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Liver-targeted siRNA delivery by polyethylenimine (PEI)-pullulan carrier

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ABSTRACT

Recently, small interfering RNA (siRNA)-based therapeutics have been used to treat diseases. Efficient and stable siRNA delivery into disease cells is important in the use of this agent for treatment. In the present study, pullulan was introduced into polyethylenimine (PEI) for liver targeting. PEI/siRNA or pullulan-containing PEI/siRNA complexes were delivered into mice through the tail vein either by a hydrodynamics- or non-hydrodynamics-based injection. The incidence of mortality was found to increase with an increase in the nitrogen/phosphorus (N/P) ratio of PEI/siRNA complexes. Moreover, the hydrodynamics-based injection increased mice mortality. Introduction of pullulan into PEI dramatically reduced mouse death after systemic injection. After systemic injection, the PEI/fluorescein-labeled siRNA complex increased the level of fluorescence in the lung and the PEI-pullulan/siRNA complex led to an increased fluorescence level in the liver. These results suggest that the PEI-pullulan polymer may be a useful, low toxic means for efficient delivery of siRNA into the liver.

1. Introduction

Small interfering RNA (siRNA)-based therapeutics, which are now recognized as a medical approach for the treatment of difficult-to-cure diseases such as viral infections and tumors, are attracting considerable attention in recent times.[1,2] However, naked siRNA is unstable in the bloodstream and is rapidly eliminated through the urinary system. Moreover, its negative charge inhibits efficient cellular uptake due to the negative charge of the cell surface. Thus, efficient and stable siRNA delivery into diseased cells is critical in this treatment modality. Many researchers have attempted to induce various chemical modifications into siRNA or to form complexes with several cationic carriers such as cationic polymers, liposomes, peptides, or proteins.[3–5]

Among cationic polymers, polyethylenimine (PEI) is the most popular synthetic polymer and has a high cationic charge density. It has been widely used to deliver siRNAs into cell lines or tissues. Naked siRNAs are unstable and are rapidly degraded, but PEI is able to form stable complexes with siRNAs, leading to the protection of genes from enzymatic degradation. Moreover, PEI shows a strong buffer capacity over a wide range of pH values; this plays an important role in the escape of genes from the endosome after endocytosis. On the other hand, the high cationic density of PEI allows for the formation of highly condensed complex with siRNAs, but complex formation with PEI can lead to cytotoxicity.[6–10] Information on the safety and biodistribution of PEI or PEI/siRNA complexes both in vitro and in vivo would contribute to improving the safety and efficiency of siRNA delivery using PEI.

In the present study, we introduced pullulan into PEI. Pullulan is a water-soluble polysaccharide consisting of three α-1,4-linked glucose polymers with different α-1,6-glucosidic linkages. It is used for liver targeting because of its high affinity for the asialoglycoprotein receptor in the liver.[11–13] We delivered PEI/siRNA or pullulan-containing PEI/siRNA complexes into mice through the tail vein by a hydrodynamics- or non-hydrodynamics-based injection. The incidence of mortality was found to increase with increasing the nitrogen/phosphorus (N/P) ratio of PEI/siRNA complexes. On the other hand, the introduction of pullulan into PEI reduced mouse mortality and increased liver-targeting efficiency.

2. Results and discussion

2.1. Polymers

A linear 22-kDa PEI was used for the synthesis of the siRNA and PEI-pullulan polymer complex (Fig. 1). The amount of pullulan in

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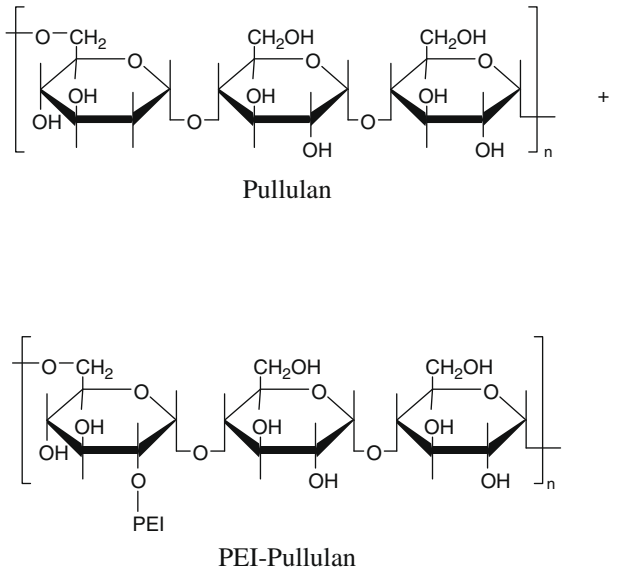

