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PAPER

Meta-tetra(hydroxyphenyl)chlorin-loaded liposomes sterically stabilised with poly(ethylene glycol) of different length and density: characterisation, *in vitro* cellular uptake and phototoxicity

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We studied the effects of density and thickness of PEG coating on *in vitro* cellular uptake, and dark- and photo-toxicity of liposomal formulations (Fospeg) of the photodynamic agent *meta*-tetrahydroxyphenyl chlorin (*m*-THPC). The cellular uptake of various Fospeg formulations was determined by flow cytometry in CCD-34Lu human normal fibroblasts and A549 lung cancer cells. Dark and light-induced cytotoxicity was measured by MTS assay after exposure to increasing concentrations of Fospeg only and followed by irradiation with red light. Intracellular localization of *m*-THPC delivered by Fospeg was determined by fluorescence microscopy. The studies were carried out in comparison with *m*-THPC delivered by the standard solvent. In the dark all Fospeg formulations were less cytotoxic than *m*-THPC in standard solvent (ethanol/poly(ethylene glycol 400/water; 20 : 30 : 50 by vol.) and cytotoxicity decreased by increasing PEGylation. *m*-THPC delivered as Fospeg was internalised by endocytosis and localised mainly in the Golgi apparatus and endoplasmic reticulum. The efficiency of cellular uptake of Fospeg was reduced by 30–40% with respect to *m*-THPC in standard solution causing a slight reduction of the phototoxicity but without serious impairment of the efficacy of the treatment. Our study suggests that PEGylated liposomes are promising nanocarriers for the delivery of photosensitisers for photodynamic therapy because they reduce dark cytotoxicity while preserving therapeutic efficacy.

Introduction

It is well established that photodynamic therapy (PDT) is a valuable method for treatment of a number of oncological, dermatological and ophthalmological diseases.[1,2] PDT is based on the light-activation of a photosensitising molecule that localises in the diseased tissue and produces reactive oxygen species (ROS), mainly singlet oxygen, to induce cell death.[3] These cytotoxic species are short lived and cannot diffuse far from the site of production, therefore the tissue damage is limited to the proximity of their site of production. PDT is becoming widely accepted and has been approved for the treatment of several types of solid tumours. *Meta*-tetra(hydroxyphenyl)chlorin (*m*-THPC, temoporfin) is one of the most potent second generation photosensitisers and is approved in Europe for the palliative treatment of patients with advanced head and neck cancers.[4,5] Clinical trials have shown that basal cell carcinomas,[6] pancreatic[7] and prostate cancers[8] can also be treated successfully with PDT. While the clinical benefit of PDT has been demonstrated, various strategies are being considered to optimise its efficacy and selectivity. In the past, various delivery systems have been proposed to overcome the problems related to the administration of the hydrophobic photosensitisers[9] and in this context liposomes were particularly useful for their capacity to solubilise such molecules in their phospholipid bilayer. The major draw-back of these conventional liposomes is the rapid clearance from plasma caused by the recognition and uptake in the phagocytic cells of the reticulo-endothelial system (RES) following systemic administration.[10] Stealth or long-circulating liposomes, having the surface coated with a hydrophilic polymer, which prevents the adsorption of blood plasma opsonins and recognition by macrophages, appear to be promising delivery systems for targeting the tumour, passively taking advantage of the enhanced permeability and retention (EPR) effect.[11,12] It is expected that hydrophobic photosensitisers can be administered in the form of highly photoactive monomers by using stealth liposomes as delivery vehicles, which should also lead to more efficient and selective targeting of the tumour. Hopefully, the increased and

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selective accumulation of the photosensitiser in the target tissue should allow the administration of lower doses of drug with fewer side effects, in particular less skin photosensitivity.

The method largely used for the production of stealth nanoparticles and liposomes is based on the coating of their surface with poly(ethylene glycol) (PEG), which is inert and biocompatible.[13] Pre-clinical studies using the chick chorioallantoic membrane model showed that *m*-THPC encapsulated in PEGylated liposomes (Fospeg) exhibited a rate of extravasation similar to that of conventional liposomes (Foslip) but the phototrombic activity was much higher, making the Fospeg a suitable formulation for the treatment of choroidal neovascularisation associated with age-related macular degeneration.[14] PEGylated liposomal *m*-THPC was also proven to be suitable for the PDT treatment of rheumatoid arthritis, due to more favourable accumulation in the inflamed arthritic joints in comparison to *m*-THPC in standard solvent or non-PEGylated liposomes.[15] Buchholz *et al.*[16] reported that the plasma concentration and bioavailability were approx. 3 times higher with the PEGylated liposomes compared to the standard formulation of *m*-THPC. The superior pharmacokinetic properties resulted in a more favourable response to PDT of feline squamous carcinomas. Thus, it appears that PEGylated liposomes may be a useful tool to increase the therapeutic response to PDT in various pathological situations. In this context, we felt it was important to establish how the density and thickness of the PEG corona could affect the stability of the *m*-THPC liposomal formulation and its ability to deliver the photosensitizer to cultured cells. In this paper, we report the characterisation of several liposomal formulations of *m*-THPC (Fospeg) in which the PEG length and density was varied. Furthermore the efficiency of uptake of various Fospeg formulations in normal and cancer cells, as well as cytotoxicity in the dark and after irradiation with red light are reported in comparison with *m*-THPC delivered to the cells in the standard solvent.

