INTEGRATED MASTER IN BIOENGINEERING – SPECIALIZATION IN MOLECULAR BIOTECHNOLOGY

Moving into advanced nanomaterials.

Toxicity of TiO_2 nanoparticles immobilized in clay on human cell line.

Maria João Moura Gonçalves Moutinho de Bessa

President of the Jury: Maria da Conceição Santos Silva Rangel Gonçalves, PhD
(Associate Professor, Institute of Biomedical Sciences Abel Salazar, University of Porto)

Supervisor: Carla Costa, PhD
(Postdoctoral Researcher, Department of Environmental Health, National Institute of Health Doutor Ricardo Jorge, Porto)

Co-Supervisor: João Paulo Teixeira, PhD
(Researcher, Department of Environmental Health, National Institute of Health Doutor Ricardo Jorge, Porto)

Examiner: Maria João Silva, PhD
(Researcher, Department of Human Genetics, National Institute of Health Doutor Ricardo Jorge, Lisbon)

Porto, 2015

ORIGINAL PUBLICATIONS

The present work contains techniques and/or data also presented in the following scientific publications, communications and conference proceedings:

1. Publications:

Bessa, M. J., Reinosa, J. J., Fernandez, J. F., Bañares, M. A., Teixeira, J. P. and Costa, C. (2015). Understanding the toxicity outcomes of kaolinite for future nanocomposite applications. Environmental & Health International Magazine Special Issue 2015: 173-178.

Costa, C., Brandao, F., Bessa, M. J., Costa, S., Valdiglesias, V., Kilic, G., Fernandez-Bertolez, N., Quaresma, P., Pereira, E., Pasaro, E., Laffon, B. and Teixeira, J. P. (2015). In vitro cytotoxicity of superparamagnetic iron oxide nanoparticles on neuronal and glial cells. Evaluation of nanoparticle interference with viability tests. Jornal of Applied Toxicology doi: 10.1002/jat.3213 (Epub ahead of print).

Valdiglesias, V., Kilic, G., Fernandez-Bertolez, N., Costa, S., Fraga, S., Bessa, M. J., E., Pasaro, Teixeira, J. P. and Laffon, B. (2015). Toxicity of Iron Oxide Nanoparticles. Encyclopedia of nanoscience and nanotechnology, American Scientific Publishers (in press).

2. Communications:

2.1. Oral:

Global Environmental Health Forum, IFEH 1st World Academic Congress on Environmental Health, 22nd to 26th of September 2015, Coimbra, Portugal. Bessa, M. J., Reinosa, J. J., Fernandez, J. F., Bañares, M. A., Teixeira, J. P. and Costa, C.. Understanding the toxicity outcomes of kaolinite for future nanocomposite applications.

2.2. Poster:

The Society of Toxicology 55th Annual Meeting and ToxExpo, 13th to 17th of March 2016, New Orleans, United Stated. Bessa M. J., Reinosa J. J., Fernandez J. F., Bañares M., Teixeira J.P., Costa C.. In vitro toxicity of TiO_2 nanoparticles immobilized on clay to human hepatic cells (submitted).

9th Congress of Toxicology in Developing Countries and XIX Brazilian Congress of Toxicology (CBTOX), 7th to 10th of November 2015, Natal, Rio Grande do Norte state, Brazil. Bessa M. J., Reinosa J. J., Fernandez J. F., Bañares M., Teixeira J.P., Costa C..

Cytotoxicity of Clay-TiO_2 nanostructures in human HepG2 cells (accepted – to be presented).

3. Conference proceedings:

51th Congress if the European Societies of Toxicology, 13th to 16th of September 2015, Porto, Portugal. Costa, C., Brandao, F., Bessa, M. J., Costa, S., Valdiglesias, V., Kilic, G., Fernandez-Bertolez, N., Pasaro, E., Laffon, B. and Teixeira, J. P. (2015). In vitro toxicity screening of silica-coated superparamagnetic iron oxide nanoparticles in glial cells. Toxicology Letters 238: S56-S383.

50th Congress if the European Societies of Toxicology, 7th to 10th of September 2014, Edinburgh, Scotland. Costa, C., Kilic, G., Brandao, F., Bessa, M. J., Costa, S., Pasaro, E., Valdiglesias, V., Laffon, B. and Teixeira, J. P. (2014). Cytotoxicity of iron oxide nanoparticles with different coatings in human neuronal cells. Toxicology Letters 229: S199-S199.

ACKNOWLEDGEMENTS

First of all, I would like to express my special and deepest gratitude to my research supervisor, Dr. Carla Costa, for the continuous support and patient guidance, without never forgetting her friendship. Her valuable advices helped me overcome many critical situations and, without her help, I could not have succeeded. Sincerely, I could not imagine a better mentor and friend to help me through my study without ever losing faith in me and in my work.

My sincere thanks also go to my co-advisor, Dr. João Paulo Teixeira, who provided me an unforgettable opportunity to join his research team and also for the enthusiastic encouragement throughout this project.

Besides my research supervisors, I could not forget to mention and acknowledge the valuable assistance of Dr. Julian Reinosa, Prof. José Fernández and Prof. Miguel Bañares from the Electroceramic Department of Instituto de Cerámica y Vidrio (Madrid, Spain) on the preparation and characterization of the materials under evaluation.

My grateful thanks are also extended to the personnel of the Environmental health department from National Institute of Health Doutor Ricardo Jorge (Porto) for their constant motivation, kindness and friendship along this journey.

I owe my gratitude to all those people who have made this dissertation possible and made this a rewarding and priceless experience.

I would also like to mention that this work was supported by TD1204 MODENA COST Action.

ABSTRACT

Titanium dioxide nanoparticles (TiO_2 NPs) are the most extensively studied metal oxide nanoparticle worldwide. Despite the inconsistency of biological effects observed *in vitro* after exposure to TiO_2 NPs, many studies have demonstrated their capacity to induce cytotoxicity and genotoxicity in various cultured cell lines.

Nanoparticle toxicity has been associated to their small size and consequent high surface area and reactivity. In an attempt to overcome this issue and maintain the advantages of nanoparticles, immobilization of nanoparticles on the surface of inorganic supports has been recently performed, in particular on clays minerals, resulting in the creation of nanocomposites.

Taking all into account, the main goal of the present work was to evaluate *in vitro* toxicity of rutile TiO_2 NPs immobilized in high purity kaolinite clay (C-TiO_2), in a hepatocellular carcinoma human cell line (HepG2). For a full understanding of the effects of this nanocomposite and its single elements, a primary characterization was performed using field emission scanning electron microscope and dynamic light scattering. Nanomaterials uptake by HepG2 was also analyzed by means of flow cytometry. For the cytotoxicity evaluation it was initially established to use the MTT, Alamar blue, Neutral red uptake and LDH assays, however, after interference analysis, only MTT and Alamar blue were found to be suitable assays for the present materials. Cytotoxicity was evaluated after exposure to different concentrations of the three materials prepared in complete and serum free media for three exposure times (3, 6 and 24 hours). The comet assay was further used for the genotoxicity assessment (non-cytotoxic concentrations for 3 and 24 hours).

Results show that all studied materials were internalized by HepG2 cells in both media types (complete and serum free) and for both periods of exposure. In addition, analyzed materials induced significant mitochondrial and potential redox dysfunction of the hepatocytes in a dose-dependent manner. TiO_2 NPs preferentially induced decrease in viability after 24 hours of exposure in serum free medium and in almost all periods of exposure in complete medium; both nanokaolin and C-TiO_2 nanocomposite could significantly decrease the HepG2 viability for both media and also in almost all periods of treatment. Furthermore, TiO_2 NPs, nanokaolin and C-TiO_2 nanocomposites also affected DNA integrity in a dose-dependent manner both after 3 and 24 hours of exposure.

Thereby, the present results suggest that kaolinite mineral is not a desirable substrate for the immobilization of nanoparticles and that, in accordance to what has been

previously stated by other authors, rutile TiO_2 NPs may present cytotoxic and genotoxic effects.

To overcome the toxic effects observed, other organically modified kaolinite clays must be studied in order to find a suitable substrate for nanoparticle immobilization; this will be essential for the development of biocompatible and safe nanocomposites.

Keywords: nanocomposites, nanoparticles, TiO_2, kaolinite, cytotoxicity, genotoxicity, HepG2 cell line

RESUMO

Entre as nanopartículas de óxido de metal mais estudadas em todo o mundo estão as nanopartículas de dióxido de titânio (NPs TiO_2). Muitos estudos *in vitro* têm demonstrado que estas nanopartículas são capazes de induzir citotoxicidade e genotoxicidade em várias linhas celulares, embora exista alguma inconsistência relativamente aos efeitos biológicos observados.

A toxicidade das nanopartículas tem sido associada ao seu tamanho reduzido e consequente elevada área de superfície e reatividade. Na tentativa de superar este problema e manter as vantagens das nanopartículas, foi recentemente desenvolvida a técnica de imobilização de nanopartículas na superfície de suportes inorgânicos, em particular em minerais de argilas, resultando na criação de nanocompósitos.

Neste contexto, o principal objetivo do presente trabalho foi avaliar *in vitro* a toxicidade das NPs TiO_2 de rútilo imobilizadas em argila de caulinite de elevada pureza (C-TiO_2), numa linha hepatocelular de carcinoma humano (HepG2). Para compreender os efeitos destes nanocompósitos e dos seus elementos individuais, realizou-se uma caracterização primária usando as técnicas microscópio eletrónico de varredura de emissão de campo e dispersão dinâmica de luz. A internalização dos nanomateriais pelas células HepG2 foi analisada por citometria de fluxo. Para a avaliação da citotoxicidade, foi inicialmente estabelecido o uso dos ensaios MTT, Alamar Blue, internalização do vermelho neutro e LDH, mas a análise das interferências revelou que apenas os ensaios MTT e Alamar Blue seriam adequados para os materiais em estudo. A citotoxicidade foi avaliada após a exposição a diferentes concentrações dos três materiais preparados em meio completo e incompleto em três períodos de exposição (3, 6 e 24 horas). O ensaio cometa foi utilizado para a avaliação de genotoxicidade (concentrações não citotóxicas para 3 e 24 horas).

Os resultados mostram que todos os materiais estudados foram internalizados pelas células HepG2 em ambos os tipos de meio (completo e incompleto) e nos dois períodos de exposição. Além disso, todos os materiais analisados foram capazes de alterar significativamente a atividade mitocondrial e o potencial redox dos hepatócitos de uma forma dependente da dose. As NPs TiO_2 induziram preferencialmente uma diminuição da viabilidade após 24 horas de exposição em meio incompleto e em quase todos os períodos de exposição em meio completo; tanto a nanocaulinite como os nanocompósitos C-TiO_2 foram capazes de diminuir significativamente a viabilidade das HepG2 em ambos os meios e também em quase todos os períodos de tratamento. Adicionalmente, as NPs TiO_2,

nanocaulinite e os nanocompósitos C-TiO_2 também afetaram a integridade do DNA de uma forma dose-dependente após 3 e 24 horas de exposição.

Deste modo, estes resultados sugerem que o mineral caulinite não é um substrato adequado para a imobilização de nanopartículas e que, à semelhança do que já foi descrito por outros autores, as NPs TiO_2 de rútilo podem ser citotóxicas e genotóxicas.

Para superar os efeitos tóxicos observados, devem ser estudadas outras argilas de caulinite organicamente modificadas, de forma a identificar um substrato adequado para a imobilização de nanopartículas; isto será essencial para o desenvolvimento de nanocompósitos biocompatíveis e seguros.

Palavras-chave: nanocompósitos, nanopartículas, TiO_2, caulinite, citotoxicidade, genotoxicidade, linha celular HepG2

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AB	Alamar blue assay
BSA	Bovine Serum Albumin
C-TiO_2	Titanium dioxide nanoparticles immobilized in clay
DLS	Dynamic light scattering
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
ENMs	Engineered nanomaterials
FBS	Fetal bovine serum
FE-SEM	Field-emission scanning electron microscopy
FSC	Forward scatter channel
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HepG2	Hepatocellular carcinoma human cell line
HSCs	Hepatic stellate cells
INT	2-(4-Iodphenyl)-3-(4-nitrophenyl)-5-phenyltetrazoliumchlorid
JRC	European Commission's Joint Research Centre
LDH	Lactate dehydrogenase
LDV	Laser Doppler velocimetry
LMP	Low melting point
MMS	Methyl methanesulfonate
MONPs	Metal oxide nanoparticles
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
NMP	Normal melting point
NRU	Neutral red uptake
PBS	Phosphate buffered saline
ROS	Reactive oxygen species
SEM	Scanning electron microscopy

SSC	Side scatter channel
TEM	Transmission electron microscopy
tDNA	Comet tail intensity
TiO_2 NPs	Titanium dioxide nanoparticles
UV	Ultraviolet

I. INTRODUCTION

1. Statement of the problem

In recent times, nanotechnology is growing in rapid pace, discovering innovative and attractive nanomaterials with specific properties for several applications (Iavicoli *et al.*, 2011; Savolainen *et al.*, 2013). Within the existing nanomaterials, the most used are the metal oxide nanoparticles (Djurisic *et al.*, 2014), being the titanium dioxide nanoparticles (TiO_2 NPs) the most extensively studied (both on material characterization and toxic properties) due to its low cost production and unique properties, including small size, large surface area, high stability, anticorrosive, excellent optical and electrical properties and photocatalytic activity (Gupta and Tripathi, 2011; Menard *et al.*, 2011; Fresegna *et al.*, 2014). Textile industries, biotechnology, pharmaceutical and cosmetics are prominent areas of TiO_2 NPs applications, leading to the increasing worldwide distribution of these nanoparticles (Gupta and Tripathi, 2011; Marquez-Ramirez *et al.*, 2012; Botelho *et al.*, 2014). However, the available studies on the toxicity of TiO_2 NPs show contradictory results (Shi *et al.*, 2013; Iavicoli *et al.*, 2011; Iavicoli *et al.*, 2012a).

Owing to the distinctive properties derived by the nano-sized particles, some reports have been demonstrating that nanoparticles are more toxic when compared to larger micro-sized particles (Kocbek *et al.*, 2010; Roy *et al.*, 2014). Recent advances in the production of nanomaterials lead to the development of new structures, namely of nanoparticles immobilized in microstructures that, by presenting new physico-chemical features, must be test in regards to their toxic potential (Aono *et al.*, 2012). Concerning TiO_2 NPs, these have already been developed in conjugation with modified clays, but to our knowledge there are no earlier toxicological studies on these novel nanocomposites (Aranda *et al.*, 2008; Manova *et al.*, 2010). In this way, *in vitro* studies are required to evaluate the biological effects of these nanostructures in order to understand if they present increased toxicity or biocompatibility as compared to TiO_2 NPs alone.

2. Research questions

In this context, the main goal of the present work was to evaluate *in vitro* cytotoxicity and genotoxicity of a nanocomposite (here designed C-TiO_2) constituted by TiO_2 NPs (NM-104 rutile obtained from the European Commission's Joint Research Centre (JRC) (Rasmussen *et al.*, 2014)) and high purity kaolinite clay in a hepatocellular carcinoma human cell line (HepG2). For a matter of comparison, both TiO_2 and kaolinite single particles were also studied.

Thus, to achieve this main objective, the following specific goals were established:

1. Nanomaterial characterization: Characterization of primary particle size, size distribution and particle morphology using field emission scanning electron microscope (FE-SEM), as well as the evaluation of the hydrodynamic particle size, polydispersity - via dynamic light scattering (DLS) - and zeta-potential (by laser Doppler velocimetry – LDV) of the nanomaterials under evaluation (TiO_2 NPs, kaolinite clay and C-TiO_2 nanocomposites);
2. Nanomaterial uptake: Exploratory analysis of nanomaterials internalization on HepG2 cells after exposure to different concentrations and time periods by means of flow cytometry;
3. Interferences studies: Evaluation of the possible interferences between nanomaterials and the cytotoxicity (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), neutral red uptake (NRU) and Alamar blue (AB) and lactate dehydrogenase (LDH) activity assays) and genotoxicity assays under evaluation. This stage was essential to identify which of them were suitable for toxicity assessment of the studied materials, and to obviate possible interference between materials and assay reading by introducing alterations to assay protocols;
4. *In vitro* cytotoxicity and genotoxicity of C-TiO_2, kaolinite and TiO_2 nanomaterials on HepG2 cell line: Assessment of the cytotoxic and genotoxic effects of the different nanomaterials on HepG2 cell line. Cell viability was evaluated after cell exposure to different concentrations (and time periods) of the studied materials, by employing MTT and AB cytotoxicity assays. Additionally, for the genotoxicity assessment, comet assay was performed after cell exposure for the different nanomaterials non-cytotoxic concentrations and time periods.

3. Organization of the dissertation

The dissertation is constituted by the following topics:

1. State of the art, presents general concepts on nanotechnology and nanotoxicology, a literature review of the nanomaterials under study and their possible related effects as well as innovative aspects of the research;
2. Methodology, provides a detailed explanation of the methods used for the nanomaterial characterization, and the methodologies used for each cytotoxic and genotoxic assays under evaluation, after a full study of the possible nanomaterial interference on the assays;

3. Results and Discussion, debates the results obtained with basis on knowledge described in the scientific literature;
4. Conclusions and future perspectives, summarizes the main results and conclusions discussed in the previous section, presents suggestions for future work and appreciations of the work developed.

This dissertation also includes appendices with supplementary data.

II. STATE OF ART

1. The Nano World

Nanotechnology is an interdisciplinary field of science that is considered as a fundamental technology of the 21st century (Frohlich, 2013; Laroui *et al.*, 2013). The interconnection of different fields of knowledge integrating physics, chemistry, engineering, biology and medicine, allows nanotechnology to study and manipulate materials at nano scale, producing innovative structures, materials and/or devices with distinctive properties and functionalities (Laroui *et al.*, 2013; OCDE, 2014).

The industry of nanotechnology is in global expansion (Baker *et al.*, 2014; Djurisic *et al.*, 2014), being estimated that, by now, in 2015, there would be around 2 million nanotechnology workers worldwide, and the value of products incorporating nanotechnology as a key component would reach around 1 trillion dollars (Roco, 2011). With the systematic introduction of new products, the nanotechnology market is doubling every 3 years. The same author has extrapolated that, by the year of 2020, the number of nanotechnology workers worldwide and product market value will increase to 6 million people and 3 trillion dollars, respectively.

In the last years, several sectors such as electronics, textiles, food, agriculture, environmental protection, cosmetics and healthcare (including drug delivery systems, regenerative medicine and diagnostics) have been revolutionized with the introduction of different types of engineered nanomaterials (ENMs) (SCENIHR, 2007; Conde *et al.*, 2014). The European Union defines ENMs as structures, agglomerates or aggregates, where 50% or more of the particles have at least one dimension, in the size range 1-100 nm (EPC, 2011; Lin *et al.*, 2014). In addition, there is a specific definition of particle at a nano scale which includes a piece of matter with demarcated physical boundaries with the same size range as defined earlier, commonly defined as nanoparticles. The European Commission also recommends that these broad definitions should include any material with natural (e.g. forest fires, mineral composites, volcanic ash, viruses), incident (e.g. cooking smoke, diesel exhaust, industrial effluents) or engineered (quantum dots, carbon nanotubes, lipid and metallic nanoparticles) origin (Lidén, 2011). Generally, engineered nanoparticles are better characterized than the environmental nanoparticles, allowing a better correlation between their physicochemical properties and their effects in biological systems (Frohlich, 2013).

Despite the implementation of ENMs being a sustainable and profitable alternative to traditional materials, it is important to consider the possible impact on environmental and human health (Mitrano *et al.*, 2015; Martirosyan and Schneider, 2014). The large scale applications of ENMs increases the extension of exposure, influencing the life cycle of these nanomaterials. Nanomaterials can be released into the environment during different stages:

production process and manufacture, usage, recycling or disposal (Djurisic *et al.*, 2014). While the manufacturing of these materials is performed in controlled conditions, the use and disposal of these products by the consumer are life cycle stages that cannot yet be controlled (Mitrano *et al.*, 2015). Once in the environment, ENMs experience different transformations (e.g., deposition, adsorption, agglomeration, aggregation, redox reactions) that possibly interfere with their reactivity and toxicity (Martirosyan and Schneider, 2014). As such, it is necessary to conduct a complete risk assessment of these nanomaterials, which must include their entire life cycle, in order to understand their biological impact and fate in the environment (Piccinno *et al.*, 2012; Martirosyan and Schneider, 2014).

2. Nanotoxicology: looking at the risks of TiO_2 NPs exposure to human health

Nanotoxicology is a branch of bionanoscience which evaluates the potential toxic impact of nanomaterials on human and environmental health, resulting from the interaction of these materials with biological and ecological systems (Ai *et al.*, 2011; Love *et al.*, 2012; Sarkar *et al.*, 2014). Some materials, harmless in its bulk form, become cytotoxic when presented at the nano scale. This adverse phenomenon is related to the high surface to volume ratio and consequent increase surface reactivity of the nanoparticles (Frohlich, 2013). One key goal of nanotoxicology is to understand the impact of existing nanoparticles in different biological systems and ultimately to help scientists to synthetize safer nanoparticles, or at least understand the impact of existing nanoparticles in different biological systems. For this purpose, the physicochemical properties of nanomaterials must be well characterized, and the analysis of its effects on biological systems must be carried out using appropriate models (Love *et al.*, 2012).

2.1. What are TiO_2 NPs?

Metal oxide nanoparticles (MONPs) are the group of ENMs with the largest share of manufacture and application worldwide (Djurisic *et al.*, 2014). These nanoparticles offer unique optical and magnetic functionalities, and are therefore appealing for several applications, including material science, engineering and biomedical applications (Falugi *et al.*, 2012; Teske and Detweiler, 2015).

Currently, one of the most studied and most abundantly produced MONPs around the world are TiO_2 NPs (Menard *et al.*, 2011; Li *et al.*, 2014).

In its bulk form, titanium dioxide is frequently characterized as a non-combustible, thermally stable, odourless and poorly soluble particulate (Iavicoli *et al.*, 2011; Marquez-Ramirez *et al.*, 2012). Titanium dioxide is obtained from the iron mineral ilmenite, a naturally

occurring crystal that can exist in three major crystalline forms: rutile, anatase and brookite (Banerjee, 2011; Park *et al.*, 2014). Despite that, only rutile and anatase are generally used in titanium dioxide applications (Iavicoli *et al.*, 2011; Becker *et al.*, 2014). Although both rutile and anatase possess a tetragonal crystal system (Figure 1), rutile has a denser arrangement of the atoms (Iavicoli *et al.*, 2011). This phase is the most common and stable phase in nature, while anatase is metastable at room temperature, exhibiting the highest photocatalytic activity and chemical reactivity when compared to rutile form (Menard *et al.*, 2011; Becker *et al.*, 2014). Commonly, TiO_2 NPs are a mixture of both rutile and anatase forms (Becker *et al.*, 2014).

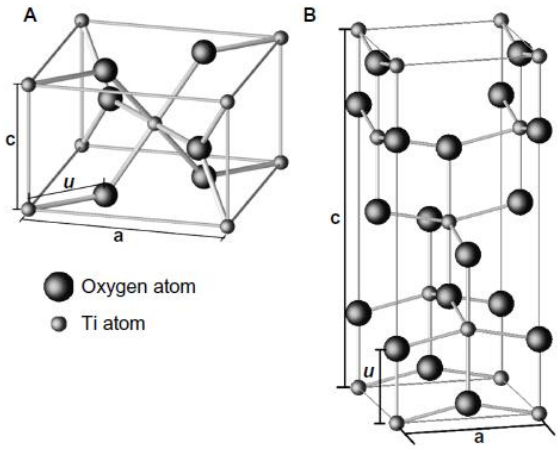


Figure 1. Tetragonal crystal strucutre of (A) rutile and (B) anatase TiO_2 forms (Banerjee, 2011).

Due to their unique and distinctive properties, including high stability, anticorrosive, biocompatibility, excellent optical performance and electrical properties, photocatalytic activity and low cost production, TiO_2 NPs are broadly used in a variety of applications (Jaeger *et al.*, 2012; Lagopati *et al.*, 2014). These include industry, waste water treatment, medicine, pharmaceuticals and cosmetics (Warheit, 2013; Botelho *et al.*, 2014). They are also applied in several industrial materials as a white pigment in paints, inks, rubber, plastics, car materials, papers and food additives (Horie *et al.*, 2010; Martirosyan and Schneider, 2014).

Cosmetics are the largest application area for TiO_2 NPs (Fig. 2.A), representing 59.4% of the total product application. These nanoparticles are commonly used in sunscreens for protecting skin cells from ultraviolet (UV) light damage, as well as white dye in toothpastes (Fenoglio *et al.*, 2009; Becker *et al.*, 2014). Additionally, they have been used in skin care products to treat some dermatologic diseases such as acne vulgaris, atopic dermatitis and hyperpigmented skin lesions, among others (Shi *et al.*, 2013).

More recently, TiO_2 NPs have been explored in several areas of biomedical research including prosthetic implants (e.g. as coating material due to their antimicrobial and mechanical properties), photodynamic therapy for cancer, drug delivery systems, cell imaging, biosensors and genetic engineering (Marquez-Ramirez *et al.*, 2012; Yin *et al.*, 2013; Martirosyan and Schneider, 2014).

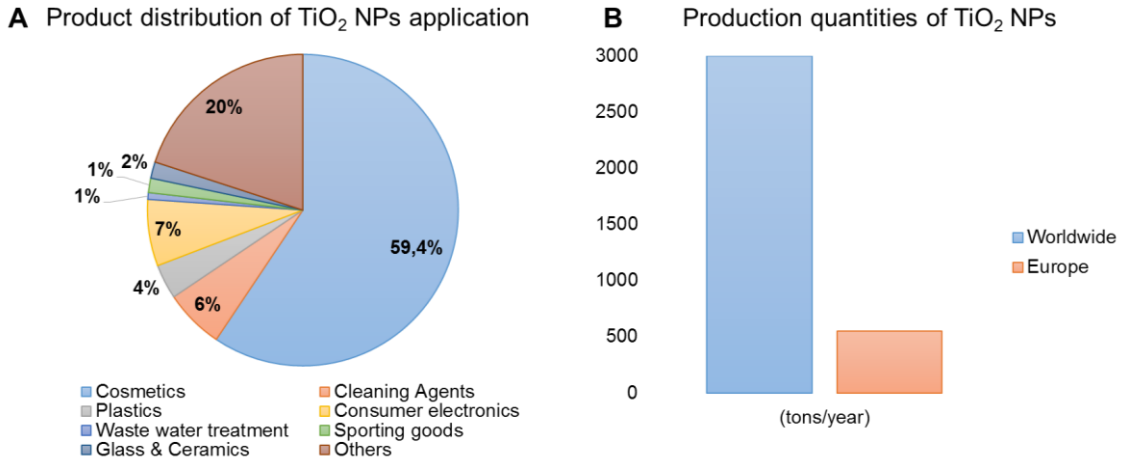


Figure 2. Percentages of product applications (A) and production quantities of TiO_2 NPs (B) (based on data presented by Sun *et al.* (2014) (graph A) and Piccinno *et al.* (2012) (graph B)).

With the increased demand for TiO_2 NPs among nanotechnology products, the amount of production of these ENMs is massive. As represented in the figure 2.B, according to Piccinno *et al.* (2012), around 3000 tons of TiO_2 NPs are produced per year worldwide, while in Europe, from these quantity, only 550 tons per year are produced. Thereby, a significant release of TiO_2 NPs into the environment is expected, increasing environmental and, consequently, human exposure (Botelho *et al.*, 2014; Djurisic *et al.*, 2014). Individuals primarily exposed to TiO_2 NPs are not only the consumers of these applications, but also the workers who may contact, directly or indirectly, with these nanoparticles during research, synthesis or further treatment and application of these materials (Klien and Godnic-Cvar, 2012).

Most of the TiO_2 NPs physicochemical properties that make them so valuable for the mentioned applications are also related to their bioavailability and toxicity in humans (Shi *et al.*, 2013). The main physicochemical properties of particles associated to their toxicity include size, shape, surface characteristics and also inner structure (Roy *et al.*, 2014; Becker *et al.*, 2014; Park *et al.*, 2014). Besides the reactivity and consequent toxicity which may be related to the small size of TiO_2 NPs (as also with the majority of nanoparticles, as mentioned above) (Wang *et al.*, 2014), is important to consider the influence of the proteins with the nanoparticles in biological conditions. Actually, nanoparticles when introduced in

biological medium will unavoidably interact with the human plasma proteins, leading to the formation of a protein corona, which result from the adsorption of these proteins to nanoparticles surface. Consequently, this protein corona will interfere with the physicochemical properties of the nanoparticles (e.g. size, agglomeration state), possibly interfering with the cellular uptake of particles (Allouni *et al.*, 2015). Therefore, researchers have been concerned about the possible harmful effects derived from TiO_2 NPs exposure in human health (Shi *et al.*, 2013).

For several applications such as sunscreens, different authors verified that surface modifications of TiO_2 NPs leads to an improvement of their properties (e.g. UV attenuation), in comparison with uncoated particles (Rahim *et al.*, 2012). Additionally, to prevent agglomeration and increase the dispersibility and stability of these nanoparticles in different organic solvents, some researchers have been employing different surface coatings (e.g. polyethylene glycol) (Rahim *et al.*, 2012; Janer *et al.*, 2014). Depending on the coatings and surface modifications applied in different applications, nanoparticles can present higher biocompatibility and possibly decrease their acute toxicity (Naqvi *et al.*, 2010).

2.2. Human Exposure to TiO_2 NPs: uptake, biodistribution and accumulation

Human exposure to TiO_2 NPs may occur during both manufacturing and use (Klien and Godnic-Cvar, 2012). Although the real effects on human health derived from nanoparticles exposure remain unclear, it is important to consider the multiple scenarios of uptake and biodistribution of the nanoparticles in the human body, to better evaluate and predict the possible toxicological effects (Magdolenova *et al.*, 2012).

The human body has several portals of entry that protect the organism against pathogens and harmful materials from the external environment (Arora *et al.*, 2012). The major portals of entry of TiO_2 NPs in the human body are the respiratory tract, the skin and hair follicles, the gastrointestinal tract and the circulatory system (Wang *et al.*, 2014) (Figure 3).

