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**Carboxylated nanodiamonds can be used as negative reference in *in vitro*  
nanogenotoxicity studies**

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## ABSTRACT

Nanodiamonds (NDs) are promising nanomaterials for biomedical applications. However, a few studies highlighted an *in vitro* genotoxic activity for detonation NDs, which was not evidenced in one of our previous work quantifying  $\gamma$ -H2Ax after 20 nm and 100 nm high-pressure high-temperature (HPHT) NDs exposure of several cell lines. To confirm these results, in the present work, we investigated the genotoxicity of the same 20 and 100 nm NDs and added intermediate-sized NDs of 50 nm. Conventional *in vitro* genotoxicity tests were used, *i.e.* the *in vitro* micronucleus and comet assays that are recommended by the French National Agency for Medicines and Health Products Safety (Afssaps, now ANSM) for the toxicological evaluation of nanomedicines. *In vitro* micronucleus and *in vitro* comet assays (standard and hOGG1-modified) were therefore performed in two human cell lines, the bronchial epithelial 16HBE14o- cells and the colon carcinoma T84 cells. Our results did not show any genotoxic activity, whatever the test, the cell line or the size of carboxylated NDs. Even though these *in vitro* results should be confirmed *in vivo*, they reinforce the potential interest of carboxylated NDs for biomedical applications or even as a negative reference nanoparticle in nanotoxicology.

Keywords: Nanodiamonds, genotoxicity, *in vitro*, micronucleus assay, comet assay.

## INTRODUCTION

Nanodiamonds (NDs) are one of the most promising carbon nanomaterials for biomedical applications (Krueger, 2011; Rosenholm et al., 2015; Shenderova and McGuire, 2015). One of their advantages for these applications consists in the presence of a large variety of carbon-related groups on their surface (Girard et al., 2010; Huang and Chang, 2004; Krueger et al., 2008; Krüger, 2006), enabling their functionalization with several biomolecules (including proteins and nucleic acids) (Krueger, 2011). ND tritium-labelling has also been developed and could be a useful tool for *in vivo* and bio distribution studies (Girard et al., 2014). Depending on their production method, NDs are scalable with sizes ranging from 100 nm down to 2 nm. Indeed, small NDs in the range of 2-8 nm are produced by detonation of carbon-containing explosives under an oxygen-deficient atmosphere (Dolmatov, 2007). NDs can be also synthesized through a milling process from HPHT (high-pressure high-temperature) (Boudou et al., 2009) or CVD (chemical vapor deposition) crystals (Neu et al., 2011). Such HPHT particles are particularly suitable for biological applications, with diameters ranging from 10 to 100 nm and a clean surface chemistry free of impurities. However, HPHT NDs generally present a higher polydispersity than detonation NDs (Paget et al., 2014).

It is now well-established that NDs are better tolerated by cells than other nanocarbon materials (Schrand et al., 2007a; Vaijayanthimala et al., 2012; Yu et al., 2005; Yuan et al., 2010; Zhang et al., 2012). Indeed, several *in vitro* (Grall et al., 2015; Liu et al., 2009; Paget et al., 2014; Schrand et al., 2007b; Vaijayanthimala et al., 2009) as well as *in vivo* (Lin et al., 2012; Mohan et al., 2010; Vaijayanthimala et al., 2012; Yuan et al., 2010) studies have shown that NDs are non-toxic for human cell lines and for rodents. Regarding genotoxicity, only few *in vitro* studies have reported that detonation NDs induce genotoxicity on embryonic stem cells (Xing et al., 2011), on glioblastoma cells (Hinzmann et al., 2014) and on human peripheral lymphocytes (Dworak et al., 2014) while carboxylated HPHT NDs did not induce genotoxicity on human cell lines (Paget et al., 2014). It should be mentioned that a recent

study has demonstrated that NDs can have bactericidal activity (Wehling et al., 2014) if they present oxidized and negatively charged surface or glycan-modified surface (Szunerits et al., 2016).

The use of standardized or recognized guideline tests is crucial for further safety of NDs used for medical applications or to place carboxylated NDs as negative references for toxicology testing. Indeed, until now and to our knowledge, no nanoparticle was clearly identified as complete negative control combining cytotoxicity and genotoxicity aspects. Several recent articles have mentioned the need of negative reference nanoparticles, in order to improve the reproducibility and reliability of nanoparticle genotoxicity studies. Moreover, this point was identified as one of the primary challenges in nanotoxicology studies (Kroll et al., 2009; McNeil, 2011; Nelson et al., 2013). Thus, the present work focused on the analysis of genotoxicity of well-characterized carboxylated HPHT NDs of 20, 50 and 100 nm diameter using recommended testing, *i.e. in vitro* micronucleus assay and *in vitro* comet assay (standard and hOGG1-modified). *In vitro* micronucleus assay is a regulatory test, and both *in vitro* micronucleus and comet assays form the *in vitro* part of the battery of tests for the toxicological evaluation of nanomedicines recommended by the French National Agency for Medicines and Health Products Safety (Afssaps, now ANSM, (Afssaps, 2011)). These assays were performed in two human cell lines: the bronchial epithelial 16HBE14o-cells and the colon carcinoma T84 cells, to mimic effects on two potential target organs after pulmonary or oral exposures. Beyond their physiological origin, the use of these 2 cell lines is based on their demonstrated internal performance in both the micronucleus test and the Comet assay.