Experimental

Materials

Rhodamine–DPPE [1,2-dipalmitoyl-*sn*-*glycero*-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl)] was purchased from Avanti Polar Lipids (Alabaster, USA). DPPC [1,2-Dipalmitoyl-*sn*-*glycero*-3-phosphocholine]; DPPG [1,2-dipalmitoyl-*sn*-*glycero*-3-phosphoglycerol]; mPEG-750-DSPE [*N*-(carbonyl-methoxypolyethyleneglycol 750)-1,2 distearoyl-*sn*-*glycero*-3-phosphoethanolamine]; mPEG-2000-DSPE [*N*-(carbonyl-methoxypolyethyleneglycol 2000)-1,2 distearoyl-*sn*-*glycero*-3-phosphoethanolamine]; and mPEG-5000-DSPE [*N*-(carbonyl-methoxypolyethyleneglycol 5000)-1,2 distearoyl-*sn*-*glycero*-3-phosphoethanolamine] were purchased from Genzyme Pharmaceuticals (Liestal, Switzerland). Temoporfin (*m*-THPC) [3,3′,3″,3‴-(2,3-dihydroporphyrin-5,10,15,20-tetrayl)tetraphenol] was provided by Biolitec AG (Jena, Germany). Rhodamine 123 (R123), *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)sphingosine (BODIPY® FL C_5-ceramide), LysoTracker Green DND-26 and ER-Tracker™ Green (glibenclamide BODIPY® FL) were purchased from Invitrogen Molecular Probes (Milan, Italy). Bicinchoninic acid (BCA) assay was from Pierce (Rockford, IL). The CellTiter 96® Aqueous One Solution Cell proliferation Assay (MTS) was from Promega Co (Madison, WI, USA). Genistein, filipin III, chlorpromazine hydrochloride, amiloride, 5-(*N*,*N*-dimethyl)-hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). Solvents and commercially available reagents were used as received.

Preparation and characterization of *m*-THPC liposomal formulations (Fospeg). Liposomes were prepared by the conventional film method. *m*-THPC was dissolved in an organic solution of phospholipids (chloroform/methanol). This mixture was dried to a thin film at 50 °C using the rotary evaporator. The obtained film was kept under vacuum (1 mbar) for 2 h at room temperature and afterwards flushed with nitrogen. The film was then hydrated with 10 mM histidine buffer pH 6.5 with 5% glucose for 30 min. Afterwards the liposome dispersion was extruded through polycarbonate membranes of different pore sizes (400 nm, 200 nm, 100 nm). Liposomal size was measured by Photon Correlation Spectroscopy (PCS) with a Zetasizer Nano S90 from Malvern Instruments GmbH (Herrenberg, Germany). *m*-THPC concentration was determined by UV-Vis spectroscopy. In Table 1, we report the list and composition of the Fospeg preparations considered in this study.

For the photophysical studies, Fospeg 2%-2000 and Fospeg 8%-2000 stock solutions were diluted in 10 mM histidine buffer, containing 5% glucose at pH 6.5, to give a 0.5 μM *m*-THPC concentration. The *m*-THPC loading was the same for both formulations with a dye : lipid ratio of 13.3. Comparative studies were carried out in dimethyl sulfoxide (DMSO, Sigma–Aldrich, UK) at the same *m*-THPC concentration.

Cell cultures. The cell line A549, derived from human lung carcinoma, and the human normal lung fibroblasts CCD-34Lu were obtained from American Type Culture Collection (ATCC, Rockville, USA). The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. A549 cells were cultured in F-12 K medium supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Gibco, Invitrogen, Milan, Italy),

Table 1 Composition of the Fospeg liposomal formulations

Formulation	DPPC	DPPG	mPEG-750-DSPE	mPEG-2000-DSPE	mPEG-5000-DSPE	*m*-THPC
Fospeg 2%-2000	18.0	2.0	—	2.0	—	1.5
Fospeg 8%-750	18.0	2.0	3.7	—	—	1.5
Fospeg 8%-2000	18.0	2.0	—	6.8	—	1.5
Fospeg 8%-5000	18.0	2.0	—	—	14.1	1.5

Values are in mg ml^{-1} of the preparation after extrusion. Watery phase in all formulations: 10 mM histidine buffer with 5% glucose. The concentrations of mPEG-DSPE correspond to 2 or 8 mol% of total phospholipids.

2 mM L-glutamine, 2.5 g l^{-1} sodium bicarbonate, 38 units ml^{-1} streptomycin and 100 units ml^{-1} penicillin G (Sigma-Aldrich). CCD-34Lu cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 3.7 g l^{-1} sodium bicarbonate, 4.5 g l^{-1} glucose, and supplemented with 38 units ml^{-1} streptomycin and 100 units ml^{-1} penicillin G, 0.1 mM MEM non essential amino acids, 0.02 M HEPES and 10% FBS. A549 and CCD-34Lu cells have a doubling time of 22 and 24 h, respectively.

Photophysical characterisation

Absorption spectra were measured using a Perkin–Elmer Lambda 25 UV/Vis spectrometer (Perkin–Elmer, Beaconsfield, UK) with quartz cuvettes. Fluorescence emission spectra were measured using a LS50B Perkin–Elmer spectrofluorimeter (Perkin–Elmer, Beaconsfield, UK). Spectra were acquired using a multimode bifurcated fibre-optic probe to provide front surface excitation/detection geometry, which is unaffected by polarization effects, unlike the conventional orthogonal excitation/detection configuration. Fluorescence lifetimes were measured using the time-correlated single photon counting method (TCSPC). For the fluorescence studies, dilute solutions were employed with an *m*-THPC concentration of 0.5 µM. The light source for TCSPC was a 405 nm picosecond laser diode (EPL-405, Edinburgh Instruments Ltd., Livingston, UK) with a pulse duration of 90 picoseconds, and a 5 MHz repetition rate. Fluorescence was detected using a fast multialkali photomultiplier module (model H5773-04, Hamamatsu Photonics UK Ltd, Hertfordshire, UK) *via* a longpass filter (OG510, Schott, Stafford, UK) and a monochromator (model M300, Bentham Instrument Ltd, Berkshire, UK). A Lyot depolarizer (Thorlabs Ltd, Ely, UK) was used to minimise any fluorescence polarization effects. TCSPC was carried out using a PC-mounted board (TimeHarp100, PicoQuant GmbH, Berlin, Germany) and lifetimes were derived using FluoFit software (PicoQuant GmbH, Berlin, Germany). The Instrument Response Function (IRF) was obtained from a non-fluorescent scattering Ludox® solution (Sigmal–Aldrich, Gillingham, UK). Optimum fitting with minimisation of the residuals was confirmed using a chi-squared value $\chi^2 < 1.4$.

