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MEDITRANS

Targeted Delivery of Nanomedicine

Integrated Project

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Final report

Publishable final activity report

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1. PROJECT EXECUTION

Project objectives

- To demonstrate the potential of *Emerging Materials* (carbon-based nanoparticles) for use as carrier materials in targeted nanomedicines
- To develop highly effective nanomedicines based on *Candidate Materials* (already used in proof-of-concept drug delivery studies in animals) by virtue of improved targeting and drug release properties
- To promote the entry of nanomedicines based on *Established Materials* into industrial exploitation activities and clinical proof-of-concept studies
- To develop high-sensitivity imaging probes properly designed for guiding drug delivery processes *in vivo*
- To formulate proprietary industrial drug molecules, already established drugs, and DNA- or RNA-based drugs into targeted nanomedicines with well-characterised and optimised physicochemical properties
- To optimise the targeting efficiency of the nanomedicines under development by *in vitro* target recognition studies
- To improve the intracellular targeting of siRNA/pDNA-loaded nanomedicines in cancer and endothelial cells
- To maximise the drug availability at the target site by means of external physical stimuli that induce drug release from the targeted nanoparticles 'on demand'
- To develop targeted nanomedicines from which release of drug/imaging probe is promoted by physiochemical characteristics of the pathological microenvironment
- To develop imaging procedures for the monitoring of the various steps in the targeted drug delivery process (nanoparticle targeting and accumulation, drug release, local level of drug and of biomarkers in response to therapy) by means of 'smart' imaging probes
- To optimise biodistribution, targeting efficiency, and therapeutic activity of the nanomedicines under development in suitable animal models of rheumatoid arthritis, Crohn's disease, multiple sclerosis and cancer
- To assess the toxicological aspects of selected MEDITRANS nanomedicines
- To enter selected prototype nanomedicines into an industrial exploitation phase to evaluate their potential to be developed into a marketable product
- To provide training courses, and access to the GALENOS-Network, provided for consortium scientists, SMEs, and key stakeholders
- To provide effective and efficient dissemination, and demonstration, of the project's results across Europe

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WP1

Work performed and end results (elaborating on the degree to which the objectives were reached)

WP1 (Nanocarrier design) aimed at identifying materials suitable for serving as drug delivery devices. Hereto, it on the one hand dealt with the synthesis and characterization of Emerging Materials, like fullerenes and nanotubes, as well as, on the other hand, with the evaluation and optimisation of already existing Candidate Materials, like pDNA- and siRNA-containing polyplexes, polymeric micelles, iron oxide nanoparticles, amino acid-based nanovesicles and stimuli-sensitive liposomes.

Regarding the Emerging materials, CEA and UU have prepared several different batches of fullerenes and carbon nanotubes (CNT), shortened CNT to sizes well below 500 nm (Figure 1A-C; Task 1.1), and functionalized the surfaces of fullerenes and CNT with hydroxyl, carboxyl and amine groups (Task 1.2). In addition, using two different strategies, the surfaces of CNT and fullerenes were modified with hydrophilic stealth polymers (Task 1.3). On the one hand, amphiphilic monomers based on a hydropobic diyne motif and a hydrophilic poly(ethylene glycol) (PEG) block, were used to adsorb to the surface of highly hydrophobic pristine CNT, and they were subsequently UV-polymerized around the nanotubes. Analogously, pyrene-containing amphiphilic block copolymers were used to coat CNT via surface adsorption, and to functionalize them with drugs, such as budesonide (Task 1.4).

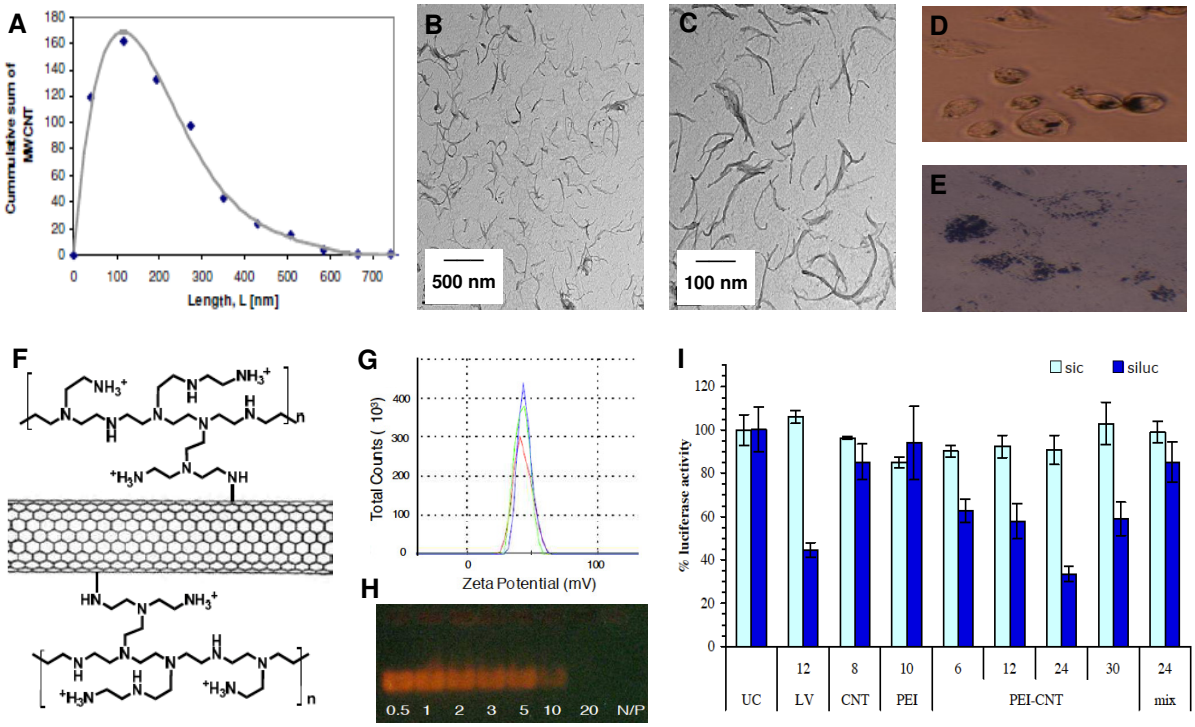


Figure 1: Overview over the most important advances made within WP1 with regard to Emerging Materials. A: Carbon nanotubes (CNT) were shortened to sizes below 500 nm using ultrasonication. B-C: TEM images of ultrasound-shortened CNT. D-E: Uptake of PEGylated CNT by J744.A1 macrophages (D) and human umbilical vein endothelial cells (HUVEC; E), after incubation for 24 h. F: Schematic structure of PEI-modified CNT. G: Zeta potential of PEI-CNT. I: Complexation of siRNA by PEI-CNT at different N/P ratios. J: Luciferase silencing in U87 cells. CNTs were mixed at a N/P ratio of 80 with unspecific control siRNA (sic) or luciferase-specific siRNA (siluc). Values indicate siRNA concentrations in nM. UC: untreated cells, LV: lipid vehicle (jet-siENDO), CNT: bare nanotube, mix: PEI and CNT

On the other hand, using appropriate surface chemistry, fullerenes and CNT were covalently modified with hydrophilic stealth polymers, i.e. with PEG-2000, PEG-5000 and PEG-10000, as well as with cationic polymers, such as poly(ethylene imine) (i.e. PEI; Figure 1F). The PEG-modified fullerenes and nanotubes were found to be well-dispersable in water and, interestingly, to be taken up by cells highly effectively, as demonstrated by Figure 1D for macrophages and by Figure 1E for endothelial cells. The PEI-containing CNT, because of their highly positive surface charge (Figure 1G), were used for siRNA delivery at both UU and CEA, and where the former failed to show efficient complexation and benefit over standard transfection agents, the latter managed to show that under certain conditions, PEI-modified CNT were able to complex siRNA (at N/P ratios >10; Figure 1H), and enabled efficient gene silencing in luciferase-transfected U87 cells, comparable to that of lipid-based transfection agents (Figure 1I; Task 1.4).

In parallel to the above experiments, as part of Tasks 1.2.1 and 1.2.2, CEA and BRACCO have established assays to evaluate the cytotoxicity of fullerenes and CNT. This turned out to be more difficult than initially envisioned, because the black suspensions and the particles sedimenting on top of the cells strongly influence absorbance, thereby interfering with the color reagent assays routinely used in viability analyses. This could be overcome by using 0.22, 0.45 and 1 µm multi-well filter plates. After 24 h of incubation of the cells with the various materials, the supernatants

were transferred to a filtering plate and then centrifuged. The recovered clear supernatants were subsequently evaluated by means of the LDH (i.e. lactate dehydrogenase) assay, and the original plates with cells were subjected to MTT (i.e. methylthiazolyldiphenyl-tetrazolium bromide) analysis. Sixteen different carbon-based carrier materials and six different cell lines were used in these viability experiments. In general, hardly any toxicity was observed at the concentrations tested (i.e. the concentrations which could be reproducibly and stably dispersed; up until 1 mg/ml). Exceptions were pristine multi-walled and certain cationic CNT (especially PEI-CNT), which resulted in up to 80% cell death at the highest concentration tested (Task 1.2.1). The hemolytic activity of all properly dispersible carbon-based carrier materials was assayed using rat and human blood. None of the tested compounds presented with hemolytic activity against human blood. Against rat blood, only carboxyl-containing multiwalled CNT induced a slight degree of hemolysis (Task 1.2.2). Together, these findings demonstrate that at the concentrations tested, certain (especially highly charged) carbon-based carrier materials display a certain degree of toxicity.

Concerning the Candidate materials, except for Task 1.3.3 dealing with molecularly imprinted particles, all Tasks were completed successfully. As part of Task 1.3.1, relatively small-sized (<100 nm) pDNA-containing polyplexes were prepared, as well as polyplexes based on bi- and trimodal carrier materials, which under certain circumstances presented with reasonable gene delivery and transfection efficacies. In Task 1.3.2, a variety of different siRNA-containing nanomedicines were synthesized, based e.g. on biodegradable cationic copolymers, on PEG-PEI, on dendrimers, on PLGA and on nanogels. The biodegradable cationic copolymers were shown to be highly useful for implementation in combination with photochemical internalization (PCI). Thiolated TMC's turned out to be relatively effective even without PCI. PEG-PEI's were evaluated with regard to silencing efficacy and biodistribution, and were found to be particularly suitable for tracheal instillation. Dendrimer-based nanoparticles were shown to be able to induce silencing only under certain specific conditions, as opposed to PLGA-based nanoparticles, which turned out to work well even under physiologically relevant conditions. And finally, efforts were invested in PEGylating siRNA-containing nanogels, and in showing that these formulations are effectively taken up by cells, and hold potential for relatively long-term gene silencing. The molecularly imprinted particles (MIP) prepared as part of Task 1.3.3 were, as already briefly mentioned above, not found to be effective, as they failed to demonstrate target-specific re-binding. Therefore, this Task was halted prematurely (i.e. in May 2009). In Task 1.3.4, one of the most successful Tasks in WP1, polymeric micelles stable in systemic circulation were prepared. As exemplified by Figure 2A-B, it could be demonstrated that upon crosslinking the core of these polymeric micelles, their in vivo circulation times could be improved substantially, leading to significantly enhanced tumor accumulation. In addition, upon covalently modifying these micelles with doxorubicin, a significant enhancement in the survival of B16F10 tumor-bearing mice could be obtained as compared to free doxorubicin (Figure 2C-D). And furthermore, upon modifying core-crosslinked micelles with nanobodies, their cellular uptake was found to be substantially enhanced (Figure 2E-F). In Task 1.3.5, several different superparamagnetic iron oxide nanoparticles (SPION) were prepared, including e.g. PEGylated and drug-containing SPION, which were shown to be able to release their payload upon hyperthermia (Figure 2G). As part of Task 1.3.6, spherical and 100-200 nanometer-sized amino acid-based drug delivery systems synthesized, which could be efficiently loaded with model compounds, such as calcein. And finally, in Task 1.3.7, several different types of stimuli-sensitive liposomes were prepared. These formulations were not only loaded with model compounds such as calcein, but also with clinically relevant drugs and imaging agents, and it could be demonstrated that content release from these formulations could be triggered by decreasing pH and by increasing temperature (Figure 2H-I).

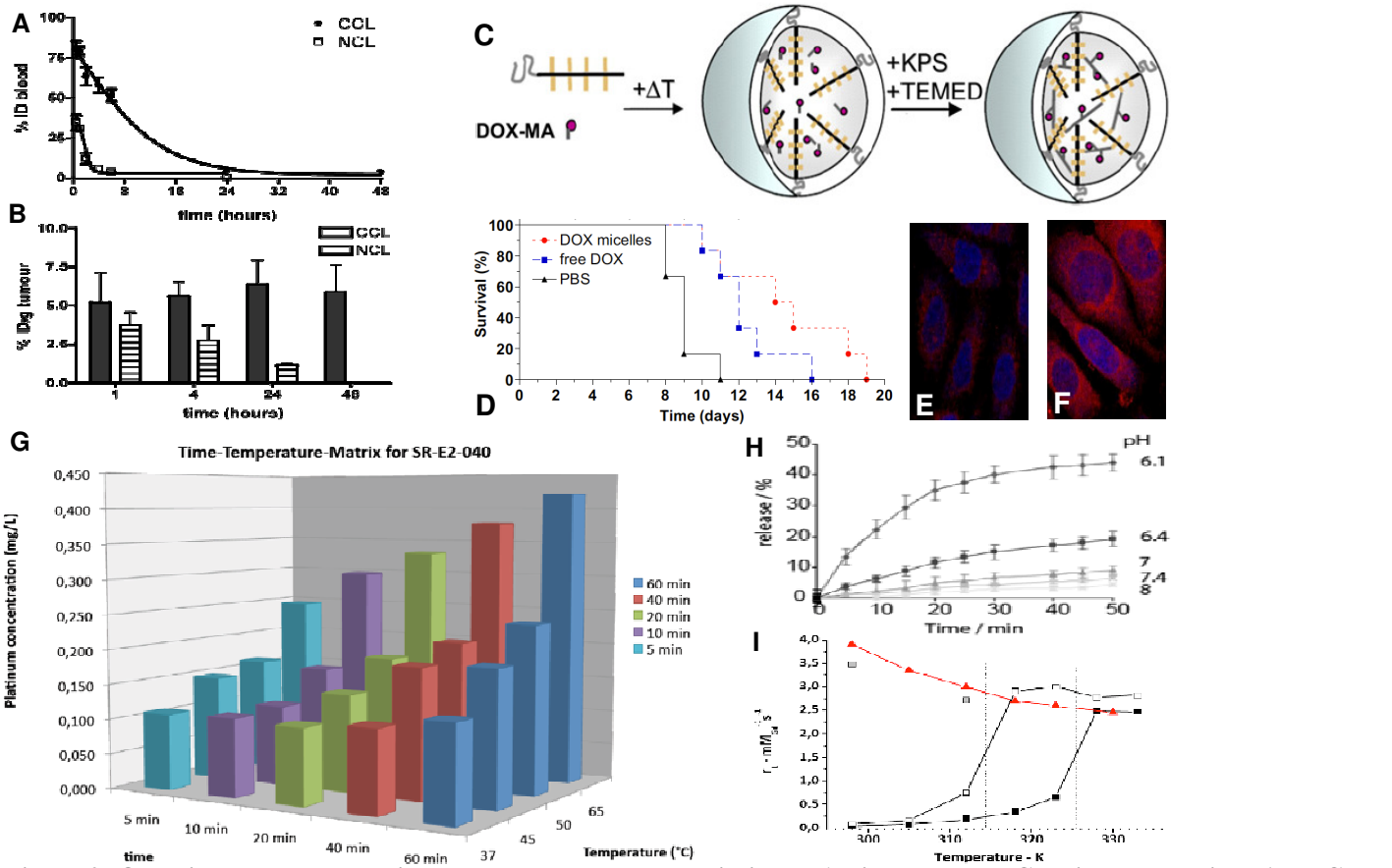


Figure 2: Overview over the most important advances made within WP1 with regard to Candidate Materials. A-B: Core-crosslinked (CCL) polymeric micelles based on block copolymers of PHPMA-Lactate and PEG circulate substantially longer (A) than non-crosslinked (NCL) polymeric micelles, and consequently accumulate in tumors significantly more effectively (B). C-D: Synthesis (C) and in vivo evaluation (D) of doxorubicin-containing CCL polymeric micelles, showing significant survival benefit. E-F: EGa1-Nanobody-targeted polymeric micelles (E) are taken up by EGFR-expressing 14C cancer cells significantly more effectively than are non-targeted micelles (F). G: Iron oxide nanoparticles loaded with cisplatin are able to release the incorporated drug upon hyperthermia in a time- and temperature-dependent manner. H-I: Stimuli-sensitive liposomes can be triggered to release their contents by means of decreasing pH (H) or increasing temperature (I)

Collectively, the above efforts show that the two primary objectives of WP1, i.e. I) To demonstrate the feasibility of the Emerging Materials (carbon–based nanoparticles) for targeted drug delivery; and II) To optimise the functionality of the Candidate Materials (polyplexes, nanogels, nanospheres, polymeric micelles, molecular imprinted particles, stimuli-sensitive liposomes) for targeted drug delivery, were both successfully completed.

Brief description of methodologies and approaches employed

A wide variety of (physico-) chemical and biological methodologies and approaches were employed in WP1. The carbon-based carrier materials were prepared using standard and laser-assisted chemical vapor deposition, they were surface-functionalized using organic chemistry techniques (e.g. via the Bingel reaction), and surface-modified and drug-functionalized using adsorptive, polymerization and conjugation techniques. The different intermediates were purified and characterized using high-pressure liquid chromatography (HPLC), gel permeation chromatography (GPC), nuclear magnetic resonance (NMR), infrared spectroscopy (IR), dynamic light scattering (DLS), zeta potential analysis and mass spectrometry (MS). The in vitro efficacy and toxicity of the Emerging Materials was tested using optimized cell culture methodologies, including e.g. the methylthiazolyldiphenyl-tetrazolium bromide (MTT) and the lactate dehydrogenase (LDH) assay, to assess cell viability on the basis of mitochondrial activity and membrane permeability, respectively. Cellular uptake was analyzed using high-resolution brightfield microscopy. Candidate Materials were prepared using standard and advanced polymer chemistry techniques (i.e. the cationic polymers for DNA and siRNA delivery, nanogels, PLGA-based nanoparticles and polymeric micelles), solvent evaporation and rehydration (stimuli-sensitive liposomes) and recombinant molecular biology-based libraries (amino acid-based nanoparticles). These were purified and characterized with similar techniques as in case of the Emerging materials, supplemented with thermogravimetric analysis (TGA), polyacrylamide gel electrophoresis (PAGE), fluorescence-activated cell sorting (FACS), confocal laser scanning microscopy (CLSM), luciferase- and calcein-based spectroscopy, magnetic resonance

relaxometry, and inductively coupled plasma mass spectrometry (ICP-MS). In addition to this, in the case of amino acid-based nanovesicles, extensive molecular modelling studies were performed.

Relationship between project achievements and state-of-the-art

The carrier materials developed as part of WP1 are either Emerging or Candidate materials, i.e. in both cases systems which have not yet been investigated in great detail before. Carbon-based carrier materials, like PEGylated fullerenes and siRNA-containing nanotubes, are still very much in their infancy, and also e.g. drug-loaded core-crosslinked polymeric micelles, surface-functionalized and temperature-responsive iron oxide nanoparticles, amino acid-based nanovesicles and stimuli-sensitive liposomes have not yet been extensively described in the literature. Therefore, the progress made and the insights obtained with regard to these novel materials (partly obtained in other WP, e.g. as part of the in vivo biodistribution and efficacy analyses in WP7-9) are considered to be of significant interest to both industry and academia, and they likely contribute substantially to the current state-of-the-art in this area of research.

Impact on its industry or research sector

As detailed above, the progress made with regard to several of the materials developed in WP1, most notably I) surface-functionalized iron oxides for magnetic fluid hyperthermia (MFH) and II) doxorubicin- and dexamethasone-loaded core-crosslinked polymeric micelles for targeted therapeutic interventions in cancer and rheumatoid arthritis, is expected to have significant impact of their respective industry and research sectors. Both systems are currently being commercialized, and the former have already been tested in a number of patients.

WP2

Work performed and end results (elaborating on the degree to which the objectives were reached)

The work performed within this WP over the full duration of the project achieved almost completely all of the planned objectives.

Objective 1) To develop high relaxivity Gd-agents including probes responsive to tissue microenvironmental pH and specific enzymatic activities.

Achievements:

- preparation of novel amphiphilic ligands able to form highly stable Gd(III) complexes endowed with high relaxivity and pH responsiveness;
- synthesis of multimeric ligands able to form neutral lanthanide(III) complexes that improve the MRI detectability of paramagnetically loaded liposomes;
- preparation of liposomes endowed with high relaxivity incorporating a highly stable macrocyclic amphiphilic Gd(III) complex;
- preparation of Gd-based carbon- and peptide-based nanotubes endowed with peculiar MRI properties
- preparation of a dual Gd-19F imaging probe as concentration-independent pH sensor
- synthesis of a novel hydrophilic Gd(III) complex endowed with high stability and relaxivity
- development of a Gd(III) complex able to report about MMPs activity.

Objective 2) To develop high sensitivity Chemical Exchange Saturation Transfer (CEST) agents including probes able to report about the level of drugs or specific biomarkers within the pathological region.

Achievements:

- preparation of non-spherical LipoCEST agents endowed with highly shifted intraliposomal water protons;
- development of safe and rapid MRI sequence devoted to the detection of CEST contrast, even in combination with MRI ^{19}F detection;
- development of post-processing analysis for improving the accuracy of the detection of CEST contrast;
- design of a temperature responsive liposomal-based probe able to report about the vesicle integrity by combining CEST and 19F-MRI measurements.
- design of LipoCEST agents for multiplex detection;
- preclinical validation of the potential of the clinically approved CT agent Iopamidol as smart concentration-independent CEST pH sensor;
- design of the first liposome-based dual ^{1}H T_1-CEST agent where the CEST contrast can be detected only after the removal, mediated by a trigger variable, of the Gd(III) complex generating the T_1 contrast

Objective 3) To develop novel iron oxide particles properly designed for applications in drug delivery processes.

Achievements:

- preparation of novel iron-based nanopowders embedded in carbon through laser pyrolysis method;
- development of novel procedures for obtaining stealth iron oxide particles through a suitable PEG-based coating;
- development of a MRI/ICP combined method to assess the sensitivity of iron oxide particles in cultured cells;

Objective 4) To develop highly sensitive Optical Imaging probes for monitoring drug delivery processes and therapeutic effects.

Achievements:

- inclusion of aggregated porphyrins (fluorescence self-quenched) in supramolecular nanosystems based on amphiphilic cyclodextrins;
- encapsulation of aggregated porphyrins (fluorescence self-quenched) in liposomes.
- preparation of dual MRI/Optical probes endowed with targeting ability towards collagen
- design of RGD-targeted dual MR/optical probes potentially utilizable for monitoring drug release;
- synthesis of a novel NIRF cyanine-like fluorescent probes;
- set-up of optical imaging procedures, mostly based on confocal microscopy, able to report about intracellular localization and release of liposomes.

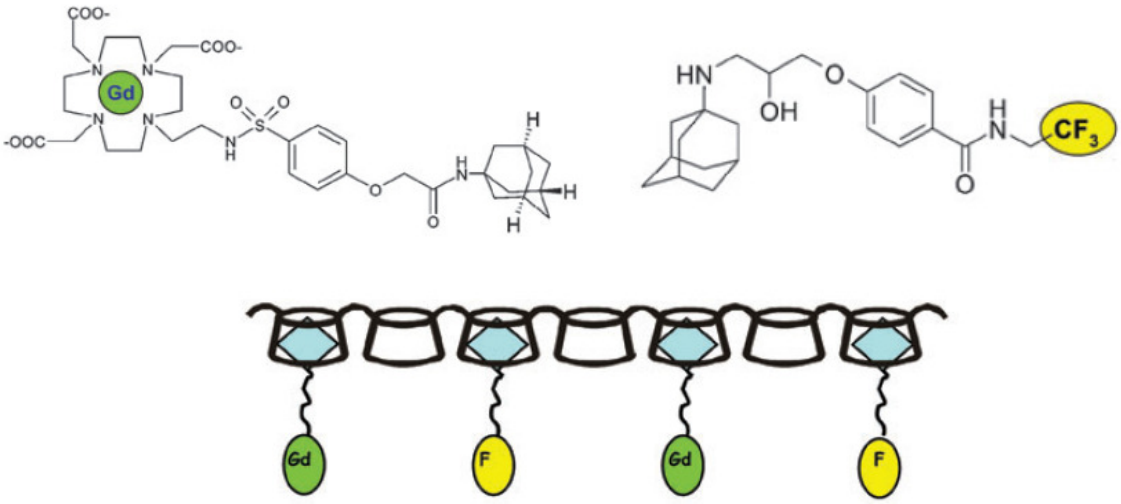
Brief description of methodologies and approaches employed

Particular attention has been paid to develop novel smart MRI agents in which the MRI response only depends on a given variable of the microenvironment that needs to be monitored (e.g. pH, enzymatic activity,…) and it is not affected by change in the local concentration of the agent.

