Research Article

Cytotoxicity Property of Nano-TiO₂ Sol and Nano-TiO₂ Powder

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A homogeneous and transparent titania (TiO₂) sol with nanosized anatase TiO₂ particles was prepared by hydrothermal synthesis method. The transmission electron microscope and X-ray diffraction were used to characterize the structure and morphology of particulates in the TiO₂ sol and purchased TiO₂ powder. The results show that the homogeneous anatase crystalline phase was formed and the size of the spindle-like particle in sol was about 20 nm in width and 150 nm in average length, and the particulates of the purchased powder were globular-like about 50 nm in diameter. In addition, a consistent set of *in vitro* experimental protocols was used to study the effects of nano-TiO₂ sol as prepared and nano-TiO₂ powder on mouse peritoneal macrophage. The cytotoxicity tests *in vitro* indicate that, with the increasing of TiO₂ sol concentration contaminated with the cells, the relative proliferation rate of macrophage cells was improved slightly after the cells contaminated for 24 h, but it reduced rapidly after contaminated for 48 h. The purchased nano-TiO₂ powder inhibited the growth of the cells obviously as cultivating with macrophage both for 24 h and 48 h.

1. Introduction

Manufactured nanoparticles (NPs) of titanium dioxide (TiO₂) have been widely used in many fields in the recent years [1–5]. During the production and application, nanoparticles will be contacted with organism inevitably. Thus, their potential toxicity gets more and more attention for human health.

Some research has shown that nanomaterials might have negative effects on the health of the organisms [6]. Mauderly et al. [7] observed pulmonary tumors in rats exposed to 2.5 mg/m³ carbon black or 6.5 mg/m³ burning products of diesel. Shvedova et al. [8] found free radicals, the accumulation of peroxide and reduction of antioxidant, and, hence, decreased cell activity and changed in the cell morphology and ultrastructure after exposure of Haca T in single-walled carbon nanotubes for 18 h. Oberdörster et al. [9] exposed rats to an environment containing 20 nm nanoparticles of polytetrafluoroethylene for 15 min and found that most of the rats died after 4 h of exposure in the air. Nevertheless, after exposure to an environment containing 130 nm of polytetrafluoroethylene particles, as the control group, were not affected. Therefore, it is essential to examine the biosafety of nanosized TiO₂ for the *in vivo* applications. However, data

from several research groups indicated that TiO₂ NPs had distinct risks to human health compared with their normal size counterparts [10–12]. TiO₂ NP toxicity has been studied with respect to reactive oxygen species (ROS) production and oxidative stress in mammalian studies [13, 14]. TiO₂ NPs induced oxidative damage to human bronchial epithelial cells [15] and to brain microglia [16]. However, some ecological studies showed that TiO₂ NP exposure in aquatic species caused oxidative damage-mediated effects [17–19]. Palomäki [20] explored immunological effects of five different nanomaterials on antigen presenting cells (APC) *in vitro*. TiO₂ and TiO₂-silica-induced dose-dependent cell death also in macrophages. Due to diverse effects on different nanomaterials on immune cells, it was suggested that each kind of new nanomaterial should be extensively studied *in vitro* and *in vivo* for risk assessment before they are used in final products.

In addition, with the rapid development of nanotechnology and the special properties of nanosized TiO₂, the application fields of nanomaterials are broadening remarkably in some cases. There are many ways to prepare nanotitania, such as sol-gel method, chemical precipitation process, hydrothermal synthesis, and magnetron sputtering technique. The advantages of sol-gel method include simplicity,

high-purity product and small particle size. However, it is very difficult to control the agglomeration of nanosized particles, resulting in the poor performance in final application. Therefore, it is of great significance to solve these problems.

In this study, a highly dispersed nanosized titania particle sol was prepared by conventional low-temperature synthesis method. The resulting sol is homogeneous, and the spindle-like particles are highly dispersed in the whole aqueous system. A consistent set of *in vitro* experimental protocols was used to study the effects of nano-TiO₂ sol as prepared and nano-TiO₂ powder on mouse peritoneal macrophage. The objective was to explore the relationship between the comparable properties with the viability response of macrophage treated *in vitro* with nano-TiO₂ colloid particles and nano-TiO₂ powder, to identify whether particle properties will impact cytotoxicity through altering intracellular oxidative conditions, and to compare the cytotoxicity degree induced by two typical different nano-TiO₂ particles at low treatment concentrations. Accordingly, cytotoxicity was sufficiently measured by the forms as follows. The relative proliferation rate, the cell morphology, the malondialdehyde (MDA), the lactate dehydrogenase (LDH), and the intracellular levels of glutathione (GSH) were determined, respectively.

