Biomaterials 30 (2009) 2632–2641

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

# Sustained transgene expression via citric acid-based polyester elastomers

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ARTICLE INFO

*Article history:*
Received 27 October 2008
Accepted 12 January 2009
Available online 5 February 2009

*Keywords:*
Surface-mediated transfection
Poly(diol citrates)
Scaffolds
Biodegradable polyesters

ABSTRACT

Polymeric scaffolds are an important tool in tissue engineering and gene delivery using porous scaffolds can be a viable approach to control tissue response. Herein we describe the use of a biodegradable polyester elastomer, poly(1,8-octanediol-co-citrate) (POC), as a substrate for plasmid immobilization and cellular transfection of colonizing cells. Plasmid (pDNA), either complexed with poly(ethyleneimine) (PEI) forming polyplexes or in its native state, was surface-immobilized onto POC scaffolds via adsorption. Polyplex-containing scaffolds showed higher loading and slower initial rates of release than naked pDNA-containing scaffolds. Seeding of HEK293 cells and porcine aortic smooth muscle cells (PASMC) onto polyplex loaded-scaffolds demonstrated cell proliferation and transfection *in vitro* up to 12 days, significantly longer relative to bolus transfection. *In vivo*, transfection was evaluated using the mouse intraperitoneal (IP) fat model. In contrast to the *in vitro* study, successful long-term transgene delivery was only achieved with the naked pDNA-containing scaffolds. In particular, naked pDNA-containing scaffolds promoted high levels of both luciferase and green fluorescent protein (GFP) expression *in vivo* for 2 weeks. The results demonstrate that POC scaffolds are a suitable material for substrate-mediated gene delivery. POC scaffolds can potentially support long-term biological cues to mediate tissue formation through non-viral gene delivery.



## 1. Introduction

The development of constructs that provide the necessary biological and mechanical signals to repair and/or integrate injured or diseased tissues in the body is a fundamental goal of tissue engineering. Toward this goal, researchers often rely on scaffolds and the delivery of cell signaling factors. The scaffolds should be biocompatible and biodegradable, have mechanical properties that are similar to native tissue, facilitate cellular processes, and provide a template and biological cues for tissue formation. In this regard, researchers have investigated the use of synthetic and natural materials [1–8]. Although there are advantages and disadvantages to both types of materials, the ease of engineering, processing, and scale up that is characteristic of synthetic materials continue to fuel research efforts to control their interactions with surrounding tissue. We have previously reported the synthesis and characterization of citric acid-based biodegradable and biocompatible elastomers, poly(diol citrates) [9,10]. These novel polyester elastomers can be synthesized by the condensation of citric acid with various diols under very mild conditions without initiators or catalysts and can be easily fabricated into films or porous scaffolds. Their mechanical properties and degradation rates can be modulated with the choice of diols, introduction of nanopores, as well as the post-polymerization conditions used to create the polyester cross-link network [9,11,12]. Specifically, poly(diol citrates) hold significant promise for use in vascular tissue engineering due to their anticoagulant properties, ability to support the adhesion, proliferation, and differentiation of endothelial cells, and reduced platelet adsorption and activation [13].

Regarding the delivery of cell signaling factors, the method used should allow for short- or long-term delivery and control of dosing without compromising the integrity and biological activity of the factor. The physical incorporation of a protein into a scaffold, followed by the slow release of the protein during scaffold degradation has traditionally been used as a preferred method to deliver proteins to the environment surrounding the scaffold [1,14,15]. However, a challenge to implementing this method *in vivo* is the

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doi:10.1016/j.biomaterials.2009.01.021

inherent inability to maintain therapeutic levels of the protein for prolonged periods due to their short half-lives, limited protein loading capacity of the scaffold, and significant loss of activity due to the processing conditions during entrapment. An alternative to direct protein delivery is to deliver genes encoding for the protein, thereby allowing seeded or infiltrating cells to internalize the DNA and express and secrete the protein, often for extended times [16–18]. In this gene transfer approach, pDNA can be delivered directly, or may be first complexed with cationic polymers, such as polyethylenimine (PEI), to form particles of approximately 50–200 nm in diameter referred to as polyplexes [7,17,19,20]. PEI is hypothesized to disrupt the endosome through the proton sponge effect and enhance the DNA delivery efficiency [21–23]. In this report, naked pDNA or polyplexes are immobilized to the scaffold surface by adsorption. The presentation of immobilized pDNA using tissue engineering scaffolds has been termed substrate-mediated delivery, as the cell-adhesive substrate delivers the pDNA directly to the cell microenvironment [17,24,25]. This strategy avoids DNA degradation caused by processing because the plasmid or polyplexes can be immobilized following scaffold fabrication, typically avoiding exposure to solvents and/or high temperatures [20]. The objective of this study was to assess whether poly(1,8 octanediol-co-citrate) can facilitate substrate-mediated gene delivery using either naked pDNA or pDNA complexed with PEI. Scaffold compatibility, pDNA loading efficacy, release kinetics, and *in vitro* and *in vivo* transfection were investigated.

## 2. Materials and methods

### 2.1. Materials

Citric acid (99.5%), 1,8-octanediol (98%), and branched PEI with molecular weight of 25 kDa were purchased from Sigma–Aldrich (St. Louis, MO). [$^{35}$S]-dATP was obtained from MP Biomedicals (Solon, OH). Nick translation kit was obtained from GE Biosciences (Piscataway, NJ). Quant-iT™ PicoGreen® dsDNA Assay kit was obtained from Invitrogen (Chicago, IL). The plasmid encoding for Luciferase and GFP combined (pEGFPLuc: 5.7 kb) was used for the *in vitro* studies. pEGFPLuc is a cotransfection marker that allows for normalization of transfection efficiencies by fluorescence microscopy of living cells or by a standard Luciferase assay. Plasmid encoding for enhanced GFP (pEGFP-C2: 4.7 kb) or Luciferase (pNGVL1: 5.7 kb) was used for the *in vivo* studies. The plasmid was transformed in *Escherichia coli* DH5α and amplified in Terrific Broth media at 37 °C overnight at 300 rpm. The plasmid was purified using an endotoxin free QIAGEN Giga plasmid purification kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. Purified DNA was dissolved in saline, and its purity and concentration were determined by ultraviolet (UV) absorbance at 260 and 280 nm. HEK293 cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FBS, streptomycin (100 μg/ml), penicillin (100 U/ml), and 4 mM L-glutamine (ATCC, Manassas, VA) at 37 °C in a humidified 5% $CO_2$-containing atmosphere. Porcine aortic smooth muscle cells (PASMC) were cultured in M199 medium supplemented with 10% FBS, streptomycin at 100 μg/ml, penicillin at 100 U/ml, 1× Non-essential amino acid solution, 2 mM L-glutamine and 0.2% (v/v) fungizone (Invitrogen, Chicago, IL 60693).

