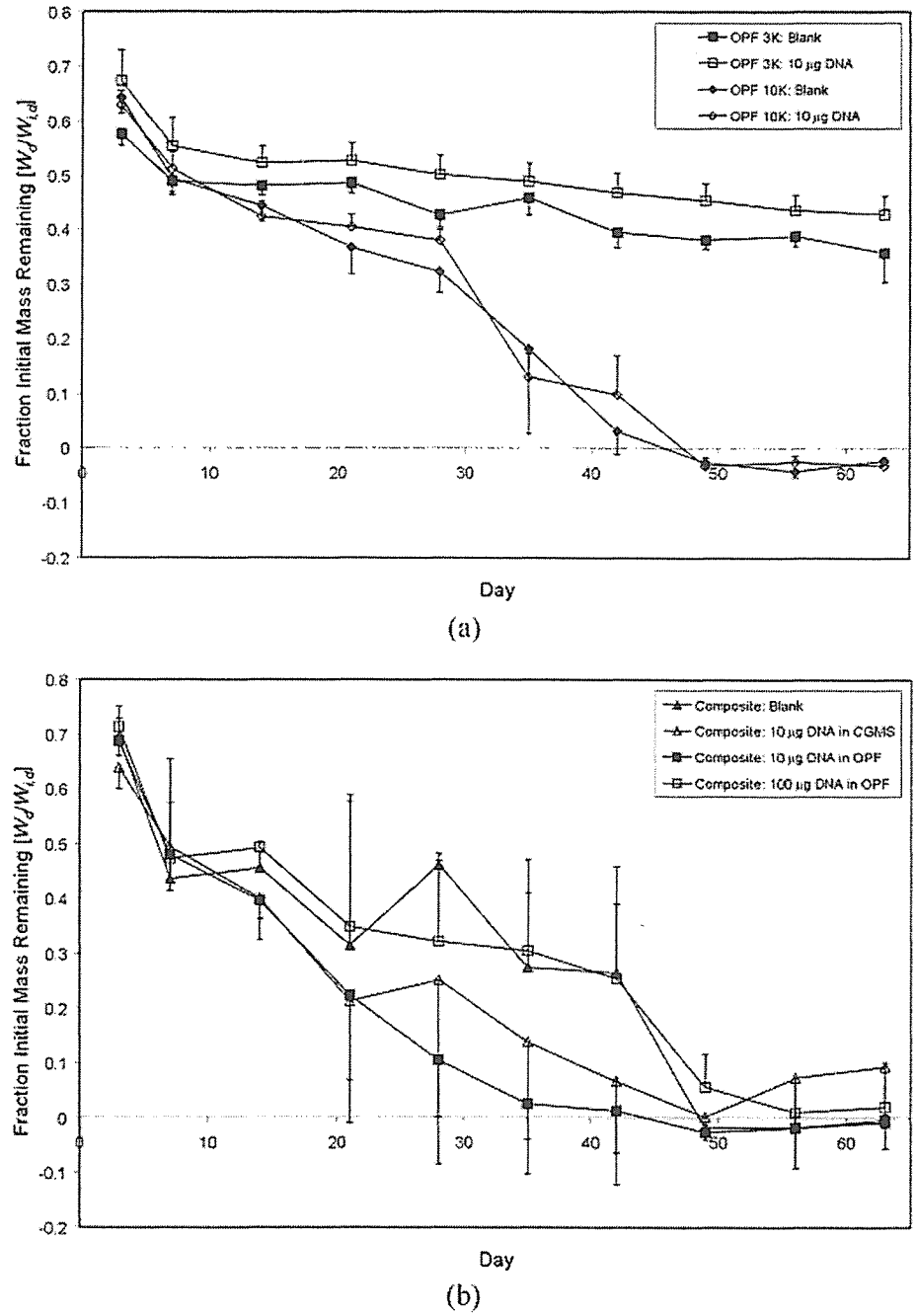

Figure 6. Profile of the fraction of initial polymer mass remaining for OPF hydrogels (a) and composites of OPF and CGMS (b) in enzymatic PBS (pH 7.4) (n = 3/time point). Error bars represent ± standard deviation.

