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Grau de Química

Treball Final de Grau

Effect of TiO_2 particle size: haemolytic activity and cell viability determination interference.

Influència de la mida de la partícula del TiO_2: activitat hemolítica i interferències en la determinació de la viabilitat cel·lular.

Laura Bescós Tenas

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B:KC Barcelona Knowledge Campus
Campus d'Excel·lència Internacional

As long as the world shall last there will be wrongs, and if no man objected and no man rebelled, those wrongs would last forever.

Clarence Darrow

M'agradaria agrair a totes les persones que d'una manera o una altra han fet possible aquest treball.

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REPORT

IDENTIFICATION AND REFLECTION ON THE SUSTAINABLE DEVELOPMENT GOALS (SDG)

The Sustainable Development Goals (SDGs) are a set of 17 interconnected global goals established by the United Nations. They mean to address various social, economic, and environmental challenges facing the world and to guide international efforts toward a more sustainable and equitable future. They are grouped into five areas: people, prosperity, planet, peace, and partnership.

The prevention of skin cancer and other skin-related diseases that are caused by the radiation is one key aspect of the well-being of society. Society plays a decisive role in the three dimensions of sustainability, which is why this project would impact the **Good Health and Well-Being** category, included in the People area. The increased public understanding of the protection offered by sunscreens and the importance of protecting ourselves from radiation has proven highly effective in reducing the mortality cases of skin cancer. It is crucial to ensure skin health and promote it for all at all ages.

This study aims to confirm that a particular UV filter, TiO_2, aside from protecting us from UV radiation, does not harm skin cells or red blood cells which could cause negative health effects. The findings of this study affirm that this TiO_2 UV filter qualifies as an exceptionally effective filter. It exhibits an extensive UV protection range without causing harm to the users, thereby improving the well-being of all.

CONTENTS

1. SUMMARY

It is well known that ultraviolet radiation harms the skin and can lead to skin cancer. The correct use of sunscreen is the most efficient way to prevent the development of this disease. The photoprotection characteristic of sunscreens is mainly attributed to their constituent UV filters, which can be organic or inorganic. A frequently used inorganic filter is TiO_2, both in its micro and nano forms. Micro-TiO_2 has a whitening effect in sunscreen formulations, decreasing their aesthetic appeal, which can be resolved by using the nano form of TiO_2. Due to the greater surface area/volume ratio, nanoparticles become more (bio)reactive giving rise to concerns about their potential toxicity, for instance generating Reactive Oxygen Species (ROS) known to be implicated in cellular damage.

This study compares the toxic behaviour of two commercially available TiO_2 at a micro and nanoscale. For this purpose, the toxicity of this compound on HaCaT epidermal keratinocytes is assessed. Prior to these cytotoxicity studies, the potential interferences when using colorimetric methods (MTT, NRU and LDH) needed to be discarded. Additionally, the haemolytic effect of TiO_2 on human red blood cells is studied and nano-TiO_2 is characterized through TEM.

Results show that neither micro-TiO_2 nor nano-TiO_2 exhibit considerable haemolysis on red blood cells, even though nanoparticles display higher haemolysis values. The particle size of nano-TiO_2 has been confirmed by TEM. No chemical interferences of TiO_2 with the reactants of the MTT, NRU or LDH methods are found, although the highly insoluble TiO_2 particles could interfere with the absorbance measurement. Finally, TiO_2 does not exhibit cytotoxicity to HaCaT epidermal keratinocytes for NRU and LDH, although a reduction in cell viability in MTT for NPs was observed.

Keywords: TiO_2 nanoparticles, UV filters, cytotoxicity, haemolytic effect.

2. RESUM

La radiació ultraviolada lesiona la pell i pot donar lloc al càncer de pell. L'ús correcte del protector solar és la manera més eficient de prevenir el desenvolupament d'aquesta malaltia. La protecció de les cremes solars s'atribueix principalment als seus filtres UV, que poden ser orgànics o inorgànics. Un filtre inorgànic freqüentment utilitzat és el TiO_2, tant en la forma micro com nano. El micro-TiO_2 provoca una coloració blanca en les formulacions de les cremes solars, disminuint el seu atractiu estètic, però es pot resoldre emprant la forma nano. A causa de l'augment de la relació superfície/volum, les nanopartícules es tornen més (bio)reactives, generant preocupacions sobre la seva possible toxicitat, com ara la generació d'espècies reactives d'oxigen (ROS) conegudes per estar implicades en la lesió cel·lular.

Aquest estudi compara el comportament tòxic de dos TiO_2 disponibles comercialment en una escala micro i nanomètrica. Amb aquesta finalitat, la toxicitat d'aquest compost sobre els queratinòcits epidèrmics HaCaT és avaluada. Abans d'aquests estudis de citotoxicitat, es van descartar les possibles interferències de les partícules en els mètodes de mesura utilitzats pels mètodes colorimètrics (MTT, NRU i LDH). Addicionalment, es va estudiar l'efecte hemolític del TiO_2 sobre els glòbuls vermells d'origen humà i s'ha caracteritzat el nano-TiO_2 mitjançant TEM.

Els resultats mostren que cap de les dues formes de TiO_2 mostren una hemòlisi considerable en els glòbuls vermells, tot i que les nanopartícules presenten valors d'hemòlisi més alts. La mida de partícula del nano-TiO_2 s'ha confirmat mitjançant TEM. No es van trobar interaccions químiques del TiO_2 amb els reactius dels mètodes MTT, NRU o LDH, tot i que les partícules molt insolubles de TiO_2 podrien interferir amb la mesura d'absorbància. Finalment, el TiO_2 no va presentar citotoxicitat sobre els queratinòcits epidèrmics HaCaT, tot i que es va observar una lleugera reducció en la viabilitat cel·lular en MTT i LDH per a les NP.

Paraules clau: nanopartícules TiO_2, filtres UV, citotoxicitat, efecte hemolític.

3. INTRODUCTION

3.1. GENERAL CONCERNS

Exposure to ultraviolet (UV) radiation can result in many harmful effects on the skin, especially skin cancer and photoaging. Skin cancers are the most diagnosed group of cancers worldwide, with an estimation of more than 1.5 million new cases for 2020. Although the risk of melanoma increases with age and incidence of sunlight, melanoma is among the most common cancers in young adults. Malignant melanomas, constituting approximately 1 in 5 of these cases, accounted for around 325,000 instances worldwide. The highest incidence rates for melanoma were observed in Australia/New Zealand (42 per 100,000 person-year, males; 31 per 100,000 person-year, females) followed by Western Europe (19 per 100,000 person-year). However, melanoma remains rare in most African and Asian countries, with incidence rates below 1 per 100,000 person-year [1].

High quality evidence has proven that sunscreen reduces the risk of developing both melanoma and nonmelanoma skin cancer. Thanks to extensive marketing promotion, there has been an increase in public awareness of the protection offered by sunscreens against photodamage to the skin. Therefore, the global sunscreen market has grown substantially.

3.2. UV FILTERS

Sunscreens are a complex mixture of active and inactive ingredients, and can be found in various forms (lotions, sprays, roll-ons). The photoprotection characteristic of sunscreens is mainly attributed to their constituent UV filters. Additionally, the exposure to these filters extends beyond sunscreens, many cosmetic products now include the filters, in response to a consumer desire for better UV protection in a very competitive marketplace. Moreover, UV filters can be used to protect those products from the damage caused by the UV component of sunlight, for instance, in hair care products to protect the colour of dyed hair.

There are two main types of UV filters: [2]

- **Chemical (organic) filters**: these filters absorb mostly UVB (290-320 nm) radiation and transform it into harmless heat. Examples include avobenzone, octocrylene and octinoxate.
- **Physical (inorganic) filters:** Initially were thought to block UVB/UVA radiation (290-320 nm for UVB, 320-400 nm for UVA) through reflection and scattering (hence the term *physical*). It has been proven however that these filters provide UV protection mostly from absorption of UV radiation and not through scattering or reflection [3]. Examples include titanium oxide and zinc oxide.

Some organic filters have been reported to cause skin irritation, limited skin penetration, photoallergy, less photostability… [4] Inorganic filters provide immediate protection upon applications and usually have a broader spectrum. Nevertheless, each UV filter has a wavelength range where it is most protective. Therefore, most sunscreens contain mixtures of UV filters to provide broad-spectrum protection, covering both UVA and UVB rays.

In Table 1, a summary of common UV filters, their protective range, and their maximum percentage concentration in sunscreens is provided [5]. As observed, only the inorganic filters can offer a broad range of protection, especially ZnO, that covers both UVA and UVB. Inorganic filters can be included safely as up to 25% of the formulation whereas organic filters exhibit a lower permitted value. In reference [5], the absorption of UV radiation based on 1% concentrations is represented. The concentration of the filter in the sunscreen affects how protective it is in its wavelength range. The higher the required concentration of a filter to protect from UV radiation, the less efficient it is. Figure 2.3 of the reference illustrates that inorganic filters, specially TiO_2, are the most inefficient in contrast to Octinoxate or PBSA (also called Ensulizole) that are the most efficient filters. In contrast, inorganic filters can be used in higher concentrations due to their lower toxicity.

There are two mechanisms of protection against UV radiation (Figure 1): as mentioned above, inorganic filters can either reflect, scatter and/or absorb UV radiation; organic filters receive the energy of UV photons and can act in three ways: undergoing conformational molecular changes, emitting radiation at higher wavelengths, or releasing incident energy as heat. This action mode is reversible; thus, the same molecule can function repeatedly [6].

