In-vitro inhalation microplastics assessments: IVIVE approaches

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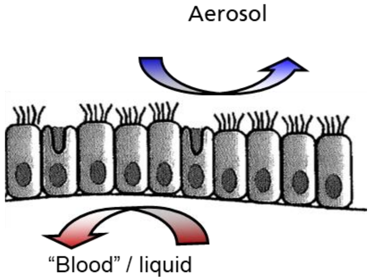
Outline

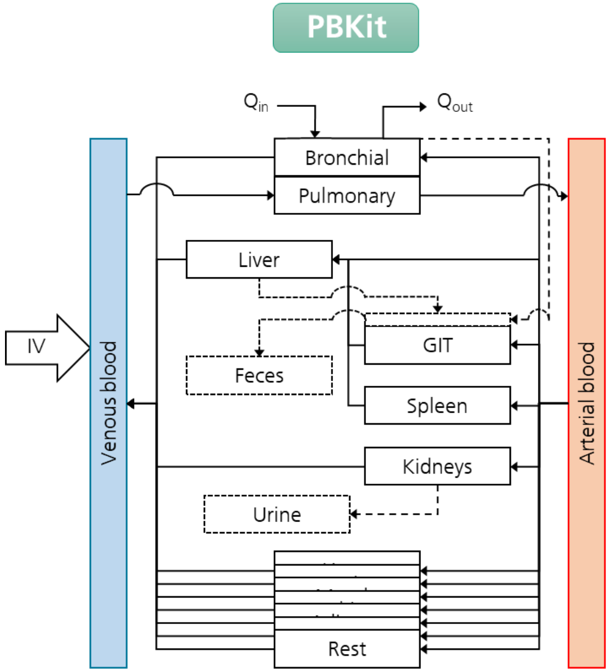
In-vitro Inhalation testing

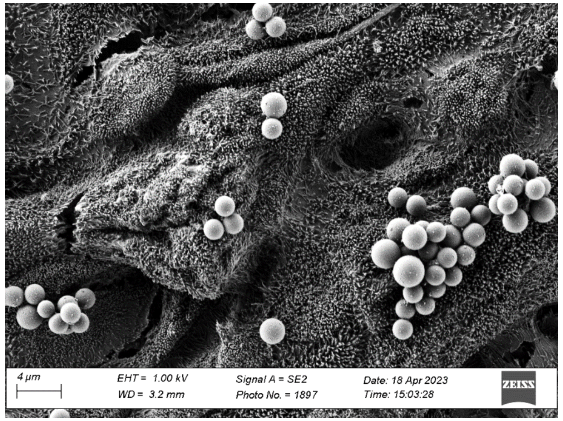
- Setup and dosimetry
- Example: prediction model
- Results with microplastic materials

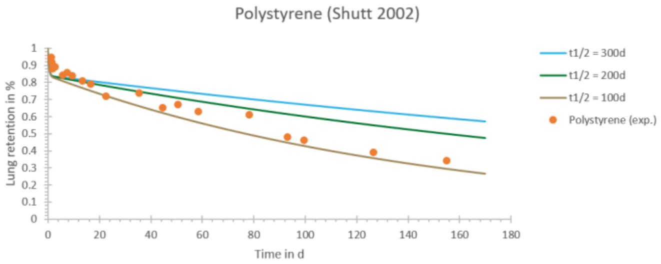
PBK modelling

- Parameterization of PBK models
- Lung barrier model
- Results with microplastic materials









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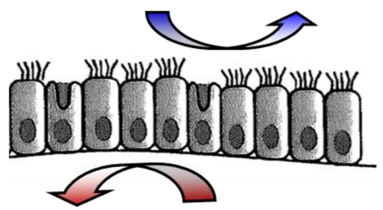
Chapter 1

In vitro inhalation testing

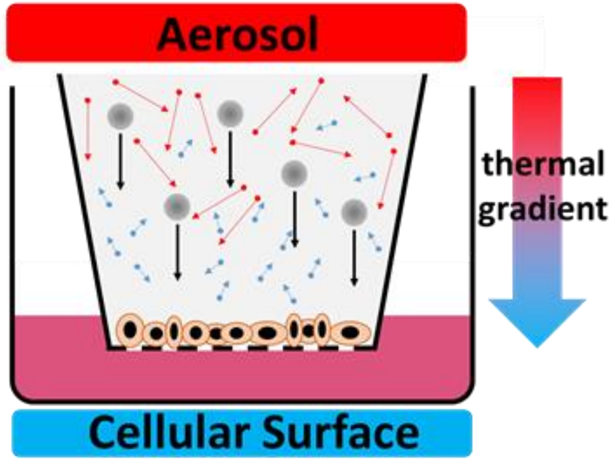
Basic dosing considerations for ALI experiments with aerosols/particles



Biological Model: Pulmonary cells or tissues at the air-liquid interface (ALI culture), e.g. Calu-3, A549

Deposition is dependent on particle size (MMAD)

Physical forces: sedimentation + impaction (3 – 10 µm),
sedimentation + diffusion (< 3 µm)
In vitro deposition rates for particles < 1µm are in the range of 1 -2 %



Thermophoresis effect for efficient particle deposition from aerosols

METHOD: In vitro dosimetry

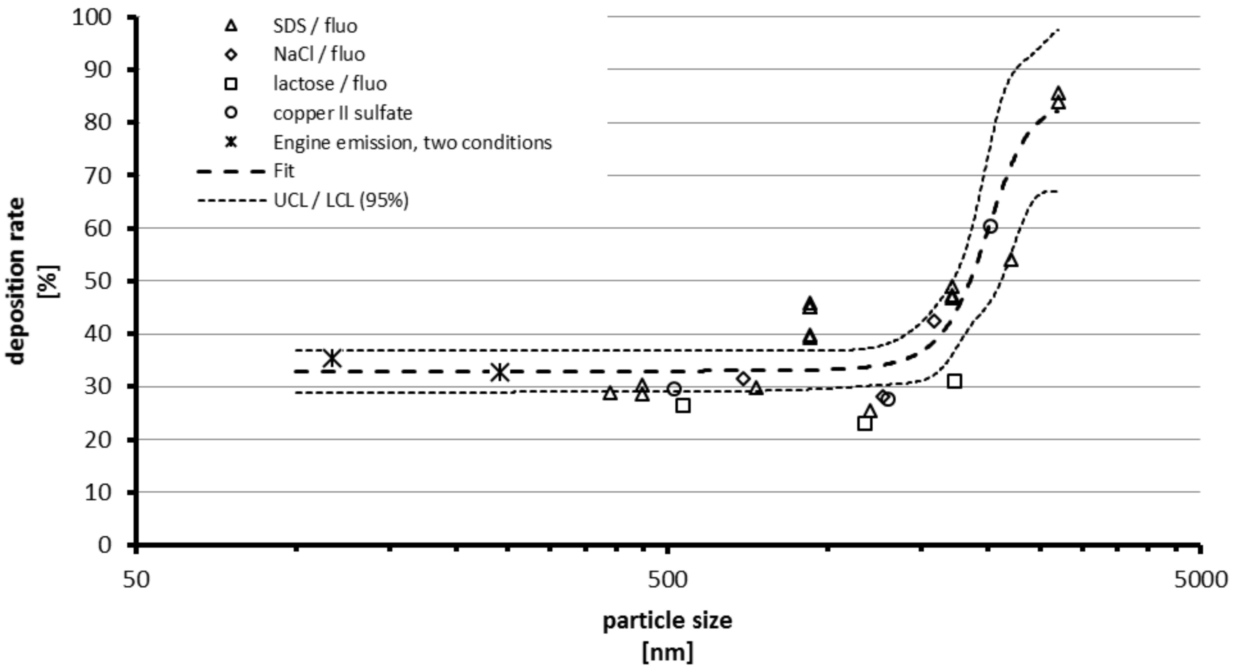
- Dry particle aerosols from droplet aerosol generation
- Engine exhausts
- Dry particle aerosols from dust aerosol generation

Methods:

CFD-Simulations

Fluorescence methods (tracing)

Analytical chemistry



Characterization of size dependent particle deposition in ExpoCube® using thermophoresis conditions

“Deposition Rate”

$$DR\ [\%] = \frac{mass\ deposited\ on\ cells[\mu g]}{mass\ conducted\ over\ cells[\mu g]} * 100$$

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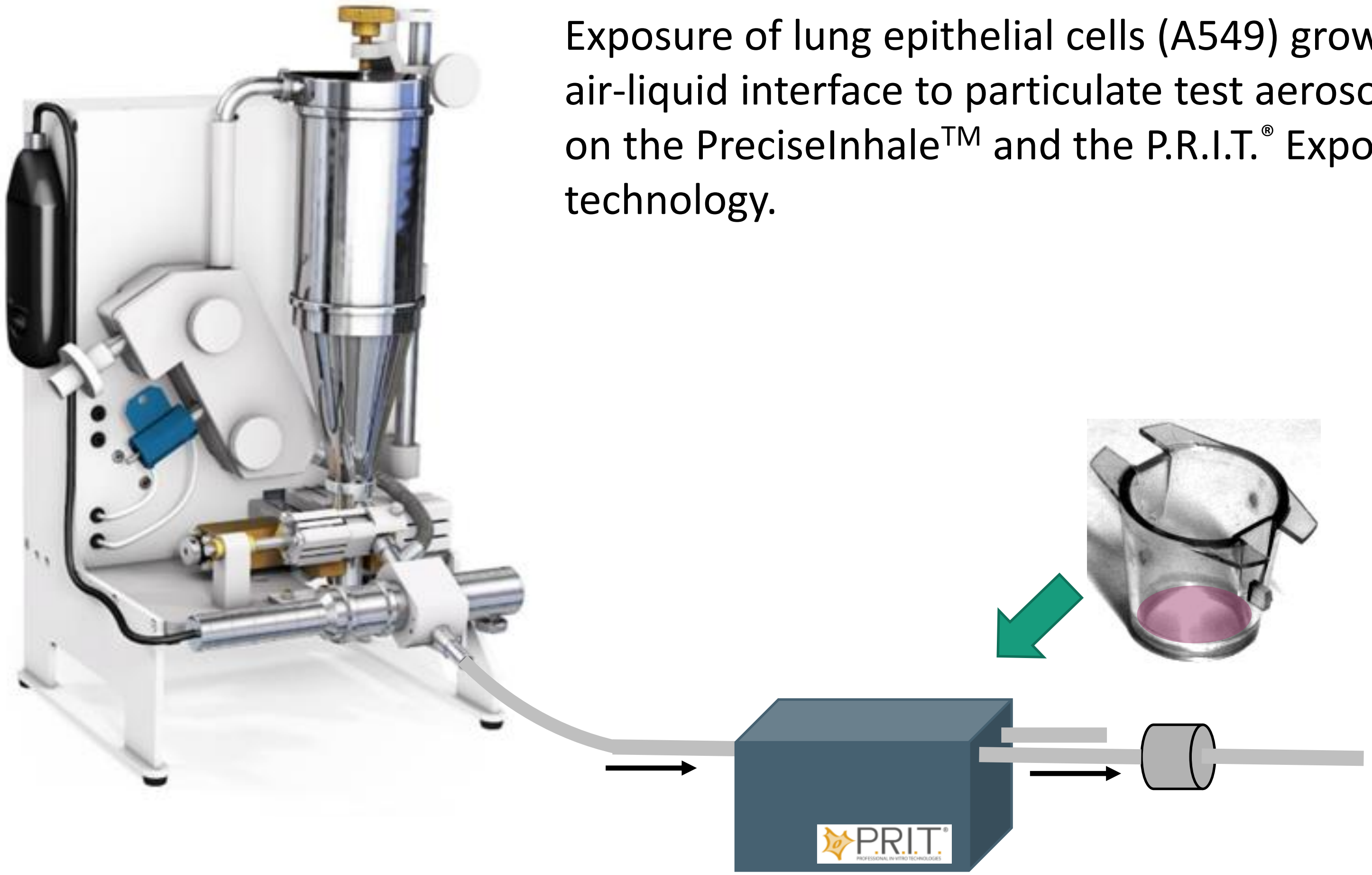
Application Example: Acute Inhalation Toxicity of Pesticides

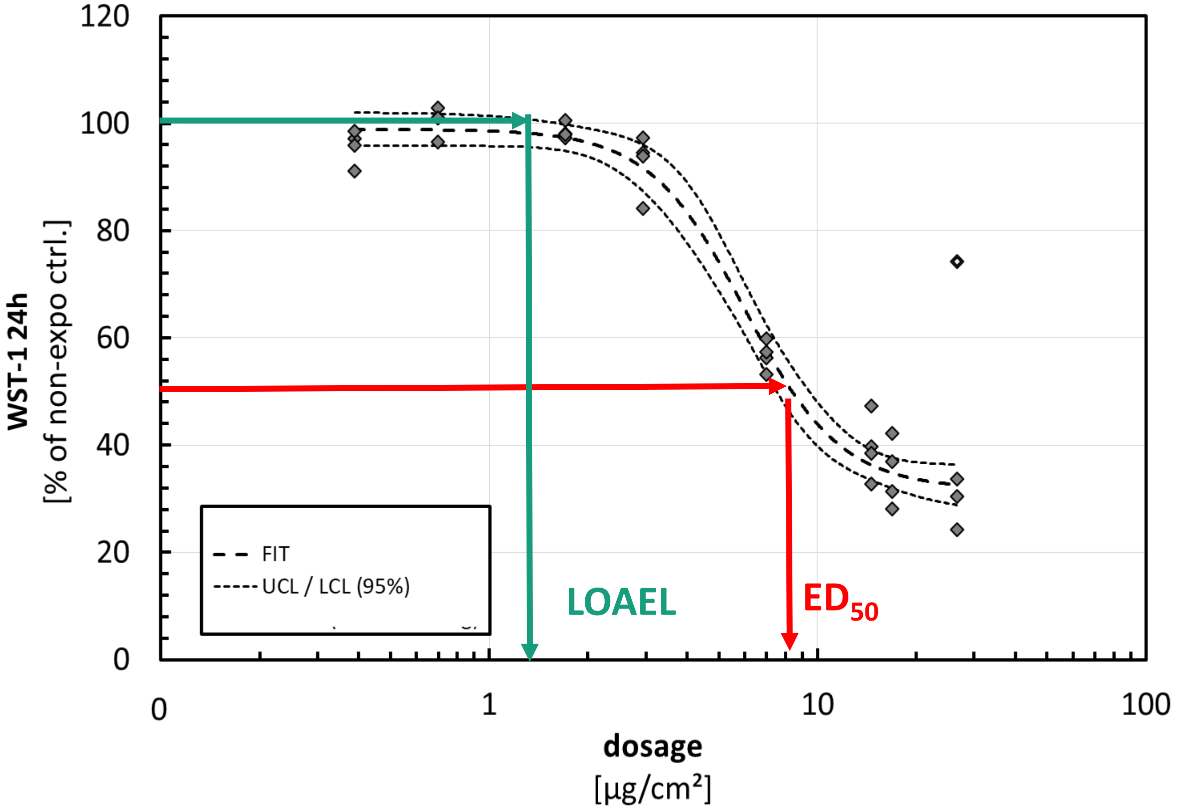
Exposure set-up for dry powder aerosols

Exposure of lung epithelial cells (A549) grown at the air-liquid interface to particulate test aerosols based on the PreciseInhale^{TM} and the P.R.I.T.$^{®}$ ExpoCube$^{®}$ technology.

