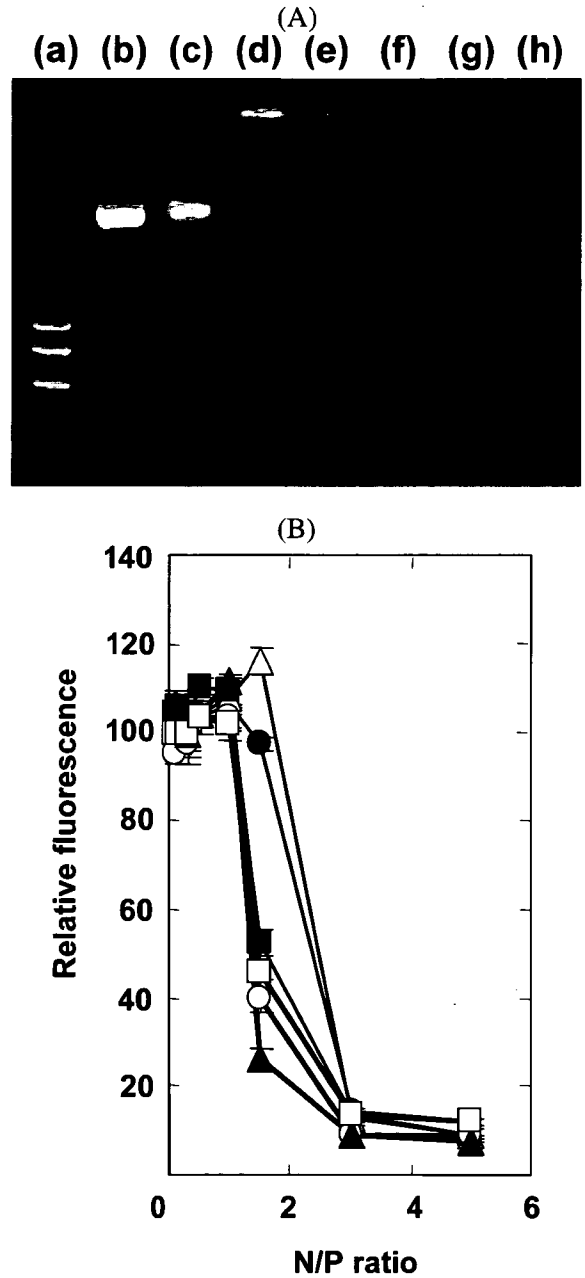

Figure 2. (A) Agarose gel electrophoresis of spermine-pullulan-plasmid DNA complexes prepared at different N/P ratios. The molecular weight of pullulan used for spermine introduction is 47.3 × 10^3. Lanes: (a) DNA marker, (b) free plasmid DNA or complexes prepared at N/P ratios of (c) 0.5, (d) 1.0, (e) 1.5, (f) 2.0, (g) 3.0 and (h) 5.0. (B) 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 ($\times 10^{-3}$): (●) 5.8, (△) 11.8, (■) 22.8, (○) 47.3, (▲) 112 and (□) 212.

Table 2.
Apparent molecular size and zeta potential of free plasmid DNA and complexes of plasmid DNA and spermine-pullulan with different molecular weights

Pullulan		Complex	
Molecular weight ($\times 10^{-3}$)	Percent introduced	Apparent molecular size (nm)	Zeta potential (mV)
5.8	12.9	1530.4 ± 727.6	+11.3 ± 0.73
11.8	12.3	1286.1 ± 0	+14.4 ± 0.47
22.8	11.0	326.5 ± 139.5	+14.3 ± 0.24
47.3	12.3	245.6 ± 56.8	+15.0 ± 0.29
112	10.7	259.3 ± 134.7	+13.4 ± 0.44
212	9.74	279.5 ± 127.0	+14.1 ± 0.68
Free plasmid DNA		409.5 ± 61.2	−14.7 ± 9.5

decreased with an increase in the N/P ratio. The pattern of fluorescence intensity decreased depended on the molecular weights of spermine-pullulan. At the N/P ratios higher than 1.5, the EtBr fluorescence intensity became low for the PIC prepared by the spermine derivatives of pullulan with molecular weights of 22.8×10^3, 47.3×10^3, 112×10^3 and 212×10^3, while that of pullulan with molecular weights of 5.9×10^3 and 11.8×10^3 exhibited fluorescence intensity decrement at the higher N/P ratio.

