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Research Paper

Heterocyclic amine-modified polyethylenimine as gene carriers for transfection of mammalian cells

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ABSTRACT

Branched polyethylenimine (PEI) is extensively used as a polycationic non-viral vector for gene delivery. Polyplexes formed with PEI are believed to be released from endocytotic vesicles by the osmotic burst mechanism in the rate-limiting step in transfection. Increasing the buffering capacity of PEI derivatives in the endosomal pH range of 4.5–7.5 should enhance transfection efficiency. In this study, PEI was derivatized by covalently attaching heterocyclic amine moieties (piperazine, pyridine and imidazole rings with p*Ka* values from 5.23 to 6.04) through amide bonds. PEI derivatives with 50% of the primary amines on PEI exhibited increased buffering capacity, increased transfection activity and decreased cytotoxicity in murine neuroblastoma (Neuro-2a) cells. The relative effectiveness in enhancing transfection efficiency was piperazine > pyridine > histidine, but each type of amine was the most effective under a particular set of conditions. Modified vectors could significantly improve transfection efficiency in murine mesenchymal stem cells. PEI_{25} derivatized at 50% with histidine administered as polyplexes in the tail veins of mice resulted in remarkably enhanced luciferase gene expression in the expected organ distribution and much lower toxicity than underivatized PEI_{25}.

1. Introduction

Human gene therapy would benefit from the availability of safe and efficient non-viral carrier systems for delivering nucleic acids into target cells [1]. Polyethylenimine (PEI) has been widely studied as a nucleic acid carrier. It is believed to act in different steps including formation of stable electrostatic complexes with the nucleic acid called polyplexes, bind to and enter the cells in vesicles by conventional endocytosis mechanisms, disruption of the endosomal vesicle and releasing the polyplexes into the cytoplasm, transport of the nucleic acid to its site of action and release from the carrier, finally it acts on cellular systems as if it were endogenous nucleic acid [1–3]. The rate-limiting step is believed to be released from endosomal vesicles [2]. Release of PEI-based polyplexes from endocytotic vesicles is believed to occur by the osmotic burst mechanism [4] in which the buffering capacity of the carrier prolongs the normal action of Na^+, K^+-ATPases to pump protons into the endosome lowering of the internal pH from 7.5 to about 5. While ATPase is pumping protons into the endosome, a chloride channel admits Cl-ions. The water of hydration needed by the added ions is followed by osmosis, swelling the endosome to the point at which it may burst, releasing the endosomal contents into the cytoplasm.

Isolated primary, secondary and tertiary amines all have p*Ka* values > 9 [5]. There are structural features in more complex amines that destabilize the protonated form, which increases the H^+ concentration needed to force protonation of affected amines, thereby lowering the p*Ka* into the endosomal pH range. The most important structural feature which lowers p*Ka* in PEI is proximity to a positive charge on an nearby amine. In a simple model compound for PEI, piperazine, the first amine is protonated at pH 9.66, but protonation of half the amines separated from it by two

Abbreviations: PEI, polyethylenimine; PAMAM, polyamidoamine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TNBS, 2,4,6-trinitrobenzenesulfonic acid; FTIR, Fourier transform infrared spectroscopy; EGFP, enhanced green fluorescent protein; EtBr, ethidium bromide; TEM, transmission electron microscopy; mMSCs, murine mesenchymal stem cells.

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methylenes occurs in the endosomal pH range (p*K*a = 5.27). Thus, in linear PEI only about half the amines are protonated when complexation with DNA occurs at neutral pH (approximately every other amine), and the remaining, unprotonated amines provide buffering capacity in the endosomal pH range. In branched PEI proximity relationships are similar but more complex resulting in a broader pH range for endosomal buffering capacity. Other structural features that lower p*K*a are delocalization of electrons around an aromatic ring, such as in pyridine and histidine; and steric stress in rings or loops from changing the C—N—C bond angle.

In this study, three heterocyclic amines with p*K*a values in the endosomal range (histidine, pyridine and piperazine) were attached to primary amines of branched PEI through amide linkages at three substitution levels. The effects on buffering capacity, cytotoxicity and *in vitro* and *in vivo* transfection efficiency were determined by comparing underivatized PEIs with heterocyclic amine derivatives of the same PEIs.

2. Materials and methods

2.1. Materials

Branched PEIs with an average molecular weight of 10 (PEI_{10}) and 25 kDa (PEI_{25}) were obtained from Polysciences, Inc. (Warrington, PA, USA). L-Histidine was purchased from Biochemical (BDH, England). 3-Pyridylacetic acid hydrochloride, piperazine-2-carboxylic acid dihydrochloride, N-hydroxybenzotriazole (HOBt), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydro chloride (EDC) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and N-(2-hydroxyethyl) piperazine-N-(2-ethanesulfonic acid) (HEPES) were obtained from Sigma–Aldrich (Munich, Germany). Dulbecco's modified Eagle's medium and fetal bovine serum (FBS) were supplied by GIBCO (Gaithersburg, USA). Calcein, AM was purchased from life technology (USA).