### 2.2. Scaffold fabrication and characterization

POC pre-polymer was synthesized according to published methods [9]. Briefly, 0.1 mol of citric acid and 0.1 mol of 1,8-octanediol were added to a 100 ml round bottom flask. The mixture was melted under vigorous stirring at 160–165 °C. Following melting, the mixture was polymerized at 140 °C for 1 h to yield POC pre-polymer (Fig. 1A). POC pre-polymer was dissolved in ethanol at 35 wt.% solution, followed by addition of sieved sodium chloride (150 ~ 250 μm), which served as a porogen (porogen weight fraction, 87.5%) (Fig. 1B). The resulting slurry was cast into a poly(tetrafluoroethylene) (PTFE) plate. After solvent evaporation for 24 h, the PTFE plate was transferred into a vacuum oven for post-polymerization (120 °C, vacuum, 2 days). The salt in the resulting composite was leached out by successive incubations in water (produced by Milli-Q water purification system, Billerica, MA, USA) for 4 days. The resulting porous scaffold was lyophilized and then stored in a dessicator under vacuum before use. Surface and cross sections were coated with gold in a sputter coater (5 nm layer) and then observed by scanning election microscopy (SEM, Hitachi S-3400, EPIC, Northwestern University). The sponge-like scaffold was cut into disk-shaped pieces (6 mm in diameter, 2.5 mm thickness) using a cork borer (Fig. 1C). The density, surface area, and porosity of these pieces were measured using mercury intrusion porosimeter (AutoPore IV 9500, Micromeritics, Norcross, GA) by Delta Analytical Instruments, Inc. (North Huntingdon, PA). Compression strength and modulus were assessed in both dry and wet conditions after compressing a cylindrical porous scaffold (6 mm height and 6 mm in diameter) at 2 mm/min at a maximum compressive strain of 60% of original height with a 10 N load cell of an Instron 5544 mechanical tester (Norwood, MA). For the mechanical test, at least six samples were tested and the mean values and standard deviation (SD) were calculated.

### 2.3. In vitro degradation of POC scaffold

The degradation of POC scaffolds (6 mm diameter × 2.5 mm thick) was assessed *in vitro* in phosphate-buffered saline (PBS), pH 7.4, at 37 °C for up to 16 weeks under static conditions. PBS was changed as necessary to ensure that the pH did not drop below 7. Prior to weighing, samples were extensively rinsed with deionized water and dried. Weight loss was calculated by comparing the initial weight ($W_0$) with the weight measured at 2, 4, 8 and 16 weeks ($W_t$) (Equation (1)). The results are presented as mean ± SD ($n = 6$):

$$\text{Mass loss } (\%) = \frac{W_0 - W_t}{W_0} \times 100\% \quad (1)$$

### 2.4. Plasmid and polyplex loading and release kinetics

EGFPLuc plasmid was labeled with [$^{35}$S]-dATP using a Nick Translation Kit according to the manufacturer's instructions. The percent incorporated and the specific activity of [$^{35}$S] pDNA were 59.1% and $2.04 \times 10^9$ cpm/μg pDNA, respectively. [$^{35}$S] labeled pDNA was then diluted with unlabeled pDNA at the ratio of 1:50. Polyplexes were prepared by mixing pDNA and PEI at N/P ratio of 10, which is the ratio of polymer amine to DNA phosphate. 80 μl of pDNA solution or polyplexes suspension containing 2 μg of pDNA were directly deposited onto POC scaffolds (6 mm in diameter, 2.5 mm thickness) (Fig. 1B), and scaffolds with no pDNA were used as controls (background readings). The scaffolds were then incubated for 4 h and washed twice using PBS. The PBS was collected and [$^{35}$S] radioactivity was measured in a scintillation counter (LS 6500, Beckman, Palo Alto, CA). pDNA loading on POC scaffolds was calculated by subtracting the pDNA content in the PBS from the initial amount of pDNA added. After PBS washes, scaffolds were placed into PBS and kept at 37 °C. At specified timepoints, PBS was collected and the amount of radioactivity that had dissociated from the scaffold was determined by scintillation counting. The data were reported as mean ± SD ($n = 4$).

### 2.5. Cell proliferation

The proliferation of cells was evaluated using the Picogreen DNA assay. Porous POC scaffolds (6 mm in diameter, 2.5 mm thickness) were sterilized and pre-wetted with cell culture medium prior to placement in the wells of Corning ultra-low attachment 24-well plates (Fisher Scientific, Pittsburgh, PA). An 80 μl volume of a suspension of HEK293 cells (ATCC, Manassas, VA) ($8.96 \times 10^5$ cells/scaffold) was added to each scaffold and allowed to incubate for 3 h to allow cell attachment. After 3 h, scaffolds were placed into new Corning ultra-low attachment 24-well plates to separate them from cells that had attached to the polystyrene rather than the scaffolds. One milliliter of supplemented DMEM medium was then added to the wells and cells were incubated at 37 °C in a humidified 5% $CO_2$-containing atmosphere for up to 12 days. The culture medium was replaced every 3 days. At predetermined times, samples were treated with 200 μl of lysis buffer (Promega Co., Madison, WI) and homogenized. The lysate was subjected to several cycles of freezing and thawing, subsequently transferred into tubes, and centrifuged for 5 min. The supernatant was collected and analyzed using a PicoGreen® dsDNA Assay kit (Invitrogen, Chicago, IL) according to the manufacturer's protocol. Scaffolds with no cells were used as controls (background readings). The data were reported as mean ± SD.

Cellular infiltration into POC scaffolds was imaged by SEM. HEK293 cells ($7.5 \times 10^5$ cells/scaffold) or PASMC ($4.6 \times 10^5$ cells/scaffold) were seeded onto sterilized POC scaffolds as described above. At predetermined times, samples were fixed with 2.5% glutaraldehyde in PBS for 24 h at 4 °C followed by dehydration sequentially in 50, 70, 95, and 100% ethanol. The fixed samples were freeze-dried and sputter-coated with gold. The morphology of cells on the surface or cross section of POC scaffold was observed via SEM.

### 2.6. In vitro transfection via POC scaffolds

Transfection studies were performed in order to determine whether cells could express the complexed or naked pDNA that was surface immobilized onto POC scaffolds. Porous POC scaffolds (6 mm in diameter, 2.5 mm thick) were sterilized and placed in the wells of Corning ultra-low attachment 24-well plates (Fig. 1D). pDNA or polyplexes (N/P ratio = 10) containing 2 μg of pDNA was loaded onto the sponge-like POC scaffold as described for the loading/release kinetics experiments above. After PBS washes, the scaffolds were air dried in a laminar flow hood. An 80 μl volume of a suspension of HEK293 cells ($7.5 \times 10^5$ cells/scaffold) or PASMC ($4.6 \times 10^5$ cells/scaffold) was seeded to each scaffold and allowed to incubate for 3 h to allow cell

