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Properties of β-glycerol phosphate/collagen/chitosan blend scaffolds for application in skin tissue engineering

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ABSTRACT: The aim of this study was to determine the properties of β-glycerol phosphate (GP)/collagen/chitosan blended films for the potential application to skin tissue engineering. Various ratios of collagen to chitosan (8:2 and 7:3) and amounts of GP (0.5, 1, and 1.5% w/w) of total polymers were blended in solution form and cast into films. According to SEM images, the casted films showed a non-porous surface. For the mechanical properties, the prepared scaffolds exhibited the maximum elongation ranging from 15–23%, which is lower than those found by other researchers. However, the maximum tensile strength values of the scaffolds made from the collagen/chitosan (ratios 7:3) crosslinked with 0.5 or 1% w/w GP were in the range of 8–10 MPa which achieve the recommended values for application in skin tissue engineering. The scaffolds showed ability to retain their structure after immersion in phosphate buffer saline solution (pH 7.4) for 1 h, and their volume increased about 20%. After incubation in collagenase solution (200 U of collagenase/5 g of collagen) at 37 °C, the scaffolds were degraded within 24 to 26 days which coincides very well with the healing time of acute wounds (about 25 days). FT-IR studies revealed the possibility of an interaction of GP with collagen/chitosan via ionic interaction that enhances the strength and stability of the prepared scaffold. The results from an in vitro culture study showed that the keratinocyte HaCaT culture could adhere well and grow on the selected scaffold with a typical morphology at 98.1 ± 1.8% of the control (cells growth on tissue culture plate) after cultivation for 5 days. The results suggest the potential of the GP/collagen/chitosan blended films for use as skin scaffolds.

KEYWORDS: skin scaffold, scaffold properties, keratinocyte

INTRODUCTION

One of the important factors in skin tissue engineering is the construction of a scaffold which functions as physical support to guide cell differentiation, proliferation, and formation of an extracellular matrix[1,2]. The ideal scaffold should provide excellent biocompatibility, controllable biodegradability, and appropriate mechanical strength[1,3,4]. In addition, it should be capable of absorbing body fluids for the delivery of cell nutrients while retaining its shape and structure[5].

Currently, a number of natural and synthetic materials are being used as tissue scaffolds. Collagen type I is one of the natural polymers that is one of the most promising materials for tissue engineering because it is biocompatible and biodegradable[6–9]. However, its fast degradation and low mechanical strength limit the use of this material. Several methods have been tried to optimize its biodegradation rate and the mechanical properties. These include blending of collagen with other natural polymers[10–12] and/or introducing other chemical agents to the collagen[13–17]. Glutaraldehyde is used as the common crosslinking agent. However, the glutaraldehyde cytotoxicity necessitates the seeking of alternative agents[18,19]. Recently the development of a promising thermosensitive hydrogel prepared from mixtures of β-glycerol phosphate salt (GP) and chitosan for application in cartilage tissue engineering has been reported[20–22]. This led to our interest in developing GP/collagen/chitosan blended films for the application to skin tissue engineering.

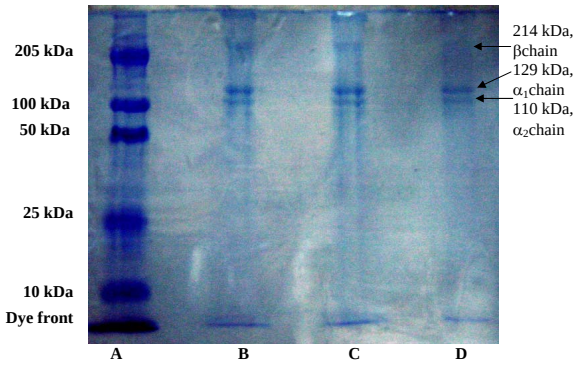

Fig. 1 SDS-PAGE protein patterns of collagen type I isolated from bovine tendon. Lane A represents protein bands of marker at different molecular weight, and lanes B to D represent protein bands of collagen type I from different isolation batches.

