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Description of the deliverable (3-5 lines)	D1.1 defines the *in vitro* and *in vivo* models to be used in the course of the whole project for both efficacy and biocompatibility studies, together with their respective analysis/characterization methods. The choice was made upon scientific literature analysis and partners' interdisciplinary modelling at various complexity levels of the aerosol-based therapeutic approach.
Key words	models, methodology, *in vitro*, *in vivo, P. aeruginosa, S. aureus,* biofilm, TCC, ASM, mouse lung infection model

DOCUMENT HISTORY

NAME	DATE	VERSION	DESCRIPTION
Santi Nonell Marrugat	20/11/20	V1	First draft
Giovanni Romano Elena Reddi Aras Kadioglu Nelly Henry	23/11/20	V1.1	First Review
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Santi Nonell Marrugat	30/11/20	V2	Final version

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DEFINITIONS & ACRONYMS

aPDT: antibacterial photodynamic therapy

ASM: Artificial Sputum Medium

CFU: Colony Forming Unit

***E. coli*:** *Escherichia coli*

LED: Light Emitting Diode

LEP: Light Emitted Particles

PDT: photodynamic therapy

***P. aeruginosa*:** *Pseudomonas aeruginosa*

RT-PCR: Real time PCR (Polymerase chain reaction)

S. aureus: *Staphylococcus aureus*

TCC: Triple Cell Culture

5-ALA: 5-aminolevulinic acid

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EXECUTIVE SUMMARY

D1.1 has the aim to define the models to be used in the course of the whole project. These will be used both to study the efficacy of the proposed therapeutic approach (luminous aerosol) and to assess its biocompatibility and possible toxicity. The chosen models for efficacy studies are characterized by an increasing complexity, starting from *in vitro* (bacterial species in planktonic and biofilm forms and artificial models for sputum - ASM) up to the *in vivo* (mouse lung infection models). Of course, all the models considered for efficacy studies share the presence of the bacterial species of *P. aeruginosa* and *S. aureus*, corresponding to the final target of the proposed therapeutic approach. The same *in vitro* – *in vivo* scheme applies to the choice of the models considered for biocompatibility/toxicity studies: *in vitro*: mammalian cells; multiple-species cell cultures – TCC; *in vivo*: the mouse model.

Being the initial choice of the bacterial species already defined, effort was devoted to the choice of the specific strains and respective infection models, based on both the analysis of literature results and the various partners' previous know-how in their respective fields.

These same criteria were used to define the models for biocompatibility/toxicity studies.

The methodologies described in this deliverable will be revised in the light of the activities carried out during months 13-24; the final methodologies and protocols will be given in D1.3.

The consortium will work on the regular revision and update of this deliverable and specially its annex.

1 INTRODUCTION

1.1 Project Overview

The FET Open project entitled **Inhalable Aerosol Light Source for Controlling Drug-Resistant Bacterial Lung Infections** (with acronym **Light4Lungs** and Grant agreement ID: 863102) proposes a novel approach to address the problem of antimicrobial resistance in the treatment of chronic lung infections, which are the leading cause of morbidity and mortality in patients with diseases such as cystic fibrosis and hospital-acquired lung infections (Johnson, 2011).

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The goal is to develop a novel therapeutic scheme for the treatment of the infections, replacing antibiotics by inhalable light sources that will excite bacterial endogenous photosensitizers (e.g., iron-free porphyrins) (Amos-Tautua, 2019), eliminating the pathogenic bacteria by the photodynamic effect (local production of cytotoxic reactive oxygen species by the combined action of light, a photosensitiser and oxygen) irrespective of its multidrug resistance profile. The aim is to have a safe treatment for the host tissue thanks to its lack of self-photosensitising ability.

