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Mitochondria-Selective Photodynamic Tumor Therapy Using Globular PEG Nanoparticles

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Abstract: In this study, we report water-soluble globular poly(ethylene glycol) (gPEG) nanoparticles for targeting mitochondria and for improved photodynamic tumor therapy. Here, gPEG (prepared after all of the π-π carbon bonds in fullerene (as a hollow core structure) were chemically combined with poly(ethylene glycol) (PEG)) was chemically linked with chlorin e6 (Ce6, as a photosensitizing anticancer drug) and iodomethyltriphenylphosphonium (IMTP). In particular, IMTP acted as a mitochondria-targeting molecule, accelerating the localization of nanoparticles into the mitochondria of tumor cells. From an *in vitro* evaluation, these nanoparticles exhibited improved singlet oxygen generation and significantly increased photodynamic tumor ablation. We believe that this nanoparticle provides a promising pathway for photodynamic drug delivery.

Keywords: globular poly(ethylene glycol), mitochondria-selective, photodynamic tumor therapy, water-soluble nanoparticle, iodomethyltriphenylphosphonium (IMTP).

Introduction

Recent research trials in photodynamic tumor therapy (PDT) involve the exploitation of a new class of photosensitizing drug and biologically-adjusted light source probe.[1-5] It is interesting to note that because of several limitations, such as poor tumor targetability and limited functionality of photosensitizing drugs, nano-sized polymeric drug carrier systems have been widely accepted as emerging tools for improving photodynamic drug therapeutics.[5-14] In particular, a broad range of drug carrier design methods for efficient photodynamic tumor therapy have been investigated based on the structural, chemical, and biological aspects of tumor cells.[15-18] For example, mitochondria targeting is one of the current challenges for highly efficient PDT.[5,15-17] It is known that mitochondria regulate vital and lethal functions of cells such as ATP synthesis, reactive oxygen species (ROS) production, programmed cell death (apoptosis), and oxidative phosphorylation.[19] Mitochondrial damage can lead to extensive cell death mechanisms, including bioenergetic dysfunctions and the cytosolic release of pro-apoptotic proteins.[5,15-19] Therefore, pursuing the versatile design of mitochondria-targeting nano-sized drug carriers may be crucial for advanceing PDT. Recently, there have been several efforts to engineer mitochondria-targeting drug carrier systems.[5,15-17] However, even in the case of sophisticatedly designed drug carrier systems, drug delivery efficiency and tumor ablation efficiency has been a major concern.

In this study, we developed water-soluble globular poly(ethylene glycol) (gPEG) particles for highly efficient mitochondria targeting and for advanced PDT. gPEG[12] with a small-sized hollow core (originated from the hollow structure of fullerene[14]) and hydrophilic poly(ethylene glycol) (PEG) shell[12] were coupled with a photosensitizing drug (chlorin e6: Ce6) and mitochondria-targetable molecule (iodomethyltriphenylphosphonium: IMTP) (Figure 1(a)). A lipophilic cation, IMTP is effective in permeating the lipid bilayer and localizing particles into mitochondria.[10] We expect that gPEG-Ce6-IMTP improves the mitochondria membrane permeability of Ce6 due to its extremely small size (~10 nm)[19] and the presence of IMTP.[10] In particular, this drug carrier design is based on the premise that fatal ROS on mitochondria allows for the significant enhancement of tumor inhibition. Here, we preferentially investigated *in vitro* mitochondria-targeting ability and the *in vitro* photodynamic tumor ablation efficacy of gPEG-Ce6-IMTP.

