Journal of
Nanoscience and Nanotechnology
Vol. 6, 2853–2859, 2006

Liver Targeting of Plasmid DNA with a Cationized Pullulan for Tumor Suppression

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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 in the mice liver. Pullulan with a weight-average molecular weight of 22,800 was cationized by the chemical introduction of spermine (spermine-pullulan). The cationized pullulan derivative was complexed with a plasmid DNA and intravenously injected for *in vivo* gene transfection. The level of gene expression by the spermine-pullulan in the liver depended on the extent of spermine introduced and the highest level was observed for the spermine-pullulan with an introduction extent of 5.60. When a plasmid DNA coding NK4 of a hepatocyte growth factor (HGF)/scatter factor antagonist complexed with the spermine-pullulan was intravenously injected to mice 1 day before the inoculation of RLmale1 tumor cells, the tumor-bearing mice survived for a longer time period, while the GPT level and the number of tumor cells grown in the liver were low compared with those of free plasmid DNA injection. These findings indicate that the liver targeting of NK4 plasmid DNA by complexation with the spermine-pullulan specifically enhanced the liver expression level, resulting in augmented suppression effect on tumor growth therein.

Keywords: Plasmid DNA, Liver Targeting, Tumor Suppression.

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1. INTRODUCTION

Recent advent of genomics has enabled to discover new genes which become therapeutically available for various diseases. In this connection, gene therapy is expected as a new and promising therapeutic choice.[1]

Presently, by utilizing the viral vectors of retroviruses, adenoviruses, and adeno-associated viruses, several human clinical trials are being carried out to treat tumor.[2] However, in spite of the high transfection efficiency, the trials are limited by the adverse effects of virus itself, such as immunogenicity and toxicity or the possible mutagenesis of the cells transfected. Therefore, instead of the viral vectors, non-viral vectors, such as cationized polymers[3] and cationized liposomes,[4] have been explored, and the complexation with plasmid DNAs or antisense oligonucleotides has been investigated to enhance the biological effects. Success of non-viral gene delivery fundamentally depends on the type of carrier materials to interact with plasmid the DNA and subsequently facilitate the cell-specific uptake.

In addition, the *in vivo* gene expression is greatly influenced by the body distribution of carrier-DNA complexes intravenously injected. However, little has been investigated on the body distribution of plasmid DNA injected in spite of the importance in *in vivo* gene therapy. *In vivo* gene transfection with plasmid DNA alone is simple, but often shows a very low efficiency. When a plasmid DNA is intravenously injected in the absence of the carrier materials, 60–70% of plasmid DNA injected is eventually taken up by the scavenger receptors of Kupffer cells[5,6] or the plasmid DNA is enzymatically degraded in the blood circulation.[7] On the other hand, for the carrier-DNA complex, the molecular size and surface charge of complex affect the body fate of plasmid DNA. Various cationic polymers and lipids have been widely used aiming at protection of DNA from enzymatic degradation, modification

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of the size, charge, and surface characteristics of DNA, and control of the DNA fate in the body.

Some trails have been experimentally performed to target plasmid DNA to the liver.[8–10] To facilitate the selective targeting to hepatocytes, the ligands for asialoglycoprotein receptors have been incorporated into the carrier materials of plasmid DNA.[11–15]

Pullulan is a water-soluble polysaccharide with a repeated unit of maltotriose condensed through α-1,6 linkage and was found to accumulate in the liver at significantly higher amounts than other water-soluble polymers.[16–18] We have demonstrated that chemical conjugation with this pullulan enabled interferon (IFN) to target to the liver and induce an IFN-specific enzyme.[19] The liver targeting with pullulan is also reported for a plasmid DNA.[20]

Hepatocyte growth factor (HGF) has been noted as a signal molecule that plays an important role in development, morphogenesis, and regeneration of living systems.[21–24] On the other hand, for malignant tumors, HGF plays a definitive role in invasive, angiogenic, and metastatic behavior of cancer cells by way of the c-Met receptor.[25–29] Therefore, it is highly expected that the inhibition of interaction between HGF and the c-Met receptor effectively suppresses the malignant activity of tumors. Based on this concept, Date et al.[30] prepared an antagonist for HGF. The antagonist (NK4) is composed of the NH_2-terminal hairpin domain and the subsequent four kringle domains of α-subunit of HGF. The NK4 binds to the c-Met/HGF receptor, but does not induce the tyrosine phosphorylation of c-Met.[30] NK4 competitively inhibits some biological events driven by the HGF-Met receptor binding, including the invasion and metastasis of distinct types of tumor cells.[30,31] Moreover, NK4 has an antiangiogenic activity which is independent of the activity as an HGF antagonist.[32] The recombinant protein of NK4 has been used for tumor animal models to demonstrate the *in vivo* therapeutic feasibility and the blocking effect on HGF functions.[31–33] In addition, desired antitumor effects through the stable expression of NK4 in tumor cells with recombinant adenoviruses have been reported.[34–38]

The objective of this study is to examine whether or not complexation with spermine-pullulan of cationized derivatives is effective in targeting a NK4 plasmid DNA to the liver in terms of gene expression and the consequent antitumor effect. Varied amounts of spermine were introduced to the hydroxyl groups of pullulan for cationization (spermine-pullulan). The pullulan derivatives of spermine were mixed with a plasmid DNA in aqueous solution to obtain various polyion complexes of spermine-pullulan and plasmid DNA. After the spermine-pullulan-NK4 plasmid DNA complexes were intravenously injected to mice 1 day before tumor inoculation, the suppression effect of tumor growth in the liver was assessed and compared with that of free plasmid DNA injected.

2. MATERIALS AND METHODS

2.1. Materials

Pullulan with a molecular weight of 22,800 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. RLmale 1 lymphoma cells were kindly supplied from Dr. K. Uno, Louis Pasteur Center for Medical Research, Kyoto, Japan and maintained in RPMI 1640 (Sigma Aldrich, Inc., St Louis, MO, USA) medium supplemented with 10 vol% fetal calf serum (FCS) at 37°.

2.2. Preparation of Cationized Pullulan

Spermine was chemically introduced to the hydroxyl groups of pullulan by a *N,N'*-carbonyldiimidazole (CDI) activation method.[39] Varied amounts of spermine and CDI were added to 50 ml of dehydrated dimethyl sulfoxide containing 50 mg of pullulan (Table I). Following agitation at 35 °C for 20 hr for introduction, the reaction mixture was dialyzed against ultra- pure double-distilled water (DDW) for 2 days with a dialysis membrane (Viskase Companies, Inc, Willowbrook, Illinois, the cut-off molecular weight of 12,000–14,000). Then, the solution dialyzed was freeze-dried to obtain the samples of spermine-introduced pullulan (spermine-pullulan, Fig. 1). The percent spermine introduced was determined on the basis of the conventional elemental analysis and expressed by the molar extent of spermine introduced to the hydroxyl groups of pullulan. From the molecular weight of pullulan, the number of glucose moieties and the hydroxyl groups were calculated because each glucose moiety has 3 hydroxyl groups.

2.3. Preparation of Plasmid DNA

The plasmid DNA used was a pCAGGS vector[40] coding for a firefly luciferase gene (pCAGGS-luciferase) and a NK4 gene (pCAGGS-NK4). The plasmid DNA was propagated in an E. coli (strain DH5α) and purified by QIAGEN plasmid Mega kit (Qiagen K.K., Tokyo, Japan)

Table I. Preparation and characterization of spermine-pullulan with different extents of spermine introduced.

Pullulan (mg/ml)	[CDI]/[OH][a]	[Sep]/[OH][b]	Percent introduced[c]
50	0.5	10	2.69
50	1.0	10	5.60
50	1.5	10	11.0
50	3.0	10	23.0

[a]Molar ratio of *N,N'*-carbonyldi-imodazole (CDI) initially added to the hydroxyl groups (HO) of pullulan.
[b]Molar ratio of spermine (Spe) initially added to the hydroxyl groups (HO) of pullulan.
[c]Molar percentage of spermine introduced to the hydroxylgroups of pullulan.

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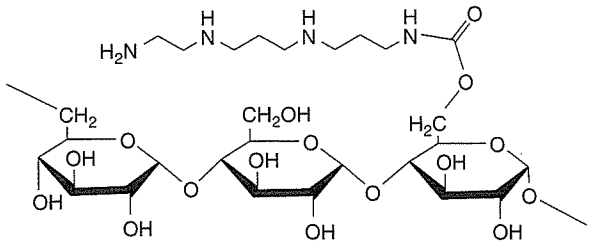

Fig. 1. Representative chemical structure of spermine-pullulan.

according to the manufacturers' instructions. When one measure plasmid DNA purity, evaluated by UV spectroscopy (Ultrospec 2000, Pharmacia Biotech, Cambridge, England), the absorbance ratio at wavelengths of 260 to 280 nm was 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, an appropriate amount of spermine-pullulan was dissolved in 100 μl of DDW and mixed with 100 μl of phosphate-buffered saline solution (10 mM PBS, pH 7.4) containing 20 μg of 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 nitrogen number of spermine-pullulan (N) per the phosphorus number of plasmid DNA (P) and expressed as the N/P ratio.

2.5. Electrophoresis of Spermine-Pullulan-Plasmid DNA Complexes

PIC were prepared in 10 mM PBS solution at a N/P ratio of 1.5. After 15 min of incubation, 10 μl of the complexes 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).

2.6. 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 1.5. 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 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-7000 AS 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.

