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Nonionic Amphiphilic Surfactant Conjuncted Polyethyleneimine as a New and Highly Efficient Non-viral Gene Carrier

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Abstract: In order to enhance the gene delivery efficiency and decrease the cytotoxicity of polyplexes, we synthesized Solutol-*g*-PEI by conjugating polyethyleneimine (PEI) to Solutol (polyoxyethylene (10) stearate), and evaluated its efficiency as a possible nonviral gene carrier candidate. Structural analysis of synthesized polymer was performed by using ^{1}H-NMR. Gel retardation assay, particle sizes and zeta potential measurement confirmed that the new gene carrier formed a compact complex with plasmid DNA. The complexes were smaller than 150 nm, which implicated its potential for intracellular delivery. It showed lower cytotoxicity in three different cell lines (Hela, MCF-7, and HepG2) than PEI 25 kDa. pGL3-lus was used as a reporter gene, and the transfection efficiency was *in vitro* measured in Hela cells. Solutol-*g*-PEI showed much higher transfection efficiency than unmodified PEI 25 kDa.

Keywords: gene delivery, polyethylenimine, polyplexes, transfection, cytotoxicity, Solutol.

Introduction

Gene therapy has become an attractive concept for a variety of biomedical applications. Viral vectors like adenovirus and retroviruses have been utilized to transfer nucleic acid constructs of pharmaceutical interest.[1,2] However, viral vectors also bear certain disadvantages such as issues of safety, immunogenicity, mutagenesis and low gene carrying capacity. Non-viral vectors are paid increasing attention to as gene delivery vehicles because of such advantages as easy manipulation, stability, low cost, safety, and high flexibility corresponding to the size of the transgene delivered.[3,4] Among non-viral gene carriers in use, the polycationic polymer, polyethylenimine (PEI), has shown high transfection efficiency both *in vitro* and *in vivo*,[5,6] probably due to its stable complex structure which is appropriate for being taken by cells and the following "proton sponge" effect from PEI facilitates the escape of the complex from an endosomal compartment. The gene delivery efficiency and cytotoxicity of PEI vary according to its molecular weight. High molecular weight PEI (HMW PEI) has a higher transfection efficiency of PEI-based complexes than low molecular weight PEI (LMW PEI). On the other hand, the huge amount of cationic charge of HMW PEI frequently results in a rather high toxicity,[7,8] which can be regarded as one of the major factors limiting its use *in vivo*. Another problem of using polycation DNA complexes is their poor solubility.[9] They may immediately precipitate out of a solution when being prepared at a higher concentration. Many *in vivo* gene administrations are limited by the volume of a solution.

In the past several years, modification of PEI to decrease its cytotoxicity and/or improve its transfection efficiency has been actively pursued. Two major vector modification strategies have been reported. The first involves the conjugation of HMW PEIs with a hydrophilic and biocompatible polymer such as poly(ethylene glycol) (PEG).[10] Copolymer of PEG modified HMW PEI can improve the solubility of macromolecules, minimize the aggregation of particulates and reduce their interaction with proteins in the physiological fluid. However, the "stealth" effect of PEG often results in a diminished transfection due to decreased cellular association and internalization.[11,12] The second vector modification strategy is to apply the chemical modification of nontoxic LMW PEI to increase its gene transfer efficiency. For example, Pluronic P123 grafted LMW PEI (M_w 2,000), in combination with unmodified Pluronic copolymers, was used to form polyplexes with plasmid DNA.[13,14] The gene transfection levels were largely decided by the formulation conditions, which suggests that the physical stability of polyplexes was a key determinant in cellular uptake and gene expression.

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However, overall gene transfection efficiency of polyplexes formulated with Pluronic grafted LMW PEI was still far lower than those formulated with HMW PEI. Molecular weight of PEI plays a crucial role in stabilizing the polyelectrolyte complex structure and subsequently in enhancing the extent of cellular uptake.

