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# Reduced Skin Photosensitivity with *meta*-Tetra(hydroxyphenyl)chlorin-Loaded Micelles Based on a Poly(2-ethyl-2-oxazoline)-*b*-poly(D,L-lactide) Diblock Copolymer in Vivo

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**Abstract:** Photodynamic therapy (PDT) is a light-induced chemical reaction that produces localized tissue damage for the treatment of cancers and other nonmalignant conditions. The activation of photosensitizers in a target tissue is accomplished with a specific light source in the presence of molecular oxygen. In the clinic, patients treated with PDT should be kept away from direct sunlight or strong indoor lighting to avoid skin phototoxicity. In this study, a photosensitizer encapsulated within a micelle was developed to overcome this problem. The pH-sensitive micelles were successfully incorporated with *meta*-tetra(hydroxyphenyl)chlorin (m-THPC), and the cytotoxicity and antitumor effects were investigated *in vitro* and *in vivo*. Our results demonstrated that PDT with m-THPC-loaded micelles had no significant adverse effects on the body weight of mice *in vivo*. Furthermore, after an extended delivery time, m-THPC-loaded micelles and free m-THPC had similar antitumor effects, but the m-THPC-loaded micelles had less skin phototoxicity. Thus, this strategy could be used as a potential nanocarrier for PDT-mediated cancer therapy.

**Keywords:** Photodynamic therapy; photosensitizer; micelle; cancer therapy; photosensitivity

## 1. Introduction

Photodynamic therapy (PDT) is a light-induced chemical reaction that produces localized tissue damage for the treatment of cancerous as well as other nonmalignant conditions. The activation of photosensitizers in the target tissue is accomplished with a specific light source in the presence of molecular oxygen.[1] Important advantages of PDT over other therapies include the ability to deliver a precisely targeted treatment through selective illumination, the potential for repeated application at the same site if needed, and that it is less invasive than surgery. Despite these significant advantages, the biodistribution of photosensitizers is unfavorable, and phototoxicity to the skin, mainly caused by an agent's hydrophobicity and nonselectivity, is a considerable limitation of their use.[2] An ideal photosensitizer should be nontoxic if it has not been irradiated. However, it should generate a

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large amount of damage to cells when exposed to a specific light source of a long wavelength, which has advantages in the treatment of deeper lesions.[3] In addition, this agent should accumulate quickly in the target tissue and demonstrate rapid clearance, and tumor selectivity must also be considered.[4]

Hydrophobic photosensitizers tend to aggregate in aqueous solution. This results in less photoactivation and generates less $^1O_2$ in solution, which subsequently influences the phototoxicity toward tumor cells.[5] Recently, nanomaterials such as polymer−drug conjugates,[6] liposomes,[7] nanoparticles,[8,9] and polymeric micelles[3,10,11] have been considered as potential carriers for hydrophobic drug delivery and may resolve the aforementioned problems. Polymeric micelles composed of amphiphilic block copolymers possess many attractive properties in drug delivery systems, such as good biocompatibility and high stability *in vitro* and *in vivo*, and can be successfully used for the encapsulation of various poorly soluble agents.[12] These nanosized micelles consist of a hydrophilic outer shell and a hydrophobic inner core that can be used to incorporate lipophilic drugs in aqueous solution for intravenous administration.[13−15] Often, these micelles are coassembled with unique macromolecules that can allow the targeted delivery to tissues of interest. The guided delivery of these cytotoxic drugs can be accomplished by a variety of means including magnetic[16] or ligand-directed methods.[17] Furthermore, the controlled release of drugs in response to environmental cues can be achieved by using micelles formed from stimuli-sensitive copolymers.[18−21]

Indeed, polymeric micelles that are pH-responsive are emerging as attractive drug delivery systems.[22] Following intravenous administration, polymeric micelles are taken up by cells via endocytosis. Subsequently, the pH value of the endocytic vesicle is gradually decreased from 7.4 to 5 due to the protons that are pumped into the lumen of the vesicle.[20] The poly(2-ethyl-2-oxazoline)-*b*-poly(D,L-lactide) (PEOz-*b*-PLA) diblock copolymer formed micelle has been demonstrated *in vitro* as a potential drug carrier with pH sensitivity, low cytotoxicity, and a favorable p$K_a$ value near neutral pH that can facilitate intracellular drug release in an acid environment.[23−25] Therefore, this delivery system allows hydrophobic drugs to be protected by micelles while cir-


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culating in the blood after intravenous administration, and they will only be released from their carriers when in the cytosol of cells.

The aim of this study was to evaluate the potential of a pH-responsive PEOz-*b*-PLA formed micelle as a photosensitizer carrier for PDT. The hydrophobic agent encapsulated was *meta*-tetra(hydroxyphenyl)chlorin (m-THPC), which is a second-generation photosensitizer with potent antitumor efficacy and has been approved for human use in Europe.[26] This agent was encapsulated in a micellar formulation, and its drug release, cytotoxicity, cellular uptake, and PDT effects were studied *in vitro*. In addition, the antitumor effect and skin phototoxicity of m-THPC and m-THPC-loaded micelles were evaluated *in vivo* with HT-29 human colorectal cancer xenografts in mice. Clearly, m-THPC-loaded micelles have great therapeutic potential for treating cancers, as well as other disease states.[27]

## 2. Materials and Methods

**2.1. Materials.** D,L-Lactide (Sigma Aldrich, St. Louis, MO) was recrystallized from acetone. 2-Ethyl-2-oxazoline (Sigma Aldrich) and methyl *p*-toluenesulfonate (Sigma Aldrich) were purified by vacuum distillation over $CaH_2$. Toluene was distilled over sodium benzophenone under dry nitrogen. Stannous octoate (Sigma Aldrich), dimethyl sulfoxide (DMSO) (Sigma Aldrich), ethanol (Tedia, Fairfield, OH), and diethyl ether (Tedia) were used as received.

