

Improving the Interferences of Methyl Thiazolyl Tetrazolium and IL-8 Assays in Assessing the Cytotoxicity of Nanoparticles

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Methyl thiazolyl tetrazolium (MTT) and interleukin-8 (IL-8) assays are common colorimetric methods to measure mitochondrial activity and drug induced pro-inflammatory factors. However, many reports have described how MTT absorbance and cytokine adsorption could limit their applicability in evaluating the cytotoxicity of nanomaterials. In this study, we used an acid-containing isopropanol complex as a substitute for dimethyl sulfoxide (DMSO) solvent to dissolve MTT formazan, which was expected to diminish the absorbance of nano-ZnO at 570 nm where maximum absorbance for the MTT formazan was detected. In addition, we used a serum-containing medium to prevent the possible effects of IL-8 protein adsorption in the nano-ZnO and nano-TiO₂. The results showed that the modified method by using acid-containing isopropanol step in MTT assay, nano-ZnO exposed to human lung epithelial cells had the lowest cell viability (from 12.5 to 50 $\mu\text{g mL}^{-1}$) and EC₅₀ value (8.4 $\mu\text{g mL}^{-1}$) comparing with the conventional MTT protocol or adding phosphate buffered saline (PBS) to wash cells. The reason for this was the acid-containing isopropanol completely dissolved nano-ZnO with no additional absorbance when compared to the background solvent at 570 nm. On the other hand, the IL-8 protein had a marked influence on the adsorption of nano-TiO₂ in the serum-free medium. While only at 100 $\mu\text{g mL}^{-1}$ of nano-ZnO, an influence on the adsorption of IL-8 was observed. This could be attributed to the different charges on the surface of nanomaterials. This problem could be overcome through the addition of fetal bovine serum (FBS) to the medium.

Keywords: MTT, IL-8, A549 Cells, Absorption Effect, Protein Adsorption.

1. INTRODUCTION

Nanomaterials have been applied in a number of various fields and used to produce a number of consumer products currently available on the market. Understanding and evaluating the degree of safety or risk posed by nanomaterials has become crucial as human exposure to nanoparticles inevitably increases. An increasing number of *in vitro* toxicity studies are using colorimetric methods to assess the toxicity of engineered nanoparticles in human cell lines.^{1–3} However, many reported cytotoxicity assays may be defective due to interaction between assay components and nanoparticles, which compromise the reliability of the data.^{4–6} For instance, the methyl thiazolyl tetrazolium (MTT) assay and the production of interleukin-8 (IL-8) inflammatory markers via enzyme-linked immunosorbent assay (ELISA) are two common methods for measuring cell mitochondrial activity and inflammatory response, respectively. However,

many reports have demonstrated that for carbon-based nanomaterials such as single-walled carbon nanotubes (SWCNTs), nanomaterial/dye interactions and/or nanomaterial adsorption of dye/dye products^{7,8} may reduce cell viability results. Researchers have reported that adsorption of cytokines or chemokines by nanoparticles could interfere with ELISA experiments, resulting in a lower indicated cytokine concentration.^{6,9} Thus, when evaluating the toxicity of target nanomaterials it is important to note that these cytotoxicity screen assays could be influenced in any number of unexpected ways.

Nano-ZnO has become a popular material in the fields of photochemistry and biology.^{10,11} However, many studies have demonstrated that nano-ZnO has higher cytotoxicity than other metal oxide nanoparticles (Al₂O₃, Fe₂O₃, Y₂O₃, SiO₂ and TiO₂)^{12,13} and might be harmful to the environment as well as the organisms living within it. In similar applications, the phase-mediated toxicity of titanium dioxide nanoparticles has been widely reported, despite the fact that it has relatively lower levels of cytotoxicity.^{14,15} An

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increasing number of *in vitro* and *in vivo* experiments have examined the toxicity and potential DNA damage caused by these materials.^{16,17}

