CeNIDE - Center for Nanointegration Duisburg-Essen

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Toxicity of Nanoparticles:

Nanoparticle-induced neurotoxic and proteomic changes at low-concentration-levels

Prof. Dr. Elke Dopp

Nano @ Duisburg-Essen

- **„Nano“ is one of the central points of strategic development at UDE**
  - Since the year 2000, the university has pursued a hiring policy to strengthen the expertise in this field.
  - This strategy has resulted in a number of coordinated research networks, such as Collaborative Research Centers (SFB) and Research Training Groups (GRK)

- **CeNIDE members**
  - 49 research groups at the University of Duisburg-Essen
  - MPI-K (Max-Planck Institute for Coal Research)
  - University Münster (Physical Chemistry)
  - IUTA (Institute for Energy and Environmental Technology)
  - ZBT (Center for Fuel Cell Technology)

Nano-expertise @ Duisburg-Essen

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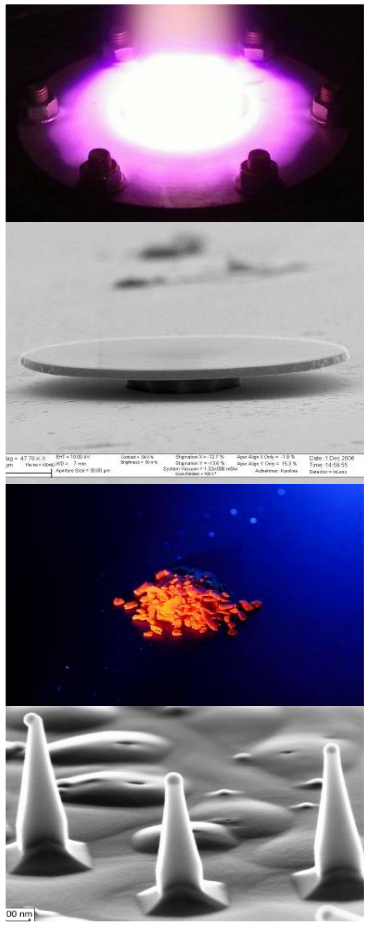
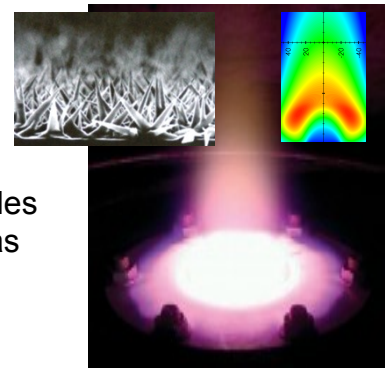
- **Synthesis**
  - new (nano) materials, new synthesis routes
- **Handling**
  - functionalizing, processing, filtering
- **Electronics, optics, photovoltaics**
  - using size effects for tailored and new properties
- **Characterization**
  - chemistry, morphology, electronic and optical properties
- **Medical implications**
  - both “good” and “bad”
- **Safety issues**
  - production, handling ...
  - Toxicity of produced nanoparticles



Research Background

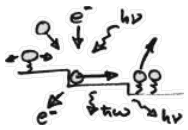
Long term research projects

Materials synthesis



CRC 445
Nanoparticles from the gas phase
Since 1999

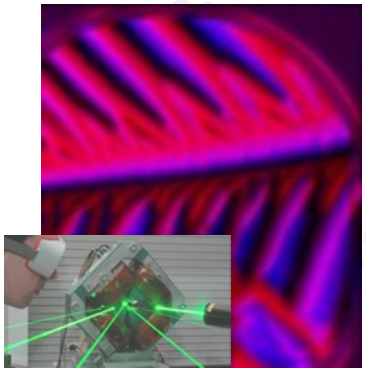
Energy on the nanoscale



CRC 616
Energy dissipation at surfaces since 2000

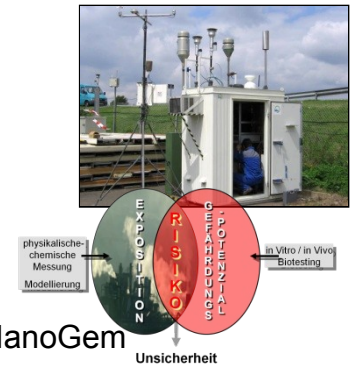
Magnetic materials and effects

SFB 491



CRC 491
Magnetic Heterolayers
Since 2000
With RUB

Sustainability



SPP 1313
Bio-Nano Responses

BMBF
NanoCare, NanoGem

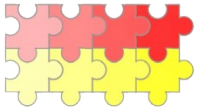
Undergraduate teaching

BA / MA course
Nano Engineering

NanoLab for High school students

Internships

Graduate program



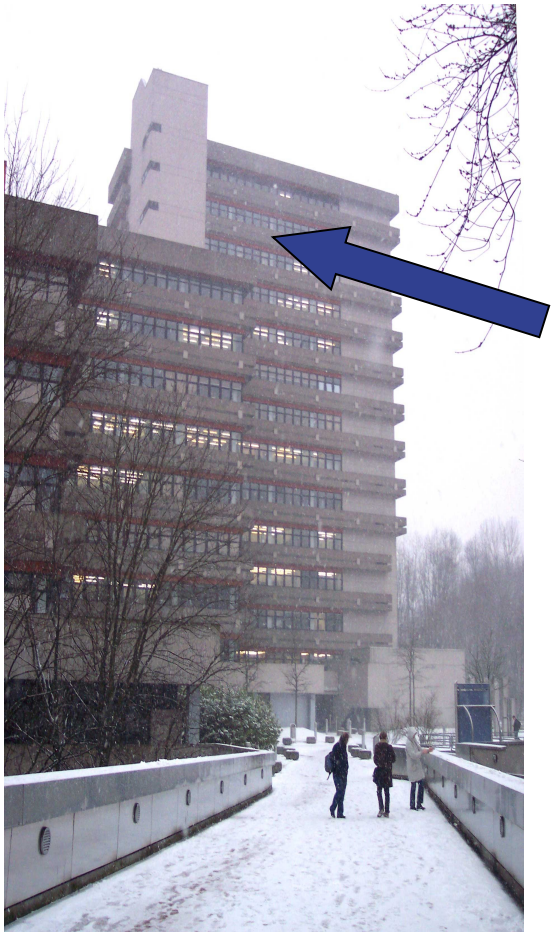
RTG1240
Nanotronics
Since 2007

ProFor

Aims and Purpose

- To initiate and to foster co-operation in the area of nanoscience and technology between different scientific units at UDE and outside partners from science, research and industrial development.
- To give support for interdisciplinary training and education
- To develop a coordinated image of the strength and competence of UDE in the area of nano-science
- To promote exchange with national and international partners
- “Science talk” with international guests known in the field
  - Prof. Harald Krug, 11/17-19/2010

Research group “Toxicology”



Research interests

- Investigation of the toxicological profile of occupationally and environmentally relevant substances (chemicals, *(nano)particles*, water related micropollutants)
- Analytical chemistry: Chemical analysis of compounds (e.g. Metal(loid)s) in biological materials
- Contributions to the establishment of threshold limit values (DFG: in the work area; UBA: environment and water)

cated at the Institute of Hygiene and Occupational Medicine, University Hospital Essen

Research group “Toxicology”

In vitro- and molecular Toxicology
(Prof. Dr. Elke Dopp)

⇒ Genetic toxicology (+ related endpoints, e.g. $[Ca^{2+}]_i$)

⇒ cyto-/genotoxicity

⇒ radical generation (extra- and intracellular)

⇒ Inflammatory responses (cytokines)

⇒ Neurotoxicity

⇒ effects at low-concentration ranges on protein expression

Toxicoproteomics
(Dr. Simone Schmitz-Spanke)

⇒ Identification of toxic responses of environmental pollutants at protein levels *in vitro*

⇒ application of proteomic approaches to environmental and occupational toxicology

⇒ better understanding of toxic mechanisms/ modes of action in response to acute/long-term exposure of cell cultures *in vitro*

⇒ identification of previously unknown protein biomarkers

Recent Research Projects

- Intracellular detection of fluorescent nanoparticles

Collaboration with the University of Heidelberg, Cellnetworks-Cluster

- Comparative study of micro- and nanoscale Fe^{+3}-containing particles for their toxicological properties *in vitro*

- Changed protein expression in co-cultures of human lung cells and macrophages after exposure to nanoparticles

Collaboration with the Toxicoproteomics group of the Institute of Hygiene and Occupational Medicine (UDE)

- Analysis of composition, function and interaction of proteins in carbon-black NP exposed human lung cells

- Effects of nanoparticles on neuronal cells

Collaboration with the University of California Davis, Neuroscience Center

Recent Research Projects

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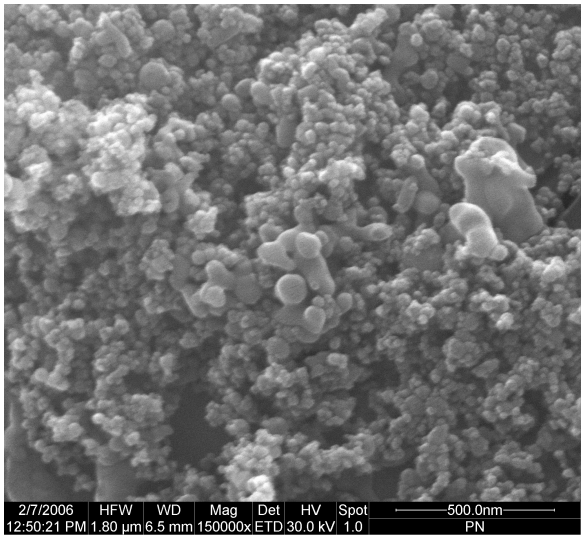
- **Effects of nanoparticles on neuronal cells**

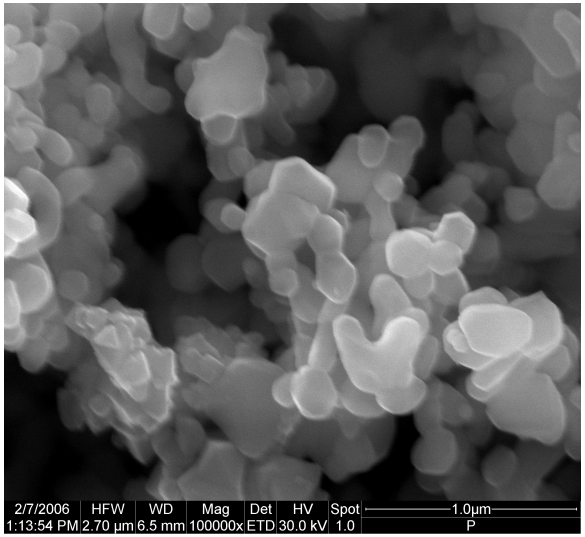
Collaboration with the University of California Davis, Neuroscience Center

1.) Comparison of micro- and nanoscale Fe^{+3}-containing particles

Particle size distribution, Agglomeration, Zeta potential

d ≤ 100 nm





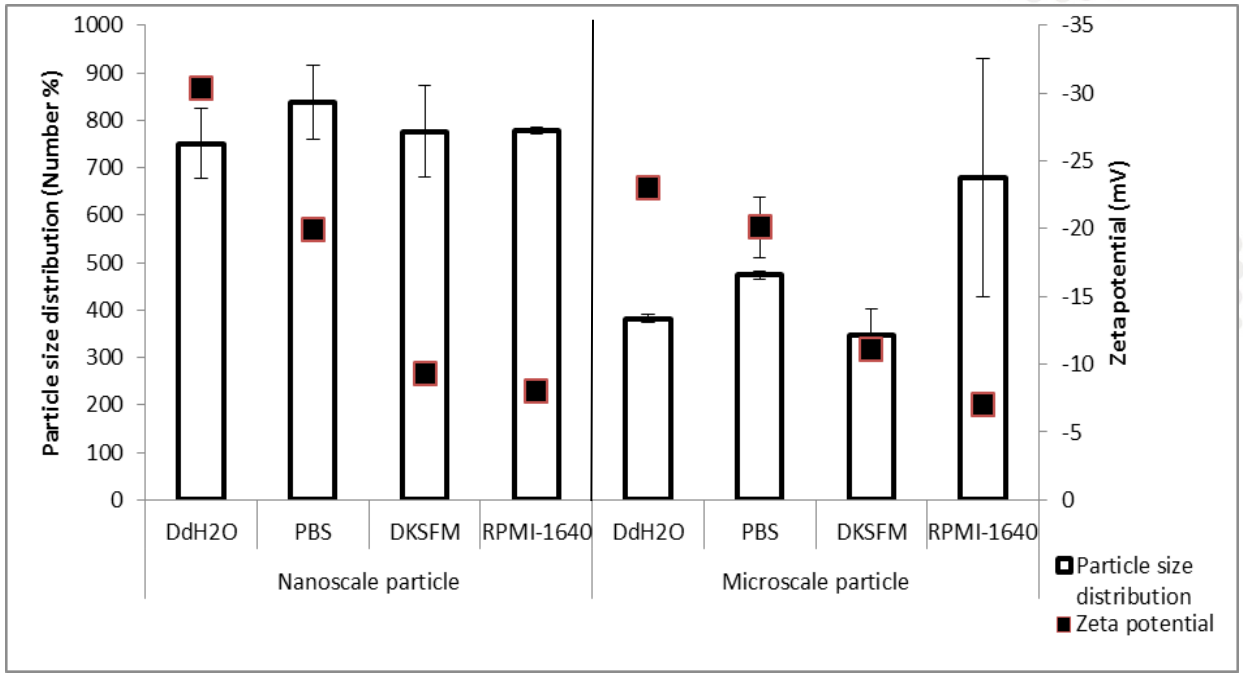
d ≤ 5 µm

Surface Elemental weight percentages	
Fe_2O_3-NP	Fe_2O_3-microscale
Fe: 78.7 %	**Fe:** 78.7 %
O: 21.3 %	**O:** 21.3 % [tky1]