In this study, Solutol (polyoxyethylene (10) stearate) was used to modify HMW branched PEI (BPEI, 25 kDa) thus formulated the copolymers of different modified degrees. Solutol, a nonionic amphiphilic surfactant, is usually used as injectable solubilization. Its molecular structure involves PEO chain and stearic acid chain. The "stealth" effect of PEO reduces toxicity and meanwhile improves the colloidal stability of the polymer/DNA complexes; and stearic acid chain is believed to be able to enhance complexes lipophilicity. It was expected that Solutol grafted HMW BPEI copolymers can decrease toxicity and maintain transfection efficiency and accordingly serve as an effective delivery system for the plasmid DNA. The copolymers were synthesized with Succinimidyl Carbonate Method.[15] Their DNA binding properties were studied with the help of electrophoresis and dynamic light scattering. The cytotoxicity of Solutol-*g*-PEI based polyplexes was evaluated by experimenting on Hela, MCF-7 and HepG2 cells. And the *in vitro* transfection activity of polyplexes was evaluated by using Hela cells.

Experimental

Materials. The Solutol ($\overline{M}_w$=1,100) was a free sample provided by BASF (Ludwigshafen, Germany). Branched PEI 25 kDa was obtained from Sigma-Aldrich (St. Louis, MO, USA), bis(trichloromethyl)carbonate, *N*-hydroxysuccinimide, triethylamine, toluene, and anhydrous dichloromethane were purchased from Sino.pharm Chemical Reagent Co.Ltd (Shanghai, China) and used without further purification. Plasmid pGFP, which contains the CMV promoter and enhanced green fluorescent protein (EGFP) gene, was a product of Systerm Biosciences (Mountain View, CA, USA). Luciferase Assay System for transfection assay *in vitro* and pGL-3 control vector with SV-40 promoter and enhancer encoding firefly (Photinus pyralis) luciferase were obtained from Promega (Madison, WI, USA). The plasmids were amplified with a competent *Escherichia coli* strain JM109 and purified by a Qiagen (Chatsworth, CA, USA) kit. The purity of the purified and concentrated DNA was determined by measuring its UV absorbance at 260 and 280 nm respectively. pGFP was used in the studies of physicochemical characterization, and pGL3 was used in transfection experiments *in vitro*.

Synthesis of Activated Solutol. Solutol (0.550 g, 0.5 mmol) was dried in vacuum at 50 °C, and then dissolved in toluene/dichloromethane (3:1, 40 mL) and treated with bis(trichloromethyl)carbonate (0.149 g, 0.5 mmol) overnight. The solution was evaporated to dryness under vacuum. The residue was re-dissolved in toluene/dichloromethane (2:1, 30 mL) and treated with solid *N*-hydroxysuccinimide (0.060 g, 0. 5 mmol) followed by anhydrous triethylamine (0.071 mL, 0.5 mmol). After 4 h, the solution was filtered and evaporated to dryness in vacuum. The product was purified on a silica gel column using stepwise elution with trichloromethane-methanol (10-15% v/v) mixtures and the yield was circa 80-85%.

Synthesis of Solutol-*g*-PEI Copolymer. PEI (0.25 g, 0.01 mmol) was dissolved in 10 mL of 10% aqueous ethanol and the predetermined amount of activated Solutol (0.01, 0.02, 0.05, 0.1, and 0.2 mmol) was added while being constantly stirred. The reaction mixture was left overnight at room temperature. The conjugate was purified from admixture of free PEI by gel-permeation chromatography on the column with Sephadex G-75, with 10% ethanol as eluent. Fractions containing the conjugate and free PEI were analyzed by color reaction with picrylsulfonic acid. The eluent was dialyzed in Amicon Ultra-4 membrane tubes (Millipore, USA) with the cutoff 10 kDa against water (twice replaced) for 12 h, followed by lyophilization. The ratio of Solutol and PEI in the copolymer samples was determined from ^{1}H-NMR (Varian 300 MHz) spectra by measuring integral values obtained for the -CH_2CH_2O- protons of Solutol and -CH_2NH- protons of PEI.

