Diogo Filipe Amaro Santana Graça

Licenciado em Biologia

Biological Effects of Acrylic Engineered Particulate-Systems

Dissertação para obtenção do Grau de Mestre em Bioquímica

Orientadora: Ana Bettencourt, Professora Doutora,
Faculdade de Farmácia, Universidade de Lisboa
Co-orientadora: Maria João Silva, Professora Doutora,
Instituto Nacional de Saúde Doutor Ricardo Jorge

Júri:

Presidente: Prof. Doutor Pedro António de Brito Tavares
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FACULDADE DE CIÊNCIAS E TECNOLOGIA
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Abstract

Polymeric particulate-systems are of great relevance due to their possible biomedical applications, among them as carriers for the nano- or microencapsulation of drugs. However, due to their unique specific properties, namely small size range, toxicity issues must be discarded before allowing its use on health-related applications.

Several polymers, as poly(methyl methacrylate) (PMMA), have proved to be suitable for the preparation of particulate-systems. However, a major drawback of its use refers to incomplete drug release from particles matrix. Recent strategies to improve PMMA release properties mention the inclusion of other acrylic polymers as Eudragit (EUD) on particles formulation. Though PMMA and EUD are accepted by the FDA as biocompatible, their safety on particle composition lacks sufficient toxicological data.

The main objective of this thesis was to evaluate the biological effects of engineered acrylic particulate-systems. Preparation, physicochemical characterization and *in vitro* toxicity evaluation were assessed on PMMA and PMMA-EUD (50:50) particles.

The emulsification-solvent evaporation methodology allowed the preparation of particles with spherical and smooth surfaces within the micrometer range (±500 nm), opposing surface charges and different levels of hydrophobicity. It was observed that particles physicochemical properties (size and charge) were influenced by biological media composition, such as serum concentration, ionic strength or pH. In what concerns to the *in vitro* toxicological studies, particle cellular uptake was observed on different cell lines (macrophages, osteoblasts and fibroblasts). Cytotoxicity effects were only found after 72 h of cells exposure to the particles, while no oxidative damage was observed neither on osteoblasts nor fibroblasts. Also, no genotoxicity was found in fibroblast using the comet assay to assess DNA damage. This observation should be further confirmed with other validated genotoxicity assays (e.g. Micronucleus Assay).

The present study suggests that the evaluated acrylic particles are biocompatible, showing promising biological properties for potential use as carriers in drug-delivery systems.

Keywords: particulate-systems; PMMA; Eudragit; particle characterization; biological effects**;** *in vitro* toxicity.

Resumo

Os sistemas de partículas poliméricas têm adquirido uma grande relevância devido às suas possíveis aplicações biomédicas, entre elas para nano- ou microencapsulação de fármacos. No entanto, as propriedades específicas destas partículas, designadamente o reduzido tamanho, tornam necessário a avaliação da sua toxicidade de modo a garantir a sua utilização segura.

Muitos polímeros têm demonstrado potencial para este tipo de aplicações, designadamente o poli(metil metacrilato) (PMMA). Contudo, a sua aplicação é muitas vezes limitada pela libertação incompleta dos fármacos através da matriz polimérica. De modo a aumentar a permeabilidade das partículas de PMMA, estratégias recentes têm vindo a incluir outros polímeros acrílicos na formulação, designadamente o Eudragit (EUD), visando melhorar a libertação dos fármacos. Apesar dos polímeros referidos serem aceites pela FDA como biocompatíveis, não existem estudos suficientes relacionados com a sua toxicidade na forma de partículas.

O principal objectivo desta tese foi avaliar os efeitos biológicos de partículas acrílicas. Em particular, realizou-se a preparação, caracterização físico-química e a avaliação de efeitos tóxicos em culturas celulares de partículas de PMMA e de PMMA-EUD (50:50).

As partículas, obtidas pelo método de emulsão-simples por evaporação de solvente, demonstraram uma distribuição de tamanhos semelhantes (±500 nm), cargas de sinal contrário e diferentes níveis de hidrofobicidade. Foi também observado que o tamanho e a carga das partículas eram influenciados pela composição do meio biológico (concentração do soro, força iónica e o pH).

Relativamente aos ensaios de toxicidade, a internalização das partículas foi confirmada em várias linhas celulares (macrófagos, osteoblastos e fibroblastos). Só foram observados efeitos citotóxicos após 72 h de exposição às partículas. Não foi detetada a produção de espécies reativas de oxigénio em osteoblastos nem em fibroblastos. Também não foram observados quaisquer efeitos genotóxicos através da avaliação de lesões no ADN de fibroblastos através do ensaio do cometa. Estes resultados devem ser confirmados através de ensaios já validados para avaliação de genotoxicidade (ex: Ensaio dos Micronúcleos).

O presente estudo sugere que as partículas acrílicas avaliadas são biocompatíveis, tendo sido demonstrado que apresentam propriedades biológicas interessantes para uma possível aplicação em veiculação de fármacos.

Palavras-chave: sistemas de partículas; PMMA; Eudragit; caracterização de partículas; efeitos biológicos; toxicidade *in vitro*.

Index

Index of Figures

Index of Tables

List of Abbreviations

ATA - Tocopherol acetate

BCA – Bicinchoninic Acid

BSA – Bovine Serum Albumin

CB – Bi-nucleated cells

CBMA - Cytokinesis-block micronucleus assay

CBMN – Number of micronuclei in bi-nucleated cells

CBPI – Cytokinesis-block proliferation index

CPZ – Chlorpromazine

DCM - Dichloromethane

DMSO – Dimethyl sulfoxide

DSB – Double-Strand Breaks

EMEA - European Medicines Agency

EUD – Eudragit RL 100

FBS – Fetal Bovine Serum

FDA – Food and Drug Administration

FF – Fast-Flow

FPG – Formamido-pyrimidine-DNA-glycosylase

FRP – Free-radical polymerization

FT-IR – Fourier-Transform Infrared Spectroscopy

HIC – Hydrophobic Interaction Chromatography

ICH - International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use

IEP – Isoelectric Point

IL-6 – Interleukin-6

LDH – Lactate Dehydrogenase

MCP-1 – Monocyte Chemoattractant Protein

MEM – Minimum Essential Medium

MMA – Methyl methacrylate

MTT - 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide

NR – Nile Red

OECD - Organization for Economic Co-operation and Development

PBMAD - Poly(butadienemaleic anhydride-co-L-DOPA)

PBS – Phosphate buffered saline

PCL - Poly-ε-caprolactone

PEG – Polyethylene glycol

PEI - Polyethileneimine

PGE-2 – Prostaglandin E2

PLGA - Poly(lactide-co-glycolide)

PLLA - Polylactide

PMA - Phorbol 12-myristate 13-acetate

PMMA – Poly(methyl methacrylate)

PMMAp – PMMA particles

PMMA-EUDp – PMMA/EUD particles (50:50)

PS – Polystyrene

PVA – Polyvinyl acetate

ROS – Reactive Oxygen Species

SCF – Supercritical Fluid

SESE – Single Emulsion by Solvent Evaporation

SDS – Sodium Dodecyl Sulphate

SSB – Single-Strand Breaks

TEM – Transmission Electron Microscopy

XTT – 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

UV – Ultraviolet

VMD – Volume Mean Diameter

YP – Yield of Production

Objectives and Thesis Structure

The thesis main objective was to evaluate the potential biological effects of engineered acrylic particulate-based systems.

Specific aims were:

- Optimization of the preparation method for obtaining two types of particles - PMMA and PMMA-Eudragit (50:50) - within the same micrometric size range and with opposite surface charges;
- Characterization of the particles physicochemical properties, as well as the effect of the biological conditions on those features;
- Evaluation of particles biological/toxicological effects by *in vitro* cellular assays.

The thesis is structured in four chapters including: *Introduction*, *Materials and Methods*, *Results and Discussion* and *Conclusions and Future Work*.

Chapter 1 – *Introduction*

Contains a brief description of the state of the art of various polymers that have already been described for particle formulation, followed by the specific case of the acrylic particulate-systems. Methods on particle formulation are then approached, as well as their influence on the particles potential toxicological effects. After, a description is made on several physicochemical properties with influence on particles' toxicity. It ends up with a general description of several *in vitro* toxicological assays.

Chapter 2 – *Materials and Methods*

This chapter is organized in three main parts. The first and second parts detail the preparation and characterization of the particles, while the third part describes the *in vitro* cellular studies, as well as the conditions in which they were conducted. All of the reagents, materials, equipment and methods used in this work are presented in detail.

Chapter 3 – *Results and Discussion*

Results concerning particles preparation and characterization in terms of size, surface charge, shape, chemical composition and hydrophobicity, as well as charge and size variation with medium composition, including protein adsorption, are presented and discussed. This is followed by a detailed discussion of the biological effects resulting from the exposure of several cell lines to the particulate-systems. Whenever available, a comparison is made between the obtained results consistency and the existent literature over the evaluated subjects on this work.

Chapter 4 – *Conclusions and Future Work*

In this chapter, the main conclusions of the project are summarized and a reflection is made over the possibilities for future work, as well as possible improvements on the established methodologies on this thesis.

Chapter 1. Introduction

1.1. Polymeric particulate-systems

Recent advances in polymer science have provided many innovations, underlining an increasing importance of polymeric particulate-systems in both therapeutic and diagnostic applications (Juneja and Joy, 2014; Papa *et al.*, 2014).

In general, nanoencapsulation of drugs in particulate-systems involves forming drug-loaded particles with diameters ranging from 1 to 1000 nm (Reis *et al.*, 2006, Naahidi *et al.*, 2013). Particulate-systems offer relevant advantages in drug delivery by targeting molecules in specific cells and controlling drug release over time aiming to solve several problems related to the drugs themselves, such as low solubility, poor stability and unwanted side effects (Choi *et al.*, 2012). The small size of these particles can have a huge impact over macro carrier systems, since it theoretically allow them to surpass barriers that other kind of carriers cannot. The use of polymeric materials for the synthesis of particles can constitute an improvement for the biomedical field, due to their flexibility in terms of size, mechanical stability, surface functionalization and hydrophilic/lipophilic properties (Papa *et al.*, 2014).

Many biocompatible polymers are available to prepare particulate-systems that are being used in biomedical applications as carrier materials of molecules like DNA, proteins and drugs, as well as in cell tracking and labelling for fluorescence or magnetic resonance imaging (Höchler *et al.*, 2012). There are multiple examples of such polymers including synthetic compounds as poly(lactide-co-glycolide) (PLGA) and poly-ε-caprolactone (PCL). For example, PLGA has been used in the preparation of microspheres (≈ 20 μm) for the sustained release of resperidone, an antipsychotic drug (D`Souza *et al.*, 2014), and PCL has been used to formulate microspheres (28 – 43 μm) and nanospheres (750 nm), for insulin controlled-release studies (Mukerjee *et al.*, 2007). Chitosan and its derivatives are another example of biocompatible polymers of natural origin used in drug delivery, as reported in the study of Gomathi *et al.* (2014), related to the preparation of particles (120-220 nm) loaded with lenalidomide, an anti-cancer drug.

In addition, the acrylic based polymers, such as poly(methyl methacrylate) (PMMA) and the Eudragit series, are a class of synthetic polymers with numerous biomedical applications.

1.1.1. PMMA particulate-systems

PMMA polymer (Figure 1), also designated as poly(methyl 2-methylpropenoate) (IUPAC name), is one of the most widely explored biomedical biomaterials because of its biocompatibility, versatility and low cost (Bettencourt and Almeida, 2012). It is a non-biodegradable synthetic homopolymer of methyl methacrylate (MMA) monomer, which is vastly applied in the biomedical field as implant

cement, in intra-ocular lenses (Pratt *et al.*, 2006), as well as prosthetic (Reis *et al.*, 2008) and mandibular dental material (Lye *et al.*, 2011; Puricelli *et al.*, 2011).

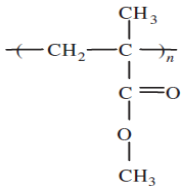

Figure 1 - Chemical structure of PMMA repeating unit.

The Eudragit polymer series is a trademark of Rohm GbmH & Co. KG. (Darmstadt, Germany) and comprise a number of (co-)polymers generally originated from polymerization reactions of acrylic and methacrylic acids or their esters. Each Eudragit compound has its own specific physicochemical properties derived from the added functional groups. Eudragit RL 100 (Figure 2) could be used in the preparation of polymeric particles because it can improve the matrix permeability, thus improving the drug release rates (Joshi, 2013).

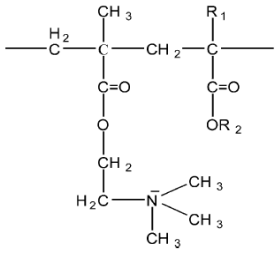

Figure 2 - Chemical structure of Eudragit RL 100.

In the last decades, the focus on the use of PMMA as a carrier material has increased, and it has been adapted as a skeletal drug delivery system for releasing the drugs from macrodevices (bone cement and beads) (Bettencourt and Almeida, 2014). Moreover, particulate-systems based on PMMA are under investigation for multiple biomedical applications. For example, in Tencomnao *et al.* (2012), magnetic PMMA core/ polyethileneimine (PEI) shell particles proved to be promising new alternatives for magnetically-assisted gene transfection. In addition, different classes of drugs are being evaluated to be loaded into PMMA particulate-systems (PMMAp), aiming various routes of administration, such as oral and topical. Examples of successful drug incorporation include antibiotics (Naves *et al.*, 2013), antioxidants like vitamin E (Bettencourt *et al.*, 2010), antidiabetics (Cui *et al.*, 2006; Dhana lekshmi *et al.*, 2010) and anticancer drugs as gemcitabine (Wang *et al.*, 2014). It is also possible to include bioactive substances, as bovine serum albumin (BSA), which can confer new properties to the PMMAp. In a relatively recent study, researchers were able to prepare protein-polymer hybrid particles composed of PMMA and BSA as a carrier system for anti-cancer drugs (Ge *et al.*, 2012).

PMMA application as a vaccine coadjuvant has also been explored. Kreuter and Speiser (1976) showed the capacity for PMMAp, with the influenza virus inserted either before or after polymerization, as new vaccine adjuvants, though the first showed to have greater efficiency than the latter. In Voltan *et al.* (2007), PMMA core/EUD shell particles (< 300 nm) were prepared with reversibly-bound proteins (trypsin and lysozyme) which are able of aiding in the antigen incorporation, protecting it from oxidation and preserving its biological activity. Caputo *et al.* (2009) showed the safety of PMMA-core particles with a Eudragit L-100-55 outer-shell (with a size range between 0.22 - 2.00 µm) with hydrophilic surface properties, to be used as HIV-1 Tat protein-based vaccines, in mice by both mucosal and systemic administration.

The interest on such applications for PMMA is mainly due to a great number of advantages related to the kinetics of drug transport into the organism, but also to the possibility for pH- and thermo-dependent release, good mechanical stability and biocompatibility (Bettencourt and Almeida, 2012).

In spite of the tremendous potential of PMMAp, a major drawback of its use refers to the incomplete drug release. To improve release profiles, recent strategies are focusing on formulating PMMA composites with permeable polymers, such as Eudragit RL 100 (Bettencourt and Almeida, 2012).

The method chosen for particle formulation influences the particles properties and, therefore, will affect its potential toxicity. With this in mind, multiple techniques exist for the purpose of preparing PMMAp for a given target application, as described in the next section.

1.1.2. Preparation techniques

PMMAp, as spheres (monolithic devices) or capsules (reservoir devices), can be conveniently prepared by different methodologies. These techniques can be divided under two main types: 1) direct polymerization of the MMA monomer using polymerization reactions and 2) formulation from pre-formed PMMA polymer.

Polymerization techniques include conventional emulsion, surfactant-free emulsion and micro/mini-emulsion. These methods always require a physical or chemical initiation step (Bettencourt and Almeida, 2012).

The second set of techniques uses a pre-formed polymer rather than a polymerization reaction. Examples are emulsion by solvent evaporation/extraction, nanoprecipitation, spray-drying, crystallization and Supercritical Fluid (SCF) methodologies (Bettencourt and Almeida, 2012). Among those techniques, single emulsion by solvent evaporation (SESE) is one of the most used and it will be explained in detail because it was the technique used in the present work.

SESE

The single emulsion by solvent evaporation method is one of the most applied techniques for particle preparation, since it is one of the easiest methods (Bettencourt and Almeida, 2014). This technique is based on the emulsification of an organic phase, where the hydrophobic polymer is solubilized, into a water phase with a surfactant agent. Then, stirring conditions are applied, generally at room temperature, as the evaporation of the organic solvent occurs. This step results in the deposition of the polymer, forming particles. Most of the particles prepared by this method acquire a spherical conformation (Bettencourt and Almeida, 2012).

Some of the advantages of the use of this technique are their easy-to-apply steps and processes and the possibility to develop various types of particles, either empty or with encapsulated compounds. It is also one of the most affordable methods for obtaining emulsions. However, since it is also necessary to apply organic solvents (as dichloromethane) and surfactant agents (as polyvinyl acetate), there could be some toxicity events if these substances are not completely eliminated from the final formulations (Soomro *et al.*, 2011; Bettencourt and Almeida, 2012).

1.1.3. Toxicological concerns

As previously mentioned, PMMAp can have a great positive impact in health-related fields and presents alternatives for numerous applications, such as drug delivery systems or the development of new vaccines. Having this in mind, an exhaustive characterization of the particles' toxicological potential needs to be made in order to ensure its safe application in biological systems.

Biocompatibility of PMMA polymer

PMMA, a Food and Drug Administration (FDA) approved polymer, has been extensively used in the past decades for both orthopaedic and ophthalmic applications showing good toxicological profiles (Fonseca *et al.*, 2013). It is a material that has been described as physiologically harmless, hence biocompatible, and it is not attacked by either moulds or enzymes, which have been some of the reasons for its numerous applications in the biomedical field. Curiously, in World War II, there were reports of PMMA fragments sticking into airplane pilots' eyes with no observable abnormal reaction by the tissue (Pratt *et al.*, 2006). In spite of its well-known biocompatibility, recent studies lead to some concerns about the material. Dansereau *et al.* (2008) observed the development of granulomas in patients submitted to cosmetic treatments with PMMA. Other studies using PMMA microspheres on soft tissue enlargement show a rare but existent risk for developing fibrosis of the treated tissue, though this side effect has been shown to be dependent on the method applied for developing the microspheres (Campos *et al.*, 2011). Also, a clinical case has raised an issue when applying PMMA as a polymer for mandibular reconstruction as it may induce necrosis in bone tissue due to the exothermic polymerization process (Cakarer *et al.*, 2010).

PMMA particulate-systems biocompatibility

In particular, when referring to PMMA in nano- and micro-size range, it is known that PMMA in the form of wear debris has been frequently present in patients' bodies, but what happens to PMMA engineered particles for drug delivery lacks considerable scientific studies (Bettencourt and Almeida, 2014). The interaction of PMMAp with cells and the extracellular environment can trigger a sequence of biological effects considerably different from the material in the macro-size form. Particularly, some concern exists regarding the non-biodegradable behaviour of the nanoparticles, which can lead to their over-accumulation near organs and, eventually, to the development of harmful effects, which turns it into a toxicity-increasing factor (Rimessi *et al.*, 2009). Besides the material toxicity, another important aspect to look for is the relevance of the preparation processes for particle safety. Preparation methods often require the use of surfactant agents and organic solvents, which may increase the risk of toxicity upon human exposure to the particles.

So far, the majority of studies that specifically address the evaluation of PMMAp biocompatibility did not find any significant toxicity. Ge *et al.* (2012) results are illustrative of this, as no toxicity from PMMAp conjugated with BSA was found with the MTT test, either *in vitro* with human colorectal cancer cells (HCT116) or *in vivo* assays with mice. The authors pinpoint the negative charge of the particles as responsible for the reduced toxicity because they interact less with the cells' membranes since they are also anionic. Furthermore, PMMA–chitosan microspheres (50-500 μm) were shown to be hemocompatible and non-cytotoxic to mouse fibroblast cells (Changerath *et al.*, 2009). Kundu *et al*. (2014) and Hazra *et al*. (2014) showed that PMMAp (<100 nm) coated with biosurfactants prepared through atomized microemulsion with and without ultrasounds, respectively, did not originate cytotoxicity on human peripheral blood mononuclear cells. Relatively to *in vivo* studies, Dhana lekshmi *et al.* (2010) concluded that no obvious toxicity was observed after oral administration of repaglinide-loaded PMMAp to albino rats.

Overall, there is insufficient data on PMMA particulate-based carriers' toxicity, as happens in general with all engineered nanoparticles for drug delivery. Lack of standard biocompatibility evaluation criteria is a subject of intense discussion and in the future it is advisable that the toxicological evaluation should become an important part in the design of such particulate-systems, as well as the establishment of standardized protocols.

1.2. Toxicological evaluation of particulate-systems

1.2.1. The role of physicochemical properties

When evaluating the toxicological effects of particulate-systems, it is extremely important to characterize the physicochemical properties that are likely to influence cell and tissue processes. In fact, specific physicochemical properties of the materials at the nano- and microscale, such as size,

charge and hydrophobicity can greatly differ from the ones of the bulk material and, thereby, can also drive unpredictable biological interactions and effects (Louro *et al*., 2014).

Size plays an important role in biological interaction since the smaller the size of the particle, the higher will be its surface reactivity and the higher will be its capability to interact with multiple organelles. Another important property of smaller particles is their capability of surpassing barriers of subcellular structures, therefore, enabling them to reach structures like the nucleus. Though this potential biological interaction may be an advantage in terms of drug delivery innovative treatments for various conditions, it has also the reverse aspect of constituting a potential toxicity induction factor (Oberdörster *et al.*, 2005).

Charge is also one of the determinant properties of biological interaction, since it relates with repulsion and attraction between different components. In fact, cationic particles have been shown to produce more effects on various cells than neutral or anionic particles. In Verma *et al.* (2008), uptake of anionic nanoparticles (≈ 6 nm) didn't cause the formation of many pores in the cell membrane and cytotoxicity was minimal, while their cationic counter-parts showed to penetrate by generating pores on the cells' membranes and produced cytotoxic effects on a mouse dendritic clone cell line. In Arvizo *et al.* (2010), it was demonstrated that the uptake of cationic gold nanoparticles caused decreased proliferation and cell viability of normal cells but not of cancer cells.

Another important property of the particles used as carriers is their hydrophobicity. This property concerns water-interactions and may affect not only the relation with the surrounding cells, but also with other compounds present in the biological media. Highly hydrophobic particles may be poorly stable and, therefore, are highly susceptible to aggregation when interacting with biological media (Ge *et al.*, 2011). In consequence, particles may also have lower clearance rates, leading to their accumulation on various organelles/cells, which in turn may cause dose-dependent toxicity (Zhu *et al.*, 2009; Murphy *et al.*, 2011).

A common interaction between particles and substances in biological media is the formation of protein corona, a layer of various proteins capable of interacting with the particles surface and even alter its properties. These can be highly influenced by charge and hydrophobicity, as well as other surface-related physicochemical properties. Particle-protein interaction is a dynamic process, as the composition of the protein layer may vary as the particles travel across different compartments in the biological system (Kane and Stroock, 2007). The resulting effect from this protein layer is generally changes in biological interaction Pelaz *et al.*, 2013).

1.2.2. *In vitro* cellular assays

At an initial stage, toxicity of particulate-systems can be evaluated through *in vitro* cellular assays.

In vitro assays allow an evaluation of cells exposure to diverse conditions, recurring to cell lines instead of a whole individual. In some cases, *in vitro* assays may be enough for toxicity assessment of a given substance, preventing the use of invasive methods on living organisms, e.g. rodents. However, their biggest downside is the difficulty of simulating a whole organism function and dynamics in aspects, such as cell-cell or surrounding tissues interactions, which may generate different effects based on parameters, as the location of the cells and their distribution over a generic area (Takhar and Mahant, 2011). Moreover, *in vitro* assays are restricted to observations at molecular and cellular levels, while *in vivo* studies account for the several layers of complexity inherent to the integrated response of a whole-organism and that may influence the outcome of the exposure to agents under study (Louro and Silva, 2010). Nevertheless, *in vitro* studies are valuable, rapid and cost-effective for the frontline toxicity assessment of new molecules or particle formulations and some limitations are being addressed with the development of 3D cultures, which is gaining increasing interest as researchers are realizing the limitations of 2D cultures (Caicedo-Carvajal *et al.*, 2011).

