PROJECT FINAL REPORT

Grant Agreement number: 201031

Project acronym: NANOPHOTO

Project title: Targeted Nanosystems for improving Photodynamic Therapy and Diagnosis of Cancer

Funding Scheme: Collaborative Project

Period covered: from month 1 to month 42

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4.1 *Final publishable summary report*

EXECUTIVE SUMMARY

The aim of NANOPHOTO was the development of nanocarriers capable of improving pharmacokinetics, biodistribution and therapeutic efficacy of the photosensitising drug *meta*-tetrahydroxyphenyl chlorine (mTHPC, Temoporfin), used in photodynamic therapy (PDT) of tumour and diminishing unwanted side effects. It is already established that well engineered nanocarriers improve the selectivity of drug delivery to tumour tissue by taking advantage of high permeability of its neovasculature and inefficient lymphatic drainage.

First generation nanocarriers like liposomes as well as more recently developed poly(lactide-co-glycolide)copolymer (PLGA) nanoparticles (NPs) and ORganically MOdified SILica (ORMOSIL) NPs were considered searching for nanocarriers guaranteeing substantial improvement of mTHPC delivery into tumour in comparison to Foscan, the clinically used formulation.

mTHPC was successfully incorporated into the three nanocarriers and the protocols to optimise drug loading were established. The incorporation of mTHPC in the nanocarriers causes little perturbation of its photophysical properties and ability to produce the reactive singlet oxygen which is important to induce PDT damage to tumour. To avoid premature leakage of the drug before cell internalisation of NPs, mTHPC was covalently bound to the matrix of ORMOSIL NPs.

Nanocarriers with stealth properties were prepared by coating their surface with a layer of polyethylene glycol (PEG) of various density and thickness. These NPs have the ability to escape the capture by phagocytic cells *in vitro* and consequently showed prolonged circulation in the bloodstream after intravenous administration.
The stealth properties of the PEGylated NPs translated into improved pharmacokinetics of the entrapped mTHPC. All the nanocarriers, administered intravenously to tumour-bearing rats, increased the bioavailability in the blood circulation of mTHPC, with respect to mTHPC in the standard formulation, and the probability of being taken up passively into the tumour tissue through the enhanced permeability and retention effect.

Improved tumour uptake and selectivity (versus skin) of mTHPC was found using in particular liposomal formulations. The three-fold increase in the uptake in tumour of mTHPC delivered with liposomes vs. standard formulation correlates with the extent of PDT damage. Maximal tumour necrosis was induced with a liposomal mTHPC dose less than 20% the original clinical dose of mTHPC in standard formulation. This promises significant potential in reducing adverse side effects of PDT, such as skin photosensitivity, and pharmaceutical costs by reducing the administered dose of drug.

The standard mTHPC formulation used in the clinics needs a drug-light interval of 4 days vs. 24 hrs of the new liposomal formulation, because maximal uptake is attained much earlier. This reduces the time of hospitalisation, with benefit for the patients, the economic costs of cancer treatment.

The studies carried out have identified a new liposomal mTHPC formulation that improve substantially and from various points of view, the treatment of cancer with PDT.
The SME Biolitec, active in the project, is contacting contract manufacturing organisations (CMOs) for the manufacturing of large batches of liposomal mTHPC formulation under GMP-conditions.

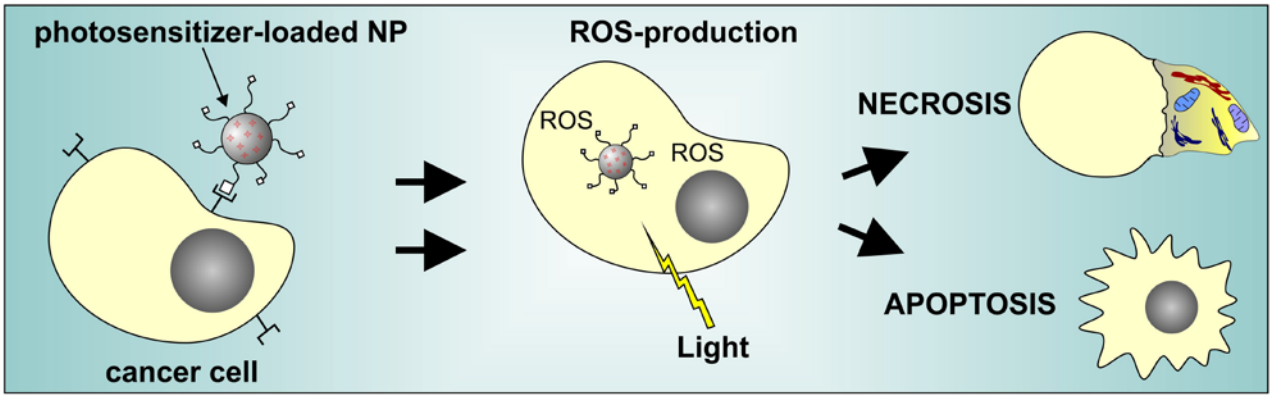
Biolitec is also planning to start the drug approval procedure for the European countries and to schedule Phase I and IIa clinical trials with mTHPC liposomal formulation.

PROJECT CONTEXT AND OBJECTIVES

In spite of several important achievements in the treatment of cancer, this disease still remains the first cause of mortality worldwide. In the last century, chemotherapy became a well established and viable modality of treating cancer and, as important mechanisms of tumour development and progression were elucidated, some agents were developed acting on specific proteins over-expressed by or pathways aberrant within the cancer cells. The general problem is that all generations of chemotherapeutics suffer from non specific biodistribution and poor bioavailability which are responsible for limited impact on patient survival.

It is now expected that nanomedicine, the application of nanotechnology to human health, will contribute to overcome the limitations of traditional chemotherapeutics. Currently nanomedicine is generating nanosized vehicles for directing the delivery of anticancer agents selectively to cancer cells since the precise delivery of drugs to pathological tissues is as important as the drug activity itself. Drug delivery systems of 10-100 nm size with hydrophilic surface can escape the capture by macrophages of liver and spleen. This property prolongs the time of circulation in the blood stream of intravenously administered nanosystems and increases the possibility of targeting the tumour taking advantage of the unique anatomical and pathophysiological abnormalities of its vasculature. The enhanced permeability and retention (EPR) effect which results from the high permeability of neovasculature and the inefficient lymphatic drainage of the tumour tissue is regarded as a gold standard for the engineering of nanosystems for the delivery of anticancer drugs. Even more exciting is the idea of engineering nanosystems simultaneously loaded with different agents able to perform different tasks like to detect the tumour, deliver drugs to kill cancer cells and then monitor the therapeutic effect.

In this context, the project NANOPHOTO aimed at the development of nanosystems for carrying the photosensitiser *meta*-tetrahydroxyphenyl-chlorin (mTHPC, Temoporfin) selectively into cancer cells. mTHPC is approved in Europe for the treatment of head and neck cancers with photodynamic therapy (PDT). PDT is a minimally invasive treatment based on the use of a light-activated photosensitizer that induces tumour necrosis/apoptosis by the production of reactive oxygen species.



Photoinduced tumour cell killing by photodynamic therapy

The formulation used in clinics, commercial name Foscan®, is prepared with polyethylene glycol and ethanol as solubilising agents of mTHPC. Biodistribution of mTHPC in normal tissues and non

selective accumulation in tumour are major drawbacks of PDT and improvements are being sought using nanomedicinal approaches. The availability of delivery systems able to modulate biodistribution of mTHPC by increasing selectivity of uptake in tumour with respect to normal tissues is highly desirable. It is expected that more selective accumulation in the tumour allows the administration of lower but still effective drug doses, thus reducing sensitivity to ambient light that is the major side effect caused by the administration of photosensitising drugs.

mTHPC is a hydrophobic molecule and can be entrapped in the phospholipid bilayer of liposomes as well as in the core of Organically MOdified SILica (ORMOSIL) and poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs).
Liposomes are clinically established nanosized lipid bilayer vesicles capable of carrying both hydrophilic and hydrophobic molecules, are biocompatible and biodegradable. When properly engineered, liposomes not only affect favourably pharmacokinetics, biodistribution and therapeutic efficacy of anticancer drugs but also decrease their unwanted cytotoxicity in non targeted organs. The NANOPHOTO consortium investigated the potential of these first generation nanosystems for achieving selective delivery of mTHPC into tumour and improve the clinical benefit of PDT. In parallel, more recently developed but still very promising PLGA NPs and ORMOSIL NPs, whose behaviour toward biological entities is largely unknown were analysed as potential carriers for mTHPC. NANOPHOTO exploited knowledge on some well established nanomedicine technologies and at the same time explored newly developed nanomedicine approaches searching for nanosystems guaranteeing substantial improvement of mTHPC delivery into tumours and selectivity of PDT treatment.

To optimise selectivity by exploiting both passive and active mechanisms of targeting, the nanosystems were equipped with ligands/antibodies binding receptors that are more abundant in cancer than normal cells and are considered biomarkers of at least some types of tumours.
The epidermal growth factor receptor (EGFR) and folate receptor (FR) are commonly up-regulated in cancer cells, due to their rapid proliferation, and therefore drug-loaded nanocarriers carrying folate or anti-EGFR antibodies exposed on their surface, should be able to interact with and, following internalisation, deliver the drug preferentially to cancer cells. Cetuximab is a chimeric antibody, binding the EGFR extracellular domain, already approved for treating selected tumours types in humans.
Analogously, the Prostate Specific Membrane Antigen (PSMA) is a markers of prostate carcinoma, a leading cause of male death in developed countries. PSMA is also expressed in the neovascular endothelium of many solid tumour types. Thus, PSMA is a very promising target molecule for anti-PSMA antibody-mediated drug delivery to attack the tumour vasculature of various different tumour types in addition to prostate carcinoma. The tumour neovasculature up-regulate also the expression of the integrin $\alpha_v\beta_3$ that facilitates invasion and aids spread of metastasis. Integrin $\alpha_v\beta_3$ binds specifically peptides containing the arginine-glycine-aspartic acid (RGD) sequence. Therefore RGD peptides may serve as an additional tool for targeting tumour vasculature. Cytokeratins and cathepsins are less studied tumour markers. Normally they are located inside the cells but in tumour cells they are translocated to the external surface and may serve as targets for specific antibodies that conjugated to nanocarriers loaded with anticancer drugs can help to increase specificity of drug delivery.

