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Novel decellularized extracellular matrix scaffolds derived from porcine costal cartilages via a combination of supercritical carbon dioxide and acidic treatments.

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Abstract

Tissue-engineering was important and popular which combine medical applications and engineering materials knowledge, just like cell extraction process was employed to remove the cellular components from porcine costal cartilages, leaving a framework of largely insoluble collagen, elastin, and tightly bound glycosaminoglycans (GAGs) in this work. A cell extraction process of supercritical carbon dioxide ($ScCO_2$) and acidic treatments was used to remove cellular components by porcine costal cartilages. Varying pore sizes and porosities of the acellular tissues were then created using enzymes and collagenase after acidic treatments. The porcine costal cartilages must remove lipids and other impurities almost by $ScCO_2$, otherwise adipose tissue will become rot immediately. For collagen scaffold samples, porcine costal cartilages were hydrolyzed by enzymes and pH reagents, in this way it can gain state completely and clearly. Electron microscopy revealed effect in decellularized extracellular matrix (ECM) of porcine costal cartilages via supercritical fluid and enzymes after acidic treatments.

Keywords: Extracellular matrix, ECM, Enzymes, Papain.

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Introduction

Recently numerous materials have been proposed, modified, combined, and employed for medical applications such as scaffolds for skin and bone tissue reconstruction. Synthetic materials, such as polyimide, polybetain, polyacrylate, polyurethane, polycarbonate, polynorborene, poly (lactide-co-glycolide), and polylactide have been used either alone or in combination with naturally derived materials including collagen, chitosan, starch and silk fibroin [1-11].

The costal cartilages are bars of hyaline cartilage that serve to prolong the ribs forward and contribute to the elasticity of the walls of the thorax. Costal cartilage is only found at the anterior ends of the ribs, providing medial extension. The objectives of the present manuscript are to provide an updated decellularization technique and their expected effects on the mechanical and biological properties of the extracellular matrix (ECM) scaffolds in orthopaedics. It's devoted to developed new biomedical materials for tissue-repairing, replace and improve human's tissue function [12-13]. Decellularized extracellular matrix (ECM) scaffolds had a lot of collagens, which constitute the main structural element of the extracellular matrix (ECM), provide tensile strength, regulate cell adhesion, support chemo taxis and migration, and direct tissue development [2]. Dense connective tissue is an abundant source of extracellular matrix (ECM) material, which can be extracted by defatting and decellularizing. The solubility changed with instrument's pressure and temperature in supercritical carbon dioxide ($ScCO_2$) procedure. Therefore, the resulting material has better permeability and stronger solubility. The fibrils are composed of collagen molecules that consist of a triple helix approximately 300 nm in length. Proteolytic cleavage of the properties results in triple helical collagen molecules that have short telopeptides at each end and can assemble into fibrils [12,13].

In this study, the procedure combined with supercritical carbon dioxide and enzymes after acidic treatments to prepare decellularized extracellular matrix (ECM) scaffolds with pore-scaffold microstructure, which show a good potential in the application for minimally invasive surgeries in orthopaedics. This study describes an effective procedure for the decellularization of porcine costal cartilages. Decellularized soft tissue such as hyaline cartilage that maintains their native collagen architecture may represent promising scaffolds for musculoskeletal tissue engineering applications. The resulting scaffolds must be characterized by Fourier transform infrared spectroscopy (FTIR) thermo-gravimetric analysis (TGA), and scanning electron microscope (SEM).

Materials and Methods

Materials

The chemicals used in the enzymes of Papain (Sigma-Aldrich Company), 3% TritonX-100 (Shanchai-Lingfeng Chemical Company), 2% NaOH (Sigma-Aldrich Company), 0.5 M acetic acid and 20% alcohol and sodium dihydrogen phosphate and disodium hydrogen phosphate (First Chemical Works Company, Taiwan).

The treatments by using supercritical carbon dioxide ($ScCO_2$)

Supercritical carbon dioxide ($ScCO_2$) was used in this study. The $ScCO_2$ was employed before alkaline or acidic treatments for removing fatty acids and tissues.

