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Zinc phthalocyanine-soybean phospholipid complex based drug carrier for switchable photoacoustic/fluorescence image, multiphase photothermal/photodynamic treatment and synergetic therapy

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ABSTRACT

For the purpose of precision theranostic of tumor, multifunctional drug delivery systems are always receiving great attentions. Here, we developed a zinc phthalocyanine-soybean phospholipid (ZnPc-SPC) complex based drug delivery system with doxorubicin (Dox) as loading cargo to achieve additional chemotherapy while the carrier itself could serve as multifunctional and switchable theranostic agent. In the early phase, the ZnPc-SPC complex assembled to nanostructure displaying photothermal therapy (PTT) and photoacoustic (PA) properties while in the late phase, the prepared NPs dis-assembled into ZnPc-SPC complex again performing photodynamic therapy (PDT) and low-background fluorescence (FL) image. With the decoration of folate receptors α (FRα) targeted MTX, Dox-loaded, MTX-decorated self-assembled ZnPc-SPC complex NPs (DZSM) was formed. *In vitro* and *in vivo* evaluations both indicated that DZSM presented high selectivity for FRα over-expressed tumor cells, excellent switchable PA/FL image, significant multiphase PTT/PDT effect, as well as great synergetic therapy potential, leading to notable inhibition of tumor growth.

1. Introduction

Cancer still remains one of the main causes of morbidity and mortality worldwide, with new cases of about 14 million in 2012 and the number is expected to rise by approximately 70% over the next 2 decades. As the second leading cause of death globally, cancer was responsible for 8.8 million deaths in 2015 which indicated that nearly 1 in 6 deaths was due to cancer [1, 2]. Generally, it involves many kinds of pathological process and single therapeutic strategy may not always cope with cancer efficiently and thoroughly. Hence, combination therapy targeting different pathways of cancers could be the potential strategy to defeat cancers [3]. Nowadays, many innovative approaches have been recently developed to achieve the consistent goals of combination therapy. For instance in our previous work, ICG-EPI nanoparticle (NP) was fabricated by direct *co-self-assembling* of chemotherapeutic drug EPI and photothermal agent ICG for multimodal imaging-guided synergistic combination therapy [4]. Dox/ZnPc-FMLM *co-loading* of Dox and ZnPc in core-interlayer-shell Fe_3O_4@mSiO2@lipid-PEG-methotrexate NP was used to ensure multimodal imaging, multistage targeting and chemo-PDT [5]. And DiR-loaded MTX-CPT NP in which methotrexate and 10-hydroxyl camptothecin *chemically conjugated* through ester linkage was applied to perform "all-in-one" carrier-free self-assemble and synergistic combination therapy for cancer treatment, *etc.* [6].

It is worthy to mention that conventional chemotherapy with PDT as an adjuvant or therapeutic combinations in which PDT serves as the core therapeutic partner has always been concerned among the researchers [7]. Since PDT could lead to a series of photochemical and

Abbreviation: Zinc phthalocyanine, ZnPc; Doxorubicin, Dox; Photothermal therapy, PTT; Photoacoustic, PA; Photodynamic therapy, PDT; Fluorescence, FL; Folate receptors α, FRα; Nanoparticle, NP; Zinc phthalocyanine-soybean phospholipid, ZnPc-SPC; ZnPc-SPC complex self-assembled NPs, ZS; Reactive oxygen species, ROS; Dox-loaded ZnPc-SPC complex self-assembled NPs, DZS; 2-diacyl-SN-glycero-3-phosphoethanolamine-N-methoxy(poly(ethyleneglycol)) -2000, DSPE-PEG; Methotrexate, MTX; DSPE-PEG-methotrexate, DSPE-PEG-MTX; Dox-loaded, PEG-decorated self-assembled ZnPc-SPC complex NPs, DZSP; Dox-loaded, MTX-decorated self-assembled ZnPc-SPC complex NPs, DZSM

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photobiologic effects causing damage to tumors when the tumor-localizing photosensitizing agent was activated by the light with specific wavelength [8]. Certain kinds of photosensitizers, such as 5-ALA, 5-aminolevulinate, photofrin, foscan, verteporfin, *etc.*, have already been approved for use in humans for various human disease, especially cancers [7]. Recent studies suggested that nanostructured porphyrin-coupled lipid-assembled-porphysomes (porphyrin-lipid conjugates were generated by an acylation reaction between lysophosphatidylcholine and pyropheophorbide) could be used to achieve optical therapeutic modality [9]. However, it primarily focused on the fluorescence self-quenching, PTT and PA properties. Rarely studies concerned about the use of second generation photosensitizer to form lipid-complex structure for other photobiologic effects such as PDT.

