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Porous PLGA scaffolds for controlled release of naked and polyethyleneimine-complexed DNA

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Abstract

The ability to precisely control delivery of single or multiple bioactive molecules is critical in tissue engineering, and controlled release of plasmid coding for growth factors and their regulators can give cell-regulated, short-term expression of these therapeutic biomolecules. In this work, porous poly(lactic-*co*-glycolic acid) (PLGA) scaffolds comprising acid-terminated chains of either low (LMW; 10 kDa) or high molecular weight (HMW; 30 kDa) were developed for controlled release of naked or polyethyleneimine (PEI)-complexed DNA. The compressive strength of blank HMW and LMW scaffolds was 6 and 2 MPa, respectively, while the strength of PEI:DNA-containing HMW and LMW scaffolds was 7 and 1 MPa, respectively. LMW scaffolds degraded more quickly than HMW scaffolds, with 80–100% and 15–30% mass loss at 30 days, respectively. Encapsulation of plasmid, particularly PEI-complexed DNA, only modestly affected degradation. Release profiles showed bi- or triphasic patterns, with early burst release of surface-associated DNA, slower diffusion-mediated release, and degradation-related release at later time points. Complexation with PEI tended to a slow release of plasmids, likely because of interaction with the carboxyl groups of PLGA. Culturing rat bone marrow cells on blank PLGA scaffolds in the presence of IGF-I resulted in growth and chondrogenic differentiation of these cells. Porous scaffolds made of PLGA with the appropriate selection of hydrophobicity and molecular weight will allow controlled delivery of naked and condensed plasmid DNA for different tissue engineering applications.

(Some figures may appear in colour only in the online journal)

1. Introduction

The ability to precisely control the delivery of single or multiple bioactive molecules is critical in tissue engineering because tissue formation involves the interplay of numerous growth factors and other signaling molecules. Biodegradable microspheres and porous scaffolds made of poly(lactic-*co*-glycolic acid) (PLGA) have been used extensively for the controlled release of therapeutics, from proteins to DNA to small interfering RNAs [9, 3]. A copolymer of lactic acid and glycolic acid, PLGA is FDA approved for certain clinical applications, and it offers the possibility of tunable mechanical and degradation properties by varying its inherent viscosity/molecular weight, molar ratio of constituent monomers, and the presence of end caps.

In an inductive approach to tissue engineering, a porous scaffold that provides space for tissue growth and differentiation is combined with local delivery of growth factors that can induce proliferation and differentiation of native precursor cells [20]. The effects of growth factors are often highly dose dependent. For example, TGF-β has

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been shown to inhibit bone formation rate and prolong mineralization lag time with high doses [8]. Another challenge with direct application of growth factors is their short half-life. Insulin-like growth factor (IGF)-I had a half-life of 240 min after intravenous injection in rats, while TGF-β_1 had a half-life of 60–163 min [32, 33]. This rapid clearance necessitates large initial loading of the delivery device with growth factors to last the entire duration of treatment.

Delivery of plasmid coding for the growth factors would be a more efficient form of therapy since the endogenous, locally transfected cells themselves can control the short-term synthesis and expression of the growth factors. Naked DNA without a carrier system, however, is highly susceptible to endonuclease degradation [17]. Plasmid DNA injected intravenously is also rapidly cleared from plasma [31]. Although transfection efficiencies with viral carriers are quite high, certain viral vectors have the disadvantage of generating an immune response in the host and the possibility of integrating exogenous DNA with the host genome. Plasmid DNA has limited issues of toxicity and immunogenicity compared to viral vectors [30].

Polyethyleneimines (PEI) are positively charged polymers due to the large number of amine groups, making them suitable to condense the negatively charged DNA molecules and facilitate their entry into cells [1, 7]. PEI–DNA complexes are engulfed by the cell, and the endosome is acidified, where the PEI acts as a proton acceptor [10]. It is thought that the interaction of PEI amines with protons triggers osmotic swelling of the complex, causing the endosome to rupture and thus release the complexes into the cytoplasm [10].

Freeze-dried PEI:DNA complexes were mixed with granular hydrophobic PLGA and compression molded to create a DNA delivery device [11]. Controlled delivery of PEI-complexed DNA encoding bone morphogenetic protein (BMP)-4 from PLGA scaffolds resulted in bone formation in a rat calvarial defect model [12]. Surface adsorption of PEI:DNA complexes on porous PLGA scaffolds has been used for gene delivery *in vitro* [15]. Porous PLGA–Pluronic F127 scaffolds incorporated with SOX plasmid DNA have been used for chondrogenesis using adipose stem cells [13].

This paper presents the investigation of two types of PLGA for controlled release of naked and PEI-complexed DNA from porous scaffolds. Addition of a freeze-dried layer of naked DNA on the surface of PLGA scaffolds was also tested to provide an initial burst release to 'jump-start' the transfection of cells attaching to the scaffolds. Degradation, mechanical properties, and controlled release profiles of low molecular weight (LMW) hydrophilic PLGA and high molecular weight (HMW) hydrophilic PLGA scaffolds were studied. Furthermore, the ability of the scaffolds to support chondrogenic differentiation of rat bone marrow cells (BMCs) in the presence of IGF-I was examined *in vitro*.

2. Materials and methods

2.1. Plasmid preparation

Plasmids pEGFP-C1 and pDsRed2-C1 (Clontech, Mountain View, CA) were propagated in DH-5α subclonal efficiency cells (Invitrogen, Carlsbad, CA). DNA from cultures of these cells was extracted using the GenElute HP Plasmid Maxiprep Kit (Sigma-Aldrich, St Louis, MO) following the manufacturer's instructions. The absorbance of the extracted DNA was measured at 260 and 280 nm using a UV spectrophotometer, and only DNA with an absorbance ratio greater than 1.8 was used. DNA concentration was determined from its absorbance at 260 nm.

Branched PEI (50% w/v aqueous solution, $M_n \sim 1200$; Sigma) was diluted to 10 mM solution for this study. PEI:DNA complexes with a nitrogen to phosphorus (N/P) ratio of 5 or 14 were prepared according to previously published protocol of Boussif *et al* [2]. Briefly, an amount of 10 mM PEI that resulted in the desired N/P ratio was added dropwise to 500 μg mL^{-1} plasmid DNA containing 1% sucrose. This PEI:DNA solution was quickly frozen to -80 °C and lyophilized for 3 days. Lyophilized PEI:DNA powder was resuspended in 700 μL of Tris-EDTA (TE) buffer. For select experiments, PEI was labeled with the amine-reactive probe Alexa488 (Molecular Probes/Invitrogen, Carlsbad, CA) using the manufacturer's protocol.

