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

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This is a postprint version of: Iglesias, T., Dusinska, M., El Yamani, N., Irache, J. M., Azqueta, A., & López de Cerain, A. (2017). In vitro evaluation of the genotoxicity of poly(anhydride) nanoparticles designed for oral drug delivery. International Journal of Pharmaceutics, 523, 418-426. Published version: doi:10.1016/j.ijpharm.2017.03.016

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) nanoparticles, 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 nanoparticles 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 nanoparticles are promising nanocarriers for oral medical administrations.

Keywords: poly(anhydride) nanoparticles, mannosamine, mouse lymphoma assay, comet assay, genotoxicity, mutagenicity.

Abbreviations

CE: Cloning efficiency; DAPI: 4,6-Diamidino-2-phenylindole; ECACC: European collection of cell cultures; FPG: Formamidopyridine DNA glycosilase; GEF: Global Evaluation Factor; GN: Gantrez® AN 119; GN-MA: Gantrez® AN 119 coated with mannosamine; HS: Heat inactivated horse serum; MF: Mutants frequency; ML: Mouse lymphoma L5178Y TK +/- clone 3.7.2C; MLA: Mouse lymphoma assay; MMS: Methyl methanesulfonate; MTT: Thiazolyl blue tetrazolium bromide; NPs: Nanoparticles; OECD: Organization for Economic Cooperation and Development; PBS: Phosphate buffered saline; PDI: Polydispersity index; PLGA-PEO: Poly-lactic-co-glycolic acid-poly-ethylene oxide copolymer; ROS: Reactive oxygen species; RSG: Relative Suspension Growth; TFT: 5-trifluorothymidine; TK: Thymidine kinase, TSG: Total Suspension Growth.

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1. Introduction

Small nanoparticles (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 and 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 glycol) (Iglesias *et al.*, 2017). This property is especially advantageous in nanocarriers designed

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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 (Iglesias *et al.*, 2017). 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 and 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 and Collins, 2011). The assay has been modified to detect oxidized bases, by incorporating lesion specific enzymes (Dusinska and 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).

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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 and 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*., 2017).

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).

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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) NPs (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) NPs 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) (see section 2.4.2. *Cleansing of TK$^{-/-}$ mutants*). 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 % CO_2 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 section 2.4.5. *Mouse lymphoma assay*).

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 (9 μg/mL thymidine, 15 μg/mL hypoxanthine, 0.3 μg/mL methotrexate and 22.5 μg/mL glycine) ~~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~~.~~, ~~N~~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. 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 thoroughly with PBS solution by centrifugation and seeded in fresh medium for the proliferation assay. Cells were counted before, after treatment, and after 48 h incubation in fresh medium using the automated cell counter (Countess™ Automated Cell Counter, Invitrogen). Untreated cells were used as negative control (control cultures). 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%.

This experiment was also performed after treating the cell with different concentrations of the GN-Polymer (7.4 - 600 µg/mL). This experiment was carried out once.

2.4.4. Alkaline comet assay in combination with FPG enzyme

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).

Each experiment consisted of a negative control (solvent), a positive control (cells treated with 4 µM of Ro-19-8022 and exposed ~~to~~for 5 min to 500 W of visible light ~~intense light~~) and five concentrations of each test compound. Six~~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 thoroughly washed with PBS by centrifugation and adjusted to 1×10^6 cells/mL. The comet assay was performed as previously described by Collins and Azqueta (2012).

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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, ~~following by 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. 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 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 then 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)~~.~~ and ~~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. T~~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-Polymer, one experiment was carried out.

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

2.4.5. 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, e~~Each experiment consisted of one negative control (without treatment), one positive control (100 µM MMS) and 5 concentrations of each test compound which were lowered by a factor of 3 (7.4 – 600 µg/mL). ~~T~~Two independent experiments were performed with GN-NP and GN-MA-NP to confirm the results. ~~Moreover the assay was also performed once using the GN-Polymer.~~Moreover, the assay was also performed once using the GN-Polymer. In the second experiment with NPs and the one with the GN-Polymer, 10 concentration of each test compound were used (0.03 – 600 µg/mL).

