Development of *N,O*-(Carboxymethyl)chitosan/Collagen Matrixes as a Wound Dressing

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In an attempt to accelerate wound healing by stimulating the recruitment of fibroblasts and improve the mechanical properties of collagen matrixes, *N*,*O*-(carboxymethyl)chitosan (NOCC) was incorporated into the backbone of a collagen (COL) matrix without or with chondroitin sulfate (CS) or an acellular dermal matrix (ADM). The result of a cell migration study demonstrated that the migration of fibroblasts was significantly enhanced by NOCC in a concentration-dependent manner. In the analysis with a dynamic mechanical analyzer, NOCC/CS/COL matrixes presented higher tensile strengths than did NOCC/ADM/COL matrixes. Skin fibroblasts cultured on the matrixes containing NOCC showed increased proliferation and secretion of three kinds of cytokines compared with the control. Results of the in vivo wound healing study showed that matrixes incorporating NOCC showed markedly enhanced wound healing compared with the control. Therefore, the above results clearly suggest that NOCC/COL matrixes containing CS or ADM can be potential wound dressings for clinical applications.

Introduction

It is well-known that wound healing is a complicated sequence of cellular and biochemical events involving inflammation, migration, the proliferation of different types of cells, the production of extracellular matrix (ECM) proteins, neovascularization, and so on. Wound healing proceeds through a series of different phases that overlap in time, each of which is guided by the action of growth factors, cytokines, and hormones.[1,2] Fibroblasts play a crucial role during the phase of granulation tissue formation; fibroblasts migrate into the wound and commence synthesis, deposition, and remodeling of the extracellular matrix. As well as macrophages and epidermal cells, fibroblasts secrete many cytokines and several proteolytic enzymes termed matrix metalloproteinases, which are responsible for controlling collagen remodeling during wound contraction.[3,4] Studies of the role of the fibroblasts in wound healing suggest that the incorporation of fibroblasts produces benefits of improving collagen deposition and remodeling, improving dermoepidermal junction formation, enhancing survival of keratinocytes, and decreasing the formation of myofibroblasts.[5−7]

Many collagen products for wound healing have been developed in the past few years, and some of them have been approved and are now commercially available.[8] Choosing collagen as a part of the matrix offers several advantages: it has well-documented structural, chemical, and physical properties. Additionally, collagen has low antigenicity, good biocompatibility, and the ability to promote cell attachment and proliferation. Considering other natural polymers frequently applied to the field of tissue engineering, both chitin and chitosan are recognized as prominent biomaterials due to their excellent physicochemical and biological properties; however, the rigid crystalline structure makes them hard to dissolve in water, which has retarded their potential for application in the biological field.[9,10] The process of wound healing can be accelerated if the healing agents are more soluble and thus accessible to the wound beds. Recent studies have shown that, by introducing carboxymethyl groups onto some of the amino and primary hydroxyl sites of the glucosamine units of the chitosan structure, a water-soluble chitosan derivative, *N*,*O*-(carboxymethyl)-chitosan (NOCC), can be prepared. NOCC is a chitosan derivative bearing carboxymethyl substituents at some of both amine and 6-hydroxyl sites of glucosamine units. In addition to being soluble in water, it also has many attractive physical and biological properties such as moisture retention, gel-forming capability, low toxicity, and good biocompatibility, all of which make it a promising biomaterial. Concerning the applications of drug delivery, a recent study showed that a hydrogel composed of NOCC and alginate can serve as a polymeric carrier for site-specific protein drug delivery in the intestine. Also, in both Caco-2 cell monolayers and rats, NOCC has proven to be a suitable polymer for the delivery and intestinal absorption of anionic macromolecular therapeutics.[11−13] In addition to its antibacterial activity, NOCC is capable of stimulating the extracellular lysozyme activity of fibroblasts, and some researchers reported that it significantly promotes the proliferation of skin fibroblasts.[14,15]

Therefore, the purpose of this study was to develop a novel collagen-based matrix for wound healing. In an attempt to accelerate the wound healing by stimulating the recruitment of fibroblasts and improve the mechanical properties of collagen matrixes, NOCC was incorporated into the backbone of a collagen (COL) matrix with or without chondroitin sulfate A (CS) or acellular dermal matrix (ADM). In the present study, the preparation of NOCC/COL matrixes is reported. The physical properties of these NOCC/COL matrixes are characterized. Finally, the enhancing effect of NOCC/COL matrixes on wound healing was examined using an in vivo animal model.

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Materials and Methods

Materials. Human skin fibroblasts and type I collagen were prepared following a method described in our previous paper.[16] NOCC (>85% degree of deacetylation, viscosity <100 mPa·s) was purchased from OHKA Enterprises (Kaohsiung, Taiwan). The ADM was obtained from our former work.[17] After lyophilization, the acellular dermal matrix was crashed into a floc-like material before use. CS was purchased from Sigma (St. Louis, MO). All other chemicals and reagents were of analytical grade.