Many studies dealing with the *in vitro* toxicity of nano-ZnO have used the MTT assay to measure cell viability. In a number of cases, cell viability slightly increased as the cells exposure to high concentrations ($\geq 30 \mu\text{g mL}^{-1}$) of nano-ZnO.^{18,19} This tendency runs contrary to the more general dose-dependent relationship; but unfortunately few reports have mentioned this phenomenon or discussed it in detail. The human lung epithelial cell line (A549) which is frequently mentioned in studies on the toxicity of nanoparticles produces IL-8 chemokines when the cells exhibit inflammation.^{14,20} However, at least one report has mentioned how the adsorption of nano-TiO₂ could disturb the detection of cytokines.⁶ Reports seldom discuss whether nano-ZnO has any adsorptive properties with regard to IL-8 protein. Therefore to realize the adsorptive characteristics of IL-8 on ZnO surfaces and to overcome the adsorption effect on TiO₂ nanoparticles are crucial.

In this study, we investigated the effects of nano-ZnO as assessing its cytotoxicity by MTT methods. An ELISA microplate reader was used to estimate the absorbance of nano-ZnO at a wavelength of 570 nm. The results which were done by conventional MTT protocol was compared with the results from two modified methods: one was adding phosphate buffered saline (PBS) to wash cells; and the other was using acid-containing isopropanolic solvent as a substitute for dimethyl sulfoxide (DMSO) solvent. We compared the variability of these three processes through realistic experiments on cytotoxicity and non-cellular tests. In addition, the effects of adsorption of IL-8 on nanoparticles (ZnO and TiO₂) were also evaluated in serum and serum-free Dulbecco's Modified Eagle's Medium (DMEM). We hope by way of our study, more accurate methods in evaluating the cytotoxicity of nanoparticles can be set up.

2. MATERIAL AND METHODS

2.1. Chemicals

ZnO commercial nanopowders (50~70 nm), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) (>99.7%) were all purchased from Sigma-Aldrich Corp., USA. Anatase-phase TiO₂ nanoparticles (ST-21) (20~40 nm) were purchased from Ishihara Sangyo Kaisha Corp., Japan. A Tergitol-type NP-40 alternative and absolute isopropanol were supplied by E. Merck, Germany.

2.2. Cell Culture and Sample Preparation

The human lung carcinoma epithelial cell line (A549) (BCRC-60074, Bioresource Collection and Research Center, Taiwan) was cultured in DMEM, supplemented

with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic and cultivated in T25 flasks at 37 °C in a humidified atmosphere of 5% CO₂/95% air.

To prepare the stock solution, ZnO nanopowder was suspended in sterile dimethyl sulfoxide at a concentration of 20 mg mL⁻¹. Prior to dilution, the solution was dispersed using a 5-W probe sonicator (Ultrasonic Cell Disruptor, Misonix, USA) for two minutes in an ice bath. To assess the cytotoxicity, the cells (4×10^4 cells mL⁻¹) were seeded in 96-well plates. To evaluate the cell number, a standard curve for the MTT assay was determined by seeding 1.56×10^3 to 1×10^5 cells mL⁻¹ in the same 96-well plates with samples. Cells intended for exposure to ZnO suspensions were treated after 20 h of cell attachment.

2.3. Cell Viability

A549 cells were exposed to ZnO concentrations ranging from 1.56 to 50 $\mu\text{g mL}^{-1}$ in serum-free DMEM for 24 hours. All suspensions were aspirated from each well and 200 μL of MTT/DMEM (0.5 mg mL⁻¹) solution was added. After the plates had been incubated for 3 h, the MTT/DMEM solution was discarded and 200 μL of DMSO was added to each well. Finally, the absorbance was measured at 570 nm by a tunable microplate reader (VersaMax, Molecular Devices, USA). The relative cell activity (%) of nanomaterial-dosed cells and cells without exposure to nanoparticles was calculated by [cell number of testing samples]/[cell number of control] \times 100. The cell viability data from the above conventional protocol was compared with those from two improved methods. The first method used two additional PBS washes per well before the second step, and the second method used an HCl/1% Tergitol-type NP-40 in isopropanolic solvent instead of the DMSO solution. In all other regards, the processes were identical to the above conventional protocol.

