Characterization of a Novel Polymeric Scaffold for Potential Application in Tendon/Ligament Tissue Engineering

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ABSTRACT

Unlike braided fabrics, knitted scaffolds have been proven to favor deposition of collagenous connective tissue matrix, which is crucial for tendon/ligament reconstruction. But cell seeding of such scaffolds often requires a gel system, which is unstable in a dynamic situation, especially in the knee joint. This study developed a novel, biodegradable nano-microfibrous polymer scaffold by electrospinning PLGA nanofibers onto a knitted PLGA scaffold in order to provide a large biomimetic surface for cell attachment. Porcine bone marrow stromal cells were seeded onto either the novel scaffolds by pipetting a cell suspension (Group I) or the knitted PLGA scaffolds by immobilizing in fibrin gel (Group II). Cell attachment at 36 hours, cell proliferation and extracellular matrix synthesis at 1 week, and mechanical properties over 2 weeks were investigated. Cell attachment was comparable and cell proliferation was faster in Group I. Moreover, cellular function was more actively exhibited in Group I, as evident by the higher expression of collagen I, decorin, and biglycan genes. Thus, this novel scaffold, facilitating cell seeding and promoting cell proliferation, function, and differentiation, could be applied with promise in tissue engineering of tendon/ligament.

INTRODUCTION

INJURIES TO TENDONS AND LIGAMENTS are among the most common injuries to the body, particularly in the young and physically active population. Associated with the problems of incomplete healing and recurrent injury, these injuries are not only responsible for large health care costs, but also result in lost work time and individual morbidity.1,2 Despite many improvements in the currently available therapies involving autografts, allografts, and tendon/ligament prostheses, there remain significant limitations in our management of these conditions. Biological grafts have drawbacks such as donor scarcity, donor-site morbidity, tissue rejection, and disease transmission, while prosthetic devices have complications such as durability and poor long-term performance. Tissue engineering holds promise in treating these conditions by replacing the injured tissue with engineered tissue with similar mechanical and functional characteristics.3

A common approach in tissue engineering involves a three-dimensional (3D) porous biodegradable scaffold loaded with specific living cells and/or tissue-inducing factors to launch tissue regeneration or replacement in a natural way, with the scaffold eventually disappearing over a period of time.4,5 A biocompatible, biodegradable, porous scaffold with optimized architecture, sufficient surface area for cell attachment, growth and proliferation, favorable mechanical properties, and suitable degradation rate so that it can eventually be replaced by newly

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orgenerated tendon/ligament is a prerequisite to achieve success with this approach.\textsuperscript{6–8} Braided or embroidered fabrics often encounter problems of nutrient transmission, poor cell seeding, infiltration, matrix production, and inadequate mechanical strength.\textsuperscript{9–11} Knitted poly (D,L-lactide–co–glycolide) (PLGA) scaffolds have been shown to possess good mechanical strength and internal communicating spaces and have been effectively used for tendon tissue engineering.\textsuperscript{12} However, this scaffold required gel systems, such as fibrin or collagen gel, for cell seeding and was found to be unsuitable for ligament reconstruction in the knee joint, because the cell-gel composite dissociated from the scaffold during motion.\textsuperscript{13} Gel systems are also likely to encounter nutrient transmission problems, and cells seeded in a 3D gel are observed to proliferate more near the surface than in the center of the gel.\textsuperscript{14,15} Recently, nanofibers produced by electrospinning technology, from a variety of biodegradable polymers, have been applied for tissue engineering of bone, blood vessel, and heart.\textsuperscript{16–22} Electrospun nanofibers have a high surface area-volume ratio, mimic the nanostructure of extracellular matrix (ECM) of natural tissue, and thus can facilitate cell attachment, proliferation, and ECM deposition. Various cell types, including bone marrow stromal cells, have been grown successfully on nanofibers scaffolds in the form of nonwoven mats, and these cells have also been induced to differentiate along osteogenic, adipogenic, and chondrogenic lineages.\textsuperscript{23–25} However, a nanofiber matrix alone would not be sufficiently strong for engineering fibrous connective tissues such as tendon and ligament.