**2.2. Synthesis and Characterization of PEOz-*b*-PLA Copolymers.** Monohydroxyl poly(2-ethyl-2-oxazoline) (PEOz−OH) was first synthesized by the cationic ring-opening polymerization of 2-ethyl-2-oxazoline (5 mL) in toluene (10 mL) at 100 °C for 3 h using methyl *p*-toluenesulfonate (92.6 $\mu$L) as the initiator. After cooling down to room temperature, 25 mL of methanolic KOH (0.1 N) was added and stirred for 1 h. The solvent of crude product was removed and then redissolved in 50 mL of ethyl acetate for flowing through silica gels. The resulting PEOz−OH was precipitated in diethyl ether and vacuum-dried for 24 h. PEOz-*b*-PLA diblock copolymer was synthesized by polymerization with D,L-lactide (2.33 g) using PEOz−OH (4 g) as an initiator. The reaction was catalyzed by stannous octoate (31 mg) in toluene (32 mL) at 140 °C for 12 h. The resulting PEOz-*b*-PLA copolymer was dialyzed (MWCO 3500, SPECTRUM) in DMSO and pure water for 2 days. The molecular weight and molecular weight distribution of PEOz−OH were analyzed by gel permeation chromatography (GPC, pump: SpectraSystem P100, TSP) with a refractive index detector (Refracto-Monitor IV, TSP). The composition of the copolymer was analyzed by $^1$H NMR (Bruker Avance-500 MHz FT-NMR) in $CDCl_3$. The critical micelle concentration (CMC) of each copolymer was determined by a fluorescence technique with pyrene as a hydrophobic probe.[28]

**2.3. Preparation of m-THPC-Loaded Micelles.** Ten milligrams of block copolymer and 1 mg of m-THPC were dissolved in 10 mL of ethanol in a round-bottom flask. The solvent was removed by rotary evaporation, and the resulting thin film was hydrated with 5 mL of PBS at room temperature to give a final aqueous suspension of 0.2 mg/mL in 10 wt %/vol PEOz-*b*-PLA drug/polymer ratio. The micelle solution was filtrated by a 0.45 $\mu$m syringe filter to remove the unloaded m-THPC. A high performance liquid chromatograph (HPLC) consisting of a Waters 510 pump and a Waters 2487 absorbance detector was used to measure the amount of loaded m-THPC in micelles. The absorbance was measured at 420 nm with a mobile phase of 1% trifluoroacetic acid aqueous solution/acetonitrile (1/1), and the flow rate was 1 mL/min. Encapsulation efficiency was calculated by the ratio of m-THPC weight before and after filtration. Particle size was measured by a Zetasizer Nano ZS (Malvern Instruments, Worcestshire, U.K.). The absorption or fluorescence emission spectra of m-THPC-loaded micelle were measured using UV−visble spectrophotometer (U-3000, Hitachi, Japan) or fluorescence spectrofluorometer (FP-6300, Jasco, Japan), respectively.

**2.4. Release Profiles of m-THPC from Micelles.** Release of m-THPC from the micelles was studied at 37 °C using a dialysis-bag diffusion technique.[29] Briefly, 1.25 mg of m-THPC-loaded micelles was dispersed in 5 mL of a buffer at pH 7.4. The buffer solution was transferred to dialysis membrane tubing, and the dialysis bag was immersed in 500 mL of release medium with continuous gentle stirring at 37 °C. At predetermined time intervals, 5 mL aliquots of the aqueous solution were withdrawn from the release medium and replaced with the same volume of fresh medium. The amount of m-THPC was estimated by HPLC as described above. All experiments were carried out in triplicate.

**2.5. Cell Lines and Culture Conditions.** Human colorectal adenocarcinoma (HT-29) cells were maintained in a humidified 5% $CO_2$ incubator at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL) and 1% antibiotics (Antibiotic-Antimycotic, Gibco BRL).

**2.6. Cellular Uptake of Photosensitizing Agents.** Cells were seeded into 96-well plates at a density of $2 \times 10^4$ cells

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per well. After 24 h, cells were then incubated with 2 $\mu$g/mL m-THPC or an equivalent amount of m-THPC-loaded micelles for various times. After washing, cells were incubated with DMSO for 15 min at 37 °C. Cell lysates were collected and measured using a fluorescence microplate reader (Gemini EM, Molecular Devices, Munich, Germany) with excitation/emission wavelengths equal to 420/625 nm, respectively. Data were obtained from at least 3 independent experiments.

**2.7. PDT Treatment of m-THPC and m-THPC-Loaded Micelles *in Vitro*.** Cells were seeded into 96-well plates at a density of $2 \times 10^4$ cells per well and cultured for 24 h. To determine the cytotoxicity of the m-THPC and m-THPC-loaded micelles, cells were incubated in medium containing different concentrations of m-THPC or m-THPC-loaded micelles for 24 h, washed, and then subjected to cell viability assays. To determine the effects of photosensitizer concentration on cytotoxicity, the cells were incubated with different concentrations of m-THPC or m-THPC-loaded micelles for 24 h at 37 °C, washed, immediately exposed to light for 60 s (0.96 J/cm$^2$), and then evaluated in cell toxicity assays after 24 h of incubation. To investigate the effects of light intensity on cytotoxicity, the cells were incubated with 0.5 $\mu$g/mL of photosensitizing agents for 24 h at 37 °C, washed, and immediately illuminated with different light intensities (0.12, 0.48, 0.96, 1.92, 2.88, and 4.8 J/cm$^2$). The illuminated cells were then evaluated in cell viability assays after 24 h of incubation as described below.