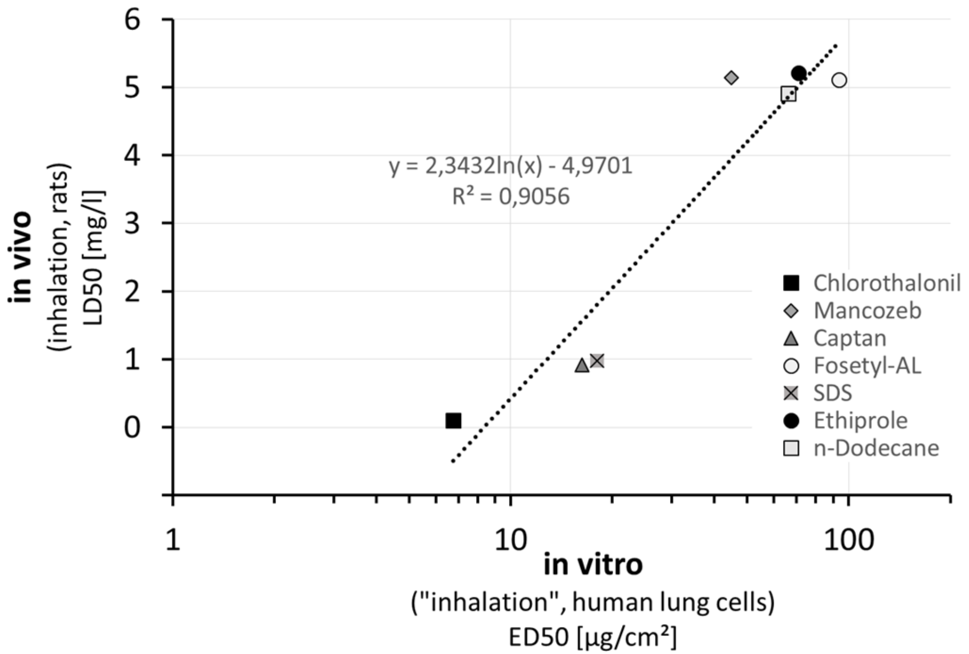


Application Example: Acute Inhalation Toxicity of Pesticides

Prediction model for dry powder aerosols



Prediction model based on comparison to reference chemicals (inhalation toxicity <u>in vivo data available</u>)



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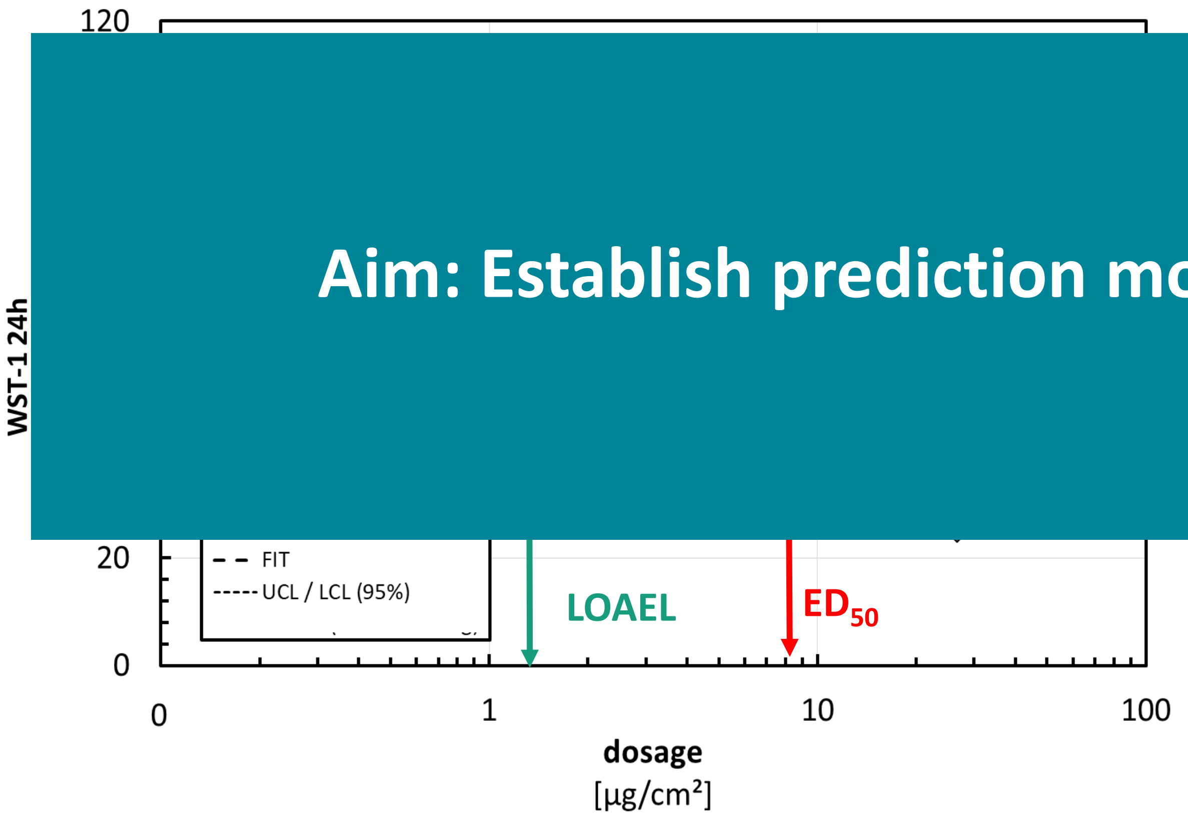
Application Example: Acute Inhalation Toxicity of Pesticides

Prediction model for dry powder aerosols



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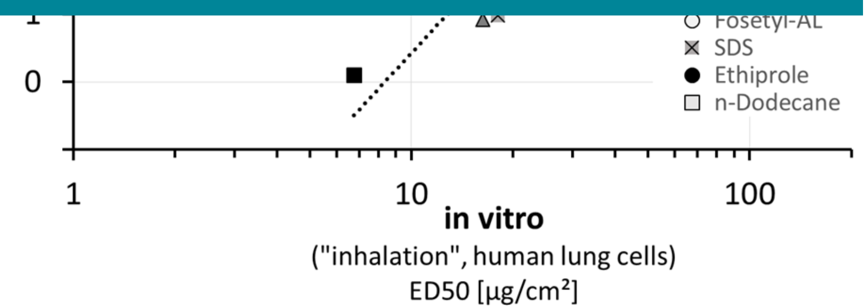

Aim: Establish prediction model for microplastic particles

Challenge 1: Generation of test material for In-vitro inhalation testing

Descriptor driven prioritization of microplastic materials (Cefic C10)

Hypotheses driven by:

Size

Density

Reactivity/charge

Chemical Composition

Polymer Selection:

Polyamide

Polyethylene

Solvent Precipitation for <1 µm

Size	PET	PA	LDPE	PVC	TPU	PS
< 1 µm	C10	C10 MOMENTUM D_{50} = 67 nm D_{50} = 37 nm	BRIGID C10	-	C10	C10 MOMENTUM D_{50} = 89 nm D_{50} = 78 nm
1-10 µm	ECO59 (sieve) C10 D_{50} = 1.37 µm D_{50} = 110 nm	InnoMatLife BRIGID D_{50} = 6.7 µm	BRIGID NIST D_{50} = 4.6 µm	BRIGID	C10 D_{50} = 1.98 µm D_{50} = 491 nm	C10* D_{50} = 2.0 µm
> 10 µm	ECO59 D_{50} = 41.3 µm	ECO59 InnoMatLife D_{50} = 42.4 µm	BAM D_{50} = 61 µm	-	ECO59 InnoMatLife D_{50} = 236 µm	BAM D_{50} = 206 µm

PA and LDPE were selected for size specific effects

*** Purchased**

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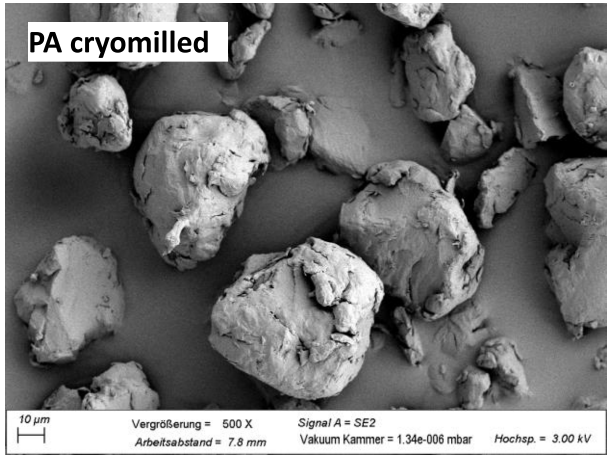
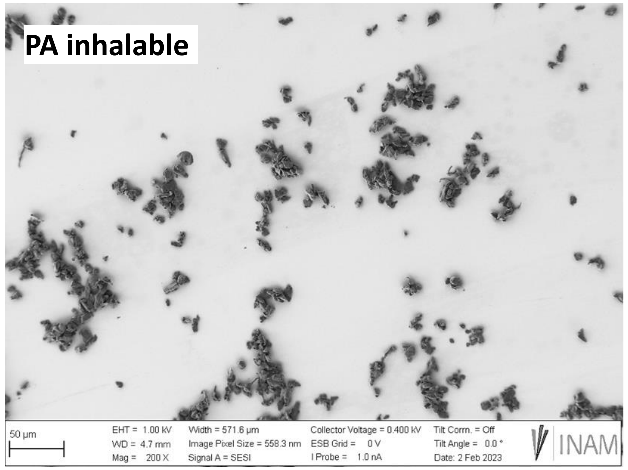
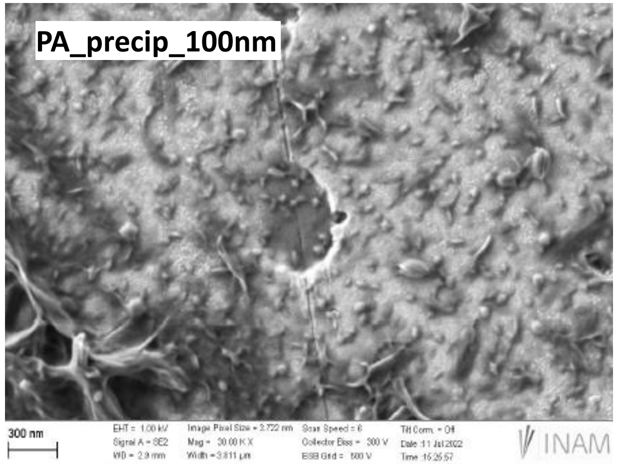
Challenge 1: Generation of test material for In-vitro inhalation testing

PA representativeness







Solidity

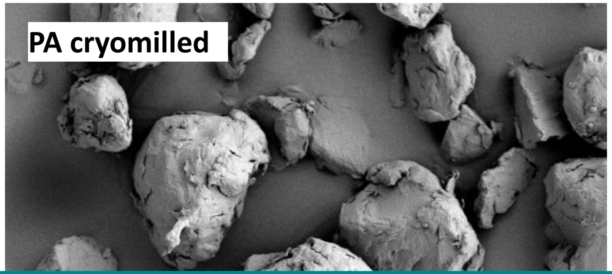
Sample Name	Crystallization Peak (°C)	Melting Pt. (°C)	Density (g/cc)	Surface Area (m^2/g)	No. Ave. (M_n, g/mol)	Wt. Ave. (M_w, g/mol)	M_w/M_n	t_{50} (sec)
PA bulk (mm)	162.2	218.5	1.14	-	19600	61900	3.2	0.0110
PA cryomilled (47 µm)	186.4	219.9	1.15	0.36	16400	57900	3.5	0.0115
PA Inhalable (7 µm)	**176.0**	**217.2**		**1.90**				
PA_precip_100nm	**175.0**	**215.4**	**1.23**	**81.8**	**13100**	**68000**	**5.2**	**0.0130**

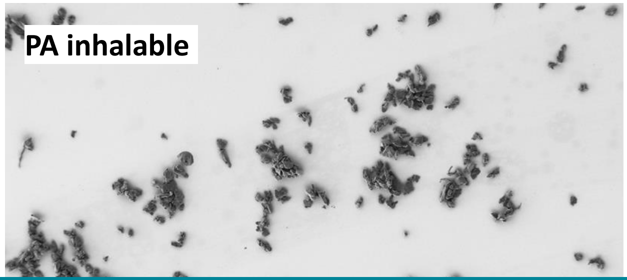
Thermal Analysis

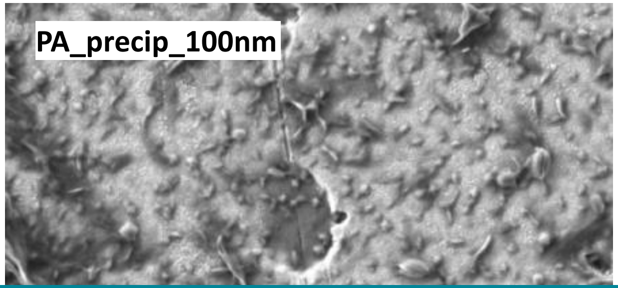
Molar Mass

Challenge 1: Generation of test material for In-vitro inhalation testing

PA representativeness







Challenge: Test materials are present as powder or suspension

S[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
P[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
PA cryomilled (47 µm)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
PA Inhalable (7 µm)	**176.0**	**217.2**		**1.90**				
PA_precip_100nm	**175.0**	**215.4**	**1.23**	**81.8**	**13100**	**68000**	**5.2**	**0.0130**

Thermal Analysis (columns 2–3); Molar Mass (columns 6–8)

Challenge: Generation of particle aerosols from suspensions/solutions

Exemplary setup for soluble substances

Test materials with different state:

