Journal of Controlled Release 133 (2009) 230–237

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Liposome encapsulated polyethylenimine/ODN polyplexes for brain targeting

Young Tag Ko [1], Raktima Bhattacharya, Ulrich Bickel *

Department of Pharmaceutical Sciences, Texas Tech University Health Science Center, Amarillo, TX 79106, United States

ARTICLE INFO

Article history:
Received 27 May 2008
Accepted 8 October 2008
Available online 26 October 2008

Keywords:
In vivo gene delivery
Oligodeoxynucleotides
PEGylated liposomes
Polyethylenimine
Blood-brain barrier

ABSTRACT

Despite high *in vitro* transfection efficiency, the use of the cationic polymer polyethylenimine (PEI) for systemic application is limited due to its rapid blood clearance and accumulation by RES sites upon intravenous administration of PEI/DNA polyplexes. Therefore, it is important to improve the properties of the PEI/DNA complex with respect to extending the systemic circulation time and suppression of RES uptake. In this study, we applied PEGylated liposome technology for systemic delivery of PEI polyplex of oligodeoxynucleotides (ODN), based on encapsulation of the PEI/ODN polyplexes into PEGylated liposomes. The PEI/ODN polyplex was prepared with a low-branched PEI with MW 2.7 kDa and 20-mer double stranded ODN and was then entrapped into PEGylated liposomes with 95% loading efficiency, leading to a virus-like structure with ~130 nm diameter. The PEG-stabilized liposome (PSL) entrapping PEI/ODN polyplexes remained stable in the presence of serum. Upon intravenous administration, the DNA in the PSL was cleared from systemic circulation at a significantly slower rate as compared to the naked PEI/ODN complex. Furthermore, targeting of the PSL with antibody specific to transferrin receptor redirected biodistribution of the entrapped ODN, leading to significant accumulation in the targeted organ, i.e. brain. Encapsulation of the PEI/ODN polyplexes within a long-circulating liposome provided a promising ODN delivery system for *in vivo* application.

1. Introduction

Among polycationic polymers, the polyethyleneimines (PEI) have been widely explored for gene delivery due to their high gene transfer efficiency [1–4]. This efficiency of PEI depends mainly on their characteristic chemical structure. PEI contain one amino group per every two carbons (ethylene group) and about 20% of the amino groups are protonated at physiological pH [5] resulting in high positive charge density. Due to this high positive charge density, PEI form dense nano-sized particulate complexes with negatively charged DNA by electrostatic interactions. The PEI/DNA complexes take overall positive charge and interact with negatively charged components of cell membranes and enter cells by endocytosis. The PEI/DNA complexes enter the cells by nonspecific adsorption-mediated endocytosis while the condensed DNA in the complexes is protected from enzymatic degradation. Upon endocytosis, PEI are subject to further protonation as the endosomal compartment acidifies. Protonation of PEI by capturing protons, the so called 'proton sponge' mechanism [1,2], leads to osmotic swelling and subsequent endosomal disruption. Hence, gene delivery using PEI is based on (i) condensation of the negatively charged DNA into compact particles by electrostatic interactions, thus protecting the DNA from enzymatic degradation, (ii) endocytosis of the particles into the cells and (iii) release of the DNA from endosomes via the 'proton sponge' mechanism.

Despite high transfection efficiency *in vitro*, PEI/DNA complexes have not shown significant therapeutic efficacy for *in vivo* application due to rapid plasma clearance and accumulation by RES sites. This instability of PEI/DNA complexes is mainly due to the overall positive charge of the complexes. While the cationic complexes interact with anionic components of cell membranes and thus trigger cellular uptake by absorptive mediated endocytosis, they also interact with blood components and are subject to clearance by the RES. As a result, PEI/DNA complexes are cleared from the circulation within a few minutes and accumulate mainly in RES organs such as liver and spleen [6]. The short blood circulation time limits the possibility of effective delivery of these complexes to target organs other than RES, e.g. central nervous system which represents a particular difficult organ for targeted delivery due to the presence of the blood-brain barrier (BBB). Therefore, it is desirable to improve the *in vivo* behavior of the PEI/DNA complexes by reducing the nonspecific charge interactions and thus prolong circulation time. Several approaches have been tried to increase stability of PEI/DNA complexes in the blood circulation [3,7], including covalent attachment of polyethylene glycol (PEG) to PEI. The PEG-PEI/DNA complexes showed reduced surface charge and non-specific interaction in blood, resulting in prolonged circulation time. Although the PEG-PEI was able to change *in vivo* biodistribution and pharmacokinetics, the stabilizing effect by PEG was modest [6,8].