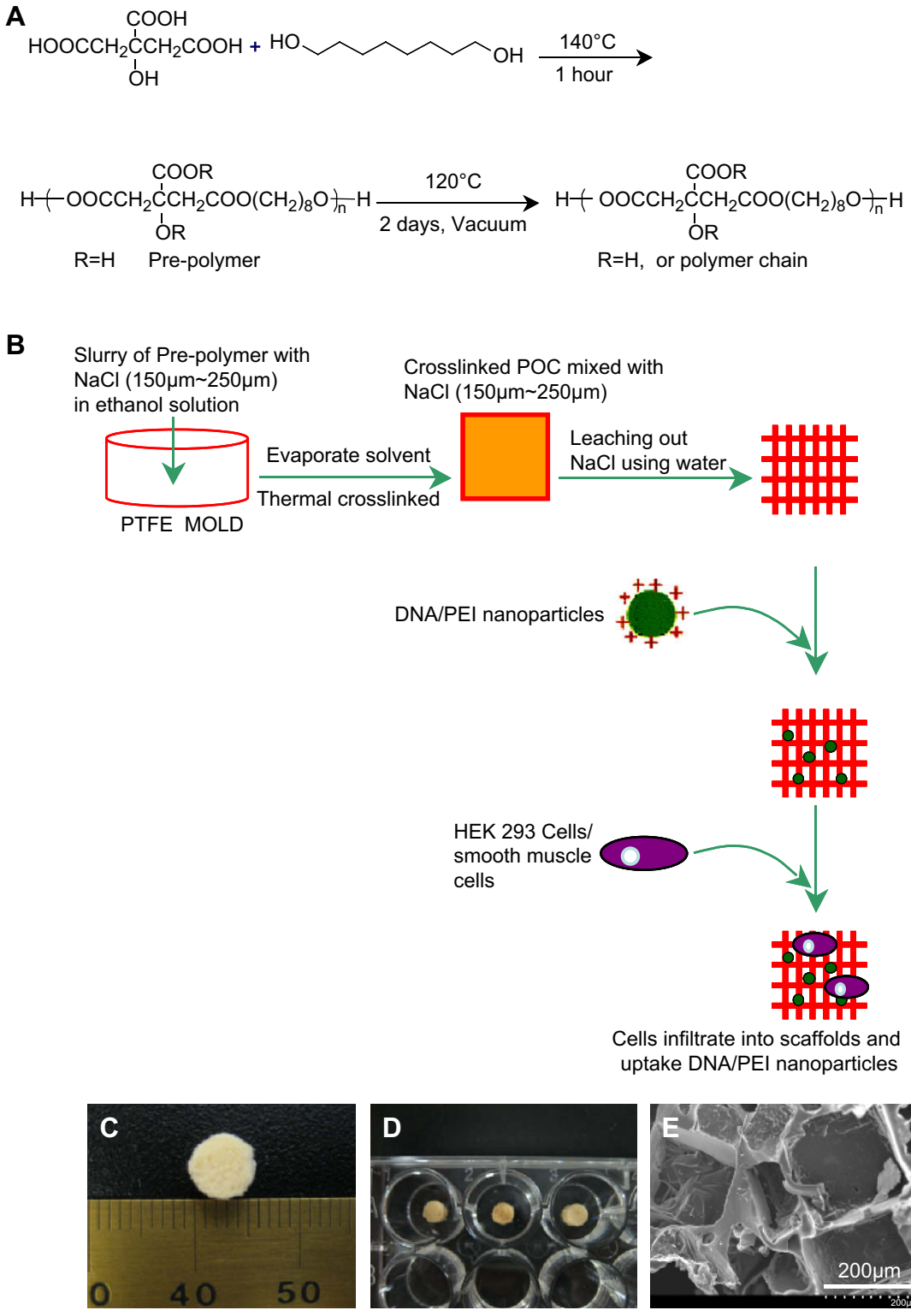



**Fig. 1.** Schematic illustration of POC polymer synthesis (A), process for the fabrication of porous POC scaffolds with surface-adsorbed PEI/DNA polyplexes (B), digital images (C, D) and SEM image (E) of disk-like POC scaffolds.

attachment. Scaffolds were then placed into new Corning ultra-low attachment 24-well plates. One milliliter of supplemented DMEM medium or M199 medium was added and cells were placed in a humidified incubator (37 °C, 5% $CO_2$, balance air) for up to 12 days. The culture medium was replaced every 3 days. At specified time-points, the samples were collected and homogenized in 200 μl of lysis buffer, and then subjected to two cycles of freezing and thawing. The lysate was centrifuged at 14,000 rpm for 5 min at 4 °C. Then, 20 μl of supernatant were added to 100 μl of Luciferase assay reagent (Promega Co., Madison, WI) and samples were measured on a luminometer for 10 s at a time. The relative light units (RLU) were normalized against protein concentration in the cell extracts, measured by a BCA protein assay kit (Pierce, Rockford, IL). Luciferase activity was expressed as relative light units (RLU/mg protein in the cell lysate). Scaffolds with no pDNA or polyplexes were used as controls (background readings). The data were reported as mean ± SD. Every transfection experiment was repeated at least two times.

As another control, a bolus delivery of polyplexes (N/P = 10) was added to HEK293 cells or PASMC. For these experiments, PASMC or HEK293 cells were seeded

into 24-well plate at a density of $1 \times 10^5$/well 24 h before transfection experiments. The same amount of pDNA (2 μg) in polyplexes (N/P = 10) was added to the serum-free medium and incubated for 4 h at 37 °C, followed by further incubation in serum-containing medium for 7 days. The culture medium was replaced every 3 days. Transfection was assessed as described for the scaffolds.

### 2.7. Assessment of in vivo transgene expression of DNA loaded POC scaffolds via intraperitoneal implantation

Naked pDNA (10 μg) or polyplexes (N/P ratio = 10, 10 μg of pDNA) were directly deposited onto the disk-shaped POC scaffold as described above. Luciferase-encoding plasmid was used for these experiments. After incubation for 4 h and extensive washes with PBS, the scaffolds were air dried. POC scaffolds loaded with pDNA were tested *in vivo* in a mouse intraperitoneal (IP) fat model. Scaffolds were loaded with polyplexes, naked pDNA, or no pDNA ($n = 4$), and implanted in mice (1 per mouse) by wrapping the IP fat pad around the scaffold before placing it back in the IP cavity [26]. The animals used were 25 male CD1 mice (Charles River, 20–22 g) and treated according to the Animal Care and Use Committee guidelines of Northwestern University. Isoflurane gas anesthesia was used during implantation and scaffolds were retrieved after 1 week and 2 weeks of implantation. To analyze Luciferase expression, the entire fat pad with scaffold was frozen, and stored in siliconized eppendorf tubes at −80 °C. The tissue and scaffold were cut with microscissors and lysed by briefly vortexing in the presence of 200 μl of lysis buffer (Cell Culture Lysis Reagent 1X, Promega, Madison WI). The tissue lysate was centrifuged at 14,000 rpm for 10 min at 4 °C and the supernatant was collected to perform a Luciferase assay (Promega). The total protein amount was measured with the enhanced test tube protocol of the BCA (bicinchoninic acid) protein assay (Pierce, Rockford, IL).