2.7. *In Vivo* Gene Transfection Experiment

All the experimental procedures were performed according to the Specifications of Guideline for Animal Experiments of Kyoto University. BALB/c female mice of 6 weeks old (body weight = 20 g, Shimizu Laboratory Supplies Co. Ltd., Kyoto, Japan) were used in this study. All the mice received one intravenous injection of 40 mM {2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid buffer with 10 wt% glucose (HEPES) solution containing free pCAGGS-luciferase plasmid DNA and the plasmid DNA complex with pullulan derivatives with different extents of spermine introduced at a N/P ratio of 1.5 in a volume of 200 μl. After intravenous injection of the complex or other agents to mice, mice were sacrificed with cervical dislocation and the liver was taken 1 days later. After washing with 10 mM PBS, the liver was homogenized in 500 μl of cell culture lysis reagent (Promega Corp., Madison, WI, USA) by TissueLyser (Retsch GmbH and Corp., Haan, Germany), 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 the supernatant, while the relative light unit (RLU) of the samples was determined by a luminometer (MicroLumatPlus LB 96 V, Berthold, Tokyo, Japan). The RLU obtained was divided by the sample weight of the liver in order to normalize the influence of weight variance of the liver on the luciferase activity. Each experimental group was carried out three times independently.

2.8. Preparation of Tumor Mouse Model

To acclimatize RLmale 1 lymphoma cells *in vivo* conditions, the cells were intravenously inoculated into mice in a concentration of 5×10^6 cells/200 μl 10 mM PBS/ mouse. The mouse liver was taken out 2 weeks later, and tumor cells were isolated from the nodules formed in the liver. The cells isolated were again inoculated similarly. Then, the resulting RLmale 1 cells were intravenously inoculated at a volume of 200 μl into mice (5×10^5 cells/mouse) to obtain mice bearing liver tumor. Previous reports experimentally demonstrate that

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the inoculation of RLmale 1 cells forms tumor nodes in the liver with high reproducibility.[41]

2.9. Suppression Effect of Polyion Complexes on Tumor Growth in the Liver

Saline or HEPES solution containing spermine-pullulan with a percentage of spermine introduced of 5.60, 20 μg of free pCAGGS-NK4 plasmid DNA, and the plasmid DNA complex with the derivative of pullulan at a N/P ratio of 1.5 was intravenously injected at the injection volume of 200 μl/mouse (10 mice/group). RLmale 1 cells were intravenously inoculated into every mouse (5×10^5 cells/200 μl HEPES/mouse) one day later. The mice were observed daily to record their survival period. Every 2 days after tumor inoculation, the blood was randomly taken from the orbital plexus of 5 mice and the plasma was extracted via centrifugation (3000 rpm, 10 min, 4 °C). Then, the liver-specific cytosolic enzyme activity of glutamic pyruvate transaminase (GPT) was analyzed on the basis of the Reitman-Frankel method (Wako Pure Chemical Industries, LTD., Osaka, Japan). The liver ($n = 3$ each) was removed 8 days after tumor inoculation, fixed with 10 wt% formalin aqueous solution, sectioned, and stained with hematoxylin and eosin to evaluate the histological responses at the tumor tissue.

2.10. 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 the statistical difference between the survival curves was determined with a logrank test and significance was accepted at $P < 0.05$.

3. RESULTS

3.1. Characterization of Spermine-Pullulan-Plasmid DNA Complexes

Spermine was introduced to the hydroxyl groups of pullulan in a dose-dependent fashion by the CDI activation method, and the percentage of spermine introduced could be changed by altering the amount of CDI added initially (Table I).

Figure 2 shows the electrophoretic patterns of polyion complexes of plasmid DNA and spemine-pullulan with different extents of spermine introduced prepared at a N/P ratio of 1.5. Migration of plasmid DNA was retarded with an increase in the introduction extent. No retardation was observed for the complex of spemine-pullulan with an introduction extent of 2.69.

Table II summarizes the physicochemical properties of polyion complexes prepared at a N/P ratio of 1.5. The apparent molecular size and zeta potential of free plasmid DNA were around 400 nm and −15 mV, respectively. On the other hand, the polyion complexes of spermine-pullulan with an introduction extent of 5.60 exhibited apparent molecular size larger than other complexes. The zeta potential became constant at introduction extents of 11.0 or higher, while the molecular size decreased to around 200 nm. Neither of molecular size nor zeta potential was obtained for the complex of the spermine-pullulan with an introduction extent of 2.69.

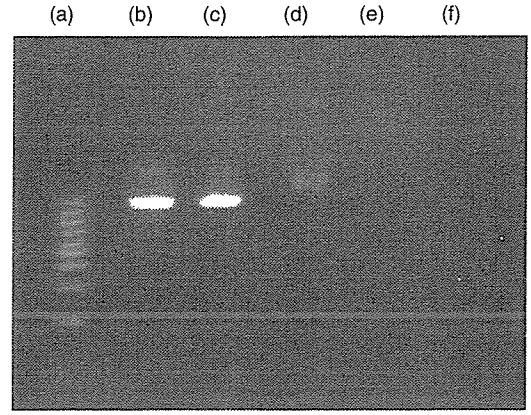

Fig. 2. Agarose gel electrophoresis of free plasmid DNA and complexes of plasmid DNA and spermine-pullulan with different extents of spermine introduced: (a) DNA marker, (b) free plasmid DNA or the complexes of spermine-pullulan with introduction extents of (c) 2.69, (d) 5.60, (e) 11.0, and (f) 23.0.

3.2. *In Vivo* Gene Transfection in the Liver

Figure 3 shows the effect of spermine introduction extent on the level of gene expression in the liver. The level of gene expression depended on the extent of spermine-pullulans used. The level for the complex of spermine-pullulan with an introduction extent of 5.60 was significantly higher than that of other complexes and free plasmid DNA. When the effect of the N/P ratio on the liver gene expression was examined, complexes prepared at N/P ratios of more than 1.5 did not enhance gene expression (data not shown). Therefore, the complex was used for the following tumor suppression experiment. No gene

Table II. Apparent molecular size and zeta potential of free plasmid DNA and complexes of plasmid DNA and spermine-pullulan with different extents of spermine indtroduced.

Sample	Percent introduced[a]	Apparent molecular size (nm)	Zeta potential (mV)
Spemine-pullulan-plasmid DNA complex	2.69	n.d.	n.d.
	5.60	2198.5 ± 0[b]	+7.00 ± 0.32[b]
	11.0	326.5 ± 139.5	+14.3 ± 0.24
	23.0	282.0 ± 166.6	+13.6 ± 0.22
Free plasmind DNA		409.5 ± 61.2	−14.7 ± 9.5

[a]Molar percentage of spermine introduced to the hydroxyl groups of pullulan.
[b]Means ± S.D.

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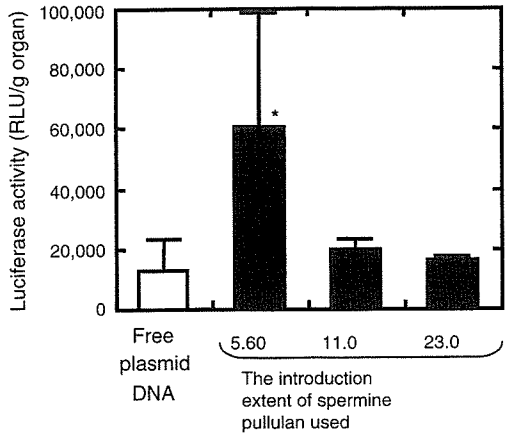

Fig. 3. Liver luciferase activity of free plasmid DNA and complexes of plasmid DNA and spermine-pullulan with different extents of spermine introduced in the liver of mice 1 day after intravenous injection. The plasmid DNA amount injected is 20 μg/mouse and the N/P ratio is 1.5. *, $p < 0.05$; versus the activity of plasmid DNA complexes prepared with other spermine-pullulans.

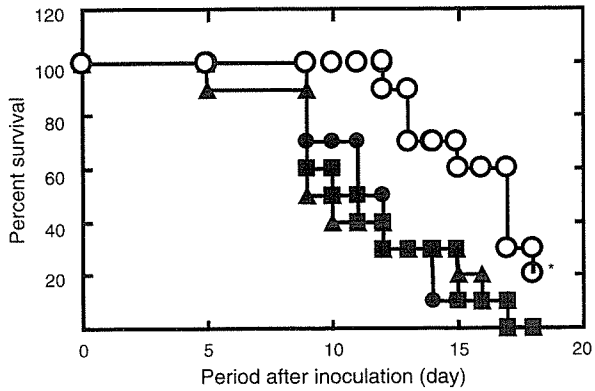

Fig. 4. Survival curves of RLmale 1 tumor-bearing mice receiving the intravenous injection of spermine-pullulan-plasmid DNA complexes: spermine-pullulan-plasmid DNA complexes containing 20 μg (○—) of NK4 plasmid DNA, 20 μg of free NK4 plasmid DNA (■—), spermine-pullulan (▲—), and saline (●—). *, $p < 0.05$; versus the survival curve of other experimental groups.

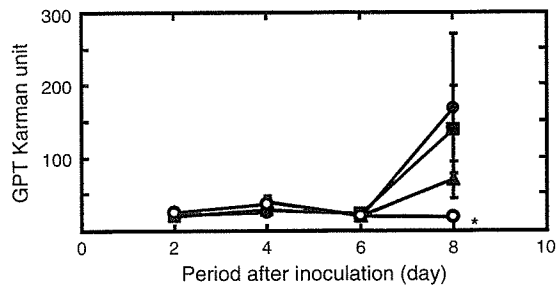

Fig. 5. Time change in the plasma GPT activity of RLmale 1 tumor-bearing mice mice receiving the intravenous injection of spermine-pullulan-plasmid DNA complexes: the spermine-pullulan-plasmid DNA complexes containing 20 μg of NK4 plasmid DNA(○—), 20 μg of free NK4 plasmid DNA (■—), spermine-pullulan (▲—), and saline (●—). *, $p < 0.05$; versus the activity of other experimental groups at the corresponding day.

