Lactose-Poly(ethylene Glycol)-Grafted Poly-L-Lysine as Hepatoma Cell-Targeted Gene Carrier

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To investigate the delivery of DNA into cells, lactose-poly(ethylene glycol)-grafted poly-L-lysine (Lac-PEG-PLL) polymers were synthesized as polymeric gene carriers. The new synthetic carriers, varying the substitution ratio of lactose-poly(ethylene glycol) (lactose-PEG), were characterized by NMR spectroscopy and size-exclusion chromatography. Electrophoretic mobility assay confirmed that the new gene carrier makes a complex with plasmid DNA. The attached poly(ethylene glycol) gives better solubility properties to gene/carrier complex. Transfection experiments showed that Lac-PEG-PLL efficiently delivers DNA to a hepatoma cell line in vitro; the best efficiency was achieved at a 1:3 weight ratio of DNA to carrier. As the lactose-PEG substitution content increased up to 30%, the transfection efficiency increased, which demonstrates that the lactose serves as a targeting moiety. No considerable cytotoxicity was observed due to Lac-PEG-PLL or its complex with DNA within the concentration range for this experiment. The use of chloroquine increased transfection efficiency that indicates the involvement of hydrolytic degradation of the system in lysosome. It is likely that plasmid DNA/Lac-PEG-PLL complexes enter the cells through a receptor-mediated endocytosis mechanism. These results show that Lac-PEG-PLL can form a complex with plasmid DNA and serve as an efficient gene delivery carrier with lower cytotoxicity compared to that of poly-L-lysine. Therefore, it is expected that our Lac-PEG-PLL carrier can be used as an in vivo gene delivery vector.

INTRODUCTION

Twenty-five years ago, Friedmann outlined prospects for human gene therapy (*1*). Since then, gene therapy has represented a new paradigm for therapy of human disease and for drug delivery (*2*). The implicit emphasis of the research so far has been on determining the safety of gene transfer procedures, sometimes with efficacy as a secondary goal (*3*). A major technical impediment to gene transfer is the lack of ideal gene delivery systems (*3*). If it were possible to deliver the gene to the appropriate specific cells in sufficient quantities, gene therapy would be efficacious. Currently, very few organs or cells can be specifically targeted for gene delivery. There are many established protocols for transferring genes into cells, including calcium phosphate precipitation, electroporation, particle bombardment, liposomal delivery, viral-vector delivery, and receptor-mediated gene delivery (*4*). Although all of these methods can be used for mammalian cultured cells, there are many difficulties in introducing genes into target cells in vivo.

Transfection methods using retroviral or adenoviral vectors (*5*) permit us to overcome some of these limitations. Retroviral vectors, in particular, have been used successfully for introducing exogenous genes into the genomes of actively dividing cells such that stable transformants are obtained (*6*). All the viral vectors use approaches that involve complementation of defective vectors by genes inserted into helper cell lines to generate the transducing infectious agent. It is well-known that the host immune response to adenoviruses limits their use to a single administration. To address this limitation, fusion peptides of the influenza virus hemagglutinin have been employed to replace adenoviruses as endosomal lytic agents, but with limited success (*7*). However, despite their high transfection efficiency in vitro, inserting genes into the host cell's genome in this method depends on the viral infection pathway. Its application for human gene therapy introduces serious concerns about endogenous virus recombinations, oncogenic effects, and inflammatory or immunologic reactions (*3*, *8*, *9*). Such concerns have limited the use of viral vectors for human gene therapy (*6*).

On the other hand, nonviral gene delivery systems such as cationic liposomes (*8*) or synthetic gene carriers, such as poly-L-lysine (PLL) (*10*), are being widely sought as alternatives (*11*). There are several theoretical advantages of nonviral therapies including their relative safety and cost of manufacture (*2*). No significant toxicity has been encountered in preclinical toxicology studies using several cationic lipid:DNA complexes (*12*, *13*) or in initial human studies (*12*, *14*). Despite a theoretical concern about raising anti-DNA antibodies (*15*), there is no evidence for the formation of antibodies against DNA or antinuclear antibodies after administration of "naked DNA" or DNA formulated with lipids or proteins in clinical gene therapy studies (*12*, *14*, *15*). The major limitation of plasmid-based approaches has been that both the efficiency of gene delivery to several important somatic targets (i.e., liver and lung) and in vivo gene expression level are lower using nonviral approaches than with viral vectors.

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It has been shown previously that the presence of polycations in culture medium can increase in vitro cellular uptake of a variety of substances, for example, albumin (*16*) and nucleic acids (*17*). These effects were shown to be dependent on size and concentration (*16, 17*). The enhancement was found to be most effective with polyornithines, but PLLs also demonstrated considerable activity. Laemmli (*18*) characterized that PLL makes condensates with DNA. Since then, PLL, modified with various substances, was used as a part of gene carrier (*4, 19*−*27*).

Recently, poly(ethylene glycol) (PEG)-grafted PLL (PEG-*g*-PLL) was synthesized in this laboratory, based on the idea that PLL modified with PEG showed high solubility, low cytotoxicity, and enhanced cell permeability. The synthesized PEG-*g*-PLL was shown to make a complex with plasmid DNA and do so with lower cytotoxicity and improved transfection efficiency to that of over PLL (*28*). In addition, Kabanov et al. (*29*) and Wolfert et al. (*11*) have developed A−B type block polycations as carriers for oligonucleotide and gene delivery. For these carriers, one hydrophilic polymer region (PEG) combines with one polycationic polymer region (PLL). This block copolymer forms a complex with DNA, maintaining a low cytotoxicity comparable to the cation alone but with high solubility.

In this paper, the grafted polymers were synthesized by introducing lactose group to the end of PEG first and covalently attaching the carboxylic acid group of lactose-PEG to the ϵ-amino group of PLL. The purpose of using lactose is to target hepatoma cells for gene delivery. The ratio of lactose-PEG and PLL can be adjusted by changing reaction conditions. The synthesized carrier, named Lac-PEG-PLL, formed a complex with pSV-β-gal plasmid DNA. PEG, grafted to PLL, resulted in better solubility and reduced cytotoxicity of the plasmid DNA/carrier complex. The gene transfection efficiency and cytotoxicity of the Lac-PEG-PLL system were investigated and compared to those of DNA complexed with PLL alone. Hep G2 cells were transfected specifically with plasmid DNA/Lac-PEG-PLL complex, indicating that lactose serves as a targeting moiety for a hepatoma cell line.

MATERIALS AND METHODS

Materials. Isobutyl chloroformate (IBCF), *N*,*N*′-dimethylformamide (DMF), triethylamine (TEA), and dimethyl sulfoxide (DMSO) were purchased from Aldrich (St. Louis, MO). PLL-hydrobromide (120 repeating units; MW = 25 000), *p*-aminophenyl-α-D-lactopyranoside, Concanavalin A (Con A)-Sepharose column, methyl-α-D-mannopyranoside, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), ethidium bromide, chloroquine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) were purchased from Sigma (St. Louis, MO). PEG diacid (MW = 550) was bought from Fluka (Ronkonkoma, NY). Plasmid pSV-β-gal, 5X reporter Lysis buffer, and DNA size marker were purchased from Promega (Madison, WI). Hep G2 cells (a human liver carcinoma cell line, ATCC accession no. HB 8065), A7R5 cells (a rat smooth muscle cell line, ATCC accession no. CRL 1444), and NIH3T3 cells (a mouse embryonic cell line, ATCC accession no. CRL 1658) were purchased from ATCC (Rockville, MD). Fetal bovine serum (FBS), minimal essential medium (MEM), Dulbecco's minimal essential medium (DMEM), 0.25% trypsin, and 1× phosphate-buffered saline (PBS) were purchased from Hyclone (Logan, UT). Lipofectin was purchased from GIBCO BRL (Gaithersburg, MD). Bigger Prep DNA purification kit was purchased from 5′-3′ Prime Inc. (Boulder, CO). Dialysis tubings (molecular weight cutoff of 1000 and 25 000) were purchased from Spectrum (Houston, TX).