Decellularization by using alkaline treatments after supercritical carbon dioxide ($ScCO_2$)

The sample, which was treated with $ScCO_2$, is soaked in 25% NH_4OH (aq) or a mixture of 2% NaOH (aq) and 3% Triton (aq) at 25°C for 2hr with magnet mixer. The resulting samples were washed with PBS (0.2g potassium dihydrogen phosphate and 1.150g disodium dihydrogen phosphate) at the intermediate interval under ultrasonic wave to remove residual fat, and organic matter (Table 1)

Decellularization by using alkaline and papain

Treatments after Supercritical Carbon Dioxide ($ScCO_2$)

The sample, which was treated with $ScCO_2$, is soaked in a mixture of 2% NaOH (aq) and 3% Triton (aq) for 2 hr with magnet mixer, followed by 0.5 U/ml Papain (aq) at 25°C for 2 h. The resulting samples were washed with PBS (0.2g potassium dihydrogen phosphate and 1.150 g disodium dihydrogen phosphate) at the intermediate interval under ultrasonic wave to remove residual fat, and organic matter (Table 1).

Decellularization by using acidic treatments after supercritical carbon dioxide ($ScCO_2$)

The sample, which was treated with $ScCO_2$, is soaked in 0.5 M acetic acid for 2 hr, followed by a mixture of 2% NaOH (aq) and 3% Triton (aq) at 25°C for 2 hr with magnet mixer. The resulting samples were further treated with 0.5 U/ml Papain (aq) at 25°C for 2 h, washed with PBS (0.2 g potassium dihydrogen phosphate and 1.150 g disodium dihydrogen phosphate) at the intermediate interval under ultrasonic wave to remove residual fat, and organic matter (Table 1).

Preclinic envalution of scarfolds

A series of scaffolds were obtained. Further, the resulting scaffolds were analyzed and characterized by SEM, TGA, FTIR.

Table 1. *Preparation of collagen scaffold.*

Sample[a])	Reagent Concentration	Temperature
SC0		
SC1	2% NaOH (aq) + 3% Triton (aq)	25℃)
SC2	2% NaOH (aq) + 3% Triton (aq)/0.5 U/ml Papain (aq)	25℃)
SC3	0.5 M Acetic Acid (aq)/2% NaOH (aq) + 3% Triton(aq)/2% NaOH (aq) + 3% Triton (aq)/	25℃)
SC4	25% NH4OH (aq)	25℃)

[a]): ScCO2 was employed before Reagent treatments.

Results and Discussion

Fourier transform infrared spectroscopy analysis of resulting scaffolds

The structural construction due to the decellularization of porcine costal cartilages was confirmed by FTIR spectroscopy, as depicted in (Figure 1). All the peaks found in the FTIR spectrum were corresponding to collagen scaffolds with ECM structure. The entire scaffolds showed characteristic amide I (1640 – 1650 cm^{-1}), amide II (1558 cm^{-1}) and amide III (1239 cm^{-1}) absorption bands of collagen.

In usual, the characteristic absorption band of lipids would be observed in the range of 1720 – 1740 cm^{-1} and 1270 – 1280 cm^{-1}, which corresponds mainly to the ester carbonyl group and – CH group. The scaffold showed the characteristic absorption band of amide A(~3331 cm^{-1}) and exhibited a broad band associated with the N – H stretching vibration of hydrogen-bonded amide groups. Amide I (~1640 cm^{-1}) corresponds to the carbonyl group, which is present within the triple helix structure in the secondary structure of the collagen protein. The N – H bending and N – H stretching vibration were assigned to amide II (1558 cm^{-1}) and amide III (1239 cm^{-1}), respectively. All scaffolds exhibited C – H stretching absorption bands at 2987 cm^{-1} and 2933 cm^{-1}, corresponding to the CH2 and CH3 in the scaffolds. The IR spectral data exhibit all the characteristic peaks corresponding to the entire scaffolds. The absorption band at ca. 3354 cm-1 δ(C-H) was attributed to the fatty acid. The FTIR spectrum of scaffold sample SC4 treated with various alkaline solutions such as NH4OH (aq) or a mixture of 2% NaOH (aq) and 3% Triton (aq) showed relative high values of fatty acids/amides I which implied high lipids residues as shown in (Figure 1a and Figure 1b). The FTIR spectrum of scaffold sample SC3 treated with an aqueous acidic solution showed the relative low values of fatty acids/amides I which implied low lipids residues in the resulting scaffold as shown in (Figure 1c).