Figure 1. Chemical structure of pullulan and PEI-pullulan. To synthesize the PEI-pullulan polymer, 48.6 mg of pullulan (M_w, 107,000; 0.3 unit mmol) and 24.3 mg of carbonyldiimidazole (CDI; 0.15 mmol) were stirred in 30 mL of anhydrous dimethylsulfoxide (DMSO) at room temperature and then 13.2 mg of linear PEI (M_w, 22 kDa; 0.3 mmol) was added to the mixture.

the polymer was estimated to be 39 mol % and molecular weight of polymer was 2.6×10^5 (see Supplementary data). The zeta potentials of polymer/siRNA complex increased with increasing N/P ratio and showed nearly neutral at N/P ratios of 48 and 96 (see Supplementary data).

2.2. Measurements of complex diameters

The complexes of polymer and siRNA were prepared at several N/P ratios (1.5, 3, 6, 12, 24, and 48) and were determined using a Zetasizer. The particle size decreased with increasing N/P ratio. PEI/siRNA complexes showed <200 nm for all N/P ratios, whereas PEI-pullulan/siRNA complexes with ratios of 12 to 48 were <200 nm (Fig. 2).

2.3. Electrophoresis of the polymer/siRNA complex

Polymers were mixed with siRNA at several N/P ratios. The complexes were analyzed by electrophoresis. Bands corresponding

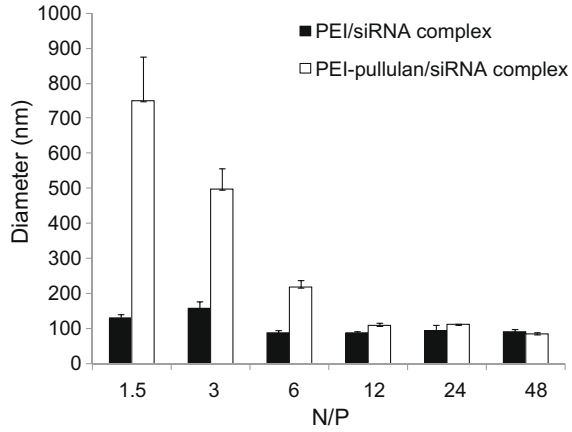

Figure 2. Diameter of the PEI/siRNA or PEI-pullulan/siRNA complexes. Polymer and siRNA complexes were simply prepared by incubating siRNA and polymer in water. The diameters of the complexes were determined using a Zetasizer.

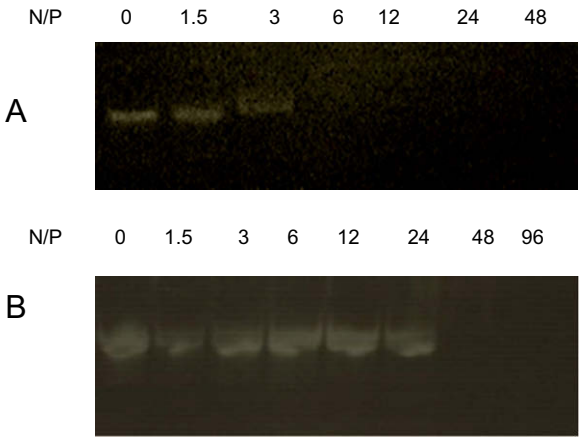

Figure 3. Electrophoresis of (A) PEI/siRNA and (B) PEI-pullulan/siRNA complexes. Various concentrations of the polymer were mixed with the siRNA and analyzed by 19% polyacrylamide gel electrophoresis. A N/P ratio of 0 implies siRNA alone.