Experimental

Materials. Chlorin e6 (Ce6) was obtained from Frontier Scientific Inc. (USA). Lithium hydroxide (LiOH), *N,N'*-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS),

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N-(2-aminoethyl)maleimide (AEM), dimethylformamide (DMF), cysteamine, triethylamine (TEA), iodomethyltriphenylphosphonium (IMTP), diethyl ether, dimethyl sulfoxide (DMSO), pyridine, toluene, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), *N*-propyl galate, glycerol, Tris-HCl, and 9,10-dimethylanthracene (DMA) were purchased from Sigma-Aldrich (USA). Fullerene (C_{60}) was purchased from NanoLab Inc. (Waltham, MA, USA). RPMI-1640, fetal bovine serum (FBS), penicillin, trypsin, EDTA (ethylene diamine tetra-acetic acid), and streptomycin were purchased from Welgene Inc. (Korea). The Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies Inc. (Japan). Mitotracker® was purchased from Invitrogen Inc. (USA). Monocarboxylated poly(ethylene glycol) (HO-PEG-COOH, M_w~2 kDa) was prepared as described in our previous reports.[7-14]

gPEG-Ce6-IMTP Synthesis. To prepare globular PEG (gPEG), all of the π-π carbon bonds in C_{60} (16 mg, as a hollow core material) were chemically conjugated with the hydroxyl terminal group of HO-PEG-COOH (2 g) using LiOH (120 mg, as a catalyst) in a DMSO (15 mL)/toluene (15 mL) co-solvent at room temperature for 3 days.[12] The toluene solvent was removed from the resulting solution using a rotary evaporator. The obtained solution was dialyzed against deionized water to remove the non-reacted chemicals using a pre-swollen dialysis membrane tube (Spectra/Por® MWCO 50 K), and was then lyophilized using a vacuum freeze dryer.[12]

Next, the carboxyl terminal groups of gPEG (0.5 g) were pre-activated using cysteamine (40 mg), DCC (10 mg), and NHS (5 mg) in DMF (20 mL) containing TEA (1 mL) and pyridine (0.5 mL) at room temperature for 12 h, yielding thiolated gPEG (gPEG-SH) (Figure 1(b)). gPEG-SH (300 mg) was reacted with IMTP (120 mg) and maleimide-modified Ce6 [Ce6-Mal (150 mg), prepared after the condensation reaction between the carboxylic group of Ce6 (150 mg) and the amine group of AEM (30 mg) in DMF (10 mL) using DCC (80 mg), NHS (60 mg), TEA (1 mL), and pyridine (0.5 mL) at room temperature for 8 h] at room temperature for 1 day, yielding gPEG with Ce6 and IMTP (gPEG-Ce6-IMTP) (Figure 1(b)). gPEG-Ce6-IMTP was recrystallized after adding excess diethyl ether to the resulting solution. gPEG-Ce6-IMTP dissolved in DMSO was further purified by dialyzing against fresh DMSO using a dialysis membrane tube (Spectra/Por® MWCO 50 K) to remove the non-reacted chemicals and then was lyophilized using a vacuum freeze dryer.

Characterization of gPEG-Ce6-IMTP. The particle size distribution of gPEG nanoparticles (0.1 mg/mL) dissolved in phosphate buffered saline (PBS, 150 mM, pH 7.4) at 37 °C was analyzed using a Zetasizer 3000 instrument (Malvern Instruments, USA) equipped with a He-Ne laser at a wavelength of 633 nm and a fixed scattering angle of 90°.[7-14] The particle size and morphology of gPEG-Ce6-IMTP was confirmed using a transmission electron microscope (TEM; JEM 1010, Japan).[11-14] The zeta-potential of gPEG nanoparticles (0.1 mg/mL) in PBS (pH 7.4) was measured with a Zetasizer 3000 (Malvern Instruments).[7-14]

***In Vitro* Photoactive Properties of gPEG-Ce6-IMTP.** The generation of singlet oxygen from gPEG nanoparticles (equivalent C_{60} 10 μg/mL) and free C_{60} (10 μg/mL, as a control) in PBS (150 mM, pH 7.4) was confirmed using fluorescent DMA (used as an extremely fast chemical trap for singlet oxygen). First, DMA (20 mmol) was mixed with each sample (equivalent C_{60} 10 μg/mL) in PBS (150 mM, pH 7.4). Then, the resulting solution was illuminated at a light intensity of 5.2 mW/cm^2 using a 670 nm laser source for 10 min. When the DMA fluorescence intensity (measured using a Shimadzu RF-5301PC spectrofluorometer at λ_{ex} 360 nm and λ_{em} 380-550 nm) reached a plateau after 1 h, the change in the DMA fluorescence intensity (F_f-F_s) was plotted after subtracting each sample's fluorescence intensity (F_s) from the full DMA fluorescence intensity (without Ce6, indicating no singlet oxygen, F_f).[7-14]

