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 $E_{260\ \mathrm{nm}}/E_{280\ \mathrm{nm}}$ 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.

Evaluation of cytotoxicity

The cytotoxicity of cationized-gelatin–plasmid DNA complexes was evaluated. Briefly, L929 cells were cultured with the complexes prepared at different N^+/P^- mixing ratios for 24 h. After that, the culture medium was discarded and 1 ml fresh culture medium containing 100 μl of a 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution, followed by 4 h incubation. Next, the MTT-containing medium was replaced with 200 μl isopropanol-HCl (0.1 M), keeping it at 37°C for 10 min to solubilize the formazan crystals. The sample solution was transferred to each well of a 96-well cell-culture plate (Corning) and the absorbance was measured at a wavelength of 570 nm. The percent cell viability of the control (non-treated) cells was taken as 100%.

Statistical analysis

All the data were expressed as the mean ± standard deviation of the mean. Statistical analysis was performed based on the unpaired Student's t-test (two-tailed) and significance was accepted at $P < 0.05$.

RESULTS

Preparation and characterization of cationized gelatin with different amine compounds

Table 1 shows the cationization extent of gelatin samples prepared through the chemical introduction of different amine compounds. The cationization extent could be controlled by changing the molar ratio of amine compounds added. GPC measurement revealed that the molecular weight of all the cationized gelatins used was approximately 1×10^5, irrespective of the type of amine groups introduced.

Table 1.
Cationization extent of gelatin prepared at different molar ratios of amine compounds added to the carboxyl groups of gelatin

Gelatin (g/ml)	Molar ratio[a]	Amino compound (mol%/mol%)[b]			
		Ethylenediamine	Putrescine	Spermidine	Spermine
2×10^{-2}	1	10.4 ± 1.2	11.5 ± 1.0	12.5 ± 1.1	14.1 ± 0.6
2×10^{-2}	10	33.0 ± 1.1	29.6 ± 1.0	32.6 ± 1.2	34.0 ± 1.3
2×10^{-2}	25	42.6 ± 0.8	40.9 ± 1.2	47.6 ± 1.1	47.0 ± 1.2
2×10^{-2}	50	50.9 ± 1.1	45.9 ± 1.0	48.1 ± 1.0	49.0 ± 1.1
2×10^{-2}	100	50.2 ± 1.5	49.8 ± 1.1	54.1 ± 1.3	55.6 ± 1.2

Values are mean ± SD.
[a] The molar ratio of ethylenediamine, putrescine, spermidine and spermine added to the carboxyl groups of gelatin.
[b] The molar percentage of amino residues introduced to the carboxyl groups of gelatin.

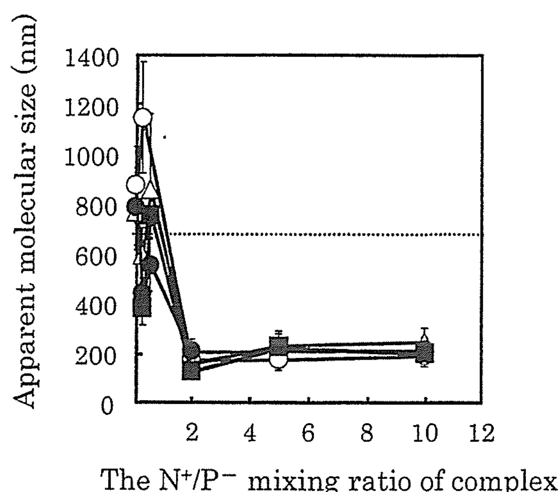

Figure 1. The apparent molecular size of cationized-gelatin–plasmid DNA complexes prepared at different mixing N^+/P^- mixing ratios (the molar number ratio of amino groups of gelatin to the phosphate groups of DNA). The amine compound used for gelatin cationization is ethylenediamine (○), putrescine (△), spermidine (■) and spermine (●). A dotted line indicates the apparent molecular size of naked plasmid DNA.

Our preliminary study revealed that the gene expression level of cationized gelatin-plasmid DNA complexes depended on the cationization extent. Based on this result, the cationization extent of 50 was used for the following experiments unless mentioned otherwise.

Figure 1 shows the apparent molecular size of cationized gelatin-plasmid DNA complexes prepared at different N^+/P^- mixing ratios. The apparent molecular size of plasmid DNA decreased by mixing with the cationized gelatin to around 200 nm, irrespective of the amine compounds introduced, when the N^+/P^- mixing ratio was 1 or higher. The apparent molecular size did not depend on the measurement angle. The zeta potential of cationized gelatin-plasmid DNA complexes prepared at different N^+/P^- mixing ratios tended to increase with increasing the N^+/P^- mixing ratio to obtain a certain zeta potential at the ratio of 2.0, irrespective of the cationized gelatin type (Fig. 2).

Figure 3 shows the electrophoretic patterns of cationized-gelatin–plasmid DNA complexes prepared at different mixing N^+/P^- mixing ratios. The band of plasmid DNA did not migrate for every type of cationized gelatin at the N^+/P^- mixing ratio of 1 or higher. However, the plasmid DNA band was migrated similarly to the original plasmid DNA at N^+/P^- mixing ratios of 0.5 or lower.

Figure 4 shows the fluorescence assay results of EtBr intercalation for various cationized-gelatin–plasmid DNA complexes in aqueous solution. At N^+/P^- mixing ratios of 1 or higher, a significant decrease in the percent fluorescent intensity was detected, irrespective of the cationized gelatin type. In addition, the percentage was significantly lower for the cationized gelatin of spermidine or spermine compared to ethylenediamine or putrescine.

Table 2 shows the K_d values of interaction between the plasmid DNA and various cationized gelatin. The K_d value for the cationized gelatin of spermine

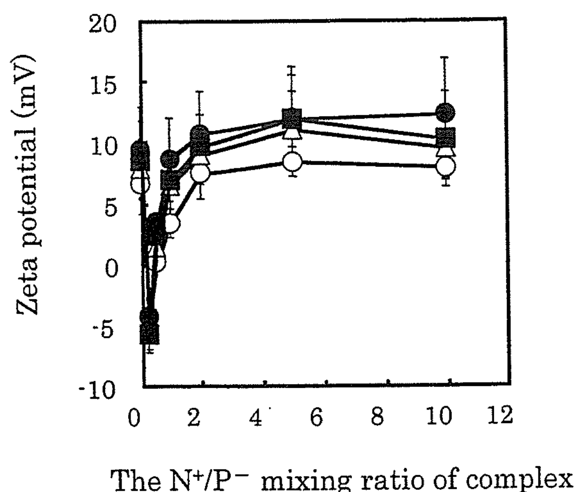

Figure 2. The zeta potential of cationized-gelatin–plasmid DNA complexes prepared at different mixing N^+/P^- mixing ratios (the molar number ratio of amino groups of gelatin to the phosphate groups of DNA). The amine compound used for gelatin cationization is ethylenediamine (○), putrescine (△), spermidine (■) and spermine (●). The zeta potential of naked plasmid DNA is -14.0 ± 2.2 mV.

was 6.4 μM, which is about 6- and 3-times lower than that of putrescine (41 μM) and ethylenediamine (20 μM), respectively.

In vitro *gene expression in L929 cells by various cationized gelatins*

Figure 5 shows the expression level of luciferase for L929 cells cultured with cationized gelatin-plasmid DNA prepared at various N^+/P^- mixing ratios. Three cationized gelatins of spermine showed higher transfection efficiency than that of other amine compounds. In addition, when the cationized gelatin of spermine was used to complex with the plasmid DNA at a N^+/P^- mixing ratio of 2, the highest transfection was achieved. However, no effect of transfection level on the N^+/P^- mixing ratio was observed for the complexes prepared from the cationized gelatin of ethylenediamine, putrescine or spermidine. Based on this result, the N^+/P^- mixing ratio was fixed at 2 for the following gene-transfection experiments. When Lipofectamine was used for transfection of plasmid DNA, the highest luciferase activity observed was 4.87×10^6 RLU/mg protein, which is significantly lower compared with that of any cationized gelatin.