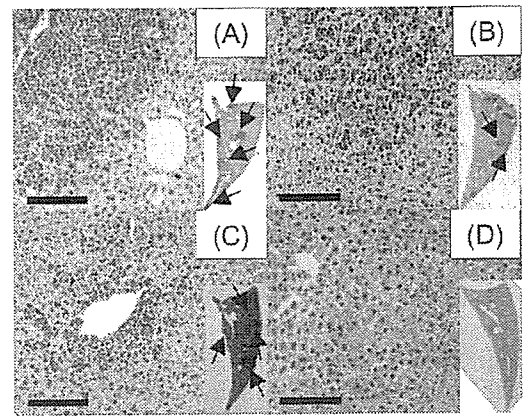

Fig. 6. Liver histological sections (inset: weak magnification) of RLmale 1 tumor-bearing mice 9 days after intravenous injection of (A) saline, (B) spermine-pullulan, (C) 20 μg of NK4 free plasmid DNA, and (D) the spermine-pullulan-plasmid DNA complexes containing 20 μg of NK4 plasmid DNA. Arrows indicate tumor cells mass. The length of scale bar is 100 μm.

expression was observed in the spleen, lung, and kidney after the intravenous injection of the complex (data not shown).

3.3. *In Vivo* Antitumor Effect of Spermine-Pullulan-NK4 Plasmid DNA Complex on RLmale 1 Tumor

Figure 4 shows the survival curve of mice applied with spermine-pullulan-NK4 plasmid DNA complexes. The survival date of mice significantly prolonged for the complex group than that of other groups. Figure 5 shows the time profile of plasma GPT activity of mice receiving the injection of spermine-pullulan-NK4 plasmid DNA complexes. It is known that glutamic pyruvate transaminase (GPT) is one of the key factors to indicate liver functions. The GPT activity is generally low in the normal state, but it becomes high when the liver function gets worse. The plasma GPT level was significantly lower for the complex group than that of other groups.

Figure 6 shows the liver histological sections of tumor-bearing mice following the intravenous injection of spermine-pullulan-NK4 plasmid DNA complexes. For the liver of mice receiving the complex application, few tumor cells was observed and the liver appearance seemed to be histologically normal.

4. DISCUSSION

The present study demonstrates that the *in vivo* gene expression of plasmid DNA in the liver was greatly enhanced by complexation with the spermine-pullulan. However, the expression level was influenced by the spermine introduction extent of spermine-pullulan used. Liver targeting of NK4 plasmid DNA with the

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spermine-pullulan suppressed tumor growth in the liver and prolonged the survival period of tumor-bearing mice.

It has been demonstrated that complexation of plasmid DNA with cationized polymers has several advantages over naked plasmid DNA. Especially, under *in vivo* conditions, complexation with the cationized polymer increases the stability of plasmid DNA. For example, some researches have experimentally indicated that polyion complexation prevents the plasmid DNA from the enzymatic degradation by DNase attack.[42–44] However, there are several drawbacks of cationized polymers to be improved, such as the low efficiency of gene transfection compared with that of viral vectors. In addition, low selectivity for the target cells is practically problematic. This is because the cationized polymer of positive charge electrostatically interacts with the cell surface of negative charge in a non-specific manner. Considering systemic gene delivery, it is important to develop a strategy for the targeting of plasmid DNA to an appropriate body site. Some trails have been experimentally performed to target plasmid DNA to the liver.[8–10] To facilitate the selective targeting to hepatocytes, the ligands for asialoglycoprotein receptors have been positively used to modify the carrier materials for plasmid DNA.[11–15] In this study, pullulan is selected because it has an inherent affinity for the liver.[16–18] Pullulan is a water-soluble, naturally occurring polysaccharide and was found to accumulate in the liver at significantly higher amounts than other water-soluble polymers conventionally used. However, since it is a nonionic polymer and cannot electrostatically interact with plasmid DNA, spermine was coupled covalently to allow pullulan to cationize (Table I). In this study, spermine was selected to cationize pullulan because it is a polyamine present in the body and showed a "buffering" effect to minimize the increase of endosomal pH.[45] Previous studies demonstrate that the cationized pullulan of spermine derivative is more effective in enhancing the *in vitro* level of gene expression than other cationized derivatives (unpublished data). As expected, plasmid DNA could be complexed with the spermine-pullulan (Table II and Fig. 1), and consequently the complexation enabled the plasmid DNA to target to the liver based on the liver affinity of pullulan (Fig. 2). No gene expression in the liver was observed following the intravenous injection of the plasmid DNA complexed with spermine at a N/P rario of 1.5 (data not shown).

Our previous study revealed that the level of *in vitro* gene expression of plasmid DNA complexed with the spermine-pullulan for HepG2 cells of a human hepatoblastoma cell lines depended on the extent of spermine introduced. The highest level was detected for the complex of spermine-pullulan with an introduction extent of 23.0 while the complex with the extent of 5.60 showed a lower level (unpublished data). On the contrary, in this study, the highest gene expression was observed at that extent. These results clearly indicate that there is difference in the optimal physicochemical properties of complexes for efficient gene expression between the *in vitro* and *in vivo* conditions. Such difference is also reported for a cationized liposome of gene carrier.[46] The highest level of gene expression was observed for the complex with the largest apparent molecular size (Fig. 2). However, the reason is unclear at present. One of the plausible reasons is that the complex had the lowest positive zeta potential among the complexes used. It is well known that highly positively-charged substances non-specifically interact with the blood components and blood vessel endothelium of negative charged when systemically injected.[47,48] It is possible that the complex with the lowest positive charge is the least susceptible to the non-specific interaction, resulting in efficient liver targeting of plasmid DNA. Some researches have demonstrated that the molecular size of the plasmid DNA complexes ranges 100 to 200 nm for cationized liposomes to augment gene expression.[48,49] The present spermine-pullulan-plasmid DNA complex showed a larger molecular size. The reason of difference in the complex size for enhanced gene expression *in vitro* or *in vivo* is not clear.

Tumor therapy with angiogenesis inhibitors has been increasingly noted.[50–53] NK4 suppresses the angiogenic actions induced by vascular endothelial growth factor and basic fibroblast growth factor.[32,54] On the basis of the bifunctional characteristics of NK4 to suppress both the tumor angiogenesis and HGF-mediated invasion, it is highly expected that NK4 can function as a bioactive molecule effective for tumor therapy. For NK4 plasmid DNA complexed with the spermine-pullulan, the survival of mice bearing RLmale 1 cells prolonged (Fig. 3). The plasma GPT level and the number of tumor cells grown in the liver were low compared with free NK4 plasmid DNA (Figs. 4 and 5). It is likely that complexation with the spermine-pullulan significantly enabled the NK4 plasmid DNA to target to the liver and consequently enhance the expression level of NK4 plasmid DNA specifically in the liver, resulting in augmented suppression of *in vivo* tumor growth thereat. The data strongly indicate that gene expression prior to tumor growth efficiently suppressed the liver engraftment of tumor cells and the subsequent growth. However, based on the present data from an animal experiment to prevent tumor growth, we cannot say that the complex system is also therapeutically effective in tumor suppression.

It is concluded that gene targeting delivery of plasmid DNA with the spermine-pullulan is a promising technology to enhance the *in vivo* gene expression at the liver. This liver targeting system is applicable to other types of plasmid DNA and oligonucleotides for *in vivo* gene expression.

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Received: 17 January 2006. Revised/Accepted: 4 April 2006.

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Journal of Controlled Release 112 (2006) 249–256
journal of controlled release
www.elsevier.com/locate/jconrel
GENE DELIVERY

Controlled release of plasmid DNA from hydrogels prepared from gelatin cationized by different amine compounds

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Received 31 August 2005; accepted 6 February 2006
Available online 20 March 2006

Abstract

This paper is an investigation to compare the in vivo controlled release of a plasmid DNA from biodegradable hydrogels prepared from gelatin cationized by different amine compounds, ethylenediamine, putrescine, spermidine, and spermine and the consequent profile of gene expression. Cationized gelatin prepared through the chemical introduction of each amine compound was crosslinked by various concentrations of glutaraldehyde to obtain cationized gelatin hydrogels for the carrier of plasmid DNA release. When the cationized gelatin hydrogels incorporating ^{125}I-labeled plasmid DNA were implanted into the femoral muscle of mice, the radioactivity remaining decreased with time and the retention period of radioactivity prolonged with a decrease in the water content of hydrogels. When ^{125}I-labeled cationized gelatin hydrogels with the higher water content was implanted, the radioactivity remaining was decreased faster with time. The remaining time profile of plasmid DNA radioactivity was in good accordance with that of hydrogel radioactivity, irrespective of the type of cationized gelatin. Following intramuscular implantation, any cationized gelatin hydrogel incorporating plasmid DNA enhanced the expression level of plasmid DNA to a significantly higher extent than the free plasmid DNA injection. In addition, prolonged time period of gene expression was observed although there was no significant difference in the expressed period between the cationized gelatin hydrogels. It was concluded that plasmid DNA of biological activity was released from every cationized gelatin hydrogel accompanied with the in vivo degradation, resulting in enhanced and prolonged gene expression.