### 2.8. Histology and immunohistochemistry

As *in vivo* quantitation of transgene expression in this study is destructive, additional three animals were implanted with POC scaffolds containing EGFP-encoding plasmid and one animal with POC containing Luciferase-encoding plasmid for histology. The Luciferase-encoding plasmid was used as a control to check for non-specific binding of the anti-EGFP antibody. Immunohistochemistry was performed on tissue sections to identify transfected cells (EGFP-positive) and assess inflammatory response to implants in the mouse IP fat model. The mice were sacrificed and tissue samples surrounding the implants were harvested with the intact implant at 2 weeks after implantation. The entire fat pad with scaffold was immediately frozen in isopentane, cryopreserved in optimum cutting temperature media (OCT) compound (Miles, Elkhart, IN), and sectioned (10 μm thick) using a cryostat. The slides were stained with hematoxylin and eosin (H&E) for morphometric analysis. Tissue samples were examined for signs of inflammation (macrophage marker F4/80) and EGFP transfection using immunohistochemical staining. Sections were post-fixed with 4% paraformaldehyde. Tissue sections for EGFP staining were permeabilized with 0.25% Triton-X100 in PBS. Immunohistochemical staining protocols were followed according to manufacturer's instructions using the Vectastain ABC-AP kit and Vector Red Substrate kit (Vector Labs, Burlingame, CA). Tissue sections were blocked with 10% normal goat serum (Invitrogen, Carlsbad, CA). Primary antibody in 1% goat serum was applied overnight at 4 °C using either anti-F4/80 (macrophage, 1:500; Serotec, Raleigh, NC) or anti-EGFP (EGFP protein, 1:200, Invitrogen, Eugene, OR). Tissue sections were mounted using Vectashield mounting medium and counterstained with DAPI (Vector Labs). Positive immunohistochemical staining was visualized using fluorescence microscopy since the Vector Red reaction product is highly fluorescent using a rhodamine excitation filter. Fluorescent images were colored to show positive EGFP staining in green and positive F4/80 staining in red.

## 3. Results

### 3.1. Scaffold fabrication and characterization

Using salt leaching, we have obtained scaffolds with pore size ranging from 150 ~ 250 μm as shown in the SEM image (Fig. 1E). This characteristic is expected to facilitate cellular infiltration and nutrient transport. The skeletal density, surface area, and porosity of these scaffolds were estimated to be 1.35 g/cm$^3$, 105 m$^2$/g and 85.2%, respectively, as determined by mercury intrusion porosimetry. Table 1 summarizes the compression strength and modulus of POC scaffolds in both dry and wet conditions. Preliminary data suggest that the porous POC scaffold is very soft and there is no difference in mechanical properties between dry and wet scaffolds. The degradation rate of the POC scaffold was low with a weight loss of approximately 3 wt.% at 4 weeks and thereafter increased significantly with a weight loss of approximately 39 wt.% at 8 weeks (Fig. 2).

**Table 1**
Compression strength and modulus of POC scaffold under dry and wet conditions.

| | Compression modulus, $E_c$ (KPa) | Compression strength $S_c$ (KPa) |
|---|---|---|
| Dry | 16.02 ± 1.35 | 1.50 ± 0.16 |
| Wet | 19.08 ± 2.16 | 1.79 ± 0.33 |

### 3.2. Loading and release of DNA and polyplexes

Initial studies were performed to optimize the N/P ratio of PEI/DNA polyplexes. Transfection mediated by polyplexes at various N/P ratios was performed in 24-well plates seeded with PASMC. The highest transgene expression occurred at N/P ratio of 10 (data not shown). Thus, PEI/DNA polyplexes prepared at N/P ratio of 10 were used for subsequent experiments. Polyplexes were directly deposited into POC scaffold and high resolution SEM was performed to confirm the presence of polyplexes on the scaffold surface. The presence of polyplexes as individuals (black arrows, ~100 nm structures) or as aggregates (black arrowheads) was confirmed (Fig. 3A). After verifying the location of the polyplexes on the scaffolds, the loading efficiencies of polyplexes and naked pDNA on the scaffolds by surface adsorption were determined (Table 2). When POC scaffolds were surface-immobilized with 2 μg naked pDNA, about 0.70 ± 0.19 μg of DNA was associated with each scaffold, leading to an estimated loading efficiency of 35.1 ± 9.4%. When POC scaffolds were surface-immobilized with 2 μg polyplexes, 1.56 ± 0.05 μg of pDNA was associated with each scaffold, leading to an estimated loading efficiency of 77.8 ± 2.6%, which was 2-fold higher loading than scaffolds with surface-immobilized naked pDNA. Losses of DNA during preparation steps were found to be minimal for surface-immobilized polyplexes, while surface-immobilized pDNA showed significant losses during wash steps.

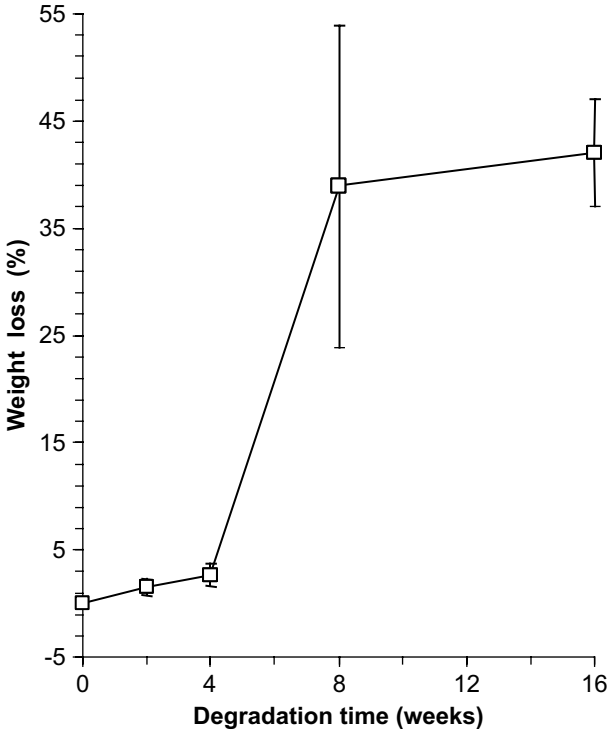
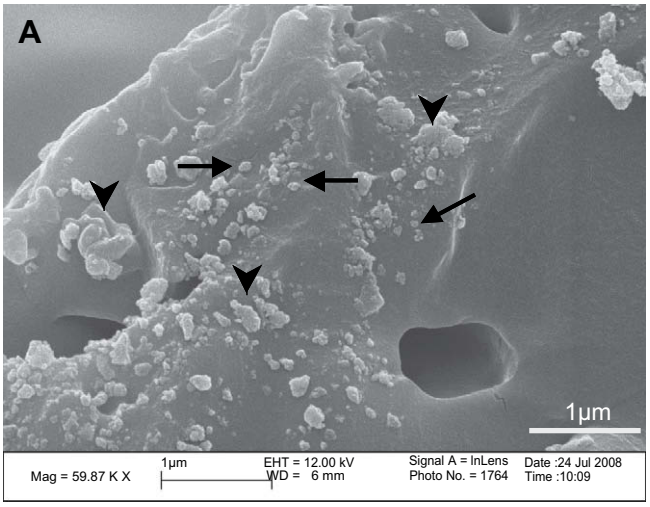



