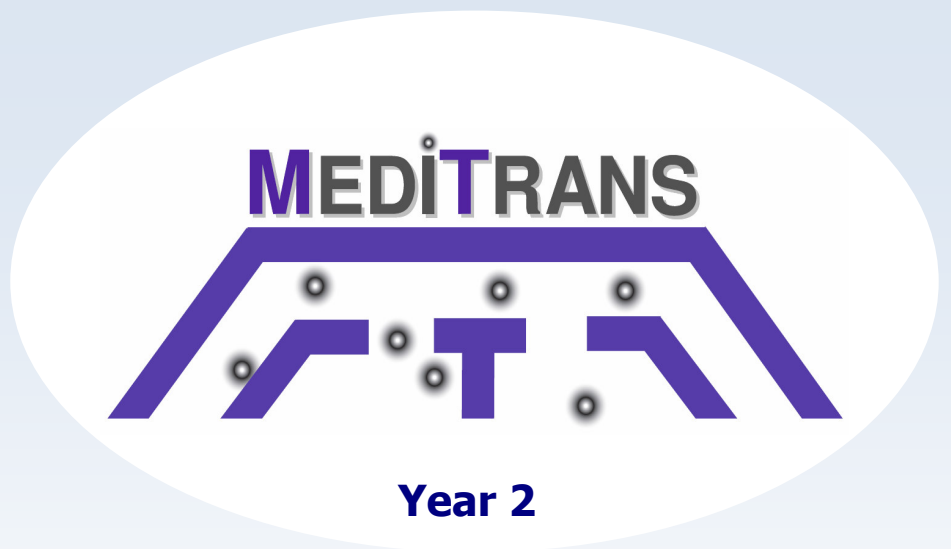
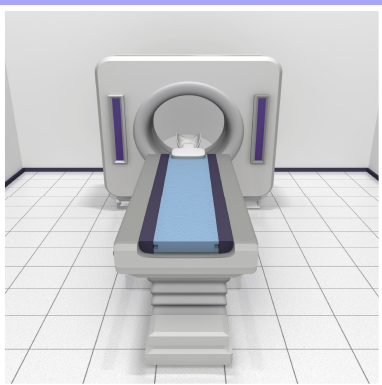
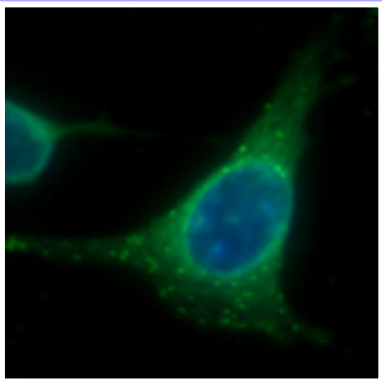
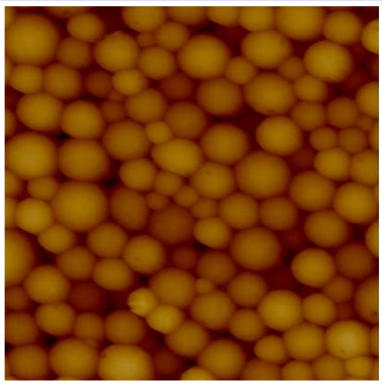
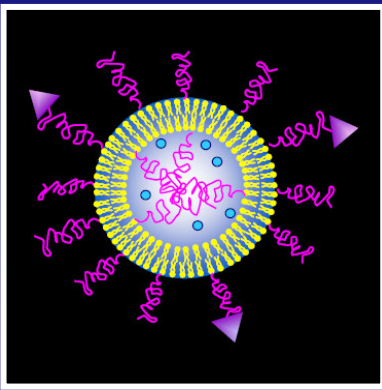




Targeted Delivery of Nanomedicine

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An Integrated Project funded by the European Commission under the 'nanotechnologies and nanosciences, knowledge-based multifunctional materials and new production processes and devices' (NMP) thematic priority of the Sixth Framework Programme

Contract Number: NMP4-CT-2006-026668

Work Package 1: Nanocarrier Design

Objectives

- To demonstrate the feasibility of the Emerging Materials (carbon-based nanoparticles) for targeted drug delivery
- To optimise the functionality of the Candidate Materials (polyplexes, nanogels, nanospheres, polymeric micelles, molecular imprinted particles, stimuli-sensitive liposomes) for targeted drug delivery

Work plan and conclusions from the second year

WP1 focuses on emerging materials, like fullerenes and carbon nanotubes, and on candidate materials, like cationic polymers for DNA and siRNA delivery, iron oxide nanoparticles, polymeric micelles, and stimuli-sensitive liposomes.

In the past few months, the scientists at CEA have succeeded in shortening carbon nanotubes to sizes below 500 nm. In follow-up experiments, these nanotubes will be surface-functionalised with carboxyl and hydroxyl groups, as well as with three different PEG moieties (PEG-2000, -5000 and -10000). To investigate the suitability of these systems to serve as drug delivery devices, the *in vitro* toxicity of surface-functionalised nanotubes will be evaluated by BRACCO, and it will be compared to the toxicity induced by established carrier materials, such as polymers and liposomes.

The majority of WP1 Tasks dealing with candidate materials are on track for achievement. As part of Task 1.3.1, extensive efforts have been undertaken at Uu to prepare polyplexes small enough to allow for nuclear translocation (*i.e.* smaller than 40 nm). Unfortunately, however, it was found that at best (*i.e.* under optimal mixing, temperature and

concentration conditions), polyplexes of ~90 nm can be obtained. This suggests that alternative strategies, like the implementation of nuclear localisation sequences, are necessary to achieve nuclear translocation of polyplexes. As part of Task 1.3.2, CU, GHENT, IDT, MARBURG and UU are co-operating to establish more effective means for delivering siRNA to and into target cells. Significant progress has been made in this regard over the past year, and several of the systems developed seem to hold significant potential for *in vivo* evaluation. Task 1.3.3 focuses on molecularly imprinted particles (MIP). Due to difficulties in properly incorporating membrane proteins into bilayers, the initial focus has shifted from MIP's targeted to ICAM1, to MIP's directed against model proteins, such as lysozyme. After promising initial results on long-circulating polymeric micelles at Uu (Task 1.3.4), attempts have been made to entrap iron oxide nanoparticles into such systems (Task 1.3.5). As shown in Figures 1A and 1B, 5-10 nm-sized oleic acid-coated iron oxide particles could be effectively encapsulated into 200-250 nm-sized mPEG-*b*-p(HPMA-Lac₂)-based polymeric micelles. The newly generated constructs retained their MR properties (Figure 1C), and they were shown to be stable for up to one week, even in the presence of serum (Figure 1D), indicating that these systems might hold significant potential for image-guided drug delivery. Finally, in Tasks 1.3.6 and 1.3.7, amino acid-based nanoparticles and stimuli-sensitive liposomes have been designed and evaluated. Proof-of-principle has been obtained for both of these classes of candidate materials, and further *in vitro* and *in vivo* studies will be performed within WP1 to further establish these systems as suitable carrier materials.

