

Revision: list of modifications

We would like to thank the reviewers for their thoughtful comments and efforts towards improving our manuscript. In the following, we address the list of the modifications made in the manuscript.

Figures:

Figure captions were corrected and completed:

Figure 1: « resulting into » was replaced by « resulting in ».

Figure 1: « that vibrates at 128 000 times per second » was removed.

Figure 2: « This Figure illustrates the reproducibility of the QCM response for independent exposures under the same conditions. 61 µL of whole porcine surfactant solution at 40 mg/mL was nebulized, and the VITROCELLR Cloud temperature was maintained at 37°C. » was added.

Figure 2: « independent » was added.

Figure 4: « TiO₂ NP are nebulized over the wells of the VITROCELL Cloud 6 System containing the NR8383 cells. » was replaced by « For each 4 biological replicate (BR), NR8383 were seeded in 5 wells, in Transwell® inserts. The next day, for each BR, the inserts containing the cells were exposed, by nebulization, back-to-back to 61 µL of whole porcine surfactant at 40 mg/mL, and to 57µL of TiO₂ NP at 30 mg/mL. After exposures, the Transwell® inserts containing the cells were kept at 37°C during 4 h, and the 5 wells (representing technical replicates, TR) of each BR were pooled for RNA extractions.».

Figure 5: « TiO₂ NP are nebulized over the wells of the VITROCELL Cloud 6 System containing the NR8383 cells. » « For each 5 biological replicate (BR), NR8383 were seeded in 4 wells, in Transwell® inserts, distributed in 4 6 well plates. The next day, for all 6 well plates, the inserts containing the cells were exposed (representing one technical replicate (TR) of each BR), by nebulization, first to 61 µL of whole porcine surfactant at 40 mg/mL, and then to 57µL of TiO₂ NP at 30 mg/mL. After exposures, the Transwell® inserts containing the cells were kept at 37°C during 4 h, and the 4 wells (TR) of each BR were pooled for RNA extractions.».

Figure 6: « (57µL of TiO₂ NP at 30 mg/mL) » and « (114µL of TiO₂ NP at 15 mg/mL) » were added.

Abstract:

Line 1: « towards stepping » was removed.

Lines 4-15: « The commercially available VITROCELL® Cloud System has been applied for the delivery of aerosolized substances to adherent cells under ALI conditions, but not yet to semi-adherent cells and surfactant. In this work, semi-adherent rat alveolar macrophages NR8383 grown at the ALI were coated with lung surfactant through nebulization using the VITROCELLR Cloud 6 System before being exposed to TiO₂ NM105 nanoparticles (NPs). The nebulization of surfactant forms a homogeneous layer over all the cells, reproducing alveolar physiological conditions. After NP exposures, RNA was extracted and its quantity and quality were measured. The results highlighted the need for adaptation of the standard operating procedure, especially concerning physiologic surfactant conditions as well as reproducibility and repeatability of NP mass deposition and RNA quality and quantity. This led us to adapt the standard operating protocol of the VITROCELL® Cloud System and to present our recommendations for in vitro ALI NP aerosolization of semi-adherent cells pre-coated with lung surfactant » was replaced by « While the commercially available VITROCELL® Cloud System has been applied for the delivery of aerosolized substances to adherent cells under ALI conditions, it has not yet been tested on lung surfactant and semi-adherent cells such as alveolar macrophages, which are playing a pivotal role in the nanoparticle-induced immune response. Objectives : In this work, we developed a comprehensive methodology for coating semi-adherent lung cells cultured at the ALI with aerosolized surfactant and subsequent dose-controlled exposure to nanoparticles (NPs). This protocol is optimized for subsequent transcriptomic studies. Methods: Semi-adherent rat alveolar macrophages NR8383 were grown at the ALI and coated with lung surfactant through nebulization using the VITROCELLR Cloud 6 System before being exposed to TiO₂ NM105 NPs. After NP exposures, RNA was extracted and its quantity and quality were measured. Results: The VITROCELL® Cloud system allowed for uniform and ultrathin coating of cells with aerosolized surfactant mimicking physiological conditions in the lung. While nebulization of 57 µL of 30 mg/mL TiO₂ and 114 µL of 15 mg/mL TiO₂ nanoparticles yielded identical cell delivered dose, the reproducibility of dose as well as the quality of RNA extracted were better for 114 µL.» (new lines: 4-17).

Introduction:

Lines 21-22: « nanomaterial containing consumer products » was replaced by « consumer products containing nanomaterials » (new lines: 23-24).

Lines 23-26: « Predictably, this list still grows over time since 2005 [3–5]. The growing presence of these nanoparticles, in all areas of production, leads to great concern regarding human exposure and, consequently, their effect on human health. » was replaced by « Predictably, this list has been growing since 2005, in all areas of production, leading to serious concerns about the effects of their exposure on human health. » (new lines: 25-27).

Line 27: « Moreover, the emergence of nanotechnologies has led to the development of new research fields such as nanomedicine, which focuses on the synthesis and engineering of nanomaterials for drugs delivery [4], which has led to novel treatment [5–8] and diagnostic options [9,10] and combinations thereof [11,12]. Consequently, the study of both adverse and therapeutic health effects of nanoparticles and new nanomaterials is a crucial issue for the development of safe nano-enabled products such as consumer products as well as medical products.» was added (new lines: 27-33).

Line 28: « disease » was replaced by « diseases » (new line: 34)

Line 33: « their » was added (new line: 39).

Line 36: « In the environment, » was added (new line: 42).

Line 38: « Indeed, » was added (new line: 43).

Line 40: « NP deposition in the respiratory tract follows specific mechanisms such as impaction, interception, sedimentation, or diffusion in the nasopharyngeal, tracheobronchial, and pulmonary regions [12,13]. » was removed.

Line 53: « Traditionally, in vitro nanotoxicology generally investigates suspension of non or poorly-soluble particles by cell culture media and incubating them with the cells » was replaced by « Traditional in vitro systems for nanotoxicology are based on the dissolution of non- or poorly-soluble particles directly in the cell culture medium, where cells are subsequently cultured. » (new lines: 57-59).

Line 55: « submerged exposure method » was replaced by « "submerged exposure method" » (new lines: 59).

Line 55 : « real-world » was replaced by « actual » (new line: 60).

Line 62: « , which differs among them in the method used to aerosolize nanoparticles » was added (new lines: 67-68).

Line 66: « particle deposition » was replaced by « the deposition of particles » (new line: 72).

Line 75: « for toxicity testing » was replaced by « for testing the toxicity » (new line: 81).

Line 80: « please refer to » was replaced by « , interested readers can refer to » (new line: 86).

Lines 82-83: « The detection and quantification of the dose of NP delivered to the exposure site is an experimental limitation of many of these systems. » was replaced by « The main limitation of most of the aforementioned systems is the detection and quantification of the dose of NP delivered to the site of exposure. » (new lines: 88-89).

Line 83: « it is » was replace by « it represents » (new line: 88).

Lines 89-94: « Quartz crystal microbalances (QCM) have been suggested for real-time measurement of the cell delivered NP dose, but its limited stability over extended periods of time have made them particularly useful for high dose rate applications (i.e. exposure times between a few minutes to hours) such as the Vitrocell®Cloud System (commercial version of ALICE Cloud system). » was replaced by « QCM are piezoelectric biosensors that detect resonance frequency variation of quartz crystal associated with mass change on their surface. During the last decade, numerous studies have reported new possible applications of QCM in biological and medical sciences including immunosensors, biosensors, microbial detectors and innovations [40–47]. Quartz crystal microbalances (QCM) have been also described as a precise device for real-time measurement of the dose-delivered in

nanotoxicology studies [26,36,48]. For these reasons, a QCM has been included in the VITROCELLR Cloud System (commercial version of ALICE Cloud system) in order to control the real-time dose-delivered during the Cloud exposure.» (new lines: 95-103).

Line 97: « like agglomeration, charge state, and type of protein corona » was replaced by « such as their agglomeration, their charge state and the type of protein corona » (new line: 106).

Line 103: « constitution » was replaced by « composition » (new line: 111).

Line 104: the space before the point was removed.

Lines 126-132: « Here, we present a methodology for pre-coating semi-adherent cells cultured at ALI with aerosolized surfactant immediately before NP exposure through the VITROCELL® Cloud 6 System and subsequent extraction of their RNA. This optimization was done to provide high quality and sufficiently efficient RNA extraction as the first step of transcriptomic analysis of cells exposed to NP in the VITROCELL® Cloud System (article previously published, Leroux M.M. et al., 2020 [42,43]), which provides important insight into the toxicological profile of NPs.» was replaced by « To sum up, in vitro studies based on ALI exposure are more and more used in toxicology studies. But researchers using such a strategy have to face some issues including 1) using a very low concentration of NP 2) applying a homogeneous layer of surfactant on cells 3) using semi-adherent cells in ALI, and 4) retrieving RNA in sufficient quantity and quality to carry out transcriptomic studies. Thus, here, we present an in-depth detailed protocol answering to these points, in which each steps are validated, after the optimization of the different parameters. For a better understanding, all experimental results are featured in the present paper. It could be of help for researchers using such a device.» (new lines: 135-143).

2.1. Chemicals and reagents

Lines 136-138: « Penicillin-streptomycin, Amphotericin B, Phosphate Buffered Saline (PBS), and Fetal Bovine Serum » was replaced by « penicillin-streptomycin (PS), amphotericin B, phosphate buffer saline (PBS), and fetal bovine serum (FBS) » (new lines: 147-148).

2.2 Cell culture

Line 148: « For VITROCELL® exposures, » was added (new line: 159).

Line 149: « Cell culture was done in » was replaced by « Cells were cultured in » (new line: 161).

Line 154: « in 1 mL of supplemented DMEM medium in the apical side of the insert, while 2 mL of supplemented DMEM medium were placed in the basolateral side of the inserts » was replaced by « in 1 mL of supplemented DMEM medium in the apical side of the insert and 2 mL in the basolateral side, » (new lines: 166-167).

Line 158 : « For fluorescence microscopy, a LA-4 murine lung epithelial cell line was cultured according to ATCC guidelines: cells were seeded at 30,000 cells/cm² in T75 flasks and split when they reached a 80% confluency. They were cultured in 10 mL of full cell medium (a mixture of F-12K medium (Gibco), 15% FBS (Fetal bovine serum, ATCC), 1% P/S (penicillin-streptomycin, Sigma), 1% NEAA (non-essential amino acids, Gibco)) in an incubator at 37°C with saturated humidity and 5% CO₂. Then, cells were seeded and observed in 35 mm μ -dishes with #1.5H glass bottom (Ibidi), cells and media covered only 1/3 of the μ -dish surface (3.5 cm²) due to the geometry of the μ -dish.» was added (new lines: 169-176).

2.3 VITROCELL® Cloud 6 System

Line 168: the space before the point was removed: « 10 ng/cm². » (new line: 185).

Line 171: « The QCMs used in the VITROCELLR Cloud systems was well described previously in Ding et al. 2020 [48]. Briefly, the QCM incorporated in the VITROCELL® Cloud 6 system has an eigenfrequency of 5 MHz, a resistance of 10 Ohm and an aerosol exposed area of 4 cm², which is close to the cell covered area in 6-well Transwell® inserts (4.2 cm²).» was added (new lines: 189-193).

2.4. Fluorescence and microscopy for homogenous deposition assessment

Line 177 : « 2.4. Fluorescence and microscopy for homogenous deposition assessment » was replaced by « 2.4. Fluorescent microscopy for the assessment of the homogeneous deposition of surfactant » (new line: 199)

Lines 178- 185: « For fluorescence microscopy, a monoculture of LA-4 murine lung epithelial cells was cultured according to ATCC guidelines: cells were seeded at 30,000 cells/cm² in T75 flasks and split when they were 80%

confluent. They were cultured in 10 mL of full cell medium (a mixture of F-12K medium (Gibco), 15% FBS (Fetal bovine serum, ATCC), 1% P/S (Penicillin-Streptomycin, Sigma), 1% NEAA (non-essential amino acids, Gibco)) in an incubator at 37°C with saturated humidity and 5% CO₂. Cells were observed in 35 mm μ -dishes with #1.5H glass bottom (Ibidi). » was suppressed (modified and moved to the section [2.2. Cell culture]).

Lines 185-193 : « In all of the surfactant nebulization or pipetting experiments (Figure 3), the cell media was first completely removed. In some cases, the surfactant was administered immediately afterwards (leaving a several 10 μ m of medium on top), whereas in others the medium was left to evaporate for a few minutes before administering the surfactant (referred to as dry cells, leaving only 10 nm – 100 nm layer of media on the cells). Surfactant was then either nebulized (6 μ L, 40 mg/mL, corresponding to 10 monolayers of surfactant on the cells, the deposition efficiency being 15%) or added by a pipette to the middle of the μ -dish (0.72 μ L, 10 mg/mL, corresponding to 10 monolayers if spread out over the whole μ -dish). » was replaced by « In order to compare the different methods for the deposition of surfactant on the cells, two different methods were used: nebulization or pipetting (Figure 3). In both cases the cell medium was completely removed before the process. However, in some cases, the surfactant was administered immediately afterwards (leaving around 10 μ m of medium on top), whereas in others the medium was left to evaporate for a few minutes before administering the surfactant (referred to as dry cells, leaving only 10 nm – 100 nm layer of media on the cells). For nebulization, 6 μ L (240 μ g) of the surfactant were deposited to the pre-wetted nebulizer and were nebulized onto the entire μ -dish (surface : 3.5 cm²). Of these 240 μ g, only c.a 7% reached the bottom surface, and c.a 2.5% (6 μ g) were deposited on the cells (which 210 are seeded only on the inner 3.5 cm² of the dish due to the geometry of the dish), corresponding to approximately 10 monolayers on the cells. When pipetting the surfactant was added by a pipette to the middle of the μ -dish (0.72 μ L, 10 mg/mL), onto the medium on cells, corresponding to 10 monolayers if spread out over the whole medium-covered area of the μ -dish (3.5 cm²).» (new lines: 200-214).

Lines 200-207: « Delivery was performed using a pipette or a pre-wetted Aeroneb® Lab Nebulizer Unit, Standard VMD (Aerogen Pro Standard VDM, Aerogen Inc., Galway, Ireland) with an estimated rate of nebulization 5 μ L/s. A tightly sealed 7 cm high cylindrical chamber with a diameter of 3 cm was adapted for live nebulization on the microscope onto a single μ -dish (see Supp Data 1A-C). We estimated that 15% of initially nebulized material reached the bottom of the μ -dish (see Supp Data 1D). The cells and surfactant were kept at 37°C at the time of surfactant delivery to assure the surfactant was as fluid as under physiological conditions when deposited on the apical surface of the cells. » was replaced by « Delivery was performed using a pipette or a pre-wetted Aeroneb® Lab Nebulizer Unit, Standard VMD (Aerogen Pro Standard VDM, Aerogen Inc., Galway, Ireland) in a tube-like setup (with the surface of the bottom being 9.6 cm², see Supp Data 1A-C), with an estimated rate of nebulization 5 μ L/s. A tightly sealed 7 cm high cylindrical chamber with a diameter of 3 cm was adapted for live nebulization on the microscope onto a single μ -dish (see Supp Data 1A-C). We estimated that 7% of initially nebulized material reached the bottom of the μ -dish (see Supp Data 1D). The cells and surfactant were kept at 37°C at the time of surfactant delivery to assure the surfactant was as fluid as under physiological conditions when deposited on the apical surface of the cells. » (new lines: 221-229).

Line 246-247 : « , then placing the inserts back into the standard multiwell plates and putting them on hold in the incubator at 37°C 5% CO₂ until the second nebulization. » was replaced by « . The inserts were then placed back into the standard multiwell plates and were incubated at 37°C, 5% CO₂, until a second nebulization » (new lines: 268-269).

Line 286 : « inserts apical and basolateral compartments were emptied. » was replaced by « the culture media was removed from the basolateral and apical compartments of the inserts. » (new line: 307).

Line 295 : « the » and « of » were added (new line: 316).

2.6. NP exposure protocol

Line 275: « resulting into » was replaced by « resulting in » (new line: 296).

3.1.1. Reproducible deposition assessment by QCM measurements

Line 309: « as seen by the rapid response of the QCM. » was removed.

Line 319: « (which, as a reminder, ranges between 0.1 and 0.9 μ m) » was added (new line: 340).

3.1.2 Homogeneous distribution assessment by fluorescence microscopy

Line 333: « The deposition of surfactant on the cells does not depend on the cell type. Therefore, the dosimetry data reported here for LA-4 cells also apply to any other cell type, including the NR8383 cells. » was added (new line: 354).

Line 335: « , fluorescence » was replaced by « . Fluorescence » (new line: 358).

3.2.2. Current improved protocol for NP nebulization in VITROCELL R Cloud 6 System

Lines 372-373: « Cells were then kept in the incubator for 4 h and then TR of each BR were pooled to proceed to the extraction of their RNA. » was replaced by « cells were then kept in the incubator for 4 h and then TR of each BR were pooled to proceed to the extraction of their RNA. » (new lines: 395-396).

4. Discussion

Lines 510-512: « Therefore, only after the NP cloud has settled onto the quartz and the top part of the exposure chamber has been removed, which allows for rapid drying of the NP layer on the QCM. » was replaced by « Therefore, directly after the NPs has settled onto the QCM, the top part of the exposure chamber is removed, which allows for rapid drying of the NP layer on the QCM. » (new lines: 533-535).

5. Conclusions

Lines 560-568: « The total RNA extracted from this VITROCELLR Cloud exposition was used to perform a complete transcriptomic study [43] including a comparison of in vivo and air-liquid interface and submerged in vitro results for TiO₂ NM105 NP exposure [42]. Owing to the specific objectives of this study, the recommended experimental procedure for operating the VITROCELLR Cloud 6 described suggests using lower nebulization volumes for the surfactant (61 µL) and the TiO₂ NP suspension (114 µL) as compared to the manufacturer-recommended 200 µL to keep the cell-deposited liquid layer as low as possible. However, the total delivered amount of liquid of 175 µL is relatively close to the 200 µL recommended by the manufacturer. To conclude, » was replaced by « The goal of this work was to refine an ALI pre-established protocol. As far we know, such a protocol has not been previously published. Therefore, after addressing each crucial steps, (i.e.. the use of very low concentrations of NP, the deposition of a homogeneous layer of surfactant, the specific use of semi-adherent cells, and finally the enhancement of the extraction yield of RNA to carry out transcriptomic studies), we present here for the first time a robust almost ready-to-use protocol. This one will be helpful for nanotoxicologists interested in developing such methodologies. This protocol is not frozen, and researchers can feel free to suggest improvements. Finally, » (new lines: 583-591).

Lines 596-599: « Finally, considering the improvement of co-culture or 3D-cultures techniques, coupled with an innovative exposure device such as the VITROCELL® Cloud, it is reasonable to think that in the future this kind of methodology will contribute to reducing the use of animals in toxicology research. » was replaced by « After the optimization of this protocol, we were able to obtain a high quality and quantity of total RNA extraction from this VITROCELLR Cloud exposition and we performed a complete transcriptomic study [64] including a comparison of in vivo and air-liquid interface and submerged in vitro results for TiO₂ NM105 NP exposure [63]. The optimization of the existing protocols is essential when moving to new or different experimental conditions (e.g., in our case of semi-adherent cell cultures, surfactant and low doses of NPs). The method that we employed to optimize/refine the previously established protocol could efficiently be transposed to 3D-cultures [87-90] or culture of organoids [91-95], which represent today the in vitro models closest to physiological conditions, considering the interactions existing between the different cell types. Furthermore, the approach that we used could serve as a basis for further developments which, in the long term, could significantly reduce the need of animal studies for toxicology research. » (new lines: 619-631).