2.2. Preparation of heterocyclic amine derivatives of PEIs

Three heterocyclic amines with a p*K*a value in the endosomal pH range (histidine, 3-pyridylacetic acid hydrochloride or piperazine-2-carboxylic acid dihydrochloride) were covalently coupled to PEI_{10} and PEI_{25} using HOBt and EDC as coupling agents. Briefly, PEI_{10} or PEI_{25} was dissolved in distilled water containing HOBt. The solution of PEI was added dropwise to a gently stirred solution in distilled water of EDC and the calculated amount of a heterocyclic amine needed to give 10%, 30% or 50% substitution of primary amines assuming a 1:2:1 ratio of primary:secondary:tertiary amines in branched PEI. The reaction was allowed to proceed at room temperature for 24 h. The final products were dialyzed against three changes of distilled water using Spectra/Por dialysis membrane (6000–10,000 Da cut-off, Spectrum Laboratories, Houston, USA) to remove unreacted materials and lyophilized. Amide bond formation was confirmed by Fourier transform infrared spectroscopy (FTIR). Proton NMR spectra of the PEI conjugates have been also obtained. The degree of substitution of primary amines on PEI was determined by 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay as described previously [6].

2.3. Buffering capacity measurements

The buffering capacities of PEIs and their derivatives were measured over a pH range of 12–2.5 by acid-base titration [7]. PEI solutions (1 ml of 0.4 mg/ml) were initially adjusted to pH 12, and the solution titrated by repeated additions of 5 μl aliquots of 0.1 M HCl until the pH was reduced to 2.5. The buffering capacity in the endosomal pH range, defined as the changes in protonation per amine at pH 7.5–4.5, was calculated as follows

$$\text{Buffering capacity} = \text{VHCl/N mol} \times 100\%$$

in which ΔVHCl is the volume of 0.1 M HCl solution which brought the pH value of PEI solutions from 7.5 to 4.5, and N mol is the total moles of protonable amine groups in the PEI solution [7,8].

2.4. Polyplex preparation

Polyplexes were prepared in the optimal range of carrier/plasmid (c/p) ratios (wt/wt) by mixing calculated concentrations of polymers in HBG buffer (HEPES-buffered glucose containing 20 mM HEPES, 5% glucose, pH 7.4) with equal volumes of the same buffer containing plasmid DNA, followed by mixing by gentle pipetting and incubating 30 min at room temperature. Two reporter genes were used in these studies, RL-CMV plasmid (pRL-CMV), which provides constitutive expression of the *Renilla* luciferase reporter gene (Promega Corp., Madison, WI, USA) or enhanced green fluorescent protein (EGFP) plasmid (pEGFP) (Promega Corp., Madison, WI, USA), which provides constitutive expression of the green fluorescent protein reporter gene.

2.5. Ethidium bromide (EtBr) exclusion assay

PEI derivatives dissolved in HBG buffer were added at different carrier/plasmid ratios (wt/wt) to a solution of pRL-CMV (5 μg/ml) and EtBr (400 ng/ml) in the same buffer. The 0% condensation standard was prepared by mixing plasmid with EtBr and measuring the resultant fluorescence emission (at excitation and emission wavelengths of 510 and 590 nm, respectively) using a Jasco FP-6200 spectrofluorimeter (Jasco Global, Hachioji, Tokyo, Japan). The fluorescence intensity of EtBr without plasmid was used as the 100% condensation standard. Results are reported as mean ± SD of samples in triplicate.

2.6. Particle size and zeta potential measurements

The particle size and zeta potential of polyplexes prepared in HBG buffer from 5 μg/ml plasmid DNA and PEI or modified PEIs were measured using dynamic light scattering (DLS) and laser Doppler velocimetry (LDV), respectively by a Malvern Nano ZS instrument and DTS software (Malvern Instruments, UK). Polyplexes were freshly prepared, and then incubated 30 min at room temperature before measurements were made. The results are reported as mean ± SD of 3 runs.

2.7. Transmission electron microscopy (TEM)

Polyplexes prepared at $c/p = 1$ (PEI carrier/plasmid DNA weight/weight ratio = 1) with the PEI derivative that had optimal transfection activity *in vitro* ($PEI_{25}His_{50\%}$) were adsorbed onto a holey carbon-coated copper grid (300 mesh). The grid was then dried at room temperature and observed with transmission electron microscope (CM120, Philips, the Netherlands, at an accelerating voltage of 80 kV).

2.8. Cell culture

Neuro-2a murine neuroblastoma cells (ATCC, CCL-131) were cultured in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated fetal bovine serum (FBS) (10% vol/vol), penicillin (100 U/ml) and streptomycin (100 μg/ml). Mesenchymal stem cells (MSCs) are useful subjects

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