2.2. PLGA microspheres

DNA-loaded microspheres were made with 50:50 PLGA (Durect Corp., Pelham, AL) using a W/O/W double-emulsion method [14]. PLGA with an inherent viscosity of 0.15–0.25 dL g^{-1} ($M_w \sim 10\,000$ Da) and acid end group has been noted as LMW hydrophilic PLGA, and PLGA with an inherent viscosity of 0.55–0.75 dL g^{-1} ($M_w \sim 30\,000$ Da) and acid end group has been noted as HMW hydrophilic PLGA. An aliquot of 500 μg mL^{-1} DNA alone or PEI:DNA solution was added to 10% w/v LMW PLGA in methylene chloride. The DNA solution was replaced with TE buffer to create blank microspheres. This suspension was then vortexed for 30 s and added dropwise to rapidly stirring 1% polyvinyl alcohol (Sigma) in deionized water to form the W/O/W emulsion. To create HMW PLGA microspheres, an Omni PDH homogenizer (Omni International, Kennesaw, GA) was used to stir the suspension at 3500 rpm for 5 min. In both cases, the emulsion was stirred overnight to evaporate methylene chloride, and the microspheres were collected using centrifugation. The collected microspheres were rinsed twice in deionized water and lyophilized for 48 h before making scaffolds. Encapsulation efficiency was determined following dissolution of microspheres in methylene chloride with subsequent measurement of DNA using the Picogreen assay (described in section 2.5).

2.3. Porous PLGA scaffolds

Porous PLGA scaffolds were prepared by mixing 42 mg of PLGA microspheres with 63 mg of 100–350 μm salt particles (40:60 w/w; ratio selected based on pilot study) for 2 min, and compressing at 1.5 tons for 2 min in a 6 mm die using a Carver press (Wabash, IN). The resulting scaffolds were 6 mm in diameter by 2 mm in height. A mass of 120 mg of PLGA microspheres was also mixed with 180 mg of salt particles (40:60 w/w) for 2 min and then compressed at 6 tons

for 2 min in a 13 mm die to create larger disks (13 mm in diameter by 2 mm in height). The microspheres were fused by incubating the compacts near the polymer's T_g for 48 h. For LMW PLGA, the 'sintering' temperature was 42 °C, while for HMW PLGA, the temperature was 49 °C. To create porous PLGA scaffolds, salt was leached from the consolidated disks by stirring six disks in 2 L of deionized water overnight, after which the leached disks were dried in vacuum. Measurement of the electrical conductance of the water solution was initially used to confirm leaching. To create disks with freeze-dried DNA on top, 40 μL of 500 μg mL^{-1} DNA solution containing 1% sucrose was added to the surface of previously prepared scaffolds and then lyophilized overnight.

For assessment of scaffold morphology, samples were coated with gold and analyzed by scanning electron microscopy (SEM, Hitachi 3200) at 20 kV in the secondary electron imaging mode. Total porosity and the pore-size distribution in scaffolds were evaluated by mercury intrusion porosimetry (Micromeritics, Norcross, GA).

2.4. Mechanical testing

The larger scaffolds (13 mm diameter × 2 mm height) were tested to failure under compressive loading using a Bose Electroforce 3300 (Eden Prairie, MN). Dimensions varying from the American Society for Testing Materials or the International Organization for Standardization standards were used to allow testing of a geometry reflecting that needed for implantation in a growth plate or cartilage defect. The displacement rate was set at 1 mm min^{-1}, and scaffolds were compressed until failure. Using the individual scaffold dimensions, stress–strain plots for different groups of scaffolds were generated, and ultimate stresses and compressive moduli were calculated.

2.5. Mass loss and plasmid release

Scaffolds (6 mm diameter) were immersed in 2 mL of phosphate-buffered saline (PBS), pH 7.4, in 12-well plates, and incubated at 37 °C with continuous shaking. PBS was replaced every 2 days. Scaffolds were collected at various time points and lyophilized for 48 h before measuring their final weights.

In vitro release experiments were conducted by incubating scaffolds in 2 mL PBS containing 2 mM EDTA, pH 7.4, at 37 °C, with continuous shaking. The supernatant was collected and replaced every 24 h. The concentration of DNA in the supernatant was determined using a fluorometric Picogreen® (Molecular Probes/Invitrogen) assay according to the manufacturer's instructions using calf thymus DNA as a standard. Release of Alexa488-labeled PEI from microspheres was measured at an excitation/emission wavelength of 488/520 nm.

2.6. BMC culture on scaffolds

BMCs were obtained from 4–6 week old male Sprague-Dawley rats using a previously described method [29]. Briefly, femurs were aseptically dissected from freshly euthanized animals. Intramedullary canals of femurs were then flushed with MEM Alpha modification medium with 10% fetal bovine serum (FBS) to collect BMCs, which were then transferred to T-75 flasks and grown to confluence. Porous LMW PLGA scaffolds were disinfected using 70% ethanol (10 min) and washed twice with sterile PBS. The scaffolds were dried in a laminar flow hood overnight before seeding cells. BMCs were carefully seeded onto each scaffold (625 000 cells/scaffold) and cultured for up to 4 weeks. 1 day after seeding, recombinant human IGF-I (R&D Systems) was added to the medium at 100 ng mL^{-1} for the treatment group, and the media was changed every 2 days. Cells growing on scaffolds were labeled, if needed, using CellTracker Green CMFDA (Invitrogen/Molecular Probes) according to the manufacturer's protocol. Cells were then visualized using a Leica TCS-SP5 confocal microscope (Leica Microsystems). At the end of the study scaffolds were washed twice with PBS, transferred to 15 mL centrifuge tubes in 1 mL PBS, and then sonicated for 30 s using an ultrasonic processor. The resulting cell lysates were used for DNA and glycosaminoglycan (GAG) analysis. Cell growth was determined by measuring the amount of DNA in the cell lysate using a fluorometric Picogreen® assay according to the manufacturer's protocol. The amount of GAG in cell/scaffold lysate was determined using Blyscan® (Biocolor, UK) reagent according to the manufacturer's protocol.

D1 cells (CRL-12424) were obtained from American Type Culture Collection. Cells were grown to confluence in T-75 flasks in DMEM/high glucose medium with 10% FBS (HyClone Laboratories). These cells were then trypsinized and grown on PEI:DNA-releasing PLGA scaffolds for transfection studies.

