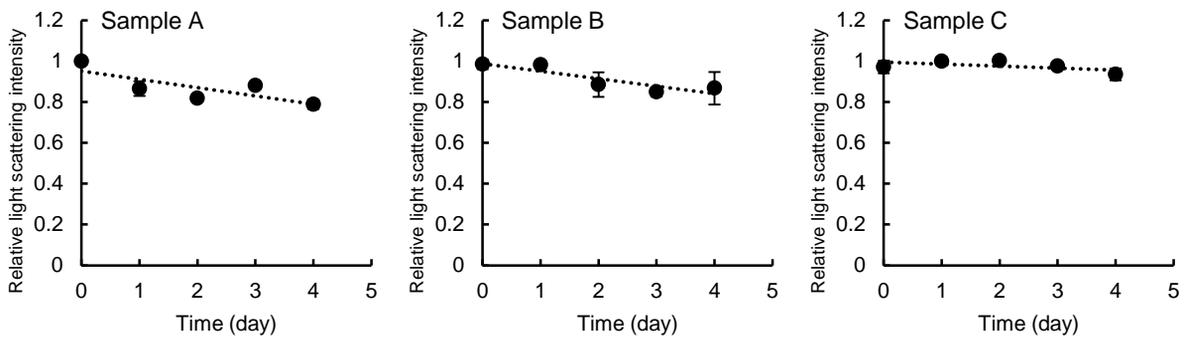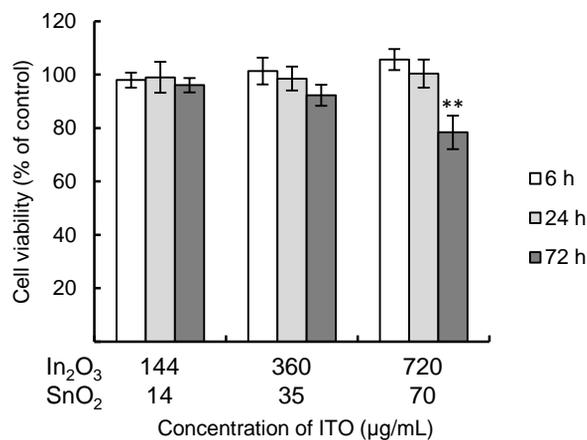
Figure 4
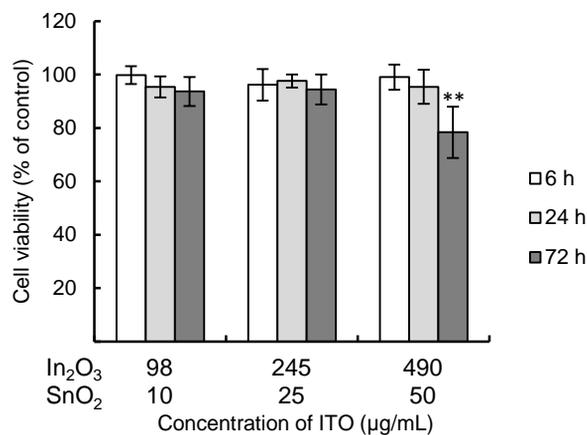
(A) Sample A
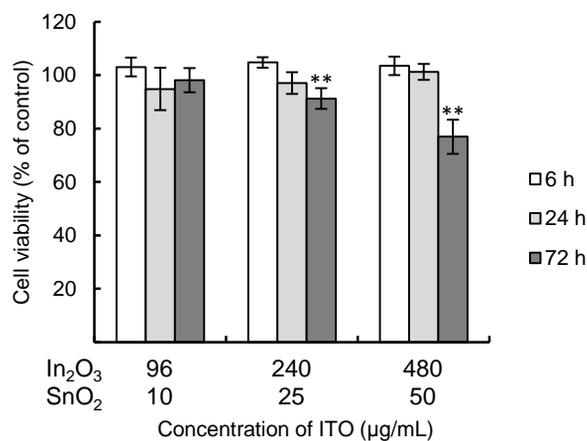
(B) Sample B
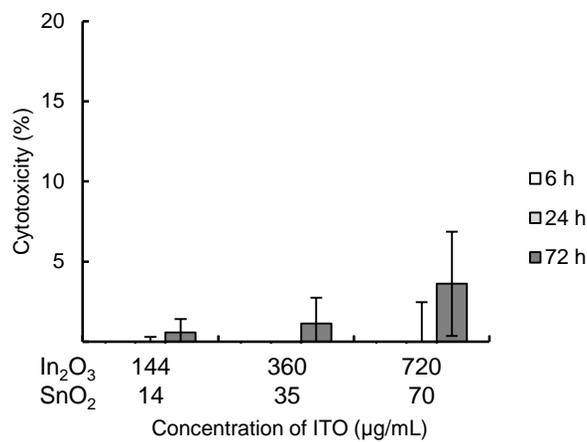
