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Nanotoxicology: study of nanomaterials' genotoxic effects in cell lines

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RESUMO

Nos dias de hoje, surge cada vez mais a necessidade de se recorrer a uma maior qualidade e quantidade de variados produtos, para que seja possível responder ao rápido crescimento da população. Assim desenvolvem-se novas tecnologias, das quais, uma das mais recentes é a utilização de nanomateriais (NMs) em diversas áreas, como na cosmética, alimentação, biomedicina, indústria, entre outras, designando-se assim de NMs manufaturados (produzidos deliberadamente pelo Homem).

Os NMs contêm propriedades distintas ao nível da estrutura levando a um aumento da área superficial relativamente ao volume e, consequentemente, a um aumento das moléculas na superfície, sendo que estas características modificam a reatividade, melhorando muitas das suas propriedades. Porém, os NMs têm suscitado grande interesse por parte dos investigadores, pois o seu efeito para a saúde humana ainda não é bem conhecido e alguns estudos sugerem a sua implicação no desenvolvimento de cancro. Apesar da grande variedade de estudos efetuados acerca da genotoxicidade dos NMs, os resultados obtidos acerca do perigo que estes podem constituir para o ser humano não são concordantes. Este facto deve-se às características que os NMs apresentam e à sua capacidade de as alterar, consoante os meios em que se encontram e das condições em que são utilizados como, por exemplo, o tamanho, estado de aglomeração/agregação, ligação a proteínas, presença de metais de transição entre outros. Assim a avaliação dos efeitos dos NMs levando em consideração as especificidades destes, tem surgido como uma nova área da toxicologia, a nanotoxicologia. Nesta área, verifica-se que existe na literatura acerca da toxicidade dos NMs falta de concordância, pelo que surge a necessidade de se efetuarem mais estudos recorrendo a metodologias padronizadas e a NMs bem caracterizados, de maneira a conseguir-se comparar resultados.

O objetivo deste trabalho foi investigar a cito- e genotoxicidade de NMs, na perspetiva da nanotoxicologia, contribuindo para a avaliação da sua segurança. O estudo envolveu NMs desenvolvidos com o intuito de aplicação médica, o Poli(metil metacrilato) (PMMA) e um novo NM recentemente desenvolvido a partir desse, o Poli(metil metacrilato)-eudragit (PMMA-eud). Foram ainda investigados dois NMs manufaturados frequentemente utilizados na indústria, um NM de dióxido de titânio (TiO_2) e outro de nanotubos de carbono de parede múltipla (MWCNTs).

Os NMs analisados foram previamente caracterizados com detalhe relativamente às suas propriedades físico-químicas e as suspensões para a exposição das linhas celulares foram preparadas de acordo com metodologias padronizadas. Para avaliação da citotoxicidade,

utilizaram-se os ensaios clonogénico e contagem de células, bem como a análise de índice replicativo. O efeito genotóxico foi avaliado através do ensaio do cometa e do ensaio do micronúcleo com bloqueio da citocinese, realizado de acordo com as orientações internacionais para testes de genotoxicidade.

O efeito do PMMA e PMMA-eud foi avaliado em fibroblastos de ratinho (células L929) através do ensaio do micronúcleo em que foram determinados também os índices proliferativo e replicativo para avaliação da citotoxicidade. Na experiência preliminar, verificou-se um atraso ou um bloqueio do ciclo celular quando as células foram expostas por 48h a PMMA-eud, pelo que se optou por uma exposição de 54h no ensaio seguinte que não revelou efeitos citotóxicos nas células expostas a nenhum dos dois NMs. Quanto à genotoxicidade destes dois, somente os PMMA induziram um aumento na frequência dos micronúcleos 54h após exposição, mas apenas em duas concentrações, sem um efeito de dose-resposta. Estes resultados sugerem que a aplicação médica de PMMA-eud pode ser vantajosa em relação ao PMMA, uma vez que apresenta menos efeitos adversos. A diferença obtida entre estes dois NMs pode ser devida à carga de superfície, que é distinta entre os dois, ou ainda a uma maior capacidade do PMMA-eud para formar mais aglomerados, relativamente à PMMA, tornando estas últimas partículas mais pequenas, podendo facilitar a entrada dentro das células.

Relativamente aos NMs manufaturados, TiO_2 e MWCNTs utilizaram-se as células do epitélio pulmonar (A549) para a sua avaliação de toxicidade, uma vez que a via mais provável de exposição é a via respiratória. A citotoxicidade destes NMs foi avaliada através do ensaio clonogénico. Após 8 dias de exposição o TiO_2 revelou-se ligeiramente citotóxico apenas numa concentração, enquanto os MWCNTs foram citotóxicos em todas as concentrações analisadas, sendo possível delinear uma curva de dose-resposta. No entanto, nos ensaios de citotoxicidade realizados após exposição de 24h ou de 48h (contagem de células e índices proliferativo e replicativo), não foram observados efeitos citotóxicos. Para a avaliação dos efeitos genotóxicos causados por estes NMs, foram avaliadas as quebras de ADN em cadeia simples e cadeia dupla após 24 horas de exposição, assim como as quebras cromossómicas durante uma exposição de 48 horas através dos ensaios do cometa e do micronúcleo respetivamente. Através do ensaio do cometa, observou-se um aumento nos danos no ADN das células expostas por 24h a TiO_2, que era dependente da concentração. No entanto não se observou genotoxicidade no ensaio do micronúcleo após exposição por 48h a este NM.

No que respeita ao TiO_2, este NM de forma cristalina anatase, mostrou causar um aumento nos danos de ADN no ensaio do cometa. Este resultado, foi coerente com outros

estudos efetuados anteriormente utilizando um outro TiO_2 na forma anatase, sugerindo que esta propriedade físico-química, é importante para a genotoxicidade deste NM. Quanto aos MWCNTs, não se verificou nenhum efeito genotóxico nos dois ensaios efetuados. Os resultados negativos obtidos nos MWCNTs, podem dever-se à forte capacidade de aglomeração.

Com este estudo, podemos concluir que a avaliação das propriedades físico-químicas dos NMs é um fator importante relativamente à avaliação dos efeitos tóxicos destes. Pequenas modificações de um NM podem condicionar o seu efeito adverso, pelo que são necessários mais estudos para compreender os mecanismos relevantes, permitindo no futuro desenvolver NMs sem efeitos negativos para a saúde humana. Por sua vez, é importante prosseguir estudos de genotoxicidade utilizando metodologias padronizadas a partir de NMs de referência para garantir a sua utilização segura.

Palavras-chave: nanomateriais, genotoxicidade, citotoxicidade, Poli(metil metacrilato), Eudragit RL 100 , dióxido de titânio, nanotubos de carbono de parede múltipla.

ABSTRACT

The nanomaterials (NMs) have distinct structural properties, namely their size and increased surface area/volume ratio, and these characteristics change their reactivity, improving the applications in biomedicine, cosmetic as well as in industry. However, these properties may also lead to different toxicological consequences, such as the development of cancer.

This project aimed to contribute to the safety evaluation of NMs that are used or being developed for human applications, using nanotoxicology approaches. The study focused in Poly(methyl methacrylate) (PMMA) and poly(methyl methacrylate)-eudragit (PMMA-eud) NMs, used in the biomedical field for drug delivery, and titanium dioxide (TiO_2) and multi-walled carbon nanotubes (MWCNTs), that are frequently used in industry.

Following the preparation of the dispersion of the NMs, previously characterized in detail, the cytotoxic effects of the NMs were analyzed in cell lines using clonogenic assay, cell counting assay, proliferation and replication indexes. The comet and the cytokinesis-blocked micronucleus assays were used to investigate genotoxicity.

The effects of PMMA and PMMA-eud were evaluated in mouse fibroblasts (L929) and after 54 hours of exposure, no impact on cell cycle progression or cytotoxicity was observed for any of the NMs. PMMA revealed genotoxic effects while PMMA-eud was negative.

Concerning TiO_2 and MWCNTs, a pulmonary cell line (A549) was used. The clonogenic assay showed high cytotoxicity of MWCNTs while TiO_2 had low cytotoxicity, 8 days after exposure. However, the cytotoxicity assays using 24 or 48 h exposure did not reveal any cytotoxicity of the NMs. TiO_2 induced genotoxicity, with a dose-dependent increase in DNA damage detected by comet assay 24 h after exposure, while MWCNTs were negative. There was no increase in the micronucleus frequency after TiO_2 or MWCNTs, showing the absence of clastogenic or aneugenic effects.

The present study showed that the NMs physicochemical properties may determine their toxicological effects.

Key words: nanomaterials, genotoxicity, cytotoxicity, Poly(methyl methacrylate), Eudragit RL 100, titanium dioxide, multi-walled carbon nanotubes.

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A549 - Human epithelial lung adenocarcinoma cell line

ATCC - American Type culture Collection

BAuA - Federal Institute for Occupational Safety and Health

BSA - Bovine serum albumin

CBPI – Cytokinesis-blocked proliferation index

CNT – Carbon nanotube(s)

DLS – Dynamic light scatering

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethyl Sulfoxide

DNA – Desoxyribonucleic acid

EDTA – Ethylenediamine Tetraacetic Acid

EMS – Ethyl Methanesulfonate

FBS – Fetal bovine serum

FPG – Formamidopyrimidine DNA Glycosylase

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid

IC50 - half maximal inhibitory concentration

L929- Mouse fibroblasts cell line

LDH – Lactate Dehydrogenase

MMC – Mitomycin C

MNBNC – Micronucleated binucleated cell

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

MWCNT – Multi-walled carbon nanotube(s)

NAC - N-acetylcysteine

NADPH – Nicotinamide Adenine Dinucleotide phosphate

NM – Nanomaterial(s)

OECD - Organization for Economic Co-operation and Development

PBS – Phosphate Buffered Saline

PMMA – Poly(methyl methacrylate)

PMMA-eud - Poly(methyl methacrylate)-eudragit

RI – Replication index

RPMI- Roswell Park Memorial Institute

ROS – Reactive oxygen species

SD – Standard deviation

SWCNT – Single-walled carbon nanotube(s)

TiO_2 – Titanium dioxide nanomaterials

1 INTRODUCTION

The term NM is defined by European Commission as "a natural material, accidently produced or manufactured that contains loose particles, aggregated or agglomerated, in which 50% or more of the particles present in the particle size distribution, have one or more external dimensions in the range 1nm-100nm" (Comission, 2011). However, other international authorities consider broader ranges of sizes for NMs and have released other definitions (SCENIHR, 2010).

NMs can have several origins: natural, resulting from volcanic eruptions and fires or produced by viruses; or anthropogenic if they originated from human activities, such as refining processes, automobile combustion and food preparation. NMs can also be synthesized deliberately by Man with a specific purpose, being then called manufactured NMs (Louro and Borges, 2013).

The NMs have distinct and attractive structural properties, such as small size and increased surface area relatively to volume and, increased number of atoms/molecules in the surface (Louro and Borges, 2013). These characteristics change the reactivity of the NMs, improve magnetic, optical and mechanical properties relatively to materials with larger dimensions, but with the same physicochemical composition (Oberdorster, 2010). Due to these characteristics, NMs have been recently used in several areas. In fact, due to the fast growth of the human population and the increase in the consumption of products, the need to improve the quantity and quality of new technologies arises. One of this technology was the development and large scale production of NMs (Louro and Borges, 2013). These have been used in the last decade in many different fields, such as pharmaceuticals, cosmetics, chemistry, computer engineering, food, paints, electronics, sports, and biomedical applications and imaging (Mittal and Pandey, 2014). For example, the colorless sunscreens contain insoluble titanium dioxide and zinc oxide nanoparticles. These sunscreens filter UV light more efficiently than microsized (>100 nm) particles. Furthermore, these particles when combined with organic UV filters has a synergistic effect of UYV scattering (particles) with the UV-absorption (organic UV filters), which permitted the development sunscreen with high (>30) sun protection factors (Nohynek and Dufour, 2012). As reported by Smolkova *et al.*, the synthetic amorphous silica (E551) has been used for many years to clear beer and wines, as an anti-caking agent. The titanium dioxide is used as an additive is categorized as E171. This additive is used as white colorant, more

specifically as white-colored sauces and dressings, and non-dairy creamers (Smolkova *et al*., 2015). On the other hand, the NMs can be used in nanomedicine, for example in drug delivery; poly(methyl methacrylate) is used to deliver antibiotics locally that have application in prevention or treatment in orthopedic infections (Bettencourt and Almeida, 2014).

The NMs have elicited more interest by scientists because their health effects are not well known. A major concern is that their specific physicochemical characteristics can lead to genotoxic effects such as an increase in the development of cancer (Andujar *et al*., 2011). As a result, more studies need to be performed, in order to be able to ensure a safe application of NMs during all of their life cycle, and to protect the environment (Louro and Borges, 2013).

1.1 NANOTOXICOLOGY: THE TOXICITY OF THE NANOMATERIALS

As referred, the distinct structural properties of the NMs change their reactivity and this fact may have implications on their biological effects. Figure 1 shows the influence of NMs properties on several cellular processes and their biological effects.

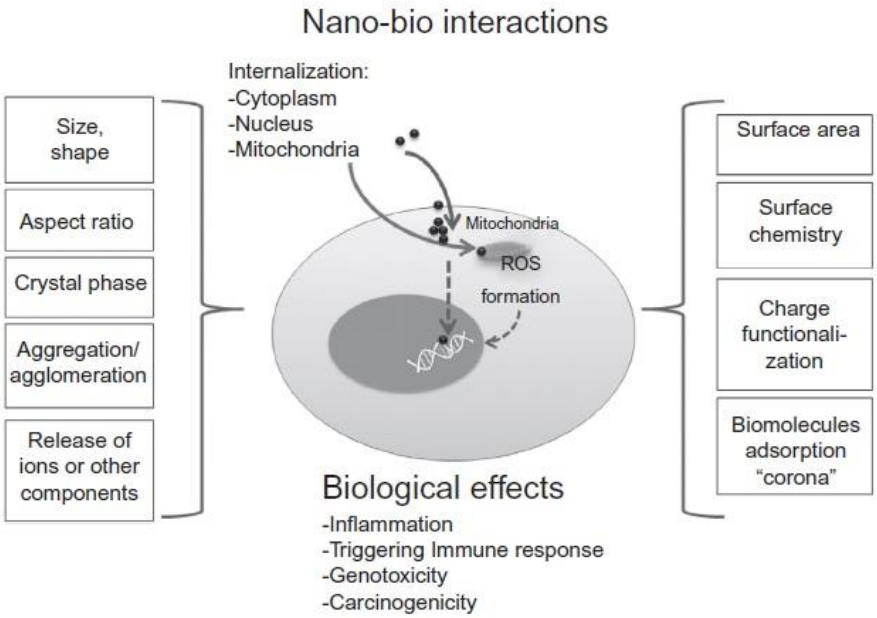

Figure 1. NMs properties and their biological effects (Louro *et al*., 2015).

Considering these specific characteristics, nanotoxicology has emerged as a recent area of toxicological science that investigate the adverse effects of NMs on living organisms and the

ecosystems. The *in vitro* and *in vivo* assays are used to identify the potential hazard and then to establish a dose-response relationship (when possible) following exposure to NMs (Oberdorster, 2010).

To understand the nanotoxicological potential it is necessary to know the characterization of each NM. It has been reported that within each group of closely related NMs, distinct biological effects (cytotoxicity and genotoxicity) occur in human cells (Tavares *et al*., 2014), suggesting the importance of testing each NM, instead of assuming a similar effect based on similar chemical composition.

Furthermore, in biological media, the surface of the NMs will get in contact with proteins and other biomolecules resulting in the formation of a dynamic protein corona whose composition varies over time due to continuous protein association and dissociation as well as changes in the environment (Louro *et al*., 2015)

The changes that occur in physicochemical properties of the NMs are very important. The aggregation or agglomeration are important factors to evaluate the toxicology of NMs. The aggregates consists of primary particles joined by strong chemical bond (covalent); the agglomerates involves the primary particles that are joint by van der Waals weak forces, their properties being strongly influenced by medium (in liquid or air) (Louro and Borges, 2013). The nanoparticles are able to interact with biomolecules such as proteins, nucleic acids, biological metabolites and lipids. Both in liquid medium and in air, it is possible to determine the actual size of nanoparticles, as well as the biological interactions or the deposition site (Oberdorster, 2010; Oberdörster *et al*., 2005).

Suspension of NM in a serum, cell culture or surfactant-coating vehicle is sometimes employed to assist disaggregation of the NMs. To reduce the aggregation/agglomeration and measure the size of NMs, a process using ultrasonication and immediate use of the NM has been established by NANOGENOTOX project (Jensen *et al*., 2011). In addition, to analyze the dispersion of NMs after the dispersion procedure, determining the average size of the particles, Dynamic Light Scattering (DLS) measurement can be used. The DLS methodology is used to measure the distribution of size of particles in suspension. It is based on the use of a light ray that focuses in suspension that contains the NMs. When the light focuses in the nanoparticles, variations occurs or “speckles” in the intensity of the scattered light. These are caused by differences in the phases of the waves scattered by different particles. Then, the variations of the intensity of the scattered light are measured through a small pinhole, and it is possible to

tell how fast the scattering particles are diffusing over a distance equal to the wavelength of scattered light (Boyd *et al*., 2011; Dhawan and Sharma, 2010).

In summary, when performing nanotoxicology studies it is essential to consider information regarding the physicochemical properties of the test NMs, but also on their behavior in the biological systems.

1.2 GENOTOXICITY OF THE NANOMATERIALS

Genotoxicity studies provide the estimation of different types of DNA damage after exposure to xenobiotics are important for risk assessment of potential carcinogens (Dobrzynska *et al*., 2014). It has been suggested that NMs can cause genotoxicity by direct interaction with DNA or indirectly by reactive oxygen species (ROS) induction or toxic ions released by soluble nanoparticles (Magdolenova *et al*., 2013). The majority of nanoparticles can cross cell membranes and some can even reach the nucleus by diffusion, or through the nuclear pores interacting directly with the DNA molecules or nuclear proteins. A study using carbon nanoparticles showed an interaction between these nanoparticles and DNA, using *Escherichia coli*. Carbon nanoparticles linked to single strain DNA, indicating that these nanoparticles could interfere with replication (Magdolenova *et al*., 2013).

When nanoparticles interact directly with DNA, they can cause genetic instability, contributing to the development of carcinogenic processes. Hypothetically, NMs penetration ability is higher than their non nanometric analogs, due to its small size, which allows them to cross cell membranes and thus reach the nucleus. In addition, the NMs that not have this ability to cross the nuclear membrane, may interact with the same nuclear DNA and proteins during the mitotic process, causing structural chromosome damage (chromosome breaks, clastogenic activity) and numerical chromosome loss (aneugenic activity) (Magdolenova *et al*., 2013). This happens by the interaction of the nanoparticles with the mitotic spindle, centrioles or associated proteins. Huang *et al*. demonstrated that TiO_2 have the ability to affect any function of the mitotic apparatus, leading to loss or gain of chromosomes in daughter cells (Huang *et al*., 2009).

