Table 1 Elemental concentrations determined by ICP-OES

Sample	Centrifugation condition		Al ppm (m/v)	Zn ppm (m/v)
	Force ($\times g$)	Time (min)	($\mu g\ mL^{-1}$)	($\mu g\ mL^{-1}$)
ZnO suspension[a]	None		0	22.0
ZnO suspension[b]	None		0	22.3
ZnO supernatant[c]	20 000	20	0	16.8
	20 000	60	0	16.9
	150 000	20	0	14.5
	150 000	60	0	14.5
ZnO supernatant[d]	150 000	60	0	14.7
ZnO supernatant[e]	20 000	20	0	25.1
Al-doped ZnO suspension[f]	None		<0.1	20.0
Al-doped ZnO supernatant[g]	20 000	60	0	12.8
ZnO suspension[h]	None		0	21.8
ZnO suspension[i]	None		0	21.6
ZnO supernatant[j]	150 000	60	0	14.3
ZnO supernatant[k]	150 000	60	0	14.5

[a] Prepared by dispersing 60 nm ZnO NPs (about 25 $\mu g\ mL^{-1}$) in medium with FBS. [b] Prepared by dispersing 60 nm ZnO NPs (about 25 $\mu g\ mL^{-1}$) in medium without FBS. [c] Collected after centrifugation of the ZnO (60 nm NPs) suspension (about 25 $\mu g\ mL^{-1}$) in medium with FBS. [d] Collected after centrifugation of the ZnO (60 nm NPs) suspension (about 25 $\mu g\ mL^{-1}$) in medium without FBS. [e] Collected after centrifugation of the ZnO (60 nm NPs) suspension (about 50 $\mu g\ mL^{-1}$) in medium with FBS. [f] Prepared by dispersing Al-doped ZnO NPs (50 nm NPs, about 25 $\mu g\ mL^{-1}$) in medium with FBS. [g] Collected after centrifugation of the Al-doped ZnO NP (50 nm NPs, about 25 $\mu g\ mL^{-1}$) suspension in medium with FBS. [h] Prepared by dispersing ~20 nm ZnO NPs (about 25 $\mu g\ mL^{-1}$) in medium with FBS. [i] Prepared by dispersing ~20 nm ZnO NPs (about 25 $\mu g\ mL^{-1}$) in medium without FBS. [j] Collected after centrifugation of the ZnO (20 nm NPs) suspension (about 25 $\mu g\ mL^{-1}$) in medium with FBS. [k] Collected after centrifugation of the ZnO (20 nm NPs) suspension (about 25 $\mu g\ mL^{-1}$) in medium without FBS.

the cytotoxicity of ZnO NP suspension as a function of the suspension concentration. The A549 cell viability (Fig. 3) suggests that there is no obvious toxic effect if the concentration of ZnO NP suspension is not higher than 25 $\mu g\ mL^{-1}$; the 25 $\mu g\ mL^{-1}$ suspension exhibited a toxic effect but not statistically important ($p < 0.05$). As shown in Fig. 4, the supernatant with an elemental Zn concentration of 25.1 ppm (Table 1), collected after the centrifugation of the ZnO NP suspension with a concentration of 50 $\mu g\ mL^{-1}$, had no obviously toxic effects on A549 cells during the exposure period of 24 h (Fig. 4a, d and f), *i.e.*, having a viability of about 95%. This observation is different from that of the 25 $\mu g\ mL^{-1}$ suspension (Fig. 3), in which possibly dissolved Zn^{2+} ions were present together with ZnO NPs. Furthermore, the observation is quite different from that of the 50 $\mu g\ mL^{-1}$ ZnO NP suspension that seriously affected the cell viability (Fig. 4c). If we assumed all the 25.1 ppm, *i.e.*, 25.1 $\mu g\ mL^{-1}$, Zn as the free Zn^{2+} ions, these Zn^{2+} ions could cause about 5% cell death. Considering the exposure of the 50 $\mu g\ mL^{-1}$ ZnO NP suspension (25.1 $\mu g\ mL^{-1}$ was assumed as free Zn^{2+}) caused about 50% cell death, we found that about 90% of the dead cells were due to the other 24.9 $\mu g\ mL^{-1}$ ZnO NPs. The analysis also indicates that the cytotoxicity was not a function of the concentration of fractions of the Zn element. Although the question is not so simple, this hypothesis reflects that the maximum toxic contribution of released Zn^{2+} ions to the cells was only about 10%. Thus, the above results suggest that free Zn cation in the cell culture medium had a negligible contribution to the cytotoxicity as compared to the non-dissolved ZnO NPs, which is in line with the other reports.[17,28] On the other hand, the addition of FBS to the medium had a remarkable influence on cell viability due to the growth factors contained in FBS (Fig. 4b and e). However, as compared to the toxic effect of ZnO NPs, the FBS function became negligible (Fig. 4c–e). Although Zn^{2+} ions are known to interact with proteins and serum albumin transports Zn^{2+} ions, the reasons for the negligible effect of the FBS might be absence of sufficient free Zn^{2+} ions and/or complicated interaction of the ZnO NPs in the biological environment.[9,10]

