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Storage stability of optimal liposome–polyethylenimine complexes (lipopolyplexes) for DNA or siRNA delivery

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ABSTRACT

The delivery of nucleic acids such as DNA or siRNA still represents a major hurdle, especially with regard to possible therapeutic applications in vivo. Much attention has been focused on the development of non-viral gene delivery vectors, including liposomes or cationic polymers. Among them, polyethylenimines (PEIs) have been widely explored for the delivery of nucleic acids and show promising results. The combination of cationic polymers and liposomes (lipopolyplexes) for gene delivery may further improve their efficacy and biocompatibility, by combining the favourable properties of lipid systems (high stability, efficient cellular uptake, low cytotoxicity) and PEIs (nucleic acid condensation, facilitated endosomal release). In this study, we systematically analyse various conditions for the preparation of liposome–polyethylenimine-based lipopolyplexes with regard to biological activity (DNA transfection efficacy, siRNA knockdown efficacy) and physicochemical properties (size, zeta potential, stability). This includes the exploration of lipopolyplex compositions containing different liposomes and different relevant branched or linear low-molecular-weight PEIs. We establish optimal parameters for lipopolyplex generation, based on various PEIs, N/P ratios, lipids, lipid/PEI ratios and preparation conditions. Importantly, we also demonstrate that certain lipopolyplexes retain their biological activity and physicochemical integrity upon prolonged storage, even at 37 °C and/or in the presence of serum, thus providing formulations with considerably higher stability as compared to polyplexes. In conclusion, we establish optimal liposome–polyethylenimine lipopolyplexes that allow storage under ambient conditions. This is the basis and an essential prerequisite for novel, promising and easy-to-handle formulations for possible therapeutic applications.

1. Introduction

The use of therapeutic nucleic acids, such as DNA and siRNA, has been a promising strategy for the treatment of genetic disorders and cancer. One of the great challenges is the delivery of nucleic acids in vivo, including their protection from nuclease degradation and their cellular uptake and intracellular release. For this purpose, viral and non-viral vectors are used. Recombinant viruses have been shown to infect cells efficiently, but revealed some disadvantages with regard to immunogenicity, inflammation, quality control and limitations in gene size and large scale manufacturing [1,2]. To overcome these problems, much attention has been focused on the development of non-viral gene delivery vectors such as cationic liposomes, cationic polymers, dendrimers, peptides and inorganic compounds [3–5].

Commonly used cationic liposomes/lipids or cationic polymers form self-assembled complexes ("polyplexes") based on electrostatic attraction with negatively charged nucleic acids, and hence protect them from degradation [6]. These non-viral systems can be divided into organic or inorganic nanoparticles (nanoplexes) [7,8]. One of the major hurdles associated with liposomes (lipoplexes) or polycationic polymers (polyplexes) is their escape from endosomal/lysosomal vesicles, where nucleases and acidic pH digest nucleic acids, into the desired cellular compartments

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(nucleus for DNA and cytoplasm for siRNA). It is assumed that lipoplexes release the nucleic acids into the cytosol via membrane fusion or destabilization by interactions of the cationic liposomes with negatively charged membrane lipids. While many cationic liposomes show high biocompatibility, they exhibit some disadvantages such as high zeta potentials and low transfection efficiencies due to inefficient protection against lysosomal nucleic acid degradation [9,10]. Therefore, many efforts have been made to synthesize new lipids and investigate the physicochemical properties of liposomes to improve the transfection efficiency [11,12]. Neutral helper lipids, e.g. DOPE, are often used as components in cationic liposomal compositions in order to neutralize high surface charges and thus reduce cytotoxicity. Furthermore, DOPE is known to destabilize lipid bilayers and favours endosomal disruption [13].

Polyethylenimine (PEI) is a widely explored cationic polymer for the delivery of nucleic acids [14–17]. PEIs are synthetic, water-soluble branched or linear polymers available in a broad range of molecular weights (0.8–800 kDa) and possess a high cationic charge density at physiological pH due to protonable amino groups in every third position [18–20]. PEI protects nucleic acids from nuclease digestion, mediates the endosomal/lysosomal release of nanoscale complexes due to the so-called "proton sponge effect" [21,22] and was shown to facilitate the DNA entry into the nucleus [23,24]. Still, transfection efficacy and cytotoxicity strongly depend on the molecular structure and molecular weight [18,25]. In general, cytotoxicity and efficiency are higher with increasing molecular weight, while linear PEIs have shown to be less toxic and more efficient in the case of DNA transfections [26,27]. Furthermore, based on their surface charge, cationic vectors display low colloidal stability and tend to aggregate in the presence of salts and serum proteins, thus hampering biological activity. For further improvement of non-viral gene vectors, particularly PEI-based formulations, different strategies have been pursued. Chemical modifications of PEI include the grafting with poly(ethylene glycol) (PEG) or other polymers like chitosan, dextran, hydroxyethyl starch or carbohydrates [28–33], or the chemical coupling of hydrophobic chains, fatty acid residues, cholesterol, hyaluronic acid, polyglycerol, amino acids or peptides [34–46].