UV filter	Protective range (UVA1, UVA2, UVB)	Maximum % concentrations (weight per volume)
Aminobenzoic acid	UVB	15
Avobenzone	UVA1	3
Ensulizole	UVB	4
Octinoxate	UVB	7.5
Oxybenzone	UVA2, UVB	6
TiO_2	UVA2, UVB	25
ZnO	UVA1, UVA2, UVB	25

Table 1. Summary of common UV filters. Data extracted from reference [5].

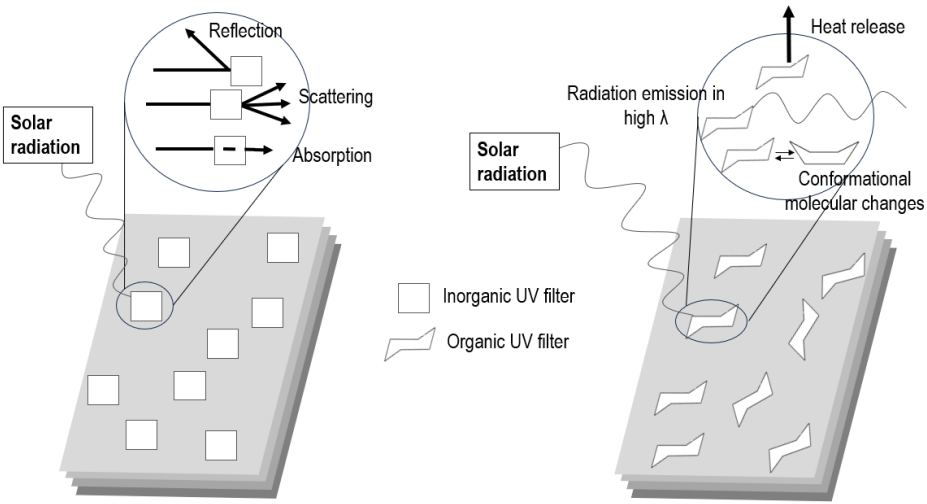

Figure 1. Mechanism of inorganic (left) and organic (right) UV filters.

The most common UV filters include titanium oxide as an inorganic filter, ethylhexyl methoxycinnamate as an organic UVB filter and butyl methoxydibenzoylmethane as an organic UVA filter. Titanium dioxide remains prevalent in facial care, powder foundation, lip colour, hair, and nail products, and is a top choice for UV protection in body care and cleansers, emphasizing its broad application in skincare and cosmetics [7].

3.3. TiO_2 AS AN UV FILTER

Titanium oxide is an inorganic compound widely used in a variety of products since the early twentieth century. Currently, this molecule has found extensive applications in various products, including paints, cosmetics, orthodontic composites, and food. In cosmetics, TiO_2 can be used either as a white pigment in its microcrystalline form or as an inorganic UV filter in sunscreens, day creams, foundations, and lip balms, to provide protection against the harmful effects of UV radiation in its nano form. In nature, TiO_2 has three polymorphs: rutile, anatase and brookite (Figure 2). Rutile is the most common and stable form of this pigment, while anatase and brookite are metastable phases.

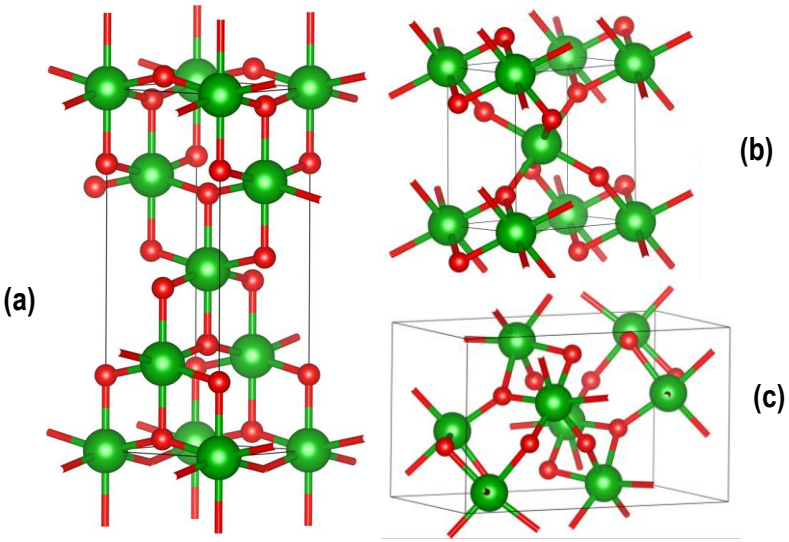

Figure 2. Schematic cells for anatase (a), rutile (b) and brookite (c) TiO_2. The green spheres represent Ti atoms, and the red spheres represent O atoms. Images represented with Vesta Program.

3.3.1. Presence of micro- and nano-TiO_2 in sunscreens

A frequently used inorganic filter is micro-TiO_2 (average size of 0.1-10.0 μm). One drawback is the potential to leave a visible white residue on the skin (white cast). In this case, the white tint in the sunscreen does not blend with darker skin and can make their skin appear slightly white. This issue can be corrected by utilising nano- instead of micro-TiO_2. It has been demonstrated that, although the opaqueness of inorganic filters in sunscreens is reduced when the particle size decreases to 10-20 nm, the UVA absorption decreases and shifts it to the UVB area [8].

Given that TiO_2 is a semiconductor, when UV radiation is directed to the inorganic filter, an electron is excited into a higher energy orbital state (from the valence band to a conduction band, Figure 3). Particle size influences the amount of UV radiation that is scattered/reflected. Larger particles reflect/scatter more UV radiation than smaller particles. Nanoparticles (NPs) reflect less visible light, therefore appearing nearly transparent, compared to the white appearance of larger particles. The Australian Therapeutic Goods Administration estimated that, in 2005, the 70% of all sunscreens containing TiO_2 and the 30% containing ZnO were formulated with NPs [9].

In summary, substituting micro-TiO_2 with NPs ensures the cosmetically desired transparency in sunscreens, but at the expense of broad UVA protection.

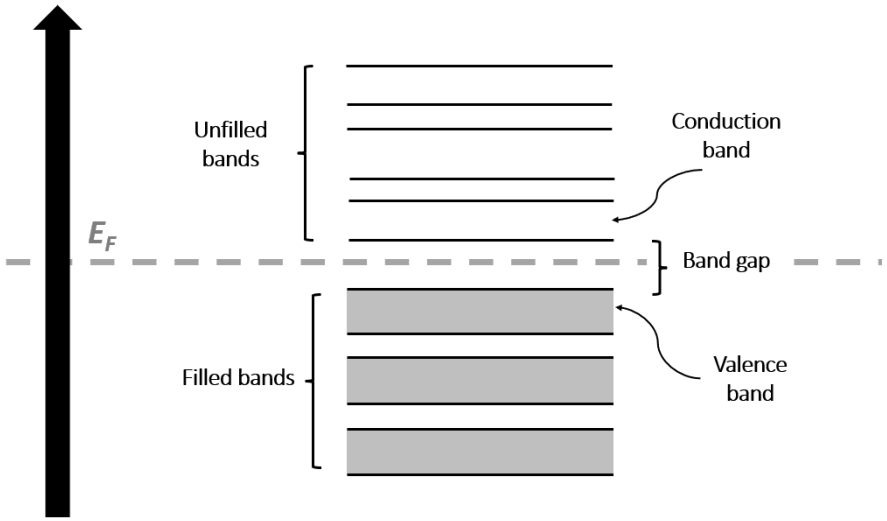

Figure 3. Semiconductor band structure.

3.3.2. Toxicity of TiO_2 NPs

Given that surface area to volume ratio of particles increases as the particle diameter decreases, NPs may be more (bio)reactive compared to normal bulk materials. That is why the safety of cosmetic products containing NPs has been frequently discussed. Firstly, nano-TiO_2 is photoreactive with a resulting increase in Reactive Oxygen Species (ROS) known to be implicated in cellular damage. ROS are highly reactive molecules that contain oxygen atoms and one or more unpaired electrons. They can cause different problems such as oxidation of phospholipids, oxidation of DNA, rupture of DNA or oxidation of proteins. Examples of ROS include the superoxide anion, $O_2^{\bullet -}$, hydrogen peroxide, H_2O_2, or hydroxyl radical, $^{\bullet}OH$. The absorption of UV light by TiO_2 (Figure 4) results in excitation of a valence band electron (e^-) to the conduction band, leaving a hole (h^+) in the valence band. The electron is transferred to molecular oxygen, generating the superoxide radical anion ($O_2^{\bullet -}$). The hydroxyl radical ($^{\bullet}OH$) is formed by the release of an electron from water hydroxyl anions (OH^-) catalysed by the holes (h^+). Additionally, in acidic media, the superoxide radical anion ($O_2^{\bullet -}$) is protonated yielding the hydroperoxyl radical ($HO_2^{\bullet}$) and ultimately hydrogen peroxide (H_2O_2). Hydrogen peroxide can also be produced by a radical chain termination step involving $OH^{\bullet}$ radicals [8].