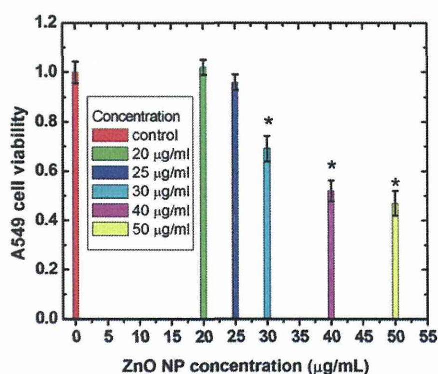

Fig. 3 A549 cell viability as a function with concentration of ZnO NP suspension of DMEM medium with FBS for 24 h exposure.

We previously reported that Al-doped ZnO NPs are more toxic than non-doped ZnO NPs and attributed the cytotoxic effects of the Al dopant to the enhancement of electrical conduction of ZnO.[27] Here, we find that the elemental Zn concentration (12.8 ppm) of the Al-doped ZnO suspension

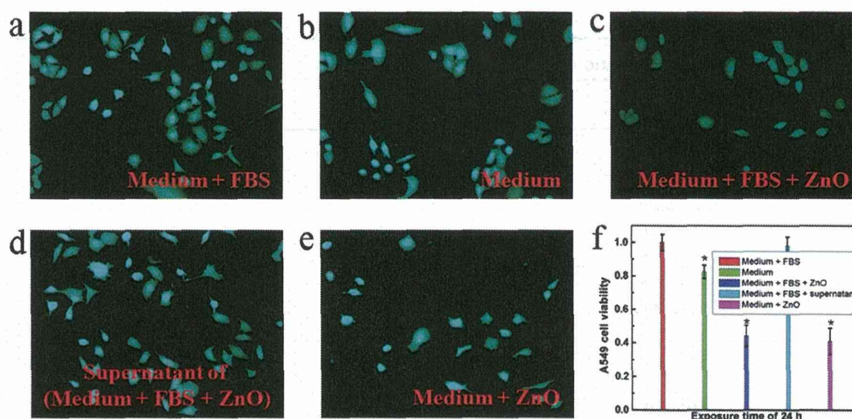

Fig. 4 Viability of A549 cells after 24 h exposure to ZnO NPs in different biological environments. (a)–(e) Live cell fluorescence images. (a) Control experiment, where A549 cells were exposed to medium supplemented with FBS but without ZnO NPs. (b) Control experiment, where A549 cells were exposed to medium without FBS and without ZnO NPs. (c) A549 cells exposed to ZnO NP suspension (50 μg mL^{-1}) in medium with FBS. (d) A549 cells exposed to the supernatant collected after centrifugation of the ZnO NP suspension (50 μg mL^{-1}) in medium with FBS. (e) A549 cells exposed to ZnO NPs suspension (50 μg mL^{-1}) in medium without FBS. (f) Statistical analysis of cell viability. *Denotes significance in comparison to control values ($p < 0.05$).

supernatant was lower than that of the non-doped ZnO supernatant (16.9 ppm) (Table 1). If we normalized a percentage of the total Zn concentration in the samples, the elemental Zn concentration of the Al-doped ZnO supernatant was about 13.3 ppm, remaining lower than that of non-doped ZnO supernatant. Together with a previous study,[27] this result suggests that the toxic effect of the Al-doped ZnO NPs and the non-doped ZnO NPs was not proportional to the concentration of elemental Zn in the samples, consistent with the above analysis. The independence of the cytotoxicity on the concentration of elemental Zn is in good agreement with the finding of Moos *et al.*,[17] that is, the cytotoxicity of ZnO NPs to RKO human colon carcinoma cells was not dependent on the Zn^{2+} concentration in the cell culture medium. Therefore, Zn ions in the medium are not the main cause of the observed cytotoxicity.[17,28,29] Also, there is no strong evidence whether the uptake of Zn^{2+} or ZnO NPs was the dominant pathway for internalizing zinc[16] or its toxic effect. Our result with the Al-doped ZnO NPs is in contrast to that of iron-doped ZnO NPs,[30] in which the reduced toxicity of iron-doped ZnO NPs, compared with ZnO NPs, was attributed to the lower solubility of the iron-doped ZnO NPs than ZnO NPs based on the analysis of Zn elemental concentration measured by ICP-MS. However, as discussed above, the concentration of free Zn^{2+} ion in supernatants could actually be lower than the data obtained by ICP-MS due to the difficulty to completely remove non-dissolved NPs. Thus, the assumption of elemental concentration as free ion concentration could overestimate the contribution of free metal ions to adverse biological effects.

