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A comparative study of the cellular uptake, localization and phototoxicity of *meta*-tetra(hydroxyphenyl) chlorin encapsulated in surface-modified submicronic oil/water carriers in HT29 tumor cells

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Abstract

The poor selectivity of photosensitizers for tumor tissue remains a drawback in photodynamic therapy (PDT) and could be improved by adapted formulations. The cellular uptake, localization and phototoxicity of *meta*-tetra(hydroxyphenyl)chlorin (*m*THPC) encapsulated in submicronic colloidal carriers have been studied in macrophage-like J774 cells and HT 29 human adenocarcinoma cells. Nanocapsules with an external layer made of poly(D,L lactic acid) (PLA NCs), PLA grafted with polyethylene glycol (PLA-PEG NCs), PLA coated with poloxamer 188 (polox PLA NCs) and oil/water nanoemulsion (NE) have been examined. The cellular uptake by J774, as determined by microspectrofluorimetry, is reduced with *m*THPC encapsulated into surface-modified NCs — PLA-PEG and polox PLA — compared with naked PLA, indicating a possible limitation of the clearance of such carriers by the reticuloendothelial system. Encapsulation also modifies the interaction between *m*THPC and HT29 cells. Compared with the manufacturer's solution (PEG, ethanol, water), the cellular uptake is strongly reduced. However, the HT29 phototoxicity is much less affected and a protecting effect against plasma proteins is observed. Fluorescence microscopy reveals a specific punctate fluorescence pattern with PLA-PEG and polox PLA NCs in contrast to a more diffuse distribution with NE and solution, indicating that photodamage targeting could be different. These findings suggest that photosensitizers encapsulated into surface-modified nanocapsules could be a promising approach for improving PDT efficacy and this has to be confirmed in vivo. ©2000 Elsevier Science S.A. All rights reserved.

Keywords: PDT; *m*THPC; Internalization; Fluorescence spectroscopy; Surface-modified nanocapsules

1. Introduction

Photodynamic therapy (PDT) of cancer is a non-invasive treatment of small and superficial tumors that is now developed in a number of countries in several medical fields [1]. The therapy is based on the systemic administration of a tumor-localizing photosensitizer followed by illumination with light of appropriate wavelength. The resulting photodynamic reactions give rise to singlet oxygen (¹O₂) and active oxygen species that lead to tumor destruction (for review, see Refs. [2–4]).

Potentially active photosensitizers, chlorin, phthalocyanines or benzoporphyrin derivatives, have been developed. Compared with hematoporphyrin derivative (HPD) and its commercial version Photofrin[®], which were first used for

clinical applications, they exhibit an improved photodynamic activity.

A good tumor affinity of the photosensitizer is a necessary condition for effective PDT. However, most of the photosensitizers of both first and second generations used in PDT have a limited selectivity for tumor tissue [5]. Large discrepancies are found in the evaluations reported in the literature, depending on the animal model, organs and experimental assays [6–8].

The lack of significant selectivity of photosensitizers for tumors thus remains a major problem. The use of well-adapted light diffusers for the illumination of the tumoral site gives PDT some spatial selectivity, the surrounding normal tissue being mostly spared. However, one of the main side effects of PDT is the long elimination time of the photosensitizer (up to several weeks), resulting in a high cutaneous photosensitivity. Systemic toxicity has also been reported in some cases. Tumor targeting is thus highly desirable, since a

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better selectivity would allow the injected photosensitizer dose to be decreased, a general challenge in pharmacology.

Most of the photosensitizers exhibiting a good affinity for neoplastic tissues are lipophilic compounds and drugs associated with lipid-based delivery vehicles (liposomes, oil/water emulsions) (for review, see Ref. [6]). Liposomal formulations, which have been extensively used for a number of hydrophilic and lipophilic anti-cancer drugs [9], have been shown to improve the efficiency and tumoral selectivity of porphyrin derivatives incorporated in a liposome bilayer [10,11]. The use of specific carriers of tumoral cell types, such as lipoproteins or monoclonal antibodies, has also been reported [6,12].

