Preparation and physical properties of chitosan scaffolds coated collagen from the skin of shark for bone tissue engineering

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Abstract

Secondary alveolar bone grafting is routinely practiced as the alveolar cleft treatment in cleft lip and cleft palate patient. Most commonly, bone for alveolar bone grafting is harvested from the iliac crests. As such, iliac crest harvesting procedures can result in paresthesia, hypersensitivity, infection and pelvic instability. In order to avoid these adverse effects, tissue engineering strategies may eliminate donor site morbidity by resorbable collagen sponge resulted in reduced donor site morbidity and decreased donor site pain intensity and frequency. The aim of this study was to investigate the effects of pepsin soluble collagen (PSC) from skin of brownbanded bamboo shark on the physical property of chitosan scaffolds. The collagen was characterized as type I collagen by Fourier transform infrared (FTIR) spectra and physical properties were studied in terms of morphology, water swelling and biodegradation of scaffolds. Physical property data were analyzed by Mann-Whitney U Test and using SPSS statistics version 16.0 program. The result of FTIR showed triple-helical structure of collagen type I. SEM demonstrated homogeneous microstructure and presence of interconnected micropores of both groups. Water swelling of PSC coated chitosan scaffolds was lower than chitosan scaffolds ($p<0.001$), whereas biodegradation tend to be lower ($p>0.05$). Biodegradation rates of both groups between different time points were statistically significant ($p<0.05$). In conclusion, PSC collagens improve physical properties of our novel chitosan scaffolds. (Supported by PSU grant # 950/427)

Keywords: Cleft lip and palate, Bone tissue engineering, Chitosan scaffold, Collagen

Introduction

The alveolar cleft is a bony defect that is present in 75% of the patients with cleft lip and palate (Aranaz et al., 2009). Secondary alveolar bone grafting is routinely practiced as the alveolar cleft treatment to allow proper eruption of the lateral or canine teeth through the cleft segment and to close the oronasal fistula. Most commonly, bone for alveolar bone grafting is harvested from the iliac crests (Cho-Lee et al., 2013; Goudy, Lott, Burton, Wheeler, & Canady, 2009; Moreau, Caccamese, Coletti, Sauk, & Fisher, 2007). Therefore, iliac crest has been described as being the gold standard for secondary grafting. However, the harvest procedure imposes a heavy burden on the patient especially at the donor site (Swan, 2006). As such, iliac crest harvesting procedures can result in paresthesia, hypersensitivity, infection and pelvic instability (Kortebein, Nelson, & Sadove, 1991; Younger & Chapman, 1989; Janssen, Weijs, Koole, Rosenberg, & Meijer, 2014). In order to avoid these adverse effects, tissue engineering strategies may eliminate donor site morbidity by bypassing the harvesting procedure, such as synthetic bone or stem cells with scaffold. Tissue engineering (TE) is a multidisciplinary field that attempts to restore the function of diseased or damaged tissues through the use of cells, biomaterials and biologically active molecules (Becker & Jakse, 2007; Drosse et al., 2008; Meyer, Joos, & Wiesmann, 2004; Vacanti & Langer, 1999). Bone tissue engineering is the

process of regeneration of functional tissue that can be used to treat a bony defect (Peppo et al., 2013). The scaffolds should act as a framework for supporting growth and functions of desired tissue as well as maintaining shape and contour of the organ (Kim, Kim, Oh, Han, & Shin, 2009). Two features of the scaffolds that influence cellular responses are 3-D architecture and the physico-chemical properties of their surfaces. The scaffolds should have the 3-D structures with highly porous and interconnected pore network for cell in-growth and transporting nutrients and metabolic waste. Chitosan presents superior tissue biocompatibility due to its structure being similar to glycosaminoglycan in an extracellular matrix (Arpornmaeklong, Suwatwirote, Pripatnanont, & Oungbho, 2007). Moreover, it has been proven to demonstrate properties needed for bone tissue engineering which include having biodegradability, a porous structure, suitability for cell ingrowth, osteoconduction, and an intrinsic antibacterial nature (Thuaksuban, Nuntanaranont, Pattanachot, Suttapreyasri, & Cheung, 2011). Collagen is a natural protein that is the main component in extracellular matrix (ECM) in tissue. Especially, in bone tissue, collagen acts as the template for calcium phosphate deposition. Collagen can enhance stability and strength of the bone (Matsuura et al., 2014). Therefore, collagen is a popular material for tissue regeneration because collagen has biological functionality that cells can recognize. Such functionality can enhance cell adhesion lead to induce tissue regeneration. Alternative sources of collagen especially from the shark skin can be used as an excellent source of collagen with the unique characteristics. The aim of this study is to produce a chitosan scaffolds coated collagen from the skin of shark for bone tissue engineering in cleft palate patients.

