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Cytotoxicity of Ultra-pure TiO_2 and ZnO Nanoparticles Generated by Laser Ablation

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This paper aims to address the cellular toxicity of ultra-pure titanium dioxide (TiO_2) and zinc oxide (ZnO) nanoparticles (NPs) frequently employed in sunscreens as inorganic physical sun blockers to provide protection against adverse effects of ultraviolet (UV) radiation including UVB (290-320 nm) and UVA (320-400 nm). In consideration that the production and the use of inorganic NPs have aroused many concerns and controversies regarding their safety and toxicity and that microsized TiO_2 and ZnO have been increasingly replaced by TiO_2 and ZnO NPs (< 100 nm), it is very important to directly investigate a main problem related to the intrinsic/inherent toxicity of these NPs and/or their incompatibility with biological objects. In the present study, we took advantage of the laser-assisted method called laser ablation for generation of TiO_2 and ZnO NPs. NPs were prepared through a physical process of irradiating solid targets in liquid phase, enabling verification of the toxicity of ultra-pure NPs with nascent surfaces free from any contamination. Our results show that TiO_2 NPs are essentially non-poisonous and ZnO NPs are more toxic than TiO_2 NPs based on the cell viability assays.

Key Words : Nanoparticle, Toxicity, Laser ablation

Introduction

Sunscreens in the forms of lotion, spray, gel, and so forth, absorb or reflect solar ultraviolet (UV) rays to protect the skin against the harmful effects. UV rays are in general divided into three: UVC for 100-290 nm, UVB for 290-320 nm, and UVA for 320-400 nm. Because the shorter wavelength range than that of UVC is filtered off from the atmosphere mainly by the ozone layer, sunscreens usually do not involve any ingredients that block UVC.

Sunscreens should provide protection against the adverse effects of UVB and UVA rays, Based on the ingredients, they are classified into two different types: organic (or chemical) and inorganic (physical) sunscreens.[1] Commercial products currently sold in markets mostly combine both organic and inorganic materials, while the protection effects vary among products depending on the combination of the materials. Materials for organic sunscreens include octyl methoxycinnamate, isoamyl *p*-methoxycinnamate, homosalate, octyl salicylate, and butyl methoxydibenzoylmethane, and inorganic sunscreens are composed of inorganic nanoparticles (NPs) as inorganic physical sun blockers.

Titanium dioxide (TiO_2) and zinc oxide (ZnO) NPs are frequently employed in sunscreens because they block UV rays by physical scattering or absorption. They are effective UV blockers, having a wider range of UV protection. Compared to organic sunscreens, inorganic sunscreens containing TiO_2 and ZnO can advantageously cover a wider spectrum range without causing photocontact dermatitis.[2] Recently, however, the toxicity and danger of TiO_2 and ZnO NPs have also come to the fore.[3,4] In consideration that TiO_2 and ZnO NPs are widely used in various areas including fabric, paint, varnish, antibacterial agents, and sanitation facilities,[5-7] it is highly important to explicitly understand the intrinsic and inherent toxicity basis of the NPs.

In the present study, we prepared TiO_2 and ZnO NPs without any surface coatings or contaminants *via* a novel method called liquid laser ablation, since the intrinsic toxicity of these NPs had not yet been directly addressed. With such ultrapure TiO_2 and ZnO NPs, we examined the toxicity of TiO_2 and ZnO NPs in comparison to Ag and Au NPs, allowing us to suggest the better NPs as inorganic ingredients for sunscreens.