However, a major drawback of colloidal vehicles is their rapid uptake by the reticuloendothelial system (RES), which results from the adsorption of opsonins (plasma proteins) onto the surface of the colloidal carriers. They are rapidly taken up by the cells of the immune system located mainly in the liver and spleen. A strategy has been proposed to decrease the adsorption of opsonins [13–15]. The interaction between the carrier and opsonins is of van der Waals and electrostatic origin and coating of liposomes, or more recently polymeric nanoparticles, with a long hydrophilic and flexible chain of polyethylene glycol (PEG) can thus prevent a close approach between opsonins and the encapsulated drug. It was shown that coating with grafted polymer confers some protection to liposomes, resulting in an increased circulation time by reducing the RES uptake. According to several studies, the microvascular permeability is enhanced in the tumor and a better tumoral selectivity could thus be expected from an increased circulation time of the drug [16].

Long-circulating liposomes (stealth[®] liposomes) have already been used for several photosensitizers [10,11,17]. The use of long-circulating nanoparticles of Zn phthalocyanines, which are potential PDT photosensitizers, has also been reported [18]. It has been shown that the RES uptake of Zn phthalocyanines in poly(D,L lactic acid) (PLA) nanosphere formulations was reduced by simple adsorption of polyethylene glycol (PEG) onto the surface of the nanospheres. An improvement of the PDT tumor response has also been observed [19]. Nanocapsules are submicronic reservoirs that consist of a thin external layer made of a biodegradable polymer, such as PLA, surrounding an inner oil compartment that is able to solubilize lipophilic compounds. The modification of the PLA nanocapsule surface was achieved in the same way as for nanospheres [20]. Poloxamer adsorbed onto PLA nanocapsules (NCs) has already been reported to reduce the liver uptake in mice and to enhance the tumor selectivity of encapsulated Zn phthalocyanine [21].

Recently, NCs have been prepared using PLA bearing a covalently attached PEG chain instead of an adsorbed chain. The interaction of these surface-modified NCs with J774 mouse macrophage-like cells, which have a high capacity of phagocytosis, has been studied. [22]. It was demonstrated that the attachment of a long PEG chain to NCs results in a

larger and more persistent decrease of the NC–cell association than when free drug is associated with PEG-adsorbed NCs. So far, such carriers, which appear to be well adapted to the delivery of lipophilic drugs, have never been used for photosensitizers. Among the second-generation photosensitizers, *meta*-tetra(hydroxyphenyl)chlorin (*m*THPC) is now currently under clinical trial and has stimulated a number of studies. However, the *m*THPC tumor selectivity is still being debated and to date, few data obtained from patients have been reported. It may be noted that tumoral:normal tissue ratios of approximately 1 to 1.5 are currently observed by optical-fiber fluorimetry on patients undergoing PDT of esophageal tumors with *m*THPC (J. Blais, unpublished results).

In the present study, submicronic colloidal carriers (PEG-grafted PLA, polox PLA, naked PLA, and nanoemulsion (NE) (oil without any polymer external wall)) have been examined as potential delivery vehicles of *m*THPC, used as a model lipophilic photosensitizer soluble in the inner core of the reservoir systems. The uptake, subcellular localization and phototoxicity of *m*THPC have been compared in macrophage-like J774 cells and HT 29 human adenocarcinoma cells. Our final goal is to find an innovative formulation for the new photosensitizers under development at the present time.

2. Experimental

2.1. Materials

The *meta*-tetra(hydroxyphenyl)chlorin (*m*THPC) was kindly provided by Scotia Quanta Nova, Guildford, UK. Miglyol 812N (caprylic/capric diglyceryl myristate), a biocompatible oil, was obtained from Huls (Puteaux, France), poloxamer 188 Symperonic PE/F68 from ICI (Clamart, France), poly(D,L-lactic acid) (PLA, MW 109kDa) from Boehringer (Ingelheim, Germany) and Epikuron 170 from Lucas Meyer (Hamburg, Germany). PLA–PEG 53–5 diblock (PLA MW 5 kDa and PEG MW 5kDa) was a gift from Dr R. Gref. It was synthesized and characterized as described by Quellec et al. [23]. RPMI 1640 without Phenol Red, phosphate-buffered saline (PBS), fetal calf serum (FCS), glutamine, antibiotics and trypsin were purchased from Bio-Media (Boussens, France).