Figure 6 shows the effect of N^+/P^- mixing ratio on the cytotoxicity of cationized gelatin-plasmid DNA complexes for L929 cells. Cell toxicity was observed for every complex when the N^+/P^- mixing ratio was 5 or higher, although it tended to be higher for the cationized gelatin of spermine compared with that of other cationized gelatins. When Lipofectamine was used for transfection of the plasmid DNA, the viability of cells was $72.1 \pm 5.2\%$, which is significantly higher compared with that of any cationized gelatin.

Figure 7 shows the effect of the cell density on the gene transfection of cationized gelatin derivatized with spermine–luciferase–plasmid DNA complexes for L929

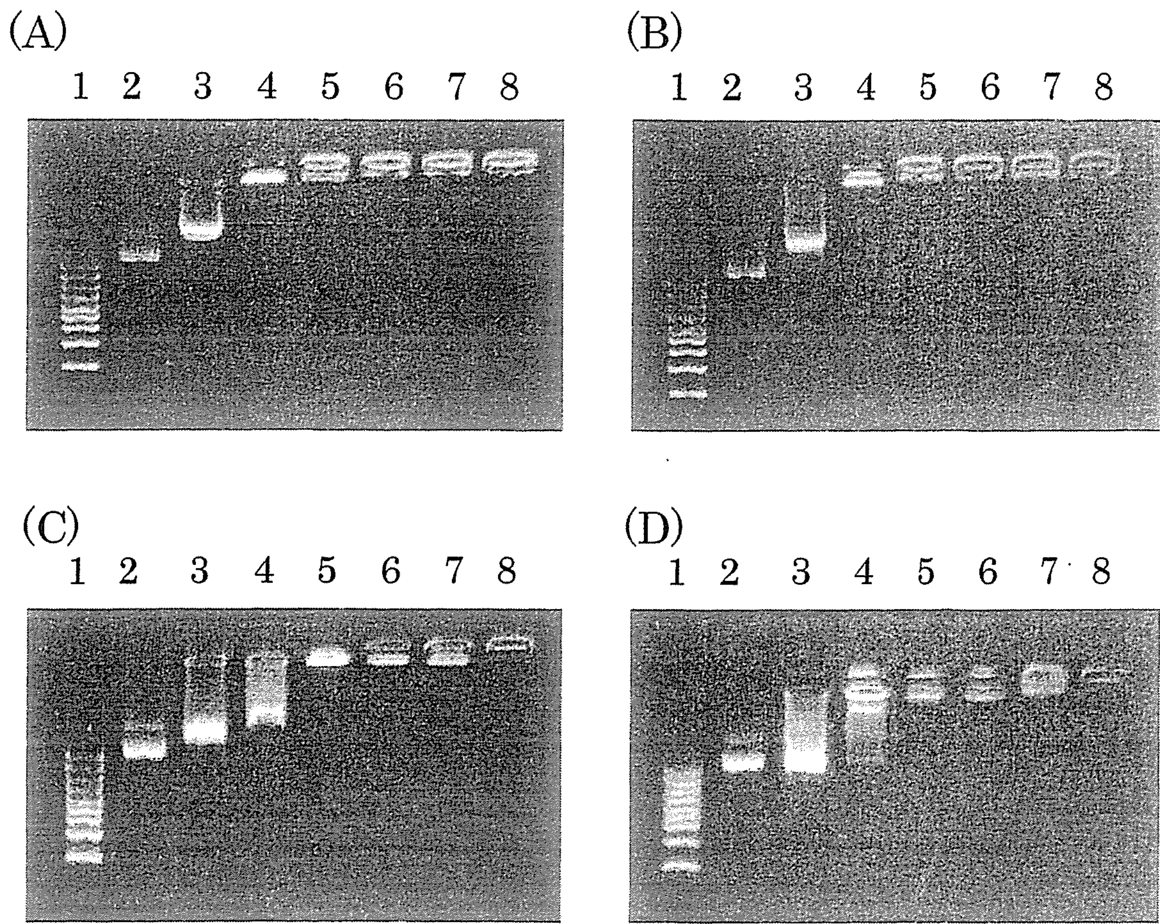

Figure 3. Electrophoretic patterns of cationized-gelatin–plasmid DNA complexes prepared at different mixing N^+/P^- mixing ratios (the molar number ratio of amino groups of gelatin to the phosphate groups of DNA). Lane 1, molecular marker; lane 2, naked plasmid DNA; lanes 3–8, plasmid DNA complexed with the cationized gelatin at N^+/P^- mixing ratios (the molar number ratio of amino groups of gelatin to the phosphate groups of DNA) of 0.25, 0.5, 1, 2, 5 and 10, respectively. The amine compound used for gelatin cationization was (A) ethylenediamine, (B) putrescine, (C) spermidine and (D) spermine.

cells. The highest gene expression level was observed at a cell seeding density of 2.5×10^4 cells/well.

DISCUSSION

In the present study, the efficacy of cationized gelatin prepared from different amine compounds as a non-viral gene delivery system was evaluated.

The cationized-gelatin–plasmid DNA complex showed an apparent molecular size in the nanometer range (Fig. 1). The complex size was around 200 nm when the N^+/P^- mixing ratio was over 1, irrespective of the type of amine compound used. It has been demonstrated that the complex with this size range can be favorably taken up by cells [29, 30]. This is also an advantageous feature of the plasmid DNA complex prepared from the cationized gelatin to enhance the transfection efficiency

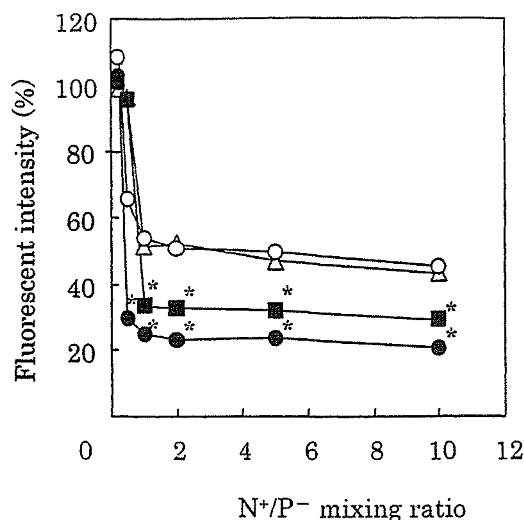

Figure 4. EtBr intercalation fluorescence assay for various cationized-gelatin–plasmid DNA complexes in aqueous solution. The fluorescence of EtBr intercalated into plasmid DNA was exclusively detected. The amine compound used for gelatin cationization is ethylenediamine (○), putrescine (△), spermidine (■) and spermine (●). The fluorescent intensity of EtBr intercalated to free plasmid DNA is defined as 100% for relative fluorescence. $*P < 0.05$, significant difference in the fluorescent intensity against the cationized gelatin prepared from ethylenediamine.

Table 2.
The K_d values of the interaction between plasmid DNA and the cationized gelatins

Amino compound	Temperature (°C)	K_d (μM)	Cationized-gelatin–plasmid DNA binding capacity (mol/mol)
Ethylenediamine	37	20.4	487.9
Putrescine	37	40.5	346.8
Spermidine	37	4.59	240.8
Spermine	37	6.35	210.4

The molar ratio of amino compound added to the carboxyl groups of gelatin was 100.

for gene expression in terms of efficient DNA condensing to a nano-order size. In addition, the surface charge of cationized-gelatin–plasmid DNA complex was tended to increase with increasing N^+/P^- mixing ratio (Fig. 2). These findings strongly suggest that the cationized-gelatin–plasmid DNA complex has a nano-size structure of which surface is covered with cationized gelatin molecules.