Based on the concepts described above, the NANOPHOTO consortium worked at the development of nanocarriers for mTHPC, possibly conjugated to ligands/antibodies binding specific markers of cancer cells or tumour neovasculature, able to deliver the drug in the tumour more efficiently than the approved mTHPC formulation (Foscan®). Therefore many studies were carried out comparing the performances of the new developed nanocarrier-based formulations of mTHPC with the standard

formulation. The consortium tried to achieve its major goal though a series of intermediate steps each associated to the achievement of specific objectives as listed below.

- Essential starting step of the work programme was the definition and optimisation of the procedures of loading the nanocarriers with mTHPC. The basic methods of incorporation of mTHPC in liposomes were already known but adaptation and optimisation was required for preparation of pegylated liposomal formulations. On the contrary with ORMOSIL and PLGA nanocarriers the best procedure for incorporation of mTHPC was defined and optimised within the project. Important objective was the definition of the characteristics of the PEG layer (density and thickness) guaranteeing stability of the particles in the aqueous biological medium. The definition of the photophysical properties of mTHPC entrapped in the nanocarriers was also important.
- To guarantee improved bioavailability of the carried drugs, nanocarriers need to escape the capture by phagocytic cells. Thus, the nanocarriers had to be characterised for their ability to escape recognition by macrophages and dendritic cells in parallel with studies on the release of the entrapped mTHPC to various types of tumour cells.
- The absence of cytotoxic effects elicited by PLGA and ORMOSIL NPs needed to be assessed to determine biocompatibility. As anticipated, with respect to liposomes, PLGA and ORMOSIL NPs are less characterised from the toxicological point of view and one of the objective was to determine cytotoxicity in various cell culture systems *in vitro*.
- The PEGylated nanocarriers loaded with mTHPC were tested *in vitro* in a number of tumour cells lines and subsequently in tumour models *in vivo*. The aim of the latter investigations was to define whether the nanocarriers could improve the efficiency and selectivity of mTHPC uptake in tumour, by exploiting passive targeting, and the therapeutic effect. The studies were carried out in comparison with the mTHPC standard formulation.
- The targeting of the nanocarriers was an important objective of the project. Selected nanocarriers were conjugated to folate, peptides or antibodies whose mission was to direct the nanocarrier specifically/preferentially to cancer cells. Protocols for conjugation of the various agents had to be defined for each class of nanocarriers and searching for optimised targeted nanocarriers the number of ligand molecules/particle and the length of the conjugating spacer were varied.
- In the subsequent step, the targeted nanocarriers were tested *in vitro* and *in vivo* to assess whether they were taken up by the cells via the specific recognition of receptors present on the cell membrane. The pharmacokinetic of the targeted nanocarriers had to be followed by experimental PDT studies to prove improved efficacy and selectivity of the treatment by delivering mTHPC with a targeted nanocarriers in comparison to the untargeted.
- At project conclusion, the objective was to undertaken the necessary activities to exploit the results. This was the major task of the SME biolitec active in the project.

SCIENTIFIC & TECHNOLOGICAL RESULTS

Preparation and characterisation of the nanocarriers

Liposomes. Liposomes are biocompatible and biodegradable lipid bilayer vesicles able to entrap both hydrophilic and lipophilic molecules. Several different methods can be used for the preparation of the various types of liposomes as MLV, SUV, LUV and FRV (i.e. multilamellar large lipid vesicles, small unilammellar vesicles, large unilammellar vesicles, freeze-drying rehydration vesicles). In order to formulate an active pharmaceutical substance, only unilammellar vesicles can be used to obtain a reliable and stable formulation. Especially by formulating a very hydrophobic drug like Temoporfin (mTHPC), unilamellarity guarantees a defined release of the incorporated substances.

Therefore the work was concentrated on unilammellar vesicles. The size of the vesicles may influence the availability of the formulated pharmaceutical substance once injected into the body and may have an effect on passive targeting. Therefore in NANOPHOTO only liposomes between 100 and 200 nm in diameter were prepared and tested. To have tight control over size, the production method chosen was the extrusion method. Briefly, phospholipids and Temoporfin were dissolved in organic solvent that was evaporated to produce a thin lipid-film on glass surface. The dried film was incubated with an aqueous solution to produce an inhomogeneous mixture of water and lipid that was extruded through polycarbonate membranes with defined pore size (400 down to 50 nm). This step of the procedure guarantees the production of liposomes of controlled size because the diameter of the pores of the membrane is controlled by laser etching. The liposomes were protected against microbiological contamination with appropriate storage.
The basic lipid components were DPPC [1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine] and DPPG [1,2-Dipalmitoyl-*sn*-glycero-3-phosphoglycerol]; DSPE [1,2 distearoyl-*sn*-glycero-3-phospho-ethanolamine] carrying a polyethylene glycol (PEG) chain was also include in the preparation to produce pegylated liposomes. The preparations were different for the length of PEG chains coating the liposomes as well as for the density of PEG lipid in the liposomal formulation. All preparations were sterilized by filtration and characterized for their long time stability.

The liposomes obtained were characterized by the following methods: photon correlation spectroscopy to measure the particle size; differential scanning calorimetry to determine phase behavior of the vesicles; UV-VIS spectroscopy to measure the content of Temoporfin; cryo transmission electron microscopy to visualise the quality of the preparation; and column size exclusion chromatography to determine the rate of incorporation.

In all preparations, the mTHPC was incorporated into the membrane of the liposomes. The analyses showed that liposomal preparations with a PEG molar concentration of 12% exhibited an enlargement of particle size distribution and short time stability because of liposomes aggregation. Consequently the PEG-lipid content of stable liposomes is limited and should not exceed a molar concentration of 8%. These preparations are stable for months concerning particle size and mTHPC content.
Comparing the different preparations, the amount of micellar structures in the liposomal preparations increased with increasing content of pegylated lipids. With increasing molecular weight of PEG the stability of the particles decreases. There is a correlation between the particle size of the liposomes and both molecular weight and concentration of PEG lipids. The higher the molecular weight of the PEG-lipid, the larger the size distribution.

PLGA nanoparticles. Non-PEGylated PLGA and PEG-PLGA nanoparticles were prepared using an emulsification-diffusion method developed in Ljubljana. Both versions of NPs were prepared either loaded with Temoporfin (mTHPC) or unloaded. PEGylated formulations were prepared with low (3.75% m/m) and with high PEG content (7.5% m/m). High PEG content formulation was established as superior in further analysis as shown by opsonisation and internalisation assays.
Further work was focused on the resuspension of lyophilized nanoparticles and the potential increase of their size due to pegylation. As measured on Zetasizer 3000, all nanoparticles increased moderately in their size after resuspension in aqueous solution, in particular, pegylation significantly increased their size after resuspension. Therefore, the lyophilisation process was improved including higher concentration of trehalose added into freezing buffer and in this way the increase in size for PEG PLGA nanoparticles was less pronounced. The test performed with MCF10A neoT cells showed that these new nanoparticles with high content of trehalose are not cytotoxic.
Besides changing the lyophilisation protocol, a method was also developed for nanoparticle preparation called nanoprecipitation. By this method smaller nanoparticles (around 150 nm before

lyophilisation) can be prepared with respect to previous ones. *In vitro* and *in vivo* investigations were carried out with PLGA NPs prepared by this method.

Silica (ORMOSIL) nanoparticles. Several methods are available to prepare silica nanoparticles generally involving base catalyzed polymerization of silicon alkoxides either in an ethanol/water solution (the Stober protocol), in reverse microemulsions, or in aqueous micellar solutions. The latter method involves the polymerization of lipophylic organosilane derivatives in the hydrophobic core of micellar aggregates. Hydrophobic molecules added to the reaction mixture are entrapped in the silica matrix during its growth. As a consequence, nanoparticles doped with organic molecules can be prepared by a mild one-pot procedure.
Such considerations led us to select micellar polymerization to prepare mTHPC loaded silica particles. Derivatives 1 (1a and 1b, Chart 1), which feature a hydrophilic PEG chain and an hydrophobic tail terminated by a trimethoxysilane group, were designed and prepared by condensation of commercially available PEG monoamine derivatives and 2-(4-chlorosulfonylphenyl) ethyltrimethoxysilane.

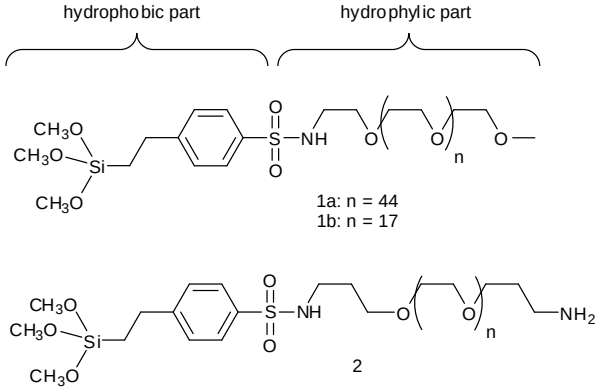
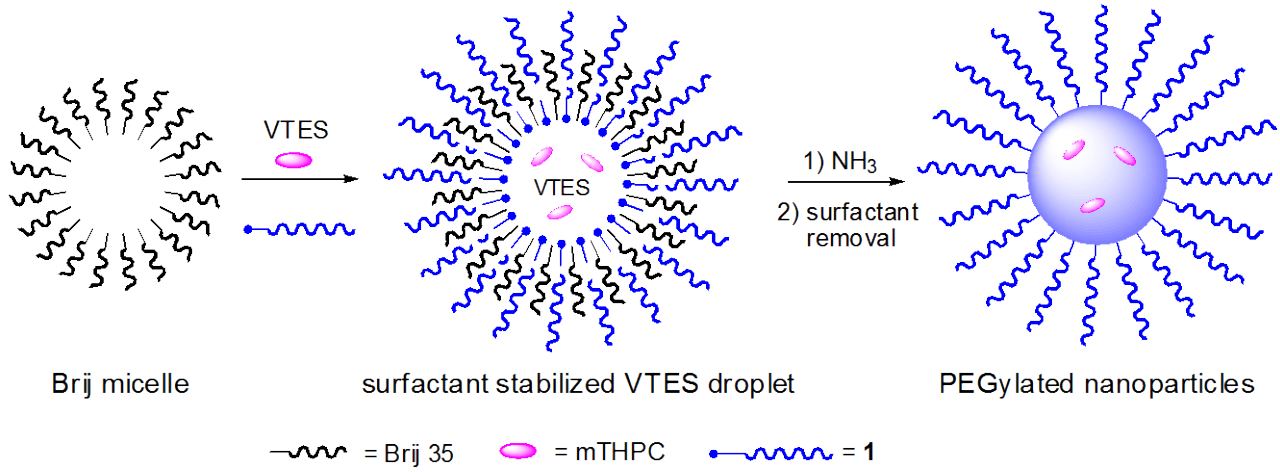


Chart 1. Alkoxysilane derivatives used for the nanoparticles preparation (Rio-Echevarria et al., J. Mater. Chem. 20:2780 (2010)

PEGylated ORMOSIL nanoparticles were hence prepared by ammonia-catalyzed co-polymerization of vinyltriethoxysilane (VTES) and 1 in aqueous solution of co-micelles of a non-ionic surfactant and *n*-butanol (Scheme 1). When added to the reaction mixture, derivative 1 places itself at the water/micellar



Scheme 1. Schematic representation of the formation of PEGylated nanoparticles. (Rio-Echevarria et al., J. Mater. Chem. 20:2780(2010)

interface, with the PEG moiety exposed to the bulk water and the trimethoxysilane group located in the hydrophobic interior, in the right position to copolymerize with the growing silica matrix (Scheme 1).

