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Selective blocking of primary amines in branched polyethylenimine with biocompatible ligand alleviates cytotoxicity and augments gene delivery efficacy in mammalian cells

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ABSTRACT

Recently, polyethylenimines (PEIs) have emerged as efficient vectors for nucleic acids delivery. However, inherent cytotoxicity has limited their in vivo applications. To address this concern as well as to incorporate hydrophobic domains for improving interactions with the lipid bilayers in the cell membranes, we have tethered varying amounts of amphiphilic pyridoxyl moieties onto bPEI to generate a small series of pyridoxyl-PEI (PyP) polymers. Spectroscopic characterization confirms the formation of PyP polymers, which subsequently form stable complexes with pDNA in nanometric range with positive surface charge. The projected modification not only accounts for a decrease in the density of 1° amines but also allows formation of relatively loose complexes with pDNA (*cf.* bPEI). Alleviation of the cytotoxicity, efficient interaction with cell membranes and easy disassembly of the pDNA complexes have led to the remarkable enhancement in the transfection efficiency of PyP/pDNA complexes in mammalian cells with one of the formulations, PyP-3/pDNA complex, showing transfection in ~68% cells compared to ~16% cells by Lipofectamine/pDNA complex. Further, the efficacy of PyP-3 vector has been established by delivering GFP-specific siRNA resulting in ~88% suppression of the target gene expression. These results demonstrate the efficacy of the projected carriers that can be used in future gene therapy applications.

1. Introduction

Polyethylenimines (PEIs) have been found to be the most extensively used vectors in gene delivery and demonstrated their ability to transfect cells from different origins [1–4]. High transfection efficiency of these vectors is mainly due to their intrinsic buffering capacity, which causes endosomal swelling and disruption leading to release of the DNA complexes in the cytosol for nuclear translocation. However, toxicity due to high charge density poses a major bottleneck while using these carriers to transfer nucleic acids in vivo [5]. Toxicity induced by PEIs is believed to be due to the charge interactions between these polymers and cell membrane [6]. It can be explained by two different mechanisms, viz., (a) early stage toxicity, the polymers bearing high cationic charge density interact with cell membranes and cause permeabilization leading to necrotic cell death [7,8], and (b) later stage toxicity, post-internalization, these polymers cause mitochondrial membrane-mediated apoptosis resulting in significant loss of mitochondrial membrane potential (MMP) [9], which is due to direct interactions and permeabilization of mitochondria. Besides, PEIs also interact with serum proteins and cause aggregation that can lead to capillary embolism [5,10]. As transfection efficiency and toxicity of PEIs significantly depend on the topology, concentration and molecular weight, high molecular weight PEIs are efficient transfection reagents but exhibit high cytotoxicity. Low molecular weight PEIs, on the other hand, are non-toxic but display poor gene delivery activity compared to high molecular weight PEIs [7]. To maintain a high transfection efficiency of bPEI (25 kDa) with minimal cytotoxic effects, several approaches have been explored to incorporate chemical modifications [1–4]. Partial blocking of primary amines (into amides or carbamates) or conversion of primary amines into secondary or tertiary amines imparts beneficial properties to the polymeric carriers [11–13]. Recently, several groups have also studied the effect of hydrophobicity on transfection efficiency mediated by non-viral vectors [1,3,14–17]. Incorporation of lipophilic ligands in bPEI by acylation increases the hydrophobicity but reduces buffering capacity at the same time. However, alkylation offers an advantage that primary and secondary amines are

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converted into secondary and tertiary amines, respectively, without altering the buffering capacity significantly. These studies have further established that introduction of hydrophobicity in the side chain exerts an additive effect, while in the backbone, it inflicts adverse effects on the transfection efficiency. Therefore, transfection efficiency significantly depends upon the molecular structure and arrangement of hydrophobic element in the polymeric backbone [13,18–21].

Taking clues from these studies, the present investigation was undertaken with the objective to incorporate hydrophobic element in the polymeric structure and evaluate its effect on the transfection efficiency, cytotoxicity and buffering capacity of the resulting polymers. For this purpose, we have selected pyridoxal hydrochloride, a natural form of vitamin B_6, which is commonly being used as one of the constituents of cell culture media. Being safe, it can be used in vitro and in vivo. Gene delivery vectors have also been prepared by condensing pyridoxal phosphate (PLP), another form of vitamin B_6, with primary amino functions of cationic polymers [22,23], however, it has been observed that phosphorylated form (PLP) is less efficient in crossing cell membrane than less polar form, pyridoxal (Py), which readily diffuses through the cell membranes [24]. Besides, polyamidoamine-vitamin B_6 bioconjugates (PAMAM-vit B_6) have been used to deliver drugs into the skin tissues and hence can be considered as potential transdermal drug delivery system in future applications [25]. Further, it has been demonstrated that amines linked to vitamin B_6 get facilitated entry into the cells by exploiting vitamin B_6 transporters [26]. In a study, Huang et al. [27] investigated the effect of phosphate on the stability of pyridoxal in presence of lysine and found that pyridoxal phosphate (PLP) was more reactive than pyridoxal but 1.5–2.0 folds less stable in aqueous medium. Moreover, phosphate group on PLP may also interact electrostatically with amines as well and generate complex structures. Hence, here, we hypothesize that pyridoxal hydrochloride, bearing reactive aldehydic function and stable in aqueous systems, would specifically react with primary amines of bPEI (main source of toxicity in cationic polymers) and after in situ reduction with sodium cyanoborohydride, convert them into secondary amines i.e. transfection-friendly charge. The presence of pyridyl ring would not only enhance pDNA condensation effectively through π–π interactions but also facilitate the interactions with the lipid bilayers present in the cell membranes leading to efficient uptake and internalization of the pDNA complexes. Thus, varying the stoichiometric ratio of pyridoxal hydrochloride and bPEI, a small series of pyridoxyl-PEI (PyP) polymers was synthesized and after characterization, PyP polymers were evaluated for their transfection efficiency and cytotoxicity in mammalian cells. We also studied the influence of degree of grafting of pyridoxal onto bPEI and weight ratios of polymer: pDNA on gene transfer efficacy. The versatility of PyP-3 was established by GFP-specific siRNA delivery to knockdown the target gene expression.