## **MATERIALS AND METHODS**

### **Chemicals**

Minimum Essential Medium (MEM), Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12), foetal calf serum (FCS) and phosphate buffered saline (PBS) were purchased from Gibco (Cergy-Pontoise, France), non-essential amino acids from Eurobio

(Courtaboeuf, France). Trypsin, Giemsa reagent, penicillin, streptomycin, amphotericin B, mitomycin C (MitoC), methyl methanesulfonate (MMS), glucose oxidase (GOx), Triton X-100, EDTA, trizma base, propidium iodide, KCl, NaCl, sodium bicarbonate, sodium pyruvate, and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France). Normal melting point agarose (NMPA) and low melting point agarose (LMPA) were purchased from Biorad (Marnes-la-Coquette, France), acetic acid from VWR (Fontenay-sous-Bois, France), human 8-oxoguanine glycosylase (hOGG1) from New England Biolabs (Evry, France), dimethyl sulfoxide (DMSO) from Acros Organics (Noisy-le-Grand, France), NaOH, L-glutamine, methanol and absolute ethanol from Merck (Darmstadt, Germany), and sterile water from Fresenius Kabi (Sèvres, France).

### **Nanodiamonds**

Nanodiamonds, synthesized from a milling process of HPHT diamond crystals, were purchased from Van Moppes. The three types of nanodiamonds studied here have been produced within the same method by the manufacturer and only differ by the final fractionation used, resulting in different mean diameters: Syndia<sup>®</sup> SYP 0-0.02: ND-20 nm, Syndia<sup>®</sup> SYP 0-0.05: ND-50 nm and Syndia<sup>®</sup> SYP 0-0.1: ND-100 nm. Extended characterization was mostly conducted on ND-50 nm which is representative of the 3 types of particles studied here. X-Ray photoelectron spectroscopy (XPS) and X-Ray Diffraction (XRD) of ND-50 nm can be found in one of our previous study (Girard et al., 2010). High Resolution Transmission Electron Microscopy (HRTEM using a FEI Tecnai F20 field-emission gun microscope operating at 200 kV) of ND-50, reveals faceted diamond particles (data not shown). However, Infrared Spectroscopy (FT-IR) has been performed on the three types of diamond particles. FT-IR spectra were measured in transmission mode using a Thermo Nicolet 8700 spectrometer. Bromide potassium (KBr) pellets were prepared with ND-20, -50 and -100 nm. KBr pellets containing NDs were dried at 350K using a primary vacuum system (103 mTorr) for 24 hours before IR measurements in a home-made IR vacuum cell equipped with KBr windows and a heater.

The stock suspension is obtained by dispersing the nanoparticles at a concentration of 5 mg/mL in ultrapure water by sonication (Hielscher UP400S, 300W, 24 kHz) for 2 hours with cooling, as previously described (Paget et al., 2014). Characterization of ND-20 nm and ND-100 nm was previously performed (Paget et al., 2014). Characterization of ND-50 nm was performed in the same conditions, *i.e.* by diluting the stock suspension at 0.5 mg/mL either in water or in complete MEM medium. Dynamic light scattering (DLS) measurements coupled with electrophoresis measurements to evaluate the surface potential ( $E_{ZETA}$ ) were performed using a Malvern NanoZS with a 173° backscattered angle at 37°C. In order to eliminate contaminants (such as bacteria) during the preparation of ND suspensions, the 5 mg/mL stock suspensions were first annealed at 95°C for 20 minutes before use in culture medium. Then, prior to cell exposure, the diluted suspensions (in complete culture medium) were first tested for 72 hours at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air to check for the absence of contaminant growth.

### **Cell culture**

*16HBE14o-* cells. 16HBE14o- (16HBE) cells were obtained from Dr D.C. Gruenert (Cochester, Vermont, USA). Cells were thawed, seeded in MEM medium supplemented with 1.6 mM L-glutamine, sodium bicarbonate 0.2%, non-essential amino acids and 10% v/v heat inactivated FCS and incubated in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>. This medium, hereafter referred to as complete MEM, was renewed twice a week. After reaching confluence, cells were trypsinized, a passage with 5x10<sup>5</sup> cells per 75 cm<sup>2</sup> flask was performed and cells were grown until confluence. Cells were then trypsinized and seeded in 6-well plates with 4x10<sup>5</sup> cells per well. Cells were allowed to attach for 24 hours before treatment. The doubling time for 16HBE cells is estimated at 22 hours (Merhi et al., 2012).