average initial dry polymer mass for each group was determined from six samples that were lyophilized immediately after fabrication. The most marked degree of mass loss in any one period occurred in the first three days. The mass loss from OPF 10K samples and from composite groups continued in a steady fashion from day 3 through day 63 (Fig. 6). All of the OPF 10K samples and some of the samples from each of the composite groups were completely degraded by day 63, whereas none of the OPF 3K samples were completely degraded at this time point. In general, the mass loss within a group was not significantly different at any given time point between a DNA loaded sample and the corresponding blank sample for OPF 10K and composite groups ($p < 0.05$). This was not the case for the OPF 3K group, in which the DNA loaded samples retained significantly more mass than the blank OPF 3K samples at each time point, except days

7, 35, and 63 ($p < 0.05$). The fraction of the initial mass remaining of OPF 3K samples loaded with DNA was significantly greater than that of OPF 10K samples loaded with DNA from day 14 onward, with the exception of day 35 ($p < 0.05$). Additionally, the fraction of the initial mass remaining of OPF 3K samples loaded with DNA was significantly higher than that of composites with 10 μg DNA loaded into the CGMS at days 14 and 42–56 and composites with 10 μg DNA or 100 μg DNA loaded into the OPF at days 14–63 and days 14 and 42–63, respectively ($p < 0.05$).

DISCUSSION

Hydrogels of OPF have been shown to release bioactive DNA in a controlled fashion[17]; yet the lack of pore volume into which tissue might infiltrate limits the potential of OPF hydrogels alone to serve as viable tissue engineering scaffolds. In a subsequent effort to create porous OPF scaffolds, composites of OPF and CGMS were fabricated and examined for controlled DNA release *in vivo*.[18] Specifically, the release of radiolabeled plasmid DNA from CGMS groups (3 and 6 m*M*), groups of composites of OPF 10K and CGMS (3 and 6 m*M*), and from OPF 10K was characterized in a subcutaneous murine model. Additionally, the degradation of radiolabeled CGMS from the same groups was examined.[18] It was shown that the composites of OPF 10K and CGMS extend the bioavailability of DNA relative to CGMS alone and injection of DNA solution.[18] Further, the encapsulation of CGMS in OPF was shown to extend the apparent persistence of the cationized gelatin, relative to injection of CGMS alone.[18] No significant difference was noted in the DNA release between the composite groups (DNA loaded into CGMS) and the OPF 10K alone.[18] It was proposed that control of the release of DNA from the composites was dominated by the degradation of the OPF network, but the characterization of the degradation of OPF *in vivo* was not undertaken in that study.[18] As a result, the present study sought to expand upon the previous work through characterization of the release of DNA from composites of OPF 10K and CGMS (6 m*M*) and characterization of the swelling and degradation properties of these composites. Thus, comparison of the observed DNA release from the composites with the degradation of the composite could be achieved, as could comparison with the release, swelling, and degradation properties of material control groups (OPF 3K and OPF 10K).

In the present study, the release of DNA from composites of OPF 10K and CGMS, in which DNA was loaded into the OPF component, and from control OPF 10K samples followed similar profiles, with the OPF 10K samples reaching a final cumulative release value slightly before the composite formulations. Although enzymes such as collagenase can readily enter the OPF network to facilitate degradation of the gelatin microspheres, the mesh size of OPF 10K hydrogels (13.6 ± 0.3 nm[23]) is much smaller than the apparent molecular size of plasmid DNA (on the order of hundreds of nanometers[4,24]) and the apparent molecular size of plasmid DNA-cationized gelatin complexes.[4] It follows that the degradation of the OPF network likely dominates the release of plasmid DNA, as size limitations presented by the small mesh size of OPF relative to the larger size of plasmid DNA and plasmid DNA-cationized gelatin microsphere complexes likely serve as a significant barrier to the release of plasmid DNA by simple diffusion.[18]

Despite the similarities between the release of DNA from composites in which DNA was loaded into the OPF and the release from the control OPF 10K, significant differences were observed between the release of plasmid DNA from composites in which the DNA was loaded into the CGMS and from the control OPF 10K. The formation of complexes between plasmid DNA and CGMS upon loading contributed to the smaller burst release of DNA at day 3, and a slower DNA release rate was observed from composites in which plasmid DNA was loaded into the CGMS (relative to OPF 10K and composites in which DNA was loaded into the OPF component) (Fig. 2). Loading of CGMS with DNA occurred through reconstitution of freeze-dried CGMS in a volume of DNA solution less than the equilibrium swelling volume for the given mass of CGMS. As a result, the solution was completely incorporated into the CGMS. It follows that the DNA was fully associated with the CGMS after loading, be it within the microspheres or on the surface of the microspheres.[18] Release of the DNA associated with the CGMS in the composite would require either dissociation of the DNA from the cationized gelatin and/or degradation of the cationized gelatin. In either case, the DNA or DNA-cationized gelatin complexes would likely be entrapped within the OPF matrix, due to previously discussed size considerations, until degradation of the network. Thus, the small burst release at day 3 of DNA from the composites in which DNA was loaded into the CGMS component likely stemmed from dissociation of DNA from CGMS at or near the surface of the composite.