2.7. Statistical analysis

The Student's *t*-test and analysis of variance were used for comparing results between different groups (SAS 9.1 NC software, Cary, NC). When significant differences were detected (p-value $\leqslant 0.05$), the Tukey–Kramer multiple comparison test was used post hoc. All figures show mean value ± standard error for each variable measured in triplicate. Mixed models with repeated measures were used to compare various release profiles.

3. Results

3.1. Microspheres and scaffolds

The resulting double-emulsion microspheres were 30–160 μm in diameter (figure 1), and they encapsulated DNA with 45% efficiency. A pilot study indicated that a 40:60 w/w ratio of PLGA microspheres to salt particles would result in mechanically stable scaffolds. Figure 1 also shows a SEM micrograph of an internal section of a scaffold. The scaffolds were characterized by an open porous structure, with pores in a wide size range. In addition, the non-porous surface layer typical of solvent casting was not observed on scaffolds prepared with this method. Mercury intrusion porosimetry showed that the scaffolds averaged 63% porosity, with 96% of the pores between 1 and 700 μm and only 4% between 3 nm and 1 μm.

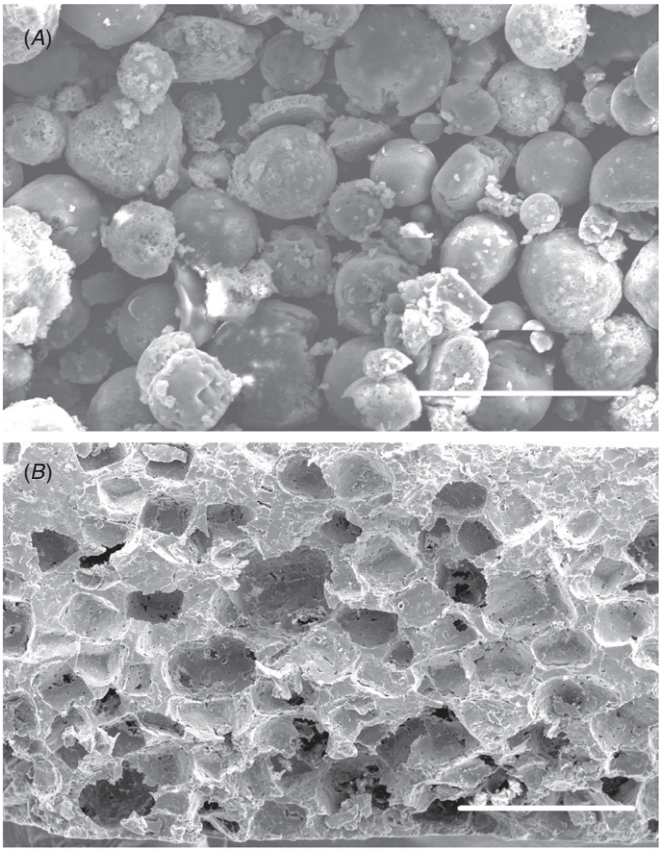

Figure 1. Representative SEM images of (*A*) microspheres and (*B*) cross section of a porous PLGA scaffold (63% porous) illustrating the uniform distribution of pores throughout the scaffold thickness. Size markers are 200 μm and 1 mm for parts (*A*) and (*B*), respectively.

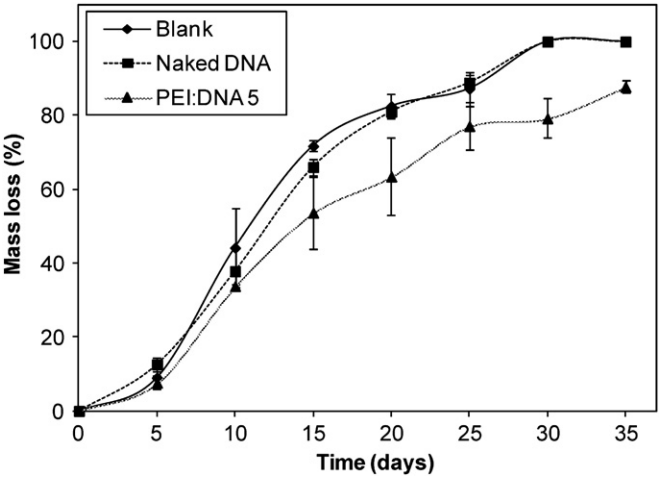

Figure 2. *In vitro* mass loss of LMW hydrophilic scaffolds. Data are mean ± standard error ($n = 3$).

3.2. Degradation/mass loss

3.2.1. LMW hydrophilic scaffolds. Figure 2 shows the *in vitro* mass loss of LMW hydrophilic scaffolds. Blank scaffolds had the highest slope, indicating the fastest mass loss, while PEI:DNA 5 scaffolds had the lowest slope, indicating the slowest mass loss. Between days 5 and 15, PEI:DNA 5 scaffolds lost their mass at an average rate of 4.6% day^{-1}, naked DNA scaffolds at the rate of 5.3% day^{-1}, and blank scaffolds at the rate of 6.2% day^{-1}. For the mixed model analysis of mass loss rates, however, there was no significant difference between the different scaffold groups. Blank and naked DNA scaffolds exhibited complete degradation by day 30, while PEI:DNA scaffolds retained 20% of their mass at that time point. The difference in masses between PEI:DNA scaffolds and blanks, and PEI:DNA scaffolds and naked DNA scaffolds was statistically significant at days 30 and 35 ($p < 0.05$).

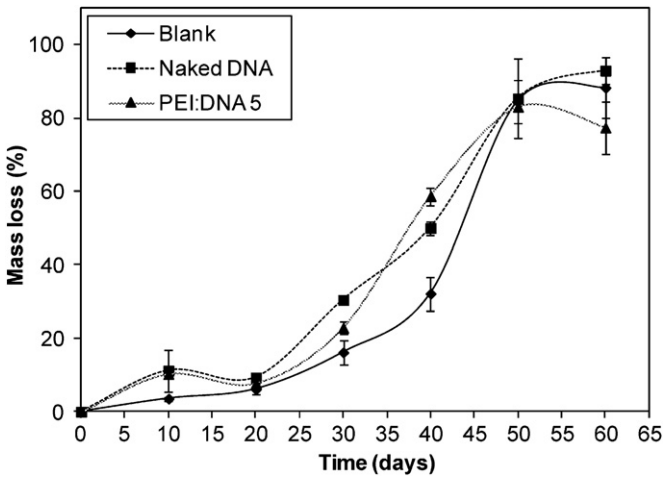

Figure 3. *In vitro* mass loss of HMW hydrophilic scaffolds. Data are mean ± standard error ($n = 3$).

