International Journal of Pharmaceutics 413 (2011) 260–270

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

# Comprehensive comparison of two new biodegradable gene carriers

Dong Zhao, Tao Gong, Di Zhu, Zhirong Zhang, Xun Sun*

*Key Laboratory of Drug Targeting and Drug Delivery Systems, Ministry of Education, West China School of Pharmacy, Sichuan University, Southern Renmin Road, Chengdu, Sichuan 610041, PR China*

ARTICLE INFO

*Article history:*
Received 10 March 2011
Received in revised form 11 April 2011
Accepted 15 April 2011
Available online 21 April 2011

*Keywords:*
Polyethylenimine
Biscarbamate linkage
Biodegradable polymers
*In vitro* and *In vivo* transfection and toxicity assays

ABSTRACT

Safety and high transfection efficiency are the prerequisites for an ideal gene vector. Polyethylenimine (PEI), especially PEI 25k (25 kDa), is a well-known cationic gene carrier with high transfection efficiency. However, the high toxicity, depended on its molecular weight, has limited its use as a potential gene carrier. In our research, for the purpose of reducing the toxicity and increasing the transfection efficiency and further to inspect where the degradation of these biodegradable polymers take place would be more beneficial, in cytoplasm or in endocytic vesicles, two kinds of degradable polymers were synthesized. One is an acid-liable PEI derivate (PEI-GA) which was cross-linked by PEI 2k with glutadialdehyde (GA) through imine linkages and the other one (PEI-TEG) was cross-linked PEI 2k with modified triethyleneglycol (TEG) through biscarbamate linkages and can be degraded at neutral environment. By the use of a series of assay methods both *in vitro* and *in vivo*, the results showed that PEI-TEG was found to be biodegradable at neutral environment and exhibit high transfection ability with low toxicity, which indicated its potential as a candidate carrier for gene therapy.



## 1. Introduction

In the past decades, non-viral gene delivery systems, such as cationic liposomes, peptides, and polymers have attracted great attention due to their superiorities over viral systems, for incidence, the lack of specific immune response, no restrictions in the size of DNA, and the ease of large scale production (Mintzer and Simanek, 2009; Thomas and Klibanov, 2003; Yu and Wagner, 2009). Among cationic polymers, polyethylenimine (PEI), a commercially available cationic polyamine first introduced by Boussif et al. (1995), is one of the most promising and widely studied gene carriers for its highly efficient delivery of DNA (Godbey et al., 1999a), which were ascribed to the buffering capacity of PEI, thus protecting DNA from nuclease degradation and facilitating endosomal escape of PEI/DNA complexes ("proton sponge hypothesis") (Boussif et al., 1995). In addition, due to its high transfection efficiency, branched PEI 25k has been used as a standard reference to compared with other newly designed polymers (Kunath et al., 2003). However, significant *in vitro* as well as *in vivo* toxicity is frequently associated with such nucleic acid carriers. The transfection efficiency and toxicity of PEI are depended on its molecular weight and it is generally accepted that PEI with a higher molecular weight (HMW) (i.e. 25 kDa) shows higher transfection efficiency and higher toxicity compared with a low molecular weight PEI (LMW) (i.e. 2 kDa) (Fischer et al., 1999; Godbey et al., 1999b).

To enhance the transfection efficiency and/or decrease the toxicity of PEI, two major vector modification strategies have been reported. The first one was based on the modification of HMW-PEI by coating with human serum albumin (Chen et al., 1994) and dextran (Tseng and Jong, 2003; Tseng et al., 2004), PEGylation (Lutz et al., 2008; Nimesh et al., 2006; Remaut et al., 2007; Sung et al., 2003; Tang et al., 2003; Zhang et al., 2008) and acylation (Forrest et al., 2004) to improve the polymer's biocompatibility. However, most of this kind of modification showed inferior transfection efficiency than non-modified PEI. By contrast, the other strategy was the direct chemical modification of nontoxic LMW-PEI to increase its gene transfer efficiency (Arote et al., 2007; Forrest et al., 2003; Jiang et al., 2007; Kim et al., 2005; Lee et al., 2003; Park et al., 2005; Tang et al., 2006; Thomas et al., 2005; Thomas and Klibanov, 2002; Wen et al., 2009; Xu et al., 2008; Zhang and Vinogradov, 2010), especially cross-linking the LMW-PEI with biodegradable linkers (Thomas and Klibanov, 2002; Forrest et al., 2003; Kim et al., 2005; Park et al., 2005; Thomas et al., 2005; Xu et al., 2008). Lower toxicity of PEI has generally been achieved by this strategy, but the improvement in transfection efficiency varied from reports (Forrest et al., 2003; Kim et al., 2005; Park et al., 2005; Thomas et al., 2005; Xu et al., 2008).

