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Full Length Research Paper

In vitro transfection of HSC-T6 cells with a novel poly(ethyleneimine) biscarbamate conjugate

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Hepatic stellate cells (HSCs) play a critical role in the fibrogenesis of liver, so they are the target cells of antifibrotic therapy. There is an increasing need for safe and effective nonviral gene delivery systems. Here, we report that gene transfer and transfection efficiency in HSC-T6 can be enhanced by the use of a novel poly(ethyleneimine) biscarbamate conjugate (PEIC) with a low molecular weight. When added to a DNA solution, PEIC condensed DNA at a w/w ratio above 1 to15 form 153 to 247 nm polyplexes with -1.18 to 22.86 mV in zeta potential. PEIC was able to effectively protect condensed DNA from DNase I degradation. Our results showed that the uptake of plasmid DNA by HSC-T6 cells depended on the N/P ratios. Treatment with PEIC at an N/P ratio of 7/1 was most effective, increasing the uptake of plasmid DNA in HSC-T6 cells by 4.5 -fold relative to PEI (branched 25 kDa), 45-fold relative to Lipofectamine 2000, and 20-fold relative to FuGENE™6 , respectively. Collectively, these results indicate that PEIC at the optimal N/P ratio might be used to safely enhance gene delivery and transfection of HSC-T6.

Key words: Poly (ethylene imine) biscarbamate conjugate (PEIC), transfection; cytotoxicity, gene delivery, hepatic stellate cells.

INTRODUCTION

Gene therapy had been realized with almost 400 clinical trials using viral vectors owing to their intrinsic high transfection efficiency (Merdan et al., 2002). Although the viral vector has high transfection efficiency, they have many serious disadvantages, such as complicated preparation process, limited loading capability, immunogenicity and potential to induce cancer (El-Aneed, 2004). In polymer-based gene delivery systems, the complexes of condensed DNA with polycations have many advantages (Thomas et al., 2003). They were easier to synthesize, and capable of carrying large amounts of genetic materials, and also had non-immunogenic, non-infectious and non-malignant transformation, hence, many researchers were interested in this field. Non-viral gene carriers generally depend on their large number of positive charges to condense plasmid DNA or antisense oligonucleotide into compact and small complexes, which enables trafficking through the diverse barriers toward the nucleus of target cells where the gene can be expressed. Polyethyleneimine (PEI) is one successful and widely used non-viral gene delivery systems because of its proton sponge effect (Boussif et al., 1995; Godbey et al., 1999a). However, high-molecular-weight PEI had been revealed to be the most effective non-viral vector based on cationic polymers owing to its high pH buffering capacity that is believed to enhance the exit of vectors from the endosomes compartment (Zhang et al., 2004). The commercially available branched PEI (25,000 g/mol) had been widely used as a “gold standard” for evaluation of transfection efficiency of other newly developed polymer- or surfactant-based gene carriers (Park, 2009). It is considered that PEI with high-

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molecular-weight PEI (≥25 kD) not only shows high transfection but also high cytotoxicity. On the contrary, low-molecular-weight PEI (≤2000) is proved to be nontoxic but displays very poor transfection activity (Godbey et al., 1999b). Many scientists reported that when the low-molecular-weight PEI is crosslinked with biodegradable bonds, such as ester, amide, etc (Kim et al., 2007), the transfection efficiency can be enhanced while the cytotoxicity remains low. In addition, the chemical structures of the PEIs and the linker seem to be relevant to their transfection activity (Anderson et al., 2004).

Hepatic stellate cells (HSCs) play a critical role in the fibrogenesis of liver, so they are the target cells of antifibrotic therapy. In normal liver, quiescent HSCs (also called Ito cells, lipocytes, fat-storing cells) are the precursor cells for myofibroblasts (MFBs), which are responsible for the dramatic increase in the synthesis of extracellular matrix proteins in cirrhotic livers (Friedman, 2000). Efficient gene delivery to cultured rHSCs and rMFBs would therefore be of great interest for studying the processes involved in hepatic fibrogenesis and for gene-therapeutic devices.