In occupational settings, inhalation is the major route of exposure to TiO_2 NPs (Shi *et al.*, 2013). The respiratory system is the portal of entry with the largest surface area in direct contact with the environment being, in the majority of exposure scenarios, the main route of entrance of nanoparticles (Kim *et al.*, 2012). After inhalation, nanoparticles can pass through the lung epithelium and reach the blood and lymph systems (Magdolenova *et al.*, 2012). In addition, inhalation of TiO_2 NPs has been also related to the deposition of these particles in the brain, since they can pass through the olfactory bulb (Huerta-Garcia *et al.*, 2014).

Dermal exposure is related to the use of cosmetics and sunscreens that contain these MNOPs (Burnett and Wang, 2011). Skin is considered the first mechanical barrier that protects the organism against toxic agents, including nanoparticles (Krug and Wick, 2011). Depending on the nanoparticle features, they can penetrate into the dermis, enter via bloodstream or lymphatic system and reach other organs that can be potentially affected (Magdolenova *et al.*, 2012). Nevertheless, several studies demonstrated no evidence of TiO_2 NPs penetration through the human skin into the body, under normal conditions (healthy skin without burns or cuts) (Shi *et al.*, 2013).

The ingestion of drug carriers, food products, water or beverages containing TiO_2 NPs are examples of sources of exposure to these nanoparticles in the gastrointestinal tract (Martirosyan and Schneider, 2014).

In medical applications, intravenous, intraperitoneal or even subcutaneous injection of TiO_2 NPs are the main route of entry into the circulatory system (Shi *et al.*, 2013). The small size of TiO_2 NPs allows internalization by cells and trancytosis across epithelial and endothelial cell barriers into the blood and lymph circulation (Huerta-Garcia *et al.*, 2014). TiO_2 NPs can form a protein corona after interacting with human plasma proteins, coagulation factors, platelets and red or white blood cells. This protein corona may interfere with the distribution, metabolism and excretion of these nanoparticles and, therefore, with their possible toxic effects (Allouni *et al.*, 2015).

After entering the systemic circulation, TiO_2 NPs can be biodistributed and bioaccumulated in organs and tissues of the body including liver, lung, spleen, kidney, heart, bone marrow and lymph nodes (Becker *et al.*, 2014; Roy *et al.*, 2014). It has also been demonstrated that high doses of TiO_2 NPs in the circulation are able to cross blood brain barrier, blood-testis and also the blood placenta barrier in pregnant women (Martirosyan and Schneider, 2014).

There are two main clearance pathways of TiO_2 NPs from the human body: via kidneys/urine and bile/faeces. A large fraction of internalized TiO_2 NPs can be rapidly excreted, however, not all of these nanoparticles will be totally eliminated, since some of them may accumulate in some tissues or organs, especially after continuous exposure. The physicochemical properties of the particles, dosage, time and route of exposure may interfere with the rate of absorption, biodistribution, metabolism and excretion of these MNOPs in the human body. Some studies reveal pathological lesions in several organs and low clearance of TiO_2 NPs after exposure to high doses and for short periods that often correspond to unrealistic particle levels of exposure. Therefore, further studies are needed

to better understand the effects of the TiO_2 NPs exposure in the normal biological function and anatomical morphology (Shi *et al.*, 2013).

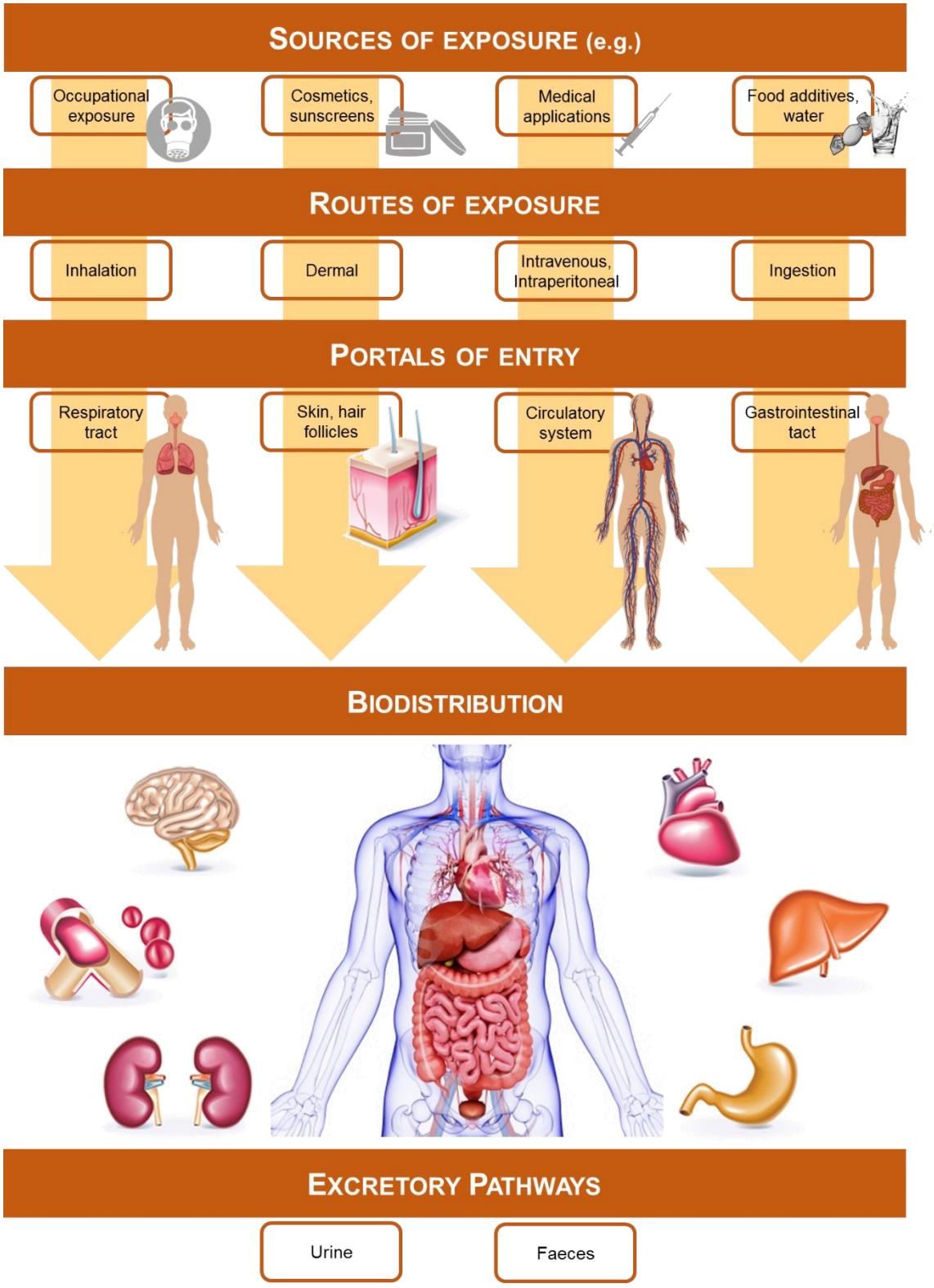


Figure 3. Toxicokinetics of TiO_2 NPs within the human body with indication of possible sources of exposure, based on the information reported along the text.

The liver is the second largest organ of the human body, playing a critical role in the metabolic (e.g. cholesterol and intestinal lipid metabolism) and clearance function (Wallace *et al.*, 2008).

The liver is mainly constituted by parenchymal cells, representing approximately 80% of the liver volume (Figure 4). About 6.5% of the remaining volume corresponds to non-parenchymal cells. Hepatocytes are the major parenchymal cells types in the liver, while the hepatic stellate cells (HSCs), liver sinusoidal endothelial cells and Kupffer cells (specialized macrophages in the liver) are the main non-parenchymal cell types (Bartneck *et al.*, 2014; Wang *et al.*, 2015). All these liver cells are promising targets for nanomedicine applications, due to their critical functions in the liver disease progression (Giannitrapani *et al.*, 2014).

This highly vascular and protective organ is involved in the detoxification of the TiO_2 NPs in the body (Roy *et al.*, 2014). Due to its physiological and anatomical characteristics, the liver is the major organ of nanoparticle accumulation and TiO_2 NPs are no exception (Shi *et al.*, 2013).

One possible reason for the nanoparticle accumulation is the high number of liver macrophages, which represents around 80-90% of total macrophages of the body. Once in the blood circulation, nanoparticles form a protein corona after interacting non-specifically with the serum proteins, which is further recognized by the Kupffer cells on the hepatic sinusoid, leading to nanoparticle accumulation. The larger the particle size, the greater the uptake by the Kupffer cells, decreasing the circulation time of these nanoparticles (Wang *et al.*, 2015). Additionally, there are other factors besides particle size that interfere with the cellular uptake of nanoparticles, for instance particles surface charge. Generally, hepatocytes are able to internalize positive charged nanoparticles, while Kupffer cells and endothelial cells tend to internalize the negative charged ones (Wang *et al.*, 2015). Beyond that, the surface charge of nanoparticles also interferes with the extracellular immobilization of nanoparticles by immune cells (Bartneck *et al.*, 2014; Giannitrapani *et al.*, 2014).

To avoid the nanoparticle accumulation on the liver and minimize nanoparticle cellular uptake by Kupffer cells, several biodegradable and biocompatible surface coating materials (e.g. polyethylene glycol) have been used (Giannitrapani *et al.*, 2014). Nonetheless, despite the numerous targeting approaches studied so far, the effect of nanoparticle properties at the cellular level in the liver is still uncertain (Wang *et al.*, 2015).

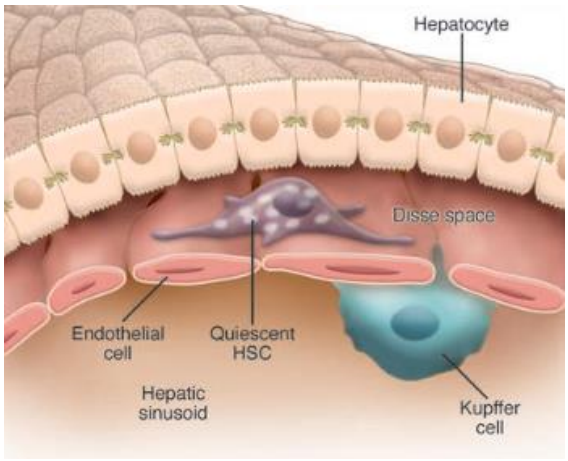


Figure 4. Major cells presented in a normal liver. Hepatocytes are parenchymal cells, while the hepatic stellate cells (HSCs), liver sinusoidal endothelial cells and Kupffer cells are non-parenchymal cells. HSCs are present in the space of Disse, which is a small region between the hepatic sinusoid and hepatocytes (adapted from Iredale (2007)).

Few studies of hepatic toxicity of TiO_2 NPs have been performed in the last years. According to Iavicoli *et al.* (2011), four studies made on hepatic cells identified the production of reactive oxygen species (ROS) as one of the predominant toxic effects related to *in vitro* TiO_2 NPs exposure. More recent studies are in agreement with these results. For instance, Shukla *et al.* (2014) demonstrated that oxidative stress is an important mechanism of toxicity after TiO_2 NPs exposure, triggering DNA damage mechanisms and initiating the expression of apoptotic proteins, resulting in hepatic injury. These confirms the results obtained in other studies reporting genotoxic effects (Petković *et al.*, 2011; Kermanizadeh *et al.*, 2012) and loss in hepatic functions (Natarajan, 2015).

It is important to take into account that *in vitro* hepatic systems might not be the more accurate liver model once these cells lose key liver specific functions and are not representative of realistic *in vivo* conditions (Kermanizadeh *et al.*, 2013). Notwithstanding, several authors claim that the use of mammalian cell line is the best option to access the toxicological effect of a certain engineered nanomaterial in cells and tissues (Suh *et al.*, 2009). Actually, the cancer cell line demonstrates increased uptake levels of nanoparticles and are more sensitive to nanomaterial toxicity, when compared with long-lived cell lines and primary cultures (Joris *et al.*, 2013). One of the widely used *in vitro* hepatic model is the HepG2 cell line. It is an extremely well characterized cell line and it is commonly used in nanotoxicological studies as a surrogate model of human hepatocytes (Jones and Grainger, 2009).

2.3. TiO_2 NPs interaction with biological systems: *in vitro* and *in vivo* evidence

As aforementioned, the unique properties of TiO_2 NPs make them an attractive option for several everyday nanotechnology-based products and applications, being the most vastly manufactured nanoparticle in the world (Liang *et al.*, 2009). Meanwhile, the major challenge in using this type of particles is to guarantee their safety to human health and environment, after inclusion in consumer products (Djurisic *et al.*, 2014; Lin *et al.*, 2014).

The main aspects to take into account during the evaluation of nanoparticle cytotoxicity and genotoxicity is the proper characterization of these materials, including morphology, crystal structure, purity, size distribution, surface area, chemical composition (surface chemistry and charge), and the degree of aggregation and agglomeration as well as the release of metal ion in biological media (Djurisic *et al.*, 2014). In addition to these, the coating and surface modification of the nanoparticle is another important feature to take into account (Klien and Godnic-Cvar, 2012). Thereby, a detailed characterization of the nanomaterial properties is essential for both *in vitro* and *in vivo* studies, to perceive their influence in the biological systems.

One of the major problems associated with *in vitro* studies with nanoparticles are related to the nanoparticles instability and their tendency to form aggregates (non-reversible grouping of nanoparticles) and agglomerates (reversible grouping of nanoparticles) in biological solutions. The same phenomenon occurs to TiO_2 NPs without surface modifications, since they tend to form aggregates and agglomerates due to their small size and also due to solution properties (e.g. conductivity and ionic strength) (Love *et al.*, 2012). Thus, to overcome this issue, is important to perform suitable medium dispersion to correctly evaluate the cytotoxic effects of the TiO_2 NPs (Fujita *et al.*, 2009).

For some researchers, TiO_2 NPs are considered to be a nontoxic mineral, being even used as negative control in many *in vitro* and *in vivo* studies (Liu *et al.*, 2013). However, others investigators demonstrated opposite effects, since their studies suggest that TiO_2 NPs may induce higher toxicity potential, especially when compared to their bulk materials (Jaeger *et al.*, 2012).

Due to their small size, TiO_2 NPs can penetrate the cells, which may disrupt their normal function (Liu *et al.*, 2013). The most studied mechanisms of nanoparticle cell internalization include endocytosis, membrane fluidity and direct passage through channels or adhesive junctions (e.g. tight junctions) (Roy *et al.*, 2014). Once inside the cells, some reports indicate the occurrence of cyto and genotoxic effects, depending on the TiO_2 NPs concentration and also their exposure time.

Despite the inconsistency of biological effects observed *in vitro* after exposure to TiO_2 NPs, many studies have demonstrated the capacity of TiO_2 NPs to induce cytotoxicity and genotoxicity in various cultured cell lines (Shi *et al.*, 2013; Prasad *et al.*, 2014). Increased ROS production, reduction in cell viability and proliferation, and DNA damage have been frequently related to these nanoparticles in mammalian cells (Huerta-Garcia *et al.*, 2014). In fact, the photocatalysis activity of TiO_2 NPs conjugated with their reduced size has been related to increased ROS production and subsequent biological damage (Fujita *et al.*, 2009; Horie *et al.*, 2010).

Additionally, other studies have reported that exposure to TiO_2 NPs leads to lipid peroxidation, caspase activation followed by micronuclei formation, DNA damage directly or indirectly via oxidative stress, chromatin condensation and inflammatory responses (Valdiglesias *et al.*, 2013; Botelho *et al.*, 2014). While some authors reported the occurrence of cell death via apoptosis, others have not demonstrated this phenomenon induced by TiO_2 NPs (Jaeger *et al.*, 2012; Roy *et al.*, 2014). Conflicting results have also been described in the study of oxidative stress response, mitochondrial dysfunction and plasma membrane damage.

In the same way as *in vitro* studies, *in vivo* studies of TiO_2 NPs exposure carried out on hamsters, rats, and mice also show inconsistent results (Iavicoli *et al.*, 2012b; Prasad *et al.*, 2014). One topic that remains controversial is the genotoxicity of TiO_2 NPs (Shi *et al.*, 2013). While some *in vivo* genotoxicity studies showed that TiO_2 NPs increase the expression of inflammatory cytokines, mRNA expression of toll like receptors and DNA deletions in different organs and also in the reproductive system, other authors does not report the same effect (Ursini *et al.*, 2014). These opposite results may be due to different TiO_2 NPs, exposure times and/or doses applied by the investigators, as previously notice for *in vitro* studies. Therefore, further *in vivo* studies should be carried out with relevant doses (which occur in occupational and environmental exposure conditions) in order to understand the real effects of TiO_2 NPs exposure (Martirosyan and Schneider, 2014).

Since most researchers use TiO_2 NPs with different crystal phase, size, surface coating and dosage, it is difficult to compare and truly understand the predictive value of some *in vitro* and *in vivo* studies (Janer *et al.*, 2014). As such, only after a proper assessment of the TiO_2 NPs properties and living models under evaluation, is possible to make a precise interpretation of results obtained in the performed studies (Frohlich, 2012; Park *et al.*, 2014). Therefore, despite the debatable aspects of TiO_2 NPs cytotoxicity and genotoxicity, these nanomaterials should be treated as potentially hazardous.

3. Moving forward with nanocomposites

3.1. The need for new materials

The fact that nanoparticles induce more damage and are more biologically active when compared to larger micro-sized particles (Roy *et al.*, 2014; Allouni *et al.*, 2015) encouraged the emergence of an innovative area of nanotechnology: nanoarchitectonics. Nanoarchitectonics is a technology system which assembles nanostructure units (discrete functional parts which have one or more dimensions of the order of 100 nm or less) (SCENIHR, 2007) into a novel structure functionality through mutual interactions among units (Aono *et al.*, 2012). This field of nanomaterials introduced innovations in the design and constructions systems in nanoelectronics, nanomachinery, energy conversion, and more recently in nanomedicine (Gonzalez-Alfaro *et al.*, 2011; Kujawa and Winnik, 2013). New nanostructured materials have been developed as electro and photocatalysts (e.g. removal of non-filterable pollutants and dissolved organic compounds from water such as dyes, halogenated organic compounds and pesticides, resulting from industrial and agricultural applications), rechargeable batteries, solar cells and fuel cells (Aranda *et al.*, 2008; Aono *et al.*, 2012). Most of these nano, or even micro scale materials have been produced utilizing synthetic techniques based on soft-chemical nanoarchitectonics, including colloid chemistry and supermolecular nanoarchitectonic processes (Aono *et al.*, 2012). Currently, colloidal systems have been studied as new nanostructure complexes for therapeutic and diagnostic applications and also for development of new materials for regenerative medicine (Kujawa and Winnik, 2013; Kim *et al.*, 2014).

Composites are materials constituted with two or more components. These can be formed through a combination of materials with different physicochemical structures which contribute to the appearance of uncommon properties difficult to obtain from the individual components. Composite materials present two main phases: continuous matrix phase and the filler material embedded to the matrix which corresponds to the dispread phase (Olad, 2011).

Nanocomposites are commonly defined as composites materials, where at least one of the phase components dimension is at the nanometre range (Jeon and Baek, 2010; Wohlleben *et al.*, 2011; Choi *et al.*, 2013). Similarly to the composites, nanocomposites can be classified as polymeric, ceramic and metallic, according to the matrix composition (Camargo *et al.*, 2009; Choi *et al.*, 2013).

Recently, the use of biocompatible and antibacterial polymeric nanocomposites in several applications has been increasing, for example, for water purification membranes,

thin films for wound dressing and biosensors (Santos *et al.*, 2012). Nanocomposites have already been considered an attractive option for several biomedical fields including tissue engineering, oral implant and orthopaedic applications (Choi *et al.*, 2013).

The degradation and consequent release of the embedded nanomaterials into the environment is a probable ending of nanocomposites life cycle (i.e., since the manufacture, consumer product use to disposal and/or recycling), representing a potential hazard aspect of these applications (Froggett *et al.*, 2014). Thereby, more studies are required to understand the potential toxicological effects derived from exposure to these nanocomposites.

In spite of the numerous advantages and potential applications regarding the polymeric nanocomposites, some researchers have identified some disadvantages associated to these structures. The main problems arise from the non-homogeneous distribution of the particles in the polymer matrix, limited degree of exfoliation and tendency to aggregation, especially in nanocomposites containing fibers, tubes or spherical particles (Keledi *et al.*, 2012).

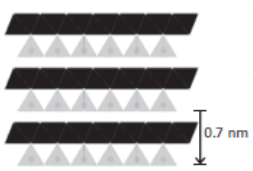
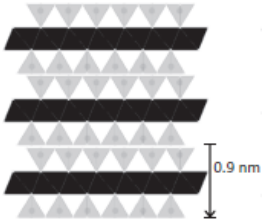
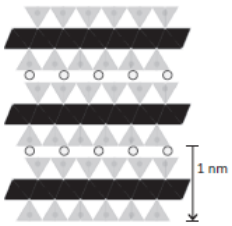
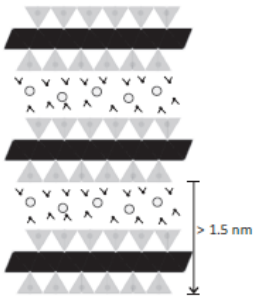
The immobilization of nanoparticles on the surface of inorganic or organic supports results in the creation of nanocomposites. These new nanoarchitectonic structures also present high surface area as well as increased porosity due to their topochemical characteristics (Aranda *et al.*, 2008). The most widely used supports include zeolites, activated carbon, silica and also natural or synthetic clay minerals like montmorillonite, vermiculite and sepiolite (Aranda *et al.*, 2008; Gonzalez-Alfaro *et al.*, 2011).

3.2. Clay minerals

Layered silicates are natural or synthetic minerals characterized by a regular laminar structure made of alumina and silica with high surface area (Pavlidou and Papaspyrides, 2008; Maisanaba *et al.*, 2015). Among the existing layered silicates, the clays are one of the most commonly used in the preparation of polymer nanocomposites since they greatly improve the polymeric matrix properties, due to their high surface to volume ratio (Arora and Padua, 2010; Olad, 2011). Clay minerals are one of the most abundant natural mineral at the surface of the earth and is presented as a nanosized layer, with a particle size less than 2 nm and layer thickness of 1 nm (Davidovic *et al.*, 2011). The layered structure of clays are built from two sheets, tetrahedral (in which a silicon atom is surrounded by four oxygen atoms) and octahedral (in which a metal like aluminium or magnesium is surrounded by eight oxygen atoms), fused together at the oxygen atoms. The rearrangements of these sheets fused together form different types of groups: kaolinite (one tetrahedral fused to one

octahedral sheet) and phyllosilicate (two tetrahedral fused to one octahedral sheet) (Table 1) (Olad, 2011; Dawson and Oreffo, 2013).

Table 1. Types of arrangements of clay layered silicates (based on Dawson and Oreffo (2013) and Maisanaba *et al.* (2015)).

Type of arrangement (tetrahedral:octahedral sheets)	Layered silicate	Structure	General formula	Layer thickness (nm)
	Kaolinite (1:1)		$Al_2Si_2O_5(OH)_5$	0.7
	Phyllosilicates (2:1) — Pyrophyllite		$Al_2Si_4O_{10}(OH)_2$	0.9
	Phyllosilicates (2:1) — Vermiculite		$(Mg,Fe^{2+},Al)_3(Al,Si)_4O_{10}(OH)_2 .4(H_2O)$	1
	Phyllosilicates (2:1) — Smectite		Montmorillonite: $M_x(Al_4-xMg_x)Si_8O_{20}(OH)_4$ Hectorite: $M_x(Mg_6-xLi_x)Si_8O_{20}(OH)_4$ Saponite: $M_xMg_6(Si_8-xAl_x)O_{20}(OH)_4$	> 1.5

Kaolinite is one relevant class of clay minerals in which the layers are held together through hydrogen bonding, as well as dipole-dipole and van der Waals interactions. This clay mineral does not tend to expand in water, but certain compounds (e.g. amides, urea), induce kaolinite swelling due to the formation of hydrogen bonds with the inter-layer surface. The high level of organization and degree of stacking of the layers allows them to be positive

charged in acidic conditions and negative charged at pH around 7 (Dawson and Oreffo, 2013; Maisanaba *et al.*, 2015).

The basic 2:1 structure without any substitution is named pyrophyllite (Pavlidou and Papaspyrides, 2008; Maisanaba *et al.*, 2015). This type of phyllosilicate is electrostatically neutral, not showing any ion within the inter layers and neither expanding in water such as kaolinite. In its turn, when the aluminium cations are partially substituted by divalent magnesium or iron cations in the neutral pyrophyllite, the smectite layered silicate is formed. The negative charge originated by this substitution is well balanced with the sodium and calcium ions presented in the inter layer (Olad, 2011). Among the different types of smectites, montmorillonite, hectorite and saponite are the most commonly used in the preparation of polymer nanocomposites (Pavlidou and Papaspyrides, 2008).

The weak forces holding the clay layers close to each other (e.g. electrostatic and van der Waals forces), the charge density and cation radius and the degree of hydration of the inter layer influences the distance between the layers, called inter layer spacing (Tang *et al.*, 2012; Mittal, 2009). The lateral dimensions of the clay and its capacity to disperse into separate layers is crucial for the interactions between the polymer matrix and the clay in a nanocomposite. As such, the thickness of the clay layer strongly interferes with the polymer properties. Several researchers reports that 1 nm of layer thickness strongly improves the polymer properties, however as the dispersing between layers increases, the internal forces for agglomeration of individual layers also rises (Olad, 2011).

3.3. TiO_2 NPs-clay nanocomposites

Titanium dioxide is considered to have excellent resistance to corrosion in different environments, a low density and also the highest strength to weight ratio, which allows many potential applications, especially for titanium-based nanocomposite materials (Shahadat *et al.*, 2015). In fact, titanium-based organic or inorganic matrix materials have been considered more advantageous comparing to other materials because of their thermal and chemical stability (Aranda *et al.*, 2008; Shahadat *et al.*, 2015). There are several applications concerning titanium-based nanocomposites, for example removal and recovery of inorganic or organic pollutants, modified electrodes, catalysts, solar and microbial fuel cells, adsorbents, gas sensors, biosensors and biomedical applications (Mallakpoura and Shahangia, 2012). Older reports have considered titanium based nanocomposites, for instance degradable polymer-TiO_2 NPs nanocomposites, as environment-friendly and safe (Reijnders, 2009).

To overcome and even restrain the release of TiO_2 NPs into the environment, it has been proposed to immobilize them in crystalline substrates, facilitating the manipulation of these nanoparticles (Tokarsky *et al.*, 2012). It has been considered that immobilization with proper mineral nanomaterials is a good strategy to diminish the possible release of these nanoparticles into the environment (Tokarčíková *et al.*, 2014).

For some applications, such as photocatalytic materials (Stathatos *et al.*, 2012; Zhang *et al.*, 2014), TiO_2 NPs can be supported on materials that should be chemically inert or participate in the chemical processes that help improving the material function. One strong example of support material for TiO_2 NPs are natural and synthetic clays (Vimonses *et al.*, 2010; Tokarčíková *et al.*, 2014). In the last years, a huge attention has been given to TiO_2 NPs supported on inorganic clay minerals, especially for photocatalysis applications (Chong *et al.*, 2009; Vimonses *et al.*, 2010). Several authors claim that the synthesis of TiO_2 NPs embedded in porous clays structures avoid the formation of aggregates (Chen *et al.*, 2013; Yang *et al.*, 2013). The small size of TiO_2 NPs (4–30 nm) leads to the formation of aggregates in suspension, decreasing its effective surface area and photocatalytic efficiency. Thereby, clay minerals are able to maintain a large surface area and enhance the photocatalytic efficiency of the photocatalysts (Chong *et al.*, 2009). Additionally, clay minerals are considered to be stable supports, protecting the TiO_2 NPs from erosion (Kibanova *et al.*, 2009) and they can also act as electron acceptors or donors (Zhang *et al.*, 2014), as well as, are capable of catalyse several chemical reactions including polymerization, reduction, decomposition or acid–base reactions (Mallakpoura and Shahangia, 2012). Resulting from its improved properties, studies have been reporting that TiO_2-clay nanocomposites exhibit more photocatalytic activity than the single TiO_2 NPs (Belessi *et al.*, 2007; Kibanova *et al.*, 2009).

Beyond the different types of clay minerals, there are various that have demonstrated to be suitable matrix for anchoring of TiO_2 NPs. Montmorillonite is the most commonly used (Vimonses *et al.*, 2010) comparing to other clay minerals, such as vermiculite, kaolinite, saponite and hectorite (Tokarčíková *et al.*, 2014). However, the interaction forces of kaolinite minerals makes this a suitable substrates for the immobilization and support of TiO_2 NPs (Chong *et al.*, 2009; Tokarčíková *et al.*, 2014) (see figure 5). It was already mentioned in the literature that this nanocomposite is not dangerous either for the environment or human health, however this must be better evaluated and clarified.

In addition to combining the best properties of both components, it has been stated that micro-sized particles induce less toxicity than the nano-size ones (Kocbek *et al.*, 2010;

Roy *et al.*, 2014). In this context, nanocomposites gain more prominence, since they are obviously larger than the single nanoparticles and, consequently, present fewer toxic effects that most nanoparticles in biological systems.

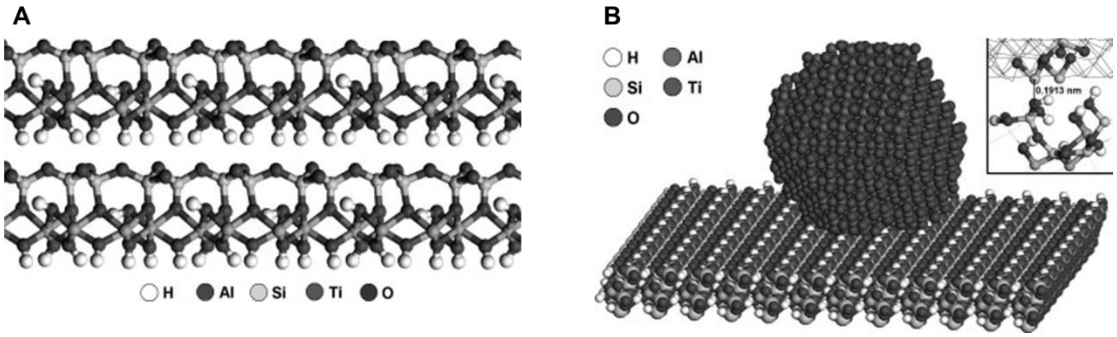


Figure 5. (A) Structure of kaolinite. (B) Model of TiO_2 nanoparticle anchored in the kaolinite clay structure. The upper right corner shows the newly formed Titanium–Oxygen bond between TiO_2 nanoparticle and the kaolinite mineral (adapted from Tokarčíková *et al.* (2014)).

