Journal of Metals, Materials and Minerals. Vol. 16 No.1 pp.37-44, 2006

Properties of Collagen/Chitosan Scaffolds for Skin Tissue Engineering

Chalonglarp TANGSADTHAKUN[1], Sorada KANOKPANONT[1], Neeracha SANCHAVANAKIT[2], Tanom BANAPRASERT[3], Siriporn DAMRONGSAKKUL[1],*

[1]Department of Chemical Engineering, Faculty of Engineering, [2]Department of Anatomy, Faculty of Dentistry, [3]Department of Otolaryngology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

Abstract

Biopolymer blends between collagen and chitosan have the potential to produce cell scaffolds with biocompatible properties. In this study, porous scaffolds were fabricated by freeze drying the solution of collagen and chitosan and crosslinked by dehydrothermal treatment (DHT). Various types of scaffolds were prepared by varying compositions of collagen and chitosan. The scaffolds were fully characterized by Fourier transform infrared (FT-IR) spectroscopy. The results proved that collagen and chitosan scaffolds in all blending compositions contained only physical but not chemical interaction in molecular level. The compressive modulus from a universal mechanical testing machine decreased with increasing the compositions of chitosan. Equilibrium swelling ratios of approximately 6-8, carried out in phosphate buffered saline (PBS) at physiological pH (7.4) were found in case of collagen dominate scaffolds. The lysozyme biodegradation test demonstrated that the presence of chitosan could significantly prolong the biodegradation of collagen/chitosan scaffolds. The collagen/chitosan scaffold was more effective to promote and accelerate L929 cell proliferation, particularly for scaffolds containing 30% of chitosan. The results elucidated that the blends of collagen with chitosan have a high possibility to be applied as new materials for skin tissue engineering.

Keywords : Collagen, Chitosan, Fibroblast, Scaffold, Skin tissue engineering

Introduction

In the past decades, many skin substitutes such as xenografts, allografts, and autografts have been employed for wound healing of large dermal defects. However, because of the antigenicity, the limitation of donors and donor sites, they cannot accomplish the complete recovery of the skin. Therefore, many studies are turning toward the tissue engineering approach to promote tissue regeneration and to sustain and regain organ functions. One crucial factor in skin tissue engineering is the construction of a scaffold. A three-dimensional scaffold provides an extracellular matrix (ECM) analog which functions as a necessary template for host infiltration and a physical support to guide the proliferation and differentiation of cells into the functional tissues or organs.(6)

Porous three-dimensional scaffolds have been used extensively as biomaterials in the field of tissue engineering for *in vitro* study of cell–scaffold interactions and tissue synthesis and *in vivo* study of induced tissue and organ regeneration. Regardless of the application, the scaffold material, as well as the three-dimensional structure of the scaffold, has a significant effect on cellular activity. They act as a physical support structure and as an insoluble regulator of biological activity that affects cell responses. Collagen is a significant constituent of the natural extracellular matrix. Scaffolds made of collagen have been used in a variety of applications due to a number of useful properties; such as hemostatic effect, low antigenicity, and good mechanical characteristics for use in soft tissue engineering applications.(11) In addition, collagen scaffolds have

*corresponding author e-mail: siriporn.d@chula.ac.th

A part of this work has been presented in medical technology at BioThailand2005: Biotechnology Challenges in the 21st Century, 2 - 5 November 2005, The Queen Sirikit National Convention Center, Bangkok, Thailand.

been observed to promote cell and tissue attachment and growth.[10-11] Collagen contains basic residues, such as lysine and arginine, and specific cell adhesion sites such as arginine-glycine-aspartate (RGD) groups. The RGD group actively induces cellular adhesion by binding to integrin receptors, and this interaction plays an important role in cell growth, and in the differentiation and overall regulation of cell functions.[13] Collagen is known to be the most promising material in tissue engineering appli - cations for their excellent biocompatibility and biodegradability. However, its fast biodegradation and the low mechanical strength are the problem issues that limit further uses of this material.[6] For this reason, the blending of biodegradable polymers has been employed to produce scaffolds. Chitosan, an amino polysaccharide (poly-1,4-D-glucosamine) derived from chitin by deacetylation, has been widely applied in biomedical applications, such as wound dressings and drug delivery systems on account of its non-toxic and biocompatible nature.[16, 18] Since chitosan composes of both reactive amino and hydroxyl groups that can be chemically or physically modified, it has a high potential in tissue engineering applications. One of the most interesting effects of chitosan on wound healing is the formation of granulation tissue with angiogenesis. It is reported that chitosan induces fibroblasts to release interleukin, which is involved in migration and proliferation of fibroblasts.[21] In the inflammatory phase chitosan has unique hemostatic properties that are independent of the normal clotting cascades.[3] Therefore, chitosan, a novel biomaterial, is introduced for fabricating collagen/chitosan scaffolds. However, it is still not clear about the complete report of the full-range blends between collagen and chitosan and their effects on both physical and biological properties of collagen/chitosan scaffolds. This study focused on the characterizations of hybrid scaffolds fabricated from porcine skin type I collagen and chitosan.