Experiments

Experimental Setup. Ti or Zn targets (99.9%, 20-mm diameter × 5-mm thickness) were irradiated by a focused laser beam in double-distilled water to produce TiO_2 and ZnO NPs. For comparison, Ag and Au NPs were also prepared according to the literature,[8] for which 7 mM NaCl aqueous solution was used to synthesize the even-sized NPs. The size of Ag or Au targets was 10-mm diameter × 1-mm thickness. Metal targets were put into 30-mm vial with 30-mL aqueous solution. A schematic diagram of the experimental setup for the production of NPs in liquid phase is depicted in Figure 1. The target was vertically irradiated by a Q-switched Nd:YAG laser (λ = 1064 nm, Continuum, Surelite I). The laser was 10-Hz pulse type with its pulse width and intensity of 6 ns and 100 mJ/pulse, respectively. The

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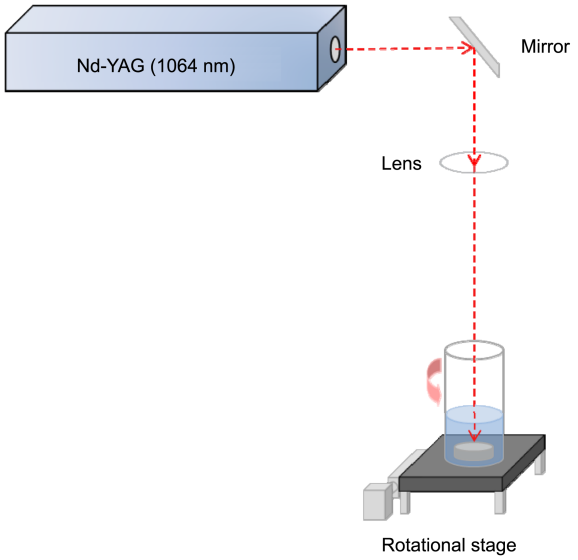


Figure 1. Experimental setup for laser ablation in liquid phase.

laser beam was focused by a lens with focal length of 25 cm and the diameter of the laser spot on the target surface was approximately 1 mm. During the experiment, the vial was rotated by a rotator to avoid any aging effect caused by the continued irradiation on a single spot.

Cell Culture and Viability Assays. HeLa uterine cervical cancer cells, MCF-7 breast cancer cells, and PC-3 prostate cancer cells were purchased from American Type Culture Collection. The cells were preserved in tissue culture plates in an atmosphere of 5% CO_2 air at 37 °C. HeLa cells were grown in MEM/EBSS media and MCF-7 and PC-3 cells were cultured in RPMI-1640 media, all supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin. In order to keep the cells in the log phase, the media were replaced twice or three times a week.

Cell viability was measured using the standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay.[9] In short, the exponentially increased cells were divided, put into an incubator at 37 °C for 24 h, and then exposed to various concentrations of TiO_2, ZnO, Ag, and Au NP solutions. After incubation, the MTT solution

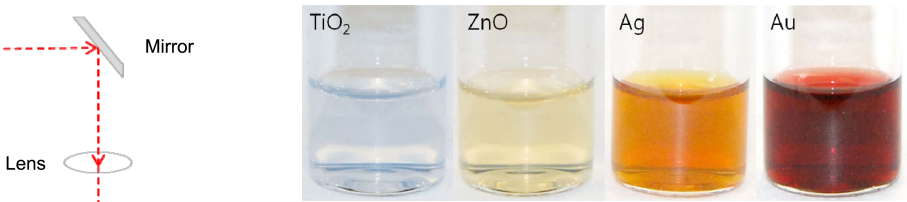


Figure 2. TiO_2, ZnO, Ag, and Au nanoparticle solutions generated by laser ablation in liquid phase. With increase in the laser irradiation time, the characteristic color of each NP solution became more apparent.