Cellular Localization Study of gPEG-Ce6-IMTP. Human nasopharyngeal epidermal carcinoma KB cells (from Korean Cell Line Bank) were maintained in RPMI-1640 medium with 1% penicillin-streptomycin, and 10% FBS in a humidified standard incubator with a 5% CO_2 atmosphere at 37 °C. Prior to testing, KB cells (1×10^5 cells/mL) were grown as a monolayer, and were harvested *via* trypsinization using a 0.25% (w/v) trypsin/0.03% (w/v) EDTA solution. The tumor cells were suspended in RPMI-1640 medium and were seeded onto well plates and cultured for 24 h prior to *in vitro* cell testing.[7-14]

The KB cells in RPMI-1640 medium were incubated with each sample (equivalent Ce6 20 μg/mL) at 37 °C (incubation time: 2 h). The treated cells were washed three times with a PBS (pH 7.4) solution and were then incubated with Mitotracker (50 nM) for 10 min and with DAPI (5 μg/mL) for 5 min.[10] The cells were again washed three times with a PBS (pH 7.4) solution and then fixed using 3.7% formaldehyde in PBS. A cover slip was mounted on a microscope slide with a drop of anti-fade mounting media (5% *N*-propyl galate, 47.5% glycerol and 47.5% Tris-HCl, pH 8.4) to reduce fluorescence photo-bleaching. The cells were examined using a confocal laser scanning microscope (CarlZeiss Meta LSM510, Germany).[10-14]

***In Vitro* Phototoxicity of gPEG-Ce6-IMTP.** The KB cells fixed in 96-well plates were treated with each sample (equivalent Ce6 1-10 μg/mL) at 37 °C (incubation time: 2 h). The cells were washed three times with PBS (pH 7.4) and then were illuminated at a light intensity of 5.2 mW/cm^2 using a 670 nm laser source for 10 min and further incubated at 37 °C for 12 h. Phototoxicities of gPEG nanoparticles for KB cells were determined using a Cell Counting Kit-8 (CCK-8) assay.[7-14] In addition, the cell viabilities of KB cells treated with gPEG-Ce6 (10-100 μg/mL) or gPEG-Ce6-IMTP (10-100 μg/mL) without light illumination for 24 h were determined using a CCK-8 assay[7-14] to evaluate the original toxicity of the gPEG nanoparticles.

Results and Discussion

Synthesis and Characterization of gPEG-Ce6-IMTP. In this study, we synthesized gPEG-Ce6-IMTP (Figure 1(b)). First, we prepared a globular PEG (gPEG) as a nano-sized backbone material with a unique hollow core and a PEG shell. Our previous reports confirmed that breaking all of the π-π carbon bonds in a fullerene[12,14] is possible using excess HO-PEG-COOH. In the presence of LiOH as a catalyst, the chemical reaction of the hydroxyl terminal group of HO-PEG-COOH and the π-π carbon bonds in fullerene resulted in that approximately 30 moles of PEG becoming completely linked to one mole of fullerene, consequently enabling the synthesis of gPEG.[12] Next, we conjugated Ce6 and IMTP to gPEG *via* a simple conjugation method, as shown in Figure 1(b). Briefly, thiolated gPEG (gPEG-SH) was reacted with maleimide-modified Ce6 (Ce6-Mal) and IMTP, which was confirmed using ^{1}H NMR peak analysis from peaks at δ 1.24 (-C**H**-, corresponding to the soccer-ball-shaped backbone), δ 4.45 (-O-C**H**$_2$-CO-, corresponding to the PEG), δ 0.95 (-C**H**$_3$, corresponding to the Ce6), and δ 7.28 (-C**H**-, corresponding to the IMTP) (Figure 2). The degree of Ce6 and IMTP substitution