The requirement for DNA to be liberated from the CGMS prior to release from the OPF network in the composite group in which DNA was loaded into the CGMS component contributes to the observed slower release rate relative to the other composite groups and the control OPF 10K. Interestingly, the total cumulative release observed from the composite group in which DNA was loaded into the CGMS component was only $71.9 \pm 5.0\%$ upon complete degradation of these samples. Potential differences between the ac-

tual DNA loading per composite and the volumetrically calculated loading values will contribute to this observation. Such differences would be expected to be most pronounced in this group, in which the DNA was loaded solely within the CGMS component of the composite. Any inhomogeneity either in the loading of DNA within the CGMS or in the distribution of the CGMS within the uncrosslinked solution prior to fabrication of the samples would affect the validity of the volumetric calculations of DNA loading. However, the use of volumetric calculations to estimate DNA loading in OPF networks without a CGMS component has been validated previously.[17] It is expected that loading of DNA within the OPF component of composites would present less margin for error in determination of DNA loading through volumetric calculation than would loading of the DNA into the CGMS component, as OPF forms the bulk of the composite, and the DNA solution should distribute homogeneously throughout the OPF solution prior to crosslinking. Interestingly, the final cumulative release of DNA from hydrogels of OPF without a CGMS component exceeded the volumetrically calculated initial DNA loading of these samples. Although an under-calculation in determination of the initial DNA loading could contribute to this observation, the exact reason for the final cumulative release surpassing the calculated initial loading for these samples was not determined. However, the release profiles and final cumulative release values obtained for these samples were highly reproducible, as evident from the small error for these groups (Fig. 2).

The release of DNA from OPF 3K hydrogels, following an initial burst at day 3, occurred in a sustained, linear fashion through day 70 (Fig. 2). From day 70 onward, the release of DNA from OPF 3K proceeded at an increased rate until complete degradation of the gels was noted at day 147. The profile of DNA release from OPF 3K hydrogels was markedly different from the DNA release profiles from OPF 10K gels and composites of OPF 10K and CGMS (Fig. 2). The data suggest that a significantly lower extent of swelling coupled with slower degradation kinetics contributed to the observed slower release of DNA from OPF 3K gels than from OPF 10K gels and composites. The swelling ratio of each release sample was tracked over the course of the study (Fig. 5). This value reflects the change in the mass of a gel at each time point, with respect to the initial mass of the gel. Positive swelling ratio values reflect an increase in the mass of a gel due to an increase in the volume of incorporated solution as the gel swells. Conversely, negative swelling ratio values reflect either a decrease in the polymer content of the gel, a decrease in the solution content of the gel, or a decrease in both the polymer and solution content of a 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. Indeed, the swelling ratio of all gels in the present study reached final values of approximately -1 (Fig. 5).

Comparison of the swelling ratio of the gels within a group (Fig. 5) with the corresponding profile of DNA release (Fig. 2) demonstrates a strong relationship between the release of DNA and the swelling ratio of the gels. The OPF 10K gels were the first to completely degrade, to reach a swelling ratio value of approximately -1, and to reach their final cumulative DNA release value, followed by the composites in which DNA was loaded in the OPF component and CGMS component, respectively (Figs. 2 and 5). The OPF 3K gels, however, exhibited a slight, steady increase in swelling ratio values from day 3 through approximately day 77, followed by a distinctly faster decrease in swelling ratio values to approach a value of -1 at day 147 (Fig. 5). A corresponding slow, sustained release of DNA from OPF 3K gels was observed from day 3 through approximately day 72, followed by a marked increase in the rate of DNA release thereafter (Fig. 2). However, the degradation of OPF 3K hydrogels and the release of DNA from OPF 3K gels occurred at a much slower rate than previously observed *in vitro*.[17] Differences with previous reports in the degradation and DNA release kinetics of OPF 3K hydrogels could be influenced by the presence of collagenase in the release buffer in the present study and variability in batches of polymer synthesis, among other factors. However, in all cases, the release of DNA from OPF hydrogels was directly related to the degradation of the hydrogels as marked by visual examination and swelling ratio values, indicating that control of the release of plasmid DNA from OPF hydrogels is dominated by the degradation of the gels.[17]

