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Studies of Novel Hyaluronic Acid-collagen Sponge Materials Composed of Two Different Species of Type I Collagen

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ABSTRACT: The authors have developed novel hyaluronic acid (HA)-collagen sponge materials (HACSMs) composed of various ratios of bird feet (BF) and pig skin (PS) collagen that are fabricated employing a combination of freezing, lyophilizing, and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) cross-linking methods. Morphology, swelling ratio, resistance to collagenase, thermal stability, tensile strength, and free amine index are determined to evaluate the physical-chemical properties of various HACSMs. Different BF: PS ratios directly vary with the physical-chemical properties of HACSMs and control their biodegradability for multiple uses. Resistance to collagenase, thermal stability, and tensile strength of HACSMs increases as the ratio of BF collagen increases. On the contrary, the higher swelling ratio, free amine index, and pore size occur in materials composed of higher ratios of PS collagen. A linear relationship between the decreased ratio of PS collagen and the increase in tensile strength and biostability are observed. The materials of B4P1HA (BF:PS:HA = 4:1:0.2) exhibit the highest value of tensile strength, but no significant difference exists between B4P1HA and B5P0HA (BF:PS:HA = 5:0:0.2). These phenomena should be closely related to the BF collagen which contains a higher amount of carboxyl groups of glutamic or aspartic acid residues and forms more amine bonds under EDC cross-linking

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when compared to PS collagen. However, these results suggest that the B4P1HA and B5P0HA materials should be produced according to highest bio-stability and mechanical strength and, furthermore they may be suitable for artificial skin or drug delivery applications.

KEY WORDS: hyaluronic acid, collagen, sponge materials, carbodiimide.

INTRODUCTION

Severely burned patients usually require alternative therapy for wound healing. Various types of wound dressing and cultured skin substitutes have been developed and used to treat skin defects. Several materials are commercially available. The representative materials are collagen-based dermis: Dermagraft (Advanced tissue Sciences Inc., La Jolla, CA, USA) and Apligraf (Organogenesis, Canton, MA, USA); bilayer matrix composed of silicone and GAG-collagen: INTEGRA® (Integra Life Sciences, Plainsboro, NJ, USA). Skin tissues are composed of cells and a largely extracellular matrix (ECM) [1]. Both collagen and hyaluronic acid (HA) are important components of ECM and serve as suitable candidates for artificial skin because they mimic the basic constituents of skin [1,2]. The HA is a linear polymer of glucuronic acid disaccharide. It plays a critical role in the regulation of several cellular functions, including cell attachment, proliferation, migration, and differentiation [3]. Collagen is the most abundant component of a mammalian connective tissue [1,2,4]. Collagen and collagen-derived peptides act as chemoattractants for cell and tissue ingrowth [4]. Considerable literature has been extensively developed using porous collagen sponge combined with HA as healing components which promotes the restoration of skin, replication of tissue cells, and biosynthesis *in vitro* and *in vivo* [5–8].

However, the rapid degradation and low mechanical strength of the non-cross-linked HA-collagen materials cannot match the demand of *in vitro* and *in vivo* application in biomedical fields [5,9]. The cross-linking procedure becomes a necessary procedure for material stabilization. Nonetheless, the cross-linking treatments with chemical agents confer remarkably high strength and durability, but also induce potential cytotoxicity or poor biocompatibility [9]. Selection of a highly biostable collagen should be an alternative way to reduce the quantity of cross-linking agent. Recently, Lin and Liu [10] compared different collagens from five animal species and detected highly enzymatic resistance collagen from pig origin; highly thermal stability collagen from bird origin that could be used as suitable material in biomedical fields [10]. However, the species-related properties of collagens are well

understood [11,12], but lack study related to composite material blending with multiple species collagens. Therefore, the authors tried to combine the advantages of thermal stability and enzyme resistance by blending bird feet (BF) with pig skin (PS) collagen and using it in sponge materials preparation. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was used as cross-linker in the present study. This reagent has been universally found to modify side-groups and to mediate the ester and amide bond formation between the hydroxyl and carboxyl group of HA and collagen with low cytotoxicity [11,13–15]. The objective of the present study was to prepare the HA-collagen sponge materials composed of different (HACSMs) blending ratios of BF and PS collagen that were fabricated employing a combination of freezing, lyophilizing, and EDC cross-linking methods and to investigate their differences in biochemical, physical, morphological, and mechanical characteristics.