Materials and methods

1. Fabrication of chitosan scaffold

To construct the scaffolds, chitosan (Sea Fresh Chitosan [Lab] Co., Thailand, DD=85%, Mw=57,000 Dalton) was dissolved in 0.2 M acetic acid in the final concentrations of 2% (v/w). Then they were injected with syringe into 1 M NaOH. Under these conditions a fibril-like chitosan was formed. The fibril-like chitosan was filtered through a sheet cloth, and placed in 15 ml centrifuge tubes and centrifuged at 4,500 rpm for 40 min then kept at 4°C for 24 hr and subsequently frozen at -20°C. After 24 hr, the scaffolds were stabilized by immersing in 96% alcohol for 1 hr, 1 M NaOH for 5 min, and 70% alcohol for 12 hr. The thus formed scaffolds were sectioned into slices with 3 mm of thickness. They were dried at 37°C for 12 hr.

2. Isolation of Pepsin soluble collagen (PSC) from the skin of brownbanded bamboo shark (Chiloscyllium punctatum)

2.1 Shark skin preparation

Skin of brownbanded bamboo shark (Chiloscyllium punctatum) with the size of 70-100 cm was obtained from Blue Ocean Food Products Co., Ltd. in Samutsakhon Province of Thailand. The frozen shark skin (10 kg) packed in polyethylene bags (1 kg/bag) was placed in ice at a ratio of skin to ice of 1:2 (w/w) using a polystyrene box as a container. The skin was kept at -20°C until use, usually within one week. To prepare collagen from shark skin, the frozen skin was thawed with running water until the core temperature reached 5°C. Thereafter, it was washed with cold tap water ($\leq$10°C). The residual meat on shark skin was removed by knife and washed with cold tap water

until any residual smell of ammonia disappeared. The clean shark skin was cut into small pieces (1.0 × 1.0 cm^2) using a pair of scissors.

2.2 Pretreatment of shark skin

To remove non-collagenous proteins, the prepared shark skin was mixed with 0.1 M NaOH to alkali solution at the ratio of 1:10 (w/v). The mixture was continuously stirred for 6 hr. The alkali solution was changed every 2 hr. Then, the deproteinised skin was washed with cold water until a neutral or faintly basic pH of wash water was reached.

2.3 Extraction of Pepsin Soluble Collagen (PSC)

Pretreated skin was soaked in 0.5 M acetic acid with a solid to solvent at the ratio of 1:15 (w/v) for 48 hr with continuous stirring using an overhead stirrer model W20 (IKA - Werke GmbH & CO.KG, Stanfen, Germany). The mixtures were filtered with two layers of cheesecloth. The residue was soaked in porcine pepsin (20 unit/g of residue) solution. The mixtures were continuously stirred at 4°C for 48 hr, followed by filtration with two layers of cheesecloth. The collagen in the filtrate was precipitated by adding NaCl to a final concentration of 2.6 M in the presence of 0.05 M tris (hydroxymethyl) aminomethane at pH 7.5. The resultant precipitate was collected by centrifugation at 20,000 g at 4°C for 60 min using a refrigerated centrifuge model Avanti J-E (Beckman Coulter, Inc., Palo Alto, CA, USA). The pellet was dissolved in a minimum volume of 0.5 M acetic acid and dialysed against 25 volumes of 0.1 M acetic acid for 12 hr. Thereafter, it was dialysed against 25 volumes of distilled water for 48 hr. The resulting dialysate was freeze-dried and referred to as ''Pepsin soluble collagen, PSC" (Benjakul et al., 2010).

