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Photosensitizer-mediated mitochondria-targeting nanosized drug carriers: Subcellular targeting, therapeutic, and imaging potentials

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ABSTRACT

Mitochondria-targeting drug carriers have considerable potential because of the presence of many molecular drug targets in the mitochondria and their pivotal roles in cellular viability, metabolism, maintenance, and death. To compare the mitochondria-targeting abilities of triphenylphosphonium (TPP) and pheophorbide a (PhA) in nanoparticles (NPs), this study prepared mitochondria-targeting NPs using mixtures of methoxy poly(ethylene glycol)-(SS-PhA)$_2$ [mPEG-(SS-PhA)$_2$ or PPA] and TPP-*b*-poly(ε-caprolactone)-*b*-TPP [TPP-*b*-PCL-*b*-TPP or TPCL], which were designated PPA$_n$-TPCL$_{4-n}$ ($0 \leq n \leq 4$) NPs. With increasing TPCL content, the formed PPA$_n$-TPCL$_{4-n}$ NPs decreased in size from 33 nm to 18 nm and increased in terms of positive zeta-potentials from −12 mV to 33 mV. Although the increased TPCL content caused some dark toxicity of the PPA$_n$-TPCL$_{4-n}$ NPs due to the intrinsic positive character of TPCL, the NPs showed strong light-induced killing effects in tumor cells. In addition, the mitochondrial distribution of the PPA$_n$-TPCL$_{4-n}$ NPs was analyzed and imaged by flow cytometry and confocal microscopy, respectively. Thus, the PhA-containing NPs specifically targeted the mitochondria, and light stimulation caused PhA-mediated therapeutic effects and imaging functions. Expanding the capabilities of these nanocarriers by incorporating other drugs should enable multiple potential applications (e.g., targeting, therapy, and imaging) for combination and synergistic treatments.

1. Introduction

Currently, the development of nanosized drug delivery systems has shifted beyond organ-, tissue-, and cell-targeted drug delivery toward targeted delivery and release in specific subcellular organelles (e.g., cytosol, nucleus, and mitochondria). The reason for this shift is that the molecular targets of many drugs are localized in these subcellular organelles (Mossalam et al., 2010; Rajendran et al., 2010; Leucuta, 2014; Maity and Stepensky, 2015, 2016; Jhaveri and Torchilin, 2016). This delivery strategy could maximize the selective accumulation and release of the delivered payloads at the drug action sites and enhance therapeutic efficacy with fewer unwanted effects than conventional targeting systems (Jean et al., 2015; Jhaveri and Torchilin, 2016; Maity and Stepensky, 2016). Specifically, interest in the mitochondria as a therapeutic target has been growing because of the role of these intracellular organelles in regulating several pivotal pathways, including proliferation, differentiation, viability/apoptosis, oxidative stress, and bioenergy generation. Furthermore, their malfunction may cause various diseases, such as cancer, neurodegenerative diseases, metabolic diseases, and aging (Szewczyk and Wojtczak, 2002; Yamada and Harashima, 2008; Frantz and Wipf, 2010; Smith et al., 2012).

To achieve selective accumulation of payloads in the mitochondria, various mitochondria-targeting peptides, chemicals, and nanostructures have been investigated. Examples of mitochondria-targeting peptides include MLSRAVCGTSRQLAPALGYLGSRQ (Shokolenko et al., 2005; Mossalam et al., 2010), MSATRMQLLSPRNVRLLSRGRSELFAGGSGGGPRVRSLISPPLSSSSPGRALSSVSATRRGLPKEKMTENGVSSRAKVLTIDT (Meton et al., 2004; Mossalam et al., 2010), MLFNLRILLNNAAFRNGHNFMVRNFRCGQPLQ (Horwich et al., 1985; Marchenko et al., 2000; Mossalam et al.,

