Enhanced Fibroblast Cellular Ligamentization Process to Polyethylene Terephthalate Artificial Ligament by Silk Fibroin Coating

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Abstract: Artificial ligaments utilized in reconstruction of anterior cruciate ligament (ACL) are usually made of polyethylene terephthalate (PET) because of its good mechanical properties in vivo. However, it was found that the deficiencies in hydrophilicity and biocompatibility of PET hindered the process of ligamentization. Therefore, surface modification of the PET is deemed as a solution in resolving such problem. Silk fibroin (SF), which is characterized by good biocompatibility and low immunogenicity in clinical applications, was utilized to prepare a coating on the PET ligament (PET+SF) in this work. At first, decrease of hydrophobicity and appearance of amino groups were found on the surface of artificial PET ligament after coating with SF. Second, mouse fibroblasts were cultured on the two different kinds of ligament in order to clarify the possible effect of SF coating. It was proved that mouse fibroblasts display better adhesion and proliferation on PET+SF than PET ligament according to the results of several technical methods including SEM observation, cell adhesive force and spread area test, and mRNA analysis. Meanwhile, methylthiazolyldiphenyl-tetrazolium bromide and DNA content tests showed that biocompatibility of PET+SF is better than PET ligament. In addition, collagen deposition tests also indicated that the quantity of collagen in PET+SF is higher than PET ligament. Based on these results, it can be concluded that SF coating is suggested to be an effective approach to modify the surface of PET ligament and enhance the “ligamentization” process in vivo accordingly. Key Words: Polyethylene terephthalate artificial ligament—Silk fibroin—Fibroblast cell—Ligamentization.

The anterior cruciate ligament (ACL) is one of the most important ligaments in the knee joint by providing stability and guiding motion. ACL is frequently injured in athletic training and accidents. It is reported that nearly 95 000 ACL reconstructions are carried out each year in the USA (1). Recently, due to the rapid development of tissue engineering, the artificial ligaments have presented a promising solution to treat ACL injuries. For example, Ligation Advanced Reinforcement System (LARS; Surgical Implants and Devices, Arc-sur-Tille, France) ligament, which is made of polyethylene terephthalate (PET), has been widely applied in clinical therapy (1). However, owing to their biological differences from natural ligaments, artificial ligaments like the LARS should undergo a series of biologic processes termed “ligamentization” (2–4), which generally refers to the healing stage following the cell adhesion and proliferation stage. During this “ligamentization” period, similar to other polymeric or metallic implants, LARS ligament could cause serious side effects such as immunological responses and recurrent instability (2,5,6). For example, we
observed that an interposed layer of fibrous scar tissue could appear at the interface between the graft and bone tunnel in ACL reconstruction using artificial ligament (7). Furthermore, one in vitro laboratory study has demonstrated that human fibroblast and osteoblast-like cells grow onto the surface of PET materials after 6 months (8). Many papers have reported the artificial PET ligaments are not conducive to cell adhesion and proliferation, vascular and collagen synthesis owing to their hydrophobicity and chemical inertness. Thus, the insufficient biocompatibility of PET materials surface has seriously stunted their “ligamentization” processes in vivo (9,10).

The surface topography of a solid substrate regulates cellular adhesion, migration, proliferation, and differentiation (11). Currently, PET ligament coating with other natural or synthetic biomaterials with good biocompatibility, such as alginic acid, hyaluronic acid, chitosan, and cationic gelatin, have received increasing interest as an alternative option to speed up the “ligamentization” process (12–15). Silk fibroin (SF) has been used as biomedical sutures for decades (16). In recent years, SF has been explored for many other biomedical applications due to its impressive biocompatibility, biodegradability, and mechanical properties. Silk as biomaterial scaffold provides support matrices for cells, including fibroblasts, osteoblasts, hepatocytes, and stem cells, as well as scaffold for tissue engineering bone, ligaments, cartilage, blood vessels, and controlled release systems for drugs and growth factors (17–24). Therefore, silk fibroin was chosen in the current study for coating on artificial ligament due to its favorable properties above. Herein, this investigation was implemented in this study, which was aimed to evaluate whether the SF on the surface of PET fiber could improve fibroblast cells’ adhesion and proliferation. It was assessed by seeding fibroblast cells in the ligament, where morphology, growth, and long-term matrix elaboration were analyzed by various technological methods.