The formation of the nanoparticles was confirmed by Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM). At difference with bare ORMOSIL nanoparticles, solutions of PEGylated nanoparticles prepared with this procedure are stable in water for months and do not aggregate in high ionic strength solutions. This may indicate that electrostatic stabilization typical of silica nanoparticles has been replaced by steric stabilization due to the PEG coating. In addition, clear evidences of surface PEGylation are provided by further spectroscopic characterization.
Translational diffusion filtered ^{1}H-NMR indicated that the PEG chains were grafted to the nanoparticles and share their reduced diffusion coefficient. The PEG signals are also relatively sharp, suggesting that the PEG chains maintain a good extent of conformational mobility at least in the part that is more exposed to the solvent.
Finally, thermogravimetric analysis (TGA) of the nanoparticles prepared with derivative 1 reveals the formation of a dense PEG coating. In the case of 20 nm diameter nanoparticles coated with derivative 1a, the residual inorganic material accounts for 23% of the particle weight: therefore, taking into account the vinyl groups linked to each silicon atom, at least 67% of the particles weight is due to the PEG chains. Of course, weight percent content of PEG is reduced by increasing particles size (38% for 55 nm nanoparticles) or reducing the length of the PEG chain (37% for 40 nm nanoparticles coated with derivative 1b).
The final size of the nanoparticles is influenced by several parameters, in particular the nature and amount of surfactant, the amount of vinyltriethoxysilane, the reaction temperature and the presence of dopants. Careful tuning of the reaction conditions allowed the preparation of the PEGylated nanoparticles in the 20-150 nm size range.
Studies on the release of the physically entrapped mTHPC showed that PEGylation of ORMOSIL nanoparticles slows down but not prevent completely the leaking of the dye from the particles in solution containing serum. For this reason, mTHPC was chemically modified by reaction with 3-(triethoxysilyl)propylisocyanate. This allows the covalent grafting of the photosensitizer to the silica network in order to prevent dye leaking.

Stealth properties of the nanocarriers

The removal of nanoparticles, and other foreign species, from the blood circulation is strongly favoured by the adsorption of plasma proteins, functioning as opsonic agents, on the surface of the particles. Opsonisation causes fast removal of the particles from the circulation because stimulates phagocytosis by macrophages and other phagocytes of the organs of the reticulo-endothelial systems. PEGylation of the nanoparticles is expected to abrogate or limit these processes and increase bioavailability of carried drugs.
Therefore, the stealth properties of the PEGylated ORMOSIL, PLGA and liposomes were analysed by determining the adsorption of serum protein on the surface of NPs, the complement activation and the phagocytosis by macrophages. Non-PEGylated NPs were used as positive controls.
We have shown that serum proteins adsorb on the surface of non-PEGylated ORMOSIL particles preventing aggregation in saline solutions and mediating the release of entrapped mTHPC (Compagnin et al. Nanotechnology 2009). Adsorption of plasma proteins was shown by the increased hydrodynamic radius of non-PEGylated ORMOSIL nanoparticles after incubation with serum, measured by Dynamic Light Scattering. This effect was not found with PEGylated nanoparticles indicating that PEGylation prevents, or at least reduces, the interaction of the particles with the serum proteins.

The pattern of nanoparticle-associated proteins was determined in PEGylated or non-PEGylated NPs after incubation with the human serum followed by separation of the free proteins by centrifugation (ORMOSIL and PLGA) or Sephadex G-25 chromatography (liposomes). SDS-PAGE analysis allowed the observation of a specific subset of human plasma proteins adsorbed on the surface of non-PEGylated ORMOSIL and PLGA nanoparticles. MALDI-TOFF analysis and mass spectroscopy of excised bands of plasma proteins adsorbed to ORMOSIL-NPs identified Apolipoprotein A1, A4, E and B and lipoprotein gln1 as the prominent polypeptides. PEGylation almost completely eliminated protein binding to ORMOSIL-NPs surface. Data proved that the surface of non-PEGylated ORMOSIL-NPs is particularly prone to bind a selected set of human (and bovine) proteins, which may play a role in mediating NPs-cell interaction (especially macrophages). However, surface PEGylation clearly eliminated these NPs-protein association.
Complement activation on the NPs surface was determined using immuno-detection with specific antibodies. Western blot analysis proved the presence of activated C3, C4, C5, C1 complement proteins: activated C3 was clearly detected on non-PEGylated ORMOSIL-NPs and slightly represented on PLGA-NPs but not on PEGylated ones.

In vitro experiments using human macrophages demonstrated that PEGylated ORMOSIL nanoparticles, contrary to their nonPEGylated form, have the ability to escape phagocytosis [Rio-Echevarria et al. J. Mater. Chem.20:2780 (2010)]. The macrophages were analyzed by confocal fluorescence microscopy and flow cytometry to quantify the amount of phago-endocytosed nanocarriers loaded with a fluorescent probe (mTHPC, fluorescein or cyanine).
Beside macrophages, whole blood leukocyte population, platelets and HUVEC endothelial cells were used to test the escape of cell-capture in the blood circulation, which may influence deeply the clearance rate of injected NPs. Capture by non-circulating cells was also tested with highly capturing immune cells like dendritic cells. Both quantitative analysis by flow-cytometry and qualitative inspection by fluorescence confocal microscopy indicated that all non-PEGylated NPs were captured by macrophages. This capture was strongly dependent on the presence of serum-derived opsonines. PEGylation abolished the engulfment of NPs to various extents: being the decrease more evident in ORMOSIL-NPs.
These observations were confirmed using monocyte-derived dendritic cells as capturing model cells.
Flow cytometry studies using the whole leukocyte population from human blood indicated that non-PEGylated ORMOSIL-NPs mostly enter monocytes, and to a minor extent PMNs, inducing the expression of coagulation promoting Tissue Factor (TF), a sign of active interaction with the cell surface preceding phagocytosis. TF-procoagulant activity induced by engulfed non-PEGylated ORMOSIL-NPs was confirmed by RT-PCR demonstrating the activation of the TF gene transcription and by functional assays with human plasma. Endothelial cells (HUVEC) and platelets were shown to capture all non-PEGylated NPs. In this case however no cell activation was observed. PEGylation decreased strongly ORMOSIL-NPs uptake by monocytes, endothelial cells and binding to platelets. PLGA and SUV uptake was only partially diminished by PEGylation [Tavano et al. Nanomedicine.**5**:881-896 (2010)]. The coating of the surface of ORMOSIL-NPs with PEG resulted in a substantial elimination of capture and concomitant activation of TF-dependent procoagulant activity. Confocal microscopy analysis showed that macrophage engulfed non-PEGylated NPs were indeed endocytosed in acidic phago/lysosomal compartments, co-stained with Lyso-tracker.

It was demonstrated that PEGylated ORMOSIL-NPs acquired excellent stealth properties in all our *in vitro* models compared with their non-PEGylated equivalent. Such a decrease of cell-capture was less evident in the case of PLGA-NPs and liposomes, possibly because of less efficient coating (maximal PEGylation 8% vs. 37% in ORMOSIL-NPs). Importantly, no sign of cell activation (typical activation markers of platelets, endothelial cells and monocytes, among which Tissue Factor) was observed with all used PEGylated-NPs.

Photophysical properties of mTHPC in the nanocarriers

Studies were performed to investigate how the photophysical properties of the photosensitiser were modified when the sensitiser was loaded into a nanocarrier and whether these properties were modified when targeting ligands were attached to the nanocarrier. The key photophysical properties investigated were absorption, fluorescence and photosensitised production of cytotoxic singlet oxygen, in comparison to the mTHPC photosensitiser dissolved as monomers in solution. The main potential problem with nanoparticle-loaded photosensitisers is aggregation e.g. dimerisation and/or self-quenching of the photosensitiser due to the high local concentration within the nanoparticle. Concentration-dependent effects on photophysical properties are manifested in several ways e.g. broadening of the Soret absorption band, and reduction in fluorescence quantum yield and lifetime due to 'self-quenching' interactions between adjacent or dimerised sensitiser molecules, and reduction in singlet oxygen quantum yields.

The steady-state and time-resolved data indicate significant sensitiser aggregation in the biodegradable liposomal and PLGA nanocarriers whether targeted or untargeted. However this aggregation is not necessarily a serious drawback, since the sensitiser can be released in photoactive monomeric form from the biodegradable nanocarriers once taken up into cells. The photodynamic therapy experiments on cells support this conclusion.

For the silica nanocarriers, the most important result is that covalent binding of the sensitiser into a silica matrix irrespective of NP size even at high loading does not significantly perturb the photophysical properties of the sensitiser. Therefore the photoactivity of the sensitiser should be maintained when the nanocarriers are taken up by cells. This conclusion applied to both targeted and untargeted NPs. One exception though was observed for the tetra-conjugated chlorin in the NPs, where strong fluorescence quenching and a lower singlet oxygen production were observed in aqueous solution, which was attributed to distortion of the chlorin macrocycle. Nevertheless the *in vivo* data showed that these NPs were still effective for PDT.

No significant degradation in photostability was observed following incorporation of the chlorin into the nanocarriers in cells or solution. These studies were carried out using the standard formulation as a reference.

Cytotoxicity of the nanocarriers

Studies were carried out to assess the safety of the PLGA and ORMOSIL nanoparticles being these nanocarriers not well characterised from the toxicological point of view.

