In conclusion, the plasmid DNA was complexed with the PEG-cationized gelatin and the *in vivo* expression level of plasmid–DNA complex was significantly increased compared with that in the solution form. This complex had another advantage as gene-delivery carrier, because the plasmid–DNA complex was protected from DNase degradation. Plasmid DNA is a macromolecule with a negative charge, irrespective of the type of the coding protein. Therefore, we can consider the plasmid DNA as one similar biological substance from the viewpoint of the electric nature. The present PEG-cationized gelatin complex is a universal gene-delivery carrier for any type of plasmid DNA. This complex is also being applied to the plasmid DNA of bioactive molecules like growth factor to demonstrate the *in vivo* biological functions at present.

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# Targeting of Plasmid DNA to Renal Interstitial Fibroblasts by Cationized Gelatin

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**Renal interstitial fibrosis is the common pathway of chronic renal disease, while it causes end-stage renal failure. A lot of cytokines and biologically active substances are well recognized to be the candidates of primary mediators to induce accumulation of extracelluar matrix (ECM) in the interstitial fibrotic area. Interstitial fibroblasts are played a crucial role in the accumulation of excess ECM during renal interstitial fibrogenesis. Therefore, the targeting of therapeutic drugs and genes to interstitial renal fibroblasts is effective in suppressing the progress of interstitial renal failure. However, despite various approaches and techniques, few successful results have been reported on the *in vivo* targeting for interstitial fibroblasts. The objective of this study is to deliver an enhanced green fluorescent protein (EGFP) plasmid DNA, as a model plasmid DNA, into renal interstitial space by a cationized gelatin. After the plasmid DNA with or without complexation of the cationized gelatin was injected to the left kidney of mice *via* the ureter, unilateral ureteral obstruction (UUO) was performed for the mice injected to induce the renal interstitial fibrosis. When the EGFP plasmid DNA complexed with the cationized gelatin was injected, EGFP expression was observed in the fibroblasts in the interstitial area of renal cortex. It is concluded that the retrograde injection of EGFP plasmid DNA complexed with the cationized gelatin is available to target the interstitial renal fibroblasts which are currently considered as the cell source responsible for excessive ECM synthesis.**

**Key words** renal interstitial fibrosis; gelatin; gene targeting

Renal fibrosis is the common pathway of chronic renal disease progressing to end-stage renal failure.[1—3] Renal fibrosis is characterized by qualitative and quantitative changes in the composition of tubular basement membranes or interstitial matrices, tubular atrophy, and the accumulation of myofibroblasts.[1—3] For chronic renal disease, persistent accumulation and deposition of extracelluar matrix (ECM) which lead to widespread tissue fibrosis, are observed.[4] Renal interstitial fibrosis is considered to be the commonly converging outcome of chronic renal diseases with a wide spectrum of diverse etiologies. For renal fibrosis, severe accumulation of ECM is observed in the renal interstitial compartment.[4—7] It is experimentally confirmed from several animal models of renal fibrosis that transforming growth factor-$\beta$ (TGF-$\beta$), heat shock protein (HSP), and other cytokines function as the primary mediators for ECM accumulation.[8—12] Therefore, it has been demonstrated that biological inhibition of TGF-$\beta$ protein by use of neutralizing antibody,[12] antisense oligonucleotide,[13] and decorin,[14,15] suppressed the accumulation of ECM in the animal models of renal fibrosis. However, there are some therapeutic limitations, for example, because protein or gene is rapidly degraded by enzyme after administration into the body. In addition, despite various approaches and techniques, there is a few trials on the *in vivo* targeting of plasmid DNA to renal interstitial cells, which have been highlighted as one cell source responsible for expressive ECM synthesis. For one strategy to overcome this problem, it is important to develop the technology and methodology of drug delivery system (DDS) for local delivery of the therapeutic drugs.

We have explored a DDS technique necessary to therapeutic drugs for the long-term and stable expression of biologically active substance.[16] As one DDS carrier for the local delivery of plasmid DNA, a cationized gelatin is used.[17—20] The electrical nature of gelatin which can be readily changed by the processing method of collagen for preparation. An acidic processing of collagen produces "basic" gelatin with an isoelectric point (IEP) of 9.0. Based on this concept, positively charged gelatin can form a polyion complex with DNA because the nature of DNA is a macromolecule of negative charges. Complexation with the biodegradable cationized gelatin enabled some plasmid DNAs to enhance their level of gene transfection *in vivo*. Complexation also reduced the DNase digestion of plasmid DNA and prolonged the *in vivo* remaining time period of plasmid DNA. In addition, the apparent molecular size of plasmid DNA was decreased and the surface charge of plasmid DNA became positive by complexation with the cationized gelatin. It is likely that these features resulted in acceleration of *in vivo* gene expression.[17—20] However, unfortunately, the complex of cationized gelatin does not have any inherent natures of targeting to a specific cell. Thus, as one practical possible way to overcome the no-targetability, contriving the administration route will be promising.

This study is a technological trial to deliver the enhanced green fluorescent protein (EGFP) plasmid DNA complexed with the cationized gelatin into renal interstitial fibroblasts which are currently considered as the cell source responsible for excessive ECM synthesis. It is well recognized that the ureteral stenosis is one of the pathogenic characteristics of renal interstitial fibrosis and responsible for the intrinsic renal pressure increase. The objective of this study is to enhance the level of gene transfection in the disease kidney with an increased renal pressure. This study experimentally shows the gene transfection of renal interstitial cells after retrograde injection of plasmid DNA complexed with cation-

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ized gelatin *via* the ureter of unilateral ureteral obstruction (UUO) mouse model. The retrograde ureteral catheterization is one of the procedures clinically capable.

## MATERIALS AND METHODS

**Preparation of Cationized Gelatin and the Complex with EGFP Plasmid DNA** A gelatin sample with an IEP of 9.0 (MW=100000), prepared by an acid process of porcine skin, was kindly supplied from Nitta Gelatin Inc., Japan. The carboxyl groups of gelatin were chemically converted by introducing amino groups for cationization of gelatin.[17—20] Briefly ethylenediamine (Wako Pure Chemical, Japan) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt (EDC, Nacalai Tesque, Japan) were added at both the molar ratios to the carboxyl groups of gelatin of 50 into 250 ml of 100 mM phosphate-buffered solution (pH 5.0) containing 5 g of gelatin. Immediately after that, the solution pH was adjusted at 5.0 by adding 5 M HCl aqueous solution. The reaction mixture was agitated at 37 °C for 18 h, and then the reaction mixture was dialyzed in a cellulose tube (the cut-off molecular weight=12000—14000, Viskase Companies, Inc.) against double-distilled water (DDW) for 48 h at room temperature and freeze-dried to obtain a cationized gelatin. When determined by the conventional 2,4,6-trinitrobenzensulfonic acid (TNBS) method,[21] the percentage of amino groups introduced into gelatin was 50.9 mol% per the carboxyl groups of gelatin. To prepare the complex between the cationized gelatin and the EGFP plasmid DNA (OligoEngine Inc., U.S.A.), 0.1 ml of 100 mM phosphate-buffered saline solution (PBS pH 7.4) containing 2 mg of cationized gelatin was mixed with 0.1 ml of PBS containing 0.4 mg of EGFP plasmid DNA. The solution was gently agitated at 37 °C for 30 min to form their complexes.

**Light Scattering Measurement** To investigate the hydrodynamic radius of EGFP plasmid DNA-cationized gelatin complex, the dynamic light scattering (DLS) measurement was carried out on a DLS 700 (Otsuka Electronics Co. Ltd., Japan) equipped with He–$Ne^-$ laser at a detection angle of 30, 90, and 120° at room temperature. The hydrodynamic diameter of EGFP plasmid DNA-cationized gelatin complex was analyzed based on the cumulants method and automatically calculated by the computer software equipped to express as the apparent molecular size. Electrophoretic light scattering (ELS) measurement was carried on an ELS-7000 (Otsuka Electronic Co. Ltd., Japan) at room temperature and an electric field strength of 100 V/cm. The complex samples were prepared similarly other than using 10 mM of phosphate-buffered solution (pH 7.4). The zeta potential was automatically calculated using the Smoluchouski equation. Each experiment was done 10—20 times independently.