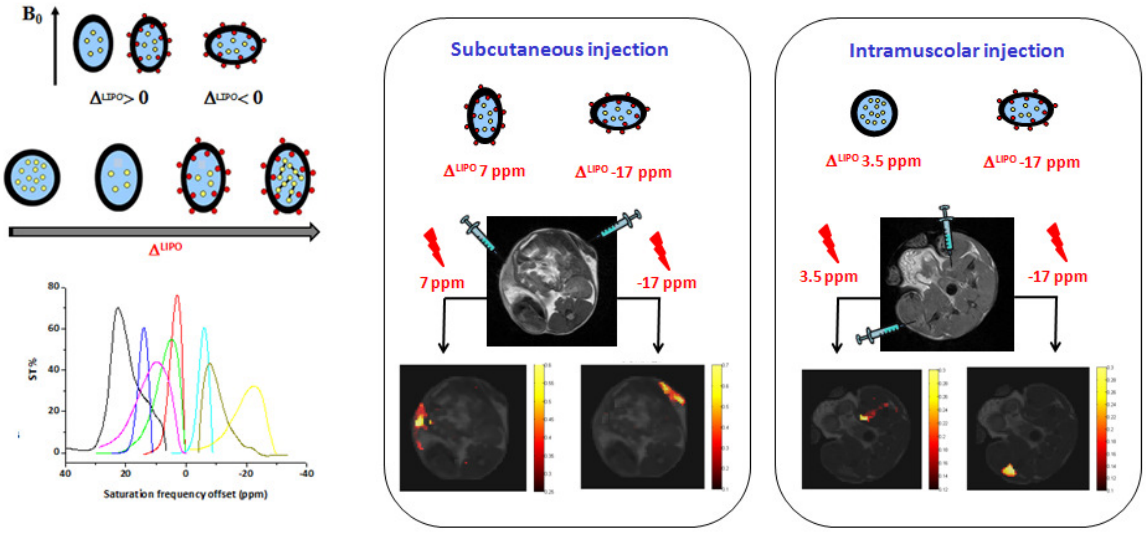
Several approaches have been proposed mainly depending on the type of contrast generated by the agent.

In case of Gd-based T_1 agents, it has been pursued the route to design dual 1H-19F probes in which the 19F detection allows the quantification of the probe and the water detection can be used to monitor the desired parameter. A representative example of this approach is represented by the combined use of pH sensitive Gd(III)-complexes with a

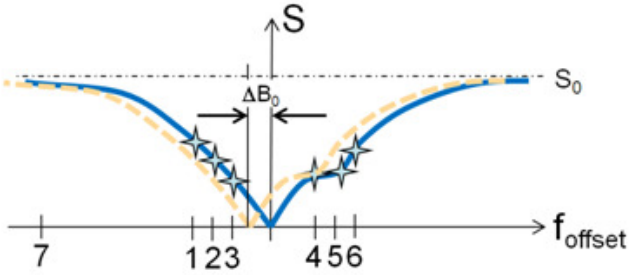
19F-labeled compound in which both the probes have been suitably functionalized to bind the cavities offered by poly-β-CD.



In the field of LipoCEST agents, a significant increase in the chemical shift values of the intraliposomal water protons has been achieved through the exploitation of bulk magnetic susceptibility contributions that implied the formation of non-spherical liposomes. The sign of the chemical shift is dependent on the specific orientation of the vesicles with respect to the static field that, in turn, depends on the anisotropy of the magnetic susceptibility of the vesicle bilayer. Hence, the orientation, and consequently the shift, can be controlled by incorporating in the bilayer paramagnetic amphiphilic complexes with a proper magnetic anisotropy. The extension of the chemical shift values in LipoCEST agents considerably facilitates their multiplex detection.

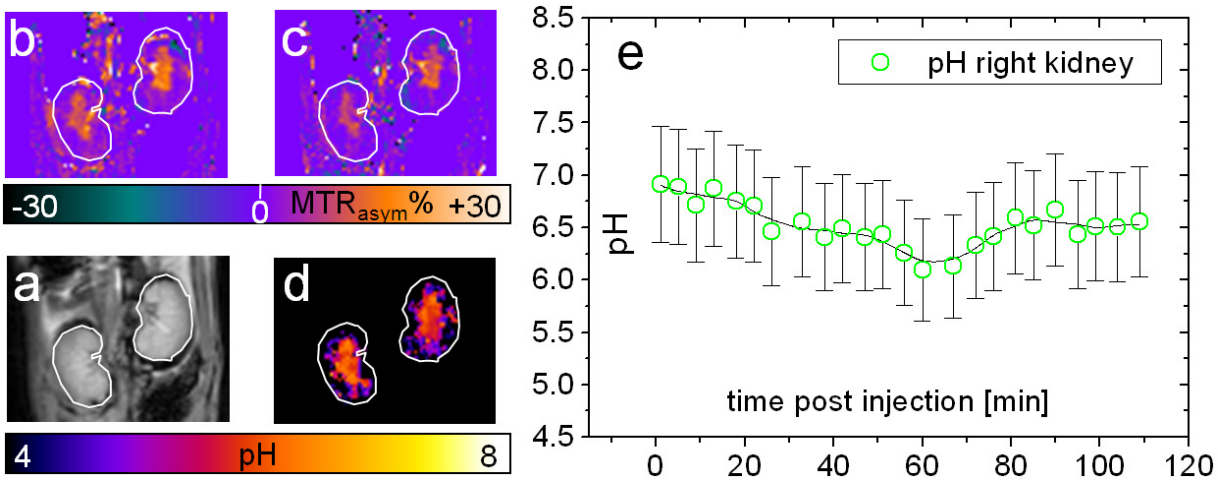


Important achievements have been also obtained in the field of MRI-CEST detection where new sequence specifically designed for improving especially the accuracy and timing of the acquisition of CEST signal.



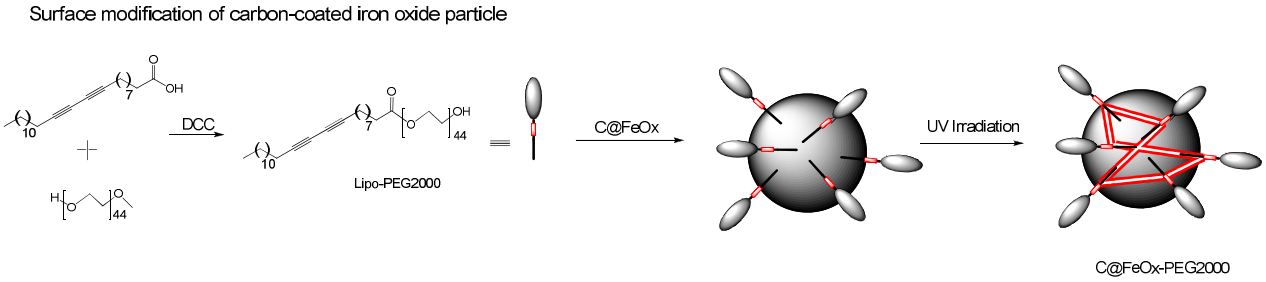
Optimization of frequency sampling for the acquisition of a Z-spectrum

All these progresses allowed the pH monitoring in kidneys after i.v. injection of Iopamidol a well-known CT agent also capable of generating MRI-CEST contrast.

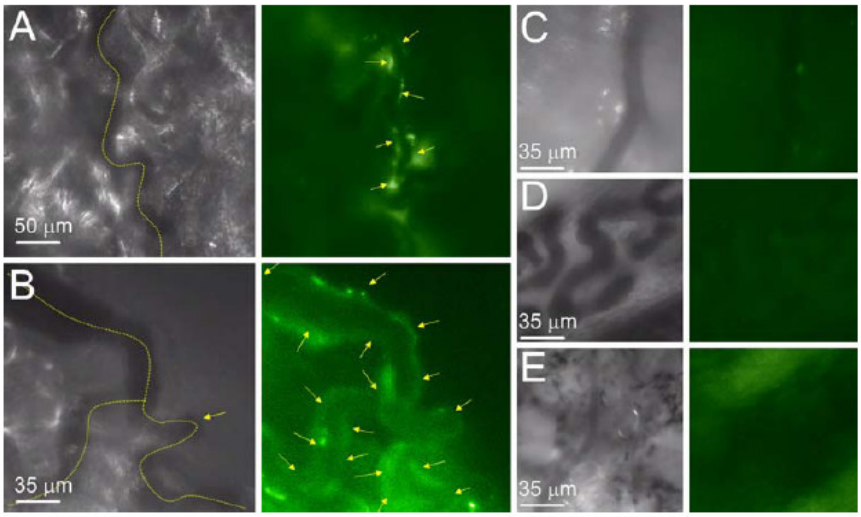


MR-CEST pH mapping of rat kidneys: The anatomical image is shown in (a) and CEST mapping at 4.2 ppm/5.5ppm in (b) and (c), respectively. The ratiometric evaluation and pH calibration allows to measure pH in both kidneys as average over all post-injection time points (d) and to monitor the time course of pH the kidney pelvis (e)

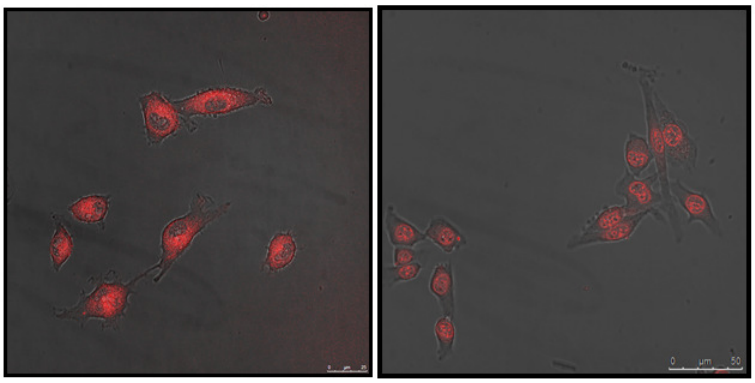
A new procedure for preparing iron oxide nanoparticles by laser pyrolysis method has been developed. The particles, coated by a carbon layer, were successively stabilized in water using several approaches among which the most efficient was the adsorption of amphiphilic PEG chains.



Several progresses have been done with the aim of developing optical imaging procedures, even combined with MRI, for the visualization of drug delivery and release processes. Most of the work focused on the preparation of liposomes (even targeted) loaded with fluorescent dye (commercially available or produced in the project) or exploiting the natural fluorescence of drugs (e.g. doxorubicin).



RGD-labeled paramagnetic quantum dots (RGD-pQDs) specifically target tumor blood vessels in vivo. Intravital microscopy of microvessels in tumor-bearing mice (C57Bl6) after intravenous injection of RGD-pQDs (A-C) and pQDs (D, E). Brightfield microscopy (left panels) was used to select blood vessels (indicated by the yellow lines). Fluorescence microscopy (right panels) revealed labeling of endothelial cells in tumor blood vessels of mice that were injected with RGD-pQDs, as indicated by the yellow arrows (A and B). Endothelial cells in blood vessels of normal tissues (skin of the ear) in the same mouse showed no accumulation of contrast agent (C). An animal that was injected with bare pQDs did not show association of contrast agent with the endothelial cells in tumor (D) or normal tissue blood vessels in the ear (E)



Confocal fluorescence images of B16 cells after 30 min of incubation with Doxil® (0.2 mgDoxorubicin/mL). Left: image acquired just after removing the non-internalized nanomedicine. Right: image acquired 1 hour after

Relationship between project achievements and state-of-the-art

The future success of image-guided therapy and personalized treatment strategies will mainly depend on the development of improved imaging probes able to really provide an *in vivo* personalized response about the pharmacokinetic and pharmacodynamic properties of a drug or to monitor and assist surgery interventions.

MRI is a very powerful technique whose main weak point is the relatively poor sensitivity. Therefore, one of the main topics that was tackled in the project is the design of highly-sensitive agents. Several strategies have been followed, also in combination, including the optimization of the determinants that control the relaxivity of paramagnetic Gd(III)-complexes (rotational tumbling, exchange rate of the metal coordinated water, occurrence of additional contribution,…). In addition, when the final task is the design of a smart agent able to monitor pH or activity of a given enzyme, also strategies for making the detected contrast independent of the local concentration of the agent need to be developed. The rational is the measurement of two observables of which one is only function of the amount of probe and the other is sensitive to the variable to be monitored. An approach developed in the project was to design a dual 19F-1H probe where 19F signal is detected for quantification and 1H signal for monitoring. To combine this approach with the required high sensitivity, it was proposed the use of two distinct probes (one a responsive Gd(III)-complex and the other a 19F-labeled compound) both functionalized to bind poly-β-CD in order to slow down the rotational tumbling of the Gd(III)-agent. Likely, the most important progresses beyond the state-of-the-art were obtained in the emerging field of CEST agents. Significantly improvements were obtained in the design of liposome-based CEST probes (LipoCEST) endowed with an extended range of chemical shift values for the intraliposomal water protons. This result was achieved by changing the shape of the liposomes exposing them to hyperosmotic condition. The change from spherical to discoidal shape allows the exploitation of a shift contribution arising from the orientation of the vesicle within the external magnetic field (bulk magnetic susceptibility). The sign of the shift is related to the orientation that, in turn, can be controlled incorporating in the vesicle bilayer paramagnetic amphiphilic agents with proper magnetic anisotropy. The extension of the chemical shift values is fundamental for the possibility of visualizing different LipoCEST agents in the same imaging experiment as it has been demonstrated *in vivo* on mice model. Very important progresses have been also done in the design of novel MRI sequences dedicated to the detection of CEST contrast and the assessment/compensation of B_0 field inhomogeneity. In parallel, also specific post-processing analysis has been developed for improving the accuracy of the determination of CEST contrast. Thanks to these achievements it has been possible to detect a CEST contrast in mice and rats kidneys after intravenous injection of the clinically approved CT agent Iopamidol, which has been also investigated as CEST probe.

Concerning the preparation of novel iron oxide particles, good progresses have been achieved using laser pyrolysis methods. The particles displayed interesting magnetic properties and their stability in aqueous solution was improved trough the absorption of amphiphilic PEG chains.

The results obtained in the field of Optical imaging has allowed the synthesis of novel NIR fluorescent dyes and the setup of optical microscopy experiments for the assessment of the intracellular localization and trafficking of liposomes loaded with drugs (e.g. doxorubicin) or optical probes.

Impact on its industry or research sector:

Some of the probe, and related imaging procedures, that have been developed in this WP could be translated in the clinical practice and indeed exploitable by companies operating in the biomedical field. Examples are hydrophilic Gd(III)-complexes endowed with high relaxivities that can be easily encapsulated in liposomes and therefore potentially useful for the imaging of liposomes biodistribution and release (see also WP6).

Another achievement that could be rather close to the clinical exploitation is the detection of the CEST contrast using agents already approved for clinical use on humans. Iopamidol is a well-established diagnostic CT agent successfully used since a long time in clinical practice. Interestingly, this compound contains two pools of mobile protons that can be selectively saturated for generating CEST contrast. In addition, the exchange rate of the two pools is different, thus allowing the setup of a ratiometric method that can be used for assessing the pH of the microenvironment in a concentration-independent manner.

WP3

Work performed and end results (elaborating on the degree to which the objectives were reached)

WP3 focuses on the formulation and physicochemical characterisation of nanocarriers loaded with drugs and imaging agents. In the **Emerging Materials**, carbon nanotubes (CNTs) have been successfully shortened to 100-200 nm and modified with surface functionalities including PEG based chemistries. Budesonide has been loaded onto the CNTs using pyrene/PEG linking chemistry. CNTs have also been loaded with siRNA using two methods. In addition, fullerenes were surface-modified with different functional groups covalently bound with PEG-based stealth polymers.

Research on **Candidate Materials** included the successful production of a large range of nanoparticles for delivering siRNA and their characterisation with a range of physiochemcial techniques. PLGA based and DOTAP modified nanoparticles, poly(amid amine) dendriplexes, nanoparticles based on DEAPA-PVA-g-PLGA complexes, PEGylated dextran nanogels and PEGylated liposomes have all been prepared.

A polyplex conjugate with the structure Fol-PEG10k(3)-PEI25k suitable for DNA complexation and folate receptor targeting has been successfully synthesised. Characterisation included particle size (170-250 nm), surface charge and morphology by atomic force microscopy (AFM) imaging.

A variety of strategies have been employed to load imaging probes containing nucleic acids. Fluorescent and radio-labelled siRNA has been used to study the cellular uptake, localisation and pharmacokinetics and biodistribution of DEAPA-PVA-g-PLGA-nanoparticles. A positively charged Gd(III) complex has been synthesised that can strongly bind nucleic acids through the formation of stable ionic pairs.

Polymeric micelles were successfully synthesised using biodegradable thermosensitive block copolymers. Dexamethasone, paclitaxel and doxorubicin were derivatised with a polymerisable methacrylate moiety and loaded into micelles. Drug release is tuneable by varying the embedded linker systems.

Stealth-coated iron-oxide (FeOx) nanoparticles have been produced consisting of a 15 nm core with a 5 nm silica shell and loaded with a model drug. IR shows successful PEG coating of these nanoparticles. Extensive physiochemical and surface characterisation of iron-oxide nanoparticles coated with flavin mononucleotide and guanosine monophosphate has been performed, finding particles 20-40 nm in size with a Fe_3O_4 structure.

"P904" is a hydroxyl modified iron oxide nanoparticle successfully developed as a MRI contrast agent for macrophage imaging. It consists of a 7 nm core with an overall size < 25 nm. Characterisation has recently focused on stability data for scale up and manufacture, and has proven stability under long-term ICH accelerated conditions.

In the **Established Materials**, research into long-circulating liposomes for IV passive drug targeting has resulted in the production of a PEGylated formulation containing dexamethasone phosphate. Liposomal compositions were modified based on packing densities and charge to optimise *in vivo* circulation time and drug loading. This formulation has been taken forward towards industrial exploitation and is currently in late stage preclinical testing, undergoing scale up towards GMP manufacture and quality control.

PLGA nanoparticles have been prepared by an emulsion-solvent evaporation method. Early on in the program, mesopram was replaced as the target drug due to its low encapsulation efficiency and rapid release. Nanoparticles containing various glucocorticoid drugs were instead formulated and evaluated for their encapsulation efficiency (highest for budesonide at 83%), particle size distribution (typically ~230 nm) and zeta potential. These systems were taken forward for in-vitro studies of drug release with the addition of various surfactants to modify the release.

Active drug targeting long-circulating PEGylated liposomes have been formulated and loaded with Neutron Capture Compounds (NCT). An antibody directed against the EGFR receptor was coupled to 100 nm PEGylated liposomes and were loaded with the NCT compound, Boron for active targeting of tumour cells. Further work has been conducted to produce neural cell adhesion molecule (NCAM)–targeted, doxorubicin loaded liposomes for enhancing drug delivery and allowing magnetic resonance imaging (MRI).

An engineered channel protein, MscL, has been successfully reconstituted into stealth liposomes. The channel was used, for the first time, for the facilitated loading and activation of liposomes with a model drug and to release on command. Successful *in vitro* studies showed the pH triggered release and that the system is ready for *in vivo* testing. The system offers fine tuning of the release, such as type of trigger and recognition (pH, wavelengths, concentration etc). Liposomes have also been loaded with siRNA.

Liposomes loaded with Gd-HPDO3A in the aqueous cavity have been selected for further development as a MR imaging probe. The basic lipidic composition is same as that used for the dexamethasone-loaded liposomes. The magnetic relaxation properties of the liposomal formulation (diameter 102 nm) were studied and the transversal relaxation efficiency increases 20-40 times. In addition, a Gd(III) complex bearing a cholesterol-like moiety has been successfully incorporated in a POPC/Cholesterol-based membrane, this is believed to be the first example of a liposome-incorporation of a Gd(III) complex containing a steroidic structure.

Brief description of methodologies and approaches employed

A wide variety of methods have been employed for loading the systems with drugs and imaging agents, including a huge variety of encapsulation, surface modification, functionalisation and conjugate chemistries. Example images and structures of systems are shown in Figure 1.

Formulations have been studied with a variety of physiochemical characterisation techniques (Figure 2). Particle sizing techniques such as dynamic light scattering (DLS) and the use of zeta-potential to measure surface charge have been used in almost all formulations to study key parameters for nanomedicine performance. Nanoparticle Tracking Analysis (NTA) has been used to study the influence of dendrimer generation and molar ratio on siRNA dendriplexes.
A range of microscopy techniques have been employed. Transmission electron microscopy (TEM) has been used on drug loaded and shortened CNTs and the morphology of iron oxide nanoparticles. Scanning electron microscopy (SEM) has been used to examine morphology and size of drug and siRNA loaded PLGA microparticles. AFM has been widely used to study surface morphology including nanogels, iron oxide nanoparticles and the changes in morphology observed as siRNA PLGA nanoparticles undergo drug release.
Examples of the use of fluorescent microscopy techniques include the addition of DOTAP during the production of PLGA nanoparticles, to show triggered release from liposomes with a pH responsive channel protein, to study the mechanism of siRNA loading into cationic dextran microgels and to prove the localisation of folic acid on the surface of PEG3k-PEI25k nanoparticles complexed with DNA.
Surface chemical techniques have provided additional insight to study the surface of coated iron oxide nanoparticles. For siRNA loaded PLGA nanoparticles, time-of-flight secondary ion mass spectroscopy (ToF-SIMS) was successfully used to prove the presence of siRNA at the outer surface. X-ray photoelectron spectroscopy (XPS) was also used to study the level of siRNA loading and functionalisation on CNTs.

Relationship between project achievements and state-of-the-art
The work completed for WP3 during the Meditrans project has resulted in a large number of publications from almost all tasks, reflecting the quality of the work, and its relevance to the state of the art in the field. Many of the systems have either not been studied in detail before, or represent entirely novel approaches. This has included both novel formulations and methods to deliver drugs and imaging agents, and their subsequent characterisation with state of the art techniques. Four of the nanomedicines have proved sufficiently stable and well characterised to proceed to further *in vitro* and *in vivo* evaluation and development towards industrial exploitation.

Impact on its industry or research sector:
WP3 has provided a large body of research into both the preparation of drug and imaging agent loaded nanomedicines and their physiochemical characterisation, impacting on both industrial and academic sectors. Four of the systems developed in Meditrans have been progressed towards industrial exploitation; FeOx nanoparticles for macrophage imaging, liposomal gadolinium for image-guided drug delivery, core-crosslinked micelles for cancer chemotherapy and liposomal dexamethasone for anti-inflammatory tumour treatment. This has involved proving these formulations are sufficiently stable and characterised to proceed to further *in vitro* and *in vivo* evaluation. More recent efforts have therefore focused on scaling up and quality control of the formulation manufacture. The results from the other systems developed are involved in a series of ongoing publications that have both industrial and academic interest, and many continue to undergo further development.

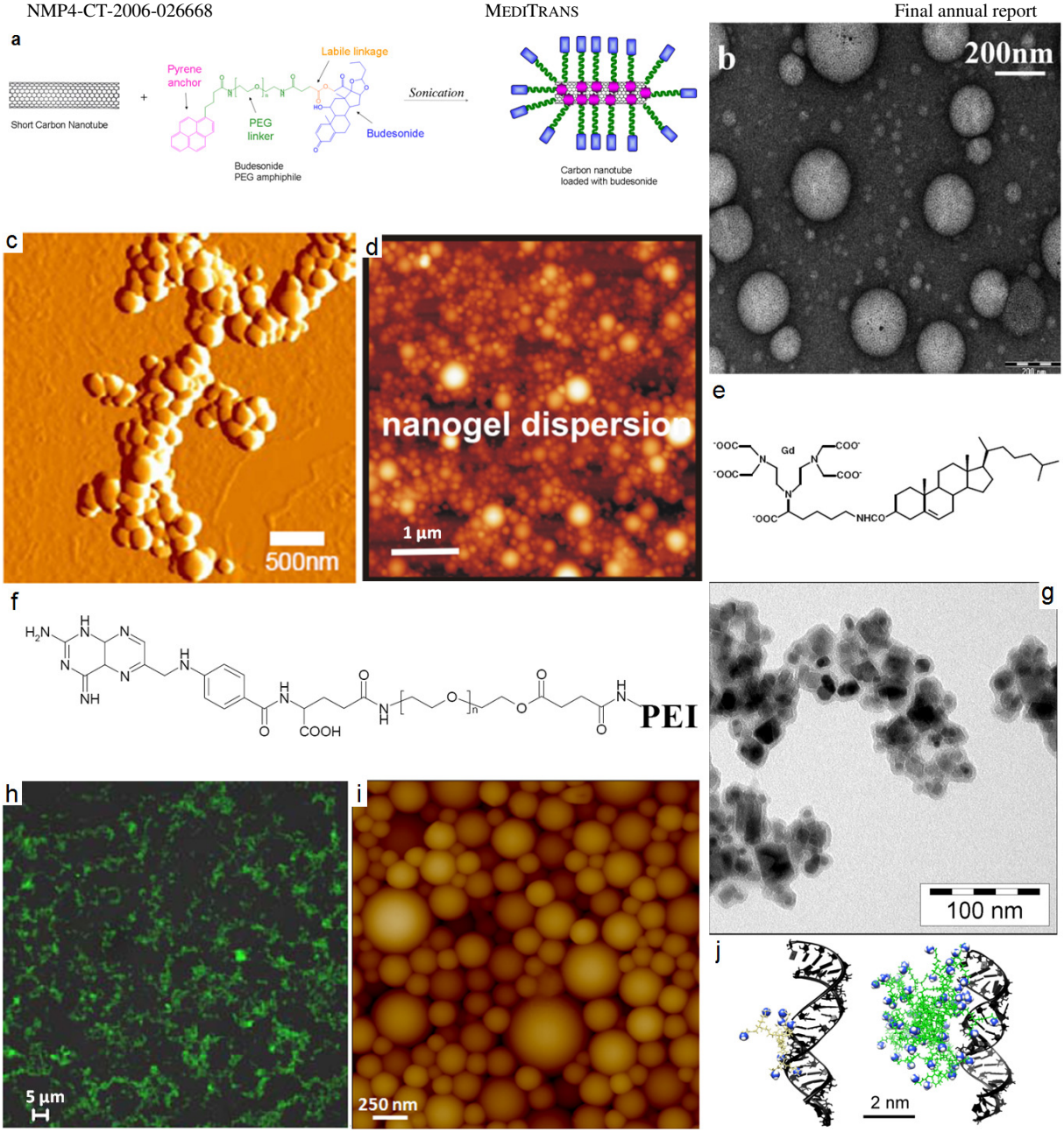


Figure 1: Imaging and structures of drug and imaging agent loaded systems. a) Functionalisation of CNT with budesonide. b) TEM of polymeric micelles loaded with 10 nm oleic acid coated FeOx. c) AFM images of folic acid-PEG3k-PEI25k –pDNA polyplexes with PLGA d) AFM image of dextran nanogel. e) Gd(III) complex with a cholesterol-like moiety incorporated into liposome membrane. f) Structure of PEG-PEI-folic acid conjugates for folate receptor targeting. g) TEM image of the FeOx-SiO2 core-shell nanoparticles. h) Fluorescence microscopy showing folic acid on the surface of DEAPA-PVA-g-PLGA nanoparticles for pDNA delivery. i) AFM image of siRNA-loaded PLGA nanoparticles. j) Molecular dynamics simulations of PAMAM dendrimer (G1 and G4) binding to siRNA

