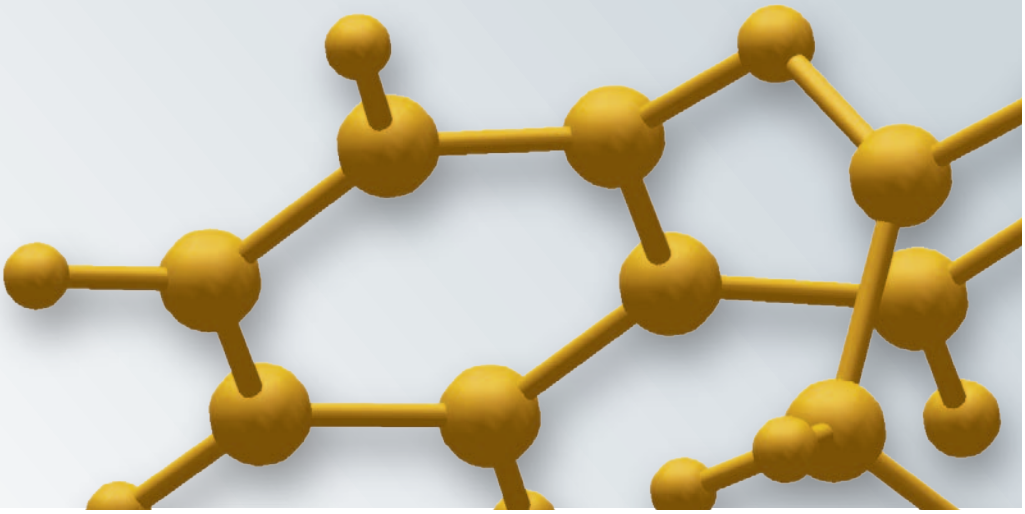
nano HEALTH

NANO-STRUCTURED MATERIALS FOR DRUG TARGETING, RELEASE AND IMAGING

www.nano-health.at



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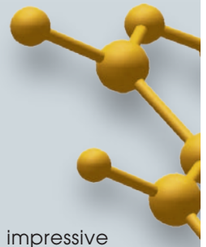
OBJECTIVES

The research project **NANOHEALTH** aimed to develop a platform for novel multifunctional nanoparticles (NPs) for use in:

- the targeted delivery of active substances for the treatment of severe and chronic diseases by various application routes
- nanodiagnostics - the NPs act as contrast media for clinical imaging via magnetic resonance imaging (MRI), and via nuclear imaging by PET/SPECT
- clinical applications, including the improved detection of atherosclerotic vascular lesions, stem cell tracking, as well as cancer diagnosis and treatment
- toxicological studies of nanostructured materials



PROJECT DESCRIPTION

In the ageing population of European society, there are high expectations for better quality of life, for which an important factor is improved, more efficient and affordable health care. Society is confronted with severe illnesses such as cardiovascular problems, diabetes, cancer, and inflammatory and infectious diseases. Focussed on these medical problems, nanotechnology offers impressive solutions. The use of NPs for early diagnosis, imaging and 'smart' drug delivery systems ("Nanocargo") ensures better and personalized health care.

The **NANOHEALTH** project aimed to **develop new generations of NPs for diagnosis, imaging and drug delivery.**

PROJECT HIGHLIGHTS

1. DEVELOPMENT OF A 'NP TOOLBOX' CONSISTING OF NEW, MULTIFUNCTIONAL NPS OF FOUR DIFFERENT TYPES

Within the project, a toolbox of nine multifunctional NPs was developed

I LIPOSOMES

I.a Sterically stabilized liposomes

Sterically stabilized liposomes were designed as drug delivery systems for the pulmonary application of vasoactive intestinal peptide (VIP). A high loading efficiency of VIP into liposomes was achieved, and the stability of the drug-in-lung surfactants was remarkably improved.

The formulations showed no toxicity at therapeutically relevant concentrations. In a pulmonary artery model, we achieved sustained release and a prolonged vasorelaxation profile for liposome-associated VIP.

I.b Magnetic liposomes

Sterically stabilized, fluorescent-labelled magnetic liposomes containing a high 'payload' of ultrasmall superparamagnetic iron oxide NPs (USPIOs) were designed, synthesized and characterized. The fluorescent labeling enabled us to visualize the nanoconstructs by both magnetic resonance imaging (MRI) and fluorescence imaging. The biodistribution of the particles in the organs of mice and rabbits was studied by MRI. Confocal laser-scanning microscopy was used for ex vivo imaging studies in mice. No toxicity was found for the magnetic liposomes.