to free siRNA in the PEI/siRNA complex were not observed when the polymer was present at N/P ratios of above 3, whereas when the N/P ratios were 1.5 and 3, bands corresponding to free siRNA were observed. In the case of the PEI-pullulan/siRNA complex, no suppression of siRNA was indentified in those complexes with N/P ratios of 1.5 to 24, while siRNA migration in complexes with N/P ratios of $\geqslant$48 was suppressed (Fig. 3). These results show that introduction of pullulan into PEI weakens the polymer and siRNA complex.

2.4. Safety of polymer/siRNA complexes in vivo

PEI alone, the PEI/siRNA complex, and the PEI-pullulan/siRNA complex were injected into mice using a hydrodynamics-based or a non-hydrodynamics-based procedure. PEI alone or the PEI/siRNA complex with high N/P ratios ($\geqslant$6.0) increased mice mortality after systemic injection using the non-hydrodynamics-based procedure (Fig. 4); note that all mice died when complexes with N/P ratios of $\geqslant$12 were injected (data not shown). Similarly, previous studies reported that the PEI/DNA complex with a N/P ratio of 6 resulted in the death of 50% of the injected mice.[14,15] However, all mice died when PEI alone or the PEI/siRNA complex with a N/P ratio of 3 was injected using the hydrodynamics-based procedure. Hydrodynamics-based transfection was developed to deliver naked DNA or RNA into the liver by intravenous injection of a large volume of DNA or RNA solution at high velocity. This is an efficient method for liver-specific in vivo gene delivery.[16,17] However, in our study, high mouse mortality was observed when the hydrodynamics-based procedure was used for the in vivo delivery of PEI/siRNA complexes.

All dead mice lapsed into dyspnea less than 30 min after injection and showed hemorrhage-like dark red regions in the lung. There was no difference in mortality between mice injected with PEI alone and those injected with the PEI/siRNA complex, but more severe hemorrhage-like dark red regions were observed in the former (Fig. 4A and B).

Concerning the death of mice after systemic injection, Fahrmeir's group suggested that free PEIs after complex formation with DNA correlate with mouse mortality.[18] Several studies showed that increased gene expression in the lung is associated with lung damage and mouse mortality after intravenous injection of PEI/DNA or modified PEI/DNA.[15,19,20] In the present study, PEI/siRNA showed a similar in vivo toxicity to PEI/DNA.

On the other hand, no mortality was observed in mice injected with PEI-pullulan/siRNA complexes with N/P ratios of 6 to 48 by the hydrodynamics-based procedure mice (Fig. 4B) and the non-hydrodynamics-based procedure (data not shown). These

