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The contribution of plasmid design and release to in vivo gene expression following delivery from cationic polymer modified scaffolds

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Abstract

Tissue engineering scaffolds capable of gene delivery can provide a structure that supports tissue formation while also inducing the expression of inductive factors. Sustained release strategies are hypothesized to maintain elevated plasmid concentrations locally that can enhance gene transfer. In this report, we investigate the relationship between plasmid release kinetics and the extent and duration of transgene expression. Scaffolds were fabricated from polymer microspheres modified with cationic polymers (polyethylenimine, poly(L-lysine), poly(allylamine hydrochloride), polydiallyldimethylammonium) or polydopamine (PD), with PD enhancing incorporation and slowing release. *In vivo* implantation of scaffolds into the peritoneal fat pad had no significant changes in the level and duration of transgene expression between PD and unmodified scaffolds. Control studies with plasmid dried onto scaffolds, which exhibited a rapid release, and scaffolds with extended leaching to reduce initial quantities released had similar levels and duration of expression. Changing the plasmid design, from a cytomegalovirus (CMV) to an ubiquitin promoter substantially altered the duration of expression. These studies suggest that the initial dose released and vector design affect the extent and duration of transgene expression, which may be sustained over several weeks, potentially leading to numerous applications in cell transplantation and regenerative medicine.

1. Introduction

Tissue engineering scaffolds are employed to control the local microenvironment and enhance the body's innate capacity to regenerate after an injury. The scaffold provides a structural support that creates and maintains a space for tissue growth, and also functions as a support for cell adhesion and migration, which facilitates cell infiltration and engraftment with the host. In addition to the structural properties, scaffolds are being developed to deliver diffusible factors that can either promote regeneration or block inhibitors of regeneration [1,2]. Factors released from the scaffold can act locally, and are available to promote regeneration yet their

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availability is time-limited to avoid aberrant cellular processes. In particular, gene delivery from the scaffold is a versatile approach to induce the expression of tissue inductive factors, in which host cells infiltrating the scaffold function as localized bioreactors. The versatility of gene delivery can enable screening of factors, and could allow the delivery of multiple factors; however, the major challenge lies in the development of effective delivery systems.

A fundamental hypothesis regarding the delivery of gene therapy vectors is that sustained release can maintain elevated concentrations of the vectors locally, which could enhance the extent and duration of gene transfer. Locally maintaining elevated concentration of plasmid is proposed to promote continued cellular internalization of plasmid, which can replace plasmid that is either cleared from the cell or silenced to prevent continued expression. Methods by which sustained release can be controlled include microsphere encapsulation [3,4], layer-by-layer assemblies with cationic polymers [5,6], and physical entrapment [7,8]. These polymeric systems have induced transgene expression that persists for days to months, though the results are dependent on the implantation site, delivery method, and dose [1,2]. Although these systems have promoted gene delivery in vivo, the contribution of release rate to gene expression in vivo has not been well defined, particularly within the context of the plasmid design and silencing of expression.

In this report, we investigated this contribution of release profile from scaffolds and plasmid design on the extent and duration of transgene expression. A layered scaffold was used, which enables efficient incorporation of plasmid into the structure [9]. The surface of the scaffold was modified with cationic polymers, which can reversibly bind the vector and thereby slow its release. The central layer of the scaffolds was modified by incubating poly(lactide-co-glycolide) (PLG) microspheres with a range of polymers, including polyethylenimine (PEI), poly(L-lysine) (PLL), poly(allylamine hydrochloride) (PAH), polydiallyldimethylammonium (PDDA), and polydopamine (PD). These components were selected based on their ability to bind DNA, their ability to provide surface coatings on PLG, or their use in layer-by-layer assemblies [5,6,10–17]. The retention of DNA within the scaffold and release profile was initially characterized. Subsequently scaffolds with varying rates of release were implanted and the extent and duration of transgene expression was characterized, along with the distribution of transfected cells around the scaffold. Plasmids with different promoters were subsequently delivered and gene expression measured, which aimed to determine whether the plasmid remained intracellular and was being silenced. Results from these studies will thus identify transgene expression as a function of the release profile and vector design, which are fundamental design parameters for gene delivery from scaffolds.