3.4. Toxicity of clay based nanocomposites

So far, few studies have been made regarding the *in vitro* characterization of cellular responses to clay materials or clay based nanocomposites (Dawson and Oreffo, 2013). Generally, available studies demonstrated the occurrence of minimal loss of cell viability or proliferation for cells exposed to clays or materials incorporating this mineral. For instance, Zhuang *et al.* (2007) shown normal stromal cells morphology and proliferation after exposure to polymeric-clay scaffolds (i.e. gelatin – montmorillonite – chitosan) and Depan *et al.* (2009) observed also normal function of fibroblast after the presence of chitosan g-lactic acid montmorillonite composites. More recent studies performed by Li *et al.* (2010) verified no genotoxicity of exfoliated montmorillonite in Chinese hamster ovary cells, as well as, no long accumulation of this clay in any specific organ, after this being absorbed into the body.

In addition, other authors have demonstrated normal cellular responses and also cell adhesion and proliferation enhancements dependent on the clay concentration on polymeric substrates (Dawson and Oreffo, 2013). Studies performed in several cell lines including HepG2, human umbilical vein endothelial cells and skin fibroblasts cultured on the surface of poly-N-isopropylacrylamide hydrogels with certain specific clay concentrations, shown that the cellular adhesion and proliferation was strongly dependent on the clay content in the hydrogel, up to a critical concentration (Haraguchi *et al.*, 2006). Moreover, Zia *et al.* (2011) also evaluated the cytotoxicity of chitin based polyurethane bio-nanocomposites with different bentonite nanoclay (clay consisting mostly of

montmorillonite) concentrations, and realized that polymers with 2% of clay content were the preferred candidate for the surgical materials that were developing. The addition of the nanoclay drastically improved the tensile strength and also decreased the elongation at break of the biomaterial.

Most of the studies that look at clay induced-cytotoxicity found that significant effects were only observed after exposure to high concentrations of this mineral. For example, Han *et al.* (2011a) verified moderate cytotoxicity of clay minerals modified with an amine functional group in different cell types only at 1000 µg/mL. However, there are authors that described significant loss of A549 lung epithelial cells viability at much lower concentrations (25 µg/mL) after 24 hours of exposure to other nanoclays (Verma *et al.*, 2012). Another study observed both decrease of cell viability and ROS generation but at even lower concentrations of nanoclay (1 µg/mL) in HepG2 liver cell line (Lordan *et al.*, 2011). These authors also mentioned that the nanoclays tended to aggregate in the cell culture medium, which appeared to be correlated to the toxicity mechanisms observed (Lordan *et al.*, 2011).

In view of this, more studies are required to elucidate the potential mechanisms of cytotoxicity of certain clay formulations and their biocompatibility in living systems. One important aspect to take into account is the possible clay interactions with the components of the cell culture media, which may interfere with cell-clay interaction, influencing the cell viability (Dawson and Oreffo, 2013). Besides, is also needed to understand what are the possible pathways of biodistribution, bioaccumulation and cellular uptake of clay minerals particles in the human organism, and also their real impact on the environment (Reijnders, 2009; Dawson and Oreffo, 2013).

III. METHODOLOGY

1. Chemicals

MTT (CAS No. 298-93-1), neutral red dye (CAS No. 553-24-2), rezasurin (CAS No. 62758-13-8), Triton X-100 (CAS No. 9002-93-1), 2-mercaptoethanol (CAS No. 60-24-2), low melting point (LMP) agarose (CAS No. 39346-81-1), Tris-HCl (CAS No. 1185-53-1), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (CAS No. 7365-45-9), methyl methanesulfonate (MMS) 99% (CAS No. 66-27-3) and bovine serum albumin (BSA) (CAS No. 9048-46-8) were purchased from Sigma-Aldrich Co. Dimethyl sulfoxide (DMSO) (CAS No. 37-68-5), acetic acid (glacial) 100% (CAS No. 64-19-7), ethanol absolute (CAS No. 64-17-5), NaOH (CAS No. 1310-73-2), NaCl (CAS No. 7647-14-5) and KOH (CAS No. 105033) were bought from Merck KGaA. Cell culture media components and SyberGold were all Invitrogen™ and purchased from Thermo Fisher Scientific Inc. LDH protein from rabbit muscle (CAS No. 9001-60-9) and Tris base (CAS No. 77-86-1) were purchased from Calbiochem, while KCl (CAS No. 7447-40-7) and Na_2EDTA (CAS No. 6381-92-6) were purchased from Prolab. Finally, high purity 2-(4-Iodphenyl)-3-(4-nitrophenyl)-5-phenyltetrazoliumchlorid (INT) (CAS No. 146-68-9), phosphate-buffered saline (PBS) buffer (CAS No. 10049-21-5) and normal melting point (NMP) agarose were supplied from VWR chemicals, Lonza Group AG and Bioline, respectively.

2. Nanomaterial preparation and characterization

Nanomaterials suspensions under study (rutile TiO_2 NPs (NM-104)), high purity kaolinite clay and TiO_2 NPs immobilized in high purity kaolinite clay (C-TiO_2)) were supplied by the Ceramic for Smart System Group of the Electroceramic Department, Instituto de Ceramica y Vidrio, CSIC, Madrid, Spain. The C-TiO_2 nanocomposite was prepared in accordance with the method previously described in the patent “Procedimiento para la dispersión de nanopartículas en seco y la obtención de estructuras jerárquicas y recubrimientos” (Fernandez *et al.*, 2010).

For the nanomaterial preparation, these were suspended in incomplete (serum-free media) and complete HepG2 cell culture media (300 µg/mL for TiO_2 NPs, 2700 µg/mL for kaolinite clay and 3000 µg/mL for C-TiO_2 nanocomposite). Afterwards, the suspensions were homogenized in a microfluidizer (Model LM10, Microfluidics International Corporation) at 12500 Psi, for three times, in order to obtain a correct homogenization. This procedure was performed in an interaction chamber with Y geometry (single-slotted) with suspensions placed in an ice-water bath.

Primary particle size, size distribution and particle morphology were characterized by Field-Emission Scanning Electron Microscopy (FE-SEM) with a resolution of 1.5 nm,

working at 20 kV with a current intensity of 10 µA (Model S-4700, Hitachi Ltd. Corporation). Information on size distribution was calculated from measuring over 100 nanoparticles in random fields of view in addition to images that show general morphology of the nanoparticles.

Average hydrodynamic size and polydispersity index (PDI) of suspensions were measured by DLS, while zeta potential was determined by Laser Doppler Velocimetry (LDV) configuration, using a Zetasizer Nano-ZS equipped with 4.0 mW, 633 nm laser (Model ZEN 3600, Malvern Instruments Ltd.). For this evaluation, the nanomaterials suspensions were diluted into a 0.01 µg/mL concentration.

Prior to each toxicity treatment and interference analysis, nanomaterials suspensions were sonicated in water bath for 5 min. Serial dilutions were carried out to obtain the different nanomaterial concentrations tested and sonicated in water bath for an additional 5 min prior to testing.

3. Cell culture

Hepatocellular carcinoma human cell line (HepG2) is a continuous cell line derived from a 15 years old Caucasian male who presented a well differentiated hepatocellular carcinoma. This cell line was obtained from the European Collection of Cell Cultures and was cultured in nutrient mixture DMEM (Dulbecco's modified Eagle's medium) medium with L-Glutamine (1%), antibiotic and antimycotic solution (1%), supplemented with 10% heat-inactivated fetal bovine serum (FBS).

Once cells reached 80% confluence in the T-flasks, they were transfer to new flasks with fresh medium. For that, culture medium was removed and the cell layer was washed with PBS. After this procedure, the confluent monolayer of cells was tripsinized (Trypsin 0.25%/EDTA 0.02% in PBS without Ca^{2+}, Mg^{2+}) and incubated at 37 ºC for approximately 1 min. After cell detachment, trypsin was inactivated with cell culture media and the suspension was transferred to a conic tube and centrifuged for 5 min at 365 x *g*. The supernatant was discarded and the pellet resuspended in cell culture medium. The suspension was transferred to new T-flasks, and fresh new complete medium was added (subcultivation ratio of 1:3). Cells were incubated in a humidified atmosphere with 5% CO_2 and 37 ºC.

All these procedures were performed in a laminar-flow chamber to ensure sterile conditions. All materials were manipulated using appropriate procedures in order to prevent external contamination.

3.1. Exposure conditions

To study nanoparticle uptake, cells were seeded at $2.5x10^4$ cells per well in 96-well plates and allowed to adhere for 24 h at 37 ºC. For the cellular internalization of the nanomaterials, 100 µL of different suspensions of nanomaterials (25 and 300 µg/mL for TiO_2 NPs; 225 and 2700 µg/mL for kaolinite clay and 250 and 3000 µg/mL for C-TiO_2 nanocomposite), both in complete (supplemented with FBS) and incomplete (without FBS) media, were incubated with cells for two periods of time (3 and 24 h). Cells not exposed to nanomaterials were used as negative control, for both media types. After the incubation period, treatment was removed from wells and cells detached with trypsin (1 min at 37 ºC). Cell culture media was used to inactivate the trypsin and, subsequently, the content of each well was collected into microtubes and centrifuged (5 min at 3200 x *g*). The resulting pellet was resuspended in PBS for cytometry for final analysis in the flow cytometer.

For cytotoxicity assessment, cells were seeded at $2.5x10^4$ cells per well in 96-well plates and allowed to adhere for 24 h at 37 ºC. For each assay and each nanomaterial, 100 µL of different suspensions of nanomaterials (5, 25, 50, 100, 200 and 300 µg/mL for TiO_2 NPs; 45, 225, 445, 900, 1800 and 2700 µg/mL for kaolinite clay and 50, 250, 500, 1000, 2000 and 3000 µg/mL for C-TiO_2 nanocomposite), both in complete and incomplete medium, for different periods of time (3, 6 and 24 h) were evaluated. Complete or incomplete media was used as negative control and Triton X-100 (1%) in complete/incomplete media was used as positive control.

For genotoxicity assessment, cells were seeded at $2x10^5$ cells per well in 12-well plates and then incubated for 24 h at 37 ºC. For each nanomaterials, different concentrations were tested (5, 25 and 50 µg/mL for TiO_2 NPs; 45, 225 and 445 µg/mL for kaolinite clay and 50, 250 and 500 µg/mL for C-TiO_2 nanocomposite) in both complete and incomplete medium, for two periods of exposure (3 and 24 h). Complete/incomplete media was used as negative control and MMS 100 µM in complete/incomplete medium was used as positive control. Afterwards, the medium was removed from the wells, followed by washing the wells with PBS and finally cells were detached with trypsin (1 min at 37 ºC). After inactivating the trypsin with cell culture media, the content of each well was collected into microtubes and three successive centrifugations were performed (3 min at 7500 x *g*). Between the 1^{st} and 2^{nd} centrifugations the pellet was resuspended in PBS and cell density was calculated in a neubauer improved counting chamber (BLAUBRAND® Neubauer improved by BRAND®). The calculated volume with $2.5x10^4$ cell/mL was transferred to a microtube with PBS and centrifuged to pellet cells.

4. Nanomaterial uptake

Cellular uptake of nanomaterials in HepG2 cells was evaluated by means of flow cytometry using a BD Accuri™ C6 Flow Cytometer equipped with BD Accuri C6 software (BD Biosciences). The analysis was carried out on the basis of the size and the complexity of the cells by measuring the forward scatter (FSC) and the side scatter (SSC). Figure 6 represents an exemplificative SSC-FSC plot; two regions were established for analysis (R4 and R5): events in R5 stand for cells that have incorporated the nanomaterials while events in R4 correspond to cells with normal inner complexity (without internalized materials).

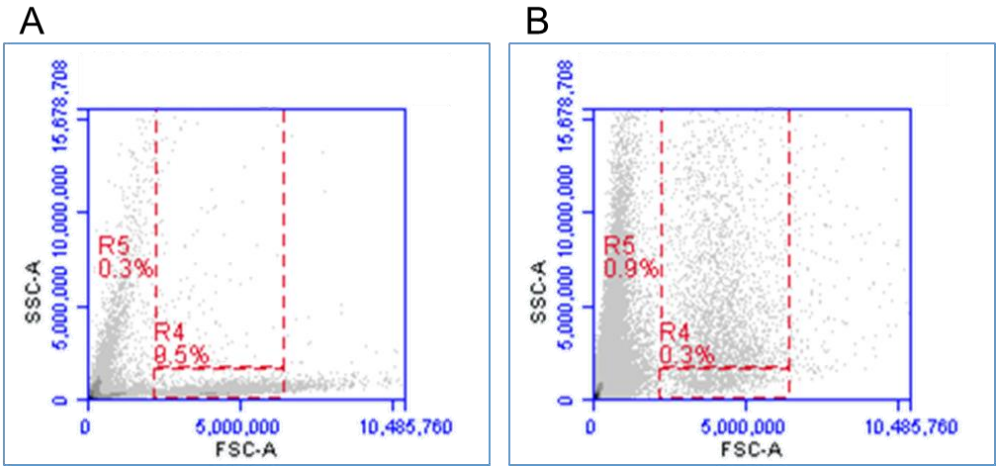


Figure 6. Plots obtained from the flow cytometry analysis: (A) Control sample (cells not exposed to nanomaterials); (B) HepG2 cells exposed to 25 µg/mL of TiO_2 NPs in incomplete medium. R4: HepG2 cells with no nanomaterial uptake; R5: HepG2 cells with internalized nanomaterials.

The percentage of cells with nanomaterials was calculated according to the following formula:

$$\%\text{Cells with Nanomaterials} = \frac{\text{Counts of events on the R5 gate}}{\text{Total counts of events on both R4 and R5 gates}} \times 100$$

5. Cellular viability and membrane integrity

In the present work, the cytotoxic effects of the different nanomaterials under study (TiO_2 NPs, kaolinite clay and C-TiO_2) were evaluated on HepG2 cell line. For the cell viability analysis it was proposed to employ MTT (Mosmann, 1983), NRU (Borenfreund and Puerner, 1985) and AB (Borra *et al.*, 2009) assays and for membrane integrity evaluation the LDH cytotoxicity assay. To evaluate if these assays were appropriate for the evaluation of nanoparticle toxicity, several interference studies were performed prior to cytotoxicity assessment.

5.1. Interference studies

To reveal possible interferences between nanomaterials and cytotoxicity assays experimental procedures, two main sets of experiments were conducted: (1) to analyze the influence of materials presence on optical measurements (light-absorption interference) and (2) to address possible nanomaterials reactivity with assay components (catalytic interference).

5.1.1. Optical interference: Evaluation of the nanomaterials interference with the light-adsorption

An exploratory analysis of nanomaterials absorbance spectrum was performed prior to the analysis of interference with early and final products of the cytotoxicity assays. Nanomaterials suspensions were prepared in water at different concentrations (0, 5, 25, 50, 100, 200 and 300 µg/mL for TiO_2 NPs; 0, 45, 225, 445, 900, 1800 and 2700 µg/mL for kaolinite clay and 0, 50, 250, 500, 1000, 2000 and 3000 µg/mL for C-TiO_2 nanocomposite) and analyzed by UV-Visible spectroscopy (ATi Unicam UV2 UV/Vis Spectrometer).

For MTT and NRU assays, light absorption interference was analyzed in the presence of dissolving agents (DMSO and fixative solution, for MTT and NRU assay, respectively) and reaction end-products (formazan dissolved in DMSO and neutral red dissolved in fixative solution). End-products were obtained by incubating live cells with MTT and NRU dyes. The nanomaterials suspensions were prepared in solvents and end-products at different concentrations (0, 25, 100 and 300 µg/mL for TiO_2 NPs; 0, 225, 900 and 2700 µg/mL for kaolinite clay and 0, 250, 1000 and 3000 µg/mL for C-TiO_2 nanocomposite). In turn, for the AB assay, the nanomaterials suspensions were prepared in both resazurin and resorufin (obtained after reduction with formic acid until obtain a pink colour).

The interference of the nanomaterials dispersions with the optical detection of the INT reduction during the LDH assay was performed by using oxidized and reduced INT. The reduced INT was obtained by adding 2-mercaptoethanol until obtain a red colour. To verify that the INT was entirely reduced, cells were exposed to Triton-X100 (1%) for 10 min and LDH assay was performed in the presence of reduced INT, prepared previously; light absorption was measured continuously at 492 nm using a Cambrex ELx808 microplate reader (Biotek, KC4) for 30 min at 37 ºC, to observe a possible increase in absorbance due to INT reduction. The nanomaterials suspensions (0, 25, 100 and 300 µg/mL for TiO_2 NPs; 0, 225, 900 and 2700 µg/mL for kaolinite clay and 0, 250, 1000 and 3000 µg/mL for C-TiO_2 nanocomposite) were prepared by serial dilution in reduced and oxidized INT.

Absorbance was measured at 570 nm and 540 nm for MTT and NRU, respectively, at 570 nm with a reference wavelength of 630 nm for the AB assay, and at 490 nm for the LDH assay using a Cambrex ELx808 microplate reader (Biotek, KC4).

5.1.2. Catalytic interference: reactivity of the nanomaterials with the assay components

Nanomaterials reactivity with dyes was determined by incubating 100 µL of nanomaterials suspensions 0, 25, 100 and 300 µg/mL for TiO_2 NPs; 0, 225, 900 and 2700 µg/mL for kaolinite clay and 0, 250, 1000 and 3000 µg/mL for C-TiO_2 nanocomposite) prepared in complete and incomplete media with MTT, NRU and AB assays reagents in the absence of cells (4 h of incubation for MTT and AB, and 3 h for NRU). The absorbance was measured at the end of the incubation.

In case of LDH assay, positive control wells with only 100 mU of LDH protein (from rabbit muscle) were also included. The assay was carried according to the kit manufacturer´s instructions (Cytotoxicity Detection Kit LDH, Cat. no. 11644793001, Roche Diagnostics Corp). Light absorption was measured continuously at 490 nm with a reference wavelength of 655 nm using a Cambrex ELx808 microplate reader (Biotek, KC4) for 1 h at 37 ºC.

5.1.3. Interference on the enzymatic activity of LDH

For the LDH assay, an additional experiment was carried out to understand the possible interference of nanomaterials on the enzymatic activity of LDH, by either inactivating or adsorbing the LDH protein. Four concentrations of nanomaterials suspensions were tested (0, 25, 100 and 300 µg/mL for TiO_2 NPs; 0, 225, 900 and 2700 µg/mL for kaolinite clay and 0, 250, 1000 and 3000 µg/mL for C-TiO_2 nanocomposite). For each concentration different units of LDH protein from rabbit muscle (20, 50, 100 and 500 mU) were tested in a 96-well plate and four periods of exposure were assessed (0, 15 min, 60 min, 3 and 24 h). After these time periods, 100 µL of the kit reaction buffer was added and the remaining assay was carried according to the kit manufacturer´s instructions (Cytotoxicity Detection Kit LDH, Cat. no. 11644793001, Roche Diagnostics Corp). Spectrophotometric absorption was taken at 490 nm with a reference wavelength of 655 nm using a Cambrex ELx808 microplate reader (Biotek, KC4).

6. Cytotoxicity assessment protocols

6.1. MTT

At the end of the exposure period, the treatment was removed and 100 µL of MTT reagent, prepared in incomplete medium (0.5 mg/mL), was added to the treated 96-well plates and incubated for 4 h at 37 ºC in the dark. After removing the MTT, the formazan was solubilized with 200 µL DMSO. Thereafter, the plates were centrifuged (Universal 320, Hettich) at 4000 x *g* for 10 min and 100 µL of the supernatant were transferred to a new plate for the final reading. Absorbance was measured at 570 nm (MTT) using a Cambrex ELx808 microplate reader (Biotek, KC4).

6.2. Alamar Blue

The Alamar Blue reagent (200 µL), prepared in incomplete medium (20 µg/mL), was added to the treated 96-well plates after removing the treatment at the end of the exposure period and then incubated for 4 h at 37 ºC in the dark. Similarly to MTT, the plates were centrifuged (Universal 320, Hettich) at 4000 x *g* for 10 min and 100 µL of the supernatant were transferred to a new plate for the final reading. Spectrophotometric absorbance was taken at 570 nm with a reference wavelength of 630, using a Cambrex ELx808 microplate reader (Biotek, KC4).

7. DNA integrity

7.1. Interference studies

To evaluate the possible interactions of nanomaterials with alkaline comet assay, two conditions were tested for each type of medium (complete or incomplete) and each type of nanomaterial (TiO_2 NPs, kaolinite clay or C-TiO_2 nanocomposite): (1) negative control (cells in cell culture medium); (2) lysis test (100 µL of nanomaterials at the highest concentrations under evaluation (300, 2700 and 3000 µg/mL for TiO_2 NPs, kaolinite clay and C-TiO_2 nanocomposite, respectively) added just before running the assay).

7.2. Genotoxicity assessment protocol: Alkaline Comet Assay

Cells (in pellet) were embedded in 200 µL 0.6% LMP agarose gel (for the lysis test in the interferences assay, 100 µL of LMP agarose at 1.2% was used as it was added 100 µL of nanomaterials) and 5 µL drops were placed on microscope slides precoated with 1% agarose (the technique was carried out using a medium throughput system 12 gel comet assay unit, Severn Biotech Ltd®). After gel solidification, the slides were placed in a coplin

jar and immersed in lysis solution (NaCl 2.5 M, Na_2EDTA 100 mM, Tris-base 10 mM, NaOH 10 M, pH 10, supplemented with Triton-X 100 1%) during 1 h, to lyse the cells and separate DNA from histones. For unwinding of DNA, all slides were immersed in electrophoresis solution (Na_2EDTA 1 mM, and NaOH 0.3 M) in the electrophoresis platform for 40 min (with a cooler underneath the platform), followed by electrophoresis for 20 min (1.15 V/cm). Neutralization was performed with cold PBS (pH 7.2) for 10 min and cold deionized water for 10 min. Slides were fixed with ethanol 70% and 96% for 15 min each at room temperature and gels were dried overnight. As represented in figure 7, just before analysis, slides were stained with SyberGold in TE buffer (Tris-HCl 10 mM and EDTA 1 mM). Slides were visualized with a Nikon Eclipse E400 microscope attached to an epi-fluorescence illuminator Nikon C-SHG1 power supply for HG 100 W, with 250x magnification (Filter G-2A). Slides were analyzed using the Comet Assay IV Software (Perceptive Instruments). At least 100 cells were scored (50 for each replicate gel). The percentage of DNA in the comet tail (% tDNA) was used as DNA damage parameter.

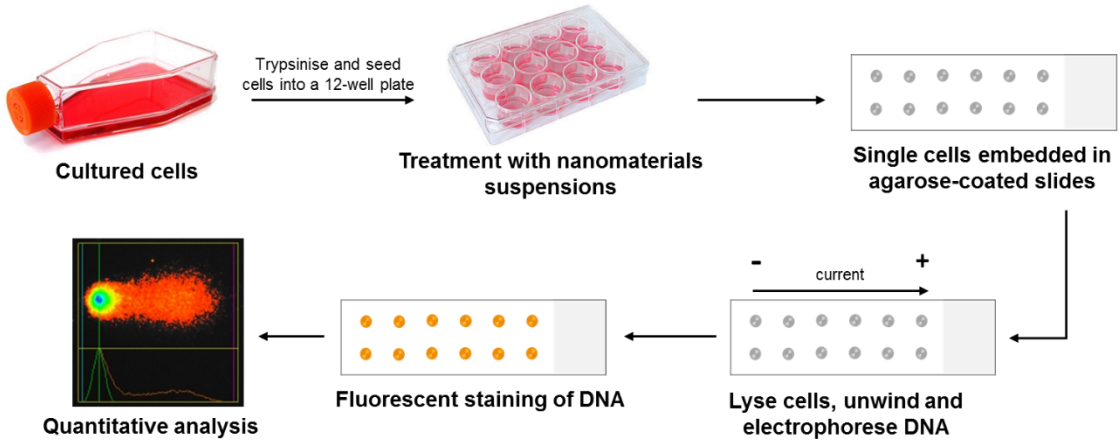


Figure 7. General procedure of the comet assay, based on the protocol described in the text.

8. Statistical analysis

Statistical analyzes were performed using SPSS for Windows statistical package (version 23.0). Non-parametric tests, Mann–Whitney U-test (differences among groups) and Spearman's correlation (associations between two variables) were used for the statistical analysis of data. A minimum of three independent experiments (in viability assays, three replicated were analyzed in each experiment) were performed for each experimental condition tested. Experimental data were expressed as mean ± standard error and a p-value of 0.05 was considered significant.

IV. RESULTS AND DISCUSSION

1. Nanomaterial characterization

With the development of nanotoxicology it has become clear that the physicochemical characterization of nanosized materials is an essential step for the risk assessment of these materials on environment and human health. A proper evaluation of the nanomaterials properties is a basic stage for screening of the mechanisms of toxicity derived from exposure to these materials (Lopez-Serrano *et al.*, 2014; Guadagnini *et al.*, 2013).

In this work, a primary characterization of C-TiO_2 nanocomposite was performed, as well as of their individual constituents: TiO_2 NPs and kaolinite mineral. Those materials were characterized after dispersion in water and biological media to understand their behaviour in the conditions as these are presented to cells. Besides, dispersion in culture media may alter original physico-chemical properties. In these conditions, nanomaterials may suffer several alterations regarding the size (e.g. altered aggregation or agglomeration state, adsorbed proteins), the particle charge, among others (Oh and Park, 2014).

The particle size is the most basic characteristic of nano-sized materials and is crucial to understand the possible mechanisms of distribution and retention of the particles in living tissues (Cho *et al.*, 2013). The most common methods for size determination are microscopy (for instance, transmission electron microscopy (TEM) and scanning electron microscopy (SEM)) and light scattering techniques, such as dynamic light scattering (DLS). While microscopy techniques allow an accurate assessment of size and morphology of the particle by image analysis, DLS measures the size and size distribution but in relevant aqueous or biological solutions (Dhawan *et al.*, 2009).

Figure 8 presents four FE-SEM micrographs of different scale magnifications of the TiO_2 NPs under evaluation. Although most existing TiO_2 NPs are a mixture of rutile anatase forms (Becker *et al.*, 2014), the present nanoparticles were only constituted by the rutile form. These nanoparticles were obtained from the JRC repository (NM-104) and are characterized as being thermal and hydrophilic. As represented in figure 8.C, these rutile TiO_2 NPs were found to be nearly spherical agglomerates with different particle size.

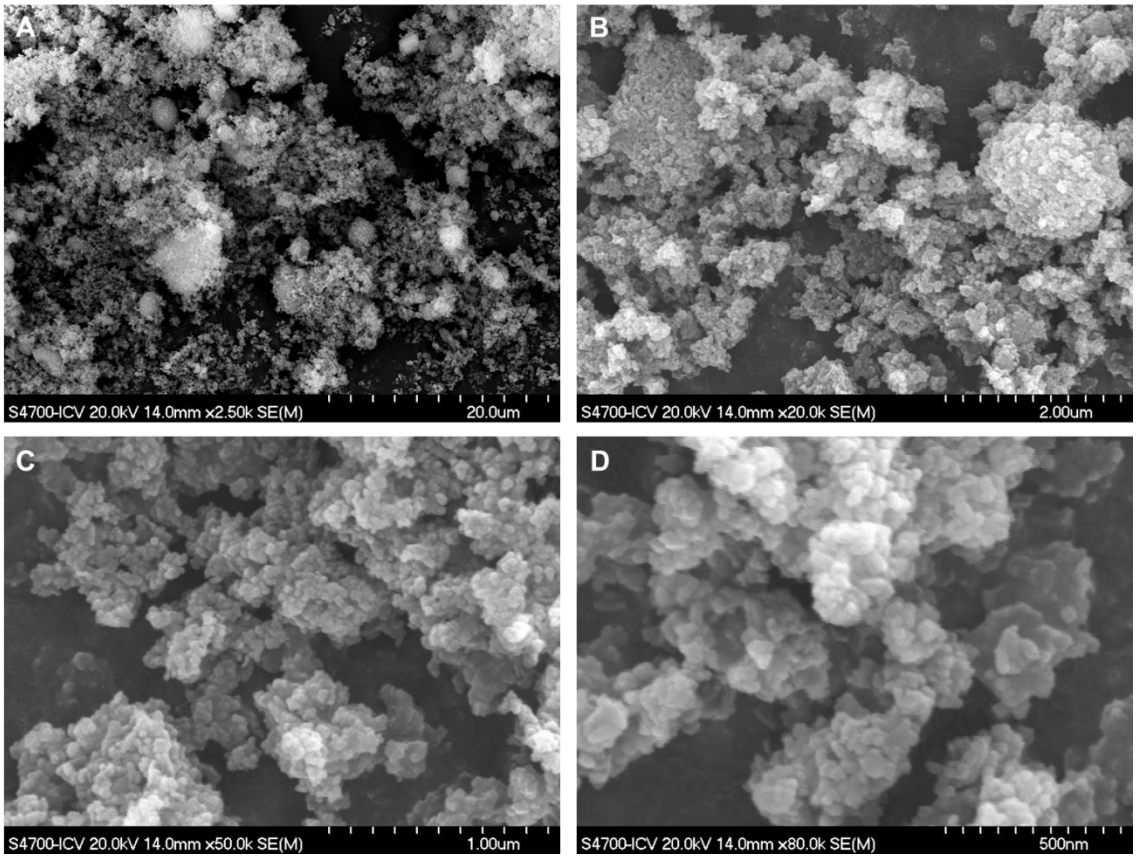


Figure 8. FE-SEM micrographs from TiO_2 NPs in four magnification scales: (A) 20.0 µm; (B) 2.0 µm; (C) 1.0 µm; and (D) 500 nm. Particles were supplied by the Ceramic for Smart System Group of the Electroceramic Department, Instituto de Ceramica y Vidrio, Madrid, Spain.

Regarding the clay mineral, it was a high purity kaolinite mineral with laminar structure of alumina and silica with a general formula of $Al_2Si_2O_5(OH)_5$, as already mentioned in previous sections. Kaolinite is a 1:1 clay mineral structure with layer thickness of 0.7 nm and its layers are held together by weak hydrogen bonding along with dipole-dipole and van der Waals interactions (Maisanaba *et al.*, 2015).

Layered silicate structures generally crystallizes into micro and nano-sized particles (Dawson and Oreffo, 2013), as was also noted in this work. The studied kaolinite clay presented agglomerates of particles with a spherical morphology and with irregular laminar shape (Fig. 9.A and 9.B). The agglomerates were formed by plates of nano-sized kaolinite (Fig. 9.C and 9.D).