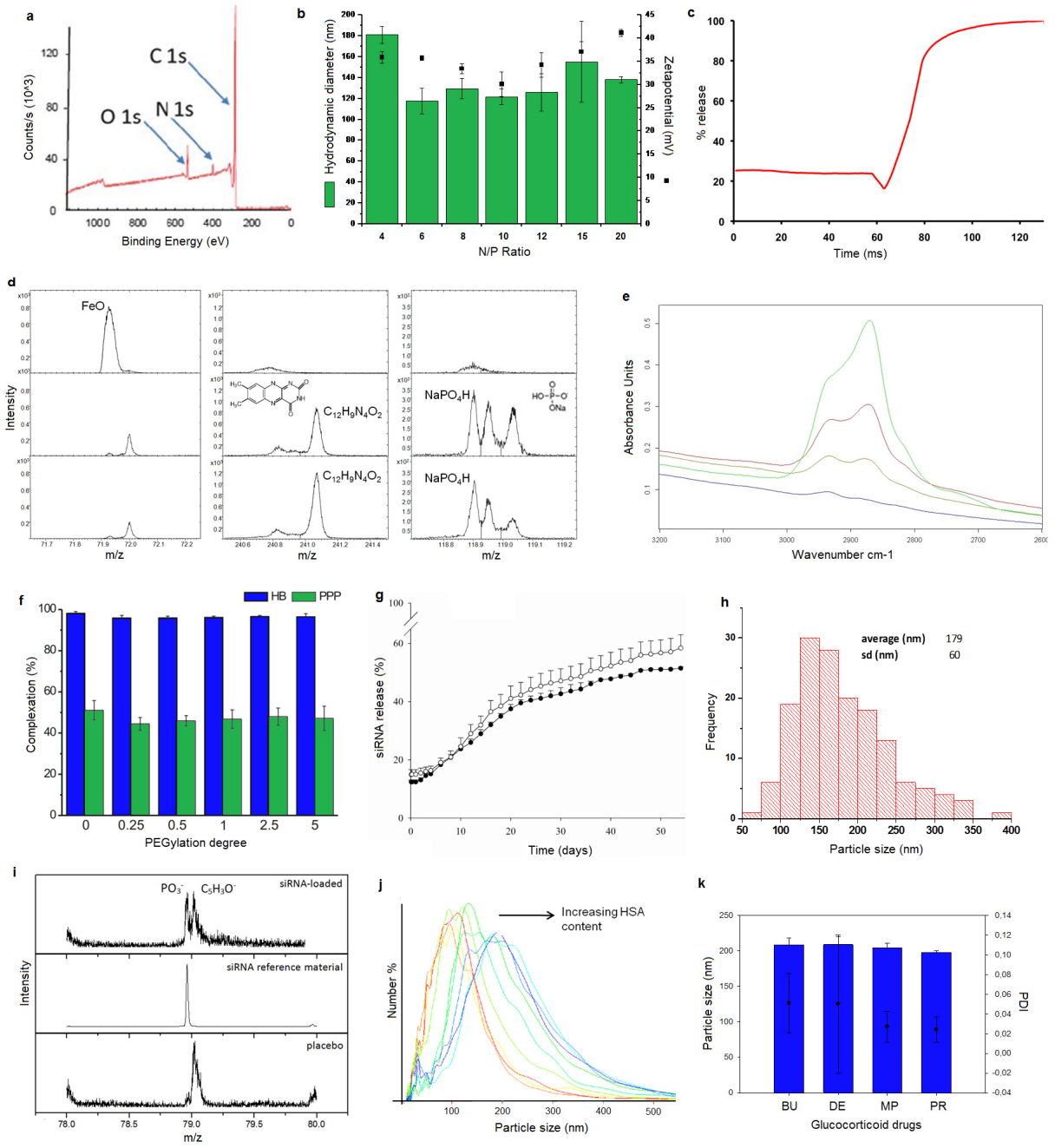


Figure 2: Overview of physiochemical characterisation. a) XPS data from PEI functionalised CNTs. b) Sizes and zetapotential of siRNA-loaded DEAPA-PVA-g-PLGA-nanoparticles at varying N/P ratio. c) Liposomal release of a model compound using a channel protein. d) ToF-SIMS mass fragments from uncoated (top) and coated (middle, bottom) FeOx nanoparticles. e) IR-spectra of FeOx nanoparticles (blue: control, brown: 20% PEG, red: 40%, green: 80%). f) Complexation of PEGylated nanogels hepes buffer (HB) and platelet poor plasma (PPP). g) Release of siRNA from PLGA nanoparticles (open 2.2; filled 0.8 µg/mg). h) Particle size distribution of PLGA nanoparticles measured from AFM imaging. i) ToF-SIMS spectra showing presence of siRNA (PO_3^- group as a marker) on PLGA nanoparticle surface. j) Nanoparticle tracking analysis of FeOx nanoparticles at varying HSA concentration. k) Particle size and PDI of drugs encapsulated in PLGA nanoparticles (dudesonide, dexamethasone, methylprednisolone, prednisolone)

WP4

Work performed and end results (elaborating on the degree to which the objectives were reached)

Briefly, the objectives of WP4 were to evaluate and optimise the active (by means of targeting ligands or imprints on their surface) or passive targeting efficiency of various nanocarriers for treatment of different pathologies, namely cancer, rheumatoid arthritis, Crohn's disease or multiple sclerosis. The targeting, binding efficiency and ability of the particles to cross the cell barriers, as well as the capacity of the target cells to internalisate the particles, were also to be studied. Whenever necessary, specialized test assays and in vitro models were to be developed.

Task 4.1: Recognition of targets in cancer. MAGFORCE generated FeOx-SiO2 core-shell particles which can be stealth coated with a PEG modified silane, thus fulfilling the task to make "long circulating iron-oxide nanoparticles". CHARITE demonstrated in C3H-RS1 mouse mammary carcinoma cells model the low toxicity, good stability and rapid uptake of these particles by the cells, achieving internalization of particles after 10 minutes. WEIZMANN generated several fluorescent transfected ovarian cancer lines and evaluated the ability to monitor the efficacy of siRNA in suppression and expression of the reporter genes EGFP and luciferase by fluorescence and bioluminescence imaging. Furthermore, they studied the passive and active (folate) targeting internalization of USPIO into MLS human ovarian carcinoma cells, CV-1 fibroblasts and JK774 macrophages, thus fulfilling the **Deliverable 27, submitted in month 25**. WEIZMANN and TUE investigated the targeting efficacy of liposomes functionalized with two angiogenic specific ligands. Optical techniques and MRI showed a dual targeting with synergistic effects, as a strongly elevated uptake of particles compared to single ligand targeting was observed. The targeting efficiency correlated well with the surface density of ligands. **Deliverables D99 and D100 were submitted in month 39. Task 4.1 finalized in month 39.**

Task 4.2 Recognition of targets in Crohn´s disease. The task of developing a cell culture in vitro model of the inflamed intestinal mucosa has been successfully fulfilled by UDS. The 3-dimensional cell culture includes the intestinal cancer cell line Caco-2, human macrophages and dendritic cells grown on permeable cell culture inserts. Reversible inflammation of the cell culture model can be achieved by addition of IL-1β to cell culture medium, as proven by the measured TEER values and release of inflammatory markers (IL-8 and TNF-α) by the cells. Furthermore, an extensive characterization of the co-culture revealed changes in the overall barrier properties, re-organization of the tight junctions, and changes in adhesion and uptake of model polystyrene nanoparticles compared to non-inflamed control. Budesonide loaded MediTrans nanomedicines (PLGA nanoparticles and liposomes) were tested in the model. PLGA nanoparticles showed an improved effect on recovering the barrier function of the cell culture compared to free drug solution while the liposomes showed an inverse effect. In parallel, CSEM designed and improved silicon nitride membranes to support epithelial cells and to permit an easy transport of the nanocarriers. CSEM developed silicon wafers containing silicon nitride membranes with micropores of 1, 2 and 3 µm size, to facilitate comparison with commercially available membranes. A special holder was designed that allows the growth of confluent Caco-2 monolayers with comparable TEER measurements as cells cultures grown on the standard membranes and high translocation rates of model nanoparticles. These membranes presented an additional advantage, as their very low background properties facilitate the microscopically evaluation of the cultures. **Deliverables D 28 was submitted in month. Deliverable D102 and D103 were submitted in month 38 and 39 respectively. The task 4.2 was finalized in month 39**.

Task 4.3 Recognitions of targets in Rheumatoid arthritis. Antiangiogenic activity of targeted nanocarriers for siRNA was evaluated in the context of cancer in task 4.1. An extravasation assay based on quantifying the uptake of fluorescent labelled nanocarriers into macrophages was successfully set up at UDS using fluorescent activated cell sorting (FACS). This technique was able to detect complete uptake of 0.1 µm model particles and partial uptake of 1 µm particles, as two different populations were identified in the splatter plot. It was also found out that 6 µm-particles are too voluminous to be taken up, instead adhering to the surface of the macrophages. A variety of liposomal formulations with different size, degree, density and structure of surface PEGylation have been evaluated through this method and correlated to their respective plasma residence time. Results show a dependence of the level of interaction on the length of the PEG chain after 90 minutes incubation, which almost vanished after 4 h exposure, although long chain PEG still reduced interaction. In vitro results did not correlate well with previously determined in vivo circulation half-lives, presumably due to differences between blood serum and foetal bovine serum used in the experiments. UU identified surface Plasmon resonance as a good tool for evaluating liposomal-protein interactions in vitro, as well as, for predicting the circulation time of liposomal formulations in vivo. Liposome interactions with plasma proteins can be evaluated allowing quantification of opsonisation and uptake by phagocytic cells from the reticuloendothelial system. It is known that plasma proteins can adhere to the outer shell of liposomes (opsonisation) facilitating its recognition by macrophages and inducing liposome clearance from the systemic circulation. Incorporation of polyethylene glycol (PEG) to the liposomes surface decreases considerably the blood clearance of the liposomes. A strong relationship between extent of protein interaction and circulation time of each liposomal formulation could be concluded. **Deliverables D27 and D101 were submitted in months 25 and 41 respectively. Task 4.3 ended in month 41**.

Task 4.4 Recognition of targets in Multiple Sclerosis. Work on assessing target binding capacity of molecularly imprinted nanocarriers for the therapy of MS was never started as the preparation of the delivery system failed. This subtask was stopped in month 25. Due to the drop out of Merck Serono from the MediTrans consortium also no in vitro screening work was conducted on actively (myelin antibody) or passively targeted nanomedicines for MS lesions in CNS. ACROSS worked on the development of a new in vitro model of the blood brain barrier in the state of inflammation. However, this task could only be partially completed. Monolayers of porcine brain endothelial cells (PBEC) have been used to start the model. The membrane grown PEBC model was successfully established and qualified for its barrier function using low (fluorescein) and high (propranolol) permeability markers and the P-Glycoprotein substrate rhodamine 123 as an indicator of the efflux activity, finding good permeability properties but only little activity of efflux transporter P-gp and low TEER values. **Deliverable D27 was submitted in month 25**. Different culture conditions were tested to improve the properties of the cell culture and collagen coating was assayed to compact the cell culture and increase the TEER values, obtaining the best improvement for the coating of the culture with 25 µl collagen IV per well. However, the achieved values were still lower than those described in the literature. Therefore, a co-culture model of the blood brain barriers was set up combining freshly isolated porcine endothelial cells with rat glia cells in a non-contact co-culture. Improved TEER values were observed, although values still fell short to literature values. A 2 fold improvement of the co-culture was also found when the permeability marker assays were conducted and a ratio of 12 was observed for digoxin efflux. In summary, an adequate in vitro model of the healthy blood barrier could be successfully set up and standardized at an industrial level by ACROSS. **Deliverable D101 was submitted in month 41 and task 4.3 was finalized in month 41**.

Brief description of methodologies and approaches employed

Task 4.1: MAGFORCE modified NanoTherm® magnetic particles with PEG optimizing the coupling-efficiency of different polyethylene glycols to the particles. The coating process was investigated by applying PEG with different molecular weights. They designed also a porous silica coating for iron oxide particles that can be PEGylated. These coatings can be easily transferred also to drug loaded particles and the resulting material can be obtained as water dispersible powder. WEIZMANN generated several fluorescently transfected ovarian cancer cell lines such as MLS transfected for EGFP and DsRed, EGFP and Luciferase and evaluated the expression of folic receptor on MLS human ovarian carcinoma cells, CV-1 fibroblasts and J774 macrophages, stained with Folic-BSA-ROX using flow cytometry. Those cell lines were tested for the USPIO uptake by staining with Prussian blue. MARBURG, WEIZMANN, UU and IDT evaluated the ability of monitor the efficacy of siRNA in suppression or expression of the reporter genes EGFP and Luciferase by fluorescence and bioluminescence imaging. CHARITE studied the passive targeting potential of the nanoparticles in cell cultures of different tumour cells (C3HRS1). TUE investigated liposomal uptake by confocal laser scanning microscopy into HUVEC incubated with different actively targeted liposomal formulations. Furthermore, MRI detection of liposomal contrast agent and antiproliferative activity in a cell cycle assay was determined.

Task 4.2: UDS prepared mesopram and budesonide loaded PLGA nanoparticles which were characterized in terms of size, zeta potential encapsulation efficiency and release of drug. Cell lines were cultivated on CSEM membranes and compared to cell growth in commercially available membranes. Cell barrier properties were evaluated by measurement of the TEER values. Macrophages and dendritic cells were seeded into a lower collagen layer and Caco-2 cells were seeded on the top of this layer. Real time PCR measurement to quantify inflammatory response was established and inflammation was induced by addition of lipopolysaccharides from *E. Coli* and *S. Typhimurium* and Il-1β. Inflammation was evaluated via the expression levels of IL-8 and TNF-α. Cell distribution and cell-particle interactions were evaluated by confocal laser scanning microscopy. Drug solution, drug loaded nanoparticles and drug loaded liposomes were incubated with the inflamed co-culture model. The pharmacological activity of the different formulations was evaluated by TEER measurements and evaluation of the IL-8 expression. CSEM fabricated silica membranes at the microscale and studied the transport of nanocarriers across biological barriers in collaboration with UDS. Silicon wafers (10 cm diameter) containing microfabricated silicon nitride membranes were produced using classical photolithographic techniques. The porous pads are firstly fabricated by depositing a thin layer of transparent material, like Si3N4 on a silicon wafer. Pores are etched in the Si3N4 by photolithography followed by a dry etch. The silicon wafer is etched from the other side to remove the entire thickness of silicon in selected areas, leaving a set of supports for the transparent porous area that remains after removal of the silicon. Several generations of specialized holders for the membranes were designed and produced.

Task 4.3: UDS established an extravasation assay using fluorescent activated cell sorting (FACS) based on quantifying the uptake of fluorescent labelled nanocarriers (polystyrene model nanoparticles and fluorescence labelled liposomes) to check particle uptake by macrophages. Two macrophage cell lines were used: MH-S and J774. UU used Plasmon resonance technology to evaluate plasma protein binding to liposomes. Macrophages and fibroblast cell cultures were incubated with different liposomal formulations and their interactions with plasma proteins such as human serum albumin, apolipoprotein E, α2-macroglobulin, β2-glycoprotein I or fibronectin were evaluated. The assay was able to distinguish between highly and poorly interacting nanocarriers.

Task 4.4: ACROSS used monolayers of porcine brain endothelial cells (PBEC) for setting up a new in vitro model of the blood brain barrier in the state of inflammation. The barrier function was evaluated using low (fluorescein) and high (propranolol) permeability markers and the P-glycoprotein substrate rhodamine 123 as an indicator of the efflux activity. Different culture conditions were tested to improve the properties of the cell culture and collagen coating was assayed to improve the TEER values. A co-culture model of the healthy blood brain barrier was set-up combining freshly isolated porcine endothelial cells with rat glia cells. In addition to previous markers, digoxin was used in this case for evaluating P-gp activity.

Relationship between project achievements and state-of-the-art

One can distinguish between two targeting principles for drug nanocarriers. Actively targeted nanocarriers have targeting ligands or imprint on their surface that enable specific binding to receptors preferentially expressed on the surface of the target cells, in particular in the state of disease. Passively targeted nanocarriers do not bear any targeting moieties on their surface. Instead, they accumulate at the site of the disease via so-called enhanced permeability and retention (EPR) effect. Here, both types of targeting have been evaluated in different cell lines and results confirm that both kind of targeting are possible, that the targeting can be modulated and that it can also be synergistic, by combination of different targeting-ligands.

Previous publications demonstrated that by coating the nanocarrier surface with inert, biocompatible polymers such as PEG, a protective layer can be formed over the carrier surface, which increases surface hydrophilicity and sterically hinders and slows down liposome recognition by opsonins or plasma proteins thus reducing recognition of nanocarriers by MPS cells (Klibanov et al. 1990, Blume et al. 1993). Here, an assay which is able to quantify the recognition by and uptake into MPS cells (e.g. splenic macrophages and blood macrophages) in the presence of opsonins or plasma proteins has been successfully implemented and confirmed as a valuable tool to predict circulation half time and the extravasation ability of nanocarriers.

In vitro screening as a precursor of animal and clinical studies allows for a cost effective, early testing of novel nanocarrier systems and may provide mechanistic insights and additional information for the optimization of formulations. Several in vitro models have been established for the physiological state of healthy intestinal tissue (Bisping et al. 2001; Toumi et al. 2004; Spottl et al. 2006) and the healthy blood-brain barrier (Gaillard et al. 2001; Perriere et al. 2007). However, in these in vitro models, pathological changes are not reflected and they therefore present only poor models for disease specific therapy approaches. Induction of inflammation in epithelial cells using lipopolysaccharides and pro-inflammatory cytokines is an often used approach (Al-Sadi and Ma 2007; Weglarz et al. 2007). Here, WP4 has succeeded in developing a ground breaking in vitro co-culture model which can be reversibly inflamed via pro-inflammatory compounds encompassing all immunocompetent cells of the intestinal tissue. Furthermore, different nanoformulations were tested using this model and demonstrating its pharmacological activity on the inflamed mucosa. Different approaches have been assayed for the set-up of a porcine brain endothelial cells (PBEC) model for the blood brain barrier. However a diseased blood brain barrier model could not be established. Instead an improved co-culture model of the healthy blood barrier presented the final outcome of this task in MediTrans combining freshly isolated porcine endothelial cells with rat glia cells.

Impact on its industry or research sector:

WP4 has fulfilled most of the objectives expected before starting the project. Others needed to be reconstructed as it was sufficiently demonstrated that the results were negative or inconsistent.

MAGFORCE succeeded in preparing long circulating-iron-oxide nanoparticles that can be modified on their surface with PEG independently of the drug loaded. CHARITE demonstrated that those particles match the most important criteria for magnetic drug carriers for passive tumour targeting: low toxicity, good stability, uptake by tumour cells.

WEIZMANN and TUE proved that it is possible to improve the targeting efficacy of liposomes by surface modification with anti-angiogenic targeting ligands. Furthermore, a synergistic targeting effect was seen when the liposomes were coupled with both ligands.

UDS and CSEM collaboration allowed the design and development of a supporting membrane where cells can grow maintaining the same properties of a cell culture grown on a commercially available membrane but allowing the transport of nanocarriers across it, without interfering in the transport. The membranes, with their 500 nm thickness, are at least 20 times thinner than the porous support nestled in the commercial well insert. The pore sizes, shapes, densities and distributions can be tuned as desired and well controlled. They exhibit high transparency in both air and water together with low intrinsic fluorescence. The porous supports can be chemically pretreated for cell culture enhancement and they are resistant to acids, bases, solvents, high temperatures and e-beam exposure (ideal for SEM observation). They withstand common sterilization procedures and they are reusable. These membranes present the drawbacks of being expensive, which can be overcome by their reuse. It was also demonstrated here that the triple co-culture established in UDS mimics the pathological situation of the inflamed mucosa and can be used for testing the effect of different nanomedicines in the restoration of the barrier function. Furthermore, among the nanoformulations

tested, budesonide loaded PLGA nanoparticles enhanced the barrier function recovery respective the free budesonide solution, demonstrating once again, the possibility of targeting by a different nanocarrier.
UU identified surface Plasmon resonance as a powerful tool for measuring liposome interactions with plasma proteins and its subsequent clearance of blood circulation by macrophages. They could also correlate the degree of PEGylation to their plasma residence time. UDS, in parallel could also develop a technique by FACS that can identify nanoparticle uptake and relate it to the nanocarrier size.
ACROSS succeeded in developing an adequate in vitro model of the healthy blood barrier could be successfully set up and standardized at an industrial level.
All these techniques are powerful tools that can be applied for evaluating further nanocarriers by different research groups. The results broaden the knowledge of particle interactions with biological systems and take many different parameters into account. The membranes developed by CSEM will be protected by a patent and commercialized as they are well standardized and present many improvements respect to the membranes available at present for studies with nanocarriers. The co-culture as model of inflamed intestinal mucosa can be used for testing new medicines for therapy of Crohn´s disease and it can also inspire new cell cultures of other physiological barriers based on other cell types. As a groundbreaking model of a diseased tissue, it was awarded with the animal welfare award of the federal state of Rhineland Palatinate.

WP5

Work performed and end results (elaborating on the degree to which the objectives were reached)

There is more and more interest in the detailed characterization of the intracellular behavior of non-viral gene delivery systems, in order to be able to improve the transfection efficiency of established and emerging materials. WP5 offers a standardised test procedure to test the association and dissociation of carriers in buffer, serum and blood, to characterize the intracellular uptake and biological activity of new carriers and to better understand carriers by the use of advanced techniques like Fluorescence Correlation Spectroscopy (FCS), Single Particle Tracking (SPT), confocal microscopy, Fluorescence Resonance Energy Transfer (FRET) and Fluorescence Recovery after Photobleaching (FRAP). Also, the use of PCI to enhance the transfection efficiency is not widely spread. The combination of these techniques and knowledge in one workpackage makes it possible to study gene delivery complexes in more detail when compared to other labs that do not have access to these advanced microscopy techniques.

The main achievements of WP5 over the period of Meditrans were:

1) Evaluation of the stability in serum and blood, the cellular uptake and biological activity of non-pegylated and pegylated nanogels loaded with siRNA and elucidating the effect of PCI on these nanocarriers. It was found that nanogels have an acceptable loading capacity and stability in serum, are sufficiently taken up by the cells and lead to acceptable biological activity, which can even further be improved by the use of PCI. Also, nanogels show limited cytotoxicity (D61.1, D62.1, D61.2, D62.2)
2) The optimization of a protocol to study the endocytic pathways and intracellular trafficking involved in the uptake and delivery of gene delivery complexes. It was found that optimization of the concentration of specific inhibitors (chlorpromazine, genistein, filipin, nystatin, cytochalasin D, methyl-β-cyclodextrin, ...) is necessary in each cell type one wishes to study to ensure that effects on uptake and biological activity result from specific downregulation of a certain pathway (clathrin dependent endocytosis, clathrin independent endocytosis, ...) and not from cytotoxic effects or unspecific inhibition.
3) The optimization of Single Particle Tracking (SPT) to determine the endocytic 'fingerprinting' of non-viral gene delivery complexes in cells. Briefly, the protocol consists of transfecting the cells with GFP-fusion proteins to label endocytic compartments or other cell organelles of interest (e.g. early endosomes, late endosomes, lysosomes, caveosomes, autophagosomes, microtubules, golgi apparatus, actin network, ...), applying red labeled fluorescent nanoparticles of interest, performing dual color colocalization microscopy and finally analyzing the obtained movies by object-based, track-based or pixel-based algorithms. The complete endocytic 'fingerprint' of p(CBA-ABOL) complexes in RPE cells has been elucidated.
4) The optimization of SPT to follow the association and dissociation of plasmid DNA to liposomes. By a dual-color labeling approach, colocalization is only found when intact complexes are present.
5) The optimization of Fluorescence Correlation Spectroscopy (FCS) and SPT to follow stability of siRNA loaded liposomes and pegylated liposomes in full blood, by following liposome aggregation and siRNA release in function of time.
6) The optimisation of FCS and SPT to follow plasmid DNA degradation in buffer. SPT can be used to determine the concentration of intact pDNA in a solution. Also, it can be used to determine whether certain carriers (eg. Liposomes) protect the pDNA against enzymatic degradation in function of time. With higher complexation degrees (e.g. charge ratio 10 instead of 5) more intact pDNA can be found indicating that the carriers give a better protection of the pDNA against nucleases in the surrounding environment.
7) Evaluation of the nuclear membrane as a barrier to pDNA delivery and if the nuclear barrier can be overcome by using chromatin targeting nanoparticles during cell division. It was found that a 2-fold enhancement of nuclear inclusion can be achieved by chromatin targeting proteins in the cell free Xenopus egg extract system. In living cells, however, nuclear targeting seems to be very difficult since microinjected nanoparticles end up in a specific perinuclear region, preventing their interaction with chromatin during cell division. Synchronised (optimally dividing) cells always show an enhanced transfection efficiency when pDNA is used, when compared to non-synchronised (randomly dividing) or arrested (not-dividing) cells. This indicates that the nuclear barrier is still one of the major barriers to pDNA delivery.
8) Evaluation of the cellular uptake, biological activity and internalization pathway of pDNA loaded cyclodextrines.
9) Evaluation of the cellular uptake, biological activity and internalization pathway of pDNA and siRNA loaded biodegradable polyesters (Marburg) (D89a, D89b).
10) Optimization of an MRI imaging method to monitor endosomal escape (D90).
11) Attaching a photosensitiser to a nanocarrier light-induced delivery of nucleic acids by photochemical internalisation. Targeting ligands have been identified that also can be used with the PCI technology (D114).

The identification of targeted nanoparticles that optimally overcome the intracellular barriers in the delivery of siRNA or pDNA to endothelial or cancer cells as was stated in D110, D111, D112 and D113 has not been completed. However, the imaging platform which has been optimized in RPE cells (epithelial cells) is also applicable to other cell types such as endothelial or cancer cells. Therefore, point 2, 3, 4, 5, 6 and 7 all contribute to the protocol needed to

answers the questions in D110, D111, D112 and D113 and Ghent is ready to study specific carriers in specific cell types if other partners would be interested to provide us with their carriers and cells.