Dark and photo toxicity of *m*-THPC delivered by standard solvent or by Fospeg

For the dark cytotoxicity experiments A549 and CCD-34Lu cells were seeded in 96-well plates (3000 cells $well^{-1}$) in 200 µl of culture medium (F-12 K and DMEM, respectively) supplemented with 10% FBS. After 24 h the medium was removed and replaced with 150 µl of fresh medium containing 3% FBS and increasing concentrations of *m*-THPC delivered in free form by standard solvent (ethanol/poly(ethylene glycol) 400/water; 20:30 : 50, by vol.) or by liposomal formulations (Fospeg). Before the addition to the cells, Fospeg was diluted 1 : 10 in sterile water and then 1 : 2 in culture medium added with 3% FBS. The cells were incubated in the dark and cell viability was measured with MTS test after 24 h of treatment (24 h) as well as after 24 h of treatment followed by additional 24 h in which the cells were kept in *m*-THPC-free medium containing 10% FBS (24 + 24 h). For MTS assay the cell medium was replaced with 100 µl of serum-free medium and 20 µl of CellTiter 96® Reagent and the wells were incubated for 1.5 h at 37 °C. The absorbance at 492 nm was measured with Biotrak II (Amersham, GE Healthcare, NJ, USA) plate reader and the viability of treated cells was expressed as percentage of the absorbance of control cells that was taken as 100% viability.

The *in vitro* phototoxic effect of *m*-THPC delivered by standard solvent or Fospeg was evaluated in A549 cells. The cells were seeded in 96-well plates and incubated for 24 h at 37 °C in the dark with increasing and non toxic concentrations of *m*-THPC (0.25–1.5 µM) as determined with the experiments of dark toxicity. At the end of the incubation time, cells were washed twice with 150 µl of PBS with Ca^{2+} and Mg^{2+}, and irradiated in PBS with 0.24 J cm^{-2} of red light (600–700 nm) emitted from a Waldmann PDT 1200 lamp (Waldmann Medizintechnik, Germany). The fluence rate at level of the cell monolayer was 12 mW cm^{-2}, as measured with the radiometer IL 1700 (International Light, Newburyport, MA). Immediately after irradiation the cells were brought back to the incubator after replacement of PBS with fresh medium containing 10% FBS. Cell viability was measured with the MTS test after additional 24 h.

Cellular uptake of *m*-THPC delivered by standard solvent or Fospeg

A549 or CCD-34Lu cells (10^5) were seeded in 2 ml of complete medium in 35 mm diameter tissue culture dishes. After 24 h, the cells were incubated with increasing concentrations of *m*-THPC (0.25–1.75 µM), delivered by standard solvent or Fospeg, in culture medium supplemented with 3% FBS. After 24 h of incubation with *m*-THPC the cells were washed twice with 2 ml of versene, detached with 500 µl of trypsin (Gibco) that was neutralized with the addition of 200 µl of FBS. Cells were centrifuged and resuspended in 700 µl of versene before measuring *m*-THPC fluorescence by flow cytometry with a BD FACSCanto™ II (Becton Dickinson, San Jose, California, USA) instrument. The blue laser at 488 nm was used as the excitation source and wavelengths longer than 670 nm (PerCP channel) were used for the detection of the *m*-THPC fluorescence. 10^5 events $sample^{-1}$ were acquired and analyzed with the FACSDiva Software. The uptake of *m*-THPC in A549 cells was also determined as a function of the incubation time, up to 24 h, with 1 µM *m*-THPC in standard solvent or Fospeg 8%-2000. The uptake of Fospeg 8%-2000 (1.5 µM *m*-THPC) was also measured after 2 h of incubation in the presence of 3 and 10% FBS or without FBS.

To study the effect of the incubation temperature on the uptake of 1 µM *m*-THPC in standard solvent and Fospeg 8%-2000, A549 cells were incubated at 4 °C and 37 °C and then analyzed by flow cytometry. Furthermore, cell samples were preincubated (30 min) with selected inhibitors of endocytosis before the addition of *m*-THPC to the culture medium. The inhibitors tested were: chlorpromazine hydrochloride (10 µg ml^{-1}), filipin III (5 µg ml^{-1}), amiloride, 5-(*N*,*N*-dimethyl)-hydrochloride (34 µg ml^{-1}) and genistein (54 µg ml^{-1}). For these experiments the cells were incubated with *m*-THPC for 2 h.

Quantification of cellular uptake of *m*-THPC

For selected concentrations of *m*-THPC in standard solvent and Fospeg 8%-2000, the data of cellular uptake measured by flow cytometry were validated with the traditional chemical extraction

method. After 24 h incubation with 0.5 or 1 μM *m*-THPC, A549 cells were washed twice with 2 ml of PBS with Ca^{2+} and Mg^{2+} and lysed with 800 μl of 2% sodium-dodecyl sulfate (SDS). Cell lysates were kept under magnetic stirring for 1 h before measuring the *m*-THPC fluorescence with a fluorescence spectrophotometer (Cary Eclipse). The intensity of the fluorescence emission spectrum of *m*-THPC in the 600–800 nm range ($\lambda_{max\ em} = 652$ nm) was registered after excitation with 420 nm light. The *m*-THPC concentrations in the cell lysates was calculated from calibration plots built up with known *m*-THPC concentrations. The solutions for the calibration plot were prepared by adding known amounts of *m*-THPC from stocks in standard solution or in Fospeg to aliquots of lysate obtained from cells not incubated with the photosensitizer and containing the same amounts of cellular protein as the samples. The protein content of the cell lysates was measured by the bicinchoninic acid (BCA) assay and was used to express the *m*-THPC uptake in cells as nmoles of *m*-THPC mg^{-1} of proteins.

