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Biodistribution and Hemolysis Study of Terplex Gene Delivery System in Mice

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Abstract: Polymeric gene delivery system attracts profound attention as it shows less toxicity, versatility, and reasonable gene expression efficiency. Terplex system, a synthetic biopolymeric gene delivery system consisting of stearyl poly-L-lysine (stearyl-PLL) and low density lipoprotein (LDL) was evaluated for its body distribution of gene expression of exogenously administered pDNA after tail-vein injection in mice. Kidney and spleen are two major organs with highest gene expression, whereas liver and heart showed marginal gene expression among the organs examined. Hemolytic effect of the terplex system was evaluated using human red blood cells, where terplex system did not cause significant hemolysis at the concentrations above the experimental ranges, although unmodified PLL or stearyl-PLL without LDL did. Serum stability of terplex system against enzymatic degradation was also significantly enhanced, presumably due to the steric stabilization from the polymrers. Based on these findings and along with its high *in vitro* transfection efficiency, terplex system could serve as a safe and efficient polymeric gene delivery system with many applications for the *in vivo* gene therapy.

Keywords: gene delivery, terplex system, stearyl-PLL, biodistribution, hemolysis.

Introduction

Gene therapy is based on the delivery of genetic material into target cells or tissues followed by gene expression. The administration of naked deoxynucleic acid (DNA), though simple and safe, has shown to be rather inefficient due to its anionic charge, size and the presence of nuclease in the blood stream that are able to degrade the DNA before reaching the target site. The use of gene carriers, or gene vectors, has been proposed to circumvent these problems.[1,2] There are two classes of gene carrier systems; virus-based and non-viral gene carrier systems. Viral gene delivery system is currently the most efficient way to transfer genes to cells. However it has big disadvantages such as its toxicity and difficulty in scale up.[3,4] Especially, safety issues have severely limited the application of viral vectors to patients.[5] This has led to the development of more effective and safe gene carrier system for gene therapy. Therefore non-viral vectors are increasingly being considered for *in vivo* gene delivery into the animal models and patients due to simplicity of use, ease of large-scale production and lack of specific immune response but need to be further optimized to reach their full potential.[6]

Among the various non-viral vectors, cationic lipids have been at the forefront of non-viral research. Lipofectin is commercially available lipid mixture of a cationic lipid *N*-1-(2,3-dioleyoxy) propyl-*N,N,N*-trimethyl ammonium chloride (DOTMA) and the co-lipid dioleoylphosphatidylethanolamine (DOPE).[7] Other commonly used cationic lipids include 1,2-dioleoyl 3-trimethyl ammonium propane (DOTAP), 2,3-dioleoyl-*N*[sperminecaroxamino]ethyl]-*N,N*-dimeth-ly-1-propanaminium (DOSPA), 3-[*N*-(*N',N'*-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol) and dimyristoyl oxypropyl dimethyl hydroxyethyl ammonium bromide (DMRIE).[8-11] However, their cationic lipids also exhibited serious limitation such as toxicity on repeated dose. Another important non-viral vectors lie in the use of cationic polymers, including poly-L-lysine (PLL), polyethylenimine (PEI) and polyamidoamide (PAMAN), and their derivatives.[12-16] Targeting ligands were also utilized for site-specific gene delivery. Terplex system which is a non-viral cationic polymeric system based on stearyl-PLL and low density lipoprotein, has been exploited for gene delivery.[17] The supramolecular gene carrier revealed higher transfection efficiency and lower cytotoxicity compared to liposome mediated gene transfer in neural cell lines. Affleck *et al.* demonstrated that this terplex/DNA complex specifically delivered genes into bovine aortic artery wall cells by receptor-mediated endocytosis.[18] This study is to evaluate the biodistribution pattern of gene expression from intravenously injected terplex system into mice and its possible effect on the hemolysis of human red blood cells.

