

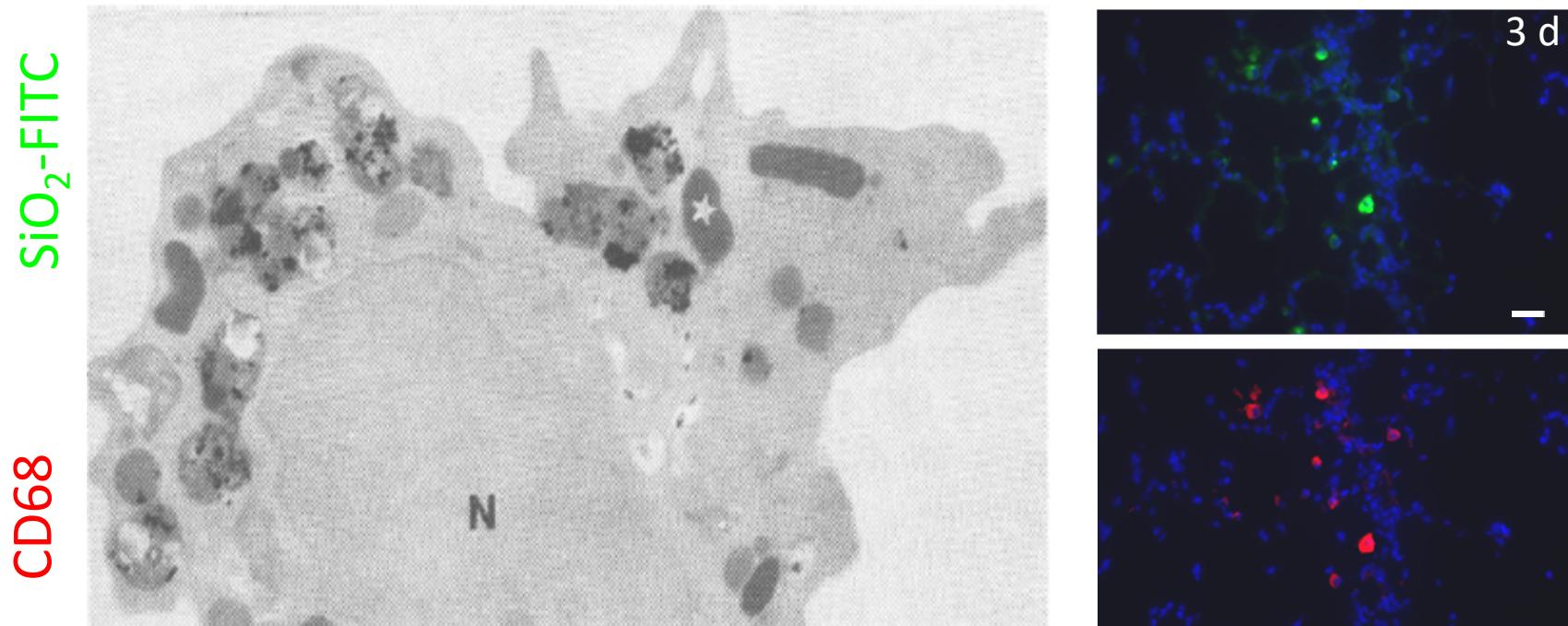
# Can We Reduce Animal Testing? A Tiered Approach Based on In Vitro Screening

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# Alveolar macrophages take up the majority of nanoparticles in the lung: colloidal amorphous silica (15 nm)



Alveolar macrophages are among the first cells to take up and concentrate inhaled NP

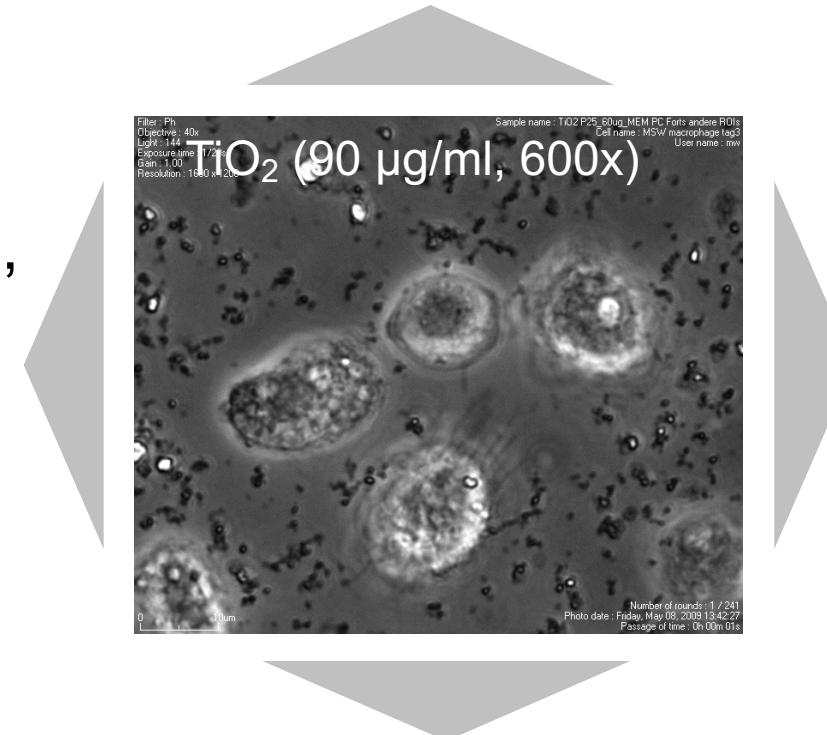
For AIOOH a cell burden of up to 90 pg/cell was found (Pauluhn et al. Toxicol. Sci. 2009)

