Deliverable 2.5 “Assessment of GFN translocation rate and of the biological responses induced at placental cell levels and evaluation of GFN impact on other barrier models”

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Summary

In the studies reported in this deliverable the interaction of graphene-related materials (GRM) with models of intestinal, placenta and blood brain barrier has been assessed. No sign of toxicity has been found in the models studied until now.
Intestinal barrier models

Assessment of the interaction of graphene-related materials (GRM) with the intestinal barrier in vitro was performed by application of the Caco-2 cell line, which is one of the gold standards for in vitro cell culture models of the intestinal tract. The Caco-2 cell line was applied in two different phenotypes. To acquire a systematic understanding of the possible biological effects induced by GRM on cells representing the intestinal epithelium, different GRM, primarily graphene oxides (GOs), from different sources and quality were investigated. The selected graphene oxides allowed comparison of commercial and research grade materials obtained by a similar production method (modified Hummers method). In addition, materials obtained from different starting materials and with different lateral dimensions were compared in their biological effects. Two research-grade GO samples were obtained by Vincenzo Palermo (CNR) from WP 10. The characterisation of the physicochemical properties of all applied GRM was performed in collaboration with partners within the WP 2 (Ester Vazquez ULCM, Emmanuel Flahaut CNRS Toulouse), WP 10 (Vincenzo Palermo CNR), as well as partners from Empa and the INM – Leibniz Institute for new Materials, Germany.

In vitro digestion

Materials entering the gastro-intestinal tract are exposed to a series of different environmental conditions. Among these, the acidic conditions in the stomach bear one of the highest potentials to induce changes in the materials properties, which in turn can lead to changes in the biological effects. Therefore, the potential impact of physiological relevant acidic conditions in vitro on the material properties as well as on the biological effects was assessed. For this purpose, two commercial GRM, graphene oxide (GO) and graphene nanoplatelets (GNP), were selected. Both materials represent GRM already on the market and show significant differences in their physicochemical properties. To mimic stomach transition both materials were incubated in 0.1 M HCl for two hours under slight agitation, followed by neutralisation, washing and transfer to ultrapure water. Characterisation of the acid-treated materials was performed by X-ray photoelectron spectroscopy (XPS), Raman spectroscopy, scanning electron microscopy (SEM) and zeta potential measurements. The results show no relevant changes in the physicochemical properties of acid-treated materials in comparison to the untreated as received materials. It should be noted that GO production methods such as modified Hummers, Staudenmaier or Brodie method involve acids such as sulfuric acid and nitric acid. The results show that pristine GO and GNP can be regarded as relatively inert materials in respect to physiological relevant acidic conditions (pH 1-2). We further investigated if non-confluent Caco-2 cells exposed to acid-treated GRM and non-acid treated GRM show any changes in the biological response. Non-confluent Caco-2 cells exposed to five different concentrations (5-80 µg GRM/ml) of GRM showed no difference in cell viability between the respective untreated and acid-treated materials. The results give evidence that acid-treatment of the pristine GRM does not result in significant changes, neither in the physicochemical properties, nor in the biological response. Therefore acid-pre-treatment is not mandatory to investigate the interaction of pristine GRM with intestinal cells. But it should be noted that this should not be necessarily valid for functionalised GRM for drug delivery or cancer treatment. In such case careful assessment of the stability of functionalisation under acidic conditions is required. Results of the in vitro digestion and exposure of
non-confluent Caco-2 cells are summarised in a joint manuscript with above mentioned partners (Kucki M. et al. under revision)

**Non confluent Caco-2 cells:**

To obtain a systematic understanding of the possible effects on cells of the intestinal tract, non-confluent Caco-2 cells were exposed to the previously mentioned selection of non-acid treated (as received) GO samples and GNP. For all GO samples, both commercial and research-grade GO, no acute toxicity could be found for an exposure time of 24 hours and 48 hours and a concentration range of 5-80 µg GO/ml. Metabolic activity was higher than 70% of unexposed control cells. By modified LDH assay no significant decrease in total cell number of adherent Caco-2 cells was found after exposure to GO for 24 hours and 48 hours and a concentration range of 5-80 µg GO/ml. Light microscopy analysis gives further evidence for the absence of acute toxicity of the four different GO samples. Despite the variations in lateral dimension of the GO samples, no size-dependent effects on the cell viability could be observed. According to the oxidative stress paradigm exposure of cells to nanomaterials can induce the formation of intracellular reactive oxygen species (ROS). ROS formation can in turn lead to inflammatory reactions, genotoxicity and cell death. The formation of ROS was measured by the dichlorofluorescein assay, based on fluorescence read-out. All four GO samples, as well as GNP, applied in a concentration range of 5-40 µg GO/ml induced the formation of ROS in non-confluent Caco-2 cells. In addition, control experiments showed that GO samples induced ROS formation in the absence of cells, which gives hints towards an intrinsic property of the materials. It should be noted that all applied assays were carefully controlled for possible interference (intrinsic absorbance and fluorescence, fluorescence quenching) by the GRM. Due to fluorescence quenching by the GRM, quantification of the ROS formation and direct comparison of different GO and GNP was not possible. Nevertheless, the level of ROS formation was not sufficient to induce cell death significantly higher than the normal cell turn-over. In summary, the applied four different GO did not reveal acute toxicity for Caco-2 cells. Nevertheless, sub-toxic and chronic effects of GO and other GRM cannot be excluded and deserve further analysis.

