Facultad de Farmacia y Nutrición

Departamento de Farmacología y Toxicología

Toxicity evaluation of poly(anhydride) nanoparticles designed for oral drug delivery

Evaluación toxicólogica de nanopartículas poly(anhidridas) diseñadas para administración oral de fármacos

Tamara Iglesias Alonso

Pamplona, 2016

TESIS DOCTORAL

Facultad de Farmacia y Nutrición

Departamento de Farmacología y Toxicología

Toxicity evaluation of poly(anhydride) nanoparticles designed for oral drug delivery

Trabajo presentado por Dña.Tamara Iglesias Alonso para obtener el Grado de Doctor

Fdo. Tamara Iglesias Alonso

Pamplona,Diciembre 2016

DÑA. ADELA LÓPEZ DE CERAIN SALSAMENDI, Decana de Farmacia de la Universidad de Navarra, y DÑA. AMAYA AZQUETA OSCOZ, Investigadora de la Universidad de Navarra,

Certifican

Que el presente trabajo, titulado "**Toxicity evaluation of poly(anhydride) nanoparticles designed for oral drug delivery**", presentado por DÑA. TAMARA IGLESIAS ALONSO para optar al Grado de Doctor, ha sido realizado bajo su dirección en el Departamento de Farmacología y Toxicología de la Universidad de Navarra. Considerando finalizado el trabajo, autorizan su presentación a fin de que pueda ser juzgado y calificado por el Tribunal correspondiente.

Y para que así conste, firman la presente:

Fdo: Dra. Adela López de Cerain Salsamendi Fdo: Dra. Amaya Azqueta Oscoz

Pamplona, Diciembre 2016

Esta tesis doctoral se ha llevado a cabo gracias a una ayuda predoctoral para la Formación de Personal Investigador adscrita al proyecto de investigación ALEXANDER (FP7-NMP-2011-LARGE 5) (2013-2015), así como a una beca predoctoral de la Asociación de Amigos de la Universidad de Navarra (2015-2016). Además, se obtuvo una ayuda de movilidad dentro del projecto QualityNano (FP7,INFRA-2010-262163) (2014-2015). El presente trabajo ha sido financiado por el proyecto ALEXANDER.

Agradecimientos

Con estas breves palabras, deseo expresar mi agradecimiento a las personas que me han acompañado a lo largo de este camino, y me han ayudado de una forma u otra en la realización de este trabajo. Gracias a todos, ya que sin vosotros no hubiera sido posible alcanzar esta meta:

A mis directoras, la Dra. Adela López de Cerain y la Dra. Amaya Azqueta, la oportunidad de realizar esta tesis doctoral. Gracias por vuestra ayuda desde el comienzo, vuestra simpatía, claridad, vuestra crítica constructiva e incansable paciencia en la lectura y corrección de esta memoria que, sin vuestra dirección y sugerencias benevolentes, hubiera sido imposible concluir. Muchísimas gracias.

A la asociación de Amigos por la aportación económica recibida a través de sus programas de ayuda para la realización de tesis doctoral.

Gracias a la Universidad de Navarra y en especial a los grandes investigadores y personal del Departamento de Farmacología y Toxicología por todos los momentos compartidos y ayuda prestada. Especial mención para los compañeros del laboratorio de Toxicología por haberme recibido con tanto cariño y sobre todo por ayudarme a desarrollarme tanto a nivel profesional como personal. Agradecer a Ana Gloria, Isma y Maite por resolverme cualquier duda respecto a los envíos de paquetes y a la anatomía de los ratones. Agradecer también a una de las mejores técnicos, Celia, mil gracias por toda tu ayuda y paciencia y por enseñarme todo lo que uno puede saber del test de Ames. Violeta, gracias por tu ayuda con el ensayo *in vivo*, correcciones del inglés y tus verduras de la huerta. Mil gracias Ariane por estar siempre dispuesta a ayudar y por tus grandes conversaciones. A mis compañeros doctorandos, Laura y Jose, gracias por los momentos vividos en el cuartito “PIF” o fuera de él, zonas bien delimitadas en el lab - ni se te ocurra plantearte usar esas pipetas… por algo están dentro de la zona perfectamente marcada “Zona ADN, ARN, NO TOCAR”.

Agradecer también al Dr. Juan Manuel Irache y al Departamento de Farmacia y Tecnología Farmacéutica por la ayuda prestada cuando la he necesitado.

I am grateful to the Health Effect Laboratory of the Norwegian Institute for Air Research (NILU), in particular to Dr. Maria Dusinska for giving me the opportunity to join their group. Thanks to those people who I met in Oslo, and especially to Naoule for your kindness and your "cake times".

A mis amigos, en especial a Laura, Naiara y Lorea. Sé que sin vuestro apoyo durante todo este tiempo y en especial “en esta última etapa” de la tesis muchísima más gente me odiaría. Gracias por toda vuestra comprensión, escucharme y darme un abrazo cuando realmente lo necesitaba.

Por último agradecer a mi familia y en particular al nuevo integrante de ésta que con una simple sonrisa hace que mi mundo se pare y por un instante todos mis problemas se desvanezcan. Gracias a todos vosotros, por enseñarme a luchar por las cosas que uno quiere y a no rendirme en las adversidades por muy pedregoso que pueda a llegar a ser el camino. En estos últimos 4 años no sabéis cuánto os he podido echar de menos, y en especial a ti Luigy, que ha sido la primera vez que he estado tanto tiempo separada de tu lado. Probablemente no pierda la costumbre… y acabe siguiéndote a Madrid. Echo de menos llegar a casa y que me cuentes como te superas a ti mismo cada día. Eres y siempre serás, mi ejemplo a seguir.

Por último, agradecer a mis abuelos por su apoyo y amor incondicional. Soy realmente afortunada de poder seguir disfrutando de vosotros, de vuestras llamadas diarias y de vuestra preocupación constante.

Gracias a todos.

Table of contents

ABBREVIATIONS

ADEX: Aminodextran

ADME/PK: Absorption, distribution, metabolism, excretion/pharmacokinetics

ALS: Alkali-labile sites

CE: Cloning efficiency

CPI: Consumer Products Inventory

DAPI: 4,6-diamidino-2-phenylindole

DEX: Dextran

DMSO: Dimethyl sulfoxide

ECACC: European Collection of Authenticated Cell Cultures

ECHA: European Chemicals Agency

ECVAM: European Centre for the Validation of Alternative Methods

EFSA: European Food Safety Authority

EMA: European Medicines Agency

EMS: Ethyl methanesulfonate

FDA: Food and Drug Administration

FPG: Formamidopyridine DNA glycosilase

GEF: Global evaluation factor

GIT: Gastrointestinal tract

GN: Gantrez® AN 119

GN-MA: Gantrez® 119 AN coated with mannosamine

GN-MA-NPAC5.5: Gantrez® AN 119 coated with mannosamine labelled with Alexa Cy5.5

GRAS: Generally Recognized As Safe

HPBCD: 2-hydroxypropyl-β-cyclodextrine

ICCVAM: Interagency Coordinating Committee on the Validation of Alternative Methods

ICH: International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use

JaCVAM: Japanese Center for the Validation of Alternative Methods

LDH: Lactate dehydrogenase

MA: Mannosamine

MF: Mutants frequency

ML: L5178Y $TK^{+/-}$ mouse lymphoma cells

MLA: Mouse lymphoma assay

MMS: Methyl methanesulfonate

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

NMs: Nanomaterials

NP(s): Nanoparticle(s)

OECD: Organization for Economic Cooperation and Development

PDI: Polydispersity index

PEG0.5: Poly-ethylene glycol 500

PEG1: Poly-ethylene glycol 1000

PEG2: Poly-ethylene glycol 2000

PEG5: Poly-ethylene glycol 5000

PEG6: Poly-ethylene glycol 6000

PEG10: Poly-ethylene glycol 10000

PLA: Poly(D,L-lactic acid)

PLGA: Poly lactic-co-glycolic acid

REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals

ROS: Reactive oxygen species

RSG: Relative suspension growth

RT: Room temperature

RV: Relative viability

SBs: Strand breaks

SCCS: Scientific Committee on Consumer Safety

SD: Standard deviation

TFT: 5-trifluorothymidine

TK: Thymidine kinase

TSG: Total suspension growth

WP: Work Package

Chapter 1: Introduction

1. Nanomaterials - a reality in today's market

Nanomaterials (NMs) are defined as *"a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm"* (EU, 2011a). Nanoparticles (NPs), which are type of NMs, have been defined as materials where the three dimensions are in the size range of 1-100 nm. However, in the medical field, and in particular in the area of drug delivery higher dimensions can be found (De Jong & Borm, 2008). Some NMs properties such as their small size, particular shape or large surface area per mass, among others, make them unique for some applications. Consequently, the nanotechnology is being expanded to a great variety of fields, and the possibility of human exposure to NMs has to be taken into account. Moreover, the fact that NMs properties are different from those of larger scale material with the same chemical composition can have major toxicological consequences (Oberdörster *et al*., 2005).

Nevertheless, NMs have increasingly been incorporated into consumer products despite the investigation is still ongoing and their potential effects on the environment and human health is not completely known. To document the penetration of nanotechnology in the consumer marketplace, the Woodrow Wilson International Centre for Scholars and the Project on Emerging Nanotechnology created the Nanotechnology Consumer Products Inventory (CPI) in 2005 (Vance *et al*., 2015). The information recovered in the CPI is a good picture of the present situation and the evolution in the period from 2005 until 2014. In 2014, the CPI described 1814 nanoproducts, which represents a thirty-fold increase over the 54 products originally listed in 2005 (Vance *et al*., 2015) (Fig. 1).

The CPI describes eight major consumer categories that are based on publicly available consumer product classification systems, which are displayed in Fig. 2. It can be seen that the largest number of available products belongs to Health and Fitness category, comprising 42 % of listed products. This category comprises supplements, sunscreens, filtration, sporting goods, cosmetics (*e.g.* long lasting make-up, anti-aging creams), clothing and personal care products (*e.g.* lotions, toothbrushes, hairstyling tools and products), being the latter the largest group of

products, with 197 from a total of 505 products (Vance *et al*., 2015). Research and development of nanoproducts in the cosmetic industry is very dynamic and the number of products advertising nanoproducts is higher than in the food sector (Contado, 2015).

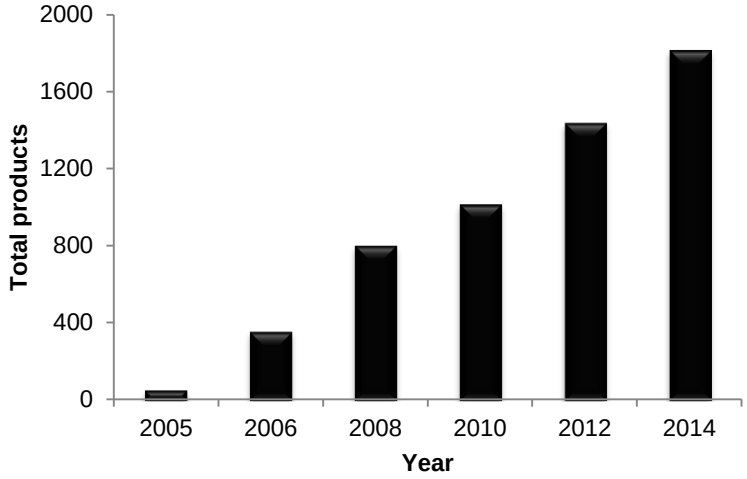


Fig. 1. Number of commercialized nanoproducts according to the CPI since 2005 until 2014. Modified from Vance *et al.* (2015).

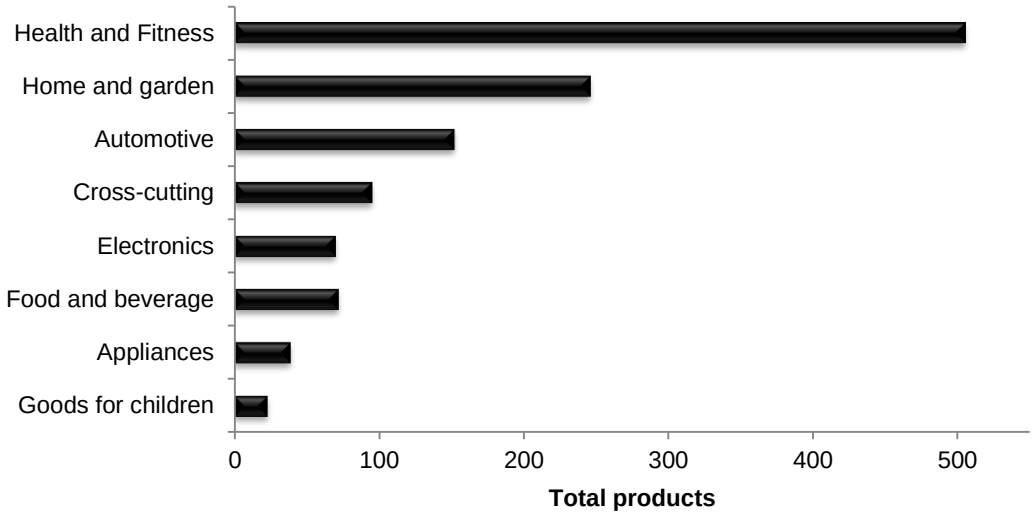


Fig. 2. Number of available nanoproducts in each major category, in 2014. Data from Vance *et al.* (2015).

Regarding NMs composition, approximately 50% of the CPI products did not advertise the composition, 40% had a metal composition and the rest were composed of carbon NMs, silicon-

based NMs, or other NMs (organic, polymers, ceramics, etc.) (Fig. 3). Within the metal category, silver NPs were the most popular NMs in the CPI (438 nanoproducts) (Vance *et al*., 2015). Surprisingly, although silver NPs are very extended, carbon black and synthetic amorphous silica are the most produced ones worldwide: according to the (EU, 2012), the global annual production of silver NPs is 0.0002 % of that of carbon black.

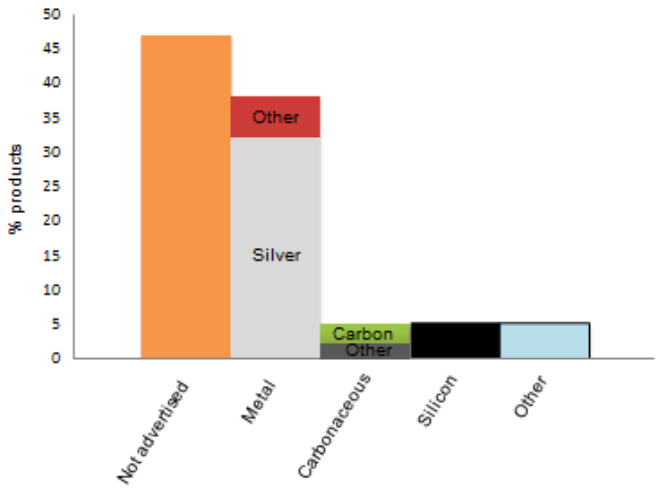


Fig. 3. NMs composition grouped in five major categories in 2014. Data acquired from Vance *et al.* (2015).

In the second Regulatory Review on NMs from the European Commission (EU, 2012), the types of commercialized NMs, together with their safety aspects were revised. An overview of these NMs can be seen in Table 1. In agreement with CPI of 2014, most of them are inorganic NMs.

Table 1. Overview of commercialized NMs. Information acquired from EU Commission (2012).

Material	EC number	Market size annually (€)
Inorganic non-metallic NMs		
Synthetic amorphous silica (SiO_2)	231-545-4	$2700x10^6$
Aluminium oxide (Al_2O_3)	215-691-6	$750x10^6$
Titanium dioxide (TiO_2)	236-675-5	
Zinc oxide (ZnO)	215-222-5	
Iron oxide (Fe_2O_3) Iron oxide (Fe_3O_3)	215-168-2 215-277-5	$20-40x10^6$
Cerium oxide (CeO_2)	215-150-4	
Zirconium oxide (ZeO_2)	215-227-2	
Calcium carbonate ($CaCO_3$)	207-439-4	
Non-oxide inorganic non-metallic NMs Aluminium nitride Silicon nitride Titanium carbonitride Tungsten carbide Tungsten sulphide		
Metals and metal alloys NMs		
Gold	231-165-9	
Silver	231-131-9	
Other metallic NPs Platinum and palladium alloy Copper nano-powders Iron NPs Titanium NPs		
Carbon based NMs		
Carbon black	215-609-9	$10000x10^6$
Fullerenes		
Carbon nanotubes		$30-40x10^6$
Carbon nanofibers		$50-60x10^6$
Graphene flakes		
Nanopolymers and dendrimers		
Quantum dots		$55x10^6$
Nanoclays		$150x10^6$
Nanocomposites		

To have a broad vision of the kind of NMs that have already been introduced in the market, the present project has only focused on the following industrial sectors: cosmetics, food and pharmaceutical/medical.

1.1 Nanomaterials in the cosmetic sector

Cosmetics contain a variety of NMs, mainly inorganic such as, synthetic amorphous silica (SiO_2), titanium dioxide (TiO_2), zinc oxide (ZnO) and metals, being SiO_2, TiO_2 and ZnO the most frequently used (EU, 2012). Interestingly enough, half of the TiO_2 global production per year (10 thousand tonnes) is exclusively used in the cosmetic industry (EU, 2012). Like TiO_2, ZnO nanoform is used as bulking, skin protector and UV absorber due to their outstanding advantages of antimicrobial properties and transparent colour for being used as sunscreens (Contado, 2015).

Also organic NMs such as liposomes or solid lipid nanoparticles are used in cosmetic industry (Raj *et al*., 2012), although several of them are at the research or development stage. L'Oreal, the world's largest cosmetics company, is devoting about 600 million dollars, of its 17 billion dollar revenues, to nanopatents, and has patented the use of dozens of liposome NPs called nanosome particles (Raj *et al*., 2012).

1.2 Nanomaterials in the food sector

Recent developments in nanotechnology are offering lots of new opportunities for innovation in the food industry. Food related applications of nanotechnologies offer a wide range of benefits to the consumer such as taste and texture improvement or the possibility of nutrients encapsulation (vitamins, omega 3 and omega 6 fatty acids, bioactive products, etc). The nanotechnology in this sector can beneficially contribute not only to primary production but also to processing and packaging, keeping food products secure during transportation, fresh for longer time and safe from microbial pathogens. For example, NMs are employed either as antimicrobial and to build highly sensitive biosensors for detecting pathogens, allergens or contaminants than can affect food quality and safety (Contado, 2015).

A wide variety of NMs compositions are used to carry food additives, biosensors, or have even been developed for new packacking strategies (Chaudhry & Castle, 2011). The most investigated NMs in the food sector are SiO_2, TiO_2, ZnO and silver NMs due their antimicrobial properties (Contado, 2015). There are various forms of SiO_2 available on the market in the food sector, mostly used as an aid for clarifying wine, beer, fruit juices, anti-caking agent in food powers, carrier for active ingredients, antifoaming agent in the manufacture of decaffeinated coffee and tea, among others (Contado, 2015).

1.3 Nanomaterials in the pharmaceutical/medical sector; drug delivery

Although NMs have several applications in this sector such as, monitorization, drug delivery, detection, diagnosis, and treatment of various diseases, this section will be focused on the field of drug delivery systems.

Currently, organic and inorganic NMs are under investigation for drug delivery (Caban *et al*., 2014), with greater attention to cancer therapy. However, the inorganic NMs present disadvantages as a slow rate of biodegradation or lack of biodegradability, which raise safety issues, especially for applications that require long-term administration. Moreover, due to the rapid progression in the development of drug delivery systems using organic NMs, fewer advances have been made for the inorganic-based ones (Grazú *et al*., 2012).

The characteristics of the organic material used for preparing the different nanocarriers should be: biodegradable, non-toxic and biocompatible. For this purpose, a wide range of organic NMs based on proteins (e.g. albumin), synthetic polymers, dendrimers, liposomes and other organic materials have been proposed (Irache *et al*., 2011). Moreover, they are suitable for the entrapment and delivery of a wide range of therapeutic agents. Undoubtedly, one of the organic NMs that have received more attention are liposomes. These molecules consist of aqueous compartments surrounded by one or more lipid bilayers comprising amphipathic phospholipids which are also present in natural cell membranes. This feature allows liposomes to fuse with the plasmatic cell membrane of target cells, thereby facilitating drug targeting at a subcellular level (Romero & Moya, 2012). Moreover, the liposomes have been widely reported to enhance the

half-life of various therapeutic agents (Romero & Moya, 2012). In fact, some of them are already on the market in liposomal formulations, as can be seen in Table 2, presenting an improved dose/effect ratio and less adverse reactions compared to the free substances at the same concentration.

However, the liposomes present problems that hamper their clinical applications, such as unsuitability for oral administration routes, toxicity or low encapsulation efficiency (Simón-Vázquez *et al*., 2012). Thus, polymeric NPs are a pharmaceutical alternative to liposomes due to their higher stability in biological systems (Irache *et al*., 2011). Recently, Marin *et al.* (2013) have revised the benefits from the biodegradable polymers being used as drug delivery carriers in seven major diseases: cancer, neurodegenerative disorders, cardiovascular disease, osteoporosis, microbial, viral and parasite infections. In this way, it is also important to mention that the FDA approved biodegradable polymeric NPs, such as poly(D,L-lactic acid) (PLA) and poly lactic-co-glycolic acid (PLGA) for human use (Grazú *et al*., 2012). Despite all these progresses, most of the substances are still in research and development phase or early stage of market development. There are still few nanodrugs available on the pharmaceutical industry, being most of them organic NMs (Table 2 and Table 3).

Table 2. Some organic drug delivery systems currently in use. Modified from (Grazú *et al*., 2012)

Product name- Company	Main component	Targeting moiety or indication	Route of administra-tion
Polymer- protein conjugates			
Adagen®- Enzon	PEG-adenosine deaminase	Severe combined immunodeficiency syndrome	i.m
Cimzia®- UCB	PEG-anti-TNFα Fab	Crohn´s disease, rheumatoid arthritis	s.c
Neulasta®- Amgen	PEG-hrGCSF (pegfilgrastim)	Chemotheray-induced neutropenia	s.c
Mircera®- Roche	PEG-EPO (poly-ethyleneglycol-epoetinbeta)	Anemia associated with chronic kidney disease	i.v / s.c
Pegasys®- Roche	PEG-interferon α 2a	Hepatitis C	s.c
Pegintron®- Schering-Plough	PEG-interferon α 2b	Hepatitis C, VIH	s.c
Oncaspar ®- Enzon	L-Asparaginase	Acute lymphocytic leukemia	i.v / i.m
Somavert®- Pfizer	Pegvisomant	Acromegaly	s.c
Zinostatin®- Astellas Pharma	Styrene maleic anhydride neocarzinostatin	Hepatocellular carcinoma	l.h
Polymer- drug conjugates			
Copaxone®- Teva	Glatiramer acetate, copolymer L-Glu, L-Ala, L-Lys, L-Tir	Multiple esclerosis	s.c
Macugen®- Eyetech	Pegaptanib sodium, selective anti-VEGF inhibitor	Age-related macular degeneration	i.v.i
Renagel®- Genzyme	Sevelamer hydrochloride phosphate binding polymer	Chronic kidney patients on hemodialysis	p.o
Welchol®- Genzyme	Colesevalam, cholesterol binding polymer	Hyperlipidemia, type II diabetes mellitus	p.o
Liposomes and lipid NPs			
Ambiosome®- Astellas Pharma	Liposomal amphotericin B	Fungal and protozoal infections	i.v
Abelcet®- Sigma-Tau Pharmaceutical	Liposomal amphotericin B	Fungal infections	i.v
Amphocil®- Beacon pharmaceuticals	Liposomal amphotericin B	Fungal infections, invasive aspergillosis	i.v
Daumoxome®- Gilead science	Liposomal daunorubicin	HIV associated Kaposi´s sacoma	i.v

e.p: Epidural; i.m: Intramuscular; i.t: Intrathecal; i.v: Intravenous; i.v.i: Intravitreal; l.h: Local via hepatic artery infusion;
p.o: Oral; s.c: Subcutaneous; top: Topical

Table 2. Some organic drug delivery systems currently in use. Modified from (Grazú *et al*., 2012) (continued)

Product name- Company	Main component	Targeting moiety or indication	Route of admininstra-tion
Liposomes and lipid NPs			
Depocyt®-Pacira Pharmaceuticals	Liposomal (nonconcentric vesicles) Cytarabine	Lymphomatous meningitis	i.t
Depodur®-EKR Therapeutics	Morphine sulfate extendedrelease liposome	Postsurgical analgesia, pain relief	e.p
Doxil®/Caelyx®-Johnson & Johnson	PEGylated liposomal doxorubicin	Breast and ovarian cancer, Kaposi's sarcoma	i.v
Epaxal®- Berna biotech	Liposomal aluminium free vaccine	Hepatitis A	i.m
Estrasorb®- Novavax	Micellar estradiol	Menopausal therapy	top
Inflexal®- Berna biotech	Virosomal adjuvant vaccine	Influenza	i.m
Myocet®- Cephalon	Doxorubicin HCL liposome	Patients with HER2 positive breast cancer	i.v
Mepact®- Takeda pharmaceutical Co.	Mifamurtide-Muramyl tripeptide phosphatidylethanolamine	Nonmetastatic sarcoma	i.v
Visudyne®- Novartis	Liposome/lipidic verteporfin	Age-related macular degeneration	i.v

e.p: Epidural; i.m: Intramuscular; i.t: Intrathecal; i.v: Intravenous; i.v.i: Intravitreal; l.h: Local via hepatic artery infusion; p.o: Oral; s.c: Subcutaneous; top: Topical

Table 3. Summary of some inorganic drug delivery systems currently in use. Modified from (Grazú *et al*., 2012)

Product name- Company	Main component	Targeting moiety or indication	Route of administra-tion
Magnetic NPs			
Cosmofer®- GRY Pharma	Dextran iron oxide	Anemia	i.v
Ferrlecit®- Sanofis-Aventis	Sodium ferric gluconate complex in sucrose	Anemia	i.v
Venofer®- Frasenius	Polynuclear iron(III)-hydroxide core superficially surrounded by noncovalently bound sucrose molecules	Iron deficiency anemia	i.v

i.v: Intravenous.

2. Nanomaterials safety: regulatory framework

As mentioned above, nowadays many NMs are utilized to produce nanoproducts in different sectors such as, cosmetics, food and pharmaceutical/medical. However, their implications in human health and in the environment are not fully understood. Thus, there is serious concern regarding the benefit and risk that NMs may pose.

Regulatory authorities carefully observe the increase and developments in this important area all over the world, trying to find a balance between consumer's safety and industry interests.

In Europe, NMs are considered ingredients or bulk materials, and the requirements are defined by the European Chemicals Agency (ECHA). Therefore, they are within the framework of the regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) (EC, 2006) and the regulation on Classification, Labelling and Packaging (CLP) (EC, 2008). Furthermore, the Scientific Committee on Consumer Safety (SCCS), the European Food Safety Authority (EFSA) and the European Medicines Agency (EMA) regulate safety of cosmetics, food, pharmaceuticals and medical devices, respectively (Wacker *et al*., 2016).

In the USA, the FDA created a nanotechnology group that has published several guidelines related to characterization and safety of NMs. These documents, which are not legally binding, only reflect the current thinking of competent authorities or notified bodies (Wacker *et al*., 2016).

In the following sections, the current legislation and guidelines dealing with NMs safety in the European Union and the United States will be presented in the three major sectors of the industry: cosmetic, food and pharmaceutical.

2.1 Cosmetic sector

Currently, the legislative framework for NMs in the cosmetic sector is more detailed than in the food and pharmaceutical ones. A summary of the regulations and guidelines in the EU and the USA is presented in Table 4.

In Europe, the SCCS adopted an opinion on the safety of NMs in cosmetic products, after the public consultation on the 14th plenary of 18 December 2007 (SCCP, 2007). The most important conclusions extracted from the summary of this document are the following:

A nanoparticle is a particle with one or more dimensions at the nanoscale (<100 nm). (…) Two principal factors cause the properties of nanomaterials to differ significantly from bulk materials: increased relative surface area, and quantum effects.

Nanoparticles can be divided into two groups: i) soluble and/or biodegradable nanoparticles which disintegrate upon application to skin into their molecular components (e.g. liposomes, microemulsions, nanoemulsions), and ii) insoluble and/or biopersistent particles (e.g. TiO_2, fullerenes, quantum dots). (…)

For the first group, conventional risk assessment methodologies based on mass metrics may be adequate, whereas for the insoluble particles other metrics, such as the number of particles, and their surface area as well as their distribution are also required. It is crucial when assessing possible risks associated with nanoparticles to consider their uptake. It is primarily for the insoluble particles that health concerns related to possible uptake arise. Should they become systemically available, translocation/transportation and eventual accumulation in secondary target organs may occur.

Some gaps of knowledge were identified and it was agreed that the safety of the insoluble NMs already used in sunscreens was required. Moreover the lack of *in vitro* assays validated for NMs was recognized.

Table 4. Summary of the regulations and recommendations dealing with the safety assessment of NMs in the cosmetic sector, in the European Union and the USA.

European Union
Regulation (EC) Nº 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products (EC, 2011).
Guidelines and other documents:
– *Opinion on Safety of Nanomaterials in Cosmetic Products* (SCCP, 2007).
– *Guidance on the Safety Assessment of Nanomaterials in Cosmetics* (SCCS, 2012).
– *Memorandum on "Relevance, Adequacy and Quality of Data in Safety Dossiers on Nanomaterials"* (SCCS, 2013).
– The *SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation, 9 th revision.* (SCCS, 2015).
United States of America
Guidelines and other documents:
– *Report of the Joint Regulator- Industry Ad Hoc Working Group: Currently Available Methods for Characterization of Nanomaterials* (ICCP, 2011).
– *Final Guidance for Industry: Considering Whether an FDA-Regulated Product Involves the Application of Nanotechnology* (FDA, 2014a).
– *Final Guidance for Industry: Safety of Nanomaterials in Cosmetic Products* (FDA, 2014b).
– **Need for updating the guidance based on experience and acquired knowledge.** – **Need for validating *in vitro* and *in vivo* test methods.**

At present, the Revised Regulation EU Nº 1223/2009 includes a premarket notification for cosmetics that contain NMs and applies to all products in the market (Wacker *et al*., 2016). The risk assessment of NMs is performed by the SCCS and it is based on the information provided by the manufacturer (Wacker, 2014). This scientific committee published a very detailed guidance on risk assessment of NMs (SCCS, 2012) and a memorandum on the relevance, adequacy and quality of the data expected in safety dossiers on NMs (SCCS, 2013). This guideline was revised three years later (SCCS, 2015).

The guidance published in 2012 about the safety assessment of NMs in cosmetics (SCCS, 2012) is meant to facilitate the preparation of safety dossiers: material identification, specification, quantity, toxicological profile, exposure estimates, and safety evaluation. All these dossiers need to be provided for any nanoform intended for its use in a cosmetic product.

During the evaluation of NMs, the SCCS observed a number of issues that had to be reviewed, especially in relation to the relevance, adequacy, and quality of the data presented in the safety dossiers. The Memorandum was therefore published and aimed to highlight the main considerations that need to be taken into account when working with NMs in the cosmetic sector (SCCS, 2013).

The SCCS/1484/12 guidance (SCCS, 2012), revised in 2015 (SCCS, 2015), includes the characterization of NMs, the assessment of their absorption, distribution, metabolism, and elimination (ADME), as well as an additional test, on mutagenicity or carcinogenicity, among others (SCCS, 2012; 2015). This guideline recommended the use of more than one method for the measurement of particle size, distribution and particle imaging because the analytical methods used for chemical substances have not yet been validated for NMs (SCCS, 2012).

Additionally, like other cosmetic ingredients, data based on a set of toxicological endpoints is also required for NMs, such as acute toxicity, skin and eye irritation and corrosivity, skin sensitisation, repeated dose toxicity, and mutagenicity/genotoxicity. Furthermore, since transdermal is the preferred administration route in cosmetics, studies of dermal/percutaneous absorption of NMs are required (SCCS, 2012; 2015).

However, there is considerable controversy and major concern on the methodology used for the evaluation of the safety and toxicity of NMs. Thus, the current testing methods may need certain modifications for taking into account the special features of NMs (SCCS, 2012). For instance, the Ames test for mutagenicity assessment is not appropriate for NMs due to the limited uptake by bacteria (SCCS, 2012; 2015).

Furthermore, the 76/768/EEC and the EU Nº 1223/2009 directives, prohibited the testing of cosmetic products and cosmetic ingredients on animals (testing ban) (EC, 2011). In view of the current lack of alternative methods specifically validated for NMs, the SCCS is of the opinion that, the complete ban on *in vivo* testing of cosmetic ingredients and products poses an obstacle to the risk assessment of cosmetic ingredients in general, and to ingredients in nanomaterial form in particular.

In the USA, a guideline on cosmetics was released by the FDA in June 2014 (FDA, 2014b). This document provides information on the characterization of NM including their physico-chemical properties, impurities, routes of exposure, uptake and absorption. Furthermore, it also gives recommendations regarding the toxicity assays that should be included. Moreover, alternative testing methods that could be optimized for a specific nanomaterial are also suggested. With respect to the physico-chemical characterization of NMs, this guidance refers to a number of documents such as, the Report for International Cooperation on Cosmetic Regulation, which summarizes the available analytical techniques (ICCP, 2011). In the same year, the FDA also published another guideline entitled “Considering whether an FDA-regulated product involves the application of nanotechnology” that describes FDA’s thinking on determining whether FDA-regulated products involve the application of nanotechnology (FDA, 2014a). This guidance is intended for manufacturers, suppliers, importers, and other stakeholders of pharmaceuticals, food or cosmetic sectors.

In contrast to the European Union, in the USA, cosmetics do not need to undergo risk assessment. However, manufacturers must make sure that their products are not misbranded or adulterated. In fact, all responsibility for cosmetics lies in the manufacturer (Wacker *et al*., 2016).

Finally, the absence of validated methods to assess NMs produces public controversy and concern on their safety and potential hazards in relation to human health as well as, to the environment.

2.2 Food sector

Nanoproducts also have a substantial impact on the food and feed sector, potentially offering significant benefits for industry, as well as for consumers. However, possible risks need to be considered due to the several applications of nanotechnology in this area, as food packaging, food additives or pathogens detection in food.