Gel Retardation Assay. Various amounts of Solutol-*g*-PEI dissolved in deionized water were added into the aqueous solution with a fixed amount of plasmid DNA and incubated for 30 min at room temperature. The mixture was electrophoresed on 1% (w/v) agarose gel for 60 min at 100 V. The gel was stained with ethidium bromide (0.5 g/mL) and illuminated on a UV illuminator to show the location of the DNA and various levels of polyplex formation.

Measurement of Particle Sizes and Zeta Potential. The sizes and Zeta Potential of cationic polymer/DNA complexes in PBS buffer (50 mM, pH 7.4) at 25 °C were measured by an electrophoretic light scattering spectrophotometer (Zeta Potential/Particle Sizer, 380ZLS, Nicomp™, USA), with 90° and 20° scattering angles respectively. The polyplexes were prepared by adding a solution with appropriate amount of cationic polymer based upon desired N/P ratios (250 μL of PBS) to 20 μg of DNA in 250 μL PBS while vortexing. The complexes were incubated at room temperature for 30 min before sizes and Zeta Potential measurements were carried out.

Cell Lines and Cell Culture. Hela (human cervical cancer cell line), MCF-7(human breast adenocarcinoma line), and HepG2 (human hepatocellular carcinoma cell line) were incubated in Dulbecco's modified Eagle medium (DMEM,) supplemented with 10% fetal bovine serum (FBS), streptomycin at 100 μg·mL^{-1}, penicillin at 100 U·mL^{-1}. The cells were maintained at 37 °C in a humidified 5% CO_2-containing atmosphere.

Cell Viability Assay. Hela, MCF-7 and HepG2 cells were cultured as outlined above. In cell viability assay, the cells (10,000 cells/well) were seeded into 96-well plates (Greiner Bio-One GmbH, Germany). After 24 h, culture media were replaced with fresh serum-free DMEM medium containing serial dilutions of Solutol-*g*-PEI copolymer and PEI 25 kDa. The cells were incubated for another 24 h. In cell viability assay, 20 μL sterile filtered MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Amresco, USA) stock solution (5 mg/mL) in phosphate buffered saline (PBS) was added to each well reaching a final concentration of 0.5 mg MTT/mL. After 4-h incubation at 37 °C, un-reacted dye was removed by aspiration. The formazan crystals were dissolved in 200 μL/well DMSO (Amresco, USA) and measured spectrophotometrically in an ELISA plate reader (Model 318 MC, Sanco) at a wavelength of 492 nm. The spectrophotometer was calibrated to 0 absorbance by using culture medium without cells. The cell viability (%) was calculated according to the following equation:

$$\text{Cell Viability (\%)} = (A_{test}/A_{control}) \times 100 \text{ (mean} \pm \text{SD, n = 6)}$$

where A_{test} is the absorbance of the Solutol-g-PEI or PEI 25 kDa treated cells and $A_{control}$ is the absorbance of the untreated cells.

***In Vitro* Transfection.** Hela cells were seeded in a 24-well plate at a density of 1×10^5 cells per well and incubated for 18-24 h to 60-70% confluency. A 3 μg of pGL3 were formulated with the different polymers at various N/P ratios and incubated for 30 min at room temperature. The polymer/DNA complexes were dispersed in the complexes-dispersed medium, serum-free DMEM, and then added to a 24-well plate and incubated for 5 h at 37 °C under a 5% CO_2 atmosphere. After the media were replaced with growth media containing serum, the incubation went on for another 48 h. The Luciferase assay was carried out by following manufacturer's instructions (Promega). Briefly, cells were lysed in 350 μL of 1×CCLR on ice for 30 min and then centrifuged at 13,000×g for 2 min. Supernatants were collected and the luciferase activity was analyzed. In a typical experiment 20 μL of supernatant was added to luminometric tubes containing 100 μL of luciferase substrate (Promega). Light emission was measured with a luminometer (Berthold) for a period of 5 sec. The relative light units/s (RLUs) determined in 20 μL of cell extract were converted into the amount of luciferase (pg) by using a luciferase standard curve. The standard curve was obtained by diluting the known amounts of recombinant luciferase (Promega E1701) in lysis buffer used to extract the cells. The amount of cell protein in 20 μL of cell extract was determined by a Micro-BCA protein assay kit. The transfection efficiency was expressed as relative light unit per mg of cell protein. All transfection experiments were performed in triplicate.

