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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-

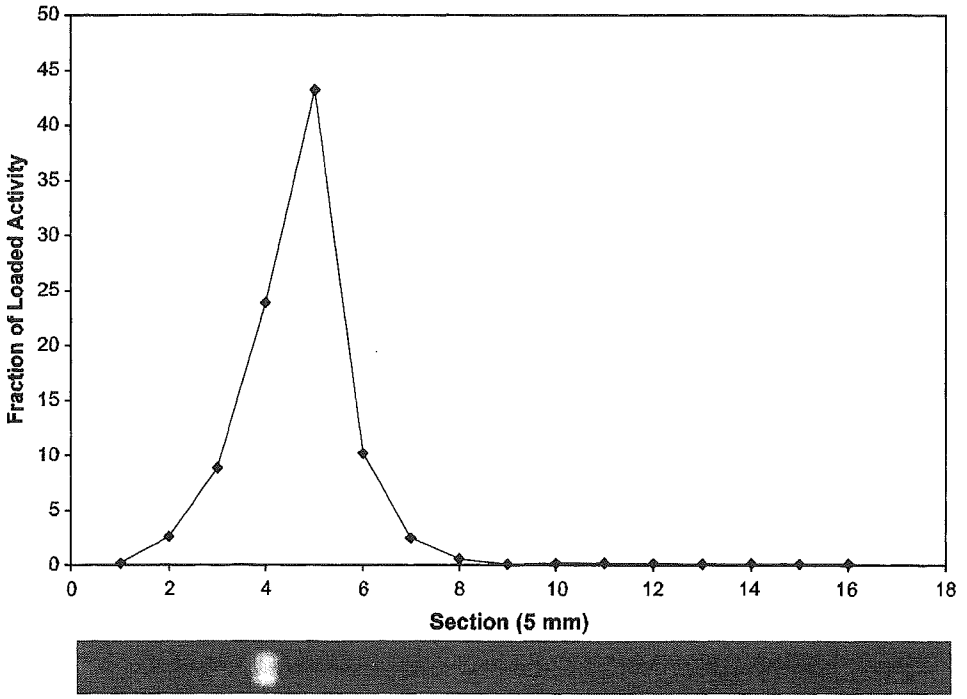

Fig. 1. Agarose gel electrophoresis of purified radioiodinated plasmid DNA and the corresponding radioactivity measured in serial 5-mm sections of the gel. The x-axis of the graph corresponds directly with the scale of the associated electrophoresis image.