Although the swelling ratio reflects the degradation of a gel with time, it does not allow for differentiation between changes due to loss of polymer mass and loss of solution mass as the swelling ratio value decreases. As a result, a set of samples were fabricated to allow for the determination of polymer mass loss with time for the various groups. Based upon the results of previous studies examining the release kinetics of DNA from composites of OPF and CGMS *in vivo* and from OPF 3K and OPF 10K *in vitro*, it was expected that all samples from each group would completely degrade by day 63.[17,18] As a result, a number of samples sufficient for characterization of the polymer mass loss through day 63 were fabricated at the onset of the study. As seen in Figure 6, all samples lost ~30–40% of the initial polymer mass by day 3. This loss of mass can be attributed to extraction of the sol fraction, composed of polymer chains that did not crosslink to incorporate into the hydrogel network.[25] The OPF 10K samples and the composite samples, in general, had approximately zero percent of the initial

polymer mass remaining by day 63, indicating complete degradation (Fig. 6). However, none of the samples from the OPF 3K groups had completely degraded by day 63, with ~35–45% of the initial polymer mass remaining (Fig. 6). The negative values observed for some groups stem from the determination of the fraction of the initial polymer mass remaining using separate samples at each time point and an average value for the initial polymer mass. Thus, the actual initial mass of a sample from a given time point may have deviated from the value determined experimentally. As a result, the swelling ratio values provide a suitable alternative to monitor hydrogel degradation, as they allow for nondestructive tracking of individual release samples over the course of the study.

The concentration of plasmid DNA in each release solution was quantified fluorescently through use of the PicoGreen and OliGreen reagents. PicoGreen and OliGreen have been applied previously toward the quantification of double-stranded DNA (dsDNA) and total DNA (both dsDNA and single-stranded DNA (ssDNA)), respectively, in release solutions.[17] The difference between the detection of DNA within a solution by the OliGreen reagent (total DNA) and PicoGreen reagent (dsDNA) can be attributed to ssDNA.[17] As expected, the amount of DNA quantified by the OliGreen reagent (total DNA) was greater than or equal to the amount quantified by the PicoGreen reagent (dsDNA) for each group at each time point in the study. This difference between the amount of DNA detected by OliGreen and PicoGreen was only significant at the time points just prior to complete degradation of the respective hydrogels in the OPF 3K and OPF 10K groups and composite groups in which DNA was loaded into the OPF component. The increased fraction of ssDNA released with time may reflect damage induced to DNA by acidic degradation products in the microenvironment as the hydrogel degrades. OPF hydrogel networks have been shown to degrade through hydrolysis of the ester bonds to form acrylic and fumaric acids among other products.[26] Indeed, similar increases in DNA damage with time of release have been demonstrated in other studies involving the release of plasmid DNA from degradable hydrogels.[17,27,28] Interestingly, the difference between the amount of DNA detected by OliGreen and PicoGreen in the composite group in which DNA was loaded into the CGMS was not statistically significant at any time point ($p < 0.05$). Potential complexation of plasmid DNA with the cationic gelatin microspheres and degradation products thereof may serve in a limited capacity to protect the DNA from degradation by acidic products of polymer degradation.[4] Further investigation, however, is required to more fully understand this observation. Although the fraction of ssDNA released from each group increased with time, the majority of DNA released from each group at every time point was dsDNA (Fig. 2).