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Experimental

Materials and Animals. Poly-L-lysine (PLL, M_w 50,000 Da), stearyl bromide, low density lipoprotein (LDL), dimethyl sulfoxide (DMSO), glutaraldehyde, osmium tetroxide and hexamethyldisilazane (HMDS) were purchased from Sigma (St. Louis, MO, USA). pCMV-β-gal was purchased from Clontech (Palo Alto, CA, USA). The plasmids were purified using Qiagen Plasmid Mega Kit (Qiagen Sciences, MD, USA) according to the manufacture's protocols. DNase and X-gal were obtained from Promega (Madison, WI, USA). Male ICR mice (6-8 weeks of age) were purchased from Samtaco (Korea).

Synthesis of Stearyl-PLL. Stearyl-PLL was synthesized by *N*-alkylation of poly-L-lysine with stearyl bromide as previously described.[17,19] Briefly, 30 mg of stearyl bromide in a solution of a mixture of 2 mL dioxane and 200 μL 1 N NaOH was added to 100 mg of PLL-HBr in 2 mL dimethylsulfoxide (DMSO). The mixture was allowed to react for 24 h at room temperature. The solution was then poured into a large excess of diethyl ether. The precipitated polymer was dissolved in DMSO and reprecipitated in diethyl ether. The formed stearyl-PLL was finally purified by dialysis against deionized water, followed by lyophilization. Terplex DNA system comprising of DNA/stearyl-PLL/LDL at a 1 : 1 : 1 ratio (wt/wt) with 60 μg of DNA in a total volume of 200 μL in PBS were prepared and incubated for at least 30 min on ice to allow complex formation.

DNase Protection Assay. DNase protection assay was carried out as described previously.[20] Naked or terplex DNA (10 μg) was incubated at 37 °C in the presence of DNase (5 unit) in 500 μL of PBS. One hundred of sample was taken at 0, 2, 5, 10 or 30 min after incubation, mixed with 100 μL of 2X stop solution (0.4 M NaCl, 80 mM EDTA and 2% SDS) and placed on ice immediately. To dissociate the DNA from terplex, the mixtures were incubated at 65 °C overnight. After phenol-chloroform extraction, the DNA was precipitated with ethanol. The pellets were dissolved in 20 μL of Tris-EDTA buffer, and analyzed by 0.8% agarose gel electrophoresis.

Hemolysis Assay. Fresh blood was purchased from the Korea Red Cross Center (Seoul, Korea). Erythrocytes were collected by centrifuging the blood three times in chilled phosphate buffered saline (PBS at 4 °C) at 1000× g for 10 min. The final pellet was resuspended in PBS to give a 2% w/v solution. Using a microtitre plate, 100 μL of the erythrocyte solution was added to dextran, PLL, stearyl-PLL or stearyl-PLL+LDL (1-1000/μg/mL) in a volume of 100 μL. Samples were then incubated for either 1 or 24 h and the microtitre plate was centrifuged then at 1000 × g for 10 min and the supernatants (100 μL) transferred into a new microtitre plate. Hemoglobin release was determined spectrophotometrically using a microtitre plate reader (absorbance at 550 nm). Dextran was used as the negative control and the detergent Triton X-100 (1% v/v) was used to produce 100% hemoglobin release. Results were expressed as the amount of released hemoglobin induced by the polymers as a percentage of the total.

Scanning Electron Microscopy (SEM). Erythrocytes were incubated with polymers (1 μg/mL) for 1 h. The media was then removed and cells were fixed in 0.25% glutaraldehyde for 24 h, followed by 1% osmium tetroxide for 1 h. Samples were then dehydrated by incubation for 5 min in PBS solutions with increasing ethanol contents. Finally they were dehydrated in HMDS and left to dry. After evaporation of HMDS, samples were gold-coated using a MSC-101 magnetron sputter coater set for 1 min at 10 mA, and erythrocytes morphology was visualized using a JSM-5410 scanning electron microscope (Japan).

