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# Elucidating the role of free polycations in gene knockdown by siRNA polyplexes

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ABSTRACT

Future improvements of non-viral vectors for siRNA delivery require better understanding of intracellular processing and vector interactions with target cells. Here, we have compared the siRNA delivery properties of a lipid derivative of bPEI 1.8 kDa (DOPE-PEI) with branched polyethyleneimine (bPEI) with average molecular weights of 1.8 kDa (bPEI 1.8 kDa) and 25 kDa (bPEI 25 kDa). We find mechanistic differences between the DOPE-PEI conjugate and bPEI regarding siRNA condensation and intracellular processing. bPEI 1.8 kDa and bPEI 25 kDa have similar properties with respect to condensation capability, but are very different regarding siRNA decondensation, cellular internalization and induction of reporter gene knockdown. Lipid conjugation of bPEI 1.8 kDa improves the siRNA delivery properties, but with markedly different formulation requirements and mechanisms of action compared to conventional PEIs. Interestingly, strong knockdown using bPEI 25 kDa is dependent on the presence of a free vector fraction which does not increase siRNA uptake. Finally, we have investigated the effect on lysosomal pH induced by these vectors to elucidate the differences in the proton sponge effect between lipid conjugated PEI and conventional PEI: Neither DOPE-PEI nor bPEI 25 kDa affected lysosomal pH as a function of time, underlining that the possible proton sponge effect is not associated with changes in lysosomal pH.

**Statement of Significance**

Gene silencing therapy has the potential to treat diseases which are beyond the reach of current small molecule-based medicines. However, delivery of the small interfering RNAs (siRNAs) remains a bottleneck to clinical implementation, and the development of safe and efficient delivery systems would be one of the most important achievements in medicine today.
A major reason for the lack of progress is insufficient understanding of cell–polyplex interaction. We investigate siRNA delivery using polyethyleneimine (PEI) based vectors and examine how crucial formulation parameters determine the challenges associated with PEI as a delivery vector. We further evaluate how lipid conjugation of PEI influences formulation, cytotoxicity and polymer interaction with cells and cargo as well as the proton sponge capabilities of the vectors.


## 1. Introduction

RNA interference (RNAi) based drugs are currently under intense investigation for treating viral infections, cancer and neurological disorders [1]. RNAi is a naturally occurring post-transcriptional gene-silencing mechanism for regulation of gene expression through degradation or translation-arrest of the target mRNA [2]. The most extensively described form of RNAi acts through small interfering RNAs (siRNAs), which have very high (≈100%) sequence similarity with the target mRNA. The siRNAs can be transfected into cells in their short active form or generated intracellularly from longer double stranded RNAs (dsRNAs) [3,4].

The safe and efficient delivery of siRNA specifically to target cells is essential to RNAi-based therapy, but remains a major challenge [5]. Many attempts have relied on modified viruses, which – while very efficient – suffer from safety, immunogenicity and scalability issues [6]. As a consequence, non-viral vectors,

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which are characterized by low host immunogenicity and easy manufacturability, have attracted considerable attention and successful development of a safe and efficient non-viral delivery system would be one of the most important achievements in medicine today [7,8].

Cationic polymers are attractive for delivery of siRNA (and DNA) since they are particularly easy to formulate with poly-anionic nucleic acids. The electrostatic interactions between the anionic phosphates in siRNA and cationic moieties in the polymers can assemble the siRNA into nanoparticles – polyplexes – suitable for cellular uptake. Vectors based on polyethylenimine (PEI) were among the first to emerge and are still considered the gold standard in polymer based DNA delivery [9–12] and have also turned out to be efficient siRNA delivery agents *in vitro* [13–15].

In spite of the huge activity in the field and the broad use of cationic polymers as siRNA delivery agents, several aspects about the mode of action remain unclear. Polyplexes are generally believed to enter the cell via endocytosis, thus a primary research focus has been to ensure endosomal escape to avoid degradation in the lysosomes [16,17] and achieve release of the cargo into the cytosol. One of the key advantages of cationic polymers is their alleged ability to facilitate the so-called proton sponge effect [9], which is believed to be associated with endosomal escape of the polyplexes or their components. In PEI, the many protonated amines facilitate polyplex formation, while the unprotonated amines are able to absorb protons when the complex enters acidic compartments (endosomes and lysosomes). The proton sponge effect is believed to result in excessive V-ATPase mediated $H^+$ influx, which leads to increased influx of $Cl^-$ and water, and in turn this may lead to osmotic swelling, destabilization and/or rupture of the lysosomal membrane and release of the siRNA into the cytosol [18]. However, the proton sponge effect is debated and incontrovertible experimental evidence remains elusive. An alternative hypothesis is that polyplexes are internalized via the caveolar pathway that bypasses the endolysosomal pathway altogether [19], but the existence of a physiologically relevant caveolar pathway with a role in constitutive endocytic trafficking is equally debated [20–22].

