2\textsuperscript{nd} International Conference on Nanotoxicology

Book of Abstracts – Poster Session

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1 - Material Properties (related to Biology)
1.01 - Optimized dispersion of nanoparticles for biological in vitro and in vivo studies

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Background: For in vitro exposure of cells as well as for systemic administration in vivo, nanoparticles should be dispersed in physiological solutions. Nanoparticles, however, tend to form agglomerates in solutions with physiological pH and salt concentration. Therefore, the aim of our study was to establish and validate a practical method to disperse nanoparticles in physiological solutions for biological in vitro and in vivo studies.

Methods and Results: TiO₂ (rutile) dispersions were prepared in distilled water, PBS, or RPMI 1640 cell culture medium. Different ultrasound energies (2 x 10⁴ - 1.65 x 10⁶ kJ/m³), various dispersion agents (human and mouse serum albumin, Tween 80), distinct concentrations of the dispersion agents (0.0015 mg/ml - 15 mg/ml), and different sequences of dispersion steps were applied. Size distribution of the dispersed nanoparticles was analyzed by dynamic light scattering and zeta potential by phase analysis light scattering (Zetasizer Nano ZS, Malvern). Nanoparticle size was also verified by transmission electron microscopy. A specific ultrasound energy of 2 - 4 x 10⁵ kJ/m³ was sufficient to disaggregate TiO₂ (rutile) nanoparticles, higher energy input did not improve size reduction. The optimal sequence was first to sonicate the nanoparticles in water, than add the dispersion agent, and at the end give buffered salt solution to the dispersion. The formation of coarse TiO₂ (rutile) agglomerates in PBS or RPMI (average diameter 912.1 ± 47.5 nm and 1120.3 ± 47.4 nm, respectively) was prevented by addition of 0.15 % human serum albumin prior to the addition of buffered salt solution (average diameter 186.4 ± 9.9 nm and 168.6 ± 4.9 nm, respectively). Human serum albumin functioned well as dispersion agent at a concentration range of 0.015 mg/ml – 1.5 mg/ml. The TiO₂ (rutile) particle dispersions prepared with this method were stable for up to 1 week. This method was also suitable for preparing dispersions without coarse agglomerates (< 275 nm) from nanosized ZnO, Ag, and diesel SRM2975 particulate matter.

Conclusion: The optimized dispersion method presented here appears to be effective and practicable for preparing dispersions of nanoparticles in physiological solutions without creating coarse agglomerates.
1.02 - Potential for different carbon nanotubes to induce reactive oxygen species in a cell free assay and in lung cells

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Little is known about the potential health effects of inhaled carbon nanotubes (CNT), which are produced by manufacturing industries in high amounts. Recent studies indicate that the geometry of the CNT influences phagocytosis by macrophages and the subsequent induction of cellular responses. Straight nanofibres undergo “frustrated phagocytosis” and are more potent in stimulating the production of proinflammatory mediators than tangled fibres. Phagocytosis of particles and foreign material is a first important step in the production of reactive oxygen species (ROS), which mediates the toxicity of nanoparticles. The aim of this study was to examine the oxidative potential of straight and tangled CNT in a cell free assay and to compare the results obtained with the formation of ROS in macrophages in vitro studied in a semi-quantitative way by laser scanning microscopy.

In the cell free system, ROS production by the tangled fibres was significantly higher than by straight CNT. Also in J774 cells, a mouse macrophage cell line, 17% (SD 7%) of the cells treated for 30min with the tangled fibres showed ROS production compared with 7% (SD 5%) ROS positive cells incubated with straight fibres or with control cells (6% (SD 2%)). However, after 4 hours particle incubation there was a significant increase in ROS production in cells in the presence of the straight CNTs (27% (SD 13%)), whereas the percentage of ROS positive cells incubated with the tangled fibres did not change.

In conclusion, the tangled fibres generated significantly more ROS in a cell-free system than the straight CNT, however, in cells the tangled generated a rapid increase in intracellular ROS, while the straight CNT generated a slower but more prolonged effect. These findings indicate that the long straight CNT, which demonstrated "frustrated phagocytosis", might induce continuous damage at the cell surface initiating an immune reaction.
1.03 - Stability and interactions of radiolabelled clay nanoparticles with algae

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To understand the interactions of engineered nanoparticles with biological systems, it is critical that stable methodologies to label these nanoparticles are established so as not to alter their native physical and biological characteristics. Radioisotopic labelling techniques offer a plethora of advantages for labelling nanoparticles, including ultra-high sensitivity as well as compatible radiosynthetic methods that allow radioisotope incorporation into the nanoparticle that does not compromise the nanoparticle structural integrity and basic function but yet allows non-invasive monitoring of its movement in vitro and in vivo. Layered double hydroxide (LDH) nanoparticles are an important class of layered inorganic clay, which have applications as drug and gene delivery vehicles, catalyst supports and composite fillers [1]. Radioisotopic labelling and stability of the LDHs with ⁵⁷Co and ⁶⁷Ga has been investigated along with their environmental persistence and accumulation behaviour in aquatic algae.

The ⁵⁷Co and ⁶⁷Ga species incorporated into the nanoparticles were found to be chemically stable, and radioisotope leaching has been observed to correlate well with nanoparticle dissolution. For the first time we have been able to quantitatively follow the rate of nanoparticle dissolution at a range pHs by using an instant thin layer chromatography (ITLC) separation methodology and quantitative gamma counting technique. Dual labelling of the nanoparticles has yielded further mechanistic information as to the route of nanoparticle dissolution under various pH. Additionally, the interactions of LDH nanoparticles with algae have been examined and the behaviour of the nanoparticles documented. The controlled synthesis and fractionation of LDHs has been achieved recently and aqueous LDH nanoparticle dispersions of tailored sizes obtained. The effect of LDH nanoparticle size on their biological interactions will also be investigated in the near future.

1.06 - Effect of covalent and non-covalent functionalization on carbon nanotube interactions with AML12 hepatocytes: cellular uptake, localization, and cytotoxicity

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Brown University, Providence, RI, United States

Recently Liu et al. demonstrated that carbon nanotubes (CNTs) conjugated with a ligand peptide that recognizes tumor cell surface receptors are able to accumulate in tumors in vivo, suggesting a potential role for CNT-based drug delivery in cancer therapy. (Liu et al., Nat Nanotech 2007) Nanoparticles including CNTs have been reported to be taken up by the reticuloendothelial system (liver, spleen, bone marrow) when injected into mice and are slowly excreted in the bile and urine (Liu et al., PNAS 2008), motivating studies of liver cell response and possible toxicity. The emerging literature on nanotoxicology includes several studies reporting reactive oxygen species generation and/or oxidative damage associated with CNTs [Sayes et al., Biomaterials 2005; Shvedova et al., Am J Physiol Lung Cell Mol Physiol 2005; Kagan et al., Toxicol Lett 2006; Lin et al., Chem Mater 2007]. For example, commercial multi-wall CNTs (MWNTs) have been observed to release bioavailable, redox-active iron, leading to catalysis of free radical production that causes single-strand-breaks in plasmid DNA. [Guo et al., Chem Mater 2007].

The present study systematically investigates the cellular uptake, localization, and cytotoxicity of modified MWNTs using murine AML12 hepatocytes as target cells. Naturally hydrophobic commercial MWNTs have been modified by two covalent functionalization schemes (negative aryl-sulfonate groups and positive aryl-amine groups) and non-covalent interactions (non-ionic phospholipid DPPC and anti-oxidant TPGS [Yan et al., Carbon 2007]), which enable MWNTs to completely disperse in aqueous phases including cell culture media. The AML12 cell line is immortalized, nontumorigenic, and maintains differentiated properties of adult liver [Wu et al., PNAS 1994]. This cellular model system is useful for investigating the influence of MWNT surface properties (charge, steric repulsion, and anti-oxidant activity) on cellular interactions and toxicity.
1.07 - Characterisation of intracellular compartmentalisation patterns in different cell types using size tuned quantum dots.

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How the living organism interacts with foreign particles is very much dependent on the size and shape of the particle. Engineered nanoparticles are found in an increasing number of applications in the biomedical sciences. Semiconductor nanocrystals or quantum dots (QDs) in particular may be very useful in the tracking of molecules in vivo. Changing conditions during synthesis such as temperature, duration and the addition of functional groups can vary the size and shape of QDs and therefore their properties. Furthermore nanoparticles are similar in scale with biomolecules and are smaller than animal cells and most microorganisms. However the potential harm of these nanoparticles is still poorly understood. Recent studies have shown that change in NP size and shape can alter interactions with living cells and thereby affect toxicity.

In this report using QDs of various sizes we examine the compartmentalisation of the QDs in three different cell lineages namely THP-1 (macrophage) cells, HEp-2 (epithelial) cells and AGS (endothelial) cells, that would be representative of the most likely environmental exposure routes in humans. The cells were fixed and permeabilised prior to the addition of the QDs thereby eliminating any effects due to active QD uptake mechanisms or to specificity of signaling routes in different cell types. All assays were performed using a High Content Analysis (HCA) platform, thereby getting robust data on large cell populations.

We demonstrate that while the smaller QDs enter the nuclei and locate to the nucleoli in all three cell types, the rate and passage differ depending on cell type. Furthermore as QD size is increased, penetration into the cell is reduced but each cell line had its own cut-off size reflecting cell-type determined nuclear pore size specificity. This gives rise to an important consideration regarding the susceptibility of certain organs, tissues and cells to QDs and may be of prime importance for biomedical imaging and drug delivery studies.
1.08 - Synthesis and biocompatibility of fullerene nanofibers

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Fullerene nanowhiskers (FNWs) are fine single crystalline fibers composed of fullerene molecules such as C60 and C70. FNWs are semiconductors and can be synthesized at room temperature using the liquid-liquid interfacial precipitation method (LLIP method) [1]. The diameter of FNWs ranges from about 100 nm to several hundred nanometres. Their application to field-effect transistors and photovoltaic cells have been investigated.

Tubular fullerene nanowhiskers, “fullerene nanotubes (FNTs)”, are also prepared by the LLIP method. FNTs can incorporate various water or alcohol solutions of nanomaterials in their holes by the capillary phenomenon. A TE-buffer with plasmid DNA encoding firefly luciferase were successfully deposited into the C60 FNTs (C60NTs) [2].

The above fullerene nanofibers (fullerene nanowhiskers and fullerene nanotubes) are composed only of fullerene molecules and contain no impurity metals such as Fe, Ni, Co and W that are usually used for the preparation of carbon nanotubes. Thus, one of the most important aspects of fullerene nanofiber is that it is a pure carbon material. Another important aspect is that the fullerene molecules are weakly bonded via van der Waals forces. This bonding property may enable the fullerene nanofibers be destroyed into short peaces through the phagocytosis by macrophages and make them harmless in a body. Hence, the fullerene nanofibers are potential materials for biological uses such as DNA vector, filler of plastics for medical uses, containers of medicines and so forth.

C60 nanofibers were prepared by the LLIP method using a C60-saturated pyridine solution and isopropyl alcohol. The quantity of mRNA of HSP70B’ were measured using the HeLa S3 cells that were exposed to the C60 nanofibers in a D-MEM culture medium added with 10% FBS. No induction of mRNA by the C60 nanofibers was observed. This result suggests that the C60 nanofibers can be utilized as new biocompatible material.

1.09 - Use of micelle nanoparticles to reduce side effect of molecular agent for photodynamic therapy

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PhotoDynamic Therapy (PDT) principle is largely based on the use of organic molecules such as Photofrin or Foscan that are able to produce Reactive Oxygen Species (ROS) with surrounding media upon irradiation with appropriate laser light. When ROS are release within cells, they lead to irreversible damages and most likely to cell death. Photofrin and Foscan compounds are currently formulated for Intra Venous (IV) injection to target tumour cells. However, despite interesting biodistribution, a significant amount of molecules still accumulate within skin which requires that the patient stays at least 4 or 6 weeks in dark after drug administration to prevent skin toxicity when exposed to light.

Nanobiotix objective is to encapsulate the active molecule within micelle nanoparticles in order to improve the performance of the PDT by reducing side effect via an optimized biodistribution corresponding to the reduction or the elimination of drug accumulation within skin. In addition, the micelle nanoparticles must guarantee the following properties:

(1) a good solubility of the organic drug within the micelle for a relevant PDT answer (at least keeping treatment efficacy similar to current PDT),

(2) an efficient protection of the organic molecule from biological media until its localisation within targeted tumour cell and PDT activation.

Successful encapsulation of PDT molecule within micelle nanoparticles has been achieved with the following properties:

(1) Increase stability of the organic molecule toward the external biological media via
   a. the consolidation of the hydrophobic/hydrophilic micelle interface by a silicone layer
   b. and/or the addition of excipient that is able to move to the interface and to bring additional stability .

   Indeed, In Vitro experiments have shown the ability of the micelle nanoparticles to kill tumour cells.

(2) Improve biodistribution of the micelle nanoparticles via both
   a. a fine tuning of the micelle nanoparticles size at the nanometre scale,
   b. the addition of appropriate excipient.

Qualitative biodistribution was used to monitor the difference of biodistribution of an organic molecule and its encapsulated form. The organic molecule alone is immediately trapped within liver whereas micelle nanoparticles accumulate preferentially within tumour in less than 6 hours. Quantitative biodistribution performed on PDT molecule encapsulated within micelle nanoparticle has shown that around 6% of the injected micelle nanoparticles are found within tumour and none within skin. At last, VIVO efficacy (based on IV injection) has shown equivalent effect of PDT using either photofrin (with best efficacy seen 24 hours after injection with skin toxicity) or micelle nanoparticles (best efficacy seen 2 hours after injection with no skin toxicity).

Conclusion: PDT molecule encapsulated within micelle nanoparticles seems to be a promising way to enhance performance of PDT by reducing skin toxicity side effect. This performance is linked to a better biodistribution (no significant skin accumulation of the molecule) together with a good stability of the encapsulated molecule, with no apparent toxicity seen during In Vitro or In Vivo tests.
1.10 - Synthesis of arbitrary compositions of uniform oxide, salt and metal nanoparticles for nanotoxicology studies

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It has been shown that for a significant interpretation of cytotoxicity assays thoroughly characterized nanoparticles are a basic requirement. In a further aspect it has been suggested that nanoparticle-nanoparticle comparison studies are only reasonable if the different nanoparticle compositions used in a study feature similar appearance, e.g. particle size, particle size distribution, morphology etc. Certainly, it would be ideal for comparison studies if such nanoparticles could be produced by one single technology only to rule out any process varieties.

In this work we would like to present a synthesis method yielding nanoparticles which are exceptionally suited for nanotoxicology research (Limbach et al. 2005, 2007, Brunner et al. 2006). By flame spray pyrolysis (FSP) nanoparticles with any given composition can be readily produced (single oxides, mixed oxides, salts and metals). Besides the freedom of composition the here presented synthesis technology yields particles with similar morphology, very narrow size distributions and high purity. A further advantage is that FSP is derived from a common industrial process that is used to annually produce multitons of some of the most widespread nanomaterials, e.g. carbon black, silica or titania.

References:


1.11 - High content analysis study examining the interaction of magnetic nickel nanowires with THP-1 cells and their biocompatibility.

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The interaction between nanowires and biological specimens has been attracting much interest due to the magnetic properties of nanowires and ability to be functionalised with various antibodies. It is thus important to evaluate the interaction and biocompatibility between nanowires and biological entities for future scientific and clinical applications. Magnetic nickel nanowires, 200 nm diameter and 20 μm in length, were synthesised by chemical electrodeposition into alumina templates. Once deposition was complete, the membrane was dissolved in 1M NaOH to obtain free-standing nickel nanowires. Complete characterisation on these nanowires was achieved using SQUID, XRD and SEM. The nanowires had a face centered cubic structure with a lattice parameter ao=3.53 x 10-10m and a saturation magnetisation of 40Am2/kg. A time course study implementing High Content Analysis (KineticScan Reader, Cellomics) was carried out to evaluate the possible cytotoxic effects of nickel nanowires on differentiated THP-1 cell line-derived macrophages and also the possible mechanism of their interaction. The time points selected were 3, 6, 24, 72 hours with matching negative and positive controls. The nanowire to cell ratios used were 1:1, 10:1, 100:1, 500:1. A multiparameter analysis was implemented to evaluate and identify the critical time points and concentrations of nanowires on THP-1 cellular response. The full content analysis involved examining cell viability, nuclear size, membrane permeability, lysosomal mass-pH. From the results it was seen that, there was an inhibition of cell growth response due to the increased phagocytic behaviour of the THP-1 cells. Nickel nanowires appeared to have no substantial effect on THP-1 cellular response after short incubation times regardless of nanowire concentration. However, there was a decrease in cell viability after 24 hours at high nanowire concentrations. The lethal dose time occurred at 72 hours when there was a 50% loss in cell viability for 100 nanowires plated to every cell. Loss of cell membrane integrity is another common phenotypic feature of cytotoxicity. In this study membrane permeability data was the most conclusive in indicating apoptosis after 24 and 72 hours at high nanowire concentrations. This study showed that THP-1 cells’ natural phagocytic response to nickel nanowires resulted in an adverse effect on cellular growth cycle at high nanowire concentrations.
1.12 - Citotoxicity of small maghemite nanoparticles in a renal proximal tubular cell line

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Biocompatible ferrofluids are stable suspensions of superparamagnetic nanoparticles (NP), which can be used as part of a delivery system for anticancer agents in loco-regional tumour therapy, called ‘magnetic drug targeting’ [1]. Through this form of target directed drug application, one attempts to concentrate a pharmacological agent and its site of action in order to minimize unwanted side effects in the organism. Small superparamagnetic nanoparticles can be made resonantly respond to a time-varying magnetic field, so the particle can be made to heat up to targeted bodies such as tumours, which leads to their use as hyperthermia agents. Additionally, these particles can act as MRI contrast agents for diagnostic.

Here we report on the molecularly controlled preparation of superparamagnetic iron oxides nanoparticles having sizes ranging from a few up to tens of nanometers, which places them at dimensions comparable to those of a biological entity of interest. Magnetic properties in nanoparticles are greatly influenced by factors such as shape, mean size, and size distribution. The particles are prepared within a polyvinylpyridine (PVP) matrix which naturally prevents aggregation [2]. The composite is then treated to attach polyethylene glycol (PEG) and have it dispersed in PBS fluid at pH = 7.4. The resulting ferrofluid is then formed by magnetic nanoparticles encapsulated by PVP and coated by PEG.

Cytotoxicity of these NPs was assayed in the Opossum Kidney (OK) cell line, an American opossum cellular model of renal proximal tubule. Quantitation of LC50 was determined according to the activity of cellular lactate dehydrogenase in culture medium after several days of incubation: At day 1 LC50 was 420 mg Fe2O3 / liter; at day 2, 66 mg/l, and at days 3 through 7 LC50 dropped to 34 mg/l. At 10 mg/l cell death was small but significant at day 7th. These results were confirmed by ethidium bromide / acridine orange staining and fluorescence inverted microscopy, and are extended to apoptosis assays and oxidative stress evaluation.

References


Silver nanoparticles (AgNPs), which are commercialized for their antibacterial properties, were considered as one of priority researched nanomaterials when nanomaterial toxicity became an issue. AgNPs toxicity is believed to be due to Ag⁺ ions contained in AgNPs solution but there are few reports that have quantified and controlled Ag⁺ ion concentration. This research focused on deionizing Ag⁺ ions in AgNPs suspension solution and evaluating potential toxicity by AgNPs, not Ag⁺ ion. Potential toxicity of deionized AgNPs was compared with AgNPs containing Ag⁺ ion, which ion ratio was controlled. In addition, the electrochemical property of Ag⁺ ion ratio was compared with the physical property of hydrodynamic diameter distribution. For this purpose, AgNPs suspension solution was prepared by the water stirring method, which excluded any chemicals such as solvent, surfactant, stabilizer or reducing agent. It should be analyzed and confirmed nanomaterials properties before toxicity evaluation. Through characterization of physico-chemical properties such as shape, crystal structure, agglomeration characteristics, and surface chemistry, it can get reliability of toxicity identification and ensure to figure out the toxicity mechanism. Ag⁺ ion was measured by ISE (ion selective electrode) and eliminated by the potentiostatic method in typical three electrode electrochemical cell. Silver wire and platinum wire were used as pseudo-reference electrode and counter electrode, respectively. For electrochemical reduction of Ag⁺ ions, -0.3 V electrical potential was applied on gold electrode. AgNPs suspension samples contained 40 ~ 60 % of Ag⁺ ion to total AgNPs concentration (about 20 mg/L). Deionized AgNPs was applied on toxicity evaluation by an early-life stage toxicity test, a short-term toxicity test on fish embryos and sac-fry stages, and fish embryo toxicity test.
1.14 - Nanoparticle synthesis, characterisation and labelling for toxicological studies: problems and pitfalls

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In recent years it has become apparent that the novel properties of nanomaterials may predispose them to hitherto unknown potential for toxicity. A number of recent toxicological studies of nanomaterials exist, but these appear to be fragmented and often contradictive. Such discrepancies may be, at least in part, due to poor description of the nanomaterial or incomplete characterisation, including failure to recognise impurities, surface modifications or other important physicochemical aspects.

Our proposed approach is to generate good quality, well characterised sets of nanoparticles to be made available to the toxicological community and we have begun synthesis of such materials. We will be discussing three case studies of synthesis, TiO₂, ZnO and SiO₂, each of which presents different challenges in the selection and application of synthesis protocols.

Firstly we will discuss different synthesis methodologies and their effects on the physicochemical properties of the produced nanoparticles, emphasising that the chosen methodology may influence the reactivity and hence toxicity of the product. We will present pitfalls in the synthesis protocols and published information. We will justify our approach, which is to study tailor-made and industrially produced nanoparticles in tandem, and explain our synthesis principles which are based on selecting simple, reproducible and reliable protocols.

We will then present a range of methods of characterisation of the synthesised nanoparticles, emphasising the importance of using a combination of spectroscopic, microscopic and chemical techniques, and of characterising both in-situ and ex-situ in a variety of media.

Finally we will explain the ideas and principles that we apply when labelling the nanoparticles we synthesise.
2 - Exposure Scenarios
2.1 - Assessing consumer exposure to nanoparticulate material in cosmetics and personal care products

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Nanomaterials exhibit novel properties which allow for applications with new characteristics in many industrial sectors. Accordingly, nanomaterial-containing applications are conquering the market, including a significant amount of consumer products, such as UV-filtering and odour-preventing textiles, sports equipment, or antimicrobial household appliances. Two categories of consumer products which can contain engineered nanoparticles (ENPs) - mostly silver, gold, titanium dioxide, zinc oxide or carbon - are cosmetics and personal care products (PCPs). Consumers use cosmetics and PCPs on a regular basis and are therefore potentially exposed to ENPs during everyday life. Considering their novelty, consumer products containing ENPs need to be subjected to a thorough risk assessment to guarantee product safety. In order to carry out such a risk assessment, it is of importance to know the situations which may lead to consumer exposure, as well as the exposure levels and the number of exposed individuals. Based on these needs for information, we carried out a modelling study investigating consumer exposure to ENPs through the use of cosmetics and PCPs.

First, a literature and internet research was carried out to identify relevant products, i.e. products with nanoparticulate content, and the materials commonly used in these products. Second, exposure models were set up to reflect typical product usage during everyday situations. This was done by using literature data about behaviour patterns and body characteristics of consumers as well as data relating to product usage and ingredients. Third, external exposure was modelled for the three potential uptake routes skin, lung and gastrointestinal tract. For each exposure situation, three scenarios have been assessed: one high, intermediate and low exposure scenario illustrating an upper, intermediate and low level of exposure, respectively. Finally, it has been estimated which fraction of the population might come into contact with the products and which consumer groups were the most exposed to the products under investigation.

Overall, the results of this work provide a first estimation of the order of magnitude of consumer exposure to engineered nanoparticles in cosmetics and personal care products.
2.2 - Exposure investigations related to engineered nanomaterials in Al2O3 and TiO2 work place environments

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The production of nanostructured materials has gained huge momentum and is seen as one of the main future technologies. Therefore a sustainable approach towards this technology is necessary to ensure safe production, and use of materials and devices containing nanostructured materials. One of the main building blocks in nanostructured materials are nanoparticles, that are particles with at least one dimension of 100 nm or less. The health effects of particles less than 100 nm, especially engineered nanoparticles, are currently discussed by toxicologist to be of concern.

In order to improve the necessary knowledge to assess the possible implications of nanoparticles it is necessary to be able to detect and quantify nanoparticles in the work place environment. Different measurement and sampling techniques are necessary as well as task specific strategies to identify and quantitatively determine nanoparticles.

The possible release of engineered nanoparticles was investigated at several work places at Evonik Degussa, Rheinfelden to allow for a first assessment of the exposure of work-ers. For the determination of nanoparticles in workplace environments two sets of SMPS with concurrent measurements in the work area and a comparison site with the following measurement and data analysis routine were used:

1) Measurements in work area without work activities (inside) and a comparison site (outside), 2) Measurements in work area with work activities and a comparison site, 3) Display and analysis of the continuous data to identify ‘events’ with subsequent test of consistency, 4) Ratio generation of particle size distributions for inside and outside, 5) Calculation of the particle release through the production process (ratio and absolute).

Data interpretation is based on the assumption that measurements at the comparison site mirror the surrounding of the work place to be investigated. This includes that particle concentrations (size distributions) at the work places during no work activity can be calculated from those at the comparison site. The comparison site therefore has to be representative of the surrounding. In the poster presentation, the measurement strategy the corresponding results from measurements at Al2O3- and TiO2-work places will be presented.
2.3 - Assessment of particular matter pollution in non-industrial occupational environment

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Introduction. The adequate indoor air quality at the non-industrial workplaces is important factor for mental and physical well being of employees. Therefore the assessment of dust (particular matter; respirable – PM$_{2.5}$) and nanoparticles (carbon nanoparticles from office equipment) is significant in Latvia.

The objective of this study was to evaluate concentration of different size particular matter in the non-industrial occupational environment.

