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Preparation and characterization of *m*THPC-loaded solid lipid nanoparticles for photodynamic therapy

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ABSTRACT

Among various attempts to enhance the therapeutic efficacy of photodynamic therapy (PDT), the specific delivery of photosensitizer (PS) in the tumor tissue is expected to improve its clinical applications. The aim of this study was to engineer lipid nanoparticles (LNP) with different sizes and various PS contents, using simple solvent-free and easily scale up manufacturing processes. Meso-(tetrahydroxyphenyl) chlorin (*m*THPC) is one of the most potent photoactive compounds for clinical use. We demonstrated that *m*THPC was efficiently incorporated into the lipid core of LNP, leading to a large range of stable and reproducible *m*THPC-loaded LNP with narrow size distribution. Photophysical and physico-chemical properties of *m*THPC-loaded LNP were assessed as well as absorption spectra and singlet oxygen emission, colloidal stability, particle size and zeta potential. The photocytotoxicity of selected *m*THPC-loaded solid LNP was demonstrated on MCF-7 cells under irradiation at 652 nm with a range of light fluence from 1.0 to 10 J/cm^2. All physico-chemical, photophysical and biological results allow us to conclude that solid LNP appear as a very promising nano-*m*THPC delivery system for PDT.

1. Introduction

The use of photodynamic therapy (PDT) is more and more attractive, especially since it has been shown that this therapeutic strategy can be performed as an alternative or adjuvant to other therapy modalities, such as radiotherapy, surgery and chemotherapy [1]. During the past 30 years, PDT has been applied in the treatment of many tumor types, and its effectiveness as a curative and palliative therapy is well documented [2,3]. Despite a lot of advantages, a substantial limitation for PDT clinical applications is the photoactive drug itself which has to be designed to possess adequate chemical and photophysical properties [4]. Indeed, to be effective, the PS should: (i) be chemically stable in biological fluids, (ii) be non-toxic in the dark after its systemic injection, (iii) possess a strong absorption peak at light wavelengths with optimal tissue penetration, and (iv) selectively be retained in the targeted tissue in high concentrations [5,6]. Furthermore, even if the PDT-induced reactive-oxygen species (ROS) production can destroy tumors through different mechanisms such as apoptosis and necrosis induction, damages of the tumor vasculature or immunological effects [7–9], the main cytotoxic species, the singlet oxygen ($^1O_2$), has only a short lifetime in biological media and consequently its radius of action remains close to its distribution sites within the cell [10–12].

Porphyrins (including chlorins) are the most frequently used PS with high efficiency *in vivo*. However, a substantial limitation for clinical applications is their low water solubility, which impairs drug administration and efficacy. The chlorin investigated in this study, *m*THPC (commercial formulation Foscan®), is a highly potent, second generation PS that exhibits several favorable characteristics for PDT [13] and has been approved for the treatment of head and neck cancers in the Europe and Japan [14]. This PS, a highly hydrophobic molecule and electrically neutral at physiological pH values, can be excited at 652 nm corresponding to a light penetration at about 7 mm [15,16]. *m*THPC-induced cell photo damage is mainly mediated by $^1O_2$

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[17,18] and although the $^1O_2$ quantum yield of *m*THPC is comparable to other chlorins, only low drug and light doses (0.1 mg/kg; 20 J/cm^2, respectively) are needed to achieve photodynamic responses equivalent to other commercially PS [0,16] such as Photofrin®. Being a chlorin it exhibits stronger absorption than porphyrins at longer wavelengths; however, *m*THPC is hydrophobic and is prone to aggregation, which presents problems for optimizing its formulation [19]. Moreover, the aggregated form of *m*THPC is less photoactive and binds strongly to serum proteins [20]. Rapid uptake of aggregates by cells of the reticuloendothelial system may lead to higher inter-patient variability of Foscan® accumulation in tumor tissue.

