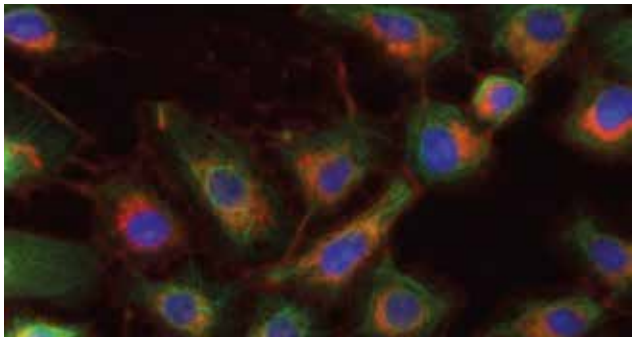
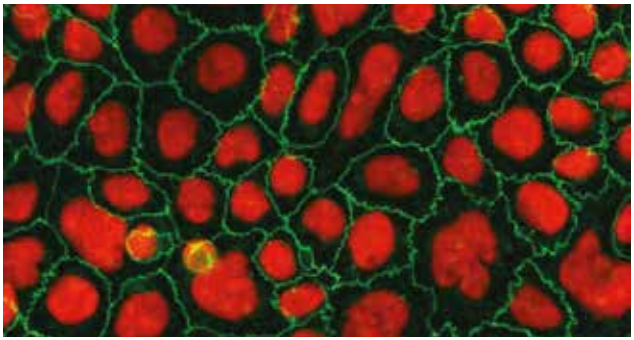
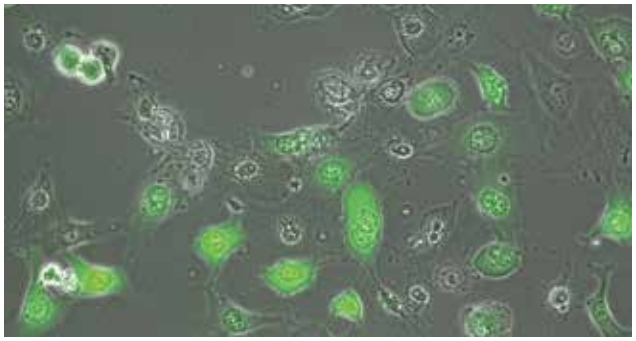
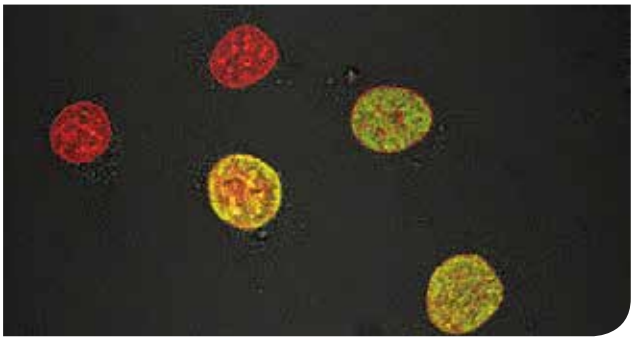
EURO NANOTOX

Assessment of Toxicological Effects by in vitro and in vivo Assays and Open Flow Microperfusion

BIO NANONET

EURO NANOTOX

4th revised and expanded edition (2015)

Content

EURO-NanoTox-Letters

EURO-NanoTox-Letters is designed in such a way that different subsets of the content may be useful to upper-level undergraduate and postgraduate students, teaching and research professors in academic programs, scientists and research managers in industry, and to professionals with an active interest in nanotoxicologic research. EURO-NanoTox-Letters also includes review and background information useful to scientists entering the field of Nanotoxicology. Abstracts of the articles can be read without registration. Registration is necessary to read the full article.

The aim of EURO-NanoTox-Letters is to increase knowledge on interactions of nanoparticles in the physiological context by investigating adsorption, distribution, metabolism and elimination of nanoparticles in order to find out to which extent toxicity testing guidelines of drug products for nanoparticles can be used for the assessment of nanoparticle toxicity.

The following top-level category structure is proposed for EURO-NanoTox-Letters:

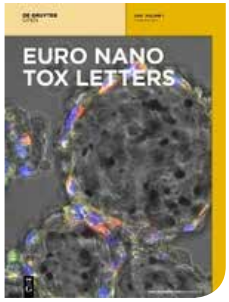
- Permeation of nanoparticles across biological barriers
- Interaction of nanoparticles with cells and effects of nanoparticles on cells
- Changes of nanoparticles by interaction with physiological fluids
- Absorption, distribution, metabolisation and excretion of nanoparticles
- Bio-persistance of nanoparticles
- Interference with test systems
- Methods for physico-chemical characterization of nanoparticles

The journal publishes original articles on all aspects of nanotoxicology; survey papers, which inform readers of the latest advances in nanotoxicology and short papers, which feature exciting research breakthroughs in the field are available resources. The journal includes in the Web-of-science database after few years depending on the success of publishing high impact papers regularly. We are looking forward to receiving high quality papers from all our colleagues to make this journal a leading publication in the field of nanotoxicology.

CrossCheck Plagiarism Screening System

The editorial board is participating in a growing community of CrossCheck System's users in order to ensure that the content published is original and trustworthy. CrossCheck is a medium that allows for comprehensive manuscripts screening, aimed to eliminate plagiarism and provide a high standard and quality peer-review process.

The editors are looking forward to receiving high quality papers from experts in the field of nanotoxicology and nanomedicine in order to make this journal a leading publication in the field.



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Introduction

The assessment of toxicological effects induced by conventional drugs, nanoparticles or medical devices includes a series of in vitro and in vivo tests.

The European Center for Nanotoxicology (EURO-NanoTox) is a growing open platform and includes the following partners:

- AIT - Austrian Institute of Technology GmbH
  - Health & Environment Department
- BioMed-zet Life Science GmbH
- BioNanoNet Forschungsgesellschaft mbH
- JOANNEUM RESEARCH ForschungsGmbH
  - HEALTH - Institute for Biomedicine and Health Sciences
- Medical University of Graz
  - Biobank Graz
  - CAM Lab, Centre of Molecular Medicine
  - Institute of Pathophysiology and Immunology
  - Center for Medical Research
  - Institute of Biophysics
  - Institute of Cell Biology, Histology & Embryology
- Messerli Research Institute of the University of Veterinary Medicine Vienna, the Medical University of Vienna and the University of Vienna
- University of Graz
  - Department of Pharmaceutical Technology
  - Institute of Chemistry, Analytical Chemistry
- University of Vienna
  - Department of Pharmaceutical Technology and Biopharmaceutics

The EURO-NanoTox partners offer a first assessment of toxicological effects for producers of chemical substances, especially of nanoparticles, and regulatory expertise on nanomaterials.

Nanotechnology is one of the key technologies of the 21st century and is associated with high expectations. Products with completely new properties for application in medicine, science, industry and various techniques are designed.

However, the larger surface area of nanoparticles makes them highly reactive compared to larger sized particles of the same chemistry resulting in both, desirable and undesirable effects.

The need for toxicological data has become increasingly important, thus several international projects are ongoing throughout the European Union. The question concerning the risks for the health and environment should not be disregarded.

The **European Center for Nanotoxicology ("EURO-NanoTox")** has been founded as an Austria-wide and internationally-visible contact point to address all aspects of nanotoxicology.

The center is active in the following areas:

1. Development and structuring of the field of nanotoxicology in Austria

2. The development, establishment and implementation of standardized in vitro and in vivo toxicological methods for nano-structured material

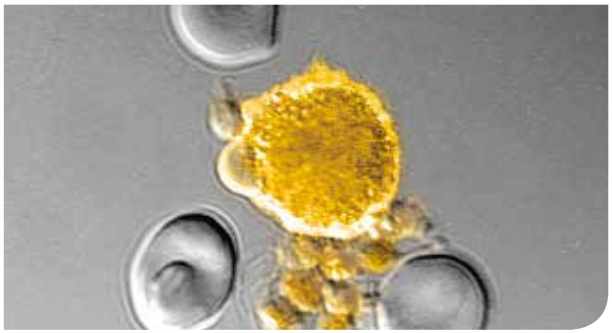
3. The development of national and international research projects on nanotoxicology

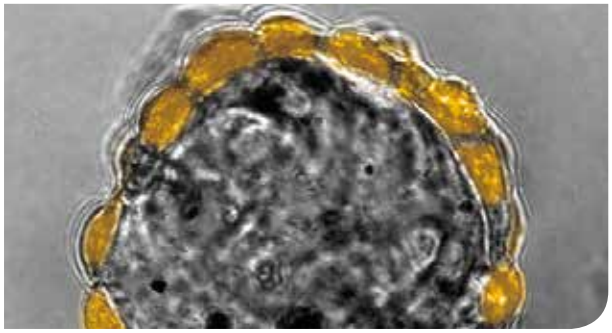
4. Provision to industry of a "tool kit" of methods for the in vitro and in vivo measurement of the toxicological potential of nano-structured materials and the carrying out and interpretation of these tests

5. The active establishment of international contacts with key players in the area of nanotoxicology

6. The development of an international scientific expert committee as review team within the European nanotox and nanosafety hub-system

7. The active monitoring of relevant literature and provision of an "information point" for interested scientists and industry partners

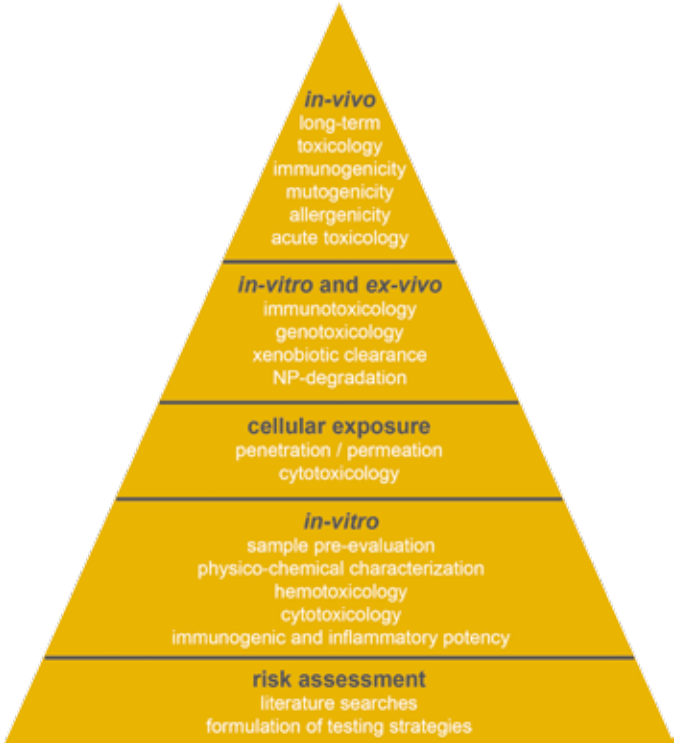




Risk Assessment for Nano-structured Materials

The toxicological profile of a given nano-structured material is determined by multiple parameters, including, amongst many others, size, payload, composition and geometrical structure. Because of this, it is essential to develop, in each case, an individual toxicology strategy tailored to each individual nano-structured material. This strategy should reflect current literature-based knowledge and enable an approach to this theme that is both cost-effective and well structured.

