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Long-Term *In Vivo* Gene Expression via Delivery of PEI–DNA Condensates from Porous Polymer Scaffolds

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ABSTRACT

Nonviral delivery vectors are attractive for gene therapy approaches in tissue engineering, but suffer from low transfection efficiency and short-term gene expression. We hypothesized that the sustained delivery of poly(ethylenimine) (PEI)-condensed DNA from three-dimensional biodegradable scaffolds that encourage cell infiltration could greatly enhance gene expression. To test this hypothesis, a PEI-condensed plasmid encoding β-galactosidase was incorporated into porous poly(lactide-co-glycolide) (PLG) scaffolds, using a gas foaming process. Four conditions were examined: condensed DNA and uncondensed DNA encapsulated into PLG scaffolds, blank scaffolds, and bolus delivery of condensed DNA in combination with implantation of PLG scaffolds. Implantation of scaffolds incorporating condensed β-galactosidase plasmid into the subcutaneous tissue of rats resulted in a high level of gene expression for the entire 15-week duration of the experiment, as exemplified by extensive positive staining for β-galactosidase gene expression observed on the exterior surface and throughout the cross-sections of the explanted scaffolds. No positive staining could be observed for the control conditions either on the exterior surface or in the cross-section at 8- and 15-week time points. In addition, a high percentage (55–60%) of cells within scaffolds incorporating condensed DNA at 15 weeks demonstrated expression of the DNA, confirming the sustained uptake and expression of the encapsulated plasmid DNA. Quantitative analysis of β-galactosidase gene expression revealed that expression levels in scaffolds incorporating condensed DNA were one order of magnitude higher than those of other conditions at the 2-week time point and nearly two orders of magnitude higher than those of the control conditions at the 8- and 15-week time points. This study demonstrated that the sustained delivery of PEI–condensed plasmid DNA from PLG scaffolds led to an *in vivo* long-term and high level of gene expression, and this system may find application in areas such as bone tissue engineering.

OVERVIEW SUMMARY

Gene therapy approaches to tissue engineering using nonviral vectors have great potential, but low transfection efficiency and short-term gene expression limit practical applications. We propose that increased transfection can be achieved through local and sustained delivery of condensed plasmid DNA from three-dimensional biodegradable polymer scaffolds that promote cell infiltration. To test this, PEI-condensed and uncondensed β-galactosidase plasmid was either incorporated into poly(lactide-co-glycolide) scaffolds and implanted into the subcutaneous tissue of rats, or injected into the site at the time of implantation (bolus delivery). Sustained delivery of condensed DNA led to extensive gene transfer, and an approximately two orders of magnitude increase in β-galactosidase expression, as compared with the other conditions. Further, whereas bolus delivery led to transient expression, sustained delivery maintained a high expression level for the 15-week duration of the experiment. These results indicate that combining DNA condensation with sustained delivery from a polymer can allow long-term and efficient gene transfer *in vivo*.

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INTRODUCTION

Gene therapy approaches to tissue engineering have been investigated using a variety of systems, including the delivery of genetically modified cells, injection of viral microparticles, and delivery of plasmid-based systems (Murphy and Mooney, 1999; Shea *et al*., 1999; Bonadio, 2000; Cohen *et al*., 2000; Hidaka *et al*., 2002; Lee *et al*., 2002; Huard *et al*., 2003; Wu *et al*., 2003). Viral gene delivery systems rely on genetically engineered viruses to deliver the gene to the target cells (Ledley *et al*., 1987; Hafenrichter *et al*., 1994a,b; Kay *et al*., 1995, 1997; Barry *et al*., 1996; Kessler *et al*., 1996; Nabel, 1999). Nonviral gene delivery systems use plasmid DNA that can be formulated with a variety of carrier molecules (Zhou *et al*., 1991; Boussif *et al*., 1996; Fominaya and Wels, 1996; Lee and Huang, 1996; Alila *et al*., 1997; Hara *et al*., 1997; Jong *et al*., 1997; Kircheis *et al*., 1997; Kim *et al*., 1998). Viral vectors generally display high efficiency. However, their application has been hampered by a variety of issues, including a limitation on the size of inserted DNA for certain viruses, uncontrollable expression of inserted genes, high immunogenicity that could result in patient death, and the potential for the insertion of the new gene into the host cell genome in a manner that leads to mutagenesis (Bonadio *et al*., 1998). Delivery of plasmid DNA has been pursued as an alternative approach to circumvent these issues. Plasmid DNA is economical and relatively simple to manufacture, and has a chemistry that lends itself to polymer-based drug delivery systems. Furthermore, plasmid DNA has limited safety issues associated with toxicity and immunogenicity (Winn *et al*., 2000). In addition, issues associated with direct protein delivery, such as instability and lack of posttranslational modification of recombinantly produced proteins, are circumvented as plasmid DNA is inherently stable and transfected cells possess the biosynthetic capability for functional protein production (Yin *et al*., 1995; Fang *et al*., 1996). Complexes between the therapeutic DNA and nonviral delivery systems are easy to construct and theoretically there is no limit as to the size of the gene that can be used with this approach (Weidner, 2002; Wolff and Herweijer, 2003). The plasmid DNA can be designed to encode the gene of interest, as well as the promoter and enhancer elements needed to achieve appropriate gene expression. However, the low transfection efficiency of plasmid DNA has been a major limitation in gene delivery applications.