Furthermore, damage to DNA bases can occur such as modification (oxidation) adducts in DNA, double strand breakage, crosslinks or structural changes (Magdolenova *et al*., 2013).

The most investigated type lesion at the nuclear level is the DNA oxidation, due to the fact that in many studies, an increase in the production of ROS following exposure to NMs has been observed. The main product resulting from this oxidation is 8-oxoguanine (8-OxoG)

produced during oxidative stress, which is highly mutagenic and consequently a potential carcinogen. NMs can also interact with proteins involved in processed such as DNA replication, transcription or repair (Magdolenova *et al*., 2013).

Indirect interactions with DNA, for example, NMs deposition in tissues can lead to the recruitment of neutrophils and macrophages to the site of contact, causing inflammatory response. This inflammatory response causes oxidative stress in cells, leading to the production of ROS; this reaction may in turn, cause changes in the genome of adjacent cells, producing secondary genotoxic effects. ROS may interact with DNA, causing breaks or lesions to purines and pyrimidines. These lesions may lead to mutations due to incorrect pairing during replication, being a potential carcinogen (Magdolenova *et al*., 2013).

In situations where the inflammation is chronic, the genotoxic stress will be lasting, resulting in an accumulation of genetic changes that facilitate the process of cell transformation leading to malignant phenotype. However, NMs do not necessarily cause an inflammatory response. In the absence of this, NMs can induce genotoxic effects primarily through interaction with cellular components, such as mitochondria (inducing the formation of ROS) and NADPH oxidases linked to the cell membrane, or even across the depletion of oxidants (Louro and Borges, 2013).

To evaluate the effects of the NMs, both *in vitro* and *in vivo* assays are performed, requiring the collection of the NMs most relevant physical and chemical characteristics (Louro and Borges, 2013). As described in the previous section (nanotoxicology), this information is critical when choosing which studies and routes of administration are to be used.

The great diversity and nonconformity of results between the various genotoxicity studies done to date, is due to several features such as the origin of NM, the method of preparation, the protocols used, the experimental conditions (physical and chemical specifications such as pH, temperature, presence of impurities or irradiation), the treatment regimen, the type of cell line or animal model that is used, the concentration and the exposure time (Shukla *et al*., 2011). An important aspect to retain is the adsorption of proteins on the nanoparticle surface and this complex nanoparticle-protein is commonly designated as the nanoparticle-protein corona. This complex can influence the biological reactivity of the nanoparticles. The corona is formed due to a multifactorial process and not only depends on the characteristics of nanoparticles, but also on the interacting proteins and the medium. For example, the pre-coating of pulmonary surfactant proteins has been shown to influence the

subsequent adsorption of plasma proteins on the surface of multi-walled carbon nanotubes (Saptarshi *et al*., 2013).

Due to the lack of agreement among the several studies, there has been a combined effort by international organizations such as the Center for Disease Control (CDC), the Organization for Economic Co-operation and Development (OECD) and the European Union (EU), in order to promote projects and working groups focused on ensuring the correct use of NMs (Louro and Borges, 2013). Thus, the area of nanotoxicology has been developed, as an important component of the field of public health in order to assess the adverse effects of NMs on the human body and the environment (Oberdorster, 2010). OECD recommended a battery of assays to support regulatory approval of pharmaceutical and chemical compounds, but to the assays for testing NMs are not yet well defined. Due to physicochemical characteristics of NMs, some assays that are commonly used to test genotoxicity have been changed, because the increased reactivity of NMs may potentially increase the probability of interactions and interference with these assays (OECD, 2014).

Among the most commonly used tests to investigate the genotoxicity of NMs are the *in vitro* Mammalian Cell Micronucleus Test (OECD, 2010b) and the comet assay (Landsiedel *et al*., 2009).

The micronucleus consists in nuclear material deriving from the total or partial loss of chromosomes. At the telophase, a nuclear envelope forms around the chromosomes and their fragments, which then assume a morphology similar to, but smaller than the major nuclei; this structure is designated micronucleus and provides information regarding both break of chromosomes as the loss thereof. There is also the possibility that nucleoplasmatic bridges are formed between the nuclei of a binucleated cell. This is probably due to the formation of dicentric chromosomes: the two centromeres are pulled to opposite poles of the cell, resulting in bridges covered by the nuclear membrane (Fenech, 2000). An increase in the frequency of micronucleated cells after exposure to a chemical, as compared with the basal frequency of micronucleated cells in unexposed control cells is an indicative of a genotoxic effect. Furthermore, an increased risk of cancer development has been related to a higher frequency of micronucleated cells (Bonassi *et al*., 2011).

Although the micronucleus assay is commonly recommended for genotoxicity testing, it has been discussed if it could be applied to NMs. Due to some factors, the NMs can interfere with this assay so, some adaptations have been done in the micronucleus assay. The

cytochalasin B is a chemical agent that has the effect to block the cytokinesis, because it inhibits actin polymerization that is required for the formation of the microfilament ring, which constricts the cytoplasm and prevents separation the daughter nuclei cells after mitosis, forming binucleated cells. The majority of binucleated cells can show one, or more micronuclei indicating losses or breakage of chromosomes (OECD, 2010b; Fenech, 2000). In addition, some authors reported that cytochalasin B also blocks the endocytosis, inhibiting the uptake of nanomaterials into the cells, and due to this fact, the cells were incubated with NMs 6 hours before addition of cytochalasin B to ensure their uptake (Magdolenova *et al*., 2012). OECD recommend that the chemicals that are being studied are removed after cytochalasin B addition; with NMs it is not possible to do this, because NMs remain adsorbed to cells after being washes (Magdolenova *et al*., 2012)

To evaluate genotoxicity at DNA level, a straightforward methodology use the single-cell gel-electrophoresis assay, or comet assay; this method represents a technically simple, relatively cheap, fast and sensitive technique and can be applied to virtually all cell types without the need of cell culture (Collins *et al*., 2008). It detects single and double strand breaks in DNA (Tice *et al*., 2000); additionally it may be modified to detect oxidative damage of bases and even DNA repair (Collins *et al*., 2008). In the comet assay, the cells are embedded in agarose on a glass slide, and are lysed to remove membranes and soluble components, leaving DNA attached to the nuclear matrix, designated “nucleoid”. Electrophoresis (a very alkaline solution) causes DNA loops containing breaks to extend toward the anode as a “comet tail”. The percentage of DNA in the tail is directly related with the frequency of DNA breaks (Louro *et al*., 2015). In order to determine the presence of oxidative DNA lesions, specific bacterial enzymes are used in the modified comet assay, such as endonuclease-III (Endo-III) and formamidopyrimidine-DNA-glycosylase (FPG) (Collins *et al*., 2008). The FPG allows the conversion of oxidative lesions in strand breaks that are detected with the comet assay, increasing its sensitivity. However, some authors state that the NMs can interfere with the comet assay (Magdolenova *et al*., 2012; Stone *et al*., 2009).

Cytotoxicity may interfere with the outcome of the genotoxicity studies since it can mislead interpretation of the results of the comet or micronucleus assay. For that reason, it is generally recommended to perform cytotoxicity assays not only to complement information from genotoxicity studies, but also to define the dose-range to investigate.

1.3 NANOMATERIALSS FOR MEDICAL APPLICATIONS (PMMA AND PMMA-EUDRAGIT NMS)

As mentioned above, the pharmaceutical area has invested in the development of new NMs.

In the latest years, polymeric nanoparticles have been widely used in the therapeutic and diagnostic areas, and their biomedical impact depends on their size, surface and composition. These particles have a great value in drug delivery because they "are biocompatibile, present colloidal stability in physiological medium and have the ability to encapsulate active agents, targeting specific cells or tissues" (Juneja and Roy, 2014).

Poly(methyl methacrylate) (PMMA) is a manufactured and biocompatible polymer and is very hydrophobic, but becomes less hydrophobic when in contact with water. It "is a non-biodegradable synthetic homopolymer of methylmethacrylate monomer (MMA)" (Figure 2) (Bettencourt and Almeida, 2014).

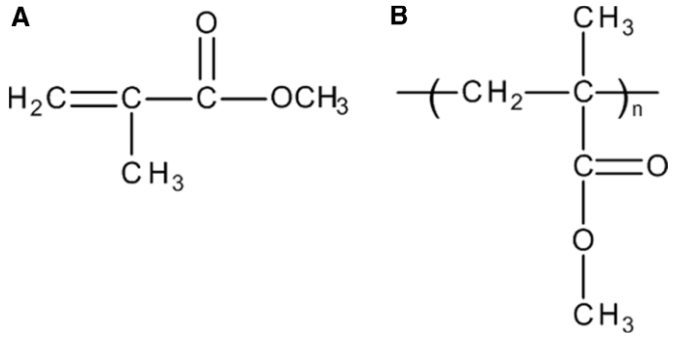

Figure 2. Chemical structure of (A) MMA monomer and (B) PMMA monomer (Bettencourt and Almeida, 2014)

PMMA began being used as a particulate transporter material and in the development of nanoparticles for vaccination (Bettencourt and Almeida, 2012; Juneja and Roy, 2014). Currently, there are other biomedical applications for PMMA, as a permanent implant for intraocular lens subsequent cataract surgery and prosthetic material in dental and mandibular corrections (Juneja and Roy, 2014). In addition, PMMA is used as a transporter for local delivery of antibiotics to local infections (Bettencourt and Almeida, 2014). Furthermore, particles of PMMA can carry many drugs, such as anti-inflammatory, antioxidants, antihypertensive, antidiabetics, anti-histamines and antibiotics (Bettencourt and Almeida, 2012).

It has been shown that the release of the drugs through the PMMA particles is not complete. This may be due to the hydrophobic character of these particles, as well as the fact

that PMMA have a low porosity, which in turn difficult the diffusion of water into the matrix (Bettencourt and Almeida, 2014). Thus several strategies have been developed to try to overcome this problem; one of these strategies was mixture two polymers, for example powders PMMA and Eudragit RL 100 (eud). This polymer has the capacity to increase its volume (swelling) and in physiologic pH values is insoluble, becoming a good polymer for a better drug release from PMMA (Bettencourt and Almeida, 2012; Ferreira *et al*., 2015).

Eudragit is a poly(meth)acrylate and is used as a pharmaceutical excipient. Blends of PMMA-eud have a greater potential as carrier materials than PMMA NMs alone, thus will allowed a higher release of drug from the NMs. Therefore, PMMA-eud seems to be promising NM to be used in the biomedical area. However, before such application its safety should be analyzed, according to the guidelines of international Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human use (European Medicines Agency, 1998; ICH, 1995) and of WHO/IPCS (Eastmond *et al*., 2009).

The characterization of PMMA and PMMA-eud (constituted by 70% of PMMA and 30% of eud) used in present study involved several parameters such as particles size distribution, surface morphology, surface charge evaluation, hydrophobicity and chemical composition, has been describe elsewhere (Graça, 2014) and is presented in Materials and Methods section.

It is very important to consider biocompatibility in order to guarantee the safe use of PMMA (Caputo *et al*., 2009). Some studies revealed possible inflammatory reactions of PMMA when these are applied in dental and ocular areas, and this is due either to the fact that PMMA is non-biodegradable or to the release of non-polymerization additives (Bettencourt and Almeida, 2014). “Therefore, as referred in Bettencourt and Almeida, strategies were developed so that there biocompatibility of these particles with the host, for example, to suppress damage caused by free radical, adding antioxidant aminoacid derivate as N-acetylcysteine (NAC)” (Bettencourt and Almeida, 2014).

PMMA particles have been used for drug delivery since the 60s, so it is often found in the human body and the issue on what happens to PMMA-engineered particles needs more scientific studies.

Some concern exists regarding these polymers’ slow biodegradability, which can cause effects due to accumulation in chronic treatments. The interaction of PMMA particles with cells and the extracellular environment can generate a sequence of biological effects considerably

different from the material in the macro size form and even when evaluated as debris resulting from orthopedic procedures (Bettencourt and Almeida, 2014).

As reviewed by Bettencourt and Almeida (2014) many studies have been done both *in vivo* as *in vitro* and so far, the majority has been satisfactory when evaluating the cytotoxic and genotoxic effects. Kreuter and Speiser (1976), did not find histological abnormalities at the injection site in guinea pigs, one year after intramuscular injection of PMMA nonparticle-containing influenza vaccine. PMMA-chitosan microspheres were hemocompatible and non-cytotoxic to mouse fibroblasts cells (Bettencourt and Almeida, 2014). Also, no cytotoxic effect was observed in human leukemic cells when PMMA nanoparticles obtained by mini emulsion polymerization technique with aim to encapsulation of antitumor agents were tested (Bettencourt and Almeida, 2014).

The capacity of NMs to cross the cellular membrane, is an important factor that contributes to their toxicity. Studies done in male albino rats shown that nanosized particles can cross small intestine by per absorption and further can be distributed into the blood, brain, lung, heart, kidney, spleen, liver, intestine and stomach (Hillye and Abrecht, 2001).

Once inside the cell, the NMs can induced the ROS production. A study done by Hazra and his colleagues verified that PMMA induce DNA damage in Gram-positive bacterial cells. Also, confirmed that PMMAs nanoparticles are internalized by bacterial cells and induce a significant stress oxidative that can lead to genotoxicity and cytotoxicity in *B. subtilis* (Hazra *et al*., 2014). In addition, a study, that used micronucleus assay in human peripheral blood lymphocytes comparing PMMA, PMMA + MMA, PMMA + MMA + HA (hydroxyapatite) and metallic materials (namely, Ti), revealed that PMMA exhibited more cytotoxicity, and reveled a highest percentage in relation to all other tested compounds. This study also confirmed that the surface properties are directly related to cell proliferation, differentiation and apoptosis. Other study done by Bigatti *et al.* (1994), using also micronucleus assay, verified that PMMA induced a highly significant increase in micronuclei frequency using human lymphocytes.

Some preliminary data has been published in respect to genotoxicity of PMMA-eud as described in Graça (2014).

1.4 MANUFACTURED NANOMATERIALS USED IN CONSUMER PRODUCTS

1.4.1 Titanium dioxide Nanomaterials

In recent years titanium dioxide NMs (TiO_2) has been used in industrial and consumer products.

TiO_2 is a white pigment and due of its high stability, anticorrosiveness and photocatalytic properties, in addition to low solubility, can be used in paints, coatings and plastics, as well as in areas such as medicine (as a component for articulating prosthetic implants), pharmaceutics, food and cosmetics (essentially in toothpastes and sunscreens) (Aueviriyavit *et al*., 2012; Olmedo *et al*., 2008; Shi *et al*., 2013). Furthermore, TiO_2 can contribute to the bioactivity of implant interfaces and enhanced cell adhesion (Louro *et al*., 2015; Shi *et al*., 2013). It can be used in catalytic reactions, such as semiconductor photocatalysis, in the treatment of water contaminated with hazardous industrial by-products, and in nanocrystalline solar cells as a photoactive material (Karlsson *et al*., 2008).

There are two crystalline forms of TiO_2 in nature that are mainly used in human consumer products: rutile and anatase (figure 3). Some studies suggested that the crystalline form of anatase has a greater toxic potential (Shi *et al*., 2013).

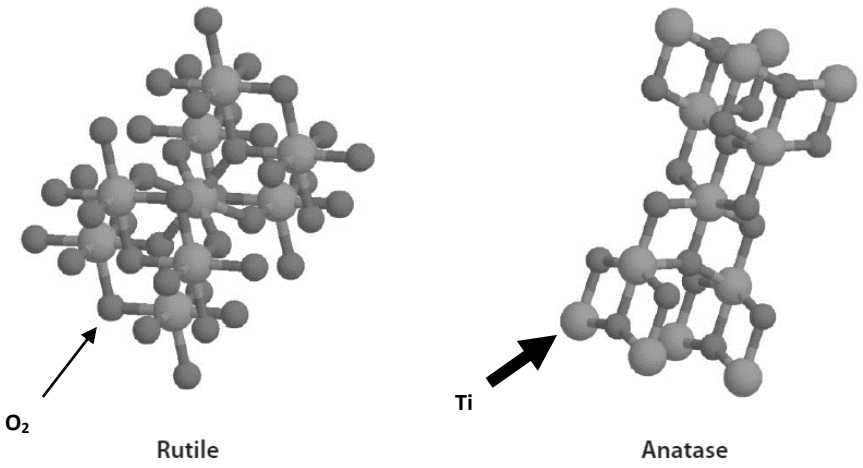

Figure 3. Rutile and anatase crystalline structures ((NIOSH), 2011)

TiO_2 is a crystalline, solid, odorless powder and nanocombustible. It is also insoluble in an aqueous medium or alcohol, and it is soluble in hot concentrated sulfuric acid or alkali ((NIOSH), 2011; Shi *et al*., 2013).

The presence of impurities in NMs is an aspect that can determine the toxicity, as well as the presence of coatings, catalysts, specific surface area and aspect ratio. The composition of TiO_2 was analyzed by semi-quantitative energy dispersive X-ray spectroscopy (EDS) (Nanogenotox Joint Action, 2013).

TiO_2 can enter the human body through several potential routes, such as inhalation, ingestion and skin contact. For this reason, many studies have been done about genotoxicity of this NM. A study, using micronucleus assay in human lymphocytes revealed a significant increase in the frequency of micronucleus in binucleated cells in some types of TiO_2 that were tested (Tavares *et al*., 2014).

As mentioned above, the TiO_2 is used in paints and these nanoparticles can be inhaled so, it is important evaluated the genotoxicity in respiratory cells. A study done by Aueviriyavit *et al.* (2012), using A549 cells shown that TiO_2 both the anatase and rutile forms significantly increased the intracellular ROS level at concentration of 100 µg/mL. Other study, verified a genotoxic potential of TiO_2 in comet and micronucleus assay using BEAS-2B cells (Prasad *et al*., 2014). On the other hand, a study *in vivo*, using comet assay, do not verified an increased in the percentage of DNA tail in mouse Crl: CD (SD) lung cells after 3 and 24 hours exposure (Naya *et al*., 2012). In other study, A549 cells were exposed to several types of TiO_2, and all of these TiO_2 revealed a significant increase in the level of DNA breaks 4 hours after exposure. This level increase more 24 hours after exposure but this time only in some TiO_2 using comet assay. In the same study also evaluated the number of micronuclei frequency and did not observed an increase these (Jugan *et al*., 2012).

As the TiO_2 is used in some cosmetic products and sunscreens, so the dermal adsorption of TiO_2 NMs have an interest in the evaluated to the genotoxicity of this NM. It is important worth noting that the majority of cosmetics and sunscreens that containing TiO_2 are normally used in intact skin. Thus, skin penetration studies of TiO_2 are usually investigated *in vivo* and *in vitro* both intact skin (Shi *et al*., 2013). Shi *et al.* (2013), concluded that TiO_2 NMs did not penetrate the intact human skin. Other authors verified various size s of TiO_2 cannot penetrate through skin cells 24 hours after exposure in porcine skin, but 30 days exposure could penetrate through the horny layer on pig ear (Wu *et al*., 2009). Reeves *et al.* (2008), tested the genotoxicity of TiO_2 in GFSk-S1 cells (primary cell line developed from the skin of goldfish) using comet assay. They verified a significant increases in oxidative DNA damage in modified comet assay in all doses.