Materials and Methods. Total dusts and respirable dusts (Particular Matter – PM$_{2.5}$) were detected by dusts monitor “Split2” and personal sampling pumps on filters with pore size 0.025 μm to collect nanoparticles at bank premises.

Results. The concentrations of total dust were detected in range 0.09 – 0.86 mg/m$^3$. The concentrations of total and respirable dusts in the premises show, that dusts are important problem especially in the premises with carpet covered floor. The concentrations of respirable dusts (particular matter – PM$_{2.5}$ including ultrafine particles) were detected in range 0.05 – 0.74 mg/m$^3$. The concentrations of respirable dusts were 2 - 3 times lower (0.13 – 0.16 mg/m$^3$) in the premises with renovated ventilation systems and low number of printing equipment.

Conclusions. 1) The high dust concentrations were detected in the premises with high speed job activities, lack of adequate ventilation system and floor covered with carpet. 2) The respirable fractions, including ultra fine particles are general dusts fractions in the non-industrial indoor air of the offices. 3) It is necessary to implement scientific grounded methods, guidelines and rules for indoor air quality assessment (include nanoparticles) and to research toxicology of nanoparticles of non-industrial occupational environment.
2.4 - Applying nanotoxicology – A non-toxicologist’s point of view

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While fundamental research is going on to identify and characterize biotransformation and interaction of nanomaterials with biological systems, manufacturers have to produce today. Since the “nano-industry” has a considerable share of SMEs, the resulting problem of product liability is of crucial importance for all of them: How can one responsibly produce substances without being completely aware of all possible future relevant safety issues? In this article we want to address this question in two aspects:

1) What are the relevant exposure scenarios with respect to products, esp. consumer products, over the whole life-cycle? Shall one perform extensive and expensive toxicological tests for materials which might then behave differently in contact with sweat or spittle, e.g.? Can we really assume that “fixing in a matrix” will prevent the release of nanomaterials and how could we prove that this is really the case?

2) What must then be measured in face of this situation? Obviously the interests of toxicologists and product managers will differ here in many aspects. If the “most important physicochemical (PC) properties” of nanomaterials are discussed which “must be measured”, the meaning of the word ‘important’ should be clarified before. Here a more phenomenological and simplified approach is proposed for characterizing nanoparticles as an important example of nanomaterials. It is based on mainly thermodynamic properties and does not claim to be of fundamental but might be hands-on and come up to the interests of researchers and producers as well.
3 - Biokinetiks
(Agglomeration / Deagglomeration)
3.1 - Developing and testing a bio-mathematical model to describe particle size-specific clearance and translocation of nano particles in rats.

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Investigations of the translocation of nanoparticles throughout the body are normally carried out through animal experiments. Animals, usually rats, are exposed to a specified dose of particles, either through inhalation, IT instillation or IV injection, and the mass of particles in certain organs is determined. Using data from these studies it is possible to model the passage of nano particles through the body.

Biological information about the major organs; lung, brain, heart, kidney, spleen, liver and GI tract, and their relationships to one another was used to draw a model of the compartments of the body. Given the initial dose, method of exposure and particle size used in an experiment estimates were obtained of the amount of particles present in each organ, through a set of differential equations. Most of the model parameters, such as organ weights, were obtained from published literature. Optimal estimates of the unknown parameters were obtained through minimisation of the model mean square error.

The optimisation was carried out, using Matlab, given data from two different studies; Semmler et al. (2004) and Takenaka et al. (2001). Different optimal estimates of the unknown parameters were obtained for the two studies as they used different particle sizes, different methods of exposure; endotracheal instillation v inhalation and results were given for different organs. The optimal estimates resulted in $R^2$ values of 99 and 98%, respectively; plots confirmed how closely the fitted values matched the actual data.

The model describes the movement of nanoparticles throughout the body well but the parameter estimates obtained are dependent on the method of exposure. The next step, validation of the model using new data, is currently being carried out.


Takenaka et al. (2001) Pulmonary and systemic distribution of inhaled ultrafine silver particles in rats. Environmental Health Perspectives: 109(suppl 4); 547-551.
3.2 - Detection of protein Binding to metaloxide nanomaterial using high resolution ultrasound technology and analytical ultracentrifugation

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Physiological liquids and buffers for in-vitro cell culture assays contain proteins, with which the nanoparticles interact and therefore the cellular reaction might be altered. Furthermore, aqueous nanoparticle suspensions have been stabilized to prevent aggregation by addition of BSA and FCS. This effect was thought to result from a BSA nanoparticle surface coverage.

In the present study we go beyond our last contribution(1) and implement three complementary physical measuring principles (sonic, hydrodynamic and optical). We quantified the binding of proteins to various metal oxide nanoparticles using a new methodological approach with high resolution ultrasound velocimetry (Ultrasonic Resonator Technology, URT). These studies were compared and complemented by Analytical Ultracentrifugation, where the concentration of dispersed proteins and nanoparticles, and their respective molar mass distribution and agglomeration / deagglomeration potential are characterized. Finally the independent method of fluorescence correlation spectroscopy with Alexa-tagged proteins in a confocal microscope setup was evaluated, but failed due to interference of the nanoparticle surface with the optical readout.

BSA (bovine serum albumin) or FCS (fetal calf serum) and inorganic metal oxide nanoparticles with different composition were used in buffer solutions or water as model systems to investigate binding and binding capacity. The Metal oxide nanoparticles (provided from partners of BMBF-Project NanoCare) were dispersed according to a protocol developed within the NanoCare project.

We find a protein adsorption that depends both on the available inner surface of the nano-suspension and on the chemistry of the nanoparticles.

3.3 - Strong size dependency of biodistribution of gold nanoparticles (NP) between 1.4 and 200 nm administered either to the lungs, blood or gut of rats

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Currently, the translocation of insoluble nanoparticles (NP) across membranes like the air-blood, vasculature and intestinal barrier into secondary target organs is debated. The key questions are related to which NP parameters determine translocation and which biological mechanisms are involved. Here we present experimental in vivo data elucidating the role of NP size using 1.4, 2.5, 5, 18, 80 and 200 nm gold NP each coated with a negatively charged ionic ligand and compare to positively charged 2.5 nm NP.

Healthy female adult WKY rats received either intratracheally (IT) or intravenously (jugular vein, IV) or intra-oesophageally (IO) 50 µg of non-agglomerated, radio-labelled gold NP suspended in 50 µl of saline. Gold was previously neutron activated receiving a \textsuperscript{198}Au radio label. The rats were killed 24-hours after NP administration and all organs and tissue samples as well as the remainder and excretion were analyzed gamma-spectrometrically in a low-background well-type detector balancing the administered dose.

After 24 hours of translocation across the air-blood or intestinal membranes, the accumulation in secondary target organs (STO) showed a strong negative correlation with NP size: the translocated fraction was about 5% (0.4%) and 0.02% (0.07%) of the IT (IO) administered 1.4 nm and 80 nm NP, respectively. Hence, there were clear differences between IT and IO. Furthermore, similar to STO accumulation after IV injection, liver was the most prominent retention site but in all other STO like brain, heart, uterus, spleen and kidneys and blood detectable fractions of NP were found. Furthermore, negatively charged NP were significantly more translocated than their negative counterparts.

These systematic and quantitative studies demonstrate the important role of size for the biokinetics of incorporated NP and the significance of the different routes of entry.
4 - Lung and Inhalation
4.1 - *In vitro* toxicity of cobalt particles on human alveolar tape-II cells.

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The production of engineered nanoparticles has dramatically increased during the last years. One of the main routes of exposure in humans is the respiratory tract, where nanoparticles can accumulate and induce inflammation. *In vivo* studies are primarily used to investigate the risk following exposure to nanoparticles. However, due to the complexity of the respiratory tract and due to the steadily increasing number of nanoparticles to investigate, a rapid and cost-effective evaluation of the toxicological effect induced by engineered nanoparticles in the lung is still missing. In this study, the toxicity induced by cobalt nanoparticles was investigated in *in vitro* cell culture model systems using the human alveolar type-II (ATII) cell lines A549 and NCI-H441, which exhibit many of the characteristics of primary human lung epithelial cells. A549 and NCI-H441 monocultures were exposed to different concentrations of cobalt nanoparticles (Co-NPs) for 4-72hr and the toxicity of Co-NPs was evaluated by classical cytotoxicity assays. The cell viability decreased in a dose-dependent manner very rapidly, beginning 4hr after exposure to Co-NPs as demonstrated by the MTT assay, which is an indicator of the mitochondrial activity. The effects of Co-NPs were further investigated by Ki-67 assay and crystal violet dye elution, both markers for cell proliferation, and lactate dehydrogenase (LDH) release, a marker for cell membrane integrity. Interestingly, in both cell lines investigated, the Ki-67 expression could be detected after exposure to cobalt nanoparticles. Crystal violet demonstrated that the number of A549 and NCI-H441 cells in culture decreased significantly only after 48-72hr exposure to Co-NPs, while the LDH release in the culture medium increased. Since cobalt ions could still be present as residues in the cobalt nanoparticles and exert an effect when nanoparticles are dispersed in cell culture media, cobalt chloride was utilized as a control. In conclusion, it appears that Co-NPs damage and reduce the viability of human ATII-like cells *in vitro*, but their toxicity is lower than the toxicity detected after treatment with cobalt chloride.
4.2 - Cyto and genotoxicity of nanoparticles and carbon nanotubes: is oxidative stress responsible?

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Increasing worldwide production of nanoparticles and carbon nanotubes for industrial purposes leads to considerable concern about their potential human and environmental toxicity.

This study focused on TiO₂, Al₂O₃, Au nanoparticles and CVD-produced carbon nanotubes. Their cyto- and genotoxicity were investigated on A549 alveolar lung cells, since this organ is directly exposed to nanomaterials in case of air contamination. For cytotoxicity, several assays were employed in order to get rid of nanomaterial-assay interferences, known to lead to false-positive results. Three complementary assays were performed to assess nanomaterial genotoxicity. Subsequent reactive oxygen species production was searched, and the activity of enzymes implicated in oxidative balance maintenance was evaluated.

Our results indicate that these nanomaterials exert a small but significant toxicity to lung cells, which is not dependent on particle size, but may be related to crystal phase and chemical composition. The presence of metal impurities in carbon nanotubes does not seem to be responsible for their biological impact. Intracellular nanomaterial uptake was demonstrated. The results show that high concentrations of some of these nanomaterials are responsible for single strand breaks and/or alkali-labile sites and/or oxidative damage to DNA, and thus are genotoxic. Cyto- and genotoxicity can be the consequence of oxidative stress generation after intracellular accumulation.

The originality of this study lies on the panel of nanomaterials that were tested on the same cell line. Moreover nanomaterial colloidal stability was evaluated at each step of the experiments. All these data lead to a better understanding of nanomaterial toxicity and dangers for health.
4.3 - A new exposure system to evaluate the toxicity of scooter emission in lung cells in vitro

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It is known that diesel exhaust particles (DEP) have the potential to induce adverse health effects associated with pulmonary and cardiovascular diseases by inducing oxidative stress, inflammatory reactions, and there is a link between exposure to diesel soot and lung cancer. The toxicity of DEP was studied by using an epithelial airway model. We have shown that DEP in suspension resulted in an increase of both of reactive oxygen species and of the pro-inflammatory chemokine, the tumor-necrosis factor alpha (TNFα).

For a realistic exposure of cell cultures, a box was developed in which these cultures can be exposed at an air-liquid interface directly to exhaust emissions of scooters, the small two-wheelers, which are very popular nowadays. The exhaust is discharged and directed to a mass regulator, where it is diluted 1:100 to 1:1000 with absolute clean air. Before passing the cell cultures in an exposure chamber which was developed especially for exhaust exposure, the diluted exhaust emission is heated to 37°C, enriched with CO₂ to an end concentration of 5% CO₂ and humidified to a relative humidity of 80%. Directly before the entering to the exposure chamber control measurements (CO and CO₂ concentration, temperature, pressure, humidity) are conducted. On the top of the round exposure chamber, which is located in the isolated and heated box (37°C), the scooter exhaust enters with a flow between 2-10 l/min and is spread evenly over the four exposed 6-well plates. The air is sucked at the bottom of the exposure chamber and again CO₂ concentration, temperature, pressure and humidity are measured. Parallel to the exhaust exposure experiments control experiments are conducted in a reference exposure chamber, where cell cultures are exposed to absolute clean air enriched in CO₂, humidified and heated.

It is planned to expose air-liquid cultures of the epithelial airway barrier model to scooter emissions and to evaluate the toxic reactions by measuring the oxidative stress as well as the inflammatory reactions. Preliminary results of the particle characterisation and the cellular analysis of cellular reactions will be presented.
4.4 - Biopersistence of inhaled and intratracheal instilled nanoparticles in rat lungs

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Biopersistences of nanoparticles in rat lungs were determined as an indicator of pulmonary response in an inhalation and an intratracheal instillation studies.

In the inhalation study, Wistar male rats are exposed to nickel oxide (NiO) nanoparticles for 4 weeks (6hr/d). The geometric mean diameter of the particles and the daily average exposure concentration in the exposure chamber were 139 ± 12 nm and 1.0 ± 0.5 × 10^5 particles/cc, respectively. At 4 days, 1 and 3 months after the inhalation, rats were sacrificed and NiO nanoparticles deposited in the lung were determined by ICP-AES after microwave digestion. The deposited amount of NiO in the rat lungs at 4 days after the inhalation was 29 ± 4 μg. The retained particle amount in the rat lungs after the inhalation exponentially decreased and the calculated biological half time (biopersistence) was 62 days.

In the intratracheal instillation study, Wistar male rats are instilled 0.1 and 0.2 mg of the same NiO nanoparticles suspended in 0.4ml distilled water. The count median diameter of the instilled particles was 26 nm. The control group received the same volume of distilled water. Rats of both groups were sacrificed at 3days, 1week and 1, 3, 6 months after the instillation. The amount of NiO in each lung was determined in the same method in the inhalation study and the calculated biological half time were 1.5 and 2.4 months, respectively.

In this study, the biopersistences in the inhalation and the instillation study were almost the same.
4.5 - From inhalation exposure to effective dose during nanoparticle synthesis

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Nanoparticle inhalation is a major concern in the area of nanomaterials and nanotechnology safety. Assessing the potential health impacts should encompass not only the characterization of exposure levels, but also the effective internal dose by considering particle intake through lung deposition and, equally importantly, the fate of the deposited particles. We present an integrated source-to-biological dose assessment study in which all above points are combined based on real monitoring data collected in a nanoparticle synthesis setting. Experimental monitoring data of airborne exposure levels during nanoparticle synthesis in a research laboratory is coupled with a transport and deposition model considering aerosol dynamics to translate the exposure concentration to lung deposition based on the physical-chemical properties of the investigated particles. In a subsequent step the lung deposition mathematical model is coupled with a mathematical model of particle retention and clearance to provide the effective biological dose in target organs by inhalation. Specifically, this study investigates the effective dose following exposure during the production of CaSO₄ and BiPO₄ nanoparticles. During the synthesis of these compounds using the flame-spray pyrolysis technique, the airborne submicron particle number concentrations rose by more than an order of magnitude compared to background conditions and the size distributions displayed peaks between 115 and 170 nm. The lung deposition mathematical model then solves in an Eulerian framework the general dynamic equation for polydisperse aerosols. Deposition is determined throughout the whole respiratory tract. The employed modelling approach is shown to be specifically appropriate for the study of the lung deposition of nanoparticles. Thereafter, the particle retention and clearance mathematical model describes the distribution of the internalised dose in different target systems beyond the portal of entry organ, in this case the lung. Additionally the model describes the time course of the build up of the target organ dose after exposure. The model has been calibrated with data from various experimental studies. The combination of the exposure data, the deposition model and the retention and clearance model completes the description of the pathway from external exposure to internal dose. To our knowledge this is the first time that such realistic biological uptake data are provided, associated to real conditions prevailing during manufacturing of engineered nanoparticles.
4.6 - Single-walled carbon nanotubes vs. crocidolite asbestos - Immunomodulating effects on lung epithelial cells in vitro and the role of lung surfactant -

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Due to their enormous popularity and the wide range of potential applications, occupational and public exposure to single walled carbon nanotubes (SWCNT) will increase dramatically in the near future. One of the first tissues potentially coming into contact with aerosolised SWCNT is the lung, and therefore, the human lung epithelial carcinoma cell line A549 and normal human bronchial primary epithelial cells (NHBE) were chosen for this study. Cell viability and proliferation assays, A549-luciferase reporter-gene assays, ELISA and real-time RT-PCR analysis were employed in order to assess effects on a multitude of inflammatory mediators (interleukin-8, interleukin-6, prostaglandin E₂, cyclooxygenase-2, tumour necrosis factor-α (TNF-α), nuclear factor-κ-B and monocyte chemotactant protein-1). Production of reactive oxygen species was evaluated using DCF-DA. Exposures were carried out on 0.2 – 50 µg/ml of unrefined SWCNT samples suspended in cell culture medium supplemented with 10% foetal bovine serum. In parallel, particles were dispersed in dipalmitoylphosphatidylcholine (DPPC), the major component of lung lining fluid, in order to assess the role of lung surfactant on the toxicity of particles in vitro. All assay systems were tested for their compatibility with carbon nanotubes. Cells were exposed to particles alone as well as co-stimulated with known pro-inflammatory stimuli such as TNF-α and lipopolysaccharide (LPS). Due to their large aspect ratio, SWCNT have often been compared to asbestos, and therefore, crocidolite was included as a well standardised control particle. Exposure to HiPco SWCNT as well as crocidolite did not result in significant reductions of cell viability of any cell line tested. However, SWCNT exposure caused time-dependent decreases in cell proliferation as measured by ³H-labelled thymidine incorporation. SWCNT were also able to significantly decrease the production of all inflammatory mediators tested in a time- and concentration dependent manner. This was also true for cells co-stimulated with TNF-α and LPS. Particle dispersion in DPPC augmented this effect. In contrast, asbestos selectively increased cell proliferation and decrease of inflammatory responses could only be observed following DPPC dispersion. Therefore, this study shows differences between SWCNT and asbestos exposures and highlights the immunomodulating potential of SWCNT exposure in vitro.
4.7 - Acute effects of aerosol exposure of different titanium dioxide types on airway inflammation in mice

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The knowledge of the potential health effects of engineered nanomaterials and the relevant parameters of nanomaterials when assessing toxicity is still limited. We studied the effect of four different forms of titanium dioxides (TiO₂) on pulmonary inflammation.

BALB/c mice were exposed with aerosols of different size and forms of TiO₂ for two hours, two hours on four consecutive days or two hours on four consecutive days for four weeks. The commercial TiO₂ materials studied were; fine-sized (initial particle size ca. 1µm) rutile, nano-sized (ca. 30 nm) rutile, nano-sized (ca. 15 nm) anatase and silicon coated nano-sized (ca. 10x40 nm) rutile. In addition, inhalation exposure was carried out with nano-sized (ca. 10 nm) anatase TiO₂ generated in a gas-to-particle conversion machine. Aerosol properties were characterized for size distribution, number concentration, mass concentration, surface area, and for shape and composition of agglomerates. Titanium concentration of the lung tissue was analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Pulmonary inflammation was assessed by characterizing inflammatory cell infiltration from bronchoalveolar lavage (BAL) and analyzing the expression of cytokines and chemokines relevant to inflammation in the lung tissue.

Inflammatory responses were seen after aerosol exposure to rutile nano-sized titanium dioxide coated with silicon. The number of neutrophils in BAL and the expression of neutrophil attracting chemokine, CXCL5, were increased dose-dependently showing induction already after 4 days exposure. There was no evidence of pulmonary inflammation after exposure to other forms of titanium dioxide. The morphology of silicon coated TiO₂ is needle-like and differs from the spherical morphology of all the other TiO₂ particles studied. Diverse morphology of a single particle generates agglomerates with divergent properties and thus also different responses in organisms. The results demonstrate that exposure to different forms of titanium dioxide can produce differential effects of pulmonary inflammation, suggesting that the pulmonary toxicities of particles appear to correlate better with crystal structure and surface area than with particle size.

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4.8 - Particle-macrophage-interactions: Oxidative stress response and inflammatory mediators

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Macrophages (MPs) are effector cells of the innate immune system and play an important role in the phagocytosis and elimination of pathological invaders. Uptake of (nano)particles may lead to MP activation and subsequent release of inflammatory mediators like cytokines, chemokines and reactive oxygen species (ROS). However, the precise mechanisms of particle uptake and generation of inflammatory mediators by MPs are currently still poorly understood. The aim of this study is to investigate the interactions between particles and MPs and their associated inflammatory effects in relation to particle size and chemical properties. Therefore, NR8383 rat lung macrophages were exposed either to DQ12 quartz (Ø 960 nm), fine (f)TiO2 (Ø 250 nm) or ultrafine (uf)TiO2 (Ø 30 nm). Flowcytometrical analysis of cellular granularity (side scatter) revealed a concentration-dependent (10, 20, 40 µg/cm²) increase in uptake of all tested particles by NR8383 cells. For DQ12 and ufTiO2 toxicity (WST-1 assay) was observed at concentrations ≥ 20 µg/cm², whereas fTiO2 did not show any toxicity up to 80 µg/cm². In line with this, DQ12 and ufTiO2 also induced the generation of ROS (electron paramagnetic resonance) as well as an upregulation of the mRNA expression (qRT-PCR) of the stress response gene heme oxygenase-1 (HO-1). However, ufTiO2 but not DQ12 caused a marked upregulation of inducible nitric oxide synthase (iNOS) mRNA. DQ12 particles were found to trigger the release of TNF-α and IL-1β from MPs in a clear concentration-dependent manner (ELISA). The release of TNF-α after ufTiO2 treatment was only observed at the highest concentration tested (80 µg/cm²), but not of IL-1β. Neither ROS generation, induction of mRNA expression of HO-1 and iNOS nor an increased release of TNF-α, and IL-1β were found in MPs treated with fTiO2. Taken together, although different types of particles are rapidly taken up by MPs, marked differences exist in their cytotoxic effects, their ability to trigger ROS generation and the release of inflammatory cytokines, as well as to induce the mRNA expression of HO-1 and iNOS. The mechanisms of particle uptake and subsequent release of inflammatory mediators by macrophages in dependence of particle size and chemistry are currently under investigation.

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5 - Immunology
5.1 - Do novel engineered nanomaterials have hazardous effects on the immune system?

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Engineered nanomaterials (EN) from various sources are widely used in biomedical and nanotechnological applications and the list of promising nanomaterials is growing rapidly. As a consequence, the industrial production of EN will increase dramatically in the near future. This entails the potential for a widespread exposure to these novel materials during both manufacturing and use and raises concerns about their possible hazardous effects on human health and the environment.

As nanomaterials are very small in size and easily become airborne, they can enter the human body by many different routes including the lung and skin. Once deposited, nanoparticles may rapidly be dispersed via the circulation and lymphatic system. Therefore, it is likely that EN will interact with cells of the immune system with the potential to stimulate and/or suppress the immune defence against diseases. Yet, to date, very little is known about the effects of EN on the immune system.

In order to provide information about the immunotoxicity of EN, we studied the effects of some industrial scale, biopersistent EN on immune cells using several in vitro toxicity tests. Our preliminary work shows that multiwalled carbon nanotubes (MWCNT) do not induce acute T-cell death at concentrations up to 50 $\mu$g/ml. However, some measurements (ROS production, population growth) indicate that MWCNT might affect the basic functionality and immunocompetence of T-cells. In further studies we will investigate additional immunobiological endpoints as well as various types of immune cells and nanomaterials to gain a comprehensive view about the immunotoxicity of EN.
5.2 - Immunomodulatory responses triggered by mesoporous silica particles: A study on human dendritic cells

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Mesoporous materials such as mesoporous silica particles are being utilized in a variety of bio-related applications as a result of their high specific surface areas, internal pore volumes, tailorable surfaces and high chemical and thermal stabilities. There is however very little information available concerning their toxicity and what influence such particles have on the immune system, e.g. on dendritic cells (DC). DC are the most efficient type of antigen presenting cells having a capacity to initiate both primary and secondary immune responses. DC decide whether an immune response should be initiated or not and are able to affect the development of T-cells into Th1-, Th2 or Treg-cells depending on their cytokines produced and their expression of co-stimulatory molecules. We addressed the question whether mesoporous silica particles of 270 nm or 2.5 µm affect DC, looking at viability, uptake, and expression of cytokines and of co-stimulatory and antigen presenting molecules. This was assessed by using human monocyte derived DC together with various techniques including confocal microscopy, flow cytometry, ELISA and ELISpot. Experiments revealed size-and concentration dependent effects, where the smaller silica particles and lower concentrations affected DC to a lower degree compared to the larger particles and higher concentrations, both in terms of viability, uptake and immune regulatory markers. These findings support the further development of mesoporous silica particles in vaccine delivery systems and the particles are therefore now investigated for stability and have also been coated with the model allergen ovalbumin (OVA) for both in vitro and in vivo studies.
5.3 - Pulmonary effects and systemic immune function alterations following multiwalled carbon nanotube inhalation exposure

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The purpose of the following studies was to assess immune function alterations and pulmonary response to inhaled multiwalled carbon nanotubes. Male mice were exposed to atmospheres containing 0.3, 1, or 5 mg/m³ for 6 hours/day for 14 consecutive days in whole body inhalation exposure chambers. Approximately 18 hours after exposure end mice were euthanized and pulmonary endpoints were assessed along with systemic immune function analysis at the site of the spleen. Few pulmonary effects were observed, however, systemic immune function was compromised with 1mg/m³ MWCNT exposure. Splenocytes from exposed animals were less able to produce antibody in response to antigenic stimuli and exhibited decreased T cell proliferation when co-cultured with a mitogen (Concanavalin A). Furthermore, splenocytes from exposed animals had increased gene expression of Interleukin 10 (IL-10) and prostaglandin synthase enzymes, known T cell suppressors. Therefore an additional inhalation exposure was conducted in which a separate group of mice were included that received Prostaglandin Synthase 2 (PTGS2 or COX2) antagonist, ibuprofen, in their drinking water. Animals that were exposed to MWCNT atmospheres but that simultaneously received oral doses of ibuprofen exhibited significant rescue from MWCNT-induced immunosuppression indicating involvement of prostaglandins in observed immune function alterations. Future studies will include the use of COX2 (PTGS2) knockout animals in the inhalation exposure system as well as identification of upstream modulators that may be involved in activation of the prostaglandin pathway. This work was supported by NIEHS (P30 ES-012072) and EPA (RD-83252701).
5.4 - Activation of innate immunity in mice by C\textsubscript{60}(OH)\textsubscript{x}

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Many biomedical applications of fullerene and its derivatives have been proposed, however, when used into the body the immune activities of these kind of nanoparticles remain unclear. In this work, the immunomodulatory activity of C\textsubscript{60}(OH)\textsubscript{x} was investigated both \textit{in vivo} and \textit{in vitro}.