Acknowledgements:

Line 617: « and Poerio Aurelia (Institut Jean Lamour, Nancy, France) » was added (new line: 649).

In addition:

All « vitrocell » were uniformized by « VITROCELL® »

All « homogenous » were replaced by « homogeneous »

All « i.e » were replaced by « i.e. »

All « polled » were replaced by « pooled »

Typos were corrected

Article

Aerosol-cell exposure system applied to semi-adherent cells for aerosolization of lung surfactant and nanoparticles followed by high quality RNA extraction.

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Abstract: Nanoparticle toxicity assessments have moved towards stepping closer to physiological conditions while trying to avoid the use of animal models. An example of new *in vitro* exposure techniques developed is the exposure of cells grown at the air-liquid interface (ALI), particularly in the case of respiratory airways. The commercially available VITROCELL® Cloud System has been applied for aerosolized substance delivery to adherent cells under ALI conditions, but not yet to semi-adherent cells and surfactant. In this work, semi-adherent rat alveolar macrophages NR8383 grown at the ALI were coated with lung surfactant through nebulization using the VITROCELL® Cloud System before being exposed to TiO₂ NM105 nanoparticles (NPs). The nebulization of surfactant forms a homogeneous layer over all the cells, reproducing alveolar physiological conditions. After NP exposures, RNA was extracted and its quantity and quality were measured. The results highlighted the need for adaptation of the standard operating procedure, especially concerning physiologic surfactant conditions as well as reproducibility and repeatability of NP mass deposition and RNA quality and quantity. This led us to adapt the standard operating protocol of the VITROCELL® Cloud System and to present our recommendations for *in vitro* ALI NP aerosolization of semi-adherent cells pre-coated with lung surfactant.

Keywords: air-liquid interface; nanoparticles; VITROCELL® Cloud System; RNA extraction; macrophages; surfactant; NR8383

1. Introduction

During the last years, the development of nanotechnologies and their many possible applications have led to their incorporation into consumer products. Indeed, in 2013, the nanotechnology Consumer Products Inventory (CPI) listed 1814 nanomaterial-containing consumer products from 622 companies in 32 countries, including health and fitness products, medicine, cosmetics, food, and electronics. Predictably, this list still grows over time since 2005 [1–3]. The growing presence of these nanoparticles, in all areas of production, leads to great concern regarding human exposure and, consequently, their health effects.

Generally, nanoparticle (NP) exposure is associated with an increased risk of cardiorespiratory morbidity and mortality, cancers, asthma, pulmonary disease, fibrosis, or allergies. At the cellular scale, NPs can induce oxidative stress, inflammation, genotoxicity, membrane alterations, mitochondrial dysfunction, cell cycle arrest, and cell death [4–6].

Numerous studies have shown that the health effects of NPs do not depend only on their chemical and physical properties such as size, shape, composition, surface area, or solubility, but also on the conditions of exposure including the type of exposed populations, as well as duration, doses, repetitions, and routes of exposure [7].

The main route of exposure to nanoparticles is the respiratory route, although effects on the skin, eyes, or gastrointestinal tract need to be taken into consideration. Because of their small size and chemical properties, NPs can reach the alveoli and cross the blood-air barrier thus infiltrating the bloodstream and other organs, for example the gastrointestinal tract, the heart, or the brain [8–11]. ~~NP deposition in the respiratory tract follows specific mechanisms such as impaction, interception, sedimentation, or diffusion in the nasopharyngeal, tracheobronchial, and pulmonary regions [12,13].~~ Over the last two decades, several attempts have been made toward reducing and replacing *in vivo* experimentation with novel *in vitro* models trying to mimic physiological conditions. These models tend to move closer to organ structures, and in the case of the respiratory airways, to bronchial/alveoli architecture. Among these models, lung cells cultured at the air-liquid interface (ALI) appear to be a promising technique as it offers many possibilities for mimicking parts of the respiratory tract. In the ALI culture, cells are grown on the microporous membrane of cell culture inserts which are often placed on multi-well plates. The basolateral compartment is filled with medium and once the cells adhere to the microporous membrane, the medium contained in the apical side of the insert is removed, establishing ALI conditions [14].

~~Traditionally, *in vitro* nanotoxicology generally investigates suspension of non- or poorly soluble particles by cell culture media and incubating them with the cells [15,16].~~ This so-called submerged exposure method is not representative of real-world exposure to airborne particles regarding the behavior of particles in the air [13] and implies numerous disadvantages including poor control of cell-delivered dose and interactions of the NPs with the medium [17,18]. That is why scientists and companies have developed new methods to expose cells to NPs through a gaseous phase including roller bottles, inverted cell monolayer, and smoking machines [19,20].

For inhalation toxicology, the most pertinent methodologies include ALI cell cultures and aerosol-cell exposure devices. For example, the Versatile Aerosol Concentration Enrichment System (VACES) uses a condensation process to aerosolize NPs [21]. The Electrostatic Aerosol In Vitro Exposure System (EAVES) guides aerosol particles horizontally across cell culture inserts (where cells are grown) and uses electrostatically enhanced diffusion and sedimentation for ~~particle deposition~~ on the cells [22]. The CULTEX® stagnation point flow system deposits aerosolized particles *via* diffusion and sedimentation directly onto the cells (Cultex laboratories GmbH, Hannover, Germany) [23,24], and the Nano Aerosol Chamber In Vitro Toxicology (NACIVT) [25] uses a stagnation point flow system and an electrostatic precipitation system to deposit aerosolized NPs onto the cells. A recently developed device by Inhalation Sciences (Huddinge, Sweden), called XposeALI, uses a high-pressure aerosol generator to aerosolize dry NP powder [26] and utilizes diffusion and sedimentation for aerosol-cell deposition in a stagnation point flow setting. For continuous flow exposure, the VITROCELL® Automated Exposure Station was described for ~~toxicity testing~~ of combustion aerosols at the air-liquid interface [27]. Moreover, nebulization of nanoparticle suspensions into an exposure chamber with subsequent cloud settling has been introduced as ALICE and ALICE Cloud technology for dose-controlled and efficient NP-cell delivery at high dose rates by Lenz *et al.*, 2009, and Lenz *et al.*, 2014, respectively [28,29]. For more details on the various types of ALI exposure systems please refer to Ehrmann *et al.*, 2020 and Paur *et al.*, 2011 [30,31].

~~The detection and quantification of the dose of NP delivered to the exposure site is an experimental limitation of many of these systems.~~ However, it is one of the most important criteria for the assessment of particle-related risks [7]. Indeed, most studies provide precise data on the concentration of exposure to particles, i.e. the mass of

particles per volume of air ($\mu\text{g}/\text{m}^3$) or - for cell assays immersed *in vitro* - per volume of cell culture medium ($\mu\text{g}/\text{mL}$), but the most relevant measure - the dose delivered to the site of exposure, e.g. lung epithelium (*in vivo*) or lung cell culture (*in vitro*) - is often overlooked. Quartz crystal microbalances (QCM) have been suggested for real-time measurement of the cell delivered NP dose, but its limited stability over extended periods of time have made them particularly useful for high dose rate applications (i.e. exposure times between a few minutes to hours) such as the Vitrocell® Cloud System (commercial version of ALICE Cloud system) with a typical exposure time of a few minutes [18].

Finally, the alveolar lining fluid, which contains innate surfactant and covers the alveolar epithelium, can interfere with nanoparticles and modify their physico-chemical properties like agglomeration, charge state, and type of protein corona [32–34]. The alveolar surfactant, which is produced by alveolar type II epithelial cells [35–37], is an aqueous mixture of lipids and proteins, lining the surface of pulmonary epithelial cells in the alveolar region, whose function is to maintain sufficient tension and protect the alveolar sacs from collapsing at the end of each exhalation phase. The surfactant is composed of 92% lipid mass, mostly phospholipids, and 8% protein mass. The constitution of the surfactant and its amphiphilic bilayer structure makes it possible to modify the ability of external compounds to reach the pulmonary epithelium [38–40]. Alveolar macrophages, which are covered with alveolar surfactant and capable of neutralizing NPs in the lung by phagocytic uptake, are often considered as the most important defense mechanism against NPs deposited in the alveolar region. Recent *in vitro* experiments have shown that surfactant decreases the cellular uptake of silica nanoparticles by up to two orders of magnitude [41]. Since the alveolar lining fluid and the alveolar macrophages are the first and second line of defense against NPs deposited in the alveolar region, a semi-adherent alveolar macrophage cell line pre-coated with a thin film of alveolar surfactant fluid before the exposure to NPs is used in our study.

This study reports the optimization of an innovative air-liquid interface system using a cloud settling exposure system by VITROCELL Systems for pre-coating of the cells with surfactant lung lining fluid and subsequent NP exposure, combined with an accurate micrometric balance to measure cell exposure in real-time. The VITROCELL® Cloud System (VITROCELL® Systems GmbH, Waldkirch, Germany) [29] is a refined version of the ALICE system, which has been developed especially for aerosolized NPs exposure [28]. This VITROCELL® Cloud system involves a vibrating membrane nebulizer, which uses a vibrating, piezoelectrically controlled, perforated membrane to induce acoustic pressure waves that squeeze the NP suspension through the pores of the membrane. This leads to the production of a dense, uniform cloud of droplets that deposits uniformly onto the cells through a process called cloud settling, resulting in an efficient and controlled dosimetry which is monitored in real-time with a highly sensitive quartz crystal microbalance.

Here, we present a methodology for pre-coating semi-adherent cells cultured at ALI with aerosolized surfactant immediately before NP exposure through the VITROCELL® Cloud 6 System and subsequent extraction of their RNA. This optimization was done to provide high quality and sufficiently efficient RNA extraction as the first step of transcriptomic analysis of cells exposed to NP in the VITROCELL® Cloud System (article previously published, Leroux M.M. *et al.*, 2020 [42,43]), which provides important insight into the toxicological profile of NPs.

2. Materials and Methods

2.1. Chemicals and reagents

Chemicals and reagents were obtained from the following sources: Duplecco's Modified Eagle Medium (DMEM), L-glutamine, Penicillin-streptomycin, Amphotericin B, Phosphate Buffered Saline (PBS), and Fetal Bovine Serum from Sigma-Aldrich (Saint-Louis, MO, USA); Transwell® polyester culture membrane from Corning (Wiesbaden,

Germany). Whole porcine surfactant was a generous gift from J Perez-Gil (Faculty of Biology, Complutense University, Madrid, Spain), which includes all of the water-soluble and non-water-soluble surfactant proteins. Propan-2-ol, chloroform, absolute ethanol were bought from Carlo Erba Reagents (Val-de-Reuil, France). RNA-Solv reagent was purchased from Omega Bio-tek (Norcross, GA, USA). TiO₂ NM105 NPs (JRC reference materials, primary NP diameter 21.5 ± 7.2 nm; secondary NP diameter (agglomerate, DLS measurement) 170 ± 0.7 , BET surface area $51 \text{ m}^2/\text{g}$, Zeta potential 11.1 ± 0.7 mV) were provided by JRC.

2.2. Cell culture

NR8383 cells (CRL2192TM) are a rat alveolar macrophage cell line purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). ~~Cell culture was done in~~ DMEM-high glucose medium supplemented with 15% FBS, 4 mM L-glutamine, 100 U/mL penicillin, 100 g/mL streptomycin, and 0.25 $\mu\text{g}/\text{mL}$ of amphotericin B at 37°C, in a 5% CO₂ atmosphere. Cells were grown in 75 cm² flasks (Sarstedt, Nümbrecht, Germany) and then passaged and seeded on 0.4 μm pore size microporous Transwell® inserts at a density of 1.5×10^5 cells/mL or 3×10^5 cells/mL ~~in 1 mL of supplemented DMEM medium in the apical side of the insert, while 2 mL of supplemented DMEM medium were placed in the basolateral side of the inserts,~~ and kept at the incubator at 37°C, 5% CO₂, for 24h. Before exposure, the apical medium was removed to establish ALI.

2.3. VITROCELL® Cloud 6 System

The VITROCELL® Cloud 6 System (VITROCELL® Systems, Waldkirch, Germany) is a commercially available device aimed at exposing cells at the air-liquid interface to inhaled aerosolized toxins through cloud settling, which mimics realistic inhalation exposure scenarios of alveolar macrophages to nanoparticles in physiological conditions. The device is schematically depicted in Figure 1. The stainless-steel base module of this device comprises 5 electrically heated wells in which 13 mL of medium and the inserts are placed. The temperature in the wells is maintained at 37 °C. A sixth well contains a quartz crystal microbalance (QCM balance) for cell-delivered dose measurements with a resolution of $10 \text{ ng}/\text{cm}^2$, at a sampling rate of 1 Hz and a manufacturer-specified zero noise level of $20 \text{ ng}/\text{cm}^2$ (VITROCELL® Systems, Waldkirch, Germany) that allows to assess not only cell-delivered dose but also reproducibility and repeatability of the exposures. The upper part of the device is a 2250 cm³ polycarbonate removable exposure chamber pierced on the top with a hole to place the nebulizer. The transwell inserts (Corning® Transwell®-Clear 6-well Inserts, 10 μm thick Polyester (PET) membrane, product number 3450) used for culturing cells under ALI conditions are composed of a microporous (0.4 μm pore size) polyethylene terephthalate (PET) membrane of a surface of 4.67 cm^2 .

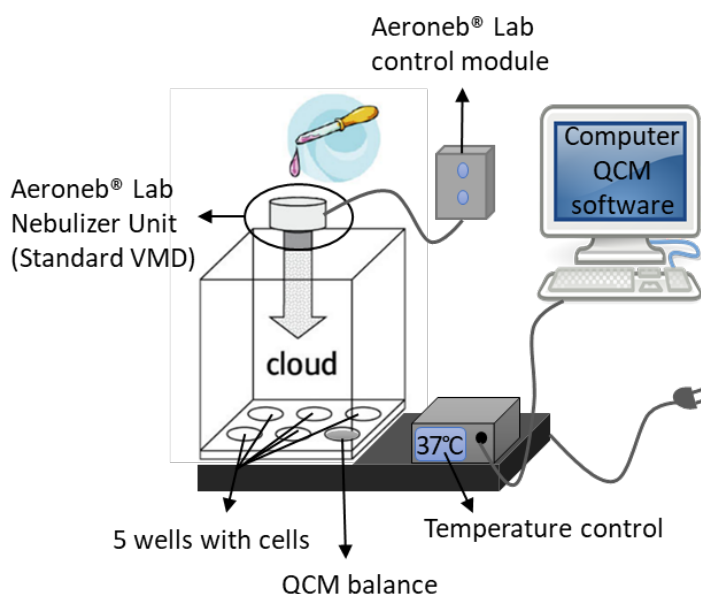


Figure 1. Schematic of the VITROCELL® 6 Cloud device. The stainless-steel base module of this device comprises 5 electrically heated wells in which 13 mL of medium and the inserts are placed. The temperature in the wells is maintained at 37 °C. A sixth well contains a quartz crystal microbalance (QCM balance) for cell-delivered dose measurements with a resolution of 10 ng/cm², at a sampling rate of 1 Hz and a manufacturer-specified zero noise level of 20 ng/cm² (VITROCELL® Systems, Waldkirch, Germany), measurements are analysed and visualized using the computer QCM software. The upper part of the device is a 2250 cm³ polycarbonate removable exposure chamber pierced on the top with a hole to place the nebulizer. The nebulizer system is composed of the Aeroneb® Lab Nebulizer Unit, Standard VMD and a Aeroneb® Lab Control Module (Aerogen Inc., Galway, Ireland). The nebulization relies on a perforated piezoelectrically controlled vibrating mesh to generate acoustic pressure waves releasing liquid droplets at a high frequency (128 kHz). This nebulizer is constituted of a palladium mesh pierced with *ca.* 1 000 holes, that vibrates at 128 000 times per second, releasing between 0.3 and 0.8 mL of liquid per minute through the holes, resulting into a stream of droplets precisely controlled for size by the diameter of the apertures (4 – 6 µm droplet diameter for the Standard VDM nebulizer).

2.4. Fluorescence and microscopy for homogenous deposition assessment

For fluorescence microscopy, a monoculture of LA 4 murine lung epithelial cells was cultured according to ATCC guidelines: cells were seeded at 30,000 cells/cm² in T75 flasks and split when they were 80% confluent. They were cultured in 10 mL of full cell medium (a mixture of F-12K medium (Gibco), 15% FBS (Fetal bovine serum, ATCC), 1% P/S (Penicillin-Streptomycin, Sigma), 1% NEAA (non-essential amino acids, Gibco)) in an incubator at 37°C with saturated humidity and 5% CO₂. Cells were observed in 35 mm µ dishes with #1.5H glass bottom (Ibidi).

In all of the surfactant nebulization or pipetting experiments (Figure 3), the cell media was first completely removed. In some cases, the surfactant was administered immediately afterwards (leaving a several 10 µm of medium on top), whereas in others the medium was left to evaporate for a few minutes before administering the surfactant (referred to as dry cells, leaving only 10 nm – 100 nm layer of media on the cells). Surfactant was then either nebulized (6 µL, 40 mg/mL, corresponding to 10 monolayers of surfactant on the cells, the deposition efficiency being 15%) or added by a pipette to the middle of the µ dish (0.72 µL, 10 mg/mL, corresponding to 10 monolayers if spread out over the whole µ dish).

The surfactant used was either whole native surfactant (extract of porcine BAL, contains all surfactant proteins (SP-A, SP-B, SP-C and SP-D), kindly provided by J. Perez-Gil) or Curosurf® (poractant alfa, the extract of porcine lung surfactant, consisting of

99% polar lipids and 1% SP-B and SP-C, by Chiesi). The surfactant in all experiments was labelled with STAR RED-DPPE (Abberior) using a Nlabel:Nlipid = 1:1000 labelling ratio and was dispersed in PBS to final concentration.

~~Delivery was performed using a pipette or a pre-wetted Aeroneb® Lab Nebulizer Unit, Standard VMD (Aerogen Pro Standard VDM, Aerogen Inc., Galway, Ireland) with an estimated rate of nebulization 5 μ l/s. A tightly sealed 7 cm high cylindrical chamber with a diameter of 3 cm was adapted for live nebulization on the microscope onto a single μ -dish (see Supp Data 1A-C). We estimated that 15% of initially nebulized material reached the bottom of the μ -dish (see Supp Data 1D). The cells and surfactant were kept at 37°C at the time of surfactant delivery to assure the surfactant was as fluid as under physiological conditions when deposited on the apical surface of the cells.~~

The TiO₂ nanotubes, characterized and fluorescently labelled as described in Kokot *et al.*, 2020 and Urbančič *et al.*, 2018 [44,45], were dispersed in deionized H₂O and concentrated to concentration of 33 mg/mL in a centrifuge at 21 000 rpm for 20 min (74000g RCF) (initial volume of 1 mL and concentration 1 mg/mL). Nanoparticles were nebulized either to a dry μ -dish for the panoramas or to cells with a previously nebulized layer of surfactant. The same nebulization setup as described above was used (3 μ l, 33 mg/mL, corresponding to a surface area dose of nanoparticles 1:1, i.e. 1 cm² of NP / per cm² cells).