2. 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. 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. Both plasmids have a cytomegalovirus (CMV) promoter. A second pLuc plasmid contained an ubiquitin C (UbC) promoter. The 1.2-kb human ubiquitin C (UbC) promoter was amplified from pLenti6/UbC/V5-DEST (gift from Dr. J.S. Jeruss, Northwestern University) using PCR with primers containing *Nhe*I and *Xho*I restriction sites flanking the promoter fragment. This *Nhe*I–*Xho*I UbC promoter fragment was cloned into pCMV-Luc replacing the CMV promoter.

2.2 Microsphere preparation and characterization

Poly(lactide-co-glycolide) (PLG) microspheres are used as the building block to make the scaffolds using a gas foaming technique. Microspheres were prepared as previously described [3,9]. 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 microspheres were collected by centrifugation, washed 3 times with deionized water to remove residual poly (vinyl alcohol), and lyophilized overnight.

To modify the microspheres, microspheres (20 mg, 2% (w/w)) were incubated in 0.5 ml of the cationic polymer solution. For the polymers poly(allylamine hydrochloride) (PAH) (MW=56 kDa, Sigma-Aldrich), polydiallyldimethylammonium (PDDA) (MW=200–350 kDa, Sigma-Aldrich), polyethylenimine (PEI) (MW=25 kDa, Sigma-Aldrich), and poly(L-lysine) (PLL) (MW=56 kDa, Sigma-Aldrich), the microspheres were incubated for 2 hours in a 2 mg/ml solution in phosphate buffer saline (PBS). For polydopamine (PD), the microspheres were incubated in a 2 mg/ml solution of dopamine hydrochloride (Sigma-Aldrich) in 10 mM Tris-HCl buffer pH 8.5 for 2, 8, and 24 hours. The microspheres were washed 3 times with deionized water by centrifugation to remove any residual polymer solution, and lyophilized overnight. Zeta potential and particle size of the microspheres were characterized using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) and a Multisizer3 Coulter Counter (Beckman Coulter, Fullerton, CA).

2.3. Scaffold fabrication

Layered scaffolds were fabricated in order to decouple the requirements for cell infiltration from those for sustained DNA release [9]. In this approach, the plasmid is entrapped within a central layer of polymer (~200 μ m thick), and the porous aspects of the scaffolds surround the central polymer allowing for cell infiltration. The central layer of the scaffold can then be manipulated to regulate DNA release. This layer can also be altered without influencing the structural integrity of the scaffold to support cell transplantation and cell infiltration. Layered DNA-loaded scaffolds were fabricated using a previously described gas foaming/particulate leaching process [18–20]. A non-porous center layer for DNA loading was employed as previously discussed [9]. The scaffold outer layers were constructed by mixing 1 mg of 6% PLG microspheres with 33 mg of NaCl (250 μ m < d < 425 μ m) and then compressing the mixture in a 5 mm KBr die at 1500 psi using a Carver press. To prepare the center layer, 2 mg of 2% PLG microspheres were reconstituted in a 100 μ l solution containing plasmid (400 μ g) and lactose (1 mg), and then lyophilized. This lyophilized product was then sandwiched between two outer layers and compressed at 1500 psi. Scaffolds without a central layer were formed by mixing 2.5 mg of 6% PLG microspheres with 82 mg of NaCl (250 μ m < d < 425 μ m) and then compressing the mixture as previously described. The composite scaffolds are then equilibrated with high pressure CO₂ 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 2 mL of water for 1 h while shaking.

2.4 DNA incorporation and *in vitro* release

The incorporation and release of DNA was determined using DNA that was radiolabeled with α -³²P dATP using a nick translation kit (Amersham Pharmacia Biotech, Piscataway, NJ), as previously reported [21]. Scaffolds were loaded with 10 μ g of ³²P labeled DNA. The DNA incorporation efficiency is defined as the mass of DNA left in the scaffold after the leaching step divided by the initial mass of DNA. To determine the *in vitro* release kinetics of DNA, scaffolds were placed in 1 mL of PBS (pH 7.4) at 37°C, and the solution was replaced at each time-point. The samples were immersed in a scintillation cocktail (Biosafe II, Research

Products International Corp, Mt. Prospect, Illinois) to measure DNA concentrations. At the end of the study, the scaffolds were immersed in scintillation cocktail to determine residual DNA. The cumulative release is reported as the total DNA released up to that time point divided by the total DNA incorporated into the scaffold.