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2010), and MLSCTSPLLRGACHNMGAAKALRLRWTVPPAVLIALGSGA-LYTTSGQTLYYKNSVQQTD (Takaya et al., 2009; Mossalam et al., 2010), which are mostly derived from proteins that are present in the mitochondria. It has been shown that these peptides utilize the protein import machinery (e.g., the translocase of the outer membrane [TOM]/translocase of the inner membrane [TIM] complex) for their mitochondrial accumulation (Yamada and Harashima, 2008; Mossalam et al., 2010). However, these long peptides have limited use because of their cost of synthesis and possible immunogenicity. Recently, shorter synthetic peptides, such as (cyclohexyl alanine-arginine)$_n$ (n = 3–6), have been designed for the delivery of various chemical drugs, and the number of repeating units (i.e., cyclohexyl alanine-arginine) was found to strongly influence their mitochondrial accumulation as well as their cytotoxicity (Horton et al., 2012; Chamberlain et al., 2013). Chemical candidates for mitochondrial targeting are dequalinium (Weissig et al., 1998, 2001; Weissig and Torchilin, 2000; Mossalam et al., 2010; Wang et al., 2015), rhodamine β (Ngen et al., 2009; Mossalam et al., 2010), pheophorbide a (PhA) derivatives (Kim et al., 2004; Tang et al., 2006; Rapozzi et al., 2010), pyropheophorbide a (PPhA) derivatives (MacDonald et al., 1999), and triphenylphosphonium (TPP) derivatives (Adlam et al., 2005; Boddapati et al., 2008; Jiang et al., 2009; Mossalam et al., 2010; Cho et al., 2015). Their mitochondrial translocation is mostly driven by mitochondrial membrane potentials (Yamada and Harashima, 2008; Mossalam et al., 2010). Additionally, as nanostructural moieties, single-walled carbon nanotubes (Zhou et al., 2010) and graphene oxide (Wei et al., 2016) have been shown to target the mitochondria. However, it is unclear whether graphene oxide is a true mitochondrial-targeting moiety, because the targeting systems included PhA, which is a known mitochondria-targeting compound.

Among these peptides, chemicals, and nanostructures for mitochondrial drug delivery, our interest was in mitochondria-targeting moieties with multifunctionalities (e.g., subcellular targeting, imaging, therapeutic effects). However, separate from their mitochondria-targeting function, the potential imaging and therapeutic applications of these moieties in drug delivery systems have rarely been studied, except for reports on TPP-mediated anti-tumor effects (Cho et al., 2015) and rhodamine β-based imaging effects (Rajaputra et al., 2013). With recent reports that PhA (Tang et al., 2006; Cho et al., 2014; Choi et al., 2014; Kim et al., 2014), its derivative (e.g., DH-I-180-3, which is derived from pheophytin a from silkworm feces) (Kim et al., 2004), and its PEGylated conjugate (Rapozzi et al., 2010) were localized in the mitochondria, mitochondria-targeting photosensitizers have garnered attention for multiple purposes because they possess both therapeutic and imaging potentials. However, our literature survey of these photosensitizers raised some concerns about their mitochondria-targeting abilities. Although the chemical structure of PPhA is similar to that of PhA, the length of the substituted alkyl ether groups on the PPhA derivatives affected their subcellular accumulation (MacDonald et al., 1999). The propyl-to-heptyl ether PPhAs were mainly localized in the mitochondria, whereas the octyl-to-dodecyl ether PPhAs were detected in the lysosomes at low concentrations but in the mitochondria at high concentrations (MacDonald et al., 1999). For chlorin photosensitizers, it was reported that *N*-aspartyl chlorin e6 targeted the lysosomes (Liu et al., 2011), and Foscan® (*meta*-tetra[hydroxyphenyl]chlorin) mainly accumulated in the endoplasmic reticulum (ER) and the Golgi apparatus (Teiten et al., 2003). In addition, the preferential mitochondrial localization of PhA derivatives to other subcellular organelles has been observed by fluorescent and confocal microscopy (Kim et al., 2004, 2014; Tang et al., 2006; Rapozzi et al., 2010; Cho et al., 2014; Choi et al., 2014). However, it is still difficult to demonstrate whether the photosensitizers are really present in the mitochondria because the organelles are spread throughout the cytoplasm and because mitochondria-sensing probes could not image all space in the mitochondria. Furthermore, although the TPP-mediated, mitochondria-targeting activities of TPP-decorated nanoparticles (NPs) have been investigated (Marrache and Dhar, 2012; Cho et al., 2015), PhA-mediated mitochondria-targeting activities of PhA-incorporated nanosized drug carriers have been rarely studied.