After the addition of photosensitizing agents, all the procedures were carried out in subdued light. The light source, which consisted of an array of light emitting diodes (LEDs; covering a spectral region of 650−670 nm with the peak intensity at about 660 nm) for activating m-THPC was obtained from ITRI (Hsin Chu, Taiwan) and the fluence rate was 16 mW/cm$^2$.

**2.8. Cell Viability Assay.** The cell viability was determined by using the MTT assay.[30] The assay was performed in triplicate on cells seeded onto 96-well plates. After treatment, cell media was replaced with fresh medium containing 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which was followed by incubation at 37 °C for 3 h. Subsequently, wells underwent as complete an aspiration as possible without disturbing the formazan crystals and cells on the plastic surface. Finally, 100 $\mu$L of DMSO was added to each well, which was followed by shaking of the plates on a plate shaker for 15 min. Colorimetric measurements were performed using a scanning enzyme-linked immunosorbent assay (ELISA) reader (550 nm; Microplate Autoreader EL311, Bio-Tek Instruments Inc., Winooski, VT).

**2.9. Tumor Response of PDT *in Vivo*.** All animal studies were approved by the Institutional Animal Care Committee. Female BALB/cAnN.Cg-*Foxn1*$^{nu}$/CrlNarl mice (4−5 weeks old, 19−21 g) were purchased from the National Laboratory Animal Center (Taipei, Taiwan). The HT-29 cell line ($5 \times 10^6$ cells/0.1 mL DMEM) was implanted subcutaneously (sc) into the right hind leg of each mouse. When the tumors reached 3 to 5 mm in diameter, a total of 24 healthy mice were randomly divided into five groups. Mice in groups 1 and 2 received intravenous (iv) injection of 0.3 mg/kg free m-THPC (BioLITEC PHARMA, Edinburgh, U.K.). Mice in group 3 and group 4 were injected intravenously with 0.3 mg of micellar m-THPC equivalents/kg. Four mice in group 5 received iv injection of sterilized PBS (150 $\mu$L/mouse) as a control. Twenty-four hours (groups 1, 3, and 5) or 48 h (groups 2 and 4) after drug injection, mice were treated with light using a 652 nm diode laser (Applied Optronics Corp., South Plainfield, NJ) to give a single dose of 10 J/cm$^2$, and the light spot diameter was 1 cm.[31]

The day of photosensitizer injection was considered as day 0. The body weight and tumor volumes of the mice were measured every 2−3 days for a total of 19 days after treatment. Relative tumor volume was calculated as the ratio $V/V_0$, where $V_0$ was the initial tumor volume and $V$ was the tumor volume monitored after treatment. Tumor volume was calculated using the following formula: $V = \pi/6(abc)$, where $a$ and $b$ were two perpendicular axes, and $c$ was the height measured using a caliper. The effectiveness of each treatment was determined by comparison of the mean tumor volume doubling time of each group, defined as the number of days the tumor required to double its pretreatment volume.

**2.10. *In Vivo* Skin Photosensitivity.** PDT-related skin photosensitivity was evaluated in mice treated with m-THPC or m-THPC-loaded micelles. A quantitative skin scoring system was utilized to evaluate the degree of normal skin damage induced by PDT.[32] The left hind leg of each animal was treated with PDT in a manner identical to that utilized in the tumor response studies. A minimum of five mice were evaluated at each drug concentration with a 10 J/cm$^2$ light dose.

**2.11. Statistical Analysis.** All data were expressed as mean ± standard deviation. To test the difference in fluorescence intensity at a specific time-point, an independent $t$ test was used. For cell viability at a specific concentration of m-THPC, the differences among all groups were tested by a linear mixed model; meanwhile, the Bonferroni test was implemented for post-hoc analysis. The linear mixed model with repeated measures was performed to examine the effects of treatment group, dose of irradiation, and time after these variables. The difference among groups with a given time or dose was performed using a Bonferroni test with an

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***Scheme 1.*** Synthesis and Preparation of Photosensitizer-Loaded PEOz-*b*-PLA Formed Micelle

