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Enzyme-responsive destabilization of stabilized plasmid-lipid nanoparticles as an efficient gene delivery

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ABSTRACT

Stabilized plasmid-lipid particles (SPLPs) have been developed to overcome the low stability issue of cationic liposomes, however, SPLPs that are too stable result in unsatisfactory transfection efficiency. In this article, we prepared enzyme-responsive SPLPs (eSPLPs) composed of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and mPEG-GLFG-K-$(C_{16})_2$, a PEG lipid with an enzymatically-cleavable linker (glycine-phenylalanine-leucine-glycine, GFLG). eSPLPs were successfully prepared with plasmid DNA (pDNA) encapsulation efficiency of over 80%, using the detergent dialysis method. The PEG shell stabilized eSPLPs and maintained a hydrodynamic diameter of around 200 nm. Although typical SPLPs were relatively intact in endosomal condition, the PEG shell of eSPLPs was cleaved following the degradation of the GFLG linker by cathepsin B in the endosome. Then, eSPLPs collapsed and induced endosomal disruption triggering the controlled release of the encapsulated pDNA into cytoplasm. Owing to the enzyme-responsive destabilization, eSPLPs showed a 10 to 100-fold higher transfection efficiency than control SPLPs, which was confirmed using luciferase assay. These results suggest that eSPLPs might be promising candidates for practical use as gene delivery systems, with both stability and high transfection efficiency for future *in vivo* applications.
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1. Introduction

Liposome is an attractive candidate for a successful gene delivery carrier (Ropert, 1999; Templeton and Lasic, 1999). In particular, cationic liposomes are one of the most widely used non-viral gene delivery carriers in current studies (Gao and Huang, 1995; Hirko et al., 2003; Shim et al., 2013). Cationic liposomes can condense anionic genetic materials such as pDNA and siRNA by electrostatic interaction to form liposome-based complexes (lipoplexes) for facilitating gene delivery. Although various lipoplexes have shown outstanding transfection efficiency *in vitro*, the majority have not accomplished satisfactory delivery results *in vivo*, likely due to the several known drawbacks of lipoplexes (Kim et al., 2013). Since the electrostatic interaction between the cationic liposomes and the anionic genetic material is reduced in physiological fluids, the genetic cargos are prematurely released from the lipoplexes prior to arrival at the target site (Zelphati et al., 1998). The surface of lipoplexes with an excess positive charge can seriously damage the plasma membrane (Lv et al., 2006), and interact with serum proteins to form large irregular aggregates (Hofland et al., 1997). Furthermore, positively charged aggregates are rapidly cleared from the blood circulation *in vivo* through filtration by 'first-pass' organs such as the lungs, liver, and spleen (Schmidt-Wolf and Schmidt-Wolf, 2003).

One of the advanced liposomal systems is stabilized plasmid-lipid particles (SPLPs) featured with cargo encapsulation and surface stabilization (Wheeler et al., 1999). The genetic cargo molecules are encapsulated into the liposomes by dialysis, and the surface of the liposomes is stabilized by neutral hydrophilic polymers such as polyethylene glycol (PEG) (Tirosh et al., 1998). The full encapsulation inhibits the premature release of genetic material, and the PEG-coated surface prevents the aggregation of the cationic liposomes and the anionic genetic molecules or serum proteins (Veronese et al., 2005). In addition, the reduced surface charge contributes to the lower cytotoxicity and longer circulation time of SPLPs (Zhang et al., 1999; Fenske et al., 2002). Moreover, other therapeutic reagents or targeting ligands can be easily incorporated into SPLPs along with pDNA as multi-functional delivery carriers (Choi et al., 2003; Ambegia et al., 2005).