ORMOSIL nanoparticles. The *in vitro* cytotoxicity studies were performed using different cell lines and applying various tests to detect the cellular responses elicited by ORMOSIL nanoparticles. For a number of studies ORMOSIL not loaded with mTHPC were used to test the toxicity of the vehicle without the incorporated drug.

The human lung carcinoma cells A549 and the normal lung fibroblasts CCD-34Lu were used as cell models. The cytotoxicity of PEGylated ORMOSIL NPs was studied by measuring proliferation/viability of the cells exposed to increasing concentrations (up to 1.5 mg/ml) of NPs with the MTS assay as well as the release of the enzyme lactate dehydrogenase (LDH) that detects damage of the cell membrane. Both assays indicated that only carcinoma cells were damaged following ORMOSIL NPs exposure and cell death occurred predominantly via necrosis as a

consequence of plasma membrane damage. In normal fibroblasts only 10-15% of the cells showed damaged plasma membrane after 24 h treatment with the highest NPs concentration. Consistent with these findings, morphological changes were detected only in carcinoma cells and not in fibroblasts.

To elucidate the mechanism of ORMOSIL cytotoxicity, we measured the production of reactive oxygen species (ROS) by flow cytometry after staining the cells, exposed and not exposed to NPs, with a specific probe for detection of intracellular ROS. In A549 cells, we could detect a production of ROS above the basal level at all time points considered (5, 24 and 24 + 24 h), while in CCD-34Lu cells ROS exceeded the basal level only at the end of the 24 h of NP exposure. In fact, 24 h after the removal of NPs from the medium, CCD-34Lu cells had already restored the basal oxidative stress level. We identified the type of ROS by measuring the amount of intracellular ROS in the presence of superoxide dismutase (SOD) and catalase (CAT) alone or in combination. In general, CAT alone was much more effective than SOD alone in reducing the level of ROS suggesting that hydrogen peroxide was the most abundant among the ROS produced. SOD and CAT together gave the highest reduction of ROS. While SOD and CAT were effective in reducing the level of intracellular ROS they were unable to protect A549 cells from death induced by NPs. Therefore we could conclude that the oxidative stress is not the major determinant of cytotoxicity.
We investigated whether ORMOSIL NPs were internalized to a similar extent in A549 and CCD-34Lu cells. For these investigation we used fluorescent NPs (FITC-NPs) and measured their intracellular uptake by flow cytometry and their localization by fluorescence microscopy.
The results showed that A549 and CCD-34Lu cells accumulated very similar amounts of NPs and for both cell lines, fluorescence microscopy showed that NPs were in the cell cytoplasm with a punctuated distribution suggesting a localisation in the acidic compartments, such as endosomes and lysosomes (Moret et al submitted)

For a more comprehensive understanding of the different cellular responses elicited by PEGylated ORMOSIL NPs in A549 and CCD-34Lu cells, we performed a gene expression profile and gene ontology (GO) analysis. Gene expression profiles were significantly altered only in A549 cells, with 133 and 45 genes differentially expressed after 24 h incubation and at 24 + 24 h (24 h after removal of NPs from the medium). No differentially expressed genes were found in NP-treated fibroblasts with respect to controls, confirming the unresponsiveness of this type of cells to NP treatments also from a transcriptional point of view.
The gene ontology analysis showed that in A549 cells, the genes differentially expressed at the end of the 24 h of NP incubation belonged to 5 major biological processes, namely, signal transduction, cell death, cell proliferation, response to stimulus and inflammation. It appeared that cell surface receptors, or enzyme-linked receptor proteins stimulating intracellular signaling cascades that lead to the activation of inflammation and cell death are very likely involved.

Our data show that the addition of the PEG corona imparts ‘stealth’ properties to ORMOSIL NPs but does not abolish completely the toxicity of these NPs toward all types of cells. However the NP concentrations used in these studies were very high (1.5 mg/ml). In A549 cells the cellular responses are very likely determined by interactions of NPs with components of the cell membrane, which activate intracellular signalling cascades and inflammatory response, and at high concentrations, membrane damages are produced leading to cell death via necrosis. More data on the cytotoxic effects of PEGylated ORMOSIL should be carried out on various different types of cells possibly associated to *in vivo* studies.

mTHPC-loaded ORMOSIL NPs.
The effects in the dark of mTHPC-loaded NPs on cell survival were compared to those induced by the same concentrations of mTHPC delivered by the standard vehicle. NPs with low loading of

mTHPC caused some dark toxicity in A549 that was however substantially lower that that caused by free mTHPC. NPs with high loading of mTHPC were not toxic in the dark. The lower dark toxicity is very likely explained with the low efficiency of cellular uptake of mTHPC-loaded NPs. In any case the amount of internalised mTHPC was sufficient to produce cytotoxic effects with light and this may suggest that PEGylated ORMOSIL NPs can be considered as nanocarriers for the delivery of photosensitizing drug for PDT. The *in vivo* data obtained with rats bearing tumour are consistent with these *in vitro* data.

PLGA nanoparticles
MTS and LDH assays showed that empty pegylated PLGA are not toxic to the cells up to 0.1 mg/ml with an incubation time of 24 h. MTS assay showed that the number of metabolically active cells at the end of the incubation (24 h) and 24 h after the end of the incubation (24 + 24 h) was not reduced with respect to unexposed cells. The LDH assay confirmed the biocompatibility of PLGA showing the absence of plasma membrane damage after exposure to PLGA. The concentrations of PLGA used in these investigations are much higher than those used to deliver the highest mTHPC concentration of 5 μM to cancer cells. The tests were carried out in four cell lines. Illumination with red light of the cells incubated with empty PLGA did not affect cell survival.

The dark toxicity of mTHPC-loaded PEG-PLGA was evaluated in A549 and CCD-34Lu cells after 24 h incubation. The typical loading of mTHPC in PLGA was 7% and the concentrations tested corresponded to 1-5 μM mTHPC. While no decrease of viability was observed immediately after the end of incubation, a reduction was found at 24 +24h. However; it is important to underline that, at both time points investigated, the observed dark toxicity is much lower than that of equivalent concentrations of mTHPC in the standard vehicle. The efficiency of mTHPC uptake in cells was not reduced significantly by the PLGA delivery and the *in vitro* PDT experiments showed that the toxic effects induced by irradiation are the same whether mTHPC is delivered in the standard vehicle or Pegylated PLGA.
We conclude that PLGA are useful carrier for mTHPC because this formulation diminishes dark toxicity of mTHPC, which is relevant for healthy tissues, while preserves its photosensitising efficiency toward cancer cells.

In vitro cellular uptake and phototoxicity of mTHPC-loaded nanocarriers

Liposomes
The uptake of m-THPC delivered with liposomal formulations (Fospeg), in comparison to standard formulation, was determined in A549 cells and CCD-34Lu. The uptake studies were performed with an incubation of 24 h and m-THPC concentrations below the threshold concentration of dark toxicity. Various Fospeg formulations different for the length and the density of the PEG chains coating the surface of the liposomes were tested. The amount of m-THPC taken up by the cells was measured by flow cytometry and validated with chemical extraction method followed by fluorescence reading of the cell lysates. In both cell lines the uptake of m-THPC delivered with the liposomal formulations approached a plateau at concentrations above 1.5 μM while steadily increased with standard formulation. This difference suggested a different modality of m-THPC uptake with standard formulation in comparison to liposomes. The time-dependent uptake of liposomal m-THPC was biphasic while that of m-THPC in standard solvent steadily increased with time suggesting again some differences in the mode of cell internalisation. The lowering of the temperature from 37°C to 4°C during the incubation, reduced the uptake by about 95% suggesting that the internalisation of both formulations occurred via endocytosis (Compagnin et al Photochem. Photobiol. Sci. 2011).

Fluorescence microscopy of cells incubated with m-THPC standard formulation or Fospeg showed that m-THPC was mainly localised in Golgi apparatus and endoplasmic reticulum. On the contrary PEGylated liposomes with the same composition of Fospeg localised in lysosomes and suggested that once internalised the liposomes released m-THPC.
We performed the dark toxicity studies and found that all Fospeg formulations were less toxic than m-THPC standard and the decreased toxicity was dependent on the degree of PEGylation. Cell toxicity in the dark was abolished when PEGylation was increased to 8% with PEG chain length ranging from 750 to 5000.
The phototoxicity of the Fospeg formulations was determined in A549 carcinoma cells in comparison with free m-THPC in standard solvent. The cells incubated with m-THPC (0.25-1.5 μM) for 24 h were irradiated with 0.24 J/cm^2 of red light. The dose-response curves showed that the tested Fospeg formulations had very similar phototoxic effects on A549 cells and were only slightly less phototoxic than free m-THPC.
These studies demonstrated that the uptake of mTHPC from Fospeg was lower than that of m-THPC in standard solvent but the photo-toxicity was only slightly reduced. On the contrary, the dark toxicity of Fospeg was strongly reduced especially with the formulations containing 8% PEG. These findings suggest that liposome-based formulations are promising for the delivery of photosensitising drugs. The *in vivo* studies with tumor-bearing animals confirmed this conclusion.

PLGA nanoparticles
The cellular uptake of mTHPC-loaded PLGA NPs was evaluated in human lung carcinoma A549, human transformed breast MCF10A neoT and human pro-monocytic differentiated U937 cells at four time points. MCF10A neoT cells internalized larger amounts of drug than the other cell lines, especially following delivery with PLGA NPs. Moreover, the uptake of mTHPC was significantly increased when delivered by nonPEGylated PLGA NPs compared to PEGylated PLGA NPs in all cell lines at 4 h and 24 h.
The data obtained from experiments with the non proliferating U937 cells were useful in clarifying the fate of PLGA NPs after their cellular uptake. Increase of mTHPC fluorescence observed in U937 cells, even after cell washing with PBS and incubation in NP-free medium indicated intracellular degradation of the PLGA matrix. The results indicate that internalization of mTHPC-loaded PLGA NPs depends on the PEGylation of the NPs. The ratio between the fluorescence intensity for non PEGylated and PEGylated PLGA NPs clearly shows a lower uptake of PEGylated PLGA NPs than the non-PEGylated version in all the tested cell lines after 4 and 24 h of incubation. U937 cells exhibit the highest fluorescence intensity PLGA/PEG-PLGA ratio after 4 h of incubation, followed by MCF10A neoT cells, while the ratio is close to 1 for A549 cells. After 24 h of incubation, the PLGA/PEG-PLGA ratio decreases in U937 cells, while in A549 and MCF10A neoT cells the ratio increases significantly compared to the 4 h time point.