Cells, at a density of 5×10^6 cells/tube, were treated for 24 h at 37 °C using gently shaking and without metabolic activation.

~~For this assay, cells were seeded in different test tubes for each test concentration, the negative control and 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~~ cells ~~culture~~ were~~as~~ 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 for 48 h. Cells were counted after 24 h to adjust them to 2×10^5 cells/mL. The following plates were seeded in order to calculate the ~~. Cells were then incubated at 37 °C during 48 h. Cells were counted after 24 h to adjust them to 2×10^5 cells/mL if necessary.~~

~~M~~mutant frequency (MF) (see section 2.4.4.1. Calculations):

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~~analysis and cloning efficiency (CE) start 48 h 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) Selection~~MF~~ plates ~~(TFT resistant)~~: 5-trifluorothymidine (TFT)-treated cells (400 µg/mL) cells were seeded at 2000 cells per well in a 96 well/plate (200 µL/well). Two identical plates were prepared per condition and incubated at 37 °C, 5% CO_2 during 11-13 days (until colonies reached appropriate sizes).

~~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) ~~CE (v~~Viability ~~plates)~~: Two cells/well ~~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,~~ 2.5 mg/mL MTT were added to each well to stain the colonies and ~~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. P~~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.

Normal cell appearance was checked by microscopy after the treatment.

2.4.4.1. Calculations

The different calculations were carried out according to the OECD guideline 490. The cloning efficiency (CE) for selection and viability plates is calculated as indicated in the next equation:

~~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" is the number of empty wells, ~~and~~ "B" ~~are~~is ~~the number of empty wells and~~ the number of wells with colonies, and ~~, respectively, whereas~~ "C" is the ~~represents the~~ number of seeded cells per well.

For determining the

~~In the case of~~ mutant frequency (MF) after each treatment condition the next formula was used:~~,~~ ~~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= ($CE_{Selection}$~~Mutant~~ / $CE_{Viability}$)

MF for small, large and total colonies was discerned.

In all cases the me~~an~~dia of the ~~was obtained of the~~ 2 plates of each condition was obtained.

2.4.4.2. Test acceptance criteria

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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 and . 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; a 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 Global Evaluation Factor (GEF) (i.e. 126×10^{-6} for the microwell version) and (3) the increase in MF is concentration-related.

. 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

Results are included in Table 1. The functionalization of bare NPs (GN-NP) with mannosamine (GN-MA-NP) increased the size of the resulting nanocarriers (198 ± 1 *vs* 276 ± 2, respectively). The preparative process was adequate to produce very homogeneous batches; the PDI of GN-NP and GN-MA-NP were 0.163 ± 0.024 and 0.138 ± 0.056, respectively. GN-NP presented a smoother surface than GN-MA-NP ~~(Fig. 1)~~.

3.2. Effects on cell proliferation

The effect of GN-NP and GN-MA-NP, as well as GN-Polymer, on the proliferation of ML cells was evaluated using the proliferation assay. GN-NP and GN-MA-NP did not affect the viability of cells since RSG was above 90% in all condition tested (Fig. ~~2~~1). However, the highest tested concentration of GN-Polymer (600 µg/mL) did affect the viability of cells, presenting an RSG of approximately 30%.

Preliminary studies showed that 1000 and 2000 µg/mL of GN-NP and of GN-MA-NP were cytotoxic for ML cells (RSG < 10%) (data not shown).

3.3. 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 h of treatment.

Fig. ~~3~~ 2 shows the effect of GN-NP and GN-MA-NP and their polymer on SBs+ALS and FPG-sensitive sites in ML cells treated for 24 h; 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 tested which presented a RSG much lower than 70% (Fig. 1~~2~~). Positive and negative controls showed the expected results.

