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Research article

Poly-ε-caprolactone electrospun nanofiber mesh as a gene delivery tool

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Abstract: Poly-ε-caprolactone (PCL) is a biodegradable aliphatic polyester which plays critical roles in tissue engineering, such as scaffolds, drug and protein delivery vehicles. PCL nanofiber meshes fabricated by electrospinning technology have been widely used in recent decade. The objective of this study intends to develop a gene-tethering PCL-nanofiber mesh that can be used as a wrapping material during surgical removal of primary bone tumors, and as a gene delivery tool to provide therapeutic means for tumor recurrence. Non-viral plasmid vector encoding green fluorescent protein (eGFP) was incorporated into PCL nanofibers by electron-spinning technique to form multilayer nano-meshes. Our data demonstrated that PCL nanofiber mesh possessed benign biocompatibility in vitro. More importantly, pCMVb-GFP plasmid-linked electrospun nanofiber mesh successfully released the GFP marker gene and incorporated into the co-cultured fibroblast cells, and consequently expressed the transgene product at transcriptional and translational levels. Further investigation is warranted to characterize the therapeutic influence and long-term safety issue of the PCL nanofiber mesh as a gene delivery tool and therapeutic device in orthopedic oncology.

Keywords: electrospun nanofiber; EGFP; PCL; tissue engineering; gene delivery

1. Introduction

Osteosarcoma is the most common primary malignant bone tumor in adolescent and young adults. The most frequent primary locations are long bones such as distal femur and proximal tibia. The main cause of death is distant metastasis [1]. The current therapeutic regime includes surgical removal of primary tumor, accompanying systemic chemotherapy and radiotherapy. However, all the people that experience chemotherapy and radiotherapy have to suffer from not only physical pain but also psychological torment. In addition, increasingly resistance of chemotherapeutic drugs and radiotherapy often happens during the therapy sessions, which leads to the failure of the treatment and high mortality [2]. It is still an urgent need to develop new therapeutic strategies for this deadly malignancy.

Gene therapy, emerging as a promising treatment strategy for many acquired or inherited diseases including cancers, is a milestone of bio-medical field [3,4]. Gene delivery methodology and techniques are critical in the development of gene modification regimes. Although viral vectors are well-known to possess higher transduction efficiency, they often suffer serious safety limitations including immunogenicity and unpredictable mutation, which may diminish their possibility on clinical applications [5]. Non-viral gene delivery system such as synthetic and natural compounds and polymers, which often has lower transfection efficiency compared to viral vectors [6,7], have showed less toxicity and low immunogenicity and may offer flexible choice of gene delivery means in clinical situations. In recent years, many copolymers have been investigated to delivery genes, drugs and proteins such as PCL, poly(lactide-co-glycolide) (PLGA) random copolymer, poly(D,L-lactide)-poly(ethyleneglycol) (PLA-PEG) block copolymer, polycations (poly-L-lysine) (PLL) and polyethylenimine (PEI) [6–10]. Among these copolymers, PCL appears a FDA-approved biomaterial for some clinical applications that is well-known for benign biocompatibility and low immunogenicity [11,12,13]. In order to overcome the barrier of low transfection of the non-viral vectors, biomechanical techniques such as electroporation, sonoporation, magnetofection, and hydroporation have been studied [14].

Using an electrospinning technique, the objective of this study was to characterize a PCL-nanofiber mesh tethering non-viral vector which may serve as a tissue wrapping/filling material during surgical removal of primary tumor and a potential gene delivery tool for residue tumor cell eradication.

2. Materials and Methods

2.1. Isolation of pCMVb-GFP plasmid DNA

Plasmid DNA (Addgene, Cambridge, MA USA) encoding eGFP with CMV promoter was amplified in *E. coli* which grew in Luria Broth (LB) containing ampicillin on a shaker at 37 °C overnight [15]. The bacteria were pelleted down next day at 4,000 rpm at 4 °C for 5 min, followed by adding 0.2 ml ice-cold solution I (50 mM glucose, 25 mM Tris-HCl PH 8.0, 10 mM EDTA PH 8.0), 0.4 ml solution II (1% SDS, 0.2N NaOH), 0.3ml ice-cold solution III (3M KCl, 5M Acetate). The mixture was in micro-centrifuge tubes on ice for 10 min before centrifugation at 12,000 rpm at 4 °C for 5 min. The supernatant was transferred to fresh Eppendorf tubes and filled with equal volume of isopropanol for 10 min on ice, followed by centrifugation at 12,000 rpm at 4 °C for 5 min.