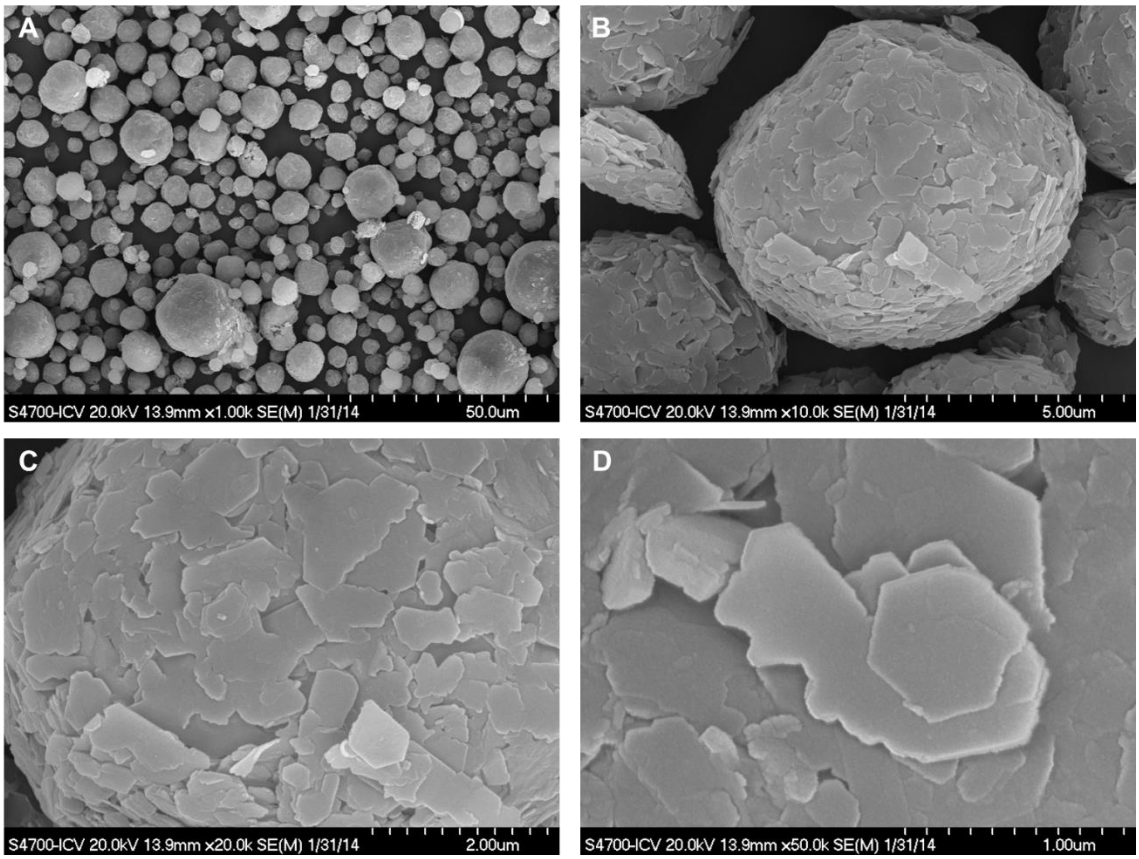


Figure 9. FE-SEM micrographs from kaolinite agglomerate particles in four magnification scales: (A) 50.0 µm; (B) 5.0 µm; (C) 2.0 µm; and (D) 1.0 µm. Particles were supplied by the Ceramic for Smart System Group of the Electroceramic Department, Instituto de Ceramica y Vidrio, Madrid, Spain.

The nanokaolin layered silicates represented 90% of the C-TiO_2 nanocomposite constitution. The remaining 10% were for the rutile TiO_2 NPs, immobilized on the nanokaolin for the formation of these nanocomposites. Figure 10 illustrates the FE-SEM images obtained from the C-TiO_2 nanocomposites. Through the magnifications of the figures 10.B and 10.C it is possible to distinguish the nanokaolin clay (Fig. 10.B.2) from the TiO_2 NPs (Fig.10.B.1), as well as the TiO_2 NPs immobilized on the kaolinite laminar structures (Fig.10.B.1 and Fig.10.C.1). These micrographs show that these nanocomposites were not spherical.

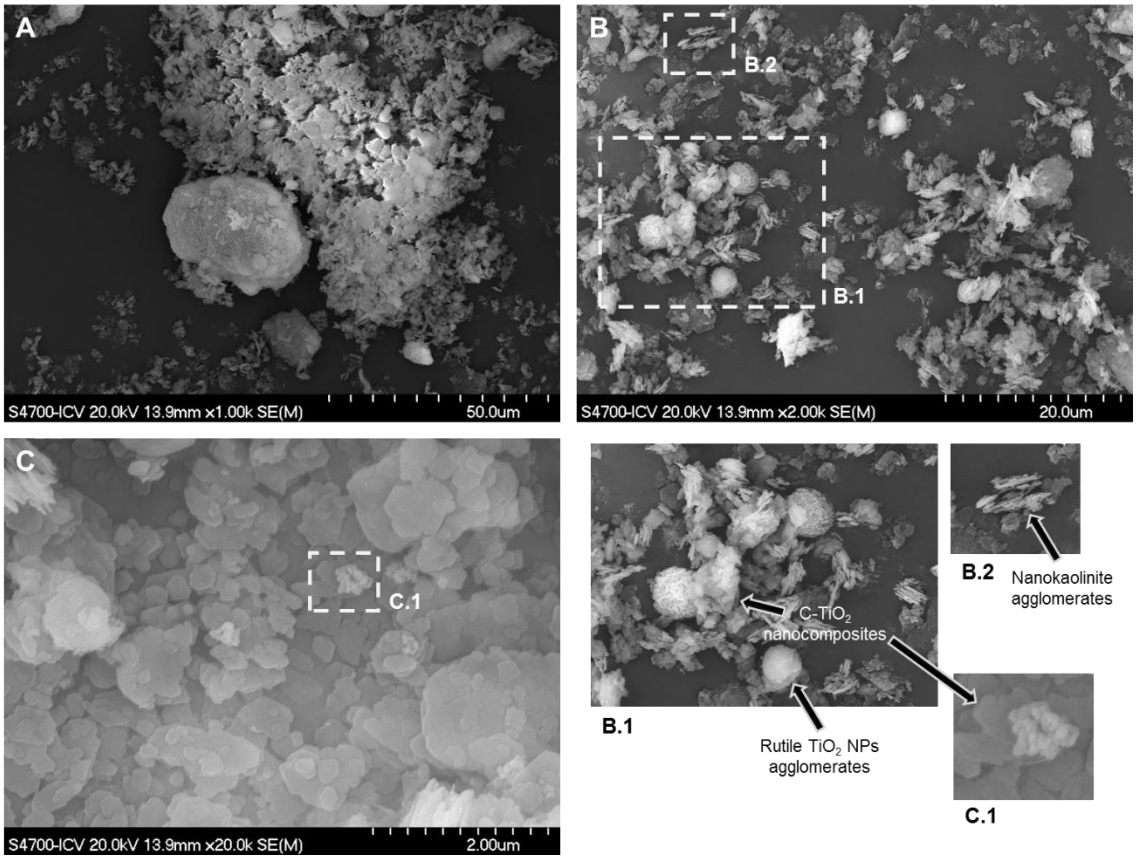


Figure 10. FE-SEM micrographs from C-TiO_2 nanocomposites in four magnification scales: (A) 50.0 µm; (B) 20.0 µm; and (C) 2.0 µm. Composites were supplied by the Ceramic for Smart System Group of the Electroceramic Department, Instituto de Ceramica y Vidrio, Madrid, Spain.

From the TiO_2 NPs and kaolinite micrographs (Figures 8 and 9) it was also possible to determine their size distribution, as represented in the figure 11. The TiO_2 NPs presented a size range from 0.01 to 0.1 µm, being the 0.04, 0.05 and 0.06 µm the most prevalent sizes (Fig. 11.A). This size range is coincident with the one reported by the JRC, that characterize the NM-104 nanoparticles with a particle size from 0.01 to 0.03 µm (Rasmussen *et al.*, 2014). The present analysis also confirms that this examined material is qualified as an ENM according to the European Commission definition (EPC, 2011).

On the other hand, the kaolinite particle agglomerates exhibited a superior size range between 0.1 to 1.4 µm, with most of them showing a predominant size 0.6 and 0.7 µm (Fig. 11.B).

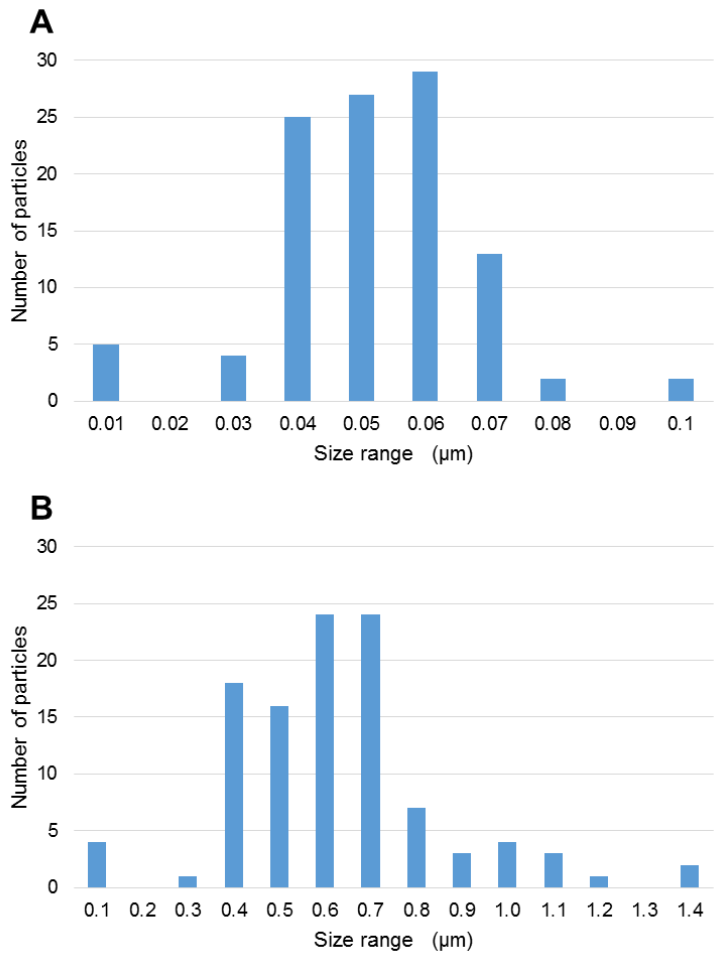


Figure 11. Size distribution of (A) TiO_2 NPs and (B) kaolinite mineral obtained from image analysis of FE-SEM micrographs.

The primary particle size is sometimes a misleading parameter particularly when considering the aggregation and/or agglomeration propensity of most nanomaterials in biological medium (due to the salts and proteins in their constitution) (Verma *et al.*, 2012). Therefore, the hydrodynamic size, polyspersity index (PDI) and zeta-potential of the C-TiO_2 nanocomposite and its individual components were evaluated by DLS to understand the nanomaterials behaviour and aggregation after dispersion in liquids.

DLS measures the Brownian motion which determines the hydrodynamic diameter of particles. The Brownian motion is defined as “the random movement of particles in a liquid due to the bombardment by molecules that surround them” (Malvern, 2004). It is the speed of particles movements into the liquid that enables the determination of size (Appendix A; Fig. A 1). While small particles move quickly, the large ones move slowly in the liquid (Malvern, 2004). As particles tend to aggregate in aqueous state, DLS size measurements show higher size than individual particles (Dhawan *et al.*, 2009). Another parameter given by the DLS technique is the PDI, that indicates if a nanoparticle sample is

monodisperse (small PDI values) or polydisperse (high PDI values) in the liquid solution (Appendix A; Fig. A 2).

Several studies demonstrate that nanoparticles stability varies in different liquid media, depending on the ionic strength and protein composition of the solution. For instance, nanoparticles stable in water can form large aggregates in physiological solutions or similar conditions, reducing their stability (Pavlin and Bregar, 2012). For this reason, nanomaterials characterization in relevant media is essential for toxicity evaluation, since interaction with biological fluids components may influence nanomaterial behaviour and communication with cells (Balog *et al.*, 2015; Allouni *et al.*, 2015). Culture media or FBS contain simple ions and peptides that can exhibit different charge groups at physiological conditions, contributing to a formation of a transient or permanent protein corona (Pavlin and Bregar, 2012). Pavlin and Bregar (2012) and his co-workers establish that proteins from the media strongly affects the stability of the nanoparticles, i.e., the protein corona formation and opsonisation around nanoparticles may destabilized the nanoparticles suspensions and contribute to a certain degree of flocculation and sedimentation of the nanoparticles.

In this regard, the DLS is a useful technique to evaluate the nanomaterials properties in relevant biological media, giving more "realistic" information of the nanomaterials behaviour than others techniques, for example TEM or SEM (Balog *et al.*, 2015).

In addition to hydrodynamic size, Zetasizer Nano-ZS also provides the value of zeta potential in solutions with different pH and ionic strength measured by the LDV configuration. Zeta potential plays an important role in the nanoparticle characterization since it delivers an indirect measure of the net charge of the particle and is also a parameter of particle diffusion degree in the medium (Dukhovich, 2004). In fact, the zeta potential is defined as the existing potential between particle surface and the dispersing liquid, giving the potential stability of the colloidal system (Malvern, 2004; Salgın *et al.*, 2012) (Appendix A; Fig. A 3). The larger the absolute values of zeta, the greater the ability of nano-sized particles to disperse; conversely, as the zeta potential is close to zero, the particles become unstable and tend to aggregate (Murdock *et al.*, 2008). Basically, the high absolute value of zeta potential enables a stable suspension of the materials due to the strength derived from the electrostatic repulsion force (Verma *et al.*, 2012).

The studied nanomaterials were characterized after dispersion in water, the cell culture media supplemented with bovine serum and also in serum-free cell culture media. In all aqueous solutions, TiO_2 NPs exhibited the smallest hydrodynamic size, when compared to the remaining materials (Table 2). As expected, C-TiO_2 nanocomposite presented higher size than the TiO_2 NPs and nanokaolin.

Curiously, the C-TiO_2 nanocomposite tended to agglomerate more in water than in the both tested biological media, presenting the size of 7289 nm and a PDI of 1, which means that, in water, the C-TiO_2 nanomaterials are strongly polydispersed. In cell culture media, it presented higher size in serum free medium (2419 nm) than in complete media (470 nm); however the PDI was very similar in both media.

Similarly to the nanocomposite, both TiO_2 NPs and kaolinite clay presented higher hydrodynamic sizes in incomplete medium than in complete medium. These results suggest that the absence of serum leads to an increase in the agglomeration state, i.e., serum proteins allowed a better dispersion of these materials, which can be proven by the obtained PDI for each material. Actually, they appeared to be more stable and monodispersed in complete medium than in serum free medium.

Table 2. TiO_2 NPs, kaolinite mineral and C-TiO_2 nanomaterial characterization. Average hydrodynamic size and polydispersity index (PDI) of particles in suspension in water, incomplete and complete HepG2 culture media were determined by dynamic light scattering (DLS), while ζ – potential was measured by laser Doppler velocimetry (LDV) configuration.

		DLS		**LDV**	
		Average hydrodynamic size (nm)	PDI	ζ – potential	pH
TiO_2 NPs	H2O*	196.7	0.308	41.6	6.63
	HepG2 incomplete medium*	447.2	0.635	**	7.58
	HepG2 complete medium*	236.6	0.223	**	7.80
Kaolinite	H2O*	508.4	0.244	-39.0	6.86
	HepG2 incomplete medium*	2675.0	0.617	**	7.72
	HepG2 complete medium*	447.5	0.474	**	7.91
C-TiO_2	H2O*	7289.0	1.000	10.4	6.56
	HepG2 incomplete medium*	2419.0	0.433	**	7.62
	HepG2 complete medium*	470.0	0.471	**	7.82

* measured at 0.01 µg/mL
** media components did not allow a correct assessment of the ζ – potential of the materials

As reported in the literature, proteins adsorbed by nanomaterials to their surface may influence key parameters such as their hydrodynamic size and, sometimes, colloid stability (Baalousha, 2009). As observed in the present work, other previously published studies have demonstrated that TiO_2 NPs create larger agglomerates in cell culture medium than in water, which result in increased hydrodynamic size (Valdiglesias *et al.*, 2013; Wang *et al.*, 2014). Adsorption of proteins may, on one hand, increase the size of nanoparticles

but also contribute to the stability of the dispersion as observed in this work, since the TiO_2 NPs presented lower values in complete medium than in incomplete medium.

Regarding the zeta potential in water, the TiO_2 NPs showed the largest absolute value (ζ – potential of 41.6) and, therefore, were found to be the most stable suspension, while the C-TiO_2 nanocomposite were the less stable (ζ – potential of 10.4).

When attempting to perform zeta potential measurements in the LDV configuration, it was found that the ionic strength of the solution impacted measurement reproducibility as the high salt content would interfere with the electrodes in the capillary tube. Additionally, when other media components, such as fetal bovine serum, were present, it would give a very broad distribution of charges with a peak close to 0 mV due to the wide distribution of charges from both particles and proteins (Murdock *et al.*, 2008).

2. Nanomaterial uptake

An exploratory analysis of the uptake of studied nanomaterials by HepG2 was performed to better understand the possible cytotoxic effect assessed further on. Two periods of exposure (3 and 24 h) and two media types (serum free and FBS supplemented) were evaluated to clarify the influence of serum proteins on nanomaterial internalization by hepatocytes over time.

Results obtained show that after 3 hours of exposure to TiO_2 NPs, these were able to be internalized by HepG2 cells in both media (Figs. 12.A and 12.B). However, in incomplete medium it was observed a significantly higher percentage of material uptake for the lowest concentration (~75%) than the one observed for the same conditions in complete medium (~10%) suggesting that the presence of FBS strongly interferes with TiO_2 NPs internalization by preventing it when nanoparticles are present in small amounts.

Additionally, for incomplete medium (and 300 µg/mL in complete medium) it was observed higher TiO_2 NPs uptake after 3 hours of exposure than after 24 hours period. This can be either due the fact that HepG2 cells started to identify these nanoparticles, avoiding their internalization or due to a dynamic equilibrium that has been reached after this period of exposure (Murugan *et al.*, 2015; Oh and Park, 2014).

Cell internalization of TiO_2 NPs has been described in different human cell lines (Valdiglesias *et al.*, 2013; Jaeger *et al.*, 2012), namely HepG2 (Shukla *et al.*, 2013; Petković *et al.*, 2011). Some authors support that internalization of TiO_2 NPs with different range of primary sizes, shapes or crystal phases (anatase, rutile or both forms), may occur in several mammalian cells (Janer *et al.*, 2014). These previous publications describe concentration

dependent increase of TiO_2 NPs cellular internalization over time (after 3 and 6 h) in complete medium (Valdiglesias *et al.*, 2013; Shukla *et al.*, 2013); yet, none of these authors performed an analysis of the internalization pattern in complete and incomplete media.

Regarding the nanokaolin, it was observed increased cellular uptake in complete medium than in serum free medium (Figs. 12.C and 12.D), suggesting that the presence of serum proteins improves kaolinite internalization by HepG2.

Results obtained for the internalization of C-TiO_2 nanocomposites in complete medium were very similar to the ones observed for the nanokaolin (Figs. 12.D and 12.F). Once these nanocomposites are mostly constituted by kaolinite, it is in fact expected that these behave similarly.

However, in incomplete medium, increased uptake of C-TiO_2 nanomaterials in HepG2 was observed when compared to what was seen for kaolinite. This can be related to differences in size and stability of the nanocomposite in complete and incomplete media.

In fact, the size of the nanomaterial is an important parameter for the uptake (Tenzer *et al.*, 2011). Usually smaller particles sizes or even smaller agglomerates have higher cellular uptake than larger agglomerates (Prasad *et al.*, 2014).

In addition to the size parameter, the real influence of the protein corona in the cellular internalization mechanism of nanomaterials is not fully understood. Human serum albumin and γ-globulins included in FBS used to supplement the complete medium often coat micro-sized materials forming a protein corona, modifying the size, agglomeration state, surface charge of the nanomaterials, potentially interfering with their cellular uptake (Allouni *et al.*, 2015). These proteins are recognized as safe by the HepG2 cells, simplifying the entrance of nano and micro-sized particles. In this context, some authors report that nanoparticles suspended in the presence of serum proteins show higher uptake while others observed the same phenomenon but in serum free biological solution (Allouni *et al.*, 2015). In the present work, this same singularity was found: TiO_2 NPs were better internalized by HepG2 cells in incomplete medium while for nanokaolin and C-TiO_2 nanocomposite the opposite was observed.

Protein adsorption to nanomaterials is a dynamic process that tends to increase over time of exposure. In *in vitro* systems models, as used herein, cells are attached to the bottom of the well, which leads to less contact of the adsorbed materials with cells since increased protein adsorption retains the particle in the biological suspensions for longer periods. Thereby, serum free medium will increase the fast sedimentation of the nanomaterials, providing an early cell-nanomaterial contact (Allouni *et al.*, 2015). In the

absence of serum proteins, the cellular uptake may be resultant from direct recognition of the materials at the cell surface instead of the recognition of the protein corona in media supplemented with serum proteins (Allouni *et al.*, 2015), as possibly happened with the TiO_2 NPs for shorter times of exposure.

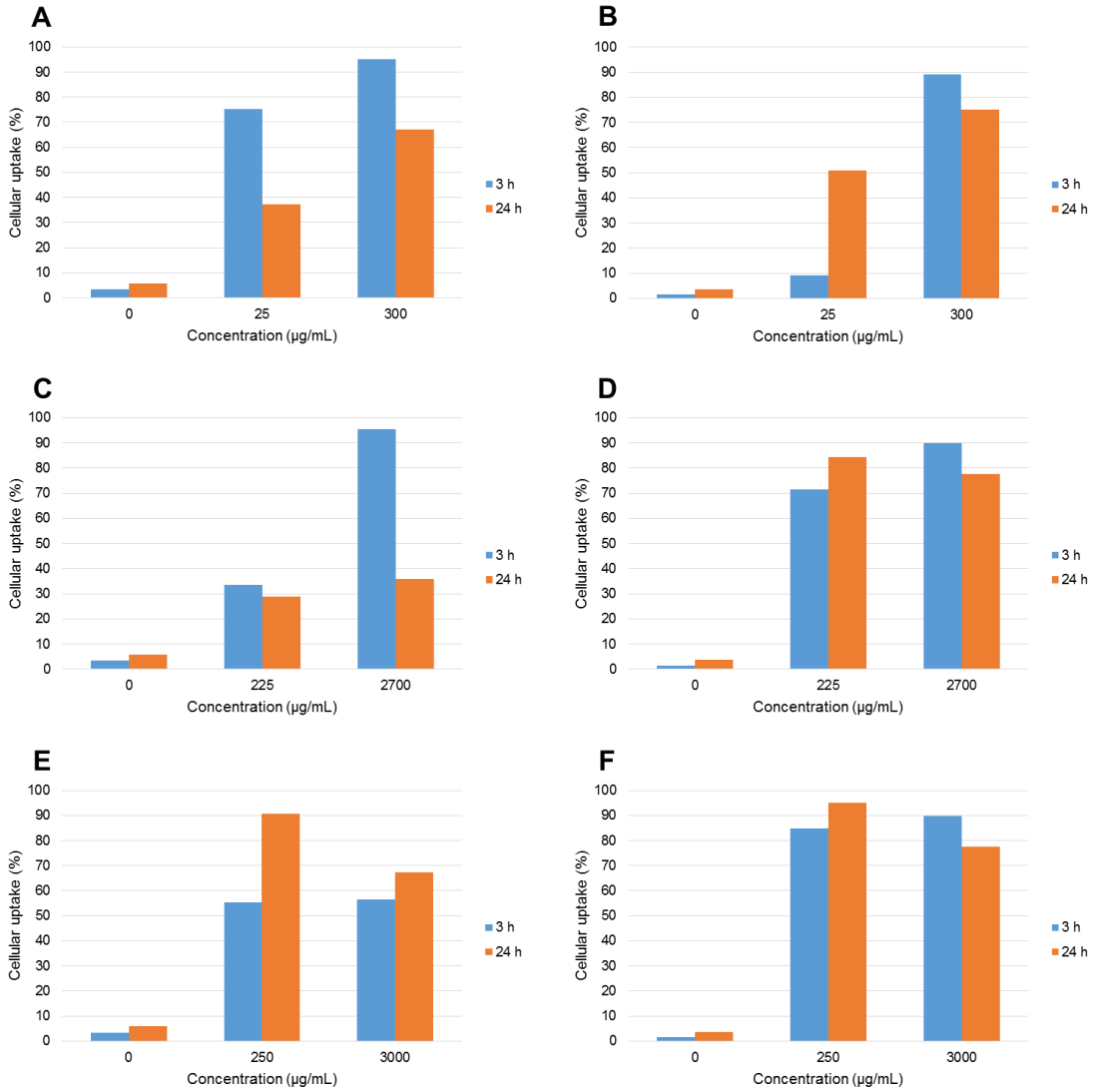


Figure 12. Cellular uptake of TiO_2 NPs, nanokaolin and C-TiO_2 nanocomposites by HepG2 cells as analyzed by flow cytometry. (A) TiO_2 NPs in incomplete medium; (B) TiO_2 NPs in complete medium; (C) Nanokaolin in incomplete medium; (D) Nanokaolin in complete medium; (E) C-TiO_2 nanocomposite in incomplete medium; (F) C-TiO_2 nanocomposite in complete medium.

Still, concerning the kaolinite mineral, possibly due to their constitution, this material could induce more protein adsorption than the TiO_2 NPs, which can be related to their increase cellular uptake by the HepG2 cells in FBS supplemented medium. This explains why the C-TiO_2 nanocomposite, mostly constituted by kaolinite, were better internalized in complete medium.

3. *In vitro* cytotoxicity of the nanomaterials in the HepG2 cell line

In the present work, it was intended to evaluate the cytotoxic effects of different nanomaterials (TiO_2 NPs, nanokaolin and C-TiO_2 nanocomposite) on hepatocellular carcinoma human cell line. For this purpose, four cytotoxic assays were chosen: MTT, NRU and AB assays for cellular viability and LDH assay for membrane integrity evaluation.

The most common method for cell viability determination is the MTT assay (Love *et al.*, 2012). MTT is a sensitive, quantitative and reliable colorimetric assay (Vega-Avila and Pugsley, 2011) that allows the detection of mitochondrial activity (Kroll *et al.*, 2009). Dehydrogenase is a mitochondrial enzyme capable to reduce the water soluble MTT reagent (yellow) into formazan (dark purple) crystals, which are subsequently solubilized by DMSO and quantitated by colorimetry (Takhar and Mahant, 2011) (schematic representation of the MTT principle presented in appendix B, Fig. B 1).

Another assay also widely used for measure the cellular viability is the AB assay (Love *et al.*, 2012). Alamar Blue is a cell viability indicator of the redox potential of cells (Takhar and Mahant, 2011). The main reagent of this technique is resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one). Resazurin is water soluble, non-toxic and stable in culture medium and that easily crosses the cell membrane (Rampersad, 2012). Viable cells reduce resazurin (blue) into resorufin (red), generating a quantitative measure for cytotoxicity of the cells (Takhar and Mahant, 2011) (see schematic representation of the AB principle in the appendix B, Fig. B 2).

AB works similarly as MTT since it is based on the conversion of resazurin to resorufin by mitochondrial enzymes (e.g. NADPH, NADH and cytochromes). Nonetheless, in addition to mitochondrial reductases, other enzymes (e.g. diaphorases, flavin reductase, among others) located in the cytoplasm and the mitochondria may also be able to reduce resazurin. Thereby, AB assay may imply an impairment of cellular metabolism but it is not necessarily related to mitochondrial dysfunction (Rampersad, 2012).

Since nanoparticles are frequently internalized and stored inside the lysosomes, this organelle is, consequently, one main target for nanoparticle accumulation possibly leading to lysosomal dysfunction. This possible destabilization of the lysosome membrane can induce oxidative stress through ROS generation, mitochondria damage and cell death (Frohlich, 2013). As such, lysosome dysfunction is often evaluated using the NRU cytotoxicity assay.

Neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) is a weak cationic dye that evaluates the integrity of the lysosomes (Kroll *et al.*, 2009; Takhar and

Mahant, 2011). Neutral red is able to enter in the cells by non-ionic diffusion and accumulate in the lysosomes of viable cells (Kroll *et al.*, 2009; Takhar and Mahant, 2011), where it becomes positively charge and is thus retained inside these organelles. When the integrity of the lysosome membrane is changed, it loses the ability to retain the dye inside (see schematic representation of the NRU principle in the appendix B, Fig. B 3). Therefore, this sensitive assay evaluates cytotoxicity through neutral red reduction, evaluating the cell integrity and growth inhibition, providing an estimation of the number of viable cells in culture (Repetto *et al.*, 2008).

The determination of total LDH content in the extracellular medium is the principle of the LDH assay and intends to evaluate the cytoplasmic membrane integrity of cells and, subsequently, is an indicator of apoptosis and/or necrosis though physical damage of the cell membrane (Love *et al.*, 2012). This colorimetric and enzymatic assay measures the level of LDH released from damaged cells (Han *et al.*, 2011b) that catalyse the reaction represented in the appendix B Fig. B 4. NAD+ is reduced to NADH/H+ catalysed by the LDH conversion of lactate to pyruvate. Secondly, H/H+ from NADH/H+ is transferred to the INT yellow salt which is oxidized to a red formazan (Kroll *et al.*, 2009; Takhar and Mahant, 2011).

Most of the cytotoxic assays were developed for the evaluation of conventional drug compounds *in vitro*. In fact, researchers have realized that not all cytotoxicity assays are appropriate for the evaluation of nanoparticle toxicity (Frohlich, 2013) as several nanoparticles are capable to interfere with these methods (Love *et al.*, 2012). For instance, the binding and possible inactivation of assay components involved and the interference with colorimetric detection are examples of nanoparticle interference on the cytotoxic assays (Frohlich, 2013). For a correct assessment of the cytotoxicity of the nanoparticles under evaluation, possible nanoparticle-assay interactions need to be identified.