The gel retardation assay revealed that the cationized gelatin electrostatically interacted with the plasmid DNA. With an increase in the N^+/P^- mixing ratio, the plasmid DNA band did not migrate in electrophoresis (Fig. 3). It is possible that the negative charge of plasmid DNA is neutralized by complexation with the cationized gelatin and the molecular size of plasmid DNA increases with the cationized gelatin complexation, resulting in reduced electrophoretic migration of plasmid DNA. From the EtBr intercalation assay, relative fluorescence percentage of the complex from cationized gelatin prepared from ethylenediamine or putrescine was larger than that of spermidine or spermine (Fig. 4). The cationized gelatin prepared from

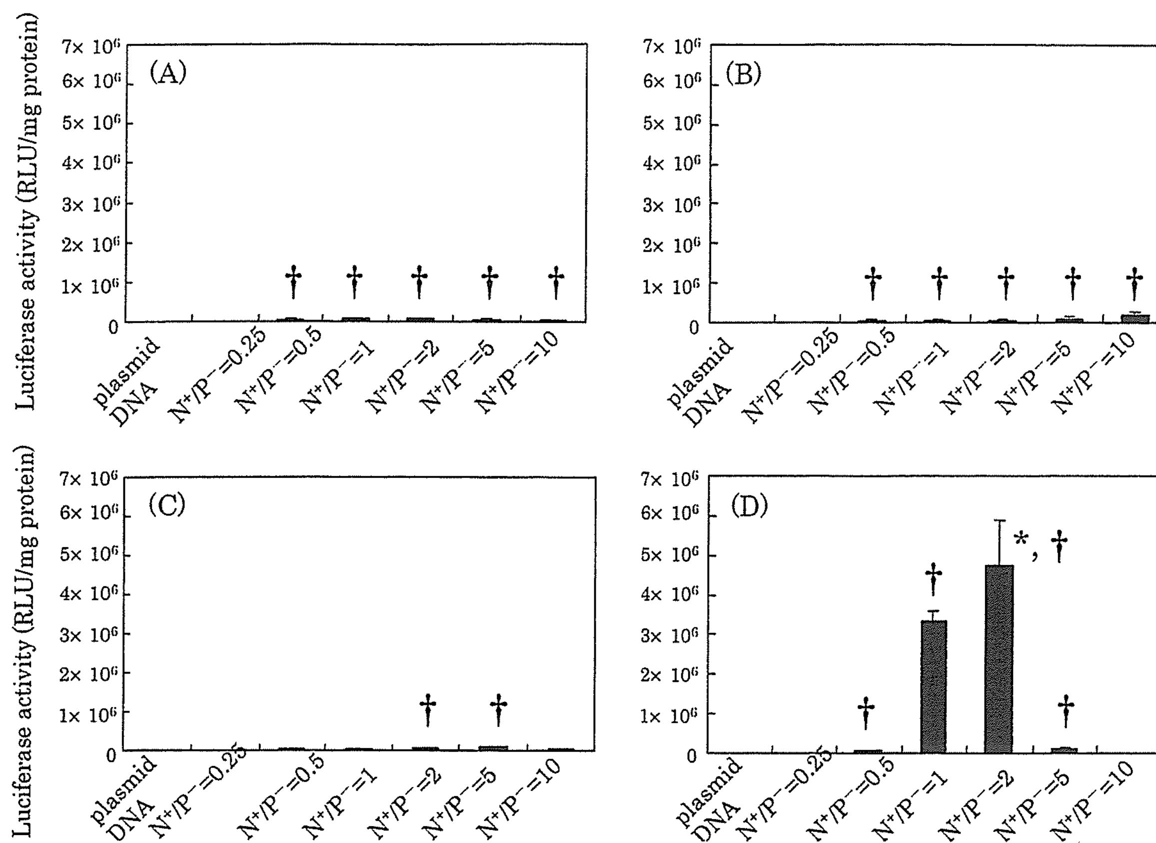

Figure 5. Effect of N^+/P^- mixing ratio (the molar number ratio of amino groups of gelatin to the phosphate groups of DNA) on the luciferase activity of L929 cells transfected by cationized-gelatins–luciferase–plasmid DNA complexes. The amine compound used for gelatin cationization was (A) ethylenediamine, (B) putrescine, (C) spermidine and (D) spermine. $^*P < 0.05$, significant difference in the luciferase activity against the complexes prepared at other N^+/P^- mixing ratios; $^\dagger P < 0.05$, significant difference in the luciferase activity against free plasmid DNA.

ethylenediamine or putrescine was intercalated about 2-fold more easily by EtBr than that of spermidine or spermine at a N^+/P^- mixing ratio of 2, indicating that the affinity of the cationized gelatin prepared from ethylenediamine or putrescine for the plasmid DNA was explicitly smaller than that of spermidine or spermine. For cationic polymers, it is conceivable that electrostatic interaction with the plasmid DNA inhibits the EtBr intercalation, resulting in a large decrease in the fluorescence intensity. The Scatchard plot analysis also demonstrates that the interaction of plasmid DNA with the cationized gelatin derivatized with ethylenediamine or putrescine was weaker than that with spermidine or spermine (Table 2). This experimentally supports the result of EtBr intercalation assay.

The transfection conditions of plasmid-DNA–cationized gelatin complexes for L929 cells, such as N^+/P^- mixing ratio, the cytotoxicity and the cell density, were optimized (Figs 5–7). From these results, the cationized gelatin prepared from spermine was suitable to obtain high transfection efficiency at a N^+/P^- mixing ratio of 2 and cell density of 70%. In addition, the cationized gelatin prepared from spermine showed a large EtBr fluorescence decrease compared with that of

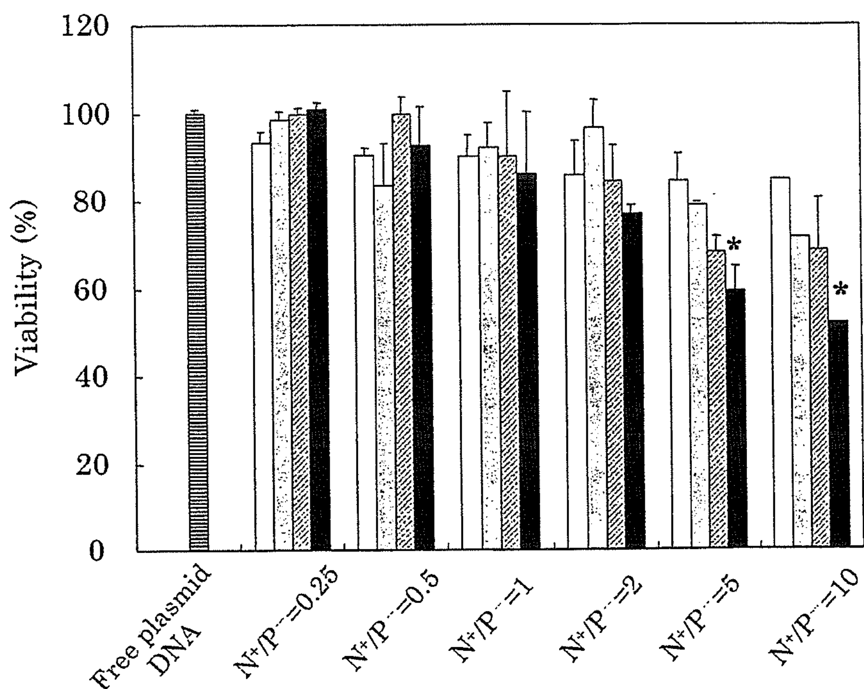

Figure 6. Effect of the N^+/P^- mixing ratio (the molar number ratio of amino groups of gelatin to the phosphate groups of DNA) on the cytotoxicity of L929 cells incubated with cationized-gelatin–luciferase–plasmid DNA complexes. The amine compound used for gelatin cationization was ethylenediamine (□), putrescine (▩), spermidine (▨) and spermine (■).