2.4 Fabrication of PSC coat 2% chitosan scaffold

2% chitosan scaffold was transferred to 0.05% PSC solution in 24 well-plate (PSC was dissolved in acetic acid and adjusted volume by PBS), kept at room temperature for 4 hr, then kept at 4°C for 24 hr, subsequently frozen at -20°C for 24 hr and transferred to freeze-dried machine (SCANVAC) for 24 hr.

3. Scaffolds characterization

3.1 Fourier-Transformed Infra-Red Spectroscopy (FTIR)

Infra-red spectra of the prepared collagen was obtained in a spectrometer (Spectrum 100 PERKIN ELMER) at the range of 4000 to 650 cm^{-1}. Spectra were registered using attenuated total reflection infra-red spectroscopy (ATR-FTIR).

3.2 Scanning Electron Microscope (SEM)

The morphology of the scaffolds were observed using a JEOL JSM 6460 LV microscope under 20 kV. All samples were coated with a thin gold layer using a EMITECH K550 sputter coater.

3.3 Water swelling of scaffold

PSC coated chitosan scaffolds for test group and uncoated-PSC chitosan scaffolds for control group (n/group =9) were weighed using an electronic balance and placed in PBS for 5 min. After this period, all excessive water was removed and the scaffolds were weighed again. Water swelling of scaffolds were determined by using the following equation:

Swelling ratio = (Ww-Wd)/ Wd

Wd represents initial dry weight and Ww represents wet weight of scaffold.

3.4 Biodegradation of scaffold

To analyze the biodegradation of scaffold, they were incubated in a solution containing lysozyme, an enzyme taken to be essential in the dissolvement of chitosan. Scaffolds (n=9) were incubated for 7,14 and 21 days in 1×10^4 U/ml lysozyme in PBS (pH 7.4) at 37°C. At the different

time intervals, the scaffolds were washed with double distilled water and were dried at 37°C 48 hr. All scaffolds were weighed again. Biodegradation of scaffold were determined by using the following equation:

Percentage weight loss = (Wo-Wt)/ Wo × 100

Wo represents the original weight and Wt represents the weight at the different time interval.

Results and Discussion

Scaffolds characterization

1. Fourier-Transformed Infra-Red Spectroscopy (FTIR)

FTIR spectra of PSC from the shark skin is shown in Figure1. The major peaks in the spectra of PSC from the skin of brownbanded bamboo shark were similar to those of collagen from others fish species (Muyonga, Cole, & Duodu, 2004; Nagai, Suzuki, & Nagashima, 2008; Wang, An, Xin, Zhao, & Hu, 2007). The amide A band PSC was found at 3299 cm^{-1}, respectively. This band is generally associated with the N-H stretching vibration and shows the existence of hydrogen bonds. When the NH group of a peptide is involved in a hydrogen bond, the position is shifted to lower frequencies. Amide B band of both collagens was observed at 2939 cm^{-1}, in agreement with that reported by Nagai et al. The sharp amide I band of PSC was observed at 1630 cm^{-1}, respectively. This band is associated with C=O stretching vibration or hydrogen bond coupled with COO^- (Payne & Veis, 1988). The amide I peak underwent a decrease in absorbance, followed by a broadening accompanied by the appearance of additional shoulders when collagen was heated at higher temperature (Bryan et al., 2007). This was reconfirmed by the ratio of approximately 1 between amide III and 1454 cm^{-1} band of both collagens. Ratio of approximately 1 revealed the triple-helical structure of collagen (Krimm & Bandekar, 1986). The amide II of collagens appeared at 1546 cm^{-1}, resulting from N-H bending vibration coupled with CN stretching vibration (Yan et al., 2010).