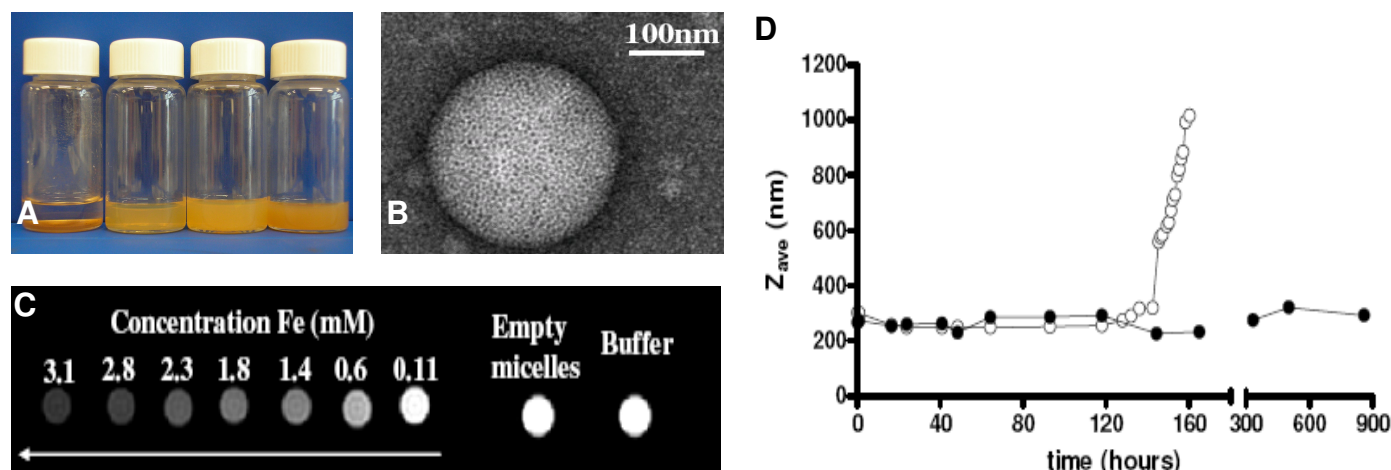
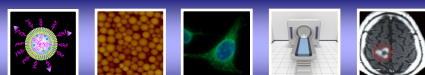


Figure 1: Hydrophobic (oleic acid-coated) iron oxide particles can be effectively encapsulated in mPEG-*b*-p(HPMA-Lac₂)-based polymeric micelles. See text for details

WP1 Leader: UU - Prof. Dr Wim Hennink (w.e.hennink@uu.nl)

WP1 Participants: UU, CEA, BIOMADE, MAGFORCE, GHENT, MARBURG, RBM, BRACCO, UNITO, CU, RUG



Work Package 2: Development of High Sensitivity Imaging Probes for Guiding Drug Delivery Processes

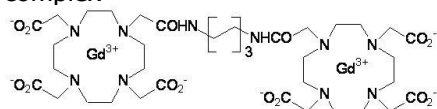
Objectives

- To develop high relaxivity Gd-agents including probes responsive to tissue microenvironmental pH and specific enzymatic activities
- 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
- To develop novel iron oxide particles properly designed for applications in drug delivery processes
- To develop highly sensitive Optical Imaging probes for monitoring drug delivery processes and therapeutic effects

Work plan and conclusions from the second year

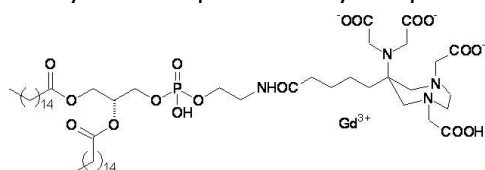
Task 2.1 – Highly sensitive Gd(III)-based agents

BRACCO and UNITO synthesised and characterised several new Gd(III)-based complexes aimed at preparing high relaxivity MRI nanovesicular probes. This Task was accomplished either by designing high relaxivity hydrophilic complexes to be encapsulated in the vesicle, or by designing novel and efficient amphiphilic chelates to be incorporated in the vesicle membrane. To the former class belongs this neutral dimeric complex



where the contrast efficiency is guaranteed by the increased intraliposomal concentration of paramagnetic centres.

To the class of amphiphilic complexes the most important system is represented by this product



in which the highly efficient Gd-AAZTA chelate has been conjugated to a phospholipid-like moiety. The relaxivity of this complex, incorporated in the liposomal membrane, is ca. 50 % higher than the best currently used amphiphilic probes.

Task 2.2 – Highly sensitive Chemical Exchange Saturation Transfer (CEST) agents

During this second year UNITO, PHILIPS, and PHILIPSD focused their efforts to design strategies for developing improved CEST agents characterised by high sensitivity and increased chemical shift offsets. PHILIPS and PHILIPSD produced and tested *in vitro* several dendrimer generations loaded with lanthanide(III) complexes acting as CEST probes. These systems displayed good sensitivity and pH

responsiveness. UNITO developed highly sensitive liposome-based CEST agents endowed with highly shifted intraliposomal water protons. This important result was achieved by inducing an osmotic shrinkage of the nanovesicle in order to exploit an additional shift contribution arising from bulk magnetic susceptibility.

Task 2.3 – Novel iron-oxide based probes

MAGFORCE produced nanosized iron oxide particles for application in cancer therapy by MFH (Magnetic Fluid Hyperthermia). The particles are applied by intratumoural injection. Currently, the high intratumoural concentration of nanoparticles causes susceptibility artefacts in MRI. However, current investigations demonstrated that in the near future the amount of iron needed for hyperthermia can be significantly reduced due to improvements in therapy-instrumentation.

The laser pyrolysis method was used by CEA to produce some iron containing nanopowders for biomedical applications. Laser pyrolysis is a very versatile process which allows the synthesis of a wide range of nanoparticles. The process parameters (precursors, solvents, gases, laser parameters) were varied to study their effect on the properties of the recovered nanopowders. Studies are ongoing.

GUERBET has developed protocols to assess the amount of iron oxide particles by means of MRI/ICP combined methods.

Task 2.4 – Optical imaging probes

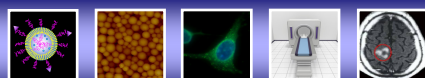
TUE is working on the Task of employing micelles equipped with a dual, MRI and Near Infrared (NIR), lipid probe (NIR664) and the collagen binding protein CNA35, in a mouse model which develops both a stable and an unstable atherosclerotic plaque. Paramagnetic micelles were conjugated to CNA35, or the non-binding mutant-CNA35. The micelles have a hydrodynamic diameter of 24 ± 3 nm, r_1 of $10.3 \text{ mM}^{-1} \text{ s}^{-1}$ at 60 MHz and 37 °C, a critical micelle concentration of 0.09 mM, and showed strong binding affinity. Histological sections were studied with fluorescence microscopy and stained with H&E, Oil Red O, Picrosirius Red, for macrophages (CD68) and vascular smooth muscle cells (α -actin).