The plasmids were washed once in 1 ml of ice-cold 70% ethanol, and resuspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer. The concentration of plasmid DNA was detected from the UV absorbance at a wavelength of 260 nm (A260).

2.2. Fabrication of nanofiber

The nanofiber gene-delivery mesh was fabricated by dissolving PCL in acetonitrile and mixed with pCMVb-GFP plasmid (3.5 mg/ml in TE buffer). The solution was allowed to equilibrate at 50 °C for 4 hours with constant stirring to generate 85% w/w polymer solution containing 700 µg plasmid DNA in 200 µl TE buffer. The solution was transferred to a 10 ml syringe with a 20 gauge plastic needle and set up in the electrospinning apparatus. The flow of polymer solution from the syringe was controlled by a programmable syringe pump. Nanofiber was electrospun at ~25 KV with flow rate of 20 µl/min. An aluminum foil was fixed at ~25 cm far from the needle to collect the nanofiber mesh. Figure 1 illustrates the schematic process of the electrospinning fabrication.

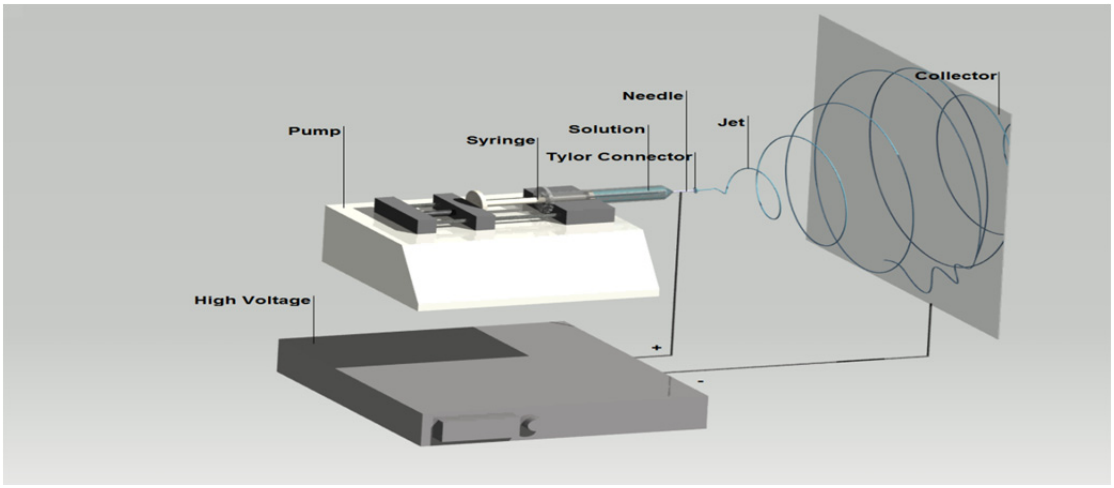

Figure 1. A schematic illustration of an electrospinning process. The bottom machine supplies strong electric field through generating high voltage.

2.3. SEM characterization of the electrospun fibers

A scanning electron microscope (SEM, Model JEOL JSM-6406LV, Japan) was used to characterize the fabricated PCL-nanofiber membranes. The operating conditions of the SEM are accelerating voltage of 10 Kev, Spot Size of 4, and a work distance of 12 mm. Images are taken at various magnifications and across various areas for each sample. Resolution can easily go below 10 nm.