If sufficient Zn^{2+} ions are present either outside or inside cells, it can certainly cause adverse effect on cells due to various mechanisms such as disruption of cellular Zn homeostasis. The question is how much ZnO NP can be dissolved in the cell culture medium. In general, the solubility of metal oxides is highly dependent on the pH of the solution,[15] where the oxides are dispersed, and the method used to disperse the oxides. ZnO and CuO is nearly insoluble in de-ionized water and in an extracellular solution,[15,19] but solubility increases in acidic environments[31] and in the presence of chelators.[19] Although there is still no direct and reliable technique for measuring the concentrations of free ions in a suspension,[32–34] we have demonstrated a protocol here for determining whether there are non-dissolved NPs in a collected supernatant, highlighting the need to establish relevant characterization techniques. Also, a simple comparison of toxicity between NPs and the solution of metal salts[14,21] such as $ZnCl_2$ and $ZnNO_3$ is not enough to elucidate the potential ill effects of NPs because of the variety of local biological environments, such as pH value, between NPs and salt solutions.[19]

The above analysis clarifies the contribution of free Zn^{2+} ions to the observed cytotoxicity and suggests that there are other dominant factors contributing to the cytotoxicity. One of the factors is the electronic properties[2,20,24,35] of nano-oxides. There are singular evidences showing the influence of the electronic properties of metal oxides[20,24] and carbon nanotubes[35] on toxicological potential[36] at cellular and whole animal levels since our work.[27] Furthermore, electronic properties have been recently developed as a paradigm for predicting the generation of oxidative stress and acute pulmonary inflammation associated with metal oxides.[20]

4 Conclusions

We found that it is difficult to completely remove ZnO NPs dispersed in a cell culture medium by centrifugation, leading to the overestimation of the concentration of free ions when determined by ICP-MS relevant techniques. We also showed that the cytotoxicity of ZnO NPs is not proportional to the concentration of Zn elemental concentration. The maximum toxic contribution of released free Zn^{2+} ions to the A549 cell lines was estimated to be about 10%. Our results indicate that other factors including electronic properties of the ZnO NPs, rather than the Zn concentration, play a dominant role in the

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cytotoxicity, and identification of the key physicochemical characteristics of NMs which govern the potential toxic effect remains a challenge.

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REVIEW

Biological Interpretation of the *In Vitro* Assessment of Nanotoxicity

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Synopsis
Even though nanomaterials are used as constituents of many manufactured articles, there are no unified criteria for evaluating their safety or toxicity. Even the same nanomaterial can cause different sensitivities in different cell types. In addition, differences in physiological conditions can cause varied sensitivity of the same cell type to a specific nanomaterial. Generally, nanomaterials form aggregates or agglomerates in the culture medium, resulting in a range of size variations. Therefore, the response of each cell differs depending on the amount of nanomaterial it absorbs. Furthermore, it is also necessary to consider the effects when a nanomaterial releases metal ions. The most complicated scenario for the *in vitro* evaluation of a nanomaterial is that it affects growth, apoptosis and necrosis collectively. Nanomaterial toxicity arises from generation of reactive oxygen species (ROS) inside the cell. However, many types of cells possess ROS elimination systems. In cells that have the ability to completely remove ROS, cytotoxicity is normally not observed even after exposure to a toxic nanomaterial. However, for cells that are not able to cope with ROS, cytotoxicity occurs. A number of factors determine which specific molecular species of intracellular ROS quenchers or scavengers act inside cells, including cell type, cellular physiological state and the type and amount of nanomaterial present. In this review, we discuss the biological interpretation of nanomaterial toxicity evaluation data.

Key words: *nanotoxicity, assessment, released ions, reactive oxygen species*

Introduction

Nanotechnology has become an essential underlying technology in the generation of new information and communications, the environment, energy and medical industries. In particular, nanomaterials, which are produced by nanotechnology and possess novel features that are not displayed by conventional bulk materials, are currently utilized in the production of widely available nano-derived products such as sporting goods, tires, anti-bacterial fabrics, anti-UV products, cosmetics, electronic appliances, and others. In addition, extensive research has been carried out towards the utilization of nanomaterials for biomedical applications such as diagnosis, imaging, drug delivery, and others, with many products already being used in clinical applications or in the pre-clinical stage of development [1]. Due to their wide-ranging applications, nanomaterials are expected to be used more widely in the future. It is noteworthy, however, that the novelty of nanomaterials also contributes to their unidentified effects to human health and the environment. The impact of nanomaterials on human health is not fully understood. In many cases, therefore, safety concerns outweigh the benefits of nanotechnology [2-7], which has resulted in a slowdown in the use of nanotechnology for industrial applications.

Currently, because there are no formal guidelines for the evaluation of safety and toxicity of nanomaterials, each researcher or manufacturer performs such evaluations by employing diverse methods. This has resulted, for example, in differing results that have been reported on the nanomaterial toxicity of carbon nanotubes (CNTs) or TiO_2 nanoparticles [8-12]. Although it is believed that these differences are mainly caused by insufficient characterization of the physicochemical properties of the nanomaterials, differences in evaluation method and interpretation of results may also give rise to such variation. In this review, we describe the biological interpretation and problems related to nanomaterial toxicity evaluation based on cell activity or viability, and ROS as the main cause of toxicity.