# Effects of (nano) particles on alveolar macrophages in vitro

## General cytotoxicity: LDH Assay

Lysosomal damage, activation,  
release of lytic enzymes:  
**Glucuronidase Assay (GLU)**

ROS formation,  
oxidative burst:  
**Amplex Red Assay  
( $H_2O_2$ )**



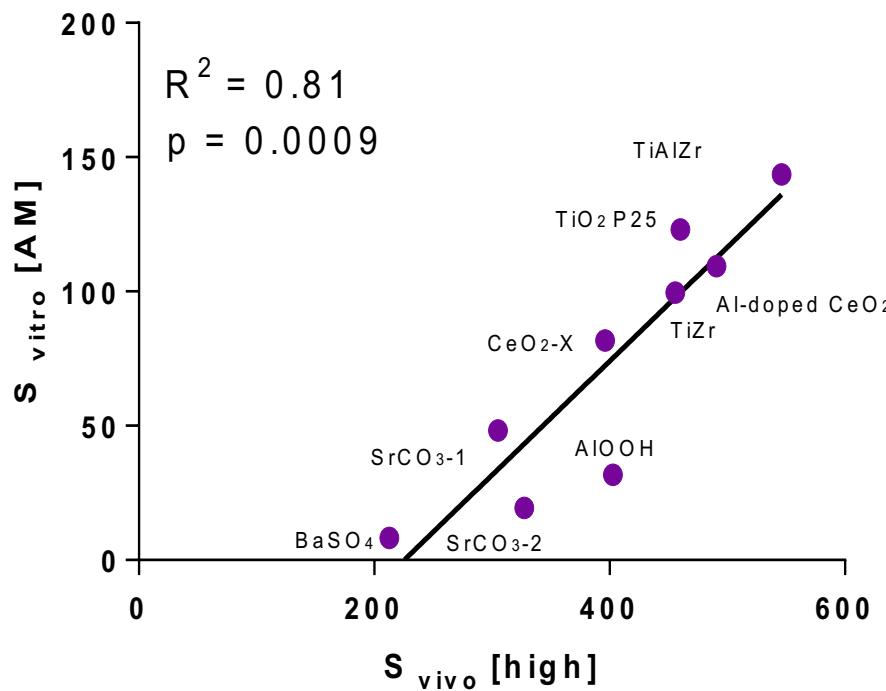
Release of **TNF $\alpha$**  as a major pro-inflammatory  
cytokine: ELISA (or L-929 Fibroblast Reporter Assay)

## In vitro - in vivo Correlation for PSP (BET surface 40-60 m<sup>2</sup>/g)

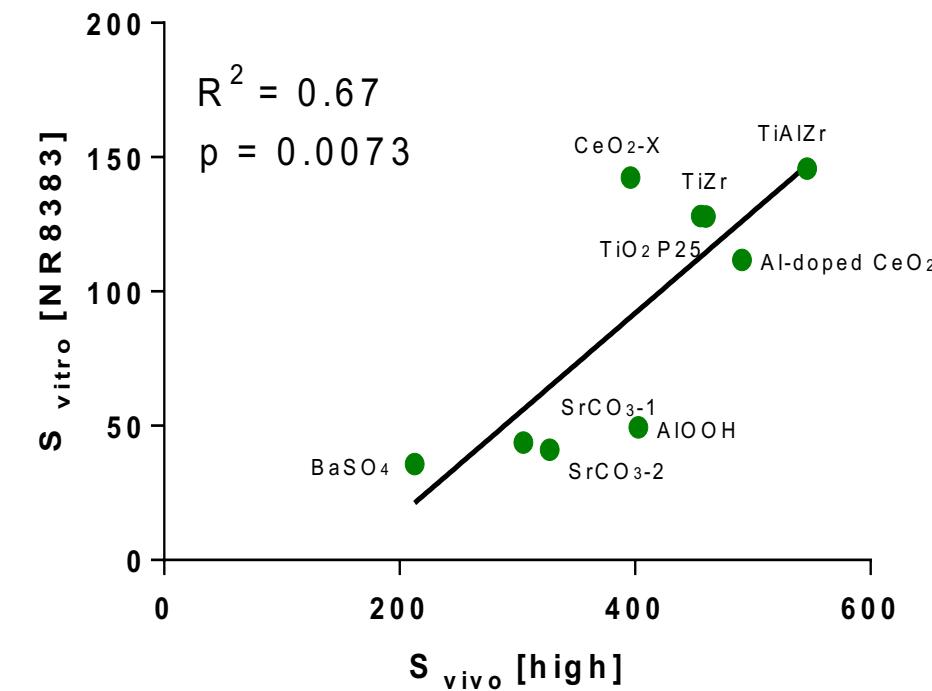
Sum Index S<sub>vitro</sub> (LDH, GLU, TNF, H<sub>2</sub>O<sub>2</sub>) vs.