Fluorescence microscopy

The intracellular localization of *m*-THPC in A549 cells was determined by fluorescence microscopy taking advantage of its red fluorescence. 10^5 cells were seeded in complete medium in 35 mm diameter tissue culture dishes containing a glass coverslip. After 24 h, the cells were incubated at 37 °C for 5 h with fresh F-12 K medium supplemented with 3% FBS and 1.5 μM *m*-THPC delivered by standard solvent or Fospeg 8%-2000. The intracellular localization of the PEGylated liposomes was determined in cells incubated with liposomes with the same composition as Fospeg 8%-2000 and labeled with the fluorescent phospholipid rhodamine–DPPE. At the end of the incubation time, the cells were washed twice with 2 ml of PBS and observed with the Olympus IMT-2 microscope equipped with a refrigerated CCD camera (Micromax, Princeton Instruments) and a 75 W xenon lamp. Fluorescence images obtained with 60× 1.4 NA oil immersion objective (Olympus) were acquired and analyzed with the imaging software Metamorph (Universal Imaging). The cellular distribution of the fluorescence of *m*-THPC or rhodamine-DPPE was compared with that of R123, LysoTracker Green DND-26, BODIPY® FL C_5-ceramide and ER-Tracker™ Green, used as markers for mitochondria, lysosomes, Golgi apparatus and endoplasmic reticulum, respectively. R123 (0.2 μM), Lysotracker Green (75 nM), BODIPY® FL C_5-ceramide (5 μM) and ER-Tracker™ Green (1 μM) were added to the cell monolayers 15 min before completing the incubation with *m*-THPC or fluorescent liposomes. For the *m*-THPC fluorescence detection a set of filters with 400 nm excitation and 620 nm emission were used, whereas 475 nm excitation and 520 nm emission were used for the other probes.

Results

Characterisation of Fospeg formulations

Each liposomal formulation used during this study was prepared with the same method and identical devices. The composition of each formulation as determined after extrusion is reported in Table 1.

To evaluate particle size and storage stability, size measurements were carried out after extrusion and after storage at 25 °C for 6 months. Directly after extrusion, the mean particle size (PCS *z*-average) of the liposomes was between 105 and 125 nm. Polydispersity indices (PDI) were between 0.04 and 0.15 in all cases, indicating narrow size distributions.

The liposomes have been found to be stable with respect to size and optical appearance over the storage time of 6 months. A small decrease in size (about 5%) was only detected in liposomes containing PEG 2000 or 5000, whereas the PEG 750 preparations have been found to be completely stable in size. Further, no drug precipitates or aggregates were observed in light microscopy.

Fluorescence spectroscopic studies

The fluorescence emission spectra and lifetimes were recorded for the liposomes at 2 and 8% PEGylation and identical *m*-THPC loading diluted in buffer. Peak emission using 423 nm excitation was recorded at 652 nm in each case but a higher peak intensity was noted for the 8% PEGylation: the ratio of fluorescence intensities for the solutions containing 8 *vs.* 2% PEGylated liposomes was 1.25. Peak absorption in the Soret band was at 423 nm for both liposomes.

In DMSO, a mono-exponential decay was observed with a lifetime of 9.6 ns. In liposomes, multi-exponential decays with significantly shorter lifetimes were observed for the PEGylated liposomes. Using tri-exponential fitting, both 2% and 8% PEGylated liposomes exhibited long lived lifetime components of 7.3 and 9.8 ns, respectively but the pre-exponential factor (normalized to unity) was significantly larger at 0.22 for 8% compared with 0.02 for 2% PEGylation. For a mono-exponential decay, the pre-exponential factor or effectively the 'weighting' is unity, whereas for multi-exponential decays the sum of various pre-exponential factors is unity. Therefore pre-exponential factors of 0.22 and 0.02 for these longer lifetimes show that they are minor components in the decay profile. Both liposomes exhibited two similar short-lived lifetime components with comparable pre-exponential factors: for 2% PEGylation, 2.5 and 0.9 ns with corresponding pre-exponential factors of 0.53 and 0.45, and for 8% PEGylation, 3.0 and 1.1 ns with pre-exponential factors of 0.35 and 0.43.

Dark cytotoxicity of Fospeg *vs.* *m*-THPC in standard solvent

The Fospeg formulations listed in Table 1 were used to evaluate the cytotoxicity of liposomal *m*-THPC in the dark toward the A549 tumour cells and the CCD-34Lu normal fibroblasts in comparison to *m*-THPC in standard solvent. The cells were incubated with concentrations of *m*-THPC up to 5 μM for 24 h. The MTS test showed that all formulations were non-toxic to the cells with *m*-THPC concentrations lower than 1 μM. However, higher concentrations of *m*-THPC caused a reduction of the cell viability that was dependent on the concentration and the formulation. Five micromolar *m*-THPC in standard solvent reduced the viability of both cell lines to about 20% (Fig. 1, A and C) at 24 h and close to zero, especially in CCD-34Lu, at 24 + 24 h (Fig. 1, B and D). All liposomal formulations were less toxic than the *m*-THPC standard and the decreased toxicity was dependent on the degree of PEGylation. Fospeg 2%-2000 reduced the viability of A549 cells to about 70% at 24 and 24 + 24 h and to about 30% in