**Fig. 2.** *In vitro* weight loss of POC scaffolds at 2, 4, 8 and 16 weeks (PBS at 37 °C).



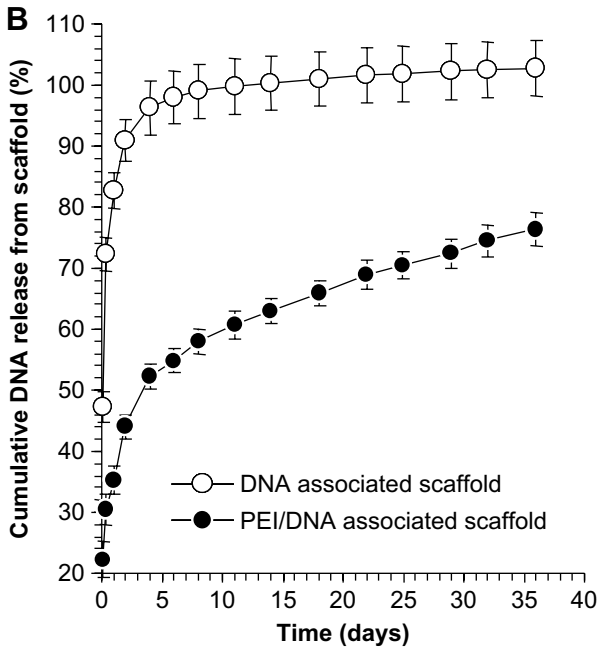




**Fig. 3.** SEM image of POC scaffold with surface-immobilized PEI/DNA polyplexes (60,000×) (A) and release profiles of naked pDNA and polyplexes from POC scaffolds (B). Error bars denote standard error of the mean ($n = 4$).

The release rates of the pDNA, either complexed with PEI or uncomplexed, were determined by measuring the amount of radiolabeled pDNA released into PBS at 37 °C. A burst release of pDNA was observed from naked pDNA-containing POC scaffolds. Naked pDNA surface immobilized onto POC scaffolds rapidly dissociated from the scaffolds; over 70% (0.49 μg) of the adsorbed pDNA was released from POC scaffold after 8 h of incubation in PBS and over 90% (0.63 μg) released after 2 days of incubation (Fig. 3B). In contrast, polyplexes surface-immobilized onto scaffolds remained tightly associated with POC scaffolds over the time course of the experiment. Approximately 48% (0.75 μg) of complexed pDNA remained scaffold-associated even after 6 days incubation of POC scaffolds in PBS. The initial burst pDNA release for polyplex-associated POC scaffolds was contained to approximately 44% (0.69 μg) in the first 2 days (Fig. 3B). Beyond that area, pDNA was released from polyplex-containing scaffolds in a linear manner.

Although the percentage of pDNA released from polyplex-containing POC scaffolds was lower than that from naked DNA-containing scaffolds, the absolute amount of pDNA released from polyplex containing-POC scaffolds at each time point was larger or equal when compared to that of naked pDNA-containing scaffolds. This is the case because the pDNA loading of polyplex-containing POC scaffolds was much higher compared with that of naked pDNA-containing scaffolds. Therefore, the polyplex-containing POC scaffolds released higher amounts of pDNA in a sustained manner.

**Table 2**
Summary of DNA loading into POC scaffolds by surface immobilization as determined by [$^{35}$S]-labeled DNA.

| | DNA bound to scaffold (μg) | DNA on scaffold (%) |
|---|---|---|
| pDNA-containing scaffold | 0.70 ± 0.19 | 35.1 ± 9.4 |
| polyplex-containing scaffold | 1.56 ± 0.05 | 77.8 ± 2.6 |

### 3.3. Cell proliferation

Cell proliferation within the scaffolds was confirmed as assessed by cellular DNA increase over time (Fig. 4). SEM confirmed that POC scaffolds supported cell attachment and spreading (Fig. 5). Both PASMC and HEK293 cells adhered to and spread on the scaffold surface and migrated into the scaffolds over time. At 12 days, both surface and cross section of POC scaffold were covered by layers of cells.

### 3.4. In vitro transfection profiles

Scaffolds with surface-associated polyplexes showed sustained transgene expression in the long-term, reaching the highest level of expression of $10^6$ RLU/mg protein at day 9 for PASMC and day 12 for HEK293 cells (Fig. 6). Furthermore, there was no significant drop in terms of transfection in PASMC at day 12. The highest expression levels of surface-associated polyplexes were similar to those achieved by bolus delivery of polyplexes, which peaked at day 1. However, polyplexes showed sustained Luciferase expression for at least 2 weeks when surface-adsorbed onto POC scaffold, while the transfection mediated by bolus delivery of polyplexes decreased by 1000-fold within 7 days. For scaffolds with surface-associated pDNA not complexed with PEI, low transfection that was similar to background was observed. The bolus delivery of naked pDNA led to low Luciferase expression, which was close to background at all timepoints (data not shown).

