Sustained viral gene delivery from a micro-fibrous, elastomeric cardiac patch to the ischemic rat heart

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Abstract

Biodegradable and elastomeric patches have been applied to the surface of infarcted hearts as temporary mechanical supports to effectively alter adverse left ventricular remodeling processes. In this report, recombinant adeno-associated virus (AAV), known for its persistent transgene expression and low pathogenicity, was incorporated into elastomeric polyester urethane urea (PEUU) and polyester ether urethane urea (PEEUU) and processed by electrospinning into two formats (solid fibers and core-sheath fibers) designed to influence the controlled release behavior. The extended release of AAV encoding green fluorescent protein (GFP) was assessed *in vitro*. Sustained and localized viral particle delivery was achieved over 2 months *in vitro*. The biodegradable cardiac patches with or without AAV-GFP were implanted over rat left ventricular lesions three days following myocardial infarction to evaluate the transduction effect of released viral vectors. AAV particles were directly injected into the infarcted hearts as a control. Cardiac function and remodeling were significantly improved for 12 weeks after patch implantation compared to AAV injection. More GFP genes were expressed in the AAV patch group than AAV injection group, with both α -SMA positive cells and cardiac troponin T positive cells transduced in the patch group. Overall, the extended release behavior, prolonged transgene expression, and elastomeric mechanical properties make the AAV-loaded scaffold an attractive option for cardiac tissue engineering where both gene delivery and appropriate mechanical support are desired.

Keywords: Gene delivery; AAV; Cardiac patch; Electrospun scaffold ; Myocardial infarction

1. Introduction

The loss of functional myocardium following myocardial infarction (MI) reduces cardiac systolic capacity concurrent with cardiac wall thinning, increased left ventricular (LV) chamber sphericity and dilatation to compensate for decreased pumping efficiency. Looking at this complex remodeling process from a mechanical perspective, a feedback loop of wall thinning leading to higher LV wall stress, and this leading to further remodeling toward thinning, is implicated in propelling the infarcted heart down a pathway towards end-stage ischemic cardiomyopathy [1]. To interrupt this process investigators have looked at biomaterial- and device-based approaches to mechanically support the dilating heart with and without coordinated pharmacologic interventions [2-5]. Cell therapy approaches, including those incorporating a mechanical component, have also been investigated extensively [6-8]. Another localized approach has been to pursue gene therapy for the damaged and at-risk cardiac tissue. Although preclinical studies have been promising, clinical gene therapy trials to date have shown mixed results, with limited efficacy attributed to low gene transduction efficiencies, insufficient gene expression levels, and the presence of neutralizing antibodies against viral vectors [9]. Yet gene therapy remains a potentially attractive therapeutic modality if the right combination of efficacy, safety and ease of application can be achieved, and translational studies for cardiac failure are ongoing [10] .

Several strategies for cardiac gene therapy have been reported. Among them, increasing vascular density has been frequently pursued to provide pseudo-physiological blood flow to ischemic myocardium through local angiogenesis, induced by genes encoding growth factors such as vascular endothelial growth factor (VEGF) [11], hepatocyte growth factor (HGF)[12, 13], and fibroblast growth factor (FGF) [14]. More recently, building off hypothesized stem cell mechanisms for cardiac repair and increased vascularization, stromal cell-derived factor 1 (SDF-1) transfection has been pursued clinically [15]. Other therapeutic strategies include improving

calcium handling by augmenting expression and activity of myocardial sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (SERCA2a), phospholamban, S100A1, and parvalbumin [16]. In addition, modulation of β -adrenergic receptor signaling by inhibiting G protein-coupled receptor kinase 2 or increasing adenylyl cyclase VI expression has been reported [17]. A more recent strategy for gene therapy has sought to improve cardiac function by increasing cardiomyocyte numbers derived from the conversion of local non-cardiomyocytes (e.g. fibroblasts). Central to this approach is the finding that cardiac fibroblasts can be reprogrammed into cardiomyocyte-like cells both *in vitro* and *in vivo* by the addition of defined transcription factors [18-20] or a combination of microRNAs [21].

Gene delivery vehicles can be separated into nonviral and viral vectors. Nonviral vectors consist of plasmid DNA or small nucleic acids that are delivered to target tissues either directly in their naked form or using some physical or chemical packaging (lipid particles or nanoparticles) to facilitate delivery. A principle advantage with this approach is low immunogenicity and high loading capacity for genetic material. Disadvantages are low levels of transgene expression due to inefficient delivery and transient gene expression [22]. Among the viral vehicles, recombinant adeno-associated viral (AAV) vectors have emerged as the most promising gene delivery vehicles today, with less immunogenicity than other viral vectors, an ability to infect quiescent and dividing cells and the potential to sustain gene expression over extended periods of time [23, 24]. Moreover, AAV can transduce a wide variety of tissues and cells *in vivo* due to the existence of different serotypes of the virus. To date, AAV-based therapeutic strategies have been tested in over 162 clinical trials worldwide [25].

Building upon the current status of mechanical and gene therapy interventions for ischemic cardiomyopathy, the objective of this study was to combine these functionalities in the form of an elastic epicardial patch with the controlled release of AAV. Previous reports have shown positive

effects on cardiac function when a biodegradable elastomeric cardiac patch has been applied to the epicardial surface as a temporary mechanical support following MI in rat and porcine models [3, 26-28]. Here, micro-fibrous, biodegradable polyurethane elastomeric scaffolds were electrospun from a single stream of polymer and AAV vectors mixed together, or from a coaxial stream with polyurethane as the outer layer and polyethylene glycol mixed with AAV as the inner layer. AAV encoding the reporter gene, green fluorescent protein (GFP) was utilized to evaluate the transduction effect from these materials. After evaluating several design options for AAV delivery, including single or coaxial stream and two types of biodegradable polyurethane, the most effective patch design was placed on rat infarcts three days post-MI. The animals were followed for 12 weeks with echocardiographic function, and at study termination ventricular histology and AAV transduction efficiency were assessed.