Confocal laser scanning microscopy revealed a NIR664 signal in the plaque in case of CNA35-micelles. Because of their size, high relaxivity, and strong binding to collagen, Gd-containing CNA35-micelles can be suitable as a MRI contrast agent for collagen imaging in atherosclerotic plaques, allowing optical validation using the NIR moiety.

UNITO is currently preparing fluorescent liposomes loaded with different dyes in order to set-up *in vitro* and *ex-vivo* imaging protocols for investigating the intracellular trafficking and fate of the nanovesicles in macrophages.

WP2 Leader: UNITO - Prof. Dr Silvio Aime (silvio.aime@unito.it)

WP2 Participants: CEA, MAGFORCE, TUE, PHILIPS, BRACCO, UNITO, UNED, GUERBET, PHILIPSD



Work Package 3: Formulation of Drugs and Imaging Agents into Carriers / Physicochemical Characterisation

Objectives

- To load the nanocarriers with biologically active compounds and imaging agents
- To study the physicochemical characteristics of the nanomedicines which are relevant for the overall aims of MEDI TRANS

Work plan and conclusions from the second year

Work package 3 encompasses the formulation of nanocarriers, based on established, candidate and emerging systems, loaded with drugs and imaging agents, and the subsequent characterisation of those formulations.

The formulation of nanocarriers with drugs, will be focused on loading with drugs for the treatment of Crohn's disease, cancer and multiple sclerosis.

Established nanocarrier systems

Significant progress has been made on the formulation and characterisation of established nanocarrier systems, in particular long-circulating liposomes for passive targeting loaded with corticosteroids have demonstrated favourable size and sufficient encapsulation, and are currently being investigated for *in vitro* and *in vivo* response. Promising results have been obtained, with early indications suggesting that enhanced tumour efficacy is the result of the inhibition of pro-angiogenic factors by tumour cells and tumour-associated macrophages. In addition long-circulating liposomes for active targeting have been formulated with an antibody to target EGFR-overexpressing ovarian carcinoma cells. These have been loaded with boron (NCT) and have already shown to be effective in *in vitro* studies. The formulation of long-circulation liposomes for active drug targeting to MS lesions is in progress with the supply and distribution drug from RBM (Merck-Serono).

Proteoliposomes have been successfully developed, demonstrating channel activity in response to a model chemical activator (MTSET). Initial studies have been based around calcein (self quenching fluorescent compound). Currently, channel protein liposomes are being formulated to contain corticosteroids.

UNITO have effectively formulated liposomal nanocarriers with MR imaging probes based on paramagnetic lanthanide (III) complexes.

UDS have formulated PLGA nanoparticles for the treatment of Crohn's disease. The drug loaded PLGA nanoparticles show favourable size, sub-200 nm, encapsulation efficiencies of greater than 70 % and stability during storage. Further optimisation of the formulation is required in order to achieve sustained release.

Candidate nanocarrier systems

A range of nanoparticles for intravenous si-RNA delivery have been developed.

CU have focused on the dendrimers terminated with different functional groups. Amino-terminated PPI dendrimers have shown to be most effective regarding transfection efficiency. Dendrimer nanocarriers are 200 nm in size and have demonstrated short term stability. Formulations are currently being developed with targeting moieties and stealth properties.

Biodegradable amine modified branched polyesters (based on DEAPA-68-10) have been formulated with si-RNA (MARBURG). The nanocarriers have been developed with a particle size of 150-225 nm, zeta potential of 40-50 mV, and are sufficiently stable for endocytotic uptake. During *in vitro* studies the formulation has demonstrated efficient knockdown of the Luciferase reporter gene, with minor or no cytotoxicity.

For the PEG-PEI-Fluc si-RNA formulations, successful uptake into a lung cancer cell line has been achieved, with complexes releasing all siRNA in only a few minutes. Selective folate receptor-mediated uptake for a PEG-Folic acid conjugate into FR-positive cells has been achieved. Current studies are focused on developing the targeting folate receptor in PEG-PEI systems.

Dextran microgels and nanogels with si-RNA have been produced by GHENT. Homogenous loading of the si-RNA has been achieved in microgels. Nanogels have been formulated with a particle size of 200 nm and zeta potential of 0-30 mV. Furthermore, high loading of si-RNA can be achieved without inducing aggregation.

Nanoparticles for intravenous pDNA delivery has seen the development of protocols for the formulation of cationic polymer/pDNA complex dispersions. Imaging probes containing nucleic acid have been explored.

Polymeric micelles have been formulated to contain 10 nm oleic coated iron oxide particles, and are currently in the process of being loaded with drugs.

Unfortunately technical issues have been encountered in the development of dextran nanoparticles with a molecular imprint and work is ongoing.

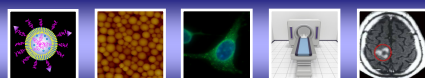
Iron-oxide particles with a stealth coating have been developed where PEG and aminosilanes are coupled to the iron oxide particles.

Emerging nanocarrier systems

For emerging materials, nanotubes and fullerenes will be loaded (by covalent coupling) with corticosteroids (prednisolone, dexamethasone), and possibly other chemotherapeutic agents, and Gd-chelates. Characterisation will include size, amount of loading, and surface morphology.

WP3 Leader: MOLPROF - Dr Andrew Parker (aparker@molprofiles.co.uk)

WP3 Participants: UU, BIOMADE, MAGFORCE, FOM, GHENT, ORGANON, MOLPROF, UDS, MARBURG, BSP, PHILIPS, RBM, UNITO, CU, PHILIPSD, IDT, RUG



Work Package 4: Recognition of Targets: Cells, Tissues, Organs

Objectives

- To optimise the targeting efficiency of the nanomedicines with respect to their disease specific application (cancer, rheumatoid arthritis (RA), Crohn's disease (CD), multiple sclerosis (MS))
- To explore and optimise new principles of active, ligand-mediated targeting (*e.g.* to angiogenic endothelial cells in tumours and RA, to myelin and ICAM-1 in MS lesions)
- To transfer the principle of passive targeting ("EPR effect") to new disease specific applications (*e.g.* targeting to inflamed colonic mucosa in Crohn's disease, and to MS lesions in MS)
- Development of disease-specific *in vitro* models of cellular targets and barriers, reflecting relevant pathological changes (*e.g.* intestinal mucosa and blood brain barrier, respectively, in the state of inflammation)

Work plan and conclusions from the second year

Task 4.1 - Recognition of targets in cancer

Successful active targeting of the hyaluronic acid receptor was demonstrated for HA-IRIS5-amino complexes in uptake and transfection experiments with MLS-EGFP ovarian carcinoma cells (Figure 1). CHARITE investigated the behaviour of different nanosized iron oxide particles for application in cancer therapy by MFH (Magnetic Fluid Hyperthermia). Particles were prepared and surface functionalised with amino functions and different PEG coatings by MAGFORCE and evaluated in human tumour cell lines RUSIRS1 (human glioblastoma), WiDr (human colon carcinoma), and BT 20 (human mamma carcinoma).

