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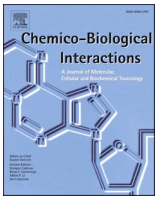

Research paper

Genotoxicity evaluation of graphene derivatives by a battery of *in vitro* assays

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ABSTRACT

The interest of graphene materials has increased markedly in the recent years for their promising applications in many fields as food packing. These new applications have caused some concern regarding their safety for consumers since the intake of these materials may increase. In this sense, a battery of *in vitro* test is required before its use as a food contact material. Then, the aim of this study was to assess the potential mutagenicity and genotoxicity of graphene oxide (GO) and reduced-graphene oxide (rGO) following the recommendations of the European Food Safety Authority (EFSA). Thus, the mouse lymphoma assay (MLA) and the micronucleus test (MN) were performed in L5178YTk ± cells, and the Caco-2 cells were used for the standard and modified comet assays. The results indicated that GO (0–250 μg/mL) was not mutagenic in the MLA. However, rGO revealed mutagenic activity from 250 μg/mL and 125 μg/mL after 4h and 24h of exposure, respectively. In the MN test, negative results were obtained for both compounds at the concentrations assayed (0–250 μg/mL) for GO/rGO. Moreover, no DNA strand breaks, or oxidative DNA damage were detected in Caco-2 cells exposed to GO (0–250 μg/mL) and rGO (0–176.3 μg/mL for 24h and 0–166.5 μg/mL for 48h). Considering the mutagenic potential of rGO observed further investigation is needed to describe its toxic profile.

1. Introduction

Graphene was described for the first time in 2004 and since then it has gained prominence among nanomaterials studies. Graphene is defined as a single carbon layer of the graphite structure, in which planar sheets of carbon atoms are arranged in a benzene-ring structure [1]. Graphene has two main derivatives, namely graphene oxide (GO) and reduced-graphene oxide (rGO). GO could be obtained by oxidation of graphite, and it has a layered carbon structure with a variety of oxygen functional groups (as carboxyl, hydroxyl, epoxy groups) attached to both sides of the layer as well as the edges of the plane, which not only expand the interlayer distance but also make GO hydrophilic. That allows the attachment of biomolecules to its structure for a wide range of bio-applications [2]. rGO is produced by removing part of the oxygen content groups from the graphene oxide structure through chemical or thermal processes. This procedure makes rGO less hydrophilic than GO, decreasing its dispersibility and stability in physiological environments [3]. Both graphene materials have attracted an ever-increasing amount of interest over the past few years due to their versatility and unique properties. Moreover, they have many promising applications in numerous fields, as electrochemical biosensing, drug delivery, medical implants, or food packaging [4]. In particular, both graphene derivatives have demonstrated to be highly promising for food packaging application. Actually, rGO films have been shown to have better properties than GO-films in this field [5]. For some of these applications to become a reality, it is necessary and scientifically relevant to evaluate their potential toxicity. Specifically, its use in the food industry is causing some concern to the consumers since the intake of these nanomaterials may increase. Recently, we have demonstrated that GO and rGO were internalized by Caco-2 cells, causing significant intracellular alterations as mitochondria damage [6]. However, different results were observed in regard to cytotoxicity. In this sense, rGO induced cytotoxicity, while GO did not show any effect, in agreement with the results of scientific literature in the same cell line [7,8]. Actually, concentrations that induce rGO cytotoxicity in Caco-2 cells are in the range of the limit established by the regulation of plastic materials and articles intended to

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come into contact with food [6,9].

Nonetheless, apart from those toxic effects, genotoxicity and mutagenicity are an important aspect to be evaluated for hazard identification purposes. Genotoxicity is defined as the process which alters the structure, information content or segregation of DNA, meanwhile mutagenicity refers to the induction of permanent transmissible changes in the amount or structure of the genetic material of cells or organisms [10]. It is of great interest since it is required by the European Food Safety Authority (EFSA) for the authorization of additives [11], plan protection products [12], food contact materials [13] or pharmaceuticals [14], for instance. According to the guidance on risk assessment of nanomaterials to be applied in the food and feed chain published by EFSA 2021 [13], the minimum requirements for the assessment of genotoxicity are a bacterial reverse mutation test (Ames test) (OECD 471, 2020) [15] and a micronucleus test (MN) (OECD 487, 2016) [16]. Thus, the three critical endpoints of genotoxicity, gene mutation, structural, and numerical chromosome aberrations, are included. However, the Ames test is not recommended to assess the mutagenicity of nanoparticles, because they may not be able to penetrate the bacterial cell wall and because bacterial cells do not have the ability to internalise, and this could induce false negative results [13]. In this sense, to assess the genotoxicity of GO and rGO, the Ames test can be replaced by the mouse lymphoma assay (MLA) (OECD 490, 2016) [17]. The MLA employs the thymidine kinase gene of L5178Y Tk $^{\pm}$ to detect a wide spectrum of genetic damage, including both gene and chromosomal mutations, in contrast with other gene-mutation assays [17]. Concerning the MN test, it is one of the preferred methods for assessing chromosome damage because it detects clastogenic and aneugenic chromosome aberrations. In the case of contradictory or inconclusive results from these two *in vitro* tests, it may be appropriated to conduct further *in vitro* tests, such as the comet assay, to optimize any subsequent *in vivo* testing, or to provide additional useful mechanistic data [10].

Nowadays, the use of the comet assay performed under alkaline conditions (pH $>$ 13) is considered the optimal version for identifying agents with genotoxic activity. The alkaline comet assay is capable of detecting DNA double-strand breaks and single-strand breaks, since the alkaline treatment converts single-stranded lesions into double stranded lesions. Moreover, it detects alkali-labile sites, DNA-DNA/DNA-protein cross-linking, and incomplete excision repair sites [18]. Probably, on top of its advantages is the versatility and flexibility of the assay [19]. Besides, the DNA damage is measured at the level of individual cells, allowing the detection of cell populations with different levels of DNA damage [19]. While SBs are a valid indicator of potentially carcinogenic DNA damage, they do not provide a comprehensive assessment of such damage since many genotoxic agents cause alterations to the DNA that do not break the phosphodiester backbone.

Uptake of graphene materials by cells could be associated to toxicity including genotoxicity. However, studies have suggested that the genotoxic effect of GO/rGO might be attributed to oxidative stress by increased ROS production [20–26] or by the direct interaction between graphene and nuclear DNA in a size-dependent manner [27–29]. In particular, Cebadero et al. [6], observed that GO and rGO altered oxidative stress markers (ROS and GSH) in human colon carcinoma cells (Caco-2). Similarly, Kucki et al. [30] also observed ROS induction for GO in the same cell line. For this reason, to clarify whether graphene materials have a direct or indirect genotoxicity mechanism of action, the use of the modified version of the alkaline comet assay is of interest. Thus, the incorporation of several enzymes such as formamidopyrimidine DNA glycosylase (FPG) and endonuclease III (Endo III), in combination with the comet assay, allows the measurement of oxidized purines and pyrimidines, respectively [31]. These are the enzymes most used nowadays [32]. Regarding graphene nanomaterials, their genotoxicity has been investigated in the scientific literature, but not extensively, and for rGO data are particularly scarce. Moreover, regarding to GO, in most cases the genotoxicity has been evaluated using only one assay (MN or Comet assay) and no studies have employed the *in vitro* tests battery required by the EFSA. In regard to mutagenicity, the assays recommended by EFSA are the *in vitro* mammalian cell gene mutation tests using the Hprt and xprt genes (OECD TG 476 (OECD, 2016b)) [33] or the *in vitro* mammalian cell gene mutation test using the thymidine kinase gene (OECD TG 490 (OECD, 2016e) [17]. Most of studies in the scientific literature used the Comet assay, although it is not included among the tests primarily recommended by EFSA [34]. Moreover, for this assay contradictory results were reported *in vitro* in human intestinal models (colon carcinoma cell line) [35] for GO. This means that results could not be generalized and more information about genotoxicity is required.