MATERIALS AND METHODS

Preparation of artificial ligaments

PET artificial ligament with a size of $3 \times 1.5$ cm$^2$ (taken from LARS artificial ligaments, Surgical Implants and Devices, Arc-sur-Tille, France) was cleaned by sonication in acetone and ethanol for 20 min, respectively. After drying in a vacuum oven at room temperature for 24 h, the samples were treated with argon plasma (50V, 2A) for 2 min, and then exposed to air for 10 min. SF solution was prepared according to previously reported methods (25,26), and the typical process was as follows: the silk cocoon was degummed in 0.5% (w/w) Na$_2$CO$_3$ aqueous solution for about 40 min to remove the sericin. The degummed silk was then dissolved in 9.3 mol/L LiBr aqueous solution. After being filtered, the fibroin solution was dialyzed against deionized water for 72 h at room temperature with a 12 000–14 000 molecular weight cutoff dialysis membrane to remove the salt. The dialyzed solution was then clarified by spinning in a centrifuge at 6000 rpm for about 4 min. The supernatant SF solution was collected and carefully stored at 4°C. After that, the PET fiber samples were immersed in 6 mol/L LiBr aqueous solution for 10 min, rinsed repeatedly with deionized water, and finally impregnated in 15% (w/w) SF solution at 4°C prior to completely cast at room temperature. The air-cast ligaments were dried in a vacuum oven at room temperature for 24 h and named as PET+SF for further experiment. Simultaneously, PET artificial ligaments that went through all treatments above except immersing in the SF solution established the blank control group marked as PET.

Characterization of artificial ligaments

All fourier transform infrared spectroscopy (FTIR) spectra were obtained with a NEXUS-470 ATR spectrometer (Nicolet Instruments, Madison, WI, USA). The measured wave number range was from 1800 to 1400 cm$^{-1}$. Each sample was measured with 128 scans, and the spectral resolution was 4 cm$^{-1}$.

Surface wettability of the testing samples was assessed by the water contact angle. In this process, we prepared the model by dropping ultrapure water on the surface of PET and PET+SF artificial ligaments and, respectively. Images of droplets on the ligaments were visualized through the image analyzer (OCA40, Dataphysics, Stuttgart, Germany) to gauge the contact angles. In order to acquire precise results, each angle was measured in the mean-value of the data gained from five different positions, and temperature was conditioned to 25°C.

Cell seeding and culture on artificial ligaments in vitro

BALB/C CL7 mouse fibroblast cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in α-MEM supplemented with 10% fetal bovine serum (Mediatech, Herndon, VA, USA), L-glutamine and 1% antibiotics (Life Technologies, Invitrogen, CA, USA), and maintained at 37°C and 5% CO$_2$.

Prior to cell seeding, all testing materials were sterilized through a conventional gas sterilization technique using ethylene oxide gas. The cells in the
exponential phase were seeded on the ligaments at a density of 400,000 cells/sample (2900 cells/cm²) and grew in supplemented α-MEM at 37°C and 5% CO₂. The culture lasted for up to 7 days.

**Morphology of ligament before and after cell culture**

Cell growth and morphology were evaluated at 1, 3, 5, 7, and 14 days using scanning electron microscopy (SEM). Prior to SEM analysis, the cells were fixed in glutaraldehyde and dehydrated through a series of ethanol dilutions. The samples were sputter-coated with gold (Denton Desk-1 Sputter Coater, Denton Vacuum, Inc., Moorestown, NJ, USA). Cell growth and morphology were examined using an SEM system (JEOL JSM-5600LV, JEOL, Tokyo, Japan). Ligaments without cell culture was also observed by SEM after sputter-coating with gold.