2. Experimental Section

2.1. Polymer synthesis and characterization

Polycaprolactone diol (PCL, $M_n=2000$), poly(ethylene glycol) (PEG, $M_n=2,000$), poly(ethylene oxide) PEO ($M_v \sim 900,000$), 1,4-diisocyanatobutane (BDI), putrescine, and tin (II) 2-ethylhexanoate were purchased from Sigma-Aldrich. 1,1,1,6,6,6-hexafluoroisopropanol (HFIP) was purchased from Oakwood Inc. Polyester urethane urea (PEUU) and polyester ether urethane urea (PEEUU) were synthesized from polycaprolactone diol, butyl diisocyanate and putrescine using a 2-step one-pot synthetic strategy [29]. The stoichiometry of the reaction was 2:1:1 of BDI:(PCL + PEG): putrescine. PCL₁₀₀ PEUU designates polyurethane urea with only PCL as the soft segment. PCL₇₅-PEG₂₅ PEEUU designates polyurethane urea consisting of PCL and PEG blocks with a feed molar ratio of 75:25.

^1H NMR spectra of the samples were collected using a Bruker 300 MHz spectrometer with samples dissolved in DMSO- d_6 solution. Thermal properties were measured by differential scanning calorimetry (DSC, DSC-60, Shimadzu). All samples were heated to 200°C to erase thermal history, then cooled to -150°C, and heated again to 200°C. All heating and cooling rates were 10°C /min. Values reported for glass transition temperatures (T_g), melting temperatures (T_m), and enthalpy of melting (ΔH_m) were taken from the second heating trace. To characterize water absorption of the polymers, solvent cast films were immersed in water at room temperature for 4 h. Water uptake was quantified using the following equation: water uptake (%) = $(m_w - m_d)/m_d \times 100\%$, where m_d and m_w were the mass of the dry and the wet samples, respectively.

2.2. Scaffold fabrication by electrospinning

AAV encoding for GFP gene with a cytomegalovirus (CMV) promoter was manufactured using CsCl gradient ultracentrifugation [30]. AAV serotype 2 (AAV2-cmv-GFP, 5×10^{12} GC/mL) was used for *in vitro* studies, and AAV serotype 9 (AAV9-cmv-GFP, 2.5×10^{12} GC/mL) was used for *in vivo* testing. Viral particles and polyurethanes were combined and electrospun into micro-fibrous mats with 1 of 2 architectures: solid fibrous scaffolds, and core-sheath fibrous scaffolds (**Fig. S1**).

Scaffolds comprised of solid fibers were made by single stream electrospinning, i.e. by dispersing AAV in polyurethane solution (12 w/v % in HFIP) and feeding this solution through a charged capillary at a rate of 1.5 mL/h. Scaffolds with core-sheath fibers were made by co-axial electrospinning where AAV was mixed in 1% PEO in PBS as the core feed solution and PEEUU solution (12 w/v% in 70:30 ratio of DCM:HFIP) as the feed for the sheath. Flow rates for core and sheath solutions were set at 0.6 and 1.5 mL/h, respectively. Electrospun fibers were collected onto a rotating and translating stainless steel mandrel located 17 cm from the tip of the discharging capillary. The voltage between the capillary and mandrel was 19 kV. The electrospun mat was cut into individual samples of 1 cm $\times$ 1 cm $\times$ 100 μm ($\sim 10^{11}$ GC/sample) for *in vitro* characterization.

To verify that the AAV had been efficiently encapsulated in the core-sheath fibers, AAV was conjugated with amine-reactive cyanine 3 (Cy3 dye, GE Healthcare Biosciences) [31]. Unconjugated dye molecules were separated from labeled viral particles using a slide-a-lyzer (10,000 molecular weight) dialysis cassette. The viral particles were then eluted with PBS, and used as part of the core feed stream for coaxial electrospinning as described above.

2.3. Scaffold characterization

2.3.1. Microstructure

The surface and cross sectional morphology of electrospun scaffolds were observed by scanning electron microscopy (SEM; JSM-6330F, JEOL USA). Samples were freeze fractured in liquid nitrogen for cross section imaging. All samples were sputter-coated with gold/palladium prior to SEM analysis.

2.3.2. Tensile tests

For tensile testing, the scaffolds were first cut into a dumbbell geometry (ASTM D1708) using a custom made dogbone cutting die, with width of 2.5 mm, gauge length of 10 mm, and total length of 20 mm. The ultimate tensile properties of scaffolds in the wet state were evaluated by uniaxial tensile testing employing an MTS Insight with a 10 N (0.01 N resolution) load cell at room temperature. The samples were immersed in PBS for 1 h and then extensionally deformed at 10 mm/min, according to ASTM D638M. Young's modulus was calculated by finding the initial slope of the stress versus strain curve ($0 < \epsilon < 10\%$) using linear regression. The ultimate stress was determined as the maximum stress. This experiment was replicated four times for each sample type.

The evolution of tensile mechanical properties during degradation was also measured as above. For the degradation period each sample was placed into an individual vial (20 mL) containing 15

mL of PBS and agitated at 75 rpm in an incubator shaker at 37°C. The buffer was changed every week. At predetermined degradation time points, three samples were retrieved and tested.

