Enhanced Nuclear Localization of Photosensitizer Using Artificial Oil Bodies for Photodynamic Therapy

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ABSTRACT
Photodynamic therapy (PDT) is a light-activated photochemical reaction that induces localized tissue damage for the treatments of cancers and other nonmalignant conditions through the generation of reactive oxygen species. In the clinic, patients treated with PDT reveal strong skin photosensitivity and thus should be kept away from direct sunlight. In our previous study, we demonstrated that the skin phototoxicity of meta-tetra(hydroxyphenyl)chlorin (m-THPC), a photosensitizer used in the clinic, can be significantly reduced after micellar encapsulation. In this present work, m-THPC was loaded into artificial oil bodies (AOBs) to improve its biocompatibility with self-aggregation. Our results show that the m-THPC-loaded oil bodies with particle size around 100 nm significantly enhanced the nuclear localization of m-THPC, whereas m-THPC-loaded Pluronic® F68 micelles were only observed in the cytosol. In addition, m-THPC-loaded oil bodies had better PDT efficacy than those m-THPC-loaded polymeric micelles. It is speculated that the nuclear accumulation of m-THPC using AOBs as drug carriers significantly enhanced the PDT effects in cancer cells. Thus, m-THPC-loaded oil bodies with improved anticancer efficacy have a great potential for developing nanotechnology-based PDT.

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1. Introduction
Photodynamic therapy (PDT) is a light-induced chemical reaction resulting in local damage by reactive oxygen species generation (ROS) following activation of the photosensitizers (PSs) with a specific light source. PDT is currently used in the treatment of cancer and various nonmalignant conditions but with skin photosensitivity which limits the clinical applications of this strategy.[1,2] In addition, hydrophobic PSs tend to aggregate in aqueous solution, thereby reducing PDT’s efficacy because of the

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less ROS generation, [3] the strategy of providing a suitable carrier for reducing the adverse effects and enhance the therapeutic efficiency had a giant potential in the development of PDT-mediated cancer therapy.[4]

meta-Tetra-hydroxyphenyl-chlorin (m-THPC, Foscan®) is a second-generation PSs with potent antitumor efficacy for clinical use in Europe.[5] Although m-THPC is less phototoxic to the skin than the first generation of PSs, patients receiving m-THPC still need to avoid direct sunlight for more than two weeks.[6] Recently, nanotechnology-based PSs delivery systems, such as polymer–drug conjugates,[7] nanoparticles,[8,9] and polymeric micelles,[10–14] have been developed at our group for improving hydrophobic PSs delivery without aforementioned problems.

Using the artificial oil bodies (AOBs) as drug hydrophobic carriers in this study was inspired from the sesame oil body. The triacylglycerol (TAG) stored in sesame oil body is the nutrition for germination and growth and several structural proteins, such as oleosin, caleosin, and sterolesin, can stabilize the structure of sesame oil body.[15–19] In this study, AOBs with self-assembled property were used as a drug carrier for the delivery of m-THPC. In addition, the recombinant caleosin protein, a minor protein in oil bodies of angiosperm seeds, was used to increase the stability of AOBs. We found that the self-assembly m-THPC-loaded AOBs revealed a particle size less than 120 nm with narrow polydispersity. The intracellular distribution and phototoxicity of m-THPC-loaded AOBs were also evaluated in this study. Our results clearly show that m-THPC-loaded AOBs have great therapeutic potential for treating cancers.

2. Experiment

2.1. Materials

1,2-Dioleoyl-Sn-glycerol-3-phosphocholine (DOPC), methanol, disodium hydrogen phosphate (Na₂HPO₄) and sodium dihydrogen phosphate (NaH₂PO₄), Pluronic F68, tetrachloro-p-benzoquinone, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (Milwaukee, WI, U.S.A.). Dimethyl sulfoxide (DMSO) and tetrahydrofuran (THF) were purchased from Tedia Inc. (Fairfield, OH, U.S.A.). m-THPC was obtained from Biolitec Pharma Ltd. Caleosin was prepared as previous report.[17] TAG was extracted from sesame oil body as previous report.[16]

2.2. Cell Line

Human cervical cancer HeLa cells were maintained in a humidified 5% CO₂ incubator at 37 °C in DMEM (GIBCO BRL, Gaithersburg, MD, U.S.A.) supplemented with 10% heat-activated fetal bovine serum (FBS).