C\textsubscript{60}(OH)\textsubscript{x} (0.2, 1.0, 2.0 mg/kg b.w.) was administered intraperitoneally to Kunming mice once daily for 7 days. The carbon clearance test was employed to test the whole phagocytic clearance capacity of the reticulo-endothelial system (RES) of mouse. The results show that the phagocytic index K increases after treatment with 2.0 mg/kg b.w. C\textsubscript{60}(OH)\textsubscript{x} (p < 0.05). Meanwhile, the chicken red blood cell (CRBC) phagocytosis test is used to determine the phagocytic capacity of peritoneal macrophages. The phagocytic rate (PR) of CRBC is significantly elevated after 1.0, 2.0 mg/kg b.w. treatment (p < 0.001). And, the activity of arginase (Acp) and acid phosphatase (Arg) of the peritoneal macrophages were determined. Both of the lysosomal enzyme activities are significantly elevated (p < 0.001) by the three tested dose of C\textsubscript{60}(OH)\textsubscript{x}. These results indicate that C\textsubscript{60}(OH)\textsubscript{x} treatment significantly activates the innate immunity of mice, especially the macrophages.

Moreover, peritoneal macrophages (obtained from Brewer thioglycollate broth stimulated mice) were treated with C\textsubscript{60}(OH)\textsubscript{x} (15, 30, 60 \textmu g/ml) \textit{in vitro}. The TNF-\alpha secretion of macrophages increases significantly via the C\textsubscript{60}(OH)\textsubscript{x} treatment at all the three doses, which might further modulate the down-stream immune response.

Macrophages are the first defence cells to intercept xenobiotic nanoparticles. Activated macrophages accomplish innate immune function through phagocytosis and possess diverse functions in the regulation of immune and inflammatory processes. Optimistically, the interaction between macrophages and C\textsubscript{60}(OH)\textsubscript{x} may lead to beneficial effects (such as antitumor function, which is under investigation in our lab) superpose onto the toxicity. Further studies on the down-stream immune response need to be carried out.
5.5 - Investigation of immunomodulatory effects caused by engineered nanoparticles

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The importance of risk assessment for engineered nanoparticles (NPs) has increased. The 6th Framework EU-project DIPNA (www.dipna.eu) aims at creating and validating appropriate instruments and bioassays for the detection and evaluation of occupational nano-toxicity in order to promote prevention and nano-safety in manufacturing and handling. Our scientific objective within the DIPNA project is to define in vitro systems to characterize interactions between engineered NPs and living cells. We are specifically interested in the relationship of NP properties such as size, shape, dispersion, surface state and charge with the cellular responses after exposure. Metal and metal oxide NPs, that are currently used in the industry, pharmacy and medicine, were chemically synthesized and placed in solution to ensure that the NPs were monodispersed. These suspensions were used in a reporter gene assay using a panel of stably transfected lung epithelial (A549) and T cells (Jurkat) containing different cytokine promoter sequences linked to the luciferase gene. In addition, secretion of cytokines by A549 cells and primary human bronchial epithelial cells was analysed by ELISA and by using Multiplex beads. Cytotoxicity of the NPs and their solvents was determined and all experiments were performed in the absence and presence of a pro-inflammatory stimulus which induces cellular stress. The results show that there is a clear difference between cytotoxicity and immunotoxicity on the different cell lines, whereby the immunotoxic effects are generally stronger. In addition, the cytokine promoters tested are affected differentially and the same promoters in different cell lines also give varying results. Interestingly, the effects of the solvents are usually more pronounced than those observed for the particles, indicating that dissolved chemicals (as present in the solvents) affect cells stronger than monodispersed nanoparticles. Overall, the results show that the study of immunotoxic effects of NPs requires careful controls to avoid bystander effects.
5.6 - Maturation response of human CD34\(^+\) progenitor-derived dendritic cells exposed to engineered nanoparticles

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The EU funded project DIPNA aims to develop an integrated platform to assess the toxicity and ecotoxicity of nanoparticles (NP). VITO N.V. mainly contributes to workpackage 2 which focuses on the impact of engineered NP on human immune cells. The goal of our study was to assess the effect of NP on dendritic cell maturation.

To study the potential immune-stimulating effect of engineered NP, human primary immature CD34\(^+\) progenitor-derived dendritic cells (CD34-DC) were exposed to spherical gold (4.3 and 13 nm), iron oxide (6 nm) or cobalt (4 nm) NP for 24 and 48 hours. Mono-dispersed NP in solution and freshly resuspended nanopowders were used at the same concentration per type of NP. The maturation response of CD34-DC was measured by determination of cell surface expression of the DC surface markers HLA-DR, CD86, CD83 and CD54 using flow cytometry. Additionally, potential inhibition of cellular growth by the NP was analysed by means of the alamarBlue\textsuperscript{TM} and WST-1 assays.

Microscopically, freshly resuspended nanopowders were observed to be more likely to form aggregates, compared with NP in dispersion. No reduction of CD34-DC growth was observed in response to all NP tested at different concentrations. At the highest concentration tested, ranging from 2.4\(\times\)10\(^{11}\) to 2.0\(\times\)10\(^{13}\) NP per ml for the different particle types, neither the mono-dispersed NP, nor the freshly resuspended NP could induce maturation of CD34-DC after 24 and 48 hours of exposure, i.e. no significantly increased expression of the DC maturation markers HLA-DR, CD86, CD83 and CD54 was measured when compared to control conditions. When the cells were co-treated with the cytokines tumor necrosis factor-\(\alpha\) and interleukin-1\(\beta\) (5 ng/ml), which are known to mature CD34-DC, none of the mono-dispersed NP was able to further enhance the induced maturation response, rather they inhibited CD34-DC stimulation.

Small spherical, engineered NP were found not to be cytotoxic to CD34-DC when added either as mono-dispersed NP solution or freshly resuspended nanopowders. Furthermore, they were not able to potently trigger DC maturation. Comparison of our data with other toxicological endpoints and different NP (size, composition, surface coating, shape, …) is warranted.
6 - Genotoxicity
6.01 - Genotoxic potential of synthetic metal-oxide nanoparticles

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Synthetic nanoparticles show a broad range of usage in science, technology and medicine. Nanoparticles are produced on industrial scale and used as additives for drugs, cosmetics, printer toners, textiles, and many other applications. However, for most of the new nanomaterials little is known about biological consequences and possible adverse effects. Nanoparticles containing transition metals are expected to be especially prone to modify DNA because of their high capability to produce reactive oxygen species via Fenton reactions. DNA damage can have fatal consequences (apoptosis, carcinogenesis); therefore, knowledge about the possible genotoxic potential of nanoparticles is strongly required.

In our study, the potential of synthetic metal-oxide nanoparticles to affect DNA was assessed using a human lung epithelial cell line (A549). Production of reactive oxygen species was measured fluorimetrically by dihydrorhodamine conversion. DNA damage was analyzed by comet assay and micronucleus test.

Vanadium oxides are used e.g. as pigments and catalysts; moreover, ultrafine particles containing vanadium oxide are released into the atmosphere by the combustion of vanadium-rich petroleum. We used both bulk and nanoscaled V₂O₃ and V₂O₅. Biological effects on lung cells were varying for different vanadium oxide species. Only soluble vanadium oxides were found to be able to generate reactive oxygen species both in cells and in a cell-free system. An increased DNA damage was observed in cells treated with nanoscale V₂O₃ and bulk V₂O₅ for 36 h and 48 h, but not with bulk V₂O₃ and nanoscale V₂O₅.

Thus, bulk material and nanoparticles of the same chemical origin exert different effects on cells. Furthermore, some metal-oxide nanoparticles may possess a genotoxic potential. These findings emphasize the importance of thorough toxicity testing for nanomaterials in order to prevent threats to human health.
6.02 - DNA damage in human cells after indirect exposure to CoCr nano-particles through a placental cell barrier

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Humans are exposed to nano-sized chromium particles from cobalt chrome orthopaedic joint replacements. In previous studies we have shown that human fibroblasts show DNA and cytogenetic damage in tissue culture after exposure to CoCr particles and ions. In this novel study we have asked the question whether human cells might also show DNA damage if separated from the metal by a placental cell barrier.

We created a barrier 3-5 cells in thickness of BeWo cells (as a standard model of a placental barrier) and exposed the barrier to nanometer (29nm) and micron (3.4µm) sized CoCr particles, as well as ions of Co^{2+} and Cr^{6+} individually or in combination. We monitored DNA damage in BJ fibroblasts beneath the barrier with the alkaline gel electrophoresis comet assay and with \( \gamma \)H2AX staining.

The results showed evidence of DNA damage after all types of exposure. The indirect damage (through the barrier) was equal to the direct damage at the concentrations tested. The integrity of the barriers was checked with measurements of electrical resistance (TEER values) and permeability to sodium fluorescein (376Da) and found to be intact.

In light of these results and with the knowledge that BeWo cells express cx43, we tested the theory that a damaging signal was being relayed via gap junctions or hemi channels in the BeWo cells to the underlying fibroblasts. We used the connexin mimetic peptides Gap19 and Gap26 (known to selectively block hemichannels and gap junctions respectively) and 18\( \alpha \)-glycyrrhetinic acid (non-selective gap junction blocker). All of these compounds completely obliterated the indirect damaging effect seen in our previous experiments.

We conclude that CoCr particles can cause DNA damage through a seemingly intact barrier, and that this damage occurs via a bystander mechanism. It would be of interest to test whether other barriers (e.g. pleura, peritoneal) are ‘leaky’ to DNA damaging effects of nanoparticles and whether this is simply a tissue culture effect or could be seen \textit{in vivo}. 
6.03 - Comparison of cytotoxic and transforming effects of metal oxide nano and micro-particles

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In vivo and in vitro studies suggest that metal oxide particles in the nano-size range are more toxic than their micro-size range counterparts. It has been hypothesized that the large specific surface area of nanoparticles may enhance free radical activity at the surface of particles. The aim of this study was to evaluate and to compare the carcinogenic potential effects of metal oxide particles in nano and micro-size ranges. Anatase and rutile titanium dioxide (TiO₂) and iron oxide (Fe₂O₃ and Fe₃O₄) nano and micro-particles were studied for cytotoxicity (cell proliferation and oxidative stress), genotoxicity (micronuclei formation), and morphological transformation using Syrian hamster embryo (SHE) cells. For all particles, chemical composition, crystal structure, size distribution, surface area and free radical activity have been characterized. Particle uptake in SHE cells was assessed by transmission electronic microscopy. The first results of this study indicated that anatase and rutile TiO₂ and Fe₂O₃ nanoparticles induced a higher inhibitory effect on cell proliferation than micro-size particles of same chemical composition. In addition, nano-size forms of anatase TiO₂ and Fe₂O₃ particles caused more cellular oxidative stress than micro-size forms. However, none of titanium dioxide and iron oxide particles induced micronuclei formation or morphological transformation.
6.04 - Nanosilver toxicity detected through a toxicogenomic approach on zebrafish embryos

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Nanosilver is a well known antimicrobial compound. For this reason, it has been widely employed in the food industry, as a component of food containers, as well as a stabilizing ingredient of commercial drinking water, soft drinks and fruit juices. Although its antimicrobial effect can be highly beneficial for various industrial application, its innocuity for human health still remains to be fully demonstrated. In this work we have developed an in vitro test based on a toxicogenomic approach, to analyse the general broad spectrum effects of nanosilver on the entire genome of zebrafish embryos. We compared the traditional morphological changes on embryos development caused by contact and injection of 5 different concentrations of nanosilver (0.1 to 5 ppm) to the gene expression pattern obtained by hybridizing the treated embryos cDNA to zebrafish DNA chips. Morphological changes could be detected only at the highest concentration tested; however, gene expression changes were evident also at lower concentrations. The increased expression of genes relevant in detoxification, regulation or response against oxidative damage was confirmed by RT-PCR. Nanosilver affected gene response in a dose dependant manner.

To this date, a study on the effects of NPs exposure on the entire genome of any model organism is still missing and this hampers greatly the full understanding of the potential toxic effects that NPs could produce. Our work, besides revealing a general toxic effect of nanosilver at the concentrations tested, sets the basis for elucidating the toxic mechanisms of nanoparticles and at an ultimate level will be instrumental for developing a rapid toxicity assay for nanoparticles with possible utility in the food industry.
Zinc oxide nanoparticles (ZnO NPs) are being widely used in electronics, cosmetics and various dermatological preparations. However, there is concern amongst environment and health scientists regarding the safety of these nanoparticles. This apprehension is primarily based on the fact that at nanoscale, the reactivity of particles and ability to cross various biological barriers increases allowing them to interact with macromolecules including DNA. Although some studies have been conducted using ZnO NPs, its genotoxic potential and mechanism by which it induces genotoxicity is not well understood. Due to its use as a sunscreen ingredient and in many dermatological preparations, skin provides the major and first portal of entry. Moreover, all exogenous compounds that enter blood circulation pass through liver. This study was therefore conducted to assess the cytotoxic and genotoxic potential of ZnO NPs in human skin (A431) and liver cells (HepG2) and to understand the probable mechanism involved.

The cells were exposed to ZnO NPs concentrations ranging from 0.008 to 20 µg/ml and various endpoints of cytotoxicity including mitochondrial function, neutral red uptake and lactate dehydrogenase (LDH) release, were measured at 3, 6, 24, 48 hrs post exposure. A significant (p < 0.05) increase in cytotoxicity was observed as a function of both ZnO NP concentrations and exposure time. Our studies also demonstrate a genotoxic potential of these particles as assessed by single cell gel electrophoresis (Comet assay) in A431 cells where a statistically significant increase (p < 0.05) in OTM was observed after NPs exposure (2.33 ± 0.32) as compared to control (OTM 1.3 ± 0.12) at a concentration of 5 µg/ml. Results of genotoxicity in HepG2 cells also showed a positive response. As there are previous reports of nanoparticles eliciting toxicity through generation of reactive oxygen species, it was prudent to examine the generation of oxidative stress, if any, in ZnO NPs exposed cells. A significant depletion (p<0.05) in the glutathione level, superoxide dismutase and catalase level at 0.8 µg/ml and 0.08 µg/ml on 24 hr exposure was observed indicating oxidative stress in A431 cells. The enhancement of released LDH as well as lipid peroxide formation indicated the membrane damage on exposure to ZnO NPs.

In conclusion, our study demonstrates that exposure to ZnO NPs result in a concentration as well as time dependent cytotoxicity in human cell lines. The results further reveal that ZnO NPs possess DNA damaging potential which might be mediated through generation of reactive oxygen species.
6.06 - Copper oxide nanoparticles are highly toxic in vitro - a comparison between different nanoparticles

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Since the manufacture and use of nanoparticles are increasing in society, humans are more likely to be exposed at their work, via consumer products or via the environment. However, so far toxicity data for most manufactured nanoparticles are limited. The aim of this study was to investigate and compare different nanoparticles and nanotubes, regarding cytotoxicity, genotoxicity and oxidative stress. The study was focused on different metal oxides (CuO, TiO₂, ZnO, CuZnFe₂O₄, Fe₃O₄, Fe₂O₃), and the toxicity was compared to that of carbon nanoparticles and carbon nanotubes (multi-walled i.e MWCNT). The human lung epithelial cell line A549 was exposed to the particles and cytotoxicity was analyzed using trypan blue staining, genotoxicity and oxidative lesions were determined using the comet assay and intracellular reactive oxygen species (ROS) production was measured using the oxidation-sensitive fluorprobe 2',7'-dichlorofluorescin diacetate (DCFH-DA). The results showed that there was a high variation among different nanoparticles regarding their ability to cause toxic effects. CuO nanoparticles were most potent regarding cytotoxic and genotoxic effects. These particles also caused oxidative lesions and were the only particles showing an almost significant increase (p=0.058) in intracellular ROS. The toxic effects were nanosize specific, since micrometer-sized CuO showed much less toxicity, and were likely not explained by soluble Cu-ions. A key mechanism behind the toxicity may be the ability to damage the mitochondria, since CuO nanoparticles caused mitochondrial depolarization analyzed using the fluorprobe tetramethylrhodamine ethyl ester (TMRE). ZnO showed mainly cytotoxic effects whereas TiO₂ (a mix of rutile and anatase) particles were genotoxic. Iron oxide particles (Fe₃O₄, Fe₂O₃) showed no or low toxicity but CuZnFe₂O₄ particles were rather potent to induce DNA lesions. Finally, the carbon nanotubes showed cytotoxic effects and was the only sample that caused genotoxicity in the lowest dose tested. In conclusion, this study highlights the toxicity of CuO nanoparticles in cultured human lung cells.
6.07 - Genotoxicity evaluation of carbon nanotubes in human lymphocytes and in the RAW 264.7 cell line

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The dramatic increase in the production and use of nanomaterials is raising concerns about possible health effects, including genotoxicity. As a part of a National Research Project aimed at validating a battery of bioassays to assess the potential genotoxicity of nanomaterials, we evaluated the in vitro induction of DNA and chromosome damage in cells playing different roles during the inflammatory response. Human peripheral blood leukocytes (HL) and macrophage cell line (murine RAW 264.7) were incubated with Carbon Nanotubes (CNTs) and the genotoxic effects were assessed by means of the micronucleus test and the Comet assay. HL were treated with two different types of commercial CNTs at doses ranging from $10^{-4}$ to $10^{-9}$ g/ml: a mixture of SWCNT and MWCNT (S/MWCNT-$\Sigma$) and MWCNT alone (>95% pure, MWCNT-$\Sigma$). Both S/MWCNT-$\Sigma$ and MWCNT-$\Sigma$ did not increase the micronuclei frequency even at higher doses. The Comet assay revealed weak genotoxic effects at the DNA level induced by S/MWCNT-$\Sigma$, whereas MWCNT-$\Sigma$ showed genotoxic damage only at intermediate concentrations ($p<0.05$). Conversely, the murine cell line incubated with both S/MWCNT-$\Sigma$ and MWCNT-$\Sigma$ showed a concentration-dependent increase in micronuclei frequency. The Comet assay revealed a weak genotoxic effects induced by S/MWCNT-$\Sigma$ and a significant increase in DNA damage at the higher concentration for MWCNT-$\Sigma$ ($P<0.01$). As a whole, these data show that CNTs per se can determine in vitro chromosome and DNA damage. However, in cells characterized by phagocytic functions, the extent of damage is more relevant. Moreover, the employment of different cell types can help to a better understanding of the mechanisms underlying the potential genotoxicity of nanomaterials.

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6.08 - Bioerodible polymeric biomaterials and relative nanoformulates for the targeted and controlled delivery of bioactive agents: Analysis of the potential genotoxicity

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The preparation of nanomaterials opens new perspectives in terms of biomedical applications and several research branches are looking forward to the development of nanodevices that could act as carriers for specific drugs, proteins, peptides and genes. Biodegradable polymeric nanoparticles (NPs) based on polylactides, polyglycolides, poly(lactide-co-glycolide), polyanhydrides and polyesters have attracted considerable attention for the controlled release of drugs. However, while the number of nanoparticles (NPs) types and applications continues to increase, the knowledge on the health effects of NPs exposure and on their toxicological mechanism of action are still limited, including genotoxic ones. Hence understanding the properties of NPs and their effect on the body is crucial before biomedical use can occur. Aim of this study is to detect the potential genotoxic effects a class of polymeric biomaterials, namely 2-methoxyethanol hemiester of poly(maleic anhydride-alt-butyl vinyl ether) (VAM41) and relative NPs by means of the micronucleus and comet assay on human peripheral leukocytes (HL). According to previous data obtained on Balb/3T3 Clone A31 cell line (Chiellini F. et al., 2008) showing that cytotoxicity decreases with the reduction of molecular weight (Mw) and with the introduction of poly(ethylene glycol) (PEG) chains, preliminary comet assay experiments were performed by treating HL with three different Mw VAM41 and with the VAM41-PEG grafted form. The genotoxicity results were in agreement with the cytotoxicity data, displaying a minor DNA damage for the polymers with low Mw and for the PEG grafted form. The genotoxicity evaluation of polymeric nanoformulations and of other polymers is currently part of the ongoing research. Taken together, the results of this project will allow for the obtainment of important indications on the biocompatibility of the prepared polymers and nanoparticles for a safer and responsible use in the biomedical and pharmaceutical fields.

Chiellini F, Piras AM, Gazzarri M, Bartoli C, Ferri M, Paolini L, Chiellini E.

6.09 - Macrophages in vitro models (RAW 264.7) for the study of cytotoxic effects and genotoxicity by single (SWCNT) and multiwall carbon nanotubes (MWCNT)

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When considering nanoparticles it must be asked how man-made nanostructures can interact with or influence biological systems (Worle-Knirsch JM et al, 2006). Carbon nanotubes (CNTs) are an example of a carbon-based nanomaterial, which has won a huge spreading in nanotechnology. There are two forms of carbon nanotubes: single wall carbon nanotubes SWCNT (graphene sheets rolled up to form cylindrical tubes with a variety of structure with a well defined diameter) and multiwall carbon nanotubes MWCNT (several concentric graphene tubes with diameter of up 100 nm). The incorporation of CNTs in living systems has raised many concern because of their hydrophobicity and tendency to aggregate and accumulate into cells, organs, and tissue with dangerous effects. On the other hand, macrophages play a key role in the cellular response to particles that deposit in the lungs. Macrophages could be affected by nanoparticles in various ways that can be studied in vitro through a variety of assays. In this work cell proliferation inhibition and genotoxicity of SWCNT and MWCNTs treated RAW 264.7 (a mouse macrophages cell line) were measured respectively by counting cells after trypan blue exclusion, MTS test (for cellular metabolism evaluation) and by micronuclei, Comet and chromosomal aberrations tests. CNTs reduced cellular proliferation and determined nuclear damage as revealed by micronuclei and comet induction. These assay pointed out that CNTs induce increase of DNA damage and loss of cellular viability depending on concentration. The results presented here demonstrate that RAW model seems to be suitable for in vitro testing genotoxicity of carbon nanomaterials. The observed genotoxicity results from intracellular events since MWCNT ans SWCNT were found inside the cells by means of TEM microscopy. Moreover MWCNT and SWCNT have shown risk potential for producing detectable levels of TNF-α (an inflammation mediator) upon cells exposure to 24h ad 48h.