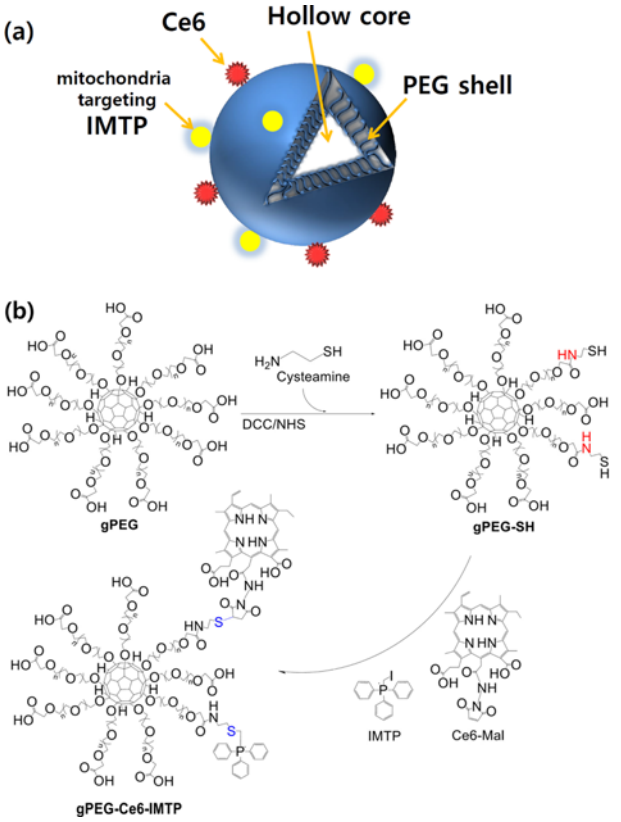

Figure 1. (a) Schematic concept for a proposed globular PEG nanoparticle with Ce6 and IMTP molecules (gPEG-Ce6-IMTP) for targeting mitochondria and for photodynamic tumor therapy (see the text for more detail). (b) Synthesis scheme of globular gPEG-Ce6-IMTP.

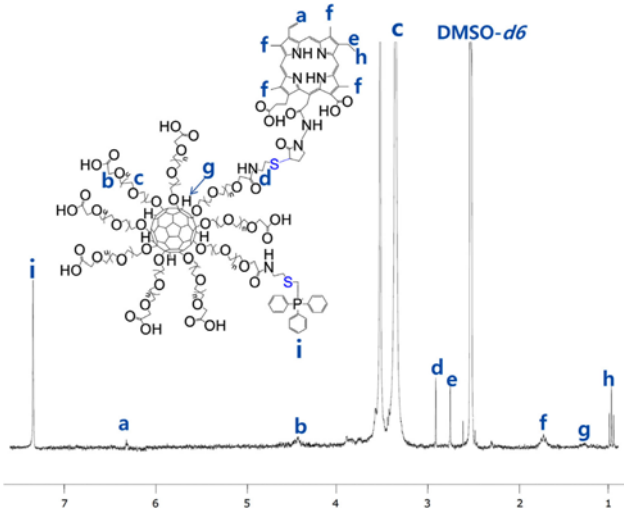

Figure 2. ^{1}H NMR peaks of gPEG-Ce6-IMTP.

[defined as the number of each moiety (Ce6 and IMTP) per one molecule of gPEG] were 9.4±1.2 (n=3) and 3.8±0.9 (n=3), respectively, as estimated by comparing the integration ratios of the ^{1}H NMR peaks. We also synthesized gPEG-Ce6 for control experiments *via* the same synthesis procedure without IMTP. It was found that the degree of Ce6 in gPEG-Ce6 was 8.5±1.5 (n=3) (data not shown).

Figure 3(a) shows that the average particle size of gPEG-Ce6-IMTP (prepared after simply dissolving gPEG-Ce6-IMTP into PBS) was approximately 10 nm in diameter, indicating that gPEG-Ce6-IMTP did not need any special particle preparation method such as diafilteration, film rehydration, water emulsion, or the supercritical fluid method.[20] The image obtained from TEM illustrates that the extremely small-sized gPEG-Ce6-IMTP were almost spherical (Figure 3(b)).

