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Reinforced Bioartificial Dermis Constructed with Collagen Threads

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Abstract In this work, a novel type of composite scaffold was designed, which has the suitability of both high biocompatibility and strong mechanical properties, for use in bioartificial dermis applications. The reinforced scaffold consisted of a lyophilized collagen sponge formed around a cross-linked collagen meshwork with an average thread diameter of approximately 55 μm. Fibroblasts were cultured in the reinforced collagen sponge for 7 days, during which time the pores in the sponge became filled with cells that secreted extracellular matrix (ECM) to form a bioartificial dermis. Results of ultimate tensile strength (UTS) measurements and compression tests indicated that the bioartificial dermis formed around the reinforced collagen sponge showed about ten times the strength of the bioartificial dermis formed around a typical collagen sponge (1.5 ± 0.05 vs. 0.15 ± 0.05 and 2.5 ± 0.1 vs. 0.2 ± 0.08 MPa, respectively). As a result, reinforced collagen mesh improved mechanical properties and this technique will be possible to make stronger scaffolds, not only for artificial skin applications but also various artificial tissues, such as synthetic cartilage, bone, and blood vessels. © KSBB

Keywords: reinforce, collagen thread, collagen sponge, tissue engineering

INTRODUCTION

Various types of skin equivalents have been developed to cover skin defects caused by burns and other types of trauma. Rheinwald and Green developed a culture of keratinocytes using a 3T3 feeder layer, but the survival rate of cultured epithelial autografts (CEAs) in full-thickness skin defects is relatively low. The graft can fail to take when CEAs and autografts are applied to a wound surface in poor condition without a dermis. Moreover, there is a risk of poor epithelization due to the absence of a dermal component at the recipient site. Therefore, a dermal equivalent is required to overcome this problem. Scar tissue formation can be reduced with the use of a living skin equivalent consisting of combined dermal equivalent and cultured epithelial layers.

The non-cellular components of the dermis, which primarily consist of extracellular matrix (ECM) proteins and collagen, have been shown to be relatively non-immunogenic. The acellular human dermis (*i.e.* AlloDerm) is the first commercially available human collagen material in sheet form, which offers the real possibility for use as a collagen scaffold and can be replaced by native collagen [1]. The original method of Yannas and Burke for the use of artificial skin has been commercialized (Integra™, Integra Life Sciences Co., San Diego, CA, USA). Their artificial skin was a bilayer membrane, composed of a dermal portion consisting of a porous matrix formed from lyophilized collagen cross-linked with chondroitin-6-sulphate [2-4]. The other commercially available wound-healing product is Dermagraft™ (Advanced Tissue Science, Inc., La Jolla, CA, USA), which is currently been marketed in Canada, the United Kingdom, and other European countries for the treatment of diabetic foot ulcers. Dermagraft™ is a metabolically active dermal skin replacement, containing various ECM components and grow th factors normally found in human dermis. This artificial skin product is produced by seeding dermal allogenic fibroblasts onto a three-dimensional scaffold consisting of polyglycolic acid (PGA) and polyglactin-910 (PLA) fibers [5,6].

Previous tissue engineering studies have been performed using a combination of cells, growth factors, and scaffolds [7,8]. Wound healing is achieved by the synergistic effect of these three components, all of which play important roles. Scaffolds provide the substrates to which the cells can attach during the initial phase, and degrade after the completion of wound healing. Consequently, various scaffolds having been investigated, including gelatin, alginate, chitosan, hyaluro-

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nate, collagen, amniotic membrane, and SIS [9,10]. However, more effective substrates are still required. To improve the wound-healing efficacy of these scaffolds, various studies have been performed where growth factors were added or the scaffolds were coated with fibronectin or other ECM components [11-13].

Recently, Okochi *et al.* [14] tried to increase wound healing through enhanced structure stability using collagen sponges. A collagen sponge reinforced with polyglycolic acid (PGA) was developed, which increased hair formation and growth compared with a typical collagen sponge. Epidermal cycts and ectopic hairs formed on the collagen sponge without PGA, and the volume of the sponge decreased as a result of humidity after the seeding of the cell suspension. Thus, the pores of the collagen sponge shrank, and this deflated sponge might have inhibited the movement of the transferred cells resulting in epidermal cysts forming at the grafted site and inducing cell leakage and lose. However, the collagen sponge reinforced with PGA demonstrated less shrinkage, thereby maintaining the size of the pore structures, so the grafted cells could be retained [14]. Previous research revealed that growth factors and ECM are important, but it is also apparent that stability of the three-dimensional structure is also essential.