Brief description of methodologies and approaches employed
The main techniques used are flow cytometry (cellular uptake, determination of endocytic pathways, determination of biological activity), Fluorescence Correlation Spectroscopy (siRNA loading in nanogels, nanogel stability in blood, pDNA stability and pDNA complexation), Single Particle Tracking (dual-color colocalization studies for the endocytic 'fingerprinting', following pDNA complexation and dissociation of pDNA from the carrier, following pDNA degradation in buffer and following pDNA protection when complexed to certain gene delivery vehicles). Photochemical internalisation (PCI) is used to elucidate the effect of an enhanced endosomal escape on the biological activity of nanogels or to enhance the contrast of magnetic resonance imaging.

Relationship between project achievements and state-of-the-art
More and more effort is being made to elucidate why certain gene delivery carriers work better than others, by studying the relation between the physicochemical properties of carriers and their obtained biological effect in a certain cell type, rather than a trial and error approach. The use of advanced microscopy techniques such as Fluorescence Correlation Spectroscopy (FCS) and Single Particle Tracking (SPT) to study cellular uptake, intracellular trafficking, endosomal escape, delivery to the nucleus and degradation of nucleic acids in the cytosol is rather new. In fact, there are only limiting methods available to monitor nucleic acid dissociation of the carrier in living cells, as well as the nucleic acid degradation which results in the observation that many of these steps are still not fully understood. We believe that WP5 contributed to the development of new advanced microscopy techniques and new approaches to better understand the intracellular behaviour of nanoparticles in living cells. Also, WP5 succeeded in the design and synthesis of nanoparticles that can be used for light-triggered intracellular delivery of nucleic acids.

Impact on its industry or research sector:
The techniques developed and used in WP5 are expected to give more insight in the most critical steps in the delivery of siRNA and plasmid DNA to the target cells. Also, this can contribute to the understanding of the (biophysical) behaviour of siRNA / pDNA nanoparticles in cancer and endothelial cells. This knowledge allows to design nanoparticles which successfully deliver nucleic acids to cancer and endothelial cells *in vivo*, since it is clear that the succesfull delivery of nucleic acids strongly depends on the architecture of the nanoparticles that are used to delivery them. Therefore, an imaging platform as was developed in WP5, in which different nucleic acids containing nanoparticles can be easily screened in the target cells, is expected to facilitate the optimization of non-viral gene delivery systems.

WP6

Objectives

- To maximise availability of nanoparticle-bound drugs to target cells by using external stimuli to induce drug release from the targeted nanocarriers 'on demand'
- To optimise the release of the drug / imaging probe payload from the nanocarrier in response to physicochemical characteristics of the biological microenvironment
- To develop MRI procedures for the quantitation of in situ drug availability and delivery by means of "smart" imaging probes

Work performed and end results (elaborating on the degree to which the objectives were reached)

Several targeted nanocarriers that release their drug "on demand" by external stimuli were developed during the Meditrans project:

- Amino-functionalised FeOx-SiO2 particles that can induce local heating or can release drugs like cisplatin by using external magnetic fields [MAGFORCE]
- Nile red polymeric micelles for triggered release with High intensity focused ultrasound [UMC Utrecht]
- MR-contrast agent containing liposomes for image-guided triggered release with ultrasound [UMC Utrecht, UNITO]
- HIFU-sensitive liposomes that showed a release at 42°C under the influence of MR guided HIFU [PHILIPS, UNITO].
- DSPC-based thermo-sensitive stealth liposomes that release their MR imaging agents by the use of ultrasound heating or acoustic pressure [UNITO]

Several nanocarriers were developed and optimised to release their drug or imaging probe in response to physicochemical characteristics of the biological microenvironment:

- pH responsive liposomes that release their content in the endosomes of cells due to the lower pH of 5.5 [UNITO, BRACCO]
- Liposome formulation containing an amphiphilic lipopeptide acting as a matrix metallo proteinase (MMP) substrate and can release its content in the presence of this enzyme which is over expressed in several diseases (e.g. melanoma) [UNITO]
- Liposome-based channel protein technology for triggerable drug release after light and pH stimuli. Therefore, a liposome with a remote controlled valve, a mechanosensitive channel of large conductance (MscL) has been engineered [RUG]. Opening and closing of the channel could be controlled on command by both light and pH at the same time (see Fig. 1).
- A lipopeptide acting as Matrix MetalloProteinases (MMPs) substrate was developed incorporated in liposomes encapsulating the clinically approved Gd-agent ProHance® [UNITO]. The release of the liposomal content will be triggered by the presence of MMP's, overexpressed in several diseases.

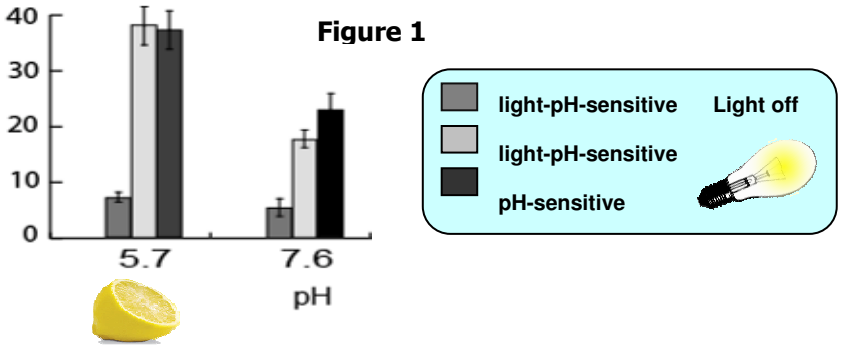


Figure 1

A number of MRI procedures for the quantitation of in situ drug availability and delivery by means of "smart" imaging probes were developed:

- An in vivo cerebral tumour model for the evaluation of extracellular pH effects in the delivery of chemotherapeutic drugs from pH sensitive liposomes/particles [CSIC].
- A dual 19F/1H-MRI land other CEST liposomal probes with contrast properties that are sensitive to temperature [PHILIPS, UNITO]
- An optimised magnetic resonance-guided HIFU system and an MR compatible HIFU system was designed and constructed, taking into account all requirements for performing ablation as well as hyperthermia experiments in small animals [UMC UTRECHT].

- A bifunctionalised carborane bearing a lipophilic chain and a MRI probe (Gadolinium; Gd) was synthesized by UNITO. The probe strongly binds to Low Density Lipoproteins (LDL) that are exploited to deliver the system to tumour cells.
- Liposomes loaded with a paramagnetic Ln(III)-based complexes namelyYb(III)-DOTAMGly (a PARACEST agent) were developed, in order to be used as induced drug release reporter when entrapped into the hydrophilic compartment.
- A boron/Gd/LDL complex was investigated for MR imageable boron neutron capture therapy [UNITO]

Methodologies and approaches employed:

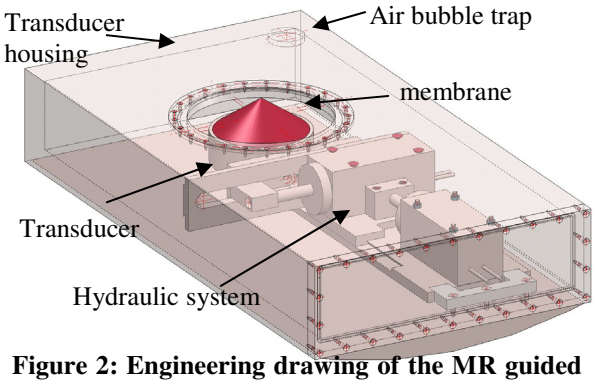


Figure 2: Engineering drawing of the MR guided HIFU system

An MRI guided HIFU system was build and optimized that allows for automatic control of the HIFU energy deposition (UMC Utrecht; Fig. 2). This system ensures that tissue temperature at the targeted location follows a predefined temperature evolution. The HIFU system is used for the investigation of HIFU-sensitive nanocarriers.

An in vivo cerebral tumour model for the evaluation of extracellular pH effects in the delivery of chemotherapeutic drugs from pH sensitive liposomes/particles was developed (Fig. 3). Therefore, rats were implanted stereotaxically with C6 glioma tumor cells in the caudate nucleus. Tumor growth after implantation was followed by T1 weighted MRI at 7 Tesla. The pH in the tumor was measured by 1H Magnetic resonance spectroscopy imaging (MRSI). The molecule imidazol 1-yl succinic acid (ISUCA) gives different signals at different pH of the tumor. ISUCA was administered in the rat and via MRSI measurements of the molecule the extracellular pH (pHe) of the tumor was defined. This method was used to investigate pH sensitive liposomes. Release of the gadolinium imaging probes from the pH-sensitive liposomes was observed in the same region were the pHe was below 6 (MRI signal intensity map).

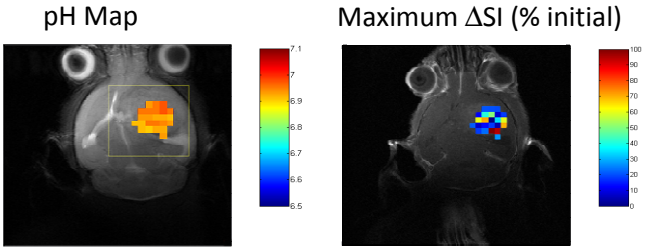
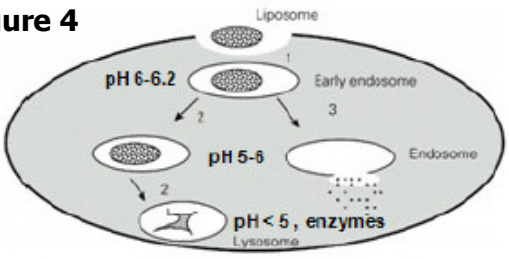


Figure 3: Representative pHe map of the model of glioblastoma multiforme in a mouse head obtained from the chemical shift variations of the H2 resonance from ISUCA in vivo. In the second figure the MRI signal intensity is increased by the release of gadolinium from the liposomes

A second system was designed by UNITO and Bracco to release its content upon the action of a specific enzyme. To this aim, an amphiphilic compound made of a stearic acid linked to an octapeptide was synthesized by solid phase synthesis and incorporated in non-stealth liposomes. The release of an imaging MR probe, in the presence of an enzyme (MMP-1) able to hydrolyze the oligopeptide, has been demonstrated in vitro and in vivo.

Figure 4



The fluorescent BSA-ROX undergoes receptor-mediated endocytosis via the caveolae pathway (Fig. 4). The vesicle membrane operates as a barrier, which masks the water adjacent the vesicle from the contrast material (CM) within it. Therefore, MR relaxivity inside the endosome is extremely low. The effect of Poly-Lys-Mellitin-RGD GdDOTA(En) on endosomal escape of BSA-ROX was investigated [Weizmann, UNITO]. A positive effect on the release of BSA-ROX was shown.

Nanoparticles loaded with Gd/B containing molecules for boron neutron capture therapy were developed (Fig. 5). A dual probe containing a carbonate unit and a Gd-containing complex (C2B10-Gd-complex) has been synthesized in order to evaluate the boron concentration by MRI.

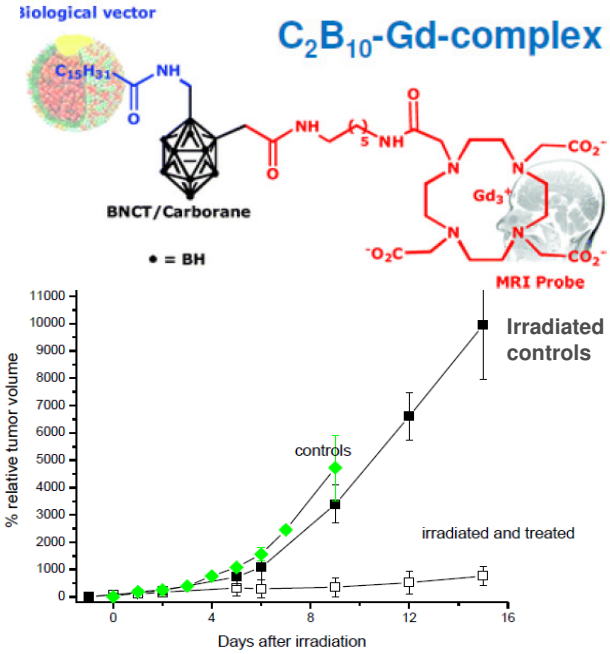
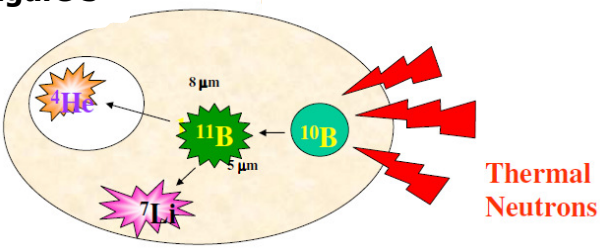


Figure 5



This probe was functionalized with lipophilic arm allowing its binding to Low Density Lipoproteins (LDL) used as a specifc vector for tumor cells. The ability to internalize the tumor cells with the complex was tested in different cell lines and in mice with implanted melanoma. The control group and the neutron irradiated group without the boron treatment showed persistent growth of the tumor. In the treated group tumor growth was arrested.

Relationship between project achievements and state-of-the-art

Image-guided therapy and individualized treatment strategies constitute one of the major research topics. The results obtained in this WP are of additional value to these important research topics and are in accordance with the planned objectives. Several approaches were accomplished in order to develop MRI probes able to report about triggered release of the nanocarrier payload; especially the new generation MRI probes like CEST are promising agents for image-guided therapy. Also fluorescence and radioactive imaging probes were used for reseach and development of the nanocarriers. Concerning the use of external stimuli, most of the attention was paid to investigate the release properties of liposomes after heating with or without HIFU. A more new concept in drug release is the release by cavitation induced with HIFU. During this period also the iron-oxide particles for drug-delivery were investigated and tested in detail. It can be expected that such systems can be tested in the clinic in the near future. As far as endogenous stimuli is concerned, liposomes that are able to release their content upon changing pH, presence of a specific enzyme and/or light stimulus were developed and tested on a animal tumor model. This new generation of nanocarriers can release their content on demand after a specific stimulus and are innovative examples of image-guided therapy and individualized treatment strategy.

Impact on its industry or research sector:

Important achievements are the development of thermosensitive and HIFU-sensitive nanocarriers, and drug releasing nanocarriers that are triggered by a specific enzyme. These particles will be the next generation of image-guided therapies. Furthermore, iron-oxide particles were developed and tested for efficacy on human colon carcinoma cell-line. An in vivo cerebral tumor model for the evaluation of extracellular pH effects was developed and validated, and tested with pH sensitive liposomes. Safety and detailed efficacy studies have to be performed for clinical use. These pH sensitive liposomes were also equipped with light-sensitive nanovalves to make them more sensitive for release on demand by external triggering. An optimized high intensity focused ultrasound system for ablation and hyperthermia was developed and was used for investigating of the HIFU sensitive micelles. The HIFU systems are currently tested in the clinic.

WP7

Work performed and end results (elaborating on the degree to which the objectives were reached)

This WP evaluated and explored the preparation, characterisation and application of several nano-particle systems for the diagnostics and treatment of chronic inflammation-related diseases like RA and CD. At the start of MEDITRANS, the partners in this WP already had considerable expertise in this area and there were extensive links with several other WPs. While the proposed nanomedicines containing corticosteroids had already been preclinically evaluated in RA models, the *in vivo* imaging of drug treatment effects and drug delivery in cvcvcvrelevant mouse models was new. The application of these formulations to treat CD is also a major innovation. The other therapeutic approaches that were proposed for preclinical evaluation were also so far unexplored. Regarding the imaging-assisted drug delivery part, procedures for the incorporation of paramagnetic chelates for MRI-based detection of liposomal nano-particles were available, but had to be established for other formulations, in particular those that were to be delivered orally e.g. PLGA nanoparticles. MRI methods for the detection of inflammatory activity in relation to RA and CD models likewise had to be optimised for the detection of the inflamed region and treatment effect. A key aspect was the MRI-based monitoring of local drug release, which is based on the use of responsive MRI contrast agents. In particular, CEST agents are promising in this respect. Complementary methods for nano-particle detection, notably optical imaging with bimodal probes and molecular imaging mass spectrometry were partly available and were exploited as well as further developed in view of their high specificity and sensitivity.

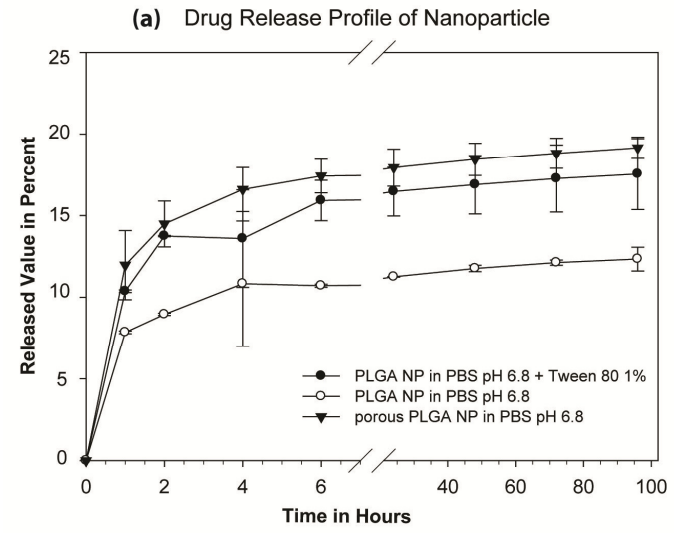
The anti-inflammatory glucocorticoid budesonide used to treat Crohn's disease via the oral route was incorporated in biodegradable (polylactic-co-glycolic) acid (PLGA) nanoparticles. The nanoparticles were characterized with regards to size distribution and surface charge, stability and aggregation on short-term storage, encapsulation efficiency and in vitro release from the formulation.

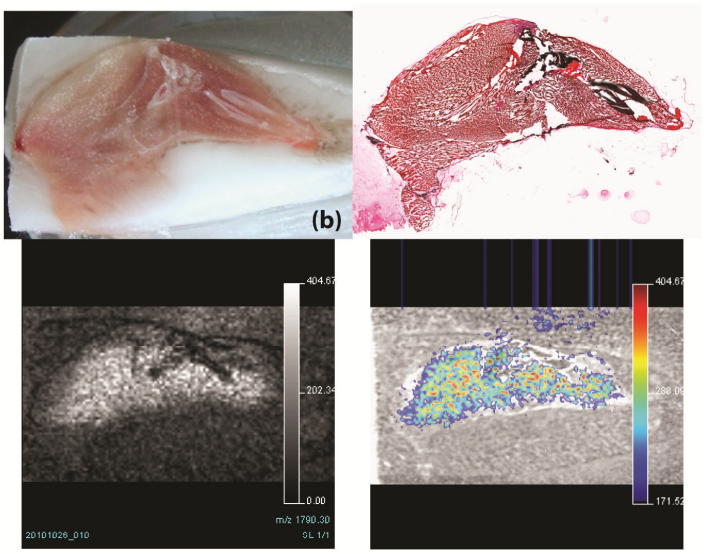
Oral use of PLGA-based nanomedicines to treat Crohn's disease (CD) was studied. Data from these glucocorticosteriod-loaded nanoparticles look promising and further investigations (e.g. to combine therapy with imaging) in an animal model were therefore done. The MSI technique was optimized to determine the different bio-molecules in the cryo-sections of RA diseased tissue. Liposome-based agents that can simultaneously act as highly-sensitive T_2-susceptibility and CEST MRI contrast agents were characterized. *Ex vivo* MRI images of the paws of RA mice, acquired at different stages of the disease progress, showed very good correlation with the disease progress score. These encouraging preclinical results obtained with the MRI tools were subsequently used for the evaluation of therapy.

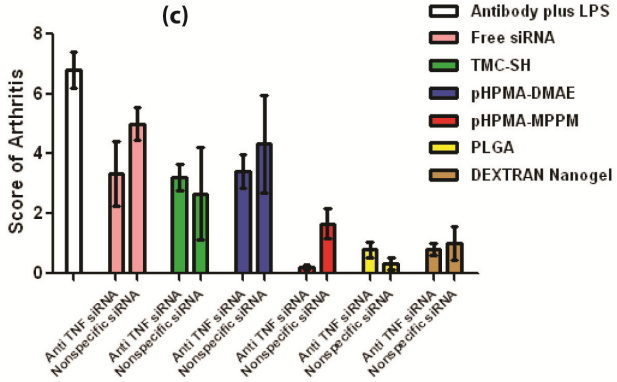
The development of CEST agents able to report about drug concentration is a very challenging task. This important and highly innovative aim was tackled using two different approaches. In the first case a PARACEST agent was synthesized to directly coordinate a drug (PLP). The binding affinity was strong, but the CEST properties of the binary adduct formed appeared not very suitable for its CEST detection. Only the reduction of the CEST contrast generated by the free agent was detectable in response to the presence of the drug. In the second approach, the possibility to use SupraCEST adducts was explored, but it was not possible to find the condition in which the supramolecular adduct formed between the cationic protein protamine, the anionic shift reagent [TmDOTP]4- and the drug PLP is stable and its CEST properties dependent on the drug concentration.

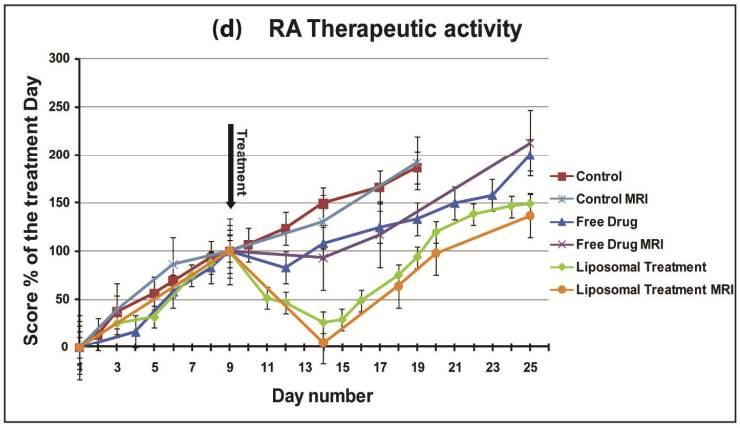
The chemically modified dicer substrate siRNA (DsiRNA) was designed and synthesized, and was used for targeting mouse TNF-α and to that aim formulated into nanoparticles, for testing in the mouse model of RA. The degree of synovitis was assessed from the MRI analyses by evaluating the volume of the knee joints after administration of 1, 5 or 10 µg siRNA loaded in PLGA nanoparticles. The current data provides a preclinical proof-of-concept for treatment of RA by siRNA-mediated TNF-α silencing upon local delivery with PLGA nanoparticles. Further experiments are needed to evaluate if the effects are a result of an active RNAi process. Whether the synovial macrophages are indeed the target cells *in vivo* for the siRNA-loaded PLGA particles should also be addressed. The study demonstrates the importance of careful siRNA dose titration because non-specific effects of the siRNA or the carrier may dominate at high concentrations and thereby mask the RNAi effect. Whether the synovial macrophages are indeed the target cells *in vivo* for the siRNA-loaded PLGA particles should also be addressed.

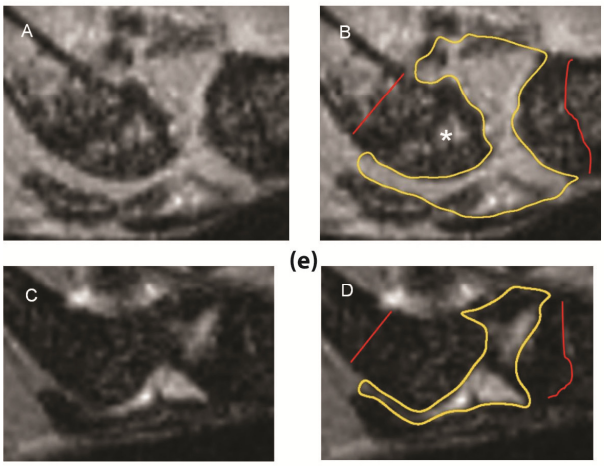
Initial studies were started to study the efficacy of liposomal corticosteroids in CD and the MRI protocol was optimized to monitor the disease progress in a mouse model of CD. Preliminary data show that colitis before and after treatment may be distinguished from each other with contrast-enhanced MRI and the clinically approved MRI contrast agent Prohance. MSI techniques optimized to determine a broad range of bio-molecules in cryo sections of diseased tissue.