Localization of mTHPC-loaded PEGylated PLGA NPs was visualized in MCF10A neoT cells using fluorescence microscopy after various incubation times. After 1 h of incubation the red fluorescence corresponding to the internalized Temoporfin was distributed throughout the cell cytoplasm. However, it was more intense in regions corresponding to endoplasmic reticulum and Golgi apparatus. Prolonging the incubation increasing numbers of red spots appeared, corresponding to accumulated mTHPC or mTHPC-loaded nanoparticles in lysosomal-endosomal compartments. Similar intracellular localization was reported for non PEGylated PLGA NPs.

Phototoxicity studies were carried out with mTHPC-loaded PEGylated PLGA NPs and with mTHPC standard solution. The cells were irradiated with red light (600-700 nm, 0.24 J/cm^2) after incubation with mTHPC for 24 h in the dark. Cell survival, measured 24 h after irradiation decreased with the mTHPC dose, but was similar for both mTHPC formulations. To assess whether the comparable

phototoxic effect correlated with a similar intracellular drug concentration at the time of irradiation, the amount of mTHPC internalized by the cells was determined by flow cytometry. We found that the cellular uptake of mTHPC delivered by standard solvent was more than two times higher than when delivered by PEGylated PLGA NPs. However, mTHPC delivered by PEGylated NPs can be completely converted to the very photoactive monomers which may compensate the reduction of the uptake. The empty PEGylated PLGA NPs exhibited no cytotoxic effects on cells after irradiation, confirming that they are photo-chemically inert and therefore, any contribution of the drug delivery system to the phototoxic effect of mTHPC-loaded PEGylated PLGA NPs can be ruled out.
In conclusion, we developed a stealth and biodegradable nanosystem for the systemic delivery and prolonged intracellular release of mTHPC. We demonstrated higher *in vitro* phototherapeutic effectiveness of mTHPC delivered by PEGylated NPs than in standard solution. Importantly, the dark cytotoxicity of mTHPC in the PLGA formulation is substantially lower than in standard formulation (Foscan®), with the consequence that fewer side effects of PDT are envisaged by delivering mTHPC in PLGA NPs.

ORMOSIL nanoparticles
Pegylated ORMOSIL NPs with an average diameter of 80 nm were loaded with the photosensitiser mTHPC which was physically entrapped or covalently linked to the NPs matrix as detailed above. The uptake of mTHPC-loaded ORMOSIL NPs was determined in human lung carcinoma A549 cells and the normal lung fibroblasts CCD-34Lu. Particles with low loading (~0.2%) and high loading (~2%) of covalently linked mTHPC were tested. In general, the cells were incubated for 24 h with increasing concentrations of mTHPC loaded NPs and internalisation was measured by flow cytometry using the mTHPC fluorescence. In parallel, experiments were performed with mTHPC delivery with the standard vehicle as in Foscan. The efficiency of mTHPC uptake and nanoparticles internalisation were similar in A549 carcinoma cells and CCD-34Lu fibroblasts. The uptake of mTHPC (0.2% loading) physically entrapped in ORMOSIL appeared slightly higher than that covalently linked but it was only approximately 3% with respect to that measured after delivery with the standard vehicle. The uptake of mTHPC was increased by 2-3 fold with 2% loading of the nanoparticles. However, the uptake of mTHPC in ORMOSIL NPs remained very low with respect to the uptake in standard formulation.
Fluorescence microscopy was used to determine the localisation of mTHPC inside the cells. The physically entrapped mTHPC showed an intracellular fluorescence distribution typical of a localisation in the Golgi apparatus and endoplasmic reticulum as for mTHPC delivered by the standard solvent. On the contrary the covalently-linked mTHPC appeared slightly different with some punctuate fluorescence typical of a localisation in endosomes and lysosomes, suggesting that ORMOSIL NPs are internalised by endocytosis. The different intracellular distribution of physically entrapped and covalently linked mTHPC indicates the release of mTHPC from the NPs in the former case, which may occur after internalisation of the nanoparticles or in the culture medium outside the cells as it was shown with the fluorescence resonance energy transfer experiments we have published in Nanotechnology [**20** (2009) 345101]. The overall results indicate that untargeted highly PEGylated ORMOSIL nanoparticles are not internalised by the cell very efficiently and modification of NPs surface is desirable to favour internalisation.
However *in vitro* PDT was carried out in A549 cells after delivery of mTHPC covalently linked to ORMOSIL and, with a ten-fold higher light dose, it was possible to induce a cell killing comparable to that of mTHPC in standard solvent. This result is very important because it shows that mTHPC covalently linked to the NP matrix is still able to produce ROS that escapes the NP interior and cause cell damage.

Production of antibodies

The following antibodies were produced during the project for the subsequent conjugation to nanocarriers. The aim was to produce nanocarriers able to recognise and bind receptor over-expressed on the surface of cancer cells for increasing selectivity of mTHPC delivery.

Anti- PSMA antibodies
The whole (conventional) antibody molecules was obtained by hybridoma technology following established protocols. Briefly, Balb/c mice were immunized i.p. with a PSMA positive cell lysate and then re-challenged with 2 injections i.v. of the purified recombinant form of the antigen, produced in bacteria. Before cell fusion, mouse serum was assayed to assess that the antibody response against PSMA antigen had taken place; affinity and specificity of antibodies obtained from cloned hybridomas were studied by flow cytometry on PSMA-positive and -negative cells and by ELISA on recombinant PSMA and non related antigens. Hybridoma cells producing the best monoclonal antibody (mAb) were selected and adapted to grow in hybridoma medium deprived of foetal calf serum to avoid contamination with calf immunoglobulins; mAb was produced in CELLline classic 350 two-compartment Bioreactor and purification was performed on Protein G Sepharose columns.
The scFv (single chain variable fragment) of the anti-PSMA mAb was obtained by RT-PCR performed on cDNA synthesized from anti-PSMA hybridoma cells; the scFv was subcloned in the expression vector pHEN2. The soluble scFv was produced in *E. Coli* and then purified with one passage on a Protein G Sepharose column. Gel filtration analysis and SDS-PAGE showed the high purity of the recombinant protein. The ability of scFv protein to specifically recognize PSMA antigen was investigated by flow cytometry on PSMA positive and negative cell lines.

Anti-cytokeratin antibodies
The anti-CK mAb was prepared using breast cancer cell line MCF-7 for immunizing mice. Briefly, a stable hybridoma cell line was established by the fusion of mouse myeloma cells with spleen cells of BALB/c mice immunized with tumour cell extract proteins. The mAb was selected by the limited dilution method and indirect ELISA using MCF-7 cell lysate immobilized on microtiter plates. Stable hybridoma cells were grown in cell culture flasks and stirrer reactors. The mAb produced was purified on a Protein G Sepharose column and the yield was quantified. The antibody recognizes a specific cytokeratin profile (cytokeratines 1, 2, 8, 10, 18) as determined by 2D electrophoresis, immunoblot and mass spectroscopy in a number of breast cancer cell lines (MCF-7 and MCF-10A neoT). For fluorescent microscopy and flow cytometry studies the antibody was labelled with Alexa FluorR-546 fluorescent dye according to the manufacturer's instructions. Besides binding cytokeratins on the cell surface of tumour cells the antibody was shown to inhibit plasmin generation and consequently, invasion potential of cells *(Kos et al., Inactivation of harmful tumour-associated proteolysis by nanoparticulate system. Int. J. Pharmaceutics, 2009, 381, 106-112).*

The stable hybridoma cell line, producing mouse anti-cytokeratin mAb, was used for preparation of chimeric mouse-human mAb according to the procedure of *Kopitar Jerala et al., Pflugers Arch 2000, 439:79-80*. For this purpose, immunoglobulin constant domains of mouse mAb Cγ and Cκ, were substituted by the human Cγ1 and Cκ. The variable heavy and light chain regions were PCR amplified from hybridoma RNA and sequenced. Mouse variable V_H and V_L regions were joint to human IgG1 and κ constant regions and subcloned into pcDNA3 expression vectors. The Sp2/0 murine myeloma cells were transfected with expression vectors pcDNA3L and pcDNA3H and the reactivity of the chimeric antibody was tested by indirect ELISA using B16F1 murine melanoma cells as well as MCF7 human breast cancer cells.

Anti-cathepsin B antibodies.
Cathepsin B specific mouse 2A2 mAb capable of inhibiting its proteolytic activity was prepared. The hybridoma cell line was obtained by the fusion of splenocytes from BALB⁄c mice immunized with recombinant human cathepsin B with NS1 ⁄ 1-Ag4-1 myeloma cells according to the method of Kohler and Milstein. Screening for clones producing the most potent inhibitory antibodies was performed with the substrate Z-RR-AMC. Stable hybridoma cells were grown in cell culture flasks and stirrer reactors. The growth medium was filtered, concentrated on ultrafilter and applied on Protein A Sepharose to separate mAb. The mAb yield was quantified by Biorad Protein Methods and spectrophotometrically. From 1.5 l of growth medium 15 mg of the antibody were isolated. In further studies the biochemical properties of the mAb were determined. The results suggest that, upon binding, the 2A2 monoclonal antibody induces a conformational change in cathepsin B, stabilizing its exopeptidase conformation and thus disabling its harmful action associated with its endopeptidase activity (*Mirkovic, B., Kos, J., et al, Regulation of cathepsin B activity by 2A2 monoclonal antibody. FEBS J. 2009, 276, 4739-4751*). Therefore, 2A2 mAb can be used for specific labelling of cathepsin B and as selective inhibitor of cathepsin B endopeptidase activity.

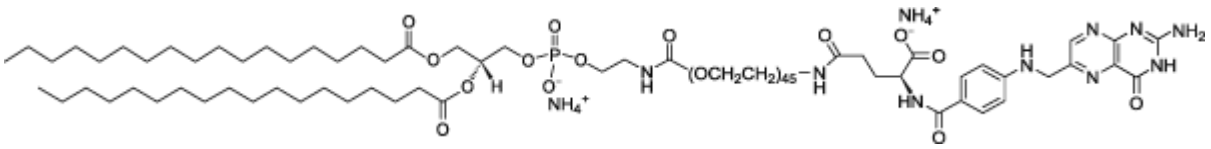
Cathepsin B specific chimeric mouse-human mAb was prepared from 2A2 mAb hybridoma cell line according to the procedure of *Fan et al., 2002, 383:1817-182*. The chimeric mAb was constructed by fusing murine V_H and V_L variable regions of the inhibitory antibody with human Ig1 and κ constant regions, respectively. Chinese hamster ovary K1 cells were co-transfected with expression vectors of pcDNA3L and pcDNA3H and the reactivity of the isolated chimeric antibody was tested by ELISA and Western blot. The chimeric antibody was affinity purified on Protein A Sepharose. It showed strong staining reactivity with cathepsin B as immobilised antigen in ELISA.