Different Aeroneb® Lab Nebulizer Units were used for nebulizing lipids and nanoparticles and they were thoroughly cleaned in between exposures. The images were acquired with an Abberior Instruments STED microscope equipped with a 60x water immersion objective (Olympus) and the associated software Inspector (version 16.3.11462-metadata-win64). The fluorescence was excited with two pulsed lasers at 561 and 640 nm, whereas two avalanche photodiodes at 580–625 nm and 655–720 nm respectively, were used for fluorescence detection (filters by Semrock).

Panoramic images were combined from side-by-side recorded 0.7x0.7 mm large images with a 0.05 mm overlap and pixel size of 1 μ m. The images were acquired over more than 1 cm using an air 10x objective (Olympus). The illumination over such a large field of view is not completely homogenous, hence the darker edges on the images. Wolfram Mathematica was used to overlay and analyze the panoramic images. Power of excitation lasers was 5 μ W.

The time series in Figure 3 D was acquired using a pixel size of 100 nm, dwell-time 10 μ s, and 561 nm and 640 nm laser powers at around 5 μ W while simultaneously imaging using two-photon excitation with wavelength at 950 nm, dwell-time 400 μ s, and power at around 10 mW (the two-photon excitation and the entire time-lapse are shown in Supp Data). The surfactant structure following nebulization of 100 monolayers (2.4 mg of Curosurf) in Figure 3 E was acquired using two-photon excitation at 950 nm, pixel size of 250 nm, and dwell-time 8 ns.

2.5. Pre-coating of ALI cells with surfactant

The freeze-dried whole surfactant powder extract from pig lungs was dissolved in 100 mL ultrapure water at room temperature, yielding a concentration of 70 mg/mL, and sonicated 30 seconds at 37 °C in ultrasonic cleaner. It was then diluted in PBS to reach a working concentration of 40 mg/mL, sonicated 30 seconds at 37 °C in ultrasonic cleaner, and left at 37 °C in the incubator for 1h. After the removal of the apical medium on the Transwell® inserts, 61 μ L of the surfactant solution were nebulized with the VITROCELL® Cloud 6 System allowing for cloud settling time. In the initial protocol, the nebulization of surfactant was done just before the NP nebulization, while, for the current protocol, surfactant nebulization was done for all together (control and NP exposed), then placing the inserts back into the standard multiwell plates and putting them on hold in the incubator at 37°C 5% CO₂ until the second nebulization (NP exposure, or - for controls - water exposure) is performed with the VITROCELL®Cloud system.

2.6. NP exposure protocol

TiO₂ NM105 NPs were suspended in 5 mL of ultrapure water at a concentration of 30 mg/mL and sonicated for 10 minutes in an ultrasonic cleaner (VWR International, Radnor, PA, USA) just before exposure with the VITROCELL® Cloud 6 System. As described above, NR8383 cells were seeded on the apical side of Transwell® inserts at a concentration of 1.5×10^5 cells/mL (initial protocol) or 3×10^5 cells/mL (current protocol) in 1 mL of supplemented DMEM medium for 24 hours. In each well of the VITROCELL® Cloud 6 System, 13 mL of medium were added and inserts were placed over the wells to set up the air-liquid configuration, 61 μ L of surfactant were then nebulized on the inserts after removal of the apical medium. Subsequently, the cells were exposed to 57 μ L of aerosolized nanoparticle suspension or an equivalent volume of DMEM medium or ultrapure water (controls). After exposure, the cells were placed in the incubator for 4 h and then recovered to extract their RNA. In this study, the nebulizer is rinsed between each exposure by nebulizing 1 mL of ultrapure water (Ultrapure (Type1) Water Simplicity®, filter Millipak® 20 Millipore 0.22 μ m) followed by 1 mL of PBS 1X. The nebulizer was also sonicated from time to time if it was necessary to address the clogging of the mesh.

The nebulization of the nanoparticles and surfactant suspensions was performed with an Aeroneb® Lab Micropump Nebulizer (Aerogen Inc., Galway, Ireland). The nebulizer system is composed of the Aeroneb® Lab Nebulizer Unit, Standard VMD and an Aeroneb® Lab Control Module (Aerogen Inc., Galway, Ireland) (Figure 1). The nebulization relies on a perforated piezoelectrically controlled vibrating mesh to generate acoustic pressure waves releasing liquid droplets at a high frequency (128 kHz). This nebulizer can be used *in vitro*, *in vivo*, or in clinical settings [46,47] and is constituted of a palladium mesh pierced with *ca.* 1 000 holes, that vibrates at 128 000 times per second, releasing between 0.3 and 0.8 mL of liquid per minute through the holes, resulting into a stream of droplets precisely controlled for size by the diameter of the apertures (4 – 6 μ m droplet diameter for the Standard VMD nebulizer) [47]. For more information on the Aeroneb® nebulizer system please refer to the manufacturer website (Aerogen Technology).

2.7. RNA extraction

Here we present the first step of transcriptomic study [42,43] i.e. RNA extraction. Excellent quality and sufficient quantity of RNA is a prerequisite for transcriptomic assay. Indeed, the concentration of total RNA should be more than 40 ng/ μ L and the quality is defined by the ratios of optical densities (OD) 260/230 and 260/280 which must be between 1.8 and 2.2.

4 hours after exposures, ~~inserts apical and basolateral compartments were emptied.~~ Cells were lysed by flushing 1 mL of RNA-Solv reagent directly onto the inserts membrane and then delicately scrapped from the membrane with a pipet tip to detach every single cell. Every technical replicate (TR) originating from the same biological replicate (BR) were pooled. Samples were then stored at -80°C over-night. The next day, 200 μ L of chloroform per tube were added and samples were centrifuged at 12000 g for 15 minutes at 4°C. 175 μ L of propan-2-ol were added to 350 μ L of supernatant and centrifugation was again proceeded at 12000 g for 20 minutes. Pellets were then subjected to two successive ethanol washing steps and then dried at 60 °C for 10 minutes to remove excess ethanol. Finally, dried pellets were diluted in 25 μ L of RNase-free water. RNA quantity and purity were assessed using BioSpec-nano spectrophotometer (Shimadzu, Marne-la-Vallée, France)

3. Results

3.1. A uniform deposition of surfactant by aerosolization with the nebulizer

3.1.1. Reproducible deposition assessment by QCM measurements

The thickness of the alveolar lung lining fluid in rat lungs ranges between 0.1 μm and 0.9 μm . The alveolar lining layer appears continuous, submerging epithelial cell microvilli and intercellular junctional ridges, and serves to smoothen the alveolar air-liquid interface in inflated lungs [48]. The standard operating procedure of the VITROCELL® Cloud System provided by VITROCELL® Systems recommends nebulization of 200 μL of liquid, which results in *ca.* 5.6 μL of liquid aerosol deposited per 6-well (4.5 cm^2) transwell® insert (the values can vary depending on nebulizer performance). This corresponds to a *ca.* 12 μm thin liquid film, which evaporates within a few seconds from the QCM after opening the exposure chamber ~~as seen by the rapid response of the QCM~~. It has been shown that a thin aqueous layer like this has no adverse effects on cellular response [28]. To adapt the thickness of the *in vitro* surfactant coating to the *in vivo* conditions, we only nebulized 61 μL of surfactant solution (at 40 $\mu\text{g}/\mu\text{L}$) or 57 μL of nanoparticle suspension (in our initial protocol, against 114 μL of NP suspension in the current protocol). It reflects the minimum volume of nebulized liquid (*ca.* 60 μL) to still allow for relatively uniform cloud mixing in the VITROCELL® Cloud System, which is a prerequisite for reliable operation. As the surface of the VITROCELL® Cloud chamber is 143 cm^2 , the surfactant mass initially nebulized corresponds to 76.5 $\mu\text{g}/\text{insert}$ and 1.9 $\mu\text{L}/\text{insert}$ (17 $\mu\text{g}/\text{cm}^2$ and 0.42 $\mu\text{L}/\text{cm}^2$), resulting (theoretically) in a 4 μm aqueous layer. Although slightly higher than *in vivo* conditions, this is the least we can obtain within the limitation of nebulization of at least 60 μL for uniformity of aerosol deposition in the VITROCELL® Cloud 6 system. However, the QCM has not provided reliable measurements of deposited surfactant mass as the reported QCM signal showed a continuous growth rather than the typically observed peak value after a few minutes and subsequent adjustment to a constant asymptotic value. This could be related to the fact that alveolar surfactant contains a high concentration of surface-active proteins, which inhibits evaporation of the aqueous phase. Indeed, in the presence of water or any other liquid, the QCM signal is more representative of the viscosity of the liquid than the deposited mass [28,49]. Nevertheless, the QCM signal demonstrates the high degree of reproducibility of surfactant deposition on a qualitative level, but the reported mass dose does not reflect surfactant mass (Figure 2).

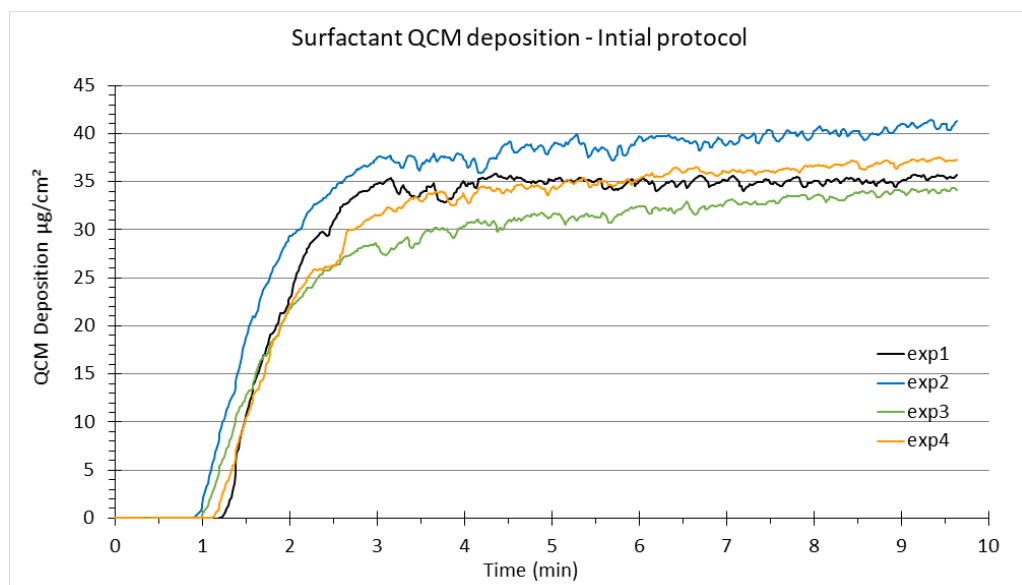


Figure 2. QCM curves showing the cloud deposition of surfactant (qualitatively) in the VITROCELL® Cloud System. The nebulization starts between 30 sec and 1 min after start of data acquisition. Depicted are the four exposures performed in the present study before NP nebulization.

3.1.2. Homogeneous distribution assessment by fluorescence microscopy

In parallel, the homogenous distribution of nebulized surfactant over the cells was assessed by fluorescence microscopy. Surfactant was labelled with STAR RED-DPPE fluorescent dye and nebulized or pipetted on a monoculture of LA-4 murine lung epithelial cells in ALI conditions. A fluorescence top-view panoramic scan was then used to observe the surfactant deposition (Figure 3). As confirmed using a fluorescence top-view panoramic scan over 1 cm (one half of the sample diameter), the nebulization of surfactant (Figure 3 A iii, B iii) covers the entire sample with a homogeneous surfactant layer. In contrast, an alternative method of surfactant application, *i.e.* pipetting the surfactant directly onto the cells at the ALI (Figure 3 A i, B i) or onto cells slightly covered with cell medium (Figure 3 A ii, B ii), does not provide a uniform surfactant deposition. Moreover, the pipetted surfactant does not spread over the whole sample, leaving the majority of the cells without surfactant. The fluorescence panoramic scan of nebulized nanomaterial confirms that nebulization also delivers the nanomaterial uniformly over the entire sample (Figure 3 C). As shown in Figure 3 D, nebulization of surfactant onto submerged cells forms a homogeneous layer of surfactant on the air-liquid interface, with the remainder of the surfactant forming structures in the underlying aqueous cell medium (Figure 3 E). These results confirm that, when subsequent nebulization of surfactant and nanoparticles is performed, the resulting sample is uniform both in terms of surfactant and the cell-delivered dose of nanomaterial (Figure 3 F). This is crucial for replicating the physiological conditions.

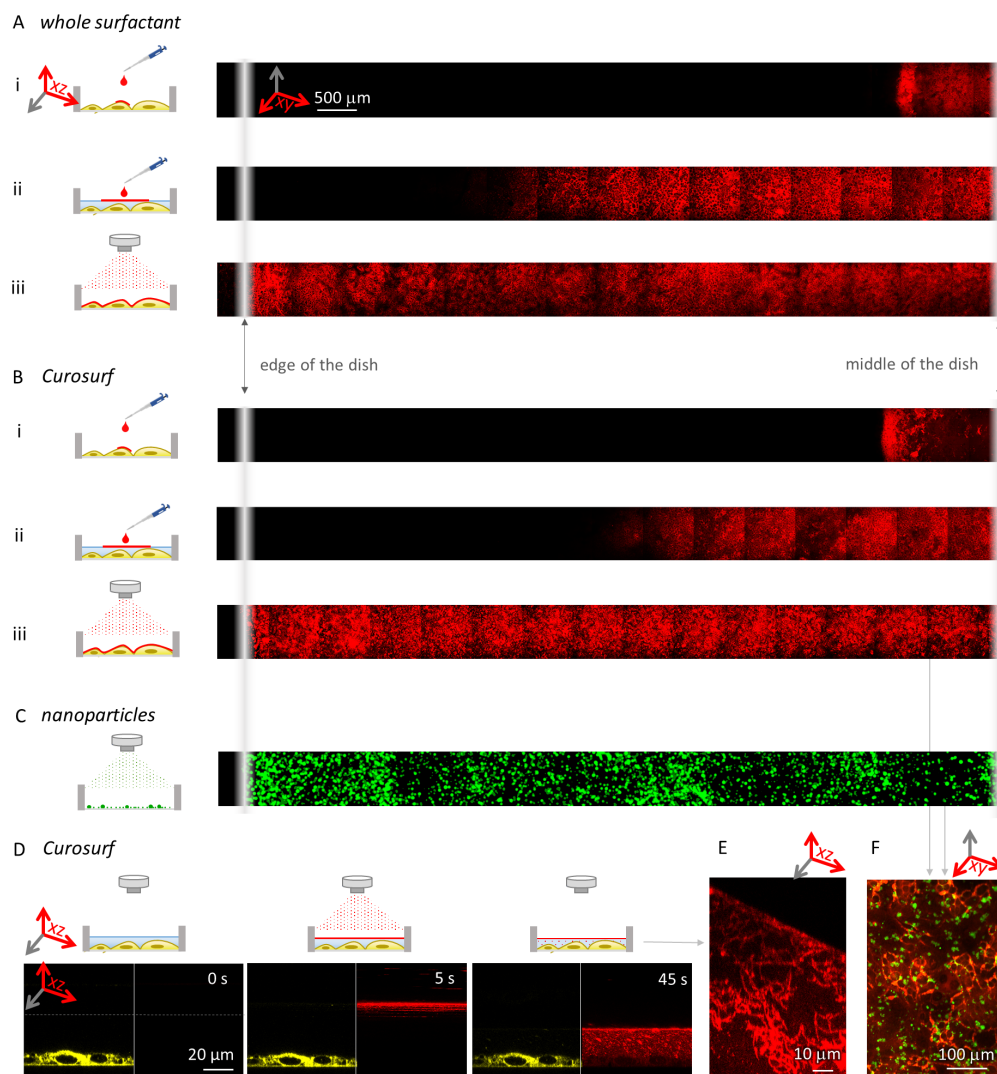


Figure 3. The deposition of nebulized and pipetted surfactant. 10 monolayers of fluorescently labelled **A)** the whole surfactant and **B)** Curosurf were administered onto cells using different methods. As shown in the top-view panoramas, in both cases **i)** surfactant, pipetted onto cells with removed medium (simulating ALI conditions), spreads only over a fraction of the sample, **ii)** surfactant pipetted onto cells slightly covered with cell medium spreads further, but still does not cover the entire sample, and **iii)** nebulized surfactant evenly covers the entire sample (fluorescence intensities are reported in Supp. Data 3). Note that the intensities are not comparable between measurements and the dark vertical lines are the consequence of uneven illumination. **C)** Nebulization of fluorescently labelled TiO_2 nanotubes (green) evenly deposits the material over the sample – shown here in a top-view panorama for nebulization directly onto a glass surface. Note that due to low signal, the image was filtered using a Gauss filter, and the intensity was scaled from 0 to 3 counts. **D)** A time series of nebulization of 10 monolayers of Curosurf (red, fluorescently labelled with STAR RED-DPPE) onto lung alveolar cells (green, labelled with CellMask Orange), (see Supp. Data 2 for complete time series). **E)** A side-view of the surfactant structure just below the surface after nebulizing 100 monolayers of surfactant (red) onto submerged cells. The surface of the medium is tilted due to capillary effects near the edge of the dish. **F)** A fluorescence top-view micrograph of 10 monolayers of nebulized surfactant (red) and subsequently nebulized 1:1 surface dose of nanomaterial (green) onto lung alveolar cells.

3.2. Improvement of the standard initial protocol

3.2.1. Standard initial protocol for NP nebulization in VITROCELL® Cloud 6 System

The initial exposure protocol that we used was set up as follows: 40 inserts were seeded with NR8383 cells out of four biological replicates (BR, 10 inserts per BR, 150 000 cells/insert) and incubated 24 h at 37 °C, 5% CO₂. After the removal of their supernatant and the exposure to surfactant, establishing ALI, half of the inserts (5 inserts for each BR, representing 5 technical replicates (TR) for each BR exposed) were exposed to ultrapure water as control and the other half to NM105 NP (Figure 4). Cells were then put back into the incubator for 4 h and their RNA was finally extracted. Running this very first protocol, we encountered a few issues and realized it had a few flaws that could be corrected to be more biologically rigorous and to optimize RNA yield and quality.