(C) Sample C
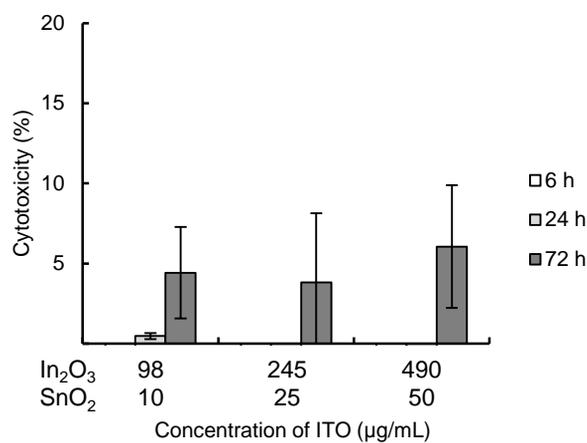
Figure 5
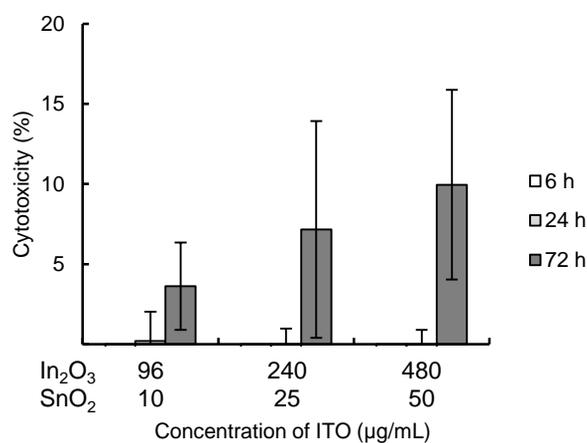
(A) TEM
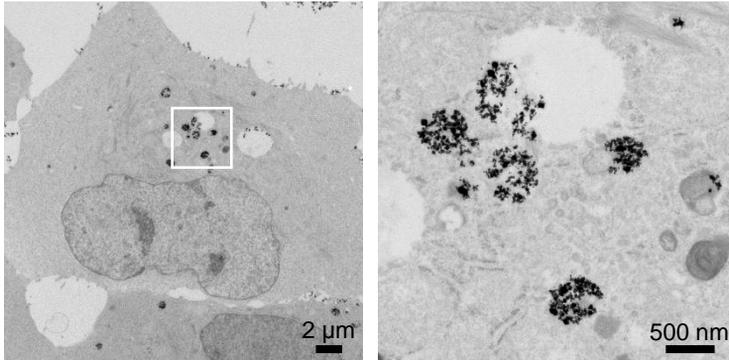
(B) Uptake of indium
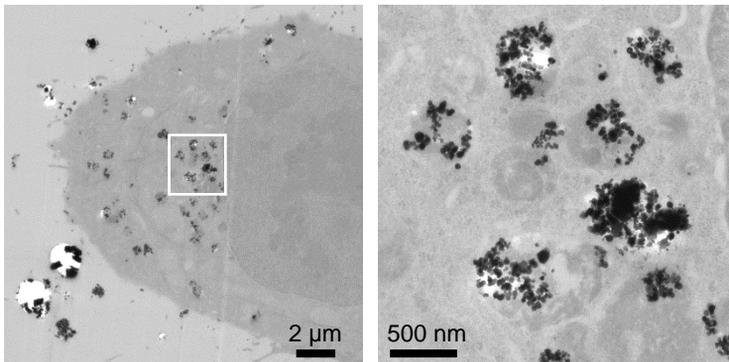
Sample A