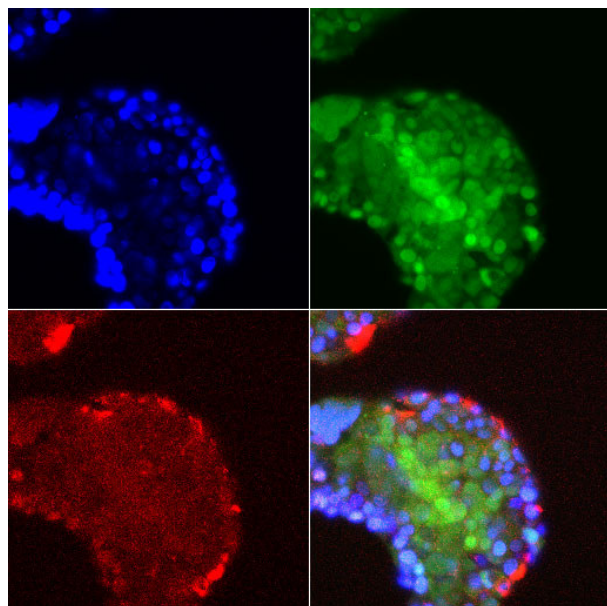


Figure 1: HA-IRIS5-amino (5-10 µg/ml) uptake by MLS ovarian carcinoma cells; blue: DAPI, green: EGFP, red: IRIS5 (WEIZMANN and UNITO)

Task 4.2 - Recognition of targets in CD

Intestinal epithelial Caco-2 cells were combined with macrophages and dendritic cells derived from peripheral blood mononuclear cells to form a three dimensional model of the inflamed intestinal mucosa (UDs).

Grown on a collagen layer in which the macrophages and dendritic cells had been imbedded, Caco-2 cells differentiated into tight monolayers with TEER values comparable to normal Caco-2 monocultures.

Induction of inflammation using a combination of lipopolysaccharides and interleukin 1-beta resulted in a reversible decrease of TEER comparable to a breakdown of barrier function in the inflamed intestine *in vivo*.

The model will now be used to test efficacy and passive targeting properties of MEDITRANS formulations directed against CD. Adaptation of the system to miniaturised membrane supports (CSEM) is being envisioned.

Task 4.3 - Recognition of targets in RA

Methods to image and quantify the uptake of nanocarriers into macrophages have been set up at UDS allowing differentiation between particles adhering to the macrophages and those internalised (Figure 2). These assays will be used to determine the uptake of different stealth liposomal formulations prepared at UU. *In vitro* data will be correlated with *in vivo* circulation half times to predict *in vivo* extravasation ability of stealth carriers.

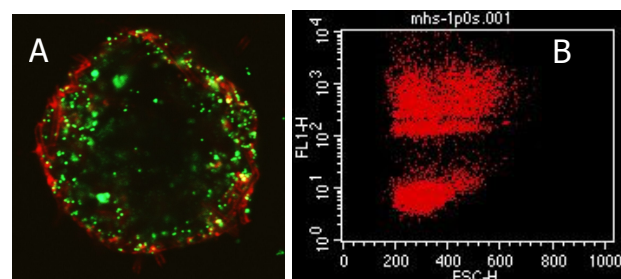


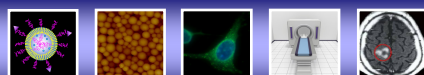
Figure 2: Imaging (A) and quantification (B) of uptake of fluorescent polystyrene model particles (200 nm and 1µm, respectively) into mouse macrophages using CLSM and FACS

Task 4.4 - Recognition of targets in MS

Using a similar approach as in the inflamed intestinal model ACROSS is setting up an *in vitro* model of the blood brain barrier in the state of inflammation initiating inflammation in porcine brain endothelial cells *via* pro-inflammatory cytokines. The model is currently being characterised with regards to barrier properties (transepithelial electrical resistance) and active and passive transport properties.

WP4 Leader: UDS - Prof. Dr Claus-Michael Lehr (lehr@mx.uni-saarland.de)

WP4 Participants: UU, MAGFORCE, CHARITE, CSEM, UDS, BSP, ACROSS, RBM, WEIZMANN



Work Package 5: Target Cell Uptake and Intracellular Trafficking

Objectives

- Contribute to the understanding of the (biophysical) behaviour of siRNA / pDNA nanoparticles in cancer and endothelial cells
- Especially, identifying the most critical step in the delivery of siRNA / plasmid DNA to the target cells, which may strongly depend on the architecture of the nanoparticles
- Use this knowledge to allow WP1 to design nanoparticles which successfully deliver *in vivo* nucleic acids to cancer and endothelial cells
- Design and synthesise nanoparticles that can be used for light-triggered intracellular delivery of nucleic acids

Work plan and conclusions from the second year

In the first year, we were able to demonstrate the loading of siRNA in dextran based microgels. Also, we were able to demonstrate the gene silencing activity of these microgels with siRNA targeted against GFP.

In the second year, we proved that siRNA can also be loaded in dextran based nanogels (typically 200 nm). These nanogels are efficiently taken up by cells and can slowly release their siRNA based on the degradation properties of the dextran nanogels (Figure 1).

Consequently, different dextran nanogels were evaluated *in vitro* for their efficiency of endosomal escape. Without any help, the non-biodegradable dextran nanogels escape poorly from the endosomal compartment, resulting in a lack of biological activity. PCI (photochemical internalisation), a technology for light-directed drug delivery by triggered endosomal release, significantly enhances the endosomal escape, and the biological activity, of the non-degradable nanogels.

When biodegradable nanogels are used, these nanogels can to a certain extent escape the endosomes and result in a biological effect. This effect can be, however, enhanced by the use of PCI or fusogenic peptides that improve the endosomal escape (Figure 2).

In conclusion, we can say that we are able to prepare cationic dextran nanogels for the loading of siRNA, and that the biodegradable nanogels result in a biological activity, which can be enhanced by the use of PCI. These data are encouraging for optimising the time-controlled intracellular delivery of siRNA.