Aequous dispersion (PA_prec),

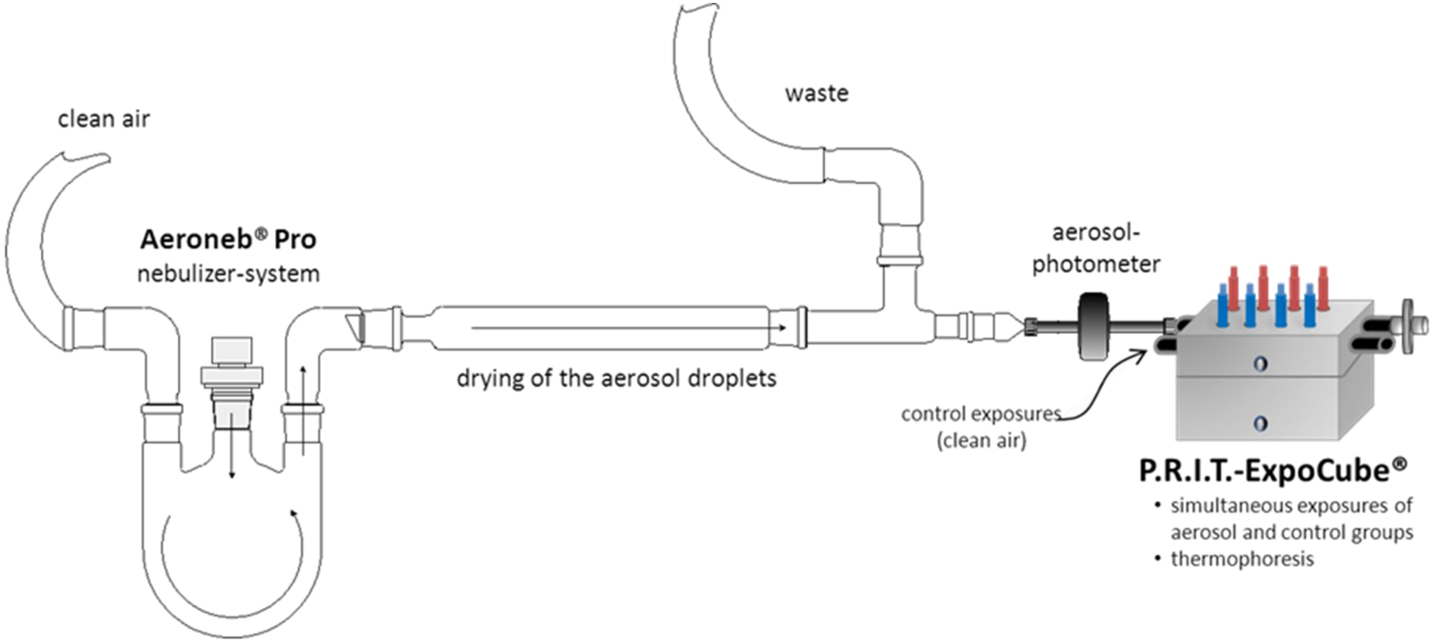
Powder (PA inhalable),

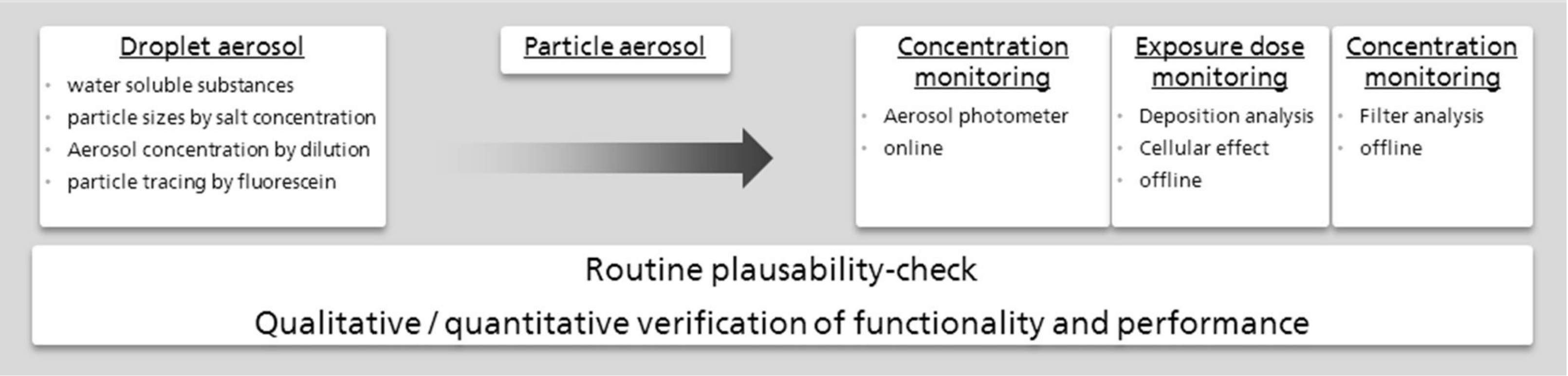
Reference materials:

Carbopol (powder)

Kaolin (powder)

SDS (solution)





Generation of particle aerosols from suspensions/solutions

Positive control: SDS

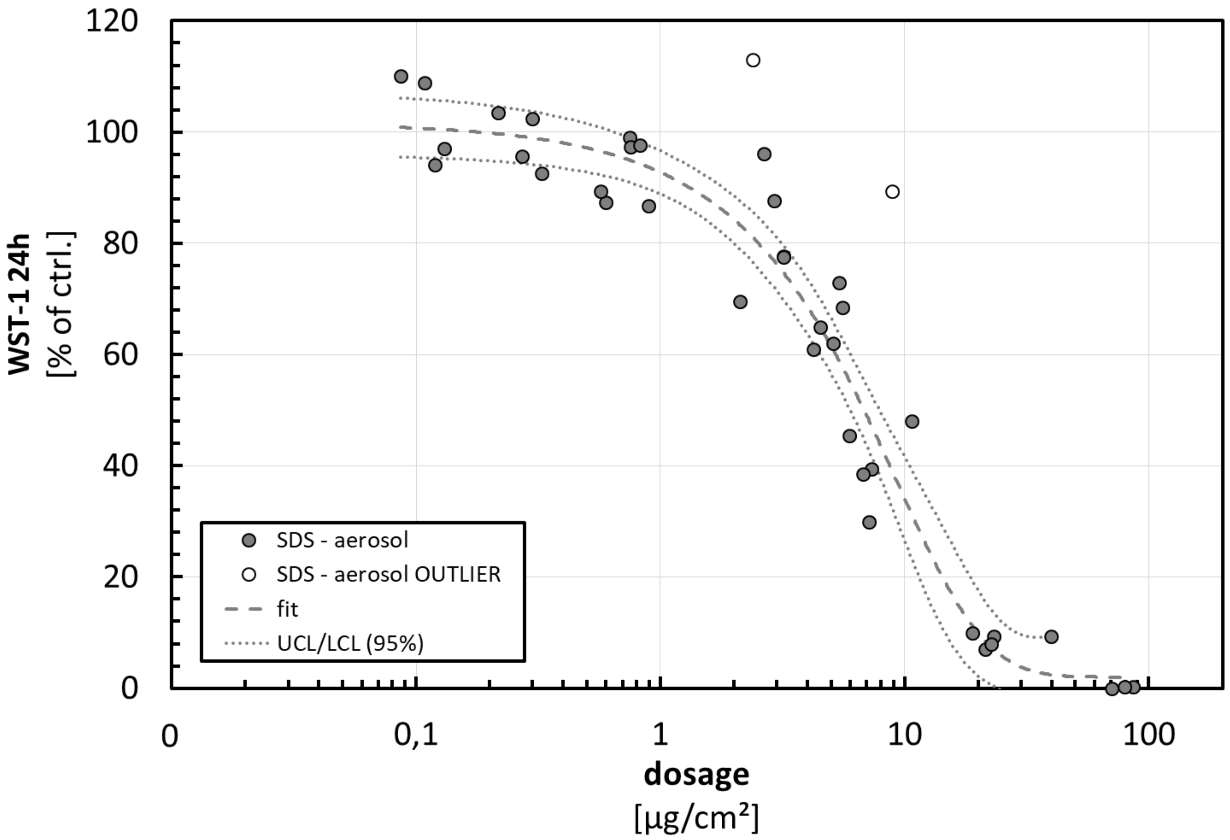
A549 cells

ALI exposure

Suspension, mesh nebulizer

ED$_{50}$: 6.8 µg/cm²

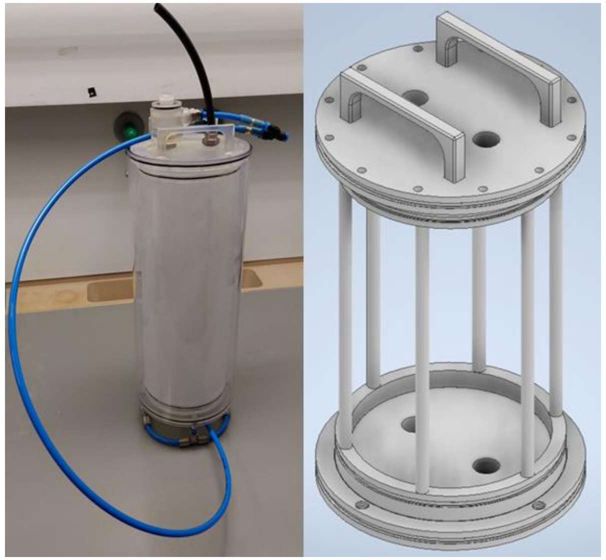
Highly toxic

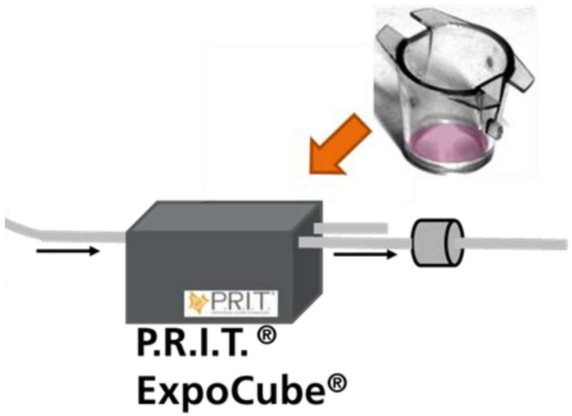


Generation of particle aerosols from suspensions/solutions

Microplastic material: PA_prec

- Aersolisation by means of a mesh nebulizer and active drying
- Developed a new membrane dryer for aerosols for higher concentration
- Waterdiffusion through hydro active membrane
- Results were: ~4x higher concentration
- Outlook: further improvement of dryer

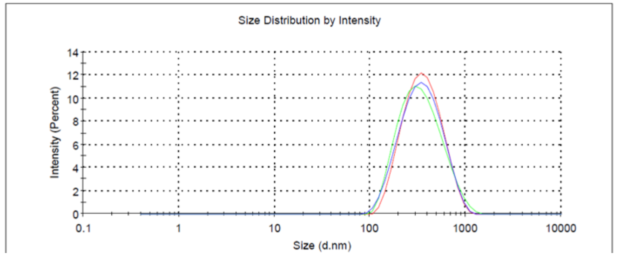




Particel size and stability tested by DLS:

Results

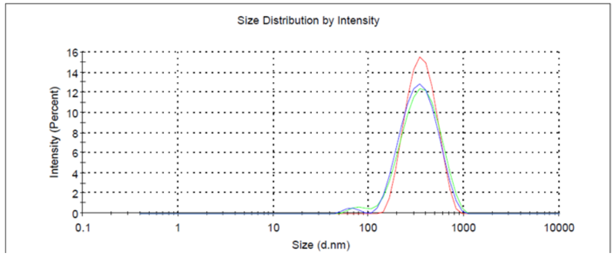
			Size (d.n...	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	304,4	Peak 1:	383,4	100,0	172,6
PdI:	0,194	Peak 2:	0,000	0,0	0,000
Intercept:	0,923	Peak 3:	0,000	0,0	0,000
Result quality	Good				



Freshly sonicated

Results

			Size (d.n...	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	308,3	Peak 1:	372,6	100,0	129,7
PdI:	0,168	Peak 2:	0,000	0,0	0,000
Intercept:	0,919	Peak 3:	0,000	0,0	0,000
Result quality	Good				