2. Material and Methods

2.1. Preparation of Titania Sol. 1.1 mL of titanium chloride was added to 10 mL distilled water with ice bath. Aqueous ammonia (10%, in mass, the same below) was dropped into the solution with continual stirring (600 r/min) to form white Ti(OH)₄ precipitate until the pH value reached 9.0 [21]. After further stirring for another 0.5 h, the precipitates were washed with distilled water and centrifugally filtered for 5 min (4000 r/min). This cleaning process was repeated four times to remove the NH₄⁺ and SO₄²⁻ byproducts formed in the reaction. Subsequently, 10 mL (30%) aqueous hydrogen peroxide were poured into the precipitates under constant stirring (400 rpm) to dissolve and disperse the products homogeneously, then the obtained solution was diluted with distilled water until the total volume reached 100 mL. The light yellow solution was added into a reactor and incubated at 120°C for 24 h. Finally, a transparent, homogeneous and light blue colloid as shown in Figure 1 was obtained.

2.2. Characterization of Nano-TiO₂. The dried-crystal powder from titania sol (NP1) and the nano-TiO₂ power (NP2) purchased from Huzheng Nano Technology was analyzed by X-ray powder diffraction (XRD) pattern obtained from D8-Advance X-ray diffractometer with CuKα radiation ($\lambda = 0.15418$ nm) with voltage of 40 kV and current of 30 mA in the region $h = 10\text{--}90^\circ$ with a step size of 0.04° .

The FT-IR spectra of the NP1 and NP2 were collected by Nexus 670 FT-IR spectrometry from the manufacturer of Thermo Nicolet Company and samples were dispersed in pressed KBr disks.

The TEM characterization of the NP1 and NP2 were carried out on an FEI-Techai 1200 instrument with 200 kV accelerating voltage.

FIGURE 1: Optical photographs of the sol.

2.3. In Vitro Culture Cell. Macrophage was collected from normal mouse and washed three times with phosphate-buffered solution (PBS) and modulated cell concentration to 1×10^6 cells·mL⁻¹ with RPMI-1640 medium containing 10 units/mL penicillin, 10 units/mL streptomycin, and 10% fetal bovine serum (FBS) [22]. The collected cells in this manner were viable at least 95% by trypan blue dye exclusion test. The cells were seeded in a 96-well plate directly at a density of 2.0×10^4 cells per well in 200 μ L culture medium, cultured 4 h later at 37°C under a 5% CO₂ atmosphere and humid chamber till adherent to the surface of the cell culture dish.

2.4. In Vitro Assay for Cytotoxic Activity of Nano-TiO₂

2.4.1. Cell Morphology Characterization. Photographs of the interaction of living cells with different concentrations of NP1 and NP2 for 24 h and 48 h were taken, respectively. The morphological microstructure (100 $\times$) was observed using an Olympus IX51 inverted fluorescence microscope.

2.4.2. Cell Viability Assay. After adherent to the surface of the cell culture dish, the supernatant substance were piped out and washed with PBS, and fresh RPMI-1640 medium with nano-TiO₂ NP1 and NP2 diluted to appropriate concentrations (2, 20, 60, and 100 μ g·mL⁻¹) with the culture medium freshly was added into every well, respectively, and incubated for 24 h and 48 h separately. The wells in which cells were not treated with nanoparticles served as positive controls and that without cells but nanoparticles as negative controls in each group of experiments.