MATERIALS AND METHODS

Preparation of HACSMs

The telopeptide-poor type I collagen from BF and PS was prepared by the Lin and Liu [10] method. The HA (sodium salt, MW 2,000,000; Shiseido, Yokohama, Japan) was kindly provided by Professor Kuroyanagi (Kitasato University, Japan). The different formulations are indicated in Table 1. The HA and collagen powder were mixed to a weight ratio of 10:1, the collagen was proportionally adjusted to a weight ratio of BF: PS collagen as 0:5, 1:4, 2:3, 1:1, 3:2, 4:1, and 5:0; and these preparations were represented as B0P5HA, B1P4HA, B2P3HA, B1P1HA, B3P2HA, B4P1HA, and B5P0HA. The mixtures were dissolved in 100 mL 0.5 M acetic acid by stirring at 4°C overnight.

Table 1. Preparation of HACSMs.

Material types	BF collagen (g)	PS collagen (g)	HA (g)
B0P5HA	0	2.0	0.2
B1P4HA	0.4	1.6	0.2
B2P3HA	0.8	1.2	0.2
B1P1HA	1.0	1.0	0.2
B3P2HA	1.2	0.8	0.2
B4P1HA	1.6	0.4	0.2
B5P0HA	2.0	0	0.2

The mixtures of HA and collagen were dissolved in 100 mL of 0.5 M acetic acid.

The mixed solutions were poured into 8-well (well size: 26×33 mm) rectangular polystyrene multidishes and sequentially frozen at -20°C for 4 h and -70°C for 24 h. The sponge materials were obtained by lyophilization. Then 50 mM EDC/95% alcohol solution was added dropwise for cross-linking and promoted reaction at 50°C for 24 h. In order to reduce the residual EDC, the cross-linked sponge materials were washed thrice with deionized water and freeze-dried under the same conditions. Finally, both surfaces of the materials were irradiated in an ultraviolet chamber (312 nm) for 1 h to produce intermolecular cross-linking of the collagen molecules.

Morphology of Materials

To observe the morphology of materials and collagenase degraded materials, specimens were coated with gold-palladium and examined under a scanning electron microscope (SEM, TOPCON ABT-150S).

Swelling Ratio

The pieces of cross-linked materials ($6 \times 8 \times 2$ mm³) were weighed and immersed in a phosphate-buffered saline (PBS) buffer (pH 7.5) at room temperature for 60 s and the unabsorbable solution was removed by aspiration. Finally, the wet materials were weighed and calculated according to the following equation:

$$\text{Swelling ratio (\%)} = \left[\left(\frac{W_w - W_d}{W_d} \right) \right] \times 100$$

where W_d is the weight of dry matrix and W_w is the weight of wet material.

Tensile Strength

The sponge materials were cut into pieces of $6 \times 8 \times 2$ mm³. The tensile strengths of the pieces of matrix were determined by a universal test machine (Shimadazu, Japan) at room temperature with an extension rate of 5 mm/min until rupture of the specimen occurred.

Resistance to Collagenase

Materials degrading in bacterial collagenase (EC 3.4.24.3) solution were measured to estimate the enzymatic sensitivity.

5 mg samples of different materials were incubated at 37°C with 5 mL of 5 unit collagenase solution (0.01 M Tris-HCl, pH 7.2, containing 50 mM CaCl₂) for 4 h. For isolation of the degraded product, the solution was filtered with a Whatmen No. 2 (Toyo) filter paper. The 4-hydroxyproline (4 Hyp.) and uronic acid concentration of the filtrate were determined by Reddy and Enwemeka [16] and Bitter and Muir's [17] method, respectively. Higher amounts of 4-Hyp. and uronic acid release value indicate more collagen molecules and HA degraded within the collagenase solution.

Thermal Stability

Thermal stability of the materials was evaluated by differential scanning calorimetry (DSC, Seiko DSC131, Japan). For the DSC evaluation, materials were analyzed immediately after cross-linking within 1 month (at room temperature). The 3–5 mg matrix was sealed in aluminum pans and subjected to DSC. The heat rate was 5°C/min and the temperature was increased from 5–200°C. Three temperatures were measured: the onset temperature (T_o), the maximal peak 1 (T_p1), and the maximal peak 2 (T_p2) as characteristics of the denaturation process of different materials.