Some workers can be exposed by inhalation to NMs, because of this, it is important to evaluate the exposition of these workers to TiO_2, once this NM can be found in paints, as mentioned above. Pelclova *et al.* (2015), found anatase and rutile TiO_2 particles in workers that were exposed for their work.

As can be observed in several studies mentioned above, discrepancies exist, so it is necessary to continue to investigate the risk assessment of NMs.

1.4.2 Multi-walled carbon nanotubes

Carbon nanotubes (CNTs) have been used in ever increasing amounts for several industrial applications, as batteries, biotechnology, clothing and as organic materials for tissue engineering applications. They offering good choices for scaffold fabrication and delivering of siRNA and DNA, oligonucleotides and proteins into cancer cells because they are able to cross cell membranes by endocytosis and thus, may have a potential application in chemotherapy (Liu *et al*., 2012; Louro *et al*., 2015; Migliore *et al*., 2010; Nymark *et al*., 2014; Zhao and Liu, 2012).

CNTs are formed by graphene layers and the composition may vary from one to one hundred cylindrical tubes. Considering this constitution, CNTs can have different names: single-walled carbon nanotubes (SWCNTs) are constituted only by one graphene layer, while multi-walled carbon nanotubes (MWCNTs) contain more than one graphene layer (figure 4). The CNTs structure is endowed with very advantageous chemical, physical and mechanical features, due their low density, extraordinary conductivity, high ductility and mechanical strength (Migliore *et al*., 2010; Zhao and Liu, 2012).

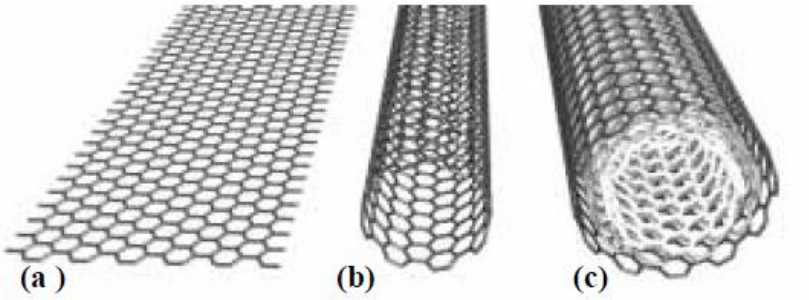

Figure 4. Representation of Carbon nanotubes. (A) Graphene sheets; (B) Single-walled carbon nanotube; (C) Multi-walled carbon nanotube (Kreupl *et al*., 2004).

CNTs are practically insoluble in any solvent, including in biological fluids. Their insolubility not only severely hinders research on their chemical properties, but significantly

restricts the applications in every field. Therefore, many procedures have been developed to modify their chemical properties and to increase the solubility of these NMs (Lam *et al*., 2006; Zhao and Liu, 2012)

It has been suggested that because the CNTs have high aspect ratio (i.e., ratio between length and diameter), they, may be able to induce lung cancer and mesothelioma in as asbestos do. Studies *in vivo* have been shown that both MWCNTs and SWCNTs can induce oxidative stress, inflammation, fibrosis and granulomas (Lam *et al*., 2006; Lindberg *et al*., 2009).

The most probable route of exposure to CNTs is inhalation, due the use of these NMs in the industry field, it is important identify if this NMs could act as lung carcinogens (Kisin *et al*., 2007). Lindberg *et al.*, evaluated the genotoxicity of CNTs using comet and micronucleus assays in BEAS-2B. This author verified that 24 hours after exposure to CNTs, these can induce a dose-dependent increase in DNA damage; in contrast, the micronucleus assay not revealed a significantly increased in the micronuclei frequency. These last results may be due the increased size of the agglomerates at higher concentrations levels (Lindberg *et al*., 2009). In a study using the V79 cells (Chinese hamster lung fibroblast) no increase in the micronuclei frequency was observed in any concentration after 24 hours exposure to SWCNTs; the author suggested that this result may be due a low degree of SWCNT uptake by V79 cells. On the other hand, in the same assay it can be observed a significantly increase in the percentage of DNA in tail length in concentration-dependent (Kisin *et al*., 2007). Guo *et al.* (2011), evaluated the genotoxic effect of MWCNTs using human umbilical vein endothelial cells, and verified an increase in DNA damage and cause apoptosis(Guo *et al*., 2011). Studies *in vivo* using mice models, detected that MWCNTs can persist in the lungs and produced an inflammatory response, fibrosis and granulomas formation (Ma-Hock *et al*., 2009; Muller *et al*., 2005).

As mentioned above, the genotoxic effects may be primary or secondary. The secondary genotoxicity response may be due the induction of inflammation accompanied by oxidative stress, leading to DNA damage. Some authors demonstrated that carbon black particles have a secondary genotoxic effect lead a chronic inflammation followed by ROS production leading to DNA damage (Kisin *et al*., 2007). A study done by Jacobsen *et al.* (2008), reported that a significant increase of ROS production in FE1 Muta^{TM} mouse epithelial cell line exposed to SWCNTs, but this production of ROS do not verified at high concentrations. These results may be due to the agglomeration of SWCNTs. Furthermore, in the same study, the researchers measure the DNA damage using FPG enzyme and they not verified an induction of strand breaks.

All of these results revealed that it is necessary to keep studying the safety of NM, due to the disagreement that exists. In each study, it is necessary have knowledge about the physicochemical properties of each NM.

2. OBJETIVES

This project aims to contribute to the safety evaluation of nanomaterials that are used or being developed for human applications, using well-characterized NMs and standardized procedures for NM preparation and for the investigation of their toxic effects.

The specific aims of this thesis were to use *in vitro* methodologies for:

i) Evaluation of genotoxic effects of poly(methyl methacrylate) and poly(methyl methacrylate)-Eudragit, that are under development to be used as drug delivery carriers for human medicine.

ii) Analysis of the cytotoxic and genotoxic potential of a titanium dioxide NM, as well as a multi-walled carbon nanotube, both manufactured NMs used in cosmetics and industrial applications.

3 MATERIALS AND METHODS

In toxicology to obtain the results in a short period of time and to reduce the number of animal tests necessary, it is usually use in vitro assays in order to evaluate the toxicity of many agents, including the nanomaterials. Also, *in vitro* testing has a relatively lower cost, as well as simplicity to perform, control and interpret the results, when compared with *in vivo* tests (Stone *et al* ., 2009). However, in *in vitro* assays it is not possible to fully replicate the complex interaction that occur between multiple cell types *in vivo*, both within an organ and also between organs; furthermore cell culture it is cannot be used to identify the targets of exposure within the body (Stone *et al*., 2009). The choice of each cell line should be selected according the aim of the study and the characteristics of the cells when growing in culture medium can influence their susceptibility to the chemicals or, in this case, to the particles, as their metabolism may be altered due to the changes of medium or to cell density (Stone *et al*., 2009).

The evaluation of the genotoxicity of PMMA and PMMA-eud was performed on the cell line L929, obtained by American Type culture Collection (ATCC® CCL-1™).

This cell line was isolated from mouse fibroblasts (*Mus musculus*) of a 100 days male. This cell line had an adherent property (figure 5) (ATCC, 2015). L929 fibroblasts were chosen because are a model usually used in the biocompatibility studies of biomaterials as it is recommended by the ISO 10993-5 (“Biological evaluation of medical devices – Part 5: Tests for *in vitro* cytotoxicity”).

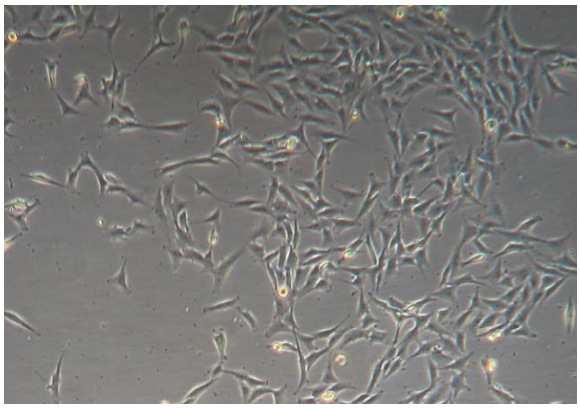

Figure 5. L929 cells in RPMI-1640 culture medium

The growth medium used for L929 cell cultures was RPMI 1640 culture medium supplemented with 10% Fetal Bovine Serum and 1% Pen/Strep. When the cells reached about 75% confluence, subculture was performed: the medium was removed and the cells were washed with trypsin-EDTA, trypsin-EDTA was added to the flask and incubated for 5 minutes at 37^{o}C. When the cells were detached from the flask, fresh culture medium was added to inactivate the trypsin-EDTA and subsequently transferred to new flasks (25 cm^{2}) at a tenth of its original volume and incubated in the same conditions as before. All of these reagents were provided by Gibco (Scotland, UK).

To evaluate the cytotoxicity and genotoxicity of TiO2 and MWCNTs the cell line A549 (figure 6) from Bundesanstalt für Arbeitsschutz und Arbeitsmedizin (BAuA, in English, Federal Institute for Occupational Safety and Health; Berlin) was used.

This cell line it was isolated from a human lung carcinoma epithelial (BAuA, 2015) and have important molecules active in the detoxification of the cells, such as P450 cytocrome, allowing the incorporation of metabolic pathways in the pulmonary epithelium (Foster *et al.*, 1998).

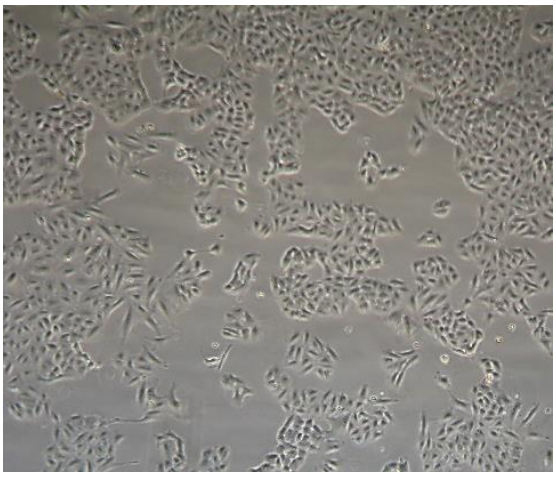

Figure 6. A549 cells in DMEM culture medium.

The growth medium used for the cell cultures was DMEM (with stable glutamine), supplemented with 10% heat-inactivated Hyclone Fetal Bovine Serum, 1% Pen/Strep, 1% Fungizone and 2.5% HEPES. When cells reached about 80% confluence, a subculture was performed: the culture medium was removed, the cells were washed twice with warm 4 mL PBS, and then incubated for 5 minutes in an incubator at 37ºC with trypsin-EDTA (0.05%). When the cells were detached from the flask, viable cells were counted and seeded at the density of 1x10^{6} cells/ flasks (75 cm^{2}) with warm culture medium. All of these reagents were provided by Gibco

(Scotland, UK), except the Hyclone Fetal Bovine Serum (FBS), provided by Thermo Scientific (Waltham, MA, USA).

3.1 NANOMATERIALS PREPARATION

In context of the project of "Biological Effects of Acrylic Engineered Particulate-Systems" (research project EXCL/CTM-NAN/0166/2012). in collaboration with Faculdade de Farmácia, two different nanomaterials were studied: PMMA and PMMA-eudragit. These particles were prepared using the method SESE, as was mentioned above, and were tested in parallel in L929 cells. The characteristics evaluated in the previous work can be seen in the table 1:

Table 1. PMMA and PMMA-eud characteristics (Graça, 2014).

		PMMA (nm)	**PMMA-Eudragit (nm)**
Size (mean ± SD)		572 ± 20	508.9 ± 8
morphology		spherical	spherical
Surface charge (mean ± SD)		-32.7 ± 1.04	+31.8 ± 1.66
Hydrophobicity*	**Sepharose – FF (%)**	16 ± 2.7	20.3 ± 2.9
	Butyl Sepharose – FF (%)	27.1 ± 0.4	84.5 ± 4.4
	Octyl Sepharose – FF (%)	17.2 ± 1.5	78.5 ± 3.5

*Hydrophobicity assay results as a percentage of sample retention in the resins sorted by increasing hydrophobicity

The PMMA and PMMA-eud white powders were weighed in a precision scale inside a glass scintillation vial. Then, the stock particle dispersion were obtained with a sterile H_2O in a final concentration of 20 mg/mL, since these NMs are partially soluble. Careful homogenization through and sample inversion was performed until no aggregation was visually detected. PMMA and PMMA-eud stock dispersions were prepared immediately before use. For both nanoparticles the same concentration were used: 0.1, 0.5, 1, 2 and 5 mg/mL. The two highest concentrations were diluted from stock solution. The samples ate the concentration of 1 mg/mL was prepared from dilution of the samples at the concentration of 2 mg/mL and the two lower

concentrations were prepared by diluting the 1mg/mL sample (showed in figure 7). All concentrations were prepared in culture medium.

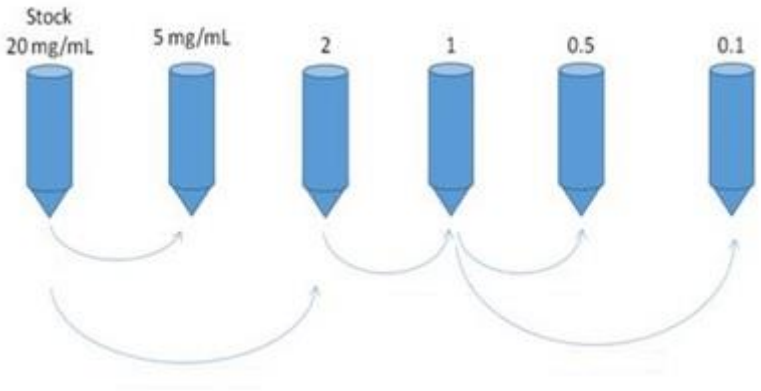

Figure 7. Preparation of NM dilutions for cell exposure (Graça, 2014)

In the project NANoREG, two different manufactured nanomaterials were analyzed: TiO_2 labeled (NM-1001) and MWCNTs (NM-4000). These NMs were produced, characterized and provided by the Joint Research Centre Repository (Institute for Health and Consumer Protection, European Commission, Ispra, Italy).

Both NM-1001 and NM-4000 were tested in A549 cells.

The NM-1001 (or NM-101) is poorly soluble in aqueous media and is has a form of white powder. The physicochemical characteristics of this nanomaterial it was already been studied in NANOGENOTOX Joint Action ("*Safety Evaluation of Manufactured Nanomaterials by Characterization of their Potential Genotoxic Hazard*") and are show in the table 2:

Table2: Geometric mean Feret's minimum and maximum diameter and aspect ratio of primary particles and boundaries of typical aggregate and agglomerate size for NM-1001 nanoparticles (JRC, 2014b).

NM	Phase	Impurities / coatings	Specific surface	Primary particles			Aggregates/Agglomerates			
				Feret min ± SD	Feret max ± SD	Aspect ratio ± SD	A*	25%	median	75%
NM-1001	anatase	Al, Na, P, S, Zr	169.5 ± 8.5	25.7 ± 22.5	38.8 ± 33.9	1.52 ± 0.33	1802	14.1	22.6	45.8

* Particles that measurement.

The NM-4000 (or NM-400), had already been studied genotoxicity in NANOGENOTOX project, using lymphocytes and do not present any cytotoxicity or genotoxicity. It is an insoluble NM shown a black powder before dispersion, and their physicochemical characteristics were analyzed by JRC and are represented in the following table:

Table 3: Geometric mean thickness, geodesic length and aspect ratio of multi-walled carbon nanotubes (JRC, 2014a)**.**

MWCNTs	Specific surfasse área (m^2/g)	Tickness ± SD (nm)	Geodesic lenght ± SD (nm)	Aspect ratio ± SD	N[c]
NM-4000	254	11 ± 3	846 ± 446	79 ± 50	20

[c] Number of measured primary particles.

Since these NMs are insoluble, their preparation in liquid medium for cell exposure involves the dispersion instead of dissolution (OECD- ORGANIZATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT – Guidance on sample preparation and dosimetry for the safety testing of manufactured nanomaterials. In the NMs dispersions ultrasonication may contribute to produce stable dispersions.

Therefore, to disperse large agglomerate and aggregates of TiO_2 and MWCNTs it was necessary to proceed a pre-sonication after pre-wetting the NM with 0.5% ethanol and suspension in sterile-filtered 0.05% wt% Bovine Serum Albumin (BSA; 95% of the volume of the final solution; from Sigma Aldrich, St Louis, MO, USA). The standardized protocol of NANOGENOTOX project for the dispersion of the nanomaterials was used (NANOGENOTOX). A precision scale was used to weigh the nanomaterials, inside a glass scintillation vial; and a 2.56 mg/mL (stock dispersion was prepared by prewetting powder with 96% ethanol (0.5% of the volume of the final solution), followed by addition of sterile-filtered 0.05 wt% BSA. This stock was selected based on the dispersibility of the nanomaterials (Jensen *et al*., 2011; Tavares *et al*., 2014).

The vial with nanomaterials dispersion was placed in an insulated box and partly submerged in ice; was sonicated at 400 W with 10% of amplitude for 16 minutes using a Branson Sonifier S-450D with 13 mm disruptor horn (Branson Ultrasonics Corporation, Danbury, USA).

The batch dispersion prepared in BSA/water was used to prepare a working solution at 0.64 mg/mL and then diluted in appropriated amount of complete cell medium for exposure of A549 cells. The two highest test concentrations were diluted directly from batch dispersion at 2.56 mg/mL.

3.2 CELLS EXPOSURE

In all assays were used a negative control (cell culture incubated only with culture medium) and a positive control (in case of cell counting and comet assay, the Ethyl Methanesulfonate; EMS, and Mitomycin C; MMC, both from Sigma-Aldrich; St. Louis, MO, USA; it was used), to compare the concentrations that were tested.

In respect to the dose-range selection for PMMA and PMMA-eud, since the previous tests showed no relevant cytotoxicity up to the concentration of 2 mg/mL, it decided to proceed with an evaluation of toxicity on the maximum concentration recommended by (OECD, 2010a) guidelines, which chosen the highest concentration (5mg/mL).

3.3 CYTOTOXICITY ANALYSIS

The cytotoxicity assays are important to measure the impact on cell death after exposure to the test compounds, including NMs. These assays are usually the first tests before genotoxic assays to allow the determination of the dose-range, avoiding concentrations that yield high levels of toxicity that may mislead the results of genotoxicity testing. The cell proliferation and the number of viable cells in exposed cultures as compared with negative controls are usually the evaluated parameters.

The most commonly used colorimetric assays (neutral red uptake or MTT assay) are not feasible due to the interference of many NMs with the assay. Alternative methodologies include the cell counting assay, that it characterized by counting viable cells and non-viable cells by a dye (in this case Trypan Blue); clonogenic assay that evaluated the proliferative potential of cells, counting the form of colonies after exposure and CBPI and RI that analyzed the viability of cells using the cytokinesis-blocked proliferation and replication indexes.

