# Establishment of human-based *in vitro* models to evaluate neurotoxicity

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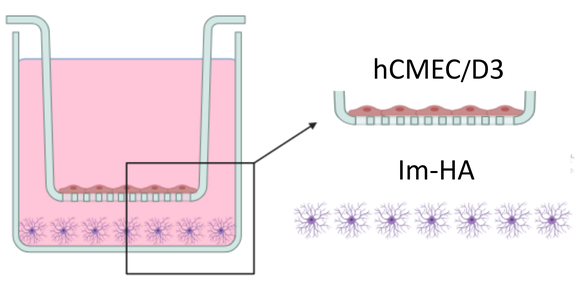
## Introduction

Currently, there is a gap in the availability of realistic models of the human central nervous system (CNS) for evaluating the neurotoxicity of Advanced Materials, including nanomaterials. To address these challenges, the iCare project focused on developing advanced in vitro models of the CNS. Specifically, we aimed to create models of the blood-brain barrier (BBB) and co-culture models to assess neurotoxicity (2D and 3D brain spheroids, also referred to as "mini-brains"). These innovative models are designed to provide more realistic and physiologically relevant systems for studying the interactions between nanomaterials and the CNS.

## Static BBB model

**Static human BBB model** using **cerebral endothelial hCMEC/D3** cells and **Im-HA astrocytes.** Endothelial cells formed a homogeneous tight junctions when seeded onto **PET Transwells** (0.4 µm pore size) for **7 days (Figure 1)**.



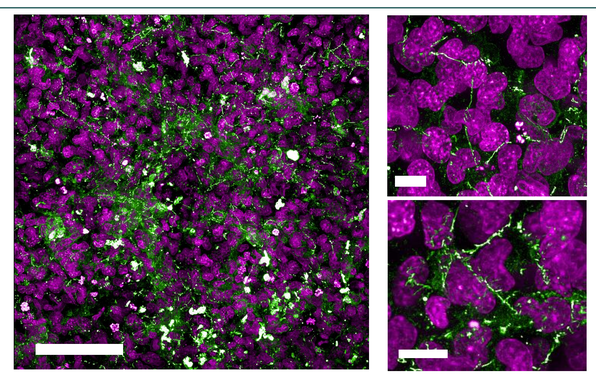
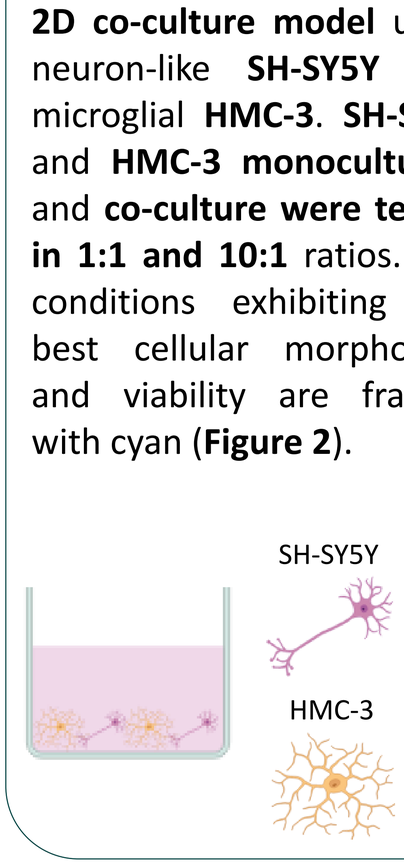



**Figure 1. Tight junctions of human BBB model**. Immunofluorescence analysis of ZO-1 (green; nuclei: magenta) in the static BBB model after 7 days of culture. Scale bars = 100 µm and 25 µm (magnified images).

## 2D neurotoxicity co-culture model

**2D co-culture model** using neuron-like **SH-SY5Y** and microglial **HMC-3**. **SH-SY5Y** and **HMC-3 monocultures,** and **co-culture were tested in 1:1 and 10:1** ratios. The conditions exhibiting the best cellular morphology and viability are framed with cyan (**Figure 2**).