Free Amine Index

The free amine index was used as efficiency of cross-linking. Free amine is liable to react with Ninhydrin agent and produce chromophore. The free amine index was determined by the method of Doi et al. [18]. The approximate 3–5 mg materials were immersed in 300 μL of 0.5 M acetic acid and placed at room temperature for 1 h, and 1 mL of Ninhydrin solution was sequentially added and heated at 100°C for 20 min. Samples were transferred to an ice-cold water bath and distilled by 5 mL of isopropyl alcohol and recorded at 570 nm with a spectrophotometer (Hitachi U2001, Japan). The results were calculated according to the following equation:

$$\text{Free amine index} = \frac{A_{570}}{W_d(g)}$$

where A_{570} is absorbance at 570 nm, W_d is the weight of dry material.

Statistical Analysis

The entire protocol was repeated six times for each sample. All data in this experiment were analyzed by GLM program and compared by Duncan's new multiple ranges test contained in the SAS system (Version 8.00, SAS institute Inc., Cary, NC, USA). The values indicated represent mean \pm standard deviation and a *p*-value <0.05 was considered significant.

RESULTS

Morphology of Materials

In the present study, insoluble sponge materials were obtained by freezing, lyophilizing, and EDC cross-linking methods. The cross-sectional morphology of the materials was observed under an SEM and is shown in Figure 1. The sponge materials had similar lattice structures and the size varied from approximately 50 to 200 μm . The pore sizes increased with a tendency to increase in proportion to the content of PS collagen. After collagenase degradation for 4 h, the materials of B5P0HA were almost completely degraded and could

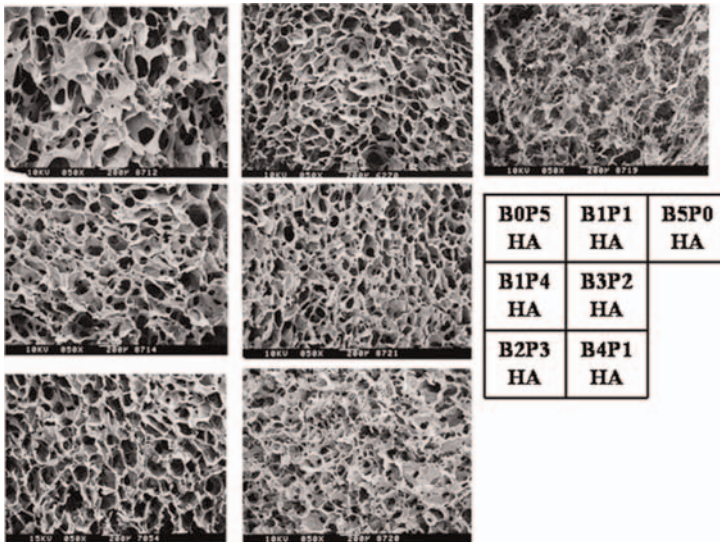


Figure 1. Microstructure of HACSMs composed of different ratios of BF collagen to PS collagen (magnification 50 \times).

not prepare the SEM specimen. The other materials exhibited an eroded lattice structure under collagenase degradation for 4 h (Figure 2). Moreover, the materials of B1P4HA and B2P3HA revealed a thinner pore wall and collapsed structures.

Swelling Ratio

The form stability under physiological condition was compared by swelling ratio after being soaked with PBS buffer (pH 7.5) and is illustrated in Figure 3. The swelling ratio of materials increased as the content of PS collagen increased. However, the materials composed of a higher ratio of BF collagen (B4P1HA and B5P0HA) resulted in a significantly ($p < 0.05$) decreased swelling ratio when compared to other materials (B0P5HA, B1P4HA, B2P3HA, and B3P2HA).

