

The Effect of Chemical Treatment Conditions of Titanium Dioxide Sols on Their Dispersion and Cytotoxic Properties

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The effect of neutralization conditions of TiO₂ acid sol obtained by hydrolysis of titanium tetraisopropoxide (the nature of monovalent cation (Li⁺, Na⁺, NH₄⁺) of electrolyte, chemical nature of a dialyzing solution, and surface modification of nanoparticles with glycidyl isopropyl ether (GE)) on TiO₂ dispersion and cytotoxic properties was investigated. The initial TiO₂ and sols after treatment were examined by a set of physicochemical methods: XRD, CS, SAXS, AFM, and TEM. The in vitro toxic effect of nanoparticles was studied on a monolayer of MDCK (Madin-Darby canine kidney) cells by trypan blue staining. It was shown that chemical nature of the electrolyte cation affects sol agglomeration, which increases in a series Li⁺ > Na⁺ > NH₄⁺, and the use of phosphate buffer for sol dialysis facilitates the formation of more uniform disperse systems. Surface modification of TiO₂ nanoparticles (TiO₂-NPs) with glycidyl isopropyl ether (GE) was found to decrease its toxic effect on the cells.

1. Introduction

The development of new forms of biosafe nanomaterials is among the most topical problems of modern biology and fundamental medicine, solution of this problem allowing a wide use of such materials in practical medicine. Nanoscale titanium dioxide is a promising object of investigation; it can be used for treatment of oncological (Zhang and Sun, 2004; Ferrari, 2005; Thevenot et al., 2008) and viral (Mazurkova et al., 2010) diseases, for fighting against bacteria (Tsuang et al., 2008; Asahara et al., 2009), and for delivery of therapeutic agents to a target gene in the damaged cells (Paunesku et al. 2003, 2007).

Wide application of TiO₂-NPs in practical medicine is hindered by their possible toxic effect on the living organisms (Landsiedel et al., 2010). According to numerous studies, toxic effect of TiO₂-NPs depends on their shape, size, agglomeration state, surface chemistry and concentration (Nel et al., 2006; Wang et al., 2007; Duan et al., 2010). Some papers reported different influence of rutile and anatase TiO₂ on cell viability (Warheit et al., 2007; Falck et al., 2009; Jin et al., 2011). In our earlier work, the effect of TiO₂ structure (amorphous, anatase, brookite, rutile) on response of the NPs treated cells was elucidated (Ryabchikova et al., 2010). It was shown that different crystalline modifications could induce different chemical reactions, and thus alter macromolecular structure of cell membrane differently.

Another problem of NPs application under conditions close to physiological ones (neutral pH, water-soluble state of nanoparticles, presence of salts and proteins) is their tendency to agglomeration and morphological changes (Bihari et al., 2008; Murdock et al., 2008). A low aggregative stability of extremely small NPs and their toxic properties make it necessary to search for special methods of their synthesis in order to improve their dispersion stability and biosafety.

The present work is aimed at searching for possible ways to decrease toxic properties of TiO₂-NPs and increase dispersion stability of the sols in physiological media by optimizing the conditions of TiO₂ sol chemical treatment.

2. Experimental

2.1 Materials

Chemicals and materials were obtained from commercial suppliers: Ti(O-i-C₃H₇)₄ (98 %) (Alfa Aesar), 2-isopropanol (Sigma-Aldrich), LiOH, NaOH, NH₄OH, HNO₃ (Merck), cellulose membrane (ZelluTrans T1, Roth).

2.2 Preparation of TiO₂ nanoparticles

TiO₂ sol was synthesized by the earlier developed technique from titanium tetraisopropoxide (Ti(O-i-C₃H₇)₄, TIP) in the presence of acidic catalyst (Ryabchikova et al., 2010). Then the sol was subjected to chemical treatment: neutralization in 0.2 M aqueous solutions of LiOH, NaOH and NH₄OH, and dialysis against water or a phosphate buffer solution. Some sols were supplemented with GE to provide surface modification of titanium dioxide nanoparticles. The resulting sols had pH close to 7. In the text, samples are designated as A-B-C or A-C, where A is Li or Na or NH₄ (electrolytes used in the work), B is GE, C is W or P (dialysis against water or phosphate buffer).

2.3 Physicochemical examination of TiO₂ samples

X-ray diffraction (XRD) patterns were recorded over a $2\theta = 20 - 85^\circ$ range using an HZG-4C diffractometer with CoK_α ($\lambda = 1.59021\text{\AA}$) radiation.

Small-angle X-ray scattering (SAXS) patterns from the samples of colloidal solutions and sols were measured on the Siemens (Germany) and Hecus (Austria) small-angle X-ray diffractometers. X-ray tubes with copper anode were used as a source of radiation ($\lambda_{\text{CuK}\alpha} = 1.54\text{ \AA}$).

