Application of Polyglycerol Coating to Plasmid DNA Lipoplex for the Evasion of the Accelerated Blood Clearance Phenomenon in Nucleic Acid Delivery

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ABSTRACT: Cationic liposomes (CLs) have shown promise as nonviral delivery systems. To achieve *in vivo* stability and long circulation, most liposomes are modified with hydrophilic polymer polyethylene glycol (PEG). However, we have reported that repeated administration of PEG-coated CLs containing plasmid DNA (pDNA; PEGylated lipoplexes) induces what is referred to as "the accelerated blood clearance (ABC) phenomenon" and, consequently, subsequently administered lipoplexes lose their prolonged circulation characteristics. Anti-PEG IgM produced in response to the first dose of PEG-coated pDNA–lipoplexes (PEG–DCL) has proven to be a major cause of the ABC phenomenon. In this study, to evade and/or attenuate this unexpected immune response, we modified the surface of a lipoplex with polyglycerol (PG)-derived lipid. The PG-coated pDNA–lipoplex (PG–DCL) attenuated the production of anti-polymer IgM, whereas PEG-coated pDNA–lipoplex (PEG–DCL) did not. In addition, a second dose of PG–DCL maintained the accumulation level in the tumor tissue of a tumor-bearing mouse model, comparable to that of the first dose, whereas the tumor accumulation level of a second dose of PEG–DCL was significantly compromised, compared with the first dose of PEG–DCL. Our results indicate that surface modification of lipoplex with PG represents a viable means for the attenuation, and/or evasion, of the ABC phenomenon that is encountered upon repeated administrations of nucleic acids containing PEG-coated nanocarriers. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:557–566, 2014

Keywords: accelerated blood clearance (ABC) phenomenon; anti-PEG IgM; clearance; lipoplexes; non-viral gene delivery; PEGylation; plasmid DNA (pDNA); polyethylene glycol (PEG); polyglycerol (PG)

INTRODUCTION

Gene therapy is a promising strategy for the treatment of several inherited and acquired human diseases, including cancer.[1] The efficient delivery of nucleic acids to target sites and then across a cellular membrane to the intracellular site of action is a key determinant for the success of gene therapy.[2,3] Nucleic acids exhibit a short half-life following intravenous administration because of their rapid degradation by circulating and intracellular nucleases.[4] In addition to rapid systemic clearance, low selectivity for the desired tissue and poor cellular uptake limit their clinical applications.[5,6] Therefore, it is imperative that a safe and efficient gene delivery system is developed to circumvent all the above-mentioned obstacles.

Cationic liposomes (CLs) have proven to be an effective tool for gene delivery both *in vitro* and *in vivo*.[7–9] They strongly interact electrostatically with nucleic acids, such as plasmid DNA (pDNA), yielding a net positively charged complex (pDNA–lipoplex). This net positive charge of pDNA–lipoplex, on the up side assures the efficient uptake of lipoplex by target cells via electrostatic interaction with the negatively charged plasma membranes.[9] On the down side, following intravenous administration, pDNA–lipoplexes easily aggregate with negatively charged blood components such as serum proteins and are rapidly eliminated from the circulation, with a resultant of reduced systemic availability.[10]

PEGylation, which amounts to the coating of liposome surface with the hydrophilic polymer polyethylene glycol (PEG), is considered an efficient approach to avoid the interaction of liposomes with serum opsonins and cells of the mononuclear phagocyte system, thus prolonging plasma circulation time.[11,12] However, an unexpected phenomenon, referred to as the "accelerated blood clearance (ABC) phenomenon" has been extensively reported upon repeated administration of PEG-coated liposomes.[13–16] We have proven that anti-PEG IgM produced in response to the first dose of PEG-coated liposomes plays a predominant role in triggering the rapid clearance of the second dose.[17,18] A similar phenomenon was also reported upon repeated administration of PEG-coated lipoplexes, and the nucleic acids (pDNA and siRNA) encapsulated/complexed with the PEG-coated CLs further enhanced the production of anti-PEG IgM, and, consequently, enhanced the ABC phenomenon.[19–21]

Abbreviations used: ABC, accelerated blood clearance; DC-6-14, O,O′-ditetradecanoyl-N-(alpha-trimethyl ammonioacetyl) diethanolamine chloride; DiD, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate; ELISA, enzyme-linked immunosorbent assay; IL-6, interleukin 6; IFN-γ, interferon gama; $mPEG_{2000}$-DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000]; pDNA, plasmid DNA; PEG, polyethylene glycol; PG, polyglycerol; PG_{760}-DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[(polyglycerine)-760]; PL, phospholipid; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; Rh-PE, rhodamine B-sulfonyl phosphatidylethanolamine; TLR, Toll-like receptor; TNF-α, tumor necrosis factor alpha.

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This immunogenicity of PEGylated nanocarriers, therefore, constitutes a major impediment upon the repeated delivery of encapsulated therapeutic agents to target tissues.[22–25]

Recently, we showed that modification of the liposome surface with a polyglycerol (PG)-derived lipid (Suppl. Fig. 1) elicited no immunogenic response upon repeated administration, as manifested by the lack of anti-PG IgM production and an absence of the ABC phenomenon.[26] In the present study, we expanded our work to investigate whether surface modification of pDNA–lipoplex with PG, instead of PEG, could prevent and/or attenuate the production of anti-polymer IgM and thus abrogate the occurrence of the ABC phenomenon.