Table I. Characteristics of Prepared Solutol-*g*-PEI

Copolymer Name[a]	Polyether: PEI[b]	Modification Degree[c] (%)	Molecular Mass[c] (kDa)
Solutol-1g-PEI	1	0.18	26.1
Solutol-2g-PEI	2	0.35	27.2
Solutol-5g-PEI	4.5	0.80	30
Solutol-10g-PEI	9.7	1.72	35.7
Solutol-20g-PEI	19.3	3.40	46.3

[a]The nomenclature for the Solutol-grafted PEI, the Solutol:PEI ratio is shown by number. [b]As determined by ^{1}H-NMR analysis of the copolymer samples. [c]Calculated based on ^{1}H-NMR data assuming that all polyether chains in the copolymer samples are linked to the PEI.

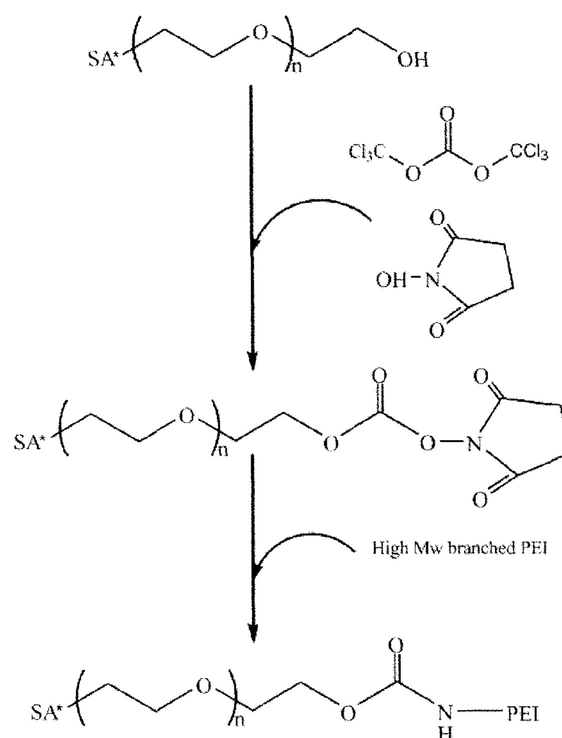

Figure 1. Synthetic scheme of Solutol-g-PEI (SA: stearate).

Results and Discussion

Synthesis of Solutol-*g*-PEI Conjugates. Cationic copolymers were synthesized by grafting polyether chains of Solutol to the amino groups of PEI. The nomenclature and characteristics of the synthesized cationic copolymers are presented in Table I. In all cases, free hydroxyl groups of the polyether chains of Solutol were activated by succinimidyl carbonate and then linked to the amino groups of the PEI, as shown in Figure 1. The copolymers were separated from unconjugated polymers by gel permeation chromatography (GPC) and then the ratios of the polyether and PEI chains in these samples were determined from ^{1}H-NMR spectra by measuring integral values obtained for the -CH_2CH_2O- pro-

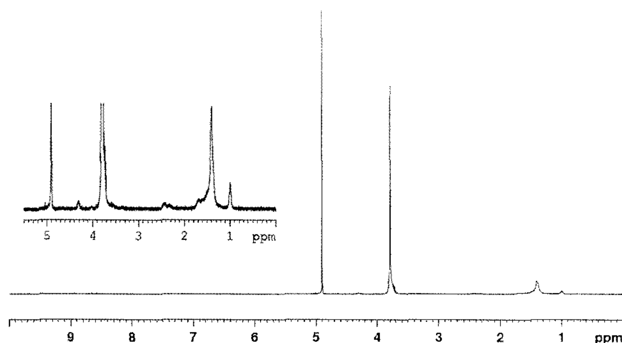

Figure 2. ^{1}H-NMR spectra of Solutol-*g*-PEI in D_2O.

tons (Solutol) and -CH_2CH_2NH- protons (PEI). Figure 2 show the ^{1}H-NMR spectra of Solutol-*g*-PEI in D_2O, where -CH_2CH_2O- proton peaks appear at δ3.6-3.9 ppm and -CH_2CH_2NH- protons peaks appear at δ2.2-2.6 ppm.