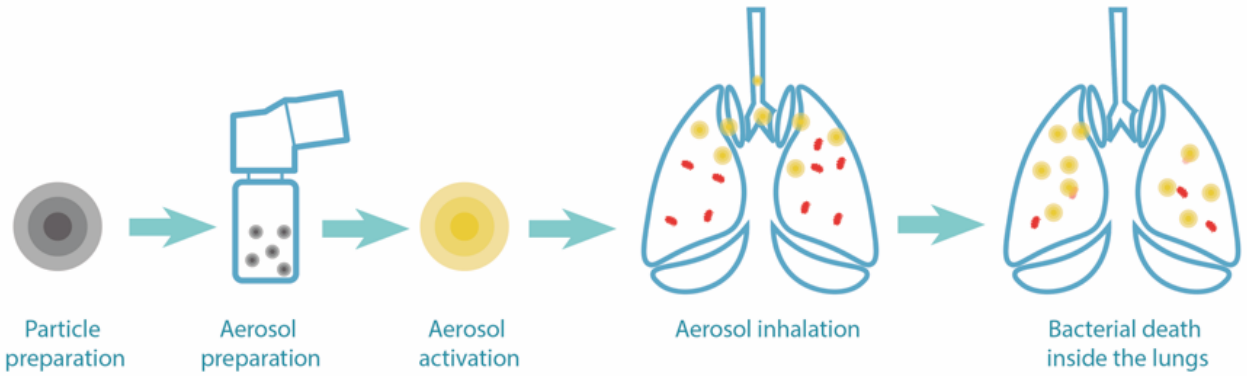

Figure 1. The Light4Lungs 5-step concept

This project is carried out by an international consortium formed by the following institutions:

1. INSTITUT QUIMIC DE SARRIA (IQS-URL
2. UNIVERSITA DEGLI STUDI DI FIRENZE (UNIFI)
3. SORBONNE UNIVERSITE (SU)
4. FUNDACIO PRIVADA INSTITUT CATALA D'INVESTIGACIO QUIMICA (ICIQ)
5. UNIVERSITA DEGLI STUDI DI PADOVA (UNIPD)
6. CARDIFF SCINTIGRAPHICS LIMITED (CSL)
7. THE UNIVERSITY OF LIVERPOOL (UNILIV)
8. WEDO PROJECT INTELLIGENCE MADE EASY SL (WeDo)

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1.2 Light-Emitting Particles and Aerosol

Development of light-emitting particles (LEPs) with persistent luminescence is the core of the project. Delivery of LEPs to the lungs is expected to achieve extensive photodynamic killing of the bacteria responsible for the infection, notably without invasive illumination devices or drugs. To achieve this goal, LEPs must meet a number of requirements concerning their optical, photophysical and chemophysical properties, will be outlined in the following sections. Equally important is the delivery of the LEPs to the lungs, which will be achieved by the use of aerosol technologies. The requirements for such aerosol delivery systems are also outlined in the sections below.

2 *IN VITRO* MODELS

The *in vitro* models considered in the Light4Lungs project are the following: (i) bacterial strains; (ii) the ASM model; (iii) the TCC model; (iv) mammalian cells. Cases (i), (ii) and (iii) will be considered to assess the efficacy of the aerosol-based aPDT approach; cases (iii) and (iv) will be used to study its biocompatibility/toxicity.

2.1 Bacterial strains, *in vitro* planktonic cultures and biofilm

In general, a minimum of two strains of *P. aeruginosa* and *S. aureus* respectively, will be considered for both *in vitro* and *in vivo* experiments. For each bacterial species, one reference strain and a clinical isolate will be selected. Literature results show that both the species of *P. aeruginosa* and *S. aureus* are well known to be naturally photolabile. Nevertheless, it is well-known that the specific concentration of endogenous porphyrins and porphyrin type(s) depends on many different factors, such as the specific strain and growing conditions. Therefore, the choice of the specific strains was demanded first to their importance and representativeness from a microbiology/clinical microbiology point of view and, in a second instance, to their specific porphyrin content.

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Being aPDT based on the presence of endogenous porphyrins in bacterial species, bacterial porphyrin content will be acknowledged by qualitative and quantitative measurements.

Proposed strains to be tested in in vitro experiments (first panel)

Species	Strain	Features	Genome sequence
P. aeruginosa	LESB65	Liverpool epidemic strain from cystic fibrosis. Used in *in vivo* model.	https://pseudomonas.com/strain/show/107
P. aeruginosa	PAO1	Laboratory strain, originally isolated from a wound.	https://pseudomonas.com/strain/show/668
S. aureus	USA300		
S. aureus	MRSA	Clinical strain obtained from patient	

Biofilm *in vitro* models will be developed first, due to their higher proximity to *in vivo* models of lung colonization/infection respect to planktonic cell models. *P. aeruginosa* and *S. aureus* biofilms will be grown on the Nunc-TSP lid system (Thermo Fisher Scientific, Waltham, MA, USA), allowing standardized high-throughput biofilm experiments. Preformed biofilms will be exposed to illumination to perform photokilling experiments.

