Repeated long-term exposures of multi-walled carbon nanotubes to the 3D human lung model EpiAlveolar™ to predict the onset of fibrosis

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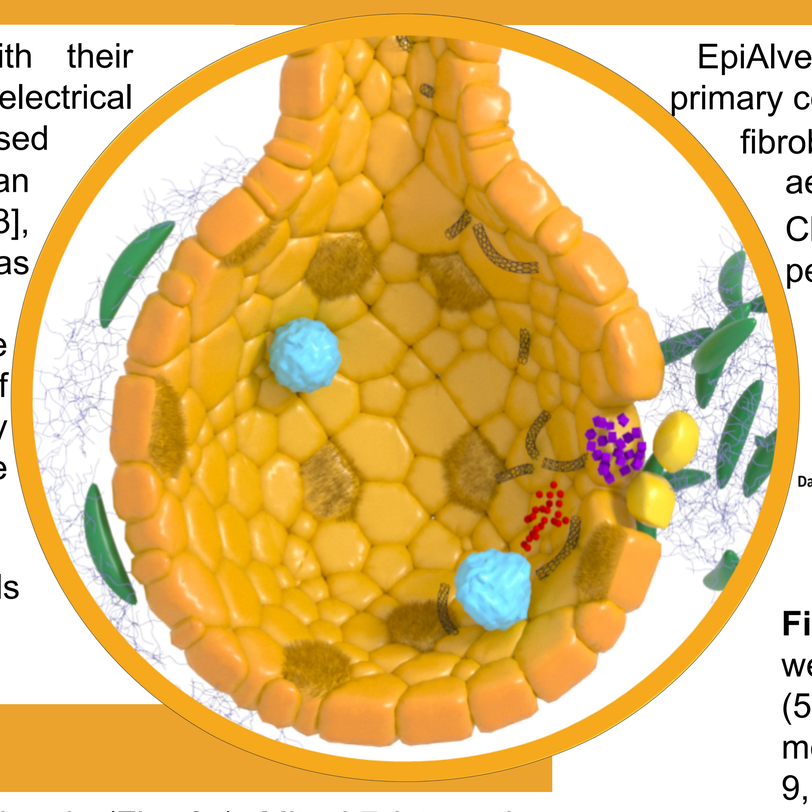
Background

Multi-walled carbon nanotubes (MWCNTs) with their extraordinary properties (e.g. stiffness, electrical conductivity, etc.) are among the most commonly used nanomaterials [1]. Human exposure to MWCNTs can occur throughout their life-cycle *via* inhalation [2, 3], and may lead to potential adverse effects, such as pulmonary fibrosis [4]. Therefore, there is a need to design human-relevant *in vitro* testing and exposure strategies to assess the biological effects of MWCNTs. The process that leads to pulmonary fibrosis can be organized into an adverse outcome pathway (AOP) framework. Starting with a molecular initiating event (MIE), AOPs sequentially link the key events (KEs) that occur at different levels of biological organization, leading to an adverse outcome (AO) [5].



Methods

EpiAlveolar™ tissues (MatTek Corporation) consisting of human primary cells, i.e. lung endothelial and alveolar epithelial cells and fibroblasts, were exposed at the air-liquid interface to aerosolized Mitsui-7 MWCNTs using the VITROCELL© Cloud system. Repeated, low doses exposures were performed based on Fig. 1 scenario.

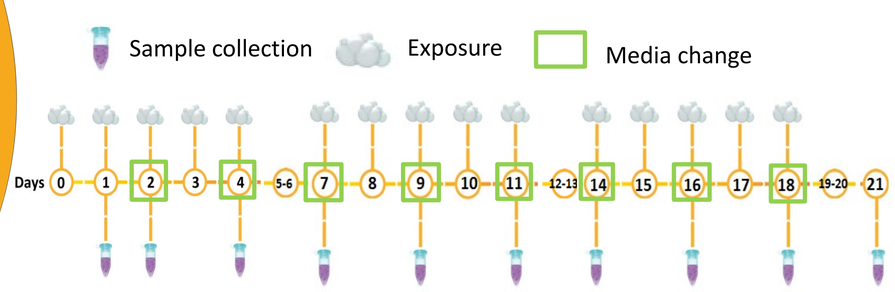


Fig. 1: Graphical scheme of exposure scenario. Tissues were exposed every working day from Monday to Friday (5 times per week) for three consecutive weeks. The medium was collected for further analysis at D1, 2, 4, 7, 9, 11, 14, 16, 18 and 21.