EURO-NanoTox prepares such testing strategies, accompanied by an overview of the relevant published information, on a service basis. This risk assessment, and the development of a strategy for the determination of the nanotoxicological profile, should constitute the first step in the toxicological testing of each novel nano-structured material.

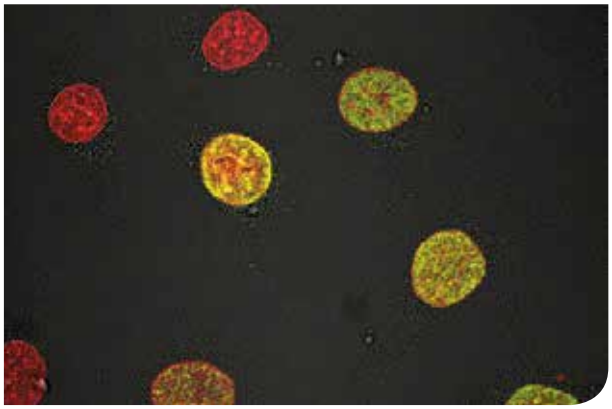


In vitro Evaluation

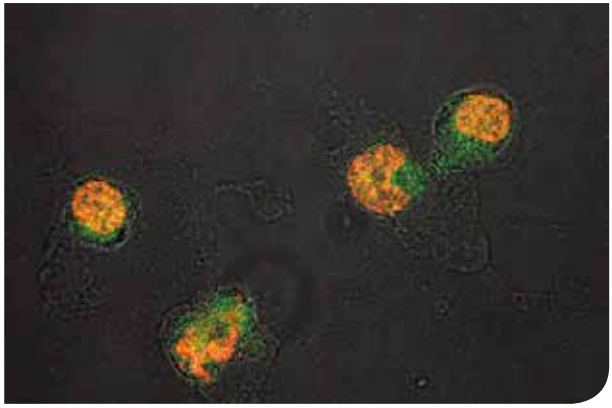
Cytocompatibility

Assays for screening the cell viability and cell number based on colorimetric, fluorescent and chemoluminescent detection are offered. Specific assays for membrane damage, generation of oxidative stress, mitochondrial damage, proliferation and apoptosis allow insight into the mechanism of the toxic effect.

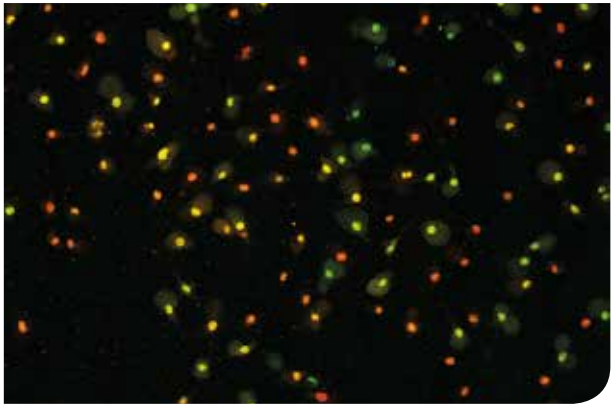
Various microscopic techniques (phase contrast, epifluorescence, differential interference contrast, confocal fluorescence microscopy) and automated image analysis in combination with radiometric and photometric read-outs are used. Co-localization of cell damage and particle-uptake and mapping of particles to organelles is possible.



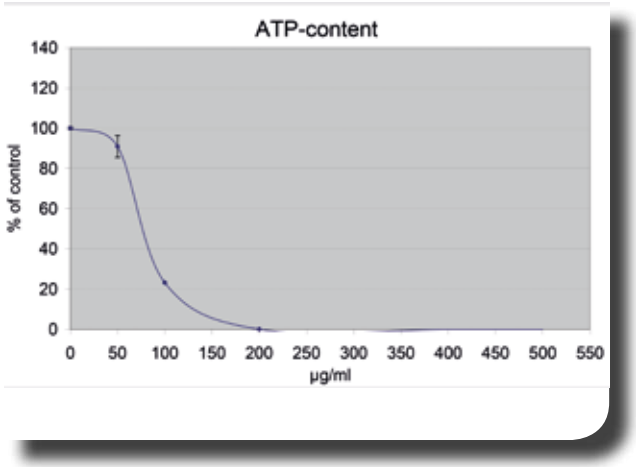
Identification of proliferating cells by BrdU incorporation and detection with anti-BrdU-antibody green).



Cells damaged by 26 nm carboxyl-polystyrene beads (green) are identified by uptake of the nuclear dye propidium iodide which stained the nucleus of affected cells red.



Identification of cell damage with nuclear dyes: YO-PRO-1 (green nuclei) is taken up in apoptotic cells and propidium iodide (red nuclei) in necrotic cells.



Typical dose-response curve observed upon incubation with a noxious agent, in this case nanoparticles.

List of available assays:

Viability screening: ATP content, LDH-release, Adenylate kinase-release, Neutral Red uptake, Calcein AM/ethidium-staining, MTT assay, XTT-assay, WST1 assay

Proliferation: Thymidine uptake, BrdU-uptake

Membrane damage: PI-exclusion, YO-PRO/PI-staining

Oxidative stress: oxidation of DCF or DHE

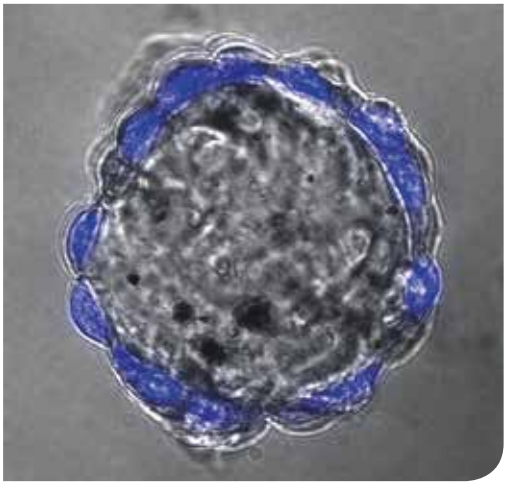
Mitochondrial damage: JC-1and TMRM uptake

Apoptosis: caspase 3/7 activation, ApoBrdU-staining, M30 staining, cleaved caspase 3 staining

Cytotoxicity Testing upon Prolonged Exposure

Nanoparticles have a tendency to accumulate in cells. To identify potential cell and organelle damage by this accumulation, cells are cultured on microbeads (Global Eukaryotic Microcarriers with different coatings) in a benchtop bioreactor and exposed to nanoparticles for 4 weeks. After the exposure cell numbers and viability is assessed.

For the detailed analysis of lysosomes as an organelle, where nanoparticle accumulation typically is seen, assays for pH, enzyme activity, membrane integrity (e.g. LysoSensor, acridine orange, cathepsin B activity, Lucifer yellow) are used.



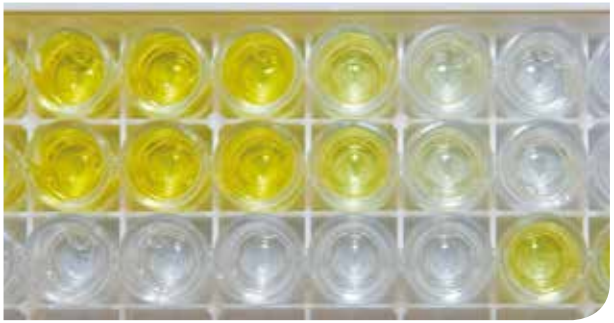
Endothelial cells that have populated a microbead

Hemocompatibility

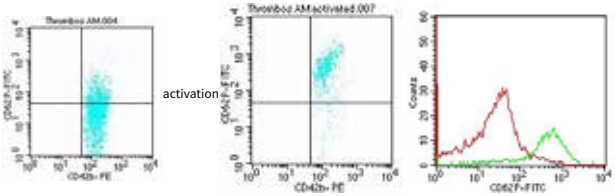
Assays for screening assess the ability to induce hemolysis and clotting in human blood.

Assays elucidating the mode of action include thrombocyte activation, leukocyte activation and lymphocyte proliferation. Microscopy, photometric-, radiometric-, FACS- and ELISA-based assays are used.

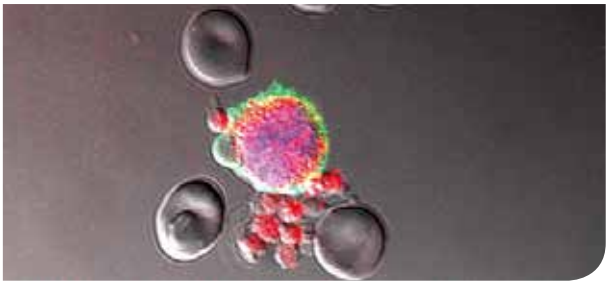
Dose-dependent increase in the degree of hemolysis induced by nanoparticles



Quantification of fibrinogen by ELISA. Similar formats are used for other clotting assays and for complement activation



Detection of thrombocyte activation by CD62P (activated thrombocytes)/CD42b (thrombocyte marker) staining. Upon activation CD62P-positive cells shift from the lower right quadrant to the upper right quadrant in the dot plot and from the red (no activation) to the green (activation) curve in the overlay.



