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Development of 2D and 3D human *in vitro* models to evaluate neurotoxicity of nanomaterials

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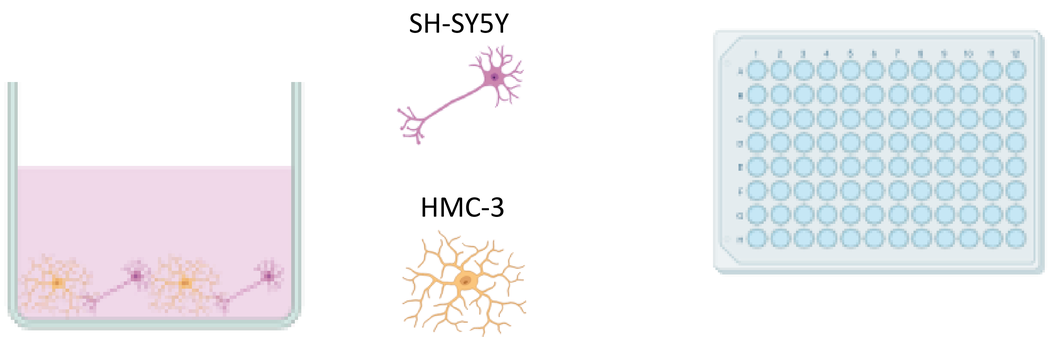
Introduction

Currently, there is a **lack of realistic human-based *in vitro* models** to assess the neurotoxicity of advanced materials (AdMa) and nanoparticles (NPs). Specifically, **nanometric particles** may enter the body through routes like inhalation, potentially **translocating to the Central Nervous System (CNS)** by crossing the **Blood-Brain Barrier (BBB)** and reaching the **brain**. Once within the CNS, these materials could cause **damage to brain tissue**. However, the existing *in vitro* models do not represent the *in vivo* microenvironment of the human CNS.

2D co-culture neurotoxicity model

2D co-culture model using neuron-like **SH-SY5Y** and microglial **HMC-3** cell lines. **SH-SY5Y** and **HMC-3 monocultures,** and **co-culture were tested in 1:1 and 10:1** ratios.



***In vitro* 2D model** was **exposed** to a range of **concentrations of graphene, silver (Ag) and SiO_2 NPs** (from enviromentally relevant to high concentrations: **0.005 to 50 µg/mL**) for **24h**.

Cell viability (Alamar Blue), **oxidative stress** (ROS production) and **inflammatory response** (IL-6 and IL-8 levels) were analyzed.

The **best cell culture conditions** were achieved using **1:1 and 10:1** ratios of cells seeded on **collagen** or **fibronectin** coated wells (**Figure 2**).

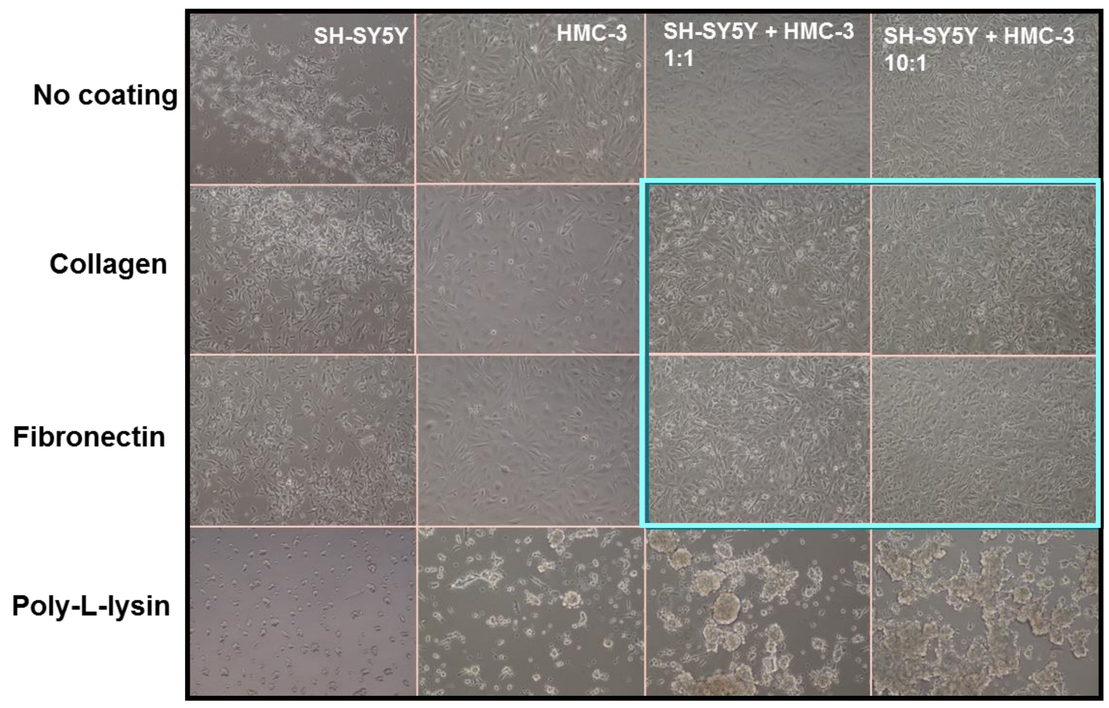


Figure 1. 2D neurotoxicity model. Images of monoculture and co-culture models 24h after seeding on uncoated and on collagen, fibronectin or poly-L-lysine coated microplates.