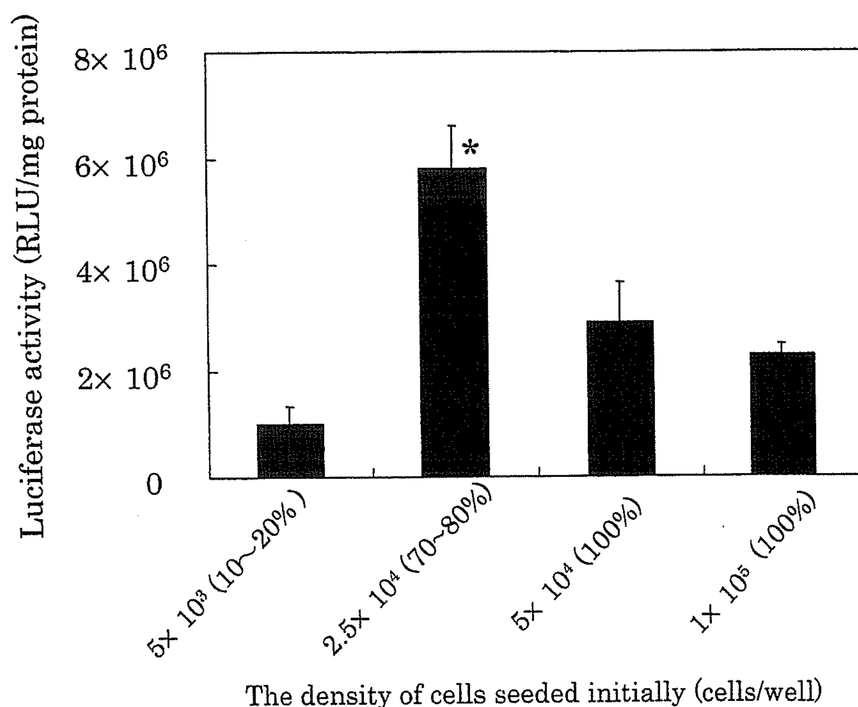

Figure 7. Effect of the cell density on the gene expression of the cationized gelatin derivatized with spermine–luciferase plasmid DNA complex prepared at a N^+/P^- mixing ratio of 2 for L929 cells. Cells were cultured for 24 h before complex addition, while the apparent cell confluency is indicated as the number in parentheses. $*P < 0.05$, significant difference in the luciferase activity against other groups at the corresponding N^+/P^- mixing ratio.

other amine compounds (Fig. 4). The lowest intensity for the cationized gelatin prepared from spermine was observed at a N^+/P^- mixing ratio of 2, indicating the sufficiently interaction with the DNA. The luciferase expression enhancement was higher for the cationized gelatin prepared from spermine than that of other amine compounds (Fig. 5). This can be explained in terms of the buffering capacity of complexes in addition to their molecular size and charge. Our previous study with the same four types of cationized gelatins revealed that the cationized gelatin prepared from spermine possessed the highest buffering effect among all the cationized gelatins used [31]. Both the condensed structure and a net positive charge of cationized-polymer–plasmid DNA complexes have been reported to be key for gene transfection [32]. However, as the N^+/P^- mixing ratio of cationized gelatin prepared from spermine increased, the viability of transfected cells decreased (Fig. 6). There was no difference in the molecular size and charge of complexes between the cationized gelatins of spermine and other amines (Figs 1 and 2). The reason of higher cytotoxicity for spermine-introduced cationized gelatin is not clear at present.

In conclusion, the plasmid DNA was complexed with cationized gelatin prepared from spermine and the *in vitro* expression level of plasmid DNA complexed increased significantly compared with that prepared from other amine compounds at optimal conditions determined. Plasmid DNA is a macromolecule with a negative charge, irrespective of the type of the coded protein. Therefore, it is practically possible to think of the plasmid DNA as one type of biological substance with the similar nature of charge, an anionic macromolecule. This cationized gelatin is being applied as the non-viral carrier for the plasmid DNA of bioactive molecules like growth factor to demonstrate enhancement of the *ex vivo* biological functions at present.

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Characterization of DNA release from composites of oligo(poly(ethylene glycol) fumarate) and cationized gelatin microspheres *in vitro*

F. Kurtis Kasper,[1] Erin Jerkins,[1] Kazuhiro Tanahashi,[1] Michael A. Barry,[1,2] Yasuhiko Tabata,[3] Antonios G. Mikos[1]
[1]*Department of Bioengineering, Rice University, PO Box 1892, MS-142, Houston, Texas 77251-1892*
[2]*Center for Cell and Gene Therapy, Baylor College of Medicine, One Baylor Plaza, N1002, Houston, Texas 77030*
[3]*Department of Biomaterials, Institute for Frontier Medical Sciences, Kyoto University, 53 Kawara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan*

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Abstract: This research investigates the release of plasmid DNA from novel hydrogel composites of oligo(poly(ethylene glycol) fumarate) (OPF) and cationized gelatin microspheres (CGMS), as well as the swelling and degradation of these materials *in vitro*. The release of total DNA and of double-stranded DNA was measured fluorescently, and the swelling properties and polymer mass loss of the hydrogels were assessed. Further, the structural integrity of the released DNA was determined through electrophoresis. It was found that plasmid DNA can be released in a sustained fashion over the course of up to 49–140 days *in vitro* from hydrogels of OPF synthesized from poly(ethylene glycol) of nominal molecular weights of 10 kDa and 3 kDa, respectively, with the release kinetics depending upon the material composition and the method of DNA loading. Released DNA was predominately double-stranded DNA (dsDNA) in structure and of the open-circular conformation. The results suggest that DNA release from hydrogel composites of OPF and CGMS is dominated by the degradation of the OPF component of the gels. Electrophoresis results indicate that the released DNA retains suitable conformation for potential bioactivity over the course of at least 63 days of release. Thus, these studies demonstrate the potential of composites of OPF and CGMS in controlled gene delivery applications. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 78A: 823–835, 2006

Key words: controlled release; degradable hydrogel; gelatin microspheres; plasmid DNA; tissue engineering

INTRODUCTION

Tissue engineering strategies for regeneration of compromised tissues generally comprise a scaffold, cells, and/or bioactive molecules. Employment of a scaffold material is often necessary to provide support and guidance to the growing tissue. Additionally, cells and/or bioactive molecules may be used in conjunction with a scaffold to direct tissue development toward a desired pathway. Although therapeutic proteins may be delivered with and released from tissue engineering scaffolds, it has been proposed that the instability of the proteins themselves raises the greatest concern regarding the success of these systems for long-term (days to months) controlled release.[1]

However, the delivery of plasmid DNA encoding a therapeutic protein presents a promising alternative to the outright delivery of the protein. Indeed, the chemical stability of plasmid DNA facilitates the application of traditional methods established for the controlled release of proteins toward achieving controlled gene delivery.[2] The success of therapeutic gene delivery from a tissue engineering scaffold, however, requires a system that provides control of DNA release, facilitates cellular uptake of DNA, maintains gene expression, and provides support for tissue infiltration while presenting void volume into which tissue may grow.

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.[3–6] A

Correspondence to: A. G. Mikos; e-mail: mikos@rice.edu
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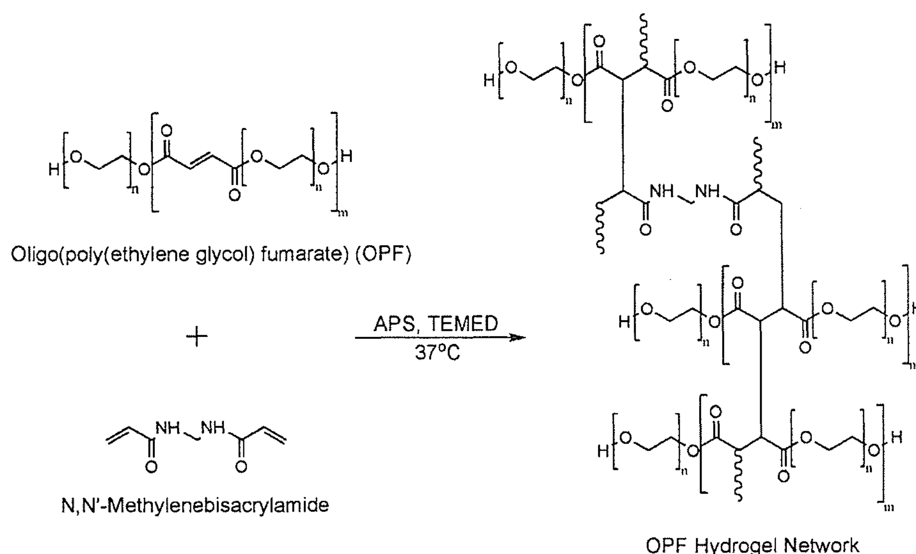