MATERIALS AND METHODS

Isolation of bovine collagen type I

Collagen type I was isolated using a described procedure[3]. Briefly, after removing the fat and muscle impurities, the bovine tendon was cut into small pieces and digested with 0.25% trypsin (analytical grade, GIBCO-BRL) solution at 37 °C for 24 h. The pieces of tendon were removed and incubated with 0.5 M acetic acid (analytical grade, VWR International) at 4 °C for 48 h. The swollen tendon was agitated and the collagen solution was centrifuged to separate the insoluble parts. The supernatant was collected and precipitated by addition of 5% NaCl (analytical grade, VWR International) solution. The precipitated collagen was dialysed with deionized water for 72 h and then lyophilized. SDS-PAGE revealed the purification of the isolated collagen with distinct polypeptide bands of molecular weight 110 and 129 kDa corresponding to the two α_1 chains and the α_2 chains, respectively (Fig. 1).

Preparation of the β-glycerol phosphate/collagen/chitosan film blends

The total amount of the polymer used was fixed at 3% w/v. The weight ratios of collagen and chitosan (molecular weight of 100 000 and more than 90% degree of deacetylation, Aqua Premier, Chonburi, Thailand) were 8:2 or 7:3. To prepare the film blend, type I collagen and chitosan were separately dissolved in 0.5 M acetic acid solution. After complete dissolution the collagen solution was mixed with the chitosan solution for 30 min. Then the aqueous solution of β-glycerol phosphate disodium salt pentahydrate (GP, analytical grade, Fluka) in an amount of 0.5–1.5% of the total polymer weight (calculated from nonhydrated GP) was added to the solution. The pH of the casting solution was 5.5–6. The casting solutions were stirred at room temperature for 30 min before being poured on a clear dry glass Petri dish in a dust-free atmosphere, and allowed to dry at room temperature. The dried scaffold film was peeled off from the Petri dish, placed in a plastic sheet, vacuum sealed, and kept at room temperature for further evaluation. The thickness of all prepared films was controlled to be in the range of 60 ± 10 µm.

Surface morphology

After drying of the selected film, its morphology of the top and side surfaces was observed using a scanning electron microscope (SEM, Model 1455VP, LEO Electron Microscopy, Cambridge). All the tested films were coated with an ultra-thin gold layer and their surface morphologies were observed at a magnification of 370 000.

Mechanical properties

A tensometer (Model 3342, Instron, Buckinghamshire, UK) was used for measuring the tensile strength and elongation until break of the prepared scaffold. The tested scaffold film was cut into rectangular (5 mm × 40 mm) pieces. The thickness of an individual film was the average value of four separate measurements taken along the middle of the 40 mm section using a micrometer (Mitutoyo Asia Pacific Pte Ltd). The cross-sectional area of the tested patch was calculated by multiplying the mean thickness by the gauge width. To measure the elongation, the film was clamped between two grips provided with a 100-N load cell before being pulled at the rate of 12.5 mm/min. Three determinations were performed for each sample. The atmosphere of the experimental room was 23 ± 2 °C with 40–70% relative humidity.

Swellability

Circularly cut scaffold samples were used to obtain the initial thickness (T_0) and diameter (D_0). The samples were then incubated in phosphate buffered saline (PBS, pH 7.4) at 37 °C under full immersion of the film. The scaffold size was measured hourly to obtain the swelling rate, the final thickness (T_t), and diameter (D_t). The swelling degree was calculated from $100(D_t/D_0)^2(T_t/T_0)$. The study was performed in triplicate.

Enzymatic degradation

The study method followed that of the previous studies with modification[23,24]. Briefly, the scaffold samples were incubated at 37 °C in 10 ml PBS solution (pH 7.4) containing collagenase at the concentration of 200 U/5 g of collagen. All supernatants were removed after incubation times of 2, 4, 6, 16, 24, 48, and 168 h. The collected supernatants were hydrolysed with 6 M hydrochloric acid at 120 °C for 12 h. The concentration of hydroxyproline (HyP) was then measured with an UV spectrophotometer (Cary 1 E, Varian Inc., Palo Alto) at a wavelength of 202 nm. The amount of released HyP at each time was calculated as a percentage of the initial amount of HyP in the scaffold.

Fourier transform infrared spectroscopy (FT-IR)

The infrared spectra (wavenumber 4000–600 cm^{-1}) of GP and the films of collagen, chitosan, chitosan/collagen, and GP/chitosan/collagen blends were recorded by FT-IR spectrometry (Model GX series, Perkin Elmer). The sample was mixed with potassium bromide powder and compressed to a thin pellet for infrared examination.