Figure 3 shows the time-course profile of relative absorbance of spermine-pullulan-plasmid DNA complexes after addition of RCA120 lectin, which recognizes galactose residues. The relative absorbance increased time-dependently. The pattern of relative absorbance increased depended on the molecular weights of spermine-pullulan (Fig. 3A). The extent of relative absorbance increased for the PIC prepared by the spermine derivatives of pullulan with a molecular weight of 47.3×10^3 was higher than that of pullulan with other molecular weights. In addition, the extent of relative absorbance increased for the PIC prepared by the spermine derivatives of pullulan with a molecular weight of 47.3×10^3 decreased by addition of galactose, which was dependent of the concentration of galactose. (Fig. 3B).

In vitro *gene transfection of spermine-pullulan-plasmid DNA complexes*

Figure 4 shows the effect of pullulan molecular weight on the level of gene expression for HepG2 cells. The level of gene expression was different among the spermine-pullulan derivatives used. The highest level was observed for the complex of spermine derivative of pullulan with a molecular weight of 47.3×10^3.

Figure 5A and 5B shows the effect of the N/P ratio and the plasmid DNA amount on the level of gene expression of spermine-pullulan-plasmid DNA complexes for HepG2 cells, respectively. The maximum level was observed for the complexes prepared at the N/P ratio of 3.0 (Fig. 5A), while the level reached a plateau at 2.5 μg/ml plasmid (Fig. 5B).

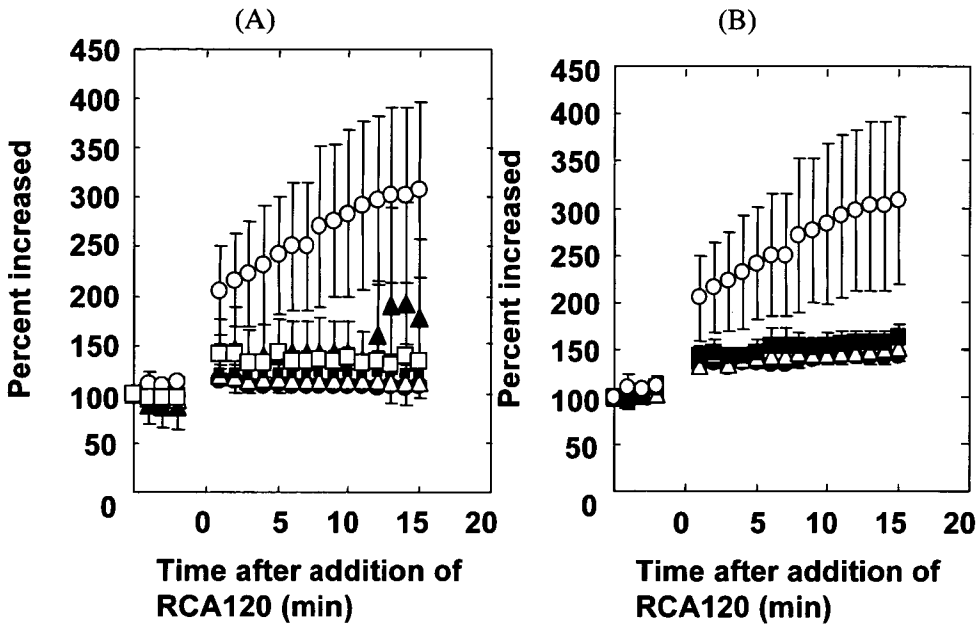

Figure 3. (A) Time-course of the turbidity change of spermine-pullulan-plasmid DNA complexes prepared at a N/P ratio of 3 after RCA 120 addition. The molecular weight of pullulan used for spermine introduction is ($\times 10^{-3}$): (●) 5.8, (△) 11.8, (■) 22.8, (○) 47.3, (▲) 112 and (□) 212. (B) Time-course of the turbidity change of spermine-pullulan-plasmid DNA complexes prepared at a N/P ratio of 3 after RCA 120 addition in the presence of galactose with different concentrations. The molecular weight of pullulan used for spermine introduction is 47.3×10^3. The galactose concentration is (●) 150, (△) 100, (■) 50 or (○) 0 mg/ml.

Figure 6 shows the effect of asialofetuin addition on the level of gene expression of spermine-pullulan-plasmid DNA complexes for HepG2 cells. The level decreased in the presence of asialofetuin pre-treated.

Cytotoxicity of spermine-pullulan-plasmid DNA complexes

Figure 7 shows the viability of cells 3 h after exposure of PIC prepared at the N/P ratio of 3.0. The cell viability decreased with the increased amount of plasmid DNA added, although it tended to decrease with an increase in the molecular weight of pullulan complexed. However, the same cytotoxicity was observed for every complex at a concentration of 0.25 μg/ml, at which the cell-transfection experiments are performed.