2.4. AAV release *in vitro*

2.4.1. Cell transduction

HEK 293 cells (passage 34-38) were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). A monolayer of 10^4 cells were cultured at the bottom of 12-well plates. AAV-loaded scaffolds (n=3) were placed on a transwell membrane (40 μ m pore size, Fisherband), which was used to prevent direct contact of the scaffolds with the bottom cell monolayer and cell detachment. At predetermined time points, GFP-positive cells were imaged using fluorescent microscopy. Then cells were trypsinized, centrifuged, and resuspended in 1 mL PBS and the percentages of GFP-expressing cells was quantified by counting 10,000 cells in each measurement using flow cytometry (FACScan, Becton Dickinson). The AAV-containing scaffolds were then transferred to new wells with a cell monolayer pre-cultured at the bottom. Cell culture medium was changed twice per week.

In another cell transduction study, the virus-containing scaffolds were used to infect cells seeded on the scaffolds. Scaffolds (n=3) were incubated in medium and removed for cell seeding at predetermined time points. A suspension of 10^4 cells in 1 mL cell culture medium was seeded onto the scaffold and incubated for 4 h to allow cell attachment. The scaffolds were then transferred into new wells and cultured for 7 days. GFP-positive cells were imaged using confocal microscopy. The cell transduction efficiency was analyzed using flow cytometry after trypsinization of cells from the scaffold, centrifugation, and resuspension in 1 mL PBS. A scheme of these cell culture procedures can be found in **Fig. S2**.

2.4.2. DNA quantification

The release kinetics for AAV were directly studied by quantifying virus particles in the supernatant. AAV-encapsulated scaffolds (n=3) were incubated in 1 mL of medium at 37°C. Supernatant was removed and replenished at predetermined time points. AAV DNA was extracted using a Wako DNA Extractor SP kit (Wako Chemicals, Richmond, VA) and quantified by RT-PCR. Specific DNA PCR primers for GFP were custom made by Invitrogen (GFP forward: 5'CTTCTTCAAGTCCGCCATGC3' GFP reverse: 5'GTCGCCCTCGAACTTCA3'). AAV DNA was mixed with the primers and iQ SYBR Green Supermix (2X, BioRad, Hercules, CA), and the reactions were monitored using an iQ5 Real-Time Detection System (BioRad). The percentage of virus release over time was estimated by dividing the amount of virus release determined by RT-PCR over the calculated initial quantity of virus loaded into each scaffold.

2.5. Left ventricular infarction model

Adult female Lewis rats (Harlan Sprague Dawley, Inc.) 10-12 wk old, weighing 180-220 were used for this study (n=5 rats per group). The research protocol followed National Institutes of Health (NIH) guidelines for animal care and was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Rats were anesthetized by 3.0% isoflurane inhalation with 100% oxygen. After anesthesia, each rat was intubated and mechanical ventilation (683 Ventilator, Harvard Apparatus, Holliston, MA) was started with a total volume of 2 mL and 60-70 breaths/min. Rats were placed on a warming pad in the supine position, and the chest was shaved and sterilized with povidone-iodine solution. The heart was exposed through 4th left thoracotomy, and the proximal left anterior descending artery was ligated with 5-0 polypropylene. Myocardial infarction was confirmed by decreasing wall motion of the left heart, then the incision was closed. 10 mg/kg of lidocaine was injected intramuscularly to mitigate fatal arrhythmias before and after coronary artery ligation. For postoperative analgesic treatment, 0.1 mg/kg of buprenorphine was injected twice daily for 3 days after surgery. For prophylaxis of surgical site infection, 100 mg/kg of cefazolin was injected twice daily for 3 days post-procedure.

2.6. AAV injection and patch implantation

Cardiac patches were obtained using a 6 mm diameter surgical punch to cut electrospun PEEUU core-sheath fibrous scaffolds with or without AAV. For the former, individual patches contained $\sim 5 \times 10^{10}$ GC. Three days after myocardial infarction, animals were anesthetized and screened by echocardiography to measure the percentage of infarcted area. Animals with infarctions greater than 25% of the left ventricular free wall were utilized for this study and were randomly divided into 3 groups: patch implantation (PEEUU core-sheath fibers without AAV), patch with AAV implantation, and AAV injection. Under mechanical ventilation, the heart was exposed through 5th left thoracotomy. The infarcted area was easily detected visually by color and motion. The surface of the infarcted region was lightly abraded before patch implantation to provide a direct contact of the patch with the myocardial area. The patch was implanted using 7-0 polypropylene with over-and-over peripheral continuous sutures to cover the infarcted area. For the direct AAV injection group, 100 μ L of AAV (5×10^{10} GC) was injected into the infarcted wall using a 27G needle and three spaced injections into the infarcted area. After patch implantation or injection, the incision was closed.

2.7. Echocardiography

Echocardiography was performed before myocardial infarction, as well as 3 days after infarction (before patch implantation), and 1, 2, 4, 8 and 12 wk after patch implantation. Animals were anesthetized with 1.5% isoflurane inhalation with 100% oxygen and standard transthoracic echocardiography was performed using an Acuson Sequoia C256 system with 13-MHz liner ultrasonic transducer (15L8; Acuson Corporation, Mountain View, CA) in a phased array format. A left ventricular short axis view was evaluated at the papillary muscle level. The end diastolic area (EDA), end systolic area (ESA), end diastolic volume (EDV), and end systolic volume (ESV) for the left ventricle were measured. The fractional area change (FAC%) was estimated as FAC%

= (EDA-ESA)/EDA x 100%. The ejection fraction (EF%) was calculated as EF% = (EDV-ESV)/EDV x 100%.

