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Phospholipid-functionalized mesoporous silica nanocarriers for selective photodynamic therapy of cancer

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ABSTRACT

This paper describes the fabrication of a highly efficient, non-cytotoxic drug delivery platform designed for photodynamic therapy (PDT): phospholipid-capped, protoporphyrin IX–loaded and FITC-sensitized mesoporous silica nanocarriers (Lipo-FMSNs/PpIX). After derivatization with folate on the phospholipid-capped FMSNs (denoted fa-Lipo-FMSNs/PpIX, the so-called nanoPDT system), we confirmed the nanoPDT systems' selective targeting of and entry into the folic acid receptor-overexpressed HeLa cells by means of cell viability assessment and confocal microscopic analysis. The decrease in the unfavorable dark toxicity of fa-Lipo-FMSNs/PpIX enabled the delivery of high concentrations of PpIX into cells. Moreover, the cellular uptake of the nanoPDT systems was greater than that of free PpIX. Upon irradiation with visible light, the nanoPDT system generated singlet oxygen efficaciously in aqueous environments—a decisive factor affecting its therapeutic applicability in PDT, demonstrating enhanced *in vitro* photocytotoxicity. Furthermore, an *in vivo* study of subcutaneous melanoma in nude mice inoculated with B16F10 cells revealed the capability for the nanoPDT system to mitigate nearly 65% of tumor growth.

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1. Introduction

Cancer is a life-threatening disease not only because of the high mortality rate of the disease itself but also the risk caused by current available clinical treatments, including chemotherapy, radiotherapy, and surgery. For decades, researchers have searched for more affordable and efficient cancer treatments. Photodynamic therapy (PDT), considered as a promising and minimally-invasive treatment strategy, involves the participation of harmless visible light, photosensitizers, and tissue oxygen [1]. The nature of photosensitizers used in PDT is generally lipophilic, enabling themselves to penetrate the hydrophobic lipid bilayer and accumulate preferentially at relevant sites to induce apoptosis or necroptosis after production of cytotoxic reactive oxygen species (ROS) upon light irradiation [2]. However, the hydrophobicity of these photosensitizers raises concern that low solubility may cause adverse side effects, limiting their applications under physiological conditions. In attempting to solve the problems, nanoformulation of PDT drugs prevails in the fabrication of drug delivery systems (DDSs) to undergo cancer treatment. The development of nanoscale DDSs is having an increasingly significant improvement in PDT. Various nanomaterials and devices have been used and engineered as delivery vehicles to meet specific criteria (i.e., stimuli-responsive controlled release, targeted drug delivery, degradable mechanism) [3–6]. Furthermore, a variety of new DDSs based on liposomes [7,8], micelles [9], dendrimers [10,11] polymeric nanoparticles [12], and polymer–drug [13] and lipid–polymer [14] conjugates have exhibited an ability to import PDT therapeutic molecules to cancer tissue effectively. Mesoporous silica nanoparticles (MSNs) are emerging as one of the most appealing candidates for nanoscale DDS [15–18] owing to their high surface area, uniform pore size and

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large pore volume. They allow encapsulation/loading with vast amounts of drugs and/or photosensitizers to achieve high drug payload and superior PDT efficacy. Functionalization of MSN with mannose for PDT applications was reported by Durand et al. (2009) on improved efficiency of PDT for breast cancer cells [19]. Additionally as demonstrated by Ren and co-workers (2012), a strategy for enhancement of PDT efficacy by endolysosomal escape highlighted the potential of using multifunctional MSNs for cancer therapy [20].

We have reported previously that phospholipid-modification of MSN nanocarriers can decrease self-aggregation in salt-containing aqueous environments and minimize nonspecific binding with proteins commonly presented in physiological fluids [21]. Stabilized nanocarriers are able to circulate in the bloodstream for a significant amount of time, resulting in enhanced delivery [22] or improved tumor accumulation and mitigated side effects of conventional chemotherapy.

To a certain extent, the most important merit of the phospholipid lies in its modifiability. One can readily sensitize the phospholipid layer on the nanocarriers with various ligands (e.g., folic acid, PEG, monoclonal antibody) to enhance accumulation of the nanocarriers within cancer cells (namely, the active targeting) [23]; whereas passive targeting, attributed to the enhanced permeability and retention (EPR) effect [24,25] leads to less selective accumulation in cancer cells. Aside of maneuvering targeting selectivity, the size of DDSs decisively affects their performance. A size of 10–200 nm is generally required in order to avoid being excreted from urine [26,27], or obstructed by sinusoids in the spleen and fenestra of the liver [28].