**Preparation of a Mouse Model with Interstitial Renal Fibrosis and Evaluation of Distribution of EGFP Plasmid DNA-Cationized Gelatin Complex** A mouse model of interstitial renal fibrosis was prepared while the distribution of EGFP plasmid DNA-cationized gelatin complex was evaluated after retrograded injection *via* the ureter.[22,23] Male C57BL/6 mice, six-week-old (Nihon SLC, Japan), were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg) while the left kidney and ureter were surgically exposed by a mid-line incision. EGFP plasmid DNA-cationized gelatin complex or free EGFP plasmid DNA at the PBS volume of 50 $\mu$l were retrogradely injected into the left kidney *via* the ureter directly by use of HAMILTON™ syringe with 30 G needle while the left renal vein was clamped to apply the pressure aiming at the increasing transfection efficiency (5 mice). Immediately after injection, the mouse ureter was completely obstructed by a silk thread. The kidneys were perfused with cold autoclaved PBS and the cortex samples were taken 3 d later. Frozen sections (5 $\mu$m thickness) of cortex samples were incubated with first antibodies for 1 h at room temperature, followed by incubation with a rhodamine isothiocyanate (RITC)-conjugated second antibody for 1 h at room temperature. The first antibodies used were a monoclonal antibody to mouse laminin (a marker for tubular basement membrane, Sigma, U.S.A.), MOMA-2 (a marker for macrophages, BMA Biomedicals, Switzerland), and ER-TR7 (a marker for reticular fibroblasts, BMA Biomedicals, Switzerland). The ER-TR7 is an antibody specific for reticular fibroblasts, but not for myofibroblasts, in the kidney.[24] The microphotographs of green fluorescence of EGFP expressed and the red fluorescence of RITC were taken by double exposure (Olympus AX-80, Olympus, Japan).

## RESULTS

The cationization extent of cationized gelatin prepared was controllable by changing the addition molar ratio of amine molecules to the carboxyl groups of gelatin.[17] We have demonstrated that the gene expression level is influenced by the cationization extent of gelatin complexed with plasmid DNA. The highest level of gene expression by the complex of plasmid DNA with cationized gelatin was observed at a cationization extent of 50.9 mol%.[17] Thus, in this study, the cationization extent for cationized gelatin was selected for all the experiments.

From the DLS measurement, the apparent molecular size of free EGFP plasmid DNA itself was 552±83 nm. On the contrary, that of EGFP plasmid DNA complexed with cationized gelatin was 229±49 nm, because of the condensation of EGFP plasmid DNA in molecular size. The apparent molecular size did not depend on the measurement angle. The zeta potential of free EGFP plasmid DNA was −14.7± 2.8 mV, but increased up to 9.3±1.4 mV by complexation with the cationized gelatin.

EGFP expression after injection of EGFP plasmid DNA complexed with cationized gelatin was observed in the interstitial cells 3 d after transfection (Fig. 1a). On the contrary, a few EGFP-positive cells were observed after injection of free EGFP plasmid DNA (Fig. 1b). To examine the cellular localization of EGFP plasmid DNA transfected, the basement membrane was stained by an anti-laminin first antibody and a RITC-conjugated secondary antibody (Fig. 1c). EGFP-positive cells (green) were observed outside the basement membrane (red). When double staining with an antibody of MOMA-2 (a marker for macrophages) or ER-TR7 (a marker for reticular fibroblasts) for cell identification and RITC-conjugated secondary antibody was performed, the gene expression was not observed in macrophages (red, Figs. 1d (×100) and 1e (×400)), but in interstitial reticular fibroblasts (red, Figs. 1f (×100) and 1g (×400)).

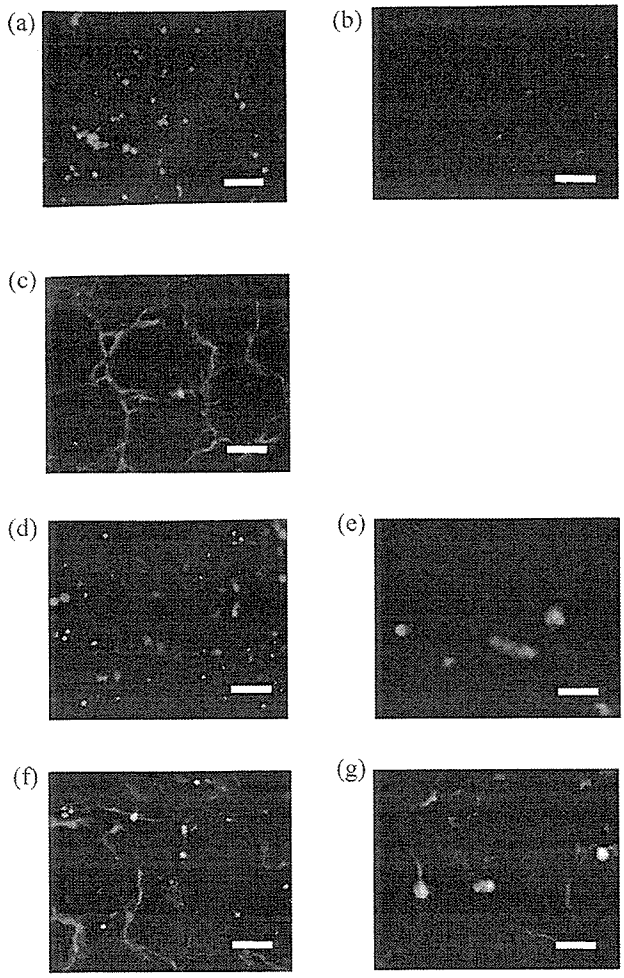


Fig. 1. The Cellular Localization of EGFP Expression 3 d after Injection of Complex of EGFP Plasmid DNA and the Cationized Gelatin into the Left Kidney *via* Ureter of UUO Mice

Magnification; (a, b, d, f)×100, (c)×200, (e, g)×400. The bar length is 200 (a, b, d, f), 100 (c) or 30 (e, g) $\mu$m. EGFP expression after injection of EGFP plasmid DNA complexed with cationized gelatin was observed in the interstitial cells 3 d after transfection (a). On the contrary, a few EGFP-positive cells were observed after injection of free EGFP plasmid DNA (b). When the basement membrane was immunologically stained with an anti-mouse laminin antibody (red) (c), EGFP-positive cells (green) were observed in the interstitial side of basement membrane. To investigate the localization of macrophages and interstitial reticular fibroblasts, immunological staining with MOMA-2 (d, e) and ER-TR7 antibodies (f, g) was performed. EGFP expression was detected in fibroblast-like cells, but not in macrophages.

## DISCUSSION

Despite various approaches and techniques, few successful results have been reported on the *in vivo* transfection for interstitial reticular fibroblasts, which play an important role in the increase of ECM synthesis.[25] The present study is the first clear demonstration that introduction of EGFP plasmid DNA into the interstitial reticular fibroblasts of mice with UUO by use of the cationized gelatin.

Zhu *et al.* have reported successful transfection into the interstitial compartment by an adenoviral vector.[26] In that work, the expression of $\beta$-galactosidase gene was observed in the interstitial vasculature including arteries of the outer medulla in both the outer and inner stripes and in the periglomerular and peritubular capillaries of cortex. In addition, Tsujie *et al.* report that TGF-$\beta$1 antisense oligonucleotides were introduced neither into macrophages nor tubular cells, but fibroblasts, suggesting that TGF-$\beta$1 antisense oligonucleotides could directly affect TGF-$\beta$1 expression in interstitial fibroblasts.[27] However, the clinical trials are quite limited by the adverse effects of virus vector itself, such as immunogenicity and toxicity or the possible mutagenesis of cells transfected. Gelatin has been extensively used for industrial, pharmaceutical, and medical applications. The biosafety has been proved through its long clinical usage as the surgical biomaterials and drug ingredients. Therefore, if gelatin is chemically modified for the carrier of gene transfection, gelatin will be one of the material candidates useful and available for clinical gene therapy.

The present data suggest that the DNA solution retrogradely injected could enter into the interstitial area by slipping through between papilla epithelial cells, and thereafter distribute diffusely into the cortical interstitial space (Fig. 1). We have performed that the EGFP plasmid DNA complexed with the biodegradable cationized gelatin enhanced gene transfection because the apparent molecular size of plasmid DNA decreased to 200 nm by complexation with the cationized gelatin.[28] It is possible that the complex of small size retrogradely injected can easily infiltrate into the interstitial area by slipping through between epithelial cells, and subsequently distribute in the cortical interstitial space by simple diffusion.[29] When the EGFP plasmid DNA complexed with the cationized gelatin was retrogradely injected *via* the ureter, it is conceivable that the intrinsic renal pressure increases by the injection procedure. The increase in the intrinsic renal or pyelic pressure might enable the complex to penetrate between papilla epithelial cells or tubular epithelial cells. In addition, vascular permeability of macromolecules is facilitated by the pressure. As a result, it is conceivable that the complex easily diffuses into the interstitial area to distribute into the cortical interstitial space. It is demonstrated that the complex with this size can be favorably taken up by cells.[30,31] This is an additional advantage of complex prepared from cationized gelatin for enhanced gene expression in terms of efficient DNA packing to nano-size particles. Moreover, the plasmid DNA-cationized gelatin complex of positive charge readily interacted with the cell surface of negative charge. It is well recognized that the ureteral stenosis is one of the pathogenic characteristics of renal interstitial fibrosis and responsible for the intrinsic renal pressure increase. The procedure of ureteral ligation in the UUO model is severe for the renal functions compared with the renal stenosis, but can induce the subsequent increase of renal pressure with good reproducibility. However, the reason why the interstitial fibroblasts, but not tublar epithelial cells, were selectively transfected is unclear at present.