2.5 In vitro transgene expression

Cell culture wells were coated with PD to investigate the impact of PD on gene transfer. Transfection studies were performed with HEK 293T cells cultured at 37°C in Dubelcco's Modified Eagle Medium (DMEM) cell growth media supplemented with 1% sodium pyruvate, 1% penicillin-streptomycin, 1.5 g/L NaHCO₃, and 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). A 2 mg/ml dopamine solution in 100 mM Tris-HCL buffer (pH 8.5) was added for 24 hours to coat the wells with PD. Cells were seeded onto PD modified and unmodified wells at a loading of 10,000 cells per well. DNA complexes were formed by mixing DNA with Transfast Reagent (Promega, Madison, WI) at a ratio of 1 µg of DNA to 1.5 µl of Transfast. The complexes were incubated for 10 minutes and added to the wells 24 hours after the initial seeding of the cells at a dose of 0.1, 0.5, or 1.0 µg of DNA per well. After 48 hours, the media was removed and the cells were washed with PBS. Lysis buffer was added and incubated for 15 minutes at room temperature, the plate was frozen at -80 C for 20 minutes, thawed, and the supernatant was removed and centrifuged at 14,000 rpm for 1 minute. The supernatant was mixed with luciferase assay reagent (Promega) and luciferase activity was measured with a luminometer using a 10 s integration time.

2.6. 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. Layered scaffolds loaded with luciferase-encoding plasmid (400 µg) under a CMV promoter were leached, immersed in 70% ethanol for 30 seconds, washed, dried overnight, and then implanted into intraperitoneal fat of CD1 male mice (Charles Rives, 30–33 g), as previously described [22]. For studies of release rate, unmodified scaffolds and PD modified scaffolds were used to provide a rapid and slow release respectively. At 3, 7, 14, and 22 days post-implantation, scaffolds were retrieved and frozen over dry ice. As a control, DNA (100 µg) was dried overnight onto scaffolds without a central layer. At 3 and 7 days post-implantation the scaffolds were retrieved and frozen over dry ice. As a second control, layered scaffolds loaded with 400 µg of DNA were incubated for 24 hours post-leaching in PBS at 37°C. At 3 days post-implantation the 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 vortex for mixing. The samples were incubated for 15 minutes at room temperature, and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was mixed with luciferase assay reagent (Promega). 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).

In order to compare promoters within the DNA vector, layered scaffolds loaded with 400 µg of Luciferase encoding gene under a CMV or UbC promoter were implanted at the IP fat site. In vivo luciferase expression was monitored using an IVIS imaging system (Xenogen Corp., Alameda, CA). For imaging, the animals were injected IP with d-luciferin (Molecular Therapeutics Inc., MI, 150 mg/kg body weight, 20 mg/mL in PBS) using 28G insulin syringes. The animals were placed in a light-tight chamber and bioluminescence images were acquired (every 5 min for a total of 20 min) until the peak light emission was confirmed. Gray scale and bioluminescence images were superimposed using the Living Image software (Xenogen Corp., CA). A constant size region of interest (ROI) was drawn over the scaffold implantation site and at another site on top of the animal as a background. The signal intensity was reported as

an integrated light flux (photons/s) subtracting background, which was determined by IGOR software (WaveMetrics, OR).

2.7. Histological analysis and immunohistochemistry

Histological analysis was performed to determine the cellular distribution and identity of transfected cells. Scaffolds loaded with 400 µg of GFP plasmid were retrieved 3 days post-implantation and frozen in an isopentane bath cooled over dry ice to -50°C . 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 3 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 along with GFP antibodies 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.

2.8 Statistical analysis

Statistical analyses were done using statistical package JMP (SAS, Cary, NC). A t-test was performed to analyze differences between individual pairs with a p-level of 0.05. Error bars represent standard deviations in all figures.