Monocytes identified by CD14 staining (green) are phagotizing 26 nm carboxyl-polystyrene beads (red). Erythrocytes do not accumulate beads.

List of available assays:

Hemolysis: hemoglobin release

Clotting: concentrations of prothrombin fragments F1.2, TAT and D-dimer

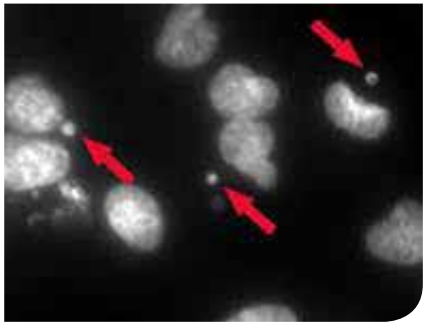
Thrombocyte activation: morphology, CD62P/CD42b labelling, quantification of PF-4 and serotonin release

Inflammation: CD11b/CD15-labelling, quantification of myeloperoxidase

Immunogenicity: complement C3a, complement C5a, mitogen activated lymphocyte proliferation, macrophage nitric oxide production

Genotoxicity / Mutagenicity

Genotoxic effects are identified by the assessment of changes in the structure of chromosomes and DNA.



Detection of micronuclei in V79 cells exposed to fullerenes

List of available assays:

Chromosomal changes: in-vitro micronucleus test, hprt mutation assay, yH2AX staining

Changes in DNA: detection of 8-OHdG

Ames test: The Salmonella typhimurium reverse mutation test can be performed to determine a possible mutagenic action of nanoparticles. This test is sensitive to frameshift mutations as well as to base pair mutations.
The performance of the test with and without an external metabolising system enables the detection of the mutagenic action of the test substance itself as well as of its metabolites.

Immunotoxicity and Cellular Stress

Nanomaterials can activate or suppress the immune system, there are many ways in which nanomaterials may drive these immune responses, through the generation of potentially damaging substances such as reactive oxygen species, or by exerting direct mechanical stress on cellular membranes or interactions with DNA, but also through indirect effects, where induction of chemical messages from other cell types leads to pro-inflammatory conditions and activated immune cells. Effects may be general or restricted to specific conditions, for example an ongoing cell activation leading to a diseased state.

The modulatory effects of nanomaterials is highly variable, and therefore no single endpoint is solely suitable when evaluating the immunosafety of nanomaterials, and, at this time, there is a need for standardized protocols that provide inclusive assessment of nanomaterial toxicological issues, which would allow the inference of relative risk evaluations, and a reliable risk assessment.

Hence, robust, cheap and reproducible tests which provide information on a number of relevant endpoints are developed, to give a clear account of immune modulation. When nanomaterials enter the body they will interact with a combination of numerous cell systems, whether it be the constituents of the respiratory tract, or that of the immune system. Numerous defense mechanisms may be elicited, including proinflammatory conditions and antioxidant defenses, which may lead to cellular stress, acute and chronic inflammation, genotoxicity, and potentially carcinogenesis.

EURO-NanoTox partners have established a panel of stable human reporter cell lines which express Luciferase, GFP, or RFP, under the control of selected promoters. These can be used to evaluate effects of chemicals and nanoparticles on cell stress induction and expression of inflammatory cytokines and chemokines, to determine the molecular pathway that has been activated, and to assess the method of cellular stress that has occurred. Luciferase, GFP, and RFP as reporter genes are highly sensitive and easy to test using a standard plate reader with a capacity for luminescence and fluorescence measurements.

Reporter assays are suitable for medium-to-high throughput formats. Positive results from screening assays are confirmed using various methods to detect gene transcripts and proteins both in cell lines and in relevant primary cells, in particular lung epithelial cells, monocytes/macrophages and T-cells. Our experiences show that most suspected effects detected in screening are confirmed by direct testing of primary human cells.

EURO-NanoTox-partners are privileged to be part of multidisciplinary EU projects, which provides our team with first-hand experience in many aspects of nanotoxicology and nanosafety. We are working with other industry and academic leaders in this field towards assembling standardized protocols for nanotoxicity research, and therefore can provide expertise, in both an advisory and a practical capacity, on nanoparticle synthesis, characterization, and toxicology, but also upon the health and safety issues and risk assessment of exposure to nanomaterials.

List of available assays:

Cytotoxicity and Proliferation: Alamar Blue®, Toxilight®, CytoTox 96®, Cell Titer Blue®, MTT, ^{3}[H]-Thymidine

Luc reporter cell lines: In A549 human lung epitelial cells IL-6, IL-8, TNF-α, NF-κB. In Jurkat human T cells IL-4, IL-6, IL-8, IL-13, IFN-γ, TGF-ß, TNF-α, NF-κB. In HeLa IL-8. In HEK Eotaxin-1.

GFP cell line: In A549 IL-8, other proinflammatory cytokines and transcription factors (in progress), and markers of oxidative stress.

Follow-up tests: qRT-PCR, ELISA, Luminex, flow cytometry

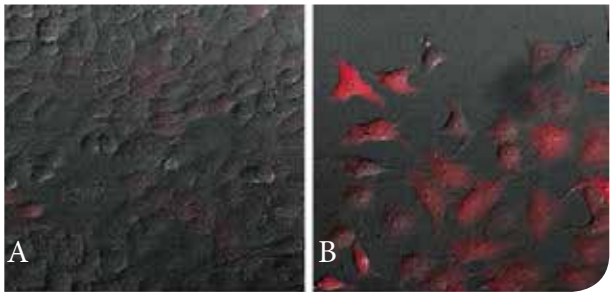


Figure I: Reporter gene cell lines allow screening of different substances in different concentrations at different time points. The figure shows the induction of the IL-8 promoter activity in RFP transfected human lung epithelial cells (A549), either (A) unstimulated or (B) stimulated with 20ng/ml TNF-α.

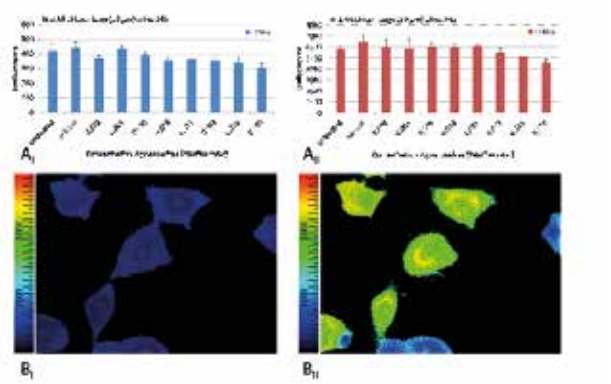
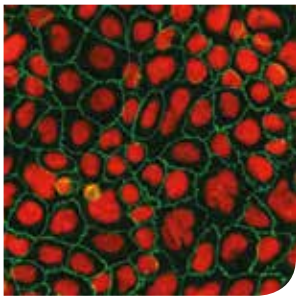


Figure II: The investigation of nanomaterial effects requires the parallel analysis of different endpoints: here it is shown that with the treatment of A549 cells with 12µm silver nanowires (A) there is no induction of IL-8 gene expression (assessed using the luciferase reporter cells), while the same length of silver wire (B) induces considerable release of an intracellular signaling molecule (Ca2+), represented as (BI) untreated and (BII) particle treated; therefore highlighting the need for numerous endpoints when assessing nanotoxicology.

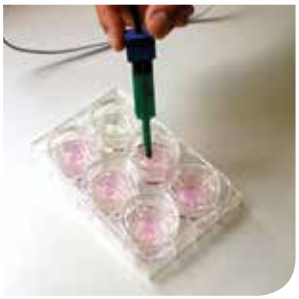
Evaluation at the Tissue Level

At the interface between development and clinics, tissue models of the human intestine (Caco-2) and human bladder(SV-HUC1) represent a well standardized and reproducible alternative to animal trials.

As opposed to single cells, this set-up offers information about tightness and viability of the artificial tissue as well as permeability of the particulate matter.



Immunofluorescent staining of tight junctions (green) and nuclei (red) of Caco-2 monolayers



TEER-measurement of monolayer cultures

List of available assays:

Proliferation: BrdU-assay

Tightness: TEER-measurement, tight junctional staining

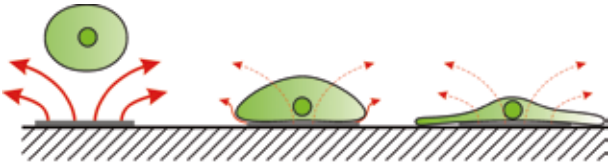
Viability: Formazan bioreduction, FDA/ethidium bromide staining for live/dead discrimination

Cell morphology: Immunofluorescent staining of cell structures

Adherent Cell Cultures

The development of drugs, medical products or chemicals demands high throughput procedures for the active substance testing at cells and tissues. This sensory system allows a high volume production and a non invasive monitoring of permanent cultures. Wireless and batteryless insets for state-of-the-art microtiter plates are realized.

Alterations of the cell metabolism which are reflected by changes of the surrounding electrical impedance are measured by the sensors of the insets. This non-invasive monitoring system allows tracking effects of toxic substances within the same cell culture up to weeks.



Features and applications

The CellMonitor

- uses standard cell culture equipment,
- is integrable in existing incubators in a space-saving way,
- has low maintenance demands and allows
- the continuous measurement of long term behaviour of cell cultures over weeks,
- proliferation and toxicity tests,
- the reduction of material and labor costs com pared to end point assay results.

If cells settle onto the sensor of the inset they cause a characteristic change in the electrical response. The provided signal is used for monitoring adherence, growth and death.