2.4. Non-Cellular Experiments

To evaluate the absorbance of nano-ZnO at 570 nm, ZnO concentrations ranging from 100 to 6.25 $\mu\text{g mL}^{-1}$ in DMSO were used as a reference optical density (O.D.). Nano-ZnO concentrations ranging from 100 to 6.25 $\mu\text{g mL}^{-1}$ in serum-free DMEM (200 μL) were first added to each well with no cells attached and incubated for 24 h. Second, all suspensions were aspirated from each well, and each well was washed with PBS (200 μL) for once or twice. The next steps were the same as in the conventional MTT protocol. The absorbance of ZnO residues in all wells were measured at 570 nm.

To assess the dissolution of ZnO by acid-containing isopropanol, we measured the absorbance of nano-ZnO (50 $\mu\text{g mL}^{-1}$) in HCl/1% Tergitol-type NP-40 ranging from 40 to 1.25 mM in isopropanol. We then selected an

appropriate HCl concentration for the isopropanolic system to measure the absorbance of ZnO at 570 nm at concentrations of 12.5 to 100 $\mu\text{g mL}^{-1}$.

2.5. IL-8 Adsorption Measurement

Human IL-8 ELISA (Human IL-8 ELISA Kit and buffer, PeproTech, USA) was used to evaluate the adsorption of pro-inflammation chemokine, IL-8, on nanoparticles (ZnO and TiO₂). First, standard IL-8 proteins (400 $\mu\text{g mL}^{-1}$) were added to the ZnO and TiO₂ suspensions (25, 50, and 100 $\mu\text{g mL}^{-1}$) in DMEM with 0% and 10% FBS. After incubation for 24 h, all suspensions were centrifuged at 250 g for 4 minutes to separate the nanoparticles from the medium. Finally, 100 μL of each sample was used for triplicate IL-8 analysis.

2.6. Zeta Potential Measurement

The surface charges of ZnO and TiO₂ nanoparticles in DMEM were monitored by a Zetasizer Nano ZS (Malvern Instruments Inc., UK). The dispersion of nanoparticles was prepared at 100 $\mu\text{g mL}^{-1}$ in medium. To decrease the state of agglomeration, the dispersions were sonicated in an ice bath by ultrasonic probe (8 W, 22 KHz) for 5 min.

2.7. Statistics

The adsorption of IL-8 protein on nanoparticles was statistically analyzed to a $p < 0.05$ significance level using a two-tailed Student's *t*-test. The EC50 values from the MTT dose-response curves were calculated using GraphPad Prism 5.02 (GraphPad Software, USA) and the following equation.

$$y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{x - \log \text{EC}_{50}}) \quad (1)$$

3. RESULTS

3.1. Absorption Effect of Nano-ZnO

During treatment of human lung epithelial cells (A549) with nano-ZnO for 24 h in the original process described above, a steeper decrease in cell viability appeared from 0~25 $\mu\text{g mL}^{-1}$. At a dose exposure of 50 $\mu\text{g mL}^{-1}$, however, the cell viability increased slightly to 12% (Fig. 1). Moreover, the EC50 value of nano-ZnO was 9.8 $\mu\text{g mL}^{-1}$. Particle residue in the 96-well plate that blocked the passage of UV-vis light might explain this. To confirm this explanation, noncellular experiments were used as a reference. Nano-ZnO showed a concentration dependent absorbance in DMSO even at 570 nm (nano-ZnO has a maximum absorbance at about 370 nm) (Fig. 2). The probable O.D. of nano-ZnO after the conventional MTT processes appears in Figure 2. This study found that the absorbance of nano-ZnO in the conventional MTT