In the present study, we found that 800Da PEI linked through bisbutanediol carbamate (PEIC) exhibited remarkably high gene transfection activity as compared with PEI of various average molecular weights at various polymer/DNA ratios. Transfection was performed with the commercially available cationic liposome reagents Lipofectamine2000, the lipid-based reagent FuGENE™6, and PEI (25 kD), respectively. This result suggests potential usages of biscarbamate linkages in assembling efficient polycationic gene carrier *in vitro* transfection of HSC-T6 cells.

MATERIALS AND METHODS

Reagents

PEIC was a gift from Prof Jin, School of Pharmacy, JiaoTong University (Shanghai, China). Branched PEI (25 kDa) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine2000 was from Invitrogen (Life Technologies, USA) and FuGENE™ 6 was from Roche (Vienna, Austria). MicroBCA protein assay kit was obtained from Pierce (Rockford, IL, USA). Luciferase assay kit was purchased from Promega (Madison, WI, USA).

The plasmids used in the study were pGL3-Control (Promega, Madison, WI, USA) coding for luciferase DNA and pGCsi-U6/neo/GFP (Genechem Biotechnology Co Shanghai, China) coding for EGFP DNA. The plasmid was transformed in Escherichia coli DH5α and amplified in terrific broth media at 37℃ with stirring overnight. The amplified plasmid DNA was purified by an endotoxinfree Giga plasmid purification kit (Qiagen, Valencia, CA, USA). Purified plasmid DNA was dissolved in Tris- EDTA (TE) buffer, and its purity and concentration were determined by ultraviolet (UV) absorbance at 260 and 280 nm. Rat hepatic stellate cell line (HSC-T6) was obtained from the Institute of Liver Disease, Shanghai University of Traditional Chinese Medicine (Shanghai, China). Other solvents and reagents were all of analytical grade.

Cell lines and cell culture

HSC-T6 (Rat hepatic stellate cell line) were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Paris, France), supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, Utah), streptomycin at 100 μg/ml, and penicillin at 100 U/ml. All cells were incubated at 37℃ in humidified 5% CO_2 atmosphere. Cells were split using trypsin/EDTA medium when almost confluent.

Preparation of PEIC/DNA complexes

All PEIC/DNA complexes were freshly prepared before use. Charge ratio (N/P) of the PEIC/DNA complexes was expressed as the ratio of moles of the amine groups of copolymer to moles of phosphates of DNA. Complexes were prepared by adding copolymer solution to equal volumes of pEGFP DNA (for size and zeta potential measurements), to pGL3 (for luciferase assay), or to pEGFP (for in vitro GFP transfection) with gentle vortexing and incubated at room temperature for 30 min.

Characterization of PEIC/DNA complexes

The size and zeta potential of PEIC/DNA complexes were analyzed using a Zetasizer 3000HS (Malvern, Worcestershire, UK). The complexes were prepared in water at N/P ratios of 1 to 15, and the DNA concentrations were fixed at 20 μg/ml for particle size and zeta potential measurements, respectively. At each N/P ratio, the complexes were prepared in triplicate and measured. The average of these measurements was used. The morphologies of PEIC/DNA complexes at N/P ratios of 7 were observed using energy-filtering transmission electronmicroscopy (EF-TEM) (LIBRA 120, Carl Zeiss, Germany).

Gel retardation assay

The agarose gel electrophoresis was used to determine the capability of condensing negatively charged nucleonic acid and protecting DNA from degradation. To confirm DNA condensation ability of the copolymer, electrophoresis was performed. Complex formation was induced at various N/P ratios from1 to 15, and the final volume with the 6× agarose gel loading dye mixture (Biosesang, Korea) was 12 μl. The complexes were loaded onto 0.8% agarose gels with EtBr (0.1 μg/ml) and run with Tris-acetate (TAE) buffer at 80 V for 60 min. DNA retardation was observed by irradiation with UV light and assayed with Camcom software.