In fact, the EGFP plasmid DNA used in this study was a pSUPER$^{TM}$ plasmid DNA system of RNA interference (RNAi) (OligoEngine Inc., U.S.A.) which has been used for efficient and sequence-specific gene silencing, resulting in the functional inactivation of gene targeted. Therefore, the present targeting system of plasmid DNA to interstitial renal reticular fibroblasts by the cationized gelatin may be useful for an efficient silencing of TGF-$\beta$, HSP or other cytokines' function in genetic level which are well known to be the primary mediators for ECM accumulation. In addition, the pro-

gression of renal interstitial fibrosis might be delayed by selectively removal of interstitial fibroblasts which produce ECM. From this viewpoint, gene therapy for cell-specific induced apoptosis by use of the cationized gelatin will be useful.

In conclusion, the present data demonstrate that plasmid DNA complexed with cationized gelatin could be delivered into renal interstitial fibroblasts, which play an important role in the interstitial renal disease. Moreover, it should be noted that the retrograde ureteral catheterization is a common clinical procedure. In the clinical setting, it is no practically problematic to clamp the ureter for a few minutes after injection. Therefore, this new technique of gene transfer to interstitial cells could be a potential therapeutic strategy in the interstitial renal disease.

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# Controlled Release Technology Suppresses The Progression of Disseminated Pancreatic Cancer Cells

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**Keywords:** controlled release, cationized gelatin, microspheres, pancreatic cancer, NK4

**Abstract**

NK4, composed of the $NH_2$-terminal hairpin and subsequent four-kringle domains of hepatocyte growth factor (HGF), acts as a potent angiogenesis inhibitor. This study is an investigation to evaluate the feasibility of controlled release of NK4 plasmid DNA in suppressing tumor growth. Controlled release by a biodegradable hydrogel enabled the NK4 plasmid DNA to enhance the tumor suppression effects. Biodegradable microspheres of cationized gelatin were prepared for the controlled release of a NK4 plasmid DNA. The cationized gelatin microspheres incorporating NK4 plasmid DNA were subcutaneously injected to tumor-bearing mice to evaluate the suppressive effects on tumor angiogenesis and growth. The cationized gelatin microspheres incorporating NK4 plasmid DNA could release over 28 days. When the cationized gelatin microspheres incorporating NK4 plasmid DNA were injected into the subcutaneous tissue of mice intraperitoneally inoculated with pancreatic cancer cells, their survival time period was prolonged. Tumor growth was suppressed to a significantly greater extent than free NK4 plasmid DNA. The controlled release of NK4 plasmid DNA suppressed angiogenesis and increased cell apoptosis in the tumor tissue, while it enhanced and prolonged the serum level of NK4 protein. We conclude that the controlled release technology was promising to enhance the tumor suppression effects of NK4 plasmid DNA.

## Introduction

Hepatocyte growth factor (HGF) has been noted as the signal molecule which plays an important role in development, differentiation, and morphogenesis of living systems (1-3). On the other hand, HGF often acts in an autocrine fashion to induce and enhance the invasive, angiogenic, and metastatic functions of malignant tumors by way of the c-Met/HGF receptor (4-7). Therefore, it is highly expected that the molecular blocking of c-Met/HGF receptor effectively suppresses the invasive, angiogenic, and metastatic functions of tumor cells. Based on this concept, Date *et al.* have prepared an antagonist for HGF which composes of the $NH_2$-terminal hairpin domain of HGF α-subunit and the subsequent four kringles domains (NK4) (8). The NK4 binds to the c-Met/HGF receptor, but does not induce tyrosine phosphorylation of c-Met. NK4 competitively inhibits some biological events driven by the HGF-Met receptor binding, such as the invasion and metastasis of distinct types of tumor cells and angiogenesis (8-10).

Based on recent advent of genomics, new genes have been discovered and will become therapeutically available for various diseases in near future. In this connection, gene therapy is expected as a new and promising therapeutic choice. Presently, several human clinical trials are being proceeded to treat cancer by utilizing the viral vectors of retroviruses, adenoviruses, and adeno-associated viruses. In spite of the high transfection efficiency, the therapeutic trials are limited by the adverse effects of virus itself, such as immunogenicity and toxicity or the possible mutagenesis of cells transfected. As the non-viral vectors, many types of cationized polymers (11) and cationized liposomes (12) have been explored. This approach is to enable the plasmid DNA to neutralize the anionic charge as well as to reduce the molecular size for enhanced efficiency of plasmid DNA transfection which causes an increase in the gene expression. However, the shorter duration and the lower level of gene expression than viral vectors are important issues to be technologically improved. One of the possible ways to tackle the issues is to permit the controlled release of plasmid DNA by combining with an appropriate carrier.

Gelatin has been extensively used for industrial, pharmaceutical, and medical applications and the bio-safety is proved through its long clinical usage as the surgical biomaterials and drug ingredients. Another unique advantage of gelatin is variation in the electrical nature, while the electric nature can be readily changed by the processing method of collagen (13). We have designed and explored the controlled release system of drugs on the basis of drug release governed by degradation of drug carrier. Drugs are immobilized into the biodegradable hydrogel of gelatin on the way of physicochemical interaction forces between the drug

and gelatin molecules. In this release system, the drug immobilized is not released from the hydrogel unless the hydrogel carrier is degraded to generate water-soluble gelatin fragments. The drug release can be controlled only by changing the hydrogel degradation (14). In addition, the cationized gelatin of positive charge can readily be prepared by introducing amine residues to the carboxyl groups of gelatin. The plasmid DNA polyionically immobilized in the cationized gelatin hydrogel is released from the hydrogel only if the hydrogel is degraded to generate the water-soluble gelatin fragments (15, 16). The release mechanism driven by degradation of release carrier is quite different from that of plasmid DNA diffusion from the release carrier which has been reported as the conventional release system of plasmid DNA (17, 18). This study indicates that the cationized gelatin hydrogel enabled a NK4 plasmid DNA to achieve the controlled release and consequently exert the tumor suppressive effects which are not observed for the plasmid DNA solution.

In this study, the cationized gelatin hydrogel was applied to the controlled release of expression plasmid for human NK4, to evaluate the suppressive effects on tumor angiogenesis and growth in tumor-bearing mice.

**Results and Discussion**

Radiotracing experiment revealed that NK4 plasmid DNA was retained around the injected site of cationized gelatin microspheres incorporating NK4 plasmid DNA over the time period of 28 days, whereas free NK4 plasmid DNA injected was excreted more rapidly (**Figure 1**).

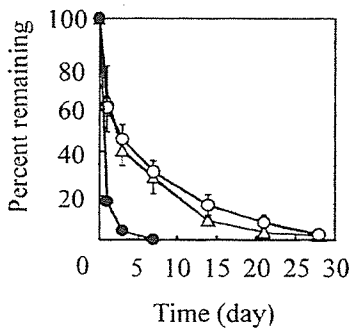


**Figure 1**

*The time course of radioactivity remaining of cationized gelatin microspheres incorporating $^{125}$I-labeled NK4 plasmid DNA (○) and free $^{125}$I-labeled NK4 plasmid DNA (●) or $^{125}$I-labeled cationized gelatin microspheres (△) after the subcutaneous injection into the back of mice. The microspheres enabled NK4 plasmid DNA to retain in the injected site for a longer time period than in the solution form. The in vivo retention profile of NK4 plasmid DNA was in good accordance with that of microspheres as the release carrier, indicating the controlled release of NK4 plasmid DNA accompanied with the carrier degradation.*

A good correlation in the time profile of *in vivo* retention was observed between the NK4 plasmid DNA incorporated and microspheres. This indicates that the controlled release of NK4 plasmid DNA was governed by microsphere degradation. When the cationized gelatin microspheres incorporating NK4 plasmid DNA were injected into the subcutaneous tissue of mice intraperitoneally inoculated with pancreatic cancer cells, their survival time period was prolonged (**Figure 2**).

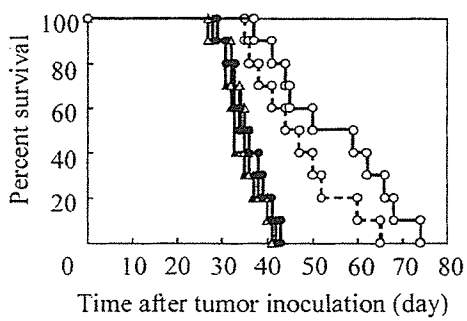


**Figure 2**

*Survival curves of tumor-bearing mice following the single injection of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA into the subcutaneous tissue: cationized gelatin microspheres incorporating 100 (○---)* and 200 μg of NK4 plasmid DNA (○—)*, 200 μg of free NK4 plasmid DNA (●—), empty cationized gelatin microspheres (△), and saline (▲). Irrespective of the NK4 plasmid DNA dose, the injection of microspheres incorporating NK4 plasmid DNA significantly prolonged the survival time period of tumor-bearing mice, in contrast to that of free NK4 plasmid DNA. *, $p<0.05$: significant against the survival curve of saline-injected, control mice.*

The subcutaneous injection of cationized gelatin microspheres incorporating NK4 plasmid DNA significantly suppressed the tumor progression of the pancreatic cancer cells disseminated in the peritoneal cavity of nude mice. Generally, it is known that the vessel formation in the tumor tissue is in progress after metastasis of tumor cells. The prolonged expression of NK4 results in significant suppression of increase in the number and total weight of disseminated nodules (**Figures 3A and 3B**). Continuous exposure of NK4 protein to the tumor cells is effective in suppressing the vessel formation. We believe that suppression of angiogenesis at least enables tumor to maintain the dormant state rather than to biologically eradicate. Therefore, in terms of tumor dormancy, tumor gene therapy based on continuous release of NK4 plasmid DNA from cationized gelatin microspheres may be an attractive new approach for treatment of advanced tumor patients.