Hence, the aim of this work was to explore the potential mutagenicity and genotoxicity of two graphene derivatives, a commercial rGO and GO developed by the Technological Institute of Packaging, Transport, and Logistics (ITENE). rGO was selected due to the scarce genotoxicity data available and GO for comparative purposes and in order to elucidate its genotoxic effects as contradictory reports are available. This research could support the future use of these graphene derivatives as food contact materials in a risk assessment frame. For this purpose, a battery of different *in vitro* assays recommended by EFSA Scientific Committee was used: MLA assay [17] and MN test [16] on L5178Y Tk $^{\pm}$ mouse lymphoma cell-line. These studies were completed with the standard comet assay to investigate the possible DNA damage in human intestinal Caco-2 cells. Moreover, the enzyme modified comet assay was also included to detect oxidative DNA damage using the digestion of DNA with lesion-specific enzymes, FPG and Endo III.

2. Materials and methods

2.1. Chemicals and reagents

GO was synthetized by ITENE following the Hummers' method [36] and rGO was purchased from Graphitene, Ltd (Flixborough, UK). Both graphene materials were previously characterized as described in Cebadero-Domínguez et al. [6]. The characterization showed an interlayer distance to 0.75 nm, and 0.32 nm in GO and rGO, respectively. rGO presented higher C/O ratio (6.35) than GO (1.99), indicating a lower oxygen content. Besides, both graphenes have irregular layers and wrinkled structures.

Chemicals for different assays were supplied by Gibco (Biomol, Sevilla, Spain), Sigma-Aldrich (Madrid, Spain), and C-Viral S.L. (Sevilla, Spain).

2.2. Cells and culture conditions

L5178Y Tk $^{\pm}$ mouse lymphoma cells (ATCC® CRL-9518) were used for MLA and MN test (Passages 10–25). Previously, L5178Y/Tk $^{\pm}$ cells were subjected to cleansing to purge excess possible Tk$^{-/-}$ mutants by culturing cells for 24 h in THMG medium (thymidine 9 mg/mL, hypoxantine 15 mg/mL, methotrexate 0.3 mg/mL, glycine 22.5 mg/mL). Afterwards, cells were transferred to THG medium (THMG without methotrexate) for 2 days. The purged cultures were checked for a low background of Tk$-/-$ mutants and were stored in liquid nitrogen.

Caco-2 cell line (Passage 10–20) was used for standard and enzyme-modified comet assays, and derives from a human colon adenocarcinoma (ATCC© HTB-37). Caco-2 cells were cultured in a medium consisting of minimum essential medium (MEM) supplemented with 20% fetal bovine serum (FBS), 1% non-essential amino acids, 50 g/mL gentamicin, 2 mM L-glutamine and 1 mM pyruvate. L5178Y Tk $^{\pm}$ were cultured in suspension in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum, 2 mM l-glutamine, 10000 U/mL penicillin, 10 mg/mL streptomycin and 1 mM sodium pyruvate. Both cultures were maintained in a humidified incubator at 37 °C with 5% CO_2 and 95% relative humidity.

2.3. Test solutions

For MLA, the test concentrations were chosen in accordance with previous tests performed to define the cytotoxicity of GO and rGO by the relative total growth (RTG) after a treatment of 4 and 24 h. RTG did not exceed more than 20%, which is usually accepted as the maximum level of toxicity in the OECD 490 guideline. According to these results, we selected 250 μg/mL as the highest concentration.

The test concentrations for MN were chosen considering their solubility in the culture medium [16]. Also, a trypan blue exclusion test was performed to confirm that the concentrations did not induce cytotoxicity in L5178Y Tk $^{\pm}$ cells. GO and rGO did not showed statistical differences versus control at the concentration range assayed. Thus, 250 μg/mL was selected as the highest concentration for the main test.

For the standard and enzyme-modified comet assays, the tested concentrations were chosen based on previous cytotoxicity studies using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium reduction assay [6]. GO did not show a significant viability reduction, then, the exposure concentrations were 62.5, 125, and 250 μg/mL. For rGO, the highest exposure concentration for the comet assay in Caco-2 cell was the mean effective concentration (EC_{50}) for 24 h (EC_{50}, 176.3 ± 7.6 μg/mL) and for 48 h (166.5 ± 21.9 μg/mL), along with the fractions $EC_{50}/2$ and $EC_{50}/4$.

Previously to the exposure, graphene samples were sonicated for 1h.

2.4. Mouse lymphoma thymidine-kinase assay

The MLA was carried out according to OECD Guideline 490 [17] and Puerto et al. [37]. RPMI medium was used as negative control, methylmethanesulfonate (MMS 10 μg/mL) was used as positive control, and five concentrations of GO and rGO in the range 0–250 μg/mL (for 4 h and 24 h-assays).

To assess the viability and mutagenicity, cells were seeded at a density of 10^4 cells/mL in 96-well plates (two replicates per experimental group in each case). For the mutation assessment, trifluorothymidine (TFT) was added to the replicates at a final concentration of 4 μg/mL. The viability plates and the mutagenicity plates were incubated at 37 °C and 5% CO_2 for 12 days. After incubation, viable colonies were counted. To assist the scoring of mutant colonies, thiazolyl blue tetrazolium (MTT) salt (2.5 mg/mL) was added to mutagenicity plates and they were incubated for 4 h.

Afterwards, colony size was estimated in a similar manner to that described by Puerto et al. [37]. The induced mutant frequency (IMF) was determined according to the formula IMF = MF- SMF, where MF is the test culture mutant frequency and SMF is the spontaneous mutant frequency. Positive responses are determined as those that for any treatment meet or exceed the global evaluation factor (GEF, 126 for the microwell assay) and also when a positive trend is obtained.

2.5. In vitro micronucleus assay

This assay was performed according to the OECD Guideline 487 [16] and EFSA recommendations [13]. L5178Y Tk $^{\pm}$ cells were seeded at 2.0 × 10^5 cell/mL and treated with the same five different concentrations for both GO and rGO (15.6; 31.25; 62.5; 125; 250 μg/mL) for 24 h. RPMI medium was used as negative control. Concurrent positive controls were used to identify clastogens (0.0625 μg/mL mitomycin C) and aneugens (0.025 μg/mL colchicine). After 24 h of exposure, cells were exposed to cythochalasin B (6 μg/mL) for 20 h. Then, cultures were centrifuged, and the pellets were subjected to a hypotonic treatment with KCl. Afterwards, the cells were again centrifuged and fixed. The resultant pellets were resuspended, dropped on microscope slides, and stained with giemsa 10%. Quantification of binucleated cells with micronuclei (BNMN) was determined analysing at least 2000 binucleated cells/concentration and the nuclear division index (NDI) were calculated in at least 1000 cells/concentration.

2.6. In vitro standard alkaline comet assay

Standard alkaline comet assay was carried out to detect DNA strand breaks as previously described by Azqueta and Collins [19]. and Medrano-Padial et al. [38] with modifications. Caco-2 cells were seeded at 3.5x10^5 cells/mL into 24-well tissue culture-treated plates (Corning Costar Corporation, New York, USA). After 24 h and 48 h, cells were incubated with different concentrations of GO (62.5, 125 and 250 μg/mL) and rGO for 24h (44.1, 88.2, and 176.3 μg/mL) and 48 h (41.6, 83.3, and 166.5 μg/mL). Medium-treated cells were used as a negative control and cells treated with a solution of 100 μM H_2O_2 for 5 min were used as positive control. After the exposure, Caco-2 cells were washed, trypsinized and resuspended in phosphate buffer saline (PBS) at 2.5 × 10^5 cell/mL. Subsequently, the cell suspensions were mixed with 1% (w/v) low-melting-point agarose, and samples were placed on agarose precoated glass slides. Slides were placed on the bottom metal holder of the 12-Gel Comet Assay Unit™ (NorGenoTech, Oslo, Norway). Afterwards, the slides were incubated in lysis buffer (2.5 M NaCl, 0.1 M Na_4EDTA-$2H_2O$, 10 mM Tris, 1% Triton X-100, pH 10) at 4 °C for at least 1 h. Later on, electrophoresis was carried out at 0.81 V/cm (300 mA) for 20 min. Slides were washed in PBS and distilled water for 10 min, and fixed with 70% ethanol and absolute ethanol for 15 min.