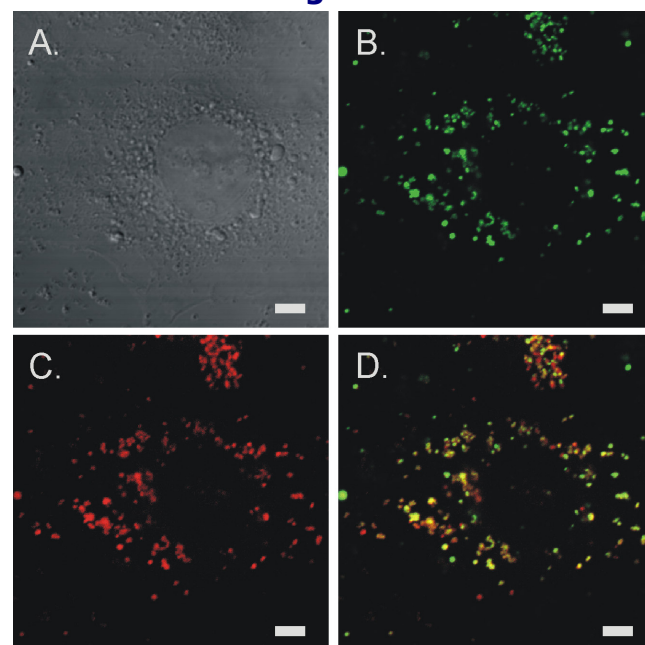


Figure 1: Uptake of siRNA loaded nanogels in Huh-7 cells. (A) transmission image (B) uptake of nanogels loaded with Alexa-488 labelled siRNA (green) (C) lysotracker red colours the endosomal compartment and (D) overlay which shows that most of the nanogels are present in the endosomal compartment

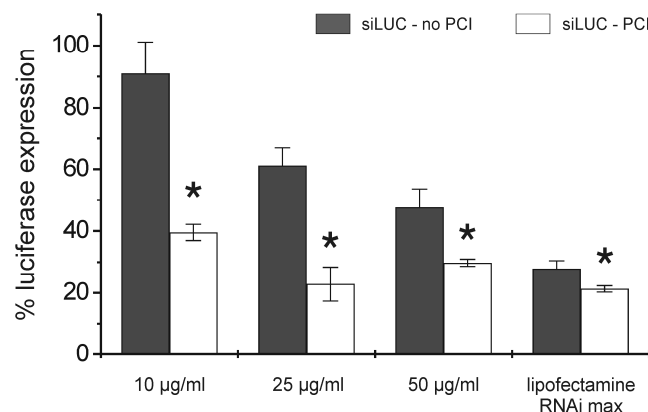


Figure 2: Biological activity of siRNA loaded biodegradable nanogels without (grey bars) and with (white bars) PCI. It can be seen that PCI significantly enhances the downregulation of the luciferase target protein. The x-axis shows different concentration of nanogels used, compared to the lipofectamine control sample

Work Package 6: Stimulus Induced Release / Activation

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 plan and conclusions from the second year

Task 6.1 – Externally applied stimuli

MAGFORCE produces nanosized iron oxide particles for application in cancer therapy by MFH (Magnetic Fluid Hyperthermia). After coating the surface with thermosensible drug release systems, those particles can be activated by heating them in an oscillating magnetic field applicator. Currently, MAGFORCE is providing a variety of magnetic core materials to the project partners which can be encapsulated in thermolabile polymers, micelles or liposomes. Furthermore, after mixing with magnetic nanoparticles, externally induced release from thermolabile liposomes filled with fluorescent dyes as model substances for cytotoxic drugs could be demonstrated in the magnetic field applicator (MFH®-12-TS A). These preliminary results indicate that hyperthermia and magnetically/thermally induced drug release could be used in combination.

Before the CNRS ceased to work on the project, they thoroughly investigated (in collaboration with GUERBET) the use of ultrasound (US) for triggered drug release from nano-carriers, as well as the role of MR contrast agents in US triggered drug delivery. Studies on the use of US contrast agents for increased cavitation have been shown to enhance the uptake of nanosized MR contrast agent in rabbit liver. Furthermore, it has been observed that the ultrasound protocol needs to be adapted to the particular nano-carrier being used since the wall stability is highly variable and depends on the particular chemical composition. In addition, some types of contrast material should be added for evaluation of the local delivery with the contrast agent acting as a convenient reporter molecule for the drug.

PHILIPS and PHILIPSD developed dual ¹⁹F/¹H-MRI CEST liposomal probes with contrast properties that are sensitive to temperature. By using a liposome formulation with a gel-to-liquid transition temperature of 39-42 °C, the vesicle can release its content upon mild hyperthermia conditions. Before the stimulus, the probe acts as a CEST agent, whereas upon heating

the CEST contrast vanishes and the ¹⁹F contrast is switched on.

Task 6.2 – Endogeneous stimuli

UNITO prepared pH-sensitive paramagnetic Gd(III)-based liposomes with improved performance.

The basic liposome formulation consisted of POPE (palmitoyl-oleyl-phosphatidylethanolamine), THS (tocopherol-hemisuccinate) and cholesterol, and the liposomes were encapsulated with different Gd(III) complexes. At physiological pH, the relaxivity of the system is controlled by the water permeability of the liposome, whereas upon acidification, the protonation of THS simultaneously induces the aggregation of the liposomes and a destabilisation of the membrane with the consequent release of the entrapped MRI agent, thus resulting in a relaxivity enhancement (see Figure 1 below).

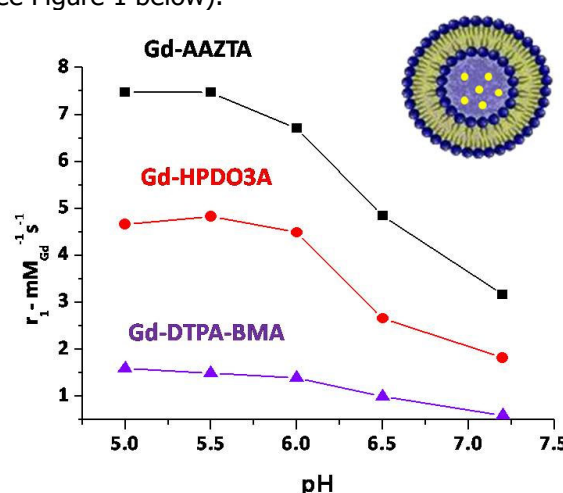


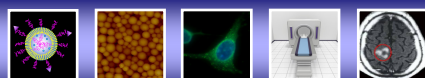
Figure 1: See text for details

pH sensitive liposomes encapsulating the clinically approved Gd-HPDO3A complex displayed a higher pH responsiveness, than the systems reported in the literature, when loaded with Gd-DTPA-BMA, and the performance can be further improved by encapsulating the more efficient agent Gd-AAZTA.

Work is ongoing in collaboration with CSIC in order to test the performance of these probes in a tumour animal model.

A research line focused on the design of MRI probes, activable by enzymatic triggering, is in progress. Liposomes incorporating lipopeptides that can destabilise the vesicle upon cleavage of the peptide are currently under examination.

