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Morphology of Colloid-Derived Nanostructures and Structure-Induced Cytotoxicity under Electric Potential Stress

Cheng-Han Chao[1], *Yu-Cheng Chang*[2,3], *Yi-Chieh Hsu*[2], *Fu-Ken Liu*[3], *Feng-Chih Chang*[4,5], *Pi-Chuan Lin*[6], *Fu-Hsiang Ko*[2,*]

[1] Division of Nephrology, Department of Internal Medicine, National Taiwan University Hospital Hsin-Chu Branch, Hsinchu 300, Taiwan
[2] Department of Materials Science and Engineering, National Chiao Tung University, Hsinchu 300, Taiwan
[3] Department of Applied Chemistry, National University of Kaohsiung, Kaohsiung 804, Taiwan
[4] Department of Materials and Optoelectronic Science, National Sun Yat-Sen University, Kaohsiung, Taiwan
[5] R&D Center for Membrane Technology, Chung Yuan Christian University, Chungli, Taoyuan, Taiwan
[6] Division of Pharmacy, National Taiwan University Hospital Chu-Tung Branch, Hsinchu County 310, Taiwan
[*]E-mail: fuhsiangko@yahoo.com.tw

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In this study, the morphology of two sizes of nanoparticles under different preparation solvents is evaluated. The vendor marked 5 nm sphere-like nanomaterials are not dispersed in water and toluene solvents, while the vendor marked 1-2 μm nanoparticles are well-dispersed in the water and environments. The behavior of solvent-induced dispersion or aggregation of TiO_2 nanomaterials has less relationship with the solvent polarity, but the dimension of nanomaterials has significant effect on the aggregation or dispersion behaviors. The morphology of nanoparticles from these solvents is, therefore, used to explain the observed cytotoxicity. The cytotoxicity of TiO_2 particles with different sizes and concentrations are evaluated by MTT assay using murine embryotic fibroblast (NIH/3T3 cells). Most of the TiO_2 materials of interest are not cytotoxicity, except for the minor toxic effect to 5 nm spherical nanomaterials at the concentration ranging from $5x10^{-6}$ to $5x10^{-2}$ μg/mL. These spherical nanoparticles with 1-2 μm dimension demonstrate no cytotoxicity for dosages ranging from $5x10^{-6}$ to 50 μg/mL, irrespective of the dosing time and dispersion behavior. The cytotoxicity of applied electrical potential is strongly dependent on the initial size of nanoparticles. The smallest 5 nm nanomaterials seems more to toxicity with duration of electric potential from 10 V stress. On the contrary, the vendor marked 1-2 μm nanoparticles owing to largest sizes demonstrates no significant cytotoxicity.

Keywords: Dispersion, Aggregation, TiO_2 Nanostructure, Cytotoxicity, electric stress.

1. INTRODUCTION

While nanotechnology and the development of nanoparticle-derived materials have emerged as the new global focus for a wide spectrum of basic sciences and applied engineering, it has caused a corresponding concern for the potential health risks both in the manufacturing microenvironments and the ambient air. How may people be exposed to engineered nanoparticles and in what quantities? For example, the nanoparticles can be used for killing localized or deeply seated cancer cells [1]. But, the effect of the nanoparticles on the viability of normal cell is still unclear and a critical challenged issue. All substances, from arsenic, antimony to table salt are toxic to cells, animals or people at some exposure level [2-3]. Prior to interpreting toxicological data, it is thus essential to characterize the expected concentrations and morphologies of engineered nanoparticles. For example, asbestos is classified as a Group 1 human carcinogen by the International Agency for Research on Cancer [4]. Industrial exposures to asbestos have usually been to mixed types of fiber, especially where manufacturing and application are undertaken, for example, textiles, insulation and asbestos cement, and have also occurred in the immediate vicinity. Thin, long fibers (less than 0.5 μm in width and more than 10 μm in length) seem to be most active in producing tumors and are linked with the respiratory ailments [5]. Fibers in these target ranges are the ones most easily inhaled through the respiratory tract into the lungs. In addition, the anthropogenic occurrences of carbon nanotubes and related nanoparticulate aggregates and exposures in various microclimates may contribute to allergies and/or asthma in humans, especially for long-term exposure [6].

