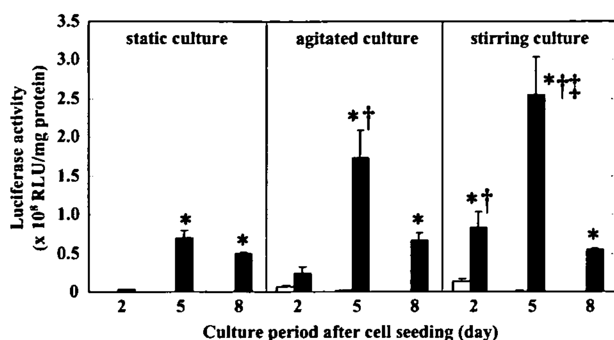

FIG. 4. Time course of luciferase expression level of mesenchymal stem cells (MSCs) transfected using the conventional (open columns) and reverse methods (solid columns) in static, agitation, and stirring cultures in polyethylene terephthalate non-woven fabric: (open columns) the plasmid deoxyribonucleic acid-cationized pullulan complex in the absence of fetal calf serum (FCS) and (solid columns) the complex in the presence of FCS. $^*p < 0.05$ versus the level in the presence of FCS using the conventional method at the corresponding time. $^\dagger p < 0.05$ versus the level in the absence of FCS using the reverse method in static culture at the corresponding time. $^\ddagger p < 0.05$ versus the level in the absence of FCS using the reverse method in agitation culture at the corresponding time. RLU, relative light unit.

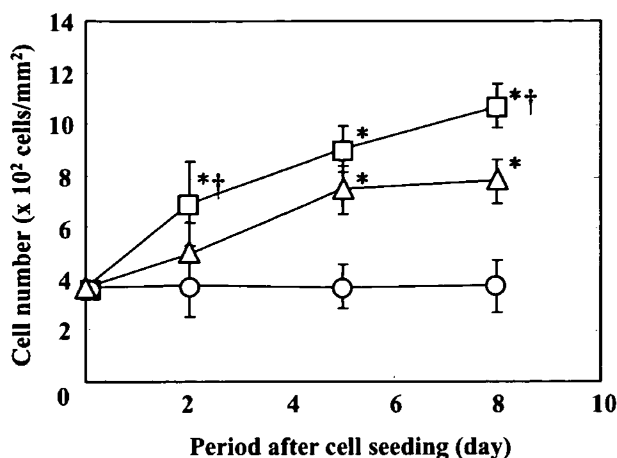

FIG. 5. Time course of proliferation of mesenchymal stem cells (MSCs) transfected using the reverse method in polyethylene terephthalate-non-woven fabric transfection in static (○), agitation (△), and stirring cultures (□). $^*p < 0.05$ significant against the cell number in static culture at the corresponding time. $^\dagger p < 0.05$ significant against the cell number in agitation culture at the corresponding time.

Irrespective of the culture method, the level and duration of gene expression were enhanced significantly more using the reverse transfection than the conventional one. The enhanced and prolonged extents of gene expression depended on the type of culture method, although the time profile of transfected level was similar. The extent increased from the static to the agitated to the stirring culture method. Using reverse transfection culture, the level of gene expression increased for 5 days after cell seeding but thereafter decreased. This was different from the time profile of gene expression using the conventional transfection culture, with which there was a maximum level on the second day for any culture method. The agitated and stirring cultures tended to increase the level of gene expression. Fig. 5 shows the time course of MSC proliferation during the reverse transfection culture. The number of MSCs that proliferated in the PET fabrics became larger from the static to the agitated to the stirring culture methods, although little cell proliferation was observed in the static culture.

DISCUSSION

The present study clearly demonstrates that the reverse transfection method was more effective in enhancing the level and duration time of gene expression than the conventional method. Considering the positioning of cells and the complex, it is likely that the complex always exists near the cells to be transfected. In addition, Pronectin achieves cell adhesion to the surface of complex-coated substrate. It is highly conceivable that the continuous exposure of complex to cells and the adhesion between cells and the substrate, which minimizes the serum influence on the transfection activity of complex, results in the enhanced and prolonged gene expression.

There are 5 key steps for expression of plasmid DNA: (1) the attachment of plasmid DNA onto the cell surface, (2) the internalization of plasmid DNA into the cell, (3) the endosomal escape of plasmid DNA, (4) the transfer of plasmid DNA to the nucleus, and (5) the internalization of plasmid DNA into the nucleus. Different from the viral vector, the non-viral carrier essentially does not have any specific mechanisms to accelerate and facilitate the above steps. Several trials have been performed to enhance the efficiency of each step. For steps (1) and (2), it has been attempted to use receptor-mediated endocytosis mechanism.[17–38] In this study, pullulan was selected as the material for a non-viral carrier because it is thought that it is internalized into cells by way of the sugar-recognizable receptor. We have demonstrated that the receptor internalizes the pullulan carrier and enhances the *in vitro* level of gene expression more than the cationized polymer carrier, which the receptor-independent mechanism is known to internalize.[12] Spermine was used for cationization of pullulan for 2 reasons. One is the need for the amine groups to allow the plasmid DNA to complex ionically. The other is an inherent property of the "buffering effect" for better endosomal escape of plasmid DNA than other amino compounds.[39] It is conceivable that the spermine-pullulan-plasmid DNA complex travels in the cytosol by simple diffusion, because it does not have any nucleus transfer mechanisms. Once the complex reaches the nuclear membrane, internalization into the nucleus will be necessary for

gene transfection. It has been recognized that non-viral carriers do not have any mechanism for nuclear internalization. Therefore, it is possible that, when the nuclear membrane disappears in cell division, the complex of non-viral carrier and plasmid DNA eventually enters the nucleus for gene transfection. Based on this mechanism, the transfection efficiency of plasmid DNA by the non-viral carrier will increase as the proliferation of cells to be transfected increases. In the reverse transfection method, cells are cultured in the presence of serum. It is possible that the presence of serum enables cells to proliferate more efficiently under better culture conditions, resulting in enhanced gene transfection. Cell viability after reverse transfection culture was significantly higher than with conventional transfection culture in the absence of FCS. This strongly indicates that reverse transfection is performed under culture conditions good for cell activity. Cells always make contact with the complex in reverse transfection culture, which is different from the contact time of 6 h in conventional transfection culture.

To allow cells to proliferate under *in vitro* culture conditions, it is necessary to contrive the local environment of cells, including the medium, the substrate of cell attachment and proliferation, the supply of oxygen and nutrients, and the excretion of waste. Some trials have been performed to accelerate the *in vitro* proliferation of MSCs. MSCs proliferated more efficiently with the addition of basic fibroblast growth factor to the culture medium[40,41] and the surface modification of culture substrates.[41] Because MSC proliferation is substrate-dependent, it is preferable to increase the surface area of culture substrate. Several 3-dimensional substrates, so-called scaffolds, have been designed to demonstrate their feasibility in proliferation enhancement.[42–44] In adddition, culture methods have been designed from the viewpoint of the supply of oxygen and nutrients and the excretion of waste. Stirring and perfusion culture methods were effective in enhancing the rate of MSC proliferation compared with the static culture method.[16,39]

For the agitated and stirring culture methods, because the culture medium is circulated, oxygen and nutrients are supplied to MSCs, and cellular wastes are excreted more efficiently than with the static culture method without active medium circulation. The plasmid DNA-carrier complex does not have any inherent potential to allow the plasmid DNA to integrate positively into the genome of cells. Considering the gene transfection mechanism, it is likely that the plasmid DNA has a chance to internalize into the nucleus of cells for gene expression only when the nuclear membrane of cells disappears in cell division. The medium-circulated culture method promoted cell proliferation (Fig. 5). Taken together, it is highly conceivable that more-efficient proliferation of MSCs under better biological conditions promotes the internalization of complex without any lethal damage to cells, resulting in enhanced gene expression. In conclusion, the reverse transfection method combined with the stirring cell culture method in the presence of the adhesion substance is a promising technology to enhance the efficiency of gene expression for stem cells. This technology is applicable to any type of cell. It is expected that this transfection method with the non-viral pullulan carrier can be applied to genetic engineering for cell therapy as well as basic research into stem cell biology and medicine.