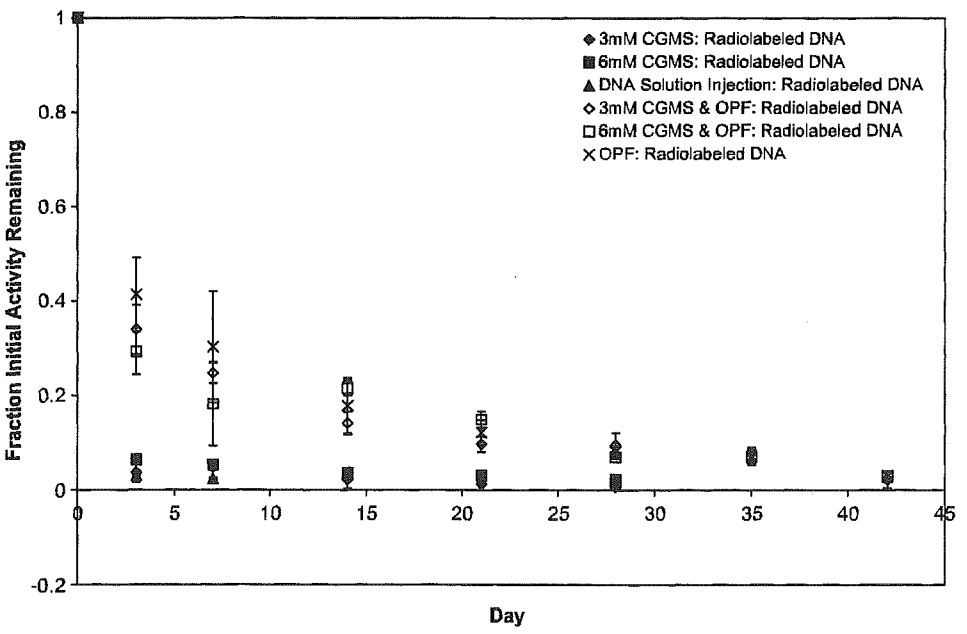

Fig. 2. Fraction of the initial radioactivity remaining in subjects receiving implants of ^{125}I-labeled plasmid DNA loaded in 3 mM cationized gelatin microspheres (CGMS) (◆), 6 mM CGMS (■), composites of 3 mM CGMS and oligo(poly(ethylene glycol) fumarate) (OPF) (◇), composites of 6 mM CGMS and OPF (□), 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.

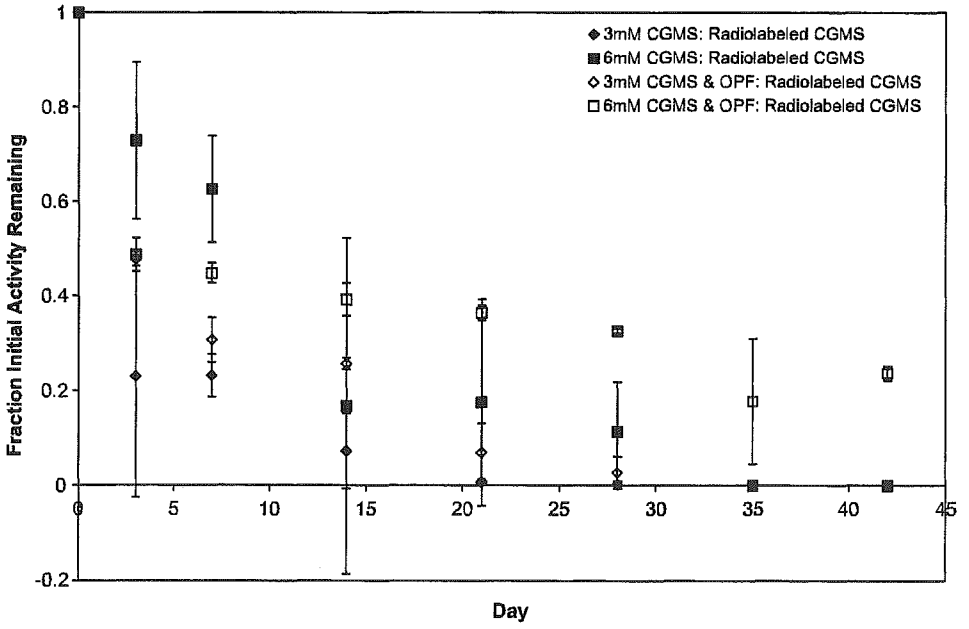

Fig. 3. Fraction of the initial radioactivity remaining in subjects receiving implants of ^{125}I-labeled cationized gelatin microspheres (CGMS) either alone (3 mM CGMS (◆) or 6 mM CGMS (■)) or as composites with oligo(poly(ethylene glycol) fumarate) (OPF) (3 mM CGMS and OPF (◇) or 6 mM CGMS and OPF (□)). 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-

