Few-Layer Graphene Induces Both Primary and Secondary Genotoxicity in in Vitro Alveolar Barrier Models

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Abstract

**Background:** Toxicological evaluation of engineered nanomaterials (ENMs) is essential for occupational health and safety, particularly where bulk manufactured ENMs such as few-layer graphene (FLG) are concerned. Additionally, there is a necessity to develop advanced *in vitro* models when testing ENMs to provide a physiologically relevant alternative to invasive animal experimentation. The aim of this study was to determine the genotoxicity of non-functionalised (neutral), amine- and carboxyl-functionalised FLG upon both human-transformed type-I (TT1) alveolar epithelial cell monocultures, as well as co-cultures of TT1 and differentiated THP-1 monocytes (d.THP-1 (macrophages)).

**Results:** In monocultures, TT1 and d.THP-1 macrophages showed a statistically significant (*p*<0.05) cytotoxic response with each ENM following 24-hour exposures. Monoculture genotoxicity measured by the *in vitro* cytokinesis blocked micronucleus (CBMN) assay revealed significant (*p*<0.05) micronuclei induction at 8µg/ml for amine- and carboxyl-FLG. Transmission electron microscopy (TEM) revealed ENMs were internalised by TT1 cells within membrane-bound vesicles. In the co-cultures, ENMs induced genotoxicity in the absence of cytotoxic effects. Co-cultures pre-exposed to 1.5mM N-acetylcysteine (NAC), showed baseline levels of micronuclei induction, indicating that the genotoxicity observed was driven by oxidative stress.

**Conclusions:** Therefore, FLG genotoxicity when examined in monocultures, results in primary-indirect DNA damage; whereas co-cultured cells reveal secondary mechanisms of DNA damage.

Introduction

ENMs have unique physico-chemical features which provide the basis for novel applications in medicine, engineering and other such disciplines (Subbiah et al., 2010). However, these unique physico-chemical features (size, shape, surface area, reactivity) cause concern as to their impact upon human health (Journeay et al., 2008). The graphene family of nanomaterials (GFNs) have received much attention due to their near-2-dimensional (2D) morphology and physico-chemical characteristics granting their use in energy storage and nanocomposites (Fadeel et al., 2018). Despite the plethora of applications, there are still concerns as to the occupational and consumer exposure risks associated with graphene-based ENMs (Burgum et al., 2020). As shown in the work by Burgum and colleagues, FLG which can be bulk manufactured, can pose a risk to lung cells with DNA damage being related to the specific surface characterisation of the materials.

ENMs have extensively been reported to induce damage DNA which could, in turn, promote carcinogenesis (Singh et al., 2009, Nagai et al., 2011, Luanpitpong et al., 2016). Evaluation of the mechanisms underlying ENM DNA damage *in vitro* can be accomplished utilising monocultured cells to detect primary (indirect) genotoxicity or advanced co-culture models, which allow detection of secondary genotoxic mechanisms (Evans et al., 2017). Primary genotoxic mechanisms are sub-classified into primary-direct and -indirect genotoxicity. Primary-direct genotoxicity is associated with the exogenous
agent inducing DNA damage through physical interaction with DNA molecule itself. For instance, graphene oxide (GO) has been shown to intercalate into DNA and when combined with copper ions, inducing DNA scission (Ren et al., 2010). Primary-indirect mechanisms represent those where the exogenous agent damages key molecules involved in DNA replication; for example, DNA repair enzymes, replication machinery, cell cycle proteins or spindle formation. Primary-indirect genotoxicity can also be attributed to mitochondrial damage, antioxidant depletion and generation of reactive oxygen/nitrogen species (ROS/RNS) (Doak et al., 2012, Evans et al., 2019, Burgum et al., 2020). These indirect pathways for damage ultimately contribute to DNA lesions, double strand breaks, aneuploidy and oxidative DNA damage. Secondary genotoxicity is typically observed in vivo and is the result of DNA damage induced through a (sub)-chronic immune response, involving immune cell activation and recruitment, heightened inflammatory activity and subsequent oxidative stress, promoting genotoxicity in the surrounding epithelial cells (Elespuru et al., 2018).