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GENE DELIVERY

Expression profile of plasmid DNA by spermine derivatives of pullulan with different extents of spermine introduced

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Abstract

The objective of this study is to prepare a novel gene carrier from pullulan, a polysaccharide with an inherent affinity for the liver, and evaluate the feasibility in gene transfection. Various amounts of spermine were chemically introduced into pullulan with molecular weights of 22,800, 47,300, and 112,000 to prepare cationized pullulan derivatives with different percentages of spermine introduced. Each cationized pullulan derivative was complexed with a plasmid DNA at various ratios and applied to HepG2 cells for in vitro gene transfection. The level of gene expression depended on the percent spermine introduced of cationized pullulan derivatives and the molecular weight of pullulan. However, when compared at the complexation molar ratio of pullulan derivative to the plasmid DNA, the expression level became maximum around the ratio of 10^2, irrespective of the pullulan molecular weight. Pre-treatment of cells with asialofetuin of asialoglycoprotein receptor ligand decreased the level of gene expression by the complexes. The cationized pullulan derivative with an appropriate physicochemical character is a promising non-viral carrier which promotes the receptor-mediated internalization of plasmid DNA and consequently enhances the expression level.
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Keywords: Pullulan; Spermine; Introduction extent; Asialoglycoprotein receptor; Gene expression

1. Introduction

Gene therapy is one of the promising approaches to treat human diseases where genes or antisense sequence oligo deoxynucleotides and small interference RNA (siRNA) are delivered into the target cells to produce desired proteins or inhibit the expression of harmful genes [1]. For successful gene therapy, efficient and safe gene delivery systems are necessary. Generally speaking, the gene delivery carrier is classified into viral and non-viral types.

Viral carrier of adenovirus [2] and retrovirus [3] has a high transfection efficiency, but there are some issues to be resolved for the clinical applications, such as the mutagenesis, carcinogenesis, and immune responsiveness. Although with lower transfection efficiency than the viral carrier, the non-viral carrier has some advantages in terms of the biological safety, lower immunogenicity, no limitation of gene size to deliver, and the easy preparation. From these practical viewpoints, researches about the non-viral carriers have been widely performed. Many types of cationized polymers [4] and cationized liposomes [5–7] have been designed [8–10], while complexation with them enabled plasmid DNA to neutralize the anionic charge as well as to reduce the particle size, which is preferable to enhance the efficiency of plasmid DNA transfection. Although the expression level of plasmid DNA was enhanced by complexation with the cationized carriers, the electrostatic interaction of the positively charged complexes with the cell surface of negative charge is not cell-specific. Considering the in vivo application, it is necessary to technologically improve the selectivity of complexes for target cells.

Several approaches for the cell-specific gene delivery have been investigated by making use of cell-surface receptors [11] (peptide-recognition receptor [12–15] or sugar chain-recognition

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receptor [16–20]). Generally, the ligand for the receptor is covalently linked to cationized polymers. For example, the liver targeting of a plasmid DNA is achieved through the covalent conjugation of high-molecular-weight polylysine with asialoorosomucoid [21,22]. Coupling of galactose residues enabled polyethylenimine to selectively deliver genes to hepatocytes via the asialoglycoprotein receptor-mediated pathway [23,24].

Gene delivery system has been investigated by making use of cationized polysaccharides, such as cationized dextran [25–27], schizophyllan [28], and chitosan [29]. Advantages of the polysaccharide-based carrier over other cationized polymers are the presence of hydroxyl groups available for simple chemical modification and the cell internalization possibly accelerated by a sugar-recognition receptor of cell surface.

Pullulan is a water-soluble polysaccharide with a repeated unit of maltotriose condensed through α-1,6 linkage and has an inherent ability to accumulate in the liver [30] at higher amounts via the asialoglycoprotein receptor than other water-soluble polymers [31,32]. We have demonstrated that chemical conjugation with this pullulan enabled interferon (IFN) to target to the liver and consequently induce the IFN-specific enzyme thereat [33]. The liver targeting was also observed for a plasmid DNA [34]. Spermine is one of the naturally occurring polyamines, and cationization with this spermine converted gelatin to a non-viral carrier of plasmid DNA with higher transfection efficiency than that with other amine compounds [35].

It is well recognized that the level of gene expression is greatly influenced by the physicochemical properties of cationized polymer carriers for gene complexation, including the molecular weight and the type or the percent introduced of amine compounds for cationization. Among them, the percentage of cationized residues introduced to the carrier polymer has been recognized as one factor contributing to the level of gene expression [36–39]. In this study, pullulan with several molecular weights was cationized by chemical introduction of spermine to obtain cationized pullulan with different percentages of spermine introduced. A plasmid DNA was complexed with the cationized pullulan derivatives in aqueous solution, and the apparent particle size and zeta potential of the complexes were measured. In addition, their physicochemical properties were evaluated in terms of the solution turbidity, gel retardation, dissociation resistance by heparin, lectin binding, and ethidium bromide (EtBr) intercalation assays. In vitro gene expression by the complexes for HepG2 cells of a human hepatoma cell line was investigated to assess the effect of complex physicochemical properties.

2. Materials and methods

2.1. Materials

Pullulan with different weight-average molecular weights, 22,800, 47,300, and 112,000 was purchased from Hayashibara Biochemical Laboratories, Inc., Okayama, Japan. Spermine was purchased from Sigma Chemical Co., St. Louis, MO, USA. Other chemicals were obtained from Nacalai Tesque. Inc., Kyoto, Japan and used without further purification.

2.2. Preparation of cationized pullulan derivatives

Spermine was introduced to the hydroxyl groups of pullulan by a *N,N'*-carbonyldiimidazole (CDI) activation method [40]. Spermine and CDI were added to 50 ml of dehydrated dimethyl sulfoxide containing 50 mg of pullulan (Table 1). Following agitation using magnetic stirrer at 35 °C for 20 h, the reaction mixture was dialyzed against ultra-pure double-distilled water (DDW) for 2 days with a dialysis membrane (the cut-off molecular weight of dialysis membrane is 12,000–14,000, Viskase Companies, Inc, Willowbrook, Illinois). Then, the solution dialyzed was freeze-dried to obtain the samples of spermine-introduced pullulan (spermine–pullulan). The spermine introduction was determined from the conventional elemental analysis and expressed by the molar percentage of spermine introduced to the hydroxyl groups of pullulan.

2.3. Preparation of plasmid DNA

The plasmid DNA used was the pGL3 vector (5.26 kb) coding for a firefly luciferase gene (Luciferase Reporter Vectors-pGL3, Promega, USA). The plasmid DNA was propagated in an *E. coli* (strain DH5α) and purified by QIAGEN plasmid Mega kit (Qiagen K.K., Tokyo, Japan) according to the manufacturers' instructions. Both the yield and purity of the plasmid DNA were evaluated by UV spectroscopy (Ultrospec 2000, Pharmacia Biotech, Cambridge, England). The absorbance ratio at wavelengths of 260 to 280 nm for plasmid DNA solution was measured to be between 1.8 and 2.0.

2.4. Preparation of polyion complexes

Polyion complexes were prepared by mixing an aqueous solution of spermine–pullulan with that of plasmid DNA. Briefly, varied amounts of spermine–pullulan were dissolved in 50 μl of DDW and mixed with 50 μl of phosphate-buffered saline solution (10 mM PBS, pH 7.4) containing 100 μg of plasmid DNA, and left for 15 min at room temperature to obtain various polyion complexes (PIC) of spermine–pullulan and plasmid DNA. The PIC composition was calculated on the basis of the nitrogen number of spermine–pullulan (*N*) per the phosphorus number of plasmid DNA (*P*) and expressed as the *N*/*P* ratio.

Table 1
Preparation and characterization of spermine–pullulan with different percentages of spermine introduced

Pullulan	[CDI]/[OH] [a]				
Molecular weight	0.5	1.0	1.5	3.0	5.0
22,800	2.69 [b]	5.60	11.0	23.0	32.5
47,300	1.07	5.95	12.3	20.4	32.9
112,000	2.19	7.35	10.7	26.3	33.1

[a] Molar ratio of *N,N'*-carbonyldiimidazole (CDI) initially added to the hydroxyl groups (OH) of pullulan.
[b] Molar percentage of spermine introduced to the hydroxyl groups of pullulan.

2.5. Turbidity measurement for spermine–pullulan–plasmid DNA complexes

To evaluate the PIC formation of plasmid DNA with the spermine–pullulan, the solution turbidity was measured at a wavelength of 500 nm in 100 mM sodium phosphate-buffered solution (pH 7.4) at different ionic strengths. The concentration of plasmid DNA solution was fixed at 100 μg/ml for the turbidity measurement.

2.6. Electrophoresis of spermine–pullulan–plasmid DNA complexes

PIC were prepared in 10 mM PBS solution at different *N/P* ratios. After 15 min of incubation, 10 μl of the complex was added to 3 μl of a loading buffer (0.1% sodium dodecyl sulfate, 5% glycerol, and 0.005% bromophenol blue) and applied on an 1 wt.% agarose gel in Tris-borate-ethylenediaminetetraacetic acid buffer solution (TBE, pH 8.3) containing 0.1 mg/ml ethidium bromide (EtBr). Electrophoretic evaluation of the PIC was carried out in TBE solution at 100 V for 30 min. The gel was imaged with a UV transilluminator (Gel Doc 2000, BIO-RAD laboratories, Segrate, Italy). To 100 μl of the PIC, 900 μl of heparin at concentrations of 6.25 and 12.5 μg/ml was added, followed by incubation at room temperature for 30 min, and then the similar electrophoresis analysis was performed to evaluate the dissociation resistance of PIC against heparin.