3.2.2. HMW hydrophilic scaffolds. Degradation of HMW hydrophilic scaffolds in PBS exhibited a two-phase trend (figure 3). In the initial phase through 20 days, HMW PLGA scaffolds lost only about 10% of their initial mass, with an average rate of mass loss at 0.38% day^{-1}. In the second phase, between days 20 and 60, the average mass loss rate was 2.05% day^{-1} for the blank scaffolds, 2.09% day^{-1} for the DNA scaffolds, and 1.74% day^{-1} for PEI:DNA scaffolds. However, per the mixed model analysis, there was no statistical difference between mass loss rate of blank, naked DNA, and PEI:DNA scaffolds during either phase. By day 60, total mass loss for blank scaffolds (88%), PEI:DNA scaffolds (77%), and naked DNA scaffolds (93%) was statistically comparable.

3.3. Mechanical properties

Figure 4(*A*) is a representative stress–strain plot from the mechanical testing of LMW PLGA scaffolds. Both the HMW and LMW PLGA scaffolds showed initial linear stress–strain relationships prior to failure/collapse, but they differed in their ultimate compressive strength (UCS) as well as compressive modulus, as evidenced in figure 4(*B*). The average compressive modulus of blank scaffolds made with HMW PLGA (78 MPa) was 169% higher ($p < 0.05$) than that for blank scaffolds made with LMW PLGA (29 MPa). Similarly, the average compressive modulus of PEI:DNA scaffolds made with HMW PLGA (81 MPa) was 211% higher ($p < 0.05$) than that for blank scaffolds made with LMW PLGA (26 MPa).

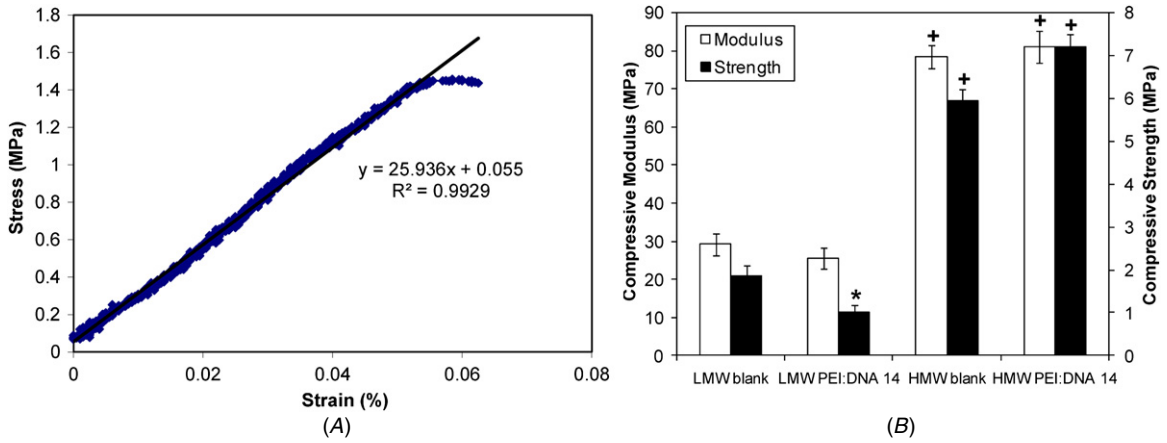

Figure 4. (*A*) Representative stress–strain curve for LMW hydrophilic scaffold. (*B*) UCS and compressive moduli of PLGA scaffolds. Data are mean ± standard error ($n = 3$). * denotes $p < 0.05$ between LMW blank and LMW PEI:DNA 14; + denotes $p < 0.05$ between corresponding LMW and HMW scaffolds.

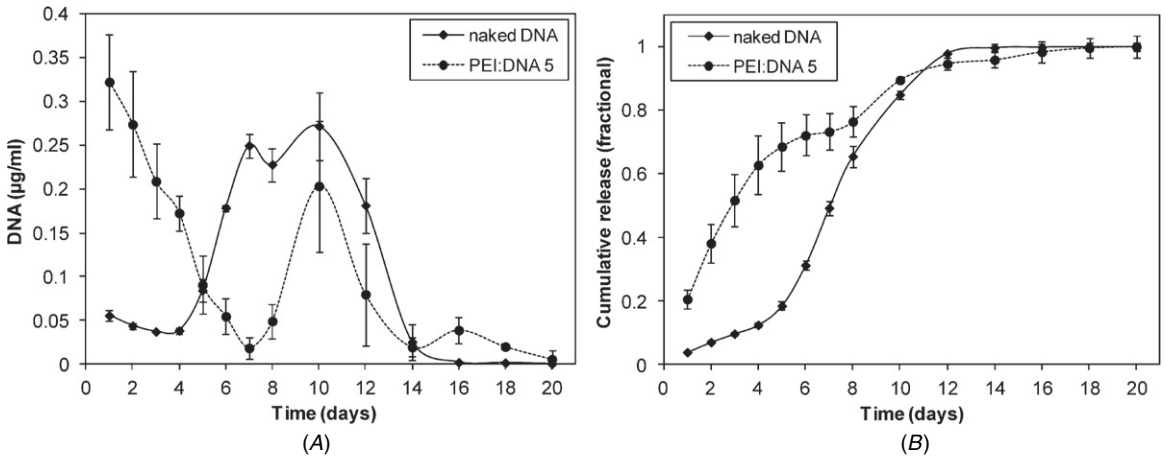

Figure 5. (*A*) Instantaneous and (*B*) cumulative fractional release of naked and PEI-complexed DNA from LMW PLGA scaffolds. Data are mean ± standard error ($n = 3$).

However, encapsulating PEI:DNA did not significantly affect the compressive modulus of the LMW or HMW PLGA scaffolds.

As shown in figure 4(*B*), the ultimate strength of blank scaffolds made with HMW PLGA (6 MPa) was 200% higher ($p < 0.05$) than that of blank scaffolds made with LMW PLGA (2 MPa). Similarly, the ultimate strength of PEI:DNA HMW PLGA scaffolds (7 MPa) was 600% higher ($p < 0.05$) than that of PEI:DNA LMW PLGA scaffolds (1 MPa). Although PEI:DNA encapsulation decreased the strength of LMW PLGA scaffolds ($p < 0.05$), the effect of PEI:DNA encapsulation on the strength of HMW PLGA scaffolds was not significant.