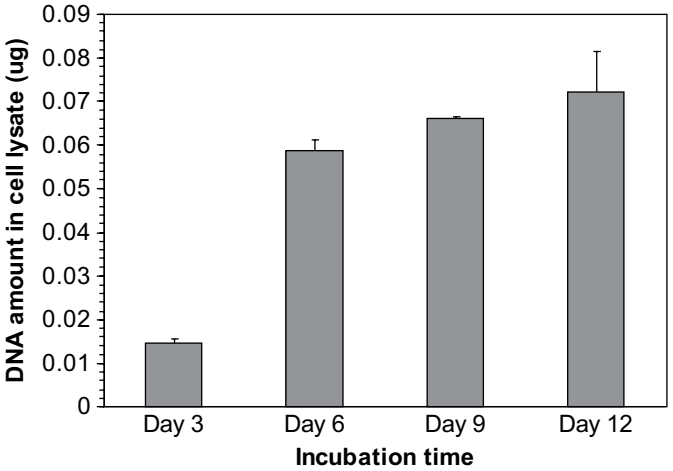



**Fig. 4.** Proliferation of HEK293 cells within POC scaffolds. The data were reported as mean ± standard deviation.

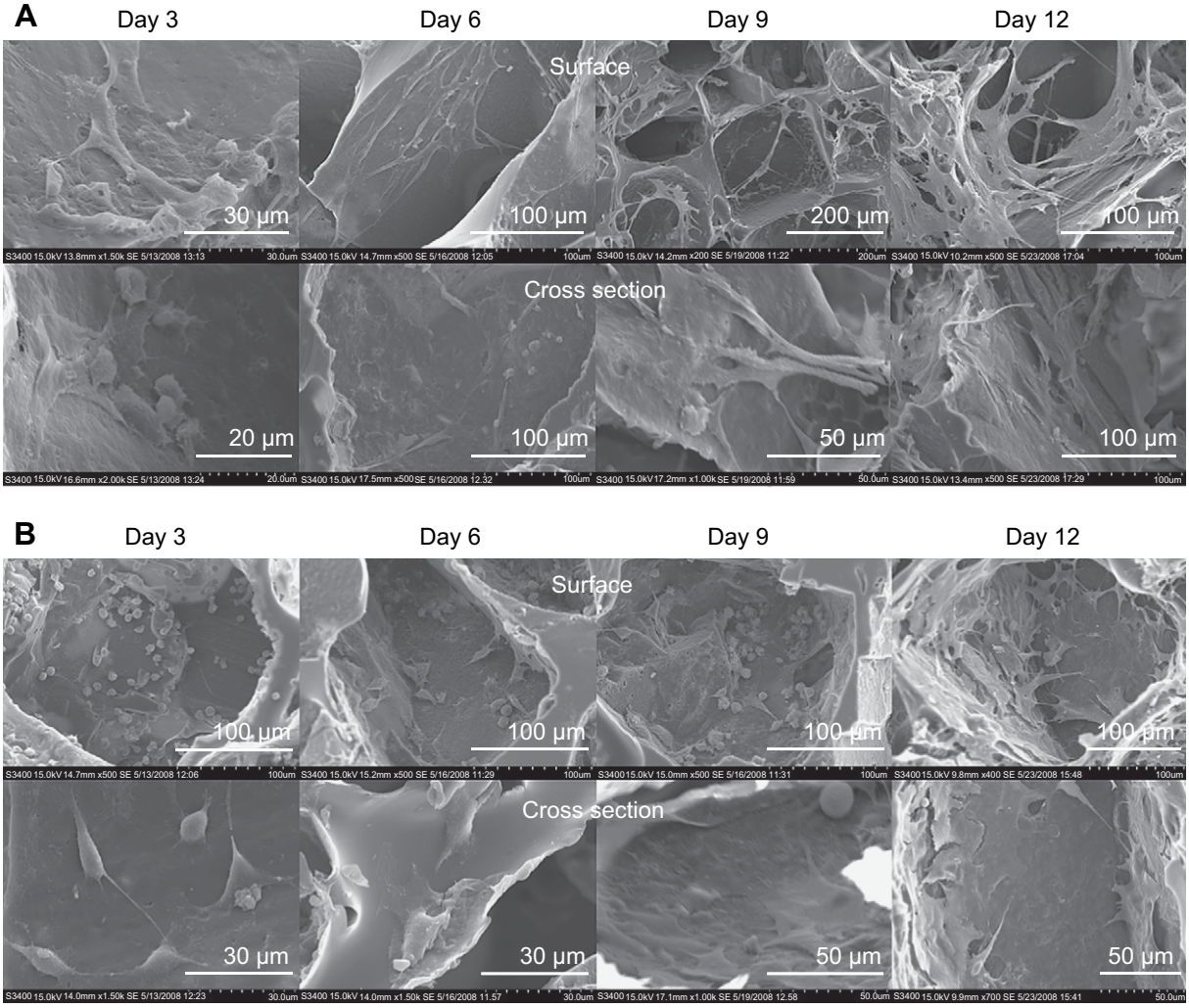



**Fig. 5.** SEM images of POC scaffolds seeded with PASMC (A) and HEK293 cells (B) demonstrating cellular infiltration into the scaffolds at day 3, 6, 9 and 12.

### *3.5. In vivo transgene expression of DNA loaded POC scaffolds via intraperitoneal implantation*

Upon gross examination, all scaffolds integrated very well with surrounding tissue. POC scaffolds loaded with naked pDNA resulted in high levels of transgene expression *in vivo* for at least two weeks (Fig. 7). However, scaffolds loaded with polyplexes did not induce transgene expression after implantation in the mouse IP fat. Immunohistochemical staining was performed to confirm the *in vivo* expression and cellular infiltration mediated by pDNA-loaded POC scaffolds. In all cases, cells migrated into the pores of the POC scaffolds (Fig. 8A). This result is consistent with the *in vitro* cellular infiltration images obtained by SEM (Fig. 5). Within the scaffold pores, many cell nuclei are visible throughout the implant. Positive EGFP transfection was also observed in cells that infiltrated the POC scaffolds containing surface-adsorbed EGFP plasmid (Fig. 8B). Cells positive for EGFP were predominantly near the surface of the pore walls with fewer cells staining positive at the center of the pore suggesting localized pDNA uptake (green stain). Immunohistochemical staining using EGFP antibodies was negative for the Luciferase-encoding POC scaffolds (Fig. 8C), confirming that the antibody specifically bound to EGFP produced by transfected cells within the POC scaffold. The identity of some of the cells located near the pore walls within the scaffold was confirmed to be macrophages as per the positive red stain with the macrophage marker, F4/80 (Fig. 8D).

## 4. Discussion

The modulation of tissue formation *in vitro* and *in vivo* is an important goal of tissue engineering and regenerative medicine. The ability to affect tissue formation can also be applicable to attenuating the foreign body response to implantable devices such as vascular grafts and catheters. Hence, the development of methods to control or address this process is important and an area of active research. One approach to confer or replace tissue function is to use a biodegradable porous scaffold that would serve as a temporary structure to provide mechanical and biological cues. As for mechanical cues, the effect of mechanical stimulation and scaffold/substrate mechanical properties on cell processes and tissue formation has been documented by several research groups [27–29]. In this regard, poly(diol citrates) are a promising class of biodegradable elastomers because their composition allows for design flexibility of mechanical and degradation properties [5,9,10].

Biological cues from the scaffold can be provided in the form of the delivery of proteins such as growth factors and small compounds such as anti-inflammatory drugs [1,30]. Strategies to deliver proteins include entrapping the protein within micro or nanoparticles that are distributed throughout the scaffold or directly adding the protein to the scaffold during or after its fabrication [1,31,32]. In these cases, protein denaturation or a fast release and wash-out of the protein may occur. These processes

