Publishable executive summary

The goal of the NANOEAR consortium is to develop novel multifunctional nanoparticles (MFNPs), which are targetable to selected cell populations, biodegradable, traceable in-vivo, and equipped with controlled drug release. With over 44 million EU citizens with hearing loss and 40 000 profoundly deaf who could immediately benefit from a MFNP-based novel drug carrier system and drug coated cochlear implant, the inner ear is a unique target. Inner ear is a clinically vital target, well representing the central nervous system and other difficult-to-access body sites; it is isolated, multicompartmental with neural, supporting and vascular targets, and relatively immunoprivileged. Measures of function and structural integrity are quantitative, precise, and objective, permitting detection of loss of a single sensory cell. Moreover, the population of profoundly deaf with cochlear implants, and commercial partners concerned with improving the benefits of sensory neuroprostheses through tissue engineering strategies, provide a direct pathway to eventual clinical application of NP-drug complexes produced by this research consortium.

Novel, - and for the first time - multifunctional, highly penetrating delivery vehicles will be created to carry and release drug/gene precisely to targeted tissue sites and selected cells. In this research consortium multifunctional lipolexes, dendrimers, micelles, mesoporous NPs, polymer-protein complexes, nanocapsules and nano-layers have been constructed to test delivery of molecules/drugs/genes. To target the NPs we are screening of seleted tissues of inner ear (receptor, nerve and vascular cells) to find suitable epitopes against which the targeting peptides will be designed. Four EU/FDA approved degradable biomaterials (PLGA, PCL, Chitosan, Silica) will be tested for targeting, coating, toxicity and payload carrying and release capacity. In addition designed lipoplexies, encapsulated by polyethylene glycol (mPEG), impregnated with drugs, and decorated with targeting ligands and signaling molecules (gadolinium) will be assessed for benchmarking purposes. These data will provide the reference for evaluating the efficacy of different novel MFNPs.

This IP consist of 8 different WPs for design, manufacturing, encapsulation, delivery, targeting, kinetics, toxicity, stability, absorption, bioefficacy and tracktability of drugs. These features will all be specifically assessed and significantly enhanced through nanotechnology-based systems. The fabricated NPs will be applicable to wide variety of drugs (e.g. conventional therapeutics, growth factors, proteins, nucleic acids). Five commercial SMEs are partners in this research consortium, each bringing special knowledge and specific market concerns. We also asses the in-vivo trackability so that drug targeting can be visualized in organs with conventional MRI.

As a demonstration milestone this IP will produce a novel human cochlear implant promoting improved cochlear nerve-implant integration. In this novel demonstration the implant will include a MFNP drug reservoir providing continuous drug delivery and MFNP electrode coatings providing targets for nerve growth.
Concerning current status of the program several sets of NPs have been provided by each producer. Also several different coating techniques have been tried and are under progress. The coating is especialy important as different ligands, signaling molecules and markers can be attached to the surface. In incorporation process, for testing purposes, the NPs are equipped with markers allowing to in´dentify them with histological methods. Of particular interest is GFP gene that is used to demonstrate the efficacy for infection rate of the NPs conceringn cell inocculation and entering the nucleus of the cells. Furhtermore we have worked on two plasmids to demonstrate their efficacy for entering the speicfic cells (PC12 cells) vitro and in rat cochlea in vivo. We have established model data sheets for NPs to be tested and model test protocols for toxicity testing and determining the efficacy of infecting the cells.