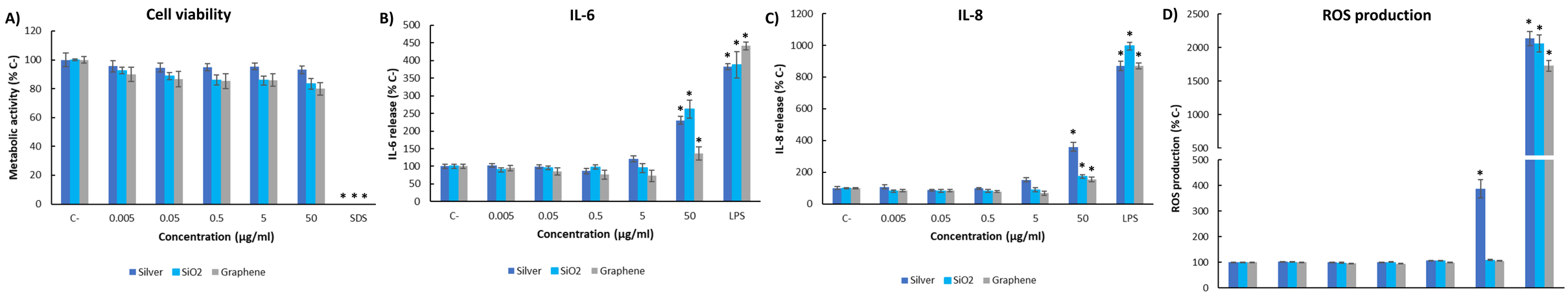
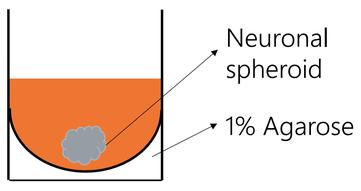


Figure 2. A) Cell viability, B) IL-6 and C) IL-8 response. Neurotoxicity 2D model exposed for 24h to Ag, SiO_2 and graphene NPs. **D) ROS production.** Neurotoxicity 2D model exposed for 3h to Ag, SiO_2 and graphene NPs. Mean % ± SEM. * Significant differences respect to C- ($p < 0.05$).

Overall, the results obtained indicated **slight dose-dependent cytotoxic effects** for all the tested compounds. In addition, an **induction of IL-6 and IL-8** released was observed after the exposure of the 2D model to the **highest concentration of the NPs**. Finally, **ROS production** increased only for the **highest concentration of Ag NPs**.

3D co-culture neurotoxicity 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.



Optical and confocal microscopy analyses were conducted **after 24 and 48 hours**. After **48h**, the spheroids were **solid and well-defined (Figure 3)** in the co-culture systems compared to monotypic cultures, but the spheroids' low handling versatility and lack of biological response made it challenging to use this model effectively.

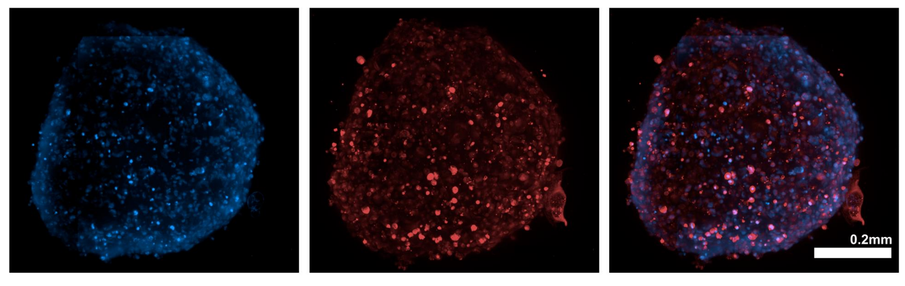


Figure 3. Neuronal spheroids. Confocal fluorescence microscopy of brain spheroids after 48h of co-culture of SH-SY5Y and HMC-3 (nuclei of both cell lines in blue, cell membranes in red). Scale bar = 0.2mm.

Conclusions

Overall, the results from 2D co-culture model showed high sensitivity to the particles, particularly at higher concentrations, highlighting the potential of this model for assessing human neurotoxic effects and emphasizing the need for novel human in vitro models to explore the neurotoxicity of advanced materials. In contrast, neuronal spheroids showed limitations making it challenging to use this model for neurotoxicity assessment.

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