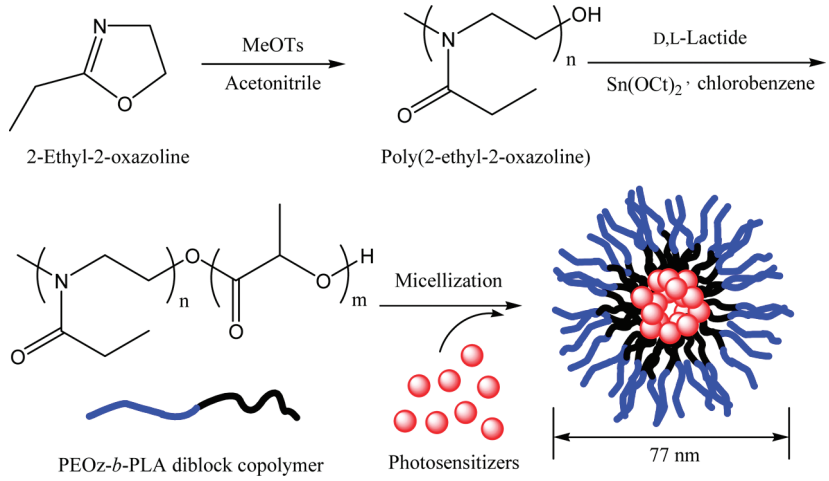


***Table 1.*** Characterizations of PEOz or PEOz-*b*-PLA Polymers

| polymer code | $M_n$ | polydispersity index[a] | yield (%) | CMC (wt %) |
|---|---|---|---|---|
| PEOz | 5940[a] | 1.1 | 95 | |
| PEOz-*b*-PLA | 7086[b] | 1.2 | 89 | $7 \times 10^{-4}$ |

[a] Estimated by GPC. [b] Estimated by $^1$H NMR.

adjusted alpha value of 0.01. All statistics were performed by SAS software (version 9.1.3, SAS Inc., Cary, NC), and all figures were generated by Sigma plot software (version 8.0, SPSS Inc., Chicago, IL). A $p$ value less than or equal to 0.05 was considered significant.

## 3. Results

**3.1. Preparation and Characterization of m-THPC-Loaded Micelles.** In order to obtain the water-soluble micellar m-THPC formulation, the PEOz-*b*-PLA copolymer was synthesized and characterized as described previously.[20,24,33] First, the PEOz−OH macromonomers were synthesized by cationic ring-opening polymerization. Subsequently, the diblock copolymers consisting of the biodegradable PLA block and the water-soluble PEOz block were synthesized by ring-opening polymerization from PEOz−OH and D,L-lactide using stannous octoate as a catalyst (Scheme 1). The composition and molecular weight of the PEOz-*b*-PLA diblock copolymer were determined by comparing the integral peak area associated with the methyl groups of PLA (1.54 ppm) and that of PEOz (1.09 ppm) (data not shown).[23] The molecular weight ($M_w$) of PEOz-*b*-PLA as determined by $^1$H NMR was 7086, and the polydispersity index was 1.2 as determined by GPC (see Table 1). The CMC of the PEOz-*b*-PLA diblock copolymer was measured by a fluorescence spectrophotometer using pyrene as a hydrophobic probe. In the presence of micelles, pyrene is transferred from the aqueous environment to the nonpolar region of the micelle interior core, which results in significant spectroscopic changes of this fluorophore. Consequentially, a red shift in the excitation spectrum of pyrene was observed when the copolymer concentration was increased, and the CMC of the copolymers was estimated based on the relative excitation fluorescence intensity ratio of $I_{337.5}/I_{335.5}$. The synthesized PEOz-*b*-PLA exhibited a low CMC (0.0007 wt %), which may provide relative stability when in systemic circulation.

Drug encapsulation was performed in parallel with micelle formation using the thin film method. At a 1:10 feed ratio of drug to PEOz-*b*-PLA diblock polymer, 92.5% of m-THPC was loaded into micelles with an average approximate diameter of 77 nm, and the polydispersity of the resulting micelles was 0.28 as estimated by GPC. For *in vitro* and *in vivo* studies, the m-THPC-loaded micelles were prepared at an approximate concentration of 0.185 mg/mL. The characterization of m-THPC-loaded micelle is summarized in Table 2. The absorption and fluorescence spectra of m-THPC-loaded micelle are shown in Figure S1 in the Supporting Information. It is obviously that free m-THPC, a lipophobic photosensitizer, showed strong fluorescence in DMSO. However, free m-THPC exhibited a relatively broad and weak fluorescence in aqueous solution, while the fluorescence of m-THPC-loaded micelle was strong and reached a maximum at 655 nm. Thus, the m-THPC which was loaded into hydrophobic core of polymeric micelle may generate efficient $^1O_2$ in aqueous solution.

**3.2. m-THPC Release Profile from Micelles.** The *in vitro* release behavior of m-THPC-loaded micelles in two different buffered solutions (pH 7.4 and pH 5.0) at 37 °C was studied, and the results are shown in Figure 1. As expected, the pH value considerably affected the release of m-THPC from the micelles. In comparison with the release at pH 5.0, the m-THPC release from micelles at pH 7.4 was effectively suppressed. Beyond the initial burst, the release profile reached a plateau, suggesting that the m-THPC-loaded micelles were relatively stable at this physiological pH. In

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***Table 2.*** Characterizations of m-THPC-Loaded Micelles

| | formulation (P/D) | size of micelles (nm) | PDI | loading efficiency (%) | drug content (mg/mL) |
|---|---|---|---|---|---|
| *m*-THPC-loaded micelles | 10/1 | 77.1 | 0.28 | 92.5 | 0.185 |

contrast, micelles examined at pH 5.0 exhibited a rapid initial release (approximately 50% release within the first 24 h) followed by a second phase of release into the acidic buffer solution. Eventually, over 60% of m-THPC was released from micelles at 48 h, which was most likely due to compromised micellar structure.

**3.3. Cellular Uptake of m-THPC and m-THPC-Loaded Micelles.** To assess the cellular uptake of the photosensitizing agent, the amount of m-THPC was determined using spectrofluorometric methods. Figure 2 shows the uptake of free m-THPC and m-THPC-loaded micelles by HT-29 cells at a constant photosensitizer concentration with different incubation times. The cellular uptake was examined over a 24 h period, and the cellular uptake of free m-THPC was significantly higher than that of m-THPC-loaded micelles ($p < 0.001$). Interestingly, the m-THPC-loaded micelles approached a steady-state in cellular uptake, which was reached after the first several hours. In contrast, the cell uptake of free m-THPC steadily increased after a prolonged incubation and did not appear to saturate.