Analysis of Complexes Using Agarose Gel Electrophoresis. In this paper, we use N/P ratio, the ratio of concentrations of total nitrogen atoms (N) of the polycation to the phosphate groups (P) of DNA, as the characteristic of the complex composition. Complexes of plasmid DNA and cationic copolymers were prepared at various N/P ratios and analyzed by agarose gel electrophoresis. The typical result obtained during electrophoresis experiments for all copolymers is presented in Figure 3. In the experiment, the movement of the plasmid DNA in the gel was retarded as the amount of the cationic copolymer increased. As N/P ratios exceeded the neutralization composition, the complexes migrated slightly toward the anode, which suggested that they had a small positive charge. For unconjugated PEI, DNA was retarded at N/P ratio of 2.5. For Solutol-1*g*-PEI, Solutol-2*g*-PEI and Solutol-5*g*-PEI, DNA was retarded at N/P ratio of 4. However, for Solutol-10*g*-PEI and Solutol-20*g*-PEI, a higher N/P ratio at 5 and 6 was needed respectively for complete neutralization of DNA. PEI modified by more Solutol could shield the positive charge of the surface of complexes. Thus, N/P value increased to the degree that DNA was able to be completely retarded. The assay established the polymer/DNA ratio required for complete condensation of DNA.

Measurement of Particle Sizes and Zeta Potential. In order to investigate the effect of cationic copolymer structure on the size of complexes, each cationic copolymer was allowed to form complexes at different N/P ratios. Figure 4(a) demonstrates a significant increase in the average particle size at low N/P ratios, most probably due to their low surface charge. With the increase of N/P ratios, the particle size of complexes gradually reduced. All of the average particle size of complexes were small (below 250 nm), which profited the efficient endocytosis and gene transfer.[16] Within the range of concentration studied, no precipitation was observed at any N/P ratios. This was in contrast with unmodified PEI/DNA complexes, which grew into large aggregates at N/P>3.[17] Obviously, the presence of Solutol led to a good stability of complexes. The structure of Solutol contained