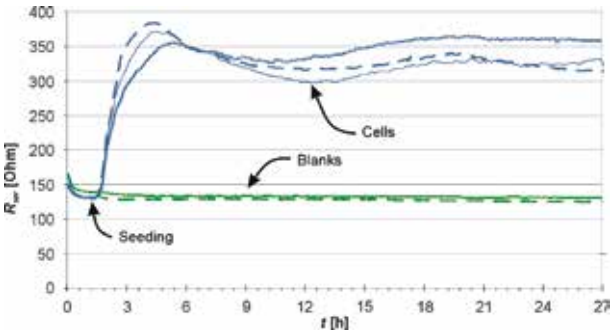


Image of a Franz diffusion cell, consisting of a donor and a receiver compartment. Between the compartments a synthetic or biological membrane is inserted and fixed.

Key benefits

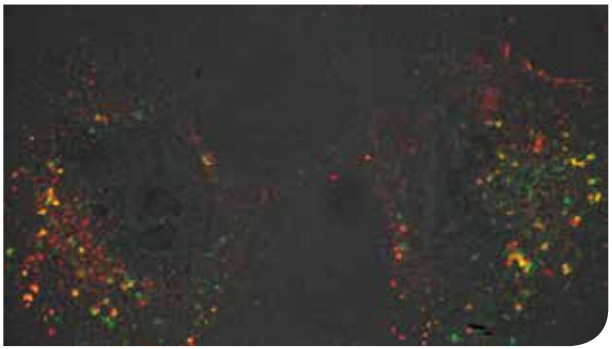
- Non-invasive: The measurement system does not interfere or harm the cell cultures
- Label-free: No labels or reporters are necessary
- Real-time: The measured data reflects the actual state of the cell culture with a sampling rate down to 1 sample/minute

- Easy to handle: CellMonitor bases on standard cell culture materials and dimensions
- Extendable: Cascading of several CellMonitor sys tems inside the incubator
- Flexible: The detachable sensor devices can be adapted to different cell cultures
- Low Maintenance: The sensors are battery-less
- Space-saving: Integration in existing cell culture laboratories
- Comfortable: MS Windows-based control software with online-protocol ability

Sensor tags in a 6-well microtiter plate and a NFC reader system for energy and data transfer

Additional Services

Endotoxin testing, live cell imaging of uptake of fluorescence-labelled substances/particles, Co-localization studies of nanoparticles and organelles.



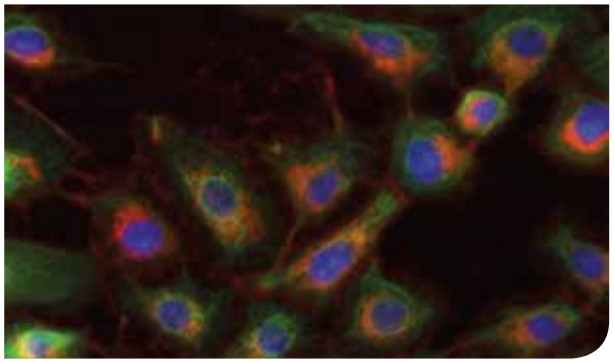
26 nm carboxyl-polystyrene beads (red) are taken up into endothelial cells. Co-localization with lysosomes (green) is seen in yellow.

In-vitro and ex-vivo mimicking of the urothelium

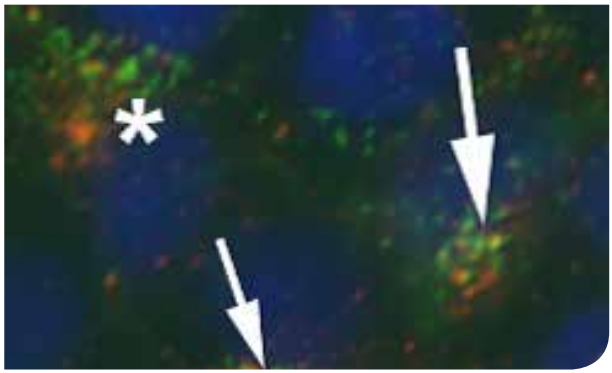
The urothelium is the lining tissue of the lower urinary tract and efficiently inhibits diffusion of the urine into the surrounding tissue. The unique structural and functional configuration makes it the hardest to penetrate epithelial barrier in the human body. Nevertheless, the urothelium is the focal point of widespread diseases such as urinary tract infections and neoplastic disorders. Although it is well accepted that size below 10nm and chemical composition determine the renal clearence of nanoparticles, currently no literature is available about the interaction of bladder cells with nanoparticles.

For mimicking the healthy human urothelial barrier in-vitro the Sv-HUC 1 cell line is used which forms monolayers of moderate tightness. Apart from that cell lines of different malignant grading (5637, grade 2; HT-1376 and T24, grade 3) featuring altered glycosylation patterns are in use as single cells and monolayers cultivated on glass and plastic labware to assess binding and uptake of particles.

The analytical techniques applied are flow cytometry, fluorescence- and deconvolution microscopy and fluorescence based quantification techniques in addition to established toxicity assays. For ex-vivo investigation of the interaction between particles and urothelium a protocol has been established for isolation and propagation of porcine primary cells as well as monolayers cultivated on transwells.



Human urothelial primary cell culture isolated from donor material without history of malignant transformation after cultivation for 5d. Intermediate filaments stained in green confirming epithelial origin, nuclei stained in blue and accumulation of added wheat germ agglutinin in vesicles in the perinuclear region as well as at the cell border in red



Partial lysosomal accumulation of fluorescent nanoconjugates by co-localisation with LAMP 2 in artificial human urothelial (Sv-HUC) monolayers (arrows)

Moreover, porcine bladder specimens serve as a nearly human-like model for binding and uptake studies of particulate drug delivery systems. Human urothelial primary cell culture. Partial lysosomal accumulation of fluorescent nanoconjugates by colocalisation with LAMP 2 in Sv-HUC monolayers (arrows).

Animal in vitro / ex vivo Cell-Barrier Models

Permeation of Nanostructured Materials across the Oral Mucosa

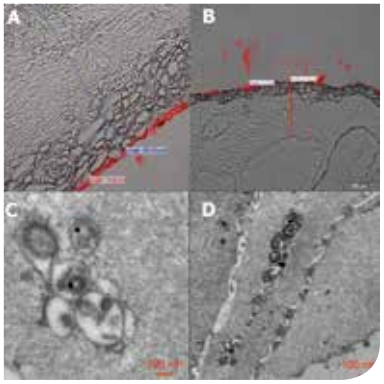
The oral mucosa offers good opportunity for drugs to be absorbed; therefore, it is possible that nanostructured material can permeate across this tissue too.

The ex vivo models used for the investigation of the permeability/penetration of NMs are static Franz type diffusion cells. They consist of a donor and of a receiver compartment. Between these compartments the buccal mucosa of the pig is inserted and fixed with retainer clips in such a way that the epithelium faces the donor and the connective tissue region faces the receiver compartment. The porcine mucosa is most similar to the human one in ultrastructure as well as in enzyme activity; it is a well-known tissue used for the evaluation of drug-penetration/permeation in ex vivo studies.

The viability of the mucosa prior and during the experiment is evaluated via MTT assay.

The graph above shows the formation of a 100 % confluent layer of 3T3 mouse fibroblasts after seeding. Blanks are monitored simultaneously.

Fluorescent labelled NMs are visualized after histopathological slicing, carried out by a cryo-microtom Microm HM560, by fluorescence microscopy. Metal nanoparticels (Silber) are visualized by TEM and element analyses (EFTEM, EDS).



Histological sections of the oral mucosa charged with A) 20 nm CP particles (100 µg/ml) B) 200 nm CP particles (100 µg/ml) C) and D) 35 nm Silber particles (20 µg/ml)

High resistance epithelial cell barriers restrict passage of different compounds. The high resistance is due to the formation of intercellular lipid barriers (from membrane coating granules) and the differentiation of the cells into squamous epithelium.

Human TR 146 cells are a widely applied cell line for the performance of in vitro transport studies. To monitor intact barrier behavior of the cell monolayer the transepithelial electric resistance (TEER) is measured (EVOM Voltmeter, MPI).The TEER value is a parameter that increases with time as cells form a tight monolayer and decreases when integrity is insufficient. The uptake and outflow of NM is characterised by fluorescence detection.

Overview of techniques:

Characterisation of NM in buccal and/or gastric/intestinal mucosa (size, size distribution, surface-charge, zeta potential); purification of NM (Ultracentrifugation); viability structural integrity for porcine mucosa (viability assays for the determination of tissue viability - MTT, methylene blue/PBS solutions with and without EDTA); permeability ex vivo (Franz diffusion cell, established for buccal mucosa); permeability in vitro (Transwell Systems TR 146 cells, established for buccal and intestinal mucosa).

Dermal Absorption / Percutaneous Penetration

In this assay integrity checked dermatomed pig skins are inserted in static penetration cells (Franz-cells). The nanoparticles are applied topically to the horny layer of the skin. After 48 hours the stratum corneum is removed by repeated stripping with adhesive tapes to obtain the adsorbed test substance. The remaining skin is taken to determine the absorbed test substance.

The penetration is calculated from the mass of the nanoparticles in the receptor fluid, consisting of phosphate buffered saline. The overall amount of bioavailable nanoparticles is defined as the sum of absorbed and penetrated quantities.

Epiderm Skin Irritation / Corrosivity Test

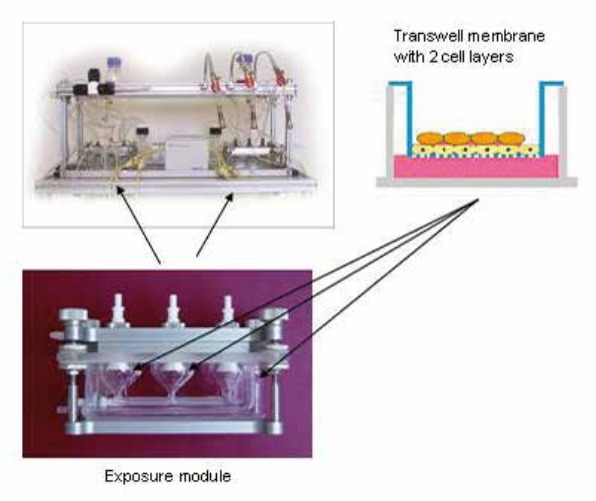
The Epiderm Skin Corrosivity/Irritation Test reveals possible irreversible tissue damages of the skin following the application of nanoparticles. The test substance is topically applied for to the epidermal surfaces of three-dimensional human epidermis models, followed by immediate determination of the cytotoxic effect. If there is no corrosive effect observed, the test substance is topically applied to the epidermal surfaces of three-dimensional human epidermis models and after a post-incubation of 42 hours, a cell viability test is performed.