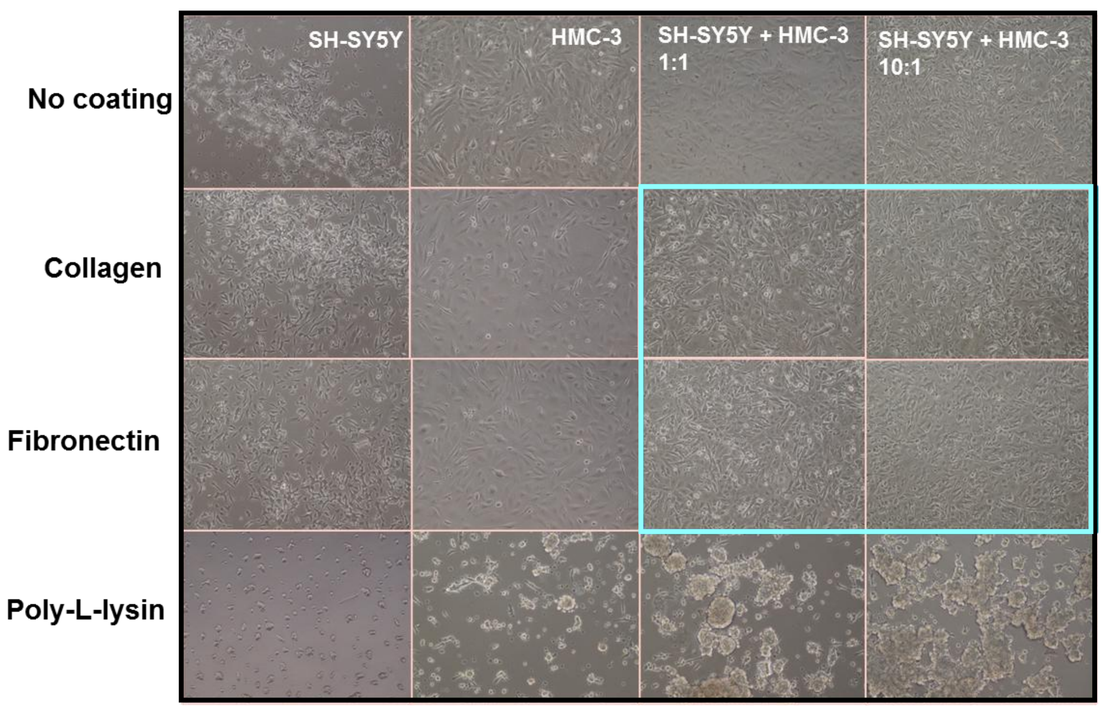
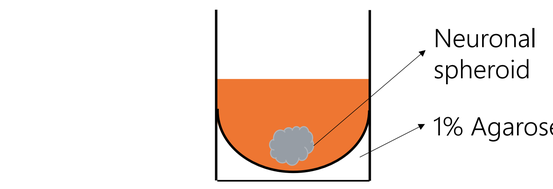




**Figure 2. 2D neurotoxicity model**. Images of monoculture and co-culture models 24h after seeding on uncoated and on collagen, fibronectin or poly-L-lysine coated microplates.

## 3D neurotoxicity co-culture model

**3D co-culture model (neuronal spheroids)** using neuron-like **SH-SY5Y** and microglial **HMC-3**. Cells were co-cultured in 1:1 ratio on wells pre-coated with 1% agarose to force them to remain in suspension. After **48h**, the spheroids were **well-defined (Figure 3)**.



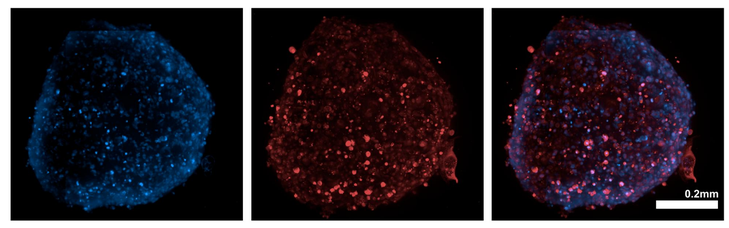




**Figure 3. Neuronal spheroids**. Confocal fluorescence microscopy of brain spheroids after 48h of co-culture of SH-SY5Y and HMC-3 (nuclei of both cell lines in blue, cell membranes in red). Scale bar = 0.2mm.