Respiratory models are common in nanotoxicology due to the primary route of ENM exposure being inhalation, especially in an occupational environment (Christensen et al., 2010). A graphene nanoplatelet with a lateral diameter of 20 µm was theorised by Schinwald and colleagues to possess an aerodynamic diameter of < 2.5 µm, representing a size fraction of particulate matter that is capable of reaching the alveolar region (Schinwald et al., 2012). To the authors knowledge, little is known about the potential of FLG materials to elicit genotoxicity in advanced in vitro models of the lung. Burgum et al investigated the genotoxic effects that FLG materials functionalised with no specific groups, amine, or carboxyl groups had on bronchial epithelial (16HBE14o−) cells (Burgum et al., 2020). Each FLG proved capable of eliciting significant oxidative and mitochondrial stress however only the carboxyl-FLG was shown to be non-genotoxic at concentrations of 100 µg/ml. The authors concluded this may be due to potential oxidative radical-scavenging properties of the carboxyl groups. It is unclear however if the same effects could be proved using alveolar models and furthermore, co-culture models. These models could highlight secondary mechanisms of FLG toxicity which can go unseen using in vitro monocultures which, constitute the bulk of the literature surrounding FLG genotoxicity. The current state of the literature pertaining to FLG genotoxicity is sparse, with no known studies conducted involving lung co-cultures to model FLG occupational hazard. The biological response of bone marrow-derived macrophages from mice were recently exposed to FLG at 3, 10, 30 and 100 µg/ml for 24 hours in the study by Malanagahalli et al. FLG uptake by the macrophages was reported and confirmed by TEM, the authors reported no significant cytotoxic effects (measure with flow cytometry), as well as no significant rise in (pro)-inflammatory mediators (Malanagahalli et al., 2020). The authors did conclude that whilst the results appear promising, more evidence is required to elucidate other immunological pathways and investigate the mechanistic effects of FLG.

This study aimed to assess the genotoxic potential of FLG in monocultured TT1 cells and a co-culture of TT1/d.THPI cells to evaluate secondary genotoxic mechanisms. It is important to note these cells were chosen as they represent ideal models of alveolar barrier function; TT1 cells were selected for their physiology (structure & function) and macrophages are professional phagocytes, thus representing a
good model of the alveolar region. Additionally, this is to the authors knowledge the first study to investigate FLG impact upon an alveolar co-culture. Understanding the mechanisms contributing to the induction of DNA damage was also explored, focusing on the role of oxidative stress and (pro)-inflammatory response. It was hypothesised that in monoculture exposures, the physico-chemical features of the graphene would highlight differences between materials based upon the biological response.

Materials & Methods

Preparation of ENMs. Neutral-, amine-, and carboxyl-FLG were manufactured via dielectric barrier discharge of mined graphite by Perpetus Carbon Technologies (PCT, UK). CB particles were sourced from (FLAMMRUSS 101, Lamp Black #8235102) Evonik Degussa Inorganic Materials, Frankfurt. All ENMs were supplied as powder and were suspended at a stock concentration of 10 mg/ml in double distilled water, which were then titrated to final concentrations with cell culture medium. Prior to exposures ENMs were sonicated in a 90W Ultrasonic Bath (Fisher Scientific #FB15046) for 20 minutes at 37 °C to encourage destabilisation of agglomerate material.

ENM characterisation. Each test ENM had previously been characterised by Burgum et al (Burgum et al., 2020). Briefly, neutral-FLG, amine-FLG, carboxyl-FLG (as well as, to a lesser extent, CB particles) particle and agglomerate sizes were investigated with several techniques including; plunge-freeze scanning electron microscopy (SEM), dynamic light scattering (DLS), atomic force microscopy (AFM) and Raman spectroscopy. The agglomerate analysis suggested a difference in size between the particle types when suspended in cell culture media with the additional surface groups of amine-FLG and carboxyl-FLG increasing the average diameter by ~300 and ~400 nm respectively. The addition of surface groups reduced the average thickness of FLG layer number when measured by AFM, the greatest effect produced by carboxyl groups which lowered the calculated layer number from 50 to 4 atomic layers of graphene.