On the other hand, for the design of biodegradable gene delivery polymers, many questions remained. One of the most important questions was where the degradation of these polymers take place would be more beneficial, in cytoplasm or in endocytic vesicles.

* Corresponding author. Tel.: +86 28 85502307; fax: +86 28 85502307.
*E-mail address:* xunsun22@gmail.com (X. Sun).

0378-5173/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2011.04.040

Up to now, hundreds of newly designed polymers have been synthesized as carriers for the gene delivery. However, no research has really compared these two kinds of degradation pattern in parallel. To address this, we synthesized two kinds of polymers PEI-GA (polymer A) and PEI-TEG (polymer B). Among them, PEI-GA was cross-linked by PEI 2k with glutadialdehyde (GA) through imine linkages and could be degraded in endocytic vesicles (Kim et al., 2005). The other one PEI-TEG was cross-linked PEI 2k by biscarbamate linkages, which can be degraded at neutral environment. This polymer was newly designed and synthesized in our group. Currently, the most commonly used linkages for biodegradable PEI derivates were ester bonds (Thomas and Klibanov, 2002; Forrest et al., 2003; Park et al., 2005; Thomas et al., 2005). However, the ester bond could generate acids upon degradation, which was disfavored for buffering the endosomal environment. Thus, we synthesized the polymer PEI-TEG. Unlike the ester bond, the degradation of each biscarbamate linkage only generated alcohols, $CO_2$ and two amino groups (Xu et al., 2008).

In this work, polymer PEI-GA and polymer PEI-TEG were tested by acid–base titration, DNA-binding assay, *in vitro* cell transfection studies, cytotoxicity assays, lactate dehydrogenase (LDH) release measurement, hemolytic activity assay and *in vivo* transfection and toxicity assays, and compared with PEI 25k and lipofectamine 2000.

## 2. Materials and methods

### 2.1. Materials

Branched PEI 2k, branched PEI 25k, 4-nitrophenyl chloroformate, 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), agarose, HEPES, Tris were procured from Sigma Chemical Co., St. Louis, MO, USA. Gold-view was purchased from Solarbio (Beijing, China). Triethyleneglycol (TEG) was purchased from Kelong (Chengdu, China). The pORF-lacZ and pGL3 plasmid DNA were isolated and purified from DH5-α *Escherichia coli* using the Qiagen Giga Endo-free plasmid purification kit (CA, USA). DNA concentration and purity were quantified by UV absorbance at 260 and 280 nm on a Varian CARY 100 Conc UV Spectrophotometer. Cell culture medium DMEM was obtained from Gibco Co. (USA). β-Gal assay kit was from Invitrogen (USA). BCA assay kit was from KeyGEN (Nanjing, China). The 293T, HepG2 and L929 cell lines were obtained from Shanghai Cell Institute, China Academy of Sciences. All the other chemicals and reagents used were of the analytical grade obtained commercially.

### 2.2. Synthesis and characterization of polymers

#### 2.2.1. Glutadialdehyde as the crosslinker

The synthesis method of the acid-liable PEI derivate was presented as Kim et al. (2005). Briefly, 2.0 g PEI 2k was introduced in 20 mL anhydrous ethylene dichloride (EDC) solution and stirred vigorously to dissolve. After PEI became clearly dissolved in the reaction solution, 0.1 g glutadialdehyde (GA) dissolved in 20 mL anhydrous EDC was dropwise added with vigorous stirring at room temperature. After stirring for another 4 h, the solution was evaporated to remove the solvent. The viscous residue was dissolved again in water and dialyzed through a cellulose membrane of molecular weight cut off of 3500 in deionized water for 3 days, and then, after 2 days of lyophilization, we got yellowish product as semisolid with high viscosity. The product was named as A or PEI-GA.