Tensile Strength

The results of tensile tests were determined by a universal test machine and illustrated in Figure 4. The tensile strength value of B4P1HA and B5P0HA were significantly ($p < 0.05$) higher than that of

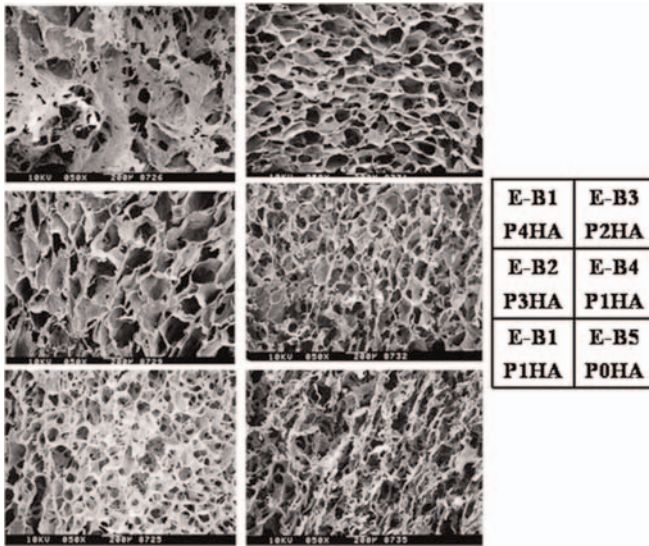


Figure 2. Microstructure of HACSMs composed of different ratios of BF collagen to PS collagen after 4 h degradation by collagenase (magnification 50×).

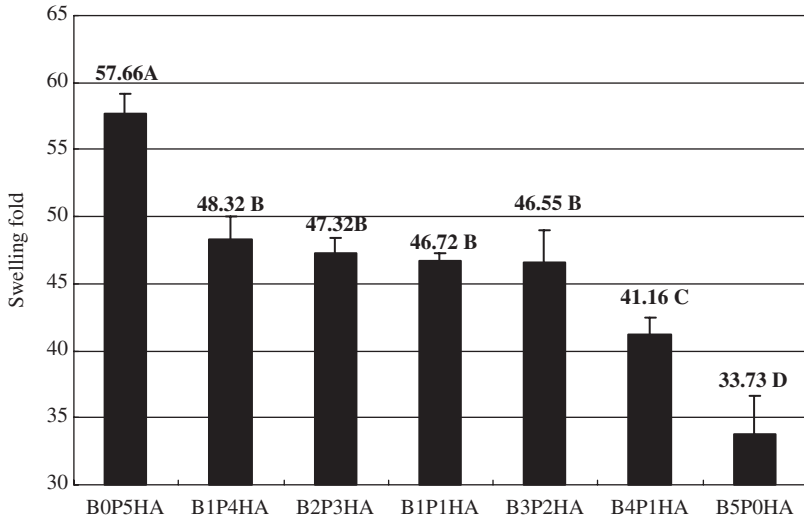


Figure 3. Swelling ratio of HACSMs composed of different ratios of BF collagen to PS collagen. A, B, C, D means without the same superscript are significantly different ($p < 0.05$).

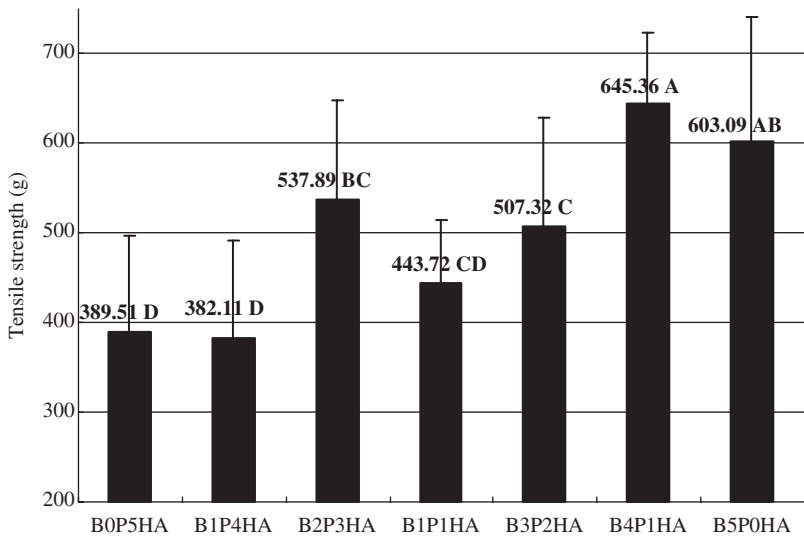


Figure 4. Tensile strength of HACSMs composed of different ratios of BF collagen to PS collagen. A, B, C, D means without the same superscript are significantly different ($p < 0.05$).

other materials (B0P5HA, B1P4HA, B2P3HA, B1P1HA, and B3P2HA) and proved to be significantly increased by the addition of BF collagen.