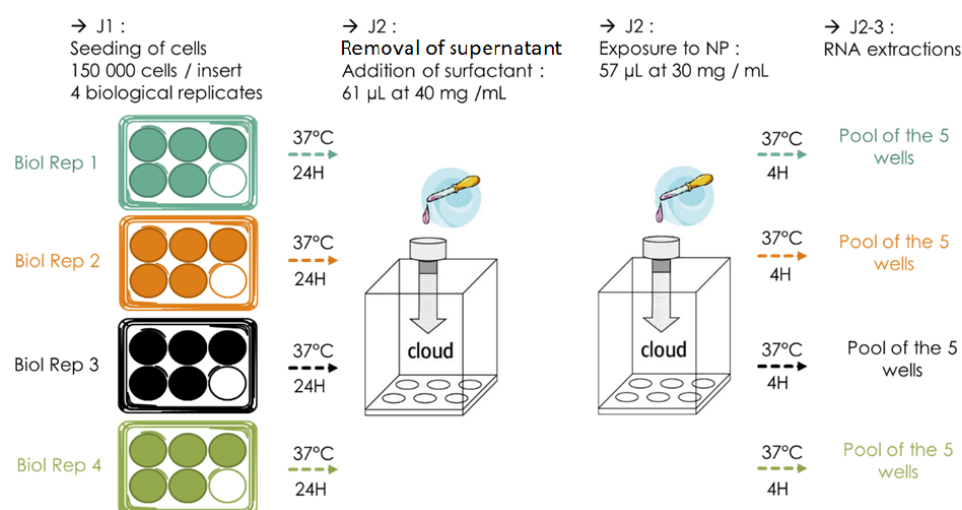


Figure 4. Initial exposure protocol. ~~TiO₂ NP are nebulized over the wells of the VITROCELL® Cloud 6 System containing the NR8383 cells.~~

3.2.2. Current improved protocol for NP nebulization in VITROCELL® Cloud 6 System

The current exposure protocol makes use of 5 BR of which 8 TR were generated. For each BR, half of the inserts (4 inserts for each BR, representing 4 TR) were exposed to ultrapure water as control and the other half to NM105 NP (Figure 5). 300 000 cells were seeded in 1 mL of complete growth medium by inserts and allowed to incubate for 24 h at 37°C, 5% CO₂. Before exposure, the apical medium contained in the inserts was removed to establish the ALI configuration, 61 µL of surfactant were then nebulized over the inserts, and the inserts were placed back into the incubator. Once each insert was exposed to surfactant, 114 µL of TiO₂ NP at a concentration of 15 mg/mL were nebulized over the cells. ~~Cells were then kept in the incubator for 4 h and then TR of each BR were pooled to proceed to the extraction of their RNA.~~

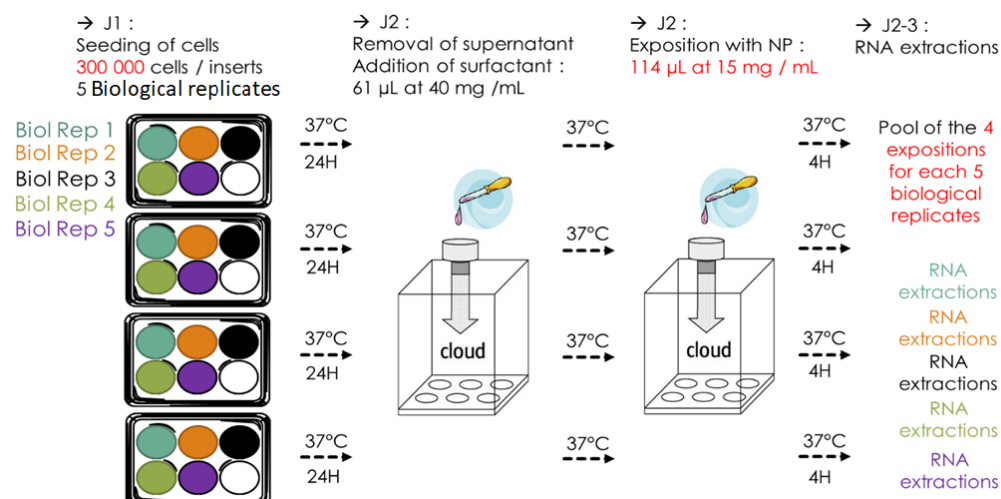


Figure 5. Current exposure protocol. ~~TiO₂ NP are nebulized over the wells of the VITROCELL® Cloud 6 System containing the NR8383 cells.~~

3.3. Troubleshooting flaws of the initial protocol

3.3.1. Preventing rapid drying of NPs on the nebulizer mesh by modifying the volume of nebulized liquid

The first issue encountered with these exposure parameters was that the TiO₂ NP suspension tends to rapidly dry out on the mesh of the nebulizer, which is likely a result of partial clogging of the pores of the mesh, making the TiO₂ NP depositions variable in time and quantity (Table 1 ; Figure 6 A). For a concentration of 30 mg/mL of NM105 TiO₂ NP suspended in 57 μ L of ultrapure water, we observed a mass deposition ranging from 2.1 to 7.4 μ g/cm² and the nebulization time needed for a stable QCM signal varied from 6 minutes to 24 minutes (Table 1). To address these high variations, we increased the volume to 114 μ L and reduced the concentration to 15 mg/mL (current protocol) by doubling the nebulized volume and reducing the TiO₂ NP concentration by two, so the same dose was present in the nebulizer as for the initial condition. This was aimed at maintaining the cell-delivered mass dose but reducing the range of variations in mass dose and in exposure time (Table 1 ; Figure 6 B). This improved the conditions from 5.1 +/- 2.7 μ g/cm² to 5.4 +/- 0.8 μ g/cm² and from 10.5 +/- 9.0 min to 5 min for 30 mg/mL and 15 mg/mL, respectively. Hence, the mass deposition was less variable, which can also be seen from the QCM signals (Table 1). It is noteworthy that unlike previous studies with the VITROCELL® Cloud 6 system we did not lift the exposure chamber after 3 – 5 min of cloud settling, since we did not want to disturb the potentially slower cloud settling of highly concentrated surfactant and TiO₂ NM105 aerosol. This prolongs the time for evaporation of the liquid layer on the QCM and hence extends the time for reaching an asymptotic QCM value as compared to previous studies [28,49].

Table 1. TiO₂ NP mass deposition comparison between the initial exposure protocol and the current protocol (asymptotic values at the end are reported). Exp1, exp2, exp3 and exp4 are abbreviations for the replicates of the exposures.

Exposition	Initial protocol (57 μ L, 30 mg/mL NP)		Current protocol (114 μ L, 15 mg/mL)	
	Mass deposited (μ g/cm ²)	Time (min)	Mass deposited (μ g/cm ²)	Time (min)
exp1	2.1	6	4.2	5
exp2	7.4	24	6.1	5
exp3	7.4	6	5.7	5
exp4	3.5	6	5.5	5
Mean	5.1	10.5	5.4	5.0
<i>SD</i>	2.7	9.0	0.8	0.0

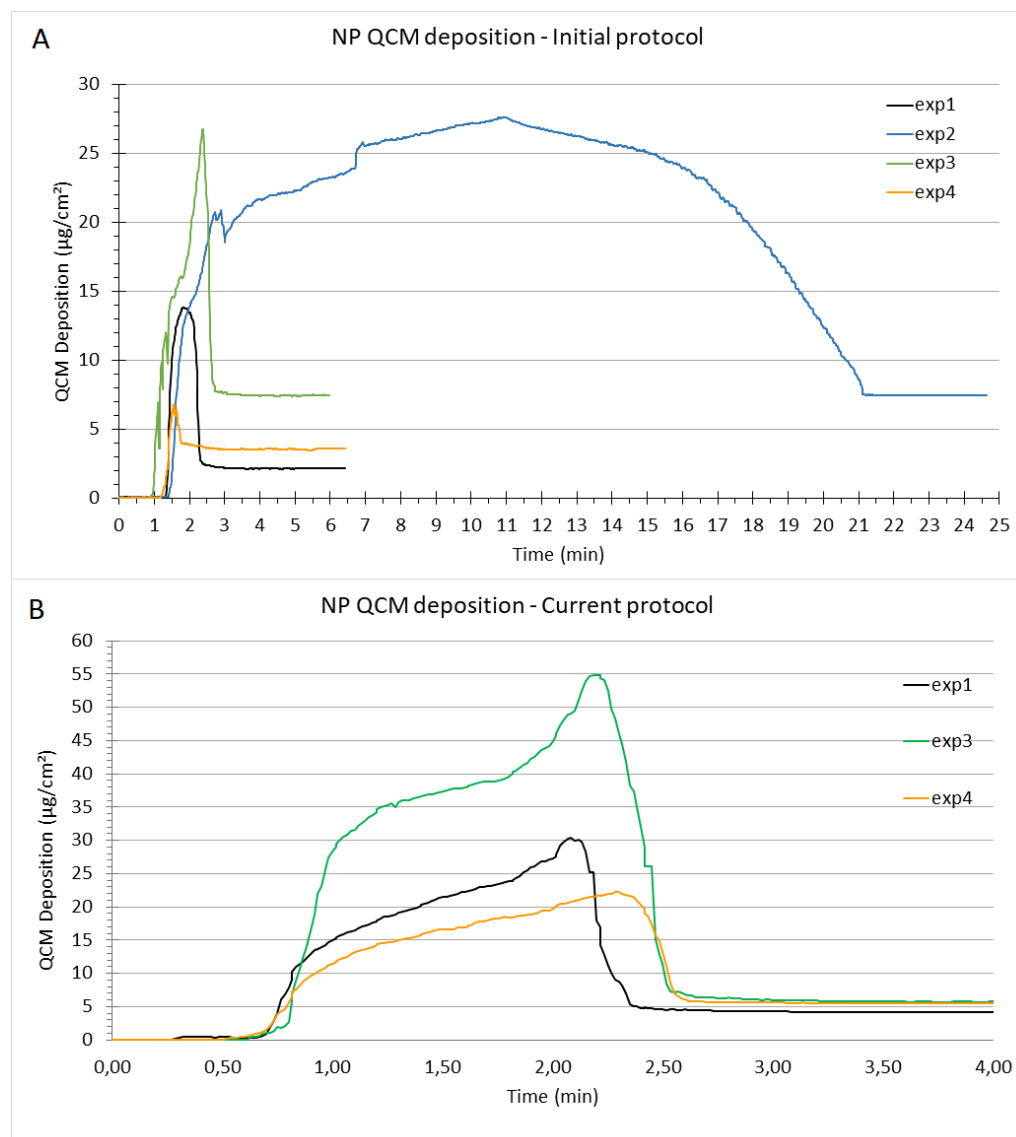


Figure 6. QCM curves showing in the first part the dynamics of cloud deposition in the VITRO-CELL® Cloud System followed by an asymptotic (constant) value, which represents the mass of the deposited TiO_2 NPs. The nebulization starts between 30 sec and 1 min after start of data acquisition. Depicted are A) the four exposures following the initial protocol and B) three exposures following the current protocol, the second nebulization (exp2) is not shown because of data backup failure (see Table 1).

3.3.2. Loss of cells and poor RNA quality

NR8383 cells are a semi-adherent cell line, indeed half of the cell population adheres to the membrane of the insert whereas the other half stays in suspension. The issue is that the supernatant has to be removed before exposing cells to surfactant to reach ALI, leading to a loss of about 50% of cells (75 000 with the initial protocol). This issue sometimes resulted in a too-small amount of RNA and the loss of the pellet while performing washing steps during the RNA extraction. The low number of cells also forced us to retrieve a bigger fraction of the aqueous phase after adding chloroform, increasing the risk of protein or salt contaminations (optical density (OD) ratios should be between 1.8 and 2.2) (Table 2). To fix this issue, we doubled the cell density in the inserts, going from 150 000 cells/insert to 300 000 cells/insert and this led to an improvement in reproducibility and RNA quality. Mean RNA quantity went, indeed, from 83.61 ± 73.27

ng/ μ L to 50.46 ± 26.34 ng/ μ L, and OD_{260/230} ratio increased from 1.33 ± 0.70 to 2.16 ± 0.59 (Table 2).

Table 2. Quality and quantity of RNA extracted from NR8383 cells exposed to TiO₂ NP through nebulization following the initial or current protocol. Results in red indicate a poor RNA quality or quantity preventing the use of the sample.

Biological replicate	Exposure	Initial protocol			Current protocol		
		RNA quantity (ng/μL)	OD 260/280	OD 260/230	RNA quantity (ng/μL)	OD 260/280	OD 260/230
1	H ₂ O	89.04	1.66	1.84	32.31	2.09	2.09
2		7.99	2.25	0.46	55.30	2.01	1.80
3		121.07	2.12	1.14	69.60	2.13	2.18
4		208.10	2.04	2.32	16.09	2.08	1.43
5		/	/	/	48.94	2.18	1.98
1	TiO ₂ NP	3.30	1.86	0.26	3.61	1.83	3.72
2		96.96	2.03	1.81	61.96	2.14	2.16
3		8.01	2.48	1.25	91.57	1.99	2.05
4		134.43	2.08	1.55	58.93	2.10	2.02
5		/	/	/	66.30	2.06	2.17
Mean		83.61	2.07	1.33	50.46	2.06	2.16
SD		73.27	0.24	0.70	26.34	0.10	0.59

3.3.3. Increasing the reproducibility and repeatability

TiO₂ NP mass deposition variability was also addressed by a change in the organization of the exposures. With the initial protocol, we used 4 biological replicates (BR) divided into 5 technical replicates (TR) (Figure 4). Each TR of a BR was exposed to TiO₂ NPs at the same time in the VITROCELL® Cloud chamber, during the same nebulization (Figure 4). This initial protocol was less rigorous as it implied that the 4 BR would be exposed during different nebulizations and consequently, potentially to a different dose of TiO₂ NPs because of the variability of mass deposition between each nebulization, introducing a bias in the comparison of the effects of TiO₂ NP on each BR. To reduce this bias, we worked with 4 TR from 5 BR. One insert (TR) of each BR is placed in a well of the VITROCELL® Cloud System and exposed to TiO₂ NP Nebulization. This was realized for each of the 4 TR, and the 4 TR of each BR were pooled to proceed to the RNA extraction (Figure 5). This allowed us to smoothen the nebulization variation bias on every BR, as each would receive the very same dose of TiO₂ NP, thus the mass deposition variations would be the same for every BR (Figure 7).

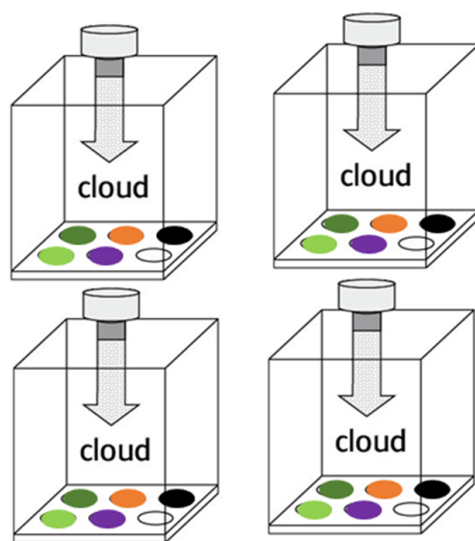


Figure 7. Methodology of NP exposure. One insert of each BR is placed in a well of the VITRO-CELL® Cloud System for each of the NP exposures. After pooling of all TR this ensures that all BR received an identical NP dose.

4. Discussion

Air-liquid exposure systems do represent closer-to-life scenarios in comparison to the traditional and currently most widely used submerged exposure methods, and this is not limited only to the Cloud systems. One of the main disadvantages of most aerosol-cell exposure devices (VACES, EAVES, CULTEX, NACIVT) is the lack of mass deposition control and - maybe more importantly - low dose rate, which often requires days to weeks of exposure time to observe any biological response *in vitro* cell culture models [31]. The choice of using VITROCELL® Cloud was based on short exposure times (a few minutes) and accurate control over the deposited NP mass dose on the cells using the quartz crystal microbalance. The latter enables the possibility to report the deposition variations on the results and to normalize the effects of NP dose on cell viability and gene expression. This control allows even more precise interpretations of the results. Moreover, the VITROCELL® Cloud system allows for surfactant coating of lung cells by nebulization before NP exposure, which is particularly relevant for cell models without innate surfactant secretion (*e.g.* alveolar macrophages). The use of surfactant provides the environment, closer to the lung physiological conditions, with controlled surface ratios between cells, surfactant, and nanomaterial.

It has been shown that surfactant can alter the physico-chemical properties of NPs and hence modulate their toxicity [32–34]. Since the nebulized surfactant completely covers the entire sample surface, the underlying cells are never directly exposed to the nanoparticles. Thus, the here-described method prevents possible artefacts arising from the direct interaction of the nanoparticles with the cells without surfactant. Moreover, nebulization of nanoparticles also prevents possible unrealistically high local doses of nanoparticles, the so called “hot spots” as described for *in vivo* experiments when instillation is used instead of inhalation [50]. Therefore, the nebulization of surfactant helps to produce results that tend to be closer to reality and are therefore more predictive for human health outcomes, which may ultimately lead to more reliable regulatory control of exposure to NPs.

It is important to consider that our problems with the initial protocol are probably a combination of several issues. Notably, it can be due to a partial clogging of the nebulizer pores for high NP concentrations (as used here, $30 \mu\text{g}/\mu\text{L}$), which then reduces the nebulizer output rate, enhances the nebulization time and then leads to partial liquid evaporation. Di Cristo *et al.* have published an interesting paper, with doses close to those we used, comparing the deposition efficiency of low volume ($30 \mu\text{L}$) of two TiO_2

NPs (NM100 and NM101), at lower concentration ($<100 \mu\text{g/mL}$) nebulized with the Aeroneb® Pro Nebulizer Sytem directly upon the insert (while we nebulized $57 \mu\text{L}$ at 30 mg/mL , or $114 \mu\text{L}$ at 15 mg/mL of NM105 TiO_2 NPs with Aeroneb® Lab Standard VMD Nebulizer, in VITROCELL® Cloud System). They showed that the deposition of NP depends on the type of NP. Indeed, the deposition of NM-100 was homogenous, whereas NM-101 formed small and large aggregates when nebulized. For NM-100, the doses delivered by aerosol were very similar to the initial concentration deposited in the nebulizer (90% of deposition efficiency, efficiency i.e. 90% of invested dose deposited on the bottom of the VITROCELL Cloud system), whereas the deposition efficiency was very low for NM-101 (values below 50%). Such an observation can be associated with the formation of NP aggregates in the suspension that could remain trapped erratically on the nebulizer mesh. Indeed, the dispersion of NPs during nebulization is strongly dependent on the aggregation phenomenon occurring before and during the biological experiments [51].

Moreover, using the same nebulizer for surfactant coating and then for NP exposure increased the risk of clogging the mesh. Indeed, in the initial protocol, we nebulized back-to-back surfactant and NP in each exposition, and we observed a frequent clogging of the mesh and variation of the NP mass deposition, even if the nebulizer was cleaned between each nebulization. The clogging probably occurs because lipids stick NPs together acting like a cross-linker. We have observed that labelled surfactant stuck to TiO_2 NTs after just the TiO_2 NTs were nebulized subsequent to surfactant nebulization (no measurements available), without in-between cleaning. This was diminished when we exposed in a first time all the 40 wells to the surfactant, and in the second time to the NP after cleaning and sonicating the nebulizer (current protocol). Hence, a rigorous cleaning procedure for the nebulizer is essential for the reliable operation of the VITROCELL® Cloud system. If possible, different nebulizers should be used if two or more substances are to be nebulized back-to-back, since this alleviates the cleaning requirements and prevent possible cross-contaminations of the nebulized entities. But even if clogging occurs, the fact that the actual dose delivered to the cells is measured with the QCM during each exposure allows for reliable dose-response curves independent of the degree of clogging.