2.8. Histology and immunohistochemistry

Whole heart explants were harvested 12 weeks following patch implantation, and other organs (kidney, lung, liver and skeletal muscles) were harvested 4 weeks following patch implantation. Tissues were fixed in 4% paraformaldehyde for three days at 4°C, embedded with paraffin and sectioned at 8 µm thickness. Then the thin sections were mounted on glass slides and stained with Masson's trichrome. For the LV wall thickness measurement, photographs were taken of transected hearts at the time of explant. The wall thickness of the infarcted anterior wall (with patch implantation) for patched and infarction control groups and the normal wall thickness for healthy rats was analyzed using ImageJ software (n=5 rats/group).

For immunohistochemistry assessment, explants (n=5/group) were fixed in 4% paraformaldehyde for three days at 4°C, dehydrated in 30% sucrose, embedded with OCT compound (Tissue-Tek, Torrance, CA) and frozen at -80 °C until sectioning at 8 µm in the LV transverse direction. Sections were mounted on glass slides and blocked with 5% goat serum in PBS solution for 1 h at room temperature. To quantify GFP expression, sections were immunolabeled with rabbit polyclonal antibodies against GFP (ab290; 1:500, Abcam) followed by a goat anti-rabbit secondary antibody Alexa Fluor[®] 488 (A-11008; 1:1000, Thermo Fisher Scientific). Sections were also stained with mouse primary antibody against α -SMA (ab7817, 1:250, Abcam), with secondary antibodies of Alexa Fluor[®] goat anti-mouse 594 (A-11005; 1:1000, Thermo Fisher Scientific). Vessels were identified as tubular structures positively stained for anti α -SMA. To define rat cardiomyocytes, sections were stained with rabbit anti-cardiac troponin T antibody (cTnT, ab45932, 1:400, Abcam), with secondary antibodies of Alexa Fluor[®] goat anti-rabbit 594 (A-11015; 1:1000, Thermo Fisher Scientific). To quantify macrophage infiltration, sections were stained with rabbit primary antibody against CD68 (ab125212; 1:500, Abcam) as a pan-

macrophage marker, followed by goat anti-rabbit secondary antibody Alexa Fluor® 488 (A-1108; 1:1500, Thermo Fisher Scientific). In addition, a mouse primary antibody against CD163 (sc-58965; 1:100, Santa Cruz) was utilized as a polarized M2 macrophage phenotype marker with the secondary anti-mouse antibody Alexa Fluor® 594 (A-11032; 1:200, Invitrogen). Finally, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; 1:10000, Sigma). For each sample, 5 different microscopic fields at 200x magnification were photographed for GFP, α -SMA, cTnT, CD68, and CD163 positive structures. Microscopic images were taken under fluorescence microscopy and assessed with ImageJ. Values are reported as the area (pixels) per 200x magnification of high powered field (HPF) for GFP-positive structures.

2.9. Statistical Analyses

All results are presented as mean $\pm$ standard deviation. The data were analyzed by one-way ANOVA with Student-Newman-Keuls' test applied for specific comparisons. $P < 0.05$ was considered to represent a significant difference.

3. Results

3.1. Polymer and scaffold characterization

To tailor the release kinetics of the encapsulated viral vectors, two polyurethane materials and two electroprocessing techniques were employed. Successful synthesis of PEUU and PEEUU with PCL and PEG soft segments was confirmed by ^1H NMR spectra. The molar ratio of PCL/PEG was quantified by comparing the integration value of proton signal of PCL at $\delta = 2.32$ ppm ($-(\text{C}=\text{O})-(\text{CH}_2)_4-\text{CH}_2-\text{O}-$) to that of PEG at $\delta = 3.65$ ppm ($-\text{CH}_2-\text{CH}_2-\text{O}-$). The PCL/PEG feed ratio was close to what was observed in the resulting product (**Table 1**). PEUU and PEEUU showed glass transition temperatures (T_g) of -57°C and -62°C , and melting temperatures (T_m) of the soft

segment crystals at 40°C and 31°C, respectively. Introducing 25% PEG blocks into the polyurethane backbone was associated with an increase in water uptake from 1.1% to 26.6%.

Core-shell fiber structures were successfully achieved via co-axial electrospinning. The freeze fractured solid fibers fabricated by single stream electrospinning showed an average diameter of 1-2 μm . PEUU core-sheath fibers had an average outer diameter ranging from 2–3 μm , with the core diameter of 1 μm . PEEUU core-sheath fibers had an average outer diameter of 15 μm , and core diameters of ~ 10 μm . A population of smaller, solid fibers with diameters of ~ 1 μm were present in the PEEUU core-sheath fibrous scaffolds (**Fig. 1a**). The pores size for PEEUU core-sheath fibrous scaffolds were 40 μm , while that of the other three scaffolds was less than 5 μm (**Fig. 1b**). As shown in **Fig. S3**, Cy3-labeled AAV was successfully encapsulated throughout the PEUU core-sheath fibers.

3.2. AAV release in vitro

3.2.1. HEK293 transduction by supernatant

After a 7-day incubation, close to 70% of cells were transduced by the supernatant from PEEUU core-sheath scaffolds, followed by a significant drop to $\sim 20\%$ transduction of fresh cells at week 2 (**Fig. 2**). PEUU core-sheath fibers infected about 30% of HEK293 cells at 7 days. The infection efficiency of the core-shell fibrous mats remained almost constant at 20% from weeks 2-8, after which a significant decrease was observed at week 11 ($p < 0.05$ vs. week 8 for both core-sheath types), where $\sim 2\%$ cells were transfected. The two solid fibrous scaffolds exhibited low levels of cell transduction ($< 10\%$) throughout the culture period.