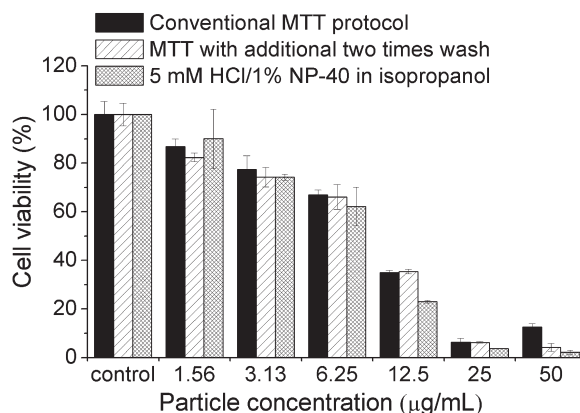


Fig. 1. MTT activity of the A549 cells after 24 h exposure to nano-ZnO via different working processes. The EC50 values of nano-ZnO from these three systems were 9.8 $\mu\text{g mL}^{-1}$ with the conventional process, 11.1 $\mu\text{g mL}^{-1}$ with two additional washing processes, and 8.4 $\mu\text{g mL}^{-1}$ in the acid-containing isopropanol system.

process had only 0.02 difference at 25~100 $\mu\text{g mL}^{-1}$ compared with the reference. This demonstrates that cell viability at higher dosage concentrations might have been overestimated.

3.2. Improvement of Methods

In view of the absorption effects resulting from particle, we used two methods to eliminate the absorbance of nano-ZnO. First, we used an additional two washes with PBS prior to the addition of MTT/DMEM, which had been applied in many study.^{12,21} Figure 2 shows that nearly two-thirds of the nano-ZnO (at 50 $\mu\text{g mL}^{-1}$) was removed by the additional washing. Furthermore, the results are in strong agreement with realistic MTT activity: the cell viability of A549 cells decreased from 12.6% to 4.2% at 50 $\mu\text{g mL}^{-1}$ (Fig. 1). Although a steeper decrease in cell viability appeared at dose exposures of 0 to 50 $\mu\text{g mL}^{-1}$, we were still unable to completely reduce the

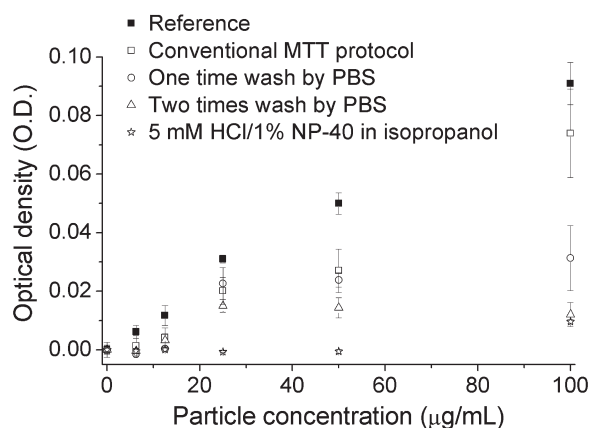


Fig. 2. Absorbance of different concentrations of nano-ZnO at 570 nm in DMSO (with zero, one, and two washes with PBS) and in acid-containing isopropanol.

effect of nano-ZnO absorbance at concentrations higher than $12.5 \mu\text{g mL}^{-1}$.

In consideration of this limitation we used an acid isopropanol system to replace the DMSO used to dissolve the MTT formazan and cell membrane. We first measured the nano-ZnO absorbance at a 4~40 mM HCl concentration in a 1% NP-40/isopropanol solvent. All the HCl concentrations were able to reduce the absorbance of nano-ZnO ($50 \mu\text{g mL}^{-1}$) almost completely (data not shown). Because the reduced MTT was pH dependent,²² we selected a working HCl concentration of 5 mM. Figure 2 shows that when 5 mM acid-containing isopropanol was used, almost no nano-ZnO absorbance occurred at concentrations of $50 \mu\text{g mL}^{-1}$ or less. Real experiments on cytotoxicity revealed that at 12.5, 25 and $50 \mu\text{g mL}^{-1}$, the cell viability was less when acid isopropanolic solvent was used than that when the other two processes were applied (Fig. 1). The EC₅₀ value of this system decreased to $8.4 \mu\text{g mL}^{-1}$. This meant that nearly all of the residual particles in the 96 wells had dissolved to the ionic state. The elimination of the absorption effect of nanoZnO yielded more reliable data in evaluation of the cytotoxicity of nano-ZnO.