Synthesis of Lac-PEG-PLL. The reaction scheme for synthesis of Lac-PEG-PLL is shown on Figure 1a. It consists of two reactions, synthesis of lactose-PEG diacid and grafting of lactose-PEG diacid to PLL. The lactose-PEG diacid is synthesized and purified according to a similar reported procedure (*50*). Six hundred milligrams (1.0 mmol) of PEG diacid (MW = 550) was dissolved in 1.5 mL of dried DMF containing 1.0 mmol of TEA in an ice−water bath under nitrogen. One millimole of IBCF dissolved in 1.0 mL of dried DMF was added dropwise, and the mixture was stirred for 15 min in an ice−water bath. IBCF served as the activator of carboxylic acid. Then *p*-aminophenyl-α-D-lactopyranoside (1.0 mmol) dissolved in 1.0 mL of DMF was added, and the mixture was stirred 30 min in an ice−water bath. It was stirred for additional 3 h at room temperature. The product was precipitated in an excess of dry ether (100 mL). Then the precipitate was dissolved in 20 mL of distilled water, dialyzed against distilled water (dialysis tubing with MWCO 1000), and freeze-dried. The product was purified by removing unreacted PEG diacid on a Con A-Sepharose column. Forty milligrams of the lyophilized product was dissolved in 40 mL of 0.02 M Tris buffer (pH 7.4, 0.5 M NaCl, 3 mM $CaCl_2$, and 3 mM $MnCl_2$) and loaded on Con A-Sepharose column at a flow rate of 0.10 mL/min. The column was eluted until absorbance at 280 nm dropped below 0.01, and then identical buffer containing 0.1 M methyl-α-D-mannopyranoside was applied. The specifically eluted peak of lactose-PEG and lactose-PEG-lactose mixture was detected by monitoring at 280 nm, collected, dialyzed against distilled water, and finally freeze-dried. The content of lactose was assayed by UV using *p*-aminophenyl-α-D-lactopyranoside as standard. The content of carboxylic acid was measured by titration. The mole ratio of lactose-PEG to lactose-PEG-lactose was about 2:1.

From the lactose-PEG and PLL, four different grafted copolymers Lac-PEG-PLLs with various mole ratios of lactose-PEG to ϵ-amino groups of PLL (mol/mol %) were further synthesized. As an example, the synthesis of Lac-PEG-PLL with 30% (mol/mol %) lactose-PEG is described. Lactose-PEG and lactose-PEG-lactose mixture (51.1 mg; containing 0.042 mmol of lactose-PEG) was dissolved in 0.5 mL of dried DMF under nitrogen in an ice−water bath. IBCF (0.036 mmol) dissolved in 0.5 mL of DMF was added to the above solution dropwise. The mixture was stirred 30 min in an ice−water bath, then added dropwise to 1 mL of dried DMSO containing 25 mg of PLL-HBr and 10 μL of TEA which had been purged with nitrogen for 10 min in an ice−water bath, then stirred an additional 3 h at room temperature. The product was precipitated in 100 mL of dried ether, and the precipitate was dissolved in 10 mL of distilled water. It was dialyzed (dialysis tubing with MWCO 25 000) first against 0.01 M $NaHCO_3$ solution, second against 0.01 M HCl solution, and finally against distilled water. The dialyzed product was finally freeze-dried. The mole ratios of lactose-PEG to the amino group in PLL were calculated from ^{1}H NMR.

Size-Exclusion Chromatography Lac-PEG-PLL. The relative distribution of Lac-PEG covalentely grafted to PLL was examined by size-exclusion chromatography (SEC) on a analytical Superose 12 column (10 mm × 30 cm) (Pharmacia AB) using FPLC. The column was eluted with 0.05 M PBS buffer (pH 7.3) containing 0.5 M NaCl at a flow rate 0.3 mL/min at 25 °C. A 200 μL sample (2

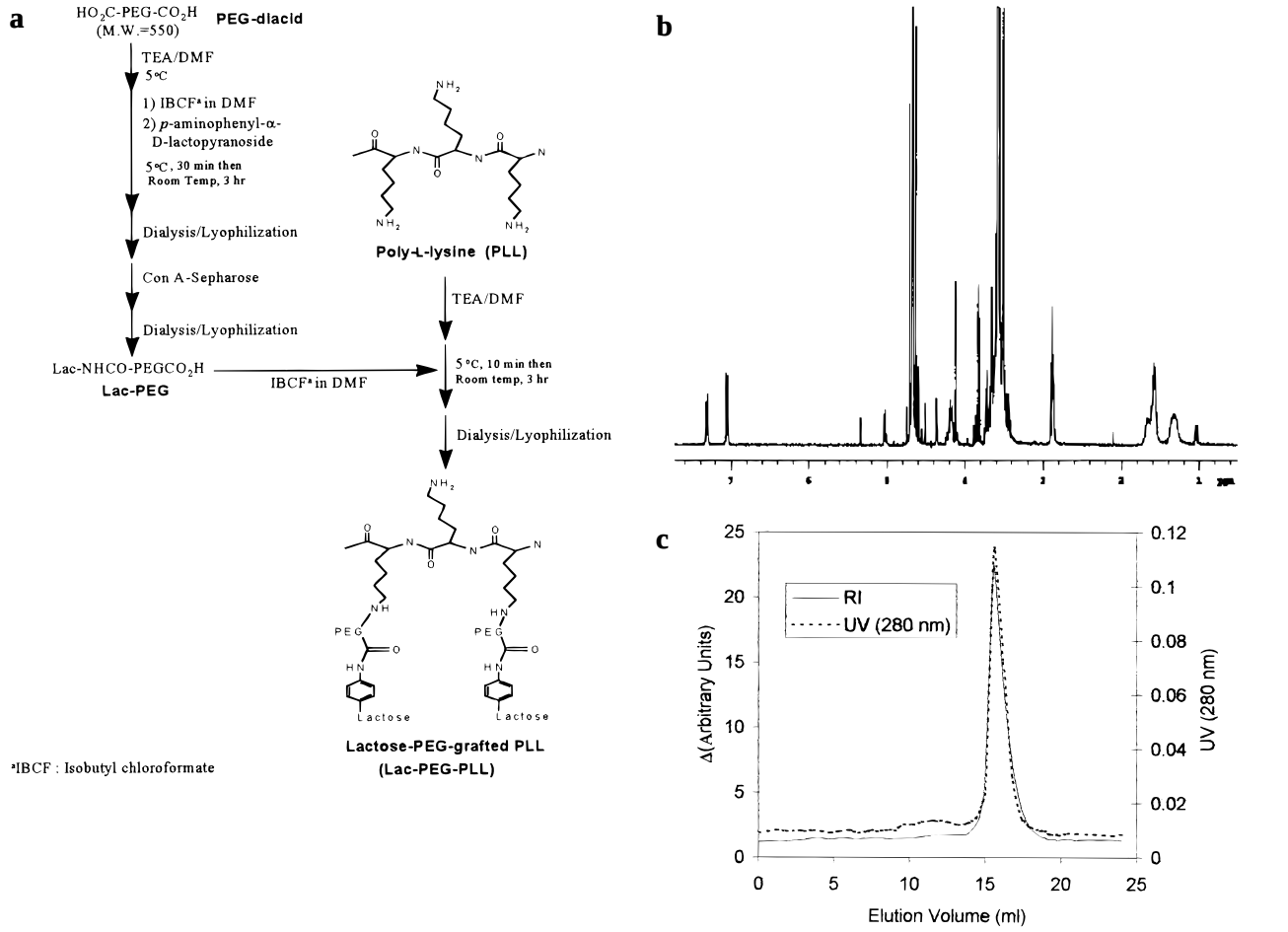

Figure 1. (a) Synthesis scheme of Lac-PEG-PLL. The ratio of lactose-PEG-attached amine group to total amine group was calculated from the peak intensities in the ^{1}H-NMR spectrum. (b) ^{1}H-NMR analysis of Lac-PEG-PLL. The ^{1}H-NMR spectrum of 30 mol % Lac-PEG-PLL was obtained in H_2O. The peak at about 3.5 ppm designates the existence of PEG. (c) SEC of Lac-PEG-PLL on an analytical Superose 12 column. The 30 mol % Lac-PEG-PLL was used. The column was equilibrated with PBS buffer (0.05 M, pH 7.3) containing 0.5 M of NaCl at a flow rate 0.3 mL/min. Sample (200 μL, 2 mg/mL) was injected.

mg of Lac-PEG-PLL/mL) was injected and the refractive index (RI) and A_{280} of the elute were continuously monitored.