In the EC Nº178/2002 regulation (EC, 2002), the general principles and requirements of the European food framework were defined, laying down procedures in matters of food safety. Thus, the EFSA was created, which is responsible for carrying out the legislation in the European market in this sector (Wacker, 2014). More specific to NMs, the EU Nº 1169/2011 regulation describing the provision of food information to consumers was approved (EU, 2011b). Importantly, the term engineered NM that has been used by the EFSA in this regulation, is not identical to the one proposed by the EU on 2011 (EFSA, 2012). A further refinement of this regulation was published in 2013, although it was considered null just one week after being published.

As shown in Table 5, the EFSA had to set up specific guidelines for NMs in food, feed products and food contact materials (Wacker *et al*., 2016). EFSA guidelines provide the methodology for the evaluation of NMs in food products: physico-chemical characterization, hazards identification and risk assessment (EFSA, 2012). In addition it distinguishes two categories:

• Nanoforms of already approved non-nanoforms with the same intended use in food/feed.
• New NMs without corresponding approved non-nanoforms

Moreover, when high exposure is expected, specific risk assessment on the nanoform has to be undertaken (Wacker, 2014). Although time- and money-consuming, all these data need to be collected before the authorization for the commercialization of a NM-containing product can be evaluated in the food and feed areas (EFSA, 2011), including food additives (EFSA, 2012), enzymes, flavourings, food contact materials, novel foods, feed additives and pesticides.

Table 5. Summary of the regulations and recommendations dealing with the risk assessment of NMs in the food/feed sector, in the European Union and the USA.

European Union
Regulation (EU) Nº 1169/2011 of the European Parliament and of the Council of 25 October 2011, on the provision on food information to consumers (EU, 2011b).
Guidelines and other documents:
– *Guidance on the Risk Assessment of the Application of Nanoscience and Nanotechnologies in the Food and Feed Chain* (EFSA, 2011).
– *Guidance for Submission for Food Additive Evaluations* (EFSA, 2012).
– *Annual Report of the EFSA Scientific Network of Risk Assessment of Nanotechnologies in Food and Feed for 2015* (EFSA, 2015).
United States of America
– *Nanotechnology: A Report of the US Food and Drug Administration Nanotechnology Task Force"* (FDA, 2007).
– *Final Guidance for Industry: Assessing the Effects of Significant Manufacturing Process Changes, Including Emerging Technologies, on the Safety and Regulatory Status of Food Ingredients and Food Contact Substances, Including Food Ingredients that are Colour Additives"* (FDA, 2014c).
– **Need for updating the guidance based on experience and acquired knowledge.** – **Need for validating *in vitro* an *in vivo* test method.**

On June 2016, the EFSA published a scientific opinion entitled "Priority Topics for the Development of Risk Assessment Guidance by EFSA's Scientific Committee in 2016-2018" (EFSA, 2016). It was agreed that the guidance document for risk assessment of nanomaterials (EFSA, 2011) required updating to stay aligned with fast developments in both scientific innovations and legal requeriments in the area of nanotechnology.

In the USA, food products are evaluated by the FDA and they do not undergo systematic safety assessments. A brief summary of the recommendations dealing with the safety assessment of NMs in this area is showed in the table 5.

In 2007, a report on nanotechnology did not recommend any changes in the regulatory framework. According to the FDA, food products are not subject to premarket approval if their

safety has been confirmed by quality experts or by common knowledge (Generally Recognized As Safe (GRAS)), except when being used as colour additives (Wacker *et al*., 2016).

In 2014, the FDA published a guideline to evaluate the NMs present in food products. Safety evaluation includes characterization of the chemical, conducting *in vitro* and *in vivo* studies, among others. Similar to cosmetic products, the responsibility lies in the manufacturer.

In the same way to cosmetics sector, there are several uncertainties related to the identification, characterisation and risk assessment of NMs. In fact, they are related to the lack of suitable and validated test methods to cover all possible applications, aspects and properties of NMs. It is crucial to overcome the existing regulatory gap between commercial developments and public expectations about regulatory protections for nanotechnologies as soon as possible.

2.3 Pharmaceutical/medical sector

In the EU and USA, the regulatory organism responsible for the scientific evaluation of new premarketing pharmaceuticals is the EMA and FDA, respectively. Specific information on every product is revised by the appropriate authority on a case-by-case basis.

As shown in Table 6, both authorities have proposed a safety assessment on a case-by-case basis. Nevertheless, they have provided reflections papers, including the characterization of physico-chemical properties, and evaluation of benefits-risks, assessment of ADME properties, and the implementation of a pharmacovigilance system (Wacker, 2014). All of this, in order to support manufacturers, importers and other interest parties in the understanding of NMs. In contrast to food sector, manufacturers of pharmaceuticals products must provide the above information for every drug formulation, as well as, their specific indication on the market.

Table 6. Summary of the regulations and recommendations dealing with the safety assessment of NMs in the pharmaceutical/medical sector, in the European Union and the USA.

European Union
Regulation on a case-by -case basis <u>Reflection papers:</u> – *Reflection paper on Nanotechnology-based Medicinal Products for Human Use* (EMA, 2006). – *Reflection paper on Non-clinical Studies for Generic Nanoparticle Iron Medicinal Product Applications* (EMA, 2011). – *Reflection paper on Surface Coatings: General Issues for Consideration Regarding Parenteral Administration of Coated Nanomedicine Products"* (EMA, 2013a). – *Reflection paper on the Data Requirements for Intravenous Liposomal Products Developed with Reference to an Innovator Liposomal Product"* (EMA, 2013b). – *Joint MHLW/EMA Reflection paper on the Development of 5 Block Copolymer Micelle Medicinal Products* (EMA, 2013c). – *Reflection paper on the Data Requirements for Intravenous Iron-based Nano-colloidal Products Developed with Reference to an Innovator Medicinal Product"* (EMA, 2015).
United States of America
Regulation on a case-by -case basis <u>Documents:</u> – *Final Guidance for Industry: Considering Whether an FDA-Regulated Products involves the Application of Nanotechnology* (FDA, 2014a). – *Liposome Drug Products-chemistry, Manufacture and Controls; Human Pharmacokinetics and Bioavailability and Labelling Documentation* (FDA, 2015).
– **Need for the specific guidance.** – **Need for validating *in vitro* an *in vivo* test method.**

The EMA has issued several reflection papers with a view to developing guidelines for manufacturers of pharmaceutical products. These documents cover the development of new nanomedicines and products for human use (EMA, 2006), including coated nanomedicine

products (EMA, 2013a), liposomal formulation (EMA, 2013b), polymeric micelle formulation (EMA, 2013c) and iron based preparations (EMA, 2011; 2015). These "rules", which cover the drug delivery devices, define the procedures for a more specific characterization, helping to generation of relevant data which ensures the quality and safety of these products.

In the USA, pharmaceuticals underlie similar requirements. The existing legislation allows the authorities to demand any information that is necessary for safety assessment on a case by case basis and there is no need to additionally defined nanospecific procedures.

In parallel to the EMA, the FDA published a draft guidance in 2014 to support the manufacturers in identifying the NMs (FDA, 2014a). In addition, the FDA has discussed the manufacture and evaluation of liposomes (FDA, 2015).

In spite of the fact that in the pharmaceutical sector new medicines have always been the subject of extensive physico-chemical and toxicological evaluation, it is recommended to validate assays (*in vitro* and *in vivo*) for an adequate safety assessment of NMs.

2.4 General comments

In general terms it can be concluded that in recent years, regulatory authorities have made great efforts to establish a framework for assessing the nanosafety in order to obtain higher quality data and provide greater customer information about the nanoproducts on the market.

However, the legislative framework is challenged by the complexities of nanotechnologies and it is thought that risk assessment research is not progressing at a sufficient rate to deal with advancements in nanotechnologies.

The EU and the USA have progressively increased the assessment requirements in cosmetics, food and feed products to ensure consumer safety. However, in the pharmaceutical sector the impact has been lower because they have always been subjected to a physico-chemical and

toxicological evaluation very thorough. Even so, in view of the singular and variable behaviour of NMs, several of the current evaluation methods for chemicals cannot be used for NMs.

Therefore, there are still several uncertainties relating to the identification, characterization and risk assessment of NMs, and a validation of methods for appropriate safety assessment of NMs is still needed.

3. ALEXANDER project

This thesis is part of a project of the 7th EU Framework Programme, within the theme [NMP.2011.1.2-2] "*New targeted therapy using nanotechnology for transport of macro-molecules across biological barriers*", that was entitled "Mucus Permeating Nanoparticulate Drug Delivery Systems" (Project acronym: ALEXANDER).

The objective of the ALEXANDER project was the identification of novel strategies (e.g., proteolytic enzyme strategy, thiomer strategy, zeta potential changing systems, SNEDDS strategy) and the optimization of existing strategies (e.g., disulfide breaking strategy and slippery surface strategy) for the efficient transport of nanocarriers through the mucus gel layer (e.g., intestinal, nasal, ocular, vaginal, buccal, pulmonary). In particular, Rerearch & Development activities had focused on the synthesis of functionalized nanocarriers capable of permeating the mucus gel layer and delivering their therapeutic payload to the epithelium. The nanocarriers had been characterized with respect to their physico-chemical properties, ability to cross the mucus gel layer, *in vitro* and *in vivo* toxicity.

ALEXANDER has been a collaborative project integrated by five Universities, one Research Institute, and seven companies, apart from DECHEMA, that coordinated the project. It was structured in seven complementary work packages (WP):

- WP1: Development of nanocarriers
- WP2: Nanocarrier permeation studies in mucus gel layer
- WP3: Cytotoxicity studies
- WP4: Development of oral delivery systems
- WP5: Development of ocular delivery systems
- WP6: Dissemination training and exploitation
- WP7: Project management

The WP3 that was related to nanoparticles toxicity and biodistribution studies, was co-leaded by the Josef Stefan Institute (Slovenia) and the University of Navarra (Spain), but other participants

were also implicated. We will focus on this work package, since the work that has been carried out in this thesis was part of it.

The objective of this work package (WP3) has been the toxicological screening of the nanocarriers synthesized in WP1 with respect to cytotoxicity, immunogenicity and genotoxicity. The examination of the biocompatibility and biodegradability of the new formulations was also determined, together with the assessment of the macromolecular drug/carrier biodistribution. Moreover, an important issue was the determination of the target organ toxicity of the selected nanoparticles, by applying a wide variety of *in vivo* tests. The final aim of this WP was to provide a crucial assessment of the suitability of the nanocarriers developed in WP1 as delivery systems for therapeutic macromolecules in order to be used at clinical level. This thesis has been carried out as part of this WP.

The first objective of this WP was to establish a sequential strategy, integrated by several *in vitro* assays, in order to select the most promising candidates. Decisions were based on the nanoparticles permeability capacity and their *in vitro* toxicological profile. The scheme presented in Fig. 4 shows the strategy that was devised and agreed between the ALEXANDER project partners. It is a sequential integrated strategy for assessing the cytotoxicity, genotoxicity and immunogenicity of nanocarriers before performing the *in vivo* toxicity studies.

Over the course of this thesis, slight variations of this strategy have been carried out. For example, *in vivo* evaluation was performed in rodent specie mice because in the case of using rat, considerable quantities of NMs would be required to get a preliminary toxicological information.

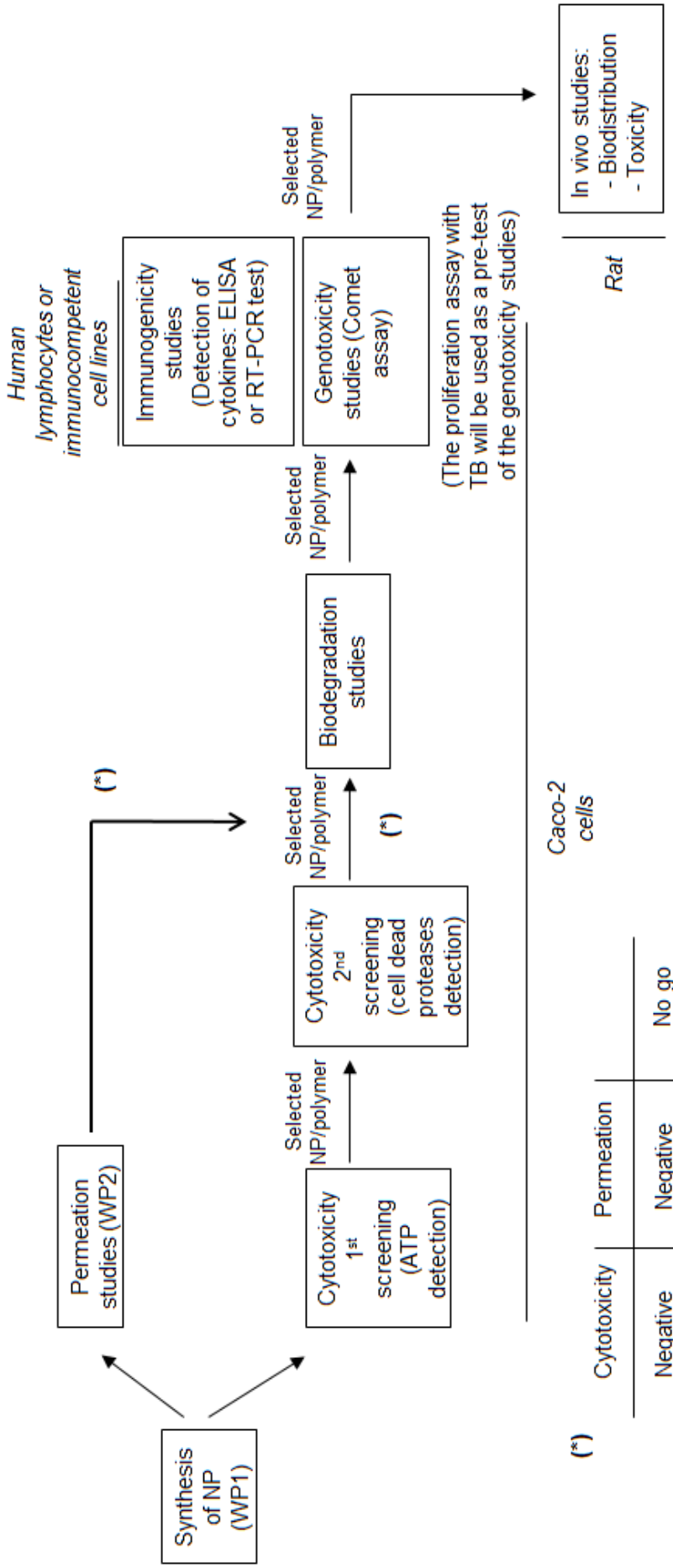


Fig. 4. Strategy to evaluate the toxicity of nanocarriers in the ALEXANDER project

4.References

Caban, S., Aytekin, E., Sahin, A., Capan, Y., 2014. Nanosystems for drug delivery. OA Drug Design & Delivery. 2 (1), 2-9.

Chaudhry, Q & Castle, L., 2011. Food applications of nanotechnologies: An overview of opportunities and challenges for developing countries. Trends in Food Science & Technology. 22 (11), 595-603.

Contado, C., 2015. Nanomaterials in consumer products: A challenging analytical problem. Frontiers in Chemistry. 3, 48-68.

De Jong, W.H & Borm, P.J., 2008. Drug delivery and nanoparticles: Applications and hazards. International Journal of Nanomedicine. 3 (2), 133-149.

EC, 2002. Regulation (EC) No 178/2002 laying down the general principles and requirements of food law, establishing the European Food, Safety Authority and laying down procedures in matters of food safety.OJ L. 31, 1-24.

EC, 2006. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. OJ L 396, 1-852.

EC, 2008. Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006 (Text with EEA relevance).OJ L. 353 (020), 1-1355.

EC, 2011. Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products (Text with EEA relevance). OJ L. 342, 59-209.

EFSA, 2011. Guidance on the risk assessment of the aplication of nanoscience and nanotechnologies in the food and feed chain. EFSA Journal. 9 (5), 2140-2176.

EFSA, 2012. Guidance for submission for food additive evaluations. EFSA Journal. 10 (7), 2817-2826.

EFSA, 2015. Annual report of the EFSA Scientific Network of risk assessment of nanotechnologies in food and feed for 2015. EFSA Supporting Publications. 13 (1), 939-960.

EFSA, 2016. Priority topics for the development of risk assessment guidance by EFSA´s Scientific Committee in 2016-2018. EFSA Journal. 14 (6), 4502-4511.

EMA, 2006. Reflection paper on nanotechnology-based medicinal products for human use. EMEA/CHMP/79769/2006.

EMA, 2011. Reflection paper on non-clinical studies for generic nanoparticle iron medicinal product apllications. EMA/CHMP/100094/2011.

EMA, 2013a. Reflection paper on surface coatings: general issues for consideration regarding parenteral administration of coated nanomedicine products. EMA/325027/2013.

EMA, 2013b. Reflection paper on the data requirements for intravenous liposomal products developed with reference to an innovator liposomal product. EMA/CHMP/806058/2009/Rev.02.

EMA, 2013c. Joint MHLW/EMA reflection paper on the development of 5 block copolymer micelle medicinal products. EMA/CHMP/13099/2013.

EMA, 2015. Reflection paper on the data requirements for intravenous iron-based nano-colloidal products developed with reference to an innovator medicinal product. EMA/CHMP/SWP/620008/2012.

EU, 2011a. Commission recommendation of 18 October 2011 on the definition of nanomaterials (Text with EEA relevance) (2011/696/EU). OJ L. 275, 28-40.

EU, 2011b. Regulation (EU) No 1169/2011 on the provision the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004 Text with EEA relevance. OJ L 304,18-63.

EU, 2012. Commission staff working paper: Types and uses of nanomaterials, including safety aspects. Accompanying the communication from the Commission to the European Parliament, the Council and the European Economic and Social Committee on the Second Regulatory Review on Nanomaterials

FDA, 2007. Nanotechnology- a report of the US Food and Drug Administration Nanotechnology Task Force.

FDA, 2014a. Final Guidance for Industry: Considering whether an FDA-Regulated products involves the aplication of nanotechnology.

FDA, 2014b. Final guidance for industry: Safety of nanomaterials in cosmetic products.

FDA, 2014c. Final Guidance for Industry: Assessing the effects of significant manufacturating process changes, including emerging technologies, on the safety and regulatory status of food ingredients and food contact subtances, including food ingredients thar are color additives.

FDA, 2015. Liposome drug products-chemistry, manufacturing and controls; human pharmacokinetics and bioavailability and labeling documentation. Draft guidance for industry.

Grazú, V., Moros, M., Sánchez-Espinel, C., 2012. Nanocarriers as Nanomedicines: Design concepts and recent advances. In: de la Fuente, J.M., Grazú, V (Eds)., Nanobiotechnology: Inorganic nanoparticles vs organic nanoparticles. E-publishing Inc., Oxford, Elsevier, pp. 337-440.

ICCP, 2011. Report of the joint regulator- Industry Ad Hoc Working Group: Currently available-methods for characterization of nanomaterials.

Irache, J.M., Esparza, I., Gamazo, C., Agüeros, M., Espuelas, S., 2011. Nanomedicine: Novel approaches in human and veterinary therapeutics. Veterinary Parasitology. 180 (1-2), 47-71.

Marin, E., Briceño, M.I., Caballero-George, C., 2013. Critical evaluation of biodegradable polymers used in nanodrugs. International Journal of Nanomedicine. 8, 3071-3091.

Oberdörster, G., Oberdörster, E., Oberdörster, J., 2005. Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles. Environmental Health Perspectives. 113 (7), 823-839.

Raj, S., Jose, S., Sumod, U., Sabitha, M., 2012. Nanotechnology in cosmetics: Opportunities and challenges. Journal of Pharmacy & Bioallied Sciences. 4 (3), 186-193.

Romero, G & Moya, S.E., 2012. Synthesis of organic nanoparticles. In: de la Fuente, J.M., Grazú, V (Eds)., Nanobiotechnology: Inorganic nanoparticles vs organic nanoparticles. E-publishing Inc., Oxford, Elsevier, pp.115-141.

SCCP, 2007. Opinion on safety of nanomaterials in cosmetic products. SCCP/1147/2007

SCCS, 2012. Guidance on the safety assessment of nanomaterials in cosmetics. SCCS/1484/12..

SCCS, 2013. Memorandum on relevance and quality of data in safety dossiers on nanomaterials. SCCS/1524/13, revision of 27 March 2014.

SCCS, 2015. The SCCS notes of guidance for the testing of cosmetic ingredients and their safety evaluation, 9 th revision. SCCS/1564/15.

Simón-Vázquez, R., Peleteiro, M., Lozano, T., González-Fernández, Á., Casal, A., 2012. Nanotoxicology. In: de la Fuente, J.M., Grazú, V (Eds)., Nanobiotechnology: Inorganic nanoparticles vs organic nanoparticles. E-publishing Inc., Oxford, Elsevier, pp. 443-485

Vance, M.E., Kuiken, T., Vejerano, E.P., McGinnis, S.P., Hochella, M.F., Rejeski, D., Hull, M.S., 2015. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. Beilstein Journal of Nanotechnology. 6, 1769-1780.

Wacker, M.G., 2014. Nanotherapeutics-product development along the nanomaterial discussion. Journal of Pharmaceutical Sciences. 103 (3), 777-784.

Wacker, M.G., Proykova, A., Santos, G.M.L., 2016. Dealing with nanosafety around the globe-regulation *vs* innovation. International Journal of Pharmaceutics. 509 (1-2), 95-106.

Ojer P, Iglesias T, Azqueta A, Irache JM, López de Cerain A. Toxicity evaluation of nanocarriers for the oral delivery of macromolecular drugs. European Journal of Pharmaceutics and Biopharmaceutics, 2015, 97(Pt A): 206-217.
http://doi.org/10.1016/j.ejpb.2015.10.005

Chapter 2:
Aim & Objectives

Many polymeric NPs have demonstrated great potential as oral drug delivery systems. However, the use of this nanotechnology may present potential risks due to the use of novel materials in novel ways. Thus, toxicity evaluation must be carried out to identify and quantify possible harmful effects.

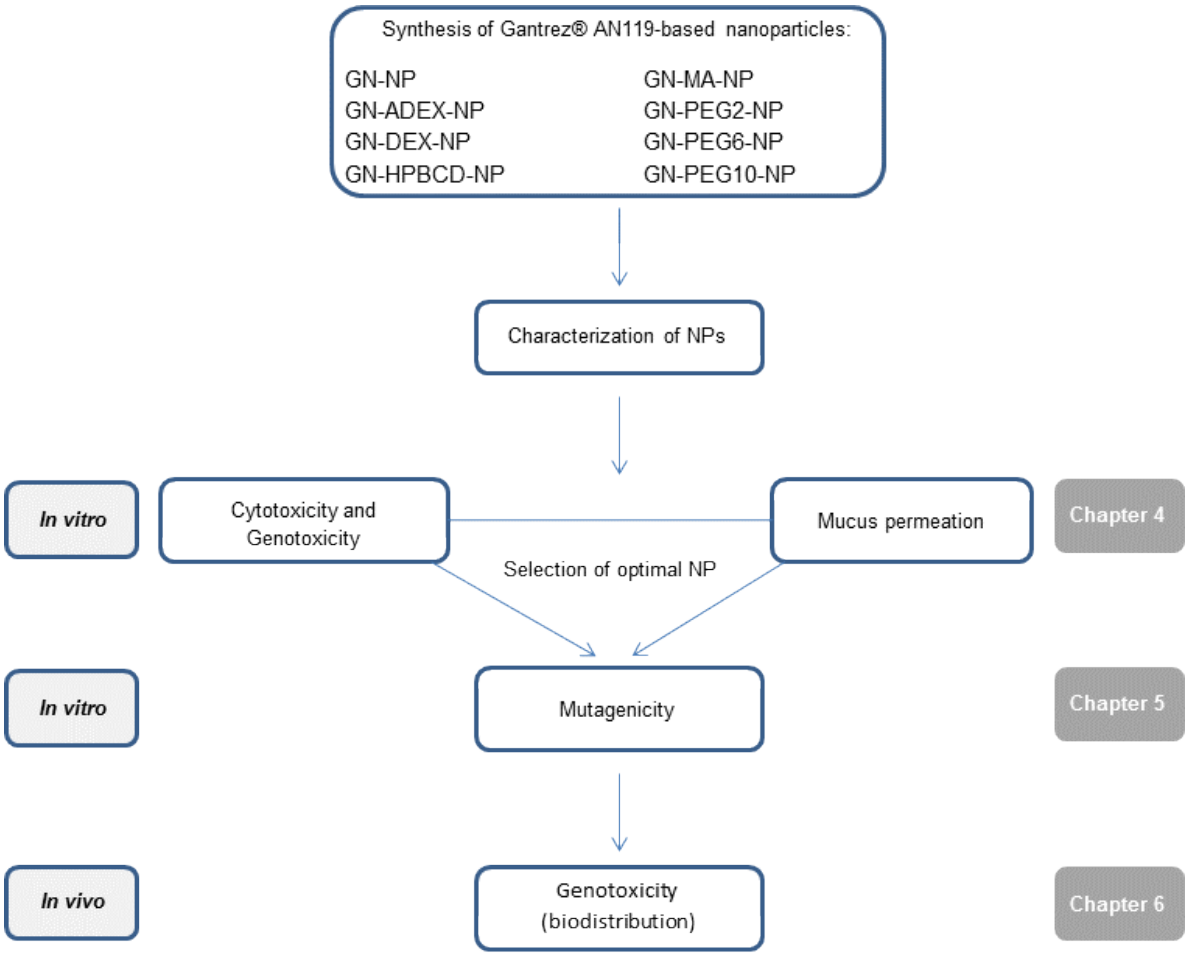
In this context, an extensive research carried out in the department of Pharmacy and Pharmaceutical Technology of the University of Navarra, has proven that poly(anhydride) NPs prepared using the copolymer of methyl vinyl ether and maleic anhydride (Gantrez® AN 119), are ideal candidates as vehicles for oral delivery of pharmaceutical drugs. Thus, the study of their toxicity was considered highly interesting.

Then, the general aim of this research project was to evaluate the toxicity of different poly(anhydride) NPs intended for oral delivery. For this purpose, the following objectives had to be accomplished:

1. Synthesis and characterization of Gantrez® AN 119-based NPs (GN-NP) coated with aminodextran (GN-ADEX-NP), dextran (GN-DEX-NP), cyclodextrin (GN-HPBCD-NP), mannosamine (GN-MA-NP) or poly-ethylene glycol (GN-PEG2-NP, GN-PEG6-NP and GN-PEG10-NP).
2. Evaluation of the *in vitro* toxicity of Gantrez® AN 119-based NPs in Caco-2 and HT29-MTX cells, using two different cytotoxicity assays: proteases leakage and ATP production.
3. Setting up an *in vitro* mucus permeability assay using a transwell technique and a natural gastrointestinal mucus obtained from pig digestive tube.
4. Evaluation of the *in vitro* permeability of Gantrez® AN 119-based NPs, using this transwell diffusion technique.
5. Assessment of the *in vitro* genotoxicity of Gantrez® AN 119-based NPs in Caco-2 and L5178Y $TK^{+/-}$ cells, using the comet assay in combination with the formamidopyridine DNA glycosilase (FPG) enzyme.

6. Setting up the mouse lymphoma assay in L5178Y $TK^{+/-}$ cells, according to the OECD guideline number 490.
7. Evaluation of the *in vitro* mutagenicity of naked Gantrez® AN 119 NPs and Gantrez® AN 119 NPs coated with mannosamine, using the mouse lymphoma assay.
8. Setting up the comet assay in different tissues of the gastrointestinal tract of mice, according to the OECD guideline number 489.
9. Assessment of the *in vivo* genotoxicity of Gantrez® AN 119 NPs coated with mannosamine, given to mice by oral route.

Chapter 3:
Experimental design


Synthesis of Gantrez® AN119-based nanoparticles:
GN-NP
GN-ADEX-NP
GN-DEX-NP
GN-HPBCD-NP
GN-MA-NP
GN-PEG2-NP
GN-PEG6-NP
GN-PEG10-NP
Characterization of NPs
In vitro
Cytotoxicity and Genotoxicity
Mucus permeation
Chapter 4
Selection of optimal NP
In vitro
Mutagenicity
Chapter 5
In vivo
Genotoxicity (biodistribution)
Chapter 6

Chapter 4: Evaluation of the cytotoxicity, genotoxicity and mucus permeation capacity of several surface modified poly(anhydride) nanoparticles designed for oral drug delivery

Evaluation of the cytotoxicity, genotoxicity and mucus permeation capacity of several surface modified poly(anhydride) nanoparticles designed for oral drug delivery.

Iglesias T[1], López de Cerain A[1,2], Juan Manuel Irache[3], Martín-Arbella N[3], Wilcox M[4], Pearson J[4], Azqueta A[1,2*]

[1]Department of Pharmacology and Toxicology, Faculty of Pharmacy and Nutrition, University of Navarra. Irunlarrea 1, 31008, Pamplona, Spain.

[2]IdiSNA, Navarra Institute for Health Research

[3]Pharmacy and Pharmaceutical Technology Department, Faculty of Pharmacy and Nutrition, University of Navarra.

Irunlarrea 1, 31008, Pamplona, Spain.

[4]Institute for Cell and Molecular Bioscience, Medical School, Newcastle University, Framlington Place, Newcastle Upon Tyne, NE2 4HH, UK.

Abstract

The main concerns with drugs designed for oral administration are their inactivation or degradation in the harsh conditions of the gastrointestinal tract, their poor solubility through the gastrointestinal mucus gel layer, the poor intestinal epithelium permeability that limits their absorption, and their toxicity. In this context, poly(anhydride) nanoparticles are capable of protecting the drug from the harsh environment, reduce the drug's toxicity and, by virtue of surface modification, to enhance or reduce their mucus permeability and the bioadhesion to specific target cells.

The copolymers between methyl vinyl ether and maleic anhydride (commercialized as Gantrez® AN 119) are part of the poly(anhydride) nanoparticles. These biocompatible and biodegradable nanoparticles (NPs) can be modified by using different ligands. Their usefulness as drug carriers and their bioadhesion with components of the intestinal mucosa have been described. However, their toxicity, genotoxicity and mucus permeation capacity has not been thoroughly studied.

The aim of this work was to evaluate and compare the *in vitro* toxicity, cell viability and genotoxicity of the bioadhesive empty Gantrez® AN 119 NPs modified with dextran 7000, aminodextran, 2-hydroxypropyl-β-cyclodextrin, mannosamine and poly-ethylene glycol of different molecular weights.

Results showed that, in general, coated NPs exhibit better mucus permeability than the bare ones, those coated with mannosamine being the most permeable ones. The NPs studied did not affect cell metabolism, membrane integrity or viability of Caco-2 cells at the different conditions tested. Moreover, they did not induce a relevant level of DNA strand breaks and FPG-sensitive sites (as detected with the comet assay).

Manuscript under revision in the "International Journal of Pharmaceutics"

1. Introduction

Recently, nanoparticles (NPs) have increasingly found practical applications in technology, research and medicine. Their wide use has given rise to a new area of medicine and research, called nanomedicine. In this context, NPs can be utilized in disease prevention, diagnosis, monitoring, treatment (Ahmad *et al*., 2008; Jain *et al*., 2011) (e.g. as drug carriers) and mitigation of pain (Mittal *et al*., 2007). NPs have been defined according to a nanometre scale of size between 0.1 and 100 nm, although in the case of pharmaceutical NPs, the dimension may be higher (De Jong & Borm, 2008).

Oral administration is the most commonly used and accepted route of drug administration. However the main concern is the inactivation or degradation of the drug in the harsh conditions of the gastrointestinal tract. In this context, to prevent rapid pre-systemic degradation of the drug due to the digestive enzymes of the gastrointestinal tract and the low pH, the NPs are used to enhance the drug's absorption (Hunter *et al.*, 2012).

The poor intestinal epithelium permeability also limits the absorption of drugs (Bernkop-Schnürch, 2013). The diffusion of poorly soluble drugs through the mucus gel layer is crucial to ensure an adequate serum concentration. This mucus layer is secreted by mucosal glands and some cells, such as goblet cells. Its main function is to protect the mucosal tissues (Lai, Wang & Hanes, 2009) and it consists of several negatively charged glycoproteins, which form a stable three-dimensional matrix (Friedl *et al*., 2013). Some strategies currently under investigation aim to solve this problem; an example is the development of slippery-surface NPs (Zabaleta *et al*., 2012).

Poly(anhydride) NPs have been considered promising platforms for drug delivery and other applications in the treatment of various diseases, such as tuberculosis (Ahmad *et al*., 2008), bacterial infections (Zaki & Hafaez, 2012) and cancer (Jain *et al*., 2011), among others. These NPs are biodegradable, surface modifiable to enhance or reduce bioadhesion to specific target cells (Ensign, Cone & Hanes, 2012) and capable of sustained drug release. The copolymers between methyl vinyl ether and maleic anhydride (commercialized as Gantrez® AN 119) are an

excellent example of the group of poly(anhydride) NPs (Arbós *et al*., 2002). Their surface can be modified with different ligands in order to alter their physico-chemical properties, as well as their distribution *in vivo* (Agüeros *et al*., 2009; Inchaurraga *et al*., 2015). It has been demonstrated that Gantrez® AN 119 NPs coated with different ligands have the ability to develop strong bioadhesive interactions with components of the intestinal mucosa (Agüeros *et al*., 2009, 2010; Arbós *et al*., 2002, 2004; Porfire *et al*., 2010; Salman *et al*., 2005, 2006; Salman, Irache & Gamazo, 2009; Yoncheva *et al*., 2005). Salman *et al.* (2006) observed that Gantrez® AN 119 NPs coated with mannosamine were uptaked by Peyer's patches while bare NPs were just localized in the outer layer, probably due to the presence of mannose receptor in this lymphoid tissue. Moreover, Gantrez® AN 119 based NPs are capable of establishing bioadhesive interactions with Caco-2 cells without being internalized (Ojer *et al*., 2013).

Gantrez® AN 119 based NPs, as biodegradable and biocompatible NPs, are considered to be of low or no toxicity to the organism (Landsiedel *et al*., 2012). Consequently, fewer studies have focused on potential adverse effects of these types of nanomaterials. However, bioadhesive NPs can affect membrane stability either directly (physical damage) or indirectly (oxidation) which can lead to apoptosis and finally, cell death. Actually, oxidative stress has been established as one of the crucial factors determining the toxicity of several NPs (Ahmad, Yokoi & Hanaoka, 2012; Kumar *et al*., 2011; Nel *et al*., 2006). Usually, the oxidative stress is generated by an increase in intracellular reactive oxygen species (ROS), highly reactive molecules that can react with cell biomolecules including the DNA.

