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Characterization of UV-irradiated Dense/porous Collagen Membranes: Morphology, Enzymatic Degradation, and Mechanical Properties

Jong-Eun Lee[1], Jong-Chul Park[1], Yu-Shik Hwang[1], Jeong Koo Kim[2], Joong-Gon Kim[3], and Hwal Suh[1]

[1]Department of Medical Engineering, Yonsei University College of Medicine, Seoul;
[2]Department of Biomedical Engineering, College of Biomedical Science and Engineering, Inje University, Kimhae;
[3]Department of Pharmaceutical Research Division, Hanwha Group R&E Center, Taejon, Korea.

Collagen-based membranous materials of various shapes (gel, film, sponge) are known to be the most promising materials in terms of facilitating the regeneration of dermal defects. In this study, dense and porous collagen membranes were fabricated using air-drying and freeze-drying processes, respectively, and the effect of ultraviolet (UV) radiation on the degree of membrane crosslinking was evaluated by *in vitro* biodegradation and mechanical testing. A non-irradiated membrane group was used as the negative control and a glutaraldehyde (GA) treated group as the positive control. Scanning electron microscopy showed that, as the freezing temperature decreased to -196℃, the resultant mean pore sizes also decreased; optimal pore size was obtained at a freezing temperature of -70℃. *In vitro* biodegradation and mechanical testing demonstrated that GA treatment or 4 hours of exposure to UV radiation significantly increased both resistance to collagenase and mechanical strength versus the untreated controls, regardless of the collagen membrane type (dense or porous). Our results suggest that UV treatment is a useful tool for the fabrication of collagen membranes designed to be used as dermal dressings.

Key Words: Collagen, dense/porous membrane, biodegradation, mechanical properties, crosslinking, ultraviolet treatment

INTRODUCTION

Collagen is the major protein of the extracellular matrix (ECM), and has been used clinically as an effective biomaterial for wound healing and skin regeneration.[1-3] It has been processed into various shapes, such as, gel, film, sponge, and fiber. Although these biomedical products have been investigated over a period of 20 years, studies on collagen-based products have proceeded continuously because of a diversity of manufacturing methods and potential end uses. Among these various types of collagen-based products, porous and dense collagen membranes have been favorably applied to healing of skin defects.[2,4] Porous collagen membrane was designed as a permanent wound dressing or a scaffold for tissue engineering.[4-6] Porous membrane has been reported to canalize fibroblast migration from the wound edge because of its porous structure, which leads to the production of newly synthesized ECM by cells.[7] Since ECM reconstruction is a very important process in the healing of skin defects involving the dermis, membrane porous structures have received much attention from researchers. Many studies related to pore morphology have been undertaken to obtain pore interconnections and the proper pore size for cellular infiltration.[8,9] In general, the porous structures of collagen membranes have been achieved using the freeze-drying technique. The microstructure of the membrane was reported to be altered by a change in the freezing rate.[8,10] Dense collagen membrane has been used as a temporary wound dressing, because it functions as an effective physical barrier, which prevents protein and fluid loss and the bacterial invasion of skin defects.[11,12] However, the application of collagen-based membranes for permanent or temporary coverage of open wounds has some limitations because they are particularly susceptible to bacterial colonization

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and enzymatic digestion.[13,14] The collagen molecule is specifically degraded by collagenase and its degradation products are spontaneously denatured to gelatin at physiologic temperature. These gelatinized fragments are then cleaved by several nonspecific proteases. Concomitantly, cells infiltrating the membrane should synthesize new ECM components for tissue regeneration. The balance between the rates of these two processes is considered to be an important feature of wound healing.[15] When one considers that non-crosslinked collagen-based materials rapidly degrade into wound fluids, it becomes apparent that membrane crosslinking is required to control the biodegradation rate. Collagen molecules are usually crosslinked by inducing further covalent bonding by glutaraldehyde (GA), formaldehyde, di-isocyanate or acyl azide treatment.[16-19] GA treatment, in particular, is an effective crosslinking method for collagen-based biomaterials used specifically for implantation. However, this treatment has encountered problems of cytotoxicity, heterogenous crosslinking, and the requirement to thoroughly remove residual aldehydes by washing. In order to ensure the absence of remaining toxic materials, physical crosslinking methods, such as ultraviolet (UV) or dehydrothermal (DHT) treatment, have been used as alternatives.[20-23]