Cytotoxicity Evaluation Method

The cytotoxicity evaluation method can be divided into measurements of the activity of certain cellular enzymes and of dead cells. For the former measure, the 3-(4,5-dimetylthial-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is the most widely used. The MTT assay is based on the correlation between the number of viable cells and the level of formazan dye that is produced by degradation of tetrazolium salt via succinate-tetrazolium reductase that is present in the mitochondrial respiratory chain [13-15]. However, in the presence of toxic materials, the value obtained by the MTT assay does not necessarily correlate with the number of viable cells. When the amount of formazan formed in the MTT assay in the presence of a toxic material is reduced by 50% compared to control cells, the succinate-tetrazolium reductase activity of the entire cell population is reduced by 50% on average. It is not known whether the actual number of living cells is also reduced by 50% or not. Generally, the value obtained from enzyme activity measurements such as the MTT assay is expressed as cell viability. On the other hand, the trypan blue [16], propidium iodide (PI) staining [17-20], EthD-1 staining and lactate dehydrogenase (LDH) activity assay [21, 22] methods evaluate cytotoxicity by detecting or measuring non-living cells. Trypan blue, propidium iodide and EthD-1 are assay methods that utilize the incorporation of dyes into cells, which is facilitated by cell membrane damage. Because the values obtained by these methods correlate with the number of non-viable cells, it is possible to determine the ratio between the number of viable and non-viable cells. Although mitochondrial damage can reduce the amount of formazan produced, if the cells are viable, then the ratio of the number of viable to non-viable cells will be greater than the relative value of cell viability.

Interpretation of toxicity evaluation

The evaluation of cytotoxicity involves many complex factors. As an example, when comparing cultured cells exposed to a toxic material for a certain period of time with an unexposed control, one may observe a 50% reduction in the number of viable cells in the exposed group, but there are several possible ways to interpret this result. First, the toxic material may not have induced cell death but rather slowed down cell proliferation (growth inhibition). Second, the toxic material may have caused the death of 50% of the cell population. And third, the toxic material may have induced both cell death and growth inhibition.

Theoretically, when cell exposure to a toxic material is uniform, just as with water, and the physiological state of all cells is the same, each cell should generate the same response. Therefore, a 50% reduction in the number of viable cells in the presence of a water-soluble toxic material should be interpreted as a 50% average reduction in growth rate. This interpretation is possible if the toxic nanomaterial is uniformly dispersed in the culture medium as a water-soluble substance and behaves uniformly in all cells.

In the case of a toxic material that behaves uniformly in all cells, the next item to consider is what meaning, for example, a 50% reduction in the number of viable cells carries. This number depends on the cultivation time in the presence of the toxic material. For example, cells seeded in the absence of a toxic material at an initial density of 5,000 cells/cm^2 may expand to 20,000 and 80,000 cells/cm^2 in 24 and 48 hours, respectively (with a doubling time in this culture condition of 12 hours), but may reach only 10,000 and 20,000 cells/cm^2, respectively, in the presence of the toxic material due to a growth

inhibition effect (with a doubling time of 24 hours). In this case, viable cell count was reduced by 50% and 75% at the 24-hour and 48-hour incubation period, respectively. In other words, the degree of toxicity changes with the incubation time. This phenomenon is not caused by an increase in toxicity over time but rather because the doubling time became two times slower in the presence of the toxic material. The correct interpretation should be that the toxic material lowered the cell growth rate by 50%. With this interpretation, the level of toxicity remains constant and does not change with time.

As described above, theoretically, all cells are uniformly affected in the case of a toxic material that is water-soluble or homogenously dispersed. However, many nanomaterials form aggregates or agglomerates of various sizes in the culture medium. This means that although a material's primary size may be uniform, its hydrodynamic size may not be (Figure 1). Cells that absorb large sized aggregates and those that absorb small size aggregates retain different intracellular levels of the nanomaterials, which can cause differences in cellular response. Because cells die after absorbing high levels of nanomaterials, while those that absorb low levels survive, the growth rate of an exposed culture will appear to be lower. When the number of viable cells is reduced by 50% due to nanomaterial toxicity, the cause may stem from two factors: cell death or a decrease in growth rate.

Furthermore, nanomaterial-induced cell death can be caused either by apoptosis or necrosis. Apoptosis is programmed cell death that follows the activation of certain signal transduction pathways, and can be considered as a reaction that occurs when a cell determines that it is not able to cope with the toxicity of a nanomaterial. In other words, the cell determines that death is more advantageous than survival. In contrast, necrosis is unregulated cell death caused by damage to various cellular functions by the nanomaterial, leading to a state where metabolic activity drops to zero. Thus, nanomaterials that are not homogenous in the culture medium can impact cells in three complex ways: growth inhibition, apoptosis and necrosis.