Cell culture. The TT1 cell line was kindly donated by Professor Terry Tetley of Imperial College London, UK. This alveolar cell line had been previously transformed, immortalised and characterised by Kemp and colleagues as a model for ATI cells (Kemp et al., 2008). TT1 cells were thawed from liquid nitrogen and transferred to T25 flasks to initiate proliferation for 48 hours or until reaching 70–80% confluency before sub-culturing into a T75. TT1 cells were cultured with DCCM-1 Media (Geneow Ltd, UK) supplemented with 1% Penicillin/Streptomycin/L-Glutamine (Sigma, UK) and 10% New-born Calf Serum (NCS) (Sigma, UK). Sub-culturing of TT1 cells began with removing existing media and discarding, washing the adherent cells twice with pre-warmed PBS before adding 4 ml of thawed Trypsin-EDTA and incubated flat at 37 °C for 5 minutes to disassociate. Once cells detached, complete media was added (6 ml) to deactivate the Trypsin-EDTA solution and the cells transferred to 15 ml Falcon tubes to be centrifuged at 340 g for 10 minutes. The resulting supernatant was discarded, cells re-suspended and seeded at $1 \times 10^5$ and returned to the incubator at 37 °C. When seeding TT1 cells for toxicological assays, cells were counted by re-suspending and taking 10 µl and adding to 90 µl of fresh media then pipetting 10 µl into a Neubauer chamber. Cells in the four corners of the chamber were counted, averaged and multiplied by
for the number of cells per ml. TT1 cells were sub-cultured every 3 days, reaching 90–100% confluency in that time. TT1 cells were not used in toxicological assays once exceeding 20 passages. THP-1 cells and differentiation were sourced and cultured as described by Evans and colleagues (Evans et al., 2019). Briefly, THP-1 monocytes were differentiated with 50 nM of PMA, incubated for 24 hours before a complete media change was performed allowing the now differentiated THP-1 macrophages to recover. On the day of seeding co-cultures, d.THP-1 macrophages were resuspended at $1 \times 10^5$ cells/ml in DCCM-1 media.

THP-1 differentiation. To differentiate, THP-1 cells were centrifuged at 130 g for 5 minutes at room temperature, following this the cells were resuspended in a 15 ml Falcon tube at a concentration of $5 \times 10^5$ cells/ml with 50 nM of phorbol-12-myristate-13-acetate (PMA). This suspension was transferred to a T75 flask and incubated at 37 °C for 24 hours. Cells were checked for morphology via light microscopy; differentiation was evident when cells did not detach from flask over gentle agitation.

Construction of co-culture model. The alveolar lung epithelial co-culture model was comprised of TT1 alveolar cells (seeded at $1 \times 10^6$ cells/ml) and d.THP-1 macrophages ($1 \times 10^5$ cells/ml per 6-well insert). These co-cultures were constructed upon the apical side of PET track-etched 4.2 cm² transwell inserts (3 µm pores) supported by a 6-well companion plate (Coming, Germany). TT1 cells were seeded on day one and left to proliferate for one week. At day five, THP-1 cells were differentiated into macrophages using PMA and left for 24 hours. On day six, d.THP-1 macrophages were lifted with Acutase and transferred to the TT1 monolayer and left to adhere for one day. The completed co-culture was then ready to be used on day seven, this was confirmed with confocal laser scanning microscopy (LSM (images not shown)).

Confirmation of cellular interaction/entry using Transmission Electron Microscopy (TEM). ENM cellular uptake was confirmed by TEM imaging. TT1 cells, the primary focus of this study were exposed to ENM were fixed, embedded, sectioned and imaged as previously described (Wills et al., 2016). The analysis was performed with a FEI Titan3 Themis G2 operating at 300 kV fitted with 4 EDX silicon drift detectors, and a Gatan One-View CCD. EDX spectroscopy and mapping to identify potential heavy metal contamination was undertaken using Bruker Esprit v1.9 software.

TEM analysis of FLG lattice planes. In conjunction with TEM uptake imaging, confirmation of graphitic materials was confirmed through fast Fourier transform (FFT) using ImageJ. To begin analysis, firstly an intracellular vesicle containing an FLG crystal oriented on its z-axis was located, the image was captured and saved for ImageJ analysis. Using ImageJ software, the captured image was uploaded, the scale bar was set, and the lattice plane focused upon and analysed with FFT to produce an image (Supplementary Fig. 1) which reveals the interlayer spacing of FLG.

Cytotoxicity by relative population doubling (RPD) & genotoxicity. TT1 cells were seeded at $1.0 \times 10^5$ cells/ml and allowed to adhere for 24 hours after which the cells were then treated with ENMs for 24 hours. Cell culture media was added as the negative control, mitomycin-C (MMC) at 0.01 µg/ml was used
as the positive control, the assay was performed as previously detailed by (Evans et al., 2019). All experiments were performed in triplicate ($N=3$) and 2000 binucleate (BN) cells per replicate were scored for the presence of micronuclei per concentration (6000 BN cells in total).

d.THP-1 cytotoxicity. Cytotoxicity of d.THP-1 macrophages (seeded at $1 \times 10^5$ cells/ml) was assessed by trypan blue exclusion following 24-hour exposure to ENMs as described by Evans and colleagues (Evans et al., 2019). Following exposure cells were lifted gently with Acutase and live cells scored with a haemocytometer (1:5 dilution). This was performed in triplicate.