2.4. Biocompatibility and cytotoxicity assay of PCL nanofiber mesh

Sterile PCL-nanofibers with or without plasmid tethering in size of 2 cm × 2 cm were put in 2 ml-Eppendorf tubes submerged with dulbecco's modified eagle medium (DMEM, ATCC, Virginia, USA) containing 5% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml

streptomycin. Soaking media were collected at first, fourth, and seventh days and replaced with fresh medium. Murine fibroblasts (3T3 cells, ATCC) were seeded in a 96-well plate at the density of 5 × 10^4 cells/well at 37 °C, 5% CO_2 atmosphere. The soaking media were added to the cells at various concentrations, respectively, for 3 days. Then 20 μl 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well. After six hours, cells were cracked by 10% Sodium Dodecyl Sulfonate (SDS) solution, followed by reading in a spectrophotometer (SpectraMax® Plus 384, Molecular Devices, Sunnyvale, CA, USA) at OD 590 nm.

2.5. DNA release assay

The electrospun nanofiber meshes were cut into 2 cm × 2 cm pieces and each piece was immersed with 1 ml TE buffer in Eppendorf tubes at 37 °C. The amount of DNA released into solution was quantified using PicoGreen® dsDNA quantitation assay (Molecular Probes, Eugene, OR). Briefly, the elution TE buffers collected at 15 min, 30 min, 1 h, 2 h, 4 h, and 1, 3, 7 days were labeled with PicoGreen® working solution to detect DNA content. Six samples per time point were examined. After 5 minutes incubation at dark, the elution solutions were measured at 520 nm (with excitation at 480 nm) in a UV Microplate Reader (CytoFlour® Series 4000, Applied Biosystems). DNA standard solution (100 μg/ml in TE buffer) was sequentially diluted to make a standard curve for calculation of the plasmid DNA concentration in each sample.

2.6. Cell culture and transfection

To determine the exogenous gene transfection efficiency, 3T3 cells were seeded into petri-dishes at a density of 10^5 cells/dish and incubated for 48 h to facilitate cell attachment before transfection. PCL-pCMVb-GFP-nanofiber meshes in size of 2 cm × 2 cm were added to the dishes and co-cultured with 3T3 cells for 48 h in a standard incubator. The petri-dishes were allocated into four groups: normal controls; sonication treatment for 15 min; lower pH (7.35–7.45) transfection medium; and sonication in lower pH medium. The green fluorescent signals in cells were detected 48 h later under a laser scanning confocal microscopy (Leica Microsystems TCS Sp5-II, German).

2.7. Polymerase chain reaction and electrophoresis

The transfected cells were harvested for transgene (eGFP) quantification using real-time polymerase chain reaction (RT-PCR) with left primer-GACGTAAACGGCCACAAGTT, right primer-AAGTCGTGCTGCTTCATGTG (product size 188 bps). RNA was extracted from the cells of four groups with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) in accordance with manufacturer's protocol. Extracted total RNA was treated with deoxyribonuclease I to eliminate genomic DNA, and reversed transcribed in a Veriti™ 96-well thermal cycler (Applied Biosystems, Grand Island, NY, USA). The quantification of GFP transgene was then performed using a StepOnePlus™ real-time PCR system (Applied Biosystems). The PCR product was electrophoresed on 2.0% agarose gels at 95 V for 1 h to confirm the single band of GFP PCR product in the transfected cells.

2.8. Statistical analysis

The data from 5 independent experiments were collected for this study. One-way ANOVA and Student's T-tests were performed to compare the group difference using SPSS 19.0 software package (SPSS, Chicago, IL, USA). P values less than 0.05 were considered as significant difference. Data were presented as the means ± standard errors of the means.

3. Results

3.1. Morphological characteristics of PCL nanofiber meshes

The characteristics of PCL nanofiber meshes are degradable and permeable. The pore size of PCL nanofiber meshes averages 530 ± 265 nm (Figure 2), which can easily let DNA molecules pass through. Six layers of PCL nanofiber meshes were examined for their mechanical properties, and resulted in a maximum stress of 0.158 MPa at 0.137 strain value (Figure 2B).