Hanagata *et al.* [23] reported that copper oxide nanoparticles (CuO NPs) can cause growth inhibition, apoptosis and necrosis of A549 human lung epithelial cells. In this study,

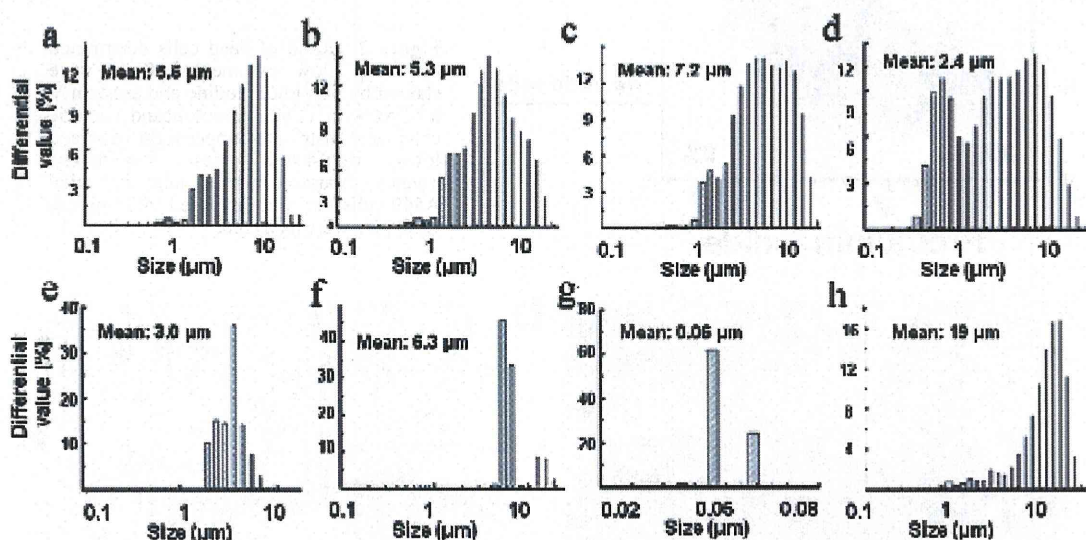

Figure 1 Hydrodynamic size distribution of nano-oxides in cell culture medium with concentration of ~ 50 µg/mL. **(a)** ZnO (~ 60 nm) NPs. **(b)** ZnO (~20 nm) NPs. **(c)** Al-doped ZnO (~ 50 nm) NPs. **(d)** CeO (~ 20 nm) NPs. **(e)** Porous SiO_2 (~ 60 nm). **(f)** non-porous SiO_2 (~ 15 nm). **(g)** Al_2O_3 (~ 15 nm) NPs. **(h)** Al_2O_3 NW. **(i)** CuO NPs. **(j)** Al-doped CuO NPs. The nano-oxides exhibited large size distribution in the cell culture medium and the mean size is much larger than the primary size measured in dry condition.

CuO NPs with an average size of 50 nm added to the culture medium at a concentration of 25μg/mL formed aggregates with an average hydrodynamic size of 300 nm. After culturing for 48 hours followed by exposure to 25μg/mL CuO NPs for 24-hours, viable cell count dropped to 34% of the control level. This reduction was found to be caused by a combination of growth arrest, apoptosis and necrosis [23]. About 18% of the cell population that was exposed to the CuO NPs had experienced cell death, with apoptosis and necrosis accounting for 9% each (Figure 2). Most of the other cells had arrested in the G1 or G2 phase of the cell cycle. After separating viable and non-viable cells followed by transmission electron microscope (TEM) observation of the CuO NPs absorbed by the cells, results clearly showed a higher level of CuO NPs in the non-viable cells vs. viable cells (Figure 3), indicating that the amount of absorbed nanomaterial is one of the factors that determines cellular fate.

Evaluation on the effect of metal ion release

Nanomaterial toxicity measurements become complex when the materials contain metal ions such as Zn, Cu or Ag due to the release by these metal or metal-oxide nanomaterials of metal ions into the medium. It has been reported that the release of metal ions from nanomaterials containing Zn, Cu or Ag to the medium occurs within as little as a few hours to 24 hours [23, 24]. It is believed that if these nanomaterials are absorbed quickly into the cells, the effects of ion release are minimal. However, cells are normally affected by both the ions released by the nanoparticles prior to their absorption and the absorbed nanoparticles themselves. The cyto-