Synthesis of PEG-*g*-PLL. The procedure for synthesis of PEG-*g*-PLL was previously described (*28*).

Preparation of pSV-*β*-gal Plasmid DNA. The plasmid pSV-*β*-gal was prepared as previously reported (*28*).

Band Retardation Assay. Various amounts of Lac-PEG-PLL, ranging from 0.1 to 10 μg, were added to 1 μg of plasmid DNA in HEPES buffer (20 mM, 0.15 M NaCl, pH 7.4) and incubated for 30 min at room temperature. Gel electrophoresis sample buffer was added to each sample, and it was electrophoresed on 1% (w/v) agarose gel for 90 min at 100 V using the Easy-Cast Electrophoresis System (Owl Scientific Inc., Woburn, MA). TBE (45 mM Tris-Borate and 1 mM EDTA, pH 8.0) buffer was used as electrophoresis buffer. The gel was stained with ethidium bromide (0.5 μg/mL) for 30 min and illuminated with an UV illuminator to show the location of the DNA. Lambda DNA, which was digested by *Hin*dIII, was used as a DNA size marker.

Solubility Test. The solubility of the carrier/plasmid DNA complex was determined by a method which was originally developed by Wadhwa et al. (*4*) with a slight modification. pSV-*β*-gal plasmid DNA and carrier were mixed in 1 mL of HEPES buffer (20 mM and 0.15 M NaCl, pH 7.4) with final concentration of 50 μg/mL in 1.5 mL centrifuge tubes. After incubation for 30 min at room temperature, the tubes were centrifuged for 5 min at 10 000 rpm. The supernatant was taken, and its absorbance at 260 nm was measured for the determining of the DNA remaining in solution. A DNA sample without carrier was used as a standard.

Cell Culture. Hep G2 cells were maintained in MEM medium supplemented with 1 mM sodium pyruvate and 10% FBS at 37 °C in a 5% CO_2 Napco incubator (Precision Scientific Inc., Chicago, IL). A7R5 cells were similarly maintained in DMEM medium supplemented with 10% FBS. NIH3T3 cells were likewise maintained in DMEM medium supplemented with 4.5 g/L glucose and 10% FBS. Cells were normally grown in 25 cm^2 (for Hep G2 cells) or 75 cm^2 (for the A7R5 and NIH3T3 cells) polystyrene tissue culture flasks (Becton Dickenson, Franklin Lakes, NJ), until they became approximately 80% confluent as assessed by light microscopy.

Transfection. In vitro transfection was performed as follows. For the transfection and cytotoxicity studies, Hep G2 cells were seeded at a density of 2×10^4 cells/each well in 96-well flat-bottomed microassay plates (Becton Dickenson, Franklin Lakes, NJ). The cells were incubated for 24 h before the addition of either the plasmid DNA/carrier complex or the carrier only. For A7R5 and NIH3T3 cells, the seeding concentration was 5×10^3 cells/well and 2×10^4 cells/well, respectively.

All reagents used in the transfection experiments were sterilized by filtering through 0.22 μm polycarbonate

membrane filters. Plasmid pSV-β-gal/Lac-PEG-PLL complexes were prepared by mixing 1 μg of pSV-β-gal and various amounts of carrier in 100 μL of FBS-free cell culture medium and incubated for 30 min at room temperature. FBS and chloroquine were added at a final concentration of 10% (v/v) and 100 μM, respectively. Medium from each well of the 96-well plate was replaced with 100 μL of transfection mixture. The cells were then incubated for 4 h at 37 °C in a 5% CO_2 incubator. After 4 h, the transfection mixtures were removed and 100 μL of fresh medium containing 10% FBS was added to each well. Cells were incubated for an additional 48 h at 37 °C.

Assay for Expressed β-Galactosidase. The β-galactosidase activity in transfected cells was determined spectrophotometrically at 405 nm using ONPG with a slight modification (*30*). Briefly, the growth medium was removed from the cells in 96-well plates to be assayed, and the cells were washed twice with 1× PBS carefully to prevent adherent cell detachment. Cells were lysed by adding 60 μL of 1X reporter lysis buffer (Promega, Madison, WI) to the cells and incubating for 30 min at room temperature, rocking the plate halfway through the incubation period, followed by scraping the cells. Sixty microliters of ONPG (1.33 mg/mL) in 2× assay buffer (Promega, Madison, WI) was added to the lysed cells, and the cells were incubated at 37 °C for 4 h. The reaction was terminated by adding 150 μL of 1 M sodium carbonate solution to each well and the absorbance at 405 nm was read with a Bio-Tek EL-311, the microplate reader (Bio-Tek Instrument Co., Winooski, VT) for the β-galactosidase activity.

Cell Viability Assay. Evaluation of cytotoxicity was performed as previously reported (*28*).

RESULTS

Synthesis of Gene Carriers. The synthesis of Lac-PEG-PLL consists of two reactions, synthesis of lactose-PEG diacid and synthesis of comb-shaped polymer Lac-PEG-PLL from lactose-PEG diacid and PLL. In the first reaction, IBCF and a carboxylic acid in PEG diacid formed a mixed anhydride, and a molecule of hydrochloric acid was released. TEA had a role as a base to neutralize hydrochloric acid which is formed during this reaction. The amino group in *p*-aminophenyl-α-D-lactopyranoside reacted with the carbonyl atom carbon on PEG-side, and an amide bond was formed between the sugar and PEG. After a 3.5 h reaction, the products were precipitated in an excess of ether. The precipitates were dissolved in distilled water and dialyzed using a dialysis membrane, 1000 MWCO, to remove unreacted *p*-aminophenyl-α-D-lactopyranoside. Con A-Sepharose column was used to remove unreacted PEG. Then lactose-PEG diacid was dissociated from the Con A-Sepharose column when 0.1 M methyl-α-D-mannopyranoside-containing buffer was applied. In that fraction, lactose-PEG-lactose, which means both carboxylic acid sides of PEG diacid had been modified to lactose moiety, was also eluted with lactose-PEG diacid, but no further purification was required because lactose-PEG-lactose will not take part in the second reaction. The content of carboxylic acid is determined by titration (*50*). Also, the lactose-PEG-lactose (MW = about 1400) was removed completely during the dialysis in the second reaction. In the second reaction, the carboxylic acid was activated by IBCF to form a mixed anhydride then the anhydride reacted with amino group on PLL.

The ^{1}H-NMR spectrum of 30 mol % Lac-PEG-PLL is shown on Figure 1b. The content of PEG was calculated from the NMR spectrum by relating the PEG ($-CH_2-CH_2-$, *s*, 3.4−3.7 ppm) peak and side chain of PLL ($-CH_2CH_2CH_2-$, *m*, 1.1−1.8 ppm) peak. From this method, the lactose-PEG content ratios of the four carriers were verified as follows: 6, 12, 20, and 30 mol %, respectively.