The well-known and commonly studied second-generation photosensitizer zinc phthalocyanine (ZnPc) exhibits many advantages in PDT, although its poor water solubility, unfavorable pharmacokinetics, inadequate accumulation in tumor tissues need to be overcome using the novel drug delivery systems [10–13]. In our previous work, we have developed ZnPc-SPC complex (photosensitizer and phospholipid were complexed by hydrogen bond and intermolecular interaction) which could self-assembled to NPs (also known as phytosome) to achieve supreme switchable PDT effect with high drug loading compared with other delivery methods of ZnPc. While in present work, the property of ZnPc-SPC complex have been further developed. ZnPc-SPC complex self-assembled NPs (ZS) could not only serve as the carrier of drug cargo with other treatment mechanism, but also the multifunctional theranostic agent. It appeared to be a novel strategy of combination therapy to enroll the carrier itself in cancer treatment. Moreover, on the one hand, when ZnPc-SPC complex self-assembled into intact NPs, due to the strong π-stacking causing light-to-heat energy conversion, the NPs could itself perform early-phase PTT effect under the irradiation of 638 nm laser for tumor ablation w/wo the loading of chemical drug. As known, photoacoustic signal generation is related to thermal expansion, thus the intact assemble NPs could generate strong PA signals utilized for PA image. On the other hand, when the NPs dis-assembled into ZnPc-SPC complex monomers again, reactive oxygen species (ROS) could be produced again when triggered by 638 nm laser to activate the late-phase PDT to achieve tumor cell apoptosis. Besides, the monomers could be utilized to perform low-background *in vivo* fluorescence precisely. Furthermore, anti-cancer chemotherapy drug Dox as the loading cargo with considerable loading efficiency, could be delivered to tumor cells and released in a pH-depended manner to accurately treat tumor cells to perform the synergetic treatment.

The Dox-loaded ZnPc-SPC complex self-assembled NPs was determined as DZS. To enhance *in vivo* pharmacokinetics, 2-diacyl-SN-glycero-3-phosphoethanolamine-N-[methoxy(poly(ethyleneglycol))-2000 (DSPE-PEG) was further included and the decorated DZS was named as DZSP [14]. Additionally, the affinity and the relative accumulation in tumor region were acquired to permit the signal readouts and curative targeted treatment [15, 16]. Previously synthesized DSPE-PEG-methotrexate (DSPE-PEG-MTX) was also inserted to improve the targeting ability to folate receptors α (FRα) over-expressed tumor cells and finally formed Dox-loaded, MTX-decorated self-assembled ZnPc-SPC complex NP, abbreviating as DZSM. This novel prepared DZSM could potentially serve as the candidate for synergetic tumor theranostic due to its excellent tumor-targeting ability, switchable PA/FL image, multiphase PTT&PDT effect and chemotherapy.