Animal Study. Terplex DNA complex or naked plasmid DNA (pCMV-β-gal) was intravenously injected into ICR mice (6-8 weeks old) via tail vein at a dose of 60 μg of DNA/mouse in a total volume of 200 μL. At indicated times, mice were sacrificed by cervical dislocation and heart, liver, spleen and kidney were taken out. These organs were rinsed with ice-cold PBS, frozen in liquid nitrogen and embedded in O.C.T compound (Miles Scientific, Naperville, IL, USA). Cryostat sections (10 μm) were sampled at 100-300 μm intervals onto gelatin-coated glass slides and dried at room temperature over night. Preparations were fixed at room temperature in 4% paraformaldehyde in PBS containing 2 mM $MgCl_2$ for 30 min, rinsed with PBS containing 2 mM $MgCl_2$ for a total of 30 min, and incubated at 37 °C overnight in 0.5 mg/mL of X-gal, 10 mM potassium ferricyanide, 10 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.02% Nonidet P-40, and 0.01% sodium deoxycholate in PBS. After staining, sections were counterstained with hematoxylin and eosin. For the assessment of beta-gal expression, X-gal staining was performed on cryostat sections.

Results

Hemolytic Testing *in vitro*. Cationic polymers have been known to interact with negatively charged cell surface.[21,22] To investigate the capability of the polymers to do so with negatively charged red blood cells (RBCs), we monitored the release of hemoglobin from RBCs. As shown in Figure 1, hemolysis was expressed as a percentage of the released hemoglobin induced by Triton X-100 (1% v/v). PLL and stearyl-PLL were used as controls. Polymers were added in a range of 1 ~ 1,000 μg/mL and allowed to interact with the RBC for 1 or 24 h.

After 1 h incubation, stearyl-PLL alone, a control polymer, caused intensive lysis, and the extent of hemoglobin release being dependent on polymer concentration. However, terplex system was not hemolytic up to concentration of 100 μg/mL. At the concentration of 100 μg/mL, stearyl-PLL induced complete liberation of hemoglobin though little lysis

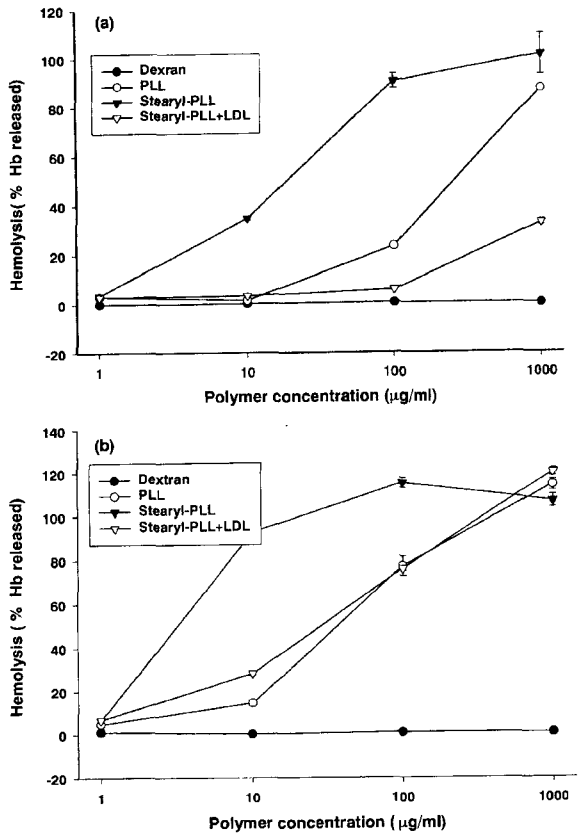

Figure 1. Effect of stearyl-PLL or stearyl-PLL+LDL on red blood cell lysis at (a) 1 h and (b) 24 h. Values are mean ± S.D.(n = 3).

(< 30% lysis) was seen in the case of terplex system.

When the incubation time was extended to 24 h, stearyl-PLL showed almost complete hemolysis even at the lower concentration of 10 μg/mL. However, the terplex system caused about 65% red blood cell lysis only at a very high concentration of 100 μg/mL.