After 5 days

Generation of particle aerosols from suspensions/solutions

Microplastic material: PA_prec

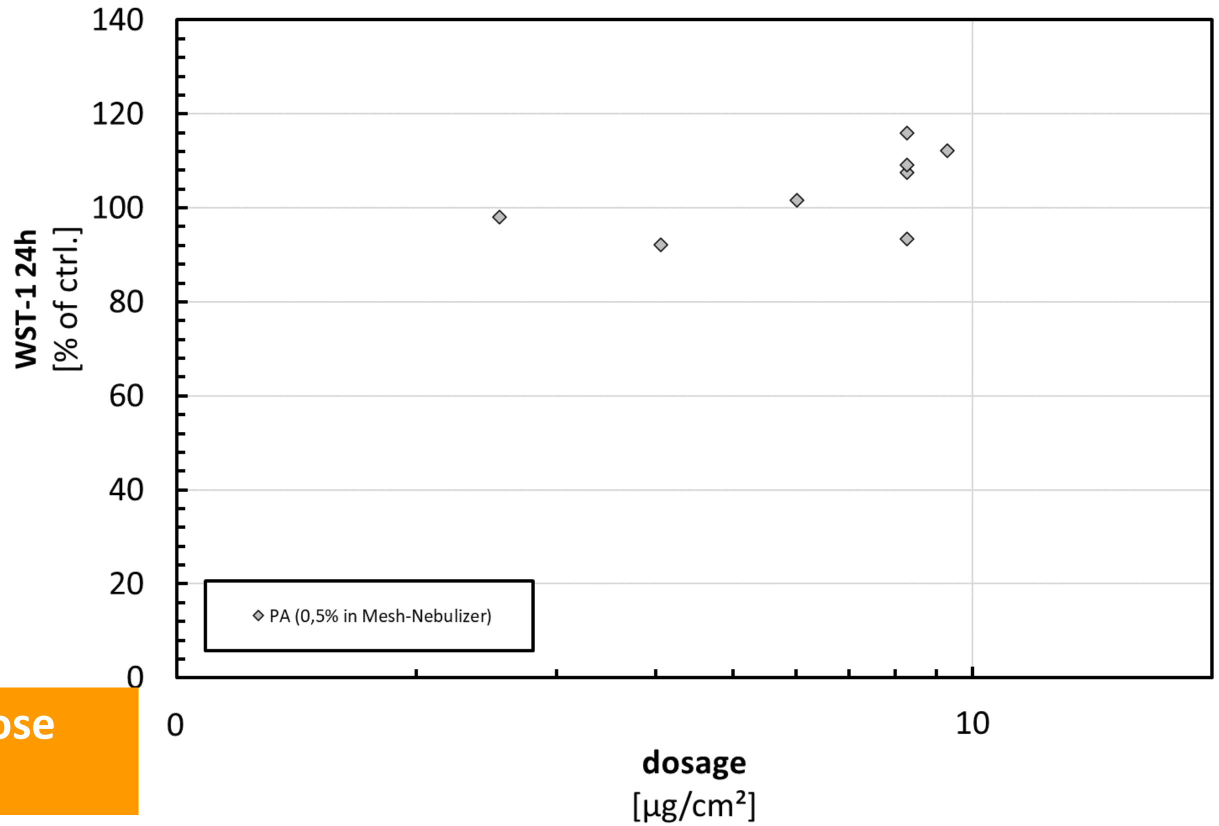
Calu-3 cells

ALI exposure

0.5% suspension, mesh nebulizer

Preliminay results, limited dose range

Non-toxic in the dose range tested



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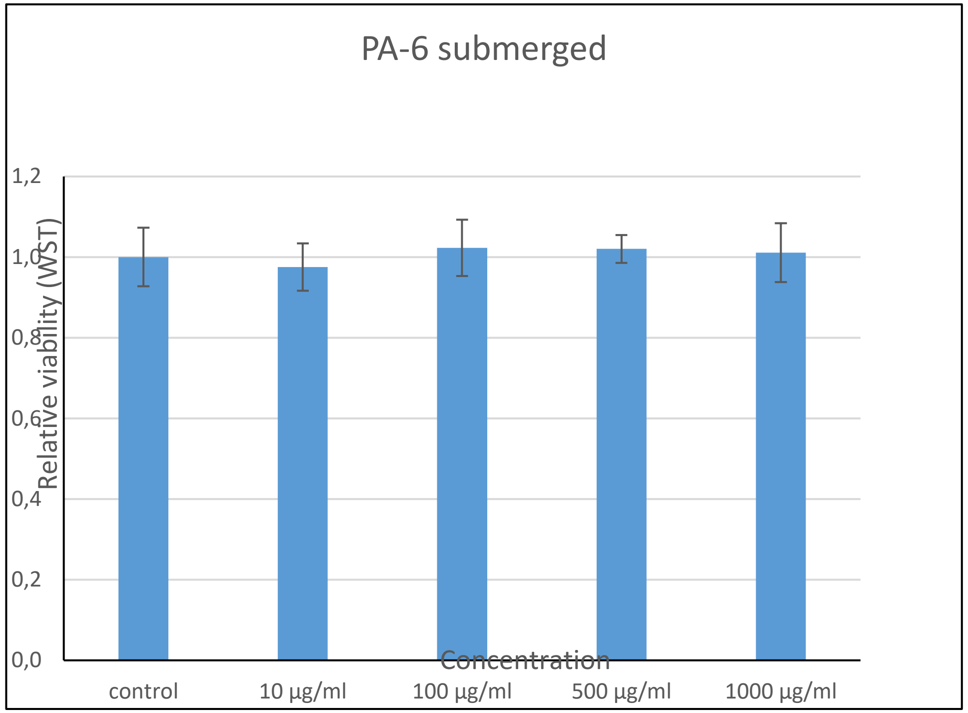
Submerged testing of microplastic materials

Microplastic material: PA_prec

Calu-3 cells

submerged exposure

Up to 1000 µg/mL



Non-toxic in the dose range tested

Generation of particle aerosols from suspensions/solutions

Reference material: Kaolin (negative control)

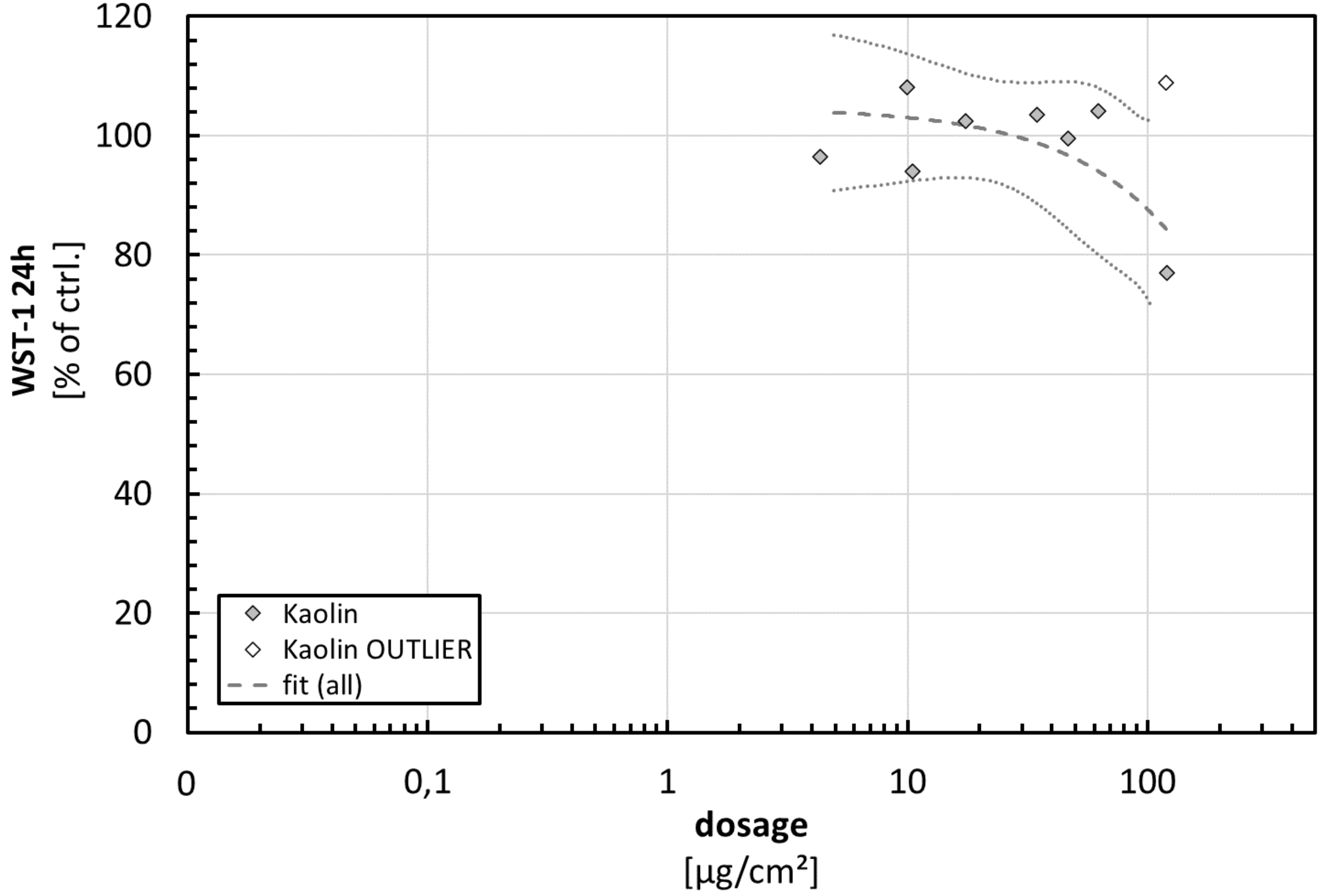
Soft white clay, porceain ingredient

Calu-3 cells

ALI exposure to dry powder aerosol (PreciseInhale)

ED_{50}: >>100 µg/cm²

Non-toxic



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Generation of particle aerosols from suspensions/solutions

Reference material: Carbopol (negative control)

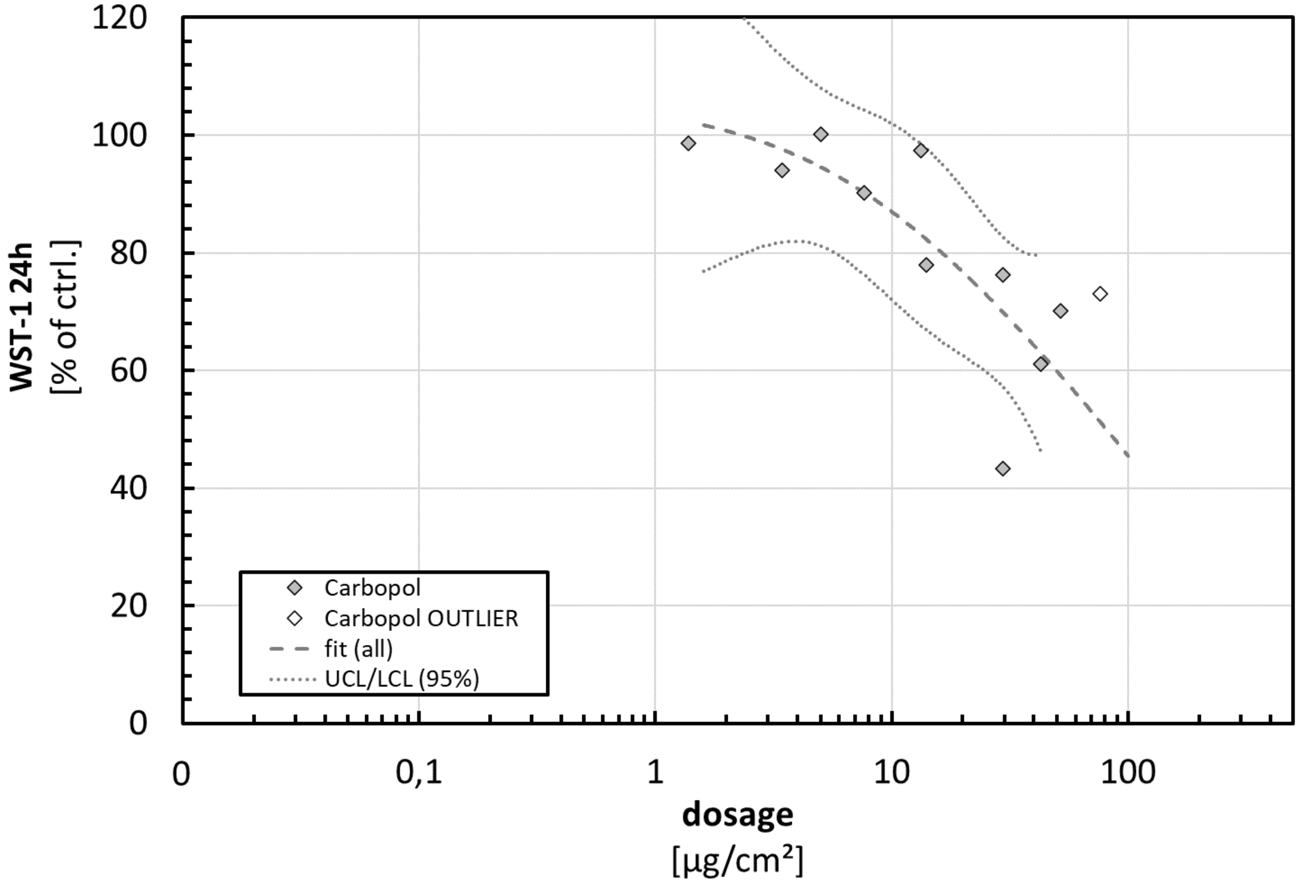
Water soluble polymer

Calu-3 cells

ALI exposure to dry powder aerosol (PreciseInhale)

ED_{50}: 81 µg/cm²

Harmless



Summary Part 1

In-vitro inhalation

- ALI testing with pulmonary cells is relevant and predictivity for local toxicity is proven for particle aerosols
- The P.R.I.T.Expocube setup enables deposition rates of roughly 30% for particles < 1.5 µm
- Micronized microplastic material generated by solvent precipitation is representative for the bulk material
- Particle aerosols can be generated from suspensions/solutions by nebulization and drying
- SDS is suitable as toxic positive control, Kaolin and Carbopol are negative reference materials
- PA particles showed no cytotoxicity in Calu-3 cells at concentrations up to
  - 1000 µg/mL (submerged)
  - 9.29 µg/cm² (ALI) – higher concentrations could not be achieved so far

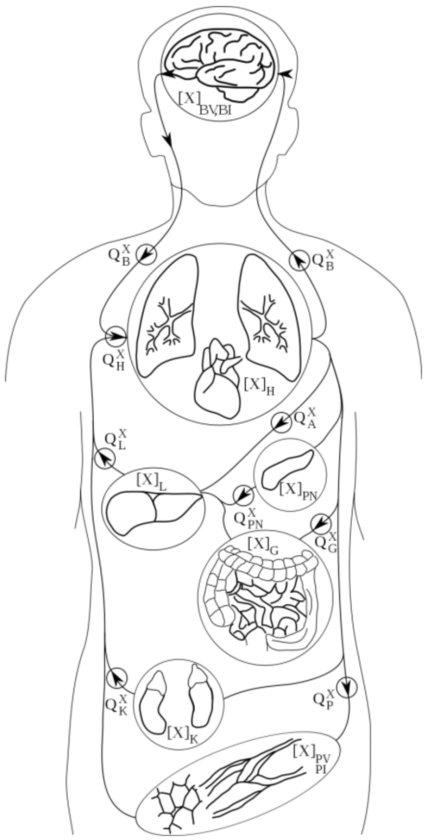
Chapter 2

PBK modelling

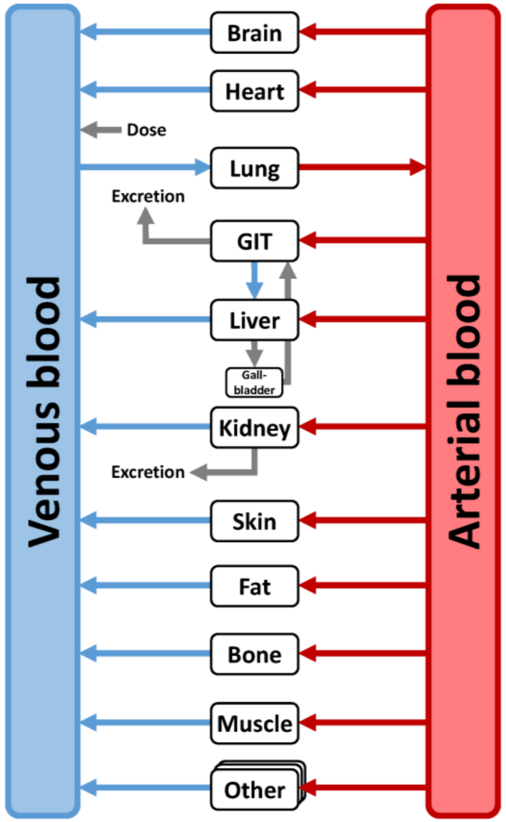
Physiologically based kinetic (PBK) modelling

Concept

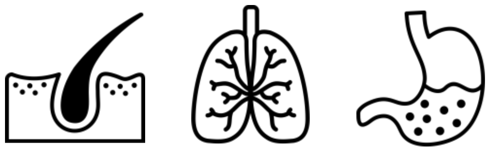
Reality



Model



Different routes

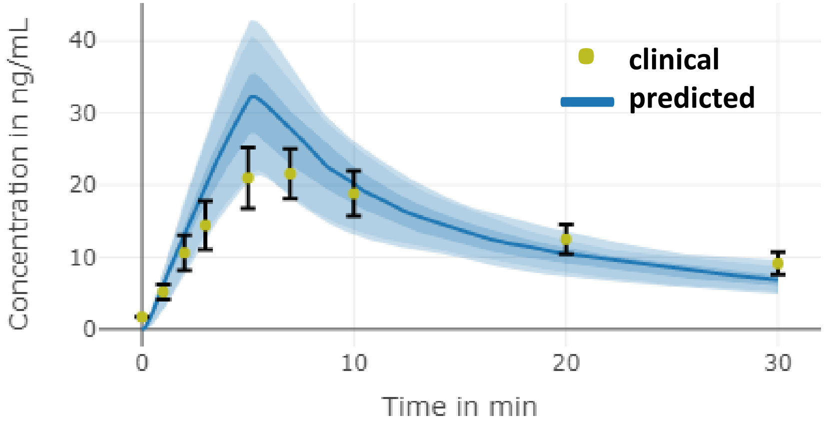


dermal inhalation ingestion

Different sources

occupation consumer environment

Prediction
(e.g. venous blood)