2.7. Characterization of PIC by dynamic light scattering (DLS) and electrophoretic light scattering (ELS)

PIC were prepared in 10 mM PBS solution at an *N/P* ratio of 3.0. DLS measurement was carried out using a DLS-DPA-60HD instrument (Otsuka Electronic Co. Ltd., Osaka, Japan) equipped with an Ar^+ laser at a detection angle of 90° at 25 °C for 15 min. In the present study, the autocorrelation function of samples was analyzed based on the cumulant method and the R_s value was calculated automatically by the equipped computer software and expressed as the apparent particle size of samples. On the other hand, the zeta potential was measured using ELS-7000AS instrument (Otsuka Electronic Co. Ltd., Osaka, Japan) at 25 °C and an electric field strength of 100 V/cm. From the determined electrophoretic mobility, the zeta potential was automatically calculated using the Smoluchouski equation. Light scattering measurement was done three times for every sample. The PIC used in this measurement was diluted enough to prevent multiple scattering.

2.8. Ethidium bromide intercalation assay

A sample containing plasmid DNA (20 μg/ml) and EtBr (0.4 mg/ml) (plasmid DNA-EtBr complex) was used to calibrate to 100% fluorescence. Spermine–pullulan was added to an aqueous solution of EtBr at different *N/P* ratios. The fluorescence intensity of the samples (excitation: 510 nm, emission: 590 nm) was measured 15 min later by Gemini EM fluorescent microplate reader (Molecular Devices, sunnyvale CA, USA). The results were expressed as a relative fluorescence intensity (percent decreased against plasmid DNA-EtBr complex).

2.9. Lectin-induced aggregation of spermine–pullulan–plasmid DNA complexes

PIC were prepared in PBS at an *N/P* of 3.0, followed by incubation at room temperature for 15 min. To 100 μl of the PIC solution, 100 μl of Ricinus communis agglutinin of lectin recognizable to galactose (RCA120, Seikagaku Corporation, Tokyo, Japan, 500 μg/ml) was added, followed by incubation at room temperature. The time profile of solution turbidity change was measured at a wavelength of 500 nm. To confirm the specificity of lectin–sugar interaction, RCA120 containing galactose was added to the PIC solution and then the similar turbidity measurement was performed. The results were expressed as the percentage of the solution absorbance to that of spermine–pullulan–plasmid DNA complex alone.

2.10. In vitro gene transfection experiment

Transfection experiments were performed independently in triplicate. HepG2 cells of a human hepatoma cell line were purchased from American Type Culture Collection, Manassas VA, USA and maintained in Minimal Essential Medium supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acid solution (Invitrogen Corp., Carlsbad, CA, USA), and 10 vol.% fetal calf serum (Hyclone laboratories, Inc., Utah, USA) (MEM-FCS) at 37 °C. Cells were seeded on each well of six-well cluster dish (Corning, NY, USA) at a density of 4×10^5 cells/well and cultivated in 2 ml of MEM-FCS for 24 h. PIC were formed by mixing 50 μl of DDW containing the spermine–pullulan and 50 μl of PBS containing 5 μg of the pGL3-luciferase plasmid DNA at different *N/P* ratios. Immediately after the medium was exchanged by fresh Opti MEM medium (Invitrogen Corp., Carlsbad, CA, USA), 100 μl of the PIC solution was added and incubated for 15 min at room temperature, followed by 6 h incubation for cell transfection. Then, the medium was changed to MEM-FCS and cells were incubated further for 42 h.

Cells were washed with PBS once, lysed in 200 μl of a cell culture lysis reagent (Promega Corp., Madison, WI, USA), transferred into a micro reaction tube, and the cell debris was separated by centrifugation (14,000 rpm, 20 min). Then, 100 μl of luciferase assay reagent (Promega Corp., Madison, WI, USA) was added to 20 μl of supernatant while the relative light unit (RLU) of the samples was determined by a luminometer (MicroLumatPlus LB 96V, Berthold, Tokyo, Japan). The total protein of each well was determined by bicinchonic acid (BCA) Protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions in order to normalize the influence of number variance of cells on the luciferase activity. Each experimental group was carried out three times independently. Asialofetuin (Sigma Chemical Co., St. Louis, MO, USA, 1 mg/ml) which is a natural ligand for the asialoglycoprotein receptor (ASGPR), was pre-incubated for 1 h, and then the similar

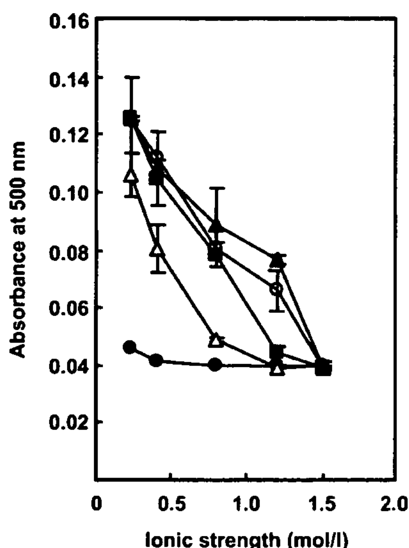

Fig. 1. Solution turbidity of spermine–pullulan–plasmid DNA complexes prepared at an N/P ratio of 3.0 as a function of the solution ionic strength in complex preparation. The molecular weight of pullulan used for spermine introduction is 47,300. The [CDI]/[OH] ratio of spermine-pullulan used for cationization is (●) 0.5, (△) 1.0, (■) 1.5, (○) 3.0 or (▲) 5.0.

transfection experiment was performed to evaluate the suppression effect on the level of gene expression.

2.11. Cell viability

Cytotoxicity was assayed using a cell counting kit (Nacalai tesque, Inc., Kyoto, Japan). Cells were seeded on each well of 96-well cluster dish (Corning, NY, USA) at a density of 1×10^4 cells/well and cultivated in MEM-FCS for 24 h. The medium was changed to the fresh Opti MEM medium, and 10 μl of the PIC solution was applied to each well, follow by 6 h incubation. Then, the medium was changed to MEM-FCS and 100 μl of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2*H*-tetrazolium (WST-8) solution was added and the cells were incubated further for 3 h. The absorbance of samples was measured at 450 nm by VERSAmax microplate reader (Molecular Devices, sunnyvale CA, USA). The percent cell viability was expressed as 100% for control, non-treated cells.

2.12. Statistical analysis

All the data were expressed as the mean±the standard deviation of the mean. Statistical analysis was performed based on the ANOVA, followed by Fisher's PLSD and significance was accepted at $p<0.05$.

3. Results and discussion

The present study demonstrates that the in vitro gene expression of plasmid DNA was greatly enhanced by complexation with the spermine–pullulan derivatives although it was influenced by the percent spermine introduced of pullulan. In general, for efficient non-viral gene delivery, it is important to design carriers that can electrostatically bind to a genetic material, molecularly condense the material size to a diameter range of several hundred nanometers, protect the material from enzymatic degradation, and facilitate the cell internalization. However, such a non-specific cellular internalization via the electrostatic interaction between the genetic material–carrier complex and the cell does not always result in the in vivo enhancement of gene expression specifically to the cell to be transfected. Therefore, it is necessary to develop a carrier for cell-specific delivery system, which allows genes to deliver specifically to the target cell and enhance the expression level. It is found that pullulan accumulates in the liver at significantly higher amounts via the asialoglycoprotein receptor (ASGPR) than other water-soluble polymers [31,32]. Our previous study has demonstrated that the liver targeting of interferon-beta (IFN-β) by the conjugation with pullulan enhanced liver induction of IFN-β-induced antiviral enzyme to a significantly greater extent than that by free IFN-β [41,42]. The liver targeting was also observed for a plasmid DNA [34]. In this study, complexation of plasmid DNA with spermine–pullulan derivatives could enhance the level of gene expression and the level decreased by adding asialofetuin that can competitively bind the ASGPR of hepatocytes. Taken together, it is likely that pullulan is recognized by the ASGPR to enhance the internalization of plasmid DNA into cells, resulting in enhanced gene expression.

3.1. Cationization of pullulan

Since pullulan itself has no cationic charges, it is required to cationize pullulan for complexation with negatively charged plasmid DNA. In this study, spermine was introduced to pullulan because spermine is one of the polyamines present in the body, and cationization with spermine converted gelatin to a non-viral carrier of plasmid DNA with transfection efficiency higher than that with other amine compounds [35]. Spermine was introduced to the hydroxyl groups of pullulan by the CDI activation method (Table 1). The extent of spermine introduced could be changed by altering the amount of CDI added initially.