2.2. Nanocapsule preparation and characterization

2.2.1. Preparation

PLA nanocapsules without poloxamer (PLA NCs), PLA nanocapsules coated with poloxamer (polox PLA NCs) and PEG-grafted PLA nanocapsules (PLA–PEG NCs) were prepared by a solvent displacement process as described by Fessi et al. [20]. Briefly, 35 mg of polymer were first dissolved in acetone (8 ml). Lipophilic surfactant (Epikuron 170) was dissolved in acetone (2 ml). The *m*THPC powder was dissolved directly in Miglyol (0.5 mg/ml), stirred and left in

the dark for 24 h at room temperature. After complete dissolution, 0.25 ml of Miglyol containing *m*-THPC was added to the acetone solution. The resulting organic solution was poured into 20 ml of an aqueous solution containing hydrophilic surfactant (Symperonic PE/F68) only in the preparation of polox PLA NCs, and magnetically stirred under moderate agitation. The colloidal suspension was concentrated to the desired final volume (10 ml) by removal of excess solvent under reduced pressure. Oil/water nanoemulsions (NEs) were prepared in the same way as polox PLA NCs without addition of polymer. For all these formulations, the final *m*THPC concentration was 12.5 µg/ml. For formulation referred to hereafter as 'mTHPC solution', stock *m*THPC solution (1 mg/ml), was made according to the manufacturer's recommendations (ethanol 20%, PEG 30%, H₂O 50%). Further dilutions were performed in RPMI or PBS. Respective formulation contents are summarized in Table 1.

The free drug concentration was determined in the clear supernatant following separation of nanocapsules from aqueous medium by a combined ultrafiltration (Microcon 100, Amicon Inc, USA) and centrifugation technique. The total amount of *m*THPC was measured after complete dissolution of the nanocapsules in acetonitrile. The *m*THPC content of nanocapsules was calculated from the difference between the total and free estimated drug amounts in the nanocapsule suspension and supernatant, respectively. The *m*THPC concentration was determined by high-performance liquid chromatography (HPLC) [24].

2.2.2. Physical properties of nanocapsules

The size of the NCs was measured by quasi-elastic light scattering with a Nanosizer (Coulter model N4 Plus, Coulter Electronic Inc., Hialeah, FL, USA). Zeta potential measurements were carried out with a Zetasizer 4 (Malvern Instruments, UK) after dilution of the NCs by a constant factor 1:250.

The physical characteristics of the NCs are given in Table 1. They appeared as a homogeneous population of particles with a mean diameter of 180 ± 60 nm and an *m*THPC incorporation rate greater than 99%. The negative zeta potential values resulted from the addition of Epikuron. NCs were stable over several months, as monitored by measuring the size and the encapsulation rate but, in order to avoid contamination, fresh formulations were prepared before each experiment.

Table 1
Physical characteristics of *m*THPC colloidal carriers

	PLA 109 K	PLA-PEG 53-5 K	Poloxamer 188	Mean size ± SD (nm)	Polydispersity	Zeta potential ± SD (mV)
PLA	×	–	–	182.8 ± 58.5	0.232	– 53.6 ± 0.5
Polox PLA	×	–	×	186.0 ± 57.0	0.132	– 51.6 ± 0.3
PLA-PEG	–	×	–	191.0 ± 50.7	0.237	– 47.6 ± 0.6
Nanoemulsion	–	–	×	170.7 ± 50.8	0.190	– 48.8 ± 0.8

2.3. Cell culture

Human colorectal adenocarcinoma (HT29) cells were allowed to grow to confluence in RPMI 1640 without Phenol Red and supplemented with 10% FCS, glutamine and antibiotics. Cells were subcultured by dispersal with 0.25% trypsin and replated (10⁵ cells/ml). The same procedure was used with J774 cells. For microspectrofluorimetric and fluorescence imaging experiments, cells were grown in plastic Petri dishes. They were washed twice with PBS after incubation and prior to measurements.