Table 1. Physicochemical Properties of Liposomal Formulations

Formulation	Particle Size (nm)	Zeta Potential (mv)
CL	107.9 ± 1.5	23.62 ± 3.5
PEG-coated CL (PEG–CL)	110.6 ± 1.5	15.66 ± 1.3
PG-coated CL (PG–CL)	123.7 ± 9.9	27.75 ± 2.2
pDNA–lipoplex (DCL)	245.4 ± 45.0	23.75 ± 1.8
PEG-coated pDNA–lipoplex (PEG–DCL)	164.8 ± 27.9	14.31 ± 0.9
PG-coated pDNA–lipoplex (PG–DCL)	192.2 ± 46.2	23.47 ± 2.5

Data were obtained with three liposome preparations, which were prepared independently.

MATERIALS AND METHODS

Materials

1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000] ($mPEG_{2000}$-DSPE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-(polyglycerine-760) (PG_{760}-DSPE), and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) were generously donated by NOF (Tokyo, Japan). A cationic lipid, O,O′-ditetradecanoyl-N-(alpha-trimethyl ammonioacetyl) diethanolamine chloride (DC-6-14) was purchased from Sogo Pharmaceutical (Tokyo, Japan). All lipids were used without further purification. The hydrophobic fluorescent dyes, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[lissamine rhodamine B-sulfonyl (Rh-PE)] were purchased from Invitrogen (Carlsbad, California). pDNA, pEGFP-N1, which contains the CpG motifs [4733 bp, 321 CpG points (13.56%)], was purchased from Clontech (Mountain View, California). All other reagents were of analytical grade.

Animals

Male Wistar rats weighing 200–250 g, male BALB/c and ddY mice (5–6 weeks) were purchased from Japan Clea (Tokyo, Japan). The animals were housed in the animal research facility and had free access to water and animal chow. All animal studies were carried out in compliance with the health guidelines for animal care and use approved by the Animal and Ethics Review Committee of The University of Tokushima.

Preparation of Liposomes

The CLs were composed of DC-6-14/POPC (50:50, molar ratio). For cellular uptake experiments, 1 mol % of the dye Rh-PE was incorporated in the lipid mixture. For *in vivo* imaging experiments, 1 mol % of the fluorescent dye DiD was incorporated in the lipid mixture. The liposomes were prepared according to a method described earlier.[27] Values for the mean diameter and zeta potential of the prepared CLs were 107.9 ± 1.5 nm and 23.6 ± 3.5 mV ($n = 3$), respectively, as determined using a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, santa Bardera, California).

Preparation of PEG/PG-Coated pDNA–Lipoplexes and PEG/PG-Coated Empty CLs

For the formulation of pDNA–lipoplexes, pDNA and CLs were mixed at a 3.82 (+/−) charge ratio and incubated at room temperature for 20 min. For surface modification of liposomes with either PEG_{2000}-DSPE or PG_{760}-DSPE, a post-insertion technique was used as described previously.[28] The mean diameters and zeta potentials of the different formulations are summarized in Table 1. The concentration of phospholipids (PLs) was determined by colorimetric assay.[29]

In vivo Fluorescence Imaging Study of the First and Second Dose in Tumor-Bearing Mice

C26 cells (2×10^6), obtained from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University), were inoculated subcutaneously in the backs of the BALB/c mice. On day 7 after tumor inoculation, when the tumor size reached approximately 100 mm^3, mice were treated with either DiD-labeled pDNA–lipoplexes (DCL), DiD-labeled PEG-coated pDNA–lipoplexes (PEG-DCL), or DiD-labeled PG-coated pDNA–lipoplexes (PG-DCL) at a dose of 0.4 μM PL/mouse and 10 μg pDNA/mouse. At different time intervals post-injection (8, 12, and 24 h), the mice were anesthetized with isoflurane (FORANE, Abott Japan, Osaka, Japan), and fluorescence imaging was conducted using a Fluorescence Image Analyzer LAS-4000IR (Fujifilm, Tokyo, Japan).

To follow the tumor accumulation of the second test dose of lipoplexes, mice were pretreated with either DCL, PEG–DCL, or PG–DCL at a dose of 0.4 μM PL/mouse and 10 μg pDNA/mouse on day 7 after tumor inoculation. Five days later, the mice were intravenously administered a second test dose of either DiD-labeled DCL, DiD-labeled PEG–DCL, or DiD-labeled PG–DCL at a dose of 0.4 μM PL/mouse and 10 μg pDNA/mouse. At 8, 12, and 24 h after test dose administration, fluorescence imaging was performed, as described above. The fluorescence intensity of DiD-labeled PEG–DCL or DiD-labeled PG–DCL accumulated within the tumor tissue was quantitatively analyzed using Fluorescence Image Analyzer software (Fujifilm, Tokyo, Japan).

Detection of Anti-polymer IgM

A simple enzyme-linked immunosorbent assay (ELISA) procedure was employed to detect IgM against either the PEG–lipid conjugate or the PG–lipid conjugate according to the method described earlier[27] with a minor modification that the 96-well plates (EIA/RIA plate; Corning Inc., Corning, New York) were coated with either 10 nmol of $mPEG_{2000}$-DSPE or PG_{760}–DSPE in 50 μL of ethanol. All incubations were carried out at room temperature.