As a first stage to assess particles toxicity it is important to evaluate their cellular uptake. For that evaluation, particles loaded with fluorescent compounds can be prepared. Some of the most commonly used fluorescent probes for this purpose are organic fluorescent dyes, such as Nile Red, which is widely used due to its absorption/emission wavelengths, high solubility on organic solvents and easy applicability on almost any particle formulation methods (Rose, 2010).

The use of fluorescent probes is an important tool for the identification of sites of interest and allows the visualization and evaluation of some effects over specific locations as far as the nuclear level (Forster *et al.*, 2012).

Cellular toxicity can be mediated by diverse mechanisms, including nutrient receptors blockage, DNA damage, oxidative stress, protein synthesis inhibition and many others. Hence, there is a need for multiple-parameter testing to evaluate toxicity of a given tested material. Moreover, a toxic effect includes many types of toxicity, e.g.: immunotoxicity, cytotoxicity, genotoxicity, among others. For the purpose of safety evaluation of new drugs, genotoxicity assessment is, unequivocally, one of the most important aspects given that genotoxicity is intimately related to carcinogenicity. Cytotoxicity and genotoxicity are distinguishable adverse effects given that the former is related with damage on the cellular level and the latter at the genetic level (O'Brien and Haskins, 2006). Both types may result from a direct effect or from a stress response of the cell due to the production of reactive species.

1.2.2.1. Cytotoxicity

Cell viability is the most commonly investigated parameter in cytotoxicity testing. As cell viability is determined by various cellular processes, different endpoints are currently used to assess the actual state of the cells *in vitro*, such as the detection of mitochondrial activity and cellular membrane integrity (Kroll *et al.*, 2009). One of the most common assays for the detection of mitochondrial activity is the MTT, a method first developed by Mosmann *et al*. (1983) to evaluate cell proliferation that enables a fast and quantitative measurement of living cells allowing the analysis of multiple samples at the same time. It is achieved through the reaction of of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), a yellow, water-soluble tetrazolium dye, which is converted by the viable cells' mitochondrial dehydrogenases into a water-insoluble, purple compound (formazan) by cleavage of the tetrazolium ring (Figure 3).

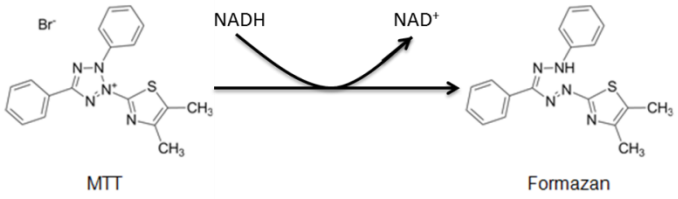

Figure 3 - MTT conversion into formazan crystals inside the mitochondria (adapted from Riss *et al*., 2013).

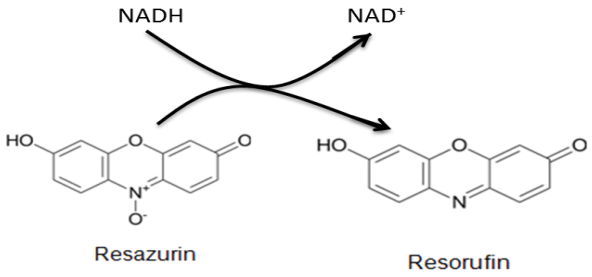

Figure 4 - Conversion of resazurin into resorufin by viable cells (adapted from Riss *et al*., 2013).

Another cytotoxicity test is the Alamar Blue Assay. This method is a more accessible alternative to MTT test and it is based on the conversion of 7-Hydroxy-3H-phenoxazin-3-one 10-oxide (resazurin), a weakly fluorescent blue dye, into resorufin, a highly red fluorescent pink coloured compound. In this redox reaction, the transference of electrons from NADH, NADPH, FADH or FMNH, as well as some cytochromes, by mitochondrial enzymes to rezasurin reduces it into resorufin (Figure 4). This technique has the disadvantage of requiring a spectrophotometer with specific filters, which may be financially incompatible with many laboratories (Borra *et al.*, 2009; Rampersad, 2012).

Other method, which may be applied to evaluate cell viability, is by measuring membrane integrity using the Trypan Blue exclusion assay. The main principle applied in this methodology is that living cells possess intact cell membranes which exclude the internalization of certain dyes, such as trypan blue (Strober, 2001). Intact/viable cells will be presented as white/non-coloured, while damaged cells should be presented as blue cells. However, care should be taken in result interpretation as not always does the uptake of the dye indicate that a cell is unviable. In fact, as shown by Tran *et al.* (2011), some toxins may originate pores over the cellular membrane, therefore, increasing its permeability to the dye, even though the cell is still viable as its recovery mechanisms are capable of repairing most of the damage. This was shown by comparing cells coloured after 2 h of exposure to the toxin with cells to which the toxin was removed after that period and left to recover for 24 h. Furthermore, metabolic activity was found to be active since cells were still able to produce ATP.

Therefore, one must take into account the cell repair mechanisms since a cell membrane may be able to recover from damage, hence the cell may still viable. Also, the dye itself may induce damage to the cells after a long period of exposure, which ultimately leads to false positives. However, it has the advantage of being more accessible than other existing alternatives for cytotoxicity evaluation, such as MTT or Alamar Blue assays.

1.2.2.2. Genotoxicity

Genotoxicity can be derived from either primary or secondary effects, where the first can be sub-divided into direct and indirect effects.

Within the primary genotoxic effects, a direct effect derives from a direct interaction between the exogenous compounds and DNA, or any other molecule or process responsible for regulating its integrity. It might be caused, for instance, by the direct contact of particles with DNA, either through physical or chemical processes. An indirect effect will arise from oxidative stress generated from by-products of the reaction between particles and other organelles, either through generated reactive oxygen species (ROS) which can deplete the available anti-oxidants (Donaldson *et al.*, 2010) or from ionic species resulting from soluble particles (Magdolenova *et al.*, 2014).

Secondary genotoxicity is associated with an inflammatory response to the presence/aggregation/accumulation of exogenous particles. This process can also lead to oxidative stress and subsequently cellular and DNA damage. Moreover, persistent oxidative stress can occur, leading to the sum of DNA damage and, eventually, to carcinogenicity (Donaldson *et al.*, 2010).

Each type of particle has its own set of properties, as described above, which can determine the potential for genotoxicity as well as the type of effect induced (Magdolenova *et al.*, 2014). For a particle to produce primary direct genotoxicity it must have the potential to enter the nucleus and

interact with DNA, which can occur by various mechanisms. According to the literature, each particle may have its own mechanisms, though most particles show size-dependent entry (Chen and von Mikecz, 2005; Geiser *et al.,* 2005; Nel *et al.*, 2006).

Several studies (Park *et al.*, 2008; Li *et al.*, 2009; Wang *et al.*, 2009) indicate that nanoparticles may decrease significantly the amount of anti-oxidants inside the cell, hence increasing the amount of free radicals and other reactive species which may affect DNA integrity. One of the by-products of mitochondrial processes, namely mitochondrial respiration, that can reproduce this mechanism is H_2O_2 originated by dismutation of superoxide anions, either spontaneously or through superoxide dismutase activity. The inhibition of the DNA repair mechanism has also been reported for metal ions that can be released from metallic nanoparticles, which seem to inhibit the activity of some of the proteins involved in this biological process (Donaldson *et al.*, 2010; Magdolenova *et al.*, 2014).

The same kind of effects may also underlie indirect genotoxicity when particles small enough to allocate near the nucleus are able to drag other small molecular weight compounds or reactive species generated on its surface, which can then diffuse into the nucleus and induce DNA damage. According to literature (Gedik *et al.*, 2002; Schins *et al.*, 2002), guanine is one of the most susceptible nucleotides to suffer damage from ROS forming 8-oxo-guanine adducts. The presence of these DNA adducts may be detected through the use of the enzyme formamido-pyrimidine-DNA-glycosylase (FPG) in a modified version of the comet assay (Donaldson *et al.*, 2010).

ROS can also result from an inflammatory process, resulting in secondary genotoxicity as previously mentioned. This process can induce a high rate of oxygen consumption which can lead to the activation of NADPH-oxidase whose reaction can produce an elevated number of ROS and add to the already existing reactive species from other processes. Though these are produced as a self-defence mechanism they can produce damage in the surrounding cells and may transverse the nuclear membrane, causing oxidative damage in the DNA molecule (Donaldson *et al.*, 2010).

Most studies pinpoint surface area and its reactivity as the two main factors involved in dose-dependent genotoxic events. As previously stated, a protein corona may be formed on the particles surface, which could imply a variation on their genotoxic potential, while making it hard to identify the source of the effect. It is also difficult to study this process of corona formation *in vitro* since, in the native organism, the particles can transverse different combinations of proteins, impossible to simulate in the cell culture environment (Donaldson *et al.*, 2010).

Depending on particle size, aggregation can also produce genotoxic effects upon entering the cell, since larger particles can accumulate outside the nucleus and deform it, while smaller particles can translocate its barrier through pores and accumulate inside, where it can lead to physical damage to the chromosomes (Magdolenova *et al.*, 2014).

Genotoxicity evaluation

The assessment of genotoxic effects of a given substance generally follows the cytotoxicity analysis in order to evaluate only non-cytotoxic doses since cell death can increase the levels of DNA fragmentation, leading to false positive results. There are a few methods that can be employed for assessment of the genotoxicity of manufactured nanomaterials, e.g. the comet and the micronucleus assays. In addition, attention has to be given to the choice of the cell line since it should be biologically relevant and must not be affected by the method itself (Donaldson *et al.*, 2010). Another issue in testing the genotoxic effects produced by nanoparticles is the choice of relevant positive and negative controls. Since most assays focus on the oxidative damage to DNA, some of the most employed positive controls are H_2O_2 and UV radiation. However, a number of other positive control candidates are being tested (Donaldson *et al.*, 2010).

There are a number of available methods for genotoxicity evaluation either for *in vitro* or *in vivo* conditions. The two most commonly used are the comet assay and the micronucleus assays.

The comet assay, also known as single-cell gel electrophoresis, is based on the evaluation of breaks in the DNA double-helix, either on both strands (double-strand breaks, DSBs) or on a single strand (single-strand breaks, SSBs). For this test, the cells are exposed to the material in evaluation for a certain period of time and then the cells are lysed in order to obtain the nucleoids. Then this organelles are put under unwinding conditions in such a way that any breaks in the helix will migrate further than the remaining of the intact chains through gel electrophoresis under alkaline conditions. The visualization of this effect through fluorescence resembles a comet, hence the name of this assay. The experimental overlay of the traditional comet assay is presented on Figure 5. The evaluation consists on analysing the percentage of DNA in the tail of these “comets”, which indicates the percentage of damaged DNA, as the intact DNA remains in the “head” of the comet. In order to analyse DNA oxidative damage enzymes, such as Endonuclease III or FPG, can be used to detect oxidation of pyrimidines and purines respectively. It could be argued that the nanoparticles may interact with FPG and inhibit its effect, yet a study by Magdolenova *et al.* (2012) was able to dismiss this hypothesis.

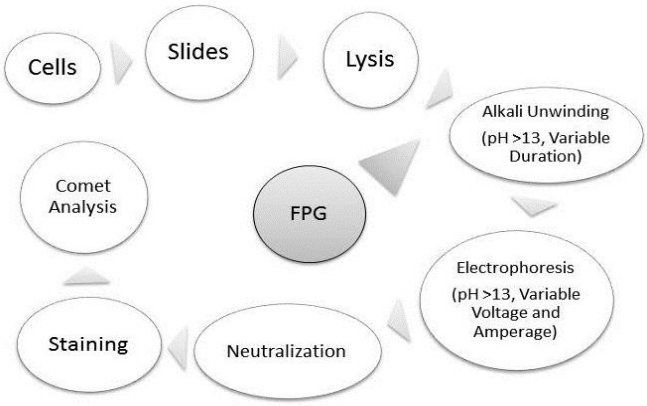

Figure 5 - Critical steps for the comet assay on alkaline conditions (Adapted from Tice *et al*., 2000).

The micronucleus assay is based on the formation of very small nuclei during anaphase, when the nucleus divides forming two separate nuclei. It evaluates chromosomal abnormalities. Micronuclei contain either whole chromosomes or fragments which do not incorporate in none of the originated nuclei. It can be detected in the cytoplasm upon staining. This assay can be improved by adding a cytokinesis blocker with no effect over mitosis, such as Cytochalasin B, which allows obtaining multiple bi-nucleated cells and, therefore, by analysing only these, excluding interference from micronuclei generated before the assay. This method is also known as cytokinesis-block micronucleus assay (CBMA) and the possible occurrences on the cells are presented on Figure 6 (Fenech, 2000; Magdolenova *et al.*, 2014).

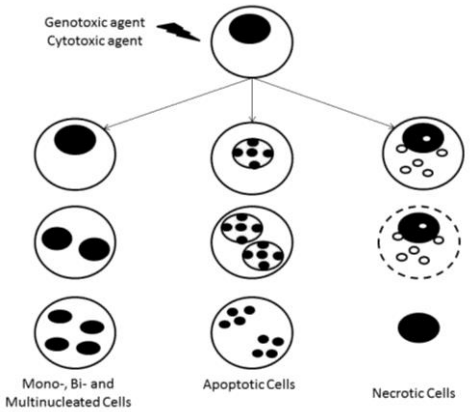

Figure 6 - Potential fates of cells during CBMA after exposure to a potential genotoxic agent (Adapted from Fenech, 2000).

Other methods widely used in genotoxicity assessment include the Chromosomal Aberration assay, Ames test, HPRT gene mutation and the H2AX assay.

Each assay can have its own strengths and weaknesses as each is focused on the evaluation of different endpoints. The comet assay can measure in detail low-levels of damaged DNA and can be

adapted to identify specific lesions on the chain, such as the ones produced through oxidative damage, but has the downside of being a somewhat subjective assay since it generally requires an operator to manually identify the comets. If not careful, an operator can be biased by knowing which sample was treated or not which can lead to ignoring some of the comets simply because they don't fit with collected data resulting in a sort-of manipulation of the results. As for the micronucleus assay, it is a less quantitative assay than the comet assay (Magdolenova *et al.*, 2014).

There are a number of variables which can influence the results obtained through genotoxicity-evaluating assays. From the method applied for the preparation of nanoparticles, to the choice of biologically significant concentrations, through the existence of impurities over the analysed samples, to the choice of the cell lines themselves, or even the way the particles are taken up at a certain cellular level (Magdolenova *et al.*, 2014).

The vehicles used on particle dispersion may, in some cases, alter particles properties, such as their surface charge or hydrophobicity, which can have a direct or indirect impact over their potential for generating genotoxic responses. Hence, it must be a parameter to take into account when evaluating toxic effects. Care must also be taken while using very high concentrations of particles for they tend to agglomerate which may affect the amount of particles available for cell intake and, therefore, result in false positive/negative assessments (Magdolenova *et al.*, 2014).

Another element which may affect these results is the presence of impurities or other chemical species mixed with the particles, such as free ionic species, ROS or proteins which promote agglomeration, since they can produce changes on the particles or simply have a direct effect over the cells during exposure, which could lead to false positive results (Magdolenova *et al.*, 2014).

One more variable is the cell line itself, since the same particles may interact differently with distinct cell lines, even if the cells derive from the same tissue origin. Factors as the receptors and transporter proteins present on the cells surface or even the amount of antioxidants and the metabolic pathways of each cell line may induce different responses when in contact with the materials/particles, since there are some lines more prone to damage than others (Magdolenova *et al.*, 2014).

Apart from these issues, operator experience also plays a role on assay variability and it is often desirable that a set of assays are performed by the same operator, minimizing experimental variation, or following specific guidelines, such as the ones described by Fenech (2000) for the micronucleus assay.

Finally, it is very important to guaranty that the particles themselves do not interfere with the chosen assay in order to achieve reproducible results (Magdolenova *et al.*, 2014).

There are some differences between what can be achieved, in terms of toxicity evaluation, through *in vitro* or *in vivo* assays. The main limitation of *in vitro* assays is that they can only evaluate primary genotoxicity due to the fact that it is very hard to reproduce all the *in vivo* conditions, in which secondary genotoxicity from the ROS originated through the inflammatory mechanism. However, *in vitro* studies can be useful to study concentrations, which may be excessive for many organisms. For biological relevance purposes the time of exposure for *in vitro* studies should be longer than the cell cycle period in order to maximize the accessibility of the cells to the particles. Also, the protein content must be taken into account since there are major differences between these systems in terms of both concentration and variability of proteins (Magdolenova *et al.*, 2014).

Having all of the above in mind, PMMAp and PMMA-EUDp potential genotoxic effects were evaluated through the combination of Comet and Micronucleus in a fibroblast cell line.

1.2.2.3. Stress response

Both cyto- and genotoxicity are often mediated by oxidative stress from ROS or their excessive accumulation as reactional by-products. Several authors implicate the generation of ROS as early signs of nanoparticle-induced cellular damage. Some of the underlying mechanisms which originate oxidative stress are mitochondrial respiration and apoptosis, NADPH oxidase system activation, interference in calcium homeostasis or depletion of antioxidant enzymes. Also, ROS biological responses include the activation of signalling pathways, expression of inflammatory cytokines and chemokines, and increased transcription of inflammatory, genotoxicity, fibrosis and cancer associated factors (Manke *et al.*, 2013).

Hence, oxidative stress is one of the most relevant outcomes of particles interaction with the biological medium. In fact, it is thought as one of the main reason for *in vivo* nanotoxicity (Zolnik *et al.*, 2010; Chompoosor *et al.*, 2010), especially if the particles can be sequestered by phagocytic cells in the reticuloendothelial system, which will target both liver and spleen (Schipper *et al.*, 2009; Stark, 2011).

Cellular stress response, evaluated through the measurement of ROS, is often investigated with 2',7'-Dichlorofluorescein diacetate (H2DCF-DA), which is a non-fluorescent cell-permeable compound used as a marker of oxidative stress. Once inside the cell, it is cleaved by endogenous esterases to H2DCF, thus preventing the back-diffusion of the dye into the extracellular space. The de-esterified product becomes the highly fluorescent compound 2',7'-Dichlorofluorescein (DCF) on oxidation by ROS (Wardman, 2007).

Chapter 2. Materials and Methods

PMMA and PMMA-EUD particles will be hereinafter represented by PMMAp and PMMA-EUDp, respectively.

2.1. Particles preparation

2.1.1. Materials

PMMA (M_w=120000) and polyvinyl alcohol (PVA, M_w=13000 – 23000; 87-89 % hydrolysed) were provided by Sigma-Aldrich (UK). Dichloromethane (DCM), D(+)-Sucrose, Nile Red was purchased from Applichem (Germany). Poly(ethyl acrylate-co-methyl methacrylate-co-trimethylammonioethyl methacrylate chloride) (Eudragit RL 100, EUD; *M*w=32000) was kindly provided by Evonik Degussa International AG (Zaragoza, Spain).

Sterile water used in the experiments was filtered through 0.22 µm pore size (Whatman filter).

2.1.2. Methods

Both particles were prepared at room temperature by single-emulsion (oil-in-water) through solvent evaporation (SESE). Preliminary assays were performed in order to optimize size distribution. After the preparation of each batch, size distribution was evaluated in order to verify changes in particles mean size and ensure that unimodal size populations were obtained.

During this work we aimed to prepare particles with a size range around 500 nm in order to exclude different cellular effects due to this property. To achieve this goal, different formulation conditions were tested (Table 1).

Batches for PMMAp and PMMA-EUDp (50:50 polymer ratios) were prepared in pairs. Batches nº 1, 4 and 6 represent PMMAp, while batches nº 2, 5 and 7 correspond to PMMA-EUDp. Batch nº 3 corresponds to 100% EUD particles preparation.

Considering the best results in what refers to size distribution the selected protocol was as shown by Figure 7

PMMA and Eudragit 100 RL powders were weighted and added directly to glass flasks and dissolved in an organic solvent (DCM). After complete dissolution of the polymers, the solution was mixed with the PVA (5 %) on a Silverson mixer emulsifier (L5M, Silverson, UK) during 10 min at high-shear velocity, to obtain the w/o emulsion. After this step, the emulsion was put under magnetic stirring conditions (350 rpm) (Multipoint 15, Varomag, UK) so that the organic solvent was evaporated per 4 h. After DCM had been completely evaporated, particles were purified by two

successive centrifugations (64R, Beckman, USA) of 20000×g, during 20 min, for PMMAp and 5723×g, during 10 min, for PMMA-EUDp, both at 4 ºC. The particles were washed two times with 10 % D(+)-Sucrose.

Table 1 - Different tested conditions for particles preparation.

Batch nº(composition)	PMMA (mg)	Eudragit RL 100 (mg)	Total Mass (mg)	DCM (mL)	PVA (5%)(mL)	Time for emulsion at Silverson (min)	Centrifugation (×g, 4 ºC,Time)
1(PMMA)	25	-	25	3.5	10	10	2×20000, 20 min
2(PMMA-EUD)	62.5	62.5	125	5	30	10	5723, 10 min 20000, 20 min
3(EUD)*	-	125	125	5	30	10	5723, 20 min 20000, 20 min
4(PMMA)	125	-	125	5	30	10	2×20000, 20 min
5(PMMA-EUD)	62.5	62.5	125	5	30	10	2×5723, 10 min
6(PMMA)	65	-	65	7.5	30	10	2×20000, 20 min
7(PMMA-EUD)	62.5	62.5	125	5	30	10	2×5723, 10 min

***Note: Eudragit (100%) particles were not possible to be prepared.**

When the particles were prepared with intent to be lyophilized they were resuspended in 5 mL of filtered H_2O and 1 mL of sucrose (0.5%).

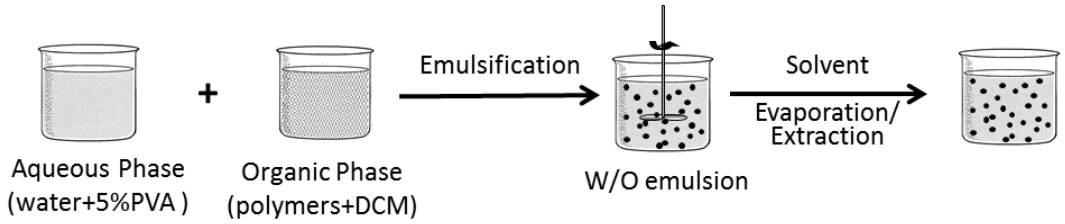

Figure 7 - Schematic representation of the experimental protocol for particles preparation (Adapted from Bettencourt and Almeida, 2012).

In order to evaluate the Yield of Production (YP), aliquots were taken and lyophilized and weighted as a powder. The yield was calculated as follows: YP (%) = (Practical Yield/ Theoretical Yield) × 100.

Lyophilized particles were obtained after frozen samples submitted through a Freeze-Dryer (Alpha 1-4 (100-400), Christ, Germany) (n = 3).

Particles loaded with the fluorophore Nile Red (0.1 mM) were obtained using the same protocol as unloaded particles except that, after dissolving the polymers with DCM, 100 µL of the compound was added to the solution immediately before the emulsification step.

2.2. Particles characterization

2.2.1. Particles size distribution

Particle size distribution was evaluated by Laser Diffraction.

In Laser Diffraction the intensity of light, which is diffracted by a particle or set of particles is measured over a set of detectors distributed under a predetermined set of angles in accordance with Lorenz-Mie Theory, which states that light intensity is inversely proportional to the analysed particles size. Still there's an associated residual error when using this technic, which must be minimized through an iterative process of measurements (Stojanović and Marković, 2012).

For Mie's theory to be applied two parameters need to be known: the refraction index and the absorbance. The first can be obtained through a refractometer and the latter is obtained by observing the dispersed particles though microscopy. Before measurements the most suitable method must be chosen since results can be affected by a variety of factors, such as temperature, sample's stability through time, agglomeration, among others. Two methods are available depending on sample properties: wet dispersion (used in the present study) and dry dispersion, the first easier to control and with higher signal to noise ratio and the latter faster and cheaper (Stojanović and Marković, 2012).