Bhattacharya et al., Tox. Sci., submitted

1.) Comparison of micro- and nanoscale Fe^{+3}-containing particles

- Particle size distribution
- Zeta potential
- Polydispersity index



⇒ Agglomeration of particles depends on content of biomolecules (amino acids, proteins, carbohydrates, free ions, trace elements etc.) in the medium

⇒ Higher agglomeration of NP compared to microscale particles

Bhattacharya et al., Tox. Sci., submitted

1.) Comparison of micro- and nanoscale Fe^{+3}-containing particles

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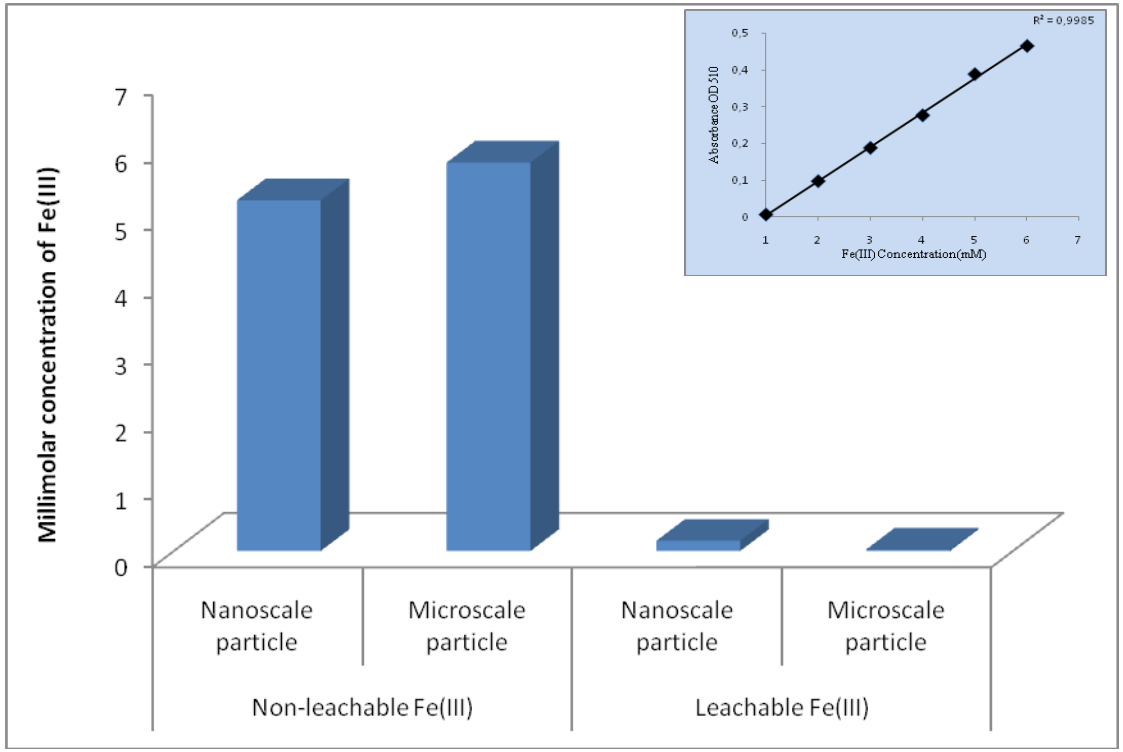
Leachable iron

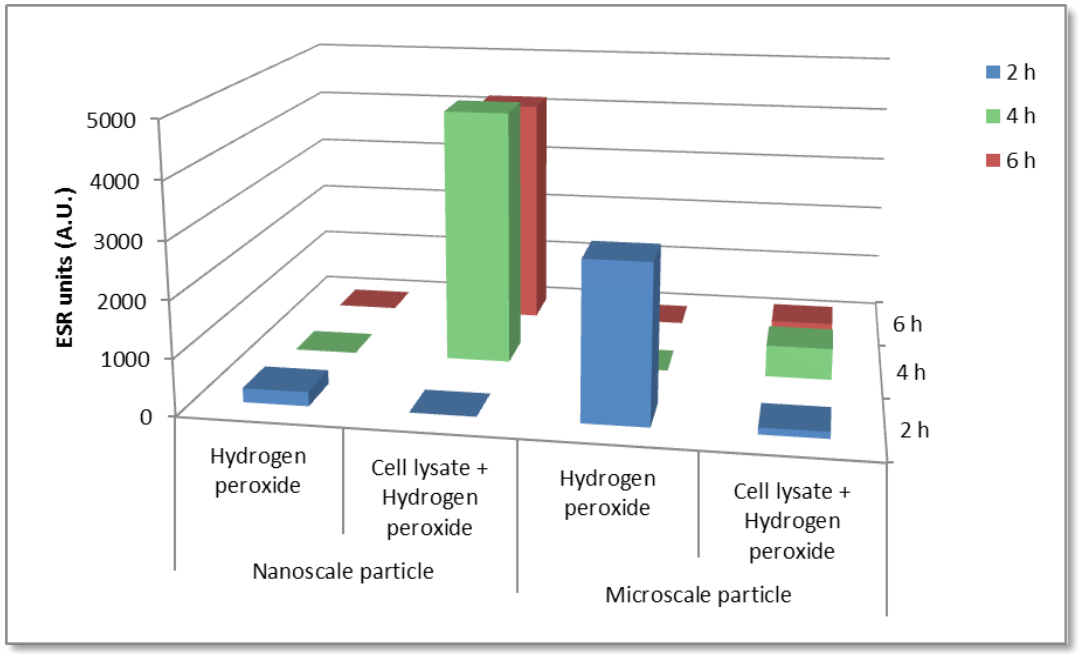


Leachable iron on the surface of NP is 7.5x higher compared to microscale particles (dependence upon available surface area)

Bhattacharya et al., Tox. Sci., submitted

1.) Comparison of micro- and nanoscale Fe^{+3}-containing particles

Extracellular radical generation



-Electron spin resonance measurement showed an early effect of microscale and a delayed effect of nanoscale particles
-a reduction of Fe(III) to Fe(II) is necessary before high generation of free radicals

Bhattacharya et al., Tox. Sci., submitted

1.) Comparison of micro- and nanoscale Fe^{+3}-containing particles

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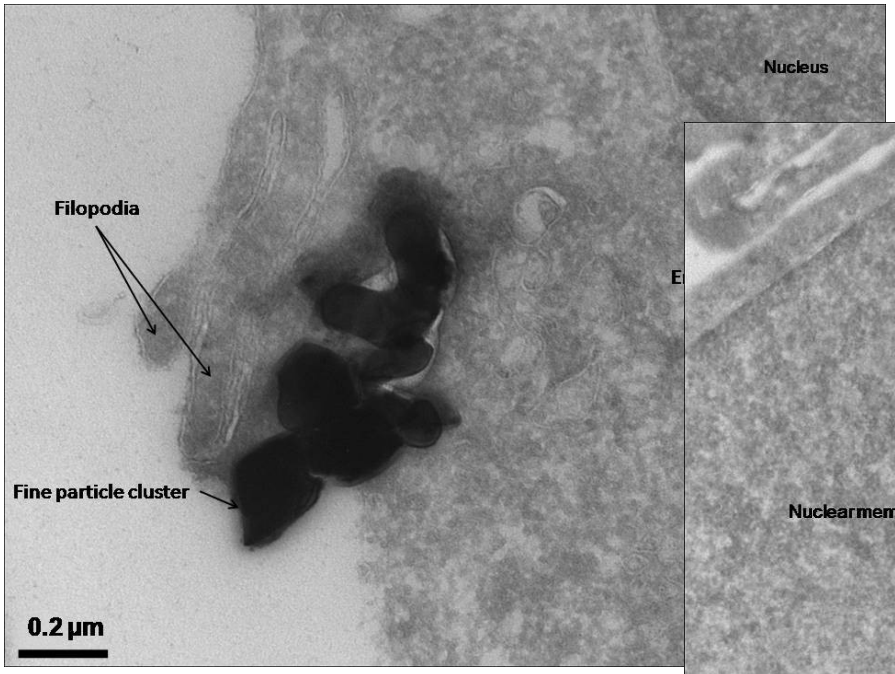
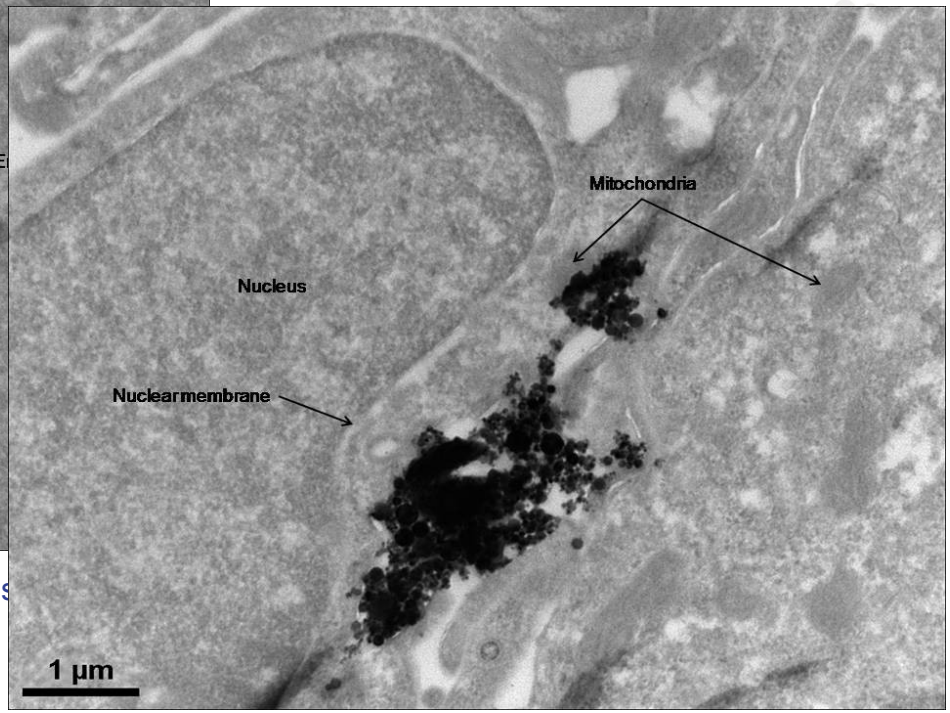
Transmission Electron Microscopy



Human lung cells: filopodia assisted endocytosis
agglomerates

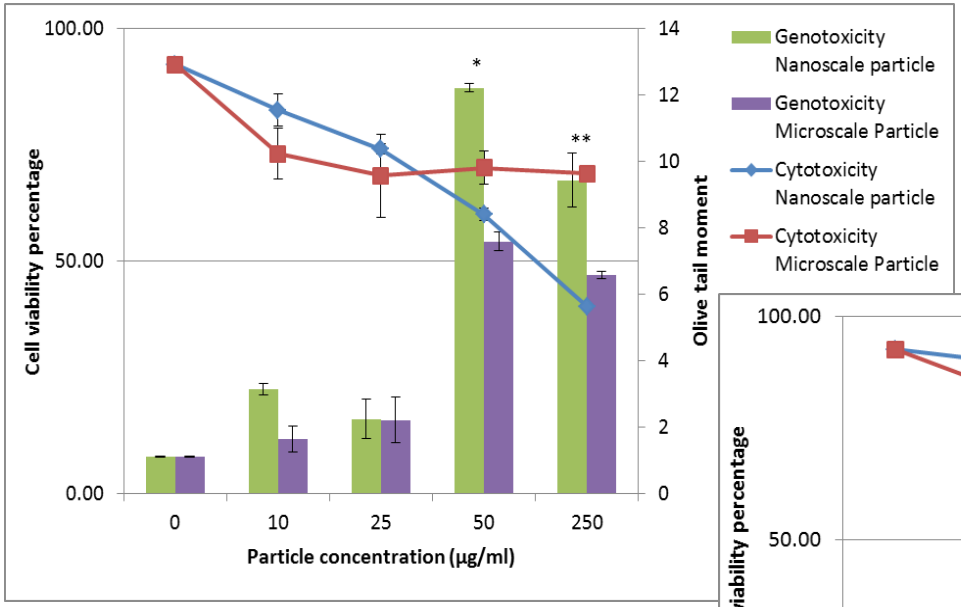


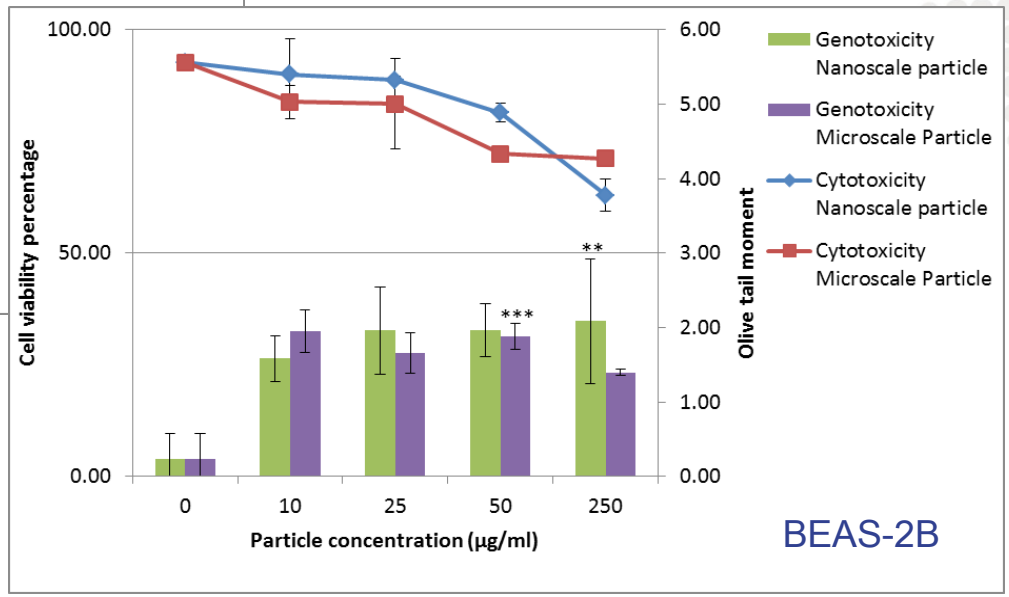
Intracellular translocation in the peri-nuclear region, in the endoplasmic reticulum, in lysosomes but NOT in the nucleus and in mitochondria

Bhattacharya et al., Particle and Fibre Tox., 2009

1.) Comparison of micro- and nanoscale Fe^{+3}-containing particles

Cyto- and Genotoxicity





Exposure time: 24 h
Positive control: DQ12; OTM 8.0 ± 1.2 in IMR-90 and 2.0 ± 0.5 in BEAS-2B cells at 250 µg/ml

1.) Comparison of micro- and nanoscale Fe^{+3}-containing particles –CONCLUSIONS-

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-Agglomeration of particles depends on composition of surrounding medium, especially on protein content

-higher leachable iron content for NP, toxicity of free ions?