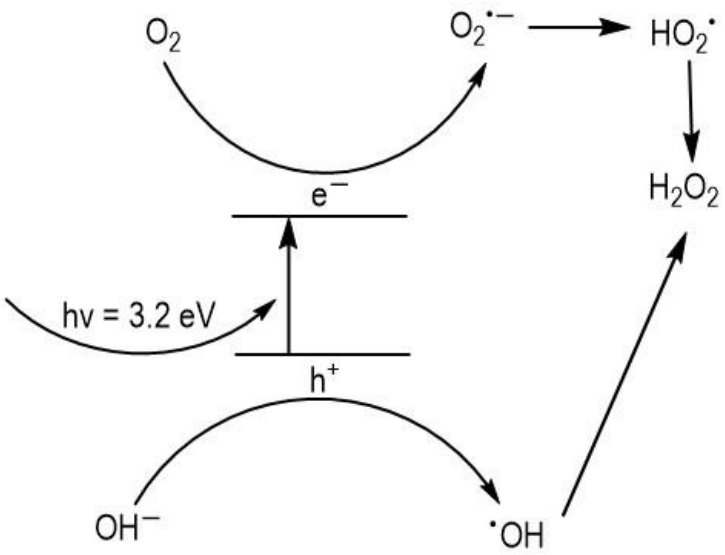

Figure 4. Schematic representation of the reactions that lead to the production of ROS.

This issue has been solved by coating nanoparticles with alumina or silica to quench the production of ROS. Furthermore, coating the NPs not only addresses the ROS production issue but also enhances the dispersion of TiO_2 nanoparticles and their compatibility with other ingredients in sunscreens formulations. As a result, nano-TiO_2 is always used in its coated form in cosmetics [10].

Yet another important concern revolves around the size of nano-TiO_2, raising suspicion regarding its ability to penetrate dermal, respiratory, or gastrointestinal barriers, disseminate in the body and therefore to constitute a potential risk to the consumer. While sunscreens and other cosmetics offering UV protection are typically applied to the skin, sometimes they are also available in sprayable forms, potentially exposing the consumers to nano-TiO_2 through inhalation. Furthermore, some manufacturers may also incorporate titanium oxide into UV-protective lip balms that could be incidentally ingested. Therefore, the potential harmful effects of the NPs in cosmetics need to be considered in the context of inhalation and oral ingestion, in addition to the possible dermal penetration.

The skin is composed of the epidermis and dermis, the former containing the *stratum corneum*, which is the upper epidermal layer and the skin's primary protection against percutaneous penetration. Certain substances have the ability to penetrate the skin's layers and reach underlying tissues, bloodstream… Chemicals may be absorbed through skin by different pathways such as through sweat glands or through hair follicles. Skin diseases can increase the risk of penetration, leading to a possible sensitization [9]. Sensitization has symptoms similar to irritation, but they are caused by the body's immune reactivity to the product, whereas irritation is caused by the product itself. Moreover, other factors such as gender, skin thickness, hair follicle density, and age may influence the skin barrier function.

The data regarding the potential of dermal absorption and/or penetration of TiO_2 NPs from sunscreens have exhibited controversial results. According to the review of Larese Filon [9], NPs can penetrate the skin dependent on their size: NPs ≤ 4 nm can penetrate and permeate intact skin; 4<NPs<20 nm can potentially permeate intact and damaged skin, 21<NPs<45 nm can penetrate and permeate only damaged skin, and finally, NPs > 45 nm cannot penetrate nor permeate skin. In contrast, many studies analysed in 2013-2014 by The Scientific Committee on Consumer Safety (SCCS), an official European committee that provides opinions on health and

safety risks of a variety of products, concluded that nano-TiO_2 stays on the skin after application of the sunscreen, and only a small proportion of NPs were likely to penetrate deeper into the *stratum corneum*, not reaching the deeper epidermis or dermis cells. The SCCS concluded in 2020, in its final version of the Opinion on TiO_2, that TiO_2 is of low acute toxicity by the oral and dermal routes, and exposure results in slight or no irritation to skin and mucous membranes [11].

As said before, a not intact *stratum corneum* increases the permeability of the skin, enabling NPs to penetrate the different layers of the skin. The *stratum corneum* can be altered by different factors, such as mechanical damage, chemical irritants, UV exposure and inflammatory infiltrates. Disruptions in skin integrity (e.g. atopic dermatitis and psoriasis) allow NPs to penetrate deeper dermal layers. This raises concerns about the potential implications of applying TiO_2-containing cosmetics to all skin types [12]. Moreover, some studies found that, after a prolonged application of TiO_2 NPs sunscreens in healthy human skin, these NPs were detected in the epidermis and dermis, indicating that the TiO_2 NPs could, in fact, penetrate deeper layers of the skin [13].

Another important aspect was to determine whether these NPs could have harmful effects after inhalation. In light of this, the SCCS classified this oxide is as carcinogen via inhalation and therefore is considered unsafe to use in a hair styling aerosol spray product for either general consumers or hairdressers. The lungs are unable to clear nanoparticles raising the probability of high concentrations in the alveolae and potential absorption into the bloodstream. If this were to occur, there is potential for damage to internal organs [14]. Consequently, TiO_2 is only permitted up to a maximum concentration of 1.4% for general consumers and 1.1% for hairdressers. The safety assessment conducted by the SCCS demonstrated that the use of TiO_2 in loose powder up to a maximum concentration of 25% in a face make-up application is safe for the general consumer. The SCCS also noted that, while the inhalation is also possible through a powder product, in this products TiO_2 is only a minor constituent and does not entail any risk [11].

In summary, although SCCS considered TiO_2 NPs to be safe for use in cosmetics for application on healthy, intact, or sunburnt skin, its penetration remains unclear. Moreover, it is considered unsafe, and it is not recommended to use in aerosol form.

4. OBJECTIVES

The present study is focused on comparing the toxic behaviour of two commercially available titanium (IV) oxides at a micro and nanoscale. To this end, the toxicity of titanium (IV) oxide on human red blood cells and skin cells is assessed.

The aim of this *in vitro* study is four-fold:

- Characterize TiO_2 nanoparticles through Transmission Electron Microscopy (TEM).
- Evaluate the haemolytic effect of both micro and nanoparticles at different concentrations on human red blood cells.
- Discard potential interferences when using colorimetric methods to evaluate cytotoxicity.
- Examine the cytotoxicity of the particles on skin cells.

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5. MATERIALS AND METHODS

5.1. REAGENTS

TiO_2 nano and micro sized and Dimethyl sulfoxide (DMSO), were supplied by Sigma-Aldrich (Madrid, Spain). Thiazolyl Blue Tetrazolium Bromide (MTT), Neutral Red Solution (NRU), Trypan Blue Solution and Ethanol and Acetic Acid, used to prepare the extinction solution, all of them were acquired from Sigma-Aldrich (Madrid, Spain). Dulbecco's Modified Eagle Medium (DMEM) 4.5 g/L glucose, supplemented with L-glutamine 2 mM, penicillin-streptomycin solution (10,000 U/mL penicillin and 10 mg/mL streptomycin), and Sterile Phosphate Buffer Saline Solution (PBS) were purchased at Lonza (Verviers, Belgium). 10% Hyclone Fetal Bovine Serum (FBS) was supplied by BioLab (Barcelona, Spain).

5.2. CHARACTERIZATION OF NANOPARTICLES BY TEM

Transmission electron microscopy (TEM) is an analytical technique used to visualize the smallest structures. It can provide valuable information about the chemical composition, electronic structure, and other properties of materials.

TiO_2 particles were observed after a dilution of 100 µg/mL in distilled H_2O. This sample was placed in an ultrasonic bath for 3 minutes and one drop of the suspension was deposited on a Holey Carbon-Copper grid and let dry. These samples were observed and analysed in a JEOL JEM 2100 microscope at "Centres Científics i Tecnològics de la Universitat de Barcelona".

5.3. HAEMOLYSIS ASSAY

The haemolysis assay is frequently employed in biomedical research, particularly in the fields of toxicology and pharmacology, to evaluate the potential cytotoxic effects of various

substances on erythrocytes. The haemolysis effect is the process of breaking the cell membrane of erythrocytes, releasing haemoglobin.

Fresh human blood was extracted from healthy non-smokers, non-alcohol consuming and not undergoing any medication volunteers (according to the protocol approved by the Bioethics Committee at University of Barcelona, Spain on 15 February 2021) and was introduced into tubes containing Ethylendiamine Tetraacetic Acid (EDTA). Red Blood Cells (RBC), also known as erythrocytes, were isolated from blood by removing plasma and leukocytes through washes with PBS at pH 7.4 (3000 rpm, 10 min, x3).

Prior to haemolysis assays, it is essential to reach a 50% of suspension of RBC in PBS. To achieve this, the absorbance of 25 µL RBC in 975 µL of deionized water (100% haemolysis) was measured. If the absorbance was between 1.8-2.1, the concentration of erythrocytes was ideal for analysis; if it was lower than 1.8, the sample needed to be centrifuged another time to remove PBS, and an ideal amount of PBS added; or else if the absorbance was higher than 2.1, PBS should be added to the suspension.

Once the concentration of RBC was suitable for analysis, TiO_2 micro and nano size solutions at 1, 0.5 and 0.25 mg/mL were prepared by triplicate. The incubation was conducted at room temperature for 24 h. Then, the samples were centrifuged (10,000 rpm, 5 min.) and the absorbance at 540 nm was measured. These samples of micro-TiO_2 and nano-TiO_2 were prepared by triplicate for 3 different concentrations: 0.25, 0.5 and 1 mg/mL from stocks of 2.5, 5 and 10 mg/mL, respectively. Additionally, two control conditions were prepared: a positive control (C+), consisting in a 100% haemolysis; and the negative control (C-) for a 0% haemolysis. This is summarised in Table 2.

A 100% haemolysis is reached by treating RBC with distilled H_2O. In a hypotonic environment (a solution with lower solute concentration than inside the cell), water enters the cells by osmosis. The influx of water causes the cells to swell and the cell membrane to rupture, leading to haemolysis and releasing haemoglobin to the medium. In contrast, PBS is an isotonic solution, meaning that it has a solute concentration similar to that inside the cell. Because of that, there is no osmosis so RBC neither swell nor shrink significantly, avoiding haemolysis.