There are many studies that support the usefulness of this type of NPs as drug carriers (Agüeros *et al*., 2010; Arbós *et al*., 2004; Salman, Irache & Gamazo, 2009); however, the toxicity and the mucus permeation capability have not been thoroughly investigated. In this study the *in vitro* toxicity, genotoxicity and the mucus permeability of empty Gantrez® AN 119 NP modified with dextran (DEX), aminodextran (ADEX), cyclodextrin (HPBCD), mannosamine (MA) and poly-ethylene glycol (PEG) as ligands are evaluated. DEX and ADEX are known to be used as a stabilizing coating material to protect metal NPs from oxidation and improve their biocompatibility (Easo & Mohanan, 2013). DEX coating has been described as a muco-

penetration enhancer across the intestinal mucus barrier (Beloqui *et al*., 2014) but it produces an uncommon but significant acute renal failure (Brooks, Okeefe & Buncke, 2001). Several studies have shown that NPs based on HPBCD reduce their toxicity as well as improves the permeability of drugs (Nagai *et al*., 2014; Jaiswal *et al*., 2015; Wu, Shen & Fang, 2013). Furthermore, it has been demonstrated that some HPBCD derivatives do not exert adverse effects in humans after oral or intravenous administration (Stella & He, 2008). Mannose and its derivatives are interesting surface ligands due to their capability to link with the mannose receptors, highly expressed in the cells of the mucosal immune system (i.e. macrophages and dendritic cells) (Carrillo-Conde *et al*., 2011). Similarly, PEG coating of NPs surfaces has been demonstrated to be an effective strategy to ensure rapid NP transport through the mucus (*Li et al*., 2015; Liu *et al.*, 2013).

Therefore, the aim of this study was to evaluate and compare the *in vitro* toxicity, cell viability and genotoxicity of the bioadhesive empty Gantrez® AN 119 NP modified with DEX, ADEX, HPBCD, MA and different PEG as ligands in human colon cell lines. Moreover, the diffusion capability through gastrointestinal natural mucus was evaluated using an *in vitro* transwell diffusion technique.

2. Material and methods

2.1. Materials

The copolymer of methyl vinyl ether and maleic anhydride (Gantrez® AN 119; MW: 200000) was provided by Ashland (Barcelona, Spain). Poly-ethylene glycol 2000 (PEG2), poly-ethylene glycol 6000 (PEG6), poly-ethylene glycol 10000 (PEG10) were provided by Fluka (Switzerland). 2-hydroxypropyl-β-cyclodextrin (HPBCD) and Dextran (DEX, MW: 70000) were provided by Sigma-Aldrich (Steinheim, Germany). Mannosamine (MA) was purchased from Sigma (Spain). Aminodextran (ADEX, MW: 70000) was obtained from Invitrogen (Spain). Lumogen® Red F 305 was supplied by BASF (North America).

Acetone was obtained from VWR Prolabo (Fontenay-sous-Bois, France). Deionized water (18.2 Ω resistivity) was prepared by a water purification system (Wasserlab, Pamplona, Spain).

Nitrogen gas (ultra-pure, > 99 %) was produced using an Alltech nitrogen generator (Ingeniería Analítica, Barcelona, Spain).

2.2. Preparation of NPs

In this study seven types of modified NPs were produced and evaluated. In all cases, NPs were prepared from an acetone phase containing the copolymer of methyl vinyl ether and maleic anhydride (Gantrez® AN 119) and a hydrophilic compound, by a desolvation procedure with a mixture of ethanol and water. The following hydrophilic compounds were employed to produce the different types of NPs: ADEX and DEX, HPBCD, MA and PEG2, PEG6 and PEG10. The resulting NPs were also purified and, finally, dried by spray-drying.

For the preparation of NPs containing dextrans (Porfire *et al*., 2010), 400 mg of the copolymer (Gantrez® AN 119) were dissolved in 20 mL acetone containing either ADEX (1.2 mg) or DEX (80 mg). Then, NPs were obtained by the addition of 40 mL of an ethanol:water mixture (1:1, v/v) and the mixture was incubated at room temperature (RT) under magnetic agitation for 30 min. The organic solvents were eliminated under reduced pressure evaporation and the resulting nanosuspension was purified by tangential filtration using Vivaspin (3000 rpm, 5 min, 4°C). Finally, the NPs were diluted with 12 mL of an aqueous solution of lactose (2% v/v) and dried in a mini Spray dryer Büchi B290 (Büchi Labortechnik AG, Switzerland). These NPs were named as GN-ADEX-NP (when NPs containing ADEX) and GN-DEX-NP (for NPs prepared in the presence of DEX).

For NPs containing HPBCD (Agüeros, Campanero & Irache, 2005), 400 mg of the copolymer (Gantrez® AN 119) were dissolved and stirred in 10 mL of acetone. In parallel, 10 mL of acetone containing 100 mg of the HPBCD were added to the polymer solution under magnetic stirring and incubated for 30 min. NPs were obtained by the addition of 20 mL of a hydroalcoholic mixture (water: ethanol, 1:1, v/v) under magnetic stirring to the organic phase. Finally, the resulting NPs were purified and dried as described above. These NPs were named as GN-HPBCD-NP

For mannosylated NPs, 100 mg of the copolymer (Gantrez® AN 119) were dissolved and stirred in 5 mL of acetone. At the same time, 10 mg of MA were dissolved in 5 mL of water. Then, 10 mL of ethanol was added to the acetone solution and mixed with 5 mL of the water solution. The mixture was incubated for 30 min and resulting NPs were purified and dried as above. These NPs were called GN-MA-NP. The production was modified from Salman *et al*. (2006).

Finally, pegylated NPs were prepared by the incubation between 400 mg Gantrez® AN 119 and 50 mg of a poly-ethylene glycol (PEG2, PEG6 or PEG10) in acetone for 1 hour at RT (Yoncheva, Doytchinova & Irache, 2010). NPs were obtained by the addition of 20 mL of a hydroalcoholic mixture (water:ethanol, 1:1, v/v) under magnetic stirring. Finally, as for the other batches, NPs were purified and, dried by Spray-drying. These NPs were identified as GN-PEG2-NP (when prepared in the presence of PEG2), GN-PEG6-NP (when PEG6 was used) and GN-PEG10-NP (for those containing PEG10).

Bare Gantrez® AN 119 (GN-NP) were prepared in the absence of any type of hydrophilic excipient and used as control (Irache *et al*., 2005). In all cases, the resulting powder containing the NPs was stored hermetically closed at room temperature.

2.3. Preparation of fluorescently labelled NPs

For the permeability studies, NPs were fluorescently labelled with red Lumogen®. For this purpose, 10 mg Lumogen® Red F 305 were dissolved for every 100 mL of acetone containing the copolymer and, eventually, the hydrophilic excipient. The different formulations were prepared as described above.

2.4. Physico-chemical characterization of NPs

<u>Size, zeta potential, morphology and pH measurements</u>

The mean size, polydispersity index (PDI) and the zeta potential of NPs were determined by photon correlation spectroscopy (PCS) and electrophoretic laser Doppler anemometry, respectively, using a Zetaplus apparatus (Brookhaven Instruments Corporation, Holtsville, USA). In all cases, the size was measured after dispersion of NPs in ultrapurified water (1/10)

and measured at room temperature using a scattering angle of 90°. The zeta potential was determined by diluting the samples in 0.1 mM KCl solution adjusted to pH 7.4. The pH was measured in MEM exposure medium (see section 2.5. Cell line and cell culture) using pH indicator paper (Nahita, Auxilab, Beriáin, Spain).

The morphology of the NPs was observed using a scanning electron microscope (Zeiss DSM 940A, Oberkochen, Germany) coupled with a digital image system (DISS, Point Electronic GmBh). Before examining the NPs, they were diluted in purified water, centrifuged to eliminate sugar and shaded with a 12 nm gold layer in a Hemitech K 550 Sputter-Coater.

Stability studies

For the stability assays, stock solutions of NPs (2 mg/mL) were prepared in MEM exposure medium (see section 2.5. Cell lines and cell culture), PBS without calcium and magnesium, and ultrapure water. The particle size and the PDI of the formulations were measured as described above at different time-points after dispersion of NPs in water. A NP formulation was considered stable if (i) PDI was lower than 0.3 and (ii) if PDI and size did not substantially changed during the incubation period.

2.5. Cell lines and cell culture

Two cell lines derived from human colon carcinoma have been used: Caco-2 and HT29-MTX. Caco-2 cells were obtained from the ATCC collection (Maryland, United States). HT29-MTX cells were a gift from Dr. Jeffrey Pearson (Institute for Cell and Molecular Bioscience, Newcastle University, UK). This cell line maintains the capability to produce mucus.

Caco-2 cells were grown in minimum essential medium (MEM) with L-glutamine supplemented with 20 % fetal bovine serum (FBS), 1 % non-essential amino acids, 1 % sodium pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco® by life tecnologies™, USA) (MEM culture medium). During the exposure period, MEM with L-glutamine with or without phenol red, depending on the assay, supplemented with 10 % FBS was used (MEM exposure medium). HT29-MTX cells were grown in Dulbecco's modified eagle's medium (DMEM) (Gibco®, USA)

supplemented with 10 % FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (DMEM culture medium). During the exposure period, DMEM with L-glutamine supplemented with 5 % FBS was used (DMEM exposure medium).

Cells were maintained in a humidified atmosphere with 5 % CO_2 at 37 °C and subcultured by trypsinization (TryPLE™ Select (1X), Gibco® by life tecnologies™, USA). Adequate care was taken with the density of cells, as it is important for maintaining the cells in exponential growth. Cells were kept on culture for a maximum of two months (approximately, 24 passages); when this period was reached a new vial of cells was used.

2.6. Cytotoxicity assays – ATP and proteases activity detection

Cytotoxicity of NPs was evaluated by measuring ATP and proteases. The amount of ATP is an indication of the number of metabolically active cells remaining after the treatment. The proteases remaining in live cells after the treatment are an indication of the membrane cell integrity. Two commercial kits both based on chemoluminiscence have been used: “CellTiter-Glo® Luminescent Cell Viability Assay” for ATP detection and “CytoTox-Glo™ Cytotoxicity Assay” for dead cells protease detection. Both kits were purchased from Promega (Madison, USA). The method 2 of CytoTox-Glo™ (i.e. lysing the living cells to measure the protease activity) was used because preliminary studies carried out in our laboratory indicated that NPs interfered in the measurement when method 1 (i.e. measuring the protease activity in the cell culture medium) was used.

Experiments were performed using sub-confluent cells in the logarithmic growth phase. According to preliminary studies, cells were seeded in 96-well plates (White plate, Costar®, Corning Incorporated Corning, NY) at 125 x10^4 cells/mL in 100 µL of medium and incubated for 24 h at 37 ºC before starting the treatment.

Before the addition of NPs, cell monolayers were washed with PBS without calcium and magnesium. Then, 100 µL of NPs solutions prepared in exposure medium without phenol red, were added. Cells were treated for 24 h at the following final concentrations: 0, 0.00002;

0.0002; 0.002; 0.02; 0.2 and 2 mg/mL. Cells treated with 0.2 mg/mL Digitonin were include as positive control in each assay. Background luminescence was determined in cell culture medium without cells or NPs. Three replicates of each condition were tested per experiment and three independent experiments were performed. The general appearance of the cells after treatment was always observed by microscope (Nikon ECLIPSE TS100). The luminescence was measured in a multimode detector (Beckman Coulter® DTX800).

The relative cell viability was determined by the following formula:

[(luminescence treated cells - background luminescence) / (luminescence negative control - background luminescence)] x 100

Cell survival values above 80 % were considered as non-cytotoxic.

Cytotoxicity determinations were carried out in Caco-2 cells. To confirm the results, the highest concentration of NPs (2 mg/mL) was tested in the HT29-MTX cell line using the ATP detection assay.

2.7. Viability assay – Proliferation assay

Viability of the cells treated with NPs was evaluated using the proliferation assay. Experiments were performed using sub-confluent cells in the logarithmic growth phase. Caco-2 cells were seeded in 6-well plates at a concentration of 120×10^3 cell/mL in 3 mL. After 48 h, they were treated with NPs at 0, 0.5, 1 and 2 mg/mL concentrations for 3 h in an incubator at 37 °C and 5% CO_2. After exposure, cultures were washed twice with PBS solution, detached by trypsinization and neutralized with 4 mL of cell culture medium. Three milliliters were seeded in a 6-well plate for the proliferation assay and the rest was used for performing the comet assay (see below).

Cells were counted before and after treatment, and after 48 h of incubation in fresh medium using the automated cell counter (Countess™ Automated Cell Counter, Invitrogen). Three independent experiments were performed.

The total suspension growth (TSG) and the relative suspension growth (RSG) of each condition were calculated as follow:

TSG= number of cells at 48 h post-treatment / number of cells before the treatment

RSG= (TSG in exposed cultures / TSG in unexposed control cultures) x 100

It was considered that the viability was affected when a decrease of more than 30% RSG was obtained.

GN-NP and GN-MA-NP, were also evaluated after 24 h of treatment.

2.8. Genotoxicity – Comet assay

he genotoxicity of NPs was evaluated using the alkaline comet assay (single-cell gel electrophoresis), in combination with the enzyme formamidopyrimidine DNA-glycosylase (FPG), as previously described by Collins & Azqueta (2012). The assay was performed in parallel with the proliferation assay (see section 2.7. Viability assay – proliferation assay). Treated cells were washed with PBS,centrifuged at 1500 rpm and 4 °C for 5 min, and suspended in PBS to obtain 1×10^{6} cells/mL. Ninety microliters of this cell suspension were mixed with 420 µL of 1 % low melting point agarose (Sigma) and 2 drops of 70 µL of the mixture were added onto agarose (Sigma) pre-coated slides. Three identical slides were prepared per condition. Following 5 min of solidification on ice, slides were immersed in lysis buffer (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1 % Triton X-100, pH 10) at 4°C overnight. After lysis, 2 of the slides were washed three times with enzyme reaction buffer (Buffer F) (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8) and incubated with either buffer F or FPG for 30 min at 37 °C in a humidified chamber. Meanwhile, the other slide remained in the lysis buffer. .All slides were then immersed in electrophoresis buffer (1 mM EDTA, 0.3 M NaOH, pH > 13) for 40 min at 4 °C before

performing electrophoresis at 25 V and 4 ºC for 20 min. Gels were neutralized by washing the slides twice with PBS at 4 ºC for 10 min and rinsed with water before leaving them to dry at room temperature.

Gels were stained with 1 µg/mL of 4,6-diamidino-2-phenylindole (DAPI) solution and examined by fluorescent microscope (Nikon Eclipse 50 i, Japan). Comets were analyzed using the image analysis system Comet Assay IV (Perceptive Instruments). The percentage of DNA in tail (% tail DNA) was used as DNA damage indicator. A total of 100 randomly selected comets (50/gel) were analyzed per slide and the median of the % tail DNA was calculated. SBs (plus ALS) were assessed by determining the % tail DNA in lysis buffer slides (i.e. the ones which remained in lysis buffer until alkaline treatment). To calculate the net FPG-sensitive sites, the median % tail DNA in the slide treated with buffer F was subtracted from the % tail DNA in slide treated with FPG. Two positive controls were included in each experiment: cells treated with 100 µM of H_2O_2 for 5 min for DNA strand breaks, and cells treated with 0.1 µM of Ro 19-8022 plus 5 min of 500 W visible light for FPG-sensitive sites. Three independent experiments were performed.

GN-NP and GN-MA-NP, were also evaluated after 24 h of treatment.

2.9. Collection and preparation of gastrointestinal mucus

The intestines of freshly slaughtered pigs were collected from a local slaughterhouse and transported on ice to the laboratory. The small intestines were incised longitudinally, opened out and scraped with a microscope slide to collect the mucus. Digesta debris was removed from the mucus by gentle agitation (stirring < 40 rpm) followed by centrifugation as follows; 5 mL of 0.1 M sodium chloride was added to 1 g of mucus, and agitated for 1 h after which the suspension was centrifuged (13.125 g) for 2 h. The clean portion of the pellet was retained. This pellet was re-suspended in 0.1 M sodium chloride at the same ratio as above and the centrifugation repeated. The resulting pellet was the clean mucus and was stored at -20◦C until required.

2.10. Permeability – *In vitro* diffusion study

The capacity of NPs to permeate across a natural mucus layer was determined by using the technique described by Friedl *et al.* (2013) with some modifications.

The experiments were performed in 24-well plates (Greiner bio-one, Germany) using polystyrene and poly-ethylene terephthalate capillary pore membranes transwells (translucent membrane with a pore size of 3 µm, ThinCerts™, Greiner bio-one, Germany). The transwell surface was covered with 50 mg of gastrointestinal mucus collected from freshly slaughtered pigs. The basolateral chamber of transwells was filled with 1600 µL of MEM exposure medium without phenol red, whereas the apical transwell area received 250 µL of each fluorescently labelled NPs (prepared in MEM exposure medium without phenol red). The transwells were then placed on a shaking board (Rotator PS-3D, Grant Bio, UK) at 37°C in a humidified atmosphere. At different times, aliquots of 100 µL from the basolateral chamber were taken and the fluorescence was measured in a fluorimeter (Beckman Coulter DTX 800) at an excitation wavelength of 578 nm and an emission wavelength of 613 nm. In all cases, the removed aliquot was replaced by the same volume of fresh medium. The influence of the NP concentration on the permeability was also evaluated by testing 0.25, 0.5, 1 and 2 mg/mL of each formulation.

For each NP formulation and concentration a blank and maximum of florescence (100% permeation) were included. The blank consisted on 100 µL of the MEM supplemented culture medium. The maximum of fluorescence consisted on 100 µL of the following mixture: 1600 µL of MEM supplemented culture medium plus 250 µL of each fluorescently labelled NP suspension. All NP formulations were also tested in the absence of the mucus layer.

The fluorescence obtained in each time sampling had to be corrected since 100 µL were removed from the basolateral chamber at each time point. Cumulative corrections were made by applying the following formula:

Corrected fluorescence = fluorescence – blank + (previous corrected fluorescence x 100 /1600)

The percentage of permeability at each time point and condition was calculated with respect to the maximum of fluorescence by the following formula:

% of permeability = (corrected fluorescence / maximum fluorescence) x 100

Three experimental replicates were used.

2.11. Statistical analysis

All the experiments were performed in triplicate and results were presented as mean ± standard deviation. Statistical comparison of the % tail DNA were performed using non-parametric Kruskal Wallis test followed by multiple comparison to the negative control U-Mann Whitney test using SPPS (SPSS® version 15.0). Differences at $p < 0.05$ were considered statistically significant.

3. Results

3.1. Physico-chemical characterization and stability

The main physico-chemical characteristics of the NP formulations evaluated in this work are summarized in Table 1. Overall, the mean size of the different NPs was around 200 nm; although some differences were found depending on the hydrophilic excipient used during the preparation of these nanocarriers. Thus, HPCD-NP presented a mean size of about 160 nm, whereas mannosylated NPs displayed a size close to 260 nm. Bare NPs (GN-NP) showed a mean size of 220 nm. In any case, all the different batches presented a low PDI (range between 0.041 ± 0.023 and 0.161 ± 0.024), which is good evidence of the monodisperse character of all the prepared formulations. In the same way, all the nanocarriers displayed a negative zeta potential of between -50 mV, for GN-ADEX-NP and -24 mV for GN-PEG10-NP.

The morphological analysis by scanning microscopy of GN-NP, GN-ADEX-NP, GN-DEX-NP, GN-HPBCD-NP, GN-MA-NP and GN-PEG2-NP are shown in Fig. 1. All of them showed a spherical shape. GN-NP presented a smooth surface while the rest were rough.

NPs labelled with red Lumogen® showed similar particle size, PDI and zeta potential to the non-labelled ones (data not shown).

The pH of aqueous dispersions of NPs in MEM exposure medium was neutral in all cases (pH 7), except for GN-DEX-NP which presented a slightly acid pH.

As a preliminary step to performing the *in vitro* assays, the stability of the NPs was determined by measuring the size and the PDI in the media used to perform the cytotoxicity and genotoxicity assays (ultrapure water, PBS and MEM exposure medium). NP solutions were prepared at 2 mg/mL, the maximum concentration tested in cytotoxicity and genotoxicity assays, and samples were taken after 0, 15, 30, 45 min, 1, 2, 3 and 24 h. Results are shown in table 1 (see supplementary material for raw data). All NPs were stable for at least 24 h in MEM exposure medium and 15 min in PBS. Regarding the ultrapure water all NPs were stable for at least 3 h, except GN-PEG10-NP which showed changes in size and PDI in 15 min.

Table 1. Physico-chemical characteristics of NPs and maximum time at which they are stable in MEM exposure medium, PBS and ultrapure water. Size, PDI and zeta potential are expressed as mean ± standard deviation (n=3).

NP	**Size (nm)**	**PDI**	**Zeta potential (mV)**	**pH**	**Maximum time of stability (2 mg/mL)**		
					Cell medium (h)	**PBS (min)**	**Ultrapure H_2O (h)**
GN-NP	219 ± 3	0.084 ± 0.049	-32.0 ± 2.00	7	24	15	3
GN-ADEX-NP	244 ± 1	0.161 ± 0.024	-50.0 ± 0.70	7	24	15	3
GN-DEX-NP	187 ± 1	0.041 ± 0.023	-21.6 ± 0.92	6	24	15	3
GN-HPBCD-NP	160 ± 1	0.098 ± 0.051	-25.7 ± 1.51	7	24	15	3
GN-MA-NP	262 ± 4	0.090 ± 0.031	-40.4 ± 0.50	7	24	15	3
GN-PEG2-NP	184 ± 2	0.098 ± 0.013	-35.4 ± 1.31	7	24	15	3
GN-PEG6-NP	176 ± 1	0.129 ± 0.016	-34.7 ± 0.96	7	24	15	3
GN-PEG10-NP	212 ± 1	0.074 ± 0.009	-24.0 ± 1.30	7	24	15	0.25

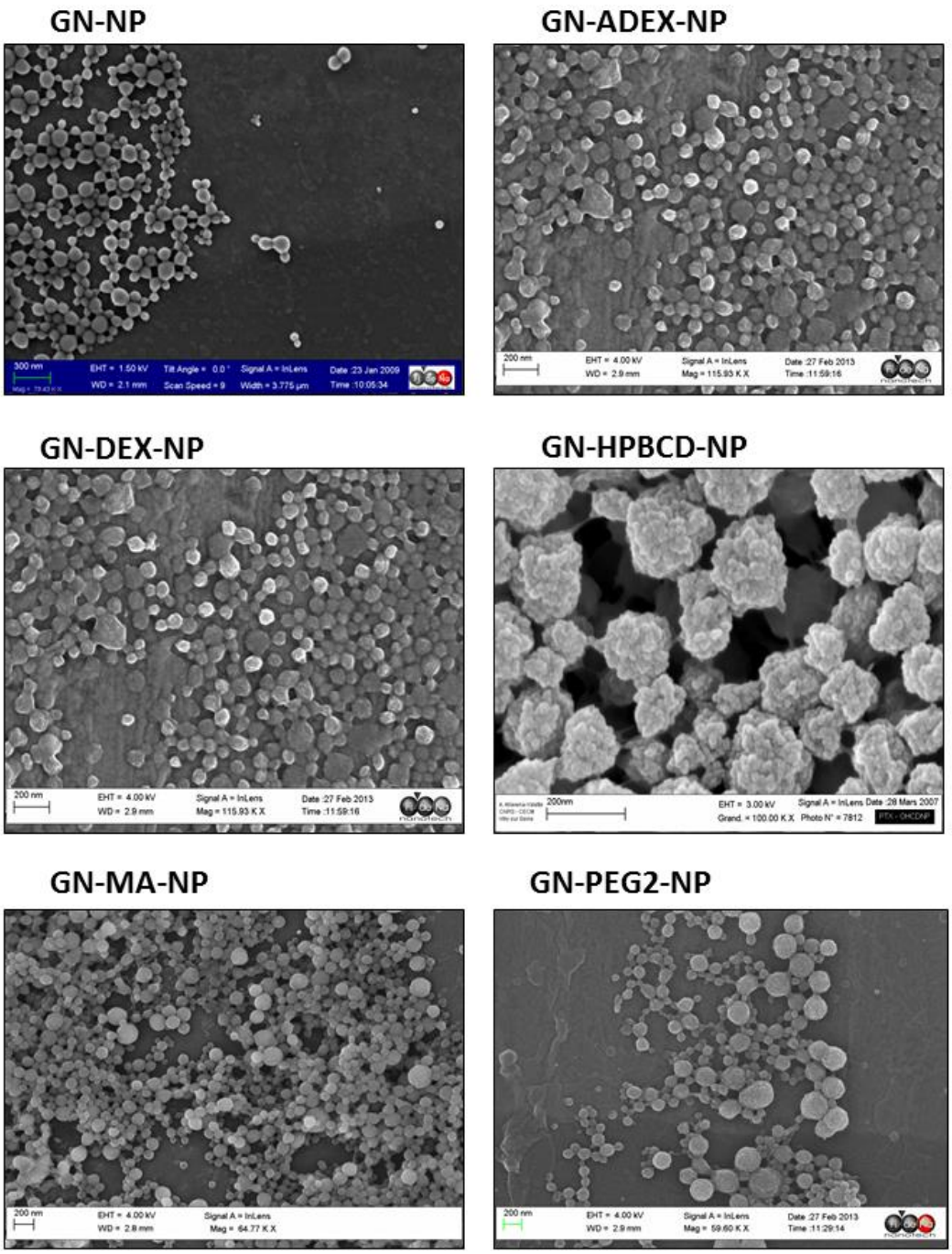


Fig. 1. SEM photographs of NPs.

3.2. ATP and protease activity detection

Cytotoxicity was evaluated by determining mitochondrial function (measuring cellular ATP) and membrane integrity (measuring cell proteases). A range of concentrations was assayed in Caco-2 cells using both assays after 24 h of treatment. Only the maximum concentration was tested in HT29-MTX using the ATP detection assay.

Results in Caco-2 cells are presented in Fig. 2. Regarding the ATP detection assay, cell viability was higher than 80% in nearly all the conditions tested (Fig. 2). Only GN-PEG6-NP showed a percentage of survival slightly lower than 80% at the highest concentration tested (78 ± 6 %).

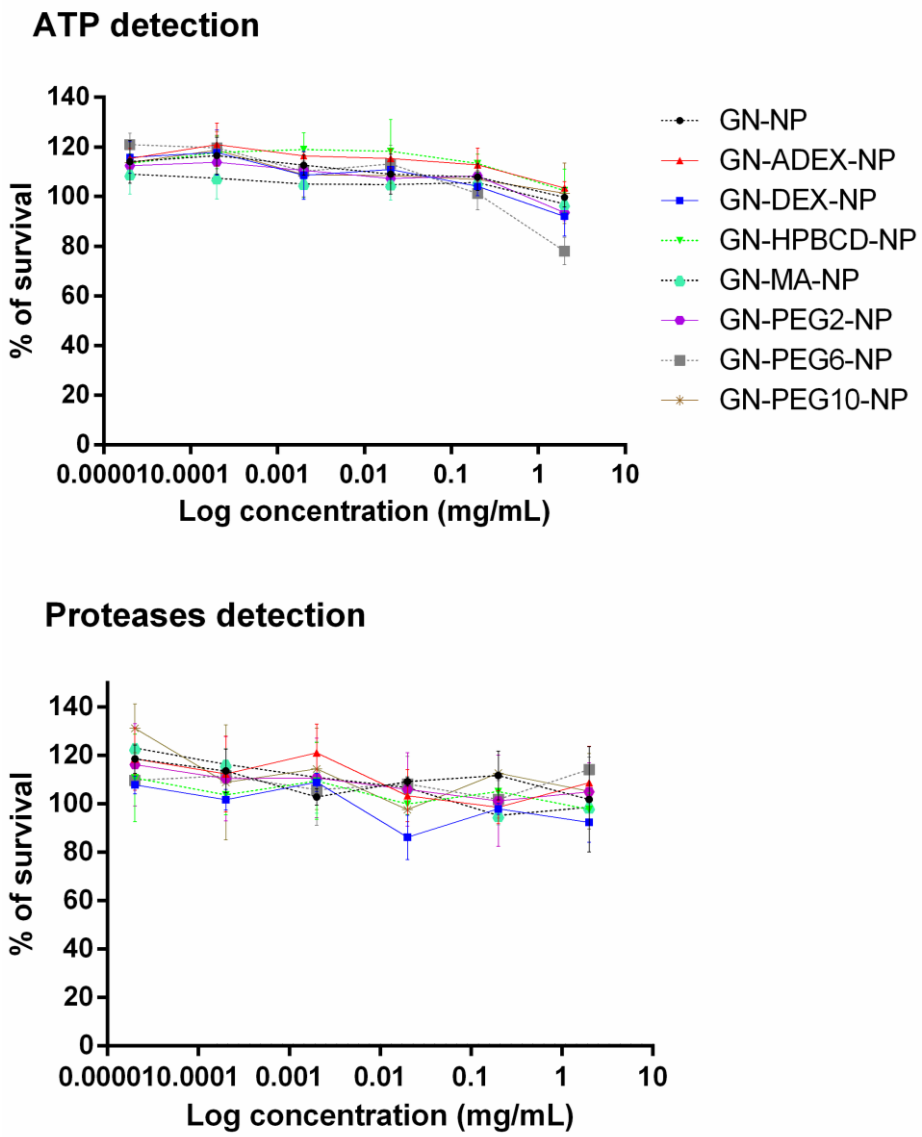


Fig 2. Cytotoxicity at 24 h. Cytotoxicity of the different NP formulations on Caco-2 cells after 24 h of treatment measured by the ATP detection assay and the dead cell proteases assay. Results presented as percentage of survival and expressed as the mean ± SD. Three independent experiments.

These results were confirmed in the HT29-MTX cell line at the highest concentration (data not shown). The percentage of cell viability was above 80% in all conditions when the dead cell

proteases assay was applied (Fig. 2). A normal cell appearance was observed by microscopy in all cases.

3.3. Cell proliferation

The cell proliferation assay was carried out by counting treated cells after 48 h of incubation. It was performed in parallel with the alkaline comet assay to correctly interpret the comet assay outcome.

As is shown in Fig. 3, all NPs tested did not show changes in the rate of proliferation of Caco-2 cells. The RSG was above 80% in all conditions tested.

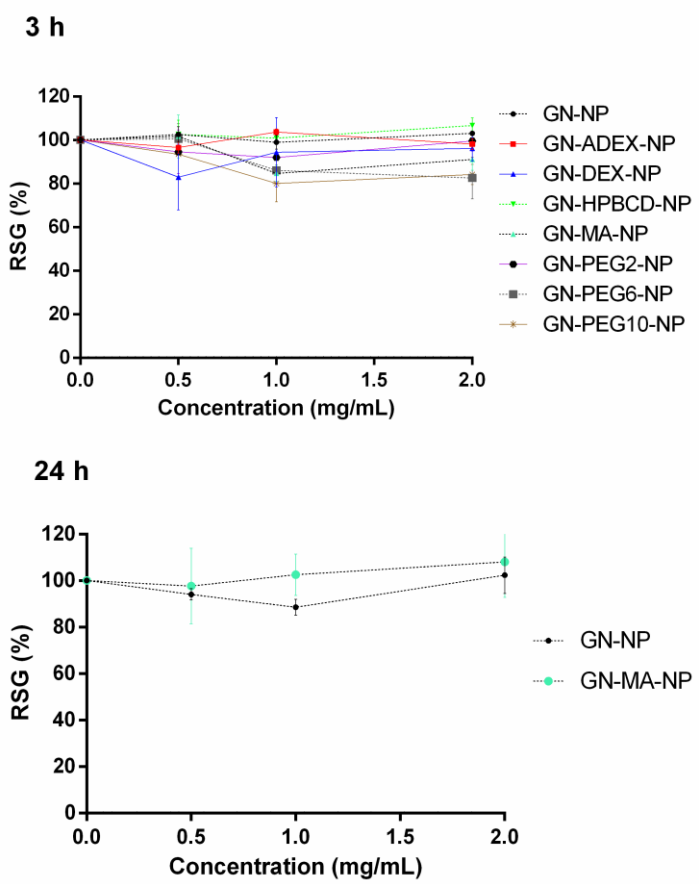


Fig. 3. Viability at 3 and 24 h. Effect of the different NP formulations on the viability of Caco-2 cells treated for 3 h and 24 h using the proliferation assay. Results presented as RSG and expressed as the mean ± SD. Three independent experiments.

Moreover, a normal cell appearance was observed by microscopy after the treatment and after the incubation period.

3.4. Detection of DNA damage by the alkaline comet assay in combination with FPG

The genotoxicity of 8 NPs was evaluated by the alkaline comet assay in combination with the enzyme FPG in Caco-2 cells. Cells were treated for 3 h with the different NPs and for 24 h with GN-NP and GN-MA-NP.

Fig. 4 shows the induction of DNA strand breaks and FPG-sensitive sites by NPs in Caco-2 cells. A statistically significant effect was seen in the case of cells treated with 2 mg/mL of GN-DEX-NP. The rest of the NPs did not significantly increase the % tail DNA after 3 h of treatment. In all cases the % tail DNA was lower than 5%.

GN-NP and GN-MA-NP induced a slight concentration-dependent increase in net-FPG sensitive sites after 24 h of treatment (Fig. 5); this effect was statistically significant ($p \leq 0.05$) at the maximum concentration tested of GN-NP. In all cases, the values obtained were very low.

Both positive controls (Ro 19-8022 plus 5 min of 500 W visible light and H_2O_2) showed the expected results in all the experiments.

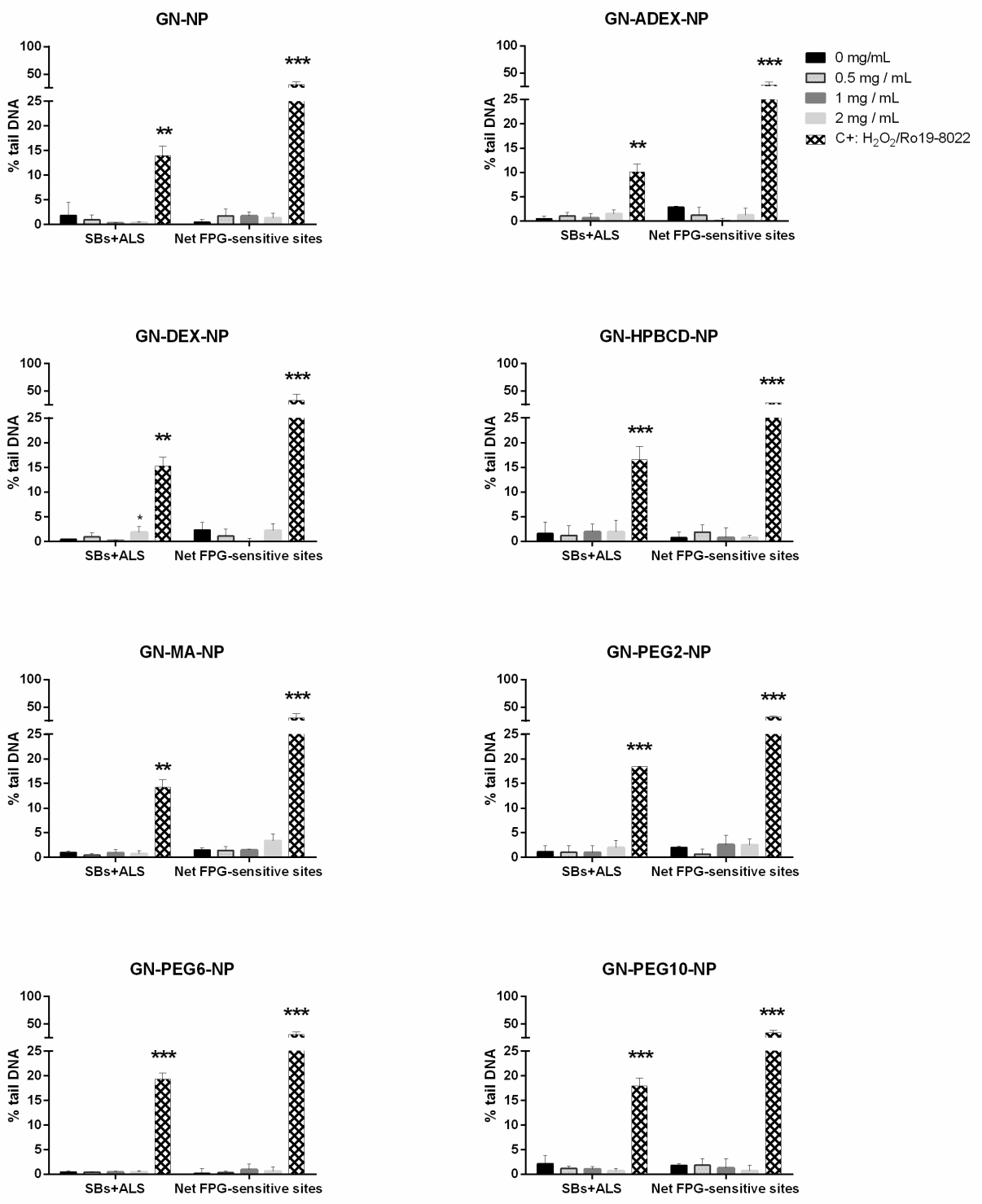


Fig. 4. Genotoxicity at 3 h. Effect of the different NP formulations on DNA strand breaks and net FPG-sensitive sites in Caco-2 cells treated for 3 h, measured using the alkaline comet assay in combination with the FPG. Cells treated with 100 µM of H_2O_2 were used as positive control for DNA strand breaks and cells treated with 0.1 µM of Ro 19-8022 plus visible light for FPG-sensitive sites. Results presented as % tail DNA and expressed as mean ± SD. Three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, significantly different from negative control.

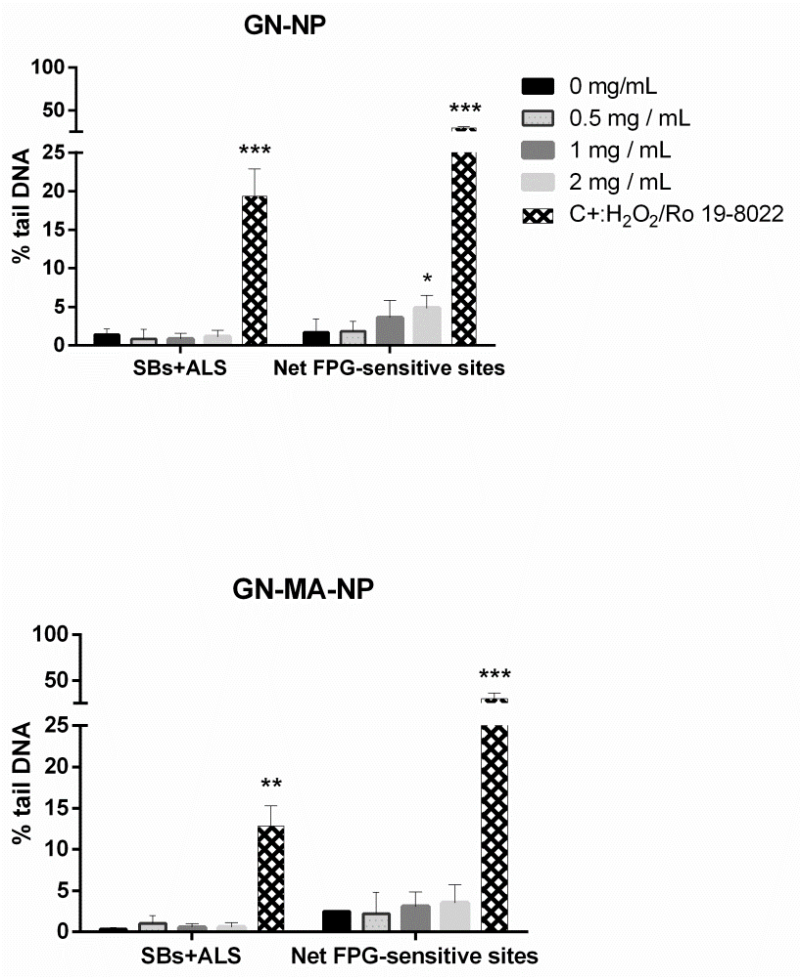


Fig. 5. Genotoxicity at 24 h. Effect of GN-NP and GN-MA-NP on DNA strand breaks and net FPG-sentitive sites of Caco-2 cells treated for 24 h using the alkaline comet assay in combination with FPG. Cells treated with 100 µM of H2O2 were used as positive control for DNA strand breaks and cells treated with 0.1 µM of Ro 19-8022 plus visible light for FPG-sensitive sites. Results presented as % tail DNA and expressed as mean ± SD. Three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, significantly different from negative control.

3.5. *In vitro* mucus permeability

To evaluate the capability of NPs to diffuse through the gastrointestinal epithelium, fluorescently labelled nanocarriers were incubated in a transwell system covered with natural gastrointestinal mucus. Four different concentrations of NPs were used: 0.25, 0.5, 1 and 2 mg/mL. In general, by increasing the concentration of NPs their permeability clearly decreased; the highest values of permeation were obtained at the concentration of 0.5 mg/mL; results al 0.25 mg/mL showed different patterns depending on the type of NP (data not shown).

Results regarding 0.5 mg/mL of NPs are shown Fig. 6. GN-HPBC-NP, GN-MA-NP, GN-PEG2-NP and GN-PEG6-NP and GN-PEG10-NP showed a higher percentage of permeability than GN-NP after 6 h of incubation.

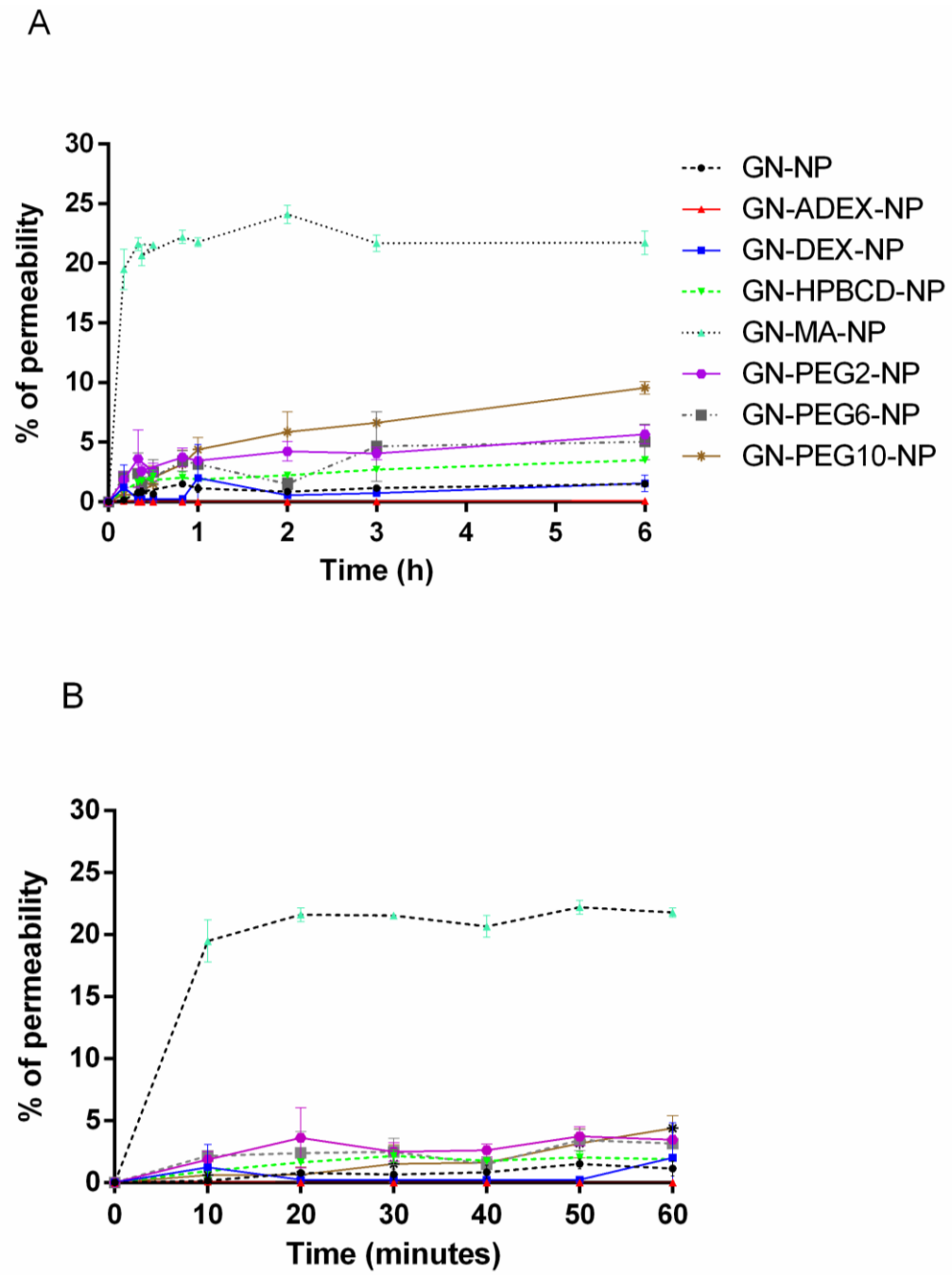


Fig. 6. Permeability study. Permeability of 0.5 mg/mL of the different NP formulations measured by a transwell technique using natural gastrointestinal mucus from pigs. Results presented as percentage of permeability during 6 h and during the first hour. Results expressed as the mean of three experimental replicates.

GN-MA-NP showed the highest percentage of permeability (21.7%) followed by GN-PEG10-NP (9.6%), GN-PEG2-NP (5.7%), GN-PEG6-NP (5%) and GN-HPBC-NP (3.52%). The bare NP (GN-NP) showed a percentage of permeability of 1.5%. GN-DEX-NP showed the same percentage of permeability as the bare NP while GN-ADEX-NP showed the lowest percentage of permeability (0.1%).

Data obtained from the 3 technical replicates was very similar in all cases (data not shown).

4. Discussion

Among the various drug-delivery routes, oral administration is the most comfortable and widely used as it offers numerous advantages such as being painless and easily self-administrable. The absorption of orally-delivered drugs occurs mainly along the gastrointestinal tract. However, the mucus barrier can prevent drug penetration and thus reduce absorption. In order to overcome these limitations, various formulations of NPs are being developed. In this context, one of the most important properties of poly(anhydride) NPs is their ability to develop strong bioadhesive interactions with components of the gut mucosa. In addition, their surface can be easily modified by simple incubation with different ligands in order to modify their *in vivo* distribution and even to increase their affinity for the intestinal mucosa (Arbós *et al*., 2002; Salman *et al*., 2005, 2006, 2008; Salman, Irache & Gamazo, 2009; Yoncheva *et al*., 2005). Nevertheless, modifications of their surface can also alter the biological properties of the NPs and their toxicity. This last aspect is a matter of concern in nanomedicine, limiting the potential use of many of the developed drug carrier NPs.

In this work the cytotoxicity, *in vitro* genotoxicity and mucus permeation capacity of different surface modifications of NPs obtained from the copolymer between methyl vinyl ether and maleic anhydride (Gantrez® AN 119) are studied. The different compounds used to modify the Gantrez® AN 119 NPs (GN-NP) are: DEX, ADEX, HPBCD, MA, PEG2, PEG6 and PEG10.

Tested NPs presented a homogeneous size around 200 nm, a narrow size distribution (PDI < 0.25) and a negative surface charge. Chemical properties of NPs can change drastically in

contact with different solutions and so influence their physico-chemical characteristics and their potential toxicity (Handy, Owen & Valsami-Jones, 2008). According to our results, NPs were stable in culture medium for at least 24 h and so they maintain their properties during the cytotoxicity and *in vitro* genotoxicity studies. Moreover, NPs did not change the neutral pH of the cell culture medium. Red lumogen® labelled NPs showed the same characteristics as non-labelled ones regarding size and polydispersion. Red Lumogen® is encapsulated in the inner part of the NPs so their physico-chemical properties as well as their stability should not change in comparison with the non-labelled ones. Red Lumogen® labelled NPs were used in the mucus permeability studies.

The first step to evaluate the safety of NPs is to elucidate their cytotoxicity. A crucial point in obtaining valuable results that complement *in vivo* studies is to choose an appropriate cell line. In this case Caco-2 and HT29-MTX were chosen; Caco-2 cells are derived from a human colon carcinoma, and HT29-MTX is a mucus secreting cell line composed of endocrine cells and M cells, with the mucus-secreting goblet cells representing the second most frequent cell type (Huet *et al*., 1987; Lesuffleur *et al*., 1990). HT29-MTX cells have been used to confirm the response of Caco-2 cells.

Another crucial point is the interference of NPs with the assays (Lu, Qi & Wu, 2012). In our case, the kit for the proteases activity detection can be applied in the cell culture medium after treatment (method 1), or in lysed cells after treatment (method 2), using sequential luminescent measures. Very high luminescent values were obtained in treated cells compared with cells treated with a detergent (0.2 mg/mL Digitonin, positive control) when using method 1 (data not shown). This indicated an interference in the measurement due to the light scattering produced by the NPs present in the cell culture medium or adhering to the cells. In contrast, the luminescent signal collected after performing the method 2, where NPs are removed before lysing the cells, indicated no dispersion of the measured luminescence.

Tested NPs did not affect the metabolism of Caco-2 and HT29-MTX cells after 24 h of treatment even at very high concentrations. Neither did they affect the cell membrane integrity of Caco-2

cells, although they are bioadhesive and some of them (GN-NP, GN-HPBCD-NP and GN-PEG6-NP) have been demonstrated to remain attached to the cell membrane (Ojer *et al*., 2013). Moreover, GN-NP, GN-HPBCD-NP and GN-PEG6-NP did not affect mitochondrial function, measured by the MTS assay, or membrane integrity, measured by the LDH assay, of HepG2 and Caco-2 cells after 24 h of treatment (Ojer *et al*., 2013).

The effect of NPs on cell viability was measured using the proliferation assay in Caco-2 cells treated for 3 h. These results, though having a great value by themselves, are crucial to the correct interpretation of the genotoxicity assay outcome (since concentrations that induce cell death can lead to false positive results). NPs did not induce any alteration in the proliferation of Caco-2 cells in any of the conditions tested so they did not affect the viability of the cells.

Genotoxicity is an important aspect in evaluating the safety of chemicals as well as NPs and, as far as we know, this is the first paper studying the genotoxicity of polymer NPs produced using the copolymer methyl vinyl ether and maleic anhydride (Gantrez® AN 119). The alkaline comet assay is a simple and widely used method for detection of DNA damage in cells (Azqueta & Collins, 2013). This method detects SBs and specific DNA lesions, such as oxidized purines and pyrimidines, when combined with lesion specific enzymes. The alkaline comet assay is considered a very useful method for *in vitro* and *in vivo* genotoxicity testing and, so far, is the most used method in nanogenotoxicology due to its robustness, versatility and reliability (Azqueta & Dusinska, 2015). The alkaline comet assay in combination with FPG was used to detect not only SBs but oxidized bases or alkylated bases. Since NPs did not affect the viability of the Caco-2 cells in any of the conditions tested, comet assay results are relevant. A concentration of 2 mg/mL GN-DEX-NP induced a statistically significant increase in SBs after 3 h of incubation. Nevertheless the level of SBs is so low (1.82 ± 1.19% tail DNA) that it may not represent any biological meaning. It is worth mentioning that the concentrations of NPs used in this work were very high; the lack of cytotoxicity and the viability of the cells at very high concentrations, determined the concentrations used in the comet assay.

Commonly, synthetic mucins provide the basis for transport and diffusion capability of low molecular weight drugs (Gniewek & Kolinski, 2012). However, they do not represent a realistic model of how NPs will interact with the intestinal epithelium. Hence, the transwell technique using a natural gastrointestinal mucus from freshly slaughtered pigs was used to evaluate the diffusion of poly(anhydride) NPs. Moreover, this novel test bears the advantage of a single membrane system, which is much closer to the *in vivo* situation in comparison with other destructive assays (i.e. models in which sectioning of the mucus filled devices is needed for the detection of the NPs) (Friedl *et al.*, 2013).

Diffusion studies showed a concentration and time-dependent behavior of the formulations tested. The diffusion of NPs increases with time. Four concentrations of NPs were tested, 0.25, 0.5, 1 and 2 mg/mL. From 0.5 to 2 mg/mL the diffusion decreases as the concentration increase. This reveals a saturation of the assay probably due to the obstruction of the membrane pores. Results obtained at 0.5 mg/mL were taken into account to elucidate the NPs mucus permeability potential.

NPs coated with different ligands showed higher mucus permeability potential than the naked ones (GN-NP), with the exception of GN-DEX-NP, which showed the same, and GN-ADEX-NP, which shower a lower potential (although the permeability of these 3 NPs was very similar). This was an expected result since the hydrophilic character of the coated NPs decreases the interactions with mucus and makes the NPs more capable of sliding. The low amount of ADEX used to produce the GN-ADEX-NP can explain the lack of mucus permeability potential of this NP.

GN-MA-NP showed the highest mucus permeability capacity followed by the pegylated ones. The mucus permeability capacity of GN-MA-NP has not been studied until now; however, pegylation is a widely used strategy to enhance the mucus permeability of NPs. Our results showed that pegylation increased the mucus permeation capacity of the studied NPs. Moreover, the mucus permeability potential of PEG NPs depends on the chain length; GN-PEG10-NP showed the highest mucus permeability potential followed by GN-PEG2-NP and GN-PEG6-NP,

with very similar values. Similar results were shown by Li *et al.* (2015) using in situ single-pass intestinal infusion in rats. In this case nanomicelles pegylated with PEG1 showed higher mucus permeability than nanomicelles-PEG0.5. Nevertheless, nanomicelles-PEG2 and -PEG5 showed less mucus permeability than nanomicelles-PEG1. The authors conclude that the PEG chain cannot exceed a key threshold in order to improve the mucus permeation. On the contrary, Inchaurraga *et al.* (2015) showed that NPs produced using the copolymer methyl vinyl ether and maleic anhydride (Gantrez® AN 119) and PEG2 and PEG6 as ligands (similar to GN-PEG2-NP and GN-PEG6-NP) showed similar mucus permeating capacity but higher than the ones coated with PEG10 (similar to GN-PEG10-NP) in an *in vivo* study. The study clearly demonstrated that in rats, the capability of Gantrez® AN 119 NPs to reach the intestinal epithelium was significantly higher when these nanocarriers were pegylated with PEG2 and PEG6 than with PEG10. From all these studies it could be concluded that the PEG chain length has an effect on the results of permeability tests, but the experimental model (*in vitro versus in vivo*; human versus rat) may also have an influence.

The genotoxicity of GN-MA-NP, as showing the highest mucus permeability capacity, was also studied after 24 h of treatment. GN-NP was also checked as a control. Although both NPs did not affect the viability of Caco-2 cells after 24 h of incubation, they induce a slight but concentration dependent increase of FPG-sensitive sites. This increase was only significant in cells treated with 2 mg/mL of GN-NP and in all cases the % of DNA obtained was very low (below 7%). The lowest concentration tested (0.5 mg/mL) did not induce any effect. Taking into account the nature of the NPs tested, the FPG-sensitive sites are due to the presence of oxidized bases in the DNA. Moreover, since NPs are not internalized into the cells but remain attached to the cell membrane (Ojer *et al*., 2013), the most probable hypothesis is that DNA is oxidized through oxidative stress rather than a direct interaction with the DNA. The fact that the naked NPs induce oxidized bases imply that probably all NPs containing the copolymer methyl vinyl ether and maleic anhydride (Gantrez® AN 119) may induce oxidized bases at high concentration. In any case, the biological meaning may be insignificant since the level of DNA damage was very low and the concentration inducing such a small amount of damage is very high.

It is worth stating that possible adverse effects of empty NPs have to be balanced with other factors such as their drug loading capacity or the drug toxicity. For example, a NP with a small loading capacity would need a higher amount than a NP with a large loading capacity. Therefore the dose to be used may be different and also the toxicity.

In conclusion, empty GN-NP and GN-NP modified using DEX, ADEX, HPBCD, MA and different PEG as ligands did not affect cell metabolism, membrane integrity or viability of Caco-2 cells at the different conditions tested. Moreover, they did not induce relevant genotoxic lesions (i.e. DNA strand breaks and FPG-sensitive sites) after 3 h of incubation. Oxidized DNA bases induced by GN-NP after 24 h of treatment may not have any biological relevance due to the low level of the damage and the high concentration required. The use of MA drastically increases the permeable capability through a mucus layer.

5. Acknowledgments

This work was supported by the ALEXANDER project (FP7-2011-NMP-280761). TI was also financially supported by a grant from the ALEXANDER project. AA thanks the Ministerio de Economía y Competitividad ('Ramón y Cajal' programme, RYC-2013-14370) of the Spanish Government for personal support We thank Professor Andrew R. Collins (University of Oslo) for the gift of the enzyme FPG and Hoffmann-La Roche (Basel, Switzerland) for providing the photosensitiser (Ro 19-8022).

6. References

Agüeros, M., Campanero, M.A & lrache, J.M., 2005. Simultaneous quantification of different cyclodextrins and Gantrez by HPLC with evaporative light scattering detection. Journal of Pharmaceutical and Biomedical Analysis. 39 (3-4), 495-502.

doi: http://dx.doi.org/10.1016/j.jpba.2005.04.034

Agüeros, M., Areses, P., Campanero, M.A., Salman, H., Quincoces, G., Peñuelas, I., Irache, J.M., 2009. Bioadhesive properties and biodistribution of cyclodextrin-poly(anhydride) nanoparticles. European Journal of Pharmaceutical Sciences. 37 (3-4), 231-240.

doi: http://dx.doi.org/10.1016/j.ejps.2009.02.010

Agüeros, M., Zabaleta, V., Espuelas, S., Campanero, M.A., Irache, J.M., 2010. Increased oral bioavailability of paclitaxel by its encapsulation through complex formation with cyclodextrins in poly(anhydride) nanoparticles. Journal of Controlled Release. 145 (1), 2-8.

doi: http://dx.doi.org/10.1016/j.jconrel.2010.03.012

Ahmad, Z., Pandey, R., Sharma, S., Khuller, G.K., 2008. Novel chemotherapy for tuberculosis: chemotherapeutic potential of econazole- and moxifloxacin-loaded PLG nanoparticles. International Journal of Antimicrobial Agents. 31 (2),142-146.

doi:http://dx.doi.org/10.1016/j.ijantimicag.2007.10.017

Ahmad, S.I., Yokoi, M & Hanaoka, F., 2012. Identification of new scavengers for hydroxyl radicals and superoxide dismutase by utilising ultraviolet A photoreaction of 8- methoxypsoralen and a variety of mutants of Escherichia coli: Implications on certain diseases of DNA repair deficiency. Journal of Photochemistry and Photobiology B: Biology. 116, 30-36.

doi: http://dx.doi.org/10.1016/j.jphotobiol.2012.07.004

Arbós, P., Wirth, M., Arangoa, M.A., Gabor, F., Irache, J.M., 2002. Gantrez® AN as a new polymer for the preparation of ligand–nanoparticle conjugates. Journal of Controlled Release. 83 (3), 321-330.

doi: http://dx.doi.org/10.1016/S0168-3659(02)00015-9

Arbós, P., Campanero, M.A., Arangoa, M.A., Irache, J.M., 2004. Nanoparticles with specific bioadhesive properties to circumvent the pre-systemic degradation of fluorinated pyrimidines. Journal of Controlled Release. 96 (1), 55-65.

doi: http://dx.doi.org/10.1016/j.jconrel.2004.01.006

Azqueta, A & Collins, A.R., 2013. The essential comet assay: a comprehensive guide to measuring DNA damage and repair. Archives of Toxicoly. 87 (6), 949-968.

doi: 10.1007/s00204-013-1070-0

Azqueta, A & Dusinska, M., 2015. The use of the comet assay for the evaluation of the genotoxicity of nanomaterials. Frontiers in Genetic. 6, 239-243.

doi: 10.3389/fgene.2015.00239

Beloqui, A., Solinís, M.Á, Rieux, A.d., Préat, V., Rodríguez-Gascón, A., 2014. Dextran-protamine coated nanostructured lipid carriers as mucus-penetrating nanoparticles for lipophilic drugs. International Journal of Pharmaceutics. 468 (1-2), 105-111.

doi: http://dx.doi.org/10.1016/j.ijpharm.2014.04.027

Bernkop-Schnürch, A., 2013. Nanocarrier systems for oral drug delivery: Do we really need them?. European Journal of Pharmaceutical Sciences. 49 (2), 272-277.

doi: http://dx.doi.org/10.1016/j.ejps.2013.03.008

Brooks, D., Okeefe, P & Buncke, H.J., 2001. Dextran induced acute renal failure after microvascular muscle transplantion. Plastic and Reconstructive Surgery. 108 (7), 2057-2060.

Carrillo-Conde, B., Song, E.H., Chavez-Santoscoy, A., Phanse, Y., Ramer-Tait, A.E., Pohl, N.L., Wannemuehler, M.J., Bellaire, B.H., Narasimhan, B., 2011. Mannose-functionalized pathogen-like polyanhydride nanoparticles target c-type lectin receptors on dendritic cells. Molecular Pharmaceutics. 8 (5), 1877-1886.

doi: 10.1021/mp200213r

Collins, A.R & Azqueta, A., 2012. Single-cell gel electrophoresis combined with lesion-specific enzymes to measure oxidative damage to DNA. Methods in Cell Biology. 112, 69-92.

De Jong, W.H & Borm, P.J., 2008. Drug delivery and nanoparticles: Applications and hazards. International Journal of Nanomedicine. 3 (2), 133-149.

Easo, S.L & Mohanan, P.V., 2013. Dextran stabilized iron oxide nanoparticles: Synthesis, characterization and *in vitro* studies. Carbohydrate Polymers. 92 (1), 726-732.

doi: http://dx.doi.org/10.1016/j.carbpol.2012.09.098

Ensign, L.M., Cone, R & Hanes, J., 2012. Oral drug delivery with polymeric nanoparticles: The gastrointestinal mucus barriers. Advanced Drug Delivery Reviews. 64 (6), 557-570.

doi: http://dx.doi.org/10.1016/j.addr.2011.12.009

Friedl, H., Dünnhaupt, S., Hintzen, F., Waldner, C., Parikh, S., Pearson, J.P., Wilcox, M.D., Bernkop-Schnürch, A., 2013. Development and evaluation of a novel mucus diffusion test system approved by self-nanoemulsifying drug delivery systems. Journal of Pharmaceutical Science. 102 (12), 4406-4419.

doi: http://dx.doi.org/10.1002/jps.23757

Gniewek, P & Kolinski, A., 2012. Coarse-grained modeling of mucus barrier properties. Biophysical Journal. 102 (2), 195-200.

doi: http://dx.doi.org/10.1016/j.bpj.2011.11.4010

Handy, R.D., Owen, R & Valsami-Jones, E., 2008. The ecotoxicology of nanoparticles and nanomaterials: current status, knowledge gaps, challenges, and future needs. Ecotoxicology. 17 (5), 315-325.

doi: 10.1007/s10646-008-0206-0

Huet, C., Sahuquillo-Merino, C., Coudrier,E., Louvard, D., 1987. Absorptive and mucus-secreting subclones isolated from a multipotent intestinal cell line (HT-29) provide new models for cell polarity and terminal differentiation. Journal of Cell Biology. 105 (1), 345-402.

Hunter, A.C., Elsom, J., Wibroe, P.P., Moghimi, S.M., 2012. Polymeric particulate technologies for oral drug delivery and targeting: a pathophysiological perspective. Nanomedicine: Nanotechnology, Biology and Medicine. 8 (1), 5-20.

doi:http://dx.doi.org/10.1016/j.nano.2012.07.005

Inchaurraga, L., Martín-Arbella, N., Zabaleta, V., Quincoces, G., Peñuelas, I., Irache, J.M., 2015. *In vivo* study of the mucus-permeating properties of PEG-coated nanoparticles following oral administration. European Journal of Pharmaceutics and Biopharmaceutics. 97 (Pt A), 280-289.

doi: http://dx.doi.org/10.1016/j.ejpb.2014.12.021

Irache, J., Huici, M., Konecny, M., Espuelas, S., Campanero, M., Arbós, P., 2005. Bioadhesive properties of Gantrez nanoparticles. Molecules. 10 (1) ,126-145.

doi: 10.3390/10010126

Jain, A.K., Swarnakar, N.K., Godugu, C., Singh, R.P., Jain, S., 2011.The effect of the oral administration of polymeric nanoparticles on the efficacy and toxicity of tamoxifen. Biomaterials 32 (2), 503-515.

doi: http://2091/10.1016/j.biomaterials.2010.09.037

Jaiswal, S., Bhattacharya, K., McHale, P., Duffy, B., 2015. Dual effects of β-cyclodextrin-stabilised silver nanoparticles: enhanced biofilm inhibition and reduced cytotoxicity. Journal of Materials Science: Materials in Medicine. 26 (1), 5367-5377.

doi: 10.1007/s10856-014-5367-1

Kumar, M., Trivedi, N., Reddy, C.R.K., Jha, B., 2011. Toxic effects of imidazolium ionic liquids on the green seaweed ulva lactuca: Oxidative stress and DNA damage. Chemical Research in Toxicology. 24 (11), 1882-1890.

doi:10.1021/tx200228c

Lai, S.K., Wang, Y & Hanes, J., 2009. Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. Advanced Drug Delivery Reviews. 61 (2), 158-171.

doi: http://dx.doi.org/10.1016/j.addr.2008.11.002

Landsiedel, R., Fabian, E., Ma-Hock, L., Wohlleben, W., Wiench, K., Oesch, F., Van Ravenzwaay, B., 2012. Toxico/biokinetics of nanomaterials. Archives of Toxicology. 86 (7), 1021-1060.

doi: 10.1007/s00204-012-0858-7

Lesuffleur, T., Barbat, A., Dussaulx, E., Zweibaum. A., 1990. Growth adaptation to methotrexate of HT-29 human colon carcinoma cells is associated with their ability to differentiate into columnar absorptive and mucus-secreting cells. Cancer Research. 50 (19), 6334-6377.

Li, Z., Han, X., Zhai, Y., Lian, H., Zhang, D., Zhang, W., Wang, Y., He, Z., Liu, Z., Sun, J., 2015. Critical determinant of intestinal permeability and oral bioavailability of pegylated all trans-retinoic acid prodrug-based nanomicelles: Chain length of poly-ethylene glycol corona. Colloids Surf B: Biointerfaces. 130,133-140.

doi: http://dx.doi.org/10.1016/j.colsurfb.2015.03.036

Liu, H., Liu, T., Wang, H., Li, L., Tan, L., Fu, C., Nie, G., Chen, D., Tang, F., 2013. Impact of PEGylation on the biological effects and light heat conversion of gold nanoshells on silicananorattles. Biomaterials. 34 (28), 6967-6975.

doi: 10.1016/j.biomaterials.2013.05.059

Lu, Y., Qi, J & Wu, W., 2012. Absorption disposition and pharmacokinetics of nanoemulsions. Current Drug Metabolism. 13 (4), 396-417.

Mittal, G., Sahana, D.K., Bhardwaj, V., Ravi Kumar, M.N., 2007. Estradiol loaded PLGA nanoparticles for oral administration: Effect of polymer molecular weight and copolymer composition on release behaviour *in vitro* and *in vivo*. Journal of Controlled Release. 119 (1), 77-85.

doi: http://dx.doi.org/10.1016/j.jconrel.2007.01.016

Nagai, N., Ono, H., Hashino, M., Ito, Y., Okamoto, N., Shimomura, Y., 2014. Improved corneal toxicity and permeability of tranilast by the preparation of ophthalmic formulations containing its nanoparticles. Journal of Oleo Science. 63 (2), 177-186.

doi: http://doi.org/10.5650/jos.ess13082

Nel, A., Xia, T., Mädler, L., Li, N., 2006. Toxic potential of materials at the nanolevel. Science. 311 (5761), 622-627.

doi: 10.1126/science.1114397

Ojer, P., Neutsch, L., Gabor, F., Irache, J., López de Cerain, A., 2013. Cytotoxicity and cell interaction studies of bioadhesive poly(anhydride) nanoparticles for oral antigen/drug delivery. Journal of Biomedical Nanotechnology. 9 (11), 1891-1903.

doi: http://dx.doi.org/10.1166/jbn.2013.1695

Porfire, A.S., Zabaleta, V., Gamazo, C., Leucuta, S.E., Irache, J.M., 2010. Influence of dextran on the bioadhesive properties of poly(anhydride) nanoparticles. International Journal of Pharmaceutics. 390 (1), 37-44.

doi: http://dx.doi.org/10.1016/j.ijpharm.2009.08.017

Salman, H.H., Gamazo, C., Campanero, M.A., Irache, J.M., 2005. Salmonella-like bioadhesive nanoparticles. Journal of Controlled Release. 106 (1-2), 1-13.

doi: http://dx.doi.org/10.1016/j.jconrel.2005.03.033

Salman, H.H., Gamazo, C., Campanero, M., Irache, J.M., 2006. Bioadhesive mannosylated nanoparticles for oral drug delivery. Journal of Nanoscience and Nanotechnology. 6 (9-10), 3203-3209.