Experimental Procedure

Materials

Type I collagen from porcine skin was purchased from Nitta Gelatin Inc. (Osaka, Japan). Middle-viscous chitosans from crab shell and lysozyme from hen egg-white (70,000 Unit/mg) were purchased from Fluka (Germany). The chitosan used has a viscosity-averaged molecular weight (M_v) of 320k and degree of deacetylation of 84.43% as determined by the viscometric method[14,20] and FT-IR spectroscopy[8] respectively. The viscosity-averaged molecular weight was calculated using the classical Mark-Houwink equation $[\eta] = KM_v^a$, where the constants $K = 3.5\times10^{-3}$ and $a = 0.76$.

Fabrication of Collagen/Chitosan Scaffolds

Collagen (0.5% (w/w)) and chitosan (0.5% (w/w)) solution in 0.5 M acetic acid were blended at different weight ratios. To obtain a homogeneous blend, the solution was stirred for 1 h and degassed under vacuum. Degassed solution was pipetted into each well of 24-well cell culture plates. The samples were frozen at -40°C for 24 h prior to lyophilization under vacuum pressure (<100 mTorr) at the condenser temperature of -40°C for 24 h to generate a porous structure. The lyophilized samples were then crosslinked via dehydrothermal treatment (DHT) at thetemperature of 105°C for 48 h.

Determination of Scaffold Properties

Fourier Transform Infrared (FT-IR) Spectroscopy

Films of collagen and chitosan blends were obtained by casting solution onto a teflon plate. After solvent evaporation for 24 h at room temperature, the samples were further crosslinked by vacuum-heating at 105°C for 48 h. Theinformation on structural contribution was collected in the FT-IR analysis using a Perkin Elmer Spectrum GX (FT-IR system). Films used in the infrared tests were about 10 μm thickness. The FT-IR analysis was based on the identification of absorption bands concerned with the vibrations of functional groups presented in macromolecules.

Compressive Modulus

A universal mechanical testing machine (INSTRON 5567, NY, USA) was used to determine the compressive modulus of scaffolds by compressing the sample discs (13 mm in diameter and 3 mm in thickness) at a constant deformation rate of 0.5 mm/min. The slopes of compressive stress-strain curves at 5 to 35% deformation were used to calculate the compressive modulus and reported values are the mean of seven specimens.

Equilibrium Swelling Ratio Determination

The equilibrium swelling ratio (E_s) was measured by the conventional gravimetric method. The dry weight of scaffold was measured before immersing in 0.05 M phosphate buffer saline (PBS) pH 7.4 at a temperature of 37°C and excess surface phosphate buffer saline was blotted out with absorbent paper. The wet weight (W_s) of the scaffold was determined after being incubated for 24 h. The equilibrium swelling ratio of the scaffolds was defined as the ratio of weight increase (W_s-W_d) with respect to the initial weight (W_d) of dry samples. Each value was averaged from three parallel measurements. Es was calculated using the following equation:

$$E_s = \frac{W_s - W_d}{W_d} \qquad (1)$$

where W_s and W_d denote the weights of swollen and dry samples, respectively.

Morphology

The morphology of collagen, collagen/ chitosan, and chitosan scaffolds was analyzed using a canning electron microscope (SEM, Joel JSM 5400) at an accelerating voltage of 12–15 kV. Dry scaffolds were sputter-coated with gold at 40 mA prior to observing under SEM.