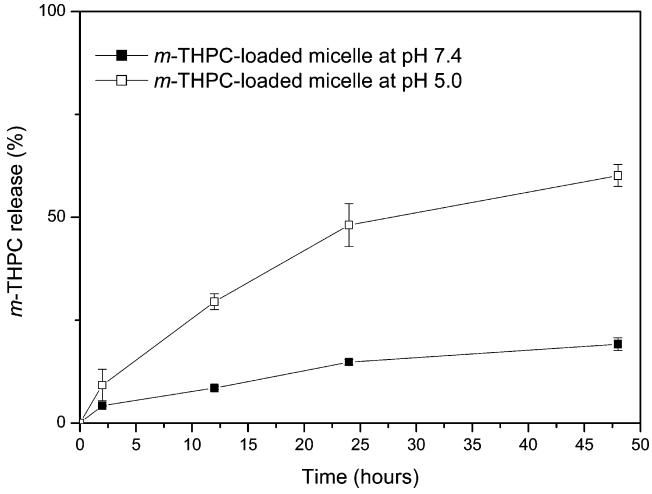


***Figure 1.*** Time course of m-THPC release profiles in different buffer solutions. Data are presented as mean ± standard deviation.

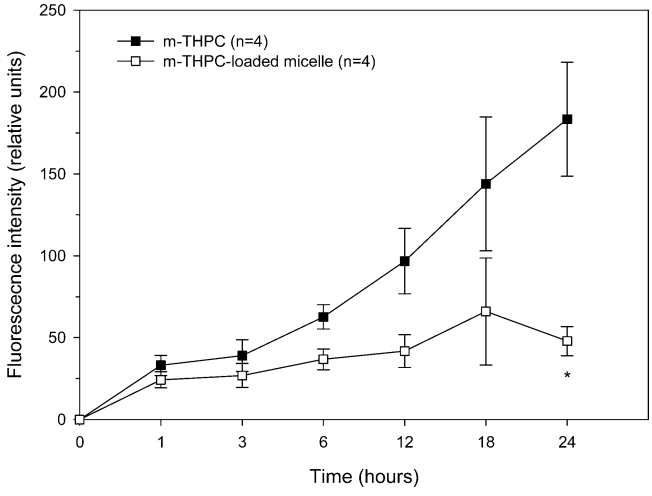


***Figure 2.*** Cellular uptake of m-THPC and m-THPC-loaded micelles at each time-point. Data are presented as mean ± standard deviation. *Significantly different between groups at the indicated time-point ($p < 0.05$).

**3.4. Cytotoxicity and Phototoxicity.** The dose-dependent cytotoxicity and phototoxicity of the photosensitizing agents with a 0.96 J/cm$^2$ light dose in HT-29 cells is shown in Figure 3A. At a concentration of 5 $\mu$g/mL, about 95% and 85% of cells were killed by free m-THPC and m-THPC-loaded micelles, respectively. Importantly, the cytoxicity of both of the light treated groups was significantly greater than that of the light-free groups (all $p < 0.01$). The light only group exhibited no significant phototoxicity in HT-29 cells (data not shown). Figure 3B shows the differential cell viability of m-THPC and m-THPC-loaded micelles at different doses of irradiation. Overall, a clear difference between the two groups was found ($p < 0.001$), and the phototoxicity increased with longer durations of irradiation ($p < 0.001$). When the differences at each dose were tested, m-THPC-loaded micelles exhibited significantly less phototoxicity than m-THPC at the 60th and 180th second ($p = 0.002$ and $p = 0.001$, respectively). After irradiation for 60 s, about 79% and 42% of cells were killed by free m-THPC and m-THPC-loaded micelles, respectively. After irradiation for 180 s, about 90% and 77% of cells were killed by free m-THPC and m-THPC-loaded micelles, respectively.

**3.5. Photodynamic Therapy and Skin Photosensitivity *in Vivo*.** To show therapeutic efficacy *in vivo*, free m-THPC or m-THPC-loaded micelles (0.3 mg m-THPC equivalents/kg) in PBS were injected intravenously into a mouse tumor model, which was followed by light treatment at 24 or 48 h postinjection with a fluence of 10 J/cm$^2$. Figure 4 shows the relative weight curves of nude mice after PDT treatments. The weight differences among the five treatment groups were revealed by a linear mixed model after controlling the effect of time ($p = 0.019$). When compared with the control group, weight reductions were similar in the m-THPC-loaded micelle−PDT group at 24 and 48 h postinjection ($p = 0.007$ and $p = 0.010$, respectively). In addition, the antitumor efficacy of micellar-PDT was evaluated by measuring tumor growth rates. Figure 5 shows the regrowth curves of HT-29 tumors in all groups. Overall, tumor volume increased in parallel with time after treatment ($p < 0.001$). The biggest tumor size was observed in the control group, which was followed by PDT-treated groups at 48 and 24 h after injection (all $p < 0.01$). Of note, light treatments at 24 or 48 h