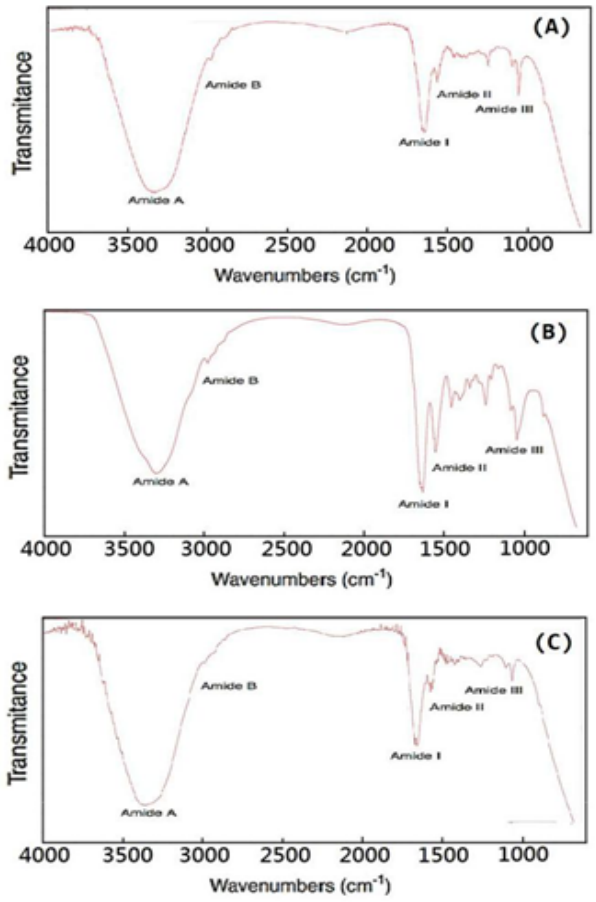

Figure 1. *FTIR spectrum of the resulting scaffold sample: (a) SC2, (b) SC3 and (c) SC4.*

The microstructure of resulting scaffolds

The microstructures of resulting scaffolds were characterized by scanning electron microscope(SEM). Scanning electron micrographs of decellularized samples were showed in (Figure 2). However, the pore-scaffold microstructure could not be remarkably observed in the samples such as SC0, SC2, and SC4 (Figure 3), derived from porcine costal cartilages by using alkaline treatments such as a mixture of 2% NaOH (aq) and 3% Triton (aq) or an aqueous solution of NH_4OH after supercritical carbon dioxide ($ScCO_2$). Even papain was further employed to move the residue of small lipid and protein molecules such as sample SC3, the pore-scaffold microstructure could not be remarkably observed. These behaviours could be due to the relative high compacted microstructures of porcine costal cartilages. It is quite difficult to remove the small molecules such as lipids and proteins.