During aerosol-cell exposure experiments, aerosol deposition is influenced by several factors, including the aerosol-generating system, aerosol characteristics (particle size, shape, density, etc.) and the inhalation pattern (flow rate and volume) [51,52]. The VITROCELL® Cloud system is unique in the sense that it uses air-less (no flow rate) cloud dynamics (not single aerosol dynamics) for rapid and spatially uniform dose delivery leveraging cloud sedimentation, which only depends on a high enough aerosol/liquid volume per air volume (cloud density) [29]. Under those conditions, aerosol-cell delivery is independent of aerosol size, shape and density provided the cloud density is high enough to marginalize single aerosol deposition mechanisms as compared to cloud settling. For the conditions of the VITROCELL® Cloud system, $200 \mu\text{L}$ of nebulized liquid ensures high enough liquid content of the air in the exposure chamber to ensure cloud rather than single aerosol settling conditions [29]. Since this may not be the case, if too small of a liquid volume is nebulized, it is recommended to operate the VITROCELL® Cloud 6 system with $200 \mu\text{L}$. However, we have shown here that also $110 \mu\text{L}$ can be sufficient for reproducible NP delivery. In addition, Ding *et al.* [49] have recently demonstrated that the QCM signal corresponds to mass only if the deposited NP layer is perfectly coupled to the quartz crystal. In the presence of water (even more if there is a high volume nebulized), there is viscoelastic decoupling of the NPs from the quartz and the signal is more representative of the viscosity of the deposited NP suspension layer than of NP mass. ~~Therefore, only after the NP cloud has settled onto the quartz and the top part of the exposure chamber has been removed, which allows for rapid drying of the NP layer on the QCM.~~

One might raise the issues on realistic dosimetry and exposure routes, *e.g.* whether the dose is too high or delivered too fast with the VITROCELL® Cloud 6 system as compared to ambient or occupational exposure scenarios. Moreover, in real-life exposure scenarios nanoparticles do not enter human lungs as liquid droplet suspension but rather in dry form, and the NP crosses the entire respiratory tract before reaching the lung cells. However, upon deposition onto the lung lining fluid all initially dry NPs will get in contact with the liquid phase of the lining fluid. After all, nebulizers are currently the most developed system for aerosol lung delivery used as well as in research experiments and in the clinic [47,53]. Dubus *et al.* have demonstrated that such a nebulizer (Aeroneb® Pro Nebulizer) provided 12% to 14% dose deposition in macaque lung, *i.e.* a 25 fold greater deposition than another nebulizer (MistyNeb 0.5%), this information is useful to compare the dose delivery in ALI exposure cell-monolayer and *in vivo* lung exposure.

Considering the bulk of studies about TiO₂ nanoparticles (NP) toxicity, there are few publications comparing *in vitro* and *in vivo* exposures, and even fewer comparing air-liquid interface exposure (ALI) with other *in vitro* and *in vivo* exposures. Therefore, after ALI cell exposure to TiO₂ NP and RNA extractions, described in this article, we aimed to identify early specific markers of lung exposure to TiO₂ NP, by a large study of genic expression modifications, mainly transcriptomics, and comparing classical submerged *in vitro*, ALI and *in vivo* exposures [42,43]. Knowing that nanoparticles can reach the alveoli [13], NR8383 cells are an appropriate model because they are alveolar macrophage precursors, which are the first implicated cells in the alveolar clearance of nanoparticles [54–56]. Moreover, this cell line has already been studied and validated as a model in the field of nanotoxicology and is relevant for their immune functions [57–59].

Nevertheless, our methodology could be improved by using ALI co-cultures of cell lines instead of monocultures, to be even closer to physiological conditions, considering the interactions that would exist between the different cell types. The first ALI co-culture was described in the 1980s with the growth of endothelial and epithelial cells on a cellulose membrane [60]. The simplest and most used ALI model is the cell monoculture (with cell lines or primary cells) and this model was largely used for studying transepithelial electrical resistance (TEER) [61–64].

Recently, Kletting showed that by cultivating differentiated m0 macrophages (THP-1) in the presence of alveolar epithelial cells (hAELVi) at the air-liquid interface, an extracellular matrix was secreted at the surface and their transport study showed a functional air-blood diffusion barrier phenotype [65]. Such a phenotype would greatly serve our pulmonary NP toxicity study, as it would mimic even more accurately the pulmonary physiological conditions. Another study conducted by Klein *et al.* [66] highlighted that SiO₂ NP exposures in ALI conditions induced a higher ROS generation in monocultures than co-cultures (macrophages + endothelial cells + bronchial epithelial cells + mast cells). It showed that the effects encountered in monocultures tend to be over evaluated compared to results obtained when culture conditions step closer to physiological conditions [66]. Currently, the best way to replicate pulmonary environment in ALI is to set up co-cultures or 3D cultures [67]. Finally, some aerosol cell exposure systems, recently developed, include continuous airflow (VITROCELL® Automated Exposure Station) [27,30,31] and even cyclic stretch, to simulate *in vitro* the mechano-elastic stimulation by continuous inhalation and exhalation [68–70].

5. Conclusions

~~The total RNA extracted from this VITROCELL® Cloud exposition was used to perform a complete transcriptomic study [43] including a comparison of *in vivo* and air-liquid interface and submerged *in vitro* results for TiO₂ NM105 NP exposure [42]. Owing to the specific objectives of this study, the recommended experimental procedure for operating the VITROCELL® Cloud 6 described suggests using lower nebulization volumes for the surfactant (61 µL) and the TiO₂ NP suspension (114 µL) as compared to~~

the manufacturer recommended 200 μL to keep the cell deposited liquid layer as low as possible. However, the total delivered amount of liquid of 175 μL is relatively close to the 200 μL recommended by the manufacturer. To conclude, we do not recommend replacing the existing standard operating procedure [29], but we present four optimization points when exposing semi-adherent cells to NPs under ALI conditions with the VITROCELL® Cloud 6 system (1); if low volumes and high concentrations are nebulized (2); when two sequential aerosol exposures are performed back-to-back (here: lung surfactant and NPs in 61 μL and 114 μL , respectively) (3); and how to arrange the wells to reduce the variability between the biological replicates (BR) (4):

1) When using semi adherent cells, the number of cells seeded on the insert has to be increased (as compared to submerged culture conditions) to ensure that enough cells stay on the membrane after discarding the apical medium to reach a satisfying RNA yield.

2) To avoid any clogging of the mesh of the nebulizer and to obtain a reproducible cell-delivered dose, the manufacturer recommendations for cleaning of the nebulizer should be adhered to and the TiO_2 NP concentration has to be decreased by increasing the nebulized suspension volume (from 57 μL of 30 mg/mL to 114 μL of 15 mg/mL in this case for TiO_2 NM105 NP). This will prevent the NP suspension from both drying out on the nebulizer mesh and alteration of the NP deposition on the cells due to low cloud density in the VITROCELL® Cloud exposure chamber.

3) When two sequential aerosol exposures are performed back-to-back, when applicable, we suggest using a different nebulizer for each liquid nebulized. If the same nebulizer is used, we suggest to nebulize all replicas with the first type of aerosol and then with the second, and to clean the nebulizer between each sequence of nebulization thoroughly as recommended by the manufacturer.

4) If it is necessary to proceed to several expositions, we recommend including each BR in each exposure session (divided into technical replicates (TR), one TR per exposition). By doing this and subsequent pooling of the TRs of each BR, the variability in deposited dose amongst expositions will be spread over every TR in the same manner for each BR.

Finally, considering the improvement of co-culture or 3D cultures techniques, coupled with an innovative exposure device such as the VITROCELL® Cloud, it is reasonable to think that in the future this kind of methodology will contribute to reducing the use of animals in toxicology research.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2079-4991/11/1/0/s1>, Figure S1: Nebulizer/tube/ μ -dish setup for microscopy; Figure S2 : Time series of nebulizing Curosurf-StarRed, fluorescence microscopy at 561nm, 640, and 950nm. Most of the material is nebulized in the first 30 seconds, and the system settles in a few minutes; Figure S3 : Intensity distribution of Curosurf-StarRed fluorescence, under different conditions

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Conflicts of Interest: Otmar Schmid is one of the inventors and patent holders of the ALICE Cloud technology, which was licensed to VITROCELL® Systems which made it commercially available as VITROCELL® Cloud. The other authors declare that they have no conflict of interests. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

MDPI	Multidisciplinary Digital Publishing Institute
NP	Nanoparticles
ALI	Air-liquid interface
QCM	Quartz crystal microbalance
BAL	Bronchoalveolar lavage
BR	Biological replicate
TR	Technical replicate

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Article

Aerosol-cell exposure system applied to semi-adherent cells for aerosolization of lung surfactant and nanoparticles followed by high quality RNA extraction.

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Abstract: Nanoparticle toxicity assessments have moved closer to physiological conditions while trying to avoid the use of animal models. An example of new *in vitro* exposure techniques developed is the exposure of cultured cells at the air-liquid interface (ALI), particularly in the case of respiratory airways. While the commercially available VITROCELL® Cloud System has been applied for the delivery of aerosolized substances to adherent cells under ALI conditions, it has not yet been tested on lung surfactant and semi-adherent cells such as alveolar macrophages, which are playing a pivotal role in the nanoparticle-induced immune response. Objectives : In this work, we developed a comprehensive methodology for coating semi-adherent lung cells cultured at the ALI with aerosolized surfactant and subsequent dose-controlled exposure to nanoparticles (NPs). This protocol is optimized for subsequent transcriptomic studies. Methods: Semi-adherent rat alveolar macrophages NR8383 were grown at the ALI and coated with lung surfactant through nebulization using the VITROCELL® Cloud 6 System before being exposed to TiO₂ NM105 NPs. After NP exposures, RNA was extracted and its quantity and quality were measured. Results: The VITROCELL® Cloud system allowed for uniform and ultrathin coating of cells with aerosolized surfactant mimicking physiological conditions in the lung. While nebulization of 57 µL of 30 mg/mL TiO₂ and 114 µL of 15 mg/mL TiO₂ nanoparticles yielded identical cell delivered dose, the reproducibility of dose as well as the quality of RNA extracted were better for 114 µL.

Keywords: air-liquid interface; nanoparticles; VITROCELL® Cloud System; RNA extraction; macrophages; surfactant; NR8383; transcriptomic

1. Introduction

During the last years, the development of nanotechnologies and their many possible applications have led to their incorporation into consumer products. Indeed, in 2013, the nanotechnology Consumer Products Inventory (CPI) listed 1814 consumer products containing nanomaterials from 622 companies in 32 countries, including health and fitness products, medicine, cosmetics, food, and electronics. Predictably, this list has been growing since 2005, in all areas of production, leading to serious concerns about the effects of their exposure on human health. [1–3] Moreover, the emergence of nanotechnologies has led to the development of new research fields such as nanomedicine, which focuses on the synthesis and engineering of nanomaterials for drugs delivery [4], which has led to novel treatment [5–8] and diagnostic options [9,10] and combinations thereof [11,12]. Consequently, the study of both adverse and therapeutic health effects

of nanoparticles and new nanomaterials is a crucial issue for the development of safe nano-enabled products such as consumer products as well as medical products.

Generally, nanoparticle (NP) exposure is associated with an increased risk of cardiorespiratory morbidity and mortality, cancers, asthma, pulmonary diseases, fibrosis, or allergies. At the cellular scale, NPs can induce oxidative stress, inflammation, genotoxicity, membrane alterations, mitochondrial dysfunction, cell cycle arrest, and cell death [13–15]. Numerous studies have shown that the health effects of NPs do not depend only on their chemical and physical properties such as their size, shape, composition, surface area, or solubility, but also on the conditions of exposure including the type of exposed populations, as well as duration, doses, repetitions, and routes of exposure [16]. In the environment, the main route of exposure to nanoparticles is the respiratory route, although effects on the skin, eyes, or gastrointestinal tract need to be taken into consideration. Indeed, because of their small size and chemical properties, NPs can reach the alveoli and cross the blood-air barrier thus infiltrating the bloodstream and other organs, for example the gastrointestinal tract, the heart, or the brain [17–20].

Over the last two decades, several attempts have been made toward reducing and replacing *in vivo* experimentation with novel *in vitro* models trying to mimic physiological conditions. These models tend to move closer to organ structures, and in the case of the respiratory airways, to bronchial/alveoli architecture. Among these models, lung cells cultured at the air-liquid interface (ALI) appear to be a promising technique as it offers many possibilities for mimicking parts of the respiratory tract. In the ALI culture, cells are grown on the microporous membrane of cell culture inserts which are often placed on multi-well plates. The basolateral compartment is filled with medium and once the cells adhere to the microporous membrane, the medium contained in the apical side of the insert is removed, establishing ALI conditions [21].

Traditional *in vitro* systems for nanotoxicology studies are based on the dissolution of non- or poorly-soluble particles directly in the cell culture medium, where cells are subsequently cultured [22,23]. This so-called "submerged exposure method" is not representative of actual exposure to airborne particles regarding the behavior of particles in the air [24] and implies numerous disadvantages including poor control of cell-delivered dose and interactions of the NPs with the medium [25,26]. That is why scientists and companies have developed new methods to expose cells to NPs through a gaseous phase including roller bottles, inverted cell monolayer, and smoking machines [27,28].

For inhalation toxicology, the most pertinent methodologies include ALI cell cultures and aerosol-cell exposure devices, which differs among them in the method used to aerosolize nanoparticles. For example, the Versatile Aerosol Concentration Enrichment System (VACES) uses a condensation process to aerosolize NPs [29]. The Electrostatic Aerosol In Vitro Exposure System (EAVES) guides aerosol particles horizontally across cell culture inserts (where cells are grown) and uses electrostatically enhanced diffusion and sedimentation for the deposition of particles on the cells [30]. The CULTEX® stagnation point flow system deposits aerosolized particles *via* diffusion and sedimentation directly onto the cells (Cultex laboratories GmbH, Hannover, Germany) [31,32], and the Nano Aerosol Chamber In Vitro Toxicology (NACIVT) [33] uses a stagnation point flow system and an electrostatic precipitation system to deposit aerosolized NPs onto the cells. A recently developed device by Inhalation Sciences (Huddinge, Sweden), called XposeALI, uses a high-pressure aerosol generator to aerosolize dry NP powder [34] and utilizes diffusion and sedimentation for aerosol-cell deposition in a stagnation point flow setting. For continuous flow exposure, the VITROCELL® Automated Exposure Station was described for testing the toxicity of combustion aerosols at the air-liquid interface [35]. Moreover, nebulization of nanoparticle suspensions into an exposure chamber with subsequent cloud settling has been introduced as ALICE and ALICE Cloud technology for dose-controlled and efficient NP-cell delivery at high dose rates by Lenz *et al.*, 2009, and Lenz *et al.*, 2014, respectively [36,37]. For more details on the various types of ALI

86 exposure systems, interested readers can refer to Ehrmann *et al.*, 2020 and Paur *et al.*,
87 2011 [38,39].

88 The main limitation of most of the aforementioned systems is the detection and
89 quantification of the dose of NP delivered to the site of exposure. However, it represents
90 one of the most important criteria for the assessment of particle-related risks [16]. Indeed,
91 most studies provide precise data on the concentration of exposure to particles, *i.e.* the
92 mass of particles per volume of air ($\mu\text{g}/\text{m}^3$) or - for cell assays immersed *in vitro* -
93 per volume of cell culture medium ($\mu\text{g}/\text{mL}$), but the most relevant measure - the dose
94 delivered to the site of exposure, e.g. lung epithelium (*in vivo*) or lung cell culture (*in vitro*)
95 - is often overlooked. QCM are piezoelectric biosensors that detect resonance frequency
96 variation of quartz crystal associated with mass change on their surface. During the last
97 decade, numerous studies have reported new possible applications of QCM in biological
98 and medical sciences including immunosensors, biosensors, microbial detectors and
99 innovations [40–47]. Quartz crystal microbalances (QCM) have been also described as a
100 precise device for real-time measurement of the dose-delivered in nanotoxicology studies
101 [26,36,48]. For these reasons, a QCM has been included in the VITROCELL® Cloud
102 System (commercial version of ALICE Cloud system) in order to control the real-time
103 NP dose-delivered during the Cloud exposure.

104 Finally, the alveolar lining fluid, which contains innate surfactant and covers the
105 alveolar epithelium, can interfere with nanoparticles and modify their physico-chemical
106 properties such as their agglomeration, their charge state and the type of protein corona
107 [49–51]. The alveolar surfactant, which is produced by alveolar type II epithelial cells
108 [52–54], is an aqueous mixture of lipids and proteins, lining the surface of pulmonary
109 epithelial cells in the alveolar region, whose function is to maintain sufficient tension
110 and protect the alveolar sacs from collapsing at the end of each exhalation phase. The
111 surfactant is composed of 92% lipid mass, mostly phospholipids, and 8% protein mass.
112 The composition of the surfactant and its amphiphilic bilayer structure makes it possible
113 to modify the ability of external compounds to reach the pulmonary epithelium [55–
114 57]. Alveolar macrophages, which are covered with alveolar surfactant and capable of
115 neutralizing NPs in the lung by phagocytic uptake, are often considered as the most
116 important defense mechanism against NPs deposited in the alveolar region. Recent
117 *in vitro* experiments have shown that surfactant decreases the cellular uptake of silica
118 nanoparticles by up to two orders of magnitude [58]. Since the alveolar lining fluid and
119 the alveolar macrophages are the first and second line of defense against NPs deposited
120 in the alveolar region, a semi-adherent alveolar macrophage cell line pre-coated with a
121 thin film of alveolar surfactant fluid before the exposure to NPs is used in our study.

122 This study reports the optimization of an innovative air-liquid interface system
123 using a cloud settling exposure system by VITROCELL® Systems for pre-coating of the
124 cells with surfactant lung lining fluid and subsequent NP exposure, combined with an
125 accurate micrometric balance to measure cell exposure in real-time. The VITROCELL®
126 Cloud System (VITROCELL® Systems GmbH, Waldkirch, Germany) [37] is a refined
127 version of the ALICE system, which has been developed especially for aerosolized
128 NPs exposure [36]. This VITROCELL® Cloud system involves a vibrating membrane
129 nebulizer, which uses a vibrating, piezoelectrically controlled, perforated membrane
130 to induce acoustic pressure waves that squeeze the NP suspension through the pores
131 of the membrane. This leads to the production of a dense, uniform cloud of droplets
132 that deposits uniformly onto the cells through a process called cloud settling, resulting
133 in an efficient and controlled dosimetry which is monitored in real-time with a highly
134 sensitive quartz crystal microbalance.

135 To sum up, *in vitro* studies based on ALI exposure are more and more used in
136 toxicology studies. But researchers using such a strategy have to face some issues
137 including 1) using a very low concentration of NP 2) applying a homogeneous layer of
138 surfactant on cells 3) using semi-adherent cells in ALI, and 4) retrieving RNA in sufficient
139 quantity and quality to carry out transcriptomic studies. Thus, here, we present an in-

depth detailed protocol answering to these points, in which each steps are validated, after the optimization of the different parameters. For a better understanding, all experimental results are featured in the present paper. It could be of help for researchers using such a device.

2. Materials and Methods

2.1. Chemicals and reagents

Chemicals and reagents were obtained from the following sources: Dublecco's Modified Eagle Medium (DMEM), L-glutamine, penicillin-streptomycin (PS), amphotericin B, phosphate buffer saline (PBS), and fetal bovine serum (FBS) from Sigma-Aldrich (Saint-Louis, MO, USA); Transwell® polyester culture membrane from Corning (Wiesbaden, Germany). Whole porcine surfactant was a generous gift from J Perez-Gil (Faculty of Biology, Complutense University, Madrid, Spain), which includes all of the water-soluble and non-water-soluble surfactant proteins. Propan-2-ol, chloroform, absolute ethanol were bought from Carlo Erba Reagents (Val-de-Reuil, France). RNA-Solv reagent was purchased from Omega Bio-tek (Norcross, GA, USA). TiO₂ NM105 NPs (JRC reference materials, primary NP diameter 21.5 ± 7.2 nm; secondary NP diameter (agglomerate, DLS measurement) 170 ± 0.7 , BET surface area $51 \text{ m}^2/\text{g}$, Zeta potential 11.1 ± 0.7 mV) were provided by JRC.