## Graphene materials – 2D Neurotoxicity co-culture model

The exposure of the neurotoxicity co-culture model to COG+ and COG+ ink formulation products induced **significant cytotoxic effects after 24h**. However, this effect was only observed for the **highest concentration of COG+,** whereas **COG+ ink formulation was significantly cytotoxic for all concentrations** tested. In addition, the **exposure** to up to **0.5 µg/ml COG+ for 3h** induced a significant **increase of ROS production;** similar results were observed for **all the tested concentrations of COG+ ink formulation**. Finally, a significant release of **IL-6 and IL-8** was observed after 24h of exposure to **50 µg/ml COG+.**

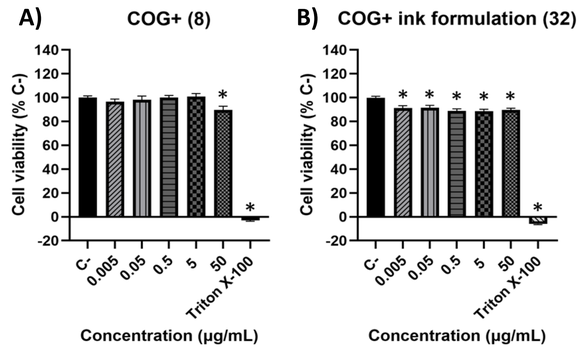



**Figure 4. Cell viability.** Neurotoxicity model exposed for 24h to A) COG+ and B) COG+ ink formulation. Mean % ± SEM. * Significant differences respect to C- ($p < 0.05$).

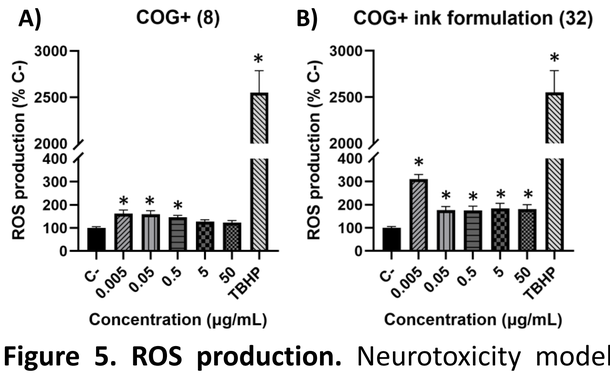



**Figure 5. ROS production.** Neurotoxicity model exposed for 3h to A) COG+ and B) COG+ ink formulation. Mean % ± SEM. * Significant differences respect to C- ($p < 0.05$).

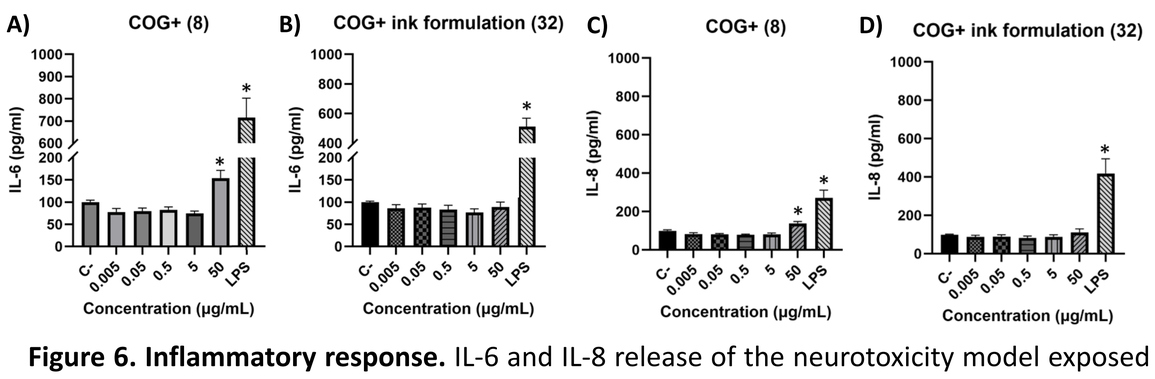



**Figure 6. Inflammatory response.** IL-6 and IL-8 release of the neurotoxicity model exposed for 24h to A) and C) COG+ and B) and D) COG+ ink formulation. Mean % ± SEM. * Significant differences respect to C- ($p < 0.05$).

## Conclusions

As conclusion, we successfully developed a human-based in vitro blood-brain barrier (BBB) model and two co-culture models (3D and 2D) for neuro-nanotoxicity studies. The BBB model showed stable barrier formation and good sensitivity to inflammatory markers. The 3D brain spheroid co-culture model, combining neuronal cells and microglia, showed improved integration and viability compared to single-cell spheroids, and the 2D co-culture model exhibited good sensitivity to nanomaterials like Ag NPs, SiO2 NPs, and graphene.

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