Characterization of *N*,*O*-(Carboxymethyl)chitosan. Determination of the molecular weight and the polydispersity of *N*,*O*-(carboxymethyl)-chitosan was performed by size exclusion chromatography (SEC) employing a triple detector (ViscoTek TriSEC model 270 dual detector including a viscometer and light scattering refractometer (RI detector, ERC-7512, ERC)). A TSK gel column ($GMPW_{XL}$ = 7.8 × 30.0 mm, TOSOH) loaded with 0.1% (w/v) *N*,*O*-(carboxymethyl)chitosan was eluted with a mobile phase containing 0.01 M sodium acetate (AcONa), pH 5.5, at a flow rate of 1.0 mL min^{-1} with the column temperature maintained at 35 °C. Results indicated that the M_w, weight-average molecular weight, of *N*,*O*-(carboxymethyl)chitosan was 192000, the M_n, number-average molecular weight, was 194100, and the Pd, polydispersity, was 1.25.

Preparation of Functional Matrixes. Preparation of the NOCC/COL matrixes was carried out as follows. Collagen (1%) was dissolved in 0.01 M, pH 4.5 acetic acid solutions to prepare a homogeneous solution. NOCC gel solutions (0.5%) were prepared as aforementioned. Subsequently, the NOCC gel solution was slowly dripped into the collagen solution in a volume ratio of 1:1, and then the pH value of the mixture was adjusted to pH 3. Powdered chondroitin sulfate (2 mg/mL) or acellular dermal matrix (5 mg/mL) was added to the mixture. After homogenization, the mixture was poured into plastic molds, frozen at −40 °C, and then lyophilized to obtain porous matrixes. The NOCC/COL matrixes containing chondroitin sulfate were denoted NOCC/CS/COL, and if the matrixes contained acellular dermal matrix, they were denoted NOCC/ADM/COL. Chemical cross-linking was performed using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). Matrixes were immersed in a 50 mM EDC solution (water:ethanol = 5:95) for 12 h. The excess chemicals in the matrixes were washed out by deionized water with sonication, and then lyophilized again to obtain the resultant matrixes.

Fibroblast Cell Migration Assay. Fibroblast cell migration in response to NOCC was examined using a modification of the method described earlier.[18] Briefly, 6.5 mm Transwell inserts (Corning Costar, Cambridge, MA) containing 8.0 μm pore size polycarbonate membranes were coated with gelatin. These inserts were overlaid onto certain wells of the plate. Fibroblast cells (4×10^5) were loaded onto the upper side of the insert, and medium containing different concentrations of NOCC was placed into the well. After incubating for 12 h, MTT was added and then cells were incubated for an additional 2 h. Cells remaining on the top of the insert (residual cells) and those migrating to the well (migrating cells) were received in 200 μL of DMSO. Following 1 h of incubation, the absorbance was determined at 570 nm using an ELISA plate reader (FLUOstar, BMG, Offenburg, Germany). The percent migratory activity was calculated as percent migration = $\{A/[A + B]\} \times 100$, where *A* represents the absorbance of the migrating cells and *B* represents the absorbance of the residual cells.

Characterization of the NOCC/COL Matrix. A dynamic mechanical analyzer (DMA7e, Perkin-Elmer, Wellesley, MA) was used to measure the mechanical strength of the matrix by recording the time−modulus curve. The tested sample was mounted on a stainless steel, parallel extension kit, and measurements were taken at ambient temperature. The initial applied force was 50 mN, with an extension rate of 200 mN/min. The modulus and stress of the collagen matrixes at the break point were monitored. The tensile strength was calculated as the maximum stress reached before breakage of the material, as indicated by a sudden decrease in the recorded load.

The denaturation temperatures (T_d) of the matrixes were measured by a differential scanning calorimeter (Perkin-Elmer DSC pyres-1). Samples at 5 ± 2 mg were sealed in aluminum pans, and empty pans were used as references. Measurements were performed from 30 to 300 °C with a heating rate of 30 °C/min.

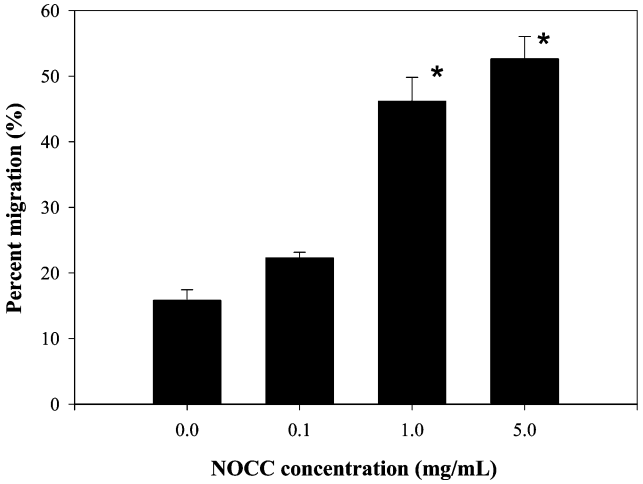

Figure 1. Migration of fibroblast cells in response to NOCC. Values are percentages of migrated cells ± SD (n = 3). The asterisks represent $p < 0.05$, a significant difference.

The content of free amine groups was determined by the modified method of Bubnis.[19] The absorbance was measured at 345 nm. The cross-linking degree was calculated by the following equation:

$$\text{crosslinking degree} = 1 - (\text{absorption}_s/\text{mass}_s)/(\text{absorption}_{ncl}/\text{mass}_{ncl})$$

where "s" represents the sample and "ncl" the non-cross-linked sample.