*T84* cells. T84 cells were obtained from ATCC (Manassas, VA, USA). Cells were thawed, seeded in DMEM/F12 medium supplemented with 10% v/v heat inactivated FCS,

penicillin (200 UI/mL) and streptomycin (0.1 mg/mL) and incubated in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>. This medium, hereafter referred to as complete DMEM, was renewed twice a week. After reaching confluence, cells were trypsinized and seeded in 6-well plates with 2x10<sup>5</sup> cells per well and grown to reach approximately 80% or 50% confluence before treatment for comet or micronucleus assays respectively. The doubling time for T84 cells is estimated at 60 hours (Ghartey-Tagoe et al., 2004)

### **Cell treatment**

16HBE and T84 cells were incubated in appropriate complete medium with different concentrations of ND-20 nm, ND-50 nm and ND-100 nm, solvent (sterile water) or positive controls. Treatment suspensions were diluted 1:9 v/v in 5 mL culture medium per wells, to obtain ND final concentrations of 12.5 – 25 – 50 – 100 µg/mL in the same range of doses used in other studies from literature (Paget et al., 2014; Schrand et al., 2007b; Xing et al., 2011; Yu et al., 2005). Cells were then incubated for 4 hours in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>.

Positive controls were MMS (10 µg/mL, 4-hour treatment) in standard comet assays, GOx (0.0075 or 0.01 U/mL in appropriate culture medium without serum, 30-minute treatment) in hOGG1-modified comet assays and mitomycin C (0.5 µg/mL, 4-hour treatment) in micronucleus assays.

### ***In vitro* micronucleus assay**

At the end of the 4-hour treatment, cells were washed and reincubated for a 1.5 to 2-cell cycle recovery period; 16HBE cells with fresh culture medium for 44 hours and T84 cells with fresh culture medium supplemented with cytochalasin B (3 µg/mL) for 96 hours. At the end of this recovery period, cells were washed and trypsinized. 16HBE cells were counted using a haemocytometer; cell viability was assessed using the Trypan blue exclusion method. After centrifugation at 1000 rpm for 6 minutes, supernatant was discarded and cells were treated with a hypotonic solution (appropriate culture medium diluted 1:1 in distilled water) for

10 minutes (16HBE cells) or 4 minutes (T84 cells). After the hypotonic shock, a pre-fixation step was performed by adding cold Carnoy's fixative (methanol/glacial acetic acid, 3:1 v/v). Cells were then centrifuged and suspended in Carnoy's fixative for 10 minutes. After another centrifugation, cells were resuspended and spread on duplicate glass slides. Slides were air-dried at least over-night and stained for 10 minutes with 4% v/v Giemsa in water.

Replication indexes were calculated for T84 cells to assess cell viability. Slides were independently coded. Micronuclei, identified according to recommended criteria (Fenech et al., 2003), were scored at 500x magnification in 1000 intact mononucleated 16HBE cells per slide, or 500 binucleated and 500 mononucleated T84 cells per slide. Results are presented as the mean +/- standard deviation from 2 cultures.

#### ***In vitro* alkaline comet assay**

The comet assay was performed under alkaline conditions (pH>13) in compliance with previously described protocol (Singh et al., 1988; Tice et al., 2000) and the hOGG1-modified comet assay was carried out to specifically detect oxidative DNA damage.

At the end of the 4-hour treatment, cells were washed and trypsinized. Trypsin was inactivated by adding complete medium. Viable cells were counted using Trypan blue exclusion and  $8 \times 10^4$  viable cells were mixed with 0.5% w/v LMPA kept at 37°C.

Cells embedded in LMPA were spread onto regular precoated microscopic slides (1.5% and 0.8% w/v NMPA). For each concentration and culture, four replicate slides were prepared: two slides to be treated with hOGG1 and two slides without hOGG1. All the following steps were sheltered from daylight to prevent the occurrence of additional DNA damage. Slides were immersed for at least 1 hour at 4°C in a cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma Base, pH 10, supplemented with 1% v/v Triton X-100 and 10% v/v DMSO). After lysis, two slides were washed in distilled water while the other two slides were washed for 2 x 5 minutes in enzyme buffer (40 mM HEPES, 100 mM KCl, 0.5 mM EDTA, 0.2 mg/ml BSA; pH 8) and incubated with hOGG1 (0.12 U/slide) and

coverslips at 37°C for 10 minutes. At the end of this enzymatic step, slides were quickly rinsed with cold PBS.

All the slides were then placed in a horizontal tank filled with fresh electrophoresis solution (1 mM EDTA and 300 mM NaOH, pH>13) for 20 minutes to allow DNA unwinding and expression of strand breaks and alkali-labile sites. Next, electrophoresis was performed for 20 minutes using an electric current of 0.7 V/cm. Slides were then placed for 10 minutes in a neutralization solution (0.4 M Trizma base, pH 7.5) and gels were dehydrated by immersion in absolute ethanol for 5 minutes. Finally, slides were air-dried and stored at room temperature.