In vitro Inhalation Exposure

The in vitro inhalation system CULTEX® enables the investigation of various celltypes or co-cultures exposed at the air/liquid interface. Cells are seeded on transwell membranes and incubated in companion plates in CO2 incubators. After removal of the cell culture medium the membranes are transferred into the exposure module. Then the cells are exposed on their superficial surface with the test atmosphere containing naonoparticles, while they are supplied with nutrient medium through the membrane below.

Thus, an in vitro system of human cells that simulates the exposure situation in human lungs is generated. Preferred celltypes used in this system are human lung epithelial cells and macrophages in co culture.

The influence of the test atmosphere on the cells can be determined by the observation of e.g. the integrity of the cell layer by the measurement of transepithelial electrical resistance (TEER), cytotoxicity (e.g. MTT assay, WST1, neutral red uptake), expression of proinflammatory cytokines and oxidative stress reaction.



CULTEX® exposure unit

Human in vitro Cell-Barrier Models

The effect of nano particles (NP) in human cells can be tested in various cell models:

- The EpiDerm™ Skin Model was proposed to assess the potential dermal irritation of test particles according to the EU classification system (R38). Using this model Zet life Science participated successful in an international validation study of the EpiDerm™ skin irritation test in 2007 together with three further partners. Finally, this model was fully accepted from the European Centre for the Validation of Alternative Methods (ECVAM) as alternative model to the Draize Eye Test (animal testing).

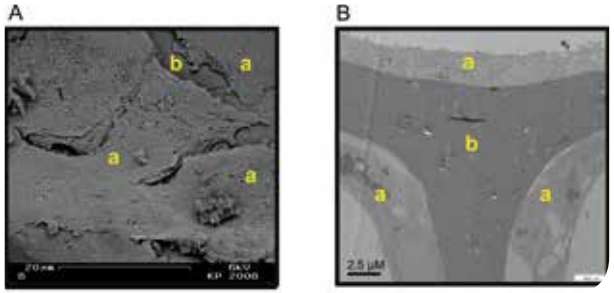
- MucilAir™, an in vitro test system of primary human respiratory epithelium, mimics the in vivo tissue of the human respiratory epithelium and it can be kept in culture for more than 12 months. This is a unique property and therefore this system is the only one and the first one which has the potential to replace or to reduce the use of the in vivo rodent-2-year-bioassay (OECD-guideline 451) for the identification of carcinogenic substances.

- Our established human 3D liver cell model for drug metabolism and hepatotoxicity studies consists of polystyrene scaffolds and HepaRG cells and resembles more closely the in vivo situation. It has many improved functions compared to a 2D liver cell model, where HepaRG cells grow flat.IV) The intestinal barrier model using Caco-2 cell lines for substance screening and transport and permeability studies.

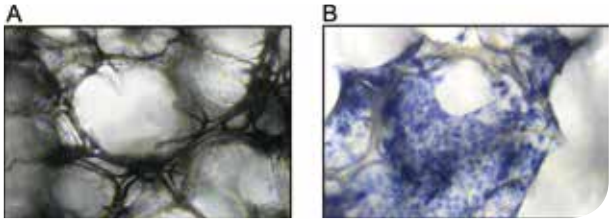
3D Liver Cell Model

HepaRG cells (Inserm, France) will be cultured on microporous scaffolds. The DMSO-containing medium is used to differentiate the HepaRG cells in vitro into polarized and functional hepatocytes. The differentiated HepaRG cells display typical hepatocyte-like gene and protein expression and functions, for instance albumin secretion, and phase I drug-metabolizing enzyme activity (cytochrome P450 isoforms, CYPs).

Thus, this 3D liver cell model is a reliable and powerful tool for studying the response under conditions of acute, repeated and continuous exposure to NP. For testing long-term liver toxicity, low concentrations of the NP must ideally be applied over weeks to months to functionally and metabolically active hepatocytes. The microporous substrate optimally ensures long-time hepatocyte culture.



Investigation of cell morphology and attachment of HepaRG cells onto the polystyrene scaffold using (A) scanning (SEM) and (B) transmission electron microscopy (TEM).
A: HepaRG cells, B: polystyrene scaffold.
(Electron microscopy pictures were arranged by Prof. Walter Pfaller, Innsbruck Medical University, Division of Physiology, Austria)
Visualisation of the HepaRG cell growth (colonisation) in a small area of the polystyrene scaffolds by haematoxylin staining. A: Area of an unseeded polystyrene scaffold. B: HepaRG cells grown within the polystyrene scaffold. Cell nuclei stained blue, 200 x fold magnification. Cell staining revealed an uniform distribution of HepaRG cells in the polystyrene sponge.



Visualisation of HepaRG cell growth (colonisation) in polystyrene scaffolds by haematoxylin staining. A: Empty polystyrene scaffold. B: HepaRG grown within the polystyrene scaffold. Cell nuclei stained blue, 200 x fold magnification. Cell staining revealed an uniform distribution of HepaRG cells in the polystyrene sponge.

Cellular Responses to NP in 3D Liver Cell Model

The chemical reactivity both of particles and of impurities found in particle preparations and the physical interaction of particles with cellular structures involved in the catalysis of biological reduction-oxidation (redox) processes may mechanistically be responsible for the enhancement of different cellular responses generation by NP.

List of available assays:

Cell viability: MTT assay

Cell toxicity: LDH assay/Resazurin

Reactive oxygen species generation: Rhodamine 123 assay

Mitochondrial membrane potential: JC-1 assay

Lipid peroxidation: Thiobarbituric acid reactive substances (TBARS) assay

Liver injury markers: Alanine aminotransferase (ALT)

Albumin depletion: ALT assay, albumin ELISA

Inflammatory response: E.g. IL-6/IL-8 secretion: ELISA assays

Glutathione status: Reduced glutathione (GSH) and oxidized glutathione (GSSG) are determined by a colorimetric/fluorometric measurement

Stress and genotoxic responses: HO-1 (heme oxygenase 1), MKP-1 (mitogen activated protein Kinase phosphates 1) and p53 expression are determined by qRT-PCR and/or western blot analysis Transcriptional activation of the HO-1 and MKP-1 gene are considered to be an adaptive response to oxidative and cellular stress and confers a protective capacity against cell and tissue injury. HO-1 and MKP-1 influence a number of cellular processes including growth, inflammation and apoptosis. Wild-type p53 is probably best recognized for its tumour suppressor function whereby p53 can induce either a cell cycle arrest or apoptosis in cells that have sustained DNA damage. The enhanced expression of p53 mRNA may be a ''secondary'' biomarker for genotoxic events directly or indirectly induced by NP.

Liver Cell Function Parameters

To quantitatively assess the effect of NP on the stability of liver specific functions, albumin secretion and ALT release, are measured over time.
To assess the influence of NP on xenobiotic metabolism, selected cytochrome-P450 (CYP450) mRNA expression and activity is measured over time using qRT-PCR and fluorogenic substrates for high-throughput screening according to Donato et al. Fluorimetric assays are rapid and highly sensitive, and allow performance of P450 activities in a microtiter plate formate.

List of available methods:

Albumin secretion: ELISA

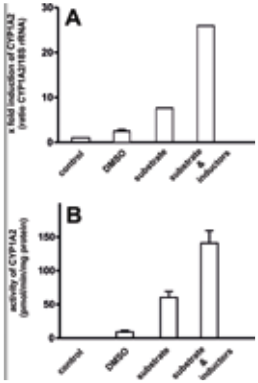
ALT release: ELISA

CYP450 mRNA expression: qRT-PCR

CYP 450 activity (xenobiotic metabolism): E.g. CYP1A2, CYP3A4 activity assay.

Intestinal Barrier Model

How NP can affect the intestinal cell membrane properties and change the net intestinal absorption of other substances present in the lumen has been poorly understood and investigated.



mRNA expression of CYP1A2 (A) was measured by quantitative real time PCR (qRT-PCR) and CYP1A2 activity (B) by means of fluorometric substrates in differentiated HepaRG cells grown under 3-D culture conditions. Substrate: Ethoxyresorufin. Inductors: Dicumarol + 3-Methylcholanthrene.

Effect of NP on the cell membrane properties across an intestinal barrier model

The influence of NP on the cell membrane properties, which may be subsequently affecting absorption processes across an intestinal barrier model, will be tested using following methods:

Cell viability: LDH /MTT assay

Membrane integrity: TEER/ impedance spectroscopy

Cell capacitance: Impedance spectroscopy

Cell surface topography: Atomic force microscopy (AFM) and Scanning electronic microscopy (SEM)

Intestinal marker, transporter, Membranproteine der Tight Junctions, post-translational modifications: Western blot analysis (WB), Immunoprecipitation (IP), immunofluoresencence microscopy (IFM), gel-shift assays

Membrane permeability: Permeability studies of different sized FITC-Dextran molecules

MDR1 transporter activity: Rh123 assay

The Human Placental Explant Culture Model

The placenta is a highly species specific organ. Only in apes is the histo-morphology and the physiology of placentation similar to the human situation. Nanoparticle transfer across biological barriers is thought to be much more dependent on the specific histological structures than the transfer of typical lipophilic drugs of low molecular weight.