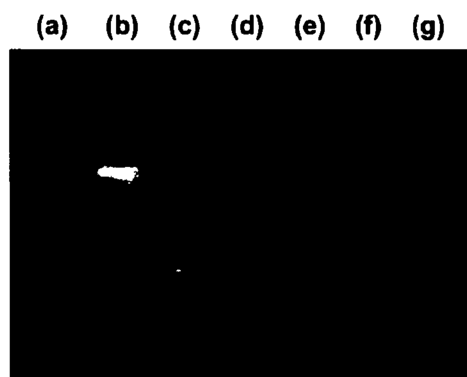

Fig. 2. Agarose gel electrophoresis of spermine–pullulan–plasmid DNA complexes prepared at an N/P ratio of 3.0. The molecular weight of pullulan used for spermine introduction is 47,300. The sample applied is (a) DNA marker, (b) free plasmid DNA or complexes of plasmid DNA and spermine–pullulan with the [CDI]/[OH] ratio of (a) 0.5, (b) 1.0, (c) 1.5, (d) 3.0 or (e) 5.0.

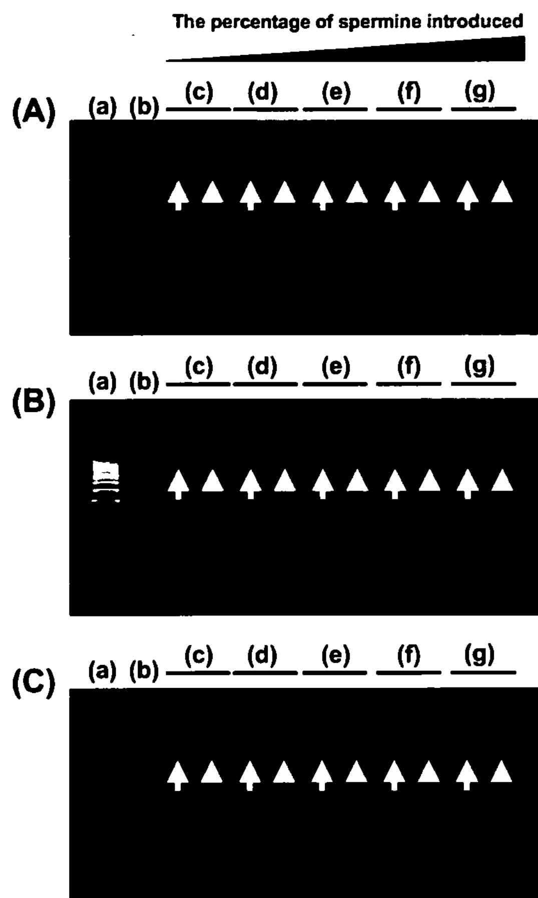

Fig. 3. Agarose gel electrophoresis of spermine–pullulan–plasmid DNA complexes prepared at an *N*/*P* ratio of 3.0 in the presence of heparin. The molecular weight of pullulan used for spermine introduction is (A) 22,800, (B) 47,300 or (C) 112,000. The sample applied is (a) DNA marker, (b) free plasmid DNA or complexes of plasmid DNA and spermine–pullulan with the [CDI]/[OH] ratio of (c) 0.5, (d) 1.0, (e) 1.5, (f) 3.0 or (g) 5.0. Heparin concentration present in the complex is (⇧) 6.25 or (△) 12.5 μg/ml.

3.2. *Characterization of spermine–pullulan–plasmid DNA complexes*

Fig. 1 shows the turbidity result of PIC solution to experimentally confirm the complexation of spermine–pullulan with a molecular weight of 47,300 and different percentages of spermine introduced with the plasmid DNA. In the turbidity experiment, increment or decrement of solution turbidity shows association or dissociation of PIC, respectively. As the ionic strength of solution became higher, the solution turbidity decreased for complexes of spermine–pullulan with percentages of spermine introduced prepared at the molar ratio of CDI initially added to the hydroxyl groups (OH) of pullulan ([CDI]/[OH]) of 1.0, 1.5, 3.0, and 5.0. However, for complexes of spermine–pullulan with the [CDI]/[OH] of 0.5, no solution turbidity was observed for all the ionic strengths studied.

Fig. 2 shows the electrophoretic patterns of polyion complexes of plasmid DNA and spermine–pullulan with a molecular weight of 47,300 and different percentages of spermine introduced prepared at an *N*/*P* ratio of 3.0. Migration of plasmid DNA was retarded with an increase in the introduction percentage. No retardation was observed for the complex of spermine-pullulan with the [CDI]/[OH] of 0.5. From the electrophoresis analysis, plasmid DNA complexes prepared by almost all of spermine–pullulan s were formed at an *N*/*P* ratio of 3.0. In this connection, complexes prepared at an *N*/*P* ratio of 3.0 were mostly used in this study.

Fig. 3 shows the electrophoretic patterns of polyion complexes of plasmid DNA and spermine–pullulan with different molecular weights and percentages of spermine introduced in the presence of different concentrations of heparin. EtBr exclusion in the presence of heparin showed that the complexes of spermine–pullulan and plasmid DNA were stable and not dissociated. Although heparin addition dissociated the plasmid DNA complexes of spermine–pullulan, the dissociation extent depended on the molecular weight of pullulan and the percent spermine introduced. While the plasmid DNA complexes of spermine–pullulan with lower molecular weights and spermine introduction percentages tended to be dissociated in the presence of heparin at 6.25 μg/ml (Fig. 3A, c–e and B and C, c–d), whereas those with higher values could not be dissociated even at 12.5 μg/ml of heparin (Fig. 3C, f and g).

Table 2 summarizes the physicochemical properties of polyion complexes prepared at an *N*/*P* ratio of 3.0. The apparent particle size and the zeta potential of free plasmid DNA were around 400 nm and −15 mV, respectively. The polyion complexation of spermine–pullulan with higher percentages of spermine introduced prepared at the [CDI]/[OH] of 1.5, 3.0 or 5.0 reduced the particle size of plasmid DNA, irrespective of pullulan molecular weight used for cationization, while the particle size oppositely became large for smaller spermine–pullulan prepared at the [CDI]/[OH] of 0.5 or 1.0. On the other hand, irrespective of the pullulan molecular weight and percent spermine introduced, the zeta potential was around 10–16 mV.

Fig. 4 shows the relative fluorescence intensity of plasmid DNA intercalated with EtBr after addition of spermine–pullulan.

Table 2
Apparent particle size and zeta potential of free plasmid DNA and complexes of plasmid DNA and spermine–pullulan with different percentages of spermine introduced

Pullulan	[CDI]/[OH] [a]				
Molecular weight	0.5	1.0	1.5	3.0	5.0
22,800	n.d.	2200±0 [b]	330±140	290±170	200±89
	n.d.	+7.0±0.3 [c]	+14±0	+14±0	+14±0
47,300	n.d.	1300±0	250±57	210±57	280±180
	n.d.	+9.0±0.2	+15±0	+16±0	+17±1
112,000	n.d.	1300±0	260±130	290±180	280±180
	n.d.	+10±0	+13±0	+11±1	+11±1
Free plasmid DNA	410±61				
	−15±10				

[a] Molar ratio of *N*,*N*′-carbonyldiimidazole (CDI) initially added to the hydroxyl groups (OH) of pullulan.
[b] Apparent particle size (nm). Means±S.D.
[c] Zeta potential (mV). Means±S.D.

GENE DELIVERY

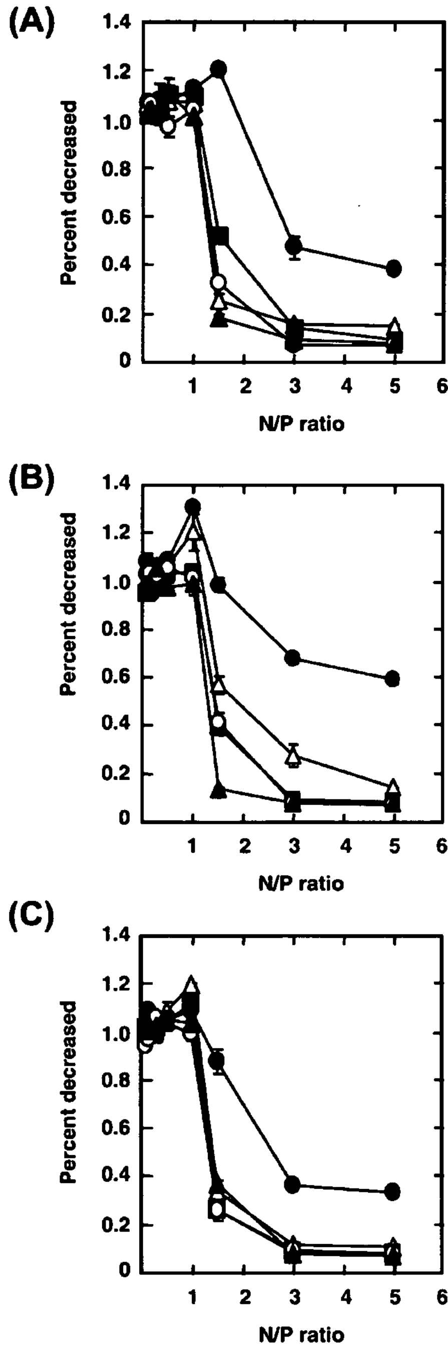

Fig. 4. Solution fluorescence of spermine–pullulan–plasmid DNA complexes prepared at different *N/P* ratios after EtBr addition. The molecular weight of pullulan used for spermine introduction is (A) 22,800, (B) 47,300 or (C) 112,000. The [CDI]/[OH] ratio of spermine–pullulan used for cationization is (●) 0.5, (△) 1.0, (■) 1.5, (○) 3.0 or (▲) 5.0.