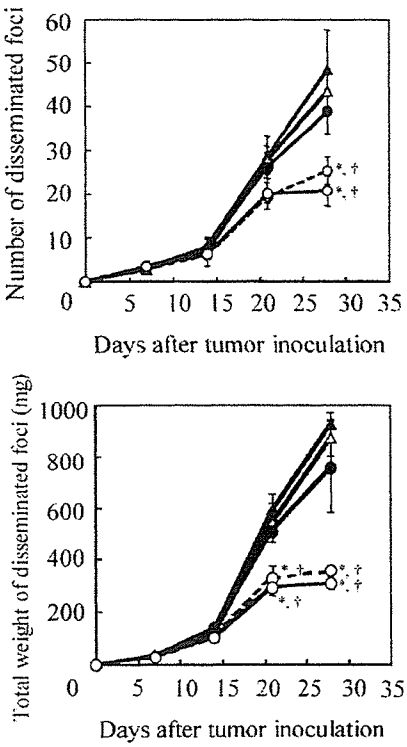


**Figure 3**

*In vivo tumor suppression effects of cationized gelatin microspheres incorporating NK4 plasmid DNA and free NK4 plasmid DNA following the single injection into the subcutaneous tissue.*

*(A) Time course of tumor number change: cationized gelatin microspheres incorporating 100 (O---) and 200 μg of NK4 plasmid DNA (O—), 200 μg of free NK4 plasmid DNA (●), empty cationized gelatin microspheres (△), and saline (▲). *, $p<0.05$: significant against the tumor number of saline-injected mice at the corresponding day. †, $p<0.05$: significant against the tumor number of mice injected with 200 μg of free NK4 plasmid DNA at the corresponding day.*

*(B) Time course of total weight of disseminated nodule: cationized gelatin microspheres incorporating 100 (O---) and 200 μg of NK4 plasmid DNA (O—), 200 μg of free NK4 plasmid DNA (●), empty cationized gelatin microspheres (△), and saline (▲).*

**, $p<0.05$: significant against the tumor weight of saline-injected mice at the corresponding day. †, $p<0.05$: significant against the tumor weight of mice injected with 200 μg of free NK4 plasmid DNA at the corresponding day.*

The injection of cationized gelatin microspheres incorporating NK4 plasmid DNA decreased the number of blood vessels in the tumor tissue and the vessel diameter compared with that of other agents. Additionally, the microspheres injection was effective in increasing the number of apoptotic cells. These findings are consistent with previous studies in which angiogenesis inhibitors suppress the tumor growth based on the increasing apoptosis of tumor cells (19, 20). We supposed that NK4 prevented the progression of disseminated tumor cells as an angiogenesis inhibitor in addition to an HGF antagonist. Improved mice survival by the injection of cationized gelatin microspheres incorporating NK4 implies that NK4 also inhibited the further extension of peritoneal dissemination.

Because of the *in vivo* instability and immunogenicity of therapeutic protein itself, it is difficult to induce the biological function and maintain it for a long time period (21). On the other hand, the plasmid DNA may achieve a prolonged biological effect by the transfected cells, although the low transfection efficiency by plasmid DNA should be improved (22). The controlled release enables the plasmid DNA to increase the concentration in the tissue over an extend time period. It is highly conceivable that the enhanced concentration increases the exposure possibility of plasmid DNA to cells, resulting in promoted gene expression. It is likely that the controlled release of the plasmid DNA prevents rapid degradation of DNA and facilitate exposure and transduction of plasmid DNA to cells, thereby increasing gene expression efficiency. Although there are still some rooms to consider as the mechanism of NK4-induced suppression effect of tumor metastasis, the present study indicates the therapeutically positive effect of NK4 plasmid DNA release on tumor suppression.

It is possible that the plasmid DNA released is condensed because of the polyion complexation with the cationized gelatin of degradation product. It has been demonstrated that plasmid DNA can be more readily taken up by cells through condensation in the molecular size of plasmid DNA through polyion complexation with cationized polymers (23, 24). This feature to induce the molecular condensation is also an advantage of the release system to enhance gene expression. Taken together, we can say with certainty that the NK4 plasmid DNA was expressed around the injection site and secreted to the systematic circulation. Our research results demonstrated that it was important for successful tumor therapy to expose NK4 to tumor cells for a long time even at a low concentration by the controlled release system, although there are still unclear points about the mechanism of tumor suppression.

We conclude that controlled release with cationized gelatin microspheres was a promising technology to enhance the *in vivo* tumor suppression effects of NK4 plasmid DNA. This release system is applicable to other types of plasmid DNA and oligonucleotide for enhanced gene expression.

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GENE DELIVERY

# In vivo release of plasmid DNA from composites of oligo(poly(ethylene glycol)fumarate) and cationized gelatin microspheres

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**Abstract**

Composites of cationized gelatin microspheres (CGMS), crosslinked with either 3 mM or 6 mM glutaraldehyde solution, and a novel hydrogel material, oligo(poly(ethylene glycol)fumarate) (OPF) were fabricated and investigated toward prolonging the release of plasmid DNA in vivo relative to the constituent materials. The composites and constituent materials were investigated in a subcutaneous murine model to assess the release of $^{125}$I-labeled plasmid DNA and $^{125}$I-labeled cationized gelatin in vivo. The time profiles of the radioactivity remaining were employed to compare the profiles of DNA release and cationized gelatin degradation. Both composite formulations (incorporating either 3 mM or 6 mM CGMS) prolonged the bioavailability of plasmid DNA relative to both injected plasmid DNA solution and the respective non-embedded cationized gelatin microspheres. Injected plasmid DNA solution persisted in the subject for only 7–10 days, whereas the persistence of DNA from composites of OPF and either 3 mM or 6 mM CGMS extended to at least day 42. The 3 mM and 6 mM CGMS each increased the persistence of DNA slightly, relative to injection of DNA solution, to between 28 and 35 days. Interestingly, the release profile of plasmid DNA from composites was not significantly different from the release of DNA from OPF alone. The release of plasmid DNA from the composites was in accord with the degradation of the microspheres within the OPF. These results show that composites of OPF and cationized gelatin microspheres are able to prolong the availability of plasmid DNA in vivo relative to cationized gelatin microspheres alone and provide a promising candidate material for the sustained, controlled release of plasmid DNA.


*Keywords:* Gene delivery; Degradable hydrogel; Cationized gelatin microspheres; Plasmid DNA; Controlled release

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## 1. Introduction

It has been suggested that the single greatest concern related to the success of systems for the long-term (days to months) controlled release of therapeutic proteins is the instability of the proteins themselves [1]. The delivery of plasmid DNA encoding a therapeutic protein presents a promising alternative to the outright delivery of the protein. The inherent chemical stability of DNA facilitates the application of traditional methods established for the controlled release of proteins toward achieving controlled gene delivery. The success of therapeutic gene delivery, however, requires a system that both facilitates cellular uptake of DNA and maintains gene expression. Although viral vectors present high transfection efficiency and sustained expression, the risks and limitations associated with their safety has placed an increasing emphasis on the exploration of non-viral methods for gene delivery.

The injection of plasmid DNA solution provides a simple non-viral means for gene delivery that has been shown to elicit expression of the encoded protein [2–4]. The injected DNA solution, however, is introduced in a bolus fashion and is rapidly cleared from the site, resulting in a short duration of bioavailability. It follows that gene expression subsequent to injection of plasmid DNA is generally limited and brief [2,5,6], often requiring repeated injections to sustain gene expression. Alternatively, the controlled release of plasmid DNA from a carrier matrix material presents the potential to sustain production of the encoded protein through prolonging the duration of the local availability of the DNA.

Although many materials have been explored toward application in the controlled release of plasmid DNA, cationized gelatin has been demonstrated to allow for prolonged and enhanced gene expression in vivo, relative to injected plasmid DNA solution [7–10]. The high density of positive charge of cationized gelatin allows for the formation of electrostatic complexes with plasmid DNA [8,11,12], a polyanionic macromolecule, such that the DNA is electrostatically bound to the matrix upon loading [7]. Thus, the release of plasmid DNA from cationized gelatin hydrogels and microspheres is driven by the enzymatic degradation of the gelatin, the kinetics of which may be controlled through the crosslinking extent of the hydrogels [7–10]. The DNA likely remains complexed with degradation fragments of the cationized gelatin upon release [7]. It has been proposed that this complexation may reduce degradation of the DNA by nucleases and may also facilitate cellular entry through interaction of the positively charged complexes with negatively charged cell membranes [8]. Thus, cationized gelatin is an attractive and effective material for controlled gene delivery.