(Pro)-inflammatory response. Supernatant from the CBMN assay was harvested following 24-hour exposure to ENMs and analysed using an IL-6 & IL-8 ELISA (DuoSet ELISA; R&D Systems Europe). ELISA’s were performed in triplicate following the manufactures instructions. The optical density (OD) was recorded at a wavelength set to 450 nm with an Omega Multimode microplate reader (BMG LABTECH Ltd, UK). This was performed in triplicate.

Mitochondrial stress. TT1 cells were seeded at $1 \times 10^5$ cells/ml (100 µl/well) into XFe24 tissue culture plates (Seahorse Bioscience). The following day, 150 µl of growth media was added and exposed to ENMs for 24 hours. The assay was performed as described in the work by Burgum et al (Burgum et al., 2020). Data was normalised through quantification of cellular protein (DC Assay (Bio-Rad, UK)) and a series of BSA (Bio-Rad, UK) prepared using RIPA buffer (Thermo Scientific, UK). This was performed in triplicate.

Co-culture in vitro CBMN assay. The standard in vitro CBMN assay was adapted for use with advanced co-culture models and performed as described in detail by Evans and colleagues (Evans et al., 2019). Following co-culture construction, cells were exposed to ENMs for 24 hours. Following exposure, a complete media change was performed with media containing 3 µg/ml of cytochalasin B before incubating for 1.5 cell cycles (24 hours). Cells were then removed with trypsin and fixed in 3% paraformaldehyde (PFA) for 24 hours at 4 °C followed by permeabilization at 4 °C with 0.2% Triton X100. Cells were stained with 1 µg/ml anti-human CD-324 (FITC fluorophore) suspended in x1 PBS and incubated at 4 °C in the dark for 45 minutes. Cells were subsequently washed in PBS before 100 µl of cells (per slide) were drop cast onto ethanol-cleaned glass slides which were left to air-dry and finally 30 µl of DAPI counterstain was applied. A total of 500BN TT1 cells were identified per replicate (1500BN total per concentration); TT1 cells were positively identified by their FITC fluorescence signal produced by e-cadherin, while d.THP-1 cells were selected against due to their absence of FITC fluorescence. Further, the present study exposures were performed with and without a two-hour incubation with 1.5 mM N-acetylcysteine (NAC) to investigate the potential role of oxidative stress. Following the NAC incubation, a complete media change was performed, and exposures were performed for 24 hours. Cellular proliferation was evaluated due to the inclusion of cytochalasin B whereby the cytokinesis block proliferation index (CBPI) was calculated by Eq. (1);
1. **CBPI** = \( \frac{(\text{Number of mononucleate Cells} + (2 \times \text{Number of binucleate cells}) + (3 \times \text{Number of multinucleate cells}))}{\text{Total number of cells}} \)

Data analysis and statistics. All data is presented as the mean +/- the standard deviation (SD). Statistical analysis was performed in SPSS statistics software (v.20 IBM, UK) where all data sets were firstly analysed for normality (Shapiro-Wilk test, \( p \leq 0.05 \)) and for equal variance \( p \leq 0.05 \)). A one-way analysis of variance (ANOVA) was performed with post hoc Dunnett’s multiple comparisons applied to evaluate pairwise statistical significance between control and concentrations; the alpha value was set to 0.05.

**Results**

**FLG induces cytotoxicity and primary-indirect DNA damage in TT1 cells**

This study aimed to evaluate the genotoxic potential of FLG and functionalised variants upon an epithelial barrier model of the lung. This was performed first in a monoculture of TT1 alveolar cells before constructing a co-culture model with d.THP-1 macrophages. Following a 24-hour exposure, statistically significant cytotoxicity and genotoxicity were induced in TT1 cells revealing a potency trend of amine-FLG, carboxyl-FLG, CB, neutral-FLG. Whilst statistically significant \( (p < 0.05) \), this is unlikely to represent biological significance as viability did not drop below 80% for any tested ENM. Genotoxicity however at the highest concentration of neutral-FLG and amine-FLG represented a 1-fold increase over baseline levels in monocultured TT1 cells at ~3.1% BN/Mn (Fig. 1).