The results clearly indicate the ability to control the release of plasmid DNA from OPF composites *in vitro*, with the majority of released DNA being double-stranded. Indeed, the structural integrity of the DNA following release is of great importance to the potential of the DNA to transfect cells and to be expressed. As a result, the structural integrity of plasmid DNA released from the materials was assessed through agarose gel electrophoresis. The results indicate that plasmid DNA converts predominately to the open-circular conformation when released from both OPF alone and from composites of OPF and CGMS (Figs. 3 and 4). DNA bands were visible for the OPF 3K group on the agarose gels, but not at every time point. This likely stems from the small amount of DNA being released from this group between days 7 and 63, relative to the other material groups. The results from PicoGreen and OliGreen clearly indicate that DNA was indeed being released from OPF 3K gels during this time frame. For some groups, bands of linear DNA were observed at later time points and can be explained by damage induced by acidic polymer degradation products. However, the majority of DNA from each group was released in either the open-circular conformation or super-coiled conformation. Although some conversion of plasmid DNA from the super-coiled to the open-circular conformation was observed, the literature suggests that little difference exists between the efficiency in transfection or transformation protocols of super-coiled or open-circular DNA, whereas linear DNA is much less efficient.[29,30] Thus, the majority of plasmid DNA released from hydrogel composites of OPF and CGMS retains potential for bioactivity.

CONCLUSIONS

Novel hydrogel composites of oligo(poly(ethylene glycol) fumarate) (OPF) and cationized gelatin microspheres (CGMS) were fabricated and studied toward the controlled release of plasmid DNA *in vitro*. Control of the release of plasmid DNA from hydrogels and hydrogel composites appeared to be dominated by the degradation of the OPF network. Additionally, the manner in which the plasmid DNA was loaded into the composites appeared to affect the DNA release kinetics, with loading into the CGMS prolonging the DNA release. The released DNA maintained suitable structural form for transfection based upon results of electrophoretic analysis. The controlled release of DNA from composites of OPF and CGMS, coupled with the potential for the creation of a porous hydrogel network upon CGMS degradation, demonstrates

the potential of the composites for controlled gene delivery in tissue engineering applications.