3. Results

3.1 Particle characterization

PLG microspheres were surface modified in order to provide for non-specific interactions with naked plasmid. In the first approach, microspheres were modified by incubation with a cationic polymer solution. The polymers PLL, PEI, PAH, and PDDA were used, since these have been used as DNA complex agents and in layer by layer assemblies [5,6,10–17]. In a second approach, the microspheres were also incubated in a solution of dopamine, which formed a PD layer around the microspheres, as observed by a color change from white to brown [23]. Zeta potential (Fig 1A) measurements indicated that the unmodified microspheres have a negative potential of -49.6 mV, and all surface modification procedures increase the zeta potential to positive values, indicating surface modification of the microspheres. The PD and PLL coatings of the microspheres produced zeta potentials that were slightly positive, and were $+0.31$ mV and $+4.25$ mV respectively. Substantially greater values for zeta potential were obtained for PAH, PDDA, and PEI, in which the zeta potential was equal to $+42.6$, $+58.9$, and $+46.1$ mV, respectively. No significant changes in particle size (Fig 1B) were observed with any of surface treatments, which indicate that the microspheres do not degrade or aggregate by the polymer treatment.

3.2 DNA Release Kinetics

We subsequently investigated the incorporation efficiency and release kinetics for plasmid that was encapsulated within layered scaffolds. Microspheres that were surface modified were combined with plasmid and lactose, lyophilized overnight, and used to form the middle layer of PLG scaffolds. Unexpectedly, the scaffolds containing the modified microspheres did not

have a significant change in DNA incorporation compared to unmodified scaffolds, suggesting that the interaction between the plasmid and polymer is not the limiting factor controlling encapsulation. With the exception of PAH, the incorporation efficiency was approximately 45% for all conditions, and did not significantly vary with the surface modification (Fig 2A). For PAH, the incorporation efficiency was approximately 33%. The release profile of plasmid from the scaffolds varied substantially with the surface modification (Fig 2B). Scaffolds with unmodified microspheres were characterized by an initial burst of plasmid (53.8% at 1 hour, 83.4% at 1 day, (Table 1)) and a maximal release at 14 days that was equal to 95.6% of the amount initially incorporated. Scaffolds modified with PEI, PLL, and PDDA also had an initial burst of plasmid exceeding 30.3% at 1 hour, and 50.3% by 1 day (Table 1). Maximal release at 14 days exceeded 74.9%. Scaffolds modified with PAH and PD had a smaller initial burst, compared to the other cases, with a plasmid release within the first hour of approximately 10%. However, PAH did not release a substantial amount of plasmid after this point, with only 14.1% released within 24 hours, and 16.1% at 14 days. PD modified scaffolds released 27.5% after 24 hours and 77.6% of the incorporated DNA at 14 days. In terms of release profiles, PD coatings produced a low initial burst of DNA (10.2 %) compared to the other polymers, and had a greater release (%/day) at subsequent time points between 1 and 14 days as shown in Table 1. PD was selected for further studies based on the release profile.

The incubation time of microspheres with dopamine was subsequently evaluated for its impact on DNA incorporation and release. Increasing the incubation time of microspheres with dopamine had an increase in the DNA incorporation efficiency (Fig 3A), with an increase from 43.2% for unmodified microspheres to 47.3%, 59.3%, and 69.9% for microspheres incubated in dopamine for 2, 8, and 24 hours, respectively. The extent of PD coating had no significant effect on DNA release; however, on average, the initial burst is reduced, with a slower release at later time points, and more DNA available for release with increasing incubation time. Microspheres incubated in dopamine for 24 hours were used in subsequent studies as they had the highest DNA incorporation (69.9%), with a slower release of DNA compared to the other cases.

3.3 Transgene expression

We subsequently investigated whether PD would impact cellular processes and thus gene transfer. Cell culture wells were modified with PD, seeded with HEK 293T cells, and then transfected by lipoplexes added to the culture media. Transgene expression did not significantly differ between the unmodified and PD modified surfaces (Fig 4). An increase in DNA dose from 0.1 μ g to 0.5 μ g significantly increased transgene expression, and further increases in DNA dose to 1 μ g did not increase expression.