2. Materials and methods

2.1. Materials

All chemical reagents were obtained from commercial suppliers and of analytical grade without further purification only if stated. Deionized (DI) water was used throughout. Zinc phthalocyanine (ZnPc) was purchased from Sigma-Aldrich (USA). Soybean phosphatidylcholine (SPC) was obtained from Degussa AG (Germany). Doxorubicin was provided by Zhejiang Hisun Pharmaceutical Co., Ltd. (Zhejiang, China). Methotrexate (MTX) was supplied by Bio Basic Inc. (Markham, Ontario, Canada). 1, 2-diacyl-SN-glycero-3-phosphoethanolamine-N-[methoxy (poly(ethyleneglycol))-2000] (DSPE-PEG-OCH_3), 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [amino (polyethylene glycol)-2000] (DSPE-PEG-NH_2), and N-hydroxysuccinimide (NHS) were purchased from Shanghai Ponsure Biotechnology (Shanghai, China). Dicyclohexylcarbodiimide (DCC) was obtained from J&K Chemical Scientific Co. Ltd. (Beijing, China). Dimethyl formamide (DMF) and tetrahydrofuran (THF) were bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). HeLa, MCF-7, and 4T1 cells were supplied by American Type Culture Collection (ATCC). RPMI medium modified and Dulbecco's minimum essential medium/high glucose (DMEM/high glucose) were purchased from GE Healthcare Life Sciences HyClone (USA). Fetal bovine serum (FBS) was obtained from Zhejiang Tianhang Biological Technology Co., Ltd. (Hangzhou, China). Trypsin and penicillin-streptomycin were bought from Beyotime Biotechnology (Shanghai, China). Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was obtained from Sigma-Aldrich. DCFH-DA were obtained from Xiamen Lulong Biotech Co. Ltd. (Xiamen, China). The dialysis bags (MWCO = 3500) were bought from Shanghai yuanye Bio-Technology Co., Ltd. (Shanghai, China). Female BALB/c nude mice and ICR mice were provided by Xiamen University Laboratory Animal Center. All animal procedures were complied with the guidelines of the Xiamen University Institutional Animal Care and Use Committee.

2.2. Preparation and characterization of ZnPc-SPC complex

ZnPc-SPC complex was synthesized by co-solvent method with modification. Firstly, 5 mg of ZnPc and 35 mg of SPC were dissolved in 10 mL of tetrahydrofuran (THF) and magnet stirred in 40 °C for at least 6 h to obtain ZnPc-SPC complex. X-ray diffractometer was performed to evaluate the phase of ZnPc-SPC complex using Phillips X' pert Pro Super, Panalytical (Almelo, Netherlands). ^{1}H NMR spectrum was displayed *via* a Bruker AV400 MHz NMR spectrometer (Bruker, Billerica, MA, USA). FTIR analysis was performed to determine the interaction between ZnPc and SPC on a Bruker IFS-55 infrared spectrometer (Bruker, Zurich, Switzerland). UV–vis-NIR absorption spectrum of ZnPc-SPC complex was scanned using a Perkin Elmer Lambda 750 UV–vis-near-infrared spectrophotometer (Perkin-Elmer, Norwalk CT).

2.3. Preparation and characterization of ZS and DZSM

ZS and DZSM was constructed by solvent evaporation method followed by a self-assemble technique. Solvent above containing 1 mg of ZnPc was taken and THF was removed later by reduced pressure evaporation using a rotary evaporator (N-1300, EYELA, Shanghai, China). A thin film of ZnPc-SPC complex was formed and 3 mL of DI water was added followed by ultrasonic dispersion using an ultrasonic bath cleaner (SK7200HP KUDOS, Shanghai, China). ZS was then formed and clear colloidal suspension was obtained. The suspension was diluted to 10 mL and ZS colloid suspension containing ZnPc equivalent to 0.1 mg/mL was obtained. Above ZnPc-SPC complex dissolved in THF containing 1 mg of ZnPc was taken and THF was removed later by reduced pressure evaporation. A thin film of ZnPc-SPC complex was formed and 3 mL of 40 μg/mL ammonium sulfate solutions was added followed by ultrasonic dispersion. The formed clear colloidal suspension was added into dialysis bag (MW = 3000) and dialyzed for at least 1 h. DSPE-PEG-MTX was synthesized through amide reaction between DSPE-PEG-NH_2 and MTX. The above dialyzed colloidal suspension, 1 mg of Dox, 0.5 mg of DSPE-PEG-MTX and 0.5 mg of DSPE-PEG-OCH_3 were mixed, diluted to 10 mL, and stirred overnight to get DZSM colloid suspension containing ZnPc equivalent to 0.1 mg/mL. DZS was synthesized with the same method except that DSPE-PEG-MTX and DSPE-PEG-OCH_3 should not be added. DZSP was synthesized with the same method except that