In the present study, we applied PEI to form polyplexes with ODN and combined it with PEG-stabilized liposomes. We hypothesized that encapsulation of PEI/ODN complexes inside PEG-stabilized liposomes

* Corresponding author. Department of Pharmaceutical Sciences, School of Pharmacy, Texas Tech University Health Sciences Center, 1300 Coulter Dr. Amarillo, TX 79106, United States. Tel.: +1 806 356 4015; fax: +1 806 356 4034.
E-mail address: Ulrich.Bickel@ttuhsc.edu (U. Bickel).
[1] Present address: Center for Pharmaceutical Biotechnology and Nanomedicine, Northeastern University, Boston, MA 02115, United States.

0168-3659/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2008.10.013

would provide a delivery system with long circulation time, thus enabling the PEI/ODN complexes to accumulate in target organs other than RES sites. In addition to the prolonged circulation time endowed by PEG-stabilized liposomes, the system entrapping PEI/ODN complexes was expected to maintain the favorable properties of PEI, such as endosomal escape and nuclear targeting upon intracellular entry. We used a low molecular weight PEI [9,10] with 2.7 kDa molecular weight to form complexes with a 20-mer double-stranded ODN containing the NF-κB cis element as a decoy for NF-κB [11]. Activation of the transcription factor NF-κB drives inflammatory processes in disorders like Experimental Autoimmune Encephalomyelitis (EAE) [12], an animal model of Multiple Sclerosis, and NF-κB inhibition has been suggested as a promising therapeutic approach in neuroinflammatory disease [13,14].

The PEI/ODN complex was then encapsulated into PEG-stabilized liposomes containing biotin at the distal end of the PEG chain for facilitated attachment of targeting moieties. The biotinyl-PEG-stabilized liposome entrapping PEI/ODN complexes (designated bioPSL) was compared to the naked PEI/ODN complexes with regard to *in vitro* cellular uptake by brain-derived endothelial cells. To obtain proof that bioPSL enable specific targeting, we took advantage of the monoclonal antibody 8D3 to target transferrin receptors, which are highly expressed on brain microvascular endothelial cells and cause receptor-mediated uptake of the antibody itself [15] and when conjugated to immunoliposomes [16]. In addition, *in vivo* delivery to brain by the systemic route was shown after i.v. administration of antibody-targeted bioPSL.

2. Materials and methods

2.1. Double stranded oligodeoxynucleotides (ODN)

The sequence of the ODN (5′-CCTTGAAGGGATTTCCCTCC-3′) and complementary strand contained the NF-κB cis element [11]. Single stranded 20-mer oligonucleotides were purchased from MWG Biotech (Highpoint, NC, USA). Double stranded ODNs were prepared by annealing equimolar amounts of the single stranded oligonucleotides at a final ODN concentration of 1 μg/μl. For tracer experiments, ODN was radioactively labeled by a 5′-end-labeling technique using [γ-^{32}P] ATP (PerkinElmer Life and Analytical Sciences, MA, USA) and T4 PNK (Promega, WI, USA) as described previously [6].

2.2. Phospholipids

1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine (POPC), 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-[Phospho-rac-91-glycerol] (POPG), 1,2-Distearoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene-glycol)-2000](DSPE-PEG2000), 1,2-Distearoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Biotinyl(Polyethylene glycol)-2000] (DSPE-(PEG2000)Biotin), cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL).

2.3. Polyethylenimine (PEI)

Low-molecular weight PEI with MW 2.7 kDa (PEI2.7) was synthesized and characterized as described previously [17], and generously provided by Dr. T. Kissel (Marburg, Germany). PEI was dissolved in HBG buffer (10 mM HEPES, 5% glucose, pH 7.4) at a final concentration of 0.9 μg/μl.