Resistance to Collagenase

The resistance against collagenase of materials was evaluated by the release amount of 4-Hyp. and galuronic acid under collagenase degradation for 4 h at 37°C (Figure 5). The release amount of 4-Hyp. and galuronic acid were increased with an increasing ratio of PS collagen in the materials. However, the B0P5HA and B1P4HA materials were difficult to lift by tweezers due to severe hydrolysis. Degraded products of B1P1HA, B3P2HA, B4P1HA, and B5P0HA materials were significantly lower ($p < 0.05$) than B0P5HA, B1P4HA, and B2P3HA materials. These results indicate that the materials composed of a higher ratio of BF collagen causes great resistance against collagenase degradation.

Thermal Stability

The DSC measurements were made to study the thermal behavior of the materials (Table 2). The thermal transition temperature of materials displayed a wide range (T_o : 59.44–91.50°C, T_p1 : 94.53–126.05°C, T_p2 : 96.44–126.05°C, respectively) and showed an increasing tendency to increase with the ratio of BF collagen content.

Free Amine Index

The free amine index provides further evidence for the cross-linking extent of the materials. The free amine index is measured by the Ninhydrin assay and is illustrated in Figure 6. Results showed a decreasing tendency as the content of BF collagen increased, displaying a wide range (41.20–83.12) and decreased in the order of B0P5HA > B1P4HA > B2P3HA > B1P1HA > B3P2HA > B4P1HA > B5POHA. The free amine index of B0P5HA and B1P4HA were significantly ($p < 0.05$) higher than that of other materials. These results indicate that the materials composed of higher ratio of BF collagen causes a higher extent of cross-linking.

DISCUSSION

Collagen-based materials formed into three-dimensional sponges serve as a wound dressing since the large pores or channels, inter-channel communications, and combinations of macromolecules of