Figure 1. Schematic of the crosslinking reaction that occurs with OPF and *N,N'*-methylenebisacrylamide, using the water soluble initiator system APS/TEMED.

primary advantage presented by cationized gelatin in DNA release applications is the formation of electrostatic complexes between the material and plasmid DNA,[4,7,8] such that the DNA is electrostatically bound to the cationized gelatin upon loading.[3] Thus, the release of plasmid DNA from hydrogels and microspheres created from cationized gelatin is driven by the enzymatic degradation of the cationized gelatin, the kinetics of which may be controlled through the crosslinking extent of the cationized gelatin.[3–6] Upon release, the DNA likely remains complexed with degradation fragments of the cationized gelatin.[3] It has been proposed that this complexation may reduce degradation of the DNA by nucleases and may facilitate cellular entry through interaction of the positively charged complexes with negatively charged cell membranes.[4] Thus, hydrogels and microspheres of cationized gelatin are attractive and effective materials for controlled gene delivery.

However, the duration of plasmid DNA release from cationized gelatin hydrogels and microspheres is limited by the enzymatic degradation of the cationized gelatin network. In the case of cationized gelatin microspheres, the observed release of plasmid DNA has generally been limited to ~3–4 weeks *in vivo*.[5,9] Applications for therapeutic gene delivery and tissue engineering, however, often call for sustained protein expression, which may require the persistence of DNA beyond a few weeks. Additionally, tissue engineering applications require the presence of a scaffold material for a period of time sufficient to support tissue growth.

A potential method to retain the benefits provided by cationized gelatin, while extending the duration of the release of DNA and the persistence of the scaffold, may be found in the formation of composites of cationized gelatin microspheres with another material, such as oligo(poly(ethylene glycol) fumarate) (OPF) (Fig. 1). 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[10,11] and have been applied toward cell attachment,[12–14] cell encapsulation,[15,16] and controlled release of plasmid DNA.[17,18] Indeed, composites of OPF and CGMS have been shown to extend the release of plasmid DNA *in vivo* relative to DNA release from CGMS alone.[18] Control of the release of plasmid DNA from the composites appeared to be dominated by the degradation of the OPF network.[18] However, the determination of the degradation kinetics of the OPF was not explored in the previous investigation.[18] Thus, the present work seeks to expand upon prior work to advance the understanding of DNA release from composites of OPF and CGMS.

The objectives of the present study were to characterize the release of plasmid DNA *in vitro* from composites of OPF and CGMS and from control hydrogels of OPF alone. As a part of the study, the release of plasmid DNA from the OPF phase and from the CGMS phase of composites was examined individually to directly assess the effect of the manner of DNA loading upon DNA release, thereby giving an indirect evaluation of the effect of potential electrostatic complexation between the DNA and CGMS upon DNA release. Additionally, the present work sought to characterize the swelling and degradation of these hydrogels *in vitro*.

MATERIALS AND METHODS

Plasmid DNA preparation

The expression vector encoding human Bone Morphogenetic Protein-2 (hBMP-2) with an upstream CMV promoter (pCMV-hBMP2, 4.9 kb) was prepared from *Escherichia coli* (*E. coli*) bacterial cultures with a QIAfilter Plasmid Giga Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The isolated plasmid DNA was dissolved in a small volume of TE buffer (10 m*M* Tris HCl; 1 m*M* EDTA, pH 7.5). 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 and 280 nm (A_{260}/A_{280}) was measured for evaluation of plasmid purity to be between 1.8 and 2.0.

Hydrogel composite fabrication

OPF was synthesized and characterized according to established methods[19] from PEG of a nominal molecular weight of either 3K (Union Carbide, New Milford, CT) or 10K (Sigma-Aldrich, St. Louis, MO) and termed "OPF 3K" and "OPF 10K," respectively. Porcine gelatin with an isoelectric point of 9.0 (MW 100,000) (Nitta Gelatin, Osaka, Japan) was cationized through the introduction of amino groups by chemical conversion of carboxyl groups of the gelatin as previously described.[3–5] Cationized gelatin microspheres (CGMS) were prepared through the chemical crosslinking of cationized gelatin in a water-in-oil emulsion state,[20] using a 6 m*M* glutaraldehyde concentration as described previously.[18] The CGMS were fractionated in size by sieves to obtain microspheres in the range of 30–90 μm in diameter.

OPF constructs and composites of OPF and CGMS with and without incorporated plasmid DNA were prepared as previously described.[18] First, OPF (300 mg) was dissolved in 790 μL of phosphate buffered saline (PBS) (4 m*M* KH_2PO_4; 18 m*M* $Na_2HPO_4 \cdot 12\,H_2O$; 125 m*M* NaCl; pH 7.4) containing 28 mg *N,N'*-methylene bisacrylamide as a crosslinking agent. Second, for composite samples in which DNA was to be loaded into the CGMS component, freeze-dried CGMS (4 mg) were reconstituted through incubation overnight at 4°C with 40 μL of PBS/DNA solution, a volume less than the known equilibrium swelling volume, to load the CGMS with DNA. Similarly, for composite samples in which the CGMS were not to contain DNA, freeze-dried CGMS (4 mg) were reconstituted through incubation at 4°C overnight with 40 μL of PBS. For composite samples in which the DNA was to be loaded into the OPF component, 196 μL of PBS and 40 μL of PBS/DNA solution were then added, along with the blank CGMS, into the OPF polymer solution. For composite samples in which the DNA was to be loaded in the CGMS component, 236 μL of PBS were added, along with the DNA-loaded CGMS, into the OPF polymer solution. In both cases, the resulting polymer solutions were vigorously mixed to disperse the microspheres in the solution. For OPF samples without a CGMS component, either 236 μL of PBS (for blank samples) or 196 μL of PBS and 40 μL of PBS/DNA solution (for DNA loaded samples) were added to the OPF polymer solution and mixed. In this way, for samples in which DNA was to be loaded into OPF, the DNA was incorporated into and dispersed throughout the OPF polymer solution prior to the formation of the crosslinked hydrogel network. Third, 102 μL of 0.3*M* tetramethylethylenediamine (TEMED) (in PBS) and 102 μL of 0.3*M* ammonium persulfate (APS) (in PBS) were added to the polymer solution and thoroughly mixed to disperse the microspheres (if present in that sample type) and initiate crosslinking of the network. Immediately after mixing, the suspension was injected into individual wells (8 mm diameter, 2 mm height) of a Teflon mold and incubated at 37°C to facilitate crosslinking of the polymer network. After 30 min, the hydrogel networks were removed from the mold, yielding discs of ~8 mm diameter and 2 mm thickness.

In vitro DNA release

Hydrogel samples were placed individually into separate containers containing 3 mL of PBS (pH 7.4) with 373 ng/mL bacterial collagenase 1A (Sigma-Aldrich, St. Louis, MO). The presence of collagenase in solution was necessary for enzymatic degradation of the CGMS, and it was incorporated into the PBS at a concentration that approximates physiologically relevant concentrations.[21] The specimens were agitated on a shaker at ~70 rpm at 37°C for the duration of the study. The release solution was completely removed from the samples and replaced with fresh solution at periodic time points.

The amount of DNA in the release solution aliquots was quantified through a fluorescent method with PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Eugene, OR) and OliGreen ssDNA Quantitation Reagent (Molecular Probes, Eugene, OR) termed PicoGreen and OliGreen, respectively, according to the manufacturer's guidelines. Briefly, for each assay, 100 μL of each sample release solution was added independently to individual wells in an opaque 96-well plate. Then 50 μL of TE buffer was added to each well. A 200-fold dilution of the respective reagent was prepared in TE buffer, and 150 μL of the diluted reagent was added to each well. After a 10-min equilibration period, the fluorescence intensity of each well was measured with a microplate fluorescence reader (FLx800, BIO-TEK Instruments, Winooski, VT) equipped with 480/525 (excitation/emission) filter sets.