Slides were independently coded and analyzed after addition of propidium iodide (20 µg/mL in distilled water) and a coverslip on each slide. Slides were then examined at 250x magnification using a fluorescence microscope (Leica Microscopy and Scientific Instruments Group, Heerbrugg, Switzerland) equipped with an excitation filter of 515-560 nm and a 590 nm barrier filter, connected through a gated CCD camera to Comet Image Analysis System software, version 4.0 (Perceptive Instruments Ltd., Haverhill, United Kingdom). One hundred randomly selected cells were scored on each slide, corresponding to 200 cells without hOGG1 and 200 cells with hOGG1 per culture. Median of tail intensity, defined as the percentage of DNA that had migrated from the head of the comet into the tail, was used as the measure of damage. Net hOGG1-sensitive sites represent additional DNA migration induced by hOGG1 and were calculated for each culture by subtracting the median of tail intensity without hOGG1 from the median of tail intensity with hOGG1. Results are presented as the mean +/- standard deviation from at least 3 cultures.

### **Statistical analysis**

For the comet assays, the non-parametric Mann-Whitney U-test was used to evaluate the statistical difference in median of tail intensity and hOGG1-sensitive sites between each concentration and the negative control. Statistical analyses were performed with StatView® Software (version 5.0, SAS Institute Inc., SAS Campus Drive, Cary, North Carolina 27513,

USA). For the micronucleus assays, the statistical significance of difference between groups was determined using the Chi<sup>2</sup>-test. In both cases, differences with a  $p < 0.05$  were considered statistically significant.

## RESULTS

### Characterization of nanoparticles

Fourier transform infrared spectroscopy (FT-IR) spectra of ND-20, 50 and 100 nm are reported in Figure 1. To limit the contribution of contaminating water, these spectra were recorded after 24 hours of drying. The three samples exhibited a similar surface chemistry, mainly characterized by the large band between 1700 and 1800  $\text{cm}^{-1}$ , attributed to C=O stretching in carboxylic groups. The large band between 1050 and 1100  $\text{cm}^{-1}$  is attributed to C-O related groups, representing both alcohol (confirmed by the large band between 3000 and 3600  $\text{cm}^{-1}$  due to O-H stretching) and/or ether groups on the surface of the nanoparticles. All these oxygen-related groups arise from the strong oxidative treatments performed by the manufacturer for the purification of the particles and to provide them a negative zeta potential in water for  $\text{pH} > 4$  (Krueger and Lang, 2012). A limited amount of amorphous carbon lying on the surface of ND-20 and ND-100 can be seen through the band between 2800 and 3000  $\text{cm}^{-1}$ .

XPS analysis, previously performed (Girard et al., 2010), revealed that no metallic impurities were measured in the samples, which were exclusively composed of carbon, oxygen and nitrogen (down to the detection limit of XPS, i.e. 0.5 at.%).

Size distribution measurements for the three sets of NDs were undertaken by dynamic light scattering (DLS) (Figure 2). Characterization of ND-20 nm and ND-100 nm was previously performed (Paget et al., 2014). For so-called ND-50 nm, 89% of the particles had diameters between 21 and 44 nm with a maximum lying around 28 nm in water and around 68 nm in complete culture medium (Figure 2). The size distribution for ND-50 nm increased in culture medium, probably because of moderate ND aggregation due to the increase in

ionic strength and the adsorption of proteins on the ND surface. For each set of NDs, values of surface potential  $E_{ZETA} = -45$  mV were measured in pure water (pH= 6.5) at 500  $\mu\text{g/mL}$ .

### ***In vitro* micronucleus assay**

The micronucleus assay was performed in 16HBE cells after a 4-hour exposure with the three sizes of NDs followed by a 44-hour recovery period. The cytokinesis-block micronucleus assay was performed in T84 cells after 4 hours of treatment with the three sizes of NDs, followed by a 96-hour recovery period. Studied ND concentrations ranged from 12.5 to 100  $\mu\text{g/mL}$ ; the latter was chosen as the highest concentration because of the increasing presence of ND aggregates on slides as a function of ND concentration interfering with the microscopic analysis. Cytotoxicity was evaluated in 16HBE cells using the relative viability compared to the negative control and in T84 cells using the replication index. None of the ND concentrations induced more than 55 +/- 5% cytotoxicity as recommended in OECD TG 487 (OECD, 2014) (Figure 3); therefore all tested concentrations were suitable for assessment of micronucleus induction.

NDs, whatever the size of the particles, did not induce any statistically significant increase in the number of micronuclei in 16HBE cells (Figure 4) or T84 cells (Figure 5). In return, mitomycin C, used as positive control, induced a statistically significant increase in the number of micronuclei in 16HBE cells and in binucleated T84 cells.

### ***In vitro* comet assay**

*In vitro* comet assay was performed in 16HBE (Figure 6) and T84 cells (Figure 7) after 4 hours of treatment with NDs of 3 different sizes, using both the standard protocol and the hOGG1-modified protocol. Concentrations were chosen according to the ones studied in the micronucleus assays, and ranged from 12.5 to 100  $\mu\text{g/mL}$ . Whatever the size, NDs did not induce any statistically significant increase in tail intensity in the standard comet assay in both cell lines. No statistically significant increase in hOGG1-sensitive sites was observed whatever the ND in both cell lines. To the contrary, MMS induced a statistically significant

increase in tail intensity in the standard comet assay and glucose oxidase induced a statistically significant increase in hOGG1-sensitive sites in both cell lines.