To investigate the morphological changes caused by polymers, RBCs were exposed to either the reference polymers or the terplex system at a concentration of 1 μg/mL. As shown in Figure 2, RBCs exposed to the terplex system showed normal, biconcave disc shape. In contrast, PLL and stearyl-PLL induced morphological changes even at a nonhemolytic concentration. Besides the presence of some intact discocytes, a wide variety of abnormal cells with many spiny protuberances were observed. This distorted morphology of RBCs is thought to be evolved towards hemolysis at higher concentrations.

Stability of the Complexes against DNase I. In order to evaluate the protective effect of complexes on the degradation of DNA by DNase I, naked DNA or terplex DNA complex was incubated with DNase I and analyzed by gel electrophoresis. Naked DNA was completely degraded into small fragments within 2 min of incubation with DNase I. On the other hand, complexation of DNA with stearyl-PLL with LDL protected DNA from DNase degradation (Figure 3). The dominant conformation of plasmid DNA on the gel is linear/open circular type due to harsh conditions for separation of plasmid DNA from the terplex DNA system and a long smear corresponding to small DNA fragments was

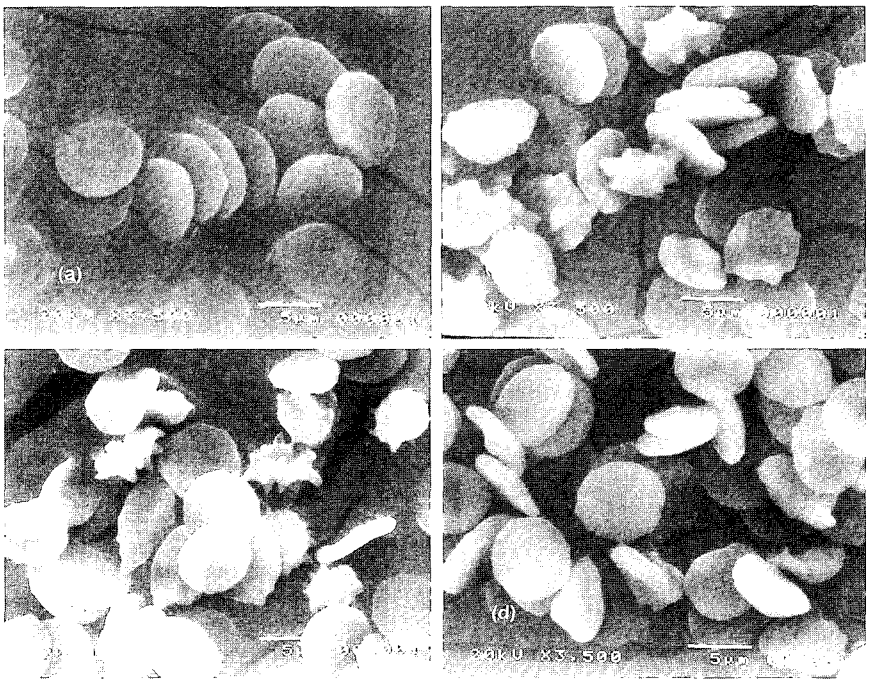

Figure 2. SEM of red blood cells exposed to dextran (a), PLL (b), stearyl-PLL (c), and LDL+stearyl-PLL (d) at a polymer concentration of 1 μg/mL for 1 h.

observed. Nevertheless, plasmid DNA still remained in the terplex DNA samples over 30min after incubation although only a small fraction of plasmid DNA was observed.

***In vivo* Expression of β-gal after Systemic Administration.** β-galactosidase expression plasmid (β-gal) was used as a reporter gene to evaluate the terplex DNA system mediated gene transfer *in vivo* by X-gal staining. After injection of terplex DNA complexes into mice via tail-vein, β-galactosidase expression was detected in the kidney (Figure 4) and spleen (Figure 5) at various time points. However, marginal β-galactosidase activity could be detected in liver and heart tissue(data not shown). The β-galactosidase gene expression with naked plasmid pCMV-β-gal alone was weakly shown only at early time point (at 1 h) in the kidney and spleen. β-gal expression of the terplex DNA in the kidney increased gradually with time and reached a peak in 12 h after injection. In the spleen, β-gal activity was detected 1 h after injection, reaching a peak level 6 h after injection and remained up to 24 h post-injection.