Previous WP6 Leader: CNRS - Dr Chrit Moonen **Interim WP6 Leader:** UNITO – Prof. Dr Silvio Aime
Likely new WP6 Leader: UMC UTRECHT – Prof. Dr Peter Lijten (P.Lijten@umcutrecht.nl)
WP6 Participants: UU, CEA, BIOMADE, MAGFORCE, PHILIPS, BRACCO, CNRS, WEIZMANN, UNITO, CSIC, GUERBET, PHILIPSD, RUG, UMC UTRECHT



Work Package 7: Application to Rheumatoid Arthritis and Crohn's Disease

Objectives

- To study pharmacokinetics, tissue distribution, targeting efficiency, and therapeutic efficacy of the developed targeted nanomedicines in suitable animal models of rheumatoid arthritis (RA) and Crohn's disease (CD)
- To evaluate and optimise targeted nanoparticles, loaded with imaging probes and drugs, for application in MRI guided imaging and therapy of inflammation

Work plan and conclusions from the second year

Four different types of liposomal corticosteroids have been synthesised and evaluated (prednisolone phosphate, methylprednisolone phosphate, dexamethasone phosphate and budesonide phosphate). It was found that liposomal budesonide was the most potent agent, both *in vitro* and *in vivo*, followed by dexamethasone, prednisolone and methylprednisolone. Based on these findings, liposomes (and micelles) containing budesonide and dexamethasone will be used in follow-up experiments to assess the efficacy of targeted corticosteroids for treating RA and CD. (Uu)

Significant progress has been made also in developing a chemically modified dicer substrate siRNA (DsiRNA) targeting mouse TNF α for RA, which can be formulated into nanoparticles, both with and without a targeting ligand. The advantage of these siRNAs is that they are first diced and then enter the RISC pathway and show enhanced potency and efficacy compared to standard Tuschl style 21-mers. Multiple 2'-O-methylation on the antisense strand has significant benefits, it gives increased stability in serum and evades activation of the innate immune response, both very important features for *in vivo* applications. Based on the TNF α DsiRNA, IDT designed and synthesised the following chemically modified duplex on a scale of 80 mg:

5' - pGUCUCAGCCUCUUCUCAUCCUGct - 3'
3' - UACAGAGUCGGAGAGAGUAAAGGACGA - 5'

Figure 1: Bold lower case letters represent 2'-deoxyribonucleotides, upper case letters represent ribonucleotides, bold underlined upper case letters represent 2'-O-methylribonucleotides and p is a phosphate residue

A macrophage cell model for TNF α expression is up and running, and will be used for the evaluation of the various nanoparticles for the *in vitro* knockdown by siRNA. (Cu)

For *ex vivo* MRI studies the male DBA1 mouse model of collagen induced RA (10 weeks old) was used. Disease severity was scored by joint examination on a scale 0-2 and animals were sacrificed at different time points of the disease progress. The legs were

preserved in formaldehyde. MRI examination was performed on a 6.3 T scanner (Bruker Biospin). The T1-weighted images (TR/TE=1000/10.2 ms) were recorded for different cross sections.

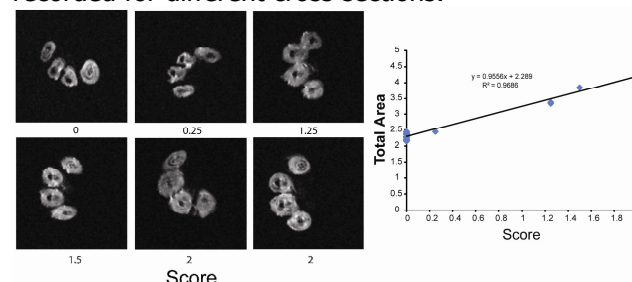


Figure 2: Left panel: the T1 weighted sagittal MRI images of the front paws of mice, taken at the same position, depicting the progression of the disease with higher score. Matrix: 256*256, FOV: 1.86*1.86 cm², Slice thickness = 0.5 mm (gap = 0.55 mm). Right panel: the total area of the inflamed mouse fingers correlates well with the disease score

The area of the sagittal MRI images of the mouse paws, acquired at different stages of the disease progress, shows very good correlation with the disease progress score (Figure 2). These encouraging preclinical results obtained with the MRI tools, for monitoring the progress of the disease, will pave the way for the *in vivo* evaluation of therapy. (TUE)

Mesopram loaded PLGA nanoparticles are to be tested in a rat colitis model for passive targeting properties and therapeutic effect. Several process parameters have been varied to achieve a better retardation of mesopram release from the nano-formulations, *e.g.* solvent, stabiliser, stirring time and homogenisation method. Analysis of new particle formulations with regards to drug release profiles is still ongoing. (UDs) However, the evaluation of efficacy of PEGylated liposomal corticosteroids in DSS-induced colitis in mice is in progress. The new data show that PEG-free liposomes with a size of ~80 nm were retained in systemic circulation as effectively as 100 nm-sized PEGylated liposomes, indicating that also non-PEGylated liposomes can be used for passive targeting purposes. (Uu)

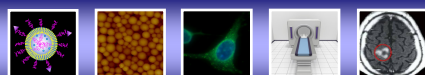
Preparation and characterisation of the new PLGA based nanoparticles for MRI are underway and hence increase the prospects of further elaboration on siRNA nanoplexes of PLGA containing contrast agents, for CD imaging. (Cu & TUE)

Mass Spectrometry Imaging (MSI) of new drug delivery systems was developed to detect and image even larger molecules such as proteins and antibodies. This work demonstrates the new capabilities of MSI technology and its use for imaging of exogenous and endogenous molecules. (FOM)

In future, the above newly developed nanoparticles, contrast agents, and techniques will be used in cellular and animal models for studies of RA and CD.

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WP7 Participants: UU, FOM, TUE, UDS, BSP, PHILIPS, RBM, UNITO, CSIC, GUERBET, CU, PHILIPSD, IDT



Work Package 8: Application to Multiple Sclerosis

Objectives

- To study pharmacokinetics, tissue distribution, targeting efficiency, and therapeutic efficacy of the developed targeted nanomedicines in suitable animal models of multiple sclerosis (MS)
- To design and optimise a carrier for imaging-guided drug release and delivery in the central nervous system (CNS)
- To evaluate, *in vivo*, the therapeutic efficacy of matrixmetalloproteinases (MMP)-inhibitors, through imaging-guided targeted and triggered delivery, in CNS lesions induced by MS-like pathology

Work plan

An initial Gadolinium-based pro-contrast agent (pro-CA) sensitive to a family of MMP active enzymes has been synthesised and tested *in vitro* proving the concept that such a pro-CA could be developed. However, its insoluble properties have been viewed as a difficulty to move to the next *in vivo* step. Thus new approaches have been assessed to develop a soluble form of a similar pro-CA agent. Various formulations have been tested and a final poly-cyclodextrin formulation has been defined as providing the optimal necessary properties while maintaining the MMP-sensitive activities *in vitro*. This phase is currently under optimisation.