3.4. DNA release

3.4.1. LMW PLGA. Figure 5(*A*) shows the release of naked and PEI-complexed DNA from LMW PLGA scaffolds. Both naked and PEI:DNA groups exhibited biphasic profiles, with DNA being detected in the supernatant from the first days of release; at day 1, the burst release from PEI:DNA scaffolds was about six times greater than that from naked DNA scaffolds. Release of naked DNA showed a peak concentration of ∼0.26 μg mL^{-1} around days 7–10, with exhaustion by day 16. In contrast, PEI:DNA scaffolds exhibited a longer initial phase of release, ending by day 7 compared to day 4 for naked DNA scaffolds. Moreover, the concentration of released PEI:DNA was also higher compared to naked DNA until day 5. The second peak of PEI:DNA release occurred on day 10, compared to a broader peak over days 7–10 for naked DNA, and release was complete by day 20.

The fractional cumulative release of naked and PEI-complexed DNA particles from LMW PLGA scaffolds is illustrated in figure 5(*B*). Scaffolds with PEI:DNA released the majority of their contents earlier than did those with naked DNA; by day 6, PEI:DNA scaffolds had released 72% of their total content, while naked DNA scaffolds had released only 31%. However, PEI:DNA scaffolds completed their release by day 20, compared to naked DNA scaffolds that completed their release by day 16. Naked DNA scaffolds had an average release rate of 2.7% day^{-1} during the first phase and 9.6% day^{-1} during the second phase of release, compared to 12.5% day^{-1} during the first phase and 4.3% day^{-1} during the second phase for PEI:DNA scaffolds. Mixed model analysis of the

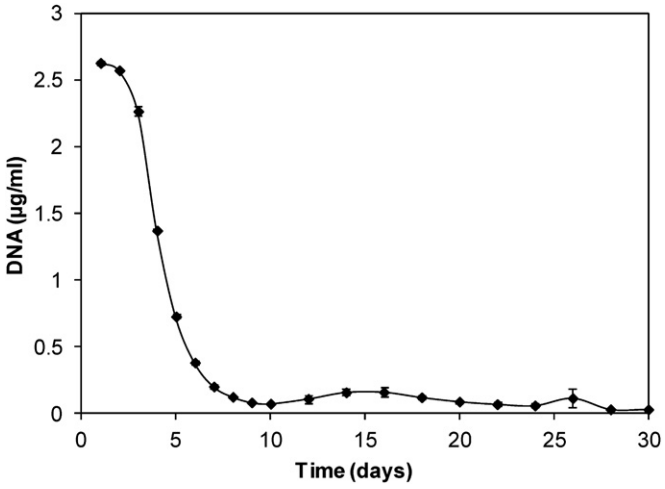

Figure 6. Release of freeze-dried and encapsulated naked DNA from LMW PLGA scaffolds. Data are mean ± standard error ($n = 3$).

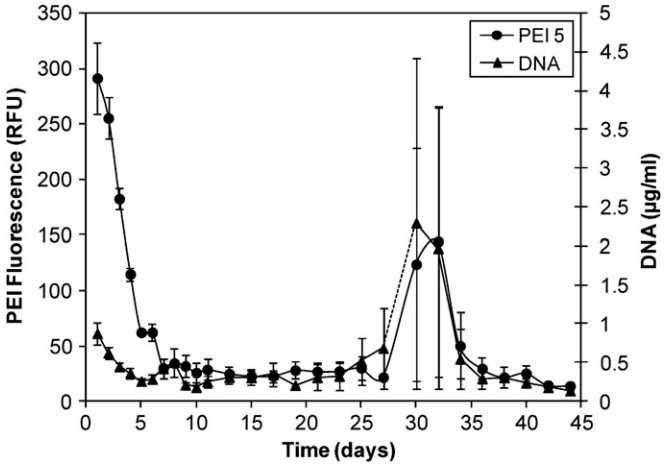

Figure 7. Superimposed release profiles of labeled PEI and DNA from LMW PLGA scaffolds. Data are mean ± standard error ($n = 3$).

DNA release rates from the two groups showed a statistically significant difference during the first phase ($p < 0.05$), while the release rates during the second phase were comparable.

The addition of a layer of naked DNA freeze-dried onto the surface of scaffolds markedly increased the initial amount of DNA released from PLGA scaffolds (figure 6). The initial amount of DNA released from scaffolds containing a freeze-dried layer of DNA was 87-fold greater and significantly higher than that from scaffolds with DNA alone (2.6 μg mL^{-1} as opposed to 0.03 μg mL^{-1}). The initial burst release of freeze-dried DNA was over by 1 week, after which the DNA release profile from scaffolds with or without the additional freeze-dried DNA layer was comparable.

Release of fluorescently labeled PEI. Complexation of DNA with PEI resulted in reduced detectability of DNA using normal DNA assays. Therefore, PEI used for making PEI:DNA complexes was labeled using Alexa488 to track its release from LMW PLGA scaffolds (figure 7). PEI was detected in the supernatant from the first day of release, and the profile showed a typical biphasic pattern. The first phase of release for scaffolds with PEI:DNA 5 began on day 1 and was over by day 7, while the second peak lasted from day 27 to day 34. In addition to tracking fluorescently labeled PEI, DNA was also assayed from the same set of scaffolds. The first phase of PEI:DNA release started on day 1 and ended by day 5, whereas the second phase of release started on day 25 and ended by day 34. The results showed excellent correlation between PEI and DNA release profiles. Variability between samples around day 30 was high as samples started disintegrating into smaller pieces.

3.4.2. HMW PLGA scaffolds. Figure 8(*A*) shows the release profiles of naked DNA and PEI:DNA complexes in HMW hydrophilic PLGA scaffolds. For naked DNA, the first phase of release started on day 1 and was over by day 10. The second phase of release started on day 26, peaked on day 36, and ended by day 50. Complexation of DNA with PEI resulted in a later second phase of release. The first phase of PEI:DNA release started on day 1 and continued until day 26. The second phase of release started on day 28, peaked around day 50, and continued until day 70. Variability between samples was high during peak release, as samples started disintegrating into smaller fragments.

Figure 8(*B*) shows the fractional cumulative release of naked and PEI-complexed DNA from HMW hydrophilic PLGA scaffolds. Both kinds of scaffolds had similar release rates during the first phase of release. PEI:DNA scaffolds had a slower release during the second phase, as evidenced by a lower slope. During the second phase of release, naked DNA scaffolds had an average release rate of 7.1% day^{-1} compared to 3.05% day^{-1} for PEI:DNA scaffolds. Mixed model analysis of this difference in release rates showed statistical significance ($p < 0.05$).