The size exclusion chromatography data of Lac-PEG-PLL is shown in Figure 1c. From the peak number and peak shape, we can see that grafted polymer Lac-PEG-PLL is chromatographically homogeneous and that the RI and A_{280} signal ratio was practically constant, which indicated that Lac-PEG was evenly distributed (conjugated) to PLL, and the Lac-PEG-PLL has certain molecular weight. Because the Lac-PEG-PLL is a grafted polymer and it is difficult to find suitable standard molecules, the molecular weight of the Lac-PEG-PLL is not calculated.

Physicochemical Characterization. Experiments were performed to investigate whether the synthesized Lac-PEG-PLL forms complexes with pSV-β-gal plasmid DNA. Figure 2a shows the result from the band retardation assay using Lac-PEG-PLL carrier for condensation with pSV-β-gal. A fixed amount of pSV-β-gal plasmid was titrated with increasing amounts of Lac-PEG-PLL resulting in complexes. The movement of pSV-β-gal (bands on lane 2) was retarded as the amount of Lac-PEG-PLL in a complex increased, suggesting that Lac-PEG-PLL forms a complex with pSV-β-gal and the negative charges of DNA were neutralized by the positive charges of Lac-PEG-PLL (lane 3−9). The pSV-β-gal/Lac-PEG-PLL complexes in lanes 6−9 did not enter the gel and show much weaker band in fluorescence intensity. It is most likely due to exclusion of ethidium bromide following formation of complex. Complete retardation was achieved at and above a 1:1 weight ratio of pSV-β-gal:Lac-PEG-PLL.

Plasmid DNA/carrier complexes were defined as soluble if centrifugation under the conditions selected failed to remove DNA from the supernatant. Figure 2b shows that complexation between pSV-β-gal and PLL at a final DNA concentration of 50 μg/mL led to the formation of a fine precipitate which sedimented upon centrifugation at 10 000 rpm for 5 min. However, when the PEG-modified PLL was used to make complex with pSV-β-gal, nearly 98% of the DNA remained in the supernatant after centrifugation. The modification of the end of the PEG group with lactose moiety did not give more than a 10% decrease when compared to that of pSV-β-gal/PEG-*g*-PLL complexes. So the attached PEG groups on PLL prevent the pSV-β-gal/carrier complexes from making fine precipitates and becoming insoluble.

Transfection and Cytotoxicity. The transfection efficiency of the synthesized Lac-PEG-PLL was evaluated using in vitro Hep G2 cell line. Hep G2 cell, a hepatoma cell line, has a specific lactose receptor on its surface. For the transfection experiments, various pSV-β-gal/Lac-PEG-PLL complexes were formulated with a fixed amount of pSV-β-gal (10 μg/mL) and increasing amounts of Lac-PEG-PLL (1−100 μg/mL). The cell culture media without FBS was used as a mixing medium.

The Lac-PEG-PLL showed transfection ability into Hep G2 cells (Figure 3). Below 1:1 weight ratio of pSV-β-gal/Lac-PEG-PLL (0.3 or 0.5 μg carriers), very weak or no transfection efficiency was observed regardless of using any lactosylated carrier. At 1:2 and 1:3 weight DNA/carrier ratios, those carriers with higher lactose quantity showed higher efficiency. The 30 mol % Lac-PEG-PLL was the best carrier among four lactosylated carriers at both of these ratios. This may be due to the targeting

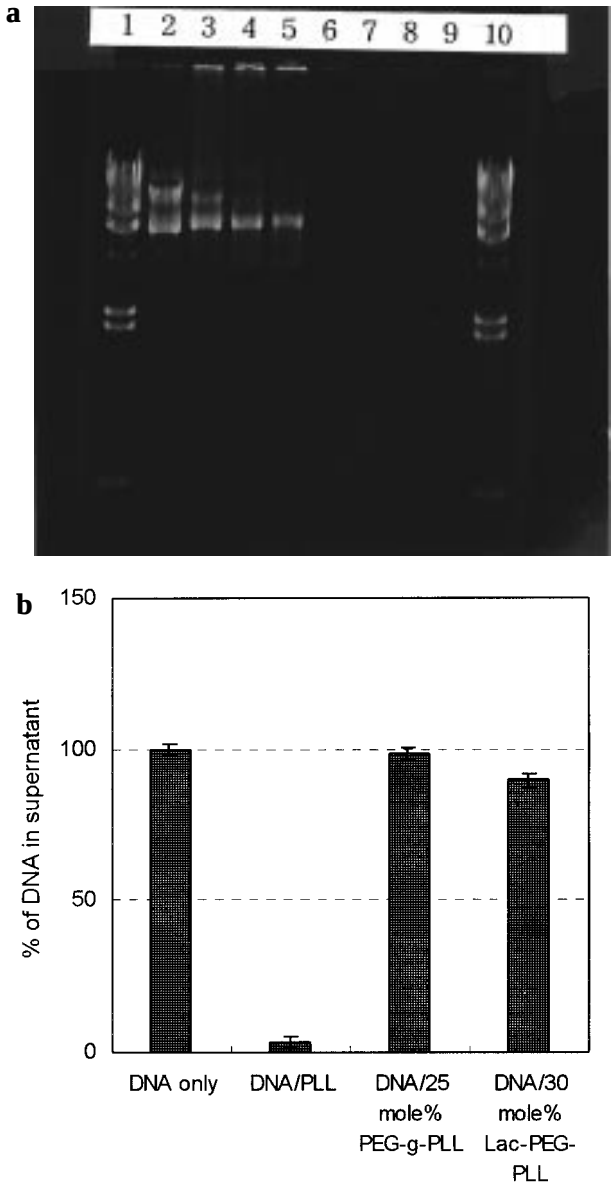

Figure 2. (a) Band retardation assay using Lac-PEG-PLL. Thirty mol % Lac-PEG-PLL was used. The band retardation showed that complete retardation was achieved at and above 1:1 weight ratio of plasmid DNA:Lac-PEG-PLL. The MWs of DNA size markers are 23 130, 9416, 6682, 4361, 2322, 2027, 564, and 265. Lanes 1 and 10: molecular weight marker. Lane 2: 1 μg of plasmid DNA. Lane 3: 1 μg of plasmid DNA + 0.1 μg of Lac-PEG-PLL. Lane 4: 1 μg of plasmid DNA + 0.3 μg of Lac-PEG-PLL. Lane 5: 1 μg of plasmid DNA + 0.5 μg of Lac-PEG-PLL. Lane 6: 1 μg of plasmid DNA + 1.0 μg of Lac-PEG-PLL. Lane 7: 1 μg of plasmid DNA + 3.0 μg of Lac-PEG-PLL. Lane 8: 1 μg of plasmid DNA + 5.0 μg of Lac-PEG-PLL. Lane 9: 1 μg of plasmid DNA + 10.0 μg of Lac-PEG-PLL. (b) Solubility test. Plasmid pSV-B-gal and carrier were mixed in 1 mL of HEPES buffer (20 mM, 0.15 M NaCl, pH 7.4) with final concentration of 50 μg/mL. After incubation for 30 min at room temperature, the tubes were centrifuged for 5 min at 10 000 rpm. The supernatant was taken and its absorbance at 260 nm was measured.