For the successful establishment of non-viral nanoparticulate systems with regard to possible therapeutic applications, the further improvement of biological activities and pharmacokinetics are still major goals. Beyond this, storage stability is another very important aspect. So far, the performance of many systems, including polymeric nanoparticles, in this regard is rather poor. More specifically, polymeric nanoparticles have a strong tendency to aggregate, leading to the complete loss of bioactivity in relatively short time. This requires the establishment and further refinement of novel or existing nanoparticle systems.

In recent years, some studies have been published on the combination of cationic polymers and liposomes (lipopolyplexes) for gene delivery. Some groups could demonstrate that lipopolyplexes, consisting of PEI and cationic liposomes, showed enhanced in vitro transfection efficiencies and improved serum stability [10,47–51]. Lipopolyplexes containing neutral, anionic or PEG-modified (phospho-) lipids, however, are more promising candidates. In vitro experiments have shown higher transfection efficiencies, lower cytotoxicities and relatively high colloidal stability against serum proteins and physiological salt concentrations. Additionally, in vivo studies indicated prolonged clearance properties, altered biodistribution and no significant toxicity [52–55].

Previously, we have introduced the 4–10 kDa branched PEI F25-LMW as a potent non-viral vector for the delivery of pDNA/siRNA in vitro and in vivo [25,56–58]. We have also established lipopolyplexes, comprising PEI F25-LMW and the neutral phospholipid dipalmitoyl-phosphatidyl-choline (DPPC) with various colipids, for enhanced DNA and siRNA delivery, reduced toxicity and altered physicochemical properties [51].

In this study, we systematically analyse various lipopolyplex preparation conditions with regard to biological activity and physicochemical properties, and extend lipopolyplex compositions towards relevant linear low-molecular weight PEIs, linPEI and linPEImax. We thus combine the favourable properties of optimal PEIs with those of lipid systems. Importantly, we also demonstrate that lipopolyplex formation retains the biological activity and physicochemical integrity of the nanoparticles upon prolonged storage, even at 37 °C and/or in the presence of serum, thus providing more stable formulations as compared to polyplexes.

2. Materials and methods

2.1. Materials

PEI F25-LMW was prepared as described previously [25]. Branched PEI 25 kDa, 1.8–2 kDa and 0.6–0.8 kDa were obtained from Sigma (Taufkirchen, Germany), branched PEI 10 kDa, linear PEI 25 kDa ("linPEI") and linear PEI max ("linPEImax") were purchased from Polysciences (Eppelheim, Germany). All PEIs were diluted in sterile distilled H_2O to a final concentration of 1 mg ml^{-1}, with the linear PEIs being adjusted to pH 4 with hydrochloric acid for improved solubility as suggested by the vendor. DPPC, *N*-[1-(2,3-dioleoyloxy)]-*N*,*N*,*N*-trimethylammonium-propane methylsulfate (DOTAP) and dipalmitoyl-phosphatidyl-ethanolamine-*n*-polyethylene glycol 5000 (DPPEmPEG5k) were obtained from Avanti Polar Lipids (Alabaster, USA). The luciferase expression plasmid pGL3 (Promega, Mannheim, Germany) and the β-galactosidase expression plasmid (pSELECT-zeo-LacZ, InvivoGen) were propagated in *Escherichia coli* DH5α. Plasmid DNA was isolated from an overnight culture using the Midi KIT from Macherey and Nagel (Düren, Germany) according to the manufacturer's protocol. Chemically synthesized siRNA duplexes directed against luciferase siLuc3 (sense: 5′-CUUACGCUGAGUACUUCGAdTdT-3′, antisense: 3′-dTdTGAAUGCGACUCAUGAAGCU-5′) and siLuc2 as negative control (sense: 5′-CGUACGCGGAAUACUUCGATTdTdT-3′; antisense: 3′-dTdTGCAUGCGCCUUAUGAAGCU-5′) were purchased from MWG (Ebersberg, Germany). Wildtype cell lines (SKOV-3 ovarian carcinoma, SW620 colon carcinoma, MCF-7 mamma carcinoma and PC-3 prostate carcinoma cells; see the ATCC website for extensive documentation) were obtained from the American Type Culture Collection (ATCC/LGC Promochem, Wesel, Germany) and constitutive luciferase expressing SKOV-3-LUC cells were described previously [25].

2.2. Liposome and PEI complex preparation

Three different liposomal formulations were prepared using the thin lipid film method; DPPC, DPPC/DOTAP (92:8 molar ratio) and DPPC/DPPEmPEG5k (95:5 molar ratio). To this end, 5 mg of phospholipid or lipid formulation dissolved in chloroform/methanol (2:1, v/v) was mixed in a 5 ml round-bottom flask. The solvent was evaporated at 55 °C on a rotary evaporator by a programmable vacuum pump and well-defined time/pressure steps (0 s/1000 mbar, 30 s/800 mbar, 5 min/500 mbar, 30 min/0 mbar). The thin lipid film was hydrated with 1 ml sterile dH_2O and incubated for 2 min at 55 °C in an ultrasound bath sonicator. Subsequently, the liposome suspension was extruded 11 times through a 200 nm polycarbonate membrane using a preheated Mini-Extruder (Avanti Polar Lipids, Alabaster, USA).

The PEI polyplexes were prepared as described previously [25] at PEI/nucleic acid ratios as indicated in the figure and the text.