Mastersizer 2000 - Hydro 2000S (Malvern Instruments, UK) was used to measure particles size distribution. This apparatus employs 50 lasers at various angles in order to identify and analyse PMMAp and PMMA-EUDp size distribution, which was determined per volume unit. Previously to each measurement the samples were homogenized and then dispersed in purified water in the reservoir until obscuration criteria (between 5 and 10 %), were met. PMMA-EUDp were also measured under 50 % sonication due to their tendency to aggregate. Solvent's refraction index was set as 1.361 and room temperature was applied.

2.2.2. Surface morphology

Surface morphology was evaluated by Transmission Electron Microscopy (TEM). This technique is based on focusing an electron beam through a measurement chamber using electromagnetic lenses in a manner, which allows synthesizing an image with very high resolution. The system is run on vacuum and the scattering effect is produced by the electronic beam hitting any gas particles present in the solution (Höcherl, 2012).

The morphological characterization of PMMAp and PMMA-EUDp was performed by image analysis obtained by TEM in a Hitachi H8100 (Hitachi High-Technologies Europe GmbH, Germany) using an applied voltage of 200 kV, equipped with digital image acquisition with a CCD MegaView II bottom-mounted camera. To analyse the samples, a droplet of the suspension was deposited on the copper grid with a formvar film and dried at room temperature.

2.2.3. Surface charge evaluation

The surface charge of the particles was evaluated through ζ-potential measurement (Malvern Zetasizer Nano Z, Zen 2600, Malvern Instruments, UK). ζ-potential is based on dynamic light scattering methodologies while applying an electrical field that will polarize an electrophoretic cell and the surface charges will be evaluated due to electrophoretic mobility/velocity in the cell through the correlation equation of Smoluchowski which relates the velocity of the molecule through the electrical field with its charge (Domingues *et al.*, 2009).

Standard electrophoretic cell was used with gold electrodes to apply the electric field. Termocaps were used to limit temperature variation. Samples were diluted in 3 mL filtered water and the cell was filled verifying for the existence of bubbles that could cause interference in the ζ-potential measurements.

2.2.4. Chemical composition

The chemical composition of the particles was assessed by FT-IR (Fourier transform infrared spectroscopy).

Lyophilized particles were mixed with potassium bromide (KBr) (Merck, Germany) (in a 2:200 mg ratio) in an agate mortar. A pellet was obtained by compressing the powder mixture into discs in a hydraulic press, under a pressure of 10 Ton for 3 min. FT-IR spectra were obtained with IRAffinity-1 spectrophotometer (Shimadzu, Kyoto, Japan) at 400–4000 cm^{-1} scanning range. The pellet was placed in a light path and the spectra obtained were the results of the average of thirty scans. PMMA and EUD powders used during the preparation of the particles were used as controls of the structure of each polymer.

2.2.5. Hydrophobicity

Hydrophobicity was evaluated by hydrophobic interaction chromatography (HIC) for each type of particles.

HIC is generally used as a purification/separation method, usually for proteins, based on their different hydrophobicity. A compound which possesses a higher hydrophobic surface can be retained for a longer period of time in a column packed with a resin presenting many hydrophobic receptors than other compounds with less hydrophobic surfaces. This may happen when the first interacts by forming stronger bonds to the column's surface where a selective resin (stationary phase) is immobilized. In HIC the substances are eluted several times with a neutral saline solution (mobile phase) until all different compounds have been separated. Strongly bonded compounds may only be released through an elution with a detergent, such as Triton X-100. However, it may occur that a compound presents such a strong bond with the chosen column package that it bounds to it irreversibly (Alpar and Almeida, 1994; Murphy *et al.*, 2011).

This method may also be applied in order to relatively evaluate and compare particles with different hydrophobicity values (Alpar and Almeida, 1994). In order to do so, three types of column packages are usually used, each with increasing hydrophobicity in order to distinguish different bonding capabilities (Murphy *et al.*, 2011).

In the present work, vertical disposable columns were used with three resins with different hydrophobicity as follows: Sepharose 4-FF < Butyl Sepharose 4-FF < Octyl Sepharose 4-FF, all provided by Ge Healthcare (UK). These resins were used as packaging for the columns after successive centrifugations (Megafuge 1.0R; Heraeus, UK), at 900 rpm for 10 min, at room temperature, in order to wash the preservation liquid (ethanol) in which they were suspended. For the third washing cycle the resins were re-suspended in mobile phase, a 0.6 M NaCl (Applichem, Germany) solution previously prepared. After the final centrifugation the resins were re-suspended in the mentioned mobile phase until 15 mL were obtained and sonicated (250, Branson, USA) for 10 min. Then 1 mL of resin suspension was applied to each empty column. The columns were washed with 10 mL mobile phase and then stored at 4-8 ºC overnight.

The next day, 1 mL of the particles samples (20 mg/mL) was applied to each column (n = 3 per resin, per particle type) and the resulting elution was captured at vials for each column (Figure 8). This step was repeated for elutions with mobile phase, for partially retained particles, and 0.1% (*w*/*V*) Triton X-100 (Applichem, Germany), for strongly bound particles. 200 μL from each vial were then transferred to a 96-well flat bottom microplate and OD_{600nm} was measured (FLUOstar Omega, BMG Labtech, Germany). Previously, samples from both particles dispersions were diluted until OD_{600nm} reached 0.5 with this value being taken as the total of particles for statistical purposes.

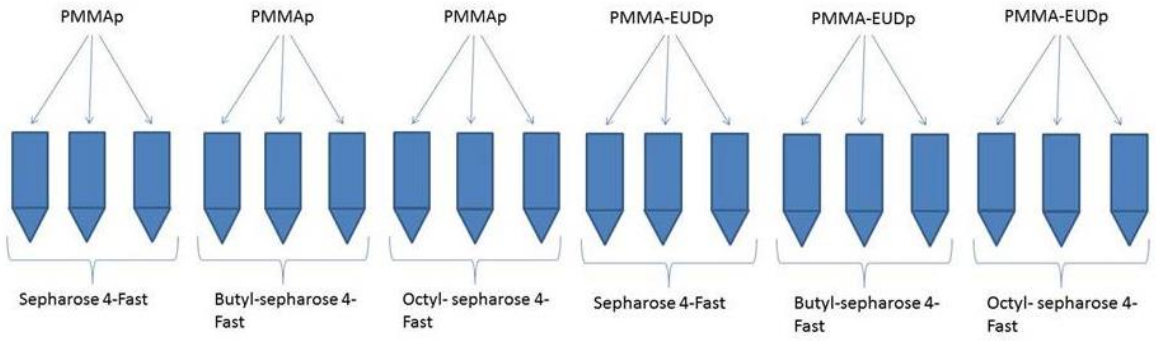

Figure 8 - Experimental overlay for HIC assay.

2.2.6. Study of the interactions between biological parameters and particles

2.2.6.1. Surface charge as a function of ionic strength, pH and serum concentration

To evaluate the ionic strength effects, aqueous solutions of NaCl were set at 0.0008, 0.0015, 0.008, 0.015, 0.08 and 0.15 M though successive dilutions.

To evaluate the serum percentage effect, solutions of Fetal Bovine Serum (FBS) (Life Technologies, UK) were prepared by successive dilutions in water and culture medium (RPMI 1640, Life Technologies, UK). The chosen concentrations were 0.01, 0.1, 1, 2, 5, and 10 % (*V/V*).

To evaluate the pH effect on the particles, sterile water was used and pH was adjusted with a pH measuring device (inoLab730, WTW, Germany) with 0.1 N HCl to pH=1.6, 2.3, 3.4, 5, 7.1 and with 0.1 N NaOH to pH=9.4, both provided by Merck (Germany).

All particle dispersions for the different evaluated conditions were prepared by adding 10 μL of particles stock suspension (20 mg/mL) to 1 mL of testing condition (n = 3). The ζ-potential of the samples was then measured with Zetasizer Nano Z.

2.2.6.2. Effect of serum concentration on size distribution

Two particles suspensions of PMMAp and PMMA-EUDp (in culture medium containing 10% FBS) were incubated at 37 ºC. Samples were taken at 0, 1, 24, 48 and 72 h and introduced into Mastersizer 2000 - Hydro 2000S for particle size measuring. Mean particle size and standard deviation result from the mean of five consecutive measurements at constant stirring conditions. Measurements for PMMA-EUDp were made with and without 100 % sonication for aggregate stability evaluation.

2.2.6.3. Protein adsorption assays

Bovine Serum Albumin (BSA) (Sigma-Aldrich, UK) was selected as the model protein for the adsorption studies. Initially, the UV-Vis spectrum of BSA was obtained in a microplate reader

(FLUOstar Omega, BMG Labtech, Germany) and the highest absorbance peaks were chosen for BSA adsorption analysis by measuring the OD at wavelengths 230 and 280 nm for BSA specific detection and 562 nm for total protein amount detection through the BCA method.

Particles dispersion (20 mg/mL) and BSA stock (1 mg/mL) solutions were prepared with 10 mM PBS (pH 7.4, Sigma-Aldrich, UK). Particles suspension with BSA were prepared in 1.5 mL microtubes at various concentrations depending on the assay and incubated at 37 ºC (Incubator INB 500, Memmert, Germany). Controls for BSA and both particles were also prepared in microtubes directly from the stock solutions. At pre-determined time points the samples were collected and then centrifuged (R11288 Sigma 112, Sigma-Aldrich, UK). The supernatants were transferred to 96-wells plates specially designed for UV measurements and non-adsorbed BSA was detected by UV-vis spectroscopy, as previously described.

In addition, a biochemical assay (bicinchoninic acid assay, BCA assay, Sigma-Aldrich, UK) for the detection of the BSA in the supernatants was also performed. The method was executed as follows: concentration of PMMAp and PMMA-EUDp was changed until OD_{600nm} = 0.5, having been chosen [PMMAp] = 500 µg/mL and [PMMA-EUDp] = 202 µg/mL. Then different studies were performed: i) BSA adsorption variation with time, ii) adsorption of particles at different concentration of BSA and iii) adsorption of BSA at different concentration of particles.

i) For BSA adsorption variation with time, dispersions of particles at the previously discussed concentrations were incubated with [BSA] = 1 mg/mL, at 37 ºC with steering conditions and analysed at 1, 2, 4, 6, 24 and 48 h.

ii) For [BSA] variation particles were incubated with 59.7; 67.6; 92.6; 113.6; 146.9; 208.3; 476.2 and 909.1 µg/mL of BSA for 24 h at 37 ºC.

iii) For [particles] variation, [BSA] was fixed at 1 mg/mL and was added to particles dispersions at 0.5, 1 and 2 mg/mL which were incubated for 24 h at 37 ºC.

After incubation, each sample was centrifuged (R11288 Sigma 112, Sigma-Aldrich, UK) for 15 min at 12000 rpm and 50 µL of the resulting supernatant were transferred into 96 wells plates specially designed for UV measurements. Then reaction was induced with 50 µL of BCA solution Kit (1:20 ratio between Copper (II) and Biocinchoninic Acid) which specifically identifies proteins through the reduction of Cu^{2+} to Cu^{+}, which forms a purple chromogenic compound with absorbance maximum at 562 nm. The plates were incubated for 15 min at 37 ºC and then OD_{562nm} was measured with the FLUOstar Omega microplate reader.

2.3. *In vitro* cellular assays

2.3.1. Cell lines, cell maintenance and particles dispersions

Cell lines

Cell lines L929 (Mouse fibroblast cell line, ATCC® CCL-1™) and MG63 (human osteoblast cell line, ATCC CRL-1427™) were chosen for uptake evaluation, cytotoxicity and oxidative stress response. L929 alone was used for genotoxicity assays due to time concerns. THP1 cells (human monocytic cell line, ATCC TIB-202™) were also used for uptake evaluation because it can be differentiated into macrophages.

Cell maintenance

Each cell line was kept at optimal conditions for growth, i.e., incubated in RPMI 1640 culture medium supplemented with 10% FBS, 100 units of penicillin G (sodium salt), 100 µg of streptomycin sulfate and 2 mM L-glutamine, at 37 ºC with 5 % CO_2, until confluence levels reached at least 75 %. At this point cell lines were trypsinized with 1 mL of enzyme (TrypLE™Express) and subsequently transferred to new T-flasks at a tenth of its original volume. All the mentioned products were acquired from Life Technologies (UK).

The THP1 cell line was differentiated to macrophages for 3 days with 200nM of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich; UK) before exposition to the particles.

Particles dispersion

Stock particles dispersions were obtained by weighting an adequate amount of particles in purified sterile H_2O to a final concentration of 20 mg/mL. Careful homogenization through sample inversion was performed until no aggregation was visually detected. PMMA-EUDp stock dispersions were prepared immediately before its use due to their rapid aggregating properties. PMMAp stock solutions were prepared and used as seen fit since these didn't show any physical signs of aggregation.

All concentrations (µg/mL) were converted into µg of particles in the applied volume (100 µL and 500 µL in 96 or 24-well plates, respectively) per well area (0.34 and 1.80 cm^2 in 96 or 24-well plates, respectively). This conversion is shown on Table 2.

Table 2 - Relationship between particles concentrations.

Particle concentration (µg/mL)	96-well plates (µg/cm^2)	24-well plates (µg/cm^2)
10	3	2.8
100	30	28
500	147	139
1000	294	278
2000	588	556
5000	1471	1389

2.3.2. Cell Uptake Assays

Nile Red-loaded particles were used in these studies.

Cells were seeded in sterile flat bottom 96-well tissue culture plates (Greiner, Germany) at a cell density of 2×10^5, 1×10^5 and 2.5×10^5 per well, respectively for L929, MG63 and THP1 cell lines. Cells were then exposed to 3, 30 and 147 µg/cm^2 of each type of particles. Negative control refers to culture media-only.

After 24 h for L929 and MG63 or 72 h for THP1, culture medium was replaced with 100 µL of Nile Red loaded particles. The analysis for each concentration was made on 5 wells. The cells with the treatment medium were incubated for 1 and 24 h, after which they were washed three times with 250 µL of PBS containing 20 mM Glycine (Bio Rad, USA) at pH 7.4 at 37 ^{0}C and then fluorescence was immediately measured at excitation wavelength 485 nm and emission 520 nm. PBS was then removed and the cells were disrupted with 100 µL of 1 % Triton X-100 after which fluorescence was again measured to determine the amount of internalized particles.

Confocal microscopy analysis was performed further for confirmation of cellular uptake.

Cells (MG63 and L929) were grown at 8-well chamber slides (Nalgen Nunc, Denmark) for immunocytochemistry assays. After incubation, cells were rinsed with 10 mM PBS containing 20 mM Glycine at pH 7.4, before and after being fixed for 15 min (in the dark, at room temperature) with paraformaldehyde (4 % *w*/*V* in PBS, Applichem, Germany). Slides were then mounted in fluorescent mounting medium ProLong® Gold antifade reagent with DAPI (Life Technologies, UK) and their images of fluorescence were recorded with Leica TCS-SPE confocal microscope and processed with Leica Software (Leica, Germay).

2.3.3. Cytotoxicity Assays

The effect of particles on cell viability was first evaluated by two distinct methods which measure the cell mitochondrial activity, MTT and Alamar Blue assays.

Cells were seeded as in the uptake assay at a cell density of 2×10^5 and 1×10^5 per well, respectively for L929 and MG63 cell lines. Cells were then exposed to 3, 30, 147, 294 and 588 µg/cm^2 of both types of particles. Negative control refers to culture media-only and Sodium Dodecyl Sulphate (SDS) (Merck, Germany) at 1 mg/mL was chosen as positive control. After 24 h, fresh media was applied with the different treatments and five wells per concentration were analysed. Cell viability was assessed after 24, 48 and 72 h of incubation at 37 ºC and 5 % CO_2. After each time of incubation, culture medium was replaced with medium containing 5 mM of resazurin (Applichem, Germany), or 0.25 mg/mL of MTT (Sigma-Aldrich, UK). This was followed by further 3 h of incubation in a fluorescence microplate reader after which fluorescence was measured at 530 nm for excitation wavelength and 590 nm for emission wavelength for the Alamar Blue assay.

For the plates containing MTT, the media was removed and intracellular formazan crystals were solubilized and extracted with 100 µL DMSO (Merck, Germany). After 15 min at room temperature absorbance was measured at 570 nm.

The relative cell viability (%) by comparison to the control cells was then calculated through the formulas [Fluorescence]sample/[Fluorescence]control x 100 for the Alamar Blue assay and [Absorvance $_{570nm}$]sample/[Absorvance $_{570nm}$]control x 100 for the MTT assay.

Alamar Blue assays had particle interference at the given fluorescence excitation and absorbance wavelengths and, therefore, results were not consistent and won't be presented.

The Trypan Blue assay was further used to evaluate cell membrane integrity. Cell seeding was done as for the cytotoxicity assays previously described and the same for particle exposure but only for 24 h. For the Trypan Blue cell counting, a sample was taken from each well and a dilution factor of 5 was employed, by adding 200 µL of Trypan Blue (Sigma-Aldrich, UK) to 50 µL of the samples. Cells were then counted with a Neubauer chamber on an inverted microscope with phase contrast (TC5400, Meiji Techno). The number of unviable cells was compared to the number of total cells.

2.3.4. Genotoxicity Assays

2.3.4.1. Materials

a) Comet Assay

RPMI 1640 w/Glutamax, FBSi, Penicillin-Streptomycin (10000 U/mL), 0.05% Trypsin-EDTA, DMSO, Tris-HCl (10mM), Trypan Blue (0.4%), PBS and 10 mM Tris-HCL were provided by Life

Technologies (UK); Triton X-100, Hydrogen Peroxide, 10 mg/mL Ethidium Bromide (EtBr), Trizma Base (0.4 M Tris), 0.5 mM Acid EDTA, 8.8M Hydrogen Peroxide (H_2O_2), 0.2 mg/mL BSA and Low Melting Point agarose were provided by Sigma-Aldrich, UK); FPG was kindly provided by A.R. Collins (University of Oslo, Norway); 2.5 M NaCl, 10 M NaOH, 4 M HCl and 100 mM KCl were provided by Merck (Germany); 100 mM $Na_2EDTA.2H_2O$ was provided by Calbiochem (Germany) and Normal Melting Point agarose was provided by Amersham (UK).

Microscopy-related material was provided by Hirschman (Germany), Thermo Scientific (UK) and Immuno-Cell (Belgium).

b) Micronucleus assay

Mitomycin C, Cytochalasin B, and 96% Methanol were provided by Sigma-Aldrich (UK); 3% Acetic Acid, KCl, Giemsa Stock Solution and Entellan Mounting Medium were provided by Merck (Germany); Gurr's phosphate buffer (pH 6.8) was provided by Life Technologies (UK); 6-well flat bottom tissue culture plates were provided by Greiner (Germany)

2.3.4.2. Methods

Though we had done the previous assays with at least two cell lines, the evaluation of the genotoxic effects were studied on the L929 cell line only, since it was the one recommended on ISO 10993-5. In respect to the dose-range selection, since the previous tests showed no relevant cytotoxicity up to the concentration of 2 mg/mL (or 556 µg/cm^2), we decided to proceed with an evaluation on the maximum concentration recommended by OECD (2010) guidelines, which is 5 mg/mL (or 1389 µg/cm^2).

a) Comet assay

Cells were grown on 24 well flat bottom plates and incubated for 24 h at 37 ºC and 5.5 % CO_2 with a cell concentration of 1.5×10^5. Particle dispersion (20 mg/mL) of the powdered particles in fresh purified H_2O was used for making the treatment mediums at concentrations 28, 139, 278, 556 and 1389 µg/cm^2 by successive dilutions with culture medium. For PMMA-EUDp the stock dispersion needed to be freshly prepared due to agglomeration concerns. After the initial incubation period, culture medium was replaced with treatment medium, two wells per concentration, and re-incubated at previous conditions for 3 and 24 h. Culture medium was added to negative control wells and 200 µM H_2O_2 was added as positive control 30 min before the end of the incubation periods. Cells were then extracted by trypsinization and transferred into microtubes where they were centrifuged for 10 min at 1200 rpm and 4 ºC. Cells were then embedded in low melting agarose (1 % *w/V*) and spread onto previously agarose-coated microscope slides, four per concentration. These were then immersed in lysis solution (2.5 M NaCl, 100 mM $Na_2EDTA.2H_2O$, 10 mM Tris-HCl, 10 M NaOH, 10 % DMSO and 1 % Triton X-100, pH 10) overnight.

Next, the slides were rinsed three times, 5 min each, with enzyme buffer (40 mM HEPES, 100 mM KCl, 0.5 mM Acid EDTA and 0.2 mg/mL BSA, pH 8) and then 50 µL of FPG was added to half of the slides, while to the others was added enzyme buffer (control), for 30 min. Then, slides were submerged in electrophoresis buffer (300 mM NaOH and 10 $Na_2EDTA.2H_2O$, pH 13), for DNA unwinding and exposure of alkali-labile sites, for 30 min. Electrophoresis was then run for 25 min, 28 V and 300 mA). Finally, the slides were submerged in neutralization buffer (0.4 M Tris and 4 M HCl, pH 7.5) for 10 min, air dried at room temperature for several days and stained with EtBr.

For evaluation of DNA damage, one hundred randomly selected nucleoids were analysed using Axioplan2 Imaging epifluorescence microscope equipped with high resolution camera (Carl Zeiss, Germany). DNA in the comets' tails was scored with Comet Imager 2.2 software (MetaSystems, Germany). For statistical purposes the median of the percentage of DNA in tail was registered and used to extrapolate the DNA damage level since it is linearly related with breaks on DNA strands.

b) Micronucleus assay

Cells at a concentration of 2.5×10^5 were incubated in 6-wells plates for 24 h before exposure. After that, the medium was removed and cells were treated with the same particle concentrations as in the comet assay, with 3 mL of treatment medium being added per well. Mitomycin C was chosen as the positive control for this assay. After overnight exposure, Cytochalasin A was added to inhibit cell cytokinesis, therefore, entrapping any formed micronuclei inside the dividing cells' membranes, for 30 h. A fixing solution was prepared, with a 3:1 ratio of methanol and acetic acid, and maintained at -20 ºC until necessary. The cells were then extracted, transferred onto 15 mL tubes and centrifuged at 1200 rpm for 5 min. The supernatant was then discarded and hypotonic shock was induced with 5 mL of KCl 0.1 M added drop-by-drop while vortexing. The solution was re-centrifuged at 1200 rpm for 5 min and supernatant discarded by pipetting. Then, the cells were fixed with 1 mL of fixing solution while vortexing and spread over microscope slides with a cytocentrifuge. The slides were kept at room temperature for several days in order to be air-dried. A solution of 4 % Giemsa was then prepared by dilution with Gurr's phosphate buffer (pH 6.8). Three recipients were prepared for the staining procedure: one with the diluted Giemsa solution and two with Gurr's phosphate buffer (1:25). Slides were then submerged in phosphate buffer for 4 min, then on Giemsa solution for 13 min and finally washed twice in phosphate buffer. The slides were then air-dried at room temperature and mounted with entellan with three drops per slide.

2.3.5. Stress Response Assays

L929 and MG63 sub-confluent cells grown in 96 well plates were incubated for 30 min with 20 µM of 2-7' dichlorodihydrofluorescein diacetate (H_2DCFDA, Life Technologies, UK) in the dark at 37 ºC. Medium was removed and fresh medium was added before exposing the cells to the different concentrations of particles for 1 and 2 h. H_2O_2 (0.5 mM) was used as positive control for the

induction of ROS. After exposure, ROS levels were determined at excitation 485 nm and emission 520 nm wavelengths using a florescence microplate reader (FLUOstar BMGLabtech, Germany).

Data from 5 replicates was reported as relative fluorescence units (RFU) percentage and expressed as mean fluorescence ratio (fluorescence of exposed cells/fluorescence of unexposed control from the same experiment x 100).

2.3.6. Statistical Analysis

Data are expressed as mean and standard deviation (mean ± SD) of separate experiments (n = 5 for uptake, cytotoxicity and stress response assays and n = 3 for genotoxicity assays). Statistical evaluation of data from uptake, cytotoxicity and stress response assays was performed using one-way analysis of variance (ANOVA). Tukey's multiple comparison test (GraphPad PRISM 5 software, USA) was used to compare the significance of the difference between the groups, a p-*value*<0.05 was accepted as significant.