Once identified the most efficient irradiation parameters, *in vitro* experiments will also be performed with biofilms grown on membrane filters (0.2 µm pore size), as described previously (Tahrioui 2019). Filters containing preformed biofilm will be removed from the agar plates and exposed to irradiation. This approach will be used to confirm obtained data by using a different biofilm model that, even if more susceptible to sedimentation and not appropriate for high-throughput screening, would allow a more homogenous irradiation of biofilms.

Finally, the most efficient irradiation parameters will also be used to challenge *P. aeruginosa* and *S. aureus* planktonic culture, in both exponential and stationary phase of growth.

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Post-irradiation analysis will be carried on by CFU counting from both biofilm and planktonic cell cultures. For biofilms grown on the Nunc-TSP lid system, sessile cells will be removed from pegs by sonication (in tryptic soy broth – TSB, supplemented with 0.1% Tween 20) and appropriate dilutions plated onto tryptic soy agar (TSA) and incubating for 24-48 h at 35 °C. Biofilms grown on filters will be removed by scraping and vortexing, before proceeding to CFU determination.

According to the specific irradiation methods and protocols, post-irradiation analysis will be mediated over a minimum of 3 technical replicates for each fixed set of irradiation parameters, and further mediated over a minimum of 3 biological replicates associated to independent biofilm/planktonic culture preparations.

CFUs will be associated to the delivered dose (J/cm^2), obtaining the final CFUs vs dose curves depicting the photokilling efficiency in each specified irradiation conditions.

2.2 *In vitro* models for biofilm study with optical methods

For the biofilm measurements by the SU partner, *E. Coli* strains will be used as preliminary *in vitro* models to tune the experimental setup and refine the methodology for the study of biofilm illumination and response to light. In particular, *E. coli* strains will be previously incubated with porphyrin precursors (e.g. 5-ALA), enabling to mimic the presence of endogenous photosensitizers in controlled conditions. This choice was also prompted by the SU partner's expertise in the precise control of *E. coli* biofilm formation and properties. This strategic planning step was considered as the most effective to establish the methodology before transferring its application to *P. aeruginosa* and *S. aureus* strains.

In general, biofilm cultivation will be performed under controlled physio-chemical conditions, i.e., in 1 mm side length square milli-fluidic channel under continuous flow of nutrients. The setup is conceived to be settled on a microscope stage to be imaged in real time by video-microscopy (Figure 2) for optical properties characterization, and in particular for the measurement of: (i) biofilm optical properties; (ii) biofilm fluorescence confocal imaging; (iii) biofilm inactivation upon illumination (Thomen, 2017); (iv) oxygen mapping (Monmeyran, 2018).

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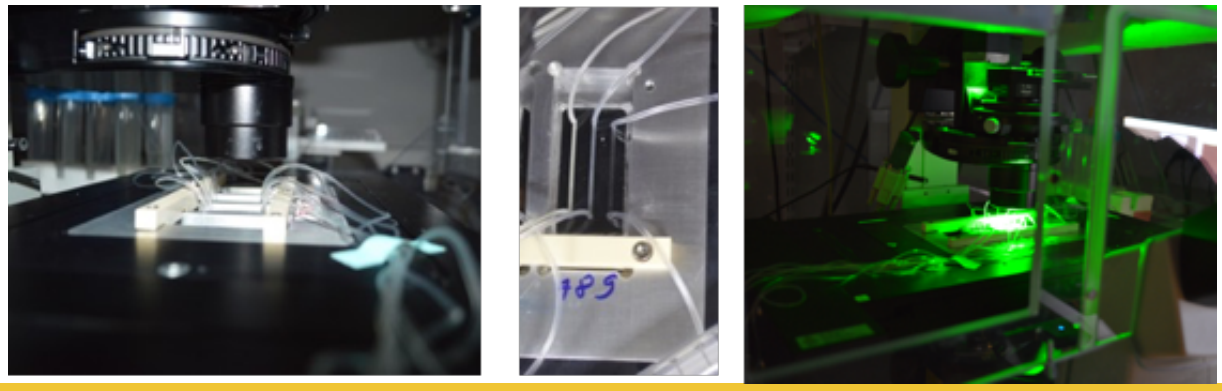

Figure 2: Microscopy setup for real time monitoring of living biofilms

2.3 Artificial Sputum Medium model (ASM)

In the CF lung, bacteria grow as self-aggregating biofilms embedded largely within mucus plugs. Artificial sputum medium (ASM) is a media designed to recapitulate the major components of the sputum of people with cystic fibrosis (CF) (Figure 3). The media is prepared from key constituents including free amino acids, extracellular DNA and mucin. The assay has been developed into a microtitre plate format in which the biofilm can be studied for up to 7 days initially. Bacterial isolates (10^5 cfu/ml) will be inoculated and growth, metabolic activity and phenotypic characteristics will be assessed.