3.5. BMC culture on scaffolds

Rat BMCs were cultured on blank hydrophilic LMW scaffolds for 4 weeks with the addition of IGF-I to verify the ability of the porous scaffolds to support cell growth and differentiation. Figure 9(*A*) shows the cross section of a blank LMW scaffold with CellTracker-labeled BMCs growing across the entire cross section of the scaffold after 14 days of culture. Figure 9(*B*) shows CellTracker-labeled D1 cells that were transfected with PEI:DNA particles released from PLGA scaffolds that the cells grew on.

Figure 10(*A*) shows that the DNA content of IGF-I treated LMW scaffolds was statistically significantly higher at 187% compared to the control group that did not receive IGF-I ($p < 0.05$). Similarly, the GAG content of IGF-I treated scaffolds was 19% higher as compared to the untreated scaffolds after 4 weeks of culture, as shown in figure 10(*B*) ($p < 0.05$).

4. Discussion

4.1. Effect of PEI on degradation

PEI complexation of DNA reduced the degradation rate of PLGA scaffolds compared to DNA-free scaffolds or those containing naked DNA, although this was not statistically

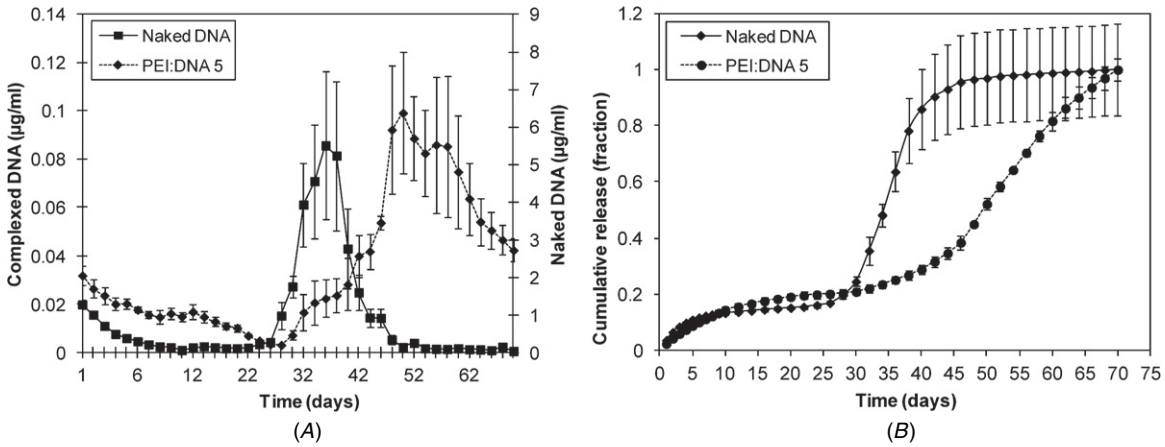

Figure 8. (*A*) Instantaneous and (*B*) cumulative fractional release of naked DNA and PEI:DNA 5 complexes from HMW PLGA scaffolds. Data are mean ± standard error ($n = 3$).

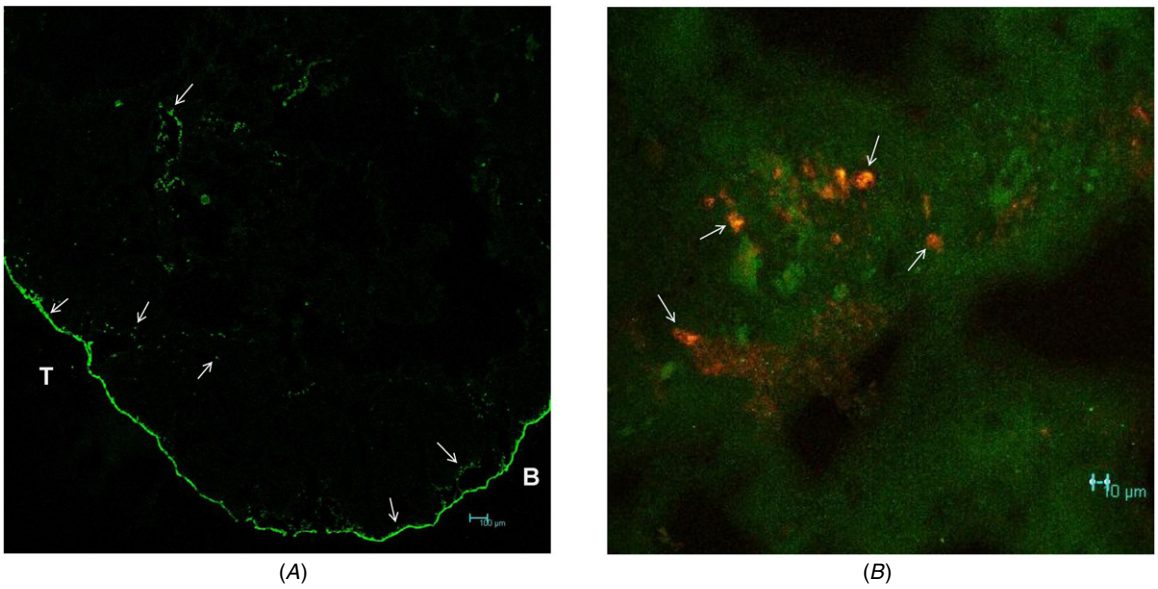

Figure 9. (*A*) CellTracker-labeled rat BMCs growing on a blank hydrophilic scaffold transversely sectioned after 14 days of culture. T and B denote top and bottom of a discoid scaffold that appears more rounded because of swelling in an unconfined state. (*B*) CellTracker-labeled D1 cells growing on PEI:DNA-loaded hydrophilic PLGA scaffold for 6 days. Scaffolds contained dsRed:PEI complexes. Transfected cells appear red (indicated by arrows), and non-transfected cells appear green (not marked). The white arrows indicate some of the cells.