The exact mechanisms involved in the RAW 264.7 response to nanostructures remains to be better determined. A full understanding of the hazard of CNTs will make a major contribution to the risk assessment that is so urgently needed to ensure that products that utilize CNTs are made safely.
6.10 - Oxidative stress and DNA damage in human colon epithelial cells by engineered nanoparticles

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Inhalation and ingestion are considered to be the major exposure routes for nanoparticles. Nanosized particles in ambient air have been shown to cause inflammation in the respiratory tract and their roles in chronic inflammatory diseases as well as risk of cancer development have been discussed. The new features of engineered nanoparticles (ENP) offer many advantages to different fields of research and industry, and can for instance be applied to optimize food and food packaging. Presently, very little is known about the potential toxic effects of ENP within the gastrointestinal tract and their possible effects on inflammatory and malignant diseases, such as ulcerative colitis, Crohn’s disease and colon cancer. In the present study we have investigated the oxidative and genotoxic effects of a panel of ENP on the human colon epithelial cell line Caco-2. Cytotoxicity was analysed by two independent assays, i.e. LDH and WST-1, whereas total cellular glutathione (GSH) was determined as a marker of oxidative stress. The formamidopyrimidine glycosylase (fpg)-modified comet assay was used to investigate DNA strand breaks as well as oxidative DNA damage. To determine the potential effects of ENP in the inflamed colon, we also investigated their ability to activate human neutrophils, by using lucigenin-enhanced chemiluminescence. Our current results revealed significant DNA strand breakage and oxidative DNA damage induction by TiO₂ and ZnO in the Caco-2 cells. Among all particles tested, SiO₂ and ZnO showed the highest cytotoxicity. Most of the particles were also found to activate neutrophils. Further research is needed to unravel the responsible mechanisms and to determine their relevance in relation to actual human exposure levels to specific ENP.
6.11 - Toxicogenomic analysis of Silver nanoparticles on Japanese medaka by using cDNA microarray

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cDNA microarray can be used as a powerful and efficient tool for searching genes that respond to toxic and potentially hazardous chemicals. We have conducted isolation of ESTs from medaka liver and characterized unique 575 cDNAs, cloned after sequencing and redundancy check from more than 2000 cDNAs obtained in randomly generated ESTs. Silver nanopowder (Ag-NPow) (<200 nm) and silver nanoparticles (Ag-NPs) (~10 nm) were exposed to Medaka fish. The maximum distribution of these toxicants occurred in fish liver, therefore, hepatic gene expression analysis with 575 cDNAs was performed using cDNA microarray. The transcription profiles of fish exposed to Ag-NPs and Ag-NPow were compared with that of AgNO₃ at the equivalent concentration of silver to draw up toxicity that specifically caused by nanoparticles of silver. Furthermore, a similar approach will be adapted to assess the potential genotoxicity of carbon nanotubes (CNT) and related nanomaterials in aquatic environment.
6.12 - Genotoxicity of single and multiwall carbon nanotubes in human bronchial epithelial cells and mesothelial cells in vitro

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Although carbon nanomaterials are increasingly utilized in various industrial applications, information on the possible genotoxicity is still scanty. The genotoxic potential of nanomaterials can be mediated either through direct interaction with DNA or indirectly via oxidative stress and inflammatory responses. The aim of the present study was to examine in vitro the potential genotoxicity of two commercially available carbon nanomaterials: singlewall (SWCNT; length 1-5 µm, outer diameter <2 nm; SES Research) and multiwall (MWCNT; length 1-2 µm, outer diameter 10-30 nm; SES Research) carbon nanotubes. Genotoxicity was assessed by the analysis of DNA damage and micronuclei (MN) in human bronchial epithelial cells (BEAS 2B) and mesothelial cells (MeT 5A). The cells were cultured with various doses (5-80 µg/cm²) of SWCNTs and MWCNTs. The single cell gel electrophoresis (comet) assay was applied to study DNA damage, and the induction of MN was examined by the cytokinesis-block method. In the comet assay, the nanomaterial treatment lasted for 24 or 48 h. Our preliminary results indicate that the 24-h and 48-h treatments with SWCNTs induce DNA damage in BEAS 2B cells, with a dose-dependent effect after the 48-h treatment. With MWCNT, an increase in DNA damage was observed in BEAS 2B cells only after the 24-h treatment, but no dose-dependency was seen. In MeT 5A cells, both 24-h and 48-h treatments with SWCNTs increased DNA damage, with a significant dependence on dose in the 48-h treatment. MWCNTs produced DNA damage in MeT 5A cells both after the 24-h and 48-h treatments in a dose-dependent manner. The analysis of the micronucleus data (48-h and 72-h exposures) is presently in progress. In conclusion, our preliminary results suggest that both single and multiwall carbon nanotubes have genotoxic potential both in human epithelial and mesothelial cells in vitro. [Supported by NMP4-CT-2006-032777]
6.13 - Genotoxicity of TiO₂ in human bronchial epithelial cells in vitro

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TiO₂ nanoparticles have been reported to cause oxidative stress, inflammation, and cytotoxicity in various mammalian systems. Information on the potential genotoxicity of TiO₂ is, on the other hand, presently limited. In this study, we aimed at evaluating the potential genotoxic effects of two types of commercial TiO₂ nanoparticles on bronchial epithelial cells (BEAS 2B) in vitro. Nanosized titanium(IV) oxide rutile (>95%; crystal size 10 x 40 nm; <5% SiO₂ coating) and anatase (99.7%, <25 nm) were assayed in two separate experiments. Fine titanium(IV) oxide rutile (99.9%; particle size <5 µm, average 1 µm) was studied for comparison in both series. The cells were cultured in the presence of several doses of each TiO₂ for 24, 48, and 72 h. The alkaline Comet assay was used to evaluate DNA strand breaks and alkaline labile sites and the cytokinesis-block micronucleus (MN) assay chromosomal damage. After the 24-h treatment, a significant increase in DNA damage was seen at 80 µg/cm² of nanosized rutile and at 1, 5, and 60 µg/cm² of fine rutile. In the 48-h treatment, nanosized rutile had no effect, but fine rutile caused a dose-dependent increase in DNA damage, with a statistically significant influence at 60 and 100 µg/cm². The 72-h treatment resulted in a significant increase in DNA damage at the highest dose of nanosized rutile tested (100 µg/cm²) and at six doses of fine rutile. Generally, the fine form of rutile was more effective than the nanosized rutile in inducing DNA damage. Nanosized anatase and fine rutile induced a significant increase in DNA damage at several doses in the 24-h and 48-h treatments and at the six highest doses, with a dose-dependent effect after the 72-h treatment. In the MN assay, none of the TiO₂ samples increased the frequency of micronuclei, except for nanosized anatase at 10 and 60 µg/cm² after the 72-h treatment. At increasing doses, the MN analysis became more difficult, due to the presence of TiO₂ on the microscopic slides. In conclusion, our results showed that, in human bronchial epithelial BEAS 2B cells in vitro, both nanosized TiO₂ (rutile and anatase) and fine TiO₂ induced DNA damage, while only anatase could increase the frequency of MN. [Funded by the European Commission, NANOSH, NMP4-CT-2006-032777]
6.14 - Strategies for in vivo genotoxicity studies of nanomaterials

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Indirect mechanisms involving lung inflammation and generation of reactive oxygen and nitrogen species are considered to play an important role in particle carcinogenesis and probably also in particle genotoxicity. As many of the mechanisms operating in inflammation cannot reliably be reconstructed in vitro, substances acting via such pathways may not efficiently be detected in short term tests utilizing cultured cells. This suggests that in vivo assays could have more relevance than in vitro tests in assessing the genotoxicity of nanomaterials. Genotoxic small particles exert their primary effects on the route of entry, especially the lungs. However, the present routine in vivo genotoxicity tests have been designed to reveal reactive chemicals that have systemic genotoxic effects, covering only the bone marrow and the liver as target organs. Although the genotoxicity of some nanomaterials may be detected by such approaches, many others are expected to be missed. In vivo genotoxicity assays utilizing cells that are involved in lung regeneration, such as type II pneumocytes and Clara cells are especially interesting in this respect, and there are techniques by which genotoxic effects in such cells can be examined. However, the in vivo genotoxicity assessment of nanomaterials involves several open questions. Issues such as the association between inflammatory and genotoxic effects of nanomaterials and the possible role of nanomaterial accumulation in tissues are insufficiently understood. In general, it is not known which nanomaterial characteristics besides chemical reactivity could be important in determining their genotoxicity and carcinogenicity. It is presently also unclear, whether the possible genotoxic effects of inhaled nanomaterials could be evaluated in humans exposed to such materials, although some strategies for such studies could be envisaged. [Supported by NMP4-CT-2006-032777]
6.15 - Genotoxicity testing on nanoclays used in biopolymers for food packaging

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Plastics produced from biopolymers are of increasing commercial interest as they are manufactured from renewable resources such as agricultural crop wastes and have the potential to meet environmental and health requirements. Biopolymers that are strengthened using reinforcing nanoscale fillers (natural clay silicates and metal hydroxides), have the potential to improve packaging quality by increasing barrier properties and heat-resistance properties. However, the potential use of nanoclays in biopolymers cannot be fully appreciated yet because of lack of sufficient knowledge about the potential health effects of nanoclays. Chemicals in food contact materials may migrate into the food and thus be ingested by consumers and it is very likely that nanoparticles will also migrate. This study aims at testing different types of nanoclays that are advantageous in polymers for food packaging. This part of the study will focus on the genotoxic potential of a selected nanoclay and by applying one to two surface modifications The Salmonella/microsome assay (Ames test) will be used to investigate the potential of inducing point mutations by testing with strains TA 98 and TA 100, with and without metabolic activation. The Ames test will provide information of frameshift and base-pair mutations and on direct/indirect mutagenicity. In addition, the genotoxic potential using the comet assay will be measured as DNA strand breaks in Caco-2 cells, a human colon cancer cell line.
7 - Biological Effects and Mechanisms
7.01 - Study of the biological activity of fine SiC powders.

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Silicon carbide powders are produced and used for the elaboration of different materials such as refractory materials, tools, particle filters and many other applications. After the synthesis, powders are collected, grinded and processed. At each step of this multi-stages process, fraction of powders can be inhaled by workers, this is the reason why we have to know if particles are safe for health. So, as a precaution, industrials have decided to check the impact of dusts inhalation on health.

Several studies have been carried out to evaluate powders toxicity. The potential toxicity has been studied on lung cells, principally on alveolar macrophages. Briefly, when a particle penetrates in the alveolar space, it reacts with cells and is engulfed by macrophages. The activation, at the molecular and cellular level, is characterized by the secretion of chemical species such as cytokines, enzymes and proteins. According to these studies, various parameters influence powders toxicity such as: granulometry, specific area, impurities, crystallographic structure, nature of bonds on the surface, powders capacity to generate free radicals in biological medium.

The aim of the study is to characterize and to interpret the biological activity of fine SiC powders synthesized by different ways (Acheson process, laser pyrolysis, sol-gel). Toxicity will be tested on line cell (RAW 264.7). Some substances, characteristic of the inflammation state such as LDH, TNF-α and H₂O₂, will be assayed during phagocytosis. Thus, we wish to establish a "vector model" as a tool to determine the global toxicity of each powder. This study is expected to develop our knowledge in this field and to better understand the powder-induced cellular responses.
The German BMBF-funded project NanoCare focuses on health aspects of engineered nanoparticles, especially synthetic metal oxides. The work package „cell types and cellular reactions in vitro“ examines the toxicological effects of synthetic nanoparticles on mammalian cell lines. Main focus of these analyses is the biological impact of nanoparticles on lung cells. The human lung epithelial cell line A549 was selected as standard cell line for toxicological investigations. For the examination of the biological endpoints “cell viability”, “inflammation” and “oxidative stress” the following methods were used in order to examine the cellular reactions after exposition of cells with nanoparticle dispersions: the cell viability assays WST-1 and LDH, an ELISA-test for the proinflammatory cytokine IL-8, the DCF test for the intracellular measurement of reactive oxygen species (ROS), and Western blot analyses of the oxidative stress proteins cyclooxygenase-2 (COX-2) and hemeoxygenase-1 (HO-1). In the present study, the following nanomaterials, that have been well-characterized for their physico-chemical properties, were investigated: the reference materials TiO₂ and Carbon Black (CB14), as well as AlOOH, CeO₂ and ZrO₂.

TiO₂ and CB14 induced the loss of A549 cell viability at very high particle concentrations and stimulated the intracellular formation of ROS (CB14>>TiO₂). AlOOH, CeO₂ and ZrO₂ had no effect on these parameters. All particles except ZrO₂ induced an increased formation of IL-8 by A549 cells in the order CB14>TiO₂>CeO₂>AlOOH. Exposure of the murine macrophage cell line RAW 264.7 with TiO₂, CB14, CeO₂ and ZrO₂ resulted in an increased expression of COX-2. AlOOH did not induce COX-2 expression. Only TiO₂ and CB14 induced the expression of HO-1 in RAW 264.7 cells.

The cellular effects after submerse exposure to nanoparticles are dose and time-dependent. While a loss of cell viability only occurs at the highest particle concentrations of the applied dose range (0.1 – 100 µg/cm²), ROS formation, an increased IL-8 secretion and the induction of COX-2 and HO-1 expression were already observed at lower particle concentrations. Further investigations will be performed to clarify the correlation between particle characteristics (material, size, surface characteristics, etc.) and biological effects.
7.03 - Toxicity and imaging of multi-walled carbon nanotubes

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Multi-walled carbon nanotubes (MWNT) have been proposed for many applications. However, exposure to these nanotubes has generated health concerns due to their physical similarity to asbestos fibres. As numerous published studies have yielded contradictory results on their toxicity, an ideal approach is to combine a cell viability assay with nanometer scale imaging to elucidate the detailed physiological and structural effects of cellular exposure to nanoparticles. In our studies, we used bright and dark-field transmission electron microscopy (TEM), confocal microscopy, and cell viability assays to understand the interactions between the nanotubes and cells.

Human macrophage cells were treated with MWNTs for 1, 2, or 4 days with 20 µg/ml as the highest concentration. Using the TEM, we found nanotubes traversing the plasma membrane into the cytoplasm and nucleus via both active and passive transport. We verified these nanoparticle spatial distributions with the confocal microscope, where cells were imaged alive and three-dimensional images recreated. Cellular toxicity was further assessed with the neutral red, MTT, and live dead assays. Although the MTT assay tended to inflate cytotoxic responses, we observed a time and dose dependent cytotoxic response for all assays; the greatest toxicity was ~30% cell death. Furthermore, as various studies suggested that the toxicity was due to the residual iron catalyst used to produce the nanotubes, we applied the cell viability assays to the MWNTs as produced (unpurified) and MWNTs purified via heat treatment. We found that the cytotoxic response in the MWNTs was from the nanotubes themselves and not the iron catalyst.
7.04 - Particle clearance – indispensable, but a serious barrier for advanced pulmonary drug delivery

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The human body has developed sophisticated systems to protect the fragile pulmonary epithelial structures against inhaled particles and to inhibit their translocation into the blood stream. Even if the two major clearance mechanisms in the lung - macrophage mediated clearance and mucociliary clearance - are very effective, they represent a real obstacle for the therapeutic application of advanced aerosols. From a pharmaceutical point of view it would be very desirable to circumvent these pulmonary clearance mechanisms to achieve longer resident time and consequently retarded drug release of pharmaceutical aerosol formulations. With the aid of an embryonic chicken trachea model it is possible to investigate the clearance velocity of particles. Different sized fluorescent stained polystyrene particles were applied with a microsprayer on excised embryonic chicken trachea. A high speed fluorescence microscope was used to visualize and quantify the transport rate of the deposited particles. The same particles were incubated with immortalized mouse alveolar macrophages to investigate also the macrophage clearance. In contrast to the mucociliary clearance, the macrophages showed a size selective clearance rate. Macrophages expressed an increased absorption rate for small particles (200nm) in comparision to microparticles (1µm) of the same material. Furthermore carboxylated polystyrene nanoparticles are underlying more the macrophage clearance than plain nanoparticles of the same size. In case of carboxylated particles 70.5% ± 22.1% of all macrophages are participating on the uptake of particles with a diameter of 200nm, however only 10.5% ± 6.7% of the macrophages are involved in the uptake of 1µm particles. Not only the number of macrophages involved in the clearance of particles is dependent on the particles size and surface but also the total amount of particles taken up. As expected small plain nanoparticles (200nm) are incorporated in higher numbers (7.68 ± 1.88) in the macrophages than particles with a diameter of 1µm (1.25 ± 0.84). The prefered uptake of carboxylated particles was confirmed also regarding the number of incorporated particles. Three times higher numbers of microparticles were phagocytosed if the particles carried carboxyl groups on their surface. Beside size and zeta potential also other particle properties like lipophilicity, surface roughness, and rigidity could be important for the clearance of particles from the human lung. Knowledge about the interactions between particles and the pulmonary clearance systems is not only necessary to understand the toxicity of particulate matter but also to design drug carriers for advanced pulmonary drug delivery.
7.05 - Uptake and transport of nanoparticles in cells: Mechanism and toxicological aspects

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New synthetic nanoparticles show a broad range of usage. In contrast to the growing knowledge about the application of nanotechnological products, little information exists about uptake mechanisms and biological effects.

The incorporation of particles via the lung presupposes their uptake mainly by epithelial cells or phagocytes. This process is strongly dependent on the size and the surface characteristics of the nanoparticles. In any case, nanoparticles have various possibilities to find their way across cell membranes. Different options exist for this transport: phagocytosis, endocytosis via caveolae or clathrin-coated pits or macropinocytosis. To date, most of the mechanistic aspects of nanoparticle-uptake into cells are still unclear.

We investigated the uptake and toxicological aspects of synthetic silica nanoparticles and different metal oxides. Analyses were carried out in different cell lines using fluorescence microscopy, FACS and transmission electron microscopy. Acute toxic effects were studied by cell viability assays.

For the silica nanoparticles, no acute toxic effect could be observed. We could demonstrate that silica particles were taken up into cells and localized in the cytoplasm, partly in agglomerates, but never in the nucleus. TEM analyses confirmed cellular uptake of CeO₂, TiO₂ and silica nanoparticles. FACS analyses demonstrated a dose and time-dependent uptake of different sized nanoparticles. The mechanism of uptake seems to be energy-dependent, as different inhibitors of phagocytic- and endocytic pathways showed an effect on uptake of the nanoparticles. A size dependent choice of phagocytic-receptors can be assumed. Furthermore, uptake studies at 4°C or after dinitrophenol treatment showed a decrease in particle ingestion up to 90%.

These results provide experimental evidence that synthetic silica nanoparticles are taken up into cells in a dose- and time-dependent manner and that the mechanism of uptake in most cases is energy-dependent. The observed effects seem to be size and cell-line dependent. These studies suggest the importance to investigate the uptake mechanisms in different cell lines and for different types of nanoparticles, regarding different sizes and material properties as well as the biological responses of cells to these particles.
7.06 - Subtoxical concentrations of single walled carbon nanotubes (SWCNT) affect cell adhesion of epithelial cells (A549)

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Single walled carbon nanotubes (SWCNT) possess unique physical and chemical properties and therefore these materials were already used in various industrial applications, such as composites, electronics and in the medical field. The epithelial cells (A549) were chosen as a very simplified air wall system and provide the best possible comparison to existing data. In this study it was demonstrated that SWCNT raw material (SWCNT rm) and purified SWCNT (SWCNT bundles) at subtoxic concentrations affected cell adhesion. Prior the measurements of cell adhesion, the influence of SWCNT bundles and SWCNT rm on cell proliferation and apoptosis/necrosis had been measured in order to evaluate the subtoxic concentrations.

Under in vitro conditions transmembrane proteins like integrins bind to the RGD-sequence of proteins, such as collagens, which are adsorbed to the substrate. Dissolved carbon nanotubes in the culture medium could probably possess similar as collagen fibres and can probably bind to integrins. Cell adherence of epithelial cells (A549) was estimated by measuring the centrifugal force necessary to detach the cells from the surface. Cell adherence decreased in presence of SWCNT’s. The effects were dependent from the purity of SWCNT (bundles or rm). SWCNT rm affected adherence of epithelial cells in a higher manner than the SWCNT bundles did, and lead to a higher decrease of cell adherence. Cell adherence decreased with increasing concentration of SWCNT and with increasing incubation time in presence of SWCNT.

It’s known that the RGD-sequences of peptides were binding to transmembrane proteins (integrins) and lead to a weaker attachment of the cells to the surface. A549 epithelial cells, which were preincubated in presence of a small peptide, consisting of 7 amino acids including a RGD-sequence, showed a weaker adherence to the substrate, than the control cells, which had been preincubated in absence of the small peptide. Thus, SWCNT affected cell adhesion in a similar way as the small peptide with the RGD-sequence did.

Decrease of adherence of epithelial cells (A549) grown in presence of dissolved SWCNT might occur long before other cell parameters, such as cell proliferation, cell spreading, assembly of stress fibres, apoptosis/necrosis and cell migration were severely affected. Therefore cell adhesion can be used as parameter to estimate cytotoxicological effects in a subtoxical level.
The unique physical, chemical and optical properties of carbon nanotubes (CNTs) suggest enormous potential for many areas of research and application. The increasing use of CNTs in consumer products and medical applications underlines the importance of understanding their potential toxic effects on human health and the environment. As there is only little knowledge about possible neurotoxic effects of CNTs we studied the effects of single-walled CNTs (SWCNTs) with different degrees of agglomeration on primary mixed neuron-gliala cultures. These cells were isolated from embryonic chicken ventral spinal cord or dorsal root ganglia (DRG) allowing us to differentiate between effects on central nervous system neurons or sensory neurons, respectively. We found that SWCNTs significantly reduce the total DNA content of the mixed cultures independent of cell origin. However more agglomerated SWCNT had a more pronounced effect than SWCNT-bundles at identical concentrations. To assess the contribution of different cell types to the observed DNA reduction we used a neuron- and glia-specific ELISA, respectively as well as purified glial cultures and found that glial cells are attacked in the first place. Electrophysiological properties of the neurons were assessed by whole-cell patch-clamp recordings. While we could not detect any influence on spinal cord derived neurons, SWCNTs negatively affected the resting membrane potential as well as the ionic conductance of DRG neurons. Additionally their cell surface was reduced indicating a negative effect on cellular growth. These data suggest that SWCNTs can have an acute adverse effect on glial cells as well as on certain neuronal subtypes.
Although multiwalled carbon nanotubes (MWCNT) are increasingly used, their effects on biological systems are only partially characterized. However, experimental evidence suggests that MWCNT are hazardous materials, with the respiratory system being the main target following exposure. We have recently found that the exposure to MWCNT, but not to amorphous carbon nanoparticles (Carbon Black), impairs the barrier function of differentiated monolayers of Calu-3 human airway epithelial cells, as demonstrated by decreased trans-epithelial electrical resistance and increased permeability to the paracellular solute mannitol. These changes do not appear to involve gross alterations in monolayer viability, as assessed with the fluorimetric dye resazurin. To characterize the interaction of commercial MWCNT with human airway epithelial cells, we carried out a morpho-functional study using confocal microscopy. Cells were grown on permeable filters and tight monolayers were exposed for up to 8 days to MWCNT (100 μg/ml), added on the apical side. When suspended in the culture medium, the nanomaterials formed aggregates on the monolayer surface which were visualized through the detection of reflected light at long wavelengths. Live cells were visualized in situ through calcein loading, while dead cells were detected from the positivity to propidium iodide (PI). The results indicated that some cells had already adhered to MWCNT aggregates after 24h of exposure. Even at this early time, several MWCNT-adherent cells were PI-positive, indicating that the contact with the nanomaterials triggered significant cytotoxicity, while the rest of monolayer was roughly normal. With longer exposures, epithelial cells almost covered the nanofibers but the number of PI-positive cells increased, suggesting a role for bio-persistence in MWCNT cytotoxicity. However, the tight junction protein occludin was detectable also in cells adherent to MWCNT aggregates. In conclusion, MWCNT-exposed airway epithelial cells adhere to MWCNT bundles and tend to cover them, including the nanomaterials in the monolayer, but exhibit overt signs of cytotoxicity that are restricted to MWCNT-adherent cells. These results are consistent with alterations in the airway barrier function referable to MWCNT exposure.

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7.09 - Placenta perfusion system: A human ex vivo model system to study the maternal – fetal barrier capacity for nanosized materials

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Background The recent research dealing with potential health hazards of nanosized materials has focused on cells and tissue barriers like air / blood, skin and brain / blood barrier. One important barrier was not yet took into consideration: the human placenta which is responsible for the nutrients, gas and waste exchange between fetal and maternal blood. Because of the unique anatomy of the human placenta no equivalent animal system is available. The ex vivo dual perfusion system can address therefore the questions of pharmacokinetics of endogenous substances, xenobiotics and nanoparticles in maternal/fetal circulation.

Objective is to establish and validate the placenta perfusion system for studying the translocation, accumulation and potential modification of nanoparticles during perfusion.

Methods Fluorescent polystyrene beads of 50-500 nm were perfused during 6h in dual perfusion system.

Results Beads of 50 nm were able to cross the placenta tissue within 3h whereas beads of 500 nm were mostly retained in the placenta tissue. During the experiment no reduction of the placenta-tissue viability (measured by glucose/lactate, hCG, leptin) or of the permeability (measured by 14[C]antipyrine transfer) were observed in both cases.

Conclusion First preliminary experiments address the translocation of polystyrene beads through the placenta. So far a translocation was observed without reducing the viability of the placenta. The pharmacological, toxicological as well as therapeutical impact of the results will be discussed.
7.10 - *In vitro* clonogenic assay to compare carbon nanoparticle toxicity

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Carbon nanoparticles are relatively inert and thus comparing cellular toxicity of a range of nanoparticles is often difficult using well established *in vitro* cytotoxicity assays like e.g. the MTT-test. At high dose the presence of particles in the culture may interfere with e.g. photometric assays. We are developing a clonogenic assay where we can expose an endothelial cell line to a variety of carbon based nanoparticles presented to the cultures as stable suspensions or total particulates. Cells are seeded in standard cell culture dishes and particles can be added before cell seeding, simultaneously or later. After appropriate exposure the cultures are stained with Giemsa and analyzed. A number of indicators can easily be recorded, e.g. number and size of colonies, number of cells in a colony as well as other morphological characteristics. The test will differentiate between particles like single wall carbon nanotubes (SWCNT), carbon black and even different batches of chemically equivalent carbon particles. Plating efficiency was reduced by 30-90% of control after dosing of 50 µg/ml particles in suspension and a dose-response have been demonstrated. The test is easy to perform and images can be preserved for further analysis or reference.
7.11 - Platelet adhesion and fibrinogen deposition in murine microvessels upon both intraarterial administration and inhalation of nanosized carbon particles

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Background: The translocation of nanoparticles in the lung toward effector organs via the circulation is considered an important direct pathway for systemic effects of nanoparticles after inhalation. Recently, we have reported that a moderate dose of systemically administered nanosized carbon black particles (10^7 particles, 60 % < 100 nm, in buffer + 0.15 % HSA as bolus over 5 min) exerted thrombogenic effects in healthy mice, i.e., GPIIb/IIIa-mediated platelet adhesion and fibrinogen deposition on the endothelium of hepatic microvessels, whereas leukocyte-endothelial cell interactions or endothelial P-selectin expression were not induced. In the present study, we addressed the question of whether similar effects were also evoked upon inhalation of the particles.

Methods and Results: 2 h and 8 h upon a 24-h exposure to either clean air or to spark discharge generated carbon nanoparticles (CNP) with a count median diameter of 45 nm at a number concentration of 10^7 cm^-3, in vivo fluorescence microscopy of the hepatic microcirculation was performed in anesthetized C57Bl/6 mice (n = 6 each). Whereas leukocyte-endothelial cell interactions (numbers of rolling and firmly adherent leukocytes) as well as sinusoidal perfusion did not differ between controls and CNP-exposed mice, the numbers of platelets adherent in hepatic microvessels were significantly increased in CNP-exposed mice as compared to controls at both 2 h (p < 0.05 in arterioles) and 8 h (p < 0.05 in venules) after exposure. Interestingly, fibrinogen deposition was detected by immunohistochemistry in both hepatic and cardiac microvessels from CNP-exposed but not in those from control mice.

Conclusion: Taken together, the findings of these two studies suggest that exposure to moderate doses of nanosized carbon black particles exerts thrombogenic effects in the microcirculation of healthy mice independent of the route of administration, i.e., inhalation or intraarterial administration.
7.12 - Intracellular localisation and cellular response in an epithelial airway model after exposure to fluorescent and magnetic hybrid nanoparticles

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The design of core-shell nanoparticles has attracted increasing interest, since it allows combining different characteristics of the components and surface modifications. Nanoparticles which show simultaneously fluorescent and magnetic features are of particular interest for pharmaceutical and biomedical applications. However, little is known about their potentially adverse health effects in humans and we have started to investigate potential effects on cells using the polymer coated iron-platinum nanoparticles with ATTO 590 dye embedded in the polymer shell (FePt-PMA-ATTO 2\%).