The concentrations that were tested in this work were: 0, 1, 3, 10, 30 and 75 μg/cm^2 for NM-1001; 0, 8, 16, 32, 64 and 128 μg/cm^2 for NM-4000.

3.3.1 Cell counting assay with Trypan Blue dye

Trypan Blue is a dye that allows for the distinction between viable and non- viable cells, because this dye can enter the cells which membranes have been compromised, thus non-viable

cells become blue. Using the Neubauer chamber, the number of viable and non-viable cells is be counted. By comparing several concentrations and the negative control, we can determine the cytotoxicity of the tested particle (Collins *et al*., 2008a).

The cells were exposed to each nanomaterial concentrations for 24 hours. It was plated $0.5x10^5$ cells per well in a 24-well plate. Cells were exposed to positive control (EMS in a final concentration of 5mM and incubated for 1 hour before harvesting. Following the exposure period, cells were detached from the flasks, and a small volume of cell suspension was diluted 1:1 in Trypan blue dye, placed in Neubauer chamber and counted. Then, the result was multiplied by the dilution factor used and the cell concentration was obtained as cell/mL. The cell concentration obtained for each concentration of nanomaterials (or EMS) was compared with the negative control and the percentage of viability was determined.

3.3.2 Clonogenic assay

The clonogenic assay allows to evaluate the proliferative potential of cells, measuring the percentage of cells in population capable to form a colony after exposure to compounds (Herzog *et al*., 2007). The cytotoxicity of tested agents, or in this case, nanomaterials, is evaluated and calculated by comparing the number of cells plated initially with the number of colonies formed after treatment period, relative to the plating efficiency in unexposed controls. The clonogenic assay allows measure the effect of concentration of an agent on cell survival (Buch *et al*., 2012; Herzog *et al*., 2007; Longo-Sorbello *et al*., 2006).

In this thesis, it was only possible to perform this assay in A549 cells; L929 cells don't have the capacity to form colonies.

The A549 cells were plated in a density approximately 150 cells per well, in a 6-well plate and allowed to attach for 18 hours before exposure. The attachment period was shorter (18 hours) than the doubling time of the cells, in order to guarantee that the cells were attached but not divided ate the time of the treatment with nanomaterials. Then, the cells were exposed to the concentrations of the nanomaterials, mentioned above. The plates were then incubated for 8 days, at 37°C, with 5% CO_2. MMC was used as positive control at a concentration of 0.05 µg/mL and was incubated for 6 hours only and was removed after that period and replaced for culture medium.

After 8 days of exposure to the treatment, the cells were washed twice with PBS and fixed with absolute cold methanol (Merck; Darmstadt, Germany) for 10 minutes. Then, the

cells plates were dried and the colonies was stained with 10% Giemsa (Merck; Darmstadt, Germany) for 10 minutes, washed twice with Gurr's phosphate buffer and allowed to dry. The colonies were counted, and several parameters were analyzed, using the following equations (Buch *et al*., 2012):

$$\text{Plating Efficiency} = \frac{\text{Number of colonies in the negative control}}{\text{Number of cells plated in each well}}$$

$$\text{Surviving Fraction} = \frac{\text{Number of colonies exposed to the treatment}}{\text{Number of colonies in the negative control}}$$

$$\text{Cytotoxicity} = 100 - (\text{Surviving Fraction} \times 100)$$

3.3.3 Proliferation and replication indexes

During the micronucleus assay, the viability of the cell lines exposed to the several nanomaterials were analyzed using the cytokinesis-blocked proliferation index (CBPI) and the replication index (RI), based on the proportion of mon-, bi- and multinucleated cells. These will be further explained bellow.

3.4 GENOTOXICITY

In this work two genotoxicity assays were used: the comet assay, for detection of breaks of DNA, and the micronucleus assay, that allows detection of chromosomal breaks, or full chromosomes unable to approach to the poles during mitosis.

3.4.1 Comet assay

The comet assay allows measure primary DNA damage, such as DNA strand breaks and oxidative damage inflicted by ROS. This damage can be detected ate the level of the individual cells, and an increases in damage as a result of occupational or environmental exposure to compounds can means un increase the risk of cancer (Collins, 2013).

Using this assay, after being exposed to the test compound, the cell suspension is embedded in agarose, treated with lysis solution and submitted to electrophoretic migration

under alkaline conditions (pH > 13). The lysis solution leads to disruption of the membranes, allowing the diffusion of the soluble and nuclear components. At this time, the cells are called nucleoids. The alkaline conditions cause DNA unwinding, required to reveal single-strand breaks (SSB) and double-strand breaks (DBS). Under electrophoretic conditions, the DNA that contain breaks migrates at a higher rate through the agarose gel, forming a tail (where are present the DNA fragments) with a head (undamaged DNA molecule) looking a comet, when viewed by fluorescence microscope following staining with a DNA-binding fluorescent dye (e.g. ethidium bromide) (see figure 8) (Collins *et al*., 2008a; Collins, 2013; Collins and Azqueta, 2011; Tice *et al*., 2000).

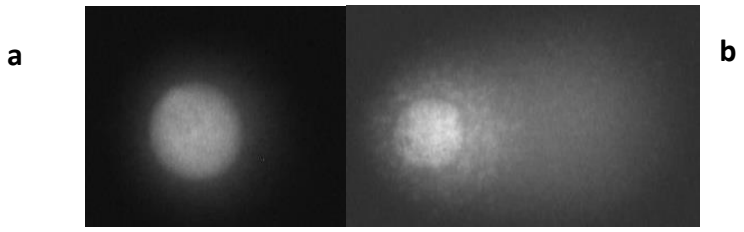

Figure 8. Examples of nucleoids obtained using Comet Assay. **a** – undamaged DNA, **b** – high level of DNA damage.

The basic comet assay procedure can be modified to detect the oxidative lesions through the presence of oxidized purines and pyrimidines. In this modification of the method, an incubation of the nucleoids embedded in agarose gels with a bacterial DNA repair enzymes (after lysis) is used. This enzyme, formamidopyrimidine DNA glycosilase (FPG) combines a specific glycosylase activity, removing the damage base and creating an apurinic/apyrimidinic (AP) site and then, an AP lyase converts the AP site to a break (Collins, 2013). This process increases the assay sensitivity and by comparing the results with or without FPG incubation, oxidative DNA lesions can be inferred (Collins *et al*., 2008; Collins and Azqueta, 2011). In this thesis, all of comet assay was performed with and without this enzyme. However, during the assays, it was concluded that the FPG activity was too low, even in the positive control cells, possibly due to a problem with the freezer where it was stored. Therefore, the results using FPG-modified comet assay were not considered valid and are not presented.

Some researchers suggested that the nanomaterials may interfere with the comet assay in several ways (Magdolenova *et al*., 2012; Stone *et al*., 2009):

i) the NMs can aggregated to DNA of nucleoids, that can interfere with the DNA migration during electrophoresis;

ii) when the NMs aggregated to the nucleoids can cause DNA break;

iii) these aggregates can interfere with the measurement in DNA tail;

iv) The NMs can interfere with FPG action.

However, as referred by Magdolenova, these events don't be relevant considering the available data (Magdolenova *et al*., 2012).

L929 cells were analyzed using comet assay in the work by (Graça, 2014) and in this work only NM-1001 and NM-4000 were used for comet assay.

The A549 cells were plated at the density of $0.5x10^5$ cells per well in a 24 well plate and allowed to grow for 24 hours. Then, were exposed for 24 hours to the 0-75 µg/cm^2 of NM-1001 or to 0-128 µg/cm^2 of NM-4000. The EMS (positive control) was diluted in PBS in a final concentration of 5mM for 1 hour before harvesting. At the end of exposure, the cells were washed twice with PBS and detached with trypsin-EDTA, removed from the plate and counted. The cell concentration obtained, was adjusted to a $1.35x10^5$ cells/mL. Then, 15 µL were embedded in agarose and placed on microscope slides previously coated with 1% normal melting point agarose.

The slides were allowed to dry and the agarose to solidify on a cold surface. Then, the slides were submerged in lysis solution ($Na_2EDTA.H_2O$ 100 mM; from Calbiochem (Darmstadt, Germany; NaCl 2.5 M, NaOH until pH=10; from Merck, Darmstadt, Germany; Tris-HCl 10 mM, from Invitrogen; Carlsband, CA, USA; 10% DMSO and 1% Triton-X100; from Sigma Aldrich) in a coplin jar for approximately 1 hour at 4ºC.

The slides were washed twice for 10 min in F buffer (HEPES 40 mM, , BSA 0.2 mg/mL from Sigma-Aldrich KCl 100 mM, acid EDTA 0.5 mM, KOH until pH=8 from Merck). Then, FPG enzyme (kindly provided by Dr. A. R. Collins, University of Oslo, Norway) diluted in F buffer, or F buffer only was added to each mini-gel and covered with a flexible cover slip, and the slides were placed in a humidified atmosphere in an incubator (37^{o}C) for 30 min.

Flexible cover slips were removed and the slides were immersed in electrophoresis buffer (NaOH 0.3, $Na_2EDTA.H_2O$ 1 mM; pH=13) for 30 minutes, allowing the DNA to unwind. Electrophoresis was performed for 25 minutes ate 28 V and 300 mA and the slides were washed for 10 min, first in cold PBS and then, in a cold dH_2O for the pH to be neutralized. The slides were allowed to dry at room temperature, overnight and were stained with 6.25 µg/mL ethidium bromide. Analysis of the slides was done in a fluorescence microscope (Axioplan2 Imaging, Zeiss), with the assistance of specific image-analysis software (Comet Imager 2.2, from Metasystems, GmbH). In each slide ten mini-gels were placed, two mini-gels of each

concentration of treatment. Fifty nucleoids were analyzed per mini-gel and 100 per treatment. Two independent cultures were made for this assay.

To evaluate the genotoxicity of nanomaterials through comet assay, the median value of the percentage of DNA in the tail was calculated. To obtain the oxidative damage, it was compared the percentage in the tail of the nucleoids from FPG-treated with the percentage of DNA in the tail of nucleoids using the following equation:

$$\text{Oxidative damage} = \%\ \text{DNA}_{\text{FPG}} - \%\ \text{DNA}_{\text{without FPG}}$$

3.4.2 Cytokinesis-blocked micronucleus assay

The cytokinesis-blocked micronucleus assay can detect chromosome loss and chromosome breakage (Bonassi *et al*., 2011). The micronuclei are chromosome fragments or whole chromosomes that have been lost during mitosis, being expressed in cells that presented chromosomes break, or chromosomes unable to migrate the poles during mitosis (Fenech, 2000). Furthermore, in this assay, other abnormal events can be observed such as nucleoplasmatic bridge between nucleus in a binucleated cell (formed due to the exposure to clastogens), or nuclear buds where micronuclei were not fully separated from the nucleus. Also, it is possible to detect the apoptotic cells due to the presence of a nuclear fragmentation (Fenech, 2000). The micronuclei are a good indicator of cancer an increase in this number is associated to an increase in cancer risk (Bonassi *et al*., 2011; Sargent *et al*., 2010).

Thus, in this work, for the micronucleus assay it was used a modified protocol to NMs exposure. The cytochalasin B was added 6 hours after NMs exposure and these, were not removed (Gonzalez *et al*., 2011).

For micronucleus assay in L929 cells, cells were seeded in 6 well plates at a density of 2.5×10^5 cells per well, and incubated for 24 hours at 37°C with 5% CO_2. The cells were exposed to the PMMA and PMMA-eud in the concentrations mentioned above and incubated again for 48 hours at 37°C with 5% CO_2. The positive control was the same used for NM-1001 and NM-4000 (MMC) which was prepared in PBS and in normal culture medium with a final concentration 0.1 µg/mL.

At 21 hours of exposure, Cytochalasin-B was added. The selection of this time point intended to avoid interference of this chemical with nanomaterials uptake. Two consecutive experiments have been conducted that vary in total incubation time as show in table 4:

Table 4: Exposure times used in each micronucleus experiment

	Time of exposure until Cytochalasin B addition	Time of exposure after Cytochalasin B	Total time of cells exposure to the NM
1st assay	21h	27h	48h
2nd assay	21h	33h	54h

The basis for extending the duration of cells exposure to the PMMA and PMMA-eud in the second experiment is given below in the results section.

After the referred total time of 48h or 54h since the start of the exposure to both nanomaterials, were added trypsin-EDTA, as described for this cell line. The cell suspension was centrifuged for 5 minutes at 1200 rpm. Then, supernatant was then discarded and hypotonic shock was induced with of KCl 0.1 M added drop by drop while vortexing. Then, the solution was centrifuged again for 5 minutes at 1200 rpm and supernatant discarded by pipetting. Then, the cells were fixed with cold fixing solution: 3 parts of methanol and 1 part of acetic acid. The cell suspensions were spread in microscope slides using a cytocentrifuge (Cytospin 3, Shandon). The slides were air-dried and stained with Giemsa for 13 minutes (4% in Gurr's phosphate buffer). Then, the slides were washes twice in Gurr's in the same buffer. After air drying, the slides were mounted with Entellan and cover slips.

The A549 cells were seeded in 6-well plates at a density of $2x10^5$ cells per well, and incubated for 24 hours at 37°C with 5% CO_2. The cells were exposed to the NM-1001 and NM-4000 in the concentrations mentioned above and incubated again for 48 hours at 37°C with 5% CO_2. The positive control was the same used in clonogenic assay (MMC) which was prepared in PBS and in culture medium in a final concentration 0.1 µg/mL. Cytochalasin-B was add to each well after six hours of exposure to the treatment (final concentration of 6 µg/mL), and cells were incubated again.

After 48 hours of treatment, the cells were washed with PBS twice and added trypsin-EDTA, as described above for this cell line. The suspension was centrifuged for 5 minutes at 1200

rpm, the supernatant was discarded and the cell pellet was ressuspended in culture medium. After that, the cells were submitted to a hypotonic shock with a solution of 73.5% sterile injectable bidistilled water, 24.5% of culture medium and 2% of inactivated FBS, added drop by drop vortexing. The cells were centrifuged for 5 minutes at 1200 rpm again, the supernatant was discarded and then the cells were ressuspended in culture medium. Two drops of cell suspension were placed on microscope slides. For each treatment, three/ four slides were prepared.

After the slides dried, they were immersed in a cold fixing solution: 3 parts of methanol and 1 part of acetic acid for 20 minutes to fix the cells. In the next day (or more), the slides were stained with Giemsa. First, the slides were immersed in Gurr's phosphate buffer (VWR, Radnor, PA, USA) for 4 minutes, then in a solution with 4% Giemsa (prepared in a Gurr's phosphate buffer) for 15 minutes and finally washed twice the same buffer. Then, slides were allowed to dry and mounted with Entellan and cover slips.

In micronucleus assay in A549 and L929 cells, coded slides were "blind" analyzed under a bright field microscope and micronuclei were scored in, at least, 2000 binucleated cells from two independent cultures. The diameter of micronucleus may vary between $1/16^{th}$ and $1/3^{rd}$ of the mean diameter of the main nuclei and must have a round or oval shape (see figure 9) (Fenech, 2000).

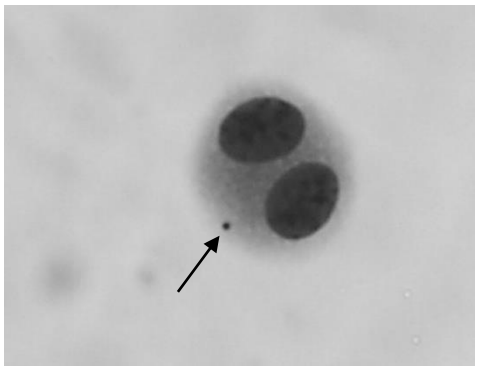

Figure 9. An example of the micronuclei diameter in L929 cells exposed to MMC (1000x).

For assessing the cell cycle progression and cytotoxicity in the cells, the proportion of mono- (MC), bi(BC) or multinucleated cells (MTC) was determined in a total of 1000 cells and the CBPI was calculated as follows (OECD, 2010b):

$$CBPI = \frac{\text{Nº mononucleated cells} + 2x \text{ nº binucleated cells} + 3 \times \text{nº multinucleated cells}}{\text{Total number of viable cells}}$$

The replication index (RI) of nanomaterials treated cultures, relative to vehicle control cultures, was also calculated by the formula:

$$RI = \frac{\left(\frac{\text{Nº binucleated cells} + 2 \times \text{nº multinucleated cells}}{\text{Total number of cells}}\right)_{\text{treated cultures}}}{\left(\frac{\text{Nº binucleated cells} + 2 \times \text{nº multinucleated cells}}{\text{Total number of cells}}\right)_{\text{Control cultures}}}$$

3.5 STATISTICAL ANALYSIS

All of statistical analyzes were performed in IBM SPSS Statistics 22.

In the cytotoxicity results, for cell counting method it was used Student's t-test; the results from clonogenic assay were analyzed by One-Way ANOVA test and the CBPI and RI were evaluated by Kruskal-Wallis test and Mann-Whitney test. One-Way ANOVA tests is used when the results assumed a normal distribution; on the other hand, when the results don't follow a normal distribution, the non-parametric Kruskal-Wallis test is used.

In the genotoxicity results, in the comet assay the results were analyzed by One-Way ANOVA test, comparing the several concentrations that were used with the negative control. This test was used, because the results presented a normal distribution. In the micronucleus assay, the Two-sided Fisher's exact test was used for comparing the frequency of micronucleated binucleated cells in several concentrations that were exposed cultures with the negative control.

In addition, the existence of a dose-response relationship in all assays was explored by regression analysis.

4 RESULTS

4.1 NANOMATERIALS FOR MEDICAL APPLICATIONS (PMMA AND PMMA-EUDRAGIT NANOMATERIALS)

4.1.1 Cytotoxicity

The cytotoxic effects of PMMA and PMMA-eud have been described previously in Graça, (2014). In this work it was evaluated by MTT assay and three exposure periods were analyzed (24, 48 and 72 hours). Graça verified that cell viability showed a slight but significant decrease with the two highest PMMA concentrations (624 μg/cm^2 and 1559 μg/cm^2) after 48 hours exposure. After 72 hours for the same concentrations of PMMA cell viability further decreased. For PMMA-eud, a slight significantly decrease in the cell viability was observed after 24 hours to exposure for the same concentrations of PMMA: 624 μg/cm^2 and 1559 μg/cm^2, after 72 hours of all PMMA-eud concentrations significantly lowered cell viability values. Yet, the lower value 59.98 ± 8.55%.

Therefore, in general neither NMs show major toxicity effects in the concentration range studied (Graça, 2014) since the decrease in cell viability was always above 50%.

Since the micronucleus assay also offers information about cytotoxicity by evaluating the cytokinesis-blocked proliferation and replications indexes (CBPI and RI), these indexes were determined in the present work and are represented in Figure 10 and Tables A1 and A2 in the Annexes.