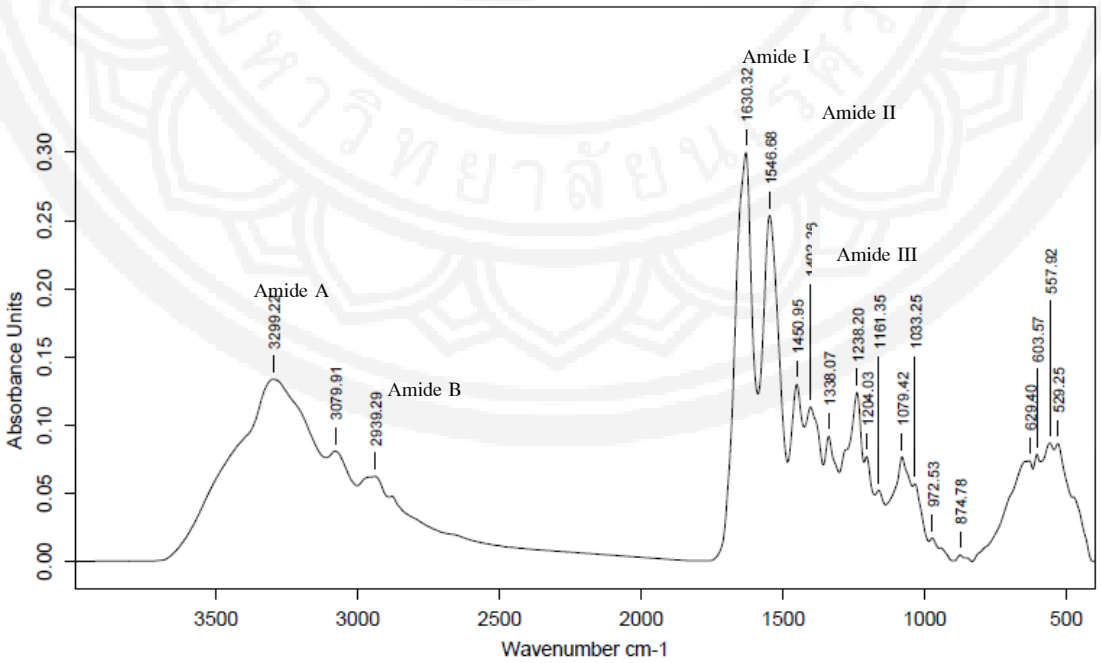
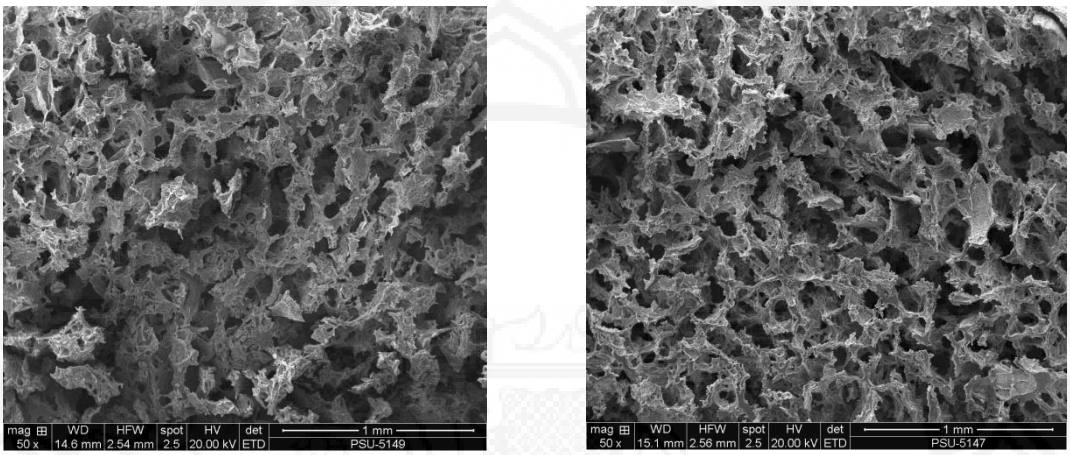


Figure 1 FTIR spectra of PSC from the skin of brownbanded bamboo shark.

2. Scanning Electron Microscope (SEM)

Figure 2 shows SEM micrographs of the chitosan and PSC coated chitosan scaffolds. Open pore structure with a high degree of interconnectivity can be observed. An ideal scaffold used for tissue engineering should possess the characteristic of a homogenous microstructure and suitable pore aperture. Scaffolds must be porous to allow ingrowths of cells and migration of vascular tissue. Since the addition of collagen increased chitosan pore sizes, cell viability and proliferation in these scaffolds might be improved.