Tab. 1: Mitsui-7 MWCNTs deposition during the experiment

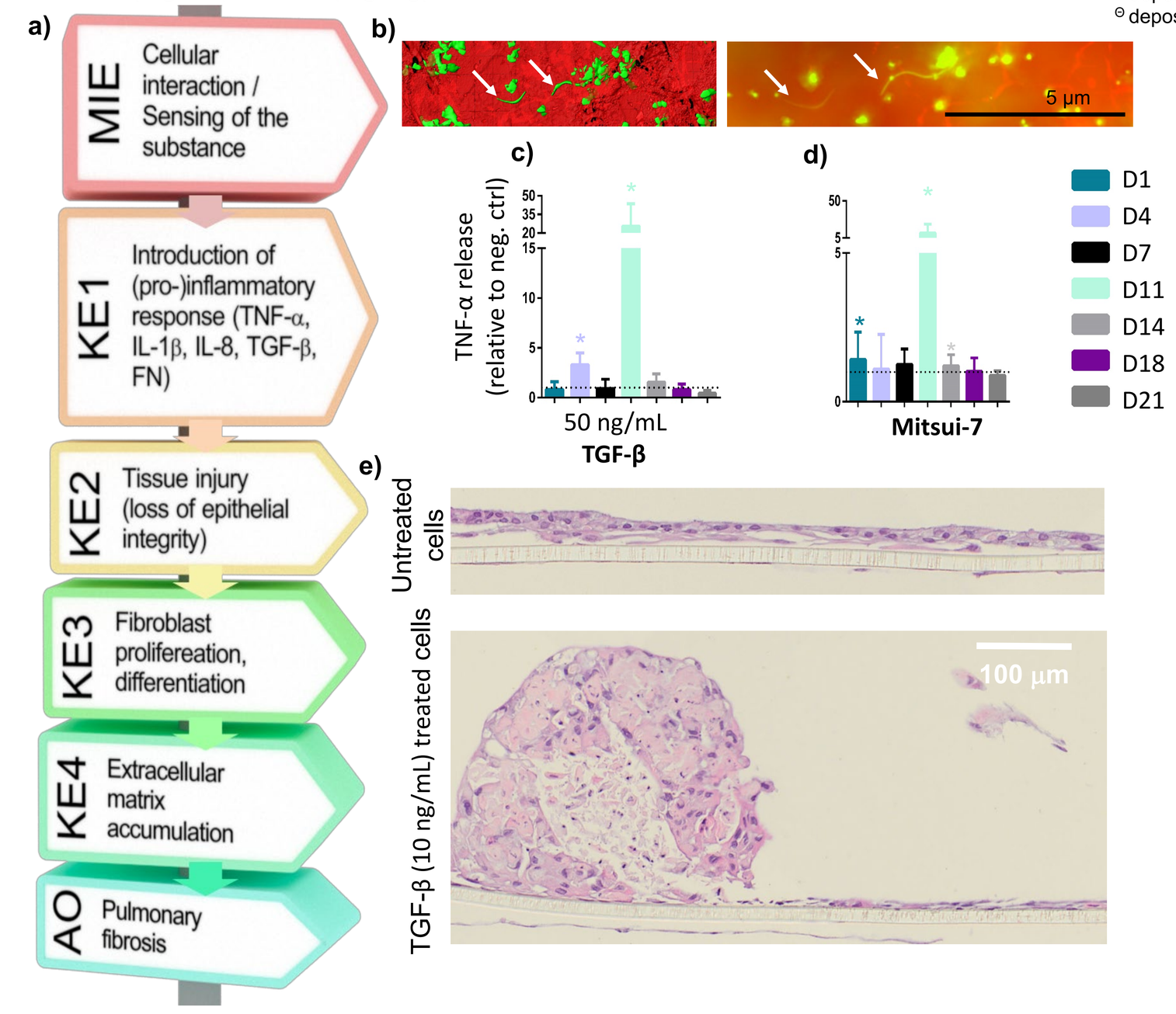
Material	Daily deposition (µg/cm²)Ψ	Weekly deposition (µg/cm²)θ	Total deposition after 21 D (µg/cm²) θ	Dispersant
Mitsui-7	1.98 ± 0.42	9.88 ± 2.10	29.63 ± 6.30	0.1 % BSA