In the present study, a novel biodegradable nano-microfibrous polymer scaffold has been developed by electrospinning PLGA nanofibers onto the surfaces of a knitted PLGA scaffold. It is expected that this technique can combine the superior mechanical strength and integrity of the knitted microfibers and the large surface area and the better hydrophilicity of the nanofibers, thus, facilitating cell attachment, new ECM deposition, and tissue formation.

**MATERIALS AND METHODS**

**Scaffold fabrication**

Knitted scaffolds of 20 × 40 mm dimension were fabricated from PLGA (10:90 PLA:PGA, 3 yarns, 20 filaments/yarn, 25 \( \mu \)m diameter of filament; Shanghai Tianqing Biomaterial, Shanghai, China) fibers. These scaffolds were kept uncurled on custom-made U-shaped stainless steel wire frames fabricated from 1 mm diameter K wires.

Hybrid nano-microfibrous scaffolds were fabricated by electrospinning PLGA nanofibers onto the surfaces of the knitted scaffold. Briefly, a 25% (w/v) solution of PLGA (65:35 PLA: PGA, Birmingham Polymers, Birmingham, AL) in a solvent mixture (1:1) of Tetrahydrofuran (Te-dia, Fairfield, OH) and N, N-Dimethylformamide (Sigma Chemicals, St. Louis, MO) was delivered at a rate of 0.10–0.20 mL/h to a 18 G stainless steel needle, maintained at a potential of 10–15 kV by a high-voltage power supply unit (RR 30-2P/DDPM, Gamma High-Voltage Research, Ormond Beach, FL). Nanofibers were collected on the knitted scaffolds placed on a grounded aluminum foil at a distance of 15 cm from the needle. After 1 h, the scaffolds were turned over and the process repeated on the other surface. The scaffolds thus fabricated were characterized by phase-contrast inverted light microscopy, scanning electron microscopy, and in vitro degradation and mechanical tests.

**Scaffold morphology: Phase contrast microscopy and scanning electron microscopy**

Some nanofibers were collected on glass cover slips and their diameter was determined by phase contrast microscopy and using an image analysis software (MicroImage v4.5.1, Olympus Optical, Hamburg, Germany). The nano-microscaffolds were sputter coated with gold, and their morphology was observed by scanning electron microscopy (SEM, JSM-5800LV, JEOL, Tokyo, Japan).

**Degradation and mechanical testing**

Scaffolds were sterilized by exposure to formaldehyde gas. Hydrolytic degradation of the scaffolds was carried out in isotonic phosphate buffered saline solution (PBS, 1 \( \times \)) at 37°C and pH 7.4 over 2 weeks. Degradation was studied primarily by assessing the scaffolds’ mechanical properties, which is vital for functional replacement of tendons and ligaments. Mechanical testing was performed using a universal testing machine (Instron 3345 Tester, Instron, Norwood, MA). Test specimens of 20 mm breadth and 20 mm gauge length were kept moist by spraying PBS and then stretched to failure at a crosshead speed of 10 mm/min, without any pretension or preconditioning. The load (N) and extension (mm) were recorded, and the failure load, elastic region stiffness, and toe region stiffness were determined after plotting the load displacement curves.

**Isolation and culture of bone marrow stromal cells**

Bone marrow stromal cells (BMSC) were harvested and cultured by previously described techniques.\textsuperscript{12} In brief, bone marrow was aspirated from the iliac crest of an anesthetized 30 kg Yorkshire pig and mononuclear cells were concentrated by Ficoll gradient centrifugation and re-suspended in complete culture medium containing Dulbecco’s modified Eagle’s medium with low glu-
cose (Gibco, Invitrogen, Carlsbad, CA), 10% fetal bovine serum, L-glutamine, penicillin, and streptomycin. BMSCs were selected due to their property of short-term adherence to tissue culture polystyrene when incubated at 37°C with humidified 5% CO₂. After 24 h, nonadherent cells were discarded and adherent cells cultured, changing the medium every 3 days. When culture flasks became nearly confluent after about 7 days, the cells were detached and serially subcultured. Semi-confluent cells, obtained after sufficient expansion through three passages, were used for seeding scaffolds.