In this study, dense and porous membranes were fabricated using air-drying and freeze-drying processes, respectively. In order to prepare an optimal collagen membrane to use as a dermal dressing, the effect of freezing temperatures on the pore sizes of the collagen membranes, and the effect of UV irradiation on the crosslinking of the collagen membrane was determined using biodegradation and mechanical testing.

MATERIALS AND METHODS

Preparation of dense/porous collagen membrane

Type I atelocollagen was extracted from calf skin by pepsin treatment and salt precipitation, as previously described.[24] The collagen precipitate was lyophilized at -40℃ and kept at 4℃, until use. The material was then dissolved in 0.001N HCl solution at 4℃ and adjusted to pH 7.4, and the resulting fibrous precipitate centrifuged at 3,000 g for 15 minutes at 4℃ and collected as a concentrated collagen precipitate, with a density of 0.53 ± 0.04%. In order to fabricate the porous membrane, 5 ml of the precipitate concentrate was poured onto a polystyrene Petri-dish (diameter 5 cm), and quickly frozen at -20, -70, and -196℃, and then lyophilized at -50℃ for 24 hours. Alternatively, to fabricate a dense membrane, 5 ml of the precipitate concentrate was poured onto a polystyrene Petri-dish (diameter 5cm), and air-dried at 4℃ for 24 hours.

Crosslinking of collagen membranes

Collagen membranes were crosslinked either by immersion in 0.625% (v/v) GA solution or using UV radiation (wavelength, 254 nm). GA solution was freshly prepared in 0.05 M phosphate and 0.15 M NaCl (pH 7.4). The collagen membranes were immersed in this solution for 24 hours at 4℃. After crosslinking, specimens were rinsed thoroughly with 0.9% NaCl. UV irradiation was conducted by placing the membranes in transparent polymethylmethacrylate (PMMA) cases, and placed in a self-designed UV chamber (Daeil DBO231S, Seoul, Korea), in which the specimens were exposed to surrounding eight 10W UV bulbs for 1/2, 2, 4, 8, or 24 hours at 4℃. The distance between the light source and sample was 5 inches, and the intensity of UV was 1.66 mW/cm^2 equivalent to 93.3 $mJ/cm^2/min$.

Scanning electron microscope (SEM) observations

The morphology of the collagen membrane was examined using a SEM (JEOL, JSM 54300, Japan). A prepared collagen membrane was fixed on an adhesive tape and coated with gold/palladium. The average pore size was measured by area analysis using a computerized image analyzer (Bum-Mi Universe Co., Ltd., Ansan, Korea).

In vitro degradation of collagen membranes

The degradation test upon crosslinked membrane was performed using bacterial collagenase

(Type IA, Sigma Chemical Co. St. Louis, MO, USA).[25] 10 mg of collagen membrane was incubated for 4 hours at 37℃ in a collagenase solution (10 units/ml, 0.05 mM Tris-HCl buffer, 10 mM $CaCl_2$, pH 7.4). The extent of crosslinking was determined by measuring the amount of dissolved collagen in solution, using the Bradford assay;25 values determined were statistically analyzed using the paired students t-test.