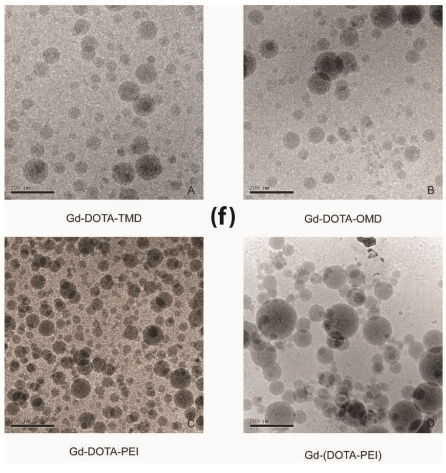












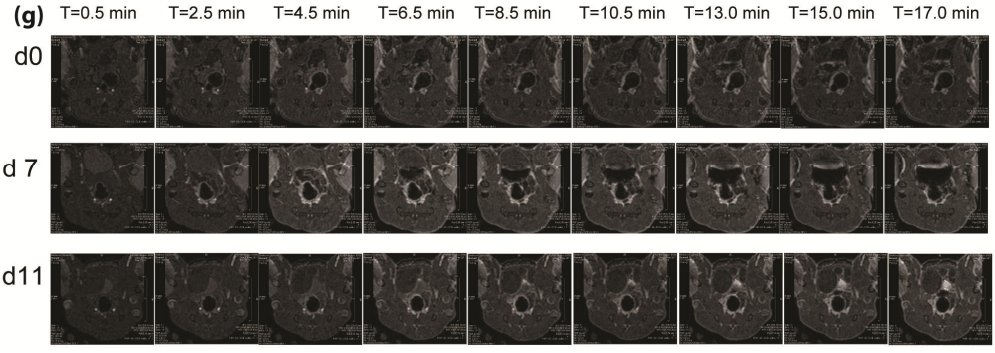


Figure 1: (a) In vitro drug release profiles of budesonide from PLGA nanoparticles (15 mg budesonide, PVA2%, EA), prepared using a solvent evaporation method; nanoparticles were separated from non-encapsulated drug by centrifugation

and washed three times using vivaspin 20 columns; mean ± SD, n = 4 (b) Clockwise: A ex vivo knee sample, the H&E stained specimen, the MSI profile image of the molecular distribution of ACTIN m/z 1790.30 and an overlay: co-registration of optical image with MSI image. (c) Scores of Arthritis in 4 limbs of collagen antibody-induced arthritis (CAIA) mouse model, after intra-articular injection of siRNA complexes. The score of RA for each limb is ranged from 0 to 4, therefore the score of RA for each animal is ranged from 0 to 16. (d) The graph shows paw inflammation clinical scores and total area of the MRI images of the paws of mice after single treatment. Score was set at 100 % at the day of treatment, indicated by arrow. In vivo MRI indices and clinical score correlate well with each other. Symbols: (■, ✳) saline group; (▲, ◆) 10 mg/kg of free Dexamethasone and (✕, ●) 10 mg/kg of Dexamethasone-PEG-Liposomes. (e) Sagittal MR images (central (8th) slice) of arthritic (HEPES treatment group) (A and B) and healthy (C and D) mouse knee joints (group 8 baseline). Example of delineation of the capsular border of the joint (yellow) and the epiphyseal border of the tibia and the transverse line connecting the ends of the cortical lines of the long femoral bone. *Highly intense region probably corresponding to bone edema. (f) Cryo-TEM images of Gd-DOTA-PEI-modified PLGA nanoparticles. (g) Time-course of contrast uptake in the colonic wall of a mouse after injection of Prohance at three time points: d0, before induction of colitis (upper row), d7, after induction of colitis, d11 after treatment with sulfasalazin per os (2.5 mg per day)

Brief description of methodologies and approaches employed

For Mass Spectroscopic Imaging (MSI)-based tryptic molecule detection and for valid molecular identification, matrix assisted laser desorption/ionization (MALDI)-MS was used with a Synapt HDMS from Waters equipped with a MALDI source. The instrument was used in MS positive-mode to measure the whole mass spectrum of molecules ionized from tissue. It was also used in MS/MS-mode (or tandem-MS) to measure the fragments mass spectrum associated with the dissociation of parent ions.

The research at UniTO on MRI methodology was focused on the quantitative assessment of the kinetic rate constant of the processes involved in the intratumor trafficking of liposomes. In particular, starting from the analysis of the evolution of the three contrast modes associated to paramagnetically loaded liposomes (T_1, T_2 and CEST), a kinetic model to quantitatively analyze the measured contrast responses was developed and used for the simultaneous fit of all the data.

In the mouse RA model, at the lowest concentration of siRNA tested, a significant decrease in joint volume was observed at day 10 for the group treated with TNF-α specific siRNA, compared to the negative control siRNA ($p < 0.001$). In addition, treatment with non-loaded nanoparticles at a dose similar to the dose for the nanoparticles loaded with 1 µg siRNA tended to be different from the treatment with specific siRNA ($p = 0.088$). The joint volume following specific treatment with PLGA nanoparticles was also reduced compared to treatment with naked TNF-α siRNA ($p<0.05$). No difference between the treatment groups was observed at higher doses of siRNA, which was in agreement with the results of the initial in vivo experiment, and the same tendency was observed at day 7. The degree of edema in the tibial and femoral head as well as the RA score of the limbs did not show any differences between the treatment groups.

Biodegradable PLGA nanospheres have been successfully labeled with gadolinium chelates for contrast enhancement in MRI. Aided by three different types of spacers the two important chelating ligands DTPA and DOTA, were covalently coupled to the particle surface and loaded with the Gd^{3+}. The modified nanoparticles were of spherical shape with mean diameters of 150 to 200 nm and narrow size distributions as determined by DLS and cryo-TEM. By contrast to the tested linear diamine spacers, branched polyethyleneimine enabled the immobilization of high amounts of gadolinium on the particle surface. ICP-OES analysis of the gadolinium content and HPLC examination of the PLGA content revealed up to 150 µg gadolinium per mg PLGA for Gd-DOTA-PEI-modified particles and even 236 µg mg^{-1} for Gd-DTPA-PEI-modified particles. The particles were characterized by high relaxivities of up to 17.5 $mM^{-1}s^{-1}$ at 25 °C and 1.41 T for Gd-DOTA-PEI-PLGA-NPs, rendering them powerful T1 lowering contrast agents. Nuclear magnetic relaxation dispersion profiles confirmed that the Gd-chelates are non-rigidly linked to the exterior of the nanoparticle. By combining the present approach with existing strategies for the encapsulation of drugs, multifunctional PLGA carriers might be designed for use in image-assisted therapy applications.

CSIC developed 3D MRI visualization approaches for dynamically monitoring the progression of Gd (III) doped contrast materials through the gastrointestinal tract in live mice. These studies are critical preparations for the use of MRI in longitudinal *in vivo* measurements in CD mice. Isofluorane anesthetized adult C57/BL mice (1%) received an intragastric administration of 0.4 mL Gd(III)DTPA and the passage of the contrast agent through the GI tract was followed by MRI during the next four hours. Acquisitions conditions were: Spin-echo MRI sequence, echo time TE=10ms, repetition time TR=450ms, slice thickness = 1mm, voxel dimensions = 192x18x256 voxels.

At TUE, a MRI protocol for in vivo CD experiments was developed. To estimate T1 in the colonic wall before contrast agent administration, a 3D T1-weighted FLASH (RF- and gradient-spoiled) sequence was applied with the following imaging parameters: FOV=20x18x20 mm^3, matrix size= 150x143x150, TR=10 msec, TE=2.8 msec and multiple flip angles α equal to 2°, 5°, 7°, 10°, 15° and 20°.

Relationship between project achievements and state-of-the-art

The study of chronic inflammation-related diseases (such as RA and CD) and their early diagnosis to initiate timely treatment is very much needed. The development of image-guided therapy and individualized treatment strategies plays a very important role in achieving this target. The results obtained in this WP contribute to this goal and are in accordance with the planned objectives. Several approaches were accomplished in order to diagnose RA and CD in mouse models during the early stages of disease development and during therapeutic interventions, using different MRI techniques and different nano-particle formulations. Several new generation MRI probes were developed and tested and showed promising characteristics for use in image-guided therapy. It can be expected that such systems may eventually be translated for use in the clinic. Irrespective of the success of the uncertain clinical translation, the nanomedical tools developed in WP7 are likely to find widespread use in preclinical image-guided therapy studies aimed at the development of improved and more effective treatments of chronic inflammation and a wide variety of other disorders that benefit from nanoparticle-based drug formulations.

Impact on its industry or research sector:

Important achievements are the development of theradiagnostic approaches using nanocarriers and non invasive *in vivo* visualisation techniques. The next generation of image-guided methodologies for two major inflammatory diseases were tested and this provides important openings for future use in the research as well as pharmaceutical industry setting. Furthermore, PLGA particles were developed and tested for their efficacy as a nano-sized drug carrier as well as MRI imaging agent. *In vivo* models of CIA-induced Rheumatoid Arthritis model and DSS-induced Crohn's disease for the monitoring of disease progression and controlled drug efficacy studies were developed and validated, and tested with different nanocarriers as well as MRI protocols. Safety and detailed efficacy studies were not part of WP7 activities and would obviously have to be performed prior to considering their clinical use.

WP8

Work performed and end results (elaborating on the degree to which the objectives were reached)

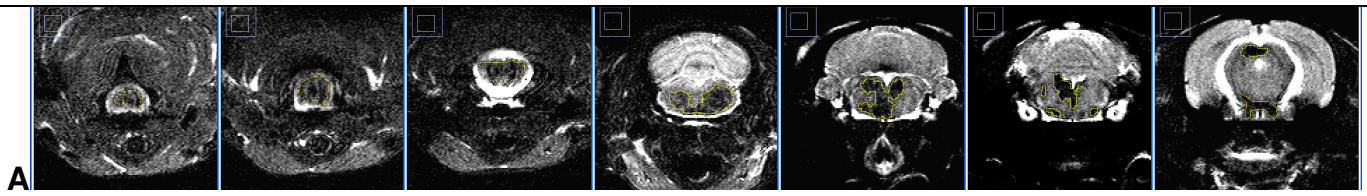
The objectives of WP8 at the beginning of the MEDITRANS project were:

1. To study pharmacokinetics, tissue distribution, targeting efficiency, and therapeutic efficacy of the developed targeted nanomedicines in suitable animal models of MS. (Task 8.1 – Passive targeting approach; Task 8.2 active targeting approach)
2. To design and optimize a carrier for imaging-guided drug release and delivery in the Central Nervous System (CNS) (Task 8.3 - MRI guided drug delivery)
3. To evaluate, *in vivo*, the therapeutic efficacy of MMP-inhibitors, through imaging-guided targeted and triggered delivery, in CNS lesions induced by MS-like pathology (Task 8.3 - MRI guided drug delivery)

Final decision from RBM (renamed MSSA after major company reorganisation) to withdraw from the MEDITRANS consortium (last quarter of 2009) has made it mandatory to revise the objectives, workplan and deliverables of WP8, as detailed throughout this document.

Task 8.1. The set-up and characterization of the experimental autoimmune encephalomyelitis (EAE) mouse model for Relapsing-Remitting (RR) MS-like pathology was addressed since the beginning of the project. The EAE model was finally obtained by immunizing SJL female mice (day=0) by s.c. injection in each flank of proteolipid protein in Complete Freund's Adjuvant (CFA) containing mycobacterium tuberculosis. Immediately after, animals received a solution of pertussis toxin by i.p. injection. Animals become progressively paralysed from the tail, through the back legs to the front legs due to progressive degeneration of myelin caused by infiltrating inflammatory immune cells into the spinal cord and brain. Starting from day 7 post-immunization, clinical signs arise whose severity was assessed using a scale of clinical score ranging from 0 to 5. The profile of cytokines in plasma was then assessed at several stages of the disease.

EAE is characterized by a major central nervous system infiltration mainly composed of T, B cells and macrophages, and this infiltration is paralleled by blood brain barrier leakage. To evaluate the permeability of the BBB during the progression of EAE, macrophage trafficking in the CNS was monitored by MRI. T_{2w} images were acquired 24h post administration of Sinerem (300µmol/kg) on a 7T-MRI and the area of USPIO uptake was measured in comparison to pre-USPIO images in a selection of brain sections acquired in EAE rats at different disease phases (onset, peak pathology and remission). Significant USPIO positive infiltration was measured at time of onset of the pathology (Figure 1) and decreased with remission. The major brain structure affected are concentrated within the spinal cord, pontine and cerebellar areas with however extensions toward the forebrain. This is the first time that the presence of USPIO positive areas has been described to this extent in a rat acute EAE mode. Moreover this data highlights the importance of not only lower CNS pathways in this model but also the involvement of more central brain structures.



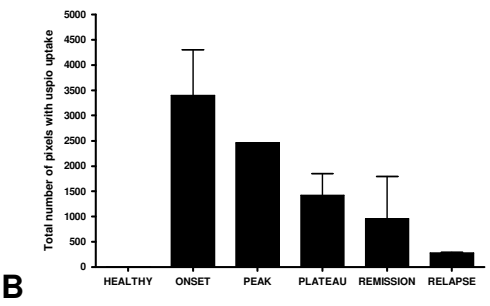


Figure 1A: Representative serial coronal sections of rat acute EAE presenting large infiltration of USPIO infiltrations at onset of the pathology

Figure 1B: Quantification of USPIO positive area at different rat acute EAE disease stage

The demonstration of substantial BBB leakage (especially in the first stages of the disease) obtained by the MRI study allows one to predict that the delivery of nanomedicines to demyelinated areas is viable. Corticosteroids are often used to improve the rate of recovery from acute exacerbation in multiple sclerosis (MS) patients. Non-targeted stealth liposomes loaded with corticosteroiods were therefore tested on EAE animals to assess whether or not liposomal encapsulation can lead to enhanced efficacy of corticosteroids in treatment of EAE. Two corticosteroids (prednisolone phosphate PLP, and dexamethasone phosphate DXP) have been adminstered as free compounds or liposomal formulations (BC18PLP: 3.0 mg/mL PLP containing liposome, 100 nm diameter; BC18DXP: 3.9 mg/mL DXP containing liposomes, 110 nm diameter) to EAE rats and their efficacy in the treatment of EAE evaluated. At the first

peak of the disease (clinical score ~ 1-2), significant clinical differences were observed in animals receiving a single injection of BC18PLP or BC18DXP compared to vehicle-treated mice (BC18BPS: drug free liposomes). These differences were maintained for four consecutive days (Figure 2A) during the acute phase of the disease. At a later time, no relevant differences were found between animals treated with BC18PLP/BC18DXP or BC18BPS. No significant clinical differences were found with the single injection of free corticosteroids compared to vehicle treated mice. Compared to these free drugs, disease severity was reduced with BC18PLP and BC18DXP (Figure 2B-C).

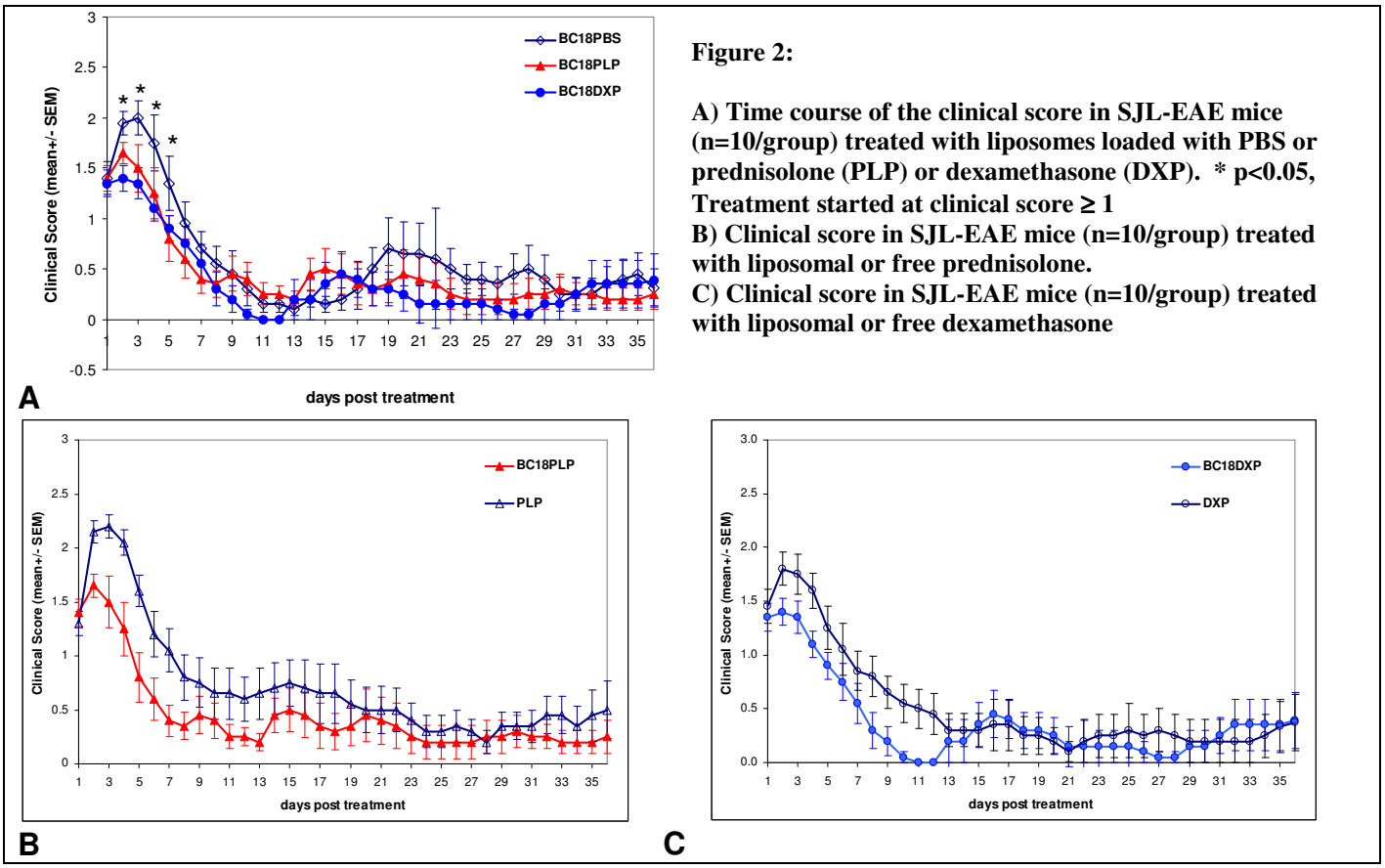
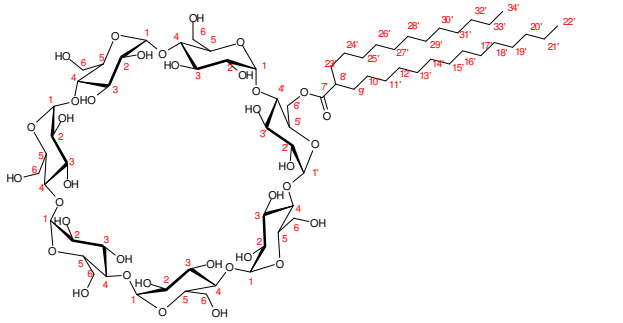


Figure 2:

A) Time course of the clinical score in SJL-EAE mice (n=10/group) treated with liposomes loaded with PBS or prednisolone (PLP) or dexamethasone (DXP). * p<0.05, Treatment started at clinical score ≥ 1
B) Clinical score in SJL-EAE mice (n=10/group) treated with liposomal or free prednisolone.
C) Clinical score in SJL-EAE mice (n=10/group) treated with liposomal or free dexamethasone

In conclusion, compared to the control group, a significant decrease of disease severity was observed for a short period (4 days) after the single treatment of corticosteroid-loaded liposomes (PLP or DXP). Furthermore, compared to free corticosteroids, the clinical benefits were more pronounced with the liposomal drugs. This study represents a significant achievement of the objectives of Task 8.1 (D44). Further testing of non-targeted nanomedicines proved difficult, as MSSA (formerly RBM), the MEDITRANS partner who had key competence and expertise in the pharmacological evaluation of the EAE model, withdrew from the consortium because of major company re-organization.

Task 8.2. Work on assessing active targeting for the therapy of MS (subtask 8.2.2: molecular imprinted-targetd nanoparticles; and subtask 8.2.3: VEGF silencing) was stopped early at month 34, with minor exploitable results. The main reason for this was the failure in the synthesis of targeted systems with suitable stability or the unexpected difficulties in the scale-up of the synthesis for *in vivo* work (as in the case of siRNA directed against the VEGF receptors Flt1 and Kdr). Drop out of MSSA from the MediTrans consortium also contributed to the decision to stop efforts on this task. Under sub-Task 8.2.1 (Ligand targetd liposomes), a nanosized system targeted to $\alpha_v\beta_3$ integrin as a target for neuroinflammation has been developed based upon liposomes bearing ligand-accessible β-cyclodextrin (β-CD) cavities on their surface. To insert β–CDs into liposomes, β–CD molecules have been made amphiphilic by grafting one 2-dodecylhexadecanoate group at position 6 per β-cyclodextrin molecule. These vesicles can be though of as a very versatile docking platform that can be loaded with suitably functionalized contrast agents for MRI, vectors targeting inflammation, and therapeutics. A targeting vector based upon the cRGDfK peptide sequence functionalized with adamantane for the interaction with cyclodextrin and a MRI contrast agent responsive to Matrix Metalloproteinases (MMPs, see below) also bearing an adamantane moiety were used to load the liposomes (Figure 3). The characterization of this system *in vitro* showed that this nanosized assembly, although conceptually very interesting, has some stability issues limitating application *in vivo*.



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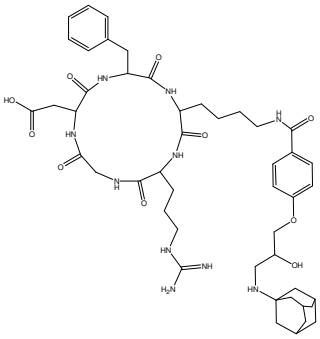

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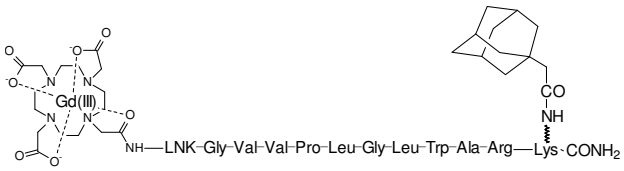
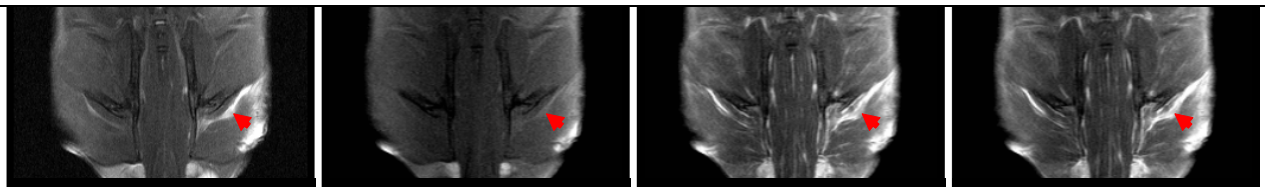


C

Figure 3: Building blocks for β–CD based liposomes targeted to integrins. A) Modified β–CD that inserts into the liposome membrane; B) Structure of the vector targeting $\alpha_v\beta_3$ integrin; C) Structure of the Gd(III) based MMP responsive contrast agent (Gd-KADA)

Task 8.3. To achieve a system for MRI drug delivery, activities focused on the development of MRI contrast agents (CAs) to assess the activity of metalloproteinases *in vivo*. The MMPs activity in neuroinflamed/neurodegenerating regions is in fact strongly related to the staging of these pathological processes. The first approach to such a MRI probe was based on the "on-off" approach. A peptide sequence containing a cleavage site for MMP (namely the PLG*LWAR) has been conjugated to Gd-DOTA on the N-terminus and an alkyl chain on the C-terminus. Complexation with Gd(III) in organic solvent and basic pH afforded an insoluble CA (Gd-PLG). In the insoluble form the CA is MRI silent (no contrast enhancement, "off" state). Upon cleavage by MMPs, a soluble Gd-contining fragment is released, that gives contrast enhancement in images ("on" state). Responsivity of this system to a subset of MMPs involved in MS, was demonstrated *in vitro*, in MMP-expressing astrocyte cultures and in the serum of EAE mice as well (D46, D47). Due to difficulties in the formulation of this CA for in vivo studies, the "on-off" approach had to be replaced by a dynamic contrast enhancement (DCE) approach. The new strategy for the visualization *in vivo* of MMP activity by MRI is based upon the Gd-K11N molecular probe (Figure 4), an amphiphilic Gd(III)-based contrast agents (CA) that can be cleaved at a known site by a number of MMPs. Upon cleavage, Gd-K11N undergoes to an amphiphilic-to-hydrophilic transformation, which yields a sharp change of its pharmacokinetic profile (D126m). A first proof-of-concept of the responsivity of Gd-K11N to the activity of MMPs was successfully obtained *in vivo* in a tumor model. Then, Gd-K11N has been tested on the sciatic nerve crush model of neurodegeneration/regeneration, that is well-established for the study of degenerating and regenerating axonal pathways in the mammalian nervous system, and shows (like MS) a profile of MMP expression that is dependent on the stage of the disease. Experiments with nerve crushed rodents demonstrated that Gd-K11N does accumulate in areas of injured tissues, where high MMP activity is expected, only when inflammation is ongoing. Other aspecific Gd based CAs such as B22596 or Gd-HPDO3A showed a faster washout from the injuried tissue



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A

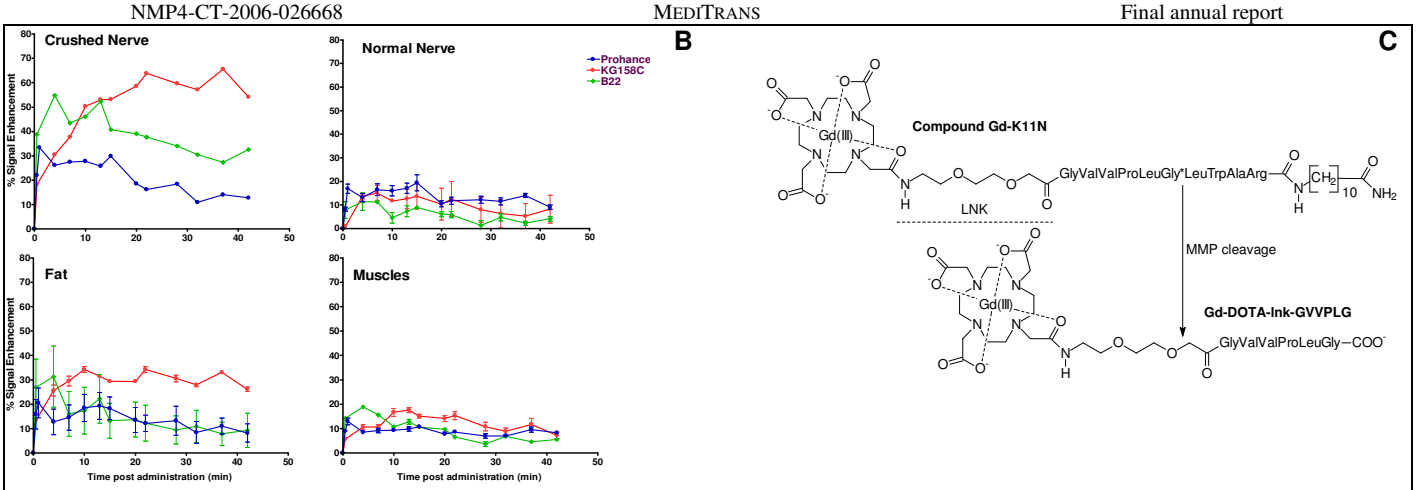
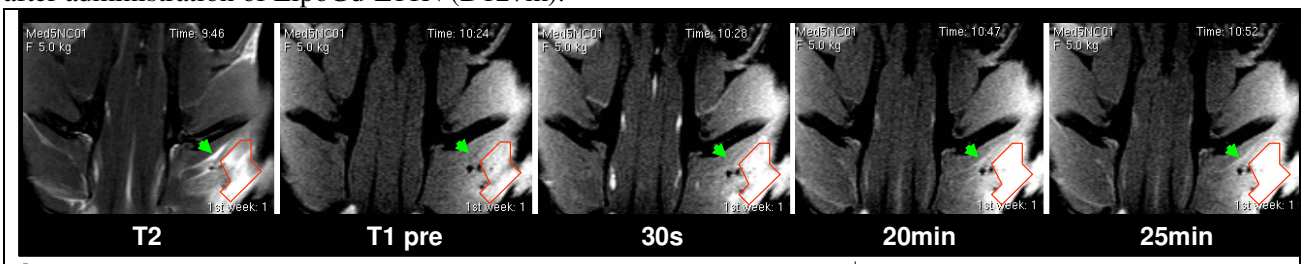


Figure 4: A) T1 weighted images of the sciatic nerve in a mouse at 1st week from crush (red arrow), pre and post-injection of Gd-K11N (i.v. at 30 µmol/kg). B) Time course of the signal enhancement of Gd-K11N (red), Gd-HPDO3A (blue) and B22596 (green) measured in different regions (crushed nerve, contra lateral nerve, muscles and fatty tissues). C) Structure of the MMP-responsive MRI CA (Gd-K11N)

Task 8.4.