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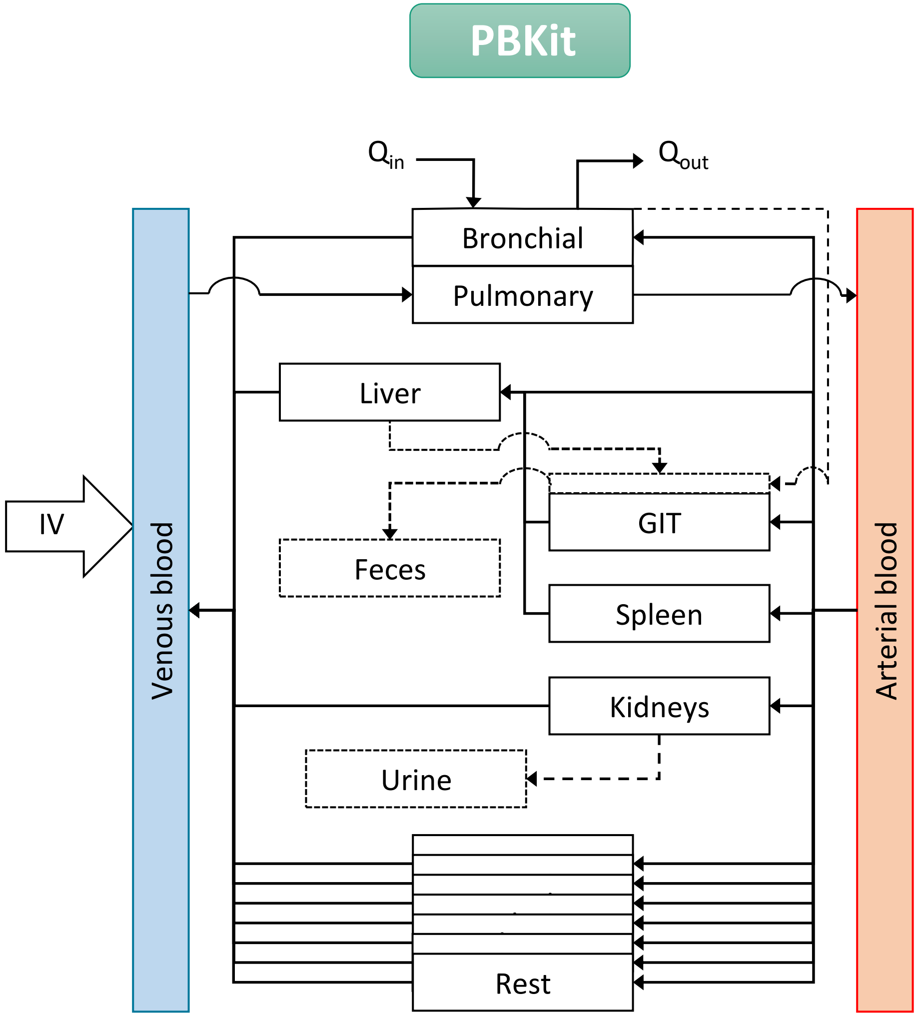
The PBKit model

Design goals

- Generic and joined model framework applicable for
  - gases/vapours,
  - aerosols (liquid or solid),
- Only in silico and in vitro informed
- Prediction of systemic concentrations but also
- local concentrations within the lung (i.e. lining fluid or tissue)

➢ **Requires multicompartmental lung**



*Heart, Brain, Muscle, Skin, Bone, Adipose, Rest

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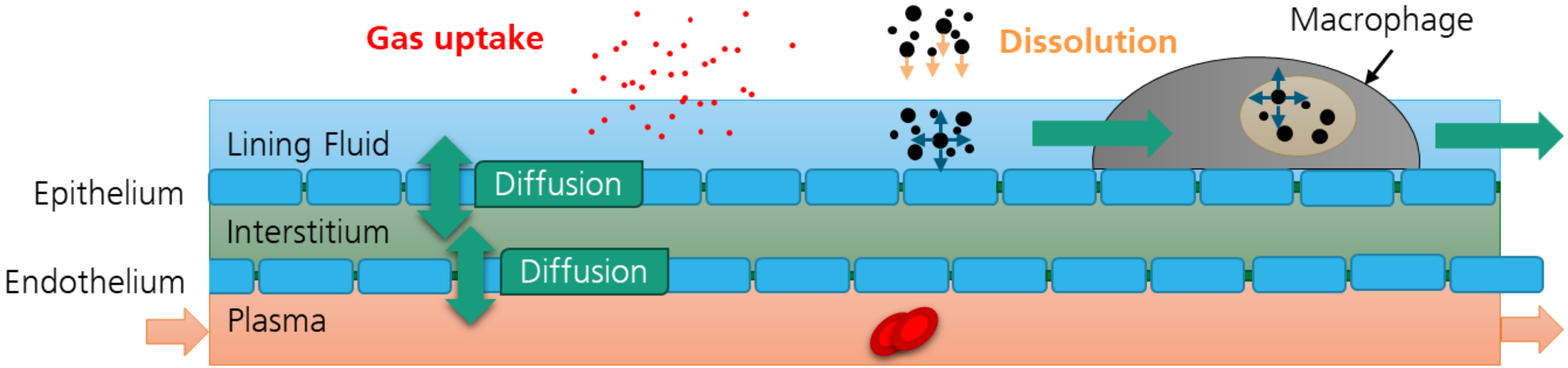
The PBKit model

Processes Relevant for Respiratory Clearance and Uptake

- deposition and uptake (aerosols and gases) (dissolution)
- retention / clearance (macrophages and mucociliary clearance) and
- systemic uptake (diffusion / permeability)



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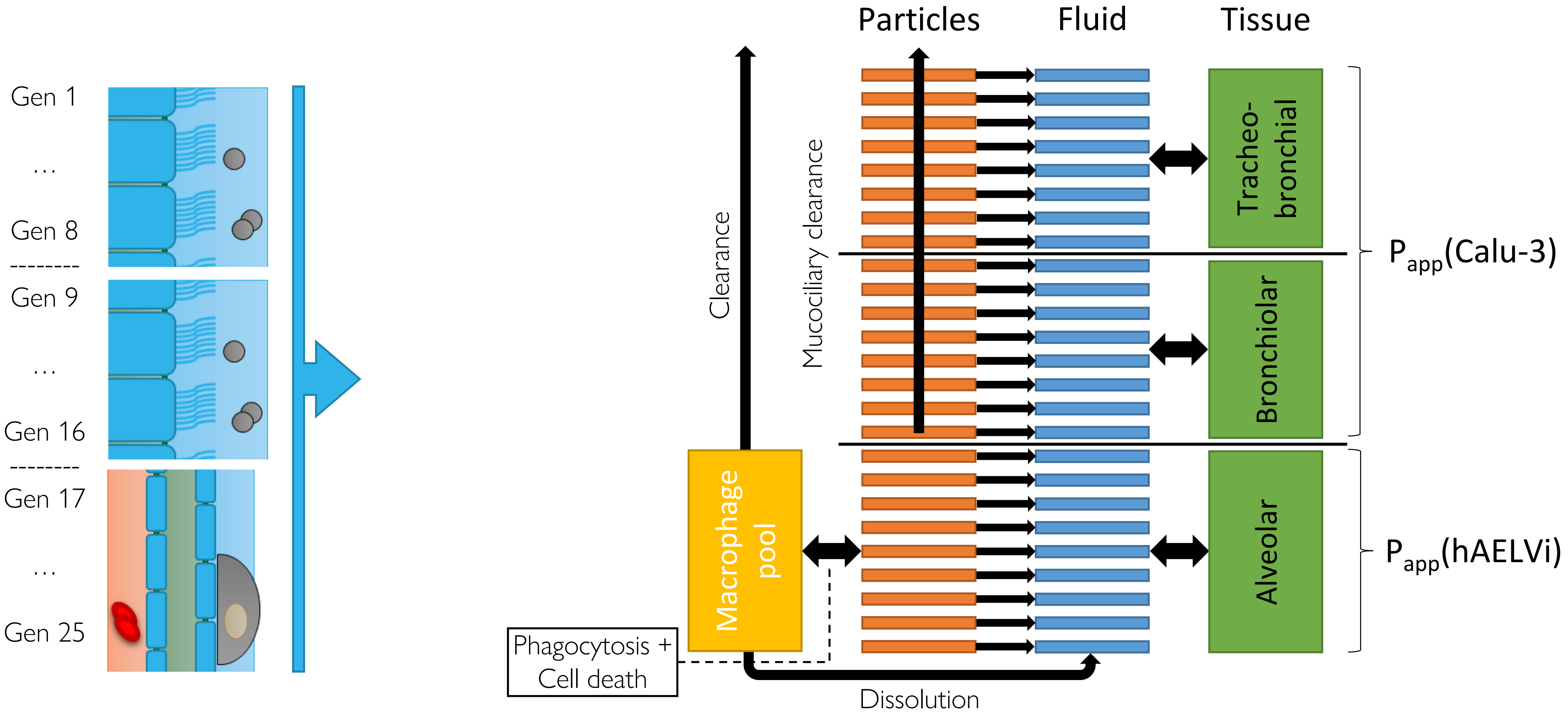
The PBKit model- Model structure

Division of the Lung based on Clearance Processes



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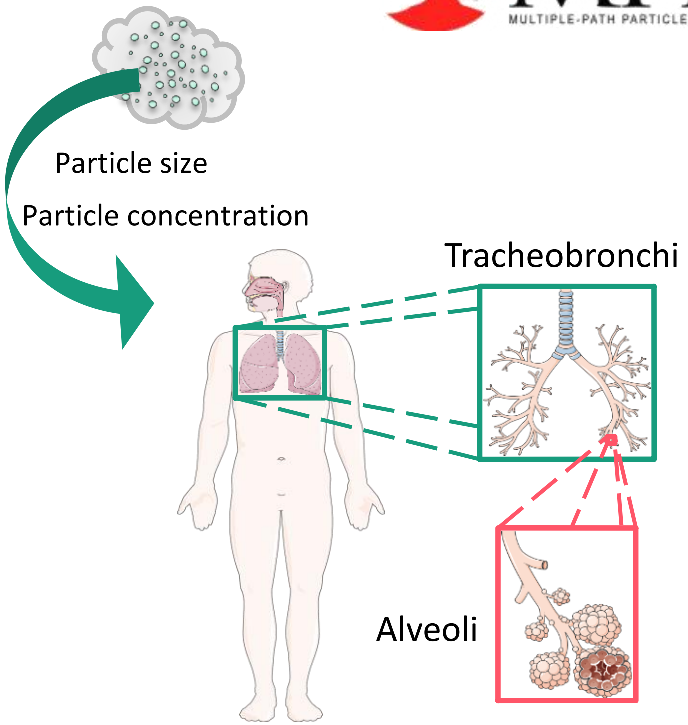
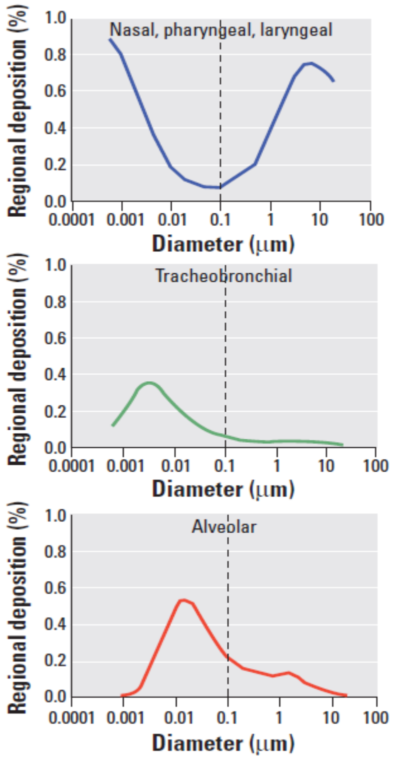
The PBKit model– Uptake processes

Aerosol Deposition

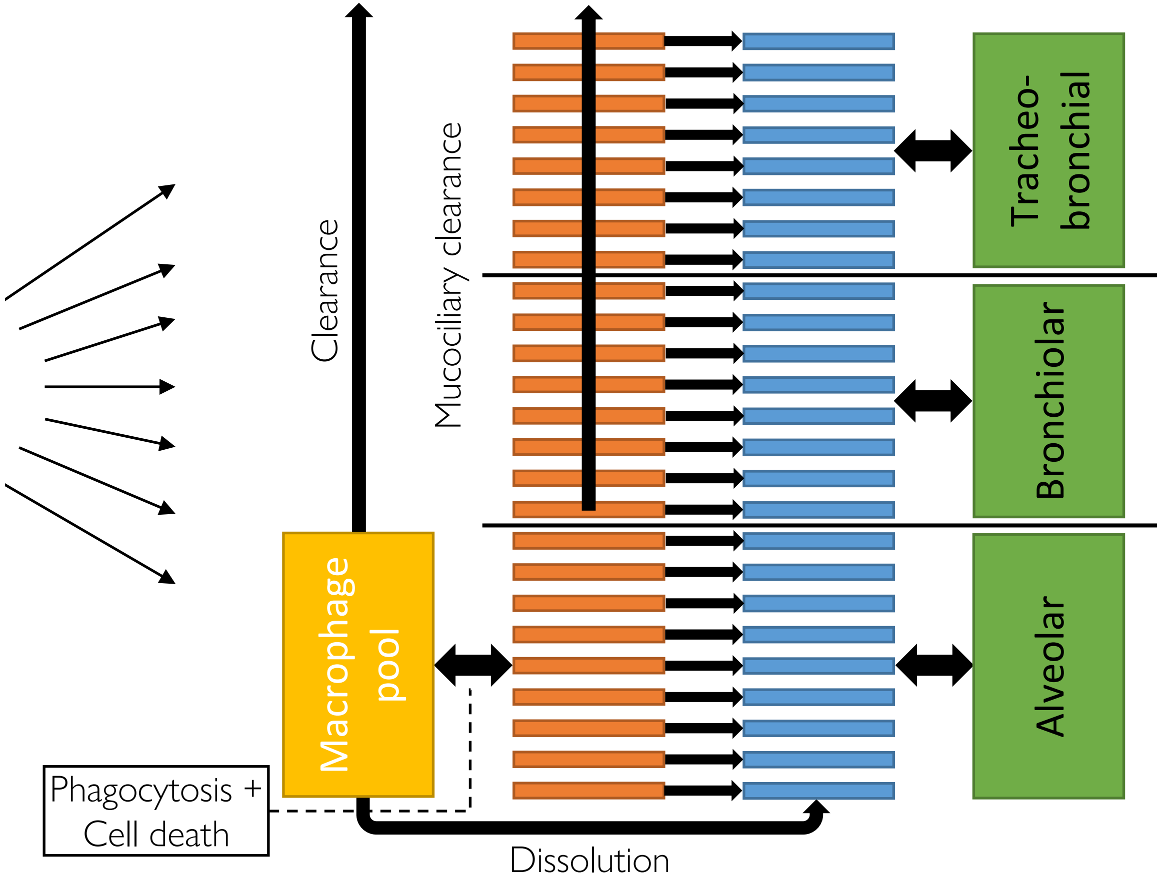
Regional aerosol deposition: Multiple-Path Particle Dosimetry Model