3.1. Interference studies

To reveal possible interferences between materials and cytotoxicity assays experimental procedure, three sets of experiments were conducted: (1) light-absorption interference of the nanomaterials in water; (2) light absorption interference with the early and final assay components; and (3) interference on the catalytic activity of the assay (Wang *et al.*, 2012; Guadagnini *et al.*, 2013). The first two experiments intended to analyze the influence of materials presence on absorbance measurements while the third addressed possible nanomaterials reactivity with assay components. For the LDH assay, an additional experiment was conducted, evaluating the interference of nanomaterials on the enzymatic activity of LDH (Han *et al.*, 2011b; Kroll *et al.*, 2012; Holder *et al.*, 2012).

In this first set of experiments, all nanomaterials seemed to interfere with light-absorption when suspended in water, as the absorbance increased with the increase of nanomaterial concentration (Figure 13). However, for nanokaolin and C-TiO_2, this tendency is observed only up to the 4th concentration for nanokaolin (900 µg/mL) and the 3th concentration for C-TiO_2 (500 µg/mL); at higher concentrations the signal decreases or stabilizes. This exploratory analysis suggested that both nanokaolin and C-TiO_2 nanomaterials interfere with light-absorption in a concentration dependent manner at the lower tested concentrations. Light absorption of rutile TiO_2 NPs was also concentration dependent but was found to be lower than the observed for the remaining nanomaterials under evaluation (with the exception of the highest tested concentration, in which the signal was similar to the other nanomaterials).

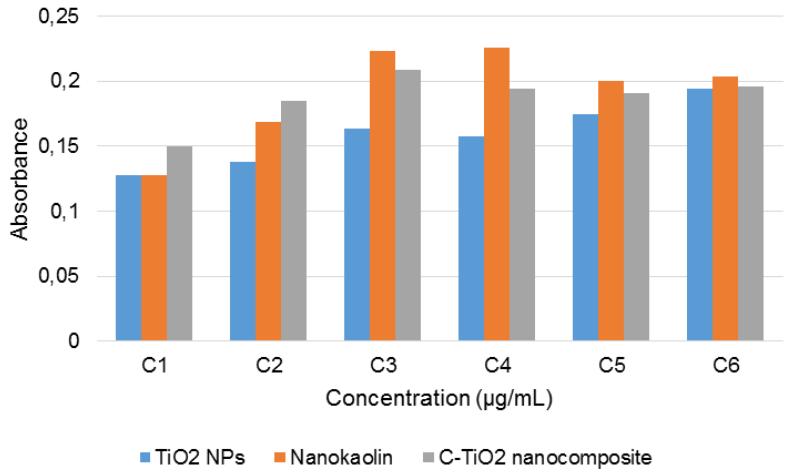


Figure 13. Evaluation of the nanomaterials interference with the light-adsorption in water. Concentrations (µg/mL): TiO_2 NPs: C1 – 5, C2 – 25, C3 – 50, C4 – 100, C5 – 200, C6 – 300; Nanokaolin: C1 – 45, C2 – 225, C3 – 450, C4 – 900, C5 – 1800, C6 – 2700; C-TiO_2: C1 – 50, C2 – 250, C3 – 500, C4 – 1000, C5 – 2000, C6 – 3000.

The remaining interferences studies were carried out separately for the cellular viability and the membrane integrity assays.

In all viability assays under evaluation (MTT, AB and NRU), the tested materials were capable to interfere with both early and final products of the respective assays (Figure 14) introducing bias in the final result.

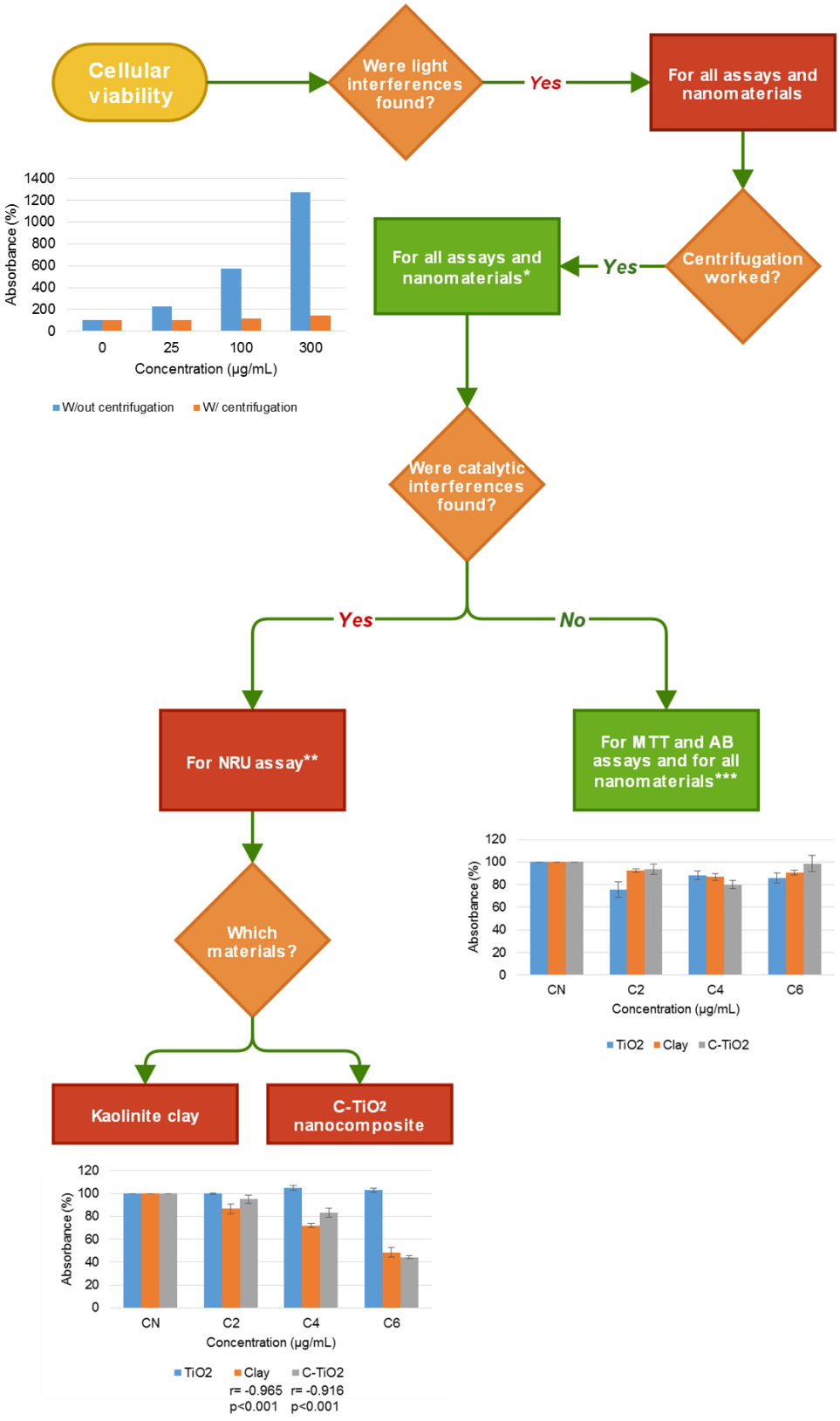


Figure 14. Schematic representation of the cellular viability (MTT, AB and NRU assays) interference studies. *As results were found to be similar in early and final products, only MTT results for the final product (DMSO) are presented; **Since the results in incomplete and complete medium were similar, only results in complete media were presented; ***Only MTT results in complete media are shown since results for MTT and AB catalytic interference were found to be similar.

To obviate this interference due to nanomaterial presence in assay reading, the plates were centrifuged before absorbance measurements. This procedure was enough to diminish or completely eliminate the optical interference of these nanomaterials since, after centrifugation, the absorbance obtained was similar to the one of negative controls (without nanomaterials in the early and final products of the assays).

Regarding the nanomaterials reactivity with the assay components, all nanomaterials concentrations exhibited a similar signal compared to the negative control for the MTT and AB assays in both media, which attested that no catalytic interference was found for all nanomaterials under evaluation. In opposition, for the NRU assay, the nanokaolin and C-TiO_2 nanocomposite could react with the NRU assays components, possibly adsorbing to them, leading to a decrease in signal. Since there is no possible alteration of the protocol to eliminate the catalytic interference, the NRU assay could not be applied to evaluate cell viability of HepG2 cells when exposed to the nanomaterials under evaluation.

Concerning the LDH assay, no optical interference was found for the TiO_2 NPs when suspended in both oxidized and reduced INT (early and final components of the LDH assay) (see figure 15). However, kaolinite layered silicate and C-TiO_2 nanocomposite were capable of interfere with light absorbance measurements. Similarly to the previous studies for the viability assays, to overcome such interferences, a centrifugation step was added before absorbance measurements. Despite the signal lowered tremendously, the centrifugation was not enough to eliminate the nanokaolin and C-TiO_2 light interference with this cytotoxicity assay as a decrease in absorbance was still observed with the increase in nanomaterial concentrations.

None of the presented materials shown catalytic interferences with the LDH assays over an hour of continuous measurements.

As this is an enzymatic assay, it was also evaluated if the nanomaterials could inactivate or adsorb the LDH protein present in the supernatant, decreasing the final measured absorbance. To do so, nanomaterials were suspended in incomplete and complete media and different units of LDH protein from rabbit muscle were added to the suspensions and absorbance was measured over time. In the absence of serum proteins, all nanomaterials were capable of interfering with enzymatic activity of the LDH protein over time, decreasing its signal, irrespective of the units of LDH protein present on the media tested. Possibly, LDH proteins were adsorbed to materials surface and ceased to be able to reduce the INT compound, inducing false negatives, i.e., the assay would indicate that the integrity of the cellular membrane was not damaged when, in the reality, the LDH was

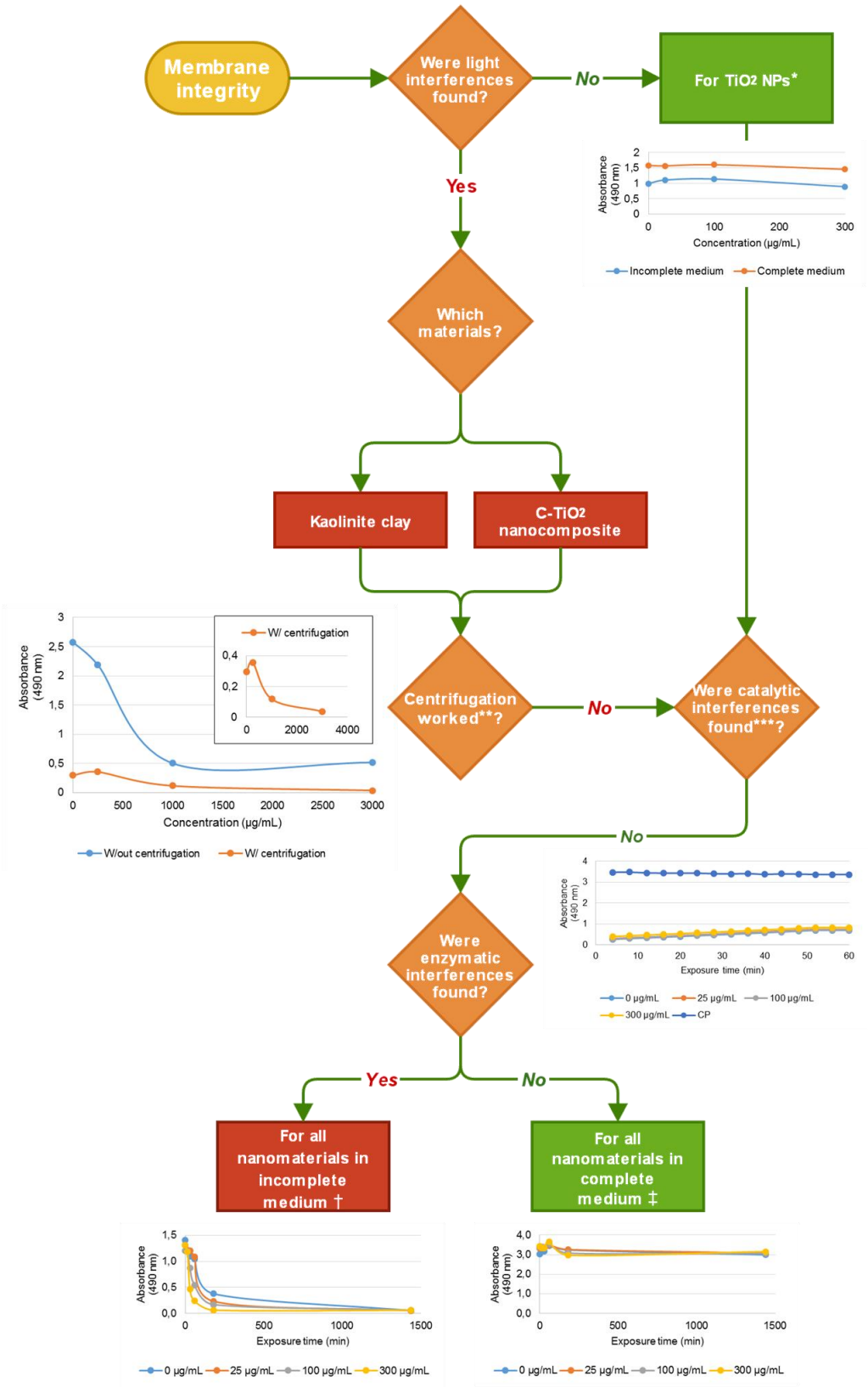


Figure 15. Schematic representation of the membrane integrity (LDH assay) interference studies. *,** As results were found to be similar in early and final products, only reduced INT results are presented; ***Only TiO_2 NPs results in complete media are shown since all nanomaterials exhibited similar catalytic interference; †,‡ Since the results were similar for all nanomaterials and mU of LDH protein, only some results were presented.

simply not available to convert lactate to pyruvate due to nanomaterials adsorption. In complete medium (with FBS proteins) this effect was not observed, possibly due to the presence of a protein corona formed by the FBS proteins around the materials that avoided LDH adsorption.

Results obtained in interference studies suggest that nanokaolin and C-TiO_2 nanocomposites were able to adsorb NRU and LDH assay components, decreasing the signal in both assays with the increase of particles concentrations. As these interferences could not be eliminated by protocol alterations, only MTT and AB assays were found to be suitable for further *in vitro* cytotoxicity study.

Current reports specify that MONPs interact with assay components or dyes or even with optical readouts due to the high adsorption or scattering of nanoparticles, distorting the assay outcome on the viability assessment (Kroll *et al.*, 2012). Different factors such as size, shape, crystallinity and concentration of the nanomaterial might influence the light detection of the colorimetric assays, owing to the nanomaterial interaction with the dyes, modifying their emission intensity. Generally, optical interference depends on the particle composition and also with its concentrations (the higher the concentration, the greater the interference) (Kroll *et al.*, 2012; Guadagnini *et al.*, 2013). These sort of interference has already been stated for TiO_2 NPs. A recent work reported that TiO_2 NPs could interfere with the NRU and MTT assays, increasing the signal reading for these dyes, which could be related to the light-absorbing properties of this nanoparticle (Guadagnini *et al.*, 2013).

Some authors demonstrated that LDH could be adsorbed and even inactivated for different metal nanoparticles, with special emphasis on TiO_2 NPs (Han *et al.*, 2011b; Guadagnini *et al.*, 2013). Nanoparticles may interact with proteins present on the biological solutions which may allow an increased reactivity with cytokines or enzymes form the cytotoxic assays, such as the LDH. With regard to metal based nanoparticles, they could induce a metal-catalysed oxidation of the LDH protein, non-specifically inactivating this molecule (Han *et al.*, 2011b). This occurrence may impair the correct evaluation of the membrane integrity assessment after nanomaterial exposure (Guadagnini *et al.*, 2013).

In respect of nanokaolin or even TiO_2 NPs immobilized in clay minerals, in accordance with the found literature, no interference studies regarding the toxicity assays have been made so far for these materials. Hence, the present work presents innovative results regarding the nanokaolin and nanocomposite interference on the proposed cytotoxicity assays (MTT, AB, NRU and LDH assays).

Is of paramount importance to assess possible nanomaterial interference with the toxicity assays methods prior to analysis of cellular responses in mammalian cell lines such

as the HepG2. The necessary protocol modifications must be introduced to eliminate or reduce the observed interferences in order to obtain a correct toxicological evaluation (Guadagnini *et al.*, 2013).

3.2. Cellular viability

After studying the nanomaterial capacity to interfere with the assays, a deeper analysis was carried out concerning the mitochondrial activity and potential redox of HepG2 hepatocytes after exposure to rutile TiO_2 NPs, nanokaolin clay and C-TiO_2 nanocomposite. The purpose of the present dissertation was to apprehend if the C-TiO_2 was a biocompatible nanomaterial for human and environmental applications. In addition, this study also evaluated toxicity of kaolinite mineral and TiO_2 NPs.

TiO_2 NPs induced a significant dose-dependent decrease in viability of the liver cells only after 24 hours of exposure to these nanoparticles ($p<0.014$) in incomplete medium, measured by the MTT assay (Fig. 16.A).

In complete medium, a significant dose-dependent decrease in viability of HepG2 cells was observed in all studied periods of exposure to TiO_2 NPs ($p<0.001$) (Fig. 16.B). This means that, the presence of FBS proteins in the medium was associated to more evident alterations in the mitochondrial activity and, consequently, lower viability of these hepatic cells.

Regarding the obtained results in AB assay (Figure 17), TiO_2 NPs induced a significant dose-dependent decrease in HepG2 viability only after 24 hours of treatment in incomplete medium ($r= -0.563$; $p<0.001$), consistent with the results obtained on the MTT assay.

In complete medium, a decrease in viability was observed only for the first two periods of exposure ($p<0.001$ and $p=0.002$ for 3 and 6 h, respectively). These results suggest that the hepatic cells could possibly recover their redox potential functionality after a 24 hours period, in the presence of FBS proteins. However, as previously observed, the mitochondrial activity was still compromised.

Despite the inconsistent *in vitro* biological effects related to TiO_2 NPs, many studies have demonstrated the capacity of TiO_2 NPs to induce cytotoxicity in various cultured cell lines (Shi *et al.*, 2013; Prasad *et al.*, 2014).

Towards the toxicity evaluation on hepatocytes cells, Gaiser *et al.* (2013) found no cytotoxic on C3A hepatocytes after exposure to rutile with minor anatase TiO_2 NPs up to 625 $\mu g/cm^2$, using the Alamar Blue and LDH assays. Decrease in the cell viability was only

detected for high concentrations. These authors concluded that these TiO_2 NPs were non cytotoxic for C3A hepatocytes up to high doses (Gaiser *et al.*, 2013). These results were consistent with the ones obtained by Kermanizadeh *et al.* (2013) for the same cell line and using exclusively mitochondrial activity assays.

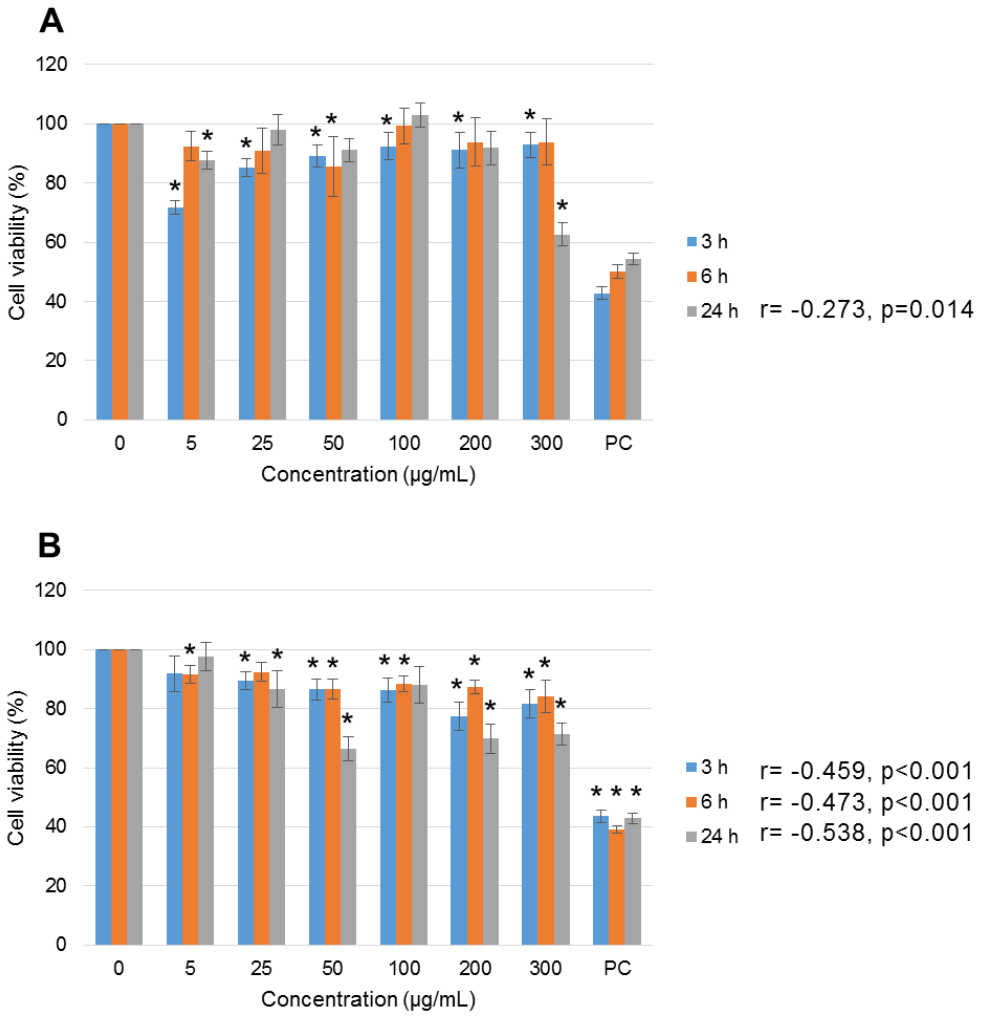


Figure 16. MTT assay performed in HepG2 cells after exposure to TiO_2 NPs suspended in (A) incomplete and (B) complete media. PC: Positive control (Triton X-100 1%). Bars present standard error of the mean. Values were normalized considering negative control as 100%. * p <0.05, significant difference with regard to the corresponding negative control. For each period was performed a correlation analysis between TiO_2 NPs concentrations and cellular viability were r value represent the correlation coefficient and p value the statistical significance of the correlation.

With respect to studies performed on HepG2 cell line, some demonstrated that TiO_2 NPs (mainly constituted with anatase) did not induce significant decrease in cell viability in medium supplemented with FBS in any concentration tested (until 100 µg/mL) after 24 hours of exposure (Prasad *et al.*, 2014). Others demonstrated negligible loss in the HepG2 viability after 6 hours of exposure while, for longer time periods (24 and 48 h) only concentrations of 20 to 80 µg/mL induce significant cytotoxicity through MTT and NRU

assays assessment (Shukla *et al.*, 2013). However, previous reports showed opposite results relating no reduced viability of HepG2 cells for either anatase or rutile TiO_2 NPs (Petkovic *et al.*, 2011).

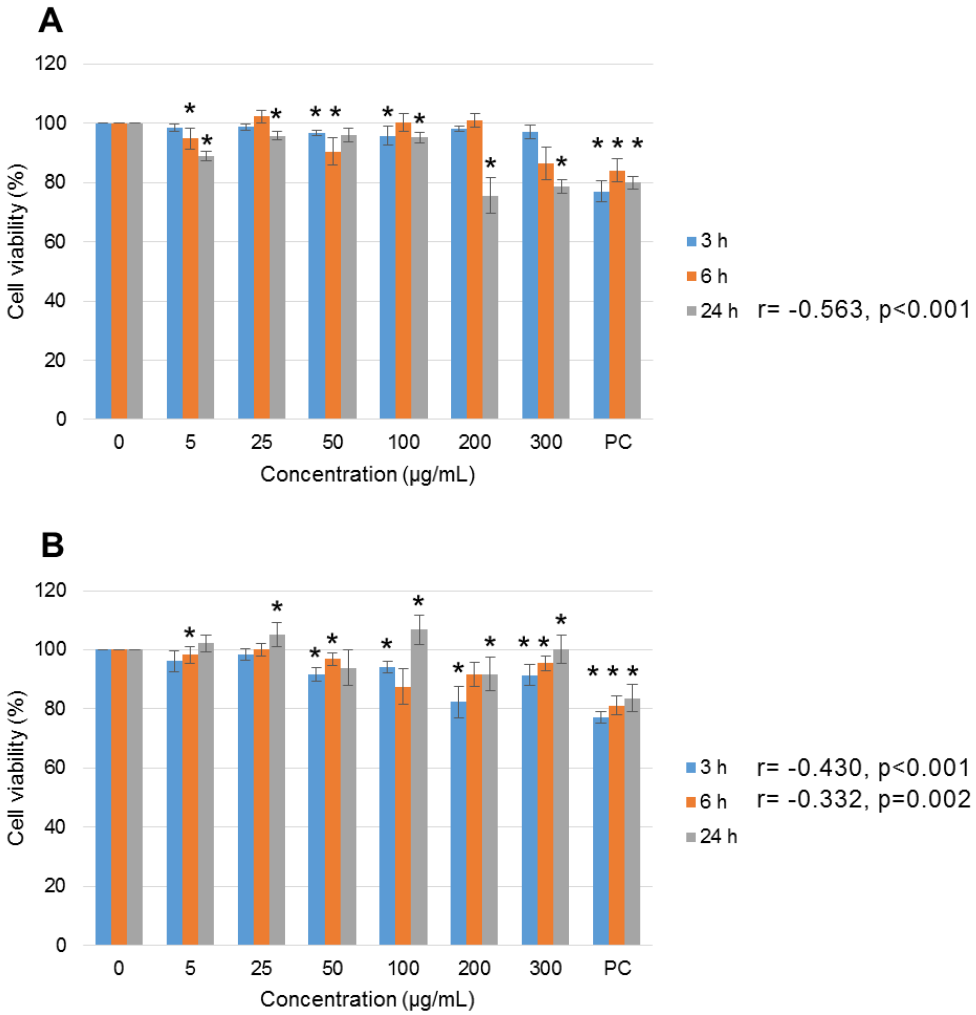


Figure 17. AB assay performed in HepG2 cells after exposure to TiO_2 NPs suspended in (A) incomplete and (B) complete media. PC: Positive control (Triton X-100 1%). Bars present standard error of the mean. Values were normalized considering negative control as 100%. * p <0.05, significant difference with regard to the corresponding negative control. For each period was performed a correlation analysis between TiO_2 NPs concentrations and cellular viability were r value represent the correlation coefficient and p value the statistical significance of the correlation.

As far as it is known, there are not many papers reporting cytotoxic studies with rutile TiO_2 NPs on hepatic cell lines. According to the previous papers, TiO_2 NPs with a mixture of both rutile and anatase presented some similar results to the ones observed on this work, i.e., for longer periods of exposure and for high doses these nanoparticles could induce *in vitro* damage on hepatic cells. However, the rutile TiO_2 NPs used here also exhibited adverse effects on the mitochondrial activity for short periods of exposure and in several

cases low concentrations of these nanoparticles could induce damage for both mitochondrial and potential redox activities.

Differences between human cell lines plays an important part in TiO_2 NPs toxicity assessment (Prasad *et al.*, 2014). While studies performed on A549 lung cell line shown lack of cytotoxic effects regarding the cell viability and membrane damage, other studies on BEAS-2B bronchial epithelial cell line found high cytotoxicity also in cell viability reduction and membrane integrity after exposure to TiO_2 NPs (mainly constituted by anatase) (Ursini *et al.*, 2014). These results were coherent with the ones obtained by Ekstrand-Hammarstrom *et al.* (2012) that obtained lower toxicity on five different types of TiO_2 NPs (either anatase or rutile), and also with Moschini *et al.* (2013) for MTT and NRU analysis, despite the efficient internalization in A549 in both studies.

In neuronal cell lines, such as SHSY5Y, no effect on viability was also measured by MTT and NRU assays after exposure to two types of TiO_2 NPs (Valdiglesias *et al.*, 2013).

The differences between studies can be related to the variation on the production and preparation of the TiO_2 NPs, as well as the comparison of nanoparticle's toxicity with different particle size, degree of aggregation, incubation and/or exposure conditions (i.e. dosage and periods of treatment) (Iavicoli *et al.*, 2011). The conjugation of the mention factors may lead to contradictory results. Therefore, the knowledge concerning the toxicity of TiO_2 NPs is still far for been completely understood.

Taking into consideration the results obtained in the present work, and despite some reports that did not observe cytotoxic effects derived from TiO_2 NPs exposure, results here obtained suggest that these nanoparticles should not be considered as negative controls in nanotoxicological studies, as some authors have suggested (Liu *et al.*, 2013).

In relation to the clay mineral under evaluation, nanokaolin induced a significant dose-dependent decrease in viability of HepG2 cells (assessed by MTT assay), in all studied periods of exposure in incomplete medium ($p<0.001$), in contrast to the observed for TiO_2 NPs exposure (Fig. 18.A). These results advocate that kaolinite clay is able to alter mitochondrial activity rapidly (just after 3 h of exposure) and continuously.

A similar tendency was observed in complete medium, with the exception of the 6 hours of kaolinite exposure (Fig. 18.B).