Water soluble fullerene derivatives are essential for many emerging biomedical technologies which exploit the unique chemical properties and physical structure of C60. Sayes *et al.* indicates various surface derivatizations on fullerene molecules can significantly affect the human cell viability [7]. The oxidative damage to the cell membranes is responsible for fullerene exposure led to cell death. Titanium dioxide (TiO_2) which is often used as a cosmetic sunscreen has several crystal forms and sizes [8], and also been used as a photocatalyst to eliminate pollutants in the atmosphere [9]. They scatter UV light more than the visible wavelength, preventing sunburn whilst remaining invisible on the skin. Moreover, TiO_2 also absorbs UV light efficiently, catalyzing the formation of superoxides and hydroxyl radicals which can initiate oxidation for pollutant elimination. The International Agency for Research on Cancer [4] proposes the TiO_2 is not mutagenic and hence is safety. However, Uchino *et al.* find the cell viability decreases with the TiO_2 concentration [10]. They attribute the formation of OH radicals upon UV irradiation may induce the cytotoxicity. Yamamoto *et al.* have studied TiO_2 with sizes of 85 nm, 140 nm and 1400 nm, and suggest the cytotoxicity of the larger pieces of particles tended to be higher than that of the smaller ones [11]. However, Oberdorster and co-workers [12] predict the enhanced inflammatory responses for ultrafine nanoparticles when compare to larger sized particles of identical chemical composition at equivalent mass concentrations. Tao's group mentions the relative proliferation rate of macrophage cells is improved slightly after the cells contaminated for 24 h, but it reduced rapidly after 48 hr contamination [13]. The above controversial results seem to reflect the complexities of nanomaterial's sizes on the cytotoxicity.

As we know that the nanomaterials in the medium can be exhibited from well dispersion, semi-dispersion to agglomeration [14-15]. The toxic effect of the same nanoparticle in the dispersion state or

agglomeration state may be quit difference. Hence, the morphology of the nanoparticles has some relationship with the cytotoxicity. Moreover, the shape such as sphere or rod also plays an important role on the cytotoxicity. Prior to investigate the cytotoxicity of nanoparticles, it is inevitable to analyze the nanoparticle-derived nanostructures. The dispersion or agglomeration morphology of nanoscale materials can help to explain the observation of cytotoxicity. In addition, the dissolved metal ion from nanomaterials also carries the toxic effect to cell proliferation [16-17]. Gray and coworkers indicate cell viability could be reduced under strong electric field due to physical damage effect [18]. The understanding of the size- and potential stress-induced cell proliferation is still an important issue to the cytotoxicity.

In this paper, we prepare two sizes of nanomaterials from water and organic phases. We identify the morphology of nanomaterials by scanning electron microscopy (SEM) in order to evaluate the aggregation or dispersion state in the solution. The cytotoxicity of nanostructures at various concentrations is also evaluated by the standard cell protocol. Finally, various concentrations of nanomaterials are used to study the cytotoxicity under different electric potential stresses.

2. EXPERIMENTAL

2.1 Preparation and characterization tools for TiO_2 nanoscale materials

Two different TiO_2 nanomaterials were used in this study. Spherical TiO_2 nanomaterial of 1-2 μm was purchased from Showa Chemical, and the smaller sizes of 5 nm TiO_2 nanomaterial was purchased from Seedchem Company. The TiO_2 nanomaterials mentioned above were put into different polarities of solvent, including polar type (i.e., water) and nonpolar type (i.e., toluene) solvents, and to evaluate the dispersion state of the TiO_2 particles. Then, these samples were dried and characterized by SEM (JEOL JSM-6500F). The instrumental parameters for SEM are operated at 15 kV accelerating potential and 4 mm working distance.

2.2 Biological sample preparation and cell viability for cytotoxicity study

NIH/3T3 cells (murine embryotic fibroblast) were cultured in 96-well flat-bottom plates ($1.2x10^4$/well) in the presence of various nanomaterials (i.e., 5 nm and 1-2 μm) and different TiO_2 concentrations (i.e., $5x10^{-6}$ μg/mL, $5x10^{-4}$ μg/mL, $5x10^{-2}$ μg/mL, $5x10^{-1}$ μg/mL, 5 μg/mL and 50 μg/mL). For the cell line, the cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. Controls were incubated with medium, and with neither vehicle nor compound. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/10% fetal cattle serum (FCS), $5x10^{-5}$ M 2-mercaptoethanol, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2 mM glutamine. After 48 hr of incubation, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay, 5 μg/mL in H_2O, Sigma-Aldrich Co., St. Louis, MO) was added and the cells were incubated for an additional 3 hours at which time 100 μL of supernatant was removed and 100 μL of lysis buffer, containing 10 M HCl in 2-propanol, was added. After several minutes, the MTT crystals formed were solubilized with gentle pipetting and the content of dissolved MTT crystals was measured with a

Molecular Devices VersaMax^{TM} tunable microplate reader set at 570nm. Cell viability assessments or mitochondria/activity of living cells were made by measuring the relative absorbance or optical density for mitochondrial dehydrogenase-transformed formazan (or color product). The cell viability (in %) was initially determined by MTT assay in triplicate for each condition. The experiment of electrical potential stress was operated in a home-made vessel as mentioned early [19]. The cell was subjected to electric voltage of 0, 5 and 10 V for different duration. Then, all the samples were again incubated for additional 24 hr in prior to cell counting.