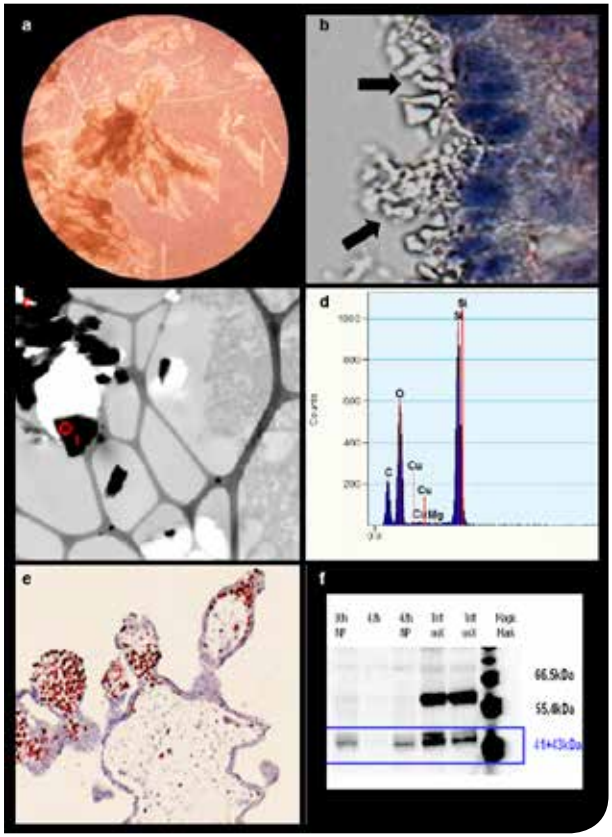
Therefore, to evaluate a possible impact of nanoparticles on human placental development and to check whether the human embryo or fetus would be exposed to nanoparticles as well, we employ the first trimester as well as term human placental explant culture model as a comparatively cheap and valuable approach towards nano REPRO tox.

Placenta material from elective terminations of pregnancy or at the time of delivery is collected, approved by the local Ethics Committee. Little branches are cut from the villous tree and cultivated under physiologic oxygen pressure (3% for first trimester and 8% for term explants) for up to 3 days according to the recommendations of Miller et.al. (Miller RK, Genbacev O, Turner MA, Aplin JD, Caniggia I, Huppertz B. Human placental explants in culture: approaches and assessments. Placenta. 2005 Jul;26(6):439-48).

In the culture supernatant we measure ß hCG as a marker for the secretory capacity of the villi, and LDH release as a viabilitymarker for the explants.

The tissue is then used for routine morphologic examination, immunohistochemistry, electron microscopy or biochemical analysis e.g. Western blotting. Thereby we analyze a possible impact of nanoparticles on the structural integrity of the tissue and the cells, as well as on proliferation and on apoptosis in the human placenta.

In addition we focus on tracking nanoparticles and we try to determine to which extend they penetrate into the tissue. We analyze how nanoparticles cross the placental barrier during pregnancy and whether or not we have to assume that they also reach the embryonic or fetal capillaries in the villi, suggesting a possible embryonic or fetal exposure.



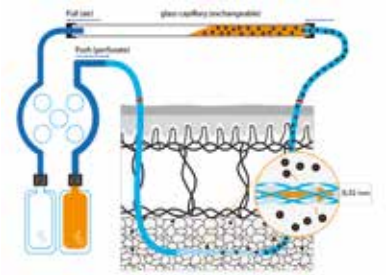
Picture a) shows placental villi of first trimester human placenta in the culture dish (phase contrast microscopy).
In b) arrows indicate silica crystals of about 2µm attached to the outer surface of the villous surface, the syncytiotrophoblast, in a routine histological section of embedded explants, stained with hematoxylin and eosin.
c) represents an electron microscopic image used for element analysis based on the EDX technology.
d) the EDX – results from the red focus (1): the crystals seen in c) seem to consist predominantly of the elements silicon and oxygen. e) shows an immunohistochemical staining pattern for Ki67 in a section from an explant, indicating proliferation in some of the villi.
In f), the comparison of band intensities of a Western blot stained for caspase 8 reveals increased expression of this marker for apoptosis in nanoparticle exposed explants, compared to controls.

Open Flow Microperfusion

Reliable sampling and quantification methods are essential to adequately assess nanotoxicity levels. One important aspect to fully understand the concept of nanotoxicity is the assessment of various effects of nanoparticles in different tissues. Tissue-specific effects of nanoparticles greatly depend on the local nanoparticle concentration. Therefore, reliable sampling methods are essential to quantify nanoparticles.

Open flow microperfusion (OFM) offers reliable sampling and quantification of nanoparticles in different tissues, such as skin, brain and adipose tissue. OFM uses a steel mesh with macroscopic openings in combination with a peristaltic pump in push/pull mode to achieve stable continuous sampling.

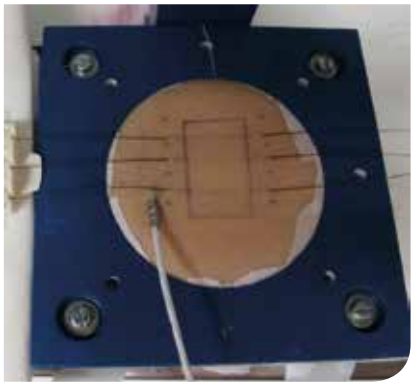
The macroscopic openings allow an unhampered exchange of all substances (incl. nanoparticles) between the interstitial fluid and the OFM sample regardless of substance size, lipophilicity, or charge. Thus, OFM samples represent an unfiltered, merely diluted sample of the interstitial tissue fluid.



Schematic figure of the OFM system with a linear membrane-free OFM probe. The inserted OFM probe is connected to a peristaltic pump via push–pull tubing. The OFM pump simultaneously pushes the perfusate into the OFM probe and pulls the OFM sample into an easily exchangeable vial. At the exchange area, substances are freely exchanged between the ISF and the perfusate.

Ex vivo

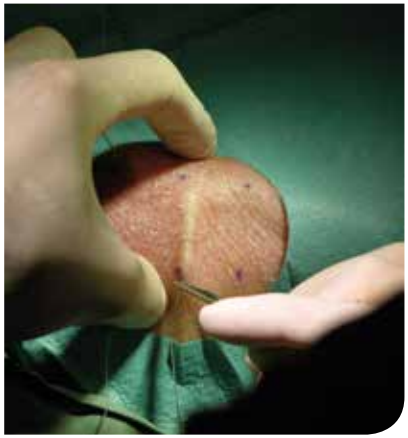
Ex vivo studies are used for a first assessment of nanoparticle pharmacokinetics. OFM has been successfully used in freshly explanted human skin to study nanoparticle transport after topical application on the dermis.



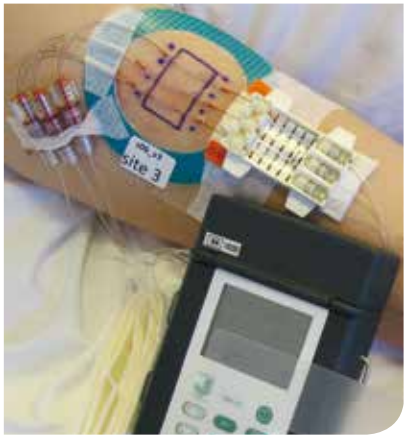
The OFM system can be operated in explanted human and pig skin. This setup allows quantitative assessment of substance penetration through the skin without a clinical study.

In vivo

For in vivo studies OFM sampling can be used to study the pharmacokinetics and pharmacodynamics in animals (preclinical) and humans (clinical). Nanotoxicity can be assessed in muscle, skin, brain and adipose tissue. In combination with the latest analytical methods (Orbitrap, Triple-Quad-MS, GC-MS, HPLC, metabolomics) OFM can provide information about the toxicological effect of nanoparticles on tissue metabolism.



OFM probes are inserted into the dermal layer of the skin at a depth of 0.6 mm.



Skin sampling site on forearm with three inserted dOFM probes. The site is stabilized for a 36 h investigation by using a self-adhesive ring. A rectangular zone within the site is marked with the insertion pattern. Dermal ISF is sampled continuously using the portable OFM pump, a push–pull tubing set and a sampling unit with glass capillaries as sampling vials. Portable equipment enables patient mobility without interrupting the sampling protocol.

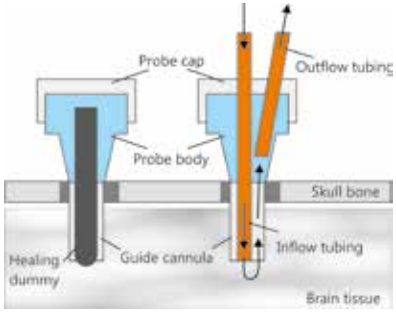
Adipose OFM (aOFM): Subcutaneous adipose tissue is an important endocrine organ that modulates metabolism, immunity and satiety. An accumulation of nanoparticles can contribute to pathophysiological processes and lead to autoimmune diseases. aOFM can be used to quantitatively assess nanoparticles in fat tissue and evaluate their toxicological effects.

Dermal OFM (dOFM)

The skin forms a major barrier to environmental nanoparticles and is increasingly exposed to topically applied substances that contain nanoparticles. dOFM allows a quantitative measurement of nanoparticles which enter the body through the skin.

Cerebral OFM (cOFM)

Nanoparticles are used to transport drugs across the blood-brain barrier directly into the brain. But there is little data available about the behavior of these carrier nanoparticles once they have crossed the blood-brain barrier. cOFM can sample and quantify nanoparticles in various regions of the brain and thus be used to assess potential toxicological effects.



The cerebral application of OFM (cOFM) enables sampling in physiological, non-traumatized brain tissue with an intact blood-brain barrier.

The open structure of cOFM puts perfusate in direct contact with brain tissue and brain ISF for sampling of lipophilic and high molecular weight substances.

Ex vivo Evaluation

Ex vivo Perfusion Model for the Human Third Trimester Placenta

The human placenta is a multicellular organ that connects the maternal with the fetal circulation and is responsible for the supply of nutrients, the removal of waste products and the protection of the fetus against harmful substances. It is segmented into 15 – 30 feto-maternal functional units, called cotyledons. Within one cotyledon, treelike structures are formed as key barrier by the syncytiotrophoblast layer, a multinucleated, epithelial derived syncytium which is in contact with maternal blood.

