# An integrated testing approach to generate validated, lung-relevant toxicity data on graphene nanoplatelets

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## INTRODUCTION

Graphene family nanoplatelets (GFNs) are a group of carbon-based engineered nanomaterials with special properties, which enable innovation in nanomedicine and technology. Within the general framework of safety assessment of nanomaterials (NMs) during their lifecycle, the main objective of the PLATOX project (funded by EU FP7-SIINN ERA-NET Programme) is to apply a tiered approach to assess the toxicity of GFNs and identify the derived no-effect level (DNEL) parameter for inhalation exposure.

## OBJECTIVES

- Selection of GFNs with different chemistry suggesting a varying toxic outcome
- *In vitro* testing: cytotoxicity, direct genotoxicity, cytokine and eicosanoid release in order to identify the candidates with the highest and lowest biological activity
- Validation of *in vitro* results with an *in vivo* 28-day inhalation test (on two selected GFNs, most and least active)
- To evaluate available information on physicochemical properties of the tested GFNs and compare it with the toxicological *in vitro* and *in vivo* results and exposure data
- Perform risk assessment and derivation of DNEL for the two selected GFNs according to current regulatory procedures. The workflow proposed for the risk assessment integrates the physicochemical characterization, *in vitro* and *in vivo* methodologies (**Figure 1**)

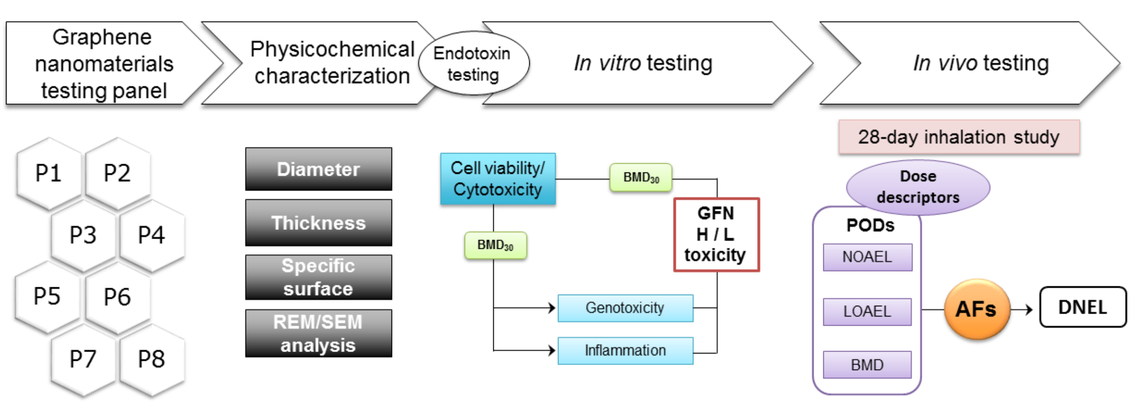


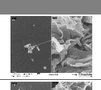
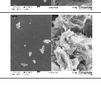
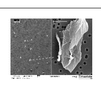
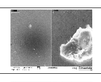
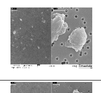
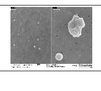

**Figure 1.** Workflow for risk assessment

## MATERIALS AND METHODS

**Test items:** Six different commercially available GFNs (P1-P6) and reference pristine graphene nanoplatelets (P7) were selected for *in vitro* testing (**Table I**). Carbon-black particles (P8) are used as spherical reference material.

**Dispersion of GFNs and exposure:** Materials were dispersed in serum-containing cell culture medium, 3 x 5 min of ultrasonication on ice, 1 min breaks, Bandelin Sonoplus HD2070 (70 W) with sonotrode VS70T (Ø 13 mm), 90% cycles (9/10), amplitude 80 $\mu m_{ss}$. Cells were incubated with the test items of increasing concentrations up to 50 $\mu g/cm^2$ for 24h or 48h.