3.2.2. HEK293 transduction when seeded on scaffolds

Seeded cells on both core-sheath fibrous scaffolds (**Fig. 3**) expressed a higher level of transgene expression for two months compared with cells transduced with AAV supernatant (**Fig. 2**). More than 80% of cells were transduced by PEEUU core-sheath fibrous scaffolds during the first week,

and then the transduction efficiencies gradually decreased to ~40%, 30% and 10% at week 2, 5, and 8, respectively. PEUU core-sheath fibrous scaffolds showed a similar trend. As observed in the above supernatant method, the two solid fibrous scaffolds showed a minimal level of transgene expression. All four scaffolds showed substantial cell proliferation with confluent cell layers achieved within one week.

3.2.3. AAV particle release in supernatant

AAV DNA quantification in the supernatant collected from incubation with the four scaffold types follows a similar trend with the cell transduction results presented above (**Fig. 4**). Over the examined period, a total of 60% and 40% of the AAV was released from the PEEUU and PEUU core-sheath fibrous scaffolds, respectively, while the total amount release in solid fibrous scaffolds was approximately 5%.

3.3. Mechanical properties of scaffolds

Representative tensile stress-strain curves for the wet polymer scaffolds are shown in **Fig. 5**, with average mechanical parameters summarized in **Table 2**. All 4 scaffolds demonstrated typical elastomeric behavior with Young's moduli of 1.0-1.6 MPa, tensile strengths of 1.3-7.9 MPa and breaking strains of 194-594%. The evolution of mechanical properties for samples degraded over time in PBS are presented in **Fig. S4**. The Young's moduli gradually decreased from 1.0-1.6 MPa to ~0.5 MPa at month 5 for all fiber types. For PEUU solid fibrous scaffolds, the ultimate stress decreased from 7.9 MPa to 3.4 MPa at 2 months and then to 0.8 MPa at month 5. For the rest of the scaffolds, the ultimate stress gradually decreased from ~1.5 MPa to ~0.8 MPa at month 5. Slight decreases in breaking strains were observed, with this parameter exceeding 150% at 5 months for all scaffold types.

3.4. Cardiac wall histology after infarction and intervention

The surface of the PEEUU patch material was macroscopically observed in situ to be covered with connective tissue with a vaguely delineated edge after the 12-week implant period. The patch was contiguous with the cardiac tissue and restored the LV wall thickness relative to hearts from the AAV injection group. The LV wall for the patch and AAV patch implantation groups were found to be significantly thicker than that in the infarction control group, but thinner than for healthy hearts. (**Fig. S5**)

An extensive cellular infiltrate with connective tissue occupied the implant area 12 wk after patch implantation as evidenced by Masson's trichrome staining. In the direct injection group, the infarcted ventricular wall was generally replaced with thin fibrous tissue, while in some areas residual myocardial tissue remained (**Fig. 6a**). In PEEUU scaffold groups, the majority of the PEEUU patch material appeared to have degraded and macrophages and fibroblasts penetrated into the remnant patch material (**Fig. 6b and 6c**).

3.5. EDA and %FAC by echocardiography

Echocardiographic assessment showed significantly lower EDA, higher %FAC and higher EF% in the PEEUU patch groups versus the AAV injection group (EDA and EF% from 8 wk onward, %FAC from 4 wk onward) (**Fig. 7**). There was no significant difference in each functional parameter between PEEUU patch group and PEEUU patch group containing AAV.

3.6. Immunohistochemistry

Immunohistochemical analysis revealed that individual GFP positive cardiomyocytes were present in the infarct border zone after AAV9-GFP particle injection into the ventricular wall. These cells contained striations characteristic of cardiomyocytes (**Fig. 8a**). A few α -SMA positive cells were visible in the AAV injection group. α -SMA positive muscle bundles were apparent underneath the remnant patch 3 months after patch application. Both GFP and α -SMA were expressed in the patch containing AAV particles, and GFP expression patterns colocalized with non-vascular α -

SMA positive cells (**Fig. 8b**). A few cardiomyocytes also expressed GFP in the infarct border zone underneath the patch (**Fig. 8a**). Quantitatively, the AAV patch group had significantly higher GFP positive cells (10900 ± 7400 pixels per HPF) compared to AAV injection group (1200 ± 1400 pixels per HPF), while the patch only group has no expression of GFP, as expected (16 ± 7 pixels per HPF) (**Fig. 8c**). After being injected into healthy rat hearts and recovering those hearts at 4 wk, AAV9-GFP provided robust expression in cardiomyocytes (54000 ± 9500 pixels per HPF), significantly larger than the other three groups (**Fig. S6**). The density of α -SMA positive vascular structures was similar between the patch-only group and the AAV patch group at 12 wk (**Fig. S7**). The macrophage response did not appear to be altered with the presence of AAV-cmv-GFP particles and the expressed GFP protein as well. No significant difference was found in the CD68 (pan-macrophage marker)-positive area, the CD163-positive (M2 macrophage marker) and the CD163/CD68 ratio for the patched walls with or without AAV (**Fig. S8**). At 4 weeks, no GFP expression was found in kidney, lung, liver, although sparse skeletal muscles cells, with the exception of sparse skeletal muscle cells transduction. (**Fig. S9**).