However, if SPLPs are too stable, this can result in lower transfection efficiency compared with simple lipoplexes, likely due to the limited endosomal escape of SPLPs (Kumar et al., 2003). Therefore, recent studies have focused on the control of PEG-coated surfaces for high stability during circulation, as well as high transfection efficiency (Blenke et al., 2013). Successful enhancement of the transfection efficiency of SPLPs had been achieved by deshielding the PEG following after their cellular internalization (Zhang et al., 2015). Linkers between PEG and a lipid molecule that is degradable at endosomal pH have been actively researched (Li et al., 2005). In addition, a reduction in pH is not only observed in the process of endosomal maturation, but also in tumor

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microenvironment, inflammatory and osteoclastic processes (Blenke et al., 2013; Kato et al., 2013; Muzylak et al., 2007). Therefore, a degradable linker that is more specific to endosomal signals is required for the delicate control of PEG deshielding.

In the present study, our aim was to introduce the enzymatically-cleavable peptide linker, GFLG (Gly-Phe-Leu-Gly), between PEG and K (Lys) conjugated to two palmitic acid molecules, to create an enzyme-responsive SPLPs (eSPLPs) using the PEG-GFLG-K-(lipid)$_2$ (Fig. 1). The GFLG linker can be degraded by the endo-lysosomal enzyme, cathepsin B, detaching the PEG shielding. Two palmitic acid anchors can be incorporated into the liposome with the PEG shell. We hypothesized that the cleavage of the linker would facilitate the endosomal escape of SPLPs to enhance the transfection efficiency. (Fig. 2) Moreover, as cathepsin B has been widely implicated in tumor progression (Yan et al., 1998; Gondi and Rao, 2013), the cathepsin B-responsive SPLPs would be a potential gene delivery carrier for cancer gene therapy.

2. Materials and methods

1,2-dioleoyl-*sn*-glycero-3-phoshphoethanolamine (DOPE) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). α-aminopropyl-ω-methoxy, polyoxyethylene (mPEG-NH$_2$, $M_W = 5000$) was purchased from Sunbright (Tokyo, Japan). *N*-α-Fmoc-glycine (Fmoc-Gly-OH), *N*-α-Fmoc-L-leucine (Fmoc-Leu-OH), *N*-α-Fmoc-L-phenylalanine (Fmoc-Phe-OH), and *N*-hydroxybenzotriazole (HOBt) were obtained from Anaspec (Fremont, CA, USA). *N*-α,ε-di-Fmoc-L-lysine (Fmoc-Lys(Fmoc)-OH) and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexaafluorophosphate (HBTU) were purchased from Novabiochem (Darmstadt, Germany). Octyl β-D-glucoyranoside (OGP), cathepsin B, piperidine, *N*,*N*-dimethylformamide (DMF), *N*,*N*-diisopropylethylamine (DIPEA), thiazolyl blue tetrazolium bromide (MTT), reduced L-glutathione, dimethyl sulfoxide-d$_6$, DEAE–Sepharose (CL-6B), *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), 2,5-dihydroxybenzoic acid (DHB), and sodium acetate were obtained from Sigma-Aldrich (St Louis, MO). The luciferase kit was from Promega (Madison, WI, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), 100× antibiotic-antimycotic agent, and Dulbecco's phosphate buffered saline (DPBS) were purchased from Gibco (Gaithersburg, MD, USA). Acetic acid was obtained from Merck (Darmstadt, Germany). 500 mM ethylenediaminetetraacetic acid (EDTA) solution and sodium chloride (NaCl) were purchased from Calbiochem (San Diego, CA, USA). Palmitic acid was obtained from JUNSEI (Tokyo, Japan). SPECTRA/POR 7 (MWCO = 10,000) and SPECTRA/POR 7 (MWCO = 3500) were purchased from Spectrum Labs (Rancho Dominguez, CA, USA). Triton X-100 was purchased from Shinyo Pure Chemicals (Osaka, Japan). The PicoGreen assay kit and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA, USA). The Micro BCA protein assay kit was purchased from Thermo Scientific (Hudson, NH, USA). The luciferase expression plasmid DNA (pCN-luc) was prepared as previously reported.