***In vitro* Degradation**

The scaffolds of known dry weights were sterilized by immersing in 70% ethanol. and digested in 0.05 M phosphate buffer saline (PBS, pH 7.4) at 37°C containing 1.6 μg/ml (112 Units/ml) lysozyme (hen egg-white). The concentration of lysozyme used corresponded to the concentration in human serum.[1, 12] The lysozyme solution was refreshed daily to ensure continuous enzyme activity. After 7, 14, 21 and 28 days, samples were removed from the medium, rinsed with distilled water, frozen, lyophilized, and weighed. The experiment was done in triplicates for each scaffold. The extent of degradation was expressed as a percentage of weight remained of the dried scaffold after lysozyme digestion. To separate between enzymatic degradation and dissolution, control samples were stored for 28 days under the same conditions without addition of lysozyme. The percentage of weight remained was calculated using the following equation:

$$\text{Weight remained} = \frac{W_i - W_f}{W_i} \times 100 \qquad (2)$$

where Wi represents the initial weight of scaffolds and W_f represents the weight of digested scaffolds.

Cells Adhesion and Proliferation Tests

A mouse connective tissue fibroblast, L929, was selected to evaluate adhesion and proliferation as a direct contact test. The collagen/ chitosan scaffolds (as matrices of 13 mm in diameter, 2 mm in thickness) were immersed in 70% ethanol for 5 min for sterilization, followed with solvent exchange by deionized water. The scaffolds were then placed on a 24-well polystyrene plate and culture medium was added to each well before cell seeding. Cells were allowed to initially attach for 5 h. For proliferation testing, cells were seeded onto each of the matrices and cultures were harvested after 5, 24, and 72 h. The attached or proliferated cells were then quantified by the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay.[9] MTT solution (0.5 mg/ml in Dulbecco's modified eagle medium (DMEM) without phenol red, filter-sterilized) were added to each culture well. After incubation for 5 h, MTT reaction medium was removed, and 900 μl of dimethylsulfoxide and 100 μl of glycine buffer (pH 10.5) were added. The optical densities were determined by a spectrophotometer (Genesis 10 UV scanning, NY, USA) at the wavelength of 570 nm.

Statistical Analysis

Significant levels were determined by the paired t-test. All statistical calculations were performed on the SPSS system for Windows (version 13.0, Statistical Package for Social Sciences (SPSS), Chicago, IL, USA). P-values of <0.05 were significantly considered.

Results and Discussions

FT-IR Spectrophotometric Analysis

FT-IR spectra obtained from pure collagen, collagen/chitosan, and pure chitosan films was shown in Figure 1. In the spectrum of pure collagen, five characteristic absorption bands at the frequencies of 3439, 3324, 1659, 1550, and 1274 cm^{-1} could be observed. Generally, amide I bands

(1659 cm^{-1}) originated from C=O stretching vibrations coupled to N–H bending vibrations. The amide II bands (1550 cm^{-1}) arised from the N–H bending vibrations coupled to C–N stretching vibrations. The amide III represented the combination peaks between N-H deformation and C–N stretching vibrations. The other two bands, arising from the stretching vibrations of N–H group in free, of a medium to weak intensity, appeared at 3324 cm^{-1} and the vibrations of hydroxyl group, –OH, appeared at 3440 cm^{-1}.[16] For the spectrum of pure MMW chitosan, the characteristic absorption bands of chitosan were observed at six locations. The vibrations of hydroxyl and free amine groups appeared at 3439 and 3300 cm^{-1}, respectively. The absorption bands at 1655, 1560, and 1381 cm^{-1} indicated C=O stretching, $–NH_2$ bending, and C–O stretching of primary alcohol groups, respectively. The last one at 1152 cm^{-1} represented -C-O-C- glycosidic linkage between chitosan monomers.[16, 17] FT-IR spectra of collagen/LMW chitosan blends at various blending compositions illustrated similar characteristic peaks of the parent molecules. For example, the intensity of amide I peak at 1650 cm^{-1} started to decrease gradually when increasing the proportion of chitosan. On the other hand, the characteristic intense peak of chitosan, such as glycosidic linkages, appeared more clearly when the composition of chitosan was increased. The results suggested the possible domination of physical interactions between collagen molecules and chitosan molecules and minimum chemical interactions in the blends. The similar results were reported by.[16]

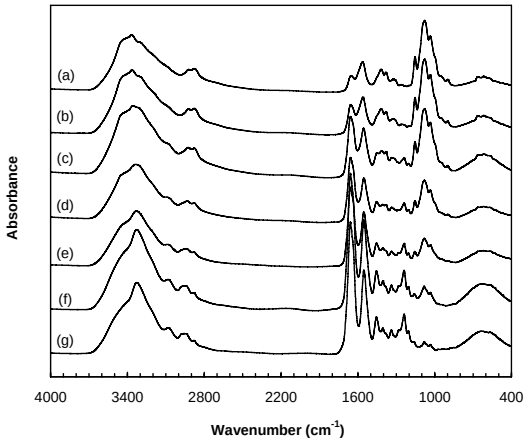

Figure 1. FT-IR spectra of collagen/chitosan scaffolds with different blending compositions : (a) 0/100, (b)10/90, (c)30/70, (d)50/50, (e)70/30, (f)90/10,and(g)100/0collagen/chitosan).