#### 2.2.2. Triethyleneglycol as the crosslinker

The new PEI derivate cross-linked by triethyleneglycol through biscarbamate linkages. Briefly, 0.15 g anhydrous triethyleneglycol (TEG, 1.0 mmol) was dissolved in anhydrous ethylene dichloride (25 mL) and afterwards 604 mg 4-nitrophenyl chloroformate (NPC, 3.0 mmol) was added. The solution was stirred and 415 μL triethylamine (3.0 mmol) was added drop-wise through a syringe over a period of 5 min. The reaction mixture was allowed to stir at room temperature for 6 h. Subsequently, it was washed with saturated sodium chloride solution and the organic phase was collected and dried over anhydrous sodium sulfate. Then the solvent was dropwise added to the ethylene dichloride solution of PEI 2k (2.0 g). After stirring for another 16 h, the solution was evaporated to remove the solvent. The viscous residue was dissolved again in double-distilled water and dialyzed through a cellulose membrane of molecular weight cut off of 3500 for 3 days, and then, after 2 days of lyophilization, we got yellowish product or albicans product as solid or semisolid with high viscosity. The product was named as B or PEI-TEG.

### 2.3. Characterization and measurements of the new PEI derivates

To confirm the synthesis of the conjugations, NMR and FT-IR were used. $^{13}C$ NMR was recorded on liquid samples ($D_2O$, Sigma–Aldrich) in an AVANCE AV II-400 MHz NMR Spectrometer (Bruker, German). To avoid any influence of the Nuclear Overhauser effect (NOE), all $^{13}C$ spectra used for quantitative analysis were recorded using inverse gated decouplig pulse sequences. The $^{1}H$ NMR experiment of PEI-TEG was also collected on liquid sample ($D_2O$, Sigma–Aldrich) in a Varian UNITY INOVA400 NMR Spectrometer at 400 MHz. FTIR spectra were recorded on VECTOR 22 FT-IR (Bruker, German), with the following scan parameters: scan range 4400–400 $cm^{-1}$: number of scan 16: resolution 4.0 $cm^{-1}$: interval 1.0 $cm^{-1}$: units %T. Nitrogen content was assayed by elemental analyzer (Euro EA 3000, Euro Vector S.P.A.).

The molecular weight and polydispersity of the PEI derivates were determined by GPC relative to PEO standards (Polymer Labs) (Lin et al., 2007). In short, GPC measurements were performed using a Waters 515 isocratic HPLC pump and thermostated (40 °C) OHpak SB 803 gel-permeation chromatography column (Shodex, Japan). Data were collected using a differential refractometer (model 2410).

The buffering capacity of the new synthesized PEI derivates from pH 10 to 2.5 was determined by acid–base titration. Briefly, each polymer was dissolved in 10 mL of 0.15 M sodium chloride aqueous solution to give a final concentration of total amino groups in the polymer of 5 mM, the pH of the polymer solution was brought to 10 with NaOH, and the solution was subsequently titrated with 0.1 M HCl at 25 °C. The pH values were recorded with a pH Meter (Five Easy, Mettler Toledo, Switzerland). The buffering capacity was defined as the percentage of amine groups becoming protonated from pH 7.4 to 5.1 (mimic the pH change from the extracellular environment to the lower pH of the endosomes), and can be calculated from the following equation:

$$\text{Buffer capacity}\,(\%) = \frac{\Delta V \times 0.1M}{\text{N mol}} \times 100\%$$

wherein $\Delta V$ is the volume of HCl solution (0.1 M) required to bring the pH value of the polymer solution from 7.4 to 5.1, and $N$ mol (0.05 mmol) is the total moles of protonable amine groups in the known amount of cationic polymers.

### 2.4. Polyplex preparation

All PEI derivates/DNA complexes were freshly prepared before use. Plasmid DNA was condensed with standard PEI 25k or the new PEI derivates at various mass ratios. PEI/DNA complexes were prepared at a final DNA concentration of 40 μg/mL as described by Kloeckner et al. (2006). Briefly, indicated amounts of plasmid DNA and PEI were each diluted in 5% glucose solution to the same vol-