2.4. Photocytotoxicity

HT29 cells were seeded into 96-well plates at 4 × 10⁴ cells/ml (0.2 ml per well) and allowed to grow for 24 h in an incubator (5% CO₂, 37°C, humidified atmosphere) (Jouan, France). On the day of the experiment, the culture medium was removed and 200 µl of fresh RPMI without FCS containing *m*THPC at a final concentration of 0.125–1.25 µg/ml were added to each well. Cells were incubated for 5 h and then washed twice with PBS, and fresh complete medium was added.

Irradiations were performed at 514 nm with an Ar⁺ laser (Spectra Physics-2020) in sterile conditions. The light was transmitted by a silica optical fiber (core diameter 200 µm, N.A. 0.22) equipped with a diffuser (Medlight, Switzerland) in order to obtain a uniform light delivery to the whole plate (spot diameter of 130 mm). The power was calibrated with an energy meter (Spectra Physics-407), and irradiation times were adjusted in order to obtain fluences of 5, 10 and 25 J/cm². Controls were as follows: wells containing cells treated with photosensitizer but not exposed to light, wells containing cells without photosensitizer and without light, wells containing cells without photosensitizer and exposed to light.

Cell viability was measured 24 h later by the determination of mitochondrial activity using a colorimetric MTT assay according to the method described by Mosmann [25]. At the time of counting, 100 µl of an RPMI-MTT (0.5 mg/ml) solution were added to each well and replaced by 200 µl of dimethyl sulfoxide (DMSO) 4 h later. Optical densities of microplates were determined at 570 and 640 nm using a Labsystem[®] instrument.

2.5. Microspectrofluorimetry

Fluorescence measurements were performed with a microspectrofluorimeter previously described [26]. Excitation was performed with the 514 nm line of an Ar⁺ laser and a long-pass filter (MTO J560) was used on the emission path. The excitation beam was focused on a cell area of 1 μm^2 with a $\times 63$ Zeiss plan Neofluar objective (N.A. 1.25), directly immersed in RPMI solution. A diaphragm was adjusted on the analysis path, in such a way that the analyzed cellular volume was about 10 μm^3 , which was smaller than the cell volume but large enough to take into account the heterogeneity of the cellular drug distribution. The fluorescence spectra were recorded in the 550–830 nm spectral region with an accumulation time of 0.5–1 s. The values reported for the maximum fluorescence intensity were obtained after numerical filtration of the experimental spectra involving fast Fourier transformation (FFT). For each experiment, measurements were carried out on 30 individual cells.

2.6. Fluorescence imaging microscopy

Fluorescence microscopy was carried out on a Nikon epi-fluorescence microscope (Optiphot-2) equipped with a Nipkow wheel coaxial–confocal attachment (Technical Instruments, model K2 BIO). Living cells were in Petri dishes and viewed with a $\times 63$ objective immersed in PBS. Fluorescence images were detected by a cooled CCD camera (RTEA 1317 K1CCD, Princeton Instruments). A standard rhodamine filter set (BP546, FT590, LP600) was used in our experiments. Specific organelle-staining probes were used. C₆NBD ceramide (1 μM) was used to visualize the Golgi apparatus. Endoplasmic reticulum was stained with 0.1 μM of 3,3-dihexyloxycarbocyanine (DiOC6). LysoTraker Green (1 μM) was used as a lysosome marker. Mitochondria were stained with 1 μM Rhodamine 123. Cells were incubated for 1 min in all cases. The probes were purchased from Molecular Probes Inc. (USA). The rhodamine filter set was used with the rhodamine 123 probe, whereas the FITC filter set (BP450-FT510, 520–570) was used with the other probes.