It was important to elucidate any key (pro)-inflammatory events in monocultured exposures prior to investigating secondary mechanisms in co-cultures. Therefore, IL-6 and IL-8, two key inflammatory mediators were quantified following 24-hour exposures to both TT1 alveolar cells and d.THP-1 cells. TT1 IL-6 levels revealed a concentration-dependent increase, revealing an initial significant response at 8 µg/ml induced by amine-FLG at 66.2 pg/ml with a 12-fold increase over control levels (5.32 pg/ml). The concentration of IL-6 protein then steadily increased over the concentration range of 10–100 µg/ml with amine- and carboxyl-FLG the greatest response was at 100 µg/ml with levels of 458.3 and 458.7 pg/ml respectively, a 90-fold change. The initial significant IL-8 response was attributed to exposure with neutral-FLG at 10 µg/ml before each ENM elicited significant IL-8 at 20–100 µg/ml. At 20 µg/ml Neutral-, amine, Carboxyl-FLG and CB particles elicited an IL-8 protein concentration of 822.9, 930, 1038.5 & 1035.7 pg/ml respectively, demonstrating a 7-, 8-, 9- and 9-fold increase over control IL-8 levels (106.34 pg/ml) respectively (Fig. 2).

TT1 cells exposed to the ENMs showed a small, non-significant \( (p > 0.05) \) increase in mitochondrial function as the concentration of ENMs increased to 20 µg/ml for each ENM. Each ENM exerted a 3-fold increase in proton leak over control levels (8.3pmoles/min/mg protein) up to 20 µg/ml. Similarly, TT1 cells exposed to neutral-FLG underwent a 5-fold increase in non-mitochondrial respiration whilst amine-FLG, carboxyl-FLG and CB particles raised the non-mitochondrial respiration levels 2-fold over control
levels. At 20 µg/ml basal levels were raised two-fold when exposed to neutral-FLG, amine-FLG and carboxyl-FLG. Exposures at 50 µg/ml however revealed a depletion in basal respiration levels with respect to 20 µg/ml which may indicate agglomeration of ENMs preventing further elevation taking place. At 10 µg/ml a depletion in ATP production from control levels was observed for each ENM with an average OCR decrease of 10pmoles/min/mg protein, most prominent with cells exposed to neutral-FLG. ATP production spiked at 20 µg/ml for all ENMs, carboxyl-FLG elicited the greatest response with a 2-fold increase. Maximal respiration in TT1 cells at 20 µg/ml yielded a 2-, 2.4-, 3- and 2-fold increase over control levels following respective exposures to neutral-FLG, amine-FLG, carboxyl-FLG and CB particles. At 50 µg/ml, neutral-FLG and CB particles demonstrated a concentration-dependent increase by raising the reserve respiratory capacity by 40.3 and 38.8pmoles/min/mg protein respectively. Conversely, amine- and carboxyl-FLG depleted the reserve respiratory capacity by 40.9 and 87pmoles/min/mg protein with respect to data recorded at 20 µg/ml (Fig. 3).

**FLG induces significant cytotoxicity in d.THP-1 macrophages**

Neutral-FLG exposure to d.THP-1 cells revealed a concentration-dependent decrease in viability up to 10 µg/ml, however concentrations of 20, 50 and 100 µg/ml promoted a 1.4, 1.4 and 1.6-fold decrease from control levels with cell viability falling to 72, 70 and 62%. This effect was also observed with exposures to amine-FLG however the final % viability here was 73, 71 and 64% at concentrations of 20, 50 and 100 µg/ml respectively. THP-1 cells exposed to carboxyl-FLG for 24-hours showed greater sensitivity with the initial significant decrease in viability at 10 µg/ml whereby the cell viability was at 77%. A concentration-dependent trend was observed with viability recorded at 59 and 51% at 50 and 100 µg/ml, thus demonstrating the most potent effect on d.THP-1 viability with a 2-fold decrease at the highest exposure concentration. Lastly, d.THP-1 cells exposed to CB particles revealed no significant cytotoxicity however a small trend can be observed at 20, 50 and 100 µg/ml. The d.THP-1 cells reached significant IL-8 levels at 100 µg/ml for each FLG material, while CB particles elicited significant IL-8 levels at 50 µg/ml (Fig. 4).

Following a 24-hour ENM exposure, TT1 cells were harvested, resin-embedded and sectioned for interaction and uptake confirmation. Electron micrographs of TT1 cells revealed each test ENM could be internalised by the epithelial cells. ENMs were always present within an endocytic vesicle and were not freely available in the cytoplasm indicative of mediated endocytosis and not passive uptake. Surface functionalisation appeared to be non-decisive in facilitating uptake as other publications have reported, whereby amine coatings (positively charged) can encourage cellular uptake. CB particles however did appear to be present in TT1 cells in lower concentrations than the FLG materials (Fig. 5), it is unclear if this is a direct result of ENM morphology, however.

