PHYSICO-CHEMICAL CHARACTERIZATION AND IN VITRO HEMOLYSIS EVALUATION OF TITANIUM DIOXIDE NANOPARTICLES

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Abstract

People have always come into contact with nanoparticles of various chemical structures that entered the human organism by different - respiratory, ingestive, transdermal - paths. The small size of these particles makes them able to penetrate the cell walls; however, their effects and mechanisms of action are far from being known. The benefits of nanoparticles in medicine, as well as their potential adverse effects, depend on their localization and accumulation in the body. In this work, titanium dioxide (TiO₂) anatase nanoparticles were characterized using various techniques: dynamic light scattering (DLS), zeta potential, transmission electron microscopy (TEM) and energy dispersive spectroscopy (EDAX). The size of the TiO₂ nanoparticles dispersed in different aqueous solutions was around 30 nm, and the zeta potential revealed their agglomeration tendency. Hemolysis test indicates nanoparticle concentrations over 400 μg/mL, inducing increased hemolysis effects (>10%).

Keywords: TiO₂ nanoparticles, zeta potential, aggregation, hemolysis

1. INTRODUCTION

Nanoscience and nanotechnology are playing an ever-increasing role in our lives; newer and diverse applications of nanomaterials emerge every day [1]. Nanotechnology deals with small structures/materials with dimensions below 100 nm, of various forms and composition, conferring to them unique (mechanical, thermal, bio-medical and catalytic) properties and recommending them for different applications [2-4]. An evaluation made in 2008 has already established a hierarchical list of nanomaterials that come into contact with and are introduced in the human body by different paths. Titanium oxide (TiO₂) nanoparticles identified as E171 are used as a pigment in paints, as well as in glazes, enamels, plastics, paper, fibers, foods, pharmaceuticals, cosmetics, and toothpastes [5,6]. Titanium, the ninth most abundant element in earth’s crust, continues to be one of the most promising biomaterials for implants, especially as hard tissue replacements, as well as in cardiac and cardiovascular applications. [7]. Titanium does not exist in metallic state in nature, due to its high affinity for oxygen and other elements (the most common oxidation state of Ti is +4) [8]. TiO₂ has long been used as a component for articulating prosthetic implants, especially for hip and knee [9]. Adverse effects of titanium dioxide nanoparticles have recently been uncovered. Latest studies show that human exposure to titanium dioxide nanoparticles occurs through inhalation (inflammation and possible link to asthma), oral and dermal routes [10-12]. Due to their size, these nanoparticles can easily cross the cell membranes and disturb their correct development [13]. For example, small TiO₂ nanoparticles with different geometry can lead to unwanted local and systemic reactions, and may cause cytotoxic effects, which are dose-, cell type- and treatment time-dependent. Their small size and large surface area may cause particles aggregation, making physical handling of nanoparticles difficult in both liquid and dry forms. The key to understanding the toxicity of nanoparticles is that their size, smaller than that
of cells and cellular organelles, allows them to penetrate these basic biological structures, disturbing their normal function; toxic effects include tissue inflammation and altered cellular redox balance toward oxidation, causing abnormal function or cell death [14]. TiO$_2$ nanoparticles are mostly found in aggregate form rather than alone, their aggregation representing an important factor in understanding potential cytotoxicity [15,16]. The effect of the aggregate size of nanoparticles on cells remains unclear. Most of the studies referring to the safe implementation of innovative nanoscience and nanotechnology show that new and rapid strategies and methods should be implemented for TiO$_2$ nanoparticles risk assessment on human health. Hazard identification for risk assessment of TiO$_2$ nanoparticles mainly implies *in vitro* cell-based assays and *in vivo* animal experiments. Present strategies are using *in vitro* toxicological assessment methods focusing on cellular models and using mainly cells from representative organs [17]. Recent studies [18] suggest that, in parallels with the cellular models for nanotoxicity assessment, the direct relationship with the mode of exposure to nanomaterials by evaluating the effect (*in vivo*) on animal models (mice, rats and pigs) should be also tested, in order to define the target organs and to establish a direct relation between nanomaterials’ effect and their accumulation. The toxicological potential of TiO$_2$ nanomaterials is strictly related to their physico-chemical properties, such as shape, size, charge and chemical composition, which requires a high number of tests; therefore, some authors [19] recommended the exclusive use of efficient and reliable methods, which produce results in a short time, and observe ethical issues. In this study, the size and zeta-potential of TiO$_2$ nanoparticles dispersed in different aqueous solutions were determined. The effects of pH on the state of dispersions and the effects of nanoparticles on blood were evaluated using a hemolysis test.