3. RESULTS AND DISCUSSION

3.1 Morphology of TiO_2 nanomaterials in water phase and organic phase

The dimensions of nanomaterials reported by the vendors are 5 nm and 1-2 μm, respectively. The solution type is critical for the nanomaterial's aggregation or dispersion. SEM morphology onto the silicon substrate is an important means to observe the aggregation or dispersion. Figures 1a and 1b are the SEM morphology for the vendor marked 5 nm TiO_2 nanoparticles. The 5 nm nanoparticles agglomerate into a ~1 μm cluster, and cannot disperse in the water media. This phenomenon seems to reflect that the smallest nanoparticles should minimize the surface activity through the serious aggregation. The dispersion behavior of water solution for 1-2 μm particles is quit difference with the 5 nm particles. Figure 2a indicates the SEM morphology of vendor marked 1-2 μm TiO_2 particles. The image clearly suggests the particles with relatively large dimension can be well dispersed from the water media.

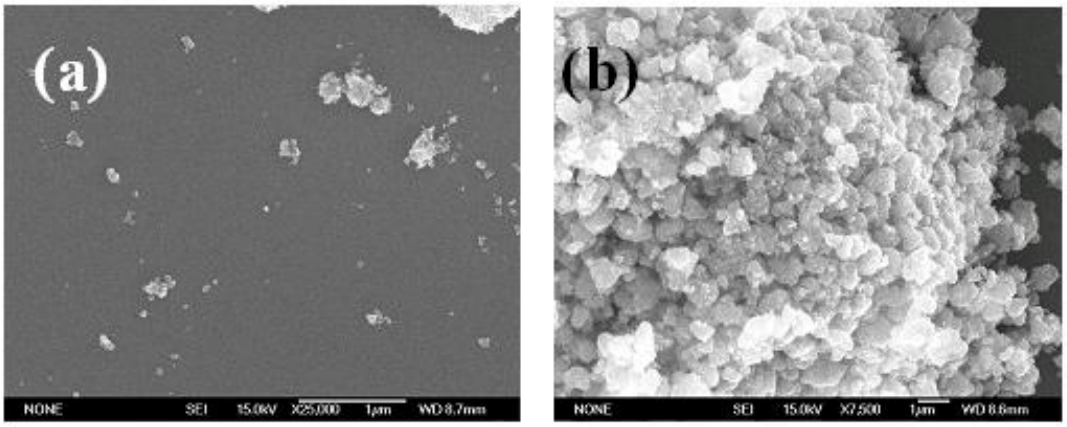

Figure 1. The SEM morphologies for vendor marked 5 nm spherical TiO_2 nanoparticles from water solution: (a) in edge region, (b) in center region.

The actual size distribution is plotted in Fig. 2b, and the size distribution can be used to calculate the average diameter and standard deviation. The actual size estimated from the size distribution is 747±240 nm of which is significantly smaller than the vendor's prediction of 1-2 μm. This observation, in together with the SEM morphology in Figs. 1 and 2a, suggests the smallest 5 nm particles are not easily disperse in the water phase, but the largest 747 nm particles can be well

dispersion in the water. Table 1 summarizes the comparison of two different sizes of TiO_2 nanomaterials from the water phase. We find the TiO_2 nanoparticles with sizes of less than 10 nm can be aggregated to a large cluster from water, even the formation of larger than 1 μm colloid. This result for nanomaterials with various dimensions and behaviors will be more relevant to the explanation of cytotoxicity later. On the contrary, the TiO_2 particles with sizes larger than 700 nm exhibit very good dispersion behavior from the water phase.