Overview of participating groups and their field of research

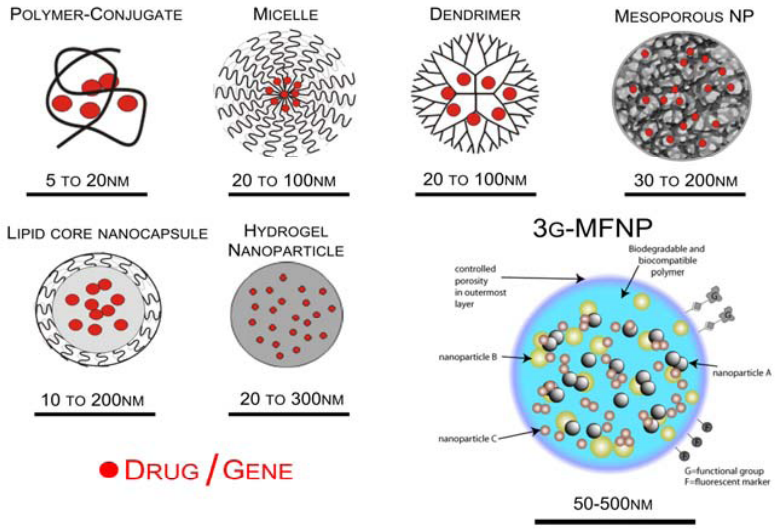
Organisation name	Short nam	Group leader	Country	Field of activity
University of Tampere	UTA	Finland	ENT-department,	in vivo testing, MRI-testing, PAMPA technology,
Tampere University of Technology	TUT	Finland	Biomaterials,	Chitosan, Professor
ÅAU Academy University	ÅAU	Finland	Biomaterial,	silica,
Kungliga Tekniska Högskola	KTH	Sweden	Biomaterial,	biopolymers,
Univertity of Angers	UA	France	Biomaterial,	lipid core nanocapsule
Medical University of Hannove	MHH	Germany	ENT-department,	in-vivo testing, CI-testin
University of Rostock	IBMT	Germany	Toxicology,	NP-toxicity
Rheinisch Westphälische Technische Hochschule	RWTH-Aachen	Germany	Biomaterial,	Pegylation, hyperbranched polymers,
University of Heidelberg	UHD	Germany	Biiomaterial,	CI-electrode coating,
Ecole Polytechnique Federale d Lausanne	EPFL	Switzerland	Biomaterial,	Pegylation,
Medizinische Universität Innsbruck	MUI	Austria	ENT-department,	Cochlear fluid spaces,
University of Ferrara,	UFE	Italy	ENT department,	In-vitro tests, proteometrics,
Academy of Science of the Cze Republic	IEM	Czeck Academy	Hearing Research Institute,	in-vi testing, aging inner ear,
University of Uppsala	UU	Sweden	ENT-department,	in-vitro testing, professo
University of Athen	UA	Greece	Internal Medicine,	Kidney secretion,
Inserm University of Montpelli	INSERM UMR	France	Inner ear research dept,	cochlear sclices, in vivo,
University of Southampton	SOTON	United Kingdom	Brain Research Institute,	Dendrimers,
MedEl AGhb	MedEl	Austria	MedEl Industry,	cochlear implants, CEO,
Aequotech	Aequotech	Italy	Signalling substance,	CEO, Dr
Yorkshire Bioscience SMI	Yorbio	United Kingdom	Ligands, peroteomics,	
Hemoteq AG	Hemoteq	Germany	Electrode coating,	
NS-Gene	NS-Gene	Danmark	Proteomics, genes,	

Scientific coordinator:
Prof. Ilmari Pyykkö
Depatment of Otolaryngology
University of Tampere
Teiskontie 35
Tampere 33520 Finland
Tel +358-3-311 66387

Website and contact information
Nanoear Website: www.nanoear.org
Contact information: ilmari.pyykko@uta.fi
Andrey.Mikhailov @uta.fi
Piia.Reku@uta.fi

Summary of work performed, results achieved so far and expected end results.