Protection and release assay of DNA

Protection and release of DNA in complexes were carried out by electrophoresis, according to the modified method of Wang et al. (2002). Briefly, 1 μl of DNase I (5 units) or PBS in DNase/Mg2+ digestion buffer (50 mM Tris–Cl, pH 7.6 and 10 mM MgCl2) was added to 200 μl of polyplex solution or to 4 μg of naked plasmid DNA, and incubated at 37℃ with shaking at 100 rpm for 60 min. For DNase I inactivation, all samples were treated with 100 ul (pH 8.0, 400 mM NaCl and 100 mM EDTA) for 10 min and mixed with 10% sodium dodecyl sulfate (SDS)12 ul. The final samples were incubated at 65℃ for 12 h, and electrophoresis was performed in 0.8% agarose gel with TAE running buffer for 30 min at 100 V.

In vitro cytotoxicity tests

The cytotoxicity of PEIC was examined by percent viability of HSC-

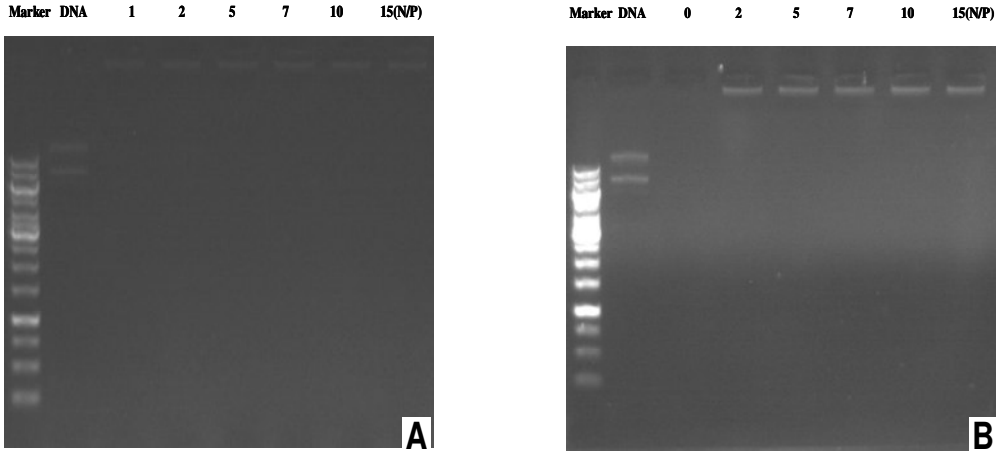


Figure 1. (A) DNA gel retardation assay of PEIC. Lanes: (1) marker; (2) naked DNA as control; (3) N/P: 1/1; (4) N/P:2/1; (5) N/P:5/1; (6) N/P:7/1; (7) N/P:10/1; (8) N/P:15/1; (B) Agarose gel electrophoresis retardation assay of polymer/DNA complexes was prepared at different N/P ratio after DNase I digestion. Lanes: (1) marker; (2) naked DNA as control; (3) N/P: 0; (4) N/P:2/1; (5) N/P:5/1; (6) N/P:7/1; (7) N/P:10/1; (8) N/P:15/1.

T6 cells. To determine cell viability using MTT assay, the HSC-T6 cells were seeded in a 96-well plate and incubated at 37℃ for 24 h prior to addition of the solutions of the polycationic carriers of desired concentrations. After the additional 4 h incubation, the supernatant containing carrier molecules was replaced by fresh DMEM, and 25 µl of MTT solution (5 mg/ml in PBS buffer) was added for determination of cell viability. Finally, after further incubation for 6 h, the absorbance was measured at 570 nm, using an ELISA plate reader (MK3 Thermo Labsystem, Finland) to measure the metabolic activity of the cells. Cell viability (%) was calculated according to the following equation: Cell viability (%) = (OD570 (sample) / OD570 (control)) × 100, where OD570 (sample) represents a measurement from a well treated with polymer and OD570 (control) from a well treated with PBS buffer only.

Cell transfection-luciferase activity assay

Cells were seeded in 48-well plates at an initial density of 5×10^4 cells/well in 1 ml of growth medium. After incubation for 24 h (to reach 70% confluence at the time of transfection), the media were replaced with serum-free or 10% serum containing media with polymer/pGL3-control (0.5 µg) complexes at various N/P ratios and additionally incubated for 4 h. Then the media were exchanged for fresh media, containing serum, and allowed to incubate for 48 h at 37℃. The luciferase assay was performed according to the manufacturer's protocols. Relative light units (RLUs) were measured with a Luminometer (Autolumat LB953, EG and G, Berthold, Germany). Protein quantification was determined by the BCA method, and RLUs were normalized to protein concentration in the cell extracts. Each transfection experiment was carried out in triplicate, and transfection activity was expressed as relative light units.