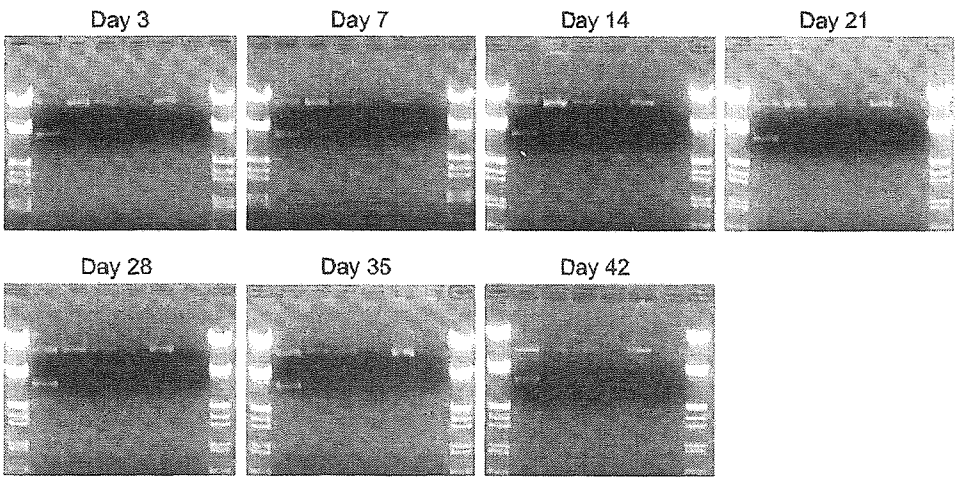

Fig. 4. Agarose gels following electrophoresis of plasmid DNA released from OPF and composites of OPF and CGMS in vitro. The lanes on each gel are as follows (left to right): (1) molecular weight marker; (2) unencapsulated plasmid DNA solution; (3) plasmid DNA released from composites of OPF and 6 mM 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.

ment within OPF gels of cationized gelatin microspheres loaded with plasmid DNA prolongs the release and subsequent bioavailability of the DNA compared to both the release of DNA from non-entrapped cationized gelatin microspheres and the persistence of injected DNA solution. A direct correspondence is apparent between the degradation of the cationized gelatin microspheres of either formulation embedded within OPF and the release of plasmid DNA from these composites (Figs. 2 and 3). Complete degradation of cationized gelatin microspheres embedded within OPF apparently required more time than for microspheres not embedded within OPF. It is likely that the slower apparent degradation of cationized gelatin microspheres embedded within OPF was due to the retention of cationized gelatin degradation fragments complexed with plasmid DNA within the OPF network.

Interestingly, the release of DNA from OPF alone and from either composite formulation was not significantly different. This result indicates that the OPF network controlled the rate of plasmid DNA release once the DNA was liberated from the cationized gelatin microspheres embedded within it. Indeed, the mesh size of OPF 10 K hydrogels is 13.6 ± 0.3 nm [22], whereas the apparent molecular size of plasmid DNA is much larger, on the order of hundreds of nanometers [8,29], as is the apparent molecular size of plasmid DNA–cationized gelatin complexes (164 ± 21.3 nm) [8]. Enzymes, however, are smaller than the mesh size of the OPF and can readily enter the hydrogels. Indeed, the entry of enzymes such as collagenase into OPF is required to degrade the CGMS component of the composites. Thus, the release of plasmid DNA from within the OPF network is likely dominated by the degradation of the OPF itself, as the small mesh size of OPF in relation to the size of plasmid DNA and of plasmid DNA-cationized gelatin complexes likely presents a significant barrier to the release of plasmid DNA by simple diffusion. However, as radiolabeling of the OPF was not feasible for this study due to a lack of primary amines in OPF, the in vivo degradation kinetics of OPF could not be examined for comparison with the observed DNA release kinetics.

In the case of the non-embedded cationized gelatin microspheres, it was expected that the release of DNA would be in good accordance with the degradation of the microspheres, as has been observed in previous studies [9,10,13]. Based upon previous results, it was anticipated that approximately 40–50% of the initial plasmid DNA and of the initial cationized gelatin would be remaining by day 3 [9,10,13]. However, this was not the case in the present study, as less than 10% of the initial plasmid DNA was remaining at day 3 for either microsphere formulation. Further, the degradation of cationized gelatin microspheres and the apparent release of plasmid DNA from the microspheres were not in accord in the present study for either microsphere formulation.

The use of radiolabeled materials required the assumption that no unbound radioisotope was present in the samples initially and that the radiolabel remained bound to the DNA or cationized gelatin throughout the duration of the study. This assumption was valid based upon experience from previous studies involving the release of radioiodinated plasmid DNA from CGMS [9,10,13]. Further, as seen in Fig. 1, the results of the radioiodination control study indicate that the radiolabel was associated with the plasmid DNA, with little to no unbound radioisotope present after purification. With respect to the possible dissociation of radioiodine from the plasmid, the primary mechanism for dissociation, in general, would be damage to the DNA induced by the hydrolytic action of nucleases or by comparable conditions, such that the plasmid is cleaved into small fragments, some of which contain the radiolabel. Release of these small fragments would result in an apparent release of the plasmid DNA. In the event that a small amount of unassociated radioisotope survived the purification procedure and was present in the samples initially or that radiolabel dissociated from the DNA or cationized gelatin during the course of the study, an apparent burst release or degradation effect would be observed.