I.c Targeted liposomes

Targeting sequences such as interleukin-10 (IL-10) were covalently coupled to sterically stabilized liposomes in order to specifically recognize atherosclerotic plaques. The immunogenic potential was investigated in the Balb/c mouse model. Immunizations via oral, intravenous or subcutaneous routes induced only liposome-specific IgM antibodies in serum, as has already been previously reported. Regarding the humoral immune response, IL-10-coated liposomes can be used for the imaging of atherosclerotic plaques.

I.d Thiomer-coated liposomes

By combining the protective nature of liposomes with the mucoadhesive and permeation-enhancing properties of thiomers, thiomer-coated liposomes for oral drug delivery were developed. These new NPs were found to be non-toxic and stable throughout the stomach. Their encapsulated drugs were slowly released while being closely attached to the intestinal mucus. As permeation was increased by the thiomer, a relatively high proportion of drug was able to pass through the intestinal barrier.

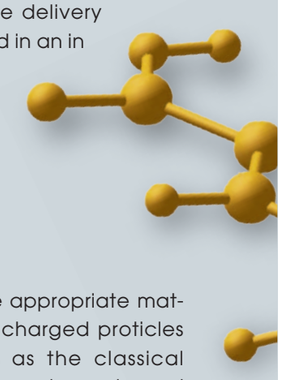
II PROTICLES

II.a Proticles as allergen carrier

Protamine-based NPs ('proticles') complexed with CpG-oligodesoxynucleotides (ODN) and allergen Ara h 2 from peanut were developed as an innovative vaccination strategy against food allergy. They induced a favourable Th1-response after subcutaneous injection in Balb/c mice. Granuloma formation was completely absent, and biodistribution of Ara h 2 was markedly decelerated when complexed to proticles. In summary, biodegradable proticles with CpG-ODN present a novel carrier system for allergy immunotherapy.

II.b Proticles for BBB drug transport

Proticles were developed and investigated as a drug delivery vehicle to overcome the blood-brain barrier. Adsorption of apoA-I onto the surface of proticles resulted in significantly improved uptake and transcytosis properties relative to uncoated proticles. The apoA-I coating enhanced proticle delivery to astrocytes almost twofold in an in vitro model of the BBB.



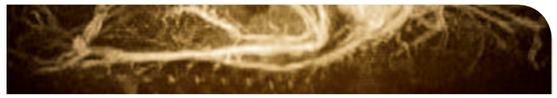
II.c VIP-loaded Proticles

Proticles were developed as a depot formulation including vasoactive intestinal peptide (VIP) as active pharmaceutical ingredient (API). We found good encapsulation efficiency, VIP release, and an appropriate NP size (177-251 nm). Investigations on rat pulmonary arteries showed a modified VIP response of proticle-associated VIP. We observed differences in the profile of artery relaxation, in that VIP proticles led to a 20-30% lower relaxation maximum than aqueous VIP solutions, followed by prolonged vasodilation.

II.d Thiomer proticles

Thiomers were found to be appropriate materials to form cationically charged proticles without oligonucleotides as the classical anionic compound. Thiomers showed good potential for the formation of proticles in a capillary microreactor, which was then successfully scaled up to achieve a production yield of 1L/hour.

We observed a prolonged bioadhesion of proticles, most likely due to the thiol content and the cationic nature of the protamine compound.



II.e Targeted proticles

Proticles were developed for the drug targeting of lung-cancer cells and for the localization of atherosclerotic plaques. VIP-loaded proticles were observed to specifically target lung-cancer tumor cells, such as cells overexpressing VPAC1-, VPAC2-, and PAC1-receptors. Furthermore, VIP-releasing proticle depots were distributed in rat tissue and human tumors.

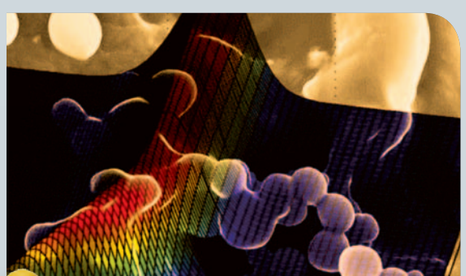
Concerning the atherosclerotic plaque targeting, we found that globular Adiponectn (gAd)-targeted sterically stabilized liposomes generated a strong signal enhancement by accumulation at the surface of AS-plaques, while gAd-targeted proticles became internalized and showed spottier plaque-staining.