Using an \textit{in vitro} model of the epithelial airway wall consisting of epithelial cells, macrophages and dendritic cells we studied the cell-particle interactions and the potential of the particles to induce an inflammatory response by interleukin-8 determination with ELISA. The intracellular localization of these particles within the three cell types was investigated by laser scanning microscopy and transmission electron microscopy. We exposed the cell cultures to different particle concentrations (control, 1.5x10\textsuperscript{12}, 7.5x10\textsuperscript{12}, 1.5x10\textsuperscript{13} particles/mL) for 24 hours.

The fluorescence of the FePt-PMA-ATTO 2\% - nanoparticles was visualized by laser scanning microscopy and the electron dense core with transmission electron microscopy. With both microscopy methods we found particles intracellular in all three cell types, i.e. in epithelial cells, macrophages and dendritic cells. First experiments showed that there is a dose-dependent increase of interleukin-8 in the supernatants.

We have shown that FePt-PMA-ATTO 2\% - nanoparticles enter different cell types and that they have the potential to induce an inflammatory reaction at high concentrations. Functional aspects and the potentially adverse effects of nanoparticles will be studied in more detail by using the same nanoparticle type with modified surfaces.
7.13 - Interaction of carbon nanotubes with macrophages: chemical imaging by synchrotron X-ray fluorescence microscopy

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Carbon nanotubes (CNT) are cylinders with diameters in the nanoscale range, possessing unique properties that make them candidates to promising applications. The forecast increase of CNT use in manufacture, which will lead to an increase of human exposure, highlights the urgent need for a better understanding of the potential human health impact of CNT. Since the respiratory tract could be the main route of exposure to these volatile materials, studies of the effects of CNT on cells involved in the immune respiratory response—murin macrophages in our study—are of special relevance.

Characterization of CNT interaction with cells is a difficult issue since CNT, and single-walled CNT in particular, are difficult to visualize in biological environments. Therefore, an alternative method to electron microscopy imaging or near-infra red fluorescence imaging is proposed here using residual iron catalyst bound to CNT: synchrotron-based X-ray fluorescence microscopy (microXRF). We will show that it allows studying CNT cellular localization and beyond, CNT-cell interactions.

The analysis of the fluorescence signal of iron catalyst nanoparticles allowed detecting the incorporation of both multiwalled carbon nanotubes (MWNT) and single-walled carbon nanotubes (SWNT) inside cells. CNT (i.e. purified SWCNT) with less than 2wt% of remaining iron catalyst particles could thus be detected due to the high sensitivity of microXRF. A dose-response effect was measured for the cellular iron signal in CNT-exposed cells (100 vs 10 µg/mL).

Chemical element imaging by microXRF makes it also possible to investigate concomitant intracellular chemical modifications due to CNT exposure. Elemental mapping of calcium showed increased concentrations of calcium in some of the cells exposed to CNT, especially those with the higher iron content (MWCNT and Raw-SWCNT) as compared to non-exposed macrophages. Furthermore, complementary pharmacological assays with calcium antagonists (chelator and inhibitor) have demonstrated that calcium plays a key role in the cytotoxicity and inflammation both induced by the CNT exposure.

These results show for the first time that, in the field of chemical imaging, microXRF can be a very promising tool for toxicological investigations of non-labelzied and non-coated CNT interactions with cells. They allow us to demonstrate the role of calcium in CNT cytotoxicity.
7.14 - Cobalt nanoparticles: evaluation of possible effects on gene expression and oxidative protein modification

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Magnetic nanoparticles (NPs), such as cobalt-based NPs, have shown great potential for applications not only in catalysis and magnetic recording, but also in medical sensors and biomedicine, as contrast enhancement agents for magnetic resonance imaging (MRI) or site-specific drug delivery systems for cancer therapies. The same characteristics that make cobalt-based NPs so attractive, also raise serious questions about their safety, underlining the urgent need to understand their biological effects and potential toxicity. In this contest, we have focused our interest on the study of cobalt (Co) and cobalt oxide (Co3O4) NP toxicity.

As it concerns Co-NPs, we worked on a research project in collaboration with ECVAM (European Commission for the Validation of Alternative Methods). Differences in the mRNA expression of BALB/3T3 fibroblasts exposed or not exposed for 72 h to 1 µM of cobalt microparticles, NPs, and ions were first evaluated using differential display technique and then confirmed by real time PCR. With this approach, we identified six transcripts that were modified by the treatment; these genes represent good candidate biomarkers to evaluate Co-NP exposure. Moreover, our results are consistent with the possibility that Co-NPs, due to their large surface area, once inside the cell release cobalt ions acting as a permanent source.

Since we are interested in determining the potential interaction of NPs with human cells and tissues, two human cell lines (HEPG-2 and ECV-304) were used to study Co3O4 NP toxicity. CoCl2 was used to compare the effects of ions and NPs. The effects of the two tested compounds on cellular viability were first evaluated using the MTT assay. Our results showed that both Co3O4 NPs and CoCl2 caused a dose-dependent reduction of MTT metabolism after 72 h of exposure. Cellular viability will also be determined by measurement of lactate dehydrogenase (LDH) release into the extracellular fluid as an indicator of cell membrane integrity.

Another objective of our study is to determine whether the exposure to Co3O4 NPs results in a potential oxidative damage. For this purpose, we have analysed some biomarkers of oxidative stress, such as changes in the glutathione/glutathione disulphide (GSH/GSSG) redox couple, S-glutathionylated and carbonylated proteins. Our preliminary results showed an increased in S-glutathionylated proteins, but not in carbonylated ones, in both Co3O4 NP and CoCl2 samples.

Believing that the characterisation of NP physical-chemical properties is relevant for the study of their biological activity, Co3O4 NPs have been analysed by atomic force microscopy and transmission electron microscopy (TEM) in order to define their aggregation, size, shape and surface texture, which may have significant influence on Co3O4 NP biological effects. TEM have also been used to evaluate the cellular uptake of NPs with the aim to obtain new insights regarding the mechanisms of entry and distribution into the cellular environment.

Moreover, to clarify if the toxic effects observed are due to a potential dissolution of NPs prior to cellular uptake, spontaneous dissolution of Co3O4 NPs has been evaluated by atomic adsorption spectroscopy. Our preliminary experiments indicated that, after 24 h, cobalt ion release was about 1‰ for all the tested concentrations (87.5 to 350 mg/L of Cobalt).
7.15 - Size dependent cytotoxicity and inhibition of differentiation of mouse embryonic stem cells by well-characterized SiO2 nanoparticles.

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As applications of nanotechnology emerge, it is anticipated that systemic exposure of humans to engineered nanoparticles will increase proportionately in time. Their distribution to and persistence in different body compartments is largely unknown. It has been demonstrated that certain nanoparticles cross the blood-brain and blood-testis barriers. Published data demonstrating the ability of nanoparticles to cross the blood-placenta barrier are scarce and contradictory. Increased systemic exposure combined with unknown exposure and persistence of nanoparticles in the uterus and placenta raise concerns over the potential risk for the developing embryo. Few data are available to support whether nanoparticles are capable of interfering with differentiating tissues, such as those present in a developing embryo.

The stem cell differentiation test is an in vitro screening assay used to investigate the embryotoxic potential of chemicals. The embryotoxic potential is determined by a chemical’s ability to inhibit differentiation of embryonic stem cells into cardiac myocytes and is quantified by microscopic observation of contracting cells. To our knowledge, this assay has not previously been applied to test nanoparticles.

The embryotoxic potential of well-characterized, amorphous SiO₂ nanoparticles of three sizes (nominal diameters 30, 80 and 400nm) in the stem cell differentiation test was investigated. Mouse embryonic stem cells were exposed to three sizes of nanoparticles at concentrations from 1 to 100μg/mL. Particles of nominal size 30nm showed a dose dependent inhibition of differentiation, while the two larger particles showed no effect. The inhibition of differentiation by the nominally 30nm nanoparticles was observed at near cytotoxic concentrations, as determined with the WST-1 cytotoxicity assay.

Amorphous SiO2 nanoparticles are generally considered non-toxic and have been demonstrated to have low cytotoxicity in many cell lines. They are used for a wide number of applications, potentially resulting in a relatively high systemic exposure. The observation of cytotoxicity and inhibition of differentiation of stem cells by such widely used particles warrants further investigation into the mechanism underlying these effects and the potential of these nanoparticles to migrate into the uterus, placenta and embryo.

placenta and embryo/fetus
7.16 - Completely manufactured fullerenic nanoparticles take classic processes across cellular membrane and the intracellular location

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Manufactured fullerene nanoparticles easily enter into cells and hence have rapidly developed for biomedical uses. However, it is generally unknown which route the nanoparticles undergo when crossing cell membranes and where they localize to the intracellular compartments. Herein we have used both microscopic imaging and biological techniques to determine the processes of \([C_{60}(C(COOH)_{2})_2]_n\) nanoparticles across cellular membrane and their intracellular translocation in 3T3 L1 and RH-35 living cells. \([C_{60}(C(COOH)_{2})_2]_n\) tends to aggregate in aqueous surroundings, and forms nanoscale \([C_{60}(C(COOH)_{2})_2]_n\) particles with average diameters of 125 nm as determined by AFM and DLS. The fullerene nanoparticles are quickly internalized by the cells and then routed to the cytoplasm with punctuated localization. Co-localization of FITC-C60(C(COOH)2)2 in Lyso Tracker Red and Mito Tracker Red punctate identified [C60(C(COOH)2)2]n nanoparticles in cellular localization determined by LCSM. The nanoparticles entering cells are mainly via endocytosis with time-, temperature- and energy-dependent manners. When the \([C_{60}(C(COOH)_{2})_2]_n\) at the cell surface attach to the membrane via some receptor molecules, this area of the plasma membrane with the clathrin and other coat proteins forming the endosomal vesicle in the cytoplasm. So, the cellular membrane leave over many obvious invagination structures, whose size is comparable with the size of the \([C_{60}(C(COOH)_{2})_2]_n\) nanoparticles. The integrity of cell membranes during this process remains intact. In addition, these invagination structures on the membrane are the result of endocytosing foreign nanoparticles by cells. Cellular lysosomes from the cell cytoplasm travel to endosomal vesicles containing \([C_{60}(C(COOH)_{2})_2]_n\) nanoparticles and fuse with them to form phagolysosomal vesicles. Depletion of potassium or hypertonicity, which is known to inhibit clathrin-mediated endocytosis, efficiently restrains the uptake of \([C_{60}(C(COOH)_{2})_2]_n\) nanoparticles. Therefore, the cellular uptake of the \([C_{60}(C(COOH)_{2})_2]_n\) nanoparticles was found to be clathrin-mediated but not caveolae-mediated endocytosis. The endocytosis mechanism and the subcellular target location provide key information for understanding and predicting the biomedical function of fullerene nanoparticles inside cells. Evidence of lysosomal distribution and oxidative stress response after fullerene nanoparticles endocytosis points to a need for basic research on their interactions with subcellular structure in the future.
7.17 - Inhaled Toxicity and Neurotoxicity of Copper Nanoparticles

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With the rapid developments of nanotechnology and its extensive applications, manufactured nanomaterials are increasing and coming into our daily life in great quantity. Nanomaterials have some properties which are different from their micron ones, for instance, the very small size, very huge surface area, high chemical reactivity, quantum effects, etc.. These properties may change the interaction with biological systems by more efficient approaches compared with micron ones even they having the same chemical components. The purpose of this study is to evaluate the potential health impact on mice after nasal instillation of nano-sized copper particles (23.5nm, hereinafter refer to as “nano-copper”) and discuss the possible mechanism of neurotoxicity that caused by nano-copper. The distribution results showed that Cu was mainly accumulated in liver, kidney and olfactory bulb, which were the target organs of nano-copper particles by nasal instillation. The pathological examination showed that there was serious swelling in the renal glomerulus and obvious hydropic degeneration around the central vein. The olfactory cells had severe lesions and the quantity of olfactory cells decreased obviously, and the samdwiches of olfactory were dilapidated. Immunochemical features results demonstrated that GFAP protein expression numbers were significant increased in the experimental groups, especially in nano-groups. The TEM observed displayed there were chromatin aggregation in the karyon and histiocyte damnify. The micro-distributions of nano- and micro- copper in the olfactory bulb and cerebrum of mice after nasal inhalation were investigated by microbeam SRXRF mapping techniques. The results showed that nano-copper particles could be translocated to the olfactory bulb through the olfactory nerve system after inhalation. The mechanism of neurotoxicity of nano-copper by nasal instillation needs further investigation. But more attention should be paid on the potential toxicity induced by nano-copper.
7.18 - Engineered titanium dioxide nanoparticle bioactivity and toxicity are dependent on particles length and shape

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While the application and benefits of manufacturing nanoparticles is highly promising, their adverse effects have not been fully investigated. For these studies, long TiO2 nanowires (anatase, diameter=80 nm, length=20-25 μm) were synthesized using sol-gel process directed by a porous anodic aluminum oxide template. Short TiO2 nanowires (< 5 μm) and TiO2 nanospheres, both with the same 80 nm diameter, were used as comparison particles. We conducted in vitro studies using alveolar macrophages (AM) isolated from both C57Bl/6 mice assessing toxicity with 4 hr suspension incubations by trypan blue exclusion and apoptosis using Cell Death ELISA. TiO2 nanospheres and short nanowires caused no toxicity or apoptosis up to 200 μg/ml. In contrast, long TiO2 nanowires caused significant and marked dose-dependent toxicity and increase in apoptosis. Furthermore, the long TiO2 nanowires, but not nanospheres or short nanowires increased alveolar macrophage antigen presenting activity (using ovalbumin and T cells from OT-II mice), similar to crystalline silica. Using MARCO null mice AM, it was determined that the MARCO receptor is critical for TiO2 nanosphere binding, but not involved in either nanowire binding. The mechanism of toxicity for the long nanowires is hypothesized to be excessive lipid peroxidation on the cell membrane, which we have imaged using BODIPY 581/591 C11 lipid probe. For in vivo studies we exposed C57Bl/6 mice by pharyngeal aspiration to the same TiO2 nanoparticles (0-80 μg/mouse) and examined lung and brain responses at one-day and seven-day post-exposure. Examining the lung lavage fluid, exposure to long TiO2 nanowires induced dose-dependent increases in pulmonary inflammation (PMN influx, protein increase, and LDH release) in addition to increased expression of the inflammatory mediators TNF-α (1.8- to 5-fold), MIP-2 (4- to 33-fold) and CCL2 (7- to 30-fold). In the brain, pulmonary exposure to long TiO2 nanowires induced expression of the endothelial cell adhesion molecule E-selectin in olfactory bulb (4-6 fold), suggestive of altered blood brain barrier permeability. Unlike the dose-dependent pulmonary effects, the neural responses were elicited only by higher doses of the long nanowires. Both the in vitro and in vivo data indicate that the relative bioactivity/toxicity of TiO2 anatase nanoparticles is nanospheres < short nanowires < long nanowires. Taken together, the data suggest that exposure to long TiO2 nanowires may result in adverse health outcomes. Supported in part by grants ES-015497 and RR-017670 (A. Holian, PI).
Regulation of antioxidative responses by engineered and combustion-derived nanoparticles in lung cells

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Inhalation of engineered nanoparticles may induce similar responses in the lung as combustion derived nanoparticles, which have been shown by epidemiologic studies to cause adverse health effects. However, the mechanisms relevant for the biological effects of both types of nanoparticles are still insufficiently known.

Here we compare the effects of engineered nanoparticles (Carbon Black) with those of fly ash particles. As target cells we used human lung epithelial cell lines BEAS-2B and A549 as well as mouse macrophages RAW264.7 and peripheral blood monocyte derived macrophages (MDM). Biological endpoints were viability, generation of reactive oxygen species (ROS), induction of oxidative stress (glutathione status), and regulation of anti-oxidant enzymes on mRNA and protein levels.

Fly ash and nano-Carbon Black (nano-CB) induced the formation of intracellular ROS as measured by oxidation of the fluorescent dye H$_2$DCF. However, only fly ash exposure resulted in an increase of the cellular glutathione level after 24 hours while nano-CB exposure resulted in a decrease. Fly ash exposure also increased nuclear accumulation of the transcription factor Nrf2, a key regulator of genes encoding anti-oxidant enzymes. Indeed, expression of heme oxygenase-1 (HO-1) and $\gamma$-glutamate-cysteine ligase ($\gamma$GCL) was enhanced by fly ash. Only the insoluble but not the water-soluble fraction of the fly ash increased the levels of ROS, glutathione and HO-1 indicating a pivotal role of particulate matter in these responses. Another signalling cascade triggered by ROS is the mitogen activated protein kinase (MAPK) pathway. Fly ash induced phosphorylation of the MAPKs ERK1/2 and JNK. Treatment with the antioxidant N-acetyl cysteine (NAC) prevented fly ash- induced activation of Nrf2, MAPKs and downstream target gene activation. Surprisingly, although nano-CB increases ROS levels it did not induce HO-1.

These data demonstrate that the generation of intracellular ROS by nanoparticle exposure may differentially regulate cellular responses such as changes in glutathione levels and expression of anti-oxidant enzymes. The reasons for these differences are not yet known. We suppose that in the case of fly ash a combination of different metals may trigger the observed induction of oxidative stress and anti-oxidative responses.
7.20 - Biodistribution and toxicity of systemically-introduced nanoscale ceria

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Objective: To characterize in the rat the distribution and toxicity of nanoscale ceria that had entered blood. Ceria was chosen as a model insoluble, stable, metal oxide tracer with extensive engineered nanomaterial (ENM) applications.

Methods: A 5% crystalline ceria dispersion with a mean particle size ~ 30 nm (light scattering); crystal size ~ 3 to 5 nm (HR-TEM); surface area ~13 m²/g; was used. Saline and 10% sucrose caused agglomeration in vitro. Fresh blood incubated with ceria for 1 hour showed primary and agglomerated ceria by HR-TEM/STEM. Ceria was given to un-anesthetized rats i.v. (0, 50, 250 or 750 mg/kg) to assess its biodistribution and potential to produce toxicity once it reaches systemic circulation. Repeated blood sampling showed a t½ of << 1 hour. Five minutes prior to termination (~ 20 hours after dosing) anesthetized rats were given i.v. Evans blue [EB]-albumin and Na fluorescein [Na₂F] as blood-brain barrier integrity markers. Brain EB and F slightly increased in ceria-treated rats. Tissue [Ce], by ICP-AES, was dose-dependent (spleen > liver > brain > blood serum). 50 to 60% of the total ceria dose was in these organs. Intracellular ceria was seen. LM and EM revealed some hepato- and nephrotoxicity, but little BBB damage. The oxidative stress marker 4-hydroxy-2-nonenal (HNE) increased in the hippocampus, 3-nitrotyrosine (3-NT) changed little, and protein carbonyls decreased in the cerebellum.

Conclusions: Ceria was cleared by peripheral tissues. Much less ceria entered the brain than peripheral organs. These results provide a foundation to study the impact of physico-chemical properties of ENMs on their peripheral organ and brain entry, and their resultant neuroprotective or neurotoxic activity.

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7.21 - Influence of size and surface charge on the hemocompatibility of polystyrene particles

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Data on environmental exposures to nanoparticles as well as in vivo and in vitro studies suggest that the interaction with blood compounds (red and white blood cells and serum proteins) determines the biological effect of nanoparticle to a high extent. On the one hand the physicochemical parameters of particles are changed by the binding of plasma proteins and on the other the particles may influence hemolysis, clotting and immunological responses.

We investigated the action of quartz particles and polystyrene particles of different sizes and surface charges on hemolysis, on clotting, on complement activation and on mitogen-stimulated lymphocyte proliferation.

Hemolysis was induced especially by small size and less by positive charge. Plasmatic coagulation and platelet activation were studied separately. Although many particles induced clotting by the plasmatic coagulation system this induction was more pronounced upon contact with positively charged particles than with neutral or negatively charged ones. Pronounced thrombocyte activation was measured neither by small particles nor by positively charged ones which leads to the assumption that clotting induced by particles is mediated predominantly by thrombin generation. The degree of complement activation was correlated to positive surface charge and not to particle size. Mitogen-stimulated lymphocyte proliferation was increased in the presence of particles independent of particles size or charge when samples contained more than 5% monocytes. Positively charged particles induced proliferation also in these samples also in absence of the mitogen.

Size and surface charge of particles determine hemocompatibility in a very complex manner; positively charged particles appear to be less hemocompatible than negatively charged or uncharged ones. Small particle size is a decisive parameter only for the induction of hemolysis but not for the other parameters of hemocompatibility investigated in this study.
7.22 - Nanoparticle-induced signaling pathways in lung epithelial cells

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The interaction of nanoparticles with target cells of the lung leads to specific cellular and molecular reactions that are not elicited by fine and coarse particles of the same material. It is suggested that these events contribute to nanoparticle-specific pathogenic outcomes. Mitogen-activated protein (MAP) kinases, as sensors of extracellular stress, seem to play a central role in the induction of cellular reactions specifically induced by nanoparticles. In order to study the molecular mechanisms of particle cell interaction we investigate the impact of different types of nanoparticles in lung epithelial cells.

Experiments using human and rat lung epithelial cell lines show that apoptosis and proliferation are induced independently from each other by different types of model nanoparticles. Both endpoints are mediated via specific MAP-kinases. Apoptosis is regulated on the level of the Jun-kinases Jnk1/2, while the activation of the extracellular regulated kinases Erk1/2 is specific for proliferation. Interestingly, the decision which of these both endpoints is induced in an individual cell is determined on the level of membrane receptors. Inhibitor studies reveal that both endpoints are triggered by EGF-R activation. Proliferative Erk1/2 signaling, however, requires an additional integrin-dependent signal. Further analyses of this signaling pathway demonstrate that PI3-kinase-mediated activation of Akt is rather relevant than the pathway via Ras and Raf in nanoparticle specific Erk1/2 activation and proliferation. Studies focusing on the oxidative capacity of nanoparticles show, that reactive oxygen species (ROS) play an important role in eliciting the initial events of membrane receptor-coupled signaling.

In addition, as a proof of principle, a group of low molecular weight substances which stabilize proteins and membrane structures by facilitating the biophysical mechanism of preferential exclusion was tested in vitro and in vivo. This preventive treatment leads to a specific decrease of the Erk1/2 mediated nanoparticle-specific endpoints including proliferation and IL-8 production. These results document the importance of membrane dependent signaling in nanoparticle-induced health effects. Additionally, these membrane and receptor structure-stabilizing substances may be used as a preventive strategy against environmental nanoparticle-induced effects e.g. in susceptible individuals.
7.23 - Interaction of a series of quantum dots with different surface characteristics with macrophages in *vitro*

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The aim of this study was to investigate the impact of a series of surface modified quantum dots (QD) on macrophages. Qdots® (545ITK, California, USA) with organic, carboxylated (COOH) or amino (NH₂) polyethylene glycol (PEG) surfaces, along with carboxylate-modified fluorescent polystyrene beads (PB) of 20 and 200nm diameter were studied. The J774 murine monocytic cell line was treated with QD (40nM) and PB (50μg.ml⁻¹) with 10% FCS prior to assessment of cellular uptake via confocal microscopy, flow cytometry and SEM. Cytotoxicity was determined via the MTT assay and lactate dehydrogenase (LDH) assays, while oxidative stress was determined via glutathione (GSH) depletion, and pro-inflammatory response by TNF-α production. The sub-lethal concentrations of COOH and NH₂ (PEG) QD entered macrophages within 30min of exposure. NH₂ (PEG) QD uptake could not be observed in single z-plane images, however uptake was clear following 3D image restoration. Live cell confocal analysis suggested that the organic QD entered the cells in low quantities up to 45min, after which fluorescence declined, but fixed cell imaging did not generate reproducible images due to significant cytotoxicity. Both 20nm and 200nm PB were found to enter the cell within 30mins at this sub-lethal concentration. Uptake of the 20nm PB was more extensive than for the 200nm particles. No changes in cell morphology were evident by SEM with any of the NP except the organic QD, which caused severe disruption to the cell structure. All QD and PB induced GSH depletion, and subsequent TNFα production at 24hrs. Both sized PB were co-localised with the early endosome marker (EEA-1) after 30mins, remaining there for up to 120mins. However, COOH and NH₂ (PEG) QD were not detected in endosomes. The COOH QD and NH₂ (PEG) QD, as well as both sized PB were found to be co-localised in lysosomes (Lysotracker) and the mitochondria (Mitotracker) at 30, 60 and 120mins. In conclusion, the QD differ in their impact on macrophages according to their composition, with the organic being the most toxic. At sub-lethal concentrations the COOH and NH₂ (PEG) QD enter cells resulting in GSH depletion and TNF-α production. Interestingly the QD share some similarities with PB, but differ in terms of intracellular localisation.
7.24 - Mechanism of Nanoparticle Induced Toxicological Effects on Bronchial Epithelial Cells: A Comparative Approach

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Despite over a decade of research our understanding of the mechanisms by which nanoparticles (NPs) may exert a negative impact on human respiratory health remains limited.

In an attempt to better understand the mechanisms by which NP’s might alter respiratory function we have performed a comparative study of the effects of two chemically distinct NPs, carbon black (CB) and TiO₂ (diameter 13 and 15nm diameter respectively), on the human bronchial epithelial cell line (16HBE14o-). The endpoints we have studied are abiotic Reactive Oxygen Species (ROS) production, intracellular oxidative stress, inflammation and cell death.

Particle behaviour in cell culture medium was characterized by measuring the granulometry and zeta potentials. NPs exhibited an aggregated state in the cell culture medium. NPs were internalised by the cells in a dose dependent manner as revealed by flow cytometry. Both, CB and TiO₂ NPs, induced propidium iodide incorporation in a time and dose dependent manner. The two types of NPs behaved differently under abiotic and biotic conditions to produce ROS. Only CB NPs were able to induce abiotic ROS production whereas both types of NPs produced an intracellular oxidative stress (superoxide anion) in a dose dependent manner. NPs were able to induce apoptosis characterized by phosphatidyl serine exposition at the cell surface and caspase activation, independent of the chemical nature of the NPs. These cytotoxic effects of NPs depended on the oxidative stress produced by the particles.