The cytotoxicity of NP1 and NP2 on macrophage was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] spectrophotometry. 20 μ L MTT (5 mg·mL⁻¹ in PBS solution) was added to each well treated with NP1 and NP2 and incubated at 37°C for 4 h in incubator thermostat (HEPA-class 100). The medium was

FIGURE 2: XRD pattern of NP1 and NP2.

then removed from each well, and DMSO (150 μ L) was added. The plates were then shaken at Micro Oscillator (Analysis Medical Instrument Factory of Shanghai) and the light absorptions of the formazan (the products of MTT reacted with succinate dehydrogenase and cytochrome C in mitochondria of macrophages) were measured at the wavelength of 490 nm using ELISA detector (BIORAD-Model 680, Japan). The effect of nanoparticles on macrophage proliferation was expressed as the percentage of cell viability compared with the controls or relative proliferation rate (RPR), which is calculated as the following formula:

$$\text{RPR}(\%) = \frac{A - A_N}{A_P - A_N} \times 100\%, \quad (1)$$

where A represents the absorbance of each different concentration group; A_N is the absorbance of negative control group; A_P is the absorbance of positive control group.

2.4.3. Enzymatic Activity Assay. Malondialdehyde (MDA) and glutathione (GSH) reagent kits (purchased from Jiancheng Bioengineering Co. Ltd, Nanjing, China) were employed to indicate the oxidative damage, lactate dehydrogenase (LDH) kit (Jiancheng Bioengineering Co. Ltd, Nanjing, China) to detect the integrity of cell membrane structural caused by nano-TiO₂. Macrophage cells were plated into twenty-four-well plates at a density of 1.0×10^6 cells per well in 2 mL culture medium and cultured 6 h before contaminated. Cells were treated with the nano-TiO₂ NP1 and NP2, respectively, at concentrations of 0, 2, 20, 60, and 100 $\mu\text{g} \cdot \text{mL}^{-1}$ for 24 h and 48 h. Then, collected the macrophage cell supernatant and centrifuged (150 $\times$ g, 10 min), then MDA, GSH, and LDH were, respectively, measured using the reagent kits according to the manufacturer's instructions, and the optical density (OD) was measured using a UV-visible spectrophotometer (UV-9200); the results

FIGURE 3: IR spectrum of NP1 and NP2.

were expressed as percentage viability compared with the untreated controls.

2.5. Statistics Analysis. All experiments were performed in duplicate and repeated at least three times. The statistical significance of the data was expressed as mean $\pm$ SD. Statistical differences among groups were determined by one-way analysis of variance with SPSS 13.0.

3. Results and Discussion

3.1. Characterization of Titania Sol. The optical photographs of the sol are shown in Figure 1. The titania sol is light blue and transparent homogeneously. XRD pattern of the as-prepared nanophase TiO₂ sol (NP1) and nano-TiO₂ power (NP2) is shown in Figure 2. All diffraction peaks of NP1 can be assigned to anatase TiO₂, and the diffraction data are in good agreement with the JCPDS card for titania (JCPDS no. 73-1764). The five major peaks locate at about 25.2°, 37.8°, 47.9°, 54.0°, and 62.7° [23]. And most of the diffraction peaks of NP2 were in accordance with anatase TiO₂, but relatively small amounts of diffraction peaks were assigned to rutile.

Figure 3 shows the IR spectrum of the obtained anatase-TiO₂ particles in colloid and purchased nano-TiO₂ powder. In both of the IR spectra, a broad absorption band in the region 3000–3600 cm^{-1} and the other absorption band in the region 1200–1700 cm^{-1} are characteristic of the OH stretching vibrations (peak at 3421 cm^{-1}) and bending vibrations (peak at 1636 cm^{-1}) of free and hydrogen-bonded surface hydroxyl groups, respectively, [24, 25]. Molecules of water can strongly or weakly be attached to titania surface forming a number of OH stretching and bending vibrations. The vibration modes of anatase skeletal Ti-O-Ti bonds were observed in the range of 500–800 cm^{-1} [26]. No other absorption band was observed in the midinfrared range.

Figures 4 and 5 show the TEM photographs of the sol and purchased nano-TiO₂ powder. Apparently, the particulates of the powder were globular-like, about 50 nm in diameter. The

FIGURE 4: TEM photographs of the sol.

FIGURE 5: TEM photographs of the powder.

particles in the sol were spindle-like, about 20 nm in width and 150 nm in average length, are highly dispersed in the whole aqueous solution. All TiO_2 crystal structures consist of TiO_6^{2-} octahedra, which share edges and corners in different manners that result in forming different crystal phases. Octahedra in anatase share four edges and are arranged in zigzag chains along [221], while rutile octahedra share only two edges and form linear chains parallel to [001] [27]. According to this, the spindle-like particulates are formed in zigzag chains.