## DISCUSSION

Surface chemistry of each set of NDs was characterized by Fourier transform infrared spectroscopy (FT-IR) (Figure 1). This characterization revealed that the three sets of NDs exhibited a similar oxidized surface chemistry, dominated by carboxylic groups, with a very limited contamination by amorphous carbon. The size distribution measurements of the initial suspensions in water and of the final suspensions in culture medium, undertaken by DLS, showed that these three sets of NDs presented three distinct populations of particles. The surface potential values ( $E_{ZETA}$ ) for each set of NDs measured in pure water were similar (-45 mV). These negative values are consistent with the presence of surface carboxylic groups revealed by FT-IR, as at such a pH ( $\approx 6.5$ ) the carboxylate form prevails and therefore confers a negative surface charge on the particles (Girard et al., 2011).

Regarding the genotoxicity of diamond nanoparticles, only few studies are available in the literature, most of them studying detonation NDs. In one of them, the authors focused on detonation NDs with a size smaller than 10 nm, and studied chromosome damage through a micronucleus assay and primary DNA damage through a comet assay on human lymphocytes after 72 hours of treatment (Dworak et al., 2014). In this study, NDs induced DNA strand breaks from the lowest concentration studied of 1  $\mu\text{g}/\text{mL}$ . NDs also increased 8-oxoG level suggesting oxidative DNA damage. An increase in micronuclei formation was observed at the concentration of 10  $\mu\text{g}/\text{mL}$ , and an aneugenic activity was suggested by an increased centromeric signal in micronuclei (Dworak et al., 2014). Detonation NDs with a size distribution between 4 nm and 5 nm also induced DNA fragmentation in the comet assay in a glioblastoma cell line in another study (Hinzmann et al., 2014). Moreover, Xing et al. (Xing et al., 2011) showed that detonation NDs with similar sizes caused increased expression of DNA repair proteins in mouse embryonic stem cells.

To the contrary, we showed in a previous study that two of the three sizes of carboxylated HPHT NDs studied in the current study (ND-20 nm and ND-100 nm) were neither cytotoxic nor genotoxic up to 500 µg/mL in the  $\gamma$ -H2Ax foci quantification test in six cell lines (Paget et al., 2014). The cell lines used in this previous study were from human origin and representative of different organs: liver (HepG2 and Hep3B), kidney (Caki-1 and Hek-293), intestine (HT29) and lung (A549). We also reported in two of our previous works that no interference was observed between NDs and *in vitro* assays. This point was verified for HPHT NDs (Paget et al., 2014) and for detonation NDs as well (Grall et al., 2015).

In the current study, we performed more conventional *in vitro* genotoxicity assays, i.e. micronucleus assay and comet assay (standard and hOGG1-modified). Both these tests are indeed recommended by the French National Agency for Medicines and Health Products Safety (Afssaps, now ANSM) for the *in vitro* evaluation of the genotoxicity of nanomaterials (Afssaps, 2011). They were carried out in two human cell lines representative of lung (bronchial epithelial 16HBE14o- cells) and intestine (colon carcinoma T84 cells) using NDs of three sizes: 20, 50 and 100 nm. Results in both the standard and modified comet assays did not highlight any primary DNA damage either in 16HBE or T84 cells, whatever the size of NDs. Moreover, no significant induction of chromosomal damage was observed in the micronucleus assays in either cell line, for any of the three sizes of NDs.

Our results therefore differ from those of above-mentioned studies (Dworak et al., 2014; Hinzmann et al., 2014; Xing et al., 2011), which reported evidences of an *in vitro* genotoxic activity. Whereas in our work we used HPHT NDs, these other studies used detonation NDs, characterized by different surface chemistry and a smaller size than HPHT NDs (Krueger, 2011). These differences in physicochemical properties could be hypothesized to contribute to the differences in results observed between studies. If NDs surface chemistry and size seem to be key points in NDs biological effects, this hypothesis should be confirmed using additional physical and chemical characterization and standardized methods, as different tests or cell types were used in each study to evaluate genotoxicity. Otherwise, these results confirm that these HPHT NDs could represent an

interesting candidate as negative reference nanoparticles in *in vitro* genotoxicity assays, as already suggested for nanotoxicology in a more general way in our previous study (Paget et al., 2014).

The HPHT NDs studied here are devoid of genotoxic activity when using the recommended battery of two *in vitro* tests allowing coverage of a broad spectrum of genetic events, namely primary DNA damage, oxidative DNA damage or chromosomal damage. According to the French recommendations (Afssaps, 2011), only one *in vivo* micronucleus test on the selected relevant target organ should be performed. For this, the choice of the target organ mainly depends on the route of human exposure which is not known for NDs at that time (e.g. colon if oral route, bone marrow or circulating lymphocytes if intravenous). For ethical reasons, the *in vivo* test was thus not performed in the current study but should be carried out to ensure the lack of genotoxic activity (direct or indirect) in order to dismiss any genotoxic concern.