**Cell seeding and culture on scaffolds**

Scaffolds were sterilized by exposure to formaldehyde gas. BMSCs were then seeded at a density of 3.3 × 10⁵ cells/scaffold onto each of the scaffolds, placed in the wells of a custom-made culture chamber. The nano-microscaffolds (Group I) were seeded by simple pipetting of 1 ml of cell medium suspension onto the scaffolds. For control experiments, the knitted scaffolds (Group II) were seeded with BMSC suspended in 1 ml of fibrin gel (Tisseel Kit Two-Component Fibrin Sealant, Baxter Healthcare, Deerfield, IL). The BMSC-seeded scaffolds were grown *in vitro* in a 5% CO₂ incubator at 37°C for 2 weeks, with the medium being replaced every 3 days. The “engineered tissues” were then assessed for cell attachment, proliferation, ECM synthesis, and mechanical properties at various time points over the 2 weeks.

**Cell seeding efficiency and cell proliferation assay**

After incubating the cell-seeded scaffolds (*n* = 3, 2.5 × 10⁵ cells/scaffold) for 36 h, the culture medium was collected from the wells into separate centrifuge tubes and cell count performed. The cell seeding effi-

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 1</th>
<th>Sequence 2</th>
<th>Bp</th>
<th>A.T. (C)</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen-I</td>
<td>1 GAT CCT GCT GAC GTG GCC AT</td>
<td>2 ACT CGT GCA GCC GTC GTA GA</td>
<td>212</td>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td>Decorin</td>
<td>1 AGT GCG AAA GGC TGT GTT CA</td>
<td>2 GCG ATG CCG ATG TAG GAG AG</td>
<td>132</td>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td>Tenascin-C</td>
<td>1 CAT CGT GAC AGA GTG GAC GGA AGA</td>
<td>2 GTG GCC ACC CTG GCG CTG ACA GGA</td>
<td>260</td>
<td>69</td>
<td>30</td>
</tr>
<tr>
<td>Biglycan</td>
<td>1 GAG CCGCAC TTG GAC AAC A</td>
<td>2 AAG TCA TTG ACG CCC ACC TT</td>
<td>119</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>GAPDH</td>
<td>1 TCA CCA TCT TCC AGG AGC GA</td>
<td>2 CAC AAT GCC GAA GTG GTC GT</td>
<td>293</td>
<td>55</td>
<td>29</td>
</tr>
</tbody>
</table>

1Forward primer.  
2Reverse primer.  
Bp, base pairs; AT, annealing temperature; cycle, number of PCR cycles.

**FIG. 1.** (A) Phase contrast (magnification × 40) and (B) SEM views of nano-microscaffold showing nanofibers randomly oriented between the microfibers.
Efficiency was expressed as the number of cells attached to the scaffold as a percentage of the number of cells seeded. Scaffolds of each group were assayed for cell proliferation using the colorimetric MTS assay (CellTiter 96 AQueous Assay, Promega, Madison, WI) on days 2 and 7. The absorbances of the culture media at 490 nm were normalized to the average absorbance of Group II scaffolds on day 2 to obtain the relative rates of cell proliferation in the scaffolds.

**Histology and electron microscopy**

At the end of 1 week, scaffolds from both groups were assessed by inverted light microscopy after hematoxylin and eosin staining, by laser scanning confocal microscopy after live cell staining with 5-chloromethylfluorescein diacetate (CMFDA), and also by SEM.