	TiO_2 (µL) 2.5 mg/mL	TiO_2 (µL) 5.0 mg/mL	TiO_2 (µL) 10 mg/mL	PBS (µL)	H_2O (µL)	RBC (µL)
C- (0%)	-	-	-	975	-	25
0.25 mg/mL	100	-	-	875	-	25
0.5 mg/mL	-	100	-	875	-	25
1 mg/mL	-	-	100	875	-	25
C+ (100%)	-	-	-	-	975	25

Table 2. Composition of the haemolysis assays.

The formula for calculating the haemolytic effect was:

$$haemolysis = \frac{A_{sample} - A_{0\%}}{A_{100\%} - A_{0\%}} \cdot 100$$

where A is absorbance; 0% is negative control and 100% is positive control.

5.4. INTERFERENCES OF TiO_2 WITH CYTOTOXICITY METHODS

The potential interferences caused by TiO_2 particles in the assessment of cytotoxicity using colorimetric techniques such as the MTT, LDH, or NRU assays need to be considered.

5.4.1. MTT assay

The MTT assay is one of the most used colorimetric methods for assessing cell metabolic activity. It was developed in 1983 by Tim Mosmann and adapted for chemosensitivity assessment in 1986 by Françoise Denizot and Rita Lang. It determines cell survival against a potentially cytotoxic agent. The assay relies on the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), a tetrazolium salt which is commonly soluble and colourless, into (*E,Z*)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan, popularly known as

formazan (Figure 5), an insoluble purple compound. Formazans are compounds of the general formula [R-N=N-CR'=N-NH-R'']. The reduction occurs as a result of the enzyme succinate dehydrogenase (SDH) present within the mitochondria of viable cells, thereby serving as an indicator of mitochondrial function or dysfunction within these cells. Provided that the cytotoxic compound does harm the mitochondria, the reduction will not take place, therefore, the amount of formazan is directly proportional to the number of viable cells. In order to determine the absorbance at 550 nm of formazan it is necessary to solubilize it with a solvent such as DMSO, acidified isopropanol or ethanol… [15]

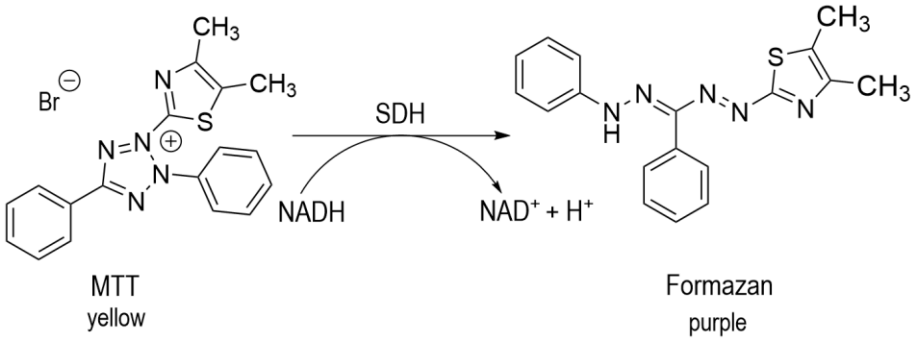

Figure 5. Representation of the reduction of MTT to Formazan. SDH stands for the enzyme succinate dehydrogenase.

The procedure was executed under similar conditions to the original methods involving cells, ensuring a sterile environment to eliminate any external factors that could potentially influence the results. A stock solution of 5 mg/mL of MTT in sterile PBS was diluted 1:10 in DMEM (5% FBS, 1% Glu, 1% Strep-pen). Solutions of TiO_2 nano and micro at 1 mg/mL were prepared in PBS and proceeded to do a 1:2 dilution in MTT-DMEM solution resulting in 9 different solutions of concentrations between 500 – 1.95 µg/mL TiO_2. 100 µL of both nano and micro solutions were placed and incubated for 3h at 37 ºC in a 96 well plate (Figure 6). In the plate, a control of MTT solution was added in the second column and PBS was added in the empty wells.

The solution was then removed and 100 µL of DMSO was added to dissolve the precipitated formazan. After shaking the plate for about 5 minutes, the absorbance was again measured at 550 nm.

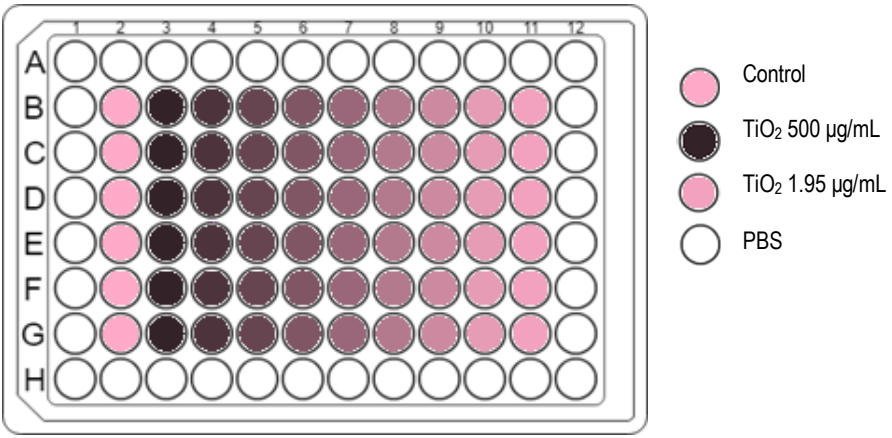

Figure 6. Schematic representation of a 96 well plate. As it can be seen, only 60 wells contain a colourful solution, whereas the other 36 contain PBS.

5.4.2. NRU assay

Another test for assessing cell viability is the Neutral Red Uptake (NRU). The NRU assay is often used to evaluate the cytotoxic effects of various compounds, such as drugs, chemicals, or environmental agents. It was developed at Rockefeller University by Ellen Borenfreund and James A. Puerner in 1984. It is based on the ability of viable cells to incorporate and bind the neutral red dye (NR), which is a pH indicator that changes colour at pH = 6.8 (Figure 7). This dye, at physiologic pH is not charged and can penetrate cell membranes by non-ionic passive diffusion and accumulate in lysosomes, which are membrane-bound organelles within cells. The pH of the lysosomes is lower than of the cytoplasm, which causes the dye to protonate and become retained inside the lysosomes. Living cells with intact lysosomes will take up the dye; in contrast, non-living cells will not take up the dye, therefore, the amount of retained dye is proportional to the number of viable cells. Analysing the intensity of neutral red dye within cells allows for the evaluation of a substance's effects on cell viability, distinguishing between viable, damaged, or deceased cells. The dye is then extracted from the viable cells using an acidified ethanol solution and the absorbance of the solubilized dye is quantified at 550 nm [16].

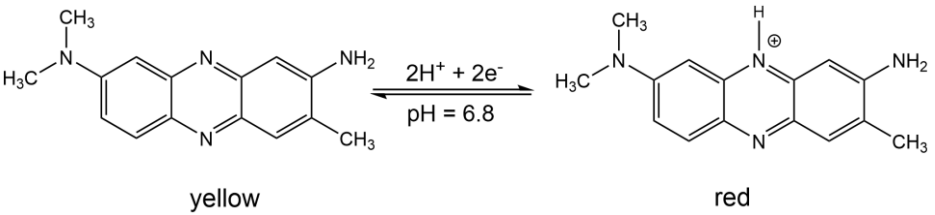

Figure 7. Acid-base reaction of neutral red dye.

The procedure was executed very similarly to the MTT assay. A solution of 0.05 mg/mL neutral red dye (NR) in DMEM (5% FBS, 1% Glu, 1% Strep-pen) was prepared from a stock of 3.3 mg/mL. The same 9 solutions of TiO_2 micro and nano were prepared in NR-DMEM and placed and incubated for 3h at 37 ºC in a 96 well plate. In the plate, a control of NRU solution was added in the second column and PBS was added in the empty wells.

The absorbance was measured at 550 nm, and, after removing the solution from the wells, 100 µL of a mixture of 50% ethanol, 49% deionized water and 1% acetic acid was added. After shaking the plate for about 5 minutes, the absorbance was again measured at 550 nm.

5.4.3. LDH assay

The Lactate Dehydrogenase (LDH) assay is a simple colorimetric method for quantitating cytotoxicity by measuring LDH activity released from damaged cells. The presence of dead cells that have lost membrane integrity can be detected by measuring markers that escape from the cytoplasm into the culture medium. The most common marker used for this type of assay is LDH. As opposed to NRU and MTT assay, that measure cellular survival, the LDH measures cellular damage. LDH is a stable enzyme present in the cytosol of a wide variety of organisms. It is rapidly released into the cell culture supernatant when the plasma membrane is damaged [17].

The enzyme LDH catalyses the oxidation of lactate to pyruvate, reducing the Nicotinamide Adenine Dinucleotide, NAD^+ to NADH + H^+, that reverts to NAD^+ in contact with an iodonitrotetrazolium salt (INT) which reduces into a formazan chloride in presence of the catalyst Diaphorase (Figure 8) [18]. Provided that the cytotoxic compound harms the mitochondria, this reduction will take place; therefore, the amount of formazan is directly proportional to the number of damaged cells.