**Table I.** Physicochemical properties of the investigated GFNs

| No. | GFN | Diameter (µm) | Thickness (nm) | BET surface area ($m^2/g$) | Endotoxin content (ng/mg) | SEM image | Preparation/ Properties |
|---|---|---|---|---|---|---|---|
| **P1** | Single layer graphene powder | ~ 5 | 2 – 10 | 278 (400-1000)* | 0.0553 |  | Thermal exfoliation reduction + Hydrogen reduction |
| **P2** | Single layer graphene (graphene factory series) | 0.5 – 5 | 2 – 10 | 620 (650-750)* | 0.0467 |  | 1-5 atomic layer graphene nanosheets |
| **P3** | Carboxyl graphene | 1 – 5 | 0.8 – 1.2 | 1.5 | <0.005 |  | 1) Modified Hummer's method to make graphene oxide 2) Convert –OH and C-O-C into –COOH. Carboxyl ratio: 5% |
| **P4** | Graphene nanoplatelets | ~ 5 | 2 – 10 | 15 (20-40)* | <0.005 |  | Stacks of multi-layer graphene, with a high aspect ratio, width to thickness |
| **P5** | Single layer graphene oxide powder (S method) | 1-15 | 0.8 – 1.2 | 5.2 (5-10)* | <0.005 |  | Stauden-Maier method; oxygen content: 35% |
| **P6** | Graphite oxide powder | 0.5 – 5 | 1 – 3 | 2.7 | 0.142 |  | Modified Hummer's method; oxygen content: 35% |
| **P7** | Reference pristine graphene nanoplatelets (GR1) | 2 | 3 | 195 (70)* | 0.0343 |  | No XPS (low defects by RAMAN) all C1s carbons; 8±0,5 atomic layer graphene |
| **P8** | Reference carbon black particles (Printex 90®) | 14 | - | 317 (337)* | <0.005 |  | Specified as >99% pure Carbon Black, PAH=0,039 ppm |

*range given by the supplier

**Test systems:** RAW 264.7 murine blood macrophage cell line (University of Aveiro - UAVR), primary rat alveolar macrophages (Fraunhofer ITEM), MRC-5 human lung fibroblasts (both laboratories).

**Endpoints and assays:** Membrane damage (LDH assay), cell viability (AlamarBlue® assay), proliferation (relative increase in cell count, RICC), cytokine release (ELISA), unstimulated/stimulated eicosanoid pattern (highly sensitive and specific competitive EIA), direct genotoxicity by measuring DNA-strand breaks and oxidative DNA-damage (hOGG1-modified alkaline comet assay), cell cycle dynamics/ploidy level (flow cytometry).