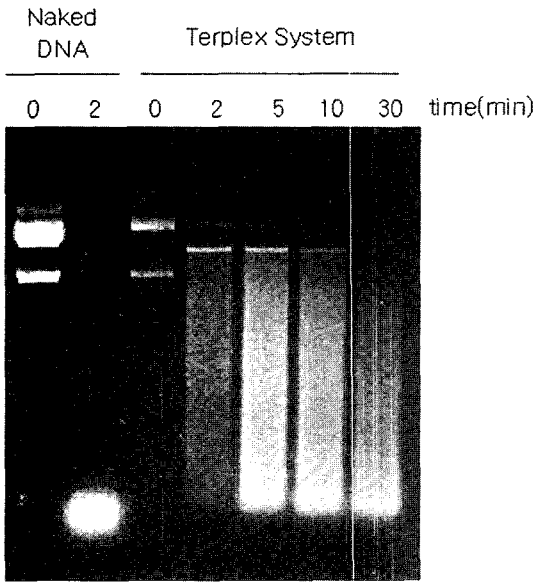

Figure 3. Enzymatic degradation assay using DNase I. The extracted DNA was analyzed on 0.8% agarose gel after 0~30 min incubation with enzyme.

Discussion

Development of synthetic non-viral gene carrier is an emerging area in human gene therapy especially after the death of a patient at the University of Pennsylvania, USA, in 1999 during the adenovirus-mediated gene therapy. Polymeric gene delivery systems are very attractive in a sense that they are easily synthesized and less toxic though gene expression efficiency still remains as a main drawback. As the terplex system was found to be very efficient polymeric gene delivery system *in vitro* previously, it was further evaluated for its *in vivo* biodistribution and the possible toxicity in mice in this study. Non-hemolytic characteristic of terplex

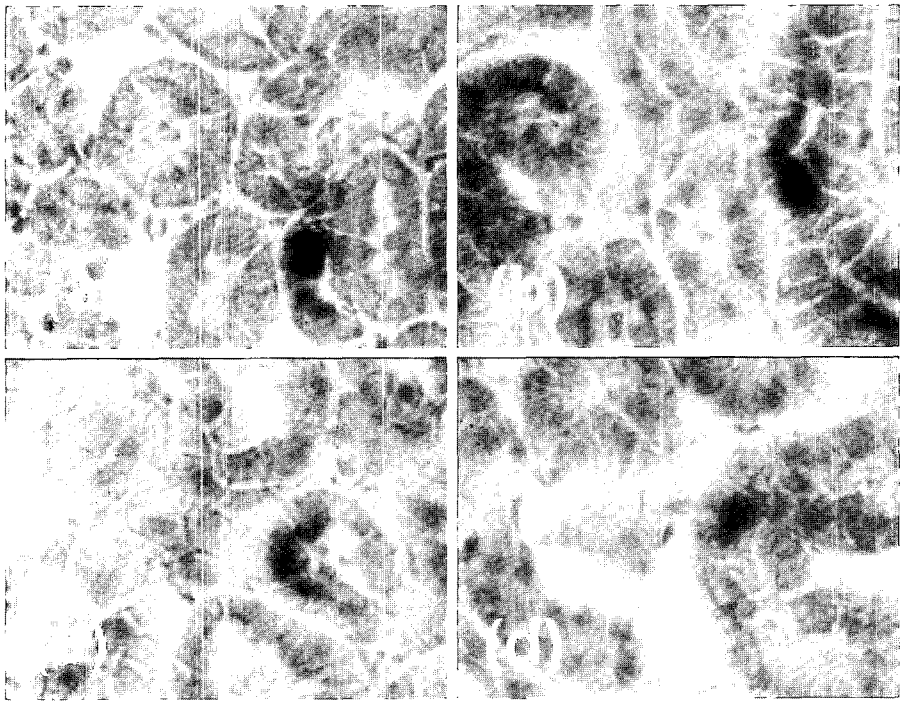

Figure 4. Histochemical analysis of β-Gal expression in kidney at 1 h (a), 6 h (b), 12 h (c), and 24 h (d) after intravenous administration of the terplex DNA into mice (magnificantion, 200×).