3.2. Cytotoxicity Evaluation

3.2.1. Morphologic Changes. Macrophage cells adhered to the plastic surface of the cell culture plate transparently and regularly in spindle shape before added nano- TiO_2 sol (NP1) and nano- TiO_2 powder (NP2) in suspension of PBS (see Figures 6(a) and 6(d), Figures 7(a), and 7(d)). But the particulates in sol were uptaken into the cytoplasm rapidly after added into the medium of macrophage cells for 24 h and 48 h, respectively, (see Figures 6(b) and 6(e)). Then the intracellular aggregated particulates of nano- TiO_2 resided mainly around the nuclear membrane, not in the cytoplasmic region. Some of the particulates of NP2 formed aggregates out of the cell, and some dispersed particulates entered into the cells and resided around the membrane (see

Figures 7(b) and 7(e)). Cell contraction and deformation in variational degree were observed with different concentration of the NP1 and NP2 in the culture medium. It suggested the reduced cell proliferation or cell activity and even cell death after 24 h and 48 h contamination with NP1 and NP2.

3.2.2. Relative Proliferation Rate of Macrophage Cells. The RPR of macrophage cells was used to evaluate the cytotoxicity of nano- TiO_2 sol (NP1) and nano- TiO_2 powder in suspension of PBS (NP2) according to the MTT assay data. The results were given in Table 1. The relative proliferation rate of macrophage cells improved slightly with the increase of concentration of NP1 after contaminated 24 h, but reduced rapidly after contaminated 48 h. It seemed that NP1 had some proliferative effect to macrophage cells in 24 h, but some apparent proliferation inhibition to macrophage cells after contaminated with NP1 in a longer time. And the relative proliferation rate of macrophage cells contaminated with NP2 reduced obviously both in 24 h and 48 h. It suggested that NP2 had apparent and instantaneous proliferation inhibition to macrophage cells.

With the variations of the contacted times and the concentration of NP1 and NP2, the relative proliferation rate of macrophage cells changed. As exogenous substances, nano- TiO_2 particulate in sol could stimulate growth of the macrophage cells during in a short time, thus, shows no apparently toxicity, but when it contacted with nano- TiO_2 sol continuously, most of them were taken up into the cells, even into the nucleus. It may be damaged the membrane or even made oxidative stress and interacted with a number of biological macromolecules, leading to the macrophage death. But in the same way as a foreign substance, the nano- TiO_2 particulate suspended in PBS (NP2) inhibited the growth of the macrophage cells instantly in 24 h and continuously in 48 h. The NP2 without surface treatment was easy to form aggregates outside the cells, which could make cells elicit an immune response and produce a large number of free radicals, leading to the decrease in the cell activity and even to death.

3.2.3. Enzymatic Activity Assay. MDA was an intermediate product of lipid peroxidation, which determined the degree of lipid peroxidation and cell damage indirectly. Normal organism keeps a dynamic balance through its own oxidase system on the free radical oxidation and antioxidant regulation. The lipid peroxidation level increased when the NP1 or NP2 acted with macrophage cells in 24 h and 48 h (see Table 2). But compared with the control group in the 24 h group, MDA content had no significant difference ($P > 0.05$), which suggested that there was no lipid peroxidation during the process of NP1 reacted with the macrophage cells in 24 h. In the 48 h group, MDA content indicated significant difference at 0.05 level or 0.01 level ($P < 0.05$, $P < 0.01$); consequently lipid peroxidation happened. As NP2 acted with macrophage cells, the lipid peroxidation level increased both in the 24 h and 48 h experimental groups. Furthermore, compared with the control groups, MDA content indicated significant difference at 0.05 level or 0.01 level ($P < 0.05$,

FIGURE 6: Morphology of macrophages before and after adding nano-TiO₂ sol with 24 h (a–c) and 48 h (d–f) (400×) (a, d) without nano-TiO₂ sol, (b, e) 60 µg·mL⁻¹, (c, f) 60 µg·mL⁻¹ (after 4 hours incubated with MTT).