In Vitro Culture Studies. Various matrixes serially cut into suitable sizes were sterilized by submersion in 70% alcohol and a gentamycin solution for 12 h. After being washed with PBS buffer, matrixes were placed in 48-well plates. Fibroblasts at a concentration of 4×10^4 cells/well were seeded onto the surface of the matrixes and then cultured for a total of 5 days. Culture media were taken out at scheduled times for analysis during the culture. The amounts of three cytokines, bFGF, VEGF, and TGF-β, released by fibroblasts were measured by an enzyme-linked immunosorbent assay (ELISA). The matrixes were also taken for histological analysis.

In Vivo Wound Healing Study. Three kinds of dressings, the NOCC/COL, NOCC/CS/COL, and NOCC/ADM/COL matrixes, were investigated for the treatment of full-thickness wounds. Briefly, male Wistar rats weighting 300−350 g were used in this study. After anesthetization with pentobarbital, the dorsal hairs of the rats were shaved off with an electric razor. On the dorsal areas of the rats, four equal mirror image areas were marked along the spinal cord, 1 cm from the spinal cord and with 2.0 cm between any two areas. Four pieces of full-thickness skin, each with a surface area of about 1.0 × 1.0 cm, were excised. Prior to the lesions being covered, the blood residues were cleaned with a 0.9% saline solution and wiped to dryness with gauze. Randomly, one of the lesions was chosen to be covered with the collagen-only matrix as the control, and the other lesions were each covered with different dressings. Treated rats were placed in individual cages with an air-filtering device. On the 3rd, 7th, 14th, and 21st days after surgery, the areas of the wounds were measured, and wound tissues were excised. The method of our previous work to calculate the wound area was employed.[20] In total, 12 rats were included in this study. The statistical significance of any difference was analyzed by paired Student's t tests.

Histological Analysis. Matrixes and wound tissue specimens were taken at scheduled time points. Specimens were dehydrated with a tissue autotreatment device (RH-12E, Sakura, Japan) and embedded in paraffin with a paraffin dispenser (Shadon Lipshow, Pittsburgh, PA). Sections

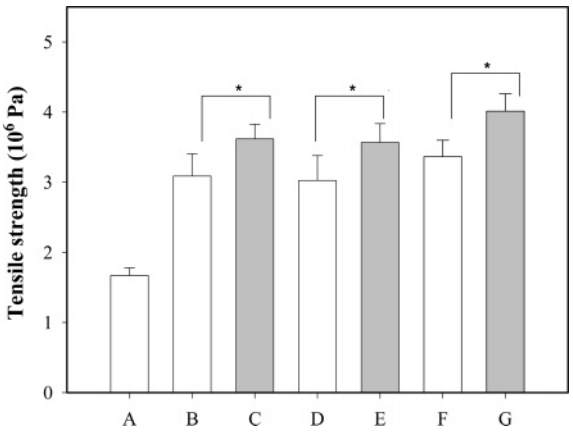

Figure 2. Tensile strength of different matrixes: (A) collagen-only matrix, (B) NOCC/COL matrix, (C) NOCC/COL matrix cross-linked with EDC, (D) NOCC/ADM/COL matrix, (E) NOCC/ADM/COL matrix cross-linked with EDC, (F) NOCC/CS/COL matrix, (G) NOCC/CS/COL matrix cross-linked with EDC ($n = 5$). The asterisks indicate a significant difference, $p < 0.05$, was noted between various matrixes.

of an appropriate thickness (about 3 μm) were sliced and stained with hematoxylin and eosin (H&E).

Results

Migration Study. As presented in Figure 1, a higher percentage of migrating cells was observed when the concentration of NOCC contained in the medium increased. The result demonstrated that the migration of fibroblasts was significantly enhanced by NOCC in a concentration-dependent manner.

Characterization of the NOCC/COL Matrix. The resultant NOCC/COL matrixes exhibited higher tensile strengths than did the collagen-only matrix (Figure 2). The tensile strengths of the matrixes were significantly affected by EDC cross-linking, which caused an increase of from 3.02×10^5 to 3.57×10^5 Pa in NOCC/ADM/COL matrixes and from 3.36×10^5 to 4.01×10^5 Pa in NOCC/CS/COL matrixes. For comparison, chemically cross-linked NOCC/CS/COL matrixes demonstrated higher tensile strengths than did NOCC/ADM/COL matrixes. Denaturation temperature (T_d) values and the cross-linking degrees of different matrixes are summarized in Table 1. Among the matrixes tested, the highest cross-linking degree (67.98%) was observed for the EDC cross-linked NOCC/CS/COL matrix. The DSC profiles for those samples examined in this study are shown in Figure 3. The figure reveals that T_d values of the matrixes all shifted toward higher temperatures after EDC cross-linking. In addition, the T_d of the cross-linked NOCC/CS/COL matrix recorded at around 124 °C was the highest, while both before and after cross-linking the T_d values of the NOCC/ADM/COL and NOCC/COL matrixes were similar.