Treatment parameters will be studied over various time points by altering the irradiation and dosing conditions. This dose dependent antimicrobial efficacy will be determined using two isolates each of *P. aeruginosa* and *S. aureus* under biofilm growth conditions.

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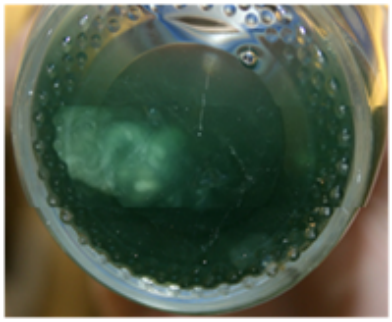

Figure 3. *P. aeruginosa* growing as a self-aggregating biofilm in artificial sputum medium. The media appears green due to the accumulation of pyocyanin and other extracellular products from the mature biofilm. The bacterial biofilm mass appears as a free-floating plug, similar to that seen in the lungs of people with cystic fibrosis.

2.4 Triple Cell Culture model (TCC)

We will next test light therapy in a novel Triple Cell Co-Culture (TCC) model that closely replicates the human epithelial airway barrier that both *P. aeruginosa* and *S. aureus* first encounter during colonisation of the respiratory tract. The model consists of a layer of human-derived lung epithelial cells (A549), co-cultured with human monocyte-derived macrophages and dendritic cells and thus captures key structural and immunological features of the airways. The model will be assembled as shown in Figure 4, before establishing infection through addition of bacteria to the epithelial cell surface at a multiplicity of infection of 5 bacteria per epithelial cell. Infection leads to a transient increase in epithelial barrier integrity, and bacterial translocation from the apical to the basal side of the model can be used as a proxy measure for the severity of the infection. Before testing the full bacterial strain panel, appropriate wavelengths and dosages of light will be determined, as described above.

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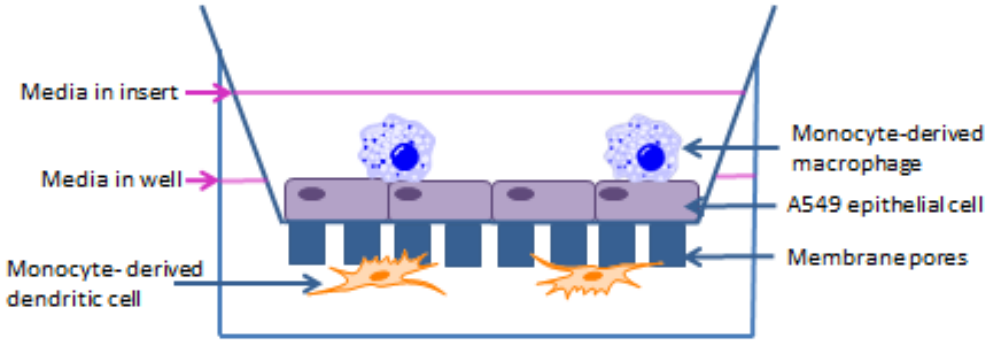

Figure 4. Schematic of the TCC lung model. The TCC lung model is established by seeding lung epithelial cells on a tissue culture insert and allowing them to form a monolayer. Monocyte-derived dendritic cells are seeded onto the basal side of the insert porous membrane. Monocyte-derived macrophages are seeded on the apical side of the insert, on top of the epithelial monolayer. Tissue culture media is added into both the tissue culture well and into the insert to support cell growth and function. Bacteria are added to the apical side of the insert for infection experiments.

In the TCC model, viable bacterial counts on the apical and basal sides of the epithelial barrier following light treatment will be the primary endpoint, but immune parameters and epithelial barrier integrity will also be assessed. Cytokine production in media will be determined by ELISA, multiplex assay, or gene expression analysis, as appropriate. Epithelial barrier integrity will be determined by measurement of trans-epithelial electrical resistance (TEER). Data from these experiments will inform the design of subsequent *in vivo* infection models and all experiments will be performed to achieve at least 3 biological replicates.

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2.5 Mammalian cell model

The biocompatibility of the aerosol nanoparticles will be assessed by performing tests with different mono- and co-cultures of human cell lines modelling the three main parts of the respiratory tract being the infection localized in both upper and lower respiratory track.