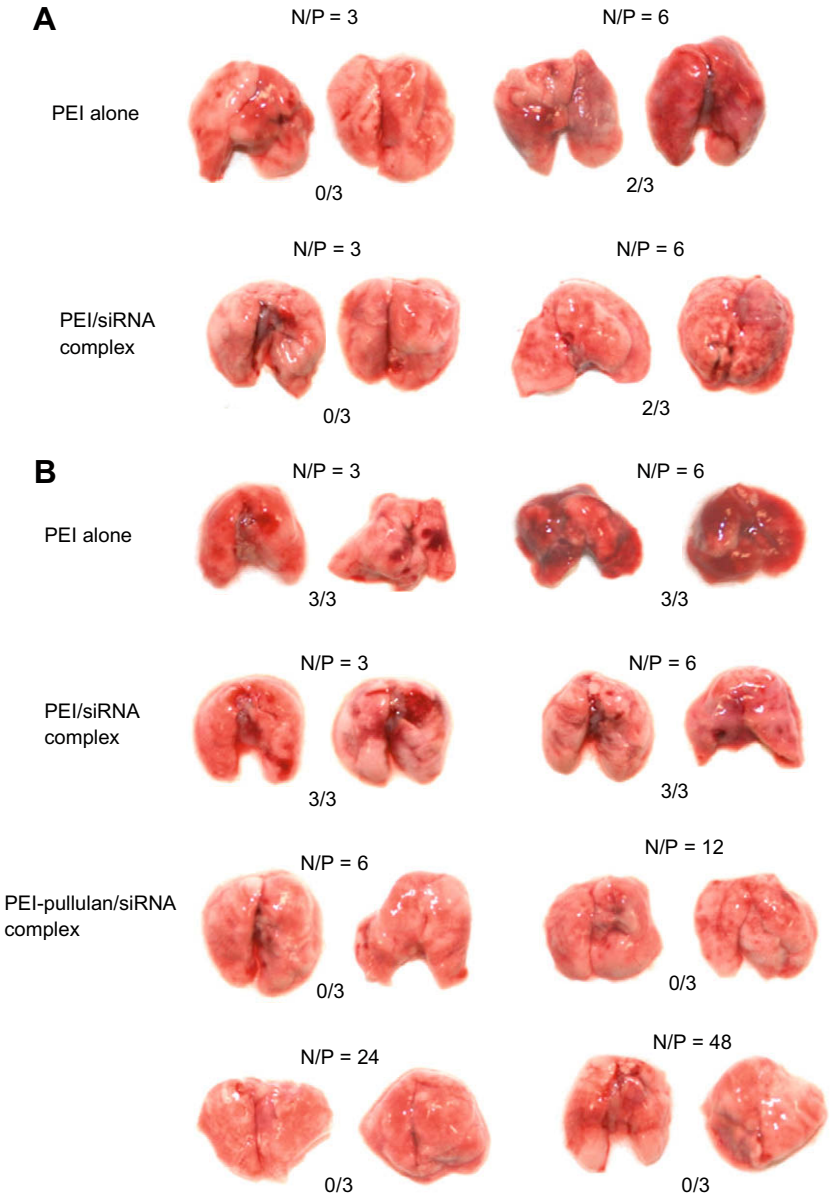

Figure 4. Delivery of PEI alone or polymer/siRNA complexes into mice by using the (A) non-hydrodynamics- or (B) hydrodynamics-based procedure. Numbers of dead mice per total mice are described below.

results suggest that intravenous injection with PEI alone or the PEI/siRNA complex at high N/P ratios can increase mortality, but introduction of pullulan into PEI results in low mortality. Moreover, hydrodynamics-based injection can increase the mouse mortality rate, compared to non-hydrodynamics-based injection. High in vivo toxicity or mortality caused by systemic injection of the PEI-based complex is an obstacle to be overcome. Many research efforts such as the introduction of poly(ethylene glycol) (PEG)[15] and removal of free PEIs after complex formation[18] were reported to efficiently reduce in vivo toxicity or mortality. In the present study, introduction of pullulan to PEI dramatically reduced in vivo toxicity and mortality.

2.5. Biodistribution after injection of the polymer/siRNA complex into mice

siRNA formed a complex with PEI at a N/P ratio of 3 and with PEI-pullulan at a N/P ratio of 48. Complexes were injected into the mice via the tail vein using the non-hydrodynamics-based procedure. The fluorescence in each tissue (heart, lung, liver, spleen, and kidney) was detected at 1 or 3 h after the injection. At 1 h after the injection of the PEI/siRNA or PEI-pullulan/siRNA complex, fluorescence was identified mainly in the lung and kidney. At 3 h, fluorescence increased in the livers of the PEI-pullulan/siRNA complex-injected mice, but was barely found in the livers of the PEI/siRNA-injected mice (Fig. 5).