Sum index in S<sub>vivo</sub> (Instillation, BALF (3d): PMN, AM, protein, fibronectin)

Primary Alveolar Macrophages

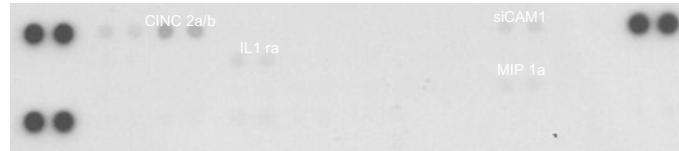


Cultured AM (NR8383 cells)

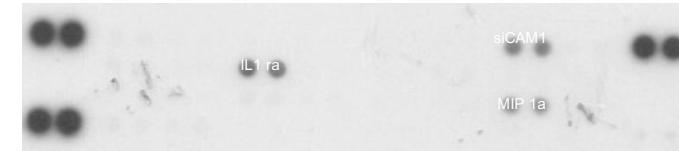


## NR8383 vs. primary AM: Comparison of Cytokine response

Rat Alveolar Macrophages  
Control



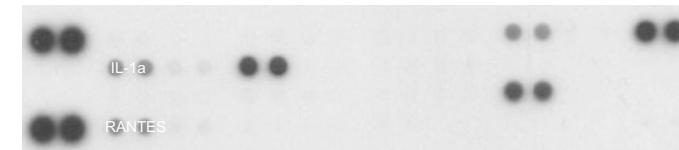
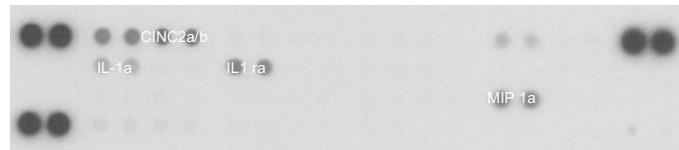
Rat NR8383 cells



NR8383 cells preferred (3R principles!):

- well established cell line (Helmke et al. 1987, 1989)
- cells phagocytose particles under serum-free conditions
- cells generate  $H_2O_2$
- interleukine response similar to primary AM (IL-1 $\alpha$ , IL-1 $\beta$ , TNF $\alpha$ , MIP1 $\alpha$ , IL10, CINC1, CINC2ab, RANTES ...)

DQ12



# Towards assay validation with inhalation studies:

## Short Term Inhalation Study (STIS): General Protocol (GLP conditions)



Study day	1	2	3	4	5	6	7	8	9 - 27	28
Study phase	x	x	x	x	x	P	P	P	P	P
Examinations					H			L		H+L

**X Generation of aerosol** with a brush or swinging bed dust generator, **Head-nose exposure** to aerosols for **6 h/d, 5d** (0.1-50 mg/m<sup>3</sup>)

**P** Post-exposure period

**H Histology** of selected organs, **Organ burden** (lung, mediastinal lymph nodes, liver, kidney, spleen and basal brain with olfactory bulb),

**L Broncho-alveolar lavage fluid** (BALF) Differential cell counts and biochemical parameters

# Short Term Inhalation Studies (STIS) to differentiate passive and active nanomaterials (NM)

No adverse effects observed up to 10-50 mg/m<sup>3</sup>

BaSO<sub>4</sub>, SiO<sub>2</sub>-PEG, SiO<sub>2</sub>-phosphate, SiO<sub>2</sub>-amino, nano-ZrO<sub>2</sub>, ZrO<sub>2</sub>-TODA, ZrO<sub>2</sub>-acrylate, SiO<sub>2</sub>-acrylate, graphite nanoplatelets , low surface area carbon black, Pigment Orange (nano), Pigment Blue 15

“passive NM”

no adverse effects  
>10 mg/m<sup>3</sup>

Adverse effects observed at 10 mg/m<sup>3</sup>

SiO<sub>2</sub>-unmodified, Graphene, Pigment Orange (bulk)

“active NM”

Adverse effects observed at approx. 0.5 mg/m<sup>3</sup>

nano-CeO<sub>2</sub>, Al-doped nano-CeO<sub>2</sub>, coated nano-ZnO, coated nano-TiO<sub>2</sub> , uncoated nano-TiO<sub>2</sub>

adverse effects  
≤10 mg/m<sup>3</sup>

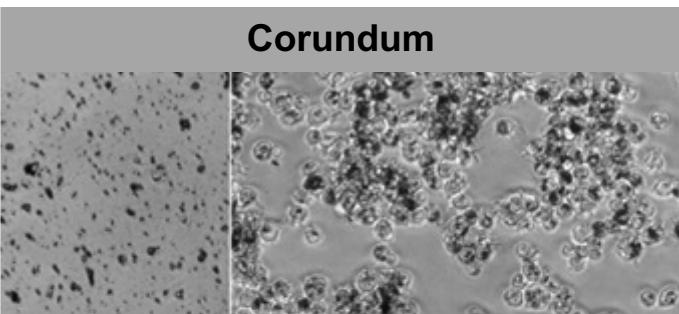
NOAEC levels < 0.5 mg/m<sup>3</sup> and progressive effects

MWCNT , quartz

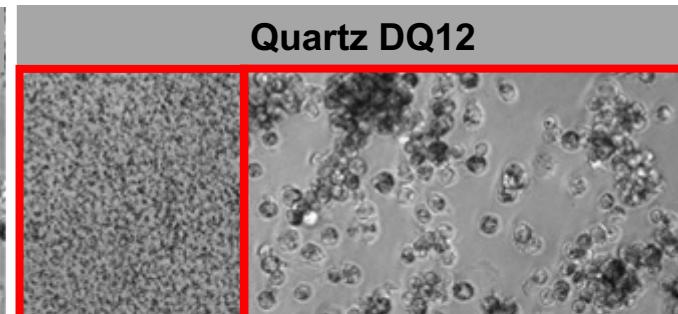
# Particle settling and uptake by NR8383 cells under serum-free conditions