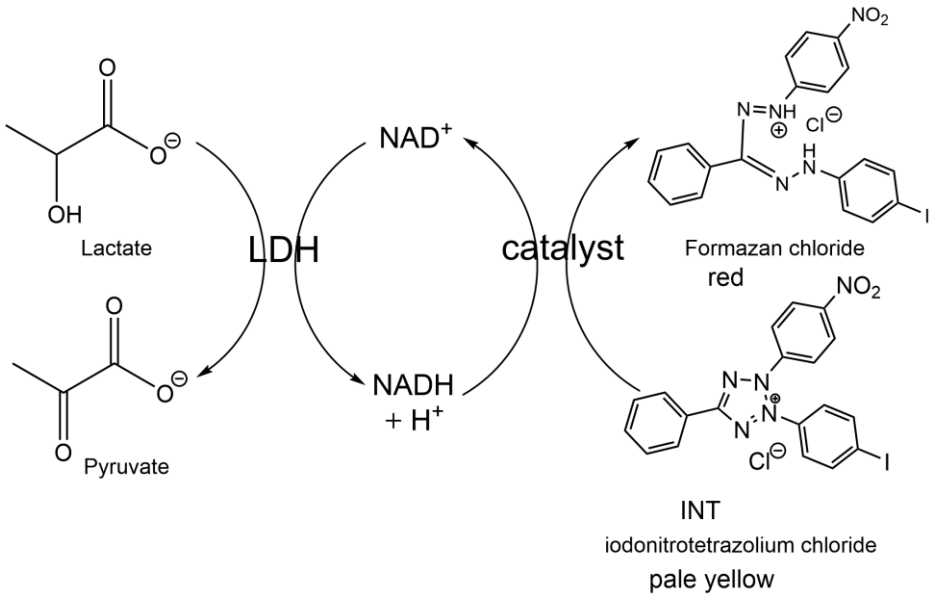

Figure 8. Mechanism of the LDH assay.

The LDH assay was performed using the Cytotoxicity Detection Kit from Roche [18]. The solutions of the same concentrations of TiO_2 as the previous assays in PBS were prepared and placed in a 96 well plate. The LDH kit consisted in two vials: the first containing the catalyst and the second containing a ready-to-use dye solution. 1 ml of deionized water had to be added into the first vial to reconstruct the solution. Then, a fresh reaction mixture of both vials was prepared adding 150 µL of vial 1 and 6.75 ml of vial 2 (for 60 wells assay) and added into the 96 well plate and incubated for 30 min at room temperature protected from light. In the plate, a control of LDH solution was added in the second column and PBS was added in the empty wells. Then, the absorbance was measured at 492 nm with a reference wavelength of 600 nm.

5.5. PREPARATION OF CELLS FOR CYTOTOXICITY ASSAYS

The cells used in the cytotoxicity assay were HaCat (Celltec-Universitat de Barcelona, Spain), a human epidermal keratinocyte cell line.

5.5.1. Trypsinization

The protocol was performed while maintaining aseptic environment using a laminar vertical flow cabinet. The enzyme trypsin, culture medium (DMEM 10% FBS, 1% Glu, 1% Strep-pen) and PBS were pre-warmed in a water bath at 37 ºC while the cells were examined under a microscope, ensuring that they were healthy, free of contamination and had grown about 80% of the surface of the flask from the previous seeding. Then, the culture medium was removed and discarded from the flask and the cells, that were adhered to the lower surface of the flask were rinsed with 10 ml of PBS twice to discard the potential Ca^{2+} and Mg^{2+} ions that could affect the trypsinization process. Afterwards, 2.5 ml of trypsin was added to the flask as a way to detach the cells from the surface. The flask was then left incubating for 6 min. It is worth mentioning that incubation time varies according to the cell line used: firmly adherent cells can be detached quickly at 37 °C. Also, cells should not be exposed to trypsin solution for longer periods than 10 min. After the incubation, the cells were examined to ensure that they were detached from the surface. When that was guaranteed, trypsin deactivation was achieved by adding three times the initial volume of trypsin (7.5 ml) of complete culture medium with intense homogenization. Lastly, the desired amount (about 1.2 ml) of cell suspension was pipetted into a new flask with culture medium (18.8 ml) so that the total volume of the flask was 20 ml and placed in the incubator [19].

A representation of the steps for reseeding cells appears in Annex 1.

5.5.2. Cell counting and cell seeding

It is necessary to know the cellular density of the suspension to be able to do any analysis. This is done using an hemocytometer or Neubauer chamber, which is the most common method used for cell counting. The Neubauer chamber (Figure 9) is divided into 2 chambers: the upper chamber and the lower chamber. There is also a glass cover, which is placed on the top of the Neubauer chamber, covering the central area.

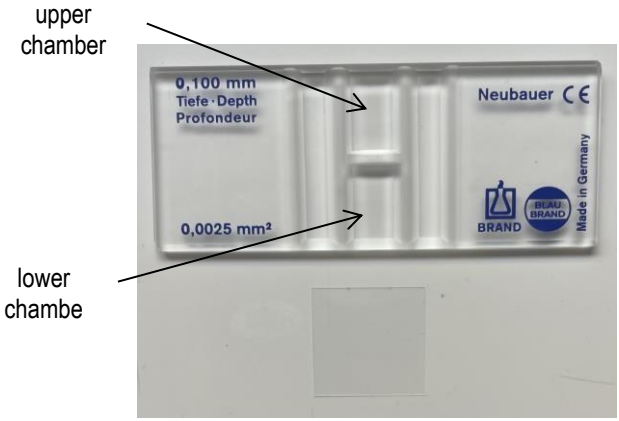

Figure 9. Image of a Neubauer Chamber and a glass cover.

Each chamber is divided into 9 squares, each of them divided in other ones by different lines. We use the 4 corner squares that are divided into 16 smaller squares (Figure 10). Only the cells inside of the 4 squares or touching the upper and right edges of the square were counted.

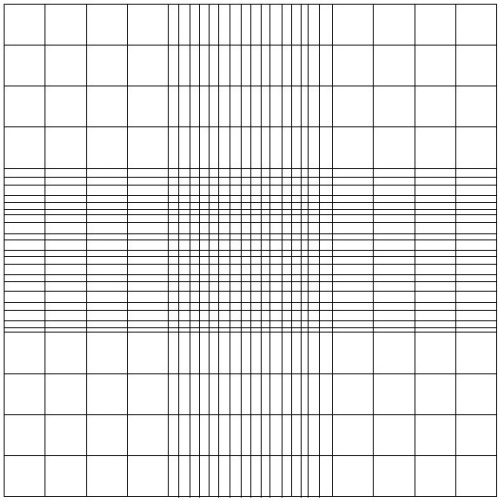

Figure 10. Representation of a chamber of an hemocytometer.

In order to count the cells present in the cell suspension, this suspension was diluted in PBS and Trypan Blue to achieve a 1:10 dilution. Trypan Blue is one of several stains recommended for use in dye exclusion procedures for viable cell counting. This method relies on the principle that live (viable) cells maintain intact cell membranes, preventing the uptake of specific dyes like trypan blue, while non-viable cells lack this membrane exclusion. Staining enhances the visualization of cell morphology, ensuring that only viable cells are visible and counted.

The following formula was used to calculate the cellular density:

$$\frac{N_{cells}}{4{\cdot}10^{-4}\,mL} \cdot 10 = \frac{N_{cells}}{4} \cdot 10^5 \frac{cells}{mL}$$

where N_{cells} is the sum number of cells in the four chambers and each chamber has a volume of 10^{-4} mL.

To prepare the well plates for analysis, the desired cellular density was $1{\cdot}10^5$ cells/mL, to ensure an 80% confluence after 24h of incubation. Therefore, a solution of the cell suspension in DMEM (10% FBS, 1% Glu, 1% Strep-pen) had to be prepared. Then, 100 µL of this solution was added to the plate in a way in which 60 centred wells of the plate contained cells and the other 36 contained PBS to maintain cell humidity. The plate was incubated for 24h (under 5% CO_2 at 37ºC).

5.5.3. Treatment of the cells with TiO_2

After the incubation the cells had to be exposed to TiO_2 micro and nanosized. A stock solution was prepared and then diluted to a concentration of 100 µg/mL of TiO_2 in DMEM (5% FBS, 1% Glu, 1% Strep-pen). Subsequently, 1:2 dilutions were performed resulting in 9 concentrations ranging from 100 to 3.906 µg/mL. After discarding the culture medium from the well plate, these solutions treatments were added to a 96-well plate (one plate for each TiO_2), from which the culture medium had been previously aspirated (Figure 11).

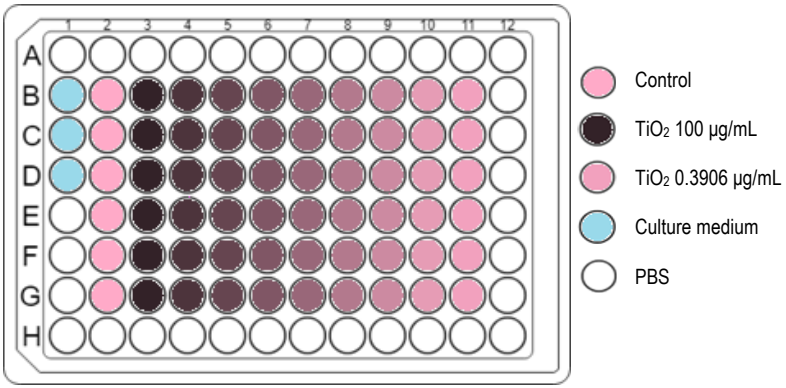

Figure 11. Schematic representation of a 96 well plate. 60 wells contain cells.

The plate was afterwards incubated for 24h (under 5% CO_2 at 37ºC). Each plate also included control wells, consisting of wells with no treatment but with cells (control), and wells with neither cells nor treatment (culture medium).