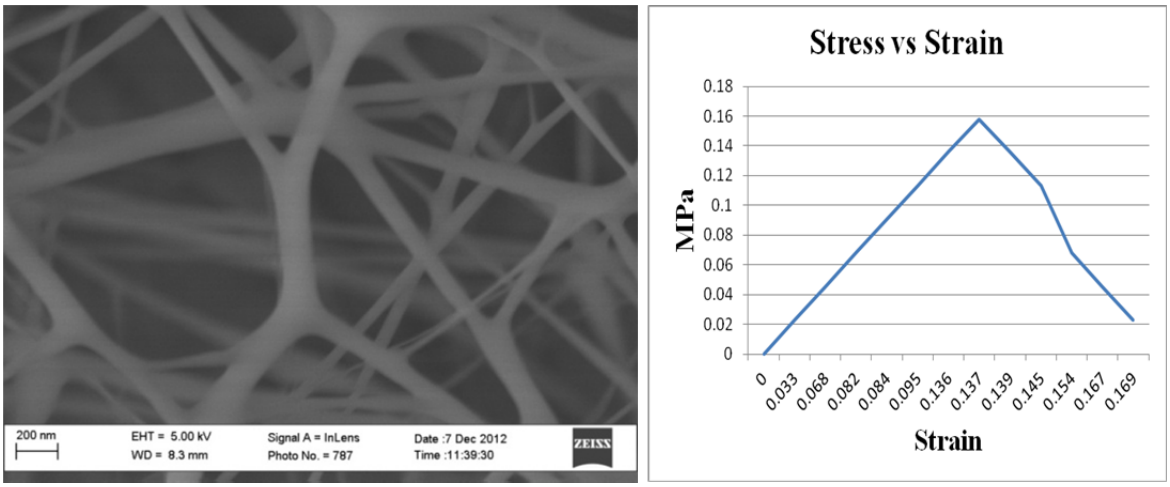

Figure 2. (A) a SEM image of the PCL-nanofiber mesh in six layers. (B) An actual stress mechanical test plot indicating the maximum strength of the 6-layer electrospun nanofiber membrane.

3.2. Biocompatibility and cytotoxicity assay of PCL nanofiber mesh

Elution of the gene-tethering and plasmid-free nanofiber meshes was applied into 3T3 cell cultures for 3 days, followed by MTT assay for cell proliferation determination. There is no apparent cytotoxicity in any of the testing group, and no significant difference in cell proliferation patterns among different time points of the two testing nanofiber elution in comparison with the normal medium control (100%). 10% SDS was used as a toxic group (Figure 3).

3.3. DNA release assay

PicoGreen® is ultra-sensitive to double-stranded DNA, but not to single-stranded DNA and oligonucleotides which can detect as little as 250 pg/ml double-strand DNA using a fluorescence microplate. We collected the TE buffer at the predefined point. The data indicate that DNA release

was detected in the beginning of 15 min and accumulated to a sustained release through the whole test period (Figure 4).

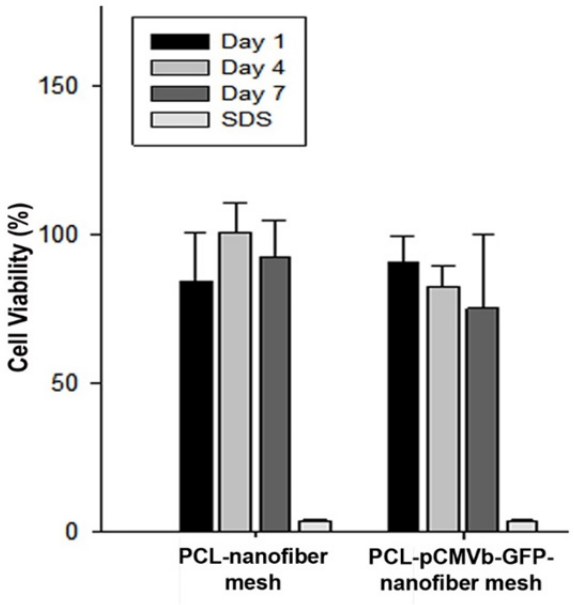

Figure 3. MTT assay was performed to detect the cell viability. The plot was an average of 5 set of data. There was no significant difference in cell viability among cells with elution media at various concentrations. No cell toxicity was noticed.

