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# Layered PLG scaffolds for *in vivo* plasmid delivery

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## ARTICLE INFO

## ABSTRACT

Gene delivery from tissue engineering scaffolds can induce localized expression of tissue inductive factors to direct the function of progenitor cells, either endogenous or transplanted. In this report, we developed a layering approach for fabricating scaffolds with encapsulated plasmid, and investigated *in vivo* gene transfer following implantation into intraperitoneal fat, a widely used site for cell transplantation. Porous poly(lactide-*co*-glycolide) (PLG) scaffolds were fabricated using a gas foaming method, in which a non-porous layer containing plasmid was inserted between two porous polymer layers. The layered scaffold design decouples the scaffold structural requirements from its function as a drug delivery vehicle, and significantly increased the plasmid incorporation efficiency relative to scaffolds formed without layers. For multiple plasmid doses (200, 400, and 800 μg), transgene expression levels peaked during the first few days and then declined over a period of 1–2 weeks. Transfected cells were observed both in the surrounding adipose tissue and within the scaffold interior. Macrophages were identified as an abundantly transfected cell type. Scaffolds delivering plasmid encoding fibroblast growth factor-2 (FGF-2) stimulated a 40% increase in the total vascular volume fraction relative to controls at 2 weeks. Scaffold-based gene delivery systems capable of localized transgene expression provide a platform for inductive and cell transplantation approaches in regenerative medicine.

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## 1. Introduction

The fundamental goal of tissue engineering is to develop novel strategies for the replacement of diseased or injured tissues [1]. Most approaches utilize biomaterials to create a three-dimensional structure, or scaffold, that will support and guide new tissue formation from progenitor cells, either endogenous or transplanted [2,3]. Scaffolds are typically fabricated from biocompatible and biodegradable polymers, and exhibit a highly porous structure that allows for cellular infiltration and integration of the scaffold with host tissue. In cell-based therapies, scaffolds can also serve as a vehicle for delivering transplanted cells to specific sites, and must ultimately create an environment that supports cell survival and promotes their function [4]. This environment can be controlled through localized delivery of tissue inductive factors from the scaffold to regulate key cellular events involved in tissue development or repair (e.g. differentiation, proliferation, migration) [5]. For example, the delivery of angiogenic factors from scaffolds has been widely investigated to promote new blood vessel formation [6], which is basic requirement for establishing a vascular network within the developing tissue [7]. Additionally, scaffolds can deliver factors that act directly on transplanted cells.

Gene delivery from scaffolds offers a versatile approach for manipulating soluble signals present within the local tissue microenvironment, and has the potential to provide prolonged expression of desired proteins at effective levels [8–10]. The versatility arises, in part, because plasmids have similar physical properties despite changes in the nucleic acid sequence [11], thereby allowing delivery of multiple genes with a single delivery system. Previous studies have demonstrated that plasmid delivery from both collagen and poly(lactide-*co*-glycolide) (PLG) scaffolds can achieve localized transfection of cells, and induce sufficient protein production to stimulate new tissue formation [12–16]. For porous PLG scaffolds, we have observed transgene expression that

persisted for months at a subcutaneous site; however, the level and duration of transgene expression have been limited at other anatomical sites. Additionally, incorporating plasmid throughout the entire three-dimensional space of a highly porous scaffold has been relatively inefficient and dependent on the scaffold structure.

In this report, we investigated a layering approach to fabricate plasmid-releasing scaffolds that provide localized transgene expression following implantation into intraperitoneal fat, a model site for cell transplantation [17]. Porous poly(lactide-co-glycolide) (PLG) scaffolds were fabricated using a gas foaming method [14,18,19], in which a thin non-porous layer containing plasmid was inserted between two porous polymer layers. The layered scaffold design decouples the scaffold structural requirements from its function as a drug delivery vehicle. The plasmid incorporation efficiency and release rate were characterized in vitro, and in vivo transgene expression levels were measured by luciferase assay for multiple DNA doses. The distribution and identity of transfected cells were determined by immunohistochemistry. Finally, the ability of scaffolds to induce angiogenesis by providing expression of fibroblast growth factor-2 (FGF-2) was evaluated using contrast-enhanced microcomputed tomography. Scaffold-based gene delivery systems capable of localized transgene expression provide a platform for inductive and cell transplantation approaches in regenerative medicine.