The DNA was stained with SYBR Gold nuclei acid gel stain (Invitrogen, Life Technologies, USA) and visualized with an Olympus BX61 fluorescence microscope coupled via a Charge Coupled-Device camera to an image-analysis system (DP controller-DP manager). Images of randomly selected nuclei (>100) per experimental point were analyzed with image analysis software (Comet Assay IV, Perceptive Instruments, UK). The results were expressed as mean % DNA in tail respect to the negative control group.

2.7. Titration and in vitro enzyme-modified comet assay

Before performing the modified comet assay, the titration of FPG and Endo III was carried out. For the titration of FPG, Caco-2 cells treated with Ro19-8022 (2.5 μM) and white light (2.5 min) on an ice bath was employed as positive control to generate predominantly 8-oxo-7,8-dihydroguanine [38].

FPG was prepared by making serial dilutions of 0.01, 0.03, 0.1, 0.3, 1 and 3.33 U/mL using the reaction buffer F (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL bovine serum albumin, pH 8.0 adjusted with 6 M KOH). For Endo III, Caco-2 cells were exposed to 500 μM H_2O_2 for 5 min and seeded in MEM for 1 h and 45 min to repair the strand breaks and leave only the oxidized lesions [32]. Cells were incubated with different concentrations of the enzyme with buffer F previously prepared (3.33, 10, 15, 21.7 and 33.3 U/mL).

For the enzyme-modified comet assay, after incubation in lysis buffer, slides were washed 3 times for 5 min each with buffer F. Slides were then placed on a cold metal plate to add the buffer lysis, buffer F, FPG and Endo III in each gel. The 12-Gel Comet Assay Unit™ were then transferred to a pre-heated moist box and placed in the incubator at 37 °C for 1 h.

Electrophoresis, neutralizing, fixation, dying and quantification of the nuclei were carried out as previously described for the standard assay.

2.8. Statistical analysis

In the MLA, the MF analyses were performed using IBM SPSS Statistics (Madrid, Spain). The statistical analysis of MN assay was performed using Kruskal-Wallis test followed by Dunn's multiple comparisons test. For the standard comet assay, significant differences about % DNA in tail were calculated by one way-Analysis of Variance (ANOVA) test followed by Tukey-Kramer multiple comparisons test. For the modified comet assay, differences in mean values between groups were compared using the Kruskal-Wallis test followed by Dunn's

multiple comparisons test.

For MN and comet assays, the analyses of results were carried out using Graph-Pad Prisma 9 version 9.0.0 software (Madrid, Spain). Differences were considered significant at *p < 0.05, **p < 0.01, ***p < 0.001.

3. Results

3.1. Mouse lymphoma thymidine-kinase assay (MLA)

GO and rGO were analyzed for their general mutagenicity with L5178Y Tk ±cells using the MLA. This test detects point mutations and different types of chromosomal mutations. Tables 1 and 2 show the results obtained in MLA after the treatment of L5178Y Tk ±cells with GO and rGO. In the case of GO, no increase was observed in the frequency of mutation for any of the concentrations or exposure times (4 h and 24 h) assayed as compared to control group (Table 1). Also, this absence of mutagenic response was corroborated with the absence of significant changes in small and large colonies and in the IMF values.

When cells were treated with rGO after 4h (Table 2), a significant increase in mutant frequency was observed from 125 μg/mL. Regarding to the IMF, the result exceeded the values of 126 × 10^{-6} from 62.5 μg/mL and 125 μg/mL after 4 h and 24 h of exposure respectively. However, reproducible increase in IMF was evident at 250 μg/mL (in both experiments) for 4 h. These results demonstrated that rGO is mutagenic.

Two independent experiments were performed with both graphene materials and a concordance was observed between them. Also, positive controls treated with MMS (10 μg/mL) were run in parallel, demonstrating an absolute increase in total MF.

3.2. Micronucleus assay (MN)

The genotoxicity of GO and rGO were determined using the MN test, which detects chemical-exposure induced formation of micronuclei in the cytoplasm of interphase cells. Regarding to the induction of micronuclei by GO and rGO, no increase in the frequency of BNMN was observed at any concentration assayed compared to the control group (Table 3). Next, the NDI was calculated to quantify the efficacy of cell division, where values above 2 indicates that all cells have experienced one division, while values below 1 indicates no cell division. In this case, no statistical significance was observed at any concentrations tested for any graphene material. The exposure to positive controls for aneugens (colchicine) and clastogens (MMC) significantly increased the number of BNMN (*p < 0.05, **p < 0.01, ***p < 0.001).

3.3. In vitro standard comet assay

With the aim to determine the GO and rGO induced DNA damage, both double and single-strand DNA breaks by the alkaline comet assay were quantified in exposed cells.

Compared to the negative control, Caco-2 cells exposed to GO did not undergo DNA breaks from 62.5 to 250 μg/mL after 24 h and 48 h of exposure (Fig. 1). Same results were obtained after rGO exposure. This material did not induce significant increases of % DNA in tail in Caco-2 cells at any concentration tested (EC50/4, EC50/2 and EC50) after both exposure times.

In all the experiments, the positive control (H_2O_2) showed significant increases of % DNA in tail (***p < 0.001) under the conditions tested.

3.4. Titration and in vitro enzyme-modified comet assay

Modified comet assay with the incorporation of FPG and Endo III enzymes was used to detect oxidative DNA damage. Based on experiments of titration, the selected enzyme concentrations were 0.3 U/mL and 15 U/mL for commercial FPG and Endo III, respectively (data not shown).

In Caco-2 cells exposed to GO or rGO and post-treatment with FPG, no significant variations in DNA strand breaks were detected after 24 h or 48 h of exposure (Fig. 2). Based on the results of Fig. 3, no increase of % DNA in tail was observed when Endo III was used in the cells exposed to GO and rGO after 24 h and 48 h.

In cells treated with Ro19–8022 or H_2O_2 (positive controls), a

Table 1
Toxicity and mutagenicity of GO in L5178YTk ± cells after 4 h (a) and 24 h (b) without S9 fraction by the MLA. Positive controls: methylmethanesulfonate, MMS 10 μg/mL[a] Total mutant frequency divided into small/large (S/L) colony mutant frequencies. The induced mutant frequency (IMF) was determined according to the formula IMF = MF-SMF, where MF is the test culture mutant frequency and SMF is the spontaneous mutant frequency. The significance levels observed is d ***p < 0.001 in comparison to negative control group values (medium).

a) In absence of S9 (4 h).

Concentration (μg/mL)	Relative total growth		Percent plating efficiency		Mutant frequency (x 10^{-6})		MF (S/L)[a]		IMF (MF-SMF) (x 10^{-6})	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2
0	100	100	76	106	128	98	40/88	38/60	–	–
15.6	62	96	106	76	142	167	54/88	60/107	13.9	69
31.2	64	53	118	64	64	166	29/35	76/90	−64	68
62.5	65	60	68	108	124	146	40/84	76/70	−3.8	48
125	27	57	108	80	133	198	47/86	99/99	4.5	100
250	34	38	87	81	166	172	44/122	95/77	3.8	75
MMS (10 μg/mL)	84	51	56	70	641***	1150***	359/282	717/433	513	982

b) In absence of S9 (24 h).

Concentration (μg/mL)	Relative total growth		Percent plating efficiency		Mutant frequency (x 10^{-6})		MF (S/L)[a]		IMF (MF-SMF) (x 10^{-6})	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2
0	100	100	65	69	135	132	49/86	62/70	–	–
15.6	47	78	88	75	230	216	156/74	114/102	95	84
31.2	33	88	118	71	178	154	122/56	109/45	43	23
62.5	39	70	127	93	121	151	57/64	64/87	−1.3	20
125	59	61	67	81	214	217	120/94	110/107	79	86
250	49	34	75	77	178	156	115/63	49/107	43	24
MMS (10 μg/mL)	27	39	44	61	2200***	1950***	926/1274	1118/832	2060	1820

***p < 0.001.

Table 2
Toxicity and mutagenicity of rOG in L5178YTk ± cells after 4 h (a) and 24 h (b) without S9 fraction by the MLA. Positive controls: methylmethanesulfonate, MMS 10 μg/mL[a] Total mutant frequency divided into small/large (S/L) colony mutant frequencies. The induced mutant frequency (IMF) was determined according to the formula IMF = MF-SMF, where MF is the test culture mutant frequency and SMF is the spontaneous mutant frequency. The significance levels observed are **p < 0.01 and ***p < 0.001 in comparison to negative control group values (medium).

a) In absence of S9 (4 h)

Concentration (μg/mL)	Relative total growth		Percent plating efficiency		Mutant frequency (x 10^{-6})		MF (S/L)[a]		IMF (MF-SMF) (x 10^{-6})	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2
0	100	100	104	74	116	124	63/53	50/74	–	–
15.6	78	94	85	106	203	191	107/96	75/116	87	67
31.2	58	84	102	80	155	235	84/71	87/148	39	111
62.5	46	80	135	82	250	205	189/61	126/79	134	81
125	35	54	118	159	220	336**	133/87	205/131	105	212
250	36	51	95	121	271	447***	227/44	349/98	156	323
MMS (10 μg/mL)	56	84	88	77	606**	609***	331/275	306/303	491	485

b) In absence of S9 (24 h).

