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Liposome–polyethylenimine complexes for enhanced DNA and siRNA delivery

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ABSTRACT

The efficient delivery of nucleic acids into cells is critical for successful gene therapy or gene knockdown. Polyethylenimines (PEIs) are positively charged polymers which complex and deliver DNA for gene transfection or small interfering RNAs (siRNAs) for the induction of RNA interference (RNAi), and mediate their endosomal release. Likewise, various liposomes act as transfection reagents, with some lipids showing increased endocytosis and influencing endosomal escape. This study combines the favourable properties of PEI and lipid systems for DNA and siRNA delivery. Various lipids and lipid combinations, which cover a broad range of physicochemical properties and form optimal liposomes, are assessed. By addition of the liposomes to pre-formed polyplexes, based on the low molecular weight PEI F25-LMW, we establish liposome–PEI complexes (lipopolyplexes) and characterise them in comparison to their 'parent' polyplexes and liposomes regarding size, shape and zeta-potential. Furthermore, while the lipidation of polyplexes generally decreases their toxicity, our studies on DNA transfection and siRNA-mediated knockdown also establish certain lipopolyplexes based on rigid, negatively charged lipids as particularly efficient vehicles for nucleic acid delivery, further improving DNA transfection. The analysis of their mechanism and kinetics of cellular uptake confirms that the biological properties of lipopolyplexes are mainly determined by the liposome shell. We conclude that certain lipopolyplexes show improved biological properties over PEI complexes, thus representing potentially attractive non-viral vectors for gene therapy and RNAi.

1. Introduction

Successful gene therapy or gene knockdown depends on the delivery of nucleic acid molecules into cells [1]. While viral vectors have shown high gene transfer efficacies, their drawbacks include immunogenic/inflammatory responses, limited loading capacity and issues with regard to large scale manufacturing and quality control (for a review see Ref. [2]). Consequently, numerous non-viral gene delivery vectors have been developed and reported in recent years; the majority of these contain (poly)cationic structures, enabling inter-polyelectrolyte complexes with the negatively-charged nucleic acid molecules [3]. These non-viral systems can be divided into liposomes (lipoplexes), polycationic polymers (polyplexes) and organic or inorganic nanoparticles (nanoplexes) [4,5]. General issues in non-viral nucleic acid delivery include the protection of the sensitive payload from degradation, efficient cellular uptake and release from the endosomal compartment as well as release of the nucleic acid from its formulation and translocation into the nucleus (in the case of DNA therapy) or incorporation into the RNA-induced silencing complex (RISC) in the case of RNA interference (RNAi). RNAi [6] is a powerful method that has been successfully applied for the down-regulation of genes in both functional genetic analysis and gene therapy. It relies on small interfering RNAs (siRNAs) [7–9], and since all other components of the RNAi machinery are provided by the target cell/target tissue, the efficient siRNA delivery is of critical importance and has proven to be the bottleneck for successful RNAi applications [10].

Polyethylenimine (PEI) holds a prominent position among the polycationic polymers used for DNA delivery, because of its (relatively) high gene transfer efficacy [11,12]. PEIs are water-soluble, synthetic, linear or branched polymers with a protonable amino group in every third position [13,14]. Due to their high cationic charge density at physiological pH, PEIs are able to form non-covalent complexes with DNA and, more recently, have been reported to also facilitate the delivery of siRNA to induce RNAi [15–17]. The intracellular release of PEI-complexed nucleic acids from endosomes is hypothesized to rely on the protonation of amines in the PEI molecule, the so-called "proton sponge effect",

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leading to osmotic swelling and subsequent burst of the endosomes without the need for an additional endosomolytic agent [18,19]. Moreover, PEIs also facilitate the DNA entry into the nucleus [20,21]. In a recent study, a novel low molecular weight 4–10 kDa branched PEI F25-LMW was introduced as an efficient and biocompatible DNA and siRNA delivery reagent [17]. *In vitro* data demonstrated low toxicity and high biological activity [17,22], and, probably more importantly, PEI F25-LMW-based complexes were successful in mouse tumor xenograft models for siRNA delivery, resulting in anti-tumor effects based on the knockdown of tumor-relevant genes [23,59].