The overall goal of this part of the project was to finally test this pro-CA in a disease animal model of Multiple Sclerosis, namely the Experimental Autoimmune Encephalomyelitis (EAE). However, before testing the molecule in EAE animals, where lesions are widespread in the CNS and of different sizes, a proof of concept animal model presenting focal large CNS lesions enriched in MMP expression was set-up and characterised through MRI (T2 and T1-BOPTA) and *in situ* zymography, a biochemical method assessing MMP activities. Middle cerebral artery occlusion (MCAO) in rats provided large infarct zones in the cortico-striatal areas that presented very large blood-brain barrier leakage by 72 hours post – ischemia (see Figure 1). *In situ* zymography measures in matched brain sections. It showed a significant increase in MMP activities in peri-infarct zones. Further characterisation will be needed.

Conclusions from the second year

This second year has led to the development of methods for optimisation of the pro-contrast agent sensitive to MMP activities as well as the characterisation of a proof of concept animal model to assess *in vivo* the efficacy of our molecules. These activities are providing the necessary support for the next steps of the project.

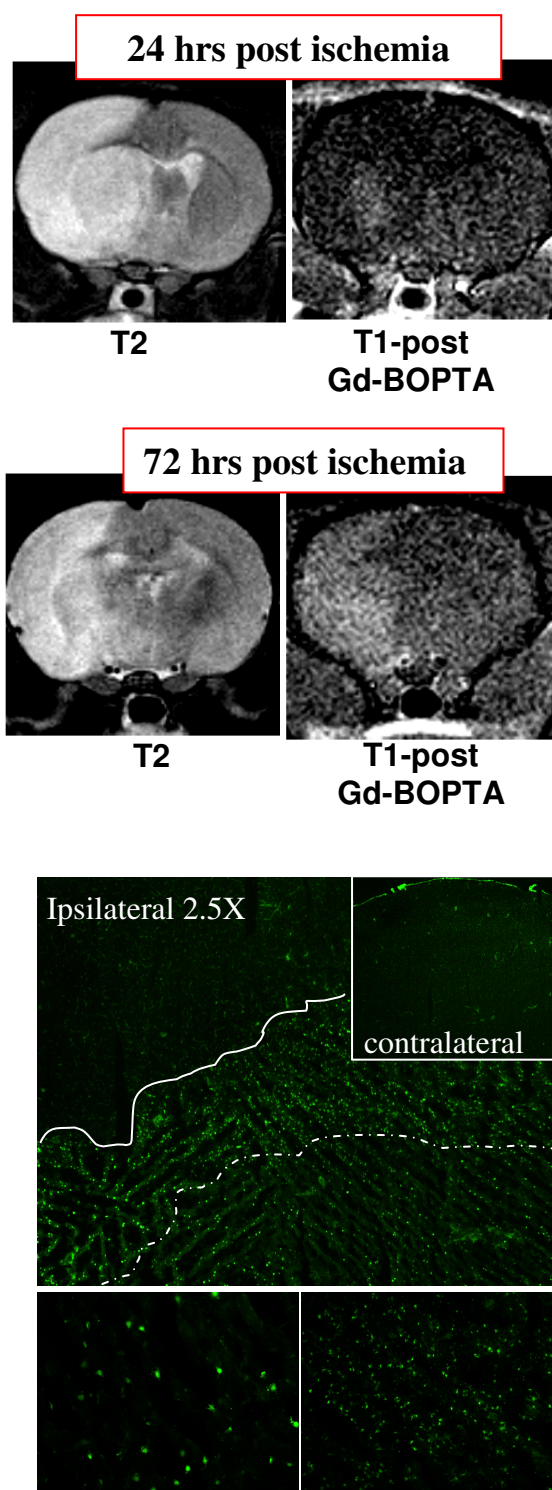
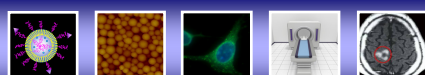


Figure 1: See text for details

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WP8 Participants: UU, FOM, PHILIPS, RBM, BRACCO, CNRS, UNITO, GUERBET, PHILIPSD, IDT, UMC UTRECHT



Work Package 9: Application to Cancer

Objectives

- 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

Work plan and conclusions from the second year

Task 9.1 – Passive targeting approach

As part of Task 9.1.1., several long-circulating liposomes and micelles were loaded with corticosteroids. Prolonged circulation times and highly effective tumour accumulation were achieved. By inhibiting tumour-associated inflammation and angiogenesis, liposomal corticosteroids have also been shown to inhibit tumour growth (Uu).

As part of Task 9.1.2., iron oxide particles have been entrapped in polymeric micelles. Systems with sizes of ~200 nm could be reproducibly obtained, and the particles were stable for more than 1 week, even in the presence of serum. The resulting nanoparticles were also shown to have proper R2 and R2* relaxivities, indicating that these systems might hold significant potential for image-guided drug delivery (Uu).

In Task 9.1.3 the MscL channel protein was modified with chemical modulators. Also, an engineered channel was reconstituted into PEG-liposomes. Cisplatin and methotrexate were chosen to optimise the loading capacity and composition of liposomes (BIOMADE).

Task 9.2 – Active targeting approach

IDT has provided WEIZMANN with 3 highly potent unmodified DsiRNAs targeting hVEGF. VEGF is highly upregulated in tumours and is a proven oncology target. Of these, one DsiRNAs was demonstrated to be more potent than the other two in suppressing the expression of VEGF in tumour cells.

Non-targeted delivery to tumours of siRNA targeting GFP and luciferase were evaluated for suppression of reporter gene expression, as detected by BLI and intravital fluorescence microscopy (IDT, MARBURG, UU and WEIZMANN).

Task 9.3 – MRI guided (triggered) drug delivery and biomarkers evaluation for assessing therapeutic effects

MRI was used to monitor the influence of US contrast agent destruction for local delivery of a MRI blood pool contrast agent in the rat liver (CNRS and GUERBET).

The $\alpha\beta3$ integrin is a potent biomarker for angiogenesis. Particles loaded with high amounts of superparamagnetic iron oxide (SPIO) were functionalised with RGD to target $\alpha\beta3$ integrins. *In vitro* analysis showed uptake by Human Umbilical Vein Endothelial Cells (TUE and GUERBET). TUE studied the accumulation and treatment effect of prednisolone

phosphate (PLP) encapsulated into long circulating liposomes (Gd-PLP-L).