As useful models for the studies, the following cells lines have been selected for their features recapitulating in whole or in part those of the respiratory epithelium formed by polarized cells connected by tight junctions and producing mucus/surfactant.

- Calu-3 lung adenocarcinoma, HBEC3-KT and 16HBE14o immortalized bronchial (models of trachea and bronchi epithelium)
- NCI-H441 adenocarcinoma (model of bronchioli epithelium)
- A549 lung adenocarcinoma with alveolar type II characteristics and hAELVi immortalized alveolar type I (models of alveoli)

Calu-3 cells form tight junctions, produce mucus and grow easily in vitro, but being transformed cells may respond differently with respect to normal cells to stressing conditions. Therefore, normal immortalized HBEC3-KT and 16HBE14o cells were also selected as models of trachea and bronchi epithelium. For bronchioli modelling the only commercially available cell line is the NCI-H441 adenocarcinoma; these cells are polarized and form a barrier. For alveolar modelling, A549 adenocarcinoma cells are selected as representative of the alveolar type II cells producing lung surfactant. However, type II cells represent a minor fraction of the alveolar cells. Therefore, hAELVi immortalized alveolar type I are also included in the studies as major representative of the alveolar cells.

These cell lines will be used as monocultures in preliminary biocompatibility tests and some of them in co-cultures in advanced tests.

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Preliminary biocompatibility tests

At the beginning of biocompatibility investigations**, cell monolayers in submerged conditions** will be exposed for increasing time periods (up to 24h) at increasing concentrations of nanoparticles containing the luminous emitters. Biocompatibility will be assessed by measuring cell proliferation/viability with the CellTiter 96® AQ_{ueous}One Solution Cell Proliferation assay (MTS, metabolic activity-based test) and membrane damages using propidium iodide staining.

Based on results obtained with submerged cultures, conditions will be selected for treating **cell monolayers** grown in cell culture inserts and at **air-liquid interface** (ALI) **conditions.** ALI cultures are expensive and time consuming, but results obtained with these models are more predictive of an in vivo response to aerosol nanoparticle induced stress. ALI cultures will be exposed on the apical side to increasing concentrations of the luminous nanospheres in the form of aerosol. At various times after exposure the effects will be evaluated by measuring: i) changes of Trans Epithelial Electrical Resistance (TEER); ii) changes of the epithelium permeability following diffusion of fluorescent probes from the apical to the basal side and iii) release of LDH in the basal and apical side; iv) histological examination and v) immunodetection of tight junctions alterations.

Advanced tests

Co-cultures of different types of cells of the respiratory tract wall, including fibroblasts and cell of the immune system (macrophages and dendritic cells derived monocytes) together with the epithelial cells (HBEC3-KT and hAELVi), will be cultured in ALI conditions and exposed to various concentrations of luminous nanoparticles in the form of aerosol for various time periods. The changes of epithelium permeability and TEER will be evaluated together with measurements of cytokine release on the apical and basal side, evaluation of changes of gene expression profiles by RT-PCR or NGS and electron microscopy analysis to detect morphological and ultrastructural alterations.

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3 *IN VIVO* MODELS

3.1 Mouse models

We will use our well-established mouse models of respiratory infection using laboratory and clinical strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus.* Bacteria will be administered by inhaled delivery leading to either shorter term acute infections or longer-term chronic infections (Fothergill, 2014; Waters, 2017; Bricio-Moreno, 2018; Gallagher, 2020). Once administered, bacteria infect the lower respiratory tract, including the conductive and respiratory zones (terminal and respiratory bronchioles) and alveolar sacs (Moore, 2017). Bacterial colony forming units (CFUs) will be determined post infection in mice over time from either total lung homogenates or by lung bronchoalveolar lavage. At various timepoints post-challenge blood samples will be collected from the tail vein (or by exsanguination by cardiac puncture under general anaesthesia). Tissues, organs and lung homogenate and or lavage samples from these mice will be removed and together with collected blood samples will be used for molecular and microbiological analysis, pathology and bacterial CFU counts. Bacterial load kinetics will be performed in nasopharyngeal and lung tissue, liver, spleen and blood samples.

The mouse models will be used for determining the irradiation protocols, the biocompatibility of the aerosol treatment, and analysis of the possible build-up effects due to repeated treatments. Furthermore, measurements of the photokilling dose-response effect in both acute and chronic infection conditions in both bacterial species will be investigated. Bacterial CFUs over time post aerosol exposure, host survival and immune responses will be determined following established protocols in-house.

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