2. MATERIALS AND METHODS

2.1 Materials

Titanium oxide (TiO$_2$, Anatase) nanoparticles – Stock number US 3493, purchased from US Research Nanomaterials, Inc., USA, were transported to the laboratory and stored in a clean and dry location. Sodium chloride (NaCl), sodium hydroxide (NaOH), and hydrogen chloride (HCl) were purchased from Sigma Aldrich.

### 2.2. Nanoparticle characterization

**Morphological characterization**

Morphological characterization of nanoparticles was performed using transmission electron microscopy (TEM) (Hitachi HT7700, Tokyo, Japan). Quantitative analysis was performed using an EDAX system connected to a Quanta 200-type scanning electron microscope (SEM). The samples were prepared by fixing the powder particles onto the microscope holder, with a conducting carbon strip.

Nanoparticles size, granulometric distribution and zeta-potential were determined in triplicate at 25°C, in NaCl solutions with the same ionic strength (0.15 M) and different pH values (3, 5.5 and 7.2), by adding HCl, NaCl, or NaOH, while the mean diameters were determined by laser diffractometry, on samples dispersed in aqueous solutions with different pH values. The zeta potential of nanoparticles was determined using a Malvern Zetasizer Nano ZS equipment, by electrophoresis.

**Haemolysis Assay**

Haemolysis experiments have been performed according to the method of Parameswara Rao Vuddanda et al. [20]. The fresh human blood samples used were obtained from (healthy, non-smoker) volunteers. Initially, 5 mL blood was centrifuged at 1,600 rpm for 5 min. Supernatant plasma surface layer was removed and sediment red blood cells (RBC) pellets separated and washed thoroughly with a normal saline solution. The RBC pellets were diluted with 25 mL of normal saline solution. TiO$_2$ nanoparticle suspensions in saline solution (2 ml) (concentrations between 25µg/ml and 400µg/ml) were added in 2 mL of RBC suspension. Positive (100% lysis) and negative (0% lysis) control samples were prepared. The samples were incubated at 37°C for 6 hrs and slightly shaken once every 30 min. After 2, 4 and 6 hrs, the samples were centrifuged at 1,600 rpm for 5 min. Oxyhemoglobin absorbance has been
measured spectrophotometrically at 540 nm. The percentage of hemolysis was calculated for each sample with equation:

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\text{Hemolysis (\%)} = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{0\% \text{ hemolysis}}}{\text{Abs}_{100\% \text{ hemolysis}} - \text{Abs}_{0\% \text{ hemolysis}}} \times 100
\]

where \( \text{Abs}_{\text{sample}} \) is the absorbance of the supernatant of erythrocytes incubated with nanoparticles suspension, \( \text{Abs}_{0\% \text{ hemolysis}} \) is the absorbance of the supernatant of erythrocytes incubated with saline solution and \( \text{Abs}_{100\% \text{ hemolysis}} \) is the absorbance of the supernatant of the erythrocytes incubated with a Triton X-100 (1\%) saline solution. All samples were analyzed in duplicate.

3. RESULTS AND DISCUSSION

3.1 TiO\(_2\) nanoparticle size and dimensional polydispersity

According to the DLVO theory [21,22], the attraction between particles is due to the van der Waals force. After dispersing TiO\(_2\) nanoparticles in solution, they can remain as such, they may form agglomerates or remain as aggregates, surrounded by a double electrical layer (Fig. 1).