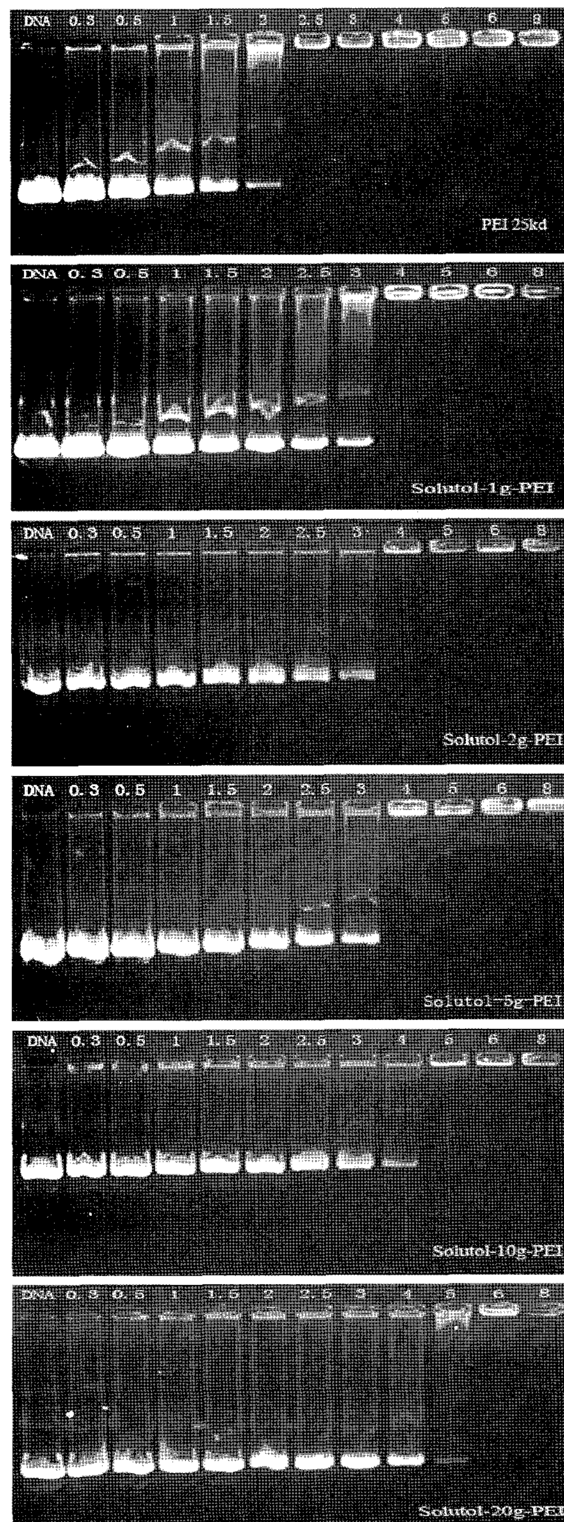

Figure 3. Agarose gel electrophoresis of polymer/DNA (pGFP) complexes at various N/P ratios.

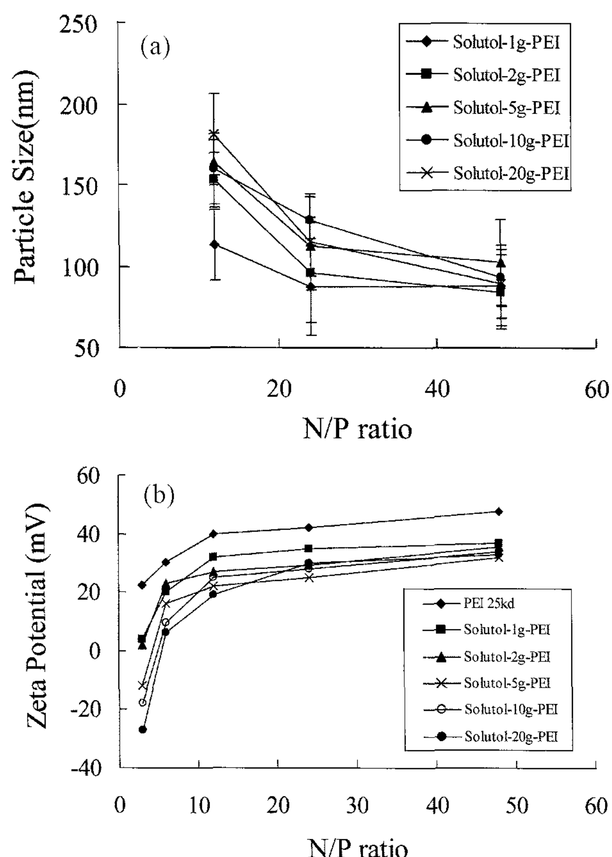

Figure 4. Particle sizes (a) and zeta-potential (b) of complexes at various N/P ratios.

the hydrophilic PEO chain, which can improve the solubility of the polycation/DNA complexes.

Changes in the charge of the complexes upon addition of the cationic copolymers were further characterized by measurement of the zeta potential for various N/P ratios. Zeta potentials of polymer/DNA complexes are closely related to cellular uptake.[18] Also, strong cationic charges of the complexes are often cytotoxic. Figure 4(b) shows the zeta potentials of complexes at different N/P ratios. The zeta potential of Solutol-g-PEI was significantly lower than those of unmodified PEI 25 kDa observed at various N/P ratios. The zeta potential of Solutol-5g-PEI, Solutol-10g-PEI or Solutol-20g-PEI was negative at low N/P ratios because the polymer was not able to condense DNA effectively. The zeta potential of Solutol-1g-PEI or Solutol-2g-PEI, on the other hand, was a slightly positive value. Particularly, the zeta potential reached -27 mV for Solutol-20g-PEI, which can be ascribed to the shielding of the positive surface charge of the complexes by the PEO of Solutol. With the increase of N/P ratio, zeta potentials of the complexes also increased, which demonstrated the progressive neutralization of the DNA phosphate groups by the charged amino groups of the polycation. Thus, the zeta potential measurements were consistent with the results of the agarose gel