**Adhesive force measurements**

Adhesive force was measured as described previously (27). Briefly, after incubation for 3, 6, 9, 12, or 24 h, both PET and PET+SF plates seeded with fibroblast cells were fixed to the holder and completely submerged into a chamber, respectively. In this experiment, adhesive force was calculated as the maximum deflection of the glass plate observed. Force ($F$) is given by $F = 3IE\omega L^{-3}$, where $E$ is Young's modulus, $I$ is the moment of inertia of the area, $L$ is the length from the fixed edge of a leaf spring to a cell, and $\omega$ is the maximum deflection at $L$, which can vary in accordance with the attachment position of a micropipette.

**Measurement of cell spreading area**

Fibroblast cells were seeded onto both PET and PET+SF substrates as described above, respectively. After incubation for 3, 6, 9, 12, or 24 h, the cells were washed twice with phosphate buffer solution, and then fixed with 4% PFA solution for 15 min at 37°C. The cells were then stained with 1% (wt/vol) Coomassie brilliant blue solution for 2 min and washed twice with phosphate buffer solution. Digital images of the cells were acquired by microscopy. Cell spreading area was measured by analyzing the binarized images using ImageJ (http://imagej.nih.gov/ij/download.html).

**Real-time PCR**

The potential influence on cellular functions and behaviors by coating of SF on artificial PET ligament was determined at the mRNA level. We targeted the detection of a series of marker gene expression by the cells cultured on ligaments compared with their counterparts grown on culture dishes by real-time PCR (RT-PCR). The genes of interest were fibronectin (FN), epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) and Cadherin 11 (CDH11). These genes are closely related to the normal fibroblast functions. Cells were first cultured in PET+SF for several time intervals, and then harvested by trypsinization. Total RNA extraction was performed with commercially available RNeasy mini kit (Qiagen, Valencia, CA, USA). Real-time PCR was performed in a thermocycler (LightCycler, Roche, Mannheim, Germany) using the QuantiTect RT-PCR Kit (Qiagen). The target PCR products were about 100 bp. At least three replicates were performed on each sample, and each experimental gene was tested by three PCR runs. In each PCR run, the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the reference. The primers for real-time-PCR are shown in Table 1.

**MTT assay and DNA content analysis**

A modified methylthiazolyldiphenyl-tetrazolium bromide (MTT) (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) test, in which the yellow MTT is reduced to a purple formazan by

### Table 1. Real-time RT-PCR primer sequences

<table>
<thead>
<tr>
<th>Gene no.</th>
<th>Forward primer sequences (5'-3')</th>
<th>Reverse primer sequences (5'-3')</th>
<th>PCR product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF NM_001287056.1</td>
<td>TCCAGGAGTACCCCGACGAGATAAG</td>
<td>GTGCTGGCTTTGTTGAGTGTTGAT</td>
<td>164</td>
</tr>
<tr>
<td>EGFR NM_207655.2</td>
<td>GGTGGCCGGCTATGTCCTCA</td>
<td>CCCCAGCTCGTTGTTGTC</td>
<td>149</td>
</tr>
<tr>
<td>FN NM_010233.2</td>
<td>TCTACGGGTCCGGACTGAAGG</td>
<td>GTGCTGGCTGGTGTTGGAAT</td>
<td>162</td>
</tr>
<tr>
<td>CDH11 NM_000966.4</td>
<td>CTTTGCGCTCGTCTCATTCTTCTT</td>
<td>TCACCACCCCCTCCTCATTAG</td>
<td>141</td>
</tr>
<tr>
<td>GAPDH NM_008084.3</td>
<td>CTGACGTGCGGCTCGTGGAG</td>
<td>CCCGGCATCGAAGGTGGAAG</td>
<td>162</td>
</tr>
</tbody>
</table>
mitochondrial dehydrogenase in cells, was used to assess the cell viability. After 12, 24, 36, 72, 120, and 168 h incubation in different mediums, the viability of fibroblasts cells was assessed. Briefly, fibroblasts cells were washed three times with culture medium. The culture medium in each well of the plate was added 100 μL MTT (5 mg/mL in PBS). After 4 h incubation at 37°C, the reaction solution was carefully removed from each well, and 200 μL dimethyl sulfoxide was added. The plates were gently agitated until the formazan precipitate was dissolved, followed by measurement of OD values by spectrophotometry at 490 nm with an ElX-800 Microelisa reader (Bio-Tek, Inc., Burlington, VT, USA). As for DNA content, the PET+SF scaffold at the same above time intervals were washed in PBS, and then were lysed in 0.2% (wt/vol) Triton X-100 and 5 mM MgCl2. Pico Green assay (Molecular Probes, Eugene, OR, USA) was used to measure the total amount of DNA.