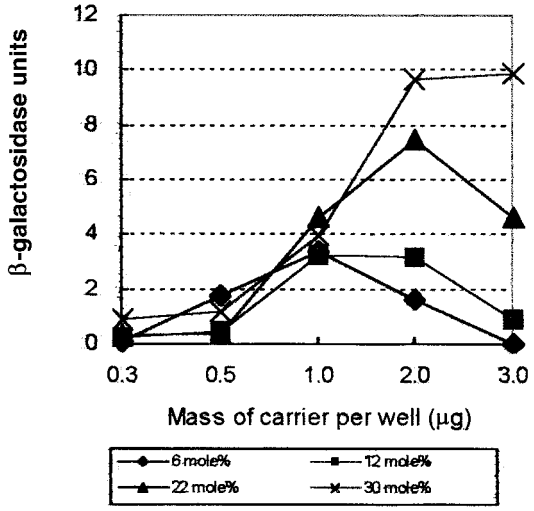

Figure 3. Optimization of transfection using different lactose-PEG substitution degree of Lac-PEG-PLL. "6 mol %" means 6 mol % Lac-PEG-PLL. The amount of each Lac-PEG-PLL was varied from 0.3 to 3.0 μg with 1 μg of pSV-B-gal in the presence of 10% FBS and 100 μM chloroquine. After subsequent culture for 48 h, the cells were lysed and assayed for β-galactosidase activity.

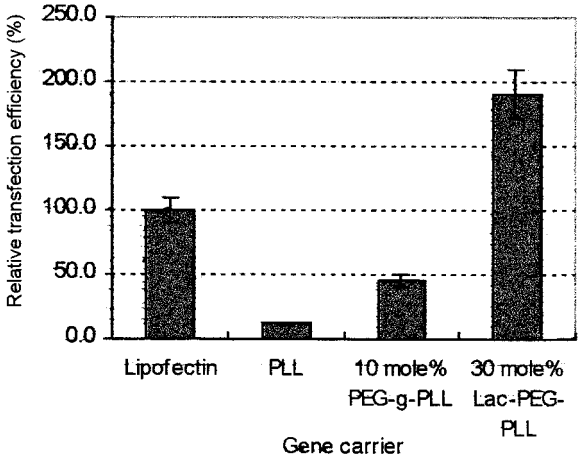

Figure 4. Comparison of transfection efficiencies of different polymeric gene carriers in Hep G2 cells. The concentration of gene carrier (except for lipofectin) and pSV-β-gal were 3 μg and 1 μg/well of 96-well plate, respectively (30 μg/mL and 10 μg/mL). The concentration of lipofectin was 1 μg/mL. Error bar shows standard deviation from six experiments.

ability of lactose to Hep G2 cells, which reveals a rather different tendency from the case of PEG-*g*-PLL. As previously reported, 10 mol % PEG-*g*-PLL showed a higher efficiency than 25 mol % PEG-*g*-PLL (*28*). Using 30 mol % Lac-PEG-PLL carrier, the highest transfection efficiency was achieved at 1:3 weight ratio, but the 1:2 weight ratio revealed transfection of almost the same magnitude. It was shown that the higher the lactose-PEG substitution degree in the carrier, the greater the quantity of carrier required to reach the highest transfection efficiency. When we used 6 mol % Lac-PEG-PLL, a 1:1 ratio complex showed the highest efficiency. In the case of 30 mol % Lac-PEG-PLL, 1:3 ratio complex showed the highest efficiency. This may be explained by the ability of the gene carrier to make complexes with pSV-β-gal. Higher substituted Lac-PEG-PLL has less free amine to interact with negative charge on DNA. Therefore, more carrier is needed to make complex with DNA to the same extent.

Figure 4 summarizes the results for the transfection using PLL and modified PLL. Lipofectin was used for the comparison of transfection efficiency. Among the 3 kinds of carrier—PLL, PEG-*g*-PLL, and Lac-PEG-PLL—and lipofectin, Lac-PEG-PLL showed the best efficiency for Hep G2 cells.

Cytotoxicity of the synthesized carriers for Hep G2 cells is shown in Figure 5. Hep G2 cells were incubated in the presence of 30 μg/mL of gene carrier for 4 h. The 30

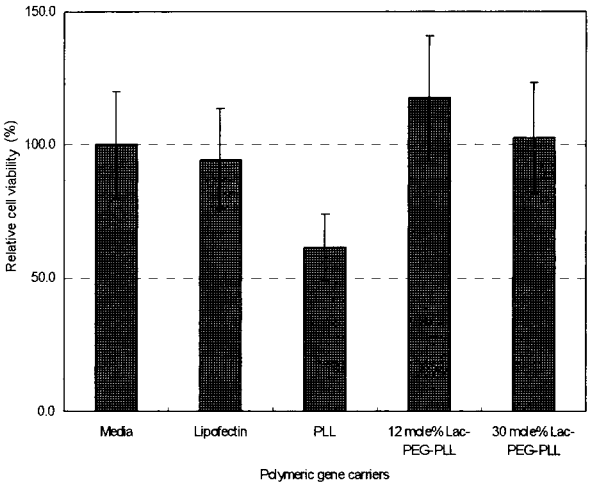

Figure 5. Comparison of cytotoxicity of various gene carriers in Hep G2 cells. The concentration of gene carrier was 3 μg/well of 96-well plate (30 μg/mL in cell culture medium). The gene carriers were incubated for 4 h with cells at 37 °C, then cell viability was determined using MTT assay method.

μg/mL concentration was chosen because PEG-*g*-PLL and Lac-PEG-PLL showed the best transfection efficiency at that concentration. For Lac-PEG-PLL, no cytotoxicity was observed in Hep G2 cells, whereas PLL showed moderate to high cytotoxicity. The difference in lactose-PEG substitution ratio gave no significant change in cell viability. It was further supported by the phase contrast microscopic observation that the incubation of Lac-PEG-PLL with Hep G2 cells did not give any significant influence in cell viability. The incubation of PLL for 4 h with the cells transformed cell morphology, but PEG-*g*-PLL or Lac-PEG-PLL at the same concentration and incubation time did not significantly alter the morphology (data not shown).

To investigate the function of attached lactose moiety, the competition experiments with free sugars were performed. The pSV-β-gal/Lac-PEG-PLL complexes were formed and transfected into Hep G2 cells on the presence of free sugar for 4 h. After this, the transfection mixture including free sugar was removed and the cells were incubated for additional 48 h. Four sugars were used: galactose, glucose, lactose, and lactobionic acid. In the competition using free lactose, 0.5 mM lactose lowered the transfection efficiency up to 61.3% compared to that of no lactose (Figure 6a). Lactobionic acid was also used because of its greater solubility than lactose. Lactobionic acid showed a very close competition ability to that of lactose within the experimental range of lactose competition (0.01−0.05 mM). When the concentration of lactobionic acid was raised to 10 mM, the transfection was inhibited more than 80%. Over the concentration of 50 mM of lactobionic acid, almost no transfection activity was observed to Hep G2 cells, but the cell viability was also dramatically dropped. Below 10 mM concentration, free lactobionic acid did not give considerable cytotoxic effect to Hep G2 cells. Galactose and glucose also showed blockage of transfection derived from pSV-β-gal/Lac-PEG-PLL complex, though their blocking strengths were lower than that of lactose or lactobionic acid. It can be concluded from these observations that the pSV-β-gal/Lac-PEG-PLL complex is delivered to Hep G2 cells via a receptor-mediated endocytosis mechanism using lactose receptors existing on the cells.

