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Functional nanosome for enhanced mitochondria-targeted gene delivery and expression

Yoonhee Bae[b], Min Kyo Jung[f], Su Jeong Song[a], Eric S. Green[g], Seulgi Lee[a], Hyun-Sook Park[h], Seung Hun Jeong[b], Jin Han[b], Ji Young Mun[d,e,*], Kyung Soo Ko[c,**], Joon Sig Choi[a,***]

[a] Department of Biochemistry, College of Natural Sciences, Chungnam National University, Daejeon 305-764, Republic of Korea
[b] Department of Physiology, College of Medicine, Cardiovascular and Metabolic Disease Center, Inje University, Busan 614-735, Republic of Korea
[c] Department of Internal Medicine, Sanggye Paik Hospital, Cardiovascular and Metabolic Disease Center, Inje University, Seoul 139-707, Republic of Korea
[d] Department of Biomedical Laboratory Science, College of Health Science, Eulji University, Seongnam, Gyeonggi-Do 461-713, Republic of Korea
[e] BK21 Plus Program, Department of Senior Healthcare, Graduate School, Eulji University, Republic of Korea
[f] Department of Life Sciences, Korea University, Seoul 136-701, Republic of Korea
[g] Salt Lake Community College, Salt Lake City, UT, USA
[h] Cell engineering for origin Research Center 46-21, Susong-dong, Jongno-gu, Seoul 110-140, Republic of Korea

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ABSTRACT

Mitochondria dysfunction plays a role in many human diseases. Therapeutic techniques for these disorders require novel delivery systems that can specifically target and penetrate mitochondria. In this study, we report a novel nanosome composed of dequalinium-DOTAP-DOPE (1,2 dioleoyl-3-trimethylammonium-propane-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) (DQA80s) as a potential mitochondria-targeting delivery vector. The functional DQAsome, DQA80s, showed enhanced transfection efficiency compared to a vector DQAsomes in HeLa cells and dermal fibroblasts. In addition, DQA80s/pDNA complexes exhibited rapid escape from the endosome into the cytosol. We observed the delivery of pDNA to mitochondria in living cells using flow cytometry, confocal microscopy, and TME imaging. More specifically, we confirmed our results by co-localization of hmtZsGreen constructs to mitochondria when delivered via DQAsomes and DQA80s in living cells. The mitochondria-targeting DQAsomes and DQA80s induced mitochondrial dysfunction through depolarization of mitochondrial membrane potential. Our data demonstrate that DQA80s show promise for use as a mitochondria-targeted carrier system for treatment of mitochondria diseases in vivo.

1. Introduction

Mitochondria are highly complex cellular organelles, in both structure and function. The principal function of mitochondria is ATP synthesis via oxidative phosphorylation, which is essential for virtually all cellular processes (Green and Reed, 1998; Heller et al., 2012). In addition, mitochondria are involved in cellular differentiation, cell growth, the regulation of the cell cycle, and cell death (Cosentino and Garcia-Saez, 2014; Lopez-Mejia and Fajas, 2014). Mitochondrial dysfunction plays a role in many human disorders, including neurodegenerative disease, obesity, ischemia-reperfusion injury, cancer, and inherited mitochondrial disease (D'Souza et al., 2011; Vaidya et al., 2009). Effective medical therapies for mitochondria-related diseases will ultimately require an intra-mitochondrial delivery system for both small drug molecules and macromolecules. Promising results have been found for certain vectors, including reports that DQAsomes induce cell death at higher rates in cancer cells than normal cells through mitochondrial targeting (Lasch et al., 1999). However, a mitochondrial delivery system must overcome many intracellular obstacles, such as the risk of degradation by enzymes, and the necessity for crossing the mitochondrial double membrane. Moreover, multiple processes must be accounted for, including regulated cellular uptake via endocytosis, endosomal escape, release into the cytosol, and targeting to a specific organelle. Despite these challenges, mitochondria-targeted drugs and gene delivery systems continue to undergo improvements to yield the desired therapeutic effects (Lechardeur et al., 2005; Malhi and Murthy, 2012).