Statistical evaluation of data from genotoxicity assays was performed using one-way analysis of variance (ANOVA) and Tukey's multiple comparison test (SPSS Statistics 22.0, IBM Software, USA) was used to compare the significance of the difference between treated and untreated cells. The Mann-Whitney test was used to compare the statistical difference between the positive and negative controls. A p-*value*<0.05 was accepted as significant.

Chapter 3. Results and Discussion

3.1. Particles preparation

The first step of the present work was the optimization of the particles preparation method.

Both PMMAp and PMMA-EUDp were obtained through simple emulsion solvent evaporation methodology (SESE). This technique is often reported in the literature for the encapsulation of bioactive principles in acrylic particles (Zydowicz *et al.*, 2002; Bettencourt *et al.*, 2010). The method is based on the emulsification of an organic phase in an aqueous phase, containing a surfactant. After the emulsification process, the volatile organic solvent is evaporated, resulting in the precipitation of the polymer and the formation of particles (Zigoneanu *et al.*, 2008).This method assures a faster and easier way to prepare the particles than other time-consuming and more costly methods as the polymerization techniques, therefore, minimizing the production costs and the time needed for this purpose (Bettencourt and Almeida, 2012). Moreover, the use of SESE allows the exclusion of toxicity issues related to the polymerization methods, such as the presence of residual monomers or oligomers in the final product. Nevertheless, the use of dichloromethane (DCM), a Class 2 solvent, according to International Conference on Harmonization (ICH) for which the maximum residual concentration allowed is 600 ppm (EMEA, 2006) could be of some concern. Its use is justified by the high volatility of DCM that induces very low residual solvent rates after particles drying (Bettencourt and Almeida, 2012). In fact, Florindo *et al.* (2008) found that the residual DCM amount present in polymeric particles after freeze-drying, quantified using nuclear magnetic resonance spectroscopy technique was well below the safety limit. Furthermore, the residual DCM content in PMMAp prepared by Kwon *et al.* (2002) was less than 10 ppm, measured by gas chromatography.

PMMA and Eudragit RL 100 were chosen as the polymers of the formulations as both are biocompatible, with a long history of application in the human body. PMMA has been largely described as a safe-to-apply, FDA-approved polymer, with low toxicity related effects. Also, it is a non-biodegradable polymer and many authors suggest its utility for controlled drug release over a specific target area or system as this feature allows particles long-term tracking in the physiological milieu, without contamination with any degradation product (Jung *et al.*, 2007; Bettencourt and Almeida, 2012; Papa *et al.*, 2014; Juneja and Roy, 2014). As for Eudragit RL 100, it is well known that this polymer can improve the efficiency of both the cellular uptake and the delivery of a target drug in acrylic polymer formulations (Joshi, 2013).

The formulation of both PMMAp and PMMA-EUDp has shown to be highly influenced by a number of parameters like polymer mass, surfactant and organic solvent volume, time of emulsifying and the purification process, among others. For example, emulsifying time can have an effect in the size, because it will affect the distribution of the polymers in the solution, and the forces applied during the emulsion step will be unbalanced. This fact will result in particles with different characteristics. The DCM volume can also influence the resulting particle size distribution. In our study we aimed to achieve reproducible narrow size distributions in the 500 nm range. This size was selected since, in preliminary studies (data not shown), it was found to be the lowest size that could be achieved for both types of particles using SESE. While trying to optimize the most suitable conditions, we verified that size was highly influenced by both the mass of each polymer and the volume of DCM used to dissolve the polymer. The purification step, with centrifugation at different velocities, was a key stage to obtain the most suitable particle sizes since, after the emulsion process, a complex suspension of particles with heterogeneous sizes was obtained.

Although the above described conditions were the best for our purposes, it is important to consider that the method should be always optimized when the particles are to be used as carriers for the delivery of drugs. Depending on the drug of interest to incorporate, adaptations of the described method may be necessary, as each compound may have its own specific points, that will always need to be taken into account.

Some studies that report the toxicity of PMMAp point to the formulation methodology as one possible cause of cytotoxicity. In Colombo *et al.* (2013), PMMAp were synthesized using either SDS or Tween80 as surfactant agents. The authors proved that the chosen method for particles formulation and minor factors, such as the composition of the surfactants, can have great impact over the particles biocompatibility. In this study, particles synthesized using SDS have shown to produce cytotoxic effects on murine mammary tumour cells, while particles with Tween80 have shown no decrease in cell viability. The possible explanation given by the authors is that the main difference that could provide this variation would be the sulphate groups provided by the SDS.

Results of the YP for each type of particle show that PMMAp were easier obtained than PMMA-EUDp (Table 3). Results for PMMAp are similar of those obtained by Bettencourt *et al.*, (2010) (YP≈ 95 %) in which the same kind of unloaded particles were formulated by the same method. The lower YP values for PMMA-EUDp may be explained by the inclusion of the EUD polymer in the formulation. EUD has hindered the emulsification step and induced the adhesion of the particles to the glass and plastic containers.

This justification was suggested by our attempt of obtaining EUD (100 %) particles. In this case it was not possible to formulate the particles as we could not obtain a stable pellet, in the purification steps, as those obtained for PMMAp. In this case it was clear that EUD polymer caused adhesion of

the particles to the glass flasks used for containing the particles dispersions, sometimes even forming films on their surface.

Table 3 - Yield of production per particle type (n = 3)

Particles	YP (%, *w/w*)
PMMAp	92.31 ± 15.38
PMMA-EUDp	32.43 ± 0.15

3.2. Particles characterization

At present, it is accepted by both the scientific community and the regulatory agencies that physicochemical data should be included in any toxicology study (Fubini *et al.*, 2010), as several of these properties affect the particles biological behaviour. Therefore, different properties of the particles, such as size distribution, shape, charge, hydrophobicity and chemical composition, were evaluated and will be subsequently presented.

3.2.1. Particles size distribution

One of the most important physicochemical properties of particulate-systems is particle size distribution. This property will influence many other characteristics either intrinsic to the particles or related with their interaction with the biological surroundings. Size is directly related to the particle surface area with obvious impact on their biological reactivity. Also, it is the main characteristic that will determine the physical barriers that the carriers can transpose either limiting or increasing access to certain organs/tissues. It will also determine the bioavailability of the particles and their pathway of internalization into cells (Vollrath *et al.*, 2012).

Table 4 - Average results from size distributions of different batches (corresponding to different formulation conditions) evaluated through Laser Diffraction (n = 3).

Batch nº(Composition)	Obscuration	VMD (µm)	Span
1(PMMAp)	5.3±0.07	0.449±0.014	0.994±0.035
2(PMMA-EUDp)	5.2±0.01	0.512±0.000	1.055±0.002
3(EUDp)	No Data Available		
4(PMMAp)	5.7±0.04	0.899±0.047	0.867±0.060
5(PMMA-EUDp)	5.9±0.10	0.703±0.260	1.269±0.253
6(PMMAp)	5.7±0.10	0.573±0.020	1.031±0.030
7(PMMA-EUDp)	5.6±0.03	0.509±0.080	1.043±0.006

Notes: Obscuration reflects the concentration of particles in solution. VMD stands for volume mean diameter. Span is a measure which evaluates the uniformity of size distributions.

Particles average size of the different prepared batches is displayed on Table 4. Particles from batches 6(PMMAp) and 7(PMMA-EUDp) were the chosen for further characterization and for all subsequent assays, since they displayed the most comparable and reproducible sizes.

Also, as shown on Figure 9 and 10, all batches are monodisperse since they present unimodal populations, evidencing the high reproducibility of the preparation process. This can also be verified by the Span values on Table 4 since, the closer these are to 1, the narrower will be the size distributions and fewer populations will be present within the analysed sample.

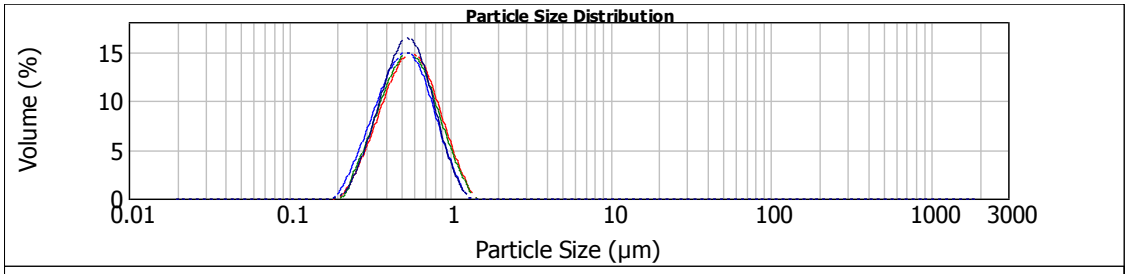

Figure 9 - Representative Particle Size Distribution for PMMAp (n = 3).

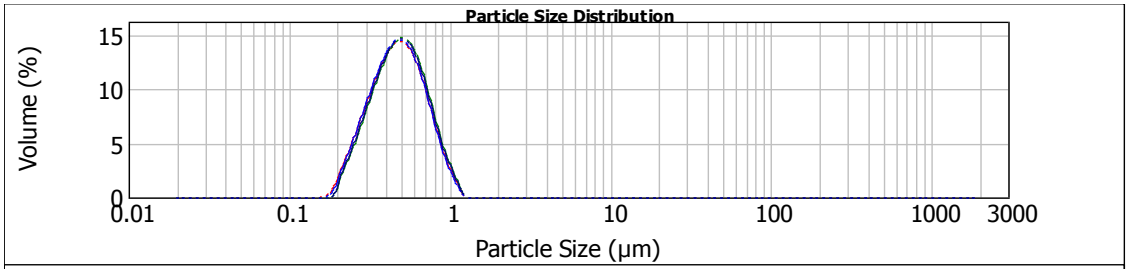

Figure 10 - Representative Particle Size Distribution for PMMA-EUDp (n = 3).

Overall, the SESE methodology allowed the preparation of particles within the micrometric range. The formulation procedure which allowed the preparation of particles with the size distribution that proved to be suitable to our propose goals (PMMAp = 572.7 ± 20 nm; PMMA-EUDp = 508.9 ± 8 nm) was elected to proceed for further evaluation.

3.2.2. Surface morphology

Shape of particles has also been shown to have a pronounced effect on the biological activity of particulate-systems (Arora *et al.*, 2012). For example, Chithrani *et al.* (2006) reported better uptake of spherical gold nanoparticles than gold nanorods in HeLa cells.

Figure 11 illustrates common TEM images of PMMAp and PMMA-EUDp. Particles exhibited very similar spherical morphology.

TEM was also used to evaluate if the loading of the fluorophore affected particle size. As the fluorescence emitted by the probe interferes with the optical properties of the particles, other methods as Laser Diffraction or Dynamic Light Scattering are not feasible. In the present study, TEM showed that the particles were able to incorporate Nile Red and maintain their size range after being loaded with this fluorescent compound (Figure 11).

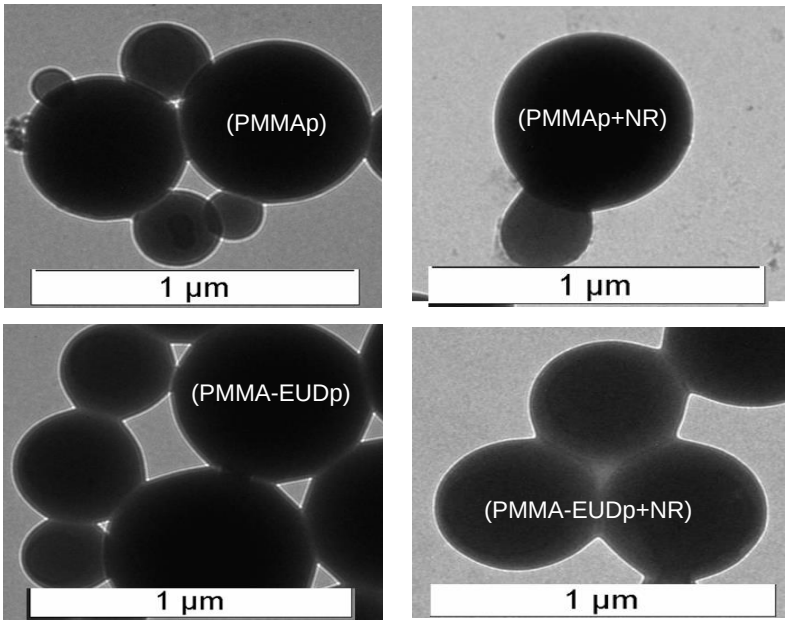

Figure 11 - Panels showing particles shape from images taken with TEM spectroscopy for PMMAp and PMMA-EUDp, with and without Nile Red (NR).

3.2.3. Surface charge evaluation

Surface charge in addition to size and shape plays a decisive role in the interaction between particles and the cells or organisms.

Our data has shown that surface charge modification, as measured through ζ-potential, was considerably changed after inclusion of Eudragit in the formulation (Table 5). PMMAp were strongly negative (-32.7 ± 1.04 mV), whereas PMMA-EUDp were strongly positive (+31.8 ± 1.66 mV).. These results suggest that particles could have different interactions with cells, which could lead to diverse toxicity effects, considered further on this thesis (Section 3.3.). Also, the fact that both particles showed absolute ζ-potential values above 30 mV should indicate high stability in dispersions because, the higher these values are, the less will be the tendency for particles to aggregate since particle repulsion will increase due to similar surface charges (Mendes *et al.*, 2012).

Similar results for ζ-potential of PMMAp were obtained in other studies, even when different preparation methods (e.g. polymerization) were applied for particle formulation (Höcherl *et al.*, 2012; Papa *et al.*, 2014). A note of interest is presented in a study by Sitia *et al.* (2014) in which opposing charged PMMAp were synthesized which could be an element of comparison with our PMMA-EUD composite particles.

Since most cells present negative surface charge, it would be most likely that particles with positive charge, such as the PMMA-EUDp, would develop more electrostatic interactions than those with a negative one. By studying two structurally very similar particles, with opposing charges, we can evaluate this phenomenon and infer as to the suitability of the charge inversion and its effects on toxicity.

Surface charge was also evaluated for the fluorescent particles containing Nile Red, as previously explained. The results have shown only slight variations of this property within the standard deviation acceptable values, hence, in combination with the TEM imaging results, it was considered that the loading of the fluorescent compound did not affect the particles size and charge.

Table 5 - Surface charge of the particles diluted in purified H_2O (n = 3).

Batch	PMMAp	PMMAp (+Nile Red)	PMMA-EUDp	PMMA-EUDp (+Nile Red)
ζ-Potential (mV)	-32.7±1.04	-27.0±0.65	31.8±1.66	29.6±0.45

3.2.4. Hydrophobicity

The hydrophobicity, which refers to the water-attracting/water-repelling properties of a particulate-system, is a major factor determining how particles become dispersed or agglomerated in aqueous solutions (Kroll *et al.*, 2009). Also, wetting properties are of paramount importance in the interaction of the particles with biological elements, influencing proteins adsorption and cellular responses (e.g. uptake, cytotoxicity, DNA damage) (Fubini *et al.*, 2010, Tran and Webster, 2013). Kim *et al.* (2013) points that hydrophobicity of particles is as important as the charge in dictating the genotoxicity of particles.

The hydrophobicity of the particles was tested by the HIC method. A similar assessment was made by Blunk *et al.*, (1993) in order to identify the levels of hydrophobicity of colloidal particles but through a more automated chromatography approach. Instead, we manually switched the containers with the eluted samples and used a colorimetric method by measuring OD=600 nm before and after elution through the packed columns.

Analysis of collected data (Table 6) has shown that PMMA-EUDp have higher retention rates than PMMAp for all of the resins, being that the degree of the hydrophobicity of the resin surfaces can be represented as Sepharose < Butyl Sepharose < Octyl Sepharose (sorted as increasingly hydrophobicity).

Table 6 - Hydrophobicity assay results as a percentage of sample retention in the resins sorted by increasing hydrophobicity (n = 3).

	Sepharose-FF (%)	Butyl Sepharose-FF (%)	Octyl Sepharose-FF (%)
PMMAp	16.0±2.7	27.1±0.4	17.2±1.5
PMMA-EUDp	20.3±2.9	84.5±4.4	78.5±3.5

HIC results showed that when interacting with the hydrophobic resins the number and distribution of surface-exposed hydrophobic groups are more abundant in the case of PMMA-EUDp than PMMAp. Also, since all resins used in the assays did not contain any charged groups and have a low content of ionisable groups, the hypothesis that electrostatic interactions will be responsible for PMMA-EUDp adherence to the resins, rather than to hydrophobic interactions between the columns and the applied samples, is unlikely. Sepharose resin, as expected retained a low % of the particles since this resin does not have the proper functional groups (alkyl residues) to influence the hydrophobic interaction with the particles, being used as a control for the assay. Therefore, although both types of particles demonstrate some hydrophobicity, our results showed that PMMA-EUDp have clearly stronger hydrophobic behaviour than PMMAp.

Interestingly, no differences were found between butyl- and octyl- resins meaning that in this case the chain length did not influence the hydrophobic interactions. Also, PMMA-EUDp form strong irreversible bonds since most particles remain on the columns even after washing with a strong detergent (Triton X-100).

Finally, it is necessary to point out that the terms hydrophilic/hydrophobic are comparative for a row of different compounds and that different experimental methods exist for assessing the hydrophobic nature of the materials. This approach consists in a relative measure of this property and, therefore, should not be used as a comparison outside the applied experimental conditions detailed in this work. It would be interesting to further evaluate this property using different approaches namely by the measurement of the contact angle between the particles and biological fluids.

3.2.5. Chemical composition

Fourier-Transform Infrared Spectroscopy (FT-IR) spectral data was used to evaluate if any changes took place on the chemical composition of the particles, compared to those of the raw polymers or between both types of particles, for example, by the appearance of new chemical bonds.

FT-IR spectra (Figure 12) showed that the main characteristic peaks of both PMMA and EUD polymers remained unchanged for both particles. Therefore, we concluded that the PMMA-EUD formulation did not yielded new chemical liaisons between both polymers.

Obtained data is in accordance with literature for PMMA and EUD (Mendes *et al.*, 2012; *Evonik technical report*):

- signals around 2990 and 2950 cm^{-1} were assigned to axial deformation of CH bonds of aliphatic carbons;
- the sharp and intense signal at 1724 cm^{-1} was assigned to the axial deformation of the ester carbonyl group (C=O);
- the region comprised in the range of 1250-1000 cm^{-1} showed signals related to the angular deformation of C=O in ester bonds and to the C-N stretch. The latter are more intense and well-defined on the PMMA-EUDp spectra because the C-N stretch, from EUD aliphatic amines, overlaps the C=O signal of ester bonds for both PMMA and EUD polymers.

The similarities between both spectra imply that differences on the biological effects cannot be explained, for these particles, by FT-IR analysis, since most of the EUD vibration peaks are coincident with PMMA peaks.

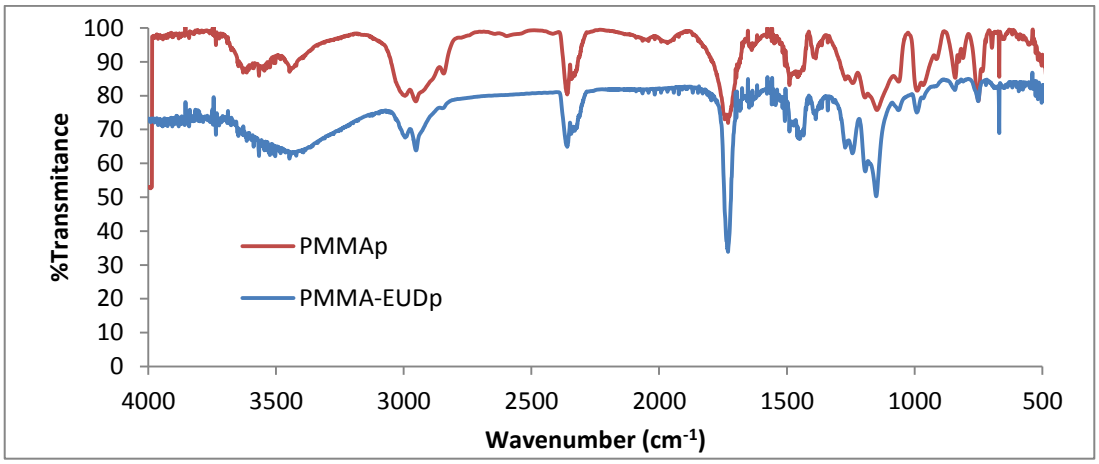

Figure 12 - FT-IR spectra with particles' characteristic vibration peaks correspondent to the vibration frequencies of various identifiable bonds on their chemical composition.

3.2.6. Effect of biological conditions on particles properties

Besides a complete physicochemical characterization of the particulate-systems, their interaction with biological media is essential for reliable studies (Landsiedel *et al.*, 2010). Phenomena occurring during the contact between the particles and cellular media or biological fluids (dispersion, agglomeration/aggregation, protein adsorption) can be highly relevant changing intrinsic particles properties, such as size and charge (Fubini *et al.*, 2010).

The evaluation of all biological factors (e.g. ionic strength, pH, protein content) with impact on the physicochemical properties of the particles is a complex task. As the main focus of this thesis was

to evaluate the biological effect of two distinct acrylic particles, within the same size range and opposite charges, we decided to investigate some of the cell culture conditions, specifically ionic strength, pH and FBS concentration that would have a direct impact on particles charge.

Also, it was found important to evaluate the effect of FBS in the size of the particles mimicking the conditions used in the cell culture assays. It was of interest to check if the assays were actually performed on particles within the same size range rather than on aggregates and/or agglomerates of the particles. Finally, an attempt was made to assess the adsorption of a model protein onto our particles. Proteins may significantly change particles properties and consequently, the cellular responses to the material (Fubini *et al.*, 2010). Protein adsorption is an important factor to be taken into consideration when testing biological responses to particulate-systems (Allouni *et al.*, 2009).

3.2.6.1. Effect of the ionic strength, serum concentration and pH value on particles surface charge

Effect of Ionic strength

The ionic strength of a physiological solution is a measure of the ions concentration in that solution. The effect of increasing the ionic strength of a solution was evaluated by measuring the surface charge of the particles suspensions with increasing concentration of a strong electrolyte (NaCl).

Results showed a significant reduction in the absolute surface charge values of the particles with the increase in the ionic strength of the media (Figure 13). It should be pointed that at 0.12 M, which is the reported salt concentration in physiological solutions (Allouni *et al*., 2009), both particles showed a surface charge close to zero.

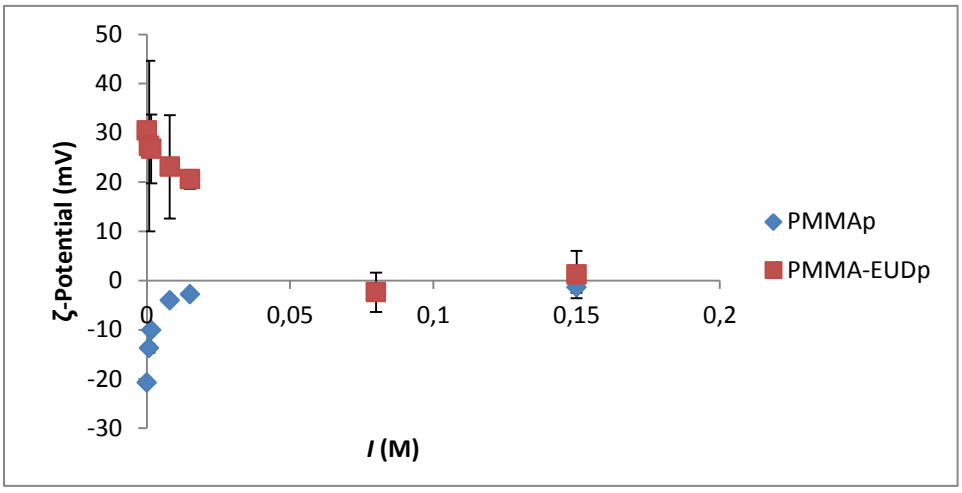

Figure 13 - Surface charge as a function of Ionic strength (n = 3).