Flow cytometry

The HSC-T6 cells were plated in a 24-well plate at 1×10^5 cells/well and incubated for 24 h. Then the media were replaced with serum-free media, containing polymer/pEGFP (1 µg) complex at vary N/P ratios. After incubation for 4 h, serum-free media were changed with fresh media, containing serum. After 44 h incubation, cells were washed once with PBS and detached with 0.25% trypsin/EDTA. Transfection efficiency was evaluated by scoring the percentage of cells expressing GFP, using a FACS Calibur System from Becton-Dickinson (Franklin Lakes, NJ, USA). Fluorescence parameters from 10,000 cells were acquired, and transfection was carried out in triplicate.

Statistical analysis

All experiments were performed in replicates (n = 3) for validity of statistical analysis. Results were expressed as mean ± SD. Student t-tests were performed on the data sets generated using SPSS11.0. Differences were considered significant for p values < 0.05.

RESULTS

Characteristics of polyplexes

The formation of polyplexes was confirmed using agarose gel electrophoresis, particle sizing and electron microscopy (TEM). Electrophoresis of DNA is completely retarded when mixed with PEIC at 1/1 weight ratio (carrier/gene), indicating that polyplex was formed (Figure 1A). At 37℃ under the action of DNase I naked DNA degraded, and PEIC formed a stable complex with DNA at the same time protecting DNA from digestion (Figure 1B). The particle sizes of the polyplexes formed with PEIC and luciferase plasmid at various polymer/DNA w/w ratios were also examined using a zetasizer and summarized in (Figure 2A). The average particle size was about from153 to 247 nm. At the same time, the zeta potential of the particles increased from 1.18 to 22.86 mV (Figure 2B). This result agrees with the TEM observation in which particles, 100 to 200 nm in diameter, were

