PROJECT ACRONYM

CUPIDO

PROJECT TITLE

Cardio Ultraefficient nanoParticles for Inhalation of Drug prOducts

Deliverable D3.2

In vitro assessment of nanoparticle behaviour at/across the pulmonary barrier (M24)

CALL ID			H2020-NMBP-2016-2017
GA No.			720834
MAIN CONTACT			**Prof Terry Tetley** Email: t.tetley@imperial.ac.uk
NATURE	Report (R)	DISSEMINATION LEVEL	PU
DUE DATE	31/01/2019	ACTUAL DELIVERY DATE	31/01/2019
AUTHOR(S)			Prof Terry Tetley and Dr Michele Chiappi

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 720834.

Table of Revisions

REVISION NO.	DATE	WORK PERFORMED	CONTRIBUTOR(S)
1	06/01/2019	Original version	Michele Chiappi
2	07/01/2019	Editing	Terry Tetley
3	07/01/2019	Revision	Daniele Catalucci
4	08/01/2019	Revision	Terry Tetley & Michele Chiappi
5	17/01/2019	Approval	Ethics Board
6	25/01/2019	Approval	CCG
7	31/01/2019	Revision & Approval	IPR team

Table of Contents

Index of Figures and Tables

1. Executive summary

IMP performed mechanistic and kinetic studies of cellular uptake and translocation/fate of fluorescent nanobeads (FluoSphere™) deposited at the apical alveolar gas-liquid interface, using a human lung cell model of human alveolar epithelial type 1 cells in vitro (TT1). TT1 cell monolayers were cultured on porous membranes (Transwell membrane inserts) under static or dynamic conditions. 24 hours after nanoparticle exposure, nanoparticle concentrations were calculated in the different compartments of the model: apical, extracellular (upper well), intracellular uptake and basal, translocated nanoparticles (lower well). Study under static conditions utilised the as-purchased Transwell insert-plate system whilst study under dynamic conditions utilised a bioreactor device developed by CNR-IEIIT.
These studies established which membranes were best to study fluorescent nanoparticle uptake and translocation under static and flow conditions. They show that the nanoparticles were taken up apically and translocated across the static model of the air-liquid barrier at measurable concentrations. The investigations of nanoparticle translocation under dynamic conditions were very promising and now require further optimisation.

Key deliverable achievements:

1. Established best growth conditions of TT1 cell monolayers on porous membranes under static conditions.
2. Established standard curve for FluoSphere™ polystyrene nanobeads, used for proof of concept studies, to accurately quantify intra- and extra-cellular nanoparticle concentrations and translocation through the cell-membrane barrier.
3. Measured translocation of FluoSphere™ translocation through the porous Transwell membranes, with and without cells, using a range of imaging techniques.
4. Design and generation of an air-liquid bioreactor device for cell culture.
5. Set up bioreactor for nanoparticle treatment studies under flow conditions and performed preliminary feasibility studies of epithelial cell monolayer integrity.

2. Cooperation between participants

1. IMP, CNR-IEIIT, and CNR-IRGB collaboration in developing and upgrading bioreactor device to mimic the gas-blood interface of the lung.

2. Manufacture and provision of fluid-flow bioreactor devices by CNR-IEIIT to perform preliminary dynamic particle translocation studies.

3. *In vitro* assessment of nanoparticle behaviour at/across the pulmonary barrier

3.1. Translocation of fluorescent nanoparticles across human alveolar epithelial type 1 cell (TT1) monolayers

Uptake and translocation/fate of nanoparticles across the alveolar gas-liquid interface (TT1 cell grown on transwell membrane inserts) was measured using fluorescent nanobeads (FluoSpheres™, ThermoFisher scientific), with features of CaP (size and surface charge) as a proof of concept. The aim was to optimise conditions prior to using the CUPIDO project fluorescent CaP nanoparticle formulations. FluoSphere™ are carboxylate-modified, 40nm diameter, red fluorescent (580/605 nm wavelengths), negative surface charge 0.6631 meq/g.

3.1.1. Comparison of FluoSphere™ translocation across acellular and cell-populated transwell membranes with different pore sizes

FluoSphere™, suspended in serum-free RPMI medium at different concentrations, were deposited onto naked and cell-populated transwell inserts (PET membrane) and measured after 24h to determine the degree of nanoparticle passage through the pores (Figure 2) under static conditions. Different pore size membranes (M1, M2, and M3) were tested.

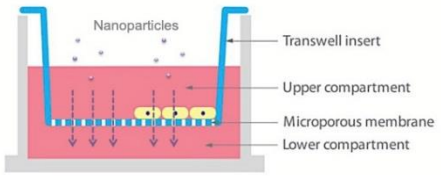

Figure 1. Diagram of the commercial transwell insert model system (Corning-Costar and Falcon) to optimize exposure conditions to determine FluoSphere™ translocation and fate across the naked, cell free membrane (left) and in the cellular alveolar air-liquid interface model (in yellow, right).