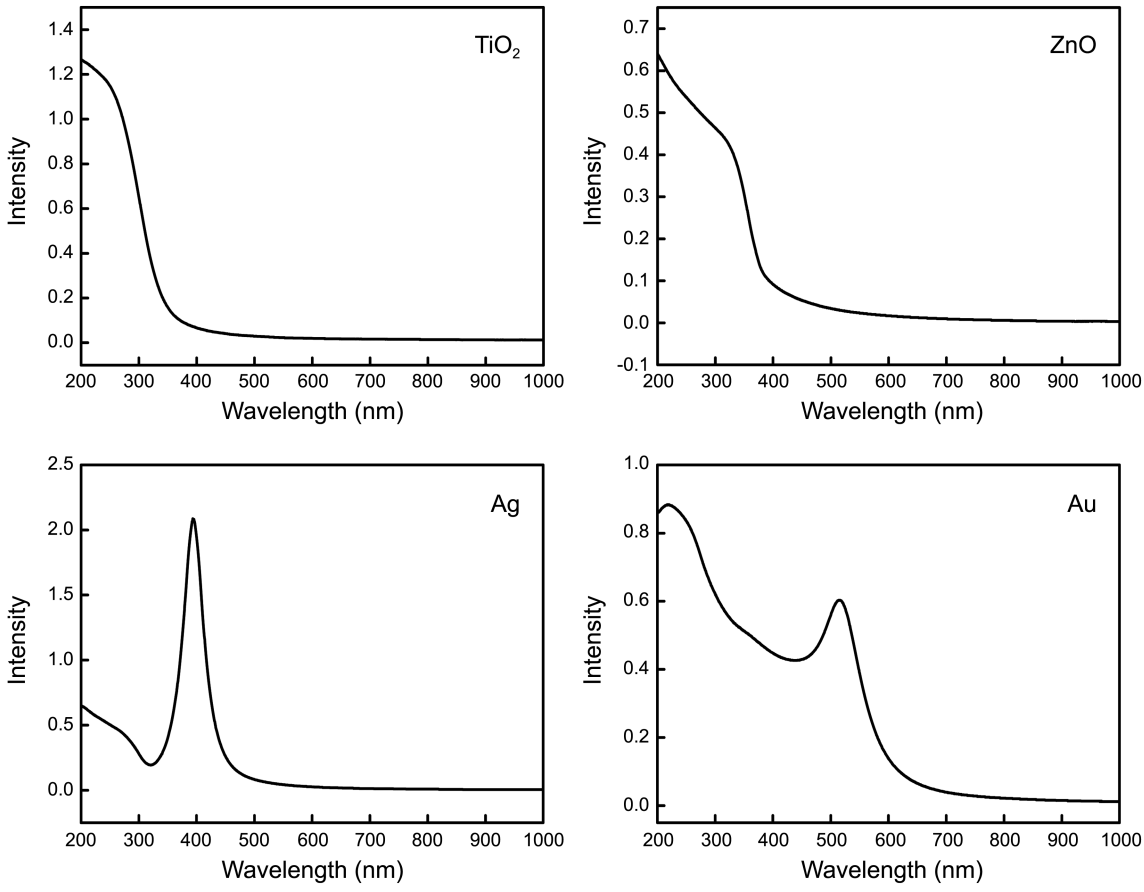


Figure 3. UV-Vis spectra of TiO_2, ZnO, Ag, and Au nanoparticle solutions.

was added and further incubated at 37 ºC for 4 h. The quantity of formazan product obtained was determined using a multiplate reader at 570 nm. The cell survival fractions were calculated as percentage of the untreated control. The experimental data were transformed to sigmoidal dose-response curves using nonlinear regression analysis (SigmaPlot) to calculate the corresponding IC_{50} values.

Results and Discussion

Two different methods have been usually used for the synthesis of NPs: physical and chemical methods. Among the physical methods, laser ablation is a new emerging technique, while chemical reduction method is mostly employed as chemical methods. Recently, laser ablation has attracted great attention as a clean and environment-friendly method for production of NPs because ultra-pure NPs can be obtained differently from chemical reduction method. Whereas many chemical reagents are involved in the synthesis of NPs by chemical reduction method, laser ablation method uses only metal targets and aqueous solution, enabling the generation of no chemical waste.[10,11] In addition, ultra-pure NPs prepared by laser ablation are stable enough to be used without any further surface modification due to the inherent high zeta potential values of the resulting NP solutions, in comparison that surface treatment is usually required for the unstable NPs prepared by chemical reduction method. TiO_2, ZnO, Ag, and Au NP solutions produced by liquid laser ablation are displayed in Figure 2.