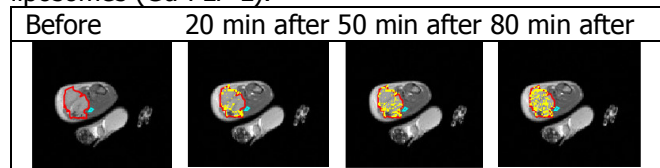


Figure 1: T1-weighted (TR/TE=800/8.8ms) Representative images of a slice through the tumour bearing mouse before and at different time points after Gd-PLP-L administration. Pixels in the tumour with signal enhancement (5x mean noise level) are yellow coded (TUE)

The potential of paramagnetic labelled stealth liposomes for imaging the delivery of antitumoural drugs was assessed. Liposomes suitably formulated for acting as T₁, T₂, and CEST agents were locally injected in mice bearing melanoma B16 xenografts. The kinetic behavior among the three contrast modalities may be interpreted as a fast cellular uptake and endosomal entrapment, followed by release of the vesicle content. *Ex-vivo* fluorescence confocal microscopy revealed co-localisation between the nanoprobe and tumour associated macrophages (UNITO).

A collaboration evaluated the feasibility of detecting and imaging an USPIO (P904) internalised in a tumour by SIMS. Carcinoma KB cells loaded with P904 were subcutaneously injected in the flank of a mouse. Tumours were removed and immediately frozen for SIMS analysis. Images show iron accumulation at the periphery of the tumour, which correlated with histological staining of iron (FOM and GUERBET).



Figure 2: Left) SIMS imaging of a P904 loaded-tumour slice (m/z 55.92 raster size 97x97 μm) (scale bar= 97μm). Right) Optical imaging of the same P904 loaded-tumour slice

ErbB-2 (HER2) receptor has been widely researched as a clinical biomarker and a therapeutic target in breast cancer. An HER2-targeted-iron oxide particle (P1164) that preferentially bound mammary cancer cells (SK-BR-3) that overexpress the receptor, as compared to low HER2-expressed MDA-MB-231 cells, was synthesised. This particle is available for *in vivo* studies on animal models that express high levels of HER2 (GUERBET).

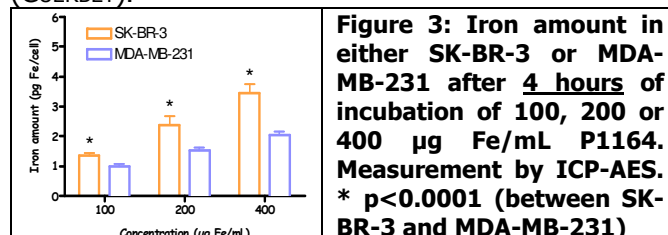
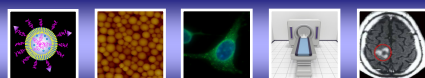


Figure 3: Iron amount in either SK-BR-3 or MDA-MB-231 after 4 hours of incubation of 100, 200 or 400 μg Fe/mL P1164. Measurement by ICP-AES. * p<0.0001 (between SK-BR-3 and MDA-MB-231)

WP9 Leader: WEIZMANN - Prof. Dr Michal Neeman (michal.neeman@weizmann.ac.il)

WP9 Participants: UU, CHARITE, FOM, PCI, UL, ORGANON, TUE, MARBURG, BSP, PHILIPS, CNRS, WEIZMANN, UNITO, CSIC, GUERBET, CU, PHILIPSD, IDT, UMC UTRECHT



Work Package 10: Preclinical Toxicology Commenced in December 2008

Objectives

- Assess the safety risks of selected prototype nanomedicines and provide guidance in the selection process of the final products
- Assess the toxicology aspects of the nanomedicines developed in MEDI TRANS in conjunction with NANOSAFE 2 and other EC projects

Work Package 11: Industrial Exploitation Commenced in December 2008

Objectives

- To convert academic concepts into products
- To guide the prototype carrier systems through the predevelopment phase

Work Package 12: Training

Objectives

- To provide advanced drug delivery courses for partners
- To develop the MEDI TRANS website as a platform for education and training, and to facilitate the exchange of MEDI TRANS' young scientists
- To provide access for MEDI TRANS' scientists to the training programme and events organised by the GALENOS-Network, and to create synergies
- To monitor and improve the efficiency of the training courses

Work plan

Advanced drug delivery courses

The second course on 'MRI Technologies for Drug Delivery' will be held in March 2009 as part of the MEDI TRANS Second Annual Meeting at WEIZMANN in Rehovot, Israel. It is advertised on the MEDI TRANS website (see www.meditrans-ip.net). MEDI TRANS partners will be encouraged to participate in other existing courses.

Internet-based training and a forum for scientific exchange

The MEDI TRANS' website has a forum for the exchange of information on training and education.

Training MEDI TRANS' and other young scientists

The exchange programme has seen the secondment of young scientists to other partner's laboratories for intensive training. This continues to be organised *via* the MEDI TRANS' website. At the end of each secondment, the secondees have prepared exchange reports on "lessons learned".

Partner participation in the GALENOS-Network

All partners have been formally invited to join the GALENOS Network and to attend 10-day training courses on drug delivery, and on management. Graduate students have applied for a Euro PhD.

Conclusions from the second year

The first Drug Targeting Systems course was held on

23rd February 2008 at Saarland University.

The website training page with sections on forthcoming, planned and recommended training courses, training course material, staff exchanges, PhD student networking and on the GALENOS Network has received >1000 hits in 2008.

MEDI TRANS' staff have been on exchange and Galenos activities have been widely promoted.

Work Package 13: Dissemination

Objectives

- To demonstrate non-confidential technologies to MEDI TRANS' partners and other interested groups
- To develop, co-ordinate, and review appropriate dissemination strategies amongst the partners throughout the whole project duration
- To develop and maintain effective supporting channels of communication, including the dissemination contacts database, enquiry mechanisms and website
- To ensure effective dissemination of the project's results to interested stakeholders and the general public

Work plan

Website development

The goal is for the website to be a powerful dissemination tool, and the interface between the project and the external community interested in research on targeted delivery of nanomedicines. A Contacts Database will be developed and maintained to support and assist in the co-ordination of all dissemination activities.

Project promotion

It is vital that the conclusions from MEDI TRANS are disseminated to as wide an audience as possible to increase the impact of the project's deliverables.

Glossy promotional leaflets will be distributed widely by partners at conferences, symposia, *etc.*, and to their normal business contacts.

Conclusions from the second year

The MEDI TRANS website www.meditrans-ip.net has been continually updated and now has pages on MEDI TRANS news, links, public awareness, partner links and on MEDI TRANS publications.

The database of contacts who will receive MEDI TRANS' disseminated results has reached the screening and selection process.

Images courtesy of: **1:** Dr R. Schiffelers (Utrecht University), **2:** Prof. Dr C.-M. Lehr (Saarland University), **3:** Dr K. Fischer (Bayer Schering Pharma AG), **4:** www.istockphoto.com, **5:** The MRI brain image is from: Dousset *et al.* (2006). *American Journal of NeuroRadiology* **27**: 1000-1005.

WP12 Leader: UDS

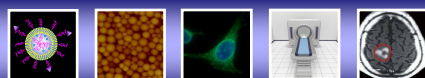
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