Mechanical testing of collagen membranes

The mechanical properties of the membranes crosslinked by UV irradiation and GA treatment were determined to investigate the extent of crosslinking. Collagen membranes were prepared in strips (10 × 4 mm), and uniaxial tensile stress testing was performed using a material testing machine (Micro Bionix, MTS Systems Co. Tryton, MN, USA). Both ends the specimen were fixed by Teflon grips and abrasive paper to prevent gliding failure at the grips, as described previously.[26] The thicknesses of individual membranes were measured using a micrometer (Mitutoyo. Co. Knagawa, Japan). Each measurement was made in triplicate at different points on the membrane using a 10 N (MTS Systems Co, Tryton, MN, USA) load cell. Preconditioning of the specimen was performed by repeated loading and unloading at a crosshead speed of 10 mm/minutes for 5 cycles. The sample was then stretched to failure. All tests were conducted at room temperature. The data from the load cell and was stored on the Data Acquisition Software of Teststar 4.0 (MTS Systems Co, Tryton, MN, USA). From the load-displacement relationship obtained using the above, the stress-strain curve was calculated. Measured values were statistically analyzed using the paired students t-test.

RESULTS

SEM observations

Fig. 1 illustrates the crosssectional morphology of the dense membrane that was fabricated by an air-drying process, and fig. 2 shows that of a porous membrane fabricated using the freeze-drying process at the different freeze dry temperatures. Decreased pore size correlated with a decrease in the freezing temperature (Fig. 2a, b, c), as collagen membranes fabricated at 20, -70, and -196C had mean pore diameters of 197, 115, and 31 m, respectively (Table 1). These non-crosslinked porous membranes showed three-dimensional porosity and an interconnected network. After crosslinking, the pores of the GA-treated membrane (Fig. 3a) tended to collapse, whereas that of UV-irradiated membrane (Fig. 3b) was similar to the non-crosslinked collagen control (Fig. 2b).

In vitro biodegradability

The degree of crosslinking was measured by assaying the amount of dissolved collagen in the resulting collagenase solution. Regardless of the

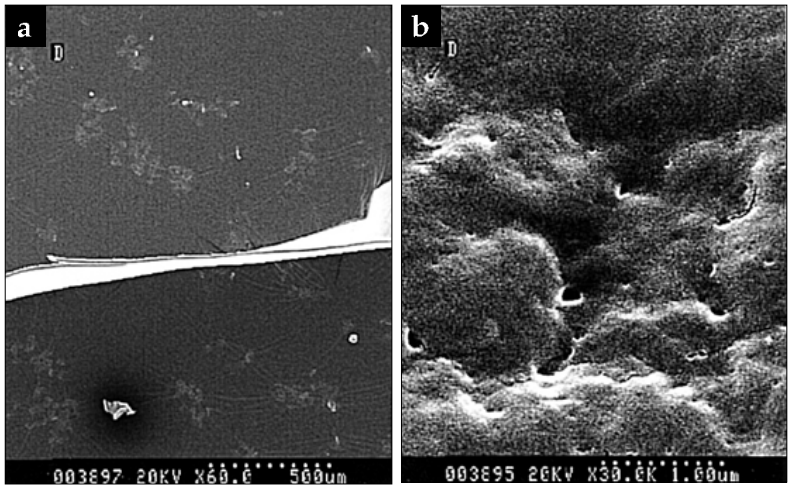

Fig. 1. Morphological observations of a dense membrane: (a) ×60, (b) ×30,000.

