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Constructed silk fibroin scaffolds to mimic adipose tissue as engineered implantation materials in post-subcutaneous tumor removal

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ABSTRACT

Treatment in post-subcutaneous tumor removal is a clinical problem that needs effective surgery. This research proposes a good performance scaffold to regenerate new tissue. Silk fibroin scaffolds (SFSs) were fabricated by the salt leaching method and were constructed by soaking in 0.25% and 0.50% (W/V) silk fibroin solutions before freeze-drying. Then, the SFSs were seeded with 3T3-L1. Structural organization of the SFSs was characterized by Fourier transform infrared spectroscopy, differential scanning calorimetry, and X-ray diffraction. The physical stability and mechanical performance were analyzed by weight loss and compressive testing. The morphology of the SFSs with and without cell culturing was observed by scanning electron microscopy and transmission electron microscopy. The proliferation and histology of the cultured cells in the SFSs were observed. The results showed constructed SFSs that had molecular and physical stability. The constructed SFSs demonstrated a morphology that was able to support cell adhesion, spreading, elongation, and aggregation. This study indicated that the constructed SFSs exhibit suitable structural and physical stability, and mechanical and biological performance for subcutaneous tissue engineering. In conclusion, the obtained results suggest that constructed SFSs had performance and promise for tissue engineering, particularly in cases of post-subcutaneous tumor removal.

1. Introduction

Many patients suffer from skin contour defects after tissue removal that includes the layer of adipose tissue. Especially in the case of breast tumor tissue, these defects not only have emotional effects but they also affect daily social functions. Scientists have developed soft tissue replacement strategies for small volume and large volume defects. In the cases of small defects, they can be corrected by collagen injections or hyaluronic acid fillers and autologous fat tissue transplantations (lipofiller) [1,2]. However, the fillers rapidly degrade and injected autologous fat has a 40–60% volume loss. Repeated treatments are required to maintain the desired volume [3].

In cases of large volume soft tissue loss, one strategy was autologous tissue transfer using vascularized flaps that contained skin, fat, and muscle [4]. These techniques required considerable surgical skill and donor site morbidity and deformity problems required hospitalization and prolonged operating times, but the results were superior to those obtained from synthetic implants for subcutaneous tissue. Therefore, to create a suitable scaffold for subcutaneous tissue particularly in adipose tissue engineering is an attractive choice to solve those problems.

Generally, in adipose tissue engineering, the adipocytes require high metabolic activity and waste removal [5,6]. An extracellular matrix (ECM) that acts as a native scaffold generally showed a loose network [7]. The loose network structure of an ECM is suitable for cell migration, nutrients, and waste transportation. Principally, artificial scaffolds for adipose tissue engineering should also have a loose network with the structure and function of native ECM that supply oxygen and nutrient diffusion. The pore size of those scaffolds is around 50 to 400 μm [3,8]. Moreover, those scaffolds should have the physical stability to maintain their structure and mechanical performance with the surrounding tissue during new tissue growth [5,9,10]. The challenge is to create the proper structure of scaffolds that have the physical stability and maintain high penetration capability for cell migration, nutrients, and waste transport [5].

Mimicking is an attractive approach for application in many fields. Tissue engineering uses mimicking to modify the structure and function of scaffolds. In the case of bio-functional mimicking, some cell recognition receptors were immobilized on a tissue engineering scaffold to enhance cell adhesion, proliferation, and differentiation [11]. For structural mimicking, synthesis molecules created the structure of an extracellular matrix that could maintain the shape of the tissue and induce cell adhesion which led to enhanced tissue regeneration [12].

Silk fibroin has been used in the biomaterial field for decades because of its superior mechanical properties, biocompatibilities, low

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inflammatory responses, and slow degradation. Silk fibroin has other potential uses as a biomaterial for tissue engineering [13]. Three-dimensional porous scaffolds have advantages over the two-dimensional scaffolds that include the promotion of cell attachment, cell proliferation, and the promotion of cells to differentiate [5]. However, the permeability and elasticity of an extracellular matrix are also important in cell behavior. Optimizing the pore size and porous structure for each cell type is important in scaffold fabrications. In particular, mature adipocytes have various cell sizes that depend on the intracellular triglyceride accumulation [14,15]. Therefore, in this research, silk fibroin scaffolds were constructed into a loose network that had the suitable pore size and porous structure for adipose tissue engineering.

Generally, silk fibroin scaffolds are fabricated into high porosity structures with large pores for adipose tissue engineering [16]. Such a structure is important for nutrient penetration and oxygen exchange during cultivation that is suitable for adipose tissue engineering. However, those structures often have less surface area and physical stability [17]. Therefore, it is challenging to construct scaffolds with suitable physical stability, pore size, and porous structure as a native extracellular matrix (ECM) for adipose tissue engineering.

This research presents mimicked silk fibroin scaffolds that have a suitable pore structure as an extracellular matrix of native adipose tissue. The scaffolds were created and the structural organization, morphology, and physical stability were characterized, observed, and tested. The potential performance of mimicked scaffolds was evaluated. The aim of this research is to propose the performance of silk fibroin scaffolds for adipose tissue engineering particularly in post-subcutaneous tumor removal.