Table 1. Physicochemical Characteristics of the Test Matrixes[a]

matrix	cross-linking method	T_d (°C)	cross-linking degree (%)	tensile strength (10^6 Pa)
collagen		92.5 ± 1.7		1.67 ± 0.11
NOCC/COL		95.5 ± 1.3		3.09 ± 0.32
NOCC/COL	EDC	112.7 ± 1.3	57.37 ± 3.69	3.61 ± 0.21
NOCC/CS/COL		98.1 ± 1.2		3.36 ± 0.24
NOCC/CS/COL	EDC	123.9 ± 1.1	67.98 ± 1.49	4.01 ± 0.25
NOCC/ADM/COL		96.4 ± 1.6		3.02 ± 0.35
NOCC/ADM/COL	EDC	110.3 ± 2.4	54.36 ± 1.66	3.57 ± 0.27

[a] T_d values and the cross-linking degree are presented as the mean ± SD ($n = 3$).

In Vitro Culture Studies. The amounts of the three cytokines, bFGF, VEGF, and TGF-β, released into the medium from fibroblasts cultured on the matrixes were measured, and the results are shown in Figure 4. The amounts of VEGF released from fibroblasts cultured on the NOCC/CS/COL, NOCC/ADM/COL, and NOCC/COL matrixes showed no significant differences, while that of the control, collagen-only matrix was lower compared to those of the above three kinds of matrixes. Fibroblasts cultured on the NOCC/CS/COL matrix released higher amounts of bFGF and TGF-β than did the other matrixes. On the whole, the amounts of bFGF, VEGF, and TGF-β released

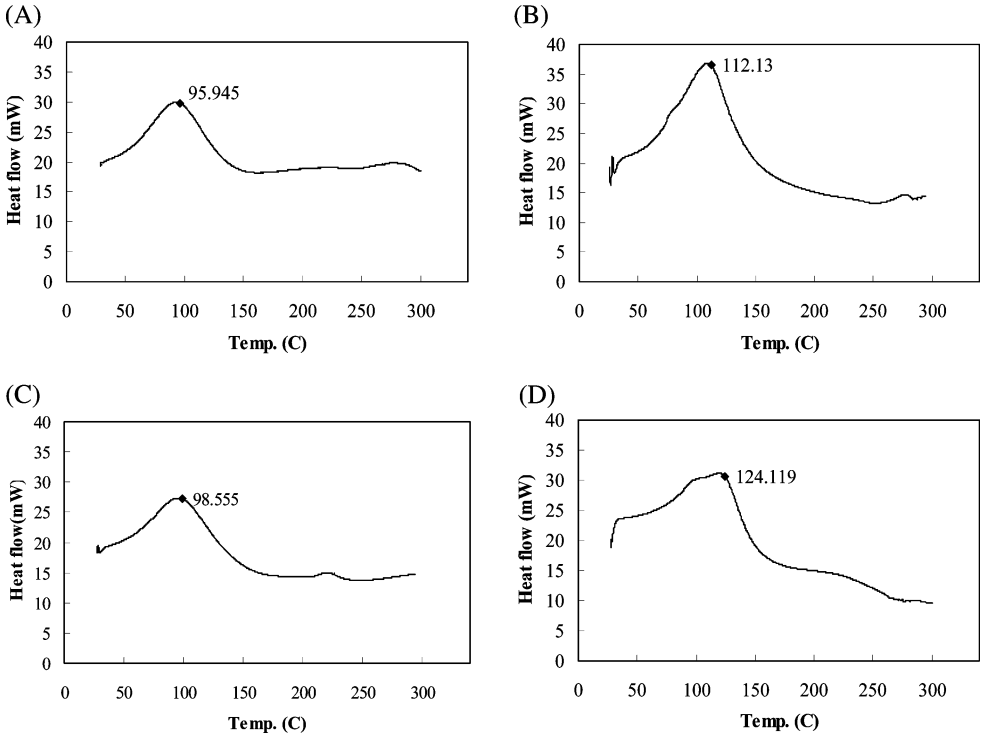

Figure 3. DSC profiles of matrixes: (A) NOCC/COL matrix, (B) NOCC/COL matrix cross-linked with EDC, (C) NOCC/CS/COL matrix, (D) NOCC/CS/COL matrix cross-linked with EDC.