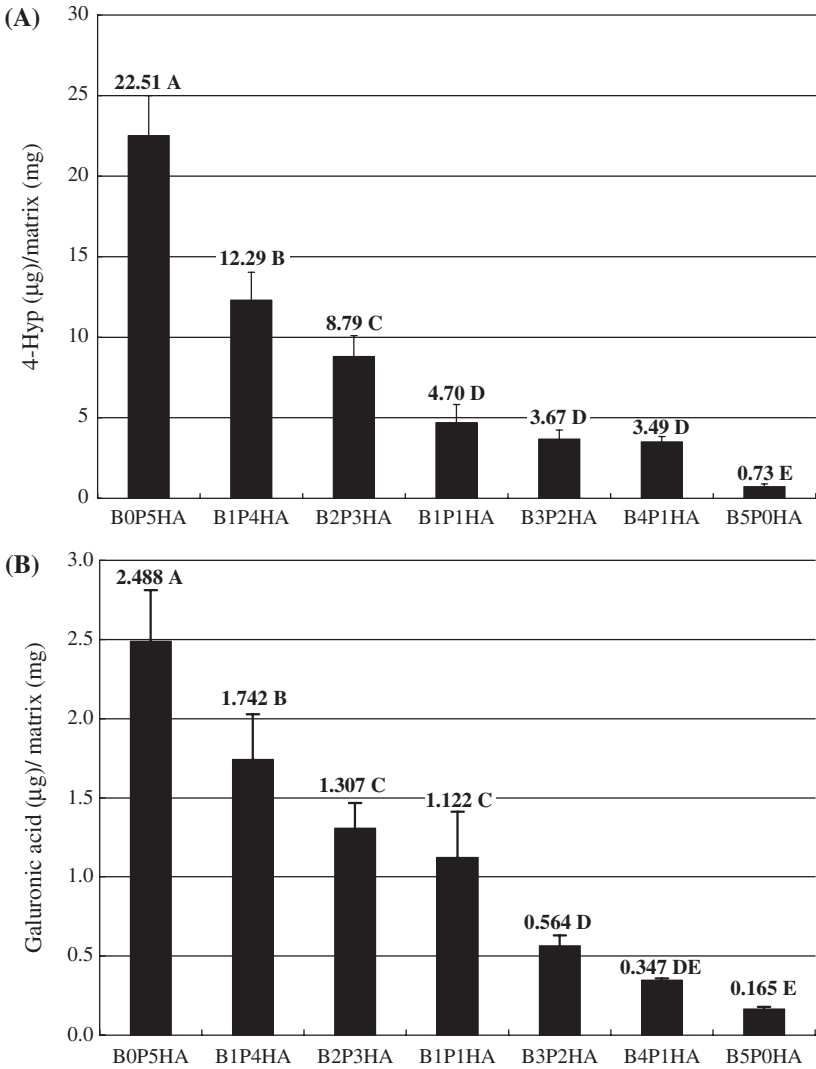


Figure 5. (A) 4-Hyp. and (B) galuronic acid release amount of HACSM composed of different ratios of BF collagen to PS collagen after 4 h degradation by collagenase. A, B, C, D, E means, without the same superscript are significantly different ($p < 0.05$).

connective tissue enhance the wound tissue infiltration *in vivo* and cell growth *in vitro* [19]. The cross section images showed that the porous structures of sponge materials were preserved after sequential freezing, lyophilizing, and EDC cross-linking procedures (Figure 1). All HACSMs

Table 2. Thermal transition temperatures of HACSMs composed of different ratio of BF collagen to PS collagen.

Material types	Thermal transition temperatures (°C)*		
	T_o	T_{p1}	T_{p2}
B0P5HA	59.44	94.53	96.44
B1P4HA	64.27	107.75	108.34
B2P3HA	75.71	108.19	108.85
B1P1HA	81.25	117.24	117.78
B3P2HA	85.51	115.64	117.29
B4P1HA	89.80	118.77	118.85
B5P0HA	91.50	126.05	126.05

*Three temperatures were measured: the onset temperature (T_o), the maximal peak 1 (T_{p1}) and the maximal peak 2 (T_{p2}) as characteristics of the denaturation process of various matrices.

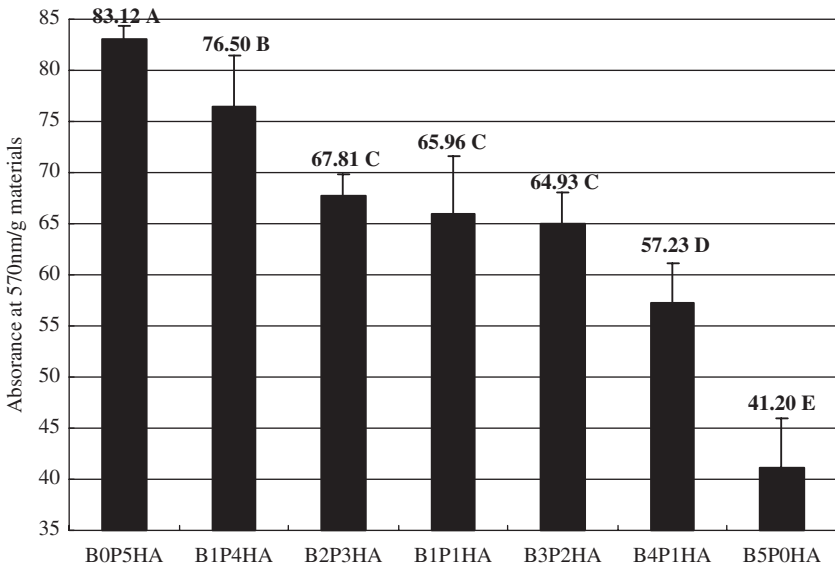


Figure 6. Free amine index of HACSMs composed of different ratios of BF collagen to PS collagen. A, B, C, D, E means without the same superscript are significantly different ($p < 0.05$).