Further, in the event that the plasmid DNA was not fully incorporated into the cationized gelatin microspheres, it is possible that an apparent burst release of DNA from the microspheres would be observed, as in the present study. However, as the volume of DNA solution added to the freeze-dried CGMS for loading was lower than the equilibrium swelling volume for the CGMS, the solution was completely incorporated into the CGMS, as in previous studies [9,10,13]. It follows that any DNA that did not incorporate into the

CGMS with the solution was associated with the surfaces of the CGMS. Therefore, the DNA was fully associated with the CGMS following loading, be it within the microspheres or on the surface of the microspheres.

The results indicate that a high degree of plasmid DNA was not fully incorporated within the cationized gelatin microspheres. Any DNA not incorporated within the CGMS would be either associated with the surface of the microspheres or injected in solution with the microspheres and would be expected to persist at the site of injection in a manner as observed for the injection of DNA solution alone. Indeed, no significant difference was observed between the release of plasmid DNA from non-embedded cationized gelatin microspheres and the persistence of injected DNA solution through day 7, whereas large differences were observed between released DNA and injected DNA over this time period in previous studies [9,10,13]. However, as DNA continued to be released beyond the limit of the observed persistence of injected DNA solution, it follows that some plasmid DNA was indeed incorporated within the cationized gelatin microspheres and subsequently released in a controlled manner.

With respect to the composites of OPF and cationized gelatin microspheres, any effect of unincorporated plasmid DNA on the observed release would be expected to be minimal, as one would anticipate unincorporated DNA to be released from the OPF scaffold in the same manner as DNA that was incorporated into the microspheres, based upon previously discussed size considerations. Although it is possible that the salt content of serum could accelerate dissociation of plasmid DNA from the CGMS relative to what one might observe in the absence of serum, the large size of plasmid DNA relative to the mesh size of OPF would result in retention of the plasmid within the OPF network of composites. Indeed, the observed release of plasmid DNA occurred in a sustained fashion in good accordance with the degradation of the embedded microspheres. Further investigation is warranted to more fully elucidate the apparent limitations on DNA incorporation within the cationized gelatin microspheres faced in the present study. Taking the effects of potentially unincorporated plasmid DNA upon the observed release kinetics into consideration, especially in the case of non-embedded cationized gelatin microspheres, it follows that the actual increases in the availability of DNA provided by cationized gelatin microspheres and by composites of the microspheres with OPF may be greater than those observed in the present study.

Although a prolonged bioavailability of therapeutic plasmid DNA is important to the success of a controlled release gene therapy system, the structural integrity of the DNA following release is of great importance to the potential of the DNA to transfect cells and be expressed. As characterization of the structural integrity of plasmid DNA released in vivo was not feasible, the structural integrity of plasmid DNA released from materials in vitro was assessed through agarose gel electrophoresis. The samples were housed in PBS containing the enzyme collagenase 1A, an enzyme known to digest gelatin through recognition of the sequence –X–Gly–Pro–R–, where X is a neutral amino acid [30]. The presence of collagenase was necessary to degrade the CGMS and to simulate physiological conditions. The collagenase 1A was present in the PBS at a concentration that approximates physiologically relevant concentrations [31].

The electrophoresis results indicate that plasmid DNA converts predominantly to the open-circular conformation when released from both the OPF alone and from composites of OPF and CGMS. The structure of the released DNA, however, does not change appreciably from the open-circular conformation with time of release for any of the material formulations examined. Although some conversion of plasmid DNA from the super-coiled to the open-circular conformation was observed, the literature suggests that there is little difference between the efficiency in transfection or transformation protocols of super-coiled and open-circular plasmid DNA, whereas linear DNA is much less efficient [32,33]. Although the integrity of plasmid DNA following release from composites of OPF and 3 mM CGMS was not assessed in vitro, the results would not be expected to differ from those of composites of OPF and 6 mM CGMS, as the only difference between the two groups is the higher concentration of glutaraldehyde employed in the crosslinking of 6 mM CGMS. Thus, the in vitro results demonstrate that plasmid DNA released from OPF and composites of OPF

and CGMS maintains viable structural integrity over the course of 42 days of release.