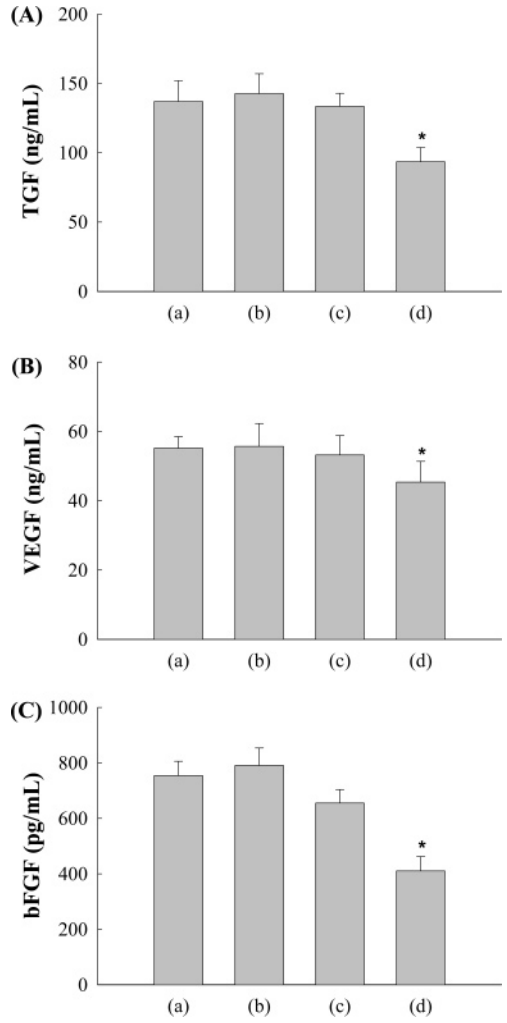

Figure 4. Amounts of three kinds of cytokines released in the medium for 5 days of culture: (A) NOCC/ADM/COL matrix, (B) NOCC/CS/COL matrix, (C) NOCC/COL matrix, (D) collagen-alone matrix (n = 5). The asterisks indicate a statistical difference, $p < 0.05$, was noted.

into the culture medium of the collagen-alone matrix were the lowest compared to those of the media of the other matrixes. Furthermore, in the analysis of histological observations (Figure 5), it was found that fibroblasts attached, proliferated, and covered the surface of the NOCC/CS/COL matrix, and some of the cells further migrated into the layer near the surface of the matrix. Similar results were also observed for the NOCC/COL and NOCC/ADM/COL matrixes. However, on the top of the control, collagen-only matrix, fibroblasts only grew on the surface and yet covered the entire surface of the matrix.

Wound Healing Study. On the 3rd, 7th, and 21st days after surgery, changes in the wound area covered with the NOCC/CS/COL, NOCC/ADM/COL, NOCC/COL, and collagen-only matrixes were examined. The average wound size at different time points is shown in Figure 6. The figure demonstrates that the average sizes of the wound area covered with the NOCC/COL, NOCC/CS/COL, and NOCC/ADM/COL matrixes at all of the scheduled time points were smaller than those covered with the collagen-only matrix, and most of the wounds had completely healed by the 21st day except for wounds covered with the collagen-only matrix. In addition, there were no significant differences among wounds covered with the NOCC/CS/COL, NOCC/ADM/COL, and NOCC/COL matrixes in terms of the rate of wound size reduction. As shown in Figures 7 and 8, on the third day after the operation, the infiltration of

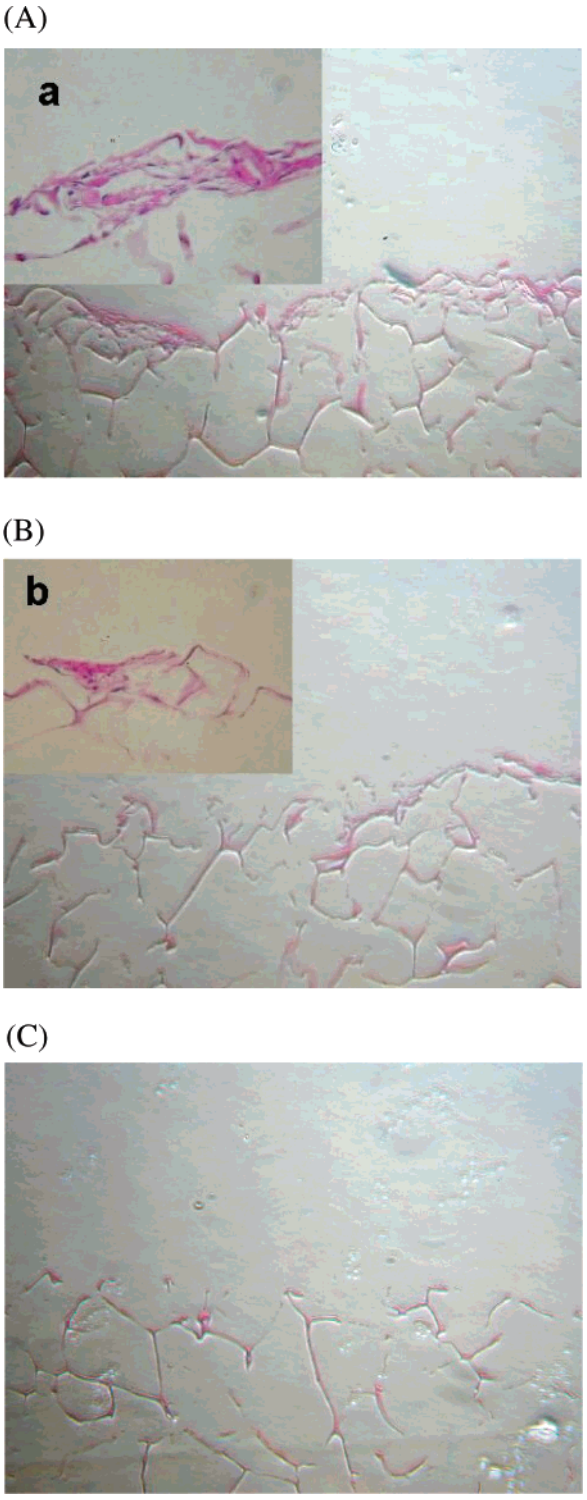

Figure 5. Histological cross-section of various matrixes. Fibroblasts were seeded onto the top of these matrixes and cultured for 5 days. Key: (A) NOCC/CS/COL matrix, (B) collagen-alone matrix, (C) NOCC/CS/COL matrix without seeding fibroblasts. The inset images a and b show certain parts of the corresponding images A and B at higher magnification.