Cell culture

The selected scaffold was soaked in 10% NH_4OH to neutralize any acidity, and rinsed 3 times with sterilized distilled water. It was then immersed in 75% ethanol for 24 h for sterilization followed by rinsing with sterile PBS 3–4 times and immersed in the culture medium for 4–10 h. Thereafter the scaffold (0.25 cm^2) was placed into a 96-well plate containing a suspension of nontumorigenic human keratinocyte HaCaT (Lot No. 300493-524, Cell Lines Service, Eppelheim, Germany) with a density of 1×10^4 cells cm^{-2} and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cell line passage numbers of 3 to 7 were used in this study. The culture medium was changed on day 3. After culturing for 5 days, the scaffold was rinsed with sterilized PBS to remove the dead cells. It was then placed in a new well and 200 µl of fresh medium was added. Cell viability was evaluated by adding 50 µl of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT, Boehringer Mannheim) as labelling reagent and then the absorbance was measured at 490 nm after incubation at 37 °C in a humidified atmosphere containing 5% CO_2 for 4 h. The absorbance value obtained from the cells seeded on the scaffold, which is directly proportional to the number of living cells, was normalized to the control (100% cells directly seeded in the tissue culture plate). The study was performed in triplicate.

Morphology of cell adhesion on the scaffold

After 5 days of cultivation, the HaCaT cells adhering to the scaffold were washed with PBS (pH 7.4) and then fixed with buffer solution containing 2.5% glutaraldehyde for 12 h at 4 °C. The sample was then washed with PBS to remove the residual glutaraldehyde. After the sample had been post fixed with OsO_4 for 2 h, it was washed with PBS and dehydrated through a series of ethanol washes (30, 50, 70, 90, 100%) for 15 min. It was then soaked in acetone for 15 min twice. Once the sample was dried using the critical point drying method, it was coated with an ultrathin gold layer and observed by SEM.

Statistical analysis

Independent samples t-test was used to compare two groups of samples at a significance level of 5%. The data are expressed as mean $\pm$ standard deviation (SD).

RESULTS

Mechanical properties and morphology

According to our preliminary study, using only collagen or films consisting of a high ratio of collagen/chitosan (9:1) resulted in rather weak and brittle scaffolds which were difficult to remove from the glass Petri dish. An increase of the ratio of chitosan together with incorporating GP was able to strengthen the weak scaffold (data not shown).

The SEM images of the surface of the prepared scaffold revealed a non-porous or sheet-like structure (Fig. 2). The maximum tensile strength and the percentage of elongation at break values of the scaffolds containing various amounts of GP are shown in Fig. 3. Increasing the GP content tended to increase the tensile strength value significantly, but the increase was not large. However, the scaffolds made from the collagen/chitosan ratio of 7:3 had a significantly higher strength compared to those made from the collagen/chitosan ratio of 8:2. The scaffolds made from the collagen/chitosan (7:3) and blended with GP at the amount of 1.5% w/w of total polymer were found to have the highest tensile strength among the tested scaffolds.

Increasing of the amount of GP tended to decrease the elongation value with the scaffolds made from 7:3 collagen/chitosan mixtures, whereas the percentage of elongation values were not different among the scaffolds made from the 8:2 collagen/chitosan mixtures (Fig. 3). At GP concentrations of 0.5 and 1%

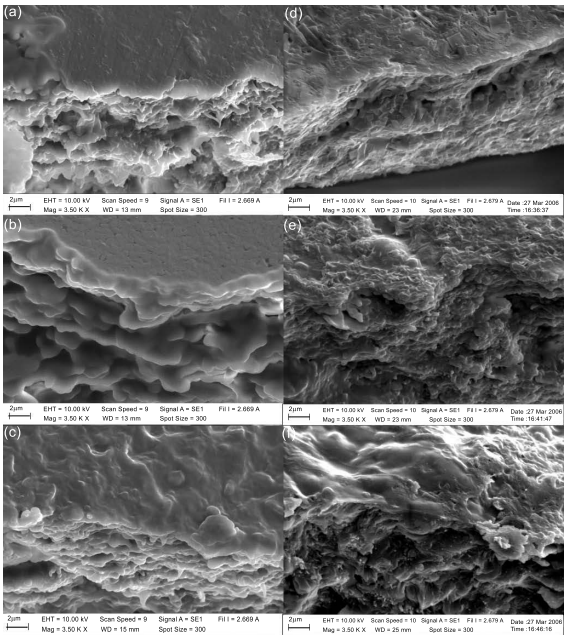

Fig. 2 SEM images of top and side-surfaces of collagen/chitosan scaffolds at ratios of 8:2 (a–c) and 7:3 (d–f) incorporating GP at concentrations of (a,d) 0.5 (b,e) 1 (c,f) 1.5% w/w of total polymer.