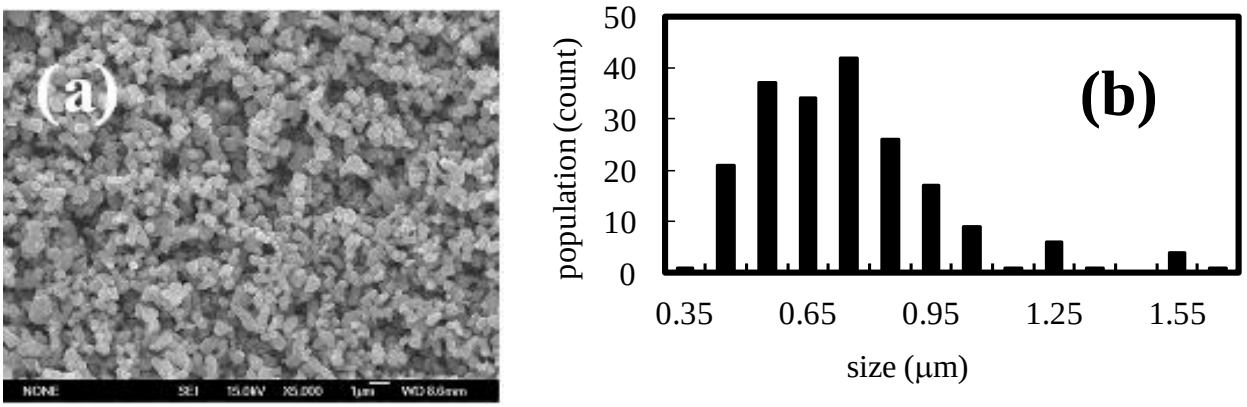

Figure 2. (a) SEM morphologies for vendor marked 1-2 μm spherical TiO_2 nanoparticles from water solution, (b) size distribution of the nanoparticles from water solution.

Table 1. Comparison of vendor marked size of 5 nm and 1-2 μm spherical TiO_2 materials with our SEM characterization size from water and toluene solutions.

solution	vendor marked size (nm)	average size from SEM (nm)	aggregation/dispersion
water	5	-	aggregation
water	1000-2000	747±240	dispersion
toluene	5	-	aggregation
toluene	1000-2000	593±160	dispersion

The dispersion performance of TiO_2 nanomaterials in polar solvent (water medium) has been addressed above. The effect of nonpolar toluene solvent on the TiO_2 dispersion also needs careful consideration. Figures 3a and 3b demonstrate the SEM morphology for the vendor marked 5 nm TiO_2 nanoparticles from the toluene solvent. In similar with the behavior of polar solvent, the 5 nm nanoparticles also seriouly agglomerate into a ~1 μm colloid, and cannot disperse in the toluene solvent. The dispersion behavior of 1-2 μm particles is quit difference with the 5 nm nanoparticles in the organic phase. Figure 4a indicates the SEM morphology of vendor-marked 1-2 μm TiO_2 particles from the toluene solvent. The image clearly suggests the particles can be well dispersed from the toluene media. Although the particles can pack into porous multilayered structures, the size

distribution in Fig. 4b can be used to calculate the average diameter and standard deviation. We find the actual diameter for the particles is 593±160 nm of which is significantly smaller than the vendor's prediction of 1-2 μm. This observation, in together with the SEM morphology in Figs. 3 and 4a, suggests the smallest 5 nm particles are not easily disperse in the organic phase, but the largest 593 nm particles can be well dispersion in the toluent. We find the TiO_2 nanoparticles with sizes of less than 10 nm can be aggregated into a large colloid from nonpolar toluene solvent. On the contrary, the TiO_2 particles with sizes larger than 500 nm exhibit very good dispersion behavior from the toluene. This observation for TiO_2 nanomaterials in polar and nonpolar media suggests the dispersion/aggregation has less relationship with the solvent system. On the other hand, the variation of solvent system can not affect the surface morphology of TiO_2 nanomaterials, while the size dimension has significant effect on the aggregation or dispersion behaviors. This phenomenon reflects that the smallest particles suffers from serious aggregation due to higher surface activity.

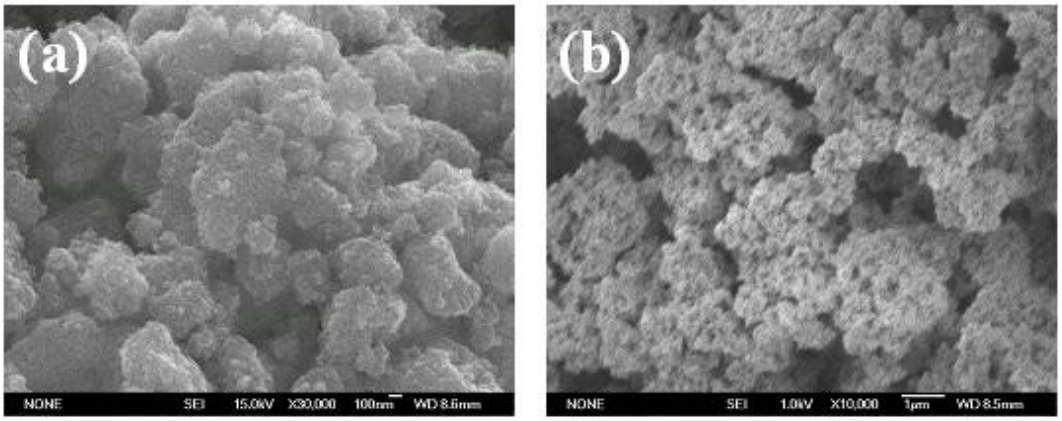

Figure 3. The SEM morphologies for vendor marked 5 nm spherical TiO_2 nanoparticles from toluene solution: (a) in edge region, (b) in center region.