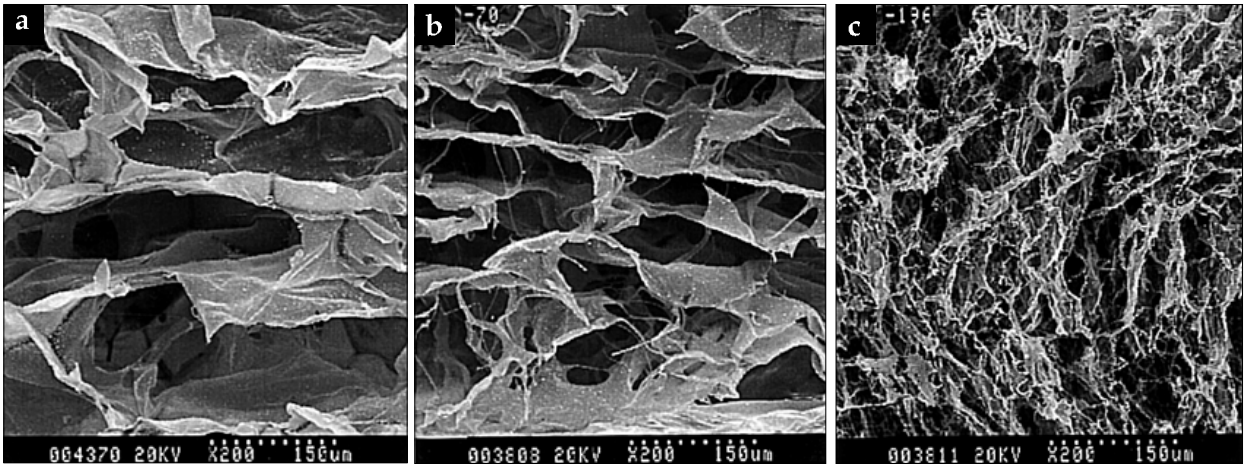

Fig. 2. Morphological observations of a freeze-dried porous membrane: (a) at -20℃, (b) at -70℃, (c) and at -196℃.

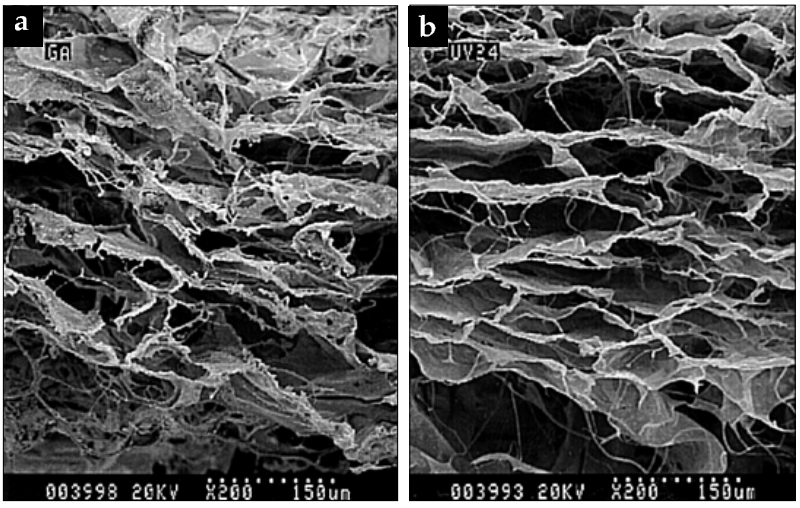

Fig. 3. Morphological observation of porous membrane: (a) GA treated after freeze-drying at -70℃, (b) UV treated after freeze-drying at -70℃.

Table 1. Characteristics of Pores According to Different Freezing Temperatures and Crosslinking Processes

Test materials	Freezing temperature (℃)	Crosslinking process	Pore size (mm)
M-20-NC	-20	no crosslinking	196.9±47.7
M-70-NC	-70	no crosslinking	114.6±30.2
M-196-NC	-196	no crosslinking	31.4±12.8
M-GA	-70	GA treatment	100.5±40.5
M-UV	-70	UV treatment	110.6±32.2

fabricated' membrane type, the UV-exposed membranes produced less dissolved collagen than the non-exposed controls (Table 2). Dense membranes demonstrated higher resistance to collagenase digestion than porous membranes in all groups. A treatment time of 4 hours produced membranes with similar dissolved collagen levels as the equivalent GA treated membranes, for dense and porous types.