Concentration (μg/mL)	Relative total growth		Percent plating efficiency		Mutant frequency (x 10^{-6})		MF (S/L)[a]		IMF (MF-SMF) (x 10^{-6})	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2
0	100	100	113	113	149	115	81/68	65/50	–	–
15.6	93	64	80	100	212	72.8	157/55	30/42	62.5	−42.3
31.2	91	53	116	113	253	152	169/84	102/50	104	37.4
62.5	86	50	111	84	260	219	189/71	147/72	111	104
125	43	50	96	47	285	275	181/104	140/135	135	160
250	23	38	106	59	443	377	283/160	246/131	294	262
MMS (10 μg/mL)	39	30	32	27	2840***	3160***	1491/1349	1457/1703	2690	3040

***p < 0.001.

Table 3
Percentage of binucleated cells with micronuclei (BNMN) and the nuclear division index (NDI) in cultured mouse lymphoma cells L5178YTk ± exposed to GO (a) and rGO (b). The genotoxicity assay was performed in absence of the metabolic fraction S9. Clastogen and aneugen positive controls were mitomicyn C (0.0625 μg/mL) and colchicine (0.0125 μg/mL), respectively. The values are expressed as mean ± SD. The significance levels observed are *p < 0.05, **p < 0.01 and ***p < 0.001 in comparison to control group values (negative control = medium).

a)

	Exposure Time	BNMN (%) ± SD	NDI ± SD
Control - *GO*	24 h	0.50 ± 0.35	1.59 ± 0.03
15.6 μg/mL	24 h	0.30 ± 0.26	1.58 ± 0.02
31.25 μg/mL		0.65 ± 0.38	1.60 ± 0.04
62.5 μg/mL		0.70 ± 0.26	1.59 ± 0.02
125.0 μg/Ml		0.40 ± 0.37	1.59 ± 0.04
250.0 μg/mL		0.45 ± 0.19	1.52 ± 0.05
Mit C (0.0625) μg/mL)	24 h	3.85 ± 0.34 *	1.53 ± 0.06
Colch (0.0125 μg/mL)		4.30 ± 0.84 **	1.52 ± 0.04

b)

	Exposure Time	BNMN (%) ± SD	NDI ± SD
Control - *rGO*	24 h	0.97 ± 0.23	1.59 ± 0.02
15.6 μg/mL	24 h	1.60 ± 0.43	1.59 ± 0.04
31.3 μg/mL		1.15 ± 0.1	1.55 ± 0.03
62.5 μg/mL		1.60 ± 0.63	1.56 ± 0.02
125.0 μg/mL		0.70 ± 0.26	1.65 ± 0.05
250.0 μg/mL		0.55 ± 0.34	1.59 ± 0.04
Mit C (0.0625) μg/mL)	24 h	6.10 ± 0.95 ***	1.50 ± 0.04
Colch (0.0125 μg/mL)		6.60 ± 0.28 ***	1.72 ± 0.04

significant increase in DNA breakage was observed when they were incubated with FPG and Endo III, respectively (Figs. 2 and 3).

4. Discussion

The rapid development and widespread application of graphene-based nanoparticles raises concern about increased human exposure and the potential risks [39]. In this sense, genotoxic effects of GO and rGO *in vitro* has been detected by different authors and reviewed by Cebadero-Dominguez et al. [34]. However, none of these two derivatives have been evaluated using the *in vitro* tests battery required by the EFSA. [13], and no single assay have the capacity to detect all genotoxic agents, nor all types of genetic damage. In our studies, several tests covering the main genotoxic mechanisms were performed, including MLA, MN test and standard and enzyme-modified comet assays. The extracellular metabolic activation system (S9-mix) was not used in any of the tests as most poorly soluble nanomaterials are not metabolized, and S9-mix could interfere with the assay reducing the nanomaterial bioavailability [13].

The TK ±mutation assay is considered by EFSA [10] as one of the most commonly used *in vitro* methods to investigate gene mutations. This test can detect a wide range of genetic events including gene mutations (point mutations, frameshift mutations, small deletions, etc.) and chromosomal events (large deletions, chromosome rearrangements and mitotic recombination) [40]. Despite its importance, there is a lack of data evaluating the mutation of the thymidine kinase locus as a target in general, and in particular after GO and rGO exposure. The results obtained with regard to the MLA revealed that GO produced no mutagenicity after 4 h of exposure on L5178Y Tk ±mouse lymphoma cells. To accomplish with the International Conference on Harmonisation (ICH) recommendations [14], a treatment without metabolic activation for approximately 24 h was included. Similarly, GO did not induce significant increase in the mutation frequency after 24 h treatment, indicating that prolonged exposure time is not needed for mutations to occur. Demir and Marcos [41] also demonstrated that graphene nanoplatelets showed absence of alterations in the mutation frequency in the L5178Y

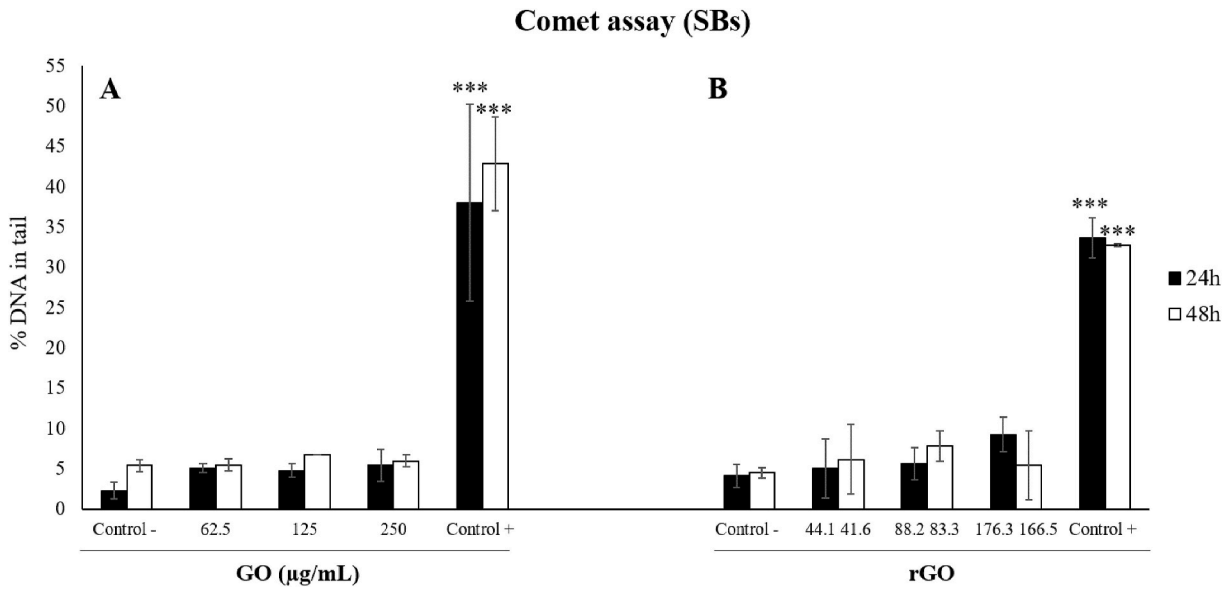

Fig. 1. DNA damage measured in Caco-2 cells expressed as the formation of strand breaks (SBs). A, % DNA in tail after 24 h and 48 h of exposure to GO (µg/mL). B, % DNA in tail after 24 h and 48 h of exposure to rGO (µg/mL). All values are expressed as mean ± SD. The significance levels observed are ***$p < 0.001$ in comparison with the negative control group values (medium). Positive controls were exposed to H_2O_2 (100 µM).