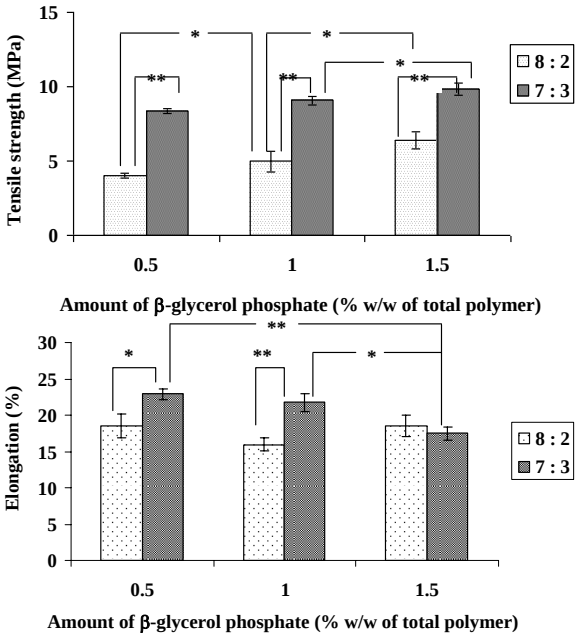

Fig. 3 Effect of GP concentration on scaffold maximum tensile strength elongation at break. Each bar represents mean $\pm$ SD of triplicate studies. Significant differences indicated by * = $P < 0.05$, ** = $P < 0.01$.

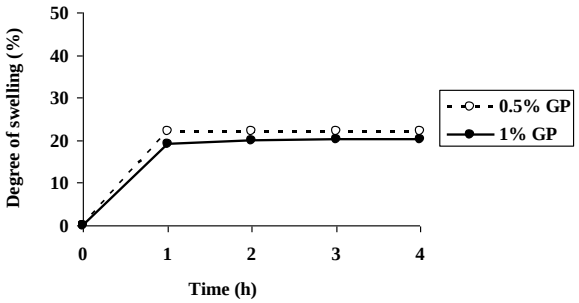

Fig. 4 Effect of GP on the equilibrium swelling characteristics of the scaffolds prepared from 7:3 collagen/chitosan.

w/w of total polymer, increasing the ratio of chitosan significantly decreased the percentage of elongation value.

In summary, the scaffolds made from 7:3 collagen/chitosan mixtures blended with 0.5 or 1% GP had the highest strength and elongation. These compositions were therefore selected for the remaining studies.

Swellability and enzymatic degradability

The swelling degrees of the collagen/chitosan (7:3) scaffolds with 0.5% and 1% GP are shown in Fig. 4. The results show that the swelling degree and rate of the 1% GP scaffold were lower than that of the 0.5% GP scaffold. The scaffold in PBS solution swelled in volume by about 20% within 1 h and retained its structure. The cross-section SEM image of the scaffold after immersion in PBS solution for 1 h revealed a rough pore wall beneath the non-porous surface of the scaffold (Fig. 5). The non-GP scaffold made from just collagen/chitosan (7:3) showed rapid water absorption and then ruptured after immersion in PBS solution for 1 h (data not shown).

After incubation in collagenase solution, the scaffold prepared from GP-free collagen/chitosan was biodegraded within 48 h. After addition of 0.5 or 1% GP to the films, the stability of the scaffold was enhanced, with the amounts of HyP remaining after 1 week incubation equalling 70 and 73% of the initial HyP value, respectively (Fig. 6). From these results it was assumed that the scaffolds prepared from collagen/chitosan and crosslinked with 0.5 and 1% GP were biodegraded within 24 and 26 days, respectively. Our study also indicated that the difference in the biodegradation rate did not differ between the scaffolds blended with 0.5 and 1% GP. Therefore, the scaffold prepared from the blending the collagen/chitosan (7:3) mixture and adding GP in an amount of 0.5% w/w was selected for the in vitro