3. Results and discussion

3.1. Cellular uptake by J774 and HT29 cells

The cellular uptake of encapsulated *m*THPC emulsion was determined on the basis of the cellular fluorescence intensity. The cellular fluorescence of the cytoplasmic area of treated cells was typical of *m*THPC emission (Fig. 1), with a main peak maximum at 654 nm and a weak band around 720 nm. It was found to be identical for all the formulations used. It must be noted that, in our experimental conditions (see Section 2), the cellular emission only results from the internalized fluorophores. The contribution of cellular autofluorescence (maximum around 570 nm) only affected the

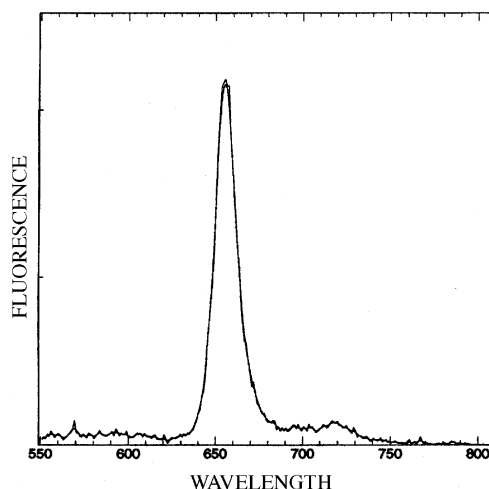


Fig. 1. Typical fluorescence spectrum from a cytoplasmic area of a single HT29 living cell incubated (300 min) with *m*THPC (0.25 $\mu\text{g}/\text{ml}$) encapsulated in PLA–PEG NCs. The contribution of cellular autofluorescence observed around 570 nm was negligible except for shorter incubation times and was taken into account in our intensity measurements (excitation = 514 nm, accumulation time 0.5 s).

fluorescence intensity for short incubation times and its contribution was subtracted when necessary. The *m*THPC uptake was evaluated from the fluorescence intensity measured at 654 nm.

The interaction of encapsulated *m*THPC with macrophage-like J774 cells was investigated with the aim of evaluating, *in vitro*, the capability of surface-modified NCs to reduce phagocytosis. As seen in Fig. 2, compared with naked PLA, the drug uptake rate of *m*THPC encapsulated in poloxamer-coated colloid carriers (polox PLA and NE) or PLA–PEG NCs was significantly lowered, the larger effect being with the latter. This is consistent with our previous study [22], which demonstrated that the interaction between surface-modified PLA NCs loaded with a lipophilic fluorescent probe and J774 cells was reduced when PLA NCs with a long PEG

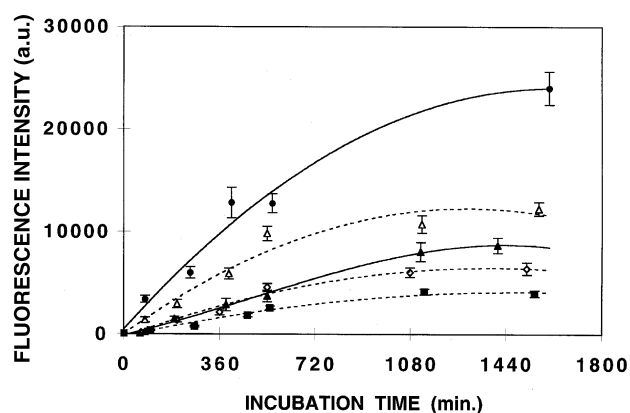


Fig. 2. Uptake of *m*THPC (0.25 $\mu\text{g}/\text{ml}$) encapsulated in PLA NCs (●), NE (△), solution (▲), polox PLA NCs (◇) and PLA–PEG NCs (■) by macrophage-like J774 cells as determined by microspectrofluorimetry. The cellular fluorescence intensities were measured at 654 nm. For each experiment, data have been averaged from intensity values determined on 30 individual living cells. Experiments have been carried out in triplicate. Bars, SE.