UV-Vis absorption spectra of TiO_2, ZnO, Ag, and Au NPs are shown in Figure 3. While TiO_2 and ZnO NP solutions do not show any obvious absorption peaks, the characteristic absorption peaks for Ag and Au NP solutions are clear: 393.5 nm for Ag NPs and 219.5 nm and 515 nm for Au NPs, where the absorption peaks at 393.5 nm and 515 nm reflect the surface plasmon absorption of Ag and Au NPs, respectively, related to the collective vibration of the surface electrons. The 219.5 nm peak of Au NPs is due to the interband transition. The location of the surface plasmon peaks and its width are known to be highly dependent on the size and shape of NPs.[10,11]

Figure 4 shows the transmission electron microscopy (TEM) images of the prepared TiO_2 and ZnO NPs and their size distributions. The TiO_2 NPs are mostly circular and their

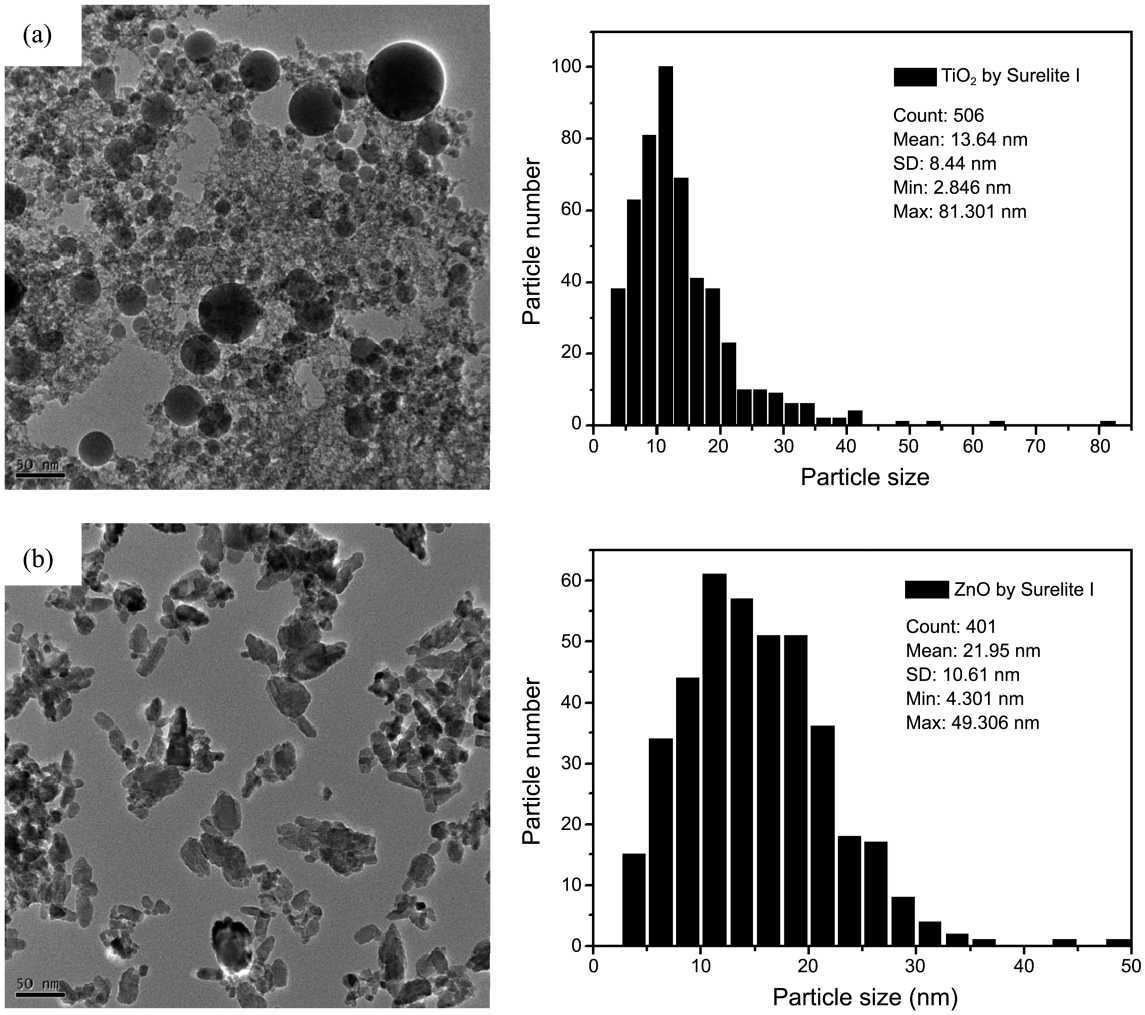