The zeta potential of gPEG, gPEG-Ce6, and gPEG-Ce6-IMTP were approximately -22.3, -32.4, and -23.0 mV, respectively (Figure 3(c)), revealing the good stability of these colloidal dispersions.[21] It was thought that the presence of residual carboxylic acid groups in Ce6 molecules decreased the zeta potential of particles (gPEG-Ce6). However, the effect of Ce6 on the zeta potential was offset by the presence of lipophilic IMTP (gPEG-Ce6-IMTP).

Singlet Oxygen Generation. Figure 4 shows the photoactivity of gPEG-Ce6-IMTP under light illumination (5.2 mW/cm^2 at 670 nm for 10 min).[7-14] In particular, we evaluated the degree of singlet oxygen generation of each nanoparticle under light illumination. As expected, free Ce6 typically displayed low singlet oxygen generation. It is known that Ce6 molecules aggregated in PBS shorten the distance between Ce6 molecules,[7-14] resulting in increasing the auto-quenching effect of the Ce6 molecules. However, water-soluble, extremely small-sized gPEG-Ce6 and gPEG-Ce6-IMTP exhibited highly increased singlet oxygen generation under light illumination. The good water-dispersion of Ce6 molecules in gPEG-Ce6

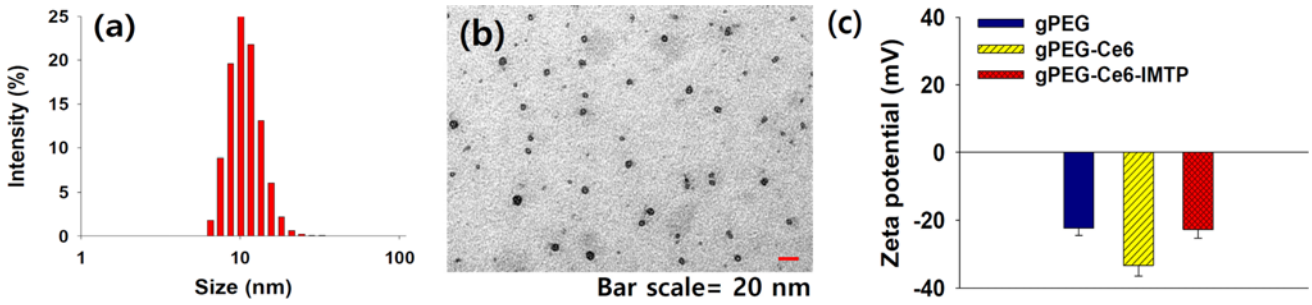

Figure 3. (a) Particle size distribution and (b) TEM image of gPEG-Ce6-IMTP (0.1 mg/mL) in PBS (150 mM, pH 7.4). (c) Zeta potential values of gPEG, gPEG-Ce6, and gPEG-Ce6-IMTP (0.1 mg/mL, 150 mM, pH 7.4) (n=3).

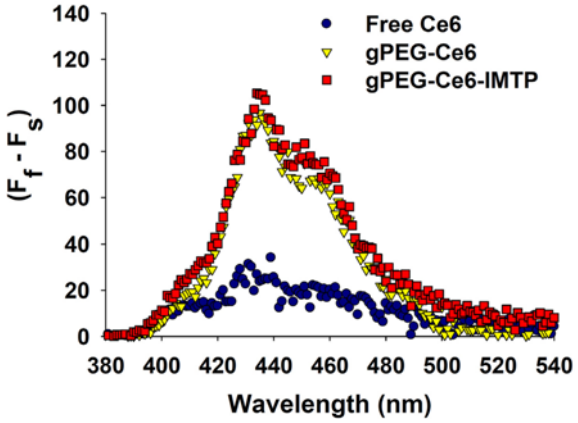

Figure 4. The 9,10-dimethylanthracene (DMA) fluorescence change (at λ_{ex} 360 nm and λ_{em} 380-550 nm) of free Ce6 (10 μg/mL), gPEG-Ce6 (equivalent Ce6 10 μg/mL), and gPEG-Ce6-IMTP (equivalent Ce6 10 μg/mL) in PBS (150 mM, pH 7.4). Singlet oxygen generation is indicated by the DMA fluorescence intensity change (F_f-F_s). All samples were illuminated for 10 min at a light intensity of 5.2 mW/cm^2 using a 670 nm laser source.