4. Discussion

A variety of polymeric matrices have been employed to provide sustained and localized viral vector delivery including from formats such as microparticles [32] and electrospun scaffolds [33, 34]. In terms of electrospun materials, the generation of coaxial fibers for the delivery of bioactive agents has been used to overcome some of the limitations associated with conventional single stream electrospinning [35, 36]. The inclusion of bioactive agents in hydrophilic cores encapsulated by a hydrophobic sheath can protect the agents from direct exposure to harsh organic solvents necessary for structural polymer dissolution. Furthermore, controlled release behavior can be tuned by modifying the thickness and structure of the sheath layer.

There are a few reports utilizing coaxial electrospinning techniques for gene delivery using either non-viral [37-40] or viral vectors [33]. In this study, employing a core of PEO/PBS with AAV encoding GFP, and a sheath layer of polyurethane, sustained release was observed from core-sheath fibers for two months, while solid fibers generated using single stream electrospinning showed minimal release. This may be attributable to loss of viral activity upon organic solvent exposure during electrospinning or a slower diffusion rate from the solid fibers. (**Fig.2-4**). PEG has been used as a porogen in the fiber sheath to facilitate the release of gene vectors in the core. By varying the molecular weights and concentration of the PEG, the period of release could be extended from several days to one month [33, 38]. Here, PEG was incorporated into the backbone of the polyurethane to tailor the release kinetics of AAV. As expected, PEEUU fibers showed more rapid AAV release than PEUU at short time points, presumably due to the hydrophilicity of PEG blocks and thereby faster diffusion of viral particles through the relatively swollen polymer in an aqueous environment. Cells seeded on the virus-containing scaffolds demonstrated higher levels of transgene expression than cells transduced by the scaffold supernatant, indicating higher efficiency of local delivery and transduction. This phenomenon was consistent with previous reports on viral and non-viral vectors [33, 41]. The PEUU core-sheath fibrous mats showed a significant decrease in wet mechanical properties compared with PEUU solid fibrous mats, possibly due to the hydrophilicity of the PEO core. The PEEUU core-sheath fibrous mats showed comparable Young's modulus and ultimate stress, but much lower strain-at-break compared with PEEUU solid fibrous mats (**Fig. 5**).

The pore size of 3D scaffolds has direct implications for cell proliferation and migration [42]. In this study, the cardiac patch made from PEEUU core-sheath fibrous mats had pore sizes of $\sim 40\ \mu\text{m}$, and exhibited extensive cellular infiltration (**Fig. 6**). The tissue morphology associated with the patch appeared to be consistent with previous reports using porous PEUU patches processed by salt leaching or thermally-induced phase separation, which possess pores ranging from 75 to 100

µm. The common features within the patch area included the presence of extracellular matrix components and cellular infiltration [3, 28]. In contrast, the solid fibrous scaffolds fabricated by single stream electrospinning had a mean pore size of 3 µm and showed minimal cellular ingress into the scaffolds [43, 44].

For the direct injection group, GFP-encoding AAV, was injected into the infarcted myocardium. With no mechanical support and no functional benefit from the GFP reporter gene, a lack of functional effect was expected. The EDA and %FAC values for the AAV injection group were consistent with previous reports for myocardial infarction control groups using the same rat model [3, 28]. Similarly, the functional parameters for the patch only group and AAV patch group, where positive functional outcomes were seen, were comparable with that previously reported for porous PEUU patches processed by salt leaching or thermally-induced phase separation [3, 28]. There were no significant differences in functional parameters between the patch only and AAV patch groups (**Fig. 7**), given no expected therapeutic efficacy from GFP.

AAV tissue tropism/specificity is determined by the structure of the capsid protein. Among a total of 13 AAV serotypes, AAV1, 6 and 9 have been observed to be the most cardiotropic, and predominantly transduce cardiomyocytes in the heart [9]. Among these three serotypes, AAV9 yielded the highest transfection efficiency for cardiac gene transfer compared to the other serotypes, at least in murine models [45-47]. However, the cardiotropic properties of AAV9 in murine models may not translate to larger animal models and humans. Clinically, AAV1-SERCA2a has been used to treat patients with heart failure and has demonstrated encouraging results with improvements in functional, symptomatic, biomarker and LV function/remodeling parameters, with no apparent adverse immune events [48-50]. However, a recent phase 2b trial did not show improved clinical outcome in patients with heart failure and reduced ejection fraction compared to a placebo [51]. The reason for this outcome remains unclear. It was postulated that

cardiomyocytes in humans may not be efficiently transduced by intracoronary delivery of the AAV1 vector, based on the fact that anterograde delivery with AAV1 encoding SERCA2a resulted in low expression in cardiomyocytes in porcine models [52].