Whatever the method used, we conclude that carboxylated NDs are not genotoxic in *in vitro* test systems and confirm the huge potential of HPHT carboxylated NDs for medical applications but also their suitability in nanotoxicology studies as negative reference as already suggested in one of our previous work (Paget et al., 2014).

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Figures

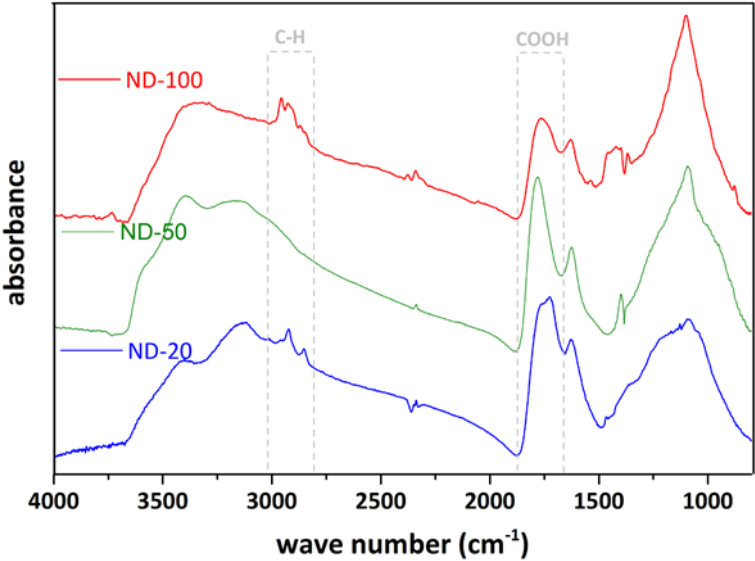


Figure 1. FT-IR spectra of ND-20 nm (blue curve), ND-50 nm (green curve) and ND 100 nm (red curve) recorded in transmittance mode.

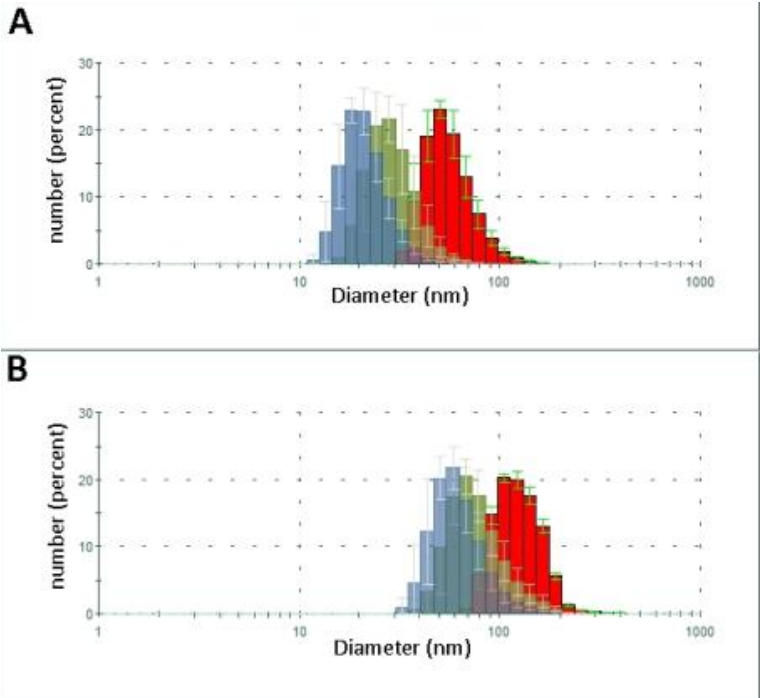


Figure 2. Size distribution of ND-20 nm (blue), ND-50 nm (green) and ND-100 nm (red) measured by DLS. A: suspensions in water, B: suspensions in complete MEM medium.

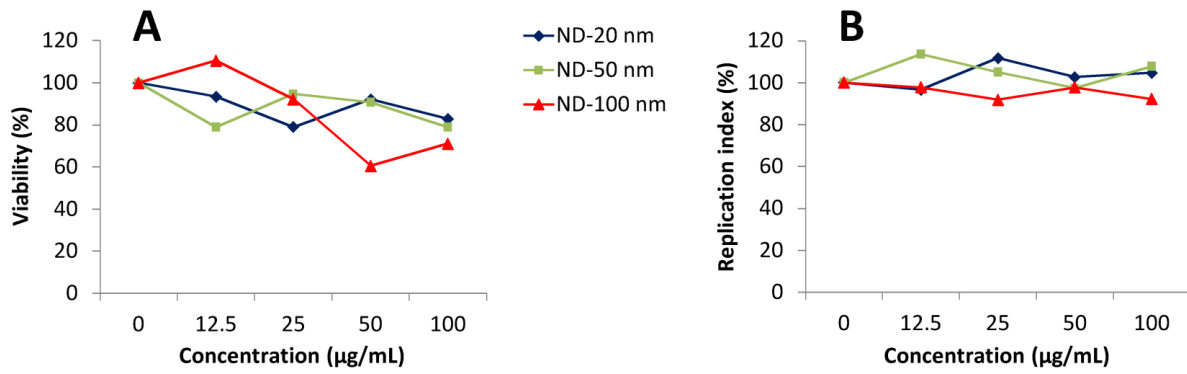


Figure 3. Evaluation of viability in 16HBE (A) and T84 (B) cells exposed to ND-20 nm, ND-50 nm or ND-100 nm for 4 hours. A: In 16HBE cells, viability was assessed with the percentage of viable cells compared to negative control at the end of the 44-hour recovery period. B: In T84 cells, viability was assessed with the replication index compared to negative control at the end of the 96-hour recovery period.