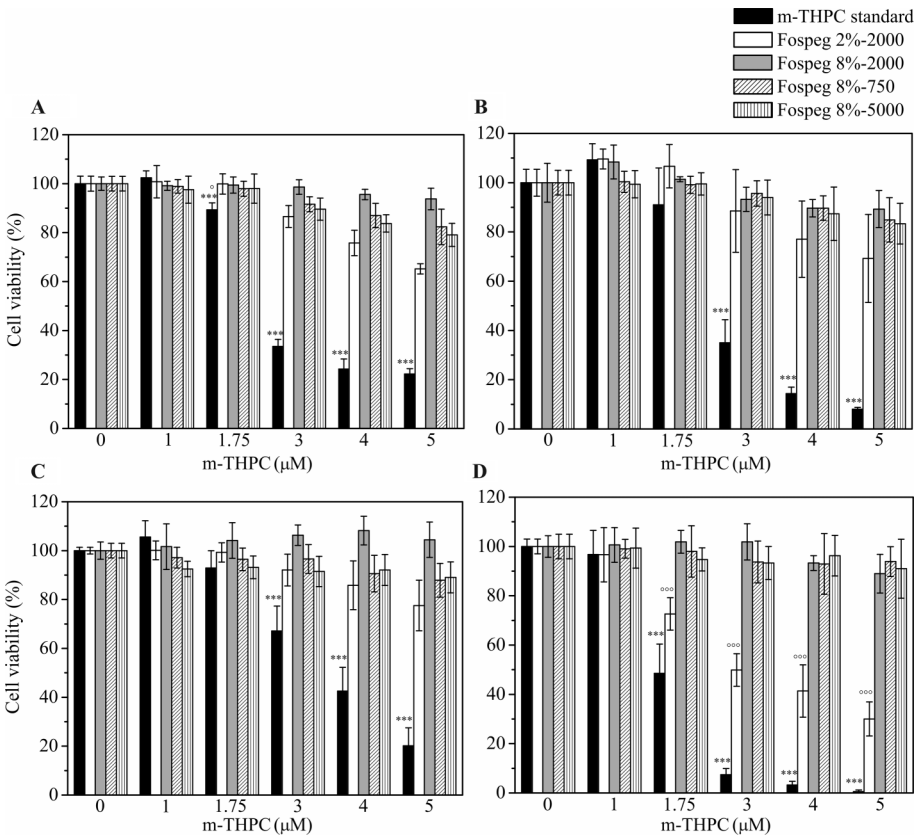

Fig. 1 Viability of A549 (A, B) and CCD-34Lu (C, D) cells after exposure to *m*-THPC standard formulation and different Fospeg formulations. Cell viability was measured by MTS assay at the end of 24 h of incubation in the dark (24 h) with increasing concentrations of *m*-THPC, delivered by standard formulation or Fospeg (A, C), and 24 h after the release in *m*-THPC-free complete medium (24 + 24 h) (B, D). Data are presented as means ± S.D. (n = 9) from three independent triplicate experiments. A and B: °, $p < 0.01$ *vs.* Fospeg 8%-5000; ***, $p < 0.001$ *vs.* Fospeg 2%-2000, 8%-2000, -750, -5000. C and D: °°°, $p < 0.001$ *vs.* Fospeg 8%-2000, -750, -5000; ***, $p < 0.001$ Fospeg 2%-2000, 8%-2000, -750, -5000 (Student t test).

CCD-34Lu fibroblasts. Cell toxicity was abolished when PEGylation was increased to 8% with PEG ranging from 750 to 5000.

Cellular uptake of Fospeg *vs.* *m*-THPC in standard solvent

The uptake of *m*-THPC delivered as Fospeg in comparison to standard formulation was determined in A549 cells and CCD-34Lu fibroblasts after 24 h of incubation in medium with 3% FCS. The uptake studies were performed with *m*-THPC concentrations up to 1.75 μM because below this concentration none of the formulations showed dark toxicity at 24 h (Fig. 2). The relative amount of *m*-THPC taken up by the cells was determined by flow cytometry. The data showed less uptake (about 30%) of *m*-THPC in the CCD-34Lu in comparison to A549 cells with all formulations. In both cell lines the uptake of *m*-THPC delivered with the Fospeg formulations reached or approached a plateau as with Fospeg 2% 2000, at concentrations above 1.5 μM while the uptake steadily increased with the standard formulation. This difference could suggest a different modality of *m*-THPC uptake with the standard formulation in comparison to Fospeg or the presence of scarcely fluorescent aggregates of *m*-THPC in the Fospeg formulations with the consequent underestimate of its intracellular concentration when measured by flow cytometry. To rule out the latter possibility, in selected samples of cells incubated with Foscan or Fospeg, cell lysates were analysed by spectrofluorimetry to determine the intracellular concentration of *m*-THPC. The results (Table 2) confirmed that, especially at the high concentrations, *m*-THPC standard formulation is taken up more efficiently than Fospeg.

In A549 cells, we measured the time-dependent uptake of *m*-THPC standard formulation and Fospeg 8%-2000 and found that uptake of *m*-THPC in standard solvent steadily increased with time while with Fospeg was biphasic. At short times the rate of uptake of Fospeg was lower than that of free *m*-THPC standard

Table 2 Uptake in A549 cells of 0.5 and 1 μM *m*-THPC delivered with standard formulation, Fospeg 2%-2000 and Fospeg 8%-2000 quantified by the chemical extraction method

	nmol *m*-THPC/mg cell proteins	
Formulation	*m*-THPC 0.5 μM	*m*-THPC 1 μM
Standard	0.62 ± 0.02	1.52 ± 0.27
Fospeg 2%-2000	0.46 ± 0.01	0.90 ± 0.02
Fospeg 8%-2000	0.52 ± 0.09	0.94 ± 0.18

The cellular uptake was determined after 24 h of incubation at 37 °C in culture medium containing 3% FBS and expressed as nmoles of *m*-THPC mg^{-1} of cell proteins. The data represent means ±S.D. from three independent experiments.

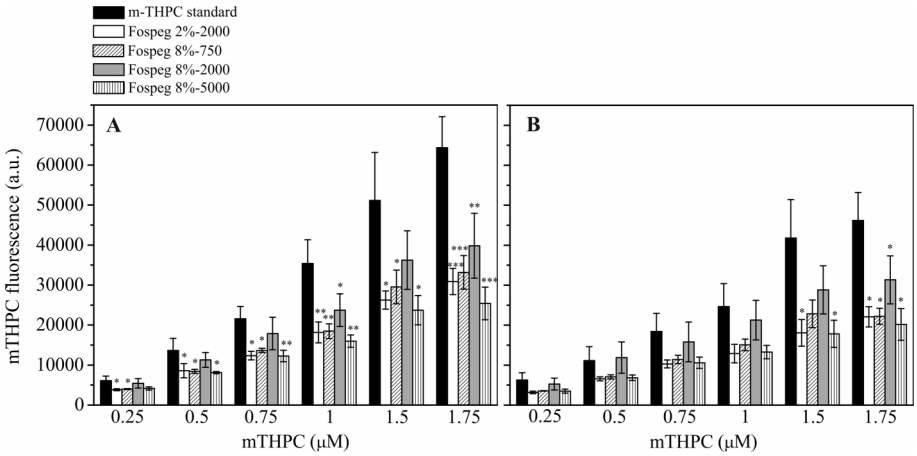

Fig. 2 Cellular uptake of increasing concentrations of *m*-THPC delivered by standard formulation or Fospeg in A549 (A) and CCD-34Lu (B) cells. Flow cytometry was used for measuring *m*-THPC fluorescence signals after 24 h of incubation. Data are presented as means ± S.D. ($n = 3$) from three independent experiments. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$ compared to *m*-THPC in standard solvent (Student t test).

formulation and a plateau could be detected around 7 h, but later the uptake raised again with no sign of plateau up to 24 h (Fig. 3).