The healthy heart is comprised of four major cell types; fibroblasts, cardiomyocytes, endothelial cells, and vascular smooth muscle cells [53]. Among resident cardiac cells, cardiomyocytes have been the primary therapeutic target of cardiac gene therapy [54]. Direct intramyocardial injection of AAV9-cmv-GFP, resulted in a high density of gene transfer in cardiomyocytes around the injection site (shown in **Fig. S6**), and limited gene expression beyond this region [55, 56]. Transduction of smooth muscle cells and endothelial cells by AAV has been described *in vitro* [57-59] and *in vivo* [60-63], but high titers or peptide-modification of the viral capsid are often employed to achieve high efficiency. With respect to cardiac fibroblasts, following myocardial infarction, these cells become activated and migrate to the injury site and proliferate [64]. Since cardiac fibroblasts account for 60-70% of the cells in the heart [65], these cells would be a viable target for cardiac gene therapies. It has been reported that AAV can infect human corneal fibroblasts *in vitro* [66], and modification of tyrosine on the AAV2 capsid increased viral transduction of murine fibroblasts *in vitro* [67]. However fibroblasts have not been effectively transduced by AAV *in vivo* so far. The data from this study show that rat smooth muscle cells can be transduced by AAV9-cmv-GFP *in vitro* with gene expression substantially increased at day 15, while rat cardiac fibroblasts showed minimal expression following transduction. (**Fig. S10**) Myofibroblasts are not part of normal cardiac tissue and appear only following cardiac injury, with positive smooth muscle actin staining. Using a mouse model of liver fibrosis, Piras et al. showed that AAV6 effectively transduced myofibroblasts, reprogramming them to fully functional hepatocytes *in vivo* and successfully reducing liver fibrosis [68]. In a mouse cardiac infarction model, systemic injection of AAV9 provided higher transduction efficiency than AAV6, with gene expression apparent in cardiac myofibroblast-like lineage located in the infarct and border zone

post-MI [69]. In this study it was shown that both cardiac troponin T and α -SMA-positive cells were transduced by applying the AAV-containing cardiac patch epicardially (**Fig. 8**), suggesting that both cardiomyocytes and myofibroblasts are being infected.

While controlled release approaches have previously been reported using other families of gene transfer vectors, only a relatively limited number of studies have investigated the feasibility of releasing rAAV vectors from biomaterials to achieve a localized, effective vector delivery. In this report, sustained and localized viral particle delivery was achieved over 2 months in vitro. Lee et al. reported encapsulation of rAAV3.45-cmv-GFP within electrospun nanofibrous scaffolds composed of blended mixtures of elastin-like polypeptides (ELP) and polycaprolactone [34]. The viral particles were released within 7 days, as quantified by qPCR. Fibrin glue scaffolds [70] and a peptide hydrogel combined with hyaluronic acid [71] have been utilized to deliver AAV2, with the hydrogels showing 100% release at 2 weeks. 3D porous scaffolds, as shown in this report, provide a structural and mechanical support that can maintain a space for tissue growth upon implantation, as the porosity supports cell infiltration throughout the scaffold. Lentivirus has been loaded onto microporous scaffolds processed from poly(lactide-co-glycolide) [72], and poly(1,8-octanediol citrate) and poly(glycerol-sebacate) [73] by simple absorption into the scaffolds. The high efficiency of the vector enables substantial transgene expression that persists for at least 4 weeks after being implanted subcutaneously into murine models, although rapid release occurred in vitro due to the low affinity of the virus to the scaffolds. There are few reports utilizing biomaterial scaffolds that directly deliver therapeutic genes for cardiac tissue engineering. An injectable methacrylated gelatin hydrogel was developed to deliver a nanocomplex of polyethylenimine functionalized graphene oxide and vascular endothelial growth factor-165 gene to a rat model with acute myocardial infarction.[74] A significant increase in myocardial capillary density and higher cardiac performance were noted at the injected peri-infarct region with nanocomposite hydrogel treatment 7 and 14 days post injection, respectively.

Several limitations in this study should be noted. First, the transduction efficiency *in vivo* was relatively low in the rat MI model. The low expression of the transgene might be attributed to low loading of viral particles in each patch, the relative insensitivity of some rat cell types (e.g. fibroblasts) to infection by the AAV utilized, or the limited permeability of the epicardial layer to AAV diffusion. To address hypothesized barrier effects, trypsin might be co-incorporated in the scaffold with the viral particles to facilitate mural gene transfer through an enzymatically degraded matrix [75]. Another approach to enhance the expression levels might be to replace the CMV promoter with a stronger promoter in cardiac myocytes, such as a combination of MCK to C5-12 promoter [76]. Second, the patch method for AAV delivery in the rat demonstrated an advantage of transgene expression in the target cardiac region in combination with low systemic contamination. (**Fig. S9**). This is in contrast to other studies reporting systemic AAV delivery [45, 77]. To further prevent the expression in skeletal muscles or other organs and achieve cardiac-specific expression, a cardiac troponin T (cTnT) promoter might be employed [78]. Thirdly, the AAV employed carried a reporter gene. With the controlled release and transduction capacity demonstrated for this method, a therapeutic gene compatible with AAV packaging, such as vascular endothelial growth factor, would be a next step to provide further functional improvement in the treated, infarcted heart. For such a study, it might be desirable to explore beyond a 12 week follow up window. Sustained gene expression at extended time points, and further scaffold degradation and tissue remodeling would be of interest. Furthermore, a potential limitation of the patch AAV delivery method is the ability for viral particles to penetrate the epicardial fat often found on the epicardium in humans. It may not be realistic to remove or move the fat at the time of patch placement, because such fat is mainly located around coronary vessels, and removal of the fat might increase the risk of bleeding or other complications, especially in an MI patient. It might be feasible to lightly abrade, under visualization, the epicardium and associated adipose regions before placing the patch. This might facilitate viral transport. This question could be addressed in a

large animal model, such as the pig, which has epicardial fat and could thereby better capture the scale and complexity of the human physiology [79]. In addition, in a large animal model the use of CT or MRI for imaging would be possible, as would be the case in a clinical study. However, for this small animal model, where the heart is small, the pulse is rapid and the infarct geometries are relatively consistent across the groups, echocardiography is more practical.