Synthetic biomaterials such as PGA and poly-L-lactic acid (PLLA) have been commonly used in clinical applications. The mechanical properties of PGA are better than collagen, but the biocompatibility of PGA is not as good. PGA has been shown to increase proliferation of T cells and subsequently induce an inflammatory reaction [15].

In this work, a novel type of composite scaffold was designed, which has the suitability of both high biocompatibility and strong mechanical properties. A collagen sponge reinforced with a collagen mesh was made by combining lyophilized and cross-linking methods. Skin fibroblasts were cultured on the reinforced collagen sponge and then this bioartificial dermis was characterized via H&E and MT staining, SEM, and mechanical testing.

MATERIALS AND METHODS

Preparation of Collagen Threads and Mesh

Collagen threads were made from type I calfskin collagen (KOKEN, Japan) according to the method described by Cavallaro and Kemp [16] with slight modification. A 10 mg/mL collagen solution in 0.05 M acetic acid was loaded into a injection pump set to infuse at 2.0 mL/min. Silicone tubing (2.0 mm I.D.) connected to an 20-gauge blunt stainless-steel needle was immersed in coagulation solution containing 20% polyethylene glycol (PEG, MW 8000, Duksan Co., Korea) in 94 mM sodium phosphate dibasic and 24 mM sodium phosphate monobasic at pH 7.55.

The coagulation solution was maintained at a flow of 4 mm/s in a pump driven circulation bath (Haake Bucher Instrument Inc., No. 426-2000, USA), and then the 0.5% (w/v) collagen solution was injected through the injection needle into the circulation bath containing the flowing coagulation solution. The collagen gelled on contact with the neutral pH solution, and the nascent thread began to dehydrate due to the osmotic gradient formed between the collagen and the PEG solution. The thread had a residence time in the bath of approximately 10 min. As the thread accumulated, it was transferred to a rinse bath filled with 5.5 mM sodium phosphate dibasic, 0.5 mM potassium phosphate monobasic, and 75 mM NaCl, at pH 7.10 and remained there for 10 min. The threads were then partially dehydrated in isopropanol at room temperature for 16 h and dried under tension of their own weight at 40°C for 30 min inside an incubator heated with air blowers.

One layer of collagen mesh was fabricated by cross joining nine collagen threads to another nine threads to form a single layer. Five of these layers were stacked on top of each other and then dried. The collagen mesh was then formed by cross-linking using 254 nm ultraviolet (UV) irradiation for 12 h.

Preparation of Collagen Sponge and Reinforced Collagen Sponge

Collagen sponges were made using a similar method to that employed by Yannas and Boyce [17,18]. A type I atelocollagen powder (Bioland, Korea) was dissolved in 0.05 M acetic acid, at a concentration of 10 mg/mL, and coprecipitated by the dropwise addition of chondroitin-6-sulfate (CS, Sigma Chemical Company, St. Louis, MO, USA) while stirring in a homogenizer. The CS was dissolved in 0.05 M acetic acid, at a concentration of 10 mg/mL; the amount added was about 10 mg per 100 mg of the total dispersed solids.

The resulting collagen-CS solution was placed in a freezer (Sanyo Corp., Japan) at −80°C for 6 h and was then lyophilized by freeze drying (Samwon Freezing Engineering Co., Korea) at −80°C for 48 h. Subsequently, the collagen sponges were placed in a vacuum oven (Fisher Scientific, Pennsylvania, USA) and subjected to a vacuum of 1.3 in of Hg at 105°C for 24 h. The collagen sponges were then incubated in 20 mL of 40% (v/v) ethanol, containing 50 mM 2-morpholineoethane sulfonic acid (MES, Fluka Chemic AG) (pH 5.5), for 30 min at room temperature. Next, the collagen sponges were immersed in 20 mL of 40% (v/v) ethanol, containing 50 mM MES (pH 5.5), 24 mM 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (Fluka Chemic AG), and 5 mM *N*-hydroxysuccinimide (Fluka Chemic AG), for 12 h at room temperature. After completion of the reaction, the sponges were washed twice in 0.1 M Na_2HPO_4 (pH 9.0) for 12 h. Finally, the sponges were washed twice in 1 M NaCl for 6 h and 2 M NaCl for 2 days, and then they were rinsed with distilled water [19,20], lyophilized by freeze drying, and sterilized with γ-irradiation at 10 KGy.