In PDT, liposomes have been widely used in recent years due to their suitability for incorporating hydrophobic PS such as *m*THPC into their lipid shells [21–23]. This avoids aggregation of PS molecules, allowing them to conserve their monomeric state, providing thus potent photocytotoxic efficiency. Recently, our group designed solid lipid nanoparticles (LNP) as promising nanocarriers enabling of all at once diagnostic, therapeutic agents delivery, and monitoring treatment. Thanks to their lipid composition, they offer for instance, a smart biocompatible alternative to quantum dots for *in vivo* fluorescence imaging [24–27]. These lipid nanoparticles, composed of a hydrophobic inner core surrounded by a hydrophilic shell, are dedicated to the encapsulation of highly lipophilic compounds [26,28] and display an outstanding colloidal stability [29]. Such LNP could therefore be a more promising nanocarrier for *m*THPC than liposomes, considering not only their higher proportion of lipids, prone to entrap PS molecules. Nanostructured lipid carriers are generally known to be more stable than liposomes in biological environment. Indeed, we recently published that loaded LNP describe a long-term shelf colloidal and optical stabilities in ready-to-use storage conditions in aqueous buffer more than 6 months at 4 °C [30]. Moreover, the high-energy process allowed reaching sized-nanometer particles with tunable hydrodynamic diameter in the range from 30 to 120 nm depending on the surfactant to lipid ratio [29]. In this study, LNP were considered as potential nanocarrier for PDT purpose through *m*THPC encapsulation. The photocytotoxicity efficiency of selected *m*THPC-loaded LNP was assessed on MCF-7 cells under irradiation at 652 nm with a range of light fluence from 1.0 to 10 J/cm^2. We highlighted the key parameters leading us to PDT efficiency when such lipid nanoparticles are considered as carriers for PS.

2. Experimental

2.1. Synthesis of 5,10,15,20-tetrakis(meta-hydroxyphenyl) chlorin (*m*THPC)

Synthesis of 5,10,15,20-tetrakis(meta-methoxyphenyl) porphyrin **(1)** (*m*THPP-OMe): In the absence of light, freshly distilled pyrrole (0.70 mL, 5 mmol) and 3-methoxybenzaldehyde (0.61 mL, 5 mmol) were stirred in CH_2Cl_2 (700 mL) at room temperature (RT). After 15 min, $BF_3{\cdot}OEt_2$ (120 μL) was added and the reaction mixture was kept under stirring for 2 h at RT. *p*-chloranil (1.98 g) was then added and the mixture was refluxed at 40 °C for 2 h. After cooling to RT, the mixture was treated with silica to remove undesired linear polymers, filtered and concentrated in vacuo. The crude product obtained was purified by column chromatography using CH_2Cl_2/hexanes (gradient 1:1 to 9:1). After solvent evaporation, *mTHPP-OMe* led to 0.602 g (16.4%) as a dark purple solid. ^{1}H NMR ($CDCl_3$): δ 8.95 (m, 8H, CH β-pyrrole), 7.65 (m, 12H, CH^{Ar}), 7.35 (3, 4H, CH^{Ar}), 3.99 (s, 9H, OCH_3), −2.78 (s, 2H, NH).

Synthesis of 5,10,15,20-tetrakis(meta-hydroxyphenyl) porphyrin **(2)** (*m*THPP): A three-necked flask was freshly taken from the oven at 100 °C and was allowed to cool under N_2 flow. Ph_2S_2 (0.716 g, 3.3 mmol) and CaH_2 (0.345 g, 8.2 mmol) were stirred in anhydrous NMP (2 mL) at 100 °C for 15 min. After cooling to RT, a solution of *m*THPP-OMe (0.602 g, 0.82 mmol) in NMP (2 mL) was added and the mixture was allowed to stir during 2 h at 202 °C. The reaction mixture was then cooled to RT and then poured into 10 mL of a 5% of aqueous NaOH solution. The aqueous phase was washed with Et_2O (2 × 5 mL), neutralized with 0.1 N aqueous HCl and extracted with CH_2Cl_2 (3 × 10 mL). The combined CH_2Cl_2 layers were dried over Na_2SO_4. The crude product was purified by gradient flash chromatography (CH_2Cl_2, then acetone, then MeOH/acetone 5:95), yielding **(2)** (80%). ^{1}H NMR (DMSO-d_6): δ 9.87 (s, 4H, OH), 8.88 (br, 8H, CH β-pyrrole), 7.62–7.57 (m, 12H, CH^{Ar}), 7.24 (m, 4H, CH^{Ar}), −2.97 (2s, 2H, NH).