Keywords: Plasmid DNA; Cationized gelatin hydrogel; Controlled release; Hydrogel degradation; Gene expression

1. Introduction

Recently, gene therapy has been increasingly noted for the clinical applications to cancer and congenital immunological diseases [1,2]. To this end, many basic and clinical researches have been performed to develop viral and non-viral carriers for enhanced gene expression [3,4]. The former carrier is widely used for gene therapy, because of the high efficiency of gene expression. However, there are some problems to be resolved, e.g. the virus antigenicity. On the other hand, for the latter carrier, less transfection efficiency than the viral carrier is one of the largest issues although it has several advantages in terms of easy production, safety, and low cost. As trials to improve the transfection efficiency, several technologies have been added to chemically and biologically design the carrier, while physical stimuli, such as electricity and ultrasound, have been tried to co-use [5–7].

Once a carrier-plasmid DNA complex in the solution was injected into the body, the gene expression cannot be always expected because of the easy diffusion away from the injected site. Drug delivery system (DDS) is a promising technology to overcome the poor gene expression. For example, it is practically possible to achieve the controlled release of plasmid DNA over an extended time period by incorporating plasmid DNA into a carrier matrix. It is conceivable that the release technology enables the plasmid DNA to enhance the transfection probability at the applied site, resulting in promoted gene expression. It has been demonstrated that the release of plasmid DNA with a biodegradable poly(lactic acid) matrix augmented

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doi:10.1016/j.jconrel.2006.02.003

the expression efficiency [8]. A collagen minipellet is reported to be effective in prolonging the period of gene expression [9]. However, little has been investigated on the influence of the property of release matrices on the level and time profile of gene expression induced by plasmid DNA released.

We have prepared biodegradable hydrogels from gelatin cationized by ethylenediamine as a release carrier of a plasmid DNA and succeeded in enhancing their in vivo biological activities which cannot be always detected only by the administration in the solution form [10–12]. In this hydrogel system, negatively charged plasmid DNA physicochemically immobilized in the cationized gelatin hydrogel can be released only when the hydrogel is degraded to generate water-soluble cationized gelatin fragments [10–12]. However, there was no basic information about the effect of diamine compounds to be used for gelatin cationization on the hydrogel-induced gene expression. In this study, hydrogels with different biodegradabilities were prepared from gelatin cationized by various amine compounds and the characteristics of gene expression was compared in terms of the type of cationization and the hydrogel degradability. We also examine inflammatory response to every cationized gelatin hydrogel in terms of interleukin 1β (IL-1β) production.

2. Materials and methods

2.1. Materials

A gelatin sample, prepared through an acid process of pig skin type I collagen, was kindly supplied from Nitta Gelatin Inc., Osaka, Japan. Ethylenediamine [$NH_2(CH_2)_2NH_2$] was purchased from Wako Pure Chemical, Ltd., Osaka, Japan. Putrescine [$NH_2(CH_2)_4NH_2$], spermidine [$NH_2(CH_2)_4NH(CH_2)_3NH_2$], and spermine [$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$] were purchased from Sigma-Aldrich Co. (MO, USA). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt (EDC), 2,4,6-trinitrobenzensulfonic acid, β-alanine, and glutaraldehyde were purchased from Nacalai Tesque Inc., Kyoto, Japan. *N*-succinimidyl-3-(4-hydroxy-3,5-di [^{125}I] iodophenyl) propionate (^{125}I-Bolton-Hunter Reagent, NEX-120H, 147 MBq/ml in anhydrous benzene) was purchased from NEN Research Products, DuPont, Wilmington, DE.

2.2. Preparation of cationized gelatin

Ethylenediamine, putrescine, spermidine or spermine and 5 g of EDC were added into 250 ml of 100 mM phosphate-buffered solution containing 5 g of gelatin at their molar ratio to the carboxyl groups of gelatin of 50. Immediately after that, the pH of solution was adjusted at 5.0 by adding 5 M of HCl. The reaction mixture was agitated at 37 °C for 18 h, and then dialyzed against double-distilled water (DDW) for 48 h at 25 °C. The dialyzed solution was freeze-dried to obtain a cationized gelatin. The percentage of amino groups introduced into gelatin was determined by the conventional trinitrobenzene sulfonate method [13]. After calculation based on the calibration curve prepared by using β-alanine, the percentage of cationized gelatin prepared was 50.9±1.1, 45.9±1.0, 48.1±1.0, and 49.0±1.1 mol/mol% of carboxyl groups of gelatin when ethylenediamine, putrescine, spermidine, and spermine were used, respectively.

2.3. Preparation of cationized gelatin hydrogels

An aqueous solution of 10 wt.% each cationized gelatin prepared (800 μl) was cast into a polytetrafluoroethylene mold (2×2 cm^2, 0.8 mm depth), followed by leaving at 4 °C overnight for gelation. The cationized gelatin gelled was crosslinked in mixed HCl/acetone (3:7 vol/vol) solution containing various amounts of glutaraldehyde. Crosslinking reaction was allowed to proceed for 24 h at 4 °C and then, the resulting hydrogel sheets were immersed in 100 mM glycine aqueous solution at 4 °C for 24 h to block the residual aldehyde groups of glutaraldehyde. The hydrogel sheets were cut out to obtain hydrogel discs (5×5×1 mm^3) and rinsed 3 times with DDW at 4 °C and freeze-dried. The freeze-dried hydrogels (2 mg) were sterilized by ethyleneoxide gas. No change in the hydrogel shape was observed before and after the freeze-drying and sterilization processes. The water content of cationized gelatin hydrogels prepared was determined as a measure to compare their crosslinking extent. After the freeze-dried hydrogel was swollen at 37 °C for 24 h in 100 mM of phosphate-buffered saline solution (PBS, pH 7.4), the weight of swollen hydrogel (W_s) was measured. The weight of freeze-dried hydrogel (W_d) was measured and the water content, which is defined by $((W_s - W_d)/W_s) \times 100$, was calculated from the W_s and W_d values.

2.4. Radiolabeling of cationized gelatin hydrogels

Cationized gelatin hydrogels prepared from different amine compounds were radiolabeled by use of ^{125}I-Bolton–Hunter reagent. Briefly, 100 μl of ^{125}I-Bolton–Hunter reagent solution in anhydrous benzene was bubbled with dry nitrogen gas until benzene evaporation was completed. Then, 125 μl of 0.1 M sodium borate-buffered solution (pH 8.5) was added to the dried reagent, followed by pipetting to prepare aqueous ^{125}I-Bolton–Hunter solution. The freeze-dried discs of cationized gelatin hydrogels (2 mg) were impregnated with the aqueous solution prepared at a volume of 20 μl per disc. The radiolabeled cationized gelatin hydrogel discs were rinsed with DDW by exchanging it periodically at 4 °C for 4 days to exclude non-coupled, free ^{125}I-labeled reagent from ^{125}I-labeled cationized gelatin hydrogels. When measured periodically, the radioactivity of DDW returned to the back ground level after rinsing for 3 days. No shape change of swollen hydrogels was observed during the radiolabeling and the subsequent rinsing process.

2.5. DNA isolation

The plasmid DNA used was the expression vector consisting of coding sequence of lacZ and a SV40 promoter inserted at the

upstream (7.9 kbp). The plasmid DNA was purified by column chromatography with Qiagen EndoFree™ plasmid kit (Qiagen, USA). When measured to ascertain the purity of plasmid DNA prepared by UV spectroscopy, the absorption ratio of wavelength 260 to 280 nm was 1.8–1.9.

2.6. Radiolabeling of lacZ plasmid DNA

PBS containing 2.5 mg/ml of lacZ plasmid DNA (1.0 ml) was added to the ^{125}I-Bolton–Hunter reagent nitrogen dried. The resulting solution was kept at 37 °C overnight to introduce ^{125}I residue into the amino groups of lacZ plasmid DNA. Non-coupled, free ^{125}I-labeled reagent was separated from ^{125}I-labeled lacZ plasmid DNA by gel filtration with a PD-10 column (Amersham Pharmacia Biotech K.K, Tokyo, Japan).

2.7. Preparation of cationized gelatin hydrogels incorporating lacZ plasmid DNA

Impregnation of lacZ plasmid DNA into freeze-dried cationized gelatin hydrogels (2 mg) was carried out by use of PBS containing 100 μg of lacZ plasmid DNA. LacZ plasmid DNA-free, empty cationized gelatin hydrogels were prepared in a similar way except for using lacZ plasmid DNA-free PBS. Briefly, 20 μl of PBS with or without lacZ plasmid DNA was dropped onto the dried hydrogel discs, followed by leaving at 4 °C overnight for swelling to obtain cationized gelatin hydrogels incorporating lacZ plasmid DNA or those without lacZ plasmid DNA incorporation. The lacZ plasmid DNA solution was fully sorbed into the dried cationized gelatin hydrogels during the swelling process because the solution volume was much less than that theoretically impregnated into each hydrogel, irrespective of the water content of hydrogels. Similarly, an aqueous solution of ^{125}I-labeled lacZ plasmid DNA was sorbed into freeze-dried cationized gelatin hydrogel discs to prepare cationized gelatin hydrogels incorporating ^{125}I-labeled lacZ plasmid DNA. Every hydrogel disc prepared by this procedure had similar appearance during the radiolabeling.

2.8. Estimation of in vivo degradation of cationized gelatin hydrogels

^{125}I-labeled cationized gelatin hydrogels with different water contents were implanted into the femoral muscle of ddY mice, 6–7 weeks old (Nihon SLC, Japan). At 1, 3, 7, 10, 14, and 21 days after hydrogel implantation, the mouse muscle containing the implanted hydrogel ($1\times1\times1$ cm^3) was taken out to measure the radioactivity on a gamma counter (ARC-301B, Aloka Co., Ltd., Tokyo, Japan). The radioactivity ratio of the muscle sample to the hydrogel implanted initially was measured to express as the percentage of remaining activity in the hydrogel.