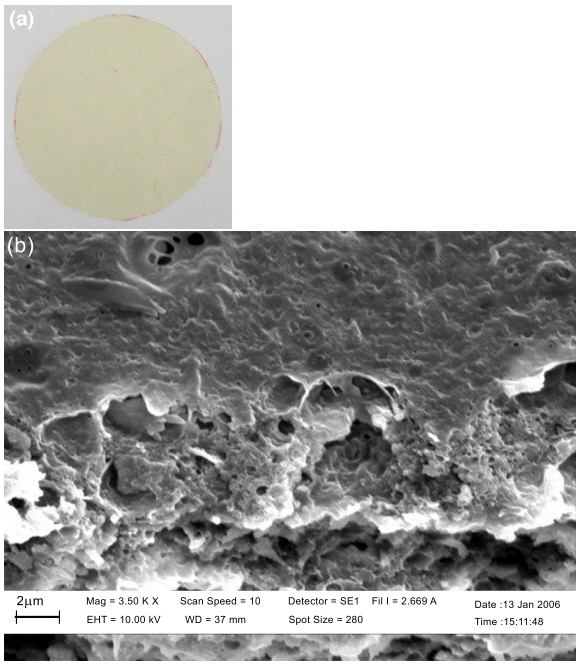

Fig. 5 GP/collagen/chitosan scaffold after immersion in PBS solution for 1 h: (a) photo (b) SEM image.

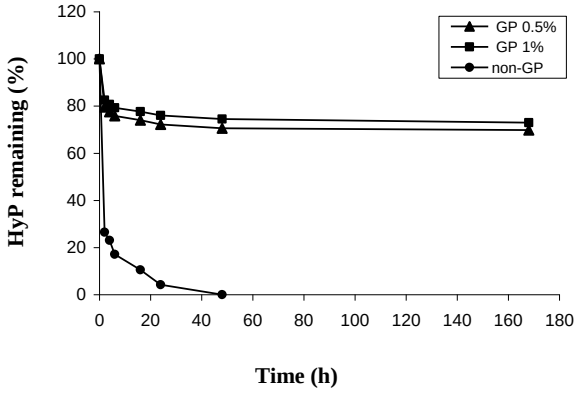

Fig. 6 Percentage HyP remaining for scaffolds prepared GP/collagen/chitosan after incubation in collagenase.

culture study.

FT-IR spectra

The FT-IR spectrum of a chitosan film is shown in Fig. 7. The absorption band in the region of 3500–3400 cm^{-1} shows the amino group, but it is masked by the broad absorption band from the $-OH$ group. The absorption band at 2881 cm^{-1} represents the $-CH_2$ and $-CH_3$ aliphatic groups. The peak at 1655 cm^{-1} is assigned to the C=O stretch of the amide bond and the N−H bending of the amide bond appears at

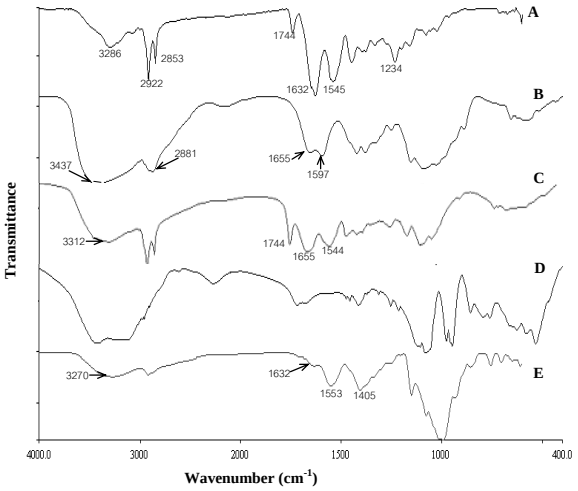

Fig. 7 Wide scan FT-IR spectra for films of (a) collagen (b) chitosan (c) collagen/chitosan (7:3) (d) GP (e) collagen/chitosan (7:3) with 0.5% GP.

1597 cm^{-1} [24].

In the FT-IR spectrum of the collagen film, the amide A, B, I, II, and III bands appear at wavenumbers of 3326, 3074, 1650, 1549, and 1240 cm^{-1}, respectively. It also shows characteristic peaks at 2921 and 2852 cm^{-1} which represent the aliphatic $-CH_3$ and $-CH_2$ groups, respectively.

The FT-IR spectrum of collagen/chitosan (7:3) scaffolds without GP is characterized by a C=O stretch of amide I, and N−H bend and the C−N stretching of the amide II bond at 1655, and 1544 cm^{-1}, respectively. However, the amide A (N−H stretch from the CO−NH portion) band in the spectrum shifts to 3312 cm^{-1} with increasing absorbance.