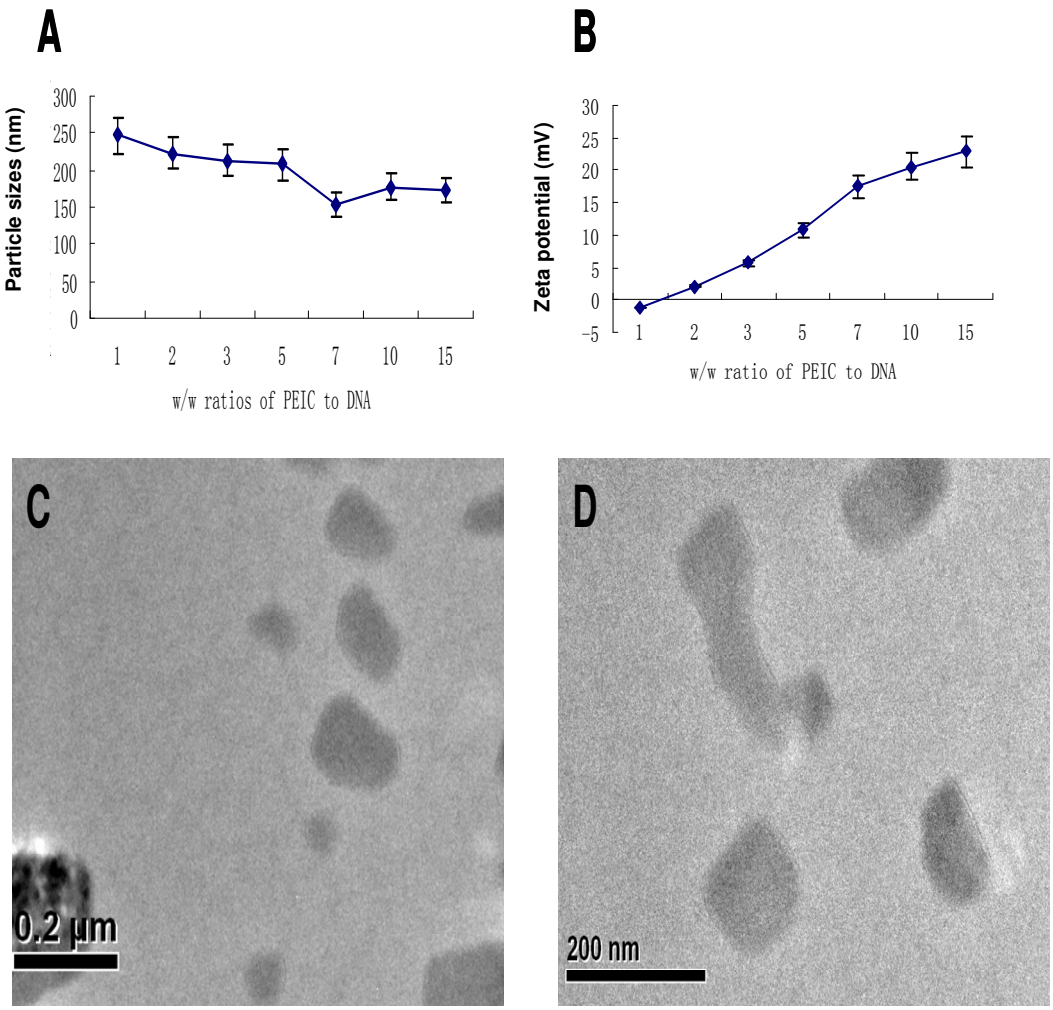


Figure 2. Morphology and size distribution of polyplexes formed of PEIC and DNA plasmids. (A) Particle size distribution and (B) zeta potential of polyplexes formed of PEIC and DNA plasmids at various carrier/gene ratios. (C-D) Transparent electron microscopic image of polyplex form of PEIC and DNA plasmids. These micrographs are obtained at magnification of 750000× and 1500000, respectively.

imaged (Figure 2C to D).

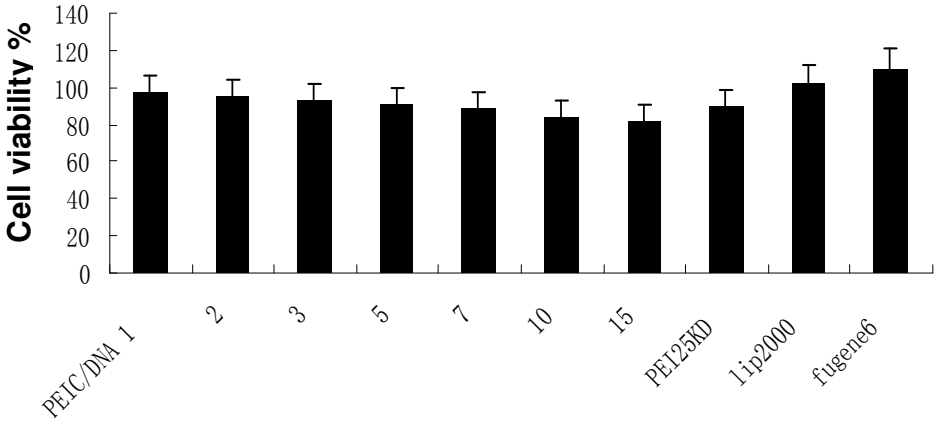
Cytotoxicities

Cytotoxicity is a major hurdle for clinical feasibility of polycatioinc gene carriers (Lv et al., 2006). Based on the viability of HSC-T6 cells incubated together with various polycationic carrier molecules, PEIC showed low cytotoxicity within the experimental concentrations (up to 15 μg/ml) in the cell culture, similar to PEI 25 KD, Lipofectamine2000, and FuGENE™6 (Figure 3).

Gene transfection activities

The gene transfection activity of PEIC was compared with PEI 25 kDa, Lipofectamine2000, and FuGENE™6 in luciferase expression in HSC-T6 cells. The result is summarized in Figure 4. For PEI 25 kDa, activity (1087±977 RLU/mg protein) for luciferase gene expression was achieved. In the case of PEIC, the gene expression rate at the same carrier to gene ratio (7/1) was 4903 ± 1747 RLU/mg protein, higher than PEI 25 kDa ($p<0.05$). Such gene transfection activity was higher than Lipofectamine 2000 (109 ± 42 RLU/mg protein) and FuGENE™6 (236±275RLU/mg protein), respectively ($p<0.01$) (Figure 4).