numerous polymorphonuclear leukocytes into the wound area was observed. However, compared to the wound covered with the collagen-only matrix, wounds covered with the other three kinds of matrixes displayed increased levels of cellular infiltration. Also, epithelialization was observed on the wound margin. On day 7, the inflammatory response seemed to have gradually subsided. The granulation tissue was found to have invaded the

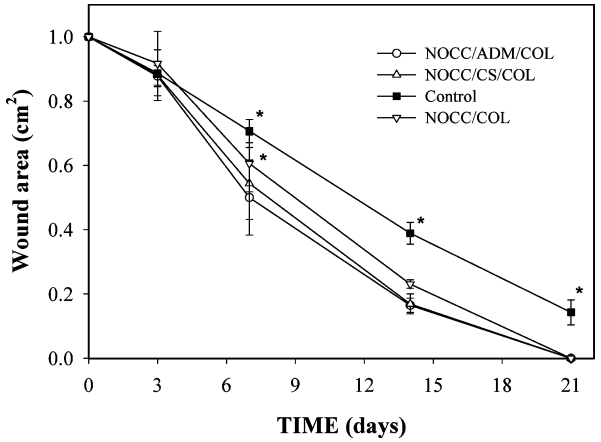

Figure 6. Comparison of average area changes of wounds covered by various matrixes at scheduled time points ($n = 3$). The asterisks indicate a significant difference at $p < 0.05$ by the paired t test.

wound space, and numerous new capillaries also appeared. On day 14, it was observed that the angiogenesis of the wounds covered with the NOCC/CS/COL, NOCC/ADM/COL, and NOCC/COL matrixes had slowly ceased, and some of the vessels had disintegrated as a result of apoptosis. On day 21, except for the wound covered with the collagen-only matrix, most of the wounds covered with the other matrixes had healed, the area of scarring had seemingly contracted, and capillary vessels had been lost.

Discussion

Blends of *N*,*O*-(carboxymethyl)chitosan and collagen gel solutions were used to design polymeric matrixes as wound dressings. Both gel solutions appeared homogeneous at pH 4.5. However, when blended together, the viscosity of the mixture decreased and the pH value rose slightly to 4.7, which showed that interactions had occurred between components. After the pH was adjusted back to 4, the viscosity of the mixture increased due to electrostatic repellent forces between the collagen chains with partial protonation. It is known that cross-linking with EDC involves activation of carboxylic acid groups to give *O*-acylisourea groups, which form cross-links with free amine groups. Both collagen and NOCC have plenty of carboxylic acid and amine groups present in their polymeric chains; hence, the cross-linking reaction catalyzed by EDC should be accessible. The results of the DMA analysis indicated that, as a whole, the tensile strengths of the matrixes significantly improved after treatment with EDC. Among the matrixes treated with EDC in this study, the highest tensile strength was observed for the NOCC/CS/COL matrix. This observation can be explained by the cooperation of chondroitin sulfate. This resulted in an increase in the number of available reactive sites for the cross-linking reaction, consequently forming more diverse and longer cross-linking chains; that is, the carboxyl groups of collagen, NOCC, and chondroitin sulfate A were all available for activation by EDC. Likewise, previous research has reported the effect of chondroitin sulfate as a cross-linking agent in a collagenous sponge which led to the sponge having a higher elastic modulus and greater resistance to enzymatic degradation.[21] Moreover, the above facts were further confirmed by the results of the analysis of the cross-linking degree. The analysis revealed that, after EDC treatment, the cross-linking degree of the NOCC/CS/COL matrix (67.98%) was higher than those of the NOCC/ADM/COL (54.36%) and NOCC/COL matrixes (57.37%). On the other hand, as shown by the results