For each experimental group, three mice were sacrificed at each time point for in vivo evaluation unless otherwise mentioned. All the animal experiments were done according to the Institutional Guidance of Kyoto University on animal experimentation.

2.9. Estimation of in vivo lacZ plasmid DNA release from cationized gelatin hydrogels incorporating lacZ plasmid DNA

Following implantation of ^{125}I-labeled lacZ plasmid DNA-incorporated cationized gelatin hydrogels with different water contents into the femoral muscle of mice, at different time intervals, the mouse muscle containing the cationized gelatin hydrogel was taken out. As control, the solution of ^{125}I-labeled lacZ plasmid DNA in PBS was slowly injected into the femoral muscle (100 μl/site). The radioactivity of the muscle sample was measured on the gamma counter and the ratio to the initial radioactivity of lacZ plasmid DNA in the hydrogel-incorporated or solution form was expressed as the percentage of radioactivity remaining.

2.10. In vivo assessment of gene expression following implantation of cationized gelatin hydrogels incorporating lacZ plasmid DNA

LacZ plasmid DNA-incorporated cationized gelatin hydrogels with different water contents were implanted into the femoral muscle of mice. As a control, 100 μl of lacZ plasmid DNA solution was slowly injected into the femoral muscle. The lacZ plasmid DNA dose was 100 μg/mouse and 6 mice were used at each time point for every experimental group. The mice were sacrificed 1, 3, 7, 14, and 21 days after lacZ plasmid DNA treatment to evaluate gene expression.

For evaluation of gene expression at the treated muscle, β-galactosidase activity was measured by use of X-gal stain kit (Invitrogen Co. USA). Briefly, the muscle samples were immersed and homogenized in a lysis buffer (0.1 M Tris–HCl, 2 mM EDTA, 0.1% Triton X-100) at the lysis buffer volume (ml)/sample weight (mg) ratio of 4 to 1 in order to normalize the influence of weight variance on the β-galactosidase assay. The sample lysate (2 ml) was transferred to a centrifuge tube, followed by freeze-and-thaw 3 times and centrifugation at 15,000 ×g at 4 °C for 5 min. The supernatant (30 μl) was mixed with 70 μl of aqueous solution containing 4 mg/ml o-nitrophenyl beta-D-galactopyranoside (ONPG) and 200 μl of cleavage buffer (60 mM Na_2HPO_4–$7H_2O$, 40 mM NaH_2PO_4–H_2O, 10 mM KCl, and 1 mM $MgSO_4$–$7H_2O$, pH 7) in a fresh microcentrifuge tube. After incubation at 37 °C for 30 min, 500 μl of 1 M sodium carbonate solution was added to the solution mixture. The solution absorbance was measured at the wavelength of 420 nm to evaluate the β-galactosidase activity. The number of muscle samples was four for each experimental group.

2.11. Evaluation of IL-1β level at the muscle tissue of mice after implantation of cationized gelatin hydrogels

As a measure of inflammation response to the cationized gelatin hydrogels, the tissue level of IL-1β was determined. After implantation of the hydrogel into the femoral muscle, the muscle around the implanted site was taken out and lysed. An enzyme-linked immunosorbent assay (ELISA) kit (R&D

Table 1
Preparation and characterization of cationized gelatin hydrogels used

Code	Concentration of glutaraldehyde (μg/ml)	Water content (wt.%) of cationized gelatin hydrogels			
		Ethylenediamine NH_2–$(CH_2)_2$–NH_2	Putrescine NH_2–$(CH_2)_4$–NH_2	Spermidine NH_2–$(CH_2)_3$–NH–$(CH_2)_4$–NH_2	Spermine NH_2–$(CH_2)_3$–NH–$(CH_2)_4$–NH–$(CH_2)_3$–NH_2
I	31.3	96.4±0.5[a]	95.8±1.1	95.2±1.5	96.2±0.8
II	0.78	97.4±0.9	97.6±0.6	97.3±0.8	97.4±0.8
III	0.31	98.3±1.1	98.1±0.8	98.8±1.2	98.3±0.6
IV	0.16	99.7±1.0	99.1±0.3	99.3±1.1	99.2±1.1

Hydrogel was prepared by glutaraldehyde crosslinking of cationized gelatin which is chemically derivatized by different amine compounds, ethylenediamine, putrescine, spermidine, and spermine.
[a] The mean±SD.

Systems, Inc., USA) was used according to the manufacturer's specifications. This assay is based on the quantitative sandwich enzyme immunoassay technique. The lysate was centrifuged (14,000 rpm, 15 min, 4 °C) to prepare the supernatant. An affinity purified polyclonal antibody specific for mouse IL-1β was pre-coated onto each well of 96-multiwell microplate. Then, the supernatant prepared and a standard IL-1β sample were pipetted into the wells. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse IL-1β was added to the wells. After the unbound antibody-enzyme reagent was removed completely, a substrate solution was added to the wells. The intensity of solution color was measured at the wavelength of 540 nm while it is in proportion to the amount of mouse IL-1β bound in the initial step. The IL-1β amount was obtained by the calibration curve obtained with the standard sample.

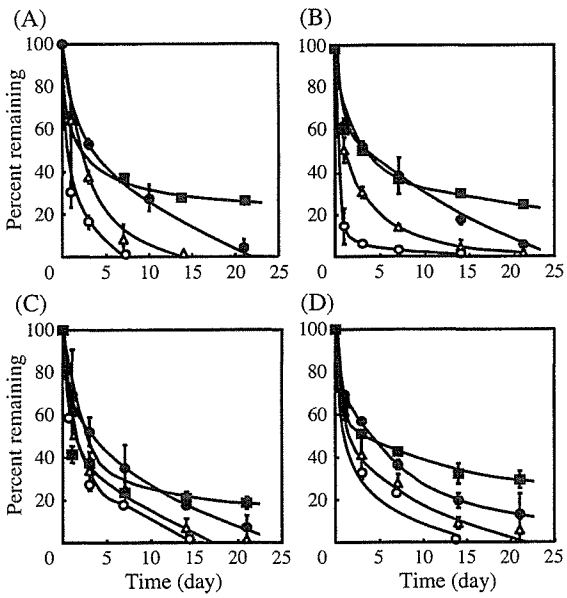

Fig. 1. The time course of the radioactivity remaining after implantation of hydrogels prepared from ^{125}I-labeled gelatin cationized by ethylenediamine (A), putrescine (B), spermidine (C), and spermine (D) into the femoral muscle of mice: (○) code I, (△) II, (●) III, and (■) IV hydrogels.

2.12. Statistical analysis

All the data were analyzed by Students' *t*-test and results were expressed as means±the standard derivation of the means. Statistical significance was accepted at $p<0.05$.

3. Results

3.1. Characterization of cationized gelatin hydrogels

Table 1 shows the water content of hydrogels prepared from gelatin cationized by different amine compounds at various concentrations of glutaraldehyde. When the glutaraldehyde concentration was changed in hydrogel preparation, the water content of cationized gelatin hydrogels could be changed: the higher the concentration of glutaraldehyde, the lower the water

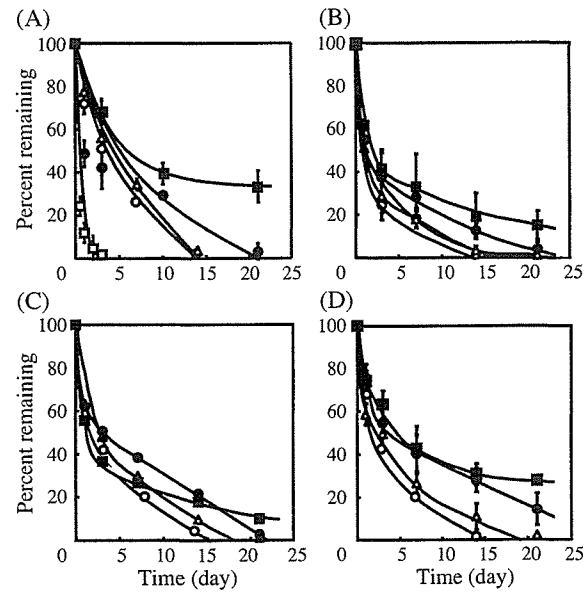

Fig. 2. The time course of the radioactivity remaining after implantation of ^{125}I-labeled lacZ plasmid DNA-incorporated hydrogels prepared from gelatin cationized by ethylenediamine (A), putrescine (B), spermidine (C), and spermine (D) into the femoral muscle of mice: (○) code I, (△) II, (●) III, and (■) IV hydrogels. (□) Indicates the radioactivity remaining after the intramuscular injection of ^{125}I-labeled lacZ plasmid DNA in the solution form.

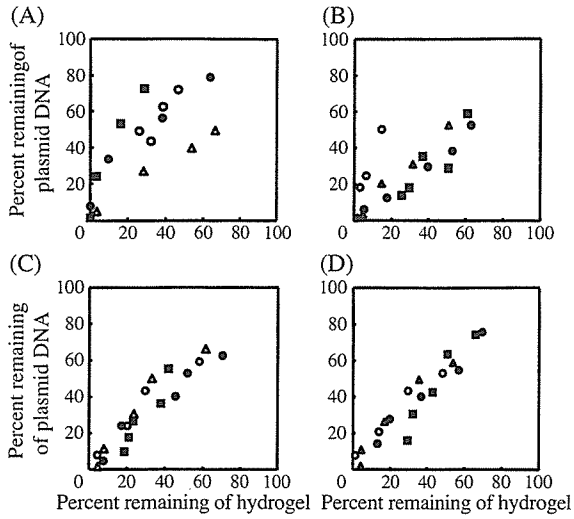

Fig. 3. The radioactivity remaining of cationized gelatin hydrogels incorporating ^{125}I-labeled lacZ plasmid DNA plotted against that of ^{125}I-labeled cationized gelatin hydrogels after implantation into the femoral muscle of mice: (○) code I, (△) II, (●) III, and (■) IV hydrogels. The hydrogels were prepared from gelatin cationized by ethylenediamine (A), putrescine (B), spermidine (C), and spermine (D).

content of hydrogels. Irrespective of the type of amine compounds introduced, there was no difference in the water content between the cationized gelatin hydrogels.