5.6. CYTOTOXICITY ASSAYS

The cytotoxicity assays were conducted similarly to those conducted without cells. The cytotoxicity was determined using the following formula [20]:

For MTT and NRU analysis the cellular viability is calculated:

$$cell\ viability\ = \frac{experimental\ value}{control\ value} \cdot 100$$

For LDH analysis the cytotoxicity is calculated:

$$cytotoxicity = \frac{experimental\ value - low\ control}{high\ control - low\ control} \cdot 100$$

Triplicates of each condition were treated to perform the three different methods. Cells adhered to the well-plate surface were used to perform MTT and NRU assays to assess cellular viability. Meanwhile, 50 µL of supernatant from wells were transferred to a new plate to study the cytotoxicity produced through the release of LDH from cells.

Firstly, a high control of cytotoxicity had to be prepared for LDH assay: a 2% solution of a highly cytotoxic agent (Triton X-100) was added to 3 wells of control with cells and centrifuged (3000 rpm, 10 min). The well plates were centrifuged to avoid cellular residues in supernatants that could interfere with the reagents used. Upon this step, 50 µL of the supernatant from each condition by triplicate was transferred into another plate to perform the LDH method.

5.6.1. MTT assay

Remaining supernatants from wells were removed and 100 µL of a 500 µg/ml MTT solution was added. The plate was then incubated for 3h (under 5% CO_2 at 37ºC). Subsequently, the contents of the wells were removed and DMSO was added, and the plate was centrifuged (150 rpm, 5 min). Afterwards, the supernatant was transferred into a new plate to avoid interferences of precipitated TiO_2. Finally, the absorbance was measured at 550 nm.

5.6.2. NRU assay

Remaining supernatants from wells were removed and 100 µL of a 500 µg/ml NRU solution was added. The plate was then incubated for 3h (under 5% CO_2 at 37 ºC). Subsequently, the contents of the wells were removed and a mixture of 50% ethanol, 49% deionized water and 1% acetic acid was added, and the plate was centrifuged (150 rpm, 5 min). Afterwards, the supernatant was transferred into a new plate to avoid interferences of precipitated TiO_2. Finally, the absorbance was measured at 550 nm.

5.6.3. LDH assay

Supernatants were mixed with 50 µL of the reaction mixture (catalyst and dye solution). After incubating for 15 min at room temperature under light protection, the absorbance was measured at 492 nm (reference wavelength 600 nm) according to protocol outlined in reference [18].

6. RESULTS AND DISCUSSION

Nano-TiO_2 was characterized by TEM, and the cytotoxic behaviour of TiO_2 micro and nano sized on skin cells was analysed through MTT, NRU and LDH assays. However, prior to this, potential interferences of titanium oxide with these assays had to be ruled out. Additionally, the cytotoxicity of this compound to RBC was examined.

6.1. CHARACTERIZATION OF TiO_2 BY TEM

Figure 12 displays images of TiO_2 nanoparticles obtained through TEM. According to technical information of the commercial supplier, nano-TiO_2 has a particle size of 21 nm and is a mixture of 80% anatase and 20% rutile. The particle size was verified through the TEM analysis. TiO_2 in rutile phase consist of both spherical and rod shape; on the contrary, the particle of TiO_2 in anatase phase has mostly spherical morphology [21]. The mixture of 80% anatase and 20% rutile is then confirmed given that the NPs have in general a spherical form.

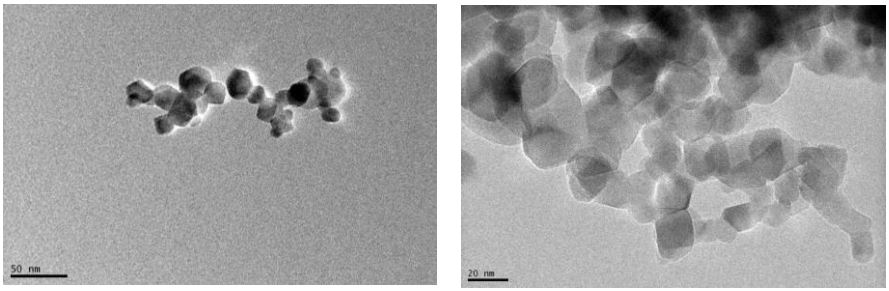

Figure 12. TEM images of TiO_2 nanoparticles in distilled H_2O recorded in "Centres Científics i Tecnològics de la Universitat de Barcelona".

The TEM analysis was also done with TiO_2 in DMEM (5% FBS, 1% Glu and 1% Strep-Pen). It was found very much difficult to visualize the nanoparticles. This medium contained numerous proteins and growth factors, as well as other components, which are thought to be the cause for

such low quality of visualization. In Appendix 2 there is a representation of the TiO_2 nanoparticles in DMEM (5% FBS, 1% Glu and 1% Strep-Pen) after diluting the initial sample 5 times in distilled H_2O.

6.2. HAEMOLYSIS STUDY

The haemolysis assay was executed for both micro-TiO_2 and nano-TiO_2 at 3 different concentrations: 0.25, 0.50 and 1 mg/mL. The incubation was done for 24h at room temperature in dark conditions. The absorbance value accounted for the amount of haemoglobin release induced by TiO_2.

The results showed a considerable difference in the haemolytic behaviour between micro- and nano-oxides (Figure 13): the haemolytic effect increased in both cases with the concentration of TiO_2, doubling when the concentration was multiplied by four. Nonetheless, the overall effect of NPs of TiO_2 was superior, approximately five times greater than the effect of the microparticles. In Figure 13, the colour of the solutions can be compared: 1mg/mL nano-TiO_2 has a red colour, which is related to the haemolytic effect of the particles. In contrast, micro-TiO_2 does not exhibit any red appearance, meaning that it does not have any haemolytic effect. This variation in the haemolytic effect observed among particles of different sizes can be attributed to the larger total surface area offered by the nanoparticles. This increased surface area, especially at higher concentrations, enhances the likelihood of interaction with the membrane of the RBC, consequently leading to haemolysis [22]. The precise mechanisms through these nanoparticles induce haemolysis in erythrocytes under physiological conditions remain incomplete.

Additionally, Figure 14 shows the Visible spectra of haemoglobin. The band at 439.0 nm is the Soret band, attributed to $\Pi \rightarrow \Pi^*$ transition of the porphyrin ring. The two bands at 576.5 and 542.5 nm are attributed to the manifestation of the oxyhaemoglobin (HbO_2) [23].

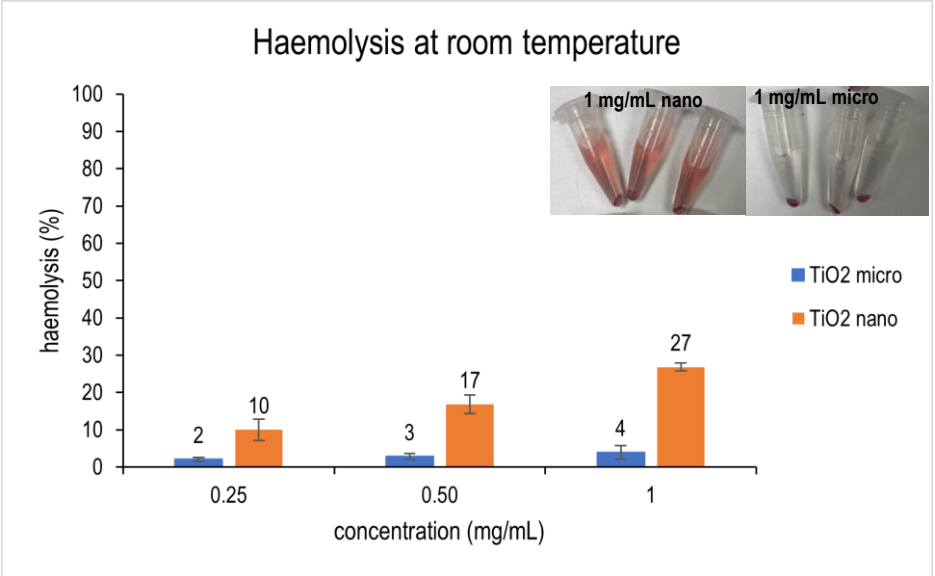

Figure 13. Haemolysis of TiO_2 micro and nano samples at room temperature, figure in inset showing for 1 mg/mL. Results are expressed as mean ± ES of three independent experiments.

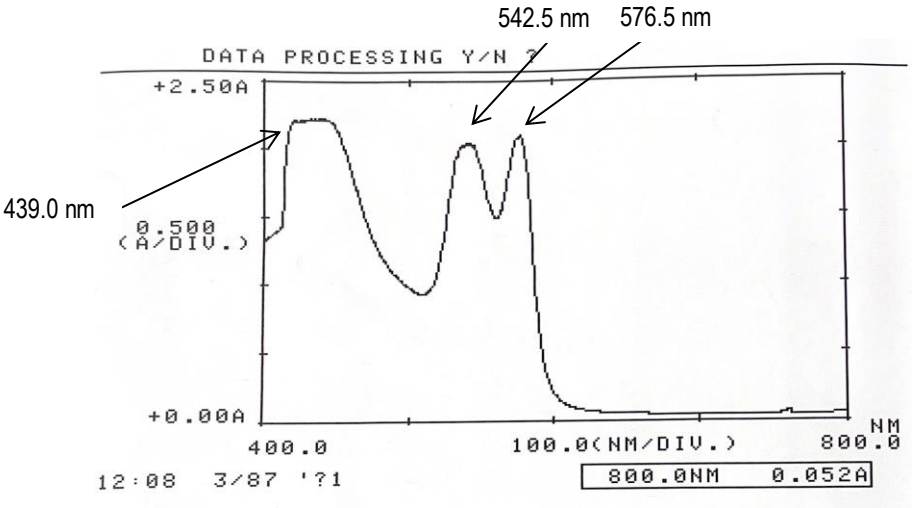

Figure 14. Visible spectra of haemoglobin.