Three independent wells were prepared from the release solution of each release specimen at each time point. Five standard solutions of known DNA concentrations were prepared from the DNA stock solution used in the fabrication of the hydrogels. The stock solution was housed with the release samples at 37°C under agitation at ~70 rpm. An aliquot of the stock solution was taken at each time point and was serially diluted in PBS with 373 ng/mL collagenase to form the calibration standards used to analyze the release solutions from the respective time point. The average fluorescence of release solutions from hydrogels not incorporating plasmid DNA was subtracted from the fluorescence values of respective samples with DNA to account for any effects of the polymer on the assay. The cumulative fraction

release for each specimen was normalized with respect to the volumetrically calculated initial DNA content. The sample number, n, for all DNA release groups was 6.

Gel electrophoresis of released DNA

A sample of plasmid DNA released from each hydrogel group was analyzed on 1% wt. agarose gels prepared with 50 mL TAE buffer (40 m*M* Tris-acetate, 1 m*M* EDTA, pH 8.3) and 25 μg ethidium bromide for days 3–63. For each group, released plasmid DNA was isolated from the combined release solution aliquots of the polymer mass loss samples for that group at a given time point, using ethanol precipitation. Briefly, 500 μL ethanol and 20 μL 5*M* NaCl were added to 200 μL of the combined release solution in a microcentrifuge tube. The tube was inverted to mix the solution and spun at 13,000 rpm for 30 min at room temperature. The supernatant was aspirated carefully from the tube, and the DNA pellet was allowed to air dry for ~15 min. The pellet was resuspended in 10 μL TE and 2 μL of 6× agarose gel loading dye was added. Samples of a 1 μg/mL dilution of the DNA standard solution aliquot from each time point (days 3–63) were prepared in the same fashion. Lanes of the gel were loaded with 10 μL of the sample/dye solution. Additionally, a lambda DNA/EcoRI+HindIII marker was run on each gel to allow for comparison of DNA migration distances between gels. All gels were run at 80 V for 60 min in 1× TAE buffer, followed by digital imaging in a UV transillumination box.

Hydrogel swelling and degradation

The initial weight (W_i) of all samples was measured immediately after fabrication, but before the addition of the initial 3 mL of PBS/collagenase solution. Thus, W_i represents the mass of the hydrogel immediately after fabrication and prior to equilibrium swelling. Additionally, the wet weight (W_w) of all release samples and polymer mass loss samples were measured at the pertinent time point immediately upon removal of the release solution. The swelling ratio of the hydrogels at each time point was calculated accordingly from the following equation:

$$\text{Swelling Ratio} = \frac{W_w - W_i}{W_i} \quad (1)$$

This value reflects the change in weight of the hydrogel at each time point with respect to the initial weight of the gel. As a hydrogel degrades, the wet weight of the gel approaches zero. Thus, the value of the swelling ratio approaches a value of −1 upon hydrogel degradation.

The polymer mass loss was calculated for three samples from each group at each time point for days 3–63. Immediately after fabrication, six samples from each group were lyophilized, and the dry polymer mass was measured for each. The average mass of the six samples for each group was calculated and used as the initial dry polymer weight ($W_{i,d}$) for that group in subsequent calculations. At each time point, three samples from each group were lyophilized immediately after aspiration of the release solution, and the dry polymer mass (W_d) was measured for each. The fraction of initial polymer mass remaining for each sample was then calculated from the following equation:

$$\text{Fraction Initial Polymer Mass Remaining} = \frac{W_d}{W_{i,d}} \quad (2)$$

Complete degradation of a hydrogel sample was noted visually when the presence of the disk or fragments of the disk were no longer apparent.

TABLE I
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)

	M_n	M_w
PEG 3K	4,000 ± 0,000	5,000 ± 0,000
PEG 10K	12,000 ± 0,000	14,000 ± 0,000
OPF 3K	14,000 ± 1,000	83,000 ± 0,000
OPF 10K	24,000 ± 1,000	63,000 ± 5,000

Statistical analysis

The cumulative fraction release values of plasmid DNA were statistically compared between assay types (OliGreen and PicoGreen) within material formulations (OPF 3K, OPF 10K, and composite groups) as well as between material formulations within an assay type at each time point, using Student's t-test ($p < 0.05$). Similarly, the swelling ratio and polymer mass loss values were statistically compared between loading treatment groups (blank and with DNA) within material formulations (OPF 3K, OPF 10K, and composite groups) as well as between material formulations within loading treatment groups, using Student's t-test ($p < 0.05$). Other statistical comparisons were made as described. Error propagation methods were employed where appropriate to determine standard deviations.[22] All values are reported as average ± standard deviation.

RESULTS

Gel permeation chromatography

The number average and weight average molecular weights of the initial PEG and resulting OPF are reported in Table I for both OPF formulations.

In vitro DNA release

The release of plasmid DNA from OPF hydrogels [Fig. 2(a)] and composites of OPF and CGMS [Fig.

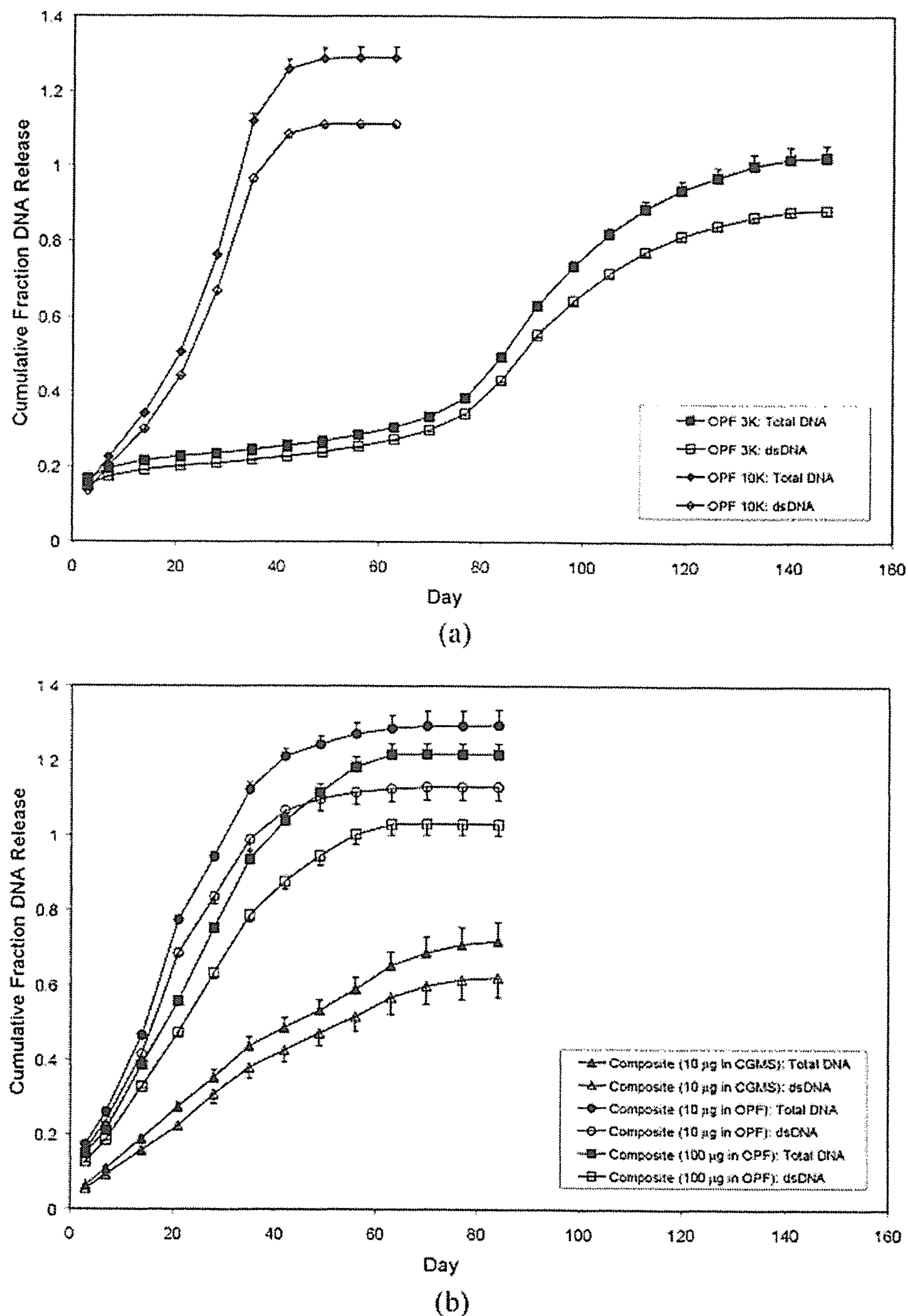

Figure 2. Release profiles of total DNA and double-stranded DNA (dsDNA) from OPF hydrogels (a) and from hydrogel composites of OPF and CGMS (b) in enzymatic PBS (pH 7.4) ($n = 6$). Error bars represent ± standard deviation.