The relationship between release rate and transgene expression was investigated using the layered scaffolds with and without PD modification. For the PD condition, microspheres incubated in dopamine for 24 hours were used, which had provided the greatest incorporation efficiency and slowest release. Scaffolds were loaded with 400 μ g of pLuc with a CMV promoter and implanted into the peritoneal fat pad. The greatest transgene expression was observed at 3 days post-implantation, and expression declined rapidly between days 7 and 14, with low levels quantified through 3 weeks of implantation (Fig 5A). Transgene expression did not vary significantly between PD modified and unmodified layered scaffolds (Fig 5A) at any time point. These results indicate that the extent and duration of transgene expression is not a function of release rate at this implantation site.

A control study was performed to identify the extent of transgene expression that would be obtained with an initial release of plasmid. The luciferase encoding plasmid (100 μ g) was dried onto PD and unmodified scaffolds without a central layer, from which a release study indicates that greater than 96% of this plasmid is released within 24 hours (data not shown). Transgene

expression at 3 and 7 days did not vary significantly between the encapsulated plasmid and the dried plasmid for both PD modified (Fig 5B) and unmodified scaffolds (not shown). These results indicate that the plasmid released within the initial 24 hours can account for the extent and duration of transgene expression.

An additional control study was performed to investigate whether the DNA released from the scaffold after 24 hours is capable of inducing transgene expression *in vivo*. Plasmid was encapsulated within PD and unmodified layered scaffolds and then incubated in PBS for 24 hours post leaching, leaving approximately 74.9% and 16.6% of the encapsulated DNA respectively (Table 1). Transgene expression was measured 3 days post-implantation, with no significant differences in transgene expression observed for both PD and unmodified scaffolds. These results indicate that the quantity of DNA remaining in the scaffold after the initial 24 hours is active and able to promote transgene expression.

The design of the plasmid was subsequently investigated by varying the promoter within the vector. Unmodified layered scaffolds were loaded with a pLuc plasmid with either a CMV or UbC promoters and implanted. Transfection was monitored over time using a bioluminescence system. Mice that received scaffolds loaded with the pLuc plasmid containing a CMV promoter had substantial transgene expression at day 3, with expression decreasing over time (Fig. 6), consistent with previous observations (Fig. 5). At 14 days, expression decreased to background levels. Mice with scaffolds containing the pLuc plasmid with an UbC promoter had comparable expression as those with a CMV promoter at day 3. However, transgene expression increased from day 3 to day 7, and was sustained throughout the duration of the study (21 days) (Fig. 6). These results suggest that expression is not limited by delivery of the plasmid, and the short-term expression observed with the CMV plasmid likely results from silencing or clearance of the plasmid.

3.4 Histological analysis and immunohistochemistry

PD modified and unmodified scaffolds implanted with 400 µg of GFP encoding plasmid were retrieved at 3 days post-implantation, which is the time of greatest expression. The scaffolds maintained structural integrity, with some cell infiltration within the porous structure of the scaffold at 3 days. Immunohistochemical staining was performed to determine the localization, distribution, and identity of the transfected cells, using an antibody directed against GFP (transgene product) and F4/80 (macrophage surface marker).

For both PD and unmodified (not shown) scaffolds, GFP positive staining (green) was typically localized in the tissue immediately adjacent to the scaffold, with some positive staining within the scaffold (Figs. 7A and 7B). Co-localization of both GFP and the nuclei staining (Hoechst, blue) indicates some cell infiltration within the scaffold by 3 days, and that GFP positive cells are predominately seen on the periphery of the scaffold (Figs. 7A, and 7B). Most of the cells stained positive for GFP also stained positive for the macrophage marker F4/80 (red) (Figs. 7C and 7D). Previous reports have identified macrophages as the predominant cell type transfected at the IP fat site [9, 24].

4. Discussion

In this report, we investigated the relationship between the release of plasmid from the scaffold and gene expression *in vivo*. The release rate of plasmid was controlled by coating of microspheres with PD prior to fabrication of the scaffold using the gas foaming/particular leach method. Dopamine has a catechol and an amine group, and under alkaline conditions spontaneously forms a PD coating [23]. PD forms as a layer on surfaces, with the thickness of the layer dependent on the incubation time. A layer with a thickness between 10 to 20 nm forms within 2 hours, and plateaus at a thickness of approximately 50 nm within 24 hours [23]. The

PD coating increased incorporation and also slowed release of plasmid from the scaffold. PD coated surfaces did not capture plasmid from solution (data not shown), suggesting that the coating does not directly bind plasmid. Thus, the increased incorporation and slower release likely results from PD providing an additional diffusion barrier that limits release.