In any case, given that the haemolysis is less than 50%, it can be stated that none of the samples analysed have a significant haemolytic effect. Additionally, a dose-dependent haemolytic activity can be observed for TiO_2.

6.3. INTERFERENCES OF TiO_2 WITH CYTOTOXICITY METHODS

The potential interferences of TiO_2 with MTT, NRU and LDH methods have been studied in acellular conditions under concentrations of micro- and nano-TiO_2 between 500 – 1.95 µg/mL. The aim of these analyses is to determine whether the TiO_2 particles interact with the methods in a way that could interfere with the response (e.g. reducing MTT to formazan or taking up the NR).

6.3.1. MTT analysis

The optical density is represented in Figure 15 as a percentage of control (optical density of MTT reduced to formazan in the absence of TiO_2). These results indicate an increase in optical density (OD) with the concentration of micro-TiO_2, reaching a final value of 22% at 500 µg/mL. The cause for this OD is believed to be a physical rather than a chemical interference, where the particles are insoluble and can precipitate in the wells. As for nano-TiO_2, no or not significant OD was found at any concentration.

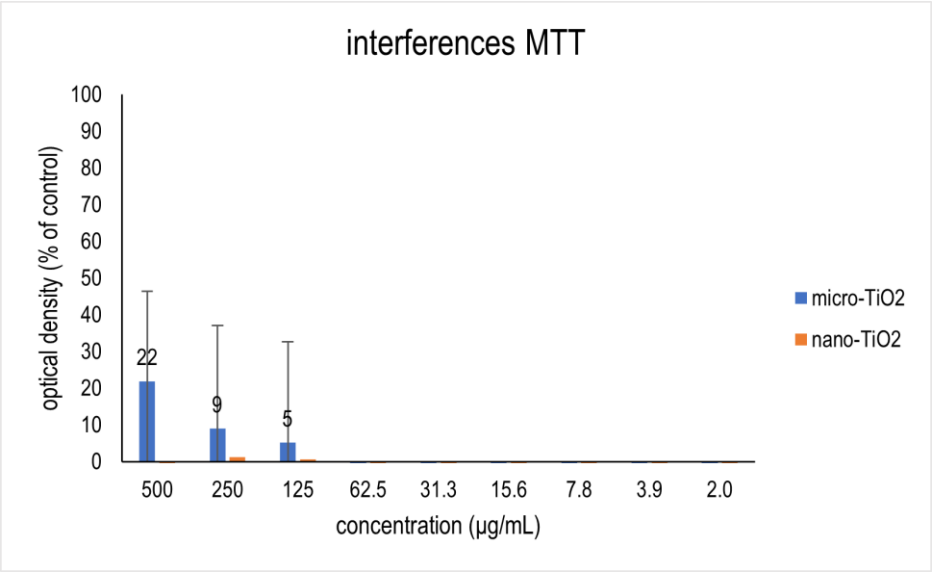

Figure 15. Interferences of TiO_2 micro and nanoparticles with MTT. Results are represented as percentage of control. Results are expressed as mean ± ES of three independent experiments.

6.3.2. NRU analysis

The OD for both micro-TiO_2 and nano-TiO_2 is represented in Figure 16 as the percentage of control (OD of NR in the absence of TiO_2). In both cases, the OD increases with the concentration of TiO_2. The OD of micro-TiO_2 in the NRU does not differ from the value obtained in the MTT assay. However, nano-TiO_2 exhibits a substantially higher value, in contrast to the MTT assay, where nanoparticles exhibited almost no OD. One explanation for this could be that the NR may increase nano-TiO_2 precipitation. One hypothesis is that TiO_2 NPs remain in suspension in DMSO, the solvent used in the MTT assay. This fact could prevent the precipitation of the NPs, keeping them in suspension and not interfering with the OD measurement. Nevertheless, this still needs to be studied more thoroughly.

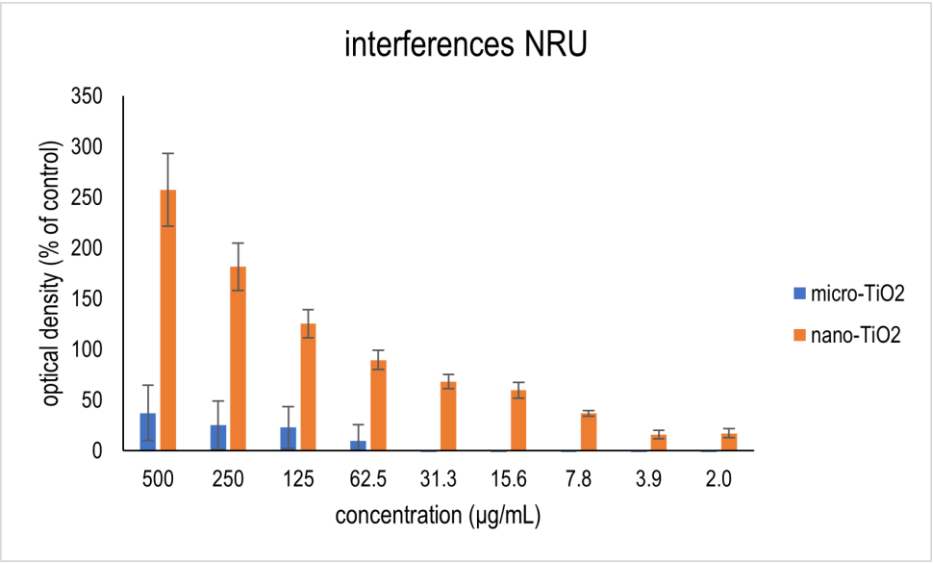

Figure 16. Interferences of TiO_2 nanoparticles with NR. Results are represented as percentage of control. Results are expressed as mean ± ES of three independent experiments.

6.3.3. LDH analysis

The results indicate no or no meaningful OD for both micro and nanoparticles with the LDH assay. The LDH control exhibits an average OD of 0.02, similar to the OD of micro-TiO_2 and

nano-TiO_2 at any concentration. This suggests that not only does the OD not increase with concentration of TiO_2, but there are also no interferences with this reagent.

In conclusion, the only possible interference found for TiO_2 is a physical one, where the micro- or nanoparticles can precipitate due to their insolubility. Therefore, it is important for cytotoxicity assays to use a low concentration of particles to avoid precipitate of TiO_2 as much as possible in order to reduce the risk of interferences. This may be achieved, for example, by extensive washing, ultracentrifugation, transferring supernatants or adaptation of gating [24].

It is worth mentioning that although no interferences were found in this study, the photocatalytic interferences should also be examined. The study by A.R. Lupu and T. Popescu [25] revealed that TiO_2 can interact with MTT in acellular conditions. Their results showed that, after 30 min of UV irradiation, the obtained OD was almost three times higher than without irradiation. Additionally, incubation times could also affect the interferences: increasing the MTT incubation time increases OD levels which are not fruit of interferences but of the incubation time.

6.4. CYTOTOXICITY ASSAYS

The risk of interferences was eliminated given that the highest concentration of this analysis was 100 µg/mL, in contrast to the 500 µg/mL of the interferences analysis. Additionally, the supernatant was transferred into a new empty 96-well plate.

As previously commented, cytotoxicity to epidermal HaCaT cells is represented by a decrease in cellular viability in MTT and NRU analysis in contrast to the LDH analysis, in which cytotoxicity is directly calculated and increases with the concentration of LDH. In this case, however, the cellular viability is represented for the LDH assay, in order to facilitate the comparison with the MTT and NRU assays. Both micro-TiO_2 and nano-TiO_2 (21 nm) are examined in Figures 17, 18 and 19.

Observing the results, micro-TiO_2 does not alter the mitochondria (MTT), lysosomes (NRU) or the plasma membrane (LDH). Nevertheless, nano-TiO_2 at high concentrations, a cytotoxic behaviour is detected in the MTT assay since a decrease in the cellular viability is observed (<80% cellular viability). This suggests that nano-TiO_2 can damage the mitochondria, preventing the conversion of MTT into formazan. An explanation for this can be that MTT is more sensitive

than NRU and LDH. Cytotoxicity is influenced not only by the characteristics of a given product but also by the method used to assess it [26].

The difference in cytotoxicity of micro- and nano-TiO_2 can be attributed to the fact that NPs have a greater surface area than microparticles. This increased surface area, especially at higher concentrations, raises the possibility of interaction with the cells and potentially harming them. Additionally, the electronic properties of nano-oxides, determined by their chemical compositions and modulated by crystalline structure, size, shape, and morphology, may play an important role in cytotoxicity [26].

In summary, given that the cellular viability is higher than 80%, neither micro-TiO_2 nor nano-TiO_2 exhibit considerable cytotoxicity to HaCaT epidermal keratinocytes for NRU and LDH assays. However, a cytotoxic activity in MTT for NPs is observed. This experiment should be continued with pulmonary cells, given that TiO_2 is considered unsafe through inhalation.