Several studies have reported that linear and branched PEI/gene complexes show different biodistribution and transfection efficiency.[6–9] The linear PEI/gene complex exhibits more efficient transgene expression in the lung when injected intravenously, as compared to the branched PEI/gene complex;[6,7,9,14,21] however the transgene expression of the branched PEI/gene complex may be more efficient in other tissues (e.g., kidney).[9,22] Further, although PEI cytotoxicity depends on molecular weight and N/P ratios, the branched PEI/gene complex is found to have higher toxicity or cause more tissue damage as compared to the linear PEI/gene complex.[8,9,23]

In the present study, we used a linear 22-kDa PEI for complex formation with siRNA and for synthesizing the PEI-pullulan

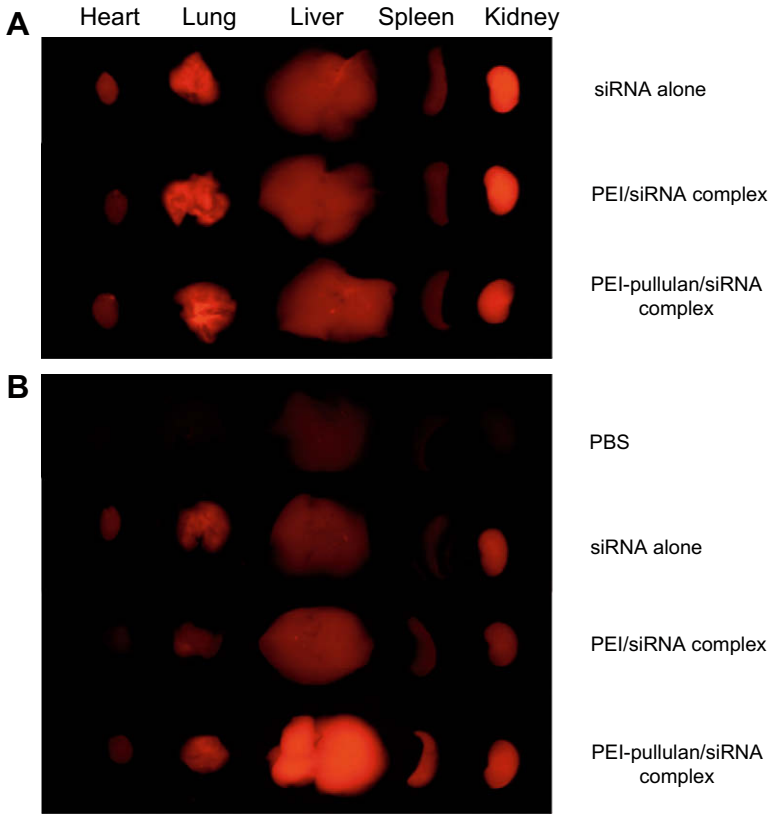

Figure 5. Biodistribution after injection of PBS, siRNA alone, or polymer/fluorescein-labeled siRNA complexes. The siRNA was bound with PEI at a N/P ratio of 3 and with PEI-pullulan at a N/P ratio of 48. The fluorescence in each tissue (heart, lung, liver, spleen, and kidney) was detected (A) 1 or (B) 3 h after the injection.

polymer. When the 22-kDa linear PEI/gene complexes were transfected via systemic administration, the main target was the lung and lower levels of transfection were found in the brain, heart, liver, spleen, and kidney.[14] High transgene expression in the lungs may relate to rapid crossing of the pulmonary endothelial barrier by the PEI/gene complexes.[21] Similarly, we found the highest level of fluorescence in the lung compared to other tissues (heart, liver, and spleen) at 1 h after intravenous injection of the PEI/siRNA complex at a N/P ratio of 3 (Fig. 5). Fluorescence in the kidney may be caused by elimination of biodegraded free fluorescein from the system.

siRNA-based therapeutics are recognized as a useful approach for liver (hepatic) diseases such as hepatitis B and C, but development of liver-targeted siRNA delivery system is an important problem to solve.[1] In the present study, pullulan, a water-soluble polysaccharide, was introduced into PEI to increase liver-targeting efficiency. At 3 h after the injection, we found highest level of fluorescence in the livers of the PEI-pullulan/siRNA complex-injected mice (Fig. 5). Thus, our system may be a useful means for efficient delivery of siRNA into the liver.

3. Conclusions

We found that introduction of pullulan to PEI increased the level of fluorescence in the liver. This finding may be explained by the fact that pullulan has a high affinity for asialoglycoprotein receptors in the liver.[11–13] Moreover, systemic delivery of PEI-pullulan polymer dramatically reduced mouse death. These results suggest that the PEI-pullulan polymer may be an efficient and low toxic means for siRNA delivery into the liver.