Negative control

Corundum

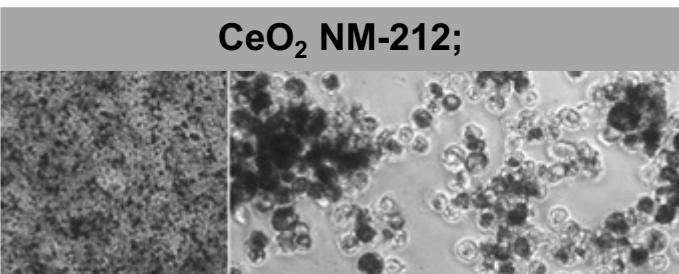


Quartz DQ12

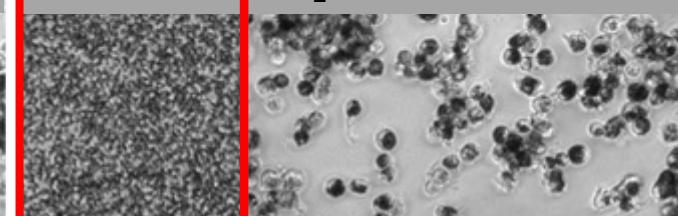


Positive control

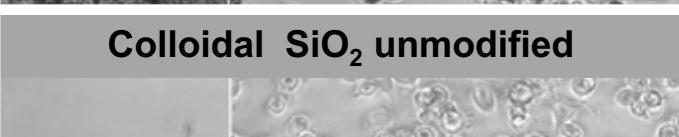
CeO<sub>2</sub> NM-212;



TiO<sub>2</sub> NM-105



Colloidal SiO<sub>2</sub> unmodified



BaSO<sub>4</sub> NM-220;

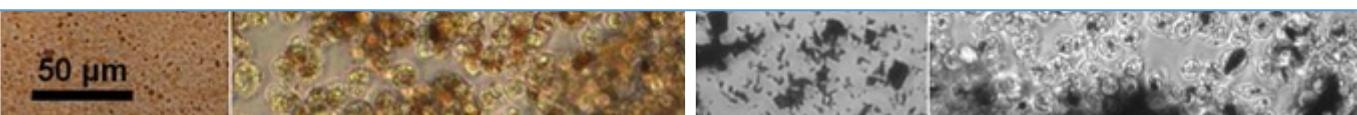


Incubation conditions:

3 × 10<sup>5</sup> cells per well, 200 µl particle suspension

Complete uptake (ideal conditions):

22.5 – 45 - 90 -180 µg/mL → 15 – 30 – 60 – 120 pg/cell



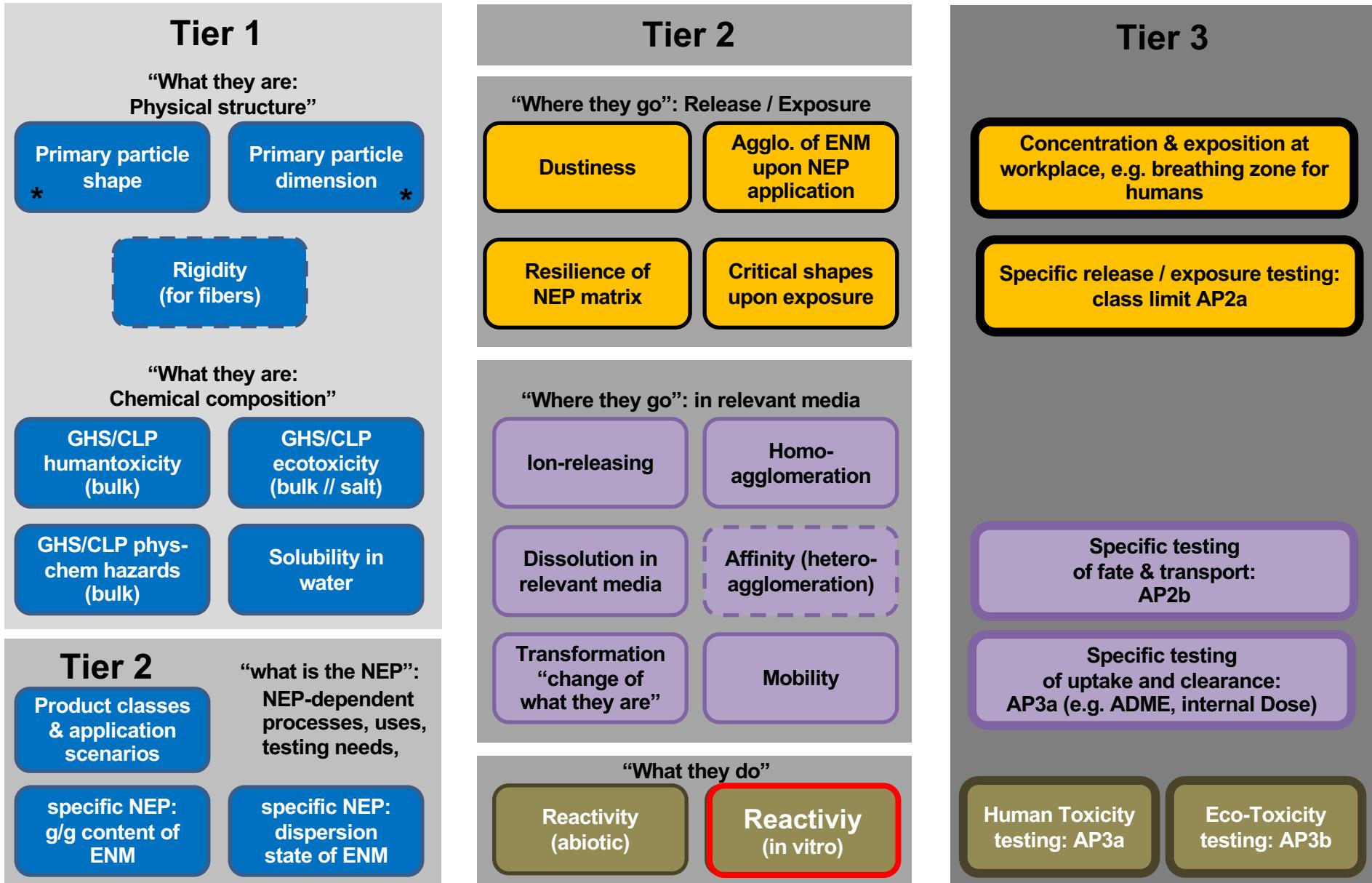
## Comparison of in vitro (NR cells) and STIS results and assay evaluation

