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Research paper

Polyethylenimine PEI F25-LMW allows the long-term storage of frozen complexes as fully active reagents in siRNA-mediated gene targeting and DNA delivery

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Abstract

Background: Polyethylenimines (PEIs) are synthetic, charged polymers which function as transfection reagents based on their ability to compact DNA into complexes. Recently, PEI-mediated delivery of nucleic acids has been extended towards small interfering RNAs (siRNAs) which are instrumental in the induction of RNA interference (RNAi). Since RNAi represents a powerful method for specific gene silencing, the PEI-based delivery of siRNAs is a promising tool for novel putative therapeutic strategies. *Aim:* For therapeutic use, major requirements are the development of formulations which (i) are sufficiently stable in the presence of serum, and which can be (ii) easily and reproducibly manufactured and (iii) stored for a prolonged time with full retention of their integrity and bioactivity. In this paper, we explore the potential of PEI F25-LMW, a low-molecular weight PEI with superior transfection efficacy and low toxicity, towards these goals. *Results:* We have systematically analyzed and determined optimal DNA and siRNA complexation conditions with regard to various parameters including buffer concentration, ionic strength, pH and incubation time. As opposed to 22 kDa linear PEI (L-PEI), the low-molecular weight (4–10 kDa) PEI F25-LMW performs DNA transfection and siRNA gene targeting with identical efficacies in the presence of serum, thus emphasizing its usefulness in vivo. Furthermore, in contrast to other polyethylenimines, PEI F25-LMW-based DNA or siRNA complexes allow freeze/thawing and frozen storage for several months. Their activity is fully retained without requiring specific buffer conditions or the addition of any lyoprotectant. Physicochemical analysis and atomic force microscopy reveal a distinct size pattern with the presence of two complex subgroups and show that frozen PEI F25-LMW complexes remain stable with little increase in complex size, no changes regarding their zeta potential and cytotoxicity, and full retention of nucleic acid protection. *Conclusions:* Frozen PEI F25-LMW-based complexes represent efficient and stable ready-to-use formulations of DNA- or siRNA-based gene therapy products.

Keywords: RNAi; siRNAs; PEI; Polyethylenimine; Frozen complexes; Nucleic acid delivery

1. Introduction

Polyethylenimines (PEIs) are branched or linear synthetic polymers with the different available products characterized by their molecular weights, ranging from low-molecular weight (<1000 Da) to high-molecular weight PEIs (>1000 kDa). Due to a protonable amino group in every third position [1,2], they possess a high cationic

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charge density which leads to the condensation of nucleic acids into complexes. Since this complex formation allows the efficient cellular uptake through endocytosis, PEIs have been demonstrated to mediate effective in vitro and in vivo gene transfer in a variety of cell lines and in animals for DNA delivery (for review, see e.g., [3–7] and references therein).

More recently, the use of polyethylenimines or PEI derivatives has been extended towards the complexation and delivery of small RNA molecules including all-RNA ribozymes [8–10] and small interfering RNAs (siRNAs) ([11–16], see [17,18] for review). siRNAs are 21–25 nt double-stranded RNA molecules playing a pivotal role in RNA interference (RNAi), since RNAi is based on the incorporation of an siRNA into an 'RNA-induced silencing complex' (RISC), with the siRNA sequence-specifically guiding RISC to the target RNA and inducing endonucleolytic cleavage of the mRNA strand within the target site [19,20]. RNAi is commonly used as a powerful tool in biological and biomedical research providing the relatively easy reduction of the expression of a target gene in functional studies of various genes. This also offers the possibility of therapeutic intervention based on the targeting of pathologically relevant genes (see e.g., [17,21,22] for review).

One critical factor that determines the success of siRNA-based RNAi approaches is the ability to deliver intact siRNAs efficiently into the cells. While chemically unmodified RNA molecules are very instable and prone to rapid degradation, upon PEI complexation they are efficiently condensed and thus fully covered and protected against enzymatic or non-enzymatic degradation [8,11]. PEI-mediated cellular DNA or siRNA delivery is dependent on the PEI, on the complexation procedure and on the transfection conditions. Among others, this includes the PEI's molecular weight and degree of branching as well as the ionic strength of the complexation solution, the presence of serum proteins, the complexation time and the N/P ratio (defined as the ratio of the nitrogen atoms of PEI to nucleic acid phosphates in the complex). In general, low-molecular weight PEIs display higher transfection efficacy which may be due to a more efficient uptake of the complexes, a better intracellular release of the DNA and/or their generally lower in vitro cytotoxicity [23–28]. On the other hand, cytotoxicity and low transfection efficacy may, among others, rely on the formation of larger aggregates which prevents their endocytosis and, when formed on the cell surface, impairs membrane functions finally leading to cell necrosis [24]. For the commercially available low-molecular weight 22 kDa linear PEI (L-PEI), used as siRNA delivery reagent, several studies have shown contradicting results [11,12,29,30]. Additionally, to improve the biocompatibility and efficiency in vitro as well as to influence the biodistribution and circulation times in vivo, modifications have been introduced including PEGylation, i.e., grafting with the hydrophilic polymer poly(ethylene glycol) (for review, see [3,7,31,32]). While PEGylation has been shown to influence the size, zeta potential, morphology and stability of DNA and siRNA complexes as well as the efficacy of DNA or siRNA complexation, protection and delivery, data still seem to be somewhat contradictory and are strongly dependent on the PEI and its degree and pattern of PEGylation. Although it was hypothesized that PEGylation enhances siRNA release in the cytoplasm, leading to increased biological activity [33], other studies show that PEG grafting seems to exert negative effects on intracellular release of nucleic acids [34]. More recently, we have shown in several PEG-PEIs that, in contrast to DNA, PEG-PEIs are rather comparable to non-PEGylated PEIs with regard to siRNA transfection and toxicity in vitro [32], and siRNA delivery, complex circulation times, biodistribution and biocompatibility in vivo (Malek and Aigner, unpublished data), and previous in vivo studies have successfully utilized non-PEGylated PEIs for in vivo siRNA delivery ([11,12], Höbel et al., submitted for publication). Thus, it appears that for siRNA delivery non-PEGylated PEIs are well suited. More recently, we introduced the novel low-molecular weight polyethylenimine 'PEI F25-LMW' derived through the fractionation of a commercially available 25 kDa branched PEI by size exclusion chromatography. PEI F25-LMW demonstrates high DNA and siRNA delivery efficacies and low toxicity in various cell lines and under various conditions [35].

Non-viral vectors like polyethylenimines may offer promising strategies for the delivery of DNA or RNA molecules, but they often suffer from poor physical stability and comparably low transfection efficacy especially in the presence of serum, and their application as medical products will require the development of formulations which can be stored for a prolonged time. While freezing or freeze-drying may be the important strategies for preserving transfection efficacies of complexes, they so far require the complex formation under highly specific and restricted medium conditions, the addition of lyoprotectants and/or conjugate formation by chemical coupling of ligands (see e.g., [35–37] and references therein).

In this paper, we show that the DNA transfection efficacy as well as siRNA gene targeting efficiency of PEI F25-LMW complexes, but not of L-PEI complexes, is independent of the presence of serum, and explore the optimal complexation conditions for PEI F25-LMW. Furthermore, we introduce and characterize simple freezing of PEI F25-LMW-based DNA or siRNA complexes for the generation of efficient ready-to-use formulations of DNA- or siRNA-based gene therapy products.

2. Materials and methods

2.1. Preparation and storage of PEI/DNA and PEI/siRNA complexes

Polyethylenimine PEI F25-LMW was prepared as described previously [35]. Briefly, 100 mg 25 kDa