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

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Preliminary studies showed that concentrations higher than 600 µg/mL, that was the highest concentration used, interfered with the comet assay scoring (data not shown).

3.4. Mutagenicity of poly(anhydride) NPs

MLA was carried out in ML cells treated with GN-NP, GN-MA-NP and GN-Polymer for 24 h 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. 34. 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. Results obtained at lower concentrations (0.03 - 7.4 µg/mL) showed the same pattern of results (data not shown).

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,

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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 < 0.2) (Table 1). In addition, a previous study has demonstrated the negative surface charge of these NPs (Iglesias *et al.*, 2017).

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 (Iglesias *et al.*, 2017). 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

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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 24 h in culture medium (Iglesias *et al.*, 2017). Nevertheless, in preliminary studies 12000 and ~~1~~2000 µg/mL of GN-NP and GN-MA-NP were cytotoxic for ML cells (RSG < 10%). We, therefore, decided to use 600 µg/mL as our highest concentration; higher concentrations can not be tested in the comet assay (i.e. RSG should be at least to 60%) and, on the other hand, testing higher concentrations is not relevant 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).

~~Cells were to be thoroughly washed in order to remove the NPs to avoid the interference with counting cells and scoring comets.~~ 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).

As it has been pointed out before, previous studies have demonstrated the absence of genotoxicity in Caco-2 cells exposed to GN-NP and GN-MA-NP (Iglesias *et al.*, 2017). Nevertheless, a very slight increase of the FPG-sensitive sites after 24 h of treatment was observed at very high and non-relevant concentrations (i.e. 1 and 2 mg/mL). Taking into account that ML cells were more sensitive to NPs than Caco-2 cells, the comet assay was repeated in this cell line to ensure the absence of genotoxicity. 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 SBs 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 and 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, 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 poly-lactic-co-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 MLA 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

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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 and 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 90% 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.

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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. *In vivo* studies will be needed to complete the safety assessment of these NPs.

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).

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FIGURES

Table 1. Physicochemical characteristics of NPs and TEM images. Data expressed as the mean ± SD.

~~**Fig. 1.** SEM photographs of NPs.~~

Fig. 12. Effect of the GN-NP, GN-MA-NP and GN-Polymer on the proliferation of ML cells treated for 24 h. Results presented as RSG and expressed as the mean ± SD of three independent experiments (n=3) except for GN-Polymer (n=1).

Fig. 23. Analysis of DNA strand breaks and net FPG-sensitive sites in ML 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

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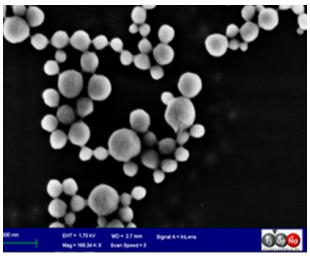
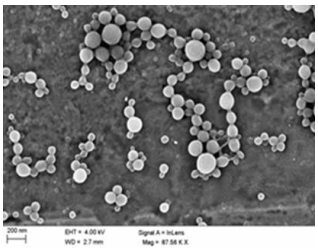
independent experiments (n=3) except for GN-Polymer (n=1). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, significantly different from negative control.

Fig. 34. 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).

ID NP	GN-NP	GN-MA-NP
Size (nm)	219.5 ± 3.1	261.9 ± 4.2
PDI	0,084 ± 0.049	0.090 ± 0.031
Zeta potential (mV)	-32,0 ±2,00	-40,4 ± 0,50
TEM		

This is a postprint version of: Iglesias, T., Dusinska, M., El Yamani, N., Irache, J. M., Azqueta, A., & López de Cerain, A. (2017).
In vitro evaluation of the genotoxicity of poly(anhydride) nanoparticles designed for oral drug delivery.
International Journal of Pharmaceutics, 523, 418-426. Published version: doi:10.1016/j.ijpharm.2017.03.016