One element of many tissue-engineering applications is the fabrication of a biocompatible three-dimensional scaffold material to replace the structural and functional characteristics of damaged tissues. Synthetic materials are an attractive choice for scaffold fabrication because of their reproducibility, and ability to be produced on a large scale. In addition, their mechanical properties and degradation rates can be readily manipulated so that they can be designed for specific applications (Kim and Mooney, 1998a). In this approach, the polymer scaffolds define the space for tissue development, serve as the delivery vehicle for growth factors to specific anatomic sites, and can also be used for simultaneous cell transplantation (Chaignaud *et al*., 1997; Wong and Mooney, 1997; Murphy and Mooney, 1999). Poly(lactic-co-glycolic acid) (PLGA) is one class of synthetic material that is often used because of its ability to be customized to meet a particular absorption time requirement, and PLGA has a history of clinical use in various applications (Burg *et al*., 2000). In addition, investigators have effectively used PLGA for tissue-engineering purposes and demonstrated compatibility *in vitro* and *in vivo* (Kim *et al*., 1994; Mooney *et al*., 1997; Haar *et al*., 1998; Kim and Mooney, 1998b; Vunjak-Novakovic *et al*., 1998; Thomson *et al*., 1999) in a number of situations, including applications involving the localized delivery of plasmid DNA for *in vivo* transfection (Shea *et al*., 1999). The delivery of plasmid DNA through localized polymeric scaffolds is an attractive approach to direct tissue development in tissue-engineering applications. The major advantage of this approach over bolus or intravenous injection and virus-based approaches is that it bypasses the side effects associated with the systemic delivery of genetic materials and the disadvantages associated with the common use of viral vectors. Further, polymeric gene delivery vehicles have the potential to effectively and safely deliver genes to target cells. PLGA scaffolds incorporating plasmid DNA allow cell infiltration and uptake of DNA for subsequent production of proteins involved in tissue formation (Bonadio *et al*., 1999; Shea *et al*., 1999). The application of localized gene delivery with plasmid DNA in tissue engineering was originally proposed as a gene-activated matrix (GAM), using collagen as the polymeric scaffold, and this system has been applied to bone regeneration (Goldstein and Bonadio, 1998; Bonadio *et al*., 1999, 2000). In addition, PLGA scaffolds fabricated by the gas foaming process have been utilized to deliver plasmid encoding platelet-derived growth factor, and this resulted in increased blood vessel and granulation tissue formation (Shea *et al*., 1999). This latter approach enables control over temporal gene expression as the new tissues form, and undesired activity is presumably lost after the polymeric vehicle is exhausted of plasmid and tissue formation is complete.

Despite potential advantages of plasmid delivery approaches in tissue regeneration, plasmid DNA typically demonstrates low transfection efficiency *in vivo*. This is a result of its chemical, enzymatic, and colloidal instability, macrophage uptake, and nonspecific targeting (Mahato, 1999). Therefore, gene therapy success is largely dependent on the development of vectors that allow efficient delivery to targeted sites at a high expression level (Li and Huang, 2000). The low transfection efficiency of plasmid DNA in these types of systems can potentially be overcome by condensing plasmid DNA with polycationic condensing agents, including poly(ethylenimine) (PEI) (Boussif *et al*., 1995; Blessing *et al*., 2001; Gautam *et al*., 2001; Lee *et al*., 2001; Lemkine and Demeneix, 2001; O'Neill *et al*., 2001). The condensing agents form positively charged small particles that facilitate cellular entry, enhance the stability of plasmid DNA, and increase transfection efficiency. PEI has been shown to be highly effective as a nonviral delivery vehicle, without additional endosomal buffering agents such as chloroquine, both *in vitro* and *in vivo* (Boussif *et al*., 1995; Boletta *et al*., 1997; Gautam *et al*., 2001; Lemkine and Demeneix, 2001).

We hypothesized that the sustained delivery of plasmid DNA condensed with PEI from porous PLGA scaffolds would lead to increased and long-term transfection *in vivo*. We have previously developed an approach to encapsulate and deliver PEI-condensed DNA from three-dimensional biodegradable PLGA scaffolds fabricated by a gas foaming process (Huang *et al*., 2003). The parameters for *in vivo* transfection utilized in this study were obtained from the optimized *in vitro* parameters (200 μg of plasmid DNA and a PEI–DNA charge ratio of 7) found