Salman, H.H., Gamazo, C., Smidt, P.C., Russell-Jones, G., Irache, J.M., 2008. Evaluation of bioadhesive capacity and immunoadjuvant properties of vitamin B (12)-Gantrez nanoparticles. Pharmaceutical Research. 25, 2859-2868.

doi: 10.1007/s11095-008-9657-5

Salman, H.H., Irache, J.M & Gamazo, C., 2009. Immunoadjuvant capacity of flagellin and mannosamine-coated poly(anhydride) nanoparticles in oral vaccination. Vaccinne. 27 (35), 4784-4790.

doi: 10.1016/j.vaccine.2009.05.091

Stella, V.J & He, Q., 2008. Cyclodextrins. Toxicologic pathology 36, 30-42.

doi: 10.1177/0192623307310945.

Wu, J., Shen, Q & Fang, L., 2013. Sulfobutylether-β-cyclodextrin/chitosan nanoparticles enhance the oral permeability and bioavailability of docetaxel. Drug Development and Industrial Pharmacy. 39 (7), 1010-1019.

doi: http://dx.doi.org/10.3109/03639045.2012.694588

Yoncheva, K., Gómez, S., Campanero, M.A., Gamazo, C., Irache, J.M., 2005. Bioadhesive properties of pegylated nanoparticles. Expert Opinion on Drug Delivery. 2 (2), 205-218.

doi: http://dx.doi.org/10.1517/17425247.2.2.205

Yoncheva, K., Doytchinova, I & Irache, J.M., 2010. Different approaches for determination of the attachment degree of polyethylene glycols to poly(anhydride) nanoparticles. Drug Development and Industrial Pharmacy. 36 (6), 676-680.

doi: http://dx.doi.org/10.3109/03639040903443434

Zabaleta, V., Ponchel, G., Salman, H., Agüeros, M., Vauthier, C., Irache, J.M., 2012. Oral administration of paclitaxel with pegylated poly(anhydride) nanoparticles: Permeability and pharmacokinetic study. European Journal of Pharmaceutics and Biopharmaceutics. 81 (3), 514-523.

doi: http://dx.doi.org/10.1016/j.ejpb.2012.04.001

Zaki, N.M & Hafaez, M.M., 2012. Enhanced antibacterial of ceftriaxone sodium- loades chitosan nanoparticles against intracellular Salmonella typhimurium. AAPS PharmSciTech. 13 (2), 411-421.

doi: 10.1208/s12249-012-9758-7

Chapter 5: *In vitro* evaluation of the genotoxicity of poly(anhydride) nanoparticles designed for oral drug delivery

In vitro evaluation of the genotoxicity of poly(anhydride) nanoparticles designed for oral drug delivery.

Iglesias T[1], Azqueta A[1,2], Dusinska M[3], El Yamani N[3], Irache JM[4], López de Cerain A[1,2*]

[1]Department of Pharmacology and Toxicology. Faculty of Pharmacy and Nutrition, University of Navarra, Pamplona, Spain.

[2]IdiSNA, Navarra Institute for Health Research

[3]Health Effects Laboratory, Department of Environmental Chemistry, Norwegian Institute for Air Research, Kjeller, Norway.

[4]Pharmacy and Pharmaceutical Technology Department. Faculty of Pharmacy and Nutrition. University of Navarra. Irunlarrea 1, 31008, Pamplona, Spain.

Abstract

In the last years, the development of nanomaterials has significantly increased due to the immense variety of potential applications in technological sectors, such as medicine, pharmacy and food safety. Focusing on the nanodevices for oral drug delivery, poly(anhydride) nanoparticles have received extensive attention due to their unique properties, such us their capability to develop intense adhesive interactions within the gut mucosa, their modifiable surface and their biodegradable and easy-to-produce profile. However, current knowledge of the possible adverse health effects as well as, toxicological information, is still exceedingly limited.

Thus, we investigated the capacity of two poly(anhydride) NPs, Gantrez® AN 119-NP (GN-NP) and Gantrez® AN 119 covered with mannosamine (GN-MA-NP), and their main bulk material (Gantrez® AN 119-Polymer), to induce DNA damage and thymidine kinase ($TK^{+/-}$) mutations in L5178Y $TK^{+/-}$ mouse lymphoma cells after 24 h of exposure.

The results showed that GN-NP, GN-MA-NP and their polymer did not induce DNA strand breaks or oxidative damage at concentrations ranging from 7.4 to 600 µg/mL. Besides, the mutagenic potential of these NPs and their polymer revealed no significant or biologically

relevant gene mutation induction at concentrations up to 600 µg/mL under our experimental settings.

Considering the non-genotoxic effects of GN-NP and GN-MA-NP, as well as their exceptional properties, these NPs are promising nanocarriers for oral medical administrations.

1. Introduction

Nanomaterials (NMs) are an increasingly important nanotechnological product. Several properties of NMs make them particularly attractive for several technological and pharmaceutical industries applications such as, small size, standardized shape among each type, large surface area and surface reactivity. However, these features can also contribute to their toxicological profile by diverse mechanisms. Oxidative stress, inflammation, immunotoxicity and genotoxicity are the main mechanisms of nanoparticle (NPs) toxicity (Dusinska *et al*., 2015).

Small NPs are able to reach the nucleus and directly interact with the DNA causing genetic damage (Magdolenova *et al*., 2014). However, NPs do not need to be in direct contact with the DNA to induce genotoxic effects. NPs can negatively interact with cellular proteins, as well as with proteins involved in DNA replication, transcription or repair, cell division or mitotic spindle formation and generate high amounts of reactive oxygen species (ROS) inside the cells, which may cause indirect DNA damage (Magdolenova *et al*., 2014). Moreover, it has been shown that some NPs are deposited on the cellular surface, or inside the cell, and induce oxidative stress signaling cascades (Manke *et al*., 2013). Nowadays, it is known that oxidative stress is a crucial factor in NP toxicity (Ahmad *et al*., 2012; Kumar *et al*., 2011; Nel *et al*., 2006). Moreover, increased DNA damage has been associated with higher frequency of cancer (Hoeijmakers, 2009) and other health issues, including infertility and genetic disorders (Aitken & Krausz, 2001). Therefore, evaluation of the genotoxic potential of NPs should be exhaustive.

Poly(anhydride) NPs have been considered promising carriers for oral drug delivery (Agüeros *et al*., 2011; Calleja *et al*., 2015; Zhang *et al*., 2015). These NPs have received widespread attention due to their singular properties, such us their modifiable surface, which can enhance or reduce bioadhesion to specific target cells (Ensign *et al*., 2012). Furthermore, poly(anhydride) NPs are biocompatible, biodegradable, and capable of releasing drugs in a sustained way (Calleja *et al*., 2015). The copolymers between methyl vinyl ether and maleic anhydride (commercialized as Gantrez® AN 119) are an excellent example of this group of poly(anhydride) NPs (Arbós *et al*., 2002). Their surface can be modified with different ligands in order to modify their physico-chemical properties to improve *in vivo* distribution (Agüeros *et al*.,

2009; Inchaurraga *et al*., 2015; Salman *et al*., 2006). For example, when Gantrez® AN 119 NPs are coated with mannosamine, their already strong bioadhesive interactions with the intestinal mucosa are enhanced (Salman *et al*., 2005; 2009). It has also been shown that NPs of Gantrez® AN 119 coated with mannosamine presented the highest ability to diffuse through a mucus layer, when compared to Gantrez® AN 119 NPs coated with other ligands (i.e., dextran, aminodextran, cyclodextrin or poly-ethylene glicol) (Chapter 4). This property is especially advantageous in nanocarriers designed for oral drug delivery, since the residence time of the drug in the organism, as well as, its availability will be greater.

It has also been demonstrated that Gantrez® AN 119 based NPs, when orally administered, remain localized in the lumen of the gastrointestinal tract, indicating that these NPs are not absorbed or translocated (Agüeros *et al*., 2009; Arbós *et al*., 2002; Inchaurraga *et al*., 2015; Porfire *et al*., 2010). Furthermore, previous studies showed that Gantrez® AN 119 nanoforms are capable of establishing adhesive interactions with Caco-2 cells without being internalized (Ojer *et al*., 2013). However, Salman *et al.* (2006) observed that this nanoform in combination with mannosamine was uptaken by Peyer´s patches, probably due to the presence of mannose receptor in this tissue

Commercial bulk Gantrez® AN 119, as well as, bulk mannosamine have been recognized as safe for human health (Moreno *et al*., 2014). Nevertheless, the safety of Gantrez® AN 119 based-NPs and their different ligands have not been thoroughly studied, although some studies showed no effect on viability, cell metabolism, membrane integrity or DNA in Caco-2 cells after 24 h exposure at high concentration (Chapter 4). In general, the toxicity of Gantrez® AN 119 nanoforms is considered low or even innocuous to the organism since these NPs are biodegradable and biocompatible (Landsiedel *et al*., 2012). However, their safety has not been thoroughly studied.

Nowadays, detection of chromosome or DNA damage represents an important tool for prioritizing compounds early in the drug development process since DNA alterations are clearly related to cancer development (Hoeijmakers, 2009). The comet assay is the most commonly

used method in nanogenotoxicity studies (Azqueta & Dusinska, 2015). It is a simple method for measuring DNA damage, such as single strand breaks and double strand breaks, and alkali-labile sites (purinic and apyrimidinic) (Azqueta & Collins, 2011). The assay has been modified to detect oxidized bases, by incorporating lesion specific enzymes (Dusinska & Collins, 1996). The use of these repair enzymes increases the sensitivity and specificity of the assay; recognizing specific base damages and creating additional DNA breaks which increases the amount of DNA that migrates from the nucleoids (Azqueta *et al*., 2013).

The use of mammalian genotoxicity tests as, the mouse lymphoma test (MLA) and the Ames test, were recommended by the OECD Working Party on Manufactured Nanomaterials in 2009 (OECD 476, 1997). The Ames test is not suitable for testing NPs due to the limited or no uptake through the bacterial wall (Azqueta & Dusinska, 2015). However, MLA could be a useful tool for genotoxicity assessment in NPs since it is performed on eukaryotic cells. MLA uses the endogenous thymidine kinase (TK) locus transcription to detect a wide spectrum of genetic damage, including both, point mutations and chromosomal alterations. This assay has been validated as a component of the genotoxic testing battery used for evaluating the mutagenicity potential of chemicals (ICH, 2011), and the Organisation for Economic Co-operation and Development (OECD) has recently updated the guideline for this assay (OECD 490, 2015). It has already been used for the assessment of mutagenicity of NMs in some studies (Gábelová *et al*., 2016).

Therefore, the aim of the present study was to explore the *in vitro* genotoxicity activity associated with the exposure of two poly(anhydride) NPs, Gantrez® AN 119 (GN-NP) and Gantrez® AN 119 covered with mannosamine (GN-MA-NP), after 24 h treatment using the alkaline comet assay and the MLA in L5178Y TK $^{+/-}$ cells. Furthermore, Gantrez® AN 119-polymer (GN-Polymer) was tested as an additional control to distinguish the possible genotoxic potential of the NPs from their bulk material form. Moreover, viability of the cells treated with NPs was evaluated using the proliferation assay.

2. Material and methods

2.1. Chemicals and reagents

NPs preparation: poly methyl vinyl ether-co-maleic anhydride or poly(anhydride) (Gantrez® AN 119; MW: 200000 g/mol) was provided by ISP (Spain). Mannosamine was purchased from Sigma (Spain). Acetone was obtained from VWR Prolabo (France). Deionized water (18.2 Ω resistivity) was obtained by a water purification system by Wasserlab (Spain). Nitrogen gas (ultra-pure, > 99 %) was produced using an Alltech Nitrogen generator by Ingeniería Analítica (Spain).

Comet and mouse lymphoma assays: Fischer's medium, glutamine, sodium pyruvate, penicillin, streptomycin, phosphate buffer saline and heat-inactivated horse serum (HS) were purchased from Invitrogen (Spain). Hypoxantine, glycine, methotrexate, sodium carbonate anhydrous, tymidine and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltatrazolium bromide (MTT), methyl methanesulfonate (MMS) , and 5-trifluorothymidine (TFT) were obtained from Sigma-Aldrich (Spain).

In addition, only for the comet assay low-melting point agarose, standard agarose, Triton X-100, Tris, HEPES, EDTA and BSA were provided by Sigma. NaCl, NaOH and KCl were purchased from Panreac. Photosensitiser Ro 19-8022 kindly supplied by Hoffmann-La Roche (Switzerland). Formamidopypiridine DNA-glycosylase (FPG) was kindly provided by Professor Andrew Collins (Department of Nutrition, University of Oslo, Norway).

2.2. Preparation and characterization

2.2.1. Conventional poly(anhydride) nanoparticles (GN-NP)

The setup of this formulation was carried out as previously reported with slight modifications (Irache *et al*., 2005; Ojer *et al*., 2012, 2013).

Briefly, 600 mg of the copolymer (Gantrez® AN 119) were dissolved in 60 mL acetone and desolvated by the addition of a hydroalcoholic mixture under magnetic stirring at room

temperature. The NPs were purified and spray-dried. The recovered powder was stored at room temperature.

2.2.2. Poly(anhydride) nanoparticles coated with mannosamine (GN-MA-NP)

The preparation of these NPs was carried out as previously reported with slight modifications (Arbós *et al.*, 2004; Salman *et al.*, 2009).

Briefly, 10 mg of mannosamine dissolved in 5 mL of water was incubated in 5 mL of acetone containing 100 mg of Gantrez® AN 119 for 30 min under magnetic stirring. NPs were obtained by the addition of 10 mL of absolute ethanol under magnetic stirring. The organic solvents were evaporated under reduced pressure. Finally, the NPs were purified and spray-dried. The recovered powder was stored at room temperature.

2.3. Characterization of NPs

Particle size and polydispersity index (PDI) of NPs were determined by photon correlation spectroscopy using the zeta potential analyzer ZetaPlus with 90 Plus/BI-MAS multi angle particle sizing option (Brookhaven Instruments Corporation, Holtsville, NY). NP diameter was determined after dispersion in ultra-purified water (1/10) and measured at room temperature using a 90° scattering angle.

NPs were diluted in purified water, centrifuged to remove sugars, and shaded with a 12 nm Gold layer (Hemitech K 550 Sputter-Coater) before being observed through an electron microscope (Zeiss DSM 940A, Oberkochen, Germany) which was coupled with a digital image system (DISS, Point Electronic GmBh).

2.4. Genotoxicity and mutagenicity studies

2.4.1. Cell lines and cell culture

Mouse lymphoma L5178Y TK $^{+/-}$ clone 3.7.2C (ML) cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC) and cleansed from TK $^{-/-}$ mutants (Sawyer *et al.*, 1985). This cell line is derived from a thymic tumour induced in a DBA/2 mouse by

methylcholanthrene treatment. ML cells have several properties which are advantageous for genotoxicity and mutagenicity studies, such as being heterozygous at the normally diploid thymidine kinase (TK) locus, presenting stable spontaneous mutation frequency, and being capable to grow in suspension culture, which is similar to the way cells circulate in the human body.ML cells were grown in Fischer's medium supplemented with 2 mM glutamine, 1% sodium pyruvate, 100 U/mL penicillin,100 µg/mL streptomycin and 10% heat-inactivated HS. Cells were maintained in a humidified atmosphere with 5 % CO2 at 37°C.

Cell culture medium was supplemented with 5% HS while exposing cells to NPs. However, cell culture medium was supplemented with 20% HS for viability and mutant frequency assessment and for maintaining the culture during the expression period in the MLA (see below).

The empiric average doubling time of ML cells in culture was 10-12 h. Cultures were maintained at an average density of 1×10^4 - 3×10^5 cells/mL by manual counting and diluting of cells every 2 to 3 days.

Cell cultures lasted for a maximum of two months, when this period was reached a new vial of cells was used.

2.4.2. Cleansing of TK$^{-/-}$ mutants

L5178Y TK $^{+/-}$ heterozygote cells spontaneously mutate at a low but significant rates into homozygous mutants (TK $^{-/-}$). They were removed before carrying out any of the experiments.
As recommended by Fellows and colleagues (Fellows *et al*., 2014), cells were grown in THMG medium for 24 h. This medium contains 9 µg/mL thymidine, 15 µg/mL hypoxanthine, 0.3 µg/mL methotrexate and 22.5 µg/mL glycine. Accordingly, TK $^{+/-}$ cells can grow in this medium since they phosphorylate the exogenous thymidine. Nevertheless, mutant TK $^{-/-}$ cells cannot, thus reducing the “spontaneous” mutant frequency.

After the 24 h incubation, cells were then centrifuged, re-suspended in thymidine-hyponxantine-glycine medium (without methotrexate) for one day and finally diluted with normal growth

medium. Stock of cleansed cells was frozen down in 1 mL aliquots at a concentration between 1- 3×10^6 cells/mL.

2.4.3. Detection of DNA damage

Genotoxicity of two poly(anhydride) NPs and GN-Polymer were evaluated using the alkaline comet assay in combination with the enzyme FPG in order to detect altered bases in addition to DNA strand breaks (SBs) and alkali-labile sites (ALS). The proliferation assay was performed in parallel in order to interpret correctly the comet assay outcome (see section 2.4.3.2).

2.4.3.1. Alkaline comet assay in combination with FPG enzyme

Each experiment consisted of a negative control (solvent), a positive control (Ro-19-8022 exposed to intense light) and five concentrations of each test compound. Seven tubes with 10 mL of ML cells at 5×10^5 cells/mL were prepared. Each tube was treated with the corresponding concentration of NPs (7.4 - 600 µg/mL) or solvent with gentle shaking in an incubator at 37°C during 24 h. Cells treated with 4 µM of Ro 19-8022 were irradiated for 5 min with 500W of visible light and used as positive control. After treatment, cells were washed three times with PBS and adjusted to 1×10^6 cells/mL. The comet assay was performed as previously described by Collins & Azqueta (2012). Ninety µL of cell suspension were mixed with 420 µL of 1% low-melting point agarose and 2 drops of 70 µL of the mix were placed onto 1% standard agarose pre-coated slides. On top of each drop, one 2x2 cm cover slide was placed in order to form 2x2 cm gels. Three identical slides were prepared per condition. Following 5 min of solidification on ice, the cover slides were removed and slides were immersed in lysis buffer (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, pH 10, 1 % Triton X-100) and kept overnight at 4 °C. Two of the slides per condition were then washed three times (5 min each) with enzyme reaction buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8) while the other one was kept in lysis buffer. After washing, 45 µL/gel of enzyme reaction buffer were added to one of the slides and another 45 µL/gel of FPG enzyme were added to the other one; gels were then incubated for 30 min at 37 °C in a humidified chamber. All slides, including the ones remaining in lysis buffer, were immersed in electrophoresis buffer (1 mM EDTA, 0.3 M NaOH, pH 13) for 40 min at 4 °C for alkaline treatment before performing the electrophoresis at 1.2 V/cm, 300 mA and 4 °C

during 20 min. Afterwards, gels were neutralized by washing twice with PBS followed by distilled water for 10 min at 4 ºC each wash. Prior to analysis, gels were stained with 1 µg/mL of 4,6-diamidino-2-phenylindole (DAPI) solution. Comets were examined by fluorescent microscope (Nikon Eclipse 50 i, Japan) using the image analysis system Comet Assay IV (Perceptive instruments, UK). The software is designed to differentiate comet head from tail, and to measure a variety of parameters, including % of DNA in tail, tail length, % of total fluorescence in head and tail, and tail moment. The percentage of DNA in tail (% tail DNA) was used as DNA damage indicator. A total of randomly selected 100 cells were analysed per slide (50 comets/gel). SBs (plus ALS) were assessed by determining the % tail DNA in lysis buffer slides (i.e. the ones which remained in lysis buffer until alkaline treatment). To calculate the net FPG-sensitive sites, the median % tail DNA in the slide treated with buffer F was subtracted from the % tail DNA in the slide treated with FPG.

Three independent experiments were performed with GN-NP and GN-MA-NP for testing the *in vitro* genotoxicity. In the case of GN bulk polymer, one experiment was carried out.

In addition, normal cell appearance was checked by microscopy after the treatments.

2.4.3.2 Proliferation assay

ML cells were seeded in tubes at a concentration of 5×10^5 cell/mL in 10 mL and were treated with different concentrations of NPs (7.4 - 600 µg/mL) during 24 h under gentle shaking in an incubator at 37 °C. After exposure, cultures were washed three times with PBS solution. From each cell suspension, 5 mL were further seeded in tubes for the proliferation assay and the rest were used for performing the comet assay (see below). Cells were counted before, after treatment, and after 48 h incubation in fresh medium using the automated cell counter (Countess™ Automated Cell Counter, Invitrogen). Three independent experiments were performed.

The total suspension growth (TSG) and the relative suspension growth (RSG) of each condition were calculated as follows:

TSG= number of cells at 48 h post-treatment / number of cells before treatment

RSG= (TSG of exposed cultures / TSG of control cultures) x 100

Cell viability was considered affected when RSG value was below 70%.

2.4.4. Mouse lymphoma assay

Mutagenicity of GN-NP, GN-MA-NP and GN-Polymer were evaluated using the microwell version of MLA. This assay was conducted according to the procedure described by the OECD guideline 490 (Adopted 28 July 2015) (OECD 490, 2015) with slight modifications. In agreement with this guideline, each experiment consisted of one negative control (without treatment), one positive control (100 µM MMS) and ten concentrations of each test compound were lowered by a factor of 3. In all cases, the highest concentration tested was 600 µg/mL. Two independent experiments were performed with GN-NP and GN-MA-NP and only one in the case of GN-Polymer.

For this assay, cells were seeded in different test tubes for each test concentration, for the negative control and also for the positive control. Densities of 5×10^6 cells/tube were seeded in each tube at the beginning of the treatment., which was carried out by gentle shaking in an incubator at 37 °C during 24 h without metabolic activation (-S9).

After the treatment, each cell culture was washed twice with PBS and diluted to 2×10^5 cells/mL. Ten mL of each cell culture were transferred to 25 cm^2 culture flasks and maintained at 37 ºC and 5% CO_2 in a humidified atmosphere. Cells were counted after 24 and 48 h using an automated cell counter (Countess™ Automated Cell Counter, Invitrogen) for RSG calculation. In addition, cell density was again adjusted to 2×105 cells/mL after the first count.

Mutant frequency (MF) analysis and cloning efficiency (CE) start 48h after treatment by seeding known numbers of cells in medium containing a selective agent to detect mutant colonies (5-trifluorothymidine or TFT), and in medium without the selective agent to determine CE.

1) TFT resistant: an aliquot of the cell suspension was used in order to obtain a second cell suspension of $1x10^4$ cells/mL (44 mL). For hundred µg/mL of TFT were added to each cell suspension and 2000 cells/well were seeded in 96 well/plates (200 µL/well). Two identical plates were prepared per condition and incubated at 37 °C, 5 % CO_2 during 11-13 days, until colonies reach appropriate sizes.

2) Cloning efficiency (viability): another aliquot of the cell suspension was used in order to obtain a second cell suspension of 10 cells/mL (44 mL) in non-selective medium. Two cells/well were seeded in 96 well/plates (200 µL/well). Two identical plates were prepared per condition and incubated at 37 ºC for the same time as TFT resistant plates.

In both cases, TFT resistant and viability, the scoring procedure followed the same protocol in which colonies were stained and count. To stain the colonies, freshly made MTT (2.5 mg/mL) was added to each well. Plates were then incubated during 4 h at 37 °C and 5 % CO_2. Colonies were scored by eye using qualitative judgement. Only in the case of plates of TFT resistant, small and large colonies were discerned. Colony size was estimated as previously reported by Honma *et al.* (1999); small colony was defined as a colony having a size less than one-fourth of the well diameter.

Two independent experiments were performed to confirm the results.

Normal cell appearance was checked by microscopy.

2.4.4.1. Calculations

The different calculations were carried out according to the OECD guideline 490. For determining the mutant frequency (MF), the cloning efficiency (CE) of the mutant colonies in selective medium is related to the CE of the non-selective medium (MF= $CE_{Mutant}/CE_{Viability}$) as indicated in tne next equation:

CE: (-ln (A)/(B))/C

In which “A” and “B” are the number of empty wells and the number of wells with colonies, respectively, whereas “C” represents the number of seeded cells per well.

In the case of mutant frequency (MF), small and large colonies were discerned; therefore CE for small, large and total colonies was calculated. Thus, MF was calculated by the next formula:

$$MF= (CE_{Mutant} / CE_{Viability}) /\text{seeded cell with TFT/well} \times 10^{6}$$

In all cases the media was obtained of the 2 plates of each condition.

2.4.4.2. Test acceptance criteria

OECD 490 Guideline (OECD 490, 2015) provides specific recommendations for determining the acceptability of the results. MF values of the solvent control must range between 50×10^{-6} and 170×10^{-6} else the study must be rejected. The reasoning for this is that greater values may lead increased number of mutants in the tested concentrations due of the high mutation rates already present at basal conditions and not due to genotoxicity of the tested compound. Values lower than 50×10^{-6} usually indicates poor recovery of small colonies. In addition, CE for solvent controls must be between 65-120 % for the assay to be considered valid.

Biological relevance must be also considered in order to define a positive or negative result. For this purpose a Global Evaluation Factor (GEF) has been defined, being 126×10^{-6} in the microwell version. Thus, the test sample is considered to be mutagenic in the assay if (1) acceptance criteria of the solvent control are met, (2) any of the experimental conditions examined showed a MF higher than the one of the solvent control plus the GEF and (3) the increase in MF is concentration-related.

2.5 Statistical analysis

Comet assay results were presented as mean ± standard deviation (SD) of the median of each concentration including negative and positive controls. The comparisons were performed using the Kruskal-Wallis test. Statistical significance was set at $p \leq 0.05$.

In MLA, data are given as mean values with SD of the two plates used per condition.

Graph plots were executed in GraphPad Prism® program (GraphPad Prism®, version 3.0, United States).

3. Results

3.1 Characterization of NPs

The principal physico-chemical characteristics of GN-NP and GN-MA-NP are summarized in Table 1. The functionalization of bare nanoparticles (GN-NP) with mannosamine (GN-MA-NP) increased both the size of the resulting nanocarriers (198 *vs* 276, respectively); although GN-NP presented a smoother surface than GN-MA-NP (Fig. 1). Interestingly, for both types of nanoparticles, the preparative process was adequate to produce very homogeneous batches (PDI lower than 0.2, Table 1).

Table 1. Physico-chemical characteristics of NPs. Data expressed as the mean ± SD.

NP	Size (nm)	PDI
GN-NP	198 ± 1	0.163 ± 0.024
GN-MA-NP	276 ± 2	0.138 ± 0.056

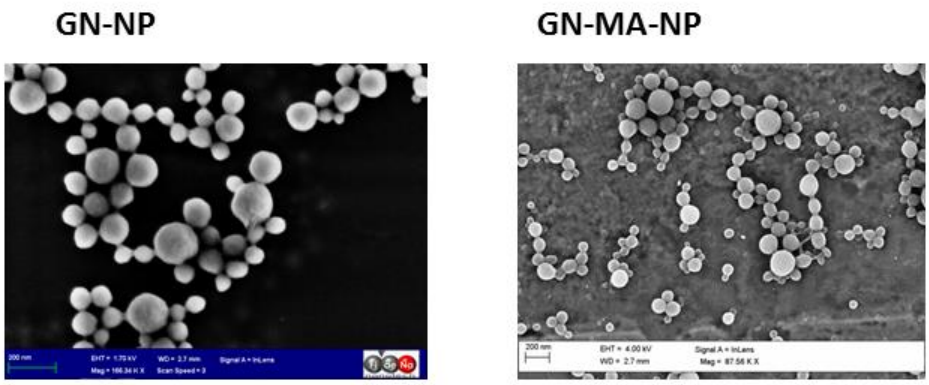


Fig. 1. SEM photographs of NPs.

3.2. Detection of DNA damage

The genotoxicity of GN-NP and GN-MA-NP, as well as GN-Polymer, was evaluated in ML cells using the alkaline comet assay in combination with the FPG. DNA damage was quantified as

the % tail DNA after 24 hours of treatment. Furthermore, cell proliferation was assessed in parallel by counting treated cells after 48 h of incubation at 37 °C.

Fig. 2 shows the effect of GN-NP and GN-MA-NP and their polymer on the viability, SBs+ALS, and FPG-sensitive sites in ML cells treated for 24 h. GN-NP and GN-MA-NP did not affect the viability of cells since RSG was above 70% in all condition tested. However, the highest tested concentration of GN-Polymer (600µg/mL) did affect the viability of cells, presenting an RSG of approximately 30%. Moreover, any of the tested NPs did not significantly induce SBs + ALS and net FPG-sensitive sites. In contrast, GN-Polymer showed an increase in the net FPG-sensitive sites at the highest concentration. Positive and negative controls showed the expected results.

Normal appearance of the cells was observed by microscopy after treatment.

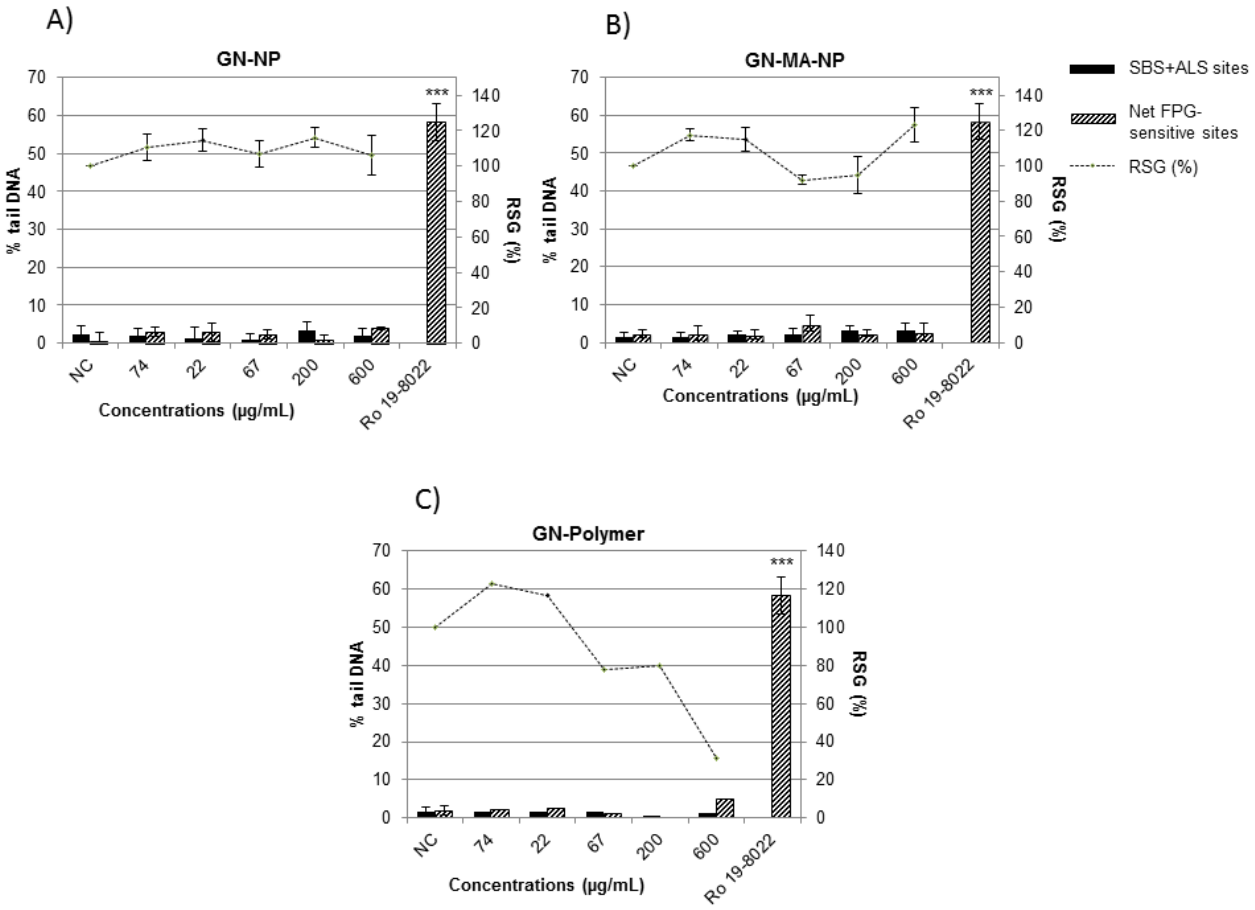


Fig. 2. Analysis of DNA strand breaks and net FPG-sensitive sites in L5178Y TK$^{+/-}$ cells treated for 24 h with GN-NP **(A)** and GN-MA-NP **(B)** and GN-Polymer **(C)** using the alkaline comet assay in combination

with the FPG. The total of DNA strand breaks and net FPG-sensitive sites are presented by using the percentage of DNA in tail. Data are expressed as the mean ± SD of three independent experiments (n=3) except for GN-Polymer (n=1). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, significantly different from negative control.

3.3. Mutagenicity of poly(anhydride) NPs

MLA was carried out in ML cells treated with GN-NP, GN-MA-NP and GN-Polymer for 24h using the microwell version of the assay. The highest concentration used was 600 µg/mL.

Results regarding the mutagenicity of NPs and their polymer are shown Fig. 3.