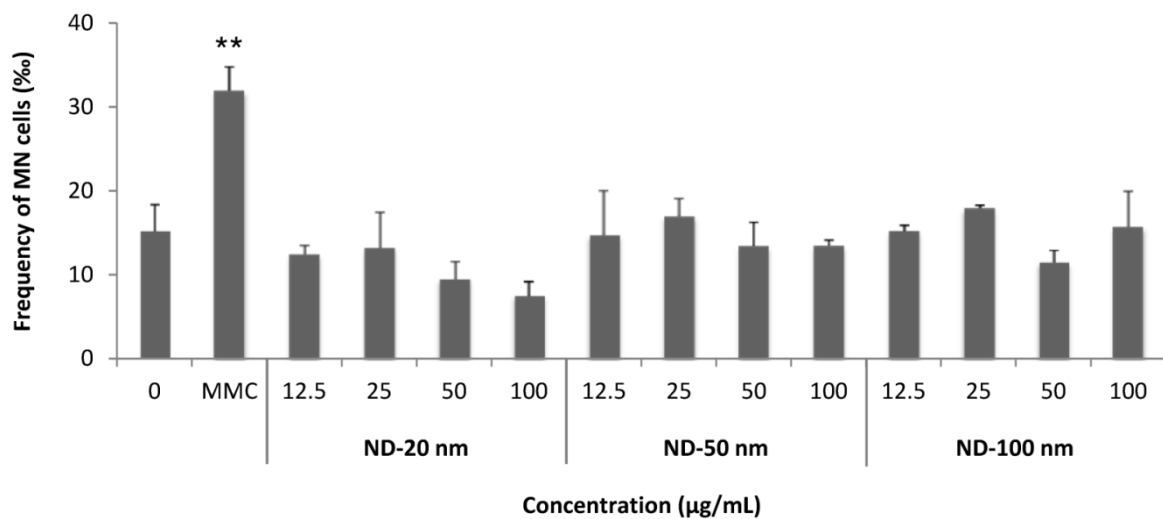


Figure 4. Frequency of MN cells in 16HBE cells exposed for 4 hours to different concentrations of ND-20 nm, ND-50 nm or ND-100 nm followed by a 44-hour recovery period. Mitomycin C (MMC, 0.5 µg/mL) was used as positive control. Mean +/- SD from 2 cultures. \*\* p<0.001.

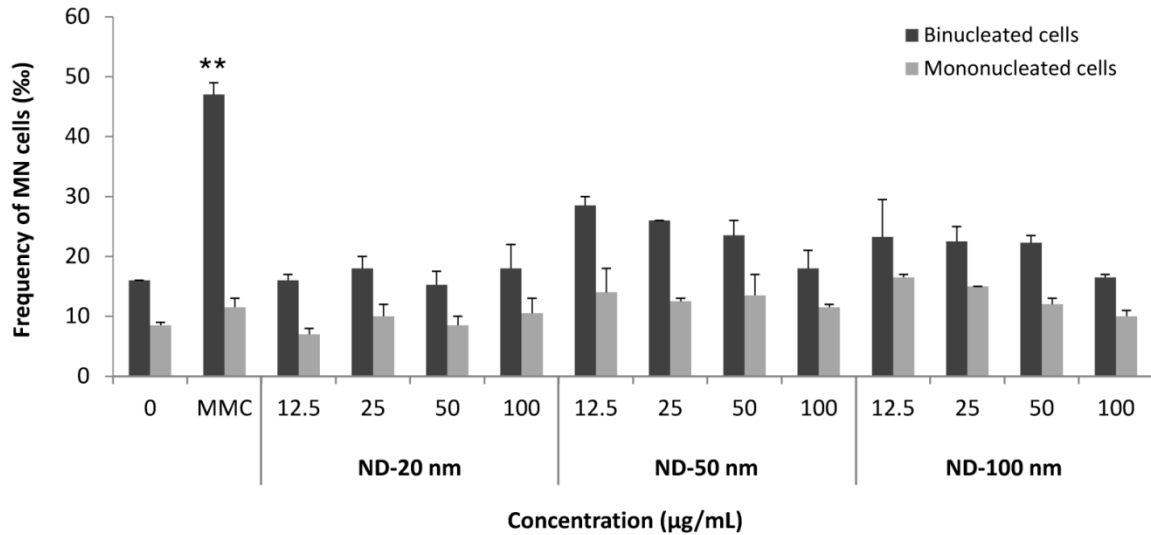


Figure 5. Frequency of MN cells in T84 cells exposed for 4 hours to different concentrations of ND-20 nm, ND-50 nm or ND-100 nm followed by a 96-hour recovery period in the presence of cytochalasin B (3 µg/mL). Mitomycin C (MMC, 0.5 µg/mL) was used as positive control. Mean +/- SD from 2 cultures. \*\* p<0.001.