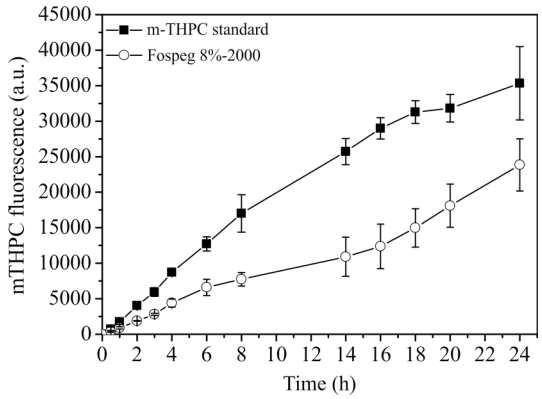

Fig. 3 Time-dependent uptake in A549 cells of 1 μM *m*-THPC in standard solvent or Fospeg 8%-2000. The uptake was measured by flow cytometry at various times up to 24 h of incubation. Data are presented as means ± S.D. from three independent experiments.

In the absence of FBS in the incubation medium the uptake of Fospeg 8%-2000 was about twice that in the presence of 3% FBS and about three times higher than with 10% FBS (Table 3).

The uptake of Fospeg 8%-2000 and free *m*-THPC was inhibited by about 95% by lowering the temperature from 37 °C to 4 °C during the incubation, suggesting that the internalisation of both formulations occurred *via* endocytosis. The addition of 10 μg ml^{-1} of chlorpromazine, an inhibitor of the clathrin-mediated endocytosis, to the cell medium 30 min before starting the incubation, reduced the uptake of free *m*-THPC and Fospeg 8%-2000 by about 30 and 20%, respectively. On the contrary, the addition of filipin III or genistein, used as inhibitors of the caveolae-mediated endocytosis, and amiloride, an inhibitor of macropinocytosis, did not reduce the uptake of free *m*-THPC or Fospeg 8%-2000 in A549 carcinoma cells (data not shown).

Table 3 Uptake in A549 cells of *m*-THPC (1.5 μM) delivered as Fospeg 8%-20000 in absence and presence of 3 and 10% FBS

FBS (%)	*m*-THPC fluorescence (a.u.)
0	3299 ± 116
3	1776 ± 532
10	1130 ± 71

The intracellular uptake was determined by flow cytometry after 2 h of incubation at 37 °C. The data represent means ± S.D. from three independent experiments.

Fluorescence microscopy studies

Fluorescence microscopy analyses of A549 cells incubated for 5 h with 1.5 μM *m*-THPC standard formulation or Fospeg 8%-2000 showed an intracellular distribution of *m*-THPC fluorescence, which co-localised with that of BODIPY® FL C5-ceramide and ER-Tracker™ Green (Fig. 4), indicating that the Golgi apparatus and endoplasmic reticulum were the major sites of localisation of *m*-THPC. In the cells incubated with Fospeg 8%-2000 in absence of FBS, the intracellular localisation of *m*-THPC was the same as in cells incubated in presence of 3 or 10% FBS (not shown). PEGylated liposomes with the same composition as in Fospeg 8%-2000 and containing rhodamine–DPPE were used to assess the intracellular localisation of the liposomes in comparison to *m*-THPC. After 5 h of incubation at 37 °C the liposome fluorescence appeared punctuated throughout the cytoplasm and colocalised with Lysotracker Green suggesting an endosome/lysosome localisation (Fig. 5).

Phototoxicity of Fospeg formulations *vs. m*-THPC in standard solvent

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 0.25–1.5 μM *m*-THPC for 24 h were irradiated with 0.24 J cm^{-2} of red light. The dose-response curves (Fig. 6) showed that the tested Fospeg formulations had very similar phototoxic effects on A549 cells and were slightly less phototoxic than free *m*-THPC. The lower

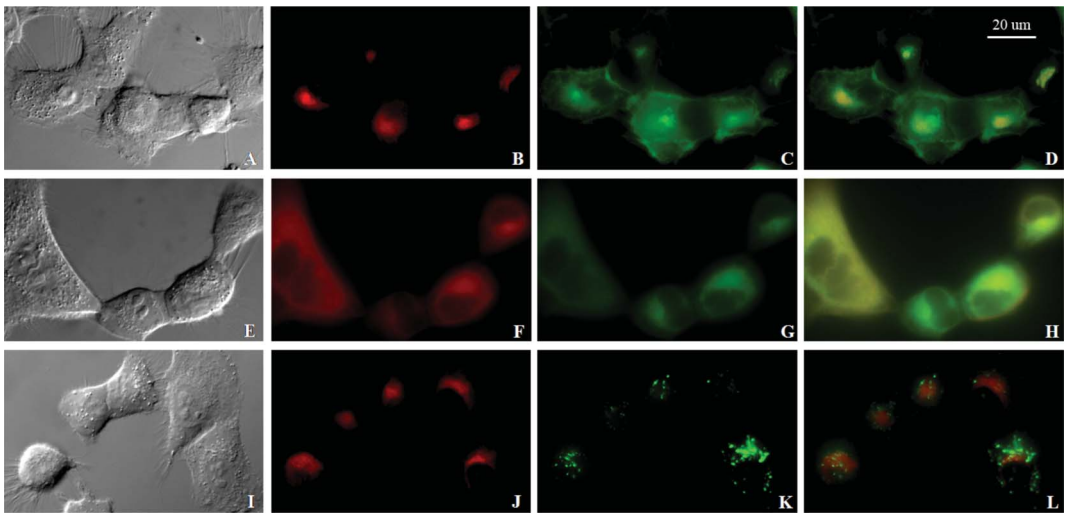

Fig. 4 Subcellular distribution of the red fluorescence of *m*-THPC (1.5 μM) delivered as Fospeg 8%-2000 (B, F and J) in A549 cells after 5 h of incubation at 37 °C. The *m*-THPC fluorescence co-localised with the green fluorescence of BODIPY® FL C_5-ceramide(C) and ER-Tracker™ Green (G), but not with LysoTracker Green DND-26 (K), used respectively as markers for the Golgi apparatus, endoplasmic reticulum and lysosomes. The Differential Interference Contrast images of cells are shown in A, E and I; the merge images for each probe are shown in D, H, and L.