## 2. Materials and methods

### 2.1. DNA sources

Plasmids were purified from bacteria culture using Qiagen reagents (Santa Clara, CA), and stored in Tris–EDTA (TE) buffer at 4 °C. All plasmids used in this study have a cytomegalovirus (CMV) promoter. The pLuc plasmid contains the firefly luciferase gene within the pNGVL vector backbone (National Gene Vector Labs, University of Michigan). The pEGFP-C2 plasmid (CLONTECH, Palo Alto, CA) encodes green fluorescent protein. The pFGF-2 plasmid was kindly provided by Dr. Claudia Heilmann (University Hospital, Freiburg, Germany), and contains the cDNA for human fibroblast growth factor-2 (18 kDa) within the pCI-neo expression vector (Promega) [20].

### 2.2. Scaffold fabrication

DNA-loaded scaffolds were fabricated using a previously described gas foaming/particulate leaching process [14,18,19], with a modified scaffold design containing a non-porous center layer for DNA loading. PLG (75% D,L-lactide/25% glycolide, i.v. = 0.76 dL/g) (Lakeshore Biomaterials, Birmingham, AL) was dissolved in dichloromethane to make either a 2% (w/w) or 6% (w/w) solution, which was then emulsified in 1% poly(vinyl alcohol) to create microspheres. The scaffold outer layers were constructed by mixing 1.5 mg of 6% PLG microspheres with 50 mg of NaCl (250 μm < d < 425 μm) and then compressing the mixture in a 5 mm KBr die at 1500 psi using a Carver press. To make the center layer, 2 mg of 2% PLG microspheres were reconstituted in a solution containing plasmid (200, 400, or 800 μg) and lactose (1 mg), and then lyophilized. This lyophilized product was then sandwiched between two outer layers and compressed at 200 psi. The composite scaffold was then equilibrated with high pressure $CO_2$ gas (800 psi) for 16 h in a custom-made pressure vessel. Afterwards, the pressure was released over a period of 25 min, which serves to fuse adjacent microspheres creating a continuous polymer structure. To remove the salt, each scaffold was leached in 4 mL of water for 2.5 h while shaking at 110 rpm, with fresh water replacement after 2 h.

### 2.3. Scanning electron microscopy (SEM)

Structural characteristics of scaffolds were imaged with a scanning electron microscope (Hitachi S-3400N-II) using the variable pressure mode and an ESED detector. The microscope was operated at an electron voltage of 15 kV.

### 2.4. DNA incorporation and in vitro release

The incorporation efficiency and in vitro release were determined as a function of DNA loading. The DNA incorporation efficiency is defined as the mass of DNA left in the scaffold after the leaching step divided by the mass of DNA initially inputted. Hereafter, the amount of input DNA will be referred to as the dose. Scaffolds were loaded with 200, 400, or 800 μg of pLuc. After leaching, scaffolds were dissolved in chloroform (600 μL), and the DNA was extracted from the organic solution. TE Buffer (400 μL) was added to the organic phase, vortexed, and centrifuged at 14,000 rpm for 3 min. The aqueous layer was collected, and two more extraction cycles were performed to maximize DNA recovery. The amount of DNA was

quantified using a fluorometer and the fluorescent dye Hoechst 33258. To determine the in vitro release kinetics of DNA, scaffolds were placed in 500 μL of phosphate-buffered saline (PBS) (pH 7.4) at 37 °C, and the solution was replaced at each time-point. The conformation of the released DNA was analyzed by agarose gel electrophoresis. A digital image of the gel was taken and NIH image software was used to evaluate the fraction of DNA remaining in the supercoiled conformation as previously described [21].