TiO\(_2\) nanoparticles were dispersed in NaCl solutions with the same ionic strength (0.15 M) and different pH values (3, 5.5 and 7.2), at a concentration of 25 \( \mu \)g/ml, and then sonicated for 15 min with a probe sonicator.

It was found out that, for TiO\(_2\) nanoparticles in NaCl solutions, probe sonication can break agglomerates, while also contributing to the agglomeration caused by the enhanced interactions between particles (Fig. 2).

The mean diameter of nanoparticles was around 30 nm, the samples exhibiting a monomodal distribution. It was observed that, with increasing sonication time, the agglomerates size initially decreased, reaching the real size (30 nm), then increased.
3.2 Nanoparticle zeta potential

The zeta-potential test has been applied to investigate the stability of TiO$_2$ nanoparticles dispersion in aqueous solutions. The zeta-potential was measured in an aqueous solution with pH =3, pH=5.5 and pH=7.2, respectively, to simulate physiological conditions. The electrolyte solution (NaCl) was used to determine nanoparticles behaviour in a constant ionic strength environment (0.15 M). The first observation made was that, in an acidic medium (pH=3), the nanoparticles have a positive surface charge and, conversely, at a slightly alkaline pH, a negative surface charge (Fig. 3). At low pH values, the nanoparticles have a higher agglomeration tendency, also reflected in the lower value of the zeta potential. In a slightly alkaline medium, the agglomeration tendency is reduced, and nanoparticles suspension is more stable. One of the major factors involved in the agglomeration process is electrostatic stabilization.

3.3 Nanoparticle morphology

The morphology of TiO$_2$ nanoparticles was evidenced by transmission electron microscopy (TEM), as presented in Figure 4. The nanoparticles are approximately spherical, presenting a strong tendency to form aggregates, favored by the zeta potential values. Their diameter takes values in the range of tens of nanometers, which agrees with laser diffractometry results (3.1).
Energy dispersive X-ray spectrometry (EDX) analysis (Fig. 5) of TiO$_2$ nanoparticles shows peaks for element Ti and oxygen. Such a quantitative analysis proved high titan contents (48%) in the examined samples. Also observed was the presence of oxygen (37%) and carbon (15%), respectively. No traces of any other impurities, which could be seen within the detection limit of EDX, were observed.

3.4 Haemolysis Assay

Another important element in the development of nanoparticulate systems is to determine their ability to cause hemolysis. The interaction of materials with blood components can lead to lysis in human erythrocytes. For this reason, the effects of nanoparticles on blood were evaluated with a hemolysis test. The results of hemolysis assay are shown in Figure 6. The test indicates that almost all samples had permissible hemolysis, except the nanoparticles with a concentration of 400 μg/mL, which showed higher hemolysis values, exceeding the limits (>10%), when compared to the positive control [24].
4. CONCLUSIONS

The key to understanding the toxicity of nanoparticles is that their size, smaller than that of cells and cellular organelles, allows them to penetrate these basic biological structures, disturbing their normal functions; toxic effects include tissue inflammation and altered cellular redox balance towards oxidation, causing abnormal function or cell death. The present study examined and discussed the important parameters governing the state and stability of TiO$_2$ nanoparticle dispersions - including size, solution ionic strength, pH and surface charge. The obtained results have important impact in further toxicological studies. Transmission electron microscopy (TEM) and laser diffractometry analysis revealed that nanoparticles are almost spherical and have a diameter of about 30 nm. The zeta potential showed that the agglomeration tendency of the TiO$_2$ nanoparticles in aqueous solutions decreases with increasing the pH of the dispersing medium. Hemolysis test indicated that almost all samples had permissible hemolysis, except the nanoparticles with a concentration of 400 μg/mL, which showed higher hemolysis, exceeding the limits (>10%), comparatively with the positive control.

References

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