The reinforced collagen sponge was prepared by immersing the collagen mesh into the collagen-CS solution and prepared following the same methods of freeze drying, cross-linking, and sterilization as above.

Preparation of Bioartificial Dermis and Reinforce Bioartificial Dermis

Normal human skin fibroblasts were aseptically isolated

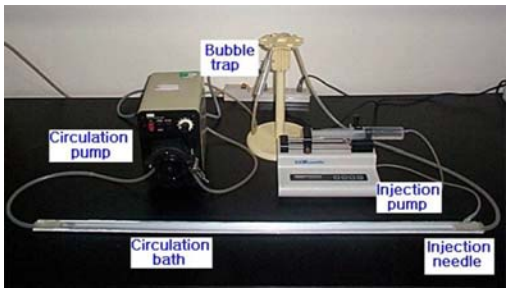

Fig. 1. Photograph of the equipment used for making collagen threads. Equipment consisted of a circulation pump, a bubble trap, an injection pump, an injection needle, and a circulation bath. The coagulation solution maintained its flow in the circulation bath via the circulation pump. The 1% (w/v) collagen solution was injected through the needle into the circulation bath containing flowing coagulation solution.

from a circumcised neonatal foreskin at Chung-Ang University Hospital (Yong San, Korea). The donated foreskin was immersed in Dulbecco's modified Eagle medium (DMEM, Invitrogen Gibco, Grand Island, NY, USA), containing antibiotic-antimycotic agents (100 unit/mL penicillin G sodium, 100 unit/mL streptomycin sulfate, and 0.25 μg/mL amphotericine B; WellGen, Korea), at 4°C. The piece of foreskin was then immersed in DMEM, containing 1.4 units/mL Protease (Dispase, Sigma Chemical Company), for 16 h at 4°C. Thereafter, the epidermal layers were mechanically stripped and the normal fibroblasts were isolated from the dermis using 362 unit/mL type I collagenase (type IA, Sigma Chemical Company) for 90 min at 37°C. Following this enzymatic treatment, DMEM supplemented with 10% fetal bovine serum (FBS, BioWhiteker™, Cambrex Bioscience Walkersville, Inc., MD, USA) was added to the cellular suspension. This diluted suspension was centrifuged for 5 min at 1,000 rpm. The cultivated fibroblasts were then inoculated into the culture medium. Fibroblasts were routinely cultured in DMEM supplemented with 10% FBS at 37°C in a 5% CO_2 incubator.

Human skin dermal fibroblasts, suspended in DMEM supplemented with 10% FBS, were seeded onto the unreinforced and reinforced collagen sponges (diameter 15 mm, thickness 2.0 mm). To obtain a very high cell seeding density, the cells were seeded onto the sponges in a dried condition. Sponges were placed in 12-well plates (the diameter of the wells was 16 mm) and 500 μL of a harvested suspension containing 5×10^6 fibroblasts was seeded onto each sponge. After 30 min, 500 μL of DMEM supplemented with 10% FBS was added to the plate wells. After a further 1 h, the sponges were transferred to an 80 cm petri dish, and the medium was replaced every 3 days for 7 days.

Morphological Examination

The bioartificial dermis and reinforced bioartificial dermis were fixed for 2 h at 4°C using 10% neutral buffered formalin. The fixed samples were embedded in paraffin, and 4~5 μm sections were stained with hematoxylin & eosin (H&E) and Masson trichrome (MT).

The MT staining was performed on 4~5 μm thick serial sections mounted on poly L-lysine coated slides. The sections were deparaffinized and mordanted in Bouin's solution, microwave 1 min, and allowed to stand 15 min. After washing with distilled water for 5 min, sections were treated with Weigert's working hematoxylin for 10 min, rinsed in distilled water for 5 min, soaked in Biebrich scarlet solution, and then treated with phosphotungstic/phosphomolybdic acid. The sections were transferred and maintained in aniline blue solution for 5 min. Finally, they were washed with 1% acetic acid for 1 min, followed by distilled water.