MPPD
MULTIPLE-PATH PARTICLE DOSIMETRY MODEL





Oberdörster et al. (2005)



The PBKit model– Clearance processes

Case Study – Mucociliary clearance inert particles

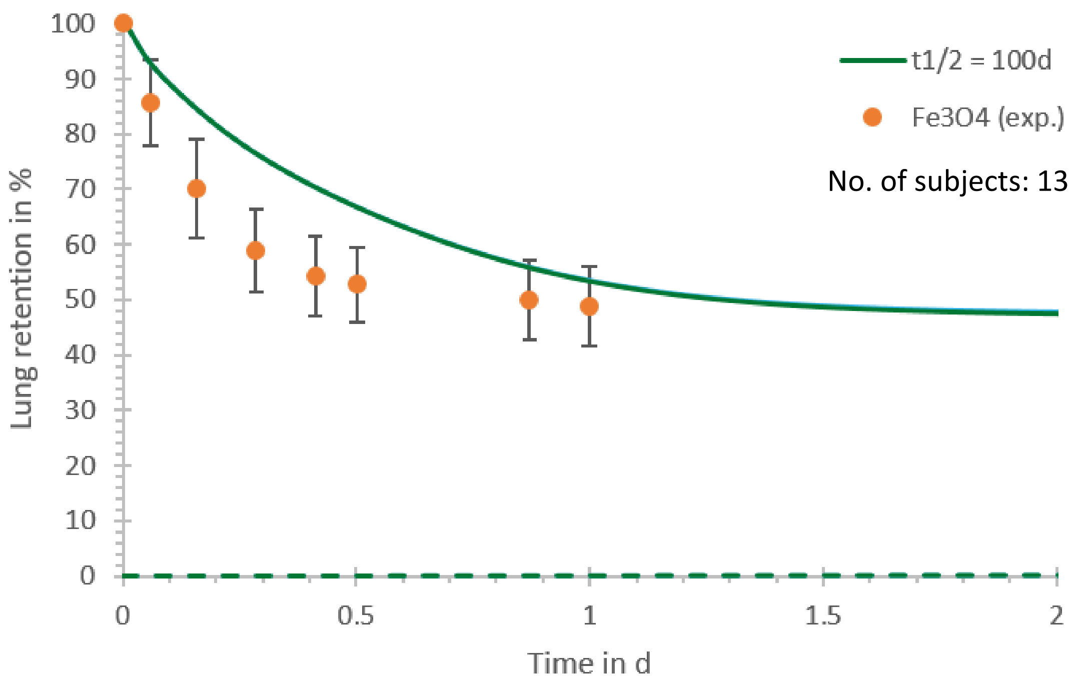
Fast short term clearance

—

The upper airways are lined with ciliated cells and mucus which compose the mucociliary "escalator" by which pathogens are cleared from the lung. This mechanical transport takes approximately 24 h to complete.

Once removed from the lung, particles are subsequently swallowed.

Exact clearance pattern is heavily dependent on the predicted deposition pattern in the upper lung airways



The PBKit model– Clearance processes

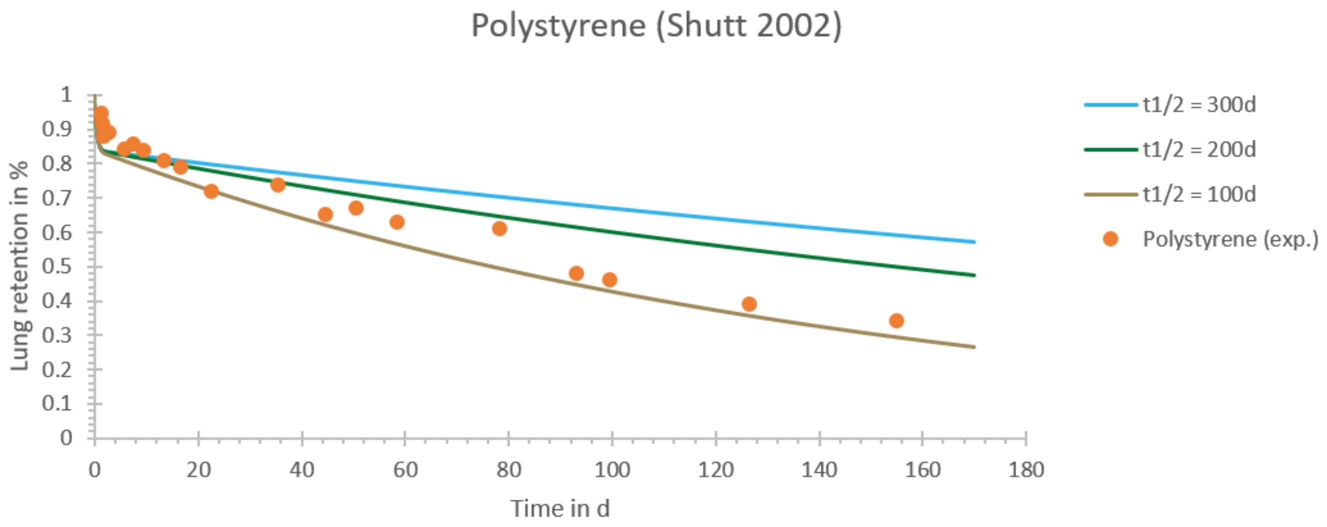
Case Study – Macrophage clearance inert polystyrene particles

Long term clearance

—

Slowly dissolving or bioinert particles are removed by mucociliary clearance in the upper airways or phagocytized by alveolar macrophages in the pulmonary region. Phagocytized particles are then either dissolved inside macrophages, released back to LLF due to apoptosis or cleared by slow removal to the upper respiratory tract.

Typical half-times are about 250 days.



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Chapter 2

PBK modelling
In-vitro uptake studies with microplastic

Exposure Scenarios – general considerations

Apparent permeability (P_{app}) of the lung barrier – ALI setup

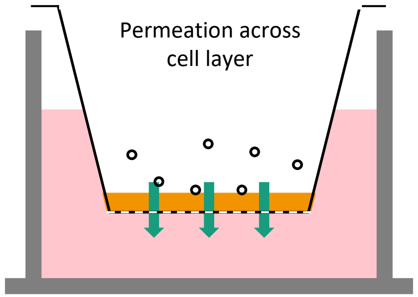
Apparent permeability (P_{app})

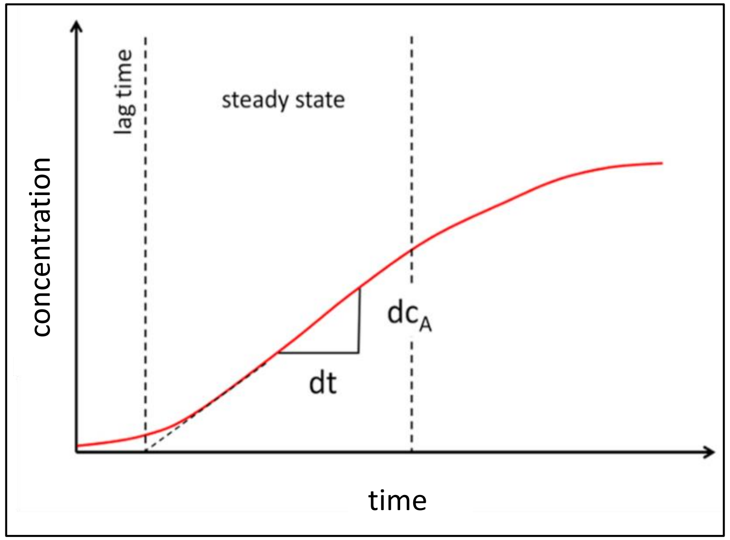
Measuring the apparent permeability of a substance through lung epithelial cells (e.g. Calu-3, hAELVi or primary BEC).

Cells are grown in transwells and are exposed by air.

Substance transfer from the apical to the basolateral compartment is measured

$$P_{app} = \frac{\Delta M}{\Delta t * A * c_0}$$





Experimental challenges:

Relevant exposure at ALI with dose control

Relevant cell model with barrier formation and control of the lining fluid thickness

Analytics to quantify the test compound

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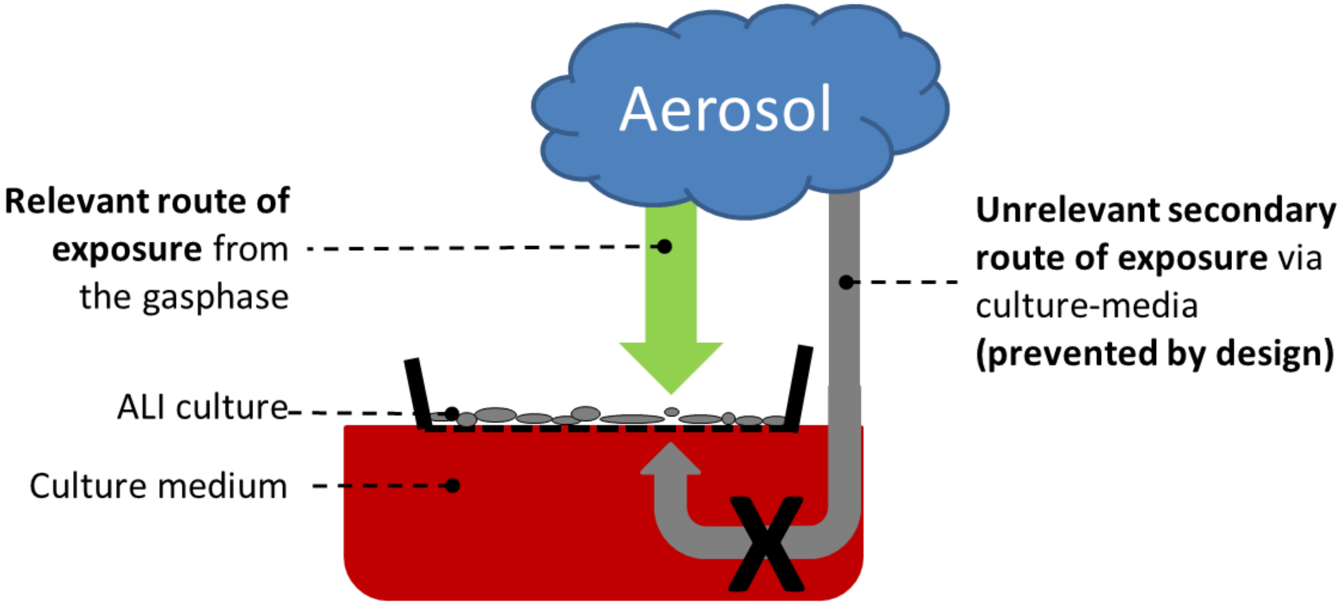
Exposure device
Air-liquid exposure (ALI)

Potential shortcut for
VOCs, gases and aerosols



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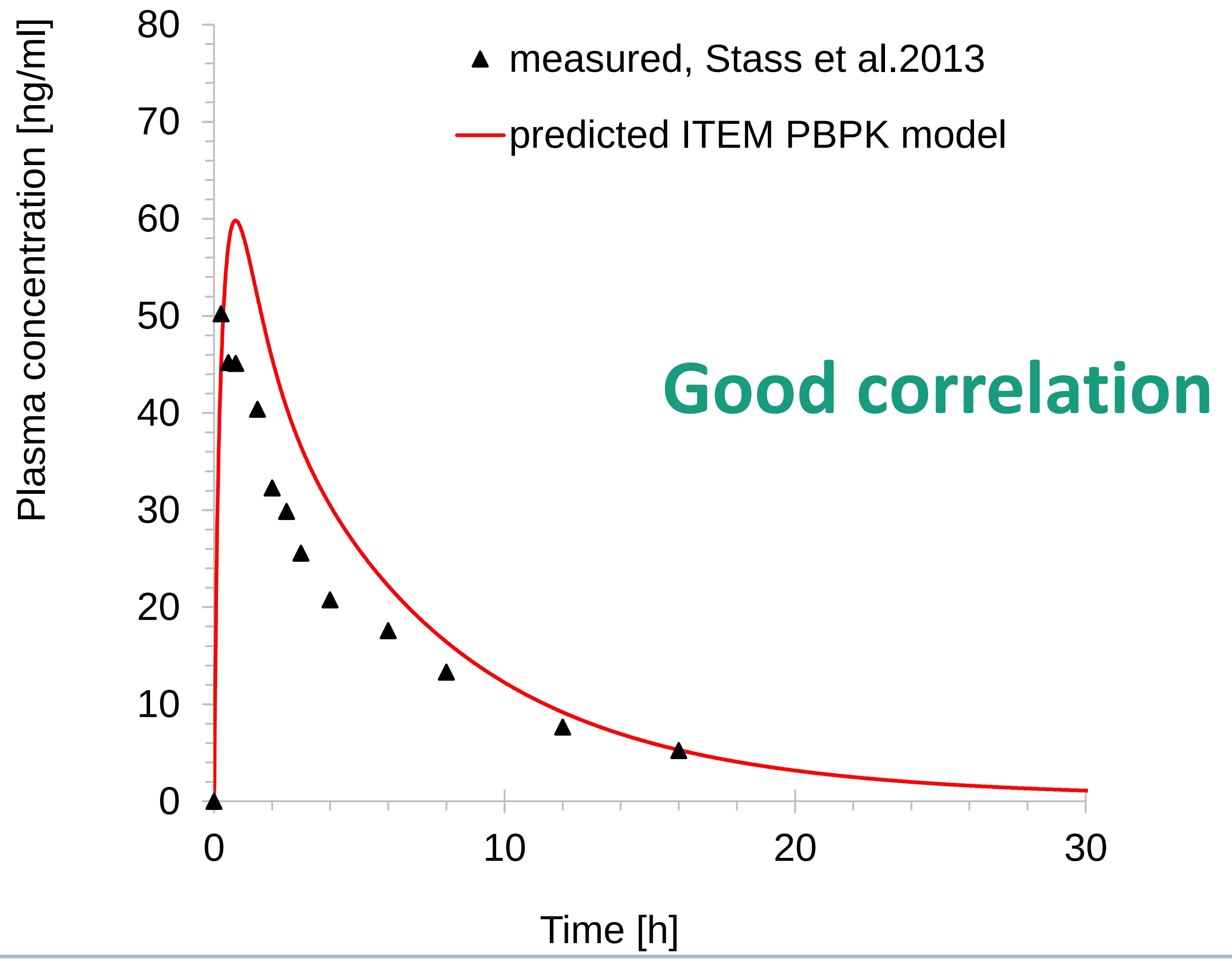
The PBKit model– Papp to model pulmonary uptake