- Consider particle surface: BET surface x LOAEC
- Compare the value to an overload threshold:  $\leq 4000 \mu\text{m}^2/\text{cell}$  ( $\rightarrow 6000 \text{ mm}^2/\text{mL}$ )
- Apply the “Two-out-of-four criterion“ to distinguish “active“ and “passive“ nanomaterials

Test materials			In vitro NR8383 AM assay								STIS			
Class	Name	BET [m <sup>2</sup> /g]	LOAEC [μg/mL]				LOAEC [mm <sup>2</sup> /mL]				NOAEC [mg/m <sup>3</sup> ] threshold <10	LOAEC [mg/m <sup>3</sup> ] <sup>a</sup> threshold <10	Ref. <sup>d</sup>	
			LDH	GLU	TNF-α	ROS H <sub>2</sub> O <sub>2</sub>	LDH	GLU	TNF-α	ROS H <sub>2</sub> O <sub>2</sub>				
Micron-sized crystalline silica	Quartz DQ12	8	90	90	45	n.s.	720	720	360	n.s.	3	0.1	1.0	[106]
Active metal oxide NMs	TiO <sub>2</sub> NM-105	47	90	90	90	n.s.	4230	4230	4230	n.s.	3	<2	2.0	[10]
	ZnO NM-111	15	5.6	90	22.5	n.s.	84	1350	338	n.s.	3	0.5	2.5	[11]
	nano-CeO <sub>2</sub>	33	90	180	45	n.s.	2970	n.s.	1485	n.s.	2	0.5	2.5	[11]
	Al-doped CeO <sub>2</sub>	46	45								3	0.5	2.0	[11]
	CeO <sub>2</sub> NM-211	66	90								2	<0.5	5.0	[111]
	CeO <sub>2</sub> NM-212	27	90								2	<0.5	5.0	[111]
Amorphous SiO <sub>2</sub> NMs	SiO <sub>2</sub> .naked	200	22.								0	2	2.5	[11]
	SiO <sub>2</sub> .PEG	200	90								0	0	≥50	n.r.
	SiO <sub>2</sub> .amino	200	45								0	1	≥50	n.r.
	SiO <sub>2</sub> .phosphate	200	90								0	0	≥50	n.r.
	SiO <sub>2</sub> NM-200	189	22.								3	1	5	[80]
	SiO <sub>2</sub> NM-203	200	22.								3	1	5	[80]
Passive metal oxide and metal sulphate NMs	AlOOH	105	90								0	(3 <sup>b</sup> )	(28 <sup>b</sup> )	[58]
	BaSO <sub>4</sub>	41	n.s.								0	0	≥50	n.r.
	Fe <sub>2</sub> O <sub>3</sub> (hematite)	98	n.s.								0	0	≥30	n.r.
	ZrO <sub>2</sub> .TODA	117	45								30	1	≥50	n.r.
	ZrO <sub>2</sub> .acrylate	117	70.								97	0	≥50	n.r.
Nanosized organic pigments	DPP Orange N	64	n.s.	n.s.	45	n.s.	n.s.	n.s.	2880	n.s.	1	≥30	n.r.	[79]
	Pigment Blue 15:1	53	90	90	n.d. <sup>c</sup>	n.s.	4770	4770	n.d. <sup>c</sup>	n.s.	2	≥30	n.r.	[79]
Carbonaceous NM	Graphite nanoplatelets	74	n.s.	45	90	n.s.	n.s.	3330	6660	n.s.	1	≥10	n.r.	[82]