**Detection of secondary mechanisms of DNA damage in TT1/d.THP-1 co-cultures**
Following the monoculture exposures, TT1 alveolar cells and d.THP-1 cells were co-cultured together to determine whether secondary mechanisms of genotoxicity could be observed. Cell proliferation and DNA damage in the co-culture was assessed by the in vitro CBMN assay adapted for use with co-cultures as detailed by Evans and colleagues (Evans et al., 2019). No significant cytotoxic responses were observed in co-cultures however significant DNA damage was observed with the lowest observed genotoxic effect level (LOGEL) was 10 µg/ml for neutral-FLG, amine-FLG and CB particles whilst the LOGEL for carboxyl-FLG was observed at 20 µg/ml. At 50 µg/ml TT1 cell %BN/Mn reached a ~ 2-fold increase over control levels (Fig. 6). Given there was a ~ 2-fold increase in concentrations of ENMs in co-culture exposures compared to monocultured TT1 exposures, secondary mechanisms were hypothesised to be present. To investigate these effects, co-culture exposures were repeated following a two-hour NAC (1.5 mM) exposure which would theoretically provide a means for oxidative radicals to be scavenged before they could cause DNA damage. Co-culture ENM exposure resulted in no significant changes in cell viability or DNA damage as compared to the untreated control. Crucially, at the highest test concentration of 50 µg/ml the %BN/Mn had decreased from an average 3.65% in experiments without NAC to 2.18% in co-cultures which had the NAC pre-treatment (Fig. 7). This result indicates that the genotoxicity observed was, if at least in part, mediated by oxidative stress when considering both FLG and CB in the co-culture. The NAC treatment appeared therefore to scavenge oxidative products produced by the d.THP-1 macrophages before they could damage surrounding epithelial TT1 cells. A summary of these results is provided in Table 1.
### Table 1

Summary of conclusions when comparing data generated in *in vitro* monocultures and co-culture models. A hazard ranking appears where direct comparisons were possible to differentiate between the test ENMs. Not applicable (N/A) appears in the table where a test was not performed or could not be optimised in the timeframe.

| Cytotoxicity | Genotoxicity | (Pro)-inflammatory response | Mitochondrial stress | Uptake |
|--------------|--------------|------------------------------|----------------------|--------|
| **Monocultured TT1** | Significant ($p < 0.05$) effect observed with all ENMs (Amine-FLG & carboxyl-FLG, CB, neutral-FLG) | Significant ($p < 0.05$) effect observed with all ENMs (Amine-FLG, carboxyl-FLG, CB, neutral-FLG) | Significant ($p < 0.05$) effect observed with IL-6 & IL-8 with all ENMs | Uptake observed with each test ENM |
| **Monocultured d.THP-1** | Significant ($p < 0.05$) effect observed with FLG materials (Carboxyl-FLG, neutral-FLG, amine-FLG) | N/A | Significant ($p < 0.05$) effect observed with IL-8 with all ENMs | N/A |
| **Co-cultured TT1/d.THP-1** | Non-significant ($p > 0.05$) effect observed | Significant ($p < 0.05$) effect observed without NAC with all ENMs Non-significant ($p > 0.05$) effect observed with NAC with all ENMs | N/A | N/A |

### Discussion

This study aimed to evaluate the scope of genotoxic mechanisms induced by FLG with varying surface chemistries within an advanced co-culture of TT1/d.THP1 cells. It is crucial to understand the fundamental hazard posed by FLG materials given their tremendous potential in reinforced nanocomposites which have large-scale consumer demand. As previously stated, studies focused upon FLG exposures to lung cells are sparse in the literature and furthermore the underlying mechanisms can be overlooked. It was imperative that the present study seek to understand how to distinguish primary and secondary mechanisms of genotoxicity using the relevant models. Secondly, to incorporate an understanding of the physico-chemical features of the materials and how they relate to the observed biological responses in each endpoint. This investigation will (to the authors knowledge) be the first to
evaluate FLG impact upon an advanced alveolar barrier model, thus providing a foundation for further research attempting to elucidate mechanisms of toxicity in similar models.