Figure 4. TEM image of (a) TiO_2 and (b) ZnO nanoparticles and their size distribution.

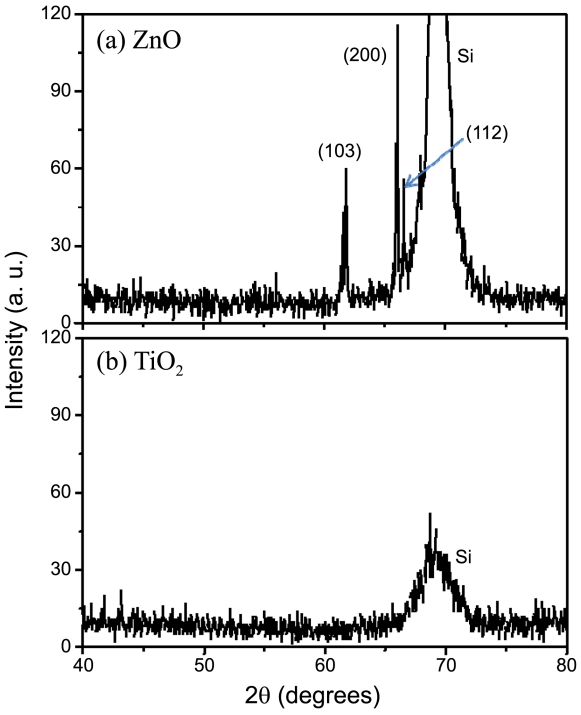


Figure 5. XRD spectrum of (a) ZnO NPs and (b) TiO_2 NPs.

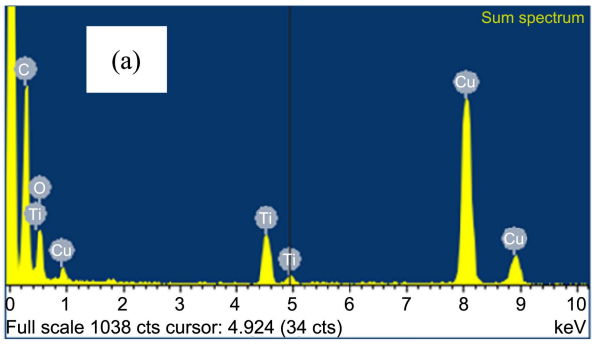
average size is 13.6 ± 3.4 nm, whereas the ZnO NPs exhibit oval shapes with the size of 21.9 ± 10.6 nm (long axis).

ZnO NPs are known to exist as two types of crystalline structures: hexagonal wurtzite crystal and cubic zinc-blende. The TiO_2 NPs are reported to have three different structures: anatase, rutile, and amorphous.[12] The ZnO NPs prepared by laser ablation method in the present study were determined to be wurtzite crystal based on the X-ray diffraction (XRD) results, and TiO_2 NPs were amorphous, as clearly indicated in Figure 5. The atomic compositions of the TiO_2 and ZnO NPs shown in Figure 6 were determined by energy dispersive X-ray (EDX) spectroscopy.