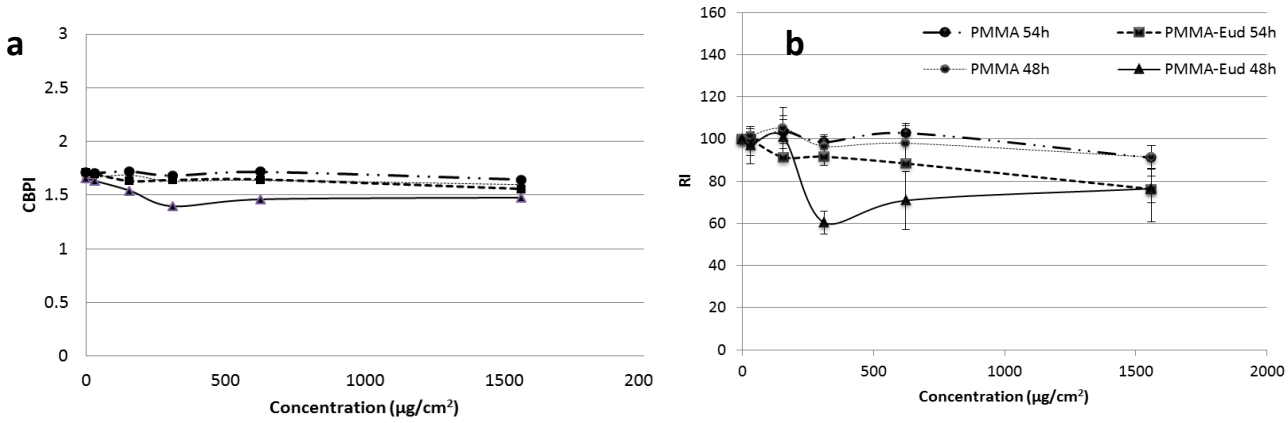

Figure 10. Results of the CBPI and RI of L929 cells exposed for 48h and 54h to PMMA and PMMA-eud: a - CBPI; b - RI. The results of the positive control (MMC) can be found in the annexes.

In L929 cells exposed to PMMA and PMMA-eud there were no differences in CBPI and RI as compared to negative controls, in any of the experiments (48 and 54 hours) or concentrations tested (p>0.05, Student's t-test). The only exception was the RI after the exposure of cells during 48 hours to 312 µg/cm^2 of PMMA-eud (p=0.000133, Student's t-test), showing a delay in the cell cycle progression. By increasing the exposure time to 54 hours this delay was no longer observed.

In the positive control exposure (MMC) a significant decrease in the RI and CBPI was observed at 48 and 54 hours (Table A1 and A2 in the annexes) (Student's *t*-test).

4.1.2 Genotoxic effects

In Graça (2014), the genotoxicity it was studied using the comet assay, with two exposure periods (3 and 24 hours). No significant were DNA damage was observed in either time points for any of the NMs, with or without FPG.

In respect to the micronucleus assay, that was performed twice during this work. In the first experiment the cells were exposed during 48 hours to PMMA or PMMA-eud and results are presented in Figure 11 and Tables A1 and A2 in the annexes.

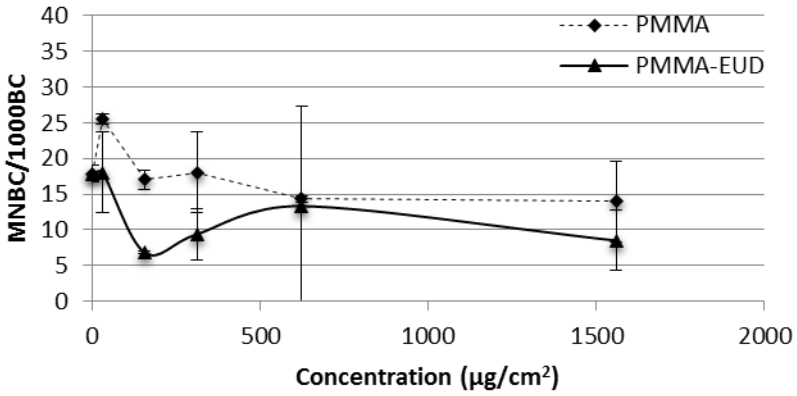

Figure 11. Mean micronucleated binucleated cells (MNBNC) after exposure for 48 hours to PMMA and PMMA-eud. Bars represent standard deviation.

In the experiment with 48 hours exposure, there were no significant increases in the micronucleus frequency in L929 cells exposed to any of the concentrations of PMMA except of the lowest concentration (31 µg/cm^2; $p<0.0001$, Fisher's exact test). In cells exposed to PMMA-eud for 48 hours the MNBNC were significant lower after 156, 312 and 1559 µg/cm^2 ($p= 0.0002$, 0.020 and 0.019, respectively). However, this latter finding may be due to the impact of the NMs with cell cycle progression that was seen through RI analysis.

In fact, in this experiment was not possible to analyze 2000 binucleated cells in L929 cells exposed to highest concentrations of PMMA-eud, due to damage of the cytoplasm. In figure 12 it is possible to observe an interference of the PMMA-eud with the integrity of the cytoplasm, making difficult to observe the micronuclei.

Following MMC exposure, a 2.5-fold increase in MNBNC was observed, showing a significant genotoxic effect ($p<0.001$; Fischer's test).

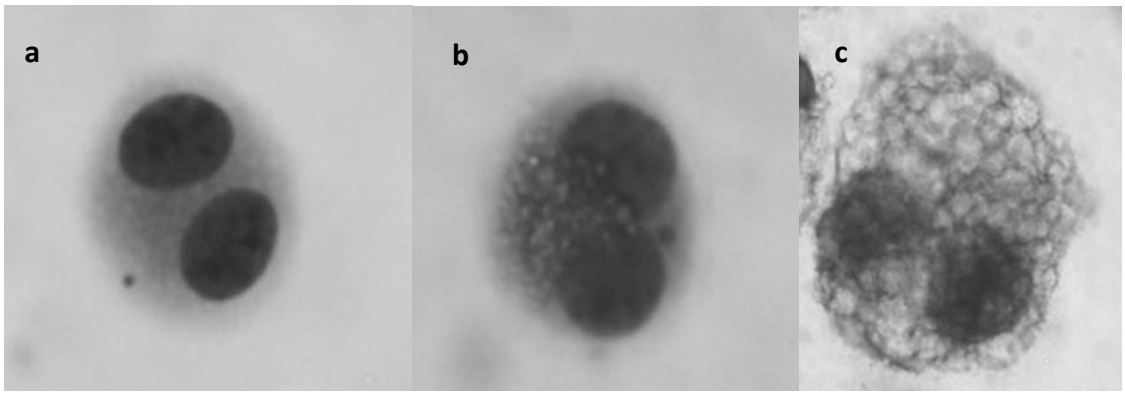

Figure 12. Microphotographs of L929 binucleated cells exposed to PMMA and PMMA-eud: a- negative control, b- cells exposed to PMMA, c- cells exposed to PMMA-EUD. In figure and b it is possible to see a micronucleus in a binucleated cell.

Considering, that the 54 hours exposure period showed no impact on cell cycle progression, only the results of this exposure were considered valid and are shown in Figure 13 and Tables A1 and A2 in the annexes:

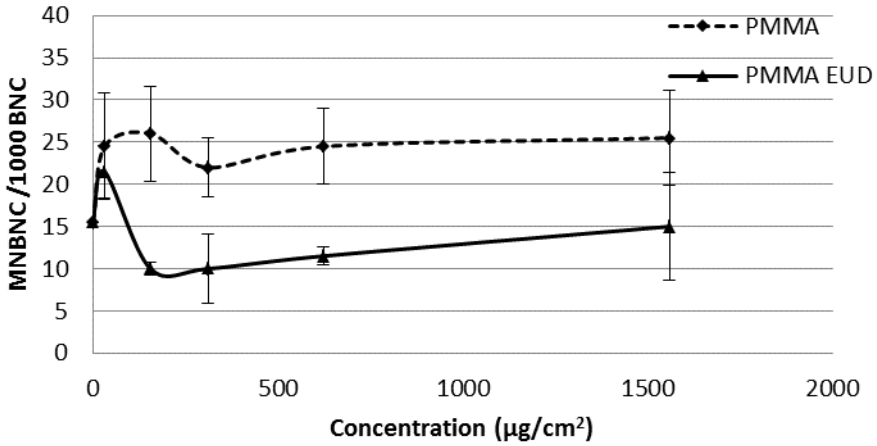

Figure 13. Mean micronucleated binucleated cells (MNBNC) after 54 hours exposure for to PMMA and PMMA-eud. Bars represent standard deviation.

In the second experiment (54 hours exposure), there were increases in the micronuclei frequency in L929 cells exposed to PMMA, that were significant in the concentrations of 156 and 1559 µg/cm^2 ($p<0.05$; Fischer's test) and almost significant in the concentrations of 30 and 624 µg/cm^2 (both with $p=0.054$; Fischer's test). No increase in the micronuclei frequency was observed in L929 cells exposed to PMMA-eud.

The positive control (MMC) caused a 3.7 fold significant increase in the micronucleus frequencies ($p<0.0001$, Fischer's exact test).

Furthermore, regression analysis using the data from 54 hours exposure did not reveal any dose-response curve that could be fitted to these data.

In conclusion, PMMA revealed genotoxic effects in L929 cells exposed for 54 hours while PMMA-eud did not induce increased micronuclei frequencies.

4.2 MANUFACTURED NANOMATERIALS USED IN CONSUMER PRODUCTS (TiO_2, MWCNTS)

4.2.1 Cytotoxic effects

Due to difficulty of using cytotoxicity assays for NMs investigation, several assays were used to produce complementary information on the toxic effects of the manufactured NMs.

The results of the clonogenic assay in A549 cells exposed to the nanomaterial NM-1001 and NM-4000 for 8 days, can be observed in figure A3 and A5 in the annexes.

The Figure 14 shows the aspect of the wells in the plates with the cell colonies, from the negative control to the highest concentration in both nanomaterials (figure 14-a, 14-b). While in Figure 14-a no major difference is apparent, in Figure 14-b it can be seen a clear decrease in the number of the colonies in all concentrations when compared with the negative control, corresponding to increase in cytotoxicity.

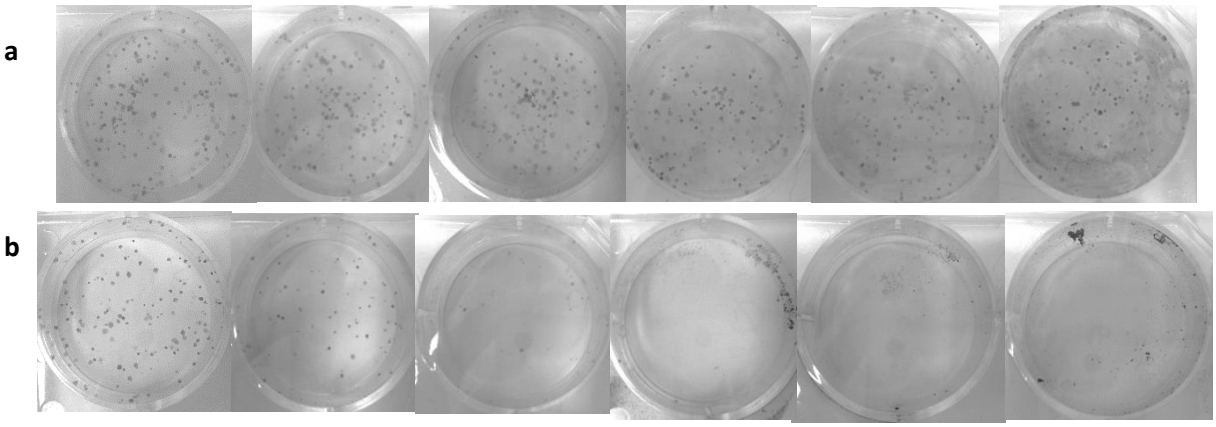

Figure 14. Clonogenic assay results after exposure to NM-1001 and NM4000: **a**- all concentrations that were tested of NM-1001; **b**- all concentrations that were tested of NM-4000; (left to right: negative control up to the highest concentration).

The clonogenic assay results are presented in Figure 14 and Tables A3 and A5 in the Annexes. An increase in cytotoxicity was observed after NM-1001, that was significant after exposure to 30 µg/cm^2 *(p*=0.033, Kruskal- Wallis test).After MWCNTs exposure, it was verified a highly cytotoxic effect in all concentrations (p<0.01, Kruskal- Wallis test).

In cells exposed to MMC (0.1 µg/mL) a significant decrease in cytotoxicity was observed (Table A3 and A5 in the annexes).

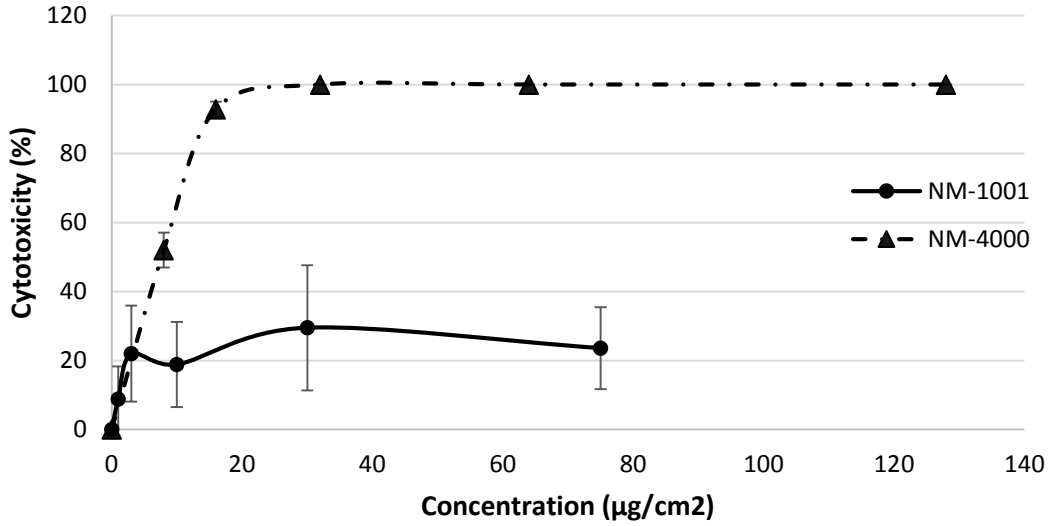

Figure 15. Results means clonogenic assay in A549 cells exposed to NM-1001 and NM-4000 for 8 days. Standard deviations of four replicates are presented.

In spite of this results relatively to NM-4000 did not show correlation to a mathematical model, considering only the lowest concentrations a best-fit could be found and is represented in Figure 16. Using this analysis it was possible calculate the half maximal inhibitory concentration, IC50 (figure 16).

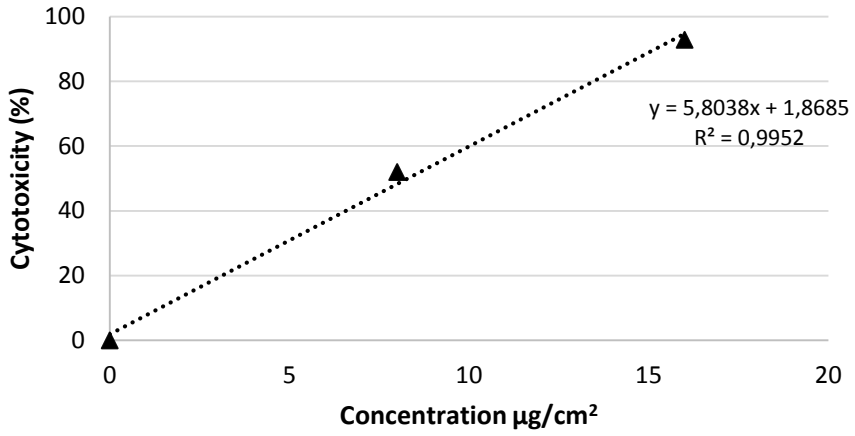

Figure 16: Determination of IC50 by clonogenic assay in A549 cells exposed to NM-4000

Using the linear model with R^2=0.995 and through of equation we obtained the IC50= 8.3 µg/cm^2 for NM-4000.

Cell counting assay also allowed analyzing the cytotoxicity of NMs. The results are presented in Figure 16 (and table A4 and A6 in the annexes) and presented high variations. After 24 hours of exposure of A549 cells to NM-1001, no cytotoxicity was observed when compared with the negative control.

When the A549 cells were exposed to NM-4000 for 24 hours, these presented a decrease in viability only at the highest concentration (fig. 17), but not significantly different from the negative control ($p>0.05$, Student's t-test).

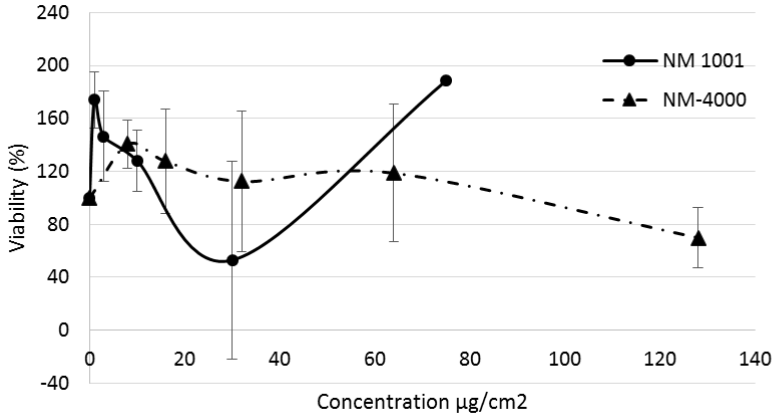

Figure 17. Results of cell counting in A549 exposed to NM-1001 and NM-4000 for 24 hours.

Finally, the evaluation of the cytokinesis-blocked proliferation and replications indexes (CBPI and RI), during micronucleus assay is presented in figure 18 and Tables A9 and A10.

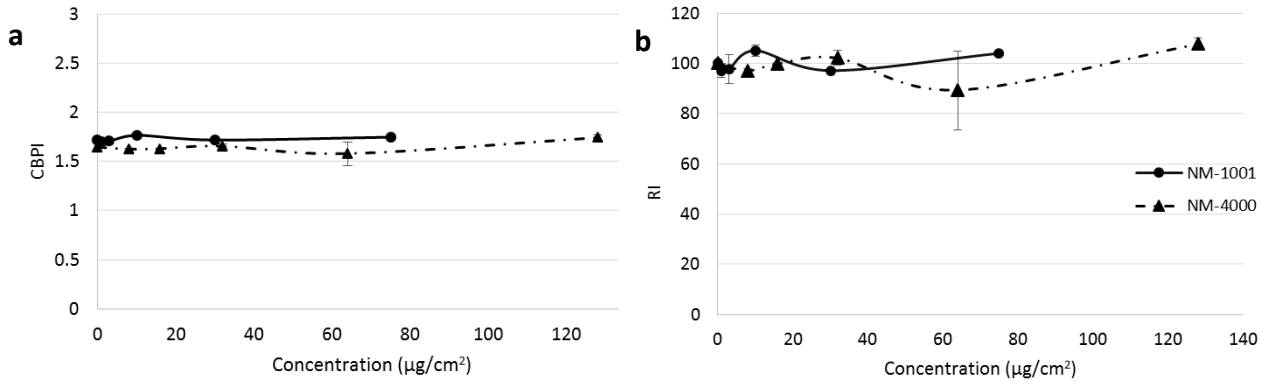

Figure 18. Results of the CBPI and RI of A549 cells exposed for 48 hours to NM-1001 and NM-4000: a – CBPI; b – RI.