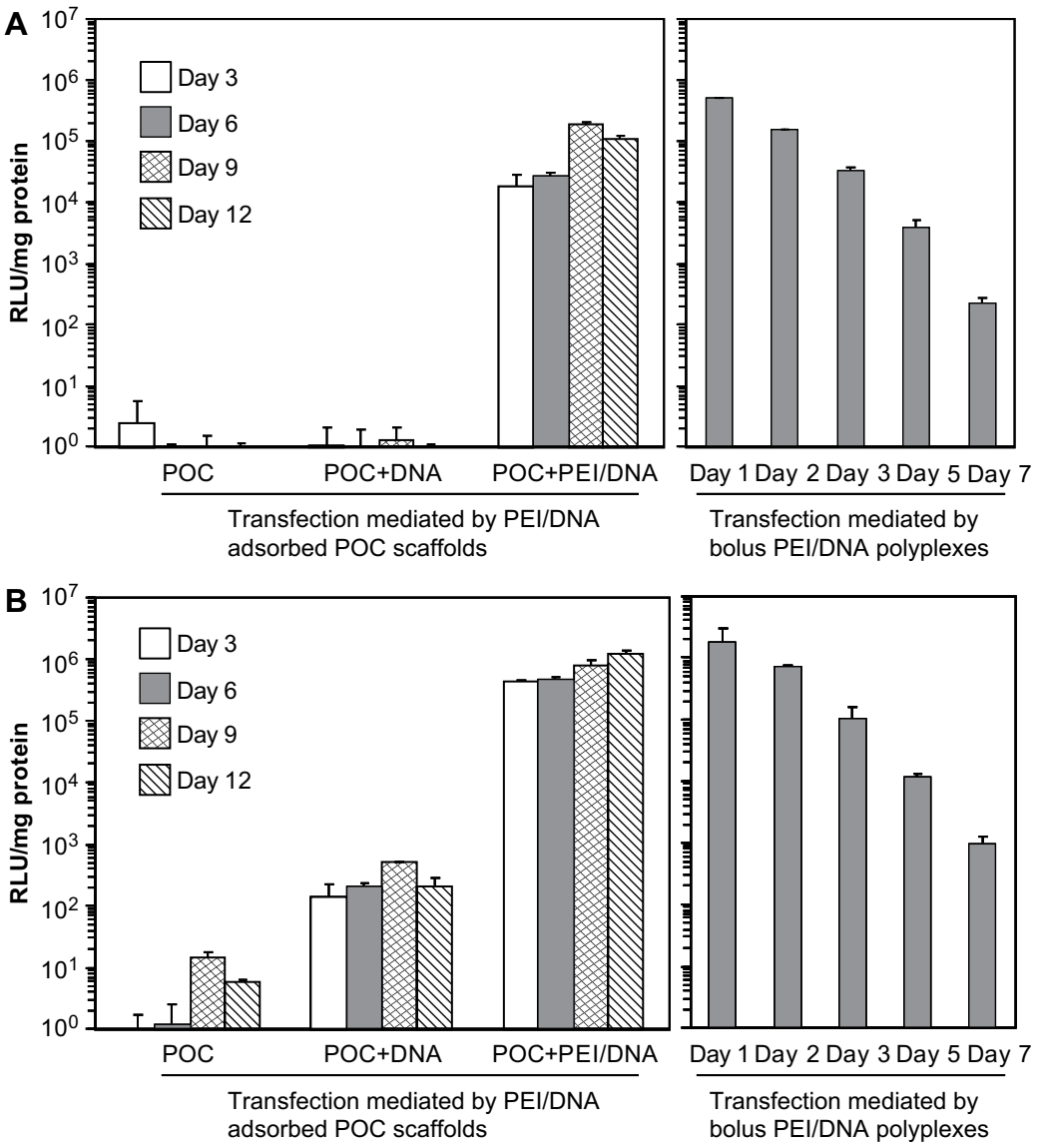



**Fig. 6.** Long-term luciferase expression in PASMC (A) and HEK293 cells (B) that were seeded on pDNA- and polyplex-containing POC scaffolds (mean ± standard deviation).

lead to significant loss of protein activity and low efficiency, which can be very costly if significant quantities of the protein of interest are required for prolonged periods. An alternative approach involves the use of gene delivery vectors in order to transfect exogenous or endogenous cells that are within the scaffold. The use of viral vectors has been investigated by many researchers and safety concerns continue to dampen enthusiasm for this approach [33]. Non-viral gene delivery approaches are perceived as being safer, but there can be significant variability regarding efficacy *in vitro* and *in vivo* [16,34,35]. Nevertheless, the fact that a gene product can be delivered without the use of a virus remains appealing. As with protein delivery, entrapment of pDNA within the scaffold would lead to significant degradation of the pDNA. Therefore, methods to deliver the pDNA using adsorption surface phenomena and DNA condensation reagents are key to controlling substrate-mediated non-viral transfection.

Naked and complexed plasmid have been encapsulated or adsorbed onto porous scaffolds [7,8,18,20,36]. However, the mechanical and degradation properties of the scaffolds used to date cannot be easily tailored to specific applications. Furthermore, they are not suitable for vascular applications that require contact with blood as they are thrombogenic. In this regard, the ability to achieve substrate-mediated gene transfection using poly(diol citrates) would be a significant contribution to the field. Plasmid, either naked or complexed with PEI, was loaded onto POC porous scaffolds via surface adsorption and evaluated for sustained transgene expression *in vitro* and *in vivo*. The presence of DNA and polyplexes within the POC scaffold was confirmed via [$^{35}$S]-labeled DNA and high resolution SEM. Polyplex containing scaffolds showed higher loading and slower initial rates of release than naked pDNA-containing scaffolds. Polyplexes-containing scaffolds exhibited a burst release of pDNA (days 1–3) followed by zero order release kinetics after day 5. POC scaffolds were able to retain polyplexes likely due to the electrostatic interactions between the charge properties of the POC scaffold's surface and polyplexes. The abundance of carboxyl groups in POC scaffolds supports tight association of positively charged PEI/DNA polyplexes. Retention of native pDNA onto POC scaffolds is likely mediated through hydrogen bonding and van der Waals interactions resulting in a more loose association of the pDNA that can be easily removed by PBS washes [20,24]. In either case, POC with pDNA adsorbed to its surface was able to support cell adhesion, proliferation, and migration.

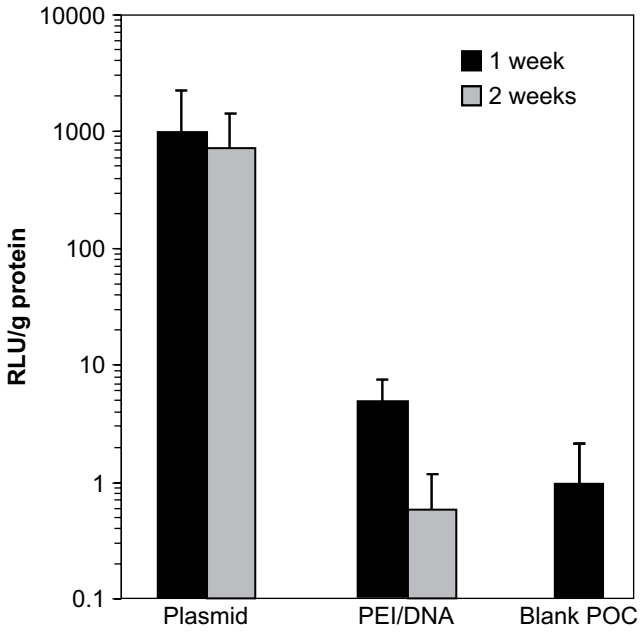



**Fig. 7.** *In vivo* luciferase expression mediated by pDNA-containing POC scaffolds loaded with PEI/DNA polyplexes, plasmid, or no DNA ($n = 4$), were implanted in separate mice by wrapping the IP fat around the scaffold before replacing it in the IP cavity. The entire fat pad containing the scaffold was retrieved and analyzed for luciferase expression.