Our results showed that the increase of ionic strength has led to a neutralization of the particles' surface charge. This may be explained by the electrostatic interactions between the ions in solution (Na^+ and Cl^-) and the particles. Since PMMAp present a high negative charge, Na^+ ions are strongly attracted to these particles, thereby neutralizing their surface charge. On the other hand, PMMA-EUDp have a high positive charge which leads to the attraction of Cl^- ions, thereby neutralizing their surface charge. This effect can have some impact *in vivo* where the charge of the particles can end up being relatively irrelevant for the interaction with cells and other properties, as the hydrophobicity or size, will have a higher relevance.

Effect of serum concentration

FBS with a concentration in the range of 10 % (*V/V*) is generally added to cell culture medium for optimal cell growth. Therefore, its effect on the surface charge of particles is highly relevant (Merhi *et al.*, 2012).

Our results indicate that particles did not retain their original charge once they were put in contact with FBS either on water (Figure 14) or culture medium (Figure 15). A complete inversion of PMMA-EUDp surface charge was observed when exposed to FBS, even at low concentrations (as low as 0.01 %), while PMMAp surface charge tends to neutrality. The standard deviation for both analyses was within acceptable values.

Figure 15 shows that the dispersion of particles in culture medium alone (a complex mixture of various ionic salts, low amounts of amino acids, vitamins, and glucose) is enough to lower particles absolute charge. FBS dissolved in water (Figure 14) or culture medium (Figure 15) has the same dose-dependent effect on particle charge. The source of variation could then be attributed to the adsorption of some of the components present in serum or in the culture medium onto the particles surfaces.

Similar results were found in a study by Merhi *et al.* (2012), in which positively charged polysaccharide nanoparticles were dispersed either in PBS or MEM (culture medium similar to RPMI 1960), and the addition of either FBS or BSA was conducted to evaluate its biological effects. Zeta potential concentration-dependent (0 – 10% serum) decrease was found to occur with lower particle concentrations (1250 µg/mL) but not with very high concentrations (5000 µg/mL). Although the effects may vary with particle composition and with the proteins present in the serum, these results are in accordance with the ones presented in this work in the matter that the constituents of serum can alter particles surface charge. The extent of this alteration is probably dependent on the particle-serum interactions, as well as the chemical composition of both.

In summary, our results show that FBS in distinct media (water or culture medium) can induce considerable variation on particles surface charge. Since our cellular assays were conducted with 10% FBS we should assume that our particles will actually be near neutrality, in the case of PMMAp, and negatively charged for PMMA-EUDp.

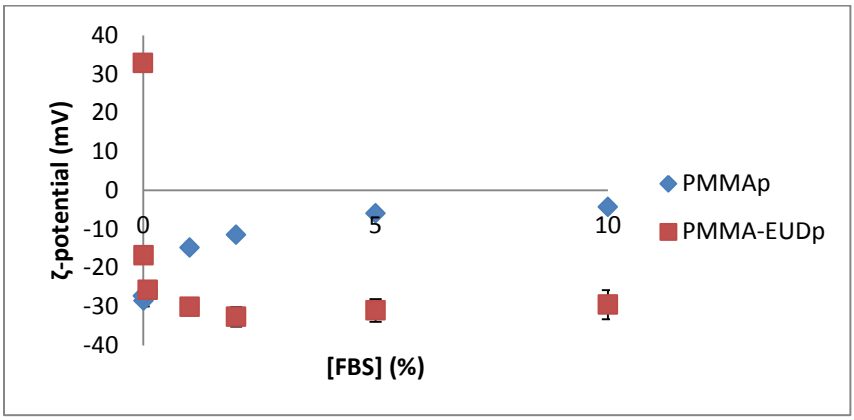

Figure 14 - Effect of FBS concentration (*V*/*V*, in water) on the surface charge (n = 3).

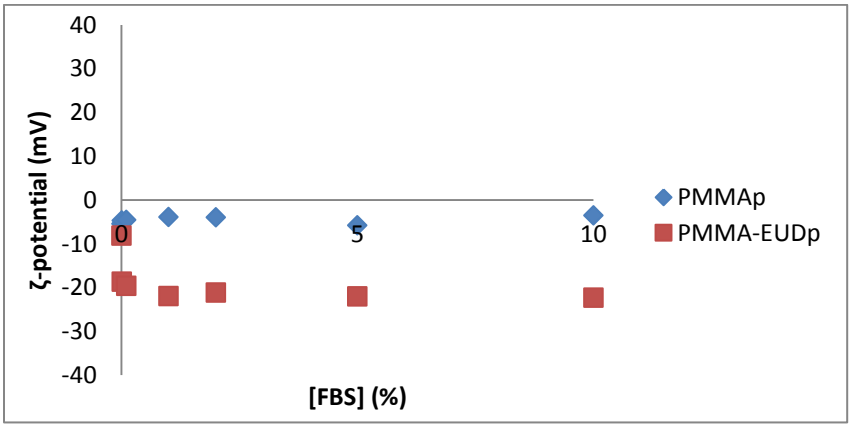

Figure 15 - Effect of FBS concentration (*V*/*V*, culture medium) on the surface charge (n = 3).

Effect of pH

Although physiological pH value is mostly constant (7.35 – 7.45), some organs work at a different pH range, as the stomach. Therefore, the different behaviour of the particles at different pH values should always be analysed. As shown by our results, pH can alter the surface charge of the particles. At pH ≈ 7, PMMA-EUDp appear positively charged, while PMMAp present a negative surface charge (Figure 16).

Also, analysis of the obtained data (Figure 16) has shown a tendency for the decrease of absolute surface charge values in PMMA-EUDp along with a pH increase. As for PMMAp, surface charge shows a decrease in absolute surface charge for pH ≥ 5.

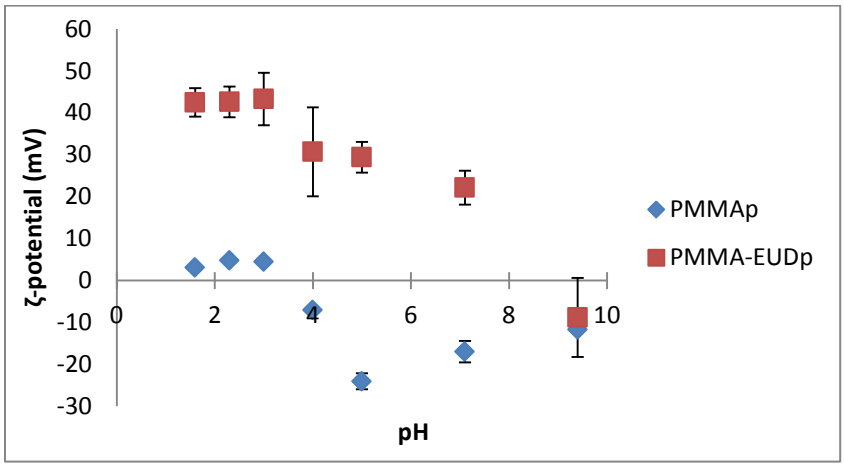

Figure 16 - Surface charge as a function of pH for 0.12 mg of particles suspended in water solutions with different pH (n = 3).

The isoelectric point, sometimes abbreviated to IEP, is the pH at which a particular molecule or surface has no net electrical charge. The different behaviour of the particles in function of pH was also shown due to the particles opposing isoelectric points - pH_{IEP} = 3.5 for PMMAp and pH_{IEP} = 8.7 for PMMA-EUDp - which demonstrates that the particles may favour different pH values, in terms of stability. For example, our results suggests that PMMAp will show a higher tendency to aggregate in the stomach (pH ranging between 1 and 3) than PMMA-EUDp. This fact could lead to toxicity-related events by the excessive accumulation of particles (Pandit, 2007).

3.2.6.2. Effect of serum concentration on size distribution

Due to their surface reactivity particles may interact with each other leading to the formation of agglomerates. This phenomenon may be checked by evaluating variation on size distribution and has been shown to occur on several studies with various types of particles (Lamprecht *et al.*, 2001; Hackenberg *et al.*, 2010; Merhi *et al.*, 2012). As such, the particles stability in culture media was monitored maintaining the particles at 37 °C in the same medium used for the cellular assays.

Our results show that PMMAp did not reveal any alteration in terms of size and span until 48 h, meaning that no aggregation took place and, consequently, that PMMAp are stable in the selected medium during this time (Table 7). At 72 h, we verified a slight increase in particle size distribution due to the formation of small agglomerates, probably due to particle deposition over the glass materials used for the laser diffraction process.

Table 7 – Particles size (VMD) variation (on culture medium containing FBS) with time.

Time (h)	PMMAp (µm)	Span	PMMA-EUDp w/o sonication (µm)	Span	PMMA-EUDp w/ sonication (µm)	Span
Negative Control (H_2O)	0.599±0.030	1.031±0.030	0.531±0.027	1.043±0.006	-	-
0	0.588±0.002	1.095±0.012	38.910±8.478	20.078±5.246	41.576±14.978	12.045±5.115
1	0.587±0.001	1.348±0.265	84.701±7.537	5.766±0.217	61.825±15.992	11.129±5.716
24	0.586±0.001	1.061±0.004	75.171±4.909	5.783±0.260	35.348±7.541	3.533±0.142
48	0.588±0.000	1.052±0.002	56.281±38.888	6.359±1.256	48.879±4.729	6.278±0.629
72	2.262±0.247	1.484±0.046	82.147±5.038	13.262±1.113	27.680±2.630	4.853±0.781

Notes: The samples' homogeny is presented by the Span values. Only PMMA-EUDp were submitted to sonication to test the aggregate stability (w/o = without; w/ = with).

Overall, PMMAp showed to be very stable in the selected medium. Our results are in accordance with Lazzari *et al*. (2012), who found that PMMA nanoparticles (in the range 100-200 nm) remained stable in different fluids as saliva, gastric juice, intestinal and lysosomal fluid, serum and tissue homogenates.

However, for PMMA-EUDp, an increase of particle size occurs and very quickly the particles showed signs of aggregation, with the formation of several different particle populations and an increase of mean size distribution from 500 nm to 42 µm as well as extremely high heterogeneity. A similar result was demonstrated in Ehrenberg *et al.* (2009), in which serum proteins rapidly covered polystyrene nanoparticles immersed in culture medium.

The formation of these agglomerates may have great implications in terms of biological applications, since it reduces the bioavailability of the carrier, and, therefore, the amount of drug being transported, in the cases of drug delivery applications.

Another relevant fact is that, after 72 h, we continually evaluated the size of PMMA-EUDp with ultrasounds for 1 h and verified a decrease in the mean size of the particles, as well as an increase over the population related with the original size, though a threshold was reached after 30 min of sonication (Table 8). This could be a proof that these agglomerates are a result of weak interactions and, therefore, a more effective treatment with ultrasounds could be used for restitution purposes of conditions more similar to those of the original particles than the ones we were able to obtain.

Table 8 - PMMA-EUDp size variation with continous sonication in culture medium with FBS (10 %).

Time with sonication (min)	VMD (µm)	Span
0	27.680 ± 2.630	4.853 ± 0.781
10	7.217 ± 0.610	13.568 ± 0.612
20	5.392 ± 0.422	13.894 ± 0.452
30	3.097 ± 0.196	10.589 ± 0.437
40	3.113 ± 0.127	11.234 ± 0.373

Notes: VMD stands for volume mean diameter. The samples' homogeny is presented by the Span values.

3.2.6.3. Protein adsorption assays

Proteins are a major component of cellular media. Estimating the proteins adsorption to particles is critical for designing optimal toxicological studies, as proteins may deeply modify the agglomeration behaviour of the particles and, consequently, the cellular responses to the material (Fubini *et al.*, 2010).

A spectrophotometric assay was initially chosen for the evaluation of protein adsorption onto particles, which was selected in accordance with the literature, as well as the model protein (BSA) (Hu *et al.*, 2005). Through the analysis of BSA absorption spectrum (Figure 17), the concentration of 1 mg/mL was chosen as the best concentration and two wavelengths were identified as optimal for the assays: 230 and 280nm. Preliminary experiments were conducted aiming to evaluate the adsorption of BSA as a function of time, protein and particles concentration.

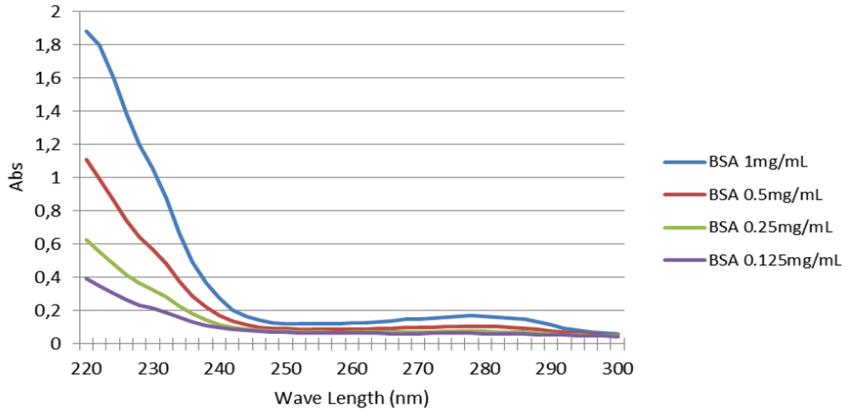

Figure 17 - BSA absorbance spectrum (concentration ranging between 0.125 – 1 mg/mL).

The chosen spectrophotometric assays were unable to verify any adsorption of BSA by the particles due to different experimental problems. At 230 nm, remaining particles present in the supernatants, due to their optical properties, directly influenced the readout by significantly increasing the light

absorption. At 280 nm, the absorbance levels for control BSA were as low as the ones obtained for the particles' control, greatly reducing the assay's sensitivity.

As we were unable to perform a spectrophotometric assay we decided to try a different technique, the BCA method, aiming to have less interference from the particles as it is a method based on a specific reaction with the proteins. However, no improvement on the procedure was observed, as no significant adsorption was possible to quantify.

Although some degree of adsorption is always expected on particles surface, we were predicting no significant adsorption for PMMAp, since BSA, like the particles, is described as negatively charged at the physiological pH (Fologea *et al.*, 2007). Also, Höcherl *et al.* (2012) proved that PMMAp have low interaction levels with the surrounding proteins. Finally, from the results of size variation in the presence of FBS, no significant change in size was observed, indicating no protein adsorption.

The adsorption of proteins is a very complex phenomena and it is difficult to compare results with others if the experimental conditions are different. For example, Hu *et al.*, (2005) was able to evaluate the adsorption of BSA but on modified PMMA microspheres. It is not possible to compare these studies with ours as particles were modified by introducing sulfonate groups on the surface of the particles and size was much higher (≈ 3.4 µm).

In contrast to PMMAp, PMMA-EUDp have a positive charge, strong hydrophobic behaviour and their size is greatly influenced by the presence of FBS as already discussed, which would predict a high degree of BSA adsorption. The fact that we weren't able to quantify any significant BSA adsorption onto PMMA-EUDp, leads us to the conclusion that the experimental procedure should be modified. One of the problems to solve is related to the centrifugation step, that was unable to remove all the particles from the supernatants, causing interference with the spectrophotometric assays. In addition, in this step the protein adsorbed to the particles could be physically desorbed causing an overestimation of non-adsorbed BSA in the supernatants, leading to the false conclusion that there was no adsorption to the particles. Also, the particles or BSA concentrations could not have been appropriate for the selected methodology.

3.3. *In vitro* cellular assays

Biological evaluation is of utmost importance in assessing the potential benefit of nano/microparticles systems for human use. *In vitro* cellular studies can be an important first step when considering the safety assessment of these systems. In fact, particulate-systems interacting with cells and the extracellular environment can trigger a sequence of biological effects. These effects largely depend on the dynamic physicochemical characteristics of the particles (previously discussed), which determine the biocompatibility and efficacy of the intended outcomes (Naadhi *et*

al., 2013). Despite the large interest of acrylic particles for medical applications lack of toxicological data still persists (Bettencourt *et al*., 2012).

In order to get further insights on the biological effects of the different prepared and characterized acrylic particles, three different cell lines were used namely mouse fibroblasts (L929), human osteoblasts (MG63) and monocytes/macrophages (THP1).

L929 fibroblasts are a cell model often used in the biocompatibility studies of biomaterials as it is recommended by the ISO 10993-5. As bone infections would be a potential therapeutic target for these acrylic particles (Bettencourt and Almeida 2012), human osteoblasts were also selected because particles are supposed to be in contact with bone forming cells, and there is some literature pointing to the toxicological effects of acrylic cement wear debris (in the nano and microsize range) in these same type of cells (Lohmann *et al.*, 2002). The use of macrophages is fully justified since these cells are primary mediators of the chronic foreign body response to implanted materials.

Different tests commonly used to evaluate the biological effects of the particles were assessed namely, cell uptake, mitochondrial enzyme activity (MTT assay), plasmatic membrane damage (Trypan blue exclusion assay), genotoxicity and stress response assays. The combination of different methods with specific targets, within the structure of the cell, is highly recommended since it provides a more reliable final evaluation of toxicity. Also, in order to obtain robust data from these assays the user must determine the correlation between what is measured and cell viability, since each assay has its own specifications and limitations that must be taken into account when choosing the ones that will best correlate with the reality. The type of cell in study also plays its part on the chosen methods since some are more suited for adherent cell and others to cells that grow in suspension (Rampersad, 2012).

3.3.1. Cell uptake assays

Certain therapeutic strategies require a more direct approach on drug delivery to certain cellular targets. In this context, it is of the upmost importance to study the internalization of these particles by the cells. Also, some toxic effects as genotoxicity may only occur if the particles are internalized. To date, there aren't many studies assessing PMMAp internalization. Still, some of the existing studies could provide insight on this problematic and on the properties involved in particle endo/exocytosis. Papa *et al.* (2014) proved that PEGylation and surface charge intervene on the selective internalization of PMMAp by LPS-activated microglia.

For cell uptake assays, and as previously mentioned, the particles were stained with a fluorescent dye, Nile Red (NR), with known excitation and absorbance wavelengths on which the amount of fluorescence was measured after cell lysis.

Assays were then conducted on the three previously described cell lines (L929, MG63 and THP1). A thorough analysis of the obtained data allowed the comparison of uptake values over its variation with time, particle type and concentration.

In L929, no significant differences with increasing concentration were detectable for internalized PMMAp, after either 1 or 24 h exposure (Figure 18). In contrast, PMMA-EUDp showed significant and consistent increase in uptake values between each concentration after both 1 and 24 h. After 24 h of exposure, PMMA-EUDp have shown their highest uptake for the maximum tested concentration.

In MG63 and THP1 cells, PMMA-EUDp showed significant increase in the uptake values with increasing concentration for both exposure times. For PMMAp, a slight increase was observed for the 2 highest concentrations for both times but comparatively lower than the ones presented with PMMA-EUDp (Figure 18).

Overall, both particles were internalized by the evaluated cells, though with greater efficiency for the PMMA-EUDp, since these presented higher uptake values (clearly concentration and time dependent) than PMMAp.

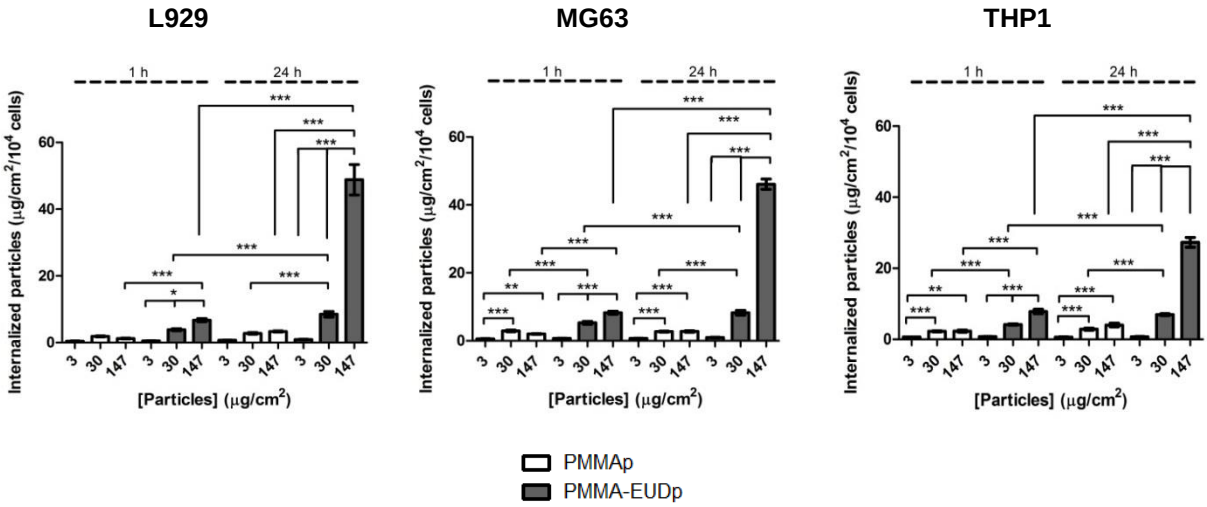

Figure 18 - Results from uptake assays after particle exposure for 1 and 24 h; Note – *, ** and * correspond to *p*<0.05, *p*<0.01 and *p*<0.001, respectively (n = 5).**

The comparison between both particles showed that PMMA-EUDp could be more easily internalized than PMMAp for all the tested cell lines, with higher differences observed for the 24 h assays. The reason for these differences may be related to their specific physicochemical features. One possible explanation could be the difference between the particles charges since positive charges are expected to be easily internalized in consideration of the attraction exerted by the negatively charged cytoplasmic membrane of the cells.

However, our results have indicated that the particles charge was heavily altered by the medium and that PMMA-EUDp even inverted their surface charge from positive to negative. Yet, a possible explanation to our results could be that these particles charge variation would be due to adsorption of various components on their surface which would mask their original charge but that when they were put in contact with the cells, these components could easily dissociate from the particles due to low interactions, returning the particles to their original surface charge. Our results are in accordance with Papa *et al.* (2014) who found that positively charged PMMAp had faster uptake rates that negatively charged ones. In that study, charge was the main difference between particles due to different levels of PEGylation. However, in the mentioned paper no evaluation of the effect of serum on the charge of the particles was conducted, so it is not known if the particles evaluated by Papa *et al.* (2014) would eventually have the change in charge as the ones presented in this study. The study also showed that PMMAp probable uptake mechanism should be a clathrin-mediated one since an inhibitor of this pathway chlorpromazine was able to decrease the uptake values.

Besides charge, it has been shown that cellular uptake depends on the hydrophobicity of the nanoparticles. This can be attributed to hydrophobic interaction between the surface of the nanoparticles and the lipophilic cellular membrane (Wu *et al*., 2012; Juneja and Roy, 2014). PMMA-EUDp showed to be highly hydrophobic which could account for favoring particles internalization rather than the charge effect. Our results are in accordance with the study of Juneja and Roy (2014), who concluded that coating PMMA nanoparticles with hydrophilic polymers, such as PVA and polyacrylic acid, decreased nanoparticles uptake by lung carcinoma cells. Also, it has been shown on the literature that the adhesion force between particles and cell membranes can increase with enhancement of particles surface hydrophobicity, creating a favorable situation for the internalization of particles into the cells (Liu *et al*., 2013).

Human monocyte-derived macrophages presented the lowest uptake values after the 24 h exposure assays. This could either be simply due to their duplication time being higher than for the other two cell lines or because of cell internal saturation (Papa *et al.*, 2014).

To get further insights on particles uptake, confocal microscopy studies were carried out on L929 and MG63 cell lines. Due to macrophages well-known capacity for particles internalization we do not consider relevant to perform these studies with THP1 cells.

Figure 19 show PMMA and PMMA-EUD particles loaded with Nile Red. As observed, PMMAp seem to be more dispersed on the solution since they are less present around the cells than PMMA-EUDp. Intense markings corresponding to PMMA-EUDp can be seen around the cells and, in some cases, inside the cells. This is valid for both cell lines although for MG63 there appear to be a smaller number of both cells and particles.

The fact that PMMA-EUDp were visible near and around the cells, while PMMAp are more scattered, is consistent with the hypothesis that positively charged particles will produce greater interactions than negatively charged ones. Confocal microscopy results are also in accordance with our uptake studies and could confirm the hypothesis that particles' charge has great importance over the particles internalization, since for the tested lines the positively charged PMMA-EUDp had higher internalization values than the negatively charged PMMAp as previously discussed.