(a) (b)

Figure 2 SEM micrographs a) PSC coated chitosan scaffold b) chitosan scaffold

3. Water swelling of scaffold

Swelling ratio is shown in Figure 3. The chitosan scaffold showed the high water binding capacity and this property plays an important role in tissue regeneration (Park, Lee, Lee, & Suh, 2003). It could preserve a high volume of liquid within the porous structure, maintained their dimension, and further enhance the penetration of cells into the inner area of scaffold (Ngamwongsatit, Banada, Panbangred, & Bhunia, 2008; Park, Park, Kim, Song, & Suh, 2002; Shanmugasundaram et al., 2001). The PSC coated chitosan scaffold seem to decreasthe swelling ratio. As the morphological structure of scaffolds had fibril network structure in the porous. Such fibril network effected on decreasing of swelling ratio. This result was similar to the previous study that the swelling property of sponge-like matrice dependent on the network porous structure and microstructure of scaffold (Park et al., 2002).

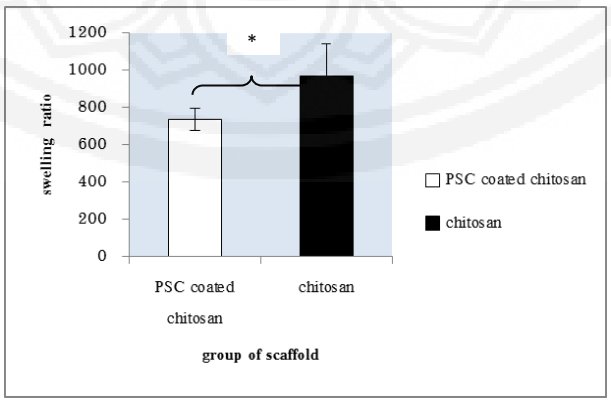


Figure 3 The swelling ratio of scaffold in each group. Compairisons among group, analyzed by Mann-Whitney U test, n=9, (*P<0.05)

4. Biodegradation

The PSC coated chitosan scaffold had more stability from biodegradation than the chitosan scaffold. The result indicted that collagen could improve biodegradation of scaffold. Chitosan is mainly degraded by lysozyme (Vårum, Myhr, Hjerde, & Smidsrød, 1997) which is present in various human body fluids and tissue (Köse, Kenar, Hasirci, & Hasirci, 2003). The rate of scaffold degradation should mirror the rate of new tissue formation or be adequate for the controlled release of bioactive molecules(Aranaz et al., 2009).

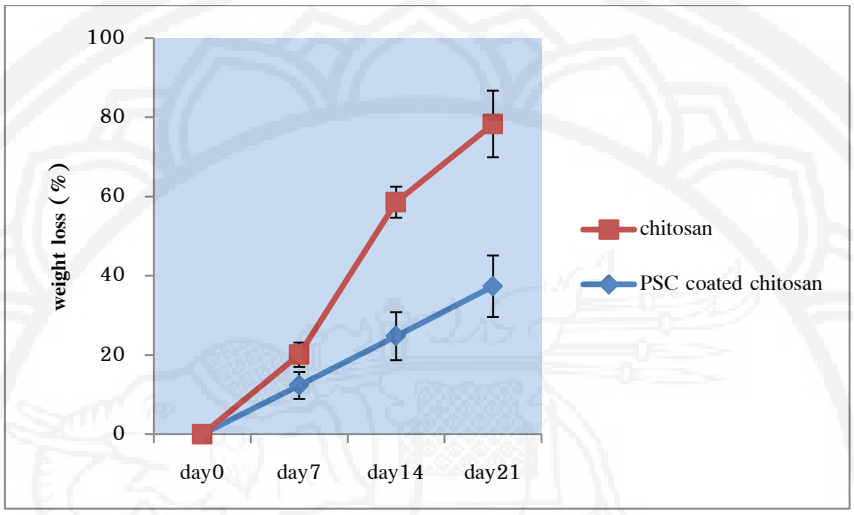


Figure 4 Biodegradation of scaffold after digestion with lysozyme at Day 7, 14 and 21. Between different time point in each group, P< 0.05, analyzed by Kruskal Wallis test, n=9

Conclusions

By using the centrifuge method porous chitosan scaffold was successfully produced. Extraction of collagens from the skin of brownbanded bamboo shark could be achieved by pepsin solubilization. Modification of chitosan scaffolds by coatings with PSC from shark skin for bone tissue engineering was proposed in this study. PSC improved our novel chitosan scaffold physical properties. Further biological property investigations need to be carried out prior to *in vivo* studies in animal and cleft palate patients for future clinical applications.

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