To enhance the mitochondria-targeting capacity of a liposome-

* Corresponding author at: Department of Biomedical Laboratory Science, College of Health Science, Eulji University, Seongnam, Gyeonggi-Do 461-713, Republic of Korea.
** Correspondence to: Kyung Soo Ko, Department of Internal Medicine, Sanggye Paik Hospital, Cardiovascular and Metabolic Disease Center, Inje University, Seoul 139-707, Republic of Korea.
*** Correspondence to: Joon Sig Choi, Department of Biochemistry, College of Natural Sciences, Chungnam National University, Daejeon 305-764, Republic of Korea.
E-mail addresses: jiyoung.mun@eulji.ac.kr (J.Y. Mun), kskomd@paik.ac.kr (K.S. Ko), joonsig@cnu.ac.kr (J.S. Choi).

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based carrier system, a more efficient and site-specific vehicle must be constructed. Currently, some well-known mitochondria-targeted drug delivery systems include the lipophilic triphenylphosphonium cation (TPP) cation molecule, dequalinium (dicationic units in DQAsomes), and mitochondria-targeted signal peptides (MTSs) (Kolevzon et al., 2011; Weissig et al., 2007; Yamada et al., 2007). Among them, DQAsomes are known as delocalized, lipophilic cation molecules that can self-assemble when prepared from dequalinium chloride. They accumulate in mitochondria in response to the electrochemical gradient across the mitochondrial membrane system (D'Souza et al., 2003; Weiss et al., 1987). The referenced report, suggests that the DQAsomes may achieve localization to mitochondria through a endocytosis pathway, and enter by non-specific electrostatic interactions between the surface charge of the DQAsomes and the mitochondrial membrane. In addition, the mitochondrial-targeting properties of the DQAsomes has shown that DQAsomes/DNA complexes (DQAplexes) may be taken into the cell via non-specific endocytosis pathways and then accumulate in mitochondria, crossing the outer mitochondrial membrane (OMM). This delivery has been demonstrated to be successful, but is hampered by lack of efficiency (D'Souza et al., 2005; D'Souza et al., 2003). The DQAsomes have also been used as a topical antimicrobial or antitumor agent, demonstrating anticancer activity due to accumulation in the mitochondria driven by a highly negative mitochondrial membrane potential that appears to be altered in cancer cells. However, the underlying mechanisms of anti-tumor activity are not yet well understood (Galeano et al., 2005; Pajuelo et al., 2011). Several studies involving DQAsomes show their antitumor effects as resulting from mitochondrial dysfunction, due to disruption of the mitochondrial membrane potential, production of reactive oxygen species (ROS), and inhibiting of ATP synthesis (Garcia-Perez et al., 2011; Sancho et al., 2007). In the present study, in order to establish unique properties of the DQAsomes and enhance the mitochondria-targeting capacity of existing DNA delivery systems, we constructed a nanosome made of dequalinium-DOTAP-DOPE (1,2 dioleoyl-3-trimethylammonium-propane,-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine), called DQA80s, a liposome-based carrier system and vector suited for transport into the mitochondria of living cells.

For the primary characterization of DQA80s, we performed dynamic light scattering, zeta potential measurements, PicoGreen assay, and TEM imaging in HeLa cells. DQAsomes and DQA80s were compared for their cytotoxicity, transfection efficiency, cellular uptake, endosomal escape, and mitochondrial distribution. In addition, when using the mini-mitochondrial construct hmtZsGreen plasmid, DQA80s showed dramatically efficient localization and expression at mitochondria sites. Furthermore, we analyzed the anticancer activity by monitoring cell cycle distribution and caspase 3 activity, and mitochondria dysfunction by studying mitochondrial membrane potential using JC-1 and intracellular glutathione (GSH) levels. Our results suggest DQA80s represent a significant improvement over existing nanosome delivery systems in their mitochondrial-targeting ability and excellent promise for use as carriers for the therapeutic applications in mitochondria-related disease.

