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 TiO 2 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 glycicydyl isopropyl ether (GE)) on TiO 2 dispersion and cytotoxic properties was investigated. The initial TiO 2 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 TiO 2 nanoparticles (TiO2-NPs) with glycicydyl 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., 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 TiO\textsubscript{2}-NPs and increase dispersion stability of the sols in physiological media by optimizing the conditions of TiO\textsubscript{2} sol chemical treatment.

2. Experimental

2.1 Materials
Chemicals and materials were obtained from commercial suppliers: Ti(O-i-C\textsubscript{3}H\textsubscript{7})\textsubscript{4} (98 %) (Alfa Aesar), 2-isopropanol (Sigma-Aldrich), LiOH, NaOH, NH\textsubscript{4}OH, HNO\textsubscript{3} (Merck), cellulose membrane (ZelluTrans T1, Roth).

2.2 Preparation of TiO\textsubscript{2} nanoparticles
TiO\textsubscript{2} sol was synthesized by the earlier developed technique from titanium tetraisopropoxide (Ti(O-i-C\textsubscript{3}H\textsubscript{7})\textsubscript{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 NH\textsubscript{4}OH, 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 NH\textsubscript{4} (electrolytes used in the work), B is GE, C is W or P (dialysis against water or phosphate buffer).

2.3 Physicochemical examination of TiO\textsubscript{2} samples
X-ray diffraction (XRD) patterns were recorded over a 2\(\theta\) = 20 - 85\(^\circ\) range using an HZG-4C diffractometer with CoK\(\alpha\) (\(\lambda = 1.59021\) Å) 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 (\(\lambda_{\text{CuK}\alpha} = 1.54\) Å).

Raman spectra of TiO\textsubscript{2} 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 mm\(^2\). 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 TiO\textsubscript{2} 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 TiO\textsubscript{2} sols with a concentration 200 µg/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 TiO\textsubscript{2}-NPs structure
The radiographic examination showed anatase to be the main phase of TiO\textsubscript{2} samples. The diffraction pattern of initial sample (Figure 1) clearly demonstrates typical anatase lines in the 2\(\theta\) 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 TiO\textsubscript{2} sample (Figure 2) is characterized by a set of bands at 154, 402-410, 519 and 637 cm\textsuperscript{-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 TiO\textsubscript{2}.

Figure: 1 Diffraction pattern of initial TiO\textsubscript{2}  
Figure 2: Raman spectra of initial TiO\textsubscript{2}

3.2 SAXS, AFM and TEM study of TiO\textsubscript{2} 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 TiO\textsubscript{2} 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 TiO\textsubscript{2} 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 TiO\textsubscript{2} 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 NH\textsubscript{4}-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 NH\textsubscript{4} to Li\textsuperscript{+}, 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 < NH\textsubscript{4}. 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 (NH\textsubscript{4}-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 NH\textsubscript{4}OH 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.
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
3.3 Study of cytotoxicity
Data obtained by testing the cells treated with TiO$_2$-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 TiO$_2$-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 TiO$_2$-NPs. A similar effect was observed in another work (Zhu et al., 2007), where the interaction of TiO$_2$-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 TiO$_2$-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 TiO$_2$-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 Li$^+$ > Na$^+$ > NH$_4^+$. It was shown that sols treated with LiOH and NaOH have a more uniform dispersion state. Treatment with a NH$_4$OH solution leads to formation of nonuniform disperse systems with large agglomerates up to 5 $\mu$m in size. Surface modification of TiO$_2$-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 TiO$_2$-NPs with glycidyl isopropyl ether decreases its toxic effect on the cells. Cytotoxicity of the developed TiO$_2$-NPs preparation did not exceed the level of natural cell death.

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Figure 5: Rate of the stained cells (damaged and dead) after their treatment with TiO$_2$ nanoparticles, 200 $\mu$g/mL. Test with trypan blue.
References