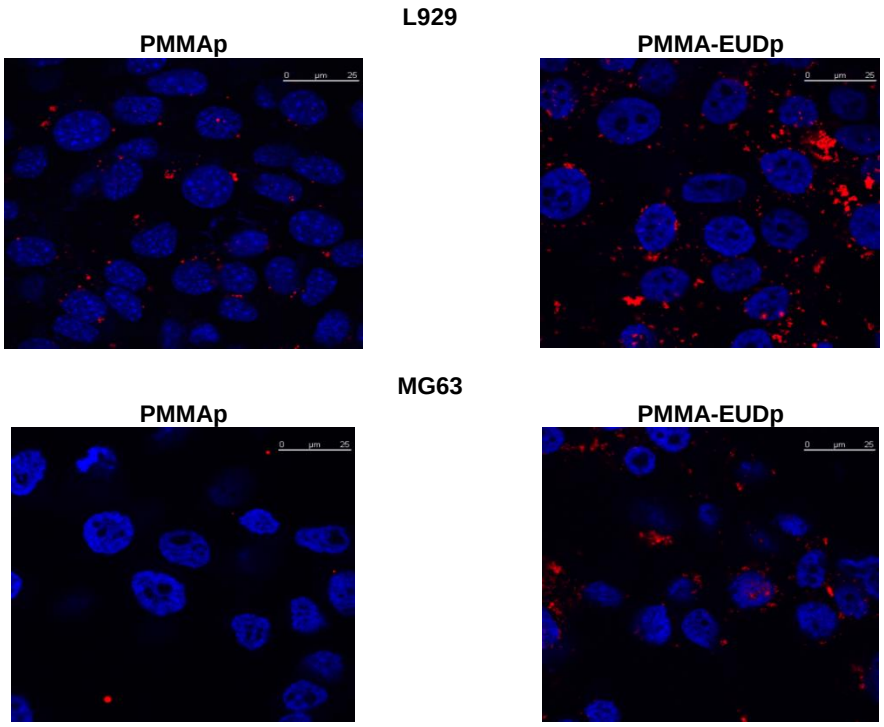

Figure 19 - Representative confocal microscopy images showing PMMAp and PMMA-EUDp (red signal) and L929 (above) and MG63 (below) cells (nuclei, blue signal) after 24 h of incubation.

3.3.2. Cytotoxicity assays

The biocompatibility of the PMMA polymer has already been investigated, including studies on cytotoxicity (Cakerer *et al.*, 2010; Gülçe Iz *et al.*, 2010; Lye *et al.*, 2011) and inflammatory potential (Horowitz *et al.*, 1988; Puricelli *et al.*, 2011). Most of the literature is related to the effects of the *in vivo* setting of the polymer and on wear debris in the nano and microsize range formed during the aging of the bone cement mantle (Goodman *et al.*, 2006; Zhu *et al.* 2010; Yang *et al.* 2011). In one of such studies, the toxicity of various MMA-based materials was evaluated. PMMA polymers showed strong cytotoxic effects on mouse fibroblasts (L929) after 48 h of exposure to the material, possibly due to incomplete polymerization during cement setting (Gülçe Iz *et al.*, 2010). Some other studies include *in vitro* tests on bone marrow cells, hepatocytes, cancer-derived and kidney cells.

There is also some literature on *in vivo* studies, mostly on model mice systems (Campos *et al.*, 2011; Dhana lekshmi and Reddy, 2012; Sitia *et al.*, 2014).

The MTT assay was performed to evaluate particles toxicity on fibroblasts and osteoblasts. As previously mentioned it is a method based on the conversion of a yellow tetrazolium salt into purple formazan crystals by the living cell's NAD(P)H-dependent mitochondrial dehydrogenases, hence giving the information of the amount of living cells (Mosmann, 1983) . This method allows the evaluation of cytotoxicity by cell exposure to the particles on different concentrations. This is generally one of the most recurrent assays made for cytotoxicity assessment, though others may also be used depending on the evaluated cells and materials like the Alamar Blue and the Neutral Red assays. The first, a method based on the conversion of rezasurin into a fluorescent dye, was also attempted but the results had great variability and the detected fluorescence of the particles control dispersion was as high as the particle-exposed cells samples (data not shown). These results suggested that particles optical properties had interference over the fluorescence measurement which made us give up the Alamar Blue assay for these specific type of particles.

By the analysis of collected data from the MTT assays represented on Figure 20, we can verify that, in L929, cell viability only showed significant, yet slight, decrease with the two highest PMMAp concentrations after 48 h of exposure (78,34.83±2.38 and 76.86±2.38 %, respectively). After 72 h, cell viability, for the same concentrations of PMMAp, further decreased (66.83±6.59 and 66.83±7.97 %). Exposure to PMMA-EUDp significantly, but slightly, reduced cell viability after 24 h, for the two highest concentrations (80.08±3.77 and 74.36±4.36 %, respectively). After 72 h, all PMMA-EUDp concentrations significantly lowered cell viability values. Yet, the lower value was 59.98±8.55%.

In MG63 (Figure 20), significant decrease in cell viability occurs after 72 h for the two highest PMMAp concentrations (53.38±3.67 and 38.87±11.12%, respectively). For PMMA-EUDp, except for the lowest concentration, there was a significant decrease in cell viability. The lowest value was 54.77±13.47, but it does not seem to be a concentration-dependent effect.

These results indicate that some cytotoxicity should be expected after 72 h for PMMAp at concentrations between 294-588 µg/cm^2 (1-2 mg/mL) and for PMMA-EUDp at concentrations between 147-588 µg/cm^2 (0.5-2 mg/mL) on both fibroblast and osteoblast cell lines.

The existing studies focusing on the toxicity of PMMAp vary on either particle composition, formulation methods or evaluated cell line, which makes it hard to consistently compare two studies. Most of these studies, in accordance with ours, suggest no evidence of high toxicity of the PMMA particulate-systems. Acosta-Torres *et al.* (2012) results didn't show any cytotoxicity (evaluated by MTT) on mouse embryonic fibroblasts (NIH-3T3) or lymphocyte (Jurkat) cells after 24 and 72 h of exposure to PMMAp (10-20 nm). Mendes *et al.* (2012) showed the safety of PMMAp with an

average size of 90 nm and negative surface charge on human leukemic cells (K562), Dhana lekshmi and Reddy (2012) verified that no significant hemolysis occurred from hepatocyte exposure to PMMAp (300 nm). Recently, Papa *et al.* (2014) found no cytotoxicity induced by PMMAp (with mean size around 92 nm) on spinal cord-derived microglia. Finally, Sitia *et al.* (2014) showed no cytotoxic events with PMMAp with different sizes (50, 100 and 200 nm) and surface charge (negative and positive) on triple-negative breast cancer (4T1) cells, though a significant reduction occurred with positively charged PMMAp (100 nm) on another similar cell line (MDA-MB231.1833).

To the best of our knowledge no published study reports the cytotoxic effect of acrylic particles (within our size range) on MG63 to be able to compare with ours. Still, there are some studies regarding particles with different sizes on several osteoblast-like cell lines. In Lohmann *et al.* (2002) no cytotoxic effect of PMMAp (2.22±0.07 µm) was found but it was proved that these particles could mediate an increase of the synthesis of alkaline phosphatase (up to 50 %) and prostaglandin E2 (PGE-2) (30-40 %) in MG63 cells. Chiu *et al.* (2006) showed, on bone marrow-extracted cells, that a more prolonged exposure to PMMAp (1-10 µm; between 5 to 15 days) could completely suppress osteoprogenitor differentiation and proliferation the cells. Recently, in a study by Shen *et al.* (2014), cytotoxic effects of PMMAp in a size range between 0.1-10 µm and concentration of 1 mg/mL were evaluated by the lactate dehydrogenase (LDH) levels on osteoblast-precursor (MC3T3-E1) cells. Similarly to our results, LDH levels showed an increase after 48 h of exposure and further elevated levels at 72 h. The increase in LDH is correlated with the decrease of cell viability and, therefore, these results are in accordance with those presented in this thesis.

Regarding the results obtained with the fibroblasts, the ones reported in this work are supported by studies with 100 and 500 nm fluorescent PMMAp where, using the XTT assay, which is similar to MTT, cell viability of L929, after exposure to nanoparticles, was similar to the negative controls. It is noticeable that these results were obtained with low particle concentrations (0.1-10 µg mL^{-1}) and only for 24 h of exposure. In addition, cell integrity was confirmed as well as the absence of haemolytic or aggregation effects on erythrocytes (Vollrath *et al.*, 2012). PMMAp safety in terms of particle cytotoxicity was also confirmed in human foreskin fibroblasts and monkey kidney cell lines, through the MTT assay, with up to 500 µg/mL of nanoparticles (114 – 169 nm) (Hoffmann *et al.*, 1997).

Other studies with human foreskin fibroblasts proved that pre-formed PMMAp (1-10 µm) are capable of mediating the release of pro-inflammatory C-C chemokines, such as MCP-1 and IL-6. Despite of this fact, no significant cytotoxicity was detected with the LDH method after exposure to several PMMAp concentrations for 24 h (Yaszay *et al.*, 2001).

Interestingly, to the best of our knowledge, no studies were reported so far addressing the evaluation by MTT or other related methodology of PMMA-EUDp cytotoxicity.

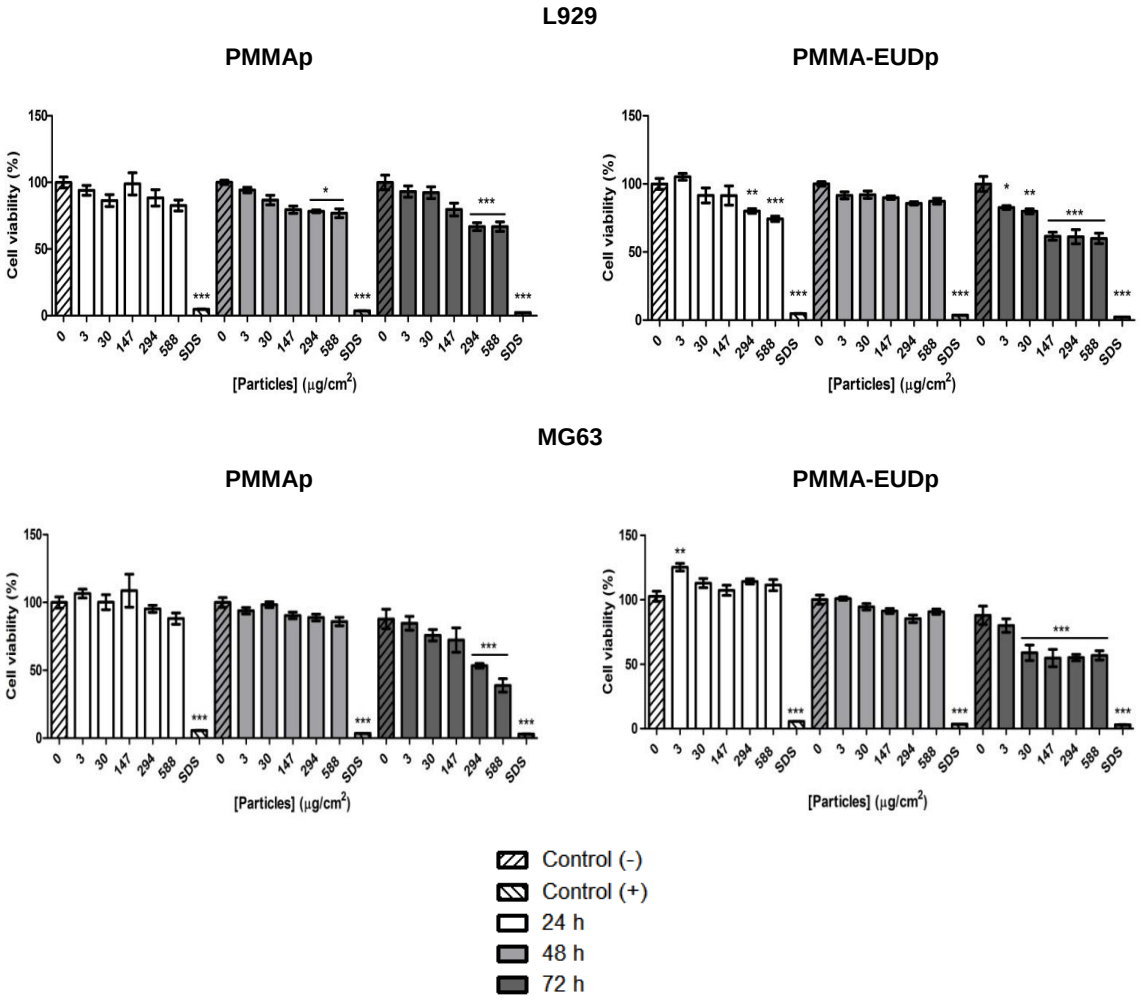

Figure 20 - MTT results on L929 and MG63 cells viability variation to particle exposure; Note: *, ** and * significantly different from negative control (*p*<0.05; *p*<0.01 and *p*<0.001, respectively) (n = 5).**

Accordingly to Dhana lekshmi and Reddy (2012), charge is crucial for toxicity. Since PMMA-EUDp present a positive charge measured in water (section 3.2.4.) it would then be expected to observe a greater impact on cell viability than the negative PMMAp, yet the differences between particles were not very pronounced on tested cell lines. This fact may be related with the change in the surface charge of PMMA-EUDp (section 3.2.6.2.) due to the effect of both the culture medium and serum. PMMA-EUDp charge became negative in cell culture conditions which could have contributed in decreasing the toxicity related to the positive charge. This could be a great advantage in terms of their applicability on the drug delivery field because of the previously described effects of the presence of Eudragit in the particles release properties, hence improving the effectiveness for drug-treatments. However, charge alone, though crucial for the effects, does not imply that given particles will be secure or otherwise cause these effects, as previously explained depend on a variety of different factors (e.g. size, hydrophobicity).

Cell membrane integrity was also assessed, using the trypan blue dye exclusion assay, which is commonly used to measure viable cells, through the incorporation of a dye on dead cells (Mehri *et al.*, 2012). Viable cells appeared refringent and dead cells incorporated trypan blue (0.4 %) due to membrane damage. Viable and non-viable cells are counted and, if there is sufficient number of cells, an assessment is made based on the ratio of viable cells, constituting a cell viability assay. However, this method doesn't allow one to distinguish between healthy cells and cells losing their cellular integrity, which constitutes a serious problem since the last may internalize the dye and, therefore, be counted as unviable when in fact their cellular repair mechanisms are able to recover from the sustained damage.

Results showed that after 24 h of exposure the number of cells permeable to the dye was very low (even for concentrations as high as 2 mg/mL) and didn't show evidence of a concentration-dependent effect for either particle type (Table 9).

Table 9 - Cell counting for membrane integrity assessment (n = 4).

PMMAp	MG63		L929	
[Particles] (mg/mL)	Non-viable Cells	Total Counted Cells	Non-viable Cells	Total Counted Cells
0	0	56	3	173
3	0	55	8	179
30	1	53	3	149
147	1	51	11	186
294	0	33	13	138
588	0	54	3	161
PMMA-EUDp	**MG63**		**L929**	
[Particles] (mg/mL)	**Non-viable Cells**	**Total Counted Cells**	**Non-viable Cells**	**Total Counted Cells**
0	0	56	3	173
3	0	38	9	148
30	1	32	6	144
147	0	24	3	168
294	0	20	6	168
588	0	29	5	141

The low number of counted cells, partly due to the dilution factor applied (1:5), didn't enable the quantification of cytotoxicity but showed that the particles did not cause damage to the cells' membranes.

This preliminary assay had relevance since the chosen subsequent *in vitro* assays were based on interactions with internal enzymes and/or organelles, which would be affected in case that the cells membranes were compromised.

3.3.3. Genotoxicity

The increasing interest in the application of nanomaterials in various fields, such as industry and medicine, has dictated a greater need for an extensive research on their potential to generate toxic effects and, particularly, genotoxicity. As the particles developed in this study have potential for application as drug carriers, their adverse effects must be prevented, especially in cases of non-cytotoxic drug delivery. Therefore, genotoxicity must be evaluated, especially considering long-term uses. Indeed, genotoxicity is frequently associated with carcinogenicity, which is time-consuming and difficult to evaluate. Thus, short-term genotoxicity assays are used as a consensual alternative. In spite of existing clear guidelines for carcinogenicity, genotoxicity and reproductive toxicity testing of pharmaceuticals for human use (ICH 2012), the particular case of the nanomaterials field of expertise is still lacking proper ones that could guide testing of the genotoxicity of such materials (Shah *et al.*, 2013). This part of the study aimed at evaluating the genotoxicity of the newly developed particles through the Comet assay and the Micronucleus assay, since, as previously described, these are two complementary assays commonly used to determine the genotoxic potential of chemicals and nanomaterials.

3.3.3.1. Comet Assay

Representative images are shown in Figure 21. Round nucleoids are seen when no damage occurs on DNA strands from exposure to a given agent. The increase in the length and intensity of fluorescence of the comet's tail is proportional to the levels of damage to DNA. In this work, most of the analysed nucleoids were similar to the first picture, i.e., without or with a very low level of DNA in the tail. Comets with longer tails, as exemplified in the last picture, are typical of positive controls. Also exemplified, is the effect produced by using the enzyme FPG on assessing oxidative damage. As the enzyme increases the sensitivity of the assay to oxidative DNA damage, usually, higher levels of DNA damage are observed.

Our results of the Comet assay in cells exposed to PMMAp and PMMA-EUDp, expressed in terms of the percentage of DNA in the comet tail, are presented in Figure 22. In general, low levels of DNA in the comets tails were observed in cells exposed to several concentrations of both test particles, independently from the exposure length (3 and 24 h), implying that these particles do not produce genotoxic events, at least under the experimental conditions applied. The FPG modification of the Comet assay was used to assess oxidative DNA damage recognized by the enzyme and converted into DNA breaks. By comparing (and calculating) the difference between the results obtained for nucleoids treated with the enzyme and their untreated counterparts, a measure

of oxidative DNA damage was obtained. No significant differences were found between FPG-treated and untreated cells, indicating that the particles under study do not produce significant oxidative DNA damage in fibroblasts. As expected, either the conventional comet assay or the FPG-modified comet assay was able to detect significant increases in DNA damage in cells exposed to hydrogen peroxide (positive control), confirming the validity of the data reported for the particles.

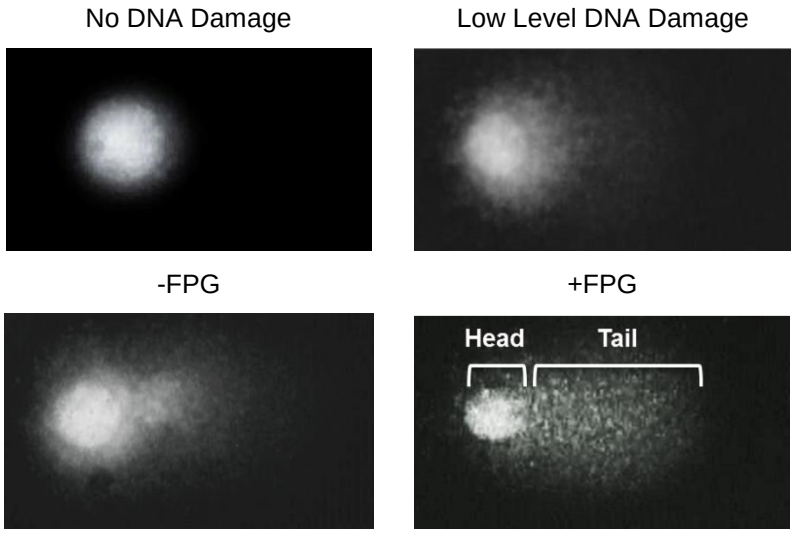

Figure 21 - Examples of increasing DNA damage that can be found during Comet Assay analysis.

Both particles gave rise to negative effects, independently from their potential to form aggregates/agglomerates. Previous results (Section 3.2.6.2.) from particle stability in culture medium with FBS, showed that PMMA-EUDp tend to agglomerate/aggregate over time when dispersed in culture medium containing 10 % FBS, while PMMAp size distribution remains steady in the same medium. Due to this different dynamic behaviour we were expecting a differential DNA damaging effect since it had been previously reported that particles aggregation may lead to an accumulation of particles near the cells interface, which could lead to the recruitment of free radicals and, consequently, to indirect primary genotoxicity (Donaldson *et al.*, 2010; Magdolenova *et al.*, 2014). This was not the case and it might even have been that aggregation reduced or even prevented its uptake into the nucleus, therefore, limiting the amount of particles which could interact with the nucleus and, consequently, the potential for direct primary genotoxicity.

Since particle size ranged from 20 and 2000 nm with a mean size of 500 nm, it is likely that very few, if any, particles would be able to transpose the nuclear barrier, which only substances below 100 nm may transpose, according to the literature (Tkachenko *et al.*, 2004)

We were only able to find one other study specifically relative to PMMA's monomer, MMA, which showed a genotoxic potential on CHO cells through the chromosome aberration and sister-chromatid exchange assays (Yang *et al.*, 2003). This could be indicative that, should the particles suffer loss of some of these monomers, the same effect could be verified after their application and, therefore, it is extremely important to confirm their stability.

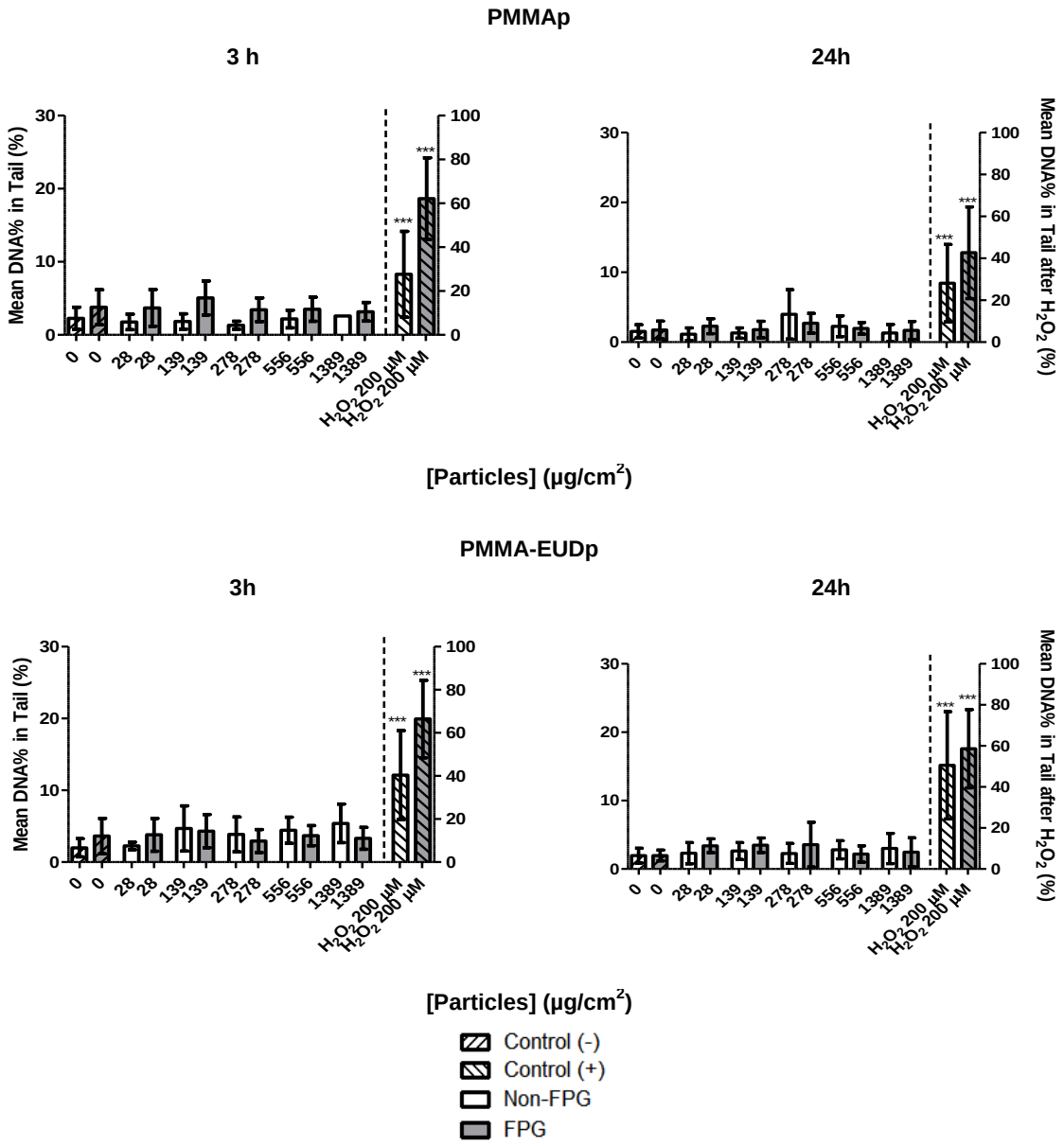

Figure 22 - Damage to the cells' DNA is expressed as the mean DNA percentage on the comets' tails. Note: * significantly different from negative control (p<0.001; n = 3).**

As previously stated, charge also plays an important role in potential interactions within a cellular environment (Magdolenova *et al.*, 2014) and particles presenting a positive net charge are expected to produce more interaction with cells (Verma and Stellacci, 2009) because of the negative surface

charge verified near the membrane and since the DNA molecule itself is negatively charged. However, the genotoxic potential of our particles does not seem to be influenced by this property since there is no relationship between the different charge of the particles in culture medium (Section 3.2.6.1.) and the amount of DNA damage. Kim *et al.* (2013) also suggested that surface hydrophobicity plays an important role in both cytotoxicity and genotoxicity but, in our study, this property was not associated with any difference in the toxicity of the tested particles.