Targeting of nanocarriers

Liposomes, PLGA and ORMOSIL NPs were targeted with small molecules or antibodies to obtain specific delivery of the carried mTHPC in cancer cells overexpressing selected receptors on their surface.

Liposomes
PEGylated liposomes were targeted with folic acid, epidermal growth factor (EGF) and the antibody against EGF receptor, cetuximab, already used in clinics for treating some types of cancers.

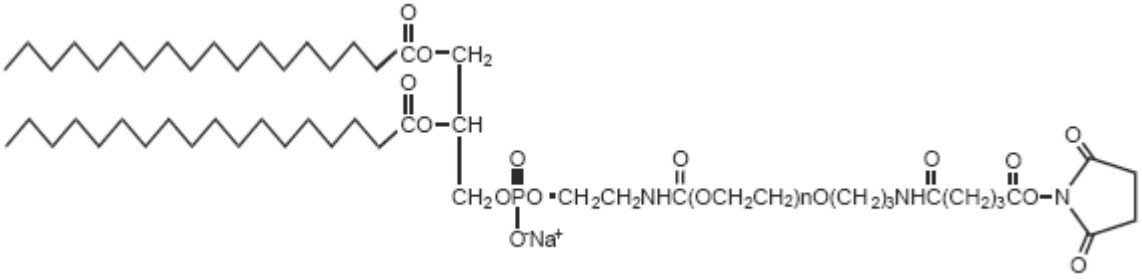
For the synthesis of folate-PEG-liposomes a spacer of PEG 2000 or PEG 5000 has been chosen. For coupling of the PEG spacer to the lipid backbone, a phosphatidylethanolamine has been used (internal NH_2-group). The compound used was: 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[folate(polyethylene glycol)-2000] (ammonium salt)



DSPE-PEG(2000)Folate

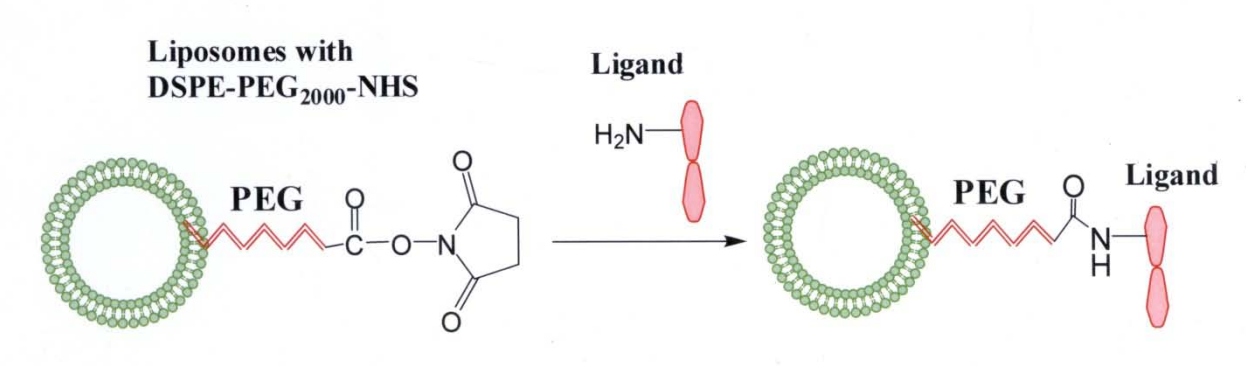
Using this compound, stable liposomes could be obtained with standard production procedures after small modifications. Test of free folic acid was performed using column chromatography. No free folic acid could be detected, confirming the suitability of the used production procedure.

Due to the proteinogenic nature of the EGF, the manufacturing procedure for the production of the liposomes had to be changed, to avoid the denaturation of the EGF during the process. Denaturation would disturb the structure and spatial orientation of the molecule and render it non-functional in respect to biological activities. Therefore, in the first step an activated nanocarrier has been manufactured, which surface was decorated with *N*-Hydroxysuccinimide (NHS)-groups. These groups are covalently connected to the liposomal surface with polyethylene glycol (PEG) chains of different length (2000 or 5000 Da).



DSPE-PEG-NHS 3-(N-succinimidyloxyglutaryl) aminopropyl, polyethyleneglycol-carbamyl distearoylphosphatidyl-ethanolamine.

The strategy for coupling is shown in the following scheme:



In the last step, the free uncoupled EGF-protein was separated from the liposomal fraction by membrane dialysis in a borate buffer system. The removal of uncoupled EGF is of outmost importance because the presence of the free EGF would impair: a) long time stability of the liposomal preparation due to the fusogenic properties of the proteins and b) the active targeting mechanism by coupling to the free receptors on target cells, therefore diminishing the number of potential binding sites for the nanocarrier system.
The characterization of the liposomal preparations in respect to long time behaviour revealed size stability over a time period of more than 60 days, which is an important aspect for the potential commercialisation of the product.

The same coupling strategy as developed for EGF has been adapted for the antibody coupling. The strategy of coupling has been established with standard IgG-antibodies to implement the techniques for the manufacturing process. The coupling of the IgG-antibody was successful but the stability of such preparation was not satisfactory because of the large size distribution that increased with time of storage. This was indicative of instability of the liposomal preparation probably caused by the fusogenic action of the protein. This phenomenon depends on the special individual structure of the protein and therefore another protein may not destabilize liposomal preparations. In the next step, we used an anti-EGFR antibody, named Cetuximab that is a recombinant human/mouse chimeric monoclonal antibody directed to the external domain of the EGFR. Cetuximab is approved for use in combination with radiotherapy for locally-advanced squamous cell carcinoma of the head and neck

(SCCHN) in the EU and US. The liposomal preparation with coupled anti-EGFR antibody revealed a sufficient stability within the time period investigated, and opened the possibility to characterize the constructs for efficiency of targeting *in vivo*.

PLGA nanoparticles

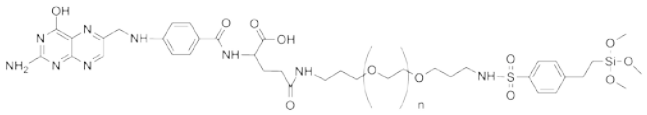
PLGA NPs were prepared by a nanoprecipitation method and loaded with mTHPC. Anti-cathepsin, anti-cytokeratins or anti-EGFR receptor (cetuximab) mAbs were covalently bound to the free carboxyl groups on the NPs activated with EDC/NHS chemistry. The antibodies were added to suspension of activated NPs. Samples were vortexed, incubated at room temperature for 2 h, centrifuged and washed with PBS.

NPs targeted with anti-cytokeratin mAb showed high ability to specifically bind to cell lysate of MCF-7 cells that overexpress cytokeratines 1, 2, 8, 10, 18. NPs targeted with anti-CK mAb were also tested for their ability to specifically internalize in MCF-7 cells.

NPs targeted with cetuximab were tested for their ability to specifically internalize compared to the untargeted NPs in A431 cells that overexpress EGFR and HeLa cells with low expression of EGFR.

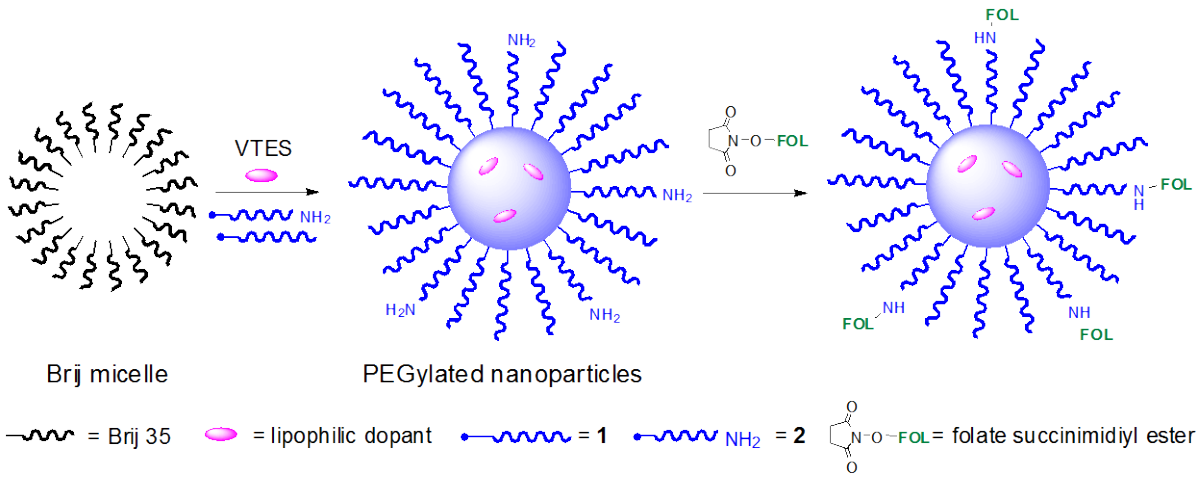
ORMOSIL nanoparticles

To produce ORMOSIL NPs targeted with folic acid, a folate-PEG2000-trimethoxysilane derivative (see below) was prepared from commercially available PEG2000-diamine.