DISCUSSION

The present study demonstrates that the *in vitro* gene expression of plasmid DNA was greatly enhanced by complexation with the spermine-pullulan derivatives and influenced by the molecular weight of pullulan. In general, for efficient non-viral

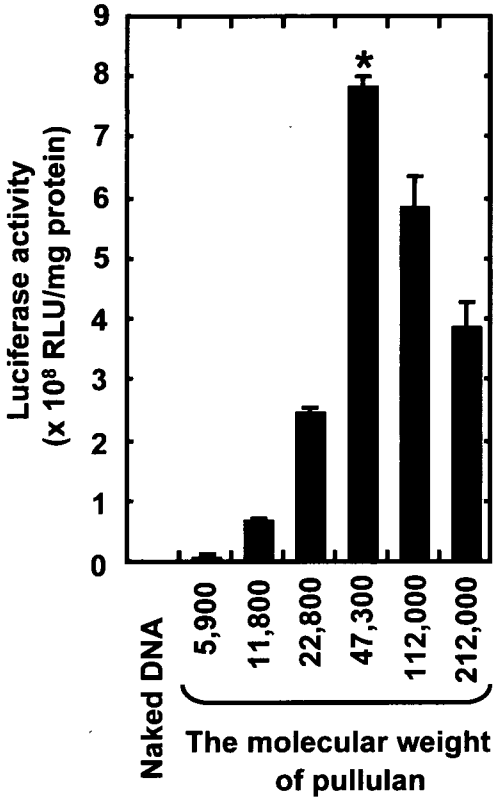

Figure 4. Effect of the pullulan molecular weight on the luciferase expression of spermine-pullulan-plasmid DNA complexes for HepG2 cells. The amount of plasmid DNA applied is 5 μg/well and the N/P ratio is 3.0. $^*P < 0.05$, *versus* the expression level of complexes prepared with other spermine-pullulans.

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 enhance the cell internalization. However, such a non-specific cellular internalization *via* electrostatic interaction does not always result in satisfactory enhancement of gene expression *in vivo*. Therefore, it is necessary to develop a carrier for a cell-specific delivery system, which allows genes to deliver specifically to the target cells 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 [35, 36]. 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 greater extent than that by free IFN-β [45, 46]. The liver targeting was also observed for a plasmid DNA [38]. In this study, complexation of plasmid DNA with spermine-pullulan derivatives could enhance the level of gene expression and the level decreased by