From: Wiemann et al. 2016, J. Nanobiotechnology, 14:16

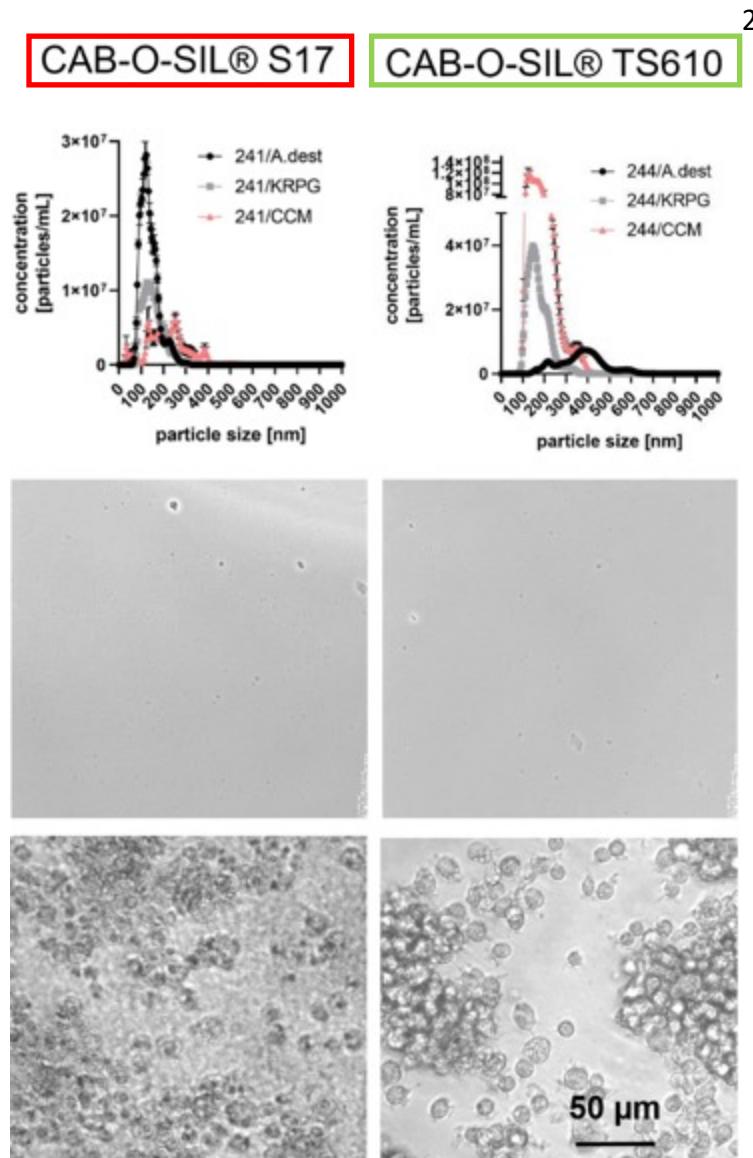
# Embedding into the tiered strategy for the risk assessment of nanomaterials



future property:  
No method yet established

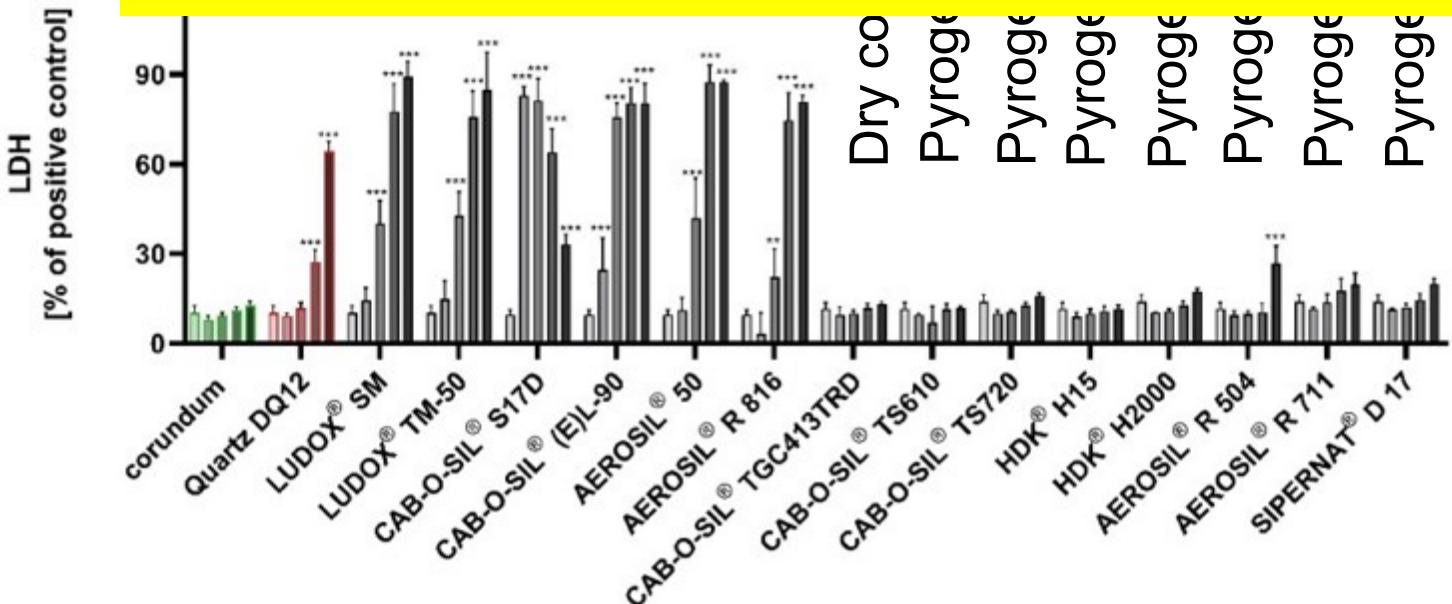
\* = also a descriptor of nanoform ranges  
NEP = nano-enabled product