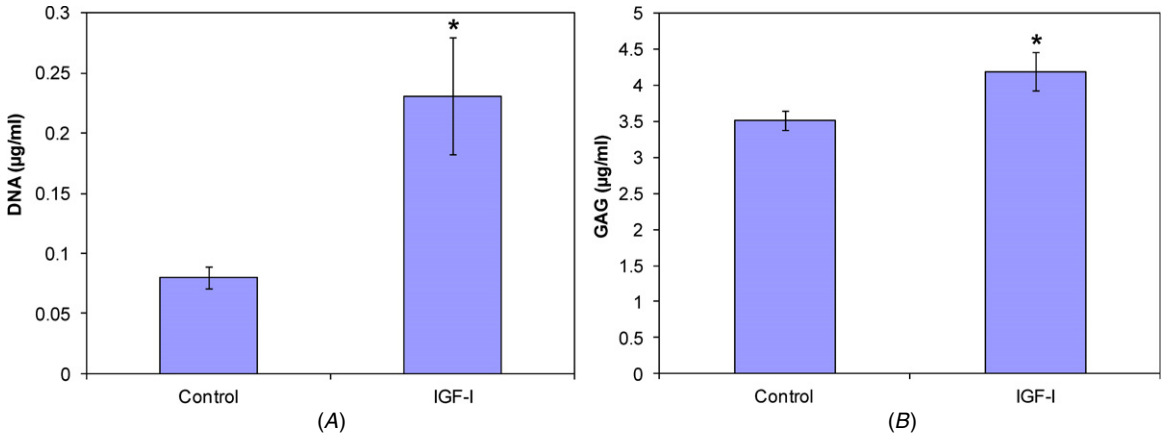

Figure 10. (*A*) DNA and (*B*) GAG contents of BMCs cultured on LMW PLGA scaffolds with and without IGF-I for 4 weeks. Data are mean ± standard error ($n = 3$). * denotes $p < 0.05$.

significant. After 30 days, PEI:DNA scaffolds lost 80% of their mass compared to naked DNA scaffolds and blank scaffolds that degraded completely. The branched PEI used in this investigation, with its structure and positive charge, likely interacted electrostatically with negatively charged acid groups on PLGA molecules and slowed their erosion. In fact, the interaction between PEI and PLGA has been used for attaching PEI to PLGA microspheres using layer-by-layer deposition. PEI deposited on PLGA microspheres was used for coating TGF-β_3-loaded nanoparticles for growth factor delivery [22].

PEI did not significantly affect the degradation rate of HMW PLGA scaffolds, in contrast to the results from LMW PLGA scaffolds. Although the mass loss rate for PEI:DNA scaffolds was slower than that of blank and naked DNA scaffolds, these differences were not statistically significant. It is possible that the electrostatic interaction between PEI and HMW PLGA is weaker compared to LMW PLGA, as the longer chain lengths, and therefore fewer chain ends, of HMW PLGA lead to less negative charge per unit mass of polymer for interacting with positively charged PEI. Schrier *et al* showed that HMW hydrophilic PLGA, with a lower acid number and less negative charge compared to LMW hydrophilic PLGA, resulted in less interaction with positively charged BMP-2 molecules [27].

There was good correlation between the degradation and release profiles for HMW scaffolds containing both naked DNA and PEI:DNA particles. Naked DNA scaffolds had the highest mass loss rate between 20 and 50 days, with the release peak observed between days 30 and 50. PEI:DNA scaffolds had the highest mass loss rates between days 30 and 50, with PEI:DNA release increasing from day 30 and peaking around day 50. In comparison, Huang *et al* reported that compression molding of freeze-dried PEI:DNA particles with PLGA granules and salt into porous scaffolds resulted in release of PEI:DNA particles within 2 days of immersion in PBS [11].

4.2. Mechanical properties

A biomaterial that is designed to replace or augment a tissue should ideally have mechanical properties that match those of the host tissue. This becomes an even greater challenge in the case of devices that are intended to replace complex tissues, such as cartilage, since the mechanical properties of cartilage depend on the zone within it. The compressive properties of cartilage are governed in large part by the proteoglycans within the extracellular matrix of cartilage [26]. The compressive modulus of articular cartilage varies between 0.079 MPa in the superficial layer and 2.1 MPa in the deepest layer [26]. Young's modulus for rabbit growth plate cartilage varied from 0.57 MPa in the reserve zone to 1.44 MPa in the mineralizing zone [24]. Young's modulus of rabbit cancellous bone was found to be 9.68 MPa in the same study [24].

Scaffold dimensions varied from the ASTM or ISO recommendation of L/D between 2.0 and 2.5. This decision was made for two reasons. First, for implantation in a growth plate or cartilage defect, the scaffolds will have L/D ratios well less than 2.0. Thus, the geometry reflects that needed for implantation. Second, the present scaffold fabrication method was designed to produce disk-shaped rather than cylindrical specimens. High L/D ratios reduce consolidation in the center of the sample and consequently compromise the mechanical integrity of the scaffolds. Although end effects contributed to a likely overestimation of the scaffold properties, the relative comparison between different scaffold compositions remains valid.

Compressive modulus for the present scaffolds varied from 26 MPa for LMW PLGA to 81 MPa for HMW PLGA. Although these values are an order of magnitude greater than the modulus for natural cartilage, these values decrease significantly when the scaffolds become wet after insertion into the implant site (results not shown). A higher initial modulus would be desirable to provide support and maintain space, which would be needed for neotissue ingrowth.

4.3. DNA release

PLGA scaffolds were used for controlled release of both adsorbed and encapsulated plasmid DNA. Controlled release from a scaffold can provide sustained release of plasmid DNA, which is not possible with bolus injections. Porous matrices made of PLGA provided sustained release of naked DNA for 10 to 30 days [28]. Sustained release of naked plasmid DNA for 21 days was achieved from PLGA scaffolds manufactured using a thermally-induced phase-separation method [5].

The present LMW scaffolds showed peak naked DNA release around days 7–10 but were depleted by day 16. Release of PEI:DNA 5 particles from LMW scaffolds differed in both time and concentration compared to the release of naked DNA. The initial phase of release lasted longer, ending by day 7, compared to day 4 for naked DNA particles. The concentration of PEI:DNA particles was also higher compared to naked DNA until day 5. The second peak of PEI:DNA release occurred on day 10, compared to days 7–10 for naked DNA. The release of PEI:DNA particles was complete by day 20. The slight delay in peak release as well as longer release duration might be from the interaction between PEI and PLGA. Electrostatic interaction between positively charged histones and negatively charged release substrates has been reported previously [18]. Less than 20% of PEI:DNA particles were released from PLGA scaffolds by 15 days, compared to 80% of naked DNA, suggesting interaction between PLGA and PEI [11]. It was also proposed that excess PEI from the making of PEI:DNA particles might interact electrostatically with PLGA [11]. At an N/P ratio of 6, approximately 86% of the PEI used to make PEI:DNA complexes remains in the free form [6]. Surface adsorption of PEI:DNA particles on PLGA scaffolds resulted in an N/P ratio-dependent release of these particles [15]. At the end of 4 days of release, 90% of adsorbed PEI:DNA 6 particles was released compared to only 40% of PEI:DNA 18 particles [15]. Electrostatic and physical interaction between the positively charged PEI or PEI:DNA particles and negatively charged PLGA molecules can slow the dissolution of PLGA scaffolds as well as release of PEI:DNA particles from PLGA scaffolds.