For scanning electron microscopy (SEM), the specimens were fixed for 2 h at room temperature in phosphate buffer (a mixture of monosodium phosphate and dipotassium phosphate, 0.2 M, pH 7.4) containing a mixture of 4% glutaraldehyde and 2% formaldehyde. After a thorough rinsing with 0.175 M phosphate buffer, the specimens were immersed in 2% osmium tetroxide (buffered with 0.2 M phosphate) for 2 h. The samples were dehydrated in HMD (E-3100, Bio-Rad, UK), sputter-coated with gold-palladium (E-5400, Bio-Rad, UK), and observed under a scanning electron microscope (JSM-840A, Jeol Inc., Japan) at 15 kV.

Mechanical Strength Test

To compare mechanical properties, ultimate tensile strength (UTS) and compression tests were performed after fibroblasts were culture for 7 days. The sponges in the form of rectangular strips (10 mm × 5 mm × 2 mm) and squares (5 mm × 5 mm × 2 mm) were tested using a material testing machine (H5KT, HOUNSFIELD, England).

For the UTS test, the gage length (starting length) was set to 4 mm and specimen width and thickness used were 5 and 2 mm, respectively. The crosshead speed was set to 2 mm/min (10% strain rate) and the chart speed was set to 2 mm/min. UTS was measured directly from the force-displacement graph.

For the compression test, the gage length was set to 5 mm and the crosshead speed was set to 2 mm/min (10% strain rate), and the chart speed was set to 2 mm/min. Compression was measured directly from the force-displacement graph.

Statistical Analysis

The data for the sponge, bioartificial dermis, and acellular artificial dermis were statistically evaluated using the Student's *t*-test. Data are presented as means ± SD. The difference between the means was considered significant when $p \leq 0.05$.

RESULTS

Structure of the Collagen Mesh and Reinforced Collagen Sponge

Fig. 2A shows a microphotograph of a collagen mesh

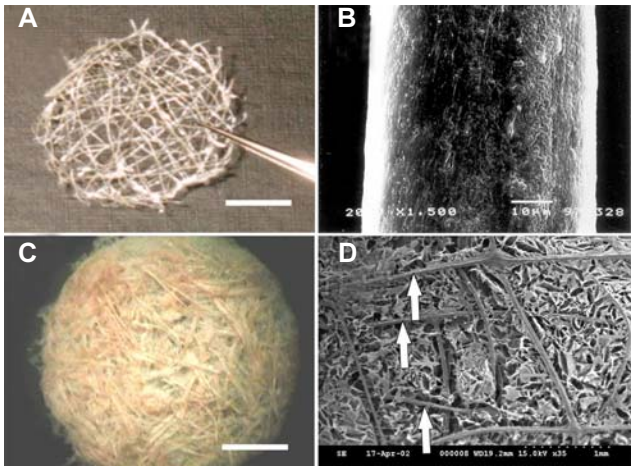

Fig. 2. Optical microscopic photographs of the collagen mesh (A, diameter = 15 mm) and reinforced collagen sponge with collagen mesh (C). Scanning electron microscopy of a collagen thread (B) and reinforced collagen sponge with collagen mesh (D). Original magnification: (B) ×1,500, (D) ×35; scale bar: (B) 10 μm, (D) 1,000 μm.

formed of collagen threads and Fig. 2B shows a scanning electron micrograph (× 1,500) of a collagen thread. The average diameter of the collagen thread is about 55 μm and formation of collagen fibril arrays on the surface of the thread was observed.

A scanning electron microphotograph depicting a cross section through a reinforced collagen sponge is presented in Fig. 2D. The figure shows that the reinforced collagen sponge was highly porous, with a loose network of collagen fibrils and an approximate pore size of 80~150 μm. The collagen threads were apparent (arrows).

Histology of Reinforced Bioartificial Dermis

On the reinforced collagen sponge, cultured for 7 days with human skin fibroblasts to form a bioartificial dermis, both cells and collagen threads were observed. Fig. 3A is a SEM (× 1,500) displaying the sponge structure and the internal distribution of the collagen mesh and cells. The reinforced bioartificial dermis was constructed of a porous collagen sponge containing collagen mesh and fibroblasts. It showed homogenous distribution of cells into the pores and on the surface of the collagen sponge and mesh. Arrows indicate collagen threads that were incorporated into the sponge.