-delayed effect of extracellular radical formation with Fe_2O_3-NP

-difference in cellular response depending upon cell type

-no particles in nucleus and mitochondria (Indirect genotoxic effects)

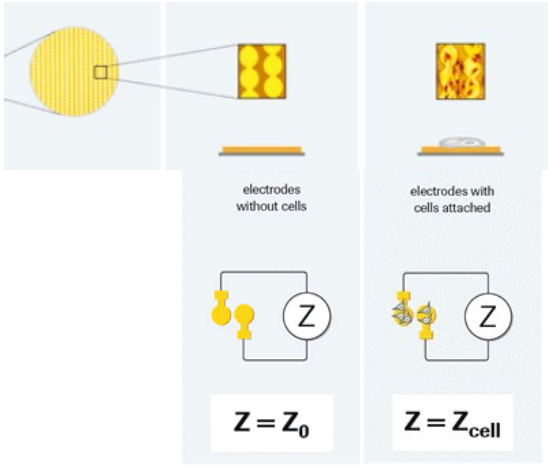
-cyto- and genotoxic effects at high concentrations (environmentally and occupationally not relevant) –cellular effects at low concentrations?

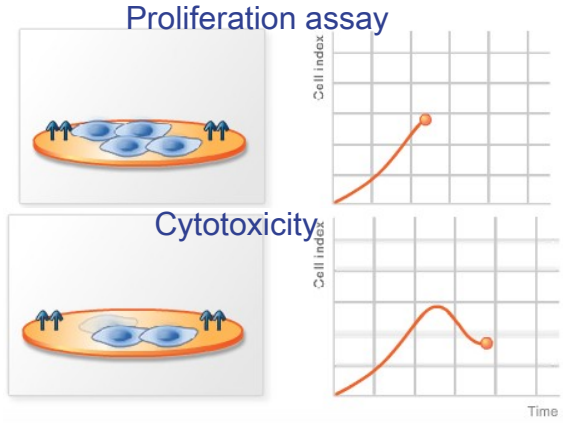
The immediate environment of the particles (biomolecules, physiological properties of the medium, protein content etc.) modulates their toxicity on the basis of agglomeration rather than their actual size.

2.) Cellular effects of NP at low-concentration levels

Methods

- Mass spectrometry (MALDI-TOF, SELDI-TOF)
- Two-dimensional gel electrophoresis (2D PAGE)
- Blue Native polyacrylamide gel electrophoresis (BNPAGE)
- Western blot, PCR, immunoprecititation, ELISA
- HPLC; GC-MS
- xCelligence (real-time cell analysis instrument)





⇒ Quantitative measure of the cell number present in a well (impedance measurement)

2.) Cellular effects of NP at low-concentration levels –methods-

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2D Gel Electrophoresis + Protein identification by Mass Spectrometry

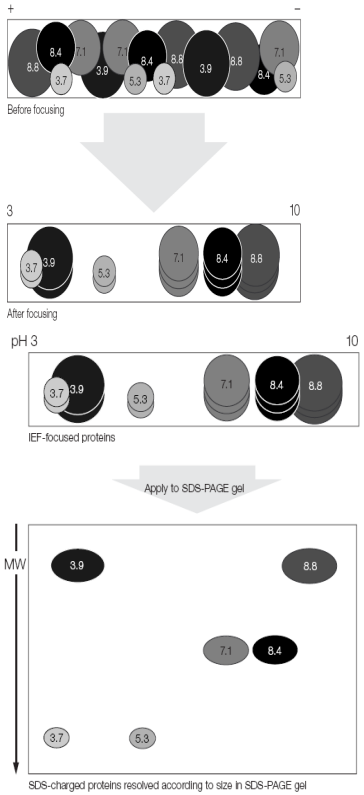
1. dimension:
separation on the basis of p*I*

isolelectric focusing

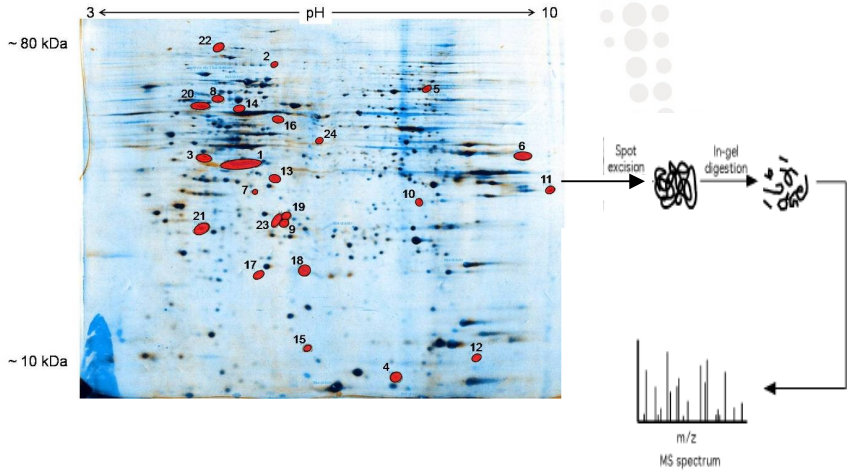
2. dimension:
separation on the basis of size

staining

protein identification



„2-D Electrophoresis for Protemoics – A Methods and Product Manual“ BIO-RAD



peptide

mass fingerprinting (PMF)

protein identification

(heterogeneous nuclear Ribonucleoprotein A3)

Contact: Simone.Schmitz-Spanke@uk-essen.de

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Toxicity of nanoparticles

Experimental setup I

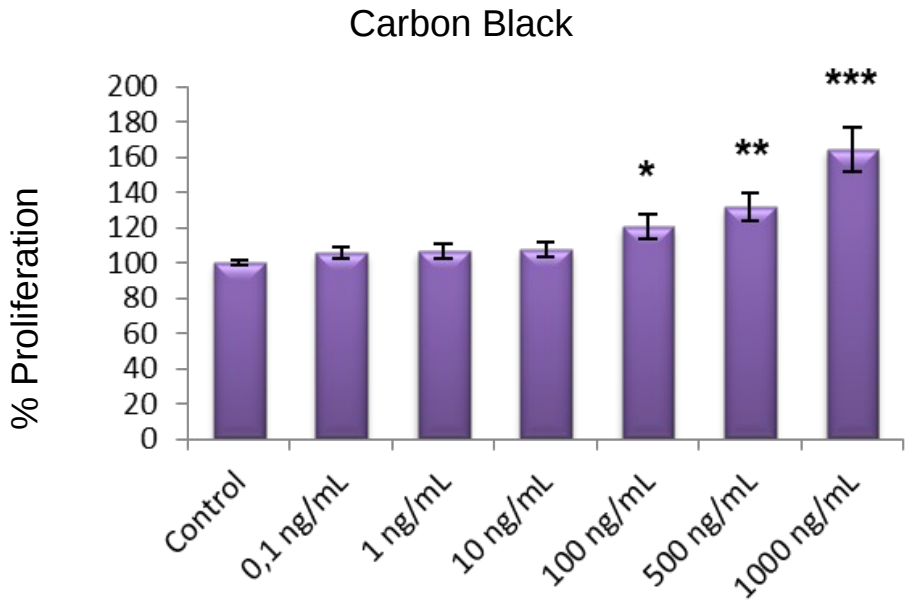
- Carbon Black (Printex® 90, CB, 70 nm)
- Cells: EA.hy926, an endothelial cell line (ATCC, CRL-2922)
- Exposure duration: 14 days (repeated exposure)
- *„classical“* cell tests (proliferation, ROS)

  Concentration range: 0.1 – 1000 ng/mL
- Proteomic analysis (2D gel electrophoresis + MS)

  Exposure conc. 100 ng/mL

2.) Cellular effects of NP at low-concentration levels –cell proliferation-

Proliferation by BrdU-staining

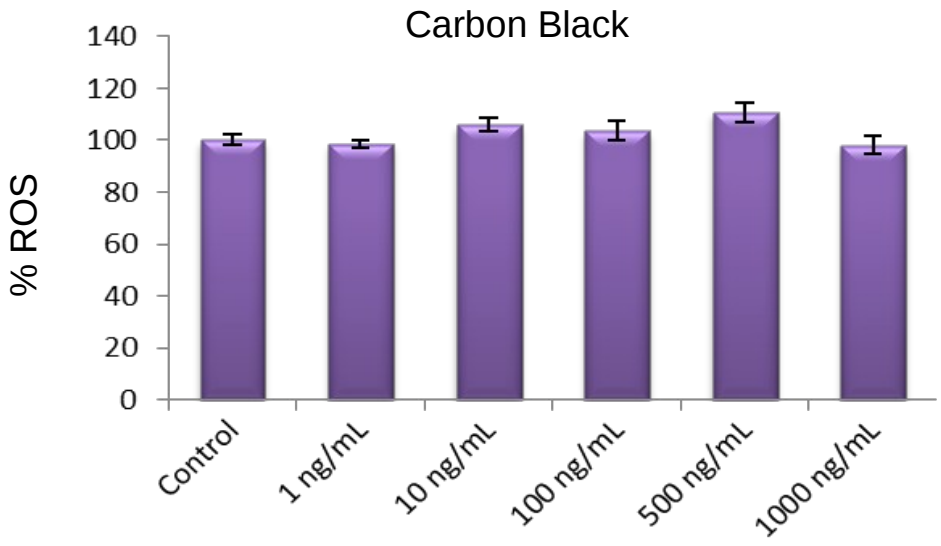


⇒ Significant increase of proliferation after repeated long-term exposure to CB

Pink et.al: *Toxicol Lett.* 2010; **196**; S273-S273

2.) Cellular effects of NP at low-concentration levels –radical formation-

Intracellular radical formation (H_2DCFDA)



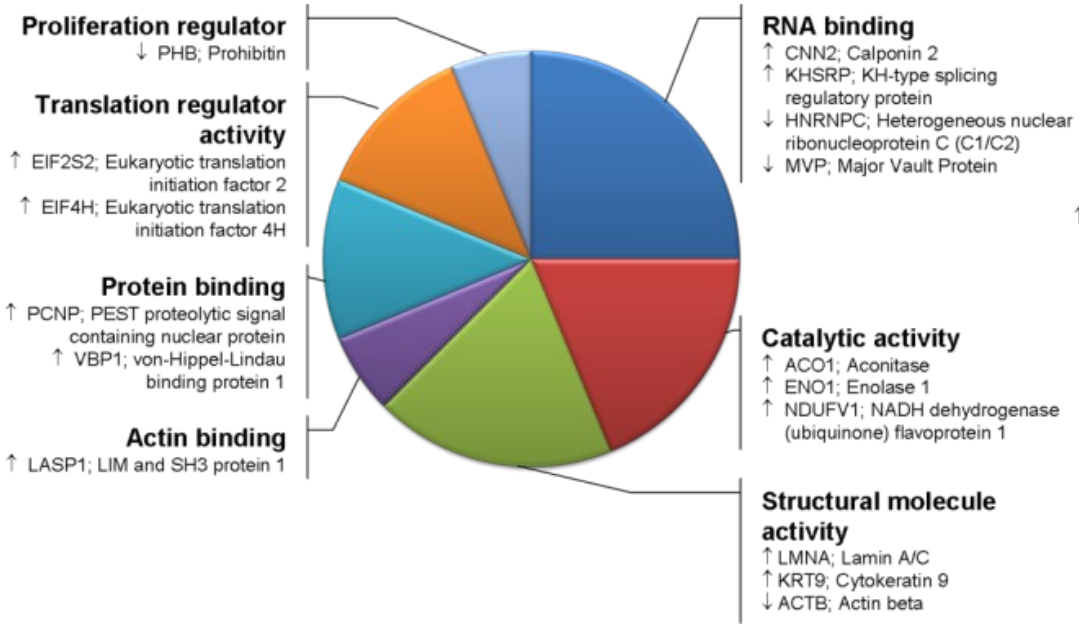
n=6
Repeated exposure for 14 days, measurement after each 48 h

- No increased radical generation at low concentration levels

Pink et al., submitted

2.) Cellular effects of NP at low-concentration levels –proteomic analysis-

Proteomic analysis – results



carbon black: Alteration of protein expression (n = 10):
Control vs. CB = 16 Proteins

http://david.abcc.ncifcrf.gov

Database as tools for investigators to understand biological meaning behind large list of genes/proteins.