Cell culture

The optimized GP/collagen/chitosan scaffold supported $98.1 \pm 1.8\%$ of the keratinocyte growth in comparison to the control after 5 days of culturing. The morphology of the HaCaT cells cultured on the collagen/chitosan (7:3) blended with 0.5% GP polymer is shown in Fig. 8. The SEM image reveals that the keratinocytes with typical morphology adhered tightly on the surface of the scaffold.

DISCUSSION

Generally, noncollagenous matrix components including glycosaminoglycans (GAGs) and noncollagenous sugar are predominantly distributed in the upper dermis where collagen fibres are located. It has been reported that the proportions (% by weight) of GAGs

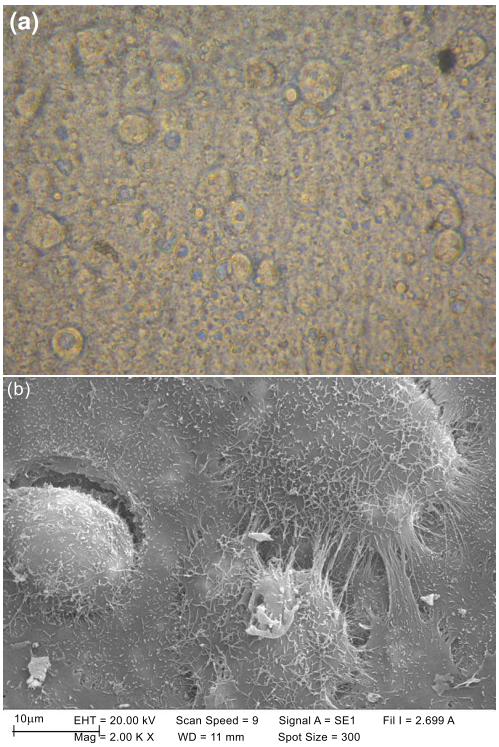

Fig. 8 Images of the HaCaT keratinocytes cultured for 5 days on GP/collagen/chitosan scaffold: (a) conventional microscope (b) SEM.

to collagens and noncollagenous sugar to collagen are 1.4 and 2.1, respectively[25]. The presence of noncollagenous matrix is important for maintaining the flexibility, elasticity, and strength of the skin. In this study we therefore developed a scaffold consisting of two biopolymers, collagen and polysaccharide chitosan, in proportions that imitate those of the components of skin dermis.

Mechanical properties and morphology

When collagen is mixed with chitosan, the long chain of the chitosan may wind around the collagen triple helix and form a complex. In collagen, the $-$OH groups of hydroxyproline, and $-$COOH and $-NH_2$ are capable of forming hydrogen bonds with the $-$OH and $-NH_2$ groups of the chitosan. For this reason, the ratio of chitosan and collagen is critical for the mechanical strength that is determined by the complex density in the scaffold. Additionally, in acidic condition, an ionic interaction can occur, taking into account the polycationic groups ($-NH_3^+$) of the collagen and/or chitosan and polyanionic groups of the GP ($-OPO(O^-)_2$)[26,27]. This charge neutralization thereby decreases electrostatic repulsions of the collagen and/or chitosan chain, leading to the possible formation of hydrogen bonds between the polymer chains. Therefore, introducing GP could increase the strength of the collagen/chitosan scaffolds.

In this study, a casting technique was used to prepare the film-like scaffolds because it is simple and cheap. As we experienced, the differences in the preparation technique, at least partially, cause the differences in the scaffold morphology which affects the mechanical properties of the scaffold. The strength of a non-porous scaffold is high but the flexibility of such material is generally low. On the contrary, the porous scaffold possesses generally low strength but it is of high extensibility as a function of pore orientation and interconnection. In this study, our scaffolds prepared with this method exhibited the maximum elongation ranging from 15–23%, which is, however, lower than those found by other researchers[28,29]. This may be caused, according to SEM examination (Fig. 2), by their sealed surface or sheet-like structure. However, the maximum tensile strength values of the scaffolds made from the collagen/chitosan (7:3) were in the range of $\sim$8 to $\sim$10 MPa which is within the range of the recommended values[13]. Therefore, these scaffolds should have a strength adequate to resist rupture during implantation and maintain integrity during in vivo/in vitro cell growth. However, it should be taken into consideration that the mechanical properties including tensile strength and percentage elongation values of the film may alter after the sterilization process. Further study is needed to clarify the effects of sterilization on the mechanical properties of the developed scaffold.