2(b)] occurred in a sustained fashion over the course of up to 49–140 days *in vitro*. The cumulative fraction of DNA released, as determined by OliGreen, increased with time relative to that determined by PicoGreen for each OPF and composite formulation. The cumulative fraction of DNA released, as determined by OliGreen, was significantly higher than that determined by PicoGreen for OPF 3K at days 112–147 ($p < 0.05$). In the case of OPF 10K, the cumulative fraction of DNA released, as determined by OliGreen, was significantly greater than that quantified by PicoGreen at days 3 and 42–63 ($p < 0.05$). Similarly, for composite groups in which DNA was loaded into the OPF component, the cumulative fraction of DNA released, as determined by OliGreen, was significantly higher than the value determined by PicoGreen at days 42–84 and

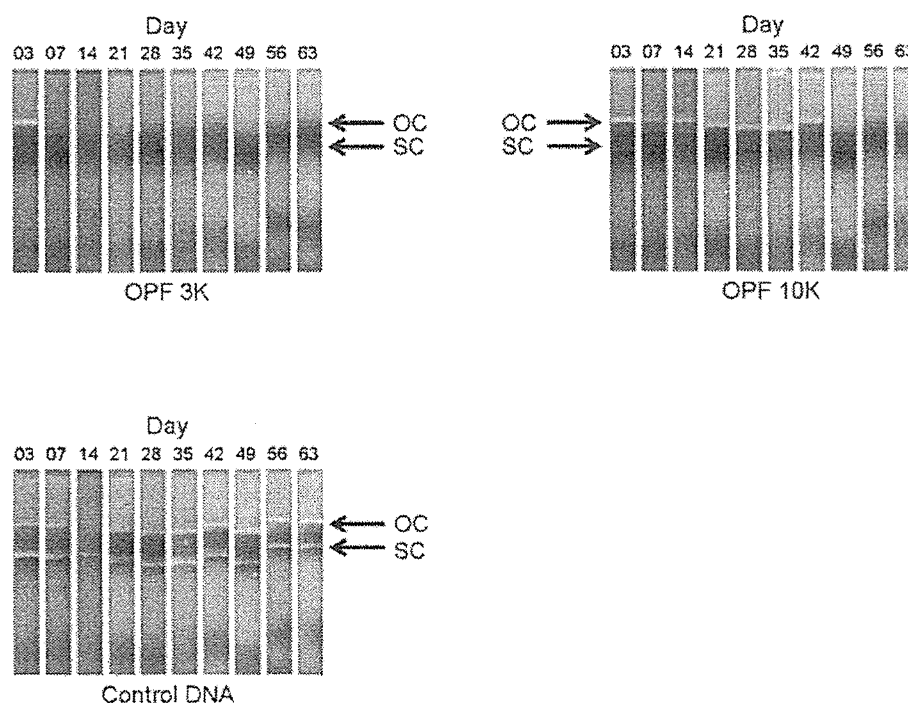

Figure 3. Agarose gels following electrophoresis of plasmid DNA released from OPF hydrogels in enzymatic PBS (pH 7.4) *in vitro* and control DNA (unencapsulated, nonreleased). The arrows indicate the migration distances for open-circular DNA (OC) and super-coiled DNA (SC).

49–84 for initial DNA loadings of 10 and 100 μg DNA, respectively ($p < 0.05$). There was no significant difference, however, in the cumulative fraction of DNA released, as determined by OliGreen or PicoGreen, at any time point in the case of composites in which DNA (10 μg) was loaded into the CGMS component ($p < 0.05$).

All of the OPF 10K release samples completely degraded by day 63. Additionally, all of the release samples from composite groups completely degraded by day 84. The OPF 3K release samples, however, persisted until day 147.

The release of DNA from OPF 10K samples and from composites in which the DNA was loaded into the OPF component presented similar profiles (Fig. 2). The release profiles of the OPF 3K and of the composites in which the DNA was loaded into the CGMS component were each unique from the release profiles of the other groups. A small initial burst release was observed from all groups at day 3, with the release from the composites in which 10 μg DNA was loaded into the CGMS component being significantly lower than the release from all other groups at this time point ($p < 0.05$). The cumulative release of DNA from composites in which 10 μg DNA was loaded into the CGMS component was significantly lower than the release from OPF 10K as well as the composites in which DNA was loaded into the OPF component at all relevant time points, as determined by PicoGreen, and at all time points except day 21 for OPF 10K and days 14 and 21 for composites (100 μg DNA in OPF), as determined by OliGreen ($p < 0.05$). The cumulative release of DNA from OPF 3K samples was significantly less than that from OPF 10K (day 14 and onward) and from composites in which 10 μg DNA (day 7 and onward) and 100 μg DNA (day 14 and onward) was loaded into the OPF component, as determined by both assays ($p < 0.05$).

Gel electrophoresis of released DNA

The structural integrity of plasmid DNA released *in vitro* from OPF 3K and OPF 10K (Fig. 3) and from composites of OPF and CGMS (Fig. 4) was assessed through agarose gel electrophoresis. The lanes on each gel corresponding to the material control groups into which no plasmid DNA was loaded did not exhibit any fluorescent bands (data not shown). The control plasmid DNA (nonentrapped, nonreleased) was present in both super-coiled and open-circular conformations at all time points (Figs. 3 and 4). Plasmid DNA released from composites of OPF and CGMS in which 100 μg DNA had been loaded into the OPF was predominately in the open-circular conformation over the course of the study, with a band of super-coiled DNA present for days 3 through 42. A band of linear

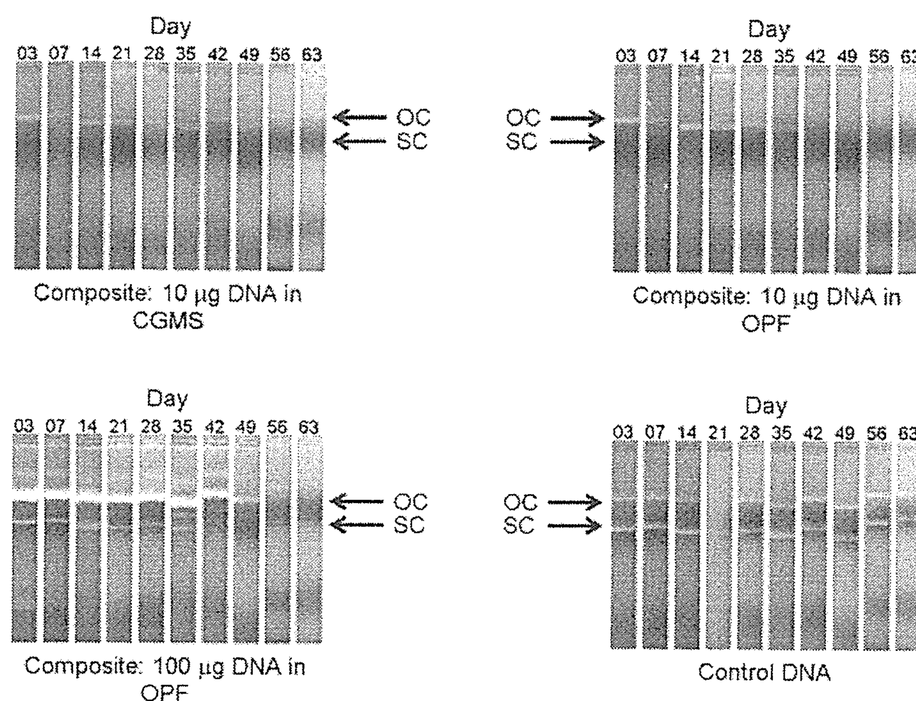

Figure 4. Agarose gels following electrophoresis of plasmid DNA released from hydrogel composites of OPF and CGMS in enzymatic PBS (pH 7.4) *in vitro* and control DNA (unencapsulated, nonreleased). The arrows indicate the migration distances for open-circular DNA (OC) and super-coiled DNA (SC).