showed highly interconnective pores with pore size that varied from approximately 50 to 200 μm (data not shown). The walls of pores contained collagen fiber and were covered by a nonfibrillar ground substance. As the PS collagen ratio increased, the pore size showed a tendency to increase. The denser structures of cross section occurred in

higher BF collagen ratio materials (B5P0HA and B4P1HA). In the present study, the morphological property appears to be caused by different mixing ratios of BF and PS collagen. Numerous factors directly influenced the extent of cross-linking and morphological property, for example collagen origin [11], EDC concentration [5,9,11,14], active agent [11,13–15], cross-linking procedure [9,19], and post-treatment [14]. Previous studies have shown that collagen-glycosaminoglycan (CG) materials used for studies of skin regeneration were inactive when the mean pore size was either lower than 20 μm or higher than 120 μm [20]. However, the pore size of HACSMs falls within an acceptable range and corresponds to the cell migrating capacity as shown in the previous studies [21,22].

After collagenase degradation for 4 h, all HACSMs exhibited an eroded lattice structure (Figure 2). The B1P4HA and B2P3HA materials revealed a high degradation of the thinner pore walls and lose initial three-dimensional structures. These results correspond with the degradation behavior shown in Figure 5 and are discussed subsequently.

For tissue engineering purposes, the sponge materials used as a cultured dermal substitute or wound dressing should also exhibit good mechanical strength to keep their shape during *in vivo* application. All cross-linked HACSMs were further assessed by measuring the swelling ratio during immersion in PBS (pH 7.5) and illustrated in Figure 3. The swelling ratio was a simulative test for that form-stability of sponge materials under physiological conditions. In general, the swelling ratio of materials decreases as the extent of cross-linking increases [25]. The swelling ratios significantly ($p < 0.05$) reduced when the ratio of BF collagen increased. These results also indicate that all the sponge materials could be bound 33–60 fold in PBS. However, the higher BF ratio materials (B5P0HA, B4P1HA, B3P2HA, B1P1HA, and B2P3HA) were significantly ($p < 0.05$) reduced in swelling ratios compared with other materials (B1P4HA, B0P5HA). This phenomenon would be explained by the morphological difference among different materials. In the present study, the cross-linking extent of B5P0HA, B4P1HA were higher than B1P4HA, B0P5HA. Highly cross-linked HA and collagen usually decrease the capacity of the matrix to absorb water [5,11,19]. As per morphological observations, materials composed of a higher BF collagen exhibited a smaller pore size and denser structure. The denser structure (B5P0HA, B4P1HA, B3P2HA, B1P1HA, and B2P3HA) materials reduced the contact area with PBS and contributed to reduce the swelling ratio. On the contrary, the larger pore size materials (B1P4HA and B0P5HA) increased the contact area with PBS and increased their swelling ratios.

In addition, the tensile strength increased as the ratio of BF collagen increased (Figure 4). The B4P1HA and B5P0HA were significantly ($p < 0.05$) higher than other materials (B0P5HA, B1P4HA, B2P3HA, B1P1HA, and B3P2HA) in this regard. These results also proved that the mechanical properties of HACSMs could be significantly increased ($p < 0.05$) by the addition of BF collagen. Improved mechanical properties as well as reduced *in vitro* or *in vivo* biodegradation rate were noted. A biostable sponge material not only prolonged the implantation time, but also reduced the amount of chronic inflammation and delay in wound healing due to excessive hydrolysis [25]. Based on the reason mentioned, B5P0HA, B4P1HA or B3P2HA should be suitable candidates for biomaterials.

The cross-linking extent was evaluated by resistance to collagenase and thermal stability. Collagenase was naturally present in healing wound and cleaves a unique site in peptide bonds in triple-helical collagen, i.e., the primary sequence of the amino acids leucine and glycine [23]. The *in vitro* degradation rate is a function of the degree of cross-linking [5,15]. Collagen and HA degraded products within collagenase solution were measured to estimate the resistance to collagenase among different materials and are illustrated in Figure 5. In the present study, an increased resistance to collagenase was observed as the ratio of BF collagen increased. Moreover, the biodegradation rates were accelerated with the materials having higher PS collagen ratio. Basic biochemical properties of different collagen origin were precisely measured in the previous study [10] and revealed that the PS collagen bounded with a relatively large amount of glycosaminoglycan and resulted in high enzymes resistance. The resistance to collagenase of HACSMs should be enhanced by PS collagen due to its relatively large amount of glycosaminoglycan, sterically hindering access to collagenase active sites. Unexpectedly, high degradation rates were observed in B0P5HA and B1P4HA materials. This result could be explained by the different swelling ratios of the materials. The swelling ratios were significantly ($p < 0.05$) reduced when the BF collagen ratio increased. The decreased ratio of degradation of higher BF collagen materials versus higher PS collagen materials is most probably due to the interference of the penetration of collagenase into the HACSMs. The reduced penetration will substantially decrease the surface area available for adsorption of collagenase in the cross-linked HA-collagen network [11,14,15].