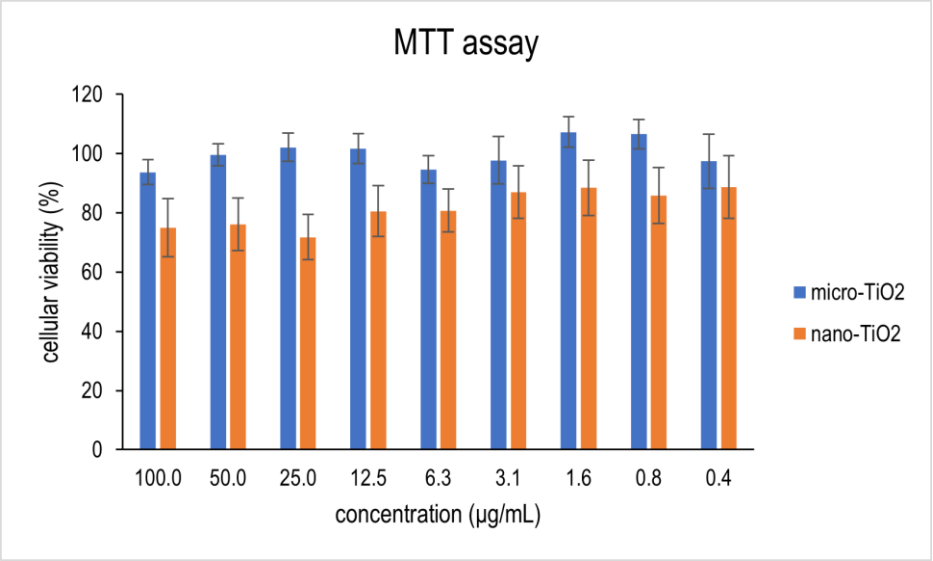

Figure 17. Cellular viability of HaCaT cells in presence of micro-TiO_2 and nano-TiO_2 of concentrations raging between 0.4-100 µg/mL for MTT. Results are expressed as mean ± ES of three independent experiments.

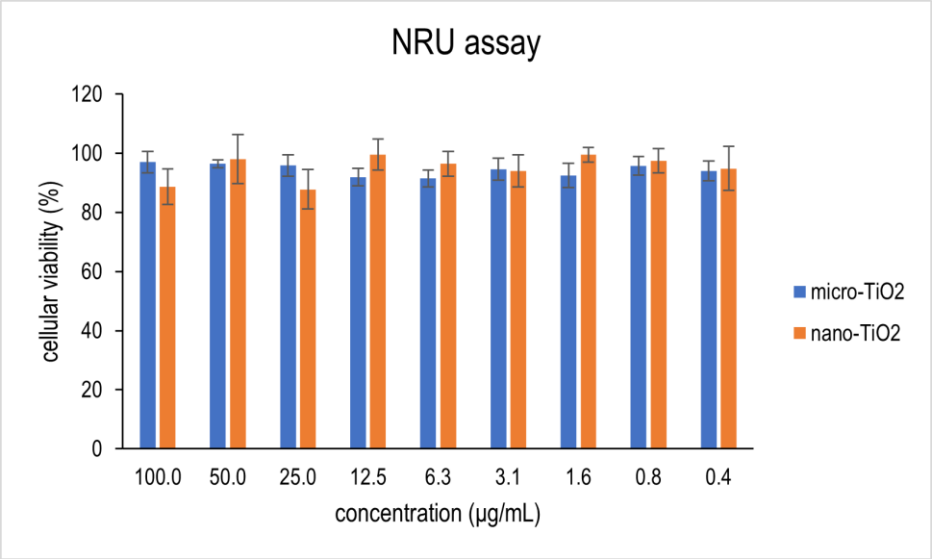

Figure 18. Cellular viability of HaCaT cells in presence of micro-TiO_2 and nano-TiO_2 of concentrations raging between 0.4-100 µg/mL for NRU. Results are expressed as mean ± ES of three independent experiments.

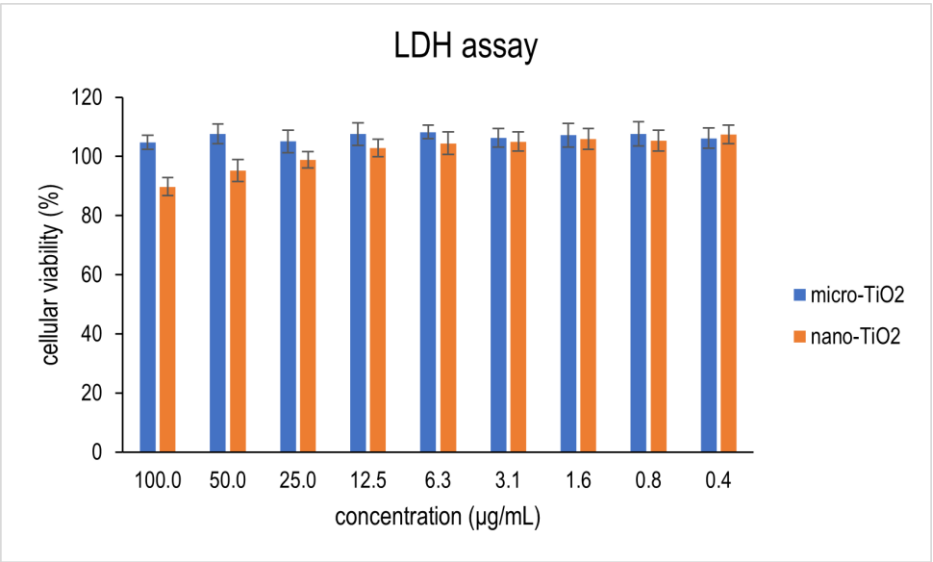

Figure 19. Cellular viability of HaCaT cells in presence of micro-TiO_2 and nano-TiO_2 of concentrations raging between 0.4-100 µg/mL for LDH. Results are expressed as mean ± ES of three independent experiments.

7. CONCLUSIONS

Based on the results presented and in alignment with the objectives set in the beginning of the study, the following conclusions can be drawn:

- The size of the TiO_2 nanoparticles has been confirmed by TEM.
- The haemolytic effect of micro and nanoparticles on human RBC has been discarded although a dose-dependent haemolytic activity was observed for both TiO_2. Additionally, higher haemolysis values were found for nano-TiO_2.
- No chemical interferences of TiO_2 with the reagents of MTT, NRU or LDH were found. Nevertheless, the highly insoluble TiO_2 particles could interfere with the absorbance measurement. Therefore, cytotoxicity assays should utilise low particle concentrations to ensure solubility and minimize TiO_2 traces by transferring the supernatant.
- TiO_2 did not exhibit cytotoxicity to HaCaT epidermal keratinocytes for NRU and LDH assays, although a reduction in the cell viability in MTT for NPs was observed.

To expand this study, cytotoxicity studies in pulmonary cells could be considered, given that TiO_2 is considered unsafe through inhalation. Additionally, photocytotoxicity studies could provide a broader understanding of the characteristics of this UV filter.

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9. ACRONYMS

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

EDTA: Ethylenediamine tetraacetic acid

FBS: Fetal Bovine Serum

Glu: Glutamine

LDH: Lactate Dehydrogenase

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAD^+ / NADH: Nicotinamide Adenine Dinucleotide (oxidised / reduced form)

NP: Nanoparticle

NRU: Neutral Red Uptake

OD: Optical density

PBS: Phosphonate Buffer Saline

RBC: Red Blood Cells

ROS: Reactive Oxygen Species

SCCS: Scientific Committee on Consumer Safety

SDH: Succinate Dehydrogenase

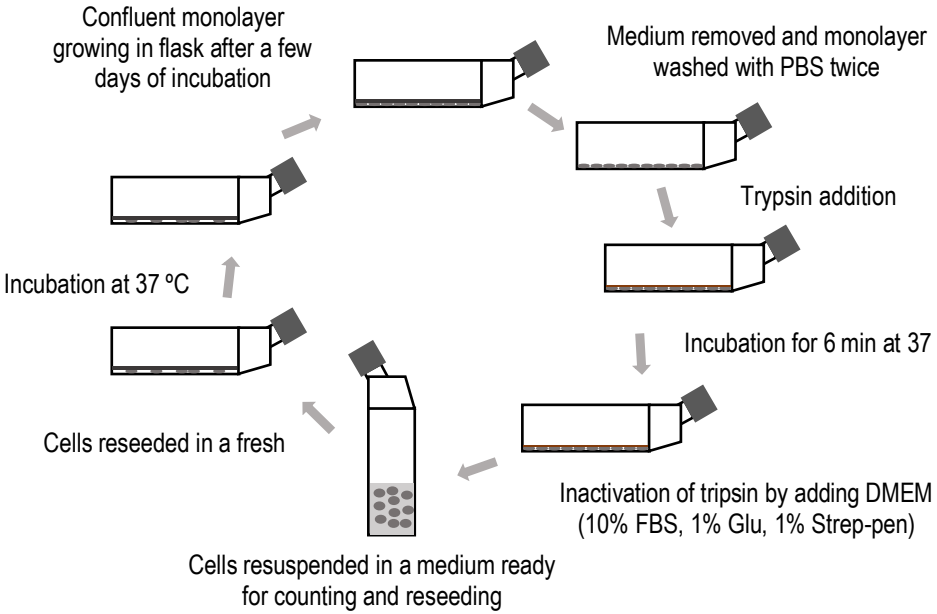
Strep-pen: Double antibiotic solution containing penicillin and streptomycin

TEM: Transmission Electron Microscopy

UV: Ultraviolet

APPENDICES

APPENDIX 1: REPRESENTATION OF CELL MAINTENANCE AND RESEEDING PROCESS



APPENDIX 2: ADDITIONAL PICTURES OF TiO_2 NANOPARTICLES IN DMEM