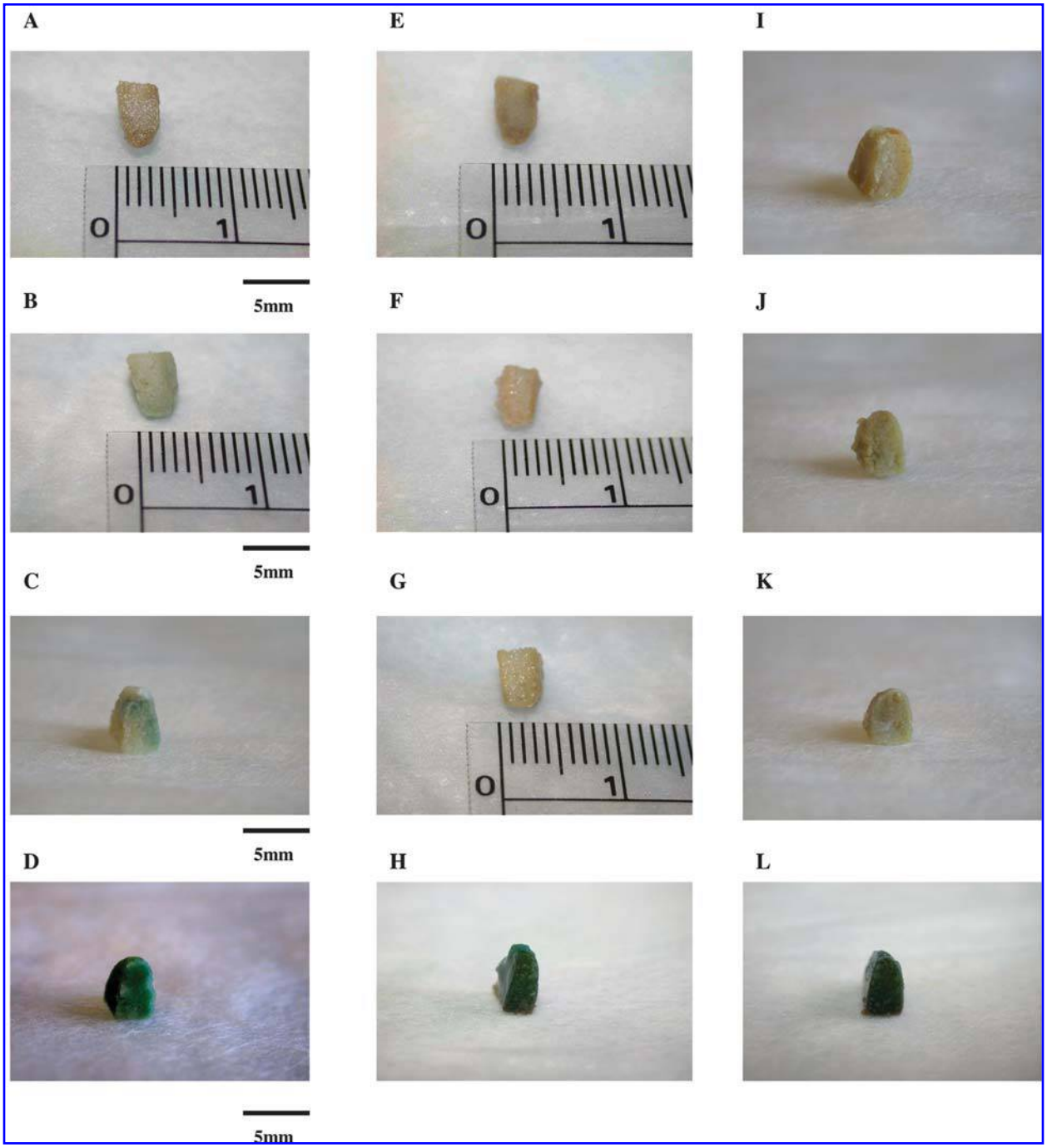

FIG. 1. Gross macroscopic photographs of (**A**, **E**, and **I**) blank scaffolds, (**B**, **F**, and **J**) scaffolds incorporating plasmid DNA, (**C**, **G**, and **K**) bolus injection of condensed DNA, and (**D**, **H**, and **L**) scaffolds incorporating condensed DNA. Scaffolds were retrieved 2 weeks (**A**–**D**), 8 weeks (**E**–**H**), and 15 weeks (**I**–**L**) after subcutaneous implantation and stained for β-Gal. At 2 weeks, the blank scaffolds displayed no positive staining in cross-section (**A**). Scaffolds encapsulating uncondensed DNA or the condition utilizing bolus injection of condensed DNA led to light staining in cross-section (**B** and **C**). Scaffolds that delivered condensed DNA demonstrated intensive positive staining in cross-section (**D**). At 8 and 15 weeks, blank scaffolds (**E** and **I**), scaffolds encapsulating uncondensed DNA (**F** and **J**), or the condition utilizing bolus injection of condensed DNA (**G** and **K**) led to no positive staining in cross-section. Scaffolds that delivered condensed DNA demonstrated intensive positive staining in cross-section (**H** and **L**). All photographs were taken at the same magnification, and a size bar is shown in (**A**–**D**).

in that study. PEI–DNA condensates were prepared by combining pCMVL plasmid and branched PEI (MW 25 kDa). The mean particle size of PEI–DNA condensates with optimal uptake was determined to be in the range of 60–150 nm. Condensates encapsulated within PLGA scaffolds, using sucrose as the porogen, tended to remain entrapped, with an efficiency of 90%, whereas uncondensed plasmid DNA exhibited a high loss of DNA (20% retention efficiency). The charge ratio strongly