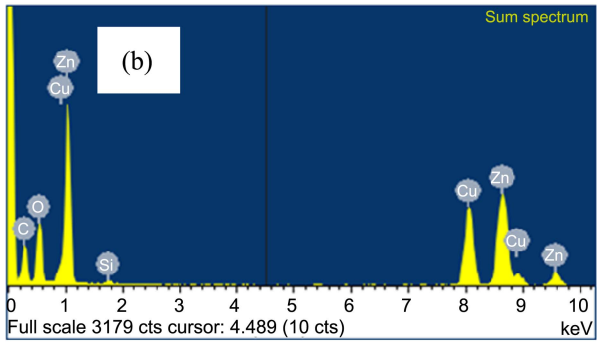
The viabilities of HeLa, MCF-7, and PC-3 cells after exposure to each NP solution are demonstrated in Figure 7. It has been reported that Au NPs are inherently non-toxic to human cells[13] and the toxicity of TiO_2 NPs is in the order of amorphous > antase > rutile with the highest toxicity caused by the amorphous TiO_2 NPs not significant.[14] Consistent with the previous reports, we observed no actual toxicity of TiO_2 and Au NPs. The cells exposed to the Ag NPs exhibited a decreasing viability tendency with increase in the concentration of NP solutions: the cell viability didn't reach 50% at the highest concentration of 200 mg/mM for HeLa and MCF-7 cells, and the IC_{50} value of Ag NPs for PC-3 cells was measured to be 65 μg/mL. This is attributed to the difference in the characteristics of the cells. We previously reported[8] that the IC_{50} values of Au and Ag NPs for PC-3 cells were 82.9 μg/mL and 88.6 μg/mL, respectively, and the difference in these cell viabilities is believed to reflect the difference in the sizes of the NPs produced through liquid laser ablation method.

Figure 7 also shows that ZnO NPs, unlike TiO_2 NPs, manifest high toxicity in HeLa, MCF-7, and PC-3 cells; the IC_{50} values were determined to be 50.0 μg/mL, 46.4 μg/mL, and 34.0 μg/mL, respectively, which are much higher than those of TiO_2 NPs. These results in the absence of UV radiation are striking because it has been reported that TiO_2 NPs is an International Agency for Research on Cancer (IARC) group 2B carcinogen and ZnO is generally recognized as safe by the FDA when used according to cosmetics directives.[15]

It is worthwhile to compare the obtained results to the literature values, since there are many contrary reports on the cytotoxicity of TiO_2 and ZnO NPs. As well reviewed in the literature,[16,17] NPs have been shown to usually interfere



Element	Weight%	Atomic%
C K	34.76	67.49
O K	6.93	10.10
Ti K	8.39	4.08
Cu K	49.93	18.33
Totals	100.00	



Element	Weight%	Atomic%
C K	9.99	28.62
O K	14.24	30.63
Si K	0.54	0.66
Cu K	32.96	17.85
Zn K	42.26	22.24
Totals	100.00	

Figure 6. The composition of (a) TiO_2 NPs and (b) ZnO NPs as measured by EDX.