Mechanical testing of collagen membranes

Both dense and porous membranes demonstrated the typical concave up characteristic of typical soft tissues stress-stain curve (Fig. 4).[27] The stress-strain curves of each membrane expectedly

Table 2. Collagen Amount Dissolved from Dense and Porous Membranes after Collagenase Digestion

Test materials	Release collagen amount (mg) per membrane (mg)	
	Dense membrane	Porous membrane
CONTROL	5.3±1.5	51.8±19.0
UV1/2	3.3±0.5*	30.7±3.8*
UV2	2.5±0.4†	23.6±0.7†
UV4	2.2±0.6‡	23.2±4.9†
UV8	2.9±0.4†	28.6±4.1†
UV24	3.1±0.4*	29.6±5.0
GA	1.9±0.2‡	20.7±1.4†

Each value represents the mean SD in five samples, *, †, and ‡: significantly different compared to the control non-treated group for each type of membrane, *p<0.05, †p<0.01, and ‡p<0.001. CONTROL: non-treated group, UV1/2: UV-treated group for 30 minutes, UV2: UV-treated group for 2 hours, UV4: UV-treated group for 4 hours, UV8: UV-treated group for 8 hours UV24 : UV-treated group for 24 hours, GA: 24 hour 0.625% glutaraldehyde pretreated group.

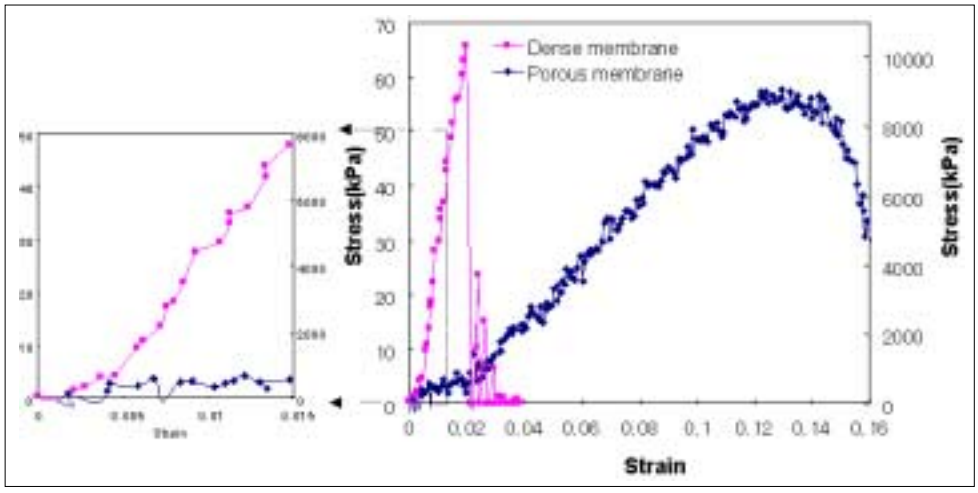

Fig. 4. Stress-strain curves of dense and porous collagen membranes.

showed that the dense membrane was stiffer than the porous membrane. The GA and UV treatments produced the crosslinking of the dense membrane and the crosslinked groups had higher ultimate tensile stress (UTS) than to the non-crosslinked group. In Particular, the collagen membrane that was exposed for 4 hours was not significantly different from the GA treated membrane in terms of UTS (Fig. 5). However, the membrane exposed to UV for 8 hours has slightly higher UTS. In the case of the porous membranes, both the GA and UV treated membranes had higher UTSs that their non-treated equivalents (Fig. 6).