NM-1001 caused a slight alteration in replication index. But the CBPI values were not different from the control (table A5 in the annexes). Likewise NM-4000 caused small fluctuations in replication index and in the CBPI there were no significant differences.

The positive control (MMC) cause a significant decrease both in CBPI and RI (table A5 and A6 in the annexes).

In conclusion, the clonogenic assay showed high cytotoxicity of MWCNTs while TiO_2 had low cytotoxicity, after 8 days exposure. However, the assay using 24 or 48 hours exposure did not reveal cytotoxicity of the NMs at these time points.

4.2.2 Genotoxic effects

4.2.2.1 Comet assay

The results of the comet assay are represented in Figure 19.

The A549 cells showed a significant increase in DNA damage 24 hours after exposure to NM-1001 (p=0.005, post hoc Tukey HSD). The concentration of 75 µg/cm^2 was significantly different from the control.

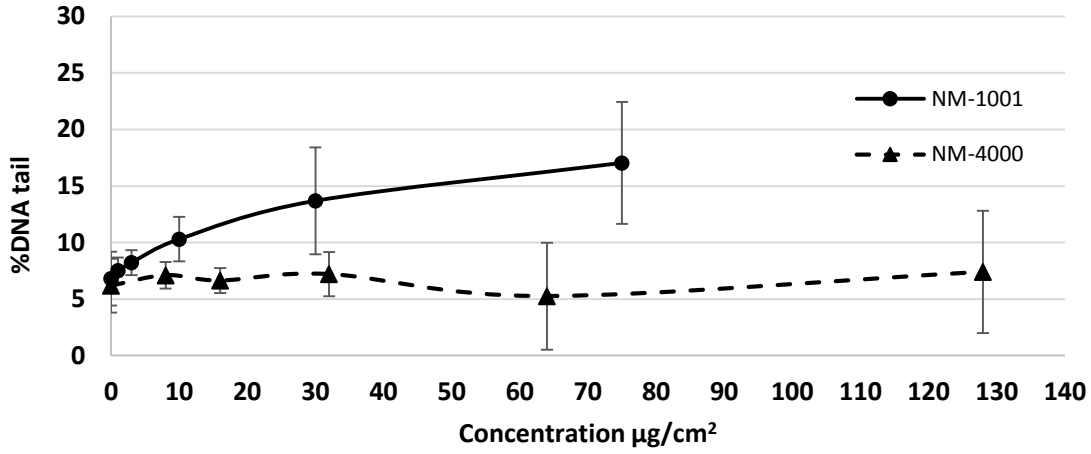

Figure 19. Results of Comet assay with NM-1001 and NM-4000. The results of positive (EMS) control can be found in the annexes.

Regression analysis using the mean of the four replicates (represented in the Figure 20) showed a concentration-response effect after NM-1001 exposure with a high correlation (R^2=0.99). On the other hand, using the four replicate values obtained for each concentration, we calculated the regression analyses by SPSS, and for a quadratic function we obtained R^2=0.631.

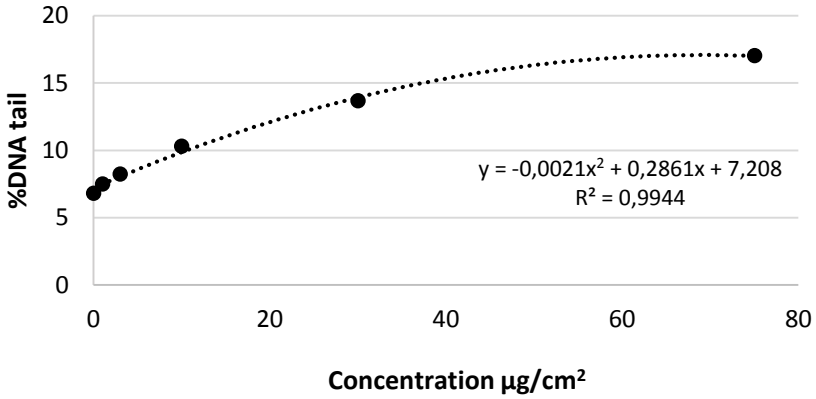

Figure 20: Relationship between concentration and percentage in DNA tail by comet assay applying the polynomial model.

When the cells were exposed to NM-4000, no significant differences were found in the levels of DNA damage detected ($p>0.005$, One-Way ANOVA) (Figure 19).

The image analysis of the comet assay was affected by the MWCNTs, because it was possible to see the comet head with MWCNTs, as observed in figure 21 that can have interfered with electrophoretic migration or with the measurement the percentage of DNA in tail.

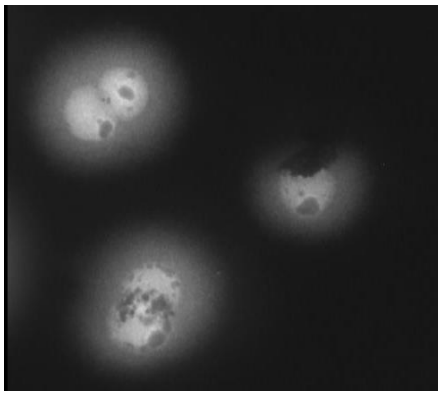

Figure 21. Photography of MWCNs in A549 cells. It is possible to see an interference of MWCNTs in cells.

In this assay, it was verified that the FPG enzyme was not working adequately, because even in the positive control, the DNA damage values were similar to these without FPG. Therefore, modified comet assay was not valid and is not presented. Further assays will be done in the future using new batch of enzyme.

4.2.2.2 Cytokinesis-blocked Micronucleus assay

The figure 22 shows the results of the Micronucleus assay in A549 cells after 48hours exposure of NM-1001 and NM-4000. The number of micronucleated binucleated cells analyzed was 2000 binucleated cells per treatment conditions.

There were no significant differences in the mean MNBNCN after TiO_2 or MWCNTs as compared to the negative controls. However, the highest concentration of NM-4000 showed a 2-fold increase over controls in micronucleus frequency that was not statistically significant.

Additionally, a 10-fold significant increase was observed in cells exposed to positive control (Tables A9 and A10 in the Annexes).

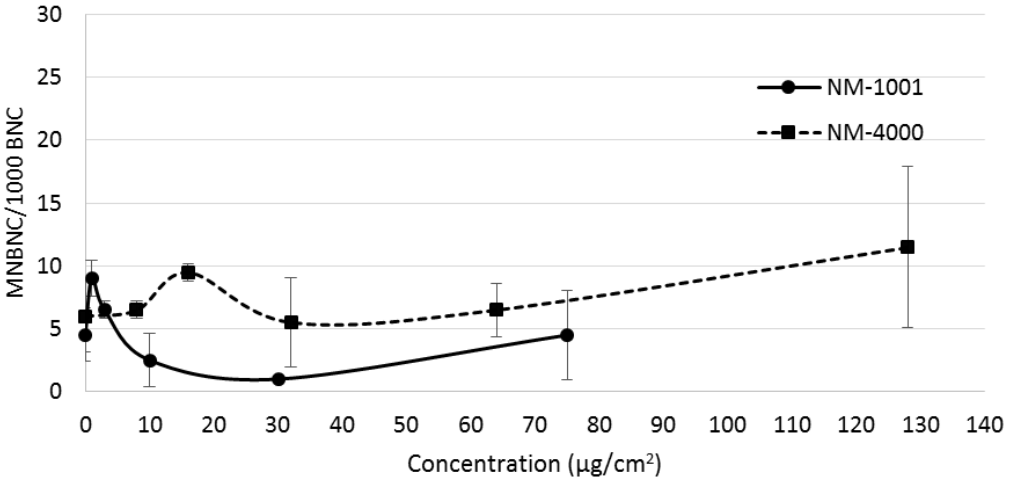

Figure 22. Results of Micronucleus assay in A549 cells exposed to TiO2 and MWCNT: MN BNC/1000 BNC. The results of the positive control (MMC) can be observed in the annexes.

It has become very difficult to observe the micronuclei after NM-1001 exposure, because the NMs deposited and aggregated on the cells, as can be seen in the figure 23.

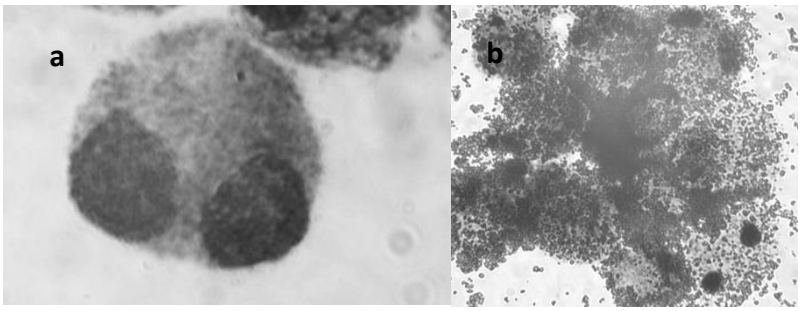

Figure 23. Microphotograph of A549 cells after 48h exposure to NM-1001: a- negative control, b- concentration 75 µg/cm².

Relatively to NM-4000, it was verified that this NM form some aggregates, but not interfered with the visualization of cytoplasm.

5 DISCUSSION

With the increase of nanomaterials and the development of nanotechnology and their used in several areas, including the pharmaceutical area, biomedical sciences, cosmetics, to paints, food, clothes, electronics (Liu and Liang, 2012; Louro *et al*., 2015; Mittal and Pandey, 2014); it is necessary to ensure the safety of these new materials, namely regarding the toxicity of these.

In the last decade many scientific reports have dealt with the toxicity of nanoparticles, both *in vivo* and *in vitro* assays, but the results are not consistent because they lack adequate characterization of the NMs or standardized methodologies. So, in this work it was used a standard procedures to evaluate the cytotoxicity and genotoxicity of three different nanomaterials, that were previously characterized in detail, using two types of cells, relevant according with probable routes of exposure. Besides considering the necessary modifications of the methodologies to analyse NMs genotoxic effects, the specific characteristics of the NMs and their suspensions were considered for the interpretation of the data, in order to use a nanotoxicology perspective.

5.1 NANOMATERIALS FOR MEDICAL APPLICATIONS

In the present work, the micronucleus assay was used to test the PMMA and PMMA-eud. The standardized methodology described in OECD 2011 was used, with the modification of adding cytochalasin B only 6 hours after beginning of the exposure. According to Magdolenova *et al.* (2013), this modification allows the contact of NMs with the cells and prevents the blockage of the NM uptake by cytochalasin addition.

According to the cytokinesis-blocked proliferation index and replications index, no cytotoxic effects of PMMA and PMMA-eud were found.

Some studies have been done to evaluate the cytotoxicity and to ensure the safe application of PMMA nanomaterials in medicine, using several cell lines and were mostly negative. Graça (2014) used the MTT assay and verified a significant, but slightly decrease at two high concentration when L929 cells exposed to PMMA after 48 and 72 hours exposure. A recent study in A549 cells did not observe cytotoxic effect after 48 hours exposure using MTT assay (Juneja and Roy, 2014). These authors compared three types of PMMA with different sizes and

surface coating and in all of these they did not verify a significant cytotoxic effect. Other study, using HCT116 cells (human colorectal cancer cells), tested the cytotoxicity of the PMMA nanoparticles by MTT assay too. The cells were incubated with PMMA for 24, 48 and 72 hours and no-cytotoxic effects were observed during any exposure periods (Ge *et al*., 2012). Papa *et al.*, reported the viability of primary cultures of microglia cells exposed to PMMA using MTS assay. The authors evaluated the toxicity of PMMA nanoparticles that were internalized selectively by LPS-activated microglia, and did not revealed a toxic effect of these particles. So, the authors concluded that PMMA nanoparticles internalized selectively by LPS will may provide benefit in different human neurologic diseases opening new perspectives to the inflammatory treatment in the central nervous system (Papa *et al*., 2014). Hazra *et al.* (2014), using a bacterial model, verified that PMMA increased reactive oxygen species inducing oxidative stress, which can lead to a cytotoxic and genotoxic effect. These authors used 0.1-0.7 g/L. Vale *et al.* (1997), verified a cytotoxic effect using MTT assay in surgical fragments of human skin after exposure for 24 hours to PMMA. Furthermore, a study using the L929 cells showed a strong cytotoxic effect after 48 hours exposure to PMMA powder (Gulçe Iz *et al*., 2010). On the other hand, other study in this cell line revealed non-cytotoxic effect after 24 hours exposure to 100mg/mL of chitosan PMMA, using the MTT assay (Changerath *et al*., 2009); in the majority of studies in vitro that evaluated the cytotoxicity potential of PMMA, they used the MTT assay. The MTT assay allows detect the mitochondrial activity (Graça, 2014). The difference between results may be due to the interference in the spectrophotometric measurements caused by NMs misleading the final results or to differences in the type of NM assayed.

The in vivo experiments are also important to verify the cytotoxicity of NMs. Dhana *et al.,* tested the toxicity of PMMA using male albino rats, analyzing the mortality and survival time, as well as by clinical picture of intoxication including behavior reactions. Rats were injected for different concentrations and, 21 days, after exposure time, all adverse reactions were observed. The authors did not observe changes in all of tested doses, not verifying toxic effects (Dhana Lekshmi *et al*., 2010). Sitia *et al.* (2014), using cell growth assay in 4T1 cells and *in vivo* assays (in female athymic Foxn1 nu/nu mice), also did not observe toxic effects.

In the studies referred, 2 were positive, while 8 did not show cytotoxic effects after PMMA exposure. However, the positive results corresponded to different forms of PMMA (powder or chitosan-coated).

In respect to the PMMA-eudragit, Graça verified the same results that were obtained for PMMA (a significant, but slightly decrease in cell viability at last two concentrations) after 48

hour exposure. When cells were exposed for 72 hours, observed a significantly decrease in cell viability at all concentrations (Graça, 2014). To our knowledge there are no more previous studies to evaluate the cytotoxicity of this NM, possible because it is a newly developed material. Therefore, the absence of cytotoxic effects of PMMA-eud adds new data to the literature.

In this study, the genotoxicity of PMMA and PMMA-eud was evaluated by micronucleus assay (OECD, 2010b) and, the most pertinent findings were obtained after 54 hours exposure. An increase in micronuclei frequency was observed after PMMA but not with PMMA-eud exposure. The two experiments performed (48 and 54 hours exposure) allowed to verify that PMMA-eud did not block the cell cycle, but caused a slight delay in cell cycle progression. Furthermore, the cells that were exposed to PMMA-eud presented disturbances in the integrity of membranes in higher concentrations as shown in results chapter. This effect could be related to an inflammatory response that might lead a ROS generation. Vale *et al.* (1997), reported an inflammatory response in surgical fragments of human skin after exposure 24 hours to PMMA, describing that this NM can injury in the antioxidant enzyme activities that can lead the production of prostaglandin, which in turn resulting in an inflammatory response. Another study done by Yang *et al.*, demonstrated an inflammatory response in rats injected with PMMA, in the same study *in vitro* cultures of PBMC (peripheral blood mononuclear cells), revealed too an inflammatory response (Yang *et al*., 2011). On the other hand, Graça (2014) did not found the production of ROS by PMMA in L929 cells after 1 and 2 hours exposure.

However, the results of Graça (2014) reported no genotoxic effects of PMMA when using the comet assay in L929 cell. The differences may reflect that PMMA cause chromosomic damage and did not cause breakage in DNA single- and double- stranded. Other hypothesis can due to the fact that the lesions primary appear after exposure to the agent that is being tested and can be repaired by the cell's DNA machinery. On the other hand, the micronuclei can emerge through clastogenic and aneugenic events and persist in the cell (Hartmann *et al*., 2001).

In respect to the evaluation of genotoxic potential of polymeric nanomaterials, namely PMMA, very few studies exist, especially in respect to de micronucleus assay.

A study performed by Gulçe Iz *et al.* (2010), evaluated the genotoxicity of PMMA exposure in human lymphocytes using micronucleus assay and these authors verified a significantly increase of MBNC frequency in cells exposed to PMMA compatible with our findings (the authors did not mentioned the concentrations that were used). Our results showed a significant increase in micronuclei frequency, after PMMA exposure at 0.1 and 5 mg/mL but it was not verified a

dose-response. Bigatti *et al.* (1994) also verified an increase in the micronuclei frequency in human lymphocytes after 5 days exposure to PMMA. In contrast, Lamberti *et al.* (1998), did not verify a genotoxic effect in human lymphocytes after 5 days exposure to PMMA.

To the best of our knowledge, we did not find studies about genotoxic effects using L929 cells. This cell line is useful in the biocompatibility studies of biomaterials and because it is thought to use PMMA-eud as a drug delivery for therapeutic in bone diseases, providing evidence of genotoxic effects in target organ. This cell line it is recommended by ISO 10993-5.

A study *in vivo* using MMA reported an increase in the micronuclei frequency after 8 hours exposure in male Wistar rats (Araújo *et al*., 2013). On the other hand, a study performed by Souto Lopes (2012) tested the MMA in two different cell types, HGF (*homo sapiens* gingival biopsy) and V79-4 cells using micronucleus assay, for 72 hours exposure and did not verified a genotoxic effect in both cell lines.

Others studies have been done using other polymer similar to PMMA such as poly(lactide-*co*-glycolide) copolymers (PLGA), using the mitotic indices in fibroblasts and lymphocyte cells, and poly(caprolactone) (PCL) using micronucleus assay in mice; are commonly used in engineering tissues and in drug delivery, such PMMA (Louro *et al*., 2015). In both polymers it was not verified a genotoxic potential (Huang *et al*., 2010; Louro *et al*., 2015). Other study, using comet and micronucleus assays in TK-6 cells (human B-lymphoblastoid cells) exposed to PLGA-PEO did not reveal a genotoxic effect; however, they verified a significant increase in micronuclei frequency in mononucleated cells in two of three concentrations that were tested (Kazimirova *et al*., 2012). In respect to the chitosan, another polymer, frequently used in tissue engineering, suitable for cell growth, antibacterial activity and bioadhesive behavior (Louro *et al*., 2015); were found a genotoxic potential using micronucleus and comet assay (in mouse bone morrow and A549 cells, respectively) (Louro *et al*., 2015); (European Medicines Agency, 1998).