Nevertheless, non-specific interactions of PEI complexes with negatively charged physiological structures, complex aggregation and suboptimal cellular uptake hamper biological efficacies. To address these issues and to further enhance biocompatibility and efficiency, modifications have been introduced into the PEI molecule, including grafting with the hydrophilic polymers, poly(ethylene glycol) (PEG)/poly(ethylene oxide) (PEO), saccharides [12,24–29] or amine modification [30,31]. Covalent coupling of various hydrophobic moieties (cholesteryl, fatty acid residues, hydrophobic chains) to PEI has also been performed [32–35], and has led to the development of "lipid nano complexes" through oleoyl coupling [35] or "water-soluble lipopolymers" (WSLP) through cholesteryl grafting of PEI1800 [36,37]. Similar approaches for hydrophobic modifications were employed for oligoethylenimines [38].

Lipids form well-defined bilayers around the DNA/RNA molecules and hence protect them efficiently from degradation [39]. Some lipids have also been reported to increase the endocytosis and to influence the endosomal escape of the gene vector [39–42]. Lipoplexes based on cationic lipids such as DOTAP or Saint® have achieved notable transfection rates *in vitro* and *in vivo* [39,40].

Non-covalent modifications of PEI complexes with liposomes have recently been evaluated. These include the encapsulation of PEI/ODN complexes into PEG-stabilised liposomes comprising a mixture of phospholipids [43] or the formation of ternary complexes (lipopolyplexes) containing PEI and different liposomes [44–46]. Lipopolyplex formulations of branched 25 kDa PEI/DNA complexes in combination with multivalent cationic lipids (DOCSPER, DOSPER) [46–48], or of PEI/DNA complexes with a mixture of cationic or anionic and neutral lipids + cholesterol and PEG lipids [49] have been shown to be promising reagents for transfection or *in vivo* delivery of DNA.

This study aimed at generating liposome–PEI complexes for improved DNA or siRNA delivery through combination of the favourable properties of PEI (nucleic acid condensation, facilitated endosomal release), namely of PEI F25-LMW [17] (high efficacy, relatively low cytotoxicity), and those of lipid systems (increased cellular uptake, low cytotoxicity). To this end, a broader range of lipids and lipid combinations, which form optimal liposomes and cover a broad range of physicochemical properties, were assessed. By addition of the liposomes to the PEI F25-LMW-based polyplexes, we establish optimized liposome–PEI complexes (lipopolyplexes) with certain liposomal shells as non-toxic and particularly efficient vehicles for nucleic acid delivery, and analyse their mechanism and kinetics of cellular uptake.

2. Materials and method

2.1. Materials

PEI F25-LMW was prepared as described previously by size exclusion chromatography of 25 kDa PEI (Sigma, Taufkirchen, Germany) [17]. Dipalmitoyl-phosphatidyl-glycerol (DPPG), *N*-[1-(2,3-dioleoyloxy)]-*N*,*N*,*N*-trimethylammonium propane methylsulphate (DOTAP) and dipalmitoyl-phosphatidyl-ethanolamine-n-polyethylene glycol 5000 (MPEG-5000-DPPE) were a gift from Lipoid AG (Cham, Switzerland). Dipalmitoyl-phosphatidyl-choline (DPPC) and cholesterol were obtained from Sigma. Phospholipon® 100H was a gift from Phospholipid (Cologne, Germany). Wildtype SKOV-3 ovarian carcinoma cells were from ATCC/LGC Promochem (Wesel, Germany) and stably luciferase expressing SKOV-3 cells were prepared as described previously [15]. Inhibitors of endocytosis pathways, filipin III and chlorpromazine, were obtained from Sigma. All water used in this study (ddH_2O) was freshly double distilled.