For the quantification of cell numbers, this study compared standard curves between acid-containing isopropanol and DMSO solutions. The standard curves for both the isopropanol and DMSO systems had good linearity ($R^2 > 0.99$), whereas the lowest cell number requirement for this MTT assay measurement increased slightly to 1950 cells cm^{-2} , about two times more than that in the original DMSO solvent (Fig. 3).

3.3. Adsorption Effect of Nanoparticles (ZnO and TiO₂)

It was important to determine the adsorption effect of IL-8 protein on nanoparticles (ZnO and TiO₂) due to a

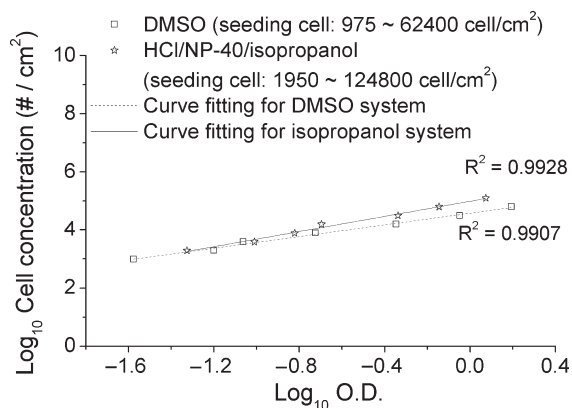


Fig. 3. Standard curves for cell viability measurement when using DMSO and 5 mM HCl-1%NP-40-isopropanol solvent for detecting MTT formazan.

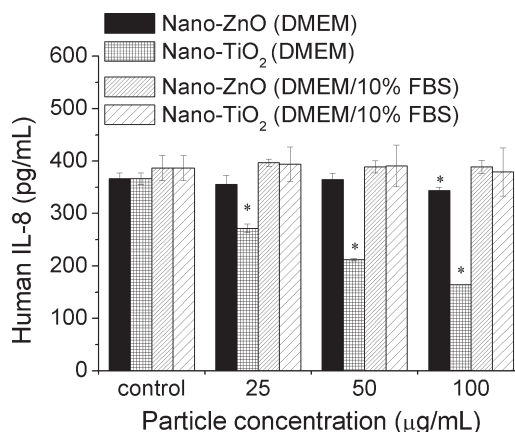


Fig. 4. IL-8 detection under the same amount of standard (400 pg mL^{-1}) with various ZnO and TiO₂ nanoparticle concentrations in serum and serum-free media. The control groups had no nanoparticles in serum and serum-free media, and had an IL-8 standard of 400 pg mL^{-1} . Asterisks (*) denote groups that differ significantly from the control groups at a 95% confidence level.

direct influence on the accuracy of the IL-8 assay, resulting in lower detection levels. In the serum-free medium, our data indicated a slight difference in the detection of IL-8 only at $100 \mu\text{g mL}^{-1}$ (344 pg mL^{-1}), compared with the control for nano-ZnO (Fig. 4). However, TiO₂ in the DMEM medium indicated that detection of IL-8 had obviously decreased as the particle concentration increased. We were able to detect only 164 pg mL^{-1} of IL-8 with a $100 \mu\text{g mL}^{-1}$ of TiO₂ suspension. On the other hand, when these substances were suspended in the serum-containing medium (DMEM/10% FBS), neither nanomaterial showed significant changes in IL-8 detection.

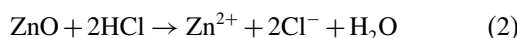
4. DISCUSSION

When human lung epithelial cells (A549) were exposed to nano-ZnO at higher concentrations the values used to determine cell viability might have been influenced by an overestimated O.D. value derived from particle residues in 96 well plates. Some reports showed a slight increase in cell viability at higher concentrations of nano-ZnO.^{18,19} Nanoparticles in cultured cells may directly influence the O.D. through increased absorbance, which had demonstrated for sodium titanate nanoparticles.²³ *In vitro* experiments lead us to expect that the absorption effect of nano-ZnO was serious because it was possible for particles to be internalized within A549 cells thereby making it difficult to wash them out at every step.