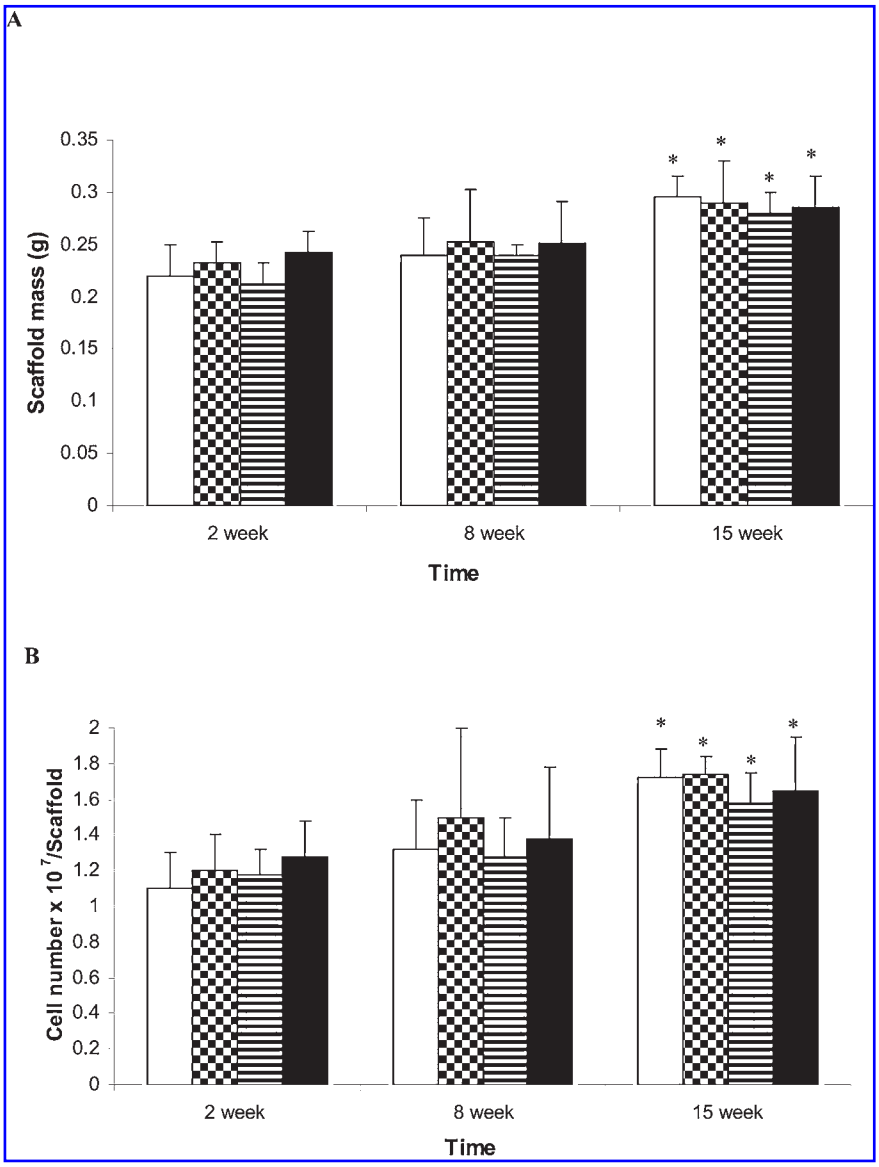

FIG. 2. (**A**) Comparison of mass of scaffolds and (**B**) comparison of cell number in scaffolds retrieved at 2, 8, and 15 weeks. Open columns, blank scaffolds; dotted columns, scaffolds incorporating plasmid DNA; hatched columns, scaffolds with bolus injection of condensed DNA; solid columns, scaffolds incorporating condensed DNA. Experimental values are reported as means ($n = 6$) ± SD. *Statistical significance relative to 2- and 8-week time points within each condition ($p < 0.05$).

influenced gene expression mediated by PEI-condensed DNA entrapped in PLGA scaffolds, with maximal gene expression at a charge ratio of 7 or higher. In addition, gene expression levels increased in proportion to the condensed DNA dose up to 200 μg, with no additional increase beyond that dose. Therefore the optimal PEI–DNA condensate charge ratio of 7 and a plasmid DNA dose of 200 μg were used in this study to evaluate *in vivo* gene expression. We also included a condition involving a one-time bolus injection of condensed DNA to contrast the characteristics of long-term delivery in this system. The applicability of this system to transfect cells *in vivo* with DNA condensates was examined by subcutaneous implantation of the scaffolds into rats.