Pink et al., submitted

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Toxicity of nanoparticles

Experimental setup II

- 5 different types of nanc
- Cells: human lung epith
- Exposure duration: 24, 4
- *„classical“* cell tests (pr

  Concentration: 10
- Mediator screening (SELDI-TOF MS)

  Exposure conc.: 100 ng/mL

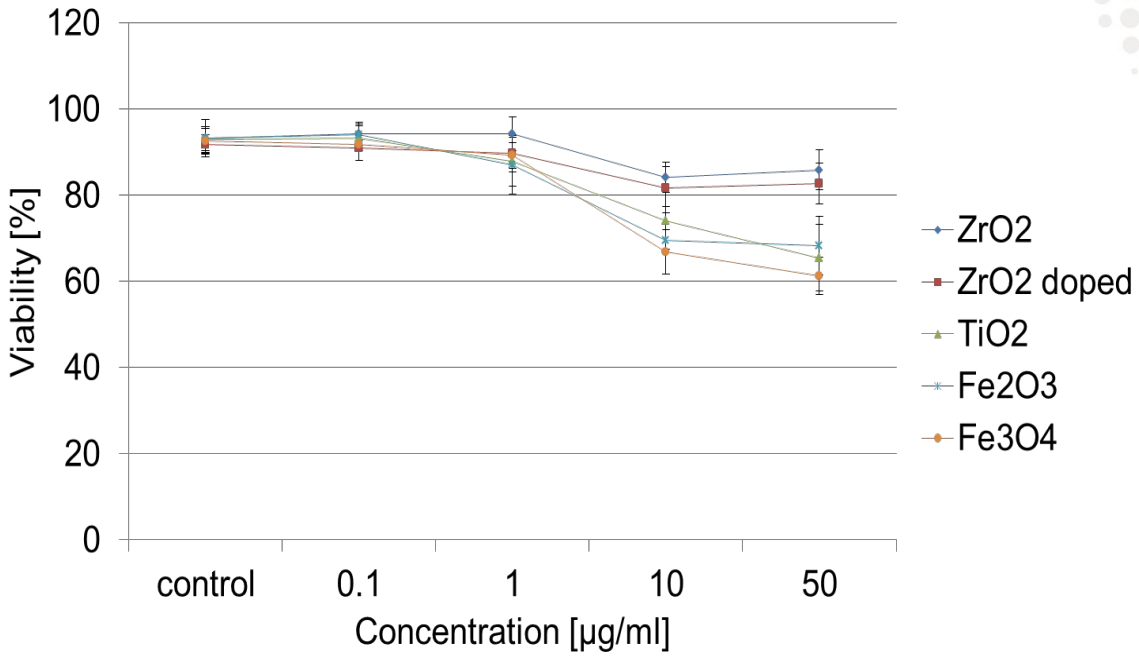
Used Nanoparticles	Formula	Specific surface area (BET)	Zeta potential (pH 7)	Particle diameter
Zirconium dioxide	ZrO_2	144.8 m²/g	-11.1	6.8 nm
Zirconium dioxide with Europium doping	ZrO_2	182.2 m²/g	-10.3	5.4 nm
Titanium dioxide	TiO_2	292.3 m²/g	-15.6	5.3 nm
Iron(III) oxide	Fe_2O_3	50.1 m²/g	25.3	22.8 nm
Iron(II,III) oxide	Fe_3O_4	67.9 m²/g	20.4	17.0 nm

Toxicity of nanoparticles

Experimental setup II

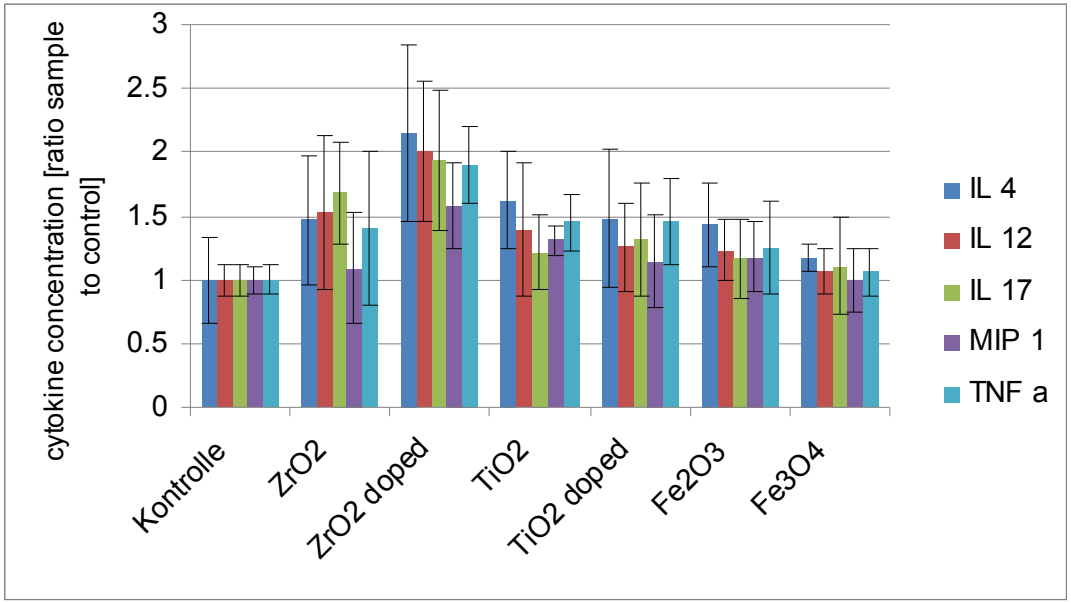
- Cytotoxicity

(Trypan blue,
exposure time: 72 h)



2.) Cellular effects of NP at low-concentration levels

Cytokine expression



Exposure time: 72 h, single exposure

-is changed in human lung cells exposed to 6 kinds of nanoparticles

-highest for zirconium dioxid (100 ng/ml)

2.) Cellular effects of NP at low-concentration levels –cell proliferation-

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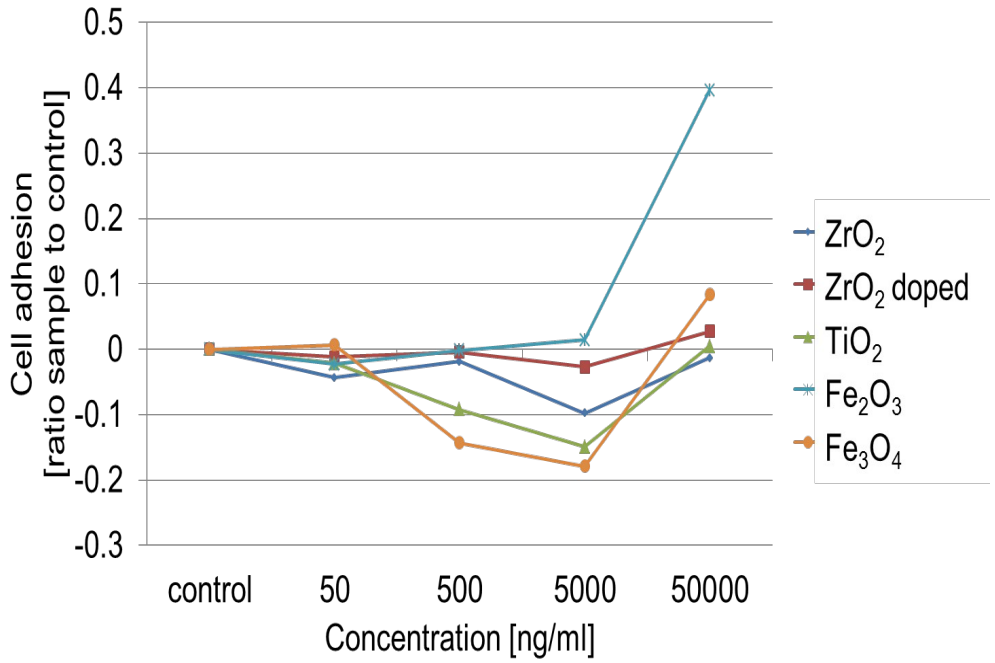
Proliferation by xCelligence technology

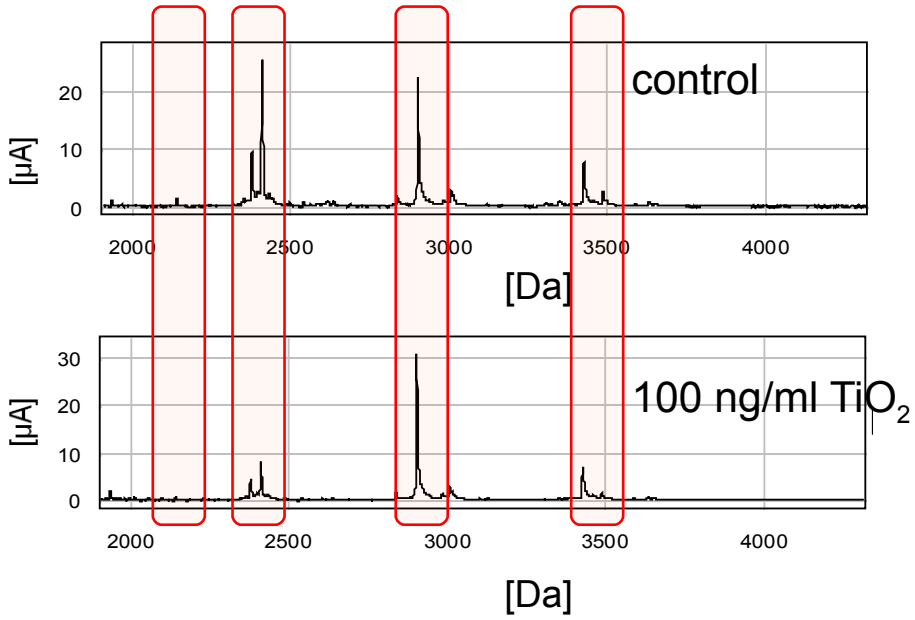


⇒ Change in cell adhesion/proliferation after single exposure of BEAS-2B cells to 5 kinds of nanoparticles starts at > 50 ng/ml (exposure time: 72 h)

2.) Cellular effects of NP at low-concentration levels

Mediator screening of culture medium

by surface-enhanced laser desorption ionization (SELDI-TOF MS)



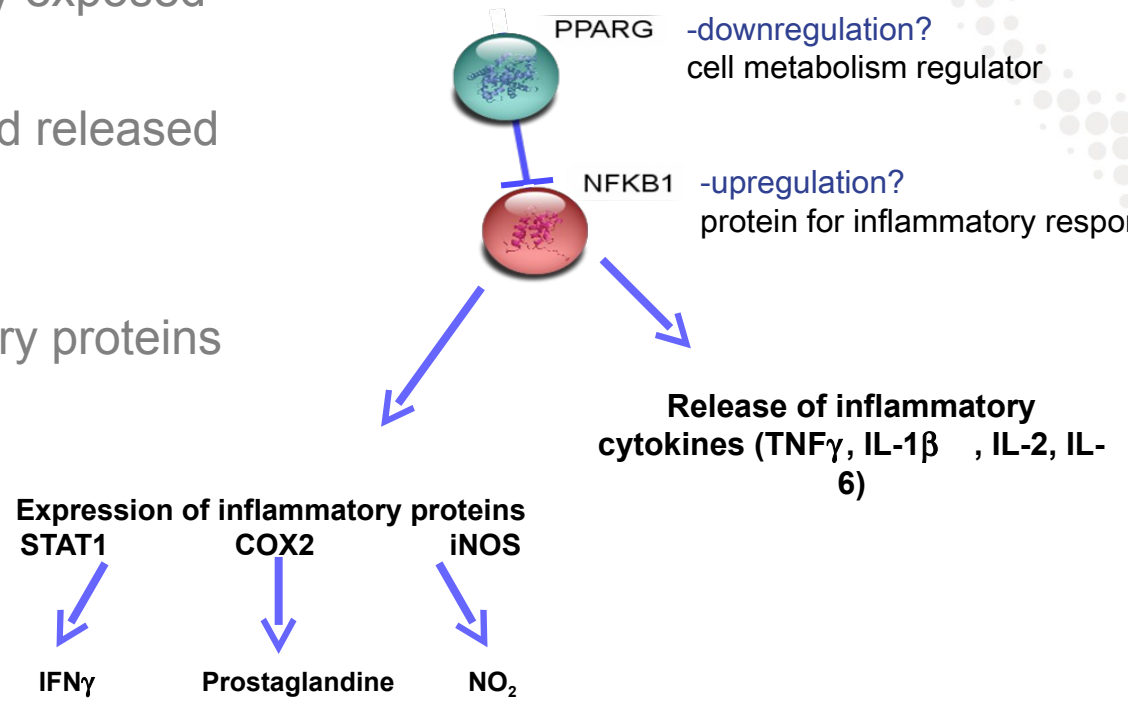
Exposed cell line: human lung cells (BEAS-2B)
Exposure time: 72 h, single exposure
Result: 4 mediators are changed (Mediators: chemokines, cytokines, proteins etc.)