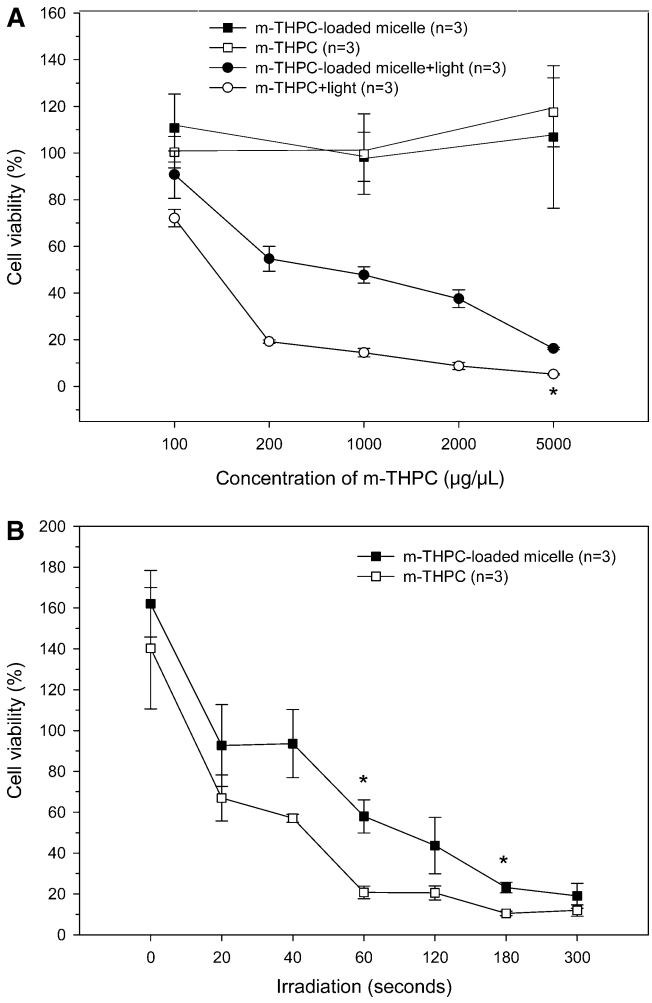


***Figure 3.*** A. Cytoxicity and phototoxicity of m-THPC and m-THPC-loaded micelles at different concentrations of m-THPC. Data are presented as mean ± standard deviation. *Significantly different among groups at the indicated concentration ($p < 0.05$). B. Cytoxicity and phototoxicity of m-THPC and m-THPC-loaded micelles at different doses of irradiation. Data are presented as mean ± standard deviation. *Significantly different between groups at the indicated dose ($p < 0.05$).

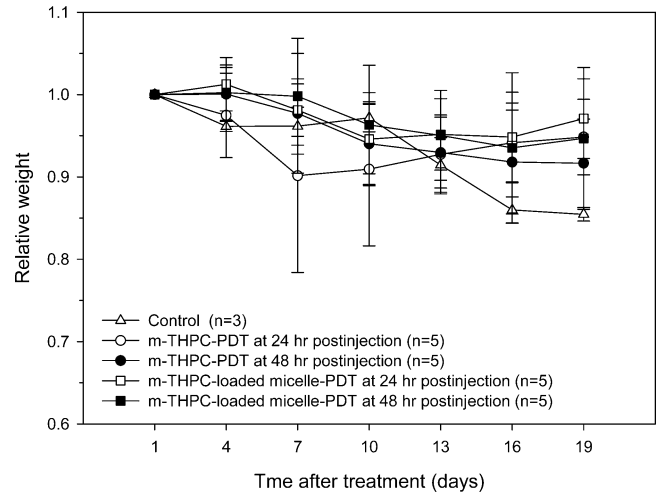


***Figure 4.*** Relative weight curves in nude mice after PDT treatment. Data are presented as mean ± standard deviation.

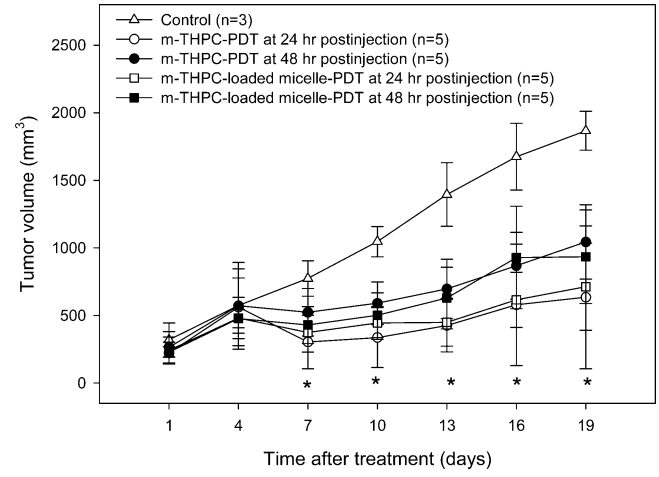


***Figure 5.*** Regrowth curves of HT-29 tumors after PDT treatment. Data are presented as mean ± standard deviation. *Significantly different among groups at the indicated time-point ($p < 0.05$).

postinjection demonstrated that the m-THPC-loaded micelle group exhibited similar antitumor effects as the free m-THPC groups (all $p > 0.05$). Nevertheless, the tumors of all PDT-treated groups showed a significant growth delay compared with the control group after day 7 ($P < 0.01$).