In view of the particle absorption effect, we first used a reported method (two extra PBS washes prior to adding MTT/DMEM). However, the influence of nano-ZnO on the scattering of radiation had not been diminished. Gojova et al. found it was difficult to fully remove particles from the cell surface by PBS washing, particularly at higher particle concentrations.¹² Nevertheless, it was still important

to remove as much of the ZnO nanoparticulate as possible from each well due to the fact that (as one report showed), metal ions such as Zn^{2+} could interfere with the MTT reduction reaction.²⁴

To completely overcome the problem this study used an HCl/1% NP-40/isopropanol system rather than a DMSO system. Many studies have applied this complex solvent for MTT assay because non-ionic surfactant NP-40 can break cytoplasmic membranes, and acid-containing isopropanol dissolves MTT formazan very effectively.^{25,26} Some studies have used TritonX-100 to replace NP-40.¹⁴ None of these researchers mentioned that the solvent might decrease the absorption of nanoparticles. The decrease could be attributed to the reaction of acid HCl with ZnO, as follows.²⁷



The dissolved zinc ions would not have influenced on the absorbance at 570 nm. Although the acid-containing isopropanol system performed better at assessing cell viability, the lowest cell number required for the measurement had slightly increased. The reason for this was that the absorbance of reduced MTT at a lower pH had decreased as measured at 570 nm,²² so more cells were required to increase its sensitivity.

Research has confirmed serum adsorption of ZnO nanoparticles,²⁸ and it would be interesting to know whether nanoparticles adsorbed IL-8 protein or not. This study found only slight effect of IL-8 adsorption on nano-ZnO suspensions at $100 \mu\text{g mL}^{-1}$ in a serum-free medium, which was related to the surface charge of ZnO particles and a net positive charge of the chemokine. IL-8 has an isoelectric point of pH 8.7, and +5 mV at pH 7.²⁹ A negative charged ZnO (-4.5 mV at DMEM) thus slightly attracted IL-8. Although adsorption did not influence IL-8 detection under lower dosage of nano-ZnO, nano-TiO₂ with a more negative zeta potential (-16 mV at DMEM) had good affinity to IL-8 protein leading to an adsorption effect. One study showed a decrease in the measured IL-6/standard ratio for TiO₂ and SiO₂ nanoparticles,⁶ but the mechanism might be preferential to attractive polar groups. Some air pollution particles, such as diesel exhaust particles (DEP), bind and concentrate IL-8 because the particles are negative charged.²⁹ The reason for a lack of IL-8 adsorption occurring in the serum-containing medium was that both nanomaterials had been coated with serum layers, and there were no active sites to bind the IL-8 protein.

It was noted that many cytotoxicity studies of nanomaterials used serum-free media in the early years, but some studies have started to use serum-containing media in exposure experiments to stabilize nanoparticle suspensions and to promote cell growth.³⁰⁻³² Our study provided one more reason to convince researchers using serum-containing media because more accurate data could

be obtained when the IL-8 assay was used for evaluation of the inflammatory response in cells induced by nanoparticles.

In this study, we demonstrated that higher concentrations of residual nano-ZnO in tissue culture plates could lead to an overestimation of cell viability using the MTT assay. This problem was almost solved by using 5 mM HCl/1% NP-40 in isopropanolic solvent. With this process, we nearly eliminated the absorbance of nano-ZnO by dissolving ZnO into an ionic state. In addition, by adding the serum to the medium, IL-8 showed no adsorption effect on nanoparticles (ZnO and TiO₂). This topic is worthy of further discussion and putting more efforts to overcome the limitations posed by colorimetric methods in the evaluation of the cytotoxicity of nanomaterials.

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