The "Navetta" *in vitro* aerosol exposure system for respiratory health monitoring as well as efficacy and safety testing of pulmonary delivered bio-based pharmaceutical formulations

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Exposure of lung epithelia to aerosols is omnipresent. The unintentional exposure to exhaled droplets expelled during speaking, sneezing, or coughing is part of our daily life. Such aerosols contribute to the airborne transmission of infectious diseases. Additionally, exposure to aerosols or airborne particulates can occur in workplaces or in environmental settings. Along this line the World Health Organization (WHO) claimed: *"In 2019, 99% of the world's population was living in places where the WHO air quality standards were not met."* (1). For instance, a significant portion for air pollution derives from car tire wear and tear with an estimated global average *per capita* emission of 0.81 kg/year accumulating to 3-7% of the particulate matter ($PM_{2.5}$) in air. In humans, the permanent exposure to polluted air leads to the development of various pulmonary diseases that become a growing problem. The top ten leading causes of deaths in 2019 included three lung diseases (chronic obstructive pulmonary disease (COPD), lower respiratory infections, lung cancer) (4). Focusing on prevention and treatment of lung diseases is a hot topic, and the development of pulmonary delivered pharmaceutics is of great interest. Budesonide, for example, is a commonly used glucocorticosteroide applied to treat asthma and COPD. The application with a dry powder (DPI) or metered dose inhaler (MDI) allows local application, downregulating inflammation in the affected lung tissue. Moreover, the thin epithelial barrier (0.1-0.2 µm) in the alveoli, the large surface area of the lung (70-100 m^2) and the good blood supply enable a swift transfer of drugs into the circulatory system, making the pulmonary administration an interesting application route, less invasive than intravenous administration and hence better-compliant. In the context of nanomedicine, carrier systems are investigated that increase therapeutic efficiency, reduce toxicity, and achieve targeting delivery. Following the 3 R principle to reduce, refine, and replace animal experiments, we developed an *in vitro* aerosol exposure system, called Navetta, to test the environmental impact of air pollution on human lung as well as efficacy and safety of therapeutic aerosols intentionally administered *via* the pulmonary route. The Navetta enables the exposure of air-liquid interface (ALI)-cultured human lung epithelial cells (here the A549 cell line was used) to a low, laminar, horizontal air flow simulating the situation in the alveoli as closely as possible. *Via* electrostatic deposition of beforehand charged aerosols this

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system allows a very efficient deposition. The efficacy of the electrostatic deposition was tested using aerosolization of a sodium fluorescein solution showing a reproducible deposition across the 4 positions inside the Navetta, as well as by FITC-labelled silica, ZnO, CeO_2, or Ag-containing nanomaterials. To generate the aerosols from an aqueous suspension a 4-jet low-flow Blaustein atomizer was used to ensure a particularly gentle aerosol generation suitable for therapeutics and biological materials. For pharmaceutic efficacy testing we established a therapeutic model simulating airway inflammation using ALI-cultured A549 cells pre-incubated with tumour necrosis factor (TNF)-α. As efficacy readout the inflammation suppression after the administration of budesonide aerosolized from dry powder was determined. For safety profiling, cell viability and immune responses after exposure were determined. Additionally, a scanning mobility particle sizer and an optical particle counter were used to determine the size distribution of the generated aerosols over a size range from 10 nm to 35 µm. The particulate number concentration mode was 50 nm, and the particulate mass concentration was below 2-5 µm resulting in a deposition in the alveolar area. This data further allowed to perform *in silico* prediction of pulmonary drug deposition (5). For optimizing the efficacy of therapeutic administration into deep lung budesonide packaging into next-generation carrier systems based on biomaterials are of interest. One type of biological material offering great drug-loading opportunities are extracellular vesicles (EVs), which are regarded as safe and effective (6). EVs are a heterogenous group of membrane vesicles secreted by eukaryotic and procaryotic cells and participate in communication and signalling. Their lipid bilayer enables the loading of EVs with therapeutics such as RNA, DNA, or small molecular weight drugs (7). Tissue targeted drug delivery can be reached due to specific surface modifications of the EVs. Here we studied bio-derived EVs, which indeed enabled efficient uptake of fluorescently double-labelled EVs into the cells, with several post-incubation times being tested for efficiency optimization. In summary, we herewith demonstrate the suitability of the Navetta *in vitro* aerosol exposure system for testing cyto- and immunotoxic pulmonary effects derived from environmental pollutants and for efficacy and safety testing of pharmaceutical applications.

1. References

(1) https://www.who.int/news-room/fact-sheets/detail/ambient-(outdoor)-air-quality-and-health

(2) Kole PJ, Löhr AJ, Van Belleghem FG, Ragas AM. Wear and tear of tyres: a stealthy source of microplastics in the environment. International journal of environmental research and public health. 2017;14(10):1265.

(3) Lolage M, Parida P, Chaskar M, Gupta A, Rautaray D. Green Silica: Industrially scalable & sustainable approach towards achieving improved “nano filler–Elastomer” interaction and reinforcement in tire tread compounds. Sustainable materials and technologies. 2020;26:e00232.

(4) https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death

(5) Hofer S, Hofstätter N, Duschl A, Himly M. SARS-CoV-2-Laden Respiratory Aerosol Deposition in the Lung Alveolar-Interstitial Region Is a Potential Risk Factor for Severe Disease: A Modeling Study. J Pers Med. 2021;11(5)

(6) Alter CL, Detampel P, Schefer RB, Lotter C, Hauswirth P, Puligilla RD, et al. High efficiency preparation of monodisperse plasma membrane derived extracellular vesicles for therapeutic applications. Communications biology. 2023;6(1):478

(7) Schürz M., Danmayr J., Jritsch M., Klinglmayr E., Melo-Benirschke H., Matea C.-T., et al. EVAnalyzer: High content imaging for rigorous characterisation of single extracellular vesicles using standard laboratory equipment and a new open-source ImageJ/Fiji plugin. J Extracell. Ves. 2022;12:12282. http://doi.org/10.1002/jev2.12282.

2. Acknowledgements

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