Raman spectra of TiO₂ samples were recorded on a BRUKER RFS 100/S spectrometer in the range of 3600-100 cm⁻¹ at a spectral resolution of 4 cm⁻¹. The 1064 nm line of Nd-YAG laser served as an excitation source. The radiating power varied in the range of 100-200 mW.

Atomic force microscopy images were obtained on a SolverP47Bio atomic force microscope in a semicontact mode. A 20 μL drop was placed on a fresh mica sheet with the surface area of ca. 25-30 mm². Adsorption was carried out for 1 h at room temperature.

TEM images were taken on a JEM 2010 electron microscope with the lattice resolution 0.14 nm at accelerating voltage 200 kV. Samples were deposited by an ultrasonic disperser onto standard carbon-coated copper grids, which were fixed in a holder and introduced in the column of electron microscope.

2.4 Cytotoxicity study

The *in vitro* toxic effect of TiO₂ sols was studied by trypan blue staining on a monolayer of MDCK cells incubated at 37 °C for 2, 4, 6 and 8 h in the presence of TiO₂ sols with a concentration 200 $\mu\text{g/mL}$. Intact cells served as the control cells. The percentage of dead cells was determined on a Leica DM 2500 microscope.

3. Results and Discussion

3.1 XRD and Raman spectroscopy study of the TiO₂-NPs structure

The radiographic examination showed anatase to be the main phase of TiO₂ samples. The diffraction pattern of initial sample (Figure 1) clearly demonstrates typical anatase lines in the 2θ angle range of 29.46, 44.22, 45.14, 56.47 and 63.54. The average size of titanium dioxide crystallite calculated by the Scherrer equation was 4.2 nm. Any variants of chemical treatment produced no changes in the anatase phase and crystallite size.

Raman spectrum of the initial TiO₂ sample (Figure 2) is characterized by a set of bands at 154, 402-410, 519 and 637 cm⁻¹ in the region of Ti-O stretching vibrations typical of the anatase phase. The Raman spectroscopy data are supplemented with XRD data confirming the anatase structure of TiO₂.

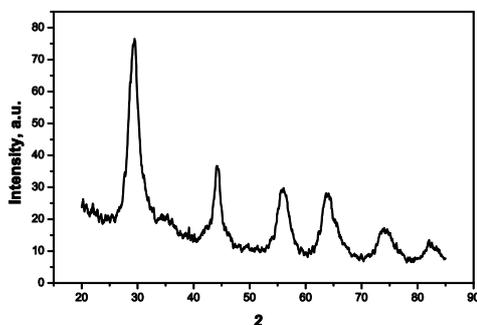


Figure 1: Diffraction pattern of initial TiO₂

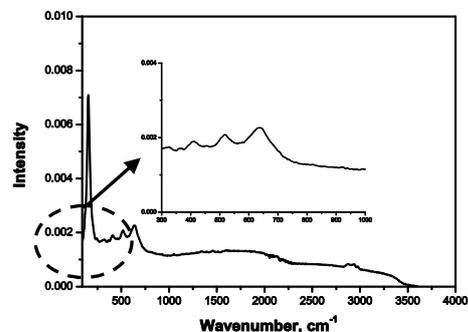


Figure 2: Raman spectra of initial TiO₂

3.2 SAXS, AFM and TEM study of TiO₂ sol dispersion

The initial sol synthesized using the acidic catalyst has a low pH value. Titanium dioxide particles are protonated and likely charged; so, in an acid medium the particles repel one another, and the sol is stable. According to SAXS, the prevailing particle size in the sol is 3-5 nm, which is close to the region of coherent scattering from TiO₂ crystallites. The sols treated with electrolytes are characterized by a similar pattern of particle size distribution and are virtually independent of the introduced electrolyte cation. However, it is known that at neutral pH values close to TiO₂ isoelectric point, the nanoparticles tend to agglomeration and sedimentation. In our experiments, sols after neutralization clouded, which indicates the formation of agglomerates. We suppose that SAXS data reflect the distribution of individual particles in loose agglomerates (but not the size of agglomerates) or primary particles isolated by a dispersion medium.