We also tried to use Lac-PEG-PLL carrier as the gene delivery carrier to other cell lines. The cell lines that we used were A7R5 (a rat smooth muscle cell line) and

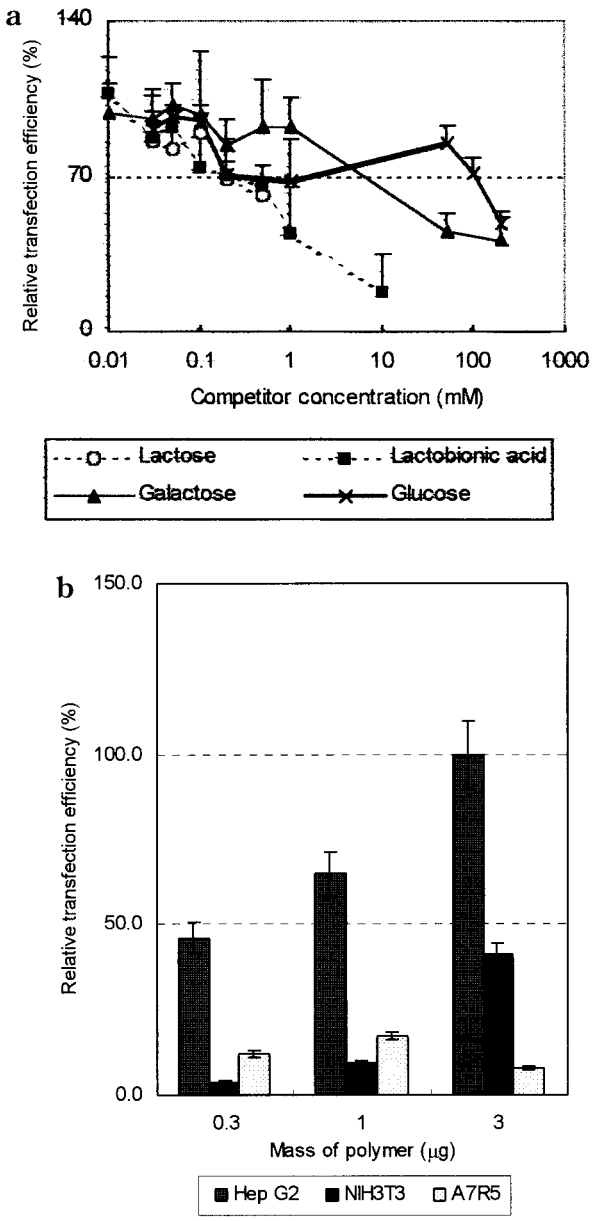

Figure 6. (a) Competition experiment with free sugar molecule. After making pSV-β-gal/Lac-PEG-PLL complex in medium, noted concentrations of each competitor were added, followed by incubation with Hep G2 cells for 4 h in the presence of 10% FBS and 100 μM chloroquine. Error bar shows standard deviation from six experiments. (b) Comparison of transfection efficiency on various cell lines. ONPG β-galactosidase enzyme assay was used for expressed enzyme quantity. The amount of enzyme at 1:3 plasmid DNA/Lac-PEG-PLL ratio (w/w), on Hep G2 cells was regarded as 100%.

NIH3T3 (a mouse embryonic cell line). Neither cell line showed detectable or remarkable transfection results (Figure 6b), regardless of the degree of cytotoxic effect. This result suggests that the lactose serves as a targeting moiety for a specific gene delivery system.

Figure 7a shows the effect of chloroquine, an endosomal disrupting agent, to the transfection efficiency. In the absence of chloroquine, the transfection efficiency of pSV-β-gal/Lac-PEG-PLL complex on Hep G2 cells was as low as that obtained by PLL. As the concentration of chloroquine increased up to 100 μM, the transfection efficiency from pSV-β-gal/Lac-PEG-PLL complex increased (Figure 7a). When 150 or 200 μM chloroquine was used, the decrease in galactosidase activity was

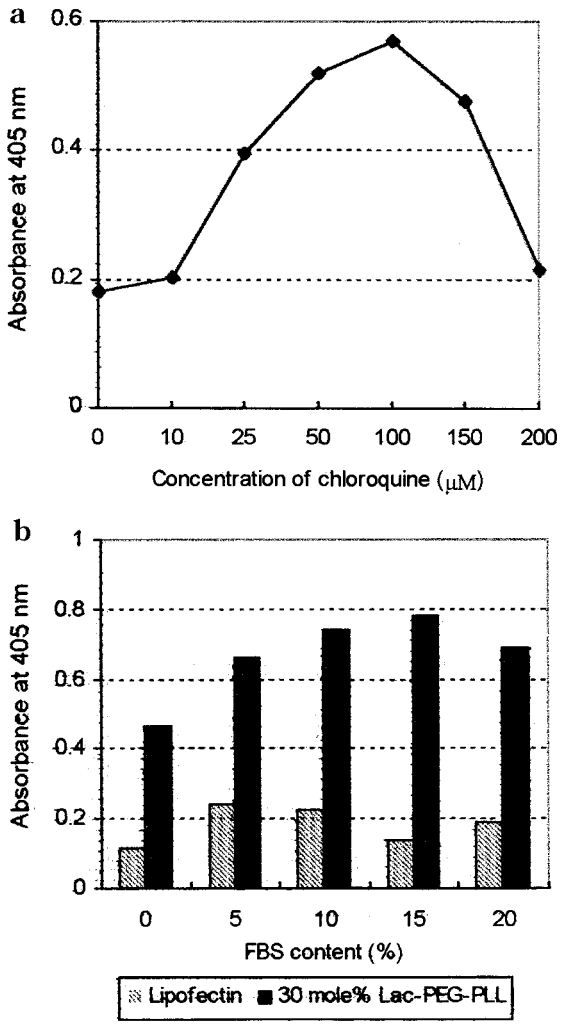

Figure 7. (a) Influence of chloroquine on gene transfection. Thirty mol % Lac-PEG-PLL was used as a gene carrier. The chloroquine at noted concentrations was added to the transfection mixture for 4 h, in the presence of 10% FBS, and the cells were incubated further for 48 h prior to assay for β-galactosidase. (b) Influence of FBS on gene transfection. Either lipofectin or 30 mol % Lac-PEG-PLL was used as a gene carrier. The FBS at noted concentrations was added to the transfection mixture for 4 h, in the presence of 100 μM of chloroquine, and the cells were incubated further for 48 h prior to assay for β-galactosidase.

observed. It is probable because large amounts of chloroquine were toxic to the cells and a morphological study showed granulated cells. From the results, it is likely that pSV-β-gal/Lac-PEG-PLL complexes enter the cells through an endocytosis mechanism.

The influence of FBS on transfection efficiency was investigated. Figure 7b shows that the presence of FBS in the transfection mixture increased transfection efficiency. The pSV-β-gal/Lac-PEG-PLL system showed about a 1.7-fold increased transfection efficiency on Hep G2 cells in the presence of 10% FBS. The best efficiency was achieved when 15% of FBS was included during the incubation of the transfection mixture with Hep G2 cells. It is not fully understood yet why transfection efficiency is decreased a little when 20% of FBS was used. It is possibly due to binding of the serum components to polycations (*31*). Lipofectin did not show any dependence of transfection efficiency on the concentration of FBS.