2.2. Cell culture

For VITROCELL® Cloud exposures, NR8383 cells (CRL2192™) are a rat alveolar macrophage cell line purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM-high glucose medium supplemented with 15% FBS, 4 mM L-glutamine, 100 U/mL penicillin, 100 g/mL streptomycin, and 0.25 µg/mL of amphotericin B at 37°C, in a 5% CO₂ atmosphere. Cells were grown in 75 cm² flasks (Sarstedt, Nümbrecht, Germany) and then passaged and seeded on 0.4 µm pore size microporous Transwell® inserts at a density of 1.5×10^5 cells/mL or 3×10^5 cells/mL in 1 mL of supplemented DMEM medium in the apical side of the insert and 2 mL in the basolateral side, and kept at the incubator at 37°C, 5% CO₂, for 24h. Before exposure, the apical medium was removed to establish ALI.

For fluorescence microscopy, a LA-4 murine lung epithelial cell line was cultured according to ATCC guidelines: cells were seeded at 30,000 cells/cm² in T75 flasks and split when they reached a 80% confluency. They were cultured in 10 mL of full cell medium (a mixture of F-12K medium (Gibco), 15% FBS (Fetal bovine serum, ATCC), 1% P/S (penicillin-streptomycin, Sigma), 1% NEAA (non-essential amino acids, Gibco)) in an incubator at 37°C with saturated humidity and 5% CO₂. Then, cells were seeded and observed in 35 mm µ-dishes with #1.5H glass bottom (Ibidi), cells and media covered only 1/3 of the µ-dish surface (3.5 cm²) due to the geometry of the µ-dish.

2.3. VITROCELL® Cloud 6 System

The VITROCELL® Cloud 6 System (VITROCELL® Systems, Waldkirch, Germany) is a commercially available device aimed at exposing cells at the air-liquid interface to inhaled aerosolized toxins through cloud settling, which mimics realistic inhalation exposure scenarios of alveolar macrophages to nanoparticles in physiological conditions. The device is schematically depicted in Figure 1. The stainless-steel base module of this device comprises 5 electrically heated wells in which 13 mL of medium and the inserts are placed. The temperature in the wells is maintained at 37°C. A sixth well contains a quartz crystal microbalance (QCM balance) for cell-delivered dose measurements with a resolution of 10 ng/cm², at a sampling rate of 1 Hz and a manufacturer-specified zero noise level of 20 ng/cm² (VITROCELL® Systems, Waldkirch, Germany) that allows to assess not only cell-delivered dose but also reproducibility and repeatability of the exposures. The QCMs used in the VITROCELL® Cloud systems was well described previously in Ding et al. 2020 [48]. Briefly, the QCM incorporated in the VITROCELL®

Cloud 6 system has an eigenfrequency of 5 MHz, a resistance of 10 Ohm and an aerosol-exposed area of 4 cm², which is close to the cell covered area in 6-well Transwell® inserts (4.2 cm²). The upper part of the device is a 2250 cm³ polycarbonate removable exposure chamber pierced on the top with a hole to place the nebulizer. The Transwell® inserts (Corning® Transwell®-Clear 6-well Inserts, 10 µm thick Polyester (PET) membrane, product number 3450) used for culturing cells under ALI conditions are composed of a microporous (0.4 µm pore size) polyethylene terephthalate (PET) membrane of a surface of 4.67 cm².

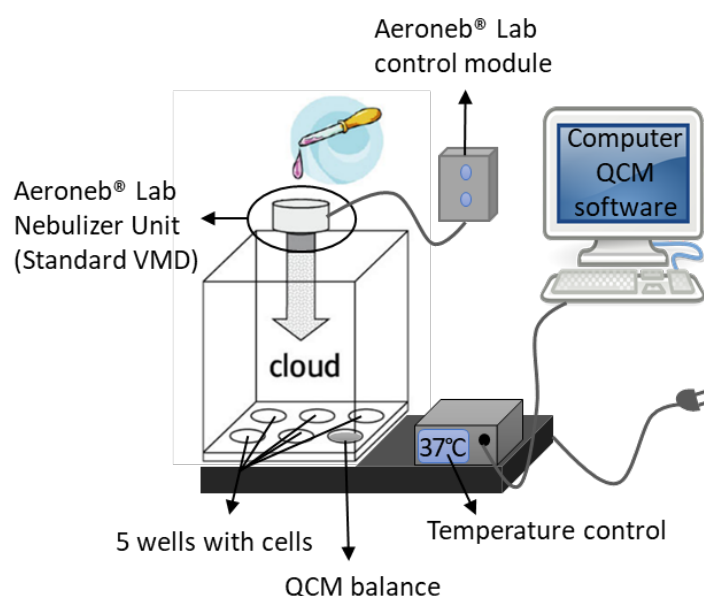


Figure 1. Schematic of the VITROCELL® 6 Cloud device. The stainless-steel base module of this device comprises 5 electrically heated wells in which 13 mL of medium and the inserts are placed. The temperature in the wells is maintained at 37°C. A sixth well contains a quartz crystal microbalance (QCM balance) for cell-delivered dose measurements with a resolution of 10 ng/cm², at a sampling rate of 1 Hz and a manufacturer-specified zero noise level of 20 ng/cm² (VITROCELL® Systems, Waldkirch, Germany), measurements are analysed and visualized using the computer QCM software. The upper part of the device is a 2250 cm³ polycarbonate removable exposure chamber pierced on the top with a hole to place the nebulizer. The nebulizer system is composed of the Aeroneb® Lab Nebulizer Unit, Standard VMD and a Aeroneb® Lab Control Module (Aerogen Inc., Galway, Ireland). The nebulization relies on a perforated piezoelectrically controlled vibrating mesh to generate acoustic pressure waves releasing liquid droplets at a high frequency (128 kHz). This nebulizer is constituted of a palladium mesh pierced with *ca.* 1 000 holes, releasing between 0.3 and 0.8 mL of liquid per minute through the holes, resulting in a stream of droplets precisely controlled for size by the diameter of the apertures (4 – 6 µm droplet diameter for the Standard VDM nebulizer).

2.4. Fluorescent microscopy for the assessment of the homogeneous deposition of surfactant

In order to compare the different methods for the deposition of surfactant on the cells, two different methods were used: nebulization or pipetting (Figure 3). In both cases the cell medium was completely removed before the process. However, in some cases, the surfactant was administered immediately afterwards (leaving around 10 µm of medium on top), whereas in others the medium was left to evaporate for a few minutes before administering the surfactant (referred to as dry cells, leaving only 10 nm – 100 nm layer of media on the cells).

For nebulization, 6 µL (240 µg) of the surfactant were deposited to the pre-wetted nebulizer and were nebulized onto the entire µ-dish (surface : 3.5 cm²). Of these 240 µg, only *c.a.* 7% reached the bottom surface, and *c.a.* 2.5% (6 µg) were deposited on the

cells (which are seeded only on the inner 3.5 cm² of the dish due to the geometry of the dish), corresponding to approximately 10 monolayers on the cells. When pipetting the surfactant was added by a pipette to the middle of the μ -dish (0.72 μ l, 10 mg/mL), onto the medium on cells, corresponding to 10 monolayers if spread out over the whole medium-covered area of the μ -dish (3.5 cm²).

The surfactant used was either whole native surfactant (extract of porcine BAL, contains all surfactant proteins (SP-A, SP-B, SP-C and SP-D), kindly provided by J. Perez-Gil) or Curosurf® (poractant alfa, the extract of porcine lung surfactant, consisting of 99% polar lipids and 1% SP-B and SP-C, by Chiesi). The surfactant in all experiments was labelled with STAR RED-DPPE (Abberior) using a Nlabel:Nlipid = 1:1000 labelling ratio and was dispersed in PBS to final concentration.

Delivery was performed using a pipette or a pre-wetted Aeroneb® Lab Nebulizer Unit, Standard VMD (Aerogen Pro Standard VDM, Aerogen Inc., Galway, Ireland) in a tube-like setup (with the surface of the bottom being 9.6 cm², see Supp Data 1A-C), with an estimated rate of nebulization 5 μ l/s. A tightly sealed 7 cm high cylindrical chamber with a diameter of 3 cm was adapted for live nebulization on the microscope onto a single μ -dish (see Supp Data 1A-C). We estimated that 7% of initially nebulized material reached the bottom of the μ -dish (see Supp Data 1D). The cells and surfactant were kept at 37°C at the time of surfactant delivery to assure the surfactant was as fluid as under physiological conditions when deposited on the apical surface of the cells.

The TiO₂ nanotubes, characterized and fluorescently labelled as described in Kokot *et al.*, 2020 and Urbančič *et al.*, 2018 [59,60], were dispersed in deionized H₂O and concentrated to concentration of 33 mg/mL in a centrifuge at 21 000 rpm for 20 min (74000g RCF) (initial volume of 1 mL and concentration 1 mg/mL). Nanoparticles were nebulized either to a dry μ -dish for the panoramas or to cells with a previously nebulized layer of surfactant. The same nebulization setup as described above was used (3 μ l, 33 mg/mL, corresponding to a surface area dose of nanoparticles 1:1, *i.e.* 1 cm² of NP/ per cm² cells).

Different Aeroneb® Lab Nebulizer Units were used for nebulizing surfactant and nanoparticles and they were thoroughly cleaned in between exposures. The images were acquired with an Abberior Instruments STED microscope equipped with a 60x water immersion objective (Olympus) and the associated software Inspector (version 16.3.11462-metadata-win64). The fluorescence was excited with two pulsed lasers at 561 and 640 nm, whereas two avalanche photodiodes at 580–625 nm and 655–720 nm respectively, were used for fluorescence detection (filters by Semrock).

Panoramic images were combined from side-by-side recorded 0.7x0.7 mm large images with a 0.05 mm overlap and pixel size of 1 μ m. The images were acquired over more than 1 cm using an air 10x objective (Olympus). The illumination over such a large field of view is not completely homogeneous, hence the darker edges on the images. Wolfram Mathematica was used to overlay and analyze the panoramic images. Power of excitation lasers was 5 μ W.

The time series in Figure 3 D was acquired using a pixel size of 100 nm, dwell-time 10 μ s, and 561 nm and 640 nm laser powers at around 5 μ W while simultaneously imaging using two-photon excitation with wavelength at 950 nm, dwell-time 400 μ s, and power at around 10 mW (the two-photon excitation and the entire time-lapse are shown in Supp Data). The surfactant structure following nebulization of 100 monolayers (2.4 mg of Curosurf) in Figure 3 E was acquired using two-photon excitation at 950 nm, pixel size of 250 nm, and dwell-time 8 ns.

2.5. Pre-coating of ALI cells with surfactant

The freeze-dried whole surfactant powder extract from pig lungs was dissolved in 100 mL ultrapure water at room temperature, yielding a concentration of 70 mg/mL, and sonicated 30 seconds at 37°C in ultrasonic cleaner. It was then diluted in PBS to reach a working concentration of 40 mg/mL, sonicated 30 seconds at 37°C in ultrasonic

cleaner, and left at 37°C in the incubator for 1h. After the removal of the apical medium on the Transwell® inserts, 61 µL of the surfactant solution were nebulized with the VITROCELL® Cloud 6 System allowing for cloud settling time. In the initial protocol, the nebulization of surfactant was done just before the NP nebulization, while, for the current protocol, surfactant nebulization was done for all together (control and NP exposed). The inserts were then placed back into the standard multiwell plates and were incubated at 37°C, 5% CO₂, until a second nebulization (NP exposure, or - for controls - water exposure) is performed with the VITROCELL®Cloud system.

2.6. NP exposure protocol

TiO₂ NM105 NPs were suspended in 5 mL of ultrapure water at a concentration of 30 mg/mL and sonicated for 10 minutes in an ultrasonic cleaner (VWR International, Radnor, PA, USA) just before exposure with the VITROCELL® Cloud 6 System. As described above, NR8383 cells were seeded on the apical side of Transwell® inserts at a concentration of 1.5×10^5 cells/mL (initial protocol) or 3×10^5 cells/mL (current protocol) in 1 mL of supplemented DMEM medium for 24 hours. In each well of the VITROCELL® Cloud 6 System, 13 mL of medium were added and inserts were placed over the wells to set up the air-liquid configuration, 61 µL of surfactant were then nebulized on the inserts after removal of the apical medium. Subsequently, the cells were exposed to 57 µL of aerosolized nanoparticle suspension or an equivalent volume of DMEM medium or ultrapure water (controls). After exposure, the cells were placed in the incubator for 4 h and then recovered to extract their RNA. In this study, the nebulizer is rinsed between each exposure by nebulizing 1 mL of ultrapure water (Ultrapure (Type1) Water Simplicity®, filter Millipak® 20 Millipore 0.22 µm) followed by 1 mL of PBS 1X. The nebulizer was also sonicated from time to time if it was necessary to address the clogging of the mesh.

The nebulization of the nanoparticles and surfactant suspensions was performed with an Aeroneb® Lab Micropump Nebulizer (Aerogen Inc., Galway, Ireland). The nebulizer system is composed of the Aeroneb® Lab Nebulizer Unit, Standard VMD and an Aeroneb® Lab Control Module (Aerogen Inc., Galway, Ireland) (Figure 1). The nebulization relies on a perforated piezoelectrically controlled vibrating mesh to generate acoustic pressure waves releasing liquid droplets at a high frequency (128 kHz). This nebulizer can be used *in vitro*, *in vivo*, or in clinical settings [61,62] and is constituted of a palladium mesh pierced with *ca.* 1 000 holes, that vibrates at 128 000 times per second, releasing between 0.3 and 0.8 mL of liquid per minute through the holes, resulting in a stream of droplets precisely controlled for size by the diameter of the apertures (4 – 6 µm droplet diameter for the Standard VMD nebulizer) [62]. For more information on the Aeroneb® nebulizer system please refer to the manufacturer website (Aerogen Technology).

2.7. RNA extraction

Here we present the first step of transcriptomic study [63,64] *i.e.* RNA extraction. Excellent quality and sufficient quantity of RNA is a prerequisite for transcriptomic assay. Indeed, the concentration of total RNA should be more than 40 ng/µL and the quality is defined by the ratios of optical densities (OD) 260/230 and 260/280 which must be between 1.8 and 2.2.

4 hours after exposures, the culture media was removed from the basolateral and apical compartments of the inserts. Cells were lysed by flushing 1 mL of RNA-Solv reagent directly onto the inserts membrane and then delicately scrapped from the membrane with a pipet tip to detach every single cell. Every technical replicate (TR) originating from the same biological replicate (BR) were pooled. Samples were then stored at -80°C over-night. The next day, 200 µL of chloroform per tube were added and samples were centrifuged at 12000 g for 15 minutes at 4°C. 175 µL of propan-2-ol were added to 350 µL of supernatant and centrifugation was again proceeded at 12000 g for 20

minutes. Pellets were then subjected to two successive ethanol washing steps and then dried at 60 °C for 10 minutes to remove the excess of ethanol. Finally, dried pellets were diluted in 25 μL of RNase-free water. RNA quantity and purity were assessed using BioSpec-nano spectrophotometer (Shimadzu, Marne-la-Vallée, France)

3. Results

3.1. A uniform deposition of surfactant by aerosolization with the nebulizer

3.1.1. Reproducible deposition assessment by QCM measurements

The thickness of the alveolar lung lining fluid in rat lungs ranges between 0.1 μm and 0.9 μm . The alveolar lining layer appears continuous, submerging epithelial cell microvilli and intercellular junctional ridges, and serves to smoothen the alveolar air-liquid interface in inflated lungs [65]. The standard operating procedure of the VITROCELL® Cloud System provided by VITROCELL® Systems recommends nebulization of 200 μL of liquid, which results in *ca.* 5.6 μL of liquid aerosol deposited per 6-well (4.5 cm^2) Transwell® insert (the values can vary depending on nebulizer performance). This corresponds to a *ca.* 12 μm thin liquid film, which evaporates within a few seconds from the QCM after opening the exposure chamber. It has been shown that a thin aqueous layer like this has no adverse effects on cellular response [36]. To adapt the thickness of the *in vitro* surfactant coating to the *in vivo* conditions, we only nebulized 61 μL of surfactant solution (at 40 $\mu\text{g}/\mu\text{L}$) or 57 μL of nanoparticle suspension (in our initial protocol, against 114 μL of NP suspension in the current protocol). It reflects the minimum volume of nebulized liquid (*ca.* 60 μL) to still allow for relatively uniform cloud mixing in the VITROCELL® Cloud System, which is a prerequisite for reliable operation. As the surface of the VITROCELL® Cloud chamber is 143 cm^2 , the surfactant mass initially nebulized corresponds to 76.5 $\mu\text{g}/\text{insert}$ and 1.9 $\mu\text{L}/\text{insert}$ (17 $\mu\text{g}/\text{cm}^2$ and 0.42 $\mu\text{L}/\text{cm}^2$), resulting (theoretically) in a 4 μm aqueous layer. Although slightly higher than *in vivo* conditions (which, as a reminder, ranges between 0.1 and 0.9 μm), this is the least we can obtain within the limitation of nebulization of at least 60 μL for uniformity of aerosol deposition in the VITROCELL® Cloud 6 system. However, the QCM has not provided reliable measurements of deposited surfactant mass as the reported QCM signal showed a continuous growth rather than the typically observed peak value after a few minutes and subsequent adjustment to a constant asymptotic value. This could be related to the fact that alveolar surfactant contains a high concentration of surface-active proteins, which inhibits evaporation of the aqueous phase. Indeed, in the presence of water or any other liquid, the QCM signal is more representative of the viscosity of the liquid than the deposited mass [36,48]. Nevertheless, the QCM signal demonstrates the high degree of reproducibility of surfactant deposition on a qualitative level, but the reported mass dose does not reflect surfactant mass (Figure 2).

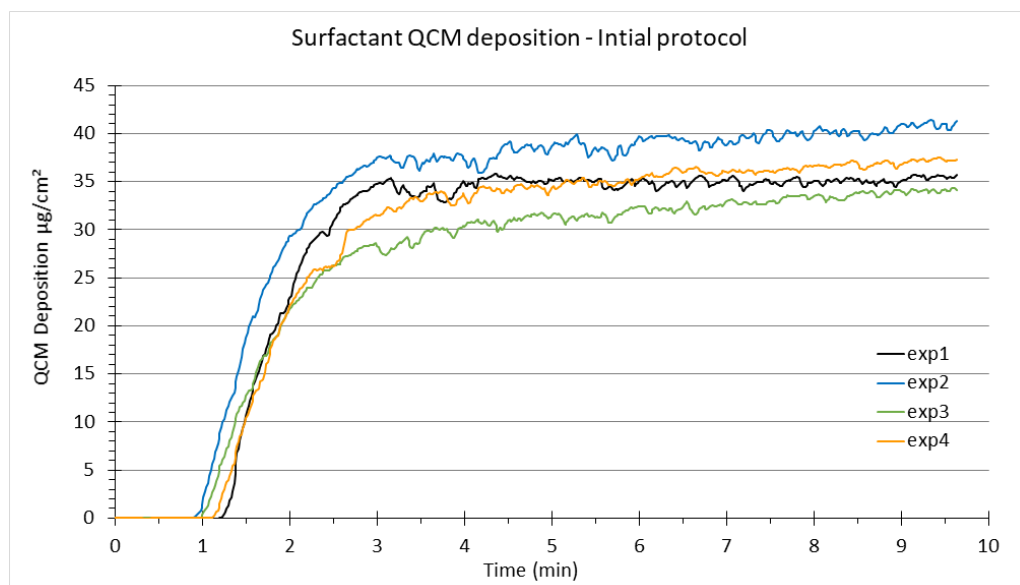


Figure 2. QCM curves showing the cloud deposition of surfactant (qualitatively) in the VITROCELL® Cloud System. This Figure illustrates the reproducibility of the QCM response for independent exposures under the same conditions. 61 μL of whole porcine surfactant solution at 40 mg/mL was nebulized, and the VITROCELL® Cloud temperature was maintained at 37°C. The nebulization starts between 30 sec and 1 min after start of data acquisition. Depicted are the four independent exposures performed in the present study before NP nebulization.