WP1-3 manufacturing and functionalisation: the routine manufacturing and delivery of NPs has now been established by all the relevant partners. University of Turku (ABO) has synthesized functionalized silica nanoparticles with a mean diameter of 90 nm containing FITC as traceable agent, coupled to a poly(ethylene imine) surface modification. The Southampton (USOU) group has prepared samples of micelles produced from block co-polymers loaded with DiI a red fluorescent dye. The uptake of which is now being studied in ex vivo cochlear implants (UU). Micelles loaded with dilsulfiram and ferrocene are now also in production (USOU). Stability and degredation of the micelles has been assessed. Another sample or labeled micelles was prepared from a coblock polymer which forms the micelles with sulforhodamine 101, a rhodamine dye. The University of Anger (UA) are manufacturing nanocapsules containing Nile red and Dio prepared in a one step reaction. Incorporation of was done in two steps. The Royal Technical University (KTH) has prepared superparamagnetic iron oxide nanoparticles (SPION) through high temperature decomposition methods. These SPIONs are covered by a layer of oleic acid molecules to endow the particles with hydrophobicity, yielding in a mean average particle size of 12.5 nm. A method for drug and traceable agent loading has been set up. TUT prepared chitosan nanoparticles using ionic gelation, labeled with a range of FITC concentrations. The FITC loaded chitosan nanoparticles have an average size of approximately 95 nm. ABO encapsulated Disulfiram in mesoporous silica particles with hydrophobically modified surfaces.. ABO have also made available mesoporous silica particles (size range of 80-200 nm as required) that are double-labelled with a fluorescent dye and streptavidin. Importantly, the particles are stable under physiological conditions due to the presence of a hyperbranched polyethyleneimin layer on the particle surfaces. Thus, the particles can directly be used for both drug and gene delivery, in combination or separately. Almost any other targeting group could also be put on the surface, as ABO can also introduce selectively COOH groups on the outer part of the dendritic surface layer if needed. Furthermore, ABO is able to selectively functionalize the mesoporous surface with a different function to that on the outer particle surface, which makes this approach very flexible as it can be tuned for a given set of drugs. All of the NPs currently in production have, or are now being tested for toxicity by the in vitro and in vivo partners.



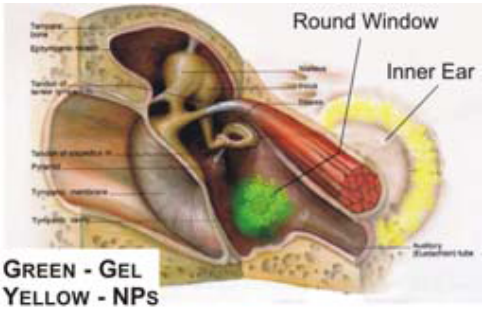
Nanoparticles on which Nanoear is working on

WP4 Toxicity testing: Within WP4 the potential toxicity of NPs had to be assessed using appropriately developed test systems such that testing can eventually be carried out on tissues relevant for the inner ear. Ideally, benchmark NPs that can serve as negative and positive controls for cytotoxicity tests would be used as recommended by Oberdörster et al. 2005 some of which show characteristics not suitable for the test systems. However as neither EMEA nor NIH can provide recommendations to benchmark with the reference materials at this time, it was decided to use commercial NPs available on the market. For NP targeting the appropriate ligands have to identified and developed, these efforts are well under way. This work uses a combination of manufacturing and molecular biological techniques with the major challenge of producing functional,

biologically active molecules that have receptors on one side and their ligands on the other. With the ultimate aim of producing NPs with slective targeting of cells in the inner ear.
As membrane penetration is of utmost importance in determining the efficacy of the NPs University of Tampere (UTA) has made efforts to develop a testing assay consisting of a miniaturized bioreactor with an isolated artificial membrane, the so called parallel artificial membrane permiability assay (PAMPA). UTA has established a prototype of this reactor and compared different characteristics of permeability of the membranes by evaluating the structure and trafficking properties of human and animal round window membranes, human amnion membrane and artificial lipid membranes. These membranes have fundamental similarities but also marked differences in composition of the material, thickness and pinocytic vesicles. UTA has further demonstrated with immunohistological methods the trafficking pathways used by NPs when passing through the round window membrane.

