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Delivery of lipophilic porphyrin by liposome vehicles: Preparation and photodynamic therapy activity against cancer cell lines

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Summary Porphyrin photosensitizers are mostly used components in photodynamic therapy (PDT). The poor solubility of porphyrins in aqueous medium is the problem to be solved for the *in vivo* applications. The delivery of photosensitizers to the tumor cells using liposome vehicles can help to overcome this problem. In this work, we have first functionalized the protoporphyrin IX with lipophilic oleylamine arms and encapsulated it into 1,2 dioleyl-*sn*-glycero-phosphatidylcholine (DOPC) liposomes. The appropriate sizes of liposomes are about 140 nm and have the characteristic Soret and Q band absorptions at 405 nm (Soret), 507 nm, 541 nm, 577 nm and 631 nm (Q bands), respectively. In the photodynamic activity studies, the liposomal porphyrins were irradiated with light (375 nm, 10 mW) in the presence of cancer cell lines, HeLa and AGS. We have found that both liposomal porphyrins and oleylamine conjugated porphyrins are much more effective than PpIX. This result can be attributed to the drug delivery characteristic of the liposomes which plays effective role in endocytosis. We also found that, in AGS cells, liposomal PpIX-Ole induced apoptosis more than HeLa cells under light conditions.
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Introduction

Photosensitizers (PS) are the essential components of the photodynamic therapy (PDT) systems. Porphyrin based photosensitizers are most commonly studied molecules for more than two decades [1—4]. Recently, many experimental studies have been performed to prepare the efficient photosensitizers in terms of their tunable solubilities, high singlet oxygen quantum yields and cell-specific affinities, *etc.* [5]. During the irradiation process under the light, the energy transfer from excited state of the porphyrin (Soret band absorption) to the HOMO orbitals (triplet state) of the molecular oxygen occurs to produce the singlet oxygen species, $^1O_2$, which are the responsible for cell death (Type II mechanism) [6,7].

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The unsubstituted porphyrin molecules have very poor solubility in aqueous media because of their highly aromatic macrocylic structures that causes some limitations for potential PDT applications. The high solubility of the PS-molecule can be achieved by having some water solubilizing groups ($-HSO_3^-$, $-COO^-$ and $-NR_4^+$) on the peripheral positions of the porphyrin molecule. The delivery of the hydrophobic PS molecules to the tumor cells is still a milestone for photodynamic therapy [8,9]. Although the water solubility is a goal for porphyrin molecules, the undesired reactions with other proteins or biomolecules in aqueous medium is still a problem in PDT applications. On the other hand, the hydrophobicity of the cell membrane can hinder the approachment of the ionized PS molecule toward to the cells. This dilemma enforced the scientist to work on the delivery of the PS molecules to the cells by the different vehicles [6,10].

Liposomes are found to be more efficient vehicles to carry the hydrophobic groups in aqueous medium [11—13]. They can easily encapsulate the hydrophobic molecules into their hydrophobic ends during bilayer formation and as well as the hydrophilic ends can provide aqueous solubility to the liposomes [14]. Therefore, the loaded liposomes can easily deliver the hydrophobic drug in aqueous medium to the target tissue and they can easily diffuse and unload the drug into the cytoplasm because of their similarity to the cell membrane in the structure [15].

Porphyrin derivatives have applications as photoactive drugs in photodynamic therapy. However, little is known about their interactions with phospholipid membranes at the molecular level [16]. In the several studies, liposomes with their high loading capacity and their flexibility to encapsulate the photosensitizers have been used as delivery systems for improving the efficacy of PDT [17—19].

In this study, we aimed to deliver the oleylamine conjugated protoporphyrin IX (PpX-Ole) to the interior of the cancer cells using 1,2 dioleyl-*sn*-glycero-phosphatidylcholine (DOPC) liposomes and investigate their photodynamic activities.

Materials and methods

Materials

Protoporphyrin IX (PpIX) was purchased from Frontier Scientific Europe Carnforth, Lancashire, LA6 1DE, United Kingdom, oleylamine was purchased from Alfa-Aesar, 1,2 dioleyl-*sn*-glycero-phosphatidylcholine (DOPC) were purchased from, Sigma—Aldrich Chemie GmbH, Germany.

Instrumentation

FT-IR spectra were obtained on a Perkin Elmer 600—4000 cm^{-1}. Absorption measurements were recorded on a UNICAM UV-spectrophotometer. SEM images were obtained on Jeol JSM-7001F/JSM-7001FA Thermal FE Scanning Electron Microscope, ^{1}H NMR spectra were obtained on Bruker Ultra NMR spectrometer 400 MHz, The size of the liposome was obtained on Malvern Zetasizer Nano ZS (Dynamic Light Scattering, DLS) The irradiation experiments were performed with 10 Philips TLK 40 W/10R lamps (λ_{max} 375 nm) having the light intensity of 10mW/cm2 that is measured by photometer.

Synthesis of oleylamine conjugated protoporphyrin IX (PpIX-Ole)

0.36 mmol (2 eq.) dicyclocarbodiimide (DCC), 0.36 mmol (2 eq.) N-hydroxysuccinimide (NHS) and 1 mg DMAP were added to a solution of 0.18 mmol (1 eq.) protoporphyrin IX in 10 ml of dimethylformamide (DMF). The mixture was stirred at room temperature for 2 h. After, 0.36 mmol (2 eq.) oleylamine in 2 ml DMF was added and continued to stirring at room temperature overnight. The mixture was filtered and the solvent was evaporated. The residue was washed with water for 3 times and the crude product was extracted from the residue with dichloromethane (CH_2Cl_2) for 2 times. The solution was dried over sodium sulfate (Na_2SO_4) and after the solvent evaporation the dark brown product was obtained (yield: 63%) (Fig. 1). The PpIX-Ole was well characterized by FT-IR and ^{1}H NMR spectroscopies. Mwt calc. 1068 g/mol.

Liposome preparation

Liposomes were prepared by using hydration-film method [20]. Briefly, 1 mg of PpIX-ole (0.94 μmol), and 7.4 mg DOPC (9.4 μmol) with the ratio of porphyrin/DOPC (10:100) were dissolved in 10 ml chloroform. After evaporation of the solvent, the thin lipid film was hydrated with 10 ml of phosphate buffer (pH 7.6) in a water bath at 30—40 °C. The resulting suspension was then sonicated for 90 min at above the phase transition temperature (T_c) which is 50 °C for DOPC. The liposome suspension was homogenized by filtering five times through sterilization micro glass filters with 0.1 μm pores. The particle sizes and the zeta potentials of the liposomes were evaluated with dynamic light scattering (DLS) and electrophoretic light scatter, respectively (Fig. 2).

Cell culture

HeLa and AGS cells were cultured in DMEM (Dulbecco's Modified Eagle Medium from GIBCO) medium containing 10% (v/v) fetal bovine serum (FBS), 1% penicillin—streptomycin, in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cells were subcultured twice a week for continuous culture.

Cell viability and proliferation assay

HeLa and AGS cell lines were seeded in 96 well plates (7.5×10^3 cells/well) and preincubated for 24 h before the experiment. The chemicals PpIX, PpIX-Ole, and PpIX-Ole-DOPC were added into the culture at final concentrations of 5 μM and 15 μM and incubated for 24 h. After incubation with chemicals, one plate was left in dark for 20 min and another plate was irradiated (λ_{max} 375 nm, 10 mW) for 20 min. Following exposure to light, 24 h proliferation assays were performed according to the manufacturer's instructions (WST-1 reagent, ROCHE). Afterwards, absorbance was measured at 450 nm using an ELISA reader. The nontoxicity

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