Histological examination (× 100) via H&E staining (Fig. 3D) and MT staining (Fig. 3C) showed that cultured skin fibroblasts and collagen threads associated with the reinforced collagen sponge. The fibroblasts adhered rapidly to the reinforced collagen sponge and attached completely in less than 24 h. These cells were cultured in the reinforced collagen sponge for 7 days, during which time the pores in the sponge became filled with fibroblasts (Fig. 3D, arrow head) that secreted ECM to form a bioartificial dermis (Fig. 3B, stars). This resulted in the virtual disappearance of the pores (Fig. 3B). Unlike monolayer cultures, where contact inhibition limits cell growth, this porous structure provides an environment for cell growth and ECM deposition to occur in a three-dimensional configuration.

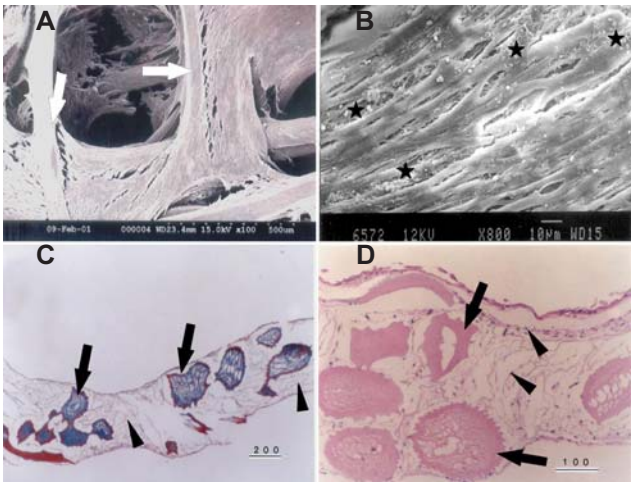

Fig. 3. Scanning electron microscopy images (A, B) and histological analysis of the bioaritificial dermis (C, D). The collagen mesh was highly porous and became filled with fibroblasts (A). The surface of the bioaritficial dermis was coved with fibroblasts and the presence of newly secreted ECM on the surface of cells was apparent (B). In the bioartificial dermis, the bluish stained substances in MT staining (C) revealed the presence of collagen threads (arrow) and the fibroblasts were indicated (arrowhead) by hematoxylin & eosin staining. Original magnification: (A) ×1,500, (B) ×800, (C) ×40, (D) ×100; scale bar: (B) 10 μm, (D) 10 μm (arrowhead: fibroblasts, arrow: cross-section of collagen threads).

Mechanical Tension Test

Both unreinforced and reinforced collagen sponges were measured for strength after 7 days of cell culturing. Results of UTS measurements indicated that sponges incorporated with collagen mesh showed about 1.5 ± 0.05 MPa, ten times the strength of reinforced collagen sponges at 0.15 ± 0.05 MPa (Fig. 4). Compression tests reveal that reinforced collagen sponges showed about 2.5 ± 0.1 MPa and reinforced collagen sponges showed about 0.2 ± 0.08 MPa (Fig. 5).

Reinforcement by collagen threads and the formation of mesh has an effect on the implant material without affecting its latent cytotoxicity. In addition to the high degree of biocompatibility, it will be possible to control the degree of strength of these scaffolds through changes in the diameter of the threads, the quantity of the threads, the thickness of the mesh, and different thread weaves used to form the mesh. Using these procedures, it will be possible to made stronger or weaker materials for use in different applications.

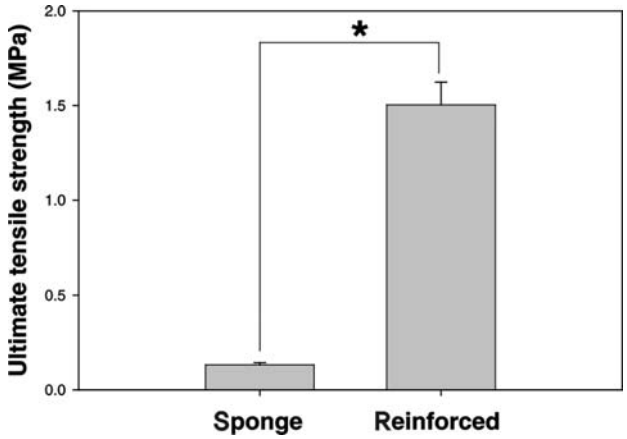

Fig. 4. Comparison of ultimate tensile strength (UTS) between the bioartificial dermis and reinforced bioartificial dermis after 2 weeks of cell culture (sponge: bioartificial dermis, reinforced: reinforced bioartificial dermis). Error bars indicate the standard deviation of the mean (n = 5, p < 0.05).