Compressive Modulus

The compressive modulus of collagen/ chitosan scaffolds was shown in Figure 2. The compressive modulus gradually decreased when the concentration of chitosan was increased. As the fraction of chitosan was as high as 90%, the compressive modulus was the same as that of pure chitosan scaffolds. This was similar to the previous work of.[19] reported on the mechanical and biological properties of collagen and chitosan blends. They proposed that the presence of chitosan induced softening to collagen scaffolds.

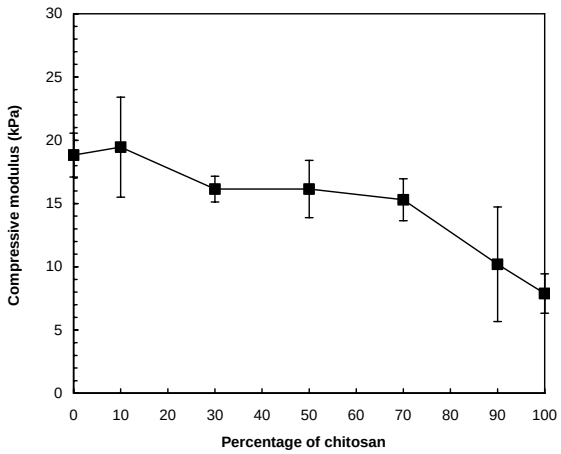

Figure 2. Compressive modulus of collagen/chitosan scaffolds with different blending composition

Equilibrium Swelling Ratio

As shown in Figure 3, the swelling ratios of collagen/chitosan scaffolds with different molecular weights could be clearly distinguished into two groups. The first group of the blends containing chitosan less than 30% (w/w) showed good swelling ratios which were similar to that of pure collagen. The other group of which the swelling ratios were as low as that of pure chitosan was the blends having chitosan composition more than 30%. This was because they lost the gel-like structure after swollen in PBS. The collagen/ chitosan scaffolds with the blending compositions of 100/0, 90/10, and 70/30 were selected to test the morphology and further biological characterization because of their excellent swelling ability, those blends were considered to have a high surface area and thus the cells can attach and grow in a three-dimensional fashion.

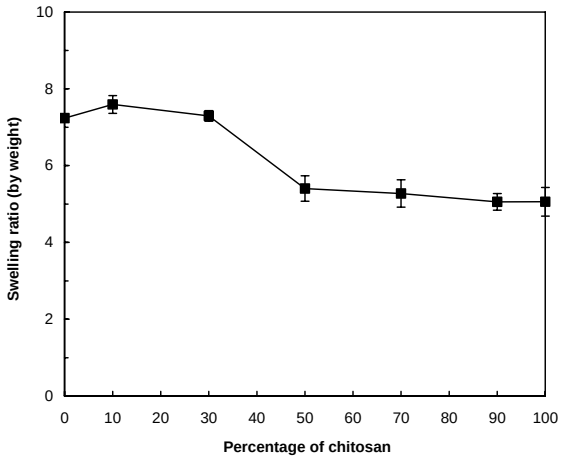

Figure 3. Equilibrium swelling ratio of collagen/ chitosan scaffolds with different blending compositions.

Morphology

Morphology of collagen and collagen/ chitosan scaffolds, revealed by SEM photographs in Figure 4, indicated the porous structure with a three-dimensional interconnection throughout the scaffolds in all compositions. The interconnection of pores could still be observed after increasing the proportion of chitosan up to 30%. On the other words, no significant difference in porous structure between collagen and collagen/chitosan scaffolds was noticed except a slight increase in pore sizes at high chitosan contents.