Sustained release has been hypothesized to promote gene transfer by maintaining elevated concentrations locally through replacement of DNA that is lost to degradation or clearance from the tissue. Maintaining these elevated concentrations can extend the opportunities for cell internalization, which may be important to counteract intracellular silencing or degradation of the plasmid. Biomaterials have been employed to achieve sustained plasmid release. Numerous biomaterials have promoted prolonged gene expression that persists for weeks to months, though expression is dependent on numerous parameters such as implantation site, the dose, and the vector [1–4,7,9,20,24–34]. Expression within muscle tissue and subcutaneous tissue tends to be more prolonged than at other implantation sites [3,4,20,28]. Though expression can persist for long times, the expression levels are either declining or steady with time. However, microspheres injected intramuscularly demonstrated increasing expression levels with time [4]. The complexities of biomaterials, implantation sites, and vectors complicated the direct testing of the hypothesis relating release rate to transgene expression.

For scaffolds implanted into the peritoneal fat, transgene expression was not a function of the release profile, despite the observation that DNA released at later times was bioactive. Expression was greatest at 3 days, and then decreased through 22 days post-implantation. Scaffolds that released most of their DNA within 24 hours in vitro induced transgene expression in vivo that was similar to scaffolds that released DNA over the course of 14 days. Importantly, unmodified and PD-modified scaffolds were incubated in vitro for an additional 24 hours in order to reduce the initial burst and determine whether the DNA released after 24 hours could induce transgene expression in vivo. Scaffolds with this 24 hr incubation produced transgene expression similar to scaffolds without the extra incubation, indicating that the quantity and integrity of DNA released at later times is active. These studies also have implications on the quantity of DNA released. Unmodified scaffolds had only 15% of their DNA remaining after the 24 hour incubation, yet were able to induce similar levels of expression. Taken together, these studies suggest that the DNA released initially is a critical determinant of transfection.

Delivery of plasmids with different promoters suggest that the locally released plasmid remains intracellularly for extended times. The CMV and other promoters of viral origin are known for inducing an initial robust expression; however, expression can substantially decrease over time due to silencing of the promoter [35]. Using the ubiquitin promoter, transgene expression obtained at 21 days was comparable to the levels observed initially. Similar prolonged expression with the ubiquitin promoter has been obtained relative to other promoters of viral origin [36–38]. CpG motifs on the plasmid may also contribute to silencing of expression with the CMV promoter [39].

Although these studies did not demonstrate an impact of sustained release on expression at the peritoneal fat site, they motivate the need for improved delivery systems for regenerative medicine. If the initial release is responsible for a majority of the gene expression, then gene delivery is likely targeting the cell types responding to the implantation procedure. Indeed, immunohistochemical staining previously demonstrated that the primary cell type transfected for scaffolds implanted into the peritoneal fat was macrophages [9,24]. Transfected cells were observed within the scaffold, though it is unclear if the cells were transfected in the surrounding tissue and migrated into the scaffold, or whether they were transfected within the scaffold due to a limited clearance of DNA from the implant site. To target gene delivery to other cell types, such as tissue progenitor cells, the delivery systems will need to retain the DNA until these cells arrive and populate the scaffold. The delivery of lipoplexes or polyplexes can alter the

release relative to plasmid [28], and can be modified with ligands with the potential to target specific cell types.

5. Conclusions

In this report, we investigated the relationship between the release profile of plasmid from scaffolds and transgene expression *in vivo*. PD modification of the scaffolds increased plasmid incorporation and provided a sustained release throughout 14 days. However, for peritoneal implantation of scaffolds, this sustained release of plasmid did not impact transgene expression relative to a rapid release, despite the observation that plasmid released at later times is bioactive. Changing the plasmid design from a CMV promoter to an ubiquitin C promoter to avoid silencing of expression enabled long term expression, indicating that the plasmid remains available and active within the cells or tissue. Thus, these results suggest that the initial amount of plasmid released, and the design of the plasmid influences the extent of transgene expression. Although sustained release did not extend transgene expression in the peritoneal fat, sustained release may function differently at other implant sites or has the potential to alter the identity and distribution of transfected cells, particularly if combined with packaging or targeting strategies. These results demonstrate the potential for scaffolds to induce transgene expression for at least 3 weeks, which would have numerous applications in regenerative medicine.