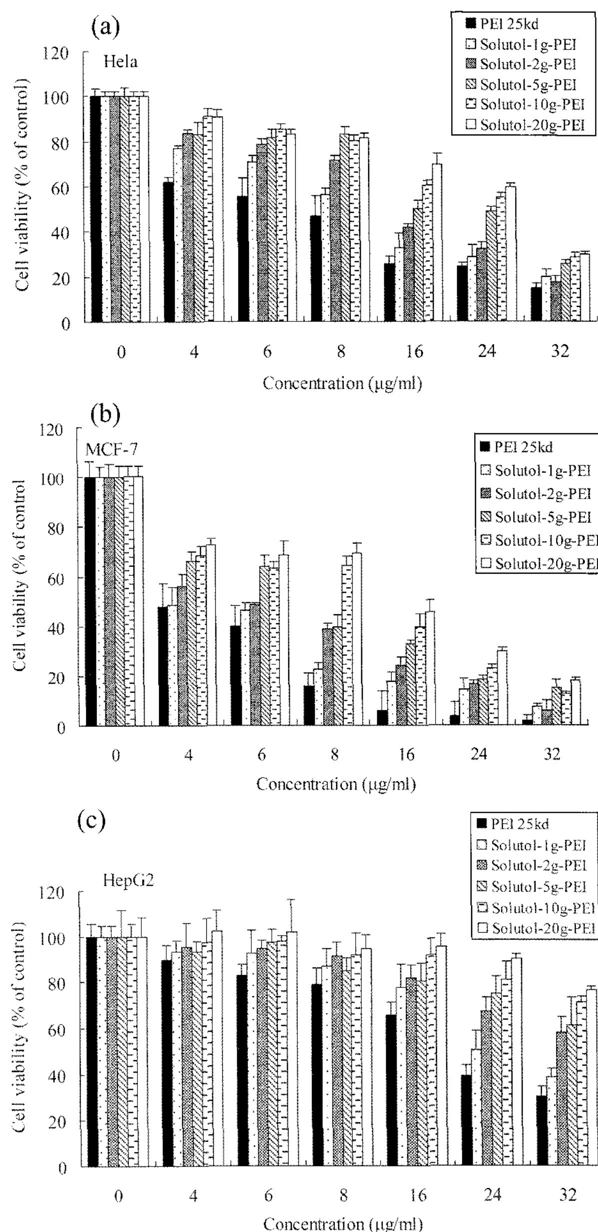

Figure 5. Cytotoxicity of polymer at various concentrations in different cell lines (a) Hela, (b) MCF-7, and (c) HepG2 (n=6, error bars represent standard deviation).

electrophoresis experiments. The zeta potentials rapidly increased up to N/P ratio 12, whereas they slowly increased from N/P ratio 12 to 48.

Cell Viability Assay. A high molecular weight PEI is more toxic than a low molecular weight PEI.[19] Accordingly, our attempt focused on reducing the cytotoxicity and increasing the transfection efficiency. Therefore, the polyoxyethylene stearate modified high molecular weight PEI was designed to reduce the cytotoxicity of cationic polymer. The cytotoxicities of the Solutol-g-PEI and the PEI 25 kDa polymer were compared with the MTT assay. Figure 5 shows cyto-

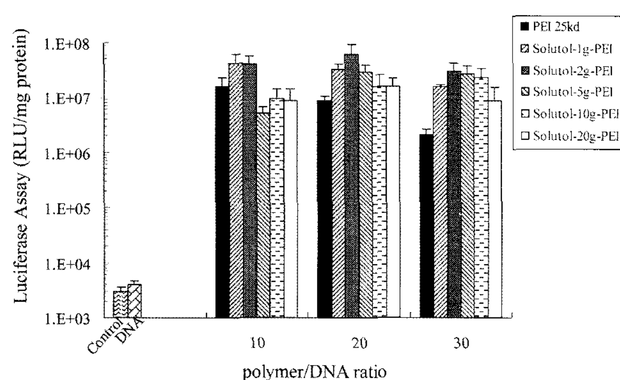

Figure 6. Transfection efficiency of polymer/DNA (pGL3-control) complexes at various N/P ratios (n=3, error bars represent standard deviation).