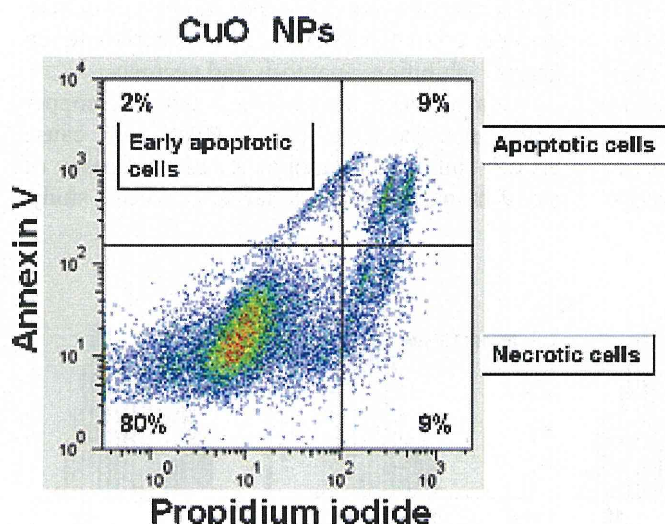

Figure 2 Ratio of dead cells determined by using flow cytometery. Cells were stained by propidium iodide and annexin V labeled with FITC. Apoptoticand necrotic cells were distributed upper right-hand and lower right-hand areas, respectively. Twenty thousand human lung epithelial A549 cells that were exposed to 25 μg/mL CuO NPs were analyzed.

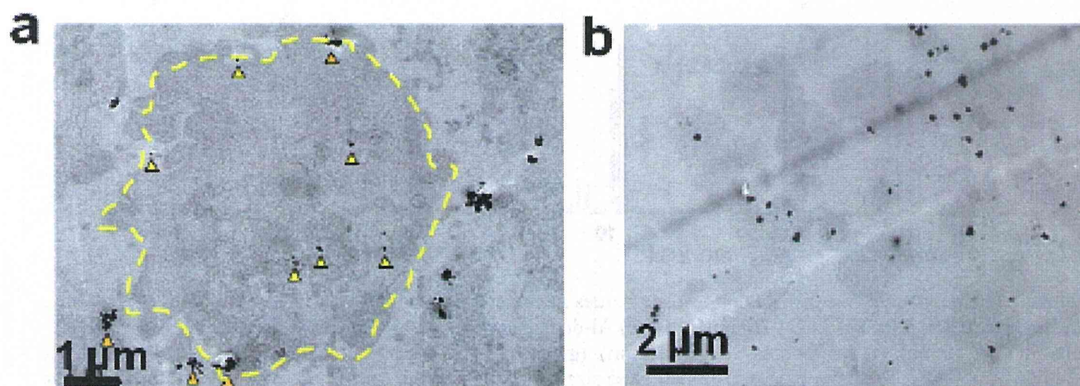

Figure 3 Internalized CuO NPs observed by transmission electron microscope. **(a)** Internalized CuO NPs (arrowhead) in living A549 cell. Dotted line indicates outline of cell. **(b)** Internalized CuO NPs in dead cell. Dead cell detached from culture tissue were observed.

toxicity of silver nanoparticles (Ag NPs) was mainly caused by Ag ions released into the medium [25]. In contrast, the toxicity of CuO NPs due to released ions was not as significant [24, 26]. Hanagata *et al.* [23] investigated the effects of Cu ions released from CuO NPs on A549 cells. CuO NPs at a concentration of 25μg/mL were added to Dulbecco modified Eagle's medium (DMEM), followed by incubation of the cells at 37°C. After 24 hours, the culture was centrifuged at 150,000 g for 60 mins and the Cu concentration in the supernatant was determined as 13μg/mL. CuO NPs were not detected when the supernatant was analyzed by a laser diffraction particle size analyzer (LDS). Further observation of the supernatant using transmission electron microscopy (TEM) and energy dispersive spectroscopy (EDS) also confirmed the absence of CuO NPs. The measured Cu present in the supernatant was therefore composed of Cu ions that had been released from the CuO NPs. Cells grown in this supernatant for 24 hours had a viable cell count of about 80% of control. Because cells grown in a medium containing 25μg/ml CuO NPs for 24 hours had a viable cell count of about 35% of control, it was estimated that the Cu ions that were released from CuO-NPs contributed a maximum of 30% towards the toxicity of CuO-NPs (= (100-80)/(100-35)). Regarding the toxicity of ZnO NPs, there are conflicting reports on the effect of Zn ions released into the culture medium. Deng *et al.* [27] reported a profound effect, whereas Lin *et al.* [28] did not. Sasidharan *et al.* [29] showed that the amount of Zn ions released from ZnO NPs depends on the pH of the medium. The release of Zn ions at pH 5.0 was significantly higher than at pH 7.0. Thus, the release of Zn ions from ZnO NPs is affected by the extracellular environment, and the amount of released Zn ions further contributes to the toxicity of ZnO NPs

Nanomaterial toxicity and reactive oxygen species generation

Much research has shown that ROS are the main contributor to the toxicity of nanomaterials that are absorbed into the cell. In addition, for CuO NPs and ZnO NPs, the released ions can also generate ROS. Therefore, ROS generation itself can likely be used as an indication of nanomaterial toxicity.