3.1.2. Homogeneous distribution assessment by fluorescence microscopy

In parallel, the homogeneous distribution of nebulized surfactant over the cells was assessed by fluorescence microscopy. The deposition of surfactant on the cells does not depend on the cell type. Therefore, the dosimetry data reported here for LA-4 cells also apply to any other cell type, including the NR8383 cells. Surfactant was labelled with STAR RED-DPPE fluorescent dye and nebulized or pipetted on a monoculture of LA-4 murine lung epithelial cells in ALI conditions. Fluorescence top-view panoramic scan was then used to observe the surfactant deposition (Figure 3). As confirmed using a fluorescence top-view panoramic scan over 1 cm (one half of the sample diameter), the nebulization of surfactant (Figure 3 A iii ,B iii) covers the entire sample with a homogeneous surfactant layer. In contrast, an alternative method of surfactant application, *i.e.* pipetting the surfactant directly onto the cells at the ALI (Figure 3 A i, B i) or onto cells slightly covered with cell medium (Figure 3 A ii, B ii), does not provide a uniform surfactant deposition. Moreover, the pipetted surfactant does not spread over the whole sample, leaving the majority of the cells without surfactant. The fluorescence panoramic scan of nebulized nanomaterial confirms that nebulization also delivers the nanomaterial uniformly over the entire sample (Figure 3 C). As shown in Figure 3 D, after nebulization onto the cells, surfactant formed a homogeneous layer on the air-liquid interface, with the remainder of the surfactant forming structures in the underlying aqueous cell medium (Figure 3 E). These results confirm that, when subsequent nebulization of surfactant and nanoparticles is performed, the resulting sample is uniform both in terms of surfactant and the cell-delivered dose of nanomaterial (Figure 3 F). This is crucial for replicating the physiological conditions.

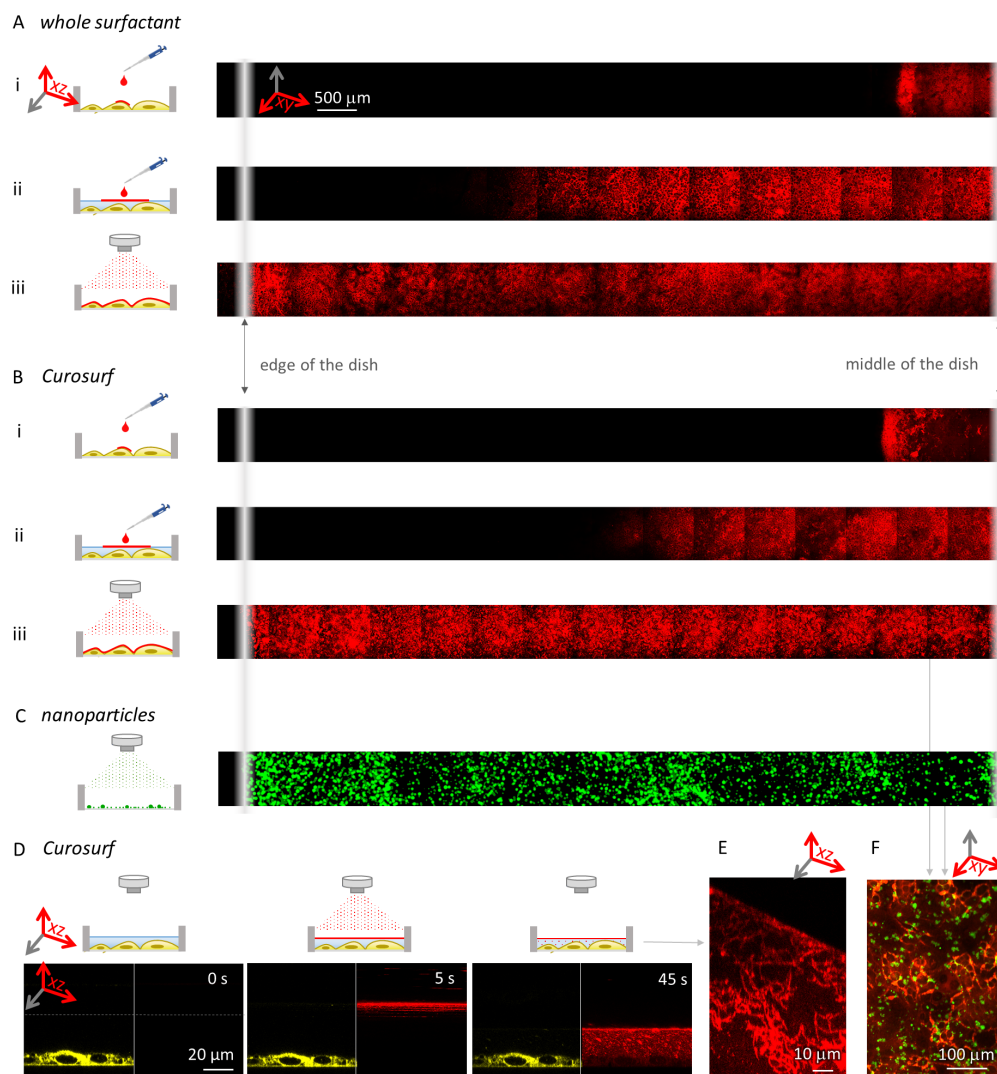


Figure 3. The deposition of nebulized and pipetted surfactant. 10 monolayers of fluorescently labelled **A)** the whole surfactant and **B)** Curosurf were administered onto cells using different methods. As shown in the top-view panoramas, in both cases **i)** surfactant, pipetted onto cells with removed medium (simulating ALI conditions), spreads only over a fraction of the sample, **ii)** surfactant pipetted onto cells slightly covered with cell medium spreads further, but still does not cover the entire sample, and **iii)** nebulized surfactant evenly covers the entire sample (fluorescence intensities are reported in Supp. Data 3). Note that the intensities are not comparable between measurements and the dark vertical lines are the consequence of uneven illumination. **C)** Nebulization of fluorescently labelled TiO_2 nanotubes (green) evenly deposits the material over the sample – shown here in a top-view panorama for nebulization directly onto a glass surface. Note that due to low signal, the image was filtered using a Gauss filter, and the intensity was scaled from 0 to 3 counts. **D)** A time series of nebulization of 10 monolayers of Curosurf (red, fluorescently labelled with STAR RED-DPPE) onto lung alveolar cells (green, labelled with CellMask Orange), (see Supp. Data 2 for complete time series). **E)** A side-view of the surfactant structure just below the surface after nebulizing 100 monolayers of surfactant (red) onto submerged cells. The surface of the medium is tilted due to capillary effects near the edge of the dish. **F)** A fluorescence top-view micrograph of 10 monolayers of nebulized surfactant (red) and subsequently nebulized 1:1 surface dose of nanomaterial (green) onto lung alveolar cells.

3.2. Improvement of the standard initial protocol

3.2.1. Standard initial protocol for NP nebulization in VITROCELL® Cloud 6 System

The initial exposure protocol that we used was set up as follows: 40 inserts were seeded with NR8383 cells out of four biological replicates (BR, 10 inserts per BR, 150 000 cells/insert) and incubated 24 h at 37°C, 5% CO₂. After the removal of their supernatant and the exposure to surfactant, establishing ALI, half of the inserts (5 inserts for each BR, representing 5 technical replicates (TR) for each BR exposed) were exposed to ultrapure water as control and the other half to NM105 NP (Figure 4). Cells were then put back into the incubator for 4 h and their RNA was finally extracted. Running this very first protocol, we encountered a few issues and realized it had a few flaws that could be corrected to be more biologically rigorous and to optimize RNA yield and quality.

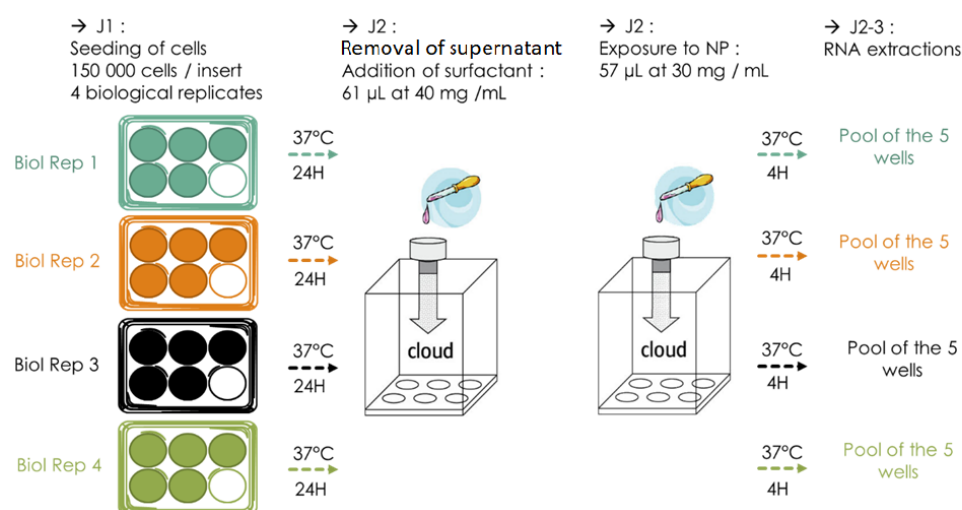


Figure 4. Initial exposure protocol. For each 4 biological replicate (BR), NR8383 were seeded in 5 wells, in Transwell® inserts. The next day, for each BR, the inserts containing the cells were exposed, by nebulization, back-to-back to 61 μ L of whole porcine surfactant at 40 mg/mL, and to 57 μ L of TiO₂ NP at 30 mg/mL. After exposures, the Transwell® insert containing the cells were kept at 37°C during 4 h, and the 5 wells (representing technical replicates, TR) of each BR were pooled for RNA extractions.

3.2.2. Current improved protocol for NP nebulization in VITROCELL® Cloud 6 System

The current exposure protocol makes use of 5 BR of which 8 TR were generated. For each BR, half of the inserts (4 inserts for each BR, representing 4 TR) were exposed to ultrapure water as control and the other half to NM105 NP (Figure 5). 300 000 cells were seeded in 1 mL of complete growth medium by inserts and allowed to incubate for 24 h at 37°C, 5% CO₂. Before exposure, the apical medium contained in the inserts was removed to establish the ALI configuration, 61 μ L of surfactant were then nebulized over the inserts, and the inserts were placed back into the incubator. Once each insert was exposed to surfactant, 114 μ L of TiO₂ NP at a concentration of 15 mg/mL were nebulized over the cells. cells where then incubated for 4 hours and the TR of each BR where pooled together for RNA extraction.

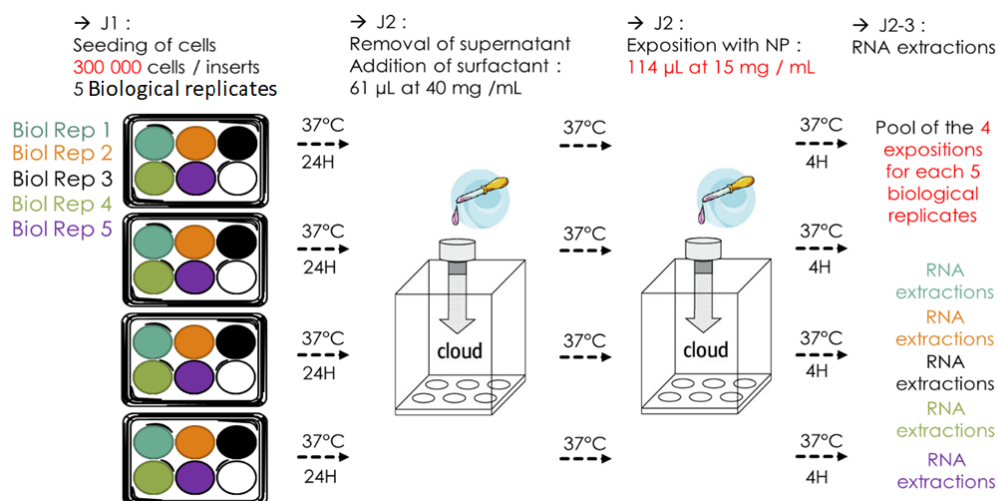


Figure 5. Current exposure protocol. For each 5 biological replicate (BR), NR8383 were seeded in 4 wells, in Transwell® inserts, distributed in 4 6 well plates. The next day, for all 6 well plates, the inserts containing the cells were exposed (representing one technical replicate (TR) of each BR), by nebulization, first to 61 μ L of whole porcine surfactant at 40 mg/mL, and then to 57 μ L of TiO₂ NP at 30 mg/mL. After exposures, the Transwell® insert containing the cells were kept at 37°C during 4 h, and the 4 wells (TR) of each BR were pooled for RNA extractions.

3.3. Troubleshooting flaws of the initial protocol

3.3.1. Preventing rapid drying of NPs on the nebulizer mesh by modifying the volume of nebulized liquid

The first issue encountered with these exposure parameters was that the TiO₂ NP suspension tends to rapidly dry out on the mesh of the nebulizer, which is likely a result of partial clogging of the pores of the mesh, making the TiO₂ NP depositions variable in time and quantity (Table 1 ; Figure 6 A). For a concentration of 30 mg/mL of NM105 TiO₂ NP suspended in 57 μ L of ultrapure water, we observed a mass deposition ranging from 2.1 to 7.4 μ g/cm² and the nebulization time needed for a stable QCM signal varied from 6 minutes to 24 minutes (Table 1). To address these high variations, we increased the volume to 114 μ L and reduced the concentration to 15 mg/mL (current protocol) by doubling the nebulized volume and reducing the TiO₂ NP concentration by two, so the same dose was present in the nebulizer as for the initial condition. This was aimed at maintaining the cell-delivered mass dose but reducing the range of variations in mass dose and in exposure time (Table 1 ; Figure 6 B). This improved the conditions from 5.1 +/- 2.7 μ g/cm² to 5.4 +/- 0.8 μ g/cm² and from 10.5 +/- 9.0 min to 5 min for 30 mg/mL and 15 mg/mL, respectively. Hence, the mass deposition was less variable, which can also be seen from the QCM signals (Table 1). It is noteworthy that unlike previous studies with the VITROCELL® Cloud 6 system we did not lift the exposure chamber after 3 – 5 min of cloud settling, since we did not want to disturb the potentially slower cloud settling of highly concentrated surfactant and TiO₂ NM105 aerosol. This prolongs the time for evaporation of the liquid layer on the QCM and hence extends the time for reaching an asymptotic QCM value as compared to previous studies [36,48].

Table 1. TiO₂ NP mass deposition comparison between the initial exposure protocol and the current protocol (asymptotic values at the end are reported). Exp1, exp2, exp3 and exp4 are abbreviations for the replicates of the exposures.

Exposition	Initial protocol (57 μ L, 30 mg/mL NP)		Current protocol (114 μ L, 15 mg/mL)	
	Mass deposited (μ g/cm ²)	Time (min)	Mass deposited (μ g/cm ²)	Time (min)
exp1	2.1	6	4.2	5
exp2	7.4	24	6.1	5
exp3	7.4	6	5.7	5
exp4	3.5	6	5.5	5
Mean	5.1	10.5	5.4	5.0
<i>SD</i>	2.7	9.0	0.8	0.0

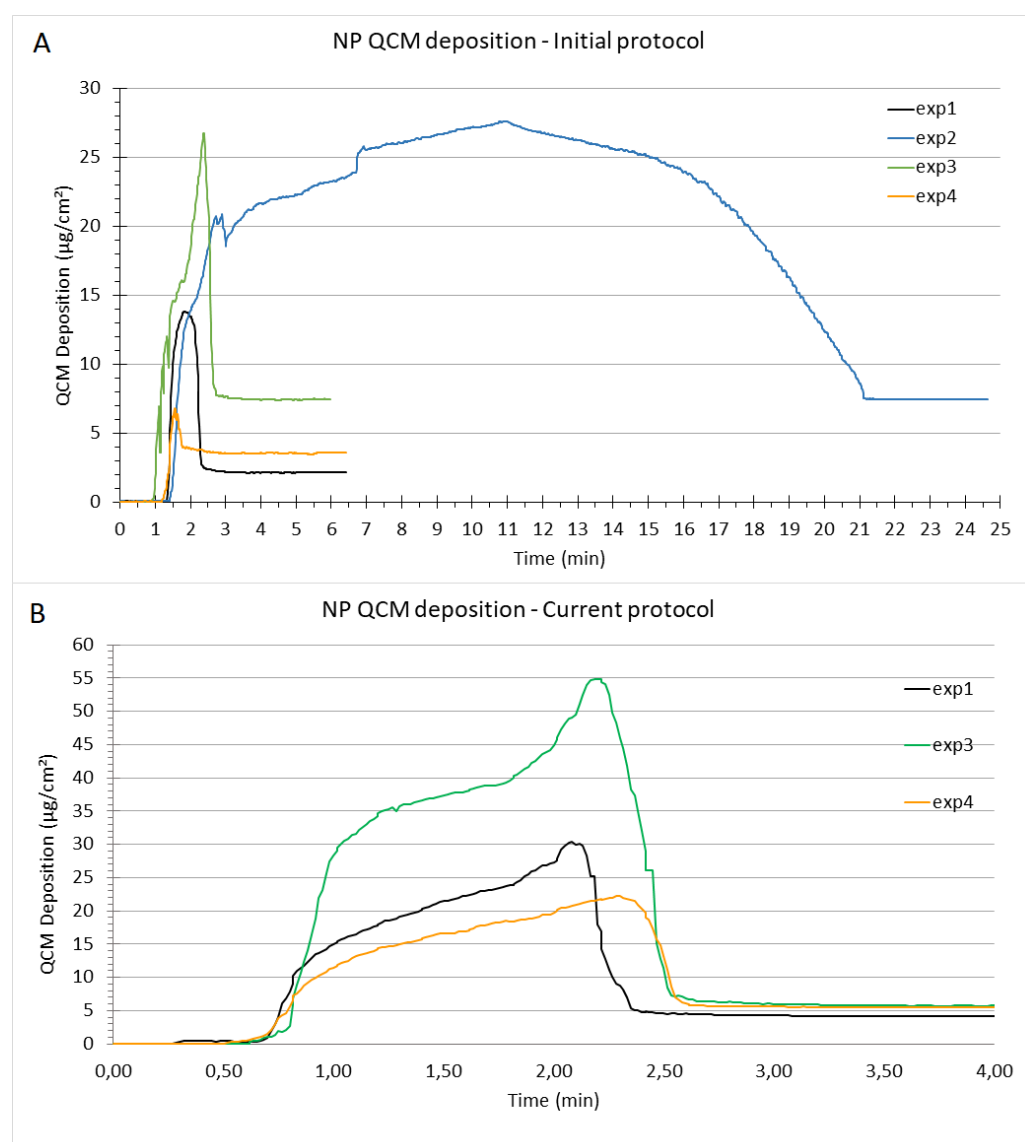


Figure 6. QCM curves showing in the first part the dynamics of cloud deposition in the VITRO-CELL® Cloud System followed by an asymptotic (constant) value, which represents the mass of the deposited TiO₂ NPs. The nebulization starts between 30 sec and 1 min after start of data acquisition. Depicted are A) the four NP exposures following the initial protocol (57 μ L of TiO₂ NP at 30 mg/mL) 2) three exposures following the current protocol (114 μ L of TiO₂ NP at 15 mg/mL), the second nebulization (exp2) is not shown because of data backup failure (see Table 1).