To assess the ability of the polyplexes or naked DNA-containing POC scaffolds to transfect cells, we first performed *in vitro* transfections with HEK293 cells and PASMCs. HEK293 cells were chosen because they are easy to transfect and represent a standard cell line used for transfection studies. PASMCs were chosen because these cells are potential targets for *in vivo* transfection to minimize their uncontrolled growth during revascularization procedures. Polyplex-containing scaffolds showed an increased Luciferase expression throughout the 12-day period of the experiment, whereas naked pDNA led to low transfection (close to background). In contrast, a bolus delivery of the same amount of pDNA (2 μg) in polyplexes (N/P = 10) resulted in a transient high Luciferase expression at day 1 followed by a 1000-fold decrease in expression within 7 days. Furthermore, POC scaffolds, containing approximately 1.5 μg of pDNA in polyplexes after washing, mediated a sustained level of Luciferase expression that was similar compared to the initial expression level for bolus delivery of 2 μg of pDNA in polyplexes. PASMC had lower transfection levels relative to HEK293 as primary cells are generally more difficult to transfect than cells lines. These transfection data support previous reports that note the advantage of surface-mediated transfection over bulk solution transfection [20,37]. The linear release profile achieved by polyplex-containing scaffolds may support the sustained levels of expression. The successful *in vitro* transfection of PASMC suggests that POC can be used as a scaffold for substrate-mediated gene delivery in the field of vascular tissue engineering.

Substrate-mediated gene transfer was further evaluated *in vivo*. Scaffolds loaded with plasmid, either complexed with PEI or naked, were implanted in a mouse intraperitoneal (IP) fat model. This model has been previously used to demonstrate cell transplantation and transgene expression in soft tissues using porous scaffolds [26,38]. Surprisingly, different expression profiles were achieved for POC scaffolds implanted *in vivo* relative to *in vitro*. Only POC scaffolds loaded with naked pDNA resulted in Luciferase (or EGFP) expression *in vivo* for at least 2 weeks. Our *in vivo* data contradicted the *in vitro* results, confirming the limited predictability of *in vitro* transfection studies. Interestingly similar findings concerning DNA transfer in the presence or absence of liposome carriers have been reported by Steinstresser et al., who described the highest gene transfer was achieved by naked DNA compared to the other liposome groups when testing in an experimental burn

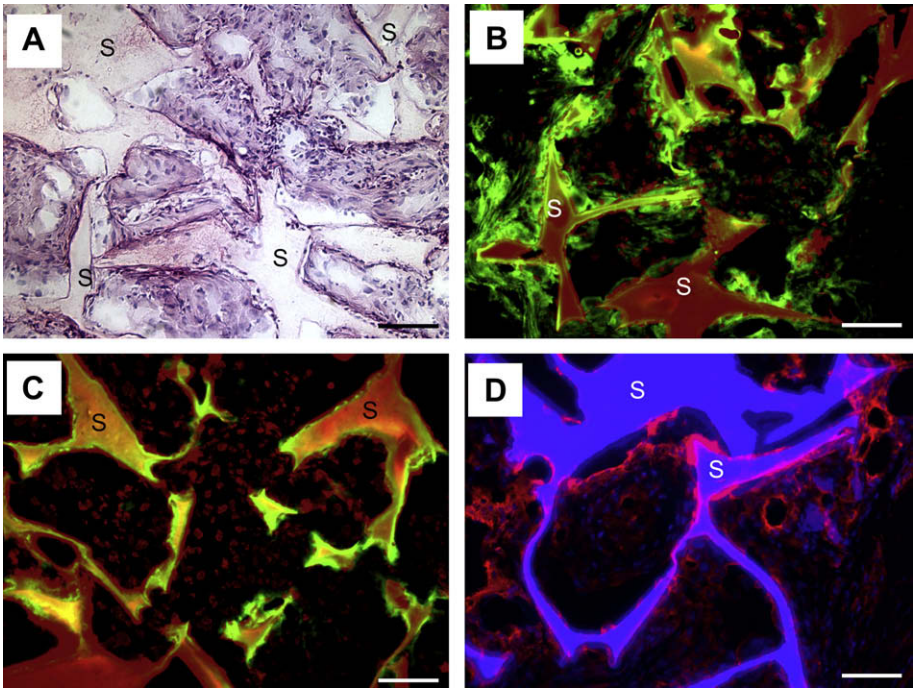



**Fig. 8.** A) Hematoxylin & eosin stain of cells within the POC scaffold. B) Anti-EGFP stain for a POC scaffold containing EGFP plasmid. Positive cells stain green, cell nuclei stain red, C) Anti-EGFP stain for a POC scaffold containing the luciferase plasmid (no positive cells, cell nuclei stain red), D) Anti-F4/80 macrophage stain of a POC scaffold. Macrophages stain red, cell nuclei stain blue. S denotes the scaffold. Scale bar = 50 μm.

wound model [34]. Huang et al. showed that naked plasmid mediated the highest level of Luciferase at day 14 in comparison with other polyplex groups after intramuscular injection [39]. Gharwan et al. found that injection of low concentrated naked DNA (3 μg) into mice livers resulted in an 11-fold lower transgene expression as compared to PEI/DNA polyplexes. The administration of higher concentrated naked DNA (9 μg) yielded a more than 3-fold increase in Luciferase expression compared to PEI/DNA polyplexes [35]. Rolland et al. discussed that the interactions between carriers and host tissue and immunity cannot be sufficiently mirrored in an *in vitro* setting as a potential cause for different results and limited predictability in gene transfer studies *in vivo* [40]. In order to confirm the transfection of naked pDNA released from POC scaffold, naked pEGFP-containing POC scaffolds were implanted in the mouse intraperitoneal (IP) fat model and analyzed by immunohistochemical staining. Positive EGFP staining was also observed within POC pores for 2 weeks with most of the positive cells located near the pore wall. Most of the transfected cells are likely to be macrophages as this cell type was also localized to the vicinity of the pore walls. Others have also noted that macrophages rapidly penetrate into scaffolds and that these cells are in fact transfected when pDNA was delivered [8,41].

## 5. Conclusions

Poly(diol citrates) are a useful class of elastomers that can be used for the fabrication of tissue engineering scaffolds. The primary goal of this study was to develop a degradable POC scaffold for substrate-mediated gene delivery to colonizing cells. We found that both naked pDNA and PEI/DNA polyplexes were efficiently associated with and released from the porous scaffold. Long-term efficient transgene levels can be achieved *in vitro* using polyplex-containing scaffolds. However, only naked pDNA-containing scaffolds can yield relevant transgene expression *in vivo* (at least in the IP cavity). More research on pDNA desorption phenomena *in vitro* and *in vivo* is required in order to better understand substrate-mediated gene delivery to cells infiltrating a scaffold.

## Acknowledgement

The authors thank Jeffrey Kuo, Haichao Zhao, and Antonio Webb for their help with setting up the experiments.

## Appendix

Figures with essential colour discrimination. The majority of figures in this article are difficult to interpret in black and white. The full colour images can be found in the online version, at doi:10.1016/j.biomaterials.2009.01.021.

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