Dry powder exposure to Ciprofloxacin

- P_{app}: inhalable drug
  - Experimental data
  - ALI exposure
  - Calu-3 and AT-1

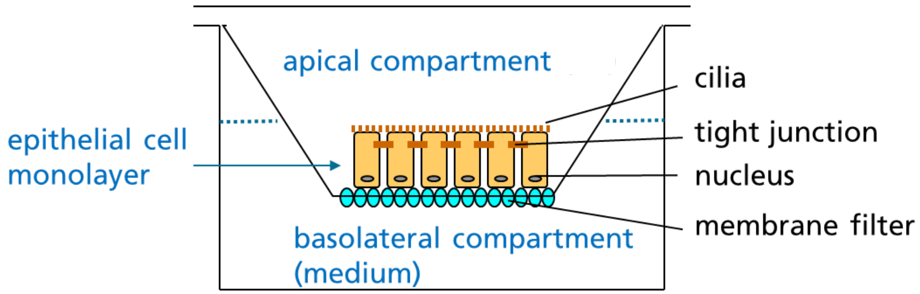


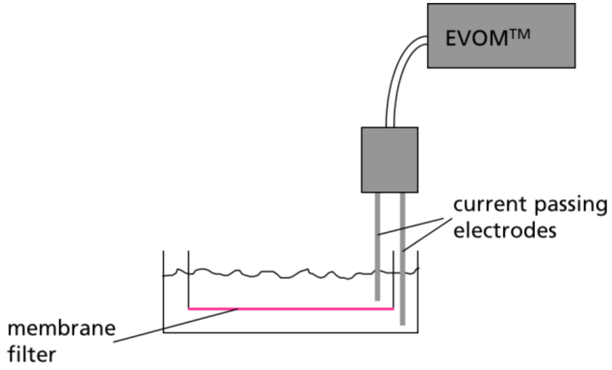
Transport of microplastic materials across the lung barrier

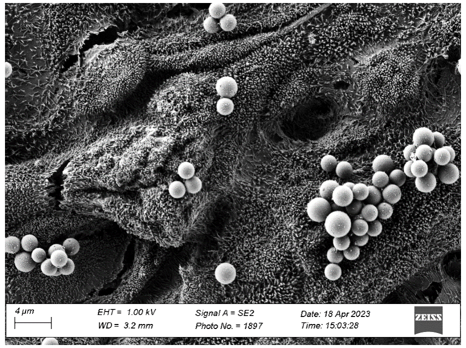
Experimental steps

Transport experiments across pulmonary epithelia

- Pre-culture of cells to ensure cellular differentiation and formation of tight monolayers.
- Cellular barrier integrity assessment (TEER)
- Determination of cytotoxicity
- Permeability (absorption) assessment:
- Apical exposure to the test compound
- Dose control (e.g. deposition for aerosols)
- **Microplastic particle localization on and in Calu-3 cells (and in the basolateral compartment)**



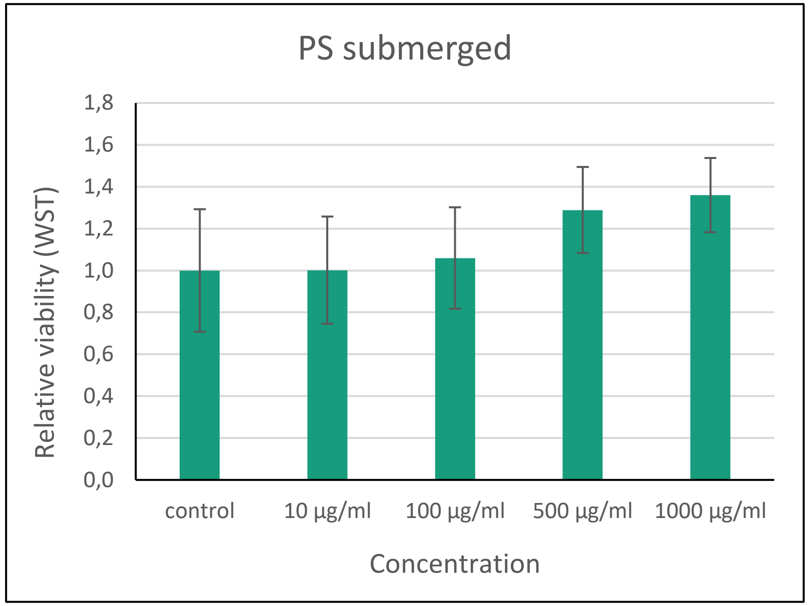


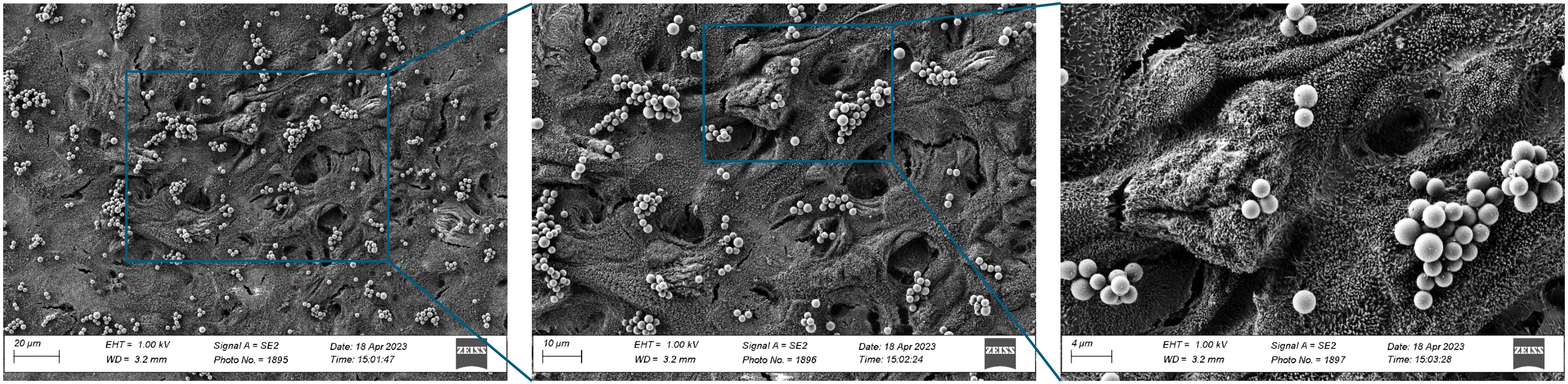


Particle localization on Calu-3 cells (SEM)

Calu-3 cells treated with PS particles (1000 µg/ml)

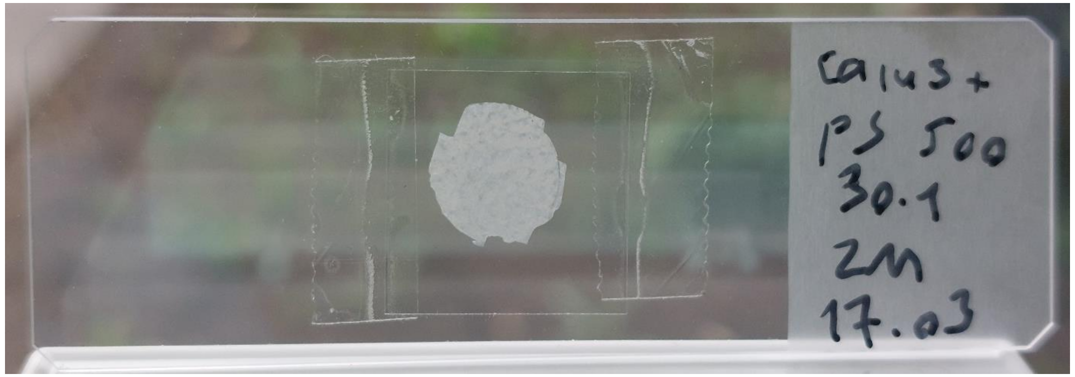
- Pre-culture and barrier integrity assessment
- Submerged exposure to PS beads (2 µm)
- Cytotoxicity testing (WST)
- Washing and fixation
- SEM analysis





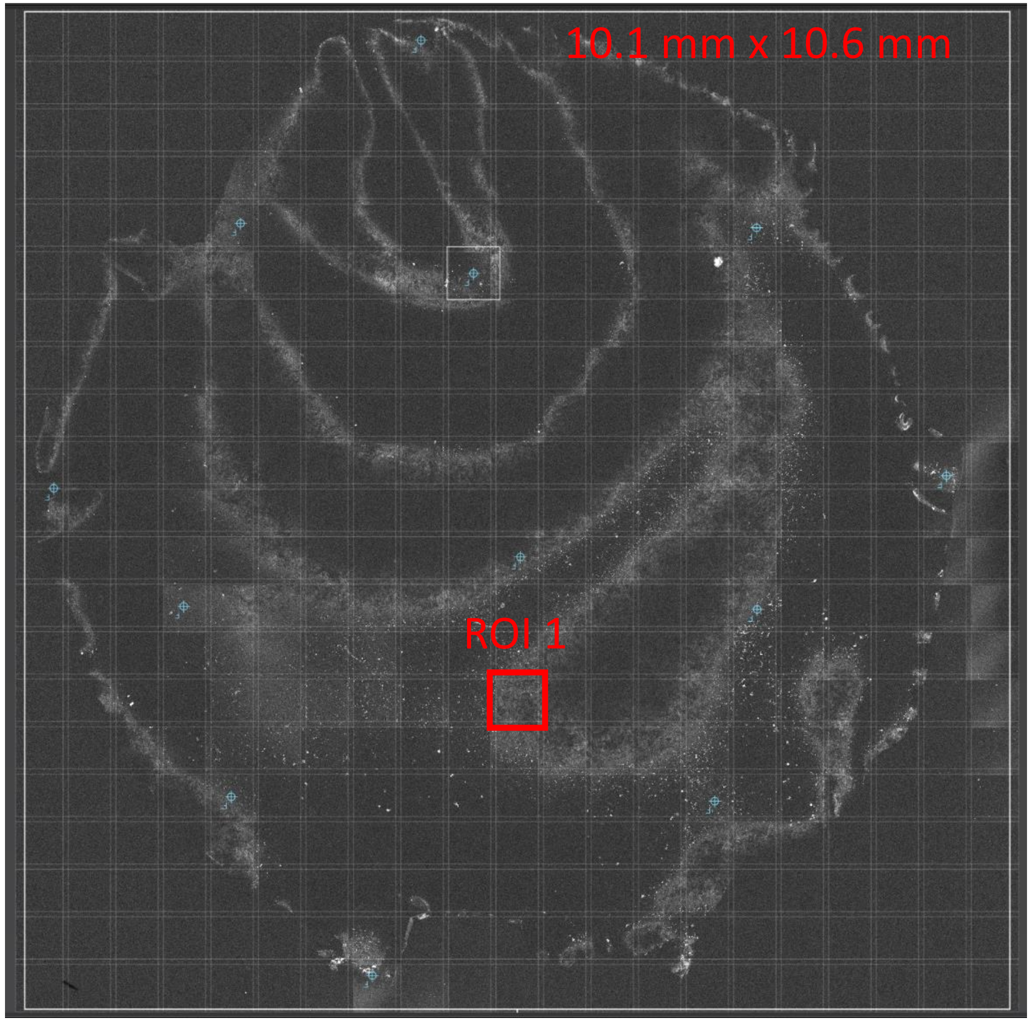
Particle localization in Calu-3 cells (SRS)

Calu-3 cells treated with PS particles (500 µg/ml)



- Stimulated Raman Scattering (SRS) mapping to detect PS particles on and inside cells
- Non-uniform PS distribution intrinsic and due to uneven surface of PET membrane
- Selected Region of interest (ROI1)
- Performed Z-Scans from cells‘ surface towards PET membrane for a 3D detection of PS particles
- Evaluated number and diameter of PS particles on and inside Calu-3 cells