3.3.2. Loss of cells and poor RNA quality

NR8383 cells are a semi-adherent cell line, indeed half of the cell population adheres to the membrane of the insert whereas the other half stays in suspension. The issue is that the supernatant has to be removed before exposing cells to surfactant to reach ALI, leading to a loss of about 50% of cells (75 000 with the initial protocol). This issue sometimes resulted in a too-small amount of RNA and the loss of the pellet while performing washing steps during the RNA extraction. The low number of cells also forced us to retrieve a bigger fraction of the aqueous phase after adding chloroform, increasing the risk of protein or salt contaminations (optical density (OD) ratios should be between 1.8 and 2.2) (Table 2). To fix this issue, we doubled the cell density in the inserts, going from 150 000 cells/insert to 300 000 cells/insert and this led to an improvement in reproducibility and RNA quality. Mean RNA quantity went, indeed, from 83.61 ± 73.27 ng/ μ L to 50.46 ± 26.34 ng/ μ L, and OD_{260/230} ratio increased from 1.33 ± 0.70 to 2.16 ± 0.59 (Table 2).

Table 2. Quality and quantity of RNA extracted from NR8383 cells exposed to TiO₂ NP through nebulization following the initial or current protocol. Results in red indicate a poor RNA quality or quantity preventing the use of the sample.

Biological replicate	Exposure	Initial protocol			Current protocol		
		RNA quantity (ng/μL)	OD 260/280	OD 260/230	RNA quantity (ng/μL)	OD 260/280	OD 260/230
1	H ₂ O	89.04	1.66	1.84	32.31	2.09	2.09
2		7.99	2.25	0.46	55.30	2.01	1.80
3		121.07	2.12	1.14	69.60	2.13	2.18
4		208.10	2.04	2.32	16.09	2.08	1.43
5		/	/	/	48.94	2.18	1.98
1	TiO ₂ NP	3.30	1.86	0.26	3.61	1.83	3.72
2		96.96	2.03	1.81	61.96	2.14	2.16
3		8.01	2.48	1.25	91.57	1.99	2.05
4		134.43	2.08	1.55	58.93	2.10	2.02
5		/	/	/	66.30	2.06	2.17
Mean		83.61	2.07	1.33	50.46	2.06	2.16
SD		73.27	0.24	0.70	26.34	0.10	0.59

3.3.3. Increasing the reproducibility and repeatability

TiO₂ NP mass deposition variability was also addressed by a change in the organization of the exposures. With the initial protocol, we used 4 biological replicates (BR) divided into 5 technical replicates (TR) (Figure 4). Each TR of a BR was exposed to TiO₂ NPs at the same time in the VITROCELL® Cloud chamber, during the same nebulization (Figure 4). This initial protocol was less rigorous as it implied that the 4 BR would be exposed during different nebulizations and consequently, potentially to a different dose of TiO₂ NPs because of the variability of mass deposition between each nebulization, introducing a bias in the comparison of the effects of TiO₂ NP on each BR. To reduce this bias, we worked with 4 TR from 5 BR. One insert (TR) of each BR is placed in a well of the VITROCELL® Cloud System and exposed to TiO₂ NP Nebulization. This was realized for each of the 4 TR, and the 4 TR of each BR were pooled to proceed to the RNA extraction (Figure 5). This allowed us to smoothen the nebulization variation bias on every BR, as each would receive the very same dose of TiO₂ NP, thus the mass deposition variations would be the same for every BR (Figure 7).

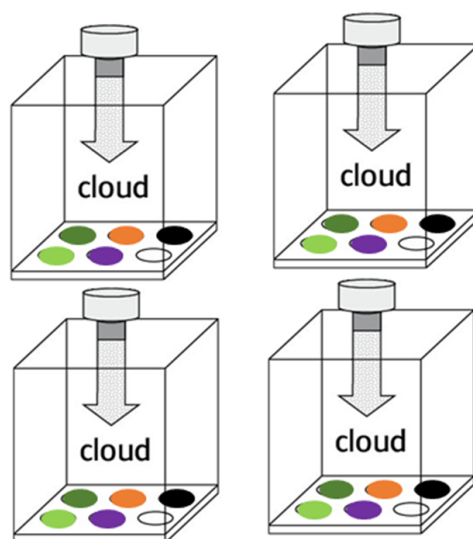


Figure 7. Methodology of NP exposure. One insert of each BR is placed in a well of the VITRO-CELL® Cloud System for each of the NP exposures. After pooling of all TR this ensures that all BR received an identical NP dose.

4. Discussion

Air-liquid exposure systems do represent closer-to-life scenarios in comparison to the traditional and currently most widely used submerged exposure methods, and this is not limited only to the Cloud systems. One of the main disadvantages of most aerosol-cell exposure devices (VACES, EAVES, CULTEX, NACIVT) is the lack of mass deposition control and - maybe more importantly - low dose rate, which often requires days to weeks of exposure time to observe any biological response *in vitro* cell culture models [39]. The choice of using VITROCELL® Cloud was based on short exposure times (a few minutes) and accurate control over the deposited NP mass dose on the cells using the quartz crystal microbalance. The latter enables the possibility to report the deposition variations on the results and to normalize the effects of NP dose on cell viability and gene expression. This control allows even more precise interpretations of the results. Moreover, the VITROCELL® Cloud system allows for surfactant coating of lung cells by nebulization before NP exposure, which is particularly relevant for cell models without innate surfactant secretion (*e.g.* alveolar macrophages). The use of surfactant provides the environment, closer to the lung physiological conditions, with controlled surface ratios between cells, surfactant, and nanomaterial.

It has been shown that surfactant can alter the physico-chemical properties of NPs and hence modulate their toxicity [49–51]. Since the nebulized surfactant completely covers the entire sample surface, the underlying cells are never directly exposed to the nanoparticles. Thus, the here-described method prevents possible artefacts arising from the direct interaction of the nanoparticles with the cells without surfactant. Moreover, nebulization of nanoparticles also prevents possible unrealistically high local doses of nanoparticles, the so called “hot spots” as described for *in vivo* experiments when instillation is used instead of inhalation [66]. Therefore, the nebulization of surfactant helps to produce results that tend to be closer to reality and are therefore more predictive for human health outcomes, which may ultimately lead to more reliable regulatory control of exposure to NPs.

It is important to consider that our problems with the initial protocol are probably a combination of several issues. Notably, it can be due to a partial clogging of the nebulizer pores for high NP concentrations (as used here, $30 \mu\text{g}/\mu\text{L}$), which then reduces the nebulizer output rate, enhances the nebulization time and then leads to partial liquid evaporation. Di Cristo *et al.* have published an interesting paper, with doses close to those we used, comparing the deposition efficiency of low volume ($30 \mu\text{L}$) of two TiO_2

483 NPs (NM100 and NM101), at lower concentration ($<100 \mu\text{g/mL}$) nebulized with the
484 Aeroneb® Pro Nebulizer Sytem directly upon the insert (while we nebulized $57 \mu\text{L}$ at
485 30 mg/mL , or $114 \mu\text{L}$ at 15 mg/mL of NM105 TiO_2 NPs with Aeroneb® Lab Standard
486 VMD Nebulizer, in VITROCELL® Cloud System). They showed that the deposition of
487 NP depends on the type of NP. Indeed, the deposition of NM-100 was homogeneous,
488 whereas NM-101 formed small and large aggregates when nebulized. For NM-100, the
489 doses delivered by aerosol were very similar to the initial concentration deposited in the
490 nebulizer (90% of deposition efficiency, efficiency *i.e.* 90% of invested dose deposited on
491 the bottom of the VITROCELL® Cloud system), whereas the deposition efficiency was
492 very low for NM-101 (values below 50%). Such an observation can be associated with
493 the formation of NP aggregates in the suspension that could remain trapped erratically
494 on the nebulizer mesh. Indeed, the dispersion of NPs during nebulization is strongly
495 dependent on the aggregation phenomenon occurring before and during the biological
496 experiments [67].

497 Moreover, using the same nebulizer for surfactant coating and then for NP exposure
498 increased the risk of clogging the mesh. Indeed, in the initial protocol, we nebulized back-
499 to-back surfactant and NP in each exposition, and we observed a frequent clogging of the
500 mesh and variation of the NP mass deposition, even if the nebulizer was cleaned between
501 each nebulization. The clogging probably occurs because lipids stick NPs together acting
502 like a cross-linker. We have observed that labelled surfactant stuck to TiO_2 NTs after just
503 the TiO_2 NTs were nebulized subsequent to surfactant nebulization (no measurements
504 available), without in-between cleaning. This was diminished when we exposed in
505 a first time all the 40 wells to the surfactant, and in the second time to the NP after
506 cleaning and sonicating the nebulizer (current protocol). Hence, a rigorous cleaning
507 procedure for the nebulizer is essential for the reliable operation of the VITROCELL®
508 Cloud system. If possible, different nebulizers should be used if two or more substances
509 are to be nebulized back-to-back, since this alleviates the cleaning requirements and
510 prevent possible cross-contaminations of the nebulized entities. But even if clogging
511 occurs, the fact that the actual dose delivered to the cells is measured with the QCM
512 during each exposure allows for reliable dose-response curves independent of the degree
513 of clogging.

514 During aerosol-cell exposure experiments, aerosol deposition is influenced by
515 several factors, including the aerosol-generating system, aerosol characteristics (particle
516 size, shape, density, etc.) and the inhalation pattern (flow rate and volume) [67,68].
517 The VITROCELL® Cloud system is unique in the sense that it uses air-less (no flow
518 rate) cloud dynamics (not single aerosol dynamics) for rapid and spatially uniform
519 dose delivery leveraging cloud sedimentation, which only depends on a high enough
520 aerosol/liquid volume per air volume (cloud density) [37]. Under those conditions,
521 aerosol-cell delivery is independent of aerosol size, shape and density provided the
522 cloud density is high enough to marginalize single aerosol deposition mechanisms as
523 compared to cloud settling. For the conditions of the VITROCELL® Cloud system, $200 \mu\text{L}$
524 of nebulized liquid ensures high enough liquid content of the air in the exposure
525 chamber to ensure cloud rather than single aerosol settling conditions [37]. Since this
526 may not be the case, if too small of a liquid volume is nebulized, it is recommended
527 to operate the VITROCELL® Cloud 6 system with $200 \mu\text{L}$. However, we have shown
528 here that also $110 \mu\text{L}$ can be sufficient for reproducible NP delivery. In addition, Ding
529 *et al.* [48] have recently demonstrated that the QCM signal corresponds to mass only
530 if the deposited NP layer is perfectly coupled to the quartz crystal. In the presence of
531 water (even more if there is a high volume nebulized), there is viscoelastic decoupling
532 of the NPs from the quartz and the signal is more representative of the viscosity of the
533 deposited NP suspension layer than of NP mass. Therefore, directly after the NPs has
534 settled onto the QCM, the top part of the exposure chamber is removed, which allows
535 for rapid drying of the NP layer on the QCM.

One might raise the issues on realistic dosimetry and exposure routes, *e.g.* whether the dose is too high or delivered too fast with the VITROCELL® Cloud 6 system as compared to ambient or occupational exposure scenarios. Moreover, in real-life exposure scenarios nanoparticles do not enter human lungs as liquid droplet suspension but rather in dry form, and the NP crosses the entire respiratory tract before reaching the lung cells. However, upon deposition onto the lung lining fluid all initially dry NPs will get in contact with the liquid phase of the lining fluid. After all, nebulizers are currently the most developed system for aerosol lung delivery used as well as in research experiments and in the clinic [62,69]. Dubus *et al.* have demonstrated that such a nebulizer (Aeroneb® Pro Nebulizer) provided 12% to 14% dose deposition in macaque lung, *i.e.* a 25 fold greater deposition than another nebulizer (MistyNeb 0.5%), this information is useful to compare the dose delivery in ALI exposure cell-monolayer and *in vivo* lung exposure.

Considering the bulk of studies about TiO₂ nanoparticles (NP) toxicity, there are few publications comparing *in vitro* and *in vivo* exposures, and even fewer comparing air-liquid interface exposure (ALI) with other *in vitro* and *in vivo* exposures. Therefore, after ALI cell exposure to TiO₂ NP and RNA extractions, described in this article, we aimed to identify early specific markers of lung exposure to TiO₂ NP, by a large study of genic expression modifications, mainly transcriptomics, and comparing classical submerged *in vitro*, ALI and *in vivo* exposures [63,64]. Knowing that nanoparticles can reach the alveoli [24], NR8383 cells are an appropriate model because they are alveolar macrophage precursors, which are the first implicated cells in the alveolar clearance of nanoparticles [70–72]. Moreover, this cell line has already been studied and validated as a model in the field of nanotoxicology and is relevant for their immune functions [73–75].

Nevertheless, our methodology could be improved by using ALI co-cultures of cell lines instead of monocultures, to be even closer to physiological conditions, considering the interactions that would exist between the different cell types. The first ALI co-culture was described in the 1980s with the growth of endothelial and epithelial cells on a cellulose membrane [76]. The simplest and most used ALI model is the cell monoculture (with cell lines or primary cells) and this model was largely used for studying transepithelial electrical resistance (TEER) [77–80].

Recently, Kletting showed that by cultivating differentiated m0 macrophages (THP-1) in the presence of alveolar epithelial cells (hAELVi) at the air-liquid interface, an extracellular matrix was secreted at the surface and their transport study showed a functional air-blood diffusion barrier phenotype [81]. Such a phenotype would greatly serve our pulmonary NP toxicity study, as it would mimic even more accurately the pulmonary physiological conditions. Another study conducted by Klein *et al.* [82] highlighted that SiO₂ NP exposures in ALI conditions induced a higher ROS generation in monocultures than co-cultures (macrophages + endothelial cells + bronchial epithelial cells + mast cells). It showed that the effects encountered in monocultures tend to be over evaluated compared to results obtained when culture conditions step closer to physiological conditions [82]. Currently, the best way to replicate pulmonary environment in ALI is to set up co-cultures or 3D cultures [83]. Finally, some aerosol cell exposure systems, recently developed, include continuous airflow (VITROCELL® Automated Exposure Station) [35,38,39] and even cyclic stretch, to simulate *in vitro* the mechano-elastic stimulation by continuous inhalation and exhalation [84–86].

5. Conclusions

The goal of this work was to refine an ALI pre-established protocol. As far we know, such a protocol has not been previously published. Therefore, after addressing each crucial steps, (*i.e.* the use of very low concentrations of NP, the deposition of a homogeneous layer of surfactant, the specific use of semi-adherent cells, and finally the enhancement of the extraction yield of RNA to carry out transcriptomic studies), we present here for the first time a robust almost ready-to-use protocol. This one will be

helpful for nanotoxicologists interested in developing such methodologies. This protocol is not frozen, and researchers can feel free to suggest improvements.

Finally, we do not recommend replacing the existing standard operating procedure [37], but we present four optimization points when exposing semi-adherent cells to NPs under ALI conditions with the VITROCELL® Cloud 6 system (1); if low volumes and high concentrations are nebulized (2); when two sequential aerosol exposures are performed back-to-back (here: lung surfactant and NPs in 61 μL and 114 μL , respectively) (3); and how to arrange the wells to reduce the variability between the biological replicates (BR) (4):

1) When using semi adherent cells, the number of cells seeded on the insert has to be increased (as compared to submerged culture conditions) to ensure that enough cells stay on the membrane after discarding the apical medium to reach a satisfying RNA yield.

2) To avoid any clogging of the mesh of the nebulizer and to obtain a reproducible cell-delivered dose, the manufacturer recommendations for cleaning of the nebulizer should be adhered to and the TiO_2 NP concentration has to be decreased by increasing the nebulized suspension volume (from 57 μL of 30 mg/mL to 114 μL of 15 mg/mL in this case for TiO_2 NM105 NP). This will prevent the NP suspension from both drying out on the nebulizer mesh and alteration of the NP deposition on the cells due to low cloud density in the VITROCELL® Cloud exposure chamber.

3) When two sequential aerosol exposures are performed back-to-back, when applicable, we suggest using a different nebulizer for each liquid nebulized. If the same nebulizer is used, we suggest to nebulize all replicas with the first type of aerosol and then with the second, and to clean the nebulizer between each sequence of nebulization thoroughly as recommended by the manufacturer.

4) If it is necessary to proceed to several expositions, we recommend including each BR in each exposure session (divided into technical replicates (TR), one TR per exposition). By doing this and subsequent pooling of the TRs of each BR, the variability in deposited dose amongst expositions will be spread over every TR in the same manner for each BR.

After the optimization of this protocol, we were able to obtain a high quality and quantity of total RNA extraction from this VITROCELL® Cloud exposition and we performed a complete transcriptomic study [64] including a comparison of *in vivo* and air-liquid interface and submerged *in vitro* results for TiO_2 NM105 NP exposure [63].

The optimization of the existing protocols is essential when moving to new or different experimental conditions (e.g., in our case of semi-adherent cell cultures, surfactant and low doses of NPs). The method that we employed to optimize/refine the previously established protocol could efficiently be transposed to 3D-cultures [87–90] or culture of organoids [91–95], which represent today the *in vitro* models closest to physiological conditions, considering the interactions existing between the different cell types. Furthermore, the approach that we used could serve as a basis for further developments which, in the long term, could significantly reduce the need of animal studies for toxicology research.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2079-4991/11/1/0/s1>, Figure S1: Nebulizer/tube/ μ -dish setup for microscopy; Figure S2 : Time series of nebulizing Curosurf-StarRed, fluorescence microscopy at 561nm, 640, and 950nm. Most of the material is nebulized in the first 30 seconds, and the system settles in a few minutes; Figure S3 : Intensity distribution of Curosurf-StarRed fluorescence, under different conditions

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 653 available as VITROCELL® Cloud. The other authors declare that they have no conflict of interests.
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 655 data; in the writing of the manuscript, or in the decision to publish the results.

656 Abbreviations

657 The following abbreviations are used in this manuscript:

658	MDPI	Multidisciplinary Digital Publishing Institute
	NP	Nanoparticles
	ALI	Air-liquid interface
659	QCM	Quartz crystal microbalance
	BAL	Bronchoalveolar lavage
	BR	Biological replicate
	TR	Technical replicate

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