To the best of our knowledge, there are no other studies involving particles with at least two similar properties for comparison purposes with our study. Yet, there are some studies with other polymeric particles concerning their genotoxic potential. No clastogenicity was found after 6, 24 and 48 h exposures of Chinese hamster lung cells to PCL-Polyethylene glycol (PEG)-PCL particles (≈ 40 nm), since no chromosomal aberrations occurred (Huang *et al*., 2010). The authors also found no genotoxicity on ICR mice exposed to these same particles, using the *in vivo* micronucleus assay. Furthermore, in Lima *et al*. (2011), no genotoxic effects were found from 72 h exposure of human lymphocytes to negatively charged PLGAp (≈ 100 nm) through the evaluation of chromosomal abnormalities.

Kazimirova *et al*. (2012) observed no genotoxic effects from negatively charged PLGA-polyethylene oxide (PEO) particles, with a bimodal distribution (143 and 180 nm), on human B-lymphoblastoid cells after exposure for 2 and 24 h, with the comet assay, and 24, 48 and 72 h with the micronucleus assay. In Jena *et al*. (2012), however, genotoxicity was found from exposure of chitosan-coated silver particles with a bimodal distribution (55 and 278 nm, respectively) and strongly positive surface charge (36.3 and 51.1 mV, respectively), though only for the highest concentrations (20 ppm). All studies were in accordance with this work on the absence of genotoxicity induced by polymeric particles as with PMMAp and PMMA-EUDp. The latter study was not in accordance with our results for the PMMA-EUDp, which were also positively charged. The reasons for this different effect could be explained from properties, such as the chemical composition of the applied polymer, particle size or even the presence of a metallic component on the studied particles, which could be more prone to oxidative reactions.

3.3.3.2. Micronucleus Assay

The Micronucleus assay was used in an effort to further evaluate the genotoxicity from exposure to PMMAp or PMMA-EUDp and to confirm the results previously obtained through the Comet assay, since it is a validated assay for regulatory purposes (ICH 2012). However, during the assay we observed that the standard technique was not directly applicable to the L929 cell line, since after treatments and hypotonic shock the cytoplasm integrity could not be attained in most.

We then proceeded with an optimization of the method by attempting different Cytochalasin B exposure intervals (36 *vs* 42 h), hypotonic shock solutions (0.1M KCl *vs* 1:1 dilution of culture

medium with sterile bi-distilled water), fixing solutions (1:3 acetic acid and methanol *vs* 3:97 of the same solutions), spreading methods (manual spreading vs cytospin-assisted) and staining time intervals with Giemsa (5, 8, 10, 13 and 15 min). In the end, we were able to accomplish optimization but, as previously justified, could not proceed with the evaluation. We assessed that the most critical parameters would be the hypotonic shock, having chosen 0.1M KCl, the fixing solution, which proved better with a 1:3 proportion between the chemicals, and the spreading method, with the cytospin-assisted spread allowing to obtain more cells with less strain on the cytoplasm integrity.

After the modification of the method, negative control samples were analysed and results are presented in Table 10:

Table 10 - Assessment of the number of micronucleus presented in untreated cells (negative control).

Concentration (µg/ml)	CBMN/1000 CB	CBPI
0	10.8	1.74

Note: CBMN - Micronucleus number in bi-nucleated cells; CB – Bi-nucleated cells; CBPI – Cytokinesis-block proliferation index.

The CBPI value gives us an indication that only about two cell cycles had passed when the cells were collected. This result allied with the fact that a high number of mononucleated cells were still found could be indicative that further optimization should be attained possibly by prolonging the time of treatment with Cytochalasin B. Furthermore, the background level of MN for L929 cell was established which allows proceeding in the future with the assay after exposure to the test particles.

To the best of our knowledge, very few studies exist on the literature about particle genotoxicity assessment in L929 cell line using the micronucleus assay (Chlopkiewicz *et al.*, 2001). This technical constraint together with the fact that the required microscopic analysis is quite time consuming prevented the test from being performed within the available time frame. Further testing should be made with this optimized method to evaluate the adequacy of the method to this cell line. No problems were found during the comet assay, as it proves to be a good alternative to the micronucleus assay.

As previously referred, by adding this particular type of EUD to the composition of PMMA particles could greatly improve its efficiency of drug encapsulation and release. Within the applied experimental conditions it was shown that this modification did not alter the genotoxic potential of these particles. Still, further research is needed to complementarily confirm this hypothesis and the particles' safety, namely the re-evaluation of the particles effects with the micronucleus assay. *In vivo* testing is also recommended, as it is impossible to completely simulate all biological condition with *in vitro* assays.

3.3.4. Oxidative Stress

The intracellular ROS production was determined using H_2DCFDA. It is a stable, non-fluorescent molecule that is hydrolysed by intracellular esterase to non-fluorescent 2-7'-dichlorodihydrofluorescein (DCFH), which is rapidly oxidized in the presence of hydroxyl radical to a highly fluorescent compound (DCF) (Crow *et al.*, 1997). This method allows the specific detection of these compounds in a solution and can be measured by fluorescence intensity which is correlated with the relative quantity of fluorescent units.

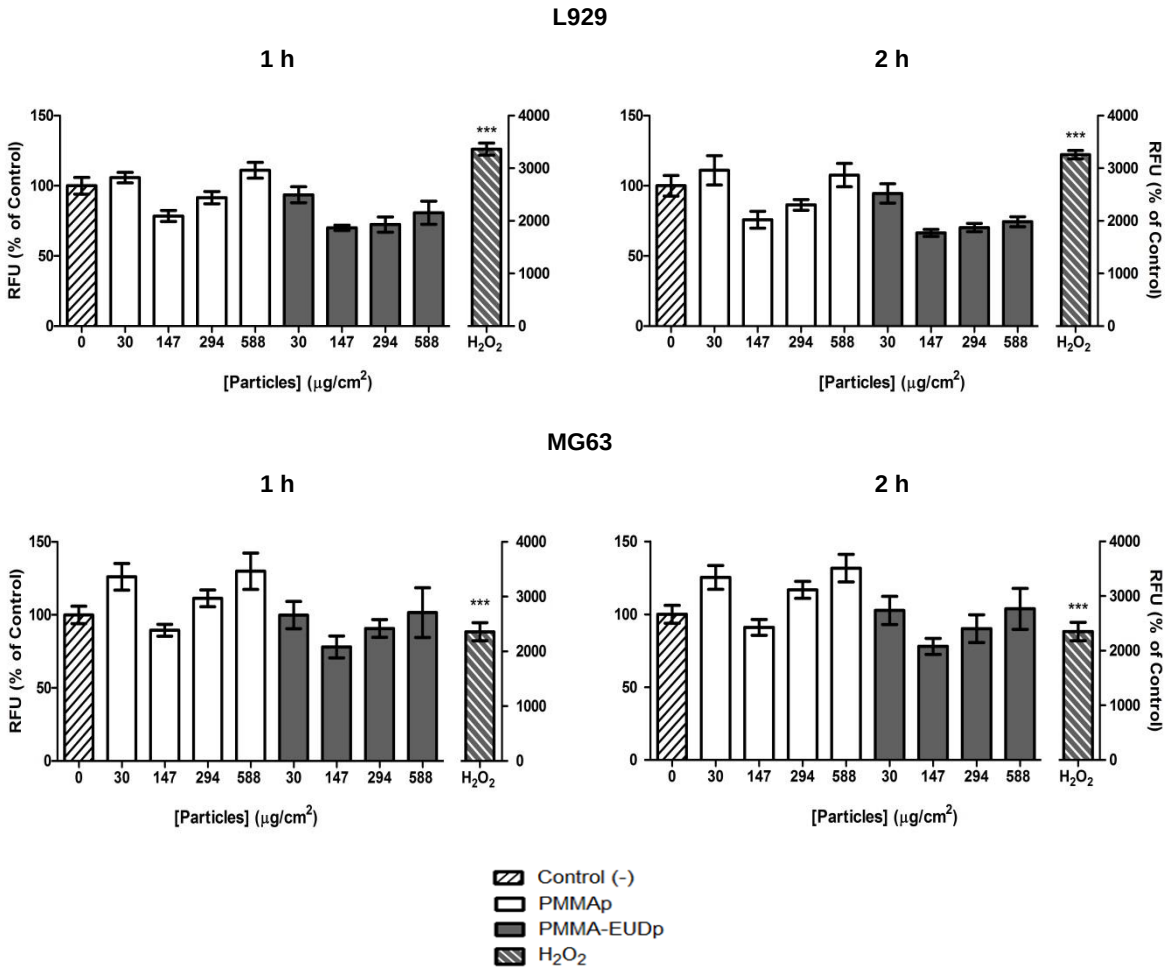

Figure 23 - Evaluation on the formation of ROS through relative fluorescence unit percentage (RFU); Note – * Significantly different from control (culture medium) (p<0.001); RFU % - Relative fluorescence units percentage.**

With this in mind, this method was applied to evaluate the intracellular formation of ROS by the particles interactions with L929 and MG63 cells. Exposure of L929 cells to various concentrations of each particle type didn't show significant variation from the values obtained with negative controls (Figure 23; P>0.05). No significant variation occurred between 1 and 2 h of exposure neither with particle type. With MG63 the same results were achieved. We can, therefore, conclude from this assay that these particles do not originate significant formation of ROS inside the tested cell lines

due to low variation of RFU percentage compared to those of negative controls. This effect was proved not be time or dose-dependent on these particles, within the established parameters of the assay.

These results are comparable and confirm those from the modified comet assay recurring to FPG, since on the mentioned assay no significant rise in DNA damage from oxidative lesions was found between treated and untreated samples with this enzyme.

Few reports that refers to the induction of free radicals by the PMMA polymer are related with the release of ROS during the *in vivo* setting of bone cement by cultured human fibroblasts (Vale *et al*., 1997) or the effect of PMMA wear debris (particle sizes range between 0.2-1 μm in diameter) by murine whole bone marrow cultures (Fang *et al*., 2011). In the case of engineered PMMA and PMMA-EUD particles, our study showed that no effect on ROS production is expected on fibroblast or osteoblast cells.

Chapter 4. Conclusions and Future Work

In summary, the objectives proposed in the beginning of this thesis of preparing, characterizing and evaluating the biological/toxicological effects of engineered acrylic particulate-systems were achieved during the timeframe of this project. SESE methodology allowed the preparation of particles with spherical and smooth surfaces within the micrometer range (PMMA = 572.7 ± 20 nm; PMMA-EUD = 508.9 ± 8 nm) with unimodal and narrow size distribution. Surface charge modification, as measured through zeta potential, was one of the main feature observed after inclusion of EUD in the formulation. Initially, PMMAp were strongly negative (-32.7±1.04 mV) whereas PMMA-EUDp were strongly positive (+31.8±1.66 mV). The formulated PMMA-EUDp have shown higher levels of hidrophobicity than the PMMAp.

Another important conclusion from our studies refers to the fact that physicochemical properties of the particles as size and surface charge were highly influenced by the media composition. The surface charge of PMMAp was shown to be neutralized by both fetal serum and ionic strength, while PMMA-EUDp surface charge was only neutralized by ionic strength but inverted by the presence of the fetal serum proteins. It was demonstrated that the evaluated particles had very different pH profiles as well as isoelectric points. Furthermore, it was verified that PMMA-EUDp may agglomerate over time in biological conditions. Protein adsorption was studied using a model protein (bovine serum albumin) through UV-Vis spectroscopy but results were inconclusive. Concerning the evaluation of the particles biological/toxicological effects assessed by *in vitro* cellular assays, it was concluded that the particles were internalized in THP1, L929 and MG63 after only 1 h of exposure. This uptake proved to be both time and dose-dependent for PMMA-EUDp but not for PMMAp. The uptake of the particles was confirmed by confocal microscopy analysis.

Cytotoxicity, evaluated by the MTT assay on L929 and MG63 was only observed after 72 h of exposure. The potential for cell membrane damage was evaluated with Trypan Blue and it was concluded that the particles did not affect its integrity.

Genotoxicity testing showed that PMMAp and PMMA-EUDp were not genotoxic *in vitro*, given that no significant induction of in DNA damage was found through the comet assay for either particle type, not significantly different from negative controls. Also, no significant oxidative DNA lesions occurred. This absence of oxidative damage was confirmed with the H_2DCFDA oxidative stress assay, since no significant rise in ROS was detected. Nevertheless, further tests with validated methods, such as the micronucleus assays that we were able to optimize for L929, would be valuable to confirm the genotoxicity results.

It can, therefore, be concluded that both PMMAp and PMMA-EUDp proved to be safe on the tested cell lines and within the conditions employed on the various assays.

Further studies need to be assessed to deeply understand the interaction of the particles with the biological environment and ensure a safe biomedical application of these acrylic particles.

The taken approach for hydrophobicity evaluation was a comparison method (HIC). This kind of methodologies has the inconvenient of depending on many variables, some of them very hard to control, such as the physical conditions of the resins and the time taken for elution on the columns. It was acknowledged that other methodologies, as the measurement of contact angles, could produce more definite results, which weren't followed for lack of time. This technique should be used to confirm our results.

It is proposed that a better characterization in terms of protein adsorbance should be conducted, preferably eliminating the centrifugation steps. Improvement in the technique should focus on removing the particles effect and presenting sensitivity to small changes in adsorption. A positive control should also be found to verify the capabilities of the method within the chosen parameters. A range of particle concentrations were tested for one BSA concentration and vice-versa, but it could not be discarded that the chosen conditions could be under the detection limit of the assays. Therefore, greater quantities of both particles and protein should be mixed in order to possibly potentiate the adsorption and achieve quantifiable results.

Another important aspect to consider in future work is the evaluation of the mechanisms by which the particles are internalized by the cells. It is an aspect of great importance, not only from a toxicity-related safety point of view, but also to understand how a pharmaceutical compound could be delivered to certain cell compartments, as well as its bioavailability. So, further evaluation of particle uptake as well as exocytosis mechanisms should be conducted in order to better understand particles biological behaviour. Moreover, to get further insights on particles toxicity, other complementary studies should be conducted. For example, the inflammatory potential and haemolytic effects will be useful.

In respect to the genotoxicity evaluation, as we were able to achieve a proper protocol for the Micronucleus assay on L929 cells, a complementary evaluation should be conducted on the particles genotoxic effects. Also, other cell lines like the MG63 could be tested to allow extrapolation of the effects of the particles to other tissues.

Although *in vitro* assays allow the specific and detailed comprehension of several biological effects and its mechanisms, it is very difficult to actually mimic all the conditions that the particles would encounter inside a living organism, as further interactions could arise from the many bio-interactions between the various tissues and circulating components. As such, as recommended by several safety regulations, it is necessary to conduct *in vivo* studies to evaluate particle safety on real biological conditions.

Overall, the present study suggests that the evaluated acrylic particles may be used in biomedical applications, showing promising properties as drug-delivery carriers. Therefore, the next logical step would be testing the toxicological particles' properties when loaded with a model drug as well as evaluating their intake release profiles and potential delivery mechanisms.

Chapter 5. Bibliography

Acosta-Torres LS, Mendieta I, Nuñez-Anita RE, Cajero-Juárez M, Castaño VM. 2012. Cytocompatible antifungal acrylic resin containing silver nanoparticles for dentures. International Journal of Nanomedicine 7: 4777-4786

Allouni ZE, Cimpan MR, Høl PJ, Skodvin T, Gjerdet NR. 2009. Agglomeration and sedimentation of TiO_2 nanoparticles in cell culture medium. Colloids and Surfaces B: Biointerfaces 68: 83-87

Alpar HO, Almeida AJ. 1994. Identification of some physico-chemical characteristics of microspheres, which influence the induction of the immune response following mucosal delivery. Eur J Pharm Biopharm 40: 198-202.

Arora S, Rajwade JM, Paknikar KM. 2012. Nanotoxicology and *in vitro* studies: The need of the hour. Toxicology and Applied Pharmacology 258: 151-165

Arvizo RR, Miranda OR, Thompson MA, Pabelick CM, Bhattacharya R, Robertson JD, Rotello VM, Prakash YS, Mukherjee P. 2010. Effect of Nanoparticle Surface Charge at the Plasma Membrane and Beyond. Nano Lett. 10: 2543-2548

Bettencourt A, Almeida AJ. 2012. Poly(methyl methacrylate) particulate carriers in drug delivery. Journal of Microencapsulation 29 (4): 353-367

Bettencourt A, Almeida AJ. 2014. Poly(methyl methacrylate) (PMMA): drug delivery carrier applications. In: Mishra MK, (ed). Encyclopedia of Biomedical Polymers and Polymeric Biomaterials. CRC Press, Taylor & Francis, (ISBN-13:9781439898796) (*In Press*)

Bettencourt A, Florindo HF, Ferreira IFS, Matos A, Monteiro J, Neves C, Lopes P, Calado A, Castro M, Almeida AJ. 2010. Incorporation of tocopherol acetate-containing particles in acrylic bone cement. Journal of Microencapsulation 27 (6): 533-541

Blunk T, Hochstrasser DF, Sanchez JC, Müller BW, Müller RH. 1993. Colloidal carriers for intravenous drug targeting: Plasma protein adsorption patterns on surface-modified latex particles evaluated by two-dimensional polyacrylamide gel electrophoresis. Electrophoresis 14: 1382-1387

Borra RC, Lotufo MA, Gagioti SM, Barros FM, Andrade PM. 2009. A simple method to measurecell viability in proliferation and cytotoxicity assays. Braz Oral Res 23 (3): 255-262

Caicedo-Carvajal CE, Liu Q, Remache Y, Goy A, Suh KS. 2011. Cancer Tissue Engineering: A Novel 3D Polystyrene Scaffold for *In Vitro* Isolation and Amplification of Lymphoma Cancer Cells from Heterogeneous Cell Mixtures. Journal of Tissue Engineering 2011: 362326

Cakarer S, Selvi F, Isler SC, Olgac V, Keskin C. 2010. Complication of Polymethylmethacrylate Bone Cement in the Mandible. J. Craniofac. Surg. 21: 1196-1198

Campos DLP, Proto RS, Santos DC, Ruiz RO, Brancaccio N, Gonella HA. 2011. Avaliação histopatológica do polimetilmetacrilato em ratos ao longo de um ano. Rev. Bras. Cir. Plást. 26 (2): 189-193

Caputo A, Castaldello A, Brocca-Cofano E, Voltan R, Bortolazzi F, Altavilla G, Sparnacci K, Laus M, Tondelli L, Gavioli R, Ensoli B. 2009. Induction of humoral and enhanced cellular immune responses by novel core–shell nanosphere- and microsphere-based vaccine formulations following systemic and mucosal administration. Vaccine 27: 3605-3615

Changerath R, Nair PD, Mathew S, Nair CPR. 2009. Poly(methyl methacrylate)-Grafted Chitosan Microspheres for Controlled Release of Ampicillin. J of Biomed Mater Res PartB: Appl Biomater 89B: 65-76

Choi SW, Zhang Y, Yeh YC, Wooten AL, Xia Y. 2012. Biodegradable porous beads and their potential applications in regenerative medicine. J. Mater. Chem. 22: 11442-11451

Chompoosor A, Saha K, Ghosh PS, Macarthy DJ, Miranda OR, Zhu ZJ, Arcaro KF, Rotello VM. 2010. The role of surface functionality on acute cytotoxicity, ROS generation and DNA damage by cationic gold nanoparticles. Small 6: 2246–2249

Chen M, von Mikecz A. 2005. Formation of nucleoplasmic protein aggregates impairs nuclear function in response to SiO_2 nanoparticles. Experimental Cell Research 305: 51-62

Chithrani BD, Ghazani AA, Chan WCW. 2006. Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells. Nano Lett. 6 (4): 662-668

Chiu R, Ma T, Smith RL, Goodman SB. 2006. Kinetics of Polymethylmethacrylate Particle-Induced Inhibition of Osteoprogenitor Differentiation and Proliferation. Journal of Orthopaedic Research 25: 450-457

Chlopkiewicz B, Ejchart A, Anuszewska E. 2001. Tofisopam – Evaluation of Mutagenic and Genotoxic Properties. Acta Poloniac Pharmaceutica – Drug Research 58 (1): 31-34

Colombo C, Lupi M, Ubezio P, Moscatelli D. 2013. Cytotoxicity of PMMA-Based Nanoparticles Synthesized Adopting SDS and Tween 80. Macromol. Symp. 324: 134-139

Crow JP. 1997. Dichlorodihydrofluorescein and dihydrorhodamine 123 are sensitive indicators of peroxynitrite in vitro: implications for intracellular measurement of reactive mitogen and oxygen species. Nitric Oxide 1(2):145-157

Cui F, Qian F, Yin C. 2006. Kinetics of Polymethylmethacrylate Particle-Induced Inhibition of Osteoprogenitor Differentiation and Proliferation. International Journal of Pharmaceutics 316: 154-161

Dansereau A, Hamilton D, Kavouni A, Neuhann-Lorenz C, Pollack S, Richards R, Roy MC, Rullan P, Tang C, Benchetrit A. 2008. A Report on the Safety of and Satisfaction With Particle-Based Fillers, Specifically Polymethylmethacrylate Microspheres Suspended in Collagen. Cosmetic Dermatology 21 (3): 151-156

Dhana lekshmi UM, Poovi G, Kishore N, Reddy PN. 2010. *In vitro* characterization and *in vivo* toxicity study of repaglinide loaded poly(methyl methacrylate) nanoparticles. International Journal of Pharmaceutics 396: 194-203

Dhana lekshmi UM, Reddy PN. 2012. Preliminary Toxicological Report of Metformin Hydrochloride Loaded Polymeric Nanoparticles. Toxicol Int 19 (3): 267-272

Domingues MM, Santiago PS, Castanho MARB, Santos NC. 2009. rBPI21 Promotes Lipopolysaccharide Aggregation and Exerts Its Antimicrobial Effects by (Hemi)fusion of PG-Containing Membranes. PLoS ONE 4(12): e8385

Donaldson K, Poland CA, Schins RPF. 2010. Possible genotoxic mechanisms of nanoparticles: Criteria for improved test strategies. Nanotoxicology 4 (4): 414-420

D`Souza S, Faraj JA, Giovagnoli S, DeLuca PP. 2014. Development of risperidone PLGA microspheres. Journal of Drug Delivery 2014: 620464

Ehrenberg MS, Friedman AE, Finkelstein JN, Oberdorster G, McGrath JL. 2009. The influence of protein adsorption on nanoparticle association with cultured endothelial cells. Biomaterials 30: 603–610.

European Medicines Agency (EMEA). 2006. Note for guidance on impurities: Residual solvents. CPMP/ICH/283/95. London: EMEA.