A real pattern of dispersion state of the neutralized sols was obtained by AFM study (Figure 3). Three-dimensional images make it possible to visualize the particles and determine their state in the sols (isolated, weakly agglomerated or agglomerated). Analysis of AFM data revealed that resistance to agglomeration of the sols depends on the chemical properties of electrolyte and dialysis solution, and on the GE additives. The initial TiO₂ sol (Figure 3a) has a high dispersion. In such sol, the main fraction of particles has a height smaller than 5 nm; the occurrence of larger species, which can be considered as medium size agglomerates (15-20 nm), is nearly 10 times lower. A comparison of AFM images of NH₄-W, Na-W, and Li-W (Figures 3b, 3c, 3d) shows that the size of agglomerates decreases from 300 to 40-70 nm in a series of NH₄⁺ to Li⁺, whereas the dispersion uniformity of sols increases. The dialysis performed against phosphate buffer produces more disperse and uniform sols as compared to dialysis against water (not shown). The same trend is observed in the sols with GE addition and water replaced by phosphate buffer for dialysis, which facilitate stabilization of the sols (Figures 3e, 3f).

TEM data on the sol particle size before and after treatment with electrolytes agree well with the AFM data: the size of agglomerates in the sols increases in a series Li < Na < NH₄. The samples differ not only in dispersion characteristics, but also in morphology.

The use of lithium hydroxide (Li-W) as an electrolyte leads to aggregates larger than 50 nm and loose dendrite splices of crystallites with the particle size of 3-7 nm (Figure 4a). Dense aggregates are virtually absent in the sols. When lithium cation is replaced by sodium (Na-W), this results in the formation of dense agglomerates and aggregates with the size of 200 nm to 2 μm (Figure 4b). With ammonium hydroxide (NH₄-W), the size of aggregates attains 5 μm (Figure 4c). The introduction of glycidyl isopropyl ether in the sols produces virtually no changes in the morphology of sols treated with LiOH and NH₄OH electrolytes (not shown), whereas in the case of NaOH an obvious dispersion with the formation of 80-100 nm agglomerates covered with organic shell (Figure 4d) is observed. As seen from the Figure, the agglomerates are merged by organic phase into extended tracks.

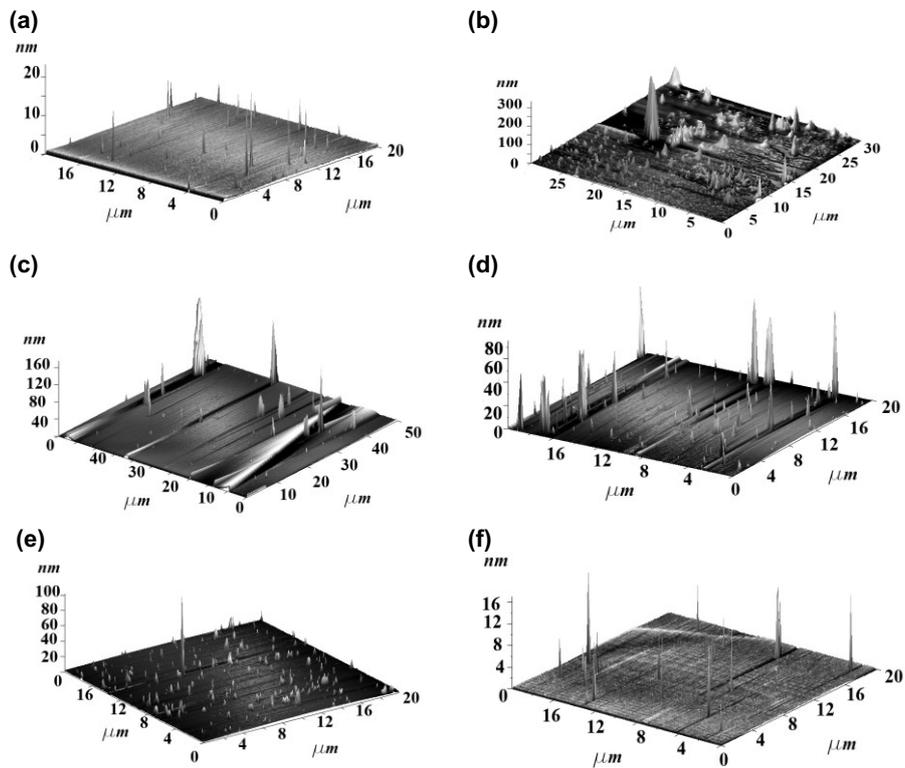


Figure 3: AFM images of TiO_2 sols: initial (a), $\text{NH}_4\text{-W}$ (b), Na-W (c), Li-W (d), Na-GE-P (e), Li-GE-P (f)