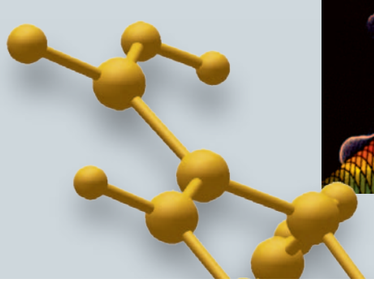
III HSA-PLA NANOPARTICLES

Nanoparticles constructed from the body's natural carrier protein (human serum albumin; HSA), were designed, synthesized and characterized physicochemically and by electron microscopy.
Crosslinked versions were developed that retained mechanical stability and integrity during passage through living cells. These particles contained HSA in its native configuration, and were thus capable of loading and transporting a wide range of pharmaceutically active agents. Signaling groups (such as chelated gadolinium ions) and targeting groups (such as oligolactosamine-specific lectins) were attached to the NPs, and used for molecular targeting and imaging; these functionalized NPs provided high imaging efficiency, allowing molecule-specific T1-weighted MRI of vascular structures (molecular Imaging). The NPs showed no toxicity in standard cytotoxicity and hemocompatibility screening assays, as listed in the EURO-Nanotox portfolio.

IV THIOMER NANOPARTICLES

Particles from synthesized thiomers such as pectin-4-aminothiophenol (Pec-ATP), chitosan thioglycolic acid (CS-TGA) and poly(acrylic acid)-cysteine (PAA-Cys) were prepared in order to investigate their potential for diagnosis, imaging and drug delivery.
In particular, PAA-Cys modified iron oxide NPs were shown to be a promising tool for the invasive in vivo tracking of transplanted cells in the host organism. Microparticles based on Pec-ATP displayed potential for colon-specific drug delivery. Furthermore, anionic and cationic unmodified and thiolated nano- and microparticles based on CS-TGA and PAA-Cys showed promising mucoadhesive properties to prolong the residence time of the incorporated drugs at the target site of the disease.





2. OPTIMIZATION OF NPS FOR EARLY DIAGNOSIS AND IMAGING IN CANCER, ATHEROSCLEROTIC PLAQUES, AND STEM-CELL MONITORING

I ATHEROSCLEROSIS

For atherosclerosis (AS) imaging, anti-inflammatory cytokines interleukin (IL)-10 and globular (g)Adiponectin were investigated for use as target molecules. Both showed promising properties for targeting AS-plaques ex vivo in rabbit.
While gAdiponectin accumulates in the fibrous cap, IL-10 is internalized by foam cells inside the plaques. Both targeting molecules showed enhanced staining signal ex vivo when coupled to fluorescence-labelled liposomes.

Finally, the liposomal nanoconstructs showed good targeting of AS-plaques in vivo when injected into the circulation of AS mice as revealed post mortem by confocal laser scanning microscopy.

II STEM CELL IMAGING FOR BLOOD-CANCER TREATMENT

Ex vivo propagation of human mesenchymal stromal/stem cells (MSCs) is considered a prerequisite for MSC therapy.
In this project, we established procedures for clinical grade MSC propagation using animal serum-free conditions in pooled human platelet lysate (pHPL). Starting from aspiration volumes of 25 mL, clinical-scale expansions could produce up to 982 million multipotent MSCs. The differentiation potential of MSC into adipo-, chondro- and osteogenic lineages in vitro, and into stable vessels and artificial human bone fragments in vivo, was verified by near-infrared imaging, µCT and MRI over time (4D imaging).
Complete genomic hybridization showed balanced profiles for all four MSC samples after expansion.

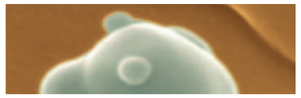
III CANCER TARGETING

After intravenous application, NPs must cross blood-tissue barriers in order to enter the tissue in which the lesion, e.g. a cancer, is situated. HSA binds to specific receptors on almost all endothelial cells and is therefore well-adapted to crossing tumour-protecting barriers.