In an attempt to not only palliate the sickness but also minimize adverse side effects and complications during therapeutic treatment of cancer, in this study we prepared folate-targeting, phospholipid-capped, photosensitizer-loaded mesoporous silica nanoparticles (denoted fa-Lipo-FMSNs/PpIX, the so-called nanoPDT system) and examined its application for PDT. We suspected that because such a nanoscale PDT system bears a high resemblance to cell membranes, they might mitigate undesirable side effects resulting from, for example, nonspecific interactions with non-targeted cells while delivering the hydrophobic photosensitizer into the targeted cells. In addition, we investigated the feasibility of folate receptor (FR)-mediated active-targeting PDT using a pair of cell lines—HeLa cells (with overexpressed folic acid receptor, FR+) and A549 cells (without overexpressed folic acid receptor, FR−)—and concurrently examined the dark-toxicity and photocytotoxicity of the nanoPDT system *in vitro*. Finally, we also evaluated the *in vivo* efficacy of the nanoPDT system in nude mice bearing B16F10 melanoma.

2. Materials and methods

2.1. Reagents, materials, and apparatus

All chemicals and organic solvents were of analytical grade or of the highest purity commercially available; they were used as received. Fluorescein isothiocyanate (FITC), aminopropyltriethoxysiliane (APTES), cetyltrimethylammonium bromide (CTAB), tetraethylorthosilicate (TEOS), sodium chloride, potassium chloride, sodium phosphate dibasic, potassium phosphate monobasic, potassium phosphate dibasic, sodium hydroxide, ethanol, dimethyl sulfoxide (DMSO), chloroform, dicyclohexylcarbodiimide (DCC), pyridine, folic acid (fa), protoporphyrin IX (PpIX), monoclonal anti-folate antibody, nutrient mixture F-12 Ham Kaighn's modification (F-12K), bovine serum albumin (BSA), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), methanol, perchloric acid, propidium iodide, trypan blue solution, potassium iodide, and ammonium molybdate were purchased from Sigma (St. Louis, MO, USA). 13-(Chlorodimethylsilylmethyl)heptacosane (CDSMH) and hydrochloric acid were obtained from Gelest (Morrisville, PA, USA) and Riedel–de Haen (Seelze, Germany), respectively. 1,2-Dihexadecanoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxypoly(ethylene glycol)-2000] ($DPPE\text{-}PEG_{2000}$), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[aminopoly(ethylene glycol)-2000] (DSPE-PEG_{2000}-amine) were acquired from Avanti Polar Lipids (Alabaster, AL, USA). IgG fraction of anti-biotin was attained from Pierce Chemical (Rockford, IL, USA). Dulbecco's modified Eagle's medium (DMEM) and trypsin-EDTA were obtained from HyClone (Logan, UT, USA). Fetal bovine serum (FBS) was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Penicillin/streptomycin (PS) was obtained from Biosource (Camarillo, MD, USA). Triton X-100 was acquired from Plusone (Uppsala, Sweden). Anti-caspase 3, anti-pRb, anti-ppRb, anti-cyclin D1, and β-actin were purchased from Bioss (Woburn, MA, USA). Anti-caspase 8 and anti-cytochrome *c* were obtained from Epitomic (Cambridge, UK). All solutions were prepared with deionized water having a resistivity no less than 18 MΩ cm (Milli-Q, Bedford, MA, USA).

Centrifugation during the cell culturing process and nanomaterial synthesis was performed at an appropriate temperature using Kubota KN-70 (Tokyo, Japan) and Hermle Z 36 HK (Wehingen, Germany) instruments, respectively. Centrifugal evaporation was performed on a SpeedVac Concentrator SAVANT SPD121P purchased from Thermo (Milford, MA USA). UV–Vis and fluorescence spectra were recorded using a Varian Cary 300 UV–Vis Bio spectrophotometer and a Varian Cary Eclipse apparatus (Palo Alto, CA, USA), respectively. Confocal microscopy images were acquired using LSM 700 confocal laser scanning microscope from Zeiss (Oberkochen, Germany). Both 460 nm and 630 nm LED lights were purchased locally. Photo illustration of our home-made LED irradiation apparatus is shown in Supporting Information (Fig. S1).