3.2. In vivo degradation of cationized gelatin hydrogels

Fig. 1 shows the time course of radioactivity remaining after implantation of ^{125}I-labeled cationized gelatin hydrogels prepared from different amine compounds into the femoral muscle of mice. Irrespective of the amine compound type, the radioactivity remaining decreased with time for every hydrogel sample, but the hydrogels with lower water contents retained the radioactivity for longer time periods than that of hydrogels with higher water contents.

3.3. In vivo release profile of lacZ plasmid DNA from cationized gelatin hydrogels incorporating lacZ plasmid DNA

Fig. 2 shows the decrement patterns of lacZ plasmid DNA radioactivity after implantation of cationized gelatin hydrogels incorporating ^{125}I-labeled lacZ plasmid DNA into the femoral muscle of mice. Irrespective of the type of cationized gelatin hydrogels, the residual radioactivity of lacZ plasmid DNA in cationized gelatin hydrogels decreased with implantation time. The decrement pattern of radioactivity greatly depended on the water content of cationized gelatin hydrogels: the lower the water content of hydrogels, the longer their radioactivity retention in the hydrogels. On the contrary, for free ^{125}I-labeled lacZ plasmid DNA, the radioactivity rapidly disappeared from the injected site within 3 days.

3.4. Relationship of remaining profile between lacZ plasmid DNA-incorporated and the cationized gelatin hydrogel

Fig. 3 shows the percent radioactivity remaining of lacZ plasmid DNA plotted as a function of that of cationized gelatin hydrogel as the release carrier. The remaining percentage of lacZ plasmid DNA incorporated in the hydrogel corresponded well with that of the hydrogel, irrespective of the hydrogel water content. Interestingly, the remaining percentage of lacZ plasmid DNA tended to be large compared with that of the corresponding cationized gelatin hydrogel.

3.5. Time course of gene expression

Fig. 4 shows the time course of gene expression following the intramuscular implantation of cationized gelatin hydrogels

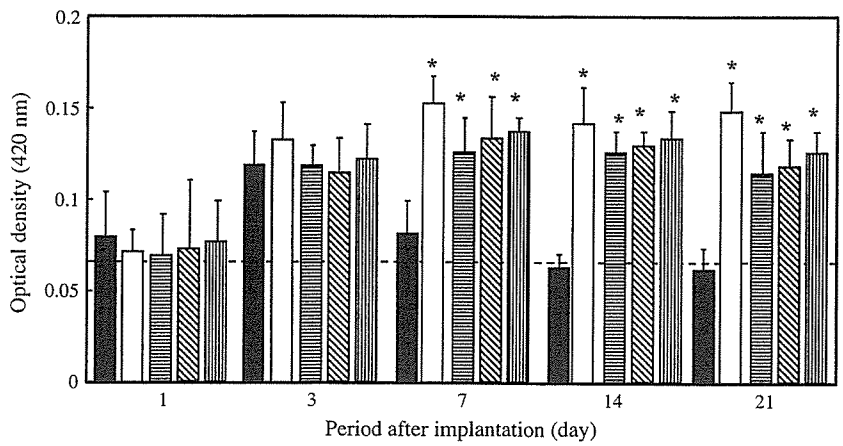

Fig. 4. The time course of lacZ gene expression after implantation of cationized gelatin Code II hydrogels prepared from gelatin cationized by ethylenediamine (□), putrescine (▤), spermidine (▨), and spermine (▥) incorporating lacZ plasmid DNA or after injection of free lacZ plasmid DNA (■) into the femoral muscle of mice. The lacZ plasmid DNA dose is 100 μg/mouse muscle. A dotted line indicates the level of gene expression after injection of saline and implantation of cationized gelatin hydrogel alone into the femoral muscle. The level was similar, irrespective of the type of cationized gelatin. *$p<0.05$: significant difference against the optical density of control group receiving the injection of free lacZ plasmid DNA.

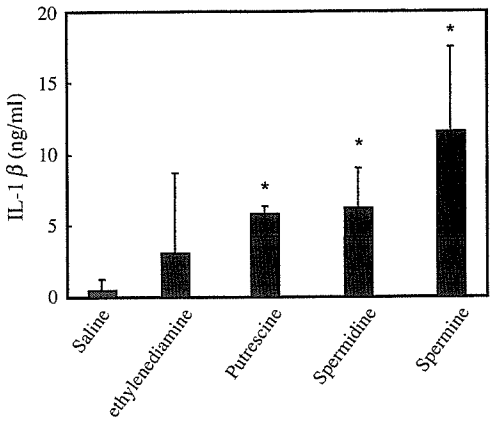

Fig. 5. The IL-1β level at the muscle tissue of mice after implantation of hydrogels prepared from gelatin cationized by different amine compounds. *$p<0.05$: significant difference against the IL-1β concentration of saline-injected control group.

incorporating lacZ plasmid DNA or intramuscular injection of PBS containing lacZ plasmid DNA. The injection of lacZ plasmid DNA solution showed significant gene expression only at 3 days after injection, but thereafter the expression level returned to the basal one. The level of gene expression was not enhanced by implantation of lacZ plasmid DNA-free, empty cationized gelatin hydrogels. On the contrary, any cationized gelatin hydrogel incorporating lacZ plasmid DNA enhanced significantly the expression level as well as prolonged the duration time period. However, no significant difference in the level of gene expression and the prolonged time profile was observed between the cationized gelatin hydrogels.

3.6. In vivo inflammatory response to cationized gelatin hydrogels

Fig. 5 shows the level of IL-1β produced following implantation of cationized gelatin hydrogels prepared from different amine compounds into the mouse muscle. The concentration of IL-1β, a biochemical maker to measure the extent of inflammation response, was significantly high for cationized gelatin hydrogels prepared from putrescine, spermidine or spermine compared with that of control mouse receiving saline injection. On the contrary, no significant increase in the IL-1β level was observed for the hydrogel of gelatin cationized by ethylenediamine.

4. Discussion

Generally, gelatin is not degraded by simple hydrolysis but by proteolysis. Therefore, in this study, the degradation of cationized gelatin hydrogels was examined in the mouse muscle to obtain the in vivo time profile of their radioactivity loss. As is apparent from Fig. 1, in vivo degradation rate of cationized gelatin hydrogels was mainly governed by their water content, irrespective of the type of amine compounds introduced. Generally, the water content of hydrogels is related to their extent of crosslinking. It is likely from the viewpoint of polymer science that the hydrogel with higher extents of crosslinking is less susceptible to enzymatic degradation, because of the steric difficulty in the approach of enzymes to the hydrogel polymer for digestion. As a result, the water content of hydrogels is one measure to normalize susceptibility of their degradation in vivo which has experimentally confirmed by our previous data [10–13].

From Figs. 2 and 3, every cationized gelatin hydrogel could release lacZ plasmid DNA and the time profile of plasmid DNA release was in good accordance with that of hydrogel degradation, irrespective of the type of cationized hydrogels. These findings clearly indicate the possibility that the lacZ plasmid DNA was released from the cationized gelatin hydrogel in the body as a result of hydrogel biodegradation. We can say with fair certainty that lacZ plasmid DNA molecules, once ionically complexed with the cationized gelatin, cannot be released from the cationized gelatin hydrogel unless hydrogel degradation takes place. It is likely that the lacZ plasmid DNA molecules are released from the hydrogels with being complexed with degraded gelatin fragments of positive charge. This is experimentally confirmed from a fluorescent study that the double staining image of lacZ plasmid DNA and cationized gelatin was detected around the hydrogel implanted [14]. In addition, it is possible when the cationized gelatin hydrogel is enzymatically degraded to generate water-soluble complexes of cationized gelatin plasmid DNA complexes, the complexes may be distributed to the tissues and organs other than the implanted site. In this study, we performed the radio-tracing test of plasmid DNA incorporated in cationized gelatin hydrogels after implantation in the mouse femoral muscle. As the result, no radioactive accumulation in the liver, kidney, thyroid grand, and other tissues was observed. These findings strongly suggest that gene expression will not be expected at the tissue other than the implanted site.

The local delivery of cells growth-promoting genes in a sustained manner is important to effectively induce tissue regeneration. Dr. Mooney et al. reported that the in vivo release of a plasmid DNA encoding platelet-derived growth factor (PDGF) gene with a carrier matrix of poly(lactide-co-glycolide) enhanced the matrix deposition on tissue and blood vessel formation [15,16]. Plasmid DNA carrying a gene fragment of human parathyroid hormone was released from a polymer matrix sponge called a gene-activated matrix (GAM) to induce tissue regeneration [17,18]. Incorporation into the GAM enabled a plasmid DNA to retain at a bone injury site and prolong the gene expression for a longer time period, resulting in reproducible and promoted regeneration of bone tissue. However, these synthetic materials are often less efficient and are highly toxic after repeated use; as a result, prolonged in vivo usage is not allowed. The controlled release of plasmid DNA with a minipellet of atelocollagen has been reported by Ochiya et al. to demonstrate enhanced gene expression and high therapeutic efficacy for some disease model animals [9]. Atelocollagen of low immunogenicity which is obtained by pepsin digestion of type I collagen and free from telopeptides is used as the carrier matrix, because it has been clinically employed for biomedical materials. It is proposed as the

mechanism of DNA release that plasmid DNA incorporated in the collagen matrix is released accompanied with matrix degradation. However, there has been no direct evidence to support the proposed mechanism. In addition, since collagen is not chemically modified to regulate the matrix, such as crosslinking and cationization, it will be practically difficult to control the degradability of collagen matrix and consequently the time period of DNA release from the matrix. Compared with collagen, it is easy for gelatin to perform the chemical modification, such as derivatization and crosslinking to modify the extent of complexation with the plasmid DNA and gelatin matrix degradation. On the contrary, collagen has a 3-dimensional helical structure and is water-insoluble. Therefore, from the viewpoint of chemical structure, it is more difficult to artificially control the chemical and crosslinking nature of collagen matrix.