In conclusion, We have shown that CB as well as TiO₂ NPs are taken up by airway epithelial cells and induce oxidative stress leading to cell death by apoptosis. Our results suggest that NP risk assessment should not rely solely on the abiotic tests to evaluate their toxic potential as we have shown different behaviours of NPs under biotic and abiotic conditions. Keeping in view the results of the present study and the huge number of products containing NPs, further research is warranted for the risk assessment of nanomaterials.
7.25 - Cytotoxicity of zinc oxide nanoparticles

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With the development of nanotechnology, more and more nanomaterials are used in daily life as consumer goods. Great concerns about their potential toxicity to environment and human beings have been aroused. Among these nanomaterials, ZnO nanoparticles are a class of the most developed and used materials. They are widely used as rubber additives, pigments and sunscreens. Besides, ZnO nanoparticles are also applied in many new areas, such as gas sensors, photocatalysis etc. Inevitably, the impact of ZnO nanoparticles on human health has attracted large interest of scientists and the public.

Here, the size effect on the uptake and toxicity of ZnO particles was investigated and the related toxicological mechanism was studied, by using rodent fibroblast NIH/3T3 cell as the model. Three ZnO nanoparticle samples with different diameters (10 nm, 30 nm, 60nm) and a commercial micronized ZnO powder (500 nm) sample were well characterized and then dispersed in cell culture medium for exposure. The uptake of ZnO was directly investigated using TEM. Cell viability and membrane damage were quantitatively assessed, and cell morphology was also recorded. To reveal the toxicological mechanism, the solubility of ZnO was measured by ICP-AES and a comparison between the toxicity of ZnO nanoparticles and zinc ions was performed.

No obvious cellular uptake of ZnO particles was observed on TEM after 24-hour co-culture. All ZnO samples show serious cytotoxicity at the exposed concentration higher than 20 μg/mL. The cell viability and membrane damage are not size-dependent, but concentration-dependent with a threshold. Morphological observations further confirm these results. Toxicological mechanism study indicates that the solubilization of ZnO particles plays an important role to induce the toxicity. These findings suggest that nanosized ZnO is safe as the substitute for the commercial micronized ZnO powder, however it should be used with caution for new applications.
7.26 - Novel method for the non-destructive detection of oxidative stress caused by nanoparticles

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Nanomaterials are innovative tools in the field of drug delivery. Thereby, inorganic nanoparticles such as silicon dioxide (SiO₂) provide promising characteristics as drug carriers. Silica exposure has also been associated with several autoimmune diseases and with the development of cancer. Since these diseases are linked with the generation of reactive oxygen species (ROS) again, it is very important to analyze in addition to cytotoxicity also the oxidative potential of nanoparticles. Because of the interaction of nanoparticles with many assays due to material properties, it is of great interest to find useful alternatives for already available techniques. Therefore, a new method for the detection of oxidative stress has been established which allows an automated non-invasive online monitoring of the oxygen concentration in a microtiter plate format (SDR SensorDish® Reader, PreSens, Regensburg, Germany), which is based on a fluorescent oxygen sensitive dye. For the establishment hydrogen peroxide (H₂O₂) served as a model substance to generate superoxide radicals (O₂⁻) in Caco-2 cells causing oxidative stress. Immediately after the addition of H₂O₂ a proportional increase in the concentration of molecular oxygen was monitored. This effect was supposed to be based on the activation of the superoxide dismutase (SOD), an antioxidant defense enzyme that catalyzes the conversion of O₂⁻ into O₂ and H₂O₂. In order to determine the involvement of the SOD, cells were first incubated for 24 hours with sodium diethyldithiocarbamate trihydrate, a specific SOD inhibitor. After addition of H₂O₂ no increase in oxygen could be detected indicative for the involvement of SOD. Experiments with this novel method were confirmed with a conventional fluorimetric assay for the detection of oxidative stress using 2', 7'-dichlorofluorescin diacetate (DCF-DA). Subsequent experiments showed that the assay is also useful for the determination of ROS caused by fluorescence-labeled silica nanoparticles, where also a concentration-dependent increase of O₂ was monitored. These results show that the novel method is a fast, convenient and non-destructive alternative to detect oxidative stress in cell cultures. Furthermore no incubation time is required because a direct effect to H₂O₂ is documented. In addition the assay is also applicable for fluorescence-labeled nanoparticles.
7.27 - Investigating the uptake, fate and functional consequences of NP exposure on hepatocytes in vitro

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The outcome of liver exposure to Nanoparticles (NPs) requires assessment as NPs accumulate within this organ after inhalation and injection. The impact of NP exposure on hepatocytes is of particular interest due to their abundance, and importance to liver function. The aim of this research was to investigate the method of NP uptake and sub-cellular distribution of NPs. The impact of NP exposure on cell viability, oxidative stress, bile formation, and inflammation was investigated to reveal the functional consequences of NP uptake. Within this study, 20nm and 200nm fluorescent carboxylated polystyrene beads (PBs) were used, in addition to the PARTICLE_RISK NP panel of 14nm CB, 260nm CB, Single Walled Carbon Nanotubes, C60, positively (QD621) and negatively (QD620) charged quantum dots. The uptake of PBs by hepatocyte cell lines (HepG2 and C3A) and primary rat hepatocyte couplets was investigated using confocal microscopy, with fixed (125µg/ml, for 10,30,60, or 240 minutes), and live (15.6µg/ml, suspended in culture medium in the presence or absence of serum, 1 hour) cell imaging. It was found that 20nm PBs were internalised by all hepatocyte cell types to a greater extent, and at earlier time points, compared to the larger particles. There was no apparent accumulation of 20nm PBs within early endosomes or lysosomes, with 20nm PBs located within the mitochondria of cell lines, but not couplets. Live imaging demonstrated that 20nm PB uptake was dependent on serum, as large PB aggregates formed in serum absence that limited their uptake. Primary hepatocyte couplets, isolated from the rat liver, are an in vitro model of bile secretion which is visualised using the fluorescent bile acid, cholyl lysyl fluorescein (CLF). Our data suggests that C3A and HepG2 cells form canalicular-like structures in which PBs (20nm) accumulate, to a limited extent, with CLF, suggesting that NPs may be eliminated in bile. Of the panel of NP investigated, QD621 were observed to negatively impact on bile secretion to the greatest extent, and it was found that QD621 and QD620 caused significant cell death after a 24hour exposure, which was correlated with a decrease in cellular GSH content. In conclusion, the toxicity of a variety of NP compositions, and structures was assessed, and it was found that the most toxic was QD621, with the other NPs in the panel observed to induce limited toxicity within hepatocytes in vitro.
7.28 - Size dependent entering of particles into A549 epithelial lung cells

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Airborne particles may enter epithelial cells (EC) of the respiratory tract. Current literature suggests that entering mechanisms of particles differ between fine particles and nanoparticles (≤0.1μm). Little is known about the quantitative relationship between particle size and particle number or surface taken up by EC.

The current study investigates the entering of differently sized fluorescent polystyrene spheres (Ø1μm, 0.5μm, 0.2μm, 0.1μm, 0.05μm) into human A549 EC in regards to intracellular particle number and surface. The cells were incubated with the particle suspension for 24h and then visualized and quantified with confocal laser scanning microscopy and image deconvolution.

The experiments provide evidence for a less pronounced entering of nanoparticles into EC. Exposure to the same number of particles resulted in significantly more intracellular fine particles as compared to nanoparticles (p<0.05). This was also observed when EC were exposed to the same total particle surface of differently sized particles (p<0.05). Furthermore, within rising particle concentration, the number of intracellular particles showed a stronger increase for fine particles as compared to nanoparticles.

These observations may partly be explained by size-dependent particle agglomeration but also by different mechanisms involved in the cellular entering of fine particles and nanoparticles. In particular, fine particles induced an increase in apical EC membrane surface (p<0.05), a phenomenon not present with nanoparticles as evidenced by stereology. The impact of this observation for nanotoxicity and -effects, as well as size dependent entering mechanisms of particles, needs to be further explored and is part of ongoing research.
7.29 - Cellular responses to carbon nanotubes are caused by media alteration through yttrium release and folate adsorption

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Nanomaterials are complex new test subjects for toxicology and may interact with cells and biological molecules in unexpected ways. We have recently conducted two studies, in which single-walled carbon nanotubes were observed to influence cell behavior at low doses by new mechanisms that do not involve direct contact between nanotubes and cells.

First, we observed that single-walled carbon nanotubes at doses as low as 1 ug/ml strongly inhibit current flow through neuronal voltage-gated calcium ion channels. Nearly identical inhibition is observed with nanotube-free supernatant after nanotube removal by centrifugation. Metal mobilization experiments and control experiments with metal salts suggest that yttrium leaching from imbedded nanotube catalyst residue is the primary source of calcium ion channel inhibition. Although the catalyst is the minority component in nanotubes, and the rare-earth element yttrium is the minority component in the catalyst (4:1 Ni/Y is used in the arc synthesis process), the biological effect is significant since yttrium is a potent inhibitor of calcium ion channels.

In a second experiment, we observed significant inhibition of HepG2 cell proliferation when the cell culture media had been previously exposed to carbon nanotubes, even when the tubes do not contain Ni or Y. Chemical profiling of the medium after nanotube exposure and removal revealed a dose-dependent depletion of amino acids and micronutrients due to physical adsorption on nanotube surfaces. Amino acid depletion occurs only at high nanotube doses (>1000 ug/ml) and increases with the increasing hydrophobicity of the amino acid side chain. In contrast, vitamin depletion can be significant at nanotube doses as low as 10 ug/ml and is especially strong for riboflavin and folate. Restoring folate to the CNT-exposed medium restored most of the cell viability, indicating that folate depletion is the primary mechanism for growth inhibition. Both of these biological responses are due to media alteration and highlight the need to carefully consider such indirect mechanisms when conducting nanotoxicity testing.
7.30 - Proinflammatory effects of nanoparticles on bronchial epithelial cells in vitro.

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In recent years, nanotechnologies have played an increasingly important role in many industries. As a consequence, the impact of nanomaterials, and particularly nanoparticles (NPs), on human health needs to be evaluated in order to define their effects on human health.

The aim of the present work was to investigate the inflammatory potential of NPs on human bronchial epithelial cells (16HBE14o- cell line). NPs of various sizes and of two different chemical natures (carbon black (CB) and titanium dioxide (TiO2)) were used. CB and TiO2 at a non-cytotoxic exposure of 10 µg/cm² induced the mRNA expression of the pro-inflammatory cytokines IL-6 (interleukin 6) and GM-CSF (Granulocyte Macrophage-Colony Stimulating Factor) as well as the growth factor Amphiregulin. CB NPs (13nm) and TiO2 (15nm diameter) NPs have different efficiencies to induce mRNA expression. Particle size also had an impact on the inflammatory response for both types of NPs as the mRNA induction increases with decreasing primary particle size and thus increasing surface area. However, we were unable to detect an increase in protein levels for IL-6, GM-CSF or for Amphiregulin in the culture supernatants using ELISAs after treatment with CB. We have shown under abiotic conditions that this was due to the fact that the secreted proteins were adsorbed by the NPs. Experiments aimed at reducing adsorption of mediators by NPs were undertaken using bovine serum albumin or foetal bovine serum, but were not conclusive as both inhibited mRNA expression of all three genes and reduced cytotoxicity in response to NPs. The use of detergents to recover proteins from NPs yielded recoveries of only 20 % of the proteins.

This study shows that NPs can alter the expression of genes thought to play important roles in the inflammatory response of human respiratory epithelial cells and warrant further investigation. Additionally, the physico-chemical interactions of NPs with biological mediators can complicate interpretation of biological responses.
Nano-sized titanium dioxide (nano-TiO\textsubscript{2}) is one widely used nanomaterial for its promising benefits in multi-purposes. Hence, increasing exposure to this nanomaterial raises serious concerns. With limited availability of toxicity data on nano-TiO\textsubscript{2}, such effects of nano-TiO\textsubscript{2} toxicity on specific stress protein expression in a human lung carcinoma cell line, A549, was explored in this study. Commercial nano-TiO\textsubscript{2}, anatase crystalline form, was used. Morphology and particle size were confirmed using TEM and DLS. Experimental data showed that agglomeration of nano-TiO\textsubscript{2} normally occurred when suspended in cell culture media. In a cell viability assay, A549 cells were exposed to different concentrations of nano-TiO\textsubscript{2} together with and without non-toxic intensity of UV light at various lengths of time. It was found that nano-TiO\textsubscript{2} with UV irradiation caused reduction in cell viability in a concentration- and time-dependent manner. At 12 h of exposure, cells treated with nano-TiO\textsubscript{2} plus UV showed an approximate IC\textsubscript{50} of 200 µg/ml, whereas nano-TiO\textsubscript{2} alone was likely non-toxic to the cells. These observations can be partly explained by the photocatalytic potential of anatase in generating reactive oxygen species that insulted the cells. It is known that exposure of environmental stress can result in changes of expression of several inducible molecular chaperones. These include the cytosolic resident heat shock proteins (HSPs) and the endoplasmic reticulum resident glucose-regulated proteins (GRPs) which involved in cellular protein quality control. Using immunoblot analysis, changes of expression level of Hsp70, Hsp90, Grp78 and Grp94 proteins were investigated. The data revealed good correlation between overexpression of Grp78 and Grp94 proteins and nano-TiO\textsubscript{2}-induced cytotoxicity. These effects suggest possible disturbance of protein folding in the cells by nano-TiO\textsubscript{2}. Thus, further studies are essential in clarifying the biological response underlying toxic effects of nano-TiO\textsubscript{2} as well as other nanomaterials.
7.33 - Accumulation and persistence of synthetic smectite clay nanoparticles by human cells

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Synthetic hectorite is a smectite clay extensively used in applications such as surface coatings, toothpastes, cosmetics, antiperspirants, dishwashing detergents, air fresheners, ceramics, latex, polymer composites, herbicides and pesticides. All these applications involve varying degrees and routes of hectorite exposure to humans. Their use in personal care products allows for direct exposure to the human body, and hence the potential for biological responses such as cellular uptake and tissue persistence. As part of an ongoing study into the biological effects of nanomaterials, we have investigated the internalisation of commercial-grade hectorite by human cells. Fluorescently-labelled hectorite (1 x 25 nm crystals) was accumulated by macrophage cells (differentiated THP-1) but not T-cells (CEM or Jurkat), epithelial cells (MCF7 or HeLa) or monocytes (undifferentiated THP-1). Uptake by macrophage cells was inhibited at 4°C and by cytochalasin D, indicative of an endocytic process. Fluorescent microscopy showed compartmentalisation of the nanoparticles within discrete intracellular vesicles. Transmission electron microscopy confirmed the internalisation of the nanomaterial in what appeared to be phagosomes. Internalised particles persisted in the cells with a half-life greater than 250 hr. The uptake of the hectorite was inhibited by serum proteins and, while initial studies have identified albumin as a major protein bound to the nanoparticle, other unidentified proteins bind to the hectorite. This study has shown that commonly used synthetic smectite clay nanoparticles are taken up by macrophages, but are not readily eliminated by these cells. The results suggest that hectorite particles that enter the systemic circulation will be taken up by ‘macrophage-like’ cells such as the reticulo-endothelial system of the liver, and may persist in these cells for extended periods.
7.34 - High dose of carbon nanotubes shows slight hepatic toxicity in vivo

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Carbon nanotubes (CNTs) are becoming promising novel materials in diverse areas like information technology, ultrastiff materials, biomedicine etc. because of their unique physical properties. The in vivo toxicological study of these materials is very imperative for the safety assessment in respect to their wide applications. However, to date this study has only been focused on the pulmonary toxicity; almost no work done on the other major organs. As the most important organ possessing detoxifying function for exotic substances, liver can trap most of the CNTs from blood and keep them without being metabolized for long time. This work aims to intensively investigate the toxicity of multi-wall carbon nanotubes (MWNTs) to the mouse liver. After mice were exposed to CNTs by single intravenous (i. v.) injection at different dosages (2, 20, 60 and 100 mg/kg) for 1, 7, 15, 30 and 60 days, various biochemical parameters were tested to evaluate CNTs’ toxicity. Compared with the control group no significant differences of serum LDH, AST, ALT and plasma tumor-necrosis-factor-α (TNF-α) level were observed. But the depressed SOD and GSH levels in liver indicate that CNTs induce the liver oxidative damage and inflammatory response. Liver sections reveal no histological alterations and TUNEL assay shows no cell apoptosis. The ultrastructural examination shows the necrosis fate of Kupffer cells and endothelial cells in liver, which remarkably engulf CNTs. This work provides the information on the administering dose for various CNTs’ therapeutic and diagnostic applications in future.
7.35 - Imaging and proteomics analysis for studying size-dependent biological behavior of nanosilicas

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Objective: The recent development of nanotechnology has facilitated a dramatic reduction in the particle size of materials. The reduction in particle size from micro to nanoscale not only provides benefits to diverse scientific fields but also poses potential risks to the environment and to human health. For the successful application of nanomaterials in bioscience, it is essential to understand the biological fate and potential toxicity of these nanoparticles. The aim of this study was to evaluate the biological distribution as well as the potential toxicity of various sizes of nanosilicas using proteomics analysis.

Methods: To analyze size-dependent the biological behaviour of nanosilicas in vitro and in vivo, we used fluorescent-labeled nanosilicas (70, 300 and 1000 nm in diameter, designated SP70, SP300, SP1000 respectively) in our experiments. For the imaging analysis, hairless mice (Hos: HR-1, 6 wks, female) were intravenously injected with nanosilica diluted in PBS. At indicated time points, optical imaging was performed using a Xenogen IVIS 200 imaging system. Cytotoxicity of nanosilica-treated mouse epidermal Langerhans cells (XS52) was evaluated by [³H]-thymidine incorporation assay. Using cells treated with nanosilicas (10 μg/ml), we performed 2-Dimensional Fluorescence Difference Gel Electrophoresis (2D-DIGE) analysis.

Results and Discussion: In vivo imaging analysis revealed that nanosilicas mainly accumulated in the gallbladder and liver, independent of particle size. However, a higher acute toxicity was observed in SP70-injected mice. On the other hand, nanosilicas exhibited dose- and size-dependent cytotoxicity against XS52 cells in vitro. The half maximal inhibitory concentrations (IC₅₀) of SP70, SP300 and SP1000 were 4.22, 32.60 and 75.03 μg/ml, respectively. Furthermore, differing, size-dependent protein expression profiles were observed by 2D-DIGE analysis of SP70- and SP1000-treated XS52 cells. Thus, our results indicate that in vivo imaging and proteomics are useful methods for analyzing the biological behaviour of nanomaterials as well as nanosilicas. Now, we are working on clarifying the mechanism of the biological behavior and identifying toxicity marker of nanosilicas.
7.36 - CdTe nanoparticles display tropism to core-histones and histone-rich cell organelles

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The mechanism by which nanoparticles interact within biological systems represents one of the key tasks in nanobiology and nanotoxicology. Quantum dots (QDs) are capable of rapid accumulation in the nuclei and nucleoli of human cells in vitro; the precise nature of these processes still remains unknown. We hypothesise that this strong tropism of the unmodified cadmium telluride (CdTe) QDs for the nucleus could be mediated by the charge-related properties of both nuclear macromolecules and the QDs. We investigated the interaction of QDs with the human phagocytic cell line THP-1, nuclear lysates, purified proteins (including core histones) and nucleic acid solutions. We used live cell confocal microscopy, fluorescent lifetime imaging (FLIM), spectroscopic methods and zeta potential measurements to probe these QD-protein interactions. The QDs preferentially bind to the positively charged core histone proteins but not to DNA or RNA. The binding of the QDs to core histones results in a dramatic shift off the absorption band, and a red-shift and decrease in their photoluminescence (PL) intensity. FLIM imaging of the QDs demonstrates an increased formation of QD-protein aggregates in the presence of the core histones, with a resulting significant reduction in the PL lifetime. Using FLIM technology we show for the first time that the localisation of negatively charged QDs to their ultimate nuclear and nucleolar destinations dramatically affects the PL lifetimes of the QDs. This method provides a sensitive readout for physical interactions between QDs and their intracellular targets. These findings strongly suggest that charge-mediated QD-histone interactions could provide the basis for localisation of QDs to the nucleus downstream of intracellular transport mechanisms.
7.37 - Interactions between cell membrane and nanoparticles determined in *in vitro* tissue system

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Manufactured nanoparticles have entered into our everyday life. This underlines the need for a better understanding of their interactions with biological systems. The aim of our work was to establish exposure protocols and identify interactions between cells and nanoparticles. We developed an *in vitro* cell-tissue-organ exposure system. Isolated digestive glands of terrestrial isopods (*Porcellio scaber*, Isopoda, Crustacea) were exposed for 18 hours to different sizes, chemical compositions (TiO₂, ZnO and fullerenes) and concentrations of nanoparticles. After incubation, affected cell membrane permeability and lipid peroxidation were measured. Cell membrane permeability was determined by a mixture of fluorescent dyes acridin orange and ethidium bromide (AO/EB). Lipid peroxidation was assessed spectrophotometrically by measuring of malondialdehyde (MDA). For detailed characterization of interactions between nanoparticles and cell membrane, a surface plasmon resonance approach was applied. The results of our experiments confirmed size effect, concentration effect and particle type effect on cell membrane. However, interactions are not necessarily in concentration- or size dependent-manner. Our *in vitro* tissue exposure protocol enables determination of effects of nanoparticles on cell membrane in highly repeatable manner. Comparison of effects between different sizes, concentrations and chemical composition of nanoparticles provides data on their reactivity against a biological system. These are discussed as biological/toxicological characteristics of nanoparticles and suggested to be added as an additional set of data to complete chemical and physical data on nanoparticles.
7.38 - Extremely rapid endocytosis and exocytosis of nanoparticles by live cells

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Interest is growing in the possibility of quantitative reproducible studies on the impact of nanomaterials on living matter. However, even for nanoparticle-cell interactions many issues are currently unresolved, with little understanding of relevant length and time scales for intracellular entry. Emphasis is placed on the role of physio-chemical control and in situ characterization of dispersions. This is one of the first efforts to explore how feasible it is to carry out semi-quantitative work. The outcome is hopeful, but indicates the enormous challenge this will require.

Systems are studied with a range of techniques, including confocal microscopy, flow cytometry and live cell imaging to identify the interactions of model nanoparticles with different cell lines. Nanoparticle uptake kinetics are studied as a function of size, in the range 50-400nm. Intracellular access of nanoparticles smaller than 70nm was found to be remarkably rapid, saturating within a matter of minutes. Nanoparticle uptake is found to compete with equally rapid exocytosis. Live cell imaging reveals that significant numbers of nanoparticles reach sub-cellular organelles within tens of seconds, but when their source vanishes, many (but not all) are exocytosed. Competition and inhibition of particle uptake are also investigated in order to elucidate the mechanisms involved. Broader implications of these observations are discussed.
7.39 - Interaction of nanoparticles with oligodendroglial cells of the rat brain

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The aim of this study is the characterization of the toxic effects of nanoparticles on oligodendroglial cells. Until now little is known about the effects and interactions of nanoparticles with glial cells.

In this study we examined nanoscaled tungsten carbide (WC) and tungsten carbide cobalt (WC-Co) as well as diamond nanoparticles which are released during the manufacturing process of tools as well as single-walled carbon nanotubes (SWCNT) which are of growing interest in technical and medical investigations.

The cell culture system used in this study is the oligodendrocyte precursor cell line OLN 93, derived from primary rat brain glial cultures. Oligodendroglial cells are the myelin forming cells of the brain, encircling and isolating the axons of the neurons with their cellular extensions. This cell line is exposed to nanoparticles and resulting effects are studied in a time and concentration dependent manner. Relevant endpoints are viability, activity of the mitochondrial membrane potential, proliferation and adhesion of the cells after exposure to nanoparticles.

The oligodendroglial cells showed different sensitivity while exposed to nanoparticles, nevertheless all examined nanoparticles caused a decrease in viability. The most toxic effects were generated by WC-Co and SWCNT. Due to comparability and interactions between particles and testing reagent different viability test systems were applied. Exposure to WC and SWCNT particles caused a reduced adhesion of the cells as well as a change of the mitochondrial membrane potential. A decreased viability and proliferation as well as a reduced adhesion of oligodendroglial cells suggest that nanoparticles interfere with the function of the cells and therefore may affect the signal transduction between the cells of the central nervous system.
7.40 - Effects of different engineered nanoparticles on primary rat neurons

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The aim of this study is to investigate the neurotoxicity of engineered nanoparticles to which humans may be exposed in the context of medical and technical applications, during manufacturing processes of tools or by using nanoparticle containing products. We focused on nanoscaled tungsten carbide (WC), tungsten carbide cobalt (WC-Co), diamond nanoparticles as well as single-walled carbon nanotubes (SWCNT).

The use of these synthetic nanoparticles is of increasing interest but limited information about their interaction with cells exists. In vivo studies have shown that nanoparticles can reach the brain via the olfactory nerve. Therefore we assessed the toxic effects of the particles on primary neurons prepared from the cerebrum of 18 day old fetal Wistar rats. The cells were exposed to well characterized and stable nanoparticle suspensions in a concentration and time dependent manner. A variety of relevant endpoints were examined such as viability, changes of the mitochondrial membrane potential, neurite outgrowth and adhesion of the neurons after exposure to nanoparticles.

Due to the different character of the particles they elicited distinct severe responses in neurons. The highest toxicity was found with SWCNT and WC-Co. The viability was not or only slightly influenced by the particles except by pure SWCNT. Depending on the time point of exposure the neurons showed different decrease in adhesion. Treatment with SWCNT caused also a change of the mitochondrial membrane potential as well as reduced neurite outgrowth.