As seen in our results, there were no cytotoxic or genotoxic effects to PMMA-eud, consistent with results obtained previously by Graça (2014) using the comet assay. The PMMA proved to be more genotoxic than the PMMA-eud, through micronucleus assay. These results can be due to the chemical properties, in this case, the surface charge modification, while PMMA presented be strongly negative (-32.7 ± 1.04 mV), the PMMA-eud were strongly positive (+31.8 ± 1.66) (Graça, 2014). A study performed by Wang *et al.*, using graphene oxide (GO) in human lung fibroblasts cells, verified that GO with a negative charge, is more toxic than a PEI-GO (Polyethylenimine functionalized graphene oxide, with positive charge). The authors referred that a mild positive charge may help GO to stay out of cells if those positive charges do not

damage the cell membrane. Furthermore, the ideal GO derivate should have lower positive electronic charge to reduce their toxic effect on cells, but this conclusion need more studies, to be confirmed (Wang *et al*., 2013). In agreement with these authors, in our study it was verified that negative PMMA it was more genotoxic than positive PMMA-eud. Another example that may influence the genotoxicity is the size and surface charge of PMMA and PMMA-eud were highly influenced by the media constitution. The surface charge of PMMA revealed to be neutralized by both fetal serum and ionic strength and PMMA-eud revealed neutralized by ionic strength but inverted by the presence of the fetal serum proteins (Graça, 2014). This data is relevant for risk analysis since an increase in micronucleus frequency has been assumed to be associated to an increase risk of cancer development (Bonassi *et al*., 2011). In view of the potential use of PMMA in the pharmaceutical area, our results provide information on its safe use under the tested conditions.

According to the guidelines of International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (European Medicines Agency, 1998; ICH, 1997) and of WHO/IPCS (Eastmond *et al*., 2009), future work for safety assessment would require the *in vivo* confirmation of these data.

5.2 MANUFACTURED NANOMATERIALS USED IN CONSUMER PRODUCTS

The increase application of manufactured nanomaterials in industry and consumer products has raised concerns their safety. The issues about the potential risks of NMs for public health arise mainly from epidemiologic studies in humans exposed to nanomaterials generated as by-products from human activity and pollution and the potential to induce cancer, suggested by some experimental studies, as seen for titanium dioxide or carbon nanotubes. To analyze in a short term the carcinogenic properties of a compound, genotoxicity assays in mammalian cell lines or models are frequently used. However, until today the investigation of the genotoxic properties of NMs has been inconclusive.

It is important evaluated the toxicity of TiO_2 and MWCNTs in respiratory cells like A549, because humans, namely, the workers from industry are possibly exposed to this by inhalation. (Pelclova *et al*., 2015), identified particles of TiO_2 rutile and anatase crystal phase, in exhaled breath condensate of exposed workers in 40% of the pre-shift and 70% of the post-shift samples. The workers may also be exposed to MWCNTs through inhalation (Lee *et al*., 2015) and some studies reported a toxic effects using several types of lung cells (Lindberg *et al*., 2009).

In the present work cytotoxicity and genotoxicity of two manufactured nanomaterials was investigated.

The cytotoxicity assays allows the detection of toxic effects of the nanomaterials and also to determine the dose-range, for the genotoxicity studies, avoiding concentrations that yield high levels of toxicity that may mislead the results of genotoxicity testing. In this work, we proceed to different assays to evaluate the cytotoxicity of TiO_2 and MWCNTs: clonogenic assay, cell counting with Trypan blue and proliferation and replication indexes.

The clonogenic assay showed high cytotoxicity of MWCNTs while TiO_2 had low cytotoxicity, after 8 days exposure. However, the other assays using 24 or 48 hours exposure did not reveal cytotoxicity of the NMs ate these time points. Therefore, the dose-range selected for the genotoxicity assays was limited only by the nanomaterial dispersibility in the medium.

Several authors have described cytotoxicity studies of TiO_2, in the same or different cell lines. Aueviriyavit *et al.* (2012), verified an increase of cytotoxicity using MTS assay in A549 cells at a concentration similar to the highest used in the present work and above, after 24 hours exposure to anatase TiO_2 NMs, with a dose-dependent effect. A recent study by (Kansara *et al*., 2015), described a cytotoxic effect in MTT and neutral red assay using A549 cells, observed at similar concentrations, 48 hours after exposure to anatase TiO_2. Using the same cellular line, Mochini *et al.* (2013), demonstrated a very little acute cytotoxicity effect of TiO_2 (not identified) in this concentration range, after 24 hours exposure. Another study reported by Hamzeh and Sunahara (2013), tested various types of TiO_2 (anatase, rutile and anatase-rutile mixture), using MTT and cell counting assays in V79 cells (Chinese hamster lung fibroblast), demonstrated a cytotoxic effect in all TiO_2 types that were tested at 10 µg/mL and 100 µg/mL after 24 and 48 hours exposure. On the other hand, Corradi *et al.* 2011), did not observe a significantly cytotoxic effect of both rutile and anatase TiO_2 in A549 cells when compared with negative control. In general, the reported assays were based on spectrophotometric measurements that may be affected by the increase in the concentration of TiO_2 in the medium. The clonogenic assay is used as an alternative method which avoids the use of any colorimetric or fluorescent indicator dye, decreasing the risk of interactions and allowing the assessment of true cytotoxicity (Herzog *et al*., 2007). Besides, differences in the TiO_2 NM analyzed and assay conditions reported may explain the different outcomes.

Concerning also exposure of other cell types to TiO_2, a study by Bhattacharya *et al.* (2009), using cell counting assay in two different type of lung cells, IMR-90 cells (human diploid fibroblasts) and BEAS-2B cells (human bronchial epithelial cells), verified that TiO_2 (anatase) did

not induced cytotoxic effects in BEAS-2B cells, whereas it had significant cytotoxic effects in IMR-90 cells, after 24 hours exposure. This result suggests that cells are more sensitive than others. Another study, in BEAS-2B cells, evaluated the cell viability using the same assay after 24, 48 and 72 hours exposure to three different TiO_2 (rutile, anatase and fine TiO_2). The decreased of cell viability when BEAS-2B cells exposed to TiO_2 anatase, starting at 304 µg/mL with all treatment times. The authors concluded that fine rutile showed the highest cytotoxicity, followed by anatase and last rutile, strengthening the importance of to know the physiochemical properties of each nanomaterial in detail (Falck *et al*., 2009).

A study using another type of TiO_2 (NM-102; another anatase), verified a decrease in viability of BEAS-2B cells using cell counting assay at last two highest concentrations, in spite of only at 256 µg/mL revealed statistically significant after 24 hours exposure. In the same study, the cytotoxicity of this NMs was analyzed in A549 cells too by clonogenic assay and no cytotoxic effect was detected in this cells (Louro, 2013).

Such as can be verified, there is extensive literature about cytotoxicity of TiO_2, but it is very difficult to establish a conclusion about their cytotoxicity. These results can depend the type of cells that are used and the physicochemical characteristics namely phase (rutile or anatase), agglomeration/aggregation, size, surface area and impurities, are also an important influence to evaluate the cytotoxicity of nanomaterials (Guichard *et al*., 2012). However, our results did not show high toxicity with the dose-range used. Thus it was decided to use the same concentrations for generality testing. Top concentrations were limited by the dispersability of the NMs. The maximum concentration was based on the availability of nanomaterials and previously conducted studies (Tavares *et al*., 2014).

In respect to the NM-4000, a marked decrease in viability of cells was verified using clonogenic assay, after 8 days exposure, while using cell counting assay and CBPI and RI indexes after shorter exposure time, no significant decrease was verified.

Some studies have reported the investigation of MWCNTs' cytotoxicity. A study realized by Simon-Deckers *et al.* 2008), used LDH (for assessment of cell membrane integrity, lactate dehydrogenase) and MTT assays in A549 cells. The authors verified a MWCNT concentration-dependent increase in LDH release from cells, suggesting increased cell membrane damage due expose to MWCNTs after 48 hours exposure at 100 µg/mL concentration. Another study, using the same assays in normal human dermal fibroblast cells, verified that MWCNTs exposure caused a significant time- and dose-dependent cytotoxicity from dose 40 µg/mL (Patlolla *et al*., 2010). SWCNTs showed cytotoxic effects even at low concentrations after 10 days exposure of

A549 cells (Herzog *et al*., 2007). The latter study suggested yet that the differences in sample preparation, sample composition and assay system used can explained the discrepancies between the studies that have been published. In spite of in our study, we used MWCNTs and the characteristics are different, we verified a high cytotoxic effect in all concentrations that were used. This study that compared if the method used to production SWCNTs might be influence the cytotoxic effect of this NMs; shown a (1.56, 6.25, 100 and 400 µg/mL; except in the colony number at 25 µg/mL concentration) for 10 days exposure. This study suggested yet that the differences in sample preparation, sample composition and assay system used can explained the discrepancies between the studies that have been done (Herzog *et al*., 2007). Relatively to SWCNTs, another study using cell counting in V79 cells and verified a decrease in viability cells at 48 and 96 µg/cm^2 after 3 and 24 hours exposure (Kisin *et al*., 2007). In our study, we verified a decrease in viability cells, when the concentrations increase in spite of results did not shown significant, may be due the variation inherent to the assay.

Cavallo *et al*. (2012), verified a cytotoxic effect from the lowest concentration (10 µg/mL) from 4 hours exposure to MWCNTs using MTT and LDH assays. These results can confirm the high cytotoxic potential induced by MWCNTs in A549 cells. Another study verified too that MWCNTs induced a concentration- and time-dependent decrease in mitochondrial metabolism by MTT assay (Tabet *et al*., 2009). A study using cell counting assay, did not reveal cytotoxic effects of MWCNTs (NM-403) in BEAS-2B. In the same study, by clonogenic assay the author verified a cytotoxic effect in A549 cells and observed a concentration-dependent after NM-402 and NM-403 exposed (Louro, 2013). Using cell counting assay, Migliore *et al.* (2010), verified a decrease of living cells and a cytotoxic effect at two last concentrations (10 and 100 µg/mL). Corradi *et al.* (2012), did not observe a cytotoxic effect of this NM of MWCNTs in A549 cells in any concentration through CBPI evaluation.

In BEAS-2B cells exposed 24, 48 and 72 hours exposure, cytotoxic effects were seen in cell counting assay but not in CBPI assay (Lindberg *et al*., 2009).

As described, much literature exists showing that the MWCNTs have cytotoxic effects. However, in other studies contradictory results were reported. Differences in the physicochemical characteristics of the various MWCNTs mentioned above may explain such contradictions.

In spite of the high cytotoxicity of NM-4000 observed in the clonogenic assay, no major cytotoxicity was observed when shorter exposure periods were used (24 and 48 hours),

therefore, the dose-range for the genotoxicity assays was only limited by the dispersability of the NM.

The assays employed for the investigation of the genotoxic effects of the NMs were the comet assay, that allows detection of DNA strand breaks (Collins *et al* ., 2013), and the cytokinesis-blocked micronucleus assay that detect chromosome loss and chromosome breakage (Bonassi *et al*., 2011). As described previously, the standardized methodology described in OECD 2011 was used, with the modification of adding cytochalasin B only 6 hours after beginning of the exposure (Magdolenova *et al*., 2013).

Relatively to NM-1001, the results obtained in comet assay, revealed a concentration dependent genotoxic effect after 24 hours exposure. We verified a high genotoxicity at highest concentration 75 μg/cm^2 corresponding 285.1 μg/mL when compared to the negative control. In fact, previous results from our group using the same concentration-range showed that another anatase TiO_2 (NM-102), with size to NM-1001, was positive in the comet assay (Louro, 2013). In agreement with our results, a study using the same cells and the same assay, demonstrated the induction of similar levels of DNA damage at 20 and 40 μg/cm^2 concentrations after 4 hours of exposure to TiO_2 (mixture of anatase and rutile) in A549 cells (Karlsson *et al*., 2008). Falck *et al.* (2009), verified an increase in DNA damage in BEAS-2B to 10, 20, 40, 60 and 80 μg/cm^2 concentrations, after 24 hours exposure to anatase TiO_2. In the same study, they tested also a rutile TiO_2 and verified an increase in DNA damage only at concentration of 80 μg/cm^2 after 24 hours exposure. These results suggested that anatase is more genotoxic than rutile and that the physicochemical characteristics are important to determine the genotoxicity of NMs. Jugan *et al.* (2012), compared several TiO_2 with different phase, shape and diameter: A12 (95% anatase, spherical, 12 nm), A25 (86% anatase, spherical 24 nm), A140 (100% anatase, spherical 142 nm), R68 (100% rutile, elongated 68 nm) and R20 (90% rutile, spherical 21 nm) using A549 cells. The authors concluded that TiO_2- A12, -A25 and –R20 an increased DNA damage after 24 hours exposure. This trend was observed for spherical TiO_2 nanoparticles with diameter smaller than 68 nm whatever their crystalline phase.

Another study using A549 cells showed an increase in DNA damage at 75 and 100 μg/mL after 6 hours exposure to TiO_2 (anatase). The authors suggested that this increase due to increased oxidative stress and ROS generation (Kansara *et al*., 2015). Ursini *et al.* (2012), demonstrated a direct and oxidative DNA damage only at 40 μg/mL concentration after 2 hours exposure and a slight induction of oxidative DNA damage at 5 μg/mL after 24 hours exposure in A549 cells. Furthermore, these authors did not verify direct or oxidative DNA damage in BEAS-

2B cells verified a significant increase DNA damage dose-dependent in A549 cells at 13, 26 and 52 µg/cm^2 after 48 hours exposure of anataseTiO$_2$ (Wang *et al*., 2015). In contrast, Louro (2013), did not verify any increase in DNA damage in BEAS-2B cells after 24 hours exposure to NM-102. In agreement with this author, Bhattacharya *et al.* (2009), did not verify a significant increase in DNA damage in IMR-90 cells (lung fibroblasts) after 24 hours exposure to anatase TiO$_2$.

The discrepancies may due to experimental factors. In fact, Karlsson *et al*., (2008) described that TiO$_2$ can form radicals in the presence of light, because this NM is photocatalytic, but the activity seems to depend on it is anatase, rutile or a mix of these two. The anatase form induces an increased production of ROS. Due to this fact, the conditions in which the assay is done are important to determine the genotoxic effect. The time of exposure is another important factor to evaluate the genotoxic effect.

Our results revealed a significant increase in DNA damage at the highest concentration using the comet assay, revealing a genotoxic effect. In addition, we verified a dose-response. Accordingly, in the majority of studies that have been done, the authors verified a genotoxic effect using the same assay. However, some studies do not demonstrate increases in the in DNA damage. It was important to retain, that the physicochemical characteristics are very important to determine the genotoxic effects of nanomaterials, as verified Falck *et al.*(2009) and Jugan *et al.* (2012); furthermore, the type of cell is important to determine the genotoxicity outcome (Guichard *et al*., 2012; Shukla *et al*., 2011).

Relatively to the cytokinesis-blocked micronucleus assay, no genotoxic effects were observed after 48 hours exposure. In this assay, we had very difficult to visualize the cytoplasm, as demonstrated in figure 22. It was observed too an agglomeration of TIO$_2$. Corradi *et al.* (2012) and Flack *et al.* (2009), verified the same problem using the same assay. They did not analyze the results because the micronuclei were obscured by TiO$_2$ NMs agglomerates covering the cells. Flack *et al* .(2009), did not observe an increase micronuclei in any concentration after 24 or 48 hours, but the authors showed an increase in the micronuclei frequency at 10 and 60 µg/cm^2 after 72 hours of exposure to TiO$_2$. These results suggested that the time of exposure is important to evaluate the genotoxic effects. Another study, using A549 and BEAS-2B cells did not observe increases in micronuclei after 48 hours exposure to TiO$_2$ anatase, in contrast, in the same study, using the human lymphocytes, the author reported an increase in the microinucleated cells frequency (Louro, 2013).

Kansara *et al.* (2015), verified a significant increase in micronucleus frequency at 75 and µg/mL after 6 hours exposure to TiO$_2$ anatase. Srivastava *et al.* (2013), also exposed A549 cells

to TiO_2 anatase for 24 hours exposure and verified a significant increase of micronuclei frequency at 10 and 50 µg/mL concentration, revealing a genotoxic effect.

Studies in other cell lines, such as in lymphocytes cells, reported an increase in micronuclei frequency in a dose-dependent of anatase-rutile TiO_2 (20, 50 and 100 µg/mL) (Kang *et al.*, 2008). A recent study, using HepG2 cells detected a significant concentration dependent of micronucleus in all concentrations (20, 40 and 80 µg/mL) (Vallabani *et al*., 2014).

As mentioned above, the NMs have the capacity adhere to cells and some studies related that NMs can cross to cell membranes (Stearns *et al*., 2001). May be this fact can explain the interferences of TiO_2 with cells that we will be found, blocking the observation of cytoplasm of cells.

Disagreements in reported genotoxic potential of TiO_2 NMs may be due to the fact of the different TiO_2 treatment regimens, the cell type used, the metabolic/antioxidant capacity of cells, as well as DNA repair capabilities (Reeves *et al*., 2008). However, our present results were performed using standardize procedures for NM preparation (Jensen *et al*., 2011) as well as accordingly to standardized micronucleus assay (Magdolenova *et al*., 2013; OECD, 2010b). In fact, the negative results obtained in the micronucleus assay for the anatase NM-1001 are in agreement with the negative results in the same assay in A549 cells exposed to another anatase TiO_2 (NM-102), with similar size (Louro, 2013).

In the next table are resumed the results obtained in this work and by other our work group (table 5) (Tavares *et al*., 2014):

Table 5: Summary of the cytotoxic and genotoxic results in A549 cells exposed TiO_2

NMs	Cytotoxicity			Genotoxicity	
	Clonogenic	Cell Counting	CBPI or RI	Comet	Micronucleus
NM-1001	(+)	-	(+)	+	-
NM-102[a]	-		-	+	-

+Positive results: the results showed a statistically significant in two or more concentrations in comparison to the control or, a statistically significant change in highest concentration.
(+)Equivocal results: statistical significant change in only one concentration
-Negative results

As mentioned above, both TiO_2 shown an increase in DNA damage. Comparing the physicochemical characteristics of these two NMs, we verified that both presented anatase phase. Furthermore, we supposed that TiO_2 that presented an aspect ratio between 1.5, may be

a factor to contribute the DNA damage. Relatively to the specific surface, we did not find a connection in respect to the genotoxicity, because the values of NM-1001 and NM-102 are much different (169.5 and 60, respectively).

When the A549 cells were exposed to MWCNTs, we no increase in DNA damage was observed in the comet assay. Previously, Louro (2013) tested two types of MWCNTs (NM-402 and NM-403) using the same procedures of the present work and observed a slight increase in DNA damage but not statistically significant after 3 or 24 hours exposure in A549 cells. In BEAS-2B, the author did not verify an increase in DNA damage. In other study, Pinhão (2014) reported also an increase in micronuclei frequency in A549 cells after 48 hours exposure to MWCNT (NM-401). However, these results may reflect the physicochemical characteristics of each NM. For example, we know that NM-401 has a surface area, length and thickness larger than the other MWCNTs which have been studied by our group.