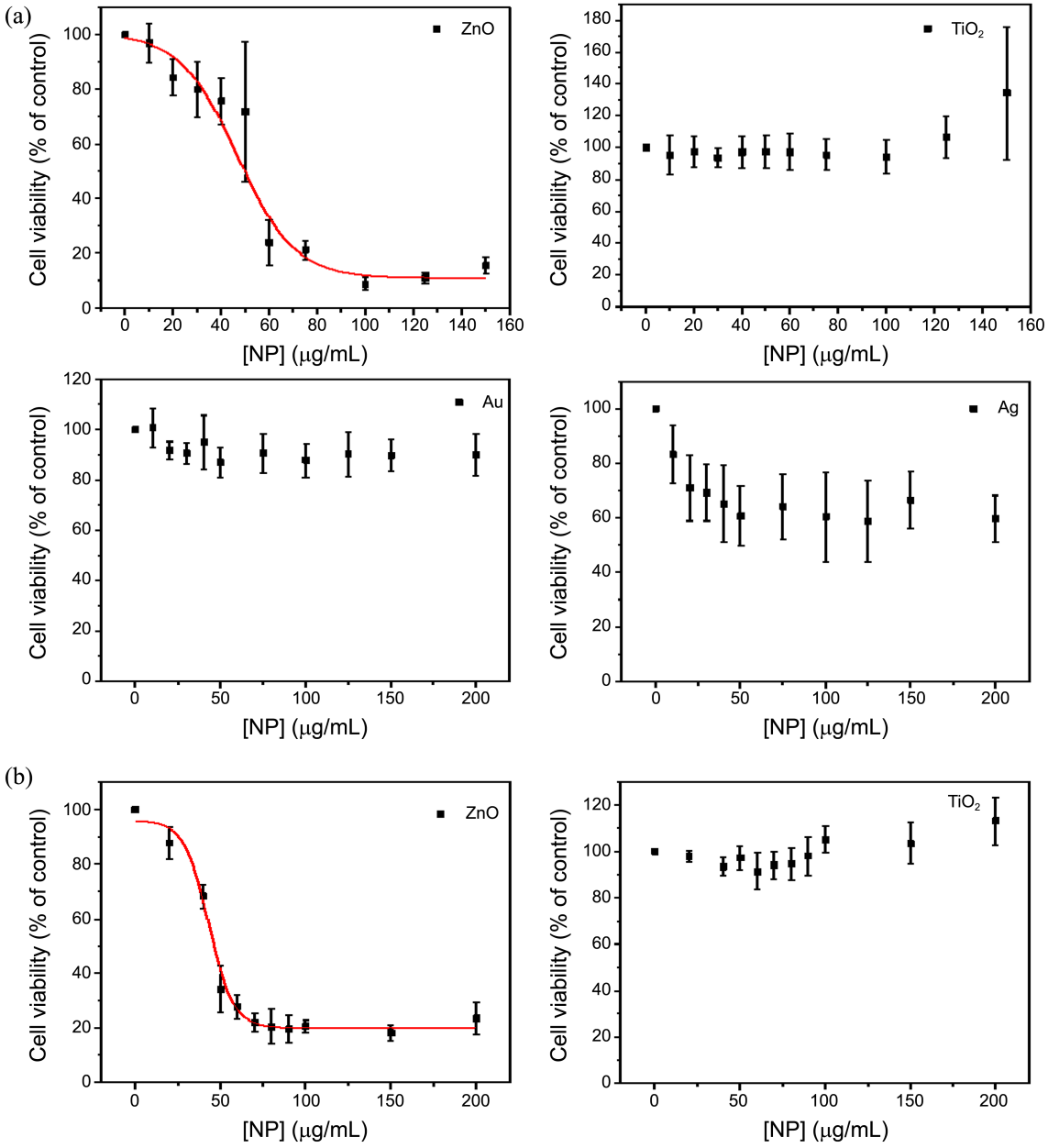


Figure 7. Cell viability of ZnO, TiO_2, Au, and Ag NPs for (a) HeLa cells, (b) MCF-7 cells, and (c) PC-3 cells.

with the antioxidant defense mechanism leading to reactive oxygen species (ROS) generation, the initiation of an inflammatory response and perturbation and destruction of the mitochondria causing apoptosis or necrosis in different mammalian cells *in vitro*.[18] TiO_2 and ZnO NPs in the solution usually generate superoxide anion radicals ($O_2^{-\bullet}$), singlet oxygen ($^1O_2$), H_2O_2 as well as hydroxyl radical (·OH) and the following activation of anti-oxidants such as superoxide dismutase, catalase, and glutathione.[4] Furthermore, it has been reported that ZnO NPs have higher toxicity than TiO_2 NPs because the ZnO NPs can generate the more Zn^{2+} ion and thus ROS although both TiO_2 and ZnO NPs produce ROS.[14] In this respect, further investigations on the free radical generation from the ultra-pure NPs based on electron spin resonance are necessary to clarify the differences in the toxicities of TiO_2 and ZnO NPs.