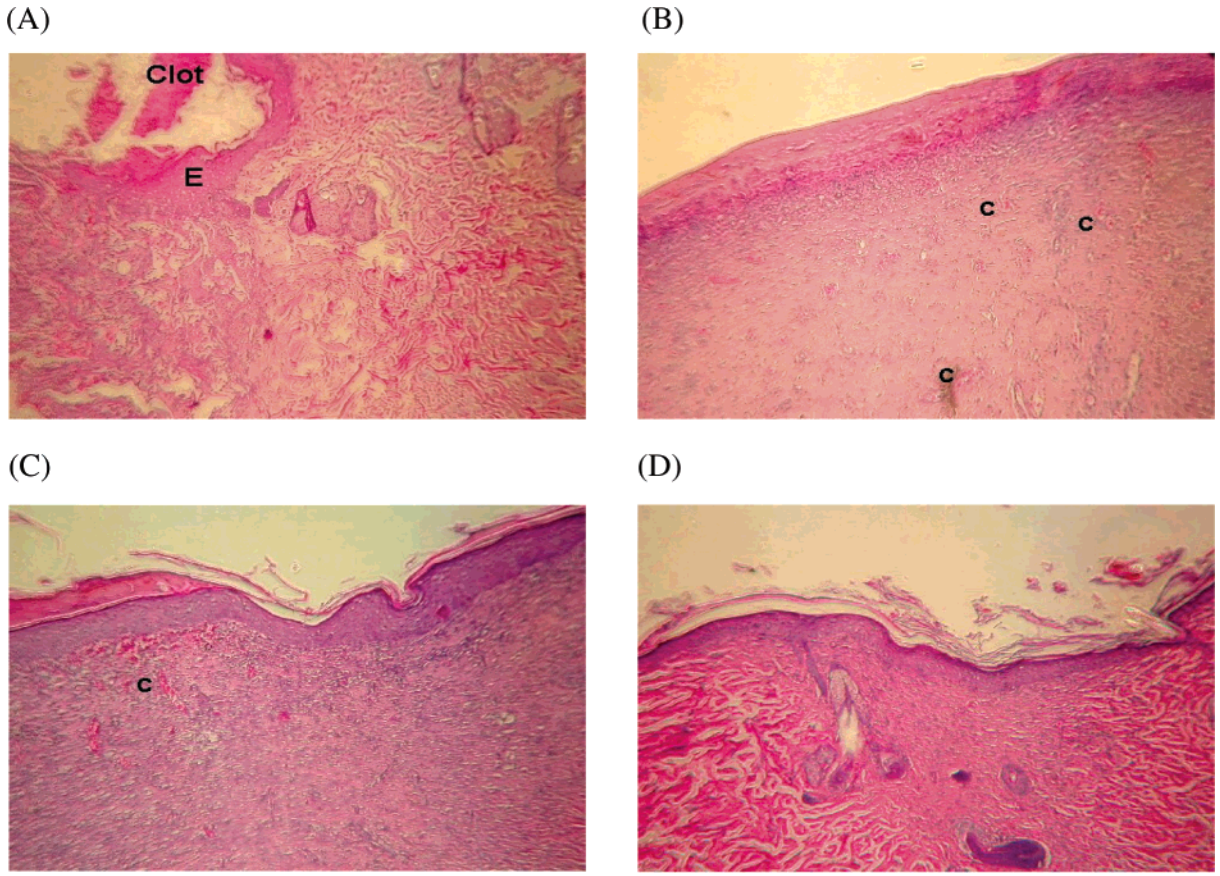

Figure 7. Photomicrographs of the wound area and its surrounding tissue covered with the NOCC/CS/COL matrix at scheduled time points: (A) day 3, (B) day 7, (C) day 14, (D) day 21. E = epidermis, and C = capillaries.