Task 8.4 was introduced after revision of WP8 (Annex I, Feb 2010 edition) to apply the MRI method developed under Task 8.3 for the visualization of the efficacy of MMP inhibitors. A major limitation of those CAs is that they are rather toxic and can therefore given at a low dosage (still with adverse effects). To circumvent these problems and to achieve a MRI method for the visualization of the effect of MMP-inhibitors on neuroinflammation two actions have been taken. Namely, *i)* the peptide moiety of the CA has been changed (from cationic to neutral, compound Gd-L11N) and *ii)* the CA has been included into liposomes (liposomes containing Gd-L11N are called LipoGd-L11N). Evaluation *in vivo* of such liposome formulation of the CA to assess MMP activity in the nerve crush model of neurodegeneration was successful (Figure 5). The liposome formulation actually abolishes toxicity, and offers the advantage that a wide variety of compounds (including therapeutics) might be inserted within the liposome carrier in addition to the contrast agent. Gd enhancement washin/washout kinetics in the nerve crush mouse model injected with LipoGd-L11N were found to be dependent on the treatment of the animal with a broad spectrum MMP inhibitor. Namely, wash-in rates are much slower when MMPs are inhibited than when they are not inhibited. The molecular mechanisms underlying the fate of the CAs within the neurological lesion deserve further investigation. However, our results show that the efficacy of therapeutics based on MMP-inhibitors can be assessed by the method based upon dynamic contrast enhancement after administration of LipoGd-L11N (D127m).



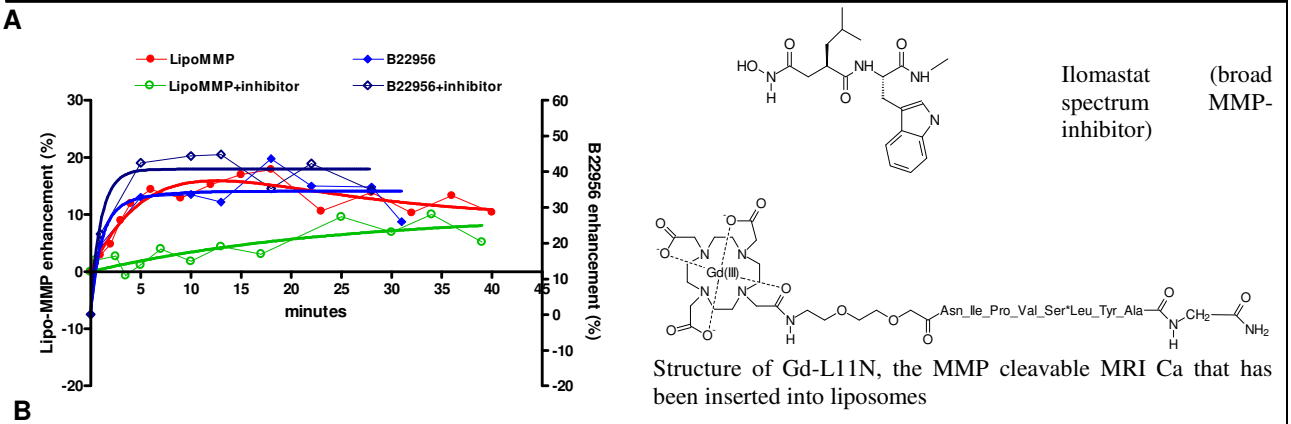


Figure 5: A) Images of the sciatic nerve in a mouse after 1 week from crush (ROI indicated by the green arrow), pre and post i.v. injection of LipoGd-L11N at 20 µmol/Kg. The ROI marked in red is the scar from surgery. B) Time courses of the signal enhancement in T_{1w} MR images of LipoGd-K11L and B22596/1 with/without administration of the broad spectrum MMP-inhibitor Ilomastat. Left panel: crushed nerve; right panel: scar

Brief description of methodologies and approaches employed

The MMP-cleavable contrast agents were prepared by Solid Phase Peptide Synthesis (SPPS), whereas a variety of intermediates (DOTA-based ligands, amphiphilic cyclodextrins, etc) were obtained by more conventional organic chemistry techniques. The purification and characterization of the synthtized products was achieved through analytical or preparative scale HPLC-UV, HPLC-ESI Mass Spectrometry, and by multinuclear multidimensional high resolution NMR spectroscopy. The magnetic properties of Gd-complexes were studied by advanced relaxometric techniques (Field Cycling Relaxometry, Nuclear Magnetic Resonance Dispersion profiles) and analytical ICP atomic emission spectrophotometry. The cleavage kinetics (MMPs) of the substrate produced was evaluated by analytical HPLC-ESI MS, UV-vis spectrophotometry or fluorescence assays. Liposomes were characterized by Dynamic Light Scattering (DLS) techniques or zeta potential analysis, and substrate/liposome interactions were evaluated by relaxometry or NMR spectroscopy. The in vitro efficacy and toxicity of candidate materials or contrast agents was tested using optimized cell culture methodologies. Evaluation of cytokines for the characterization of the animal models of neurodegeneration/neuroinflammation was done by means of a multiplex array and electrochemiluminescence. Imaging of MMP activity in vivo was achieved by Magnetic Resonance Imaging techniques at different magnetic fields (1, 3 or 7 Tesla) by acquiring T_{1w} or T_{2w} spin-eco images and T_1/T_2 maps.

Relationship between project achievements and state-of-the-art

Clinically relevant diagnostic imaging techniques such as MRI are evolving toward the visualization in vivo of the molecular biochemical processes that underlie a pathologic condition rather than the visualization of the anatomic alterations brought about by the pathology. The emerging field of molecular imaging is deemed to have a very high potential for the staging of the pathology, the assessment of the efficacy of a therapy, and the choice and calibration of a therapeutic approach. The over-expression of a subset of MMPs has been identified as a marker of several neurodegenerative diseases including MS) in humans, and increased levels of MMPs activity have been correlated with the severity of neuroinflammation and, ultimately, with the severity of the disease. MMPs have therefore become a target for anti-inflammatory therapeutics, and any imaging method able to assess the effect on MMP activity during therapy would be extremely valuable to predict the outcome of the treatment and to take corrective actions. The results of WP8 provide for the first time a MRI strategy for the assessment of MMP activity in neurodegenerative processes. Liposome systems that have been finally developed offer the advantage that both a MMP-responsive MRI contrast agent and anti-inflammatory drugs can be delivered to the neurologic lesion, paving the way for the simultaneous visualization of drug delivery and efficacy at the molecular level.

Impact on its industry or research sector:

Preclinical research aimed at the assessment of drug efficacy and optimization of drug delivery will be the main research sector which can benefit from our studies. Efficacy of drugs, either corticosteroids or MMP-inhibitors, can be monitored by MRI through the assessment of MMP activity. In principle, therapeutic efficacy can be monitored over the full duration of a treatment, allowing for the study of the relationships between effects at the molecular level, anatomical changes, and clinical evaluations. The extent of BBB leakage during neurodegeneration/inflammation has been shown to be such to allow for the delivery of nanosized medicines, which can be designed to include a MRI contrast agent as well. In this case, imaging-guided drug delivery applications can be exploited for the screening of the efficacy of different drug formulations.

WP9

Work performed and end results (elaborating on the degree to which the objectives were reached)

The objectives of WP9 at the beginning of the MEDITRANS project were:

• To study pharmacokinetics, tissue distribution, targeting efficiency, and therapeutic efficacy of the developed targeted nanomedicines in suitable animal models of cancer

• Therapeutic evaluation of MRI-guided drug delivery (triggered release) in animal models of cancer

To achieve these objectives the following tasks were performed:

Task 9.1 Passive targeting approach (UU, CHARITE, FOM, ORGANON, BSP, RUG)
- Task 9.1.1 Targeting of industrial drugs (UU, CHARITE, FOM, ORGANON, BSP)
- Task 9.1.2 Nanosized iron-oxide particles (UU, CHARITE)

Task 9.2 Active targeting approach (UU, PCI, GHENT, MARBURG, UNITO, CU, IDT)
- Task 9.2.1 Targeted siRNA delivery (UU, PCI, GHENT, CU, IDT)
- Task 9.2.2 Targeted pDNA delivery (UU, PCI, GHENT, MARBURG)
- Task 9.2.3 Targeted NCT compounds (UU, TUE, UNITO)
- Task 9.2.4 Molecular imprinting targeted nanoparticles (UU)

Task 9.3 MRI guided (triggered) drug delivery and biomarkers evaluation for assessing therapeutic effects (UU, UL, TUE, PHILIPS, WEIZMANN, UNITO, CSIC, GUERBET, PHILIPSD, UMC UTRECHT)

Task 9.1 – Passive targeting approach (UU, CHARITE, FOM, ORGANON, BSP, RUG)

Task 9.1.1: Targeting of industrial drugs (UU, CHARITE, FOM, ORGANON, BSP)

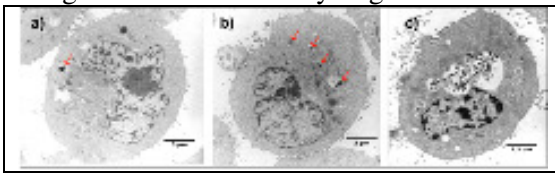
Pharmacokinetics (PK) and pharmacodynamics (PD) of liposomal formulations (ORGANON)

The aim of this research was to develop a new method for pharmacokinetics (PK) and pharmacodynamics (PD) of liposomal formulations. Liposomes are known for their drug carrying and drug targeting capacities resulting in increased efficacy and reduced side-effects compared to free drug formulations. Unfortunately, liposomes show their own disadvantages: Already numerous biodistribution studies showed large accumulations of liposomes in heavily perfused organs like the liver, spleen and kidneys (Emerson et al, 2000; Schiffelers et al, 2005; Vaage et al, 1994). Since liposomes are often used for the formulation of very toxic compounds like cytostatics this might lead to severe damage of these healthy organs. The newly developed method was found accurate, robust and reliable and is currently being applied to measure liposome encapsulated and free drug concentrations in murine plasma, whole blood, tumour, liver, spleen and kidney. Results will be completed soon and implemented into the PKPD system.

Task 9.1.2: Nanosized iron-oxide particles (UU, CHARITE)

Iron oxide nanoparticles have been equipped with different types of polymer coatings, enabling either thermally controllable drug release or prolonged circulation times. Transmission electron microscopy (TEM) analysis demonstrated that 150-200 nm clusters of iron oxides were present in the cores of the micelles. A maximum loading capacity of 40% was obtained. Magnetic resonance scanning demonstrated that the loaded micelles had high r2 and r2* relaxivities, and furthermore showed that the r2* values were always at least twice as high as the r2 values, confirming the clustering of the iron oxides in the cores of the micelles. The particles displayed excellent stability under physiological conditions, even in the presence of serum, and they therefore seem to be highly suited for image-guided drug delivery.

The TEM analysis revealed that the C3H RS1 cells take up the PEGylated SPIONs in high amounts and that they are accumulated in agglomerates in vesicular structures within the cytosol. Representative TEM micrographs after 10 min, 30 min and 24 hours incubation time. A time-dependent uptake of the PEG-NPs by C3H RS1 cells can be observed which already starts within the first 10 min after exposition. After 30 min, more of these vesicles can be seen in the images and after 24 h very large vesicles with huge amounts of nanoparticles are visible.



Time-dependent uptake of PEGylated NPs by C3H RS1 cells. a) Cell with PEGylated NP after 10 min, b) after 30 min, c) after 24 h

The obtained *in vivo* results demonstrate that the biodistribution of PEGylated SPIONs altered time-dependently and that the amount of the nanoparticles varied in different organs. The iron content in the blood decreases from 23.7 % of the injected iron 1 h post application over 11.0 %, 6 h post application to 4.9 %, 24 h post application. The iron content of the hearts, lungs and kidneys are at all taken timepoints below 1 % of the injected iron. The liver shows the highest amount of iron, which decreases over the course of time, indicating uptake of the iron oxide by the RES. Part of the SPIONs will be probably excreted after liver metabolism.

Task 9.1.3: Liposomal channel proteins (BSP, RUG)

The channel protein MscL was engineered with the pH-sensors and was reconstituted into stealth liposomes, and successfully showed for the first time its in vivo performance as a nanocarrier for image guided drug delivery to tumors. Briefly, pH-sensitive MscL containing stealth liposomes were loaded with the MRI agent Gd(III) DTPA. Intracranial C6 tumors were grown in C57BL/6 mice and when the tumor was fully developed, the pH-map of the tumors were determined by using pH_e probe ISUCA as ^{1}HMRSI spectral grids. Afterwards, pH-sensitive proteoliposomes were injected and T1 weighted images were acquired consecutively during the next 120 minutes. At the end the pH map and imaging agent release were compared. In vivo release profile of the proteoliposomes met the expectations of the in vitro experiments and the low pH voxels of the pH map in C6 glioma tumor coincided with pH-induced release of proteoliposomes. The results could be reproduced with another mice and a new study with 15 mice has been started.

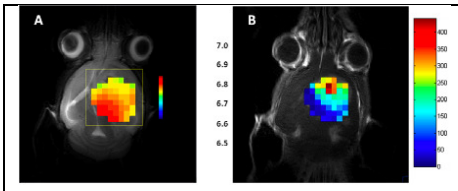


Figure 1: Representative pH map (A) and releative increase in voxel intensity (B) after the intravenous administration of a suspension of pH sentive liposomes loaded with Gd(III)DTPA. Note that the highest intensities correspond to the more acidic regions

Task 9.2 – Active targeting approach (UU, PCI, GHENT, MARBURG, UNITO, CU, IDT)

Task 9.2.1: Targeted siRNA delivery (UU, PCI, GHENT, CU, IDT)

Synthesis and analysis of siRNA

(IDT, UU, Weizmann)

IDT provided the Weizmann Institute with a complete set of DsiRNAs (10 per target) targeting mouse VEGFR-1 (Flt1) and VEGFR-2 (Kdr) in 2´-*O*-methyl modified form on the antisense strand for a complete comparison of *in vitro* activity with the set of unmodified DsiRNAs that had been previously synthesized, since initial experiments at the Weizmann Institute showed that the 2´-*O*-methyl variants (Evader DsiRNAs) were frequently less potent than the unmodified DsiRNAs. This reduction in potency seemed to be sequence specific as for some DsiRNAs the differences between the modified and unmodified variants was less pronounced. The 2´-*O*-methylation is obligatory to prevent immune stimulation and also to increase nuclease resistance of the duplex for use *in vivo*. In order to track/evaluate various nanoparticle delivery formulations by *in vivo* bioimaging IDT synthesized 10 mg of a chemically modified EGFP DsiRNA with the LI-COR IRdye 800CW covalently attached to an aminolinker on the 5´-end of the antisense strand. This near infrared fluorescent dye absorbs at 774 nm and emits at 789 nm and has an extinction coefficient of 240,000 $M^{-1}cm^{-1}$, and is ideally suited for whole body imaging of small mammals such as mice. Additionally, AlexaFluor 488 and AlexaFluor 647 tagged EGFP Evader DsiRNAs have been made available for screening uptake of targeted and non-targeted nanoparticles in cancer cells both *in vitro* and *in vivo*.

Sense and antisense strands were synthesized by the phosphoramidite method on solid-phase using optimized TBDMS chemistry, whereby the antisense strands contained heavy 2´-*O*-methylation in order to increase stability *in vivo* as well as minimize immune stimulation. Following deprotection and purification by preparative anion-exchange HPLC, the purified strands were QC´d by analytical HPLC and mass spectroscopy. The complementary strands were then annealed, desalted and the duplex was lyophilized and subjected to further QC. The DsiRNAs have already been distributed to the formulation and testing groups.

In vitro silencing of EGFP expression with DOTAP-modified PLGA-siRNA nanoparticles (CU)

To study whether the DOTAP-modified PLGA nanoparticles could enhance the delivery of siRNA *in vitro*, PLGA nanoparticles modified with various weight ratios of DOTAP (from 5 to 25%, w/w) were used for transfection of EGFP-H1299 cells, and the silencing effect was measured by flow cytometry. Particles with a positive zeta-potential (DOTAP weight percent $\geq$ 15%, w/w) showed a statistically significant gene silencing (Figure 20A) as compared to untreated cells ($p < 0.0001$), while particles displaying a negative zeta-potential (5 and 10% (w/w) DOTAP) lacked this ability, as also reported for non-modified PLGA particles. At the highest DOTAP concentration tested (25% w/w), 63% gene silencing was obtained. Importantly, the gene knock down was observed in the presence of 10% (v/v) serum, which shows that the PLGA nanoparticles are active under more physiologically relevant conditions, in contrast to serum-free conditions. Low toxicity was detected for all the formulations based on the PI staining, although there was a tendency towards slightly elevated PI levels at increased DOTAP content. Particles containing 15% (w/w) DOTAP displayed the highest potential for EGFP knock-down, while still maintaining high siRNA encapsulation efficiency and low toxicity (results not shown) and was therefore chosen for further investigation. The gene silencing activity of siRNA encapsulated in PLGA nanoparticles modified with 15% (w/w) DOTAP was dose-dependent.

Task 9.2.2: Targeted pDNA delivery (UU, PCI, GHENT, MARBURG)

Synthesis and characterization of integrin and folate receptor-targeted bioconjugates

The influence of RGD and control RAD targeted conjugates on cell viability was tested in L929 cells. Cells were treated for 24 hours with increasing amounts of conjugates and unmodified PEI as comparison. After change of media,

MTT solution was added into fresh serum-free medium and incubated for 4 h. The remaining mitochondrial enzyme activity as compared to untreated cells was determined.

Folic acid is a suitable ligand for targeting cancer cells, because of high expression levels of the folate receptor (FR), especially in ovarian cancer. Folic acid-PEG-COOH was synthesized according to a previously published method. The binding of folic acid-decorated polyplexes to the surface of FR-positive KB cells was investigated to check the targeting potential of the formulation. A significantly higher binding of the folic acid-decorated polyplexes which were prepared in glucose solution was determined in comparison to the binding of PEG-PEI-polyplexes (t-Test, p = 0.05). The binding could be increased by preparation of the formulation at slightly acid pH.

Task 9.2.3: Targeted NCT compounds (UU, TUE, UNITO)

The aim of this Task was to develop actively targeted nanocarriers which are able to specifically deliver NCT (neutron capture therapy) compounds to ovarian cancer cells upon intraperitoneal administration. It could be convincingly shown, that liposomes are suitable systems for encapsulating NCT compounds. In addition, it has been shown that in vitro, active targeting (using EGF) to EGFR-expressing ovarian carcinoma cells improves not only the binding, the uptake and the retention of liposomes loaded with NCT compounds, but - importantly - also their ability to kill cancer cells (upon exposure to boron irradiation). And furthermore, in line with this, also in vivo, liposomes actively targeted to the EGFR receptor were found to be more effective than untargeted liposomes in delivering boron to and into ovarian carcinoma cells. The vast majority of these efforts have been performed in the first two years of the project, and they have

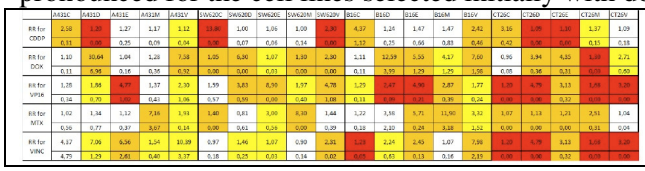
Task 9.2.4: Molecular imprinting targeted nanoparticles (UU)

Significant efforts were invested in the realization of target-specific molecularly imprinted nanoparticles. Polyacrylamide nanogel particles with varying crosslink densities were hereto prepared in the presence of myoglobin as a model protein template, as well as, later on, in the presence of several smaller oligopeptides as templates. However, after the preparation and extraction of the template, the resulting nanoparticles never presented with any specific rebinding to the imprinted nanogels (i.e. as compared to non-imprinted nanogels). Alternative approaches to obtain macroscopic or nanosized protein imprinted hydrogels, involving e.g. a semi-covalent method of imprinting using lysozyme modified with polymerizable groups via a labile linker as a template, also failed to provide convincing and/or reproducible proof-of-principle for molecular imprinting. Even attempts in which procedures published in the literature were exactly reproduced were unsuccessful. Based on these notions, and in line with the recommendations of the reviewers and the EC officers, we have decided to halt this Task early in 2009.

Task 9.3 – MRI guided (triggered) drug delivery and biomarkers evaluation for assessing therapeutic effects (UU, UL, TUE, PHILIPS, WEIZMANN, UNITO, CSIC, GUERBET, PHILIPSD, UMC UTRECHT)

MDR in tumor models (UL; See also D54)

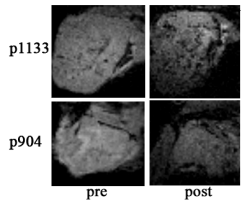
The phenotype of drug-resistant cells was characterized by a series of different experimental procedures allowing for evaluation of MDR issues at levels of mRNA expression, protein expression and function. The obvious aspects of a multidrug resistant phenotype are: i) increased resistance to the drug used and ii) resistance against drugs which were not administered before. Both features were observed in our system. The highest relative resistance (RR, ratio of IC50 value for a given drug for a resistant cell line to IC50 value for a sensitive cell line) was observed in each case for the drug used for selection of a specific cell line and in most cases the cross-resistance was also detected, being more pronounced for the cell lines selected initially with doxorubicin, etoposide and vincristine.



Resistance ratio for cell lines and drugs. Statistical significance of the sample value against control (calculated for IC50 values, one-tailed paired t-test) is color-coded: P<0.001 – red, P<0.01 – orange, P<0.05 – yellow, n=3

Folate receptor as a target in ovarian cancer: in vivo analysis GUERBET, WEIZMANN (see also D55)

USPIO contrast media (p904 and p1133; Guerbet) were administered intravenously to CD-1 nude mice bearing subcutaneous MLS human ovarian carcinoma tumor xenografts. The contrast material was found to be localized specifically in the tumour rim. The kinetics of P904 or P1133 delivery and distribution were followed by MRI. Immediately after injection we monitored the decrease in the signal intensity in the T2* weighted images in the centre of the tumour and in the periphery. After 24h, the signal in the centre of the tumour was recovered along with clearance of the contrast material from the circulation. Blood monocytes from these mice were isolated by Ficoll-gradient and adherence and were stained with Prussian blue. We observed USPIO internalization by these cells. In view of the observed induction of binding of folic-BSA-ROX after exposure of macrophages to targeted p1133 USPIO, we tried two-dose sensitization-targeting protocol. In a preliminary experiment, we injected small dose (3 µmolFe/mouse) i.v. followed 24h later by a larger dose (18 µmolFe/mouse) of USPIO. In contrast to the single dose, we found that pre-sensitization led to increased delivery of the contrast media to the centre of the tumour of mice injected with P1133.

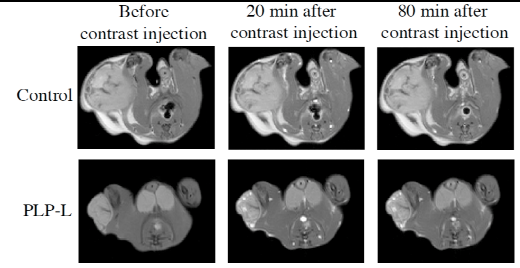


MLS xenografts bearing mice injected with folate receptor targeted P1133 and non targeted p904 (Guerbet)

Hypoxia imaging (CSIC)

Hypoxia is known to play a central role in the development of oncologic diseases and their treatment. In this period CSIC has investigated a novel protocol for the non invasive detection of hypoxia in tumors in vivo using ^{1}H Magnetic Resonance Spectroscopy. Nude mice with C6 glioblastoma cells in the flank were injected intratumorally (multisite) a solution containing a mixture of Misonidazole (300 mg/kg) and TSP (2 M). The rate of reduction of intratumoral Misonidazole in vivo was dependent on the local oxygen tension, being faster in mice breathing normal air than in mice breathing pure oxygen. These findings indicate that the oxygen dependent bioreductive process in nitromimidazoles is detectable in vivo by 1H MRS or 1H MRSI allowing, for the first time to our knowledge, the non invasive detection of tumor hypoxia using these methodologies.