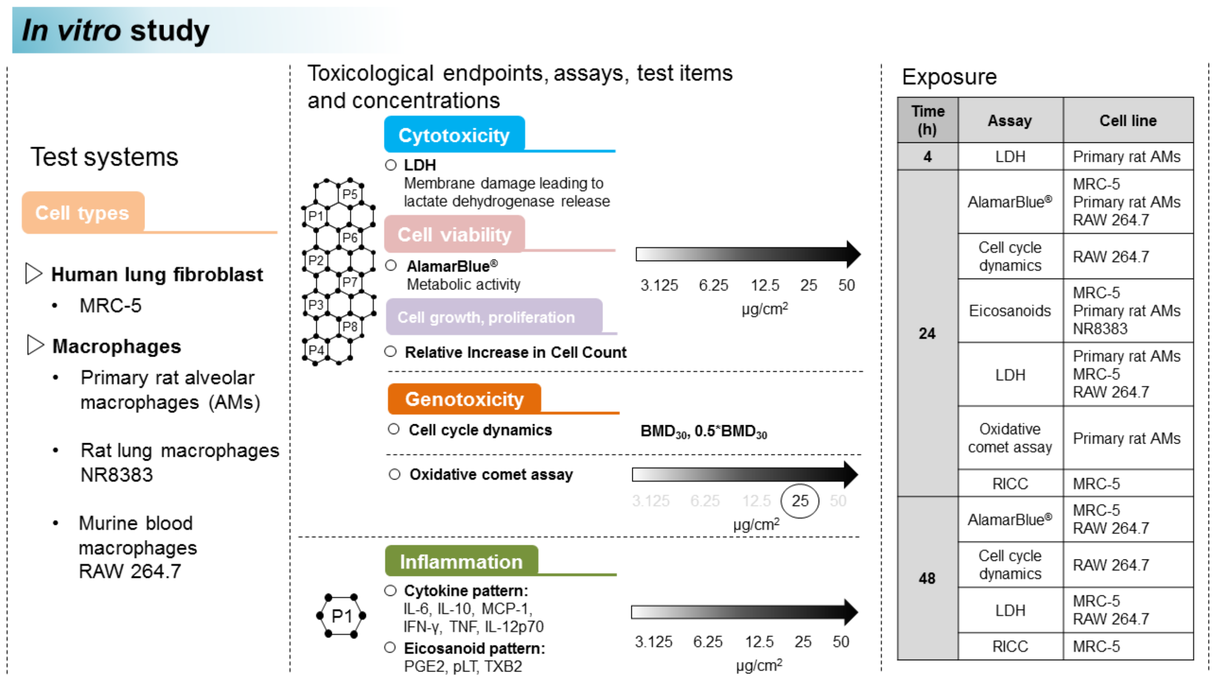



**Figure 2.** Overview of *in vitro* testing

## *IN VITRO* RESULTS

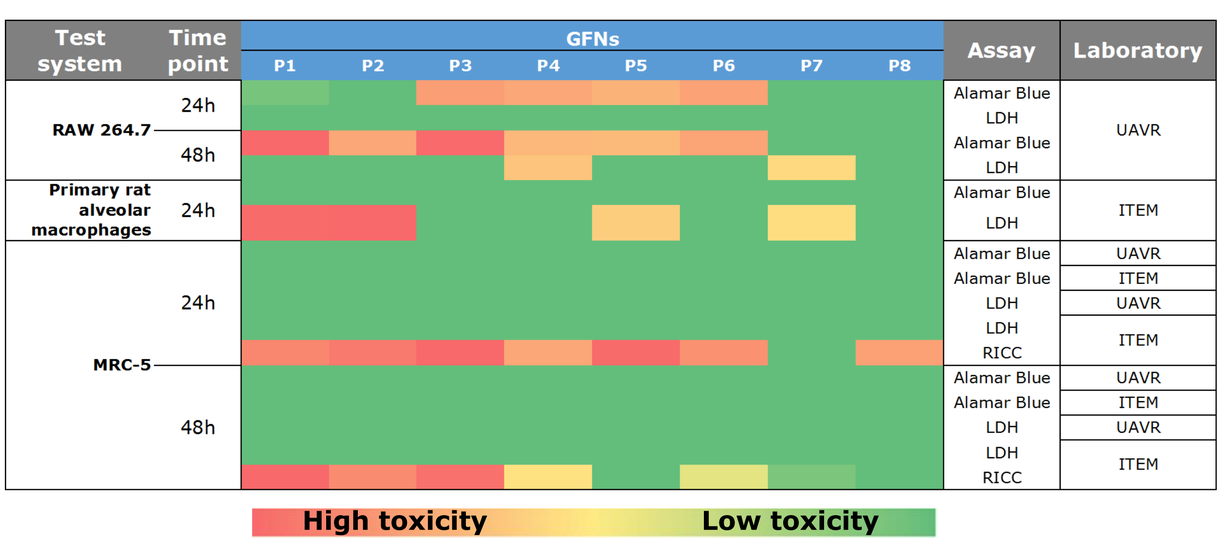



**Figure 3.** Preliminary toxicity ranking based on cytotoxicity results and comparison of $BMD_{30}$ for all GFNs

**Table II.** Ranking of toxic effects detected in *in vitro* assays at UAVR and ITEM

| Test item graphene | P1 | P2 | P3 | P4 | P5 | P6 |
|---|---|---|---|---|---|---|
| **Ranking of toxic effect** | **high** | **high** | **low** | **moderate** | **moderate** | **low** |
| **Parameter** | | | | | | |
| LDH, cytotoxicity, Primary rat alveolar macrophages 24 h | +++ | +++ | - | - | + | - |
| LDH, cytotoxicity, RAW 24 h | + | - | - | - | + | + |
| LDH, cytotoxicity, RAW 48 h | + | + | + | ++ | + | + |
| Comet assay, genotoxicity, Primary rat alveolar macrophages 24 h | ++ | ++ | + | + | + | - |
| Cell cycle, G0/G1, RAW 24h | + | - | - | + | - | - |
| Cell cycle, %G0/G1, RAW 24h | - | + | - | - | - | + |
| Cell cycle, varG0/G1, RAW 24h | ++ | ++ | - | + | + | + |
| Cytokines, TNF-α, RAW 24h | - | - | + | - | - | - |
| Cytokines, MCP-1, RAW 24h | - | - | + | - | - | - |

## DISCUSSION AND CONCLUSIONS

- Fully characterized GFNs are used in this study, in order to perform an integrated risk assessment based on *in vitro* and *in vivo* studies.
- The preliminary *in vitro* results show that P1 and P2 are the most toxic GFNs, while P3 and P6 are less toxic; a higher sensitivity of both primary rat alveolar macrophages and murine blood macrophages was observed, as compared to lung fibroblasts. However, the results on macrophages show no correlation between the two assays applied (**Figure 3 and Table II**).
- Non-toxic concentrations of GFNs were used for evaluation of additional *in vitro* parameters, genotoxicity and cytokines. Therefore the selection of the GFNs for *in vivo* validation is based on a complex *in vitro* test battery. The selected graphenes with high and low toxicity are P2, respectively P4, for the 28-days inhalation study, in order to reduce the overall number of animals used in the project. Selection relies on the clear toxicity in primary cells of the respiratory tract of the test system used for the *in vivo* experiments, chemical composition as they are pure carbon typical graphenes, by contrast to other test items which represent technical intermediates to synthesize graphene by reduction.
- In summary, the expected outcome of the project will be a toxicological ranking of the tested GFNs, providing an improved basis for risk assessment of these NMs and ensure their safety from production phase to use of medical products and applications that incorporate graphene nanoplatelets.

**Project partners**

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