By using living human tissues with intact microvasculatures, we demonstrated that the HSA-based NPs are taken up by the endothelial cells lining the microvessel walls (of placenta ex situ), are transported by these cells and deposited in the tissue behind the blood-tissue barrier. This is good evidence for the suitability of these particles for use in delivering therapeutically active agents to cancer cells sheltering behind blood-tissue barriers.

For cancer targeting, a further target molecule was attached to HSA NP. The targeting group employed was lectin LEA, which selectively binds to oligolactosamines at the apical endothelial surface: these NPs were shown by MRI and light microscopy to achieve molecular targeting of this layer (thus achieving molecular imaging).

Synthesis of the HSA-based NPs was performed in 5-gram batches - although scale-up to 20-30 gram batches is easily achievable.
Physicochemical and pharmacokinetic investigations revealed high uniformity and their clearance modes in living animals.



IV CANCER IMAGING

A variety of radiolabeled NPs (with Tc-99m, In-111, Ga-68, Y-90 and 177-Lu) were developed and characterized for potential diagnostic and therapeutic applications in oncology using SPECT and PET. Radiolabelled liposomal NPs carrying a variety of peptide-based targeting sequences (TOC, RGD, Substance P) were prepared and characterized, revealing specific tumor-cell targeting both in vitro and in vivo. Optimization of peptide loading and PEGylation was performed. Combination of the radiolabeling properties with Gd-loading for MRI and fluorescent building blocks provided the NPs with multi-functional imaging properties specifically targeting tumours.
Overall, the radiolabeling experiments provided valuable insights into targeting and imaging multifunctional NPs.

V TARGETING MOLECULE DEVELOPMENT

The application of nanostructured materials for drug targeting, release and imaging is only hindered by the (lack of) availability of functional targeting molecules. To meet the need for new targeted–drug-delivery vehicles, especially as potential therapeutic agents against neuroendocrine tumors, a novel technology platform for the de-novo design, production and screening of receptor affine peptides, with priority to somatostatine receptors (SSTR-2) localized on neuroendocrine tumors was developed. Such products might then be used for radio-diagnosis or receptor radionuclide therapy (PRRT), either alone or as part of a nanostructured targeted drug-delivery system for the treatment of gastrointestinal tumors (GEPNET). The search for new receptor-active compounds starts with an in-silico de-novo similarity design based on a database of hundreds of building blocks and their corresponding wold scores characterising their physiochemical properties. By in-silico molecular design we try to mimic the structure of known receptor agonists or antagonists using these building blocks. An iterative process of production and screening results in a set of potential candidates for further investigation.
To enable fast targeting-molecule development, high-throughput screening methods without the use of radiolabeling techniques were developed. A novel screening assay measuring the ligand-receptor interaction for all somatostatine receptor sub-types (SSTRI-V) was established. The ligand to receptor interaction is measured by use of mid-IR spectroscopy (MIR) in an ATR (attenuated total reflection) configuration on artificial membranes presenting the recombinant receptors. Tightly binding ligands are differentiated into agonists and antagonists by using another cell-culture assay measuring the Ca-efflux of agonistic compounds.

3. OPTIMIZATION AND USE OF THE MOST SUITABLE NPS FOR NON-INVASIVE DRUG DELIVERY via oral administration to the gut mucosa and via nasal administration

Highly efficient drug delivery systems were developed by attaching thiol groups to well-known polymers and formulating them to create NPs. The free thiol groups on the surface of these NPs enhanced their mucoadhesive properties substantially. Thiolated chitosan NPs, for instance, had in vivo residence times on the mucosa four times longer than the corresponding unthiolated particles. Furthermore, the oral bioavailability of the peptide drug leuprolide was significantly improved by using a similar nanoparticulate formulation.
The nanoparticulate drug delivery systems generated within this project could substantially improve the therapeutic efficacy of many existing drugs.