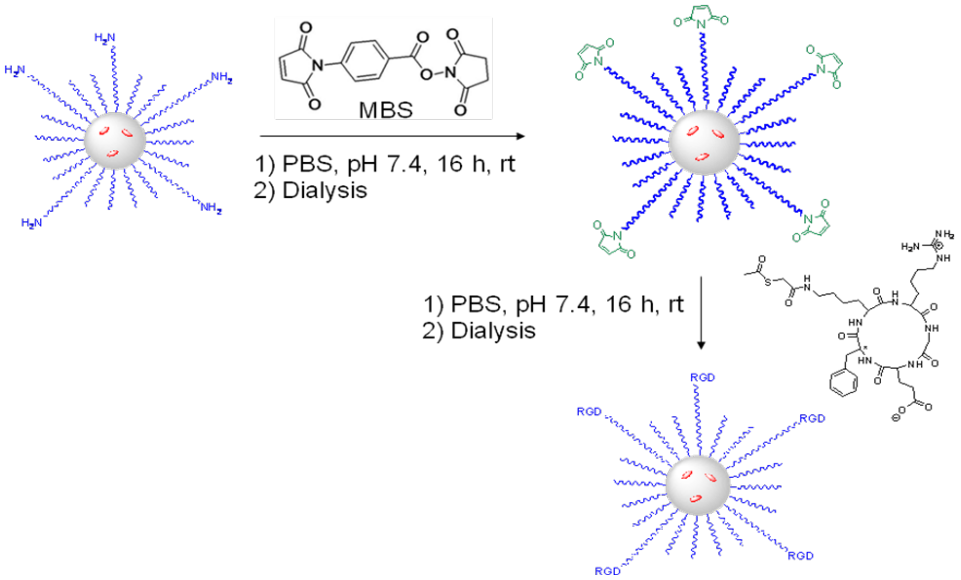
When this derivative was added in the preparation mixture of the nanoparticles, the folate groups were introduced in the PEG coating. The presence of the folate groups in the nanoparticles PEG coating (after purification) was proved by UV-Vis analysis that showed the typical absorption bands of the folate at 280 e 350 nm. Folate conjugated PEGylated NPs, loaded with covalently linked mTHPC, were prepared in different sizes (20 and 100 nm), different folate loadings (0.1%, 1%, 5% and 10% in moles of the total PEG derivatives in the coating layer) and with different lengths of unfunctionalized PEG derivative present in the coating (respectively PEG_{2000}-folate/PEG_{2000} and PEG_{2000}-folate/PEG_{700}).

In a different approach, silica nanoparticles where prepared using a mixture of the PEGylated silanes bearing a terminal amino group (see scheme below).



In this way, PEGylated nanoparticles containing primary amino groups in the coating shells where obtained (the presence of the amino group was confirmed by the fluorescamine test). Folate was then conjugated to the nanoparticles using an activated O-succinimidyl ester. After purification, the nanoparticles showed a folate content much higher than the maximum amount calculated on the basis of the nanoparticles amines content.
Control experiments performed using PEGylated nanoparticles without reactive groups revealed that folate has a strong affinity for the PEG layer of the particles, since the NPs still contained a high amount of non-specifically (adsorbed) bound folate after extensive purification.
Likely, strong non covalent association of conjugated-folate groups to the PEG coating layer resulted into a scarce solvent exposition and non ideal targeting properties.

As alternative strategy, mTHPC loaded ORMOSIL-NPs were conjugated with the RGD peptide, to target endothelial cells of the tumour neovasculature. NPs with different mTHPC loading and coated with a mixture of $PEG_{2000}OMe$ and $PEG_{3300}NH_2$ in different ratio were prepared as depicted in the scheme below.



RGD conjugation was performed through the m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) crosslinker. Successful functionalization with MBS was determined by the decrease of the concentration of amino groups measured by the fluorescamine test. The MBS-functionalized NPs were incubated with two equivalents (with respect to the nominal maleimide groups concentration) of cycloRGD in PBS at pH 7.4 for 16 hours and then purified by extensive dialysis.

mTHPC-loaded ORMOSIL were also conjugated to EGF and mAbs directed to EGF receptor (cetuximab) and PSMA (mAb, D2B).
The protocol included the derivatization of EGF or mAb with 2-Iminothiolane (2-IT) to insert reduced SH groups in the Ab molecule and derivatization of ORMOSIL-NPs with MBS to allow reaction with mAb forming a thioether chemical bond. mAb-2IT and NP-MBS were mixed and incubated at room temperature for 48 hrs and then purified to eliminate free antibody. Two different separation protocols were tested: the first based on a chromatographic approach (gel filtration on HiLoad-16/60 column) and the second based on an ultracentrifugation step. With both approaches free mAb was totally separated from mAb conjugated to NPs, but with chromatography a higher recovery (75-80%) of conjugated NPs was obtained with respect to centrifugation (recovery ~ 60-70%). Therefore chromatography was chosen as method of purification. The presence of Ab molecules on the NP surface was confirmed by flow cytometry after staining the conjugated NP with anti-mouse IgG-FITC or rabbit polyclonal anti-EGF+anti-rabbit-FITC.

In vitro studies with targeted nanocarriers

Folate targeted nanocarriers

The folate receptor (FR) mediated internalisation of different types of folate-targeted nanocarriers was studied *in vitro* in KB (FR-positive) and A549 (FR-negative) cells. The extent of expression of the folate receptor in these two cell lines was checked by flow cytometry analysis using a FITC-labelled primary monoclonal antibody against folate receptor α (FR α).

The amount of folic acid bound to the NP surface, the relative length of PEG chains used for coating or as arm for folic acid and the NP size were varied to study the effect of these parameters on the FR-mediated internalisation. The cells were incubated for different times with various concentrations of mTHPC covalently bound to the matrix of ORMOSIL NPs or entrapped in liposomes and mTHPC internalisation was measured by flow cytometry. The results obtained with ORMOSIL-NPs showed that none of the tested preparations gave satisfactory results in terms of specific internalisation mediated by folic acid receptor. We have evidences that folate, being a hydrophobic molecule, has a high affinity for PEG and therefore it is possible that folate is buried in the PEG coat of these NPs and is not sufficiently exposed to the surface to bind its receptor. It appears that this effect is less drastic in the case of liposomes because of the lower degree of pegylation (maximum 8% with respect to 37% of SiO_2-NPs). In fact, testing several different preparations we found that liposomes coated with PEG750 and PEG5000 as linker for folate, some specific internalisation could be found. In FR-positive KB cells, the uptake of mTHPC delivered with folate-targeted liposomes was twice that of the untargeted but only for short incubation times. The premature release of mTHPC from liposomes and its consequent internalisation by pathways other than the FR-mediated explain why selectivity is lost by prolonging the incubation time.

In vitro PDT experiments with cells incubated for 3 h with these liposomal formulations showed higher efficiency of killing in KB cells incubated with the folate targeted with respect the untargeted liposomes (no differences were detected in A549 cells). It was shown that, in KB cells overexpressing FR, for the same mTHPC dose, the light dose required to reduce cells survival by 50%, with folate-targeted Fospeg was approx. half that required with untargeted Fospeg. Therefore the folate targeted Fospeg might shorten the illumination time when treating tumours with high expression of folate receptor with reduction of PDT side effects.

RGD targeted nanocarriers

Another small molecule, namely the cyclic RGD peptide was used for targeting ORMOSIL NPs. Peptides with the RGD sequence bind the $\alpha_v\beta_3$ integrin and are recognised as agents for targeting the tumour neovasculature and some types of cancer cells. Human umbilical vein endothelial cells (HUVEC) were selected as model for the *in vitro* tests with RGD-targeted ORMOSIL NPs. NPs pegylated with PEG2000 and with RGD linked to PEG3300 gave the best results in particular those with 30% (as compared to 15%) of PEG carrying the peptide. As untargeted control, NPs of the same composition but carrying the peptide RAD, that is unable to bind the $\alpha_v\beta_3$ integrin, were used. In the incubation conditions tested, in general, uptake in HUVEC cells of mTHPC delivered with RGD-NPs was twice that delivered with RAD-NPs suggesting some involvement of the integrin in the internalisation process. However the higher mTHPC uptake did not correlate with higher killing of HUVEC cells after illumination.

EGF and cetuximab targeted nanocarriers

The specific internalisation of epidermal growth factor (EGF) and cetuximab targeted ORMOSIL NPs and liposomes was studied in cells with high and low expression of the EGF receptor; the cell lines were A431 with high expression and HeLa with low expression of EGF receptor.

The results suggested that EGF receptor has a very minor role in the internalisation of mTHPC delivered by liposomes targeted with EGF or cetuximab.

Contrary to liposomes, ORMOSIL targeted with EGF and even more with cetuximab accumulated in A431cells, over-expressing EGFR, with a high degree of selectivity. ORMOSIL targeted with EGF accumulated in A431 almost 10 times more than SiO_2 conjugated to bovine serum albumin (BSA) used as untargeted control. The maximum selectivity was after 1 h incubation and gradually decreased prolonging the incubation time. The uptake of ORMOSIL targeted with cetuximab in A431 cells was more than 25 times higher with respect to SiO_2 conjugated to BSA. An excess of free cetuximab inhibited the internalisation of the targeted-NPs indicating specific internalisation mediated by EGFR. However, the *in vitro* PDT experiments showed that A431 cells were killed with some degree of selectivity but the killing efficiency was very low also by using high doses of light. Overall, the results suggest that SiO_2 targeted with cetuximab are promising nanocarriers for specific delivery of mTHPC to cancer cells over-expressing EGF receptor but the efficiency of cell internalisation need to be improved to gain in PDT efficacy.

Similar results were obtained with anti-PSMA targeted ORMOSIL NPs. In PSMA over-expressing cells, the uptake of these NPs was 4.5-5 times higher with respect to the untargeted counterpart but again photodynamic killing of cells was very low. Also for these NPs strategies to increase internalisation need to be found for improving efficacy of PDT treatment.

Anti-cytokeratin and anti-cathepsin mAb targeted nanocarriers

PLGA nanoparticles, conjugated with anti-cytokeratin mAb were tested for specific recognition and internalisation on MCF10A neoT cells with high level of cytokeratins on the surface and Caco-2 enterocytic cells, expressing low levels of cytokeratins. Cellular uptake of non-coated and antibody coated-nanoparticles was investigated with a mono-culture of MCF-10A neoT cells or Caco-2 cells.
To determine specific delivery of immuno-nanoparticles MCF-10A neoT cells were co-cultured with Caco-2 cells. Nanoparticles labelled with Alexa FluorR-488 cystatin were added and the cells were observed for particle internalization. Nanoparticles incubated in the absence of anti-CK mAb were used as a control. Specific internalisation was assessed by fluorescence microscopy and images were analyzed using Cell R Imaging software. The results clearly show that PLGA nanoparticles, coated with anti-cytokeratin mAb, in co-culture of MCF10A neoT and Caco-2 cells solely recognized MCF10A neoT cells and internalised them. When non-coated PLGA nanoparticles were used as a control, they internalized both cell types (*Kos et al.,. Int. J. Pharmaceutics, 2009, 381, 106-112).*

***In vivo* investigations with mTHPC loaded nanocarriers**.

The pharmacokinetic properties of mTHPC delivered using untargeted nanocarriers were studied in a small animal model (rats) and comparative studies of PDT efficacy were carry out in comparison to mTHPC delivered in its standard formulation, Foscan.