The duration of plasmid DNA release from cationized gelatin, however, is limited by the enzymatic degradation of the gelatin matrix. In the case of cationized gelatin microspheres, the observed release of plasmid DNA has generally been limited to approximately 3–4 weeks in vivo [9,13]. Applications for therapeutic gene delivery, however, often call for sustained protein expression, which may require the persistence of DNA beyond a few weeks. A potential method to prolong the release of plasmid DNA from cationized gelatin microspheres, while maintaining the benefits of the cationized gelatin–plasmid DNA complexes, is to form composites of cationized gelatin microspheres with a biodegradable synthetic hydrogel, such as oligo(poly(ethylene glycol)fumarate) (OPF). OPF is a water-soluble material that can be crosslinked in situ under physiological conditions to form hydrogels, which have been demonstrated to be biocompatible and biodegradable [14,15] and have been applied toward cell attachment [16–18] and cell encapsulation [19,20]. Further, the formation of composites of OPF and gelatin microspheres has been shown to be effective in prolonging the controlled release of proteins, such as TGF-β, relative to the release of the protein from the microspheres alone [21,22].

The present study was undertaken to explore the potential of prolonging the release of plasmid from cationized gelatin microspheres through formation of composites with hydrogels of OPF. In the present study, composites of OPF hydrogels and cationized gelatin microspheres were fabricated and implanted subcutaneously into mice. Through the use of $^{125}$I-labeled plasmid DNA and $^{125}$I-labeled cationized gelatin microspheres, the time profiles of the radioactivity remaining were assessed to compare the profiles of DNA release and cationized gelatin degradation. Additionally, the degradation and DNA release kinetics of the constituent materials were eval-

uated and compared, where feasible, to determine the effect of microsphere encapsulation upon the material degradation and DNA release.

## 2. Materials and methods

### 2.1. Materials

Gelatin with an isoelectric point of 9.0 (MW 100,000) was prepared through an acidic processing of porcine skin type I collagen and kindly supplied by Nitta Gelatin (Osaka, Japan). Ethylenediamine (ED), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt (EDC) and olive oil were purchased from Wako Pure Chemicals, Ltd. (Osaka, Japan) and used as obtained. Glutaraldehyde (GA), 2,4,6-trinitrobenzensulfonic acid, β-alanine, chloramine-T, and sodium pyrosulfite were obtained from Nacalai Tesque (Kyoto, Japan). Poly(ethylene glycol) (PEG) of a nominal molecular weight of 10,000 Da, *N,N′*-methylene bisacrylamide (Bis), tetramethylethylenediamine (TEMED), ammonium persulfate (APS), and bacterial collagenase 1A were purchased from Sigma-Aldrich (St. Louis, MO). Fumaryl chloride (FuCl) and triethylamine (TEA) were acquired from Acros (Pittsburgh, PA). Agarose gel loading dye and TAE buffer were obtained from Fisher Scientific (Pittsburgh, PA). *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 obtained from NEN Research Products (DuPont, Wilmington, DE). Na$^{125}$I (NEZ033, 2 mCi in 0.1 M NaOH, pH 12–14) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA).

### 2.2. Synthesis of OPF

OPF was synthesized according to established methods [23] from PEG of a nominal molecular weight of 10,000 Da and termed "OPF 10 K." Briefly, PEG was dehydrated by azeotropic distillation in toluene then dissolved in anhydrous methylene chloride. TEA and distilled FuCl were added drop-wise concurrently over the course of 3–6 h to the PEG solution as it stirred vigorously in an ice bath. FuCl and TEA were diluted at least threefold by volume with anhydrous methylene chloride prior to addition to the PEG solution in a molar ratio of 0.9 PEG:1 FuCl:2 TEA. The reaction was conducted in a nitrogen environment. Once the addition of reagents was complete, the ice bath was removed, and the reaction solution was stirred vigorously at room temperature.

After 48 h, methylene chloride was removed from the polymer solution through rotary evaporation. The polymer was then dissolved in ethyl acetate and vacuum-filtered to remove the salt precipitate formed by the reaction of the chloride with TEA. The resulting product was recrystallized twice in ethyl acetate then washed three times with anhydrous ethyl ether. The resulting OPF powder was dried under vacuum for approximately 8 h to remove residual solvent. The dry, purified polymer was stored in a sealed vessel at −20 °C until use.

### 2.3. Gel permeation chromatography

The molecular weight of OPF was determined using gel permeation chromatography (GPC). Samples of the synthesized OPF and the PEG used for the reaction were dissolved in chloroform and filtered (0.45 μm) before injection into the system. A Waters Styragel HR 4E column (50–100 kDa range; Waters, Milford, MA) was used in conjunction with a system consisting of a pump (Model 510; Waters) and an injection module (Model 717; Waters) equipped with a differential refractometer (Model 410; Waters). Samples were run at a flow rate of 1 ml/min. Sample elution times in comparison to those of PEG calibration standards of known molecular weights were used to determine molecular weights of OPF using Empower GPC Software (Waters). Samples were run in triplicate.

### 2.4. Preparation of cationized gelatin

Porcine gelatin with an isoelectric point of 9.0 was cationized through the introduction of amino groups by chemical conversion of carboxyl groups of the gelatin [7–9]. Briefly, a solution of 20 g of gelatin in 500 ml of 100 mM phosphate-buffered solution (PBS) was prepared, and ED was added to the gelatin solution, such that the molar ratio of ED to the carboxyl groups of gelatin was 50. The number of carboxyl groups per mole of gelatin was 93. Immediately

thereafter, the pH of the reaction solution was adjusted to 5.0 through the addition of 5 M HCl aqueous solution. Then, EDC was added to the reaction solution at a ratio of 3 mol of EDC per mole of carboxyl groups in the gelatin, and the total volume of the reaction solution was adjusted to 1 L through the addition of 100 mM PBS. The reaction solution was agitated at 40 °C for 18 h and then dialyzed against double-distilled water (DDW) for 48 h at room temperature. Following dialysis, the solution was freeze-dried to isolate the cationized gelatin. The percentage of amino groups introduced into the gelatin was determined by the conventional trinitrobenzene sulfonate method [24] to be 43.5 mol% of the carboxyl groups of gelatin, based on a calibration curve prepared using β-alanine.

### 2.5. Preparation of cationized gelatin microspheres

Cationized gelatin microspheres (CGMS) were prepared through the chemical crosslinking of cationized gelatin in a water-in-oil emulsion state [25]. Briefly, a 10 wt.% aqueous solution of cationized gelatin was prepared under stirring and heating at 40 °C then added drop-wise to 175 ml olive oil under impeller agitation at 420 rpm at 40 °C to form a water-in-oil emulsion. After 10 min of stirring, the temperature of the emulsion was reduced to approximately 4 °C with an ice bath, and the emulsion continued to stir for an additional 30 min to allow for physical thermal gelation of the cationized gelatin. The resulting microspheres were then collected through centrifugation (5000 rpm, 4 °C, 5 min), homogenized in cold acetone (10,000 rpm, 30 s, 4 °C), washed three times by centrifugation (5000 rpm, 4 °C, 5 min) with cold acetone, fractionated in size by sieves with apertures of 30 and 90 μm, and air-dried at 4 °C. To crosslink the uncrosslinked, air-dried cationized gelatin microspheres, 50 mg of the microspheres was added to 25 ml of acetone/0.01 M HCl solution (7:3, v/v) containing either 30 or 60 μl of 25 wt.% GA solution and stirred at 4 °C for 24 h. Microspheres crosslinked with 30 and 60 μl of 25 wt.% GA solution will be termed "3 mM" and "6 mM," respectively, as these terms reflect the final concentration of GA in the respective crosslinking solutions. The crosslinked microspheres were then collected by centrifugation (5000 rpm, 4 °C, 5 min) and washed three times by centrifugation (5000 rpm, 4 °C, 5 min) with DDW. The microspheres were then added to 25 ml of 100 mM aqueous glycine solution and agitated for 1 h at room temperature to quench the residual aldehyde groups of unreacted glutaraldehyde. The microspheres again were collected by centrifugation (5000 rpm, 4 °C, 5 min) and washed three times by centrifugation (5000 rpm, 4 °C, 5 min) with DDW. The cationized gelatin microspheres were collected finally by lyophilization. Plasmid DNA was incorporated into the freeze-dried cationized gelatin microspheres (2 mg) through the addition of 20 μl of PBS (pH 7.4) containing 150 μg of pBMP-2 plasmid DNA, followed by incubation overnight at 4 °C.