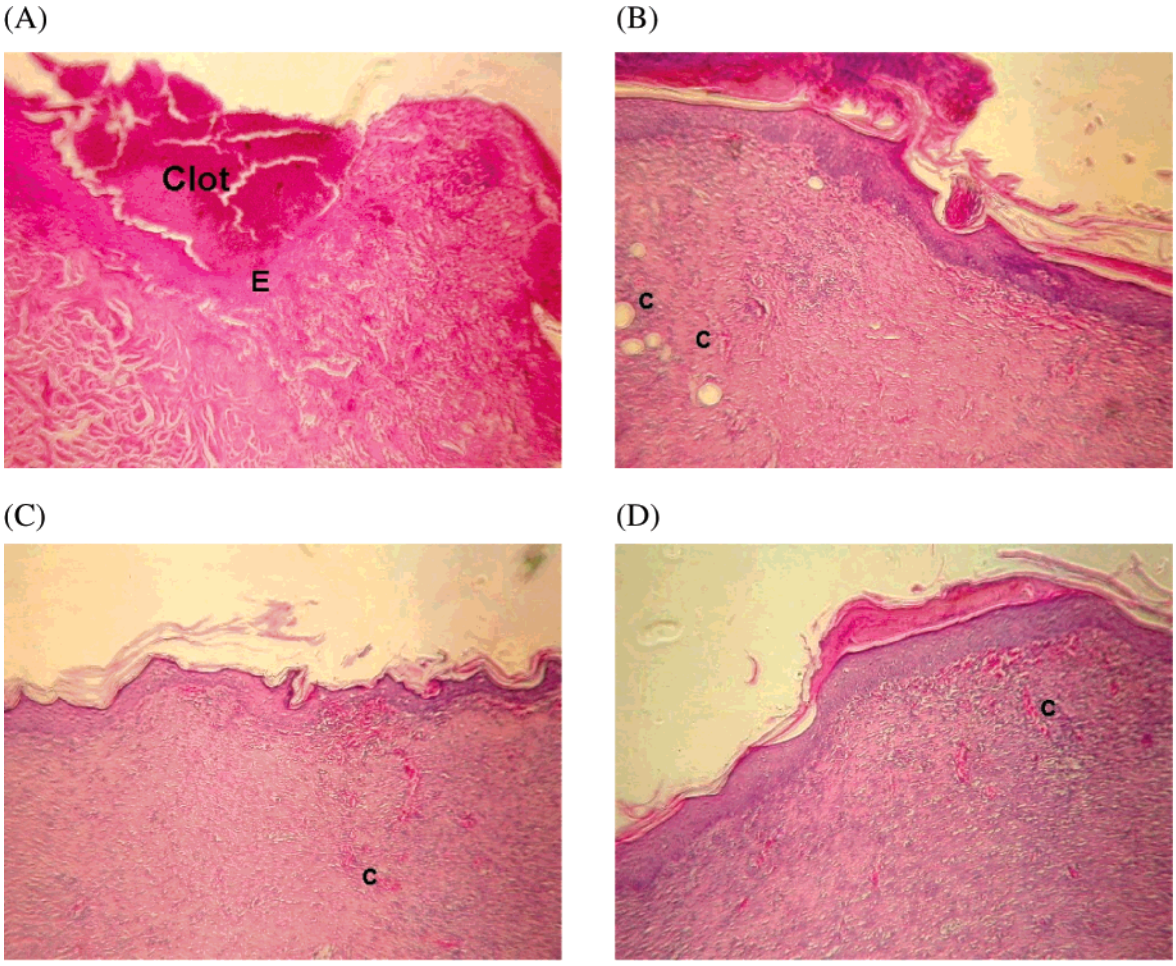

Figure 8. Photomicrographs of the wound area and its surrounding tissue covered with the collagen-only matrix at scheduled time points: (A) day 3, (B) day 7, (C) day 14, (D) day 21. E = epidermis, and C = capillaries.

of DMA and the analysis of the cross-linking degree, it was obvious that the floc of the acellular dermal matrix did not contribute to the enhancement of the tensile strength of the matrix, suggesting that the floc was insoluble in the mixed collagen gel solution due to its intact structure. DSC analysis was performed to examine the effect of EDC cross-linking on various matrixes. The results revealed that the T_d values of the corresponding matrixes shifted toward higher temperatures compared to those of the untreated matrixes; furthermore, the height of the endothermic peaks of the cross-linked matrixes was larger in comparison with that of the untreated matrix, which implied an increase in enthalpy. The reason for that may be ascribed to the increase in cross-linkages that break endothermically, and the situation in which as the number of cross-linkages increases, less water can be bound.[22] In addition, following a trend similar to that for the observations of cross-linking degree, the T_d values of the NOCC/CS/COL matrix were the highest compared those of the other treated matrixes due to a greater number of and more complex cross-linkages presented.

Earlier studies demonstrated that chitin and NOCC are able to promote the proliferation of skin fibroblasts, and additionally, chitin was proven to stimulate the migration of fibroblasts, hence accelerating wound healing.[23] In this study, we examined the effect of NOCC on skin fibroblast migration. As compared to cells incubated with medium alone, a concentration-dependent, significantly enhanced migration of fibroblasts was observed, whereas the corresponding receptor on the fibroblasts and mechanism are still left for further exploration. On the other hand, the addition of chondroitin sulfate A or the floc of the acellular dermal matrix was thought to be beneficial to its potential for use in wound healing since it is well-known that chondroitin sulfate is important for binding and modulation of growth factors and cytokines, as well as the adhesion, migration, and proliferation of cells.[24,25] Acellular dermal matrixes are derived from full-thickness skin which is treated to remove cells and cellular components but retains the critical extracellular matrix components, namely, the native dermal structure, proteoglycans, vascular channels, and so on.[26,27] Studies with the acellular dermal matrix have shown that it is capable of supporting fibroblast infiltration, neovascularization, and incorporation into host tissues.[28] Accordingly, we ground the lyophilized acellular dermal matrixes into a floc and used the mixture comprised of collagen, elastin, and proteoglycans as a supplement for the matrixes. To determine the influence of different matrixes on human skin fibroblasts, an in vitro culture study was conducted. It is known that fibroblasts release certain types of cytokines, and the amount of cytokines is dependent on the culture conditions.[29] It should be noted that the amounts of bFGF, VEGF, and TGF-β detected in the in vitro culture study were similar to the results reported by Kuroyanagi et al.[30] In addition, the fact that the amounts of bFGF, VEGF, and TGF-β released in the culture medium of the matrixes in which NOCC was incorporated were higher compared to those of the collagen-only matrix can probably be explained by the culture conditions; that is, fibroblasts seeded on the matrixes in which NOCC was incorporated markedly proliferated and secreted considerable amounts of cytokines. The results of histological observations also confirmed this explanation.

In animal wound healing studies, in view of the rate of wound reduction, the matrixes incorporating NOCC were obviously faster than the control, collagen-only matrix, suggesting that NOCC plays a key role in improving wound healing. From the histological observations, a possible reason proposed for the acceleration of wound healing is the enhancement of acute inflammation and fibroblast recruitment, and hence promotion of the differentiation of granulation tissue. We demonstrated in this study that NOCC's ability to stimulate the migration of fibroblasts might play a pivotal role.

Conclusions

In this study, we demonstrated that NOCC is able to stimulate the migration of fibroblasts. Two polymeric gel solutions, NOCC and collagen, were combined to design a novel matrix for wound healing. Characterization of the matrixes performed by DMA and DSC analysis indicated that these matrixes were successfully cross-linked by EDC, and their tensile strengths were improved to a certain extent, making them suitable for clinical applications. Results of the animal wound healing study showed that matrixes incorporating NOCC markedly enhanced wound healing compared with the control in terms of rates of wound reduction. Thus, the above results clearly suggest that NOCC/COL matrixes with the addition of chondroitin sulfate or acellular dermal matrix can be potential wound dressings for clinical applications.

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