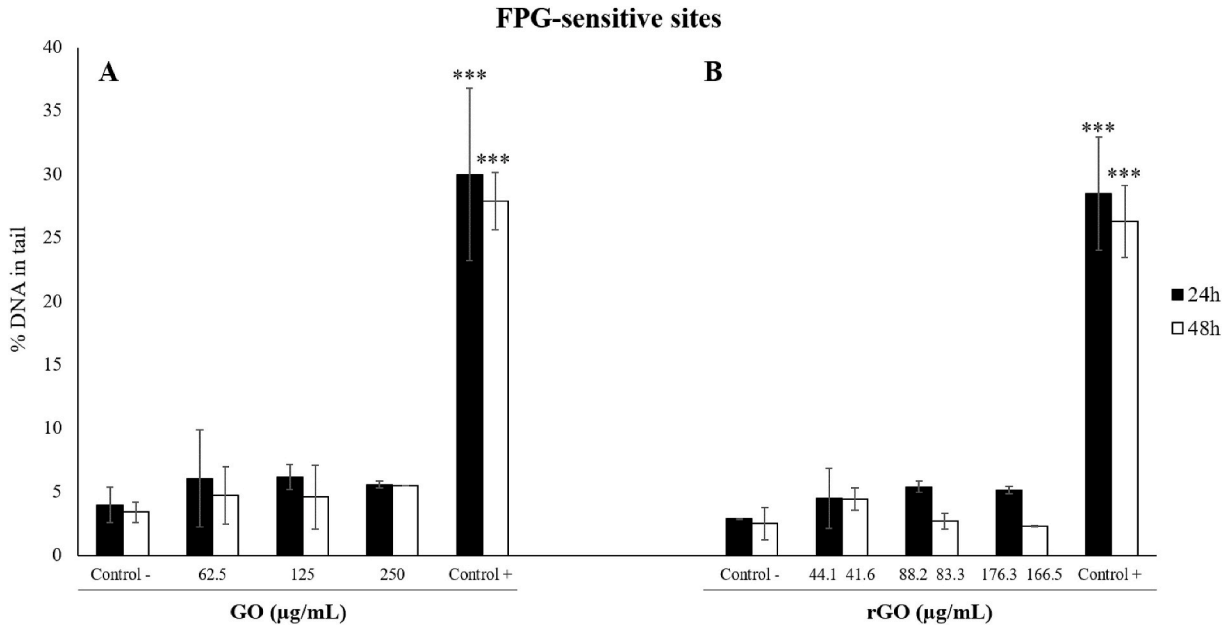

Fig. 2. Oxidative DNA damage measured in Caco-2 cells expressed as FPG-sensitive sites. A, % DNA in tail after 24 h and 48 h of exposure to GO (µg/mL). B, % DNA in tail after 24 h and 48 h of exposure to rGO (µg/mL). All values are expressed as mean ± SD. ***$p < 0.001$ is considered significantly different from the negative control. Positive controls were exposed to Ro19-8022 (2.5 µM).

Tk ±mouse lymphoma cells at concentrations from 0.01 to 250 µg/mL at 4h of exposure. Similarly, Petibone et al. [42] used the same target as in our study (TK gene) but in TK6 and NH32 cell lines exposed to oxygen functionalized graphene (f-G). No significant effects were observed in these mammalian cells lines for 4 h. These two studies did not include longer exposure times. On the other hand,. after 4h of incubation with rGO, the IMF exceeds the GEF only in one of the two replicate experiments at 62.5 µg/mL and 125 µg/mL. However, reproducible increase in IMF was evident at 250 µg/mL (in both experiments) being this concentration biological relevant. Since the increase in IMF above the concurrent background exceeds the GEF and the increase is concentration related, then rGO is considered able to induce mutation from 250 µg/mL and 125 µg/mL at 4 h or 24 h of exposure, respectively. Moreover, the observed increase in MF was related with to induction of small colonies. Landsiedel et al. [43] indicated that the small colonies arise due to large deletions, chromosome rearrangements, and mitotic recombination as well as specifically due to large damage to chromosome11b, which harbors the Tk gene. This is indicative of a clastogenic potential of rGO. In addition, increase in MF coincided with a marked increase in toxicity, as shown by decreased RTG values.

Moreover, this assay is capable to detect a wide spectrum of mutations and to use liquid media cloning in 96-microwell plates makes this method one of the most sensitive to detect mutagenicity of nanoparticles [44]. Although, the molecular initiating event that triggers the adverse

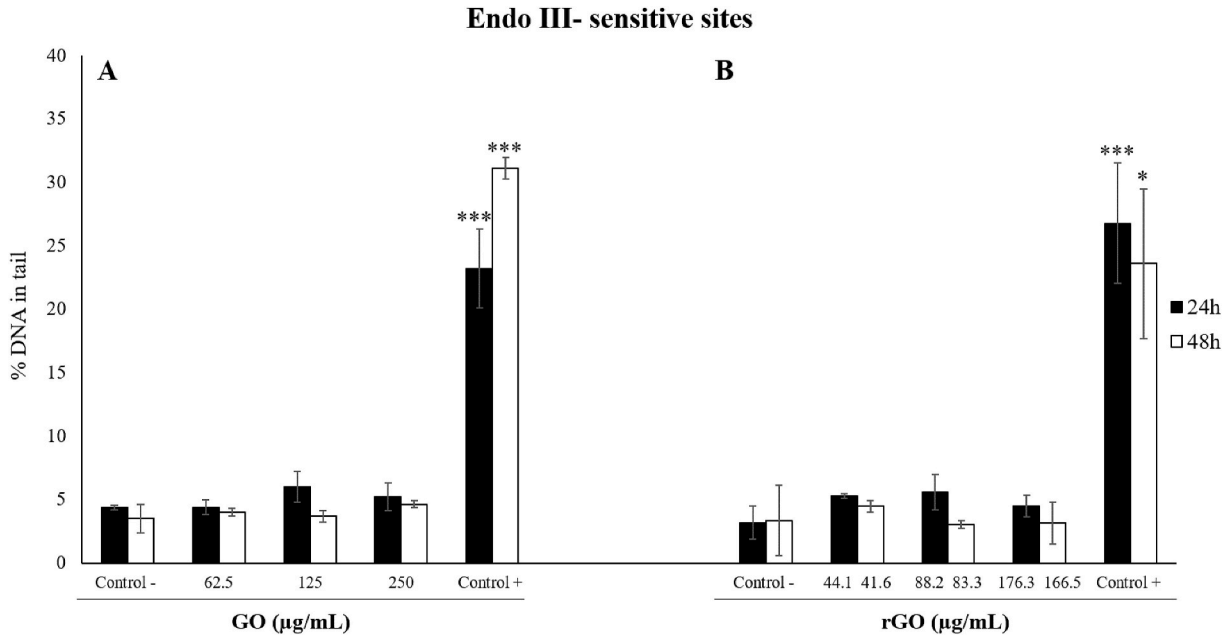

Fig. 3. The level of DNA damage measured in Caco-2 cells expressed as Endo III-sensitive sites. A, % DNA in tail after 24 h and 48 h of exposure to GO (µg/mL). B, % DNA in tail after 24 h and 48 h of exposure to rGO(µg/mL). All values are expressed as mean ± SD. *$p < 0.05$, ***$p < 0.001$ are considered significantly different from the negative control. Positive controls were exposed to H_2O_2 (100 µM).

outcome pathway is not fully elucidated, different key events such as inadequate repair of DNA strands, increasing gene mutation, chromosome break or rearrangement could lead to the mutagenicity observed [45]. Differences in mutagenicity between GO and rGO could be attributable to their different chemical composition, size, surface structure, solubility, shape, and aggregation state [46,47]. Specifically, GO reduction alter the physicochemical properties of the original material that conceivably have unique interactions with cell components and produce toxicity through very different mechanisms [48]. Actually, there are authors that have reported that GO, due to its higher amount of oxygen functional groups in its structure (-OH and –COOH), is easily cross-linked to a variety of materials, including DNA, protein, biomolecules, or polymers, which prevent aggregation in salt and other biological solutions and improve biocompatibility [3]. Nevertheless, only a few investigations have endeavoured to elucidate the influence of the oxidation level, and the associated toxicity mechanisms are yet to be fully understood [49].