2.1. Synthesis of PEG lipids

Poly(ethylene glycol) was conjugated to a lipid by liquid-phase peptide synthesis using fluoren-9-ylmethoxycarbonyl (Fmoc) chemistry (Hamley, 2014). PEG-NH$_2$ was reacted with Fmoc-Gly-OH (4 eq.) in DMF containing HOBt (4 eq.), HBTU (4 eq.) and DIPEA (8 eq.) at ambient temperature for 16 h. The glycine-conjugated PEG was precipitated using cold diethyl ether. The Fmoc group of the precipitated compound was deprotected in 30% piperidine solution (DMF:piperidine (7:3, v/v)) at ambient temperature for 1.5 h. The compound was re-precipitated

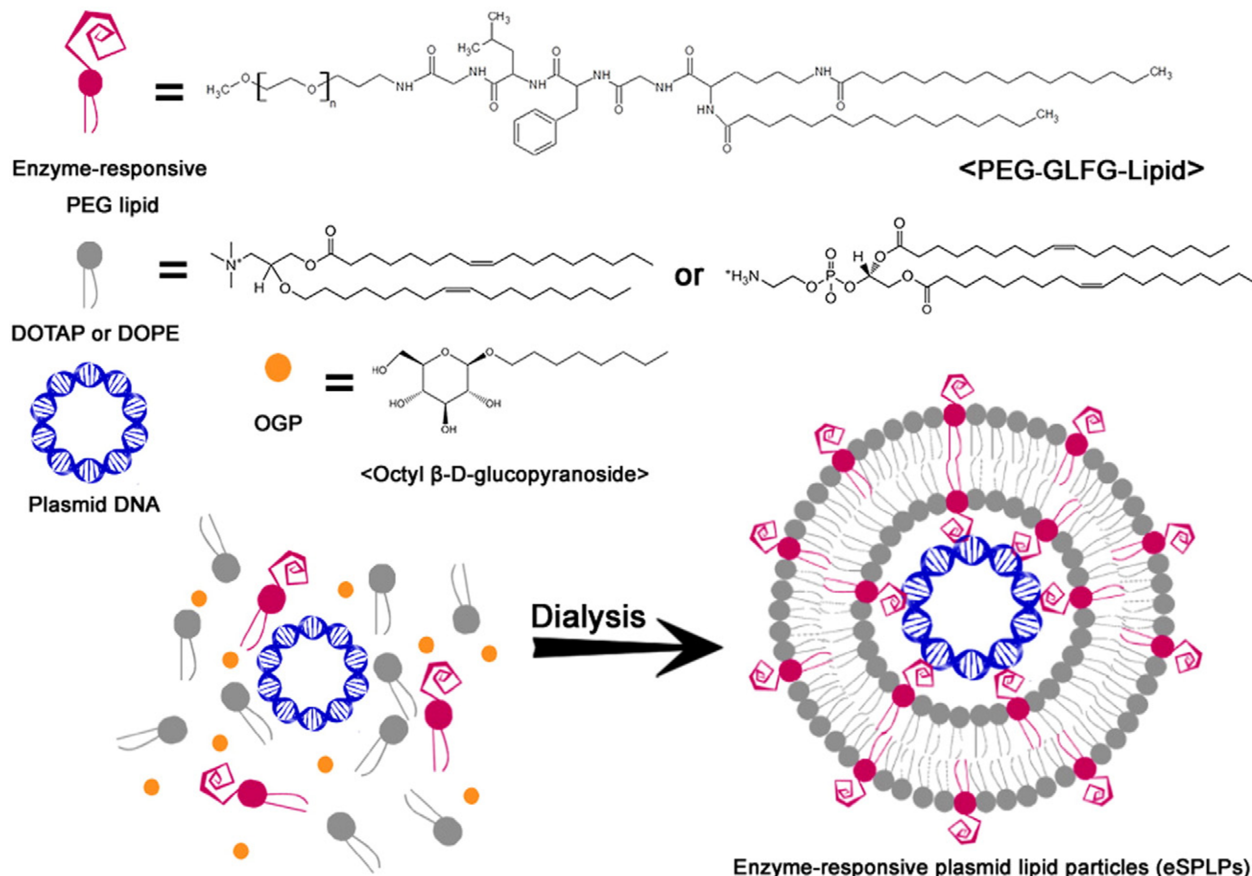

Fig. 1. Preparation of enzyme-responsive stabilized plasmid lipid particles (eSPLPs).

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