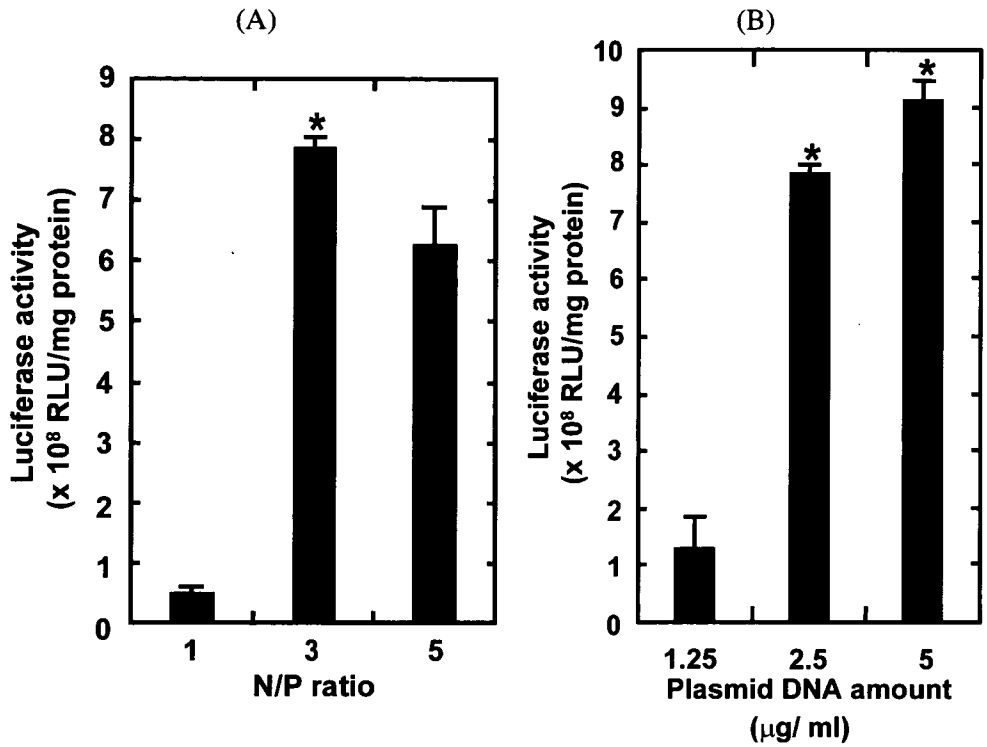

Figure 5. (A) Effect of the N/P ratio on the level of gene expression of spermine-pullulan-plasmid DNA complexes for HepG2 cells. The plasmid DNA amount is 5 μg/well. The molecular weight of pullulan used for spermine introduction is 47.3×10^3. $^*P < 0.05$, *versus* the expression level of complexes prepared at other N/P ratios. (B) Effect of the plasmid DNA amount on the level of gene expression of spermine-pullulan-plasmid DNA complexes for HepG2 cells. The N/P ratio is 3.0. The molecular weight of pullulan used for spermine introduction is 47.3×10^3. $^*P < 0.05$, *versus* the expression level of complexes at other plasmid DNA amounts.

the addition of asialofetuin that can competively bind the ASGPR of hepatocytes, used for cell pre-treatment. Taken together, it is likely that pullulan is one of the materials recognizable by the ASGPR.

However, 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 higher transfection efficiency than that with other amine compounds [40]. It has been reported that the spermine-pullulan prepared electrostatically interacted with the plasmid DNA, irrespective of the pullulan molecular weight [44]. Electrophoresis analysis revealed that retarded or no migration of plasmid DNA was observed at a N/P ratio of 1.5 (Fig. 2A). In addition, relative fluorescence of plasmid DNA intercalated with EtBr was reduced by addition of spermine-pullulan at the N/P ratio of 1.5, except for spermine derivatives

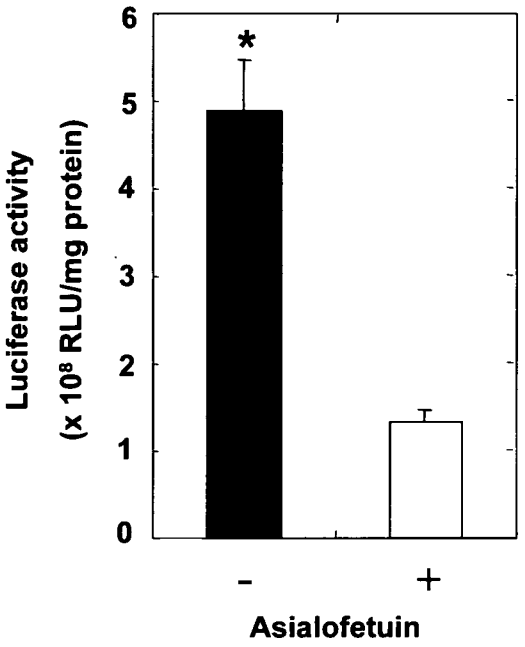

Figure 6. Effect of asialofetuin addition on the level of luciferase expression of spermine-pullulan-plasmid DNA complexes prepared at the N/P ratio of 3.0 for HepG2 cells. Cells were pre-treated with asialofetuin before usual gene transfection. The molecular weight of pullulan used for spermine introduction is 47.3×10^3 and the plasmid DNA amount is 5 μg/well. $^*P < 0.05$, *versus* the expression level of cells transfected without asialofetuin addition.

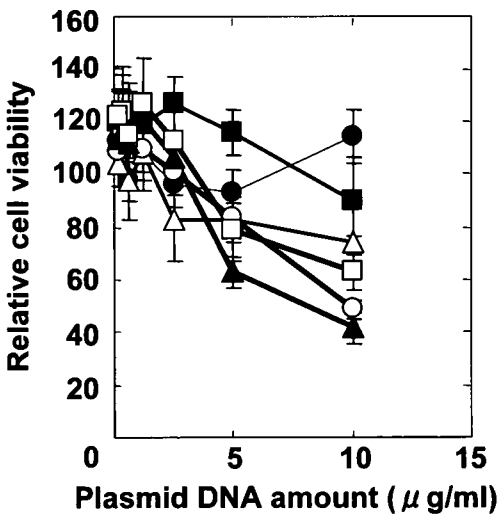

Figure 7. Effect of the plasmid DNA amount on the cell viability of HepG2 cells transfected with spermine-pullulan-plasmid DNA complexes prepared at the N/P ratio of 3.0. The molecular weight of pullulan used for spermine introduction is ($\times 10^{-3}$): (●) 5.8, (△) 11.8, (■) 22.8, (○) 47.3, (▲) 112 and (□) 212. The viability of cells without transfection is indicated as 100%.