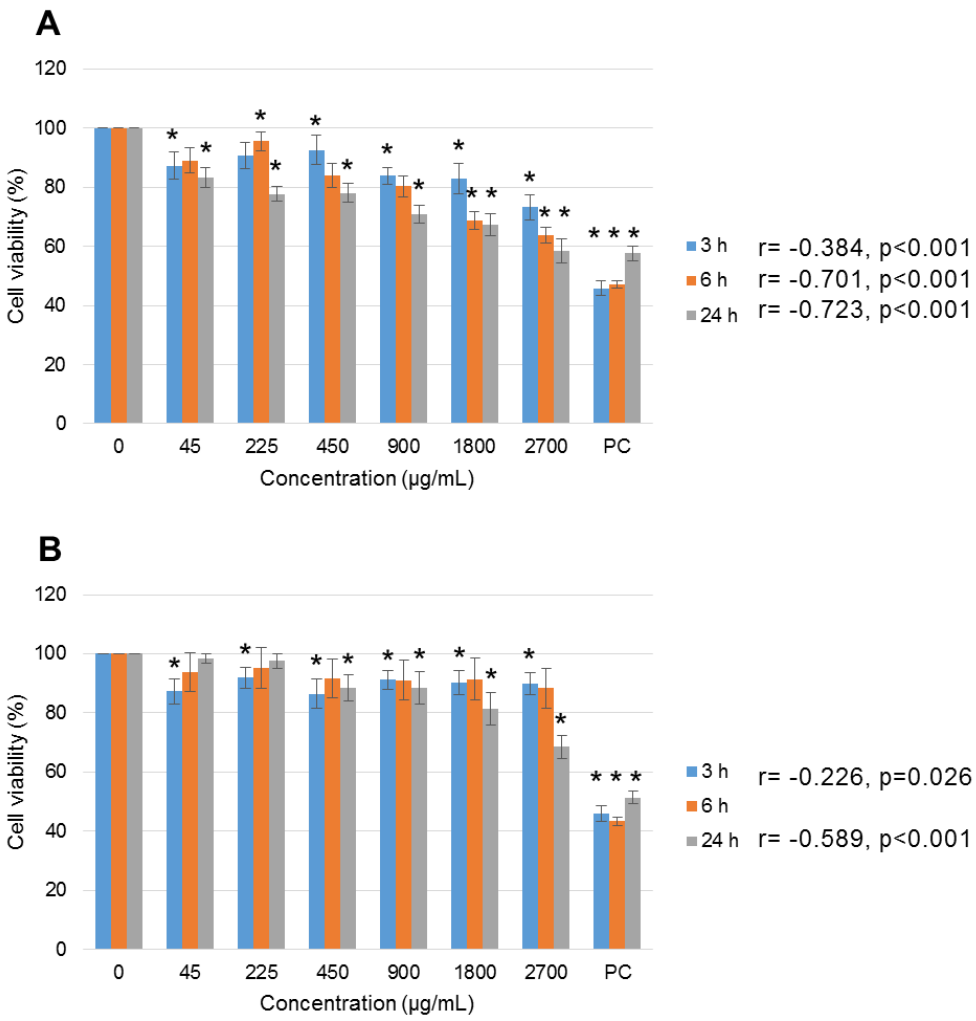


Figure 18. MTT assay performed in HepG2 cells after exposure to nanokaolin suspended in (A) incomplete and (B) complete media. PC: Positive control (Triton X-100 1%). Bars present standard error of the mean. Values were normalized considering negative control as 100%. * p <0.05, significant difference with regard to the corresponding negative control. For each period was performed a correlation analysis between nanokaolin concentrations and cellular viability were r value represent the correlation coefficient and p value the statistical significance of the correlation.

In respect to the AB assay, kaolinite induced a dose-dependent decrease in the cell viability (measured by the redox potential of the HepG2 cells) in both incomplete (Fig. 19.A) and complete medium (Fig. 19.B), which, along with the results obtain for the MTT assay, corroborates that these particles are cytotoxic to HepG2 cells.

Despite the multiple functionalities of the clay minerals, the use of nanoclays raises some concerns since there is still few information about the toxicity that these nanomaterials can induce (Jones and Grainger, 2009).

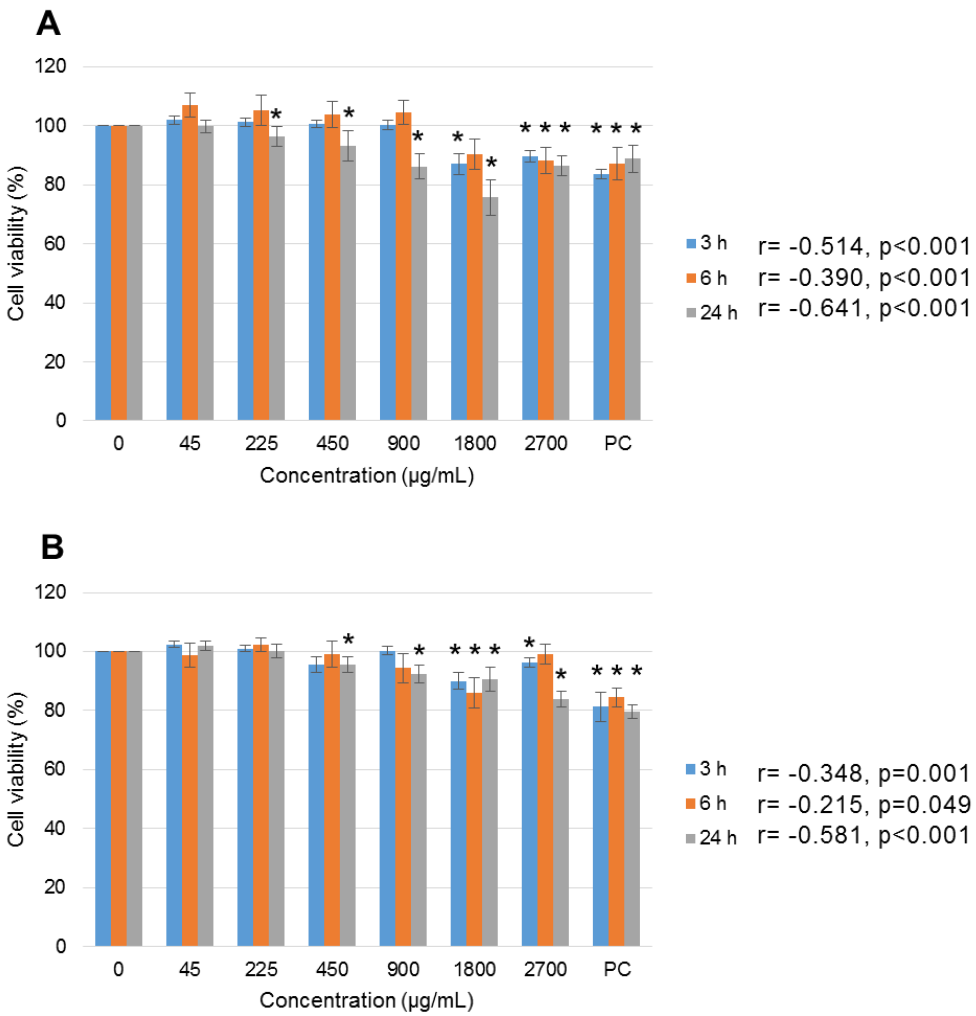


Figure 19. AB assay performed in HepG2 cells after exposure to nanokaolin suspended in (A) incomplete and (B) complete media. PC: Positive control (Triton X-100 1%). Bars present standard error of the mean. Values were normalized considering negative control as 100%. * p <0.05, significant difference with regard to the corresponding negative control. For each period was performed a correlation analysis between nanokaolin concentrations and cellular viability were r value represent the correlation coefficient and p value the statistical significance of the correlation.

Several studies already demonstrated that some types of clays are able to cause cytotoxicity in different cell lines, and even induce cell death. Most of the studies show that clay induces cytotoxicity only at high concentrations. For example, Han *et al.* (2011a) verified moderate cytotoxicity of clay minerals modified with an amine functional group in different cell types only at 1000 µg/mL (Han *et al.*, 2011a). However, other authors have described significant loss of A549 lung epithelial viability after 24 hours of exposure to different nanoclays at lower concentrations (25 µg/mL) (Verma *et al.*, 2012). Another study observed the same effect but in even inferior concentrations of nanoclay (1 µg/mL), in HepG2 liver cell line, as well as the significant generation of ROS (Lordan *et al.*, 2011). These authors also mentioned that the ultra-small sized clays particles tended to aggregate in the cell culture medium, which appeared to be correlated with the observed toxicity

mechanisms. In spite of this data, there are still few toxicological studies in the literature about clays in HepG2 cell line (Abdel-Wahhab *et al.*, 2015).

Recent reports illustrated no significant cytotoxic effect in intestinal Caco-2 cell line after being exposed to selected concentrations of a specific type of modified montmorillonite clay after 24 and 48 hours of exposure. However, the same group shown the opposite results on the same type of clay but with different organic modification, for relatively low concentrations (Maisanaba *et al.*, 2014). The same author studied the same clay particles in the HepG2 liver cell line and observed similar effects (Maisanaba *et al.*, 2013).

After evaluating the cytotoxicity of their single components, the impact of C-TiO_2 nanocomposite on the cellular viability of hepatocytes was also analyzed using the MTT and AB assays.

Results of MTT assay (Figure 20) show that C-TiO_2 induced mitochondrial imbalance for increased concentrations of these material, regardless of the presence of serum proteins in the biological media.

In the case of AB assay (Figure 21), it was demonstrated that C-TiO_2 nanocomposite lowered the cell viability of the hepatic cell line under study also in a dose-dependent way, with the exception for the shorter treatment period in complete medium. Possibly the FBS proteins have some positive influence on the redox potential of the liver cells in the first hours of exposure.

As aforementioned, the C-TiO_2 nanocomposite was mainly constituted by nanokaolin. Probably for this reason, similarly to what was observed for the kaolinite clay mineral, the C-TiO_2 induced a dose-dependent decrease in the cell viability (in both assays) of the HepG2 cell line in both serum supplemented and serum free medium.

One important aspect to take into account is the possible interactions between clay and cell culture media components, including serum (Dawson and Oreffo, 2013). Although the uptake results showed that FBS proteins apparently improved kaolinite internalization, these proteins did not protect cells from both mitochondrial or redox potential dysfunction since both nanokaolin and C-TiO_2 nanomaterials could cause cytotoxicity damage on liver cells in both biological media tested.

Altogether, results suggest that kaolinite laminar structure has the biggest contribution to the cytotoxicity of C-TiO_2 nanocomposites.

Thereby, these results suggest that kaolinite is not a suitable clay substrate for the immobilization of other nanomaterials (for instance the TiO_2 NPs), since increased

concentrations of kaolinite induce a significant decrease in the cell viability in the HepG2 cell line, which was also observed on the C-TiO_2 nanocomposites.

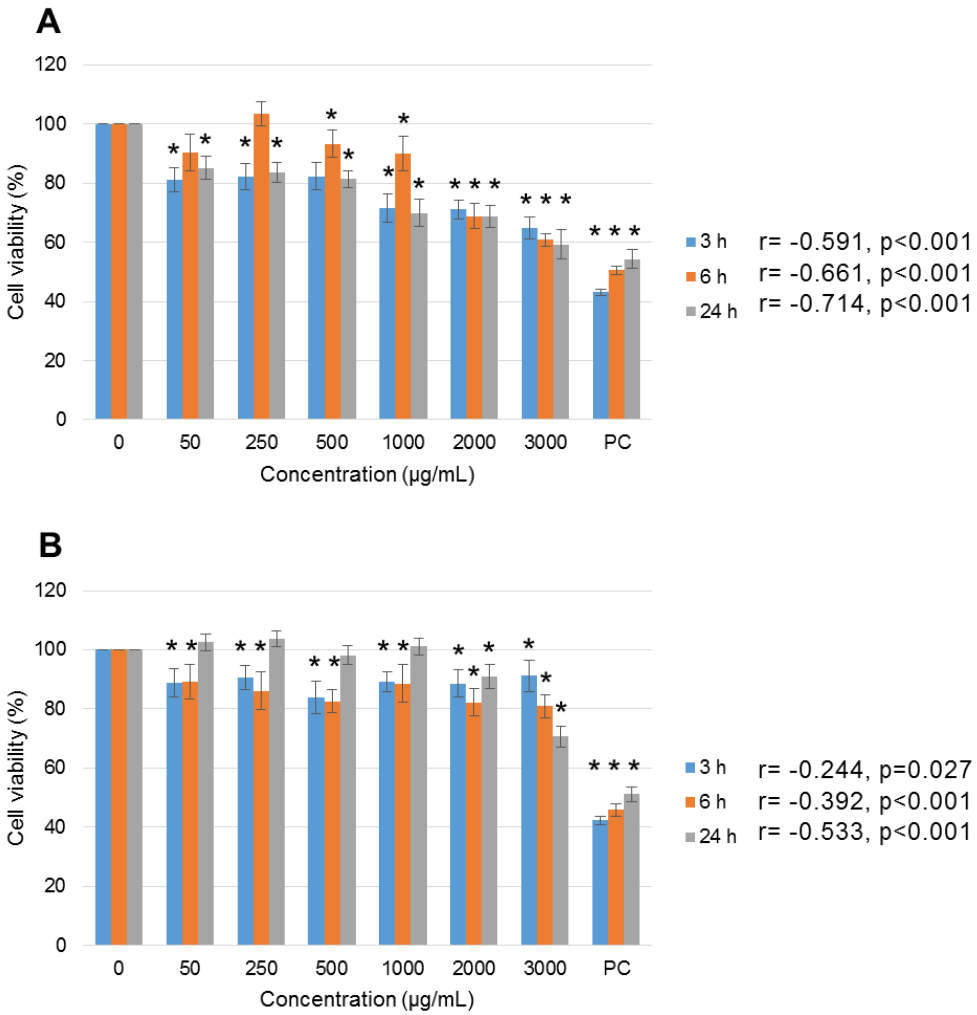


Figure 20. MTT assay performed in HepG2 cells after exposure to C-TiO_2 nanocomposites suspended in (A) incomplete and (B) complete media. PC: Positive control (Triton X-100 1%). Bars present standard error of the mean. Values were normalized considering negative control as 100%. * p <0.05, significant difference with regard to the corresponding negative control. For each period was performed a correlation analysis between C-TiO_2 concentrations and cellular viability were r value represent the correlation coefficient and p value the statistical significance of the correlation.

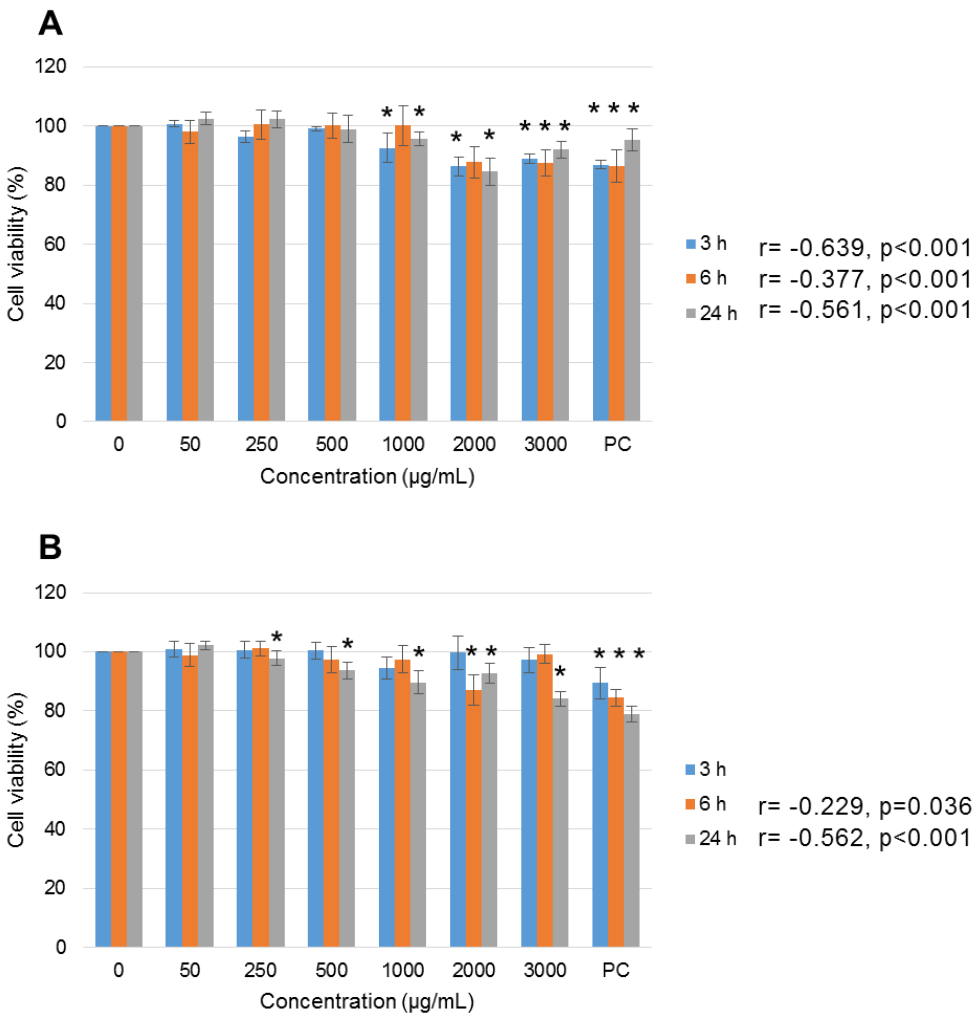


Figure 21. AB assay performed in HepG2 cells after exposure to C-TiO_2 nanocomposites suspended in (A) incomplete and (B) complete media. PC: Positive control (Triton X-100 1%). Bars present standard error of the mean. Values were normalized considering negative control as 100%. * p <0.05, significant difference with regard to the corresponding negative control. For each period was performed a correlation analysis between C-TiO_2 concentrations and cellular viability were r value represent the correlation coefficient and p value the statistical significance of the correlation.

4. *In vitro* genotoxicity of the nanomaterials in the HepG2 cell line

To understand the genotoxic potential of rutile TiO_2 NPs, nanokaolin clay and the conjugation of both nanomaterials (C-TiO_2 nanocomposite), the alkaline comet assay was selected.

Similarly to cytotoxicity assays, interference studies were performed before assessing the DNA damage derived from *in vitro* nanomaterial exposure. The following section will describe in detail the observed interferences and also the adopted strategies to avert them.

4.1. Interference studies

There are different mechanisms associated to the capacity of the nanomaterials to interfere with genotoxic comet assay, by: (1) associating to the DNA nucleoid, affecting its performance during electrophoresis and (2) inducing breaks in the naked DNA. Thereby, in order to estimate the nanomaterials capacity to damage DNA in cells it is important to evaluate the interaction of these with the alkaline comet assay. The presence of nanomaterials during the comet assay can interfere with the sensibility of the assay, misleading the results (Magdolenova *et al.*, 2012).

During the lysis step of the alkaline comet assay, cells are embedded in a solution with high concentration of NaCl and 1% of Triton X-100, responsible for membrane disruption, removing the cytoplasm and nuclear proteins and, therefore, exposing non-nucleossomal DNA. In this stage, the nanomaterials may interact with the unprotected DNA and cause additional DNA damage (Karlsson, 2010).

As represented in figure 22, when comparing the negative control (cells non-exposed to nanomaterials) in incomplete medium with the lysis test, all nanomaterials seem to cause additional damage on the DNA, interfering with the assay.

In opposition, in the presence of FBS there were no major differences between the lysis test of all materials and the negative control, showing that all tested nanomaterial did not to interfere with the alkaline comet assay in complete medium. To reduce the risk of interferences observed in serum free medium, extensive washing of the wells was performed after the nanomaterials exposure, in order to remove nanomaterials.

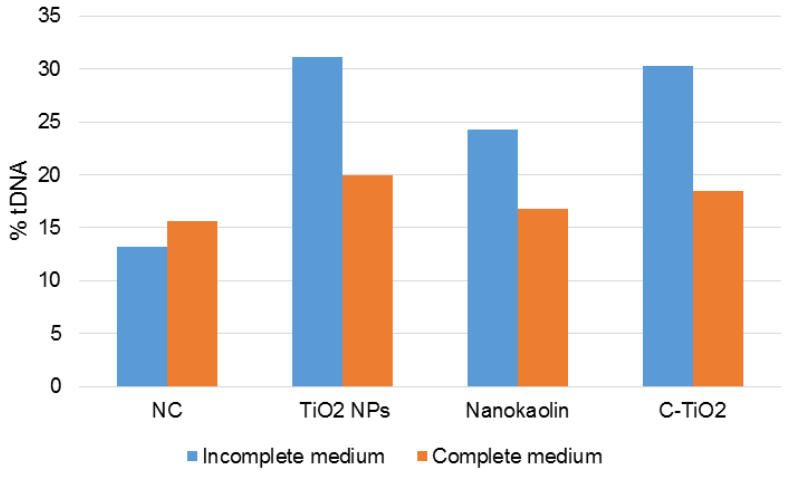


Figure 22. Possible interactions of TiO_2 NPs, nanokaolin clay and C-TiO_2 nanocomposites with alkaline comet assay in incomplete and complete media. In the lysis test, nanomaterials were added just before the assay. NC: cells non-exposed to nanomaterials; tDNA: tail intensity.

4.2. DNA integrity

The alkaline comet assay is one of the most common test systems to assess genotoxicity (Love *et al.*, 2012; Chang *et al.*, 2013). This version of comet assay allows the detection of two types of DNA damage: SSBs (single strand breaks) and ALSs (alkali-labile sites). As the name implies, SSBs denote single breaks on the DNA strands, while ALSs represent a loss of pyrimidine and purines bases from the sugar in the DNA backbone (Karlsson, 2010; Kain *et al.*, 2012).

Comet assay results showed that after exposure to TiO_2 NPs, cells presented higher levels of tail DNA in complete medium rather than in the absence of serum proteins. Regardless, increase in DNA damage with increasing dosage of TiO_2 NPs was observed for both media and periods of exposure (Figs. 23.A and 23.B).

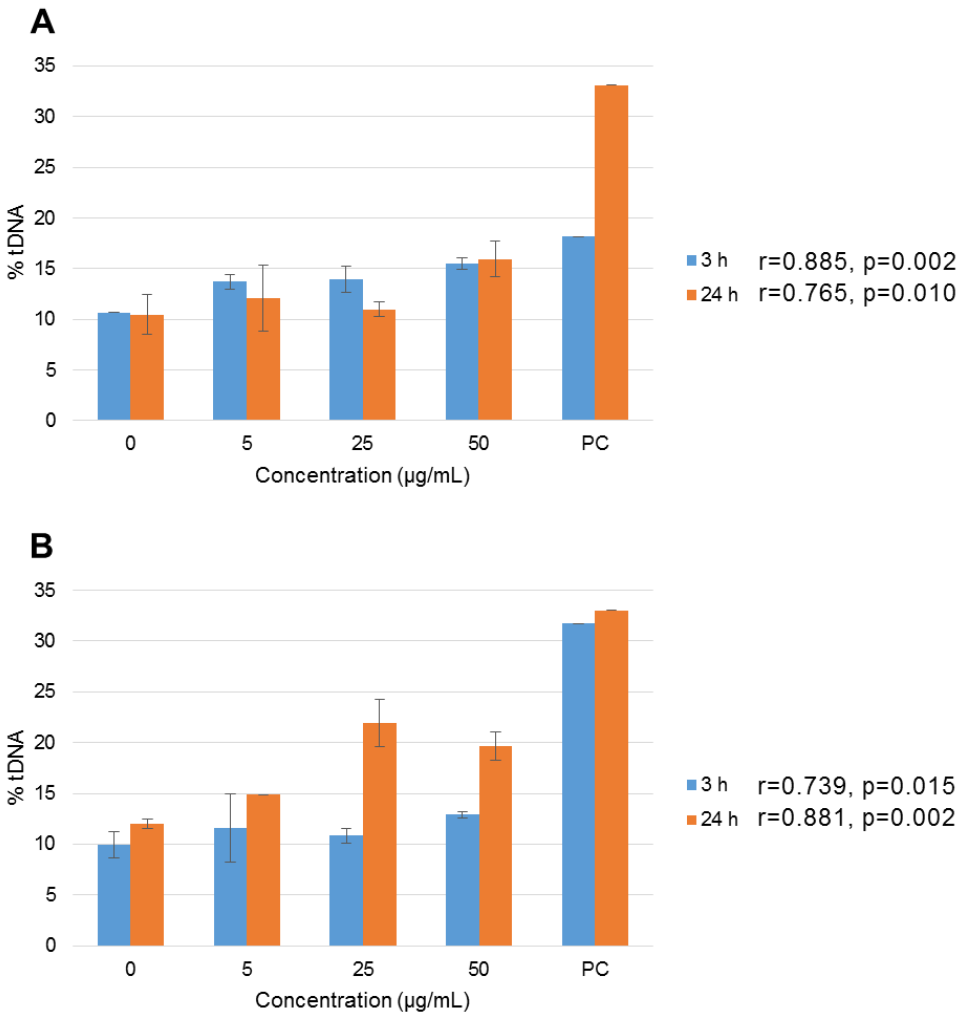


Figure 23. Effect of TiO_2 NPs on DNA damage of HepG2 cells analyzed by alkaline comet assay in (A) incomplete and (B) complete media. PC: Positive control (MMS 100 µM). Bars present standard error of the mean. tDNA: tail intensity.

Once again, the physicochemical characterization of the TiO_2 NPs plays a crucial role on their toxicity but, in this case, on the DNA damage derived from the exposure to these nanoparticles. Petkovic *et al.* (2011) shown in his work that the genotoxic potential of TiO_2 NPs depends on its particle size and crystalline structure. In fact, anatase TiO_2 NPs induced stronger DNA damage than the rutile ones in HepG2 cell line. These results were confirmed later with the same hepatocyte cell line (Petković *et al.*, 2011). Also using the HepG2 cell line, Prasad *et al.* (2014) observed that increased concentration of TiO_2 NPs mostly constituted by anatase suspended in medium supplemented with 10% of FBS induced increase DNA damage (Prasad *et al.*, 2014).

Other studies on mammalian cell lines (for instance human gastric epithelial AGS cell line, A549 lung cell line, lymphocytes) also observed genotoxic effects for different sized anatase and rutile TiO_2 NPs by using comet assay (Botelho *et al.*, 2014; Kang *et al.*, 2008; Jugan *et al.*, 2012).

According to the review authored by Karlsson (2010), TiO_2 NPs are the most well studied metal-based nanoparticle up to date and most studies on their genotoxicity using the alkaline comet assay reported an increase in damage compared with non-exposed controls, in different cell lines, including A549, lymphocytes, BEAS-2B, Caco-2, among others.

Another study looking at the genotoxicity of the TiO_2 NPs studied here (NM-104) showed that these did not cause significant DNA damage on intestinal Caco-2 cell line. In fact, the same study evaluated other TiO_2 NPs with distinct crystal phases and noticed that anatase and anatase/rutile (NM-102 and NM-105, respectively) were the most hazardous nanoparticles in comparison to NM-104, in accordance with the *in vitro* comet assay results. The only cell line in which was observed DNA damage derived from NM-104 exposure was the dermal cell line NHEK (Norppa *et al.*, 2013).

Concerning nanokaolin and C-TiO_2 nanomaterials, both induced similar effects on the DNA integrity on the hepatic cells. Similarly to TiO_2 NPs, nanokaolin clay and C-TiO_2 nanocomposite induced more damage in complete medium (Figs. 24.B and 25.B). In this case, these nanomaterials could induce more SSBs and ALSs breaks on the HepG2 DNA even after 3 hours of exposure, in contrary to what was observed in incomplete medium, where increase of DNA damage was only detected after 24 hours of exposure (Figs. 24.A and 25.A). For both materials, a high correlation coefficient and significant p value was obtained for 24 hours of exposure in incomplete medium ($r=0.867$; $p=0.001$).

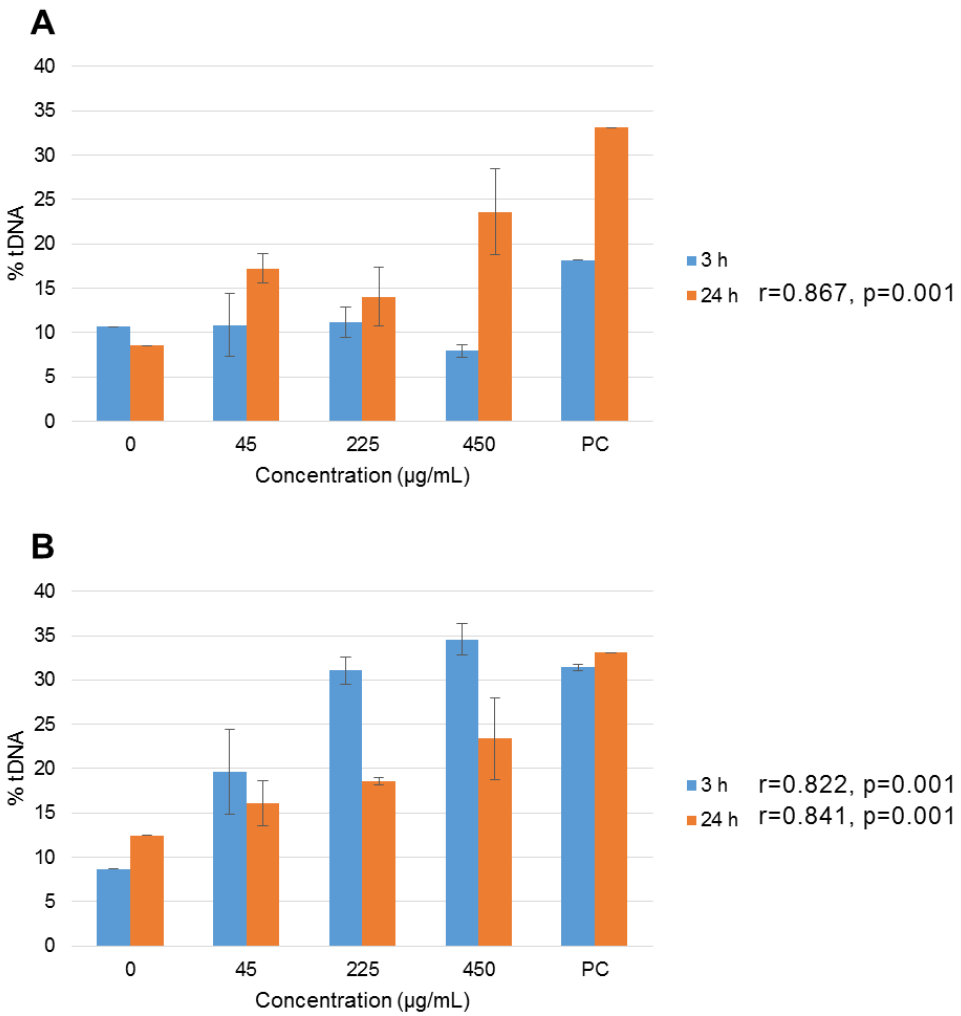


Figure 24. Effect of nanokaolin on DNA damage of HepG2 cells analyzed by alkaline comet assay in (A) incomplete and (B) complete media. PC: Positive control (MMS 100 µM). Bars present standard error of the mean. tDNA: tail intensity.

Previous studies have already demonstrated that clays commonly found in soils (e.g. smectites) can react with pesticides and modify their genotoxicity in mammalian cells; pesticides alone did not cause genotoxicity, while, when coupled to redox-modified clays, triggered environmental genotoxicity (Sorensen *et al.*, 2005). On the contrary, more recent studies performed by Li *et al.* (2010) showed no genotoxicity of exfoliated montmorillonite (a 2:1 clay mineral type). Another study also demonstrated that a chemically modified montmorillonite clay mineral (cloisite) could not induce *in vitro* damage on the DNA of Caco-2 cell line exposed for more than 24 hours (Sharma *et al.*, 2010); however, other author demonstrated the opposite effect on the same cell line (Sharma *et al.*, 2010). Two recent studies proved to occur DNA strand break on hepatic HepG2 cells in a time dependent manner, also measured by the comet assay, after exposure to organomodified cloisite clays (Maisanaba *et al.*, 2013; Houtman *et al.*, 2014).