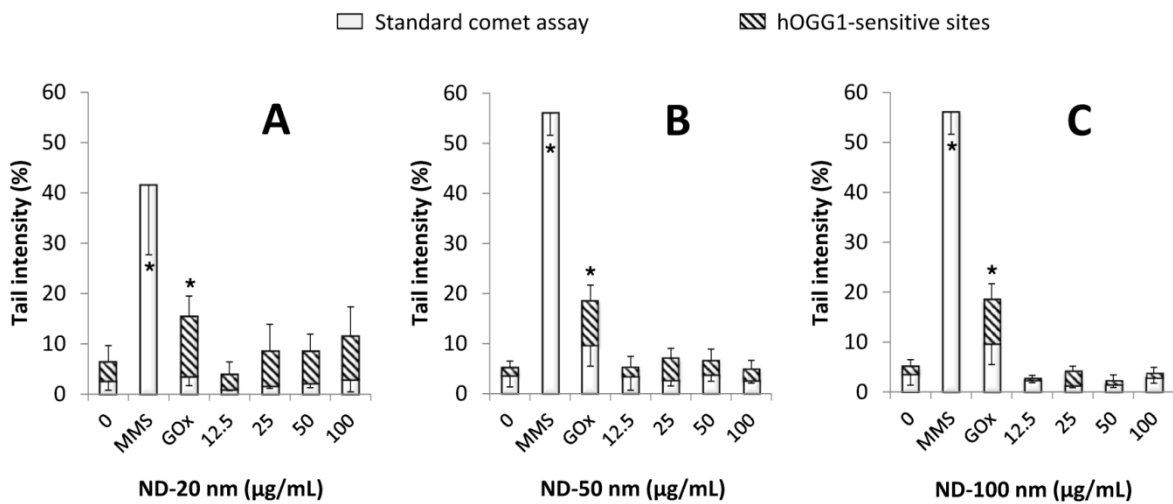


Figure 6. Comet assay in 16HBE cells incubated with ND-20 nm (A), ND-50 nm (B) or ND-100 nm (C) for 4 hours, with and without further incubation with hOGG1. MMS (10 µg/mL) and GOx, (0.0075 U/mL in A or 0.01 U/mL in B and C, 30-minute incubation) were used as positive controls. Results for ND-20 nm (A) represent the mean +/- SD of median tail intensity for 6 cultures (3 assays). All other results (B and C) represent the mean +/- SD of median tail intensity for 3 cultures (2 assays). \* p<0.05.

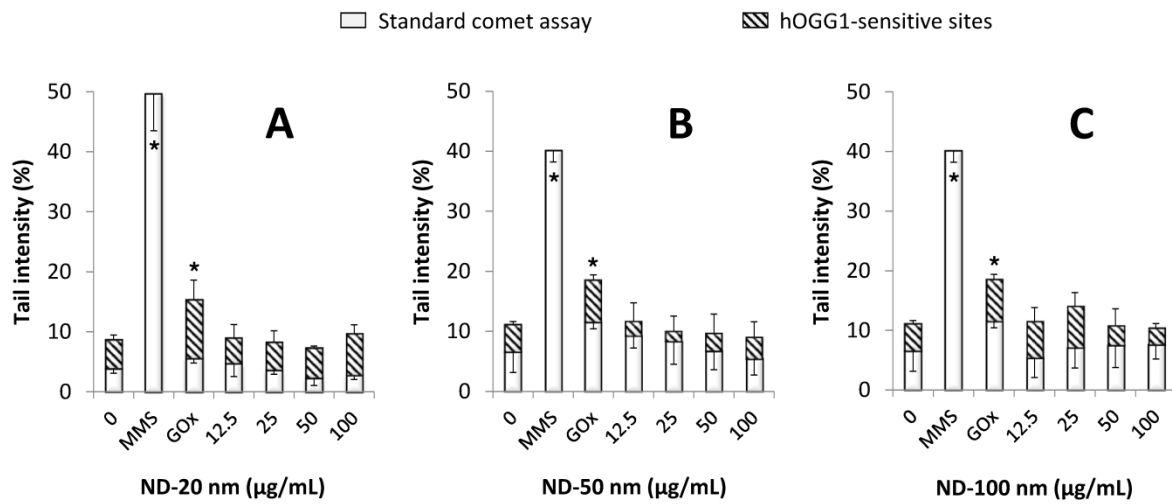


Figure 7. Comet assay in T84 cells incubated with ND-20 nm (A), ND-50 nm (B) or ND-100 nm (C) for 4 hours, with and without further incubation with hOGG1. MMS (10 µg/mL) and GOx (0.01 U/mL, 30-minute incubation) were used as positive controls. Results represent the mean  $\pm$  SD of median tail intensity for 3 cultures (2 assays). \*  $p < 0.05$ .