and gPEG-Ce6-IMTP decreased the self-quenching effect of Ce6 molecules.[7-14] Consequently, under light illumination, the up-regulated production of singlet oxygen from gPEG-Ce6 and gPEG-Ce6-IMTP was expected to cause high phototoxicity for KB tumor cells. In addition, gPEG (without Ce6) showed no production of singlet oxygen under light illumination as described in our previous report.[12]

***In Vitro* Photodynamic Tumor Ablation.** Figure 5 shows confocal images of human nasopharyngeal epidermal carcinoma KB cells treated with free Ce6, gPEG-Ce6, and gPEG-Ce6-IMTP. Here, free Ce6 led to poor cellular uptake in KB tumor cells. gPEG-Ce6 exhibited relatively increased cellular uptake, but the internalized gPEG-Ce6 (without IMTP) nanoparticles were scattered in the cells. It is interesting to note that the cellular uptake of gPEG-Ce6-IMTP was significantly increased, compared with that of gPEG-Ce6 and free Ce6. In particular, the ability of the IMTP permeating lipid bilayers enabled the highest cellular uptake of gPEG-Ce6-IMTP for KB tumor cells. Of course, gPEG-Ce6-IMTP permeating

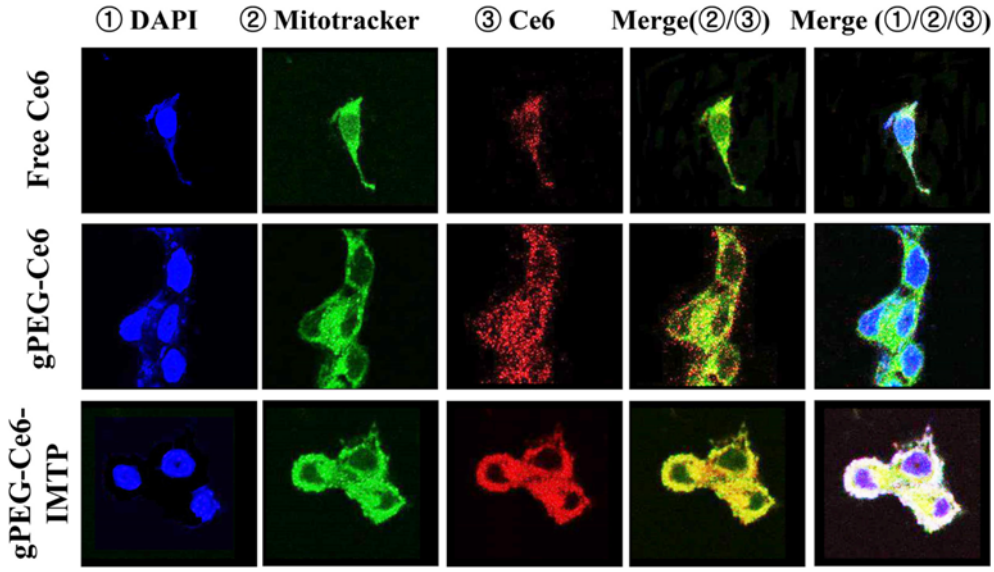

Figure 5. Confocal images of KB tumor cells treated with free Ce6 (20 μg/mL), gPEG-Ce6 (equivalent Ce6 20 μg/mL), and gPEG-Ce6-IMTP (equivalent Ce6 20 μg/mL) at 37 °C (incubation time: 2 h). The treated cells were stained using DAPI (for staining nucleus) and Mitotracker® (for staining mitochondria).