# Surface-treatment of SAS<sup>1)</sup> reduced bioactivity!



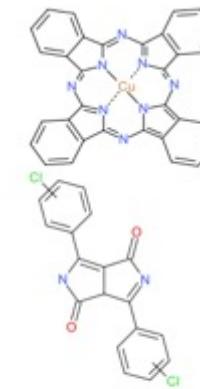
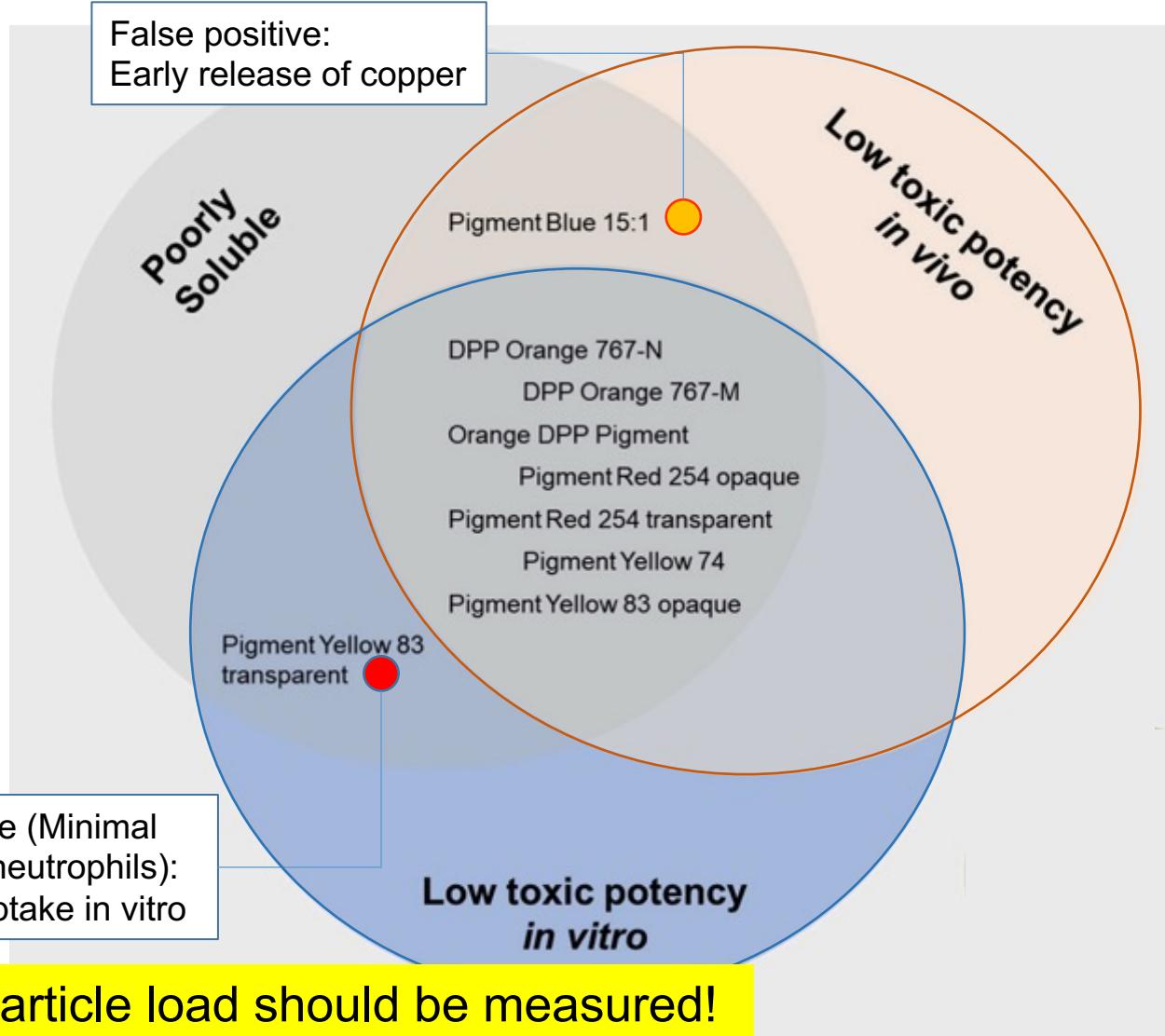
al      al      nic      nic      nic      HMDZ      MD      OMS      MDCS      MDZ      EMO+HDMZ      EMO      OMS

Effects of surface treatment on the bioactivity of a NM can be explored with the AMA!

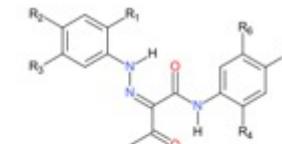


- 1) All SAS were provided by the **SASFORREACH Consortium GbR**
- 2) Very similar to AEROSIL® R 974 which was found less bioactive than CAB-O-SIL® S17 in a 90 d inhalation study.

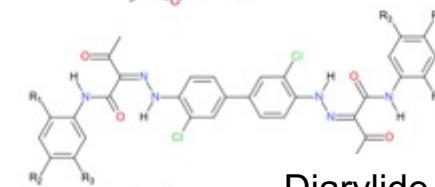
## Prediction of Lacking Toxicity of Poorly Soluble Organic Pigments



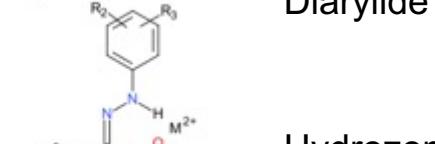
Phthalocyanine Pigments



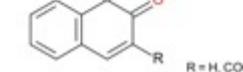
Diketopyrrolo-Pyrrole Pigments



Monohydrazone Pigments



Diarylide Pigments



Hydrozone Pigment Lakes

# Conclusions

- Macrophage responses *in vitro* (NR8383 cells) correlate with signs of acute inflammation *in vivo* (instillation and STIS).
- A panel of four *in vitro* parameters from NR8383 cells (LDH, GLU, TNF, H<sub>2</sub>O<sub>2</sub>) appears sufficient to differentiate between “active” and “passive” nanomaterials, if the specific particle surface (BET) and the 2-out-of-4 criterion is considered.
- The alveolar macrophage assay (AMA) is suggested as a screening tool in a tiered approach.
- Further validation is necessary.