The EtBr fluorescence intensity decreased with an increase in the *N/P* ratio. The pattern of fluorescence intensity decreased depended on the percent spermine introduced of pullulan. At the *N/P* ratios of 3.0 or higher, the EtBr fluorescence intensity became low for the complexes of spermine–pullulan with higher introduction percentages, while it did not clearly for the complexes of spermine–pullulan with the lowest percentage. These findings indicate that the spermine–pullulan formed a polyion complex with the plasmid DNA, although the

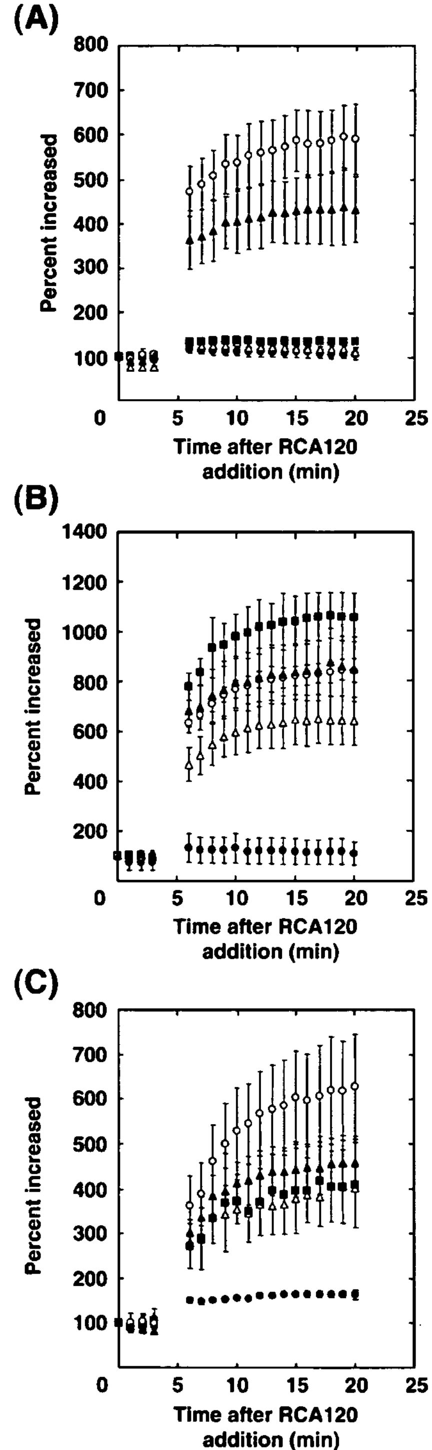

Fig. 5. Time course of the turbidity change of spermine–pullulan–plasmid DNA complexes prepared at an *N/P* ratio of 3 after RCA 120 addition. The molecular weight of pullulan used for spermine introduction is (A) 22,800, (B) 47,300 or (C) 112,000. The [CDI]/[OH] ratio of spermine–pullulan used for cationization is (●) 0.5, (△) 1.0, (■) 1.5, (○) 3.0 or (▲) 5.0.

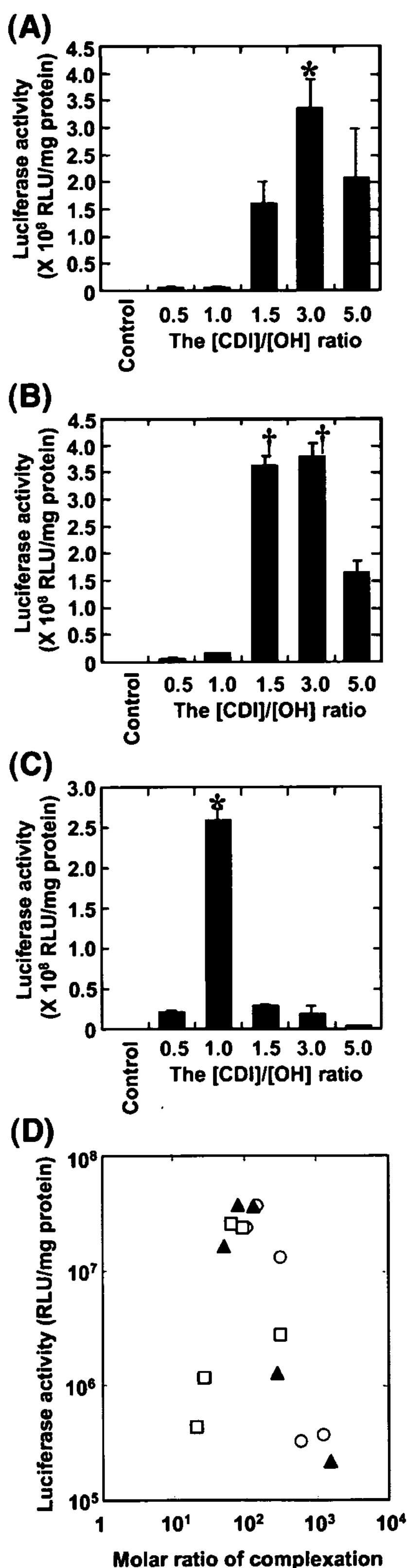

Fig. 6. Effect of the spermine introduction extent of pullulan on the luciferase expression of spermine–pullulan–plasmid DNA complexes for HepG2 cells. The molecular weight of pullulan used for spermine introduction is (A) 22,800, (B) 47,300 or (C) 112,000. The amount of plasmid DNA applied is 5 μg/well and the N/P ratio is 3.0. *$p<0.05$; versus the expression level of complexes prepared by other spermine–pullulans. †$p<0.05$; versus the expression level of complexes prepared by spermine–pullulans with the [CDI]/[OH] ratio of 0.5, 1.0, and 5.0. (D) Effect of the molar ratio of spermine–pullulan to plasmid DNA in complexation on the level of gene expression. The molecular weight of pullulan used for complexation is (○) 22,800, (▲) 47,300 or (□) 112,000.

complexation was greatly influenced by the percent spermine introduced of pullulan. Considering the reaction conditions to prepare spermine–pullulan derivatives, it is likely that spermine residues are randomly introduced at an equal distance interval on the sugar main chain. Since the total length of chain is different by the molecular weight of pullulan, the distance interval changes by the percentage of spermine introduced. When a spermine residue on the sugar chain interacts with a plasmid DNA, other residues subsequently interact with the plasmid DNA because the spermine residues are linked on the sugar chain. The longer the sugar chain of spermine-pullulan derivatives or the higher the percentage of spermine introduced, it is conceivable that the derivative interacts with the plasmid DNA interacts to form the complex more easily.

DLS and ELS measurements (Table 2) and EtBr intercalation assay (Fig. 4) revealed that the percent spermine introduced of pullulan greatly affected the physicochemical properties of plasmid DNA complexes with spermine–pullulan. It is possible that the distance interval of spermine residues on the molecular chain of spermine–pullulan with higher introduction percentages was short enough to condense the plasmid DNA. The long distance interval of spermine residues does not allow the plasmid DNA to condense in size, but form an aggregate structure. On the other hand, the zeta potential of all the complexes was a positive value about +14 mV, irrespective of the pullulan molecular weight (Table 2). This may be due to the excessive positive charge of spermine–pullulan which is not involved in the complexation with plasmid DNA.

From the results of electrophoretic patterns of spermine–pullulan–plasmid DNA complexes in the presence of heparin (Fig. 3), it is revealed that the dissociation behavior of the complex depended on the molecular weight and the percent spermine introduced of pullulan. Interestingly, compared with the DLS results (Table 2), it is found that complexes with the same particle sizes (molecular weight of 47,300 or 112,000, and [CDI]/[OH] of 5.0) showed different dissociation behaviors. It is possible that these differences greatly affected the level of gene expression.