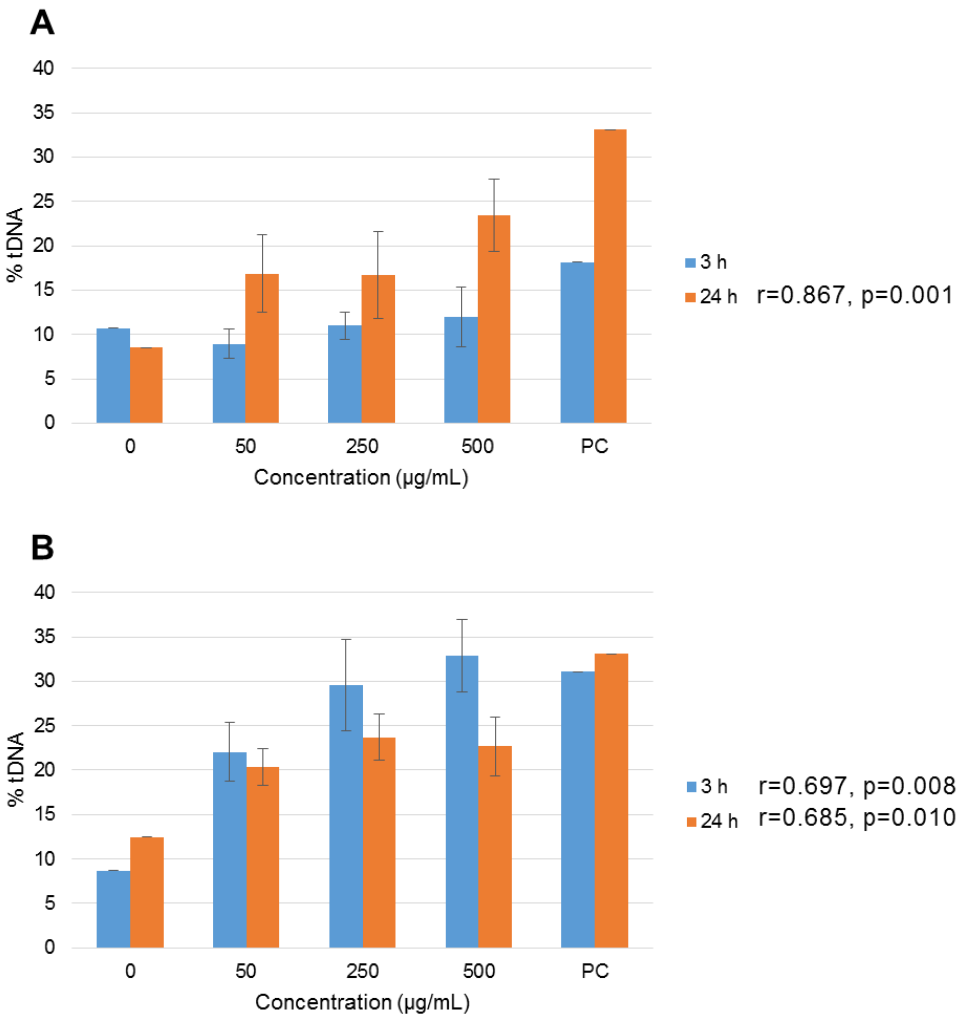


Figure 25. Effect of C-TiO_2 nanocomposites on DNA damage of HepG2 cells analyzed by alkaline comet assay in (A) incomplete and (B) complete media. PC: Positive control (MMS 100 µM). Bars present standard error of the mean. tDNA: tail intensity.

Results obtained herein indicate, once again, that kaolinite is not a suitable clay substrate for the immobilization of other nanomaterials, since, in opposition to what has been previously reported, high concentrations of clay induced a significant increase in DNA damage of HepG2 cell line.

V. CONCLUSIONS AND FUTURE PERSPECTIVES

One of the main concerns of nanotechnology applications is that nanoparticles might be more toxic, and therefore more hazardous than larger particles and bulk materials, due to their large surface area and consequent enhanced chemical reactivity and also cellular internalization (Tucci *et al.*, 2013).

In the last years, TiO_2 NPs have been extensively studied to investigate their potential toxicological effects due to their increased environmental and occupational exposure (Tucci *et al.*, 2013). In order to monitor and control the release of TiO_2 NPs, it has been developed the technique of immobilization of these nanoparticles into crystalline substrates, such as inorganic clay minerals, facilitating their manipulation, restraining their release and diminishing their threat into the environment and human health (Tokarsky *et al.*, 2012; Tokarčíková *et al.*, 2014). Clay minerals have been distinguished as the materials of the 21st century due to their relatively low cost, availability and environmental sustainability (Maisanaba *et al.*, 2015). Among the existing clay minerals, kaolinite, commonly found in soils, has been reported has an excellent substrate for immobilizing MONPs (Tokarsky *et al.*, 2012). The formation of the TiO_2 NPs supported in kaolinite mineral created nanocomposites that combines the properties of both components (Tokarsky *et al.*, 2012; Tokarčíková *et al.*, 2014).

The liver is an organ of nanoparticle accumulation and clearance and, for that reason, is susceptible to nanomaterials toxicity (Petkovic *et al.*, 2011; Prasad *et al.*, 2014). In addition, the hepatocytes include an abundance of cellular organelles associated with metabolic and secretory functions, such as high number of mitochondria that helps providing high energy levels to support the numerous metabolic function of the liver (Rolfe, 2013).

In the current work it was applied an *in vitro* model system to surrogate human hepatocytes, the HepG2 cell line. *In vitro* studies constitute an initial stage of the toxicological evaluation (Joris *et al.*, 2013) and for each type of nanomaterials is crucial to perform cytotoxicity studies because of their distinctive biological response (Lewinski *et al.*, 2008). Conflicting results found in the literature highlight the need for a greater consistency on all experimental procedures such as detailed physicochemical characterization of the materials, dispersion protocol, dosage of the materials, periods of exposure, cell culture medium constitution, interference assays and cytotoxicity assays protocols.

In this regard, the present study measured the cellular interaction and biological responses of human hepatocytes after exposure to TiO_2 NPs immobilized in nanokaolin laminar structures. To achieve this main goal, a set of experiments were performed in order to evaluate the cellular uptake, viability and DNA damage on hepatic cells. In addition, all

proposed assays were tested in regards of the possible interference of this nanocomposite and/or its individual components with optical, catalytic and even enzymatic performance.

To overcome possible interferences of the C-TiO_2 nanocomposite and its single elements on the cytotoxicity assessment, a set of experiments were performed and, when possible, alterations on the classic assays protocols were done. Only the NRU assay was not suitable for viability assessment since both nanokaolin clay and C-TiO_2 nanocomposite exhibited light and catalytic interferences; for membrane integrity assessment, LDH was also not adequate since all materials suspended in incomplete medium enzymatically interfered with the assay. Thereby, MTT and AB were the only cytotoxic assays compatible with the all TiO_2 NPs, nanokaolin and C-TiO_2 nanocomposite.

While there is an outlook that the incorporation of single nanoparticles on nanocomposites may decrease their biological damage (COST, 2012), the present *in vitro* study suggests that kaolinite is not a suitable substrate for the immobilization of nanoparticles, as well as that TiO_2 NPs may not be harmless for human applications as some previous studies reported. Both cytotoxic and genotoxic effects were observed on the HepG2 hepatic cell line after exposure to C-TiO_2 nanocomposites and its single elements. The figure 26 summarizes the main toxicological mechanisms identified in the present *in vitro* assessment. All nanomaterials could induce mitochondrial and potential redox dysfunction of the hepatocytes, after internalization in these hepatic cells. Lastly, the HepG2 DNA was also affected after longer periods of exposure to TiO_2 NPs, nanokaolin clay and C-TiO_2 nanocomposite even in the presence of serum proteins in the biological media.

A possible strategy to overcome the cytotoxic and genotoxic effects of naked kaolinite observed in this study is to chemically modify the kaolinite particles with organic compounds (Maisanaba *et al.*, 2014). Even so, further toxicity studies must be performed in organoclay particles to understand if the chemical modification allows the creation of a more compatible kaolinite mineral and, therefore, a more convenient material for the incorporation of nanoparticles for future and safer nanocomposite applications.

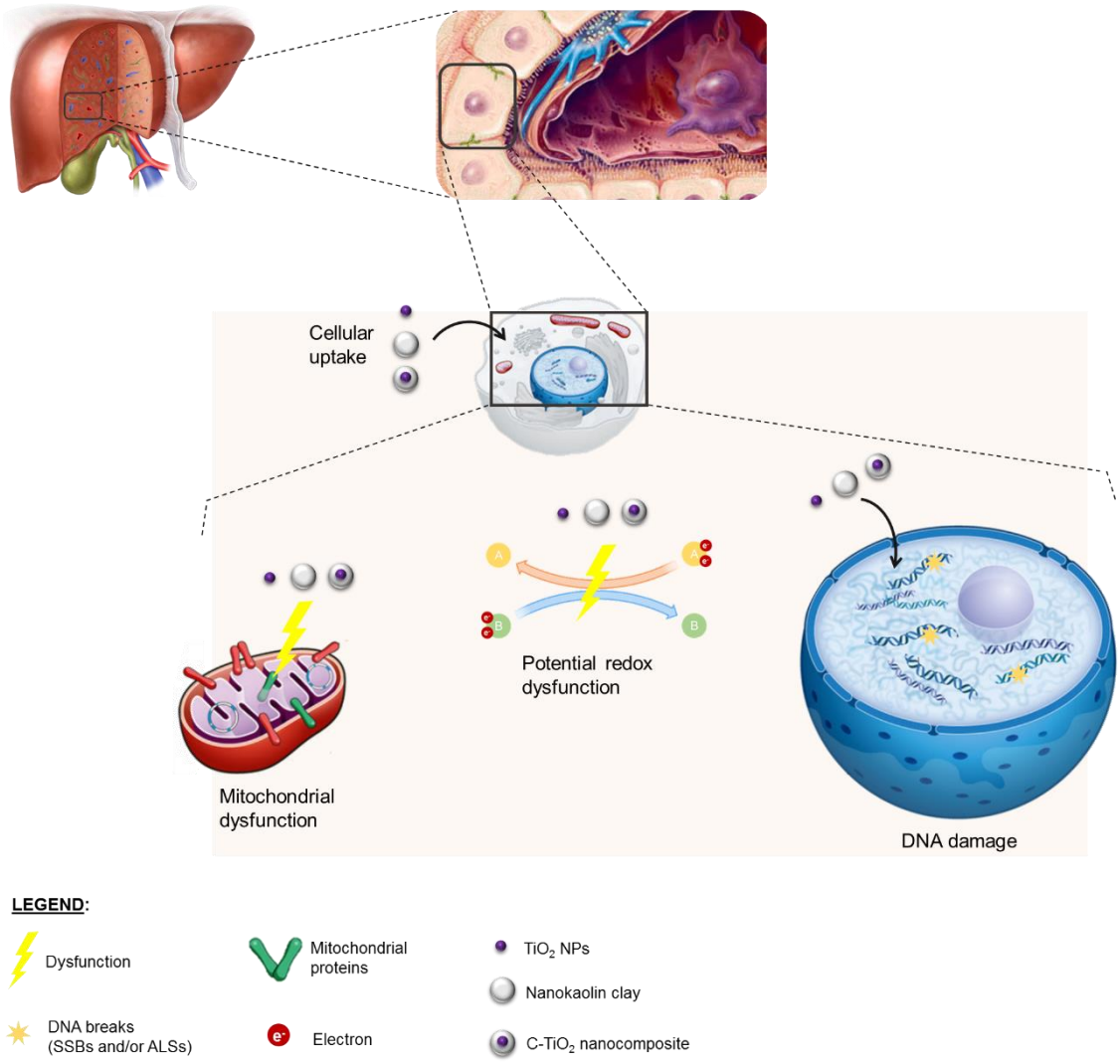


Figure 26. General mechanisms of toxicity on the hepatocyte cell line under study (HepG2) caused by TiO_2 NPs, nanokaolin clay and C-TiO_2 nanocomposite in the presence or absence of serum proteins in the biological medium.

After selecting the appropriate clay mineral for the immobilization of TiO_2 NPs or other nanoparticles, the following step is to perform *in vivo* studies. The first stage for *in vivo* studies is to choose an appropriate animal model with similar metabolic and physiological system background to humans in order to evaluate the biological effects of nanoparticles in real situations.

The current innovative study represents a primordial toxicity evaluation of the nanocomposites using mineral clays structures on human exposure, moving towards to a safer application of these new materials.

VI. REFERENCES

Abdel-Wahhab, M. A., El-Denshary, E. S., El-Nekeety, A. A., Abdel-Wahhab, K. G., Hamzawy, M. A., Elyamany, M. F., Hassan, N. S., Mannaa, F. A., Shaiea, M. N. and Gado, R. A. (2015). Efficacy of Organo-Modified Nano Montmorillonite to Protect against the Cumulative Health Risk of Aflatoxin B 1 and Ochratoxin A in Rats. Soft Nanoscience Letters 5(2): 21.

Ai, J., Biazar, E., Jafarpour, M., Montazeri, M., Majdi, A., Aminifard, S., Zafari, M., Akbari, H. R. and Rad, H. G. (2011). Nanotoxicology and nanoparticle safety in biomedical designs. Journal of International Journal of Nanomedicine 6: 1117-1127.

Allouni, Z. E., Gjerdet, N. R., Cimpan, M. R. and Hol, P. J. (2015). The effect of blood protein adsorption on cellular uptake of anatase TiO2 nanoparticles. International Journal of Nanomedicine 10: 687-695.

Aono, M., Bando, Y. and Ariga, K. (2012). Nanoarchitectonics: pioneering a new paradigm for nanotechnology in materials development. Advanced Materials 24(2): 150-151.

Aranda, P., Kun, R., Martín-Luengo, M. A., Letaïef, S., Dékány, I. and Ruiz-Hitzky, E. (2008). Titania-Sepiolite Nanocomposites Prepared by a Surfactant Templating Colloidal Route. Chemistry of Materials 20: 84–91.

Arora, A. and Padua, G. W. (2010). Review: nanocomposites in food packaging. Jornal of Food Science 75(1): 43-49.

Arora, S., Rajwade, J. M. and Paknikar, K. M. (2012). Nanotoxicology and in vitro studies: the need of the hour. Toxicology and Applied Pharmacology 258(2): 151-165.

Baalousha, M. (2009). Aggregation and disaggregation of iron oxide nanoparticles: Influence of particle concentration, pH and natural organic matter. Science of the Total Environment 407(6): 2093-2101.

Baker, T. J., Tyler, C. R. and Galloway, T. S. (2014). Impacts of metal and metal oxide nanoparticles on marine organisms. Environmental Pollution 186: 257-271.

Balog, S., Rodriguez-Lorenzo, L., Monnier, C. A., Obiols-Rabasa, M., Rothen-Rutishauser, B., Schurtenberger, P. and Petri-Fink, A. (2015). Characterizing nanoparticles in complex biological media and physiological fluids with depolarized dynamic light scattering. Nanoscale 7(14): 5991-5997.

Banerjee, A. N. (2011). The design, fabrication, and photocatalytic utility of nanostructured semiconductors: focus on TiO2-based nanostructures. Journal of Nanotechnology, Science and Applications 4: 35-65.

Bartneck, M., Warzecha, K. T. and Tacke, F. (2014). Therapeutic targeting of liver inflammation and fibrosis by nanomedicine. Hepatobiliary Surgery and Nutrition 3(6): 364-376.

Becker, K., Schroecksnadel, S., Geisler, S., Carriere, M., Gostner, J. M., Schennach, H., Herlin, N. and Fuchs, D. (2014). TiO(2) nanoparticles and bulk material stimulate human peripheral blood mononuclear cells. Food and Chemical Toxicology 65: 63-69.

Belessi, V., Lambropoulou, D., Konstantinou, I., Katsoulidis, A., Pomonis, P., Petridis, D. and Albanis, T. (2007). Structure and photocatalytic performance of TiO2/clay nanocomposites for the degradation of dimethachlor. Applied Catalysis B: Environmental 73(3–4): 292-299.

Borenfreund, E. and Puerner, J. A. (1985). Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicology letters 24(2): 119-124.

Borra, R. C., Lotufo, M. A., Gagioti, S. M., Barros, F. d. M. and Andrade, P. M. (2009). A simple method to measure cell viability in proliferation and cytotoxicity assays. Brazilian oral research 23(3): 255-262.

Botelho, M. C., Costa, C., Silva, S., Costa, S., Dhawan, A., Oliveira, P. A. and Teixeira, J. P. (2014). Effects of titanium dioxide nanoparticles in human gastric epithelial cells in vitro. Biomedicine & Pharmacotherapy 68(1): 59-64.

Burnett, M. E. and Wang, S. Q. (2011). Current sunscreen controversies: a critical review. Photodermatology, Photoimmunology & Photomedicine 27(2): 58-67.

Camargo, P. H. C., Satyanarayana, K. G. and Wypych, F. (2009). Nanocomposites: Synthesis, Structure, Properties and New Application Opportunities. Materials Research 12: 1-39.

Chang, X., Zhang, Y., Tang, M. and Wang, B. (2013). Health effects of exposure to nano-TiO2: a meta-analysis of experimental studies. Nanoscale Research Letters 8(1): 51.

Chen, D., Du, G., Zhu, Q. and Zhou, F. (2013). Synthesis and characterization of TiO2 pillared montmorillonites: application for methylene blue degradation. Journal of Colloid and Interface Science 409: 151-157.

Cho, E. J., Holback, H., Liu, K. C., Abouelmagd, S. A., Park, J. and Yeo, Y. (2013). Nanoparticle characterization: state of the art, challenges, and emerging technologies. Molecular Pharmaceutics 10(6): 2093-2110.

Choi, A. H., Ben-Nissan, B., Matinlinna, J. P. and Conway, R. C. (2013). Current perspectives: calcium phosphate nanocoatings and nanocomposite coatings in dentistry. Journal of Dental Research 92(10): 853-859.

Chong, M. N., Vimonses, V., Lei, S., Jin, B., Chow, C. and Saint, C. (2009). Synthesis and characterisation of novel titania impregnated kaolinite nano-photocatalyst. Microporous and Mesoporous Materials 117(1–2): 233-242.

Conde, J., Dias, J. T., Grazu, V., Moros, M., Baptista, P. V. and de la Fuente, J. M. (2014). Revisiting 30 years of biofunctionalization and surface chemistry of inorganic nanoparticles for nanomedicine. Frontiers in Chemistry 2: 48.

COST (2012). Memorandum of Understanding for the implementation of a European Concerted Research Action designated as COST Action TD1204: Modelling Nanomaterial Toxicity MODENA. Retrieved from http://w3.cost.eu/fileadmin/domain_files/MPNS/Action_TD1204/mou/TD1204-e.pdf in 2015.

Davidovic, M., Kutin, M., Linic, S., Mioc, U., Nedic, Z., Sredic, S., Nikolic, A., Jovanovic, D. and Pissis, P. (2011).Nanocomposite Based on Natural Materials. In Advances in Diverse Industrial Applications of Nanocomposites(Ed Dr. Boreddy Reddy). InTech.

Dawson, J. I. and Oreffo, R. O. (2013). Clay: new opportunities for tissue regeneration and biomaterial design. Advanced Materials 25(30): 4069-4086.

Depan, D., Kumar, A. P. and Singh, R. P. (2009). Cell proliferation and controlled drug release studies of nanohybrids based on chitosan-g-lactic acid and montmorillonite. Acta Biomaterialia 5(1): 93-100.

Dhawan, A., Sharma, V. and Parmar, D. (2009). Nanomaterials: A challenge for toxicologists. Nanotoxicology 3(1): 1-9.

Djurisic, A. B., Leung, Y. H., Ng, A. M., Xu, X. Y., Lee, P. K., Degger, N. and Wu, R. S. (2014). Toxicity of Metal Oxide Nanoparticles: Mechanisms, Characterization, and Avoiding Experimental Artefacts. Small.

Dukhovich, F. (2004). Molecular Recognition: Pharmacological Aspects. Nova Publishers.

Ekstrand-Hammarstrom, B., Akfur, C. M., Andersson, P. O., Lejon, C., Osterlund, L. and Bucht, A. (2012). Human primary bronchial epithelial cells respond differently to titanium dioxide nanoparticles than the lung epithelial cell lines A549 and BEAS-2B. Nanotoxicology 6(6): 623-634.

EPC (2011). European Parliament and Council Regulation Nº 1169/2011 - Provision of food information to consumers. Official Journal of the European Union L304: 26.

Falugi, C., Aluigi, M. G., Chiantore, M. C., Privitera, D., Ramoino, P., Gatti, M. A., Fabrizi, A., Pinsino, A. and Matranga, V. (2012). Toxicity of metal oxide nanoparticles in immune cells of the sea urchin. Marine Environmental Research 76: 114-121.

Fenoglio, I., Greco, G., Livraghi, S. and Fubini, B. (2009). Non-UV-induced radical reactions at the surface of TiO2 nanoparticles that may trigger toxic responses. Chemistry 15(18): 4614-4621.

Fernandez, L. J. F., Lorite, V. I., Rubio, M. F., Romero, F. J. J., Garcia, G. T. M. Á., Quesada, M. A., Martn, G. M. S. and Costa, K. J. L. (2010).Procedimiento para la dispersión de nanopartículas en seco y la obtención de estructuras jerárquicas y recubrimientos. Google Patents.

Fresegna, A., Lega, D., Antonini, A. and Fanizza, C. (2014).Cell surface modifications induced by titanium dioxide nanoparticles in A549 cells. In Microscopy: advances in scientific research and education, 8 (Ed A. Mendes Vilas).

Froggett, S. J., Clancy, S. F., Boverhof, D. R. and Canady, R. A. (2014). A review and perspective of existing research on the release of nanomaterials from solid nanocomposites. Particle and Fibre Toxicology 11: 17.

Frohlich, E. (2012). The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles. Journal of International Journal of Nanomedicine 7: 5577-5591.

Frohlich, E. (2013). Cellular targets and mechanisms in the cytotoxic action of non-biodegradable engineered nanoparticles. Current Drug Metabolism 14(9): 976-988.

Fujita, K., Horie, M., Kato, H., Endoh, S., Suzuki, M., Nakamura, A., Miyauchi, A., Yamamoto, K., Kinugasa, S., Nishio, K., Yoshida, Y., Iwahashi, H. and Nakanishi, J. (2009). Effects of ultrafine TiO2 particles on gene expression profile in human keratinocytes without illumination: involvement of extracellular matrix and cell adhesion. Toxicology Letters 191(2-3): 109-117.

Gaiser, B. K., Hirn, S., Kermanizadeh, A., Kanase, N., Fytianos, K., Wenk, A., Haberl, N., Brunelli, A., Kreyling, W. G. and Stone, V. (2013). Effects of Titanium Dioxide Nanoparticles on the liver and hepatocytes in vitro. Toxicological sciences 131(2): 537-547.

Giannitrapani, L., Soresi, M., Bondi, M. L., Montalto, G. and Cervello, M. (2014). Nanotechnology applications for the therapy of liver fibrosis. World Journal of Gastroenterology 20(23): 7242-7251.

Gonzalez-Alfaro, Y., Aranda, P., Fernandes, F. M., Wicklein, B., Darder, M. and Ruiz-Hitzky, E. (2011). Multifunctional porous materials through ferrofluids. Adv Mater 23(44): 5224-5228.

Guadagnini, R., Halamoda Kenzaoui, B., Cartwright, L., Pojana, G., Magdolenova, Z., Bilanicova, D., Saunders, M., Juillerat, L., Marcomini, A., Huk, A., Dusinska, M., Fjellsbo, L. M., Marano, F. and Boland, S. (2013). Toxicity screenings of nanomaterials: challenges due to interference with assay processes and components of classic in vitro tests. Nanotoxicology.

Gupta, S. M. and Tripathi, M. (2011). A review of TiO2 nanoparticles. Chinese Science Bulletin 56(16): 1639-1657.

Han, H. K., Lee, Y. C., Lee, M. Y., Patil, A. J. and Shin, H. J. (2011a). Magnesium and calcium organophyllosilicates: synthesis and in vitro cytotoxicity study. ACS Applied Materials & Interfaces 3(7): 2564-2572.

Han, X., Gelein, R., Corson, N., Wade-Mercer, P., Jiang, J., Biswas, P., Finkelstein, J. N., Elder, A. and Oberdorster, G. (2011b). Validation of an LDH assay for assessing nanoparticle toxicity. Toxicology 287(1-3): 99-104.

Haraguchi, K., Takehisa, T. and Ebato, M. (2006). Control of cell cultivation and cell sheet detachment on the surface of polymer/clay nanocomposite hydrogels. Biomacromolecules 7(11): 3267-3275.

Holder, A. L., Goth-Goldstein, R., Lucas, D. and Koshland, C. P. (2012). Particle-induced artifacts in the MTT and LDH viability assays. Chemical Research in Toxicology 25(9): 1885-1892.

Horie, M., Nishio, K., Fujita, K., Kato, H., Endoh, S., Suzuki, M., Nakamura, A., Miyauchi, A., Kinugasa, S., Yamamoto, K., Iwahashi, H., Murayama, H., Niki, E. and Yoshida, Y. (2010). Cellular responses by stable and uniform ultrafine titanium dioxide particles in culture-medium dispersions when secondary particle size was 100 nm or less. Toxicol In Vitro 24(6): 1629-1638.

Houtman, J., Maisanaba, S., Puerto, M., Gutiérrez-Praena, D., Jordá, M., Aucejo, S. and Jos, A. (2014). Toxicity assessment of organomodified clays used in food contact materials on human target cell lines. Applied Clay Science 90: 150-158.

Huerta-Garcia, E., Perez-Arizti, J. A., Marquez-Ramirez, S. G., Delgado-Buenrostro, N. L., Chirino, Y. I., Iglesias, G. G. and Lopez-Marure, R. (2014). Titanium dioxide nanoparticles induce strong oxidative stress and mitochondrial damage in glial cells. Free Radical Biology & Medicine 73: 84-94.

Iavicoli, I., Leso, V. and Bergamaschi, A. (2012a). Toxicological effects of titanium dioxide nanoparticles: a review of in vivo studies. Journal of Nanomaterials 2012: 5.

Iavicoli, I., Leso, V., Fontana, L. and Bergamaschi, A. (2011). Toxicological effects of titanium dioxide nanoparticles: a review of in vitro mammalian studies. European Review for Medical and Pharmacological Sciences 15(5): 481-508.

Iavicoli, P., Xu, H., Keszthelyi, T., Telegdi, J., Wurst, K., Van Averbeke, B., Saletra, W. J., Minoia, A., Beljonne, D., Lazzaroni, R., De Feyter, S. and Amabilino, D. B. (2012b). Organization of the enantiomeric and racemic forms of an amphiphilic resorcinol derivative at the air-water and graphite-1-phenyloctane interfaces. Chirality 24(2): 155-166.

Iredale, J. P. (2007). Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. Journal of Clinical Investigation 117(3): 539-548.

Jaeger, A., Weiss, D. G., Jonas, L. and Kriehuber, R. (2012). Oxidative stress-induced cytotoxic and genotoxic effects of nano-sized titanium dioxide particles in human HaCaT keratinocytes. Toxicology 296(1-3): 27-36.

Janer, G., Mas del Molino, E., Fernandez-Rosas, E., Fernandez, A. and Vazquez-Campos, S. (2014). Cell uptake and oral absorption of titanium dioxide nanoparticles. Toxicology Letters 228(2): 103-110.

Jeon, I.-Y. and Baek, J.-B. (2010). Nanocomposites Derived from Polymers and Inorganic Nanoparticles - Review. Materials 3: 3654-3674.

Jones, C. F. and Grainger, D. W. (2009). In vitro assessments of nanomaterial toxicity. Advanced Drug Delivery Reviews 61(6): 438-456.

Joris, F., Manshian, B. B., Peynshaert, K., De Smedt, S. C., Braeckmans, K. and Soenen, S. J. (2013). Assessing nanoparticle toxicity in cell-based assays: influence of cell culture parameters and optimized models for bridging the in vitro-in vivo gap. Chemical Society reviews 42(21): 8339-8359.

Jugan, M.-L., Barillet, S., Simon-Deckers, A., Herlin-Boime, N., Sauvaigo, S., Douki, T. and Carriere, M. (2012). Titanium dioxide nanoparticles exhibit genotoxicity and impair DNA repair activity in A549 cells. Nanotoxicology 6(5): 501-513.

Kain, J., Karlsson, H. L. and Moller, L. (2012). DNA damage induced by micro- and nanoparticles--interaction with FPG influences the detection of DNA oxidation in the comet assay. Mutagenesis 27(4): 491-500.

Kang, S. J., Kim, B. M., Lee, Y. J. and Chung, H. W. (2008). Titanium dioxide nanoparticles trigger p53-mediated damage response in peripheral blood lymphocytes. Environmental and Molecular Mutagenesis 49(5): 399-405.

Karlsson, H. L. (2010). The comet assay in nanotoxicology research. Analytical and bioanalytical chemistry 398(2): 651-666.

Keledi, G., Hari, J. and Pukanszky, B. (2012). Polymer nanocomposites: structure, interaction, and functionality. Nanoscale 4(6): 1919-1938.

Kermanizadeh, A., Gaiser, B. K., Hutchison, G. R. and Stone, V. (2012). An in vitro liver model-assessing oxidative stress and genotoxicity following exposure of hepatocytes to a panel of engineered nanomaterials. Particle and Fibre Toxicology 9(1): 1-13.

Kermanizadeh, A., Pojana, G., Gaiser, B. K., Birkedal, R., Bilanicova, D., Wallin, H., Jensen, K. A., Sellergren, B., Hutchison, G. R., Marcomini, A. and Stone, V. (2013). In vitro assessment of engineered nanomaterials using a hepatocyte cell line: cytotoxicity, pro-inflammatory cytokines and functional markers. Nanotoxicology 7(3): 301-313.

Kibanova, D., Cervini-Silva, J. and Destaillats, H. (2009). Efficiency of Clay−TiO2 Nanocomposites on the Photocatalytic Elimination of a Model Hydrophobic Air Pollutant. Environmental Science & Technology 43(5): 1500-1506.