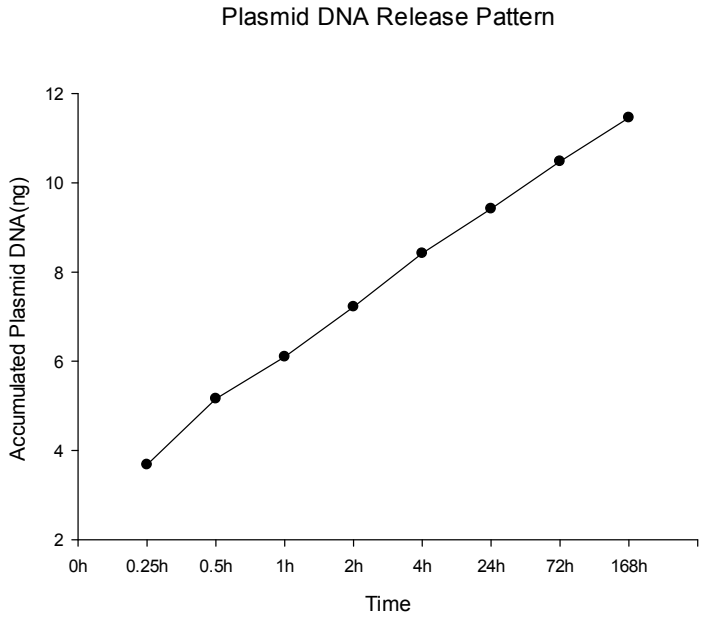

Figure 4. Accumulation of DNA release into the elution medium from the electrospun PCL-pCMV-GFP-nanofiber mesh of 2 cm × 2 cm membrane for 7 days.

3.4. Cell culture and transfection

Confocal microscope sensitively detected GFP fluorescent signals in the transfected 3T3 fibroblasts starting at day 3 after nanofiber-mesh co-cultures. Over 70% of transfection efficiency at day 7 was obtained (Figure 5). However, there was no significant difference in transfection rate between normal pH and lower pH groups, nor with the sonication treatment ($p > 0.05$).

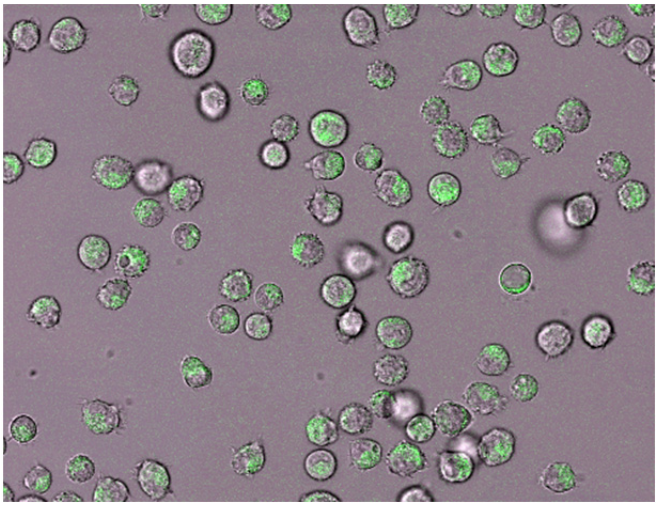

Figure 5. Fluorescent histomicrograph reveals the strong GFP signals within cells after 7-day co-culture on the PCL-pCMV-GFP-nanofiber meshes (200× magnification).

3.5. Electrophoresis of PCR products

In order to detect the GFP mRNA expressions among the four treatment groups, electrophoresis of agarose gels was performed to visualize the real-time PCR product. It appeared that lower the culture condition to pH 7.3 instead of normal pH 7.4 in combination of brief sonication significantly improved the transgene production (Figure 6).