Swellability and enzymatic degradability

The SEM images reveal a rough pore wall beneath the non-porous surface of the scaffold after swelling. These pores, at least partially, will hold the aqueous medium that is necessary for cell growth. In general, the water-binding ability of the collagen/chitosan scaffold is attributed to its hydrophilic character. The swelling degree of such scaffold decreases as the number of free hydrophilic groups (such as $-$COOH, $-NH_2$, and $-$OH) is decreased[3,30]. As a consequence, the swelling degree and swelling rate of the 1% GP containing scaffold was somewhat lower than that of the 0.5% GP containing scaffold. However, the differences were not significant. The results coincide with the mechanical study that indicated a small difference of the tensile strength of the scaffold depending on the GP content. The amount of 0.5% of GP is probably sufficient for an almost overall interaction between the available polycationic groups

($-NH_3^+$) of the chitosan or collagen and the anionic groups of the GP ($-OPO(O^-)_2$).

The degradation time of the scaffolds prepared from collagen/chitosan and crosslinked with 0.5 and 1% GP coincides very well with the healing time of acute wounds (about 25 days)[31]. From our results it is likely that not only the presence of GP but also the addition of chitosan is important for the decrease of the biodegradation time of the scaffolds.

FT-IR spectra

The FT-IR spectra of collagen/chitosan (7:3) scaffolds without GP were not distinctively different from those of the pure collagen film. The C=O stretch of amide I and N−H bend and C−N stretching of amide II bond was observed near 1655 cm^{-1} and 1544 cm^{-1}, respectively. However, the amide A (N−H stretch from CO−NH portion) band in the spectrum in the collagen/chitosan mixtures shifted to 3312 cm^{-1} with increasing in absorbance. The reason for this may be the integration between the amide bands of collagen and the −OH bands of chitosan. In addition, as the NH− group of collagen is generally involved in a hydrogen bond, the shift to a lower frequency, usually near 3300 cm^{-1}, of this peak indicates the possibility of the formation of hydrogen bonds between NH_2 of collagen and the −OH groups of chitosan[32,33].

The spectrum obtained from the 0.5% GP/collagen/chitosan film showed a broad peak around the frequency of 3270 cm^{-1}. The amide A and B bands of collagen disappear from these regions because they must have been masked by the absorption bands of the −OH group from the GP molecule. Moreover, the amide II peak displays a slight change from 1655 to 1632 cm^{-1}. This indicates that GP could induce the formation of possible hydrogen bonds and the interaction of GP with chitosan is a physical interaction[27,34]. The interaction of GP with collagen/chitosan occurs via the ionic interaction between the positively charged amino groups of collagen and chitosan and the negatively charged phosphate groups of the GP.

Cell culture

The keratinocytes growth on GP/collagen/chitosan scaffold indicates the ability and biocompatibility of such scaffolds for skin tissue engineering. Although the surface of the scaffold is non-porous, the keratinocytes could adhere tightly on the surface of the scaffold. The mechanisms of the HaCaT adhesion and proliferation on the GP/collagen/chitosan scaffold are still unclear. They may be related to the fact that, although the amino group of polymers is interacting with the phosphate group of GP, there are sufficient free amino groups remaining on the polymer chains allowing electrostatic interactions with the negative charge of the surface of cell membranes. It also means that the GP itself is not likely to interact with the cells and therefore the interactions with the polymers are more likely to influence the cell adhesion and proliferation. Another possibility is the biospecific interaction between a cell receptor with the chitosan and/or collagen molecules since regular adhesion and then proliferation of seeded cells is strongly dependent on the specific cell surface receptor used by cells to interact with the scaffold, resulting in the attachment of cells on the scaffold.

CONCLUSIONS

In the present study, films with various amounts of collagen, chitosan and GP were prepared and their strength, flexibility, swellability and biodegradability were determined. The interactions of the GP/collagen/chitosan components with each other were investigated by FT-IR. The scaffold that showed optimum properties was selected for a cell culture study with keratinocyte HaCaT. The results obtained from this study suggest the potential of the collagen/chitosan (7:3) blends with GP in an amount of 0.5% of total polymer as a suitable scaffold for applications in skin tissue engineering.

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