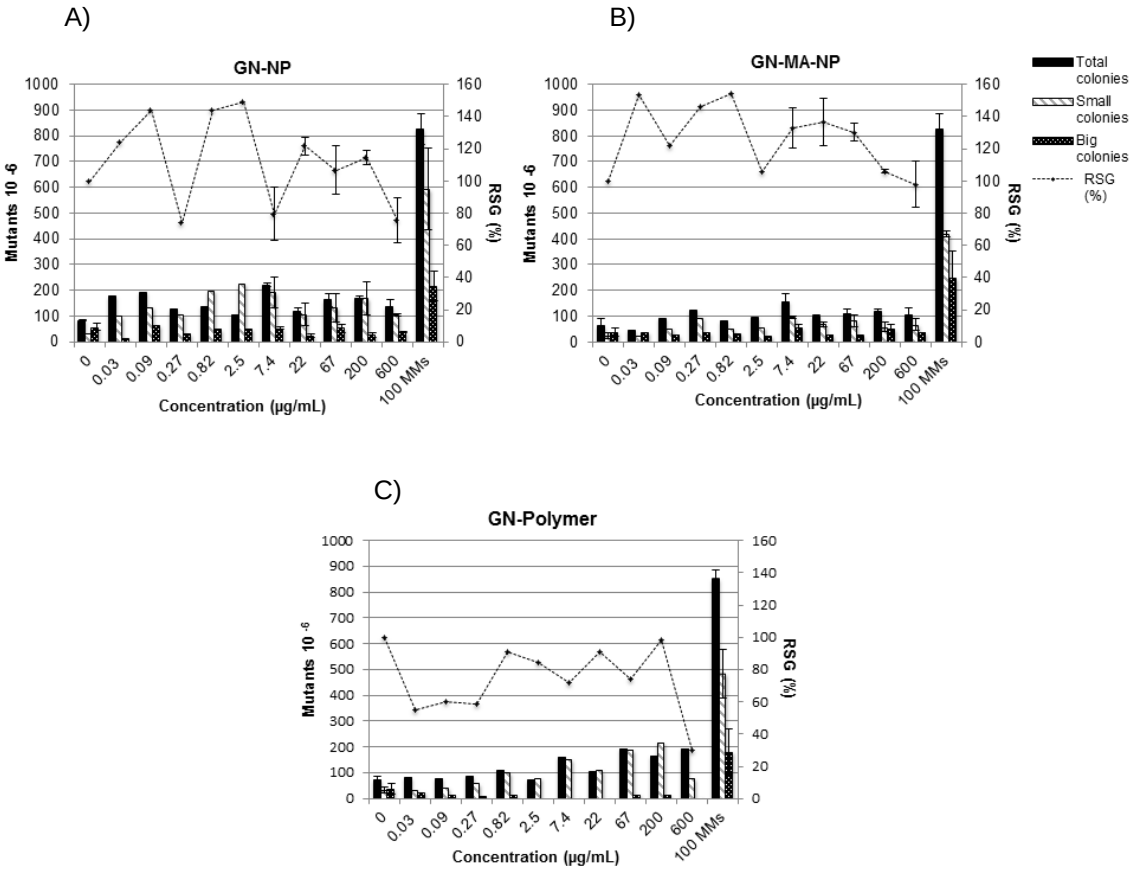


Fig. 3. Cytotoxicity represented by viability, and mutant frequency (MF) in L5178Y $TK^{+/-}$ cells treated with GN-NP **(A)** and GN-MA-NP **(B)** and GN-Polymer **(C)**. Data are expressed as the mean ± SD of two independent experiments (n=2) except for GN-Polymer (n=1)

All concentrations tested of NPs, GN-NP and GN-MA-NP, presented RSG values higher than 70 %. However, their polymer (GN-Polymer) showed a decrease of RSG in the maximum concentration tested, presenting a value of 30 %. Both NPs evaluated, GN-NP and GN-MA-NP, induce an increase of the MF of small and total colonies at all concentrations tested. Although a concentration-dependent effect was not observed. The MF of big colonies did not show an increase compared with the negative control. This increase of the MF (small and total colonies) did not exceed the MF of the negative control plus the GEF. In the case of GN-Polymer, a concentration dependent increase of the number of small and total colonies was observed, though this increase was not higher than the MF values of the negative control plus the GEF.
In agreement with the acceptance criteria for the microwell method for negative control, the MF detected in control cells was 62.14 ± 28.55 x 10^{-6}. In the case of the cell treated with 100 µM MMS, the value of MF was 827.29 ± 58.86 x 10-6, dominated by small colonies.

Normal cell appearance was observed by microscopy after the treatments.

4. Discussion

Nanotechnology is nowadays one of the fastest growing and most promising technologies in our society regarding human health. It can be applied in many areas, such as improvement of disease diagnosis, pain relief and treatment of human diseases (Ahmad *et al*., 2008; Jain *et al*., 2011). The use of polymeric NPs for medical applications encompassing oral drug delivery has attracted increasing interest due to their singular properties such biocompatibility, biodegradability, controlled release properties and their modifiable surface that facilitates the preparation of functionalized nanocarriers with specific biodistribution properties (Agüeros *et al*., 2009; Calleja *et al*., 2015; Ensign *et al*., 2012; Inchaurraga *et al*., 2015; Salman *et al*., 2006). Gantrez® AN 119 based NPs has attract a lot of attention due to their capability to develop strong adhesive interactions within the gut mucosa and, hence, to prolong the residence time of the nanocarrier form in close contact with the absorptive epithelium (Agüeros *et al*., 2009, 2010; Arbós *et al*., 2002, 2004; Porfire *et al*., 2010; Salman *et al*., 2005, 2006; 2009; Yoncheva *et al*., 2005).

Though there are some studies that demonstrate the drug loading capacity and the efficacy of GN-MA-NP and other Gantrez® AN 119 based NPs to this to transport therapeutic agent (Calleja *et al*., 2015; Salman *et al*., 2009), the safety of this NPs has not been thoroughly studied. However, the use of these nanodevices may be hampered by the biological behaviour and the toxicological properties of the new nanodrugs. Moreover, due to their unique properties, the safety assessment of nanomaterials cannot be analyzed in the same way as chemical compounds. According to the reflection papers for the development of new nanomedicine products for human use published by the European Medicines Agency, assessment of the toxicity, as well as, the characterization of NPs are crucially important for safety assessment of NPs (EMA, 2006).

Therefore, the physico-chemical parameters of 2 Gantrez® AN 119 based NPs, GN-NP and GN-MA-NP, were determined. As a result, these NPs presented a homogeneous size around 250 nm diameter and narrow size distribution (PDI < 0.1). In addition, a previous study has demonstrated the negative surface charge of these NPs (Chapter 4).

The first step to evaluate the safety of NPs is to perform *in vitro* toxicity tests. Previous studies have demonstrated the absence of cyto- and genotoxicity in Caco-2 cells exposed to GN-NP and GN-MA-NP (Chapter 4). They did not affect viability, cell metabolism, membrane integrity even at very high concentration (2 mg/mL) and 24 h of treatment. Nevertheless, they induce a very slight increase of the FPG-sensitive sites at 24 h of treatment at very high and non-relevant concentrations (i.e. 1 and 2 mg/mL). Ojer *et al.* (2013) demonstrated that GN-NP and NPs of Gantrez® AN 119 coated with cyclodextrin and poly-ethylene glycol, were non cytotoxic in HepG2 and Caco-2 after 24 h of treatment at high concentrations (2000 µg/mL) using MTT and lactate deshydrogenase assays.

The majority of *in vitro* toxicity testing to evaluate the safety of the NPs uses low concentrations of NPs since NPs tent to agglomerate in higher concentrations and this may reduce toxicity. However, in a previous study, much higher concentrations of both of our NPs (2000 µg/mL) demonstrated no agglomeration up to 24h in culture medium (Chapter 4). We, therefore,

decided to use 600 µg/mL as our highest concentration. Higher concentrations were not tested because they would not have biological relevance since such high concentrations would not be suitable for *in vivo* or clinical studies. Moreover, higher concentrations interfered with the comet assay scoring (data not shown).

In this study we explored the *in vitro* genotoxicity associated with the exposure of GN-NP, GN-MA-NP and GN-polymer using the comet assay in combination with FPG for detection of strand breaks as well as FPG-sensitive sites. The alkaline comet assay is a widely used method for *in vitro* and *in vivo* genotoxicity testing in nanotechnology due to its robustness, versatility and reliability (Azqueta & Dusinska, 2015). FPG is able to detect both alkylated and oxidised bases, mainly 8-oxo-guanine (a pre-mutagenic lesion). However, taking into account the nature of the NPs, i.e. composition and the fact that they are not internalized, FPG was used to detect oxidized purines.

GN-NP and GN-MA-NP did not affect the viability of ML cells in any of the conditions tested. However, GN-Polymer induced a decrease of about 60% in the viability of ML cell treated with 600 µg /mL. Thus, it seems that the nanoform of GN-polymer protects the cells from the decrease in the viability induced by the GN-polymer itself; it may be due to the fact that the polymer is dissolved and may be more reactive. Viability is crucial to interpret the comet assay outcome since DNA SBs can be a secondary effect of dead cells; at least 60% of viable cells should be present (Dusinska *et al*., 2012).

GN-NP, GN-MA-NP and their polymer did not significantly increase the frequency of SBs+ALS or FPG-sensitive sites in ML cells exposed to different concentrations after 24 h exposure, suggesting no genotoxic oxidative damage. Genotoxic studies of polymeric NPs are very scarce. Cowie and colleagues demonstrated a negative genotoxicity effect of polylactic glycolic acid poly-ethylene oxide polymeric NPs (PLGA-PEO) and metal NPs in human and mammalian cells of different origin using different times of incubation (30 min - 24 h) and the comet assay in an inter-laboratory study (Cowie *et al*., 2015). Tullinska *et al.* (2015) and Kazimirova *et al.* (2012) showed the same results when testing PLGA-PEO.

Despite the extensive advantages of comet assay, using one technique it is not enough to obtain all the necessary information on the potential genotoxicity. Therefore, a battery of tests that measure different endpoints to consider the potential risk of NPs to human health is required. Thus, MLA was performed, which uses the TK gene as the mutational target, detecting a broad spectrum of genetic damage, including both point mutations and chromosomal alterations. In addition, gene mutations, including small deletion, were detected by means of the mouse lymphoma assay in L5178Y TK $^{+/-}$ cells. The comet assay and the MLA assay measure different endpoints in the downstream of genotoxicity; the comet assay detects reparable lesions while the MLA detects mutations. The combination of the 2 assays gives a reliable idea of the genotoxic potential of the NPs.

In the MLA, two distinct phenotypic classes of mutants are generated. Normal growing mutants (big colonies) indicate a point mutation and slow growing mutants (small colonies) suggest clastogenic activity (Combes *et al*., 1995). Small colonies have suffered gross structural changes at the chromosomal level that involves putative growth-regulating genes near the TK locus, which results in lengthy doubling times and thus, the formation of small colonies (Amundson & Liber, 1992). This assay is widely used and it has been validated for its use as a component of the genotoxic testing battery by the OECD, which is used for evaluating the mutagenic potential of chemicals (OECD 490, 2015).

Our study was carried out according to the procedure described by OECD 490 with slight modifications for evaluation of NPs. This guideline indicates that the highest concentration should aim to achieve between 20 and 10% RSG. However, in our study a maximum concentration of 600 µg/mL was evaluated although GN-NP and GN-MA-NP presented a RSG value greater than 70% and its polymer showed a value of 30% at the highest concentration. Nevertheless, as abovementioned, we believe that higher concentrations would not have biological relevance for pharmaceutical products intended for human use.

Our results showed that in ML cells treated for 24 h at different concentrations ranging from 0.03 to 600 µg/mL, GN-NP, GN-MA-NP and GN-Polymer have no mutagenic potential *in vitro*.

However, they showed slight increase in the MF of small colonies, thus resulting in augmented number of the MF of total colonies. This was especially pronounced in samples treated with GN-Polymer, which also showed a concentration-dependent effect. Our data, therefore may suggests a clastogenic activity. However, the increased MF of small colonies in both NPs and GN-Polymer did not exceed the negative control plus the GEF, as well as, it did not show a concentration-dependent increase in the case of NPs. Thus, according with the OECD 490 guideline, this increase of MF is not biologically relevant in any of the poly(anhydride) NPs studied or in the GN-Polymer. Therefore, we can assume that these NPs and their polymer do not show clastogenic activity. Similar results were found by He *et al.* (2009), Kazimirova *et al.* (2012) and Tulinska *et al.* (2015) when testing PLGA-PEO NPs; they did not show potential mutagenic effects measured by the micronucleus test.

In conclusion, empty GN-NP and GN-MA-NP did not affect viability of ML cells at any of the different conditions tested. Moreover, they did not induce relevant genotoxic or mutagenic lesions after 24 h of exposure. Altogether, our study has provided crucially important information contributing to the overall safety profile of poly(anhydride) NPs designed for medical applications as oral drug delivery systems.

5. Acknowledgements

This work was supported by the ALEXANDER project (FP7-2011-NMP-280761), the QualityNano Research Infrastructure project (FP7, INFRA-2010-262163), a Transnational Access Grants (NILU-TAF-289), and the project *NorNANoREG (*Research Council of Norway, 239199/070). TI was also financially supported by a grant from the ALEXANDER project. AA also thanks the Ministerio de Economía y Competitividad (‘Ramón y Cajal’ programme, RYC-2013-14370) of the Spanish Government for personal support. We thank Professor Andrew AR. Collins, Oslo University, for enzyme-FPG gift and Hoffmann-La Roche (Brasilea, Switzerland) for providing the photosensitiser (Ro 19-8022).

6. References

Agüeros, M., Areses, P., Campanero, M.A., Salman, H., Quincoces, G., Peñuelas, I., Irache, J.M., 2009. Bioadhesive properties and biodistribution of cyclodextrin-poly(anhydride) nanoparticles. European Journal of Pharmaceutical Sciences. 37 (3-4), 231-240.

Agüeros, M., Zabaleta, V., Espuelas, S., Campanero, M.A., Irache, J.M., 2010. Increased oral bioavailability of paclitaxel by its encapsulation through complex formation with cyclodextrins in poly(anhydride) nanoparticles. Journal of Controlled Release. 145 (1), 2-8.

Agüeros, M., Espuelas, S., Esparza, I., Calleja, P., Peñuelas, I., Ponchel, G., Irache, J.M., 2011. Cyclodextrin-poly(anhydride) nanoparticles as new vehicles for oral drug delivery. Expert Opinion on Drug Delivery. 8 (6), 721-734.

Ahmad, Z., Pandey, R., Sharma, S., Khuller, G.K., 2008. Novel chemotherapy for tuberculosis: chemotherapeutic potential of econazole- and moxifloxacin-loaded PLG nanoparticles. International Journal of Antimicrobial Agents. 31 (2),142-146.

Ahmad, S.I., Yokoi, M., Hanaoka, F., 2012. Identification of new scavengers for hydroxyl radicals and superoxide dismutase by utilising ultraviolet A photoreaction of 8-methoxypsoralen and a variety of mutants of Escherichia coli: Implications on certain diseases of DNA repair deficiency. Journal of Photochemistry and Photobiology B: Biology. 116, 30-36.

Aitken, R & Krausz, C., 2001. Oxidative stress, DNA damage and the Y chromosome. Reproduction. 122 (4), 497-506.

Arbós, P., Wirth, M., Arangoa, M.A., Gabor, F., Irache, J.M., 2002. Gantrez® AN as a new polymer for the preparation of ligand–nanoparticle conjugates. Journal of Controlled Release. 83 (3), 321-330.

Arbós, P., Campanero, M.A., Arangoa, M.A., Irache, J.M., 2004. Nanoparticles with specific bioadhesive properties to circumvent the pre-systemic degradation of fluorinated pyrimidines. Journal of Controlled Release. 96 (1), 55-65.

Azqueta, A & Collins, A.R., 2011. The comet assay: a sensitive and quantitative method for analysis of DNA damage. In: Meyers, R.A., Wiley and Sons, J (Eds). Encyclopedia of Analysis Chemistry,pp. 1-19.

Azqueta, A., Arbillaga, L., López de Cerain, A., Collins, A.R., 2013. Enhancing the sensitivity of the comet assay as a genotoxicity test, by combining it with bacterial repair enzyme FPG. Mutagenesis. 28 (3), 271-277

Azqueta, A & Dusinska, M., 2015. The use of the comet assay for the evaluation of the genotoxicity of nanomaterials. Frontiers in Genetic. 6, 239-243.

Calleja, P., Espuelas, S., Vauthier, C., Ponchel, G., Irache, J.M., 2015. Controlled release, intestinal transport, and oral bioavailablity of paclitaxel can be considerably increased using suitably tailored pegylated poly(anhydride) nanoparticles. Journal of Pharmaceutical Sciences. 104 (9), 2877-2886.

Collins, A.R & Azqueta, A., 2012. Single-cell gel electrophoresis combined with lesion-specific enzymes to measure oxidative damage to DNA. Methods in Cell Biology. 112, 69-92.

Combes, R.D., Stopper, H., Caspary, W.J., 1995. The use of L5178Y mouse lymphoma cells to assess the mutagenic, clastogenic and aneugenic properties of chemicals. Mutagenesis. 10 (5), 403-408.

Cowie, H., Magdolenova, Z., Saunders, M., Drlickova, M., Correia Carreira, S., Halamoda Kenzaoi, B., Gombau, L., Guadagnini, R., Lorenzo, Y., Walker, L., Fjellsbø, L.M., Huk, A., Rinna, A., Tran, L., Volkovova, K., Boland, S., Juillerat-Jeanneret, L., Marano, F., Collins, A.R.,

Dusinska, M., 2015. Suitability of human and mammalian cells of different origin for the assessment of genotoxicity of metal and polymeric engineered nanoparticles. Nanotoxicology. 9, 57-65.

Dusinska, M & Collins, A., 1996. Detection of oxidised purines and UV-induced photoproducts in DNA, by inclusion of lesion-specific enzymes in the comet assay (single cell gell electrophoresis). ATLA. 24, 405-411.

Dusinska, M., Magdolenova, Z., Fjellsbø, L.M., 2012. Toxicological aspects for nanomaterial in humans. Methods in Molecular Biology. 948, 1-12.

Dusinska, M., Boland, S., Saunders, M., Juillerat-Jeanneret, L., Tran, L., Pojana, G., Marcomini, A., Volkovova, K., Tulinska, J., Knudsen, L.E., Gombau, L., Whelan, M., Collins, A.R., Marano, F., Housiadas, C., Bilanicova, D., Halamoda Kenzaoui, B., Correia Carreira, S., Magdolenova, Z., Fjellsbø, L.M., Huk, A., Handy, R., Walker, L., Barancokova, M., Bartonova, A., Burello, E., Castell, J., Cowie, H., Drlickova, M., Guadagnini, R., Harris, G., Harju, M., Heimstad, E.S., Hurbankova, M., Kazimirova, A., Kovacikova, Z., Kuricova, M., Liskova, A., Milcamps, A., Neubauerova, E., Palosaari, T., Papazafiri, P., Pilou, M., Poulsen, M.S., Ross, B., Runden-Pran, E., Sebekova, K., Staruchova, M., Vallotto, D., Worth, A., 2015. Towards an alternative testing strategy for nanomaterials used in nanomedicine: Lessons from NanoTEST. Nanotoxicology. 9, 118-132.

EMA, 2006. Reflection paper on nanotechnology-based medicinal products for human use. EMEA/CHMP/79769/2006.

Ensign, L.M., Cone, R., Hanes, J., 2012. Oral drug delivery with polymeric nanoparticles: The gastrointestinal mucus barriers. Advanced Drug Delivery Reviews. 64 (6), 557-570.

Fellows, M.D., McDermott, A., Clare, K.R., Doherty, A., Aardema, M.J., 2014. The spectral karyotype of L5178Y $TK^{+/-}$ mouse lymphoma cells clone 3.7.2C and factors affecting mutant

frequency at the thymidine kinase (tk) locus in the microtitre mouse lymphoma assay. Environmental and Molecular Mutagenesis. 55 (1), 35-42.

Gábelová, A., El Yamani, N., Iglesias, T., Buliaková, B., Srancíková, A., Bábelová, A., Pran, E., Fjellsbø, L., Elje, E., Yazdani, M., Silva, M.J., Dusinska, M., 2016. Fibrous shape underlies the mutagenic and carcinogenic potential of nanosilver while surface chemistry affects the biosafety of iron oxide nanoparticles. Mutagenesis. (in press).

He, L., Yang, L., Zhang, Z., Gong, T., Deng, L., Gu, Z., Sun, X., 2009. *In vitro* evaluation of the genotoxicity of a family of novel MeO-PEGpoly(D,L-lactic-co-glycolic acid)-PEG-OMe triblock copolymer and PLGA nanoparticles. Nanotechnology. 20 (45), 455102-455147.

Hoeijmakers, J.H.J., 2009. DNA Damage, aging, and cancer. New England Journal of Medicine. 361 (15), 1475-1485.

Honma, M., Hayashi, M., Shimada, H., Tanaka, N., Wakuri, S., Awogi, T., Yamamoto, K.I., Kodani, N., Nishi, Y., Nakadate, M., Sofuni, T., 1999. Evaluation of the mouse lymphoma tk assay (microwell method) as an alternative to the *in vitro* chromosomal aberration test. Mutagenesis. 14 (1), 5-22.

ICH, 2011. Topic S2B Genotoxicity: A standard battery for genotoxocity testing of pharmaceuticals, international conference on harmonisation of technical requirements for registration of pharmaceuticals for human use.

Inchaurraga, L., Martín-Arbella, N., Zabaleta, V., Quincoces, G., Peñuelas, I., Irache, J.M., 2015. *In vivo* study of the mucus-permeating properties of PEG-coated nanoparticles following oral administration. European Journal of Pharmaceutics and Biopharmaceutics. 97 (Pt A), 280-289.

Irache, J., Huici, M., Konecny, M., Espuelas, S., Campanero, M., Arbós, P., 2005. Bioadhesive properties of Gantrez nanoparticles. Molecules. 10 (1), 126-145.

Jain, A.K., Swarnakar, N.K., Godugu, C., Singh, R.P., Jain, S., 2011. The effect of the oral administration of polymeric nanoparticles on the efficacy and toxicity of tamoxifen. Biomaterials. 32 (2), 503-515.

Kazimirova, A., Magdolenova, Z., Barancokova, M., Staruchova, M., Volkovova, K., Dusinska, M., 2012. Genotoxicity testing of PLGA-PEO nanoparticles in TK6 cells by the comet assay and the cytokinesis-block micronucleus assay. Mutation Research. 748 (1-2), 42-47.

Kumar, M., Trivedi, N., Reddy, C.R.K., Jha, B., 2011. Toxic effects of imidazolium ionic liquids on the green seaweed ulva lactuca: oxidative stress and DNA damage. Chemical Research in Toxicology. 24 (11), 1882-1890.

Landsiedel, R., Fabian, E., Ma-Hock, L., Wohlleben, W., Wiench, K., Oesch, F., Van Ravenzwaay, B. 2012, Toxico-biokinetics of nanomaterials. 86 (7), 1021-1060.

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, 233-278.

Manke, A., Wang, L., Rojanasakul, Y., 2013. Mechanisms of nanoparticle-induced oxidative stress and toxicity. Biomed Research International. 942916-942931.

Moreno, E., Schwartz, J., Larrañeta, E., Nguewa, P.A., Sanmartín, C., Agüeros, M., Irache, J.M., Espuelas, S., 2014. Thermosensitive hydrogels of poly(methyl vinyl ether-co-maleic anhydride)- Pluronic® F127 copolymers for controlled protein release. International Journal of Pharmaceutics. 459 (1-2), 1-9.

Nel, A., Xia, T., Mädler, L., Li, N., 2006. Toxic potential of materials at the nanolevel. Science. 311 (5761), 622-627.

OECD, 1997. Test Guideline 476: *In vitro* mammalian cell gene mutation test. In: OECD Guidelines for testing of chemicals.

OECD, 2015. Test Guideline 490. *In Vitro* Mammalian Cell Gne Mutation Tests Using the Thymidine Kinase Gene. In: OECD Guidelines for testing of chemicals.

Ojer, P., de Cerain, A., Areses, P., Peñuelas, I., Irache, J.M., 2012. Toxicity studies of poly(anhydride) nanoparticles as carriers for oral drug delivery. Pharmaceutical Research. 29 (9), 2615-2627.

Ojer, P., Neutsch, L., Gabor, F., Irache, J., López de Cerain, A., 2013. Cytotoxicity and cell interaction studies of bioadhesive poly(anhydride) nanoparticles for oral antigen/drug delivery. Journal of Biomedical Nanotechnology. 9 (11),1891-1903.

Porfire, A.S., Zabaleta, V., Gamazo, C., Leucuta, S.E., Irache, J.M., 2010. Influence of dextran on the bioadhesive properties of poly(anhydride) nanoparticles. International Journal of Pharmaceutics. 390 (1), 37-44.

Salman, H.H., Gamazo, C., Campanero, M.A., Irache, J.M., 2005. Salmonella-like bioadhesive nanoparticles. Journal of Controlled Release. 106 (1-2), 1-13.

Salman, H.H., Gamazo, C., Campanero, M., Irache, J.M., 2006. Bioadhesive mannosylated nanoparticles for oral drug delivery. Journal of Nanoscience and Nanotechnology. 6 (9-10), 3203-3209.

Salman, H.H., Irache, J.M., Gamazo, C., 2009. Immunoadjuvant capacity of flagellin and mannosamine-coated poly(anhydride) nanoparticles in oral vaccination. Vaccinne. 27 (35), 4784-4790.

Sawyer, J., Moore, M.M., Clive, D., Hozier, J., 1985. Cytogenetic characterization of the L5178Y $TK^{+/-}$ 3.7.2C mouse lymphoma cell line. Mutation Research. 147 (5), 243-253.

Tulinska, J., Kazimirova, A., Kuricova, M., Barancokova, M., Liskova, A., Neubauerova, E., Drlickova, M., Ciampor, F., Vavra, I., Bilanicova, D., Pojana, G., Staruchova, M., Horvathova, M., Jahnova, E., Volkovova, K., Bartusova, M., Cagalinec, M., Dusinska, M., 2015. Immunotoxicity and genotoxicity testing of PLGA-PEO nanoparticles in human blood cell model. Nanotoxicology. 9 (Suppl 1), 33-43

Yoncheva, K., Gómez, S., Campanero, M.A., Gamazo, C., Irache, J.M., 2005. Bioadhesive properties of pegylated nanoparticles. Expert Opinion on Drug Delivery. 2 (2), 205-218.

Zhang, D., Pan, X., Wang, S., Zhai, Y., Guan, J., Fu, Q., Hao, X., Qi, W., Wang, Y., Lian, H., Liu, X., Wang, Y., Sun, Y., He, Z., Sun, J., 2015. Multifunctional poly(methyl vinyl ether-co-maleic anhydride)-graft-hydroxypropyl-β-cyclodextrin amphiphilic copolymer as an oral high-performance delivery carrier of tacrolimus. Molecular Pharmaceutics. 12 (7), 2337-2351.

Chapter 6: Genotoxic evaluation of a poly(anhydride) nanoparticle in the gastrointestinal tract of mice

Genotoxic evaluation of a poly(anhydride) nanoparticle in the gastrointestinal tract of mice.

Iglesias T[1], López de Cerain A[1,2], Gomez-Rodriguez V[1], Irache JM[3], Butinar M[4], Turk B[4], Azqueta A[1,2]*.

[1]Department of Pharmacology and Toxicology. Faculty of Pharmacy and Nutrition, University of Navarra, 31009 Pamplona, Spain.

[2]IdiSNA, Navarra Institute for Health Research

[3]Pharmacy and Pharmaceutical Technology Department. Faculty of Pharmacy and Nutrition. University of Navarra. Irunlarrea 1, 31008, Pamplona, Spain.

[4]Department of Biochemistry, Molecular and Structural Biology, Jozef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia

Abstract

Gantrez® AN 119 based NPs have been developed as oral drug carriers due to their strong bioadhesive interaction with components of the gastro intestinal mucosa and their adaptable surface. The use of mannosamine to decorate Gantrez® AN 119 based NPs results in a high mucus permeable carrier, able to reach the gastrointestinal epithelium. Although their efficacy for transport therapeutic agent has been proved, their safety has not been thoroughly studied. It has been demonstrated that they are non-cytotoxic, non-genotoxic and non-mutagenic *in vitro*; however, the *in vivo* toxicity profile has not been determined yet. In this study the *in vivo* genotoxic potential of Gantrez® AN 119 NPs coated with mannosamine (GN-MA-NP) has been determined using the *in vivo* comet assay in combination with the FPG in mice and by following the OECD test guideline 489. To determine the relevant organs to analyse and the sampling times, an *in vivo* biodistribution study was also carried out. Results showed a statistically significant induction of DNA strand breaks and oxidized bases in duodenum tissue of animals exposed to 2000 mg/kg bw. However, this effect was not observed at lower doses (i.e. 1000 and 500 mg/kg –i.e. close to the potential therapeutic dose -) or in other organs.

In conclusion, GN-MA-NP are promising nanocarriers as oral drug delivery systems.

1.Introduction

In the last two decades, the development of nanoparticles (NPs) has significantly increased due to the immense variety of potential applications in medicine, pharmacy and food safety (Salata, 2004). Among the various nanoparticles for oral drug delivery systems, polymeric NPs have received major attention due to their unique properties and their adaptable surface *(*Salman *et al*., 2006). Furthermore, they are biodegradable, biocompatible and easy to produce.

In addition, in many cases, their surface can be easily coated with different ligands in order to modify their physico-chemical properties, as well as their distribution *in vivo* (Agüeros *et al*., 2009; Inchaurraga *et al*., 2015; Salman *et al*., 2006). For instance, coating with mannosamine ligand enhances the ability of Gantrez® AN 119 to develop stronger bioadhesive interactions with components of the intestinal mucosa (Salman *et al*., 2006; 2009). In line with this result, this coating showed the best mucus permeability compared to other ligands (i.e. dextran, aminodextran, cyclodextrin, and poly-ethylene glycol (PEG) of different molecular weights) in an *in vitro* transwell system approach (Chaper 4). This property is particularly interesting and advantageous for engineered products administered orally since it results in prolonged residence time of the formulation in close contact with the absorptive mucosal epithelium and, thus, in important increments in drug absorption and bioavailability. It is worth to mention that after oral administration Gantrez® AN 119-based NPs are not absorbed or distributed to other organs but remain in the gastrointestinal tract before being eliminated (Agüeros *et al*., 2009; Arbós *et al*., 2002; Inchaurraga *et al*., 2015; Porfire *et al*., 2010; Yoncheva *et al*., 2005). Nevertheless, Gantrez® AN 119 NPs coated with mannosamine are taken up by Peyer's patches, probably due to the presence of mannose receptor in this tissue (Salman *et al*., 2006).

Though there are several studies that demonstrate the efficacy of Gantrez® AN 119 coated with mannosamine to transport therapeutic agent (Salman *et al*., 2009), the safety of these NPs has not been thoroughly studied. Commercial Gantrez® AN 119 as well as mannosamine ligand have been recognized as safe to human health (Moreno *et al*., 2014). The nano-form of their combination did not affect the cell metabolism, the membrane integrity or the viability of Caco-2 cells after 24 h exposure at high concentrations (2 mg/mL) (Chapter 4).

However, genotoxicity is one the most relevant issue in toxicology due to its relation with the development of mutations and cancer. Genotoxicity and oxidative damage have been described as the main mechanisms of toxicity of several NPs (Dusinska *et al*., 2015). Therefore, according to the reflections papers on the development of new nanomedicine products for human use published by the European Medicines Agency (EMA), as well as by several authors, the detection of DNA damage represents a crucially important endpoint for the safety assessment of NPs (Dusinska *et al*., 2015; EMA, 2006; Magdolenova *et al*., 2014). NPs do not have to react with the DNA to induce genotoxic lesions; their interaction with proteins, membranes and other cellular components can generate high amount of reactive oxygen species, which can damage DNA (Magdolenova *et al*., 2014). Moreover, reactive oxygen species can also be produced after an inflammatory reaction.

Gantrez® AN 119 NPs coated with mannosamine (GN-MA-NP) did not induce a significant level of DNA strand breaks and formamidopyridine DNA glycosilase (FPG)-sensitive sites after 3 and 24 h of incubation measured by the comet assay in Caco-2 cells. Nevertheless, it induced a very slight increase of the FPG-sensitive sites at 24 h of treatment at very high concentrations (i.e. 1 and 2 mg/mL) (Chapter 4). Furthermore, GN-MA-NP did not show potential genotoxic or mutagenic activities in mouse lymphoma cells (Chapter 5).

The evaluation of the potential *in vivo* toxicity of polymeric NPs is essential for acquiring more relevant information about human exposure to NPs. The *in vivo* mammalian alkaline comet assay is a simple method for measuring DNA damage in single cells from multiple animal tissues, usually rodents, that have been exposed to potentially genotoxic material. This assay has been validated by the Japanese Center for the Validation of Alternative Methods (JaCVAM), in conjunction with the European Centre for the Validation of Alternative Methods (ECVAM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) resulting in the OECD test guideline 489 (Morita *et al*., 2015; OECD 489, 2014). This assay detects DNA strand breaks (single and double strand) at the level of single cells. The combination of the comet assay with the use of different enzymes from the DNA repair system allows the detection of different types of DNA damages. For instance, the comet assay can be

modified for detecting oxidized purines, by incorporating the formamidopyridine DNA-glycosylase (FPG) enzyme (Dusinska & Collins, 1996). The enzymes detects base damages and cause additional DNA breaks increasing the amount of migrated DNA, improving the sensitivity and specificity of the comet assay (Azqueta *et al*., 2013).

Thus, the present study aimed at investigating the *in vivo* genotoxic potential GN-MA-NP using the *in vivo* comet assay in combination with the FPG in mice. The comet assay protocol used in our study complies with the OECD test guideline 489. Times points and organs were selected after performing an *in vivo* biodistribution study using fluorescence labelled GN-MA-NP.

2. Material and methods

2.1. Chemicals

For the preparation of the NPs, Gantrez® AN 119 and mannosamine were provided by ISP and Sigma, respectively. Acetone was obtained from VWR Prolabo. Alexa Cyanine5.5 carboxilic acid was provided by Interchim.

For the mincing solution, Mg^{++}, Ca^{++} and phenol red free Hank´s balanced salt solution were provided by Gibco, Na_2EDTA from Sigma, and dimethyl sulfoxide (DMSO) was purchased from Panreac.