Fig. 5 shows the time profile of solution absorbance change of spermine–pullulan–plasmid DNA complexes after the addition of RCA120 lectin. The solution absorbance increased time-dependently, although the increment pattern depended on the molecular weight and the percent spermine introduced of pullulan. The extent of absorbance increased was suppressed by addition of galactose in the concentration-dependent manner (data not shown).

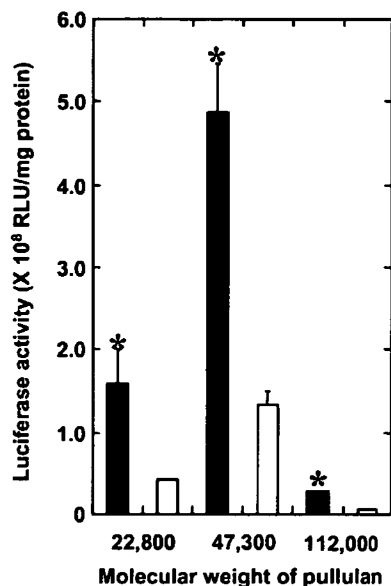

Fig. 7. Effect of asialofetuin addition on the level of luciferase expression of spermine–pullulan–plasmid DNA complexes prepared at the *N*/*P* ratio of 3.0 for HepG2 cells. Cells were pretreated with (solid columns) or without (open columns) asialofetuin before gene transfection. The plasmid DNA amount is 5 μg/well. $*p<0.05$; versus the expression level of cells transfected without asialofetuin addition at the corresponding molecular weight of pullulan.

3.3. In vitro gene transfection of spermine–pullulan–plasmid DNA complexes

Fig. 6 shows the effect of spermine introduction percentage on the level of gene expression for HepG2 cells. The level of gene expression was different among spermine–pullulan derivatives with different percentages of spermine introduced. In addition, the percentage of spermine introduced where the highest level was observed was different among the pullulan molecular weight used for spermine introduction (Fig. 6A–C).

Fig. 6D shows the relationship between the molecular ratio of spermine–pullulan to plasmid DNA and the level of gene expression. Irrespective of the molecular weight of pullulan, a maximum level was observed in the molar ratio of around 10^2.

Fig. 7 shows the effect of asialofetuin addition on the level of gene expression of spermine–pullulan–plasmid DNA complexes for HepG2 cells. The level decreased by adding the asialofetuin for any complex.

Dependence of the spermine–pullulan derivative type on the level of gene expression can be explained in terms of not only the apparent particle size, the extent of compaction, and stability of polyion complexes, but also the interaction of polyion complexes with receptors of cell surface. Firstly, it is reported that a certain range of apparent particle size of polyion complexes was effective in enhancing the in vitro gene expression [43,44]. Complexation with more spermine-introduced pullulan prepared at [CDI]/[OH] ratios of 1.5 to 5.0 reduced the particle size of plasmid DNA, whereas less spermine-introduced pullulan did not, irrespective of the pullulan molecular weight. It is possible that the compacted complex of reduced size is more readily internalized by cells, resulting in enhanced gene expression. The particle size of complex will be one of the factors affecting the level of gene expression.

Secondly, it is well known that the extent of EtBr intercalated into plasmid DNA is affected by the conformational change of plasmid DNA molecules and the molecular condensation of plasmid DNA [45]. Intercalation with the spermine–pullulan allows to change the molecular structures, resulting in EtBr release from plasmid DNA which is detected by the decrease in the fluorescent intensity. The release is accelerated as the interaction becomes stronger. The relative fluorescence intensity was not decreased after addition of spermine–pullulan with the lowest percent introduced. This suggests that the interaction force with the plasmid DNA was not strong enough to form a complete for efficient gene transfection.

Thirdly, it is recognized that the appropriate dissociation of the plasmid DNA and the carrier inside cells is necessary for the successful translation of gene transfected to [46]. From the result of the electrophoretic patterns of spermine–pullulan–plasmid DNA complexes in the presence of different concentrations of heparin (Fig. 3C), spermine–pullulan s with a molecular weight 112,000 and higher introduction percentages ([CDI]/[OH] of 3.0 or 5.0) complexed strongly with plasmid DNA and the complexes could not be dissociated even in the presence of heparin at the higher concentration. Actually, a thermodynamic model demonstrates that the dissociation probability decreases with an increase in the amino groups present on a polymer chain [47]. Since the amount of spermine introduced into one pullulan molecule increases with an increase in the percentage of spermine introduced, it may be that the dissociation probability of complex decreases with an increase in the percent spermine introduced of pullulan. From the result of gene expression (Fig. 6C), the levels of gene expression for these complexes were low. These results clearly

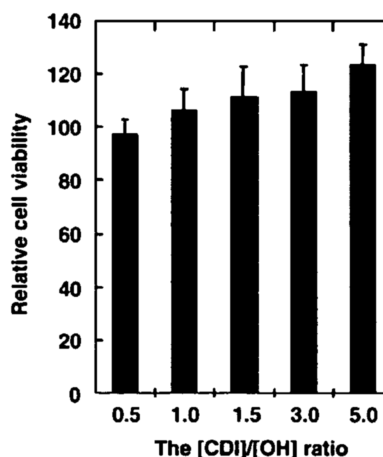

Fig. 8. Effect of the percent spermine introduced on the cell viability of HepG2 cells transfected with spermine–pullulan–plasmid DNA complexes prepared at the *N*/*P* ratio of 3.0. The molecular weight of pullulan used for spermine introduction is 47,300. The viability of cells without transfection is indicated as 100%.

demonstrate that the dissociation suppression reduced the level of gene expression. It is likely that the balance of complex stability governed by the interaction force between the plasmid DNA and spermine–pullulan results in the highest level of gene expression.

The percentage of spermine introduced where the highest expression level was observed was different among the molecular weight of pullulan used for spermine introduction (Fig. 6). The percent spermine introduction for the highest expression level tended to decrease with an increase in the molecular weight of pullulan. At the same *N/P* ratio, the number of spermine–pullulan complexed with plasmid DNA decreases with an increase in the molecular weight and percent spermine introduced of pullulan. There was an optimal molar number of the spermine–pullulan–plasmid DNA complex for enhanced gene expression at an *N/P* ratio (Fig. 6D). This result experimentally indicates that a well-balanced combination of the spermine introduction percentage and molecular weight of pullulan is necessary to enhance the level of gene expression. The complex was characterized in terms of the particle size, surface charge, lectin recognition, and dissociation stability, although it is not always done sufficiently. However, the reason of the optimal number for the maximum gene expression is not clear at present.

Finally, the lectin affinity assay revealed that the spermine–pullulan–plasmid DNA complex was recognized by the sugar-recognizable lectin (Fig. 5). When the complex was mixed with the lectin, the solution absorbance was increased. The presence of galactose suppressed the absorbance increase in the concentration-dependent manner. This is explained in terms of a sugar-specific interaction between the complex and the lectin. It is likely that the pullulan chain present on the complex surface is specifically recognized by the lectin, resulting in the formation of complex-lectin aggregates. The aggregate formation depended on the percent spermine introduced and the molecular weight of pullulan used for complexation. This is because the molecular mobility of surface pullulan chains was different between the complexes. Compared with Fig. 6, it is apparent that most complexes strongly interacted with the lectin showed the high gene expression level. Taken together, it is likely that the plasmid DNA complex with the spermine derivative of pullulan with an inherent affinity for the ASGPR is effectively internalized by cells, resulting in enhanced gene expression. In addition, it is reported that an asialoglycoprotein receptor is expressed on the surface of HepG2 cells. As shown in Fig. 7, the level of gene expression decreased by addition of asialofetuin, whereas no inhibition effect was observed for a transfection reagent commercially available, Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA) (data not shown). There have been many research papers about gene delivery systems with polymers targetable to ASGPR [15–18]. Complexation with a galactosylated polymer or an ASGPR-targeting protein-conjugated polymer enabled plasmid DNAs to selectively deliver to hepatocytes, while the gene expression was suppressed by the addition of asialofetuin. Taken together, it is conceivable that the in vitro gene transfection by the spermine–pullulan was facilitated by the ASGPR-mediated uptake.

It should be noted that there is an issue regarding the effect of spermine addition on the pullulan specificity for the liver. It has been demonstrated that some spermine–pullulan samples enabled plasmid DNA to efficiently deliver to the liver [48]. However, the liver targetability of pullulan is not investigated in terms of the spermine-introduced extent. Further study about this point is needed for the drug delivery applications.

3.4. Cytotoxicity of spermine–pullulan–plasmid DNA complexes

Fig. 8 shows the viability of cells 3 h after exposure of PIC prepared at the *N/P* ratio of 3.0. No cytotoxicity was observed, irrespective of the percent spermine introduced. Since cytotoxicity is one of the drawbacks in cationized polymer-based gene transfection, this result demonstrates that the spermine–pullulan is a feasible gene carrier for enhancement of gene expression.