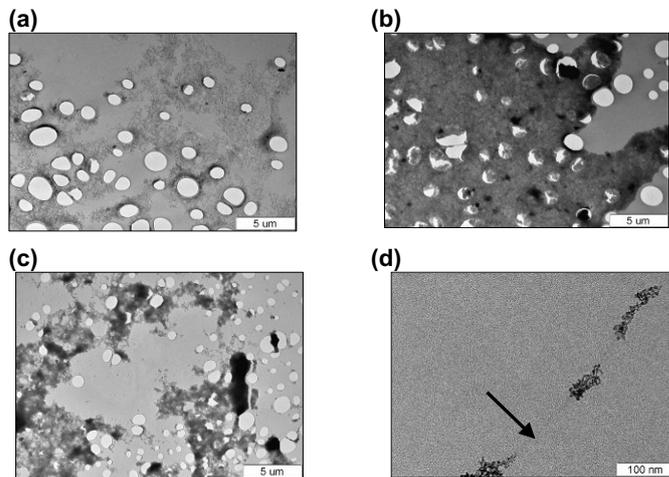


Figure 4: TEM images of TiO_2 sols after treatment: Li-W (a), Na-W (b), $\text{NH}_4\text{-W}$ (c); the Na-GE-W organic track is marked with an arrow (d)

Thus, the main factor affecting the sol resistance to agglomeration is the chemical nature of electrolyte. It can be suggested that morphology of the aggregates is affected by adsorptivity of a cation (Kunz et al., 2004), which changes as $\text{Li}^+ < \text{Na}^+ < \text{NH}_4^+$. Each succeeding ion is adsorbed better than the

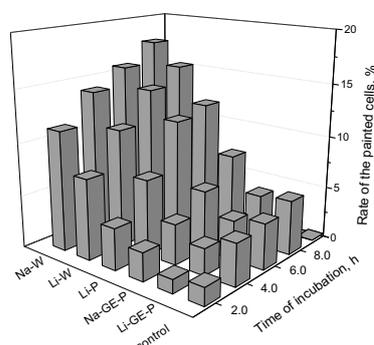


Figure 5: Rate of the stained cells (damaged and dead) after their treatment with TiO_2 nanoparticles, 200 $\mu\text{g}/\text{mL}$. Test with trypan blue

preceding one, thus raising the possibility of solution coagulation when going from lithium to ammonium. In particular, a distinctive visual feature of $\text{NH}_4\text{-W}$ sample was an increased turbidity and appearance of flocculent species with the most strong release of disperse phase as compared with Li-W and Na-W .

3.3 Study of cytotoxicity

Data obtained by testing the cells treated with $\text{TiO}_2\text{-NPs}$ preparation at a 200 mg/mL concentration and the intact (control) cells are displayed as histograms in Figure 5. The histograms show the fraction of trypan blue stained (dead) cells versus the incubation time (2, 4, 6 and 8 hours) and conditions of treatment with $\text{TiO}_2\text{-NPs}$. According to the trypan blue test, the Li-GE-P and Na-GE-P sols have the least damaging effect on MDCK culture cells, which may be related with shielding of their surface by glycidyl isopropyl ether molecules. The Li-P sol (without GE) exerted a moderate damaging effect on the cells as compared to Li-W . In this case, the use of phosphate buffer can lead to surface modification of TiO_2 with phosphate groups, which prevents undesirable TiO_2 reactions with the cell components, thus diminishing the cytotoxic properties of $\text{TiO}_2\text{-NPs}$. A similar effect was observed in another work (Zhu et al., 2007), where the interaction of $\text{TiO}_2\text{-NPs}$ with calf thymus DNA was found to occur in an aqueous medium by the mechanism of TiO_2 binding with DNA phosphate groups, and the absence of this reaction in PBS was revealed. A pronounced damaging effect of Li-W and Na-W sols on MDCK culture cells may be caused by aggressive action of unmodified $\text{TiO}_2\text{-NPs}$ on cell plasma membrane, its mechanisms being thoroughly discussed in (Ryabchikova et al., 2010).

4. Summary

The methods were developed for synthesis of anatase $\text{TiO}_2\text{-NPs}$ having high dispersion stability and low cytotoxicity, which is essential for their application in nanobiomedicine. The chemical nature of the electrolyte cation was found to affect sol agglomeration, which increases in a series $\text{Li}^+ > \text{Na}^+ > \text{NH}_4^+$. It was shown that sols treated with LiOH and NaOH have a more uniform dispersion state. Treatment with a NH_4OH solution leads to formation of nonuniform disperse systems with large agglomerates up to 5 μm in size. Surface modification of $\text{TiO}_2\text{-NPs}$ with glycidyl isopropyl ether produces a protective layer preventing sol agglomeration in a long-term storage and after their incubation with the cells. The *in vitro* cytotoxicity study in a MDCK cell culture demonstrated that surface modification of $\text{TiO}_2\text{-NPs}$ with glycidyl isopropyl ether decreases its toxic effect on the cells. Cytotoxicity of the developed $\text{TiO}_2\text{-NPs}$ preparation did not exceed the level of natural cell death.

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