2.3. Preparation of m-THPC-loaded AOBs or Micelles

In brief, empty AOBs were prepared by mixed the 20 mg of TAGs, 150 μg of DOPC, and 250 μg of caleosin in 1 ml of 10 mM sodium phosphate buffer at pH = 7.5. The m-THPC-loaded AOBs (m-THPC-AOBs) were prepared by mixed the 20 mg of photosensitizer/TAG mixture, 150 μg DOPC, and 250 μg of caleosin in 1 mL of 10 mM sodium phosphate buffer at pH = 7.5. The scheme of preparation process was shown in Figure 1. The m-THPC-loaded F68 micelles (m-THPC-F68) were prepared using solvent evaporation method as our previous report.[12, 14] The resultant solution was filtered through a 0.45-μm filter to remove unencapsulated m-THPC aggregates. The particle size of AOBs were measured using dynamic light scattering (DLS, Zetasizer Nano ZS, Trek, INC) with a 633-nm laser source with at least three independent measurements. The morphology of m-THPC-loaded AOBs was also observed by a confocal microscope (CLSM; Leica Microsystems Heidelberg GmbH, Heidelberg, Germany).

2.4. Intracellular Localization of m-THPC-loaded AOBs or m-THPC-loaded F68 Micelles in HeLa Cells

To investigate the cellular distribution of m-THPC-loaded nanoparticles, cells were seeded onto glass coverslips in 35-mm dishes for 24 h then treated with m-THPC-loaded AOBs or m-THPC-loaded F68 micelles for 1 h, respectively. The treated cells were then observed by using a confocal microscope (CLSM; Leica Microsystems Heidelberg GmbH, Heidelberg, Germany) with Ex 405 nm.

2.5. Photocytotoxicity of m-THPC-loaded Particles

To investigate the phototoxicities of the m-THPC-loaded AOBs or m-THPC-loaded F68 micelles, cells were first seeded into 96-well plates at a density of 5000 cells per well for 24 h. The nanoparticles were then added into the wells
in a total volume of 0.1 ml at 37 °C. After 2 h incubation, cells were washed twice with PBS and then were exposed to blue light (410–430 nm, light dose: 1 J/cm²). Cells were then washed and incubated for another 24 h. Cell viability was evaluated by MTT assay using a scanning multiwell ELISA reader at 485 nm (SpectraMax® M2e, Molecular Devices, U.S.A.).[20]

3. Results and Discussion

3.1. Characterization of m-THPC-loaded AOBs Without or with Caleosin

The hydrodynamic diameter of empty AOBs or m-THPC-loaded AOBs without or with caleosin co-construction was evaluated by DLS. As shown in Figure 2, AOBs with caleosin showed the particle size around 158.3 nm. All the measurements were summarized in Table 1. The particle size of AOBs was significantly reduced after the m-THPC loading may due to the increase of hydrophobic interaction between the PS and the alkyl chains of the DOPC.[21] AOBs without or with caleosin co-construction both showed narrow polydispersity less than 0.3 in aqueous solution. It is noticed that the AOBs in the presence of caleosin revealed smaller particle size around 106.7 nm than the 122.2 nm of AOBs which was without caleosin. The caleosin is the main stabilizer protein on the surface of AOBs that can stabilize the structure and avoid the aggregation of AOBs via electrical repulsion effects and thus reduced the size of AOBs.[17,22] In addition, the nanoparticles with size less than 200 nm may increase the accumulation of cytotoxic agents in tumor tissues based on enhanced permeability and retention (EPR) effects.[23] Thus, the m-THPC-loaded AOBs with particle size around 100 nm may performed as a potential carrier to increase the accumulation of PSs in tumor and thus provide efficiently killing abilities for cancer therapy.

3.2. Morphological Observation of m-THPC-loaded AOBs Using Confocal Microscopy

The PSs-loaded AOBs can exhibit fluorescent signal and can be observed using the confocal microscopy according to the fluorescent properties of m-THPC that can be activated by light wavelength at 405 nm. The morphologies of AOBs and m-THPC-AOBs observed by confocal microscopy were shown in Figure 3. The fluorescence of m-THPC can be easily observed in m-THPC-loaded AOBs which revealed spherical in shape. Thus, the hydrophobic can be successfully encapsulated into the AOBs for further biological tests. The encapsulation efficiency of m-THPC is around 80%.