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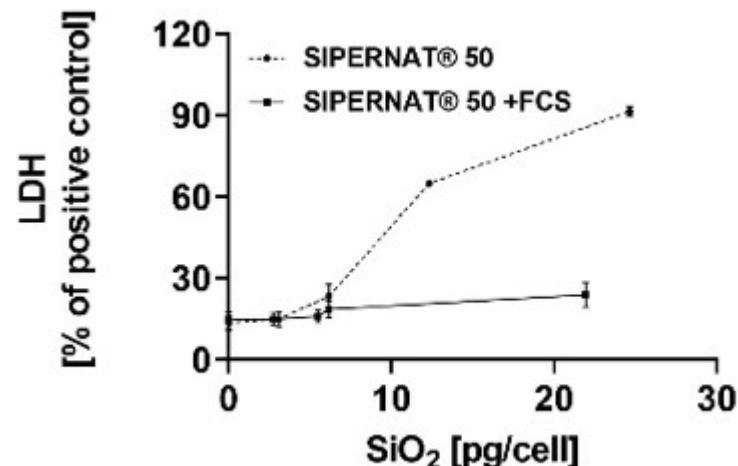
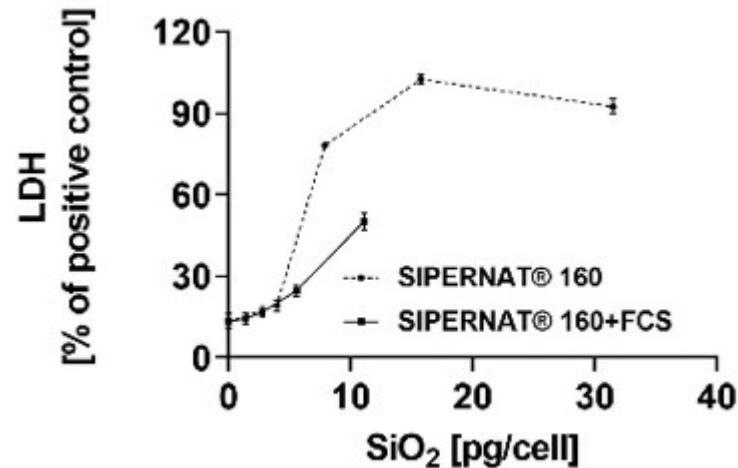
Thanks for your  
attention!



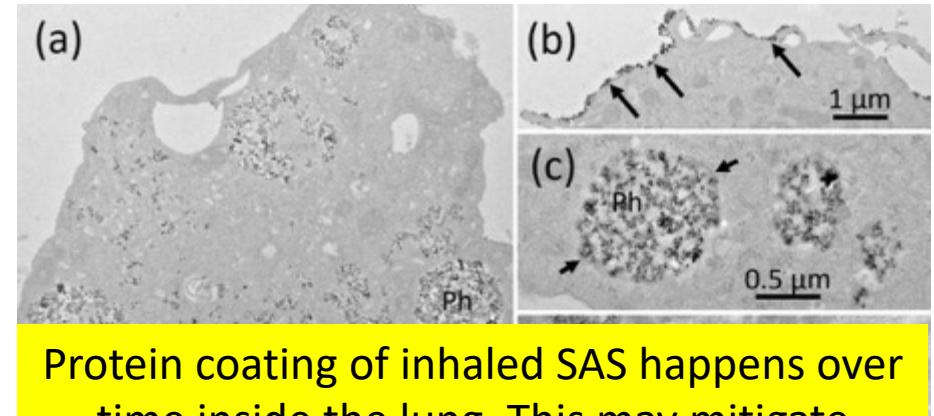
SASFORREACH Consortium GbR

## The influence of fetal calf serum (FCS) on cytotoxicity and subcellular distribution of particles

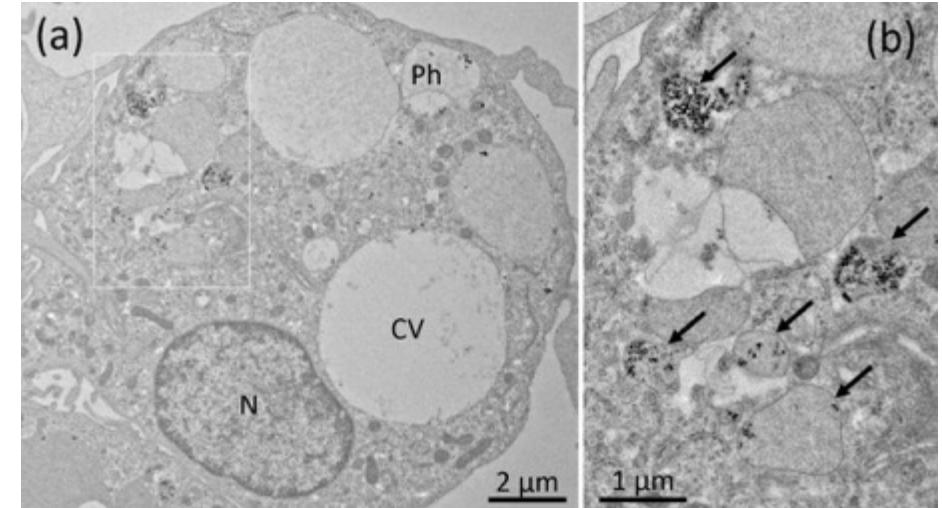
Sipernat® 160 and Sipernat® 50



Aerosil® 380F



Protein coating of inhaled SAS happens over time inside the lung. This may mitigate adverse effects of SAS!



From: Wiemann et al. 2020, Nanomaterials(Basel)

## Use of the pattern of LOAECs for grouping (Cluster Analysis)

nano**GRAVUR**