In addition to the mutagenicity study, the assessment of the genotoxic potential is another critical step that has been considered, as MLA is not appropriate to detect aneugenes. In this sense, the *in vitro* mammalian cell MN test [16] was employed, that detects structural and numerical chromosome damage. The *in vitro* MN assay was adapted for the genotoxicity assessment of nanomaterials, using a long-duration treatment (24 h) that facilitates the contact of the test items with DNA after nuclear membrane dissolution during mitosis. Moreover, cytochalasin B was used after nanomaterial treatment. It is recommended to delay its addition because of its ability to inhibit endocytosis and reduce nanomaterial cell uptake [13].

Our results did not show a significant increase in BNMN in the L5178Y Tk ±cell line after 24 h of exposure for any GO and rGO concentrations (15.6–250 µg/mL) evaluated. In general, and as it has been stated before, toxicity studies by means of *in vitro* MN tests are scarce for graphene materials [34] and there is no clear understanding on the exact mechanisms involved. In the scientific literature could be found positive results by GO and rGO in different cell lines [27,50,51].

However, in our case, we confirmed that both graphene derivatives are neither cytostasic nor aneugenic/clastogenic agents by MN test.

Regarding the comet assay, it is the technique most widely used to evaluate the genotoxicity of different graphene materials [34]. The genotoxic potential of GO using the comet assay in different cell lines has been already documented whereas only few studies were available for rGO. In this work, we investigated the potential genotoxicity of these compounds on Caco-2 cells, the most frequently used cell model of the intestinal tract [52]. To our knowledge, there are few studies using the comet assay with GO in gastrointestinal cells [8,28,35] showing contradictory results. In the present work, we have demonstrated that GO and rGO do not directly induce DNA SBs after 24 or 48 h at any concentrations assayed in Caco-2 cells. To correctly interpret the negative results, it is necessary to take into account whether the material tested has been in contact with the cells or not (EFSA, 2021). In our case, in *vitro* internalization of GO and rGO has been demonstrated in Caco-2 cell line [6], so it can be concluded that the exposure do not lead to genotoxic effects at the tested conditions.

Different authors have shown that GO and rGO do not produce genotoxicity by comet assay [20,35,53–55]. Xing et al. [56] suggested that the mechanisms by which these graphene derivatives exert their biological activities are not centred on the inestability and the genome integrity, but rather in mitochondrial metabolism and oxidative damage. In this sense, the lack of genotoxicity suggests that relative high levels of ROS generation are required to induce DNA strand breaks, as it can be seen in the positive control, where 100 µM H_2O_2 was used and the result showed significant increases of % DNA in tail. In sake of a good toxicological evaluation, studies with positive results for this same assay should be also taken into account. In particular, a different sizes of GO (induced single and double strand breaks, in HepG2 and Caco-2 cells, respectively [21,28].

Also, GO was able to induce DNA breaks in Caco-2/HT29 model of intestinal barrier and in Colon 26 cells [8,35], in HEK 293T [22], in spermatogonial stem cells (SSCs) [23], in retinol pigment epithelium (RPE) cells [24], and in THP-1 [53]. Moreover, rGO caused DNA damage in other different experimental models such as HepG2, U87, SSCs and RPE cells lines [21,23,24,54].

As mentioned above, generation of ROS induced by nanomaterials could indirectly play a vital role in genotoxicity. Then, the comet assay was completed by the use of restriction enzymes (modified comet assays) recommended by EFSA 2021 [13], to evaluate the oxidized bases

and to evidence the possible oxidative DNA damage caused by GO/rGO. In the present work, GO and rGO did not induce oxidation of purine bases at any concentration tested in Caco-2 cells. Similarly, Domenech et al. [8] reported that GO (5–50 μg/ml) in presence of FPG enzyme did not cause DNA damage in a coculture of differentiated Caco-2/HT29 cells. Moreover, the results obtained in the modified comet assay with Endo III showed that these compounds did not produce oxidation of pyrimidines bases. As far as we know, there are no other studies that explore oxidative mechanisms of genotoxicity for these graphene derivatives. According to our results, no oxidative damage in DNA bases were detected in the *in vitro* experimental model used, although an increase of ROS was previously observed by Cebadero-Domínguez et al. [6] for both test items. The absence of effects observed could be due to that ROS levels might not be high enough to produce oxidative DNA damage or an activation of nucleotide excision repair (NER) or base excision repair (BER), two oxidative DNA repair pathways [19].

5. Conclusion

The results have shown that GO and rGO did not exhibit genotoxic activity in mammalian L5178Y Tk $^{\pm}$ cells through the MN test. However, in the same eukaryotic system, rGO induced the mutation of the thymidine kinase gene (Tk $^{\pm}$ to Tk$^{-/-}$) in the MLA test from 125 μg/mL. In Caco-2 cells exposed to GO and rGO, no DNA damage was detected in the standard and modified-comet assays. Taking into account all the obtained results, rGO has demonstrated to induce mutagenic effects after application of the *in vitro* tests battery required by the EFSA. This raises concern about the toxicity profile of rGO and support a further thorough toxicological evaluation of this graphene derivative.

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Author contributions

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Concepción Medrano-Padial: formal analysis, investigation, methodology, writing-original draft.

María Puerto: investigation, methodology, supervision, writing-original draft and writing—review & editing.

Soraya Sánchez-Ballester: resources and writing—review & editing.

Ana María Cameán: funding acquisition, project administration, supervision and writing—review & editing.

Angeles Jos: methodology, funding acquisition, project administration, supervision and writing—review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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References

[1] K.S. Novoselov, A.K. Geim, S.V. Morozov, D. Jiang, Y. Zhang, S.V. Dubonos, I. V. Grigorieva, A.A. Firsov, Electric field effect in atomically thin carbon films, Science 306 (5696) (2004) 666–669, https://doi.org/10.1126/science.1102896.

[2] O.C. Compton, S.T. Nguyen, Graphene oxide, highly reduced graphene oxide, and graphene: versatile building blocks for carbon-based materials, Small 6 (6) (2010) 711–723, https://doi.org/10.1002/smll.200901934.

[3] M.G. Kim, J.Y. Par, Y. Shon, G. Shim, Y.K. Oh, Pharmaceutical applications of graphene-based nanosheets, Curr. Pharmaceut. Biotechnol. 14 (2013) 1016–1026, https://doi.org/10.2174/1389201015666140113113222.

[4] Z. Singh, Applications and toxicity of graphene family nanomaterials and their composites, Nanotechnol. Sci. Appl. 9 (2016) 15–28, https://doi.org/10.2147/NSA.S101818.

[5] K. Goh, J.K. Heising, Y. Yuan, H.E. Karahan, L. Wei, S. Zhai, J.X. Koh, N.M. Htin, F. Zhang, R. Wang, A.G. Fane, M. Dekker, F. Dehghani, Y. F. Chen, Sandwich-architectured poly(lactic acid)-graphene composite food packaging films, ACS Appl. Mater. Interfaces 8 (15) (2016) 9994–10004, https://doi.org/10.1021/acsami.6b02498.

[6] O. Cebadero-Domínguez, B. Ferrández-Gómez, B. Sánchez-Ballester, J. Moreno, A. Jos, A.M. Cameán, In vitro toxicity evaluation of graphene oxide and reduced graphene oxide on Caco-2 cells, Toxicol, For. Rep. 9 (2022) 1130–1138, https://doi.org/10.1016/j.toxrep.2022.05.010.