Utilising monocultures of alveolar TT1 cells and d.THP-1 macrophages, this study has demonstrated the capacity for FLG materials to induce (pro)-inflammatory effects and genotoxicity. ENMs have been reported to induce genotoxic and (pro)-inflammatory effects in monocultured human bronchial epithelial (16HBE14o−) cells and immune cells. For instance, in the work by Evans and colleagues who used two types of superparamagnetic iron oxide nanoparticles (SPIONs) only the Fe₂O₃ was genotoxic in monocultured 16HBE14o− cells. The other particle type, Fe₃O₄ was not capable of promoting genotoxicity; however both particle types elicited a strong tumour necrosis factor (TNF-α) and IL-8 response over a similar concentration range as the present study (Evans et al., 2019). Most recently, in the study by Burgum and colleagues the cytotoxic and genotoxic effect of the ENMs used in the present study were evaluated in monocultured 16HBE14o− cells over the same concentration range. The authors reported no cytotoxic effects with neutral-FLG and amine-FLG inducing significant DNA damage at the higher concentrations (Burgum et al., 2020). Whilst studies investigating the genotoxicity of FLG on lung cells are few, CB particle exposure is much more common. In vivo CB exposure causes malignant tumours in test animals possibly due to chronic inflammation of the lungs which could lead to carcinogenesis (Hiraku et al., 2017). To understand the mechanistic impact of CB particles, Hiraku and colleagues exposed 56 nm (CB56) and 95 nm (CB95) diameter CB particles to A549 lung epithelial cells and RAW 264.7 macrophages at 0–50 µg/ml. Both particle types induced the formation of 8-nitroguanine, a nitratine DNA legion. Further oxidative damage was elucidated in the study whereby CB95 induced greater ROS in macrophages as well as producing greater amounts of nitric oxide (NO) than did CB65. Hiraku and colleagues also demonstrated uptake in their study was through clathrin-mediated endocytosis (Hiraku et al., 2017).

IL-6 and IL-8 were both significantly elevated in the present study which is consistent with the observed genotoxicity in TT1 cells and supported by TEM-confirmation of uptake. TT1 cells have been reported capably internalising functionalised multi-walled carbon nanotubes (f-MWCNTs) however no information currently exists on their interaction with graphene (Ruenraroengsak et al., 2016). Exposures to TT1 cells revealed no significant effect on the mitochondrial function in the present study, similar work has been reported in the literature by Jantzen and colleagues who exposed diesel exhaust particles (DEP) up to 100 µg/ml to THP-1a cells. Unlike the present study, Jantzen et al/ attempted the same procedure with A549 cells however the cells OCR rate proved too low, THP-1a cells did respond with significant elevations in basal respiration, however. The authors also noted respiratory capacity was significantly elevated following exposures at concentrations of 25 and 100 µg/ml (Jantzen et al., 2012). Similarly, Burgum et al/ performed (pro)-inflammatory and mitochondrial analysis of FLG upon monocultured 16HBE14o− cells and observed significant elevation in IL-8 levels and significant depletion in mitochondrial function including ATP production. These effects were largely dependent upon surface characterisation where carboxyl groups appeared to hinder ATP production more than amine groups (Burgum et al., 2020).
Graphene-associated toxicity in d.THP-1 macrophages has been demonstrated by Li and colleagues, whereby hydrated GO (hGO) demonstrated the strongest cytotoxic effect in macrophages. The authors noted the presence of carbon radicals (introduced in the hydration of GO) caused lipid peroxidation of the cell membrane, membrane lysis and ultimately cell death (Li et al., 2018). The lack of cytotoxicity from CB particles in d.THP-1 cells can be supported by findings in the literature whereby 100 nm CB particle exposures to A549 and THP-1 cells at 16 µg/ml showed no cytotoxic effects in the Alamar Blue assay (Don Porto Carero et al., 2001). More recently, Gurunathan and colleagues were able to demonstrate immunotoxicity of GO and vanillin-functionalised GO (V-rGO) on THP-1 cells with further notable effects of loss in cell viability, proliferation in a dose-dependent manner, increased lactate dehydrogenase levels, ROS production and depletion of mitochondrial membrane potential (Gurunathan et al., 2019). Seemingly providing evidence for similar modes of action to the present study, with emphasis on the carboxyl-FLG which had the strongest cytotoxic effect of each ENM on d.THP-1 cells and bearing the closest resemblance with chemical reactivity to the V-rGO.

The co-culture model of TT1/d.THP-1 cells was constructed to better mimic the architecture of the alveolar region of the lung using a dual-cell model. Genotoxicity induced in the co-culture model occurred at lower concentrations and represented a greater %BN/Mn frequency, than levels reached in monoculture exposures of TT1 cells and in 16HBE14o− cells, tested in a separate investigation (Burgum et al., 2020). This effect was demonstrated by Evans et al/ whereby secondary mechanisms of genotoxicity elevated the frequency of DNA damage across the concentration range following dextran-coated superparamagnetic iron oxide nanoparticle (dSPION) exposures (Evans et al., 2019). NAC treatment for 2 hours prior to exposures in the present study eliminated the genotoxicity within co-cultures at all concentrations for each test FLG. This major finding supports the theory that secondary mediators such as NO were readily scavenged by excess antioxidant levels in the culture conditions.