In addition to releasing plasmid DNA in a controlled manner while maintaining the structural integrity of the released DNA, the success of a candidate system for controlled DNA release in vivo requires that the components of the system be cytocompatible. Although potentially toxic materials such as TEMED and glutaraldehyde were employed in the fabrication of the materials examined in the present study, the cytotoxicity of the chemicals associated with OPF crosslinking has been addressed in previous reports [34,35]. Indeed, OPF crosslinked by the present method has been employed in studies involving cell encapsulation [19,20]. Further, extensive measures were taken to remove and deactivate glutaraldehyde following crosslinking of the CGMS, including treatment with glycine solution and a total of six washes with water. Indeed, gelatin microspheres crosslinked with glutaraldehyde in this manner were recently explored for the encapsulation of chondrocytes within composites of gelatin microspheres and OPF [36], demonstrating the cytocompatibility of the composite system.

Despite the limitations potentially associated with loading of plasmid DNA within the cationized gelatin microspheres, the increased persistence of plasmid DNA at the site of introduction provided by the microspheres and by the composites of cationized gelatin microspheres and OPF relative to the injection of DNA solution is apparent. Additionally, the prolonged persistence of plasmid DNA when delivered through cationized gelatin microspheres embedded within OPF rather than through the microspheres alone was demonstrated. Although no difference in DNA release was observed between OPF and composites of OPF and cationized gelatin microspheres, the presence of the microspheres may serve two valuable functions. First, the microspheres may act to serve as a porogen within the OPF hydrogel, allowing for the formation upon degradation of void volume into which tissue may grow. Although the term of plasmid DNA release can be controlled through the use of cationized gelatin microspheres alone [9,10], the degradation rate of the microspheres alone is too rapid with respect to typical rates of tissue ingrowth to allow for the microspheres to act as viable tissue scaffolds. The combination of cationized gelatin microspheres with OPF, however, may provide an ideal tissue scaffold by both prolonging the period of the bioavailability of the cationized gelatin and therapeutic plasmid DNA and by creating a porous OPF network into which tissue may grow. Second, plasmid DNA has been shown to form polyionic complexes with cationized gelatin upon loading and been proposed to be released with cationized gelatin degradation fragments as a complex with net positive charge [7]. This released complex of plasmid DNA with cationized gelatin has been proposed to protect the DNA from degradation by nucleases and to facilitate cellular entry [8]. Indeed, the release of plasmid DNA from cationized gelatin has been shown to prolong the duration of gene expression relative to the injection of plasmid DNA in solution form [7,9,10,12]. Thus, composites of cationized gelatin microspheres and OPF show promise as a vehicle for the controlled, sustained delivery of plasmid DNA for enhanced and prolonged gene expression. Further study is warranted, however, to investigate the effect of the controlled release of plasmid DNA from the composites upon gene expression.

5. Conclusion

Composites of cationized gelatin microspheres and a novel hydrogel material, oligo(poly(ethylene glycol)fumarate) were fabricated and investigated toward prolonging the release of plasmid DNA in vivo relative to the constituent materials. The effectiveness of the composites in prolonging plasmid DNA bioavailability relative to both injected plasmid DNA solution and non-embedded cationized gelatin microspheres was demonstrated. Interestingly, the release of plasmid DNA from composites was not significantly different than release from OPF alone. The release of plasmid DNA from the composites appears to be controlled by the degradation of the OPF and of the embedded microspheres. Despite the lack of difference in DNA release observed from OPF and the composites, the formation of composites provides the potential for the formation of porous OPF scaffolds to facilitate tissue infiltration and for enhanced gene expression through the release of plasmid DNA complexed to cationized gelatin degradation fragments. Thus, composites of

OPF and cationized gelatin microspheres show promise for application in the long-term controlled release of plasmid DNA.

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