TUE: Prednisolone disodium phosphate (PLP) was encapsulated into long circulating DPPC-based liposomes to treat C57BL/6 mice with a subcutaneous B16F10 melanoma on the flank. In order to obtain a full picture of the disease during the course of treatment, we performed contrast enhanced T1-weighted imaging using RGD-conjugated paramagnetic liposomes as a contrast agent. Tumor size measurements and the anatomical T2-weighted images, indicated no regression of the tumor mass and continued tumor growth. Nevertheless, treatment with liposomal prednisolone phosphate significantly affected tumor volumes compared to vehicle-treated control animals. Although T2 maps showed, in the course of the therapy, an increasing tumor area having a shorter T2, indicating enhanced necrosis, the same trend was observed for the control group (data not shown). ADC histograms did not show any significant change in water diffusion, from which may be concluded that the cellularity of the tumor was not affected by the therapy. However, contrast enhanced T1-weighted imaging using RGD-conjugated paramagnetic liposomes showed a clear difference between the tumors treated with liposomal PLP and the control group. Compared to the control group, the treated tumors show a significant signal enhancement after contrast agent injection (Figure 12). Accumulation of contrast agent was found in the entire tumor volume. We hypothesize that the observed contrast enhancement is not due to a receptor-mediated association of the contrast agent but results from unspecific accumulation by increased tumor perfusion.



Representative T1-weighted MR images through the tumor of control and PLP-L-treated tumors acquired before, 20 min and 80 min after administration of the RGD-conjugated paramagnetic liposomes

Fluorine and proton MRI (PHILIPSD and PHILIPS)

PHILIPSD/PHILIPS focused on providing highly sensitive MR detection methods for ^{19}F imaging label compounds, which show multiple MR resonance frequencies like for example perfluoro-carbons (PFC), and which could be highly relevant for future clinical applications in drug delivery monitoring for cancer applications. A combination of ultra-short echo time (UTE) with a balanced steady-state free precession (SSFP) pulse sequence offers a means for highly sensitive detection of multi-resonant imaging labels like PFOB, combining the signal of near-lying chemical shift components. For the developed technique, there are no requirements for special reconstruction or correction methods. Such a robust technique will be important for the clinical translation of NP-based targeted molecular MRI. The sequence was implemented on a 3T clinical whole-body scanner (Achieva, Philips Healthcare, NL) using a $^{19}F/^{1}H$ dual-tuned transmit/receive solenoid coil (∅7 cm). With S=51 $\mu mol_{PFOB}^{-1} min^{-1/2}$, the proposed UTE-SSFP technique is superior with a sensitivity of at least twice that of other sequence types. The next best sequence is balanced SSFP with a cartesian k-space trajectory, demonstrating the value of the T1/T2 contrast, in particular for perfluoro-carbons with long T1 and for agents with shortened T2 such as those bound to molecular targets. The signal gain by constructive addition of all CF_2 lines clearly over-compensates the loss in SNR-efficiency imposed by 3D radial sampling (25%) and the FID readout, which requires twice the number of k-space lines, since all start at $k_{x,y,z}=0$. For $(CF_2)_6$, the proximate CS components lead to destructive signal overlay at larger echo time (e.g. 2.8 ms) and are difficult to separate with line selection techniques.

Brief description of methodologies and approaches employed

Multiple types of particles were generated for targeted imaging and delivery of therapy to preclinical tumor models. Targets include epitopes on the tumor cell surface, such as specific receptors (eg the folate receptor); targets on tumor endothelial cells (eg integrins) and targeting of tumor associated macrophages (uptake by phagocytosis). Therapies studied included anti-inflammatory drugs, pDNA and si-RNA.

Relationship between project achievements and state-of-the-art

The tools developed at Meditrans are at the state of the art. Novel methods were introduced for targeted delivery of novel therapeutics. In vivo imaging was developed to be active sensors of tumor progression, drug delivery and for monitoring the response to therapy.

Impact on its industry or research sector

The project led to significant advances in nanoparticles design for targeted imaging and therapy. The impact on research is significant. The tools developed are valuable for the study and manipulation, and for in vivo imaging of preclinical models of human cancers. Some of the agents are now being evaluated for their potential clinical use.

WP10

Work performed and end results (elaborating on the degree to which the objectives were reached)

The work performed in this work package aimed at studying the potential adverse effects of the selected nanomedicines, prior to clinical studies. The work plan was based on safety pharmacology tests which included pharmacokinetic, biodistribution, and toxicology studies. Four nanomedicines were selected among the various candidates investigated in MediTrans: two nanomedicines for the treatment of cancer (*i.e.* Liposomal Dexamethasone and Polymeric Micelles) and two nanomedicines for imaging-guided drug delivery (*i.e.* Liposomal Gadolinium and Ultrasmall superparamagnetic iron-oxide (P904 USPIO)). The collected data are important prior to human trials and identification of clinical monitoring parameters.

Liposomal Dexamethasone

Preclinical pharmacology studies with liposomal corticosteroids, with the aim to target tumor-associated inflammation, have become a highly relevant research topic in targeted nanomedicine. From a clinical point of view, the use of dexamethasone is attractive as it is 5 to 10 times more potent than prednisolone and has a more favorable pharmacological profile in oncology. Encapsulation of dexamethasone in liposomes has also been proven to reduce its side effects and enhance its efficacy compared to free corticosteroids. The toxicological profile of liposomal dexamethasone (Oncocort) was established through an extensive GLP repeated dose toxicity study in rats at different dosages. No animals died and clinical signs were observed in a few groups of both male and female mice. Body weight and body weight gain were affected in a dose related manner, from 1 mg/kg/injection. Food consumption decreased in all treated groups. Hemathological changes (mainly RBC and Hb increases, PLT and WBC decreases) and changes in urinalysis (mainly decreased volume, increaded presence of leukocytes, erytrocytes, ketones and bilirubin) were observed in animals treated with Oncocort at 1 and 5 mg/kg/injection. On the basis of the above preliminary data the NOAEL (No Observed Adverse Effect Level) for Oncocort intravenous administration was found to be 0.2 mg/kg/injection.

Polymeric micelles

Core-crosslinked micelles containing transiently covalently linked compounds represent a novel, highly attractive platform suitable for targeted delivery of small molecules and/or imaging agents. These polymeric micelles are based on a novel type of bioresorbable polymers and provide easy drug loading and controlled release properties. The micelles were loaded with Paclitaxel (PTX) which is one of the most effective anti-cancer drugs marketed today. The aim of this work was to evaluate the safety pharmacology of core-crosslinked micelles with covalently entrapped paclitaxel (coded PTXL2 CCL PM) according to Irwin method (evaluation of behavioral, neurological and autonomical responses, either in the absence or presence of provoking stimuli). No relevant effects on animal behaviour were observed at the investigated doses. The toxicological profile was assessed by determining the maximum tolerated dose (MTD) after single intravenous administration. Preliminary pharmacokinetic and bio-distribution data were also evaluated. Results indicated that the intravenous administration of PTXL2 CCL PM to mice was well tolerated at a PTX dose of 20 mg/kg, corresponding to about 1.5 times the MTD of Taxol. Preliminary bio-distribution data showed a significant amount of paclitaxel in the mice blood after intravenous administration of the polymeric micellar paclitaxel formulation (determination of organ distribution of PTXL2 CCL PM is ongoing). These preliminary results indicate that that the polymeric micelles are not toxic by themselves and the polymeric micellar paclitaxel could be a clinically useful chemotherapeutic formulation.

LipogadI

Gadolinium (Gd)-loaded liposomes represent an innovative diagnostic tool, yielding a marked contrast in magnetic resonance imaging. The gadolinium based contrast agents (GBCAs) currently used in the clinical practice are administered in the form of concentrated solutions. Gd-loaded liposomes may find applications either in angiographic studies or, more interestingly, as a reporter of changes in vascular permeability. LipogadI is a system based on a liposome containing, in its inner aqueous cavity, a concentrated solution of Gd-HPDO3A. The work focused on the assessment of *in vivo* toxicology, bio-distribution, and safety pharmacology (investigating the potential effects on animal behavior by Irwin test). The aim of the toxicological assessment was to determine the maximum tolerated dose (MTD) after single intravenous administration of LipogadI to rats. Doses of 0.4 and 0.6 mmol/kg mainly induced histopathological changes in liver and spleen which did not recover after a 14 day observation period. Animals were sacrificed at different time points, up to 7 days after administration. Blood, liver, spleen, skin and femur were collected and analyzed. According to the results obtained in the above studies, it can be concluded that that LipogadI induces histopathological changes in liver and spleen (vacuolation of Kupffer cells and spleen macrophages) which correlates with the amount of residual gadolinium found in the bio-distribution study. Mice were also intravenously administered with LipogadI and examined according to the Irwin method, either in absence or in presence of provoking stimuli. No significant differences were noted between animals treated with LipogadI and controls; LipogadI is thus not expected to cause alterations in behavioural, neurological and autonomical responses in human.

P904 Ultrasmall Superparamagnetic Iron Oxide

P904 is an ultrasmall superparamagnetic iron-oxide (USPIO) nanoparticle that is used as contast agent in magnetic resonance imaging. P904 is made of an iron core (maghemite) of 8 nm diameter and is coated with stable and biocompatible small hydrophilic molecules. The mean particle size of the coated nanoparticle is approximately 25 nm. P904 nanoparticles were evaluated as regards their potential toxicity. Results showed that the blood half-life of P904 in rats is about 145 minutes at a dose of 200 µmol of iron per kilogram of body weight. This extended half-life allows the uptake of P904 by the mononuclear phagocyte system (mainly activated macrophages). Thus, passive targeting of P904 to inflamed sites of tumours, multiple sclerosis, and arthritis diseases can be followed by MRI (hyposignal). P904 was found to be non-cytotoxic or pro-inflammatory and the lethal dose was > 25 mmol Fe/kg. Effect on blood coagulation time was only detected at the highest concentration tested (14 mg Fe/mL), which is several orders of magnitude higher than the clinical dose (50 µmol Fe/kg, *i.e.* 0.05 mg Fe/mL in plasma). Histopathological examination showed accumulation of a yellow-brown pigment in the spleen and liver. The preclinical data for animals treated with P904 are either not different from the controls (injected with the same volume of physiological serum) or not significant enough to suggest potential systemic toxicity in rats.

Brief description of methodologies and approaches employed

The preclinical pharmacology studies of the selected nanomedicines were performed in compliance with good laboratory practice regulations and animal procedures in accordance with the recommendations for the care and use of laboratory animals. Various parameters were monitored for the evaluation of the non-clinical safety information and include characterization of toxic effects with respect to target organs, dose dependence, relationship to exposure, and potential reversibility. Data were collected based on ophthalmoscopy, mortality and clinical signs, bodyweight and body weight gain, food consumption, haemathology, pharmacokinetic, biodistribution, toxicology, repeated dose toxicity, hemathological changes, gross pathology, and urinalysis studies.

Relationship between project achievements and state-of-the-art

The development of a pharmaceutical is a stepwise process involving the evaluation of both animal and human safety. The collected data of WP10 on the non-clinical safety of liposomal dexamethasone, polymeric micelles, liposomal gadolinium, and ultrasmall superparamagnetic iron-oxide are important for the estimation of a safe starting dose for further trials and the identification of parameters for clinical monitoring of potential adverse effects. Although limited, the nonclinical safety studies conducted in WP10 are sufficient for the characterization of the potential toxic effects of the four selected nanomedicine.

Impact on its industry or research sector

The results obtained in this project open new perspectives in the diagnosis and treatment of various diseases as some of the selected nanomedicines are likely to enter clinical evaluation in the near future. For example, the polymeric micelles platform will be developed towards clinical evaluation within the next years so are the ultrasmall superparamagnetic iron-oxide nanoparticles which are currently evaluated by Guerbet for phase II studies. These novel nanomedicines are expected have some impact on the diagnosis and therapy market by providing cost-efficient tools which should translate into lower health care costs.

WP11

Work performed and end results (elaborating on the degree to which the objectives were reached)

As a result of the withdrawal of BSP, ORGANON and RBM, from WP10 and WP11, there was a need to restructure these WPs in Year 3. The activities described below commenced as soon as possible in Year 4. The following nanomedicines have been selected for further study in WP10 and WP11:

1) liposomal dexamethasone for treatment of cancer (UU)
2) polymeric micelles for treatment of cancer (UU)
3) liposomal gadolinium for imaging guided drug delivery (BRACCO)
4) ultrasmall superparamagnetic iron-oxide (USPIO) nanoparticles for imaging guided drug delivery (GUERBET)

To bring nanomedicine-based products to the patient, many activities are necessary and include aspects like upscaling, validated analysis, packaging issues and the production of nanomedicines as sterile products. In WP11, several essential development steps have been successfully taken.

Task 11.1 Stability

Polymeric micelles: Drug loaded stabilised polymeric micelles are stable for at least 15 weeks at -20 °C and can undergo at least 4 freeze-thaw cycles without having an effect on the burst release, particle size distribution and the in vitro release profile of paclitaxel.

Liposomal gadolinium: The liposomal formulations remained substantially stable over a period of three months at 4°C.

USPIO: The colloidal solution of superparamagnetic iron oxide, P904, from two different preclinical batches is stable during 6 months at 40°C and 12 months at 25°C.

Overall, the proven stability is highly attractive in the industrial development of these nanoparticles.

Task 11.2 Upscaling

Liposomal dexamethasone: A large-scale manufacturing method for formulation and suitable for performance in a clean room was designed. The formulation prepared by an optimised method was monitored with in-process controls and by full characterisation of the final product. The results corresponded with nicely with set specifications.

Polymeric micelles: The optimal formulation for freezedrying of drug-loaded stabilised micelles comprised an ammonium acetate buffer. Upon reconstitution, particles are obtained with similar properties as compared to fresh formulations.

Several formulation steps, analytical procedures and other common procedures at put down in general documents as part of a documentation system to assure uniformity and traceability. A new synthesis route for the monomers and macroinitiator was developed. Despite being not optimal, rather inexpensive starting materials are used and intermediates could easily be isolated. The current manual batch process to prepare drug-loaded micelles is in-depth evaluated and generated an optimised prototype for the semi-continuous preparation of micelles. Detailed information is not given here to protect confidentiality.

Liposomal gadolinium: Liposomes were prepared by high pressure homogenization followed by subsequent tangential flow filtration. The procedure was optimised considering both the characteristics of the final suspensions and the aspects of up-scaling. In particular, each unit operation was carefully defined to have batches of liposomes with the same size, homogenisity, lipid concentration and gadolinium content. All analytical methods applied to perform the complete characterization of the final preparation were developed and validated.

Overall, all main objectives of this task with regards to the stability and manufacturing upscaling were achieved.

Brief description of methodologies and approaches employed

The networking and the intensive interactions of academia and industry were of high impact for the development of innovative future drug products in Europe. As a very first step, the selection process of the industrial developable nanomedicines resulting from earlier WPs was initiated. The selection criteria included accepted industrial developability criteria (e.g. stability, efficacy, safety, applicability).

The actual development was done in the individual labs according to standard guidelines, so including the upscaling, formulation as well as the corresponding analytical methods. Detailed apsects of the various upscaling, formulation and analytical routes were explored but detailed information is not given here to protect confidentiality.

Relationship between project achievements and state-of-the-art

The four different nanomedicines are significantly developed within this project. Some of the nanomedicines are so innovative, that one cannot consider really consider a state-of-the-art. With regard to the drug-loaded polymeric micelles, the freeze-thaw and freezedrying strategy was established and is clearly an advantage as compared to drug-loaded liposomes. Various other synthesis and formulation routes were explored but detailed information as compared to current state of the art is not given here to protect confidentiality.

Impact on its industry or research sector:

The preliminary scale-up activities have shown that it is possible to prepare these nanomedicines on an industrial scale. Until now, no criticalities were pointed out, even though further upscaling and long term stability studies heve to be completed in the next months. With the polymeric micelles platform technology, an actual product will be developed towards clinical evaluation within the next years. With regard to the use of liposomal gadolinium, a detailed evaluation about the return of investment is also ongoing and nothing can be said on the use of this nanodiagnostic before the conclusion of the cost analysis. The impact of a product like Lipogad is expected to be quite high because with the introduction of this product in the clinical practice, we could add an additional tool for patient selection and follow up specifically when a liposomal drug can be used. The current MRI contrast agents on the market can not be used for the same scope, owing to their difference in size and *in vivo* transport properties if compared with a nanomedicine. The USPIO was already developed before the Meditrans project and the future clinical use of USPIO is currently evaluated by Guerbet, with a potential phase II study in mind.

WP12

Work performed and end results (elaborating on the degree to which the objectives were reached)

Task 12.1: This Task was on Advanced Drug Delivery Courses. The objectives of 'providing advanced drug delivery courses for partners and key stakeholders' and 'monitoring and improving the efficiency of the training courses' were achieved in the following ways as foreseen in Annex I. D3, Initial advanced drug delivery course held and evaluated, was delivered in Month 15. The course was on "Drug Targeting Systems" and was held on 23rd February 2008 at UDS. More than 17 participants attended this successful course. D63, Second years' advanced drug delivery course held and evaluated, was delivered in Month 29. The course was on "MRI Technologies for Drug Delivery" and was held on 29th March 2009 at WEIZMANN. Approximately 45 participants attended this successful course. D91, Third years' advanced drug delivery course held and evaluated, was delivered in Month 40. The course was on "Targeting and Imaging", and was held on 22nd March 2010 at UDS. Between 100-150 participants attended this successful course. D141, Final advanced drug delivery course held an evaluated, was delivered in Month 47. The course was titled "MEDITRANS PhD student oral presentation competition", and was held on 30th October 2010 at Hotel Eden Roc, Sant Feliu de Guixols, Spain. Approximately 30 participants attended this successful course. These four courses were advertised to MEDITRANS partners and were actively promoted.

Task 12.2: This Task was on Internet Based Training and a Forum for Staff Exchange. The objective of 'developing the MEDITRANS website as a platform for education and training, and to facilitate the exchange of MEDITRANS' young scientists' was achieved in the following ways as foreseen in Annex I. The MEDITRANS forum was set up for information exchange, and as a tool for scientific exchange, training and education. It was designed to facilitate administrative tasks, staff exchange, and discussion forums. It was accessed via the MEDITRANS public website, which had a link to the MEDITRANS forum. The MEDITRANS forum contained a subsection on training. The training forum was available for all MEDITRANS partners to use between 4th June 2007 and 23rd April 2010. MEDITRANS partners preferred to use e-mail rather than the training forum. Therefore, it was "mothballed" on 23rd April 2010. The text for the MEDITRANS Public and Members' Training Webpages was prepared by UU, INFUTURIA and UDS. The webpages promoted all the activities of WP12. The "web site access indicator (*e.g.* hit counter)" provided detailed analysis of the effectiveness of the training webpages. The public MEDITRANS training webpage had good usage over the course of MEDITRANS with more than 5,127 total hits between September 2007 and March 2011. When usage of the public MEDITRANS training webpage is compared to usage of other MEDITRANS webpages, it is one of the most frequently used webpages after the forum, the home page, and the members' area. Internet based training has been a useful part of the MEDITRANS public and members' websites as validated by the website usage statistics. The scientific exchange programme was a success as 11 exchanges occurred between the MEDITRANS partners. D142, Internet-based training and a forum for scientific exchange, was delivered, on schedule, in Month 48.

Task 12.3: This Task was on Training MEDITRANS and Other Young Scientists. The objective of 'providing access for MEDITRANS' scientists to the training programme and events organised by the GALENOS-Network, and to create synergies' was achieved in the following ways as foreseen in Annex I. D4, D64, D92 and D143, the four exchange reports, were delivered, on schedule, in Months 12, 24, 36, and 48 respectively. The following was done in 2007, (1) development of the Training sections of the website, (2) regular internal advertisement of the Staff Secondment Programme, (3) the collection of the names and contact details of likely secondees, and (4) the collection and collation of completed Staff Secondment Forms. The regular advertisement of the Staff Secondment Programme, and the collection of the names and contact details of likely secondees (*i.e.* MEDITRANS PhD students) continued, as planned, throughout the duration of MEDITRANS. In 2007, there were no staff exchanges. In 2008, there were 4, in 2009, there were 2, and in 2010 there were 5 staff exchanges.

Task 12.4: This Task was on Co-ordination of partner participation in the GANENOS Network. The objective of providing access for MEDITRANS' scientists to the training programme and events organised by the GALENOS-Network, and to create synergies' was achieved in the following ways as foreseen in Annex I. D5, D65, D93 and D144, the four reports on participation in the GALENOS-Network, were delivered, on schedule, in Months 12, 24, 36, and 48 respectively. D145, Euro PhD qualified scientists, was delivered, on schedule, in Month 48. All MEDITRANS partners were invited to join the Galenos Network. In Month 29, TUE officially joined the GALENOS network as a new member. Other workshop and conference activities have been promoted *via* the MEDITRANS website.

Brief description of methodologies and approaches employed

This is not a Research and Technological Development WP.

Relationship between project achievements and state-of-the-art

This is not a Research and Technological Development WP.

Impact on its industry or research sector:

Relevant, up-to-date training provided to MEDITRANS partners implemented *via* courses, website-based information, exchange to other partners labs, and *via* the GALENOS Network. Enabled MEDITRANS employees, who participated in WP12 activities, to work as effectively as possible and pass on new skills learned to others.

WP13

Work performed and end results (elaborating on the degree to which the objectives were reached)

Task 13.1: This Task was on Demonstration of Non-Confidential Technologies. The objective of 'demonstrating non-confidential technologies to MEDITRANS' partners and other interested groups' was achieved in the following ways as foreseen in Annex I. D146, Delivery of demonstrations to partners and other interested groups was delivered in Month 49. Demonstrations and seminars, structured in a manner suitable for MEDITRANS' scientists, SMEs, and other interested stakeholders, took place at partner facilities between Months 42-48. MOLPROF held a demonstration webinar on 8.10.10. PHILIPS/PHILIPSD prepared a demonstration video based on the concept of temperature-sensitive liposomes for MR image guided delivery. GUERBET demonstrated MRI guided drug/contrast media (notably P904) delivery at an Imaging Conference organised at GUERBET. UDS produced an educational movie on the setup and handling of the 3D Crohn's model and on the extravasation assay. This was put on the MEDITRANS and UDS websites. UDS also organised a public demonstration course on 'Innovative *in vitro* models and assays in MediTrans' on 27.03.10. BRACCO demonstrated an MRI experiment at their imaging workshop on diagnostic and nanotechnologies on 14.07.10.

Task 13.2: This Task was on Website Development. The objective of 'developing and maintaining effective supporting channels of communication, including the dissemination contacts database, enquiry mechanisms and website' was achieved in the following ways as foreseen in Annex I. D1, Project website online (http://www.MEDITRANS-ip.net), was delivered, in Month 5. The project website has two main sections, the public and members' areas. The members' area has password restricted access and is only for use by MEDITRANS members and by the EC. The website text was prepared by UU with contributions from selected MEDITRANS partners. The project's members' forum www.MEDITRANS-ip.net/forum/ was also password protected. It contained forums for each WP, for end users, and for the PSC. The forum was discontinued on 23rd April 2010 as partners preferred to communicate via e-mail. Website content was reviewed and approved by the MEDITRANS consortium. This Task also involved website maintenance, development and updating throughout the project. By the end of the project, the website had >74,592 visitors, with >644,996 hits and with >209,210 page views. There were >54 visitors per day on average, with the USA and Germany being the most active countries. Of the MEDITRANS partners' websites, MAGFORCE provided most referals. Web pages added later in the project included: News, Useful links, Public awareness, MEDITRANS publications, and Partner website links. D94, web based presentation produced, was delivered in Month 36. The confidential contacts database was prepared and was used in Task 13.4.

Task 13.3: This Task was on Project Promotion. The objectives of 'ensuring effective dissemination of the project's results to interested stakeholders and the general public' and 'developing, co-ordinating, and reviewing appropriate dissemination strategies amongst the partners throughout the whole project duration' were achieved in the following ways as foreseen in Annex I. D2, D6, D66, D95 and D147, the five MEDITRANS promotional leaflets were delivered, on schedule, in Months 4, 12, 24, 36, and 48 respectively. The leaflets, which can be downloaded from the website, provide an annual overview of the projects' objectives, work plan, and of the results achieved in 2007, 2008, 2009, and 2010. Other promotional activities included a competition to design the project logo. The best of the 12 designs was by Dr Katrin Fischer from BSP. A project promotional poster, produced in Month 4, introduced MEDITRANS to the research community. It is available to download from the MEDITRANS website. Other project promotional material has also been designed and is available on the MEDITRANS website. For example, the MEDITRANS promotional slides are available on the members' area of the MEDITRANS website. For a full picture of the numerous other promotional activities carried out in MEDITRANS, please see D148, the final PUDK, which was delivered with the final reports.

Task 13.4: This Task was on Technology Transfer Workshops and Dissemination Event. The objective of 'ensuring effective dissemination of the project's results to interested stakeholders and the general public' was achieved in the following ways as foreseen in Annex I. D96 (First technology transfer workshop (EuroNanoMedicine 2009 Conference)) was delivered in Month 35. The first MEDITRANS technology transfer workshop was combined with the EuroNanoMedicine Conference (Bled, Slovenia, 28th–30th September 2009). It was jointly organised by the three IPs, MEDITRANS, NANOEAR and NANOBIOPHARMACEUTICS. MEDITRANS partners contributed to the organisational committees, to conference dissemination activities, and to session chairing. There were 50 attendees from MEDITRANS partner organisations, there were 14 MEDITRANS oral presentations and 12 MEDITRANS poster presentations. The conference transferred interesting results and was well attended. D149, Second technology transfer workshop, and D150, Dissemination event, were delivered in Month 47. These MEDITRANS events were combined with the ESF 2010 CONFERENCE, NANOMEDICINE: REALITY NOW AND SOON (Sant Feliu de Guixols, Spain, 23rd–28th October 2010). MEDITRANS partners contributed to "Session 10: Targeted Nanomedicine and the EC" (D149) and to the Poster Sessions (D150). There were 25 attendees from MEDITRANS partner organisations, there were 10 MEDITRANS oral presentations and 13 MEDITRANS poster presentations. The conference transferred interesting results and was well attended. The outcomes of Task 13.4 were summarised in D151, Technology transfer programme report, which was delivered in Month 47. Key stakeholders, listed in the dissemination contacts database, were invited to all the aforementioned events.

Brief description of methodologies and approaches employed
This is not a Research and Technological Development WP.

Relationship between project achievements and state-of-the-art
This is not a Research and Technological Development WP.