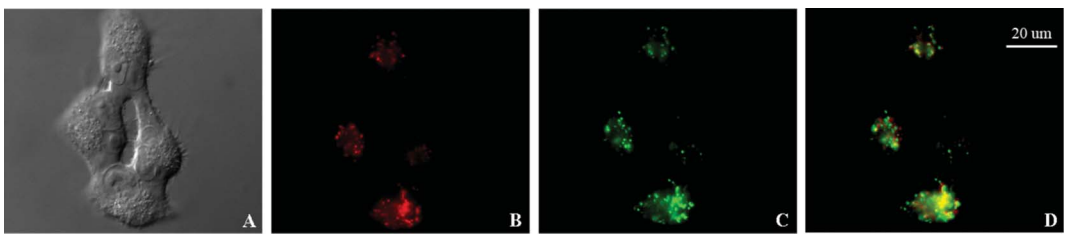

Fig. 5 Localisation in A549 cells of PEGylated liposomes with the same phospholipid composition as Fospeg 8%-2000 and labeled with rhodamine-DPPE. The punctuated red fluorescence of the rhodamine–DPPE (B) co-localised (merge image D) with that of LysoTracker Green DND-26 (C), used as marker for lysosomes. The differential interference contrast image of cells is shown in A.

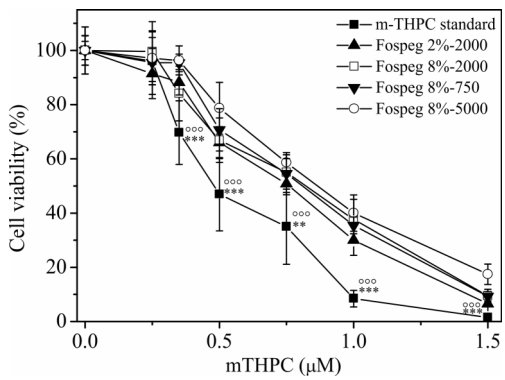

Fig. 6 Light-induced toxicity in A549 cells. Cells were irradiated with 0.24 J cm^{-2} of red light (600–700 nm) after 24 h incubation with increasing doses of *m*-THPC in standard solvent or Fospeg. Cell viability was measured 24 h post-irradiation by MTS assay and expressed as mean percentage ± S.D. (n = 12) with respect to untreated and unirradiated control cells. °°°, $p < 0.001$ *vs.* Fospeg 8%-2000 and -5000; **, $p < 0.005$; ***, $p < 0.001$ Fospeg 2%-2000 and 8%-750 (Student t test).

phototoxicity correlated with the lower uptake of the liposomal *m*-THPC in comparison to the free form.

Discussion

One of the ideal properties of a photosensitising agent for the PDT of tumours is low toxicity in the absence of light. For this reason, we have tested the dark toxicity of the Fospeg formulations towards A549 carcinoma cells and CCD-34Lu normal fibroblasts *in vitro*. We had already reported that free *m*-THPC in the standard formulation is toxic to oesophageal carcinoma cells in the dark at concentrations above 1.75 μM.[17] Here, we found a similar threshold of dark toxicity in two other cell lines and confirmed that *m*-THPC can cause cell death without irradiation at rather low concentrations. In this context, the findings reported here on the dark toxicity of Fospeg appear particularly interesting. We have found that all Fospeg formulations with 8% PEGylation did not cause any toxicity to the cells up to the *m*-THPC concentration of 5 μM (Fig. 1). On the contrary, Fospeg with a lower degree of PEGylation (2%) exhibited some dark toxicity especially to CDD-34Lu fibroblasts. In any case, the cytotoxicity of Fospeg 2%-2000 was much reduced in comparison to free *m*-THPC. Lower dark cytotoxicity of *m*-THPC liposomal formulation in comparison to Foscan was reported also by Kiesslich *et al.*, who tested Foslip, the non PEGylated liposomal formulation of *m*-THPC, on biliary tract cancer cells.[18] Similarly, Berlanda *et al.* found that a Fospeg formulation was less toxic than Foscan to epidermoid carcinoma

cells, but the characteristics of the PEG layer coating the liposome surface were not reported.[19] Our data suggest that the length of PEG chains had no influence on dark toxicity of Fospeg while the increasing density of PEG layer coating the liposomes decreased the cytotoxicity. Because of the loss of stability of the liposomal vesicles in Fospeg formulations with a degree of PEGylation higher than 8%, we could not test the effects of higher levels of PEGylation. The much lower toxicity of Fospeg in comparison to *m*-THPC in standard solvent may be attributed to multiple factors. The lower uptake of *m*-THPC in Fospeg formulation in comparison to standard solvent certainly accounts for the lower toxicity. In fact a significantly lower uptake of *m*-THPC was found in A549 and CCD-34Lu cells after 24 h of incubation with Fospeg in comparison to the standard formulation. Both flow cytometry (Fig. 2) and chemical extraction (Table 2) showed reduced uptake of Fospeg. The reduction was concentration dependent and increased by increasing concentrations of *m*-THPC. In A549 cells the uptake of Fospeg was reduced by 25–30% and about 40% at, respectively, 0.5 and 1 μM *m*-THPC. However, the lower uptake of *m*-THPC does not fully explain our dark toxicity data because the uptake of Fospeg 2%-2000 was not significantly different from that of Fospeg 8% PEG but its toxicity was higher. Thus, it appears that the increasing level of PEGylation of liposomes diminishes the dark toxicity of *m*-THPC. The different kinetics of cellular uptake of Fospeg *vs.* *m*-THPC standard formulation may also have an effect on dark toxicity of *m*-THPC. Our studies showed that the uptake of Fospeg was biphasic and slower than that of free *m*-THPC (Fig. 3), suggesting some differences in their modality of cell internalisation. Kiesslich *et al.*[18] reported that the kinetics of Foslip and extent of cellular uptake in the presence of FBS were very similar to Foscan and concluded that, in both cases *m*-THPC attached to the serum components that mediated its cellular uptake. The coating of the liposomes with a layer of PEG should limit the interaction with serum proteins and increase the probability that the photosensitiser can enter the cells while still encapsulated in the liposomes.