2.) Cellular effects of NP at low-concentration levels –CONCLUSIONS (II)-

- changed cell proliferation at low NP concentrations

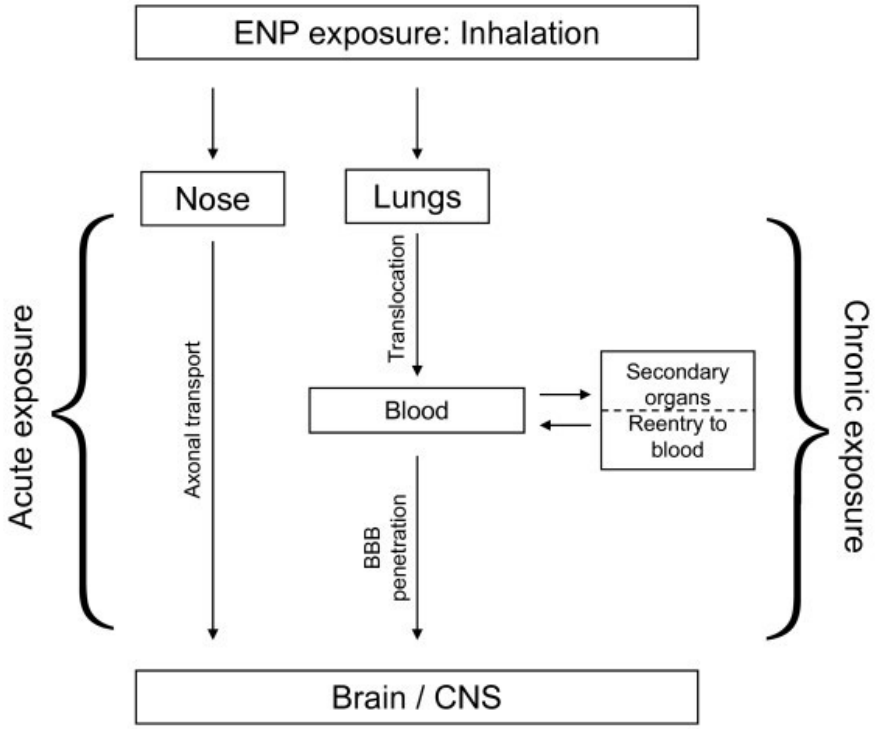
- signal mediators are released into culture medium by exposed human lung cells (identification of detected released mediators)

- Are major key regulatory proteins influenced by NP? (NFkB, PPARG)



3.) Changes of neuronal activity after NP exposure

Entry of NP into brain



-through the olfactory pathway

-close proximity of the nasal olfactory mucosa to the olfactory bulb

-intimate association between the nasal epithelium and olfactory neurons

-translocation of NP along the olfactory neurons to the olfactory bulb

-nanoparticles were found in several brain regions such as the cerebral cortex, hippocampus and cerebellum

Simko and Mattson, 2010

3.) Changes of neuronal activity after NP exposure

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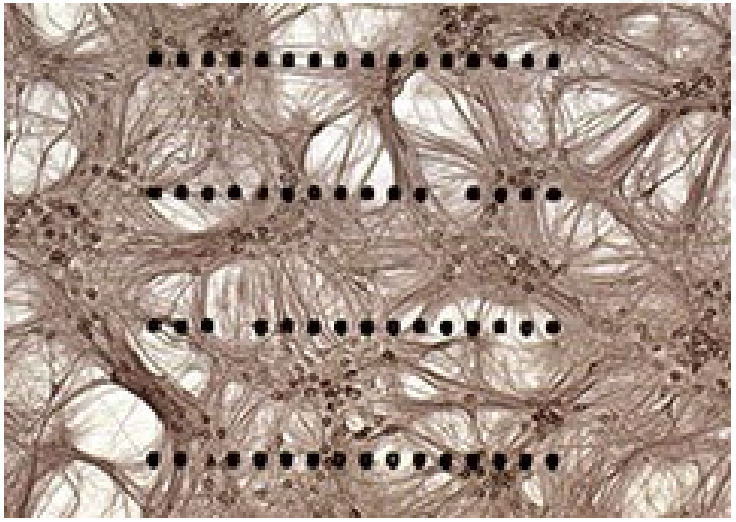
Electrical Activity of Neuronal Networks on Microelectrode Array Neurochips



Cells: - frontal cortex tissue from embryonic day 15 crl:NMRI mice
- primary frontal cortex networks was cultured on MEA neurochips
- cells develop electrical activity after 3-4 days in culture with a coordinated burst pattern and interburst spiking

Nanoparticles: TiO_2 (d<100 nm), CB (d: 55 nm), Fe_2O_3 (d<100 nm)

- tested concentration range: 0.001–300 µg/cm^2

Gramowski et al., EHP, 2010

3.) Changes of neuronal activity after NP exposure

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Properties of used nanoparticles

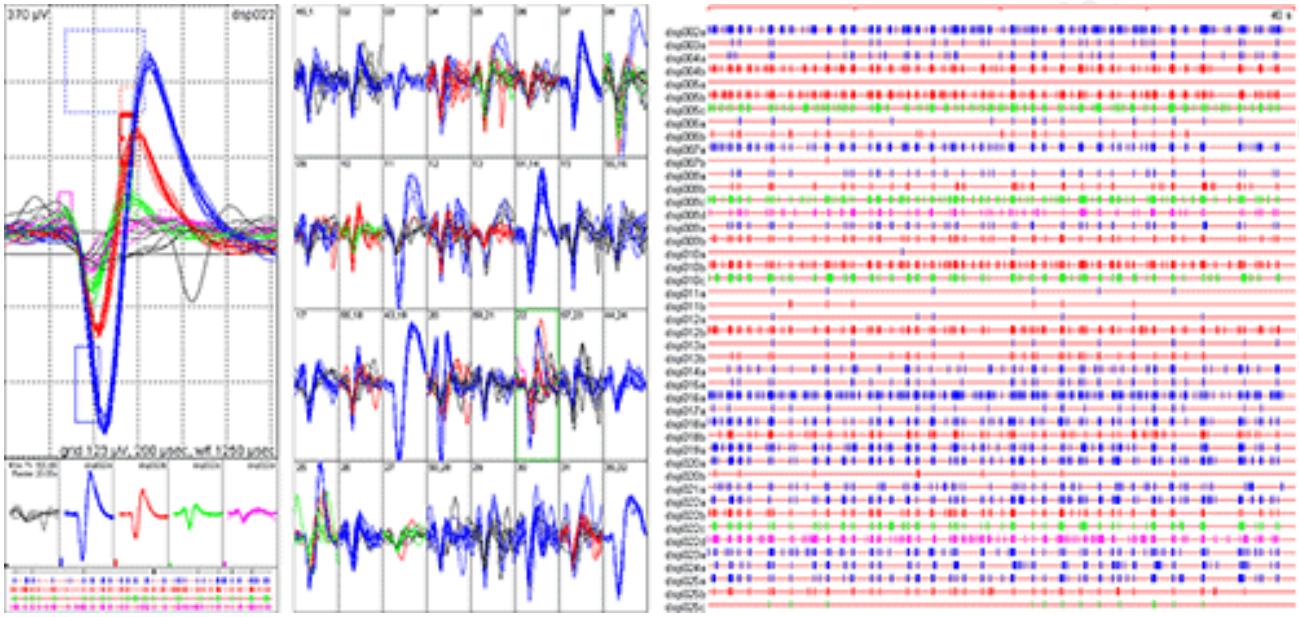
Particle type	Elements	Weight %	Diameter (REM) nm	Average Hydrodynamic Diameter, nm	Zeta Potential mV	Surface Area m^2/g
Carbon black Nanoparticles	C O S	88.6 10.8 0.65	55	575.2	- 14.5 mV	123.0 + 0.01
Hematite Nanoparticles	Fe O	78.7 21.3	< 100	50	- 28.68 mV	34.39 ± 0.17
Titanium Dioxide Nanoparticles	Ti O C	56 41 3	< 100	91	+ 48.8 mV	49.71 ± 0.19

Stable dispersions of NPs in solution occur only at zeta potentials greater than 30 mV (positive or negative).

CB NPs with a low zeta potential of 14 mV (positive or negative) showed a high degree of agglomeration, whereas the other types with zeta potentials of 29 mV and 49 mV (positive or negative) remained mono-disperse to a large extent.

3.) Changes of neuronal activity after NP exposure

Activity pattern



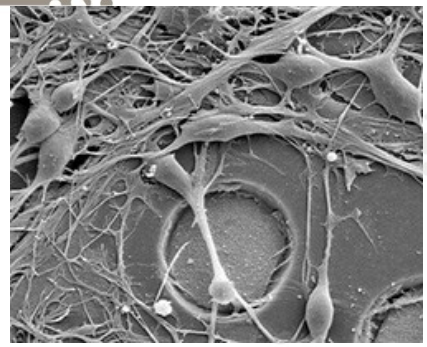
- Action potential („Spikes“) of neuronal networks are measured
- Measurements of 250 neuronal cells of the neuronal network are possible at the same time
- General activity: „Spike rate“ = number of „Spikes“ per unit
  „Bursts“ = resting and activity phase of „Spikes“

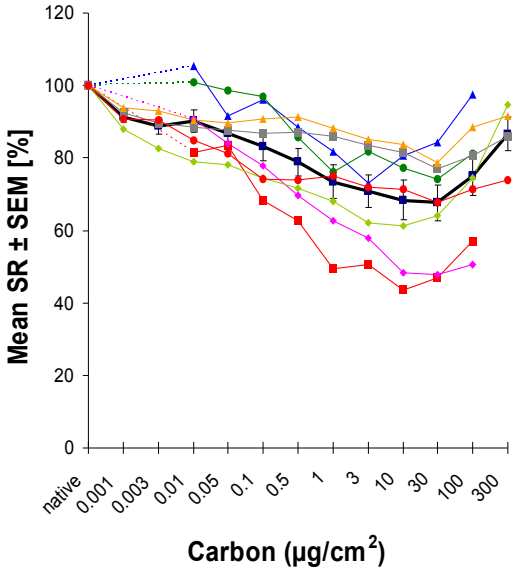
Gramowski et al., EHP, 2010

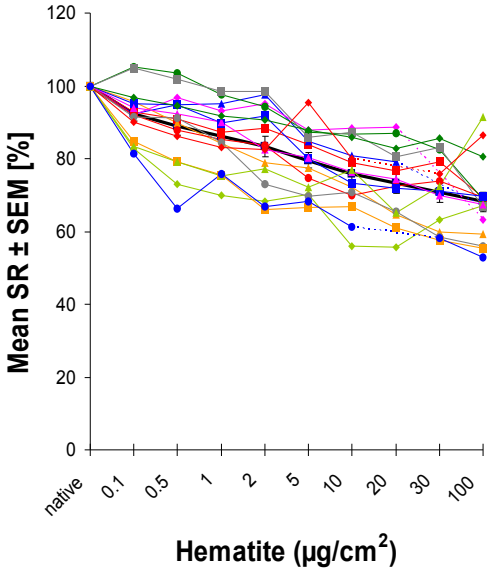
3.) Changes of neuronal activity after NP exposure –RESULTS-

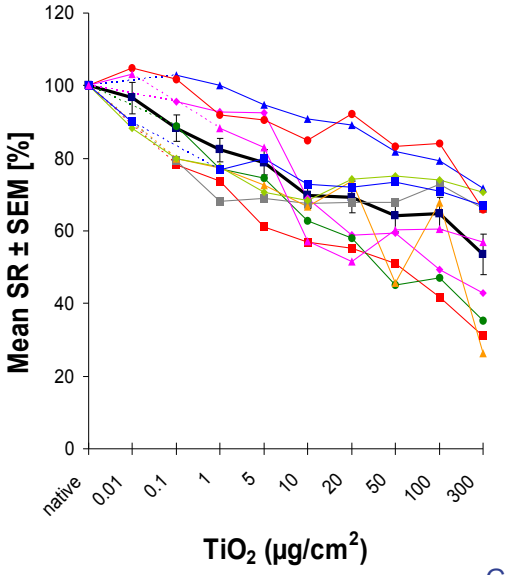
- concentration-dependent reduction of activity pattern of neuronal networks in culture

- Inhibition of neuronal activity starts already at lowest concentration measured



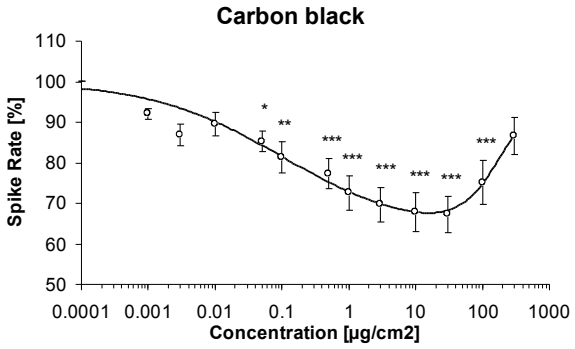


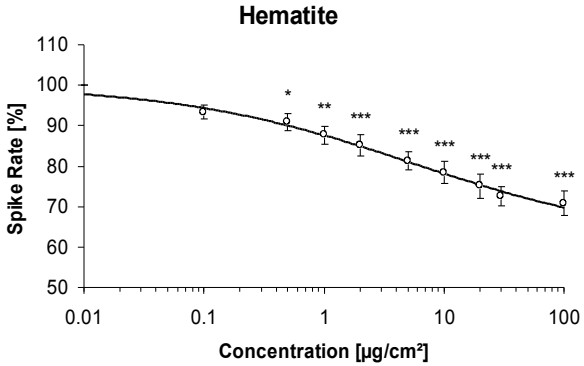


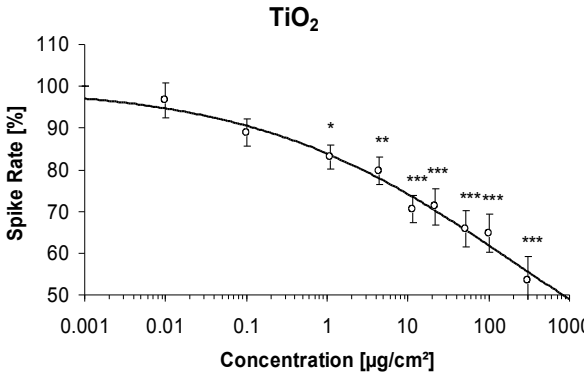


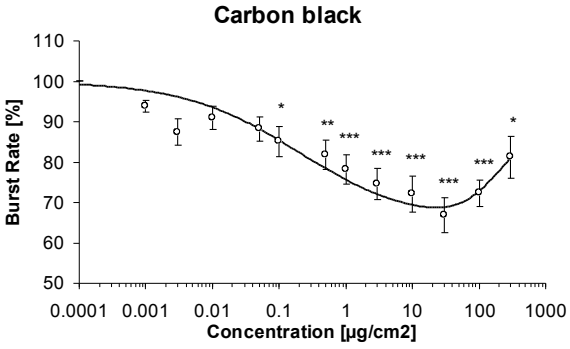
Gramowski et al., EHP, 2010

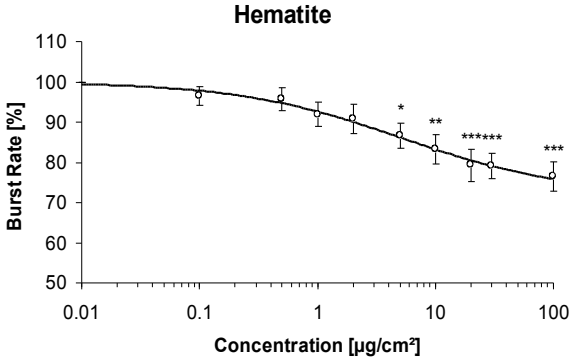
3.) Changes of neuronal activity after NP exposure –RESULTS-