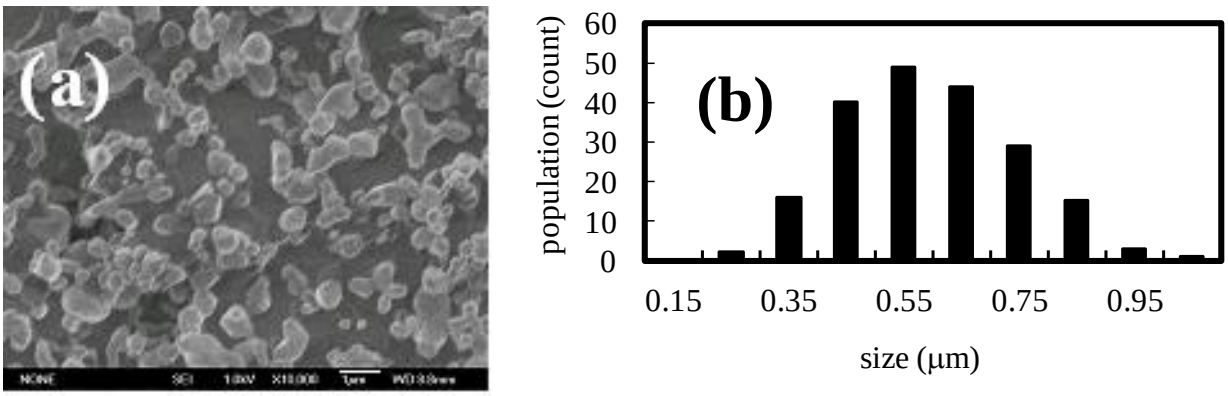

Figure 4. (a) SEM morphologies for vendor marked 1-2 μm spherical TiO_2 nanoparticles from toluene solution, (b) size distribution of the nanoparticles from toluene solution.

3.2 Cytotoxicity of TiO_2 nanomaterials in aqueous buffer solution

The cytotoxicity of nanomaterials has recently been addressed in the literatures [7,20]. Hoshino *et al.* [20] indicates the cytotoxicity of quantum dots for imaging is dependent on their surface molecules. They propose the treatment with MUA for 12 hours causes severe WTK1 cytotoxicity at doses greater than or equal to 50 μg/mL. Sayes and co-workers [7] find 50% human dermal fibroblasts cell death after 48-hour exposure as if the nano-fullerene concentration is increased to 20 ng/mL. These reports mention the nanomaterials may lead to cytotoxicity owing to special morphology or dimension. For the effect of TiO_2 concentrations and sizes on the NIH/3T3 cytotoxicity, we demonstrate the viability result in Table 2 and 3. As Table 2 indicates, the smallest 5 nm TiO_2 has a little bit cytotoxicity (74-83% viability at 24-hour incubation duration) in the concentration ranging from $5x10^{-6}$ μg/mL to $5x10^{-2}$ μg/mL. Although the 5 nm TiO_2 nanomaterials can significantly aggregate from the water or toluene phase, they may diffuse more into the cell solution at the lower TiO_2 concentration and affect the cell viability. The other effect may be related to the dissolution of smallest nanoparticles. The dissolution of nanoparticles into ions also induces the toxic effect. This observation is similar with the seeding mechanism: the agglomeration is stopped due to lack of nanomaterials at the lower concentration. The cytotoxicity is not found as the duration is 48 hours and 72 hours, even more cell proliferation. We infer the cell can adapt to the dissolved 5 nm TiO_2 and culture environment after 48-hour incubation.

The effect of vendor marked 1-2 μm TiO_2 nanparticles on the cell viability is shown in Table 3. The actual size of the nanoparticles after SEM characterization has been reported in Table 1. No significant cytotoxicity for doses ranging from $5x10^{-6}$ to 50 μg/mL is observed from Table 3, irrespective of the dosing duration and dispersion behavior for nanoparticles. In comparison of the cytotoxicity effect in Tables 2 and 3, the smallest 5 nm nanoparticle demonstrate a small portion of toxicity under lower concentration range. However, the largest 1-2 μm TiO_2 nanparticles demonstrate no cytotoxicity effect. As we mention the 5 nm nanoparticle will have serious aggregation, but the vendor marked 1-2 μm TiO_2 nanparticles should well disperse in the water media. The main effect of cytotoxicity is very complicate and requires more experimental design to verify.

Table 2. Effect of various concentrations of vendor marked 5 nm TiO_2 colloid on cell viability.

TiO_2 concentration	viability, %		
	24-hr	48-hr	72-hr
$5x10^{-6}$ μg/mL	78	98	120
$5x10^{-4}$ μg/mL	76	95	115
$5x10^{-2}$ μg/mL	80	92	109
$5x10^{-1}$ μg/mL	98	109	119
5 μg/mL	101	112	113
50 μg/mL	106	115	121

Table 3. Effect of various concentrations of vendor marked 1-2 μm TiO_2 colloid on cell viability.