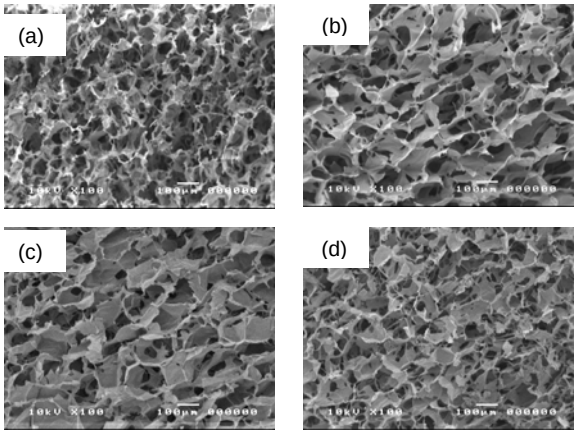

Figure 4. SEM micrographs of collagen/chitosan scaffolds at different blending compositon: (a)100/0,(b)90/10, (c)70/30, and (d)0/100.

In vitro Biodegradation

The biodegradation results were shown in Figure 5. Collagen scaffolds incubated in lysozyme had the highest weight reduction and were completely degraded after three weeks. However, the addition of chitosan reduced the degradation of scaffolds in lysozyme solution. This suggested that the physical interaction between collagen and chitosan possessed a greater steric hindrance effect to specific cleavage sites of lysozyme than that of the pure collagen.[19] Moreover, when the proportion of chitosan was increased to 30%, the remained weights were sustained after 21 days at 61%. For the scaffolds with 50% chitosan, the remained weights were approximately 77%. Regarding the stability of scaffolds which was higher than that of pure collagen scaffolds, the results proved to be the vital characteristic since it was well-known that the degradation rate of collagen scaffolds was very fast, hence the addition of chitosan could prolong the biodegradability of scaffolds.

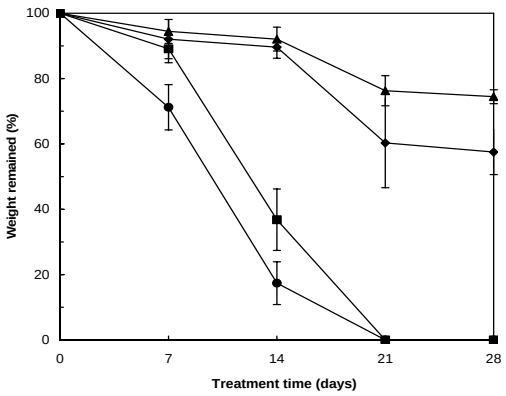

Figure 5. In vitro biodegradation of collagen/chitosan scaffolds with different blending compositions: (●) 100/0, (■) 90/10, (♦) 70/30, and (▲) 50/50 (collagen/chitosan).

Cell Adhesion and Proliferation Tests

Cell adhesion and proliferation were crucial for a scaffold to support and guide tissue regeneration. L929 cells were seeded onto substrates and cultured in DMEM medium. Figure 6a showed the initial cell adhesion at 5 h after seeding. The results showed no significant difference comparing to collagen, collagen/ chitosan, and chitosan scaffolds. Figure 6b and 6c represented the cell proliferation in FBS-supplemented medium at 24 and 72 h after seeding.

The scaffolds with 30% chitosan expressed the significant difference ($p<0.05$) in relative cell viability at 72 h compared to those of pure collagen scaffolds. The effects of chitosan on the cell behavior could be explained as follows. The specific cell binding amino acids of collagen for cell integrin receptors may be consumed after crosslinked by DHT.[21] Addition of chitosan may provide much more amino groups for cell adhesion and proliferation due to the affinity between positively charged ammonium groups of chitosan and negatively charged cell membrane surfaces.[2,7] The growth of L929 might be inhibited in the case of pure chitosan scaffolds by the extremely high affinity between cell and scaffold. The results indicated that the proliferation of L929 could be enhanced by adding chitosan to a certain degree. To confirm the stimulation of LMW chitosan on L929 mouse fibroblast proliferation, the effects of serum component were investigated. Cells were seeded onto scaffolds with mimic conditions except the presence of serum in medium. The results, in Figure 6d-6f, showed similar characteristics of cell behavior with a slightly difference in the relative cell viability respect to serum culture since cell response was delayed with the absence of serum in medium. The scaffolds with 30% chitosan still predominantly stimulated L929 cell proliferation. The results showed that the chitosan had the additional effect with growth factors or some secreted protein from cell. Bound growth factors could be slowly released by the action of lysozyme on chitosan supplying the cells with a sustained level of mitogenic signals.[3-5,15] This indicated that scaffolds fabricated from collagen and chitosan had biological activity and potential as wound healing agents or dressing materials.