The mechanism of ROS generation by nanomaterials absorbed intracellularly differs according to the chemical composition of the nanomaterials. For nanomaterials that contain transition metals such as iron, ROS generation has been suggested to occur through the Fenton reaction [30]. On the other hand, ROS generation by ZnO NPs was proposed to occur at the surface of the ZnO NPs as a result of continuous oxidation-reduction reactions caused by amino acid-Zn^{2+}/Zn^{+} redox properties, e.g., glycine-Zn^{2+}/Zn^{+} [28, 31]. Xia *et al.* [32] showed that ROS generation by ZnO NPs occurs in endosomes. Furthermore, ROS generation by nanomaterials has also been shown to arise via the change in mitochondrial function resulting from nanomaterials absorbed by the mitochondria [33, 34]. Also, CNTs absorbed by the cell have been reported to cause ROS generation [35-37], although the mechanism for this is not well understood. A main cause of ROS generation by CNTs was also reported to be impurities from the CNT manufacturing processes [38, 39].

Ostrovsky *et al.* [40] reported the existence of a correlation between nanomaterial toxicity and ROS generation. They found that ZnO NPs are toxic to human glioma cells but not to human astrocytes. Examination of ROS generation using 2,7-diachlorofluorescein diacetate showed that the amount of ROS production increased significantly in glioma cells, but not astrocytes, when cells were exposed to ZnO NPs. Furthermore, the toxicity of ZnO NPs in glioma cells was reduced when ROS generation was inhibited by the antioxidant N-acetyl cysteine (NAC), indicating that ROS generation was the cause of the toxicity. Similarly, exposure of A549 cells to CuO NPs causes ROS generation depending on the concentration of CuO NPs (Figure 4), and CuO NP toxicity was reduced in the presence of NAC [23], indicating that CuO NP toxicity is caused by ROS generation. Furthermore, Hanley *et al.* [41] reported that ZnO NPs were non-toxic to human lymphocytes but were toxic to monocytes, with the toxicity being caused by the difference in ROS generation levels. In addition, Yang *et al.* [42] proposed that in addition to ROS generation, the toxicity of 20 nm SiO_2 NPs

in RAW264.7 cells was also due to its effect in increasing intracellular Ca^{2+} concentration. Toxicity caused by increased intracellular Ca^{2+} concentration has also been reported in human bronchial epithelial cells (BEAS-2B) exposed to ZnO NPs [43] and Ag NPs [34, 44, 45]. It was suggested that inhibition of ZnO NP toxicity in BEAS-2B cells by the NAC antioxidant was the result of inhibition by NAC of the influx of Ca^{2+} from the extracellular medium to intracellular compartments, indicating that both ROS production and an increase in intracellular Ca^{2+} concentration are involved in toxicity.

ROS generation by toxic materials can be determined not only by direct quantification but also by indirect measurements. Indirect ROS generation measurements can be achieved by quantification of molecules that are related to oxidative stress such as glutathione or malondialdehyde [46-48], or by measurement of the activity of enzymes that participate in ROS elimination such as superoxide dismutase (SOD) and catalase, glutathione peroxidase (GPX), glutathione reductase, and others [46, 47, 49, 50], or by gene expression analysis of molecules that are involved in ROS elimination [23, 25, 43, 51]. However, two different results have been obtained using these respective measurement methods. In the first result, the molecules and enzymes involved in ROS elimination were damaged by the nanomaterial, which caused an increase in intracellular oxidative stress [47, 48, 50, 52]. The increase in oxidative stress led to an increase in lipid peroxidation [28, 47, 52] that further led to genotoxicity [47, 50]. The other result showed that the number of molecules and the activation of enzymes involved in ROS elimination increased in the presence of the nanomaterials, thus alleviating oxidative stress. These conflicting results may arise from differences in the nanomaterial toxicity level. Cells incubated with metal or metal oxide nanoparticles that are highly toxic such as Ag NPs, CuO NPs, or ZnO NPs were reported to effect a significant increase in the expression of genes encoding metallothionein (MT) family proteins [23, 25, 51]. MT is known as a molecule that can overcome oxidative stress caused by the presence of excess metal ions [53-60]. Therefore, an increase in the expression of MT family genes in response to Ag NPs, CuO NPs, or ZnO NPs is involved in ROS elimination. On the other hand, ZnO NPs were reported to reduce the amount of glutathione and the activities of enzymes involved in ROS elimination such as catalase and SOD [47]. Because the hydroxyl radical scavenging activity of MT is more than 300 times higher than glutathione [61], it is likely that the MT family proteins are the main factor in the cellular response to ROS generation in the presence of ZnO NPs. In other words, although the level of glutathione and the activities of catalase and/or SOD are reduced by ZnO NPs, cells can alleviate ROS toxicity via MT family proteins. When evaluating nanomaterial toxicity by quantitative measurements of molecules that are related to oxidative stress such as glutathione and malondialdehyde; by the activity of enzymes