DNA was observed for this group at days 28 through 42. Plasmid DNA released from composites of OPF and CGMS in which 10 μg DNA had been loaded into the CGMS was present predominately in the open-circular conformation over the course of the study from days 3 through 35, after which no bands were visually apparent for this group. In the case of composites of OPF and CGMS in which 10 μg DNA was loaded into the CGMS, the released DNA was consistently present in an open-circular conformation for the duration of the study (days 3–63). DNA released from OPF 10K alone was predominant in the open-circular conformation over the course of the study, with a faint super-coiled band at days 3 through 21 and a faint linear band at days 35 and 42. The DNA released from OPF 3K alone was predominately in the open-circular conformation with visible bands at days 3, 7, 28, 56, and 63.

Hydrogel swelling

The swelling ratio of each release sample was calculated over the course of the release study. All swelling ratio profiles exhibited a slight increase from an initial positive value to a maximum value, followed by a steady decrease in swelling ratio with time to approach a value of -1.0 upon complete degradation (Fig. 5). In general, no significant differences were present between the swelling ratio of samples containing DNA and their respective blanks ($p < 0.05$). The composite groups containing DNA exhibited the same general swelling ratio profiles, with no significant difference between them at any time point, with the exception of day 14 ($p < 0.05$). Although the swelling ratio profiles of the OPF 10K groups approached a value of -1.0 more rapidly than did the composite groups, few significant differences existed between the OPF 10K groups and the composite groups before day 49 ($p < 0.05$). However, the swelling ratio of the OPF 10K samples with DNA was significantly lower than that of the composites with 10 μg DNA in CGMS and 10 μg DNA in OPF at days 49–63 and the composites with 100 μg DNA in OPF at day 49 ($p < 0.05$).

The initial swelling ratios (day 3) of all OPF 10K and composite groups were significantly greater than the swelling ratios of each OPF 3K group ($p < 0.05$), with the initial swelling ratio of the OPF 3K samples being approximately half of the value for other groups. The swelling ratio of the OPF 10K and composite groups then increased to a maximum value at day 7 (day 14 for composites with 100 μg DNA loading in OPF), followed by sustained decreases in swelling ratio values until approaching a value of -1.0 upon degradation. The swelling ratio of OPF 3K samples, however, increased slowly with time to maximum values at day 77, followed by sustained decreases in swelling ratio

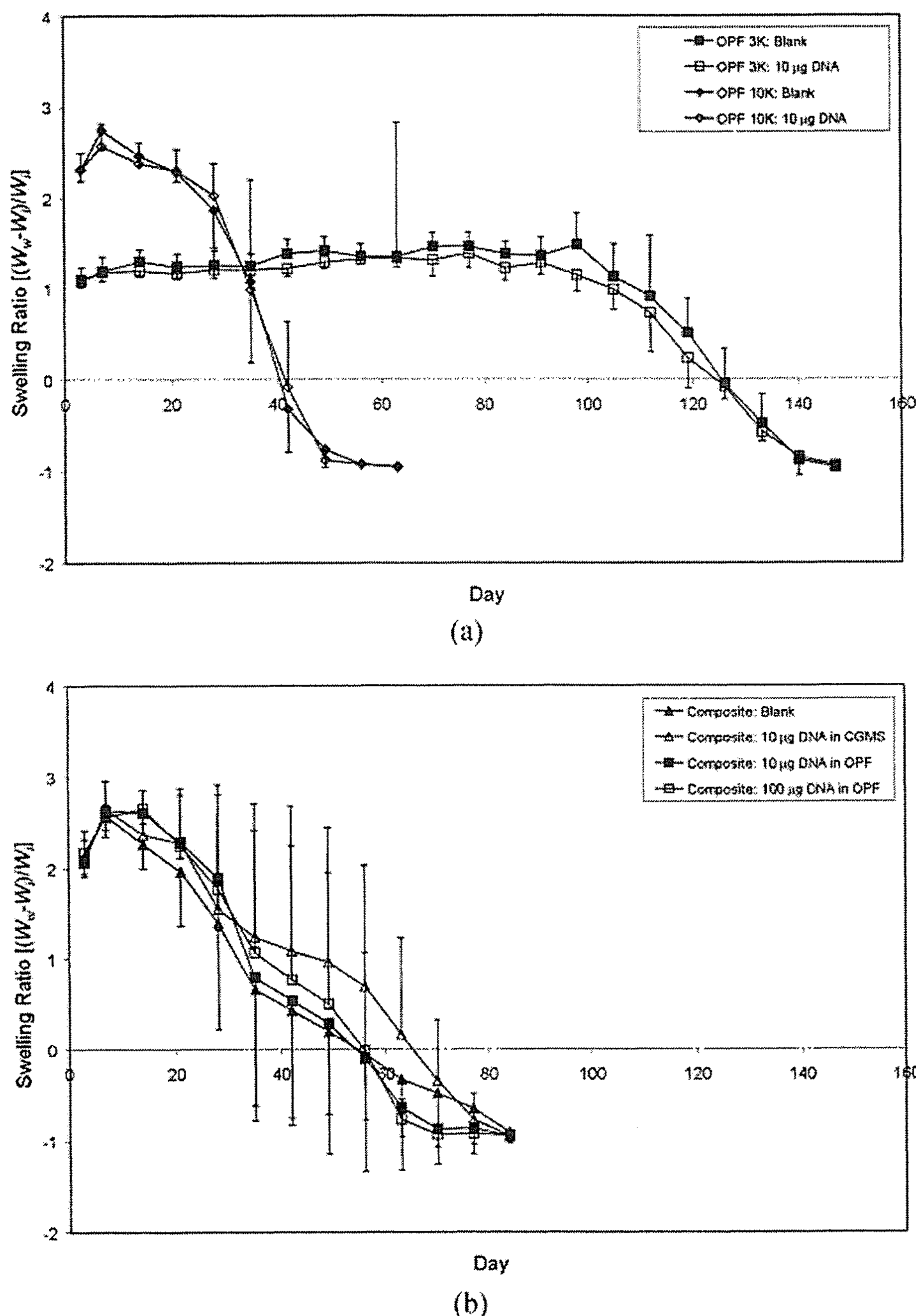

Figure 5. Swelling ratio profiles of OPF hydrogels (a) and of hydrogel composites of OPF and CGMS (b) in enzymatic PBS (pH 7.4) (n = 6). The DNA release profiles were measured from these samples. Error bars represent ± standard deviation.

values to approach a value of −1.0 upon complete degradation at day 147.

By the time the OPF 10K samples completely degraded at day 63, the swelling ratio of the blank samples and samples containing DNA reached values of approximately −1. The composite group samples required a longer amount of time for degradation, being completely degraded at day 84, at which the swelling ratios were approximately −1. The OPF 3K samples required the longest time to degrade, with complete degradation of all samples noted at day 147, with final swelling ratios of approximately −1.

Hydrogel polymer mass loss

The polymer mass loss of three samples from each group at each time point was examined (Fig. 6). The