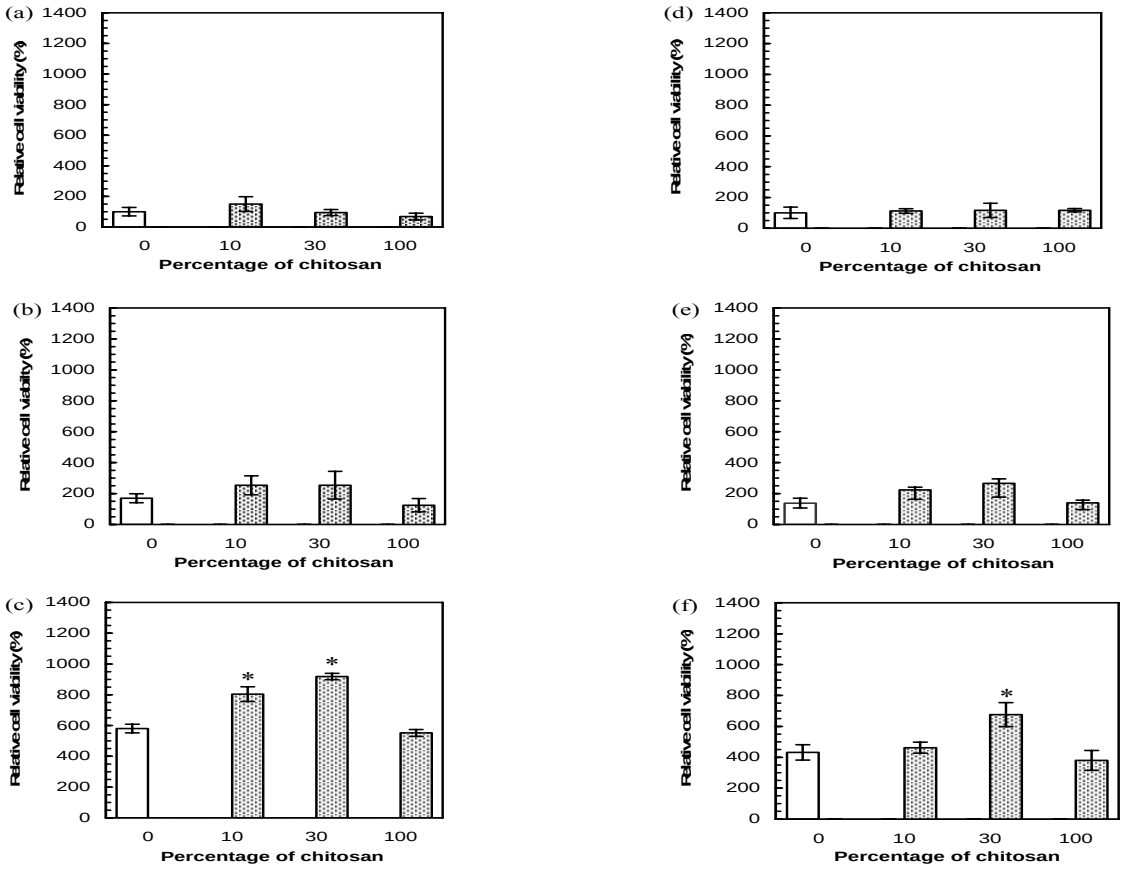

Figure 6. Relative cell viability of L929 on collagen/chitosan scaffolds with different blending compositions in FBS-supplemented medium at (a) 5, (b) 24, and (c) 72 h and serum-free medium at (d) 5, (e) 24, and (f) 72 h after cell seeding. The percentages of relative cell viability shown in the figure were averaged from six different values (n = 6, error bar = 2×SD, and * represents the significant difference $P<0.05$ relative to pure collagen) where clear and shaded blocks represented collagen (control) and collagen/chitosan scaffolds, respectively.

Conclusions

This study described the fabrication of porous scaffolds of biopolymer blends between collagen and chitosan by freeze drying and dehydrothermal crosslinking techniques. Physical interaction between collagen and chitosan could affect both physical and biological properties of scaffolds. The ability to resist lysozyme degradation of collagen scaffold was obviously augmented when blending with chitosan and thus could be manipulated by the change of blending composition of chitosan. The cell culture using collagen-based scaffolds containing 30% chitosan enhanced fibroblast proliferation compared to those of the pure collagen scaffolds. The scaffold of collagen and chitosan has promising properties of mechanical strength, biodegradable rate, and cell proliferation stimulating ability, which are crucial for a tissue engineering applications.

Acknowledgements

This work received the financial support from the National Research Council of Thailand and Affair of Commission for Higher Education-CU Graduate Thesis Grant.

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