MATERIALS AND METHODS

Chemicals

Branched PEI (MW 25 kDa) was purchased from Sigma-Aldrich (St. Louis, MO). Sucrose was purchased from Sigma (St. Louis, MO). Poly(D,L-lactic-co-glycolic acid) (PLGA) 85:15 was purchased from Alkermes (Cambridge, MA). Plasmid DNA (pNGVL, 7.5 kb) encoding the nucleus-targeting β-galactosidase (nt β-Gal) under the control of the cytomegalovirus (CMV) promoter was purified from *Escherichia coli*, using a Qiagen ultrapure column (Qiagen, Chatsworth, CA) according to the manufacturer's instruction. This approach

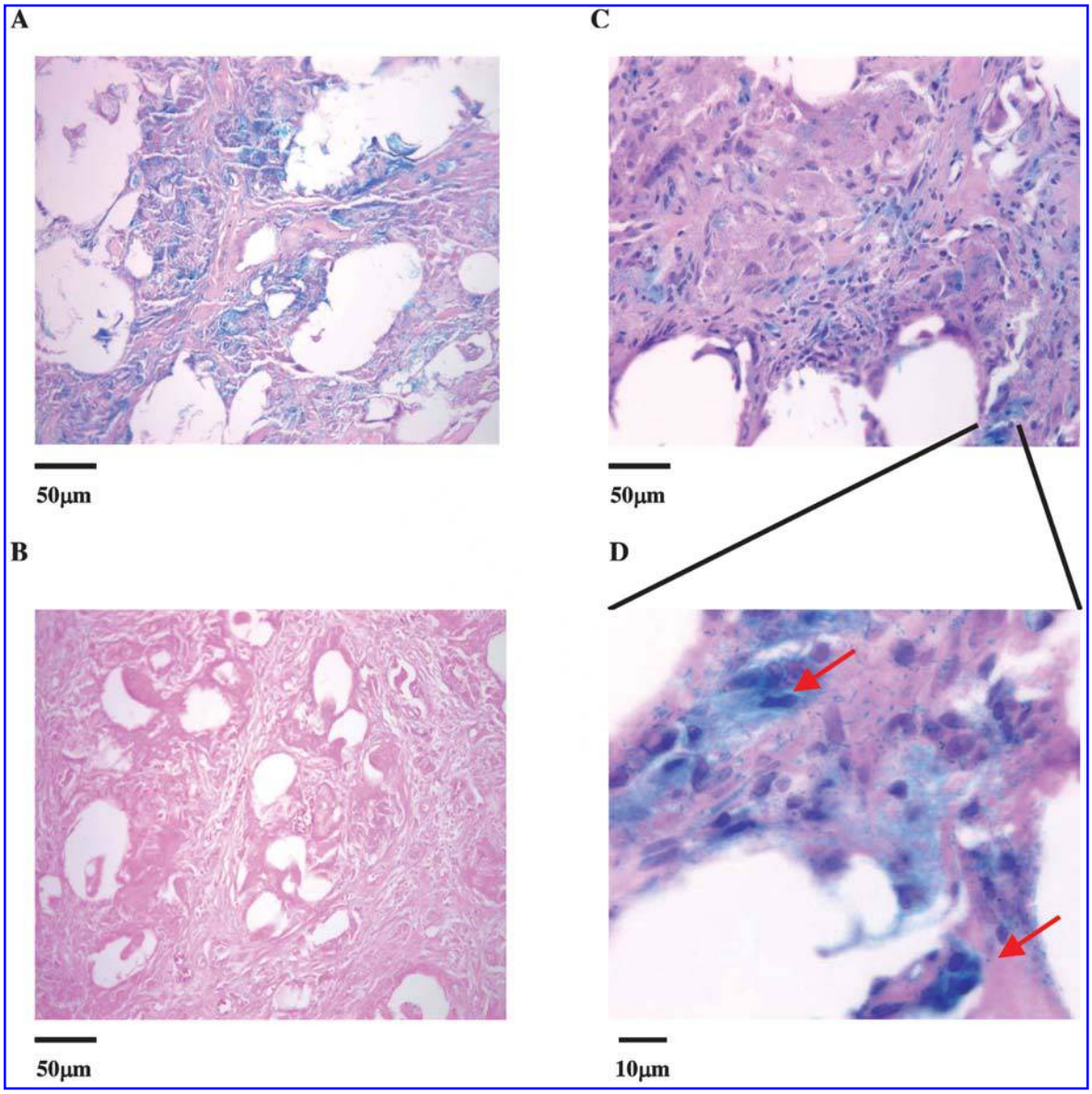

FIG. 3. Photomicrographs of histological sections of samples stained for β-galactosidase activity at 15 weeks. Scaffold incorporating condensed DNA (**A**), and scaffold incorporating plasmid DNA (**B**), stained with eosin only. (**C**) Section from scaffold incorporating condensed DNA counterstained with hematoxylin. (**D**) Higher magnification of (**C**), showing a greater intensity of nuclear staining in the transfected cells (arrows). Original magnification: (**A**–**C**) ×200; (**D**) ×400; scale bars are shown for each photomicrograph.

typically yielded plasmid DNA that was 50:50 supercoiled:open circular, as determined by agarose gel electrophoresis. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and β-galactosidase staining solutions were purchased from Specialty Media (Phillipsburg, NJ). A β-galactosidase quantification kit (Galacto-*Star*) was purchased from Applied Biosystems (Foster City, CA). Antibodies for fluorescence-activated cell-sorting (FACS) analysis, including anti-rat CD45–fluorescein isothiocyanate (FITC) (green) for leukocytes and anti-rat CD31–phycoerythrin (PE) (red) for endothelial cells, were purchased from BD Biosciences Immunocytometry Systems (San Jose, CA).