TiO_2 concentration	viability, %		
	24-hr	48-hr	72-hr
$5x10^{-6}$ μg/mL	94	103	116
$5x10^{-4}$ μg/mL	95	104	126
$5x10^{-2}$ μg/mL	96	102	128
$5x10^{-1}$ μg/mL	97	108	114
5 μg/mL	99	105	110
50 μg/mL	101	103	111

3.3 Cytotoxicity of TiO_2 nanomaterials in aqueous buffer solution under various electric potential stresses

We design the apparatus with controllable electric potential stress to evaluate the cytotoxicity effct for the various nanomaterial concentrations. As to the smallest 5 nm nanoparticles, Figs. 5 and 6 observes the significant cytotoxicity effect under respective electric field of 5 V and 10 V. In Fig. 5, the lower concentration nanoparticles exhibit higher toxicity than high concentration. This observation is related to the aggregation effect for 5 nm nanomaterials under 5 V stress. The potential stress may fluctuate the aggregate nanomaterials with lower concentration region and induce the dissolution effect. Hence, the dissolute ion from the low concentration range material will more induce toxic effect.

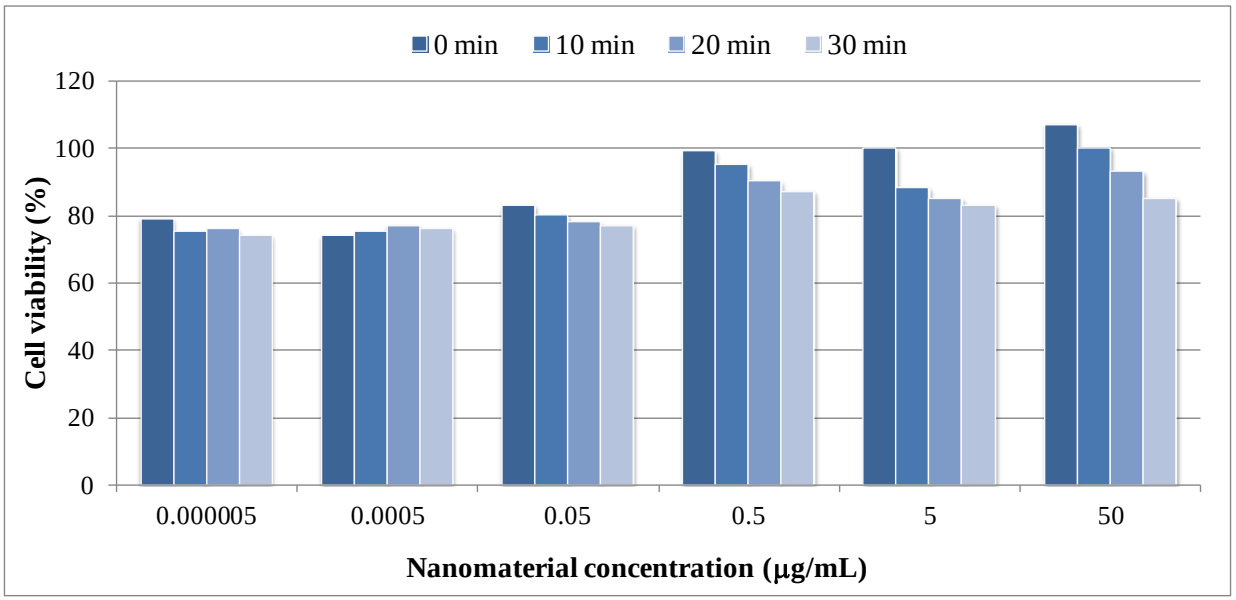

Figure 5. Cell viability under various durations (0, 10, 20 and 30 min) of electric potential circumstance (5 V) for various concentrations of "vendor marked 5 nm" TiO_2 colloids. All samples after electric field treatment are subjected to 24 hr cell incubation.

The duration of electric stress also plays important role on cytotoxicity. As to the high concentration of nanomaterials, the cytotoxicity effect is enhanced with the electric stress duration. This observation suggests the suitable electric stress strength and duration also influences the cytotoxicity effect.

However, the size-induced morphology and nanomaterial concentration change still plays the factor to vary the toxic effect.