Detection of PS particles by Raman



411 Tiles, 110 MB, 8 min 36 s, one Z level

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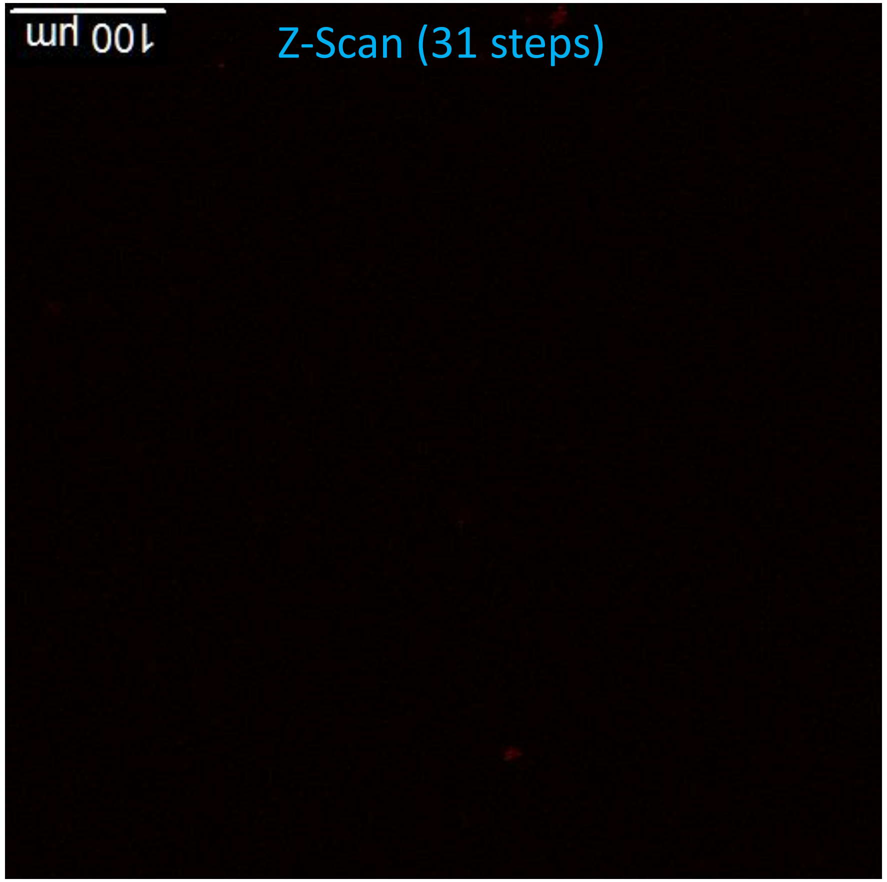
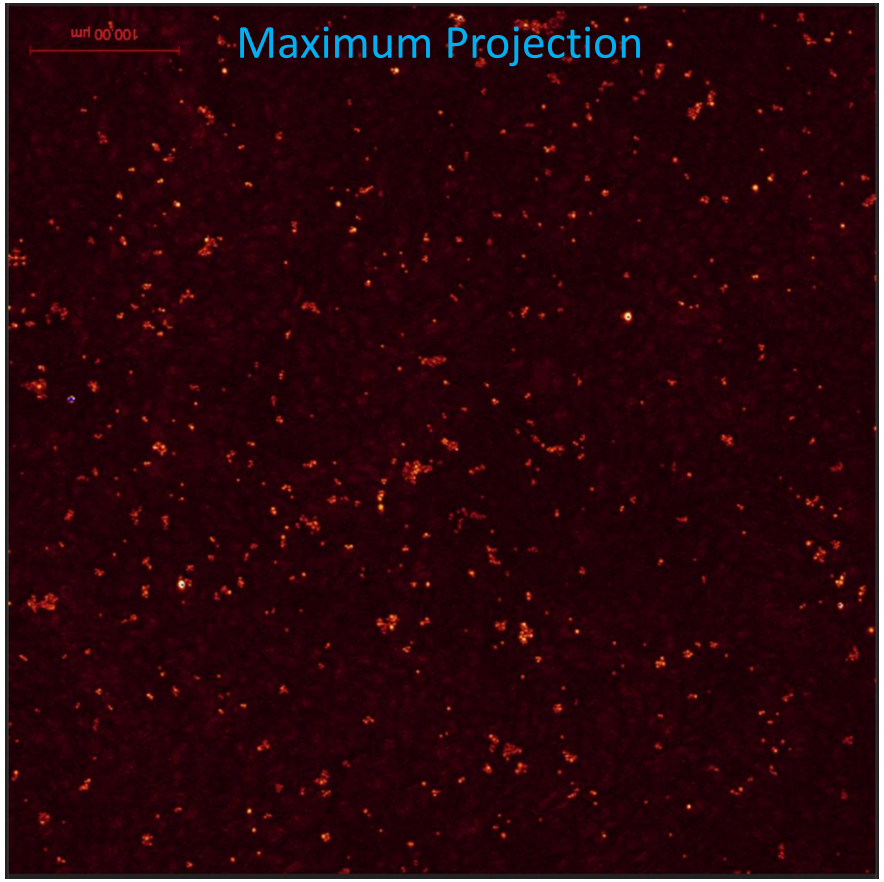
Particle localization in Calu-3 cells (SRS)

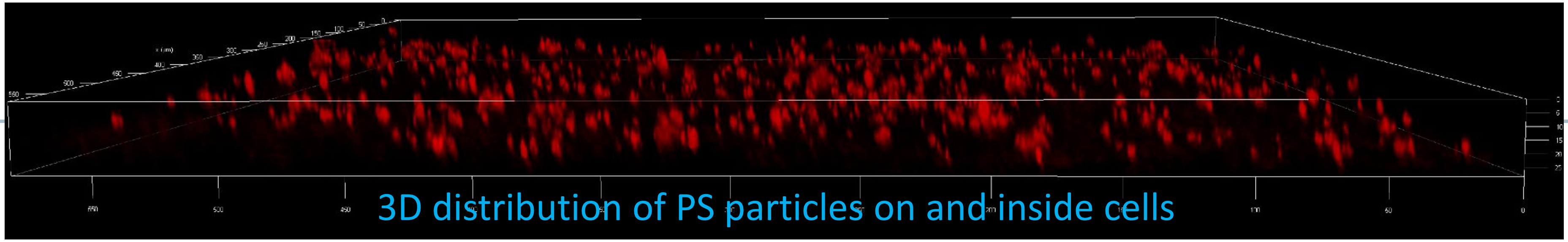
Calu-3 cells treated with PS particles



3D Z-Scan ROI 1 (XYZ)

- 581 µm x 581 µm x 27 µm
- X, Y: 1.13 µm, Z: 0.91 µm (pixel size)
- 8.1 MB, ~2 min





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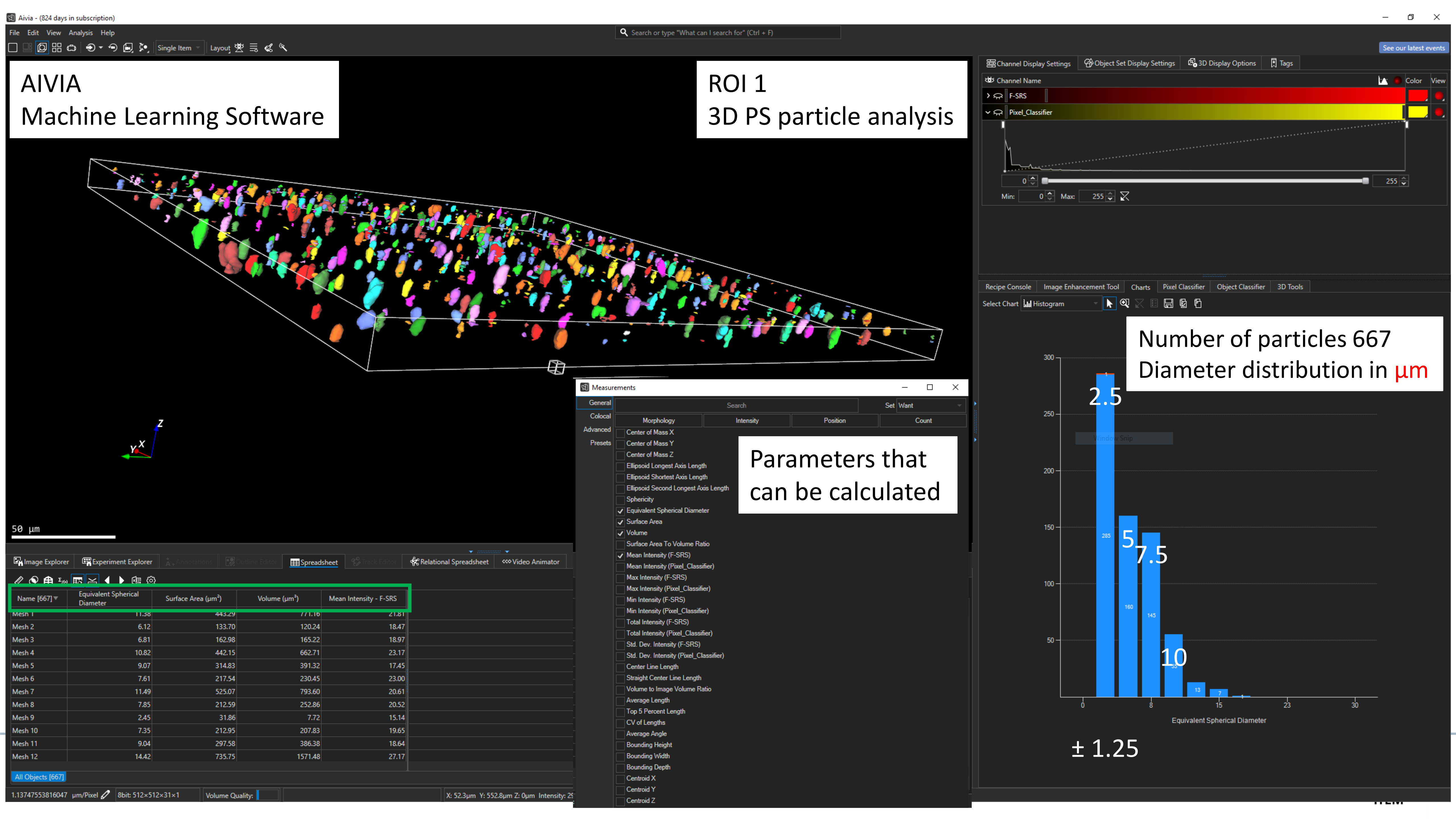


AIVIA
Machine Learning Software
ROI 1
3D PS particle analysis
Number of particles 667
Diameter distribution in µm
Parameters that can be calculated
50 µm
Measurements
General
Colocal
Advanced
Presets
Morphology
Intensity
Position
Count
Center of Mass X
Center of Mass Y
Center of Mass Z
Ellipsoid Longest Axis Length
Ellipsoid Shortest Axis Length
Ellipsoid Second Longest Axis Length
Sphericity
Equivalent Spherical Diameter
Surface Area
Volume
Surface Area To Volume Ratio
Mean Intensity (F-SRS)
Mean Intensity (Pixel_Classifier)
Max Intensity (F-SRS)
Max Intensity (Pixel_Classifier)
Min Intensity (F-SRS)
Min Intensity (Pixel_Classifier)
Total Intensity (F-SRS)
Total Intensity (Pixel_Classifier)
Std. Dev. Intensity (F-SRS)
Std. Dev. Intensity (Pixel_Classifier)
Center Line Length
Straight Center Line Length
Volume to Image Volume Ratio
Average Length
Top 5 Percent Length
CV of Lengths
Average Angle
Bounding Height
Bounding Width
Bounding Depth
Centroid X
Centroid Y
Centroid Z
Name [667] | Equivalent Spherical Diameter | Surface Area (µm²) | Volume (µm³) | Mean Intensity - F-SRS
Mesh 2 | 6.12 | 133.70 | 120.24 | 18.47
Mesh 3 | 6.81 | 162.98 | 165.22 | 18.97
Mesh 4 | 10.82 | 442.15 | 662.71 | 23.17
Mesh 5 | 9.07 | 314.83 | 391.32 | 17.45
Mesh 6 | 7.61 | 217.54 | 230.45 | 23.00
Mesh 7 | 11.49 | 525.07 | 793.60 | 20.61
Mesh 8 | 7.85 | 212.59 | 252.86 | 20.52
Mesh 9 | 2.45 | 31.86 | 7.72 | 15.14
Mesh 10 | 7.35 | 212.95 | 207.83 | 19.65
Mesh 11 | 9.04 | 297.58 | 386.38 | 18.64
Mesh 12 | 14.42 | 735.75 | 1571.48 | 27.17
All Objects [667]
2.5
5
7.5
10
285
160
145
13
7
Equivalent Spherical Diameter
± 1.25

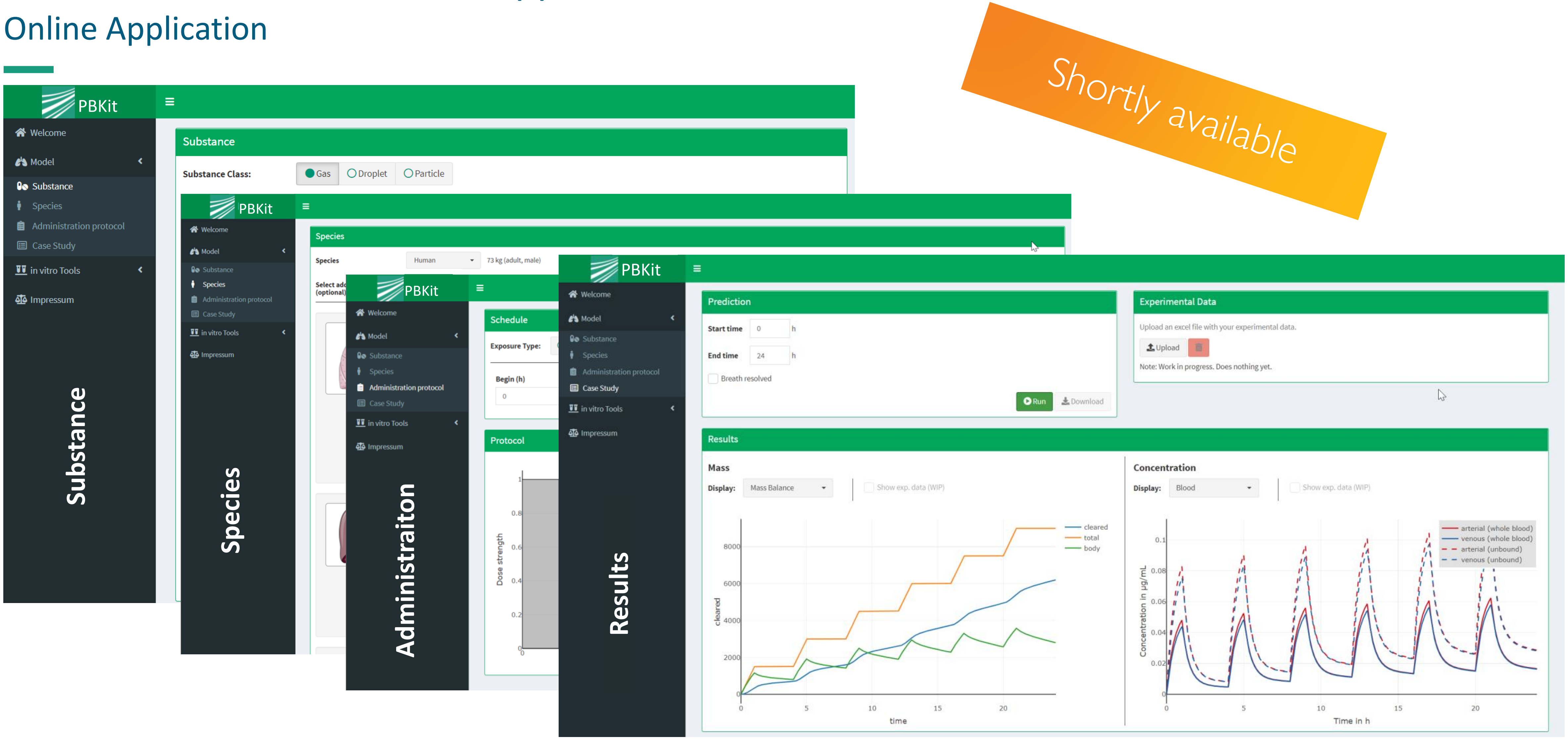
Summary Part 2

PBK modelling

- PBKit: Inhalation PBK model with multicompartmental lung
  - PBKit can be used to model particle retention
  - PBKit can be used to model uptake
- Cellular pulmonary barrier models can be used to derive P_{app} coefficients as input parameters
- Visualization and quantification of particles is challenging when studying microplastic uptake
  - Stimulated Raman Scattering (SRS) is a promising method to measue cellular uptake of microplastic particles providing 3D visualization of particle distribution inside cells

PBKit accessible as free web app

Online Application



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Thank You

Fraunhofer ITEM

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Andreas Hiemisch

Sabrina Lamsat

Tanja Schwarz

Fraunhofer IKTS

Silke Christiansen

George Sarau

Zeynab Mirzaei

BASF We create chemistry

Wendel Wohlleben

Katherine Y Santizo

Susanne Kolle

Andreas Verlohner

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Cefic LRI-B21

Cefic RI-C10

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