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Cellular effects and gene expression after exposure to amorphous silica nanoparticles in vitro

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Introduction

Much of the concerns regarding engineered nanoparticle (NP) toxicity are based on knowledge from previous studies on ambient and environmental particles. E.g., the effects of exposure to silica dust particles have been studied intensively due to the carcinogenicity of crystalline silica. However, the increasing usage of engineered silica (SiO_2) NPs, which mainly occur in the amorphous form, has emphasized the need for further mechanistic insight to predict the consequences of exposure to this type of silica NPs. Different mechanisms have been proposed to explain NP toxicity, e.g., dissolution of the NPs (Beer et al., 2011). But, reactive oxygen species (ROS) are generally considered to be a key event in NP toxicity. As there are limited human studies on NP toxicity, risk assessment is based upon *in vivo* animal studies and *in vitro* studies mostly using established human cell lines. Thus, knowledge about the differential responses to toxicants in human and murine cells from comparable organs becomes an important intermediate to allow extrapolation from mouse to human.

Objectives

1) To study the cytotoxicity of amorphous silica NPs in three pairs of human/mouse cell lines selected to explore the significance of tissue type and species.

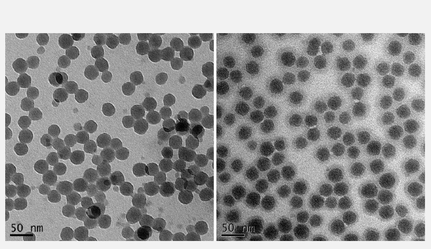
2) To investigate transcriptional regulation and oxidative stress in the human epithelial lung cell line, A549, after exposure to silica NPs.

Materials & Methods

Cell lines
The cells were selected as three pairs of human/mouse cell lines derived from lung epithelium (A549 and ASB-XIV), colon epithelium (CaCo-2 and Colon-26) and macrophages (THP-1 and J774A.1).

Nanoparticles
LUDOX® TMA colloidal silica, 34 wt % suspension in H_2O (Sigma-Aldrich #420859). The BSA-coating procedure was previously described (Wang et al., 2011). The table below displays NP characterization data.

	TMA (native NP)	TB (BSA-coated NP)
Surface area (BET)	118 m^2/g	NA
Stabilizer	None	BSA
Primary size (nm) (TEM)	32.14 ± 2.6 nm (n=106)	NA
Hydrodynamic diameter (DLS)	34.43 ± 0.23 nm (n=3)	42.06 ± 0.56 nm (n=3)
Zeta potential (mV)	-39.2	-31.6

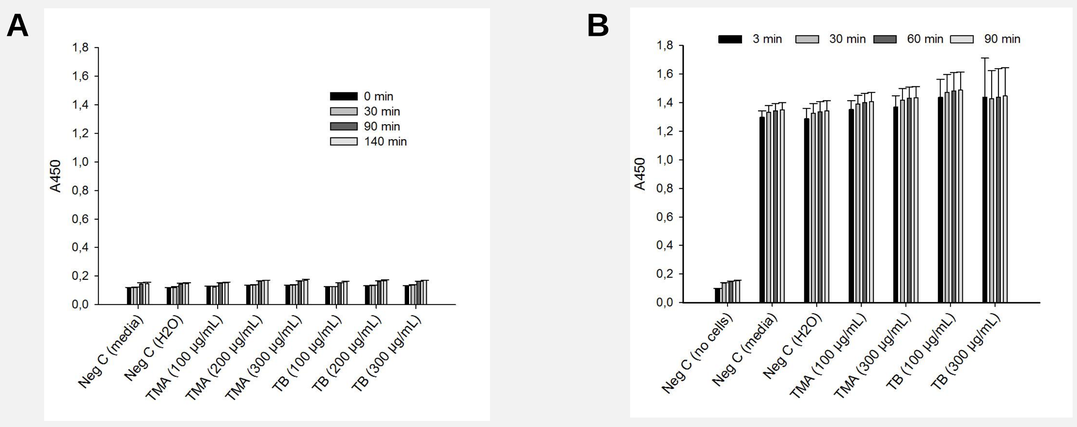


TEM images of TMA (left) and TB (right) silica NPs. Scale bars represent 50 nm.