4. Conclusions

In this study, spermine was chemically introduced to pullulan with different molecular weights to prepare cationized pullulan derivatives with different percentages of spermine introduced. Complexation with the spermine–pullulan enabled a plasmid DNA to significantly enhance the expression level of HepG2 cells although the level depended on the percent of spermine introduced and the molecular weight of pullulan. In addition, pre-treatment of cells with the asialoglycoprotein receptor ligand decreased the level of gene expression by the complexes. It is concluded that the cationized pullulan derivative with an appropriate physicochemical character is a promising non-viral carrier which promotes the receptor-mediated internalization of plasmid DNA for hepatocytes.

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Expression profile of plasmid DNA obtained using spermine derivatives of pullulan with different molecular weights

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Abstract—The objective of this study was to prepare a novel gene carrier from pullulan, a polysaccharide with an inherent affinity for the liver, and evaluate the feasibility in gene transfection. Pullulan with different molecular weights was cationized by chemical introduction of spermine. The cationized pullulan derivative was complexed with a plasmid DNA and applied to HepG2 cells for *in vitro* gene transfection. The level of gene expression depended on the molecular weight of cationized pullulan derivatives and the highest level was observed for the cationized pullulan derivative with a molecular weight of 47.3×10^3. Pre-treatment of cells with asialofetuin decreased the level of gene expression by the complexes. These findings indicate that the cationized pullulan derivative is a promising non-viral carrier of plasmid DNA which is internalized in a receptor-mediated fashion.

Key words: Pullulan; spermine; molecular weight; asialoglycoprotein receptor; gene expression.

INTRODUCTION

Gene therapy is one of the promising approaches to treat human diseases where genes or anti-sense sequence oligodeoxynucleotides and small interference RNA (siRNA) are delivered into the target cells to produce the desired proteins or inhibit the expression of harmful genes [1]. For successful gene therapy, efficient and safe gene delivery systems are necessary. Generally speaking, the gene carriers for the delivery systems are classified in viral and non-viral types.

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The viral carrier of adenovirus [2] and retrovirus [3] has a high transfection efficiency, but there are some points to be resolved before clinical applications, such as the mutagenesis, carcinogenesis and immune-responsiveness. Although with lower transfection efficiency than viral carriers, non-viral carriers have some advantages in terms of the biological safety, lower immunogenicity, no size limitation of gene to deliver and easy preparation. From this practical viewpoint, studies on non-viral carriers have been widely performed. Many types of cationized polymers [4] and cationized liposomes [5–7] have been designed [8–10] and complexation with them enabled plasmid DNA to neutralize the anionic charge, as well as to reduce the molecular size, which is preferable to enhance the efficiency of plasmid DNA transfection. Although the expression level of plasmid DNA was enhanced by complexation with the cationized carriers, the electrostatic interaction of the positively charged complexes with the cell surface of negative charge is not cell-specific. Considering the *in vivo* application, it is necessary to technologically improve the selectivity of complexes for target cells.

Several approaches to cell-specific gene delivery have been investigated by making use of cell-surface receptors [11] (peptide-recognition receptor [12–15] or sugar chain-recognition receptor [16–20]). Generally the ligand for the receptor is covalently linked to the cationized polymers. For example, the liver targeting of a plasmid DNA is achieved through the covalent conjugation of high-molecular-weight polylysine with asialoorosomucoid [21, 22]. Coupling of galactose residues enabled polyethylenimine to selectively deliver genes to hepatocytes *via* the asialoglycoprotein receptor-mediated pathway [23, 24].

Gene-delivery systems have been investigated by making use of cationized polysaccharides, such as cationized dextran [25–29] including DEAE-dextran [30], schizophyllan [31] and chitosan [32, 33]. Advantages of the polysaccharide-based carrier over other cationized polymers are the presence of hydroxyl groups available for simple chemical modification and the possible cell internalization by a sugar-recognition receptor of cell surface.

Pullulan is a water-soluble polysaccharide with a repeated unit of maltotriose condensed through α-1,6 linkage and has an inherent ability to accumulate in the liver [34] in higher amounts *via* the asialoglycoprotein receptor than *via* other water-soluble polymers [35, 36]. We have demonstrated that chemical conjugation with this pullulan enabled interferon (IFN) to target to the liver and consequently induce the IFN-specific enzyme there [37]. Liver targeting was also observed for a plasmid DNA [38, 39]. Spermine is one of the naturally-occurring polyamines, and cationization with this spermine converted gelatin to a non-viral carrier of plasmid DNA with higher transfection efficiency than that with other amine compounds [40].

It is well recognized that the level of gene expression is greatly influenced by the physicochemical properties of cationized carriers for complex, including the molecular weight and the type of amine compounds or the extent for cationization. Among them, the molecular weight of carrier polymer has been recognized as a factor contributing to the level of gene expression [32, 41, 42]. In this study,

therefore, pullulan with different molecular weights was cationized by the chemical introduction of spermine and a plasmid DNA was complexed with the cationized pullulan derivatives. The complexes were characterized in terms of the apparent molecular size and zeta potential, while the complex formation behavior was evaluated by the solution turbidity, gel retardation and ethidium bromide (EtBr) intercalation examinations. In addition, lectin-induced aggregation assays were carried out to examine the susceptibility to sugar recognition. We investigate the effect of complex physicochemical properties on the *in vitro* gene expression by the complexes was investigated for HepG2 cells of a human hepatoma cell line.

MATERIALS AND METHODS

Materials

Pullulan with different weight-average molecular weights, 5.9×10^3, 11.8×10^3, 22.8×10^3, 47.3×10^3, 112×10^3 and 212×10^3, was purchased from Hayashibara Biochemical Laboratories (Okayama, Japan). Spermine was purchased from Sigma (St. Louis, MO, USA). Other chemicals were obtained from Nacalai Tesque (Kyoto, Japan) and used without further purification.

Preparation of cationized pullulan derivatives

Spermine was introduced to the hydroxyl groups of pullulan by a N,N′-carbonyldiimidazole (CDI) activation method [43]. Spermine and CDI were added to 50 ml of dehydrated dimethyl sulfoxide containing 50 mg of pullulan (Table 1). Following agitation at 35°C for 20 h, the reaction mixture was dialyzed against ultra- pure double-distilled water (DDW) for 2 days. The cut-off molecular weight of dialysis membrane is 1×10^3 for pullulan with molecular weights of 5.9×10^3 and 11.8×10^3 (Spectrum Laboratories, Rancho Domiguez, CA, USA) and $(12–14) \times 10^3$ for pullulan with molecular weights from 22.8×10^3 to 212×10^3 (Viskase,

Table 1.
Preparation and characterization of spermine-pullulan with different molecular weights

Pullulan molecular weight ($\times 10^{-3}$)	[CDI]/[OH][a]	[Spe]/[OH][b]	Percent introduced[c]
5.8	1.5	10	12.9
11.8	1.5	10	12.3
22.8	1.5	10	11.0
47.3	1.5	10	12.3
112	1.5	10	10.7
212	1.5	10	9.74

[a]Molar ratio of N,N′-carbonyldi-imidazole (CDI) initially added to the hydroxyl groups of pullulan. [b]Molar ratio of spermine (Spe) initially added to the hydroxyl groups of pullulan. [c]Molar percentage of Spe initially added to the hydroxyl groups of pullulan.

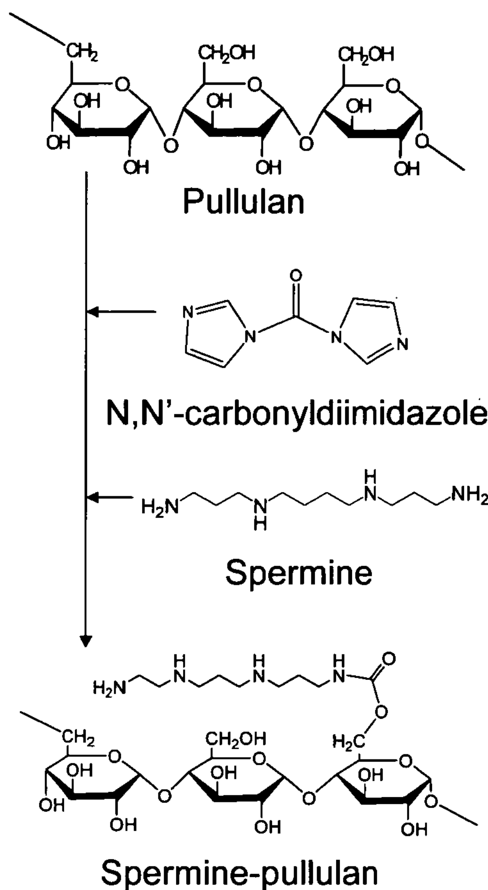

Figure 1. Schematic diagram of spermine-pullulan preparation and structure.