Preparation of PLGA sponges

PEI–DNA condensates were prepared by combining 200 μg of plasmid in 4 ml of HEPES buffer (5 m*M*, pH 7.4) with 4 ml of PEI (10 m*M*). Sucrose (1%, w/v) was added and condensates were rapidly frozen, using dry ice in ethanol, and lyophilized for 72 hr. Freeze-dried PEI–DNA condensates were combined with milled sucrose (250–425 μm) and 85:15 polylactide:glycolide copolymer particles (106–250 μm). Sponges were prepared at 91:9 weight ratio of porogen:PLGA by combining 728 mg of sucrose and PEI–DNA condensate with 72 mg of PLGA. Sponges were fabricated by compression molding 800 mg of the dry mix-

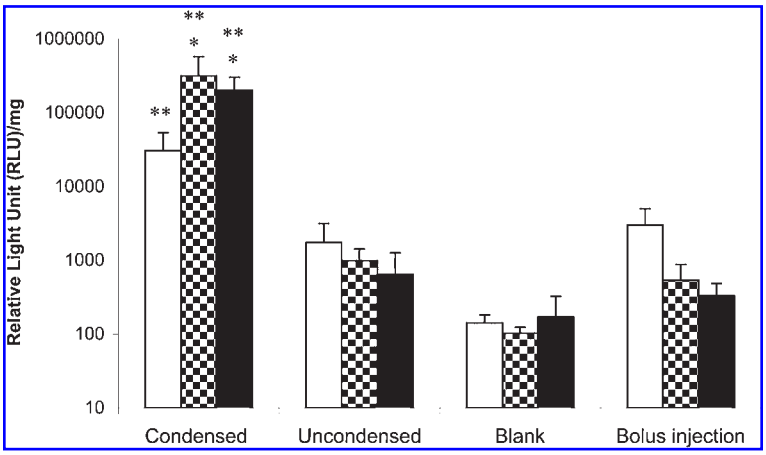

FIG. 4. Quantification of β-Gal expression in samples retrieved at 2, 8, and 15 weeks. Open columns, 2-week time point; dotted columns, 8-week time point; solid columns, 15-week time point. Experimental values are reported as means ($n = 6$) $\pm$ SD. *Statistical significance relative to the 2-week time point; **statistical significance relative to the blank scaffolds, the scaffolds incorporating plasmid DNA, and the condition involving bolus injection of condensed DNA ($p < 0.05$). Relative light units (RLU) represent a measurement of gene expression level via light emission as determined by luminometer.

ture at 1500 psi for 1 min, using a 13-mm die set from Pike Technologies (Madison, WI) and a Carver model "C" hydraulic press (Pike Technologies). The compressed pellet (2 $\times$ 13 mm) was then foamed into a scaffold via a gas foaming process (Harris *et al*., 1998) in a custom-designed stainless steel high-pressure vessel using dry CO_2 gas at 800 psi for 24 hr. A rapid reduction in pressure causes the polymer particles to expand and fuse into an interconnected structure. Sucrose was leached from the sponges by immersion in phosphate-buffered saline (PBS). Sponges containing naked plasmid DNA were also prepared by mixing 200 μg of lyophilized plasmid DNA, sucrose, and PLGA followed by the gas foaming and particulate leaching process.

In vivo *transfection studies*

The ability of the released plasmid to transfect cells *in vivo* was assessed by implantation of PLG sponges containing plasmid DNA into the subcutaneous tissue of male Lewis rats (250–300 g). Experiments were performed by implanting blank scaffolds, scaffolds encapsulating condensed plasmid DNA, and scaffolds encapsulating plasmid DNA, and by directly injecting condensed DNA (in Tris–EDTA buffer), into the subcutaneous tissue of rats. Scaffolds encapsulating plasmid DNA contained 200 μg of plasmid DNA per scaffold. Six samples were used for each condition at each time point. To implant scaffolds, Lewis rats (Charles River Laboratories, Wilmington, MA) were anesthetized via intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Midlongitudinal skin incisions approximately 15 mm in length were made on the dorsal surface of each rat, using a scalpel blade, and four subcutaneous pockets were created per animal via blunt dissection. One implant of each condition was placed per animal. Incisions were then closed with black filament nylon sutures (Ethicon, Somerville, NJ). After 2, 8, and 15 weeks, the animals were killed by cervical dislocation and the implants were retrieved. Treatment of experimental animals was in accordance with University of Michigan (Ann Arbor, MI) animal care guidelines, and all National Institutes of Health (NIH, Bethesda, MD) animal-handling procedures were observed.