4. DEVELOPMENT OF SAFE NPS - STANDARDIZED METHOD FOR TOX

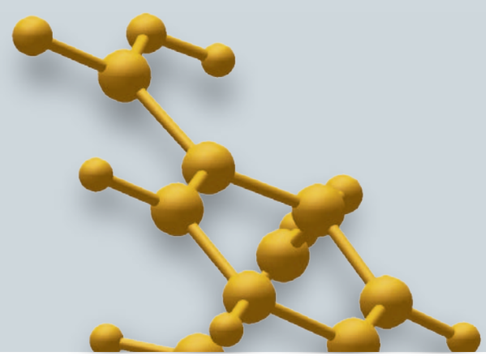
With a view to cyto- and hemocompatibility testing, new liposomes, human serum albumin particles and chitosan particles were developed and toxicologically characterized.
Standard cytotoxicity and hemocompatibility screening assays, as listed in the EURO-Nanotox portfolio, were used for the screening. In addition, genotoxicity tests (HPRT, micronucleus), immunological screening methods (nitric oxide production, chemotaxis, cytokine secretion) and a new method to identify effects of long-term NP exposure were included in the testing. Cellular exposure to polystyrene particles at non-cytotoxic concentrations for four weeks in this bioreactor system showed marked reductions of cell numbers relative to untreated controls. This result highlights the importance of chronic toxicity testing.

SUMMARY

The development of multifunctional NPs in this research project cluster (RPC) focused on **four different types of NPs**: lipid (LIPO-NP), **protamine** (proticles), **poly lactid acid - human serum albumin** (PLA-HSA) and **thiomer** based (thiomers). A 'toolbox' of nine different NPs was developed, characterized and optimized for various medical applications.
For diagnosis and imaging, two different visualization strategies were followed: **magnetic resonance (MRI)** and **radioactivity**. Coupling of these tags allowed in-vitro characterization as well as in-vivo tracking of the different types of NPs developed within the consortium.
For drug delivery, oral, inhalative and nasal delivery routes were the objectives. In this context, the focus was the **"injectable-to-non-invasive-conversion"**. Nanotoxicology is an important aspect of NPs and nanotechnology in general. Nanotoxicology, which deals with toxicological aspects of nanostructured materials, was a particular focus: numerous in-vitro models were developed to evaluate the possible toxicity of the NPs used within the consortium (ref. www.euro-nanotox.at). Multidisciplinary collaboration as well as close cooperation among industry, research centres, academia, hospitals, funding agencies and other stakeholders were key factors in our success. All of the medical universities of Austria, three Austrian universities, two non-university research organizations, four small and medium-sized enterprises and one global organization (Siemens Medical Solutions) allied to form the joint research project NANOHEALTH.
Number of RTD projects in the cluster:
RTD project cluster (calls 2004, 2005, 2006 and 2007) and one large scale project (call 2008)
Funding period: March 2005 - February 2012
Project volume: EUR 8.039.693

PUBLICATIONS

- **231** published and accepted **publications in peer-reviewed journals** (representing a total of over 669 impact points)
- **308 presentations** (invited lectures and congress lectures)
- **373 posters** on different national and international conferences



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PROJECT PARTNER

Universities:

- Biobank, Medical University of Graz
- Center of Medical Research, Medical University of Graz
- Clinical Department of Internal Medicine I, Angiology, Medical University of Innsbruck
- Clinical Institute of Medical and Chemical Laboratory Diagnostics - Medical University of Graz
- Department of Anatomy, Histology and Embryology, Medical University of Innsbruck
- Institute of Cancer Research, Medical University of Vienna
- Institute of Medical Engineering, Graz University of Technology
- Institute of Pathophysiology and Allergy Research, Medical University of Vienna
- Institute of Pharmaceutical Sciences, Pharmaceutical Technology, University of Graz
- Institute of Pharmacy, Pharmaceutical Technologies, Medical University of Innsbruck
- Max F. Perutz Laboratories, University of Vienna
- Messerli Research Institute, University of Veterinary Medicine of Vienna, Medical University of Vienna and University of Vienna
- University Clinic of Internal Medicine - Clinical Department of Haematology, Medical University of Graz
- University Clinic of Nuclear Medicine, Medical University of Innsbruck

Non-university research organisations:

- Institute for Biomedicine and Health Sciences, JOANNEUM RESEARCH Forschungsgesellschaft mbH
- Institute of Biophysics and Nanosystems Research, Austrian Academy of Sciences

Industry:

- JSW Lifesciences GmbH, Grambach
- Green River Polymers Forschungs- und EntwicklungsGmbH
- piCHEM Forschungs- u. Entwicklungs GmbH
- RCPE GmbH - Research Center of Pharmaceutical Engineering
- Siemens Medical Solutions Austria
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