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References

1. Fu K, Klibanov AM, Langer R. Protein stability in controlled-release systems. Nat Biotechnol 2000;18:24–25.
2. Goldstein SA, Bonadio J. Potential role for direct gene transfer in the enhancement of fracture healing. Clin Orthop Relat Res 1998;355 (Suppl):S154–S162.
3. Fukunaka Y, Iwanaga K, Morimoto K, Kakemi M, Tabata Y. Controlled release of plasmid DNA from cationized gelatin hydrogels based on hydrogel degradation. J Control Release 2002;80:333–343.
4. Kushibiki T, Tomoshige R, Fukunaka Y, Kakemi M, Tabata Y. In vivo release and gene expression of plasmid DNA by hydrogels of gelatin with different cationization extents. J Control Release 2003;90:207–216.
5. Kushibiki T, Matsumoto K, Nakamura T, Tabata Y. Suppression of tumor metastasis by NK4 plasmid DNA released from cationized gelatin. Gene Ther 2004;11:1205–1214.
6. Kushibiki T, Matsumoto K, Nakamura T, Tabata Y. Suppression of the progress of disseminated pancreatic cancer cells by NK4 plasmid DNA released from cationized gelatin microspheres. Pharm Res 2004;21:1109–1118.
7. Hosseinkhani H, Aoyama T, Ogawa O, Tabata Y. Ultrasound enhancement of *in vitro* transfection of plasmid DNA by a cationized gelatin. J Drug Target 2002;10:193–204.
8. Aoyama T, Hosseinkhani H, Yamamoto S, Ogawa O, Tabata Y. Enhanced expression of plasmid DNA-cationized gelatin complex by ultrasound in murine muscle. J Control Release 2002; 80(1/3):345–356.
9. Aoyama T, Yamamoto S, Kanematsu A, Ogawa O, Tabata Y. Local delivery of matrix metalloproteinase gene prevents the onset of renal sclerosis in streptozotocin-induced diabetic mice. Tissue Eng 2003;9:1289–1299.
10. Fisher JP, Vehof JW, Dean D, van der Waerden JP, Holland TA, Mikos AG, Jansen JA. Soft and hard tissue response to photocrosslinked poly(propylene fumarate) scaffolds in a rabbit model. J Biomed Mater Res 2002;59:547–556.
11. Shin H, Quinten Ruhe P, Mikos AG, Jansen JA. In vivo bone and soft tissue response to injectable, biodegradable oligo(poly(ethylene glycol) fumarate) hydrogels. Biomaterials 2003;24: 3201–3211.
12. Shin H, Zygourakis K, Farach-Carson MC, Yaszemski MJ, Mikos AG. Attachment, proliferation, and migration of marrow stromal osteoblasts cultured on biomimetic hydrogels modified with an osteopontin-derived peptide. Biomaterials 2004; 25:895–906.
13. Shin H, Jo S, Mikos AG. Modulation of marrow stromal osteoblast adhesion on biomimetic oligo[poly(ethylene glycol) fumarate] hydrogels modified with Arg-Gly-Asp peptides and a poly(ethyleneglycol) spacer. J Biomed Mater Res 2002;61:169–179.
14. Temenoff JS, Steinbis ES, Mikos AG. Effect of drying history on swelling properties and cell attachment to oligo(poly(ethylene glycol) fumarate) hydrogels for guided tissue regeneration applications. J Biomater Sci Polym Ed 2003;14:989–1004.
15. Temenoff JS, Park H, Jabbari E, Conway DE, Sheffield TL, Ambrose CG, Mikos AG. Thermally cross-linked oligo(poly-(ethylene glycol) fumarate) hydrogels support osteogenic differentiation of encapsulated marrow stromal cells *in vitro*. Biomacromolecules 2004;5(1):5–10.
16. Temenoff JS, Park H, Jabbari E, Sheffield TL, LeBaron RG, Ambrose CG, Mikos AG. In vitro osteogenic differentiation of marrow stromal cells encapsulated in biodegradable hydrogels. J Biomed Mater Res A 2004;70:235–244.
17. Kasper FK, Seidlits SK, Tang A, Crowther RS, Carney DH, Barry MA, Mikos AG. In vitro release of plasmid DNA from oligo(poly(ethylene glycol) fumarate) hydrogels. J Control Release 2005;104:521–539.
18. Kasper FK, Kushibiki T, Kimura Y, Mikos AG, Tabata Y. In vivo release of plasmid DNA from composites of oligo(poly-(ethylene glycol) fumarate) and cationized gelatin microspheres. J Control Release 2005;107:547–561.
19. Jo S, Shin H, Mikos AG. Modification of oligo(poly(ethylene glycol) fumarate) macromer with a GRGD peptide for the preparation of functionalized polymer networks. Biomacromolecules 2001;2:255–261.
20. Ikada Y, Tabata Y. Protein release from gelatin matrices. Adv Drug Deliv Rev 1998;31:287–301.
21. Yoshihara Y, Nakamura H, Obata K, Yamada H, Hayakawa T, Fujikawa K, Okada Y. Matrix metalloproteinases and tissue inhibitors of metalloproteinases in synovial fluids from patients with rheumatoid arthritis or osteoarthritis. Ann Rheum Dis 2000;59:455–461.
22. Taylor JR. An Introduction to Error Analysis. Mill Valley, CA: University Science Books; 1982.
23. Holland TA, Tabata Y, Mikos AG. In vitro release of transforming growth factor-β 1 from gelatin microparticles encapsulated in biodegradable, injectable oligo(poly(ethylene glycol) fumarate) hydrogels. J Control Release 2003;91:299–313.
24. Ledley FD. Pharmaceutical approach to somatic gene therapy. Pharm Res 1996;13:1595–1614.
25. Peppas NA, Barr-Howell BD. Characterization of the cross-linked structure of hydrogels. In: Peppas NA, editor. Hydrogels in Medicine and Pharmacy: Fundamentals. Boca Raton: CRC; 1986. p 27–56.
26. Timmer MD, Jo SB, Wang CY, Ambrose CG, Mikos AG. Characterization of the cross-linked structure of fumarate-based degradable polymer networks. Macromolecules 2002;35:4373–4379.
27. Quick DJ, Anseth KS. Gene delivery in tissue engineering: A photopolymer platform to coencapsulate cells and plasmid DNA. Pharm Res 2003;20:1730–1737.
28. Quick DJ, Anseth KS. DNA delivery from photocrosslinked PEG hydrogels: Encapsulation efficiency, release profiles, and DNA quality. J Control Release 2004;96:341–351.
29. Kimoto H, Taketo A. Studies on electrotransfer of DNA into *Escherichia coli*: Effect of molecular form of DNA. Biochim Biophys Acta 1996;1307:325–330.
30. Xie TD, Sun L, Zhao HG, Fuchs JA, Tsong TY. Study of mechanisms of electric field-induced DNA transfection. IV. Effects of DNA topology on cell uptake and transfection efficiency. Biophys J 1992;63:1026–1031.