FluoSphere™ passage through naked membranes under static conditions. 24h after introduction into the apical chamber, nanoparticle concentrations were evaluated in both the upper (non-translocated) and lower (translocated nanoparticles) chambers for each of the transwell inserts.
Two concentrations of FluoSphere™ were tested: a low concentration of 50 µg/ml (10 µg/well total) and a high concentration of 100 µg/ml (20 µg/well total) were placed into the upper compartment. Relative fluorescence intensity (FI; ~1000 for 10 µg and ~2300 for 20 µg) was determined in aliquots placed in a black 96 well plate fluorometer reader. Relative FluoSphere™ concentrations were calculated by interpolating the obtained OD values into the standard curve established as described in Figure 2.

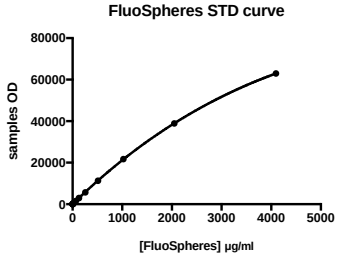

Figure 2. FluoSphere™ standard curve. Serial dilutions of nanoparticles from 0.5 to 4096 µg/ml RPMI-1640 were generated and their fluorescence intensities were measured through a fluorescence 96-well plate reader at excitation/emission wavelengths of 530/25 and 620/40 nm.

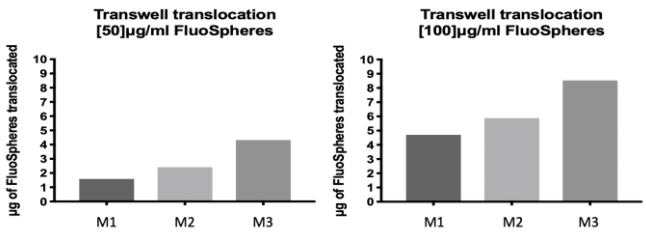

Figure 3. FluoSphereTM (50 and 100 µg/ml) translocation through naked transwell inserts. Translocation trough M1, M2, and M3 membranes showed differential results. Each condition was performed in triplicate, data are shown as the mean.

M3 membranes allowed the highest amount of nanoparticle translocation into the lower chamber (Figure 3). Interestingly, doubling the applied concentration of nanoparticles doubled the number that translocated, suggesting no saturation. For example, for M3, ~4 µg translocated at the low concentration (~40%), and ~8 µg translocated at the high concentration (~40%).

FluoSphere™ passage through confluent TT1 cell-occupied membranes under static conditions. TT1 cells were grown to confluence on porous transwell membrane inserts, in RPMI-1640 tissue culture medium (10% serum, no phenol-red). Complete confluence was essential as any cell-free space would allow uncontrolled nanoparticle translocation directly through the membrane pores invalidating the final results. Exactly the same experiments, as described above, were performed in the presence of TT1 cells (Figure 1, right) grown on the 3 types of transwell membranes. After 24h the cell uptake and translocation across the membranes of the FluoSphere™ were analysed by measuring the FI in the upper and lower chamber media and the FI associated with the lysed cells, calculated by interpolating the FI values into the standard curve.

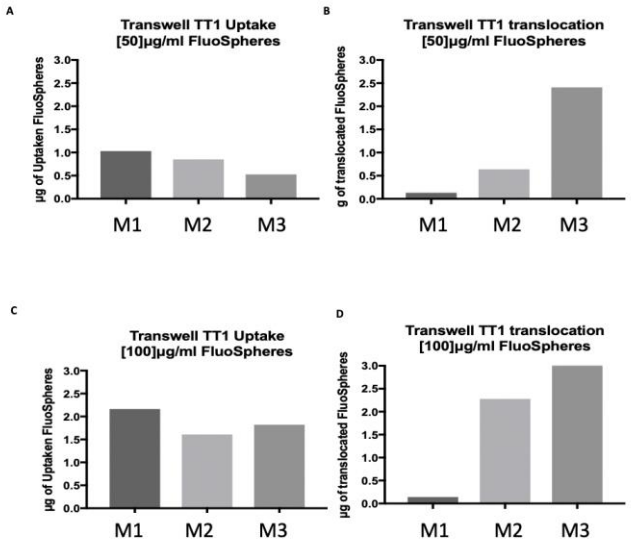

Figure 4. FluoSphereTM uptake and translocation (24h) through different Transwell inserts in the presence of TT1 cell monolayers. FluoSphereTM uptake (A) and translocation (B) at 50 µg/ml (10 µg applied), and uptake (C) and translocation (D) at 100 µg/ml (20 µg applied), are shown. Each experiment was carried out in triplicate, the data are shown as the mean.