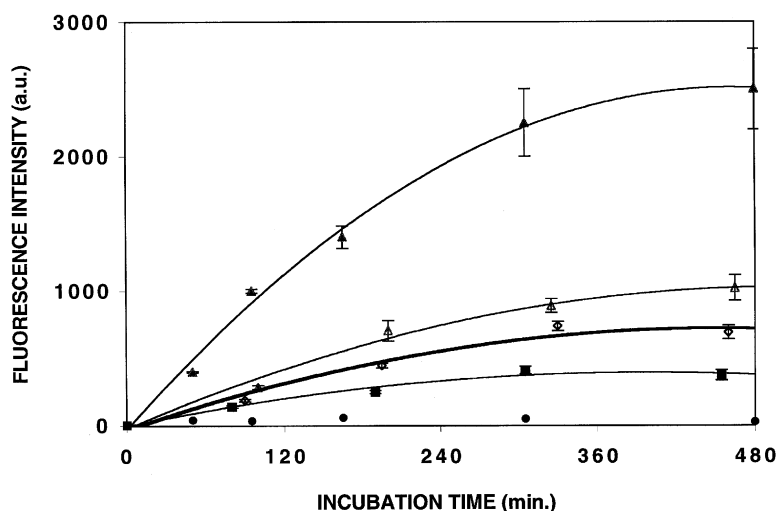


Fig. 3. Uptake of *m*THPC (0.25 µg/ml) encapsulated in NE (△), polox PLA NCs (◇), PLA-PEG NCs (■), PLA NCs (●) and solution (▲) by HT29 cells as determined by microspectrofluorimetry. The cellular fluorescence intensities were measured at 654 nm. For each experiment, data have been averaged from intensity values determined on 30 individual living cells. Experiments have been carried out in triplicate. Bars, SE.

chain attached were used. This could be considered as a first indication that RES clearance could be limited by the use of such a carrier. It must be noted that the uptake rate determined with the solution was less than that of the naked PLA. This could be explained by a possible intermolecular interaction between *m*THPC and PEG from the solvent.

Encapsulation also strongly affected the *m*THPC uptake by HT29 cells (Fig. 3). Compared with the solution, the drug penetration was lowered whatever NCs were used. The uptake rate at 300 min, as determined from Fig. 3, was decreased by factors of 2.4, 3.1 and 5.4 with NE, polox PLA and PLA-PEG NCs, respectively. The time-course dependence of *m*THPC uptake by HT29 cells was similar for all the formulations studied except for naked PLA. Surprisingly and unlike J774 cells, a specific behavior was observed in the case of PLA NCs. The drug was not internalized and only a very weak cytoplasmic fluorescence was observed.

When incubations were performed at 4°C, the cellular fluorescence intensity became very weak whatever the formulation used and could not be determined accurately, suggesting an active uptake occurring via endocytosis.

3.2. Photocytotoxicity

None of the colloidal carriers used showed any dark cytotoxicity in HT29 cells after incubation times of up to 18h. The phototoxicity of *m*THPC was much less affected by encapsulation into colloidal carriers than by the drug internalization process. As illustrated in Fig. 4, the phototoxicity of HT29 cells treated with *m*THPC encapsulated into naked PLA, NE and polox PLA and exposed to 514 nm light with fluences of 25 J/cm² was quite similar, with a cell-survival fraction of approximately 33% for a dose of 0.25 µg/ml. It was only slightly reduced compared with that of the solution (27%). A lower phototoxicity was found with *m*THPC encapsulated into PLA-PEG NCs, with a cell-survival frac-

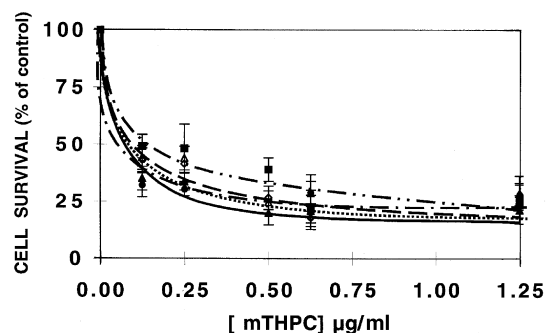


Fig. 4. Survival fractions of HT 29 cells incubated (300 min.) with *m*THPC encapsulated in PLA-PEG NCs (■), PLA NCs (●), polox PLA (◇), NE (△) and solution (▲) and followed by exposure to 514 nm light (25 J/cm²). Dark cytotoxicity was always negligible. Each experiment (4 wells/experiment) has been carried out in triplicate. Bars, SE.

tion of 42%. For a dose of 1.25 µg/ml, the cell survival fractions were not significantly different with the various formulations studied (in the range 18–25%). It may be noted that the concentrations used in these experiments were lower than those generally used in the literature for phototoxicity determinations (several µg/ml) and that the phototoxicity remained low when treated cells were exposed to fluences of 5 and 10 J/cm².