To adopt GFP as the reporter gene we studied PEIC transmission performance as a gene vector. Its expression products of green fluorescent protein in UV light would be spontaneously excited by the green fluorescence. We used flow cytometry calculate percentage of fluorescent cells and measured in transfection efficiency. As shown in Figure 5, in the N / P = 7, the green fluorescent protein plasmid expression



Figur 3. Cell percents viabilities of PEIC at different N/P ratios in HSC-T6 cells. PEI 25 kDa, Lipofectamine2000, and FuGENE™6 was used as control. Data were shown as mean±SD (n=3).

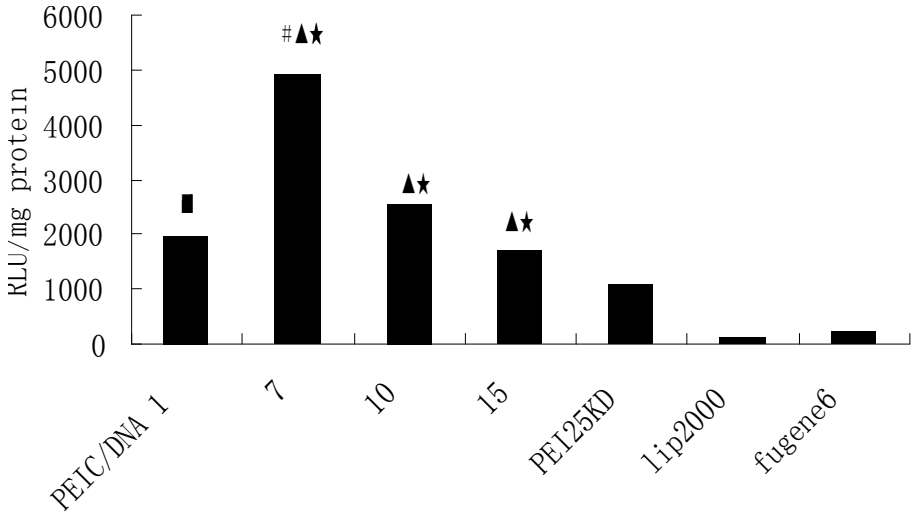


Figure 4. Comparison of relative luciferase activity of PEIC/DNA complexes at N/P ratios range from 1 to 15. PEI 25 kDa, Lipofectamine2000, and FuGENE™6 was used as control. Data were shown as mean±SD (n=3).

levels of the highest transfection efficiency of 20 ± 2.1%.

DISCUSSION

HSC has been recognized to be responsible for most of the ECM observed in chronic hepatic fibrosis (Friedman, 2008). The rat HSC-T6 cell line (Vogel et al., 2000) is constructed through transfecting SV40 into rat HSC and its phenotype is activated HSC. HSC-T6 has the stable phenotype and biochemical characters of activated HSC, expressing myogenic and neural crest cytoskeletal filaments, and the cell line had been a useful tool for studying hepatic fibrogenesis and it is also a reliable cell model for investigating antifibrotic drugs (Cheng et al., 2006). HSC-T6 is one of the hard-to-transfect cell types which may be due to the large amount of ECM secreted by HSC. The three-dimensional structure of the ECM can reversibly regulate the morphology, proliferation, and functions of the stellate cells. Molecular mechanisms in the regulation of the stellate cells by three-dimensional structure of the ECM imply cell-surface integrin binding to the matrix components, followed by signal transduction processes and cytoskeleton assembly (Senoo, 2004). Viral vectors enable stable inhibition of gene expression (Pichler et al., 2005). The study demonstrated with the use of chemically enhanced transfection methods, the highest relative efficiency was obtained with FuGENE6 gene mediated DNA transfer (approximately 6%) and adenoviral mediated transfer was a promising approach