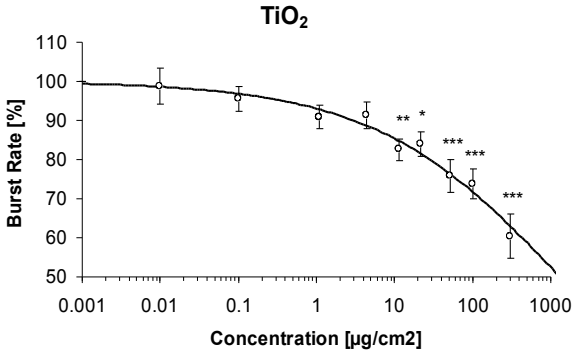












- Spike and burst rates were influenced after NP exposure

Gramowski et al., EHP, 2010

3.) Changes of neuronal activity after NP exposure –RESULTS-

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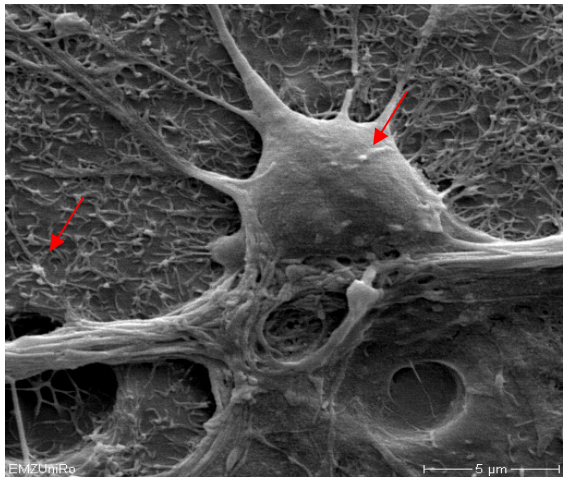
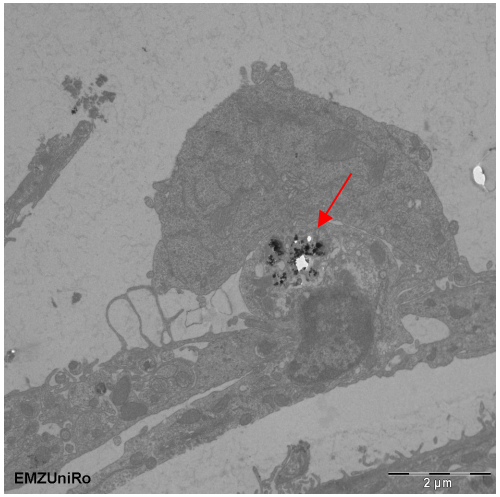
Influence of NPs on cortical network morphology

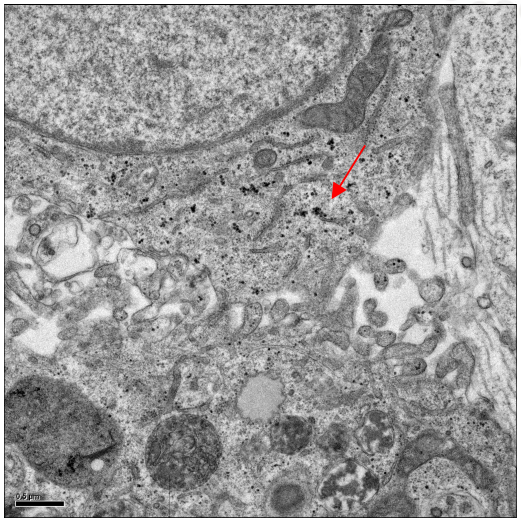
- Presence of NP (agglomorates) in neuronal cells and at the cell surface
- no obvious cell damage or injury after 24 h exposure
- Fe_2O_3 NP near the nucleus



CB-NP



CB-NP



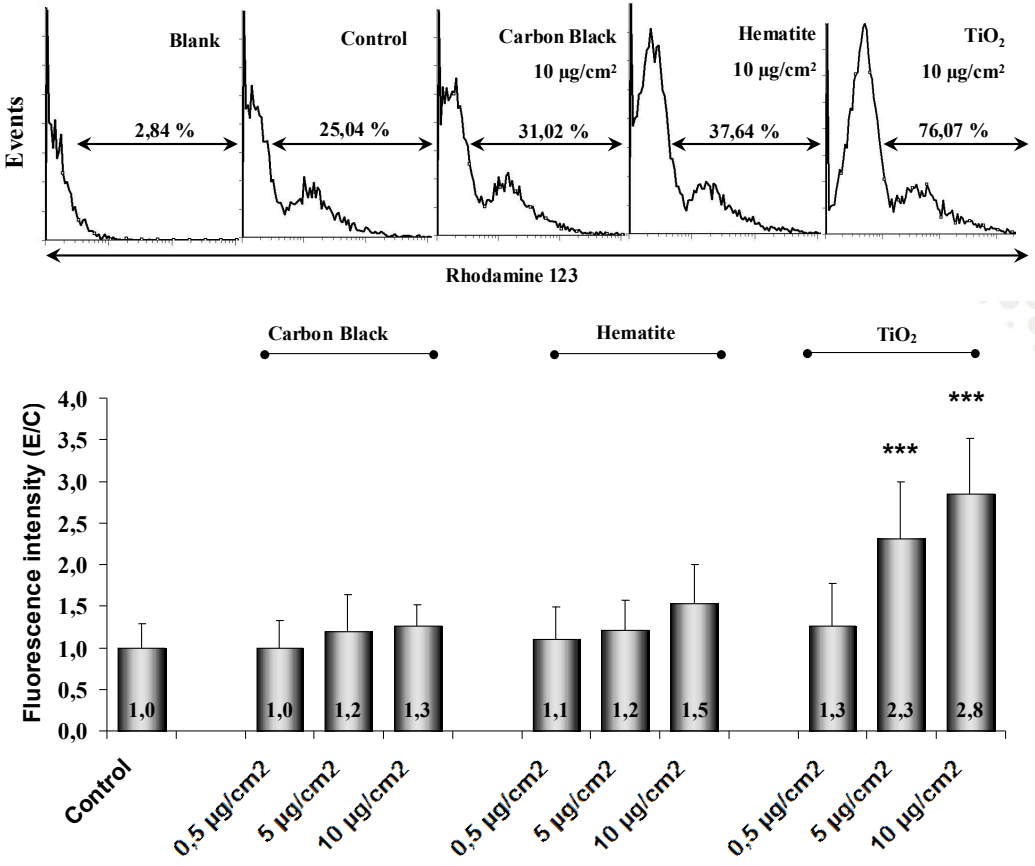
Fe_2O_3-NP

Gramowski et al., EHP, 2010

3.) Changes of neuronal activity after NP exposure –RESULTS-

Influence of NPs on intracellular ROS formation

- ROS production was detected using dihydrorhodamine 123 (DHR) staining
- no significant ROS production after exposure of neuronal network cells to CB and Fe_2O_3
- significant ROS increase just for TiO_2 at high concentrations



Gramowski et al., EHP, 2010

Exposure time: 24 h

3.) Changes of neuronal activity after NP exposure –recent project-

Influence of NPs on $K^{\pm}$ channels

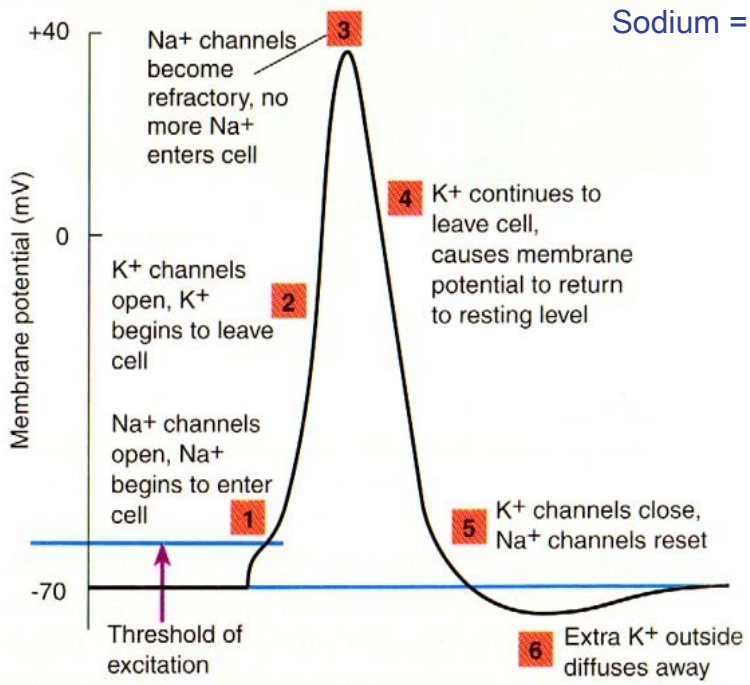
Potassium = K^+
Sodium = Na^+

Action potential with (1) rising phase and (2) falling phase

(1) Na^+-permeable channels open and sodium ions enter the cell depolarizing the membrane

If the depolarization achieves a threshold an action potential can be generated.

(2) -membrane is rapidly repolarizing
-repolarization is achieved by an efflux of potassium ions



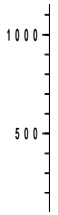
1-3 Depolarization due to opening of sodium channels, 4-6 Repolarization due to opening of potassium channels followed by the refractory phase

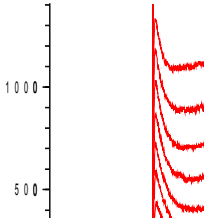
Collaboration with the University of California, Davis, Center for Neurosciences

Method: Patch-Clamp-Technique
Cells: hippocampal neurons (mice)

3.) Changes of neuronal activity after NP exposure –RESULTS-

Influence of TiO_2-NPs on potassium channels





Green: 0 min
Red: 10 min 100 ng/ml TiO_2

Green: 0 min
Red: 10 min

Control

- transient and stable parts of the potassium current are reduced after applying TiO_2 nanoparticles

- potassium channels are blocked

3.) Changes of neuronal activity after NP exposure –RESULTS-

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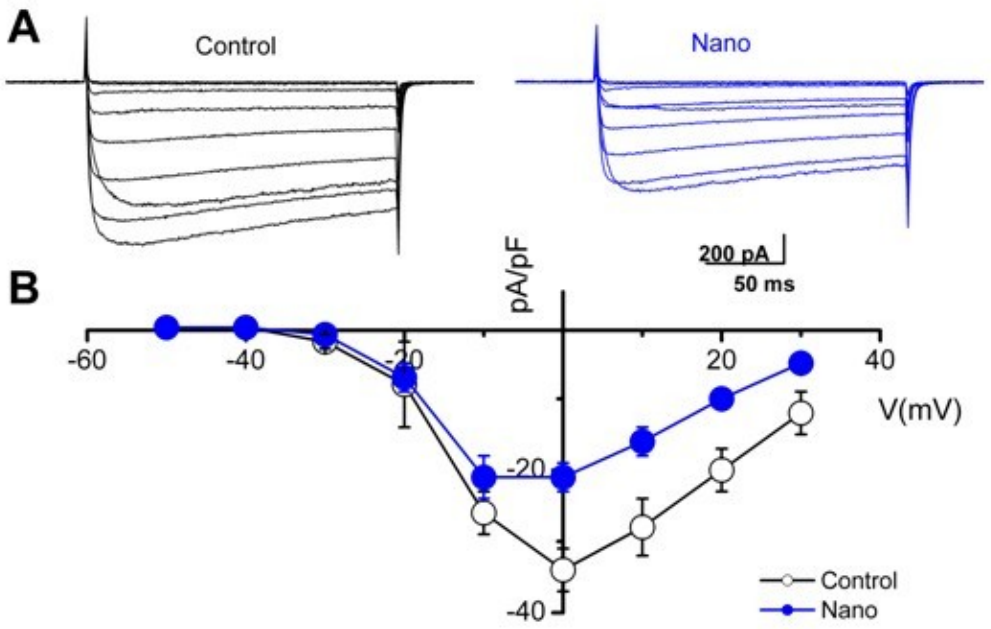
Influence of TiO_2-NPs on whole-cell Ca^{2+} currents

Calcium interacts with potassium *via* calcium-activated potassium channels

An interruption of calcium channels can have an effect on potassium channels.