of pullulan with molecular weights of 5.9×10^3 and 11.2×10^3. These findings indicate that the spermine-pullulan formed a polyion complex with the plasmid DNA at N/P ratios higher than 1.5. Considering the reaction conditions to prepare spermine-pullulan derivatives, it is likely that spermine residues are randomly introduced at equal distance intervals on the sugar main chain although the chain length is different by the molecular weight of pullulan. 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 spemine-pullulan derivatives or the higher the extent of spermine introduced, it is conceivable that the plasmid DNA interacts to form the complex more easily.

DLS and ELS measurements (Table 2) and EtBr intercalation assay (Fig. 2B) revealed that the molecular weight of pullulan greatly affected several physicochemical properties of plasmid DNA complexes with spermine-pullulan. As shown in Table 2, after complexation with spermine-pullulan of higher molecular weights ranging from 22.8×10^3 to 212×10^3, the apparent molecular size of plasmid DNA became smaller, while the apparent size of complexes using lower-molecular-weight pullulans (5.9×10^3 and 11.8×10^3) was larger in comparison with that of the free plasmid DNA. The EtBr intercalation assay revealed that the extent of compaction for polyion complexes became smaller for the pullulans with lower molecular weight (5.9×10^3 and 11.8×10^3). It is possible that the molecular chain of spermine-pullulan with higher molecular weights was long enough to condense the plasmid DNA. The short molecular chain of pullulan 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 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 complexation with plasmid DNA.

The level of gene expression depended on the type of spermine-pullulan derivatives used. This is caused not only by physicochemical properties of polyion complexes, such as the apparent molecular size, the extent of compaction and the stability of polyion complexes, but also by the interaction of polyion complexes with receptors of cell surface.

It is reported that a cetain range of apparent molecular size of polyion complexes is effective for the enhancement of *in vitro* gene expression [47, 48]. Complexation with spermine-pullulan with molecular weights ranging from 22.8×10^3 to 212×10^3 reduced the molecular size of plasmid DNA, whereas smaller spermine-pullulan did not. It is possible that the compacted complex of reduced size is internalized by cells, resulting in enhanced gene expression. The molecular size of complex will be one of the factors affecting the level of gene expression.

In addition, 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 [49]. Intercalation with the spermine-

pullulan induces the molecular changes, resulting in EtBr release from plasmid DNA which is detected by decrease in the fluorescent intensity. The release will be accelerated as the interaction becomes stronger. The relative fluorescence intensity was not decreased after addition of spermine-pullulan with lower molecular weights at a N/P ratio of 1.5. This suggests that the interaction force with the plasmid DNA was not strong enough to form a complex for efficient gene transfection.

On the other hand, it is recognized that it is necessary for successful gene transfection to dissociate the plasmid DNA and the carrier inside the cells [50]. The dissociation suppression caused by too strong interaction, the so-called complex stability, will oppositely reduce the level of gene expression. A thermodynamic model demonstrates that the dissociation probability decreases with an increase in the amino groups present in a polymer chain [51]. Since the amount of spermine introduced into one pullulan molecule increases with increasing molecular weight, it may be that the dissociation probability of complex decreases with the molecular weight of pullulan. 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 for the complex of spermine derivative of pullulan with a molecular weight of 47.3×10^3.

Lectin-induced aggregation assays revealed that the surface property of plasmid DNA complexes with the spermine-pullulan was greatly influenced by the molecular weight of pullulan. As shown in Fig. 3, the plasmid DNA complex with the spermine derivative of pullulan with a molecular weight of 47.3×10^3 could interact strongly with the RCA120, which recognizes galactose residues. It is well known that the ASGPR also recognizes the galactose residues. Taken together, it is possible that the plasmid DNA complex with the spermine derivative of pullulan with a molecular weight of 47.3×10^3 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. 6, the level of gene expression decreased in the presence of asialofetuin, whereas no decrement effect was observed for one of the transfection regents commercially available, Lipofectamine 2000 (Invitrogen) (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 addition of asialofetuin. Taken together, it is conceivable that the *in vitro* gene transfection by spermine-pullulan was facilitated by the ASGPR-mediated uptake.

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