**Collagen and glycosaminoglycan assays**

The total soluble collagen and sulphated glycosaminoglycan (GAG) synthesized and secreted into the culture medium were determined by using SirCol Assay for collagen and BlyScan Assay for GAG (Biocolor, Newtownabbey, Northern Ireland). Culture media were changed on day 5 and collected on day 7 to estimate the total amount of collagen/GAG secreted per scaffold in 2 days.

**Reverse transcriptase-mediated PCR analysis of ECM proteins**

Total RNA was extracted from both group of BMSC-seeded scaffolds on day 3 using the Qiagen RNeasy Kit. The RNA extract was assessed for its purity and concentration by spectrophotometry and stored at −80°C. Reverse transcriptase-mediated PCR (RT-PCR) was performed for collagen I, tenascin C, decorin, and biglycan, which are ECM proteins specifically present in tendons and ligaments, and GAPDH was used as the housekeeping gene. Primer sequences (Table 1) were obtained from published literature (Collagen I, GAPDH; Tenascin C; Biglycan, Decorin) and synthesized by Research Biolabs, Singapore. After electrophoretic separation on 2% agarose gel, the RT-PCR products were photographed in a gel documentation system (Gel Doc 2000, BioRad, Hercules, CA), and densitometry analysis of the bands was performed with the help of imaging software (Quality-One 4.4.0, BioRad). The average integrated density of each band was normalized to that of the GAPDH band of the respective scaffold group.

**Mechanical testing of cell scaffold constructs**

After 7 and 14 days of culture, mechanical tests were conducted on the cell scaffold constructs of both groups (n = 4/5) as done previously for the unseeded scaffolds. The failure load, elastic region stiffness, and toe region stiffness were determined.

**FIG. 2.** Phase contrast view of BMSC-seeded nano-microscaffold (day 3, magnification × 40).

**FIG. 3.** SEM image of BMSC-seeded Group I scaffold after (A) 7 days (magnification × 55) and (B) 14 days of culture (magnification × 550).
Statistical analysis

Data were analyzed by single-factor ANOVA and presented as mean ± standard deviation. For pair-wise comparisons, two-tailed, unpaired Student’s t-tests were used and \( p < 0.05 \) was accepted as significant.

RESULTS

Scaffold characterization

The average thickness of the knitted scaffold varied between 0.6–1.0 mm and that of the nano-microscaffold was between 0.8–1.3 mm. Phase contrast microscopy (Fig. 1A) and SEM (Fig. 1B) revealed that nanofibers with diameters varying from 300 to 900 nm were spread randomly over the surface and between the loops on the knitted scaffolds. The pores were interconnected, with pore size varying from 2 to 50 \( \mu \)m. Mechanical testing revealed that the nano-microscaffolds had an initial failure load of 56.3 (± 6.66) N, which fell to 1.82 (± 0.6) N by 14 days. The elastic stiffness and toe region stiffness decreased from 5.80 (± 0.7) N/mm to 0.64 (± 0.2) N/mm and from 0.34 (± 0.1) N/mm to 0.05 (± 0.01) N/mm, respectively, over the same period.

FIG. 4. Histology of (A) Group I and (B) Group II scaffolds showing BMSCs with a notably more elongated morphology on the nano-microscaffold (day 7, magnification \( \times 100 \)).

A

B

FIG. 5. Confocal images after live cell staining show a denser cell population on (A) Group I compared with (B) Group II scaffolds (day 7, magnification \( \times 45 \)).
Cell seeding efficiency

The cell seeding efficiency was found to be 88.8 (± 1.11)% for Group I scaffolds and 97.7 (± 2.02)% for fibrin-gel based delivery in Group II scaffolds.