Considering the release mechanism of cationized gelatin hydrogels, it is likely that the plasmid DNA is released from the hydrogel with being complexed with the fragment of cationized gelatin [14]. Although it is unclear that the plasmid DNA-cationized gelatin complex released has positive charge in vivo, our previous data demonstrated that the plasmid DNA complexed with cationized gelatin prepared is positively charged [6]. Therefore, the plasmid DNA-cationized gelatin complex released will have a positive in vivo. The electric charge of non-negative will facilitate the interaction of the lacZ plasmid DNA with cells and the subsequent internalization into cells because the charge of cell surface is negative. In addition, the complex released is interacted with the extra cellular matrix of negative charge around the cells [14]. Probably, such interaction would allow the complex to remain around the implanted site of hydrogels and increase the exposure frequency to cells, resulting in enhanced gene expression. There was poor correlation between the remaining percentage of plasmid DNA and gelatin hydrogel cationized by ethylenediamine or putrescine (Fig. 3). Since they have only primary amine groups, the interaction force between the plasmid DNA and the cationized derivative of gelatin would be weak compared with that of spermidine or spermine. Therefore, it is highly conceivable that the plasmid DNA was detached from the cationized gelatin of hydrogels in the body more readily.

The crosslinking extent of cationized gelatin hydrogels affects not only the water content but also the biodegradation of hydrogels. Slower degradation of cationized gelatin hydrogel with lower water contents is undoubtedly due to the higher extent of crosslinking. The present study demonstrated that lacZ plasmid DNA was released from the cationized gelatin hydrogel as a result of biodegradation of cationized gelatin, because the lacZ plasmid DNA molecules ionically complexed with cross-linked gelatin will be released only when water-soluble gelatin fragments are generated through biodegradation of gelatin hydrogels. Irrespective of the type of cationized gelatin hydrogels, the gene expression induced by the lacZ plasmid DNA incorporated in cationized gelatin hydrogels with the water content around 97 wt.% disappeared at approximately 21 days (Fig. 4). At the same time, the corresponding hydrogel was completely degraded in vivo and the lacZ plasmid DNA remaining was almost zero (Figs. 1 and 2). This finding strongly suggests that the lacZ plasmid DNA incorporated in the cationized gelatin hydrogel still maintained its transfection activity even though exposed to in vivo environment for a long time period. Interestingly, there was no difference in the time profile of gene expression between the cationized gelatin hydrogels. We have demonstrated that the cationized gelatin prepared from spermine of water-soluble-type showed the highest transfection efficiency of plasmid DNA in vitro among all the cationized gelatin prepared by introduction of ethylenediamine, putrescine, spermidine, and spermine [19]. However, unlike the water-soluble cationized gelatin, no effect of cationized type on the gene expression was observed in vivo. This difference in the gene expression between the in vitro and in vivo systems was not clear at the moment.

It is indispensable to understand the interactions between cells and biomaterials for developing implantable biomedical devices. The inflammatory responses of cells by contact with biomaterials are composed of various biological processes [20]. The cellular responses have been investigated with different kinds of cells, materials, and techniques, such as the counting of adherent cells, morphological observations, and evaluation of bioactive substances secreted from adherent cells [21–23]. Macrophage is considered to be one of the important cells contributing to the initial non-specific host response against biomedical materials and devices implanted. It is well recognized that macrophages mediate inflammation reactions by the secretion of inflammatory mediators, such as coagulation factors, lysosomal enzymes, cytokines, and growth factors. The proinflammatory cytokines, IL-1β, IL-6, and tumor necrosis factor-α are multifunctional, soluble mediators that affect various types of cells. IL-1β mediates the activation and proliferation of lymphocytes, fibroblasts, and endothelial cells [24]. Recently, many researches have been carried out to evaluate the interactions between macrophages and materials to obtain fundamental information about material biocompatibility [25–32]. The IL-1β assay revealed that a higher inflammation response was observed for cationized gelatin hydrogels prepared from diamine compounds other than ethylenediamine (Fig. 5). Since the basicity of ethylenediamine is lower than that of other amine compounds, the inflammation response of cationized gelatin prepared from ethylenediamine was lower than that from other amine compounds. Taken together, among all the diamine compounds, ethylenediamine is a compound suitable to prepare a hydrogel-type carrier of plasmid DNA for in vivo controlled release. Since the IL-1β assay revealed that a higher inflammation response was observed for cationized gelatin hydrogels prepared from diamine compounds other than ethylenediamine (Fig. 5), the plasmid DNA-cationized gelatin complex released might be rejected by immunoresponse.

The controlled release will enable the lacZ plasmid DNA not only to increase the local concentration but also to prolong the period of the enhanced concentration around the implanted site of hydrogels. It is highly conceivable that such advantages enhance the possibility of lacZ plasmid DNA to transfect, resulting in promoted and prolonged gene expression. The present study is the first report to show that the time period of

gene expression could be changed by altering that of plasmid DNA released from hydrogels of cationized gelatin prepared from different amine compounds. Since the plasmid DNA released is complexed with the cationized gelatin, this complexation functions positively for the transfection into cells. In this system, the plasmid DNA release can be regulated only by changing the hydrogel degradability which is controllable by regulating the crosslinking extent of hydrogel. This implies that the controlled release can be achieved even if the hydrogel carrier is as small as injectable microspheres. The present release concept based on polyion complexation may be applicable to any type of plasmid DNA. This release system is being presently applied to some plasmid DNAs coding growth factors to demonstrate the in vivo efficient augmentation of the biological activity. Moreover, the sequence-specific silencing of gene expression by small interference RNA (siRNA) has been augmented by the controlled release with the cationized gelatin hydrogel, which will be reported in the near future.

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J. Biomater. Sci. Polymer Edn, Vol. 17, No. 6, pp. 645–658 (2006)

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In vitro transfection of plasmid DNA by cationized gelatin prepared from different amine compounds

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Received 4 April 2005; accepted 9 December 2005

Abstract—The objective of this paper is to compare the *in vitro* transfection efficiency of a luciferase plasmid DNA using cationized gelatin prepared from different amine compounds. The compounds used here were ethylenediamine, putrescine, spermidine and spermine, chemically introduced to the carboxyl group of gelatin for the cationization. Complexation of the cationized gelatin with the plasmid DNA was performed by simply mixing the two materials at various N^+/P^- mixing ratios (the molar number ratio of amino groups of gelatin to the phosphate groups of DNA) in aqueous solution. Gel retardation studies revealed that the formation of cationized-gelatin–plasmid DNA complexes depended on the N^+/P^- mixing ratio. The stronger interaction of plasmid DNA with the cationized gelatin of spermine compared to the other cationized gelatins was observed by an ethidium bromide intercalation assay and Scatchard binding analysis. When the transfection efficiency of plasmid DNA complexed with the various cationized gelatins at different N^+/P^- mixing ratios was evaluated for mouse L929 fibroblasts, the highest transfection efficiency was observed for the complex prepared from the cationized gelatin of spermine at a N^+/P^- mixing ratio of 2. The present study indicates that there is an optimal N^+/P^- mixing ratio and a type of amine compound or cationization extent of cationized gelatin to enhance the transfection efficiency of plasmid DNA.

Key words: Cationized gelatin; gene transfection; ethylenediamine; putrescine; spermidine; spermine.

INTRODUCTION

Gene therapy involves the introduction of exogenous genes into target cells where production of the protein encoded is expected. In the case of acquired or inherited

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genetic disorders, this enables the replacement of a missing or defective gene, leading to normalization of cell functions [1–8].

DNA has been delivered or internalized into cells for gene transfection either by a viral or non-viral system. Viral carriers include adenoviruses, retroviruses, adeno-associated viruses and lentivirus [9]. Although the viral carriers possess the inherently high efficiency of gene transfection, there are some issues to be resolved for the clinical trials, such as immunological and/or toxic responses toward the carriers themselves. To tackle these problems, several genetic modulations of biological approach have been attempted for viral carriers. As another approach, non-viral carriers have been investigated to demonstrate several advantages over the viral carriers. For the non-viral transfection system, naked DNA alone or the complex of DNA with cationic liposomes, cationic lipids and cationic polymers have been utilized [10–12]. Their nature to induce toxicity and immune responses is low compared with that of viral carriers. Macromolecules, such as poly(ethylenimine) and poly(lysine), and lipids of positive charges have been designed to demonstrate their feasibility as the non-viral carrier in enhancing gene expression [13–15]. However, some points of low transfection efficacy and cytotoxicity should be improved [16].

Gelatin has extensively used for industrial, pharmaceutical and medical applications and the bio-safety has been proven through its long clinical use as biomaterial and drug ingredient [17]. Other advantages of gelatin include the availability of samples with various physicochemical natures and the simple chemical modification. For example, the positively charged, cationized gelatin can readily be prepared by introducing amine residues to the carboxyl groups of gelatin. We previously reported that when a plasmid DNA was complexed with the ethylenediamine-introduced cationized gelatin and applied to cells, the transfection efficiency of plasmid DNA was increased significantly [18–22]. However, there are only few reports on the effect of diamine compounds to be introduced on the gene expression.