FIGURE 7: Morphology of macrophages before and after adding nano-TiO₂ powder in suspension of PBS with 24 h (a–c) and 48 h (d–f) (400×) (a, d) without nano-TiO₂, (b, e) 60 µg·mL⁻¹, (c, f) 60 µg·mL⁻¹ (after 4 hours incubated with MTT).

$P < 0.01$). Therefore, NP2 caused the lipid peroxidation and even damaged the cells.

Glutathione (GSH) was an important oxidant in cells, and its primary function was to eliminate radicals. During the reaction, the glutathione might be oxidized to oxidized glutathione; consequently the antioxidant capacity of cells

was depressed. This made for the maladjustment of redox reaction and the content of active oxygen increasing and impacted the viability of cells. Almost all GSH levels exhibited some changes after 24 h and 48 h exposure to NP1 or NP2 (see Table 2). Statistically GSH contents have significant difference ($P < 0.05$) and even extremely significant

TABLE 1: Result of macrophage toxicity by MTT assay.

Concentration of nano-TiO ₂ /mg·L ⁻¹		0	2	20	60	100
NP1	Absorbance in 24 h	0.830	0.878	0.930	1.003	1.073
	Viability/%	100	106	112	121	129
NP2	Absorbance in 24 h	0.481	0.246	0.216	0.054	0.073
	Viability/%	100	51	45	11	15
NP1	Absorbance in 48 h	0.690	0.630	0.543	0.418	0.198
	Viability/%	100	91	79	61	29
NP2	Absorbance in 48 h	0.244	0.179	0.115	0.101	0.067
	Viability/%	100	73	47	42	27

TABLE 2: The content of MDA, LDH, and GSH in different concentrations of nano-TiO₂.

Time	TiO ₂ (μg/mL)	0	2	20	60	100
24 h	MDA (nmol/mL)	0.40 ± 0.02	0.44 ± 0.05	0.54 ± 0.04	0.74 ± 0.05	1.18 ± 0.08
	NP1 LDH (U/L)	1222.22 ± 93.95	1132.62 ± 79.26	681.00 ± 55.95*	741.94 ± 38.77**	358.42 ± 14.12**
	GSH (mg/L)	7.25 ± 0.59	5.08 ± 0.32**	7.15 ± 0.75*	8.46 ± 0.49	10.66 ± 0.16*
	NP2 MDA (nmol/mL)	1.24 ± 0.03	2.11 ± 0.07**	4.26 ± 0.07**	3.27 ± 0.04**	4.33 ± 0.08**
	LDH (U/L)	738.10 ± 62.99	769.84 ± 49.56	1055.56 ± 49.56**	1150.80 ± 13.75**	1325.39 ± 59.92**
	GSH (mg/L)	8.11 ± 0.22	10.97 ± 0.38**	16.45 ± 0.38**	15.50 ± 0.46**	20.25 ± 0.63**
48 h	MDA (nmol/mL)	0.56 ± 0.04	1.11 ± 0.13	1.41 ± 0.14*	1.57 ± 0.23*	1.97 ± 0.15**
	NP1 LDH (U/L)	197.39 ± 15.92	424.58 ± 27.72	581.00 ± 32.21**	849.16 ± 29.56**	1009.31 ± 46.79**
	GSH (mg/L)	27.33 ± 1.09	21.85 ± 0.88	9.95 ± 0.16**	18.87 ± 0.93*	24.58 ± 0.31*
	NP2 MDA (nmol/mL)	0.94 ± 0.14	1.10 ± 0.14	4.06 ± 0.04**	2.13 ± 0.03**	3.11 ± 0.04**
	LDH (U/L)	854.38 ± 8.62	902.69 ± 12.42*	959.28 ± 4.78**	995.17 ± 6.32**	938.58 ± 10.42**
	GSH (mg/L)	6.37 ± 0.33	8.22 ± 0.25**	11.81 ± 0.19**	9.36 ± 0.25**	10.01 ± 0.41**

* $P < 0.05$, ** $P < 0.01$ (of control).

difference ($P < 0.01$) compared with the control groups. After contaminated to the NP1 or NP2 for 24 h and 48 h at $2\sim 100\mu\text{g}\cdot\text{mL}^{-1}$, the GSH levels fluctuated compared with the control groups, which were not a dose-dependent change. In the final analysis, the GSH levels of the culture supernatant were affected by the quantity of radicals and the damaged degree of membrane. For the penetration of damaged membrane was increased, the intracellular GSH excreted into the extracellular so that the GSH levels of the culture supernatant changed and were not dose dependent markedly.