In conclusion it can be stated that some of the examined nanoparticles may have an impact on neuronal function and the signal transduction in the central nervous system when they reach the brain.
7.42 - Uptake, intracellular distribution and cytotoxicity of gold nanoparticles in MDCK and HepG2 cell lines

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The research strategy applied in this work is based on an integrated approach combining cell-based in vitro assays with specific radiochemical and physicochemical facilities. It consists in the selection, based on industrial interest, of manufactured nanoparticles (mNPs), their synthesis, the assessment of their physicochemical characterisation and toxicological profile by in vitro systems relevant for human exposure. In this work, we show preliminary results that meant to be paradigmatic of the Directorate General Joint Research Centre of the European Commission (DG-JRC) research on nanotoxicology. The in vitro study was performed by analyzing the physicochemical characteristics of Gold nanoparticles (AuNPs) before and after their neutron irradiation. In addition, we quantified their uptake, intracellular distribution and cytotoxicity in MDCK and HepG2 cell lines, which should represent two different possible detoxification routes. After 24 hrs of exposure at the concentration of 400µM the cell viability was of 80% in MDCK cells and 90% in HepG2 compared to control. The radiolabelled AuNPs (¹⁹⁸AuNPs) uptake for MDCK was 6.7 pgAu/cell, whereas parallel analysis in HepG2 showed that the ¹⁹⁸AuNPs uptake was approximately four fold increased (25.9 pgAu/cell). Moreover, to better understand the intracellular distribution of ¹⁹⁸AuNPs, experiments to localize the presence of the particles in specific intracellular compartments were performed by differential centrifugation and qualitatively verified by Transmission Electron Microscopy analysis. In this work, we demonstrated the possibility to use neutron activated AuNPs to study NPs interaction with cells giving evidence that AuNPs maintained the same physicochemical characteristics after their activation. More in general, using this approach, we can correlate the possible cytotoxic effects to the uptake and express the concentration-effect relationship as uptake-effect relationship. This latter consideration could suggest an innovative approach in order to better define the NPs in vitro toxicological profiles. In any case, the understanding of the mechanisms of toxicity requires proactive multidisciplinary research initiatives to address the impact of nanoparticles on human health.
7.43 - Comparative toxicity of nanosized and bulk form of ZnO and CuO to Saccharomyces cerevisiae

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Metal oxide nanoparticles are increasingly used in various commercial products such as cosmetics (e.g., sunscreens), dental fillings, solar-driven self-cleaning coating, textiles etc. It is generally assumed, that the changes in the physicochemical and structural properties along with a decrease in size could lead to new biological effects of engineered nanoparticles and as a result to enhanced toxic properties compared to respective non-nano analogues. Currently, the best-developed paradigm for nanoparticles toxicity is generation of reactive oxygen species (ROS) leading to oxidative stress response.

The aim of this study was to evaluate the toxic effect of nanosized ZnO (50-70 nm) and CuO (30 nm) on the growth of the yeast Saccharomyces cerevisiae – a widely used model to study oxidative stress and ageing in eukaryotes. The inhibition of the growth compared to the control was used as a toxicity endpoint. The effect of nanosized metal oxides, their bulk forms and ionic forms of respective metals were compared. The toxic effects were quantified from respective dose-effect curves as IC\(_{20}\) and IC\(_{50}\) (inhibition of the growth by 20% or 50%). To differentiate the toxic effects of particles and soluble metal ions, the bioavailable ions in culture medium were quantified by recombinant bacterial sensors responding specifically to Zn\(^{2+}\) and Cu\(^{2+}\) ions by increased bioluminescence. Both, nano and bulk ZnO were of comparable toxicity (8-h IC\(_{50}\) ~170 ppm) to S. cerevisiae. However, nano CuO was about 50-fold more toxic than bulk CuO (8-h IC\(_{50}\) ~40 and ~2000 ppm, respectively). The Zn-sensor bacteria showed that the toxicity of both nano and bulk ZnO was mainly due to solubilized Zn ions. Conversely, in case of both nano and bulk CuO the solubilized Cu ions did not explain the whole toxicity of respective formulations.

To evaluate the potential of metal oxide NPs to create the oxidative stress, the intracellular GSH level in S. cerevisiae will be determined.
7.44 - Effect of nanomaterials on the induction of autophagy

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[Introduction] Nanotechnology has produced a diverse array of nanomaterials such as carbon nanotubes, fullerene derivatives and quantum dots. The advent of nanomaterials has provided incredible opportunities for biomedical applications. For example, nano scaled silica (NS) is most commonly found in nature as sand or quartz, and used in various fields such as engineering, electronics, foods, and drugs. However, the potential adverse effects of nanomaterials on human health remain to be established. Recently, we have tried to clarify the property and safety of nanomaterials in vivo and in vitro. In this study, we examined the effects of NS against cells such as endothelial cells and macrophages.

[Methods] Cellular viability was measured in NS-treated cells by methylene blue assay. The formation of autophagic vacuoles was assessed by staining with monodansylcadaverine (MDC) and GFP-LC3 expression plasmids. The redistribution of LC3, which is associated with autophagosomal membranes, from diffuse cytosolic staining to punctate staining is a reliable marker of the induction of autophagy.

[Results and Discussion] Different sizes of NS (Diameter; 70, 300, 1000 nm) labeled with fluorescence were evaluated for their potential toxicity on four types of endothelial cells. NS showed no cytotoxicity to each endothelial cell even in the presence of high concentration (100 μg/ml) of NS. On the other hand, NS induced the cytotoxicity to macrophage cells. Especially, NS of 70 nm induced the significant cytotoxicity compared to NS of 300 nm and 1000 nm. Next, to determine whether autophagy was induced in macrophage, we first assessed by staining with MDC, which accumulates in acidic cell compartments enriched in lipids. NS treatment of macrophage resulted in the appearance of punctate structures, suggesting that NS induces the accumulation of autophagic vacuoles in these cells. In addition, after cell transfection with GFP-LC3 expression plasmids, NS treatment caused the appearance of a punctuate fluorescence pattern in macrophage cells, confirming again that NS induces an increase in autophagic structures. Now we are examining the relation between the cytotoxicity of NS and the induction of autophagy.
7.45 - Multi-centre systematic characterisation of cytotoxicity and specific cellular responses in human cell lines of various lineage to SiO₂ nanoparticles with distinct physico-chemical properties

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Rapid development of nanotechnology consistently increases the likelihood of human contact with environmentally presented and manufactured nanomaterials. However, there is still very little definitive systematic information about the consequences of interactions of nano-scale objects with human cells of diverse origin. As a non-metal oxide, silicon dioxide (SiO₂) nanoparticles have extensive use in industry, cosmetics and food products. In recent years, the use of SiO₂ nanoparticles has been also extended to the biomedical and biotechnological fields.

We present here the results of the first European multi-centre (NanoInteract FP-6) systematic characterisation of the effects of SiO₂ nanoparticles of different size and precisely defined physico-chemical characteristics on cultured human cells of various origin. The selection of cell types was based on the probability of human exposure to nanomaterials via respiratory, alimentary and parenteral routes in physiological environment. We have developed a set of standardised experimental assays that have been implemented across the different institutes, enabling the first truly comparative studies and validating the results obtained thereby. These assays included conventional cytotoxicity tests and a range of High Content Screening (HCS) assays which enable comprehensive multi-parametric investigation of effects of nanoparticles on live human cells. HCS, or image based screening, has recently emerged as a platform for compound testing for phenotypic cell-based assays in pharmacotoxicological studies. Based on automated microscopy, it permits time-resolved imaging of individual cells and cell populations exposed to nanoparticles in controlled physiological conditions.

For all the experiments implementing various reporter systems, common protocols were used to disperse the nanoparticles, and control experiments were undertaken with common serum, and cells originating from the same repository. Characterisation of the dispersions under appropriate conditions and as a function of time was performed. In our study, potential cytotoxic, apoptotic and specific responses of phagocytic cell line THP-1, alveolar epithelial A549, colonic epithelial Caco2, and hepatic HepG2 lines were evaluated in the presence of (nominally) 10, 30, 80 and 400 nm diameter SiO₂ nanoparticles over 3-72 hours exposure intervals. In distinction to several previous reports, we find (reproducibly across the multi-centre program) remarkably low degree of cytotoxicity and genotoxicity at concentrations below 500 μg/mL. Nevertheless, HCA (supported by several other forms of imaging) detected significant dose- and particle size-dependent changes in lysosomal mass/pH values, cell membrane permeability and other characteristics which also clearly reflected the nature of particular cell types.

These findings, and the reproducible multi-centre manner in which they were achieved, establish the lack of cyto- and genotoxicity of a wide variety of silica nanoparticle types, and prompt an altered focus of future investigations. Thus, the observation of more subtle biological impacts (downstream of primary membrane-and endocytosis-associated phenomena) suggests that additional investigations are merited, especially addressing the sustained exposure of human cells to SiO₂ nanoparticles.
7.46 - Assessment of hard metal nanoparticles: A combined approach of characterisation, visualisation and toxicology

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Nanoscale tungsten carbide (WC) and tungsten carbide cobalt (WC-Co) particles are used widely in hard metal industries. The assessment of the hazardous potential of these materials is relevant for workplace safety as well as for the environment. In light of this, we are investigating the impact of both types of nanoparticles to various cell lines with human and piscine origin. The particles are intensively described in respect to their characteristics and behaviour within all suspensions and physiological media used. Thus, stable suspensions with known properties are provided for in vitro hazard assessment.

We found that the presence of serum in the cell culture media influences the agglomeration behaviour dramatically. Nevertheless, electron micrographs of exposed cells, coupled with EDX-detection, were similar irrespective of the presence or absence of serum and the type of cell line. Both types of particles were found to be able to penetrate the plasma membrane of the cells and gather in the cytoplasm but not in the nucleus.

Based on the knowledge that the particles enter the cells, several toxicological endpoints have been addressed. Both WC and WC-Co nanoparticles showed little impact on cell viability although WC-Co particles were in general slightly more cytotoxic in high concentrations (33 µg/ml) than WC. The mode of action behind this effect is not completely known since comparable experiments using both elements alone (WC and Co) or in combination did not show the same response. Further investigations involve the measurement of ROS (reactive oxygen species) production and the regulation of expression of cytokines as immunological response. Results from these more subtle endpoints revealed that the cells do in fact respond to the exposure to particles. To better understand these responses as part of the cellular response network, gene expression analyses using the microarray – genechip – technology have been started. With this global approach we also aim to elucidate whether short-term, sub-lethal responses could result in chronic toxicological effects.
7.47 - Comparison of intrinsic free radical activity and oxidative ability generated by different nanoparticles

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There is an increasing concern about the potential risk of nanoparticles because of their unique physico-chemical characteristics, such as extremely small diameter, large surface area, altered electronic properties, surface reactivity, and surface derivatization. Therefore, there have been calls that nanoparticles should be assessed for adverse human and ecological toxicity before their widespread industrial application. In this study, free radical activity and radical types generated by a panel of NPs was investigated by using Electron Paramagnetic Resonance (EPR) and laser flash photolysis respectively, while their oxidative ability was evaluated by DCFH fluorescent probe (cell-free assay).

EPR results demonstrated, for individual NP types, free radical intensity increased with increasing surface area (positive relationship, $R^2=0.95$). However, there were differences in the intensity of free radical activity produced by different NP types. Free radical generation of the panel NPs in the following order NiO > CeO₂ > Co₃O₄ > CB > ZnO = TiO₂ (Anatase) = TiO₂ (Rutile) = MgO = SiO₂ = Al₂O₃. Laser flash photolysis results revealed that cation radicals and netural radicals can be generated by nanoparticles NiO and ZnO rather than Al₂O₃. The cell-free assay showed that fluorescent intensity of the NPs has time-dependent and dosage-dependent response. The oxidative ability of the panel of NPs as measured by the fluorophore DCFH probe ranked in the order NiO > Co₃O₄ = CB = TiO₂ (Rutile, Anatase) = ZnO = MgO = CeO₂ = SiO₂ = Al₂O₃. The relationship between free radical activity and oxidative ability of NPs was not predictive (correlation coefficient equals 0.825). Our results implied that physico-chemical characterization of NP might play very important role in potential toxicological assessment of nanoparticles.

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7.48 - Investigations on particle-induced cell death in macrophages

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Although apoptosis is commonly considered as a protective mechanism to eliminate damaged cells, a relationship between particle-induced lung inflammation, fibrosis and apoptosis is currently discussed. The aim of our study is to investigate the ability of different types of particles to induce apoptosis in macrophages in relation to their particle size and physico-chemical properties. Murine macrophages (RAW264.7) were treated with various particles, i.e. crystalline silica (DQ12, Ø 960 nm), amorphous silica (a-SiO₂ Ø 14 nm), ZnO (Ø 20 nm), MgO (Ø 20 nm), and two TiO₂ samples (Ø 250 and 30 nm). Staurosporine was used in all assays as positive control. In order to screen the different particles regarding their effects on cell viability, the WST-1 assay was performed upon treatment for 24, 48, or 72 h at concentrations ranging from 1 to 80 µg/cm². For the specific detection of apoptotic cells or for the discrimination between apoptotic and necrotic cell death we applied a flow cytometric detection of cells with subdiploid DNA content using the fluorescent dye 7-Aminoactinomycin D, the Annexin V-FITC/Propidium iodide staining method, and the Cell Death Detection ELISA (Roche).

Irrespective of the assay used, DQ12, a-SiO₂ and ZnO were found to have rather strong effects, while the effects of MgO and the two types of TiO₂ tended to be low to absent. Moreover, with the exception of ZnO, all samples revealed notable differences in sensitivity for the different assays used. Comparative evaluation of the two different TiO₂ types in the RAW264.7 cells revealed only slightly stronger effects of the nanosize sample when compared to the non-nano-size sample for the WST-1 assay, and hypodiploid cells were solely detected at high concentrations (≥ 40 µg/cm²). As such, each test revealed particle specific cytotoxicity data, which appeared to be more dependent on the chemical composition than the size of the particles. Although our results provide clues for particle type specific kinetics and mechanisms of cell death, they also underline the importance of using multiple apoptosis assays when addressing cell death in nanotoxicology research. Our ongoing work focuses on the elucidation of mechanisms implicated in particle-induced apoptosis and necrosis, and on how these may affect inflammation and tissue remodeling upon particle exposure.
Our daily life is being changed by nanotechnology. Nano materials which have unique physico-chemical characterization, such as its small size, chemical composition, surface structure, solubility, shape, and aggregation have been used widely in industrial application, medical imaging, and disease diagnoses, etc. However, there is concern of environmental contamination and health negative effects might caused by nanoparticles. Reports about toxicity of nano particles have been increased rapidly in recent years. However, few papers concerned about potential neural system toxicities induced by nanoparticles. In this study, neural stem cells (NSC) line (C17.2) were employed to investigate toxicity of four different kinds TiO₂ nano particles (25 nm and 80 nm Antase TiO₂, 155 nm Rutile TiO₂, and 151 nm functionalized Rutile TiO₂). Our results clearly demonstrated that after the NSC cells line exposed to TiO₂ (Anatase) and TiO₂ (Rutile) nanoparticles 24 h, their cytotoxicity (by using LDH assay), showed no significant difference with the value of cytotoxicity varied 5~20% at the mass concentration 6.25~50 μg/ml. Cell variability results (by using CCK-8 assay) claimed that there exist time-dependent response after the C17.2 exposed to TiO₂ nanoparticles, suggesting all of the four kinds TiO₂ nano particles stimulate the C17.2 cells line to have distinctive multiplication. On the base of cytotoxicity and cell variability, 80 nm Anatase TiO₂ was selected to observe location of nanopartilces in C17.2 cells. Results of Laser Scanning Confocal Microscopy and TEM observation revealed that after endocytosed by the cells, nanoTiO₂ particles aggregated near around nucleolus and relocated in endocytic vesicles.

Armed with this data, we intend to investigate oxidative ability of nano TiO₂ by using DCFH-DA assay and EPR, and try to explain its toxicological mechanism by using oxidative stress theory.

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7.50 - Carbon nanoparticle- induced intracellular signaling: The role of membrane structures and receptors

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Environmental nanoparticles have been reported to induce adverse health effects in humans. Our previous studies showed that the interaction of nanoparticles (NP) with lung epithelial cells mediates cell proliferation via the activation of MAP kinases ERK1/2. Interestingly, the membrane receptors EGFR and β1-integrins both are involved in this NP-specific signaling. The initial events, by which nanoparticles trigger this receptor-dependent signaling as well as the way how these receptors mediate the signal response, are not understood.

In order to study these early signaling events on the level of membrane receptors, possible mediators of receptor crosstalk and membrane signaling platforms have been investigated in RLE-6TN (rat lung epithelial cells) treated with carbonaceous NP (Printex 90).

Cells were preincubated with src family kinase inhibitor PP2 followed by treatment with NP. The induction of proliferative signal cascades involving phosphorylation of ERK1/2, Akt and src family kinases were studied by Western blot analyses. Furthermore, the consequence of inhibiting EGFR and β1-integrins on the phosphorylation of src kinases was analysed using specific inhibitors/antibodies. Lipid rafts as membrane structures relevant for cell signaling were investigated using density gradient centrifugation.

Treatment of cells with NPs resulted in an activation of src kinases at time points after 5min up to 8h. The src inhibitor dose-dependently prevented the activation of the NP-specific proliferative signaling pathway via ERK1/2 and Akt, demonstrating the relevance of src kinases for NP-specific signaling. Blocking of the receptors EGFR and β1-integrins both resulted in a reduction of src phosphorylation. First investigations on lipid rafts, membrane microdomains in which the described receptors as well as src kinases are located, showed an impact of NP on raft protein composition.

These results indicate an important role for src family kinases in the crosstalk of the identified membrane receptors in proliferative signaling. Moreover, NP-induced changes in lipid raft composition may be an early event in particle-cell interaction triggering NP-specific endpoints.
7.51 - Challenges of evaluating the \textit{in vitro} effects of nanoparticle exposure

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Due to their increasing global production and applications, considerable concern has been raised regarding the potential health risks associated with nanoparticles. The number of publications describing the effects of nanoparticle exposure is increasing rapidly. Unfortunately, the results of these studies are often contradictory. This paper describes the factors influencing the \textit{in vitro} toxicity of nanoparticles that might lead to data misinterpretation and/or difficulties in inter-study comparisons based on our experience in studying diesel exhaust and engineered nanoparticles such as carbon based particles and metal nanoparticles (gold, iron oxide and cobalt oxide).

Any type of particle treatment including dispersion method and use of solvents is influencing chemical and physical characteristics such as size, shape, aggregation state, and surface parameters. In addition, particle contaminants and batch-to-batch variations may also affect cellular responses. Therefore, extensive particle characterisation at the different stages of an experiment is of utmost importance. A multitude of assays, including those that have optical density, luminescence and fluorescence as read-out parameters, are used in order to study the \textit{in vitro} effects of nanoparticle exposure. However, these parameters might be affected by the different nanoparticles leading to false positive or negative results. Several ways to reduce these interactions have been investigated. Nanoparticles, particularly carbon based nanomaterials such as SWCNT, are able to adsorb a multitude of molecules onto their surfaces. On the one hand this might decrease the protein levels being analysed (i.e. cytokines, chemokines or other regulatory proteins), on the other hand it might also affect the cell culture medium by extracting important growth factors. Moreover, protein coating may influence toxicological parameters of particles. To enable correction for these factors, it is of main importance to include the appropriate controls. In conclusion, \textit{in vitro} studies on nanomaterials should be tightly controlled and well-defined in order to increase their reproducibility and enable the comparison of results between different laboratories.
7.52 - Effects of carbon nanotubes on human blood cells

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Carbon nanotubes (CNT) are one of the novel most attractive materials in nanotechnology. Due to their multiple industrial and biomedical applications, thorough analyses on their toxicity and biocompatibility are a priority to prevent possible health risks. Likely target of CNT following inhalation, accidental bruises, or on-purpose administration for nano-biomedical drug delivery applications are leukocytes; we selected human tumor leukocytes (monocytes, U937, and T lymphocytes, Jurkat) and their normal counterparts from peripheral blood (PBML). We tested CNT at different concentrations and incubation times for effects on: cytotoxicity (viability, apoptosis, necrosis); sensitization/desensitization to chemotherapy-induced apoptosis; cell proliferation and cell cycle; oxidative stress (reactive oxygen species and glutathione levels); mitochondria; intracellular Ca²⁺. Our results show that the effects depend on the target cell: on Jurkat, CNT do not affect proliferation but induce apoptosis, whereas in U937 they inhibit proliferation but do not induce cell death; U937 but not Jurkat were sensitized by CNT to chemotherapy-induced apoptosis. The analysis of intracellular parameters linked to apoptotic signalling, i.e., oxidative stress; mitochondria; Ca²⁺, showed that CNT differentially affect the two cells, providing a biochemical rationale for their different effect on apoptosis by CNT. On PBML, CNT exert a cytotoxic effect and sensitize cells to chemotherapeutic agents. These effects may have important implications, recommending much attention in terms of evaluation of exposure risks.

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7.53 - Development of innovative methods for phagocytosis quantification of microsized particles.

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Introduction: Inhaled particles exhibit variable toxicity levels which mainly depend on their physicochemical characteristics (size, morphology, crystallinity, chemical composition of the surface…). Biological effects using several classical tests (ROS, TNFα, LDH) are usually performed on alveolar macrophages collected from the respiratory system. Available publications on toxicity generally do not take into account the number of phagocytosed particles. Thus, the relationship between the potential toxicity and the amount of particles phagocytosed is rarely investigated.

The aim of this study is to develop a quantitative evaluation of phagocytosis using both direct and indirect methods in order to distinguish entirely engulfed microsized particles (beads) from those which are just adherent to the cell membrane.

Material and methods: Fluorescent beads with variable and well-characterized sizes and surface coatings are incubated with alveolar macrophages (cell line RAW 264.7) at different levels of concentration and different incubation times. The direct quantification of phagocytosis is based 1) on the measure of fluorescence of intracellular beads 2) on lysosomal enzyme analysis using pHrodo® probe (Invitrogen™). Indirect quantification of phagocytosed beads is based on cytoskeleton modifications evaluated by immunocytochemistry with fluorescent anti-actin antibodies. For all the three steps, analysis is performed using both flow cytometry and confocal microscopy coupled with 3D image processing.

Results indicate a correlation between data obtained by flow cytometry and confocal microscopy concerning the number of ingested beads and the times of incubation. Complete results will be presented for both direct and indirect quantification methods.

Perspectives: Application to non-fluorescent micro and nanosized particles should be possible after validation of the indirect method.
7.54 - Carbon nanotubes affect cells by conditioning biological fluids

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Within the growing field of nanotoxicology, we have focused our research on the effects of carbon nanotubes (CNT) on blood cells, to assess possible hazard of CNT crossing the epithelial barriers. We demonstrated deep effects on leukocytes that depend on the type of cell examined. Many cell features are altered by CNT, i.e., cell proliferation, oxidative stress, Ca2+ and others. These alterations occur in (almost) the whole CNT-treated cell population, as demonstrated by flow cytometric analyses, which allow assessing the different parameters at the single cell level. Intriguingly, TEM analysis failed to reveal any CNT internalized within cells, indicating that CNT exert their cellular effects acting from the outside. Three possible explanations are now investigated in our laboratory: a) CNT may interact with plasma membrane receptors, triggering a signal transduction chain; b) CNT may be internalized by a tiny fraction of cells, stimulating release of signaling molecules (bystander effect); c) CNT, being very reactive, may interact with the complex array of molecules composing the culture medium, altering its composition thus affecting cell behavior. Here, we show the results obtained by testing the third hypothesis. We pre-incubated CNT with culture medium; the medium was then freed of CNT, and cells were incubated for the required times. These pre-treated media exert on cells effects that are identical to those obtained by incubation with equal concentration of CNT, indicating that interaction with molecules of the culture medium is the main factor determining the cellular effects of CNT.

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7.55 - Human skin penetration of silver nanoparticles

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There is a growing interest in the debate on nanoparticles safety for topical use: silver nanoparticles are widely used in creams, textiles, topical products and surgical prosthesis, but until now we have no information about their capability to penetrate or permeate the skin. This study aims at evaluating in vitro silver nanoparticles skin penetration using the Franz cells (1975) method.

Experiments were run with intact skin as well as abraded skin following the Bronaugh and Steward (1985) protocol to estimate the effect of skin lesions on the permeation rate. Physiological solution was used as receiving phase and 70µg/cm² of silver nanoparticles dispersed in synthetic sweat were applied as donor phase to the outer surface of the skin for 24h. The receptor fluid measurements were performed by Electro Thermal Atomic Absorption Spectroscopy (ETAAS). Transmission Electronic Microscopy (TEM) was used to determine the location of silver nanoparticles in the skin layers.

In the study we applied the experience and the protocol employed during the European project EDETOX (Evaluations and predictions of DErmal absorption of TOXic chemicals) founded in 2000. Median silver concentrations respectively of 0.46 ng/cm² (range 0.43-2.23) and 2.32 ng/cm² (range 0.43-11.6) were found in the receiving solutions of cells where the nanoparticles solution was applied on intact skin (8 cells) and on damaged skin (8 cells). Evaluation of metal skin content showed a significant increase of Ag in damaged skin. TEM visualization shown Ag nanoparticles in deep stratum corneum.