[7] R. Garriga, T. Herrero-Continente, M. Palos, V.L. Cebolla, J. Osada, E. Muñoz, M. J. Rodríguez-Yoldi, Toxicity of carbon nanomaterials and their potential application as drug delivery systems: in vitro studies in caco-2 and MCF-7 cell lines, Nanomaterials 10 (8) (2020) 1617, https://doi.org/10.3390/nano10081617.

[8] J. Domenech, A. Hernández, E. Demir, R. Marcos, C. Cortés, Interactions of graphene oxide and graphene nanoplatelets with the in vitro Caco-2/HT29 model of intestinal barrier, Sci. Rep. 10 (1) (2020) 1–15, https://doi.org/10.1038/s41598-020-59755-0.

[9] COMMISSION REGULATION (EU), No 10/2011 on plastic materials and articles intended to come into contact with food. https://eur-lex.europa.eu/eli/reg/2011/10/oj. (Accessed 14 January 2011).

[10] EFSA Scientific Committee, Scientific Opinion on genotoxicity testing strategies applicable to food and feed safety assessment, EFSA J. 9 (9) (2011) 2379. Available online: htpps://doi:10.2903/j.efsa.2011.2379 www.efsa.europa.eu/efsajournal.

[11] EFSA panel on food additives and nutrient sources added to food (ANS), guidance for submission for food additive evaluations, EFSA J. 10 (7) (2012) 2760. Available online: https://doi:10.2903/j.efsa.2012.2760 www.efsa.europa.eu/efsajournal.

[12] COMMISSION REGULATION (EU), No 283/2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market, http://data.europa.eu/eli/reg/2013/283/oj, 2013. (Accessed 1 March 2013).

[13] EFSA Scientific Committee, S. More, V. Bampidis, D. Benford, C. Bragard, T. Halldorsson, A. Hernández-Jerez, S. Hougaard Bennekou, K. Koutsoumanis, C. Lambré, H. Machera, K. Naegeli, S. Nielsen, J. Schlatter, D. Schrenk, V. Silano, D. Turck, M. Younes, J. Castenmiller, Q. Chaudhry, F. Cubadda, R. Franz, D. Gott, J. Mast, A. Mortensen, A.G. Oomen, S. Weigel, E. Barthelemy, A. Rincon, J. Tarazona, R. Schoonjans, Guidance on risk assessment of nanomaterials to be applied in the food and feed chain: human and animal health, EFSA J. 19 (8) (2021) 6768, https://doi.org/10.2903/j.efsa.2021.6768.

[14] ICH S2(R1), Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use, Available at: https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-s2-r1-genotoxicity-testing-data-interpretation-pharmaceuticals-intended-human-use-step_en.pdf (accessed June 2012).

[15] Organization For Economic Co-operation and Development, OECD, Bacterial Reverse Mutation Test, OECD Guidelines for the Testing of Chemicals, vol. 4, OECD, Paris, France, 2020, https://doi.org/10.1787/9789264071247-en, 471 (No. 471).

[16] Organization For Economic Co-operation and Development, OECD, Vitro Mammalian Cell Micronucleus Test, OECD Guidelines for the Testing of Chemicals, vol. 4, OECD, Paris, France, 2016, https://doi.org/10.1787/9789264264861-en, 487 (No. 487).

[17] Organization For Economic Co-operation and Development, OECD, Vitro Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene, vol. 490, OECD, Paris, France, 2016, https://doi.org/10.1787/9789264264908-en. No. 490).

[18] X. Pu, Z. Wang, J.E. Klaunig, Alkaline comet assay for assessing DNA damage in individual cells, Curr. Protocol. Toxicol. 65 (2015) 3–12, https://doi.org/10.1002/0471140856.tx0312s65, 1–3.12.11.

[19] AR A. Azqueta, A.R. Collins, The essential comet assay: a comprehensive guide to measuring DNA damage and repair, Arch. Toxicol. 87 (2013) 949–968, https://doi.org/10.1007/s00204-013-1070-0.

[20] S. Bengtson, K. Kling, A.M. Madsen, A.W. Noergaard, N.R. Jacobsen, P.A. Causen, B. Alonso, A. Pesquera, A. Zurutuza, R. Ramos, H. Okuno, J. Dijon, H. Wallin,