The relationships between thermal stability and cross-linking efficiency were well established [9,11,12,14]. With DSC the helix-to-coil transition of collagen fiber can be measured as a

temperature-dependent endothermic signal, which indicates the extent of intermolecular cross-linking [11]. The results indicated that the difference in thermal stability of HACSM depended on the different BF : PS collagen mixing ratios and revealed a tendency to increase with ratio of BF collagen (Table 2). A previous study [10] indicated that the BF collagen had higher hydroxyproline (Hyp) + proline (Pro) value and contributed to higher molecular thermal stability. This result not only demonstrated that HACSMs can be prepared with a wide range of thermal stability (94–126°C) but also proved that the thermal stability would be enhanced by increasing the BF collagen ratio. Moreover, the higher BF collagen ratio might contribute more cross-link bridges during EDC cross-linking. These bridges not only reduced the swelling ratio and pore size but also increased the tensile strength, thermal stability, and resistance to collagenase. The EDC cross-linking systems were dependent on the primary structure of collagen. The greater amount of glutamic acid and aspartic acid in BF collagen could explain the different physical–chemical properties of the HACSMs composed of higher BF collagen ratios, for example reduction in pore size and swelling ratio, enhancement in tensile strength, resistance to collagenase, and thermal stability. The EDC cross-linking system is frequently used in the porous collagen materials [5,9,11,14,15], which has shown better biocompatibility than glutaraldehyde (GA) [9]. In principle, the EDC cross-linking takes place by the reaction between carboxyl groups of glutamic acid and aspartic acid and generated stable amide bonds [9,11,13–15,24,25]. A previous study [26] indicated that the biostability of materials could not be enhanced by external glutamic acid addition. However, in the present study the materials were cross-linked by a similar system but different in strengthening approach. The BF collagen exposure of more carboxyl groups yields residues and provides forming stable intermolecular bridges under EDC cross-linking. Further evidence for our hypothesis was obtained by examining the free amine index and is illustrated in Figure 6. The non-cross-linked amine groups are liable to react with the Ninhydrin agent and produce a chromophore. In the present study, a linear relation among the increase of thermal stability, resistance to collagenase, and decrease in free amine index were observed. The B5P0HA revealed the highest value of thermal stability, resistance to collagenase, and lowest free amine index, indicating that the highest degree of cross-linking was achieved. It is also supported that these differences among the different BF : PS collagen ratios directly introduced effects on the morphological property, tensile strength, degree of swelling ratio, resistances to collagenase, and thermal stability. This result also implied that the biodegradability

rate of novel HACSMs can be varied by a different BF:PS collagen mixing ratio. Moreover, previous studies stated that the BF collagen is more easily extracted in high yield from beet than from mammals tissue and provide a relatively low risk of possessing unknown pathogens such as bovine spongiform encephalopathy [27,28]. Recently, this collagen-based biomaterial has been widely used in drug delivery systems and has shown many advantages, such as the controlled release of spermicidal agents, antibiotics or rhBMP-2, and reduced the tissue irritation activity [29]. The novel HACSM should be a suitable candidate for artificial skin or drug delivery systems due to its controllable biodegradability.

CONCLUSION

The novel HA-collagen materials retaining porous structures were successfully fabricated by employing a combination of freeze-dry and EDC cross-linking techniques. The biostability and mechanical strength can be enhanced by a high proportion of BF collagen and closely related to the BF collagen that contained higher amounts of carboxyl groups of glutamic or aspartic acid residues, which produced more bridges under EDC cross-linking. In conclusion of these results, the authors assume that B4P1HA and B5P0HA should be produced according to high biostability and mechanical strength, and furthermore, they may be suitable for artificial skin or drug delivery applications.

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