2. Materials and Methods

2.1. Materials

Dequalinium chloride was purchased from Sigma-Aldrich (Seoul, South Korea). 1,2-dioleoyl-*sn*-glycero-3-phoshphoethanolamine (DOPE), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), and 1,2-dioleoyl-*sn*-glycero-3-phoshphoethanolamine-*N*-(carboxy-fluorescenin) were obtained from Avanti polar and lipids (Alabaster, AL, USA). The luciferase reporter plasmid DNA was prepared as previously reported (Choi et al., 2004). The luciferase assay kit and 5 × Reporter Lysis Buffer were purchased from Promega (Madison, WI, USA). The Micro Protein Assay Kit was obtained from Pierce (Rockford, IL, USA). EZ-Cytox and EZ-LDH reagent were purchased from Daeil Lab Service (Seoul, South Korea). PicoGreen reagent, LysoTracker, LysoSensor Probe, Mitoprobe JC-1 assay kit, MitoTracker Red CMXRos, NucBlue Live Ready probes, Alexa fluor 488 5-SDP ester, DMEM medium supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/mL), penicillin (100 U/mL), and Trizoi were purchased from Invitrogen (Seoul, South Korea). Glutathione Colorimetric and Caspase-3/CPP32 Colorimetric Assay kit were purchased from BioVision (Seoul, South Korea). TMRE Mitochondrial Membrane Potential Assay Kit was purchased from abcam (Seoul, South Korea). Transcriptor Universal cDNA Master was purchased from Roche (Seoul, South Korea). SYBR Green Master Mix was purchased from Bio-Rad (Seoul, South Korea).

2.2. Preparation of DQAsomes, DOTAP/DOPE, or DQA80s

DQAsomes were prepared as described previously (Weissig et al., 2000). In brief, dequalinium chloride (10 mM final) was dissolved in methanol in a round bottom flask and the organic solvent was evaporated using a rotary evaporator for 20 min. The solvent free dequalinium film was resuspended in 5 mM HEPES buffer, at pH 7.4 and sonicated for 1 h, and the suspension was centrifuged at 10,000 rpm for 10 min to remove metal particles. Finally, prepared DQAsomes were filtered using a 0.8 μm pore filter. DOTAP/DOPE and DQA80s were prepared using the transfer lipid DOTAP and helper lipid DOPE. Briefly, 10 mg of DOTAP/DOPE (molar ratio, DOTAP/DOPE = 50:50) and DQA80s (molar ratio, DOTAP/DOPE, dequalinium chloride = 10:10:80) were dissolved in chloroform in a glass bottle. The solvent was dried using a nitrogen gas stream. The solvent-free lipid films were vacuum-stored for 1 h and then 5 mM HEPES buffer, pH 7.4 was added to the lipid film and sonicated for 1 h. The suspension of DQA80s was centrifuged at 10,000 rpm for 10 min and supernatant was subsequently collected. Finally, prepared DQA80s were filtered using a 0.8 μm pore filter.

PE-CF (an ammonium salt conferring lipid fluorescence) was incorporated to fluorescently label DOTAP/DOPE, DQAsomes, or DQA80s, at a molar ratio of 1/50,000 lipid and dequalinium chloride.

2.3. HmtZsGreen plasmid

For design of the mitochondria ZsGreen expression gene, the hmtZsGreen construct was prepared as previously described (Lyrawati et al., 2011). Briefly, the mitochondria ZsGreen expression gene was synthesized and then inserted into a pET-28b (+) vector by (Cosmogenetech, Seoul, South Korea). HmtZsGreen plasmid gene was confirmed by gene sequencing (Cosmogenetech, Seoul, South Korea). Human mitochondrial DNA referenced from NC_012920, and ZsGreen gene from CQ871118.

2.4. Primary human dermal fibroblasts and HeLa cell culture

Human skin samples were obtained under the written informed consent of donors, in accordance with the ethical committee approval process of the Institutional Review Board of Chungnam National University School of Medicine (CNUH 2013-04-025). Human dermal fibroblasts were isolated from human foreskin tissues and primary cultured. Skin tissues were sterilized in 70% ethanol, minced, and treated with dispase 16 h at 4 °C. The dermis was separated from epidermis and placed in a culture dish for explant culture. Cells were grown in DMEM supplemented with 10% FBS, streptomycin (100 μg/mL), and penicillin (100 U/mL) (invitrogen). Cells passaged between 3 and 8 times were used in this study. Human HeLa cells purchased from Korean Cell Line Bank were maintained up to passage 17 in DMEM supplemented with 10% FBS, streptomycin (100 μg/mL), and penicillin (100 U/mL) under conditions of 5% CO_2 in humidified incubator.