HeLa, A549, and B16F10 cell lines were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan).

2.2. Synthesis of FMSNs and fa-Lipo-FMSNs/PpIX

The FITC-sensitized MSNs (FMSNs) and DSPE-PEG_{2000}-FA bioconjugate were synthesized as described previously (synthetic details are provided in the Supporting Information) [21]. The synthesis of fa-Lipo-FMSNs/PpIX included steps of hydrophobization of FMSN, encapsulation of PpIX, and surface modification of phospholipid molecules onto the PpIX-loaded hydrophobic FMSNs.

The hydrophobically modified FMSNs (denoted m-FMSNs) were obtained by reacting FMSN (100 mg) with CDSMH (1 mL) in $CHCl_3$ (50 mL) for 12 h. The product was repeatedly washed with hexane and centrifuged (16,000 rpm), and finally dried in air. Loading of the hydrophobic photosensitizer (PpIX) into the m-FMSNs began by mixing the m-FMSNs (15 mg) with PpIX (24 mg) in DMSO (1 mL). The mixture was stirred in the dark for 24 h, and the product was collected by repetitive washing with DMSO and centrifugation (16,000 rpm, 10 min). For the phospholipid modification of the PpIX-loaded m-FMSNs, a mixture of DPPC (14.7 mg) and DPPE-PEG_{2000} (2.3 mg) in $CHCl_3$ (7.35 mL) was first prepared. A portion of this mixture (0.918 mL) and DSPE-PEG_{2000}-FA (0.5 mg/mL in $CHCl_3$, 0.067 mL) were added to the suspension of the PpIX-loaded sample (2 mg) in $CHCl_3$ (4 mL). The mixture was sonicated for 9 min at 45 °C under N_2. After removing the organic solvent using a rotary evaporator, pre-heated PBS (45 °C, 2 mL) was added to the flask, which was then sonicated for 1 h. The resulting sample, fa-Lipo-FMSNs/PpIX, was obtained by centrifugation (16,000 rpm, 10 min) followed by repeated washing with PBS to remove any uncapped lipids. Finally, fa-Lipo-FMSNs/PpIX was re-dispersed in PBS (2 mL) and stored at 4 °C prior to use.

The modification of the folate-ligand onto the PpIX-loaded m-FMSNs was confirmed by an immunoblotting study. The amount of PpIX encapsulated in fa-Lipo-FMSNs/PpIX was determined as follows: an aliquot of fa-Lipo-FMSNs/PpIX (100 μL) was taken and centrifuged (16,000 rpm, 10 min). The supernatant liquid was decanted and then replaced with DMSO, followed by sonication for 10 min. The UV–Vis spectral absorbance of the resulting solution was examined to measure the effective concentration of PpIX in the fa-Lipo-FMSNs/PpIX suspension, in relation to a PpIX calibration curve.

2.3. Detection of singlet oxygen production

For the detection of $^1O_2$ produced by fa-Lipo-PpIX-MSNs under photoirradiation, a rapid iodometric analysis, based on the method reported by Mosinger and Mosinger in 1995 [29], was employed, in which I^- ions reacted with the ROS, resulting in the production of I_3^-. The concentration of I_3^-, which could be determined spectrophotometrically (showing an absorption peak at ca. 355 nm), was proportional to the amount of photodynamically produced $^1O_2$. A solution of the iodometric reagent was prepared from 0.12 M potassium iodide, 10 μM ammonium molybdate (as a catalyst) and 0.2 M potassium phosphate (pH 6.2), and stored in a light-protected bottle prior to use. The sample fa-Lipo-FMSNs/PpIX was reacted with the iodometric reagent at a concentration amounting to 10 μM of PpIX and subjected to irradiation with 460 nm LED light [power: 320 mW; fluence: 1.47×10^{20} photons/sec•m^2 (calculation shown in Supporting Information)] for 1, 5, or 8 min. The spectra of these solutions were acquired (340–420 nm) as the experimental groups; the same solutions that had not been irradiated served as the control group.

2.4. Cell culture and in vitro targeting selectivity

To demonstrate the selective targeting of the fa-Lipo-FMSNs/PpIX towards folic acid receptor overexpressed cells, two cell models, HeLa cells (with overexpressed

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