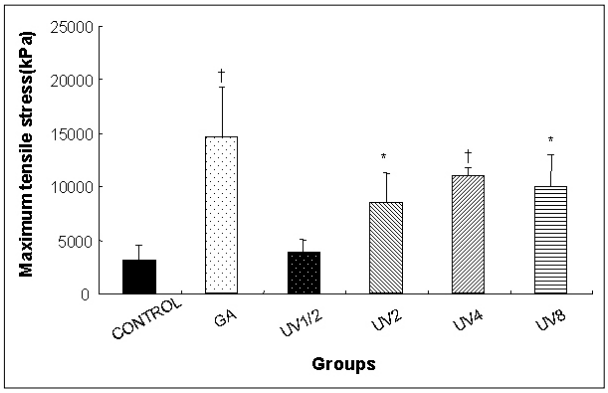

Fig. 5. Ultimate tensile stress of a dense membrane with respect to UV irradiation time. Each value represents the mean ± SD of five samples, * and † significantly different compared to the non-treated control group for each type of membrane, *p<0.05 and †p<0.01. CONTROL: non-treated group, UV1/2: UV-treated group for 30 minutes, UV2: UV-treated group for 2 hours, UV4: UV-treated group for 4 hours, UV8: UV-treated group for 8 hours, GA: 24 hour 0.625% glutaraldehyde pretreated group.

DISCUSSION

Collagen is known to be the main structural matrix protein of the tissue extracellular matrix,

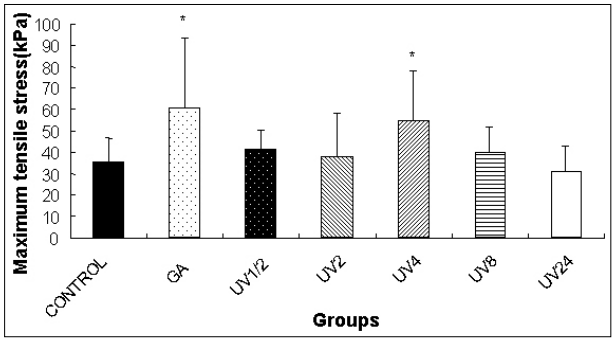

Fig. 6. Maximal tensile stress of a porous membrane with respect to UV exposure time. Each value represents the mean ± SD of eight samples, *significantly different from the non-treated control group for each type of membrane, *$p<0.05$. CONTROL: non-treated group, UV1/2: UV-treated group for 30 minutes, UV2: UV- treated group for 2 hours, UV4: UV-treated group for 4 hours, UV8: UV-treated group for 8 hours, UV24: UV-treated group for 24 hours, GA: 24 hour 0.625% glutaraldehyde pretreated group.

and of the various types of collagen, type I is the most abundant form, and is found in a variety of tissues. However, the antigenicity of collagen can cause foreign body reactions when it is applied to the human body. The immunological reaction of the host to the collagen-based implants is directed against selected sites (antigenic determinants) in the protein molecules.[2] Thus, type I atelocollagen, which is devoid of antigenic telo-peptide chains, was used in this study. The size and morphology of the pores in collagen-based biomaterial normally make a great contribution to its ability to facilitate tissue regeneration. A three-dimensionally interconnected porous structure in a collagen-based biomaterial provides vascularization and cellular colonization when in contact with living tissues.[28] The size and morphology of the pores are recognized to be dependent upon the freezing temperature of the mixture before lyophilization.[29] It has been reported that the optimal pore size ranges between 50 and 150 m, and that pores of this size can provide appropriate space for cellular infiltration and proliferation.[8,15] In our present study, this optimal pore size was obtained when the collagen precipitate was freeze-dried at -70℃.

To control the biodegradation rate these fabricated membranes need to be crosslinked. GA crosslinked collagen-based biomaterials are widely used as dermal dressings, because GA treatment can provide collagen-based biomaterials with improved mechanical properties and a reduced biodegradation rate.[28,29] GA reacts with the -amino group of lysyl residues in collagen, to induce intra- and inter-molecular crosslinking.[30] Cheung et al. showed that intramolecular crosslinking is often observed when low concentrations of GA are used, while at higher concentrations, intermolecular crosslinking is favored as under these conditions GA is polymerized.[31] GAs unique properties such as high water-solubility, high reactivity at physiologic pH, and the high stability of its reaction products, strongly favor its continuous use.[32] Nevertheless, the use of GA to crosslink collagen-based biomaterials is associated with a number of undesirable side effects, including cytotoxicity and calcification.[33,34] It has been proposed that the active form of GA is an aldehyde. Free aldehydic groups remaining after the crosslinking of the polymeric network are extremely reactive and can produce significant toxic effects.[31,33] In addition, GA is considered to be a promoter of calcification due to the action of its aldehyde group in the collagenous matrix,[34] and Revy et al. demonstrated that a pretreatment of Type I collagen subcutaneous implants with GA promoted their calcification.[34]

One possible approach to prevent of these problems might be avoiding the use of chemical crosslinking reagents such as GA, and we have investigated a crosslinking method using UV irradiation that does not introduce toxic chemicals.