Cell morphology on scaffolds

BMSCs attached and grew well on the Group I scaffolds, as evident in the phase contrast (Fig. 2) and SEM images (Fig. 3). On day 2, BMSC had attached to the nanofibers on the scaffold and had formed small cell aggregates, which increased in size (day 7) and bridged across the scaffold connecting with other cell aggregates (day 14). Abundant ECM was observed filling the pores between the nanofibers. Hematoxylin and eosin staining (Fig. 4) revealed that BMSC had a more elongated morphology in Group I compared with Group II scaffolds. Confocal microscopy (Fig. 5) after fluorescent staining with CMFDA showed a more profuse cell proliferation in the Group I scaffolds.

Cell proliferation assay

Starting with the same number of cells (2.5 × 10^5) on both scaffold groups, MTS assay results (normalized to the average absorbance of Group II scaffolds on day 2) gave...
values of 1.0 ± 0.28 and 1.2 ± 0.28 for Group I and 6.1 ± 1.03 and 11.7 ± 0.89 for Group II scaffolds on day 2 and day 7, respectively. This shows that, between the second and seventh day, the cell population increased by 92% in the Group I scaffolds and by 21% in the Group II scaffolds.

Collagen and glycosaminoglycan assays

Both group of BMSC-seeded scaffolds produced similar amounts of soluble collagen and GAG. Group I scaffolds synthesized an average of 466.59 (± 179) μg of soluble collagen and 2.37 (± 0.2) μg of GAG between the fifth to seventh days of in vitro culture, whereas Group II scaffolds synthesized 490.28 (± 556) μg of soluble collagen and 2.49 (±0.6) μg of GAG during the same period, the differences being statistically insignificant (p > 0.05).

RT-PCR analysis of ECM proteins

The concentration of RNA was determined to be 0.041 μg/μL from Group II scaffolds (A_{260:280} = 1.182) and 0.080 μg/μL from Group I scaffolds (A_{260:280} = 1.993). Gel electrophoresis images proved that BMSCs growing on the nano-microscaffold expressed collagen I, tenascin-C, decorin, and biglycan (Fig. 6A). Densitometric image analysis revealed that the mRNA expression for collagen-I, decorin, and biglycan was greater on the Group I scaffolds (Fig. 6B).

Mechanical tests

Cell seeded nano-microscaffolds on days 7 and 14 of culture possessed slightly higher values of failure load, elastic region stiffness, and toe region stiffness than unseeded scaffolds maintained in PBS under similar conditions (37°C, 5% CO₂) for the same duration (Table 2). However, owing to small sample sizes (n = 4/5), the increase was not statistically significant for most comparisons.

DISCUSSION

Development of nano-microfibrous scaffold

Though knitted scaffolds provide large 3D internal spaces for cell proliferation, nutrient and metabolic waste diffusion, and neovascularization after in vivo implantation, they require gel systems for cell seeding. These are often associated with problems of weak gel-scaffold attachment and poor nutrient transmission. Inspired by collagen type I nanofibrous architecture in natural extracellular matrix, nonwoven scaffolds have been fabricated using nanofibers in the form of thin mats, which have proven useful in engineering of tissues such as blood vessels and nerves. However, for stronger tissues like tendon and ligament, a nanofiber scaffold alone does not provide sufficient strength. In the novel nano-microfibrous scaffold, fabricated by electrospinning PLGA nanofibers onto the surfaces of a knitted PLGA scaffold, the knitted microfibers provide the mechanical integrity while the nanofibers, randomly spread over the surface and between the loops of the knitted scaffold, increase the surface area and reduce the pore size of the scaffold.

<table>
<thead>
<tr>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Group II</td>
</tr>
<tr>
<td>Failure Load (N)</td>
<td>18.11 ± 3.53</td>
</tr>
<tr>
<td>p value</td>
<td>0.29</td>
</tr>
<tr>
<td>Elastic Stiffness (Nmm)</td>
<td>3.88 ± 0.63</td>
</tr>
<tr>
<td>p value</td>
<td>0.30</td>
</tr>
<tr>
<td>Toe stiffness (Nmm)</td>
<td>0.34 ± 0.12</td>
</tr>
<tr>
<td>p value</td>
<td>0.07</td>
</tr>
</tbody>
</table>