We also investigated the influence of other factors of in vitro transfection such as quantity of pSV-β-gal, incubation time with pSV-β-gal/carrier complex, and the

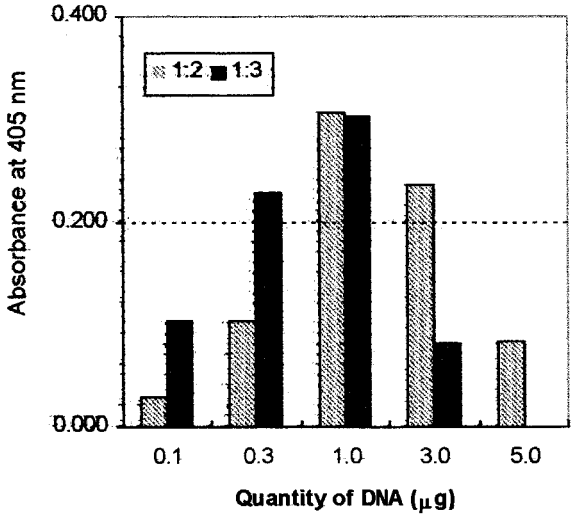

Figure 8. Optimization of pSV-β-gal quantity on transfection. Thirty mol % Lac-PEG-PLL was used. The amount of pSB-β-gal was varied from 0.1 to 5 μg with the same 1:2 or 1:3 ratio of pSB-β-gal to Lac-PEG-PLL, in the presence of 10% FBS and 100 μM chloroquine. After subsequent culture for 48 h, the cells were lysed and assayed for β-galactosidase activity.

time course of protein expression. Figure 8 shows the influence of DNA quantity. One microgram of pSV-β-gal is typically used for the transfection into cells which were grown in a well of 96-well microplate. It was already shown that at 1:2 and 1:3 ratio of pSV-β-gal/Lac-PEG-PLL complex the best transfection efficiency was achieved using 1 μg of pSV-β-gal. Therefore, we tried to optimize the quantity of pSV-β-gal per well. The results showed that the 1 μg of pSV-β-gal DNA is the best dose for a well of 96-well microplate on Hep G2 cells. It is very interesting that the pSV-β-gal/Lac-PEG-PLL complex at 1:3 ratio showed better efficiency than one at 1:2 ratio below 1 μg of pSV-β-gal dose, while the latter proved to be better over 1 μg of pSV-β-gal dose.

The optimal incubation time of the complex with Hep G2 cells was also determined (Figure 9a). It was shown that 4 h of incubation achieved the best transfection efficiency. The expression of β-galactosidase along the time course after transfection was also monitored up to 96 h (Figure 9b). The result showed that the expression of β-galactosidase in Hep G2 cells, which were transfected with pSV-β-gal/Lac-PEG-PLL complex, was maintained up to 96 h as a linear fashion. The synthesized Lac-PEG-PLL has ability to make complexes with DNA and showed enhanced transfection efficiency into Hep G2 cells.

DISCUSSION

Receptor-mediated gene delivery has its advantages and limitations (*32*). Its advantages for use in gene therapy are as follows. First, the gene delivery carrier can be designed and customized for a specific target receptor. Second, the DNA does not have to integrate into the host cell genome to be expressed. Third, the delivery system is theoretically unlimited by the size of the transgene. Finally, the technique does not involve the use of potentially infectious agents. There are also disadvantages which must be overcome before this procedure can be routinely used for human gene therapy. For example, the transgene is not integrated into the host cell chromosomes so that its expression is transient. Therefore, it will most likely be necessary to subject patients to multiple injections of a gene of interest. The

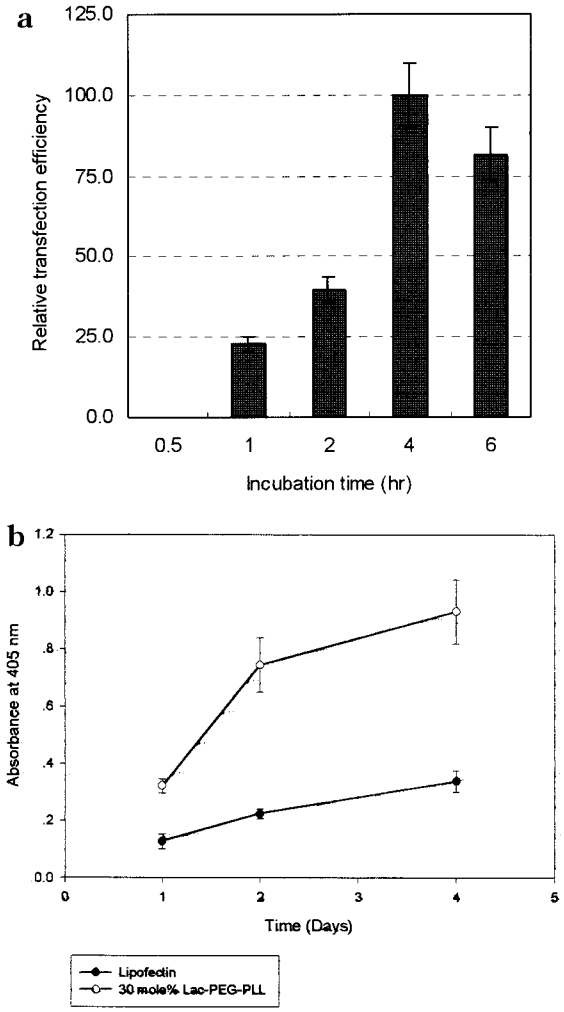

Figure 9. (a) Influence of the time of transfection. Thirty mol % Lac-PEG-PLL was used. The amount of pSV-β-gal was 1 μg with the 1:3 ratio of pSV-β-gal to Lac-PEG-PLL. The transfection time was examined from 0.5 to 6 h in the presence of 10% FBS and 100 μM chloroquine. After each time, the Hep G2 cells were incubated for 48 h, followed by β-galactosidase assay using ONPG. (b) Influence of the time of gene expression. Thirty mol % Lac-PEG-PLL was used as a gene carrier. The amount of pSV-β-gal was 1 μg with the 1:3 ratio of pSV-β-gal to Lac-PEG-PLL. After 4 h incubation of pSV-β-gal/Lac-PEG-PLL with Hep G2 cells, the transfection medium was removed and the cells were incubated. At the noted times, the cells were lysed and assayed for β-galactosidase activity.

DNA-ligand complexes are difficult to prepare and, until recently, little was known about their structure−function relationship. Also there is only a fragmentary understanding of the biological process involved in the transfer of the transgene into the cell and its subsequent expression. These and other features of this system for gene therapy have recently been reviewed in detail (*33*).

PLL itself can be used as a DNA condensate (*18*), but the use of PLL as a gene delivery carrier has several disadvantages. First, its transfection efficiency is very low because PLL has no functional group except the amine group used in charge neutralization. Also due to the negative charges of the DNA phosphate backbone, an increase in the degree of charge neutralization of the DNA often results in extensive condensation and the separation of the DNA phase in the form of insoluble compact structures (*32*, *34*, *35*).

Lac-PEG-PLL was synthesized to serve as a nonviral vector. It was used to deliver the pSV-β-gal reporter gene into Hep G2 cells with enhanced transfection efficiency, compared to that of PLL alone. Plasmid pSV-β-gal, which is a modification of pRSV-β-galactosidase vector, is often used as a reporter gene for monitoring gene expression in mammalian cells (*36*). The SV40 early promoter and enhancer drive the transcription of the *lacZ* gene in mammalian cells. β-Galactosidase is an excellent reporter enzyme (*37*) which can be assayed quickly and directly in cell extracts using a spectrophotometric assay.

It was shown that lactose-PEG-modified PLL has the ability to make a complex with pSV-β-gal from band retardation assay (Figure 2a). The shift of the pSV-β-gal/Lac-PEG-PLL band during electrophoresis depends on the pSV-β-gal:Lac-PEG-PLL ratio that alters the complex net charge as well as its size and density (*9*). The decrease in the electrophoretic mobility of pSV-β-gal/Lac-PEG-PLL complex was accompanied by the increase in the content of Lac-PEG-PLL in the system, and it is due to neutralization of the DNA negative charge by the carrier positive charge. Formation of a DNA−carrier complex was also observed in the dye displacement assay (data not shown).

As discussed earlier, one of the problems of PLL as a gene carrier is that complexation between PLL and DNA frequently results in formation of fine precipitates, limiting the concentration that can be used (*38*). In our experiment, complexation of pSV-β-gal with PLL in 20 mM HEPES (pH 7.4) with 0.15 M NaCl resulted in precipitates at 50 μg/mL of pSV-β-gal. On the other hand, PEG-*g*-PLL and Lac-PEG-PLL maintained the solubility of pSV-β-gal when they made complexes with DNA at this concentration. Therefore, it is apparent that the attached PEG moiety makes pSV-β-gal/gene carrier complexes substantially more soluble.