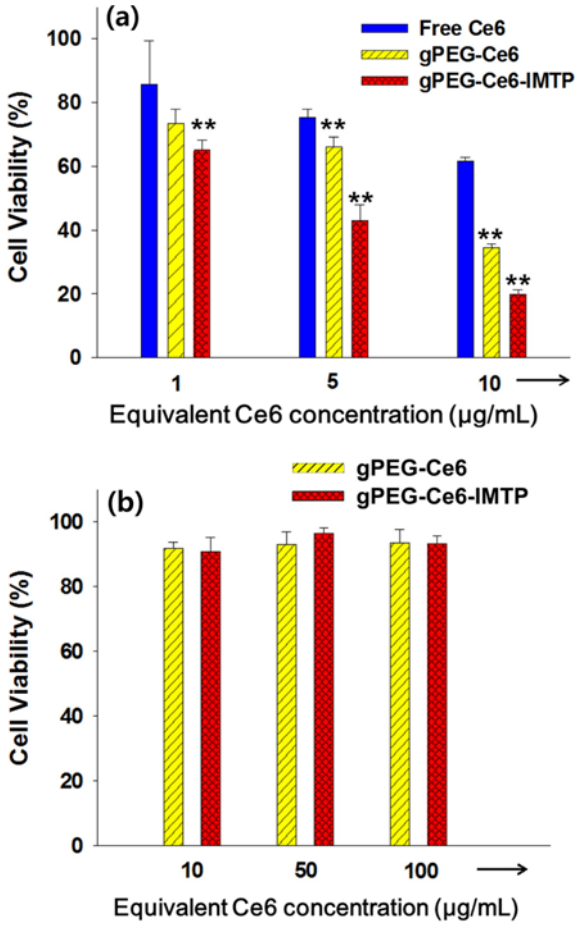

Figure 6. (a) Phototoxicities determined by a Cell Counting Kit-8 (CCK-8) of KB cells treated with free Ce6 (1-10 μg/mL), gPEG-Ce6 (equivalent Ce6 1-10 μg/mL), and gPEG-Ce6-IMTP (equivalent Ce6 1-10 μg/mL) at 37 °C (incubation time: 2 h). All of the cells were illuminated for 10 min at a light intensity of 5.2 mW/cm^2 using a 670 nm laser source and were incubated for an additional 12 h (n=7) (**p<0.01 compared to free Ce6). (b) Cell viabilities of KB cells treated with gPEG-Ce6 (10-100 μg/mL) or gPEG-Ce6-IMTP (10-100 μg/mL) without light illumination for 24 h (n=7).

lipid bilayers may be internalized into normal tissue during blood circulation. However, *in vivo* tumoral (local) light illumination will decrease the possibility of singlet oxygen generation in normal tissues. This local PDT method is expected to improve the antitumoral activity of gPEG-Ce6-IMTP. In addition, the Ce6 (red) fluorescence of gPEG-Ce6-IMTP was fully overlapped with the fluorescence (green) of Mitotracker® (staining mitochondria),[10] indicating efficient Ce6 delivery to the mitochondria.

Figure 6 provided evidence of improved phototoxicity of gPEG-Ce6-IMTP for KB tumor cells. It is assumed that the localization of gPEG-Ce6-IMTP in mitochondria enhanced cell damage under light illumination (5.2 mW/cm^2 at 670 nm for 10 min). Indeed, gPEG-Ce6-IMTP led to higher levels of tumor cell ablation, comparable with those of free Ce6 and gPEG-Ce6. In addition, gPEG nanoparticles (gPEG-Ce6 and gPEG-Ce6-IMTP), prior to light illumination, showed no cytotoxicity up to 100 μg/mL in 24 h of culture (Figure 6(b)), supporting their non-toxicity without light illumination.

Conclusions

In this study, we prepared a water-soluble, extremely small-sized gPEG with a photosensitizing drug and mitochondria-targetable molecule. The obtained gPEG nanoparticles (gPEG-Ce6-IMTP) showed highly increased singlet oxygen generation and improved tumor ablation under light illumination. Based on the results of this study, we expect that this system has great potential as an advanced PDT technology for efficient tumor therapy. A detailed *in vitro/in vivo* evaluation of gPEG-Ce6-IMPT should be performed to support the biomedical potential of gPEG-Ce6-IMTP.

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