The present study was undertaken to investigate the *in vitro* transfection efficiency of plasmid DNA by the cationized gelatin of non-viral carriers prepared from different types of amine compounds. Ethylenediamine, putrescine, spermidine and spermine were chemically introduced into the carboxyl groups of gelatin to prepare cationized gelatins. A plasmid DNA was mixed with the cationized gelatin at different ratios to form the complexes while their physicochemical properties were evaluated in terms of the apparent molecular size, the zeta potential and the interaction affinity for the plasmid DNA. We examined the *in vitro* transfection of plasmid DNA for mouse fibroblasts by the cationized gelatin of different amine compounds at various mixing ratios and evaluated the cytotoxicity.

MATERIALS AND METHODS

Materials

A gelatin sample (molecular weight 1×10^5), prepared through acid treatment of pig-skin type-I collagen, was kindly supplied by Nitta Gelatin. Ethylenediamine was

purchased from Wako. Putrescine, spermidine and spermine were purchased from Sigma-Aldrich. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt (EDC), 2,4,6-trinitrobenzensulfonic acid, β-alanine and ethidium bromide (EtBr) were purchased from Nacalai Tesque.

Preparation of cationized gelatin with different amine compounds

Ethylenediamine ($NH_2(CH_2)_2NH_2$), putrescine ($NH_2(CH_2)_4NH_2$), spermidine ($NH_2(CH_2)_4NH(CH_2)_3NH_2$) or spermine ($NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$) was added together with 3 g EDC into 250 ml 100 mM phosphate-buffered solution (pH 5.0) containing 5 g gelatin at different molar ratios to the carboxyl groups of gelatin (0, 1, 10, 25, 50 and 100). Immediately after that, the pH of solution was adjusted at 5.0 by adding 5 M of HCl aqueous solution. The reaction mixture was agitated at 37°C for 18 h and then dialyzed (cut-off molecular weight (12–14) $\times$ 10^3, Viskase) against double-distilled water (DDW) for 48 h at 25°C. The dialyzed solution was freeze-dried to obtain cationized gelatin samples. The percentage of amino groups introduced into gelatin (the cationization extent of gelatin) was determined by the conventional trinitrobenzene sulfonate method [23] based on the calibration curve prepared by using β-alanine at the pre-determined concentration. The molecular weight of all the cationized gelatins was evaluated by gel-permeation chromatography (UV-8000 system, Tosoh).

DNA isolation

The pGL3 vector (5.26 kb) encoding firefly luciferase gene (Luciferase Reporter Vectors-pGL3, Promega) was propagated in *Escherichia coli* (strain DH5α) and purified with the EndoFree™ plasmid kit (Qiagen) according to the manufacturer's instruction. The yield and purity of plasmid DNA obtained were ascertained by UV spectroscopy and the $E_{260\ nm}/E_{280\ nm}$ absorption ratio ranged between 1.8 and 1.9.

Preparation of plasmid DNA-cationized gelatin complexes

Complexation of the cationized gelatin with the luciferase plasmid DNA was performed by simply mixing the two materials at various N^+/P^- mixing ratios in aqueous solution. Briefly, 50 μl 100 mM phosphate-buffered saline solution (PBS, pH 7.4) containing 12.5, 25, 50, 100, 250 and 500 μg of cationized gelatin was slowly added to the same volume of PBS containing 10 μg of luciferase plasmid DNA at N^+/P^- mixing ratios of 0.25, 0.5, 1, 2, 5 and 10. The mixed solution was gently agitated at 37°C for 30 min to form cationized-gelatin–plasmid DNA complexes.

Dynamic and electrophoretic light scattering measurements

To investigate the hydrodynamic radius of cationized-gelatin–plasmid DNA complexes, the dynamic light scattering (DLS) measurement was carried out on a DLS

700 machine (Otsuka Electronics) equipped with a He-Ne$^-$ laser at detection angles of 30, 90 and 120° at room temperature. The cationized-gelatin–plasmid DNA complex solution was filtered by a disposable syringe filter (pore size 0.8 μm; Millipore) for DLS measurement. The hydrodynamic diameter of cationized gelatin complexed with the plasmid DNA was analyzed based on the cumulants method and calculated automatically by computer software equipped to express the values as the apparent molecular size. Each experiment was done 5–10 times independently. Electrophoretic light scattering (ELS) measurement was carried on a ELS-7000 machine (Otsuka Electronic) at room temperature and an electric field strength of 100 V/cm. The complex samples were prepared similarly using 10 mM phosphate buffer (pH 7.4). The solution was filtered through a disposable syringe filter (pore size 0.8 μm; Millipore) for ELS measurement. The zeta potential was automatically calculated using the Smoluchowski equation. Each experiment was done 10–20 times independently unless stated otherwise.

Electrophoresis of cationized-gelatin–plasmid DNA complexes

Formation of cationized-gelatin–plasmid DNA complexes was confirmed by the gel retardation assay [24]. An aliquot (5 μl) of plasmid DNA complex solution (0.1 μg/μl) was loaded into a well of an 0.8% agarose gel containing ethidium bromide and electrophoresed at 100 V for 15 min in Tris-borate-EDTA (TBE) buffer. Bands corresponding to plasmid DNA were detected under UV light and photographed.

Ethidium bromide (EtBr) intercalation assay

An EtBr intercalation assay was carried out according to the method of Xu and Szoka [25]. In brief, cationized gelatin solution (50 μl) and 20 μg/ml plasmid DNA solution (50 μl) were mixed in PBS (pH 7.4) in various N^+/P^- mixing ratios. EtBr (1.2 μg/ml, 50 μl) was added to these mixtures, and the mixed aqueous solutions (150 μl) were placed in a 96-well flat-bottomed microassay plate (Becton Dickinson). The fluorescence of EtBr intercalated to DNA was specifically monitored using a Gemini EM fluorescent microplate reader (excitation 510 nm, emission 590 nm; Molecular Devices). Free EtBr was not detected under these conditions.

Sorption experiment with Scatchard binding analysis

For the immobilization of gelatin to agarose beads, a HiTrap™ NHS-activated HP column (Amersham Bioscience) containing *N*-hydroxysuccinimide (NHS)-immobilized agarose beads in ethanol (1.0 ml) was washed three times with 1 mM cold HCl solution (5 ml). Next, 1.0 ml cationized gelatin solution (1.0 mg/ml) in a coupling buffer (0.2 M $NaHCO_3$ and 0.5 M NaCl aqueous solution, pH 7) was added to the column. Then, the column was washed three times with 2 ml NHS

deactivation buffer (0.5 M ethanolamine and 0.5 M NaCl aqueous solution, pH 8.3) three times, neutralized by 2 ml acidic buffer (0.1 M acetic acid and 0.5 M NaCl, pH 4.0) and 2 ml PBS (pH 7.4) five times. The gelatin-immobilized column prepared was stored in 2 ml PBS (pH 7.4). The amount of cationized gelatin immobilized was determined by protein assay using a Lowry kit (Nacalai Tesque) by measuring the protein amount eluted from the column and calculated from the amount of gelatin in the solution before and after immobilization reaction. Non-immobilized and NHS-deactivated columns were prepared as controls.

^{125}I-radiolabeled plasmid DNA was prepared according to the chloramine-T method reported by Chan *et al.* [26]. The sorption test of ^{125}I-radiolabeled plasmid DNA to the cationized gelatin-immobilized column was performed. Briefly, aqueous solution containing different amounts of ^{125}I-radiolabeled plasmid DNA (100 μl) was added to the cationized gelatin-immobilized column and left for 15 min at 37°C. Then, the column was washed with 2 ml PBS (pH 7.4) three times to remove non-complexed ^{125}I-radiolabeled plasmid DNA. The radioactivity of ^{125}I-radiolabeled plasmid DNA bound to the cationized gelatin-immobilized column was measured by a gamma counter. The equilibrium concentration of non-complexed ^{125}I-radiolabeled plasmid DNA cationized gelatin eluted (C_f) was also determined by measuring the radioactivity. The molar ratio of complexed plasmid DNA to cationized gelatin (r) was calculated using 3.5×10^6 and 1×10^5 as the molecular weight of plasmid DNA and gelatin. The r/C_f was plotted according to the Scatchard binding model [27]. The dissociation constant (K_d) was obtained from the slope and the intercept at $r = 0$ of the $(r/C_f) - r$ line.

In vitro *transfection study*

L929 cells of a murine fibroblast cell line were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS). The cells were seeded, 24 h prior to transfection, into each well of a 6-well cell-culture plate (Corning) at a density of 2.5×10^4 cells/well in 2 ml of the culture medium. For transfection, the culture medium was replaced with 1.9 ml of FCS-free culture medium, and then the solution of cationized gelatin-plasmid DNA complexes prepared at different N^+/P^- mixing ratios or free plasmid DNA in PBS (100 μl) was added to the well, following by incubation for 6 h. After the medium was exchanged to that containing 10% (v/v) FCS, the cells were incubated for a further 24 h. The luciferase gene expression was quantified using a commercial kit (Luciferase Assay System, Promega) and the relative light units (RLU) were determined by a luminometer (MicroLumatPlus LB 96V, Berthold). The total protein of each well was determined using the BCA Protein Assay Reagent (Pierce) in order to normalize the luciferase activity for cell number. A lipid-based reagent (Lipofectamine, Invitrogen) commercially available was used for comparison. Transfection study was carried out three times independently for every experimental group.