The syncytium at term is in close proximity to fetal endothelial cells building blood vessels and capillaries. The placental transfer of substances across this barrier depends on four different mechanisms: passive diffusion, active transport, phagocytosis/ pinocytosis and biotransformation through metabolic enzymes.

At time of delivery, the placenta is still viable and metabolic active. The human placental ex-vivo dual perfusion model allows keeping the tissue under physiological conditions in an active state. Permeability, active and passive transport, vasoactive effects and toxicity of small chemical compounds, biomolecules like lipids and nanoparticles can be studied in this human model within a native epithelial-endothelial barrier.

Several parameters are measured during the placental ex-vivo dual perfusion experiment, in order to monitor viability of the tissue and the impact of experimental conditions (substances) on the tissue.

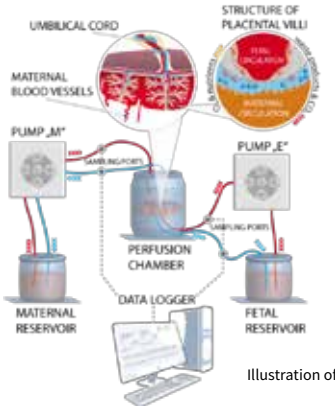


Illustration of the ex-vivo placenta perfusion system

Blood gases (pO2, pCO2) as well as pH, glucose consumption and lactate production are determined. Blood vessel pressure is online recorded by a microcatheter within the perfusion system. These data is collected by a specific software in order to fulfill GMP requirements. The small, lipophilic drug antipyrine is used as a control substance for passive diffusion, in order to determine the degree of overlap of maternal and fetal circulation, in each experiment.

Collected perfusion media samples can be used for transport kinetic studies of nanoparticles from maternal to fetal and fetal to maternal side. Furthermore biological nanoparticles >50nm can be isolated and concentrated by ultracentrifugation for testing after the performed experiment. The perfused placental tissue can be used for electron- and microscopic techniques to identify the accumulation of nanoparticles within the tissue.

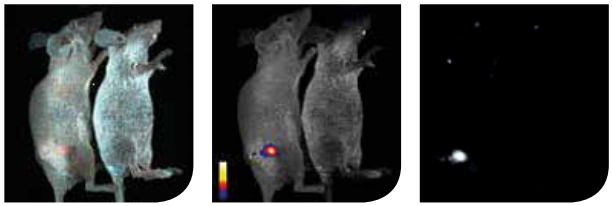
In vivo Evaluation

In vivo Imaging

The assessment of toxicological effects includes information on the bio-distribution of the substance. Especially particulate substances can be traced by their inherent physico-chemical properties or by the attachment of fluorescent tags.

Optical Imaging

Fluorescent nanoparticles are tracked with high sensitivity and quantitative analysis of the distribution by Maestro CRi. Multiple labels can be tracked in the same animal.

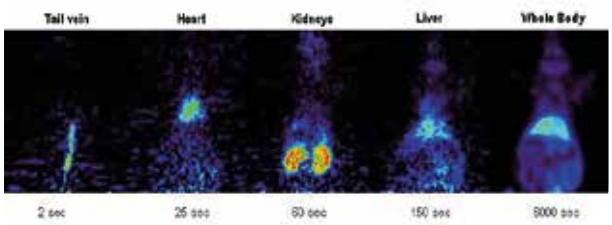


Conventional RGBImage | Quantitative Composite Image | Spectral Unmixed Image

Accumulation of the label is seen in the hint leg. Autofluorescence is removed by spectral unmixing.

Molecular Imaging with Animal PET

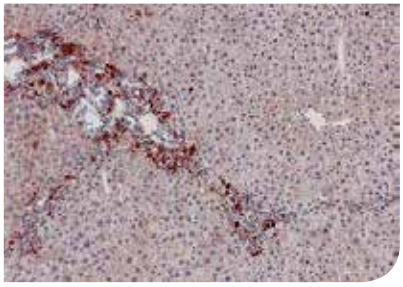
Positron Emission Tomography is an analytical imaging technology developed to use compounds labelled with positron emitting radioisotopes as molecular probes to image and measure biochemical processes in vivo.



Using PET provides quantitative determinations of the spatial and temporal distribution of radiolabeled molecules (PET-tracers) in living small animals. These PET-tracers can be used to explore various biological processes such as metabolism, enzyme-substrate reactions, specific receptor-ligant interactions, protein- or DNA-synthesis, gene-expressions, oxygen consumption and cell-membrane formations.

Toxicology

Evaluations of the in vivo effect of nanoparticles include blood count and clinical chemistry (serum parameters for liver damage, kidney function, inflammation, immune response), histopathology and immunohistochemistry addressing specific questions (proliferation, inflammation, oxidative stress etc.).



Immunohistochemical detection of activated granulocytes by anti-CD11b-staining

Following in vivo toxicological studies performed in compliance with various Guidelines (OECD, EU, ICH) can be performed:

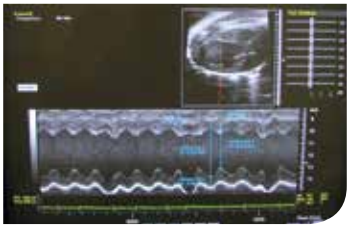
- **Short Term Toxicity**
- **Acute Toxicity**
  Routes of administration: by inhalation, peroral, intravenous, subcutaneous, dermal, intranasal
- **Skin and eye irritation**
- **Sensitization**
  Local Lymph Node Assay (LLNA), The LLNA is a mouse model developed to evaluate the skin sensitization potential of chemicals. It is an alternative approach to traditional guinea pig methods (Magnus-Klingman, Buehler, Maurer) and in comparison provides important animal welfare benefits. The assay relies on measurement of events induced during the induction phase of skin sensitization, specifically lymphocyte proliferation in the draining lymph nodes. In addition to providing a robust method for skin sensitization hazard identification, the LLNA has proven very useful in assessing the skin sensitizing potency of test chemicals, and this has provided invaluable information to risk assessors.
- **Subacute, Subchronic and Chronic Toxicity**
  (28-days, 90-days, 6-month and 12-month) Routes of administration: by inhalation, peroral, intravenous (including infusion), dermal, intranasal
- **Carcinogenicity**
  Routes of administration: by inhalation, peroral, dermal, intranasal
- **Reproduction Toxicity**
  Fertility, 1-and 2-generation studies, reproduction studies according to ICH and OECD
- **Mutagenicity**
  In vivo Micronuceus test

Additional Services

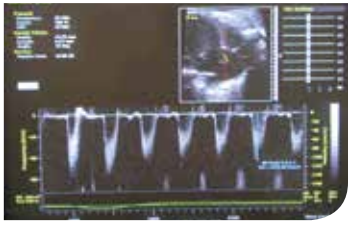
Embedding, sectioning and routine staining of tissue samples, assistance in various immunohistochemical techniques, automated analysis of immunohistochemical staining, histopathological evaluation of slides.

Physiological Measurements

Cardiovascular blood flow and blood pressure are assessed non-invasively by micro Ultrasound using equipment especially designed for imaging in small animals (Vevo 770).



Wall motion measurements of the left ventricle lumen are easily calculated (M-Mode)



Quantification of blood flow in an adult mouse aorta using Pulsed Wave Doppler

In vivo Tests – Alternatives to Animal Testings

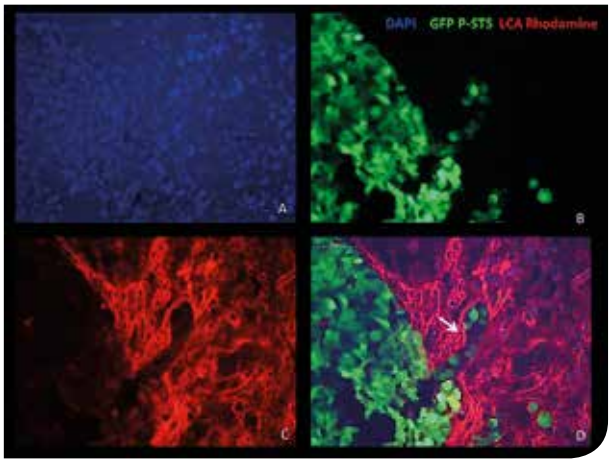
CAM assay – a simple method for many applications

The chicken chorioallantoic membrane (CAM) assay provides an alternative versatile, cost effective and ethically unobjectionable in vivo model for many applications as listed below.

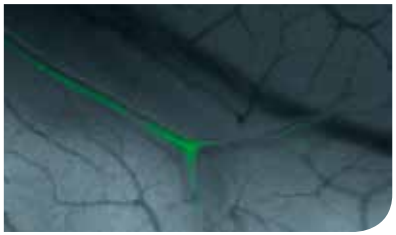
CAM is a well vascularized extra-embryonic tissue located underneath the eggshell. The ex ovo CAM assay enhances the applicability of the CAM facilitating experimental manipulation and enabling in vivo documentation.

List of feasible applications:

- Angiogenesis (induction, inhibition)
- Tumour cell behaviour (angiogenesis, proliferation, apoptosis, invasion, migration, metastasis)
- Short term tissue culture (tumour samples, small pieces of bone, liver, skin etc)
- Toxicity assay (HET-CAM)
- Testing Biomaterials and drugs

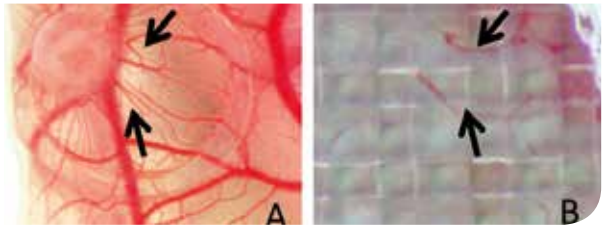


Invasive growth of GFP-tagged tumour cells on CAM tissue (arrow in D). A) DAPI staining of nuclei, B) GFP tagged cells, C) CAM tissue stained with LCA Rhodamine, D) merge



GFP tagged tumour cells can be traced in CAM vessels

GFP tagged tumour cells grafted for 3 days on CAM form distinct tumours



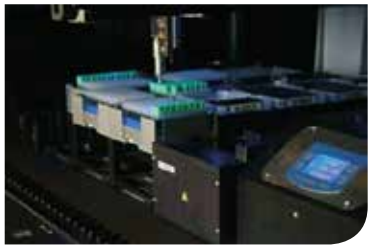
Induction of neo-angiogenesis in CAM by A) tumour cells B) supernatant of tumour cells

In situ Evaluation

Human Specimens for Nanoparticles Analysis

Knowledge about the relation of the exposure to nanoparticles and human diseases is still in its infancies. Hence, large collections of human specimens in combination to disease and further clinical information of the donors may well offer the potential to investigate such relations.