Analysis of tissue explant β-galactosidase expression

The mass of the scaffolds and the cell numbers within the scaffolds were determined after retrieval, and scaffolds were bisected into two equal pieces. One-half of each scaffold was used for β-Gal staining and subsequent histological analysis, and the other half was flash frozen in liquid nitrogen for quantification of β-Gal expression. The scaffolds were stained with X-Gal to detect β-galactosidase production, and the staining was confirmed by the nucleus-specific staining of the transfected cells. Specifically, samples used for staining were placed in a fixing solution (37.6% formaldehyde [53.1 ml/liter] and 50% glutaraldehyde [4 ml/liter] in PBS) at 4°C for 90 min. Samples were subsequently rinsed with rinse solution (EGTA [1.900 g/liter] and $MgCl_2 \cdot 6H_2O$ [0.406 g/liter] in phosphate buffer) once and then placed in rinse solution for 30 min in the dark at room temperature. After 20–25 min, the X-Gal substrate and tissue stain base solution [$MgCl_2 \cdot 6H_2O$, 0.406 g/liter; $K_3Fe(CN)_6$, 1.645 g/liter; $K_4Fe(CN)_6$, 2.110 g/liter; deoxycholate, 0.1 g/liter; and Nonidet P-40, 0.2 ml/liter in phosphate buffer] were thawed to make the staining solution. Samples were then placed in the staining solution for 1.5 hr. After staining, samples were placed in tubes containing 70% ethanol, paraffin embedded, and sectioned (Histology Core Facility, University of Michigan Dental School, Ann Arbor, MI). The percentage of transfected cells in tissue sections was estimated by counting the fraction of cells positively stained in eight randomly chosen locations per section. Positively stained and total cell numbers were determined by comparing slides stained with eosin only, and slides stained with both eosin and hematoxylin.

To quantify β-galactosidase enzyme activity, samples were either flash frozen and stored at -20°C for later analysis, or analyzed immediately. At the time of analysis, samples were washed twice with ice-cold phosphate-buffered saline (calcium and magnesium free) and then treated with lysis buffer to lyse the cells. The lysate was centrifuged to pellet cell debris, and the supernatant was added to the reagent provided in the β-galactosidase quantification kit (Galacto-*Star*). Relative light units (RLU) were recorded on a luminometer (Lumat LB 9501; Berthold Detection Systems, Pforzheim, Germany) with 10-sec integration. In addition, FACS analysis was performed to identify cell types infiltrating the scaffolds. Briefly, samples were cut into small pieces on retrieval and placed in a collagenase solution and incubated for 45 min at 37°C. After incubation, cell strainers (pore size, 45 μm) were used to remove debris remaining in the solution. Released cells were

resuspended in cold PBS and counted, followed by the addition of antibodies for surface antigen recognition, and subsequently analyzed (Flow Cytometry Core Facility, University of Michigan).

RESULTS

The ability of plasmid incorporated within polymers to transfect cells *in vivo* was assessed by implanting PLGA scaffolds encapsulating a plasmid DNA encoding nucleus-targeted β-galactosidase (β-Gal) into the subcutaneous tissue of Lewis rats. In addition, the following control conditions were examined: encapsulated uncondensed DNA, blank scaffolds, and bolus delivery of condensed DNA. The latter condition involved the direct injection of condensed DNA into the scaffold at the time of surgery, instead of its incorporation for sustained delivery. To grossly identify β-galactosidase expression, scaffolds were stained with X-Gal after being retrieved at each time point. At 2 weeks, on gross macroscopic examination, blank scaffolds displayed no positive staining either at the outer surface or in cross-section (Fig. 1A). Scaffolds encapsulating uncondensed DNA, and the condition involving bolus injection of condensed DNA, demonstrated only slight positive staining at the outer surface of the scaffolds, and light staining was observed in cross-sections of the scaffolds (Fig. 1B and C). Bolus injection of condensed DNA appeared to lead to a greater amount of positive staining than did delivery of uncondensed DNA. In contrast, scaffolds containing encapsulated condensed DNA led to positive β-galactosidase staining over the outer surface of the scaffold, and intense staining was present at least three-fourths of the distance into the scaffolds (Fig. 1D). No evidence of positive staining was observed for the blank scaffolds, scaffolds incorporating plasmid DNA, and bolus injection of condensed DNA at both 8 and 15 weeks (Fig. 1E–G and I–K). Strikingly, scaffolds encapsulating condensed DNA still displayed extensive positive staining at both 8 and 15 weeks (Fig. 1H and L). Furthermore, staining was evenly distributed at the outer surface and in the cross-section of scaffolds.

Cellular infiltration of host cells into the scaffolds under each condition was also qualitatively and quantitatively determined. The mass of each scaffold was determined, and there was an increase in mass for all conditions over the time course from 2 to 15 weeks (Fig. 2A). The samples for all conditions at 15 weeks displayed a statistically significant increase in mass relative to 2- and 8-week time points for each condition ($p < 0.05$). However, there was no statistically significant difference in mass between samples at each time point ($p > 0.05$). The number of cells infiltrating the scaffolds also increased over time for each condition (Fig. 2B). However, there were no statistically significant differences in cell number between experimental conditions at each time point ($p > 0.05$). These results indicated continuous cell infiltration and tissue growth in this delivery system. The cell types infiltrating the scaffolds, as identified by FACS, included 30% endothelial cells, 18% lymphocytes, and 52% other cell types, which were mostly fibroblasts, as based on cell morphology observed in tissue sections.