For the comet assay, low melting point agarose, standard agarose, Triton X-100, Tris, HEPES, EDTA, BSA and ethyl methanesulfonate (EMS) were provided by Sigma. NaCl, NaOH and KCl were purchased from Panreac.

For the anaesthesia of mice isofluorane was used (Forane).

2.2. Preparation of GN-MA-NP

NPs from the copolymer between methyl vinyl ether and maleic anhydride (commercialized as Gantrez® AN 119) and, subsequently, covered with mannosamine were prepared. The

fabrication of this formulation was carried out as previously reported with slight modifications (Salman *et al*., 2006).

Briefly, 100 mg of the copolymer (Gantrez® AN 119) were dissolved and stirred in 5 mL acetone. In parallel, 5 mL water containing 10 mg mannosamine were added to the polymer solution under magnetic stirring and incubated for 30 min. NPs were obtained by the addition of 10 mL of absolute ethanol under magnetic stirring. The organic solvents were evaporated under reduced pressure. Finally, the NPs were purified by tangential filtration (3000 rpm, 5 min, 4°C) and spray-dried in a mini Spray dryer Büchi B290. The resulting powder was then stored at room temperature until its use. Resulting NP was called as GN-MA-NP.

2.2.1. Preparation of fluorescently labelled NPs

For the biodistribution study, GN-MA-NP was fluorescently labelled with Alexa-Cy5.5 carboxilic acid (GN-MA-NPAC5.5). For this purpose, 2 mg Alexa-Cy5.5 carboxilic acid was dissolved per every 50 mL of acetone containing the copolymer. The formulation was prepared as described above.

2.3. Characterization of NPs

The particle size, polidispersity index (PDI) and zeta potential of GN-MA-NP were determined by photon correlation spectroscopy and electrophoretic laser Doppler anemometry, respectively, using a ZetaPlus zeta potential analyzer with 90 Plus/BI-MAS Multi Angle Particle Sizing Option (Brookhaven Instruments Corporation). The diameter of these NPs was determined after their dispersion in ultrapurified water (1/10) and measured at room temperature using a scattering angle of 90°. The zeta potential of GN-MA-NP was determined by diluting the samples in a 0.1 mM KCl solution adjusted to pH 7.4. The particle size and PDI of GN-MA-NPAC5.5 were also measured.

The morphology of the GN-MA-NP was observed using a scanning electron microscope (Zeiss DSM 940A, Oberkochen, Germany) coupled with a digital image system (DISS, Point Electronic

GmBh). Before examining the NPs, they were diluted in purified water, centrifuged to eliminate sugar and shaded with a 12 nm gold layer in a Hemitech K 550 Sputter-Coater.

2.3. Biodistribution study

Biodistribution studies were carried out in accordance with the institutional and national guidelines for the welfare of laboratory animals and were approved by the Republic of Slovenia Ministry of Agriculture and the Environment, Veterinary Administration. (Permit No. 34401-38/2012/3).

Eight week old Balb/C mice were housed in the pathogen free animal facility at the Jozef Stefan Institute (Slovenia), with the food and water *ad libitum*. Alfa-alfa free Teklad global rodent diet 2016 was used to minimize background auto-fluorescence. GN-MA-NP-AC5.5 were resuspended in MilliQ-dH_2O to a concentration of 100 mg/mL. Mice were fasted 4 h prior the application and 500 mg of resuspended nanoparticles per bw were administered by oral gavage. Mice were anesthetized immediately after the administration using isofluorane (Forane) and imaged non-invasively using an IVIS Spectrum (PerkinElmer) whole body imaging system and LivingImage® software, version 4.3.1. Spectral unmixing of GN-MA-NP-AC5.5 and background fluorescence signal was performed by recording sequences of images using the following excitation/emission filter pairs: 675/720 nm, 675/740 nm, 675/760 nm, 675/780 nm and 500/740 nm, 500/760 nm respectively. Background fluorescence was removed by using background subtraction tool (LivingImage®). Region of interest (ROI) was set manually based on the body atlas of the mice from the software, accordingly for the stomach, small intestine, cecum and colon position and set to radiant efficiency (photons s-1 cm-2 steredia-1 per µW cm-2).

Animals were anesthetized during the first 30 min and then at the following time points: 1, 2, 4, 6, 8, 24 and 32 h.

2.4. *In vivo* comet assay

2.4.1. Animals and experimental design

The genotoxicity *in vivo* experiment was approved by the Ethics Committee on Animal Experimentation of the University of Navarra. Six-week-old male ICR mice, purchased from Harlan Iberica, were used. On the day of arrival, the animals were weighed (weight variation did not exceed ± 20%), and then distributed into polycarbonate cages with stainless steel covers. During one week, animals were allowed to acclimatize to the new environmental conditions: 12 h day/night cycle, temperature 22 ± 2°C, relative humidity 55 ± 10%, standard diet (Harlan Iberica) and water ad libitum.

GN-MA-NP was freshly prepared in distilled water and administered by oral gavage. GN-MA-NP was administered in doses of 0, 500, 1000 or 2000 mg/kg bw (OECD guideline 489: limit dose). Two sampling times were selected, 2 and 4 h, according to the *in vivo* biodistribution study. Five animals per sampling time and treatment were used. Animals were randomly distributed over the different treatment groups.

Distilled water was used as a vehicle control, whereas positive control animals received 200 mg/kg bw of EMS, administer orally, 2 or 4 h before being sacrificed by cervical dislocation. The volume of administration was 20 mL/kg bw.

At necropsy, tissues of the gastrointestinal tract were collected. Details of tissue processing are described below.

2.4.2 Tissue collection and fresh sample preparation

For each animal, stomach, duodenum, jejunum, ileum and colon were collected. The preparation of the different samples was performed as previously described with slight modifications (Hobbs *et al*., 2015; Recio *et al*., 2012). The different tissues were observed macroscopically.

Briefly, the stomach was cut open and washed free from food using cold mincing buffer (Mg^{++}, Ca^{++} and phenol red free Hank´s balanced salt solution; 20 mM Na_2EDTA, pH 7.5 and 10 % of DMSO, added immediately prior to use). The glandular stomach was placed into cold mincing buffer and incubated on ice for 30 min, then the surface epithelium was gently scraped two times using a scalpel blade. This layer was discarded and the gastric mucosa washed out with cold mincing buffer. The stomach epithelium was carefully scraped 6-7 times into 500 µL mincing solution with a scalpel blade to release single cells. In a similar way, a 4-5 cm length of colon was rinsed extensively with cold mincing solution and cut open longitudinally. The colon was lightly scraped with a scalpel (1-2 times) and flushed with cold mincing solution. This layer was removed. The colon epithelium was scraped approximately 5-6 times into 500 µL mincing solution and single cells were obtained.

In regard to the small intestine, a portion of the duodenum, jejunum and ileum was washed out with cold mincing solution and then trimmed extensively before being transferred to mincing solution. The mincing solution containing the cells of each tissue was pipetted up and down several times to help cell separation. After obtaining single cells suspensions from all tissues, cells were soaked in agarose in order to form gels for the comet assay.

2.4.3. Comet assay in combination with FPG-enzyme

The comet assay was carried out following the recommendations of OECD guideline 489 (OECD 489, 2014). Moreover, this assay was performed with the enzyme FPG in order to detect altered bases in addition to DNA strand breaks (SBs) and alkali-labile sites (ALS).

Ninety µL of cell suspensions of the different tissues (i.e. stomach, duodenum, jejunum, ileum and colon), were mixed with 420 µL of 1 % low melting point agarose. Subsequently, two 70 µL drops of mixture were placed onto a 1 % standard agarose pre-coated slide. Three 3 slides were prepared per sample. A cover slide was then placed on top of each drop in order to form 2x2 cm gels and the slides were then transferred to an ice-cold surface in order to solidify the gels. After solidification, cells were lysed overnight at 4°C in lysis buffer, containing 1% Triton X-100, in order to obtain the nucleoids.

Two of the slides were used for the analysis of the FPG-sensitive sites while the other slide restrained in lysis buffer. For detecting the FPG-sensitive sites, these two slides were washed three times with the enzyme reaction buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8). Afterwards, one of the slides was incubated with the enzyme reaction buffer and the other one with FPG during 30 min at 37°C in a humidified chamber. All slides were then immersed in electrophoresis buffer (1 mM EDTA, 0.3 M NaOH, pH > 13) for 40 min at 4 °C for alkaline unwinding. Afterwards, an electrophoresis was carried out during 20 min at 1.2 V/cm (300 mA) and at 4°C. Slides were then neutralized by washing twice using PBS followed by distilled water during 10 min at 4°C each. Before the analysis, slides were stained with 1 µg/mL of 4,6-diamidino-2-phenylindole (DAPI). Slides were then examined by fluorescent microscope (Nikon Eclipse 50 i, Japan) using the image analysis system Comet assay IV (Perceptive instruments) by scoring 75 comets per gel. The software is designed to differentiate comets head from tail, and to measure a variety of parameters, including tail length, % of total fluorescence in head and tail and tail moment. From all parameters provided by the software, percentage of DNA in tail (% tail DNA) was used as DNA damage indicator.

SBs (plus ALS) were assessed by determining the % tail DNA in lysis buffer slides (i.e. the ones which remained in lysis buffer until alkaline treatment). To calculate the net FPG-sensitive sites, the median % tail DNA in the slide treated with buffer F was subtracted from the % tail DNA in the slide treated with FPG.

2.4.4. Histopathological studies

Tissue samples from gastrointestinal tract were taken during necropsy and fixed in 4% formaldehyde solution.

Following the OECD guideline 489, histopathology was performed in the samples that showed statistically significant DNA damage compared to the vehicle control group. In this case, tissues were dehydrated, embedded in paraffin, cut to obtain 4 µm sections, mounted onto glass slides, deparaffinised and stained with haematoxylin and eosin for the subsequent histopathological examination.

2.5. Statistical analyses

The % tail DNA of a total of 150 randomly selected cells were analysed per slide, animal and tissue. The median of each sample was calculated and the mean ± standard deviation (SD) of the medians of each treatment group. The comparisons were performed using the Kruskal-Wallis test. Statistical significance was set at $p \leq 0.05$. Furthermore, dose-dependent effect was evaluated using linear regression.

According with the OECD 489 (Adopted on September 2014), acceptance criteria for positive results in the *in vivo* comet assay are: (i) to have at least one statistically significant treatment group ($p < 0.05$), (ii) to get a treatment group falling outside the range of laboratory historical control data, and (iii) to obtain a statistically significant trend test ($p < 0.05$).

3. Results

3.1. Characterization of GN-MA-NP

The main physico-chemical features of GN-MA-NP were a mean size of 239 ± 1.4 nm, with a PDI of 0.136 ± 0.015 and a negative zeta potential (-44.40 ± 0.82 mV). The morphological analysis of GN-MA-NP by scanning microscopy is shown in Fig. 1. GN-MA-NP presented a spherical shape and a rough surface. The particle size of GN-MA-NPAC5.5 was 190 nm with a PDI of 0.13.

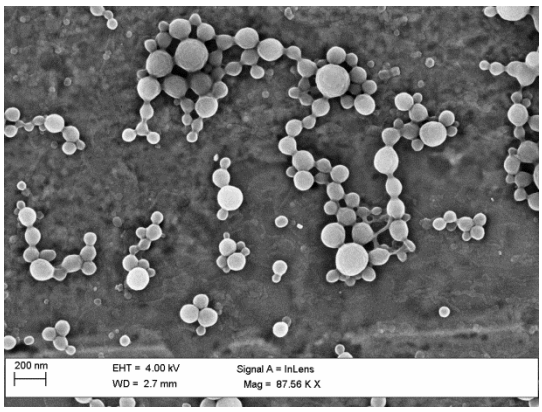


Fig. 1. SEM photographs of GN-MA-NP.

3.2. Biodistribution studies

A group of three mice was administered orally with the fluorescently labelled GN-MA-NPAC5.5 in order to determine the biodistribution profile of the NPs in mice (Fig. 2). The Alexa-Cy5.5 signal was reconstituted from a sequence of images using spectral unmixing algorithm in order to minimize the auto-fluorescence background effect as described previously (Berlec *et al.*, 2015). Radiant efficiency reached the highest signal 10 - 20 min post administration with 1.17 - 1.29 x 10^{11} (data not shown). Values then very slowly decreased over time, and reached near control level after 32 h (Fig. 2), indicating slow excretion of NPs from the gastrointestinal tract. Signal intensity values at time points up to 8 h revealed that NPs moved rapidly from stomach to small intestine (Fig. 2B and Fig. 2C); however, a significant portion of the NPs were detected in the stomach even up to 24 h post administration (Fig. 2B), suggesting that NPs exhibited mucus adhesive properties.

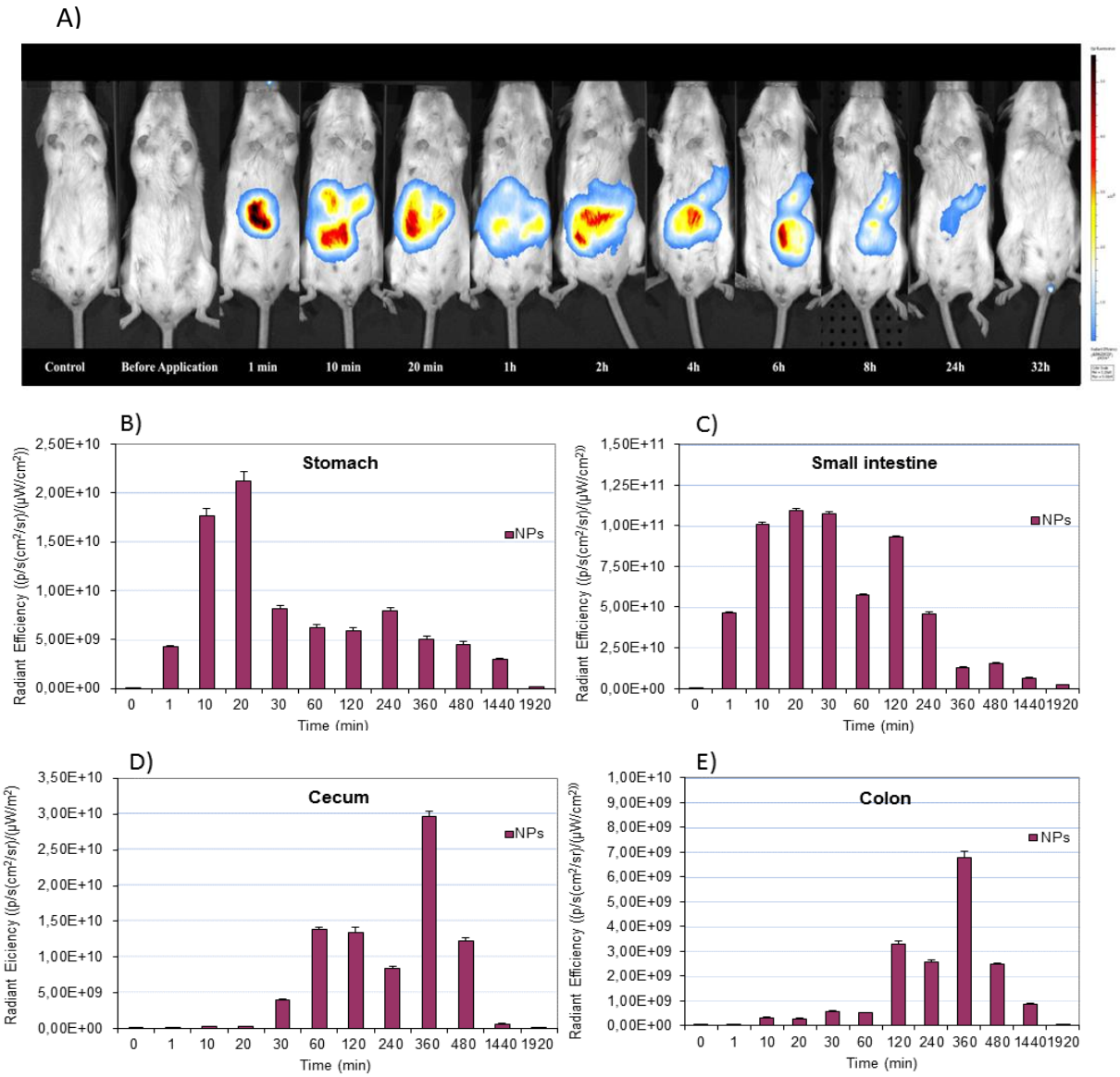


Fig. 2. Biodistribution. Time-course imaging of mice (n=3) after administration of 500 mg/kg GN-MA-NPAC5.5 labelled nanoparticles. (**A)** Representative images (epifluorescence) of mice at different time-points with localisation of signal and scale bar indicating radiant efficiency [(p/s/cm2/sr)/(µW/cm2)]. (**B-E)** Column chart for different sections of gastrointestinal tract, indicating signal intensity, retention time and cleareance of NPs in mice. Results presented as mean ± SD.

Signal intensity in the small intestine revealed that the particles can reach cecum 30 - 60 min post administration, however, due to their properties strong signal was detected for up to 6 h and then gradually dropped. In addition, signal intensity in cecum accumulated over time, reaching the strongest signal 6 - 8 h post administration (Fig. 2D). After 24 h signal intensity in cecum dropped significantly and reached near control level at 32 h (Fig. 2D). The colon section of the gastro intestinal tract was reached around 1 h post administration with the highest signal intensity 6 - 8h post administration, while the signal was present even after 24 h post administration (Fig. 2E).

Fluorescence data obtained from control animals were not include in the graphs due to the low values obtained (data not shown).

3.2. Genotoxicity in the gastrointestinal tract

DNA SBs+ALS, and net-FPG sensitive sites were evaluated with the comet assay in combination with FPG, at two different time points, in tissues of the gastrointestinal tract of mice treated orally with 500, 1000 and 2000 mg/kg bw of GN-MA-NP. There were no abnormal gross necropsy organ observations. In addition, digestive transit and stool consistency were normal in all experimental groups. Five tissues from each of five animals per treatment group were sampled: stomach, duodenum, jejunum, ileum and colon.

As shown in Fig. 3 and Fig. 4, the %tail DNA in SBs+ALS as well as the net FPG-sensitive sites significantly increased only in duodenal tissue after treatment with GN-MA-NP at 2000 mg/kg bw 2 and 4 h after dosing. This increase of SBs+ALS was higher at 2 h post exposure (13.44 ± 5.26 *vs* 3.99 ± 1.98 % tail DNA control vehicle, $p < 0.05$). Similarly, the net FPG-sensitive sites increase was also greater at 2 h of treatment (to 5.59 ± 5.04 *vs* 1.28 ± 2.03 % tail DNA control

vehicle, $p < 0.05$). Furthermore, these increases were dose-dependent, and were evaluated using linear regression analysis (Fig. 5).

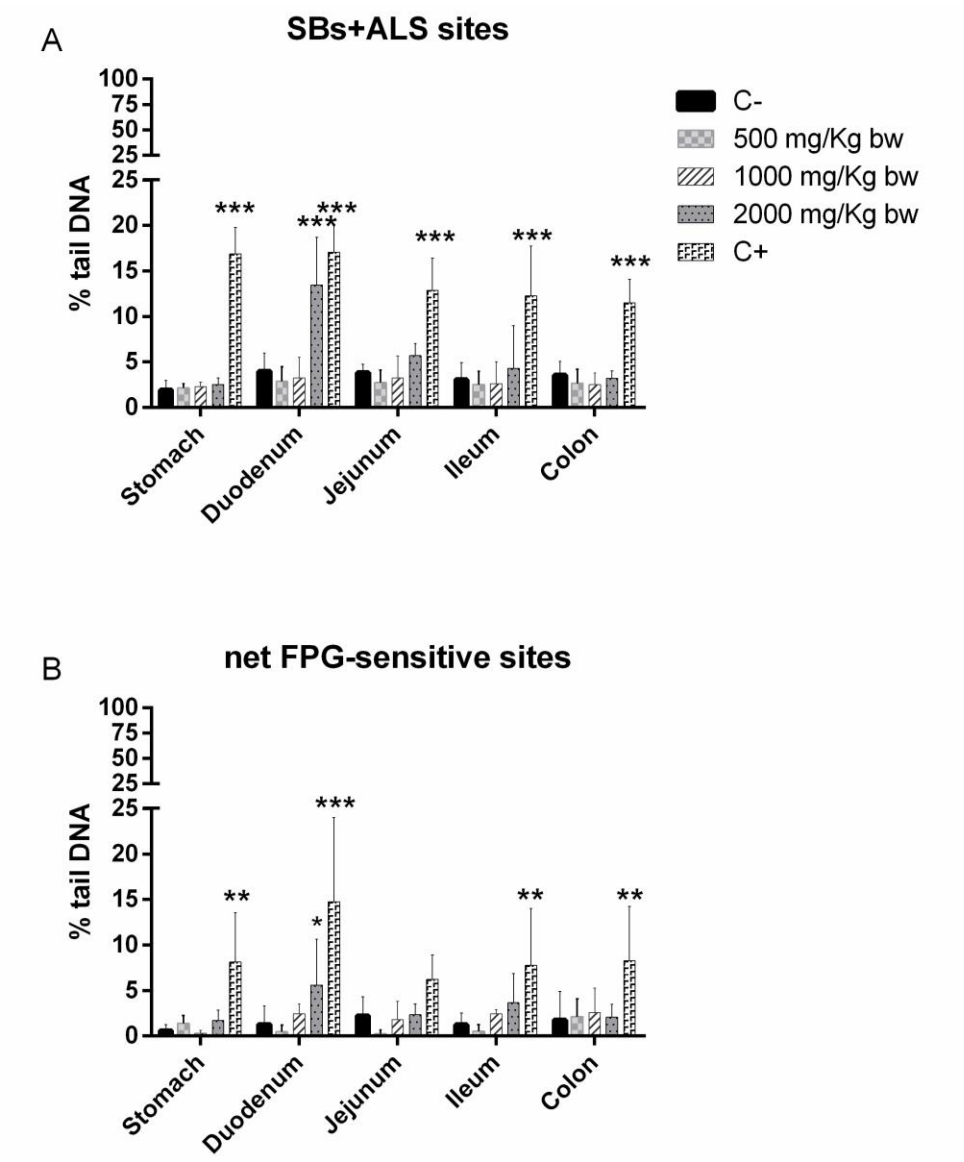


Fig. 3. Genotoxicity *in vivo*, 2 h. DNA strand breaks **(A)** and net FPG-sensitive sites **(B)** induced by the oral administration of 500, 1000 and 2000 mg/kg GN-MA-NP in the gastrointestinal tract of mice. Samples were taken 2 h after the administration. C+: animals administered with 200 mg/kg bw of EMS. Results presented as mean ± SD (n=5). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, significantly different from negative control.

No increase in the % tail DNA in SBs+ALS and net FPG-sensitive sites compared to the vehicle control group in the evaluated doses was found in other tested tissues (Fig. 3 and Fig. 4).

The positive control demonstrated a statistically significant DNA damage in all tissues investigated (Fig. 3 and Fig. 4); higher level of DNA damage was observed after 4 h treatment compared to 2 h. According to the recommendation of the OECD guideline 489, histopathology of the duodenum at the highest dose was carried out.

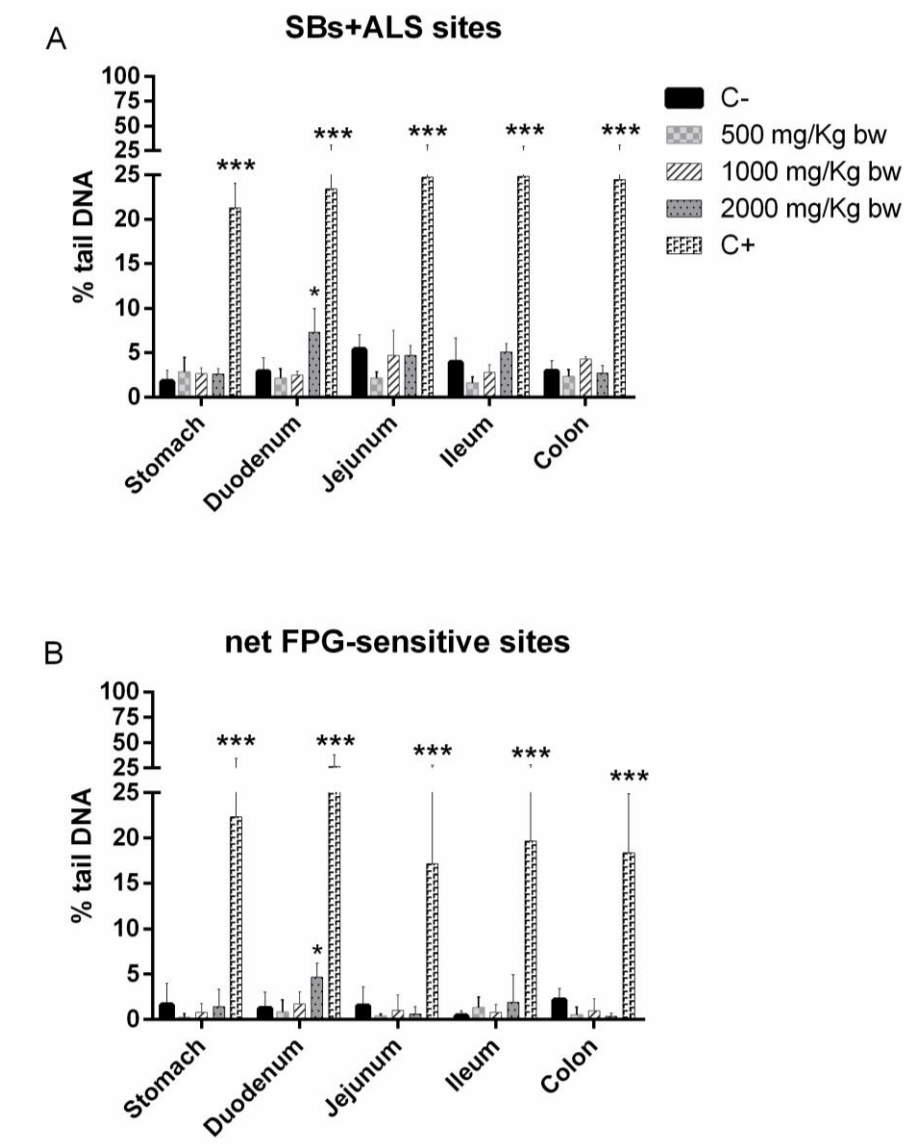


Fig. 4. Genotoxicity *in vivo*, 4 h. DNA strand breaks **(A)** and net FPG-sensitive sites **(B)** induced by the oral administration of 500, 1000 and 2000 mg/kg GN-MA-NP in the gastrointestinal tract of mice. Samples were taken 4 h after the administration. C+: animals administered with 200 mg/kg bw of EMS. Results presented as mean ± SD (n=5). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, significantly different from negative control.

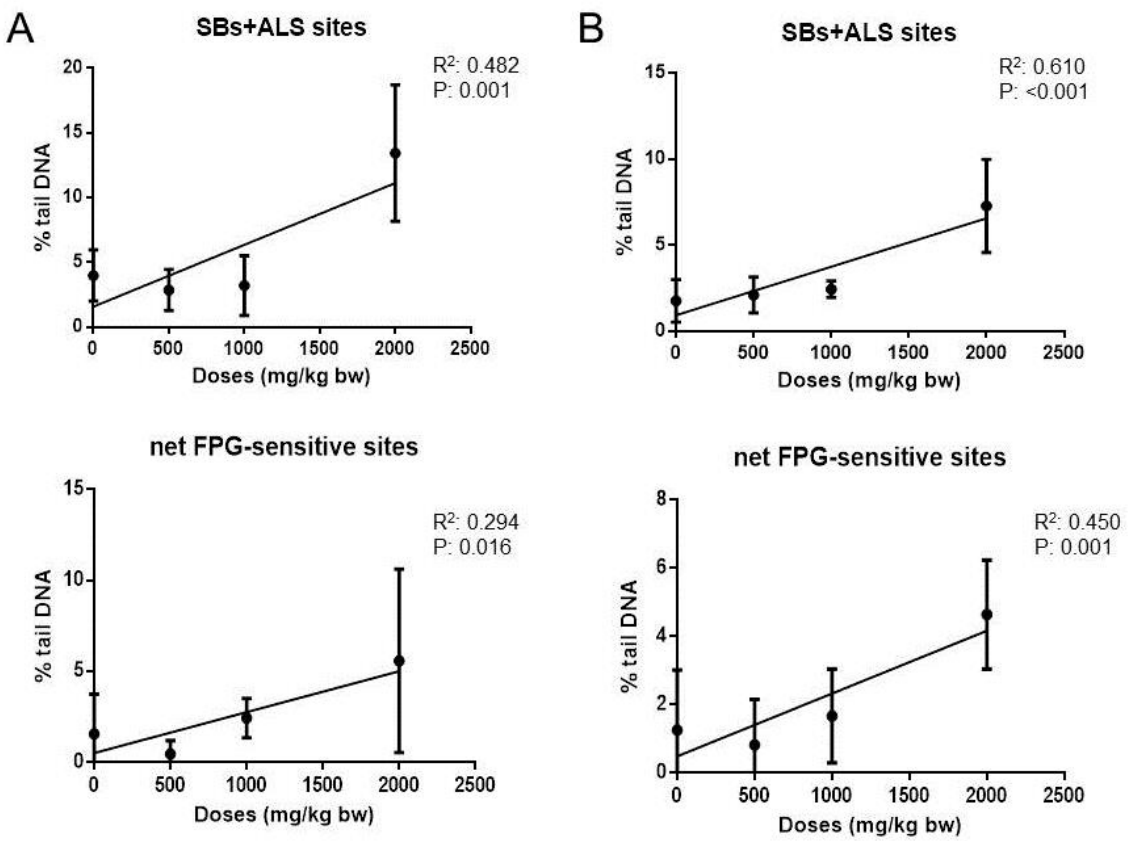


Fig. 5: Dose-dependent genotoxicity in duodenum. Liner regression of the DNA strand breaks and net FPG-sensitive sites induced by the oral administration of 500, 1000 and 2000 mg/kg GN-MA-NP in the duodenum tissue 2 h **(A)** and 4 h post-admisnistration **(B)**. Results presented as mean ± SD (n=5).

3.2. Histopathological studies

Following the OECD guideline 489, histopatology of the duodenum tissue of animals administered with 2000 mg/kg bw was carried out.

As shown in Fig. 6, the duodenum tissue of treated animals did not present histopathologic changes compared to control ones. Thus, general morphology, length and characteristics of the villi, as well as cell composition were similar to the negative control samples. Furthermore, the extent of inflammatory infiltration in the lamina propria was also similar to that observed in the negative control.

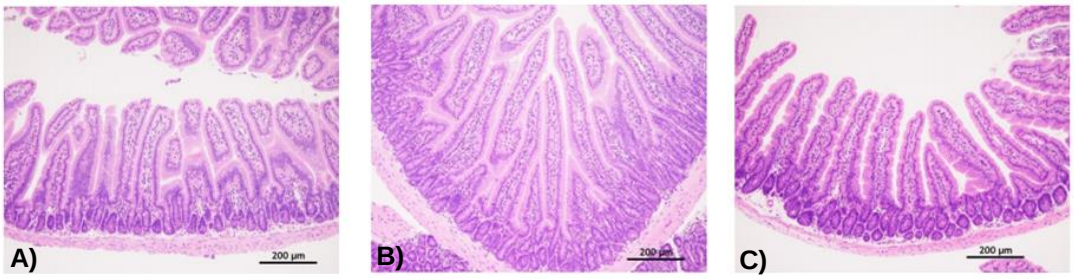


Fig. 6. Histological sections of mice terminal duodenum tissue. Negative control **(A)**, duodenum tissue exposed to 2000 mg/kg bw of GN-MA-NP at 2 h **(B)** and at 4 h **(C)**.

4. Discussion

The oral route is one of the common and preferred ways for drug delivery. However, several drugs, when administered orally, are poorly available in the blood stream. There are many facts that influence this bioavailability, but the main ones are the mucosal permeability of a drug and the restriction of the permeability of a region of the gastrointestinal tract. The use of biodegradable NPs with bioadhesive properties, such as GN-MA-NP, has arisen as a possible strategy to overcome these obstacles. However, strong interactions of such NPs with the epithelial mucosa may cause toxicity of the cells of the gastrointestinal tract (Hartmann *et al*., 2003) leading to an enormous risk for human health.

The design of an appropriate regulatory framework for the assessment of the nanomaterials safety in pharmaceuticals or medicinal products for human use is hindered by the absence of sufficient data on NPs safety. Thus, establishing the entire toxicological profile is essential for evidencing the safety of NPs for human use, which has been the main objective of the current investigation.

Previous *in vitro* studies have demonstrated the absence of cytotoxicity, genotoxicity and mutagenicity in cells exposed to GN-MA-NP (Chaper 4; Chapter 5). However, it induced a very slight and non-significant dose dependent increase of the FPG-sensitive sites at 24 h of treatment (Chapter 4). Although these results pointed to a harmless effect of GN-MA-NP *in vitro*, further studies need to be carried out in order to obtain a complete and relevant toxicological profile for these nanomaterials. A clearly defined strategy for the assessment of NPs safety in

humans has not yet been established. Anyhow, EMA presents several reflections, as well as Dusinska and colleagues, thereby proposing a testing strategy for NPs (Dusinska *et al*., 2015; EMA, 2006).

An important aspect in this evaluation is the *in vivo* studies, including *in vivo* genotoxicity studies. As out, genotoxicity is a key point in the toxicological evaluation due to its close relation with the indication of mutations and cancer. For this purpose, the *in vivo* alkaline comet assay, which is especially relevant to assess potential genotoxic hazard in nanotoxicological studies (Azqueta & Dusinska, 2015) was performed in the current work.