2. Materials and methods

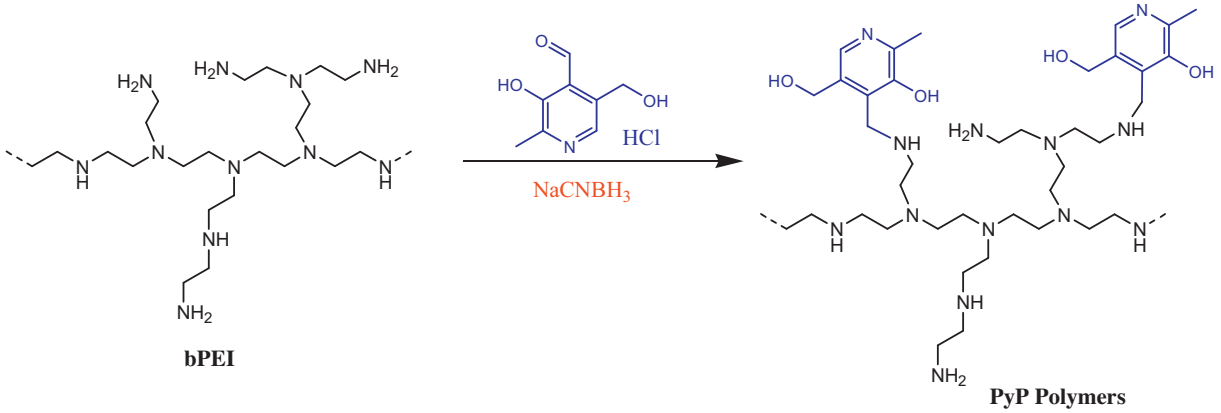
2.1. Synthesis of N-pyridoxyl-PEI (PyP) polymers

The synthesis strategy is depicted in Scheme 1. Briefly, for 30% grafting of pyridoxyl moieties on bPEI, an aqueous solution of pyridoxal hydrochloride (15.27 mg, dissolved in 500 μl of deionized water) was added dropwise to a stirred solution of bPEI (43 mg), dissolved in dH_2O (4.3 ml). After 3 h, sodium cyanoborohydride (20 mg) was added and stirring was continued for 12 h. Subsequently, the solution was transferred to a dialysis bag (12 kDa cut-off) and dialyzed against water for 48 h (6 × 8 h). The dialyzed solution was lyophilized to obtain PyP-1 polymer in ~75% yield. Similarly, PyP-2, PyP-3 and PyP-4 polymers were synthesized by varying the amount of pyridoxal hydrochloride (20.36 mg for 40%, 25.45 mg for 50% and 30.54 mg for 60% grafting) in ~70–74% yield. These polymers were then characterized by UV, FTIR and ^{1}H NMR.

2.2. In vitro transfection and quantification

MCF-7 and HeLa cells were seeded into 96-well plates 24 h prior to experiments at a density of 4×10^3 cells/cm^2 in DMEM containing 10% FBS at 37 °C in a humidified 5% CO_2 environment. Transfection assay was carried out at ~70–75% confluency in the absence and presence of serum. PyP/pDNA and bPEI/pDNA complexes were prepared at various w/w ratios (*see supplementary Information*) and diluted with DMEM with and without 10% FBS (60 μl). Similarly, pDNA complex was prepared with the commercial transfection reagent, Lipofectamine, following manufacturer's protocol. These complexes were then gently added onto the cells and kept the plates in an incubator at 37 °C. After 3 h, the media was replaced by the fresh complete medium (DMEM containing 10% FBS). The plates were kept in the incubator for 36 h and then GFP expression was observed under fluorescence microscope.

In another experiment, GFP-specific siRNA was sequentially delivered using PyP-3 polymer and the efficiency was determined by measuring the suppression in the expression of the target gene. Briefly, cells were first transfected with PyP-3/pDNA complex as above and after 3 h, PyP-3/siRNA (2 μl, 2.5 μM) complex was added onto the cells for 3 h. Subsequently, the complex was removed and the growth medium (DMEM containing 10% FBS) was added for 36 h. Cells transfected with PyP-3/pDNA complex alone served as control. Similarly, Lipofectamine/pDNA and Lipofectamine/siRNA complexes were also prepared and added onto HeLa cells. After



Scheme 1. Schematic representation of the preparation of PyP polymers.