Assays
The WST-8 assay measures cytotoxicity by dehydrogenase reduction of a tetrazolium compound to a formazan dye which is detected as absorbance at 450 nm. Data was normalized to the control and is expressed as mean ± SD. To calculate EC20 values, the NP concentrations were log10-transformed and a dose-response curve was fitted in GraphPad Prism ver. 5 by the variable slope model. Intracellular ROS levels were estimated by flowcytometry using the fluorogenic marker H_2DCF (dichlorodihydrofluorescein) as previously described (Foldbjerg et al., 2009). All assays, except gene arrays, were repeated in 3-5 independent experiments. RNA for gene arrays was isolated using the RNeasy Mini Kit (QIAGEN) and quality control, labelling and hybridization to GeneChip Human Gene 1.0 ST Arrays (Affymetrix) were performed as described previously (Foldbjerg et al, 2012). Data was quantile normalized in the GeneSpring GX 11.5 software (Agilent) using the iterPLIER16 algorithm. The gene arrays were conducted in two independent experiments. All data are publicly available on the ArrayExpress data repository at the European Bioinformatics Institute (http://www.ebi.ac.uk/arrayexpress/) under the following accession number; E-MEXP-3600. Fold change differences were calculated for all pairs of samples (each NP-treated sample compared to both control samples) and only genes exhibiting a 2-fold expression difference in all pairs of samples were used for pathway analysis in the Database for Annotation, Visualization and Integrated Discovery (DAVID). Top annotation clusters were selected based on a false discovery rate (FDR) < 0.05

Results

Validation of the WST-8 assay for SiO_2 NP testing

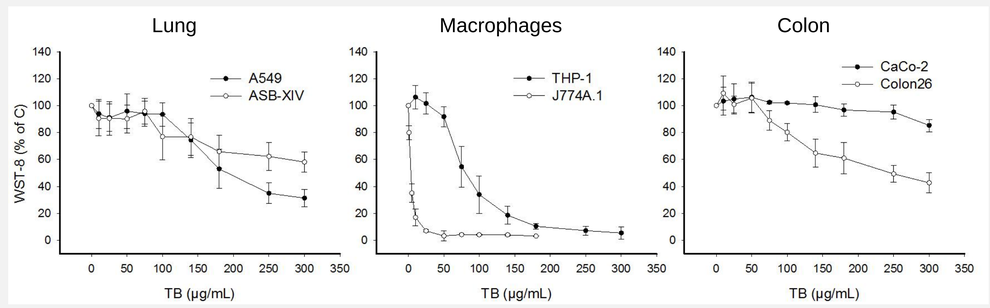


The interference test was designed to assess whether silica nanoparticles **(A)** interfere with the WST-8 reagent or **(B)** the formazan product. Absorbance values from triplicates are shown as mean ± SD. The results of both interference tests indicate that the interference of the TB and TMA silica nanoparticles with the WST-8 reagent and the WST-8 product was negligible.

Results

Cytotoxicity of TB and TMA SiO_2 NPs in 6 different cell lines

A



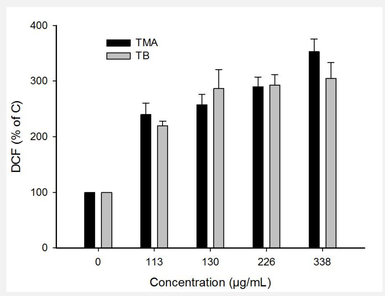
B

Origin	Cell line	TMA EC20 (µg/mL)	95% CI	TB EC20 (µg/mL)	95% CI
Human lung	A549	59,53	51,18 to 69,25	129,7	109,5 to 153,9
Mouse lung	ASB-XIV	26,64	21,43 to 33,11	106,3	74,58 to 151,5
Human macrophage	THP-1	16,87	14,74 to 19,31	55,14	46,72 to 65,07
Mouse macrophage	J774A,1	1,88	1,33 to 2,66	0,96	0,7614 to 1,21
Human colon	CaCo-2	75,58	58,01 to 98,45	321	268,0 to 384,5
Mouse colon	Colon-26	59,11	48,31 to 72,33	108,6	81,57 to 144,5

(A) Viability was measured by the WST-8 assay after 24 h exposure to TB NPs in six cell lines derived from lung, macrophage or colon (data for TMA are not shown). **(B)** The table summarizes the concentrations of TB and TMA which induce a 20 % decrease in the WST-8 assay (EC20). In the colon and macrophage cell lines, higher toxicity was found in the murine cells compared to the human counterpart whereas in the lung cell lines the dose-response curves for the two species were intertwined. Generally, the BSA-coated NPs were less toxic than the native NPs, except in J774A.1 cells.