U. Vogel, No cytotoxicity or genotoxicity of graphene and graphene oxide in murine lung epithelial FE1 cells in vitro, Environ. Mol. Mutagen. 6 (2016) 469–482, https://doi.org/10.1002/em.22017.
[21] N. Chatterjee, H.J. Eom, J. Choi, A systems toxicology approach to the surface functionality control of graphene-cell interactions, Biomaterials 35 (4) (2014) 1109–1127, https://doi.org/10.1016/j.biomaterials.2013.09.108.
[22] P.P. Jia, T. Sun, M. Junaid, et al., Nanotoxicity of different sizes of graphene (G) and graphene oxide (GO) in vitro and in vivo, Environ. Pollut. 247 (2019) 595–606, https://doi.org/10.1016/j.envpol.2019.01.072.
[23] E. Hashemi, O. Akhavan, M. Shamsara, M. Daliri, M. Dashtizad, A. Farmany, Synthesis and cyto-genotoxicity evaluation of graphene on mice spermatogonial stem cells, Colloids Surf. B Biointerfaces 146 (2016) 770–776, https://doi.org/10.1016/j.colsurfb.2016.07.019.
[24] L. Ou, X. Lv, Z. Wu, W. Xia, Y. Huang, L. Chen, W. Sun, Y. Qi, M. Yang, L. Qi, Oxygen content-related DNA damage of graphene oxide on human retinal pigment epithelium cells, J. Mater. Sci. Mater. Med. 32 (2021) 20, https://doi.org/10.1007/s10856-021-06491-0.
[25] A. Wang, K. Pu, D. Dong, Y. Liu, L. Zhang, Z. Zhang, W. Duan, Y. Zhu, Role of surface charge and oxidative stress in cytotoxicity and genotoxicity of graphene oxide towards human lung fibroblast cells, J. Appl. Toxicol. 33 (2013) 1156–1164, https://doi.org/10.1002/jat.2877.
[26] M.J. Burgum, M.J.D. Clift, S.J. Evans, N. Hondow, A. Tarat, G.J. Jenkins, S. H. Doak, Few-layer graphene induces both primary and secondary genotoxicity in epithelial barrier models in vitro, J. Nanobiotechnol. 19 (2021) 24, https://doi.org/10.1186/s12951-021-00769-9.
[27] O. Akhavan, E. Ghaderi, A. Akhavan, Size-dependent genotoxicity of graphene nanoplatelets in human stem cells, Biomaterials 33 (32) (2012) 8017–8025, https://doi.org/10.1016/j.biomaterials.2012.07.040.
[28] L. De Marzi, L. Ottaviano, F. Perrozzi, M. Nardone, S. Santucci, J. De Lapuente, M. Borras, E. Treossi, V. Palermo, A. PomaFlake size-dependent cyto and genotoxic evaluation of graphene oxide on in vitro A549, CaCo2 and vero cell lines, J. Biol. Regul. Homeost. Agents 28 (2) (2014) 281–289.
[29] O. Akhavan, E. Ghaderi, H. Emamy, F. Akhavan, Genotoxicity of graphene nanoribbons in human mesenchymal stem cells, Carbon, N. Y. 54 (2013) 419–431, https://doi.org/10.1016/j.carbon.2012.11.058.
[30] M. Kucki, P. Rupper, C. Sarrieu, M. Melucci, E. Treossi, A. Schwarz, V. León, A. Kraegeloh, E. Flahaut, E. Vázquez, V. Palermo, P. Wick, Interaction of graphene-related materials with human intestinal cells: an in vitro approach, Nanoscale 8 (16) (2016) 8749–8760, https://doi.org/10.1039/c6nr00319b.
[31] AR A.R. Collins, The comet assay for DNA damage and repair: principles, applications, and limitations, Appl. Biochem. Biotechnol. Part B Mol. Biotechnol. 26 (2004) 249–261, https://doi.org/10.1385/MB:26:3:249.
[32] D. Muruzabal, J. Sanz-Serrano, S. Sauvaigo, B. Treillard, A.K. Olsen, A. López de Cerain, A. Vettorazzi, A. Azqueta, Validation of the in vitro comet assay for DNA cross-links and altered bases detection, Arch. Toxicol. 95 (8) (2021) 2825–2838, https://doi.org/10.1007/s00204-021-03102-3.
[33] Organization For Economic Co-operation and Development, OECD, Vitro Mammalian Cell Gene Mutation Tests Using the Hprt and Xprt Genes, vol. 4, OECD, Paris, France, 2016, https://doi.org/10.1787/9789264264809-en, 476 (No. 476).
[34] O. Cebadero-Domínguez, A. Jos, A.M. Cameán, G.M. Cătunescu, Hazard characterization of graphene nanomaterials in the frame of their food risk assessment: a review, Food Chem. Toxicol. 164 (2022), 113014, https://doi.org/10.1016/j.fct.2022.113014.
[35] N. Krasteva, M. Keremidarska-Markova, K. Hristova-Panusheva, T. Andreeva, G. Speranza, D. Wang, M. Draganova-Filipova, G. Miloshev, M. Georgieva, Aminated graphene oxide as a potential new therapy for colorectal cancer, Oxid. Med. Cell. Longev. 3738980 (2019), https://doi.org/10.1155/2019/3738980.
[36] S. Hummers, R.E. Offeman, Preparation of graphitic oxide, J. Am. Chem. Soc. 80 (6) (1958) 1339, https://doi.org/10.1021/ja01539a017.
[37] M. Puerto, A.I. Prieto, S. Maisanaba, D. Gutiérrez-Praena, P. Mellado-García, A. Jos, A.M. Cameán, Mutagenic and genotoxic potential of pure Cylindrospermopsin by a battery of in vitro tests, Food Chem. Toxicol. 121 (2018) 413–422, https://doi.org/10.1016/j.fct.2018.09.013.
[38] C. Medrano-Padial, A.I. Prieto, M. Puerto, S. Pichardo, In vitro assessment of the mutagenic and genotoxic potential of a pure stilbene extract, Food Chem. Toxicol. 150 (2021), 112065, https://doi.org/10.1016/j.fct.2021.112065.
[39] H.R.H. Mohamed, M. Welson, A.E. Yaseen, A.A. El-Ghor, Estimation of genomic instability and mutation induction by graphene oxide nanoparticles in mice liver and brain tissues, Environ. Sci. Pollut. Res. Int. 27 (2020) 264–278, https://doi.org/10.1007/s11356-019-06930-0.
[40] B.B M.R. Schisler, M.M. Moore, B.B. Gollapudi, In vitro mouse lymphoma (L5178Y Tk+/-3.7.2C) forward mutation assay, Methods Mol. Biol. 1044 (2013) 27–50, https://doi.org/10.1007/978-1-62703-529-3_2.
[41] E. Demir, R. Marcos, Toxic and genotoxic effects of graphene and multi-walled carbon nanotubes, J. Toxicol. Environ. Health A. 81 (14) (2018) 645–660, https://doi.org/10.1080/15287394.2018.1477314.
[42] D.M. Petibone, T. Mustafa, S.E. Bourdo, A. Lafont, W. Ding, A. Karmakar, Z. A. Nima, F. Watanabe, D. Casciano, S.M. Morris, V.N. Dobrovolsky, A.S. Biris, p53-competent cells and p53-deficient cells display different susceptibility to oxygen functionalized graphene cytotoxicity and genotoxicity, J. Appl. Toxicol. 37 (11) (2017) 1333–1345, https://doi.org/10.1002/jat.3472.
[43] R. Landsiedel, N. Honavar, S.B. Seiffert, B. Oesch, F. Oesch, Genotoxicity testing of nanomaterials, 6, in: Wiley interdisciplinary reviews. Nanomedicine and nanobiotechnology. Wires Nanomed. Nanobi., 14, 2022, e1833, https://doi.org/10.1002/wnan.1833.
[44] T. Chem, M. Dusinskam R. Elespuru, Thymidine Kinase+/- mammalian cell mutagenicity assays for assessment of nanomaterials, Front. Toxicol. 4 (2022), 864753, https://doi.org/10.3389/ftox.2022.864753.
[45] J.C. Sasaki, A. Allemang, S.M. Bryce, L. Custer, K.L. Dearfield, Y. Dietz, A. Elhajouji, P.A. Escobar, A.J. Fornace Jr., R. Froetschl, S. Galloway, U. Hemmann, G. Hendriks, H.H. Li, M. Luijten, G. Ouedraogo, L. Peel, S. Pfuhler, D. J. Roberts, J. van Benthem, C.L. Yauk, M. Schuler, Application of the adverse outcome pathway framework to genotoxic modes of action, Environ. Mol. Mutagen. 61 (1) (2020) 114–134, https://doi.org/10.1002/em.22339.
[46] A. Nel, T. Xia, L. Mädler, N. Li, Toxic potential of materials at the nanolevel, Science 311 (5761) (2006) 622–627, https://doi.org/10.1126/science.1114397.
[47] H. Yang, C. Liu, D. Yang, H. Zhang, Z. Xi, Comparative study of cytotoxicity, oxidative stress and genotoxicity induced by four typical nanomaterials: therole of particle size, shape and composition, J. Appl. Toxicol. 29 (2009) 69–78, https://doi.org/10.1002/jat.1385.
[48] C. Liao, Y. Li, S.C. Tjong, Graphene nanomaterials: synthesis, biocompatibility, and cytotoxicity, Int. J. Mol. Sci. 19 (11) (2018) 3564, https://doi.org/10.3390/ijms19113564.
[49] T. Jiang, C.A. Amadei, Y. Lin, N. Gou, S.M. Rahman, J. Lan, C.D. Vecitis, A.Z. Gu, Dependence of graphene oxide (GO) toxicity on oxidation level, elemental composition, and size, Int. J. Mol. Sci. 22 (19) (2021), 10578, https://doi.org/10.3390/ijms221910578.
[50] A. Ivask, N.H. Voelcker, S.A. Seabrook, M. Hor, J.K. Kirby, M. Fenech, T.P. Davis, P.C. Ke, DNA melting and genotoxicity induced by silver nanoparticles and graphene, Chem. Res. Toxicol. 28 (2015) 1023–1035, https://doi.org/10.1021/acs.chemrestox.5b00052.
[51] M. Heshmati, S. Hajibabae, N. Barikrow, Genotoxicity and cytotoxicity assessment of graphene oxide nanosheets on HT29 cells, J. Kerman Univ. Med. Sci. 22 (2018), e69641, https://doi.org/10.5812/jkums.69641.
[52] Y. Sambuy, I. De Angelis, G. Ranaldi, M.L. Scarino, A. Stammati, G. Zucco, The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics, Cell Biol. Toxicol. 21 (1) (2005) 1–26, https://doi.org/10.1007/s10565-005-0085-6.
[53] E. Di Ianni, P. Møller, U.B. Vogel, N.R. Jacobsen, Pro-inflammatory response and genotoxicity caused by clay and graphene nanomaterials in A549 and THP-1 cells, Mutat. Res., Genet. Toxicol. Environ. Mutagen. 872 (2022), 503405, https://doi.org/10.1016/j.mrgentox.2021.503405.
[54] M. Hinzmann, S. Jaworski, M. Kutwin, J. Jagiełło, R. Koziński, M. Wierzbicki, M. Grodzik, L. Lipińska, E. Sawosz, A. Chwalibog, Nanoparticles containing allotropes of carbon have genotoxic effects on glioblastoma multiforme cells, Int. J. Nanomed. 15 (9) (2014) 2409–2417, https://doi.org/10.2147/IJN.S62497.
[55] M. Georgieva, B. Vasileva, G. Speranza, D. Wang, K. Stoyanov, M. Draganova-Filipova, P. Zagorchev, V. Sarafian, G. Miloshev, N. Krasteva, Amination of graphene oxide leads to increased cytotoxicity in hepatocellular carcinoma cells, Int. J. Mol. Sci. 21 (7) (2020) 2427, https://doi.org/10.3390/ijms21072427.
[56] Y. Xing, W. Xiong, L. Zhu, E. Osawa, S. Hussin, L. Dai, DNA damage in embryonic stem cells caused by nanodiamonds, ACS Nano 5 (3) (2011) 2376–2384, https://doi.org/10.1021/nn200279k.