Sample B

Sample C
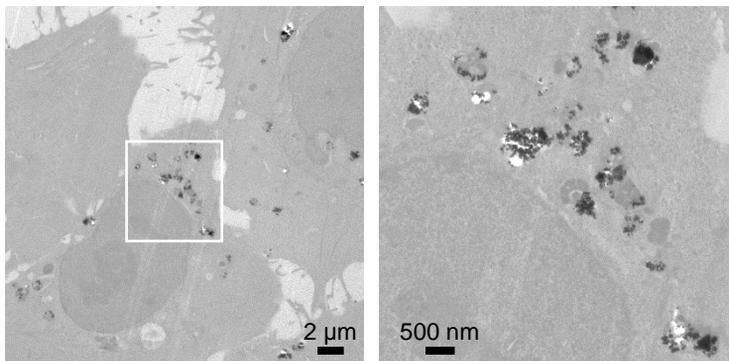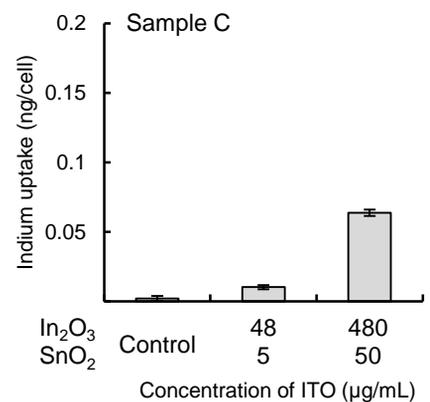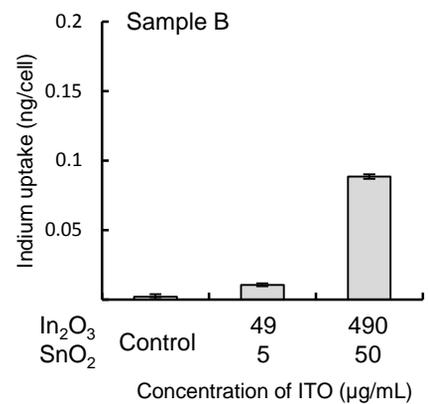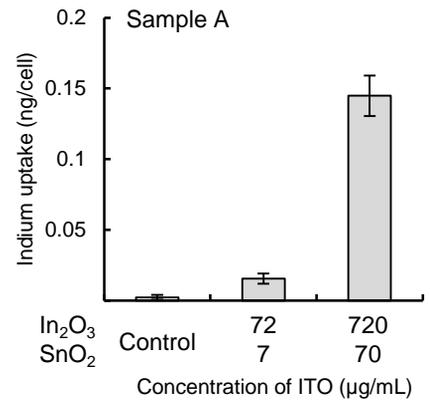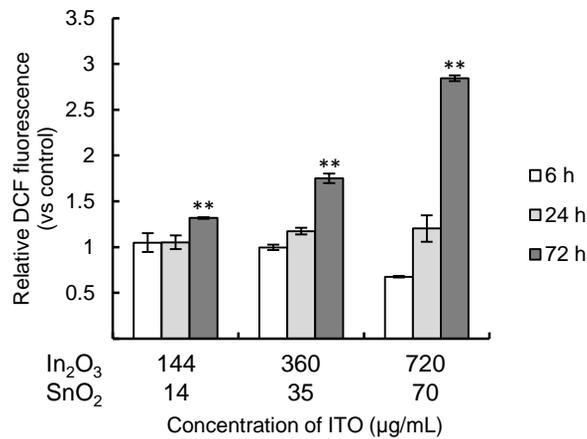
Figure 6
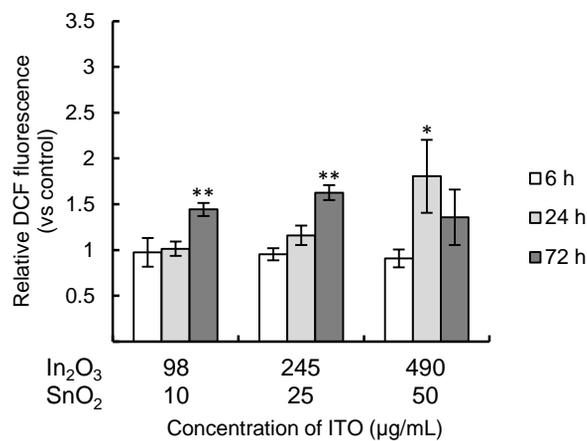
(A) Sample A
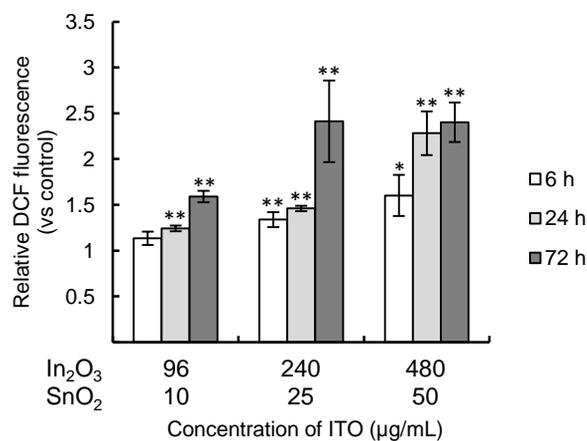
(B) Sample B

(C) Sample C

Figure 7

(A) Sample A

(B) Sample B

(C) Sample C

Figure 8

Figure 9

(A) Sample A

(B) Sample B

(C) Sample C

Figure 10