European Medicines Agency (EMEA). 2012. ICH guideline S2 (R1) on genotoxicity testing and data interpretation for pharmaceuticals intended for human use EMA/CHMP/ICH/126642/2008

Eudragit RL 100 Technical Information sheet [monograph on the internet]. Darmstadt: Evonik Industries; 2012 (cited 2014, 4 Sep) Available from: http://eudragit.evonik.com/product/eudragit/Documents/evonik-specification-eudragit-rl-100-rl-po-rs-100-rs-po.pdf

Fang Q, Wang H, Zhu S, Zhu Q. 2011. N-acetyl-L-cysteine Inhibits Wear Particle-Induced Prosthesis Loosening. Journal of Surgical Research 168 (2): 163-172

Fenech M. 2000. The in vitro micronucleus technique. Mutation Research 455: 81-95

Florindo HF, Pandit S, Gonçalves LM, Alpar HO, Almeida AJ. 2008. Streptococcus equi antigens adsorbed onto surface modified poly-epsilon-caprolactone microspheres induce humoral and cellular specific immune responses. Vaccine 26: 4168–4177

Fologea D, Ledden B, McNabb DS, Li J. 2007. Electrical characterization of protein molecules by a solid-state nanopore. Appl Phys Lett 91 (5): 053901-1 – 053901-3

Fonseca LB, Nele M, Volpato NM, Seiceira RC, Pinto JC. 2013. Production of PMMA Nanoparticles Loaded with Praziquantel Through “In Situ” Miniemulsion Polymerization. Macromol. React. Eng. 7: 54-63.

Forster S, Thumser AE, Hood SR, Plant N. 2012. Characterization of Rhodamine-123 as a Tracer Dye for Use In *In vitro* Drug Transport Assays. PLoS ONE 7 (3): e33253

Fubini B, Ghiazza M, Fenoglio I. 2010. Physico-chemical features of engineered nanoparticles relevant to their toxicity. Nanotoxicology 4 (4): 347-363

Ge J, Neofytou E, Lei J, Beygui RE, Zare RN. 2012. Protein-Polymer Hybrid Nanoparticles for Drug Delivery. Small: 1-6 DOI: 10.1002/smll.201200889

Gedik CM, Boyle SP, Wood SG, Vaughan NJ, Collins AR. 2002. Oxidative stress in humans: validation of biomarkers of DNA. Carcinogenesis 23 (9): 1441-1446

Geiser M, Rothen-Rutishauser B, Kapp N, Schürch S, Kreyling W, Schulz H, Semmler M, Hof VI, Heyder J, Gehr P. 2005. Ultrafine Particles Cross Cellular Membranes by Nonphagocytic Mechanisms in Lungs and in Cultured Cells. Environmental Health Perspectives 113 (11): 1555-1560

Gomathi T, Govindarajan C, H.R. MHR, Sudha PN, Imran PKM, Venkatesan J, Kim SK. 2014. Studies on drug-polymer interaction, *in vitro* release and cytotoxicity from chitosan particles excipient. International Journal of Pharmaceutics 468: 214-222

Goodman SB, Ma T, Chiu R, Ramachandran R, Smith RL. 2006. Effects of orthopaedic wear particles on osteoprogenitor cells. Biomaterials 27:6096-6101

Gülçe Iz S, Deliloğlu Gürhan SI, Şen BH, Endoğan T, Hasirci N. 2010. Comparison of in vitro cytotoxicity and genotoxicity of MMA-based polymeric materials and various metallic materials. Turk. J. Med. Sci. 40 (6): 905-916

Hackenberg S, Friehs G, Froelich K, Ginzkey C, Koehler C, Scherzed A, Burghartz M, Hagen R, Kleinsasser N. 2010. Intracellular distribution, geno- and cytotoxic effects of nanosized titanium dioxide particles in the anatase crystal phase on human nasal mucosa cells. Toxicology Letters 195: 9-14

Hazra C, Kundu D, Chatterjee A, Chaudhari A, Mishra S. 2014. Poly(methyl methacrylate) (core)–biosurfactant (shell) nanoparticles:Size controlled sub-100 nm synthesis, characterization, antibacterialactivity, cytotoxicity and sustained drug release behavior. Colloids and Surfaces A: Physicochem. Eng. Aspects 449: 96-113

Höcherl A. February 2012. Qualitative and Absolute Quantitative Studies of the Cell-Nanoparticle Interaction (Dissertation). Mainz (Germany): Johannes Gutenberg-Universität; 141 p.

Höchler A, Dass M, Landfester K, Mailänder V, Musyanovych A. 2012. Competitive Cellular Uptake of Nanoparticles Made From Polystyrene, Poly(methyl methacrylate), and Polylactide. Macromol. Biosci. 12: 454-464

Hoffmann F, Cinatl Jr J, Kabičková H, Cinatl J, Kreuter J, Stieneker F. 1997. Preparation, characterization and cytotoxicity of methylmethacrylate copolymer nanoparticles with a permanent positive surface charge. International Journal and Pharmaceutics 157: 189-198

Horowitz SM, Frondoza CG, Lennox DW. 1988. Effects of Polymethylmethacrylate Exposure upon Macrophages. Journal of Orthopaedic Research 6: 827-832

Hu J, Li S, Liu B. 2005. Adsorption of BSA onto sulfonated microspheres. Biochemical Engeneering Journal 23: 259-263

Huang Y, Gao H, Gou M, Ye H, Liu Y, Gao Y, Peng F, Qian Z, Cen X, Zhao Y. 2010. Acute toxicity and genotoxicity studies on poly(ε-caprolactone)-poly(ethylene glycol)-poly(ε-caprolactone) nanomaterials. Mutation Research 696: 101-106

International Standard ISO Specification 10993-5: Biological evaluation of medical devices – Part 5: Tests for in vitro cytotoxicity. 2009, 3rd edition (ISO, Geneva, Switzerland).

Jena P, Mohanty S, Mallick R, Jacob B, Sonawane A. 2012. Toxicity and antibacterial assessment of chitosan-coated silver nanoparticles on human pathogens and macrophage cells. International Journal of Nanomedicine 7: 1805-1818

Joshi M. 2013. Role of Eudragit in Targeted Drug Delivery. International Journal of Current Pharmaceutical Research 5 (2): 58-62

Juneja R, Roy I. 2014. Surface modified PMMA nanoparticles with tunable drug release and cellular uptake. RSC Adv. 4: 44472-44479.

Jung J, Lee IH, Lee E, Park J, Jon S. 2007. pH-Sensitive Polymer Nanospheres for Use as a Potential Drug Delivery Vehicle. Biomacromolecules 8: 3401-3407

Kane RS, Stroock AD. Nanobiotechnology: Protein-nanomaterial interactions. Biotech. Prog. 23: 316-319

Kazimirova A, Magdolenova Z, Barancokova M, Staruchova M, Volkovova K, Dusinska M. Genotoxicity testing of PLGA-PEO nanoparticles in TK6 cells by the comet assay and the cytokinesis-block micronucleus assay. Mutation Research 748: 42-47

Kim ST, Saha K, Kim C, Rotello VM. 2013. The Role of Surface Functionality in Determining Nanoparticle Cytotoxicity. Accounts of Chemical Research 46 (3): 681-691

Kreuter J, Speiser PP. 1976. New Adjuvantson on a Polymethylmethacrylate Base. Infection and Immunity 13 (1): 204-210

Kroll A, Pillukat MH, Hahn D, Schnekenburger J. 2009. Current *in vitro* methods in nanoparticle risk assessment: Limitations and challenges. European Journal of Pharmaceutics and Biopharmaceutics 72: 370-377

Kundu D, Hazra C, Chatterjee A, Chaudhari A, Mishra S. 2014. Sonochemical synthesis of poly(methyl methacrylate) core–surfactin shell nanoparticles for recyclable removal of heavy metal ions and its cytotoxicity. RSC Adv. 4: 24991-25004

Kwon SS, Nam YS, Lee JS, Ku BS, Han SH, Lee JY, Chang IS. 2002. Preparation and characterization of coenzyme Q_{10}-loaded PMMA nanoparticles by a new emulsification process based on microfluidization. Colloids and Surfaces A: Physicochemical and Engineering Aspects 210: 95-104

Lamprecht A, Schäfer U, Lehr CL. 2001. Size-Dependent Bioadhesion of Micro- and Nanoparticulate Carriers to the Inflamed Colonic Mucosa. Pharmaceutical Research 18 (6): 788-793

Landsiedel R, Ma-Hock L, Van Ravenzwaay B, Schulz M, Wiench K, Champ S, Schulte S, Wohlleben W, Oesch F. 2010. Gene toxicity studies on titanium dioxide and zinc oxide nanomaterials used for UV-protection in cosmetic formulations. Nanotoxicology 4 (4): 364-381

Lazzari S, Moscatelli D, Codari F, Salmona M, Morbidelli M, Diomede L. 2012. Colloidal stability of polymeric nanoparticles in biological fluids. J Nanopart Res 14: 920-929

Li KG, Chen JT, Bai SS, Wen X, Song SY, Yu Q, Li J, Wang YQ. 2009. Intracellular oxidative stress and cadmium ions release induce cytotoxicity of unmodified cadmium sulfide quantum dots. Toxicol In Vitro 23: 1007–1013.

Lima R, Pereira AES, Porto RM, Fraceto LF. 2011. Evaluation of Cyto- and Genotoxicity of Poly(lactide-co-glycolide) Nanoparticles, J Polym Environ 19: 196-202

Liu Y, Yin Y, Wang L, Zhang W, Chen X, Yang X, Xu J, Ma G. 2013. Surface hydrophobicity of microparticles modulates adjuvanticity. J. Mater. Chem. B 1: 3888-3896

Louro L, Bettencourt A, Gonçalves LM, Almeida AJ, Silva MJ. 2014. Role of nanogenotoxicology studies in safety evaluation of nanomaterials. In: S. Thomas, Y. Grohens, N. Ninan, ed. Nanotechnology Applications for Tissue Engineering. Amesterdam: Elsevier (*In Press*)

Louro H, Silva MJ. 2010. *In Vivo* Mutagenic Effects of Alkylating Agents Eliciting Different DNA-Adducts. In: DNA Adducts: Formation, Detection and Mutagenesis; Emerson Alvarez and Roberto Cunha (eds): 39-60; ISBN 978-1-60741-433-9

Lye KW, Tideman H, Wolke JCG, Merkx MAW, Chin FKC, Jansen JA. Biocompatibility and bone formation with porous modified PMMA in normal and irradiated mandibular tissue. Clin. Oral Impl. Res. 24: 100-109

Magdolenova Z, Collins A, Kumar A, Dhawan A, Stone V, Dusinska M. 2014. Mechanisms of genotoxicity. “A review of *in vitro* and *in vivo* studies with engineered nanoparticles. Nanotoxicology 8(3): 233-278

Magdolenova Z, Lorenzo Y, Collins A, Dusinska M. 2012. Can Standard Genotoxicity Tests be Applied to Nanoparticles?. Journal of Toxicology and Environmental Health, Part A: Current Issues 75:13-15, 800-806

Manke A, Wang L, Rojanasakul Y. 2013. Mechanisms of Nanoparticle-Induced Oxidative Stress and Toxicity. BioMed Research International 2013: 942916

Mendes AN, Hubber I, Siqueira M, Barbosa GM, Moreira DL, Holandino C, Pinto JC, Nele M. 2012. Preparation and Cytotoxicity of Poly(Methyl Methacrylate) Nanoparticles for Drug Encapsulation. Macromol. Symp. 319: 34-40

Merhi M, Dombu CY, Brient A, Chang J, Platel A, Curieux FL, Marzin D, Nesslany F, Betbeder D. 2012. Study of serum interaction with a cationic nanoparticle: Implications for *in vitro* endocytosis, cytotoxicity and genotoxicity. International Journal of Pharmaceutics 423: 37-44

Mosmann T. 1983. Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. Journal of Immunological Methods 65: 55-63

Mukerjee A, Sinha VR, Pruthi V. 2007. Preparation and Characterization of Poly-ε-caprolactone Particles for Controlled Insulin Delivery. Journal of Biomedical & Pharmaceutical Engineering 1: 40-44

Murphy PJM, Stone OJ, Anderson ME. 2011. Automated Hydrophobic Interaction Chromatography Column Selection for Use in Protein Purification. J. Vis. Exp. 55: e3060, DOI : 10.3791/3060

Naahidi S, Jafari M, Edalat F, Raymond K, Khademhosseini A, Chen P. 2013. Biocompatibility of engineered nanoparticles for drug delivery. Journal of Controlled Release 166: 182-194

Naves AF, Palombo RR, Carrasco LDM, Carmona-Ribeiro AM. 2013. Antimicrobial Particles from Emulsion Polymerization of Methyl Methacrylate in the Presence of Quaternary Ammonium Surfactants. Langmuir 29 (31): 9677-9684

Nel A, Xia T, Madler L, Li N. 2006. Toxic potential of materials at the nanolevel. Science 311: 622–627

Oberdörster G, Oberdörster E, Oberdörster J. 2005. Nanotoxicology: An Emerging Discipline Evolving from Studies of Ultrafine Particles. Environmental Health Perspectives 113 (7): 823-839

OECD, 2010. Guideline for the Testing of Chemicals, In Vitro Mammalian Cell Micronucleus Test Organization for Economic Co-operation and Development

O'Brien P, Haskins JR. 2006. *In vitro* cytotoxicity assessment. In K. Guiliano, D. Lansing Taylor and J.R. Haskins (eds), High-Content Screening: A Powerful Approach to Systems Cell Biology and Drug Discovery, Human a Press, Totowa, NJ, USA: 415-425

Pandit NK. 2007. Introduction to the Pharmaceutical Sciences. Baltimore, MD; 1st Edition, Lippincott Williams & Wilkins: 12-24

Papa S, Ferrari R, De Paola M, Rossi F, Mariani A, Caron I, Sammali E, Peviani M, Dell'Oro V, Colombo C, Morbidelli M, Forloni G, Perale G, Moscatelli D, Veglianese P. 2014. Polymeric nanoparticle system to target activated microglia/macrophages in spinal cord injury.

Park EJ, Yi J, Chung KH, Ryu DY, Choi J, Park K. 2008. Oxidative stress and apoptosis induced by titanium dioxide nanoparticles in cultured BEAS-2B cells. Toxicol Lett 180: 222–229

Pelaz B, Charron G, Pfeiffer C, Zhao Y, de la Fuente JM, Liang XJ, Parak WJ, Del Pino P. 2013. Interfacing engineered nanoparticles with biological systems: anticipating adverse nano-bio interactions. Small 9 (9-10): 1573-1584

Pratt CM, Barton S, McGonigle E, Kishi M, Foot PJS. 2006. The effect of ionising radiation on poly(methyl methacrylate) used in intraocular lenses. Polymer Degradation and Stability 91: 2315-2317

Puricelli E, Nácul AM, Ponzoni D, Corsetti A, Hildebrand LC, Valente DS. 2011. Intramuscular 30% polymethylmethacrylate (PMMA) implants in a non-protein vehicle: an experimental study in rats. Rev. Bras. Cir. Plást. 26 (3): 385-389

Rampersad SN. 2012. Multiple Applications of Alamar Blue as an Indicator of Metabolic Function and Cellular Health in Cell Vability Bioassays. Sensors 12: 12347-12360

Reis CP, Neufeld RJ, Ribeiro AJ, Veiga F. 2006. Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles. Nanomedicine: Nanotechnology, Biology and Medicine 2: 8-21

Reis KR, Bonfante G, Pegoraro LF, Conti PCR, Oliveira PCG, Kaiser OB. 2008. *In vitro* wear resistance of three types of polymethylmethacrylate denture teeth. J. Appl. Oral Sci. 16 (3): 176-180

Rimessi P, Sabatelli P, Fabris M, Braghetta P, Bassi E, Spitali P, Vattemi G, Tomelleri G, Mari L, Perrone D, Medici A, Neri M, Bovolenta M, Martoni E, Maraldi NM, Gualandi F, Merlini L, Ballestri M, Tondelli L, Sparnacci K, Bonaldo P, Caputo A, Laus M, Ferlini A. 2009. Cationic PMMA Nanoparticles Bind and Deliver Antisense Oligoribonucleotides Allowing Restoration of Dystrophin Expression in the *mdx* Mouse. Molecular Therapy 17 (5): 820-827

Riss TL, Moravec RA, Niles AL, Benink HA, Worzella TJ; Minor L, editor. Cell Viability Assays. 2013 May 1. In: Sittampalam GS, Gal-Edd N, Arkin M, et al., editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004. Available from: http://www.ncbi.nlm.nih.gov/books/NBK144065/ on February 21, 2014.

Rose C. October 2010. Particulate Systems for Fluorescence Imaging and Drug Delivery (Dissertation). Bavaria (Germany); Naturwissenschaftlichen Fakultät IV - Chemie und Pharmazie – der Universität Regensburg. 200 p.

Schins RPF, Duffin R, Höhr D, Knaapen AM, Shi T, Weishaupt C, Stone V, Donaldson K, Borm PJA. 2002. Surface modification of quartz inhibits toxicity, particle uptake, and oxidative DNA damage in Human lung epithelial cells. Chem. Res. Toxicol. 15: 1166-1173

Schipper ML, Iyer G, Koh AL, Cheng Z, Ebenstein Y, Aharoni A, Keren S, Bentolila LA, Li J, Rao J, Chen X, Banin U, Wu AM, Sinclair R, Weiss S, Gambhir SS. 2009. Particle size, surface coating, and PEGylation influence the biodistribution of quantum dots in living mice. Small 5 (1): 126-134

Shah V, Taratula O, Garbuzenko OB, Patil ML, Savla R, Zhang M, Minko T. 2013. Genotoxicity of different nanocarriers: possible modifications for the delivery of nucleic acids. Curr Drug Discov Technol 10 (1): 8-15

Shen Y, Wang W, Li X, Markel DC, Ren W. 2014. Mitigative effect of erythromycin on PMMA challenged preosteoblastic MC3T3-E1 cells. The Scientific World Journal 2014: 107196

Sitia L, Paolella K, Romano M, Violatto MB, Ferrari R, Fumagalli S, Colombo L, Bello E, De Simoni MG, D'Incalci M, Morbidelli M, Erba E, Salmona M, Moscatelli D, Bigini P. 2014. An integrated approach for the systematic evaluation of polymeric nanoparticles in healthy and diseased organisms. J Nanopart Res 16: 2481

Soomro S, Aziz S, Tunio AH. 2011. Characterization of PMMA Polymer Particles by Light Diffraction Method: Effect of Monomer, Initiator and Dispersion Medium Concentration on Monodispersity. Mehran University Research Journal of Engineering & Technology 30 (2): 297-306

Stark WJ. 2011. Nanoparticles in biological systems. Angew. Chem., Int. Ed., 50: 1242–1258

Stojanović Z, Marković S. 2012. Determination of Particle Size Distributions by Laser Diffraction. Technics – New Materials 21: 11-20

Strober W. 2001. Trypan blue exclusion test of cell viability. Curr Protoc Immunol. Appendix 3B: 10.1002/0471142735.ima03bs21

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

Tencomnao T, Klangthong K, Pimpha N, Chaleawlert-umpon S, Saesoo S, Woramongkolchai N, Saengkrit N. 2012. Acceleation of gene transfection efficiency in neuroblastoma cells through polyethyleneimine/poly(methyl methacrylate) core-shell magnetic nanoparticles. International Journal of Nanomedicine 7: 2783-2792

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. 2000. Single Cell Gel/Comet Assay: Guidelines for In Vitro and In Vivo Genetic Toxicology Testing. Environmental and Molecular Mutagenesis. 35: 206–221

Tkachenko AG, Xie H, Liu Y, Coleman D, Ryan J, Glomm WR, Shipton MK, Franzen S, Feldheim DL. 2004. Cellular Trajectories of Peptide-Modified Gold Particle Complexes: Comparison of Nuclear Localization Signals and Peptide Transduction Domains. Bioconjugate Chem. 15: 482-490

Tran PA, Webster TJ. 2013. Understanding the wetting properties of nanostructured selenium coatings: the role of nanostructured surface roughness and air-pocket formation. International Journal of Nanomedicine 8: 2001-2009

Tran SL, Puhar A. Ngo-Camus M, Ramarao N. 2011. Trypan Blue Dye Enters Viable Cells Incubated with the Pore-Forming Toxin HlyII of *Bacillus cereus*. PLoS ONE 6 (9): e22876

Vale FM, Castro M, Monteiro J, Couto FS, Pinto R, Rico JMGT. 1997. Acrylic bone cement induces the production of free radicals by cultured human fibroblasts. Biomaterials 18: 1133-1135

Verma A, Stellacci F. 2010. Effect of Surface Properties on Nanoparticle-Cell Interactions. Small 6 (1): 12-21

Verma A, Uzun O, Hu Y, Hu Y, Han HS, Watson N, Chen S, Irvine DJ, Stellacci F. 2008. Surface Structure-Regulated Cell Membrane Penetration by Monolayer Protected Nanoparticles. Nat. Mater. 7 (7): 588-595.

Vollrath A, Pretzel D, Pietsch C, Perevyazko I, Schubert S, Pavlov GM, Schubert US. 2012. Preparation, Cellular Internalization, and Biocompatibility of Highly Fluorescent PMMA Nanoparticles. Macromol. Rapid Commun. 33 (20): 1791-1797

Voltan R, Castaldello A, Brocca-Cofano E, Altavilla G, Caputo A, Laus M, Sparnacci K, Ensoli B, Spaccasassi S, Ballestri M, Tondelli L. 2007. Preparation and Characterization of Innovative Protein-coated Poly(Methylmethacrylate) Core-shell Nanoparticles for Vaccine Purposes. Pharmaceutical Research 24 (10): 1870-1882

Wang F, Gao F, Lan M, Yuan H, Huang Y, Liu J. 2009. Oxidative stress contributes to silica nanoparticle-induced cytotoxicity in human embryonic kidney cells. Toxicol In Vitro 23: 808–815

Wang W, Li C, Zhang J, Dong A, Kong D. 2014. Tailor-made gemcitabine prodrug nanoparticles from well-defined drug–polymer amphiphiles prepared by controlled living radical polymerization for cancer chemotherapy. J. Mater. Chem. B 2: 1891-1901

Wardman P. 2007. Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: Progress, pitfalls, and prospects. Free Radical Biology & Medicine 43: 995-1022

Wu YL, Putcha N, Ng KW, Leong DT, Lim CT, Loo SCJ, Chen X. 2012. Biophysical Responses upon the Interaction of Nanomaterials with Cellular Interfaces. Accounts of Chemical Research 46 (3): 782-791.

Yang HW, Chou LSS, Chou MY, Chang YC. 2003. Fibroblast expression of C-C chemokines in response to orthopaedic biomaterial particle challenge in vitro. Biomaterials 24: 2909-2914

Yang SY, Zhang K, Bai L, Song Z, Yu H, McQueen DA, Wooley PH. 2011. Polymethylmethacrylate and Titanium Alloy Particles Activate Peripheral Monocytes during Periprosthetic Inflammation and Osteolysis. J Orthop Res 29: 781-786

Yaszay B, Trindade MCD, Lind M, Goodman SB, Smith RL. 2001. Fibroblast expression of C-C chemokines in response to orthopaedic biomaterial particle challenge in vitro. Journal of Orthopaedic Research 19: 970-976

Zhu FB, Cai XZ, Yan SG, Zhu HX, Li R. 2010. The Effects of Local and Systemic Alendronate Delivery on Wear Debris-Induced Osteolysis In Vivo. Journal of Orthopaedic Research 28: 893-899

Zhu MT, Feng WY, Wang Y, Wang B, Wang M, Ouyang H, Zhao YL, Chai ZF. 2009. Particokinetics and Extrapulmonary Translocation of Intratracheally Instilled Ferric Oxide Nanoparticles in Rats and the Potential Health Risk Assessment. Toxicological Sciences 107 (2): 342-351

Zigoneanu IG, Astete CE, Sabliov CM. 2008. Nanoparticles with entrapped tocopherol: Synthesis, characterization, and controlled release. Nanotechnology 19: 1–8

Zolnik BS, Gonzalez-Fernandez A, Sadrieh N, Dobrovolskaia, MA. 2010. Minireview: Nanoparticles and the Immune System. Endocrinology 151: 458–465

Zydowicz N, Nzimba-Ganyanad E, Zydowicz N. 2002. PMMA microcapsules containing water-soluble dyes obtained by double emulsion/solvent evaporation technique. Polymer Bulletin 47: 457-463.