The gene delivery ability of our synthesized carriers and its specificity were investigated. Hep G2 cells were chosen for specificity test because they have specific lactose receptors on their surface. The recognition of lactose is mediated by the lactose receptors on liver cell (*39*). They are used for removal of glycoproteins from the blood. Many newly synthesized glycoproteins, such as immunoglobulins and peptide hormones, contain carbohydrate units with terminal sialic acid and galactose residues. During hours or days, depending on the particular protein, terminal sialic acid residues are removed by sialylases on the surface of blood vessels. The exposed galactose residues of these trimmed proteins are detected by the asialoglycoprotein receptors in the plasma membranes of liver cells. The complex of the asialoglycoprotein and its receptor is then internalized into the liver cell by endocytosis process to remove the trimmed glycoprotein from the blood. We predicted that the lactose moiety on the carrier could serve as a targeting material for the hepatoma cell line.

Lac-PEG-PLL showed improved transfection efficiency over PLL and PEG-*g*-PLL. Among Lac-PEG-PLLs with different modified ratios, 30 mol % Lac-PEG-PLL accomplished the highest efficiency at 1:3 weight ratio to pSV-β-gal. It demonstrates that the lactose moiety on the end of PEG renders cell targeting ability to pSV-β-gal/Lac-PEG-PLL complexes. In our opinion, the fusogenic ability of PEG may serve as another factor for increase of transfection efficiency. It is supported by the fact that PEG is known to associate with the phospholipid headgroup of cell membranes, assisting modified proteins to penetrate into cell membranes (*40*).

The free sugar competition experiment of transfection using Lac-PEG-PLL emphasizes the probability that pSV-β-gal/Lac-PEG-PLL complexes are delivered into

Hep G2 cells by a receptor-mediated endocytosis mechanism. The increasing concentrations of lactose or lactobionic acid added to the transfection medium decreased the expression of β-galactosidase. Lactose (0.5 mM) and lactobionic acid (1 mM) caused 39.0 and 55.3% inhibition in β-galactosidase activity, respectively. In the case of other sugars, the higher concentration of galactose (50 mM) or glucose (200 mM) is needed to achieve more than 50% inhibition (54.8 and 51.0%, respectively). So lactose has 50-fold more specificity into Hep G2 cells than galactose. Aring et al. (*41*) found that lactosylated human serum albumin (HSA) strongly bound to the hepatic lectins, in contrast to galactosylated and glucosylated HSA. Also, it was reported that mammalian hepatic Gal/GalNAc receptors show preference for three terminal galactose residues in the triantennary ligand (*42*). It means that the spatial arrangement of the Gal residues is very important for optimal binding. Our Lac-PEG-PLL carrier contains multiple lactose groups in it, and may bind to the receptor with greater affinity compared with the monomeric sugars.

It was further supported that the lactose on Lac-PEG-PLL was served as targeting moiety by using different cell lines in transfection. In the A7R5 and NIH3T3 cell line, which do not have lactose receptors on their surface, the transfection efficiencies were much lower than that in Hep G2 cells. Wu and Wu (*20*) used an asialoglycoprotein-PLL conjugate for gene delivery and reported that all receptor (−) cells tested, regardless of the tissue source (SK-hep-1, hepatoma; IMR-90, fibroblasts; uterine smooth muscle cells), failed to undergo gene transformation under the conditions identical to those which were successful for Hep G2 [receptor (+)] cells. Their results further support our concept in which the cell specificity of this new delivery system for gene transformation is based on the presence of unique receptors on the target cells.

That the attached sugar on PEG-*g*-PLL can serve as a targeting moiety onto specific cells can bring up the other possibility for gene delivery. Membrane lectins are present at the surface of many normal cells as well as on various tumor cells, and the sugar specificity is dependent on the cell types (*24*). For example, mannose is recognized by macrophages, liver nonparenchymal cells, and some B cells; mannose-6-phosphate by monocytes; galactose by melanoma cells; glucose by colon carcinoma cells; and *N*-acetyllactosamine by T cells. Many ligands that bind to the target receptor can be used as targeting moieties for gene delivery (for reviews, see ref *43*). Folate is indispensable for health, and therefore, its receptor is expressed on many cell types (*44*). In addition, this receptor is highly expressed on ovarian cancer cells (*45*). Several kinds of cancer cells are known to express the receptors highly for epidermal growth factor (EGF), low-density lipoprotein (LDL), and/or transferrin. Brain capillary endothelial cells express at least three types of receptors, transferrin, insulin, and insulin-like growth factor receptors, implying that these may be a suitable target for delivering gene to the brain (*46*). Fc and polymeric immunoglobulin receptors (pIgR) can be used for targeting the lung. In the lung, the Fc receptor is expressed on macrophages, the pIgR on epithelial cells (*47*).

The efficiency of gene tranefection via receptor-mediated endocytosis can be augmented by the use of pharmacologic agents that disrupt the endocytic trafficking of DNA−ligand complexes (*33*). Chloroquine is known to induce a partial neutralization of acidic cell compartments, to reduce the fusion between endosomes and lysosomes, to decrease intracellular degradation of the internalized plasmid by lysosomal enzymes, and to cause an increase in the volume of endocytic vesicles. In the presence of chloroquine, the DNA carried by Lac-PEG-PLL conjugates could escape degradation by endosome and lysosome hydrolases, allowing a correlative large number of DNA molecules to reach the cytosol and/or the nucleus (*24*). Moreover, chloroquine, which binds to DNA might also protect DNA molecules from nuclease degradation (*25*, *48*). One hundred micromolar concentration of chloroquine was found to be optimal in transfecting Hep G2 cells using pSV-β-gal/Lac-PEG-PLL complexes. At higher concentration (150 and 200 μM), the efficiency was lower in relation to a higher toxicity. It has not been definitely solved how and where DNA gets rid of the carrier chains in cell. Kabanov suggested that negatively charged membranes and/or polynucleotides in a cell may serve as components reacting with DNA/carrier complex and providing for DNA release as a result of carrier substitution (*9*).

Other factors of the in vitro transfection experiment were also optimized. The expression of β-galactosidase along the time course after transfection was also monitored up to 96 h (Figure 9b). The expression was maintained until 96 h. The maintenance of gene expression up to 96 h is an advantage of these synthesized gene carriers for transfection in cultured cells to provide a window for manipulation of transfected cells. As an opposite case, the transfected luciferase activity in human macrophage with mannosylated PLL/DNA was maximal after 24 h, then rapidly diminished to only 2% after 72 h (*49*).

In conclusion, the Lac-PEG-PLL polymers, with four ratios of lactose-PEG substitution, namely 6, 12, 22, and 30 mol %, were synthesized as gene carriers. Our synthetic comb-shaped polymers could make a complex with pSV-β-gal. The attached PEG made the complex soluble, while the complex between pSV-β-gal and PLL alone formed fine precipitate. The Lac-PEG-PLL carrier/DNA complex had more than a 10-fold increase in transfection efficiency when compared to that of a PLL complex on Hep G2 cells. This increase may be due to the cell-targeting effect of lactose moiety and partially from the fusogenic effect of PEG. The attached PEG group also allowed the Lac-PEG-PLL and the pSV-β-gal/Lac-PEG-PLL complex to be less cytotoxic than PLL. The lactose moiety specifically served as a targeting moiety for the transfection into Hep G2 cells, a human hepatoma cell line. This was indicated by two pieces of evidence. First, free lactose inhibited transfection at millimolar concentration. Second, very low transfection efficiency was monitored when other lactose receptor-deficient cell lines were used for transfection. The existence of FBS and chloroquine improved transfection, showing us that the transfection occurred via an endocytosis mechanism. Therefore, the application of these new carriers to animal studies in vivo has been concluded to be promising.

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