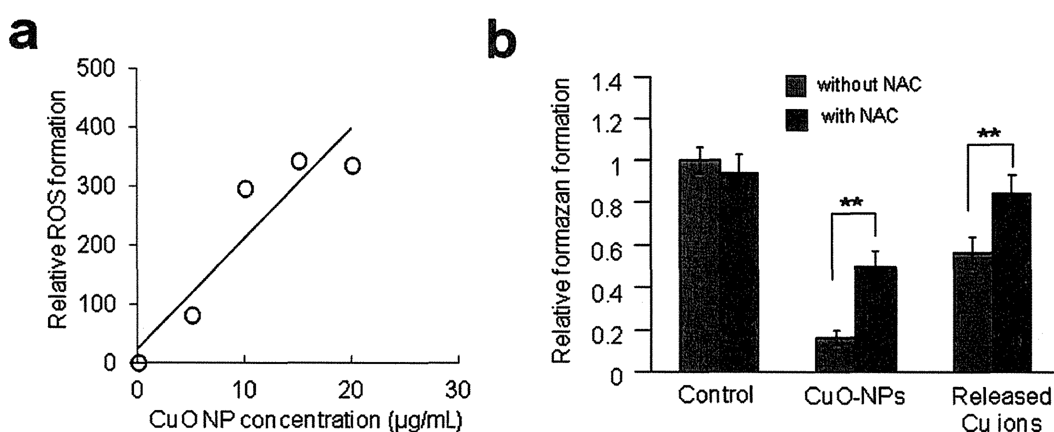

Figure 4 Toxicity of CuO NPs caused by generation of reactive oxygen species (ROS). **(a)** Relationship between CuO NP concentration and intracellular ROS generation. **(b)** NAC treatment reduces the toxicity of CuO NPs because of antioxidant action of NAC.

that participate in ROS elimination such as SOD, catalase, GPX and glutathione reductase; or by expression analysis of the genes involved in ROS elimination, it is important to assess whether or not the measured molecule(s) or enzyme(s) play a central role in the ROS pathway.

Generation of ROS by nanomaterials was reported to increase the activation of p53 and the Bax/Bcl-2 ratio, and induce the expression of apoptosis-related genes, ultimately leading to apoptosis [43, 62]. Apoptosis induction by ROS may also be explained by the fact that overexpression of MT that participates in ROS elimination can prevent cells from entering the apoptotic pathway [63]. In addition, oxidative stress due to ROS induces the inflammatory response [64, 65]. In many cases, ROS are the cause of DNA damage [66-73]. Furthermore, it has been reported that oxidative stress caused by nanoparticles induces the formation of the guanine adduct 8-hydroxy deoxyguanisine (8-OH-dG), which has been suggested to have an important role in carcinogenesis [74, 75].

The relationship between ROS generation caused by nanomaterials absorption and toxicity can be explained by the model shown in Figure 5. If the level of ROS generation by nanomaterials is low, cells can completely remove ROS by using their naturally induced ROS elimination systems. In such a case, no apparent toxicity ensues. However, if the level of ROS generation by nanomaterials cannot be overcome by the naturally induced ROS elimination systems, remaining ROS are able to cause cytotoxicity. Differences in the toxicity of the same nanomaterials in different cell types may be caused by differences in ROS elimination systems that each cell type possesses. When the amount of remaining ROS is low, cells experience growth inhibition, and when the amount of remaining ROS is high, cell death ensues. As cell death can either be apoptotic or necrotic in nature, which pathway the cell ultimately takes may depend on the remaining ROS.

Conclusions

So far, much research on the toxicity and safety of nanomaterials has been performed. Although many review articles describing the degree and mechanism of action of various nanomaterials have been published, much less attention has been paid to the interpretation of toxicity evaluation results. Evaluation of nanomaterial toxicity by the MTT assay cannot differentiate whether a decrease in cell viability is caused by a decrease in growth rate or by cell death. It has been reported that in many cases, nanomaterial toxicity is caused by ROS. However, because cells possess ROS elimination systems, the amount of ROS generation measured in assays reflects the amount of ROS that cannot be removed by such systems. Cells possess various ROS elimination systems and information detailing which systems primarily work for different nanomaterials is lacking. In order to generalize information concerning the toxicity of a particular nanomaterial in a certain cell type under certain conditions, careful biological interpretation of toxicity data is important.

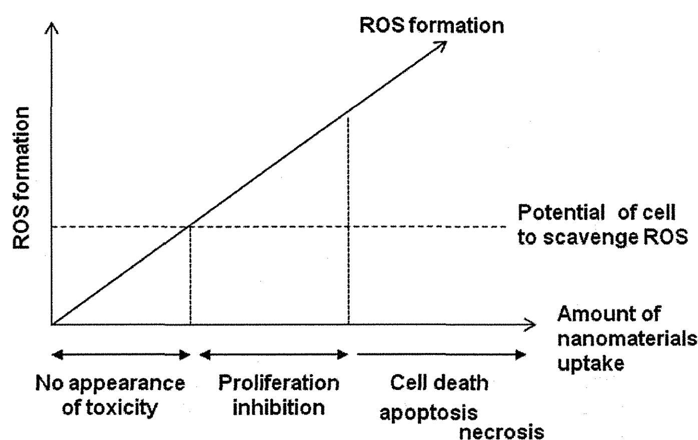

Figure 5 Proposed model for the relationship between ROS generation and toxicity.