Thus, in this study, we investigated the following: 1) whether PhA is preferentially localized in the mitochondria; 2) whether PhA drives PhA-based nanosized drug delivery systems to the mitochondria; 3) whether the mitochondria-targeting ability of PhA in nanosystems is sufficiently high, especially, relative to that of TPP, a well-known mitochondria-targeting compound; and 4) whether PhA endows triple functionalities (i.e., mitochondria targeting, therapy, and imaging) to NPs serving as nanosized drug carriers. The goal of these investigations was to extend the potential applications of PhA-based NPs. For this purpose, the mitochondrial localization of PhA was assessed using two different technologies: confocal microscopy to image the intracellular localization of PhA in the whole cytoplasm and flow cytometry to evaluate the fluorescent intensities of PhA in the isolated mitochondria. In addition, two different amphiphilic components, a bioreducible photosensitizer conjugate (i.e., methoxy poly(ethylene glycol)-(PhA)$_2$ conjugate; mPEG$_{2kDa}$-(ss-PhA)$_2$ or PPA) (Kim et al., 2014) and a mitochondria-targeting copolymer (i.e., TPP-*b*-poly(ε-caprolactone)-*b*-TPP polymers; TPP-PCL$_{1.25kDa}$-TPP or TPCL) (Cho et al., 2015), were blended to construct PPA-TPCL NPs (PPA$_n$-TPCL$_{4-n}$ NPs, where $0 \leq n \leq 4$; for example, PPA$_1$-TPCL$_3$ NPs were prepared by blending a 1:3 molar ratio of PPA:TPCL) with tunable compositions of the mitochondria-targeting moieties (Fig. 1(a)). After being internalized into the cells, the PPA$_n$-TPCL$_{4-n}$ NPs should release their mPEG chains from the PPA component via intracellular cleavage of the disulfide (S-S) bonds, and the resulting PhA$_n$-TPCL$_{4-n}$ NPs (i.e., either TPCL NPs for n = 0, TPP-decorated PCL-PhA NPs for n = 1–3, or solid PhA NPs for n = 4) would target the mitochondria, thus demonstrating both the therapeutic and imaging effects of the delivered PhA in the presence of light (Fig. 1(b)).

2. Materials and methods

2.1. Materials and cell cultures

A bioreducible bi-armed methoxy poly(ethylene glycol)-(PhA)$_2$ (mPEG$_{2kDa}$-(ss-PhA)$_2$ or PPA) conjugate (Kim et al., 2014) and a TPP-*b*-poly(ε-caprolactone)-*b*-TPP (TPP-PCL$_{1.25kDa}$-TPP or TPCL) polymer (Cho et al., 2015) were synthesized as previously reported. The number of PhA molecules in one PPA conjugate was 1.57, and the number of TPP molecules in one TPCL polymer was 2. Pheophorbide a (PhA) and MitoTracker® Green FM were obtained from Frontier Scientific, Inc. (Logan, UT, USA) and Life Technologies (Grand Island, NY, USA), respectively. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst33342, 4-(2-hydroxy-ethyl)-1-piperazine (HEPES), fetal bovine serum (FBS), RPMI1640, Dulbecco's Modified Eagle's Medium (DMEM), sodium bicarbonate, D-glucose, Ca^{2+}-free and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS), penicillin-streptomycin antibiotics, trypsin-ethylenediaminetetraacetic acid (EDTA), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A mitochondria isolation kit and Spectra/Por membranes were acquired from BioVision (Milpitas, CA, USA) and Spectrum Laboratories, Inc. (Rancho Dominguez, CA, U.S.A), respectively.

HeLa cells (a human cervical adenocarcinoma cell line) and MCF7 cells (a human breast adenocarcinoma cell line) were used