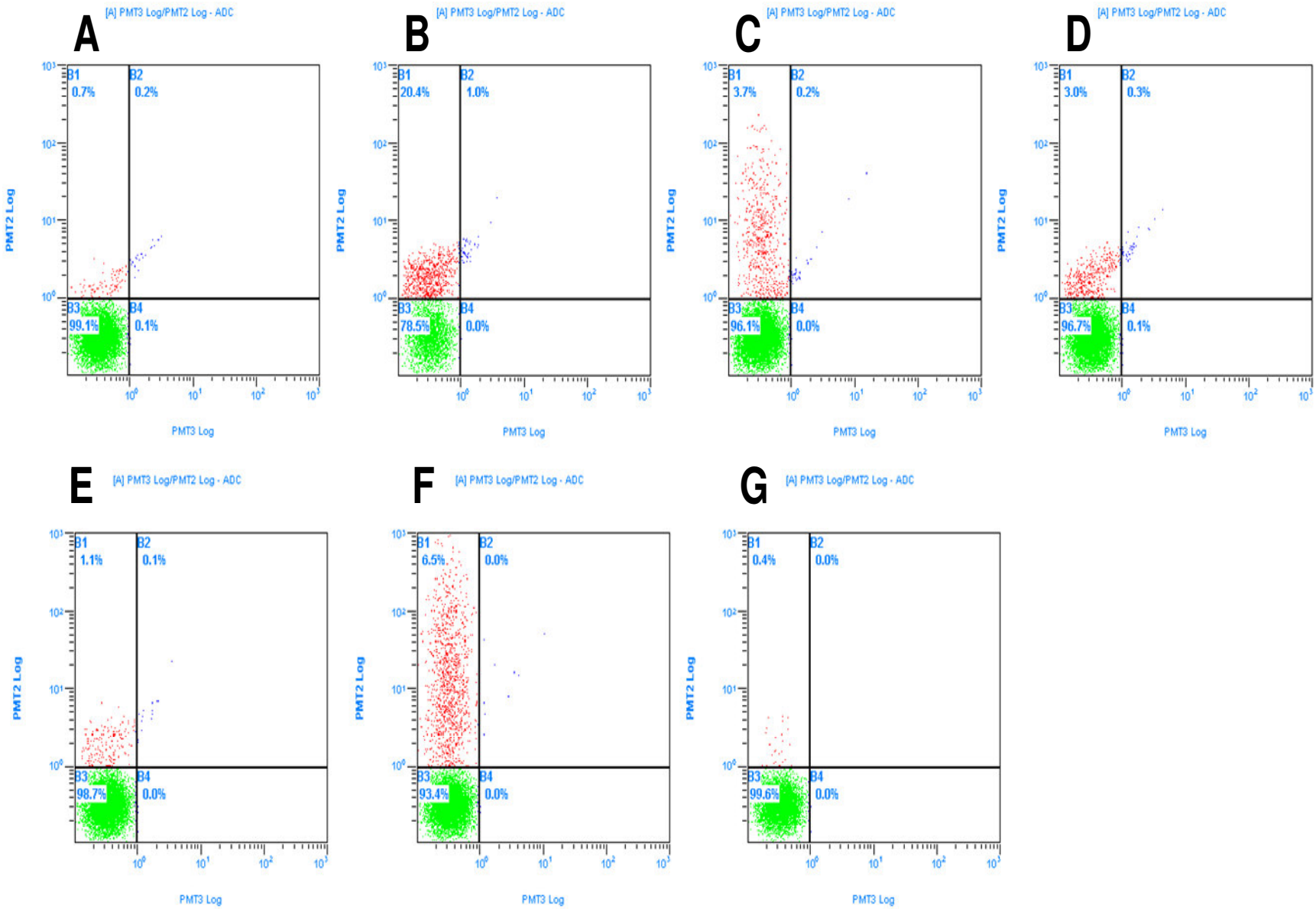


Figure 5. Transfection efficiency of PEIC/DNA complexes at N/P ratios range from 1 to 15. (A) N/P: 1/1; (B) N/P: 7/1; (C) N/P:10/1; (D) N/P:15/1; (E) PEI 25 kDa; (F) Lipofectamine2000; (G) FuGENE™6.

for gene delivery to these hepatic cells (100%) (Weiskirchen et al., 2000), however, viral vectors are associated with concerns of toxicity and immunogenicity (Schagen et al., 2004). A number of therapeutic applications require sustained gene inhibition with minimal toxicity following repeated administration of the vector. Non-viral vectors are generally considered safer than viral vectors (Aigner, 2007). Polyethyleneimine (PEI) is one successful and widely used non-viral gene delivery systems.