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Figure 1. Microsphere characterization. A) Zeta potential (mV) and B) particle size (μm) of the modified microspheres. (a statistical significance at $P < 0.01$ relative to unmodified, b statistical significance at $P < 0.01$ relative to PD and PLL.)

Figure 2. Plasmid incorporation (A) release (B) from scaffolds formed with microspheres modified with a range of polymers. The microspheres were modified by incubation in a polymer solution for 2 hours. Scaffolds were loaded with 10 μ g of 32 P labeled DNA (n=3).

Figure 3.

Plasmid incorporation (A) and release (B) from scaffolds with microspheres modified with PD. The microspheres were modified by incubation in a dopamine solution for 0, 2, 8 or 24 hours. Scaffolds were loaded with 10 μg of ^{32}P labeled DNA ($n=3$). (* statistical significance at $P < 0.05$ relative to 0 and 2 hour incubation time)

Figure 4.

Transgene expression by cells cultured on PD coated wells. Lipoplexes were added to HEK 293T (10,000 cells) seeded into and cultured on PD coated or uncoated wells. Luciferase expression was measured after 48 hours of seeding (n=3).

Figure 5.

In vivo transgene expression. A) Unmodified and PD modified scaffolds with plasmid loaded in the central layer were initially loaded with 400 μ g of luciferase encoding plasmid and implanted in the IP fat of mice. Scaffolds were retrieved 3, 7, 14, and 22 days post implantation. B) Comparison between PD modified layered scaffold (same as A) and a PD modified scaffold (i.e., no central layer) with 100 μ g of luciferase encoding plasmid dried overnight. Scaffolds were retrieved 3 and 7 days post implantation. C) Layered scaffolds (as in A) were implanted after leaching or following an additional 24-hour incubation. Scaffolds were retrieved 3 days post implantation. (n=4 at each time point). (* statistical significance at $P < 0.05$ relative to 14 and 22 days)

Figure 6.

In vivo transgene expression. Unmodified layered scaffolds were loaded with 400 μ g of a luciferase encoding plasmid under a CMV promoter (n=4) or an UbC promoter (n=5). Transgene expression was measured using an IVIS bioluminescence system.

Figure 7.

Distribution and identity of transfected cells. PD modified scaffold loaded with 400 μ g of GFP plasmid were retrieved 3 days post implantation, cryosectioned, and analyzed by immunohistochemistry. Images (100X) were captured at the interface of the scaffold with surrounding tissue (scale bars equal 100 μ m). Images were captured for (A) GFP antibody staining, (B) GFP antibody staining along with Hoechst staining for cell nuclei, (C) F4/80 (macrophage) antibody staining with Hoechst, and (D) overlay of F4/80 (macrophage) and GFP antibody staining. “S” indicates scaffold region, “T” indicates tissue, and dashed line indicates interface.

Table 1
Summary of DNA release from layered scaffolds. Data presented in terms of mean values.

Polymer	Incubation Time (h)	DNA Encapsulated (%)	Cumulative DNA Released at 14 days (%)	Initial Burst ^a (%)	Released at 1 day (%)	Release Rate (% per day) ^b		
						1–3 days	3–7 days	7–14 days
N/A	0	43.2	95.9	53.8	83.4	3.1	0.8	0.5
PEI	2	47.3	74.9	30.3	50.3	4.7	1.7	1.2
PLL	2	50.2	85.0	32.9	57.3	5.4	2.0	1.3
PAH	2	33.3	16.1	13.6	14.1	0.2	0.1	0.1
PDDA	2	50.1	91.0	45.2	70.4	4.2	1.5	0.9
PD	2	47.3	77.6	10.2	27.5	9.6	4.7	1.7
PD	8	59.2	67.2	12.2	28.7	6.7	3.3	1.7
PD	24	69.9	64.4	7.8	25.1	7.1	3.2	1.8

^aInitial burst is defined as the total DNA released in the first 4 hours divided by the total DNA incorporated.

^b% per day is defined as the total DNA released between specific dates divided by the total DNA incorporated and the number of days in the interval.