Kim, E. S., Ahn, E. H., Dvir, T. and Kim, D. H. (2014). Emerging nanotechnology approaches in tissue engineering and regenerative medicine. Journal of International Journal of Nanomedicine 9 Suppl 1: 1-5.

Kim, J. E., Shin, J. Y. and Cho, M. H. (2012). Magnetic nanoparticles: an update of application for drug delivery and possible toxic effects. Archives of Toxicology 86(5): 685-700.

Klien, K. and Godnic-Cvar, J. (2012). Genotoxicity of metal nanoparticles: focus on in vivo studies. Archives of Industrial Hygiene and Toxicology 63(2): 133-145.

Kocbek, P., Teskac, K., Kreft, M. E. and Kristl, J. (2010). Toxicological aspects of long-term treatment of keratinocytes with ZnO and TiO2 nanoparticles. Small 6(17): 1908-1917.

Kroll, A., Pillukat, M. H., Hahn, D. and Schnekenburger, J. (2009). Current in vitro methods in nanoparticle risk assessment: limitations and challenges. European journal of pharmaceutics and biopharmaceutics 72(2): 370-377.

Kroll, A., Pillukat, M. H., Hahn, D. and Schnekenburger, J. (2012). Interference of engineered nanoparticles with in vitro toxicity assays. Archives of Toxicology 86(7): 1123-1136.

Krug, H. F. and Wick, P. (2011). Nanotoxicology: an interdisciplinary challenge. Angewandte Chemie International Edition 50(6): 1260-1278.

Kujawa, P. and Winnik, F. M. (2013). Innovation in nanomedicine through materials nanoarchitectonics. Langmuir 29(24): 7354-7361.

Lagopati, N., Tsilibary, E. P., Falaras, P., Papazafiri, P., Pavlatou, E. A., Kotsopoulou, E. and Kitsiou, P. (2014). Effect of nanostructured TiO(2) crystal phase on photoinduced apoptosis of breast cancer epithelial cells. Journal of International Journal of Nanomedicine 9: 3219-3230.

Laroui, H., Rakhya, P., Xiao, B., Viennois, E. and Merlin, D. (2013). Nanotechnology in diagnostics and therapeutics for gastrointestinal disorders. Digestive and Liver Disease 45(12): 995-1002.

Lewinski, N., Colvin, V. and Drezek, R. (2008). Cytotoxicity of nanoparticles. Small 4(1): 26-49.

Li, M., Yin, J. J., Wamer, W. G. and Lo, Y. M. (2014). Mechanistic characterization of titanium dioxide nanoparticle-induced toxicity using electron spin resonance. Journal of Food and Drug Analysis 22(1): 76-85.

Li, P. R., Wei, J. C., Chiu, Y. F., Su, H. L., Peng, F. C. and Lin, J. J. (2010). Evaluation on cytotoxicity and genotoxicity of the exfoliated silicate nanoclay. ACS Applied Materials & Interfaces 2(6): 1608-1613.

Liang, G., Pu, Y., Yin, L., Liu, R., Ye, B., Su, Y. and Li, Y. (2009). Influence of different sizes of titanium dioxide nanoparticles on hepatic and renal functions in rats with correlation to oxidative stress. Journal of Toxicology and Environmental Health 72(11-12): 740-745.

Lidén, G. (2011). The European Commission Tries to Define Nanomaterials. Annals of Occupational Hygiene 55(1): 1-5.

Lin, Z., Monteiro-Riviere, N. A. and Riviere, J. E. (2014). Pharmacokinetics of metallic nanoparticles. Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology.

Liu, K., Lin, X. and Zhao, J. (2013). Toxic effects of the interaction of titanium dioxide nanoparticles with chemicals or physical factors. Journal of International Journal of Nanomedicine 8: 2509-2520.

Lopez-Serrano, A., Olivas, R. M., Landaluze, J. S. and Camara, C. (2014). Nanoparticles: a global vision. Characterization, separation, and quantification methods. Potential environmental and health impact. Analytical Methods 6(1): 38-56.

Lordan, S., Kennedy, J. E. and Higginbotham, C. L. (2011). Cytotoxic effects induced by unmodified and organically modified nanoclays in the human hepatic HepG2 cell line. Journal of Applied Toxicology 31(1): 27-35.

Love, S. A., Maurer-Jones, M. A., Thompson, J. W., Lin, Y. S. and Haynes, C. L. (2012). Assessing nanoparticle toxicity. Annual Review of Analytical Chemistry 5: 181-205.

Magdolenova, Z., Lorenzo, Y., Collins, A. and Dusinska, M. (2012). Can standard genotoxicity tests be applied to nanoparticles? Journal of toxicology and environmental health. Part A 75(13-15): 800-806.

Maisanaba, S., Gutierrez-Praena, D., Pichardo, S., Moreno, F. J., Jorda, M., Camean, A. M., Aucejo, S. and Jos, A. (2014). Toxic effects of a modified montmorillonite clay on the human intestinal cell line Caco-2. Journal of Applied Toxicology 34(6): 714-725.

Maisanaba, S., Pichardo, S., Puerto, M., Gutiérrez-Praena, D., Cameán, A. M. and Jos, A. (2015). Toxicological evaluation of clay minerals and derived nanocomposites: A review. Environmental research 138: 233-254.

Maisanaba, S., Puerto, M., Pichardo, S., Jorda, M., Moreno, F. J., Aucejo, S. and Jos, A. (2013). In vitro toxicological assessment of clays for their use in food packaging applications. Food and Chemical Toxicology 57: 266-275.

Mallakpoura, S. and Shahangia, V. (2012). Modification of Clay with L-Leucine and TiO2 with Silane Coupling Agent for Preparation of Poly(Vinyl Alcohol)/Organo-Nanoclay/Modified TiO2 Nanocomposites Film. Designed Monomers and Polymers 15(3): 329-344.

Malvern (2004).Malvern Instruments Ltd Manual, Zetasizer Nano Series User.

Manova, E., Aranda, P., Martín-Luengo, M. A., Letaïef, S. and Ruiz-Hitzky, E. (2010). New titania-clay nanostructured porous materials. Microporous and Mesoporous Materials 131 252–260.

Marquez-Ramirez, S. G., Delgado-Buenrostro, N. L., Chirino, Y. I., Iglesias, G. G. and Lopez-Marure, R. (2012). Titanium dioxide nanoparticles inhibit proliferation and induce morphological changes and apoptosis in glial cells. Toxicology 302(2-3): 146-156.

Martirosyan, A. and Schneider, Y. J. (2014). Engineered nanomaterials in food: implications for food safety and consumer health. International Journal of Environmental Research and Public Health 11(6): 5720-5750.

Menard, A., Drobne, D. and Jemec, A. (2011). Ecotoxicity of nanosized TiO2. Review of in vivo data. Environmental Pollution 159(3): 677-684.

Mitrano, D. M., Motellier, S., Clavaguera, S. and Nowack, B. (2015). Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products. Environment Internationa 77C: 132-147.

Mittal, V. (2009). Polymer Layered Silicate Nanocomposites: A Review. Materials 2: 992-1057.

Moschini, E., Gualtieri, M., Colombo, M., Fascio, U., Camatini, M. and Mantecca, P. (2013). The modality of cell-particle interactions drives the toxicity of nanosized CuO and TiO(2) in human alveolar epithelial cells. Toxicology Letters 222(2): 102-116.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of immunological methods 65(1): 55-63.

Murdock, R. C., Braydich-Stolle, L., Schrand, A. M., Schlager, J. J. and Hussain, S. M. (2008). Characterization of nanomaterial dispersion in solution prior to in vitro exposure using dynamic light scattering technique. Toxicological Sciences 101(2): 239-253.

Murugan, K., Choonara, Y. E., Kumar, P., Bijukumar, D., du Toit, L. C. and Pillay, V. (2015). Parameters and characteristics governing cellular internalization and trans-barrier trafficking of nanostructures. Journal of International Journal of Nanomedicine 10: 2191-2206.

Naqvi, S., Samim, M., Abdin, M., Ahmed, F. J., Maitra, A., Prashant, C. and Dinda, A. K. (2010). Concentration-dependent toxicity of iron oxide nanoparticles mediated by increased oxidative stress. International Journal of Nanomedicine 5: 983-989.

Natarajan, V. (2015). Titanium Dioxide Nanoparticles Trigger Loss of Hepatic Function and Perturbation of Mitochondrial Dynamics in Primary Hepatocytes. 1-71.

Norppa, H., Siivola, K., Fessard, V., Tarantini, A., Apostolova, M., Jacobsen, N. R., Wallin, H., Goetz, M. E., Fieblinger, D. and Stepnik, M. (2013). In vitro testing strategy for nanomaterials including database (Deliverable 5): final report.

OCDE (2014). OCDE Working Party on Nanotechnology (WPN): Vision Statement. Retrieved from http://www.oecd.org/sti/nano/oecdworkingpartyonnanotechnologywpnvisionstatement.htm in 2014.

Oh, N. and Park, J. H. (2014). Endocytosis and exocytosis of nanoparticles in mammalian cells. International Journal of Nanomedicine 9(Suppl 1): 51-63.

Olad, A. (2011).Polymer/Clay Nanocomposites. In Advances in Diverse Industrial Applications of Nanocomposites(Ed Dr. Boreddy Reddy). InTech.

Park, E. J., Lee, G. H., Shim, H. W., Kim, J. H., Cho, M. H. and Kim, D. W. (2014). Comparison of toxicity of different nanorod-type TiO2 polymorphs in vivo and in vitro. Journal of Applied Toxicology 34(4): 357-366.

Pavlidou, S. and Papaspyrides, C. D. (2008). A review on polymer–layered silicate nanocomposites. Progress in Polymer Science 33: 1119–1198.

Pavlin, M. and Bregar, V. B. (2012). Stability of nanoparticle suspensions in different biologically relevant media. Digest Journal of Nanomaterials and Biostructures 7(4): 1389-1400.

Petković, J., Žegura, B. and Filipič, M. (2011).Influence of TiO2 nanoparticles on cellular antioxidant defense and its involvement in genotoxicity in HepG2 cells. In Journal of Physics: Conference Series, Vol. 304, 1-8.

Petkovic, J., Zegura, B., Stevanovic, M., Drnovsek, N., Uskokovic, D., Novak, S. and Filipic, M. (2011). DNA damage and alterations in expression of DNA damage responsive genes induced by TiO2 nanoparticles in human hepatoma HepG2 cells. Nanotoxicology 5(3): 341-353.

Piccinno, F., Gottschalk, F., Seeger, S. and Nowack, B. (2012). Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. Journal of Nanoparticle Research 14(9): 1-11.

Prasad, R. Y., Simmons, S. O., Killius, M. G., Zucker, R. M., Kligerman, A. D., Blackman, C. F., Fry, R. C. and Demarini, D. M. (2014). Cellular interactions and biological responses to titanium dioxide nanoparticles in HepG2 and BEAS-2B cells: role of cell culture media. Environmental and Molecular Mutagenesis 55(4): 336-342.

Rahim, S., Sasani Ghamsari, M. and Radiman, S. (2012). Surface modification of titanium oxide nanocrystals with PEG. Scientia Iranica 19(3): 948-953.

Rampersad, S. N. (2012). Multiple applications of Alamar Blue as an indicator of metabolic function and cellular health in cell viability bioassays. Sensors (Basel) 12(9): 12347-12360.

Rasmussen, K., Mast, J., De Temmerman, P., Verleysen, E., Waegeneers, N., Van Steen, F., Pizzolon, J., De Temmerman, L., Van Doren, E. and Jensen, K. (2014). Titanium Dioxide, NM-100, NM-101, NM-102, NM-103, NM-104, NM-105: Characterisation and Physico-Chemical Properties. JRC Science and Policy Reports: 218.

Reijnders, L. (2009). The release of TiO2 and SiO2 nanoparticles from nanocomposites. Polymer Degradation and Stability 94: 873–876.

Repetto, G., del Peso, A. and Zurita, J. L. (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat Protoc 3(7): 1125-1131.

Riss, T. L., Moravec, R. A., Niles, A. L., Benink, H. A., Worzella, T. J. and Minor, L. (2004).Cell Viability Assays. In Assay Guidance Manual(Eds G. S. Sittampalam, N. Gal-Edd, M. Arkin, D. Auld, C. Austin, B. Bejcek, M. Glicksman, J. Inglese, V. Lemmon, Z. Li, J. McGee, O. McManus, L. Minor, A. Napper, T. Riss, O. J. Trask and J. Weidner). Bethesda (MD).

Roco, M. (2011).The Long View of Nanotechnology Development: The National Nanotechnology Initiative at 10 Years. In Nanotechnology Research Directions for Societal Needs in 2020, Vol. 1, 1-28: Springer Netherlands.

Rolfe, V. (2013). The anatomy of the liver: hepatocytes. Retrieved from http://www.nottingham.ac.uk/nmp/sonet/rlos/bioproc/liveranatomy/5.html in 2015.

Roy, R., Kumar, S., Tripathi, A., Das, M. and Dwivedi, P. D. (2014). Interactive threats of nanoparticles to the biological system. Immunology Letters 158(1-2): 79-87.

Salgın, S., Salgın, U. and Bahadır, S. (2012). Zeta potentials and isoelectric points of biomolecules: the effects of ion types and ionic strengths. International Journal of Electrochemical Science 7: 12404-12414.

Santos, C. M., Mangadlao, J., Ahmed, F., Leon, A., Advincula, R. C. and Rodrigues, D. F. (2012). Graphene nanocomposite for biomedical applications: fabrication, antimicrobial and cytotoxic investigations. Nanotechnology 23(39): 395101.

Sarkar, A., Ghosh, M. and Sil, P. C. (2014). Nanotoxicity: oxidative stress mediated toxicity of metal and metal oxide nanoparticles. Journal of Nanoscience and Nanotechnology 14(1): 730-743.

Savolainen, K., Backman, U., Brouwer, D., Fadeel, B., Fernandes, T., Kuhlbusch, T., Landsiedel, R., Lynch, I. and Pylkkänen, L. (2013). Nanosafety in Europe 2015-2025: Towards Safe and Sustainable Nanomaterials and Nanotechnology Innovations. Finnish Institute of Occupational Health: 218.

SCENIHR, E.-. (2007). Opinion on the scientific aspects of the existing and proposed definitions relating to products of nanoscience and nanotechnologies. . Retrieved from http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_012.pdf in 2015.

Shahadat, M., Teng, T. T., Rafatullah, M. and Arshad, M. (2015). Titanium-based nanocomposite materials: A review of recent advances and perspectives. Colloids and Surfaces B: Biointerfaces 126C: 121-137.

Sharma, A. K., Schmidt, B., Frandsen, H., Jacobsen, N. R., Larsen, E. H. and Binderup, M. L. (2010). Genotoxicity of unmodified and organo-modified montmorillonite. Mutation Research 700(1-2): 18-25.

Shi, H., Magaye, R., Castranova, V. and Zhao, J. (2013). Titanium dioxide nanoparticles: a review of current toxicological data. Particle and Fibre Toxicology 10: 15-48.

Shukla, R. K., Kumar, A., Gurbani, D., Pandey, A. K., Singh, S. and Dhawan, A. (2013). TiO(2) nanoparticles induce oxidative DNA damage and apoptosis in human liver cells. Nanotoxicology 7(1): 48-60.

Shukla, R. K., Kumar, A., Vallabani, N. S., Singh, S. and Dhawan, A. (2014). TiO2 NPs induced hepatic injury in mammals: a mechanistic approach. Molecular Cytogenetics 7(1): 1-1.

Sorensen, K. C., Stucki, J. W., Warner, R. E., Wagner, E. D. and Plewa, M. J. (2005). Modulation of the genotoxicity of pesticides reacted with redox-modified smectite clay. Environmental and Molecular Mutagenesis 46(3): 174-181.

Stathatos, E., Papoulis, D., Aggelopoulos, C. A., Panagiotaras, D. and Nikolopoulou, A. (2012). TiO2/palygorskite composite nanocrystalline films prepared by surfactant templating route: synergistic effect to the photocatalytic degradation of an azo-dye in water. Journal of Hazardous Materials 211-212: 68-76.

Suh, W. H., Suslick, K. S., Stucky, G. D. and Suh, Y. H. (2009). Nanotechnology, nanotoxicology, and neuroscience. Progress in neurobiology 87(3): 133-170.

Takhar, P. and Mahant, S. (2011). In vitro methods for Nanotoxicity Assessment: Advantages and Applications. Archives of Applied Science Research 3(2): 389-403.

Tang, X. Z., Kumar, P., Alavi, S. and Sandeep, K. P. (2012). Recent advances in biopolymers and biopolymer-based nanocomposites for food packaging materials. Critical Reviews in Food Science and Nutrition 52(5): 426-442.

Tenzer, S., Docter, D., Rosfa, S., Wlodarski, A., Kuharev, J., Rekik, A., Knauer, S. K., Bantz, C., Nawroth, T., Bier, C., Sirirattanapan, J., Mann, W., Treuel, L., Zellner, R., Maskos, M., Schild, H. and Stauber, R. H. (2011). Nanoparticle Size Is a Critical Physicochemical Determinant of the Human Blood Plasma Corona: A Comprehensive Quantitative Proteomic Analysis. ACS Nano 5(9): 7155-7167.

Teske, S. S. and Detweiler, C. S. (2015). The Biomechanisms of Metal and Metal-Oxide Nanoparticles' Interactions with Cells. International Journal of Environmental Research and Public Health 12(2): 1112-1134.

Thermo, F. S. (2015). Pierce™ LDH Cytotoxicity Assay Kit. Retrieved from https://www.thermofisher.com/order/catalog/product/88953 in 2015.

Tokarčíková, M., Tokarský, J., Čabanová, K., Matějka, V., Mamulová Kutláková, K. and Seidlerová, J. (2014). The stability of photoactive kaolinite/TiO2 composite. Composites Part B: Engineering 67(0): 262-269.

Tokarsky, J., Capkova, P. and Burda, J. V. (2012). Structure and stability of kaolinite/TiO2 nanocomposite: DFT and MM computations. Journal of Molecular Modeling 18(6): 2689-2698.

Tucci, P., Porta, G., Agostini, M., Dinsdale, D., Iavicoli, I., Cain, K., Finazzi-Agro, A., Melino, G. and Willis, A. (2013). Metabolic effects of TiO2 nanoparticles, a common component of sunscreens and cosmetics, on human keratinocytes. Cell Death & Disease - Nature 4: e549.

Ursini, C. L., Cavallo, D., Fresegna, A. M., Ciervo, A., Maiello, R., Tassone, P., Buresti, G., Casciardi, S. and Iavicoli, S. (2014). Evaluation of cytotoxic, genotoxic and inflammatory response in human alveolar and bronchial epithelial cells exposed to titanium dioxide nanoparticles. Journal of Applied Toxicology 34(11): 1209-1219.

Valdiglesias, V., Costa, C., Sharma, V., Kilic, G., Pasaro, E., Teixeira, J. P., Dhawan, A. and Laffon, B. (2013). Comparative study on effects of two different types of titanium dioxide nanoparticles on human neuronal cells. Food and Chemical Toxicology 57: 352-361.

Vega-Avila, E. and Pugsley, M. K. (2011). An overview of colorimetric assay methods used to assess survival or proliferation of mammalian cells. Proceedings of the Western Pharmacology Society 54: 10-14.

Verma, N. K., Moore, E., Blau, W., Volkov, Y. and Babu, P. R. (2012). Cytotoxicity evaluation of nanoclays in human epithelial cell line A549 using high content screening and real-time impedance analysis. Journal of Nanoparticle Research 14(9): 1-11.

Vimonses, V., Chong, M. N. and Jin, B. (2010). Evaluation of the physical properties and photodegradation ability of titania nanocrystalline impregnated onto modified kaolin. Microporous and Mesoporous Materials 132(1): 201-209.

Wallace, K., Burt, A. D. and Wright, M. C. (2008). Liver fibrosis. Biochemical Journal 411(1): 1-18.

Wang, G., Zhang, J., Dewilde, A. H., Pal, A. K., Bello, D., Therrien, J. M., Braunhut, S. J. and Marx, K. A. (2012). Understanding and correcting for carbon nanotube interferences with a commercial LDH cytotoxicity assay. Toxicology 299(2-3): 99-111.

Wang, H., Thorling, C. A., Liang, X., Bridle, K. R., Grice, J. E., Zhu, Y., Crawford, D. H., Xu, Z. P., Liu, X. and Roberts, M. S. (2015). Diagnostic imaging and therapeutic application of nanoparticles targeting the liver. Journal of Materials Chemistry B 3(6): 939-958.

Wang, Y., Cui, H., Zhou, J., Li, F., Wang, J., Chen, M. and Liu, Q. (2014). Cytotoxicity, DNA damage, and apoptosis induced by titanium dioxide nanoparticles in human non-small cell lung cancer A549 cells. Environmental Science and Pollution Research.

Warheit, D. B. (2013). How to measure hazards/risks following exposures to nanoscale or pigment-grade titanium dioxide particles. Toxicology Letters 220(2): 193-204.

Wohlleben, W., Brill, S., Meier, M. W., Mertler, M., Cox, G., Hirth, S., von Vacano, B., Strauss, V., Treumann, S., Wiench, K., Ma-Hock, L. and Landsiedel, R. (2011). On the lifecycle of nanocomposites: comparing released fragments and their in-vivo hazards from three release mechanisms and four nanocomposites. Small 7(16): 2384-2395.

Yang, S., Cui, X., Gong, J. and Deng, Y. (2013). Synthesis of TiO2-polyaniline core-shell nanofibers and their unique UV photoresponse based on different photoconductive mechanisms in oxygen and non-oxygen environments. Chemical Communications 49(41): 4676-4678.

Yin, Z. F., Wu, L., Yang, H. G. and Su, Y. H. (2013). Recent progress in biomedical applications of titanium dioxide. Physical Chemistry Chemical Physics 15(14): 4844-4858.

Zhang, P., Mo, Z., Han, L., Zhu, X., Wang, B. and Zhang, C. (2014). Preparation and Photocatalytic Performance of Magnetic TiO2/Montmorillonite/Fe3O4 Nanocomposites. Industrial & Engineering Chemistry Research 53(19): 8057-8061.

Zhuang, H., Zheng, J. P., Gao, H. and De Yao, K. (2007). In vitro biodegradation and biocompatibility of gelatin/montmorillonite-chitosan intercalated nanocomposite. Journal of Materials Science: Materials in Medicine 18(5): 951-957.

Zia, K. M., Zuber, M., Barikani, M., Hussain, R., Jamil, T. and Anjum, S. (2011). Cytotoxicity and mechanical behavior of chitin-bentonite clay based polyurethane bio-nanocomposites. International Journal of Biological Macromolecules 49(5): 1131-1136.

VII. APPENDICES

Appendix A

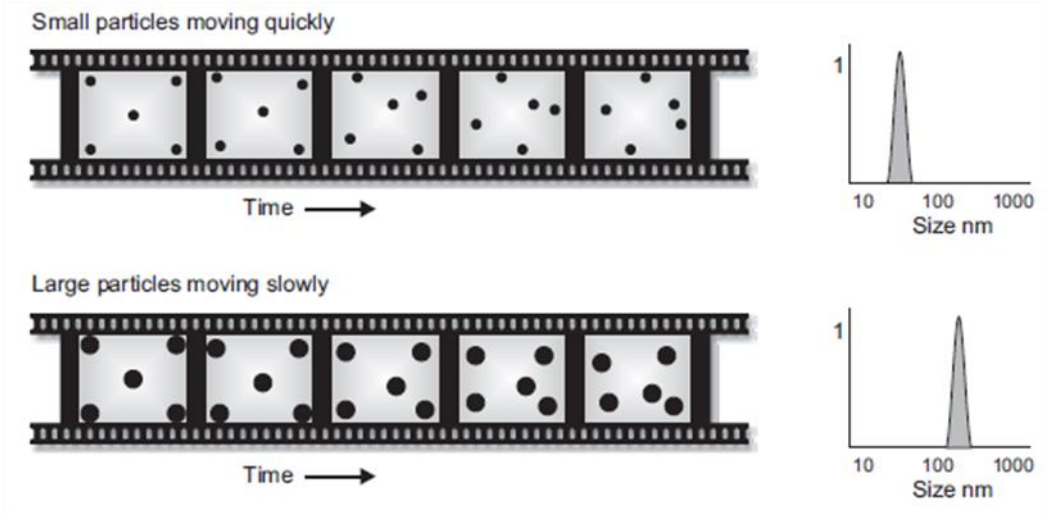


Figure A 1. Principle of hydrodynamic particle size measured by DLS (from Malvern (2004)).

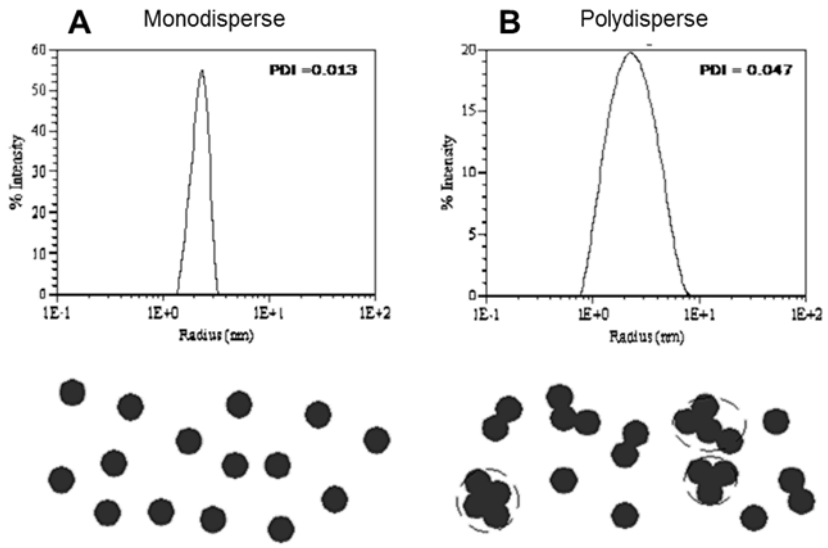


Figure A 2. Principle of polydispersity index (PDI). (A) Monodisperse sample; (B) Polydisperse sample.

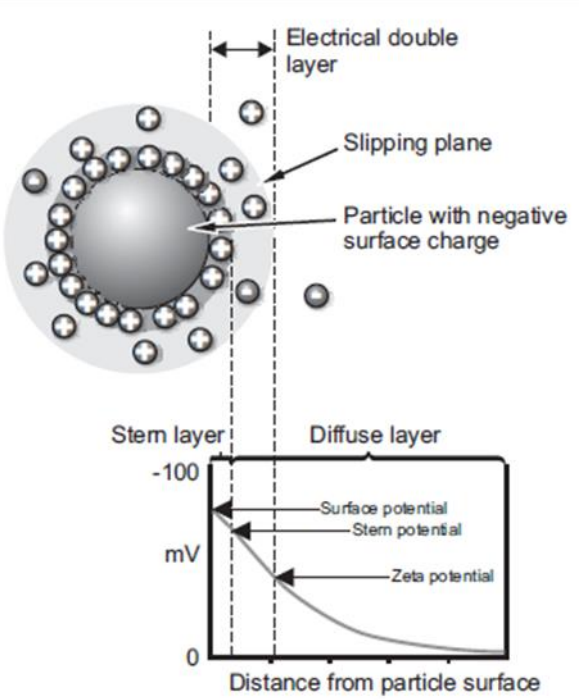


Figure A 3. Principle of zeta potential (from Malvern (2004)).

Appendix B

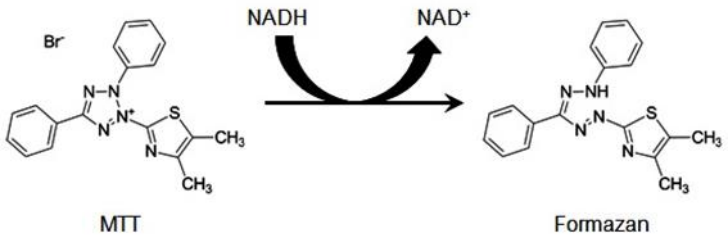


Figure B 1. Principle of the MTT methodology (from Riss *et al.* (2004)).

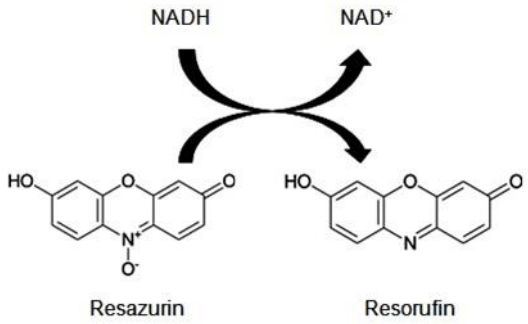


Figure B 2. Principle of the Alamar Blue methodology (from Riss *et al.* (2004)).

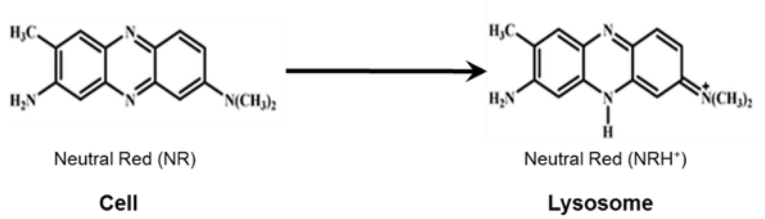


Figure B 3. Principle of the Neutral Red uptake methodology (based on Repetto *et al.* (2008)).

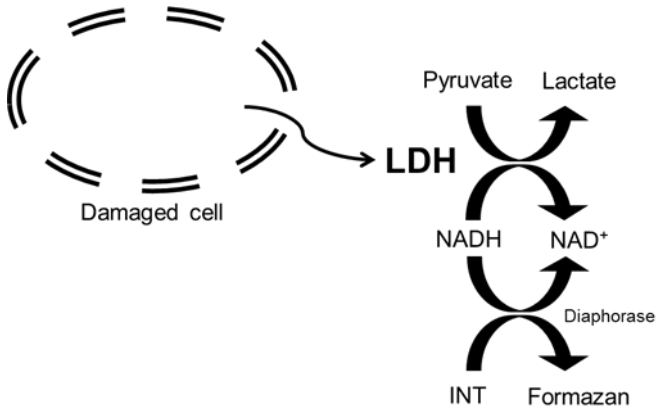


Figure B 4. Principle of the lactate dehydrogenase (LDH) reaction inside the cell (adapted from Thermo (2015)).