The fluorescence lifetime data indicate that the *m*-THPC fluorescence is strongly quenched in the liposomes, in comparison to monomeric *m*-THPC, which exhibits a longer mono-exponential lifetime of 9.6 ns in DMSO. The results are consistent with the occurrence of fluorescence self-quenching due to dimerisation in combination with energy-transfer between adjacent *m*-THPC monomers and weakly fluorescent aggregates within the liposomes. These time-resolved data are in accordance with the steady-state studies reported by Reshetov *et al.*,[20] who observed lower fluorescence yields when *m*-THPC was incorporated in both PEGylated (Fospeg 2%-2000, as used here) and unPEGylated liposomes (Foslip). We also studied *m*-THPC in Fospeg 8% and observed higher fluorescence emission by a factor of 1.25 over that from the same concentration of *m*-THPC in the Fospeg 2% solution. Although the fluorescence lifetimes are multi-exponential, the higher fluorescence efficiency of *m*-THPC in Fospeg 8% appears to correlate with the higher pre-exponential factor observed for the long-lived lifetime component with Fospeg 8%.

Since *m*-THPC is water-insoluble it should reside within the liposomal membrane but it has recently been suggested that some partitioning of *m*-THPC may also occur in the PEGylated coating of the liposomes.[20] The higher degree of PEGylation for Fospeg 8% may favour more partioning of *m*-THPC to this layer. Multi-exponential fluorescence decays usually arise from the presence of aggregates as well as monomers and/or the presence of different microenvironments. In the latter case, the measured pre-exponential factors would then correlate with the relative concentrations or populations of the fluorophore in the different microenvironments. From the fluorescence lifetime analysis, the significantly higher pre-exponential factor (0.22) observed for the long-lived lifetime component (9.8 ns) for Fospeg 8% could be explained by the presence of a higher relative population of *m*-THPC dispersed within the PEGylated layer either as aggregates or more likely in monomeric form. Our studies were carried out at 25 °C, which, as noted by Reshetov *et al.*,[20] should promote more partitioning into the PEGylated layer compared to 37 °C. However, further studies using much lower *m*-THPC loading would be required before drawing any firm conclusion.

We have previously reported that PEGylation of ORMOSIL nanoparticles limits the release of the physically entrapped *m*-THPC[17] and a similar effect could be expected also for liposomes. Contrary to this hypothesis, it was found that about 40% of *m*-THPC was released more rapidly from Fospeg 2% than from Foslip and only the remaining fraction redistributed much slower.[20] We found that the time-dependent cellular uptake of Fospeg 8% is biphasic and this might be explained by the initial slow uptake of *m*-THPC still entrapped in the liposomes while the second fast wave of uptake could depend on *m*-THPC that with time escaped from liposomes and associated to serum proteins. The fluorescence microscopy studies performed with the fluorescent labelled liposomes (rhodamine–DPPE) suggest that at least some liposomes enter the cells and localise in the endosomal/lysosomal compartments, as shown by their colocalisation with LysoTracker Green (Fig. 5). Fospeg is a DPPC-based liposomal formulation with a phase transition temperature of 41 °C and liposomes are stable also in the presence of serum proteins. In this connection, Pegaz *et al.*, in their studies on the chick chorioallantoic membrane model, found that the extravasation of *m*-THPC delivered with Fospeg was strongly reduced when compared to Visudyne®, which is based on more fluid lipids, namely dimyristoylphosphatidylcholine and egg yolk phosphatidylglycerol.[14] The authors concluded that the leakage of *m*-THPC from Fospeg was slow and its transfer to serum proteins delayed. Our studies also suggest that, following liposome internalisation, *m*-THPC was promptly released and localised mainly in the Golgi apparatus and endoplasmic reticulum (Fig. 4) as reported for other types of cells.[18,21] In fact, the red fluorescence of *m*-THPC co-localized with the green fluorescence of BODIPY® FL C5-ceramide and ER-Tracker™ but not LysoTracker Green as did the fluorescence of the labelled phospholipid (Fig. 4 and 5). The images of Fig. 4 and 5 were acquired after 5 h of incubation but a very similar *m*-THPC localisation was found at shorter and longer (up to 24 h) incubation times. Some uptake experiments with Fospeg 8%-2000 were carried out in the absence of FBS and showed a very efficient uptake of *m*-THPC (Table 3). It is reasonable to assume that, because of its lipophilicity, in the absence of FBS, *m*-THPC was internalised only entrapped in the PEGylated liposomes, but, also in this case, was localised (not shown) in the Golgi apparatus and endoplasmic reticulum, confirming its release from liposomes after internalisation. The presence of FBS clearly inhibited the uptake of *m*-THPC delivered as Fospeg (Table 3),

suggesting some competition for *m*-THPC binding, interactions between liposomes and serum components, possible release of *m*-THPC to serum components and consequent cellular internalisation as serum protein complexes. With increasing incubation time, the *m*-THPC uptake mediated by serum proteins prevails over the uptake mediated by liposomes that takes place at the beginning of the incubation. In all cases the internalisation of *m*-THPC occurred *via* endocytosis because at 4 °C the process was inhibited by 95%. The reduced *m*-THPC uptake in the presence of chlorpromazine, an inhibitor of clathrin-mediated endocytosis, suggested that some *m*-THPC enters the cells with this type of endocytosis.

The A549 carcinoma cells were irradiated with red light (0.24 J cm^{-2}) following incubation with increasing concentrations of *m*-THPC in the various formulations. As expected based on the uptake of *m*-THPC delivered by the different formulations (Fig. 2), a slightly lower phototoxic effect was found with Fospeg in comparison to standard formulation (Fig. 6). The LD_{50} for free *m*-THPC was approx 0.5 μM while it was in the range of 0.75–0.85 μM for the Fospeg formulations with no significant differences among the various types of Fospeg.

Conclusions

We have shown that Fospeg formulations with a degree of PEGylation up to 8% are stable over time with respect to the size of the liposomes and the content of *m*-THPC. The *in vitro* cellular studies demonstrated that the uptake of 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 PEGylated liposome-based formulations are promising for the delivery of photosensitising drugs, such as *m*-THPC, to cancer cells. Future investigations with tumor-bearing animals will define pharmacokinetic properties, *in vivo* PDT efficacy and side effects of Fospeg with respect to *m*-THPC in standard formulation and further assess their potential benefit for clinical PDT.

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