### 2.6. Plasmid DNA isolation

An expression vector encoding human Bone Morphogenetic Protein-2 (hBMP-2) was used (pBMP-2). The pBMP-2 was prepared from *Escherichia coli* bacterial cultures with a QIAfilter Plasmid Giga Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. Briefly, *E. coli* transformants containing pBMP-2 were grown by incubating in Luria-Bertani (LB) medium at 37 °C with vigorous shaking (140 rpm) for approximately 20 h. The bacterial cells then were harvested by centrifugation (6000×*g*, 20 min, 4 °C). The resultant bacterial cell pellet was resuspended in a resuspension buffer (50 mM Tris–HCl; 10 mM EDTA, pH 8.0) in the presence of RNase (100 μg/ml) and lysed in a lysis buffer (200 mM NaOH; 1% sodium dodecylsulfate). A 3.0 M potassium acetate solution (pH 5.5) was added to neutralize the lysate, and the resultant solution was vacuum-filtered and applied to a Qiagen-tip 10,000 column of anion-exchange resin, which had been equilibrated with an equilibration buffer (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol; 0.15% Triton X-100). A medium-salt wash buffer (1 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol) was then applied to the column to remove traces of remaining protein, RNA and other contaminants. Following the wash, the plasmid DNA was eluted through application of an elution buffer (1.25 M NaCl; 50 mM Tris–HCl, pH 8.5; 15% isopropanol). The plasmid DNA was then precipitated through addition of isopropanol and collected by centrifugation (15,000×*g*, 10 min, 4 °C). A 70% ethanol aqueous solution was then applied to

wash the pellet and replace the isopropanol. The DNA solution was centrifuged once again (15,000×$g$, 10 min, 4 °C), the ethanol solution was decanted, the pellet was air-dried for approximately 20 min, and the pellet was resuspended in a small volume of TE buffer (10 mM Tris–HCl; 1 mM EDTA, pH 8.0). The concentration of plasmid DNA in solution was determined from the UV absorbance at a wavelength of 260 nm ($A_{260}$), and the ratio of the absorbance at wavelengths of 260 nm and 280 nm ($A_{260}/A_{280}$) was measured for evaluation of plasmid purity to be between 1.8 and 2.0.

### 2.7. Radiolabeling of pBMP-2

Plasmid DNA was radiolabeled with $^{125}$I through an adaptation of established methods [9,26]. Briefly, to prepare for the radioiodination procedure, 10 μl of plasmid DNA solution in TE was placed in a 1.5-μl microfuge tube and 10 μl of $Na^{125}I$ was added. Then, 100 μl of a 0.2 mg/ml solution of chloramine-T in potassium phosphate buffer (0.5 M $K_2PO_4$, pH 7.5) was added to the tube. The resulting reaction solution was gently mixed, then left to stand at room temperature. After 1 min, 100 μl of a 4 mg/ml solution of sodium pyrosulfite in MilliQ $H_2O$ was added to quench the reaction. The reaction solution was then applied to a PD-10 gel filtration column (10-cm; Amersham Pharmacia Biotech, Tokyo, Japan) for purification. The effluent solution was collected in 1-ml fractions, and the radioactivity in each fraction was assessed to identify the fraction containing the radioiodinated DNA.

### 2.8. Electrophoresis of radioiodinated DNA

The purified $^{125}$I-labeled pBMP-2 plasmid DNA was analyzed using established methods [27] on a 1% wt. agarose gel prepared with 50 ml 1×TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.3) and 25 μg ethidium bromide. A small volume (10 μl) of the DNA sample solution was combined with 2 μl of 6× agarose gel loading dye, and 10 μl of the sample/dye solution was loaded into a lane of the gel. The gel was run at 80 V for 60 min in 1× TAE buffer followed by digital imaging under UV illumination. After imaging, the DNA lane of the gel was isolated and divided serially into 5-mm sections. Subsequently, the radioactivity of each section was counted with a gamma counter to allow for correlation of radioactivity with migration distance in the gel.

### 2.9. Radiolabeling of cationized gelatin microspheres

Cationized gelatin microspheres were radioiodinated by use of $^{125}$I-Bolton-Hunter Reagent [28]. Briefly, dry nitrogen gas was bubbled through 100 μl of $^{125}$I-Bolton-Hunter Reagent solution in anhydrous benzene. Once the benzene was completely evaporated, 200 μl of MilliQ $H_2O$ was added to the vial to resuspend the reagent. The resuspended reagent solution was added to freeze-dried cationized gelatin microspheres at a ratio of 10 μl/mg for incorporation of the solution into the microspheres. The microspheres were then stored at 4 °C overnight to allow for the reaction of the $^{125}$I with the amine groups of the gelatin. Non-coupled, free $^{125}$I-labeled reagent was removed from the $^{125}$I-labeled cationized gelatin microspheres through periodic rinses with PBS over the course of 3 days, during which the radioactivity of the PBS returned to the background level.

### 2.10. Composite and hydrogel fabrication

OPF (150 mg) was dissolved in 395 μl of PBS containing 14 mg $N,N'$-methylene bisacrylamide as a crosslinking agent. In the case of composites of OPF with cationized gelatin microspheres, 118 μl of PBS and 2 mg of cationized gelatin microspheres (impregnated with of 20 μl PBS/DNA solution) were added, followed by vigorous mixing to disperse the microspheres. In the case of OPF hydrogels without a microsphere component, 98 μl PBS and 20 μl of PBS/DNA solution were added, followed by gentle mixing. The volume of PBS/DNA solution added to the formulation for OPF without a microsphere component allowed for the theoretical loading of plasmid DNA per disc to be equivalent to that of the composites of OPF and cationized gelatin microspheres, approximately 14 μg plasmid DNA per disc. Lastly, 51 μl of 0.3 M tetramethylethylenediamine (in PBS) and 51 μl of 0.3 M ammonium persulfate (in PBS) were added to the polymer solution and thoroughly mixed to disperse the microspheres and initiate crosslinking of the network. Immediately after mixing, the suspension was injected into individual wells (6 mm diameter, 1 mm height) of a Teflon mold and incu-

bated at 37 °C to facilitate crosslinking of the polymer network. After 30 min, the hydrogel networks were removed from the mold, yielding discs of approximately 6 mm diameter and 1 mm thickness.

### 2.11. In vivo DNA release

The in vivo release of radioiodinated plasmid DNA from the following five material groups was evaluated in a murine model: (1) OPF hydrogels without a microparticle component, (2) composites of OPF and 3 mM cationized gelatin microspheres, (3) composites of OPF and 6 mM cationized gelatin microspheres, (4) 3 mM cationized gelatin microspheres, and (5) 6 mM cationized gelatin microspheres. Briefly, OPF hydrogels and composites of OPF and cationized gelatin microspheres were implanted subcutaneously into the dorsal space of 7-week-old female ddY mice (Japan SLC, Inc., Hamamatsu, Japan). Each composite of OPF and cationized gelatin microspheres theoretically contained approximately 183 μg of microspheres. The theoretical DNA loading in each OPF hydrogel and composite of OPF and cationized gelatin microspheres was approximately 14 μg DNA. Cationized gelatin microspheres (approximately 2 mg in 100 μl sterile PBS) were subcutaneously inoculated into the dorsal subcutis of mice. As a control, an additional group was examined in which 14 μg of radioiodinated pBMP-2 plasmid DNA was injected subcutaneously in solution with 100 μl of sterile PBS into the dorsal subcutis of mice. One treatment was introduced per mouse, with a total of three mice per treatment per time point. At 3, 7, 14, 21, 28, 35 and 42 days after implantation or injection, the mice corresponding to the respective sampling time were sacrificed, and the dorsal skin and remaining implant, if any, were excised. Additionally, the underlying muscle tissue was thoroughly swabbed. The radioactivity of the dorsal skin, swab and remaining implant material was measured collectively with a gamma counter (ARC-301B, Aloka, Tokyo, Japan). The ratio of the collective radioactivity in the dorsal skin, swab and remaining implant material to the radioactivity of the implant material initially was calculated and expressed as the fraction of activity remaining in the implant. All the animal experiments were conducted in accordance with the Institutional Guidance of Kyoto University on Animal Experimentation.

### 2.12. In vivo cationized gelatin microsphere degradation

The in vivo degradation of radioiodinated cationized gelatin microspheres was evaluated in a murine model for the following four groups: (1) composites of OPF and 3 mM cationized gelatin microspheres, (2) composites of OPF and 6 mM cationized gelatin microspheres, (3) 3 mM cationized gelatin microspheres, and (4) 6 mM cationized gelatin microspheres. Briefly, composites of OPF and radioiodinated cationized gelatin microspheres were implanted subcutaneously into the dorsal space of 7-week-old female ddY mice. Each composite of OPF and cationized gelatin microspheres theoretically contained approximately 183 μg of microspheres. Cationized gelatin microspheres (approximately 2 mg in 100 μl sterile PBS) were subcutaneously inoculated into the dorsal subcutis of mice. One treatment was introduced per mouse, with a total of three mice per treatment per time point. The sampling schedule and analysis procedure were the same as those employed in the evaluation of the in vivo release of radioiodinated DNA.