Collagen quantification
The amount of deposited collagen on the scaffold was quantified at 1, 3, 5, and 7 days using a Sircol collagen dye binding assay Kit (Biocolor Ltd., Newtownabbey, Ireland) following the manufacturer’s protocol. Briefly, fibroblasts cells/scaffold (n = 6) from PET and PET+SF groups were incubated with 500 mL of pepsin solutions (0.25 mg/mL) and shaken for 2 h at room temperature. Then 1 mL of dye reagent was added to 300 mL of soluble collagen and mixed for 30 min. The pellet of dyed collagen was precipitated by centrifugation for 5 min, and then dissolved by 1 mL of releasing reagent. The absorbance was measured at 540 nm. The standard curve was set up using collagen standard provided by the vendor.

Statistical analysis
All statistical analysis was carried out using the SPSS 17.0 statistical software package (SPSS, Inc., Chicago, IL, USA) and reported as the mean ± standard deviation. P < 0.05 in all cases was considered statistically significant based on one-way analysis of variance.

RESULTS

Characterization of ligament
After SF coating, the morphology of PET artificial ligament changed according to the observation of SEM (Fig. 1). PET fiber in the original ligament has a smooth surface and no cracks could be identified (Fig. 1a). These fibers are well defined among each other. However, the surface of fiber in PET+SF ligament was wrapped with a layer of SF, and part of PET fibers was bridged by SF (Fig. 1b). It can be found that surface roughness of PET ligament was increased relatively by the SF coating, which is beneficial for adhesion of fibroblast cells (28). In addition, existence of SF in the new fabricated artificial ligament was also solidified by the result of FTIR. It is shown in Fig. 2 that PET ligament displays a typical PET FTIR spectrum, which 1435 cm⁻¹ was assigned to C = C stretching vibration of benzene and 1720 cm⁻¹ to -C = O stretching vibration (29). When SF was immobilized onto PET surface through the coating method, an adsorption band at 1623 cm⁻¹ (assigned to amide I of SF β-sheet structure) and 1520 cm⁻¹ (assigned to the amide II of SF β-sheet structure) showed up, indicating the successful introduction of SF onto PET surface. Furthermore, SF molecular conformation in PET+SF differs from pure regenerated SF material that mainly displays random coil or a helix conformation after vacuum dehydration according to published articles (30). The conformation change of the SF molecule can be initiated by many factors including interactions with order domain of other polymer chains (31). Therefore, it is suggested that there exists some interaction...
between PET and SF molecular chains that contribute to both SF conformation change and adhesion of SF on PET fibers. In order to evaluate the hydrophilicity of artificial ligament before and after coating of SF, we adopted the method of measuring the water contact angle on the surface of ligament. The water contact angles of original PET and PET+SF are $132 \pm 15^\circ$ and $50 \pm 11^\circ$, respectively, after calculating based on more than five times measurements (Fig. 2b,c). The result indicated that hydrophilicity of PET ligament was significantly improved after coating of SF, which may benefit the biocompatibility of the substrate.

**Morphology of cell on the surface of ligament**

The morphology of fibroblast cells on the testing material during culture is shown in Fig. 3. There were obvious differences between PET+SF and PET in cell adhesion and spreading after the first day of incubation. Specifically, there were only some spherical fibroblast cells growing on the surface of PET+SF, whereas nothing could be observed in the PET group. The morphology of fibroblast cells could evolve to a spindle-shape along the PET+SF ligament fiber after culturing 3 days, whereas no obvious cell morphology was observed on PET. After 5 days’ incubation, the whole surface of PET+SF was already coated by a large number of spindle-shaped fibroblast cells. Cell morphology after 7 days on PET+SF ligament illustrated that extensive fibroblast cells had grown unidirectionally and aggregated into irregular cell clusters following the longitudinal axis of the fibers, and even formed large cellular networks that bridged the fibers. However, in the PET ligament, the growth orientations of fibroblast cells were found to be random and did not correspond to the underlying geometry of the PET fibers.

