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Sustained transgene expression via citric acid-based polyester elastomers

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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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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 2.04×10^9 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) (8.96×10^5 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 (7.5×10^5 cells/scaffold) or PASMC (4.6×10^5 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 (7.5×10^5 cells/scaffold) or PASMC (4.6×10^5 cells/scaffold) was seeded to each scaffold and allowed to incubate for 3 h to allow cell