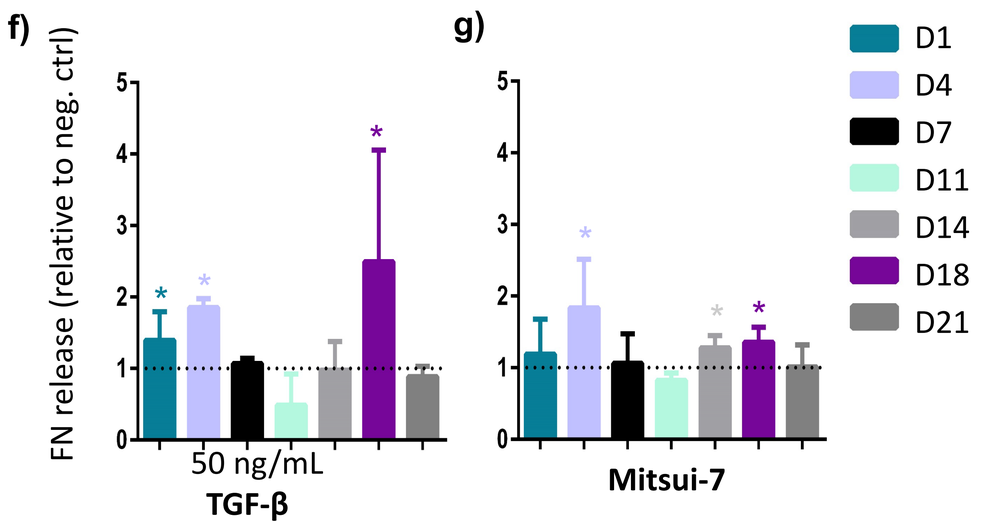
Ψ deposition measured by quartz crystal microbalance

Θ deposition calculated - daily deposition multiplied by number of exposure days

Results

The results are following the AOP for pulmonary fibrosis (Fig. 2a), Mitsui-7 interaction with EpiAlveolar™ is presented at Fig. 2b. Repeated exposures to transforming growth factor-β, used as a positive control to induce a fibrotic response, showed a statistically significant ($p<0.05$) increase in cytokines release, especially tumor necrosis factor α (TNF-α, Figure 2c) and fibronectin (FN, Figure 2f) release, as well as increase in cell layer thickness compared to untreated cells (Fig. 2e). Repeated exposures to MWCNTs (range of 1 – 30 µg/cm², Tab. 1, Fig. 3) caused also statistically significant increase in TNF-α (Fig. 2d) and FN (Fig. 2g) release.





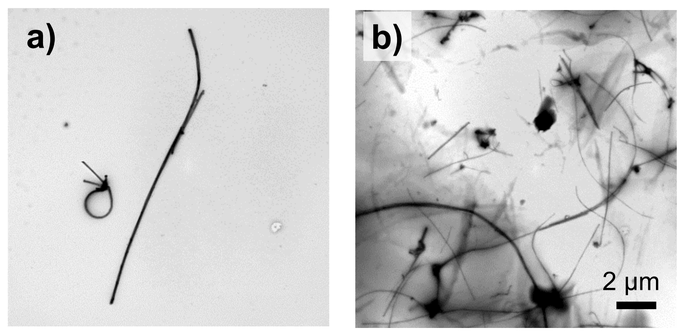


Fig. 3: TEM micrographs of deposited Mitsui-7 on TEM grid placed at the bottom of VITROCELL™ Cloud exposure chamber. (a) deposited particles after 1 exposure, (b) overall deposition during whole experiment – i.e. after 3 weeks (15 days of exposures).

Fig. 2: Results following the adverse outcome pathway (AOP) framework. (a) Schematic depicting the AOP for pulmonary fibrosis, (b) Dark-field images showing the interaction of Mitsui-7 with the tissue, white arrows show the Mitsui-7, left 3D rendered image, (c, d) KE1 (pro-)inflammatory cytokine TNF-α release, (e) KE 2 – 4, contraction of EpiAlveolar™ tissue upon exposure to TGF-β for 21 days visualized using hematoxylin and eosin staining, (f, g) extracellular matrix (Fibronectin (FN)) production upon exposures to TGF-β and Mitsui-7 MWCNTs. Results are presented as relative to negative control, (*) marks statistically significant increase (1-way ANOVA, $p<0.05$), error bars=SD, n=3.

Conclusion

The EpiAlveolar™ tissue is promising tool for predicting the development of pulmonary fibrosis upon exposure to MWCNTs. In the future, the model can be used in combination with other *in vitro* and *in silico* methods for hazard assessment of aerosolized nanomaterials.

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