**Cell adhesive force and spread area**

The time-dependent changes in adhesive force and spreading area of fibroblast cells grown on PET and PET+SF ligament are shown in Fig. 4a,b. As shown in Fig. 4a, the PET+SF tended to show higher adhesive force than the PET ligament. Specifically, the PET+SF showed significantly higher adhesive force than the PET ligament at 9, 12, and 24 h after seeding, but it did not change at 3 and 6 h. The cell spreading area as a function of culture time is shown in Fig. 4b. Significant differences were found between the PET and PET+SF since after seeding 6 h. With the increasing of incubation time, the spreading area in PET+SF rapidly increased from $464.55 \pm 0.32 \mu m^2$ at 3 h to $1340.28 \pm 7.3 \mu m^2$ at 24 h, whereas it was around $515.64 \pm 0.53 \mu m^2$ at 24 h in the PET ligament.

**MTT and DNA content analysis for cell proliferation**

MTT and DNA content analysis of fibroblast cells cultured on PET and PET+SF ligament was made (Fig. 4c,d). Both results revealed that the viability of fibroblast cells in PET+SF was significantly higher.
than those in PET after 24-h incubation. The DNA content of the PET ligament was $8.23 \pm 0.25$, $10.01 \pm 0.67$, $13.34 \pm 0.43$, $15.20 \pm 0.62$, $16.72 \pm 0.37$, and $16.78 \pm 0.85$ mg/scaffold after culturing 12, 24, 36, 72, 120, and 168 h, respectively, whereas the corresponding values were $13.65 \pm 1.31$, $18.48 \pm 1.83$, $21.32 \pm 2.57$, $36.28 \pm 3.53$, and $50.59 \pm 2.47$ mg/scaffold in the PET+SF at the same time intervals. The DNA content of the PET+SF was significantly higher than that of the PET ligament since 12 h incubation ($P < 0.05$).

Expression of the factors secreted from fibroblast cells
Owing to the great differences in cell adhesive force and spreading area during 24 h seeding between PET and PET+SF ligament, the mRNA levels of specific genes secreted by fibroblast cells were further detected and analyzed by RT-PCR. The mRNA levels of VEGF, EGFR, FN, and CDH11 in PET+SF were higher than those in PET ligament (Fig. 5). The upregulated mRNA expression of FN and CDH11 in PET+SF group confirmed that SF coated on the surface of PET should enhance cell adhesion and proliferation, because both FN and CDH11 were related to cell adhesion and cell–cell adhesion, respectively. Both VEGF and EGFR are powerful pro-angiogenic factors; their upregulated expression implied that they also improved neovascularization process, which would help collagen remodeling in later stages.

Collagen synthesis
The collagen deposition stage plays an important role in the ligamentization process following the cell adhesion and proliferation stage. Thus, the amount of deposited collagen on PET and PET+SF ligament were determined, respectively. Quantification of collagen showed that there was a higher production in PET+SF compared with PET ligament during incubation time (Fig. 6). The fibroblast cells of the PET+SF ligament produced an average of $13.85 \pm 2.43$, $98.53 \pm 15.2$, $220.72 \pm 12.26$, and $681.37 \pm 25.28$ mg collagen/scaffold at 1, 3, 5, and 7 days, respectively, whereas the fibroblast cells of PET ligament produced an average of $10.13 \pm 1.27$, $12.35 \pm 2.45$, $58.27 \pm 4.45$, and $89.67 \pm 12.25$ mg collagen/scaffold at the same time intervals ($P < 0.05$), respectively. More collagen deposition maybe is one of the reasons for better cellular adhesion and proliferation on the PET+SF ligament.