2. Materials and methods

2.1. Construction of silk fibroin scaffolds

A silk fibroin solution was prepared according to a previous report [17]. The silk scaffolds were prepared by the salt-leaching process adapted from Kim et al. [17]. The silk fibroin solution was diluted to obtain a final concentration of 6 w/v% and warmed to 50 °C for 30 min. NaCl (particle size: 500–600 μm) was characterized using a sieving tower (Retsch, Arlesheim, Switzerland) and 2 g of NaCl were added to 1 ml of the warm 6 w/v% silk fibroin solution in 24-well tissue culture polystyrene (TCPS) plates. The plates were then covered and left at room temperature for 24 h. The plates were immersed in water for over 1 day to extract the salt from the scaffolds. Air-dried scaffolds were cut to 2 mm in height. A silk fibroin solution was diluted to 0.25 and 0.5 w/v% solutions. Cut scaffolds were immersed in serial concentration solutions at 4 °C for 12 h. The scaffolds were freeze-dried for 12 h and immersed in 80% methanol (Labscan) for 45 min. The methanol was removed with distilled water for 24 h and the scaffolds were then air-dried and stored at −20 °C.

2.2. Fourier transform infrared spectroscopy (FTIR)

The molecular organization of silk fibroin in the scaffolds was measured by FTIR spectroscopy. The wave numbers of the freeze-dried samples ranged from 4000 to 400 cm^{-1} by a Fourier transform infrared spectrometer (EQUNOX55, Bruker, Germany) in the ATR mode.

2.3. Differential scanning calorimetry (DSC)

Non-constructed and constructed scaffolds were kept in a desiccator for 24 h. Samples were then cut into small pieces and weighed. Afterward, samples were put in an aluminum pan and covered. The samples were heated with a differential scanning calorimeter (DSC7, Perkin Elmer, USA) in increments of 5 °C from 25 to 300 °C.

2.4. X-ray diffraction (XRD)

The crystal structures of the non-constructed and constructed scaffolds were analyzed by XRD (X'Pert MPD (PHILIPS, Netherlands)). Samples were put in the XRD instrument and the diffraction pattern was measured over a 2 θ range of 5–90 θ with a step size of 0.05 θ and time per step of 1 s.

2.5. Scanning electron microscopy (SEM)

The freeze-dried scaffolds without cell culturing were frozen in liquid nitrogen and cut with a razor blade. Cell attachments were observed in the cell-cultured freeze-dried scaffolds at 4 h after seeding. The cell-seeded scaffolds were sequentially ethanol dehydrated in 70% ethanol for 10 min, 80% ethanol for 10 min, 90% ethanol for 10 min, and finally in absolute ethanol (critical point dried). Finally, the freeze-dried scaffolds with and without cell culturing were sputter-coated with gold. The cell morphology on the scaffolds was observed by SEM (Quanta 400, FEI, Czech Republic).

2.6. Transmission electron microscopy (TEM)

For the ultrastructure studies, the scaffolds were cut into small pieces and primary fixed in 4% paraformaldehyde and washed in 0.1 M PBS solution. Post-fixation in 1% osmium tetroxide and en-block stain in 2% uranyl acetate were performed. After that, the seeded scaffolds were sequentially dehydrated with ethanol as described previously and embedded in epoxy resin. The scaffolds were cut into semi-thin (0.5–2 μm) and ultra-thin sections with an ultra-microtome (MT-XL, RMC, USA). The ultra-thin sections were stained with uranyl acetate and lead citrate. The 3T3-L1 preadipocyte cell line pellets were prepared by putting the trypsinized 3T3-L1 cells into 4% paraformaldehyde 0.1 M phosphate buffer (pH 7.3), shook well, and fixation was carried out for 2 h. The cell cultures were separated from the fixative using a centrifugal separator (1500 rpm) for 10 min. The supernatant was removed and the cells were resuspended in 0.1 M phosphate buffer (pH 7.4) and recentrifuged as above (3 changes of 10 min each). The cells were post-fixed in 1% osmium tetroxide solution in phosphate buffer at 4 °C for 1 h. Washing was performed in two changes of distilled water for 5 min and post-fixed in 2% uranyl acetate for 30 min. Dehydration was done using an ethanol series as described previously. Propylene oxide was used for two changes of 15 min each and then infiltrated with epoxy resin (Epon-821). Each block was inserted into BEEM capsules and the blocks were filled with fresh epoxy resin before polymerization. Sectioning was cut with an ultra-microtome (MT-XL, RMC, USA) and the semi-thin sections (0.5–2 μm) were stained with 1% toluidine blue. The sections were then stained with uranyl acetate and lead citrate. Observation was conducted under an accelerating voltage of 80 kV by TEM 100-CX II (JEOL Ltd., Japan).

2.7. Evaluation of physical stability

First, the dry weights (Wd1) of the freeze-dried scaffolds were measured. The scaffolds were then immersed in distilled water at room temperature for 24 h. After the excess water was removed, the scaffolds were freeze-dried and weighed again (Wd2) [5]. The water stability was implied from the weight loss ratio (%) derived from this formula [18].

$$\text{Weight loss ratio (\%)} = ([Wd1 - Wd2] / Wd1) \times 100$$

2.8. Evaluation of mechanical performance

Dried cylinder-shaped scaffolds measuring 13.2–13.8 mm in diameter and 5.75–8.7 mm in height were used. Mechanical compression of the dried samples was performed on a universal testing machine (Lloyd instruments, LRX-Plus, AMETEK Lloyd Instrument