Impact on its industry or research sector:
Dissemination of the non-confidential results from MEDITRANS to scientists, stakeholders, and to the public. Non-confidential results from MEDITRANS disseminated.

2. DISSEMINATION AND USE

Exploitable Result n° 1: Carbon nanotubes synthesis process

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	Carbon nanotube synthesis process: use of chemicals to stop the growing of the nanotubes during the synthesis
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Medicine
Stage of development (laboratory prototype, demonstrator, industrial product...)	Laboratory
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	No
Collaborator details (type of partner sought and task to be performed)	N/A
Intellectual property rights granted or published	No
Contact details	hicham.maskrot@cea.fr

Exploitable Result n° 2: Functionalization process of nanotubes - Novel iron oxide based probes

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	Synthesis of iron oxide nanoparticles by laser pyrolysis using various precursors. + Surface modifications of these nanoparticles for biocompatibilization
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Pharmaceutical
Stage of development (laboratory prototype, demonstrator, industrial product...)	Laboratory
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	No
Collaborator details (type of partner sought and task to be performed)	N/A
Intellectual property rights granted or published	No
Contact details	eric.doris@cea.fr

Exploitable Result n° 3: Nanocarrier design

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	Design of emerging, candidate and established nanomaterials. The nanomaterials have been investigated by the specified MEDITRANS partners.
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Pharmaceutical and imaging products.
Stage of development (laboratory prototype, demonstrator, industrial product...)	Most are laboratory prototypes. Some of them have been subjected to industrial exploitation.
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	Financial support / investment.
Collaborator details (type of partner sought and task to be performed)	Large pharma for co-development.
Intellectual property rights granted or published	Some IPR has been realised.
Contact details	Prof. Dr. W. Hennink Department of Pharmaceutical Sciences, Utrecht University, Universiteitsweg 99, 3584 CG Utrecht, P.O.B. 80082, NL-3508 TB, Utrecht The Netherlands w.e.hennink@uu.nl

Exploitable Result n° 4: Nanocarriers (biodegradable polymers/nanogel, liposomes, iron oxide based material, etc.)

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	Design of emerging, candidate and established nanomaterials. The nanomaterials have been investigated by the specified MEDITRANS partners.
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Pharmaceutical and imaging products.
Stage of development (laboratory prototype, demonstrator, industrial product...)	Most are laboratory prototypes. Some of them have been subjected to industrial exploitation.
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	Financial support / investment.
Collaborator details (type of partner sought and task to be performed)	Large pharma for co-development.
Intellectual property rights granted or published	Some IPR has been realised.
Contact details	Prof. Dr. W. Hennink Department of Pharmaceutical Sciences, Utrecht University, Universiteitsweg 99, 3584 CG Utrecht, P.O.B. 80082, NL-3508 TB, Utrecht The Netherlands w.e.hennink@uu.nl

Exploitable Result n°5: Drug loaded Nanocarriers with increased plasma half-life as compared to the drug molecule alone

Exploitable result 5 cancelled by BSP.

Exploitable Result n°6: Drug loaded Nanocarriers with high affinity to tumour tissues/organs

Exploitable result 6 cancelled by BSP.

Exploitable Result n°7: Biocompatible/Biodegradable carbon based nanocarriers

Exploitable result 7 cancelled by BSP.

Exploitable Result n° 8: Commercially and regulatory viable production process of drug loaded nanocarriers

Exploitable result 8 cancelled by BSP.

Exploitable Result n° 9: Improved nanocarrier specifications addressing all relevant quality parameters including related analytical methods

Exploitable result 9 cancelled by BSP.

Exploitable Result n° 10: Improved understanding of biological clearance mechanisms in relation to nanocarrier physchem properties (RES, Liver, kidney, spleen)

Exploitable result 10 cancelled by BSP.

Exploitable Result n° 11: Imaging agents/contrast markers

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	Hydrophilic and amphiphilic Gd(III) complexes to be efficiently loaded in liposomes (improve the MRI detection sensitivity and release responsiveness). Smart Gd(III)-agents responsive to pH and enzymatic activity (responsiveness independent of the concentration of the probe).
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Image-guided therapy (mostly pharmacological and non-surgical interventional
Stage of development (laboratory prototype, demonstrator, industrial product...)	Laboratory prototype
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	Financial investment, consultancy
Collaborator details (type of partner sought and task to be performed)	Pharma companies
Intellectual property rights granted or published	Under evaluation
Contact details	Prof. Klaas Nicolay Eindhoven University of Technology Department of Biomedical Engineering Biomedical NMR PO Box 513, NLa b1.10 5600 MB Eindhoven Tel: +31 40 247 5789 Fax: +31 40 243 2598 Email: k.nicolay@tue.nl

Exploitable Result n° 12: Method of preparation of drug loaded nanoparticles (dissolved, entrapped, encapsulated)

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	Method of preparation of drug loaded nanoparticles (dissolved, entrapped, encapsulated) A MRI protocol for the assessment of Drug loaded liposomal activity in diseases.
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Diagnostics; therapy; theragnostics
Stage of development (laboratory prototype, demonstrator, industrial product...)	Laboratory prototype
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	Offered to research institutions (public or private): Sought: financial support to further develop our technology
Collaborator details (type of partner sought and task to be performed)	Pharma companies
Intellectual property rights granted or published	Under evaluation
Contact details	Prof. Klaas Nicolay Eindhoven University of Technology Department of Biomedical Engineering Biomedical NMR PO Box 513, NLa b1.10 5600 MB Eindhoven Tel: +31 40 247 5789 Fax: +31 40 243 2598 Email: k.nicolay@tue.nl

Exploitable Result n° 13: Imaging probes for guiding drug delivery and relevant procedures

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	Hydrophilic and amphiphilic Gd(III) complexes to be efficiently loaded in liposomes (improve the MRI detection sensitivity and release responsiveness). Smart Gd(III)-agents responsive to pH and enzymatic activity (responsiveness independent of the concentration of the probe). LipoCEST agents (highest sensitivity in the field of CEST agents, multiplex detection). Novel fluorescent probes (improved photophysical properties, ability to be loaded in liposomes)
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Image-guided therapy (mostly pharmacological and non-surgical interventional (e.g. HIFU))
Stage of development (laboratory prototype, demonstrator, industrial product...)	Laboratory prototype
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	Financial investment, consultancy
Collaborator details (type of partner sought and task to be performed)	Pharma companies
Intellectual property rights granted or published	Under evaluation
Contact details	Prof. Silvio Aime Department of Chemistry and Molecular & Preclinical Imaging centers University of Torino - Italy Phone: +39-011-6706451 Fax: +39-011-6706487 e-mail: silvio.aime@unito.it

Exploitable Result n° 14: Methods and tools for stimulus induced drug release/activation (ultrasounds, magnetic, PH, enzymes, etc)

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	Methods and tools for stimulus induced drug release/activation (ultrasounds, magnetic, PH, enzymes, etc) A MRI protocol for the assessment of release/activation of drug from drug loaded liposomal.
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Diagnostics; therapy; theragnostics
Stage of development (laboratory prototype, demonstrator, industrial product...)	Laboratory prototype
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	Offered to research institutions (public or private): Sought: financial support to further develop our technology
Collaborator details (type of partner sought and task to be performed)	Pharma companies
Intellectual property rights granted or published	Under evaluation
Contact details	Prof. Klaas Nicolay Eindhoven University of Technology Department of Biomedical Engineering Biomedical NMR PO Box 513, NLa b1.10 5600 MB Eindhoven Tel: +31 40 247 5789 Fax: +31 40 243 2598 Email: k.nicolay@tue.nl

Exploitable Result n° 15: Drug delivery treatment for rheumatoid arthritis

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	PEG-liposomes loaded with corticosteroids for the treatment of RA A MRI protocol for the assessment of Drug loaded liposomal activity in RA
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Diagnostics; therapy; theragnostics
Stage of development (laboratory prototype, demonstrator, industrial product...)	Laboratory prototype
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	Offered to research institutions (public or private): Sought: financial support to further develop our technology
Collaborator details (type of partner sought and task to be performed)	Partners sought: Experts in pre-clinical pharmacology on RA models Task to be performed: Clinical evaluation of the efficacy of anti-inflammatory drugs on animal models of RA
Intellectual property rights granted or published	Not at the moment
Contact details	Prof. Klaas Nicolay Eindhoven University of Technology Department of Biomedical Engineering Biomedical NMR PO Box 513, NLa b1.10 5600 MB Eindhoven Tel: +31 40 247 5789 Fax: +31 40 243 2598 Email: k.nicolay@tue.nl

Exploitable Result n° 16: Drug delivery treatment for Crohn's disease

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	PLGA loaded with corticosteroids for the treatment of CD A MRI protocol for the assessment of Drug loaded liposomal activity in CD
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Diagnostics; therapy; theragnostics
Stage of development (laboratory prototype, demonstrator, industrial product...)	Laboratory prototype
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	Offered to research institutions (public or private): Sought: financial support to further develop our technology
Collaborator details (type of partner sought and task to be performed)	Partners sought: Experts in pre-clinical pharmacology on CD models Task to be performed: Clinical evaluation of the efficacy of anti-inflammatory drugs on animal models of CD
Intellectual property rights granted or published	Not at the moment
Contact details	Prof. Klaas Nicolay Eindhoven University of Technology Department of Biomedical Engineering Biomedical NMR PO Box 513, NLa b1.10 5600 MB Eindhoven Tel: +31 40 247 5789 Fax: +31 40 243 2598 Email: k.nicolay@tue.nl

Exploitable Result n° 17: Drug delivery treatment for multiple sclerosis

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	PEG-liposomes loaded with corticosteroids for the treatment of MS A MRI protocol for the assessment of MMP activity in neurological demyelinating lesions
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Diagnostics; therapy; theragnostics
Stage of development (laboratory prototype, demonstrator, industrial product...)	Laboratory prototype
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	Offered to research institutions (public or private): technology transfer and consultancy about the MRI protocol for the assessment of MMP activity in pre-clinical evaluation of the therapeutic effect of MMP-inhibitors or other anti-inflammatory drugs Sought: financial support to further develop our technology
Collaborator details (type of partner sought and task to be performed)	Partners sought: Experts in pre-clinical pharmacology on MS models Task to be performed: Clinical evaluation of the efficacy of anti-inflammatory drugs on animal models of MS
Intellectual property rights granted or published	Not at the moment
Contact details	Prof. Silvio Aime University of Turin Via Nizza 52 10125 Turin – Italy silvio.aime@unito.it

Exploitable Result n° 18: Drug delivery treatment for cancer

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	New compositions for improved efficacy in tumor targeting. dexamethasone-containing liposomes, and core-crosslinked polymeric micelles with covalently entrapped doxorubicine.
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Pharmaceutical and medicinal products.
Stage of development (laboratory prototype, demonstrator, industrial product...)	Preclinical demonstration of efficacy completed. Clinical translation on-going.
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	Financial support / investment.
Collaborator details (type of partner sought and task to be performed)	Large pharma for co-development.
Intellectual property rights granted or published	Some IPR has been realised: dexamethasone-containing liposomes. Core-crosslinked polymeric micelles: partially covered by background IP which belonged to UU. Shortly after the end of MediTrans, i.e. 11.04.11, the UU spin off company Cristal Delivery now holds the inlicensed IPR rights.
Contact details	Prof. Dr. G. Storm & Prof. Dr. W. Hennink Department of Pharmaceutical Sciences, Utrecht University, Universiteitsweg 99, 3584 CG Utrecht, P.O.B. 80082, NL-3508 TB, Utrecht The Netherlands g.storm@uu.nl w.e.hennink@uu.nl

Exploitable Result n° 19: Training material

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	Slides, programs, posters and abstracts from different MediTrans training courses held during the duration of the project
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Academic teaching
Stage of development (laboratory prototype, demonstrator, industrial product...)	Demonstration
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	None
Collaborator details (type of partner sought and task to be performed)	None
Intellectual property rights granted or published	No intellectual properties rights to be granted. The training material was made accessible to all MediTrans members via the internal website. Participants in the training and teaching courses still hold the intellectual property rights for their own materials and slides and should be contacted directly after the end of MediTrans if further dissemination is envisioned. Furthermore it is noted that in many cases published results were shown so that the copy rights often lie with the publisher and further dissemination has to be negotiated on an individual basis.
Contact details	e.collnot@mx.uni-saarland.de

Exploitable Result n° 20: Imaging MS protocols for on tissue metabolite evaluation

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	An imaging protocol for the elemental imaging of nanoparticle based pharmaceuticals and contrast enhancement agents and their metabolites in treated tissue
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	As add-on with analytical MS instrumentation
Stage of development (laboratory prototype, demonstrator, industrial product...)	Laboratory prototype
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	No
Collaborator details (type of partner sought and task to be performed)	N.A.
Intellectual property rights granted or published	No
Contact details	Prof. dr. R.M.A. Heeren

Exploitable Result n° 21: Miniaturized cell culture system with integrated TEER measurements

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	Microfabrication allows production of cell culture support which is extremely thin (500nm) with better transport and optical properties. Integrated TEER measurement allow for in line continuous cell line monitoring. Innovative use of microfabrication to replace 10um thick polymer disposable with silicon nitride based devices (20 times thinner).
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Pharma, food industry, in vitro toxicology (nanotox), industrial R&D, life science researchers in the field of biological barriers.
Stage of development (laboratory prototype, demonstrator, industrial product...)	Pilot production, used in several academic institutions for in vitro biological barrier modelling.
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	Development of in vitro system for complex cell culture with increased mimicking properties of in vivo model.
Collaborator details (type of partner sought and task to be performed)	Academic partner, validates advantages of porous support for complex cell culture
Intellectual property rights granted or published	Patent filing pending
Contact details	Silvia Angeloni Suter, CSEM, Switzerland.

Exploitable Result n° 22: The 3D in vitro model of the inflamed colonic mucosa and the inflamed BBB model

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	First three dimensional in vitro model of the intestinal mucosa in the state of inflammation (reflecting pathophysiological changes)
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Academic and industrial research
Stage of development (laboratory prototype, demonstrator, industrial product...)	Laboratory prototype, validation versus in vivo animal models on-going
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	None
Collaborator details (type of partner sought and task to be performed)	No other partner involved
Intellectual property rights granted or published	Published in Molecular Pharmaceutics, 2010, 7 (6), pp. 2103-2119
Contact details	e.collnot@mx.uni-saarland.de

Exploitable Result n° 23: Formulated antisense oligonucleotides, siRNAs etc. for in vivo applications

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	O-methylated siRNAs for sequence specific gene silencing and for use in targeted nanocarrier materials.
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Pharma, biotech, life sciences and academic research.
Stage of development (laboratory prototype, demonstrator, industrial product...)	Industrial product obtainable from IDT.
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	No.
Collaborator details (type of partner sought and task to be performed)	n/a
Intellectual property rights granted or published	IDT owns several relevant patents dealing with siRNAs.
Contact details	http://eu.idtdna.com/home/home.aspx

Exploitable Result n°24: Method to measure by MRI laminar or turbulent blood flow in the microvasculature using paramagnetic nanotubes

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	We describe the potential use of Single Wall Carbon Nanotubes as probes to detect anisotropic water motions as tissue diffusion or perfusion
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Medicine, vascular or neovascular diseases
Stage of development (laboratory prototype, demonstrator, industrial product...)	Laboratory prototype
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	We seek finantial support or investment, information exchange
Collaborator details (type of partner sought and task to be performed)	We look for an industrial partner supporting the industrial development
Intellectual property rights granted or published	Results patented in 2008 and published 2010 (Negri et al (2010) Angw. Chem. Int. Ed. 49: 1813-5.
Contact details	pballesteros@ccia.uned.es or scerdan@iib.uam.es

Pacheco-Torres, J., Lopez-Larrubia, P., Ballesteros, P., Cerdan, S. Imaging Tumor Hipoxia by Magnetic Resonance Methods (2011) NMR in Biomedicine, 24: 1-16.

Pacheco-Torres, J., Calle, D., Lizarbe, B., Negri, V., Fayos, R., Lopez-Larrubia, P., Ballesteros, P., Cerdan, S. (2011) Envronmentally sensitive paramagnetic and diamagnetic contrast agents for nuclear magnetic resonance imaging and spectroscopy. Curr. Top. Med. Chem. 11: 115-30.

Exploitable Result n° 25: Liposomal corticosteroids (e.g. for cancer, Crohn's and MS, for RA, they are already being exploited)

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	Long-circulating liposomal corticosteroids for improving the therapeutic index in cancer and inflammatory disorders.
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Medicine and pharmaceutics.
Stage of development (laboratory prototype, demonstrator, industrial product...)	Phase I trial completed in RA. Preclinical proof of principle provided in cancer and MS.
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	Financial support / investment.
Collaborator details (type of partner sought and task to be performed)	Large pharma for co-development.
Intellectual property rights granted or published	Some IPR has been realised.
Contact details	Prof. Dr. G. Storm Department of Pharmaceutical Sciences, Utrecht University, Universiteitsweg 99, 3584 CG Utrecht, P.O.B. 80082, NL-3508 TB, Utrecht The Netherlands g.storm@uu.nl

Exploitable Result n° 26: Core-crosslinked polymeric micelles

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	Core-crosslinked polymeric micelles containing covalently entrapped chemotherapeutics and anti-inflammatory agents.
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Medicine and pharmaceutics.
Stage of development (laboratory prototype, demonstrator, industrial product...)	Preclinical proof of principle provided.
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	Financial support / investment.
Collaborator details (type of partner sought and task to be performed)	Large pharma for co-development.
Intellectual property rights granted or published	Partially covered by background IP which belonged to UU. Shortly after the end of MediTrans, i.e. 11.04.11, the UU spin off company Cristal Delivery now holds the inlicensed IPR rights.
Contact details	Prof. Dr. W. Hennink Department of Pharmaceutical Sciences, Utrecht University, Universiteitsweg 99, 3584 CG Utrecht, P.O.B. 80082, NL-3508 TB, Utrecht The Netherlands w.e.hennink@uu.nl

Exploitable Result n° 27: New design of contrast agents for drug delivery system

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	P904 is a new contrast agent for MRI that assess the inflammatory process in broad-spectrum diseases: lymph nodes metastasis, multiple sclerosis, Alzheimer disease, atherosclerosis. Its potential application will be: early diagnosis, patients stratification, therapeutic effect follow-up. The main advantages are better therapeutic effectiveness, less side effects, better therapeutic follow and increasing of medicinal security.
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	The customers of P904 will be clinicians (radiologist, oncologist, neurologist,…), patients and Biotechno/Pharma companies as imaging biomarker for drug development.
Stage of development (laboratory prototype, demonstrator, industrial product...)	Launching of Phase I in 2nd semester 2011
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	Research collaboration with CEA Funding for clinical development : Oséo
Collaborator details (type of partner sought and task to be performed)	Look for therapeutic partner to co-develop P904 for inflammatory imaging applications
Intellectual property rights granted or published	Guerbet patent
Contact details	claire.corot@guerbet-group.com

Exploitable Result n° 28: Multidrug resistant cell lines

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	We offer 4 different sets of cell lines of different tissue (skin, colon) and species (human, murine) origin, where each mother cell line is accompanied by 5 daughter cell lines established by selection with 5 different chemotherapeutics (cisplatin, doxorubicin, etoposide, methotrexate and vincristine). Each set is a valuable tool for assessment of novel drug resistance profiles as the cell lines of the same origin express different multidrug resistance proteins at different levels.
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	The main branch of economy which could be interested inuse of the product is the pharmaceutical industry.
Stage of development (laboratory prototype, demonstrator, industrial product...)	As it is a living organism, the final product is the same as the prototype – the only problem is to culture and maintain the enough number of cells.
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	Sought: information exchange. Offered: manufacturing agreement, training, consultancy.
Collaborator details (type of partner sought and task to be performed)	SME interested in advertising and distribution of the product worldwide.
Intellectual property rights granted or published	None at the moment.
Contact details	Blazej Rychlik, PhD, Laboratory of Cytometry, Division of Membrane Biophysics, Department of Molecular Biophysics, Faculty of Biology and Environmental Protection, University of Lodz, Lodz, POLAND, tel./fax +48 426 354 476, tel. +48 426 354 100, e-mail: brychlik@biol.uni.lodz.pl

Exploitable Result n° 29: Liposomal delivery devices

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	We developed a triggerable-liposomal delivery system by building remote-controlled valve in stealth liposomes as a new release mechanism. It is a novel approach in drug delivery. The valve is a pore-forming bacterial membrane protein, mechanosensitive channel of large conductance (MscL). It has been engineered such that, after being reconstituted into the liposomes, it functions as an environmental sensor and as a respons it generates pores in the liposomes and release the content. Its opening and closing could be controlled on command by the ambient pH, light and the combination of both. The main advantage of the system is its high degree of flexibility for fine-tuning of the liposome's response to its environment, which is not the case for the alternative systems.
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	It is useful for medical and pharmaceutical industry. The areas of use are triggered delivery of medicine, imaging agent and the combination of both at the same time.
Stage of development (laboratory prototype, demonstrator, industrial product...)	demonstrator
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	Financial support
Collaborator details (type of partner sought and task to be performed)	New grant application will be submitted in June 15 for market search and business plan towards finding investors
Intellectual property rights granted or published	Two international patents are owned
Contact details	Dr. A Kocer, Rijksuniversiteit Groningen, Nljenborgh 4, 9747 AG, Groningen, The Netherlands Email: a.kocer@rug.nl Tel: +31503633941

Exploitable Result n° 30: Stable high-loaded PEGylated dye-containing liposomes that are suitable for heat-triggered release by High Intensity Focused Ultrasound (HIFU)

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	Stable high-loaded PEGylated dye-containing liposomes that are suitable for heat-triggered release by High Intensity Focused Ultrasound were developed. The particles can be used for identification of tumours during operation or can be used to stain the tissue that was treated by HIFU.
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Can be used for research of HIFU treatment and as a pharmaceutical to identify tumor lesions after treatment with HIFU in patients
Stage of development (laboratory prototype, demonstrator, industrial product...)	The particles are not tested in animal studies. Proof of concept was investigated in vitro and in ex-vivo tissue.
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	We still discuss the use of these particles with the medical specialist in our hospital. Since, several grants were awarded on HIFU the particles are still of interest.
Collaborator details (type of partner sought and task to be performed)	The particles will probably be used in research of future projects.
Intellectual property rights granted or published	IP is a difficult issue since the patents around liposomes are numerous and the same counts for the drug loaded liposomes. It can be expected that a specific system that is tested in detail can be patented.
Contact details	JFW Nijsen, Heidelberglaan 100, 3584CX Utrecht, The Netherlands, 0031-887556295; f.nijsen@umcutrecht.nl

Exploitable Result n° 31: Nanoparticles from DEAPA-PVAL-PLGA containing siRNA

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	Inhalable biodegradable NP for the delivery of p-DNA and siRNA for the treatment of lung diseases including viral infections, asthma and COPD.
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Infectious lung diseases, astma, COPD, micrometastasis
Stage of development (laboratory prototype, demonstrator, industrial product...)	Laboratory
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	No
Collaborator details (type of partner sought and task to be performed)	No
Intellectual property rights granted or published	Basic patent available, specific applications to be filed
Contact details	T. Kissel: kissel@staff.uni-marburg.de

Exploitable Result n° 32: Targeted NP as above containing folate or peptides as ligands

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	Composite NP were decorated with folate to achieve more efficient uptake into cancer cells by active targeting, leading to a more selective tumor therapy of ovarian cancer.
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Oncology
Stage of development (laboratory prototype, demonstrator, industrial product...)	Laboratory
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	No
Collaborator details (type of partner sought and task to be performed)	No
Intellectual property rights granted or published	No
Contact details	T. Kissel; kissel@staff.uni-marburg.de

Exploitable Result n° 33: PEG-PEI as targeted delivery systems

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	Conjugates of PEG-PEI with folate. New linker strategy.
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	Oncology
Stage of development (laboratory prototype, demonstrator, industrial product...)	Laboratory
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	No
Collaborator details (type of partner sought and task to be performed)	No
Intellectual property rights granted or published	Basic patent protection available; specific applications to be filed
Contact details	T. Kissel; kissel@staff.uni-marburg.de

Exploitable Result n° 34: Development of methodologies to isolate, prepare and acquire physicochemical data from 'nanomedicines'

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	Standardize Protocols for NP characterization were implemented including AFM, cryo-SEM, TEM and DLS
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	none
Stage of development (laboratory prototype, demonstrator, industrial product...)	laboratory
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	No
Collaborator details (type of partner sought and task to be performed)	No
Intellectual property rights granted or published	No
Contact details	No

Exploitable Result n° 35: Analytical tool to determine encapsulated and free drug concentrations separately in plasma, whole blood, liver, spleen, kidneys and tumour during PK studies of liposome encapsulated prednisolone phosphate.

Describe the result (product(s) envisaged, functional description, main advantages, innovations)	Result: Analytical tool to determine encapsulated and free drug concentrations separately in plasma, whole blood, liver, spleen, kidneys and tumour during PK studies of liposome encapsulated prednisolone phosphate. Advantages: Until now only liposome concentrations or total drug concentrations in plasma/blood or the tissues of interest were determined during PK or biodistribution studies of liposomal formulations. Since only free drug is bioavailable and results in efficacy and toxicity, liposome or total drug concentrations can be misleading. Therefore, the developed analytical tool will gain significant insight in the PKPD, efficacy and toxicity of liposomal formulations. In combination with modelling data obtained using this analytical tool will yield numerous quantitative kinetic information, including even the local in vivo release parameters. Innovations: To our knowledge this is the first analytical tool able to measure in vivo liposomal and free drug concentrations in tissue samples.
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)	The analytical tool can help scientists in the field of liposomal corticosteroids: Compared to previous analytics, this tool will gain a much more valuable insight in the PKPD of a liposomal formulation during in vivo studies. In combination with modelling it will have a significant contribution in the selection process of the final formulation and its feasibility as a drug delivery system.
Stage of development (laboratory prototype, demonstrator, industrial product...)	The tool is validated and ready to use in a non-clinical environment.
Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other)	Feel free to contact us if this analytical tool interests you.
Collaborator details (type of partner sought and task to be performed)	
Intellectual property rights granted or published	The analytical tool will be published in several articles. Manuscripts are in preparation.
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