Synthesis of 5,10,15,20-Tetrakis(meta-hydroxyphenyl) chlorin (*m*THPC): In the dark under N_2 atmosphere, *m*THPP (0.136 g, 0.2 mmol), *p*-TSH (0.186 g, 1 mmol, 5 eq.) and anhydrous K_2CO_3 (0.276 g, 2 mmol, 10 eq.) were stirred in pyridine (20 mL) at 115 °C. The same initial amount of *p*-TSH and anhydrous K_2CO_3 were added every hour during 7 h. After stirring overnight, ethyl acetate (30 mL) and distilled water (30 mL) were added and the mixture was allowed to stir one more hour at 90 °C. The reaction mixture was then cooled to RT. The organic phase was washed with 10% of HCl and distilled water until neutrality. The presence of bacteriochlorin (band at 735 nm) was controlled by UV spectroscopy. After gradually addition of *o*-chloranil (30 mg), the bacteriochlorin was totally oxidized into chlorin. After completion of reoxydation, monitored by UV, 30 mL ultrapure water was instantaneously added to quench further reduction into the initial porphyrin. The organic phase was washed with 0.1 M aqueous NaOH solution and distilled water, dried over $MgSO_4$ and concentrated *in vacuo*. ^{1}H NMR (DMSO-d_6): δ 9.74 (s, 4H, OH), 8.62 (d, J = 2 Hz, 2H, CH β-pyrrole), 8.36 (s, 2H, CH β-pyrrole), 8.23 ((d, J = 2 Hz, 2H, CH β-pyrrole), 7.55–7.08 (m, 16H, CH^{Ar}), 4.16 (s, 4H, CH_2 β-pyrrole), −1.65 (s, 2H, NH). MS (ESI) *m/z* calculated 680.24, found 681.25 $[M+H]^+$.

*m*THPC was purified by high performance liquid chromatography (HPLC) ((Alltech, Apollo C18 preparative column Reverse-phase (5 μm; 250 mm × 10 mm), fluorescence detection (λ_{exc} = 415 nm, λ_{em} = 650 nm) and UV–visible detection (λ = 415 nm)). Gradient elution was adapted throughout the purification. The eluting solvent was started with MeOH–H_20 (85:15, v/v) to (100:0 v/v) for 15 min, followed by 100% MeOH for another 5 min. Pure *m*THPC was obtained with 60% yield. *m*THPC solution was prepared in ethanol for physico-chemical studies and was reconstituted in an aqueous solution containing 30% of ethanol and 20% of poly (ethylene glycol) (PEG) (Myrj s40) in an aqueous phase (sterile phosphate buffer saline, PBS) for biological studies, as previously described [31].

2.2. Preparation of mTHPC-loaded LNP

LNP are composed of a lipid core (soybean oil and Suppocire NB), stabilized by phospholipids (lipoid s75) and pegylated surfactants (Myrj s40) in an aqueous phase (sterile phosphate buffer saline). Super refined soybean oil and PEG stearate (brand name Myrj s40) are kind gifts from Croda Uniqema (Chocques, France) whereas Suppocire NB was donated by Gattefossé (Saint Priest, France). Lipoïd s75 (soybean lecithin at >75% phosphatidylcholine) is purchased from Lipoid GmbH (Ludwigshafen, Germany) and other chemical products are from Sigma Aldrich (Saint Quentin Fallavier, France). They are engineered as previously described [28] and their hydrodynamic diameter can be adjusted between 30 and 120 nm by modifying their composition and more particularly the lecithin/PEG surfactant or surfactant/core weight ratios in the