This study clearly indicated that although FluoSphereTM were taken up by cells grown on M1 membranes, translocation was low. In contrast, for M2 membranes, nanoparticle uptake and translocation were significant, indicating active and cell-dependent (cell-limited) nanoparticle translocation. Of the three membranes studied, the M3 showed the highest amount of nanoparticle translocation (~28%) and the M2 allowed ~15% translocation following treatment with the higher FluoSphere™ concentration (Figure 4 B and D).

3.1.2. Determination of monolayer structural integrity

TT1 cell monolayers were grown and treated with the same concentrations of FluoSphere™ as described above. These high concentrations were selected to check the integrity of the cellular barrier in more extreme conditions and to prove the high biocompatibility and non-toxicity of the FluoSphere™.

MTT viability

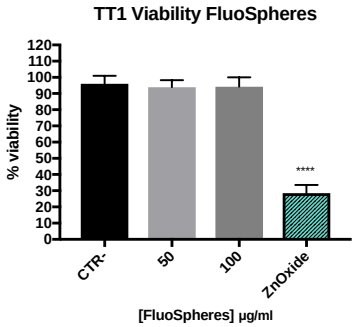

Figure 5. MTT viability of TT1 cell monolayers after 24h incubation with FluoSphereTM at 50 and 100 µg/ml. Unexposed TT1 cells were used as negative control (100% viability) and highly toxic zinc oxide nanoparticles at 10 µg/ml were used as positive control. ****, p<0.0001. n=3 separate experiments, performed in triplicate.

TT1 cell viability, measured as mitochondrial metabolic activity, was not significantly different to untreated control (CTR-). Zinc oxide (ZnOxide) nanoparticles caused 70% cell death as positive control (Figure 5). Thus, there is an intact monolayer without unwanted cell death following FluoSphere™ treatment.

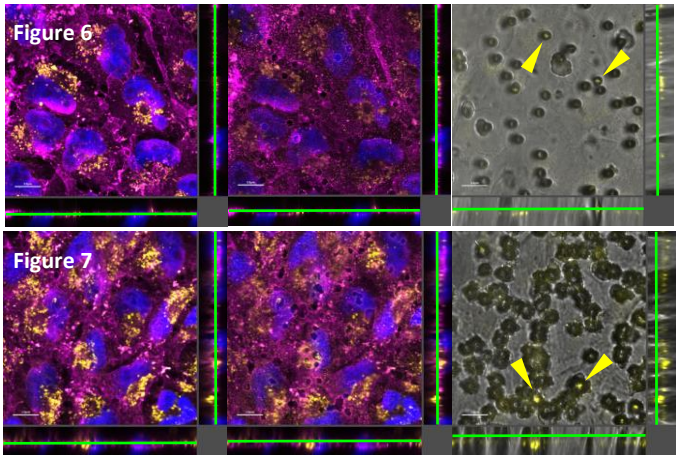

Figure 6. (upper), M2 membrane.

Figure 7. (lower), M3. Confocal Z-stack analysis of TT1 cells incubated with FluoSpheres™ for 24h. Nanoparticles in yellow. Nucleus in blue, cell membrane and endosomes purple. Same region of interest (ROI) analysed in different virtual planes (green lines, in lateral views) to visualise: FluoSphere™ localisation inside the cytoplasm along a central plane (left panel), cell membrane integrity and structure along the basolateral plane (central panel) and nanoparticle agglomeration state at the membrane pores level using a lower virtual plane cutting the transwell membrane longitudinally (right panel). Yellow arrowheads indicate small groups of FluoSphere™ within the membrane pores.

3D confocal microscopy Z-stack scans were performed and stack series were analysed by Fiji and Icy imaging software to visualize the samples along different Z planes (Figures 6 and 7).
These images show the yellow fluorescent FluoSphere™ structures (along different planes of the stack series, green lines in lateral views) within the cell cytoplasm, in clusters close to the perinuclear area and more dispersed at the basolateral cell membrane of TT1 cells. There was cell membrane (purple) integrity and continuity (no gaps at cell-cell junctions, Figure 6 and 7, left micrograph) and at the cellular basolateral interface around cell perimeters (Figure 6 and 7, central micrograph). Circular dark holes in the basal cell image (Figure 6 and 7, central micrographs) were observed perfectly overlapping the regular pores of the transwell membrane underneath (Figure 6 and 7, right micrograph). A virtual longitudinal plane cutting deeper into the membrane shows small groups of FluoSphere™ within the transwell pores (Figures 6 and 7, right micrographs, yellow arrowheads), fixed during translocating the barrier through the pores and reaching the lower transwell collection chamber.