The phototoxicity was affected in the presence of plasma protein to a different extent depending on the formulation. Whereas the effect of the addition of FCS (10%) to the culture medium during incubation slightly enhanced the phototoxicity of *m*THPC formulated in NE, it was found to be lowered with the other formulations. With naked PLA, PLA-PEG and polox PLA, for a 0.25 µg/ml dose, the phototoxicity was reduced by 80, 41 and 24%, respectively. These data clearly indicate that the association with plasma proteins depends on the surface characteristics of the NCs. The polymeric layers made of PLA-PEG and polox PLA appear to have the best protecting effect. Since FCS contains opsonins, these findings could be a clue to indicating that polox PLA

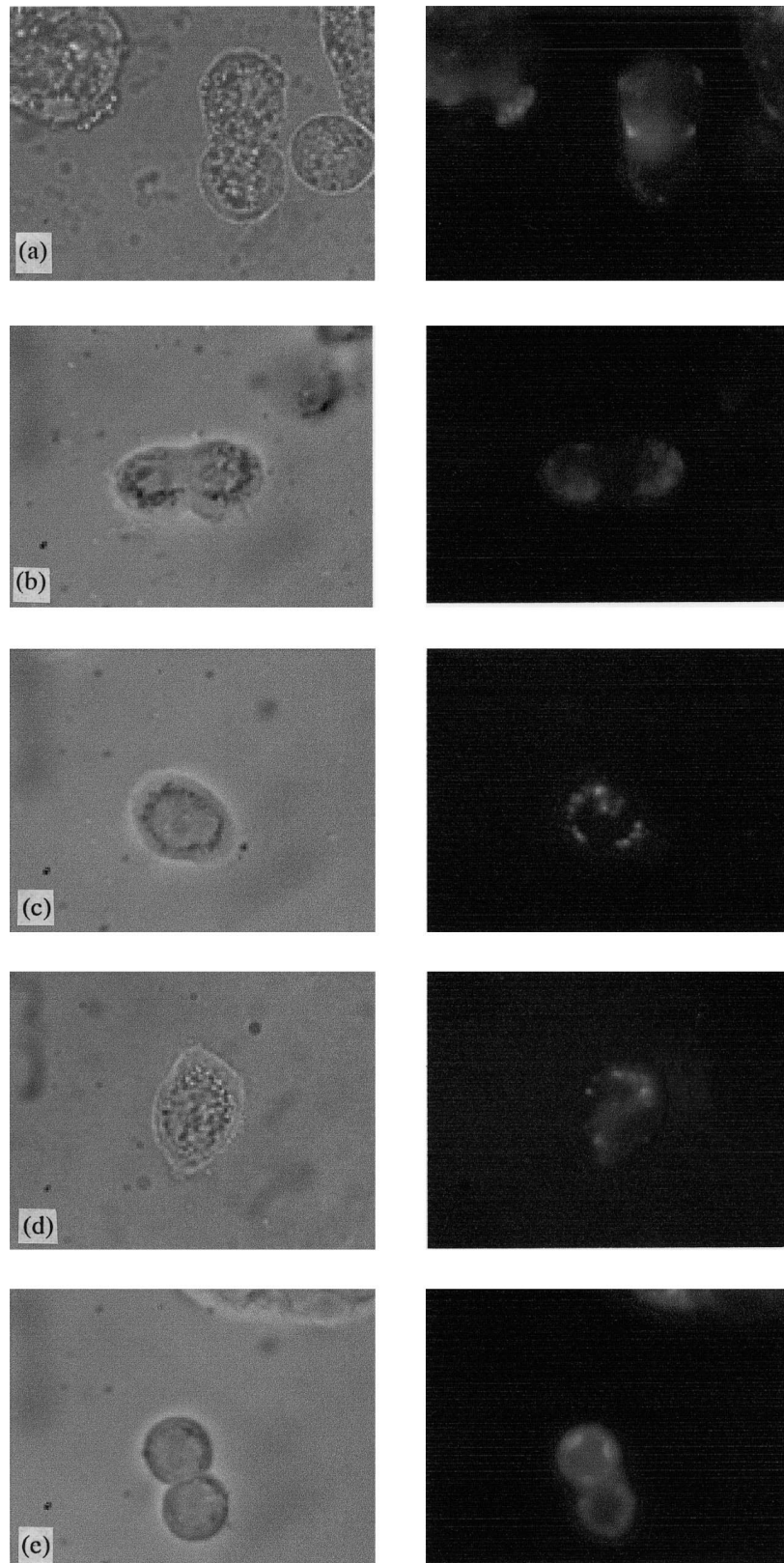


Fig. 5. Right panel: fluorescence images of HT 29 cells incubated (300 min.) with *m*THPC (0.625 $\mu\text{g}/\text{ml}$) formulated in (a) PLA NCs; (b) NE; (c) polox PLA NCs; (d) PLA-PEG NCs; and (e) solution. Left panel: corresponding brightfield images.

and PLA–PEG NCs could be effective in limiting the RES uptake.

3.3. Subcellular localization in HT29 cells

The subcellular localization of *m*THPC in HT29 cells, as studied by fluorescence microscopy, was found to be formulation dependent (Fig. 5). The cells treated with *m*THPC solution showed the fluorescence diffusively distributed throughout the cytoplasm, with a non-fluorescent nuclear area. Similar observations have been reported in V79 hamster cells [8] and in HT 29 cells [27].

The cellular distribution was markedly affected when colloidal formulations were used. In all cases, the nuclear area remained non fluorescent. A punctate fluorescence pattern, mainly perinuclear, was seen when cells were treated with *m*THPC formulated in PLA–PEG and polox PLA NCs (Fig. 5(c),(d)). Separate incubations with specific probes give indications that these intense bright spots could arise from fluorophores localized into the Golgi system. A more accurate localization cannot be ascribed because of the specific HT29 cell morphology, which is quite thick with a small cytoplasm. Moreover, the simultaneous staining of subcellular organelles with specific probes and *m*THPC was not possible in these experiments. The excitation was actually not selective enough to avoid a partial overlapping of the probe emission of high fluorescence quantum yield with that of *m*THPC of low fluorescence quantum yield (0.08 [28]).