Scanning electron microscopy (SEM) analysis revealed close interaction of the four different GO samples and the GNP with the cell surface of non-confluent Caco-2 cells. A high decoration with GO sheets was found at the cell borders of Caco-2 islets. Well exfoliated GO sheets were often found in parallel alignment to the cell surface. Similar observations were made by Russier et al (Russier J. Et al. Nanoscale. 2013 Nov 21;5(22):11234-47) and described as mask effect. In addition, SEM analysis revealed the formation of membrane waves and processes strongly interacting with the GRM. Several hints were found that point towards an active uptake mechanism as shown in Figure 1.
Uptake of GO by non-confluent Caco-2 cells was analysed by transmission electron microscopy (TEM) for two selected GO samples with different lateral dimensions. For both GO samples uptake by non-confluent Caco-2 cells could be confirmed. Small GO sheets with a lateral dimension below 1 µm were found in intracellular vesicles. Even large GO sheets of up to 10 µm in lateral dimensions were taken up by the cells. GO sheets were arranged as multi-layered structures and exhibited several wrinkles as well as sharp bends. SEM and TEM results give clear evidence for an active uptake mechanism by the cells. Furthermore, the results show that the GO sheets are deformable and not strictly rigid structures. The mechanical properties of the GO sheets allow the uptake of even large GO sheets with lateral dimension in the size range of bacteria or even larger. The detailed uptake mechanism is not known to date (presumably macropinocytosis) and deserves further investigation. Nevertheless, the results also show that the mechanical properties of the GRM are very important regarding their cellular uptake and that the lateral dimension cannot be regarded as isolated determining factor. Stacking of several GO sheets should lead to an increase in stiffness which might results in an increase in the importance of the lateral dimensions. GRM materials with highly defined physicochemical properties such as shape, lateral dimension and thickness are required to analyse the mechanical properties of GRM in biological environment and the required forces needed by cells to induce deformation of the materials during uptake in detail.

**Differentiated enterocyte-like Caco-2 cell monolayer**

The observed uptake of large GO sheets by non-confluent Caco-2 cells was surprising, as these cells are not considered as professional phagocytes. To increase the physiological relevance of the applied *in vitro model*, Caco-2 cells were differentiated to exhibit an enterocyte-like phenotype. The dependence of the cellular uptake behaviour on the Caco-2 phenotype was investigated by Scanning electron microscopy (SEM) and Transmission electron microscopy (TEM) analysis. Caco-2 cells were differentiated to enterocyte-like cells; over a period of three weeks. During differentiation Caco-2 cells undergo intensive transcriptomic, proteomic and architectural changes towards an enterocyte-like phenotype forming a tight epithelial barrier with brush border and tight junctions. After exposure of
differentiated Caco-2 cells to the previously applied GO samples (large and small sheets) TEM analysis was performed giving no evidence for cellular uptake. In addition SEM analysis showed only very low amounts of GO sheets adhered to the cellular surface which is highly enlarged and structured by thousands of dense microvilli per cell (see figure 3). (Immuno)fluorescence labelling of the Actin network and tight junction protein ZO-1 showed no difference between GO-exposed and untreated differentiated Caco-2 cells indicating no significant loss of barrier integrity. The results show that the phenotype of Caco-2 cells highly influences the cellular uptake of GO sheets. Due to the similarity between differentiated Caco-2 cells in vitro and human enterocytes in vivo, together with the low adhesion of GO sheets to the microvilli brush border, the closed-packed arrangement of microvilli and the resulting highly negatively charged cell surface, it can be assumed that the translocation of GO through intact enterocytes in the intestinal barrier is relatively unlikely. In addition, the here applied model does not include the mucus-layer which covers the enterocytes in vivo and serves as an additional barrier. Nevertheless, uptake and translocation of GRM by other cell types of the human intestinal barrier, such as antigen-sampling M-cells or mucus-producing goblet cells, cannot be excluded and deserve further investigation. The obtained results are summarised in a manuscript which is in preparation (Kucki M. et al., in preparation).