Whole cell calcium currents are changed after application of NP (100 ng/ml, exposure time: 30 min). TiO_2-NP reduced the peak total Ca^{2+} current by ~30%. (n =6).

3.) Changes of neuronal activity after NP exposure -CONCLUSIONS-

-change of neuronal activity after NP exposure (low concentrations, ng/ml)

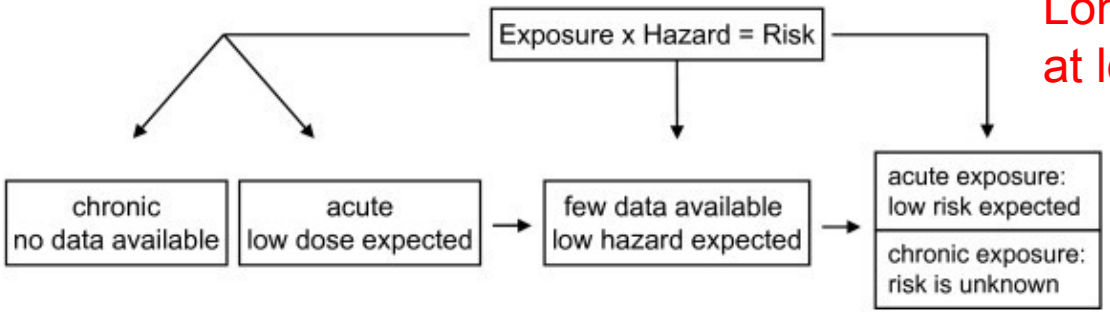
-NP are intracellularly detectable

-intracellular radical formation at higher NP concentrations (µg/ml), no increase at low concentrations (<100 ng/ml)

-potassium channels are blocked after TiO_2-NP exposure

-calcium currents are changed after application of TiO_2-NP

Risc?



Long-term studies at low concentrations

Simko and Mattson, 2010

Nanoparticle-induced neurotoxic and proteomic changes at low-concentration-levels –DISCUSSION-

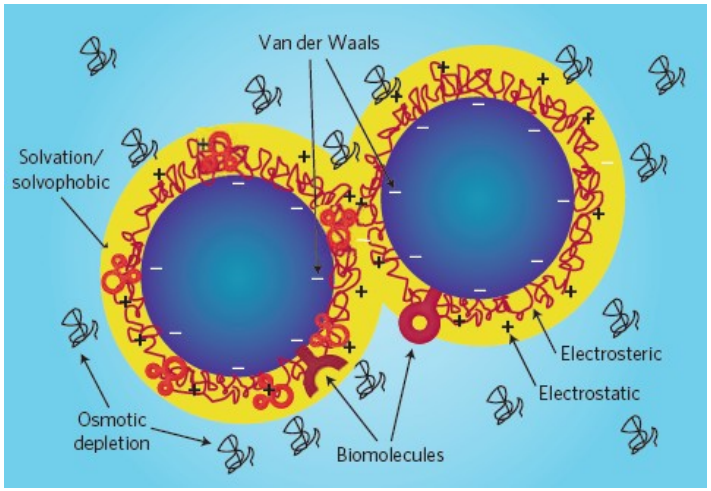
-physico-chemical properties of NP have to be investigated before biological studies are carried out

-Informations about size, shape, composition, surface charge, adsorbed species, redox-reactivity etc. are necessary

-aggregation/dissolution

-dose-response studies are needed –
also at low (relevant) concentration levels

-long-term studies (repeated exposure) are needed



Nel et al., 2009

Nanoparticle-induced neurotoxic and proteomic changes at low-concentration-levels

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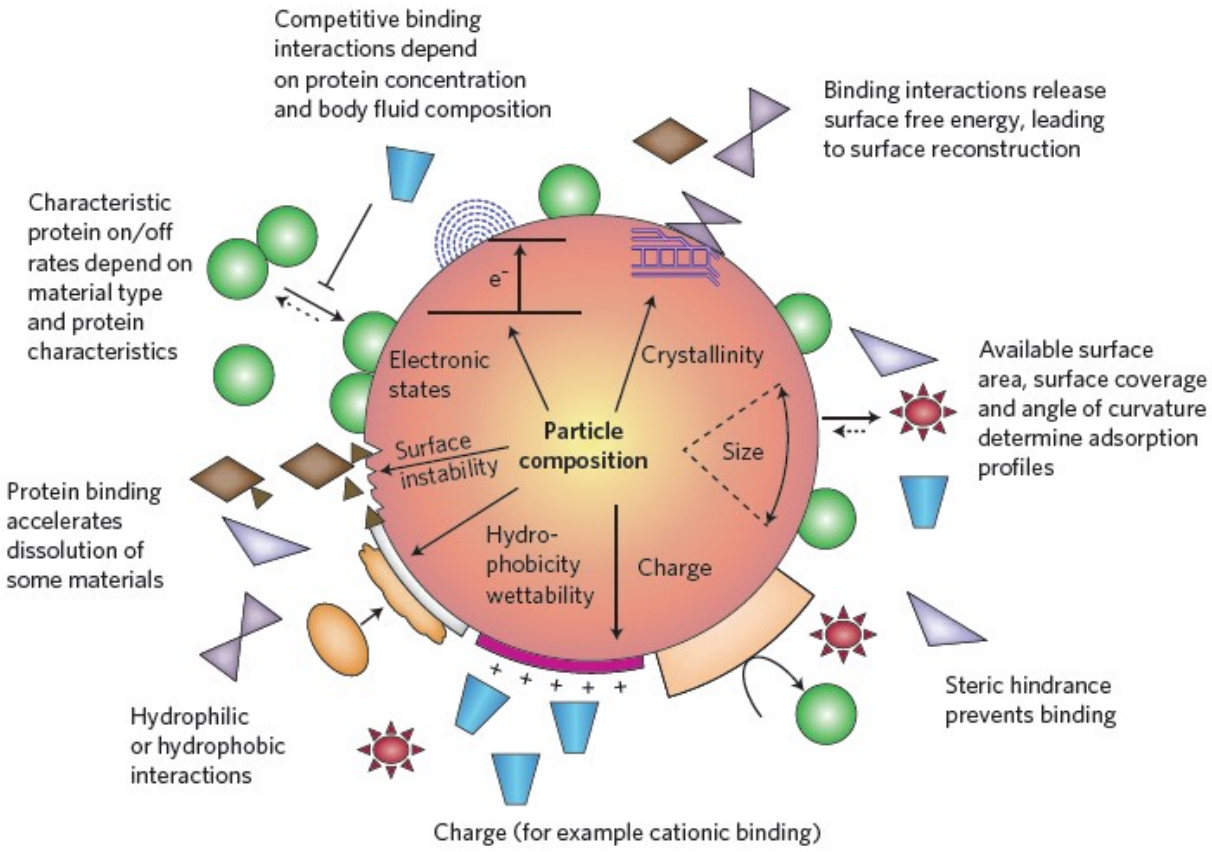
Interdisciplinary research is needed!

(Material sciences, analytical chemistry, biological sciences…..)



Net et al., 2009

Thanks!

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Deutsche Forschungsgemeinschaft DFG

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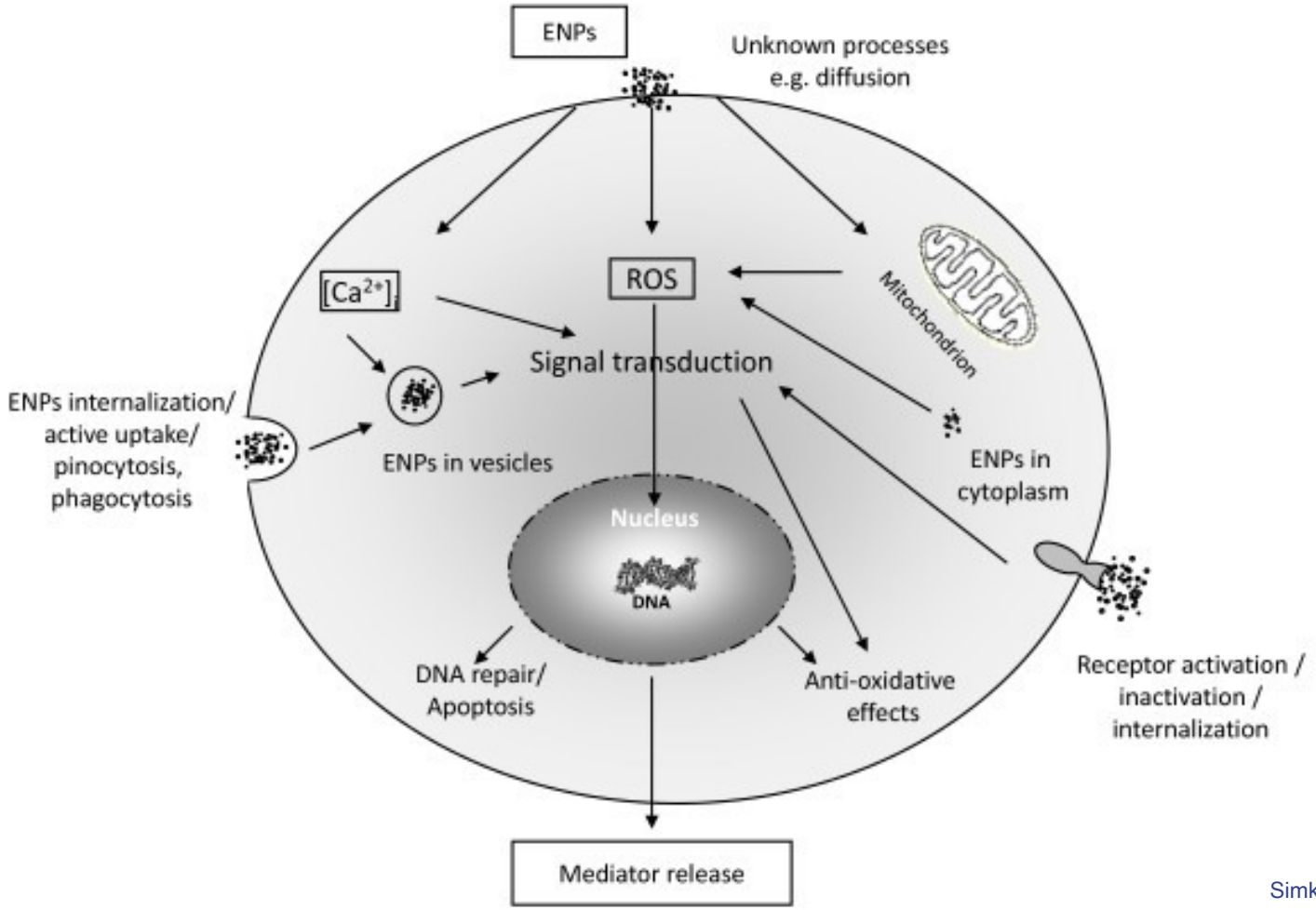
Nanoparticle-induced neurotoxic and proteomic changes at low-concentration-levels –DISCUSSION-



Simko and Mattson, 2010

Nanoparticle-induced neurotoxic and proteomic changes at low-concentration-levels –DISCUSSION-

Lowest observed concentration level

Is it relevant for health effects?

General threshold limit value for inhalable dust

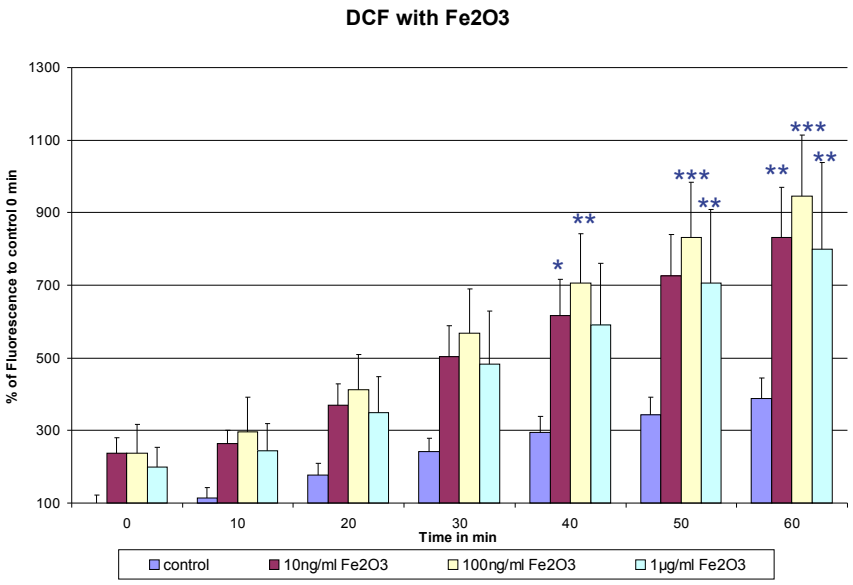
EU-value for PM10 (24 h):	50 µg/m³
Lung volume/day:	20 m³/d
Lung surface:	100 m²
Daily max. exposure:	20 x 50 µg = 1000 µg/d
Concentration at the lung surface:	1000 µg / 100 m² = **10 µg/cm²**
Max. concentration at workplaces:	0.3 mg/m³ 6000 µg / 100 m² = **60 µg/cm²**