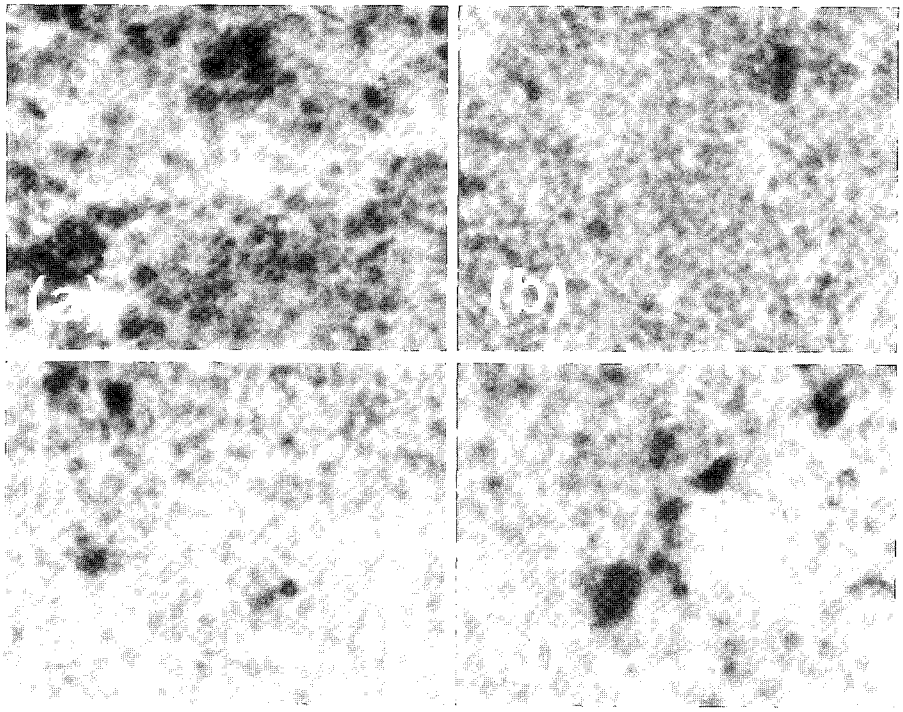

Figure 5. Histochemical analysis of β-Gal expression in spleen at 1 h (a), 6 h (b), 12 h (c), and 24 h (d) after intravenous administration of the terplex DNA into mice (magnification, 400×).

system is very attractive, especially when considering intravenous administration of this system. The presence of LDL in terplex formulation seems to be the main component which prevents this system from hemolysis. The role of LDL in protecting the system from hemolysis is not clear at this moment, however, LDL is thought to coat the surface of cationic polymer (stearyl-PLL) and result in the inhibition of direct interaction of polymer with red blood cells. Biodistribution study indicated that kidney and spleen are two main organs for *in vivo* gene expression after tail-vein injection into mice. But this result seems to be a little bit different from other data published by Yu *et al.*,[23] where liver and heart are the two major organs for gene expression in mice. This difference might have come from the various factors such as injection speed, size and zeta-potential of the terplex system, etc. It is worthy to note that the degree and pattern of both *in vitro* and *in vivo* gene expression varies a lot depending upon the experimental conditions. Further elaboration of optimal formulations for the better gene expression at specific organs in the body is under investigation.

In conclusion, terplex DNA gene delivery system is still a robust and novel artificial gene carrier system both *in vitro* and *in vivo*. Further study will be conducted to evaluate the interaction with LDL-receptors of different organs and the gene expression efficacy in disease animal models.

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