3.3. Intracellular Distribution of m-THPC-loaded AOBs in HeLa Cells

To investigate the intracellular distribution of m-THPC using AOBs carrier in vitro, cells were treated with m-THPC-loaded particles for 1 h and observed by confocal microscopy. The m-THPC-loaded F68 micelles were utilized as a control group. As shown in Figure 4(A) and (B), the fluorescence signal of m-THPC-formulated pluronic F68 micelles was observed only in the cytosol. The major intracellular localization of m-THPC is endoplasmic reticulum and Golgi bodies and our previous report also demonstrated the intracellular distribution of m-THPC-loaded polymeric micelles was in the cytosol, not nucleus.[12,14] Intriguingly, the m-THPC fluorescence in nuclei was significantly observed using AOBs carriers after 1-h incubation which indicates the intracellular fate of m-THPC was supposed to be altered via the using of drug carriers (Figure 4(C) and (D)). This finding may due to the interaction between membrane structure and AOBs. In addition, membrane blebbing was observed in the group of m-THPC-loaded AOBs after PDT treatment. Thus, the different intracellular distribution of PSs may cause different PDT efficacy in vitro.
F68 micelles with light irradiation was observed more than 600 ng/ml. The enhancement of PDT efficacy of m-THPC-loaded AOBs was mainly due to the nuclear localization of m-THPC (Figure 4). It has been demonstrated that the nuclei in living cells are very sensitive to ROS.[24,25] Thus, the nanocarriers that facilitate the nuclear accumulation of PSs may cause more efficient killing abilities for cancer treatments. Poly-L-lysine, a cationic polymer with high affinity for DNA, can deliver PSs into

3.4. Phototoxicities of m-THPC-loaded Pluronic F68 Micelles or AOBs in HeLa Cells

The PDT effects of m-THPC-loaded Pluronic F68 micelles or AOBs were evaluated using MTT assay in HeLa cells. The m-THPC-loaded AOBs-mediated PDT suppressed cell viability around 85% cancer cells at 500 ng/ml m-THPC concentration after light irradiation (Figure 5). Cells treated with m-THPC-loaded AOBs revealed IC50 around 180 ng/ml, whereas the IC50 of m-THPC-loaded F68 micelles with light irradiation was observed more than 600 ng/ml. The enhancement of PDT efficacy of m-THPC-loaded AOBs was mainly due to the nuclear localization of m-THPC (Figure 4). It has been demonstrated that the nuclei in living cells are very sensitive to ROS.[24,25] Thus, the nanocarriers that facilitate the nuclear accumulation of PSs may cause more efficient killing abilities for cancer treatments. Poly-L-lysine, a cationic polymer with high affinity for DNA, can deliver PSs into

\[ \text{Figure 3. Morphology of m-THPC-loaded AOBs observed by confocal microscopy. The wavelength of excitation is 405 nm and emission is 600 nm. (A) Bright view, (B) fluorescence, and (C) merge images of m-THPC-loaded AOBs.} \]

\[ \text{Figure 4. Intracellular distribution of m-THPC using Pluronic F68 micelles or AOBs drug carriers after 1-h incubation in HeLa cells. (A, B) m-THPC-loaded Pluronic F68 micelle or (C, D) m-THPC-loaded AOBs. Ex/Em: 405 nm/600–700 nm.} \]
nucleus with enhanced photodynamic effect. Thus, AOBs may be potential drug carriers from the natural products for nuclear delivery of m-THPC. In ongoing work, we are preparing the tumor-targetable AOBs for PSs delivery and in vivo PDT efficacy of this particle will be investigated in the near future.

4. Conclusions

In this study, we demonstrated new carrier AOBs for m-THPC delivery that can enhance the nuclear accumulation of PSs and thus increase the PDT efficacy in vitro. The caleosin in AOBs can stabilize the structure of AOBs and reduce its particle size to around 100 nm. These particles with ideal size may have the potential for further investigation in vivo with EPR effects. Thus, m-THPC-loaded AOBs with improved anticancer efficacy have a great potential for developing nanotechnology-based PDT.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Figure 5. Phototoxicity of m-THPC-loaded Pluronic F68 micelles or AOBs with 2-h incubation under blue light irradiation in HeLa cells.
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