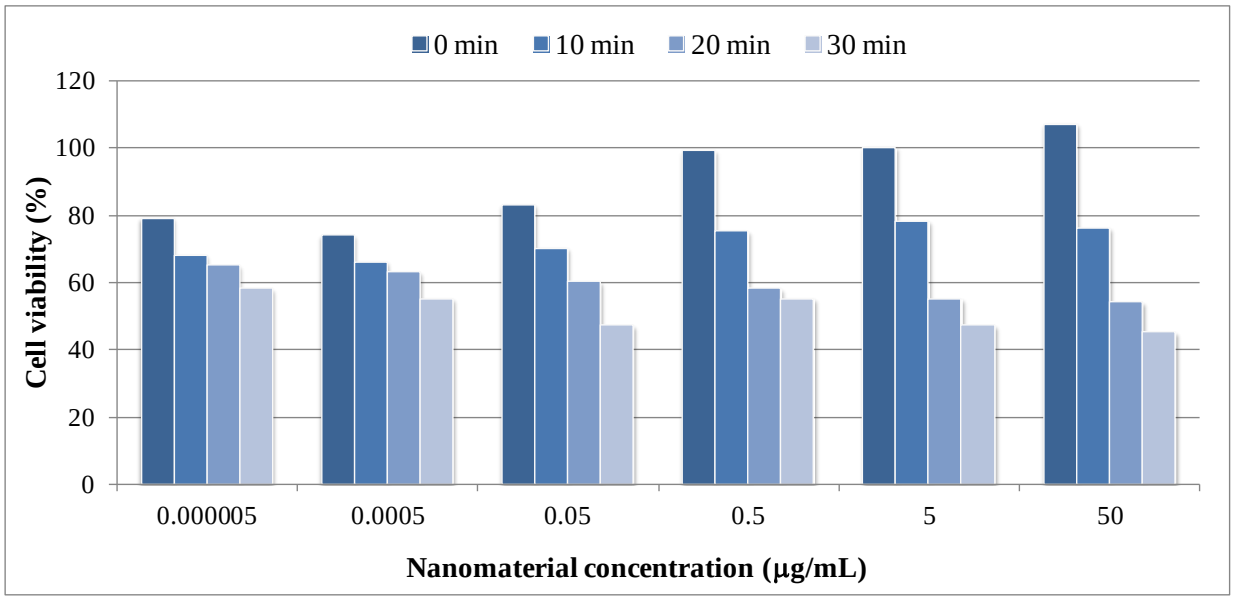

Figure 6. Cell viability under various durations (0, 10, 20 and 30 min) of electric potential circumstance (10 V) for various concentrations of "vendor marked 5 nm" TiO_2 colloids. All samples after electric field treatment are subjected to 24 hr cell incubation.

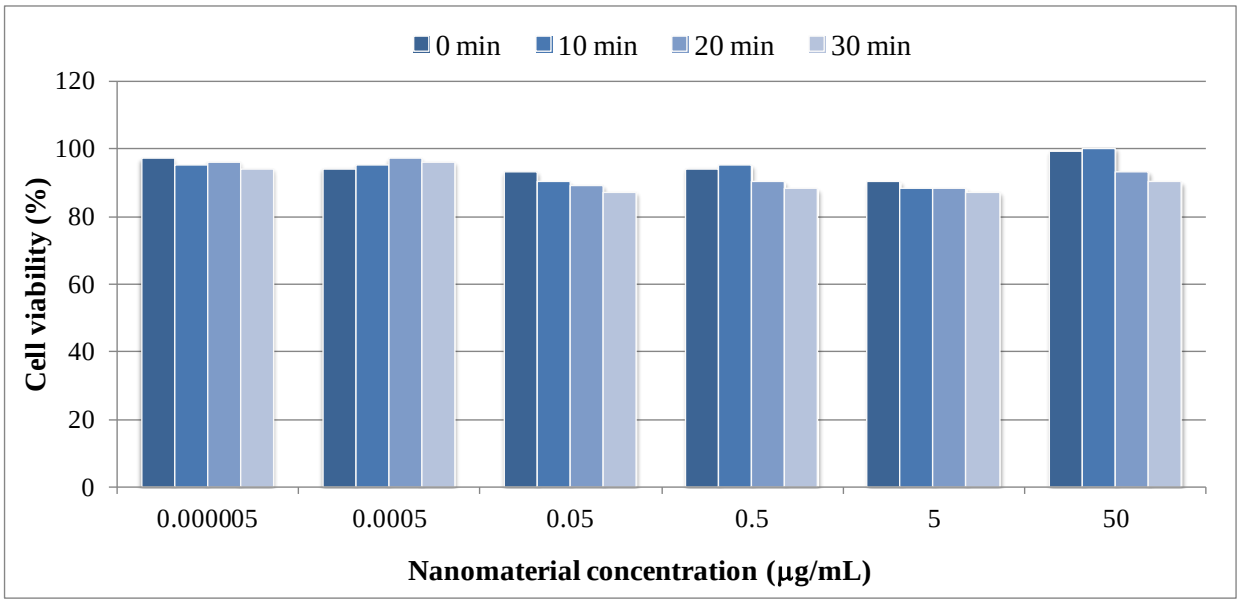

Figure 7. Cell viability under various durations (0, 10, 20 and 30 min) of electric potential circumstance (5 V) for various concentrations of "vendor marked 1-2 μm" TiO_2 colloids. All samples after electric field treatment are subjected to 24 hr cell incubation.