Placental barrier models

2D BeWo monocultures

Assessment of the interaction of GO with the placental barrier was performed in vitro by the application of the BeWo cell line, human trophoblast cells representing the epithelial layer in the human placenta. The applied GO samples consisted of the four different GO previously applied with the non-confluent Caco-2 cells (one commercial GO sample was bought from Cheaptubes Inc. USA, the other GO was obtained from Antolin), plus two different GO samples obtained from Kostas Kostarelos (UNIMAN). None of the applied GO samples showed acute toxicity to BeWo cells for an exposure time of 24 hours or 48 hours for a concentration range of 5-80 µg GO/ml. Light microscopy analysis gave no hints towards increased cell-turn over. SEM analysis of the interaction of GO sheets with the BeWo cell surface revealed highly GO decorated cell borders and several hints towards cellular uptake, similar to non-confluent Caco-2 cells. Analysis of the cellular uptake is under current investigation by TEM.

3D microtissues

A novel 3D in vitro cell culture model of the human placenta has been established. The so called microtissues composed of two different cell types were obtained by hanging drop technology. The core of the microtissues consists of human villous mesenchymal fibroblasts (HVMF), and are covered by epithelial cells of the human placenta (BeWo cells). This new type of placenta in vitro model enables the investigation of the interaction of graphene-related materials with human cells arranged in a 3D architecture. Advantage of this model is the absence of an artificial culture membrane separating the different cell types and layers. Such membranes often represent a significant barrier for the translocation of nanomaterials as summarised in (Muoth C. et al. Nanomedicine, accepted). Scanning electron
microscopy images show microtissues highly decorated with GO sheets. There are several hints towards cellular uptake of graphene oxide by the outer BeWo cell-layer. Uptake as well as the potential translocation of GO to deeper tissue layers of the microtissues is under current investigation by TEM analysis.

**Ex vivo-placenta studies**

*Ex vivo* human term placenta were obtained from healthy donors (giving informed consent) after caesarean section. Perfusions of the placentas with perfusion medium containing commercial GO sheets have been performed. Analysis of the potential translocation of GO sheets through the placental barrier is in progress by application of different methods. Nevertheless, the localisation of unlabelled graphene oxide sheets in complex biological tissue with high carbon background is challenging and deserves further investigation. Perfusions were performed with unlabelled GO due to the fact that labelling of the GO sheets is connected with changes in the physicochemical properties which can highly influence the translocation behaviour.

**Blood Brain Barrier Model**

In order to better mimic the *in vivo* scenario UCD is currently developing an endothelial-astrocyte contact cell co-culture model for the Blood Brain Barrier (BBB), which is suitable for live cell imaging. In this model, hCMEC/D3 cells are used as endothelial cells and normal human astrocytes (NHA) as astrocyte cells. Our preliminary studies revealed the need of physical separation of the two cell types, which can be achieved by the membrane of the traditional Transwell™ insert or the use of other devices, such as SiMPore CytoVu imaging slides. The separate staining of the two cell populations is also crucial. We optimised the co-culture model using Transwell™ inserts (pore size 0.4µm): hCMEC/D3 cells are seeded on collagen coated Transwell™ inserts and grown for six days. On day six endothelial cells are stained with CellTracker™ Green dye (25 µM for 30 min) then incubated in fresh media for at least 1.5-2 hr. Pre-stained astrocytes are then seeded to the basolateral side of the membrane (turning it upside down) and incubate it for 2 - 3 hr (Figure 5.A). Finally, the insert is turned back to the original position, and cells let growing for one day (Figure 5.B).

![Figure 5](image-url)

**Figure 5**: A) seeding of pre-stained NHA on the reverse Transwell™ and B) final endothelial-astrocyte co-culture in the Transwell™ and the medium fractions.

The barrier can be analysed by live cell imaging techniques (spinning disk confocal microscopy and TIRFM) coupled with computational analysis (Figure 6).
Figure 6: Confocal live cells images stained with CellTracker™ Green CMFDA Dye (hCMEC/D3 labelling, post-staining) and CellTracker Red CMTPX (labelling astrocytes (NHA), pre-staining only suitable for 1 day).

The integrity of the barriers themselves, including being assured that the biological processes are fully reconstituted, is challenging but it is progressing well. The model has been validated checking the optimized integrity of the barrier in two ways: the permeability of 4 kDa FITC-Dextran (FD4) and the Claudin-5 expression which indicates tight junctions (Figure 7).

For the permeability, cells were grown for 6 or 7 days and collagen coated Transwell™ insert without cells was used as control. Permeability was calculated according to Czupalla et al. (Czupalla CJ et al. Cerebral Angiogenesis: Methods and Protocols (2014): 415-437) and the results are in good agreement with the literature.

Figure 7: A) Cell monolayer permeability (Pe) of hCMEC/D3 and co-culture to FD4 calculated by the method of Czupalla et al. (Czupalla CJ et al. Cerebral Angiogenesis: Methods and Protocols 2014, 415-
Live cells confocal images. In green the Claudin-5 and in blue the nucleus (DAPI Stain).