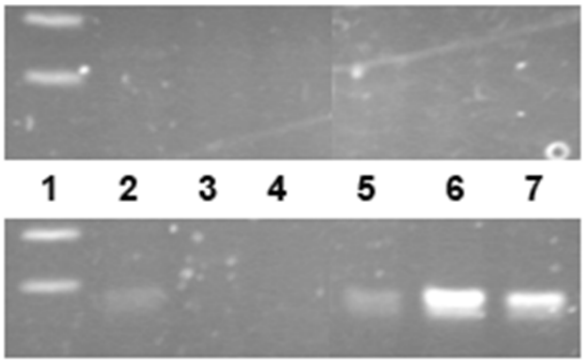

Figure 6. Agarose gel electrophoresis illustrates the real-time PCR product of GFP mRNA from the cells with various treatments. The lane 1 shows the DNA ladder for sizing purpose. The upper lanes (2–4) were control group. The upper lanes (5–7) were lower pH group. The lower lanes (2–4) were ultrasound group. The lower lanes (5–7) were the group of the combination of sonication treatment with lower pH.

4. Discussion

Osteosarcoma is a common malignant bone tumor which is prone to grow in metabolically active position such as metaphysis of long bones. The characteristics of cancer are sustaining proliferation, evading immune recognition, inducing angiogenesis and metastasis [16]. Although curettage or resection of the tumor remains the first line of the treatments besides chemo- and radiotherapies, residue tumor stem cells may become the main reason of recurrence [17]. The concept of this research is to develop a gene-tethering nanofiber mesh or membrane that can work as a wrapping or filling material during the tumor removal surgical procedures, and in the meantime, can serve as a gene delivery tool to transfer therapeutic gene such as IL-21 or CTS1 to eliminate the residue cancer cells [18,19]. During surgical osteosarcoma procedure, PCL-nanofiber meshes could be used to wrap the region after tumor tissue removed and bone filler or total joint in place. PCL is well-known as a biocompatible biomaterial in clinical practice [13,20,21]. As a good candidate for gene delivery tool, PCL is a super hydrophobic biomaterial and has a relatively slow biodegradable property, which will result slow yet sustained release of the exogenous gene while working as a wrapping/support material at the primary bone tumor site. The advantages of nanofiber mesh include less cytotoxicity and immunogenicity, flexible choice of gene size.

During the tumor growth, it has been confirmed that the extracellular pH of within human cancers was significantly lower than in normal tissue [22]. Our data suggested that significantly more transgene was expressed after the cells incubated on gene-tethering nanofiber membranes in lower pH environment, indicating a potential targeting nature of the non-viral gene therapy. More detailed investigation is warranted to explore the mechanism.

Current research on nanofiber scaffold focuses to delivery stem cells, drugs and proteins [23–26]. There is so far lack of information on applied nanofiber to deliver genes. In the current study, non-vial vector coding green fluorescent protein (GFP) gene was successfully delivered into fibroblasts and transfection efficiency was quite satisfactory. It appears that gene-tethering PCL-nanofiber mesh resulted in sustained release of the plasmid DNA and more than 70% percent of the cells were transfected. Indeed, this gene delivery material may have more broadly clinical applications. One can also apply PCL-nanofiber meshes for skin burns, diabetic ulcers, and melanoma as a wound dressing [27,28], which has good breathability and can carry therapeutic genes (like epidermal growth factor or IL-21), or antibiotics to minimize the frequency of dressing changes.

This is a proof of concept study that provides a potential useful medical device for both gene delivery tool and wound dressing/wrapping material. Detailed investigations using animal models have been performing to evaluate the therapeutic efficiency and long-term safety issue of the gene-tethering PCL-nanofiber meshes for cancer treatment.

5. Conclusion

PCL-electrospun nanofiber mesh has benign biocompatibility and can be successfully linked with non-viral vectors to serve as a gene delivery tool. The data suggested that the nanofiber mesh can continuously release plasmid DNA with initial burst release, which may be especially beneficial in orthopaedic oncology such as using as a bone scaffold wrapper after bone tumor removal and arthroplasty. It appears that manipulation of some physical treatments such as sonication and lowing pH level can effectively improve the transfection efficiency of the PCL-plasmid nanofiber meshes.

The advantages of electro-spun nanofiber scaffolds, including high surface-to-volume ratio, appropriate porosity and malleability, a wide variety of size and shapes, and good biocompatibility, may make it an ideal candidate as a gene delivery tool and therapeutic device.

Acknowledgment

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Conflict of Interest

The authors declare that they have no conflict of interest.

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