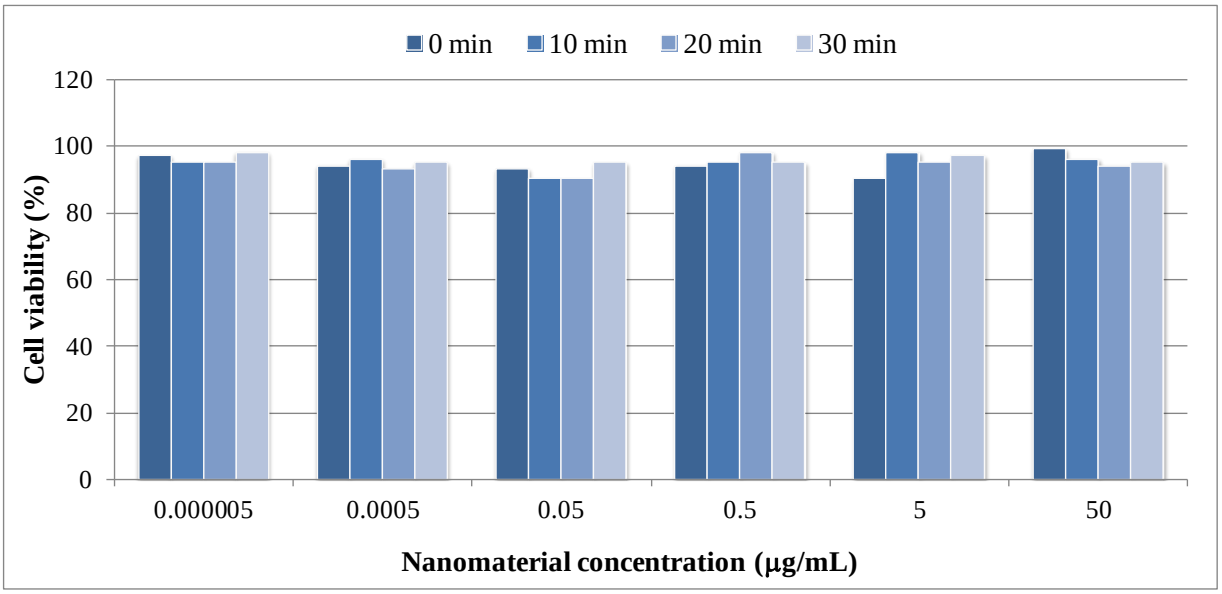

Figure 8. Cell viability under various durations (0, 10, 20 and 30 min) of electric potential circumstance (10 V) for various concentrations of "vendor marked 1-2 μm" TiO_2 colloids. All samples after electric field treatment are subjected to 24 hr cell incubation.

In order to verify the decisive parameter for affecting the cytotoxicity under potential stress, we also control the apparatus to higher 10 V stress in Figs. 7 and 8. Interestingly, the cytotoxicity for the largest nanomaterials of vendor marked 1-2 μm TiO_2 colloids does not demonstrate significant cytotoxicity effect under 5 V or 10 V stress. The electrochemical behavior of average 747 nm nanoparticles seems not to change too much for both voltage stresses. This phenomenon may be ascribed to the large size of nanoparticles. This dimension does not exhibit significant toxicity effect due to the size. The larger size of nanomaterials seems more stable to cell proliferation due to less surface activity in comparison with smallest 5 nm nanoparticles, regardless of aggregation effect. Overall, the cytotoxicity effect is related to many subtle effects such as electric field, nanomaterial morphology, nanomaterial concentration, and dispersion/aggregation state. It is inevitable for more further study to elucidate the real mechanism of cytotoxicity on the issue of nanomaterials.

4. CONCLUSIONS

In this study, we characterize the morphology of two sizes of nanoparticles from polar and apolar solvents. The sizes of TiO_2 less than 10 nm can agglomerate from water and toluene, while sizes higher than 500 nm are well dispersed from both solvents. The vendor marked smallest 5 nm TiO_2 nanoparticle has a little bit cytotoxicity in the concentration ranging from $5x10^{-6}$ μg/mL to $5x10^{-2}$ μg/mL within 24-hour dosing. The cytotoxicity of applied electrical potential is strongly dependent on the initial size of nanoparticles. The smallest 5 nm nanomaterials seems more to toxicity with duration of electric potential from 10 V stress. In contrast, the vendor marked 1-2 μm nanoparticles owing to largest sizes demonstrates no significant cytotoxicity.

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