DISCUSSION
In the last 40 years, for the appropriate materials used in the artificial ligament fabrication, several trials such as the carbon fiber, PTFE, and collagen have been reported (32–34). However, the early
Clinical results from these applications were so poor, and the postoperative complications were leveled as disabling status (35,36). As a consequence, the void of an ideal material in the synthesis and fabrication thwarted following research programs. With the widely use of the LEEDS-KEIO ligaments and the LARS ligaments (37,38), PET generally gained popularity in such area. Recently, a new type of artificial ligament made in England called the Neoligaments also adopted PET in the fabrication. However, with the long-term follow-ups after clinical application of such artificial ligaments being reported, complications caught people’s attention (39). Cases of postoperative complications were observed in the follow-ups, despite surgical techniques and combined injuries, the major problem is the poor healing or even no healing in the bone-graft interface, which could lead to the laxity of grafts and joint instability (40). After study in both in vivo and in vitro experiments, researchers believed that the hydrophobic nature of PET is the major defect of the artificial ligaments made of such materials, which is attributed to the main cause for the poor healing results (41). Therefore, what can be done to enhance the biocompatibility of the PET is an attractive issue recently. As we know, a hydrophilic environment is vital for cell proliferation and differentiation. The nature of PET as a hydrophobic fiber can be deemed as a foothold for the next step in the artificial ligament research program.

The present study demonstrated that the introduction of SF on the PET substrate ligament increased the fibroblast cells adhesion and proliferation, which is attributed to the higher adhesive force and spread area, mRNA levels, and DNA content. For example, the values of spread area and DNA content on PET+SF is about 15 and 3 times that of PET, respectively, at the same intervals. The reasons could be due to more collagen deposition on PET+SF ligament, which is also well expounded by...
experiment of collagen synthesis, namely PET+SF and PET produced an average of 681.37 ± 25.28 mg and 89.67 ± 12.25 mg collagen/scaffold at 168 h, respectively. Therefore, the results confirmed that coating with SF on PET ligament could improve the biocompatibility of PET materials and further speed the “ligamentization” process in vivo. Reason for the improvement is expounded as follows. Some previous studies have shown that PET material may cause poor “ligamentization” owing to its hydrophobicity and chemical inertness. Furthermore, modification of PET artificial ligament by increasing its hydrophilicity or introducing new chemical groups have been proved to be effective to solve the problem (27,42,43). On one hand, SF is famous as a typical amphipathic material coming from nature (44). It is assumed that the hydrophobic groups of SF molecule tend to adhere on PET surface a while hydrophilic groups of SF turn to the outside, and then the hydrophilicity of the ligament is increased accordingly. The hypothesis is verified by contact angle measurements of PET and PET+SF ligament. On the other hand, it is well accepted that the amino groups, which are repeated units in SF molecular chains, are amenable to adhesion and proliferation of living cells (45,46). FTIR test declared that the amino groups can be detected in PET+SF ligament. Under such circumstances, it is concluded that SF coating can contribute to increase the hydrophilicity and chemical activity of PET ligament, which can improve its biocompatibility and speed its “ligamentization” process in vivo. In summary, this study lays the foundation for using SF to speed up the process of “ligamentization” for artificial PET ligament in tissue engineering. Further study should focus on seeking evidence of collagen deposition and neovascularization in the PET+SF ligamentization process in vivo.

CONCLUSION

In order to improve fibroblast cellular adhesion and proliferation process of PET ligament, silk fibroin was coated on it. Appearance of amide groups was found by FTIR while contact angle decreased from 132 ± 15° to 50 ± 11° after the coating process. Therefore, successful coating process on PET ligament was well solidified. Based on observation of SEM, fibroblast cells display better adhesion and proliferation on PET+SF than PET ligament after cultivation. Furthermore, comparative results of cell adhesive force, spread area test, and mRNA analysis between the two kinds of ligaments told us the same story. Meanwhile, MTT and DNA content tests declared that biocompatibility of PET+SF is better than PET ligament, and the collagen deposition test also indicated that the quantity of collagen in PET+SF is higher than PET ligament. Therefore, owing to its amphipathic and biocompatible features, SF is well suited to be an effective coating biomaterial to modify the surface of PET ligament and enhance the “ligamentization” process in vivo accordingly.

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