WP6 Improved cochlear implants; WP6 is focussed on the development of nanotechnology based strategies for the development of targeted drug delivery by cochlear implants. A feasibility study of the three best candidate techniques is due to be carried out within the first 18 months of the project, after which one or two techniques will be further investigated. These should overcome already identified or unexpected technical limitations and qualify in principle for regulatory approval and industrial exploitation. WP6 also evaluates biologic variables with regard to penetration of NP through bordering membranes, distribution within fluid compartments, penetration through micrometer scale tissue channels, and binding and entering cells (tissue) from a fluid based surrounding. In addition fluidics in the whole inner ear is being studied with a pumped delivery system and compared with round window (RW) delivery using signalling molecules. Finally, inner ear and brain slices in continuum are to be used to study the bioefficacy and targeting of MFNP in the nervous structures inside and outside the inner ear. In addition TEM and confocal microscopy will be used to investigate the structure of RW membrane and search for alternative fluid pathways in the inner ear through which the MFNP can penetrate into the different compartments of the inner ear.

WP7 In vivo evaluation of NPs; this workpackage aims to provide essential in vivo validation of in vitro screening information from WP 4 & 6 on NP distribution, targetability, cell uptake, biocompatibility, toxicity, bioefficacy and model membrane transit in appropriate animal models. Established inner ear disease models exist in the guinea pig, mouse and rat. These are relevant to the human clinical target populations. In WP7 profoundly deaf guinea pigs (WP 7.1), moderately hearing impaired rats (WP 7.2) and mice with age related hearing loss (WP 7.3) provide the animal models. WP 7.1 is focused on hair cell and nerve degeneration, its prevention and directed regrowth of the auditory nerve. WP 7.2 is focused on repair of sensory cell damage. WP 7.3 is focused on addressing how NP toxicity and uptake may be altered in age-related hearing loss and the use of NPs in the amelioration of age related hearing loss. NP distribution, uptake, membrane transport, targeting and toxicity in sensory, neural and vascular cells will be determined in each model. NP ,Ultimately the bioefficacy will be evaluated in the prevention and repair of hearing loss in each of the animal models. The inflammatory response and possible immunological rejection of NPs and delivery systems, e.g. in situ gelling agents, will be assessed. Tissue will be investigated for a panel of markers including IL6 and TNFα and also for the presence of cellular infiltrates and changes to the extracellular matrix.. To exclude NP toxicity a functional assay of the auditory pathway will be assessed after administration of NP by testing the auditory brainstem response [ABR] after sacrifice histological and/or molecular changes of the inner ear tissues will be assessed by cytocochleograms, together with confocal, light and electron microscopy. Additionally WP 7.1 will evaluate artemin on the survival of auditory neurons explanted from neonatal rats. Artemin is one of the four members of the glial cell line-derived neurotrophic factor (GDNF) family and therefore a potential candidate for the therapy of deafness.



Our new TEM experimental data indicates that the neural structures can be accessed by round window membrane delivery, as the cochlear fluid pathways will deliver the NPs to spiral ganglion cells through holes present in the bone.

WP8 MRI visualisation of NPs; WP8 will evaluate the visibility, distribution and targeting of NPs loaded with gadolinium. The

results of distribution studies of gadolinium applied to animal round window membranes indicates that the vestibulum and semicircular canal are quickly and powerfully accessed through the membrane whereas the cochlear apex takes time to be accessed. In ham installation on gadolinium on round window will resut in first MRI visible loading of gadolinium in the vestiubule after 2 hours. After 12 hours the semicircular canals are filled with gadolinium whereas the basal turn of cochlea is filled and the middle turn starts to load. To reach the apex more time is needed.

The research consortium has two WEB sites. The first site (www.nanoear.org) is public. The main purpose of this site is to distribute information about the following topics: goal of the project, progress of the project and major achievements, - published publication (title and abstract) and major events. The site is updated regularly by the project administrations.

The second site (nanoear.ttl.fi) is for internal use and is protected individual user name and passwords to guarantee free dissemination of information in the consortium. The contents of the site consists of: timetable for the present year, protocols of all meetings (board, steering committee), deliverables, names and contact information of all people involved, test reports of all nanoparticles, discussion boards for various topics, administrative documents (quality handbook, forms, CA,TA among others). All partners have the right to add documents to the server. The server is maintained by UTA.

From the nanoear project 11 papers have been provided in intenational symposiums and in different handicap organization meetings. Four scientific papers have been accepted for publication. Two internal educational courses have been provided and four intenal meetings have been arranged.