BMSC as candidate cells for tendon/ligament tissue engineering

Because ligament and tendon are relatively hypocellular tissues, it is difficult to obtain ligament/tenon cells for clinical tissue repair. BMSCs are known to possess the ability of self-renewal and differentiation into cells of various mesenchymal lineages. Their ease of collection by needle aspiration, simplicity of isolation on the basis of their adherence to tissue culture polystyrene, and excellent properties of attachment, proliferation, and synthesis of an ECM rich in collagen type I make them suitable candidates for tissue engineering applications.10 Several previous studies12,30,31 have shown that BMSCs can improve tendon/ligament healing and were better suited for ligament tissue engineering than were cells derived from ligaments or skin.10 RT-PCR results indicate that BMSCs retain the potential to differentiate along the lineage of tendon and ligament on the novel scaffold.

Effect of nanofibrous substrate on cell adhesion and proliferation

In contrast to the tedious method of cell seeding using fibrin gel, nano-microscaffolds were easily and effectively seeded by the simple method of pipetting a cell suspension onto them. The reduced pore size (2–50 μm)
of the scaffold and the large surface area-volume ratio of nanofibers facilitated cell attachment, proliferation, and ECM deposition. BMSCs were seen to attach and proliferate well on the nano-microscaffold, forming cell aggregates that gradually increased in size and fused with adjacent cell aggregates to cover wide areas of the scaffold. Proliferation was better on the nano-microscaffolds than in the scaffolds seeded using fibrin gel.

Effect of nanofibrous substrate on cell function

The proliferating cells produced abundant ECM that filled the pores between the nanofibers. Collagen production by BMSC on the novel scaffold (466 μg/days 6 and 7) was higher than that produced by a similar number \((3 \times 10^5)\) of BMSC grown on braided PLGA scaffolds (161 μg/day 12) reported in a recent study. Furthermore, BMSCs on the nano-microscaffold exhibit a higher expression of collagen-I, decorin, and biglycan, proving their potential to differentiate into tendon/ligament tissue. Studies with aligned nanofiber scaffolds have shown that cells produced significantly more ECM on them than on random nanofiber matrices. Nanofibers also have the potential to deliver biological molecules like growth factors to stimulate cell proliferation and differentiation. Thus, the novel nano-microscaffold could be further tailored for tendon/ligament tissue engineering.

Limitations of the study

Limitations in the current scientific knowledge about specific markers for tendon/ligament cells restrict this study to only characterize MSC differentiation into tendon/ligament lineage by a list of tendon/ligament matrix components. Also some research results, such as histology and RT-PCR, could not be quantified or could only be semi-quantified. Although the study shows that BMSC-seeded novel scaffolds could differentiate into tendon/ligament lineage by synthesizing their major matrix, the mechanical properties of the scaffold were not comparable with the normal values of tendon/ligament. Use of knitted scaffolds with a different PLGA copolymer ratio, or with alternate biomaterials such as PLLA or silk, should be addressed to solve this problem in future studies. Nevertheless, this study exhibited the efficiency of this novel nanofiber-reinforced knitted scaffold for MSC delivery and synthesis of tendon/ligament matrix. It also suggested the new concept of nano-macrofibrous scaffold in which nanofibers provide a larger surface and microfibers provide greater strength.

CONCLUSION

A novel method for producing a nano-microscaffold is presented. Electrospinning nanofibers over the surface and between the loops of the knitted scaffold increase the surface area and reduces the pore size of the knitted scaffold, thereby eliminating the need of cell delivery by fibrin gel. The nanofibers also mimic the natural nano-architecture of tendon/ligament extracellular matrix, which promotes the functional capability of the bone marrow stromal cells to secrete a tendon/ligament specific matrix. Thus, this novel scaffold is able to facilitate cell seeding and promote cell proliferation and function, showing promise in tissue engineering of tendon/ligament.

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REFERENCES


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