4. Materials and methods

4.1. Fluorescein-labeled siRNA

The gene (*apoB* siRNA) used in this study was amidated and its sequence was as follows: 5′-GUCAUCACACUGAAUACCAAUdTdT-3′ (sense) and 5′-dTdTCACAGUAGUGUGACUUAUGGUUA-3′ (antisense). Alexa Fluor 750 (Invitrogen, Tokyo, Japan) was used as an amine-reactive dye. The fluorescein-labeled siRNA was dialyzed against water containing 0.1% diethylpyrocarbonate (DEPC) for 2 days in a dialysis membrane bag with a molecular weight (MW) cut-off of 3500, followed by lyophilization.

4.2. Synthesis of PEI-pullulan polymer

A mixture of 48.6 mg of pullulan (M_w, 107,000; 0.3 unit mmol) and 24.3 mg of carbonyldiimidazole (CDI; 0.15 mmol) was stirred in 30 mL of anhydrous dimethylsulfoxide (DMSO) at room temperature. After 4 h, 13.2 mg of linear polyethyleneimine (PEI; M_w, 22 kDa; 0.3 mmol) was added to the mixture and further stirred at room temperature under a nitrogen-rich atmosphere for 1 day. The mixture was dialyzed against water for 3 days in a dialysis Spectra Pore membrane bag with a molecular weight cut-off of 10,000 (Spectrum Laboratories, Inc., Rancho Dominguez, CA), followed by lyophilization to obtain a PEI-pullulan polymer powder.

The buffering capacity of the PEI-pullulan polymer from pH 12 to 3 was determined by acid-base titration. Briefly, the polymer (4.8 mg) was dissolved in 8 mL of 150 mM NaCl to a final concentration of 0.6 mg/mL and the pH of the polymer solution was set to 12 with NaOH. The solution was subsequently titrated with 0.1 M HCl.

4.3. Measurements of the diameter of complexes

Polymer and siRNA complexes were prepared by incubating both the siRNA and the polymer in water for 30 min. The final concentration of the siRNA was adjusted to 1 μg/mL using water (pH 7.3). The diameters of the complexes were determined using a Zetasizer (Malvern Instruments, Malvern, UK) with the He/Ne laser at a detection angle of 173° and a temperature of 25 °C.

4.4. Electrophoresis of the polymer/siRNA complex

For the electrophoresis experiment, various concentrations of the polymer were mixed with the siRNA in ultrapure distilled water (Invitrogen) at room temperature for 30 min, and then analyzed by 19% polyacrylamide gel electrophoresis.

4.5. Delivery of polymer/siRNA complexes into mice by direct injection

All animal studies were performed in accordance with the Guidelines for Animal Experiments, established by the Ministry of Health, Labour and Welfare of Japan, and by the National Cardiovascular Center Research Institute. Male 6-week-old BALB/c mice (CLEA Japan Inc., Osaka, Japan) weighing approximately 22 g were used in this study. The mice were maintained in a temperature-controlled room (22 °C) with a 12-h light-dark cycle and were provided with a standard pellet diet (CE-2; CLEA Japan) and water ad libitum. One week after arrival, mice were divided into two groups, the hydrodynamics injection group and the non-hydrodynamics injection group. In the hydrodynamics injection group, 2 mL of 5% glucose solution containing each polymer/siRNA complex was injected, whereas in the non-hydrodynamics injection group, 0.2 mL was injected. For the hydrodynamics-based procedure, solutions were injected over 6–8 s into the tail vein using a 27-gauge needle. The mice were sacrificed 1 or 3 h after the injections, and thereafter each tissue type (lung, heart, liver, spleen, and kidney) was excised. Images were obtained with the Maestro In Vivo Imaging System (Cambridge Research & Instrumentation, Woburn, MA, USA).

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Supplementary data

Supplementary data (Tables S1 and S2 describing molecular parameters of polymer and zeta potential of PEI-pullulan/siRNA complex, respectively) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.04.031.

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