Uptake and ROS in A549 cells exposed to TB SiO_2 NPs

A549 cells exposed to SiO2 NPs for 24 h were subjected to flow cytometry analysis to investigate ROS production measured by DCF fluorescence. Both types of silica NPs induced ROS. Data was normalized to the control and is expressed as mean ± SD.



Several processes are transcriptionally affected by SiO_2 NP exposure

A549 cells were treated with TB NPs for 24 h at the EC20 concentration (130 µg/ml). Gene expression changes were analyzed using a selection criteria of >2-fold changes compare to controls. After filtering the data, 380 genes were found to be up-regulated and 771 genes to be down-regulated. In order to explore common themes of affected genes, we employed DAVID an on-line resource that identifies enrichment of genes with specific gene ontology (GO) terms. The table displays results from the top annotation clusters (FDR p-value < 0,05). Noticeably, GO terms related to oxidative stress were not identified.

Up-regulated

Category	Term	Count	%	FDR
GOTERM_BP_FAT	Sterol metabolic process- GO:0016125	16	5	4,3E-09
GOTERM_BP_FAT	Cholesterol metabolic process - GO:0008203	15	4,6	1,7E-08
GOTERM_BP_FAT	Sterol biosynthetic process - GO:0016126	11	3,4	2,4E-08
GOTERM_BP_FAT	Steroid metabolic process - GO:0008202	19	5,9	1,5E-07
GOTERM_BP_FAT	Cholesterol biosynthetic process - GO:0006695	9	2,8	1,4E-06
KEGG_PATHWAY	Steroid biosynthesis - hsa00100	8	2,5	6,3E-06
GOTERM_BP_FAT	Steroid biosynthetic process - GO:0006694	12	3,7	0,000015
GOTERM_BP_FAT	Isoprenoid biosynthetic process - GO:0008299	7	2,2	0,00017
KEGG_PATHWAY	Terpenoid backbone biosynthesis - hsa00900	6	1,9	0,0038
GOTERM_BP_FAT	Lipid biosynthetic process - GO:0008610	17	5,3	0,0065
GOTERM_BP_FAT	Isoprenoid metabolic process - GO:0006720	7	2,2	0,029

Down-regulated

Category	Term	Count	%	FDR
GOTERM_BP_FAT	Transcription - GO:0006350	127	17,5	0,00011
GOTERM_BP_FAT	Regulation of transcription - GO:0045449	146	20,2	0,00053
GOTERM_MF_FAT	Transcription regulator activity - GO:0030528	96	13,3	0,0009
GOTERM_CC_FAT	Cell junction - GO:0030054	39	5,4	0,009
KEGG_PATHWAY	ECM-receptor interaction - hsa04512	14	1,9	0,01
GOTERM_BP_FAT	Positive regulation of transcription, DNA-dependent - GO:0045893	40	5,5	0,013
GOTERM_BP_FAT	Positive regulation of RNA metabolic process - GO:0051254	40	5,5	0,016
GOTERM_BP_FAT	Positive regulation of biosynthetic process - GO:0009891	51	7	0,025
GOTERM_CC_FAT	Adherens junction - GO:0005912	18	2,5	0,031
GOTERM_MF_FAT	Transcription factor activity - GO:0003700	64	8,8	0,033
GOTERM_BP_FAT	Embryonic morphogenesis - GO:0048598	29	4	0,037
KEGG_PATHWAY	Notch signaling pathway - hsa04330	10	1,4	0,046
GOTERM_BP_FAT	Positive regulation of macromolecule biosynthetic process - GO:0010557	48	6,6	0,049

Conclusions

- A concentration-dependent increase of cellular ROS was demonstrated in silica NP exposed A549 cells. However, induction of oxidative stress related pathways was not found in gene array studies after silica NP exposure.
- Up-regulated genes primarily related to lipid metabolism and biosynthesis whereas down-regulated genes were associated with several processes, including transcription, cell junction and extra cellular matrix (ECM)-receptor interaction.
- The differential sensitivity in the six cell lines suggests careful consideration in the choice of cell models and cautions that interspecies extrapolation may have to consider higher sensitivity in mice towards NPs

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