Lactate dehydrogenase (LDH) was an endoenzyme and could leak to the extracellular space as the membrane was damaged and its penetration was increased. So the Lactate dehydrogenase activity of the macrophage culture supernatant may be reflected the damaged degree of membrane. The NP1 and NP2 induced LDH leakage apparently from macrophage cells treated both for 48 h and 24 h, and LDH levels in cell medium were gradually elevated as particle concentrations increased (see Table 2). Also all of them were statistically significant different except the $2\mu\text{g}\cdot\text{mL}^{-1}$ groups compared with the control groups. In addition, the LDH levels of the macrophage treated with the NP1 for 24 h were not increased, which suggested that damages on the cellular membrane were not obvious. As the contacted time reached 48 h, the LDH levels were increased. Generally the NP2

damaged the cellular membrane obviously both in 24 h and 48 h.

In summary, in 24 h the NP2 had some inhibition on the growth of macrophage cells, while the NP1 had some stimulation on their proliferation to the macrophages superficially which could be seen as an immunostimulant characteristic actually. Within 48 h, both NP1 and NP2 inhibited the growth of macrophage cells significantly with the different concentrations and had some degree of cytotoxicity. The LDH test results indicated that the membrane of macrophage cells was damaged apparently contacted with the NP1 and NP2 for 48 h, but in 24 h the NP1 damages on the cellular membrane were not obvious while the NP2 damages were apparent. The variation regularity of LDH test results was in accordance with the one of relative proliferation rate of macrophage cells. The MDA and GSH test results suggested that the particulate could get through the membrane of macrophage cells and interact with some substances to damage the membrane, which resulted in oxidative stress, decreasing the activity of cells and even induced death. Overall, the MDA and GSH levels responded to the instant cytotoxicity in cells. And the LDH levels and the relative proliferation rate of cells represented the overall effect and continual cytotoxicity reaction. It suggested that the instant cytotoxicity primarily originated from the cellular internalization of foreign substance rather than physical

damage on the cellular membrane. Thus, with the toxicity of nanomaterials, size, shape, chemical composition, and other factors related to the mechanisms of cell toxicity still need further detailed study and discussion.

In contrast to the growing literatures on application of nanomaterials, the information about biological effects of nano-TiO₂ is insufficient, and the publications available on this topic are often controversial. We focused on nano-TiO₂ as examples of typical manufactured nanomaterials that are associated with environmental and occupational exposures. In the present study, we examined the effect of nano-TiO₂ sol and powder concentrations on macrophage cells. It is reasonable to suggest that, according to our results, more attention should be paid to the biosafety evaluation on the reactive metal oxide nanomaterials. Otherwise, the macrophage cells utilized in our study were primary cultured cells from mouse abdomen and could be genetically identical with normal cells *in vivo* while more sensitive to extraneous stimulating factors than cell lines. Though these qualities can help us achieve more practical results; the conclusions should be further tested *in vivo*.

4. Conclusions

A highly dispersed nanosized anatase TiO₂ particle sol was prepared with the conventional low-temperature synthesis method. The sol is transparent and homogeneous. The spindle-like particle size is about 20 nm in width and 150 nm in average length. Moreover, both the particulate in the sol and nano-TiO₂ powder purchased have some cytotoxicity to macrophage cell. The relative proliferation rate of macrophage cells improved slightly with the increasing in concentration of TiO₂ sol after contaminated 24 h, but the relative proliferation rate of cells reduced rapidly after contaminated with nano-TiO₂ sol in 48 h. The purchased nano-TiO₂ powder inhibited the growth of the cells obviously as cultivating with macrophage for 24 h and 48 h. The variation regularity of LDH test results was in accordance with the one of relative proliferation rate of macrophage cells. The MDA and GSH levels responded to the instant cytotoxicity in cells *in vitro*. According to our results, it is reasonable to suggest that more attention should be paid to the biosafety evaluation on the reactive metal oxide nanomaterials.

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