2.4. Preparation of PEI/ODN complexes

100 μl of ODN and 90 μl of PEI stock solutions were separately diluted in HBG to a final volume of 500 μl. After 10 min incubation at room temperature, the PEI solution was then transferred to the ODN solution by fast addition and vortexed immediately, resulting in 1 ml PEI/ODN complexes (N/P=6) in HBG. The amounts of PEI were calculated from the desired amine/phosphate (N/P) ratio assuming that 43.1 g/mol corresponds to each repeating unit of PEI containing one amine and 330 g/mol corresponds to each repeating unit of ODN containing one phosphate.

2.5. Preparation of biotinylated PEG-stabilized liposomes encapsulating PEI/ODN (bioPSL)

The optimal fraction of the anionic lipid POPG in the lipid composition had been determined in a pilot study based on complete extraction of PEI/ODN into an organic phase (data not shown). The following amounts of lipids were dissolved in chloroform: POPC (3.7 μmol), POPG (3.0 μmol), cholesterol (3.0 μmol), DSPE-PEG2000 (0.3 μmol) and DSPE-PEG2000-Biotin (0.03 μmol). Chloroform was removed by vacuum evaporation using a rotary evaporator (500 mmHg, 4 h). PEI/ODN complexes were prepared as described above and 1 ml of the positively charged PEI/ODN complexes was then added to the dried anionic lipid film and incubated at room temperature for 4 h with intermittent mixing, resulting in a final lipid concentration of 10 mM. The suspension was extruded 11 times through a stack of two polycarbonate membranes of 100 nm pore size employing a hand-held extruder (Avestin, Ottawa, Canada). The resulting suspension was loaded onto a 1.0×30 cm Sepharose CL4B column (GE Healthcare Biosciences, Piscataway, NJ, USA) and then eluted with HEPES buffered saline (10 mM, pH 7.4) at a flow rate of 0.4 ml/min. The column eluents were monitored by on-line absorbance measurement at 254 nm while 1 ml fractions were collected. When applicable for tracer studies, the fractions were also analyzed for radioactivity. The fractions containing liposome-entrapped PEI/ODN (bioPSL) eluted at void volume and were used for further studies.

To determine entrapment efficiency, the bioPSL was prepared with tracer ^{32}P-ODN as described and the fractions of Sepharose CL4B column eluent were analyzed for radioactivity using liquid scintillation counting (Beckmann LS6000, CA, USA). Entrapment efficiency of ODN was calculated as area percentage of total activity found in the first peak. A formulation without PEI was prepared as a control to demonstrate the effect of condensation of ODN by PEI on encapsulation efficiency.

2.6. Size distribution and zeta potential

Size distribution and zeta potential of the bioPSL were determined by dynamic light scattering (DLS) using the particle size analyzer Nicomp 380ZLS (Particle Sizing Systems, Santa Barbara, CA, USA). The scattered light was detected at 23 °C at an angle of 90° and 18.9° for size and zeta potential, respectively. A viscosity value of 0.933 mPa·s and a refractive index of 1.333 were used for the data analysis. The instrument was routinely calibrated using latex microsphere suspensions (0.09 μm, 0.26 μm; Duke Scientific Corp, Palo Alto, CA, USA).

2.7. Cryo-transmission electron microscopy

The bioPSL was incubated with streptavidin-conjugated gold colloidal particles (10 nm, EY Labs, San Mateo, CA, USA) for 30 min at room temperature. As a control, the biotin binding sites in streptavidin-conjugated gold particles were pre-saturated with excess of free biotin before mixing with the bioPSL. Samples were applied as a drop on glow-discharged Fomvar carbon-coated grids (Ted Pella, Redding, CA, USA). After 30 s at room temperature, the excess sample was removed by blotting with filter paper. The grid was then rapidly frozen in liquid ethane and then observed under cryogenic conditions using a cryo-holder in a JEOL2100F field emission gun TEM (JEOL, Tokyo. Japan).

2.8. Binding of streptavidin-conjugated 8D3 to bioPSL

A 1:1 conjugate between the anti mouse transferrin receptor monoclonal antibody 8D3 and streptavidin (dubbed 8D3SA) was