The 15-week samples were subsequently paraffin embedded and sectioned for histological examination. Sections were stained with eosin only to allow ready contrast of positively β-galactosidase-stained cells relative to negative cells. Sections taken from scaffolds with encapsulated condensed DNA showed positive β-galactosidase staining throughout the section (Fig. 3A), corroborating the extensive staining observed on gross examination of the scaffolds at that time point. No positive staining could be observed on sections from scaffolds incorporating plasmid DNA (Fig. 3B). To assess the transfection efficiency of scaffolds incorporating condensed DNA, the sections were counterstained with hematoxylin (Fig. 3C). By comparing eosin-stained sections with sections stained with both hematoxylin and eosin, it can be observed that approximately 55–60% of the total cells in the scaffolds displayed positive β-galactosidase staining. Higher magnification examination of these cells demonstrated the expected, more intense nuclear staining, confirming expression of the nucleus-targeting β-galactosidase plasmid (Fig. 3D).

The kinetics of gene expression were determined by quantification of β-galactosidase enzyme expression level at each time point (Fig. 4). Scaffolds incorporating condensed β-galactosidase plasmid DNA exhibited a significant increase in gene expression over time from 2 to 8 weeks, and this level was maintained at the 15-week time point. The highest expression level resulting from bolus delivery of condensed DNA was at the 2-week time point, and this level rapidly decreased over time. This result was in contrast to that of scaffolds encapsulating condensed DNA, and highlighted the importance of sustained delivery of DNA. The scaffolds incorporating uncondensed plasmid DNA also exhibited sustained gene expression over the period of 2 to 15 weeks, but the level was lower than that of scaffolds incorporating condensed DNA. Blank scaffolds displayed background values of gene expression at all time points. Scaffolds incorporating condensed β-galactosidase plasmid resulted in a statistically significant increase in gene expression level, as compared with the other conditions, at each time point. Furthermore, the expression level was at least one order of magnitude higher than that of other conditions at the 2-week time point, and at least two orders of magnitude higher than that of other conditions at the 8- and 15-week time points ($p < 0.05$, at all time points). The gene expression levels resulting from scaffolds incorporating condensed DNA at both the 8- and 15-week time points displayed a statistically significant increase relative to the 2-week time point ($p < 0.05$), and there was no statistically significant difference in expression level between the 8- and 15-week time points for the scaffolds incorporating condensed DNA ($p > 0.05$).

DISCUSSION

In this study, we have demonstrated the feasibility of freeze-dried PEI–DNA condensates incorporated within PLGA sponges to efficiently transfect cells *in vivo* in a sustained manner, compared with other control conditions to deliver plasmid DNA, as shown by the gross appearance of explants. Scaffolds encapsulating condensed DNA also demonstrated high transfection efficiency and gene expression levels during the course of the experimental period when compared with other conditions. The lack of significant differences in mass and cell number between experimental conditions demonstrated comparable tissue ingrowth under each condition.

A critical factor in the success of gene therapy is the extent of cellular uptake and expression of DNA. The system described here demonstrated at least 80% DNA retention efficiency within scaffolds incorporating condensed DNA after the leaching step, and sustained DNA delivery (Huang *et al*., 2003). The distinctive contrast between the extent of gene expression resulting from scaffolds incorporating condensed DNA, and both scaffolds incorporating uncondensed plasmid DNA and scaffolds subjected to bolus injection of condensed DNA over 15 weeks in this study, clearly indicates the importance of sustained delivery of condensed DNA for heightened and extended expression of transferred genes. The continuous infiltration of host cells into the scaffolds, followed by the uptake of DNA, led to long-term transfection. A high percentage (55–60%) of cells within scaffolds incorporating condensed DNA at 15 weeks demonstrated expression of DNA, further confirming this finding. The rapid drop in β-galactosidase expression levels at the 8-week time point for scaffolds implanted with a bolus injection of condensed DNA was expected, as a result of the short-term availability of the plasmid to cells in this situation. The stability of plasmid DNA incorporated by this method into PLGA scaffolds has been previously confirmed (Shea *et al*., 1999), suggesting that instability of plasmid DNA does not explain the decreased transfection over time with scaffolds encapsulating uncondensed plasmid DNA. These results highlighted the importance of sustained delivery for long-term gene expression, as previously proposed (Shea *et al*., 1999). The ability to deliver DNA locally in a sustained manner has important implications in tissue engineering, as it offers the possibility of tightly controlling the extent and duration of expression of inductive factors by host cells.

We have demonstrated that PLGA scaffolds capable of controlled and sustained delivery of condensed DNA lead to enhanced and long-term transfection *in vivo*. The system developed here combines the advantages of synthetic polymers for fabricating scaffolds (e.g., reproducibility and biocompatibility) and the enhanced transfection efficiency afforded by PEI. This system will also potentially allow much lower doses of plasmid DNA (e.g., on the order of micrograms) than are needed otherwise (e.g., on the order of milligrams) (Bonadio *et al*., 1999) to promote high levels of gene expression. Future issues that can be addressed with this system include the ability of this system to deliver DNA condensates into the surrounding tissues, as contrasted with transfecting cells that migrate into the scaffolds, and identification of the cell types that are transfected within the scaffolds. In future studies, this system could be further modified to increase cellular infiltration by modifying the polymer composition, and by incorporating growth factors that increase recruitment of host cells into the scaffolds. This scaffold delivery system will likely find great utility in a variety of tissue-engineering applications (e.g., bone regeneration) because of the high level and duration of expression achievable with this system.

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