### 2.5. Measuring in vivo transgene expression

Animal studies were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and protocols were approved by the IACUC at Northwestern University. Scaffolds loaded with luciferase-encoding plasmid (200, 400, or 800 μg) were sterilized in 70% ethanol, washed in RPMI-1640 growth medium (Gibco-BRL, Grand Island, NY) (supplemented with 10% heat inactivated fetal calf serum (Hyclone, Logan, UT), 100 U/mL penicillin-G, 100 mg/mL streptomycin sulfate, and 1 mmol/L L-glutamine) to mimic the cell transplantation procedure, and then implanted into intraperitoneal fat of 10–12 week old C57BL/6 male mice (Jackson Laboratories), as previously described [17]. The selection of plasmid doses was based on previous reports [14–16]. At 3, 7, 14, and 21 days post-implantation, scaffolds were retrieved and frozen over dry ice. The frozen tissue samples were cut into small pieces with scissors, immersed in 200 μL of cell culture lysis reagent (Promega), and placed on a rotator for 30 min. Then samples were snap frozen in liquid nitrogen, thawed in a 37 °C water bath, and centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was mixed with luciferase assay reagent (Promega) and luciferase activity was measured with a luminometer using a 10 s integration time. Samples were normalized by total protein amount, which was measured using a BCA protein assay (Pierce Biotechnology Inc., Rockford, IL).

### 2.6. Histological analysis and immunohistochemistry

Histological analysis was performed to determine the cellular distribution and identity of transfected cells. Scaffolds loaded with 400 or 800 μg of GFP plasmid were retrieved 7 and 14 days post-implantation and frozen in an isopentane bath cooled over dry ice. Tissue samples were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA) and sections were cut at 14 μm thickness using a cryostat. Prior to staining, sections were fixed with 4% paraformaldehyde for 10 min and washed in PBS. The extent of cellular infiltration into scaffolds was visualized by hematoxylin and eosin (H&E) staining of tissue sections at 7 and 14 days. The distribution of transfected cells was determined by performing immunohistochemistry using an antibody directed against green fluorescent protein (GFP). Additionally, an antibody directed against the macrophage surface marker, F4/80, was used to determine if the cell type transfected was macrophages. After blocking, the two primary antibodies (rabbit anti-GFP (1:500 dilution; Invitrogen) and rat anti-mouse F4/80 (1:100 dilution; AbD Serotec, Raleigh, NC)) were applied for 2 h at room temperature. Secondary antibodies (Alexa Fluor 546 nm goat anti-rat (1:500 dilution; Invitrogen) and Alexa Fluor 488 nm goat anti-rabbit (1:500 dilution; Invitrogen)) were used to visualize the antigens. Lastly, sections were incubated with Hoechst 33258 (Invitrogen) (10 mg/mL, 1:2000 dilution) for 5 min to allow visualization of cell nuclei.

### 2.7. Evaluation of angiogenesis using microcomputed tomography

A previously described method for contrast-enhanced microcomputed tomography was used to evaluate blood vessel formation in scaffolds delivering plasmid encoding an angiogenic factor [22]. Scaffolds were loaded with 800 μg of pFGF-2 or 800 μg of pGFP and implanted into mice as described above. Control scaffolds were loaded with pGFP, rather than no DNA, to separate any effects of plasmid delivery from the response due to expression of FGF-2. At 2 weeks post-implantation, animals were deeply anesthetized by an intraperitoneal injection of tribromoethanol and placed ventral side up on a perfusion tray. The thoracic cavity was opened and a 21-gauge needle was inserted into the left ventricle and secured in place. The right atrium was cut and a peristaltic pump was used to flush the vasculature with 25 mL of normal saline containing heparin sodium (10 U/mL) at a rate of ∼5 mL/min. The specimen was then fixed by perfusion with 75 mL of 4% paraformaldehyde. The fixative was subsequently flushed from the vasculature with heparinized saline. A radiopaque silicone rubber compound containing lead chromate (Microfil MV-122, Flow Tech Inc., Carver, MA) was mixed with a curing agent, and then manually injected into the vasculature. Specimens were stored at 4 °C overnight to allow for polymerization of the compound, and then tissue samples were surgically retrieved. Samples were stored in 4% paraformaldehyde at 4 °C until imaging.

Samples were imaged using a Scanco Micro-CT 40 system (Basserdorf, Switzerland) operated at a voltage of 45 kV and current of 88 μA. Samples were scanned in a 16.4 mm diameter sample holder at high resolution, creating a series of 2048 × 2048 voxel (volume element) reconstructed slices with isotropic voxel size of ∼8 μm. Each scan consisted of 160 slices through the center of the sample. Reconstructed serial slices were globally thresholded based on X-ray attenuation and used to create 3-D renderings of the vascular networks. The same threshold (200 on a scale from 0 to 1000, corresponding to linear attenuation coefficients from 0 to 8 $cm^{-1}$,