toxicity of polymers at various concentrations with three different cell lines. It was found that the Solutol-*g*-PEI showed significantly higher cell viability as compared with no-modified PEI 25 kDa at high concentrations, although the cytotoxicity was highly cell-dependent. Especially, Solutol-*g*-PEI with the higher modified degree showed significantly higher cell viability at various concentrations. The enhanced cell viability is undoubtedly the result of the formation of low-toxic building blocks. The PEI cytotoxicity may be due to the multiple attachment of PEI to the cell surface, thus resulting in the lysis of the cell.[20] In our case, the PEI macromolecules were conjuncted to the Solutol. As a result, the number of possible PEI attachments to cell surfaces might be reduced, thus lowering the cytotoxicity to the cell.

***In Vitro* transfection.** Complexes formed between plasmid DNA and the cationic copolymers were assessed for their transfection activity *in vitro* utilizing a transient expression of luciferase reporter in Hela cell lines used in the cell viability assay. For all cationic copolymers, the efficiency of transfection depended on the N/P ratio. As shown in Figure 6, transfection efficiency of PEI 25 kDa rapidly decreased with an increase of the N/P ratio owing to its simultaneously increasing cytotoxicity; while the transfection efficiency of Solutol-*g*-PEI increased when increasing N/P ratios (except Solutol-1*g*-PEI), which could be explained by the structural compactness of complexes. The decrease of the transfection efficiency of Solutol-1*g*-PEI was possibly related to cell toxicity caused by higher zeta potential. Solutol-*g*-PEI showed higher gene transfer ability compared with PEI 25 kDa. The highest luciferase expression level was obtained at Solutol-2*g*-PEI. Petersen *et al.*[18] reported that the low level of PEG grafting to PEI could enhance transfection, which suggested the important role of the balance between cytotoxicity and zeta potential of the complexes. These are consistent with our results obtained by Solutol-1*g*-PEI and Solutol-2*g*-PEI, whose modification degrees were 0.18% and 0.35%, respectively. Sharma *et al.*[21] reported that the aggregation process of PEI/DNA complexes was completely inhibited by 2.5% polyoxyethylene (100) stearate (POES). POES preserved the transfection efficiency of the complexes without inducing toxicity. That the enhanced transfection efficiency of Solutol-*g*-PEI is higher than PEI 25 kDa is due to the introduction of polyoxyethylene stearate segments with highly cationic PEI. Solutol is a nonionic amphiphilic surfactant with the PEO chain and the stearic acid chain. The PEO chain extends into water and sterically hinders the complexes from approaching each other and can shield the positive charge of surface of complexes. Thus, the PEO chain can reduce toxicity and meanwhile improve the colloidal stability of the polymer/DNA complexes. And stearic acid chain is believed to enhance complexes lipophilicity, which possibly possesses the ability to interact with biological membranes and to enhance the transport of complexes into the cells. Therefore, our results indicated that reducing cytotoxicity as well as increasing hydrophobicity and stability, made newly synthesized Solutol-*g*-PEI more effective in transfection compared with unmodified PEI 25 kDa itself.

Conclusions

In this work, we synthesized a new polymer, Solutol-*g*-PEI, and studied its feasibility as a gene delivery system. Through various physico-chemical methods (gel electrophoresis, size and surface charge measurement), we confirmed that Solutol-*g*-PEI had great ability to form complexes with DNA and suitable physicochemical properties for gene delivery system. Its cytotoxicity was also lower in three different cell lines (Hela, MCF-7, and HepG2) than unmodified PEI 25 kDa. These polymers exhibited much enhanced gene transfer efficiency compared with unmodified PEI 25 kDa itself. In conclusion, this new polymer might be very useful in gene delivery with low cytotoxicity and high transfection efficiency.

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