The degree of crosslinkng of collagen molecules can be evaluated indirectly by biodegradation and mechanical testing. Actually, our studies demonstrate that exposure to UV increases the resistance of collagen membranes to enzymatic digestion and improves their mechanical properties (Table 2, Fig. 5 and 6). This result is supported by studies which show that collagen fiber can be crosslinked by UV (254 nm) radiation.[21,22] UV exposure produces radicals from the nuclei of aromatic residues, such as, those in tyrosin and phenylalanin, and the binding of these radicals results in the observed crosslinking.[35-37] These crosslinks may inhibit the action of collagenase upon the collagen membrane and

reduce membrane solubility. The bacterial collagenase (*Clostrium histolyticum*) specifically hydrolyzes the peptide bond on the amino side of Gly in -X-Gly-Pro- sequence of collagen.[22] In our present study, resistance against enzymatic digestion decreased with extended UV exposure (Table 2). Moreover, crosslinking increases the tensile stress of collagen membranes. As shown in figures 5 and 6, the UTS of fabricated membranes increases with UV exposure. This result is supported by a previous study, which demonstrated that UV exposure improved mechanical properties (stiffness and tensile strength).[26] However, membranes showed decreased collagenase resistance and lower tensile properties after UV exposure for 8 and 24 hours. This result suggests that collagen molecules were degraded by UV exposure of more than 4 hours. Koide reported that collagen membranes are fragmented and denatured after 5 hours of UV irradiation.[38] GA treated fabricated membranes had better durabilities than UV exposed membranes by mechanical testing or on exposure to collagenase solution. This may be due to the relatively small number of aromatic residues present, that is, only out of the 3156 amino acid residues on the collagen molecule.[38] When one considers that GA easily penetrates the collagen fiber network and participates in the crosslinking of collagen fiber, it becomes apparent that the attenuation of UV light by collagen fiber also limits the achieved degree of crosslinking. Accordingly, thin membranes with a compact structure are probably more effectively crosslinked by UV treatment. This hypothesis was supported by the result that dense membranes showed higher levels of crosslinking rate than porous membranes. By mechanical testing, the UTS of dense membrane increased significantly from 3.2 ± 1.2 MPa to 8.5 ± 2.7 MPa after UV irradiation for 2 hours ($P<0.05$), whereas the UTS of porous membranes were not altered significantly by 2 hours of UV exposure (Fig. 5 and 6). Comparing the two types of membrane without modification, the UTS value of dense membrane was one hundred times that of the equivalent porous membrane, whereas, the amount of collagen dissolved from a dense membrane was only ten times that dissolved from a porous membrane. In this regard, it is believed that combinations of porous and dense membranes are likely to provide improved durability *in vivo*.

The pore size of porous membranes was found to be dependent on freezing temperatures, and was optimized at -70℃. Regardless of whether the collagen membrane was of the dense or porous type, both the mechanical and enzyme resistant characteristics of the collagen membranes could be controlled by altering UV exposure levels. In addition, the dense membrane demonstrated greater tensile stress and resistance to enzyme activity than the porous membrane. Combinations of dense and porous collagen membranes are currently being examined as potential tissue regeneration scaffolds or as wound dressings.

Hwal Suh, D.D.S.
Depertment of Medical Engineering, Yonsei University College of Medicine, C.P.O. Box 8044, Seoul 120-752, Korea.
Tel: 82-2-361-5406, Fax: 82-2-363-9923,
E-mail: hwal@yumc.yonsei.ac.kr

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