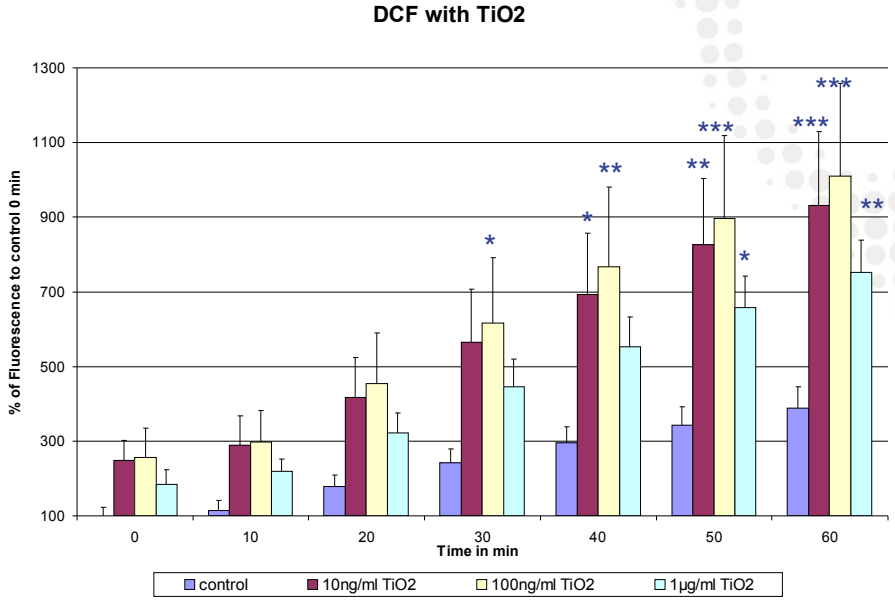
3.) Changes of neuronal activity after NP exposure –RESULTS-

Radical generation after short-term exposure (10 - 60 min)

H_2DCFDA-staining

Bhattacharya et al., PFT, 2009





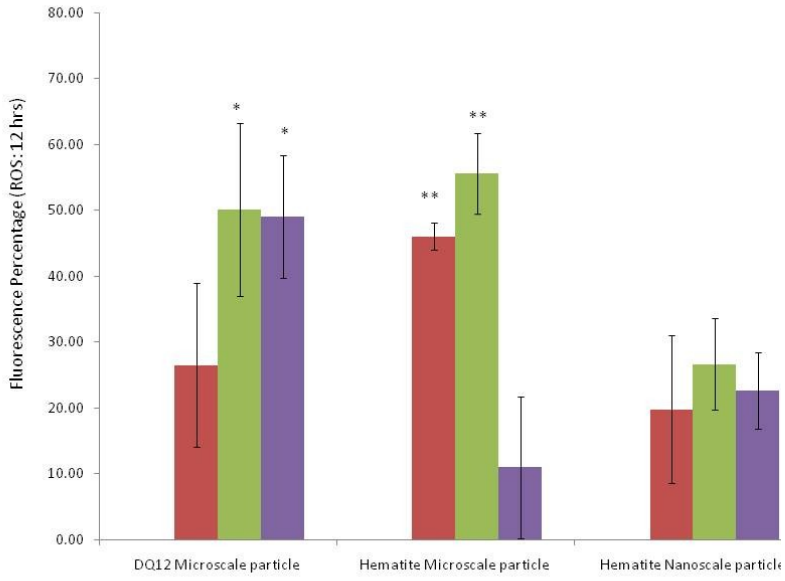
Students t-test:* $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$

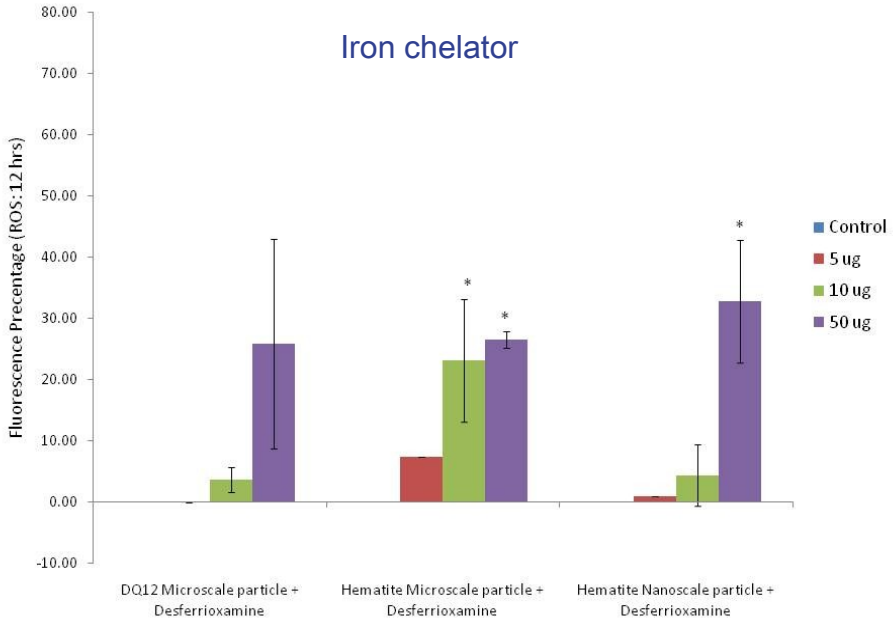
-slightly higher radical formation with TiO_2 NP

-significant effect is delayed with Fe_2O_3 NP

1.) Comparison of micro- and nanoscale Fe^{+3}-containing particles

Intracellular radical formation (Fe_2O_3)





-is higher in BEAS-2B exposed to microscale particles
-exposure time: 12 h

-desferrioxamine is able to bind iron ions
-free radical production is reduced

1.) Comparison of micro- and nanoscale Fe^{+3}-containing particles

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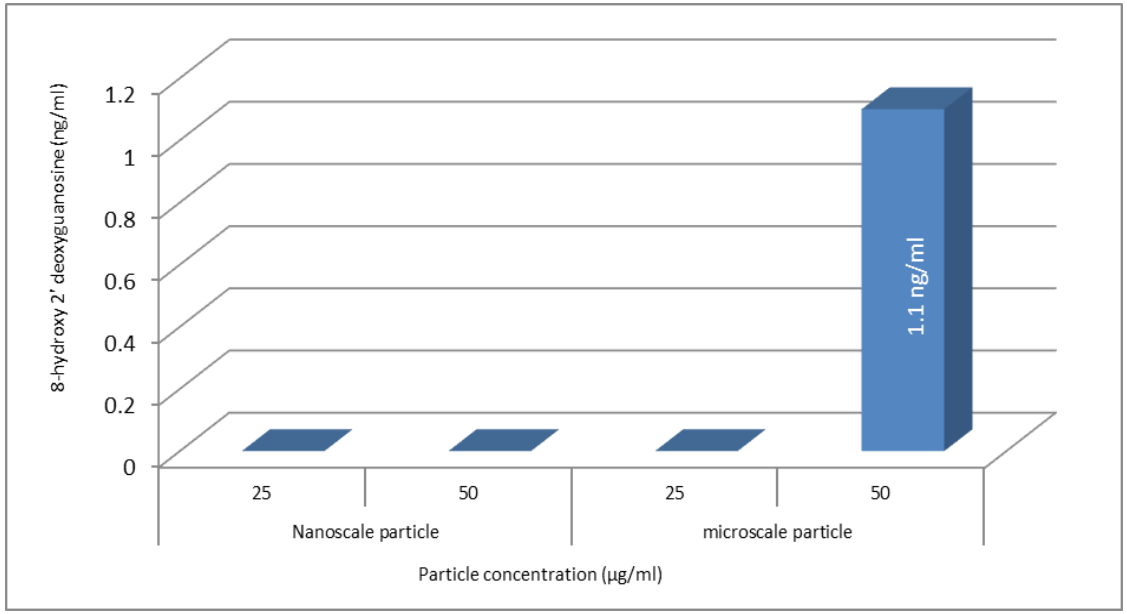
8-hydroxyl-2'-deoxyguanosine (8-OhdG adduct)

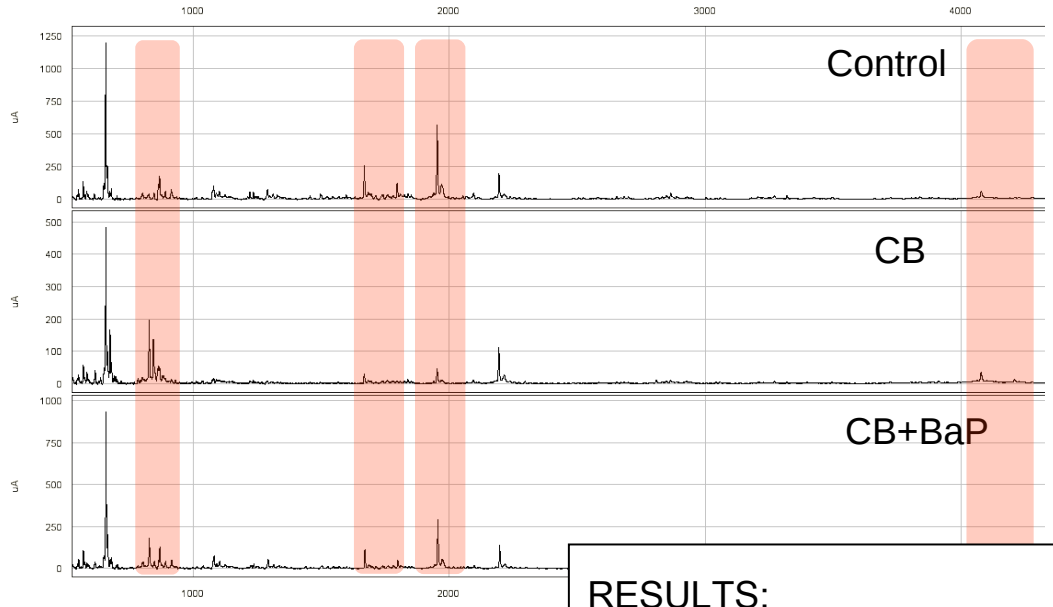


-oxidative DNA damage just at 50 µg/ml (microscale particles) detectable

2.) Cellular effects of NP at low-concentration levels

Mediator screening of culture medium

by surface-enhanced laser desorption ionization (SELDI-TOF MS)



Exposed cell line: endothelial cells
Concentration: 100 ng/ml
Exposure time: 14 days, repeated expo

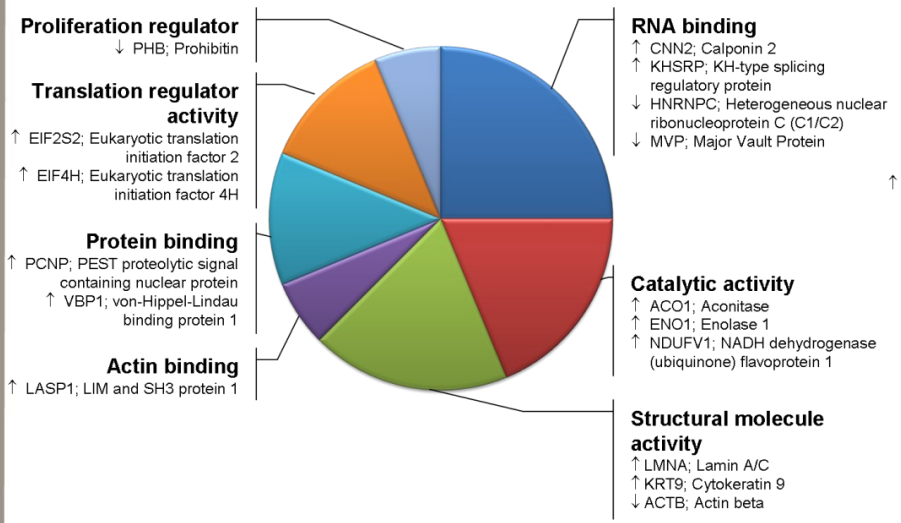
RESULTS:
CB: one mediator significantly increased
CB-BaP: 14 mediators significantly increased

-in the small molecule range (< 10 kDa)
-both n ≥ 30, p ≤ 0.05

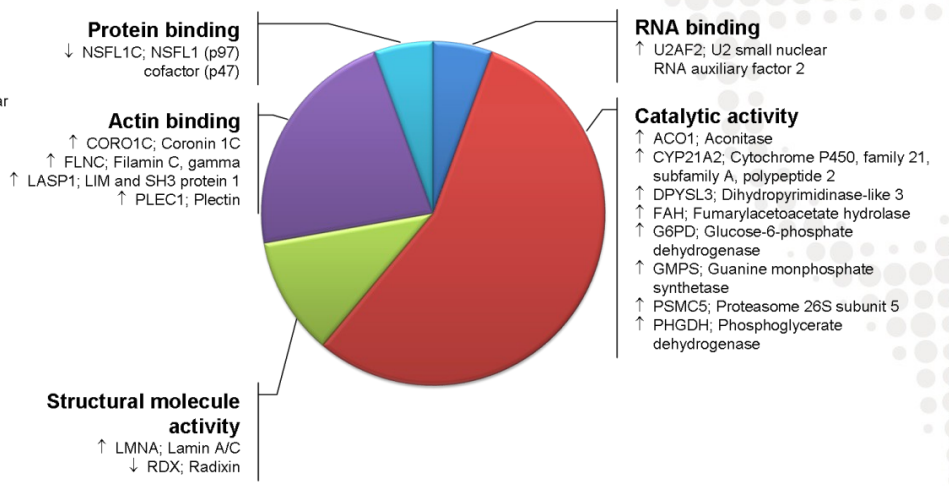
Pink et al., submitted

2.) Cellular effects of NP at low-concentration levels –proteomic analysis-

Proteomic analysis – results



carbon black



carbon black + BaP

Alteration of protein expression(n = 10):

Control vs. CB = 16 Proteins

Control vs. CB+BaP= 17 Proteins

http://david.abcc.ncifcrf.gov

Database as tools for investigators to understand biological meaning behind large list of genes/proteins.

Pink et al., submitted