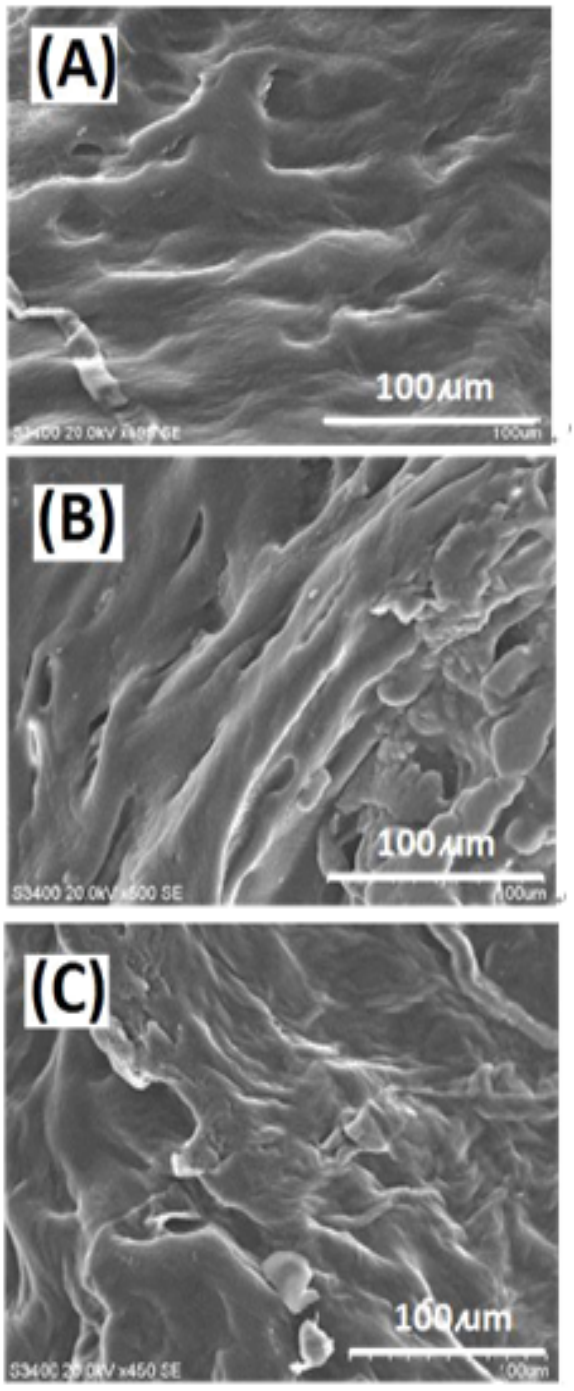

Figure 2. *Scanning electron micrographs of the sample after $ScCO_2$. Sample: (A) SC0, (B) SC1, and (C) SC2.*

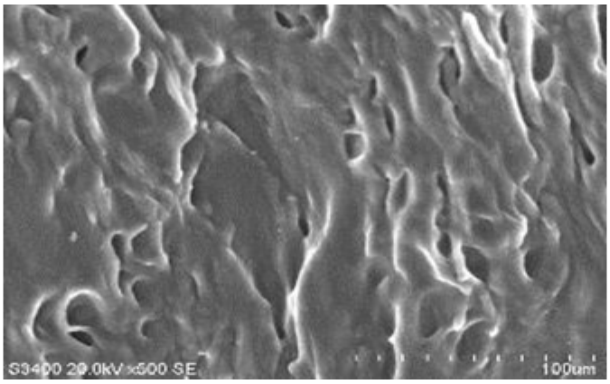

Figure 3. *Scanning electron micrographs of the decellularized sample (SC4).*

The acidic treatment was considered to solve the decellularized problems of porcine costal cartilages because the porcine costal cartilages would be swollen in the solution of acetic acid. The swollen microstructure could decrease the limitation of small molecules in ECM and provide relative good permeability and strong solubility of costal cartilages. Figure 4 showed the SEM results of SC3, which was decellularized by using an acidic treatment with an aqueous solution of acetic acid after supercritical carbon dioxide ($ScCO_2$). Then the mixture of 2% NaOH (aq) and 3% Triton (aq) and following papain (aq) were employed with the same procedures of samples SC3. The pore-scaffold microstructures could be observed. The diameter of pores could be observed in the range between 30 μm and 50 μm. From (Figure 1a and 1b) the relative peak values of fatty acids/amides I in the spectrum of sample SC3 tend to slow down, demonstrating the effectiveness of the acidic treatments after supercritical carbon dioxide treatment.