The mechanisms of PEIs are to condense the DNA into the compact particles to protect the DNA from the endosomal degradation and to target the DNA into the nucleus. The PEI/DNA complexes are taken up by the cells via a nonspecific adsorptive endocytosis. The high density of primary, secondary and tertiary amines of PEIs, expressing protonation only on every third nitrogen under physiological conditions, confers large buffering capacity to the polymers over a wide range of pH. This buffering effect causes an influx of protons into the endosomes followed by a passive chloride influx and a consequent osmotic swelling. These result in a bursting of the endosomal membrane and a release of the endocytosed PEI/DNA complexes (Kichler et al., 2001).

The PEIs are available in a wide molecular weight range (200 to 800,000 Da) and in various degrees of branching. The transfection efficiency of different PEIs seems to be dependent at least partly on both their size and structure. PEIs with higher MWs have been relatively efficient both *in vitro* and *in vivo* (Fischer et al., 2003), whereas PEIs with lower MWs have shown weaker transfection efficiency (Godbey et al., 2000). Several studies indicate also that the transfection efficiency is higher for linear than branched PEIs. On the other hand, some reports show that also the low MW PEIs can be used for gene delivery (Kunath et al., 2003; Shin et al., 2005). The low MW PEIs having a linear structure have displayed increased *in vitro* transfection efficiency in comparison to their higher MW counterparts (Breunig et al., 2005). The low molecular weight may exhibit higher gene transfection activity than high molecular weight polycations due to reduced cytotoxicity (Kunath et al., 2003).

Furthermore, we took low molecular weight PEI as the multi-amino moiety to form a polycationic gene carrier via a degradable biscarbamate linkage (Xu et al., 2008). We found that 800 Da PEI linked through bisbutanediol carbamate (PEIC) exhibited remarkably high gene transfection activity as compared with PEI of various average molecular weights at various polymer/DNA ratios. So we examined comparatively PEIC polycationic carrier, cationic liposome reagents Lipofectamine2000 and the lipid-based reagent FuGENE™6, as potential tools for efficient delivery of DNA to HSC-T6.

Different physicochemical properties of nonviral vectors, DNA and their complexes have been studied to improve the transfection efficiency. It is generally thought that the size and surface charge of gene delivery complexes are important factors in modulating their cellular uptake. These PEIC polyplexes were stable with particle sizes of 150 to 250 nm at N/P ratios from 1 to 15. The particle size of PEIC polyplexe at N/P ratio 7:1 was smallest. The zeta potentials of all formulations were from -1.18 to 22.86 mV. The zeta potentials of the complexes were negative at lower N/P (<2) and positive at higher N/P (<5) ratios. The overall percentage of transfected cells with the most efficient complexes was about 20%. The exact mechanism of this polyplexe of gene delivery is not known at present.

It seems to be due to the structural difference between these cationic gene carriers. A speculation could be that the electron-attracting carbamate structure increased the portion of un-protonated amino groups of PEI, thus the proton sponge potential of the polycationic carrier was enhanced. Degradation of the carbamate linkage may be another factor facilitating gene transfection (by releasing genes in cells). Yang et al. (2004) reported that hydrolysis of N, N-diethylethylenediamine polyurethane at pH 7.4 and 37℃ took tens of hours. Degradation of carbamate linkages may not only help to reduce cytotoxicity and release genes, but also generate additional amino groups that may enhance proton sponge potential and thus facilitate endosomal escape of genes (Wolff and Rozema, 2008).

In conclusion, the *in vitro* increment of PEIC-mediated gene transfection seems to be a common attribute which is associated with the structure than rather the size. Its gene transfection activity improved. The present study has shown a great potential of biscarbamate-linked multi-amine polycations as effective and safe non-viral gene carriers *in vitro* transfection of HSC-T6 cells.

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