Supraregional biobanks such as Biobank Graz enable such research without the need of starting a respective collection today, but offering large numbers of specimens (FFPE samples, fresh frozen tissues as well as body fluids) already collected over the last three decades.



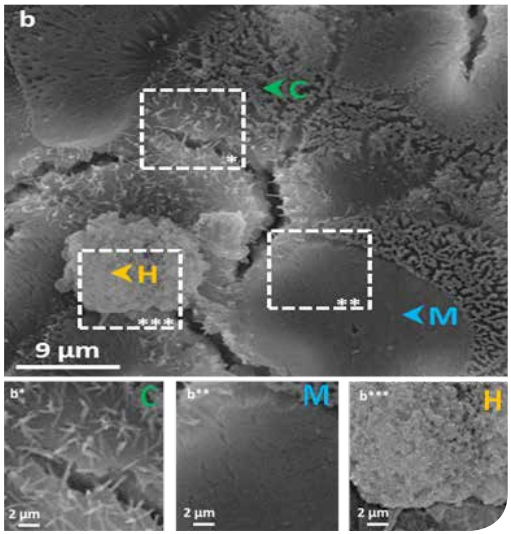
Fully automated handling of fluid samples and a collection of 2D data matrix coded FFPE samples at Biobank Graz.

Nanoparticle Characterization

In vitro and ex vivo models mimicking epithelia of the small intestine

The oro-gastrointestinal route represents a great biological barrier against the passage of chemical compounds and nanomaterials. In the small intestine, where main absorption occurs, two distinct epithelia are available: i) the epithelium of the villi, comprising enterocytes and mucus producing cells and ii) the follicle associated epithelium. The latter one is available at the interface between the luminal environment and the gut associated lymphoid tissue. It consists of enterocytes, mucus producing cells and membraneous cells (M cells), which are not covered with mucus, belong to the immune system and deliver a broad range of materials (e.g., bacteria, viruses, antigens).

These epithelia can be mimicked by in vitro co- and triple-cell-culture models and porcine ex vivo models that enable the investigation of various nanomaterials. For the co-culture model (simulating the villi-epithelium), an appropriate ratio of Caco-2 cells and HT29-MTX cells are seeded on transwells and incubated. Regarding the triple culture model, again, Caco-2 cells and HT29-MTX cells are seeded on transwells and Raji B cells are added to trigger M cell formation. The models are characterized regarding confluence, integrity, differentiation/expression of M cells and cell surface architecture. Permeability studies (calculation of permeability coefficients), transport studies (active and passive mechanisms performed with various inhibitors and marker) and cytotoxic effects (MTS assay, LDH assay, oxidative stress reaction) of model drugs and nanoparticles can be performed.



Cell architecture of the triple culture model comprising enterocytes (C), mucus producing cells (H) and M cells (M) (modified from Schimpel et al., Mol Pharm, 2014).

Ex vivo experiments are conducted with porcine intestinal tissue and dynamic diffusion cells (Ussing Chambers). For this, defined excised intestinal sheets (1 cm2) are mounted vertically between the compartments so that the epithelium faces the donor compartment and the basal tissue region faces the receiver compartment.

The receiver compartment is filled with 5 ml Krebs-Ringer buffer and the temperature is kept constant at 37°C ± 0.5°C. Experimental studies are performed at 37° to evaluate the permeability/interactions of nanoparticles/model drugs. A gas-mixture (95% O2/5% CO2) provides stirring (approximately one bubble per second).

The integrity of the porcine intestinal tissue during the experiment is evaluated by recording the resistance (R; Ω) with Ag/AgCl electrodes.

After 4 h of incubation, the mucosa is washed and fixed in 4% formalin. The samples are cut into 10 µm slices with a cryo-microtom (Microm HM560) and nanoparticle uptake is observed using fluorescence microscopy.

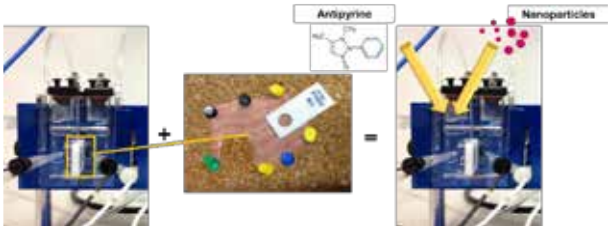


Image of an Ussing chamber consisting of a donor and receiver compartment. Between the compartments a porcine intestinal mucosa is inserted. The compartments (37°C) are supplied with solutions bubbled with 95% O2 and 5% CO2 gas. To assess tissue integrity/viability transepithelial electrical resistance (tissue integrity) is monitored.

Overview of techniques:

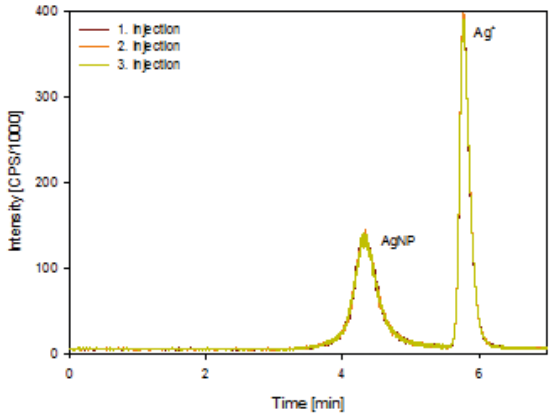
Characterization of the in vitro models regarding confluence/integrity (TEER measurements), differentiation/expression of M cells (WGA staining and alkaline phosphatase assay), mucus secretion (Alcian Blue staining) and cell surface architecture (scanning electron microscopy); viability of the in vitro model exposed to nanoparticles (formazan bioreduction via MTS assay, LDH leakage); permeability ex vivo (Ussing chamber, established for intestinal mucosa); permeability in vitro (Transwell® Systems co and triple culture models);

Chromatographic Separation of Nanoparticles

For the estimation of the total nanoparticle (NP) concentration we developed chromatographic methods that allow the separation of the NPs from the ionic forms of an element.

Together with the knowledge about the total concentration of an element the concentration of the NPs in a sample can be calculated.

For this purpose HPLC with different chromatographic conditions is employed to separate the NPs. The effluent of the chromatographic system is directly coupled to inductively coupled plasma mass spectrometry (ICPMS) which serves as an element-selective detector.



Reversed phase separation of AgNPs from ionic silver using an ICPMS as element-selective detector

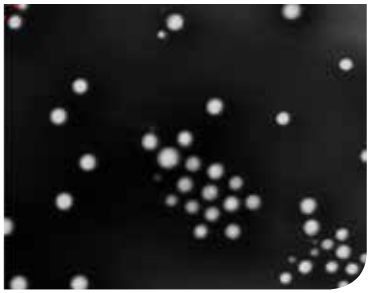
Single-Nanoparticle Determination with ICPMS

Solutions containing NPs can be directly analysed with inductively coupled plasma mass spectrometry (ICPMS) for its NP concentration.

Therefore, diluted solutions are aspirated and single NPs are ionized in the Argon plasma and detected as single events in the time resolved analysis measurement mode. From the results, different features of the NPs (for example concentration, diameter, composition and size distribution) can be calculated.

Standardization

The biodegradable and biocompatible polymer (d,l-lactide-co-glycolide) (PLGA) is approved by the WHO as well as the FDA for medical and pharmaceutical purposes. For comparison with new biomaterials, micro- and nanoparticles made from a polymer with an equal content of lactic and glycolic acid can serve as a standard for some assays.



TEM of PLGA-Nanoparticles with a mean diameter of 140nm.

Additional Services - Surface Functionalization of PLGA Micro- and Nanoparticles

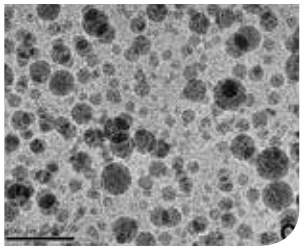
Fluorescent PLGA nanoparticles made either from covalent dye-polymer conjugates or via incorporation of dyes as well as surface modification of the nanoparticles are available for quantitative assays or microscopy.

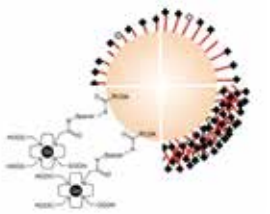
According to the actual needs, the surface of plain or drug-loaded particles can be decorated with targeting moieties such as lectins, enzymes for substitution therapy or imaging agents such as gadolinium.

Especially, the impact of different stabilizers on re-dispersibility and toxicity might be elucidated.

The techniques applied for preparation of the particles are spray drying, solvent evaporation techniques according to hydrophilicity of the drug as well as high pressure homogenization. The size and the zeta potential is determined by dynamic light scattering, nanoparticle tracking analysis or laser diffraction. HPLC-analysis is applied to determine the PLGA-content and custom SEM & TEM to get an idea of size and shape.

In order to track, detect, and quantify the particles hydrophobic fluorescent dyes are incorporated. For characterization of cell (tissue)-particle interaction, different cell lines for single cell and monolayer experiments (intestine, bladder, blood vessels, macrophages, breast and prostate cancer) are in use for binding and uptake as well as transport studies. Additionally, the impact of flow on particle - cell interaction by use of a surface acoustic wave driven chip, and the toxicity of the particles is elucidated assessing the cell number, the metabolic activity, the expression of marker enzymes or the proliferation.





Cryo-TEM and scheme of Gd–DOTA-PEI-PLGA nanoparticles for magnetic resonance imaging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