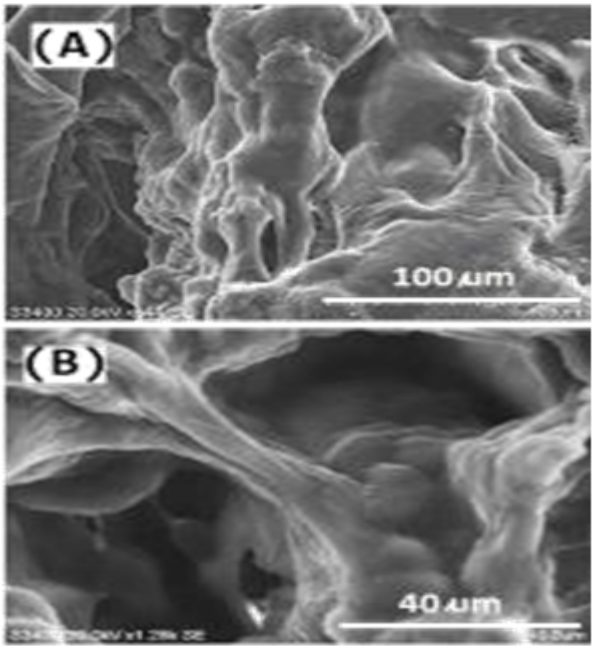

Figure 4. *Scanning electron micrographs of the decellularized sample (SC3). (A) X544 and (B) X1.28K.*

Thermal stability of resulting collagen of scaffolds

Thermal stability of resulting scaffolds could be characterized by TGA. The main loss is presented in two different temperature ranges given by I: (<150°C) and II: (150 – 450°C). The curve in I corresponded to the loss of the physisorbed and chemical water in the resulting decellularized extracellular matrix scaffold, which represented the 70 wt % of the decellularized extracellular matrix scaffold, which occurred at 80°C. The following loss, occurring in the range of temperatures II in the thermo gram, for the scaffold was observed at 335°C as shown in (Figure 5a). The losses are related to the combustion of scaffolds. From Figure 5b, the main loss is also presented in two different temperature ranges given by I: (<150°C) and II: (150 – 450°C). The 50 wt% loss of the resulting decellularized extracellular matrix scaffold after acidic treatments was observed at 80°C. The following loss, occurring in the range of temperatures II in the thermo gram, related to the combustion of scaffolds after acidic treatments was observed at 341°C as shown in (Figure 5b). The small molecules were removed and the thermal stability of resulting scaffold was improved. Relative high Td values could be obtained.

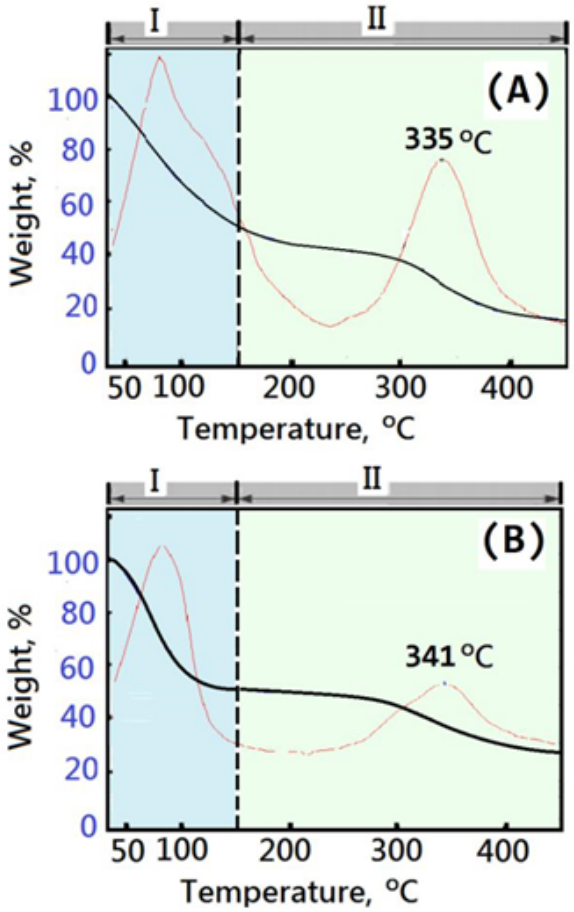

Figure 5. *Thermogravimetric analysis of decellularized sample: (A) SC0 and (B) SC3.*

Conclusion

In this study, a series of new scaffolds were obtained from porcine tissue by using supercritical carbon dioxide fluid technology. The retain extra-cellular matrix and integrity scaffold-structure was observed. The Tdmax of the resulting decellularized extracellular matrix scaffolds were observed over 300°C. The decellularized extracellular matrix scaffolds with high thermal stability were obtained. The resulting scaffolds could be a potential application for minimally invasive surgeries in orthopedics.

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