Our experimental data showed for the first time that silver nanoparticles absorption through intact and damaged skin was very low but detectable, and that in case of damaged skin it was possible an increasing permeation of silver. Further researchers are necessary to explore if silver can be absorbed in nanoparticle or in ionized form.
7.56 - *In vivo* biodistribution of aryl derivatised functionalised very thin MWNT

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Recently, the field of drug delivery has witnessed the development of a number of nanomaterials that have shown great promise for several therapeutic and diagnostic applications. One of these fascinating nanomaterials are carbon nanotubes (CNT) shown in proof-of-principle studies their ability as biosensors and delivery vectors for a variety of diagnostics and therapeutic agents. The rapid advances in the development of CNT for biomedical applications call for a fundamental understanding of the biodistribution and the pharmacokinetics of CNT *in vivo*, a critical step in the development of any pharmaceutical product. Herein we describe previously unreported the biodistribution of functionalised very thin multiwalled carbon nanotube (VTMWNT). Ammonium functionalised VTMWNT (f-VTMWNT) achieved via *in-situ* generated aryl diazonium salts of aniline derivative were used to covalently link the diethylenetriaminepentaacetic (DTPA) chelating agent which is used to cage the γ-emitting radionuclide (In-111) in order to track the *in vivo* biodistribution of f-VTMWNT. Transmission electron microscopy (TEM) showed the presence of different fractions of f-VTMWNT ranging from fully individualised nanotubes to large aggregates. We have observed early urinary excretion of CNT of what we hypothesize is the fraction of the fully individualised f-VTMWNT. Accumulation of aggregates in the lungs, the liver and spleen was indicated by quantitative radioactivity analysis and SPECT/CT imaging of live animals over a 24hr period following administration. Moreover, it was shown that tissue uptake of f-VTMWNT occurs rapidly, and that lung uptake seems to be transient as the CNT aggregates translocate from the pulmonary endothelium to the spleen at later time point. Furthermore, histopathology sections indicated the accumulation of large clusters of f-VTMWNT in the lungs and smaller ones in the spleen. This study indicated that CNT will exhibit different biodistribution profiles *in vivo*, with tissue affinities being dependent on different chemistries and different degrees of functionalisation which lead to different fractions of individualised and aggregated CNT within a single sample.
Nanotoxicology – 2nd International Conference

7.57 - Applications, cytotoxicity and pharmacokinetics of self-assembled lipid-nanocrystal vesicle hybrids

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Nanocrystals are novel nanomaterials that have been used to label cells in vitro or tissues in vivo. Seeking novel multimodal therapeutics, we have demonstrated that a variety of nanocrystals and lipid components can self-assemble into nanoscale, lipid-nanocrystal vesicle hybrids which were successfully used to label cells in vitro and tumor xenografts in vivo. We assessed the cytotoxicity of these hybrids in vitro. Moreover, the fate and the interaction of the lipid-nanocrystal vesicle hybrids were evaluated in animal models after local and systemic administration. In vitro toxicity, tissue biodistribution, and blood pharmacokinetic parameters were found to be dependent on multiple factors, primarily governed by the nanocrystal characteristics and the lipid coat components. By varying such parameters, we could develop various lipid-nanocrystal vesicle hybrid systems that can be considered as effective, biocompatible platforms for combinatorial diagnostic and therapeutic biomedical applications.
7.58 - Mutagenicity, the effects on DNA damage, ROS levels and the cell cycle in the Muta™mouse lung epithelial cell line

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Carbon black (CB), diesel exhaust particles (DEP), quartz, single-walled carbon nanotubes (SWCNT) and C₆₀ fullerenes (C₆₀) were investigated for cytotoxicity, genotoxicity, ROS production, proliferation effects and mutagenicity in the FE1-Muta™Mouse lung cell line. Mutagenicity was studied using a parallel continuous sub-culture setup. Cells were incubated for 72 h with either pure media or a test substances (100 ug/ml quartz, SWCNT or C₆₀)(75 ug/ml of CB or DEP)(37.5 ug/ml DEP) through eight subsequent exposure rounds. The cells were incubated with or without the test particle a total of eight exposure rounds, making the total exposure time 576 h. The cumulative dose was 8 mg (quartz, SWCNT and C₆₀), 6 mg (CB and DEP) or 3 mg (DEP). None of the materials were cytotoxic. However, cell proliferation was markedly slower with SWCNT with a larger fraction of cells in the G1 phase. This effect was evident throughout the treatment but disappeared after withdrawal. CB and DEP (75 ug/ml) significantly increased the mutant frequency. The other particles did not significantly affect the mutant frequency (MF). Results were as follows: CB 1.40-fold (p=0.0002), DEP₇₅ 1.55-fold (p=0.003), Quartz 1.30-fold (p= 0.14), DEP₃₇.₅ 1.28-fold (p=0.06), SWCNT 0.95-fold (p=0.64) and C₆₀ 0.92-fold (p=0.43). Genotoxicity measured by DNA strand breaks and FPG sites was greatest with CB was the most potent particle. It significantly increased the level of strand breaks (2-fold) and FPG sites (2.1-fold). SWCNT and C₆₀ did not induce strand breaks, but increased the level of FPG sites by 1.5-fold and 1.2-fold, respectively. Quartz gave no effects in the comet assay. ROS production was measured within cells and in cell free experiments following 3 h of exposure at varying low particle concentrations. CB produced the greatest ROS signal both within cells and without cells. SWCNT produced similar levels at the lowest concentrations whereas the other particles only resulted in weak ROS production. In conclusion it appears that genotoxicity determined by comet assay are closely related to the increase in MF. Since the SWCNT exposure did produce FPG lesions, the lack of mutations following this exposure may be due to the reduced proliferation.
7.59 - Mechanisms of uptake of nano-sized latex beads by human alveolar type I epithelial (ATI) cells

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There is a growing body of evidence demonstrating that increases in airborne particles, in particular the nano-sized fraction, are associated with increased cardiovascular morbidity. One proposed mechanism for this effect is translocation of inhaled nanoparticles across the alveolar epithelium into the bloodstream. We hypothesised that alveolar epithelial uptake and relocation of latex nanoparticles depends on size and charge.

Confluent human ATI cells (n=4) were exposed to 50 or 100nm fluorescent latex beads that were either charge neutral or positively or negatively charged. Particle uptake was visualised over 4 hours using live cell fluorescent microscopy and quantified using SimplePCI image analysis software.

ATI cells internalised all three types of 50nm beads whereas only negatively and positively charged 100nm beads were internalised. Negatively charged 50nm beads were internalised more rapidly and to a greater extent than negatively charged 100nm beads over 4 hours (approximately 3-fold). Conversely positively charged 100nm beads were internalised to a greater extent than 50nm beads (approximately 5-fold) although this may be due, in part, to induction of apoptosis by positively charged 50nm. Inhibitors of endocytosis were used to investigate mechanisms of differential uptake of the various particles. Inhibition of endocytic pathways, particularly clathrin mediated endocytosis, actin rearrangement and microtubule formation, significantly inhibited uptake of 100nm beads and 50nm neutral beads (P<0.001). Uptake of charged 50nm beads was not inhibited, suggesting that they enter the cell by passive diffusion. Cells were therefore exposed to particles at 4°C, thereby subduing metabolic processes. Internalisation by passive diffusion accounted for approximately 65% of particle uptake, indicating that the greater internalisation of 50nm particles compared to 100nm particles may be due to their ability to pass through the cell membrane by passive diffusion in to the cytoplasm whereas larger particles are internalised by active vesicular transport via clathrin coated pits and caveolae.
There is compelling evidence that environmental pollution causes pulmonary, cardiovascular and other systemic diseases. However, the underlying molecular and cellular processes leading to clinically evident pathology are not elucidated. In the present study, we have investigated developmental gene expression profiles in response to in utero exposure to carbon black particles.

Female C57BL/6J pregnant mice were exposed daily by inhalation to 40 mg/m$^3$ carbon black (the Danish Working Authorities require a daily 8-hour exposure limit of less than 3.5 mg/m$^3$), or to filtered air, for 60 minutes from gestation day 8 to 8 (2 days before expected delivery). Pups were sacrificed on days 2, 22 or 50 post delivery and whole liver tissue was collected. RNA was extracted from newborn liver tissue obtained from day 2 pups (male and females) and hybridized against universal mouse reference RNA to Agilent Oligo DNA microarrays containing 44,000 transcripts. A James-Stein shrinkage test (MAANOVA 2.0) identified 400 genes that were significantly differentially expressed (1.5-5 fold up or down regulated). These genes belong to number of pathways including embryogenesis, inflammation, cell cycle, apoptosis and signal transduction.

The roles of a subset of genes involved in carbon black response were analyzed in more detail. This list included carboxypeptidase Z, a secreted Zn-dependent enzyme implicated in wnt-signaling, arginase type II, a nitrogen metabolism enzyme potentially linked to anti-inflammatory effects, interleukin 1 beta, an inflammatory cytokine, and apoptotic chromatin condensation inducer 1, linked to internucleosomal DNA cleavage during apoptosis. We have further validated and confirmed the microarray results by quantitative real-time PCR. Our data indicates that the changes in gene expression were more pronounced in female pups compared to males.

Gene expression profiling provides a novel approach to study the effects of environmental pollution on susceptible stages of development starting from conception. In the present work we identify several molecular pathways affected in response to carbon black exposure and discuss their potential adverse effect.
8 - Ecotoxicology
8.1 - Impact of gold nanoparticles combined to X-Ray irradiation on bacteria

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Recent increase of multi drug-resistant bacteria represents a crucial issue of public health. As novative approaches are required to face this problem, those emerging from nanotechnology are of great interest.

In this context we propose the possibility to use gold nanoparticles combined with ionising radiation to destroy pathogenic bacteria. We investigated the potential X-Rays enhanced reduction of bacterial cell viability, following nanoparticle exposure, on a bacterial model, \textit{Escherichia coli}. Bacteria were exposed to 40-nm gold nanoparticles prepared by Turkevitch method, \textit{i.e.} citrate thermal reduction, and irradiated with a X-ray generator at 10.7 Gy/min. Our first concern was to confirm the absence of toxicity of the colloidal solution used. Toxicity was assessed and exposed bacteria were observed by transmission electron microscopy, confirming the lack of ad- or absorption of nanoparticles in bacteria. Gold nanoparticles were shown to weakly increase the efficiency of ionising radiation to induce bacteria cell death.

Gold nanoparticles submitted to X-Ray radiation appear to be a potential tool for anti-microbial proliferation system or pathogenic bacteria killing system. For example, they could be used as antibacterial gold nanocomposite coatings on materials, decontaminated by the application of X-Ray radiation. The emergence of resistant bacteria to traditional antibiotics motivates such a development of new antibacterial systems.
8.2 - Exposure, uptake and toxicity of nanoparticles from contaminated environments – inter-species assessments.

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This project links nanotoxicology and ecotoxicology to examine the potential risk nanoparticles (NP) pose to humans and the environment. Our work focuses on silver (Ag) and cerium dioxide (CeO2) NP used in applications such as bactericidal coatings (Ag) and fuel additives (CeO2). Ag nanoparticles as self-prescribed prophylactics are available for purchase. In addition, release of both particle types into the aquatic environment can be expected to increase in the near future, potentially leading to the exposure of a variety of species via the water or the food chain.

We used Ag particles with a diameter of 35 nm (NP) and 0.6-1.6 μm (bulk material), as well as CeO2 particles with a diameter of <25 nm (NP) and <5 μm (bulk material) to treat invertebrates, fish and human cells.

In a 96 h acute study of the aquatic invertebrate Daphnia magna, CeO2 did not cause any mortality, whereas Ag was toxic at concentrations of 0.1 mg/l (NP, 60 % mortality) and 1 mg/l (bulk material, 60 % mortality). A 21 day exposure is underway to assess how chronic exposure to the particles affects D. magna survival, growth and reproductive capacity.

Ag, but not CeO2, particles exhibited size- and dose-dependent cytotoxicity on the human hepatocyte cell line C3A (LD50 of 50 μg/ml for NP, and 330 μg/ml for the bulk material). In addition, a pilot study of Ag particles in primary trout hepatocytes showed a higher toxicity of the NP than the bulk material.

Ag was found in carp (Cyprius carpio) liver and gills after a 96 h aqueous exposure to 0.1 mg/l of Ag NP. A 21 d sub-chronic exposure, both to Ag NP and bulk material, is currently underway to further assess uptake of the particles into fish. Imaging as well as a thorough characterisation of the water are expected to deliver more detailed information about the way in which Ag is taken up by the fish.

Our work to date suggests that Ag particles have the potential to be harmful to invertebrates, fish and human models, with Ag NP exhibiting more harmful effects than the bulk material. In contrast, CeO2 particles did not cause significant toxicity in the human hepatocyte cell line or Daphnia magna.
8.3 - Removal of oxide nanoparticles in a model waste water treatment plant: Influence of agglomeration and surfactants on clearing efficiency

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The rapidly increasing production of engineered nanoparticles has created a demand for particle removal from industrial and communal waste water streams. Efficient removal is particularly important in view of increasing long term persistence and evidence for considerable ecotoxicity of specific nanoparticles. The present work investigates the use of a model waste water treatment plant for removal of oxide nanoparticles. While a majority of the nanoparticles could be captured through adhesion to clearing sludge, a significant fraction of the engineered nanoparticles escaped the waste water plant’s clearing system and up to 6 wt% of the model compound cerium oxide was found in the exit stream of the model plant. Our study demonstrates a significant influence of surface charge and the addition of dispersion stabilizing surfactants as routinely used in the preparation of nanoparticle derived products. A detailed investigation on the agglomeration of oxide nanoparticles in waste water streams revealed a high stabilization of the particles against clearance (adsorption on the bacteria from the sludge). This unexpected finding suggests a need to investigate nanoparticle clearance in more detail and demonstrates the complex interactions between dissolved species and the nanoparticles within the continuously changing environment of the clearing sludge.
8.4 - Analysis of toxic modes of action of nano-size materials using recombinant bioluminescent bacteria

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The toxic modes of actions by silver nanoparticles\(^\#\), carbon nanotubes, and titanium dioxide were investigated with a panel of recombinant bioluminescent bacteria. In our earlier study, the silver nanoparticles had cytotoxicity to wild-type of bacteria, and it is expected that carbon nanotubes and titanium dioxide also cause cytotoxicity to bacteria. In our previous work, using the stress-specific nature of the promoters present in bacterial biosensors, it was possible to determine that the silver nanoparticles cause protein and oxidative damage to the bacterial cells, but no DNA damage. Bioluminescence induction at a particular concentration range of silver nanoparticles was compared to silver nitrate and elucidated the differences between the toxic mechanisms of silver ions and the nano-sized silver particles. In this study, we are now elucidating the toxic mechanism of carbon nanotubes and titanium dioxide nanoparticles using similar panel of stress specific responsive bioluminescent bacteria to differentiate toxicities between two different nanomaterials.

Environmental toxicology research has taught us that chemicals of frequent use and/or high persistency may be globally distributed, transferred between environmental compartments and accumulated in sediments or food chains. Inasmuch as nanomaterials can be foreseen to be commonly applied, they may reach a similar scale of distribution and therefore require a risk assessment that takes their behaviour and potential hazards in different environmental compartments and to organisms at different levels of the food web into account. We know that particles that are released in the environment may undergo abiotic transformation processes, ranging from a change of surface charge and coating to potential dissolution. Substantial experience can be drawn from the field of colloid research and research on the interactions of common environmental chemicals with naturally occurring particles. Engineered nanoparticles may also be biotically transformed. Biological transformation may occur in external biogenic matrices, such as the mucus of the fish skin or gills, but also upon internalisation, e.g. by digestion of a nanoparticle surface coat. With regard to uptake, use of different mammalian cells allows to study the cellular uptake of nanoparticles by vertebrates in general. However, cell lines of other non-mammalian species help to investigate the role of external parameters, such as temperature and medium composition, both of which cannot easily be varied in mammalian cells. Investigations on nanoparticle uptake in bacteria and plants help to identify the role of the cell wall as a barrier.

As for chemicals of frequent use, the number of different kinds of nanoparticles being produced is huge. We therefore need to prioritize. One strategy is to focus on particles that are already or likely soon released into the environment. For ecotoxicology, this has led to many studies on metal-based nanoparticles, such as silver, which are of lesser concern to human health. Nevertheless, the basic phenomena observed in these studies may also inform human toxicology. In fact, there are many issues in nanoparticle research that concern both ecotoxicology and human toxicology, calling for an integrated nanotoxicology research.
9 - Proactive Risk Assessment
9.1 - NanoCare project – A German initiative on health aspects of synthetic nanoparticles: Establishing an information- and knowledge-base for innovative material research

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NanoCare is a German project, funded by the German Federal Ministry of Education and Research (BMBF), which aims to broaden knowledge about synthetic nanomaterials with regard to the potential impacts of nanomaterials on human health.

15 partners from industry, universities and research institutes are contributing their expertise to this partnership. The work plan of the NanoCare project is composed of three different parts: the generation, the management, and the transfer of knowledge. The production of synthetic nanoparticles, the subsequent analysis of primary particles, aggregates and agglomerates, as well as the behavior in biological media and effects on biological systems are focused in the generation of knowledge. In addition to the production and characterization of new synthetic nanoparticles (metal oxides like zirconium dioxide or zinc oxide), TiO₂ and Carbon Black will be established as reference materials. This enables the comparison of the results of all partners. Various analytical methods for characterization will be applied, for example: electron microscopy, ICP-MS, AAS and the BET method. In vitro studies will systematically investigate biological mechanisms of action of nanoparticles and the dependency on their size, shape, zeta potential and other important properties. In vitro data will be complemented by in vivo studies. Another work package deals with the measurement of working place exposure and agglomerate stabilities. Established measurement devices and methods will be developed further in order to determine aerosols and nanoparticles directly at the workplace during ongoing work processes. The stabilities of the agglomerated nanoparticle powders are additionally investigated with three different methods to assess deagglomeration probabilities which also influence the possible exposure. Data created within NanoCare will be interpreted together with information from literature and then published for the public on the World Wide Web (www.nanopartikel.info). Furthermore, the results will be presented and discussed with the interested public, politicians and NGOs at dialogue events.

Together with two other BMBF-funded projects (INOS, TRACER) NanoCare will help to standardize analytical procedures and will substantially increase knowledge about the biological activities of nanomaterials.
9.3 - Comprehensive environmental assessment of nanomaterials: Case studies with nano-titanium dioxide

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Although at this time there is limited information on adverse ecological and human health effects of nanomaterials, evaluation of such effects requires diligence in assessing risks. As with other production materials, exposure can also result from many different stages of the nanomaterial life cycle, including feedstocks, processing of feedstocks into manufactured nanomaterials, the distribution of nanoproducts, the storage of those products, the use of these products and finally the recycle or disposal of the nanomaterials and waste by-products. This product life cycle framework should be considered in combination with risk assessment for nanomaterials as part of a comprehensive environmental assessment (CEA). Using CEA, the National Center for Environmental Assessment (Office of Research and Development, US EPA) is developing case studies examining the life cycle of various nanomaterials in use today. Criteria for selection of case studies include current or future public exposure to the material, the nanosize of the material throughout the life cycle, data availability, potential ecological and health effects, and relevance to EPA programs. Following completion of the case studies they will then be reviewed by technical experts and stakeholders using expert judgement methods. The current case studies are being developed on use of nano-titanium dioxide in water treatment and in sunscreens. These case studies have highlighted data gaps, particularly in the lack of analysis of effects following disposal of these materials. This methodology is useful to determine data gaps and to highlight research needs for risk assessment of nanomaterials.

The views expressed in this abstract are that of the authors and do not represent the views and/or policies of the U.S. Environmental Protection Agency.
9.4 - Missing links in the human risk assessment of nano-particles – Nano-silver as a case study

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Silver is one of the substances used in nanoformulation in an increasing number of consumer and medical products, such as cosmetics and wound dressings. In general, nanomaterials may have different toxicological properties than the substances in bulk form and therefore their risks need to be assessed on a case by case basis. For human risk assessment sufficient data are needed with respect to exposure as well as the hazard. Therefore an inventory was made of the data available on nano-silver to identify the pivotal knowledge gaps that have to be filled for a proper risk assessment. Nano-silver is applied in different sizes (1-100 nm) and shapes (rods, dots, spheres, tubes) and can also occur as aggregates or agglomerates. Human exposure to nano-silver is difficult to assess. Hardly any data are available on characteristics, concentrations of nano-particles in and leakage out of products. With respect to potential human hazard, some relevant results have been published on toxicokinetics and toxicodynamics of nano-silver in the body. However, the characteristics of the silver nanoparticles that have been shown to influence exposure, kinetics as well as toxicology of the nano-silver substance, vary widely between studies and do not always correspond with those used in products. To conduct proper risk assessment of nano-silver, a more systematic approach is needed to collect sufficient data to assess the human health risks for every form and size of nano-silver.
9.5 - Assessing the toxicologic evidence base for medical surveillance of workers potentially exposed to engineered nanoparticles

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How do you determine whether there are appropriate and sufficient toxicological data to recommend surveillance of workers exposed to engineered nanoparticles? Moreover, what specific medical surveillance should be recommended? In this paper, the nature of toxicologic data on selected engineered nanoparticles is characterized and various approaches are suggested for how to assess these data and use them. Critical in determining the threshold for medical surveillance is the development of a sufficiently informative hazard evidence base. Because of the diversity of engineered nanoparticle types, it is not likely that a sufficient evidence base will be developed for each type of nanoparticle. Rather, it is likely that a combination of various physico-chemical parameters could be ranked for potential toxicity. On the basis for this ranking, various occupational health actions such as establishment of exposure registries or prescriptions for medical surveillance can be made. Key questions that arise in assessing physico-chemical parameters are: 1) whether nanoparticle size will be sufficient to trigger action; 2) whether other combinations of parameters are useful; 3) whether the evidence base that currently exists for incidental and engineered nanoparticles is sufficient to recommend a precautionary medical surveillance approach.
9.6 - Inductively coupled plasma mass spectroscopy for in vitro tests of nanoparticle toxicity

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In vitro assays based on models of such biological barriers as the lungs and the intestine can contribute to our understanding of the mechanisms by which nanoparticles (NPs) gain access to the body. Studies on the translocation of NPs across these barriers require a detection method for NPs with excellent accuracy and sensitivity. Optical methods such as fluorescence detection or light scattering can be extremely sensitive. However, fluorescence labeling has been found to alter the transport properties of the NPs, while scattering methods have been found to lack specificity. Inductively coupled mass spectroscopy (ICP-MS) is now being tested as an alternative to optical detection for the detection and quantification of nanoparticles.

ICP-MS is a classical technique for elemental analysis which has recently been improved to achieve higher sensitivities. This paper will report on its use in the detection and quantification of inorganic NPs. The influence of NP size and composition is studied, together with potential interference due to the presence of other NPs in the samples.

Particular attention will be paid to the use of ICP-MS in the context of high throughput in vitro screening of engineered NPs. Quantification of NPs in cell culture media is considered, as is the reduction of sample volume to achieve compatibility with a microfluidic system.

Finally, an outlook will be given towards the implementation of ICP-MS as the detection method for a miniaturised high-throughput system to study the translocation of NPs across in vitro models of lung epithelia.
9.7 - Translocation and injury of intranasal instilled TiO$_2$ nanoparticles on central nervous system

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The time-dependent translocation and potential damage of intranasal instilled TiO$_2$ nanoparticles on central nervous system were investigated. Female mice was intranasally instilled the well characterized TiO$_2$ nanoparticles. After exposure for 2, 10, 20 and 30 days, the titanium contents in the whole brain, lung and sub-brain regions, including olfactory bulb, cerebral cortex, hippocampus, cerebellum, were determined by inductively coupled plasma mass spectrometry. Results indicated that the instilled TiO$_2$ directly entered into the brain through olfactory bulb in the whole exposure process, especially deposited in the hippocampus, and only a little diffused into the lung. After exposure for 30 days, the pathology changes were found only for the kidneys and brain. The irregular arrangement of neurons in the olfactory bulb and hippocampus were detected using Nissl staining method. The oxidative damage (lipid peroxidation and decreased superoxidase dismutase activity) in the brain and the ultrastructure change of neurons in the hippocampus are mainly due to the deposition of TiO$_2$ particles. The increased TNF-α and IL-1β levels imply that the immune response was activated in the brain.
Recent mass upscaling of nanotechnologies has led to growing concern about potential risks to health and to the environment that could potentially result from exposure to nanoparticles. Over recent years, international reviews considering the potential risks to health and the environment from nanotechnology have led to global recognition that the safety of nanomaterials must be addressed due to their increasing implementation in everyday living.

The SAFENANO initiative is a framework which aims to enable industrial and academic communities to quantify and control potential risks from nanotechnology to their workforce, as well as to consumers, the general population and the environment.

A combination of novel research, review activities, expert opinion and training enables the SAFENANO initiative to provide the information necessary for assessment of risks specific to nanotechnology, and facilitate responsible development of safe nanomaterials. Key to this is the initiative’s maintenance of impartiality and independence of opinion.

Using a three-tiered approach comprising a website for dissemination of the latest advances in nanotechnology health and safety, a community to allow nanotechnologists worldwide to share and compare their experiences, and physical services available to industry, academia and beyond, SAFENANO encourages demonstration of responsible development in nanotechnology on a global scale.

SAFENANO’s multidisciplinary team, comprised of toxicologists, ecotoxicologists, materials scientists, exposure consultants, chemical risk consultants and occupational health & hygiene experts, all at the forefront of their respective disciplines, has enabled the initiative to provide comprehensive proactive assessment of risk across the breadth of the field.
9.9 - Alternative toxicity evaluation method of nanomaterials: Protein deformation induced by silver nanoparticles

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The deformation of amyloid proteins induced by silver nanoparticles was investigated. It have been attempted to adjust the traditional evaluation methods for nanomaterials. Although the current evaluation method is the best reliable solution at this time, it is necessary to develop more simple and practical methods. Because it is expected that the key factors that determine their toxic effects is very various and complex, current evaluation method seemed be time-consuming and ultimately impractical. Even if the alternative methods can not clarify the toxic effects of nanomaterials directly, they can give us the information of ‘possibility’ of their toxic effects. This will be able to shorten the screening works for determination of the toxicity. In this study, the amyloid proteins’ deformation induced by silver nanoparticles was used as an alternative toxicity evaluation method. Their deformation can cause the perturbation of important biological processes or diseases involving protein misfolding and assembly. To avoid the aggregation of nanoparticles in buffer, silver nanoparticles were immobilized on the self-assembled monolayers followed by exposure to protein solution. The deformation of proteins was detected by atomic force microscopy (AFM) and spectroscopic analysis. Through these results, the mechanism of deformation was expected. This fundamental study would accelerate commercialization of nano-products with safety by faster testing a new nanomaterials.
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