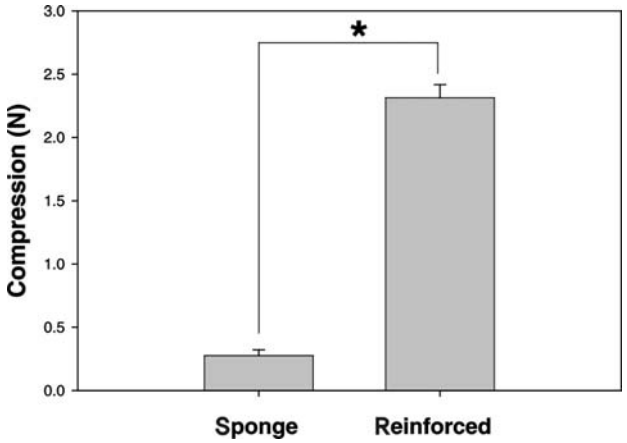

Fig. 5. Comparison of compression tests between the bioartificial dermis and reinforced bioartificial dermis after 2 weeks of cell culture (sponge: bioartificial dermis, reinforced: reinforced bioartificial dermis). Error bars indicate the standard deviation of the mean (n = 5, p < 0.05).

DISCUSSION

Synthetic materials may afford better plasticity and controlled biodegradation than grafts prepared from natural ECM materials, but they are also less biocompatible. However, natural ECM materials may be less suitable in many instances because of their weaker mechanical properties. So, many researchers have designed composite or reinforced scaffolds so as to overcome these shortcomings.

Early reinforced composite scaffolds were developed for use in urological surgery dating back to 1987. Because the scaffolds needed to have both biocompatibility and strong mechanical properties, natural and synthetic polymers were combined. These reinforced scaffolds were composed of Vicryl (Polyglactin) and collagen sponges or films. Many researchers utilized these reinforced scaffolds to repair urinary tract defects as well as bladder and kidney surfaces. These reinforced composite scaffolds were shown to be biodegradable, prevent leakage of urine, and were readily replaced by collagenous scar tissue lined with aurothelium [21-24].

Recently, a collagen sponge tube reinforced with co-poly(L-lactide/ε-caprolactone) was designed for urethral reconstruction. As indicated by animal tests, this reinforced scaffold was slightly fibrotic but completely epithelialized, and it supported the regeneration of smooth muscle layers without fistulae or stenoses [25].

Furthermore, reinforced scaffolds have been used for hard tissue regeneration. A collagen scaffold reinforced with chitin fibers was designed and cell proliferation *in vitro* and bone growth *in vivo* were evaluated. As a result of these tests, cell population and bone growth was determined to be superior to collagen scaffolds without chitin fibers. It was possible that the shrinkage suppression of the sponges in the presence of chitin fibers maintained the interspace, resulting in increase cell migration and bone formation [26,27]. A fibrinscaffold reinforced with poly glycolic acid fibers was also utilized for hard tissue regeneration [28]. Moreover, a reinforced scaffold composed of Vicryl and collagen sponge was used as dural substitutes, which should prevent cerebrospinal fluid leakage, seal fistulae, prevent cortical adhesion, be soft and easy to suture, resist ingress of infection, produced a minimal inflammatory response, and (if restorable) allow regeneration of the host dura to occur [29].

In research aimed at fabrication of artificial skin, pHEMA reinforced with nylon was developed [30]. This reinforced system increased the mechanical properties of the skin replacement, but reinforcement with a non-degradable polymer was problematic, preventing removal of the polymer after implantation. Recently, collagen sponges reinforced with PGA fibers have produced good results for regeneration of skin after wounding, as they maintain their 3-dimentional structures when implanted. However, PGA is likely to induce more inflammation (and subsequent fibrosis) when compared to collagen.

In this study, collagen was used because of its known biomaterial properties and its low-inflammatory response following implantation. We propose that the combination of collagen threads and sponges are optimal materials for solving the problems outlined above. We demonstrate the production of a reinforced collagen sponge and suggest that these products can be used not only in artificial skin but also in various other artificial tissue applications, such as synthetic cartilage, bone, and blood vessel formation.

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