### 2.13. Electrophoresis of plasmid DNA released in vitro

The structural integrity of plasmid DNA was assessed by agarose gel electrophoresis upon release in vitro from the following material groups: (1) OPF alone, (2) composites of OPF and 6 mM cationized gelatin microspheres in which plasmid DNA was loaded into the microspheres, and (3) composites of OPF and 6 mM cationized gelatin microspheres in which plasmid DNA was loaded into the OPF. The plasmid DNA was incorporated into the materials as before, with the exception that the plasmid DNA was not radioiodinated. The samples were housed individually in tubes containing 3 ml of PBS with 373 ng/ml bacterial collagenase 1A. The samples were placed at 37 °C with orbital shaking (~75 rpm). The PBS was completely aspirated from each tube at 3, 7, 14, 21, 28, 35 and 42 days and replaced with 3 ml of fresh enzyme-containing PBS. The collected release solutions were compiled for all samples within a group.

Released plasmid DNA was collected from each compiled release solution though ethanol precipitation. Additionally, plasmid DNA was isolated from

an aliquot of a stock solution of the input DNA (neither encapsulated nor released) for each time point. Briefly, 500 μl of ethanol and 20 μl of 5M NaCl were added to 200 μl of release solution in a micro-centrifuge tube. The tube was inverted to mix the solution then centrifuged at 13,000 rpm for 30 min. Following centrifugation, the supernatant was aspirated from the tube, and the pellet was resuspended in 10 μl TE buffer and 2 μl of 6× agarose gel loading dye. The resuspended DNA solutions were analyzed on 1% (w/v) agarose gels prepared with 50 ml 1× TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.3) and 25 μg ethidium bromide. Lanes of the gel were loaded with 10 μl of the sample/dye solution. Additionally, a lambda DNA/*Eco*RI+-*Hin*dIII marker was run on each gel. All gels were run at 80 V for 60 min in 1× TAE buffer followed by digital imaging through UV transillumination.

### 2.14. Statistical analysis

Values for the fraction of initial radioactivity remaining were compared statistically between two formulations within a time point using Student's *t*-test ($p<0.05$). In the case of comparison between more than two treatments within a given time point, statistical significance was determined by one-way ANOVA and post hoc Tukey HSD tests ($p<0.05$). All values are reported as average ± standard deviation.

## 3. Results

### 3.1. Gel permeation chromatography

The number average and weight average molecular weights of the initial PEG and resulting OPF are reported in Table 1.

Table 1
Number average ($M_n$) and weight average ($M_w$) molecular weight values of PEG and OPF as determined by gel permeation chromatography (each sample was run in triplicate) and reported mesh sizes of crosslinked OPF hydrogels [22]

| | $M_n$ | $M_w$ | Mesh size (nm) |
|---|---|---|---|
| PEG 10 K | 12,000 ± 0 | 14,000 ± 0 | n/a |
| OPF 10 K | 24,000 ± 1000 | 63,000 ± 5000 | 13.6 ± 0.3 |

### 3.2. Electrophoresis of radioiodinated DNA

A control study was conducted to verify the association of the radiolabel with the plasmid DNA following labeling and purification. The purified radiolabeled DNA was run on an agarose gel, and the radioactivity with migration distance in the gel was measured. One peak of radioactivity was observed in the gel, with a migration corresponding to that of the radiolabeled plasmid DNA (Fig. 1). The total activity measured in the gel accounted for greater than 93% of the radioactivity initially loaded into the gel.

### 3.3. In vivo DNA release

The release of radioiodinated plasmid DNA from cationized gelatin microspheres alone occurred rapidly in a burst fashion in the first 3 days, with little subsequent release. As seen in Fig. 2, less than 7% of the initial radioactivity remained for either microsphere formulation after 3 days of implantation, indicating a release of greater than 93% of the DNA in this time period. Approximately 3% of the initial radioactivity of the injected DNA solution persisted at day 3, and by day 14, no activity remained for this group. No significant difference in the fraction of initial radioactivity remaining was observed between the 3 mM CGMS, 6 mM CGMS and the DNA solution injection ($p<0.05$) at any time point for which remaining activity was detected in the DNA solution injection group (days 3 and 7).

The release of radioiodinated plasmid DNA from cationized gelatin microspheres of either formulation embedded within OPF was slower than the release from the respective microspheres alone, as shown in Fig. 2, although the final fraction of DNA released was not significantly different between the two groups. The fraction of initial radioactivity remaining was higher for the composites of cationized gelatin microspheres and OPF than for the respective cationized gelatin microspheres alone for both microsphere formulations at all time points, except day 7, at which there was no significant difference for the 6 mM CGMS alone vs. those in composites. Additionally, the release of DNA was prolonged from cationized gelatin microspheres in composites when compared to the microspheres alone, for both microsphere formu-

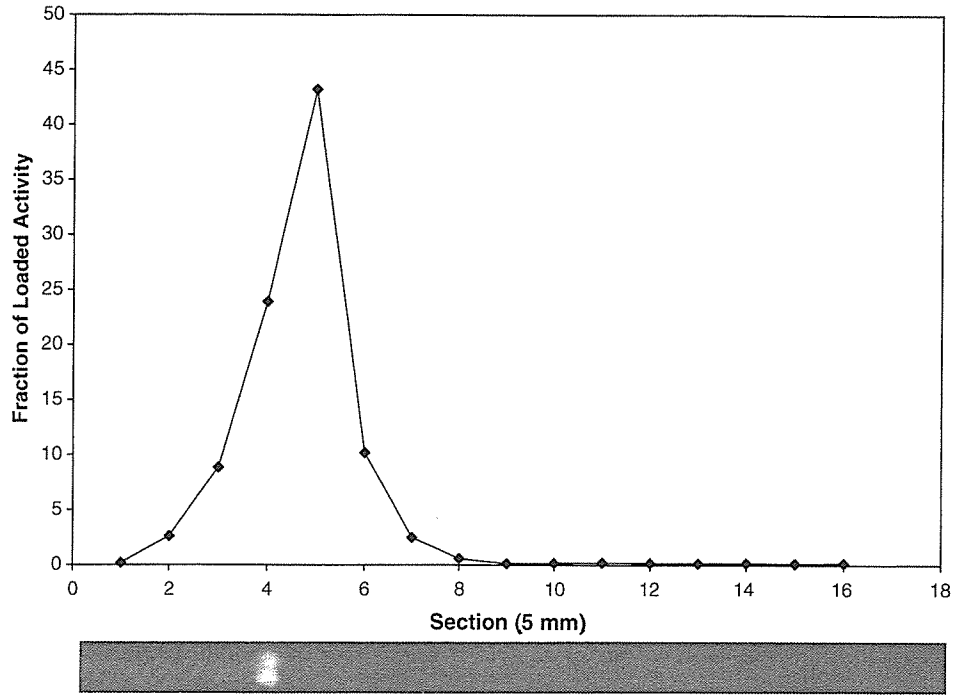


Fig. 1. Agarose gel electrophoresis of purified radioiodinated plasmid DNA and the corresponding radioactivity measured in serial 5-mm sections of the gel. The $x$-axis of the graph corresponds directly with the scale of the associated electrophoresis image.

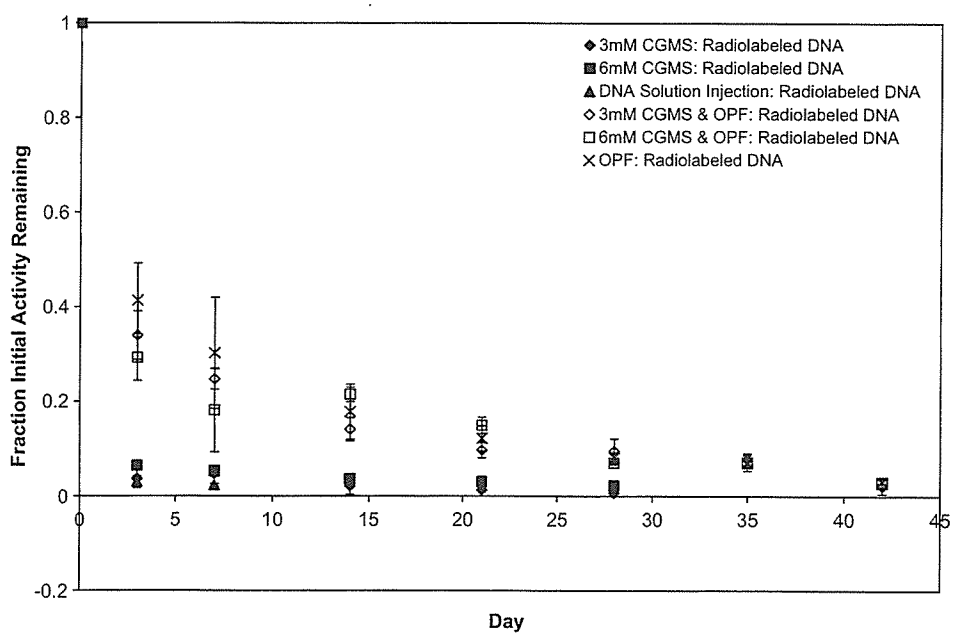


Fig. 2. Fraction of the initial radioactivity remaining in subjects receiving implants of $^{125}$I-labeled plasmid DNA loaded in 3 mM cationized gelatin microspheres (CGMS) (♦), 6 mM CGMS (■), composites of 3 mM CGMS and oligo(poly(ethylene glycol) fumarate) (OPF) (◇), composites of 6 mM CGMS and OPF (□), OPF alone (×), or an injection of $^{125}$I-labeled plasmid DNA solution (▲). To maintain simplicity of the figure, results of statistical comparisons are described in the text. Data are plotted as average ± standard deviation, $n=3$.

lations. The release of DNA from cationized gelatin microspheres alone did not persist past day 28 for either microsphere formulation, whereas the release of DNA from cationized gelatin microspheres within OPF continued through day 42 for both formulations. The release of radioiodinated plasmid DNA from composites of OPF and cationized gelatin microspheres and from OPF alone followed the same general sustained profile from days 3 to 42, as seen in Fig. 2. Indeed, the fraction of the initial radioactivity remaining was not significantly different between the composites and OPF alone at any time point, except day 3, where OPF alone had a significantly higher fraction of activity remaining, and day 21, where 6 mM CGMS composites had a significantly higher fraction of activity remaining than the 3 mM CGMS composites.