Pharmacokinetics in normal tissues and tumour model

The pharmacokinetics were measured in a range of normal tissues followed by tumour studies. The nanocarriers were administered intravenously and tissues were harvested at different time points. The levels of mTHPC were determined quantitatively using chemical extraction based on fluorescence detection. Microscopic biodistributions were studied using fluorescence microscopy. There was particular focus on uptake by organs of the reticuloendothelial system (RES), e.g. liver, spleen, which are known to scavenge nanocarriers.

The untargeted nanocarriers studied were: (a) liposomal FosPEG2% and 8% formulations; (b) PLGA, unpegylated and pegylated, and (c) pegylated ORMOSIL nanoparticles (NPs) with either small (typically 20 nm) or large diameters (typically 90 nm).

All nanocarriers exhibited prolonged plasma levels in comparison to the standard formulation Foscan. This result is consistent with previous studies using nanocarrier delivery where pegylation is employed to inhibit opsonization.
Uptake in the kidney was relatively low showing that renal clearance and toxicity were not limiting factors. Uptake in skin was comparable or lower in comparison to Foscan which is important in limiting skin photosensitivity. Significant uptake in tissue was observed for the ORMOSIL NPs despite the relatively weak uptake observed by cells grown in culture. This is attributed to different uptake mechanisms occurring *in vivo*.
No significant systemic toxicity was observed for the nanocarriers. In the case of the larger silica NPs some minor inflammation was noted in the lung but this resolved over time. Slower clearance was observed from the lung and liver compared to Foscan which is believed to result from retention in macrophages.

In tumour, significantly improved tumour uptake of mTHPC and selectivity (versus skin) was observed using the two pegylated liposomal formulations, by approximately a factor of three after 24 hr, compared to Foscan. The greater tumour uptake is consistent with the prolonged plasma levels exhibited by the nanocarriers which favours passive targeting of nanocarriers to tumour. Tumour microscopic localisation was similar for the liposomal nanocarriers vs. Foscan.
The PLGA and larger ORMOSIL NPs also elicited improved tumour uptake and selectivity. However based on fluorescence microscopy there was some uptake by tumour-associated macrophages, which would be expected especially for the non-biodegradable ORMOSIL NPs.

PDT in normal tissues and tumour model
The PDT studies were carried out using fibre-optically delivered 652 nm laser light at low power so that there was no thermal damage (as confirmed using control animals).The drug-light interval of 24 hr was selected on the basis of the pharmacokinetic studies For assessing skin photosensitivity, a lamp was used with an output spectrum that simulated sunlight based on previously published protocols. Damage was measured histologically.

Using the liposomal nanocarriers, increased tumour damage was observed in comparison to Foscan at low doses. Liposomal m-THPC showed a greater PDT effect than standard Foscan when using less that 20% of the original clinical dose of mTHPC (0.05 mg kg^{-1}). At higher doses there was less difference but this was attributed to documented resistance of the tumour margins to PDT. In normal colon we observed selectivity to the mucosa versus the underlying muscle which is a significant result for treatment of early cancers in hollow organs where sparing of muscle is important. Importantly, no adverse skin photosensitivity was observed for the liposomal formulations.

The silica NPs were also effective at inducing PDT in tumour. This is an important result since PDT using these nanocarriers has not previously been studied *in vivo*. Likewise the PLGA formulations

were effective for PDT *in vivo*. The fact that the silica NPs are less effective for killing cells *in vitro* may be due to different uptake mechanisms *in vivo*. For example unlike *in vitro* studies, damage to the microvasculature is important *in vivo* and nanocarriers due to their size would be expected to photosensitise the microvasculature.

POTENTIAL IMPACT

The major objective of NANOPHOTO was the delivery of a nanosystem-based formulation of mTHPC able to carry the drug into tumour tissue with a selectivity higher than Foscan®, i.e. the presently used mTHPC formulation. mTHPC is a very powerful photosensitising agent but it is also a fluorescent molecule. Therefore, at least in principle mTHPC can be used to kill cancer cells and to visualize them by fluorescence provided that mTHPC is taken up in sufficient amount and a suitable fluorescence imager is available.

At present PDT with Foscan (or other photosensitising agents) is efficient in the treatment of selected types of cancers in advanced stages. There are several reasons that may determine the limited clinical use of PDT. Among these the general skin photosensitivity arising after the administration of the drug. Such photosensitivity causes some discomfort in the patient for a more or less prolonged period because exposure to intense natural or artificial light sources must be avoided. This is the major side effects in patients undergoing Foscan PDT and is caused by the accumulation of relevant concentration of mTHPC not only in the tumour but also in healthy tissues. For the economic point of view the fact is important that the present formulation of Foscan needs a relatively long drug-light interval of 4 days which translates into a long hospital stay of the patients undergoing to the PDT treatment.

The work carried out during the project demonstrated that all the studied nanocarriers are potentially useful for the delivery of mTHPC to tumours. It has been found that dark toxicity of mTHPC is substantially reduced with the nanocarrier formulations and all nanocarriers are able to deliver sufficient amount of mTHPC to tumour and induce a PDT effect with illumination. However, mTHPC liposomal formulations appear particularly promising because of several important features that can improve substantially PDT with respect to the standard Foscan. Detailed investigations on the biodistribution and accumulation of mTHPC in various tissues of normal and tumour-bearing rats showed that liposomes improve tumour selectivity in comparison to Foscan. In the tumour the uptake of mTHPC in liposomes was at least three times that with Foscan and maximal tumour necrosis was induced with a liposomal mTHPC dose less than 20% the original clinical dose of mTHPC in standard formulation. The data showed also that the maximal tumour to skin ratio was attained at less than 24 h after administration and therefore a shorter drug light interval can be adopted with respect to Foscan. From the clinical point of view the main advantage of increased uptake and selectivity of liposomal mTHPC into tumour tissue is the reduction in damage to surrounding normal tissues. The enhanced efficacy of PDT to tumour administering lower doses of drug overcome or minimisation of skin photosensitivity.

Therefore mTHPC in liposomes is proposed as new drug formulation and should replace the formulation Foscan used at present in clinics. With the liposomal formulation, PDT should become a highly selective and more effective treatment. This favourably affects the outcome of PDT since the administered drug dose can be probably reduced eliminating or minimising the major side effect of the treatment, namely, the general skin photosensitivity.

Minimally invasive PDT treatments guaranteeing a high efficacy and selectivity have the potential to become a first-line treatment for primary localised tumours due to the excellent healing and preservation of function following PDT, in contrast to surgery, and without the debilitating side-effects of chemotherapy. Repeated treatments using PDT can be carried out safely unlike for example radiotherapy of head and neck tumours where bone fibrosis is a major risk factor. With the newly formulated liposomal mTHPC the expected impact on patient quality of life is therefore extremely positive. Moreover the time of hospitalisation can be relatively short and, in the future, PDT could ideally be performed when appropriate without the need for an overnight stay. NANOPHOTO has identified a delivery system for mTHPC that will minimise time spent in hospital which will be both to the patients' benefit and reduce the economic costs of cancer treatment

Nanoparticulate delivery systems developed within this project for active targeting of malignant tumours with mTHPC, can be used for delivery of other anti-tumour drugs. Protease inhibitors are promising candidates to impair tumour growth and invasiveness due to the active role of proteolytic enzymes in these processes. PLGA NPs labelled with anticytokeratin antibody and loaded with cysteine protease inhibitor cystatin have been shown to specifically recognize tumour cells and deliver inhibitor cargo to endosomes/lysosomes, which are the major source of harmful intracellular proteolytic activity (Kos et al.,. Int. J. Pharmaceutics, 2009, 381, 106-112). Besides protein inhibitors small synthetic inhibitors, specifically recognizing the active site of the particular protease, provide another possibility for cancer treatment (Mirkovic et al., ChemMedChem 2011, 6, 1351 – 1356)

Dissemination activities

The results produced during the NANOPHOTO project were disseminated with publications in peer reviewed international scientific journals of high impact factors and with presentations at scientific meetings and conferences.

Publications in scientific journals.
Because of the multidisciplinary work carried out during NANOPHOTO the results produced were published in diverse journals specialized in nanotechnology, drug delivery, nanomedicine, photobiology and material chemistry. The targeted journal was selected based on the specific type of results to be published. Nine papers are already published, one is in press and one was submitted. In addition four papers are in preparation and will be submitted in due course.

Presentations at scientific meetings and conferences
The results produced during NANOPHOTO were presented in the form of oral communications or posters at various national and international scientific meetings. The meetings were in the fields of photomedicine, nanotechnology, oncology, photobiology, chemistry. The participation at these meetings disseminated the knowledge gained during NANOPHOTO among scientists working in diverse disciplines ranging from chemistry to biology and medicine.
Fifteen oral communications were given and 9 posters were presented.

During the 6th Conference on Experimental and Translational Oncology, held in Kranjska Gora (Slovenia) on 24-28 March, 2010 and co-organised by Prof. Janko Kos a half session was dedicated to the presentation of the project results by team members of the consortium.
In the frame of the 14th Congress of the European Society for Photobiology, held in Geneva on 1-6 September 2011, the coordinator Elena Reddi and the team leader of UCL Alexander J. MacRobert organised the Symposium on "Nanotechnology: from photochemistry to photomedicine" that was

mainly devoted to presentations given by members of the Nanophoto Consortium. This was an excellent opportunity to present the most relevant project results to a large international audience.

In Padova and Ljubljana the project has been presented to public in several reports in local newspapers.

Exploitation of the results

The exploitation of the NANOPHOTO results was one of the major tasks assigned to the SME involved. Based on the fact that encapsulation into liposomal nanoparticles makes mTHPC more tolerable for the patient and, compared to the formulation in organic solvents (Foscan), shows an improved bioavailability the SME biolitec will do a lot of effort to identify possible contract manufacturing organizations for the liposomal formulation to start the drug approval procedure for the European countries.
Biolitec has already contacted about 30 possible contract manufacturing organizations (CMOs) in 8 different countries and selected one company to enter contract negotiations. Together with this company, Biolitec will establish the manufacturing process for the CMO-facility, including identifying a possible extrusion device for the main manufacturing step. Furthermore, Biolitec will establish the time schedules and specifications for the stability testing of the GMP-material and the organization of the clinical trials (first phases.)
In conclusion, Biolitec will take the following activities to enter the production/approval phase for a liposomal mTHPC-based formulation:

- Upscaling the manufacturing process to be able to produce larger batches
- Identifying a suitable device for the production of liposomal mTHPC under GMP-conditions
- Starting the scheduling of clinical trials with the liposomal formulation (Phase I and IIa)

The Consortium

The following institutions formed the Consortium:

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http://www.bio.unipd.it/nanophoto/