Conclusion

We have investigated the cytotoxicity of TiO_2 and ZnO NPs which are used as major ingredients in sunscreens. The liquid laser ablation method was employed with a goal to produce ultra-pure NPs with no surface modification and to evaluate the intrinsic cytotoxicity of the NPs, and TiO_2 NPs were found to be non-toxic while ZnO NPs exhibited clear cytotoxicity. This important approach is unique in avoiding the secondary toxicity related to the traditional chemical synthesis procedure, because incomplete characterization of NPs may hinder attempts to find a correlation between a variety of biological effects and NP properties. Further works

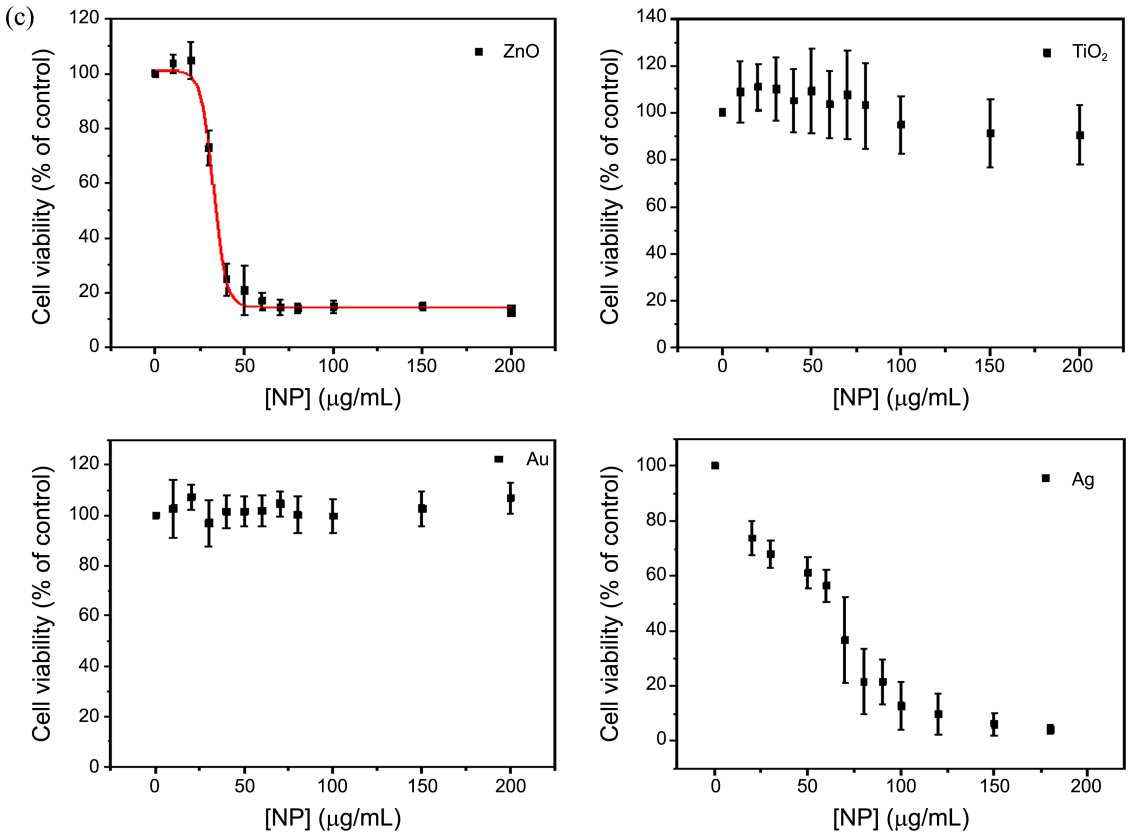


Figure 7. Continued.

on ROS produced in the absence of UV radiation by NPs in the size- or shape-dependent manner and the oxidative stress in cells are under investigation to determine the better NPs for the sunscreens,

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