Lateral view confocal microscopy reconstruction analysis of FluoSphereTM location at the transwell membrane surface and in association with pores. A more detailed analysis of TT1 cell monolayers grown on M3 membranes is illustrated in Figure 8.

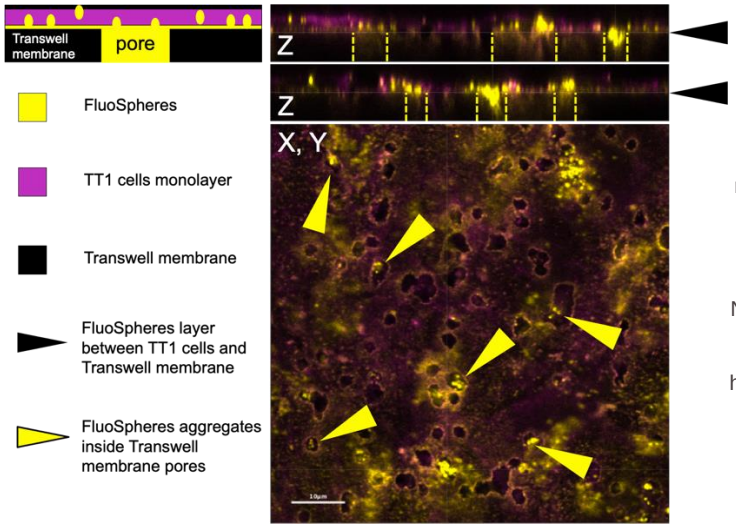

Figure 8. Lateral view confocal microscopy reconstruction analysis on FluoSphere™ location at the transwell membrane surface and associated with membrane pores. TT1 cells are purple. The M3 transwell membranes appears as a black band underneath the cells (see Z planes, top right). Nanoparticles are yellow. Black arrowheads indicate the FluoSphere™ layer deposited on the Transwell membrane in the lateral view of the Z stacks, top right; here, the aggregates of FluoSphere™ are clear as bright yellow clusters above the pores, shown within the dashed yellow lines. Yellow arrowheads indicate FluoSphere™ aggregates within the membrane pores (overhead view, X,Y aspect, lower right panel).

The specific virtual plane corresponding to the space between basal cell membrane and polystyrene membrane surface revealed the presence of a thin layer of FluoSphere™ both dispersed and in small clusters of aggregated particles. This thin layer can be observed in the overhead view square panel (Figure 8, X, Y aspect) and in the lateral views (Figure 8, Z aspect; two black arrowheads). A simplified colour-coded diagram of the lateral view (Figure 8, top left) explains the different components of the virtual transverse section of the Z-stack. The TT1 cell monolayer is represented as a purple band and the transwell membrane as a black band below. There are small groups of FluoSphere™ within the pores (Figure 8, X, Y, yellow arrowheads; pores

depicted in Figure 8. Z as dashed yellow lines. These clearly contain nanoparticle clusters, supporting TT1 cell uptake and subsequent transmembrane nanoparticle translocation.

3.1.3. Bioreactor device set up: preliminary in vitro tests

These on-going studies aim to apply proper stimuli on cell culture of TT1 cell monolayers and compare with the static model. Based on the findings above, M2 and M3 membranes were used for the bioreactor studies. Furthermore, as their plastic insert wells differ slightly in diameter and shape; these ongoing tests will also determine which best suits the bioreactor device.
Different flow rates will be set to regulate the induced fluid flow.
Preliminary results are promising and further work aims to assess the device stability and to set up the best conditions for reliable and reproducible experiments for the CaP exposure studies.

4. Conclusions

IMP has performed mechanistic and kinetic studies on TT1 cell uptake and translocation/fate of fluorescent nanoparticles (FluoSphere™) with a similar size to the CaP particles (dissolved from dpCaPs) that are being used as a drug delivery system in CUPIDO. These commercially available bright fluorescence nanoparticles were selected for these preliminary studies as a proof of concept and to establish and optimise the conditions before performing the tests on fluorescent CaPs produced by CNR-ISTEC for the CUPIDO consortium.
Cell monolayers were grown on different membrane inserts in static conditions to mimic the gas-liquid barrier of the human alveolar unit. A combination of imaging and bioassays confirmed that the FluoSphere™ were not toxic and did not modify the TT1 monolayer. Under static conditions, the data thus far indicate that the particles were internalised by TT1 cells and a proportion of these were translocated to the basolateral chamber. As we have shown that even high concentrations of dpCaPs are not toxic to TT1 cells, this is very promising in terms of using inhalation as a route of drug delivery to treat the heart. Preliminary studies are under way under conditions of flow using the bioreactor device (as developed in collaboration with CNR-IEIIT and constructed by CNR-IEIIT).