Lindberg *et al.* (2012), reported a dose-dependent increase in DNA damage in BEAS-2B cells after 24 hours exposure to carbon nanotubes and a significant increase at 1, 60, 80 and 100 $\mu g/cm^2$. The authors increased the time exposure (48 and 72 hours) and verified a clear increase in DNA damage in al concentrations. Zhu *et al.* (2007), Muller *et al.* (2008) and Yang *et al.* (2009) also reported a dose-dependent in mouse embryonic stem cells, in epithelial cells and mouse embryo fibroblasts cells. Migliore *et al.* (2010) also verified an increase in DNA damage in murine macrophage cell line RAW 247.7.

Cavallo *et al.* (2012), used A549 cells and reported a significant increase in the DNA damage at 10 µg/mL after 24 hours exposure to MWCNT. In addition, these authors verified a concentration-dependent induction that was statistically significant at 10 and 40 µg/mL after 2 hours exposure and at 5, 10 and 100 µg/mL after 4 hours exposure. On the other Karlsson *et al.* (2008) verified only an increase in DNA damage at 1 $\mu g/cm^2$ after 4 hours exposure.

Our negative results may be due the agglomerates/aggregates that it can be found when visualizing the DNA damage. These agglomerates/aggregates can have interfered in the DNA tail, preventing the software to read the percentage of DNA damage, as illustrate the figure 20 in results chapter.

Studies *in vivo* are important to determine the genotoxicity of NMs. Kato *et al.* (2012), observed in lung of mice for 3 hours treatment with MWCNTs a dose-dependent and the values of DNA tail moment were significantly increased compared with control. Another study *in vivo*, using morrow cells of Swiss-webster mice, the authors verified an increase in DNA damage after

5 days of treatment, reporting a dose-dependent (Pelclova *et al*., 2015). In contrast, Ema *et al.* (2012) did not verified an increase in DNA damage, not reveling a genotoxic effect in mouse.

Relatively to the micronucleus assay, we observed a two fold increase in the frequency, but not statistically significant may be due to the standard deviation be a little high. It could be observed some agglomerates of MWCNTs, but did not interfered with our visualization.

Kato *et al.* (2012), exposed A549 cells for 6 hours to MWCNTs and verified an increase the number of micronucleated cells in a dose-dependent (8.5% in the 200 µg/mL). Louro (2013) reported a significant increase in 2 fold in the micronuclei frequency at 125 and 256 µg/mL concentrations in A549 cells exposed to NM-402 when compared to the negative control. In contrast the NM-403 did reveal an increase in micronuclei frequency in both A549 and BEAS-2B cells. Lindberg *et al.*, used too BEAS-2B cells and did not verified an increase in micronuclei frequency after 24 or 72 hours exposure. However, they verified an increase after 48 hours exposure at 10, 60 and 100 µg/cm^2 . Srivastava *et al.* (2013), performed a study using also BEAS-2B cells and verified that at 10 µg/mL induce an increase in micronuclei frequency higher than 50 µg/mL.

In *in vivo* studies, using bone morrow cells of Swiss-webster mice, reported that MWCNTs induced a dose-related increased in micronuclei frequency after 24 hours exposure (Patlolla *et al*., 2010). On the other hand, Kim *et al* (2011) did not verify an increase in the micronuclei frequency in any concentration.

Such as been mentioned throughout this thesis, the physicochemical characteristics are important properties in the assessment of toxic effects of NMs. A study done by Kisin *et al.* (2011) compared the induction of cytotoxicity and genotoxicity between carbonanofiber (CNF) (aspect ratio: 500) and SWCNT (aspect ratio: 1000). They verified that CNT produced a stronger genotoxic effect than SWCNTs in V79 cells, using the comet assay. Magrez *et al.* (2006), confirmed that carbon nanofibers (aspect ratio: 30-40) is more toxic when compared to MWCNTs (aspect ratio: 80-90) in H596 lung tumor cells. In contrast with these authors, Poland *et al.*(2008) and Takagi *et al.* (2008) showed that high-aspect-ratio MWCNTs is more toxic and potential to induce mesothelioma than low-aspect-ratio MWCNTs. On the other hand, Kim *et al.* (2011), reported that neither the high- nor the low aspect ratio MWCNTs appeared to induce any cytotoxicity in the hematopoietic cells or genotoxicity in the mice due to their inability to translocate to the bone morrow of the femurs.

Our work group studied another MWCNTs, and the results are summarized in the table 6:

Table 6: Summary of the cytotoxic and genotoxic results in A549 cells exposed MWCNTs (Louro, 2013; Pinhão, 2014)

NMs	Cytotoxicity			Genotoxicity	
	Clonogenic	Cell Counting	CBPI or RI	Comet	Micronucleus
NM-4000	+	-	(+)	-	-
NM-401	+	(+)	+	-	+
NM-402	+	NP	-	-	+
NM-403	+	NP	-	-	-

+Positive results: the results showed a statistically significant in two or more concentrations in comparison to the control or, a statistically significant change in highest concentration.
(+)Equivocal results: statistical significant change in only one concentration
-Negative results

Pinhão (2014) verified a genotoxic effects of NM-401 and NM-402 using micronucleus assay, but Louro (2013), did not find a genotoxic effect with NM-403 in both assays. On the other hand, Pinhão (2014) verified an association with the physicochemical properties using the three nanomaterials tested above (NM-401, NM-402 and NM-403). She observed an association between the aspect ratio and the frequency of micronucleated cells. The results that were obtained in this work, with NM-4000 did not allow stablish any correlation.

As mentioned above, there are many studies on the toxicity of NMs. The disagreement that exists between the different results may be due to the physicochemical characteristics of NMs. These properties when analyzed alone may be not revealed a connection with the genotoxic effects, because of these it is important have attention all of characteristics. The cell line that is used in each study and also due to the exposure times to NMs that are used it is another important aspect to evaluate the toxicity of NMs. In this work, we verified that TiO_2 induced DNA damage but did not induce clastogenic or aneugenic effects.

As mentioned above, the results obtained may have been subjected to the interference of TiO_2 with the cells that prevents viewing the cytoplasm. This fact was reported for more authors (Corradi *et al*., 2012; Falck *et al*., 2009). Likewise, the MWCNTs results showed some interference since MWCNTs adhere to the cells (Stearns *et al*., 2001) and when we wash the wells, the cells can be adherents to the NMs and it cannot be possible to observe any colony. In spite of NMs specific characteristics, with the methodology used it was possible to analyze their genotoxic effects.

6 CONCLUSIONS

The present work, had the objective to evaluate the toxicity of nanomaterials for medical applications, the PMMA, PMMA-eud, and also two nanomaterials used in consumer products, such as titanium dioxide (NM-1001) and multi-walled carbon nanotubes (NM-4000), considering a nanotoxicology approach.

A major finding of this work is the lack of genotoxic effects of the modified PMMA-eud in fibroblast cells, suggesting that this NM provides an advantage for biomedical application as compared with PMMA. To our knowledge, this result constitutes new information that may be useful for regulatory decisions since the methodologies used were based on ISO and ICH guidelines. Future work for safety assessment would require the *in vivo* confirmation of these data.

Furthermore, the suggested association of the NM surface charge and its genotoxic effects should be further investigated since it provides a clue on the property that may be more determinant for nano-genotoxicity.

Concerning TiO2, we verified that TiO_2 induced genotoxic effects in dose-dependent way. Together with previous results, this finding suggests that the anatase form of TiO2 may be responsible for increased genotoxicity, and this relation with the crystal form of TiO2 NM should be investigated in the future, using larger panels of these NMs. There was no induction of micronucleus after TiO_2 or MWCNTs, showing the absence of In each study is necessary to know the physicochemical properties of respectively nanomaterial with the objective to compared studies with the same characteristics of nanomaterials;

For future studies, exist some aspects deserve more research, such as:

i) It is important to performed methodologies more specifics to evaluate the toxicity of nanomaterials;
ii) It is important to try understand by which mechanism that the nanomaterials act, such as evaluating the reactive oxygen species production and how nanomaterials interact with DNA;
iii) In each study is necessary to know the physicochemical properties of respectively nanomaterial with the objective to compared studies with the same characteristics of nanomaterials.

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8 ANEXES

Table A1. Results of the micronucleus assay in L929 cells exposed for 48h to the PMMA and PMMA-eud.

	Concentration		Total BC analysed	MNBNC/1000 BC (mean ± SD)	CBPI (mean ± SD)	RI (mean ± SD)
	(µg/cm²)	(mg/mL)				
PMMA	**0**	**0**	2500	17.8±1.2	1.65±0.04	100±0
	31	**0.1**	2000	25.5*±0.7	1.66±0.03	101.7±1.7
	156	**0.5**	2000	17.0±1.4	1.68±0.06	105.1±9.7
	312	**1.0**	2000	18.0±5.7	1.63±0.01	96.6±4.7
	624	**2.0**	2500	14.3±0.5	1.64±0.01	98.0±8.2
	1559	**5.0**	2000	14.0#±5.7	1.60±0.07	91.5±5.4
PMMA-Eud	**31**	**0.1**	2000	18.0±5.66	1.63±0.02	97.1±8.8
	156	**0.5**	2500	6.83±0.24	1.54±0.14	101.1±10
	312	**1.0**	1231	9.41±3.6	1.39±0.03	60.4**±5.4
	624	**2.0**	1088	13.3±13.9	1.46±0.06	70.9±13.8
	1559	**5.0**	1237	8.52±4.21	1.48±0.04	76.5±15.,7
MMC		**0.1 µg/mL**	1522	43.04**±2.69	1.14**±0.42	53**±23.2

*Significantly different from the negative control ($p \leq 0.05$, Fischer's test). The mean MNBNC/1000 BC was lower than in controls; **significantly different from the negative control ($p \leq 0.01$, Fischer's test). The mean MNBNC/1000 BC after MMC exposure was higher than in controls. MMC- positive control.

Table A2. Results of the micronucleus assay in L929 cells exposed for 54h to the PMMA and PMMA-eud.

	Concentration		Total BC analysed	MNBNC/1000 BC (mean ± SD)	CBPI (mean ± SD)	RI (mean ± SD)
	(μg/cm^2)	(mg/mL				
PMMA	**0**	**0.0**	2000	15.5±6.36	1.71±0.01	100±0.00
	31	**0.1**	2000	24.5*±0.71	1.71±0.06	98.46±6.27
	156	**0.5**	2000	26.0±2.83	1.72±0.06	103.53±5.66
	312	**1.0**	2000	22.0±1.41	1.68±0.02	98.47±3.5
	624	**2.0**	2000	24.5±0.71	1.72±0.03	102.73±4.53
	1559	**5.0**	2000	25.5*±3.54	1.64±0.06	91.15±5.62
PMMA -Eud	**31**	**0.1**	2000	21.5±4.95	1.70±0.00	99.86±3.14
	156	**0.5**	2000	10.00±1.41	1.63±0.03	91.43±0.79
	312	**1.0**	2000	10.00±4.24	1.64±0.05	91.64±4.05
	624	**2.0**	2000	11.50 ± 9.19	1.64±0.02	88.41±1.05
	1559	**5.0**	2000	15.00 ± 1.41	1.55±0.07	76.15±6.39
MMC		**0.1 μg/mL**	2000	57.5**±3.54	1.52**±0.01	70.29**±2.44

*Significantly different from the negative control ($p \leq 0.05$, Fischer's test). The mean MNBNC/1000 BC was lower than in controls; **significantly different from the negative control ($p \leq 0.01$, Fischer's test). The mean MNBNC/1000 BC after MMC exposure was higher than in controls. MMC- positive control.

Table A3. Surviving fraction of A549 cells that were exposed for 8 days to TiO_2 in Clonogenic assay

	Concentration		Surviving fraction (mean ± SD)	Cytotoxicity (mean ± SD)
	(µg/cm^2)	(µg /mL)		
NM-1001	**0**	**0**	100 ± 0.02	0 ± 0
	1	**3.8**	91 ± 9.5	8.8 ± 9.5
	3	**11.4**	78 ± 13.9	22 ± 13.9
	10	**38**	81 ± 12.3	18.9 ± 12.3
	30	**114.1**	71* ± 18.1	29.5* ± 18.1
	75	**285.1**	76 ± 11.9	23.6 ± 11.9
MMC		**0.1**	0** ± 0	100 ± 0

*Significantly different from the negative control ($p \leq 0.05$, One-Way ANOVA test); **Significantly different from the negative control ($p \leq 0.01$, One-Way ANOVA test). MMC- positive control.

Table A4. Cell counting assay after of A549 cells exposure for 24h to TiO_2.

	Concentration		Viability mean ± SD (%)
	µg/cm2	µg/mL	
NM-1001	**0**	**0**	100 ± 0
	1	**3.8**	173.9 ± 21.3
	3	**11.4**	146.4 ± 34.2
	10	**38**	128.1 ± 23.1
	30	**114.1**	52.9* ± 0
	75	**285.1**	188.6 ± 0.5

*Significantly different from the negative control ($p \leq 0.05$, One-Way ANOVA test); MMC- positive control

Table A5. Surviving fraction of A549 cells that were exposed for 8 days to MWCNTs in Clonogenic assay.

	Concentration		Viability (mean ± SD)
	(µg/cm²)	(µg/mL)	
NM-4000	**0**	**0**	100 ± 0
	8	**16**	140.7 ± 18.24
	16	**32**	127.8 ± 39.28
	32	**64**	112.5 ± 53.03
	64	**128**	118.8 ± 51,91
	128	**245**	69.8 ± 23.01

*Significantly different from the negative control ($p \leq 0.05$, One-Way ANOVA test); **Significantly different from the negative control ($p \leq 0.01$, One-Way ANOVA test). MMC- positive control.

Table A6. Cell counting assay after of A549 cells exposure for 24h to MWCNTs.

	Concentration		Surviving fraction (mean ± SD)	Cytotoxicity (mean ± SD)
	(µg/cm²)	(µg /mL)		
NM-4000	**0**	**0**	100 ± 2.82	0 ± 0
	8	**16**	47.96* ± 5.05	52.04* ± 5.05
	16	**32**	7.14** ± 2.16	92.86** ± 2.16
	32	**64**	0** ± 0	100** ± 0
	64	**128**	0** ± 0	100** ± 0
	128	**245**	0** ± 0	100** ± 0
MMC		**0.1**	11.71** ± 5.03	88.29** ± 5.03

*Significantly different from the negative control ($p \leq 0.05$, One-Way ANOVA test); MMC- positive control

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Table A7. Comet assay: percentage of DNA tail with, and without FPG and oxidative damage in A549 cells exposure for 24h to TiO_2.

	Concentration		DNA in tail (%) (mean ± SD)	Tail length (µm)	Tail moment
	(µg/cm²)	(µg/mL)			
NM-1001	0	0	6.8 ± 2.4	15.91 ± 3.1	1.45 ± 0.39
	1	3.8	7.5 ± 1.2	16.97 ± 1.51	1.67 ± 0.32
	3	11.4	8.2 ± 1.1	18.7 ± 2.13	1.8 ± 0.24
	10	38	10.3 ± 2	18.37 ± 4	2.05 ± 0.56
	30	114.1	13.7 ± 4.7	24.06 ± 5.06	2.97 ± 1.25
	75	285.1	17* ± 5.4	27.86* ± 8.6	3.8 ± 1.33
EMS	5mM		25.5** ± 5.7	28.08** ± 8.46	6.43** ± 1.79

*Significantly different from the negative control ($p \leq 0.05$, One-Way ANOVA test); **significantly different from the negative control ($p \leq 0.001$, One-Way ANOVA test). EMS- positive control.

Table A8. Comet assay: percentage of DNA tail with, and without FPG and oxidative damage in A549 cells exposure for 24h to MWCNTs.

	Concentration		DNA in tail (%) (mean ± SD)	Tail length (µm)	Tail moment
	(µg/cm2)	(µg/mL)			
NM-4000	0	0	6.2 ± 2.4	16.58 ± 2.7	1.36 ± 0.46
	8	16	7.1 ± 1.2	17.59 ± 4.16	1.43 ± 0.35
	16	32	6.6 ± 1.1	15.47 ± 2.72	1.39 ± 0.44
	32	64	7.2 ± 2	18.03 ± 4.8	1.59 ± 0.52
	64	128	5.3 ± 4.7	14.68 ± 4.65	1.2 ± 0.58
	128	256	7.4 ± 5.4	16.58 ± 6.52	1.32 ± 0.77
EMS	5mM		26.1** ± 5.7	39.11 ± 3.91	7.36 ± 1.47

*Significantly different from the negative control ($p \leq 0.05$, One-Way ANOVA test); **significantly different from the negative control ($p \leq 0.001$, One-Way ANOVA test). EMS- positive control.

Table A9. Micronucleus assay in A549 cells exposed for 48h to TiO_2.

	Concentration		Total BC analysed	MNBNC/1000 BC (mean ± SD)	CBPI (mean ± SD)	RI (mean ± SD)
	µg/cm2	µg/mL				
NM- 1001	**0**	**0**	2000	4.5 ± 2.1	1.7 ± 0.03	100 ± 0
	1	**3.8**	2000	9.0 ± 1.4	1.7 ± 0.01	97.2 ± 2.8
	3	**11.4**	2000	6.5 ± 0.7	1.7 ± 0.03	97.8 ± 5.8
	10	**38**	2000	2.5 ± 2.1	1.8 ± 0.02	105.# ± 2.3
	30	**114.1**	2000	1.0 ± 0	1.7 ± 0	97.2 ± 0.2
	75	**285.1**	2000	4.5 ± 3.5	1.7 ± 0.02	104# ± 0.9
MMC		**0.1**	2000	40.5** ± 7.8	1.74 ± 0.03	48.9$ ± 5.5

** Significantly different from the negative control ($p \leq 0.01$, Fisher's test). #significantly different from the negative control ($p \leq 0.05$, Student's *t* test). $ significantly different from the negative control ($p \leq 0.01$, Student's *t* test)

Table A10. Micronucleus assay in A549 cells exposed for 48h to MWCNTs

	Concentration		Total BC analysed	MNBNC/1000 BC (mean ± SD)	CBPI (mean ± SD)	RI (mean ± SD)
	µg/cm2	µg/mL				
NM- 4000	**0**	**0**	2000	5.0 ± 2.8	1.6 ± 0.02	100 ± 0
	8	**16**	2000	6.5 ± 0.7	1.6 ± 0.01	97.1$ ± 0.7
	16	**32**	2000	9.5 ± 0.7	1.6 ± 0	99.8 ± 0.5
	32	**64**	2000	5.5 ± 3.5	1.7 ± 0.02	102.1 ± 3
	64	**128**	2000	6.5 ± 2.1	1.6 ± 0.12	89.3 ± 15.7
	128	**256**	2000	11.5 ± 6.4	1.7 ± 0.03	107.7# ± 2.4
MMC		**0.1**	2000	59.0** ± 4.2	1.1$$ ± 0	23.2$$ ± 0.6

** Significantly different from the negative control ($p \leq 0.01$, Fisher's test). #significantly different from the negative control ($p \leq 0.05$, Student's *t* test). $ significantly different from the negative control ($p \leq 0.05$, Student's *t* test). $$ significantly different from the negative control ($p \leq 0.01$, Student's *t* test)

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