### 3.4. In vivo cationized gelatin degradation

The degradation of radiolabeled cationized gelatin microspheres both alone and in composites with OPF was evaluated in vivo (Fig. 3). The mean of the degree of degradation of 3 mM CGMS was greater than that of 3 mM CGMS embedded in OPF at each time point, although the difference between groups was not statistically significant at any time point. In both cases, no remaining radioactivity was observed for 3 mM CGMS beyond day 28, with the 3 mM CGMS alone reaching a value of approximately 0% of the initial activity remaining at day 21. Similarly, no remaining radioactivity above background was observed for 6 mM CGMS alone beyond day 28, although radioactivity persisted for the 6 mM CGMS in OPF through day 42. Thus, the persistence of CGMS in OPF was prolonged over that of the CGMS alone for both the 3 mM and 6 mM CGMS. The degree of degradation of 6 mM CGMS was lower than that of 6 mM CGMS embedded in OPF at days 3 and 7, and then higher at each subsequent time point. The only statistically significant differences observed in the fraction of initial radioactivity remaining between the 3 mM CGMS alone and those embedded in OPF were at day 7, where the remaining activity was higher in CGMS alone, and day 28, where the remaining activity in the composite was higher than in the CGMS alone.

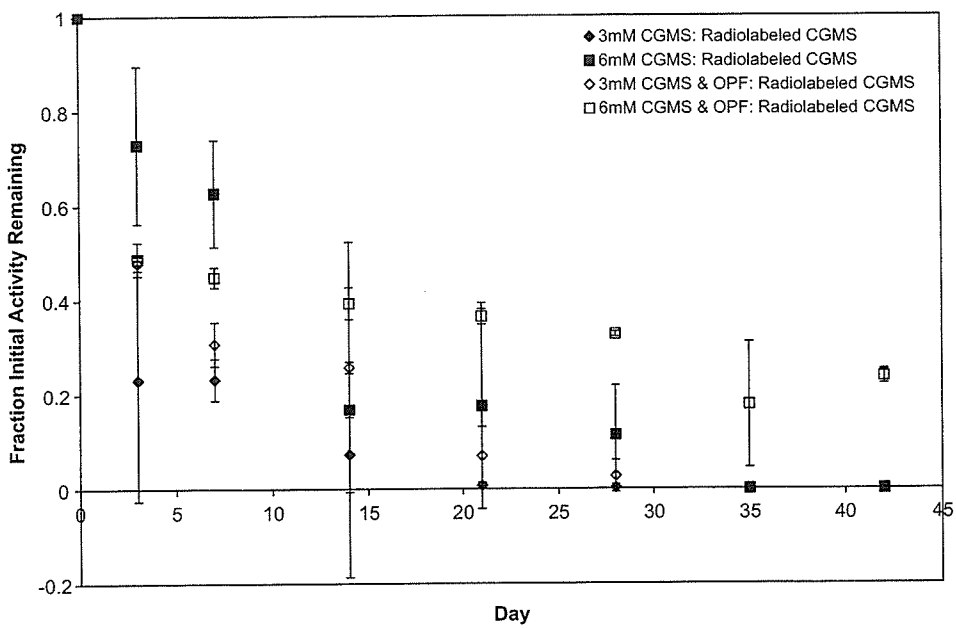


Fig. 3. Fraction of the initial radioactivity remaining in subjects receiving implants of $^{125}$I-labeled cationized gelatin microspheres (CGMS) either alone (3 mM CGMS (◆) or 6 mM CGMS (■)) or as composites with oligo(poly(ethylene glycol) fumarate) (OPF) (3 mM CGMS and OPF (◇) or 6 mM CGMS and OPF (□)). OPF was not radiolabeled in the present study. To maintain simplicity of the figure, results of statistical comparisons are described in the text. Data are plotted as average ± standard deviation, $n=3$.

### 3.5. Electrophoresis of plasmid DNA released in vitro

The structural integrity of plasmid DNA released in vitro from OPF and composites of OPF and CGMS was assessed through agarose gel electrophoresis (Fig. 4). The lanes on each gel corresponding to the material control groups (lanes 5 and 7) into which no plasmid DNA was loaded did not exhibit any fluorescent bands. The input plasmid DNA (non-entrapped, non-released) was present in both super-coiled and open-circular conformations at all time points (lane 2). Plasmid DNA released from composites of OPF and 6 mM CGMS in which the DNA had been loaded into the OPF (lane 3) was predominantly in the open-circular conformation over the course of the study, with a faint band of super-coiled DNA present for days 3–21. The DNA band for this group at day 42, although present, was very faint. Plasmid DNA released from composites of OPF and 6 mM CGMS in which the DNA had been loaded into the CGMS (lane 4) was present predominantly in the open-circular conformation over the course of the study. DNA released from OPF alone was predominantly in the open-circular conformation over the course of the study, with faint super-coiled bands at days 3 and 21 and faint linear bands at days 35 and 42.

## 4. Discussion

The release of plasmid DNA from non-embedded cationized gelatin microspheres was not significantly different between microsphere formulations at any time point. Further, the duration of plasmid DNA release from both cationized gelatin microsphere formulations exceeded the duration of the persistence of the injected DNA solution. These results indicate that cationized gelatin microspheres retained the plasmid DNA and released it in a controlled manner, thereby extending the bioavailability of the DNA beyond that achieved through injection of the DNA solution alone. When the profiles of microsphere degradation and DNA release are compared for the non-embedded cationized gelatin microspheres, no direct correlation between degradation of the microspheres and the release of plasmid DNA from the microspheres is apparent (Figs. 2 and 3), with the release of plasmid DNA occurring much more rapidly than the degradation of the microspheres.

However, the release of DNA from cationized gelatin microspheres embedded in OPF was significantly prolonged with respect to the release observed from the non-embedded microspheres, with no significant difference in the final amount of DNA released from either formulation. Thus, the entrap-

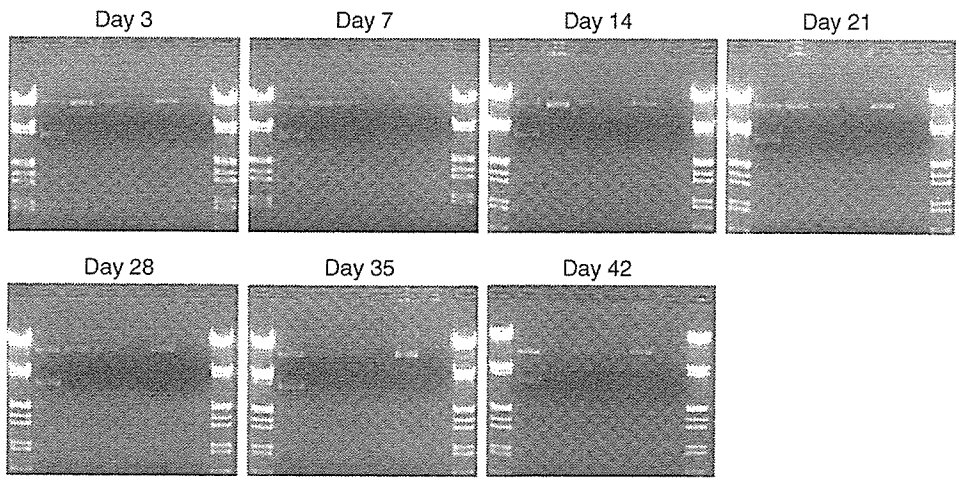


Fig. 4. Agarose gels following electrophoresis of plasmid DNA released from OPF and composites of OPF and CGMS in vitro. The lanes on each gel are as follows (left to right): (1) molecular weight marker; (2) unencapsulated plasmid DNA solution; (3) plasmid DNA released from composites of OPF and 6 mM CGMS (DNA loaded into OPF); (4) plasmid DNA released from composites of OPF and 6 mM CGMS (DNA loaded into CGMS); (5) release solution from composites of OPF and 6 mM CGMS (no DNA); (6) plasmid DNA released from OPF (no CGMS component); (7) release solution from OPF (no DNA); (8) molecular weight marker.