Willowbrook, IL, USA). Then the solution dialyzed was freeze-dried to obtain the samples of spermine-introduced pullulan (spermine-pullulan, Fig. 1). The spermine introduction was determined by conventional elemental analysis and expressed by the molar extent of spermine introduced to the hydroxyl groups of pullulan.

Preparation of plasmid DNA

The plasmid DNA used was the pGL3 vector (5.26 kb) coding for a firefly luciferase gene (Luciferase Reporter Vectors-pGL3, Promega, Madison, WI, USA). The plasmid DNA was propagated in an *Escherichia coli* strain (DH5α) and purified by Qiagen plasmid Mega kit (Qiagen K.K., Tokyo, Japan) according to the manufacturers' instructions. Both the yield and purity of the plasmid DNA were evaluated by UV spectroscopy (Ultrospec 2000, Pharmacia Biotech, Cambridge, UK). The absorbance ratio at wavelengths of 260 to 280 nm for plasmid DNA solution was measured to be between 1.8 and 2.0.

Preparation of polyion complexes

Polyion complexes were prepared by mixing an aqueous solution of spermine-pullulan with that of plasmid DNA. Briefly, varied amounts of spermine-pullulan were dissolved in 50 μl of DDW and mixed with 50 μl of 10 mM phosphate-buffered saline solution (PBS, pH 7.4) containing 100 μg plasmid DNA, followed by leaving for 15 min at room temperature to obtain various polyion complexes (PIC) of spermine-pullulan and plasmid DNA. The PIC composition was calculated on the basis of the ratio the nitrogen content of spermine-pullulan (N) and the phosphorus content of plasmid DNA (P) and expressed as the N/P ratio.

Electrophoresis of spermine-pullulan-plasmid DNA complexes

PIC were prepared in 10 mM PBS solution at different N/P ratios. After 15 min of incubation, 10 μl of the complex was added to 3 μl of a loading buffer (0.1% sodium dodecyl sulfate, 5% glycerol, 0.005% bromophenol blue) and applied on an 1 wt% agarose gel in Tris-borate-ethylenediaminetetraacetic acid buffer solution (TBE, pH 8.3) containing 0.1 mg/ml ethidium bromide (EtBr). Electrophoretic evaluation of the PIC was carried out in TBE solution at 100 V for 30 min. The gel was imaged with a UV transilluminator (Gel Doc 2000, Bio-Rad, Segrate, Italy).

Characterization of PIC by dynamic light scattering (DLS) and electrophoretic light scattering (ELS) PIC were prepared in 10 mM PBS solution at a N/P ratio of 3.0. DLS measurement was carried out using a DLS-DPA-60HD instrument (Otsuka Electronic, Osaka, Japan) equipped with an Ar^+ laser at a detection angle of 90° at 25°C for 15 min. In the present study, the autocorrelation function of samples was analyzed based on the cumulants method and the R_s value was calculated automatically by the equipped computer software and expressed as the apparent molecular size of samples. On the other hand, the zeta potential was measured using ELS-7000AS instrument (Otsuka Electronic) at 25°C and an electric field strength of 100 V/cm. From the determined electrophoretic mobility, the zeta potential was automatically calculated using the Smoluchowski equation. Light scattering measurement was done three times for every sample.

EtBr intercalation assay

A sample containing plasmid DNA (20 μg/ml) and EtBr (0.4 mg/ml) (plasmid DNA-EtBr complex) was used to calibrate to 100% fluorescence. Spermine-pullulan was added to an aqueous solution of EtBr at different N/P ratios. The fluorescence intensity of the samples (excitation 510 nm, emission 590 nm) was measured 15 min later by Gemini EM fluorescent microplate reader (Molecular Devices, Sunnyvale, CA, USA). The results were expressed as a relative fluorescence intensity (percent decreased against plasmid DNA-EtBr complex).

Lectin-induced aggregation of spermine-pullulan-plasmid DNA complexes

PIC were prepared in 10 mM PBS solution at a N/P of 3.0, followed by incubation at room temperature for 15 min. To 100 μl PIC solution, 100 μl *Ricinus communis* agglutinin (RCA120, Seikagaku, Tokyo, Japan, 500 μg/ml) was added and incubated at room temperature. Time profiles of the solution turbidity in the pre-determined time were measured at a wavelength of 500 nm. To confirm the specificity of lectin–sugar interaction, RCA120 containing galactose was added to the PIC solution and then the similar turbidity measurement was performed. The results were expressed as a relative absorbance (percent increased against spermine-pullulan-plasmid DNA complex).

In vitro *gene transfection experiment*

Transfection experiments were performed in triplicate. HepG2 cells were purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in Minimal Essential Medium supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino-acid solution (Invitrogen, Carlsbad, CA, USA), and 10 vol% fetal calf serum (Hyclone, Logan, UT, USA) (MEM-FCS) at 37°C. Cells were seeded on each well of six-well cluster dish (Corning, Corning, NY, USA) at a density of 4×10^5 cells/well and cultivated in 2 ml MEM-FCS for 24 h. PIC were formed by mixing 50 μl DDW containing spermine-pullulan and 50 μl PBS containing 5 μg pGL3-luciferase plasmid DNA at different N/P ratios. Immediately after the medium was exchanged by fresh OptiMEM medium (Invitrogen, Carlsbad, CA, USA), 100 μl of the PIC solution was added and incubated for 15 min at room temperature, followed by 6 h incubation for cell transfection. Then, the medium was changed to MEM-FCS and cells were incubated further for 42 h.

Cells were washed with PBS once, lysed in 200 μl of cell-culture lysis reagent (Promega), transferred into a micro reaction tube, and the cell debris was separated by centrifugation (14×10^3 rpm, 20 min). Then, 100 μl luciferase assay reagent (Promega) was added to 20 μl supernatant while the relative light unit (RLU) of the samples was determined by a luminometer (MicroLumatPlus LB 96V, Berthold, Tokyo, Japan). The total protein content in each well was determined by bicinchonic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturers' instructions in order to normalize the influence of number variance of cells on the luciferase activity. Each experimental group was carried out three times independently. Asialofetuin (Sigma), which is a natural ligand for the asialoglycoprotein receptor (ASGPR), with a concentration of 1 mg/ml was pre-incubated for 1 h, and then the similar transfection experiment was performed to evaluate the suppression effect on the level of gene expression.

Cell viability

Cytotoxicity was assayed using a cell counting kit (Nacalai Tesque, Kyoto, Japan). Cells were seeded in each well of a 96-well cluster dish (Corning) at a density of

1×10^4 cells/well and cultivated in MEM-FCS for 24 h. The medium was changed to the fresh OptiMEM medium, and 10 μl of the PIC solution was applied to each well, follow by 6 h incubation. Then, the medium was changed to MEM-FCS and 100 μl 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) solution was added and the cells were incubated further for 3 h. The absorbance of samples was measured at 450 nm using a VERSAmax microplate reader (Molecular Devices). The percent cell viability was expressed as 100% for control, non-treated cells.

Statistical analysis

All the data were expressed as the mean ± the standard deviation of the mean. Statistical analysis was performed based on ANOVA, followed by Fisher's PLSD and significance was accepted at $P < 0.05$.

RESULTS

Cationization of pullulan

Spermine was introduced to the hydroxyl groups of pullulan with different molecular weights by the CDI activation method (Table 1). The extent of spermine introduced could be changed by altering the amount of CDI added initially (data not shown). Irrespective of the pullulan molecular weight, the extent of spermine introduced was around 10%. A previous study revealed that the spermine-pullulan derivatives with the spermine introduction extent of 10% showed a good transfection efficiency [44]. Based on this, the spermine-pullulan derivatives with the extent around 10% were used in this study.

Characterization of spermine-pullulan-plasmid DNA complexes

Figure 2A shows the electrophoretic image of polyion complexes of plasmid DNA and spermine-pullulan prepared at various N/P ratios. Migration of plasmid DNA was retarded with an increase in the N/P ratio, but was not observed at the N/P ratio higher than a certain value.

Table 2 summarizes the physicochemical properties of polyion complexes prepared at the N/P ratio of 3.0. The apparent molecular size and the zeta potential of free plasmid DNA were around 400 nm and −15 mV, respectively. The polyion complexation of spermine-pullulan with molecular weights of 22.8×10^3, 47.3×10^3, 112×10^3 and 212×10^3 reduced the molecular size of plasmid DNA, but the molecular size oppositely became large for smaller spermine-pullulan. On the other hand, irrespective of the pullulan molecular weight, the zeta potential was around +14 mV.

Figure 2B shows the relative fluorescence intensity of plasmid DNA intercalated with EtBr after addition of spermine-pullulan. The EtBr fluorescence intensity