The transcytosis of different graphene materials (FLG, GO and exfoliated graphene) through this system is currently being analysed, where GO is graphene oxide (Antolin), FLG is few layer graphene exfoliated with melamine and PEGr is protein-exfoliated graphene.

The BBBs were exposed to GO, FLG and PEGr at the concentrations of 50 and 25 μg/ml for 4 h and 24 h. A Transwell™ without cells has been used as a control.

Prior the analysis, the toxicity of the material on the barrier was assessed by LDH cytotoxicity assay. If the cell membrane is damage and the junctions no longer are tight due to some toxicity of the material, LDH is released in the apical and basal fraction of medium. For FLG and PEGr the detected values of LDH after 4h and 24h in the basal and apical fraction are comparable to the untreated cells. For GO, the LDH detected in the apical fraction after 4h and 24h are slightly higher than the control cells, in good agreement with the results of the High Content Analysis (HCA) in which GO was found to undergo large acidic organelle formation at low serum concentrations.

After exposure, the fraction of medium in the apical and basal part of the Transwell™ were collected and analysed by UV/Vis Spectroscopy and Raman. The limit of detection for the two techniques for each material in water are respectively (Figure 8):

- UV/Vis: GO [2.5 μg/ml], FLG [2.5 μg/ml], PEGr [5 μg/ml]
- Raman: GO [2.5 μg/ml], FLG [20 μg/ml], PEGr [20 μg/ml];

The limits are slightly higher when Phenol-red free medium is used, since it partially covers the Raman peaks.

**Figure 8:** Raman spectra and UV/Vis absorption spectra for GO, FLG and PEGr in water at different concentrations.

The results are the same for the two concentrations and for the three materials with UV/Vis spectroscopy and Raman. After 24 h any graphene material is detectable in the basal fractions (for both BBB and control) whereas it can be found in the apical fractions.

The barriers have been imaged by spinning disk confocal microscopy. From the section images in bright field presented in Figure 9 we can see how FLG over the other materials presents large aggregates.
formation on top of the cells whereas for GO and PEGr smaller black spots are visible. This is a consequence of a better dispersion and stability overtime.

The flakes are only visible in bright field and only visible (in focus) in the upper section of the barrier.

For these reason we decided to use another method, that allow for a label-free detection, to study the graphene translocation across the BBB and the possible internalization in the cells of the membrane.

Figure 9: Fluorescence and bright field images of the BBB exposed to the 3 graphene materials. Cells are stained with DAPI stain for the nuclei and Texas red - X Phalloidin for the actin filaments. The images are sections seen from the top. On the bottom and at the right of each image, the vertical section of the barrier can be seen and a white line indicates at which depth of the barrier referred to the top section.

Exploiting the clear Raman signature of graphene, the material can be precisely localised by Raman-confocal thanks to a 3D mapping. The detection of graphene by Raman mapping is quite challenging because of the high cell signal background and because of the important photo-thermal degradation effect when the material is irradiated with high energy laser. The optimization of a protocol is not straightforward, especially in the case of the Transwell™ membranes that have to be removed from the well and cut in order to be analysed by Raman Spectroscopy.

We decided to establish a preliminary set-up for these measurement based on classic cell culture in a culture dish in order to optimize the acquisition parameters.

For the preliminary set up, HEK-293 (Human Embryonic Kidney) cells were seeded in a 35 mm imaging dish with an ibidi Standard Bottom. The cells were exposed to 10 μg/ml of PEGr for 24 h and then fixed with 10% formalin solution. The Raman spectra were recorded for unexposed cell in the nucleus and
cytoplasm as a control. For the PEGr exposed cells, aggregates of various sizes are visible at the optical microscope. Raman spectra recorded at different focal planes show the typical graphene signature (D, G and 2D peaks), as can be seen in Figure 10. In the lower part, Figure 10 depicts a z-stacking from -10 μm to 10 μm with a pitch of 1.5 μm where all the recorded spectra are overlapped. On the right, the distribution of the intensity of the D, G and 2D peaks is reported in relation to the z position. From these results, we can speculate that the PEGr are situated inside the cell.

Figure 10: Above, Raman spectra (532 nm laser excitation) of cells cytoplasm, nucleolus and nucleus (background) and graphene agglomerates in cell. The spectra of graphene, recorded for two different focal planes, show the typical peaks D (1350 cm⁻¹), G (1580 cm⁻¹) and 2D (2690 cm⁻¹). Below, overlapped spectra for z-stacking from -10 μm to 10 μm inside the cell. The intensities plotted vs z position for the three characteristic graphene peaks: D (green), G (red) and 2D (blue) reveal a maximum concentration of graphene at -4 μm.

This protocol is actually being transferred to the Transwell™ to study graphene translocation across the BBB.