The skin photosensitivity caused by m-THPC after nanoparticle administration was evaluated in mice injected with m-THPC (0.3 mg/kg), which were then exposed to a 10 J/cm$^2$ (652 nm) light source. As shown in Figure 6, the highest skin photosensitivity scores were observed on the fifth day in the free m-THPC group after light irradiation 24 h postinjection (group 1), which then remained stable to day 20. Reduced skin photosensitivity was observed from free m-THPC after light irradiation 48 h postinjection (group 2), except for days 8, 9, 11, and 12 (all $p < 0.01$) when compared with group 1. After micelle encapsulation of m-THPC, the skin photosensitivity was reduced to values similar to the results found in group 2. The differences in skin photosensitivity between group 1 and the m-THPC-loaded micelle group irradiated 24 h after injection were found to be significantly different except for days 3, 9, and 10 (all $p < 0.01$). Surprisingly, no skin photosensitivity was observed when the mice were exposed to light 48 h after m-THPC-loaded micelles were injected, and these time-points overlapped with the control group (all $p = 1.000$). After the skin photosensitivity reached the maximal value for the m-THPC 24 and 48 h postinjection groups, the error bars increased as well, possibly due to the inconsistency of wound healing.

## 4. Discussion

Macromolecular therapeutics can increase the accumulation of a cytotoxic agent in tumor tissue based on

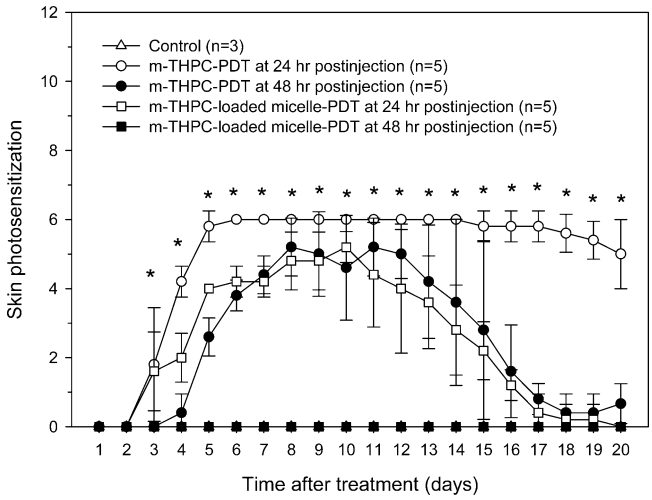


***Figure 6.*** Skin photosensitivity at various time-points after PDT treatment. Data are presented as mean ± standard deviation. *Significantly different among groups at the indicated time-point ($p < 0.05$).

enhanced permeability and retention (EPR) effects.[34] However, the mechanisms of tumor suppression by the hydrophobic free m-THPC or its hydrophilic micellar form *in vivo* have been suggested to be different. It has been demonstrated that no significant correlation exists between tumor or skin drug levels and m-THPC-mediated PDT responses.[35] Consequently, the effect of m-THPC based PDT, including free or micellar photosensitizers, on tumor and normal tissues was considered to be largely mediated by vascular damage. After micelle encapsulation, the biodistribution and pharmacokinetics of m-THPC may be altered because of the differences in particle size, hydrophobicity, and solubility of the PEOz in the micelle's outer shell.[31,35] Therefore, it can be expected that the tumor tissue damage by m-THPC-loaded micelles may be different from that observed with m-THPC alone. Nevertheless, our study demonstrated that the micellar formulation of m-THPC was extremely cytotoxic when tested *in vitro* and could significantly delay tumor growth when examined in an *in vivo* mouse model. However, the skin photosensitivity of m-THPC was greatly reduced after micelle encapsulation. This potentially offers a significant clinical advantage over free m-THPC, yet the cellular mechanisms responsible for this benefit are not entirely clear.

The pH-dependent release of a cytotoxic agent from PEOz-*b*-PLA based micelles appears to provide some control of drug release.[23] These *in vitro* data demonstrated that the PEOz-*b*-PLA diblock copolymers allowed deformation of the micelle to occur as the pH of the solution dropped below 7.4. The authors suggested this was due to the protonation of the tertiary amide belonging to PEOz, thereby inducing intra/intermolecular interactions between micelles that may have caused aggregation and collapse of the micellar outer shell.[23] Furthermore, it has been demonstrated that the pH-induced deformation was observed in PLA-PEOz-PLA triblock or PEOz-PLA diblock copolymers.[23,33] This pH-dependent releasing behavior of drugs is of particular interest for tumor targeted drug delivery using polymeric micelles.[36] In the current study, m-THPC release is expected to occur after micelles are internalized into tumor cells via endocytosis and trapped in acidic endosomal/lysosomal compartments. Consequently, the toxicity of encapsulated m-THPC is made more cell-specific due to these changes in biodistribution and pharmacokinetics, which may explain the reduced skin photosensitivity of this formulation *in vivo*. Certainly, this controlled release from endosomal or lysosomal vesicles has been supported by numerous designs in pH-dependent polymeric micelles that specifically target the acidic tumor microenvironments.[37]

In addition, differences in cellular uptake, probably due to the hydrophobic property of m-THPC, caused more permeation and accumulation in the cultured cells. In contrast to the free form of m-THPC, which can diffuse across the cell membrane and enter cells, the micellar form is most likely sequestered in acidic intracellular compartments. Therefore, one of the possible reasons for the differential cytotoxicity between the free m-THPC and the micellar form is the different amounts of cellular uptake of photosensitizing agent. The amount of internalized free m-THPC was higher than that of the internalized m-THPC-loaded micelle, which may have caused more phototoxicity in HT-29 cells. Moreover, the preferential sites of m-THPC accumulation have been shown to be in the endoplasmic reticulum (ER) and the Golgi apparatus of MCF-7 cells.[38] In contrast to free m-THPC, the subcellular localization of m-THPC-loaded micelles is predicted to be in endosome/lysosome compartments via the endocytic pathway. Thus after irradiation, the primary sites of damage by m-THPC-mediated or m-THPC-loaded micelle-mediated PDT are presumed to occur in the ER and Golgi apparatus or endosome/lysosome compartments, respectively. Therefore, another reason for the observed differences in phototoxicity may be due to the differential