5. Conclusions

In this report, AAV particles encoding GFP, have been incorporated into four fibrous scaffold types: PEUU and PEEUU solid fibrous scaffolds, fabricated by single-stream electrospinning; and PEUU and PEEUU core-sheath fibrous scaffolds, fabricated by coaxial electrospinning. Sustained transgene expression over 2 months was observed for HEK293 cells exposed to, or seeded on AAV-loaded core-sheath fibrous scaffolds, where cells seeded onto the scaffolds showed superior transduction. The application of core-sheath fibrous PEEUU cardiac patches, with or without AAV9-cmv-GFP, showed functional benefit in abrogating the effects of ischemic cardiomyopathy on functional and structural cardiac parameters versus a control group of AAV injection alone. Both α -SMA and cardiac troponin T positive cells could be successfully transduced by the cardiac patch with AAV. Overall, this study demonstrates the creation of a cardiac patch design that combines both mechanical support, through a biodegradable, microfibrous elastomeric component, with the controlled release of viral gene vectors able to infect ventricular wall cells with a reporter gene. When packaging appropriate genes, this bioactive scaffold might find application in the treatment of cardiac and other tissues where both gene delivery and appropriate mechanical support are desired.

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Supporting information Available

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Sustained viral gene delivery from a micro-fibrous,
elastomeric cardiac patch to the ischemic rat heart

Table 1. Polymer composition and material properties.

Samples	PCL/PEG Feed ratio, mol%	PCL/PEG ^a Actual ratio, mol%	T _g ^b (°C)	T _m ^b (°C)	ΔH _m ^b (J/g)	Water uptake (%)
PCL ₁₀₀ PEUU	100/0	100/0	-57	40	23	2.1 ± 0.9
PCL ₇₅ -PEG ₂₅ PEEUU	75/25	74.8/25.2	-62	31	13	26.6 ± 4.5

^a: based on ¹H-NMR

^b: based on DSC second heating

PEUU
solid fibers

PEEUU
solid fibers

PEUU
Core-sheath fibers

PEEUU
Core-sheath fibers

(a)

(b)

(c)

PCL blocks
 PEG blocks
 PEO core
 AAV

Fig. 1. Electron micrographs of the **(a)** cross sections and **(b)** surface of scaffolds. Scaffolds were freeze-dried for the cross sectional imaging. **(c)** Schematic images illustrate rAAV encapsulation strategies for a typical fiber in each corresponding scaffold. Scale bars for (a): 10 μm . Scale bars for (b): 20 μm .

Fig. 2. Transduction of HEK 293 cells incubated with AAV2-cmv-GFP fibrous scaffolds. (a) Cell transgene expression was quantified using flow cytometry. * $p < 0.05$ compared with solid fibers. # $p < 0.01$ compared with PEUU core-sheath fibers. (b) Fluorescent images of HEK 293 cells incubated with PEEUU core-sheath fibers and PEEUU solid fibers incorporating AAV2-cmv-GFP. Scale bar: 100 μm .

Fig. 3. Transduction of HEK 293 cells seeded on AAV2-cmv-GFP fibrous scaffolds. (a) Cell transgene expression was quantified using flow cytometry. * $p < 0.05$ compared with solid fibers. (b) HEK 293 cells seeded on PEEUU core-sheath fibers and PEEUU solid fibers incorporating AAV2-cmv-GFP, pics taken by confocal microscope. Scale bar: 100 μm .

Fig. 4. Release of rAAV DNA from AAV2-cmv-GFP fibrous scaffolds, as quantified by rt-PCR.
*p<0.05 compared with PEUU solid fibers.

Fig. 5. Typical stress-strain response of wet scaffolds at room temperature.

Table 2. Mechanical properties of wet scaffolds at room temperature. *p<0.05 compared with the other groups.

Samples	Young's modulus (MPa)	Ultimate stress (MPa)	Strain at break (%)
PEUU Solid Fibers	1.6 ± 0.5	$7.9 \pm 1.1 *$	296 ± 76
PEEUU Solid Fibers	1.0 ± 0.3	1.3 ± 0.4	$594 \pm 108 *$
PEUU Core-sheath Fibers	1.1 ± 0.2	1.7 ± 0.1	194 ± 2
PEEUU Core-sheath Fibers	1.1 ± 0.1	1.4 ± 0.2	292 ± 12

(a)

(b)

(c)

Fig. 6. Representative composite views of cross-sections of infarcted rat hearts harvested at 12 wk, and stained by Masson's trichrome for **(a)** direct injection of AAV9-cmv-GFP, **(b)** implantation of PEEUU fibrous scaffolds, and **(c)** implantation of PEEUU fibrous scaffolds containing AAV9-cmv-GFP. Black dashed boxes indicate higher magnification area shown in right panels. Yellow dashed lines trace the implanted material areas. Scale bars: 2 mm.

Fig. 7. Echocardiographic assessment for **(a)** end-diastolic area (EDA), **(b)** fractional area change (%FAC), and **(c)** ejection fraction (EF) after AAV injection and patch implantation 12 wk after myocardial infarction; *p<0.05.

Fig. 8. (a) Representative micrographs immunostained for GFP and cTnT 12 wk after myocardial infarction in the ventricular wall region below the patch or equivalent area for the direct injection group.

Fig. 8. (b) Representative micrographs immunostained for GFP and α -SMA 12 wk after myocardial infarction in the ventricular wall region below the patch or equivalent area for the direct injection group. Scale bar: 100 μ m.

Fig. 8. (c) The quantification for GFP-positive cells for AAV injection, AAV patch and patch only groups 12 wk after myocardial infarction; * $p < 0.05$.