2.2. Preparation of PEI/DNA and PEI/siRNA lipoplexes and lipopolyplexes

PEI complexes were prepared at optimal PEI/nucleic acid (i.e., DNA or siRNA) ratios, which are expressed as PEI/DNA or PEI/RNA equivalents (N/P ratios) on the basis of PEI nitrogen per nucleic acid phosphate (1 μg of DNA or siRNA is equivalent to 3 nmol of phosphate, and 1 μl of 0.9 mg/ml PEI solution contains 10 nmol of amine nitrogen). For complexation at N/P = 33, which was determined as optimal [22,23], 1 μg nucleic acid was dissolved in 40 μl buffer (150 mM NaCl, 10 mM HEPES, pH 7.4) and in a separate vial, 5 μg PEI (5 mg/ml solution) was dissolved in 40 μl of the same buffer. The PEI solution was then pipetted to the nucleic acid solution and after vortexing, the mixture was incubated for 30–45 min at room temperature.

Liposome–PEI complexes were prepared by incubation of the PEI/nucleic acid complexes with a dispersion of freshly prepared liposomes. The thin film hydration method was used for liposome production [40]. Briefly, for each formulation a mixture of lipids (see Table 1) was prepared from a 5 mg/ml stock solution in chloroform/methanol (2:1, v:v) and then dried to a thin film in a rotary evaporator (Heidolph, Schwabach, Germany). The lipid film was hydrated in ddH_2O to a final lipid concentration of 5 mg lipid/ml. All formulations were incubated for 5 min above the main phase transition temperature of the lipids (e.g., 41 °C for DPPC) in an ultrasound bath sonicator. Prior to addition to the PEI complex solutions, the vesicles were extruded 11 times through a 200-nm polycarbonate membrane using a heated Mini-Extruder (Avanti Polar Lipids, Alabaster, USA). Liposome–PEI complexes were stored at 4 °C.

2.3. Tissue culture, cell transfection and determination of luciferase activity

SKOV-3 cells were cultivated in IMDM medium (PAA, Cölbe, Germany) supplemented with 10% fetal calf serum (Gibco/Invitrogen, Karlsruhe, Germany) under standard conditions (37 °C, 5% CO_2 in a humid atmosphere). DNA transfection and siRNA-mediated knockdown experiments were performed in serum-containing medium as described previously [26]. Briefly, wildtype or stably luciferase expressing SKOV-3 ovarian carcinoma cells were plated at 4×10^4 cells/well in 24-well plates, and after 1 day in culture, liposome–PEI complexes were added at amounts corresponding to 0.5 μg DNA or 0.8 μg siRNA/well, respectively. PEI complexes were prepared as described above and taken from aliquots stored frozen [22]. Luciferase activity was determined 48 h after DNA transfection or 72 h after siRNA transfection, respectively, using the Luciferase assay kit from Promega (Mannheim, Germany) according to the manufacturer's protocol. Briefly, the medium was aspirated and the cells were lysed in 100 μl lysis buffer. In a luminometer tube, 25 μl substrate was mixed with 10 μl lysate and chemiluminescence was determined immediately in a luminometer (Berthold, Bad Wildbad, Germany).

To determine the time dependence of DNA transfection, cells were cultivated for 48 h after transfection and the medium containing the liposome–PEI complexes was exchanged with normal medium at the time points indicated in Fig. 6. To analyse uptake mechanisms, inhibitors of vesicular pathways (i.e., filipin III or chlorpromazine) were added at concentrations indicated in Fig. 7, 1 h prior to the start of the

Table 1
Physicochemical properties of the various liposomes.

Formulation (mol:mol)	PCS diameter ± SD [nm] (PDI)	AFM diameter ± SD [nm]	Zeta-potential ± SD [mV]
DPPC	158.7 ± 12.2 (0.12)	168.4 ± 17.4	−10.5 ± 1.2
DPPC/cholesterol (85:15)	149.3 ± 9.3 (0.14)	156.7 ± 12.3	−10.9 ± 2.2
DPPC/DOTAP (92:8)	189.3 ± 11.3 (0.11)	212.3 ± 13.4	+12.3 ± 0.9
DPPC/DPPG (92:8)	132.4 ± 8.7 (0.09)	152.4 ± 9.8	−14.5 ± 1.2
Phospholipon	121.5 ± 8.3 (0.15)	142.5 ± 10.3	−8.2 ± 1.2
DPPC/MPEG-5000-DPPE (95:5)	182.6 ± 10.6 (0.10)	173.8 ± 11.7	−24.5 ± 1.2