Biodistribution studies were carried out in order to determine the relevant tissues and the time points (Lee *et al*., 2012; Ma *et al*., 2015; Panthani *et al*., 2013). This study showed the biodistribution profile of fluorescently labelled nanoparticles through the gastro intestinal tract at different time points on which the *in vivo* genotoxicity was further based. Data revealed that GN-MA-NP-AC5.5 particles have high bioadhesive properties, since the signal intensity was high even after 24 post administration in the stomach, with prolong retention also in other parts of the small intestine, cecum and colon. However the strongest signal intensities were for the majority of the gastro intestinal tract between 2 and 4 h post administration, therefore these time points were selected for further genotoxicity experiments. Salman *et al.* (2006) reported a nearly total elimination of the Gantrez® AN 119 NPs coated with mannosamine in about 8 h. In our study NPs were still present in the gastrointestinal tract even 32 h after the administration. Differences in both studies may be due to the different experimental system used (rat *vs* mice), the different concentrations tested (45 mg/kg *vs* 500 mg/kg) and the fact that NPs are not exactly the same. Salman *et al.* (2009) demonstrated that Gantrez® AN 119 NPs coated with mannosamine were always localized in the gastrointestinal tract; they were not absorbed or distributed to other organs. A likely explanation for the NPs uptake in the small intestine is the presence of mannose receptor in the Peyer's patches.

Another critical variable is the sampling time. According to the OECD guideline 489, it is determined by the period needed for the test product to reach the maximum concentration in the

target tissue and for inducing SBs+ALS sites before those breaks are removed, repaired or have led to cell death (Hartmann *et al*., 2003; OECD, 2014). Taking into account the OECD guideline and the biodistribution data, 2 sampling times were selected at 2 and 4 hour time points.

The *in vivo* genotoxicity study was carried out on five tissues of the gastrointestinal tract of mice (i.e. stomach, duodenum, jejunum, ileum and colon) with selected time points post administration of GN-MA-NP using the comet assay in combination with FPG-enzyme for detecting SBs+ALS sites and oxidative DNA damage. Animals received 500, 1000 and 2000 mg/kg bw of GN-MA-NP by oral gavage. In a preliminary assay, the maximum tolerated dose of very similar NPs (i.e. small changes in the production process) in Balb/c mice could not be determined, proving to be greater than 2000 mg/kg bw (maximum dose tested) (unpublished results). Thus, according to the guideline 489, for a non-toxic test chemical with an administration period of less than 14 days, the procedure of the limit test was applied and a single dose of 2000 mg/kg was administered by gavage. In this study, 500 and 1000 mg/kg were also used. Doses from 167 mg/kg to 500 mg/kg of Gantrez® AN 119 NPs coated with cyclodextrin and poly-ethylene glycol were used to administer a therapeutic dose of paclitaxel in mice (Calleja *et al*., 2015).

Results showed a statistically significant induction of SBs+ALS sites and net-FPG sensitive sites in duodenum tissue of animals exposed to 2000 mg/kg bw of GN-MA-NP at both time points (Fig. 3 and Fig. 4). However, this effect was not observed at lower doses or in other organs.

The level of SBs+ALS was higher at the 2 hour time point and it decreased after 4 h, which suggests that the DNA damage has begun to be repaired. However, net-FPG sensitive sites showed similar results at both sampling times. Moreover, both types of damage, SBs+ALS sites and net-FPG sensitive sites, showed to be dose-dependent (Fig. 4).

FPG-enzyme is considered a useful addition to the standard comet assay in genotoxicity testing to detect genotoxic chemicals that do not directly induce strand breaks (Azqueta *et al*., 2013). FPG is not specific for oxidised purines, but it also detects alkylated guanines (Speit *et al*., 2004). However, taking into account the nature of the NPs tested, the FPG-sensitive sites detected in duodenum tissue are likely a consequence of the presence of oxidized bases in the DNA. As GN-MA-NP are bioadhesive NPs, base oxidation probably result from their interaction with the plasmalemma, which generate high amount of reactive oxygen species (Magdolenova *et al*., 2014).

The decrease in the level of SBs+ALS observed in the duodenum tissue (i.e from 2 to 4 h after administration) may be due to the DNA repair process; moreover, these lesions are probably formed during the repair of oxidised bases. Oxidised bases do not virtually decrease because of the balance of DNA repair and DNA oxidations since GN-MA-NP are still present.

The positive control (200 mg EMS /kg bw) demonstrated statistically significant SBs+ALS sites and oxidative damage in all tissues investigated (Fig. 3 and Fig. 4). Moreover, this damage was time-dependent, as following longer period of exposure a greater damage was observed.

To demonstrate that these increased levels of DNA damage were not due to cytotoxicity or cell death, histopathological analyses were carried out to 2000 mg/kg bw at both sampling times. In the studied animals, increased DNA migration accompanied by non-decreased viability was observed in duodenum cells of treated animals thereby suggesting that the enhanced damage observed at the level of the duodenum is directly related to GN-MA-NP genotoxicity

Gantrez® AN 119 NPs coated with mannosamine (i.e. similar to the ones tested in this work) were not absorbed or distributed to other organs but the gastrointestinal tract after oral administration, but were found in Peyer’s patches (Salman *et al*., 2009). In our study, each tissue evaluated from the intestine contained Peyer´s patches. Nevertheless, DNA damage was not observed in the ileum, where Peyer's patches are more frequent.

According to the OECD guideline 489, GN-MA-NP is clearly genotoxic, since our data showed one statistically significant treatment group (2000 mg/kg bw) and a statistically significant trend among the tested doses. However, the dose at which this damage occurs at the duodenum is the limit dose established by the guideline 489 (OECD 489, 2014). We therefore believe that exposure to such high concentrations has the potential to induce positive results, which are not relevant to real exposure levels. In order to avoid artefactual positive results, O Donovan & Burlinson pointed out the importance of the exposure levels, using doses close to the therapeutic dose and not the maximum tolerated levels or limit dose levels in the products designed not to leave the gastrointestinal tract (O'Donovan & Burlinson, 2013). In the case of nanocarriers, a relevant dose will depend on drug loading capacity which will directly depend on the type of drug. Calleja *et al.* (2014) used 167 mg/kg of Gantrez® AN 119 NPs coated with cyclodextrin or 500 mg/kg of Gantrez® AN 119 NPs coated with poly-ethylene glycol to orally administer a therapeutic dose of paclitaxel in mice. Salman *et al.* (2009) used about 11 mg of Gantrez® AN 119 NPs coated with mannosamine to orally administer 100 µg of ovalbumin, enough to induce a balanced systemic specific antibody response in mice. Defining a relevant dose in case of nanocarriers is difficult, but we can say that the lower dose used in this study is the most relevant one.

The present study has provided essential information contributing to the overall safety profile of GN-MA-NP for using it as drug carrier. Depending on the encapsulation efficiency, as well as the type of the drug, different doses of NPs will be administered. Therefore, GN-MA-NP can be administered up to a dose of 1000 mg/kg bw without entailing any health risk, whereas higher doses may represent a risk.

In conclusion, due of the absence of toxic effects at 1000 mg/kg bw, as well as its unique properties such as biodegradability, biocompatibility, strong bioadhesion to the gut mucosa, modifiable surface and easy production, GN-MA-NP are promising nanocarriers in medical applications such as drug delivery systems, when orally administered.

5. Acknowledgements

This work was supported by the ALEXANDER project (FP7-2011-NMP-280761). TI was financially supported by a grant from the Asociación de amigos at the University of Navarra. AA thanks the Ministerio de Economía y Competitividad ('Ramón y Cajal' programme, RYC-2013-14370) of the Spanish Government for personal support.We also thank Professor Andrew AR. Collins, Oslo University, for enzyme-FPG gift and Hoffmann-La Roche (Brasilea, Switzerland) for providing the photosensitiser (Ro 19-8022).

6. References

Agüeros, M., Areses, P., Campanero, M.A., Salman, H., Quincoces, G., Peñuelas, I., Irache, J.M., 2009. Bioadhesive properties and biodistribution of cyclodextrin-poly(anhydride) nanoparticles. European Journal of Pharmaceutical Sciences. 37 (3-4), 231-240.

Arbós, P., Wirth, M., Arangoa, M.A., Gabor, F., Irache, J.M., 2002. Gantrez® AN as a new polymer for the preparation of ligand–nanoparticle conjugates. Journal of Controlled Release. 83 (3), 321-330.

Azqueta, A., Arbillaga, L., López de Cerain, A., Collins, A.R., 2013. Enhancing the sensitivity of the comet assay as a genotoxicity test, by combining it with bacterial repair enzyme FPG. Mutagenesis. 28 (3), 271-277

Azqueta, A & Dusinska, M., 2015. The use of the comet assay for the evaluation of the genotoxicity of nanomaterials. Frontiers in Genetic. 6, 239-243.

Calleja, P., Espuelas, S., Vauthier, C., Ponchel, G., Irache, J.M., 2015. Controlled release, intestinal transport, and oral bioavailablity of paclitaxel can be considerably increased using suitably tailored pegylated poly(anhydride) nanoparticles. Journal of Pharmaceutical Sciences. 104 (9), 2877-2886.

Dusinska, M & Collins, A., 1996. Detection of oxidised purines and UV-induced photoproducts in DNA, by inclusion of lesion-specific enzymes in the comet assay (single cell gell electrophoresis). ATLA. 24, 405-411.

Dusinska, M., Boland, S., Saunders, M., Juillerat-Jeanneret, L., Tran, L., Pojana, G., Marcomini, A., Volkovova, K., Tulinska, J., Knudsen, L.E., Gombau, L., Whelan, M., Collins, A.R., Marano, F., Housiadas, C., Bilanicova, D., Halamoda Kenzaoui, B., Correia Carreira, S., Magdolenova, Z., Fjellsbø, L.M., Huk, A., Handy, R., Walker, L., Barancokova, M., Bartonova, A., Burello, E., Castell, J., Cowie, H., Drlickova, M., Guadagnini, R., Harris, G., Harju, M., Heimstad, E.S., Hurbankova, M., Kazimirova, A., Kovacikova, Z., Kuricova, M., Liskova, A., Milcamps, A., Neubauerova, E., Palosaari, T., Papazafiri, P., Pilou, M., Poulsen, M.S., Ross, B., Runden-Pran, E., Sebekova, K., Staruchova, M., Vallotto, D., Worth, A., 2015. Towards an alternative testing strategy for nanomaterials used in nanomedicine: Lessons from NanoTEST. Nanotoxicology. 9, 118-132.

EMA, 2006. Reflection paper on nanotechnology-based medicinal products for human use. EMEA/CHMP/79769/2006.

Hartmann, A., Agurell, E., Beevers, C., Brendler-Schwaab, S., Burlinson, B., Clay, P., Collins, A., Smith, A., Speit, G., Thybaud, V., Tice, RR., 2003. Recommendations for conducting the *in vivo* alkaline comet assay: 4th International comet assay workshop. Mutagenesis. 18 (1), 45-51.

Hobbs, C.A., Swartz, C., Maronpot, R., Davis, J., Recio, L., Koyanagi, M., Hayashi, S., 2015. Genotoxicity evaluation of the flavonoid, myricitrin, and its aglycone, myricetin. Food and Chemical Toxicology. 83, 283-292.

Inchaurraga, L., Martín-Arbella, N., Zabaleta, V., Quincoces, G., Peñuelas, I., Irache, J.M., 2015. *In vivo* study of the mucus-permeating properties of PEG-coated nanoparticles following oral administration. European Journal of Pharmaceutics and Biopharmaceutics. 97 (Pt A), 280-289.

Lee, C.M., Jeong, H.J., Yun, K.N., Kim, D.W., Sohn, M.H., Lee, J.K., Jeong, J., Lim, S.T., 2012. Optical imaging to trace near infrared fluorescent zinc oxide nanoparticles following oral exposure. International Journal of Nanomedicine. 7, 3203-3209.

Ma, Y., Fuchs, A.V., Boase, N.R.B., Rolfe, B.E., Coombes, A.G.A., Thurecht, K.J., 2015. The *in vivo* fate of nanoparticles and nanoparticle-loaded microcapsules after oral administration in mice: Evaluation of their potential for colon-specific delivery. European Journal of Pharmaceutics and Biopharmaceutics. 94, 393-403.

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, 233-278.

Moreno, E., Schwartz, J., Larrañeta, E., Nguewa, P.A., Sanmartín, C., Agüeros, M., Irache, J.M., Espuelas, S., 2014. Thermosensitive hydrogels of poly(methyl vinyl ether-co-maleic anhydride)- Pluronic® F127 copolymers for controlled protein release. International Journal of Pharmaceutics. 459 (1-2), 1-9.

Morita, T., Uno, Y., Honma, M., Kojima, H., Hayashi, M., Tice, R.R., Corvi, R., Schechtman, L., 2015. The JaCVAM international validation study on the *in vivo* comet assay: Selection of test chemicals. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 786-788, 14-44.

O'Donovan, M & Burlinson, B., 2013. Maximum dose levels for the rodent comet assay to examine damage at the site of contact or to thegastrointestinal tract. Mutagenesis. 28 (6), 621-623.

OECD, 2014. Test Guideline 489. OECD guideline for the testing of chemicals. *In vivo* mammalian alkaline comet assay. In: OECD Guidelines for testing of chemicals.

Panthani, M.G., Khan, T.A., Reid, D.K., Hellebusch, D.J., Rasch, M.R., Maynard, J.A., Korgel, B.A., 2013. *In vivo* whole animal fluorescence imaging of a microparticle-based oral vaccine containing (CuInSe(x)S(2-x)/ZnS core/shell quantum dots. Nano Letters. 13 (9), 4294-4298.

Porfire, A.S., Zabaleta, V., Gamazo, C., Leucuta, S.E., Irache, J.M., 2010. Influence of dextran on the bioadhesive properties of poly(anhydride) nanoparticles. International Journal of Pharmaceutics. 390 (1), 37-44.

Recio, L., Kissling, G.E., Hobbs, C.A., Witt, K.L., 2012. Comparison of comet assay dose-response for ethyl methanesulfonate using freshly prepared versus cryopreserved tissues. Environmental and Molecular Mutagenesis. (2), 101-113.

Salata, O., 2004. Applications of nanoparticles in biology and medicine. Journal of Nanobiotechnology. 2, 3-9.

Salman, H.H., Gamazo, C., Campanero, M., Irache, J.M., 2006. Bioadhesive mannosylated nanoparticles for oral drug delivery. Journal of Nanoscience and Nanotechnology. 6 (9-10), 3203-3209.

Salman, H.H., Irache, J.M., Gamazo, C., 2009. Immunoadjuvant capacity of flagellin and mannosamine-coated poly(anhydride) nanoparticles in oral vaccination. Vaccinne. 27 (35), 4784-4790.

Speit, G., Schutz, P., Hoffmann, H., 2004. Enhancement of genotoxic effects in the comet assay with human blood samples by aphidicolin. Toxicology Letter. 153, 303-310.

Yoncheva, K., Gómez, S., Campanero, M.A., Gamazo, C., Irache, J.M., 2005. Bioadhesive properties of pegylated nanoparticles. Expert Opinion on Drug Delivery. 2 (2), 205-218.

Chapter 7: Discussion

1. Poly(anhydride) NPs based on Gantrez® AN 119 as drug carriers

Nanotechnology is one of the fastest growing and most promising technologies in our society regarding human health. It can be applied to many areas, such as, cosmetics, food and pharmaceuticals. However, their potential effects on the environment and human health of several products containing NMs, which are already on the market or under research, are not yet fully understood. Thus, there is a serious concern about the human risk that NMs may pose.

Regarding NMs medical applications, their use in drug delivery systems may be actually hampered by their biological behaviour and their toxicological properties. So, in the last years nanotoxicology has emerged as the cornerstone for recognizing and avoiding potential health risks associated with NMs.

The oral route is widely common and it is the preferred way for drug delivery. In order to overcome the main obstacles of oral drug administrations, i.e. their poor absorption in the gastrointestinal tract, different strategies are being investigated. The use of poly(anhydride) NPs by employing Gantrez® AN 119 copolymer as drug carriers has been successfully used. Gantrez® AN 119 NP showed strong bioadhesion to the gut mucosa increasing the residence time of the formulation in close contact with the mucosal epithelium and, thus, increasing the potential drug absorption and bioavailability (Arbós *et al*., 2002; Irache *et al*., 2005). In addition, their surface can be easily modified with different ligands in order to modify their *in vivo* biodistribution and their affinity for the intestinal mucosa (Arbós *et al*., 2002; Irache *et al*., 2005; Salman *et al*., 2006, 2008; 2009; Yoncheva *et al*., 2005).

Previous studies demonstrated that Gantrez® AN 119 NPs coated with different ligands are capable of establishing bioadhesive interactions with Caco-2 cells without being internalized (Ojer *et al*., 2013). In addition, several *in vivo* assays also showed that, after oral administration, Gantrez® AN 119 -based NPs are not absorbed or distributed to other organs (Agüeros *et al*., 2009; Arbós *et al*., 2002; Inchaurraga *et al*., 2015; Porfire *et al*., 2010; Yoncheva *et al*., 2005). However, Gantrez® AN 119 NPs coated with mannosamine are taken up by Peyer's patches,

probably due to the presence of mannose receptors in the gastrointestinal tract (Salman *et al*., 2006).

In addition, the efficacy of Gantrez® AN 119-based NPs for transporting therapeutic agents has been proved (Calleja *et al*., 2014; Salman *et al*., 2009). Moreover, Salman *et al.* (2009) used about 11 mg of Gantrez® AN 119 NPs coated with mannosamine to orally administer 100 µg of ovalbumin, which was enough to induce a balanced systemic and specific antibody response in mice. In addition, Calleja *et al.* (2014) used 167 mg/kg of Gantrez® AN 119 NPs coated with cyclodextrin or about 500 mg/kg of Gantrez® AN 119 NPs coated with poly-ethylene glycol to orally administer a therapeutic dose of paclitaxel in mice.

2. Problems evaluating NPs toxicity

One of the biggest problems in nanotoxicology is to face the lack of standardized assays to evaluate NPs safety. It is very common to use standardized assays for the assessment of chemicals toxicity (i.e. OECD guidelines) and to try to adapt them to assess NPs toxicity. Nevertheless, the unique properties of NPs increase the likelihood of interfering with *in vitro* assays (Kroll *et al*., 2012). Commonly used assays such as lactate dehydrogenase, MTT, ATP detection, among others, are frequently reported to be affected by a range of different NPs (Iglesias *et al*., 2015). Preliminary studies carried out in our laboratory indicated that Gantrez® AN 119-based NPs interfered in the measurement with proteases activity detection using luminometry; the presence of NPs in the solution induces a light scattering phenomenon which produces an extremely high signal. Changing the protocol by washing cells, thus removing the NPs, and lysing the intact cells, allows to get reliable measurements.

Regarding *in vitro* genotoxicity studies, the three most used assays in regulatory studies are the Ames test, the micronucleus assay and the MLA. However, NPs interferences were reported with the first two (Azqueta & Dusisnka, 2015). On one hand, the Ames test, which is the most used *in vitro* mutagenicity test, is not suitable for testing NPs due to the limited penetration of NPs thorough the bacteria wall. On the other hand, the micronucleus test is usable but needs slight modifications in the protocol, such as the incubation with NPs before adding cytochalasin

B (cytochalasin B inhibits endocytosis, thus it is likely to prevent uptake of NPs). Nevertheless, the MLA is, with no modifications, an appropriate test to assess the mutagenicity of NPs.

The comet assay is the most used technique for assessing the *in vitro* genotoxicity of nanomaterials (Magdolenova *et al*., 2004). However some interferences have also been described; NP can be present in the gels if not well washed or uptaken by the cell (Karlsson, 2010; Stone *et al*., 2009) and even interfere with the FPG enzyme (Kain *et al*., 2012). The presence of the NPs in the gels implies that they can be in contact with the naked DNA and induce extra DNA lesions, though some authors demonstrate that the potential additional damage is not significant (Karlsson *et al*., 2015). In preliminary studies, NPs were found in the gels of ML cells treated with 1 or 2 mg/mL of GN-NP and GN-MA-NP. ML cells grow in suspension, so they are washed by centrifugation; although several centrifugations at different speed were used, it was impossible to remove the NPs. Since 1 and 2 mg/mL are very high concentrations, we decided not to test them in the definitive study. Curiously, this phenomenon was not observed when Caco-2 cells, adherent cell line, were treated with the same high concentrations.

In vivo techniques are not so troublesome. The *in vivo* micronucleus assay, followed by the *in vivo* comet assay, is the most used technique for *in vivo* genotoxicity assessment of NPs. In this study, we performed the *in vivo* comet assay since it gives the opportunity to check the DNA damage in several organs and it detects DNA damage induced by oxidative stress and inflammation. With respect to the micronucleus and the chromosome aberration tests, they are not suitable for assessing the oral genotoxicity of Gantrez® AN 119 NPs since they are not absorbed.

3. Toxicity evaluation of poly(anhydride) NPs based on Gantrez® AN 119

Nowadays, there is a lack of toxicity studies of polymeric NPs. Perhaps it is because these type of NPs are not expected to cause any damage due to its unique properties such as, biodegradability and biocompatibility. In the case of Gantrez® AN 119-based NPs, it has been demonstrated that naked Gantrez® AN 119 NPs and Gantrez® AN 119 NPs coated with 2-

hydroxypropyl-β-cyclodextrin and poly-ethylene glycol 6000 were non-cyototoxic in Caco-2 and HepG2 cells after 24 h of exposure at very high concentrations (i.e 2 mg/mL) (Ojer *et al*., 2013). In addition, acute and sub-acute toxicity (28 days) studies of these NPs orally administered to rats at doses up to 2000 mg/kg bw and 300 mg/kg bw, respectively, demonstrated the absence of adverse effects related to either the treatment or the sex of the animals (Ojer *et al*., 2012).

In the present work, Gantrez® AN 119 NPs (GN-NP) combined with the following hydrophilic ligands: aminodextran (GN-ADEX-NP), dextran (GN-DEX-NP), 2-hydroxypropil-β-cyclodextrin (GN-HPBCD-NP), mannosamine (GN-MA-NP) and poly-ethylene glicol (GN-PEG-NP), have been evaluated.

Several properties of NMs such as, small size, shape, large surface area and surface reactivity can contribute to their toxicological profile by diverse mechanisms (Iglesias *et al*., 2015). Therefore, their proper characterisation is also of utmost importance. All tested NPs presented a very similar profile with mean size of around 200 nm, low PDI, which indicates a monodisperse character, spherical form, and negative Z potential, which indicates a lower tendency to form aggregates. Naked NPs presented a smooth surface while the coated ones were rough. These characteristics were very similar when they were labelled to carry out some of the determinations (i.e. mucus permeation capacity and biodistribution).

For the selection of the most promising nanocarriers, cytotoxicity, *in vitro* genotoxicity and *in vitro* mucus permeation capacity were determined. Tested NPs did not affect the metabolism of Caco-2 and HT29-MTX cells, after 24 h of treatment even at very high concentrations (i.e. 2 mg/mL). Neither did they affect their viability nor cell membrane integrity of Caco-2 cells, despite being bioadhesive. Moreover, they did not induce DNA SBs or oxidized bases after 3 h of treatment in the same cell line. However, they showed very different mucus permeation profile being GN-MA-NP the most permeable ones.

The stability of the NPs in the conditions tested was assured even at the highest concentration tested (i.e. 2 mg/mL). This is critical since chemical properties of NPs can change in different

solutions and influence their physico-chemical characteristics and so their potential toxicity (Handy *et al*., 2008).

Genotoxicity assessment is an essential aspect in the evaluation of NPs safety; increased DNA damage has been associated with higher frequency of cancer and other health consequences. This endpoint is critical as oxidative stress and genotoxicity are the main mechanisms of NPs toxicity (Dusinska *et al*., 2015). However, genotoxicity of Gantrez® AN 119-based NPs and similar NPs have not been studied since it is not expected to observe significant toxic effects due to the characteristics of these NPs such as, biodegradability, biocompatibility and the lack of cellular internalization. Nevertheless, it has been shown that some NPs deposited on the cellular surface induce oxidative stress signaling cascades (Manke *et al*., 2013), which may induce the oxidation of the DNA among other cell components. Therefore, it is important to study the genotoxicity induced by oxidative stress of these NPs.

Selected NPs, GN-MA-NP and GN-NP (selected as a control) induced a slight increase in the level of oxidized DNA bases of Caco-2 cell after 24 h of treatment. This increase may not be of any biological relevance due to the low level of damage and the not-relevant high concentrations required (i.e. 1 and 2 mg/mL). Moreover they did not induce DNA SBs, oxidized bases or mutations in ML cells treated during 24 hour with concentrations up to 600 µg/mL.

In vivo assays are of great importance and necessary; they will verify the result of the *in vitro* assays and ensure the safe use of the products. In our case, to verify the negative genotoxic potential found in *in vitro* assay, an *in vivo* genotoxicity study was performed. GN-MA-NP induced a dose-dependent and statistically significant increase of SBs+ALS sites and oxidized bases, in duodenum tissue of animals orally exposed to 2000 mg/kg bw of GN-MA-NP, a non-relevant exposure level, for 2 and 4 hour. However, this effect was not observed at more realistic doses (i.e. 1000 and 500 mg/kg bw) or in other organs (stomach, duodenum, jejunum, ileum and colon).

After performing the present work, the following step is to improve the toxicological profile of GN-MA-NP by performing an *in vivo* genotoxicity repeated-dose study using conditions (i.e. dose and administration schedule) close to the therapeutic use.

Nowadays, the effect of these NPs in the microbiota is not known. This may be very relevant since these NPs interact with the mucus of the gastrointestinal tract. The mucus layer is a critical component in maintaining intestinal homeostasis, including the microbiota, and can be modified by external agents (Faderl *et al*., 2015). Changes in the microbiota are associated with several diseases such as cancer, neurodegenerative diseases and even mental diseases, among others (Bressan & Kramer, 2016; Ghaisas *et al*., 2016; Paul *et al*., 2016; Schroeder & Bäckhed, 2016). Moreover, these interaction can disrupt the metabolism of a drug and so its effect (Kang *et al*., 2013). The study of the microbiota is one of the key points that toxicology has to tackle in the next years.

Taking into account the results obtained in this work, GN-NP and GN-MA-NP, did not show potential cyto- or genotoxic effects, neither *in vitro* nor *in vivo*, at biological relevant concentrations, thus they can be considered to be further evaluated as a safe option to increase the absorption of oral drugs. It should be taken into account that the ‘therapeutic dose’ of the NPs will depend on the drug loading capacity and that some non-desirable effects have been seen at very high concentrations.

4. References

Agüeros, M., Areses, P., Campanero, M.A., Salman, H., Quincoces, G., Peñuelas, I., Irache, J.M., 2009. Bioadhesive properties and biodistribution of cyclodextrin-poly(anhydride) nanoparticles. European Journal of Pharmaceutical Sciences. 37 (3-4), 231-240.

Arbós, P., Wirth, M., Arangoa, M.A., Gabor, F., Irache, J.M., 2002. Gantrez® AN as a new polymer for the preparation of ligand–nanoparticle conjugates. Journal of Controlled Release. 83 (3), 321-330.

Azqueta, A & Dusinska, M., 2015. The use of the comet assay for the evaluation of the genotoxicity of nanomaterials. Frontiers in Genetic. 6, 239-243.

Bressan, P & Kramer, P., 2016. Bread and other edible agents of mental disease. Frontiers in Human Neuroscience. 10, 130-141.

Calleja, P., Espuelas, S., Vauthier, C., Ponchel, G., Irache, J.M., 2015. Controlled release, intestinal transport, and oral bioavailablity of paclitaxel can be considerably increased using suitably tailored pegylated poly(anhydride) nanoparticles. Journal of Pharmaceutical Sciences. 104 (9), 2877-2886.

Dusinska, M., Boland, S., Saunders, M., Juillerat-Jeanneret, L., Tran, L., Pojana, G., Marcomini, A., Volkovova, K., Tulinska, J., Knudsen, L.E., Gombau, L., Whelan, M., Collins, A.R., Marano, F., Housiadas, C., Bilanicova, D., Halamoda Kenzaoui, B., Correia Carreira, S., Magdolenova, Z., Fjellsbø, L.M., Huk, A., Handy, R., Walker, L., Barancokova, M., Bartonova, A., Burello, E., Castell, J., Cowie, H., Drlickova, M., Guadagnini, R., Harris, G., Harju, M., Heimstad, E.S., Hurbankova, M., Kazimirova, A., Kovacikova, Z., Kuricova, M., Liskova, A., Milcamps, A., Neubauerova, E., Palosaari, T., Papazafiri, P., Pilou, M., Poulsen, M.S., Ross, B., Runden-Pran, E., Sebekova, K., Staruchova, M., Vallotto, D., Worth, A., 2015. Towards an alternative testing strategy for nanomaterials used in nanomedicine: Lessons from NanoTEST. Nanotoxicology. 9, 118-132.

Faderl, M., Noti, M., Corazza, N., Mueller, C., 2015. Keeping bugs in check: The mucus layer as a critical component in maintaining intestinal homeostasis. International Union Biochemistry and Molecular Biology Life Journal. 67 (4), 275-285.

Ghaisas, S., Maher, J., Kanthasamy, A., 2016. Gut microbiome in health and disease: Linking the microbiome-gut-brain axis and environmental factors in the pathogenesis of systemic and neurodegenerative diseases. Pharmacology & Therapeutics. 158, 52-62.

Handy, R.D., Owen, R., Valsami-Jones, E., 2008. The ecotoxicology of nanoparticles and nanomaterials: current status, knowledge gaps, challenges, and future needs. Ecotoxicology 17 (5), 315-325.

Iglesias, T., Ojer, P., Azqueta, A., Irache, J.M., López de Cerain, A., 2015. Toxicity evaluation of nanocarriers for the oral delivery of macromolecular drugs. European Journal of Pharmaceutics and Biopharmaceutics. 97 (Pt A), 206-217.

Inchaurraga, L., Martín-Arbella, N., Zabaleta, V., Quincoces, G., Peñuelas, I., Irache, J.M., 2015. *In vivo* study of the mucus-permeating properties of PEG-coated nanoparticles following oral administration. European Journal of Pharmaceutics and Biopharmaceutics. 97 (Pt A), 280-289.

Irache, J., Huici, M., Konecny, M., Espuelas, S., Campanero, M., Arbós, P., 2005. Bioadhesive properties of Gantrez nanoparticles. Molecules. 10 (1) ,126-145.

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

Kang, M.J., Kim, H.G., Kim, J.S., Oh, D.G., Um, Y.J., Seo, C.S., Han, J.W., Cho, H.J., Kim, G.H., Jeong, T.C., Jeong, H.G., 2013. The effect of gut microbiota on drug metabolism. Expert Opinion on Drug Metabolism & Toxicology. 9 (10), 1295-1308.

Karlsson, H., 2010. The comet assay in nanotoxicology research. Anaytical and Bioanalytical Chemistry. 398 (2), 651-666.

Karlsson, H.L., Di Bucchianico, S., Collins, A.R., Dusinska, M., 2015. Can the comet assay be used reliably to detect nanoparticle-induced genotoxicity?. Environmental and Molecular Mutagenesis. 56 (2), 82-96.

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

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, 233-278.

Manke, A., Wang, L., Rojanasakul, Y., 2013. Mechanisms of nanoparticle-induced oxidative stress and toxicity. BioMed Research International. 942916-942931.

Ojer, P., de Cerain, A., Areses, P., Peñuelas, I., Irache, J.M., 2012. Toxicity studies of poly(anhydride) nanoparticles as carriers for oral drug delivery. Pharmaceutical Research. 29 (9), 2615-2627.

Ojer, P., Neutsch, L., Gabor, F., Irache, J., López de Cerain, A., 2013. Cytotoxicity and cell interaction studies of bioadhesive poly(anhydride) nanoparticles for oral antigen/drug delivery. Journal of Biomedical Nanotechnology. 9 (11), 1891-1903.

Paul, B., Barnes, S., Demark-Wahnefried, W., Morrow, C., Salvador, C., Skibola, C., Tollefsbol, T.O., 2015. Influences of diet and the gut microbiome on epigenetic modulation in cancer and other diseases. Clinical Epigenetics. 7, 112-123.

Porfire, A.S., Zabaleta, V., Gamazo, C., Leucuta, S.E., Irache, J.M., 2010. Influence of dextran on the bioadhesive properties of poly(anhydride) nanoparticles. International Journal of Pharmaceutics. 390 (1), 37-44.

Salman, H.H., Gamazo, C., Campanero, M., Irache, J.M., 2006. Bioadhesive mannosylated nanoparticles for oral drug delivery. Journal of Nanoscience and Nanotechnology. 6 (9-10), 3203-3209.

Salman, H.H., Gamazo, C., Smidt, P.C., Russell-Jones, G., Irache, J.M., 2008. Evaluation of bioadhesive capacity and immunoadjuvant properties of vitamin B (12)-Gantrez nanoparticles. Pharmaceutical Research. 25, 2859-2868.

Salman, H.H., Irache, J.M., Gamazo, C., 2009. Immunoadjuvant capacity of flagellin and mannosamine-coated poly(anhydride) nanoparticles in oral vaccination. Vaccinne. 27 (35), 4784-4790.

Schroeder, B.O & Bäckhed, F., 2016. Signals from the gut microbiota to distant organs in physiology and disease. Nature Medicine. 22 (10), 1079-1089.

Stone, V., Johnston, H., Schins, R.P.F., 2009. Development of *in vitro* systems for nanotoxicology: methodological considerations. Critical Reviews in Toxicology. 39 (7), 613-626.

Yoncheva, K., Gómez, S., Campanero, M.A., Gamazo, C., Irache, J.M., 2005. Bioadhesive properties of pegylated nanoparticles. Expert Opinion on Drug Delivery. 2 (2), 205-218.

Chapter 8: Conclusions

Results obtained in this work have led to the following conclusions:

1. Gantrez® AN 119 NPs coated with aminodextran, dextran, cyclodextrin, mannosamine or poly-ethylene glicol did not affect viability of Caco-2 and HT29-MTX cells, at the different conditions tested.
2. Gantrez® AN 119-based NPs did not induce DNA strand breaks or oxidized bases neither in Caco-2 nor in L5178Y $TK^{+/-}$ mouse lymphoma cells, at the different conditions tested.
3. Gantrez® AN 119 NPs coated with mannosamine showed the highest *in vitro* mucus permeability capacity.
4. Gantrez® AN 119 NPs coated with mannosamine were evaluated by the "*In Vitro Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene*" following the OECD guideline number 490. They did not induce mutations in L5178Y $TK^{+/-}$ mouse lymphoma cells, at the different conditions tested.
5. Gantrez® AN 119 NPs coated with mannosamine were evaluated by the "*In Vivo Mammalian Alkaline Comet Assay*" following the OECD guideline number 489. They induced DNA strand breaks or oxidized bases in duodenum tissue of mice exposed to 2000 mg/kg bw for 2 and 4 h. This effect was not observed at lower doses or in the other organs of the gastrointestinal tract.