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intracellular distribution of photosensitizing agents, which can significantly influence the mechanisms of cellular responses to PDT. Recently, this concept has been exploited to enhance the cell-specific uptake of conjugated m-THPC in a mouse tumor model.[39] Indeed, a cell-specific form of m-THPC encapsulated PEOz-*b*-PLA micelles is currently under development and should prove to be of great therapeutic value.

Our most significant discovery is that photosensitivity could be completely avoided with micellar encapsulation of m-THPC. The graphs in Figure 6 are very complex. There are at least two factors apparent from the curves: PDT timing and micellar encapsulation. The first factor is that delaying the timing of the PDT reduced the photosensistivity in all cases. Toward the end of the study time (15−20 days) we can see that the 24 h PDT treatment of the m-THPC group began to form a (skewed) bell curve, and may have completed the bell curve had we continued the experiment for 20 more days. Merely waiting until 48 h to give PDT to the m-THPC group reduced the total area under the curve in the bell curve shaped graph. Therefore, delaying PDT had some sort of effect on the intensity of the curve.

The second factor affecting photosensitivity was the use of the micellar carrier. Adding micellar carrier (24 h-PDT, micellar m-THPC group, Figure 6) had the same magnitude of effect as delaying PDT (waiting until 48 h to give PDT in the free m-THPC group, Figure 6): the curves were the same. The explanation of the effect of 48 h-PDT, micelle-m-THPC group (Figure 6), which had no photosensitivity, is the combined protective effect of delaying the PDT by 24 h plus the protective effect of the micellar carrier.

Body distribution of m-THPC after iv administration of m-THPC-loaded micelles in mice was determined (Figure S2 in the Supporting Information). Distribution profiles of m-THPC in mice receiving intravenous m-THPC-loaded micelles indicated that m-THPC was widely distributed into most tissues; the highest concentrations were detected in the spleen, followed by lung, liver, skin, heart, kidney and heart after 24 h. However, after 48 h, m-THPC concentration in spleen, liver and lung was significantly decreased. It is noticed that no significant difference about m-THPC concentration in skin was observed between m-THPC and m-THPC-loaded micelle groups. We can only speculate about the mechanisms of photosensitivity that might account for the observed changing bell curves seen in Figure 6. The bell curve itself must represent some basic process in the animals. The root cause may be related to normal versus cancer cells. Indeed one of the rationales for photodynamic therapy is that cancer cells often uptake photodynamic agents more than normal cells. Free m-THPC or m-THPC-loaded micelles were equally effective at killing tumor cells (Figure 5). However, the effect on photosensitivity, which is the effect on normal cells, was different between free and encapsulated m-THPC (Figure 6). We speculate that micellar m-THPC has a slower rate of uptake than free m-THPC, and that when the uptake of photodynamic reagent reaches high enough concentration at the timing of PDT there is not as much m-THPC in the normal cells in the micellar m-THPC group plus different intracellular distribution, making them less photosensitive.

However, this simple chemical equilibrium model does not lend itself to explaining why delaying the photodynamic treatment of the tumors has a later effect on the systemic photosensitivity in both free and micellar m-THPC groups. A more sophisticated model may involve the immune system. Both encapsulation of the m-THPC and delaying the PDT could be affecting a systemic immune response. The PDT could be sensitizing the skin similarly to the way that chemotherapy and radiation therapy sensitize mucous membranes during gastrointestinal mucositis.[40] Mucositis also shows a delayed response time as the tissues become more and more sensitive to oxidative damage, leading to focal ulcerations. The silent damage occurring during mucositis also has a healing period, and the ulcerations (analogous to the photosensitive damage in our experiments) can be avoided if the tissues are not overstimulated during the critical period of sensitivity. Because PDT is also known to induce a systemic immune response,[41] it would be reasonable to test the hypothesis that both delaying PDT by 24 h and delivering m-THPC via micellar carrier are cumulatively reducing the amount of systemic immune response while still maintaining adequate tumoricidal activity.

## 5. Conclusions

In conclusion, skin photosensitivity is a major side effect of current clinical PDT. The clinically relevant hydrophobic photosensitizer, m-THPC (Foscan), was encapsulated by a synthetic PEOz-*b*-PLA copolymer with a diameter less than 100 nm. Because of the pH sensitivity of PEOz-*b*-PLA composed micelles, m-THPC was released in buffer solutions with a low pH, which may be clinically beneficial by reducing the aforementioned side effects of free m-THPC. Furthermore, the m-THPC-loaded micelles exhibited phototoxicity *in vitro* and *in vivo*; yet, the skin photosensitivity after m-THPC−PDT was significantly reduced by micelle encapsulation. Therefore,

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these findings suggest that this micellar photosensitizer is a potential delivery system for photodynamic therapy in a clinical setting.

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**Supporting Information Available:** Figures depicting absorbance and fluorescence spectra of free m-THPC or m-THPC-loaded micelles and biodistribution of m-THPC in mice bearing HT-29 solid tumors. This material is available free of charge via the Internet at http://pubs.acs.org.

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