In the case of PLA NCs, the fluorescence was mainly located on the external side of the plasma membrane, which is consistent with the results obtained by microspectrofluorimetry. It may be pointed out that, surprisingly, the phototoxicity remains little affected.

As far as a specific subcellular localization of the photosensitizer was observed with NCs, it could be assumed that the type of photodamaged targets involved, and hence the mechanism and kinetics of cell death, are different. This could explain the absence of correlation between respective uptake rates determined with the various formulations used and the phototoxicity.

4. Conclusions

PLA–PEG and polox PLA NCs have been used for the first time as carriers for *m*THPC, a photosensitizer used in PDT. First, the reduced *m*THPC uptake by macrophage-like cells indicates that the NCs could limit the RES uptake. Secondly, although the encapsulation results in a lower *m*THPC cellular uptake, the phototoxicity remains only slightly affected. The weak inhibition of phototoxicity by plasma proteins could be considered as a key factor for the in vivo distribution. Finally, the subcellular localization pattern observed suggests a different drug targeting. Possibly different cell-death mechanisms could thus be involved.

These in vitro data clearly demonstrate that, as long as the colloidal carriers studied consist of an identical oily core and have a similar size, the modification of the nanocapsule surface is the main parameter to be considered in the interaction between encapsulated drug and cells. In vivo experiments are currently in progress to examine whether the photosensitizer biodistribution and photodynamic activity are modified by the use of PLA–PEG and polox PLA NCs as *m*THPC delivery vehicles.

5. Abbreviations

PDT	photodynamic therapy
RES	reticuloendothelial system
PLA	poly(D,L lactic acid)
PEG	polyethylene glycol
<i>m</i> THPC	<i>meta</i> -tetra(hydroxyphenyl) chlorin
PBS	phosphate-buffered saline
HPLC	high-performance liquid chromatography
NCs	nanocapsules
FCS	fetal calf serum

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