Comparison of the mass loss and release profiles for LMW scaffolds loaded with naked and PEI:DNA 5 particles showed good correlation between the time points of peak degradation and peak DNA release. The second phase of naked DNA release was seen around days 5 to 15, while peak degradation occurred around days 5–20; the second phase of PEI:DNA 5 release from the scaffolds occurred between days 5 and 25, while peak degradation was observed around days 7 to 14. A low concentration, sustained release was observed after the second phase release in most release experiments, probably from the release of any adsorbed DNA or PEI:DNA particles from the walls of the tubes in which the scaffolds were degrading. The release profile of naked and PEI-complexed DNA from PLGA has diffusive and erosive components. The smaller size of PEI:DNA particles compared to naked DNA will aid in a quicker diffusive component, as evidenced in the earlier peak release from both LMW and HMW PLGA scaffolds. The repulsive interaction of naked DNA with negatively charged PLGA chains will also aid a quicker release of such molecules compared to positively charged PEI:DNA particles from PLGA scaffolds.

The concentrations of released PEI:DNA from scaffolds were consistently higher than those of naked DNA, even though both kinds of scaffolds were manufactured identically. Some of this difference can be explained by the technique employed to measure the concentration of released DNA in the supernatant which was the Picogreen® assay. Picogreen dye binds to double-stranded DNA and fluoresces, allowing determination of concentration using a standard curve. The very property of PEI that enables condensation of DNA into toroidal particles also limits the ability to detect DNA once it has been condensed. Picogreen reagent binds less efficiently to PEI:DNA particles as the ratio of PEI to DNA increases, until it becomes impossible to detect DNA at high PEI:DNA ratios. This is the reason the PEI:DNA ratio was kept at 5 in all the present release experiments, so that the Picogreen® assay could be used to detect DNA in the release supernatants. PEI:DNA 5 concentrations were calculated using PEI:DNA 5 calibration curves, but even a small amount of free DNA in the supernatant could have artificially increased the apparent concentration of PEI:DNA 5. Most researchers report PEI:DNA release as fractional cumulative release, which circumvents this problem. Figures 6–8 show both instantaneous and cumulative release of PEI:DNA 5 from the scaffolds, with the caveat that the instantaneous release profile should be used more as an indication of time profile rather than the absolute values of DNA concentration.

Fluorescent labeling of PEI enabled tracking of its release simultaneously with DNA and showed excellent correlation between these two profiles. This also confirmed that the results obtained from using Picogreen® to track PEI:DNA 5 release were not artifacts, and that Picogreen® was effective in detecting PEI:DNA 5 in supernatant.

4.3.1. Effect of molecular weight of PLGA on DNA release. For both LMW and HMW PLGA scaffolds, complexation of DNA with PEI resulted in a later second phase of release compared to naked DNA. However, peak release of both naked and PEI:DNA complexes occurred at a later time for scaffolds made from HMW PLGA. This was expected as polymer chains of HMW PLGA will take longer to hydrolyze into oligomers that are soluble in water. Jang *et al* reported that porous scaffolds made from HMW PLGA, with an inherent viscosity of 0.6–0.8 dL g^{-1}, released about 50% of their naked DNA by 21 days, compared to 90% for scaffolds made from LMW PLGA with an inherent viscosity of 0.16–0.24 dL g^{-1} [16]. 'Hydrophilization' of 120 000 Da PLGA scaffolds by blending with Pluronic F127 led to release of DNA/PEI–PEG over approximately 8 weeks [21]. Capan *et al* also reported that microspheres made from PLGA with a molecular weight of 10 000 Da released 95% of their encapsulated poly(L-lysine) (PLL)–DNA complexes by 40 days, compared to 50% of encapsulated PLL–DNA complexes from microspheres made of PLGA with a molecular weight of 31 000 Da [4]. Similar effects were observed in the present study, with 20% of naked and PEI:DNA complexes being released from HMW PLGA scaffolds within 30 days, compared to 100% of naked and PEI:DNA 5 complexes from LMW PLGA scaffolds.

4.4. BMCs on porous LMW PLGA scaffolds

BMCs have been shown to be particularly useful in tissue engineering because of their potential to differentiate into various phenotypes depending on the culture conditions. Human BMCs cultured in alginate–collagen II capsules showed large regions of a cell-generated matrix [23]. Human BMCs cultured using a rotating wall bioreactor without any growth factors showed significant GAG synthesis at 2 weeks [25], while human bone marrow stromal cells grown on silk scaffolds with TGF-β_1, insulin, and dexamethasone in a rotating bioreactor showed significantly higher GAG/DNA content compared to control scaffolds without chondrogenic growth factors [19]. These studies demonstrate the chondrogenic potential of bone marrow stromal cells in various chondrogenic media and three-dimensional matrices.

In the present studies, rat BMCs seeded on hydrophilic LMW PLGA scaffolds continued to grow through 28 days, when the scaffolds were extensively degraded. Cell Tracker-labeled cells were seen growing across the entire volume of the scaffold 14 days after seeding. Furthermore, in the presence of IGF-I, BMC proliferation and GAG contents were significantly elevated compared to the untreated controls. It appears that IGF-I acted as a strong mitogen, driving the proliferation of seeded cells in the treated scaffold, along with synthesis of extracellular GAG. These GAGs, along with other cell-derived matrix components, enabled the cells to remain as the scaffold degraded. IGF-I during the initial periods of culture would not have aided in the differentiation of seeded BMCs until they reached the critical density, after which they would have driven differentiation and enhanced synthesis of extracellular matrix proteoglycans such as GAG. Culturing BMCs on IGF-I plasmid-releasing LMW PLGA scaffolds could produce a system whereby transfected cells produce IGF-I protein, thus driving their own proliferation and chondrogenic differentiation.

5. Conclusion

Porous scaffolds made of PLGA with the appropriate selection of hydrophobicity and molecular weight will allow tailoring of degradation profiles and mechanical properties for controlled delivery of naked and condensed plasmid DNA in tissue engineering applications. Scaffolds with layers of different hydrophobicities and molecular weight can allow sequential as well as concurrent release of plasmid DNA encoding different growth factors. This process will mimic natural tissue growth, and can be used in the generation of complex tissues, such as cartilage, bone, and blood vessels.

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