In the present study, it was hypothesised that in the co-culture exposures the d.THP-1 macrophages would phagocytose the ENMs, which in turn would result in superoxide generation and depletion of mitochondrial function. The oversaturation of the mitochondrial enzyme manganese superoxide dismutase (Mn-SOD) could then activate nicotinamide adenine dinucleotide phosphate (NADPH), dislocating cytochrome c and inducing subsequent apoptosis of d.THP-1 macrophages. An increase in the expression of p53, NF-kB and NOX2 could then be tested for. The release of (pro)-inflammatory mediators and nitric oxide NO into the co-culture environment would then exert lipid peroxidation on the TT1 cells. This provides a clearly testable hypothesis as NO can be converted in to stable products; nitrite and nitrate (NO2− & NO3−) which can then be quantified using fluorescent techniques (Dekhuijzen and van Beurden, 2006). It is known that whilst NAC boost intracellular glutathione levels, it also has reducing potential through its thiol-disulphide exchange activity. NAC may also possess other beneficial processes such as inhibiting p38 MAP kinase, activating protein-1 as well as NF-kB transcription factors (Zafarullah et al., 2003); all of which would flood the cells in a chronic (pro)-inflammatory response upon ENM exposure over 24 hours.
Fukai and colleagues investigated the genotoxic effects of multi-walled carbon nanotubes (MWCNTs) on murine lung resident (GDL1) cells and RAW 264.7 macrophages. The authors reported a stronger mutation frequency in co-cultured GDL1 cells and RAW 264.7 macrophages over monocultured GDL1 cells noting a greater IL-1α and IL-1β production when macrophages were present. The authors also reported the presence of the free-radical-induced DNA lesion, 8-hydroxydeoxyguanosine (8-OHdG), in co-cultured GDL1 cells was greater than monocultured cells indicative of secondary mechanisms (Fukai et al., 2018). In the recent work of Evans and colleagues investigating SPION toxicity in co-cultures of 16HBE14o− and d.THP-1 macrophages, the authors reported secondary genotoxicity at concentrations of 10, 50 and 100 µg/ml. This was represented by a rise in the %BN/Mn over monocultured 16HBE14o− cells (Evans et al., 2019). In the latter study, the authors also demonstrated particle uptake in both cell types with NPs contained within endocytic vesicles indicative of endocytosis rather than passive uptake. However, diesel exhaust particles (DEPs) used in the study by Jantzen and colleagues revealed greater genotoxicity in monocultured A549 cells rather than a co-culture of A549 cells grown with THP-1a cells (Jantzen et al., 2012). The current battery of tests for \textit{in vitro} nano-safety primarily focus on monocultured cells whereas this study and others before clearly demonstrated the value of combining multiple cell types to explore secondary mechanisms of genotoxicity. Whereas the THP-1a cells provided a protective role in the work by Jantzen et al, in the present study the d.THP-1 cells appear to have enhanced the genotoxic effects of FLG and CB. This demonstrates the need for more \textit{in vitro} nanosafety studies utilising advanced models to distinguish the mechanistic differences observed in the literature thus far.

**Conclusions**

Neutral-, amine-, carboxyl-FLG & CB particles each induced significant primary-indirect genotoxicity in monocultured TT1 cells. Furthermore, significant cytotoxicity was observed in d.THP-1 macrophages following a 24-hour exposure period. The ENMs were able to gain entry to the TT1 monocultured cells (evidenced by TEM) and were observed within membrane-bound vesicles. Surface functionalisation appeared to play no role in preferential uptake of FLG materials. Each test ENM promoted significant genotoxicity \textit{via} secondary mechanisms in co-cultured TT1/d.THP-1 cells. Co-culture genotoxicity was lowered to non-significant levels when the model was pre-exposed to 1.5 mM of NAC for two hours prior to exposures. This observation is evidence to support the hypothesis that oxidative stress is the driving mechanism behind the genotoxicity induced by the FLG and CB following exposure to an alveolar co-culture barrier model.

**Declarations**

**Data availability statement**

The data sets used/analysed in this manuscript are available from the corresponding author under reasonable request.
Competing Interests

The authors have declared no competing interests.

Author Contributions

MJB, MJDC and SHD designed the experiments. MJB performed cell culture and all toxicological assays described. SE helped with characterisation data interpretation. NH performed TEM uptake imaging. AT provided FLG samples and contributed to data interpretation. All authors contributed to data interpretation and preparation of manuscript. All authors have read and approved the manuscript prior to submission.

Ethics approval & consent to participate

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Consent for publication

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