One limitation of PEI as a transfection agent has been that both toxicity and delivery efficiency correlates with average molecular weight (MW) [23], and an average MW of 25 kDa is generally considered the upper limit before the toxicity becomes problematic [11]. Several strategies have been devised to preserve the low toxicity of low-MW PEI but improve the delivery capability. One promising approach has been to incorporate hydrophobic moieties onto the polymer amines of e.g. PEI 2 kDa [24] to increase the transfection efficiency, potentially by increasing the interaction of the polymer with the cell membrane. Such a beneficial effect of lipid substitution has been established in the context of plasmid DNA delivery for several cationic carriers [25,26] and more recently for delivery of siRNA [24,27–29].

An important aspect of polycation based gene delivery is the polymer/siRNA ratio necessary to obtain high transfection efficiency. This issue has been addressed thoroughly for PEI mediated DNA transfection [30–32], where it was found that a free (non-condensed) fraction of PEI is very important for achieving high transfection levels. However, as size, electrostatic charge and intracellular compartment of action differ for siRNA compared to DNA, it is not clear how a free fraction of PEI influences gene silencing. For *in vivo* use, a potential requirement for a free fraction for achieving high gene silencing efficiency is a major obstacle for the use of PEI and other polycations for non-viral gene delivery. Such systems are challenged by the fact that highly cationic entities will interact strongly with anionic blood constituents, the immune system and cells in general, and will therefore not allow for targeting of diseased tissue. In order to rationally develop efficacious siRNA carriers, it is essential that the interplay between the above-mentioned properties are understood.

In the present study we have compared lipid derivatized bPEI with conventional bPEI 1.8 kDa and bPEI 25 kDa. Our PEI derivative is a conjugate of dioleoylphosphatidylethanolamine (DOPE) and bPEI 1.8 kDa, which we refer to as DOPE-PEI throughout this text. bPEI 25 kDa serves as a point of reference as delivery efficiency of conventional PEI correlates with average MW and 25 kDa is widely accepted to be the highest useful average MW before cytotoxicity becomes problematic [11]. We establish to what extent the lipid conjugation alters the condensation properties of siRNA, cellular delivery and silencing of a reporter gene as well as to what extent the lipid conjugation reduces the toxicity of bPEI. We further investigate and compare how PEI and lipid conjugated PEI affect the pH in the lysosomes, as an increase in lysosomal pH has been associated with the proton sponge effect. Lastly, we systematically investigate the impact of lipid conjugation to bPEI in relation to the use of excess transfection agent beyond the requirement for full condensation of the siRNA (termed the "free fraction") and the silencing efficiency in comparison to bPEI itself. Although lipid conjugation affects the behavior of PEI and possibly the structure of the particles formed with siRNA, we have chosen to refer to all the used vectors as polymers forming polyplexes.

## 2. Materials and methods

### 2.1. Materials and cell lines

Branched PEI with a weight-averaged molar mass of 25,000 g/mol (bPEI 25 kDa) and 1800 g/mol (bPEI 1.8 kDa) were purchased from Sigma-Aldrich (US) and used without further purification. Before use they were dissolved in MQ water to a concentration of 15.4 mM with respect to nitrogen and further diluted in Hepes buffed glucose (HBG: 10 mM Hepes, 5% w/v glucose, pH 7.4) as needed. DOPE-PEI was synthesized from PEI 1.8 kDa and glutaryl-modified DOPE (NGPE) as described in [26], and kindly supplied by Professor Vladimir Torchilin's laboratory as a lyophilized powder. Briefly, NGPE in chloroform was activated with N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide HCl (EDCI) and N-hydroxysuccinimide (NHS) in the presence of triethylamine at room temperature (RT) for 4 h. PEI 1.8 kDa was dissolved in chloroform and the activated acid solution was added drop-wise into the PEI solution while stirring and the mixture was stirred at RT overnight. The chloroform was evaporated in a rotary evaporator and freeze-dried to remove traces of chloroform. The residue was suspended in 2 ml of deionized water ($dH_2O$) and purified by dialysis (MWCO 2 kDa) against $dH_2O$ and freeze-dried. The product was dissolved in chloroform and characterized by $^1H$ NMR using a Varian 500 MHz spectroscope. Using the standard pyrene method, the CMC of DOPE-PEI was determined to be 0.034 mg/ml (0.013 mM) [26]. Before use, DOPE-PEI was dissolved in MQ water to a concentration 15.4 mM with respect to nitrogen (1 mg/mL) and further diluted in HBG as needed. All siRNA duplexes were purchased from Eurofins MWG Operon (Germany): siRNA targeting green fluorescent protein (GFP-siRNA): 5′-GGCUACGUCCAGGAGCGCACC(dTdT)-3′ (sense) and siRNA targeting Luciferase (5′-CUUACGCUGAGUACUUCGA(dTdT)-3′ (sense). PicoGreen and Lipofectamine® RNAimax were purchased from Invitrogen (US) and Na-Heparin from porcine intestinal mucosa was purchased from Sigma-Aldrich (US). The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) was purchased from Promega (US).

The cell line HT1080 (human fibrosarcoma, ATCC No. CCL-121) was transfected to stably express luciferase and is referred to as HT1080pLUC. The cells were cultured at 37 °C, 5% $CO_2$ in RPMI 1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum